UNIVERSITAT DE BARCELONA

Role of ZEB1 in Tumor Progression: Regulation of Cell Invasion and Senescence

Oriol de Barrios Barri

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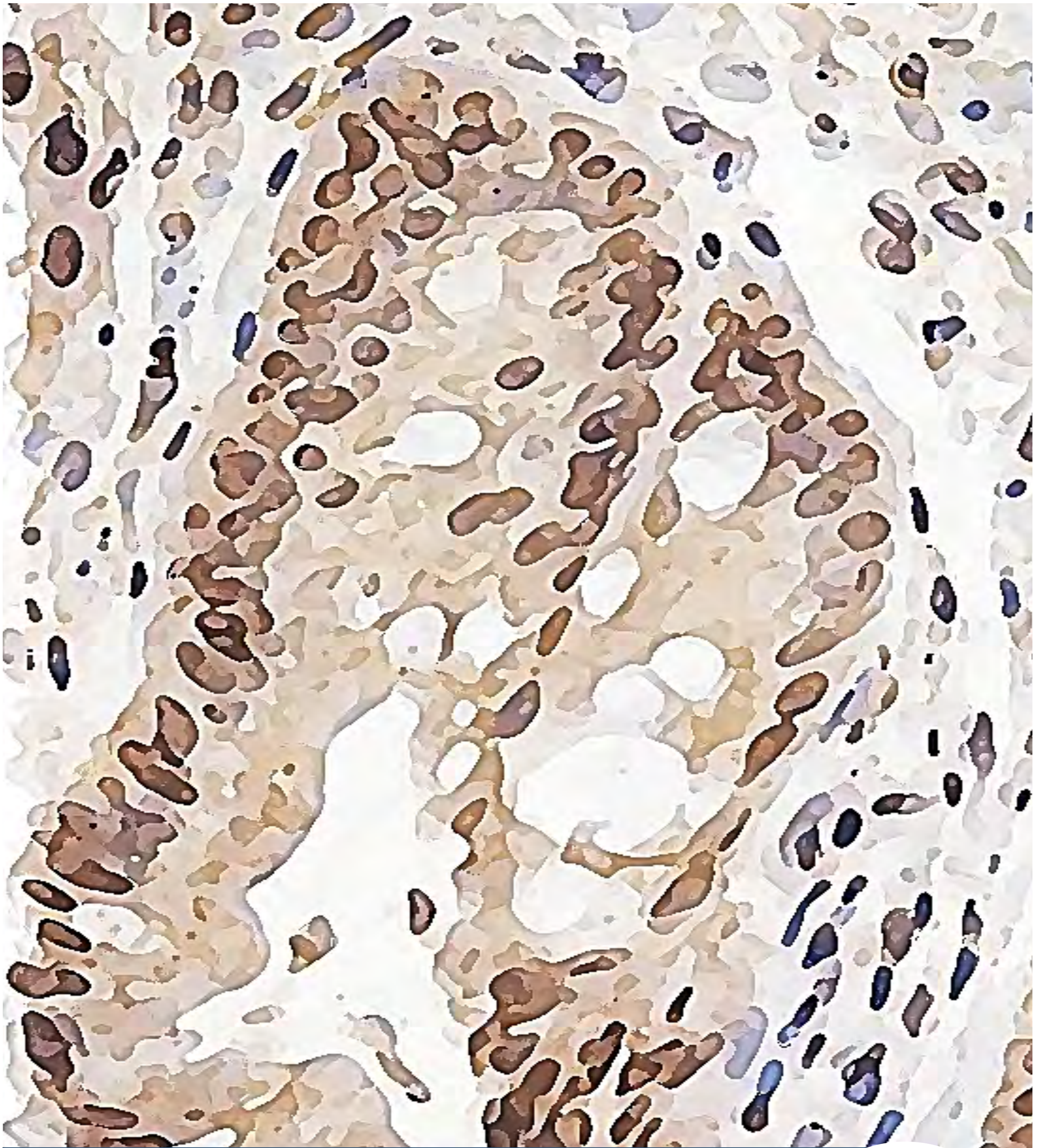

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Tesi Doctoral

Oriol de Barrios Barri

Memòria presentada per Oriol de Barrios Barri per a optar al grau
de Doctor en Biomedicina per la Universitat de Barcelona

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Aquesta Tesi Doctoral s'ha dut a terme a les instal·lacions de l'Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) i de la Facultat de Medicina (Universitat de Barcelona), sota la direcció del Dr. Antonio Postigo i del Dr. Antoni Castells

Programa de Doctorat en Biomedicina 2011-2016

El doctorand

Els directors

Oriol de Barrios Barri

Dr. Antonio Postigo

Dr. Antoni Castells

Novembre 2016

Universitat de Barcelona

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"Qui experimenta i no sap què està buscant,

mai podrà comprendre el que troba"

Claude Bernard

"Mai he conegut ningú tan ignorant

que no es pugui aprendre res d'ell"

Galileo Galilei

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Al meu pare,

"The words of the prophets are written on the subway walls
and tenement halls, and whispered in the sounds of silence"

Semblava que no arribaria mai aquest dia... sempre que hi pensava, el veia molt llunyà, però ara ja si, sembla que s'acosta el final d'aquesta etapa i em poso a escriure una de les darreres coses pendents, però no per això menys importants, com són els agraïments de la tesi. Sens dubte, mirar enrere i recordar tota la gent que m'ha ajudat a arribar fins aquí és molt especial i, segons com, fa una mica de impressió pensar-hi. Ja d'entrada demano disculpes per tota aquella gent a qui amb unes quantes paraules no li podré agraïr prou tot el que m'ha aportat durant aquests anys i, com que segur que em passarà, també demano perdó a tothom de qui m'oblidi en aquest apartat (no serà intencionadament).

En primer lloc, crec que haig de començar pels de casa... gràcies als meus pares per tantes coses que no puc ni enumerar. Són ells els que em van transmetre des de petit la importància d'estudiar i de voler aprendre coses noves, de tenir sempre alguna inquietud més enllà del dia a dia. Suposo que quan vaig decidir estudiar Farmàcia, van pensar que al cap de cinc anys tindrien algú que els ajudés i que agafaria el relleu del "negoci familiar". Però, tot i que potser els sorprengués, no em van posar mai cap pega ni cap impediment quan vaig decidir dedicar uns quants anys a la recerca i a fer una tesi doctoral. Segurament han estat més anys dels que creien llavors... En especial vull donar les gràcies a la meva mare i mostrar-li la meva admiració per com ha estat capaç de tirar endavant aquests darrers quatre anys, amb els moments difícils que ha hagut de viure, sempre donant-nos suport al meu germà i a mi perquè poguéssim continuar amb els camins que havíem triat. Gràcies també a l'Agus, el futur xef de la casa que, tot i que a vegades m'hagi posat dels nervis, suposo que també em fa entendre que m'haig de prendre les coses amb més filosofia. I també, indubtablement, en aquest moment penso (i molt) en el meu pare, que estaria molt content de veure que el camí que ell tot just va veure iniciar, ara té un bon final. Ell sempre deia que, de petit, no volia estudiar Farmàcia, i al final és just el que va acabar fent... i a mi, em va passar una mica el mateix. Si m'he dedicat a això suposo que deu ser perquè alguna cosa se'm va quedar de tant veure'l fer fórmules magistrals al laboratori de la farmàcia!

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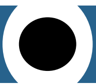
ABBREVIATIONS

ACF	aberrant crypt foci
APC	adenomatous polyposis coli
AOM	azoxymethane
BM	basement membrane
BRAF	b-raf proto oncogene
BRG1	brahma-related gene-1
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDKN2B	cyclin-dependent kinase inhibitor 2B
CIN	chromosomal instability
CRC	colorectal carcinoma
CtBP	C-terminal binding protein
DKK	dickkopf
DSS	dextran sodium suplhate
E-cadherin	epithelial-cadherin
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
EMT-TF	EMT-inducing transcription factor
FAP	familial adenomatous polyposis
Fz	frizzled receptor
GLB1	galactosidase β1
GSK-3β	glycogen synthase kinase - 3β
H2AFY	H2A histone family member Y
HD	homeodomain
HDAC	histone deacetylase
HuR	hu-antigen R
KRas-MAPK	kirsten rat sarcoma oncogene – mitogen-activated protein kinase
LAMC2	laminin subunit gamma 2
LEF	lymphoid enhancer binding factor 1
LRP	low-density lipoprotein (LDL)-related receptor protein
MDM2	mouse double minute 2, human homolog

MET	mesenchymal-to-epithelial transition
MEF	mouse embryonic fibroblast
miR-200	microRNA-200
MMP	matrix metalloproteinase
MT1-MMP	membrane type-1 matrix metalloproteinase
MTOB	2-keto-4-methylthiobutyrate
MSI	microsatellite instability
N-cadherin	neural-cadherin
NFκβ	nuclear factor κβ
p300	E1A binding protein p300
p/CAF	p300/CBP-associated factor
PAI-1	plasminogen activator inhibitor-1
PAS	plasminogen activator system
Rb	retinoblastoma-associated protein
SA-β-gal	senescence associated-β-galactosidase
SAHF	senescence associated heterochromatin foci
SASP	senescence associated secretory phenotype
sFRP	secreted frizzled-related protein
SID	smad interacting domain
TCF4	transcription factor 4
TGFβ	transforming growth factor β
TMA	tissue multiple array
TP53	tumor protein p53
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
Vn	vitronectin
Wnt	wingless-type MMTV integration site family
Wnt3a	wingless-type MMTV integration site family, member 3a
ZEB	zinc-finger enhancer-binding protein
ZFC	zinc finger cluster

INTRODUCTION



I - INTRODUCTION

Cancer is currently one of the biggest worldwide public health challenges and a major cause of mortality, accounting for more than 14 million new cases per year and more than 8 million related deaths (1). Despite all the recent data and progresses obtained due to cancer research, the rate of death associated to metastasis still stands as it was two decades ago, at 90% (2, 3).

1. Cancer: General Hallmarks

Cancer is a multistep disease driven by an interconnected myriad of cellular events. Tumor cells undergo a multistep process that triggers its ability to invade and, later on, metastasize. The basics of these mechanisms are mainly shared among different types of solid tumors (4) and they resemble the embryonic development and tissue repair processes (5, 6). However, despite the wide variety of cancer types and the diversity of molecular mechanisms that can be potentially deregulated in every single cancer cell, Hanahan and Weinberg have identified eight specific traits that are common to all tumor cells, which are: cell death resistance, sustained proliferative signaling, evasion of growth suppressors, metastasis and invasion activation, replicative immortality, angiogenesis induction, cellular metabolism deregulation and avoiding immune destruction (7, 8). They also included two consequential traits that allow the acquisition of all the previously defined hallmarks, thus enabling malignant cell transformation (tumor-promoting inflammation and genomic instability), making a total of ten hallmarks common to all (or widely most) types of cancer (8) (**Figure 1**). In collaboration with Dr. B. Györffy, we have recently established guidelines for the assessment of cancer hallmarks (9).

The sequence of cellular events that drive cancer metastasis begins with the detachment of epithelial cells and an initial local invasion, followed by an intravasation into close lymphatic vessels, which allows the cellular transit through body lymphatic system or hematological circulation, leading to an extravasation and settling in a distant organ (10). The most relevant molecular facts that take place during this so-called metastasis cascade have been largely described in literature (3, 6, 11, 12).

The main processes that primary tumors undergo are the following: ***a)*** <u>Disruption of basement membrane (BM):</u> BM is an organized matrix of glycoproteins and proteoglycans that enfolds epithelial and endothelial cells. Its proteolytic disruption enables tumor cells to initiate invasion and metastasis (13). ***b)*** <u>Poteolysis and remodelation of extracellular matrix (ECM)</u>: The degradation of the extracellular matrix that surrounds a specific tumor is essential for cancer cells to invade and intravasate in lymphatic or blood vessels (14). ***c)*** <u>Apoptosis resistance and survival</u>: Tumor cells become resistant to the apoptosis induced by loss of cell-to-cell and cell-ECM contacts (15). In this sense, it is also critical that a proportion of the ECM is maintained, since it helps tumor cells to survive by preventing the entrance in apoptotic programs (4, 6). ***d)*** <u>Proliferation</u>: Once ECM is completely or partially degraded, the cancer cells in a primary

tumor face a new microenvironment, in absence of contact with other epithelial cells and the ECM (11). In this context, they require proliferation signals from the surrounding stroma, mainly secreted by cancer associated fibroblasts (CAFs) (16). ***e)*** Immune escape: Invading cells of some cancers develop an intrinsic mechanism to escape from the tight control of the immune system, which has been designated as "immune escape" (17). ***f)*** Loss of cell-to-cell contacts: During the invasive and metastatic processes, tumor cells modify their adhesion preferences. Epithelial cells, previously attached to their neighbors, interact with stromal cells such as endothelial cells or fibroblasts. The cell adhesion molecules (CAMs) involved in this process can be classified in three main families (integrins, immunoglobulins and cadherins) (18). ***g)*** Migration and motility: Epithelial cancer cells need to disseminate in order to reach either lymphatic or hematological circulation and distant organs where settle. A collective cell movement constitutes an effective strategy in their dissemination through the organism (6).

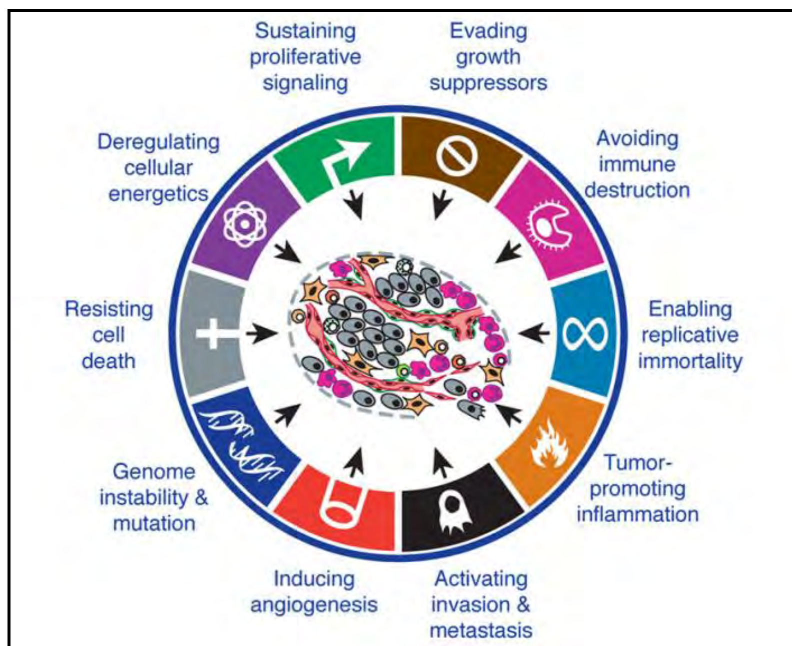


Figure 1 – The Hallmarks of Cancer. *The figure displays the eight hallmarks of cancer proposed by Hanahan and Weinberg in 2000 and 2011, as well as the enabling characteristics. Taken with permission from (8).*

Many of these outcomes occur in the context of the so-called epithelial-to-mesenchymal transition (EMT), a molecular, phenotypic and functional reprograming of cancer cells, that endows them with a more aggressive and invasive phenotype (19-22). Of note, cells undergoing EMT are capable of entering into blood circulation, whereas lymphatic metastases still retain epithelial features (23, 24). Remarkably, EMT not only influences the metastatic capacity of epithelial cancers, but it also occurs during dissemination of non-epithelial tumors (25).

2. Epithelial-to-mesenchymal transition (EMT)

The EMT is a critical mechanism, shared by cells from distinct epithelial tumors, through which invading cells lose their intercellular junctions and cell-to-cell contacts, as well as their apico-basal polarity, in order to acquire a more motile and mesenchymal phenotype (21, 25, 26). EMT was firstly described in the context of embryonic development (26), but its role in tumor progression and invasiveness has been now unveiled and broadly investigated. During the EMT, cells lose the expression of epithelial markers like E-cadherin, occludins and cytokeratins; apico-basal polarity markers (Crumbs3, PATJ, HUGL2) and tight cell junctions (claudin-7, JAM1) and acquire the expression of mesenchymal markers such as vimentin or N-cadherin (20, 22, 27, 28). In fact, EMT is reversible, and the opposite process (mesenchymal-to-epithelial transition, MET) involves the recovery of epithelial hallmarks (20, 27). Moreover, intermediate states between epithelial and mesenchymal phenotypes have been also described in mediation of organ fibrosis and dissemination of tumor cells (22, 29) (**Figure 2**).

E-cadherin is involved in the maintenance of *adherens* junctions between epithelial cells (26, 30), thereby acting as a tumor suppressor (18). Although it can be regulated by epigenetic and post-translational mechanisms, its most relevant regulation is at transcriptional level (30, 31). In fact, upon E-cadherin loss, β-catenin is released from the epithelial adhesions and is translocated into the nucleus where it activates the expression of EMT-inducing factors (19). Therefore, it is considered a tumor suppressor and its loss is associated to a poorer outcome (18, 30).

Several transcription factors have been described to repress E-cadherin, although not all of them regulate its expression by direct binding. The transcription factors that trigger EMT initiation mainly through binding to the E-cadherin gene promoter – so-called EMT-inducing transcription factors (EMT-TFs) (20, 21, 32, 33) – are grouped in distinct families: two families of zinc finger proteins (ZEB and Snail) and the Twist family of basic/helix-loop-helix (bHLH) factors (25). Beyond these families, other proteins such as Goosecoid, HMGA2, E2-2A, E2-2B or E47 (also called TCF3) repress E-cadherin transcription as well, through direct binding to its promoter (20, 34-36). The EMT-TFs have acquired more relevance, since their classical functions as promoters of cell motility and tumor invasive capacity have been expanded and different roles in the regulation of other cancer hallmarks have been reported (21, 37). In fact, they are considered independent prognostic factors for tumor aggressiveness, recurrence and poor patient survival (21). Thus, EMT-TFs have gained importance as potential therapeutic targets in cancer treatment.

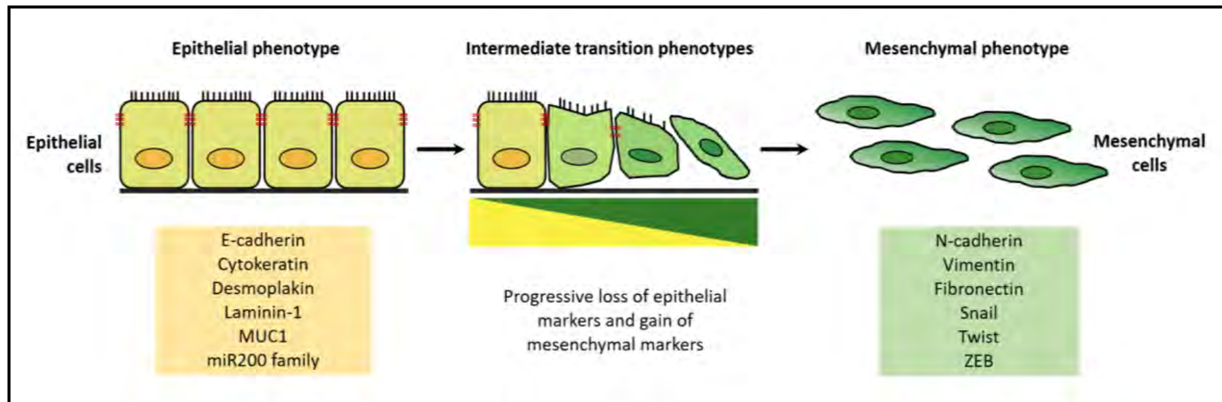


Figure 2 – Epithelial-to-Mesenchymal Transition (EMT). *The illustration shows epithelial cells progressively undergoing EMT transformation. The common markers used to define each state are specified. Intermediate phenotypes display both epithelial and mesenchymal features and express a mixture of markers from both states. Adapted with permission from (25).*

The regulation of EMT-TFs expression depends on the activation of several cellular signaling pathways. For instance, TGFβ, Wnt, Notch, hypoxia, Ras-MPAK, growth factors (such as EGF or VEGF) and NFκβ signaling routes induce EMT-TFs (26, 38). The secreted factors involved in activating these cascades are commonly released by stromal cells during developmental and disease processes. The relevance of each single pathway depends on tissue and cell context (19).

The Twist family is comprised of two members, Twist1 and Twist2, which bind to DNA through a bHLH domain. Binding of Twist proteins to regulatory regions of target genes can result either into activation (N-cadherin) (39) or repression (E-cadherin) (32). Several other transcription factors and co-factors (i.e. Runx2, NFκβ or p300/CAF) cooperate with Twist proteins in the regulation of gene expression. For instance, Twist1 binds to PRC1 and PRC2 polycomb repressor complexes at the E-cadherin promoter (40). In addition, Twist factors are upregulated during tumor progression, compared to its levels in benign neoplasia or healthy organs. It was demonstrated that knocking down Twist1 in breast cancer cells impairs metastasis in a xenograft model, whereas it does not affect the formation of a primary tumor (41). Some studies have shown that the joint expression of Twist and Snail in distinct types of cancer can involve an additive negative effect on patient survival (42, 43), suggesting that despite displaying separate roles in cancer progression, they can collaborate in enhancing tumor aggressiveness.

The family of Snail EMT-TFs is constituted by three members: Snail1 (or Snail), Snail2 (Slug) and Snail3 (Smuc). In this family, the domains that bind DNA regions in target genes promoters correspond to zinc-finger clusters, which are located at the C-terminal end of the protein (44-46). Snail proteins are considered the key factors in the initiation of EMT process, that drives the initial steps of tumor invasion and metastasis, since they repress not only E-cadherin transcription but also other epithelial markers like desmoplakin, claudins, occludins,

Mucin-1 and cytokeratins (36). In parallel to this loss of epithelial hallmarks, Snail factors also promote the acquisition of mesenchymal properties by the activation of genes that mediate cell migration (36, 47, 48). In the regulation of some genes, the action of a unique factor is not sufficient. For instance, in order to repress the vitamin D receptor, a good-prognostic marker in colorectal carcinoma (CRC), the cooperation between Snail1 and Snail2 is required (49, 50).

However, the importance of EMT in driving cancer metastasis has been recently questioned (51, 52). In a mouse model for spontaneous breast carcinomas, it was described that EMT is dispensable for the appearance of lung metastasis. Conversely, it highlighted the relevance of EMT in promoting chemotherapy resistance, enabling cell survival in already constituted metastasis (51). In parallel, another study reported that the inhibition of EMT-TFs Snail1 and Twist1 caused no alteration in the metastatic capacity of pancreatic carcinoma cells (52). Nevertheless, these studies have not ruled out the role of other EMT-TFs, including the ZEB family, in metastasis.

3. ZEB family of EMT-TFs

The ZEB family in mammalians is constituted by two members: ZEB1 (also known as δEF1) and ZEB2 (SIP1) (37). Regarding their structural organization, ZEB proteins comprise diverse independent domains that mediate the binding to DNA regions and to other cofactors, which cooperate in their repression and activation functions, but that are unable to bind DNA directly. Both ZEB1 and ZEB2 present two zinc finger clusters (ZFC), located at each protein end: N-terminal ZFC and C-terminal ZFC. These domains bind to regulatory regions of target genes containing the 5'-CANNT(G) DNA-binding sequence (where N states for either C or G nucleotide), which have been defined as ZEB boxes (E-box and E-box-like DNA sequences) (53-55) (**Figure 3**).

ZEB proteins mediate TGFβ signaling through cooperation with activated Smad transcription factors (56, 57). This binding is feasible due to the presence of a Smad Interacting Domain (SID), located between the N-terminal ZFC and the so-called homeodomain (HD). Other cofactors can bind to the N-terminal domain, depending on the cellular context and the genes targeted (**Figure 3**), such as p300 and p/CAF histone acetyltransferases (27), which cooperate in transcriptional activation. This same region can interact with other cofactors involved in chromatin reorganization. ZEB1 binds as well one of the members of the SWI/SNF remodeling complex, BRG1 (58), whereas ZEB2 binds NuRD (59) **(Figure 3)**. Both BRG1 and NuRD function as transcriptional corepressors.

Another important region for the binding of cofactors is placed between the HD and the C-terminal end. This region contains several motifs for the association with C-terminal Binding Proteins 1 and 2 (CtBP1 and CtBP2) cofactors and is referred to as CtBP Interacting Domain (CID). Moreover, ZEB1 can also interact through the C-terminal domain with transcription factor TCF4, effector of Wnt signaling and the Hippo signaling coactivator YAP1 (60, 61). In the specific case of YAP1 protein, it can also associate to the N-terminal ZFC (61) (**Figure 3**).

Due to the complex structure and diversity of available domains, combined with the variety of cofactors, ZEB proteins can either act as transcriptional repressors or activators, depending on the associated cofactors (27, 37). Their role as transcriptional repressors occurs via competition and displacement of other activating factors (62), or by direct binding to an E-box DNA sequence, which is the most common mechanism (63, 64). The transcriptional cofactors bound to ZEB proteins are recruited in a promoter-specific manner. Even though, the specific identity of the concrete corepressors or coactivators that are involved in transcriptional regulation has been only investigated for a few genes.

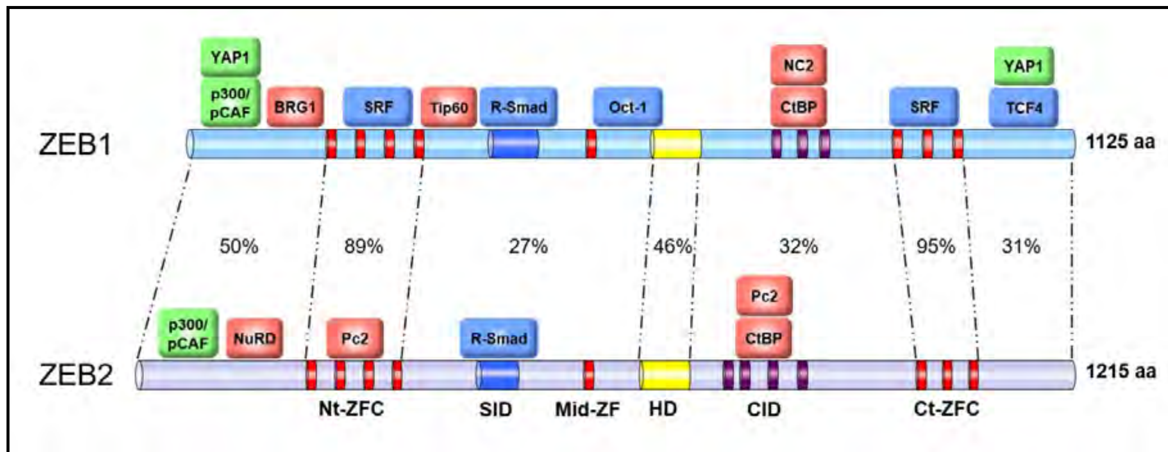


Figure 3 – Structure of ZEB transcription factors. *Schematic diagrams of the structure of ZEB proteins and the most relevant cofactors involved in their activity as transcriptional regulators. Coactivators are depicted in green, whereas corepressors are shown in red. Other transcription factors are labeled in blue. Nt-ZFC: N-terminal Zinc Finger Cluster; SID: Smad-Interacting Domain; Mid-ZF: Middle Zinc Finger; HD: homeodomain; CID. CtBP-Interacting Domain; Ct-ZFC: C-terminal Zinc Finger Cluster. Adapted with permission from (37).*

In contrast, the activity of ZEB factors as transcriptional inducers involves the interaction with coactivating proteins. The most common are the histone deacetylases p300 and p/CAF, whose binding to ZEB factors triggers a synergistic effect when TGFβ signaling is simultaneously activated and Smad proteins are also recruited (56, 57). A clear example of this cooperation is the Vitamin D Receptor gene, directly induced by ZEB1 (65). This cooperative effect also occurs in the case of Wnt signaling, where ZEB1 is converted into an activator upon binding to TCF4 (60).

The ZEB family of transcription factors, especially ZEB1, presents the strongest inverse correlation with E-cadherin expression among the three families described (66). This fact can be partially explained by the induction that both Snail and Twist proteins exert on ZEB factors (67). Snail1 induces ZEB1 through either direct transcriptional or post-transcriptional mechanisms (68). Even more, Snail1 stabilizes Twist1 protein levels, which also binds to ZEB1 promoter and enhances its expression. High throughput gene profile approaches indicate that

Snail1 and Twist1 function upstream of ZEB1 (69). Oppositely, ZEB1 acts upstream of other EMT-TFs and induces their expression, mainly that of Twist1 (70). The expression and functional coincidence of the EMT-TFs families suggests that the specificity and role of each EMT-TF may be restricted to spatial and temporal levels during tumor progression (21). As an example, ZEB2 functions as a tumor suppressor in melanoma cells, oppositely to the tumor-promoting role of its homologue ZEB1 (71).

Since ZEB transcriptional activities can be modulated indirectly, the interplay between them and the miR-200 family of microRNAs constitutes a relevant mechanism (72). This family of non-coding microRNAs, that abolishes the mesenchymal phenotype and is key in maintaining the epithelial features of cells, is transcriptionally repressed by ZEB factors (73). Thus, the targets of the miR-200 family are indirectly activated by ZEB1 and ZEB2, since their post-transcriptional repressors are inhibited. Conversely, the mRNA transcripts of ZEB1 and ZEB2 are both inhibited by miR-200 family microRNAs. Moreover, ZEB factors are involved in other regulatory loops with microRNAs, such as miR-183, miR-203 or miR-205 (21, 74, 75).

Over the last decade, a considerable amount of data has demonstrated that ZEB1 and ZEB2 participate in other processes of cancer development, beyond the induction of EMT. In fact, ZEB proteins are driving tumor progression by being involved in some of the cancer hallmarks proposed by Hanahan and Weinberg (7, 8):

- <u>Acquisition and maintenance of stemness properties</u>: ZEB1 confers stemness to cancer cells through the repression of several microRNAs that target regulators of stemness (such as *BMI1*, *KLF4* or *SOX2*) (74, 76). Moreover, it has been defined as one of the signature genes that determine the stem-like subtype of CRCs (77). In a model of pancreatic cancer, the acquisition of a mesenchymal phenotype and stemness properties mediated by ZEB1 precedes the formation of a primary tumor (78). However, in prostate cancer models, the expression of *ZEB1* appears to be independent of the tumor-initiating properties exerted by stem cells (79).

- <u>Resistance to apoptosis</u>: ZEB1 represses the transcription of pro-apoptotic cell cycle regulators, such as TAp73, impairing cell cycle arrest (80). In addition, it activates the expression of anti-apoptotic genes such as *MCL1* or *BCL2*, and represses pro-apoptotic ones like *PUMA*, *NOXA* or *BAX* (81). Instead, ZEB2 exerts a cell cycle-independent role, promoting survival through the PARP and pro-caspase 3 cleavage inhibition (82).

- <u>Angiogenesis promotion</u>: ZEB1 and ZEB2 are commonly found overexpressed by endothelial cells in the tumor stroma. ZEB2 has been directly involved in mediating angiogenesis (83). Moreover, ZEB1 upregulates vascular endothelial growth factor (VEGF), promoting angiogenesis (84).

- <u>Chemotherapy and radiotherapy resistance</u>: It has been widely reported that EMT is potentiated by resistance to DNA-damaging drugs. Likewise, ZEB1 confers resistance to treatment in several types of tumors, such as pancreatic or colorectal, as well as in

mantle cell lymphoma. In parallel, the presence of ZEB2 decreases the sensitivity of bladder cancer and squamous carcinomas to treatment (21, 37, 74, 81, 82).

- Cellular senescence inhibition: ZEB1 represses some senescence-associated genes (85). For example, it inhibits *CDKN1A*, *CDKN2A* and *CDKN2B* genes (encoding for p21, p16 and p15 proteins, respectively), effectors of TGFβ pathway that are involved in senescence response (56, 86, 87). Moreover, earlier evidence showed that mouse embryo fibroblasts (MEFs) derived from either heterozygous or homozygous deletion of ZEB1 undergo senescence earlier than wild-type MEFs, which display a normal proliferation rate (87). Interestingly, its counterpart ZEB2 has an opposite role, since it represses TERT and induces senescence (88).

4. Role of Senescence during Cancer Progression

During the process of epithelial cells transformation into malignant and tumorigenic cells, they need to overcome several processes that trigger programmed cell death or growth arrest. In fact, both cell death resistance and maintained proliferation are included among the reported hallmarks of cancer (7). Remarkably, ZEB factors are involved in growth arrest impairment (81, 82). Cellular senescence, described as an irreversible arrest of cell cycle, constitutes a barrier that cells need to skip in order to fulfill oncogenic transformation (89-91). Thus, senescence is considered a tumor suppressive mechanism and mouse models that are unable to trigger senescence response present higher tumor formation (92, 93).

The main tumor suppressors (i.e. p53, p21, p16 or pRb) mount a senescence response upon receiving oncogenic stimuli, promoting an irreversible arrest of proliferation (94). Indeed, the establishment of senescence involves the activation of several cellular processes, such as chromatin modifications, DNA damage response and induction of autophagy (90). These events include the release of several cytokines, growth factors and proteases with inflammatory properties, that has been termed as the Senescence-Associated Secretory Phenotype (SASP) (91). This phenotype provides beneficial effects, such as tissue repair and regeneration, but some of its components promote chronic inflammation and activate a paracrine proliferation signaling, among other deleterious features (91, 93).

Despite being originated by distinct molecular processes, senescent cells share some common characteristics (**Figure 4A**) such as a larger size (even doubling basal size) and flat morphology (90, 91). Moreover, they use to accumulate lysosomal vacuoles, with an increase of senescence-associated lysosomal β-galactosidase activity (SA-β-gal), which can be used as a specific marker of senescent cells (95) (**Figure 4B**). In addition, the secretory phenotype that cells release during senescence acquisition implies the overexpression of several genes that can be used as biomarkers for this phenotype (96). These biomarkers include: cell cycle genes (e.g. *CDKN2A*, *CDKN1A*), chromatin remodeling proteins (HIRA, ASF1a, PML, HP1γ, macroH2A1) and secreted proteins (IL1-A, IL-6, IL-8, MMP1, MMP3, TGFβ1) (90, 96).

Among these markers, there are some genes that are responsible for remodeling the structure of chromatin, which is a typical trait of senescent cells (97). This remodeling involves a more condensed structure, that causes transcriptional activity silencing (98). In fact, referring to senescent cells, the regions of the genome that include those genes related to proliferation induction, are relocated to areas of transcriptional repression, defined as senescence-associated heterochromatin foci (SAHF) (97). The promyelocytic leukemia (PML) nuclear bodies mediate the assembly of SAHF by recruiting heterochromatin proteins in early senescence. The formation of these SAHF supports the cell cycle exit (99). Among the distinct SAHF markers, the macroH2A1 histone (encoded by *H2AFY* gene), recalls a special interest, since it renders a key role in gene silencing (100).

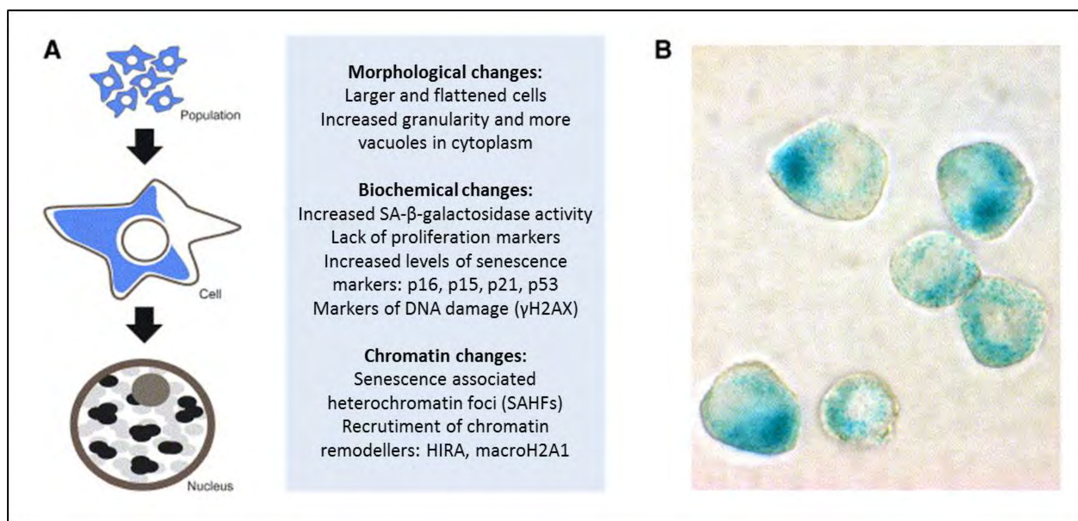


Figure 4 – Main features of cellular senescence. (A) *Scheme of cell population undergoing senescence. The main morphological, biochemical and chromatin modifications are listed.* **(B)** *Example of SA-β-gal staining in cancer cells. Taken with permission from (101).*

5. Importance of EMT and ZEB proteins in colorectal cancer

Colorectal cancer (CRC) ranks third among all types of malignancies in Western countries and approximately 1.5 million of new cases are annually diagnosed (102, 103). It is also the fourth cause of cancer-related death worldwide, accounting for almost 700,000 deaths every year. Particularly, metastatic colorectal cancer supposes a 5-year mortality rate over 90% (104). The factors that involve an increased risk of CRC appearance are diverse and present distinct origin. Some of the factors are modifiable and refer to behavioral individual matters. Instead, others depend on the existence of a genetic predisposition (103, 105).

The spontaneous CRC (including the ones associated to a previous familial history) accounts for 90-95% of all diagnosed CRCs. However, around 5% of CRCs have a genetic

hereditary cause. Among them, the most common is the Lynch syndrome, which accounts for a 3-5% of all CRC new cases. These patients have an increased risk of developing diverse types of cancer, but with the highest probability for colorectal affectation (106, 107). The familial adenomatous polyposis (FAP) is the second most frequent genetic syndrome involving appearance of a CRC. In this specific syndrome, the affected patients spontaneously develop hundreds of colorectal adenomas and an early age intervention is required (108, 109).

5.1 Molecular pathogenesis of spontaneous CRC

One of the most prevalent mutations that occur in more than 70% of CRC cases is in the *APC* gene. This alteration occurs as an early event in the multistep process previously described (103, 110) and is commonly driven by a biallelic inactivation of this gene (**Figure 5**), which in wild-type condition plays a key tumor suppressor role. The mutation and subsequent loss of function of APC triggers the activation of Wnt signaling pathway (111, 112), which will be discussed in the following section. After this initial step, the colonic crypts can suffer a slight dysplasia, leading to the appearance of an early adenomatous lesion (also called aberrant crypt foci, ACF) that can evolve into a larger adenoma in case of acquisition of new mutations or phosphorylation in the small GTPase protein KRAS (113-115). At this stage of the adenoma-carcinoma progression, the mutation of *BRAF* occurs at much lower frequency (**Figure 5**). Remarkably, *KRAS* mutations confer resistance to anti-epidermal growth factor therapy and associate to worse prognosis.

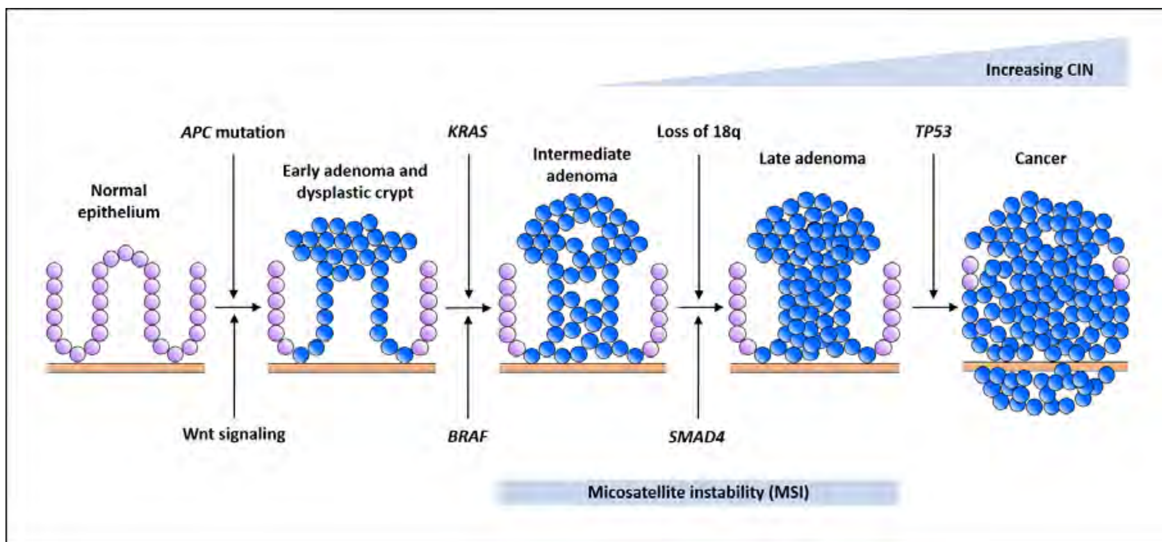


Figure 5 – Molecular pathogenesis of CRC adenoma-carcinoma progression. *Simplified model of the transition from early adenoma lesions to late colorectal carcinoma, aligned with most common genetic alterations driving each step. Model adapted with permission from (114).*

Later, an intermediate adenoma can suffer further promoting genetic alterations, such as the deletion of a fragment of chromosome 18, that includes the *SMAD4* gene, whose loss is associated to poor patient outcome (116). The definitive triggering hit of the progression from late adenoma into colorectal carcinoma is the alteration of the tumor suppressor *TP53* gene, associated to weak therapeutic response (117). These genetic losses are a clear reflection of chromosome instability (CIN). CIN may appear at the initiation of CRC, although it acquires more relevance in later stages (118). It must be also noted the relevant role of microsatellite instability (MSI), that commonly occurs at late adenoma stages (114) **(Figure 5)**.

5.2 Histological and molecular classifications of CRCs

Classification of CRCs depends on distinct histological and pathological criteria (119-121). The mainly used stages are invasion depth (T), affectation of lymph nodes (N) and presence of distant metastasis (M). Thus, the CRC classification is named as TNM (Tumor, Nodes and Metastasis) staging system (120). Stage 0 corresponds to an early adenoma step and further stages range from I to IV, where stages I and II include increasing grades of invasion depth. Stage III is assigned to patients with affected lymph nodes. Finally, stage IV is assigned to distant metastasis cases (103, 121). This classical CRC staging criteria provides worthy information and data on prognosis, but fails at predicting therapeutic response. Thus, the convenience of using this classification has become a matter of debate in the last decade (122, 123). In order to improve the prognostic capacity and the ability to determine patient's outcome, several groups have proposed new CRC classification systems, based on gene expression profiles (77, 124, 125).

The firstly proposed molecular CRC subtype classifications were published by De Sousa *et al.* and Sadanandam *et al.* (77, 125). The study from De Sousa *et al.* assigns the poorest survival prognosis to a specific subtype characterized by poorly differentiated cells and upregulation of EMT-associated genes (125). This poor-prognosis group is related to a specific type of precursor lesions, known as sessile serrated adenomas, where an aberrant TGFβ signaling promotes the induction of *ZEB1* (126). In parallel, Sadanandam *et al.* reported that the so-called "stem-like" subtype correlated with the worst disease-free survival. Remarkably, this subtype accounts for the strongest Wnt signaling and its gene signature contains *ZEB1* as the biomarker for CRC diagnosis and classification (77).

In a summary of these previous works, recent studies have connected the data from all publications and grouped the different subtypes of CRC in four consensus subtypes, characterized by specific cancer hallmarks. (127, 128). Once again, the mesenchymal subtype provides a worse overall and relapse-free survival than the other subtypes. This group of tumors displays high expression of ZEB factors (127). As a global conclusion from these new molecular CRC classification systems, CRC subtypes with an increased expression of mesenchymal phenotype markers (especially ZEB1) and active Wnt signaling present a poor overall and relapse-free survival. Thus, the following sections of this Introduction chapter will focus on the role of Wnt pathway in CRC and its connection with ZEB proteins.

6. Wnt signaling pathway and its role in CRC progression

The Wnt signaling pathway is involved in regulating several physiological processes during embryonic development, as well as in adult tissue homeostasis. The effects triggered by this molecular cascade include stimulation of cellular proliferation through mitosis activation, overcome of cellular growth arrest and death and cellular commitment towards specific differentiation. In fact, Wnt signaling is involved in development and intestinal crypts proliferation (129). However, an aberrant deregulation of the pathway leads to the appearance of diverse pathologies, particularly cancer. Once again, as in the case of EMT process, cancer arises from the aberrant deregulation of a developmental pathway (130-133). The participation of Wnt ligands and β-catenin is key in the so-called canonical pathway. Even though, other potential signaling cascades coexist with it and have been described as non-canonical pathways (134).

In homeostatic conditions, mammalian cells keep the cytoplasmic β-catenin at low levels through a β-catenin degradation complex containing glycogen synthase kinase-3β (GSK-3β), Axin2 and APC proteins, that drives β-catenin to proteasomal degradation after being phosphorylated and ubiquitylated (132, 135) (**Figure 6**). When Wnt proteins are secreted to the extracellular medium, they bind to surface receptor complexes. These complexes are commonly constituted by a member of Frizzled (Fz) receptors and another member of the low-density-lipoprotein (LDL)-related receptor protein (LRP) receptors families. In general, the LRP receptors that interact with Wnt proteins are LRP5 and LRP6 (131, 133, 135). These receptors promote the intracellular signaling transduction (**Figure 6**).

Firstly, the Dishevelled (Dsh) protein is recruited to the Fz receptor, promoting the joint recruitment of Axin2 to the membrane receptor complex, through its interaction with LRP 5/6. Once both proteins have been recruited to the membrane, the function of the β-catenin destruction complex is impaired and the ability to degrade cytoplasmic β-catenin is lost. In consequence, the remaining cytoplasmic pool of β-catenin accumulates and translocates to the nucleus (132, 135). Finally, nuclear β-catenin physically interacts with the constitutive transcription factors lymphoid enhancer-binding factor / T cell-specific transcription factor (LEF/TCF) and modulates the transcription of Wnt target genes (132) (**Figure 6**).

The LEF/TCF factors are commonly associated to transducin-like enhancer protein (TLE, also named Groucho) in the nucleus, which represses transcription by recruiting histone deacetylases (HDACs) (137, 138). The nuclear translocated β-catenin displaces TLE from the TCF/LEF factors and recruits other transcription factors such as BCL9 or histone acetyltransferase p300 (135), promoting a transcriptional switch that converts LEF/TCF factors into activators (**Figure 6**). The induction of Wnt target genes promotes the activation of Wnt signaling cellular programs, such as proliferation, survival or cell fate determination. It must be also noted that some Wnt signaling activators or antagonists are included in the group of activated targets, providing an autonomous feedback control loop on the pathway (131, 132).

Importantly, the activation of Wnt signaling cascade inhibits the induction of cellular senescence, allowing tumor cells to continue proliferating (135). For instance, Wnt1 and Wnt2, prevent the appearance of a senescent phenotype in epithelial cells and in fibroblasts, respectively (139, 140). Additionally, some Wnt receptors have been implicated in senescence repression. Specifically, a role for Fz7 receptor in impairing apoptosis and growth arrest has been reported in CRC (141).

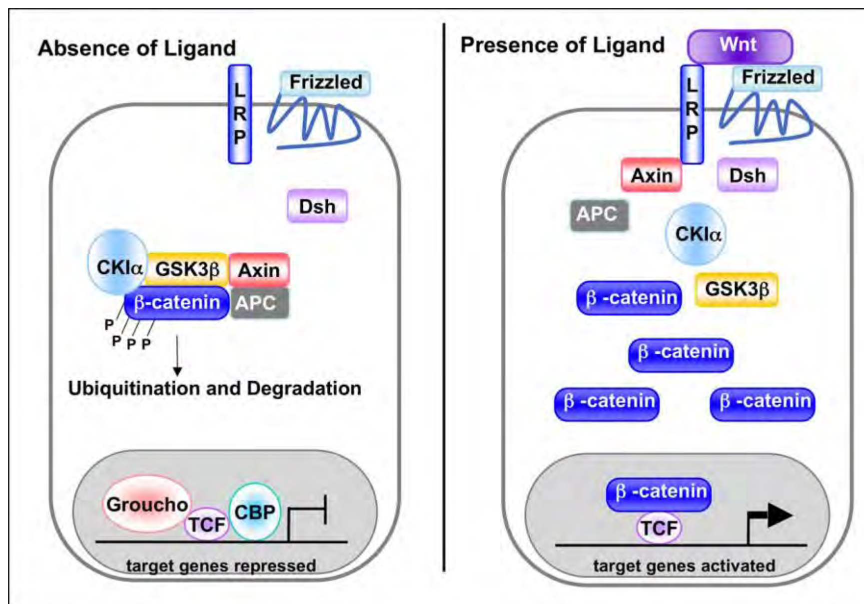


Figure 6 – Activation of canonical Wnt signaling. *The illustration shows the transduction of Wnt signaling. Left panel shows degradation of cytoplasmic β-catenin in absence of ligand. In right panel, binding of Wnt ligands to the receptor triggers Wnt signaling. Non-degraded β-catenin translocates to the nucleus, activating Wnt targets. Taken with permission from (136).*

In addition to the Wnt secreted proteins, other proteins can transduce Wnt signaling (131). For instance, leucine-rich repeat containing G-protein coupled receptors 4 and 5 (LGR4/5) have been identified to bind R-Spondin proteins at cell membrane (142, 143). These receptors are implicated in the Wnt signaling transduction, and are highly expressed in the proliferative stem cells of small intestine and colon, providing renewal of epithelial intestinal colon cells (129).

Several families of secreted proteins can act as Wnt antagonists and play a relevant role in pursuing a fine-tuned regulation of Wnt signaling, which is critical in the appropriate regulation of developmental events and prevention of pathologies. Some of the antagonists are transmembrane proteins (Shisa, Waif1, APCDD1 and Tiki1), while the others belong to families of secreted proteins. A total of 6 families have been reported up to date: secreted Frizzled-related proteins (sFRPs), Dickkopf proteins (DKKs), Wnt-inhibitory factor 1 (WIF-1),

Wise/SOST, Cereberus and insulin-like growth-factor binding protein 4 (IGFBP-4) (130, 131, 144). Some studies have reported a tumor suppressor role for WIF-1 and IGFBP-4 in osteosarcoma and prostate cancer, respectively (145, 146). However, the most deeply studied families and whose physiological role is best described are the sFRP and DKK ones.

The human family of sFRPs is composed of five members, sFRP1-5, all of them sharing an amino-terminal cysteine-rich domain (CRD), that exhibits high similarity with a specific region of the Fz receptors (147). Since Wnt proteins can recognize this domain and bind to Fz receptors, they can also interact with sFRP proteins. In this case, they are sequestered by sFRPs and become unable to trigger the Wnt pathway (144, 148). As expected for Wnt signaling antagonists, a tumor suppressor role has been reported for the sFRP family in a repertoire of carcinomas, where its expression is commonly downregulated (131).

The four members of the DKK family (DKK1-DKK4) harbor two distinct CRDs, at the amino-terminal and carboxy-terminal regions. The proteins of DKK family impair Wnt signaling cascade by high-affinity binding to the LRP5/6 receptors through the N-terminal CRD. This interaction was firstly reported for DKK1 and DKK2, although it is also shared by other family members (149, 150). The carboxy-terminal CRD motif of DKK1 and DKK2, but not DKK3 (151), binds to Kremen receptors. This interaction enhances the capacity of DKK1 to repress the transduction of Wnt signaling (**Figure 7**), being functional in developmental processes (152).

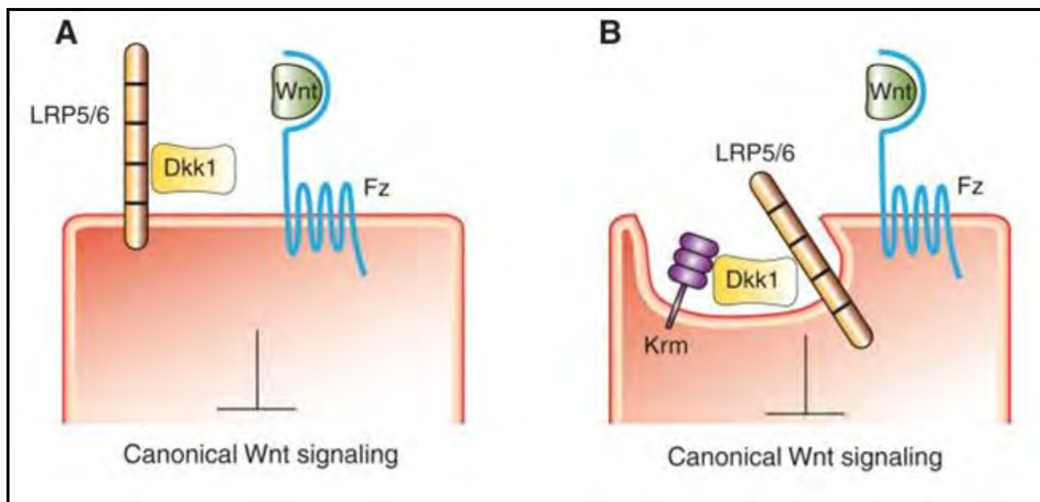


Figure 7 – Inhibition of canonical Wnt signaling by Wnt-antagonist DKK1. (A) *Binding of DKK1 to LRP5/6 receptor impairs the assembly of Fz-LRP5/6 complex.* **(B)** *Simultaneous interaction of DKK1 with both Kremen and LRP5/6 surface receptors triggers the endocytosis of LRP5/6, thus preventing Wnt signaling transduction. Taken with permission from (131).*

Consequently, regarding the potential interaction with cell surface receptors, DKK proteins mainly block the Wnt pathway by preventing the association of Wnt secreted proteins to the LRP5/6 receptors. In addition, they dissociate the Fz-LRP complexes that trigger Wnt

signal transduction (150) (**Figure 7A**). In the case of coexpression of Kremen receptors and particularly referring to DKK1, its joint interaction with LRP5/6 and Kremen promotes endocytosis of both receptors, which are eliminated from the membrane (144, 151) (**Figure 7B**). Interestingly, the expression of DKK1 is itself activated by β-catenin/TCF complex, conforming a pathway regulatory feedback loop (153, 154).

Among a variety of roles, DKK proteins play key functions in cell differentiation control during embryonic development and bone formation (131). In cancer field, DKK family members are inactivated by epigenetic silencing in some specific types of cancer, such as CRC (144, 155). DKK1 has also been reported to mediate chemotherapy-induced apoptosis (156) and programmed cell death (157). Likewise, DKK2 and DKK3 decrease cell viability and proliferation (144, 158).

6.1 Wnt signaling in CRC

Genetic APC alterations were initially found in FAP patients, where both alleles are inactive (111, 159). Apart from genetic APC alterations, other mutations in the Wnt pathway components can provoke the accumulation of cytoplasmic β-catenin and, in consequence, the uncontrolled induction of Wnt targets. For instance, Axin2, another member of the destruction complex, is mutated in one allele in some unusual CRC cases with intact APC (160). The mutant form of Axin2 adopts a dominant-negative function, turning into a β-catenin stabilizing factor (161). Interestingly, β-catenin itself can suffer point mutations in the specific motifs that bind to GSK3β, preventing its proteasomal degradation (162). Any of these mutations imposes aberrant Wnt transduction and initiates colon cells malignant transformation (163). The APC *multiple intestinal neoplasia* (APC*min*) mouse model resembles FAP syndrome and has been used as an animal model for FAP. This murine model triggers the formation of up to 100 adenomas in the small intestine, rather than in the colon, reducing the lifespan of APC*min* mice to an average of 150 days due to intestinal occlusion (164).

Wnt activity does not remain constant throughout the distinct CRC areas. For instance, central areas of the tumor present an epithelial phenotype and lack of nuclear β-catenin, which is confined to the membrane and the cytoplasm. Conversely, dedifferentiated CRC cells at the invasive front display mesenchymal features, which correlate with an increased nuclear β-catenin (165). Of note, the microenvironment surrounding tumor cells plays a relevant role in the profile of Wnt activation across the colorectal carcinoma tissue, since it contributes to the modulation of intracellular β-catenin localization (165). At the nucleus, β-catenin associates to TCF4, the most prominently expressed TCF factor in intestinal cells (166). The complex triggers a genetic program that drives proliferation of progenitor cells (167). Even though, some of the TCF4/β-catenin targets, such as the Ephrin B (*EPHB*) family genes, are only expressed during the initial steps of colon cancer, and decrease during CRC progression (168).

The pattern of expression of Wnt target genes in CRC reflects a gradient of Wnt activity between the differentiated tissue and the invasive front (165). The targets that are differentially expressed in tumor central areas are required for the tumor formation process

itself and include *CCND1* (cyclin D1), *ETS2*, *MYC* or *CLDN1* (claudin 1). Instead, the expression of invasion-related Wnt-target genes is turned off in tumor center and is only activated at invading cells. Genes such as *PLAU* (uPA), *PLAUR* (uPAR), *LAMC2*, *MMP7*, *TNC* or *ENPP2* are included in this second group and promote CRC cells invasion and metastasis (169).

6.2 Wnt-mediated activation of ZEB1 in CRC

Among the distinct EMT-TFs, ZEB1 recalls the highest relevance in the regulation of the equilibrium between both epithelial and mesenchymal states in CRC. In the central regions of primary colon tumors, ZEB1 displays a low expression, but it is induced at cells located at the interface between tumor tissue and microenvironment (i.e. invasive front), promoting the mesenchymal phenotype that confers invasive properties (170) (**Figure 8A**). Conversely, ZEB1 expression needs to be downregulated in tumor metastasizing cells, that require an epithelial switch and a redifferentiation in order to settle and grow (28, 170). The induced expression of ZEB1 at tumor front of CRCs promotes several features that are involved in malignant tumor progression. Importantly, its homologous counterpart ZEB2 has been also detected at CRC tumor front and determines poor survival (171).

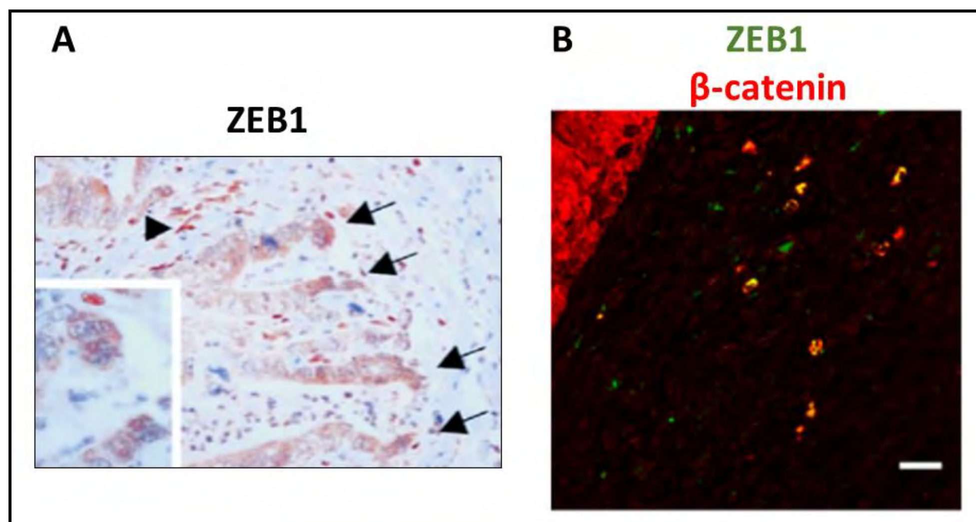


Figure 8 – ZEB1 is induced by Wnt signaling pathway at CRC invasive front. (A) *Detection of ZEB1 at nucleus of invasive front cells in CRC. Image taken with permission from (170).* **(B)** *ZEB1 is coexpressed with nuclear β-catenin at invading CRC cells. Coexpression of ZEB1 (stained in green) and β-catenin (in red) is depicted in yellow. Image taken with permission from (172).*

The studies carried out in our research group during my period as an MSc student identified a connection between the roles of ZEB1 and the Wnt signaling pathway in CRC (172). In fact, in the FAP model of genetically inherited CRC, nuclear translocated β-catenin strongly correlates with ZEB1 (**Figure 8B**). Since a homogeneous activation of Wnt signaling can be observed in these type of patients (173), there is a clear correlation between Wnt activity and

ZEB1 among epithelial CRC cells. This correlation was corroborated in spontaneous CRC samples, as well as in the APC*min* murine model. The nuclear translocation of β-catenin, driven by mutant *APC*, is inversely associated to epithelial E-cadherin expression, reinforcing the connection between Wnt and a ZEB1-triggered EMT (172).

In addition, the β-catenin/TCF4 transcriptional complex is able to induce the expression of ZEB1 by direct binding to its promoter (172). These findings placed ZEB1 as a novel target for Wnt signaling in CRC, as well as an effector of the distinct processes that it regulates in malignant transformation (28, 74, 170). In order to invade and disseminate to proximal or distant organs, CRC cells need to disrupt and remodel both the BM and the peritumoral stroma. Both processes are also mediated by some Wnt effectors (169).

7. Initial stages of CRC progression through BM and stroma

During the early stages of CRC progression, malignant cells need to overcome the extracellular surrounding barriers. The disruption of the basement membrane (BM) and the remodeling of the tumor microenvironment are key parts of this process (169, 170).

7.1 BM disruption

Epithelial tissues are mostly surrounded by an extracellular protein matrix that acts as a barrier between the epithelial cells and the connective neighbor tissue (174). Interestingly, both the epithelial tissue and surrounding stromal cells cooperate in the synthesis of BM components: type IV collagen, proteoglycans and members of the laminins family (175). The main role of this structure is to confer mechanical support to epithelial tissues (174, 176).

The family of laminins plays a pivotal role in maintaining BM structure. Specifically, laminin-5, which is built up of the α3, β3 and γ2 chains, presents anchoring functions in the BM of several epithelial tissues (177, 178). Diverse studies have shown a correlation between the expression of laminin-5 and tumor cells invasive ability. Particularly, the γ2 chain of laminin-5 (LAMC2) activates migratory properties of malignant cells (179). LAMC2 is a specific marker of invasiveness (180, 181). It promotes the dissociation of a set of poorly differentiated carcinoma cells from the neoplastic tissue, generating an invasive cellular entity defined as CRC budding (180, 182). This structure, despite losing the epithelial markers, still retains LAMC2.

In close connection with the role of laminins family, matrix metalloproteinases (MMPs), exert a prominent role in early steps of cancer invasion and metastatic processes, such as disruption of BM. MMPs share a zinc-dependent endopeptidase activity, that allows them to collectively degrade BM and extracellular matrix components (14, 183, 184). The membrane-type MMPs (a subgroup with 6 members, MT1-6 MMPs) are expressed at the surface membrane of producer cells and are responsible for the proteolytic activity in pericellular space and affect anchoring and adherence functions of epithelial cells (14, 185).

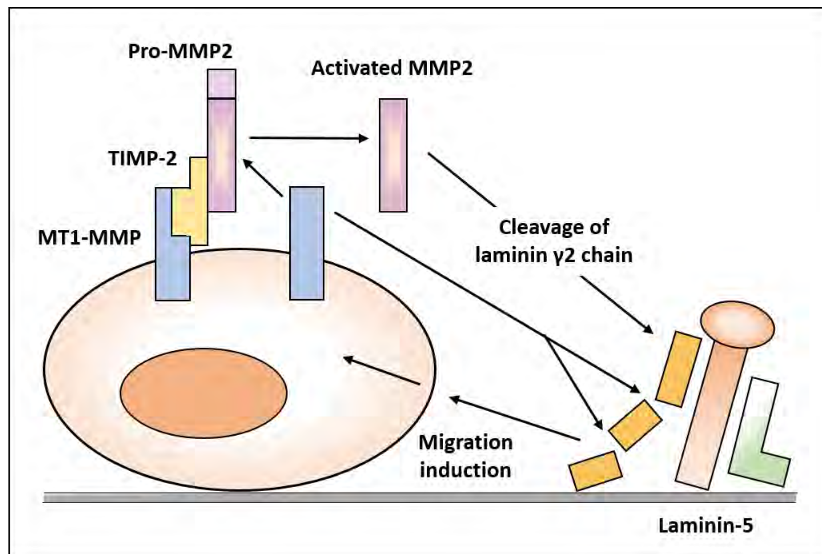


Figure 9 – LAMC2 and MT1-MMP stimulate migration at CRC invading front. *Schematic model of MT1-MMP dependent cleavage of LAMC2. Cleavage can occur through direct or indirect (involving MMP2) mechanisms. Adapted with permission from (186).*

MT1-MMP has been localized at tumor front of CRCs, coordinating pro-invasive activity of tumor cells (187). It exerts a proteolytic cleavage on laminin family member LAMC2, which stimulates migration of CRC cells (186, 187). This cleavage either occurs through a direct mechanism, by which MT1-MMP degrades laminin-5 at anchoring BM or through an indirect process involving the activation of pro-MMP2 (186, 188). Thus, co-expression of MT1-MMP and LAMC2 at invasive front of CRCs stimulates cell migration and metastasis (187) (**Figure 9**).

Both *LAMC2* and *MT1-MMP* belong to the Wnt signaling target genes set involved in triggering CRC invasiveness (169). Regarding LAMC2, it correlates with nuclear accumulation of β-catenin at CRC invading cells. Moreover, β-catenin/TCF4 complex can directly bind to its promoter region and induce its transcription (179). Therefore, *LAMC2* is considered a Wnt target in CRC cells (166, 179). In parallel, MT1-MMP has been also identified as an effector of Wnt signaling, through a direct regulation at promoter level by β-catenin/TCF4 (189). At the same time, it is strongly induced at invasive front of CRCs (184, 189).

In connection with the modulation of BM components, it had been previously reported that ZEB1 modulates the levels of collagen IV α2 chain and laminin-5 α3 chain (170). Since ZEB1 is a downstream effector of β-catenin/TCF4 complex, it was also investigated whether ZEB1 could be partially mediating the regulation of Wnt transcriptional spectrum. In fact, it was reported that ZEB1 itself induces both *MT1-MMP* and *LAMC2* in APC mutant cells. Even more, in the case of LAMC2, it was described a direct association of ZEB1 to its transcriptional region, in cooperation with TCF4 factor (60). Moreover, both proteins co-express with ZEB1 at the invasive front of *APC*-mutated CRCs (172).

7.2 Remodeling of the peritumoral stroma

Once the BM is disrupted, cancer cells need to migrate across the extracellular matrix (ECM) seeking for nearby lymphatic vessels where they can intravasate (14). The process commonly involves the distinct proteolytic systems that operate in the stromal matrix (190, 191), which can be imposed by the migrating malignant cells. However, the stromal cells cooperate with tumor cells in the production of several proteases that coordinately remodel the peritumoral matrix, by modifying epithelial adhesions (190-193). The main roles of peritumoral proteases in the promotion of cancer progression are migration, invasion, angiogenesis and supporting metastasis through enabling cell intravasation (191). Plasminogen Activation System (PAS) is one of the main protease cascades involved in these processes. The PAS has been reported to have a relevant function in both physiological and pathological events mediated by ECM remodeling. For instance, it regulates tissue regeneration and wound healing (194, 195), apart from promoting tumor progression and invasion (194, 196) (**Figure 10**). The PAS includes three main components: urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR) and the plasminogen activator inhibitors (PAI-1 and PAI-2) (194, 195). An elevated expression of PAS members correlates with poor prognosis in cancer (192, 197).

uPA is a serine protease that is secreted as a latent pro-form (pro-uPA), due to post-translational glycosylation (198). Several proteases (trypsin, kallikrein or cathepsin-B and –L) at the ECM are capable of converting pro-uPA in the active uPA protease (195, 199). Instead, other enzymes such as elastase and thrombin cleave uPA in a different pattern, yielding the secretion of an inactive amino terminal fragment (ATF). Both the active uPA and ATF proteins are able to bind uPAR (198).

Plasminogen is initially presented as a single chain inactive peptide, which is converted into the active protease plasmin by the action of plasminogen activators, mainly by uPA. In turn, plasmin degrades components of the extracellular matrix (194, 200). The activation of plasminogen is accelerated due to the existence of a positive feedback loop by which plasmin is capable of cleaving inactive pro-uPA into its active form (201). In fact, plasmin plays a central role in remodeling peritumoral tissue, by enhancing the BM and ECM disruption, thus supporting migration of tumor cells (195, 202). In addition, it can convert latent MMPs into active metalloproteinases, strengthening ECM degradation (195). The PA inhibitors (PAI-1 and PAI-2) belong to the serpins family. Among them, PAI-1 is much more effective as an inhibitor of uPA, since it reacts faster than PAI-2 and a lower amount of protein is sufficient to inhibit the plasminogen activator function (203). Despite being an inhibitor of uPA, an elevated expression of PAI-1 has been associated to a worse survival in some cancers (204, 205). Likewise, a pro-angiogenic role has been reported for PAI-1 (206).

Additionally, PAI-1 can block cells migration by binding to Vitronectin (Vn). Nevertheless, the joint expression of uPA results in the formation of uPA/PAI-1 complexes, lowering the affinity of PAI-1 for Vn. In this context, PAI-1 association to uPA allows cellular migration through the ECM (207). In summary, apart from its antitumoral effects, PAI-1 has been reported to display several unexpected pro-tumoral roles, generating some controversy about its function in tumor progression in diverse types of cancer. Therefore, tumor progression

requires a tight expression of both uPA and PAI-1, in order to achieve the optimal migration and invasion capacities. In order to migrate appropriately, tumor cells need sufficient stromal cells for their traction. In consequence, an excessive expression of uPA can involve an excessive proteolytic activity at the ECM and impair malignant cells migration. For this reason, uPA proteolytic function can even be a drawback for tumor progression and needs to be modulated by PAI-1 (205). Thus, in order to achieve an optimal migration and invasion through the ECM, cancer cells require a finely tuned equilibrium between the levels of both PAS members (200, 205).

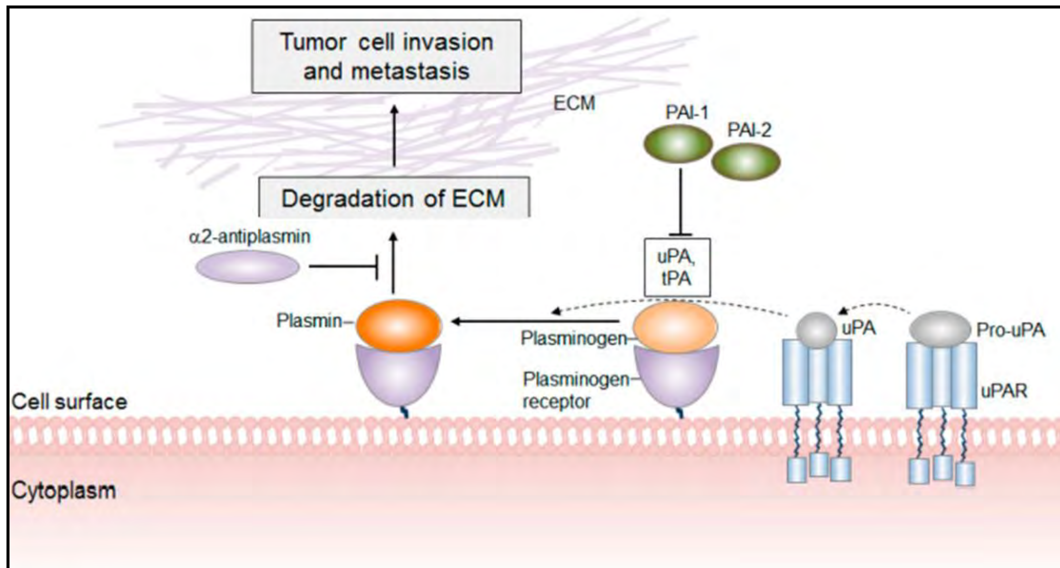


Figure 10 – Role of Plasminogen Activation System (PAS) in cancer invasion. *The illustration shows a model of PAS members' role at the interface between cell surface and ECM. Plasminogen is converted into plasmin by uPA, enhancing tumor cells invasive ability. PAI-1 and PAI-2 inhibit the function of plasminogen activators. Image taken with permission from (208).*

Expression of both uPA and PAI-1 are found to be elevated in CRC patients' samples (209, 210). Furthermore, increased expression of PAI-1 is associated to metastasis, whereas it has been reported that uPA is inactivated in liver metastasis, allowing settling and progression of secondary tumor (211, 212). In addition, uPA is associated with higher aggressiveness in colorectal tumors, and is considered a marker of poor prognosis, as well as a potential therapeutic target in this specific cancer (195, 213).

At the invasive front of CRCs, as well as in other carcinomas, uPA and PAI-1 are induced both in tumor and in stromal cells, correlating with an adverse clinical outcome (209, 212). In fact, the elimination of uPA in either tumor or stromal cells impairs tumor growth and diminishes metastasis incidence in mouse models (214). The signaling pathways that can activate both genes are mainly well defined. uPA has been identified as a direct target of the β-catenin/TCF4 transcriptional complex. Therefore, it is induced under the effect of an aberrant

Wnt signaling (215). However, it can also be induced by other pathways involved in cancer progression, such as the Notch cascade (216). In the case of PAI-1, it can be modulated by several upstream signaling cascades, like TGF-β, p53, hypoxia or insulin-like growth factors (IGFs) (217).

AIMS OF THE THESIS

II – GENERAL AIM OF THE THESIS

The general aim of this Thesis is to characterize new potential mechanisms by which ZEB1 regulates the oncogenic transformation and tumor progression in colorectal carcinoma beyond the induction of EMT.

SPECIFIC AIMS:

1. Describe the role of the transcription factor ZEB1 in the regulation of initial stages of CRC cells invasion, such as the remodeling of the ECM in the tumor microenvironment.
2. Identify new targets of ZEB1 at tumor front of CRCs and, in particular, define the specific modulation of Wnt-mediated signaling, as well as examine the *in vivo* relevance of any newly identified target.
3. Identify new cancer cell hallmarks regulated by ZEB1 that enhance its role in promoting CRC tumor progression.

RESULTS

III – RESULTS

The results obtained in this Thesis have resulted in the following published original articles:

1- Sánchez-Tilló E*, **de Barrios O***, Siles L, Amendola PG, Darling DS, Cuatrecasas M, Castells A, Postigo A. ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1. *Clin Cancer Res*, 2013. 19(5):1071-82 (* equal contribution as first author).

2- **de Barrios O**, Györffy B, Fernández-Aceñero MJ, Sánchez-Tilló E, Sanchez-Moral L, Siles L, Esteve-Arenys A, Roué G, Casal JI, Darling DS, Castells A, Postigo A. ZEB1-induced tumorigenesis requires senescence inhibition through activation of a new ZEB1-DKK1-mutant p53-Mdm2-CtBP pathway to repress macroH2A1. *Gut*, 2016. *Article in press.* doi:*10.1136/gutjnl-2015-310838*.

Results #1 *Clinical Cancer Research*, 2013. 19(5):1071-82

ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1

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Human Cancer Biology

Clinical Cancer Research

ZEB1 Promotes Invasiveness of Colorectal Carcinoma Cells through the Opposing Regulation of uPA and PAI-1

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Abstract

Purpose: Carcinoma cells enhance their invasive capacity through dedifferentiation and dissolution of intercellular adhesions. A key activator of this process is the ZEB1 transcription factor, which is induced in invading cancer cells by canonical Wnt signaling (β-catenin/TCF4). Tumor invasiveness also entails proteolytic remodeling of the peritumoral stroma. This study aimed to investigate the potential regulation by ZEB1 of the plasminogen proteolytic system constituted by the urokinase plasminogen activator (uPA), and its inhibitor, plasminogen activator inhibitor-1 (PAI-1).

Experimental Design: Through multiple experimental approaches, colorectal carcinoma (CRC) cell lines and samples from human primary CRC and *ZEB1* (−/−) mice were used to examine ZEB1-mediated regulation of uPA and PAI-1 at the protein, mRNA, and transcriptional level.

Results: ZEB1 regulates uPA and PAI-1 in opposite directions: induces uPA and inhibits PAI-1. *In vivo* expression of uPA depends on ZEB1 as it is severely reduced in the developing intestine of *ZEB1* null (−/−) mice. Optimal induction of uPA by Wnt signaling requires ZEB1 expression. ZEB1 binds to the uPA promoter and activates its transcription through a mechanism implicating the histone acetyltransferase p300. In contrast, inhibition of PAI-1 by ZEB1 does not involve transcriptional repression but rather downregulation of mRNA stability. ZEB1-mediated tumor cell migration and invasion depend on its induction of uPA. ZEB1 coexpresses with uPA in cancer cells at the invasive front of CRCs.

Conclusions: ZEB1 promotes tumor invasiveness not only via induction in cancer cells of a motile dedifferentiated phenotype but also by differential regulation of genes involved in stroma remodeling. *Clin Cancer Res; 19(5); 1071–82. ©2013 AACR.*

Introduction

During carcinoma progression, cancer cells enhance their migratory and invasive capacity through the downregulation of epithelial markers involved in the maintenance of cell polarity and intercellular adhesion—chiefly the inhibition of E-cadherin—and the acquisition of a motile dedifferentiated phenotype as part of the epithelial-to-mesenchymal transition (EMT; reviewed in refs. 1–3). At the transcriptional level, E-cadherin is repressed by factors of the ZEB, Snail, and Twist families, with ZEB1 (also known as δEF1) as final downstream effector and having the most consistent inverse correlation with E-cadherin across carcinomas (1–5). In addition to repress epithelial polarity and adhesion genes, ZEB1 activates mesenchymal and stemness markers (2). Consequently, ZEB1 expression promotes tumorigenesis and metastasis in mouse models and correlates with a poorer prognosis in human cancers, including colorectal carcinomas (CRC; refs. 2, 3, 6, 7).

ZEB1 is induced by multiple signaling pathways including TGF-β, Notch, and canonical Wnt (β-catenin/TCF4; reviewed in refs. 1–3, 8; 9–11). As a repressor of E-cadherin, ZEB1 is not expressed in normal epithelium or the tumor center of well-differentiated carcinomas (12). ZEB1 is rather upregulated at the invasive front of tumors in dedifferentiated cancer cells that have translocated β-catenin to the nucleus and therefore display active Wnt signaling (7, 11–14).

However, tumor invasiveness does not depend exclusively on intrinsic cancer cell traits and often entails a deregulation of proteolytic systems operating in the stroma (15). Proteases produced at the tumor invasive front by cancer and stromal cells remodel the peritumoral matrix modifying cancer cell adhesions and releasing active peptides that

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

E. Sánchez-Tilló and O. de Barrios contributed equally to this work.

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Translational Relevance

ZEB1 is expressed by invading carcinoma cells at the tumor front where it represses epithelial adhesion genes and induces a migratory and dedifferentiated phenotype. Consequently, ZEB1 promotes tumorigenesis and metastasis in mouse models and correlates with a poorer clinical prognosis in human cancers. We identified here a novel mechanism for the promotion of tumor invasiviness by ZEB1. Using cell lines and samples from primary human carcinomas and mice knocked out for *ZEB1*, we show that ZEB1 regulates genes involved in proteolytic remodeling of the peritumoral stroma: activating urokinase plasminogen activator (uPA) and inhibiting plasminogen activator inhibitor-1 (PAI-1). In fact, our data indicate that uPA is responsible for most of ZEB1-mediated cancer cell migration and invasion and that both proteins are coexpressed in tumor cells at the invasive front of primary carcinomas. Our results set ZEB1 as a potential biomarker of prognosis and a potential therapeutic target in colorectal carcinomas and probably other carcinomas.

promote tumor invasion. A key protease cascade involved in tumor invasiveness is the plasminogen activation system formed by the urokinase plasminogen activator (uPA) and its inhibitor, the plasminogen activator inhibitor-1 (PAI-1; ref. 16). uPA processes plasminogen into plasmin, which in turn cleaves extracellular matrix components (16). uPA is upregulated in cancer and stromal cells at the invasive front of colorectal and other carcinomas, where high expression by invading cancer cells correlates with metastasis and an adverse prognosis (17, 18). In turn, elimination of uPA in either cancer or stromal cells reduces tumor growth and the incidence of metastasis in mouse models (19, 20).

PAI-1 plays distinct roles in tumor invasion depending on the cell type expressing it and the tumor model studied. At the invasive front of tumors, including CRCs, PAI-1 is induced in cancer cells, but it is especially upregulated in stromal cells (17, 18). Paradoxically, considering its inhibitory function over uPA activity, PAI-1 expression at the tumor front of colorectal and other carcinomas correlates with an adverse clinical course (17, 18, 21). While results in mouse models vary with the experimental setting, evidence indicates that expression of PAI-1 by myofibroblasts and endothelial cells has a proinvasive effect, whereas PAI-1 expressed by cancer cells inhibits tumor growth and invasion (21). Thus, malignant keratinocytes and fibrosarcoma cells produce less invasive tumors when injected in *PAI-1*–deficient mice than in normal counterparts (19, 22). In contrast, compared with parental cells, overexpression of PAI-1 in prostate carcinoma cells decreases their invasiveness when xenotransplanted in nude mice (23).

If unchecked by PAI-1, excessive proteolysis by uPA can potentially inhibit tumor invasiveness, as tumor cells need sufficient stromal matrix for traction. At the same time, PAI-1 has proinvasive effects that are independent of its role as enzymatic inhibitor of uPA but are rather related to its regulation of cell adhesion (21). Efficient tumor invasiveness therefore requires a tight control of uPA and PAI-1 expression in cancer cells. uPA is a downstream target of Wnt (β-catenin/TCF4) and Notch pathways (24, 25), and PAI-1 is transcriptionally activated or repressed by multiple signals (e.g. p53, TGF-β, hypoxia, insulin, KLF-2; reviewed in ref. 26). Expression of uPA by cancer cells at the invasive tumor front of CRCs, where ZEB1 is also induced in response to aberrant activation of Wnt signaling (11), prompted us to investigate both the potential regulation of the uPA/PAI-1 system by ZEB1 and its contribution to ZEB1-mediated tumor invasiveness.

In this study, we show that ZEB1 controls both components of the plasminogen activation system in CRC cells, but in opposing ways; inducing uPA and inhibiting PAI-1. ZEB1 upregulates uPA through direct binding to its promoter and activation of transcription via a mechanism involving the histone acetyltransferase p300. Meanwhile, ZEB1 repressive effect on PAI-1 entails a downregulation of PAI-1 mRNA stability. Importantly, the ability of ZEB1 to promote migration and invasion of CRC cells depends on its induction effect over uPA. *In vivo* relevance of these findings was confirmed through two approaches. *In vivo* expression of uPA is critically dependent on ZEB1 as is virtually abrogated in the developing intestine of embryos from *ZEB1* null (−/−) mice. uPA also requires ZEB1 for its optimal induction by Wnt, which is hindered in *ZEB1*-deficient cells from these mice or CRC cells knocked down for ZEB1. In addition, ZEB1 coexpresses with uPA in cancer cells at the invasive front of primary CRCs but inversely correlates with PAI-1.

Materials and Methods

Antibodies, plasmids, siRNAs, and shRNAs

Description and source of antibodies, plasmids, siRNAs oligonucleotides, and short hairpin RNA (shRNA) lentivirus used in the study are detailed in Supplementary Information.

Cells

SW480 and HCT116 cells originated as in ref. (14) and Colo320 cells from the Cancer Cell Line Repository (RTICCC-PRBB, Barcelona, Spain). Mouse embryo fibroblasts (MEF) were obtained from E15.5 C57BL/6J wild-type (+/+) and δEF1 null (−/−) embryos (27). Mouse Wnt3a-containing and control-conditioned media were produced from L-cells stably carrying either an expression vector for mouse Wnt3a (L-Wnt3a cells) or its corresponding empty vector (L-Ctl cells), respectively. Details regarding cell culture, transfections, and transduction are provided in Supplementary Information.

Mouse and human tissues

Mouse tissue samples corresponded to 4 μm sections from E18.5 C57BL/6J wild-type (+/+) and δEF1 null (−/−) embryos (27). Paraffin-embedded sections of

human primary CRCs, either as individual samples or as an array, were obtained from Institut d'Investigacions Biomèdiques August Pi i Sunyer's (IDIBAPS) Tumor Bank. The tissue array contained 36 separate cores for the CRC invasive front from 12 independent patients in triplicate. Use of mouse and human samples was approved by the corresponding research ethics committees. Tissue immunostaining was conducted as described in Supplementary Information.

Determination of protein and mRNA expression and transcriptional assays

Western blot analyses, immunostaining, quantitative real-time PCR (qRT-PCR), chromatin immunoprecipitation (ChIP), and transcriptional assays were conducted as described in Supplementary Information.

Migration and invasion assays

The migration and invasion capacity of CRC cells was assessed as per standard protocols and detailed in Supplementary Information.

Statistical analysis

Statistical significance ($P \leq 0.01$) was determined by *t*- and Mann–Whitney-U tests.

Results

ZEB1 regulates endogenous uPA and PAI-1 in opposite directions

Of the transcription factors repressing E-cadherin, ZEB1 has the most consistent inverse correlation with across carcinomas (2–4, 8). Because ZEB1 is induced by Wnt signaling, constitutive nuclear accumulation of β-catenin in cells of the CRC cell line SW480 results in higher levels of ZEB1 and lower E-cadherin with respect to HCT116 cells, another CRC cell line where β-catenin is mostly membranous/cytoplasmic (11). Here, we found that, as compared with HCT116 cells, higher ZEB1 expression in SW480 cells was associated not only with lower E-cadherin but also with higher uPA expression (Supplementary Fig. S1A). This observation, along with the reported expression of uPA in cancer cells at the invasive front of carcinomas (24), prompted us to question whether ZEB1 could also promote tumor invasion by regulating the uPA/PAI-1 system.

To test this hypothesis, we explored the effect of manipulating endogenous ZEB1 expression on endogenous uPA and PAI-1 protein levels. We found that knockdown of ZEB1 in SW480 cells with two different siRNA (hereafter referred to as si1ZEB1 and si2ZEB1), but not by transfection of a siRNA control (siCtl), downregulated endogenous uPA protein (Fig. 1A). Likewise, SW480 cells where ZEB1 had been stably knocked down with a pool of specific shRNAs against ZEB1 (shZEB1), whose target sequences differ from those in si1ZEB1 and si2ZEB1, displayed lower uPA expression than SW480 cells stably transfected with control shRNA (shCtl) (Supplementary Fig. S1B). Interestingly, both transient and stable downregulation of ZEB1 in SW480 cells resulted in the opposite effect on PAI-1—lowering of ZEB1 expression increased endogenous levels of PAI-1 protein (Fig. 1A and Supplementary Fig. S1C).

Next, we investigated ZEB1 regulation of uPA and PA1-1 at the mRNA level by qRT-PCR. SW480 cells were again transiently transfected with si1ZEB1, si2ZEB1, or siCtl. Knockdown of ZEB1, but not transfection with siCtl, downregulated uPA mRNA levels while upregulating those for PAI-1 (Fig. 1B). Likewise, transient knockdown of ZEB1 in Colo320 cells, a CRC cell line that, like SW480, displays nuclear accumulation of β-catenin and high endogenous Wnt signaling activity (28), also resulted in downregulation of uPA mRNA (Supplementary Fig. S1D). Opposite regulation of uPA and PAI-1 mRNA by ZEB1 was also observed when ZEB1 was stably knocked down in SW480 cells with shZEB1 (Supplementary Fig. S1E).

As indicated above, and contrary to SW480 cells, β-catenin in CRC HCT116 cells remains mostly at the plasma

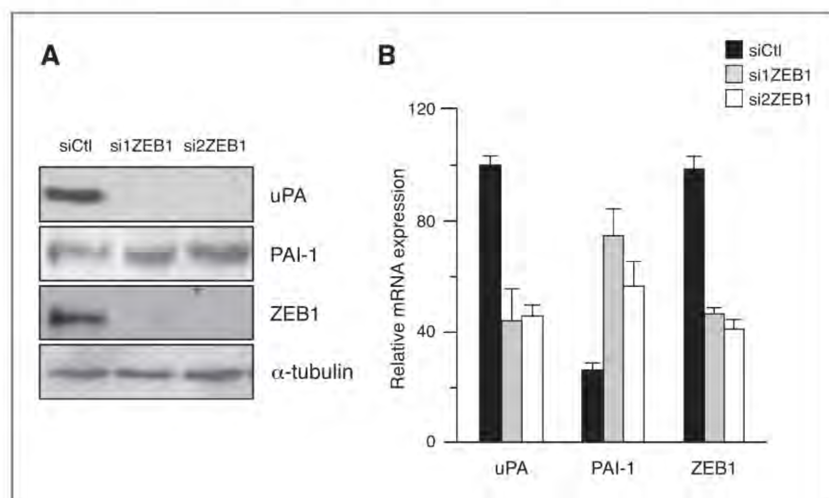
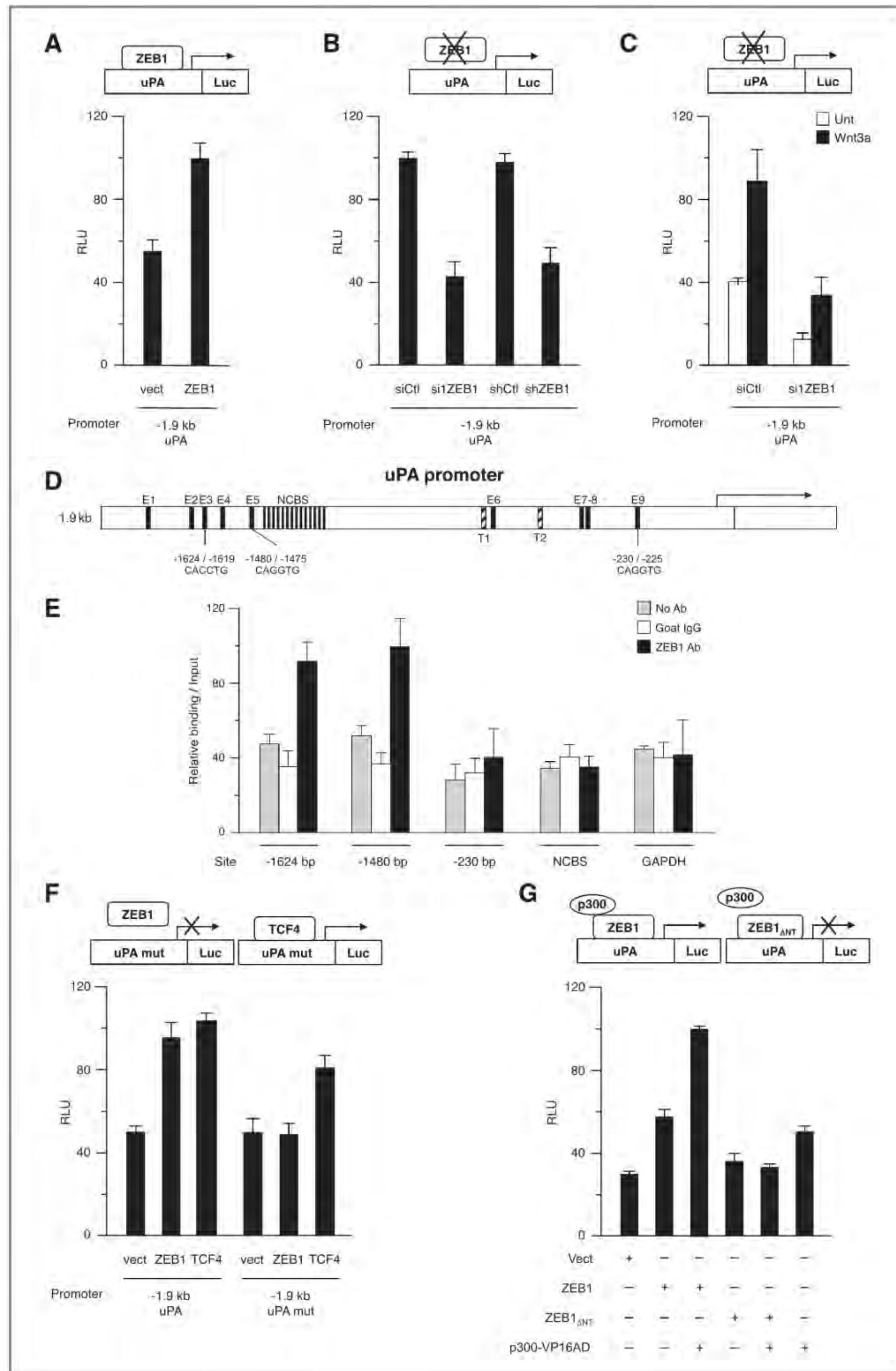


Figure 1. Opposing regulation of endogenous uPA and PAI-1 by endogenous ZEB1. A, transient knockdown of ZEB1 differentially regulates uPA and PAI-1 proteins. SW480 cells were transfected with 100 nmol/L of a siCtl, or specific siRNAs for ZEB1 (si1ZEB1 and si2ZEB1). Cell lysates were immunoblotted for uPA (3689/HD-UK1), PAI-1 (H-135), ZEB1 (H-102), along α-tubulin (B5-1-2) as loading control. B, transient knockdown of ZEB1 differentially regulates uPA and PAI-1 mRNAs. SW480 cells transfected as in A were assessed for relative mRNA levels of uPA, PAI-1, and ZEB1 by qRT-PCR with respect to GAPDH.



A
ZEB1
uPA
Luc
RLU
vect
ZEB1
Promoter
-1.9 kb uPA
B
siCtl
si1ZEB1
shCtl
shZEB1
C
Unt
Wnt3a
D
uPA promoter
1.9 kb
E1 E2 E3 E4 E5 NCBS E6 E7-8 E9
T1 T2
-1624 / -1619 CACCTG
-1480 / -1475 CAGGTG
-230 / -225 CAGGTG
E
No Ab
Goat IgG
ZEB1 Ab
Relative binding / Input
Site
-1624 bp
-1480 bp
-230 bp
NCBS
GAPDH
F
uPA mut
TCF4
-1.9 kb uPA mut
G
p300
ZEB1ΔNT
Vect
p300-VP16AD

membrane and in the cytoplasm, thus preventing activation of Wnt signaling and β-catenin–mediated transcription (11, 28). Leptomycin-B (LMB), a drug that specifically blocks CRM-1/exportin-dependent nuclear export, triggers the nuclear translocation of β-catenin in HCT116 cells and the induction of ZEB1 (11). We found here, that induction of ZEB1 mRNA upon short-time exposure of HCT116 cells to LMB also increased uPA mRNA and downregulated PAI-1 mRNA (Supplementary Fig. S1F). Similar results were obtained when HCT116 cells were treated with human recombinant Wnt3a ligand (Supplementary Fig. S1G). Altogether, these results show that ZEB1 has opposing effects on the two arms of the plasminogen activation system, inducing uPA expression and repressing PAI-1.

ZEB1 induces uPA through direct binding and transcriptional activation of its promoter via a mechanism involving p300

ZEB1 represses epithelial markers and activates mesenchymal and stemness genes by either direct binding to their regulatory regions and repression/activation of their transcription or through repression of an expanding set of microRNAs (2, 3, 8). We therefore decided to investigate the mechanism of ZEB1-mediated induction of uPA by examining the effect on uPA transcription of overexpressing or knocking down ZEB1.

Because SW480 and Colo320 cells exhibit strong β-catenin–mediated transcription and high levels of endogenous ZEB1 (11), the basal activity of a 1.9 kb fragment of the human uPA promoter proved to be relatively high when transfected in both cell types (Fig. 2A and Supplementary Fig. S2A). Still, overexpression of exogenous ZEB1 was able to further activate uPA transcription (Fig. 2A and Supplementary Fig. S2A). As found earlier for uPA protein and mRNA, transient and stable knockdown of ZEB1 in SW480 cells downregulated the basal transcriptional activity of the uPA promoter (Fig. 2B). uPA is also a direct transcriptional target of β-catenin/TCF4 (24), and overexpression or knockdown of β-catenin had similar effects on uPA transcription than those obtained for ZEB1 (Supplementary Figs. S2B and S2C). We therefore hypothesized that Wnt-mediated activation of the uPA promoter occurs not only by direct β-catenin/TCF4-mediated transcription (24) but also via ZEB1 (i.e., indirectly via Wnt-mediated induction of ZEB1; ref. 11). Accordingly, knockdown of ZEB1 not only reduced basal uPA promoter activity but also its optimal response to soluble Wnt3a ligand (Fig. 2C).

We next wondered whether the ability of ZEB1 to activate uPA transcription was mediated by direct binding of ZEB1 protein to its promoter. ZEB1 binds to a subset of E-box and E-box–like sequences on the regulatory regions of target genes (29). Examination of the first 1.9 kb of the human uPA promoter revealed the existence of several E-box sites, 3 of which (located at −1624, −1480 and −230 bp from the transcriptional start site) conforming to the reported ZEB1's optimal recognition sequence (CACCTG/CAGGTG; ref. 29; Fig. 2D). We therefore tested by ChIP assays whether endogenous ZEB1 binds to any of these sites on the uPA promoter. Indeed, it was found that an antibody against ZEB1, but not its respective control immunoglobulin G (IgG), immunoprecipitated regions of the uPA promoter containing E-boxes at −1624 bp, and −1480 bp (Fig. 2E). ZEB1 failed to bind, however, to the region containing the ZEB1 consensus binding site at −230 bp (Fig. 2E). Likewise, ZEB1 did not bind to a region of the uPA promoter lacking consensus binding sites [−1376/−1223, referred to as non-consensus binding sites (NCBS) in Fig. 2D] or to the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, whose expression is not regulated by ZEB1 (Fig. 2E).

To test the functionality of ZEB1 binding sites at −1624 bp and −1480 bp, both E-boxes were mutated to sequences known to not bind ZEB1 (29), and the resulted mutant uPA promoter was then tested for its induction by ZEB1. As shown in Fig. 2F, mutation of both sites was sufficient to abrogate uPA promoter response to ZEB1 overexpression. As in the above ChIP assays we only tested sites with optimal ZEB1 consensus binding sequence (CACCTG/CAGGTG), we could not rule out that other E-boxes in the mutant

Figure 2. ZEB1 transcriptionally activates uPA in CRC cells by direct binding to its promoter. A, ZEB1 activates uPA transcription. SW480 cells were transfected with 0.5 μg of a luciferase reporter containing 1.9 kb of the human uPA promoter along with 1 μg of an empty expression vector or the corresponding equal molar amount of a ZEB1 expression vector. Transcriptional assays and relative luciferase activity (RLU) in A–C, F, and G were conducted as described in Supplementary Materials and Methods. B, transient and stable knockdown of ZEB1 inhibits uPA transcription. SW480 cells were transiently transfected with 100 nmol/L of a siCtl or a specific siRNA for ZEB1 (si1ZEB1) or stably transfected with control (shCtl) or ZEB1-specific (shZEB1) shRNAs. C, downregulation of ZEB1 hinders Wnt-mediated transcription of uPA. SW480 cells transiently transfected as in B with siCtl or si1ZEB1 were either untreated (Unt) or exposed to 100 ng/mL of recombinant human Wnt3a during 48 hours. D, schematic representation of E-box and E-box–like sequences (E1 to E9) and TCF4 (T1 and T2) consensus binding sites in the first 1.9 kb of the human uPA promoter. E1 (-1792/-1787: CACGTT), E2 (-1665/-1660: CAGCTC), E3 (-1624/-1619: CACCTG), E4 (-1575/-1570:CAGCTG), E5 (-1480/-1475:CAGGTG), T1 (-737/-731:CTTTGTT), E6 (709-704: CACCTA), T2 (-562/-556: CTTTGTT), E7 (422-417: CAGCTC), E8 (413-408:CAGCTG), E9 (230-225:CAGGTG). NCBS refers to a region of the uPA (−1376/−1223) that contains no consensus binding sites for ZEB1. E, ZEB1 binds to the uPA promoter. qRT-PCR of fragments of the uPA and GAPDH promoters immunoprecipitated in ChIP assays from SW480 cells with ZEB1 antibody (E-20X), goat IgG, or with no antibody. Regions of the uPA promoter lacking consensus binding sites for ZEB1 (NCBS, −1376/−1223) or containing consensus ZEB1 binding sites at −1624 bp, −1480 bp, and −230 bp or from the GAPDH promoter were amplified by qRT-PCR as described in Supplementary Materials and Methods. Values represent average relative binding in relation to input from three independent experiments carried out in triplicate. F, ZEB1-binding sites are required for ZEB1, but not TCF4-mediated induction of uPA transcription. SW480 cells were transfected with 0.5 μg of a luciferase reporter containing 1.9 kb of the human uPA promoter either wild-type (−1.9 kb uPA) or mutated for ZEB1-binding sites at −1624 and −1480 bp (−1.9 kb uPA mut) along with 1 μg of an empty expression vector or the corresponding equal molar amount of either ZEB1 or TCF4 expression vectors. G, induction of uPA transcription by ZEB1 involves the histone acetyltransferase p300. As in A, SW480 cells were transfected with 0.5 μg of the human uPA promoter along with 0.5 μg of empty expression vector or the corresponding equal molar amount of full-length ZEB1 or a version of ZEB1 lacking the N-terminal region (ZEB1$_{\Delta NT}$) along with 0.5 μg p300-VP16AD.

uPA promoter, apart from −1624 bp and −1480 bp, could still bind ZEB1 and maintain its basal transcriptional activity. Together with the ChIP assays, these results show that endogenous ZEB1 binds to E-boxes at −1624 bp and −1480 bp in the uPA promoter to directly drive its transcription.

Mutation of ZEB1 sites in the uPA promoter did not impede direct Wnt-mediated transcription (by overexpression of TCF4) of the uPA promoter (Fig. 2F). The lower response of the mutant uPA promoter to TCF4 was likely due to the elimination of the ZEB1 component of Wnt-mediated induction of uPA: ZEB1 that is induced in response to TCF4 overexpression would be unable to bind to the mutant uPA promoter (see also below).

ZEB1 activates transcription through recruitment of p300 and p/CAF histone acetyltransferases to its N-terminal region (4, 30, 31). We therefore sought to investigate the involvement of p300 in ZEB1-mediated activation of uPA. Transfection of a version of p300 where its cDNA is fused to the activation domain of the herpes simplex virus VP-16 protein (p300-VP16AD), synergized with ZEB1 in the transcriptional induction of uPA (Fig. 2G). In contrast, deletion of the N-terminal region of ZEB1 (ZEB1$_{\Delta NT}$) eliminated most of the transcriptional activation effect of ZEB1 on the uPA promoter and failed to synergize with p300-VP16AD (Fig. 2G). We could therefore conclude that ZEB1 induces uPA expression through direct binding and transcriptional activation of its promoter via a mechanism involving p300.

ZEB1 inhibits PAI-1 expression by reducing the stability of its mRNA

Increased PAI-1 protein and mRNA expression upon ZEB1 knockdown indicates that, contrary to uPA, PAI-1 is under negative regulation by ZEB1. In fact, most known ZEB1 targets are transcriptionally inhibited rather than activated (2–4). We therefore proceeded to test the ability of ZEB1 to repress the human PAI-1 promoter at the transcriptional level. As shown in Fig. 3A, overexpression of ZEB1 in SW480 cells did not affect the basal transcriptional activity of a reporter containing a 0.8 kb fragment of the human PAI-1 promoter harboring most known PAI-1

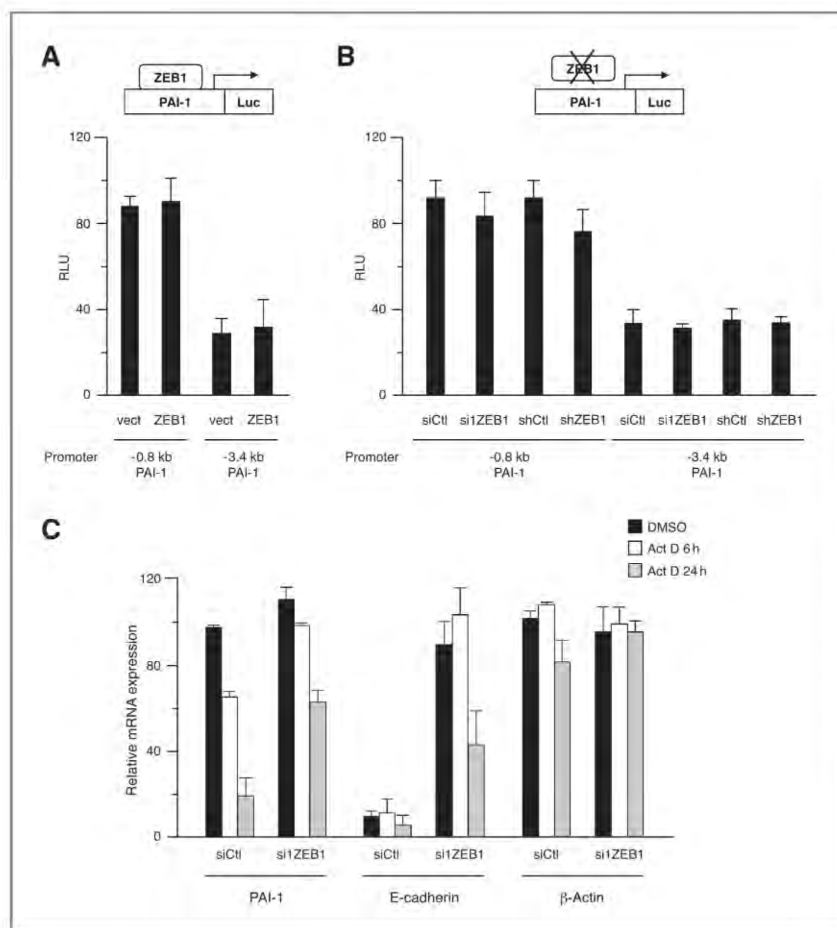


Figure 3. ZEB1 regulates PAI-1 at the posttranscriptional level. A, ZEB1 overexpression does not affect the transcription of the human PAI-1 promoter. SW480 cells were transfected with 0.5 μg of 2 different fragments of the PAI-1 promoter (−0.8 kb and −3.4 kb) along with 1 μg of an empty expression vector or the corresponding equal molar amount of a ZEB1 expression vector. B, transient and stable knockdown of ZEB1 does not alter PAI-1 transcription. As in Fig. 2B, but using −0.8 and −3.4 kb PAI-1 promoter luciferase reporters instead. C, ZEB1 expression decreases PAI-1 mRNA stability. SW480 cells transfected for 48 hours with either siCtl or a siRNA against ZEB1 (si1ZEB1) were incubated for the indicated periods with 10 μg of Act D before relative mRNA levels of PAI-1, E-cadherin, and β-actin were assessed by qRT-PCR. DMSO, dimethyl sulfoxide.

regulatory elements (reviewed in ref. 26). ZEB1 also failed to alter the basal activity of a larger 3.4 kb fragment of the PAI-1 promoter (Fig. 3A). We also found that neither transient nor stable knockdown of ZEB1 affected PAI-1 promoter transcription (Fig. 3B).

Although it cannot be ruled out that ZEB1 could transcriptionally repress PAI-1 via binding to more upstream or downstream regulatory regions, we decided to explore the potential regulation of PAI-1 by ZEB1 at an alternate level and examined whether ZEB1 expression affects PAI-1 mRNA stability. SW480 cells transiently or stably knocked down for ZEB1 were treated with actinomycin D (Act D) to inhibit RNA elongation and PAI-1 mRNA levels examined by qRT-PCR (Fig. 3C and Supplementary Fig. S3). We found that following treatment with Act D, PAI-1 mRNA remained more stable in SW480 cells where ZEB1 had been knocked down than in counterpart control cells (Fig. 3C and Supplementary Fig. S3).

Similar to parental SW480 cells, SW480 cells transfected with siCtl displayed very low levels of E-cadherin mRNA that, as expected, were drastically upregulated upon ZEB1 knockdown with si1ZEB1 (Fig. 3C). In contrast to PAI-1, E-cadherin expression is inhibited by ZEB1 through transcriptional repression of its promoter (2–4), and incubation of SW480 cells with Act D resulted in a decline of E-cadherin mRNA levels irrespective of ZEB1 knockdown with si1ZEB1 (Fig. 3C). At the incubation periods examined here, mRNA levels of the housekeeping gene β-actin, included as negative control, remained relatively stable to Act D treatment and, given that it is not regulated by ZEB1, unaffected by ZEB1 knockdown (Fig. 3C). Altogether, these results indicate that ZEB1 controls PAI-1 expression, at least in part, through regulation of its mRNA stability.

uPA expression mediates ZEB1-dependent CRC cell migration and invasion

Expression of ZEB1 and uPA by CRC cells promotes tumor cell migration, invasion, and metastasis (2, 6, 17, 18, 32). We therefore questioned whether upregulation of uPA by ZEB1 contributes to ZEB1-mediated tumor cell migration and invasion. To that effect, the SW480 cells stably infected with control (shCtl) and ZEB1-specific (shZEB1) shRNAs used in previous experiments were also stably transfected with either a cytomegalovirus (CMV) expression vector encoding the uPA cDNA (to restore loss of uPA expression due to ZEB1 knockdown) or the corresponding empty vector (Fig. 4A). The resulting stable pools were then assessed for their capacity to either actively migrate through an 8-μm pore polycarbonate membrane or invade through a Matrigel matrix. We reasoned that because ZEB1 upregulates uPA via transcriptional activation of the endogenous uPA promoter, ZEB1 knockdown should not affect uPA expression when driven by the CMV promoter.

As expected, cells knocked down for ZEB1 and expressing the empty CMV vector ("shZEB1 + vect" cells) displayed significantly lower migratory and invasive capacity than counterpart cells stably transfected with shRNA control and the empty vector ("shCtl + vect" cells; Fig. 4B and C). To ascertain whether lower levels of uPA in ZEB1 knockdown cells (Fig. 4A) contributed to their lower migratory and invasive capacities, we also tested their counterpart cells overexpressing exogenous uPA ("shCtl + uPA" and "shZEB1 + uPA" cells; Fig. 4A). We found that in cells where ZEB1 has been knocked down, forced restitution of uPA expression reinstated virtually all their migratory capacity and a significant part

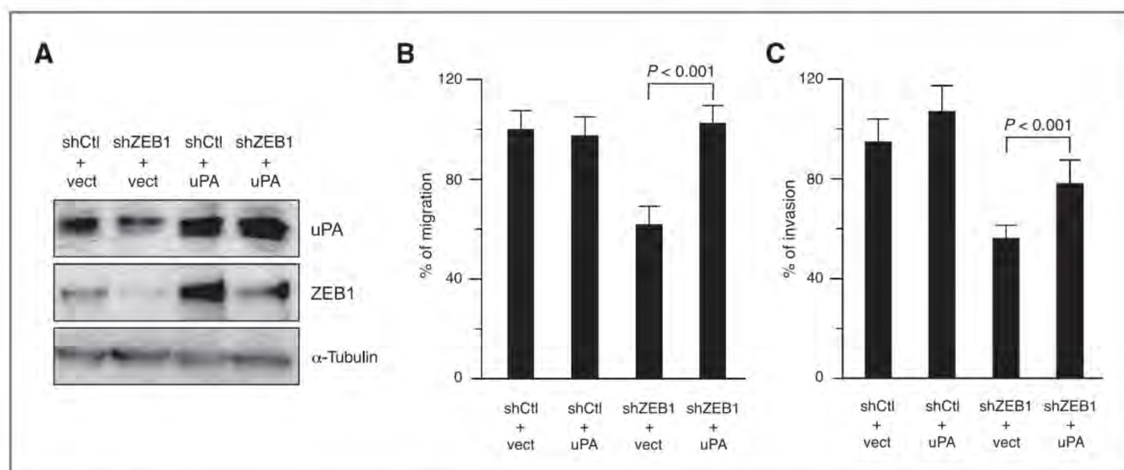
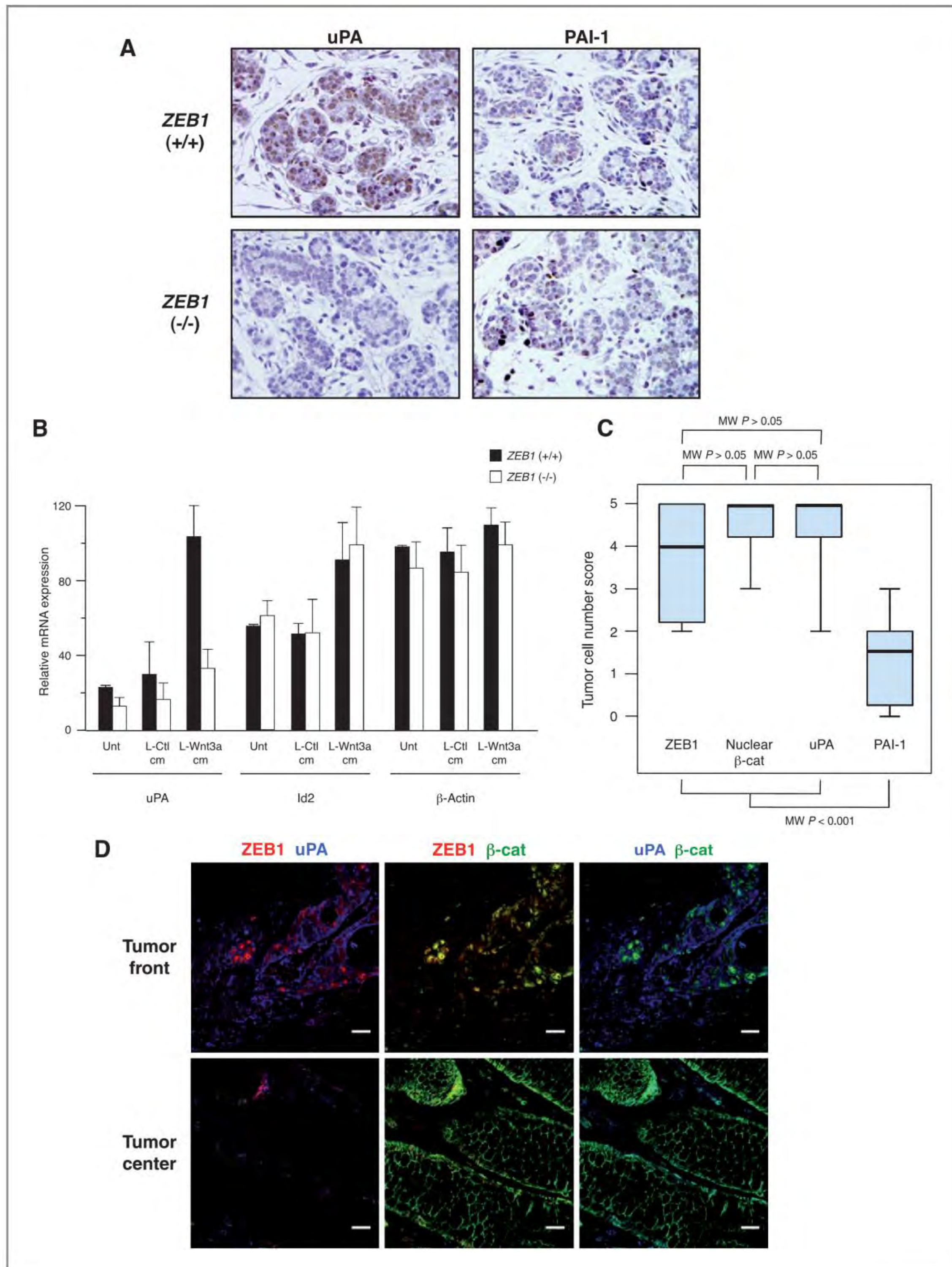


Figure 4. ZEB1 promotes migration and invasiveness of CRC cells in an uPA-dependent manner. A, SW480 cells were stably transfected with shCtl or shRNA against ZEB1 (shZEB1) plus either an empty expression vector ("shCtl + vect" and "shZEB1 + vect" cells, respectively) or an expression vector encoding human uPA cDNA ("shCtl + uPA" cells and "sh1ZEB1 + uPA" cells, respectively). Levels of ZEB1 (H-102) and uPA (3689/HD-UK1) were determined by Western blot analysis along α-tubulin (B5-1-2) as loading control. B, stable pooled cell lines as in A were tested for their ability to migrate during 6 hours through a Transwell polycarbonate membrane (migration assays) as described in Materials and Methods. A *t* test for significance of means difference was used. C, as in B, but cells were examined for their ability to invade during 24 hours through a Matrigel matrix (invasion assays).


A
uPA
PAI-1
ZEB1 (+/+)
ZEB1 (-/-)
B
ZEB1 (+/+)
ZEB1 (-/-)
Relative mRNA expression
120
80
40
0
Unt
L-Ctl cm
L-Wnt3a cm
uPA
Id2
β-Actin
C
MW P > 0.05
Tumor cell number score
ZEB1
Nuclear β-cat
uPA
PAI-1
MW P < 0.001
D
ZEB1 uPA
ZEB1 β-cat
uPA β-cat
Tumor front
Tumor center

of their invasive behavior (Fig. 4B and C and Supplementary Fig. S4A).

Given the central role of the plasminogen activation system in cancer progression, a number of specific inhibitors of uPA have been identified and/or developed (33). To confirm the involvement of uPA in ZEB1-mediated invasiveness of SW480 CRC cells, we tested the effect of the uPA inhibitor amiloride in Matrigel invasion. Treatment with amiloride reduced uPA levels and inhibited cell invasiveness in all four stable cell lines of Fig. 5A: inhibition that, as expected, was not rescued by exogenous overexpression of uPA (Supplementary Figs. S4A and S4B). Altogether, these results indicate that the well-established role of ZEB1 promoting tumor migration and invasion involves its upregulation effect over uPA.

Several reports have shown that intracellular signaling upon binding of uPA to its cell surface receptor triggers an EMT phenotype in breast and squamous carcinoma cells (e.g., refs. 34 and 35). In that line, we observed that overexpression of uPA in ZEB1 knockdown cells upregulated ZEB1 expression (Fig. 4A), whereas uPA inhibition by amiloride decreased it (Supplementary Fig. S4B).

ZEB1 is required for *in vivo* expression of uPA and its optimal induction by Wnt

We decided then to test the *in vivo* relevance of the above findings by examining uPA and PAI-1 expression in *ZEB1* (*δEF1*) null (−/−) mice. As these mice die around birth (27), we examined their expression in the developing intestine of late stage embryos (embryonic day 18.5; Fig. 5A and Supplementary Fig. S5A). We found that uPA was significantly reduced in the developing intestine of these mice indicating that *in vivo* expression of uPA requires of ZEB1 (Fig. 5A). Although in carcinomas uPA is mostly located at the cytoplasm or plasma membrane level, a large share of the uPA staining observed in the developing intestine was nuclear; a finding that is in line with evidence about uPA nuclear localization in other cell systems and its binding to transcription factors (e.g., 36, 37). In contrast, expression of PAI-1 in *ZEB1* null (−/−) embryos was not altered or only slightly upregulated (Fig. 5A). It could be argued that because uPA and PAI-1 are expressed at high levels in the developing intestine of wild-type embryos, it is more difficult to detect an increase in PAI-1 immunostaining upon loss of *ZEB1*. Overall, these results show that ZEB1 is critically required for the regulation of the plasminogen activation system *in vivo*.

As indicated earlier, expression of both ZEB1 and uPA is induced by Wnt signaling (11, 24). We therefore wondered whether ZEB1 expression is also required for uPA induction by Wnt in cells from *ZEB1* null (−/−) mice. To that effect, we tested the ability of conditioned medium containing recombinant mouse Wnt3a ligand to activate uPA expression in MEFs from the *ZEB1* (−/−) mice. Importantly, we found that induction of uPA by Wnt3a was significantly reduced in *ZEB1* null (−/−) MEFs (Fig. 5B). However, Wnt3a-mediated induction of Id2, a target of canonical Wnt signaling in CRCs (38) that is not regulated by ZEB1, was not altered by the loss of ZEB1 expression (Fig. 5B). Finally, no effect of Wnt3a-conditioned medium or of ZEB1 loss was observed in the expression of housekeeping gene β-actin (Fig. 5B). Therefore, and in line with cell line-based results above (Fig. 2C and F), we conclude that endogenous ZEB1 expression is also required for optimal Wnt-mediated induction of uPA *in vivo*, via induction of ZEB1 by Wnt and subsequent activation of uPA by ZEB1, but not for direct β-catenin/TCF4-mediated activation of uPA or of other ZEB1-independent Wnt targets.

ZEB1 coexpresses *in vivo* with uPA but not PAI-1 at the invasive front of human primary CRCs

While the tumor center of typical CRCs is relatively well differentiated, their invasive front contains progressively less-differentiated open tubular structures, small cell clusters, and eventually isolated dedifferentiated cancer cells (13). ZEB1 is never expressed by epithelial cells of the normal colonic mucosa of the tumor center of CRCs. Instead, ZEB1 is induced in CRC cells at the invasive front that have accumulated nuclear β-catenin (7, 11, 12, 14). uPA and PAI-1 are also expressed, albeit at different levels, by cancer cells at the invasive tumor front of CRCs (17, 18, 24). We therefore tested whether the expression of these genes correlates in cancer cells at the invasive front using an array of primary human CRCs. As shown in Fig. 5C, ZEB1,

Figure 5. *In vivo* expression of uPA depends on ZEB1, and both proteins are coexpressed in invading tumor cells at the leading front of CRCs. A, *in vivo* expression of uPA depends on ZEB1. Immunohistochemistry for uPA (H-140) and PAI-1 (H-135) of the developing intestine from *ZEB1* wild-type (+/+) and null (−/−) E18.5 mouse embryos. Immunostaining was conducted as described in Supplementary Materials and Methods. B, optimal induction of uPA by Wnt signaling depends on ZEB1. Relative mRNA levels of uPA, Id2, and β-actin in *ZEB1* wild-type (+/+) or null (−/−) MEFs under either basal conditions (Unt) or upon incubation for 48 hours with conditioned media from either L-cells stably transfected with an expression vector for mouse Wnt3a (L-Wnt3a cm) or its corresponding empty vector (L-Ctl cm). Relative mRNA levels were assessed by qRT-PCR with respect to GAPDH. The experiment is the average of three different experiments. C, ZEB1 correlates with uPA and nuclear β-catenin, but not with PAI-1, in invading cancer cells at the leading front of CRCs. A tissue array of primary CRCs was stained by diaminobenzidine (DAB) method for ZEB1 (H-102), β-catenin (C2206), uPA (3689/HD-UK1), and PAI-1 (H-135). Samples were scored for number of positively stained tumor cells at the invasive front as indicated in Supplementary Materials and Methods. Values represented in a quartile boxplot were assessed for significance using a nonparametric Mann–Whitney-U test. D, ZEB1 colocalizes with uPA in invading cancer cells at the tumor front of CRCs but neither protein is expressed in the tumor center. At the tumor front, ZEB1 and uPA also colocalize with nuclear β-catenin. β-catenin is mostly membranous/cytoplasmic at the tumor center. Sections of the tumor front and tumor center of a sporadic CRC were immunostained for ZEB1 (red, E-20), uPA (blue, 3689/HD-UK1), and β-catenin (green, Ab5302). Representative merge pictures are shown. Single staining for uPA, ZEB1, and β-catenin along with 4′, 6-diamidino-2-phenylindole (DAPI) is shown in Supplementary Figs. S5B and S5C. Staining of a more differentiated area of the tumor front is shown in Supplementary Figs. S5D and S5E. uPA was originally detected with Dylight 649 but converted to blue for representation. Scale bars represent 25 μm.

nuclear β-catenin, and uPA were expressed by a higher number of cancer cells than PAI-1. Statistical correlation indicated that ZEB1 expression significantly associates with uPA, but not with PAI-1, in cancer cells at the tumor front of CRCs (Fig. 5C).

To confirm the correlation between ZEB1 and uPA in the tissue array, we also examined whether both proteins coexpressed in confocal immunofluorescence analysis. High levels of ZEB1 (red) and uPA (blue) were found coexpressed by cancer cells at the invasive front of CRCs (Fig. 5D and Supplementary Figs. S5B, S5D, and S5E). These cancer cells also coexpressed nuclear β-catenin (green). In contrast, cancer cells in central areas of the same CRCs were negative for ZEB1 and uPA, whereas β-catenin was mostly membranous/cytoplasmic (Fig. 5D and Supplementary Figure S5C). At the tumor center, as in normal areas, ZEB1 and uPA were only found in nonepithelial cells of diverse origin at the stromal compartment Altogether these data show that, in line with earlier results in this study, ZEB1 and uPA are coexpressed *in vivo* in CRC cells at the invasive front.

Discussion

Efficient tumor invasiveness requires not only the loss of intercellular adhesions and the acquisition of a dedifferentiated phenotype by cancer cells, as part of the EMT, but also a remodeling of the surrounding extracellular matrix through the concerted action of cytokines, proteases and their inhibitors. Coordinated regulation of both processes involves reciprocal and dynamic influences between the tumor and its microenvironment. In this study, we showed that ZEB1, a key inducer of EMT during cancer progression, regulates the expression of both components of the plasminogen activation system, inducing uPA and repressing PAI-1. We found that ZEB1 induces uPA by direct binding and transcriptional activation of its promoter through a mechanism involving p300. The relevance of ZEB1 in the regulation of uPA is evidenced *in vivo* by its virtual loss in the developing intestine of *ZEB1* null (−/−) mouse embryos and its suboptimal induction by Wnt in *ZEB1*-deficient cells from these mice. Meantime, inhibition of PAI-1 by ZEB1 does not involve a transcriptional mechanism but rather the downregulation of PAI-1 mRNA stability. We also found that ZEB1-mediated motility of CRC cells depends on uPA and that the exogenous reconstitution of uPA restores the motility of CRC cells where uPA expression had been downregulated following ZEB1 knockdown. Finally, we showed that ZEB1 is coexpressesed with uPA, but not PAI-1, in cancer cells at the invasive front of primary CRCs.

This study has identified ZEB1 as a regulator of the plasminogen activation system that participates in the remodeling of the tumoral stroma in several human cancers, including CRCs (16, 21, 32). ZEB1 would thus promote tumor invasion not only by enhancing the motility of cancer cells via dissolution of intercellular adhesion, but also creating a path for their migration through restructuring of the tumor microenvironment. In addition, pericellular degradation of the basement membrane and stromal matrix generates proteolytic fragments that foster tumor invasion. In CRCs, ZEB1 regulates the expression of components of the epithelial basement membrane, disruption of which is a crucial step preceding tumor invasion into the peritumoral stroma (12). Proteolysis of extracellular matrix proteins by uPA-generated plasmin also creates promigratory peptides. Cleavage by plasmin of fibronectin and binding of its fragments to $\alpha5\beta1$ integrin mediate the tumor invasion- and hematogenous dissemination-promoting effects of uPA (39). Interestingly, ZEB factors induce $\alpha5\beta1$ integrin expression (40). It is therefore possible that in promoting cancer cell migration, ZEB1 not only induces the generation of fibronectin promigratory peptides through activation of the uPA/plasmin system but also activates expression of their integrin receptors.

While proteolytic remodeling of the stroma is required for tumor invasion, excessive matrix degradation could actually hinder invasion as cancer cells need sufficient substrate to sustain their motility. A tight regulation of uPA and PAI-1 levels is therefore needed for an optimal proteolytic balance. In that regard, joint but differential regulation of uPA and PAI-1 by a single gene, *ZEB1*, seems particularly relevant. Likewise, separate and distinct mechanisms of control, direct transcriptional activation and likely indirect inhibition of mRNA stability, respectively, allow ZEB1 to finely tune the expression of both genes during tumor progression. uPA and PAI-1 also regulate cancer cell migration and invasion through mechanisms independent of their regulatory role over proteolysis. For instance, PAI-1 binding to vitronectin, which releases uPA activity, modulates successive cycles of adhesion/deadhesion of cancer cells as they migrate across the stroma (reviewed in ref. 21). By regulating their expression, ZEB1 would also modulate these nonproteolytic activities of uPA and PAI-1. In addition, increased production of vitronectin by cancer cells during EMT adds another level of functional regulation of uPA/PAI-1 by ZEB1.

ZEB1 binds to selected E-box and E-box–like sequences in the regulatory regions of its targets showing higher affinity for the CACCTG/CAGGTG core sequence (29). We found that ZEB1 activates uPA expression by direct binding and activation of its promoter in a mechanism involving the histone acetyltransferase p300. Although we identified three optimal ZEB1 sites in the first 1.9 kb of the human uPA promoter, one of them (−230 bp) failed to recruit ZEB1, potentially suggesting that binding of ZEB1 to its target genes is also modulated by the identity of the nucleotides surrounding the core E-box. Given that in this study we only checked by ChIP assays the binding of ZEB1 to optimal consensus binding sites (CACCTG/CAGGTG), it is possible that ZEB1 still binds to the lower affinity sites in the mutant uPA promoter to maintain its basal transcriptional activity.

In contrast, regulation of PAI-1 by ZEB1 in CRC cells occurs at the posttranscriptional level. Knockdown of ZEB1 reduced and/or delayed the effect of Act D over PAI-1 mRNA indicating that ZEB1 inhibits PAI-1 expression, at least in part, by reducing the stability of its mRNA. PAI-1 mRNA is

known to have a very short half-life and its stability to be modulated in response to multiple stimuli (e.g., hypoxia and growth factors) by the interaction of mRNA-binding proteins, some still to be identified and characterized, to its 3′ UTR and protect or degrade PAI-1 mRNA (e.g. refs. 41, 42). It remains to be elucidated whether the inhibition of PAI-1 expression by ZEB1 involves regulation of PAI-1 mRNA-binding proteins. In this manner, ZEB1 may not only act as both a transcriptional repressor and an activator (depending on the target gene; ref. 4), but also regulate gene expression through different mechanisms.

In breast and squamous carcinoma cells, binding of uPA to its receptor triggers an EMT phenotype, including the expression of Snail1 (34, 35). In this study, we found that uPA overexpression upregulates ZEB1, whereas inhibition of uPA expression by amiloride reduces ZEB1. As ZEB1 is a downstream target of Snail1 (reviewed in ref. 3), uPA induction of ZEB1 could be mediated by Snail1, although direct activation cannot be excluded given that uPA triggers signaling via Ras-ERK, JAK-STAT, and PI3K-AKT (43), all of which are direct upstream inducers of ZEB1 (2, 8). ZEB1 has been suggested to maintain the EMT process initiated by Snail1 (3, 9), making it possible that ZEB1 mediates uPA's EMT-promoting effects. The induction of uPA by ZEB1 reported here could thus create a feed forward loop between both proteins, reinforcing their expression and a mesenchymal and dedifferentiated phenotype among invading cancer cells, thus promoting tumorigenesis and tumor invasion across the stroma. Of note, only migrating cancer cells that have lost E-cadherin expression and undergone an EMT could intravasate and disseminate through blood circulation (reviewed in ref. 44), a process that, as noted earlier, depends on uPA expression (39). In that regard, the loop between ZEB1 and uPA could be important in maintaining repression of E-cadherin and promoting ulterior intravasation of cancer cells.

Over the last few years, our understanding of ZEB1 has evolved from being a repressor of E-cadherin that promotes EMT and tumor invasiveness to a factor that induces most of the hallmarks that cancer cells need to acquire during tumor progression: thus, *inter alia*, ZEB1 contributes to overriding safeguard programs against cancer like senescence, promotes tumor angiogenesis, and cooperates with oncogenic signals (reviewed in refs. 2, 3, 8). Likewise, a number of reports indicate that uPA and PAI-1 also participate in an expanding set of functions during cancer progression beyond tumor invasiveness (32), many in line with their coordinated regulation by ZEB1. In addition, evidence of the nuclear localization of uPA and its binding to transcription factors (36, 37), supports that uPA and/or PAI-1 may be playing roles in gene regulation still to be elucidated. It is therefore tempting to speculate whether uPA/PAI-1 could mediate some of the new ZEB1 roles in cancer progression or vice versa.

In sum, this work has shown that ZEB1 promotes tumor invasiveness not only by inducing a more motile dedifferentiated phenotype in cancer cells but also through the opposing regulation of uPA and PAI-1. By expanding the mechanisms by which ZEB1 regulates tumor invasiveness, the results presented here reinforce its role as a potential therapeutic target in CRCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E. Sánchez-Tilló, O. de Barrios, A. Postigo
Development of methodology: E. Sánchez-Tilló, O. de Barrios, A. Postigo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Sánchez-Tilló, O. de Barrios, L. Siles, P.G. Amendola, D.S. Darling, M. Cuatrecasas, A. Castells
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Sánchez-Tilló, O. de Barrios, A. Postigo
Writing, review, and/or revision of the manuscript: A. Postigo
Study supervision: A. Postigo
Other: All authors provided critical review of the manuscript.

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SUPPLEMENTARY INFORMATION

for

ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1

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SUPPLEMENTARY MATERIALS AND METHODS

Cell culture

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Lonza) or RPMI (Lonza) supplemented with 10% FCS (Sigma). L-cells stably carrying an expression vector for mouse Wnt3a (L-Wnt3a cells) or its corresponding empty vector (L-Ctl cells) were cultured in the presence 0.4 mg/ml of geneticin (G418 disulfate, Fisher Scientific). Conditioned media from L-Ctl and L-Wnt3a cells was collected in the absence of geneticin and, where so denoted, added to cells during 48 h. Where indicated, cells were treated with Leptomycin-B (LMB) (Sigma, 10 ng/ml, 30 min), recombinant human Wnt3a (R&D Systems, 100 ng/ml for 24 h in the case of HCT116 cells or 48 h in SW480 cells) or amiloride (Sigma, 100 μM, 24 h).

Cell transfections

Cells were transfected with plasmids and siRNA oligonucleotides using Lipofectamine 2000 (Life Technologies) or, when exclusively transfected with siRNA oligonucleotides, using Lipofectamine RNAiMAX (Life Technologies) as described in (1). After 48 h (by default, or at the time indicated in specific experiments), cells were processed for protein, mRNA or transcriptional analyses. SW480 cells were also stably infected with lentiviral particles encoding shRNAs (see below) followed by selection in puromycin (10 μg/ml)-containing media as described below. In some experiments, the resulting pooled stables were subsequently stably transfected with pCI-neo (CMV empty expression vector) (Promega Corp.) or pCI-neo-uPA (with the cDNA encoding human uPA) followed by selection in geneticin (0.7 mg/ml)-containing media.

Antibodies

Antibodies used in this article originated as follows: ZEB1 [H-102, E-20, and E-20-X from Santa Cruz Biotechnology (SCBT) and Zfhep as in (2)], uPA (3689/HD-UK1 from American Diagnostica and H-140 from SCBT), PAI-1 (H-135 from SCBT), β-catenin (Ab6302 from Abcam, C2206 from Sigma), and α-tubulin (B5-1-2 from Sigma). Secondary antibodies were obtained from Jackson ImmunoResearch (JIR), namely, horseradish peroxidase (HRP)-conjugated donkey antimouse IgG, HRP-conjugated goat-anti-rabbit IgG, Donkey Dylight™ 488-anti-rabbit IgG, Donkey Cy3™ anti-goat IgG, Donkey Dy-light ™ 649 anti-mouse IgG, and Donkey Rho RedX ™ anti-mouse IgG. For blocking in immunostaining and as control for ChIP assays, normal donkey Ig G (JIR) or normal goat IgG (JIR, 5–8 mg⁄mL of IgG in normal serum), respectively, were used.

Plasmids

ZEB1 and $ZEB1_{\Delta NT}$ expression vectors have been previously described (1). Other expression vectors were obtained from the following researchers: β-catenin from J. Woodgett (Mount Sinai Hospital, Canada), uPA from GR Nemerow and S Huang (The Scripps Research Institute, La Jolla, CA) (3), CMV-p300-VP16AD from D Livingston (Dana Farber Cancer Institute, Boston, MA). Firefly luciferase reporters for the promoters tested here originated as follows: -1.94 kb of the human uPA from A Varro (University of Liverpool, Liverpool, UK) (4), -0.8 and -3.4 kb of human PAI-1 promoter from DE Vaughan (North Western University, Chicago) (5). pCI-neo was purchased from Promega, CMV-β-galactosidase from Clontech, and pBluescript SK from Stratagene-Agilent.

siRNAs and shRNAs

siRNAs were ordered from Invitrogen or Integrated DNA Technologies (IDT) using sequences reported elsewhere: two different siRNAs against human ZEB1 [siZEB1 as described in (6) (5'-UGAUCAGCCUCAAUC UGCA-3') and si2ZEB1 as in (7) (5'-AACUG AACCUGUGGAUUA-3')] and β-catenin (8). As negative control, we used a scramble control siRNA (5'-GGUUACGAACUAAGC UAUA-3') as well as (in Western blot/qRT-PCR studies) a siRNA against firefly luciferase as described in (9). Lentiviral particles encoding shRNAs against human ZEB1, and whose target sequences are different from those in si1ZEB1 and si2ZEB1, consisted of a pool of three shRNAs (referred as shZEB1) (5'-GAAGCAG GAUGUACAGUAA-3', 5'-GGCGAUAGAUG GUAAUGUA-3', 5'-CCAGAACAGUGUUUA UUCU-3') were purchased from SCBT (sc-38643-V). Lentiviral particles for a control shRNA (also different from the siRNA control) were also obtained from SCBT (sc-108080-V).

Western Blot assays

Western blot assays were performed as previously described (1). Briefly, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% SDS, 50 mM Tris pH 8, 2 mM EDTA plus protease inhibitors) and loaded onto 8% or 12% polyacrylamide gels. Gels were then transferred to a PVDF membrane (Immobilon-P, Millipore). Following blocking for nonspecific antibody binding with 5% nonfat milk, membranes were incubated with the corresponding primary and HRP-conjugated secondary antibodies before the reaction was developed using Pierce's ECL Western Blotting Substrate or SuperSignal West Pico Chemiluminescent Substrate (Pierce-Thermo Fisher Scientific). Western blots shown in the article are representative of at least three independent experiments.

RNA Extraction and Quantitative Real-Time PCR

Total RNA extracted with Trizol reagent (MRC Inc.) followed by RNAse free DNAse I treatment (Promega) or SV Total RNA Isolation System (Promega) was used to synthesize cDNA with random hexamers/ oligodT with a reverse transcription kit (GoScript, Promega or iScript™ Reverse Transcription, BioRad) according to manufacturer's instructions. mRNA levels were determined by quantitative real-time PCR (qRT-PCR) at 60°C using either SYBR Green/ROX (GoTaq, Promega) or SYBR®Green (Biorad) and oligonucleotide primer sequences previously described in the literature: human ZEB1 (10), human E-cadherin (11), human uPA (12), human PAI-1 (13), human GAPDH (14), human β-actin (15), mouse uPA (16), mouse Id2 (17), mouse β-actin (18) or mouse GAPDH (19). Relative mRNA levels were determined by Opticon Monitor 3.1.32 software (BioRad) by ΔCt method normalizing values of genes under study to housekeeping GAPDH as reference gene. qRT-PCR data shown in the study are the average of at least three independent experiments performed in triplicate.

Site directed mutagenesis

Identification of ZEB1 consensus binding sequences was conducted using MacVector software. ZEB1 consensus binding sites at positions −1624 bp and −1480 bp in the human uPA promoter were mutated to sequences known to not bind ZEB1 using the QuickChange Lightning site-directed mutagenesis kit (Agilent) as per manufacturer's instructions. Complementary mutant primers to opposite strands of the target DNA were purchased from IDT. For the ZEB1 binding site at −1624 bp of human uPA promoter, the upper strand oligonucleotide sequence used is 5′-TCTCCA GAAGACAGTGGGTCTATTGCCTCCCAAA AGCTGAAAGGC-3'. For the ZEB1 binding site at −1480 bp of the human uPA promoter, the

upper strand oligonucleotide sequence used is 5'-GCCTTCCTTCTGTCACTCTCTAATGGA CCCAGACCCAAGGTCCAG-3'. For the ZEB1 binding site at −230 bp of human uPA promoter, the upper strand oligonucleotide sequence used is 5′-CACTGGGGCAGGCCCCCGGCCTATT GCATGGGAGGAAGCACGGAG-3'. All three mutations were confirmed by sequencing with BigDye® Direct Cycle Sequencing Kit (Life Technologies-Applied Biosystems) with the followigng primers: 5′-TGGGAGTTTCGGGG TAAGTCCTC-3′ 5'-TAACTTGTACTTTCCC CAGCAGGC-3', and 5'-GGTCTGAGGCAGT CTTAGGCAAGTTGG-3', respectively.

Transcriptional Assays

In transcriptional experiments, cells were transfected with firefly luciferase reporter vectors and equal molar amounts of either cDNA-containing or reference control empty expression vectors. As internal control for transfection efficiency, all points included cotransfection with 0.5 μg pCMV-β-gal. Total DNA was brought to the same amount by adding promoterless pBluescript SK vector. Firefly luciferase activity was assessed with a Luciferase Assay System kit (Promega Corp.), whereas β-galactosidase was determined with Luminiscent β-galactosidase Detection Kit II (Clontech). Relative luciferase activity values (RLU) throughout the article are expressed as the mean of duplicate and are representative of at least four independent experiments.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using EpiQuick ChIP kit (Epigentek) as per manufacturer's instructions. Briefly, SW480 cells were incubated during 10 min with 1% formaldehyde solution (Electron Microscopy) at room temperature followed by incubation with 125 mM glycine. Lysates were sonicated as described (20). Antibodies used for ChIP were as follows: goat anti-ZEB1 (E-20X) and normal goat IgG (JIR). DNA fragments were quantified by qRT-PCR as detailed above. Identification of DNA binding sequences for ZEB1 and design of primers for qRT-PCR was conducted using MacVector software. For the ZEB1 binding site at position -1624 bp of the human uPA promoter, the primers used were as follows: forward 5'-CAGCAAGCACAGAAGT CTCTCCAG-3' and reverse 5'-GAGTGACAG AAGGAAGGCAGGG-3'. For the ZEB1 binding site at position -1480 bp of the human uPA promoter, the primers used were as follows: forward 5'-TTCCTCCTTCCTACCTTCCTGG-3' and reverse 5'-TTCTGTCACGCCCATCTC TTTC-3'. For the ZEB1 binding site at position -230 bp of the human uPA promoter the primers used were as follows: forward 5'-GAAGAAC TGATTAGAGGACCC-3' and reverse 5'-ACA GCGTCTGGACTGAGG-3'. For the amplification of a region of the uPA promoter with "no consensus binding sites" for ZEB1 (referred as NCBS in Figure 2D, -1376/-1223,) the following primers were used: forward 5'-TC CTCCTTCCCACTAAGAGAGC-3' and reverse 5'-CACACACACACACACGCATC-3'.Primers used for amplification of a fragment of the GAPDH promoter are included in the EpiQuick ChIP kit. In all qRT-PCRs, values shown represent relative binding in relation to input and are the average of three independent ChIP experiments, each in triplicate.

CRC cell migration and invasion assays

6.5 mm diameter/8 μm pore polycarbonate membrane Transwell™ inserts (Costar, Corning Inc.) were used directly for migration assays. For invasion assays, these Transwell inserts were first coated overnight with a 1/8 dilution of Matrigel™ matrix (BD Biosciences) in DMEM. Next, 1 x 10^5 cells in 100 μl of DMEM were added to Matrigel-Transwell inserts that were then placed on 24-well plates containing 650 μl of DMEM supplemented with 10% FCS. After incubation at 37°C during 6 h for migration or 24 h for invasion assays, cells on the upper

surface of inserts were removed by washing with PBS and a cotton swab. Migration or invasive cells on the lower side of inserts were detached by incubation (30 min, 37°C) with Trypsin-EDTA, collected and counted by staining with Trypan blue. Results shown are the average of four independent experiments with triplicates of each condition, and cells counted thrice.

Immunohistochemistry and immunofluorescence analysis

Tissue samples were first immunostained by the horseradish peroxidase and 3, 3'-diaminobenzidine (DAB) method and, where indicated, processed for mutiple immunofluorescence analysis. Antigen retrieval was performed with 10 mM sodium citrate (pH 6.0). Slides for immuno-histochemistry were then treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase, whereas slides for immuno- fluorescence staining were first incubated for 30 min with 0.1% sodium borohydride. In either case, slides were next incubated with a non-specific binding blocking solution at 37°C (5% donkey normal serum, 4% BSA and 0.5% Tween 20 in PBS) followed by the corresponding primary (overnight at 4°C) and HRP-conjugated or fluorochrome-conjugated secondary (1 h at 37 °C) antibodies. The immunohistochemistry reaction was developed with a DAB substrate Kit (Vector Labs) before slides were counterstained with hematoxylin and mounted in Di-N-butylPhthalate in Xylene solution (DPX, Sigma). The number of positively stained tumor cells in the CRC tissue array was scored by microscopic analysis at 400X magnification according to the following scale: 0 (0-10 positively stained cells), 1 (11-20), 2 (21-30), 3 (31-40), 4 (41-50), 5 (over 50). Slides for immunofluorescence were mounted in Vectashield-DAPI (Vector Labs). Immunofluorescence was examined in a TCS SP5 Spectral confocal microscope (Leica) at the Microscopy Unit of the University of Barcelona, whose staff is here acknowledged for their technical advice. Images were analyzed using ImageJ software applying a median filter of 0.5–1 pixels. We are also grateful to E Jimenez for help in assembly of immunohistochemistry figures.

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure S1

(A) Expression of ZEB1 associates to uPA in CRC cells. Relative mRNA expression for ZEB1, E-cadherin and uPA were determined in SW480 and HCT116 cells by qRT-PCR respect to GAPDH as reference gene. (B) Stable knockdown of ZEB1 downregulates uPA protein. SW480 cells were stably infected with lentiviral particles encoding shRNAs against ZEB1 (shZEB1) or a control shRNA (shCtl). Cell lysates were immunoblotted for uPA (3689/HD-UK1) or ZEB1 (H-102) along α-tubulin (B5-1-2) as loading control. (C) Stable downregulation of ZEB1 upregulates PAI-1 protein. As in (B), but with cell lysates immunobloted for PAI-1 (H-135). (D) Transient knockdown of ZEB1 inhibits uPA mRNA expression in CRC Colo320 cells. Colo320 cells were transiently transfected with 100 nM of a siRNA control (siCtl) or two specific siRNAs for ZEB1 (si1ZEB1 and si2ZEB1) and relative mRNA levels for uPA and ZEB1 determined by qRT-PCR respect to GAPDH. (E) Stable downregulation of ZEB1 differentially controls uPA and PAI-1 mRNAs. SW480 cells stably interfered with a shRNA control (shCtl) or against ZEB1 (shZEB1) and assessed for mRNA levels by qRT-PCR as described in Supplementary Materials and Methods. (F) Upregulation of endogenous ZEB1 following nuclear translocation of β-catenin increases uPA and decreases PAI-1. Increase in ZEB1 mRNA levels in HCT116 cells in response to treatment with 10 ng/ml LMB for 30 min resulted in

upregulated levels of uPA mRNA and decrease of PAI-1 mRNA. Cells were also subjected for 30 min to the same volume amount of the solvent in which LMB was resuspended (70% methanol, MeOH). Relative mRNA levels of uPA, PAI-1 and ZEB1 were determined by qRT-PCR respect to GAPDH. (G) Upregulation of endogenous ZEB1 following activation of Wnt signaling increases uPA and decreases PAI-1. HTC116 cells were either untreated (Unt) or exposed during 24 h to 100 ng/ml of recombinant human Wnt3a. Relative mRNA levels of uPA, PAI-1 and ZEB1 were determined by qRT-PCR respect to GAPDH.

Supplementary Figure S2

(A) ZEB1 activates uPA transcription in Colo320 cells. Colo320 cells were transfected with 0.5 μg of a luciferase reporter containing 1.9 kb of the human uPA promoter along with 1 μg of empty expression vector or the corresponding equal molar amount of a ZEB1 expression vector. Transcriptional assays were performed as described in Supplementary Materials and Methods. (B) β-catenin activates uPA transcription. As in (A) but using SW480 cells and either 1 μg of an empty expression vector or equal molar amount of the same vector carrying β-catenin cDNA. (C) As in (A) but replacing expression vectors with 200 nM of siRNA control (siCtl) or a siRNA against β-catenin (siβcat).

Supplementary Figure S3

ZEB1 expression decreases PAI-1 mRNA stability. SW480 cells stably transfected with a shRNA control (shCtl) or against ZEB1 (sh1ZEB1) were incubated for the indicated periods with 10 μg/ml of Actinomycin D (Act D) before relative mRNA levels of PAI-1 were analyzed by qRT-PCR respect to GAPDH as reference gene.

Supplementary Figure S4

The uPA inhibitor amiloride blocks ZEB1-mediated invasiveness. (A) SW480 stable cell lines described in Figure 4A were tested for their ability to invade through a Matrigel matrix during 24 h in the presence of either 100 μM of amiloride or equal volume of its solvent (DMSO). Invasion assays were performed as described in Materials and Methods. A t-test for significance of means difference was used. (B) Amiloride reduces uPA expression in SW480 cells. SW480 cells were treated for 24 h with either 100 μM of amiloride or equal volume of DMSO. Cells lysates were assessed by Western blot for expression of ZEB1 (H-102) and uPA (3689/HD-UK1) proteins using α-tubulin (B5-1-2) as loading control.

Supplementary Figure S5

(A) Expression of ZEB1 in the developing intestine. As in Figure 5A, immuno-histochemistry for ZEB1 (Zfhep Ab) in the intestine of *ZEB1* wild type (+/+) and null (-/-) E18.5 mouse embryos. (B) Single staining for uPA (blue, 3689/HD-UK1), ZEB1 (red, E-20) and β-catenin (green, Ab6302) corresponding to tumor front pictures in Figure 5D. uPA was originally detected with Dylight 649 (invisible far red) but converted to blue for representation. DAPI staining is also shown. Scale bars represent 25 μm. (C) As in (B) but corresponding to tumor center pictures in Figure 5D. (D) ZEB1 colocalizes with uPA in invading cancer cells at the tumor front of CRCs. As in Figure 5D but for an alternative (more differentiated area) of the tumor front. (E) As in (B), single staining for uPA, ZEB1 and β-catenin corresponding to tumor front pictures in panel (D).

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Supplementary Figures

for

ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1

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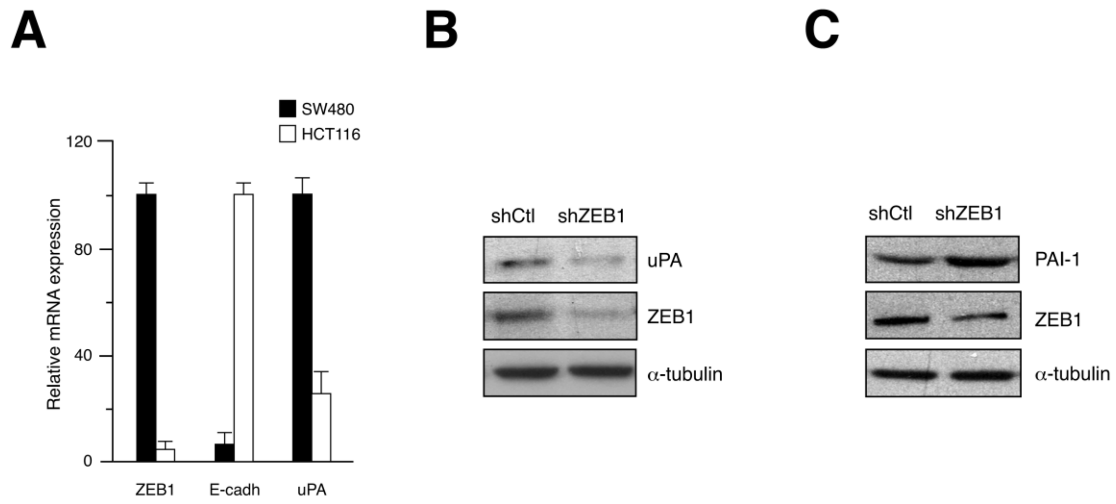
Supplementary Figure S1



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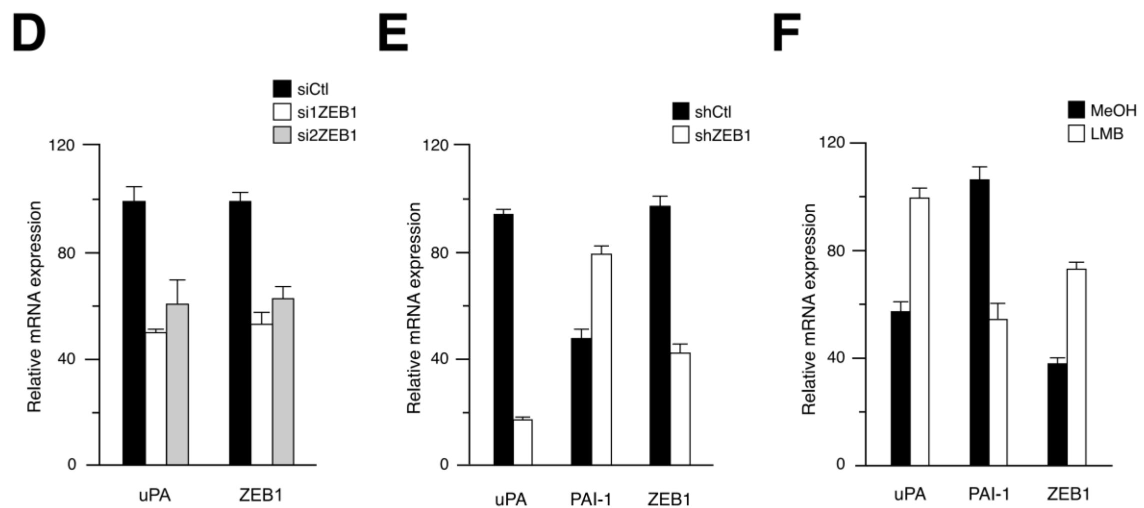

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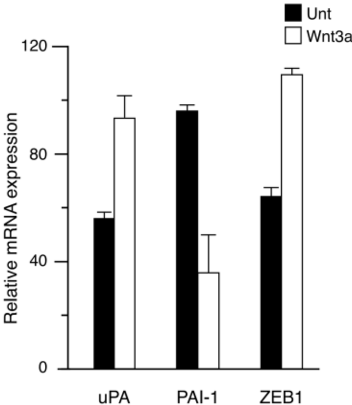

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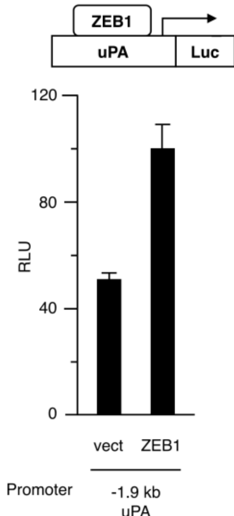

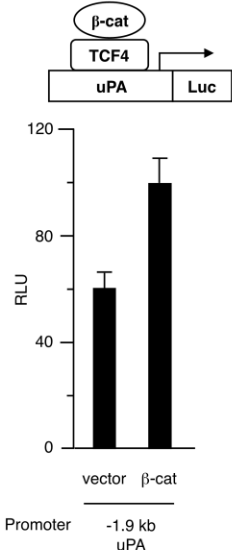

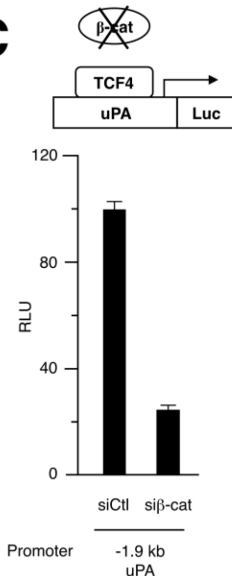

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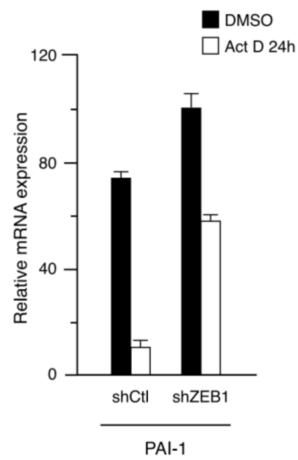

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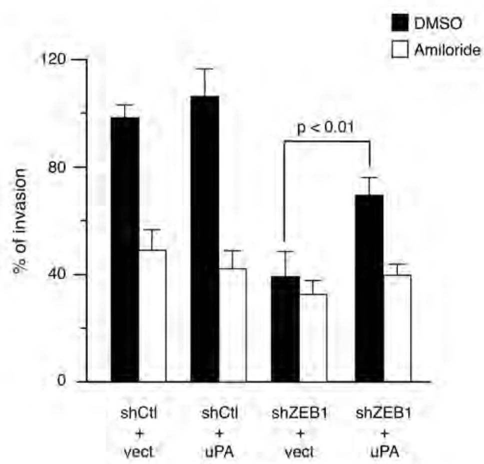

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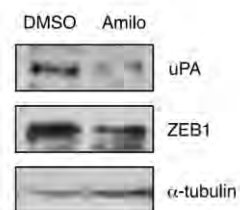

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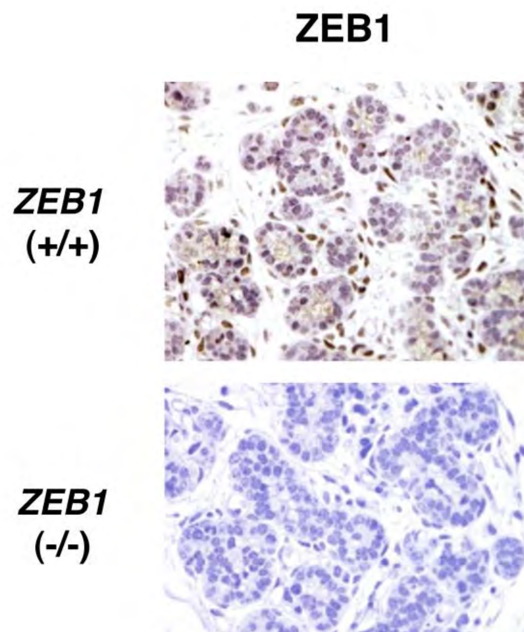

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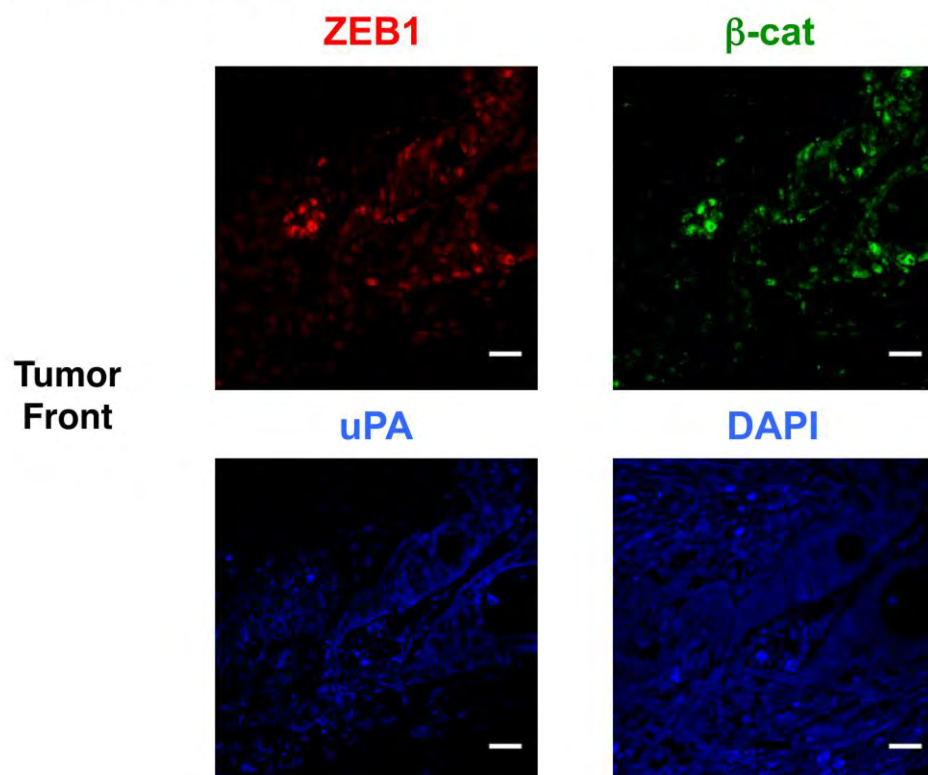

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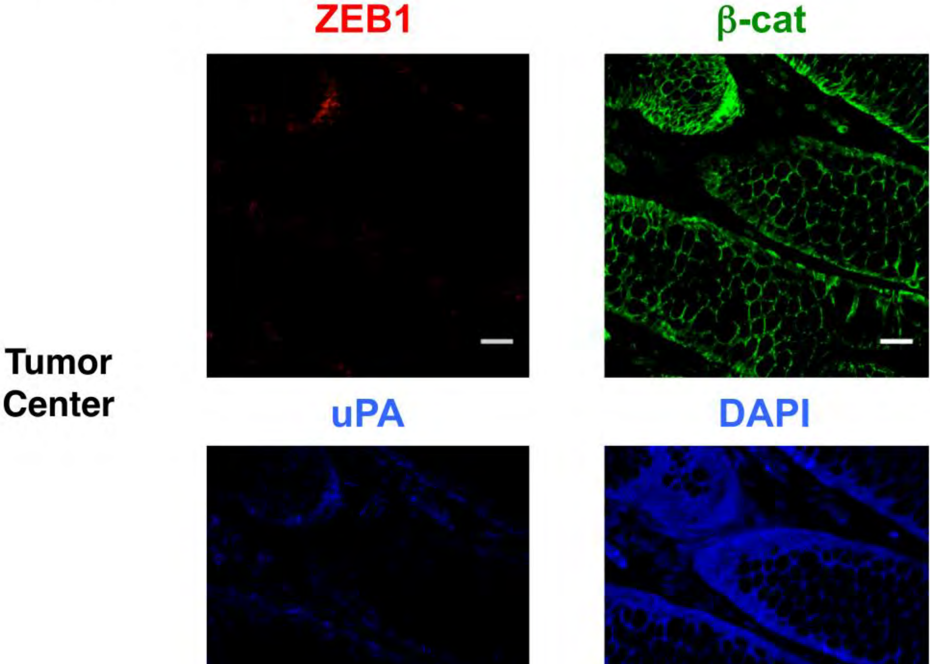

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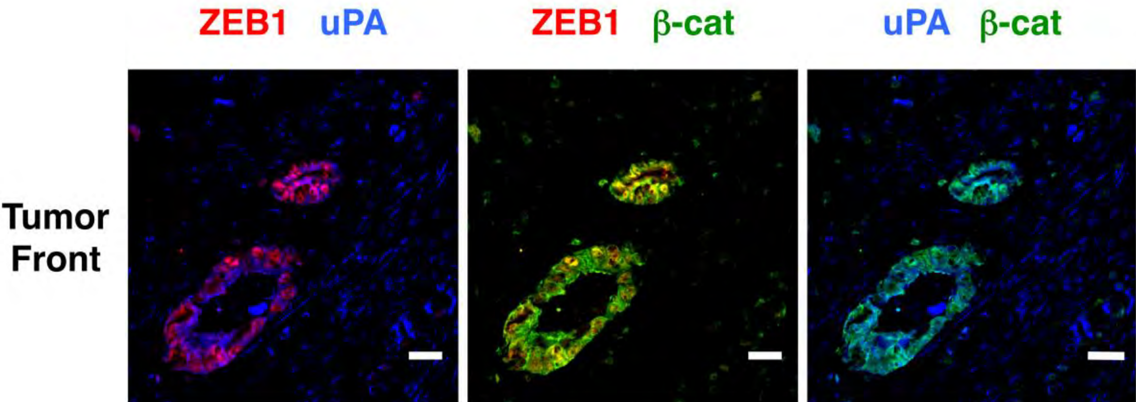

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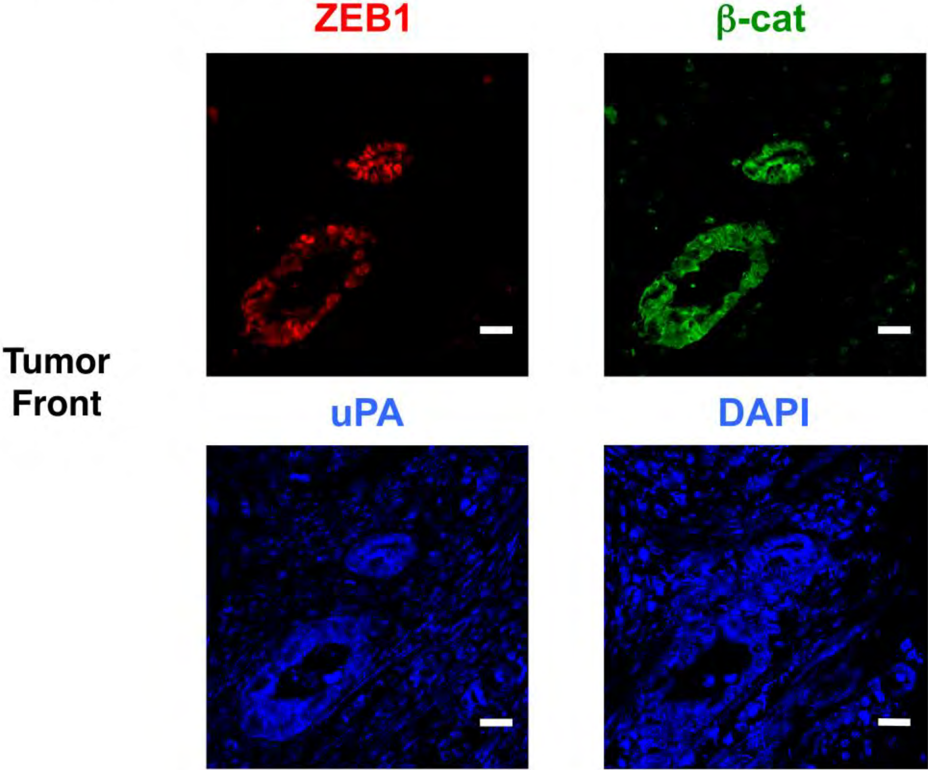

 Gut, 2016 doi:10.1136/gutjnl-2015-310838

ZEB1-induced tumorigenesis requires senescence inhibition through activation of a new ZEB1-DKK1-p53-Mdm2-CtBP pathway to repress macroH2A1

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ORIGINAL ARTICLE

ZEB1-induced tumourigenesis requires senescence inhibition via activation of DKK1/mutant p53/Mdm2/CtBP and repression of macroH2A1

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ABSTRACT

Objective Understand the role of ZEB1 in the tumour initiation and progression beyond inducing an epithelial-to-mesenchymal transition.

Design Expression of the transcription factor ZEB1 associates with a worse prognosis in most cancers, including colorectal carcinomas (CRCs). The study uses survival analysis, in vivo mouse transgenic and xenograft models, gene expression arrays, immunostaining and gene and protein regulation assays.

Results The poorer survival determined by *ZEB1* in CRCs depended on simultaneous high levels of the Wnt antagonist *DKK1*, whose expression was transcriptionally activated by ZEB1. In cancer cells with mutant *TP53*, ZEB1 blocked the formation of senescence-associated heterochromatin foci at the onset of senescence by triggering a new regulatory cascade that involves the subsequent activation of DKK1, mutant p53, Mdm2 and CtBP to ultimately repress macroH2A1 (*H2AFY*). In a transgenic mouse model of colon cancer, partial downregulation of *Zeb1* was sufficient to induce *H2afy* and to trigger in vivo tumour senescence, thus resulting in reduced tumour load and improved survival. The capacity of ZEB1 to induce tumourigenesis in a xenograft mouse model requires the repression of *H2AFY* by ZEB1. Lastly, the worst survival effect of *ZEB1* in patients with CRC ultimately depends on low expression of *H2AFY* and of senescence-associated genes.

Conclusions The tumourigenic capacity of ZEB1 depends on its inhibition of cancer cell senescence through the activation of a herein identified new molecular pathway. These results set ZEB1 as a potential target in therapeutic strategies aimed at inducing senescence.

Significance of this study

What is already known on this subject?

- Aberrant activation of Wnt is involved in the pathogenesis and progression of several human cancers, most notably colorectal carcinomas (CRCs).
- A key target of Wnt is the transcription factor ZEB1, whose expression promotes tumour initiation and progression by triggering an epithelial-to-mesenchymal transition (EMT) that induces a more motile and stem-like phenotype in cancer cells at the tumour invasive front.
- However, the role of ZEB1 in tumour initiation and progression beyond the induction of an EMT remains poorly understood.

What are the new findings?

- The worst survival effect of ZEB1 in patients with CRC depends on high levels of the Wnt antagonist DKK1. ZEB1 transcriptionally activates DKK1 and their expression correlates in human CRCs and ZEB1-deficient mice.
- ZEB1 inhibits senescence in cancer cells through activation of a newly identified ZEB1-DKK1 mutant p53-Mdm2-CtBP pathway to repress macroH2A1.
- ZEB1 and DKK1 associate in Wnt-active cells at the invasive front of CRCs in an inverse pattern with macroH2A1. In a mouse model of CRC, partial downregulation of ZEB1 is sufficient to induce macroH2A1 and trigger in vivo senescence, thus resulting in fewer tumours and better survival. In a xenograft mouse model, the tumourigenic capacity of ZEB1 requires its repression of macroH2A1.
- The worst survival effect of ZEB1 in human patients with CRC ultimately depends on low expression of senescence-inducing genes and macroH2A1.

How might it impact on clinical practice in the foreseeable future?

- The study establishes a new mechanism through which ZEB1 promotes tumour progression and sets ZEB1 as a target in therapeutic strategies aimed at inducing senescence.

INTRODUCTION

Aberrant activation of canonical Wnt signalling, most often by mutations in components of the pathway, is involved in the pathogenesis of numerous cancers.[1] Wnt signalling is also triggered by the engagement of cell surface receptors by Wnt ligands, which prompts the nuclear translocation of β-catenin that cooperates with TCF/LEF factors in the transcriptional activation of Wnt target genes. In turn, Wnt antagonists of the sFRP (sFRP1-sFRP5) and Dickkopf (DKK1-DKK4)

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families inactivate Wnt signalling by sequestering Wnt ligands or binding to Wnt receptors, respectively.[1] Wnt antagonists most commonly function as tumour suppressors and their loss of expression/function promotes tumour growth and invasiveness. However, in certain cancers and stages of tumour progression, some Wnt antagonists can promote oncogenesis.[1 2] Notably, the Wnt antagonist DKK1 is induced by TCF4/β-catenin and is overexpressed in some carcinomas.[3 4]

Senescence involves an irreversible cell cycle arrest and constitutes an important tumour suppressor mechanism.[5] Repression of proliferative genes during senescence is in part achieved through chromosome condensation into senescence-associated heterochromatin foci (SAHF).[6–8] Wnt signalling delays both replicative and oncogene-induced senescence and downregulation of the Wnt3a ligand is both necessary and sufficient to trigger SAHF assembly and to drive several cell types into senescence.[9] Nevertheless, the role of senescence in Wnt-induced cancer initiation and progression is still not fully understood.

A key target of Wnt in tumour progression is the transcription factor ZEB1 (also known as δEF1), whose expression in malignant cells at the invasive front of carcinomas triggers an epithelial-to-mesenchymal transition (EMT), endows cancer cells with a proinvasive and stem-like phenotype, and determines a worse clinical prognosis in most human cancers.[10] ZEB1 can either directly activate or repress gene expression by recruitment of coactivators (eg, p300) or corepressors (eg, CtBP).[10–12] Interestingly, ZEB1 is downstream of several classical pathways involved in oncogenesis (eg, transforming growth factor (TGF)β, Wnt, Ras), whose activity can be in turn regulated by ZEB1.[12–17] Thus, ZEB1 is induced by Wnt signalling and also activates several downstream Wnt target genes.[14 15] The binding of ZEB1 to DNA-binding transcription factors that act as effectors of some of these pathways (eg, R-Smads, TCF4) can turn ZEB1 from a transcriptional repressor into an activator.[12 13 15]

Beyond the induction of an EMT, the mechanisms by which ZEB1 promotes tumour initiation and progression remain to be elucidated. We found here that the maximum effect of *ZEB1* as a determinant of decreased survival in patients with colorectal cancer (CRC) unexpectedly depends on its coexpression with high levels of the Wnt antagonist *DKK1*. We also found that ZEB1 upregulates *DKK1* expression by direct activation of its promoter and that both genes are inversely correlated with senescence-associated genes in expression arrays of human CRCs. ZEB1, through its induction of DKK1, inhibited SAHF formation and expression of senescence-associated genes in cancer cells. In cancer cells with mutant *TP53*, we found that DKK1 upregulates the expression of *TP53*, *MDM2* and *CTBP* and that inhibition of senescence by ZEB1 ultimately depends on the DKK1/mutant p53/Mdm2/CtBP-mediated repression of histone variant macroH2A1 (encoded by *H2AFY*). Of note, activation of this pathway by DKK1 is independent of its role as a Wnt antagonist. ZEB1 and DKK1 display an inverse correlation pattern with macroH2A1 in malignant cells at the tumour front of CRCs. Using a transgenic mouse model of CRC, we found that deletion of a single *Zeb1* allele was sufficient to trigger both macroH2A1 expression and cancer cell senescence that resulted in reduced tumour load and higher survival in *Zeb1* (+/−) mice vis-à-vis *Zeb1* (+/+) littermates. Importantly, in a xenograft mouse model, we found that the capacity of ZEB1 to promote tumourigenesis requires its inhibition of macroH2A1. In parallel, the role of ZEB1 as a determinant of poorer survival in patients with CRC depends on the simultaneous low expression of *H2AFY* and senescence-associated genes.

These results establish the mechanism by which ZEB1, through the subsequent induction of DKK1, mutant p53, Mdm2 and CtBP and the downregulation of macroH2A1, inhibits cancer cell senescence and promotes tumour progression, thus offering a new entry point to interfere with ZEB1 expression and function.

METHODS

Cell lines and cell culture

The origin and culture of cell lines in the study is described in online supplementary information.

Antibodies, plasmids, oligonucleotides and short hairpin RNAs

Description and source of antibodies, plasmids, DNA and RNA oligonucleotides and short hairpin RNA (shRNA) lentivirus used in this study are detailed in online supplementary information. Transient and stable interference of gene expression by small interfering RNA (siRNA) and shRNA are also described in online supplementary information.

Gene expression array data and survival plots

Analysis of the association between *ZEB1* and *DKK1* expression in CRCs and gene signatures associated with different cohorts of patients with CRC is described in online supplementary information. Correlation between the expression of selected genes and relapse-free survival was assessed as detailed in online supplementary information.

Human primary CRC tissue

Paraffin-embedded sections of human primary CRCs were obtained as described in online supplementary information.

Transgenic mouse model of CRC and mouse tumour xenograft model

Description of the transgenic mouse model of CRC is detailed in online supplementary information. Likewise, the set up and analysis of the mouse tumour xenograft model were performed as described in online supplementary information.

Cell viability and senescence assays

Overall cell viability and senescence were assessed by MTT assays and staining for senescent-associated β-galactosidase (SA β-gal), respectively, as detailed in online supplementary information.

Determination of protein and RNA expression and transcriptional assays

Analysis of protein expression by immunostaining or Western blot is described in online supplementary information. Quantitative real-time PCR, mutagenesis of promoters as well as chromatin immunoprecipitation (ChIP) and transcriptional assays were performed as described in online supplementary information.

Statistical analysis

Statistical analysis of the data shown in this study was performed as described in online supplementary information.

RESULTS

ZEB1's role as a determinant of worse cancer survival depends on the joint coexpression of the Wnt antagonist *DKK1*

ZEB1 has an independent prognostic value for decreased survival in a number of cancers.[14] Since ZEB1 directly activates

several Wnt target genes, we hypothesised that ZEB1 would inhibit the expression of Wnt antagonists and decided to explore first whether ZEB1 and Wnt antagonists have opposing effects on survival of patients with CRC.

Examination of the relapse-free survival associated with *ZEB1* and Wnt antagonists in gene expression arrays of CRCs[18 19] revealed that, as expected, high expression of *ZEB1* correlated with poorer survival. In turn, expression of Wnt antagonists was either associated with better prognosis (*sFRP3*) or had no significant association with survival (see online supplementary table S1). Unexpectedly, we found that *DKK1* and *sFRP4* were associated with poorer prognosis, indicating a potential tumour-promoting role of these Wnt antagonists in CRCs. Given that DKK1, like ZEB1, is induced by canonical Wnt signalling,[3] we investigated whether the joint expression of *DKK1* and *ZEB1* affects overall survival in CRCs. Interestingly, using survival data for 928 patients from published databases (see Methods), we found that patients displaying high expression of both *DKK1* and *ZEB1* had a lower survival rate than those with high levels of *ZEB1* but low *DKK1* (figure 1A). In other words, the maximum effect of *ZEB1* as a predictor of reduced survival requires high levels of *DKK1*. This suggests that, at least for some of its tumour-promoting functions, *ZEB1* depends on *DKK1* expression.

In light of these results, we next investigated whether downregulation of ZEB1 and/or DKK1 affects overall cell viability of SW480 cells, a commonly used Wnt-active CRC cell line, which harbour a mutation in *APC* at 1338 bp that results in a stop codon and a truncated protein, methylated p16^{INK4A} (*CDKN2A*), phosphorylated Rb protein and mutant *TP53* (R273H and P309S). Compared with an siRNA control (siCtl), knockdown of either *ZEB1* or *DKK1* with specific siRNAs (si*ZEB1* and si*DKK1*, respectively) reduced overall cell viability in SW480 CRC cells (figure 1B and online supplementary figure S1A). Simultaneous interference of *ZEB1* and *DKK1* did not have an additional effect over *ZEB1* knockdown alone

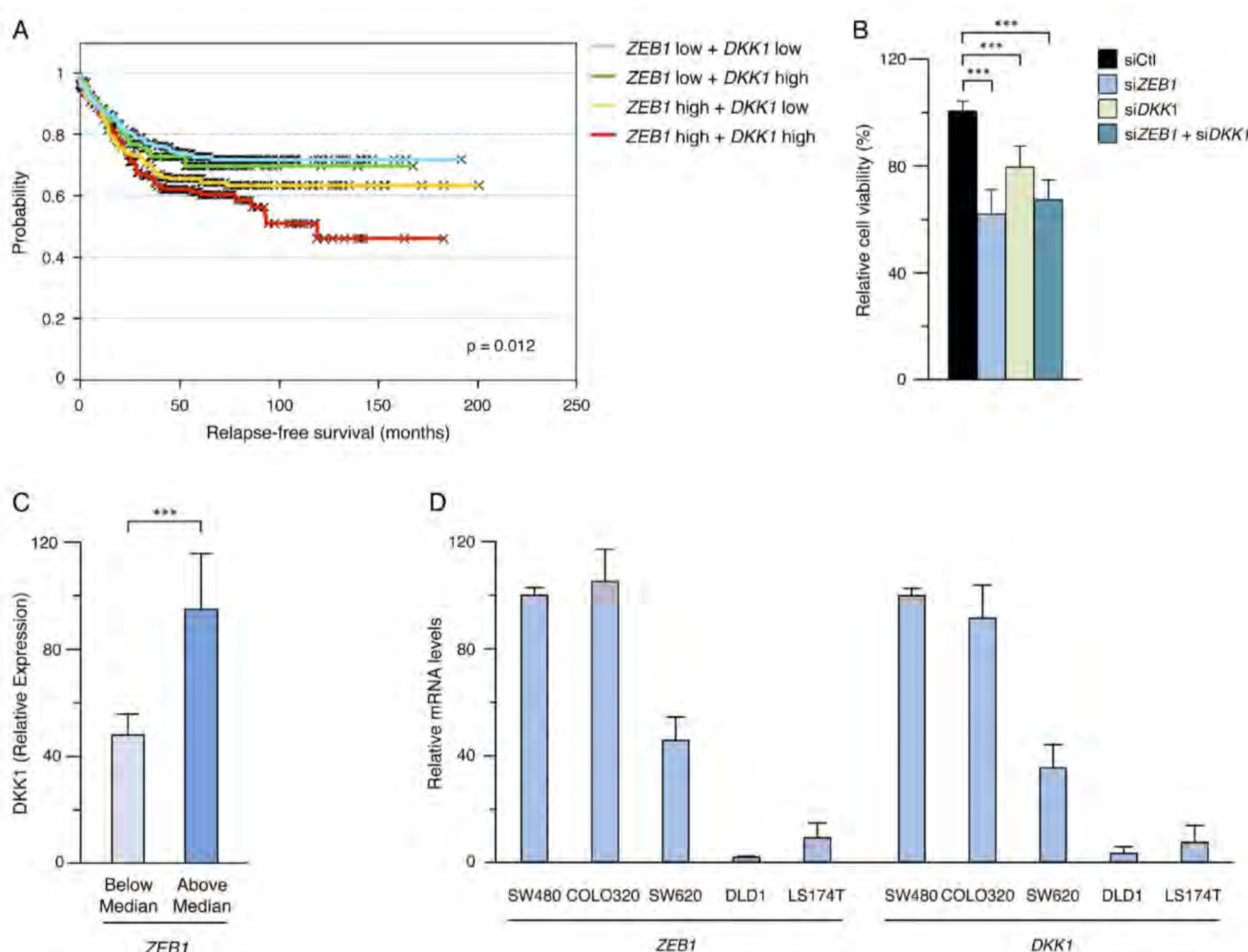

Figure 1 *ZEB1* and *DKK1* expression associate in colorectal carcinomas (CRCs) where the maximum effect of *ZEB1* as a factor of poorer survival depends on its simultaneous expression with *DKK1*. (A) Patients with the highest expression of *ZEB1* and *DKK1* (red line) have shorter relapse-free survival than those with high expression of *ZEB1* but low expression of *DKK1* (yellow line) that in turn have worse survival than those with high *DKK1* but low *ZEB1* (green line) or that display low levels of both genes (blue line). The survival probability of patients with CRC was represented in a Kaplan-Meier plot according to their median expression of *ZEB1* and *DKK1* (see Methods). (B) Expression of *ZEB1* and *DKK1* determines overall cell viability in CRC cells. *ZEB1* and *DKK1* were knocked down in SW480 cells by transient transfection of 100 nM of specific small interfering RNA (siRNAs) against *ZEB1* (si*ZEB1*-A) and/or *DKK1* (si*DKK1*) or transfected with 100–200 nM of a control siRNA (siCtl). Cell viability was determined at 96 hours by an MTT assay. (C) *ZEB1* and *DKK1* expression associate in CRCs. Expression of *ZEB1*—segregated by the median—in gene arrays of CRCs (see Methods) correlated positively with *DKK1* levels. (D) *ZEB1* and *DKK1* expression correlate in a panel of CRC cell lines. SW480, COLO320, SW620, DLD1 and LS174T CRC cells were assessed for *ZEB1* and *DKK1* mRNA levels by quantitative real-time PCR with respect to *GAPDH*.

suggesting that in cell viability—although not in relapse-free survival—DKK1 may act in the same functional pathway as ZEB1 (figure 1B). We next tested whether recombinant human DKK1 (rhDKK1) was able to revert the effect of si*DKK1* on cell viability. Despite the fact that SW480 cells have a truncated APC, they are still responsive to Wnt ligands and antagonists.[20] As control we used recombinant human DKK3 (rhDKK3), a protein homologous to DKK1 that through a different receptor and mechanism also reduces nuclear β-catenin in SW480 cells and, as other Wnt antagonists, can either activate or repress Wnt-mediated signalling depending on the cell type.[21 22] We found that although both rhDKK1 and rhDKK3 displayed similar effectiveness blocking Wnt signalling in SW480 cells, only rhDKK1 reverted the effect of si*DKK1* on cell viability (see online supplementary figures S1B and S1C).

We then examined whether *ZEB1* and *DKK1* were correlated in primary CRCs. Examination of 1557 cases from expression arrays of CRCs (see Methods) revealed that high levels of *ZEB1* associated with increased expression of *DKK1* and *vice versa* (figure 1C and online supplementary figure S1D). This positive correlation between *ZEB1* and *DKK1* was also observed in a panel of CRC cell lines (figure 1D). SW620 cells originated from CRC metastasis in the same patient than SW480 cells and although they carry identical mutations they have epigenetic differences. Expression of *DKK1* did not associate with *ZEB2*, a second member of the ZEB family and highly homologous to *ZEB1*[23] (see online supplementary figure S1E). Altogether, these results indicate that *ZEB1* and *DKK1* are coexpressed in CRCs, where they jointly determine a worse prognosis.

ZEB1 transcriptionally activates *DKK1* expression

The above data prompted us to investigate whether ZEB1 may activate DKK1 expression. We first examined whether deletion of the *Zeb1* gene alters *Dkk1* expression in the intestinal tract of *Zeb1*-deficient mice. Compared with wild-type littermates, *Dkk1* expression was reduced in the intestinal tract of *Zeb1* (+/−) mice—*Zeb1* (−/−) mice die perinatally[24]—corroborating the in vivo association between both genes (figure 2A).

We then examined the effect on endogenous *DKK1* expression of knocking down endogenous *ZEB1* in three CRC cell lines expressing high levels of ZEB1. Compared with siCtl, transient knockdown of *ZEB1* with two specific siRNAs (si*ZEB1*-A and si*ZEB1*-B) resulted in the downregulation of endogenous *DKK1* mRNA levels (figure 2B, *left panel*, and online supplementary figures S2A and S2B). Similar downregulation of *DKK1* was observed when *ZEB1* was stably knocked down in SW480 cells using two different shRNAs (to generate SW480-sh*ZEB1*-A and SW480-sh*ZEB1*-B cells) and compared with cells transfected with an shRNA control (SW480-shCtl cells) (figure 2B, *right panel*). In contrast, knockdown of *ZEB2* in ZEB2-positive COLO320 CRC cells did not affect *DKK1* mRNA levels (see online supplementary figure S2C). Positive regulation of DKK1 by ZEB1 was also examined at the protein level. Compared with control siRNA or shRNA, expression of DKK1 protein was downregulated upon transient and stable knockdown of *ZEB1* in SW480 CRC cells (figure 2C, *left* and *right panels*, respectively). Altogether, these results indicate that ZEB1 induces DKK1 expression in CRC cell lines.

ZEB1 binds to a subset of E-box and E-box-like sequences in the regulatory regions of its target genes.[10] We therefore investigated whether ZEB1-mediated induction of DKK1 occurs at the transcriptional level. Examination of the first 1 kb of the human *DKK1* promoter revealed the existence of several ZEB1 consensus binding sites including two high-affinity sequences at −490 and −880 bp. Accordingly, the transcriptional activity of serial deletions of the *DKK1* promoter increased when the site at −490 bp was included (see online supplementary figure S2D). The ability of endogenous ZEB1 to directly bind to the *DKK1* promoter was tested for the site at −490 bp by ChIP assay. An antibody against ZEB1, but not its respective matched specie IgG control, immunoprecipitated a region of the human *DKK1* promoter containing the site at −490 bp, but not a region lacking consensus binding sites for ZEB1 (figure 2D). ZEB1 also failed to bind to the *GAPDH* promoter, whose expression is not regulated by ZEB1 (figure 2D).

To test the functionality of the ZEB1 binding site at −490 bp, this sequence was mutated within the −535 bp *DKK1* promoter reporter to a sequence known not to bind ZEB1 and the resulting mutant *DKK1* promoter ($DKK1_{ZEB1mut}$) was compared with the wild-type *DKK1* promoter ($DKK1_{wt}$) for its regulation by ZEB1. Mutation of the ZEB1 site in $DKK1_{wt}$ promoter reduced its basal activity in SW480 and SW620 CRC cells (figure 2E and online supplementary figure S2E). Transient and stable knockdown of *ZEB1* in both CRC cell lines resulted in a downregulation of the activity of $DKK1_{wt}$ but not of $DKK1_{ZEB1mut}$ promoter (figure 2E, *left* and *right panels*, respectively, and online supplementary figure S2E). We also examined the response of the wild-type and mutant versions of the *DKK1* promoter to ZEB1 overexpression in SW480 and SW620 cells. While exogenous ZEB1 further activated transcription of the $DKK1_{wt}$ promoter, it had no effect on $DKK1_{ZEB1mut}$ promoter (figure 2F and online supplementary figure S2F).

ZEB1 activates transcription through recruitment of the p300 histone acetyltransferase to its N-terminal region.[12 13] Overexpression of ZEB1 upregulates Wnt target genes in a p300-dependent manner in cells with active Wnt signalling, but not in cells where this pathway is inactive.[15] Therefore, we investigated the involvement of p300 in ZEB1-mediated activation of *DKK1*—that as noted earlier is a direct Wnt target—in SW480 and SW620 cells, two Wnt-active CRC cell lines that therefore express high levels of ZEB1 (figure 1D). A version of p300 where its cDNA (*EP300*) is fused to the activation domain of the herpes simplex virus VP16 protein (*VP16-p300*) enhanced ZEB1-induced activation of $DKK1_{wt}$ promoter (figure 2G). In contrast, deletion of the N-terminal region of ZEB1 (*ZEB1*ΔNterm) eliminated most of the transcriptional activation effect of ZEB1 on the $DKK1_{wt}$ promoter (figure 2G). The recruitment of p300 by full-length ZEB1 was confirmed by a ChIP assay where a p300 antibody, but not a matched specie IgG control, precipitated the fragment of the *DKK1* promoter harbouring the ZEB1 binding site at −490 bp, but not a fragment of this promoter lacking ZEB1 binding sites (figure 2H).

ZEB1 and TCF4 mutually modulate their transcriptional activities in the regulation of selected Wnt target genes,[15] and we found here that both transcription factors cooperate in the regulation of the *DKK1* promoter in SW480 and SW620 CRC cells (figure 2I and online supplementary figure S2G). Altogether, the above results demonstrate that ZEB1 binds to the *DKK1* promoter to directly drive its transcription through a mechanism involving p300 and in cooperation with TCF4.

ZEB1, through its induction of DKK1, inhibits senescence-associated genes

The 1557 cases of CRC in figures 1C and online supplementary figure S1D were classified in four cohorts based on their expression of *ZEB1* and *DKK1* above or below the upper quartile. Analysis of the gene signature of these cohorts revealed that,

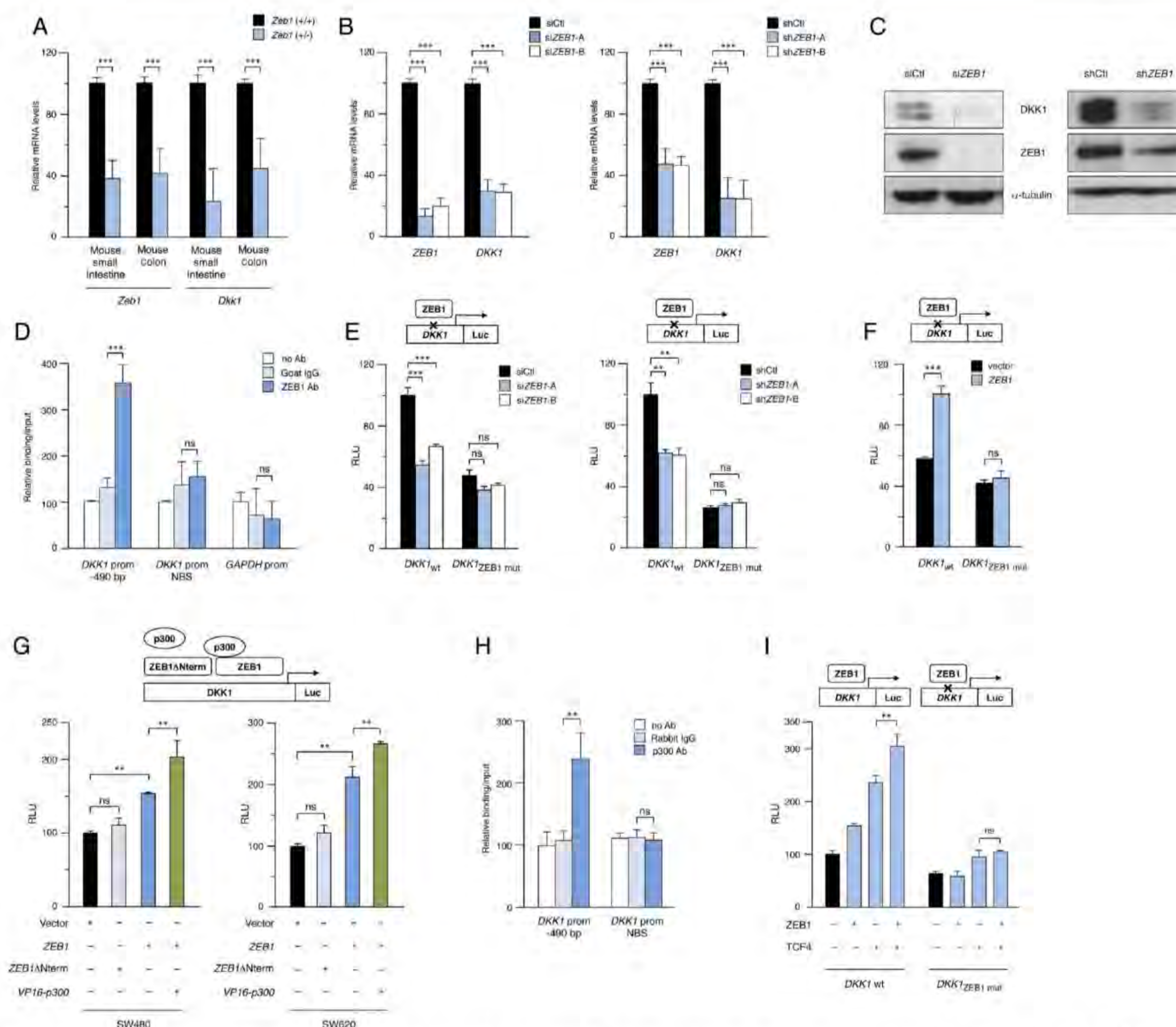

Figure 2 ZEB1 transcriptionally activates *DKK1* expression by direct binding to its promoter. (A) In vivo expression of *Dkk1* depends on *Zeb1*. mRNA levels for *Zeb1* and *Dkk1* in small intestine and colon from *Zeb1* (+/+) and (+/−) mice aged 3 months. Results shown are the average of four mice for each genotype. (B) Knockdown of endogenous *ZEB1* downregulates endogenous *DKK1* mRNA in colorectal carcinoma (CRC) cells. *Left panel*: SW480 cells were transiently transfected with 100 nM of siRNAs against *ZEB1* (si*ZEB1*-A and si*ZEB1*-B) or siCtl. Relative mRNA levels of *ZEB1* and *DKK1* were determined by quantitative real-time (qRT)-PCR. *Right panel*: as in left panel but using SW480 cells stably infected with an shRNA control (shCtl) or two independent sets of shRNAs against *ZEB1* (sh*ZEB1*-A and sh*ZEB1*-B). (C) Knockdown of endogenous *ZEB1* protein downregulates endogenous DKK1 protein in CRC cells. As in (B), SW480 cells were transiently (si*ZEB1*-A, *left panel*) or stably (sh*ZEB1*-A, *right panel*) knocked down for *ZEB1*. Cell lysates were then blotted for DKK1 (clone 2A5) and ZEB1 (H-102) along with α-tubulin (B5-1-2) as loading control. (D) ZEB1 binds to the *DKK1* promoter. ChIP assays using DNA from SW480 cells immunoprecipitated without antibody (Ab) or with Abs against ZEB1 (E-20X) or goat IgG control. Regions from the *DKK1* promoter—either lacking consensus binding sites for ZEB1 (non-binding site (NBS) −757/−631 bp) or containing a consensus ZEB1 binding site at −490 bp—or from the *GAPDH* promoter were amplified by qRT-PCR. For each promoter fragment, the condition without Ab was equalled to 100. (E) Knockdown of *ZEB1* downregulates *DKK1* transcription. *Left panel*: SW480 cells transiently knocked down for *ZEB1* (si*ZEB1*-A and si*ZEB1*-B) or transfected with siCtl were co-transfected with 0.25 μg of either wild-type *DKK1* promoter ($DKK1_{wt}$) or a version of it where the ZEB1 binding site at −490 bp has been mutated ($DKK1_{ZEB1mut}$). In all panels of this figure, transcription is assessed as relative luciferase units(RLU). *Right panel*: as in left panel but using SW480 cells stably interfered for *ZEB1* (sh*ZEB1*-A and sh*ZEB1*-B) or infected with an shRNA control (shCtl). (F) Overexpression of *ZEB1* activates *DKK1* transcription. As in (E), SW480 cells were transfected with either $DKK1_{wt}$ or $DKK1_{ZEB1mut}$ but were then cotransfected with 0.25 μg of an empty expression vector or the corresponding equal molar amount of the same vector encoding full-length *ZEB1*. (G) Transcriptional activation of the *DKK1* promoter by ZEB1 involves p300. SW480 cells were transfected with 0.25 μg of $DKK1_{wt}$ promoter along with 0.25 μg of an empty expression vector or the corresponding equal molar amount of full-length *ZEB1* or a version of *ZEB1* lacking the N-terminal region (*ZEB1*ΔNterm) in the presence or absence of 0.5 μg of *VP16-p300*. (H) p300 binds to the *DKK1* promoter in a ZEB1-dependent manner. ChIP assays using DNA from SW480 cells immunoprecipitated without Ab or with Abs against p300 (N-15) or rabbit IgG serum control. The NBS region used in (D) was included as control. For each promoter fragment, the condition without Ab was equalled to 100. (I) ZEB1 and TCF4 cooperate in the regulation of *DKK1*. As in (E), SW480 cells were transfected with 0.25 μg of either $DKK1_{wt}$ or $DKK1_{ZEB1mut}$ reporters and 0.25 μg of the expression vectors for ZEB1 and/or TCF4 (denoted by '+') or equimolar amounts of their corresponding empty vectors (denoted by '−').

compared with CRCs where both *ZEB1* and *DKK1* were above the upper quartile (cohort 1), 429 genes were upregulated when the expression of either *ZEB1* or *DKK1* was below that quartile (cohorts 2 and 3, respectively) (see online supplementary table S2). The expression of these genes was further increased when both *ZEB1* and *DKK1* levels were in the three lower quartiles (cohort 4). Interestingly, within this subset of genes upregulated when *ZEB1* and *DKK1* expression was low, around 10% of them were associated with senescence, suggesting that ZEB1 and DKK1 may cooperate in the repression of cellular senescence (figure 3A).

We therefore validated the potential regulation by *ZEB1* and/or *DKK1* of some of these senescence-related genes. To that effect, SW480-shCtl, SW480-sh*ZEB1*-A and SW480-sh*ZEB1*-B cells used in previous figures were stably transfected with an expression vector for *DKK1* (to generate SW480-shCtl+*DKK1*, SW480-sh*ZEB1*-A+*DKK1* and SW480-sh*ZEB1*-B+*DKK1* cell lines, respectively) or their corresponding empty vector equivalents (to generate SW480-shCtl+vector, SW480-sh*ZEB1*-A +vector and SW480-sh*ZEB1*-B+vector cell lines, respectively) (see online supplementary figure S3A for *ZEB1* and *DKK1* levels in these stable cell lines).

The upregulation of senescence-associated genes in CRCs with low expression of *ZEB1* and/or *DKK1* (figure 3A) suggested that ZEB1 and DKK1 are repressing their expression. Using these stable cell lines, we validated the regulation by ZEB1 and/or DKK1 of several of these senescence-associated genes. Namely, histone variant macroH2A1 (encoded by *H2AFY*)—an integral component of SAHF and whose knockdown blocks SAHF formation and cellular senescence,[6] *GLB1*—that encodes lysosomal β-galactosidase and whose mRNA levels as well as enzymatic activity at pH 6.0 increase in senescent cells, commonly referred as SA β-gal activity[25] [26]—*RPL5*, *EIF3K*, *EHMT2* and *AATF*.[27–30] Indeed, we found that mRNA

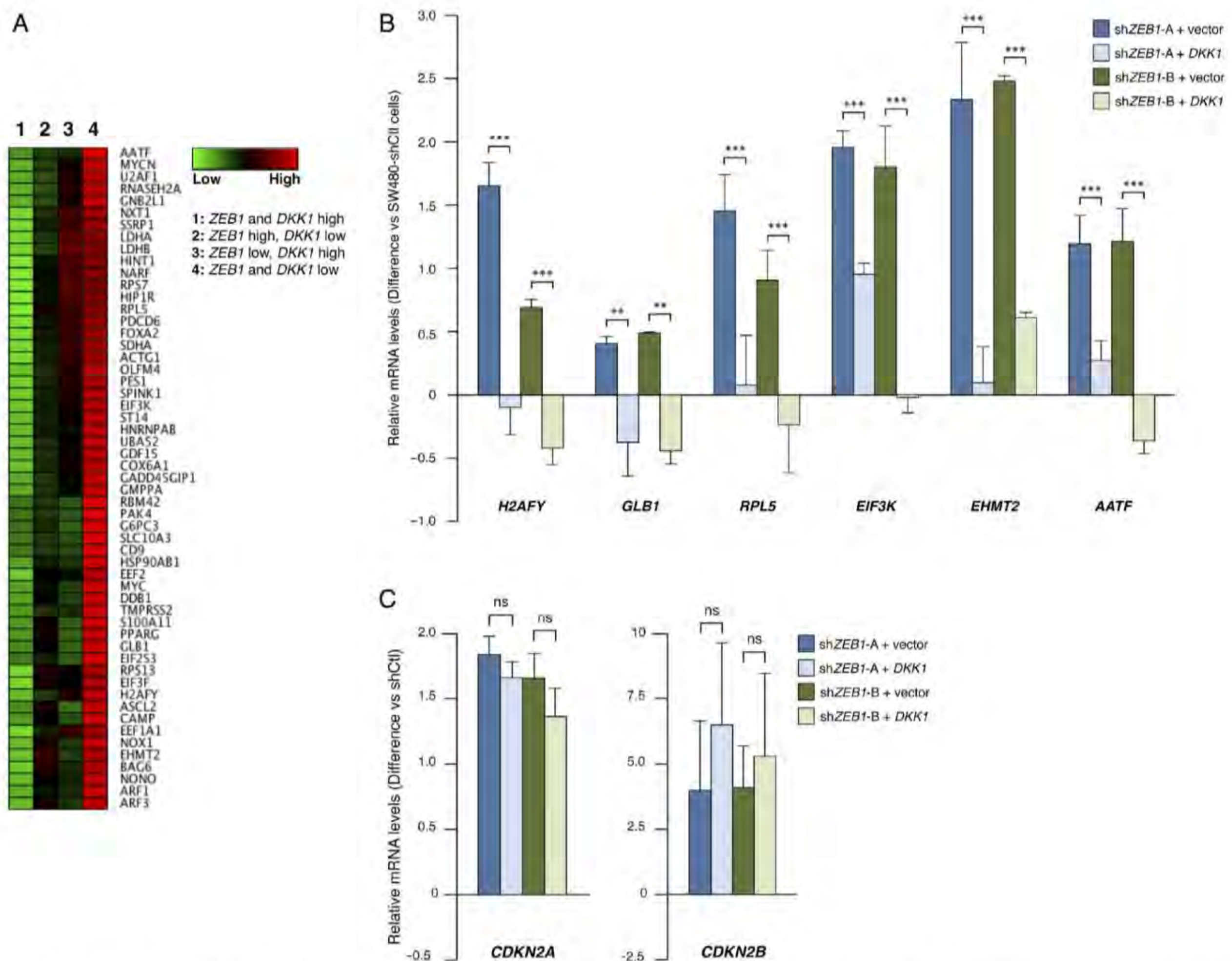

Figure 3 *ZEB1* and *DKK1* cooperate in repressing a senescence-associated gene signature. (A) Heat map of senescence-associated genes upregulated by low levels of *ZEB1* and/or *DKK1*. A total of 1157 colorectal carcinomas (CRCs) from gene arrays (see Methods) were classified in four cohorts according to their levels of *ZEB1* and/or *DKK1* expression. Cohort 1: expression of both genes in the upper quartile; cohort 2: *ZEB1* in upper quartile, *DKK1* below Q4; cohort 3: *DKK1* in the upper quartile, *ZEB1* below Q4; cohort 4: both genes below Q4. (B) *ZEB1* and *DKK1* repress senescence-associated genes. Stably interfered/transfected SW480 cell lines—namely, SW480-shCtl+vector, SW480-sh*ZEB1*-A+vector, SW480-sh*ZEB1*-A+*DKK1*, SW480-sh*ZEB1*-B+vector and SW480-sh*ZEB1*-B+*DKK1*—were assessed for relative mRNA levels of selected senescence-associated genes by quantitative real-time (qRT)-PCR. Data are represented as the difference in relative mRNA levels between each of the cell lines and shCtl-infected cells. (C) *ZEB1* represses *CDKN2A* and *CDKN2B* independently of *DKK1*. Stable SW480 cells in (B) were assessed for relative mRNA levels of *CDKN2A* ($p16^{INK4a}$) and *CDKN2B* ($p15^{INK4b}$) by qRT-PCR. Data are represented as the difference in relative mRNA levels between each cell line and shCtl-infected cells.

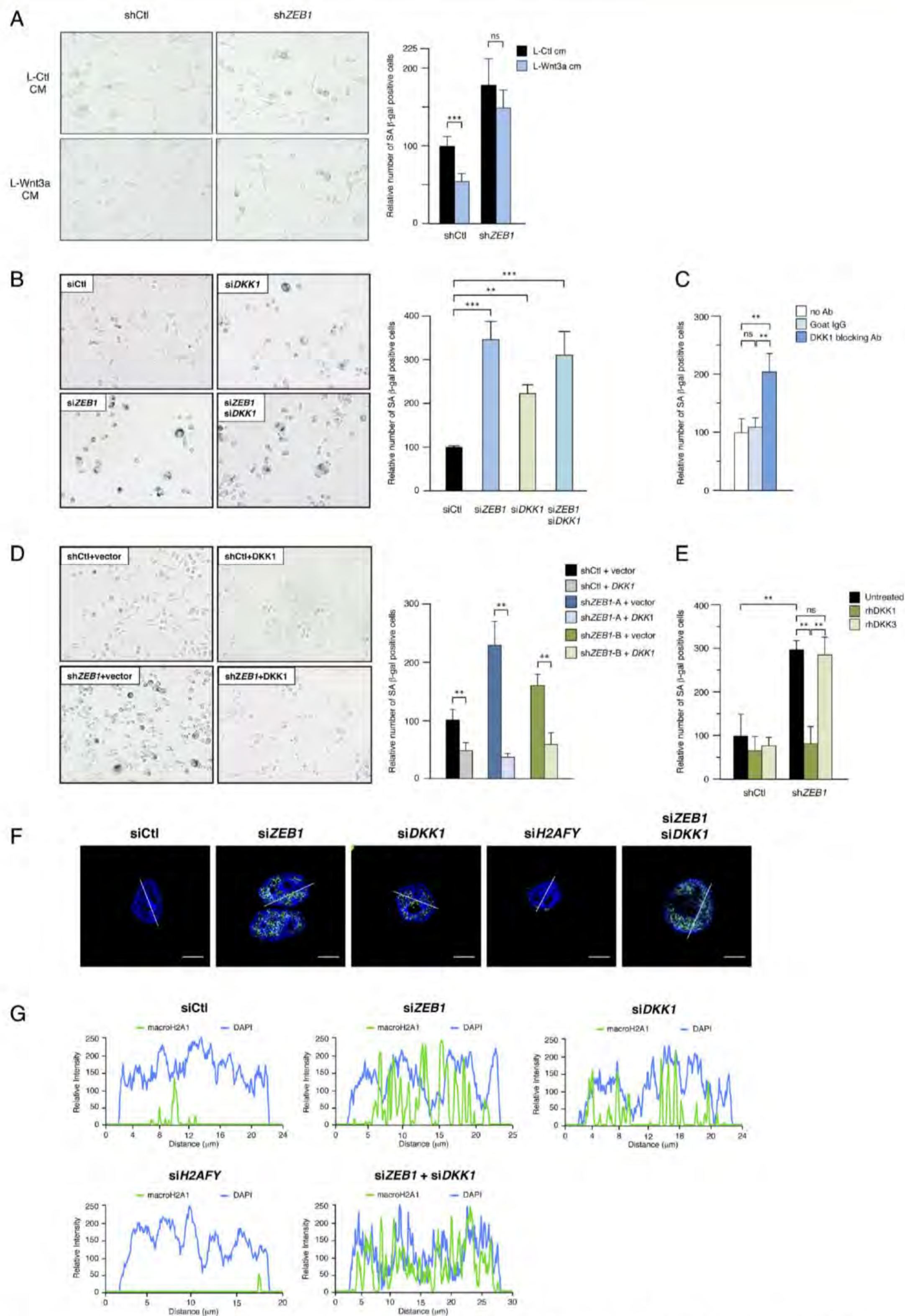

Figure 4 ZEB1, through its induction of DKK1, inhibits senescence-associated heterochromatin foci (SAHF) formation and cell senescence. (A) ZEB1 mediates Wnt-induced inhibition of cellular senescence. SW480-shCtl and SW480-sh*ZEB1*-A cells were cultured for 72 hours in the presence of conditioned medium from L cells stably transfected with a vector encoding recombinant Wnt3a (L-Wnt3a cm) or its corresponding empty vector (L-Ctl cm). Senescence was assessed by staining for senescent associated β-galactosidase (SA β-gal). Representative pictures are displayed in the *left panel* and quantification of the relative number of SA β-gal-positive cells from four independent experiments is shown in the *right panel*, where the first condition was arbitrarily set to 100. (B) ZEB1 and DKK1 inhibit senescence in colorectal carcinoma (CRC) cells. SW480 cells transiently knocked down with si*ZEB1*-A and/or si*DKK1* were assessed for SA β-gal staining. As in (A), representative pictures are shown in the *left panel* and

levels for all these genes increased in SW480-sh*ZEB1*-A and SW480-sh*ZEB1*-B cells with respect to SW480-shCtl cells (figure 3B). In turn, overexpression of *DKK1* reverted the effect of *ZEB1* knockdown (figure 3B).

Cell cycle inhibitors p16^{INK4A} (*CDKN2A*) and p15^{INK4b} (*CDKN2B*) are upstream of Rb1 and accumulate in senescent cells.[5] Although *CDKN2A* is methylated in SW480 cells, expression of *CDKN2A* and *CDKN2B* was upregulated in SW480 cells knocked down for *ZEB1* (SW480-sh*ZEB1*-A+vector and SW480-sh*ZEB1*-B+vector) compared with SW480-shCtl +vector cells (figure 3C). In that line, *CDKN2A* and *CDKN2B* are also repressed by ZEB1 in lung epithelial cells and mouse embryo fibroblasts (MEFs).[13 31] In contrast, overexpression of *DKK1* in SW480-sh*ZEB1*-A+*DKK1* and SW480-sh*ZEB1*-B +*DKK1* cells failed to revert this increase, suggesting that *CDKN2A* and *CDKN2B* are regulated by ZEB1 but not by DKK1.

ZEB1, through its induction of DKK1, inhibits SAHF formation and cell senescence

Downregulation of Wnt3a is both necessary and sufficient for the translocation of the chaperone HIRA to nuclear bodies and accelerates the assembly of SAHF, which are required to trigger senescence.[9] Since ZEB1 is both a target and an effector of Wnt signalling, we first tested whether ZEB1 could mediate Wnt3a-induced inhibition of senescence in SW480 CRC cells. As noted earlier, SW480 cells harbour a stop codon mutation in *APC*. Nevertheless, truncated forms of the APC protein can still bind β-catenin and cells carrying them are responsive to Wnt ligands.[20] SW480-shCtl and SW480-sh*ZEB1*-A cells were cultured in the presence or absence of Wnt3a and their senescence was assessed by staining for SA β-gal activity.[25] We found that *ZEB1* knockdown increased the number of SA β-gal-positive cells and that Wnt3a inhibited basal SA β-gal activity in SW480-shCtl cells but not in SW480-sh*ZEB1*-A cells (figure 4A). ZEB1 interference yielded similar results in SW620 CRC cells treated with Wnt3a (see online supplementary figure S4A). Activation of Wnt signalling by Wnt3a was confirmed by the upregulation of Wnt targets *ZEB1*, *DKK1* and *AXIN2* (see online supplementary figure S4B). These results indicate that ZEB1 mediates the Wnt-induced inhibition of senescence and that *ZEB1* knockdown triggers senescence in CRC cells.

We next investigated whether DKK1 also inhibits senescence in cancer cells. SW480 cells were transiently interfered with siCtl, specific siRNAs for either *ZEB1* (si*ZEB1*-A) or *DKK1* (si*DKK1*) or a combination of si*ZEB1*-A and si*DKK1* and then examined for SA β-gal staining. Compared to siCtl, single interference of either *ZEB1* or *DKK1* resulted in an increase in the number of SA β-gal-positive cells (figure 4B). Induction of senescence by si*DKK1* was reverted by rhDKK1, but not by rhDKK3 (see online supplementary figure S4C). These results indicate that inhibition of senescence by DKK1 occurs independently of its role as a Wnt antagonist since both rhDKK1 and rhDKK3 were equally efficient blocking Wnt signalling (ref 22 and online supplementary figure S1B). The number of senescent cells following joint knockdown of both *ZEB1* and *DKK1* was similar to that observed with just single *ZEB1* knockdown (figure 4B), thus suggesting that DKK1 inhibits senescence through the same functional pathway as that by ZEB1. Lastly, the induction of senescence by si*DKK1* was corroborated with an anti-DKK1 blocking antibody (figures 4C and online supplementary figure S4D).

We then examined whether exogenous overexpression of DKK1 could revert the senescence induced by *ZEB1* knockdown. To that effect, the stable SW480 cells used in figures 3B,C were assessed for senescence by SA β-gal staining. Like in transient interference, stable knockdown of *ZEB1* increased the number of SA β-gal-positive cells that, interestingly, was reverted by overexpression of *DKK1* (figure 4D). The increase in SA β-gal-positive cells induced by sh*ZEB1* was reverted by rhDKK1 but not by rhDKK3 (figure 4E and online supplementary figure S4E). Once again, the failure of DKK3 to inhibit senescence indicates that DKK1 mediates ZEB1-induced inhibition of senescence through a mechanism that is independent of its role as Wnt antagonist. It is worth noting that since SW480 cells are Wnt-active and express high levels of ZEB1, exogenous *DKK1* also inhibited basal levels of senescence in shCtl cells. The above data allow us to conclude that inhibition of senescence by ZEB1 is mediated, at least in part, by activating DKK1 expression.

Lastly, we examined whether manipulation of ZEB1 and/or DKK1 expression alters SAHF formation triggered by macroH2A1. We found that knockdown of *ZEB1* or *DKK1* increased the assembly of SAHF (figures 4F,G and online supplementary figure S4F). In parallel with figure 4B, simultaneous knockdown of *ZEB1* and *DKK1* did not increase SAHF formation vis-à-vis single *ZEB1* knockdown suggesting, once again, that DKK1 may exert its effects on senescence through the same functional pathway than ZEB1.

Inhibition of senescence by ZEB1 requires repression of *H2AFY*

Contrary to ZEB1, macroH2A1 suppresses tumour initiation and progression and associates with better survival in multiple types of cancer.[32] Therefore, out of all the genes validated in figure 3B, we focused on *H2AFY*. First, we confirmed the inhibition of macroH2A1 by ZEB1 and DKK1 at the protein level. Knockdown of *ZEB1* and *DKK1*—with si*ZEB1*-A and si*DKK1* —upregulated macroH2A1 protein expression (figure 5A). Repression of macroH2A1 by ZEB1 was also tested in vivo.

quantification of SA β-gal-positive cells in the *right panel*. (C) Blockade of DKK1 induces senescence. SW480 cells were incubated with either Dulbecco's Modified Eagle Medium (no antibody (Ab)), 5 μg/mL of goat IgG serum or 5 μg/mL of a goat blocking anti-DKK1 Ab (clone AF-1096). SA β-gal-positive cells were quantified after 24 hours. Representative pictures are shown in online supplementary figure S4D. (D) *Right panel*: quantification of SA β-gal-positive cells in the following stable cell lines: SW480-shCtl+vector, SW480-shCtl+*DKK1*, SW480-sh*ZEB1*-A+vector, SW480-sh*ZEB1*-A+*DKK1*, SW480-sh*ZEB1*-B+vector and SW480-sh*ZEB1*-B+*DKK1*. Representative pictures for the first four cell lines are shown in the *left panel*. (E) Induction of senescence by sh*ZEB1* is reverted by rhDKK1, but not rhDKK3. SW480-shCtl and SW480-sh*ZEB1*-A cells were cultured in complete medium alone (untreated) or supplemented with 200 ng/mL of either rhDKK1 or rhDKK3. SA β-gal-positive cells were quantified after 24 hours with the first condition arbitrarily set to 100. Representative pictures are shown in online supplementary figure S4E. (F) ZEB1 and DKK1 inhibit formation of SAHF in CRC cells. SW480 cells were transiently transfected with 100 nM of si*ZEB1*-A and/or si*DKK1* or 100–200 nM of siCtl. Cells were stained with DAPI (blue) and an Ab against macroH2A1 (clone 4827) to assess for nuclear staining and SAHF formation, respectively. Representative merged pictures are shown here, while single staining captures are shown in online supplementary figure S4F. Scale bars represent 10 μm. (G) Quantification of the relative expression of DAPI and macroH2A1 in (F). RGB profiles display relative levels of macroH2A1 (green) or DAPI (blue) across a cell section—as marked by a white line in panel (F).

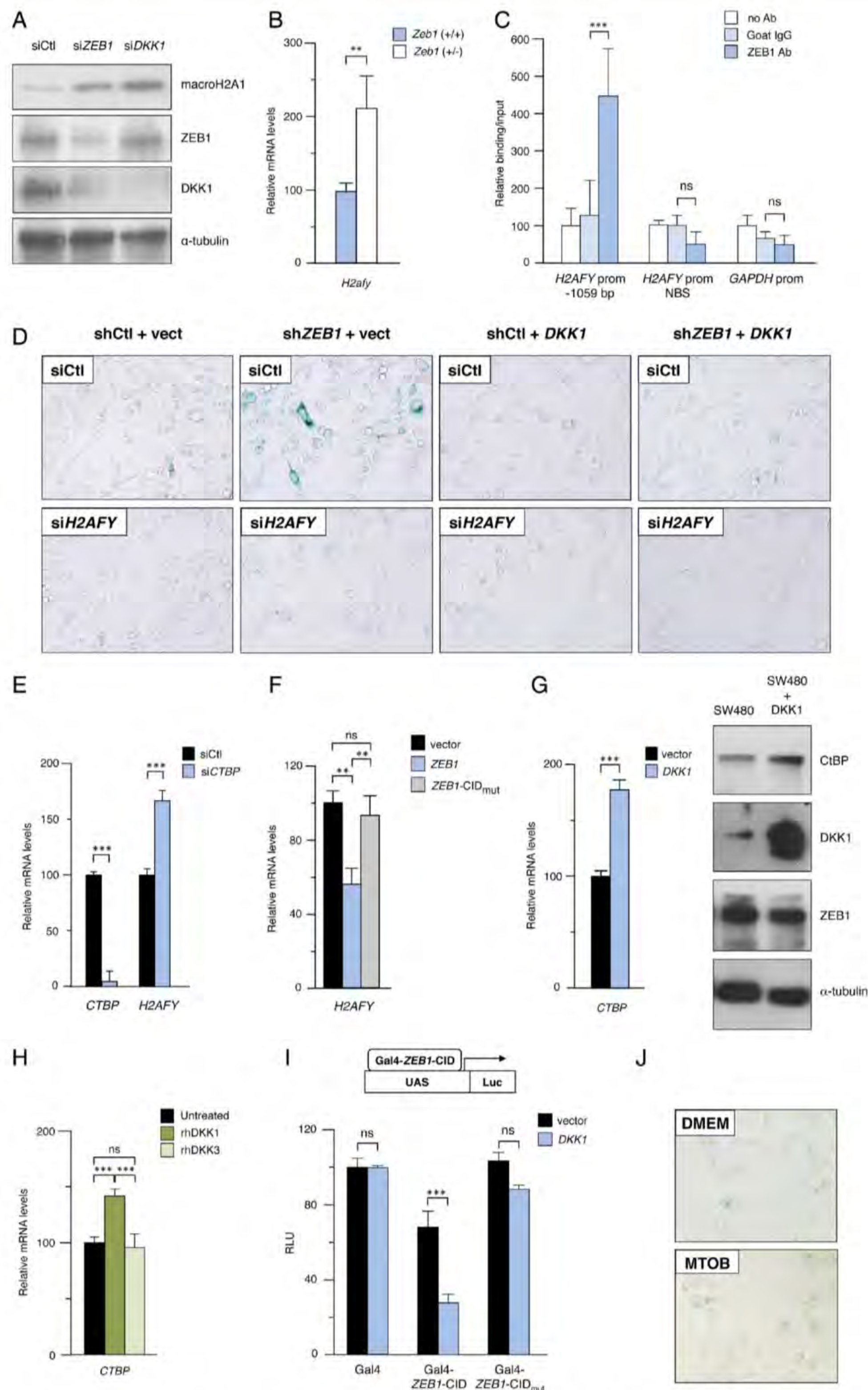

Figure 5 Inhibition of senescence by ZEB1 and/or DKK1 depends on the induction of *CTBP* to repress *H2AFY*. (A) ZEB1 and DKK1 inhibit macroH2A1 expression. SW480 cells were transiently interfered with siCtl, si*ZEB1*-A or si*DKK1*. Cell lysates were then blotted for macroH2A1 (clone 4827), ZEB1 (H-102) and DKK1 (2A5) along with α-tubulin (B5-1-2) as loading control. (B) In vivo expression of macroH2A1 depends on ZEB1. *H2afy* mRNA levels in the small intestine of *Zeb1* (+/+) and *Zeb1* (+/−) mice aged 3 months. See figure 2A for comparison of *Zeb1* and *Dkk1* levels in both genotypes. (C) *ZEB1* binds to the *H2AFY* promoter. Quantitative real-time (qRT)-PCR amplification of fragments of the *H2AFY*—either containing the consensus ZEB1 binding site at −1059 bp or with no binding sites (NBS) for ZEB1—and *GAPDH* promoters that have been immunoprecipitated in ChIP assays from SW480 cells without antibody (Ab) or with Abs against ZEB1 (E-20X) or goat IgG serum control. The condition without Ab was equalled to 100. (D) Inhibition of senescence by ZEB1 and DKK1 depends on their repression of macroH2A1. Stable

Compared with their wild-type littermates, expression of *H2afy* was upregulated in the intestine of *Zeb1* (+/−) mice (figure 5B).

Next, we investigated whether inhibition of *H2AFY* by ZEB1 involves direct binding of ZEB1 to the *H2AFY* promoter. We identified two high-affinity binding sites for ZEB1 at −1059 and −1687 bp and tested the ability of the former to recruit ZEB1 in ChIP assays (figure 5C and online supplementary figure S5A). An antibody against ZEB1, but not its corresponding matched specie IgG control, immunoprecipitated a region of the *H2AFY* promoter containing this binding site but not a region lacking consensus binding sites for ZEB1 (figure 5C). The *GAPDH* promoter, which is not regulated by ZEB1, did not recruit ZEB1.

Repression of *H2AFY* by ZEB1 and DKK1 prompted us to investigate whether inhibition of senescence by ZEB1 and DKK1 depends on macroH2A1. The stable SW480 cell lines used in figures 3B,C—with *ZEB1* and *DKK1* expression knocked down or overexpressed, respectively—were now transiently transfected with siCtl or a siRNA against *H2AFY* (si*H2AFY*) and tested for SA β-gal staining (see online supplementary figure S5B for *H2AFY* knockdown efficiency). As shown in figure 5D and online supplementary figure S5C, si*H2AFY* inhibited both basal SA β-gal staining and the senescence induced by *ZEB1* knockdown. Once again, *DKK1* overexpression reverted the senescence induced by sh*ZEB1*, which was further reduced by si*H2AFY*. These results indicate that the ability of ZEB1 and DKK1 to repress senescence depends on their repression effect on *H2AFY*.

ZEB1 represses *H2AFY* expression and senescence through the subsequent induction of DKK1, mutant p53, Mdm2 and CtBP

ZEB1 represses gene expression through the recruitment of non-DNA binding corepressors, chiefly CtBP.[11 13 33] Therefore, we first checked whether CtBP represses *H2AFY* expression. Indeed, as shown in figure 5E, transient knockdown of *CtBP* with a specific siRNA (si*CtBP*) upregulated *H2AFY* mRNA levels. Furthermore, mutation of the CtBP binding sites in *ZEB1* (*ZEB1*-CID$_{mut}$) hampered *ZEB1*'s repression of *H2AFY* (figure 5F).

The ability of DKK1 to mediate ZEB1 inhibition of *H2AFY* prompted us to investigate whether DKK1 could induce CtBP expression. Indeed, overexpression of *DKK1* upregulated CtBP expression at the mRNA and protein levels (figure 5G and online supplementary figure S5D). Likewise, addition of rhDKK1, but not rhDKK3, upregulated *CTBP* expression (figure 5H). We then examined whether the increased CtBP expression induced by DKK1 enhances ZEB1 repressor activity. The region of *ZEB1* containing the CtBP interacting domain (CID), either wild-type or mutated to a sequence unable to bind ZEB1, was fused to the DNA binding domain of the yeast protein Gal4 to create Gal4-*ZEB1*-CID and Gal4-*ZEB1*-CID$_{mut}$. Both constructs were then cotransfected with a luciferase reporter containing UAS DNA binding sites and driven by the SV40 promoter into SW480 cells stably expressing *DKK1* (SW480+*DKK1* cells) or its corresponding empty vector (SW480+vector cells). *DKK1* overexpression greatly enhanced the ability of ZEB1 to repress transcription (figure 5I).

CtBP binding to 2-keto-4-methylthiobutyrate (MTOB), an intermediate in the methionine salvage pathway, relieves CtBP-mediated transcriptional repression in breast cancer cells.[34] We found that blocking of CtBP activity in SW480 cells by MTOB induced senescence (figure 5J and online supplementary figure S5E) and upregulated *H2AFY* (see online supplementary figure S5F). Altogether, these results indicate that DKK1 cooperates with ZEB1 in the repression of *H2AFY* and senescence by upregulating CtBP.

Mdm2 is an E3 ubiquitin-ligase best known for targeting the p53 tumour suppressor for proteasomal degradation, although Mdm2 also promotes tumour progression through transcriptional regulation and histone modification in a p53-independent manner.[35–37] Induction of senescence is in fact one of the key mechanisms through which p53 suppresses tumourigenesis and, consequently, Mdm2 antagonists like nutlin-3a trigger senescence.[38 39] Of note, Mdm2 binds to and cooperates with CtBP to repress transcription.[40] This evidence prompted us to investigate whether mutant p53 and/or Mdm2 are involved in the CtBP-mediated repression of *H2AFY* and senescence by ZEB1 and DKK1.

It is now well established that mutations in *TP53* not simply eliminate wild-type *TP53* tumour suppressor functions but also confer cancer cells with oncogenic hallmarks to promote tumour progression such as overcoming of senescence.[41 42] Wild-type and mutant p53 proteins differ in their target genes and also in the direction (eg, activation or repression) and degree (eg, deficient or enhanced transcription) of their regulation.[43] Interestingly, mutant p53—including some variants with mutations in the DNA binding domain—retain the ability to bind and transcriptionally activate the *MDM2* promoter.[44 45] Thus, some mutant *TP53* cells, like SW480 CRC cells, still express *MDM2*.[46]

We found that overexpression of *DKK1* or addition of rhDKK1, but not rhDKK3, in mutant SW480 cells upregulated mutant *TP53* and *MDM2* expression in these cells (figure 6A, B). In turn, *DKK1* knockdown downregulated both mutant *TP53* and *MDM2* (figure 6C) and this downregulation was

SW480-shCtl+vector, SW480-shCtl+*DKK1*, SW480-sh*ZEB1*-A+vector and SW480-sh*ZEB1*-A+DKK1 cell lines were transiently interfered with either a siRNA specific for *H2AFY* (si*H2AFY*) or siCtl. *H2AFY* knockdown efficiency in each condition is shown in online supplementary figure S5B. Representative pictures of senescent associated β-galactosidase (SA β-gal)-positive cells are shown here and quantified in online supplementary figure S5C. (E) ZEB1 represses *H2AFY* through CtBP. SW480 cells were interfered for siCtl or a siRNA against *CTBP* (si*CTBP*) and *H2AFY* mRNA determined by qRT-PCR. (F) Repression of *H2AFY* by ZEB1 depends on CtBP. SW480 cells were transfected with an expression vector encoding full-length *ZEB1*, *ZEB1*-CID$_{mut}$ or the corresponding empty vector. *H2AFY* mRNA was determined by qRT-PCR. (G) *DKK1* induces *CTBP* mRNA and protein expression. *Left panel*: *CTBP* mRNA levels from SW480-shCtl+vector and SW480-shCtl+*DKK1* cells were assessed by qRT-PCR. *Right panel*: Lysates from these cells were blotted for CtBP (E12), DKK1 (2A5) and ZEB1 (H-102) along with α-tubulin (B5-1-2). (H) rhDKK1 but not rhDKK3 induces *CTBP* mRNA. SW480 cells were cultured during 24 hours with complete medium alone (untreated) or supplemented with 200 ng/mL of either rhDKK1 or rhDKK3 before *CTBP* mRNA expression was assessed by qRT-PCR. (I) *DKK1* enhances *ZEB1's* repressor activity; 0.25 μg of expression vectors for Gal4's DNA binding domain (Gal4) or equal molar amounts of Gal4-*ZEB1*-CID or Gal4-*ZEB1*-CID$_{mut}$ were transfected in SW480-shCtl+vector or SW480-shCtl+*DKK1* cells along with 0.25 μg of a Gal4×5-SV40-luciferase reporter. (J) Inhibition of CtBP induces senescence in colorectal carcinoma (CRC) cells. SW480 cells were culture during 24 hours in the presence or absence of 10 mM of the CtBP inhibitor methylthiobutyrate (MTOB) and stained for SA β-gal. Quantification of SA β-gal-positive cells is shown in online supplementary figure S5D. DMEM, Dulbecco's Modified Eagle Medium; RLU, relative luciferase units.

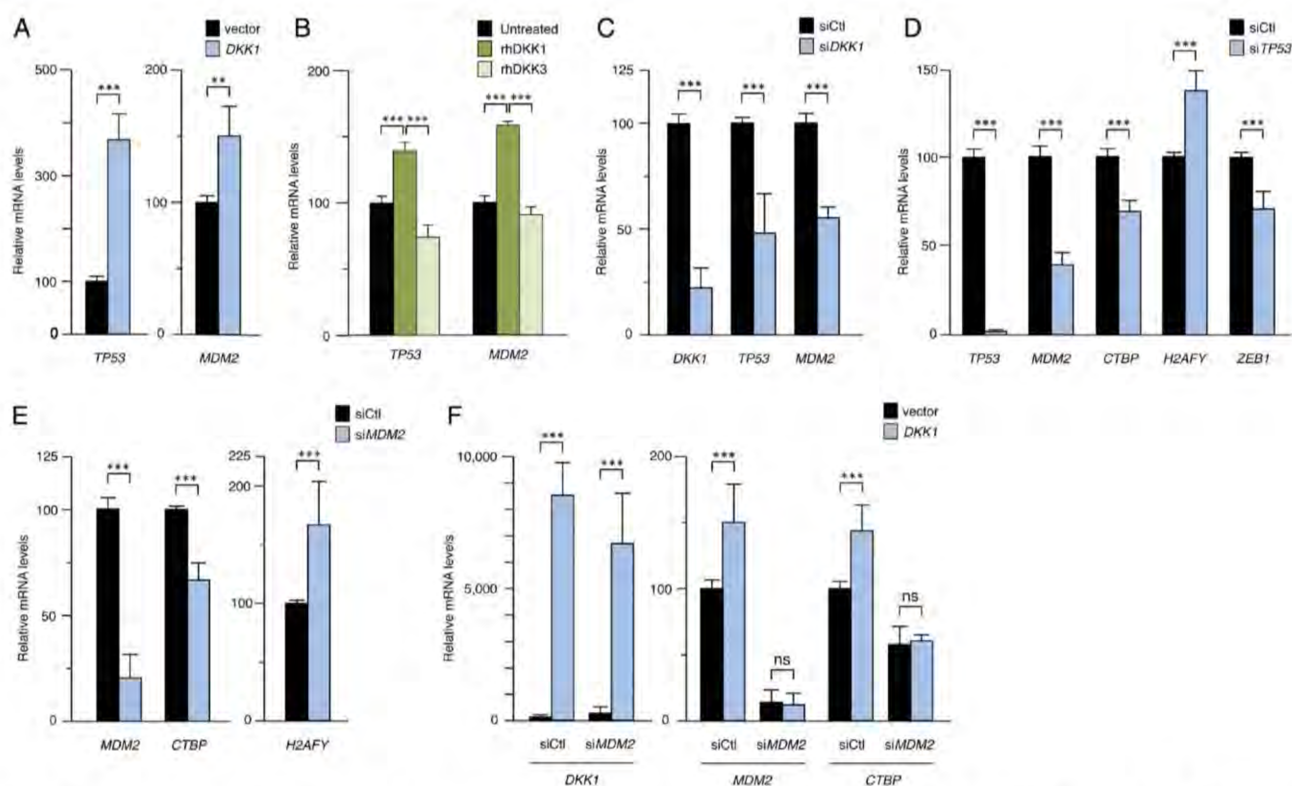

Figure 6 ZEB1 represses *H2AFY* through the subsequent induction of DKK1, mutant p53, Mdm2 and CtBP. (A) *DKK1* induces *TP53* and *MDM2*. mRNA levels for both genes were determined by quantitative real-time (qRT)-PCR in SW480-shCtl+vector and SW480-shCtl+*DKK1* cells. (B) rhDKK1 but not rhDKK3 induces mutant *TP53* and *MDM2* mRNA. Mutant *TP53* SW480 cells were cultured during 24 hours in complete medium alone (untreated) or supplemented with 200 ng/mL of either rhDKK1 or rhDKK3. *TP53* and *MDM2* mRNA expression was assessed by qRT-PCR. (C) Knockdown of *DKK1* downregulates *TP53* and *MDM2*. SW480 cells were interfered for siCtl or si*DKK1* and *TP53* and *MDM2* mRNA levels assessed by qRT-PCR. (D) Mutant *TP53* activates *MDM2* and *CTBP* and represses *H2AFY*. SW480 cells were interfered for siCtl or a siRNA against *TP53* (si*TP53*) and mRNA levels of mutant *TP53, MDM2, CTBP* and *H2AFY* were assessed by qRT-PCR. (E) *MDM2* represses *CTBP* and subsequently induces *H2AFY*. SW480 cells were interfered for siCtl or a siRNA against *MDM2* (si*MDM2*) and mRNA levels for *CTBP* (*left panel*) and *H2AFY* (*right panel*) were assessed by qRT-PCR. (F) *MDM2* is downstream of *DKK1* and induction of *CTBP* by *DKK1* requires of *MDM2*. SW480 cells were interfered with 100 nM siCtl or si*MDM2* and then transfected with either 1 μg of an expression plasmid encoding *DKK1* or an equal molar amount of its corresponding empty vector. *DKK1*, *MDM2* and *CtBP* mRNA levels were assessed by qRT-PCR.

reverted by addition of rhDKK1, but not of rhDKK3 (see online supplementary figure S6A). The downstream effect of DKK1 via mutant p53 was corroborated by the knockdown of the latter with a specific siRNA (si*TP53*). si*TP53*, but not siCtl, downregulated mRNA expression levels of *MDM2* and *CTBP* while increasing those of *H2AFY* (figure 6D).

Interestingly, wild-type and mutant p53 have been described to have opposite functions with regard to EMT.[47] Wild-type p53 inhibits the EMT by direct transactivation of miR-200c, which targets ZEB1 mRNA for decay. Meanwhile, mutant p53 proteins induce an EMT.[47] Accordingly, we found that si*TP53* downregulated ZEB1 in SW480 cells (figure 6D).

MDM2 expression was also knocked down with a specific siRNA (si*MDM2*). si*MDM2* downregulated CtBP mRNA and protein levels (figure 6E and online supplementary figure S6B) and increased *H2AFY* mRNA expression (figure 6E). *MDM2* knockdown was also used to confirm the sequencing of the regulatory cascade from DKK1 to CtBP. SW480 cells interfered with siCtl or si*MDM2* were transfected with an expression plasmid for *DKK1* or its corresponding empty vector. As shown in figure 6F, *MDM2* knockdown blocked the upregulation of *CTBP* by *DKK1* as shown in figure 5G. Altogether, the above results indicate that in mutant *TP53* cells DKK1 cooperates with ZEB1 in the repression of *H2AFY* through the subsequent induction of mutants *TP53*, *MDM2* and *CTBP*.

Downregulation of *Zeb1* is sufficient to trigger in vivo senescence, reduce tumour load and improve survival in a mouse model of colon cancer

ZEB1 is not expressed in the normal colonic mucosa or by well-differentiated cancer cells in the tumour centre of CRCs. Instead, ZEB1 is found in dedifferentiated malignant cells at the CRC invasive front.[14 48] ZEB1 is also expressed by stromal cells in both normal colorectal mucosa and CRCs.[10 14 48] In contrast, macroH2A1 is strongly expressed in normal colonic mucosa, but not in invasive CRCs (ref. [49], and online supplementary figure S7A). In light of this reverse pattern of expression, we tested the correlation of ZEB1, DKK1 and macroH2A1 at the invasive front of sporadic CRCs. Evaluation of ZEB1 and DKK1 staining in a tissue microarray of 53 primary human CRCs stages I–IV revealed a positive correlation between both proteins (Spearman's ρ=0.46). Conversely, macroH2A1 expression displayed an inverse correlation with ZEB1 and DKK1 (figures 7A, B).

We then examined whether expression of ZEB1 in CRCs represses senescence in vivo. To that effect, *Zeb1* (+/+) and *Zeb1* (+/−) mice were used in a well-established mouse model

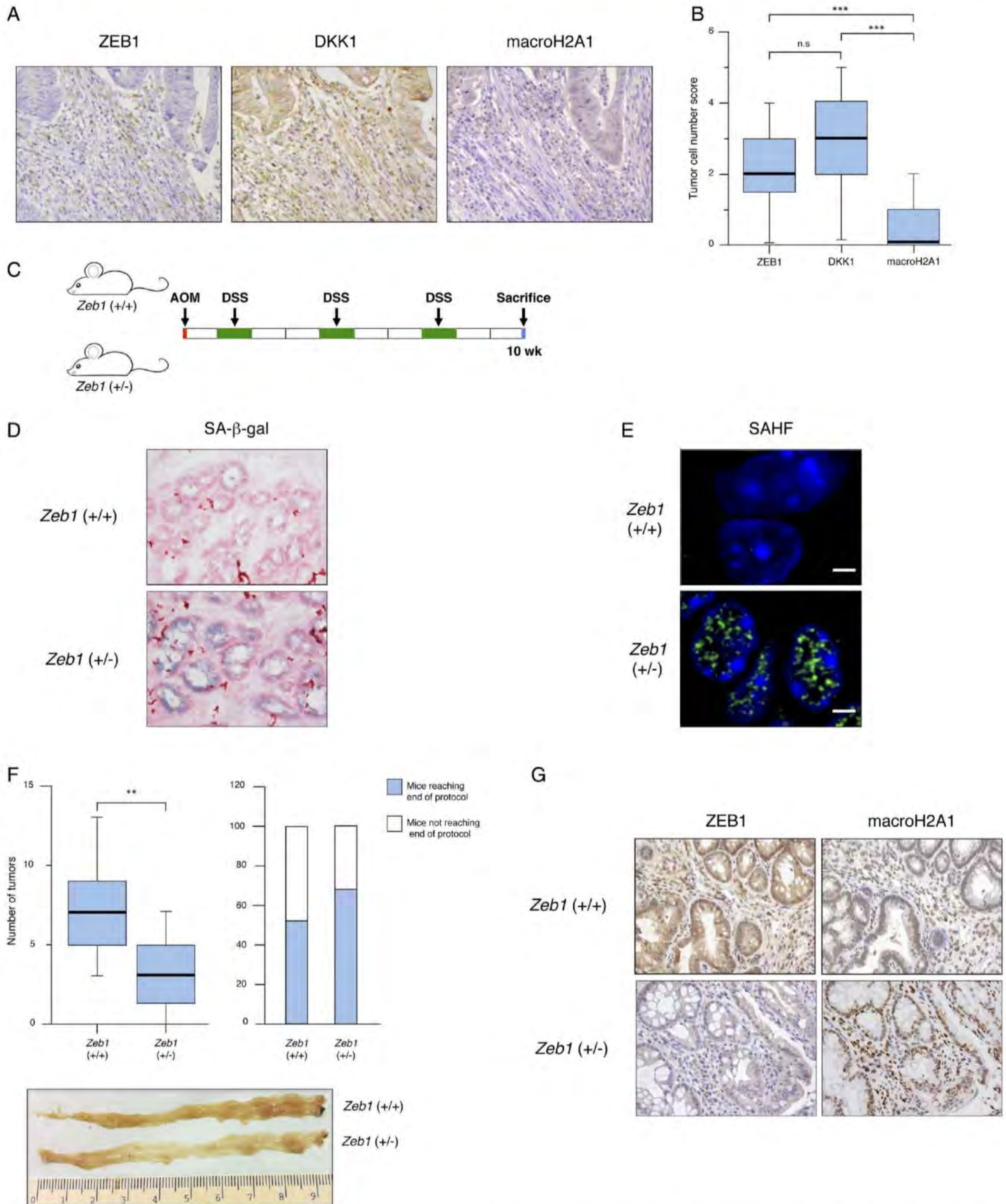

Figure 7 Downregulation of *Zeb1* induces senescence in vivo and reduces tumour load in a *Zeb1*-deficient mice. (A) ZEB1 and DKK1 inversely correlate with macroH2A1 in the invasive front of colorectal carcinomas (CRCs). A tissue array of primary CRCs was stained for ZEB1 (H-102), DKK1 (clone 2A5) and macroH2A1 (gift from M Buschbeck, see online supplementary methods). CRC cells at the tumour front were identified by their expression of pan-cytokeratin (IR053). Representative pictures of the tumour front are shown. Magnification 400×. (B) Quantification of the staining in (A). Samples were scored for the number of positively stained CRC cells at the invasive front as per the scale described in online supplementary information. (C) Scheme of the mouse model of CRC used in (D). *Zeb1* (+/+) and *Zeb1* (+/−) mice were treated azoxymethane (AOM) and cycles of dextran sodium sulfate (DSS) as detailed in the Methods section. (D) Downregulation of *Zeb1* triggers in vivo senescence in CRC tumours. Frozen sections of CRC tumours from *Zeb1* (+/+) and *Zeb1* (+/−) mice treated as in (C) were stained for senescent associated β-galactosidase (SA β-gal)

of CRC induced by administration of azoxymethane and cycles of dextran sodium sulfate (reviewed in ref. 50) (figure 7C). Colorectal tumours collected at the end of the protocol (day 70) were examined for cellular senescence. Interestingly, we found that tumours from wild-type mice barely had senescent cells, whereas those from *Zeb1*-deficient ones displayed larger number of SA β-gal-positive cells (figure 7D). Likewise, SAHF were only observed in tumours from *Zeb1* (+/−) mice but not in those from *Zeb1* (+/+) mice (figure 7E and online supplementary figure S7B). Given the role of senescence as a tumour suppressor programme, we examined mice from both genotypes for the total number of colonic tumours. In line with the above results, *Zeb1* (+/−) mice developed a lower number of tumours than *Zeb1* (+/+) mice and the percentage of *Zeb1* (+/−) mice that survived until the end of the protocol was also higher than among *Zeb1* (+/+) mice (figure 7F).

Colorectal tumours from *Zeb1* (+/+) and (+/−) mice were also stained for macroH2A1. ZEB1 was strongly expressed by cancer epithelial cells in colorectal tumours from *Zeb1* (+/+) mice but only a few epithelial cells were stained for macroH2A1 (figure 7G). In contrast, in the tumours of *Zeb1* (+/−) mice, expression of ZEB1 was greatly reduced in the malignant epithelial cells—ZEB1 was mostly confined to a few stromal cells—while they displayed strong expression of macroH2A1. In addition, tumours in *Zeb1* (+/−) mice displayed neoplastic glands with partially preserved goblet cells and polarisation and scarce signs of mitotic activity (figure 7G). In contrast, the tumours arising in *Zeb1* (+/+) mice showed altered tubular structures with almost complete loss of polarisation and atypical nuclei and mitosis homogeneously distributed throughout the gland, even reaching the lumen (figure 7G). Altogether, these data indicate that expression of ZEB1 in CRCs represses senescence. Importantly, these results also show that partial downregulation of *Zeb1*, even to only heterozygous levels, is sufficient to induce macroH2A1 expression and SAHF formation, to trigger in vivo cancer cell senescence, to reduce tumour load and to confer a more differentiated histological pattern.

The tumourigenic capacity of ZEB1 critically depends on its repression of *H2AFY*

Next, we wondered whether ZEB1's role promoting tumourigenesis requires in vivo repression of macroH2A1. The SW480-shCtl and SW480-sh*ZEB1*-A cells used above were now stably cotransfected with retroviral shRNA vector against *H2AFY* (pSUPER-sh*H2AFY*) or its corresponding shRNA control (pSUPER-shCtl) to generate the following cell lines: SW480-shCtl/shCtl, SW480-sh*ZEB1*-A/shCtl, SW480-shCtl/sh*H2AFY* and SW480-sh*ZEB1*-A/sh*H2AFY* (see protein levels of macroH2A1 and ZEB1 in these cell lines in online supplementary figure S8A). The four cell lines were then used in a xenograft mouse model by inoculation in 10-week-old SCID mice and tumour growth was monitored for up to 23 days. As expected, single knockdown of *ZEB1* (SW480-sh*ZEB1*-A/shCtl cells, *blue line* in figure 8A, B and online supplementary figure S8B) drastically reduced tumour growth. Interestingly, when cells were simultaneously knocked down for both *ZEB1* and *H2AFY* (SW480-sh*ZEB1*-A/sh*H2AFY* cells, *green line* in figures 8A, B and online supplementary figure S8B), the ability of sh*ZEB1* to reduce tumourigenesis was reversed. Lastly, single knockdown of *H2AFY* (SW480-shCtl/sh*H2AFY* cells, *red line* in figure 8A, B and online supplementary figure S8B) accelerated tumour growth compared with control cells (SW480-shCtl/shCtl cells, *yellow line* in figure 8A, B and online supplementary figure S8B). Overall, these results indicate that in vivo expression of macroH2A1 inversely correlates with ZEB1 (figure 7A, G) and ZEB1 induces tumourigenesis through repression of macroH2A1 (figure 8A).

It is worth remarking here that there was no statistical difference between the tumourigenic capacity of SW480-sh*ZEB1*-A/sh*H2AFY* and SW480-shCtl/sh*H2AFY* cells (*green line* vs *red line* in figure 8A) or between SW480-shCtl/shCtl and SW480-sh*ZEB1*-A/sh*H2AFY* cells (*yellow line* vs *green line* in figure 8A). Importantly, the lack of significant difference between these experimental condition pairs suggests that macroH2A1 is a key ZEB1 target in tumourigenesis and also that most of ZEB1's role in tumourigenesis and tumour progression is contingent on its repressor effect on macroH2A1.

ZEB1's role as determinant of worse cancer survival depends on its inhibition of *H2AFY* and senescence

In vivo evidence in human and mouse tumours indicates that senescence is associated with lower malignancy, slower tumour growth and, consequently, better clinical prognosis.[5] Therefore, we tested whether the worse survival effect of ZEB1 in CRCs depends on its inhibition of senescence.

Altogether, the results shown above indicate that ZEB1 inhibits senescence in CRCs by induction of DKK1 and repression of macroH2A1. We therefore first examined whether expression of *H2AFY* modulates the effect of *ZEB1* on the prognosis of patients with CRC. Analysis of the 928 human CRCs used in figure 1A revealed that those cases displaying high expression of *ZEB1* and low expression of *H2AFY* have lower survival probability than those with high levels of *ZEB1* but high levels of *H2AFY* (yellow and red lines in figure 8C). This implies that the maximum effect of *ZEB1* as a predictor of poorer survival in CRC requires of low levels of *H2AFY* or that, in other words, high levels of *ZEB1* are not sufficient to determine worse survival if not accompanied by low levels of *H2AFY*. Similar results were found for the effect of *H2AFY* expression on the survival probability determined by *DKK1*: high levels of *DKK1* only determined maximum levels of reduced survival when expression of *H2AFY* was low (see online supplementary figure S8C). Of note, overall survival depends on multiple variables beyond tumour growth thus contributing to explain that *H2AFY* knockdown promoted tumour growth in the xenograft mouse model independently of ZEB1 levels (*red* and *green lines* in figure 8A), while lower overall survival in patients with CRC associated with low levels of *H2AFY* is worsened by high levels of ZEB1

and counterstained with Fast Red. (E) Downregulation of *Zeb1* triggers in vivo senescence-associated heterochromatin foci (SAHF) assembly in CRC tumours. As in (D), but formalin-fixed paraffin-embedded (FFPE) tumour samples were stained for macroH2A1 and DAPI as in figure 4F. Merged pictures are shown here and individual staining are displayed in online supplementary figure S7B. Scale bars represent 2 μm. (F) *Zeb1* (+/−) mice developed lower number of tumours and survived in greater number to the end of protocol than *Zeb1* (+/+) counterparts. Twenty-three *Zeb1* (+/+) mice and 19 *Zeb1* (+/−) mice were subjected to the protocol described in (C). At the end of the procedure (day 70), mice from both genotypes were examined for the number of colorectal tumours (*upper left panel*). A representative picture of colorectal tumours in *Zeb1* (+/+) and *Zeb1* (+/−) is shown (*lower panel*). The percentage of *Zeb1* (+/−) mice that reached day 70 was larger than for *Zeb1* (+/+) mice (*upper right panel*). (G) FFPE tumour samples as in (E) were stained for ZEB1 (H-102) and macroH2A1 (ab37264). Magnification 400×.

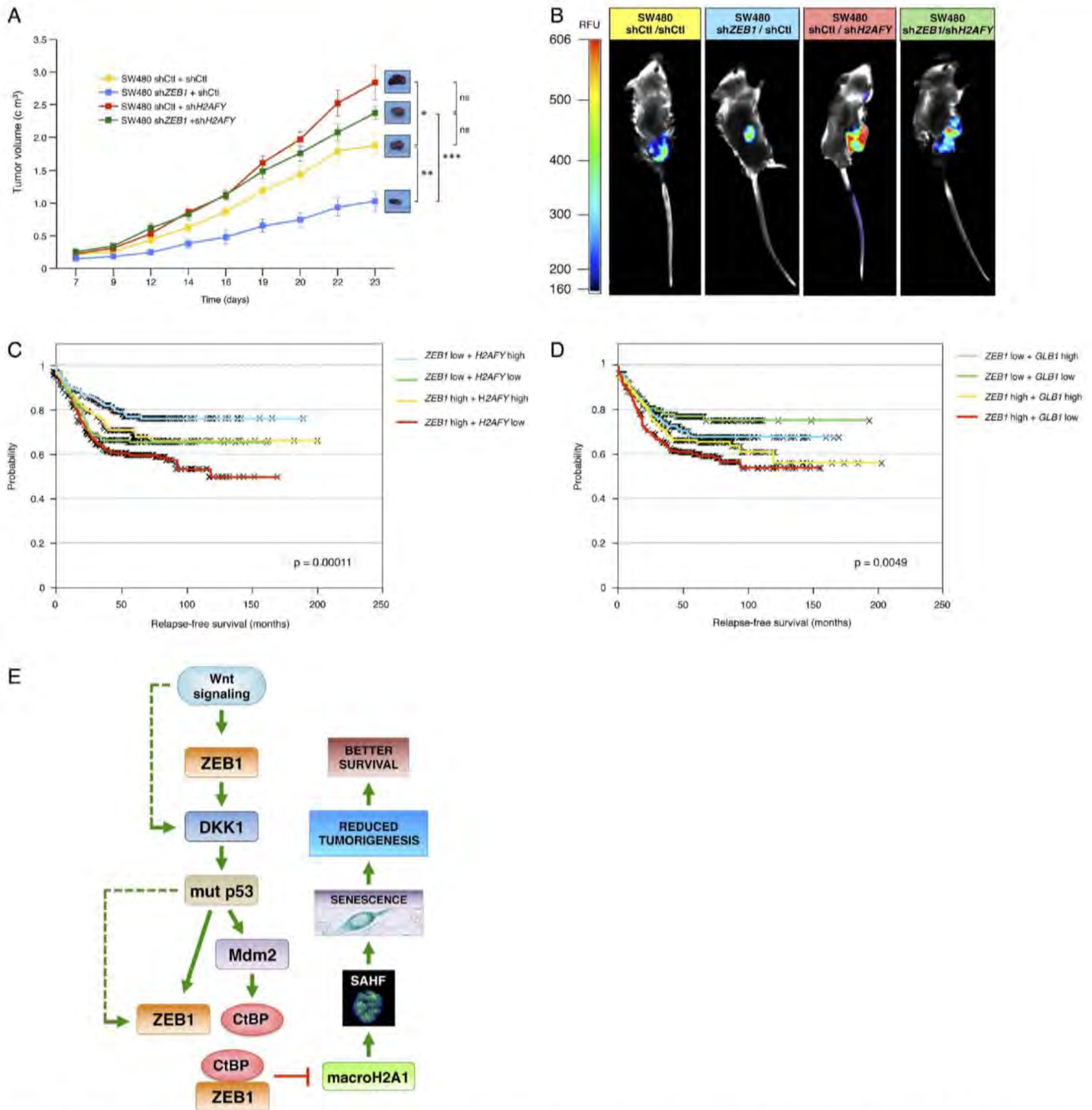

Figure 8 ZEB1-induced tumourigenesis and *ZEB1*'s role as determinant of worse survival depends on its repression of macroH2A1 and senescence. (A) *H2AFY* knockdown reverses the inhibitory effect on tumourigenesis of *ZEB1* knockdown. SW480 shCtl/shCtl, SW480 sh*ZEB1*-A/shCtl, SW480 shCtl/sh*H2AFY* and SW480 sh*ZEB1*-A/sh*H2AFY* stable cell lines were injected into SCID mice (seven to eight mice per cell line) and tumour development was monitored over time as described in the Methods section. A representative tumour for each cell line is shown with pictures of all tumours displayed in online supplementary figure S8B. See main text for details and discussion. (B) As in (A) but tumour volume was visualised by the uptake of fluorescent-labelled 2-deoxy glucose. Colour scale represents relative fluorescence units (RFU) with respect to an area where there was no tumour. A representative mouse for each condition is shown. (C) The maximum effect of *ZEB1* as a factor of worse survival in colorectal carcinomas (CRCs) depends on low expression of *H2AFY*. The relapse-free survival probability of patients with CRC was represented in a Kaplan-Meier plot segregated by the median expression of *ZEB1* and *H2AFY*. (D) As in (C) but for the *GLB1* gene instead of *H2AFY*. (E) Schematic model of the molecular mechanism by which ZEB1 promotes tumourigenesis by repressing senescence in cancer cells. ZEB1 inhibits senescence and tumourigenesis and determines worse survival through subsequent activation of DKK1, mutant p53, Mdm2 and CtBP, and repression of macroH2A1. The dashed line refers to the induction of DKK1 by Wnt signalling as described in[3] and the induction of ZEB1 by mutant p53.[47] See main text for details. RFU, relative fluorescence units; SAHF, senescence-associated heterochromatin foci.

(*red* and *green lines* in figure 8C). From all these data it could be therefore concluded that, at least for some of its tumour-promoting and worse-survival functions, *ZEB1* depends on its activation effect on *DKK1* (figure 1A) and also on its repression of *H2AFY* (figure 8C).

Likewise, we also tested whether expression of *GLB1*, encoding for SA β-gal and whose mRNA levels increase in senescent cells,[26] alters the predictor value of *ZEB1* in CRC survival. Indeed, among patients with CRC with high levels of *ZEB1*, low levels of *GLB1* determined poorer survival (figure 8D). Altogether, these results indicate that *ZEB1* promotes tumour progression and determines worse prognosis in patients with CRC, at least in part, through its effect as an inhibitor of senescence.

DISCUSSION

ZEB1 triggers an EMT in invading cancer cells at the tumour invasive front conferring malignant cells a more motile and stem-like phenotype. However, beyond the induction of an EMT, the mechanisms through which ZEB1 promotes tumour initiation and progression and determines a worse survival in most cancers remain to be elucidated. Here, we found that the maximum effect of *ZEB1* as a predictor of reduced survival in CRCs requires the joint expression of high levels of the Wnt antagonist *DKK1* and low levels of *H2AFY* and *GLB1*. We showed that *ZEB1* activates *DKK1* by direct transcriptional activation of its promoter and that both genes inversely correlate with a number of senescence-inducing genes, whose expression they repress. Inhibition of senescence by ZEB1 and DKK1 depended on their repression of histone macroH2A1 and of SAHF formation through the subsequent induction of mutant p53, Mdm2 and CtBP (see model in figure 8E). Expression of ZEB1 and DKK1 at the invasive front of CRCs displays an inverse correlation with that of macroH2A1. In a mouse model of CRC, partial deletion of *Zeb1* is sufficient to trigger senescence, SAHF formation and expression of macroH2A1. Importantly, using a tumour xenograft mouse model, we found that most of the tumourigenic effect of ZEB1 depends on its repression of macroH2A1.

Although Wnt antagonists are frequently silenced in human cancers, resulting in activation of the Wnt signalling and carcinogenesis, they can also have oncogenic roles.[2 4] In this line, DKK1 is overexpressed in several carcinomas where it promotes tumour growth and metastasis[4] and we found that high *DKK1* expression is required for *ZEB1* to impose its maximum impact on poorer survival in patients with CRC. In fact, DKK1 expression and function can be modulated during tumour progression. For instance, compared with normal prostate tissue, DKK1 expression increases in early malignant lesions and primary prostate carcinomas but declines in metastatic prostate carcinomas.[51] In colorectal tumours, DKK1 is expressed at higher levels in Wnt-active CRCs than in adenomas and it is silenced by methylation in CRCs with low Wnt activity.[52] As referred earlier, DKK1 is induced by Wnt signalling[3] and our results here indicate that DKK1 is induced, along with ZEB1, in invasive malignant cells at the tumour front of CRCs. These invasive cells are characterised by the expression of nuclear β-catenin and by the display of active Wnt signalling and a proinvasive and stem-like phenotype.[10]

Our results also showed that DKK1 mediates the repression by ZEB1 of senescence-associated genes and of senescence. In SW480 cells, DKK1 increased endogenous levels of mutant *TP53* and *MDM2* and of the corepressor *CTBP*, thus enhancing ZEB1 transcriptional repression of *H2AFY*. Notably, wild-type and mutant p53 compete in the regulation of EMT: while wild-type p53 inhibits EMT through the direct transactivation of miR-200c, which in turn targets ZEB1 mRNA for decay, mutant p53 triggers an EMT.[47] Our results in SW480 cells indicate that ZEB1, through DKK1, activates mutant *TP53* and that ZEB1 is also under positive regulation by *TP53*, indicating the existence of a positive feedback loop between both transcription factors. Of note, EMT-activating and ZEB1-activating mutations of *TP53* (at least, R175H and R273H) are common in primary CRCs and CRC cell lines, including those used in this study.[42 46] Wild-type and mutant p53 vary in their target genes and also in their transcriptional activities for a given gene with some genes being regulated in different directions (eg, activation vs repression) and others to different degree (eg, deficient or enhanced activation).[43]

We showed here that DKK1, through induction of mutant p53 and Mdm2, activates CtBP. CtBP is overexpressed in human cancers and represses a number of tumour suppressors.[53] As it does not bind to DNA, CtBP regulates gene expression through its recruitment by transcription factors, particularly ZEB1.[11 53] To the extent that many functions of ZEB1—in cancer and beyond (eg,[12 33])—depend on its repressor activity through CtBP, it could be expected that induction of CtBP by DKK1-mutant p53-Mdm2 may participate in other ZEB1-mediated processes beyond senescence such as regulation of cell differentiation, proliferation, apoptosis and/or cell migration.

Importantly, this study found that deletion of a single *Zeb1* allele is sufficient to trigger senescence in cancer cells and therefore reduce tumour growth and improve survival in a mouse model of CRC. Our results here in CRC cells support earlier evidence that MEFs with heterozygous and homozygous deletion of *ZEB1* display replicative senescence at earlier passages than wild-type ones.[31] Senescence in *Zeb1*-deficient MEFs occurs in parallel with an upregulation of *Cdkn2b* and *Cdkn1a* ($p21^{WAF1/CIP1}$), direct targets of ZEB1.[13 31] We found that while DKK1 cooperates with ZEB1 in the repression of *H2AFY*, repression of *CDKN2A and CDKN2B* by ZEB1 is independent of DKK1 levels. Evidence in the extant literature for a role of DKK1 in senescence is inconclusive as DKK1 expression decreases in senescent endothelial cells but DKK1 mediates senescence in oesophagous epithelial cells,[54 55] indicating once again that DKK1, as other Wnt antagonists, plays opposing functions under different physiological and pathological contexts. In contrast to DKK1, DKK3 failed to induce the mutant p53-Mdm2-CtBP pathway or to inhibit senescence in CRC cells. Since both DKK factors displayed similar inhibitory effect on Wnt signalling (ref 22 and online supplementary figure S1B), these results indicate that activation of the mutant p53-Mdm2-CtBP pathway and inhibition of macroH2A1 and senescence by DKK1 occurs independently of DKK1's role as a Wnt antagonist.

In contrast to ZEB1, we found that ZEB2 does not regulate DKK1. ZEB2 is structurally and functionally highly homologous to ZEB1, induces an EMT and has been associated with increased invasiveness in several cancers.[10 23] However, ZEB2 represses hTERT in hepatocarcinoma and induces replicative senescence.[56] ZEB1 and ZEB2 also play opposing roles in the regulation of TGFβ signalling during differentiation and development.[12 13] In some cancers (eg, melanomas), ZEB2 functions as tumour suppressor displaying an inverse expression pattern with ZEB1.[57] Therefore, it is possible that the divergent roles of ZEB factors in tumour progression are related to their opposing effects on senescence.

In a wide range of cancers, expression of macroH2A1 is reduced in tumours compared with their respective normal tissues and in invasive cancers with respect to cancer precursor

lesions.[32 49] Contrary to ZEB1, macroH2A1 suppresses tumour initiation and metastasis and associates with better survival. We found here that ZEB1 binds to the *H2AFY* promoter whose expression represses in a CtBP-dependent manner. In addition to the binding site we validated here, we identified other ZEB1 binding sites in the *H2AFY* gene in the ENCODE Project database (http://www.genome.gov/encode). We showed that *ZEB1* and *H2AFY* display an inverse pattern of expression in CRCs and that downregulation of *Zeb1* in a mouse model of CRC results in upregulation of macroH2A1. This correlation was validated at the mechanistic level in a xenograft model, where most of the tumourigenic activity of ZEB1 depended on its repression of macroH2A1. Of relevance, the worst survival effect of *ZEB1* in patients with CRC depends on low levels of *H2AFY*.

Senescence is a key tumour suppressor mechanism that malignant cells must bypass to sustain tumour growth. Thus, while senescent cells are abundant in preneoplastic lesions, they disappear as tumours progress into malignant stages.[5] Nevertheless, malignant cells can still undergo senescence if the expression and/or function of tumour suppressors are restored or that of oncogenes is inactivated. In that regard, senescence plays an important role in the response of cancer cells to many conventional chemotherapy agents and also a number of therapeutic strategies are currently being designed to specifically induce senescence.[58] We show here that partial downregulation of *Zeb1* is sufficient to trigger in vivo senescence in tumours and to reduce their growth and that the role of *ZEB1* as determinant of worse prognosis in CRCs depends on low levels of senescence markers *GLB1* and *H2AFY*. Therefore, ZEB1 could represent a new and important target in cancer therapy approaches aimed at inducing senescence.

This work has established a new role for ZEB1 as an inhibitor of senescence in cancer cells, thus expanding the set of cancer cell hallmarks regulated by ZEB1. By dissecting the molecular mechanisms by which ZEB1 contributes to overcome this cancer safeguard programme, the results presented here offer new opportunities to target the tumour-promoting functions of ZEB1.

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Contributors OdB performed most of the experimental work in the study, designed and interpreted experiments and wrote the manuscript. BG carried out the bioinformatics analysis of arrays for survival and gene expression. MJFA facilitated the collection of human samples of colorectal carcinomas and conducted the pathological analysis of their immunostaining. EST, LSM and LS performed some of the experimental work. AEA and GR assisted in the setting of the xenograft mouse model. JIC, DSD and AC supplied critical materials to the study. AP conceived and supervised the study, designed and interpreted experiments, obtained funding and wrote the manuscript. All authors critically reviewed the manuscript.

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SUPPLEMENTARY INFORMATION

for

ZEB1-induced tumorigenesis requires senescence inhibition through activation of a new ZEB1-DKK1-p53-Mdm2-CtBP pathway to repress macroH2A1

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Running Title: ZEB1 represses macroH2A1 to inhibit cancer cell senescence

SUPPLEMENTARY METHODS

Antibodies

The following commercial Abs were used in this study: anti-ZEB1 [Santa Cruz Biotechnology (SCBT), H-102 and E-20X], anti-DKK1 (Abnova, 2A5), anti-macroH2A1 [Cell Signaling, clone 4827; Abcam, ab37264; and (reference 1) a gift from M. Buschbeck, Institut Josep Carreras, Barcelona, Spain], anti-p300 (SCBT, N-15), anti-CtBP (SCBT, E-12), pan-cytokeratin (Dako, IR053), anti-α-tubulin (Sigma-Aldrich, B5-1-2), and blocking anti-DKK1 (R&D, AF-1096). Secondary antibodies were obtained from Jackson ImmunoResearch Europe (JIR): HRP-conjugated goat anti-rabbit IgG, HRP-conjugated donkey anti-mouse IgG, and Alexa Fluor® 488-conjugated goat anti-rabbit. Normal goat serum used in chromatin immunoprecipitation assays was also purchased from JIR.

Plasmids

Wild type -228bp, -535bp, and -1068bp fragments of the human DKK1 promoter fused to firefly luciferase were obtained from K. Katula (University of North Carolina, Greensboro, NC), TOP-FLASH and FOP-FLASH luciferase reporters from B. Vogelstein (John Hopkins University, Baltimore, MD), and (Gal4 × 5)–SV40-luc reporter from M.A. Lazar (University of Pennsylvania, Philadelphia, PA). The following expression vectors were used: *ZEB1* (CS2MT-*ZEB1*) and *ZEB1*ΔNterm (CS2MT-*ZEB1*ΔNtR) (2), CaGGS-*TCF*4 from M. Kato (University of Tsukuba, Japan), Gal4 (PM1), Gal4-*ZEB1*-CID (G-ZEB-700-776) and Gal4-*ZEB1*-CID_{mut} (G-ZEB-700-776-3mut) (3). *ZEB1*-CID_{mut} (CS2MT-*ZEB1*-CID3mut) was generated by mutation of the three CtBP binding sites in ZEB1 (CS2MT-*ZEB1*) at aminoacids 705, 734 and 767 to the sequence ASASA that does not bind to CtBP. pCS2+*DKK1*-Flag was obtained from Addgene (plasmid 16690; Cambridge, MA), *VP16-p300* (CMV-*P300*-VP16AD) from D. Livingston (Dana Farber Cancer Institute, Boston, MA). pCMV-β-galactosidase and pBluescript SK vectors were purchased from Clontech Laboratories (Mountain View, CA) and Stratagene-Agilent (Santa Clara, CA), respectively.

Gene expression array data and survival plots

Association between *ZEB1* and *DKK1* expression in CRCs and gene signatures associated to different cohorts of patients segregated by the expression of *ZEB1* and/or *DKK1* above/below the upper quartile were obtained from the analysis of published gene array databases, namely: GSE12945, GSE14333, GSE17538, GSE31595, GSE33114, GSE37892, GSE39582 and GSE41258. Gene expression heat maps were assembled using Genesis software, version 1.7.6 (Institute for Genomics and Bioinformatics, Graz University of Technology, Graz, Austria). Correlation between the expression of *ZEB1, DKK1, H2AFY* and/or *GLB1* and relapse-free survival was examined in three array databases of CRC containing survival data (GSE17538, GSE37892, GSE39582). Statistical significance was assessed by Cox proportional hazard regression and Kaplan-Meier survival plots by the best cut-off were computed as described (4).

Cell lines and cell culture

SW480, SW620, COLO320 and DLD1 cells were obtained from the Cancer Cell Line Repository at the Barcelona Biomedical Research Park (Barcelona, Spain) that conducts quality controls for authentication and mycoplasma contamination. LS174T cells were obtained from A. Muñoz (Instituto de Investigaciones Biomédicas Alberto Sols, CSIC, Madrid, Spain). SW480 and LS174T

were maintained in Dulbecco's modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland) while SW620, COLO320 and DLD1 cells were grown in RPMI 1640 medium (Lonza). Both media were supplemented with 10% fetal calf serum (Sigma-Aldrich, Merck, St. Louis, MO), 100 U/mL penicillin, and 100μg/ml streptomycin (Lonza). L-cells stably transfected with an expression vector for mouse Wnt3a (L-Wnt3a cells) or its corresponding empty vector (L-Ctl cells) were cultured in DMEM in the presence of 0.4 mg/ml of geneticin (G418 disulfate, Thermo Fisher Scientific, Waltham, MA) and used to obtain Wnt3a-containing and control conditioned medium, respectively. In selected experiments, SW480 cells were treated with 10 mM of 4-methylthio-2-oxobutyric acid (MTOB) (Sigma-Aldrich). Wherever indicated, either recombinant human DKK1 (rhDKK1) and DKK3 (rhDKK3) (Peprotech, London, UK) was added to cell cultures at 200 ng/ml during 24 h.

Transient and stable transfections and RNA interference

For transient knockdown, 100-200 nM of siRNA oligonucleotides were transfected into cells using Lipofectamine RNAiMAX (Life Technologies). siRNA oligonucleotides for the following genes have been previously described: *ZEB1* (5), *DKK1* (6), *H2AFY* (7), *TP53* (8), and *MDM2* (9). Other siRNA oligonucleotides were ordered from Sigma-Aldrich with the following sequences: *ZEB2* (5'-AAGAGAAGCUGUUUGGAGUGUAU GA-3'), *CTBP* (5'-GGGAGGACCUGGAGA AGUU-3'). A scramble siRNA 5'-GGUUAC GAACUAAGCUAUA-3' was used as negative control. 48 hours after transfection, cells were processed for functional assays. Stable transfection of cells with specific plasmids or the corresponding empty vectors was performed using Lipofectamine 2000 (Life Technologies). Positive cell clones were selected after at least 3 weeks of culture in 0.8 mg/ml geneticin (G418 disulfate, Thermo Fisher Scientific) and/or 10 μg/ml Puromycin (Sigma-Aldrich). Stable lentiviral infections of ZEB1 were performed using a pool of three shRNAs against human *ZEB1* (sc-38643V, SCBT) (sh*ZEB1*-A) (5'-GAAGCA GGAUGUACAGUAA-3', 5'-GGCGAUAG AUGGUAAUGUA-3', 5'-CCAGAACAGU GUUUAUUCU-3') or with a single shRNA from Thermo Fisher (SMARTVector 2.0, SH-006564-02-20) (sh*ZEB1*-B) (5'-TGAAT TTACGATTACACCC-3'). An shRNA control (shCtl) was also purchased from SCBT (sc-108080-V).

Cell viability assays

Cell viability (the result of proliferation minus apoptosis and senescence) was assessed by MTT assays as follows. 1 x 10^4 SW480 cells were transiently interfered for ZEB1, DKK1 or both and were plated in 96 well plates. After 96 h, 10 μl of MTT (5 mg/ml in PBS) were added and incubated for 1 h. Precipitated formazan was dissolved in 100 μl of DMSO. The colorimetric reaction was detected in a Modulus II Glomax detection system (Promega Corp., Madison, WI) at 560 nm absorbance using 750 nm as reference wavelength. Results are mean values of at least four independent experiments performed in triplicate and normalized to the siCtl condition.

Cellular senescence assays

Senescence was determined by senescent associated β-galactosidase activity (SA β-gal) following overnight incubation at 37ºC in CO_2-free conditions using a commercial kit (Cell Signaling). Pictures from representative fields are shown. Percentage of SA-β-gal positive cells was calculated by counting twice the number of stained cells out of a

total of 200 cells in 8-10 representative fields.

Transcriptional assays

Luciferase assays were performed using standard procedures. Briefly, cells were grown in 12-well plates and were transiently transfected with firefly luciferase reporters, cDNA expression vectors and/or siRNAs with 2 μl of Lipofectamine 2000 per well. As control, the corresponding expression empty vectors and/or control siRNAs were also transfected. The total amount of transfected DNA was constant adjusting with pBluescript SK. 0.25 μg of pCMV-β-galactosidase were used for normalization of transfection efficiency. Levels of luciferase and β-galactosidase activity were assayed 48 hours later with Luciferase Assay System kit (Promega Corp.) and Luminiscent β-galactosidase Detection kit II (Clontech), respectively. Relative luciferase activity (RLU) was determined using Modulus II Glomax microplate reader (Promega Corp.). Data shown correspond to a representative experiment from at least four independent ones with each transfection conducted as duplicates. When RLU values are represented in a histogram, one of the conditions is arbitrarily set to a RLU value of 100.

Mutagenesis of human DKK1 promoter

Binding sites for ZEB1 in human *DKK1* promoter were identified using MacVector 12.5 software (MacVector Inc.). Site-directed mutagenesis was performed using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as previously described (5). Site-directed mutagenesis of the ZEB1 binding site at -490 bp contained in the -535bp *DKK1* promoter luciferase reporter was performed using the following oligos: 5'-TGAGCAACTTGCAC CCGCCTTATGGCCCTCTCAGAGGCGTCTTGC-3' and 5'-GCAAGACGCCTCTGAG AGGGCCATAAGGCGGGTGCAAGTTGCTCA-3'. Mutations were confirmed by sequencing with BigDye® Direct Cycle Sequencing Kit (Life Technologies) with primer 5'-AAGCACTTTTTGCCCCTCTCCT TT-3'.

Quantitative real-time PCR

Total RNA from CRC cell lines and mouse intestinal/colonic samples was extracted using SV Total RNA Isolation System (Promega Corp.,) and Purezol (Bio-Rad, Hercules, CA), respectively. RNA was retrotranscribed with random hexamers using the standard procedure with GoScript reverse transcription kit (iScript, Bio-Rad). Quantitative PCR was run on at 60 ºC using SYBR®Green (Bio-Rad or Promega Corp.). Assays were performed in triplicate and at least three experiments were done. Results were analyzed using Opticon Monitor 3.1.32 software (Bio-Rad) by ΔΔCt method comparing values. GAPDH and β-actin were used as reference genes for normalization in human and mouse samples, respectively. Figures shown are the average of at least three independent experiments. Primers used to amplify the different genes examined in the study were as follows: human *ZEB1* (5), mouse *Zeb1* forward 5'-A ACTGCTGGCAAGACAAC-3' and reverse 5'-TTGCTGCAGAAATTCTTCCA - 3', human *DKK1* (10), mouse *Dkk1* (11), human *ZEB2* forward 5'-GAAAAGCAGTTCCCTT CTGC-3' and reverse 5'–GCCTTGAGTGC TCGATAAGG-3', human *AATF* forward 5'-GACACGGACAA AAGGTATTGCG-3' and reverse 5'-AGACCCAGTCCCTCTGAATC TT-3', human *EIF3K* forward 5'-TCCACCA CCTCTTCCT GTTC-3' and reverse 5'-GGC CTGCGTCTCTACATAGC-3', human *EHMT2* forward 5'- AGGTAGCCCGTTA CATGGTG-3'

and reverse 5'-ATCACCTCG ATGTGCTTGTG-3', human *GLB1* forward 5'-CGTATGTGCCCTGGAACTTT-3' and reverse 5'-AATCCTCCCATTTCCCACTC-3', human *H2AFY* forward 5'-AATGCAGC GAGAGACAACAA-3' and reverse 5'-CTT CTTCGCTAGCAACTCGG-3', mouse *H2afy* forward 5'-AATGCAGCAAGAGACAACA A-3' and reverse 5'-CTTCTTCGCTAGCA ACTCAG-3', human *CDKN2B* forward 5'-TC CTGGAAGCCGGCGCAGATC - 3' and reverse 5'-AGCGTGTCCAGGAAGCCTTC C-3', human *DKK1* (11), *MDM2* forward 5'-ATGAAAGCCTGGCTCTGTGT-3' and reverse 5'-TGAGTCGAGAGATTCAACTT CAAA - 3', human *RPL5* (12) human *CDKN2A* (13), human *CTBP* (14), human *TP53* (15), and human *AXIN2* (16). The nomenclature for mouse and human genes adheres to MGI (Mouse Genome Informatics, http://www.informatics. jax.org/) and HGNC (HUGO Gene Nomenclature Committee, http://www. genenames.org/), respectively.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using EpiQuick ChIP kit (Epigentek) as per manufacturer's instructions. Briefly, SW480 cells were incubated during 10 min with 1% formaldehyde solution (Electron Microscopy) at room temperature followed by incubation with 1.25 mM glycine. Lysates were sonicated as described (5). Antibodies used for ChIP were as follows: goat anti-ZEB1 (E-20X), rabbit anti-p300 (N-15), and their corresponding goat or rabbit normal goat IgG (JIR). DNA fragments were quantified by qRT-PCR as detailed above. Identification of DNA binding sequences for ZEB1 and design of primers for qRT-PCR was conducted using MacVector software (MacVector Inc, Apex, NC). For the ZEB1 binding site at position -495/-490 bp of the human *DKK1* promoter, the primers used were as follows: forward 5'-TCAGATCTGCAAACTGCGAC-3' and reverse 5'-CACACAAGCAAGTTCCCAGA -3'. For a region within DKK1 promoter without potential ZEB1 binding sites, the primers used were as follows: forward 5'-AT GGTCATTTCCCTCTTCCC-3' and reverse 5'-TGCACATCAAACGAGGGTAA-3'. For the ZEB1 binding site at position -1059 bp of the human *H2AFY* promoter, the primers used were as follows: forward 5'-TGCCTGTTCT CTTGGCTGTT- 3' and reverse 5'-CCGGG ATGCTTTACACCTT-3'. For a region within *H2AFY* promoter without potential ZEB1 binding sites, the primers used were as follows: forward 5'-ACACTCCTACCCCA GCCTTT-3' and reverse 5'-CG GAGAAGG GGAATAGGAA-3'. Primers used for amplification of a fragment of the *GAPDH* promoter, included in the EpiQuick ChIP kit, are forward 5'-ACGTAGCTCAGGCCTCA AGA-3' and reverse 5'-GCGGGCTCAATT TATAGAAAC-3'. In all qRT-PCRs, values shown represent relative binding in relation to input and are the average of at least three independent ChIP experiments, each one performed in triplicate.

Western blot

Cells were harvested, washed with ice-cold PBS and resuspended in RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 1 % NP40, 0.5 % SDS, 2 mM EDTA) containing protease inhibitors (10 µg/ml aprotinin, leupeptin, pepstatin A and PMSF) as previously described (5). Lysates were sonicated in an Ultrasonic Liquid Processor (Misonix Inc., Farmingdale, NY), clarified by centrifugation and quantified by Bradford assay. Lysates were then boiled and loaded onto 10 % polyacrylamide gels and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). Membranes were blocked with 5 % non-fat milk and blotted with the indicated primary antibodies

overnight at 4 ºC. Detection was done after incubation with HRP-conjugated secondary antibodies. Reaction was developed with Pierce's ECL Western Blotting Substrate or SuperSignal West Pico Chemilluminescent Substrate (Thermo Fisher Scientific). Images shown in the article are representative of at least three independent experiments. Full unedited gel images for all Western blots in the study are included as a separated supplementary file with the area displayed in the main Figures shown boxed. The quantification of CtBP expression with respect to α-tubulin in Supplementary Figure S5D was assessed with ImageJ software (https:// imagej.nih.gov/) (NIH, Bethesda, MD).

Human primary CRC tissue

Paraffin-embedded sections of human primary CRCs, as individual samples and as a tissue microarray from 53 patients, were obtained from the Department of Pathology at Hospital Universitario Fundación Jiménez Díaz (Madrid, Spain).

Transgenic mouse model of CRC and mouse tumor xenograft model

Normal mouse intestinal and colon samples originated from 3-month-old *Zeb1* (+/+) and *Zeb1* (+/-) mice (17). Chemically-induced CRCs in mice were generated in two stages as described (18). Briefly, 4-month-old *Zeb1* (+/+) and *Zeb1* (+/-) mice were injected intraperitoneally with 10 mg/kg of azoxymethane (Sigma-Aldrich). One week later, mice were exposed to dextran sodium sulfate as 1.5% in drinking water (mw 36-44K, Affymetrix, Thermo Fisher Scientific) followed by 2 weeks of recovery with regular drinking water. After repeating this cycle of DSS treatment twice, mice were euthanized and colon tumors extracted for further analysis. For each genotype, mice participating in the study were randomly selected for crossing and ulterior analyses. Investigators were not blinded to the group allocation and/or when assessing the outcome of animal studies. The number of mice used in each experiment is specified in the corresponding figure legend. Use of mouse and human samples were approved by the corresponding Research Ethics Committees.

Mouse tumor xenograft model

A total of 5 x 10^6 SW480-shCtl/shCtl, SW480-sh*ZEB1*-A/shCtl, SW480-shCtl/sh*H2AFY*, and SW480-sh*ZEB1*-A/sh*H2AFY* cells resuspended in 100 μl of PBS and 100 μl of Matrigel basement membrane matrix (Corning, NY) were inoculated subcutaneously into the right lower dorsum of 10-week-old SCID female mice (Janvier, Le Genest-Saint-Isle, France) according to a protocol approved by the Ethics Committee at the University of Barcelona. Tumor growth was followed up as by measuring the shortest and longest diameter of tumors with calipers at 1- to 3-day intervals. Tumor volumes in cm^3 were calculated according to the following formula: (the shortest diameter)^2 x (the longest diameter) x 0.5. Tumor growth was also assessed by the intratumoral uptake of 2-deoxy glucose. Mice were injected i.p. with 10 nmol of IRDye® 800CW-labeled–2-deoxy glucose (LI-COR, Lincoln, NE) and 72 h later fluorescence signal was collected in an Odissey infra-red scanner (LI-COR, Lincoln, NE) before they were euthanized. Fluorescence signal was analyzed using Image Studio v3.1 software (LI-COR).

Immunohistochemistry and immuno-fluorescence

For immunohistochemistry of formalin-fixed, paraffin-embedded human samples of CRCs,

slides were deparaffinized and rehydrated before being subjected to antigen retrieval with 10 mM sodium citrate pH 6.0 for 5 min. Slides were then incubated with a non-specific binding blocking solution (5% donkey normal serum plus 4% BSA in PBS, 0.5% Tween 20) followed by the corresponding primary (overnight at 4°C) and HRP-conjugated secondary (1 h at 37 °C) antibodies. The immunohistochemistry reaction was developed with a DAB substrate kit (Vector Labs, Burlingame, CA) before slides were counterstained with hematoxylin and mounted in Di-N- butylPhthalate in Xylene solution (DPX, Sigma-Aldrich). The number of CRC cells in the tissue microarray, assessed by their staining for pan-cytokeratin-positive, was scored by microscopic analysis at 400X magnification according to the following scale: 0 (0-10 positively stained cells), 1 (11-20), 2 (21-30), 3 (31-40), 4 (41-50), 5 (over 50). For immunostaining of senescent cells, frozen samples of AOM-induced mouse CRCs were embedded in OCT solution (Tissue-Tek®, Sakura-Finetek, Torrance, CA), sectioned and fixed before being processed for SA β-gal staining using the same commercial kit than for cells (Cell Signaling Technology, Danvers, MA) before being counterstained with nuclear solution (Sigma-Aldrich). For immunofluorescence staining of SAHFs, SW480 cells were first transiently interfered for ZEB1 and/or DKK1 or H2AFY during 48 hours and transferred into 8-well PermanoxTM LabTek chambers (Nalgene-Nunc, Penfield, NY), fixed for 30 min with 4% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) and permeabilized with PBS- 0.5% Triton X100. LabTek slides were then incubated with the blocking solution, primary, and fluorochrome-conjugated secondary antibodies and mounted with Prolong Gold Antifade Reagent with DAPI (Life Technologies). Immunofluorescence was examined in a TCS SP5 Spectral confocal microscope (Leica) at the Microscopy Unit of the University of Barcelona. Images were analyzed and RGB profiles generated using ImageJ software applying a median filter of 0.5 pixels.

Statistical analysis

Statistical analysis of the data shown in this study was performed using SPSS® 18.0 software (IBM, Armonk, NY) and/or Prism (GraphPad Software Inc., La Jolla, CA). Normal distribution of the data was determined with Kolmogorov-Smirnov test. Statistical significance of the normally distributed data was assessed with a t-test and with a non-parametric Mann-Whitney U Test for those with non-normal distribution. Where appropriate, relevant comparisons were labeled as either significant at the $p \leq 0.001$ (***), $p \leq 0.01$ (**) or $p \leq 0.05$ (*) levels, or non-significant (ns) for values of $p > 0.05$.

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LEGENDS TO SUPPLEMENTARY TABLE AND SUPPLEMENTARY FIGURES

SUPPLEMENTARY TABLE S1

Relapse-free survival of ZEB1 and Wnt antagonists in a published microarray of CRC. Cox regression analysis and hazard ratio survival rates of array GSE17538 were computed as described in Methods section (main text of the article).

SUPPLEMENTARY TABLE S2

Genes upregulated in cohort 4 of Figure 3A. Genes upregulated when the expression of both *ZEB1* and *DKK1* is in the three lower quartiles in the microarray of Figure 3A.

SUPPLEMENTARY FIGURE S1

***ZEB1* and *DKK1* expression associate in CRCs. (A)** As in Figure 1B, mRNA levels of *ZEB1* and *DKK1* following transient knockdown of *ZEB1* and/or *DKK1* with specific siRNAs (si*ZEB1*-A and si*DKK1*, respectively). **(B)** rhDKK1 and rhDKK3 inhibit Wnt signalling. SW480 cells were treated with different amounts of rhDKK1 and rhDKK3 for 24 h and their effect on transcriptional Wnt signalling was assessed by the TOP/FOP ratio. The TOP/FOP ratio for 200 ng/ml of rhDKK1 and rhDKK3 is shown. **(C)** rhDKK1, but not rhDKK3, reverts effect of si*DKK1* on cell viability. As in Figure 1B, SW480 cells were transfected with 100 nM of either si*DKK1* or siCtl. Forty-eight h later cells were treated with 200 ng/ml of either rhDKK1 or rhDKK3 or DMEM medium (untreated). Cell viability was determined as in Figure 1B. **(D)** Expression of *ZEB1* and *DKK1* positively associate in CRCs. *ZEB1* expression—segregated by quartiles—in the arrays of Figure 1C is displayed with respect to *DKK1* levels. **(E)** Expression of *ZEB2* does not associate with *DKK1* in a panel of CRC cell lines. SW480, COLO320, SW620, DLD1 and LS174T were assessed for mRNA levels by qRT-PCR. See Figure 1D for *DKK1* levels.

SUPPLEMENTARY FIGURE S2

ZEB1 transcriptionally activates *DKK1* expression by direct binding to its promoter. (A and B) As in the *left panel* of Figure 2B but for SW620 and COLO320 cells, respectively. **(C)** Transient knockdown of endogenous *ZEB2* has no effect on the expression of endogenous *DKK1*. ZEB2-positive COLO320 cells were transfected with 100 nM of a specific siRNA against *ZEB2* (si*ZEB2*) or a siCtl and the relative mRNA levels of *DKK1* and *ZEB2* were determined by qRT-PCR. **(D)** *Upper panel*: Scheme of the -1068/+1 fragment of the human *DKK1* promoter. Consensus sites for TCF4 (red) have been previously described in reference (19). Consensus sites for ZEB1 (blue) identified in this study within the first -1 kb of the *DKK1* promoter include four sites at -880, -590, -490 and -199 bp, two of them (those at -880 and -490bp) high affinity binding sites (darker blue). *Lower panel*: Basal transcriptional activity of different fragments of the human *DKK1* promoter. SW480 cells were transfected with 0.25 μg of luciferase reporter constructs containing the indicated deletion fragments of the human DKK1 promoter. **(E)** As in *left panel* of Figure 2E but for SW620 cells. **(F)** As in Figure 2F but for SW620 cells. **(G)** As in Figure 2I but for SW620 cells.

SUPPLEMENTARY FIGURE S3

ZEB1 and DKK1 inhibit senescence in colorectal carcinoma cells. **(A)** mRNA levels of *ZEB1* and *DKK1* following stable knockdown of *ZEB1* and/or overexpression of *DKK1* in the cell lines of Figures 3B and 3C.

SUPPLEMENTARY FIGURE S4

ZEB1, through its induction of DKK1, inhibits SAHF formation and cell senescence. (A) ZEB1 mediates Wnt-induced inhibition of cellular senescence. SW620 cells were first interfered first with either siCtl or si*ZEB1*-A. After 6 h, the medium was replaced and cells were cultured for up to 72 h in the presence of conditioned medium from L cells stably transfected with either a vector encoding recombinant Wnt3a (L-Wnt3a cm) or its corresponding empty vector (L-Ctl cm). Senescence was assessed by staining for SA β-gal. Representative pictures are displayed in the *left panel*. *Right panel*, quantification of the relative number of SA β-gal positive cells from three independent experiments. The first condition was arbitrarily set to 100. **(B)** SW480 cells were cultured during 72 h with conditioned media from L cells stably transfected with either an expression vector encoding recombinant Wnt3a (L-Wnt3a cm) or its corresponding empty vector (L-Ctl cm). mRNA levels for *ZEB1*, *DKK1* and *AXIN2* were determined by qRT-PCR. **(C)** Induction of senescence by si*DKK1* is reverted by rhDKK1, but not by rhDKK3. SW480 cells were transiently transfected with 100 nM of siCtl or si*DKK1* and 48 h later treated with 200 ng/ml of either rhDKK1 or rhDKK3 or DMEM medium (untreated). As in Figure 4B, senescence was assessed by the number of SA β-gal positive cells (*right panel*) with representative pictures shown in the *left panel*. **(D)** Representative pictures of cells stained for SA β-gal in Figure 4C. **(E)** Representative pictures of cells stained for SA β-gal in Figure 4E. **(F)** Single immunofluorescence staining for macroH2A1 (green, clone 4827) or DAPI (blue) for Figure 4F.

SUPPLEMENTARY FIGURE S5

***DKK1* induces *CTBP* to repress *H2AFY*. (A)** Scheme of the -2.0 Kb/+1 fragment of the human *H2AFY* promoter. This study found two high affinity binding sites for ZEB1, at -1687 bp (CACCTG) and -1059 (CAGGTA) (dark blue). **(B)** Expression of *H2AFY* in Figure 5D. SW480 stable cell lines used in Figure 3B and 3C were assessed for relative mRNA levels of *H2AFY* by qRT-PCR. **(C)** Quantification of the number of SA β-gal-positive cells in Figure 5D. **(D)** Quantification of the CtBP protein levels in Figure 5G (right panel), with respect to α-tubulin. **(E)** Quantification of the number of SA β-gal-positive cells in Figure 5J. **(F)** As in (E) and Figure 5J, SW480 cells were treated with 10 mM of MTOB for 24 h and the mRNA levels of *H2AFY* were determined by qRT-PCR.

SUPPLEMENTARY FIGURE S6

Inhibition of *H2AFY* by ZEB1 and/or DKK1 depends on the subsequent induction of mutant *TP53, MDM2* and *CTBP*. (A) rhDKK1 but not rhDKK3 reverts the downregulation of *TP53* and *MDM2* upon *DKK1* knockdown. Cells were interfered with siCtl or si*DKK1* as in Figure 6C. After 48 h, cells were incubated for another 24 h with DMEM or 200 ng/ml of either rhDKK1 or rhDKK3. **(B)** *MDM2* induces *CtBP* expression. SW480 cells were transfected with either siCtl or si*MDM2* and lysates were blotted for CtBP (E12) along with α-tubulin (B5-1-2).

SUPPLEMENTARY FIGURE S7

ZEB1 and DKK1 display an inverse correlation with macroH2A1 in the tumor front of CRCs. (A) Staining of normal colonic mucosa with ZEB1 (H-102), DKK1 (M11, clone 2A5) and macroH2A1 (gift from M. Buschbeck, reference 1). Magnification 400X. **(B)** Immunofluorescence staining of SAHF in a mouse model of CRC. The white box indicates the inset magnified in Figure 7E. The scale bar represents 10 μm.

SUPPLEMENTARY FIGURE S8

ZEB1's role promoting tumorigenesis and determining worse colorectal carcinoma patient survival depends on its repression of *H2AFY*. (A) Lysates from the cell lines used in the xenograft mouse model of Figures 8A and 8B were blotted for macroH2A1 (clone 4827) and ZEB1 (H-102) along with α-tubulin (B5-1-2) as loading control. **(B)** Tumors collected at day 23 from the xenograft mouse model of Figure 8A. **(C)** The maximum effect of *DKK1* as a factor of worse survival depends on low expression of *H2AFY*. Kaplan Meier relapse-free survival plot of CRC patients in Figure 1A segregated in four cohorts by the median expression of *DKK1* and *H2AFY*. Cases with high expression of *DKK1* and low expression of *H2AFY* (red line) had shorter relapse free survival than those patients with high expression of *DKK1* and *H2AFY* (yellow line), low expression of both (green line) or high *H2AFY* but low *DKK1* (blue line).

SUPPLEMENTARY TABLES AND FIGURES

for

ZEB1-induced tumorigenesis requires senescence inhibition through activation of a new ZEB1-DKK1-p53-Mdm2-CtBP pathway to repress macroH2A1

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Running Title: ZEB1 represses macroH2A1 to inhibit cancer cell senescence

SUPPLEMENTARY TABLE S1

Relapse-free survival associated to high expression of *ZEB1* and Wnt antagonists in CRCs

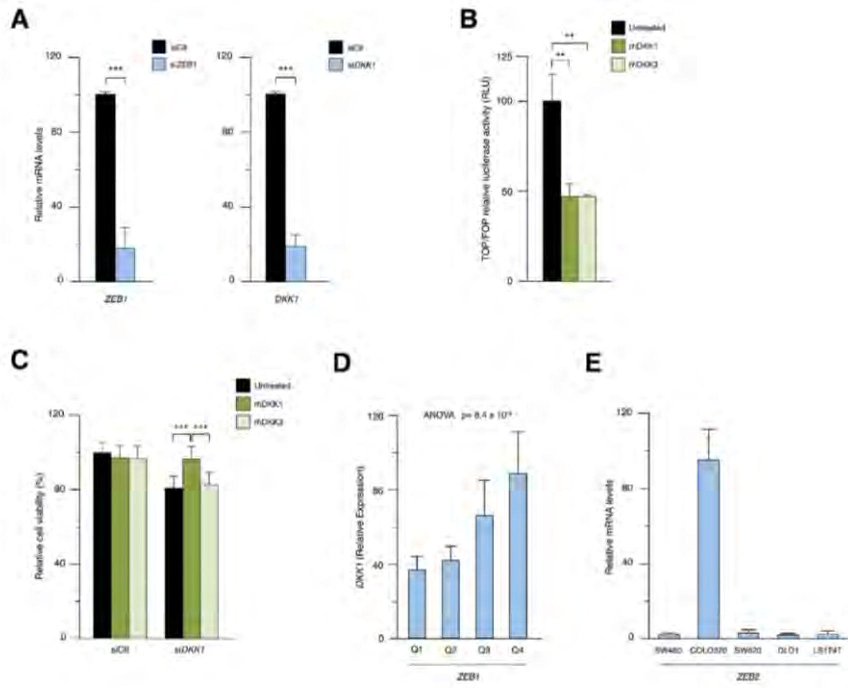
Genes	Survival	Hazard Ratio	Significance (p value)
ZEB1	Worse survival	3.50	Significant (0.0053)
DKK1	Worse survival	2.20	Significant (0.0099)
DKK2	Worse survival	1.27	Non-significant (> 0.05)
DKK3	Worse survival	1.77	Non-significant (> 0.05)
DKK4	Better survival	0.66	Non-significant (> 0.05)
sFRP1	Worse survival	1.54	Non-significant (> 0.05)
sFRP3	Better survival	0.45	Significant (0.0086)
sFRP4	Worse survival	2.30	Significant (0.0051)
sFRP5	Better survival	0.59	Non-significant (> 0.05)

SUPPLEMENTARY TABLE S2

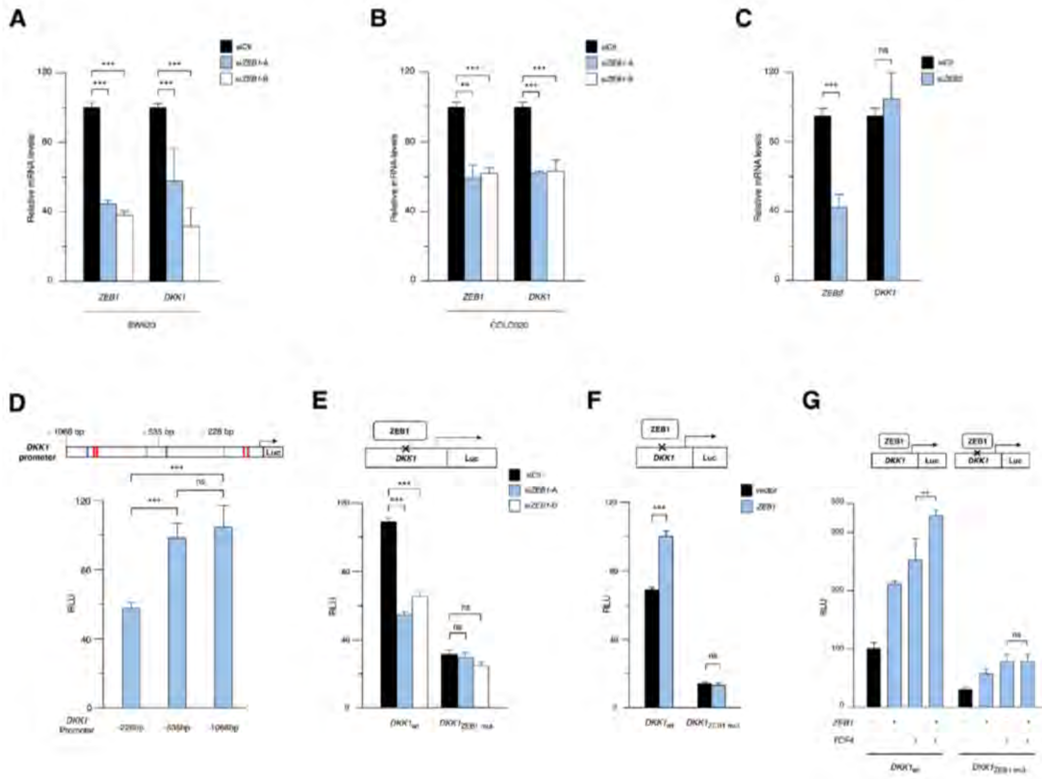
Genes upregulated in cohort 4 of Figure 3A

ACAT2	CDH1	ELF3	IRGQ	NHP2	PSMB7	RPS14	SSSCA1
ACD	CDK1	ELOVL1	KHSRP	NIPAL3	PSMC3	RPS15	ST14
ACO2	CDK2AP2	EMCB	KIF4A	NONO	PSMD3	RPS15A	STK24
ACOT13	CDS1	ERCC2	KPNA2	NOP56	PSMD4	RPS16	STOML2
ACOT7	CDX1	ETFA	KRT18	NOX1	PSMD8	RPS18	STX3
ACTB	CEACAM5	FABP5	KRT19	NRTN	PTMA	RPS19	SURF2
ACTG1	CEACAM6	FAH	KRT8	NT5C	PYGB	RPS2	TBL2
ADAP1	CETN2	FAM60A	LAMP1	NUP43	QPRT	RPS20	TBRG4
ADRM1	CFBP	FAM96B	LARS2	NUP62CL	QTRT1	RPS21	TCA
AKR1A1	CFL1	FLAD1	LAS1L	NUP93	RAE1	RPS23	TCEB2
AKR1C3	CHCHD2	FOXA2	LCN2	NXT1	RALY	RPS24	TCF7
ALDH1B1	CLDN3	FRAT2	LDHA	OLFM4	RANBP1	RPS25	TECR
ARF1	CLDN4	FTL	LDHB	OVOL1	RANGRF	RPS27	TFDP1
ARF3	CLN3	G6PC3	LDLR	P2RY2	RBBP7	RPS29	TFRC
ARHGAP44	CLPB	GADD45GIP1	LGALS3	P4HB	RBM42	RPS3A	TH1L
ARL6IP1	COPE	GAG6	LGALS3BP	PA2G4	RDH11	RPS4X	THOP1
ASCL2	COQ4	GALE	LMAN2	PAAF1	REG1B	RPS5	TIMM44
ASMTL	COQ9	GAPDH	LPCAT1	PAFAH1B3	RFC4	RPS6	TMEM106C
ASNS	COX4I1	GDF15	LPCAT4	PAICS	RFXANK	RPS7	TMPRSS2
ASPM	COX5A	GINS1	LRRC14	PAK4	RNASEH2A	RPS9	TMPRSS4
ATP2C2	COX5B	GINS3	LSM7	PC	RNPEP	RRP12	TMSB10
ATP5I	COX6A1	GLB1	LSR	PCBD1	RPL10A	RUVBL2	TMX2
ATP6V1F	COX7C	GLOD4	LYRM4	PCBP2	RPL12	S100A11	TPPP3
ATPAF2	CRYBA2	GMPPA	MCTS1	PDCD6	RPL13	S100A6	TPT1
AUP1	CS	GNB2	MDH2	PDIA6	RPL14	SCAND1	TRIM24
AURKAIP1	CSNK2A1	GNB2L1	MEA1	PEBP1	RPL18	SCD	TSPAN8
B3GNTL1	CSNK2B	GSPT2	MEST	PES1	RPL18A	SCGB1D2	TSSC4
BABAM1	CTDP1	GSR	MET	PET112	RPL19	SCO2	TST
BCAP31	CYC1	GSTM4	MIF	PEX10	RPL24	SDHA	TUBG1
BDH1	CYP51A1	H2AFY	MLF2	PFAS	RPL27	SEC61A1	TXN
BPI	DAK	H2AFZ	MMP15	PGK1	RPL27A	SF3A3	U2AF1
BSG	DBI	HDHD1	MORC4	PHF16	RPL29	SHMT2	UBA52
BUD31	DDB1	HDHD3	MPG	PIGT	RPL3	SKA1	UBAP2L
BYSL	DDC	HINT1	MRPL4	PLAGL2	RPL30	SLC10A3	UBB
C10orf116	DDT	HIP1R	MRPS15	PLK1	RPL32	SLC25A15	UBC
C16orf53	DDX39A	HIST1H1B	MRPS34	PLP2	RPL34	SLC25A3	UBE2L3
C16orf59	DGAT1	HKDC1	MRPS7	PMPCA	RPL35	SLC25A5	UCN
C16orf80	DHX34	HMG20B	MUC3A	PMVK	RPL35A	SLC25A6	UNC93B1
C19orf21	DIAPH1	HMGB1	MVD	POLA2	RPL36AL	SLC27A5	UQCR10
C20orf27	DMBT1	HMGB2	MYC	POLR2E	RPL37	SLC37A4	UXT
C9orf116	DNMT1	HNRNPAB	MYCN	PPA2	RPL37A	SLC44A4	VDAC1
C9orf16	DUOX2	HNRNPM	MYLPF	PPARG	RPL39	SLC6A6	VIL1
CAMP	DUS1L	HOXB13	N6AMT1	PPIB	RPL41	SLC9A3R1	WBP11
CARHSP1	ECH1	HPCAL1	NADSYN1	PQBP1	RPL5	SMPDL3B	WDR18
CASP5	EEF1A1	HSP90AB1	NARF	PRDX2	RPL7	SNRNP25	YWHAE
CASP6	EEF1D	HSPD1	NCAPD2	PRDX4	RPL7A	SNRPA	ZC3H3
CBLC	EEF2	HSPE1	NDUFA10	PRMT1	RPL9	SNRPB	ZNF574
CCDC59	EFNA4	IDH1	NDUFA3	PROSAPIP1	RPLP0	SNRPD2	ZNF76
CCL20	EHMT2	IDH2	NDUFA4L2	PRPF4	RPLP1	SNRPF	ZNHIT1
CCT5	EIF2S3	IFITM1	NDUFAB1	PRPF6	RPLP2	SOD1	ZNHIT2
CD9	EIF3F	IFITM3	NDUFB11	PRR13	RPS10	SPINK1	ZP2
CDC42EP4	EIF3G	IL17B	NDUFB6	PSMB10	RPS11	SPINT1	
CDC45	EIF3K	IMMT	NDUFS7	PSMB3	RPS12	SSR4	
CDC6	EIF6	IMP4	NDUFS8	PSMB4	RPS13	SSRP1	

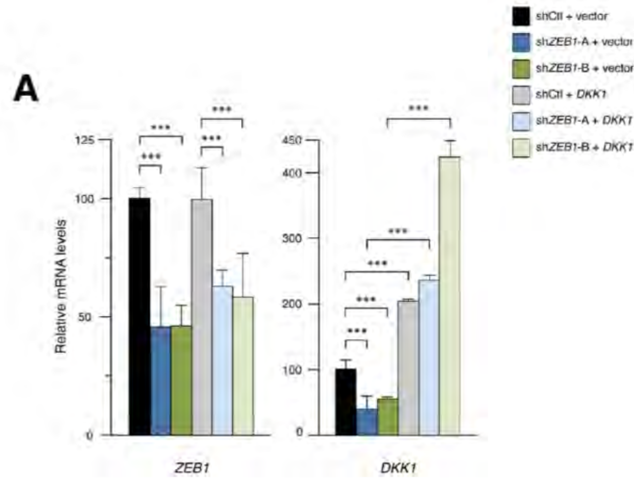
Supplementary Figure S1



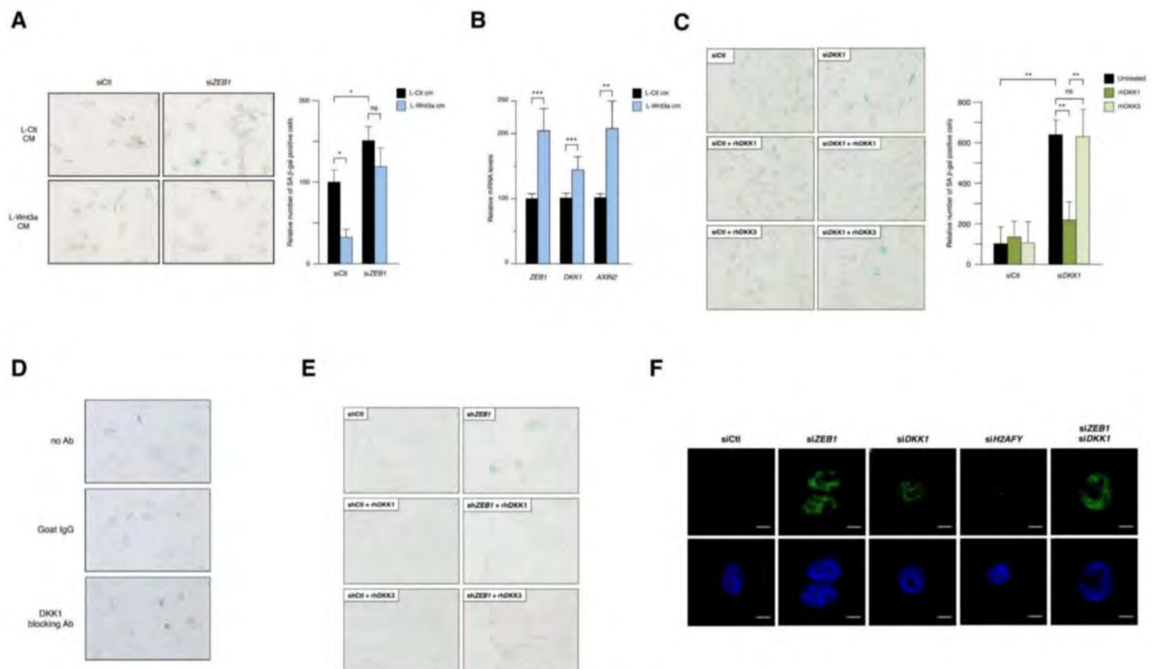
Supplementary Figure S2



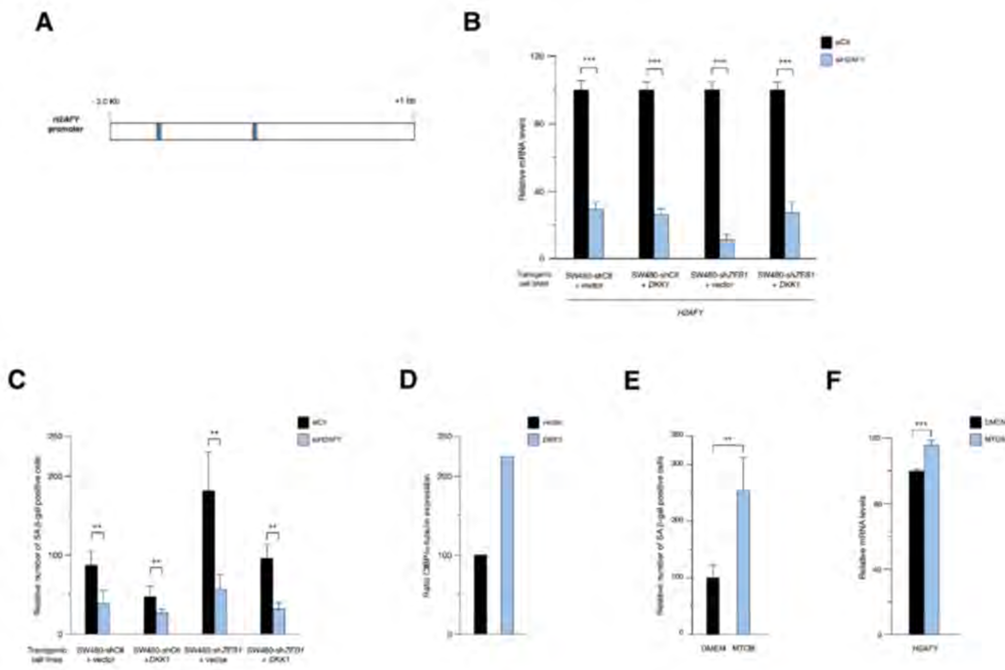
Supplementary Figure S3



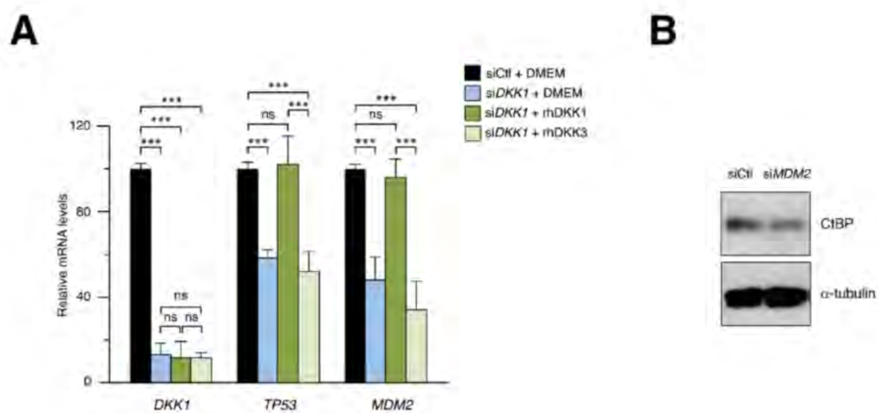
Supplementary Figure S4



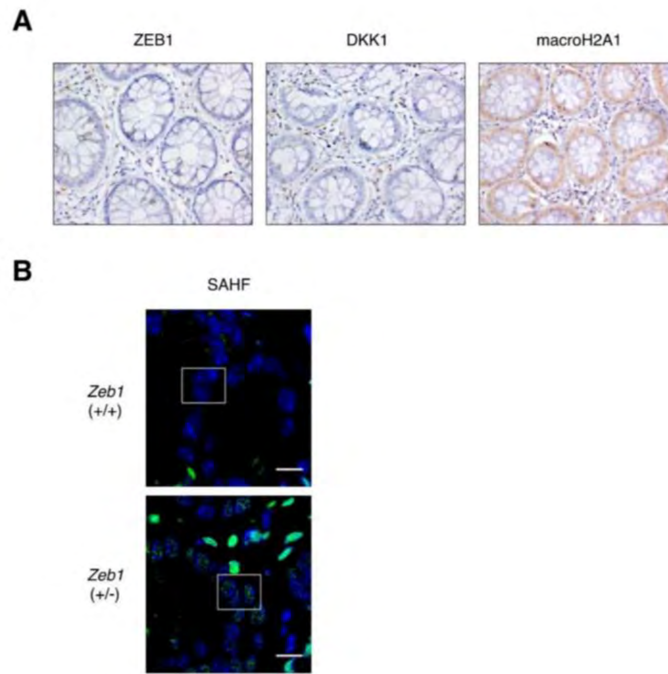
Supplementary Figure S5



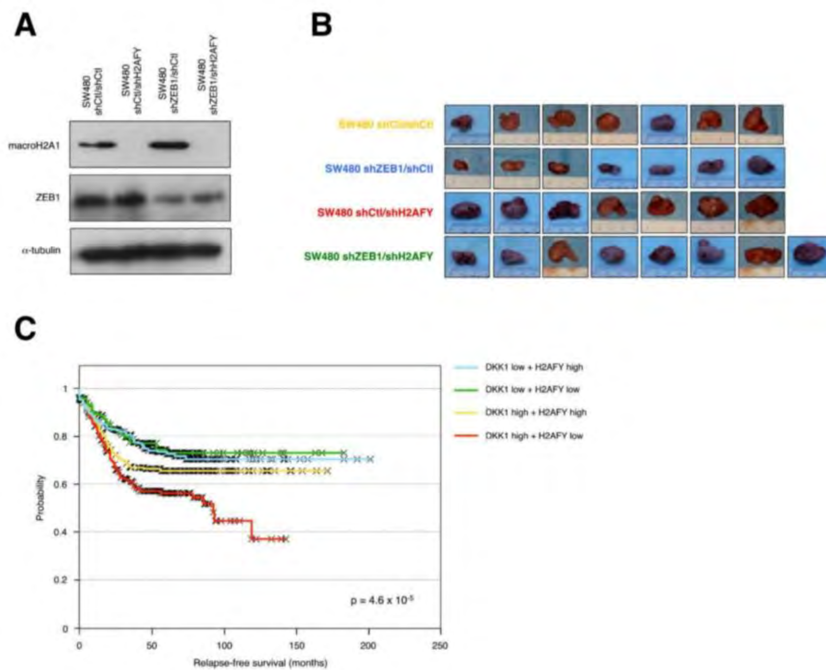
Supplementary Figure S6



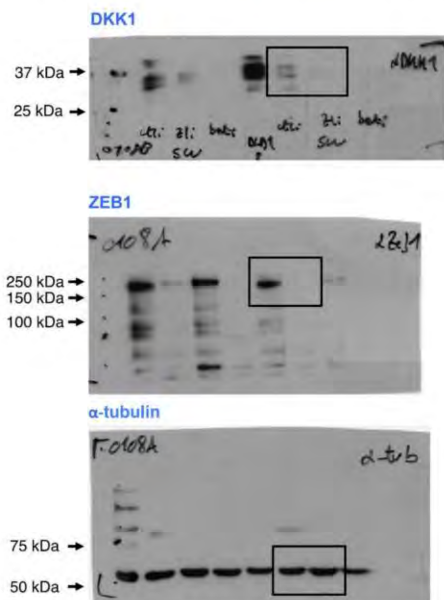
Supplementary Figure S7



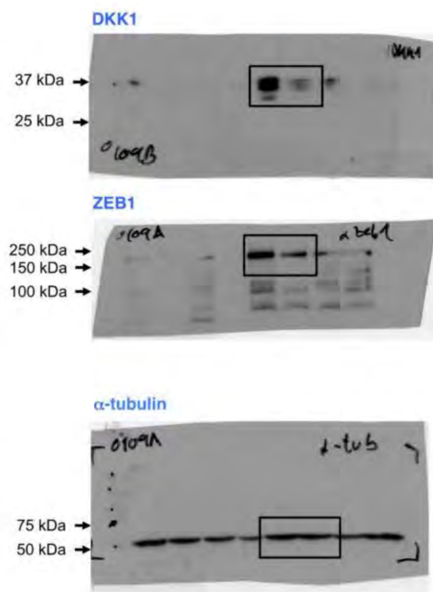
Supplementary Figure S8



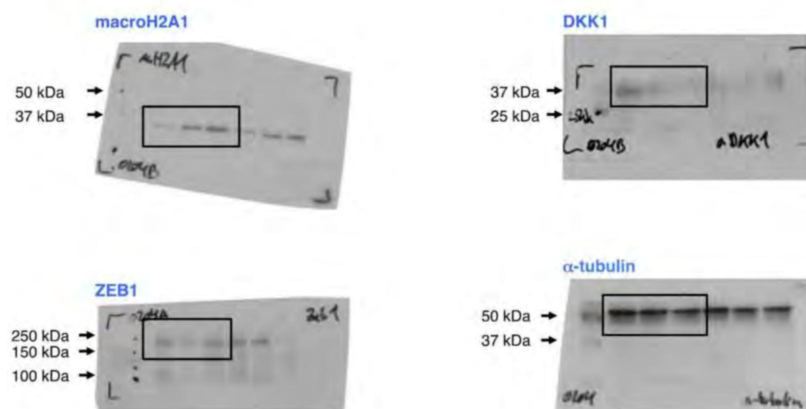
Full unedited gels for Figure 2C (*left panel*)



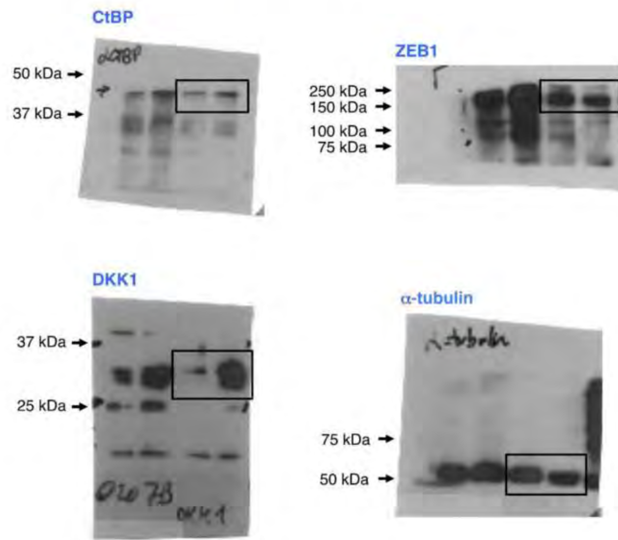
Full unedited gels for Figure 2C (*right panel*)



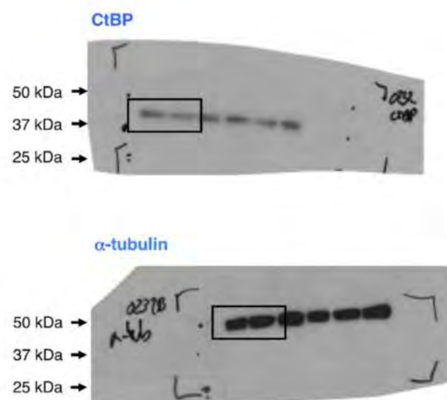
Full unedited gels for Figure 5A



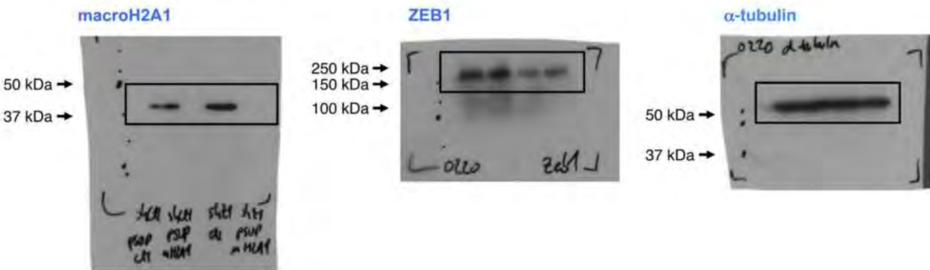
Full unedited gels for Figure 5G (*right panel*)



Full unedited gels for Supplementary Figure S6B



Full unedited gels for Supplementary Figure S8A



SUMMARY OF THE RESULTS

IV – SUMMARY OF THE RESULTS

ZEB1 regulates the expression of genes involved in the disruption of the basement membrane in CRCs (172). Therefore, we sought to investigate whether ZEB1 also regulates the remodeling of the peritumoral stroma. The following results were obtained:

a) ZEB1 regulates uPA and PAI-1 in opposite directions

We found that higher expression of ZEB1 in Wnt-positive SW480 cells associated with lower E-cadherin and higher uPA expression, while Wnt-negative HCT116 presented the opposite pattern. This correlation prompted us to hypothesize that ZEB1 may regulate uPA. To test this, we explored the effect of knocking down endogenous *ZEB1* on the endogenous levels of uPA and PAI-1 protein and mRNA. uPA levels were reduced upon both transient and stable *ZEB1* knockdown. Interestingly, downregulation of *ZEB1* in SW480 cells resulted in the opposite effect on PAI-1—ZEB1 downregulation increased endogenous levels of PAI-1 protein and mRNA. These results indicate that ZEB1 has opposing effects on the two arms of the PAS, inducing uPA expression and repressing that of PAI-1.

b) ZEB1 induces uPA expression by direct transcriptional activation

We investigated the mechanism of ZEB1-mediated induction of uPA by examining the effect of ZEB1 overexpression or knockdown on uPA promoter region. In line with the results shown above, knockdown of *ZEB1* with siRNA reduced basal uPA promoter activity and diminished its response to Wnt3a ligand. We next investigated whether the ability of ZEB1 to activate uPA transcription was mediated by direct binding of ZEB1 to its promoter. In chromatin immunoprecipitation (ChIP) assays we found that an antibody against ZEB1 specifically immunoprecipitated regions of the uPA gene promoter containing ZEB1-binding E-box sequences. Mutation of these sites suppresses response to ZEB1 overexpression. When Wnt signaling was exogenously activated through the overexpression of TCF4, the mutation of ZEB1 sites did not affect the activation of uPA promoter. However, the response was lower due to the lack of ZEB1 component. These results demonstrated that ZEB1 binds to the uPA promoter to directly drive its transcription.

c) ZEB1 inhibits PAI-1 expression by reducing the stability of its mRNA

We tested the ability of ZEB1 to repress the human PAI-1 promoter at the transcriptional level. However, it could not be ruled out that ZEB1 can transcriptionally repress PAI-1 via binding to regulatory promoter regions. We therefore explored alternative mechanisms by which ZEB1 represses PAI-1, namely mRNA stability. SW480 cells knocked down for *ZEB1* were treated with Actinomycin D to inhibit RNA elongation and PAI-1 mRNA levels examined by RT-PCR. After Actinomycin D treatment, PAI-1 mRNA remained more stable in *ZEB1*-depleted cells, compared to its controls. These results indicate that ZEB1 controls PAI-1 expression, at least partially, through regulation of its mRNA stability.

d) uPA expression mediates ZEB1-dependent CRC cells migration and invasion

We wondered whether upregulation of uPA by ZEB1 contributes to ZEB1-mediated tumor cell migration and invasion. Indeed, cells knocked down for ZEB1 displayed significantly lower migratory and invasive capacity than control cells. To evaluate the contribution of uPA loss in *ZEB1*-inhibited cells to this effect, we also tested counterpart cells overexpressing exogenous uPA. We found that forced re-expression of uPA virtually fully restored their migratory capacity and a significant part of their invasive capacity. Moreover, pharmacological uPA inhibition impaired the restoration of migratory and invasive properties, demonstrating that the effect observed was due to the specific role of uPA.

e) ZEB1 is required for the *in vivo* expression of uPA and its induction by Wnt

The *in vivo* relevance of these findings was tested by examining uPA and PAI-1 expression in the developing intestine of late stage *Zeb1*-null (-/-) mouse embryos (embryonic day 18.5). We found that uPA was significantly reduced in the developing intestine of these mice indicating that *in vivo* expression of uPA requires of ZEB1. In contrast, expression of PAI-1 in *Zeb1*-null embryos was not altered or only slightly upregulated. To examine whether ZEB1 is required in Wnt-mediated uPA induction, we tested the ability of recombinant mouse Wnt3a ligand to activate uPA expression in mouse embryonic fibroblasts (MEFs) from the *Zeb1*-null mice. Importantly, we found that induction of uPA by Wnt3a was significantly reduced in *Zeb1* null (-/-) MEFs. Overall, these results confirm that ZEB1 is critically required for the regulation of the PAS and Wnt-mediated induction of uPA *in vivo*.

f) ZEB1 co-expresses with uPA but not PAI-1 at the invasive front of CRCs

We therefore tested whether the expression of ZEB1, uPA and PAI-1 correlates in cancer cells at the invasive front using an array of primary human CRCs. We found that ZEB1, nuclear β-catenin and uPA were expressed by a higher number of cancer cells than PAI-1. Statistical correlation indicated that ZEB1 expression associates with uPA, but not with PAI-1, in CRCs tumor front. This correlation was confirmed by confocal immunofluorescence analysis. High levels of ZEB1 and uPA were found co-expressed by cancer cells at the invasive front of CRCs. Altogether these data show that ZEB1 and uPA are co-expressed *in vivo* in cells at CRCs invading front.

Next, we sought mechanisms of tumor progression regulated by ZEB1 beyond the induction of an EMT phenotype. Given that ZEB1 is induced by canonical Wnt signaling and, in turn, it activates several Wnt-target genes, we started by exploring a potential modulation of Wnt antagonists by ZEB1 during tumor progression. Firstly, we explored whether ZEB1 and Wnt antagonists have opposing effects on the survival of CRCs. The results obtained were:

a) The role of ZEB1 in survival determination depends on the co-expression of *DKK1*

Examination of the relapse-free survival associated with *ZEB1* revealed that, as expected, high expression of *ZEB1* correlated with poorer survival, while expression of most Wnt antagonists was associated with better prognosis. In contrast, we found that *DKK1* and

S*FRP4* were associated with poorer prognosis, indicating that they may have a tumor-promoting role in CRCs. Since *DKK1* is also a target of Wnt pathway (154), we investigated whether joint expression of *DKK1* and *ZEB1* affects overall survival in CRCs. Interestingly, we found that the maximum effect of *ZEB1* as a predictor of reduced survival requires high levels of DKK1. This suggests that, at least for some of its tumor-promoting functions, ZEB1 depends on DKK1 expression. We also observed a positive association between *ZEB1* and *DKK1* in primary CRC samples, as well as in a panel of CRC cell lines. Altogether these results indicate that *ZEB1* and *DKK1* are co-expressed in CRCs where they jointly determine a worse prognosis.

b) ZEB1 directly activates *DKK1* expression in CRC cells

These data led us to investigate whether ZEB1 may activate DKK1 expression. We first examined whether deletion of the *Zeb1* gene alters *Dkk1* expression in the intestinal tract of Z*eb1*-deficient mice. Compared to wild-type littermates, *Dkk1* expression was reduced in the intestinal tract of *Zeb1* (+/-) mice, indicating an *in vivo* association between both genes. In addition, we examined the effect on endogenous DKK1 expression of knocking down endogenous *ZEB1* in three CRC cell lines expressing high levels of ZEB1. Both transient and stable *ZEB1* interference led to a downregulation of DKK1 mRNA and protein levels. These results indicate that ZEB1 induces DKK1 expression in CRC cells.

Additionally, we found that ZEB1 binds to a -490 bp E-box in the promoter region of *DKK1* promoter. In order to test the functionality of this site, this sequence was mutated to a sequence known not to bind ZEB1. The mutation of the ZEB1 site reduced its basal activity in Wnt-active CRC cells. Transient and stable knockdown of *ZEB1* resulted in a downregulation of the activity of wild-type but not of mutant *DKK1* promoter. We also examined the response of both versions of the *DKK1* promoter to ZEB1 overexpression. While exogenous ZEB1 further activated transcription of the wild-type promoter, it had no effect on the mutant version. Moreover, both p300 and TCF4 cooperated with ZEB1 in the activation of DKK1 promoter. In summary, these results demonstrate that endogenous ZEB1 directly drives *DKK1* transcription.

c) ZEB1 and DKK1 mediate inhibition of cellular senescence in CRC cells

In a series of 1557 cases of CRCs, low expression of *ZEB1* and *DKK1* induces a senescence-associated gene signature. To ascertain the molecular mechanisms through which ZEB1 and DKK1 inhibit senescence in CRC cells, we validated the regulation by ZEB1 and/or DKK1 of a subset of the genes included in the signature. The *H2AFY* gene (encoding for histone macroH2A1) was one of the genes selected for validation.

Consequently, we investigated whether ZEB1 and DKK1 inhibit senescence in CRC cells. Compared to the control cells, single interference of either *ZEB1* or *DKK1* resulted in an increase in the number of senescent cells. This number was maintained upon simultaneous knockdown of *ZEB1* and *DKK1*, suggesting that DKK1 inhibits senescence through the same functional pathway that ZEB1. To test this hypothesis we investigated whether exogenous overexpression of DKK1 could revert the onset of senescence induced by ZEB1 knockdown and, of note, it succeeded in reverting it. These effects were also confirmed by the use of a

recombinant DKK1 protein and a DKK1-blocking antibody, which respectively repressed and activated senescence.

We also examined whether manipulation of *ZEB1* and/or *DKK1* expression alters SAHF formation, and found that knockdown of *ZEB1* or *DKK1* increased the assembly of SAHF. In parallel with cellular senescence, simultaneous knockdown of *ZEB1* and *DKK1* did not increase SAHF formation compared to single *ZEB1* knockdown. These results indicate that inhibition of senescence by ZEB1 is mediated, at least in part, by activation of DKK1 expression

d) ZEB1 and DKK1 inhibit cellular senescence by repression of H2AFY

Among the senescence-related genes previously selected, we focused our attention on *H2AFY*, since its expression is required for the formation of SAHF and senescence onset (100). Interference of both *ZEB1* and *DKK1* genes in SW480 cells upregulated macroH2A1 protein expression. Repression of H2AFY by ZEB1 was also tested *in vivo* and the expression of *H2afy* was upregulated in the intestine of *Zeb1* (+/-) mice. In order to confirm that the inhibition of senescence driven by ZEB1 and DKK1 was mediated by macroH2A1 modulation, CRC cells were transiently transfected with siRNA against *H2AFY* and tested for senescent cells number. As expected, siH2AFY inhibited both basal senescence and the senescence induced by ZEB1 knockdown. These results indicate that the ability of ZEB1 and DKK1 to repress senescence depends, at least in part, on their repression effect on macroH2A1.

e) Repression of *H2AFY* is mediated by mutant p53, Mdm2 and CtBP

Next, in an attempt to define the molecular mechanism underlying this effect, we explored whether ZEB1 corepressors mediated the repression of *H2AFY*. Indeed, transient knockdown of the main corepressor of ZEB1, i.e. CtBP, upregulated *H2AFY* expression. Furthermore, mutation of CtBP binding sites in ZEB1 hampered the ability of ZEB1 to repress *H2AFY* expression. Interestingly, both stable DKK1 overexpression and DKK1 recombinant protein induced the expression of CtBP. In conclusion, DKK1 cooperates with ZEB1 in the repression of *H2AFY* by upregulating the corepressor CtBP.

The Mdm2 ubiquitin-ligase cooperates with CtBP in transcriptional repression of gene promoters (218) and, in parallel, is activated by either wild-type and mutant p53 protein (219, 220). Thereby, we sought to investigate a potential involvement of both *TP53* (which is mutated in SW480 cells) and *MDM2* in the regulation of CtBP. In fact, we found that DKK1 activated the expression of both genes, inducing a sequential cascade that ends in the activation of CtBP and consequent repression of *H2AFY*.

f) *In vivo* inhibition of *Zeb1* triggers senescence and reduces CRC tumor formation

We decided to test the correlation between ZEB1, DKK1 and macroH2A1 at the invasive front of sporadic CRCs. Evaluation of ZEB1 and DKK1 staining in a tissue microarray of 53 primary human CRCs stages I to IV revealed a positive correlation between both proteins. Conversely, macroH2A1 expression displayed an inverse correlation with ZEB1 and DKK1. In addition, we examined whether expression of ZEB1 in CRCs represses senescence *in vivo*. To

that effect, wild-type (+/+) and *Zeb1* (+/-) mice were used in a well-established mouse model of chemical-induced CRC. Interestingly, we found that tumors from wild-type mice barely had senescent cells whereas those from *Zeb1*-deficient colon displayed large number of SA β-gal-stained cells. Likewise, SAHF were only observed in the tumors isolated from *Zeb1* (+/-) mice but not in those from wild-type mice. Moreover, in line with these results, *Zeb1* (+/-) mice developed lower number of tumors than wild-type mice. Finally, tumors induced in *Zeb1*-deficient mice displayed stronger expression of macroH2A1. These results indicate that expression of ZEB1 in CRCs represses senescence while its downregulation is sufficient to trigger macroH2A1 expression, SAHF formation and senescence *in vivo*.

g) The tumorigenic potential of ZEB1 depends on its role as a repressor of *H2AFY*

In order to assess whether ZEB1's tumorigenic capacity depends on the repression of *H2AFY*, we used a xenograft mouse model, where stable CRC cells with either *ZEB1* or *H2AFY* or both genes stably depleted were orthotopically injected in immune-deficient mice. We observed that the inhibition of *H2AFY* reverted the decrease in tumor volume caused by *ZEB1* depletion. Therefore, we concluded that ZEB1 mediates tumorigenesis induction through the repression of *H2AFY*.

h) The role of ZEB1 as determinant of worse survival in CRC patients depends on its role as an inhibitor of *H2AFY* and senescence

Analysis of survival in 928 human CRCs demonstrated that those displaying high expression of *ZEB1* and low expression of *H2AFY* have lower survival probability than those with high levels of *ZEB1* but joint high levels of *H2AFY*. This implies that the maximum effect of *ZEB1* as a predictor of poorer survival in CRC requires of low levels of *H2AFY*. Therefore, the role of ZEB1 depends not only on its activation effect on *DKK1* but also on its repression of *H2AFY*. Likewise, we also tested whether expression of *GLB1*, the gene encoding for SA β-gal, alters the predictor value of *ZEB1* in CRC survival. Indeed, among CRCs with high levels of *ZEB1*, patients with low levels of *GLB1* have poorer survival than those with high levels of *GLB1*. Altogether, these results indicate that ZEB1 promotes tumor progression and determines worse prognosis in CRCs, at least to a large extent, through its effect as an inhibitor of senescence.

GLOBAL DISCUSSION

V – GLOBAL DISCUSSION

The role for ZEB transcription factors in mediating progression of cancer lies on their capacity to induce cellular transition from an epithelial to a mesenchymal state (22, 27, 37). It is widely described that the acquisition of mesenchymal traits by the tumor cells provides a more aggressive phenotype and enhances the invasive capacity (19, 20, 26). In CRCs, invading cells at tumor host interface express high levels of ZEB1. This induction of ZEB1 expression is required to achieve an appropriate migration through the surrounding ECM and to initiate metastasis to distant organs (28, 170).

Recent reports have questioned the role of Snail1 and Twist1 in metastasis (51, 52). Instead, ZEB1 is involved in invasion initiation and bloodstream entering even before a primary pancreatic tumor can be histologically detected (78). In addition, it also promotes the formation of lung metastasis (221). In CRCs, the elevated expression of ZEB1 at tumor front (170) promotes cell intravasation and dissemination to the liver, where its downregulation permits the formation of metastasis (222, 223).

In this context, the distinct pieces of work presented in this Thesis show new mechanisms through which ZEB1 is mediating CRC progression, beyond classical mesenchymal transformation and metastasis induction. Therefore, the results support the key role of the EMT-TF ZEB1 in cancer progression. In fact, previous data had already connected ZEB1's role with the induction of several distinct cancer hallmarks (8, 21). For instance, it has been associated to an upregulated stemness capacity (74, 77), angiogenesis induction (84) and improved cell-death resistance (81, 82).

Since ZEB1 is best known for its role on epithelial-mesenchymal plasticity and apico-basal polarity regulation (28, 224), as well as metastasis induction (21, 225), we decided to investigate the potential involvement of ZEB1 in alternative ways of driving tumorigenesis. Thereby, the two studies that are presented in this Thesis associate the role of ZEB1 to two key processes in oncogenic progression: the disruption of extracellular matrix that precedes tumor cell invasiveness and the overcome of cell senescence, that enhances cell proliferation capacity. In fact, the results we found are tightly connected by a coordinated activation through Wnt-mediated ZEB1 induction.

The first work describes the importance of ZEB1 in the remodeling of the surrounding tumor matrix, required for an efficient invasive capacity. This process supports the acquisition of mesenchymal features by the cell, dynamically coordinating migration and invasion to neighbor tissues. The work was focused on the role of PAS in CRC, and reported a differential regulation by ZEB1 of two of its components: uPA and PAI-1. In the case of uPA, we found that it is directly induced by ZEB1, through direct association to its regulatory promoter region. Instead, PAI-1 is repressed by ZEB1, although its regulation is independent of any transcriptional mechanism and is related to a reduction of its mRNA transcript stability. As a confirmation of the results, we observed that ZEB1 and uPA were co-expressed at invading CRC cells, whereas the expression of PAI-1 presented an inverse pattern.

ZEB1 cooperates with the Wnt signaling effector complex β-catenin/TCF4 in the activation of specific Wnt targets (*LAMC2* and *MT1-MMP*) that mediate the disruption of the BM (172, 187). We found here that it is also capable to directly activate even another Wnt target, such as the PAS member uPA, whose role in ECM remodeling is preceded by the BM cleavage exerted by LAMC2 and MT1-MMP, among other laminins and metalloproteinase family members (187). Remarkably, LAMC2 and uPA, well-defined markers of invading CRC cells, are both activated by ZEB1 and their expression is correlated at CRC tumor front buddings (226). Thus, ZEB1 coordinates colorectal cancer progression through the simultaneous activation of diverse Wnt signaling targets. In fact, LAMC2 and uPA not only correlate with ZEB1 and nuclear β-catenin at the CRC tumor front (172), but the expression of both is activated by the cooperative transcriptional activity of ZEB1 and Wnt-effector TCF4 (60).

Binding of uPA to surface complexes conformed by its own receptor (uPAR) and α5 integrin stimulates the activation of Ras-MAPK signaling and, consequently, enhances cellular proliferation (227). At the same time, ZEB factors regulate the expression of both α4 and α5 integrins (63, 228, 229). Since the formation of this complex activates migration and invasion in CRC cells, once again ZEB factors are probably modulating proliferation and invasion processes through the combined activation of both the PAS system and integrins family members (230).

An interesting finding of this study is the regulation that ZEB1 exerts on PAI-1, which was found to be unrelated to its gene promoter modulation. In fact, ZEB1 was reported to inhibit PAI-1 expression through post-transcriptional mechanisms involving a reduced mRNA stability. This new function had not been previously reported, although it requires further investigation in order to define whether it exclusively occurs in the case of PAI-1 mRNA or it is extensive to other transcripts. This result is in line with previous studies that had reported a specific regulation of PAI-1 mRNA stabilization, involving RNA binding proteins such as SERBP1 (231-233). Since the expression of PAI-1 protein is tightly regulated by modulation of its mRNA stability, a potential role for ZEB1 at this point has a special interest in order to interfere with its function in CRC stromal remodeling. HuR protein is involved in the stabilization of mRNA transcripts when it is translocated from the nucleus to the cytoplasm (234, 235). Importantly, in colon cancer and melanoma cells, it has been reported that it forms a cytoplasmic mRNA stabilizing complex with β-catenin (236, 237). Therefore, translocation of β-catenin to the nucleus upon Wnt signaling activation (as it occurs in *APC*-mutant SW480 cells) reduces the activity of this complex and impairs the stabilizing function of HuR on mRNA transcripts (237). Furthermore, it must be noted that HuR binds to a specific sequence of PAI-2 mRNA, providing post-transcriptional control of its expression (238). In this sense, one could speculate that β-catenin/TCF4 complex promotes an earlier degradation of PAI-1 mRNA, not only by the induction of ZEB1 (172), but also by impairing HuR function through the reduction of β-catenin cytoplasmic levels.

In order to achieve an optimal migration through the ECM, the role of ZEB1 in the activation of uPA could be relevant since its proteolytic function enables tumor cells migration

and invasion (194). Furthermore, the simultaneous role of ZEB1-mediated inhibition of uPA repressor PAI-1 is an additional driver of cell invasion. However, PAI-1 presents high affinity for Vitronectin (Vn) and, thereby, its presence is translated into a Vn-rich stroma, which facilitates traction for cancer cells, enabling their migration through the stroma (205). In consequence, despite ZEB1 enhances tumor promotion through its opposite regulation of PAS members, other mechanisms are required to check the levels of PAI-1. Otherwise, ZEB1 could provoke an excessive degradation to the tumor surrounding ECM, which would impair an appropriate traction for migrating cells (113, 205).

Remarkably, uPA activates angiogenesis processes by induction of VEGF receptor (VEGFR) expression (239, 240). Conversely, PAI-1 displays an antiangiogenic role in melanoma cells (241), while it also blocks the binding of VEGFR2 to integrin receptors at endothelial cells (242). Of note, ZEB1 has been associated to angiogenesis promotion through the regulation of vascular endothelial growth factor (VEGF). At the same time, ZEB1 is also induced by VEGF (84, 243). In light of the results shown in this Thesis, and taking all these previous data together, it can be hypothesized that ZEB1-mediated opposite modulation of uPA and PAI-1 is not only key in the tumor stroma remodeling, but it also mediates other tumor progression roles driven by the PAS.

PAI-1 belongs to the family of Serpin proteins, whose role in brain metastasis from lung cancer has been recently reported (244). The presence of plasmin in the brain microenvironment is lethal for most of the metastasizing cells. Thus, cells expressing high levels of Serpin proteins are protected from this effect, since plasminogen is not cleaved into plasmin in the specific regions surrounded by these cells. In addition, PAI-1 is also present in liver metastatic cells from CRC primary tumors (245). In parallel, metastatic cells need to recover their epithelial traits (through MET, the reversion of EMT process) in order to settle and constitute the secondary tumor (20). Therefore, it can be suggested that a downregulation of mesenchymal features in parallel to a loss of ZEB1 in distant metastasis provides an increase in PAI-1 levels and, possibly, in other members of Serpins family, which drive the settling of metastatic cells.

Another interesting finding is the potential feedback loop that seems to exist between uPA and ZEB1 in CRC cells. In fact, the stable overexpression of uPA in SW480 cells increased ZEB1 protein expression that may, as well, support the enhanced malignant and invasive properties conferred by uPA overexpression itself. This result, that appears to be somehow unexpected, can be explained through the role of uPAR, which mediates the cell signaling triggered by uPA. In fact, the uPAR membrane domain activates distinct signaling routes, such as Ras-MAPK or PI3K-Akt (246) that, in turn, induce ZEB1 expression (21). Since both uPA and uPAR promoters are activated by the ZEB1-activator NFκβ (247-249), we sought to investigate whether uPAR could be also activated by ZEB1. Contrary to our expectancies, our preliminary non-published data indicated that uPAR was not regulated by ZEB1, although this point should be evaluated in further detail.

In parallel, the knockdown of uPA expression in breast cancer cells is associated to a decrease in the levels of Vimentin, Snail or Twist. Moreover, uPA activates some stemness-

associated genes (250). At the same time, Snail activates uPA and uPAR, in a positive feedback loop (251), that resembles the ZEB1-uPA loop here reported. Thus, and according to our results, uPA supports the role of EMT-TFs, not only by enabling mesenchymal cells to invade surrounding tissues, but also by activating its own expression and cooperating in other oncogenic functions.

In order to assess the effect of a total elimination of ZEB1 expression *in vivo,* the assays were performed either in MEFs or in the developing murine intestinal tract, where uPA expression was drastically diminished in *Zeb1*-null samples. This finding implies that ZEB1 activation on uPA expression is not only confined to cancer cells and that it may also have a role in intestinal tract developmental stages. We also found that the expression of ZEB1 is required to mediate the activation of uPA by Wnt signaling activity. The role of cancer-associated fibroblasts has acquired a high relevance in the interplay between tumor cells and surrounding ECM (252-254). According to the data presented here, *Zeb1* is indispensable for the activation of Wnt target genes, such as uPA, in murine fibroblasts. Since uPA is also expressed in stromal cells (255, 256), it is plausible to suggest that ZEB1 mediates the mechanisms through which tumor cells cooperate with the stroma in the progression of cancer.

Thereby, the results presented in the first study of this Thesis unveil a new role for ZEB1 in the promotion of cancer progression. Apart from the induction of a mesenchymal phenotype, ZEB1 modulates the stroma remodeling by an opposite regulation of uPA and PAI-1, exerted by different mechanisms. The new data presented here provides alternative options for therapeutic approaches targeting ZEB1's role in CRC stroma, enhancing its value as a potential therapeutic CRC target.

In line with the expanding variety of ZEB proteins functions in malignant transformation, the results obtained in the second study of the Thesis have assigned to ZEB1 a role in Wnt-induced repression of cellular senescence. Although previous studies had shown that *Zeb1*-null MEFs undergo senescence before wild-type ones (86, 87), the specific basis that underlies ZEB1-mediated senescence repression had not been still described. Here, we have uncovered a molecular mechanism by which ZEB1 inhibits the senescence onset in cancer. In fact, the unexpected finding that ZEB1 activates the Wnt-antagonist DKK1 and both synergize in determining a worse CRC relapse-free survival led us to uncover a senescence-associated signature repressed by the presence of high levels of both *ZEB1* and *DKK1*. Among the set of genes from this signature, the mechanism of regulation of *H2AFY* (encoding macroH2A1 histone) was further analyzed. Remarkably, this histone displays a key role in SAHF formation and is associated to a better prognosis in cancer (257, 258). Finally, we also confirmed that the low expression of senescence-associated genes (including *H2AFY*) correlates to a poorer relapse-free survival in CRC patients.

First of all, the analysis of CRC relapse-free survival associated to the distinct sFRP and DKK Wnt antagonists families members revealed that, contrary to what we expected, high levels of DKK1 were related to a worse prognosis. In general terms, Wnt antagonists exert a tumor suppressive function, since they impair the transcription of Wnt targets (144, 158, 259-

261). However, under specific conditions, some Wnt antagonists have an opposite role, thus promoting tumor progression. For instance, DKK2 drives invasion and metastasis in Ewing sarcoma (262), and DKK4 is upregulated in renal and colorectal carcinomas (263, 264). According to our results, DKK1 is associated to cell migration in hepatocellular carcinoma (265).

Since *DKK1* is transcriptionally activated by the β-catenin/TCF4 complex (154), Wnt signaling is able to modulate itself by a negative feedback loop through one of its targets. In fact, it is epigenetically inactivated in CRCs (155). However, recent data has identified DKK1 in the cell nucleus as a marker of chemotherapy resistance and as a determinant of poor survival (266). This paradoxical function of DKK1 had already been widely reported by previous researchers (267, 268). The results shown in this Thesis support the tumor-promoting role of DKK1, in light of the data about relapse-free survival and its co-expression with ZEB1 factor at the CRC invasive front. Nevertheless, in the samples we have analyzed, DKK1 is mainly stromal and cytoplasmic. Therefore, its role in the nucleus deserves further study since it may depend on the cell context.

We found that ZEB1 cooperates with TCF4 in the activation of *DKK1* promoter activity. In a published work where I participated, we demonstrated that ZEB1 mediates the induction of Wnt targets MT1-MMP and LAMC2 (172) and it is also described for the uPA promoter in the first study here reported. In Wnt-positive cells, TCF4 and ZEB1 associate through their C-terminal regions and this binding converts ZEB1 from a transcriptional repressor into an activator (60). As it is shown in the DKK1 promoter assays, the induction by ZEB1 overexpression is enhanced upon simultaneous overexpression of TCF4, resembling the reciprocal cooperation demonstrated on uPA and LAMC2 promoters (60). These assays have been performed in CRC cell lines with strong Wnt signaling (269). It is possible to speculate that an opposite effect may occur in Wnt negative cells, possibly implying a repression of DKK1 promoter. We also found that p300 histone acetyltransferase, which binds the N-terminal region of ZEB1 (56, 57), was involved in *DKK1* gene activation. Cooperation of ZEB1 with TCF4 in the activation of *DKK1* involves recruitment of p300. Since it binds to *DKK1* promoter at the same region that ZEB1, as demonstrated by ChIP assays, it can be concluded that ZEB1 is recruiting p300 coactivator to the DKK1 promoter region. Therefore, ZEB1 seems to activate DKK1 through cooperation with distinct cofactors at both protein ends. While p300 binds the N-terminal region, it is probable that (in a Wnt-active context) TCF4 is simultaneously bound at the C-terminal end (60).

The second study of the Thesis directly connects DKK1 with the repression of senescence. Previous literature is controversial at this point and murine models of accelerated aging have uncovered an augmented Wnt activity during the acquisition of senescence (270). Additionally, Wnt ligand Wnt5a induces senescence through the non-canonical Wnt pathway in ovarian cancer (136). The discrepancy is also extended to the role of Wnt antagonists. For instance, sFRP1 mediates senescence induction provoked by DNA damage (271), while DKK3 displays an antiproliferative role in lung carcinomas (272).

DKK1 expression correlates with an increased senescence in bone and esophagus cells (273, 274) and mediates the effect of p16 (275). The promoter region of *CDKN2A* (encoding for p16 protein) is methylated in SW480. Despite the low basal expression of this gene, downregulation of ZEB1 induces *CDKN2A* expression. Interestingly, we found that DKK1 overexpression after *ZEB1* knockdown caused no variation in the ZEB1-mediated upregulation of *CDKN2A* mRNA levels. Instead, around 50 genes conformed a senescence gene signature that was downregulated by *DKK1* expression. Some of the genes that were further validated in genetically-modified CRC cell lines are involved in PML bodies formation (276) or in the stabilization of p53 tumor suppressor (277). It must be noted that both cases are paradoxically opposite to the oncogenic events triggered by Wnt signaling (140, 278). Altogether, these data suggest that DKK1 may be exerting distinct roles on senescence modulation, depending on the context and the specific markers evaluated.

Wnt signaling pathway also plays a role in senescence repression. In fact, phosphorylation of HIRA in specific residues, which is mediated by GSK3β, is necessary to drive the formation of SAHF in nuclear heterochromatin (279). In consequence, the inhibition of GSK3β, with a key function as a member of the β-catenin destruction complex delays the induction of senescence, either replicative or induced by oncogenes (135, 140). In addition, downregulation of Wnt3a is necessary and sufficient to trigger the recruitment of HIRA into nuclear bodies and to drive cells into senescence by promoting the formation of SAHF (140). Our results in Wnt-active cell lines SW480 and SW620 corroborate the repression that Wnt3a exerts on the senescence onset. Moreover, knockdown of *ZEB1* eliminates Wnt3a effect, suggesting that it mediates senescence repression driven by the Wnt pathway.

The earlier growth arrest and increased senescence of *Zeb1*-null MEFs is accompanied by an upregulation of ZEB1 targets p15 and p21 (56, 87). In line with these previous works, we have corroborated the role of ZEB1 in senescence repression in the context of CRC. Apart from the already described regulation of *CDKN2B*, our results show a simultaneous elevated expression of *CDKN2A* upon *ZEB1* inhibition in CRC cells, which correlates with the senescent phenotype. In addition, ZEB1 promotes tumor progression after the functional loss of Rb tumor suppressor (86, 280), a classically reported cell cycle inhibitor and senescence inducer (281). Instead, we have found a mechanism involving ZEB1 in the senescence repression mediated by Wnt signaling in CRCs.

Importantly, ZEB1 is repressed by the tumor suppressor pathway p16/Rb (86, 282). In a model of lung adenocarcinoma, ZEB1 promotes tumor initiation through overcoming senescence. In this context, a loss of function of Rb tumor suppressor allows Ras oncogene to induce ZEB1 that, in turn, represses senescence-associated genes in a complex mechanism involving miR-200 (86). Regarding miR-200, it has been reported to drive growth arrest in endothelial cells by downregulation of ZEB1 expression (283). In line with previous data, our results indicate that ZEB1 plays a relevant role in protecting cells from entering a senescent state, as well as it has been described for other tumor suppressor mechanisms, like apoptosis.

In connection with the first study presented, PAI-1 is a classical marker of senescence (284) and is a key target of p53 in mediating the induction of senescence (285). Additionally, it

controls senescence mechanisms in accelerated aging murine models (286). As found in the first study, it is repressed by ZEB1, which stimulates a rapid decay in the stability of its mRNA transcripts. These results are in line with our data showing that ZEB1 inhibits senescence. Nevertheless, *PAI-1* was not included in the senescence signature genes because, like *CDKN2A* and *CDKN2B*, it was not regulated by DKK1.

TGF-β signaling induces PAI-1 in parallel to ZEB2 expression in kidney fibrosis (287). This work diverges from the repression effect we reported for ZEB1 on this senescence marker. However, it is not surprising that both ZEB factors display distinct roles in the regulation of senescence, considering that ZEB2 promotes replicative senescence in liver carcinomas (88). In addition, *ZEB2* and *DKK1* expression did not correlate in CRC cells, and knockdown of *ZEB2* had no effect on *DKK1* expression levels. Taking these data together, it is possible that the opposite function of both ZEB factors in senescence modulation lies on distinct modes of regulation of DKK1 and PAI-1 expression.

Interestingly, we found that DKK1 upregulates CtBP expression, which presents a key function in distinct tumor-promoting events such as metabolism, stemness and genome instability in breast cancer (288). In relation to senescence, it represses p16 in melanoma cells (289), supporting the inhibitory effect of ZEB1 reported here. In addition, we also found that the use of a CtBP inhibitor (MTOB) caused an increase of senescence, accompanied by an upregulation of another senescence marker, *H2AFY*. In breast cancer cells, MTOB has already shown to be effective in reverting the pro-tumoral functions of CtBP (288). This pharmacological inhibitor is also effective in reverting the antiapoptotic role of CtBP in CRC cells (290). Remarkably, our results support a possible therapeutic strategy aiming at CtBP pharmacological inhibition, since they connect CtBP repression with the induction of another tumor suppressor mechanism, such as senescence.

In line with a potential use of CtBP pharmacological inhibitors like MTOB (288), there is also an increasing interest in obtaining small molecule drugs against EMT-TFs, including ZEB1 (291). It has been recently published that class I histone deacetylase (HDAC) mocetinostat is effective in repressing ZEB1 and in reverting chemoresistance induced by ZEB1 (292). However, this drug may present off-target effects, since it promotes global epigenetic acetylation (292). Unfortunately, no compounds targeting exclusively ZEB1 have been reported up to date.

Cellular senescence presents a special interest in the development of new therapeutic strategies in cancer treatment (293-295). Growth arrest and the triggering of a senescent phenotype in cancer cells may involve lower side effects than current chemotherapeutic treatment and can stimulate the activity of immune system against tumor cells (294, 296). Several drugs have proved to induce senescence in cancer cell lines (294). In addition, gallotannin reinforces the induction of cellular senescence in CRCs through the induction of DNA damage (297). In light of our results, inhibiting the newly identified mutant p53-MDM2-CtBP pathway that represses macroH2A1 can be helpful in provoking cellular growth arrest. In fact, we have demonstrated that pharmacological repression of one of its components (CtBP) results in an increased number of senescent cells.

The newly identified pathway that mediates the induction of CtBP by the extracellular ligand DKK1 presents a special relevance among the results of the second study presented in this Thesis. This pathway involves the activation of *TP53* and *MDM2*. In line with our results, the use of inhibitory compounds of MDM2 (nutlin-3a) has been associated to an increased senescence in cancer models, such as the case of prostate cancer (298, 299). Since MDM2 mediates p53 destruction (300), it harbors a tumor promoting effect. For instance, it mediates the anti-apoptotic effect induced by Ras signaling in CRC cell lines (301). MDM2 has been also proposed as a potential target for CRC therapy (302). Thus, we suggest that the activation of CtBP and ulterior cooperation with ZEB1 promoter repression activities might be one of the mechanisms through which MDM2 exerts its pro-tumoral functions in CRCs.

Of note, SW480 cells harbor mutations in p53 protein (R273H and P309S), included among the most common p53 mutations in CRC patients (303). Importantly, both wild-type and mutant forms of p53 activate the *MDM2* promoter through binding to its promoter region (219, 304). However, while wild-type p53 impairs the induction of a mesenchymal phenotype by activating miR-200 (i.e. repressing ZEB1), its mutant version promotes an EMT induction. Accordingly, in line with the data presented here, the EMT-TF ZEB1 is activated by mutant p53 and repressed by the wild-type form (220). In order to investigate a differential role between the two versions of p53 in the repression of senescence in CRC cells, further experiments combining intact and mutant p53 CRC cell lines should be performed.

We have reported that the induction of p53 by DKK1 triggers the final repression of macroH2A1 histone through the sequential activation of MDM2 and CtBP. Of note, mutant p53 confers oncogenic features to cancer cells, reversing in some cases the functions of wild-type p53 (305). In fact, some specific mutant forms (including the R273H, present in SW480 cells) drive tumorigenesis and enhance tumor growth and proliferation, therefore endowing tumors with a more aggressive phenotype and associate to poorer survival in CRCs (306, 307). Our results in mutant p53 cells support these previous data, since the mutant form of p53 in SW480 cells plays a key role in the repression of senescence orchestrated by ZEB1 and DKK1.

In CRCs, the induction of senescence through microRNA-34 (miR-34) reduces tumor growth as well (308). Interestingly, miR-34 is activated by wild-type p53 (309). Therefore, the presence of mutant p53 in SW480 cells may impair miR-34 induction. In line with the results shown in the second study, mutant p53 drives senescence inhibition through the activation of *MDM2*, *CTBP* and *ZEB1*. Accordingly, the lack of effect of mutant p53 on miR-34 could be enhancing its effect on senescence overcome and corroborating the pro-tumoral effect of mutant p53 in cancer cells (305).

We found that ZEB1 and DKK1 inhibit expression of *H2AFY* through direct recruitment of ZEB1 to its promoter region. We actually found several potential binding sites for ZEB1 in the first 2kb of its promoter region. In addition, we validated one of the high-affinity binding sites by ChIP assay, demonstrating a direct binding of ZEB1. The role of ZEB1 as a transcriptional modulator depends on Wnt signaling activity (60). Several Wnt targets such as LAMC2, uPA or DKK1 are activated by ZEB1 in a Wnt-positive context like in SW480, SW620 or COLO320 cells. However, in the case of *H2AFY* we have found that ZEB1 can still function as a repressor on the

H2AFY promoter, even in CRC cells with strong Wnt activity. Interestingly, the CtBP corepressor is involved in inhibiting expression of *H2AFY* gene. It is therefore tempting to speculate that, even after being converted into an activator by TCF4, ZEB1 still can repress the activity of some specific promoters when it is bound to corepressors.

In parallel to the modulation that ZEB1 and DKK1 impose on H2AFY protein and mRNA expression, we found that GLB1, the gene encoding for SA β-gal, was also repressed by both of them. This is an important result, since most of previously published data only focuses on assessing the enzymatic activity of lysosomal SA β-gal (91, 95). Instead, very few studies have investigated the connection between GLB1 transcript levels and senescence. Even though, the depletion of GLB1 mRNA correlates with a decreased staining for SA β-gal (310, 311). According to these data, we have corroborated that the upregulation of senescence in ZEB1-downregulated cells is accompanied by an increase in GLB1 mRNA expression, whereas the overexpression of DKK1 reverts this effect.

ZEB1-mediated repression of macroH2A1 was corroborated *in vivo* through different approaches. In fact, a tissue multiple array (TMA) with more than 50 CRC patient samples was used and we found a positive correlation between ZEB1 and DKK1 at the tumor front, whereas macroH2A1 displayed an inverse pattern. Accordingly, macroH2A1 expression is also lost in advanced bladder cancer, compared to the initial stages (312). In addition, the expression of macroH2A1 is associated to a more differentiated cellular status in colon cancer (313). Therefore, macroH2A1 function is opposite to that of ZEB1 and DKK1 in invading tumors, since it is associated to the loss of cellular mesenchymal and aggressive traits.

In the AOM-DSS CRC model we found that tumors formed in *Zeb1*-deficient mice displayed more SA β-gal positive cells than tumors from their wild-type counterparts. In consequence, the lack of *Zeb1* impaired tumor formation in these mice, which, in addition, presented a lower mortality during the assay when compared to wild-type ones. Remarkably, murine models that are deficient for mounting an apoptotic response display increased tumor formation upon combined treatment with AOM and DSS (314). Additionally, the loss of the angiogenesis-mediator VEGFR2 protects from tumor formation in this model by inducing senescence (315), while an increase in cell senescence diminishes the proliferation rate in colon tumor cells (316). Taken these data together, it can be suggested that several tumor suppressor mechanisms may be involved in modulating tumor formation in mice models of AOM/DSS administration. Interestingly, ZEB1 inhibits most of these tumor suppressor functions (82, 84).

We corroborated the importance of macroH2A1 levels in tumor formation in a xenograft murine model, where CRC cells were implanted. The maximum tumor growth of CRC cells was obtained upon stable depletion of *H2AFY*, involving reduced senescence. Accordingly, the induction of senescence decays tumor growth in some cancers (317, 318). Therefore, the loss of mesenchymal features is accompanied by a senescence induction, resembling the effect we found upon ZEB1 depletion. It can be thus suggested that EMT-TFs not only modulate tumor growth *in vivo* by modifying cellular adhesions and polarity, but also through the suppression of senescence.

The results here presented report new mechanisms of CRC tumor progression that are orchestrated by ZEB1 in a context of active Wnt signaling and confirm that its role is not simply restricted to a mesenchymal transformation, but it also modulates several other cancer hallmarks. In the first study, the distinct modulation of PAS members was unveiled, constituting a new mechanism of tumor invasiveness through ECM regulated by ZEB1. The second study sets ZEB1 as a key repressor of cellular senescence, through the activation of a new signaling pathway that involves the Wnt-antagonist DKK1. Both studies expand the oncogenic functions of ZEB1 in CRC and enhance its potential as a promising therapeutic target. In fact, inhibiting ZEB1 by using global deacetylase drugs (i.e. mocetinostat) (292) or through repression of the canonical Wnt pathway (81, 319) appear as promising strategies. However, these drugs do not target ZEB1 exclusively and other chromatin remodeling proteins or Wnt-effectors may be affected. Therefore, the pharmacological inhibition of ZEB1 in cancer therapy is still not currently available and further translational studies are required. In summary, the pieces of work presented in this Thesis combined with recently published research on ZEB factors set ZEB1 as a promising therapeutic target in cancer therapy.

CONCLUSIONS

VI - CONCLUSIONS

The results described in this Thesis can be summarized as follows:

Conclusions of the first study:

1- ZEB1 modulates the migration of CRC cells through the peritumoral stroma by means of an opposite regulation of the PAS members; namely, activation of uPA expression and repression of PAI-1.

2- ZEB1 activates uPA at the transcriptional level by direct binding to its promoter region, through a mechanism that involves p300. In parallel, Wnt signaling requires ZEB1 presence to mediate uPA induction.

3- PAI-1 is repressed by ZEB1 through a transcriptional-independent mechanism. ZEB1 reduces PAI-1 mRNA stability, which represents a new mechanism of gene regulation by ZEB1.

4- uPA is required for ZEB1-mediated CRC cells migration and invasion, driven by Wnt signaling. Its repression impairs cell invasion even in the presence of ZEB1.

5- ZEB1 correlates with uPA, but not with PAI-1, at invasive front of CRCs, supporting its key role in remodeling the ECM in the tumor microenvironment.

Conclusions of the second study:

1- The maximum effect of ZEB1 in determining worse survival in CRC requires the simultaneous expression of Wnt antagonist DKK1. The expression of both genes correlates positively in CRCs.

2- ZEB1 transcriptionally activates *DKK1*, through a mechanism that involves p300 and in cooperation with the Wnt-effector TCF4.

3- Joint expression of *ZEB1* and *DKK1* inhibits a senescence signature in CRC patients. Some of these senescence-associated genes, including *H2AFY*, are repressed by a cooperative action between ZEB1 and DKK1.

4- ZEB1 requires of DKK1 expression to repress SAHF formation and cellular senescence in CRC cells.

5- The repression of macroH2A1 by ZEB1, involves direct binding to the *H2AFY* gene promoter.

6- ZEB1 represses *H2AFY* expression and senescence in CRC cells through the subsequent induction of *DKK1*, mutant *TP53*, *MDM2* and *CtBP*. The ultimate activation of CtBP cofactor enhances the repressor activity of ZEB1 on the *H2AFY* promoter.

7- ZEB1 correlates positively with DKK1 at the tumor front of invasive CRCs. Conversely, the expression of macroH2A1 is inversely correlated to both ZEB1 and DKK1.

8- Downregulation of *ZEB1* in CRC cancer cells *in vivo* is sufficient to trigger senescence, reduce tumor load and improve survival in a mouse model of colon cancer.

9- The tumorigenic capacity of ZEB1 depends on the concomitant low expression of macroH2A1.

10- The role of *ZEB1* as a determinant of worse survival in CRC depends on its inhibition of *H2AFY* and senescence markers like *GLB1*.

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APPENDIX

VIII – APPENDIX

A – Impact Factor Report

Informe del Factor d'Impacte

Com a co-directors de la Tesi Doctoral presentada per Oriol de Barrios Barri, els Drs. Antonio Postigo i Antoni Castells fem constar que els articles que conformen la secció de Resultats han estat publicats o acceptats per a publicació a les següents revistes, amb els seus respectius factors d'impacte, corresponents a l'any 2015:

- *Clinical Cancer Research*: **8,738**
- *Gut*: **14,920**

Dr. Antonio Postigo

Dr. Antoni Castells

Novembre 2016

Informe de Participació

Com a co-directors de la Tesi Doctoral presentada per Oriol de Barrios Barri, els Drs. Antonio Postigo i Antoni Castells fem constar que la participació del doctorand en els articles que conformen la secció de Resultats ha estat la següent:

- En l'article titulat "*ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1*", publicat a la revista **Clinical Cancer Research**, el doctorand ha participat en el disseny i la realització dels experiments en grau de contribució igual a la Dra. Ester Sánchez-Tilló, primera autora de l'article, i consta com a primer co-autor de l'article.

  Aquest article no ha estat utilitzat en l'elaboració d'altres Tesis Doctorals.

- En l'article titulat "*ZEB1-induced tumorigenesis requires senescence inhibition through activation of a new ZEB1-DKK1-mutant p53-Mdm2-CtBP pathway to repress macroH2A*1", acceptat per a publicació a la revista **Gut**, el doctorand ha participat en el disseny i la realització dels experiments com a principal responsable dels mateixos, i consta com a primer autor de l'article.

  Aquest article no ha estat utilitzat en l'elaboració d'altres Tesis Doctorals.

Dr. Antonio Postigo **Dr. Antoni Castells**

Novembre 2016

C – Participation in other Original Articles and Reviews

During my PhD studies period, I have contributed to the following published articles and reviews:

Original articles:

- Sánchez-Tilló E, de Barrios O, Valls E, Darling DS, Castells A, Postigo A. ZEB1 and TCF4 reciprocally modulate their transcriptional activities to regulate Wnt target gene expression. *Oncogene*, 2015. 34(46):5760-70.

- Sánchez-Tilló E, de Barrios O, Siles L, Cuatrecasas M, Castells A, Postigo A. β-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. *Proc Natl Acad Sci USA*, 2011. 108(48):19204-9.

Reviews:

- Menyhart O, Harami-Papp H, Sukumar S, Schafer R, Magnani L, de Barrios O, Gyorffy B. Guidelines for the selection of functional assays to evaluate the hallmarks of cancer. *Biochim Biophys Acta,* 2016. 1866(2):300-319.
- Sánchez-Tilló E*, Liu Y*, de Barrios O*, Siles L*, Fanlo L, Cuatrecasas M, Darling DS, Dean DC, Castells A, Postigo A. EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. *Cell Mol Life Sci*, 2012. 69(20):3429-56. (*equal contribution as first author)
- Sánchez-Tilló E, Siles L, de Barrios O, Cuatrecasas M, Vaquero EC, Castells A, Postigo A. Expanding roles of ZEB factors in tumorigenesis and tumor progression. *Am J Cancer Res*, 2011. 1(7):897-912.

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ORIGINAL ARTICLE

ZEB1 and TCF4 reciprocally modulate their transcriptional activities to regulate Wnt target gene expression

E Sánchez-Tilló[1], O de Barrios[1], E Valls[1], DS Darling[2], A Castells[3,4] and A Postigo[1,4,5,6]

The canonical Wnt pathway (TCF4/β-catenin) has important roles during normal differentiation and in disease. Some Wnt functions depend on signaling gradients requiring the pathway to be tightly regulated. A key Wnt target is the transcription factor ZEB1 whose expression by cancer cells promotes tumor invasiveness by repressing the expression of epithelial specification markers and activating mesenchymal genes, including a number of Wnt targets such as LAMC2 and uPA. The ability of ZEB1 to activate/repress its target genes depends on its recruitment of corepressors (CtBP, BRG1) or coactivators (p300) although conditions under which ZEB1 binds these cofactors are not elucidated. Here, we show that TCF4 and ZEB1 reciprocally modulate each other's transcriptional activity: ZEB1 enhances TCF4/β-catenin-mediated transcription and, in turn, Wnt signaling switches ZEB1 from a repressor into an activator. In colorectal cancer (CRC) cells with active Wnt signaling, ZEB1 enhances transcriptional activation of LAMC2 and uPA by TCF4/β-catenin. However, in CRC cells with inactive Wnt, ZEB1 represses both genes. Reciprocal modulation of ZEB1 and TCF4 activities involves their binding to DNA and mutual interaction. Wnt signaling turns ZEB1 into an activator by replacing binding of CtBP/BRG1 in favor of p300. Using a mouse model of Wnt-induced intestinal tumorigenesis, we found that downregulation of ZEB1 reduces the expression of LAMC2 *in vivo*. These results identify a mechanism through which Wnt and ZEB1 transcriptional activities are modulated, offering new approaches in cancer therapy.

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INTRODUCTION

The canonical Wnt pathway (TCF4/β-catenin) has crucial roles during embryogenesis, stem cell renewal and differentiation, whereas mutations of components of the pathway have a pathogenic role in a number of human diseases.[1] Wnt signaling is triggered by the binding of Wnt ligands to their membrane receptors, which causes the translocation of the oncoprotein β-catenin to the nucleus where it acts as a coactivator of TCF/LEF factors in transcriptional activation of Wnt target genes.[2] The Wnt pathway is tightly regulated at multiple levels, *inter alia*: subcellular localization and secretion of Wnt ligands, binding of activators and antagonists to cell surface receptors, regulation of TCF4/β-catenin transcriptional activity through degradation of β-catenin by the APC/Axin/GSK3/CKI destruction complex or binding of β-catenin to membrane or nuclear proteins.[1,2]

During embryogenesis, Wnt signaling varies significantly across tissues with temporal- and tissue-specific gradients of Wnt activity regulating tissue patterning and cell differentiation.[1,3,4] In adults, differential levels of Wnt signaling regulate hematopoietic lineage specification and normal homeostasis in epithelial tissues.[2,5,6]

Aberrant activation of the canonical Wnt pathway is directly linked to the initiation and progression of several human cancers, particularly colorectal, hepatic and cutaneous.[1] Most of these cancers follow loss-of-function mutations of components of the destruction complex, notably the APC gene. Nevertheless, evidence *in vivo* shows that mutation of APC is not sufficient to activate Wnt signaling, requiring additional exocrine signals from the peritumoral stroma.[7–9] Thus, in primary colorectal carcinomas (CRCs) with APC mutation, Wnt target genes are only expressed at the tumor front but not at the center nor in well-differentiated areas.[7,9]

A key gene induced by Wnt at the tumor front is the transcription factor ZEB1 (also known as δEF1), which triggers an epithelial-to-mesenchymal transition in cancer cells—thus promoting tumor invasiveness—and that associates with a worse clinical prognosis in most human cancers.[10,11] Like Wnt, expression of ZEB1 is also modulated during tissue patterning and cell differentiation,[12,13] and different threshold levels of ZEB1 are required for tumor initiation and progression.[14] ZEB1 can regulate gene expression by direct binding to the regulatory regions of its target genes, activating or repressing their transcription, or indirectly through feedback loops with microRNAs, particularly of the miR200 family.[11] In triggering an epithelial-to-mesenchymal transition, ZEB1 not only directly represses the transcription of a number of epithelial specification markers involved in cell adhesion and differentiation (for example, E- and P-cadherin, cell polarity markers, tight and gap junction proteins), but also activates mesenchymal genes, including a number of pro-invasive Wnt target genes such as the laminin 5 γ2 chain (LAMC2) and the urokinase plasminogen activator (uPA).[15–18] These antagonistic transcriptional activities of ZEB1 depend on its recruitment of different coactivators (p300) or corepressors (CtBP,

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BRG1).[19–22] During embryonic development, the opposing activities of ZEB1 are modulated by the TGFβ/BMP pathway.[20,21] However, it remains unclear how ZEB1 repressing and activating functions are regulated with regard to genes involved in tumor invasiveness.

In this study, we show that the ability of ZEB1 to upregulate Wnt target genes in CRC cells critically depends on the prevalence of an active canonical Wnt pathway. In the presence of Wnt signaling, ZEB1 enhances TCF4/β-catenin-mediated transcriptional activation of LAMC2 and uPA. In turn, in the absence of Wnt signaling—or when expression of TCF4 and/or β-catenin has been knocked down—ZEB1 represses LAMC2 and uPA. This reciprocal modulation of the transcriptional activities of ZEB1 and TCF4/β-catenin requires their binding to DNA, as well as of the interaction of ZEB1 and TCF4 with each other through their respective C-terminal domains. Binding of ZEB1 to TCF4 results in the replacement of ZEB1 corepressors CtBP and BRG1 by coactivator p300. In a mouse model of Wnt-induced intestinal tumorigenesis (APC (Min/+) mice), harboring a loss-of-function mutation of APC and active Wnt signaling, ZEB1 and LAMC2 are induced albeit displaying great heterogeneity from cell to cell across the tumor. Interestingly, when the expression of ZEB1 in these mice was downregulated, expression of LAMC2 in the tumors of APC (Min/+) mice closely parallel that of ZEB1. Altogether, this study provides evidence that ZEB1 and TCF4/β-catenin modulate each other's transcriptional activities in the regulation of pro-invasive Wnt target genes. These results open new avenues to interfere with the tumor-initiating and metastasis-promoting functions of ZEB1 and Wnt.

RESULTS

ZEB1 activates or represses a given Wnt target gene depending on the prevalence or absence of Wnt signaling

During epithelial-to-mesenchymal transition, ZEB1 transcriptionally represses epithelial markers while it activates mesenchymal genes, including several Wnt targets.[15–18] For instance, uPA and LAMC2, two tumor pro-invasive mesenchymal genes, are direct transcriptional targets of both TCF4/β-catenin[23,24] and ZEB1.[16–18] As Wnt signaling could also trigger a mesenchymal phenotype independently of ZEB1,[25] we investigated a potential transcriptional cooperation between Wnt and ZEB1 by exploring the regulation of these two pro-invasive genes.

We selected a panel of CRC cell lines with different levels of ZEB1 expression and of epithelial (for example, E-cadherin, miR-200c) and mesenchymal (for example, vimentin) markers (Supplementary Figures S1A to S1D). We then tested the effect of transient overexpression of ZEB1 in these CRC cell lines on the transcription of the uPA and LAMC2 promoters (Figures 1a and b). As expected, ZEB1 overexpression upregulated uPA and/or LAMC2 transcription in SW480 and SW620 CRC cells. However, ZEB1 had the opposite effect—repression of uPA and LAMC2 transcription—when overexpressed in HCT116 and HT29 CRC cells. Interestingly, the ability of ZEB1 to activate or repress these promoters nicely correlated with the endogenous Wnt activity of these CRC cell lines—namely, high in SW480 and SW620 cells and low in HCT116 and HT29 cells—as determined by the basal transcription of a Wnt-responsive reporter (Supplementary Figure S1E).

To confirm this finding, we cotransfected ZEB1, β-catenin and/or TCF4 along with the uPA and LAMC2 promoter reporters in two of these CRC cell lines: SW480 (with mutant APC and a predominantly nuclear β-catenin localization that results in active Wnt signaling) and HCT116 (where β-catenin remains primarily membranous/cytoplasmic and Wnt activity is low)[17,26] (see Supplementary Figure S1F for overexpressed protein levels of ZEB1, TCF4 and β-catenin). In SW480 cells—where levels of TCF4 constitute the main limiting factor to enhance Wnt signaling[17]—overexpression of ZEB1 not only activated uPA promoter activity but also further enhanced its transcription by TCF4 and/or β-catenin (Figure 1c). Meantime, in Wnt-low HCT116 cells—where levels of nuclear β-catenin are the limiting factor for upregulating Wnt activity[17]—while ZEB1 by itself repressed uPA promoter transcription, ZEB1 activated it when cotransfected with β-catenin (Figure 1d). Transient overexpression of β-catenin resulted in increased Wnt activity (Supplementary Figure S1G). In other words, activation of Wnt signaling in HCT116 cells by overexpression of β-catenin turned ZEB1 from a repressor into an activator of uPA transcription. Similar results were observed in SW480 and HCT116 cells with regard to the LAMC2 promoter (Figures 1e and f).

Lower levels of nuclear β-catenin and Wnt signaling activity in HCT116 cells translate into lower expression of ZEB1 in comparison with SW480 cells[17] (Supplementary Figures S1A and S1B). We therefore stably overexpressed ZEB1 and/or β-catenin in HCT116 cells (Supplementary Figure S1H) and examined their effect on the regulation of uPA and LAMC2 expression. As shown in Figures 1g and h, stable overexpression of ZEB1 in HCT116 cells reduced the mRNA levels and basal transcription of uPA and LAMC2 genes. Stable overexpression of β-catenin upregulated its own mRNA levels and increased Wnt signaling activity (Supplementary Figures S1H and S1I) and, consequently, ZEB1 mRNA levels (Supplementary Figure S1H). Stable overexpression of β-catenin also stimulated uPA and LAMC2 mRNA and transcription, whose levels—in line with results in Figures 1d and f—were further enhanced by the stable joint overexpression of β-catenin and ZEB1 (Figures 1g and h).

From the above results, it is possible to draw two main conclusions: first, ZEB1 cooperates with TCF4 and/or β-catenin in the transcriptional activation of Wnt target genes uPA and LAMC2 and, second, active Wnt signaling (endogenously in SW480 cells or resulting from transient/stable overexpression of β-catenin in HCT116 cells) transforms ZEB1 from a transcriptional repressor into an activator.

Cooperation between endogenous ZEB1 and TCF4/β-catenin in the regulation of Wnt target genes

The results so far showed that exogenously overexpressed ZEB1 cooperates with overexpressed TCF4 and/or β-catenin. We next wondered whether transcriptional cooperation between both complexes also occurs at the endogenous level. First, we examined whether exogenous ZEB1 could activate LAMC2 transcription in SW480 cells in the absence of endogenous TCF4 and/or β-catenin. Compared with an siRNA control (siCtl), transient knockdown in Wnt-active SW480 cells of endogenous TCF4 and/or β-catenin with specific siRNAs (siTCF4 and/or siβ-cat, respectively) not only inhibited the ability of ZEB1 to activate the LAMC2 promoter but also turned ZEB1 into a repressor (Figure 2a, left panel). In turn, and in line with the results shown above, transient knockdown of endogenous ZEB1 in SW480 cells only partially inhibited the transcriptional activation of LAMC2 by exogenous TCF4 and/or β-catenin (Figure 2a, right panel and Supplementary Figure S2A for efficiency of transient knockdown).

The cooperation between endogenous ZEB1, TCF4 and/or β-catenin was also tested using versions of the LAMC2 promoter mutated for TCF4 or ZEB1 sites (LAMC2-TCF4mut and LAMC2-ZEB1mut, respectively). As shown in Figure 2b, overexpression of ZEB1 repressed rather than activated the transcription of LAMC2-TCF4mut. Accordingly, transcription of LAMC2-TCF4mut was upregulated by the transient knockdown of endogenous ZEB1 but remained unaltered by the transient knockdown of endogenous TCF4 (Figure 2b, middle panel). In turn, LAMC2-ZEB1mut was unresponsive to the overexpression of ZEB1 or transient knockdown of ZEB1. However, transcription of the LAMC2-ZEB1mut

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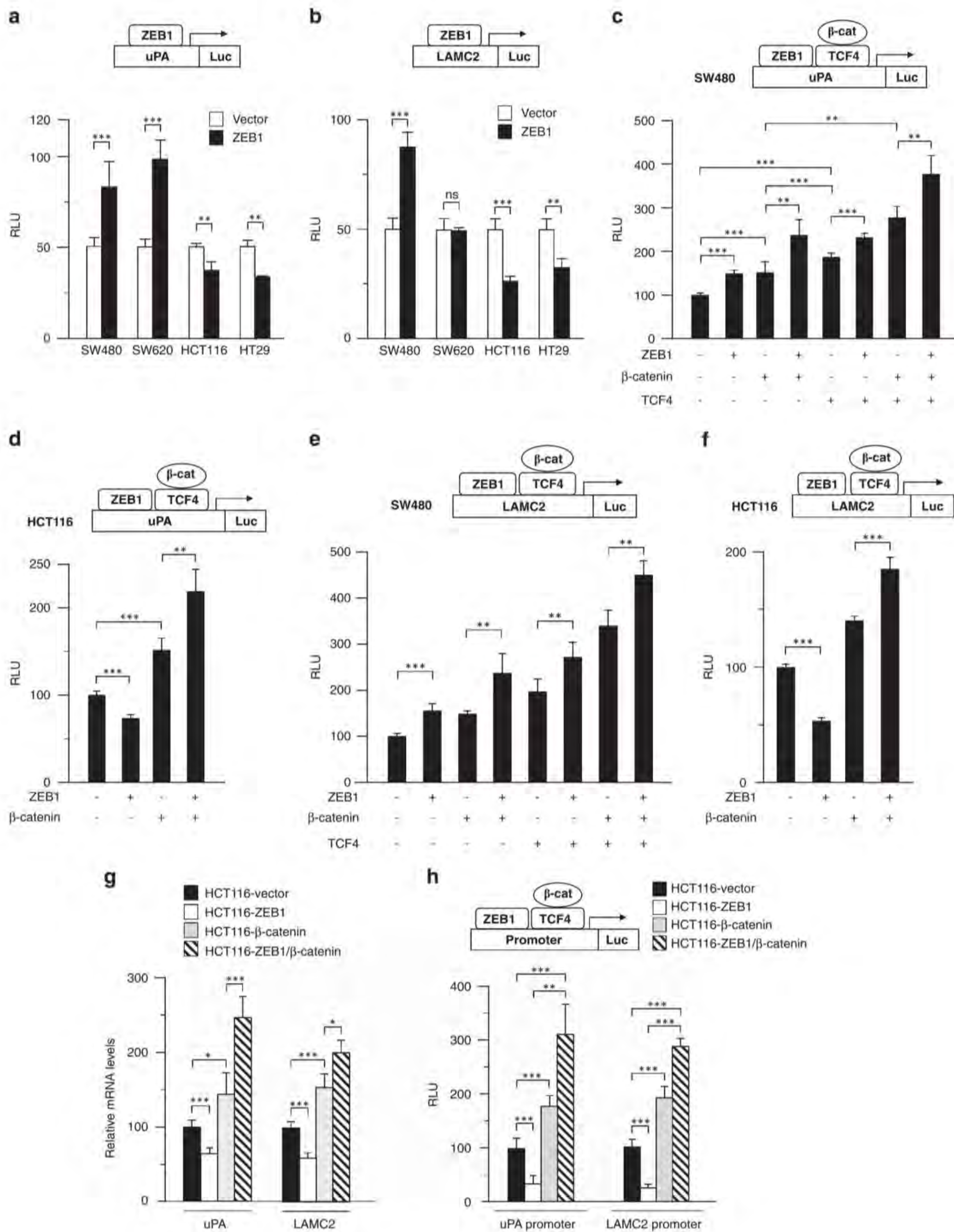
promoter was still activated by overexpression of TCF4 and downregulated by TCF4 knockdown (Figure 2b, right panel). These wild-type and mutated LAMC2 reporters were also tested in Wnt-inactive HCT116 cells (Figure 2c). In line with our results so far, LAMC2-TCF4mut was repressed by ZEB1 independently of the presence or absence of β-catenin (TCF4/β-catenin will not be able to bind to DNA) while LAMC2-ZEB1mut remained unresponsive to overexpression of ZEB1.

Cooperation between TCF4/β-catenin and ZEB1 at the endogenous level was also confirmed in Wnt active cells where either β-catenin or ZEB1 have been stably knocked down. SW480 cells were stably interfered with an shRNA control (SW480-shCtl) or two



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specific shRNAs for ZEB1 (SW480-shZEB1-A, SW480-shZEB1-B) (Supplementary Figure S2B). ZEB1 mRNA and protein expression was inhibited directly by stable ZEB1 interference and, in line with the direct activation of ZEB1 by TCF4/β-catenin,[17] indirectly through β-catenin knockdown with specific shRNAs (sh/β-cat-A and shβ-cat-B) (Supplementary Figures S2C and S2D). As expected, stable knockdown of ZEB1 and β-catenin reduced endogenous Wnt signaling activity (Supplementary Figure S2E).

Stable interference of ZEB1 or β-catenin resulted in down-regulation of the mRNA levels and transcription of LAMC2 and uPA genes (Supplementary Figures S2F and S2G). Compared with SW480-shCtl cells, SW480-shZEB1-A and SW480-shZEB1-B cells

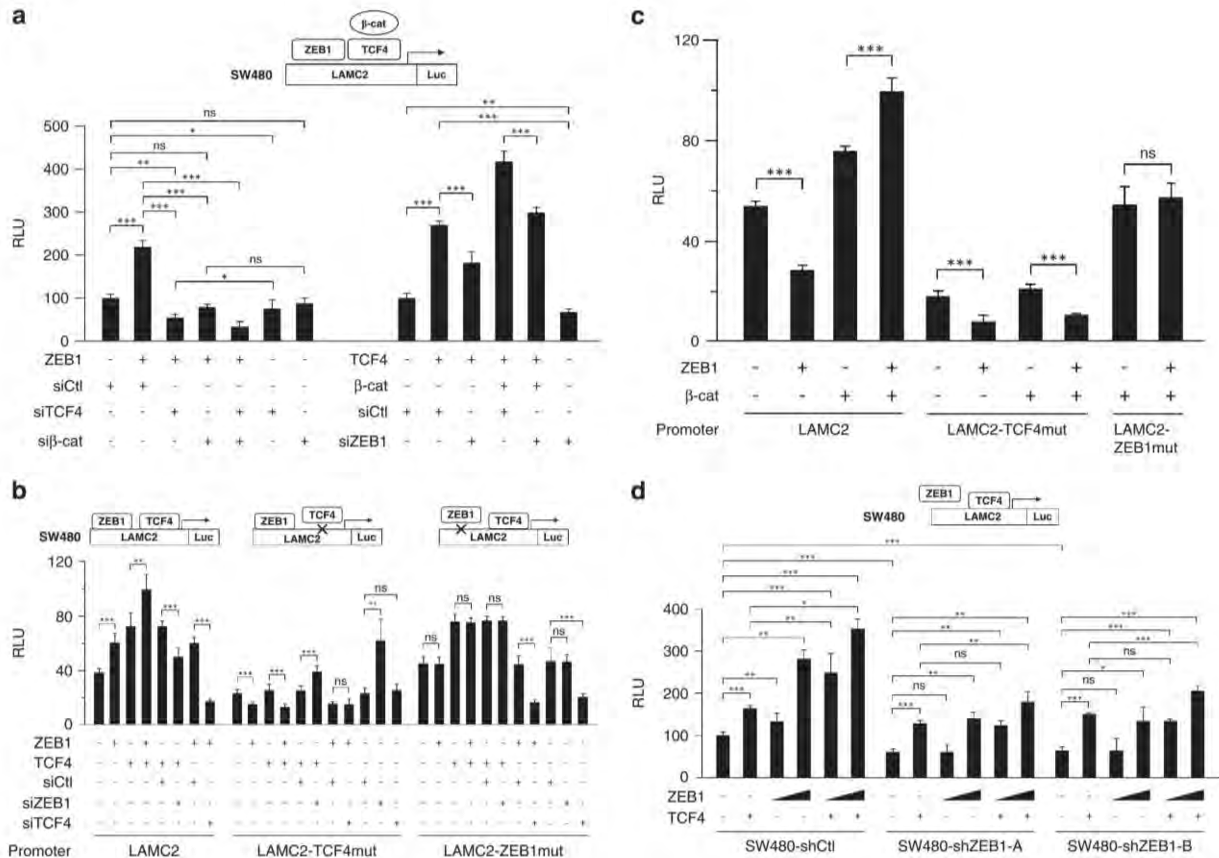

Figure 2. Endogenous ZEB1 and TCF4/β-catenin cooperate in the regulation of Wnt target genes. (**a**) SW480 cells were transfected with 0.2 μg of wild-type LAMC2 promoter and 0.3 μg of the expression vectors for ZEB1, TCF4 and/or β-catenin or equimolar amounts of the corresponding empty vectors. In addition, 100 nM siRNAs against either ZEB1 (siZEB1), TCF4 (siTCF4), β-catenin (siβ-cat) or a control siRNA (siCtl) were added. Transcriptional regulation of the promoter was analyzed 48 h after transfection as described in Supplementary Information. (**b**) SW480 cells were transfected with 0.2 μg of LAMC2 promoter either wild type or mutated for its TCF4- or ZEB1-binding sites along with 0.3 μg of the expression vectors for ZEB1, TCF4 or equimolar amounts of the corresponding empty vectors. Where indicated, 100 nM of siCtl, siZEB1 and/or siTCF4 were added. (**c**) HCT116 cells were transfected with 0.2 μg of LAMC2 promoter either wild type, mutated for its TCF4-binding sites or ZEB1-binding sites along with 0.3 μg of the expression vectors for ZEB1, β-catenin or their corresponding empty vectors. (**d**) SW480 cells stably infected with shCtl, shZEB1-A and shZEB1-B lentivirus were transfected with 0.2 μg of the LAMC2 promoter along with ZEB1 (0 μg, 0.1 μg or 0.25 μg), TCF4 (0.3 μg) or both and compared with their corresponding empty vectors. In all panels, relevant comparisons were either significant at the $P<0.001$ (***), $P<0.01$ (**) or $P<0.05$ (*) levels, or non-significant (ns) for values of $P>0.05$.

Figure 1. ZEB1 activates or represses a given target gene depending on the prevalence or absence of Wnt signaling. (**a**) ZEB1 activates transcription of the uPA promoter in different CRC cells. SW480, SW620, HCT116 and HT29 cells were transfected with 0.2 μg wild-type uPA promoter. In addition, 0.3 μg of the expression vector for ZEB1 or an equimolar amount of the empty vector were added. Luciferase assays in this figure were carried out as described in Supplementary Information and represented as relative luciferase units (RLU) activity. In all figures of this study, the RLU activity of one condition was arbitrarily set to 50 or 100. Error bars in all figures represent s.d. (see Supplementary Materials and Methods). (**b**) As in (**a**), but using a reporter containing the promoter region of human LAMC2. (**c**–**f**) Up/downregulation of human uPA and LAMC2 promoter transcription in SW480 (**c** and **e**) and HCT116 (**d** and **f**) cells. SW480 and HCT116 cells were transfected with 0.2 μg of wild-type uPA (**c** and **d**) and LAMC2 (**e** and **f**) promoters. In addition, 0.3 μg of the expression vectors for ZEB1, β-catenin and/or TCF4 (or equimolar amounts of the corresponding empty vectors) were added. (**g**) HCT116 cells stably expressing ZEB1, β-catenin or both, or their corresponding empty vectors were assessed for relative levels of uPA and LAMC2 mRNA by quantitative real-time PCR (qRT-PCR) as described in Supplementary Information. (**h**) Stable HCT116 cell lines in (**g**) were transfected with 0.2 μg of the wild-type uPA or LAMC2 promoters. In all panels, relevant comparisons were either significant at the $P<0.001$ (***), $P<0.01$ (**) or $P<0.05$ (*) levels, or non-significant (ns) for values of $P>0.05$.

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displayed reduced basal transcription of the LAMC2 promoter (Figure 2d). We then transiently overexpressed exogenous ZEB1 at a level still able to cooperate with TCF4 in SW480-shCtl cells but targeted for degradation in SW480-shZEB1-A and SW480-shZEB1-B cells (Supplementary Figure S2H). Stable knockdown of ZEB1 in these cells does not inhibit the activation of LAMC2 promoter by TCF4 overexpression, but it blocked the cooperation between ZEB1 and TCF4 (Figure 2d). High levels of exogenous ZEB1 in ZEB1 knockdown cells reverted this effect. These results further supported our conclusion that ZEB1 cooperates with TCF4/β-catenin at the endogenous level in the activation of Wnt targets but while ZEB1 requires TCF4 and/or β-catenin for this effect, TCF4/β-catenin is able to activate transcription of Wnt targets, albeit to a lower extent, even in the absence of ZEB1.

Lastly, cooperation between endogenous ZEB1 and TCF4/β-catenin complexes was also tested in regard to the expression of other known Wnt targets. Vitamin D receptor is activated by both ZEB1 and Wnt[27,28] and, consequently, transient knockdown of either ZEB1 or β-catenin in SW480 cells led to a downregulation in vitamin D receptor mRNA levels (Supplementary Figure S2I). By contrast, expression of Id2 (ID2), a known target of Wnt but not of ZEB1,[29] is inhibited by interference of β-catenin but not of ZEB1. mRNA expression of the human lethal giant larvae homologue 2 (HUGL2), which is a target of both ZEB1 and Wnt,[15,30] is upregulated upon knockdown of both β-catenin and of ZEB1. On the other hand, α4 integrin (ITGA4)—a well-established target for ZEB1 repression that has not been reported to be controlled by Wnt[30,31]—is only upregulated upon ZEB1 knockdown in Wnt-inactive U937 cells (Supplementary Figure S2I). These results indicate that ZEB1 activates the expression of a subset of Wnt targets.

Cooperation between ZEB1 and TCF4 to activate Wnt targets requires binding of both factors to DNA

Next, we investigated whether cooperation between ZEB1 and TCF4/β-catenin in the activation of Wnt targets requires binding of these factors to DNA. To that effect, we returned to the earlier experiments in Figure 2b and first examined the effect of overexpressing ZEB1, TCF4 and/or β-catenin on LAMC2-TCF4mut promoter, unable to bind TCF4. As expected, mutation of the two existing TCF4 binding sites to sequences unable to bind TCF4[24] reduced the basal transcriptional activity of the LAMC2 promoter in both SW480 and HCT116 cells (Figures 2b and c). Despite the different levels of Wnt signaling activity of SW480 and HCT116 cells, overexpression of ZEB1 repressed the basal transcription of LAMC2-TCF4mut in both CRC cell lines (Figures 2b and c). These results indicate that the ability of ZEB1 to activate LAMC2 transcription shown earlier in Figures 1b and d, respectively, depends on the prevalence of an intact and DNA-bound TCF4/β-catenin-mediated transcription. When binding of TCF4 to DNA was impaired and, consequently, downstream TCF4/β-catenin-mediated transcription is abrogated, ZEB1 functions as a repressor of LAMC2 even in Wnt-active SW480 cells.

We then explored whether mutation of previously identified ZEB1 sites in the LAMC2 and/or uPA promoters[17,18] inhibits the cooperation between ZEB1 and TCF4/β-catenin in the activation of these promoters. Overexpression of ZEB1 in SW480 cells failed to upregulate the transcription of LAMC2-ZEB1mut (Figure 2b). In turn, overexpression of ZEB1 in HCT116 cells was unable to repress transcription of the same ZEB1-mutated LAMC2 promoter (Figure 2c). Altogether, the above results indicate that the ability of ZEB1 to upregulate the transcription of LAMC2 and uPA and cooperate with TCF4/β-catenin requires the binding of both ZEB1 and TCF4 to DNA.

This requirement for DNA binding was then confirmed using a heterologous Gal4/UAS reporter system. A firefly luciferase reporter containing UAS DNA-binding sites upstream of the SV40 promoter ((Gal4 x 5)-SV40-luc) was cotransfected in SW480 cells with a version of ZEB1 fused to Gal4. As expected, Gal4-ZEB1, which binds to the UAS sequence present in the (Gal4 x 5)-SV40-luc, transcriptionally repressed the activity of this reporter (Figure 3a). In the absence of binding sites for TCF4, over-expression of TCF4 and β-catenin by themselves or in combination failed to activate the heterologous reporter or to revert repression by ZEB1 (Figure 3a). A similar result was observed using a UAS-luciferase reporter driven by the thymidine kinase promoter (data not shown). These results indicate that ZEB1 functions as a transcriptional repressor even when TCF4 and β-catenin are overexpressed as long as they are not bound to DNA.

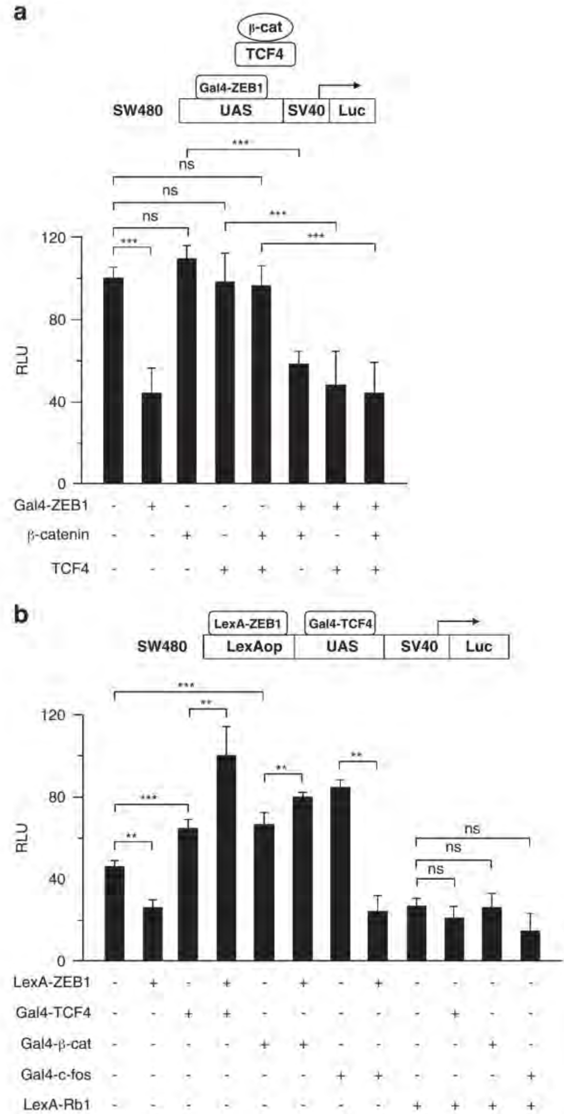

Figure 3. Transcriptional cooperation of ZEB1 and TCF4/β-catenin requires binding of factors to DNA. (**a**) SW480 cells were transfected with 0.2 μg of (Gal4 x 5)-SV40-luc reporter in combination with 0.3 μg of Gal4-ZEB1 and/or expression vectors for β-catenin or TCF4. (**b**) SW480 cells were transfected with 0.2 μg of X2G2P reporter in the presence or absence of several expression vector combinations: LexA-ZEB1, Gal4-TCF4, Gal4-β-catenin, Gal4-c-fos and LexA-Rb1. In all panels, relevant comparisons were either significant at the $P < 0.001$ (***) or $P < 0.01$ (**), or non-significant (ns) for values of $P > 0.05$.

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We then decided to bring ZEB1 and TCF4 down to DNA using a heterologous luciferase reporter that, in addition to UAS sites, also contains binding sites for bacterial LexA protein (LexA operators) and is also driven by SV40 (X2G2P). On one hand, overexpression of LexA-ZEB1 led to transcriptional repression of this reporter, but it cooperated with Gal4-TCF4 or Gal4-β-catenin activating transcription (Figure 3b). In contrast to the experiments with the (Gal4 x 5)-SV40-luc reporter in Figure 3a, when TCF4 or β-catenin are bound to DNA, LexA-ZEB1 cooperates with TCF4 and β-catenin to activate transcription (Figure 3b). Altogether, these experiments indicate that ZEB1 cooperates with TCF4 and β-catenin when both complexes are bound to DNA.

We then examined whether cooperation between ZEB1 and TCF4 also occurs between either of them with other transcription

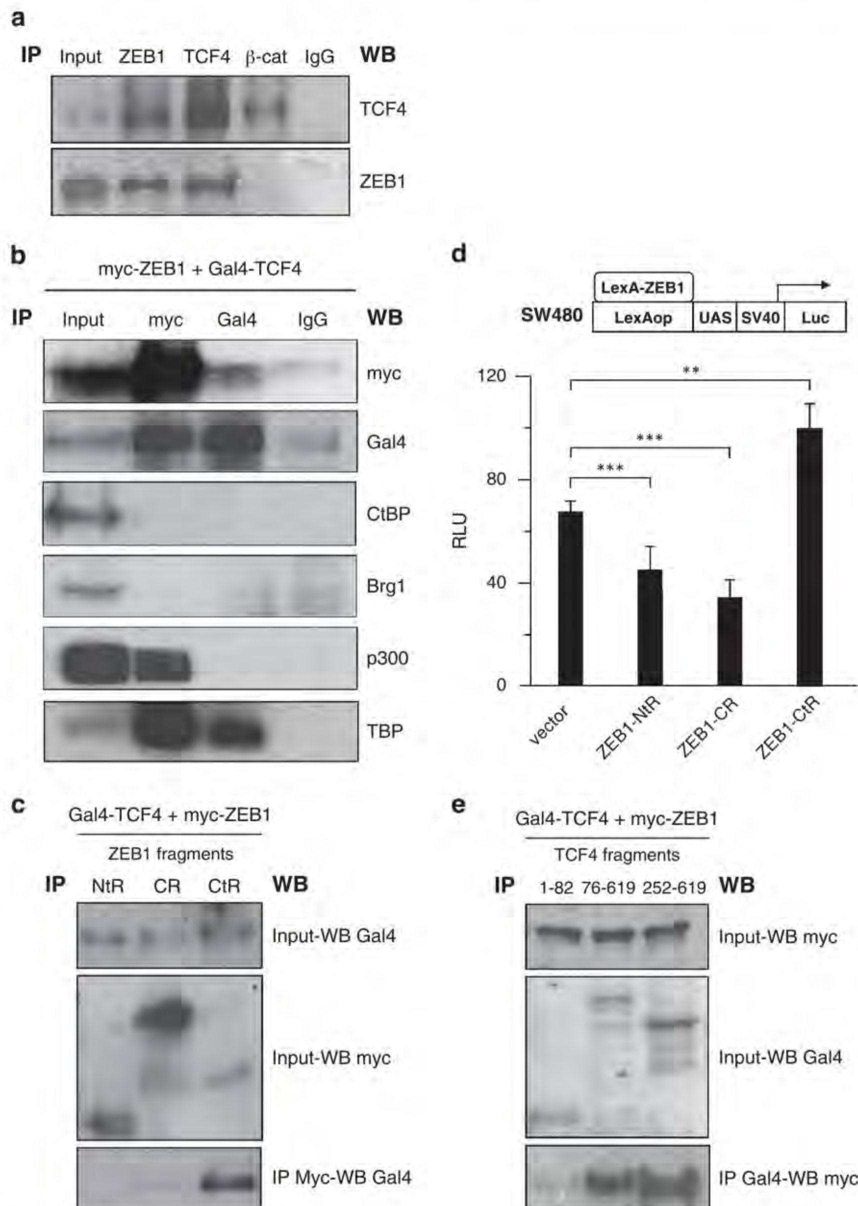

Figure 4. ZEB1 interacts with TCF4. (**a**) Endogenous interaction of ZEB1 with TCF4. Endogenous immunoprecipitation of SW480 total cell lysates with Abs against ZEB1, TCF4, β-catenin and control IgG was carried out. Lysates were immunoblotted for TCF4 and ZEB1 expression. The input lane in panels (**a**) and (**b**) represents 2.5% of total starting lysate. (**b**) Pull-down assay of overexpressed tagged ZEB1 and TCF4. 293 T cells were transfected with myc-ZEB1 and Gal4-TCF4. After 48 h, cell lysates were immunoprecipitated (IP) for myc and Gal4. Pulled-down protein was detected by western blotting (WB) with anti-myc (9E10), Gal4 (RK5C1), CtBP (E12), BRG1 (H88), p300 (1499A) and TBP (N12) antibodies. (**c**) TCF4 binds to the CtR of ZEB1. 293 T cells were transfected with Gal4-tagged full-length TCF4 in the presence of different myc-tagged ZEB1 domains: N-terminal (NtR), central region (CR) and C-terminal region (CtR). Lysates were immunoprecipitated for myc and immunoblotted against Gal4 and myc. (**d**) SW480 cells were transfected with X2G2P reporter in the presence of LexA-ZEB1-NtR, -CR, -CtR or the corresponding empty vector and assayed for transcriptional activity. Comparisons were either significant at the $P < 0.001$ (***) or $P < 0.01$ (**) levels. Luciferase assays were performed at 48 h. (**e**) ZEB1 binds to the C-terminal region of TCF4, aa 252-619. 293 T cells were transfected with myc-full length ZEB1 and several regions of Gal4-TCF4 (aa 1–82, 76–619 and 252–619). Lysates were immunoprecipitated for Gal4 and immunoblotted against myc and Gal4.

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factors. Thus, we found that LexA-ZEB1 does not cooperate with Gal4-c-fos but rather represses c-fos-mediated transcription (Figure 3b). On the other hand, we also examined whether TCF4 and/or β-catenin cooperate with other transcriptional repressors apart from ZEB1. We found that, like ZEB1, the repressor Rb1 strongly repressed the basal activity of the X2G2P reporter. However, in contrast with LexA-ZEB1, LexA-Rb1 repressed the activation by both Gal4-TCF4 and Gal4-β-catenin as well as of Gal4-c-fos (Figure 3b). Altogether, these experiments indicate that cooperation between ZEB1 and TCF4/β-catenin is quite specific because ZEB1 does not cooperate with just any transcriptional activator and TCF4/β-catenin does not transform other known transcriptional repressors into activators.

These experiments were next conducted using a luciferase reporter with UAS- and LexA-binding sites but lacking the SV40 promoter. ZEB1 repressed its transcription but, having this reporter lower basal transcriptional activity, its repressor effect was weaker. Under these conditions, LexA-ZEB1 also cooperated with Gal4-TCF4, even in a promoterless reporter (Supplementary Figure S3A). ZEB1 also repressed activation by c-fos.

Altogether, the above results allow us to conclude that: (i) ZEB1 transcriptional cooperation with TCF4/β-catenin requires binding to DNA of ZEB1 and TCF4, (ii) TCF4/β-catenin do not cooperate with all transcriptional repressors as they failed to do so with Rb1, and (iii) ZEB1 does not cooperate to enhance transcription with any transcriptional activator as it failed to do so with c-fos.

Interaction of ZEB1 and TCF4 through their respective C-terminal regions replaces CtBP and BRG1 from their binding to ZEB1 in favor of p300

Next, we investigated whether cooperation between ZEB1 and TCF4/β-catenin in the regulation of Wnt targets with binding sites for both factors involves not only binding to DNA but also interaction between ZEB1 and TCF4. To that effect, we first tested whether ZEB1 and TCF4 coimmunoprecipitated in SW480 cells that endogenously express both proteins. Indeed, immunoprecipitation with an anti-ZEB1 antibody specifically pulled down endogenous TCF4 and, conversely, an anti-TCF4 antibody coimmunoprecipitated endogenous ZEB1 (Figure 4a). These results indicate that ZEB1 and TCF4 interact *in vivo* to form an endogenous transcriptional complex. Of note, the anti-β-catenin antibody did not pull down endogenous ZEB1 suggesting that ZEB1 cooperation with the TCF4/β-catenin transcriptional complex involves interaction with TCF4 but cooperation with β-catenin is only indirect through TCF4. Interaction between ZEB1 and TCF4 was also confirmed using exogenously overexpressed tagged versions of both factors in 293 T cells. Compared with a control IgG, Gal4-TCF4 coimmunoprecipitated myc-ZEB1 and vice versa (Figure 4b).

As ZEB1 and TCF4 are both highly modular proteins with independent domains for binding to DNA and to a number of cofactors, we next mapped the regions in ZEB1 and TCF4 involved in their interaction. First, we defined the region within ZEB1 that mediates its binding to TCF4. To this end, three myc-tagged fragments of ZEB1 encompassing its N-terminal (NtR, aa 1–168), central (CR, aa 295–902) and C-terminal (CtR, aa 988–1124) regions were co-expressed in 293 T cells along with Gal4-full-length TCF4. Lysates were immunoprecipitated for myc and then blotted for Gal4. As shown in Figure 4c, TCF4 was pulled down by the CtR of ZEB1, but not by the other two domains. The NtR and CR of ZEB1 interact with the BRG1 and CtBP corepressors, respectively,[19,22] but no protein has been previously reported to bind to the CtR.

We then examined the transcriptional activity of these ZEB1 fragments using a heterologous reporter driven by the SV40 promoter (Figure 4d). In line with their binding to corepressors BRG1 and CtBP, the NtR and CR repressed transcription of this reporter. In contrast, the CtR activated transcription of the reporter suggesting that CtR acts as an autonomous activation domain (Figure 4d).

Next, we sought to identify the region of TCF4 involved in the interaction with ZEB1. Three Gal4-tagged fragments of TCF4—an N-terminal domain (aa 1–82) and two C-terminal regions (aa 76–619 and aa 252–619)—were cotransfected in 293 T cells with myc-tagged full-length ZEB1. Immunoprecipitation of cell lysates with Gal4 and subsequent blotting for myc revealed that the two C-terminal regions of TCF4 interact with ZEB1 while the N-terminal domain did not (Figure 4e). Altogether, these results indicate that ZEB1 and TCF4 interact with each other through their respective C-terminal regions.

The region aa 252–619 in TCF4 not only contains its DNA-binding domain, but also interacts with the Groucho/TLE-1 corepressor that competes with β-catenin for binding to TCF4.[32] We found that the overexpression of increasing amounts of TLE-1 inhibited the cooperation between ZEB1 and TCF4 in the transcription of the LAMC2 promoter (Supplementary Figure S4A).

We have shown above that the interaction of ZEB1 with TCF4 converts ZEB1 from a repressor into an activator. ZEB1 could either repress transcription of its target genes via recruitment of a repressor complex containing CtBP and BRG1 or activate it through its binding to p300 histone acetyl-transferase.[19,21,22] We therefore investigated whether interaction between ZEB1 and TCF4 alters the binding of ZEB1 to its cofactors. Following the reciprocal immunoprecipitation of overexpressed ZEB1 and TCF4 in Figure 4b, blots were examined for CtBP, BRG1 and p300 as well as for the general basal transcription factor TBP. Although 293 T cells express both ZEB1 co-repressors (CtBP and BRG1), these failed to immunoprecipitate with ZEB1 when the latter was bound to TCF4. Instead, ZEB1 formed a complex with p300 and TBP. These results indicate that interaction of ZEB1 and TCF4 induces a replacement of CtBP and BRG1 in favor to p300, suggesting a potential mechanism for the conversion of ZEB1 into an activator.

ZEB1 and Wnt cooperate *in vivo* in the regulation of LAMC2 in a mouse model of intestinal tumorigenesis

Over 80% of sporadic CRCs and most cases of familial adenomatous polyposis—an autosomal dominant inherited syndrome displaying intestinal adenomas that eventually evolve toward CRCs—have loss-of-function mutations of the APC gene.[33]

Figure 5. High expression of LAMC2 correlates with high expression of ZEB1 and active Wnt in APC (Min/+) mice. (**a**) Association between expression levels of ZEB1 and LAMC2 in the adenomas of APC (Min/+) mice. Normal and adenomatous mucosa from the intestine of 4-month-old APC (Min/+) mice were immunostained for ZEB1 (green, H-102), β-catenin (red, clone 14) and LAMC2 (blue, C-20). LAMC2 was originally detected with Dylight 649 and converted to blue for representation. Representative merge pictures are shown and the area included within the white square in the left panel is magnified in the center panel. Single immunofluorescence staining for the three proteins is shown in (**b**). In the right panel, an RGB profile shows relative levels of expression for ZEB1 (green), β-catenin (red) and LAMC2 (blue) versus relative distance across different cell sections (white diagonal lines). Scale bars in (**a**) and (**b**) represent 25 μm. (**b**) Single staining of normal and adenomatous mucosa from the intestine of APC (Min/+) mice corresponding to (**a**). Tissue sections were immunostained for ZEB1 (green, H-102), β-catenin (red, clone 14) and LAMC2 (blue, C-20). A merge picture is also shown. (**c**) Schematic representation of the regulation of Wnt targets by TCF4/β-catenin and ZEB1 (see Discussion for details).

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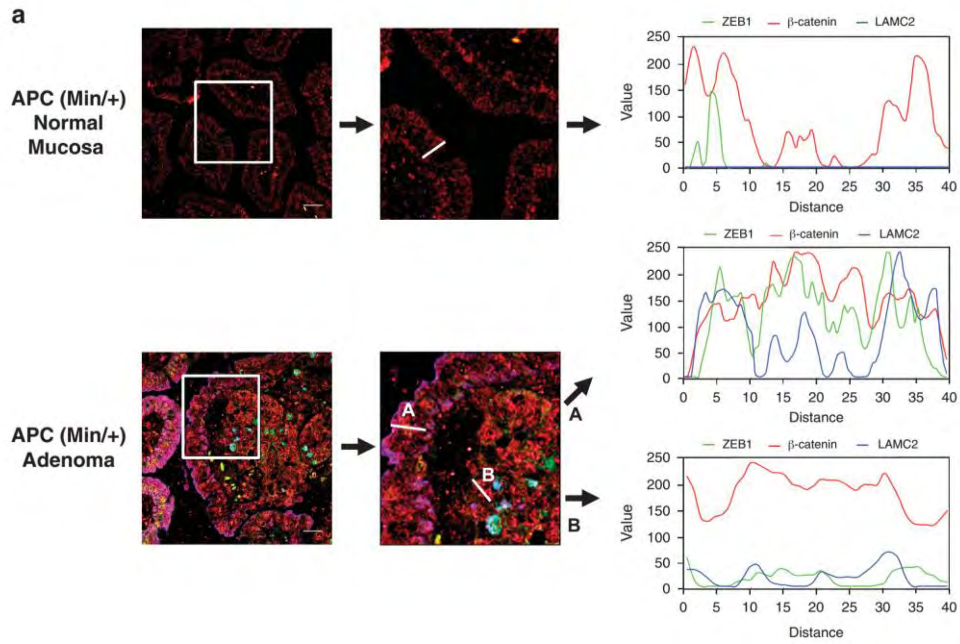

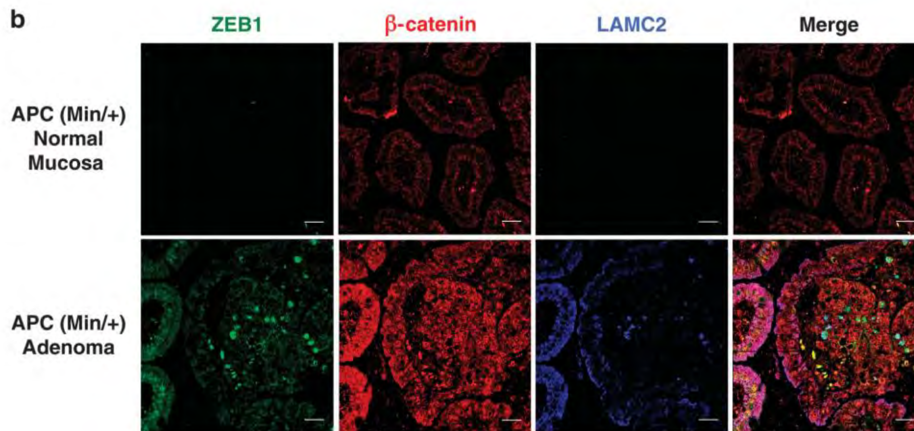

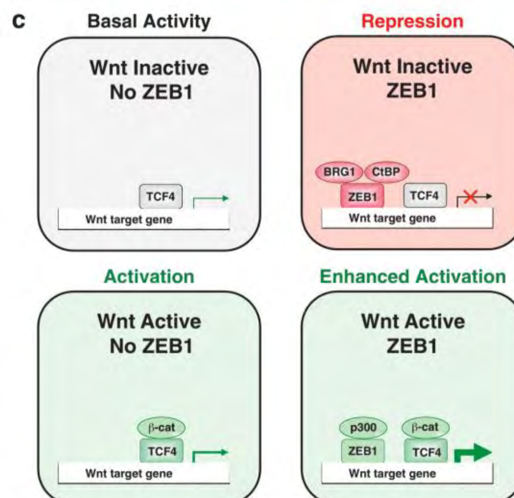

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Intestinal adenomas are also found in a mouse model of familial adenomatous polyposis carrying a heterozygous stop codon mutation in APC (APC (Min/+) mice).[34] In the normal intestinal epithelium of familial adenomatous polyposis patients and APC (Min/+) mice, β-catenin remains membranous/cytoplasmic and Wnt target genes like ZEB1 or LAMC2 are absent[17,35] (Supplementary Figure S5A). However, in the adenomas of familial adenomatous polyposis patients and APC (Min/+) mice, β-catenin levels increase and a fraction of it translocates to the nucleus where it activates Wnt target genes, including ZEB1 and LAMC2.[17,35] Expression levels of TCF4 in the normal and adenomatous intestinal mucosa are similar (Supplementary Figure S5B).

We first examined the *in vivo* expression of ZEB1, β-catenin and LAMC2 by immunofluorescence in tissue sections of normal intestinal mucosa and adenomas from APC (Min/+) mice. We found that in the epithelial cells of normal mucosa, β-catenin remained mostly membranous/cytoplasmic while ZEB1 and LAMC2 were low or not expressed (Figure 5a, upper panel and Figure 5b). In contrast, in adenoma cells, β-catenin is not only found at the membrane/cytoplasm but also at the nucleus although nuclear accumulation of β-catenin varies from cell to cell within the adenoma.[17] Nuclear expression of β-catenin in the intestinal adenomas of APC (Min/+) is accompanied by the induction of ZEB1, mostly at the nuclear level and whose expression is also heterogeneous across cells in the adenoma.[17] We found that there was association between expression the levels of ZEB1 and LAMC2: in cells with nuclear β-catenin, high levels of ZEB1 were accompanied by high levels of LAMC2 (cell section A, right panel), and conversely, low levels of ZEB1 were paralleled by low levels of LAMC2 (cell section B, right panel) (Figure 5a). These results indicate that in cells with active Wnt signaling, LAMC2 is coexpressed in line with ZEB1 levels *in vivo*.

We also examined mRNA expression of ZEB1 and LAMC2 in the intestine of wild-type and APC (Min/+) mice. As shown in Supplementary Figure S5C, mRNA levels for both genes were higher in APC (Min/+) than in APC (+/+) mice confirming that they are upregulated by active Wnt and suggesting that their expression is somehow associated. We then examined whether Wnt signaling and ZEB1 cooperate *in vivo* in the regulation of Wnt targets. As ZEB1 (−/−) mice die perinatally, we crossed APC (Min/+) mice with ZEB1 heterozygous (+/−) mice. Interestingly, compared with APC (Min/+); ZEB1 (+/+) mice, expression of LAMC2 was reduced upon downregulation of ZEB1 in APC (Min/+); ZEB1 (+/−) mice (Supplementary Figure S5C). These results indicate that *in vivo* expression of LAMC2 depends on the cooperation between ZEB1 and Wnt signaling.

DISCUSSION

Canonical Wnt signaling and ZEB1 control key genes during embryogenesis as well as during cell differentiation and tissue homeostasis in adults. This study provides evidence that TCF4/β-catenin and ZEB1 modulate each other's transcriptional activity in the regulation of tumor pro-invasive genes. In the presence of active Wnt signaling, ZEB1 cooperates with TCF4/β-catenin in the transcriptional activation of the Wnt targets LAMC2 and uPA. Conversely, in the absence of Wnt signaling, ZEB1 transcriptionally represses these very same Wnt targets (Figure 5c). Transcriptional cooperation between ZEB1 and TCF4/β-catenin requires binding of both transcriptional complexes to DNA. The molecular basis of ZEB1/Wnt cooperation lies in the direct binding of ZEB1 and TCF4 through their respective C-terminal domains. Binding of ZEB1 to TCF4 inhibits interaction of the former with co-repressors CtBP and BRG1 and instead promotes its binding to the co-activator p300. In a mouse model of intestinal tumorigenesis driven by activation of Wnt signaling (APC (Min/+) mice), ZEB1 and LAMC2 are heterogeneously induced within adenomas but their levels correlated in a given cell. When expression of ZEB1 in these mice is downregulated, expression of LAMC2 was reduced in parallel with that of ZEB1.

The pleiotropic and often antagonistic effects of Wnt signaling on a myriad of biological processes require its activity to be tightly regulated at every stage of the pathway—from the secretion of ligands to its transcription—in a highly temporal and tissue-specific manner.[1] As a result, Wnt targets are not simply expressed in an on/off manner but rather finely modulated during embryonic development and adult homeostasis[3–6] as well during cancer progression.[7–9] In addition to control Wnt signaling at the extracellular level, Wnt-mediated transcriptional activity is regulated *inter alia* by the availability of nuclear β-catenin to bind TCF/LEF factors and by the recruitment of different cofactors by either β-catenin or TCF/LEF factors (reviewed in Clevers and Nusse[1] and Lien and Fuchs[2]). Here, we showed that interaction of ZEB1 with TCF4 also modulates Wnt signaling by enhancing TCF4/β-catenin-mediated transcription. ZEB1 activates the expression of LAMC2 and uPA, two well-established Wnt targets involved in tumor invasiveness,[16–18] and our results here indicated that ZEB1 cooperates with TCF4/β-catenin to enhance their transcription. LAMC2 is expressed at the epithelial basement membrane and its cleavage by metalloproteases promotes early stages of cancer cell migration.[36] Once cancer cells reach the stroma, uPA cleaves the extracellular matrix favoring their invasiveness.[37] Expression of LAMC2 and uPA must be finely regulated during tumor progression as either deficient or excessive matrix degradation could inhibit cancer cell migration. To that effect, the ability of ZEB1 to increase Wnt-mediated activation of both genes may be important during tumor invasiveness.

Although transcriptional regulation most often involves the interplay of transcriptional repressors and activators bound to the regulatory regions of target genes, some transcription factors could function as either repressors or activators depending on the tissue, cellular status and/or promoter context.[38] These dual transcription factors can switch between transcriptional activator and repressor activities through several mechanisms such as the existence of isoforms with distinct binding affinities for cofactors, differential recruitment of coactivators or corepressors by post-translational modifications or modulation of steric DNA structure. In the absence of nuclear β-catenin, TCF-LEF factors repress transcription by binding corepressors.[2] Like TCF/LEF factors, ZEB1 can also activate or repress transcription depending on the set of cofactors it recruits. Binding of ZEB1 to co-repressors CtBP and BRG1 or to the coactivator p300 depends on the promoter context and it is regulated by TGFβ/BMP signaling during development.[20,21] Our results here showed that ZEB1 transcriptional activity is modulated by the Wnt pathway: when Wnt signaling is active, ZEB1 functions as a transcriptional activator of LAMC2 and uPA, whereas, in CRC cells with low Wnt signaling, ZEB1 represses these genes. The switch of ZEB1 from a transcriptional repressor into an activator depends on its binding to TCF4, which triggers the replacement of CtBP and BRG1 by p300.

In inducing an epithelial-to-mesenchymal transition, ZEB1 activates a number of mesenchymal markers and many of them, such as vimentin or fibronectin, also happen to be direct Wnt targets.[39,40] Although the precise molecular mechanism by which ZEB1 activates these genes remains to be determined, it is tempting to speculate that the activation of some mesenchymal genes by ZEB1 involves cooperation with Wnt.

Our results also indicate that the ability of ZEB1 to cooperate with TCF4/β-catenin and, on the other hand, of TCF4/β-catenin to transform ZEB1 into an activator depends on the binding of both transcriptional complexes to the DNA. In fact, other transcription factors cooperating with TCF4/β-catenin (for example, Smad1, c-jun, CDX2) also directly bind to the promoters of Wnt target genes.[41–43] It is therefore possible that ZEB1 and all these factors

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compete for their binding to TCF4. Alternatively, several of these factors could bind to TCF4 to form higher-level complexes and cooperate in the transcriptional activation of Wnt targets. The precise configuration of these interactions would be dictated by the location of these transcription factors in relation to TCF4 within the tertiary DNA structure of the regulatory regions. The existence of ZEB1-binding sites in a subset of Wnt target genes could be crucial to concentrate endogenous ZEB1 and TCF4 at their regulatory regions. β-Catenin interacts with other transcription factors besides TCF/LEF (for example, Sox17) to activate TCF/LEF-independent transcription.[2,44] Similar to ZEB1, and to the best of our knowledge, all DNA-binding transcription factors previously reported to cooperate with TCF4/β-catenin have to bind to the regulatory regions of Wnt targets to regulate their transcription rather than simply interacting with TCF4 and/or β-catenin. Thus, the joint presence of sites for TCF/LEF factors and for these cooperating transcription factors (including ZEB1) increases specificity in the modulation of Wnt target genes.

ZEB1 represses transcription by recruiting a CtBP-coREST-LSD1 complex that also contains BRG1.[19,22,45] We found here that binding of ZEB1 to TCF4 triggers the replacement of CtBP and BRG1 in favor of p300. But, although CtBP binds directly to ZEB1 through specific sites in its CR,[19] it still remains unclear whether recruitment of BRG1 by ZEB1 is direct or is rather mediated through BRG1-associated factors (BAF170, BAF57) known to interact with the coREST complex.[22,45,46] ZEB1 is the archetypal DNA-binding transcription factor interacting with CtBP, but many proteins mediate CtBP transcriptional effects.[47,48] For instance, binding of CtBP to wild-type APC, but not to mutant APC, indirectly inhibits Wnt signaling by forming a complex with β-catenin and prevents β-catenin binding to TCF4.[49]

Mutation of the APC gene is a key event occurring in the vast majority of CRCs.[33] Mutant APC can promote the transcriptional activation of Wnt target genes through several mechanisms. The inability of mutant APC to interact with CtBP and to participate in the ubiquitination of β-catenin allows the nuclear translocation of β-catenin and its interaction with TCF4.[1,49] In addition, active Wnt signaling triggered by mutant APC induces ZEB1 expression,[17] which we have shown here to cooperate with TCF4 to further enhance transcriptional activation of Wnt target genes. This tight multilevel transcriptional regulation of Wnt target genes may be important in creating thresholds of Wnt signaling and ZEB1 expression/function during embryogenesis, cell differentiation, and cancer initiation and progression. Altogether, this study identified a new molecular mechanism regulating the expression of pro-invasive Wnt target genes thus offering possibilities for targeting them in therapy.

MATERIALS AND METHODS

Cell lines and cell culture

All cell lines were maintained in Dulbecco's modified Eagle Medium (Lonza, Basel, Switzerland), except SW620 cells, which were grown in RPMI 1640 medium (Lonza). All media contained 10% fetal calf serum (Sigma, St Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. HEK 293 T cells were kindly provided by late S.J. Korsmeyer (DFCI, Harvard University, Boston, MA, USA), HCT116 by B. Volgestein (John Hopkins University, Baltimore, MD, USA) while SW480, SW620, HT29 and U937 cell lines were obtained from the Cancer Cell Line Repository (CCLR, IMIM-Hospital del Mar, Barcelona, Spain) that conducts quality controls for authentication and mycoplasma contamination.

Mouse tissues

C57BL/6J wild-type and APC (Min/+) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were crossed with ZEB1 (+/−) mice.[12] Normal and adenomatous intestinal samples from 3- to 4-month-old female mice were processed for immunostaining or quantitative real-time PCR as described in Supplementary Materials and Methods. Results shown originate from: three APC (+/+); ZEB1 (+/+) mice (at least three samples from the proximal small intestine and three from the distal small intestine), three APC (+/+); ZEB1 (+/−) mice (three samples from the proximal region and three from the distal), four APC (Min/+); ZEB1 (+/+) mice (two samples from the proximal region and two from the distal), four APC (Min/+); ZEB1 (+/−) mice (two samples from the proximal region and two from the distal). For each genotype, female mice participating in the study were randomly selected for crossing and ulterior analyses. Investigators were not blinded to the group allocation and/or when assessing the outcome of animal studies. Use of animals in this project was approved by and followed the guidelines of the Animal Experimentation Ethics Committee at the University of Barcelona.

Antibodies, plasmids, oligonucleotides and shRNAs

Description and source of antibodies, plasmids, oligonucleotides and shRNA lentivirus used in the study are detailed in Supplementary Information. Transient and stable interference of gene expression by siRNA and shRNA are also described in Supplementary Information.

Determination of protein and RNA expression and transcriptional assays

Analysis of protein expression by immunostaining or western blot and of protein-protein interaction by immunoprecipitation is described in Supplementary Information. Quantitative real-time PCR, mutagenesis of promoters and transcriptional assays were performed as described in Supplementary Information.

Statistical analysis

Statistical analysis of the data was carried out using SPSS 18.0 software (IBM, Armonk, NY, USA). The normal or non-normal distribution of the data was determined with Kolmogorov-Smirnov test. Statistical significance of data lacking normal distribution or with less than 50 observations was performed using a non-parametric Mann-Whitney *U* Test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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β-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness

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In most carcinomas, invasion of malignant cells into surrounding tissues involves their molecular reprogramming as part of an epithelial-to-mesenchymal transition (EMT). Mutation of the *APC* gene in most colorectal carcinomas (CRCs) contributes to the nuclear translocation of the oncoprotein β-catenin that upon binding to T-cell and lymphoid enhancer (TCF-LEF) factors triggers an EMT and a proinvasive gene expression profile. A key inducer of EMT is the ZEB1 transcription factor whose expression promotes tumorigenesis and metastasis in carcinomas. As inhibitor of the epithelial phenotype, ZEB1 is never present in the epithelium of normal colon or the tumor center of CRCs where β-catenin remains membranous. We show here that ZEB1 is expressed by epithelial cells in intestinal tumors from human patients (familial adenomatous polyposis) and mouse models ($APC^{Min/+}$) with germline mutations of *APC* that result in nuclear accumulation of β-catenin. However, ZEB1 is not expressed in the epithelium of hereditary forms of CRCs that carry wild-type *APC* and where β-catenin is excluded from the nucleus (Lynch syndrome). We found that β-catenin/TCF4 binds directly to the ZEB1 promoter and activates its transcription. Knockdown of β-catenin and TCF4 in *APC*-mutated CRC cells inhibited endogenous ZEB1, whereas forced translocation of β-catenin to the nucleus in *APC*-wild-type CRC cells induced de novo expression of ZEB1. Upregulation of MT1-MMP and LAMC2 by β-catenin/TCF4 has been linked to invasiveness in CRCs, and we show here that both proteins are activated by ZEB1 coexpressing with it in primary colorectal tumors with mutated *APC*. These results set ZEB1 as an effector of β-catenin/TCF4 signaling in EMT and tumor progression.

Loss of epithelial characteristics in favor of a mesenchymal phenotype—a process referred to as epithelial-to-mesenchymal transition (EMT)—bestows carcinoma cells at the tumor front with stem cell-like and more motile properties needed for their invasion into surrounding tissues (1). One of the signals initiating an EMT is the canonical Wnt pathway whose stimulation triggers the translocation of the oncoprotein β-catenin to the nucleus, where it acts as coactivator of T-cell and lymphoid enhancer (TCF–LEF) factors in the transcriptional activation of target genes (2). In the absence of nuclear β-catenin, TCF–LEF factors function instead as transcriptional repressors by binding to corepressors of the Groucho–TLE family. In normal epithelial cells, β-catenin associates at the cellular membrane with the adhesion molecule E-cadherin, while any free cytoplasmic β-catenin is phosphorylated and targeted for ubiquination-dependent degradation—thus preventing it from reaching the nucleus—by a protein complex formed by APC, GSK-3, CKIα, and Axin (2).

Aberrant activation of β-catenin/TCF signaling has been involved in a number of tumors, most notably colorectal carcinomas (CRCs) (2). Over 80% of sporadic CRCs and the vast majority of cases of familial adenomatous polyposis (FAP)—an autosomal dominant inherited syndrome displaying high predisposition to CRC—have loss-of-function mutations of the *APC* gene (3). A high predisposition to intestinal adenomas is also found in mice heterozygous for a stop codon mutation in *APC* ($APC^{Min/+}$ mice) (4). In the normal intestinal epithelium of FAP patients and $APC^{Min/+}$ mice, β-catenin remains membranous. However, in the tumors developed by FAP patients and $APC^{Min/+}$ mice, a fraction of β-catenin—which levels vary among and within tumors—translocates to the cytoplasm and nucleus (5–7). Nuclear accumulation of β-catenin is higher and more homogenous in FAP carcinomas than in FAP adenomas and also increases as adenomas develop in $APC^{Min/+}$ mice. In hereditary syndromes with predisposition to CRC but carrying wild-type *APC*, like the Lynch syndrome, β-catenin does not accumulate at the nucleus of CRC cells. Heterogeneity in the levels of nuclear β-catenin in the tumors of FAP patients and $APC^{Min/+}$ mice is also observed in sporadic CRCs. Although in well-differentiated areas and the tumor center of CRCs β-catenin has a membranous/cytoplasmic distribution, it becomes mostly nuclear in scattered dedifferentiated cells undergoing an active EMT at the tumor invasive front (8). Genomic location analyses in CRC cells have identified a large number of genes that bind β-catenin at their regulatory regions (9). β-catenin/TCF activates a differential pattern of gene expression in the center and front of primary CRCs with the induction of proinvasive genes taking place only at the tumor front (10).

A key initial step in the EMT is the downregulation of E-cadherin, which at the transcriptional level is repressed by several factors; namely, ZEB1, ZEB2, SNAI1, SNAI2, Twist1, Twist2, and E12/E47 (1). Of all of them, ZEB1 (also known as δEF1) has the most consistent inverse correlation with E-cadherin across different types of carcinomas (11, 12). In inducing an EMT, ZEB1 not only transcriptionally represses epithelial markers like E-cadherin but also activates mesenchymal genes. ZEB1 expression determines resistance to chemotherapy drugs in carcinoma cell lines, promotes tumorigenesis and metastasis in mouse xenograft models, and associates to poorer clinical prognosis in human carcinomas (11–13). Control by ZEB1 of these cancer hallmarks involves not only direct transcriptional activation or repression of target genes but also indirect mechanisms. In most cell types, ZEB1 represses and is repressed by microRNAs of the miR-

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The authors declare no conflict of interest.

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200 family that could also directly downregulate mesenchymal genes involved in cancer cell migration (11, 14, 15).

In normal and carcinomatous colonic mucosa, ZEB1 is mostly found in nonepithelial cells of diverse origin at the stromal compartment (16, 17). As expected from an inducer of EMT, ZEB1 is never expressed by epithelial cells of the normal mucosa or the tumor center of carcinomas. However, ZEB1 is present in the nucleus, and often also in the cytoplasm, of the above-mentioned dedifferentiated cancer cells at the tumor front of CRCs (13, 16, 18).

In this study, we show that ZEB1 is found in the epithelial cells of colorectal tumors from FAP patients and intestinal adenomas from $APC^{Min/+}$ mice where it is coexpressed with nuclear β-catenin. By contrast, ZEB1 is not detected in the epithelium of Lynch syndrome CRCs with wild-type APC and lacking nuclear β-catenin. Using established cell lines of CRC with reportedly different levels of nuclear β-catenin (19), we found that endogenous β-catenin and TCF4 bind to the promoter region of ZEB1 and induces its expression. Finally, in CRC cells with mutated APC, ZEB1 activates MT1–MMP and LAMC2, two proinvasive genes jointly regulated by β-catenin/TCF4 at the tumor front of CRCs, which we also found coexpressed with ZEB1 in colorectal tumors from FAP patients.

Results

ZEB1 Coexpresses with Nuclear β-Catenin in the Epithelium of Tumors from FAP Patients and $APC^{Min/+}$ Mice. Expression of ZEB1 by dedifferentiated nuclear β-catenin-positive cancer cells at the tumor front of sporadic CRCs led us to question whether ZEB1 expression also correlates with nuclear β-catenin in colorectal tumors from FAP patients with mutated *APC*. In FAP adenomas and carcinomas, β-catenin is found in the cytoplasm and nucleus of epithelial tumor cells. Nuclear accumulation of β-catenin varies within and among FAP tumors but is higher and more homogeneously distributed in FAP carcinomas (5, 6) (red in Fig. S1 *A* and *B*). Interestingly, we found ZEB1 in the epithelial cells of FAP tumors (green in Fig. S1 *A* and *B*), where it was coexpressed with nuclear β-catenin (yellow in Fig. 1*A* and Fig. S1*B*). ZEB1 paralleled levels of nuclear β-catenin in tumor epithelial cells —highly expressed by the majority of FAP adenomas and virtually all FAP carcinomas (Fig. 1*A* and Fig. S1*A*). Obviously, no colocalization was observed in nonepithelial stromal cells, positive for ZEB1 but negative for β-catenin (green in Fig. 1*A*). ZEB1 was not detected in Lynch syndrome carcinomas (no epithelial cells labeled in green in Fig. 1*A* and Fig. S1 *A* and *B*) with wild-type APC and where β-catenin remains membranous/cytoplasmic (red in Fig. 1*A* and Fig. S1 *A* and *B*).

Expression of ZEB1 was then examined in $APC^{Min/+}$ mice, considered as an animal model of human FAP. In the normal intestinal epithelium of wild-type and $APC^{Min/+}$ mice, β-catenin stayed membranous and ZEB1 was absent (Fig. 1*B*). By contrast, in intestinal adenomas of $APC^{Min/+}$ mice, nuclear accumulation of β-catenin was accompanied by expression of ZEB1 (Fig. 1*B*).

An important target of ZEB1 during EMT is the E-cadherin, expression of which is lost in those dedifferentiated cells at the invasive front of sporadic CRCs (Fig. S1*C*) (8). Here, we found that in epithelial cells of FAP colorectal tumors expression of ZEB1 also correlated with a loss/reduction of E-cadherin (Fig. S1*C*). Altogether, these results indicate that nuclear translocation of β-catenin in tumors with mutant *APC* correlates with ZEB1 expression and loss of E-cadherin.

β-Catenin/TCF4 Bind the Human ZEB1 Promoter and Activate Its Transcription. The above results prompted us to investigate whether β-catenin signaling could induce ZEB1. To test this hypothesis, we examined the effect on ZEB1 transcription of exogenously overexpressing β-catenin and TCF4, the most prominent TCF–LEF factor in the intestine. In these experiments, we used two CRC cell lines with different levels of nuclear β-catenin (19). On the one hand, we used SW480 cells with mutant APC and where β-catenin is predominantly nuclear (Fig. S2*A*). As expected from the correlation we found in FAP tumors between nuclear β-catenin and ZEB1, SW480 cells contain high levels of ZEB1 and low levels of E-cadherin (Fig. S2*B*). On the other hand, we used HCT116 cells that have wild-type APC and low levels of β-catenin accumulated in the nucleus (Fig. S2*A*). In line with our results in Lynch samples, low nuclear β-catenin in HTC116 cells associated with low levels of ZEB1 and high levels of E-cadherin (Fig. S2*B*). A high pool of nuclear β-catenin in SW480 cells translates into strong β-catenin/TCF-mediated transcription as assessed by the activity of the pTOPFLASH luciferase reporter (20) (Fig. S2*C*), containing

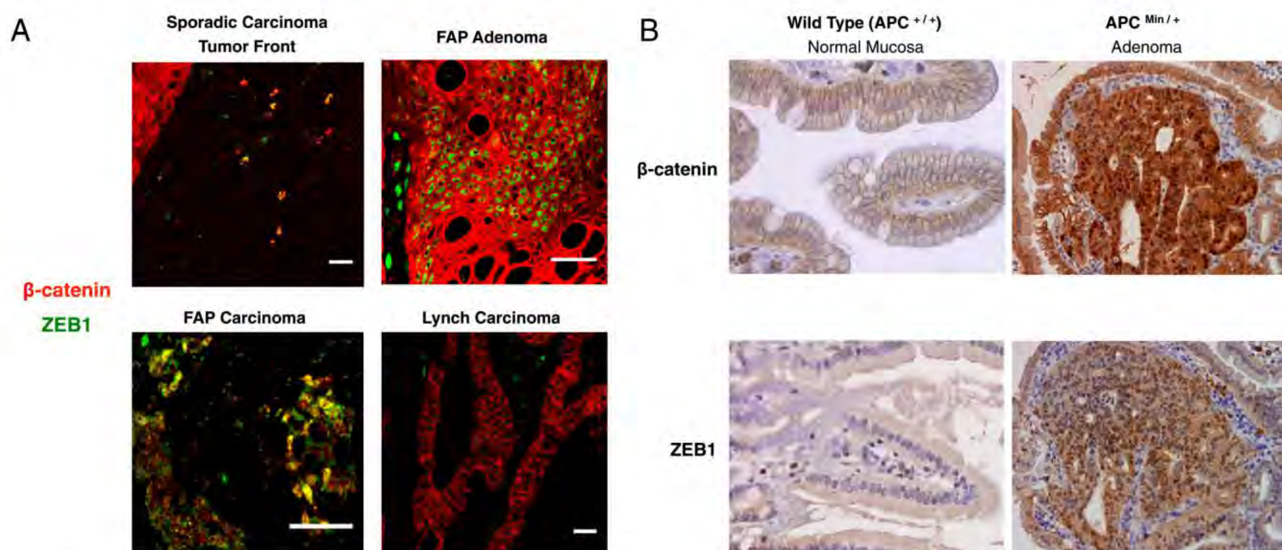

Fig. 1. ZEB1 coexpresses with nuclear β-catenin. (*A*) Immunofluorescence staining for β-catenin (red, clone 14 or Ab6302) and ZEB1 (green, H-102 or E-20) in tumor front of sporadic CRCs, FAP colorectal adenomas and carcinomas, and Lynch CRCs. A representative merge picture is shown (yellow cells for colocalization). Single staining for β-catenin, ZEB1 and DAPI is shown in Fig. S1*A* and magnified in Fig. S1*B*. Scale bar, 25 μm. (*B*) Immunohistohemistry staining for β-catenin (clone 14) and ZEB1 (H-102) in normal intestinal mucosa and adenomas from 4-mo-old wild-type APC (magnification 400×) and $APC^{Min/+}$ (200×) mice, respectively.

multimerized TCF binding sites. By contrast, in HCT116 cells Wnt signaling and pTOPFLASH transcription were low (20) (Fig. S2*C*).

Overexpression of TCF4 in SW480 cells, but not its corresponding empty vector, increased transcription of the human ZEB1 promoter (Fig. 2*A*). This effect, however, was not found when we transfected a mutant form of TCF4 ($TCF4_{d1\text{-}30}$) that lacks the β-catenin binding domain (Fig. 2*A*). These results suggest that the ability of TCF4 to activate ZEB1 transcription is dependent on the formation of a transcriptional activation complex with endogenous nuclear β-catenin.

Because SW480 cells have high endogenous levels of nuclear β-catenin, overexpression of β-catenin had no effect by itself on ZEB1 transcription (Fig. 2*B*)—presumably, most endogenous TCF4 is already bound to endogenous β-catenin. Exogenous β-catenin did synergize with TCF4 when the latter was also overexpressed, although only modestly (Fig. 2*B*). However, their synergistic activation of ZEB1 transcription was strong when TCF4 was coexpressed with a mutant and more stable form of β-catenin (β-catenin^{SA}) that is not degradable by the APC-GSK3-Axin complex (Fig. 2*B*). It is of note that the effect on ZEB1 transcription of overexpressing in SW480 cells TCF4 and/or β-catenin mirrored that obtained with the pTOPFLASH reporter (Fig. S2*D*).

The same set of experiments was then performed in HCT116 cells. Because these cells have low levels of nuclear β-catenin, overexpression of TCF4—let alone $TCF4_{d1\text{-}30}$—had by itself limited impact on the ZEB1 promoter (Fig. 2*A*). By contrast, exogenous expression of wild-type β-catenin and β-catenin^{SA} activated ZEB1 transcription (Fig. 2*C*). Again, the results obtained with the ZEB1 promoter in HTC116 cells paralleled those observed with the pTOPFLASH reporter (Fig. S2*E*).

We next wondered whether the ability of TCF4 and/or β-catenin to activate ZEB1 transcription was mediated by their direct binding to the ZEB1 promoter. Examination of the human ZEB1 promoter sequence revealed the existence of several potential TCF binding sites, two of them—located at −578 and −161 and similar to those reported in the human laminin 5 γ2 chain/LAMC2 promoter (21)—within the ZEB1 promoter reporter used above. We therefore tested through ChIP assays if endogenous β-catenin/TCF4 complexes assemble on the ZEB1 promoter. Indeed, it was found that antibodies against TCF4 and β-catenin, but not their respective control IgG, immunoprecipitated regions of the ZEB1 promoter containing the TCF binding sites (Fig. 2*D* and Fig. S2*F*). Lower binding by β-catenin vis-à-vis TCF4 is consistent with the fact that β-catenin interacts with DNA only indirectly via TCF4. TCF4 and β-catenin antibodies failed, however, to immunoprecipitate a fragment of the GAPDH promoter, which lacks TCF binding sites (Fig 2*D*). These results indicate that endogenous β-catenin/TCF4 complexes bind directly to the ZEB1 promoter.

To test whether these endogenous β-catenin/TCF4 complexes activate ZEB1 transcription, we examined the effect of eliminating both proteins. In SW480 cells, where high levels of nuclear β-catenin correlate with strong endogenous ZEB1 expression, knockdown of either endogenous β-catenin or TCF4 with specific siRNAs—but not a siRNA control—downregulated the activity of the ZEB1 promoter (Fig. 2*E*) just as it occurred with the TOPFLASH reporter (Fig. S2*G*).

β-catenin and members of the Groucho–TLE family of transcriptional corepressors competitively displace one another from their binding to TCF4. We therefore questioned whether TLE proteins could regulate ZEB1 transcription in the opposite direction. Overexpression of TLE1 in SW480 cells efficiently blocked β-catenin/TCF4-mediated transcription of the ZEB1 promoter (Fig. 2*F*) and the pTOPFLASH reporter (Fig. S2*H*).

Regulation of Endogenous ZEB1 by β-Catenin/TCF4. Next, we examined if β-catenin/TCF4 could regulate endogenous ZEB1. Knockdown of endogenous β-catenin or TCF4 in SW480 cells drastically downregulated both endogenous ZEB1 mRNA (Fig. 3*A*) and protein (Fig. 3*B*).

We also wondered whether forced nuclear translocation of endogenous β-catenin in HTC116 cells induces endogenous ZEB1. Leptomycin-B (LMB)—a drug that specifically blocks CRM1/exportin-dependent nuclear export and triggers a shift of β-catenin from the cell membrane/cytoplasm to the nucleus in

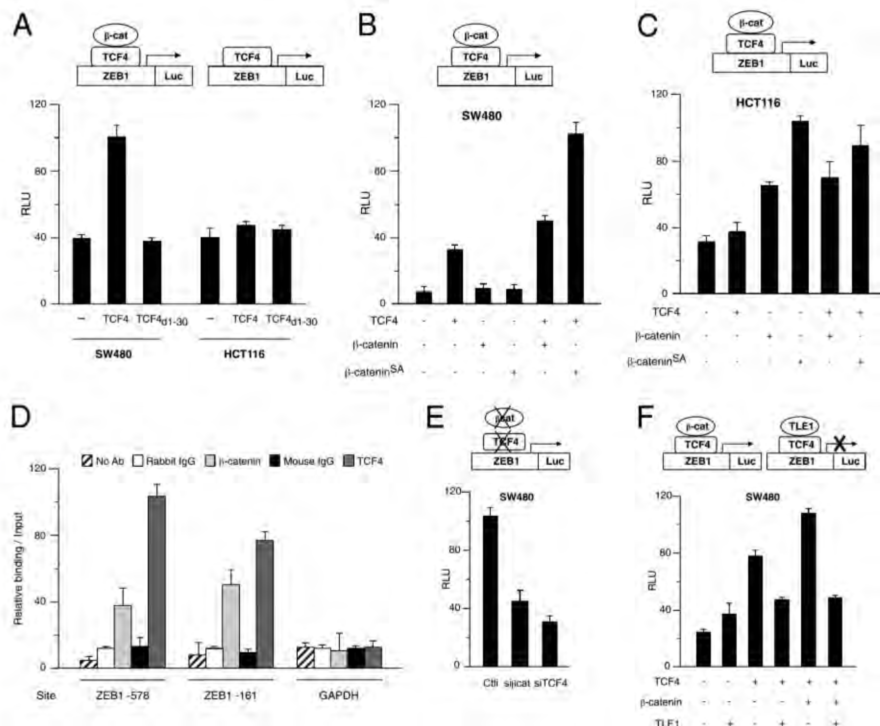

Fig. 2. β-catenin/TCF4 activates ZEB1 transcription. (*A*) SW480 and HCT116 cells were transfected with 0.5 μg of human ZEB1 promoter plus 1 μg of the empty expression vector ("−") or equal molar amounts of expression vectors for TCF4 or $TCF4_{d1\text{-}30}$ ("+"). Transcriptional assays were performed as described in *SI Discussion*. The relative luciferase activity (RLU) in the two cell lines with the empty vector was set to a common value for comparison. As for the TOPFLASH reporter (Fig. S2*C*), basal activity of the ZEB1 promoter is lower in HTC116 cells. (*B*) SW480 cells were transfected with ZEB1 promoter and combinations of 1 μg of empty expression vector or equal molar amounts of TCF4, β-catenin and/or β-catenin^{SA}. (*C*) Same as in *B*, but in HCT116 cells. (*D*) TCF4 and β-catenin bind to the ZEB1 promoter. Real-time PCR quantification (qRT-PCR) of fragments of the ZEB1 and GAPDH promoters immunoprecipitated in ChIP assays from SW480 cells with Abs against TCF4, β-catenin, their respective control IgG, and with no Ab. Amplified ZEB1 promoter regions contain TCF binding sites at −578 and −161. Values represent relative binding in relation to input. (*E*) Endogenous β-catenin and TCF4 regulate ZEB1 transcription. As in *A*, SW480 cells were transfected with the ZEB1 promoter along with 100 nM siRNAs against either β-catenin (siβcat), TCF4 (siTCF4), or a control siRNA (siCtl). (*F*) As in *B*, SW480 cells were transfected with the ZEB1 promoter and different combinations of 1 μg of empty vector or equal molar amounts of TCF4, β-catenin, and TLE1.

APC–wild-type cells (19, 20)—caused a rapid accumulation of nuclear β-catenin in HCT116 cells (Fig. S3A). Nuclear translocation of β-catenin in these cells stimulated ZEB1 transcription (Fig. 3C) with a temporal pattern that resembled the activation of the TOPFLASH reporter (Fig. S3B). Accumulation of nuclear β-catenin in HCT116 cells also induced endogenous ZEB1 mRNA and protein (Fig. 3C), and, consequently, reduced E-cadherin mRNA levels (Fig. S3C).

β-Catenin/TCF4 Signaling Regulates ZEB1 Activation of Proinvasive Markers. Activation of the β-catenin/TCF pathway upregulates the expression of proinvasive genes (10). Likewise, repression of epithelial genes and induction of mesenchymal markers by ZEB1 is required for the acquisition of an invasive phenotype by carcinoma cells at the tumor front (11, 12). E-cadherin is a direct target for ZEB1 repression, and knockdown of ZEB1 in SW480 cells induced an upregulation of endogenous E-cadherin mRNA that, in line with our results above, was also observed upon the elimination of β-catenin and TCF4 (Fig. S4A). These results are consistent with the loss of E-cadherin in cells positive for nuclear β-catenin and ZEB1 at the tumor front of sporadic CRCs and FAP adenomas and carcinomas (Fig. S1C; also see *Discussion*). On the other hand, cyclin D1 is a known target of β-catenin/TCF4 in CRCs (10) but not of ZEB1 and, accordingly, cyclin D1 mRNA levels were downregulated upon knockdown of β-catenin and TCF4 but not by a siRNA against ZEB1 (Fig. S4A).

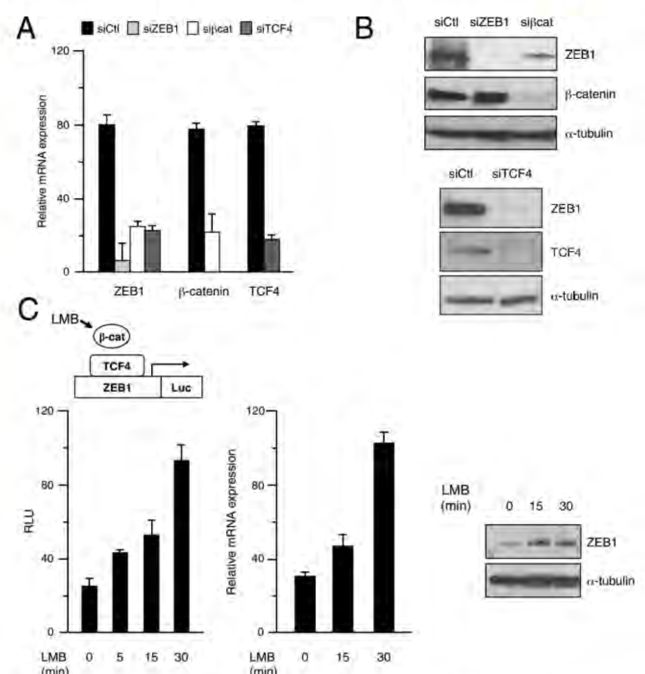

Fig. 3. Endogenous regulation of ZEB1 by β-catenin/TCF4. (*A*) Endogenous β-catenin and TCF4 regulate endogenous ZEB1 mRNA. SW480 cells were transfected with 50–100 nM of a control siRNA or specific siRNAs for ZEB1, β-catenin, and TCF4. Relative mRNA levels were determined by qRT-PCR respect to GAPDH. (*B*) Endogenous β-catenin and TCF4 regulate endogenous ZEB1 protein. SW480 cells were transiently transfected with control and specific siRNAs and cell lysates were immunoblotted for ZEB1 (H-102), β-catenin (clone 14), TCF4 (H-125), as well as α-tubulin (B5-1-2) to control for loading. (*C*) Nuclear accumulation of β-catenin induces endogenous ZEB1. (*Left*) HTC116 cells were transfected with 0.5 μg of ZEB1 promoter and, after 48 h, treated with 10 ng/mL of LMB for different periods before cell lysates were processed for luciferase activity quantification (see *Supporting Information*). (*Center*) HCT116 cells, treated with 10 ng/mL of LMB for different periods, were assessed for ZEB1 mRNA levels by qRT-PCR. (*Right*) HCT116 cells, treated with 10 ng/mL of LMB for different periods, were lysed, and protein levels were determined by Western blot with ZEB1 (H-102) and α-tubulin (B5-1-2) Abs.

The literature reveals some overlap in the gene expression pattern activated by β-catenin/TCF and ZEB1. For instance, vimentin is activated by both β-catenin/TCF and ZEB1 (22, 23), while knockdown of ZEB1 in CRC cells downregulates LAMC2 mRNA levels, a well-characterized target of β-catenin/TCF4 (16, 21).

A key mechanism by which β-catenin/TCF4 promotes invasiveness in sporadic CRCs is through the coordinated induction of MT1–MMP and LAMC2 at the tumor front (10, 16, 21, 24, 25). In addition, cleavage of LAMC2 by MT1–MMP promotes CRC cell migration (24). Given that nuclear β-catenin and ZEB1 are coexpressed at the tumor front of CRCs, we examined the ability of ZEB1 also to regulate MT1–MMP and LAMC2 in APC mutant CRC cells as well as their possible coexpression in FAP tumors.

Because SW480 cells exhibit strong β-catenin/TCF-mediated transcription and ZEB1 expression, the basal activity of the MT1–MMP promoter in these cells was high (Fig. 4A). Still, overexpression of ZEB1 was able to further activate MT1–MMP transcription. It was also found that knockdown not only of β-catenin

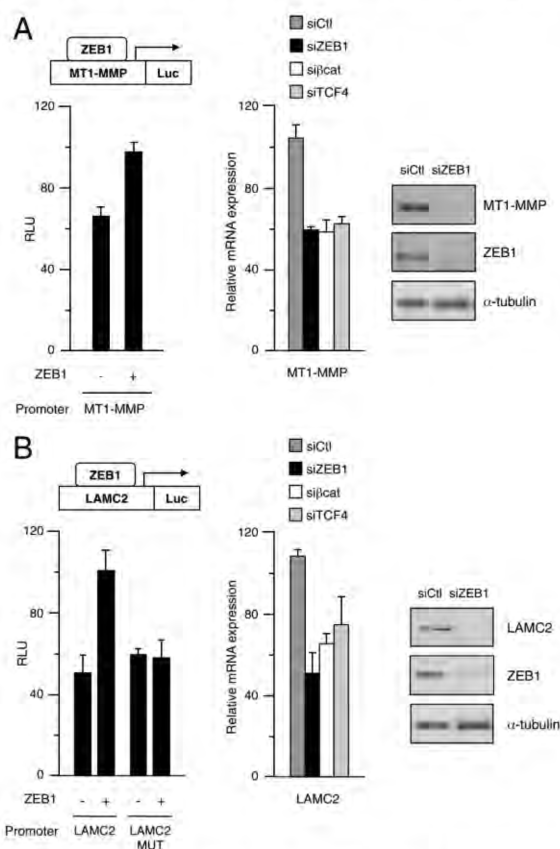

Fig. 4. Regulation of MT1–MMP and LAMC2 by ZEB1 in CRC cells with mutant APC. (*A*) ZEB1 activates MT1–MMP expression in CRC cells. (*Left*) ZEB1 activates MT1–MMP transcription. SW480 cells were transfected with 0.8 μg of the human MT1–MMP promoter along with 1.0 μg of empty expression vector or equal molar amounts of ZEB1 expression vector. (*Center*) ZEB1 activates endogenous MT1–MMP mRNA. SW480 cells transfected with a siRNA control or specific siRNAs for ZEB1, β-catenin, and TCF4 were assessed for relative levels of MT1–MMP mRNA by qRT-PCR. (*Right*) ZEB1 regulates endogenous MT1–MMP protein. SW480 cells were transiently transfected with 50 nM of a siRNA for ZEB1 or a control siRNA. Protein expression was assessed by Western blot with Abs for MT1–MMP (LEM2/15.8), ZEB1 (H-102), and α-tubulin (B5-1-2). (*B*) ZEB1 activates LAMC2 expression in CRC cells. (*Left*) ZEB1 activates LAMC2 transcription. Same as in *A*, but using both wild-type LAMC2 promoter (LAMC2) or a mutant version where two ZEB1 binding sites had been mutated (LAMC2-MUT) (*Center*) ZEB1 activates endogenous LAMC2 mRNA. Same as in *A*, but for LAMC2 mRNA. (*Right*) ZEB1 regulates endogenous LAMC2 protein. Same as in *A*, but a LAMC2 (D4B5) Ab was used.

and TCF4 but also of ZEB1 reduced mRNA levels of MT1–MMP (Fig. 4*A*). Likewise, transient and stable elimination of ZEB1 in SW480 cells downregulated endogenous MT1–MMP protein expression (Fig. 4*A* and Fig. S4*B*).

We next explored the regulation of LAMC2 by ZEB1 in CRC cells. As for MT1–MMP, despite a high basal activity of the LAMC2 promoter in SW480 cells its transcription was stimulated by overexpression of ZEB1 (Fig. 4*B*). The LAMC2 promoter contains several consensus ZEB1 binding sites, two of them—at −213 and −96—within our promoter reporter and whose mutation abrogated the ability of ZEB1 to activate LAMC2 transcription in SW480 cells (Fig. 4*B*). ChIP assays showed that endogenous ZEB1 in SW480 cells binds to the LAMC2 promoter but not to the GAPDH promoter, expression of which is not regulated by ZEB1 (Fig. S4 *C* and *D*). In the same line, elimination of endogenous ZEB1 in these cells downregulated endogenous LAMC2 mRNA and protein (Fig. 4*B* and Fig. S4*B*). These results indicate that in CRC cells ZEB1 directly activates LAMC2 expression, although these results do not exclude the possibility of concurrent indirect regulation via miR-200.

The expression of ZEB1 was then examined in tandem with that of MT1–MMP and LAMC2 in FAP adenomas and carcinomas. High levels of MT1–MMP (in red) were detected at the cell membrane and cytoplasm of tumor cells in FAP adenomas and carcinomas that also expressed ZEB1 (in green) (Fig. 5*A* and Fig. S5*A*). Despite lack of nuclear β-catenin, some expression of MT1–MMP was observed among stromal cells in Lynch CRCs where, obviously, there was no correlation with nonexistent ZEB1. Atypical nuclear localization of MT1–MMP has been associated with increased tumor aggressiveness (26). Remarkably, we found that MT1–MMP (in red) colocalized at the nucleus with ZEB1 (in green) precisely on dedifferentiated nuclear β-catenin-positive invading cancer cells at the tumor front of sporadic CRCs (yellow cells in Fig. S5*B*).

Similarly, when we examined ZEB1 and LAMC2 in FAP colorectal adenomas and carcinomas, we evidenced the coexpression of both proteins (green and red, respectively, in Fig. 5*B* and Fig. S5*C*). Again, neither expression nor correlation of ZEB1 and LAMC2 was found in CRCs from Lynch syndrome patients. Altogether these data show that ZEB1 and β-catenin/TCF4 regulate common proinvasive target genes in colorectal cancer.

Discussion

Aberrant activation of the β-catenin/TCF pathway—mostly by inactivating mutations of *APC*—is a necessary step in the genesis of many human cancers, including most CRCs. In this article, we show that ZEB1, a key inducer of EMT, is a direct target of β-catenin/TCF4. As an inhibitor of epithelial phenotype, ZEB1 is not present in the epithelium of normal colonic mucosa or in the center of sporadic CRCs but is expressed by dedifferentiated cells with nuclear β-catenin at their invasive front. In this study, ZEB1 was found in epithelial cells of intestinal tumors from FAP patients and $APC^{Min/+}$ mice, both carrying germline mutations of *APC* and displaying accumulation of nuclear β-catenin. By contrast, ZEB1 is absent in Lynch syndrome CRCs with wild-type APC and lacking nuclear β-catenin. TCF4 and β-catenin bind to the ZEB1 promoter to activate its transcription that is, in turn, repressed by TLE1. β-catenin/TCF4 complexes regulate ZEB1 at the endogenous level in *APC* mutant CRC cells as knockdown of endogenous β-catenin and TCF4 resulted in the downregulation of endogenous ZEB mRNA and protein. On the other hand, forced nuclear translocation of β-catenin in wild-type *APC* CRC cells activated ZEB1 transcription and induced endogenous ZEB1 mRNA and protein. We also showed that MT1–MMP and LAMC2—two genes whose coordinated induction by β-catenin/TCF4 contributes to the invasiveness of sporadic CRCs—are also activated by ZEB1 in CRC cells and coexpress with ZEB1 in colorectal FAP adenomas and carcinomas.

The upstream signals regulating most other EMT-inducing transcription factors (e.g., SNAI1, SNAI2, Twist1, Twist2) are well characterized (1), but it is only recently that the regulation of ZEB1 has begun to be understood. ZEB1 is induced by several pathways triggering an EMT (11, 12), and here we report that ZEB1 is directly activated by β-catenin/TCF4. Redundant regulation of ZEB1 through several EMT-inducing signals suggests that its expression is key in the initiation and/or maintenance of the EMT process.

Although in established CRC cell lines mutation of APC is accompanied by nuclear translocation of β-catenin and activation of β-catenin/TCF4 transcription, most cells in primary CRCs with mutated APC have membranous β-catenin and do not exhibit mesenchymal characteristics (8). The fact that nuclear β-catenin is only found in dedifferentiated cells at the invasive front indicates that APC mutation is a necessary but not sufficient step for nuclear translocation of β-catenin and induction of an EMT (8). Recent evidence has shown that the microenvironment at the

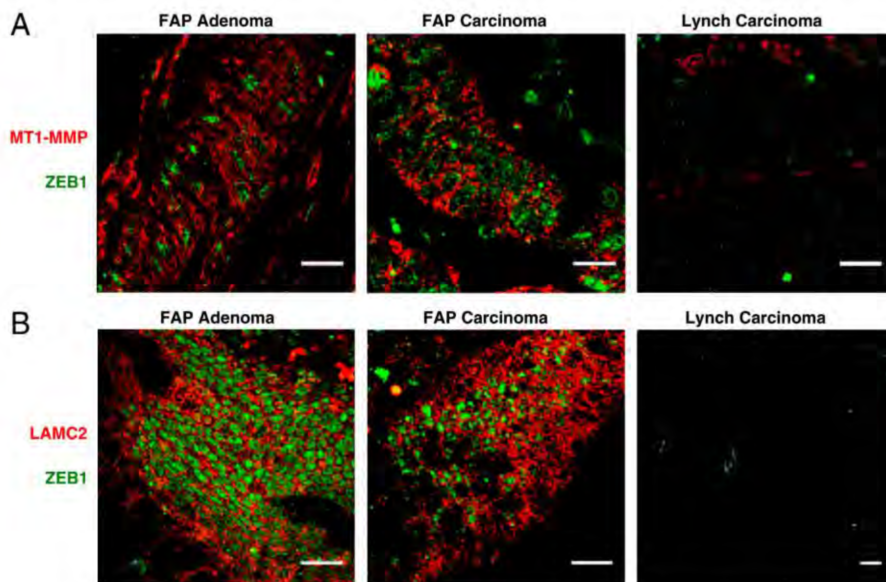

Fig. 5. ZEB1 correlates with MT1–MMP and LAMC2 in FAP adenomas and carcinomas. (*A*) FAP adenomas and carcinomas and Lynch carcinomas were immunostained for MT1–MMP (red) and ZEB1 (green). Representative merge pictures are shown. Single staining for MT1–MMP, ZEB1 and DAPI is shown in Fig. S5*A*. (*B*) Same as in *A*, but for LAMC2 (red) and ZEB1 (green). Single staining captures are shown in Fig. S5*C*. Scale bars, 25 µm.

invasive front of CRCs—and, more specifically, secretion of factors like hepatocyte growth factor by stromal myofibroblasts in the vicinity of dedifferentiated cancer cells—play an important role in the nuclear translocation of β-catenin and activation of β-catenin/TCF signaling (27). Although not as strong and homogeneous as in FAP carcinomas, β-catenin also accumulates in the nucleus—and starts inducing ZEB1 expression—in relatively well-differentiated adenomas from FAP patients and $APC^{Min/+}$ mice (5–7). This observation suggests that additional mechanisms beyond the microenvironment at the carcinoma front may regulate β-catenin translocation in FAP and $APC^{Min/+}$ adenomas. This study found that ZEB1 expression in these samples correlates with levels of nuclear β-catenin across and within tumors (see *SI Discussion*).

Lynch syndrome patients have germline mutations in mistmach repair genes resulting in microsatellite instability and increased risk of developing CRCs (3). Compared to sporadic CRCs, Lynch CRCs are less invasive and metastatic and have a more favorable prognosis. Over 90% of Lynch CRCs also present mutations of the TGFβ receptor type II compromising their ability to undergo an EMT in response to TGFβ, thus contributing to explain their lower invasiveness (28). Here, we have shown that, contrary to FAP tumors, Lynch CRCs with wild-type APC do not express ZEB1. In this regard, it is worth noting that ZEB1 is not only induced by TGFβ but ZEB1 synergizes with R-Smads to activate TGFβ-dependent genes and is required for TGFβ-mediated induction of EMT (29, 30). It is therefore possible that in the same way redundancy of upstream EMT-inducing signals would ensure ZEB1 expression during tumor progression, lower invasive and metastatic behavior in Lynch CRCs requires the loss of ZEB1 through inactivation of several of these pathways (e.g. wild-type APC plus mutation of TGFβ receptor type II).

Proteolytic cleavage of LAMC2 by MT1–MMP stimulates migration of CRC cells (24), and the coordination induction of both proteins by β-catenin/TCF4 at the front of sporadic CRCs associates to increased invasiveness (25). We found that both genes are upregulated by ZEB1 in APC mutant CRC cells and coexpress with ZEB1 in FAP tumors. (see *SI Discussion*).

Altogether, our results support a model where APC mutation, factors in the microenvironment, and, potentially, additional signals bring about the nuclear translocation of β-catenin, which upon binding to TCF4 induces ZEB1. ZEB1 would then initiate the EMT process—repressing E-cadherin and other epithelial markers and activating mesenchymal and proinvasive genes, some jointly with β-catenin/TCF4—to promote tumor progression and metastasis. An alternative model would be that induction first of ZEB1 through some unrelated mechanism would downregulate E-cadherin, thus increasing the availability of β-catenin to translocate the nucleus. However, this second possibility could be rebutted because ZEB1 is known to be unable to activate the TOPFLASH reporter (16).

In sum, this work has shown that in intestinal primary tumors and CRC cell lines with mutant APC ZEB1 is a direct target of β-catenin/TCF4 and acts also as an effector of this signaling pathway in regulating genes associated to tumor invasiveness. Given the salience of the β-catenin/TCF4 pathway in CRCs and recent evidence about the role of ZEB1 in tumorigenesis, metastasis, and chemotherapy resistance, the establishment of such link sets ZEB1 not only as an important diagnostic predictor but also as a potential therapeutic target in CRCs.

Materials and Methods

Details of the materials and experimental procedures used are given in *SI Materials and Methods*. *SI Discussion*, Figs. S1–S5, and additional references are also included in *Supporting Information*.

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Review

Guidelines for the selection of functional assays to evaluate the hallmarks of cancer

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ABSTRACT

The hallmarks of cancer capture the most essential phenotypic characteristics of malignant transformation and progression. Although numerous factors involved in this multi-step process are still unknown to date, an ever-increasing number of mutated/altered candidate genes are being identified within large-scale cancer genomic projects. Therefore, investigators need to be aware of available and appropriate techniques capable of determining characteristic features of each hallmark.

We review the methods tailored to experimental cancer researchers to evaluate cell proliferation, programmed cell death, replicative immortality, induction of angiogenesis, invasion and metastasis, genome instability, and reprogramming of energy metabolism. Selecting the ideal method is based on the investigator's goals, available equipment and also on financial constraints. Multiplexing strategies enable a more in-depth data collection from a single experiment – obtaining several results from a single procedure reduces variability and saves time and relative cost, leading to more robust conclusions compared to a single end point measurement. Each hallmark possesses characteristics that can be analyzed by immunoblot, RT-PCR, immunocytochemistry, immunoprecipitation, RNA microarray or RNA-seq. In general, flow cytometry, fluorescence microscopy, and multiwell readers are extremely versatile tools and, with proper sample preparation, allow the detection of a vast number of hallmark features. Finally, we also provide a list of hallmark-specific genes to be measured in transcriptome-level studies.

Although our list is not exhaustive, we provide a snapshot of the most widely used methods, with an emphasis on methods enabling the simultaneous evaluation of multiple hallmark features.

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1. Introduction

Tumorigenesis is a multi-step process that progressively converts normal cells into malignancies. The six hallmarks characterizing neoplasia (*sustaining proliferative signaling*, *evading growth suppressors*, *resisting cell death*, *enabling replicative immortality*, *inducing angiogenesis*, and *activating invasion and metastasis*) were delineated in Hanahan and Weinberg's seminal work compressing decades of cancer research data into a single paper [1]. Since then further features of malignant conversion have been described. *Genomic instability* generates random mutations, while *inflammation* nurtures and accelerates tumorigenesis. *Reprogramming of energy metabolism* and *avoiding immune destruction* emerged as important supporting features and epigenetics was accepted as a player of equal importance to genetic abnormalities in the initiation and progression of cancer. Finally, development of cancer can only be understood by integrating the dynamic signaling circuitry between cells and the tumor microenvironment [2].

Hallmark capabilities are regulated by partially redundant signaling pathways; the significance of these pathways depends on the tumor's underlying molecular features. Choosing an appropriate *in vitro* assay to monitor a given hallmark among the available options is a challenging task, especially for concurrent analysis of multiple hallmarks. Combining assays provides more information, but at the same time multiplexing requires compatibility for parallel or sequential measurements. The cost and speed of an assay, a need for a quantitative real-time or endpoint measurement, and the equipment required should be considered carefully to select an optimal solution. Understanding the pros and cons of each assay is crucial.

In this review we attempt to summarize the most frequently used *in vitro* assays for analyzing each hallmark in cell culture. We particularly emphasize assay combinations to study multiple hallmarks simultaneously. Animal models, *e.g.* mouse or zebrafish, are popular to reproduce hallmark features; however, the cost and the required special procedures put animal models beyond the scope of this review. Also, hallmarks involving the immune system were excluded because of the difficulty of assessing them in cell culture-based experiments.

2. Hallmarks of cancer

2.1. Sustaining proliferative signaling and evading growth suppressors

Unlimited proliferation is the predominant feature of all cancer cells. Sustaining proliferative signaling and evading growth suppressors is defined by the number of healthy cells in a sample and/or by the maintenance of proliferative ability over time. Assays are based on biochemical events specific to living cells, such as metabolic activity and cellular ATP, membrane integrity, DNA synthesis, protein content, and the impedance of living cells. An alternative way to measure cells' ability to undergo sufficient proliferation is to assess colony formation by single cells.

2.1.1. Metabolic activity

Reduction of substrates to a final product by intracellular enzyme activity in living cells can be assessed by a colorimetric assay. The degree

of color change is not directly proportional to the number of viable cells, but rather to the activity of an integrated set of enzymes of the cellular environment, which utilize the cofactor, NADH, and various other substrates [3]. This method permits only a moderately robust measure of viability; however, the ease of use and potential for high throughput analysis in multiwell plates has widely popularized it. The most frequently used tetrazolium compound (MTT) is reduced to formazan [4]. More recently-developed tetrazolium analogs (XTT, MTS, WST-1, WST-8) are used in conjunction with intermediate electron acceptors [3] and are converted to water-soluble formazan [5–8]. Eliminating the solubilization step simplifies the protocol and allows real-time measurement of metabolic activity. The deep blue Alamar Blue (resazurin) is reduced to the pink, fluorescent resorufin [9]. Acetoxymethyl ester of calcein (Calcein AM) cleaved by esterases within live cells produces green fluorescence, while dead cells exhibit red fluorescence. Quantification with a fluorescence microplate reader permits determination of the ratio of viable/dead cells in a population. Bioluminescence assays (*e.g.* CellTiter-Glo) measuring cellular ATP by the development of mutant luciferase perform with better reproducibility and sensitivity compared to MTT assays and are particularly useful when assessing cell viability in low density cell populations [10–13]. Table 1 summarizes the most frequently used enzymatic assays and their limitations.

2.1.2. Protein content

The presence of living cells is assayed by SRB (sulforhodamine B), a red fluorescent aminoxanthene dye. This compound produces a change detectable *via* colorimetry as its sulfonic group binds to basic amino acid residues in proteins, to give an estimation of total protein mass, which is directly proportional to cell number [14]. In contrast to MTT, SRB staining is not dependent on mitochondrial function; therefore less variation is observed between cell lines [15]. SRB has better linearity and higher sensitivity than other colorimetric assays, and is comparable to those achieved with standard fluorescent dye staining methods [16,17]. The method is very cheap as it requires only a multiwell reader, has a stable endpoint, and the reagent can be stored indefinitely.

2.1.3. Membrane integrity – dye exclusion assays

Several methods employ dyes which diffuse passively through damaged membranes, while they are excluded by healthy cells. First, the trypan blue exclusion assay [18] is cheap, simple and still widely used. Viable cells are counted with a hemocytometer or an automated electronic particle counter, and the percentage of viable cells is calculated [19]. Second, healthy and early apoptotic cell membranes are impermeable to the red-fluorescent propidium iodide (PI), which intercalates between base pairs of double-stranded DNA. Uptake is assessed by flow cytometry allowing single cell level analysis [20,21]. Finally, SYTOX Green Stain and YO-PRO-1 can provide more reliable results than PI-based tests in flow cytometric assays [22]. The major drawback of the dye exclusion assays is the cytotoxic nature of most probes, preventing long term use, and inaccurate automated readings if the cells tend to cluster.

Cells with a compromised membrane release substances such as lactate dehydrogenase (LDH) [23]. Fluorescence-based detection methods have been developed to perform LDH measurement directly in culture wells with a spectrophotometer containing a mixture of viable and dead cells without damaging the membranes of the viable cell population. Limitations are the low selectivity and the relatively short half-life of LDH [24].

2.1.4. DNA synthesis

Incorporation of radiolabeled DNA precursor ^{3}H-thymidine into new strands of chromosomal DNA or more recent analogs, BrdU

Table 1
Summary of intracellular metabolic assays available to measure cellular proliferation.

Assay	Substance used	Pros	Cons
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) - forms water-insoluble formazan	Very robust, suitable for most cell lines, assay stays linear from 200 to 1000 up to 50,000–100,000 cells/well. Good correlation with radioactive cell counting.	Endpoint assay. Incomplete solubilization, the presence of phenol red, overconfluent cells and the lack of glucose interferes with enzyme activity [4,263]. Reducing compounds converting MTT (such as ascorbic acid, sulfhydryl-containing compounds) can alter the results [264,265].
XTT	Sodium 3¢-[1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrat + F3 + F4:I7 – forms water-soluble formazan	Suitable for real time measurement. More sensitive and less cytotoxic than MTT.	Not suitable for cells with lower metabolic activity due to relatively elevated background levels. The presence of DTT, mercaptoethanol, L-cysteine, L-ascorbic acid, cyanide, azide, and changes in environmental oxygen distorts the results [5].
MTS	(3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) - forms water-soluble formazan	More sensitive than MTT and more stable than XTT.	Optimization advisable for each cell type [6,266,267]
WST-1	Water-soluble tetrazolium salt-1	More stable than XTT and MTS.	Optimization is required for each cell type [267].
WST-8	Water-soluble tetrazolium salt-8	Non-destructive method. Higher sensitivity compared to the other tetrazolium salts. Correlates well with the 3H-thymidine incorporation assay.	Optimization is required for each cell type [8].
Alamar Blue	Resazurin	Very sensitive, 80 cells provide a reproducible signal, linear reading in the range of 50–50,000 cells. More sensitive than tetrazolium-based methods. Fast, simple and relatively cheap. Stable signal. Allows multiplexing.	At higher cell density or at metabolically very active cells hydroresorufin is produced distorting the results. Necessary to test crossreactivity with any compound to be tested, without any cells in the medium [268,269].
Calcein AM	Acetoxymethyl ester of calcein	Is unaffected by pH change, and has a superior cellular retention [270].	Cytotoxic for several cell lines [271].
Cellular ATP content	Firefly luciferase enzyme	Higher reliability and sensitivity compared to MTT, can detect as few as 10 cells/well. Shorter protocol and less handling compared to other methods. Especially useful in low density populations.	Endpoint assay, cell lysis required. ATP varies significantly in cells [272,273].

(bromoeoxyuridin) and EdU (5-ethynyl-2 deoxyuridine), can be used to assess *de novo* DNA synthesis [25–27]. During the S phase of the cell cycle they are integrated into the nuclear DNA [28]. ^{3}H-thymidine detection requires a scintillation beta-counter, whereas BrdU can be detected by antibodies, allowing analysis by flow cytometry or by immunohistochemistry, eliminating the need for radioactive labeling. BrdU is toxic and mutagenic, alters cell cycle and the fate of the cells that incorporate it, thus appropriate controls are required [29,30]. In contrast to BrdU, the newest analog EdU does not require DNA denaturation by exposing cells to HCl, heat or DNAse, and its detection is rapid and highly sensitive [27]. BrdU and EdU are thought to provide the most reliable and direct index of proliferation.

2.1.5. Impedance of a living cell

The traditional methods, usually based on end-point analysis, provide limited information about cell physiology. The non-invasive, label-free real-time cell analyzer platforms (RTCA) are capable of continuously monitoring biological processes, such as proliferation, migration and invasion, based on the impedance of living cells grown on surfaces, thereby permitting conductivity measurements [31]. Platforms such as xCELLigence RTCA are commercially available; gold microelectrode arrays line the bottoms of the wells and create an electric field with the introduced cells acting as insulators, such that net cell adhesion can be measured. Cell Index (CI) is an arbitrary unit reflecting the electronic cell-sensor impedance that directly correlates with the number of viable cells. The RTCA is also suitable for co-culture studies. When testing cytotoxic compounds, the effects of toxic insult are often transient. RTCAs are ideal to determine the optimal time points for end-point measurements and the temporal profile of drug responsiveness [32].

2.1.6. Live-cell time-lapse imaging

Commercially available, the IncuCyte™ system allows real-time, automated monitoring of cell proliferation based on live cell time-lapse imaging. The system monitors proliferation by analyzing confluence and allows the separation of living and apoptotic cells by labeling reagents. Moreover, transfecting the nucleus with green or red fluorescent protein (GFP/RFP) tagged constructs allows the quantification of cell number and the monitoring of migration/invasion. A major advantage is the nearly unlimited real-time analysis, but one must consider the high initial cost of the setup. Performance of real-time systems (xCELLigence, IncuCyte™) was outstanding in sub-confluent cell cultures, but these were outperformed by endpoint assays at full confluence [33].

2.1.7. Colony formation assay

The clonogenic assay tests the effects of external stress signals on cell survival, namely the cell's ability to undergo sufficient proliferation to form a colony. This assay is especially useful to assess long-term effects, such as survival after irradiation. It is suitable for use with any type of cell that can be grown in culture. Sufficient colonies generally contain at least 50 cells. Colony counting is usually done manually with a pen or with automated gel count systems, such as the Oxford Optronix. These systems provide additional information, such as colony size, are able to handle high plate throughput and offer consistent counting. However, the method is also time consuming with extended incubation times, plus colony formation ability of cells differs [34]. Cell architecture and conformation influence cellular responsiveness [35,36].

Two-dimensional assays limit intercellular communication; therefore 3D culture and assay methods are under development [37]. The soft agar assay determines anchorage-independent proliferation, a characteristic of cancer cells, as normal cells undergo anoikis when they detach from the extracellular matrix. Cells are grown in soft agar, with a denser agar beneath to prevent adhesion to the culture plate, where transformed, but not normal, cells form colonies. The assay allows the study of tumor suppressive function of signaling molecules and is often predictive of tumorigenicity *in vivo* [38]. The traditional assay requires weeks for completion and does not allow cell retrieval; however incorporation of fluorimetric dyes allows quantitative, high-throughput colony counting and the use of specialized agars permits viable cell retrieval (*e.g.* CytoSelect™ multiwell transformation assays).

Assays detecting living cells and proliferation in cell cultures are summarized in Table 2. Sustained proliferation and evasion of growth suppression can also be estimated by analyzing gene expression of key enzymes involved (Table 6) [39,40].

2.2. Resisting cell death

Apoptosis is an active form of cell death, an organized process that leads to programmed self-destruction. Activation of apoptotic pathways alters morphology and cell surface, leads to genome fragmentation and mitochondrial dysfunction and fragments the dying cells in the final phases into apoptotic bodies [41]. It is a relatively swift process, from initiation to completion lasting less than 3 h. The complex signaling pathways are strongly regulated, enabling opportunities to detect and evaluate the process, although the timing of analysis must be optimized as some proteins only appear transiently. Two main apoptotic pathways are recognized. The extrinsic pathway is mediated by cell surface death receptors such as Fas or TNF-R, while the intrinsic pathway is activated by internal damage leading to mitochondrial cytochrome *c* release. The two pathways are linked, mutually influence each other and ultimately converge on a single execution pathway modulated by caspase activation [42].

2.2.1. Morphological changes

Morphological changes such as cell size reduction, nuclear condensation and fragmentation, the presence of large clear vacuoles, and membrane blebbing can be quantified by light microscopy or transmission electron microscopy. Live cell time-lapse imaging allows real-time, automated monitoring of cell proliferation and morphology by high-definition phase-contrast images [43]. Additionally, each of these cellular modifications can be rapidly measured by flow cytometry [44].

2.2.2. Genome fragmentation

During apoptosis the chromatin is fragmented by endonucleases, the nucleus destructs (karyorrhexis), and cells lose oligo-nucleosomal fragments. DNA laddering is a common end result that can be detected as genome fragments by gel electrophoresis [45]. The loss of genome fragments leads to reduced staining of apoptotic cells with DNA-intercalating fluorochromes such as propidium iodide, which is measured easily and accurately by flow cytometry [20].

The TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling) assay is a common method to detect early apoptosis. It is based on the appearance of 3′-OH DNA termini generated by endonucleases [46]. Terminal transferase (TdT) is used to add fluorescently-labeled dUTP to the free DNA double- and single-stranded breaks [47,48]. Such DNA breaks can also be detected in cell suspensions by flow cytometry or fluorescence microscopy by apoptosis enzyme-linked immunoassay based on immunostaining [49].

2.2.3. Plasma membrane alterations

Phospholipids are asymmetrically distributed on the inner and outer surfaces of the plasma membrane. Apoptotic cells are unable to maintain the phospholipid asymmetry, thus phosphatidyl-serine (PS) appears on the outer surface. PS can be detected using PS-binding anticoagulant Annexin V-FITC. FITC or other fluorescent-labeled apoptotic cells then are visualized with fluorescence microscopy or counted by flow cytometry [50,51]. Using Annexin V-FITC in combination with PI permits viable (double negative), early apoptotic (Annexin V-FITC positive/PI negative) and late apoptotic (PI positive) cells to be distinguished [51].

Table 2
Methods investigating cell viability and proliferation.

Feature	Substance	Detection method	Principle
Metabolic activity	MTT	Colorimetric	Redox reaction; colorless compounds are reduced by living cells to brightly colored metabolites
	XTT	Colorimetric	
	MTS	Colorimetric	
	WST-1	Colorimetric	
	WST-8	Colorimetric	
	Alamar Blue (resazurin)	Fluoro/colorimetric	Redox reaction, nonfluorescent resazurin is converted by reduction to a fluorophore pink resorufin
	Calcein AM	Fluorometric	Intracellular esterases convert Calcein AM to fluorescent calcein
	Intracellular ATP	Bioluminescense	Changes in ATP are correlated with viable cells
DNA synthesis	BrdU, EdU	Flow cytometry, IHC	Substances are incorporated into new strands during *de novo* DNA synthesis
	3H-thymidine	Scintillation beta-counter	
Membrane integrity	Trypan blue	Hemocytometer, Fluorometric	Dead cells or substances released from a cell take up the dye
	Propidium iodide	Fluorometric	
	SYTOX	Fluorometric	
	Yo-PRO-1	Fluorometric	
	Lactate dehydrogenase	Fluorometric	
Protein content	sulforhodamine B	Colorimetric	Binds amino acid residues in proteins through its sulfonic group and gives an estimation of total protein mass
Impedance of living cells	–	Real time cell analyzer system (xCelligence)	Microelectrode array at the bottom of the well detects impedance of living cells
Live-cell time-lapse imaging	–	IncuCyte™	Real-time, automated monitoring of cell proliferation based on live-cell time-lapse imaging
Colony formation	–	Colony counting (manually or gel count systems)	Cells grown in culture

2.2.4. Assays to monitor mitochondrial membrane alterations

Assays to monitor mitochondrial membrane alterations allow the recognition of changes in the early phase of the intrinsic pathway activation. The collapse of the electrochemical gradient across the mitochondrial outer membrane decreases mitochondrial transmembrane potential ($\Delta\Psi_m$), leading to increased membrane permeability, detectable by several sensitive cationic dyes (listed in Table 3) [52,53]. Normally accumulating in the mitochondria, during apoptosis these cationic dyes diffuse into the cytoplasm where they can be detected by altered fluorescent emission [54].

2.2.5. Intracellular calcium concentration

Altered intracellular Ca^{2+} concentration is one of the first signs that a cell is committed to apoptosis initiated by the Bcl-2 family (intrinsic pathway; see below). Release of the key apoptotic enzyme, cytochrome *c*, depends on the emission and uptake of Ca^{2+} from endoplasmic reticulum and mitochondria, respectively. The Ca^{2+} levels of the cytosol or mitochondria can be measured by selective fluorescent dyes (Table 3) [55,56].

2.2.6. Detection of apoptosis regulators

Cytochrome *c* can be detected in cell extracts by immunoprecipitation or immunoblotting with cytochrome *c*-specific monoclonal antibodies (mAbs). Subcellular detection of exogenous GFP-tagged cytochrome *c* is possible by fluorescence microscopy [57,58]. Cytochrome c is also released extracellularly from cells that are committed to apoptosis. In cell cultures it can be detected in the media in the early phase and is specific to apoptosis, but not to necrosis [59].

Another group of proteins regulating apoptosis is the Bcl-2 family that facilitates (Bcl-2 subfamily) or inhibits (Bax and BH-3-only subfamily) cell survival (Table 3) [60]. Cell surface receptors Fas (Apo-1, CD95) and their ligand (FasL) serve as mediators. Commercially available mAbs raised against human apoptotic proteins (Bcl-2, Bax, Fas, FasL) can be used in flow cytometry, immunoblot, and immunochemistry [61,62].

Members of the CDK (cyclin-dependent kinase) family play a key role in the regulation of both mitosis and apoptosis. Activation of CDK2 is required for apoptosis and is controlled by p53 and Bcl2-family proteins [63]. The detection of CDK2 along with associated proteins is based on immunoprecipitation followed by immunoblots [64].

2.2.7. Detection of caspases

Caspases, belonging to the cysteine protease family, are irreversibly activated during apoptosis and other cellular processes, such as inflammation or differentiation. An intrinsic or extrinsic death signal activates the proteolytic cascade of caspases promoting their aggregation into dimers or macromolecular complexes, resulting in controlled demolition of cellular components. With specific antibodies, caspases can be identified by immunoprecipitation or immunoblot, may be labeled with fluorescent probes, and detected by fluorescence microscopy [65], flow cytometry [66], or laser scanning cytometry [67]. The IncuCyte™ technology allows the direct addition of the caspase 3/7 apoptosis assay reagent to tissue culture wells, preventing cell loss. The inert, non-fluorescent reagent freely crosses the cell membrane, and after cleavage by caspase 3, releases a green DNA-binding dye. The fluorescent staining of the cleaved substrate can be monitored by live cell imaging [43].

Small labeled peptide-based substrates conjugated to fluorogenic or chromogenic groups are released and detected spectrophotometrically when the substrate is cleaved by a particular caspase [68–70]. Synthetic caspase inhibitors (Fluorochrome Inhibitor of Caspases; FLICA) bind to functional groups of active sites of caspases [71].

Detecting cleavage of the multiple caspase substrates inside a cell is a common method to assess caspase activity. One of the most frequently used targets, a DNA repair enzyme, Poly(ADP)ribose polymerase (PARP), is identified by specific antibodies (together with mAb for entire, uncleaved PARP) and detected by immunoblot [72], fluorescence microscopy [73] or flow cytometry [74].

A detailed comparison of apoptosis detection assays is included in Table 3. Hallmark features for resisting cell death and their applicable detection methods are presented in Fig. 1A. The occurrence of apoptosis can also be identified by analyzing the gene expression of key enzymes and regulators (Table 6) [75,76].

2.3. Enabling replicative immortality

Normal cells pass through a limited number of cell cycles, followed by an irreversible, nonproliferative state (senescence), and finally death. Telomeres protect the termini of chromosomes and are central in preventing uncontrolled proliferation [77]. The size of the telomeric DNA determines the cell's maximum capacity for division before

Table 3
Comparison of apoptosis detection assays.

Apoptosis features	Detection target	Method	Detection methods	Principle
Morphology alterations	Changes in cell morphology		Light microscopy Electron microscopy Time-lapse microscopy Flow cytometry	Cell staining [44]
Genome fragmentation	Genome fragments	DNA laddering	Gel electrophoresis	DNA staining [45]
	Loss of genome fragments	DNA content analysis	Flow cytometry	DNA staining: PI (propidium iodide), acridine orange, ethidiume bromide, Hoechst 33,342, *etc.* [20]
	Free 3′-OH DNA ends	TUNEL	Fluorescence microscopy Flow cytometry	Labeling DNA breaks by terminal transferase with fluorescently marked dUTP [46-48]
	ssDNA	Apoptosis enzyme-linked immunoassay	Fluorescence microscopy Flow cytometry	Immunostaining single-stranded DNA with monoclonal antibodies (mAbs) [49]
Plasma membrane alteration	Phospholipids in the outer side of PM	Annexin binding assay	Fluorescence microscopy Flow cytometry	PS-binding Annexin V labeled with fluorescein isothiocyanate (FITC) or phycoerythrin [50, 51]
	Intracellular enzymes in media	LDH activity assay	Spectrophotometric methods	LDH enzyme reaction linked to NADH reduction leads to absorbance changes of specific probes [274]
Mitochondrial membrane alteration	Increased mitochondrial membrane permeability	Mitochondrial membrane potential assay	Spectrophotometric methods Fluorescence microscopy flow cytometry	Cationic fluorescent probes that responds to the changes in Δψm such as: JC-1, DiOC6 , Rhodamine-123, CMXROS, TMRM, HE, NAO [52–54]
Intracellular calcium concentration	Free Ca^{2+} levels	Changes in intracellular calcium	Spectrophotometric methods Fluorescence microscopy Flow cytometry	Specific dyes consist of Ca^{2+} chelating molecules that undergo changes in their fluorescence excitation and emission properties (Fura-2; Indo-1; Rhod-2; Fluoro-3/Fluoro-4) [55,56]
Specific regulators	Cytochrome *c*	Intracellular cytochrome *c* detection	Immunoprecipitation Immunoblot Fluorescence microscopy	Using *anti*-cytochrome *c* mAbs [57,58]
		Extracellular cytochrome *c* detection	Immunoprecipitation Immunoblot Fluorescence microscopy	Using anti-cytochrome *c* mAbs, alternative protocol [59]
	Bcl-2 family, Fas and FasL, CDK family	Direct detection	Immunoprecipitation Immunoblot Flow cytometry	Using specific mAbs [61–64]
	Caspase	Direct detection	Immunoprecipitation Immunoblot Flow cytometry	Using specific mAbs for inactive (zymogen) or active form of the proteins [65–67]
			Fluorescence microscopy IncuCyte™	Live-cell imaging [43]
	Caspase activity assay	Caspase activity	Spectrophotometric methods	Small labeled peptide based substrates [68–70]
		FLICA	Spectrophotometric methods Flow cytometry	Small labeled peptide based inhibitors [71]
		PARP assay	Immunoprecipitation Immunoblot Flow cytometry Fluorescence microscopy	Using specific mAbs for cleaved or uncleaved caspase target proteins such as PARP (Poly(ADP)ribose polymerase) [72–74]

Abbreviations: PM: plasma membrane, PS: phosphatidyl-serine, LDH: lactate dehydrogenase, $\Delta\psi_m$: mitochondrial membrane potential, DIOC6: n to 3,3′ dihexyloxacarbocyanine iodide, CMXROS: chloromethyl-X-rosamine, TMRM: tetramethylrhodamine methyl ester, HE: dihydroethidine, NAO: nonyl acridine orange, mAbs: monoclonal antibodies, FLICA: fluorochrome-labeled inhibitors of caspases assay PARP: poly(ADP)ribose polymerase.

telomeres are eroded and lose their protective function. In the absence of telomeres, unprotected chromosomal termini undergo fusion resulting in karyotypic disarray (aneuploidy) and cell death. Telomerase, a specific DNA polymerase, adds telomere hexanucleotide repeats at the termini of telomeric DNA. It is nearly absent in normal cells, but its expression is up-regulated to significant levels in almost all types of malignant cells.

2.3.1. Measuring the length of telomeres

For many years, terminal restriction fragment analysis (TRF) was the gold standard quantitative method to measure telomere length; it became a reference for techniques developed thereafter. Restriction enzymes fully digest the genomic DNA but specifically avoid telomere repeats, resulting in short genomic fragments, while simultaneously leaving longer uncut telomeres. Fragments are resolved by agarose gel electrophoresis and telomeres are identified through either Southern blotting or in-gel hybridization using a labeled probe specific for telomeric DNA [78–80]. TRF requires substantial amounts of genomic DNA, prepared at least from 10^5 cells, and is relatively insensitive to very short telomeres [81].

Single Telomere Length Analysis (STELA) takes advantage of a single-stranded 3′ G-rich overhang present at the end of all telomeres [82]. Using the overhang as a specific template, an oligonucleotide linker is annealed to the 5′ terminus of the telomere. After amplification with polymerase chain reaction (PCR) the products are Southern blotted and probed with the specific subtelomeric sequence [83,84].

Quantitative fluorescence *in situ* hybridization (qFISH) of telomere repeats directly employs fluorescent-labeled$(CCTAA)_n$ peptide nucleic acid (PNA) probes on metaphase chromosomes. Once the PNAs specifically hybridize to denatured telomere DNA repeats [85,86], the detected signal is compared to standards of known telomere length with specific Q-FISH image analysis software [87]. The method allows the quantification of telomere length in distinct cell populations within a single sample by antibody staining [88,89].

2.3.2. Telomerase activity

Telomerase activity is measured by TRAP (telomere repeat amplification protocol) or real-time quantitative PCR. TRAP is a PCR-based assay based on the low substrate specificity of telomerase. During elongation, telomerase from the cell extract adds telomeric repeats to the telomere-imitating oligonucleotide, after which telomerase-

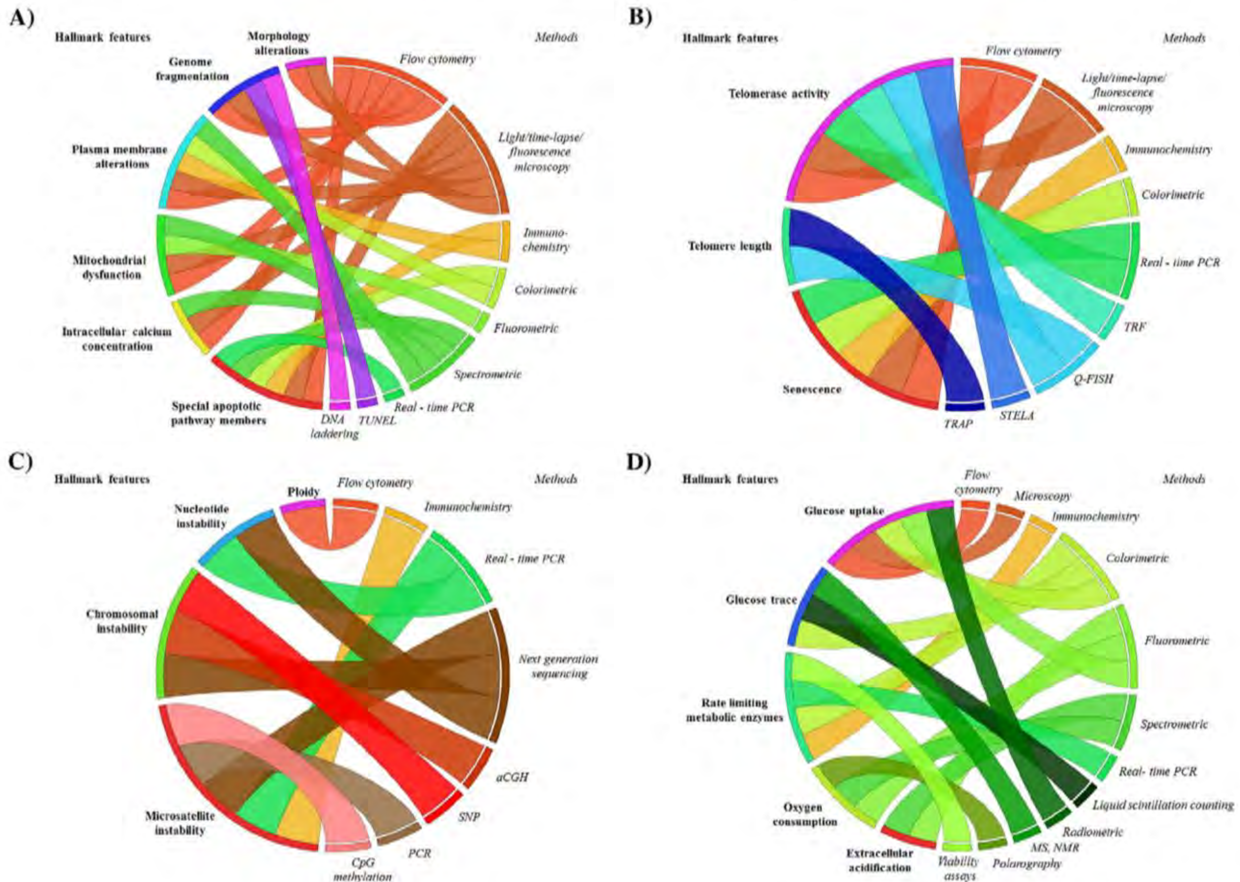

Fig. 1. Circos plot showing a panoramic view of hallmark features (bold) and *in vitro* methods (italic) available for their examination for resisting cell death (A), replicative immortality (B), genome instability (C), and reprogramming of energy metabolism (D). Please refer to the manuscript text for details regarding each component.

synthesized DNA is amplified by using specific primers (telomere-imitating and reverse) [90–92]. Different labels are incorporated into the telomerase-synthesized DNA [93]. In the final step the telomerase product is visualized. The TRAP assay can be performed in both semi-quantitative and quantitative modes [94]. Multiplexing can be achieved by the microarray TRAP (MTRAP) [95]. *In situ* TRAP (FISH-TRAP) using fluorescent telomerase primers and *in situ* PCR can detect telomerase activity in clinical specimens [96].

2.3.3. Senescence

Repeated cell division eventually leads to replicative senescence. Excessive extra- or intracellular stress, such as telomeric dysfunction, mitochondrial retrogression, oxidative stress, DNA damage, or oncogene activation also provoke stable cell-cycle arrest to prevent the spread of damage to the next generation [97,98]. The first identified marker was the lysosomal senescence-associated β-galactosidase (SA-βgal) that cleaves galactosidase from glycoproteins and is only functional at pH 6.0 in senescent cells [99]. By cleaving the chromogenic substrate X-gal at pH 6.0, senescent cells develop a blue stain detected by bright field or phase contrast microscopy. Using substrates that become fluorescent after cleavage, SA-βgal positive cells can be quantified by fluorescence microscopy, by fluorescence-activated cell sorting (FACS) for living cells, or by high throughput screening (HTS) on cell lysates in multiwell plates [100]. Senescent cells are metabolically active and secrete a complex mixture of factors referred to as the SASP (senescence-associated secretory phenotype) [101]. The secretome, regulated by transcription factors such as CEBPb and NF-kB, includes extracellular proteases, matrix components, growth factors, pro-inflammatory cytokines, and chemokines. The secretome can be evaluated by antibody arrays [102]. In some cases, cellular senescence is a potent tumor suppressive mechanism that stops the growth of cells at risk for malignant transformation [103,104]. However, some cytokines and growth factors are pro-tumoral and pro-invasive – rather than tumor suppressive – and act by changing the tissue microenvironment and promoting migration and invasion [101].

The most frequently used methods and their impacts on detecting hallmark features are illustrated in Fig. 1B. Cancer cell immortality can be assessed by analyzing expression of key enzymes and regulators as listed in Table 6 [101,102,105–107].

2.4. Inducing angiogenesis

Neoplastic cells induce angiogenesis to secure the tumor's requirements for nutrients and oxygen. A trait first attributed solely to rapidly growing macroscopic tumors, angiogenesis has later been identified in microscopic pre-malignant lesions as well, in morphologically atypical tissues with an abnormally high occurrence of cancer. A prototypical angiogenesis inducer is vascular endothelial growth factor-A (VEGF-A), that coordinates vessel growth, homeostatic survival of endothelial cells and wound healing. Up-regulation of VEGF and fibroblast growth factor (FGF) expression in tumors as compared to normal tissue is attributed to oncogenes like KRAS and hypoxia [2,108,109].

Two approaches have been widely used to simulate the complex process of angiogenesis [110,111]. First, the response to exogenous stimulatory or inhibitory agents, proliferation, differentiation, and migration of endothelial cells can be studied in cultured HUVEC (human umbilical vein endothelial cells) and HuDMEC (human dermal microvascular endothelial cells) cell lines. The proliferative ability of

these established cell cultures offers various methods to quantify angiogenesis *via* measuring endothelial proliferation [112].

Endothelial cells respond to angiogenic factors with increased motility, and move along the gradient of angiogenesis-inducing factors (*e.g.* VEGF), a process called chemotaxis. Migration is assessed with a modified Boyden-chamber assay. Endothelial cells are plated on top of a filter with a defined pore size and migrate through as a response to an attractant in a lower chamber. The assay is highly sensitive to small differences in concentration gradients [113–118].

Endothelial cells, including progenitor and immortalized cells, rapidly form capillary-like structures *in vitro* when plated on top of a basement membrane extracellular matrix [119]. The presence of fibrin, collagen or Matrigel stimulates the attachment, migration and differentiation into capillary-like tubules in a manner that mirrors the *in vivo* situation, and, to some extent, tissue architecture. Tube formation is quantified from several microscopic fields randomly selected in a given experiment, by measuring the total number of nodes, connected and unconnected tubes as well as their lengths by digital images produced by CCD cameras. These images are processed with a specific digital image-analysis system for automated and quantitative evaluation [120–123].

The second approach is based on direct detection of known factors effecting tumor cell angiogenesis [124]. It employs monoclonal/polyclonal antibodies for known angiogenesis activators (*e.g.* members of the VEGF, FGF, EGF family) by immunoblot or ELISA [125–127].

The key enzymes and regulators that allow the study of gene expression linked to angiogenesis are summarized in Table 6 [128–132].

2.5. Activating invasion and metastasis

The spread of neoplastic disease has been described as a sequential multi-step process, termed the invasion-metastatic cascade. The program by which epithelial cells acquire migratory and invasive capacity is called epithelial-mesenchymal transition (EMT) [133]. The transformation includes the loss of cell adherence proteins, alteration of cell morphology, increased motility, expression of matrix-degrading proteinases and resistance to apoptosis, representing a fundamental reprogramming of every aspect of the cell's biology. EMT, which is central to normal developmental processes such as gastrulation [134], is hijacked by cancer cells for their own dissemination. EMT can be induced by intrinsic factors, such as genetic mutations, or by extrinsic signals, such as growth factors. However, some tumors exhibit incomplete or partial EMT [135], or invade without epithelial–mesenchymal conversion [136]. The tumor microenvironment acquires increasing importance, as the crosstalk between the tumor and the surrounding stroma is capable of inducing metastasis [137,138]. Understanding the complex nature of interactions between cancer cells and the extracellular matrix (ECM) requires a systems-based approach instead of focusing merely on genetic mutations within neoplastic cells.

Migration and invasion are distinct phenomena in experimental cell biology. Migration is the directed movement of cells without passing through obstructive barriers, whereas invasion necessitates the destruction of barriers in order to pass through them and thus is of necessity accompanied by ECM remodeling [139]. However, during both migration and invasion, cells squeeze through tight interstitial spaces, and the nuclear deformation caused by the confining microenvironment requires subsequent restoration of the nuclear envelope and DNA content [140].

2.5.1. Transwell migration and invasion assay (Boyden chamber)

A double chamber is filled with two media, one with an attractant (like FBS) to trigger chemotaxis. Cells are seeded in the upper well, and migrate vertically between the chambers through a porous membrane [141,142]. Migrated cells can be visualized by cytological dyes, stained fluorescent, or lysed and assessed by a plate reader. Due to different cell dimensions, the assay must be optimized for each cell type [139]. By coating the porous filter with ECM components like type I collagen or a basement membrane-like matrix (Matrigel), the device is made suitable for testing invasion. Invasive cells degrade the matrix and move through to the bottom. Parallel measurements with ECM-coated and non-coated assays allow one to calculate an "invasive index": the rate of invasiveness *versus* migration [143]. Although providing only endpoint measurements, the transwell invasion assay is the most frequently used method due to its easy setup.

2.5.2. Scratch (wound healing) assay

This simple and popular assay allows the observation of two-dimensional (2D) cell migration in confluent, monolayer cell cultures. The surface of a cellular monolayer is wounded by scraping, and images are captured by microscopy at regular intervals to track the migration of cells from the edge of the wound until the scratch is closed [116,144,145]. Time-lapse microscopy with image analysis software enables automation. Mechanical scraping is done usually with pipette tips, needles, razors, *etc.*, although more advanced methods produce more controllable wound sizes with clearly defined edges, without damaging the underlying matrix. Electric cell-substrate impedance sensing (ECIS) systems enable automatic monitoring of cell adherence and wound closure while ablating cells with a high electric current [146,147]. Laser ablation is another method to create a well-defined scratch [148,149], however both methods generate leftover cells and debris in the wound area [150]. Dying cells release apoptotic factors, potentially effecting wound closure. The advantage of the scratch assay is its easy setup and low cost, but the experiments usually require relatively large cell and reagent quantities. Scratch assays are appropriate to study regeneration of epithelial or endothelial cells [116], and can be combined with other techniques, such as gene transfections [151].

2.5.3. Cell exclusion zone assays

Cell-free zones are created before seeding the cells, using a physical barrier such as silicone. Later, the barrier is removed, allowing cells to occupy the area [152]. Alternatively, cells are seeded inside of a ring, and migrate outward once the barrier is removed [153]. Barriers can be made of glass, silicone, metal, Teflon, microfabricated soft and elastic "stencils" or agarose gels [139,150,153,154]. Cells are visualized by photomicrography or labeled with fluorescence and measured with a microplate reader [150,152]. Migratory capacity and interaction between two different populations can be compared [139]. These assays avoid the drawbacks of scratch assays, such as uneven and irreproducible scratch areas and remnants of dying cells in the media.

2.5.4. Microcarrier bead and spheroid migration assays

Cells migrate from the surface of confluent cell-coated microcarrier beads to a 2D surface in a cell culture vessel, where cells can be fixed, stained and quantified. The close contact of cells on the bead surface mimics *in vivo* cell-to-cell contact better than flat monolayers [155]. The original version of the assay measures end-points, but cells can also be traced during migration by light microscopy [139]. Beads are expensive and some beads might not be covered sufficiently. In the *spheroid migration assay*, tumor cell spheroids are placed on a surface of cultured vessels. Tumor cell spheroids resemble *in vivo* physiologic conditions by establishing oxygen and nutrient gradients characteristic of tumors, but the method can only be performed on cells that are able to form spheroids [156]. The *spheroid invasion assays* enable the study of invasive properties of multicellular spheroids embedded into three-dimensional (3D) extracellular matrices [157]. Different scenarios including malignant and non-malignant spheroids and invasion of epithelial cells can be investigated [158,159]. In the *spheroid confrontation assay* two cell types are co-cultivated, allowing them to invade each other in a single cell pattern or collectively as a population [160]. Fluorescent labeling enables an invasion to be followed with microscopy, and flow cytometry allows quantification of invasive cells.

2.5.5. The capillary chamber migration assay

The capillary chamber migration assay consists of two chambers, one containing the cell suspension and the other a chemoattractant, connected by a linking capillary. Migratory behavior of cells is observable, and morphological responses are visualized by time-lapse microscopy in real time [161]. The small cell numbers and reagent volumes make this assay suitable for rare cell types and expensive compounds. Liquid handling and image processing can be fully automated [162].

2.5.6. Vertical assays

Cells are placed on top of an ECM, where after forming a monolayer they move down individually or collectively, as first described with lymphocytes [163]. In skin cancer, cells can also be coated with matrix and move up [164,165]. Alternatively, cell monolayers can be sandwiched between a top and bottom layer of ECM, and additional cell types might be embedded into the ECM to study their effects on cell invasion [166].

2.5.7. Motility of individual cells

Motility of individual cells can be traced by time-lapse video microscopy. New algorithms are available to follow up to a dozen cells simultaneously [167]. 3D tracking is also possible, but requires specialized microscopes and image analyzing software, as well as extensive data processing [168]. The *gelatin degradation assay* allows analysis of progressive invasion at a subcellular level. The ability to form invadopodia directly correlates with the invasive potential of tumor cells. Cells are seeded on top of a thin, fluorescent dye-labeled gelatin matrix. The actin-rich invadopodia and podosomes extend into the ECM and degrade the gelatin with matrix metalloproteinases, creating spots with decreased fluorescence [169]. Computerized methods allow the analysis of invasive properties of single cells as well as of populations [170].

2.5.8. Mechanical properties

Mechanical properties of individual tumor cells affect the way cells are able to spread. Metastatic cancerous cells are 70% less stiff than normal cells [171], and stiffness inversely correlates with migration and invasion through 3D structures, measured by a magnetic tweezer system combined with a 3D Matrigel invasion assay [172]. A recently developed high throughput microscope system is able to assess cellular mechanical properties in multiwell plates [173].

2.5.9. Real-time monitoring and live-cell imaging

The impedance based xCELLigence System (see also Section 2.1.5) permits label-free real-time monitoring of invasion and migration. Membranes can be coated with ECM to study invasive properties [174].

The IncuCyte™ Chemotaxis System, based on live-cell time-lapse imaging, allows automated visualization of labeled or label-free chemotactic cell migration and invasion even in multiwell format [175,176].

The most frequently used *in vitro* migration and invasion assays are summarized in Table 4, and the genes linked to migration and invasion are summarized in Table 6 [157,167,177–180].

2.6. Genome instability and mutation

Most cancers exhibit chromosomal instability, meaning altered chromosomal structures and high rates of rearrangements compared to normal cells. Microsatellite instability is characterized by the expansion or reduction of short nucleotide repeats present in microsatellite sequences. Nucleotide instability refers to the increased frequency of substitutions, deletions and insertions in one or several nucleotides [181]. The origin of genomic instability is still a subject of intense research [182]. Oncogene activation can induce the collapse of DNA replication forks resulting in double-strand breaks. DNA damage activates *TP53*, leading to senescence or apoptosis. In contrast, inactivating (loss-of-function) mutations in *TP53* eliminate the barrier against tumor progression [183]. In hereditary cancers, loss of a DNA repair gene is considered to drive cancer development by increasing the spontaneous mutation rate [182]. Neoplastic cells incur mutations through increased sensitivity to mutagens, by weakening the genomic maintenance machinery or by eliminating the surveillance system guarding genomic integrity.

2.6.1. Flow cytometry

Flow cytometry is a universal multicellular approach that can detect cellular ploidy and cell cycle distribution with fluorescent dyes that bind to DNA in stoichiometric fashion. Genome instability can be evaluated by comparing ploidy in the G0/G1 fraction for tumor *versus* normal cells. However, flow cytometry does not provide information about other levels of genome instability [184].

2.6.2. Karyotyping

Karyotyping is a more informative method. A DNA-binding dye intercalates into specific DNA regions of chromosomes (*e.g.* A/T-rich areas) in metaphase spreads, resulting in a specific banding pattern for each chromosome. Spectral karyotyping is a multicolored whole chromosome-painting assay using FISH probes to visualize each chromosome. Decoding the chromosomes with different fluorochromes allows specification of rearrangements by confocal microscopy. Spectral karyotyping is limited to detecting global changes in chromosomes and as such is unable to evaluate alterations at the sequence level [185,186].

2.6.3. Array of comparative genomic hybridization

Array of comparative genomic hybridization (aCGH) is a multicellular technique used to quantitatively detect and visualize chromosomal alterations. For the detection of gains, losses, amplifications and loss-of-heterozygosity, the sample DNA is compared to a normal reference genome. The differentially-labeled reference and sample DNAs are combined, and non-analogous hybridizations are visualized as changes in fluorescence intensity on metaphases. CGH detects unbalanced chromosomal abnormalities affecting copy number, but is unable to distinguish reciprocal translocations, inversions or somatic mutations [187, 188]. Single nucleotide polymorphism (SNP) arrays are also hybridization-based. Fragmented nucleic acid sequences labeled with fluorescent dyes hybridize to immobilized, allele-specific oligonucleotide probes. SNP arrays are capable of detecting copy-neutral loss of heterozygosity (so-called uniparental disomy or gene conversion) and distinguish alleles at specific polymorphic sites [189].

To detect microsatellite instability (MSI), the gold standard approach amplifies known microsatellite regions by PCR and quantifies the lengths of PCR products by gel or capillary electrophoresis using autoradiography, silver staining [190] or fluorescent methods [191,192]. The microsatellite length of the sample is compared to normal DNA to determine MSI status. Alternatively, real-time quantitative PCR (qPCR) melting point analysis is used with sequence-specific fluorescent-labeled hybridization probes [193]. MSI status can also be detected with next generation DNA sequencing (NGS) [194]. Microsatellite instability is the results of defects in mismatch repair (MMR) genes such as MSH, MLH and PMS: the expression profile of MMR genes correlates with microsatellite instability [193]. Mutant MMR genes are translated to proteins, and mutant MMR proteins can be studied by immunochemistry using mutation-specific monoclonal antibodies [195].

2.6.4. Whole genome sequencing

Whole genome sequencing is the most comprehensive and informative method to distinguish nucleotide mutations in coding, non-coding and un-annotated regions. It is also able to measure larger genomic rearrangements frequently present in cancer such as copy number variations (CNV), insertions and translocations [196]. Sanger sequencing, developed in 1977, is based on a dideoxy chain-termination method and provides high-quality, long read length, spanning 400–900 base

Table 4
Comparison of migration assays and their modifications into invasion tests.

Migration assay features	Cell type	Direction of movement	Chemokine gradient	HTS	Equipment complexity	Individual cell tracking	Data collection methods	Analysis type	Modification to invasion assay
Transwell migration assay (Boyden chamber assay) [141,142]	Adherent cells	Vertical	✔	✔	Chambers Easy setup	–	Cell counting Fluorescent staining	Endpoint	Coating chamber with ECM [143]
Scratch (wound healing) assay [116,144,147,149]	Adherent cells	Horizontal	–	✔	Plastic or glass surface Easy setup	✔	Cell counting with time-lapse microscopy	Kinetic	–
Cell exclusion zone and fence assays [150,152–154,275]	Adherent cells	Horizontal	–	✔	Plastic or glass surface Physical barrier Easy setup	✔	Cell counting with microscope Fluorescent staining	Kinetic	Coating the surface with ECM (Platypus invasion assay) [276]
Microcarrier bead assay [155] and Spheroid migration assay [156]	Adherent cells / Spheroid	Vertical and horizontal	–	✔	Plastic or glass surface microbeads technically demanding	✔	Cell counting with microscope fluorescent staining time-lapse microscopy	Endpoint/kinetic	Microbeads or spheroids placed into ECM [157,158,160]
Capillary migration assay [161,162]	Adherent and suspension cells	Horizontal	✔	✔	Glass surface Specific chambers Technically demanding	–	Cell counting with microscopy	Endpoint / kinetic	–
Single cell motility assay [167,277–279]	Adherent and suspension cells	Horizontal	✔	✔	Glass surface Advanced Expensive technical setup	✔	Cell tracking with time-lapse microscopy	Kinetic	Coating the surface with ECM [163]
Gelatin degradation assay [169]	Adherent cells	Vertical	–	–	Gelatin media	–	Cell tracking with microscopy	Endpoint	Coating the surface with ECM
Mechanical properties (stiffness, compliance) [172,173]	Adherent cells	Horizontal	–	✔	3D Force Microscope, coated magnetic beads	–	high speed video camera, video spot tracker	Endpoint	Only for invasion
xCELLigence System [174]	Adherent cells	Horizontal	–	✔ (16 well plate)	Specific chambers	–	Microelectronic cell sensor	Kinetic	Coating chamber with ECM [174]
IncuCyte™ [176]	Adherent and non-adherent cells	Horizontal	✔	✔	Specific chambers, Costly initial set up	✔	Live-cell time- lapse imaging	Kinetic	Coating chamber with ECM [175]

Abbreviations: HTS: high throughput screening, ECM: extracellular matrix.

pairs. However it is costly and is unable to provide high throughput. Next-generation sequencing (NGS) systems enable high throughput and accurate analysis along with decreased sequencing cost. The Illumina GS/HiSeq platform implements sequencing by synthesis technology providing high throughput and low reagent cost; nevertheless it is hampered by short read lengths. The Ion Torrent adopts semiconductor sequencing and provides rapid and low-cost sequencing results [197]. Sequencing the transcriptome (*RNA-seq*) also allows the discovery of special genetic aberrations, such as gene fusions; it could replace microarrays in the long run [198]. Developments in NGS and whole-genome amplification already allow single-cell sequencing [199,200]. Adequate genome coverage is critical, and each area of genome research requires markedly different sequencing depth [201].

Common methods to assess genomic instability and their association with hallmark features are illustrated in Fig. 1C. Genes participating in genome maintenance and related to cancer genome instability are listed in Table 6 [202,203].

2.7. Reprogramming energy metabolism

Actively proliferating cancer cells must regulate their energy metabolism to fuel constant cell growth. By simultaneously increasing the rate of glycolysis while limiting oxidative phosphorylation, cells enter a state termed aerobic glycolysis, also known as the Warburg effect [204,205]. Increased glycolysis can allow the diversion of glycolytic intermediates into biosynthetic pathways required for new cell assembly. Altered energy metabolism is so widespread in cancer cells that it has been included as one of the hallmarks of cancer [206].

2.7.1. Glucose uptake

Glucose is rapidly metabolized, so virtually non-metabolizable glucose analogs like FDG (fluoro-deoxyglucose), 2DG (2-deoxy-D-glucose) and 3MG (3-*O*-methylglucose) are needed to study uptake efficiency. In cell cultures, the most commonly used analogs are radioactive hexoses, quantified from cell lysates by a liquid scintillation counter [207–209]. The uptake of nonradioactive, fluorescent glucose analogs is measured by flow cytometry or fluorescence microscopy [210]. Enzymatic assays of glucose uptake are based on a dye reduction reaction connected to the first step of glycolysis and are detected spectrophotometrically [211,212].

2.7.2. Activity of rate-limiting glycolytic enzymes

Hexokinase, phosphofructokinase, and pyruvate-kinase are the main rate-limiting enzymes of glycolysis, controlling the maximum possible carbon flux. Enzyme activity is assessed by coupling them with a NAD^+/NADH- dependent enzyme reaction producing visible substrates and measuring changes in absorbance values [213]. Determination of free nuclear NADH can be assessed by two-photon microscopy to quantify the intensity of NAD(P)H autofluorescence.

2.7.3. Metabolomics and inorganic products

Metabolites are quantified by mass spectroscopy (MS) or nuclear magnetic resonance spectrometry (NMR). By using ^{13}C- or ^{3}H-labeled glucose, the amount of ^{13}C- or ^{3}H-glucose derived isotopes in downstream metabolites is linked to glycolytic activity. ^{13}C can replace any of the six carbons in the glucose molecule [214]. ^{3}H is incorporated into H_2O from a ^{3}H-labeled glucose [215]. $^{3}H_2O$ is released into the medium by the conversion catalyzed by enolase and its subsequent presence in the media of cultured cells can be measured by liquid scintillation counting [216].

2.7.4. Oxygen consumption

The rate of oxygen consumption (OCR) is a universal indicator of mitochondrial respiration, and can be measured both intra- and extracellularly. Intracellular O_2-levels provide accurate information about the metabolic activity of a cell. The measurement is based on a cell-penetrating O_2-sensitive phosphorescent probe [217]. Currently, supramolecular and nanoparticle-based intracellular probes are available with rapid intracellular accumulation allowing direct, contact-free and quantitative oxygen assessment. The assays are typically performed in microplates and measured by real-time fluorescence plate readers [218].

Extracellular oxygen consumption can be measured by polarographic respirometry (Clark's electrode) or with optical sensor probes. A Clark's electrode allows measurement of intact cells or extracted mitochondria in suspension by polarography [219]. Optical sensor probes determine oxygen consumption from the medium over a monolayer of living cells within a microplate reader. The Extracellular Flux Analyzer measures multiple metabolic parameters, such as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real time. Metabolism can also be modified by successive addition of different compounds [220]. During mitochondrial stress, the sequential addition of metabolic modulators to different parts of the electron transport chain (Fig. 2A) allows the identification of key mitochondrial parameters such as basal respiration, ATP turnover, proton leak and maximal respiration. The difference between maximal and basal respiration represents the respiratory reserve, the capacity of a cell to create ATP *via* oxidative phosphorylation in response to increased energy demand. Non-mitochondrial respiration results in substrate oxidation and cell surface oxygen consumption (Fig. 2B).

2.7.5. Extracellular acidification

Intensified glycolysis with higher glucose uptake results in excess lactate secretion into the extracellular environment. Commercially available kits are available to quantify lactate levels in cell cultures and are based on colorimetric or fluorometric reactions compatible with standard laboratory equipment, *e.g.* spectrophotometers [221,222]. Glycolytic activity is also indicated by conversion of pyruvate to lactic acid, determining the extent of extracellular acidification rate (ECAR) as measured by an extracellular flux analyzer [223]. This test is performed by adding glucose, followed by inhibitors of glycolysis and oxidative phosphorylation, and provides information on glycolytic capacity and reserve. Glycolytic reserve indicates the cell's ability to increase glycolytic rate upon increased energy demand (Fig. 2C).

2.7.6. Glutathione (GSH)

Glutathione (GSH) is a tissue antioxidant preventing cell damage from free radicals and peroxidases. Increased levels of oxidative stress generate oxidized glutathione (GSSG), causing an increased ratio of GSSG to GSH. Increased susceptibility to oxidative stress has been implicated in cancer development [224]. Alterations in the GSH molecular pathways in cancer cells can lead to enhanced chemoresistance and increased survival. Tumor glutathione levels tend to be altered, *i.e.* frequently increased compared to healthy tissue [225]. GSH and GSSG levels can be assessed rapidly and reliably spectrophotometrically in a microplate reader [226].

Assays of energy metabolism are summarized in Table 5, and their connection with hallmark features are illustrated in Fig. 1D. Expression of glucose transporters or other metabolic enzymes is rendered feasible by immunoblotting, FACS or qPCR. The bioenergetics and metabolism of a cell can also be estimated by analyzing gene expression of key enzymes (Table 6) [227,228].

2.8. Epigenetics

In recent years, epigenetics has become equally important compared to genetic mechanisms in cancer initiation and progression [229,230]. Promoter hypermethylation leads to transcriptional silencing. CpG methylation represents a key epigenetic mechanism of inactivation of tumor suppressors and MMR genes [231]. Methylation patterns can be detected by methylation-specific PCR (MSP). Treating the DNA with sodium bisulfite converts unmethylated cytosine to uracil, and during

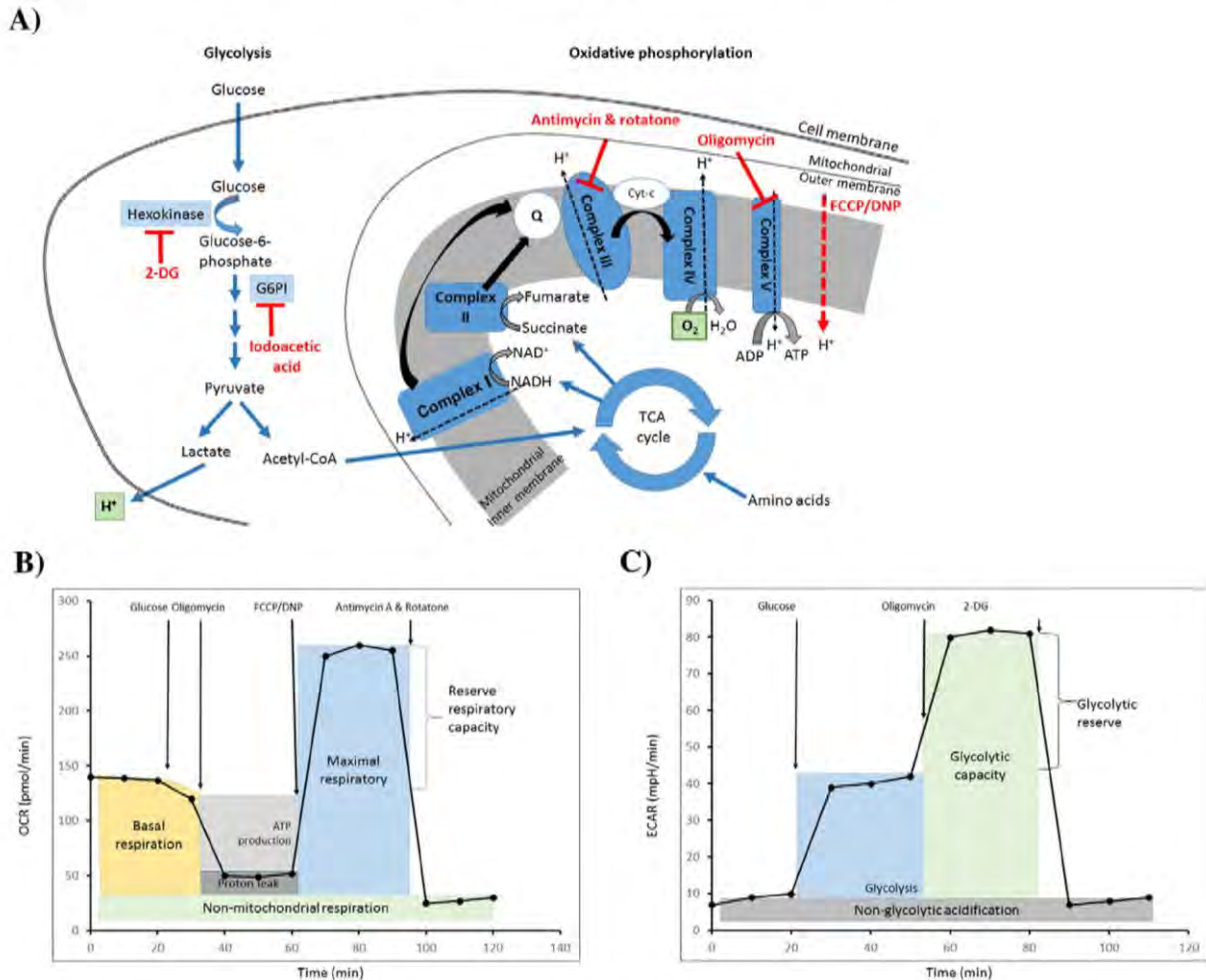

Fig. 2. Schematic representation of measured features related to the reprogramming of energy metabolism. Inhibitors used during OCR and ECAR measurements (A). Schematic graph of OCR illustrating metabolic modulators and the detected key parameters (B). Schematic graph of ECAR measurement. (C). Blue arrows represent glycolysis. Blue box represents Electron Transport Chain (ETC) members in the mitochondrial inner membrane: Complex I: NADH dehydrogenase, Complex II: Succinate dehydrogenase, Q: Coenzyme Q, Complex II: Coenzyme Q, cytochrome-c oxidoreductase, Complex IV: cytochrome-c oxidase, Complex V: ATP synthase. Black arrows represent electron flow in the mitochondrial ETC. Inhibitor abbreviations: 2-DG: 2-deoxyglucose, FCCP: trifluorocarbonylcyanide phenylhydrazone. Oligomycin is an ATP synthase inhibitor, blocks the mitochondrial respiration and pushes the cells to rely upon glycolysis for ATP production. DNP or FCCP are mitochondrial uncouplers that compel the mitochondria to raise the flow of electrons and oxygen consumption. Antimycin A & rotatone block complexes in the electron transport chain of mitochondria. Other abbreviations: TCA cycle: tricarboxylic acid cycle, Acetyl-CoA: acetyl coenzyme A, cyt c: cytochrome *c*, ATP: adenosine triphosphate, ADP: adenosine diphosphate, H^+: proton. The parameters measured with extracellular flux analyzer, the rate of oxygen (O_2) consumption and the measure of protons (H^+) released during lactate production, are depicted in green boxes.

a subsequent PCR reaction, primers anneal to either the methylated or the unmethylated sequence [232]. MSP is not quantitative and bears a significant risk of false positive and false negative results. In contrast, Quantitative Methylation-Specific PCR (qMSP) assays utilizing TaqMan probes are highly sensitive to promoter hypermethylation and available in high throughput format (*e.g.* MethyLight) [233]. Quantitative multiplex methylation-specific PCR (QM-MSP) accurately determines promoter hypermethylation for many genes simultaneously in small samples, is highly sensitive and linear over 5 orders of magnitude [234]. Pyrosequencing is a "sequencing by synthesis" technique, during which nucleotide incorporation releases a pyrophosphate molecule, generating a luciferase-catalyzed luminometric signal. Pyrosequencing can be used for quantification of DNA methylation at specific CpG sites within the target region [235]. The method provides high quality, quantitative results and is appropriate to analyze methylation of CpG sites in close proximity [236]. DNA methylation enrichment methods avoid the limitations of bisulfite-conversion. Methyl-CpG binding domain-based capture (MBDCap) uses beads coated in MBD polypeptide that bind methylated DNA; the eluted DNA is sequenced with NGS [237,238]. In addition, epigenetic studies employ chromatin immunoprecipitation (ChIP) to identify DNA sequences that are associated with a chromatin component of interest. Coupled with high-throughput methods, such as microarrays (ChIP-chip) or massively parallel sequencing (ChIP-seq) it is possible to identify binding sites of specific proteins across the entire genome [239,240].

Small (17–24 base), highly conserved, non-coding *microRNAs* (miRNA) regulate gene expression at the post-translational level by targeting complementary mRNAs and play a regulatory role in all cellular pathways [241]. About half of all miRNA genes are located in close proximity to CpG islands and thus are subject to epigenetic regulation *via* DNA methylation and chromatin remodeling. Additionally, miRNAs modulate the expression of genes linked to the epigenetic machinery [242]. Disregulated miRNA expression has been connected to cancer initiation and progression [243]. Microarray hybridization, quantitative reverse transcription PCR (RT-qPCR) and small RNA sequencing with NGS are the most common platforms to evaluate miRNA expression, although the small size of active, mature miRNAs renders expression profiling challenging. Comparing reproducibility, specificity, sensitivity and accuracy across 12 commercially available platforms showed qPCR in general to be especially suitable for low input RNA samples

Table 5
Comparison of assays suitable for measuring altered energy homeostasis.

Energy metabolism features	Target of analysis	Method	Detection	Principles	Advantages /disadvantages
Glucose uptake	Labeled glucose	Stable isotope tracking analysis	Liquid scintillation counter [207–209]	Radioactive hexoses *e.g.*: 2-deoxy-D-[1,2-3H]-glucose or 2-deoxy-D-[1-14C]-glucose	Better signal-to-noise ratio and higher specificity. Expensive and requires specialized training and equipment
		Fluorescent hexose analogs tracking analysis	Flow cytometry, fluorescence microscopy [210]	Fluorescent glucose analogs: 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG)	Single cell analysis
	Glucose analog conversion	Enzymatic assay for glucose determination	Spectrophotometric method [211,212]	2DG-P converted to 2-deoxy-6-phosphogluconate by G6PDH parallel with NADP + reduction, coupled with dye conversion to fluorescent form (resazurin to resorufin)	Simple technique
	Hexose conversion	Phenotype MicroArrays™	Spectrophotometric method [280]	Added monotype carbon nutrients, detection of NADH production by redox dye alteration	Parallel testing of several metabolic pathways
Rate-limiting glycolytic enzymes	Activity of glycolytic enzymes	Measuring activity of rate-limiting glycolytic enzymes	Spectrophotometric method [213]	Glycolytic enzyme reaction coupled with NAD +/NADH- or NADP +/NADPH-dependent enzymes by using enzymes that react with their products.	Simple technique
Metabolic results	Metabolites from glucose	Metabolomics	Mass spectroscopy (MS) nuclear magnetic resonance spectrometry (NMR) [214]	^{13}C or ^{3}H-labeled glucose derived isotopes in downstream metabolites	NMR spectroscopy is fast, quantitative and reproducible, but MS is more sensitive. MS needs metabolite separation by chromatography.
	H_2O	Stable isotope tracing	Liquid scintillation counter [215,216]	Trace of ^{3}H from ^{3}H-labeled glucose to H_2O from media	The labeled glucose must be removed from the sample
Oxygen consumption	Intracellular O_2 level	Intracellularoxygen-sensitive probes	Spectrophotometric method [217,218]	Cell-penetrating O_2-sensitive phosphorescent probes	
	Extracellular O_2 level	Clark's electrode	Polarography [219]	Oxygen dissolved in liquid or gas phase in the sample chamber is detected by polarography using a Clark-type oxygen electrode	Large amount of material, isolated mitochondria and permeabilized cells
	Extracellular O_2 level	XF Extracellular Flux Analyzer	Spectrophotometric method [223]	The dissolved oxygen is measured by solid state fluorophore probes.	Inhibitors: oligomycin, DPN or FCCP, and antimycin A & rotenone
Extracellular acidification	Extracellular lactate level	Lactate assay	Spectrophotometric method [221,222]	Lactate in the media is oxidized by added LDH to generate a substance which interacts with a probe to produce a color/fluorescent signal	Simple technique
	Extracellular pH/lactate level	XF Extracellular Flux Analyzer	Spectrophotometric method [223]	The pH in cell media is measured by solid state fluorophore probes.	
Oxidative stress	Glutathione	Measuring GSH/GSSG ratio	Spectrophotometric method [226]	Enzymatic recycling of GSH from GSSG by glutathione reductase. GSH reacts with DTNB producing TNB chromophore.	Simple technique

Abbreviations: 2DG-P: 2-deoxy-D-glucose phosphate, DPN: dinitrophenol, FCCP: trifluorocarbonylcyanide phenylhydrazone, GAPDH: glucose-6-phosphate dehydrogenase, DTNB: 5,5′-dithio-bis(2-nitrobenzoic acid).

Table 6
List of marker genes for which an expression change is linked to a hallmark feature.

Hallmark feature	Marker genes
Sustaining proliferative signaling and evading growth suppressors [39,40]	
Oncogenes	BAX, BCL2L1, CASP8, CDK4, ELK1, ETS1, HGF, JAK2, JUNB, JUND, KIT, KITLG, MCL1, MET, MOS, MYB, NFKBIA, NRAS, PIK3CA, PML, PRKCA, RAF1, RARA, REL, ROS1, RUNX1, SRC, STAT3, ZHX2
Tumor suppressor genes	ATM, BRCA1, BRCA2, CDH1, CDKN2B, CDKN3, E2F1, FHIT, FOXD3, HIC1, IGF2R, MEN1, MGMT, MLH1, NF1, NF2, RASSF1, RUNX3, S100A4, SERPINB5, SMAD4, STK11, TP53, TP73, TSC1, VHL, WT1, WWOX, XRCC1
Transcriptional factors	ABL1, BRCA1, BRCA2, CDKN2A, CTNNB1, E2F1, ELK1, ESR1, ETS1, FOS, FOXD3, HIC1, JUN, JUNB, JUND, MDM2, MEN1, MYB, MYC, MYCN, NF1, NFKB1, PML, RARA, RB1, REL, RUNX1, RUNX3, SMAD4, STAT3, TGFB1, TNF, TP53, TP73, TSC1, VHL, WT1, ZHX2
Cell cycle	ATM, BRCA1, BRCA2, CCND1, CDK4, CDKN1A, CDKN2A, CDKN2B, CDKN3, E2F1, HGF, MEN1, STK11
Multiple features	BCR, EGF, ERBB2, ESR1, FOS, HRAS, JUN, KRAS, MDM2, MYC, MYCN, NFKB1, NRAS, PIK3C2A, RB1, RET, SH3PXD2A, TGFB1, TNF, TP53
Resisting cell death [75,76]	
Apoptotic pathway members	
Bcl2 Family-regulated pathway	BCL2, BCL2L1, BCL2L2, BCL2L10-15, MCL1, BAX, BCL2A1, BAK1, BAD, BOK, BBC3, BIK, BNIP3L, HRK, BBC3, BID, BNIP3
TNF Receptor pathway	TNFRSF1A, TNFRSF1B, TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10, TNFRSF11, TNFRSF21, NGFR, TRAF1, TRADD
TNF superfamily	TNF, TNFSF4, TNFSF8-15, LTA, LTB
Fas signaling pathway (CD95)	FAS, FASLG
Caspase family	CASP1 (ICE), CASP10 (MCH4), CASP14, CASP2, CASP3, CASP4, CASP5, CASP6, CASP7, CASP8, CASP9, CFLAR (CASPER), CRADD, PYCARD (TMS1/ASC),
NF-kß signaling pathway	NFKB1, NFKB2, NFKB3, NFKBIB, NFKBIA, NFKBIE, NFKBIKB, RELB, NFKBIZ, REL, IKBKE
IAP family	DIABLO
CARD family	APAF1, BLC10, RIPK2, BIRC1-8, CRADD, CARD4-19, NALP1, PYCARD
Kinases	TBK1, DAPK1, CHUK
Other	NOXA, SOCS3, DEDD, DEDD2, PEA15, DAXX, FADD
TP53 pathway	
TP53 activators	ATM, ATR, BBC3, BRCA1, CDKN1A (p21CIP1/WAF1), CDKN2A (p16INK4), CHEK1, CHEK2 (RAD53), FOXO3, GML, MDM2, MSH2, SIRT1, TP63, TP73
TP53 regulators and cooperators	CDKN2A (p16INK4), DNMT1, HDAC1, KAT2B, MDM2, MDM4, SIRT1, TADA3, TP53AIP1, TP53BP2, TP63 (TP73L), TP73
Apoptosis pathway members	APAF1, BCL2, BCL2A1, BID, BIRC5, CASP2, CASP9, CRADD, FADD, FASLG (TNFSF6), IGF1R, MCL1, NF1, NFKB1, PIDD (LRDD), RELA, TNFRSF10D, TRAF2, WT1
Cell cycle pathway members	CCNB1, CCNE1, CCNG1, CCNH, CDK1 (CDC2), CDK4, PRC1, TSC1
DNA repair pathway members	BRCA2, MLH1, XRCC5
Transcriptional factors	BRCA2, EGR1, JUN, MYOD1, NFKB1, RELA, STAT1
Enabling replicative immortality [101,102,105–107]	
Telomere maintenance	
	ACD, BLM, DCLRE1C, DKC1, ERCC1, ERCC4, HSPA1L, MRE11A, MYC, NBN (NBS1), PARP1 (ADPRT1), PIF1, POT1, PRKDC, PTGES3, RAD50, RFC1, RTEL1, SMG6, TEP1, TERF1, TERF2, TERF2IP, TERT, TINF2, TNKS (TIN1), TNKS2, XRCC5, XRCC6 (G22P1)
Regulation of telomere length	RTEL1, TEP1, TERF1, TERF2IP, TNKS (TIN1)
Telomerase activity	
Telomerase complex	DKC1, GAR1, NHP2, NOP10, TERT, WRAP53, hTR, NOLA1-3, ACD, POT1, RAP1A, TERF1, TERF2, TINF2, SLX4, ERCC1, ERCC4, EME1, MSH2, MSH3, MUS81, PLK1, TERF2, TERF2IP
Telomerase regulation	BL1, AKT1, ATP5C1, BCL2, EGF, IGF1, KRAS, MEN1, MYC, PAX8, PINX1, PPARG, PPP2R1A, PPP2R1B, PRKCA, PRKCB, RB1, SART1, SMAD3, SP1, SSB, TGFB1, TP53, MAD1L1, MEN1, SIP1,
Senescence pathways	
	ATM, BMI1, CCND1, CCNE1, CDK2, CDK4, CDK6, CDKN1A, CDKN2A (p16INK4), CDKN2D, CHEK1, CHEK2, E2F1, E2F3, ETS1, ETS2, MDM2, RB1, RBL2, TP53, TWIST1
Senescence secretome	
Soluble factors	IL-6, IL-7, IL-I, IL-13, IL-15
Chemokines	IL-8, GRO, MCP-2, MCP-4, MIP-1, MIP-3, HCC-4, EOTAXIN, TECK, ENA-78, I-309, I-TAC,
Inflammatory factors	GM-CSE, G-CSE, IFN-γ, BLC, MIF
Growth factors	Amphiregulin, eporegulin, heregulin, EGF, Bfgf, HGF, KGF, VEGF, angiogenin, SCF, SDF-1, PIGF, NGF, IGFBP-2-7
Protease, regulators	MMP-1,-3,-10,-12,-13,-14, TIMP-1,-2, PAI-1,-2, tPA, uPA, Cathepsin B
Soluble or shed receptors or ligands	ICAM-1,-3, OPG, sTNFRI, TRAIL-R3, Fas, sTNFRII, uPAR, SGP130, EGF-R
Nonprotein soluble factors	PGE2, nitric oxide
Insoluble factors (ECM)	Fibronectin, collagens, laminin
Inducing angiogenesis [128–132]	
Growth factors and receptors	ANG, ANGPT1, ANGPT2, ANPEP, TYMP, FGF1, FGF2 (bFGF), FIGF (VEGFD), FLT1, JAG1, KDR, NRP1, NRP2, PGF, VEGFA, VEGFB, VEGFC,
Proteases, inhibitors and other matrix proteins	ANGPTL4, F3, PECAM1, PF4, PROK2, SERPINE1 (PAI-1), SERPINF1,
Adhesion molecules	BAI1, COL4A3, IL8, NRP1, NRP2
Cytokines and chemokines	BMP2, CCL15, CCL2, CSF3, CXCL11, CXCL12, CXCL13, CXCL14, CXCL3, CXCL5, CXCL6, CXCL9, IL10, IL6, IL8, PPBP, PTN, TNF
Other	HIF1A, NOS3, SPHK1, AGGF1, AMOT, ANG, ANGPT1, BTG1, EDIL3, EREG, FST, RHOB, RUNX1
Activating invasion and metastasis [157,167,177–180]	
EMT transcription factors	SNAI1, SNAI2, SNAI3, TWIST1, TWIST2, FOXOC2, TCF3, SMAD3, HIF1A, TGFB1, ZEB2, ZEB1
Cell motility	
Chemotaxis	FGF2, ITGB2, MAPK1 (ERK2), MYH10, MYH9, PLAUR (uPAR), PLD1, PRKCA, RAC2, TGFB1, VEGFA, WASF2, WIPF1,
Receptors	EGFR, IGF1R, ITGA4, ITGB1, ITGB2, ITGB3, MET, PLAUR (uPAR), RHO
Growth factors	CSF1 (MCSF), EGF, FGF2, HGF, IGF1, TGFB1, VEGFA
Cell–cell adhesion	DPP4, EGFR, EZR, ITGA4, ITGB1, ITGB2, MSN, MYH9, ROCK1, TGFB1,
Cell–matrix adhesion	ACTN1, ACTN3, CSF1 (MCSF), ILK, ITGB1, ITGB2, ITGB3, MMP14, PTEN, PTK2B, PXN, RASA1, RHOA,
Focal adhesion	ACTN1, ACTN3, ARHGEF7, BCAR1, CAPN1, CAPN2, CAV1, ENAH, ILK, ITGB1, MYL9, PTK2, PTK2B, PXN, TLN1, VASP, VCL

(continued on next page)

Table 6 (*continued*)

Hallmark feature	Marker genes
Proteolysis	AKT1, CAPN1, CAPN2, DPP4, FAP, HGF, MMP14, MMP2 (Gelatinase A), MMP9 (Gelatinase B), MYH9, PLAUR (uPAR), TIMP2
Filopodia	BAIAP2, CDC42, DIAPH1, EGFR, ENAH, EZR, MSN, RDX, SVIL, VASP,
Lamellopodia	CTTN, DPP4, EGFR, ENAH, FAP, PIK3CA (p110a), PLD1, PTK2, PXN, RDX, SVIL, VASP, VCL, WASF1, WASF2, WASL
Invasive projections	ACTR2, ACTR3, ARF6, CDC42, CFL1, CTTN, DPP4, EGF, EZR, FAP, MMP14, MMP2 (Gelatinase A), MMP9 (Gelatinase B), MSN, MYH9, PLAUR (uPAR), RAC2, RASA1, SH3PXD2A, SRC, SVIL, TGFB1, VEGFA, WASL, WIPF1
Rho family GTPases	
Rho signaling	ACTR2, ACTR3, ARHGDIA, LIMK1, MSN, MYL9, MYLK, PLCG1, PLD1, PRKCA, PTEN, PTPN1, RHO, RHOA, RHOB, RHOC, RND3, ROCK1, VIM,
Rac signaling	ACTR2, ACTR3, BAIAP2, CFL1, CRK, PAK1, PAK4, PLD1, PRKCA, RAC1, RAC2, STAT3, WASF1, WASF2, WASL,
Cdc42 signaling	ACTR2, ACTR3, CDC42, PFN1, WASF1, WASF2, WASL
Invasion	
Matrix metalloproteases	MMP10, MMP11, MMP13, MMP2, MMP3, MMP7, MMP9
Transcription factors, regulators, Other	CHD4, ETV4, EWSR1, HTATIP2, MTA1, MYC, MYCL, NR4A3, RB1, RORB, SMAD2, SMAD4, TCF20, TP53, CST7, CTSK, CTSL, CD82 (KAI1), KISS1, METAP2, NME4,
Genome instability [202,203]	
Cell cycle check point genes	ATM, ATR, ATRIP, BARD1, BRCA1, CDC25A, CHEK1, CHEK2 (RAD53), CSNK2A2, FANCD2, H2AFX, HUS1, MDC1, PARP1 (ADPRT1), RAD1, RAD17, RAD50, RAD9A, RBBP8, RNF168, RNF8, SMC1A, TOPBP1, TP53
Nucleotide excision repair	ATXN3, BRIP1, CCNH, CDK7, DDB1, DDB2, ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, ERCC6, ERCC8, LIG1, MMS19, PNKP, POLL, RAD23A, RAD23B, RPA1, RPA3, SLK, XAB2, XPA, XPC
Base excision repair	APEX1, APEX2, CCNO, LIG3, MPG, MUTYH, NEIL1, NEIL2, NEIL3, NTHL1, OGG1, PARP1, PARP2, PARP3, POLB, SMUG1, TDG, UNG, XRCC1
Mismatch repair	MLH1, MLH3, MSH2, MSH3, MSH4, MSH5, MSH6, PMS1, PMS2, POLD3, TREX1
Double strand break repair	BRCA1, BRCA2, DMC1, FEN1, LIG4, MRE11A, PRKDC, RAD21, RAD50, RAD51, RAD51C, RAD51B, RAD51D, RAD52, RAD54L, XRCC2, XRCC3, XRCC4, XRCC5, XRCC6
DNA replication checkpoint genes	BL1, CDC6, MCM2, MCM3, MCM4, MCM5, WEE1
Mitotic phase checkpoint genes	AURKB, CCNB2, CCNF, CDC25C, CDC6, CDK1 (CDC2), CDC16, CDC20, MRE11A, RAD51, STMN1
DNA repair	AIMP2, APEX1, CHEK1, MSH2, NBN, NPM1, PCNA, TERT, TP53, BRCA1, BTG2, EGFR, GADD45A, PCNA, PTTG1, BRCA2, MLH1, XRCC5
Histone acetylation	KAT5, NCOA1, NCOA3, KAT2B
Histone deacetylation	HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7
Chromatin modification molecules	ESR1, NCOA6, PPARGC1B, CBX3, NAP1L1
Other	ATM, ATR, ATRIP, ATRX, BARD1, BRIP1, CHEK2 (RAD53), CIB1, CRY1, EXO1, FANCA, FANCD2, FANCG, GADD45A, GADD45G, MGMT, RAD1, RAD17, RAD18, RAD21, RAD51B, RAD9A, RBBP8, RDC1, REV1, RNF168, RNF8, SMC1A, SUMO1, TOP2A, TOPBP1, XRCC3, XRCC6BP1
Deregulation of cellular energetics [227,228]	
Glycolysis	ALDOA, ALDOB, ALDOC, BPGM, ENO1, ENO2, ENO3, GALM, GCK, GPI, HK2, HK3, PFKL, PGAM2, PGK1, PGK2, PGM1, PGM2, PGM3, PKLR, TPI1
Gluconeogenesis	FBP1, FBP2, G6PC, G6PC3, PC, PCK1, PCK2
Regulation	PDK1, PDK2, PDK3, PDK4, PDP2, PDPR
TCA cycle	ACLY, ACO1, ACO2, CS, DLAT, DLD, DLST, FH, IDH1, IDH2, IDH3A, IDH3B, IDH3G, MDH1, MDH1B, MDH2, OGDH, PC, PCK1, PCK2, PDHA1, PDHB, SDHA, SDHB, SDHC, SDHD, SUCLA2, SUCLG1, SUCLG2
Pentose phosphate pathway	G6PD, H6PD, PGLS, PRPS1, PRPS1L1, PRPS2, RBKS, RPE, RPIA, TALDO1, TKT
Glycogen metabolism	GBE1, GYS1, GYS2, UGP2, AGL, PGM1, PGM2, PGM3, PYGL, PYGM, GSK3A, GSK3B, PHKA1, PHKB, PHKG1, PHKG2
Mitochondrial: Complex I	NDUFA1, NDUFA10, NDUFA11, NDUFA2, NDUFA3, NDUFA4, NDUFA5, NDUFA6, NDUFA8, NDUFAB1, NDUFB10, NDUFB2, NDUFB3, NDUFB4, NDUFB5, NDUFB6, NDUFB7, NDUFB8, NDUFB9, NDUFC1, NDUFC2, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS5, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, NDUFV3,
Mitochondrial: Complex II	SDHA, SDHB, SDHC, SDHD
Mitochondrial: Complex III	CYC1, UQCR11, UQCRC1, UQCRC2, UQCRFS1, UQCRH, UQCRQ
Mitochondrial: Complex IV	COX4I1, COX5A, COX5B, COX6A1, COX6A2, COX6B1, COX6C, COX7A2, COX7A2L, COX7B, COX8A
Mitochondrial: Complex V	ATP5A1, ATP5B, ATP5C1, ATP5F1, ATP5G1, ATP5G2, ATP5G3, ATP5H, ATP5I, ATP5J, ATP5J2, ATP5L, ATP5O, PPA1
Activator genes	ARRDC3, ASB1, CYB561D1, DNAJB1, EDN1, GADD45B, HSPA1A, HSPA1B, LRP5L, MitoH1, MitoH2_12106, MitoH2_14573, MitoH2_4162, MitoH2_5726, RNU11, SLC25A25

with an overall improved sensitivity and accuracy compared to hybridization platforms [244].

About 70% of the human genome transcribed to RNA will not serve as a template for protein translation. Among them the *long non-coding RNAs* (lncRNA), containing sequences of over 200 nucleotides in length, are involved in the regulation of transcriptional and post-transcriptional control of gene expression and epigenetic processes, such as chromatin remodeling and histone modification [245]. lncRNAs are deregulated in several human cancers and are frequently expressed in disease in a tissue-specific manner [246]. Serial analysis of gene expression (SAGE) and its subsequent modifications (LongSAGE and SuperSAGE) use short tags to count transcripts that later are mapped on assembled genome sequences to allow quantitative analysis and identification of new transcripts [247–249]. Cap analysis gene expression (CAGE) is based on the isolation and sequencing of short cDNA sequence tags derived from the 5′ terminus of mRNAs, thus identifying the location of transcriptional start points [250]. Functional lncRNAs have been discovered using ChIP-seq by focusing on histone modifications that are associated with active transcription [251]. RNA-seq provides the most powerful tool in lncRNA discovery and expression analysis and allows the detection of low abundance transcripts with low background noise [252]. RT-qPCR, northern blots and fluorescence *in situ* hybridizations can verify the presence of single candidate lncRNAs in different tissue and cell types identified from high-throughput data [253].

3. Outlook

Selecting the ideal analytic technique is based on the investigator's goals, available equipment and also on financial constraints. Multiplexing strategies enable a more in-depth data collection from a single experiment, yielding several values while controlling for variability, saving time and relative cost and leading to more robust conclusions.

High throughput screening (HTS) allows rapid compound analysis in low volume assay formats, such as in 384- or 1536-well plates and is gaining increasing support with the development of automated optical detection systems [254]. High content screening (HCS) is based on automated microscopy and image analysis and is able to measure multiple cell physiology characteristics in real time. HCS allows screening of enormously large libraries of molecules to discover new inhibitors of cellular physiology, thereby enhancing the drug discovery process.

Table 7
List of widely applicable assays suitable for the analysis of multiple hallmark features.

Assay	Hallmark	Hallmark feature
Flow cytometry	Sustaining proliferation	Membrane integrity, DNA synthesis
	Resisting cell death	Morphological alternations
		Genome fragmentation
		Plasma membrane integrity
		Mitochondrial membrane integrity and dysfunction
		Intracellular Ca++ concentration
		Caspase cascade detection
	Immortality	Telomere length
	Genome instability	DNA content changes
	Metabolism	Glucose uptake
Fluorescence microscopy	Resisting cell death	Genome fragmentation
		Plasma membrane integrity
		Mitochondrial membrane integrity and dysfunction
		Intracellular Ca++ concentration
		Caspase cascade detection
	Migration/invasion	Cell tracking, counting
	Genome instability	Karyotyping
	Metabolism	Glucose uptake
Multiwell reader (colorimetric, spectrometric, fluorimetric)	Sustaining proliferation	Cell viability (metabolic assay)
		Membrane integrity (LDH assay)
		Amino acid binding
	Resisting cell death	Plasma membrane integrity
		Mitochondrial membrane integrity and dysfunction
	Immortality/senescence	SA-β-gal reactivity at pH 6.0
	Metabolism	Glucose uptake/Glutathione uptake
		Glucose trace
		Rate limiting enzymes
		OCR, ECAR

HCS provides benefits over, or complements, existing methods to assess proliferation [255–257], migration [258,259] and metastases [260,261]. Cytometers based on automated fluorescence microscopy are sensitive enough to detect single cells based on the strength of a fluorescent signal [256]. Proliferation can be assessed by counting DAPI-labeled nuclei and BrdU-incorporating cells separately, then evaluating the proportion of cells in the S-phase based on the dual emission-images [258,262].

Viability and proliferation assays are relevant to evaluate additional hallmarks that involve the analysis of similar physiological processes within living cells. For example, the loss of membrane integrity is a signal of cell death, measured by dye exclusion or LDH release after a cytotoxic insult. Metabolic assays provide information not only about cell viability but also about the extent of overall metabolic change. Dyes incorporated into DNA, such as PI or BrdU, also help to gain insight into DNA content changes when evaluating genome instability.

Each and every hallmark possesses properties that can be analyzed by immunoblot, RT-PCR, immunochemistry, immunoprecipitation, RNA microarray or RNA-seq (see Table 6). In general, flow cytometry, fluorescence microscopy, and multiwell readers are also extremely versatile tools that with proper sample preparation, allow the detection of a vast number of hallmark features (summarized in Table 7).

In summary, here we have provided a link between available techniques and the recognized hallmarks of cancer. Although our list is not exhaustive, our aim is to include the most widely used methods. We encourage the reader to make an informed decision regarding assay selection to gain the maximum benefit from existing tools.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Cellular and Molecular Life Sciences

REVIEW

EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness

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Abstract Cancer is a complex multistep process involving genetic and epigenetic changes that eventually result in the activation of oncogenic pathways and/or inactivation of tumor suppressor signals. During cancer progression, cancer cells acquire a number of hallmarks that promote tumor growth and invasion. A crucial mechanism by which carcinoma cells enhance their invasive capacity is the dissolution of intercellular adhesions and the acquisition of a more motile mesenchymal phenotype as part of an epithelial-to-mesenchymal transition (EMT). Although many transcription factors can trigger it, the full molecular reprogramming occurring during an EMT is mainly orchestrated by three major groups of transcription factors: the ZEB, Snail and Twist families. Upregulated expression of these EMT-activating transcription factors (EMT-ATFs) promotes tumor invasiveness in cell lines and xenograft mice models and has been associated with poor clinical prognosis in human cancers. Evidence accumulated in the last few years indicates that EMT-ATFs also regulate an expanding set of cancer cell capabilities beyond tumor invasion. Thus, EMT-ATFs have been shown to cooperate in oncogenic transformation, regulate cancer cell stemness, override safeguard programs against cancer like apoptosis and senescence, determine resistance to chemotherapy and promote tumor angiogenesis. This article reviews the expanding portfolio of functions played by EMT-ATFs in cancer progression.

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Abbreviations

bHLH	Basic helix loop helix
CSC	Cancer stem cells
EMT	Epithelial-to-mesenchymal transition
EMT-ATF	EMT-activating transcription factors
HNSCC	Head and neck squamous cell carcinoma
MET	Mesenchymal-to-epithelial transition
NSCLC	Non-small cell lung carcinoma

Introduction

Cancer is a multipronged process that requires the acquisition by malignant cells of a number of capabilities—referred as hallmarks of cancer [1]—that ensure their survival and proliferation and, therefore, the growth of the tumor. Cancer cells in a solid tumor can detach from the main mass to invade the surrounding stroma, enter the circulation and eventually metastasize to distant organs. In most cases, invading cancer cells lose their polarity and intercellular adhesions and acquire a more motile phenotype as part of an epithelial-to-mesenchymal transition (EMT) [2–5]. While EMT was identified in the context of embryogenesis almost three decades ago, its underlying molecular mechanisms have only begun to be understood more recently with the discovery of its role in tumor invasiveness.

A hallmark of EMT is the functional loss of the adherens junction protein E-cadherin. Although transcriptional control of E-cadherin during EMT seems to be prevalent, recent studies point to the existence of intricate relationships between epigenetic, transcriptional and translational mechanisms. Although E-cadherin expression is inhibited by a number of transcription factors, only a small set are known to regulate it directly. The main groups of transcription factors that bind to the E-cadherin promoter and directly repress its transcription—which will be referred to hereafter as EMT-activating transcription factors, EMT-ATFs—are the ZEB (ZEB1, ZEB2) and Snail (Snail1, Snail2, Snail3) families of zinc finger proteins and the Twist family of bHLH factors (Twist1, Twist2) [2, 3]. While E12/E47 and Tbx3 also bind to the E-cadherin promoter [6, 7], the ability of Goosecoid and HMGA2 to repress E-cadherin expression and induce an EMT seems to be mediated by other EMT-ATFs [8, 9]. Most of these transcription factors were originally identified as regulators of embryogenesis and cell differentiation and only later recognized for their role in cancer progression. Still, the upstream regulatory signals and downstream targets of EMT-ATFs in cancer largely concur with those during embryogenesis [2, 3].

In addition to their now classical function as promoters of tumor invasiveness, over the last few years EMT-ATFs have gained new relevance in light of their role regulating several other hallmarks of cancer. EMT endows cells not only with greater ability to migrate but also with stem cell characteristics that play a role in tumorigenesis and resistance to chemotherapy. Recent evidence shows that EMT-ATFs also participate in early stages of cancer development cooperating in oncogenic transformation, overriding safeguard programs against cancer like apoptosis and senescence or promoting tumor angiogenesis. The expanding portfolio of functions of EMT-ATFs in cancer set them not only as important diagnostic and prognostic biomarkers but also as potential therapeutic targets. This article reviews the roles of EMT-ATFs in cancer and is organized as follows: The next section summarizes the EMT process in cancer. The following section reviews the main regulatory pathways and mechanisms of action of EMT-ATFs. Later sections describe the regulation of tumor invasiveness and metastasis by EMT-ATFs and overview their newly assigned roles in earlier stages of cancer progression. The final section concludes and presents EMT-ATFs as multifunctional regulators of the hallmarks of cancer.

EMT in cancer

E-cadherin mediates homotypic intercellular adhesion and interacts with intracellular proteins to establish and coordinate the morphology, polarity and function of epithelial cells [10, 11]. The downregulation of E-cadherin is inherent to EMT, but EMT also entails the downregulation of other epithelial specification genes like components of tight and gap junctions, desmosomes, cytokeratins, etc. [2, 3]. In parallel, there is an induction of mesenchymal markers (e.g., N-cadherin, cadherin-11), reorganization of the cytoskeleton (e.g., switch from cytokeratins to vimentin), and the synthesis of extracellular matrix components and metalloproteases [2, 3].

Many signals unleashing an EMT during embryogenesis are also active in cancer. Thus, stimuli such as TGFβ, FGF, EGF, IGF, HGF, PDGF, estrogens, Wnt, Shh, inflammatory cytokines or hypoxia as well as oncogenes like Ras^{V12}, ErbB2 or mutant p53 may be involved in EMT during cancer progression (reviewed in [3]). Triggering and maintenance of the mesenchymal state requires cooperation between several of these pathways through autocrine signaling loops [12].

These growth factors, inflammatory, hypoxic and oncogenic signals, along with an increasing number of microRNAs, converge in the induction of EMT-ATFs either at the transcriptional level or by increasing their protein or mRNA stability. E-cadherin expression and function could be downregulated by loss-of-function mutations, but modulation of EMT during embryogenesis and cancer progression mostly involves the participation of EMT-ATFs providing a high degree of functional redundancy and plasticity. E-cadherin has also been found to be silenced by promoter hypermethylation in a number of carcinomas. Some of the signals triggering EMT activate or repress the expression of non-coding microRNAs (miRs), which in turn modulate tumor invasion and metastasis by regulating EMT-ATFs transcripts (see below), targeting E-cadherin itself, or altering the expression of small GTPases or extracellular matrix receptors (reviewed in [13]).

Downregulation of E-cadherin is a critical initial step in EMT, not only because of the disruption of adherens junctions but also because loss of E-cadherin reinforces the EMT process by inducing the expression of Twist1 and ZEB1 in a feed-forward loop [194]. EMT-ATFs do not simply repress E-cadherin but are able to orchestrate the entire EMT program, inhibiting and activating a wide array of epithelial and mesenchymal genes, respectively [14–21].

Expression of EMT markers by primary human tumors correlates with enhanced invasiveness and poorer clinical prognosis. Cancer cells at the tumor invasive edge that have transitioned through an EMT secrete cytokines and proteases that promote angiogenesis, remodel the peritumoral extracellular matrix, and activate non-neoplastic stromal cells. In turn, stromal cells release factors that reinforce the EMT in cancer cells and foster survival, growth, and invasiveness of the tumor, thus creating a reciprocal influence between the tumor and its microenvironment [22, 23]. EMT is required for cancer cells to shift from a collective type of invasion—where cells retain E-cadherin and some intercellular adhesions—to an individual mesenchymal type of invasion, a change that depends on TGFβ signals at the tumor–host interface [24, 25]. While both models of invasion allow cancer cells to reach the lymphatic circulation, optimal hematogenous dissemination only occurs in EMT-mediated individual invasion [22, 25].

In addition to the mesenchymal switch, the molecular reprograming encompassed by the EMT also endows cancer cells with stem-like characteristics [12, 26]. Many of the signals controlling normal stem cell homeostasis are inducers of EMT and seem to contribute to the generation and maintenance of cancer stem cells (CSCs). In an influential article, Brabletz and colleagues [27] proposed a model of tumor progression where only cancer cells at the tumor–host interface, the "migrating CSCs", undergo EMT and acquire mesenchymal and stem-like characteristics and therefore migratory and self-renewal capacities. CSCs are thought be important in the genesis of primary tumors and metastasis and could also be at the root of tumoral chemoresistance and recurrence [28].

As in embryogenesis, the EMT occurring during cancer progression is a reversible process. At the site of micrometastasis, epigenetic changes and the absence of EMT-inducing signals in the microenvironment lead to the re-expression of particular sets of microRNAs and the downregulation of EMT-ATFs (see below), allowing cancer cells to regain the epithelial characteristics of the primary tumor, through a mesenchymal-to-epithelial transition (MET), and grow to form a secondary tumor [29, 30]. In fact, recent evidence also indicates that EMT and metastatic dissemination of cancer cells may in fact occur from very early in tumor progression [31].

Regulation and mechanisms of action of EMT-ATFs

ZEB1 and ZEB2

The ZEB family comprises zinc finger/homeodomain proteins that are highly conserved across species (see an excellent review in [32]). In higher organisms, the family is constituted by two members: ZEB1 and ZEB2. ZEB factors contain multiple independent domains to interact with other transcriptional regulators (Fig. 1) (reviewed in [32] and [33], see also [34–41]). ZEB proteins bind to CtBP corepressors that in turn recruit histone deacetylases and methyltransferases, polycomb, and coREST [37–43]. Transcriptional repression by ZEB1 is also mediated through recruitment of the histone acetyl transferase Tip60, the SWI/SNF chromatin remodeling ATPase BRG1 and the histone deacetylase SIRT1 [44–46]. Meantime, in addition to CtBP, ZEB2 interacts with the NuRD remodeling and deacetylase repressor complex [47]. On the other hand, ZEB factors can also activate transcription through binding to histone acetyltransferases p300/pCAF ([48–50], reviewed in [33]). ZEB1 and ZEB2 repressor activities are modulated by post-translational modifications. SUMOylation by Pc2 or acetylation by p300/pCAF disrupt ZEB factor binding to CtBP [48–51]. Although its transcriptional significance remains unclear, phosphorylation of ZEB1 varies widely among cell types [52].

ZEB1 and ZEB2 trigger an EMT by repression of epithelial markers and activation of mesenchymal ones ([14–16], [20] and reviewed in [33, 53, 54]). ZEB1 and ZEB2 could also regulate EMT through their repressor effect on miRs (see below). ZEB2 mRNA also functions as a competitive endogenous RNA (ceRNA) squelching miRs

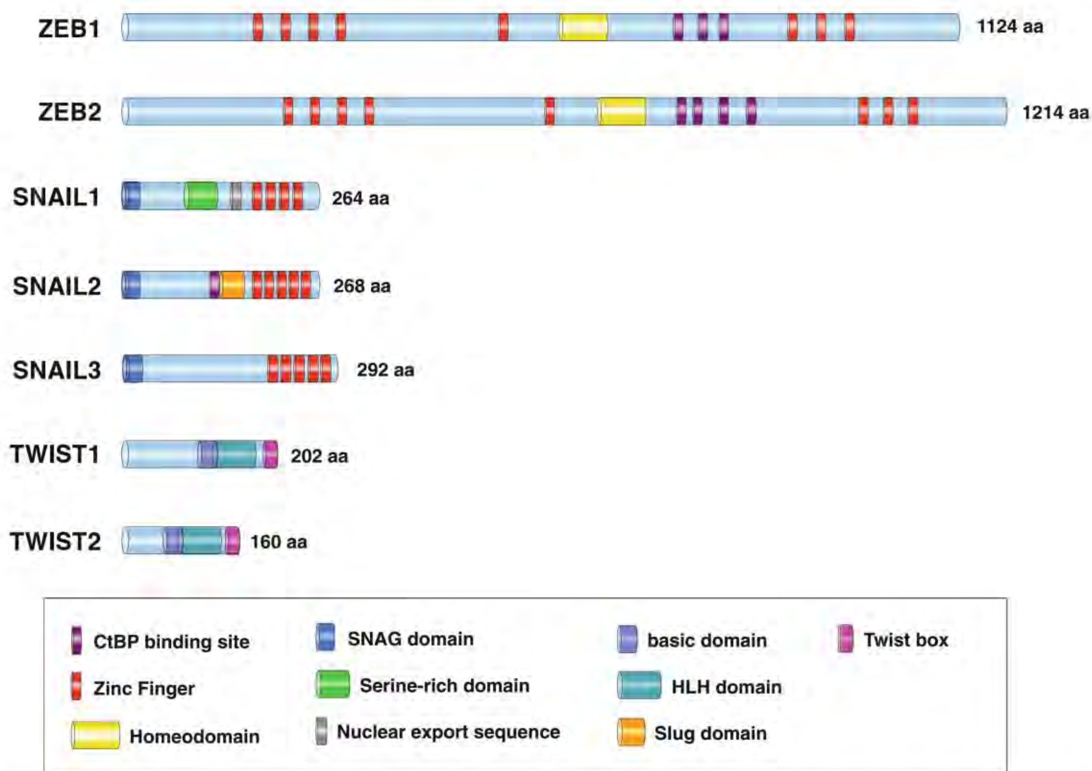

Fig. 1 Domain structure human EMT-ATFs: human ZEB1 (GenBank reference sequence AAA20602.1), human ZEB2 (AAI27103), human Snail1 (NP_005976.2), human Snail2 (NP_003059.1), human Snail3 (NP_840101.1), human Twist1 (NP_000465.1), and human Twist2 (NP_476527.1)

targeting other transcripts, thus activating their expression [55].

ZEB1 and ZEB2 have overlapping, but still distinct, patterns of expression that result from their regulation by classical signaling pathways and an expanding set of microRNAs (see excellent reviews in [53, 54, 311] and below). In most cell types, TGFβ factors induce both ZEB proteins that in turn modulate TGFβ signaling in opposing ways with ZEB1 synergizing with R-Smads and ZEB2 repressing their activity. In fact, ZEB factors mediate some TGFβ functions during development, cell differentiation, and proliferation [48, 49, 56–58]. ZEB1 and ZEB2 are induced by HIF-1α in hypoxic conditions, inflammatory cytokines, and by ligand-mediated activation (e.g., FGF, IGF-1, PDGFR) of receptor tyrosine kinases [59–66]. ZEB factors are directly activated by downstream signals frequently activated in tumors like Ras-ERK2-Fra1, NFκB, and JAK/STAT3 [61, 67, 68].

Aberrant activation of the canonical Wnt pathway by loss-of-function mutation of APC gene, as occurring in most colorectal carcinomas, induces ZEB1 [69]. ZEB1 expression is also induced by COX2/PGE2, which can activate Wnt signaling by inhibiting GSK3β [70]. The Notch pathway, deregulated in many cancers, also activates ZEB1 expression [71, 72]. In turn, ZEB1 enhances Notch activity by indirectly increasing Jag1 and its coactivators Maml2/Maml3 [72]. ZEB factors are also upregulated by growth and steroid hormones [73–75]. Hippo/YAP signaling and the tumor suppressor Rb/E2F pathway activate and repress ZEB1 transcription, respectively, without affecting ZEB2 levels [76, 77]. SIRT1 is recruited by ZEB1 to repress the E-cadherin promoter but SIRT1 itself also induces ZEB and Snail factors but not Twist [46]. Interestingly, SIRT1 and miR-200a—a microRNA that inhibits ZEB factors (see below)—are involved in a reciprocal negative feedback loop [78]. ZEB1 is repressed at the promoter level by Grainyhead-like 2, a transcription factor tightly co-regulated with E-cadherin [79]. Finally, ZEB1 and ZEB2 are downstream of Snail and Twist factors (see below).

In addition to transcriptional regulation by these upstream signals, ZEB factors expression is also controlled at the mRNA and protein level. They are regulated by a complex network of microRNAs (see below). Furthermore, YB-1, a protein associated with increased invasiveness in breast carcinomas, activates the mRNA of ZEB2 without affecting ZEB1 [80]. ZEB2 is also controlled at the protein level, and its binding to F-box FBXL14 targets it for ubiquitin-proteasome degradation [81].

Snail1, Snail2 and Snail3

The Snail family comprises three members: Snail1 (originally identified as Snail), Snail2 (Slug), and Snail3 (Smuc) (Fig. 1). All three contain a single C-terminal zinc finger cluster that bind to E-boxes in the regulatory regions of target genes. At the N-terminal end, they share a SNAG (Snail-Gfi1) domain that mediates binding to Sin3A/HDAC1/HDAC2 and Ajuba-PRMT5-PRC2 and LSD1-coREST complexes [82–85]. Only Snail2 maintains the central CtBP binding motif present in *Drosophila* that has also been implicated in recruitment of HDAC and coREST [86]. A recent report shows the recruitment of NCoR and CtBP1 to the SNAG and SLUG domains of Snail2, respectively, with the former being essential for Snail2-mediated EMT [87]. The precise mechanism by which Snail-mediated histone modifications (deacetylation, H3K27 and H4R3 methylation, H3K4 demethylation) cooperate to repress E-cadherin has not been fully elucidated. Snail1 also represses epithelial specification genes, including E-cadherin, by interacting with Smad3/Smad4 [88]. Overexpression of Snail1 decreases binding of AKT1 to the E-cadherin promoter in favor of AKT2 on which Snail1 depends for its repressor activity [89]. Conversely, activation of mesenchymal genes by Snail factors seems to be, at least in part, indirect, via E-cadherin downregulation.

Snail1 and Snail2 transcriptional activities are also modulated by post-transcriptional modifications that alter their protein stability and intracellular localization. Thus, binding of Snail1 to Sin3a is strengthened by PKA and CK2 phosphorylation [90]. In a similar manner, phosphorylation of Snail2 modulates its ability to transcriptionally repress E-cadherin [87]. Phosphorylation of Snail1 by GSK3β functionally inactivates it through both CRM1-dependent export to the cytoplasm and SCF/β-TrCP ubiquitin-mediated proteasome degradation [91]. Interaction of Snail1 with LOXL2 and LOXL3 prevents GSK3β-induced degradation [92], which is, however, fostered by phosphorylation by CK1ε [93]. Snail factors are also targeted for degradation in a GSK3β-independent manner by binding to FBXL14 [81, 94, 95] or, in the case of Snail2, through Mdm2 ubiquitination [222]. In addition, the intracellular localization of Snail factors is also controlled through import and export signals [96, 97]. In addition to GSK3β, phosphorylation of Snail1 by PKD1 also triggers its nuclear export [98]. On the other hand, phosphorylation by PAK1 promotes its nuclear localization, which is also dependent on the breast cancer-associated zinc transporter protein LIV1 [99, 100].

As with other EMT-ATFs, signals regulating Snail proteins in cancer parallel those operating during development. Snail factors are induced by TGFβ, Notch, TNFα, EGF, FGF, Wnt, Shh, SCF/c-kit, hypoxia, and estrogens [3]. Transcriptional activation of Snail1 by TGFβ requires interaction of R-Smads with HMGA2 [101]. In some cancer cell types, induction of Snail1 by TGFβ requires simultaneous oncogenic Ras signaling [102]. On its part, TGFβ-mediated induction of Snail2 depends on the downregulation of KLF4 and FOXA1 that, interestingly, form a double repressor loop with Snail2 [103]. Notch signaling induces Snail1 through direct and indirect mechanisms. In addition to direct activation of the Snail1 promoter, Notch stabilizes Snail1 through HIF-1α-mediated activation of LOX [104]. NFκB signaling by AKT, hypoxia or inflammatory cytokines transcriptionally activates Snail1. Induction of NFκB by inflammatory cytokines also stabilizes Snail1 by inhibiting GSK-3β-mediated ubiquitination [105]. Engagement of receptor tyrosine kinases signaling and COX2/PEG2 also induces expression of Snail1 by inhibition of GSK3β [70]. Snail1 is also able to repress its own transcription [106]. Finally, Snail1 is activated at the translational level by YB-1 [80].

Twist1 and Twist2

Twist1 and Twist2 share a basic/helix-loop-helix (bHLH) domain that mediates their binding to DNA and homo/hetero-dimerization (Fig. 1). At the C-terminal end, there is a "Twist box" that has been implicated in both transcriptional activation and repression (reviewed in [107] and [312]). Regulation of gene expression by Twist factors depends on their binding to other transcriptional regulators, post-translational modifications, and choice of partner for dimerization. Binding of Twist proteins to E-boxes in the promoters of target genes could either activate (e.g., N-cadherin, AKT2 or Gli1) or repress (e.g., E-cadherin) transcription [108, 109]. In some cases, Twist-mediated repression involves binding to other transcription factors and cofactors to inhibit their activity (e.g., Runx2, myogenic bHLH, NFκB, and p300/pCAF). Direct repression of E-cadherin by Twist1 entails recruitment of multiple chromatin remodeling complexes, whose composition and dynamics is currently being uncovered. Twist1 activates transcription of the PRC1 component Bmi1 and binds to polycomb repressor complexes PRC1 and PRC2 at the E-cadherin promoter [310]. Twist1 interaction with components of the NuRD complex is also required for E-cadherin repression [110], a result in line with the finding that recruitment of PRC2 is specified by NuRD histone deacetylation [111]. In addition, it was recently reported that binding of Twist1 to the H4K20 methyltransferase SET8 could simultaneously contribute to repression of E-cadherin and activation of N-cadherin [112].

Gene regulation by Twist factors is also modulated by control of their intracellular localization and the identity of

their dimerization partner. Nuclear/cytoplasm shuttling of Twist factors is modulated by integrin-mediated adhesion to the extracellular matrix, post-translational modifications, and partner dimerization [108]. In *Drosophila*, Twist homodimers activate transcription whereas heterodimers with Daughterless function as repressors. However, in mammalian systems, Twist/E12 heterodimers can both activate and repress transcription [107, 312]. Homo- or heterodimerization of Twist proteins is determined by availability of E12. Likewise, Twist can titrate away E12 from other bHLH proteins thus inhibiting their function. Phosphorylation of the bHLH domain of Twist alters not only dimerization partner choice but also binding affinity for DNA [107].

Understanding the signaling pathways upstream of Twist1 and Twist2 is not as complete as for ZEB and Snail factors. Still, Twist factors are upregulated by classical EMT-inducing pathways during development, inflammation, and cancer, such as TGFβ, Wnt, hypoxia, and ligand-binding activation of receptor tyrosine kinases and inflammatory cytokines receptors [107, 312]. EGF and IL6 induce Twist1 via activation of JAK/STAT signaling and direct binding of STAT3 to the Twist1 promoter [113, 114]. Twist1 and Twist2 form a negative loop with inflammatory cytokines, as Twist factors are transcriptionally induced by NFκB and in turn bind to the TNFα and IL1β promoters blocking NFκB transcriptional activity [115]. As discussed below, upregulation of Twist1 by HIF-1α during hypoxic conditions has significant implications in tumor invasion and angiogenesis [116].

Twist factors are also regulated at the mRNA and protein level as well by their intracellular localization. As with other EMT-ATFs, YB-1 fosters cap-independent translation of Twist1 [80]. Twist1 is also controlled post-translationally by cytoplasmic polyadenylation sites in its 3'UTR [117]. Heterodimerization of Twist1 with E12 and Hand2, fostered by phosphorylation by PKA, stabilizes Twist1 protein that is, on the other hand, targeted for degradation upon binding to FXBL14 [81, 118].

Regulatory networks between EMT-ATFs and microRNAs

In recent years, a plethora of publications have identified miRs regulating each and every step during cancer progression, from cell proliferation, cancer cell stemness, and apoptosis to angiogenesis or tumor invasiveness (reviewed in [13]). miRs regulate invasiveness and metastasis by targeting the transcripts of a large number of genes involved in EMT/MET regulation, including those of EMT-ATFs (Fig. 2).

Members of the miR-200 family (miR-200a/b/c, miR-141, and miR-429) maintain an epithelial status and prevent EMT through inhibition of ZEB1 and ZEB2 (reviewed in [54], see also [119–121], and other references in [54]). In turn, miR-200 members are transcriptionally repressed by ZEB factors—as well as Snail1—thus forming a double-negative loop that maintain cells in either an epithelial or mesenchymal state [54, 122–124]. Expression of miR-200 and ZEB factors presents an inverse pattern in a number of human cancers. Invading mesenchymal-like cancer cells that extravasate and metastasize eventually need to revert to an epithelial phenotype for the metastatic colony to grow into a secondary tumor. Cancer cells that form macroscopic metastasis have higher levels of miR-200 compared to those that invade but are not able to colonize and, paradoxically to their roles as EMT repressors: overexpression of miR-200 in mouse breast cancer isogenic cell lines fosters lung and liver mestatasis by inhibition of Sec23a, a component of the tumor secretome that blocks metastatic colonization [125–313]. The 5′CpG islands of miR-200 loci are also subjected to dynamic epigenetic regulation [126]. In epithelial cell lines, miR-200 expression is silenced by hypermethylation during TGFβ-induced EMT but reverted by demethylation in MET. Likewise, 5′CpG islands of miR-200 loci are hypermethylated in cancerous cells of primary colorectal carcinomas but remain unmethylated in epithelial cells of the normal colonic mucosa.

miR-200 members mediate the anti-EMT activities of other factors and signaling pathways. For instance, Six1 induces ZEB1 and EMT through transcriptional repression of miR-200 [127]. By contrast, the tumor suppressor p53 inhibits a mesenchymal and stem cell phenotype and/or the invasive capacity of mammary and pancreatic epithelial cells and hepatocarcinoma cell lines by upregulating miR-200—as well as miR-192—to repress ZEB1 and ZEB2 [128–130]. Conversely, oncogenic mutant forms of p53 decrease miR-200 and increase ZEB1 expression [128].

ZEB1 and ZEB2 participate in other miR regulatory networks. ZEB factors repress miR-183 and miR203, which together with miR-200, inhibit the expression of stemness factors Bmi1, Sox2 and KLF4 [131, 132]. ZEB1 and ZEB2 are also targets of miR-205 [13]. In aggressive basal-like breast carcinomas, but not the luminal type, Fra-1 activates miR-221 and miR-222 [133], miRs that have been associated with EMT and tumor invasiveness and are downregulated by EGFR inhibitors. miR-221 and miR-222 downregulate TRPS1, a GATA-like repressor, that in turn inhibits ZEB2 [133]. In contrast, in endothelial cells, ZEB2 is a direct target of miR-221 [134], confirming evidence elsewhere that miR may have different, even opposing, functions depending on the cell type.

Contrary to its inhibitory effect on E-cadherin in breast epithelial cells [135], miR-9 downregulates Snail1 expression in melanoma cells by targeting NFκB, thus

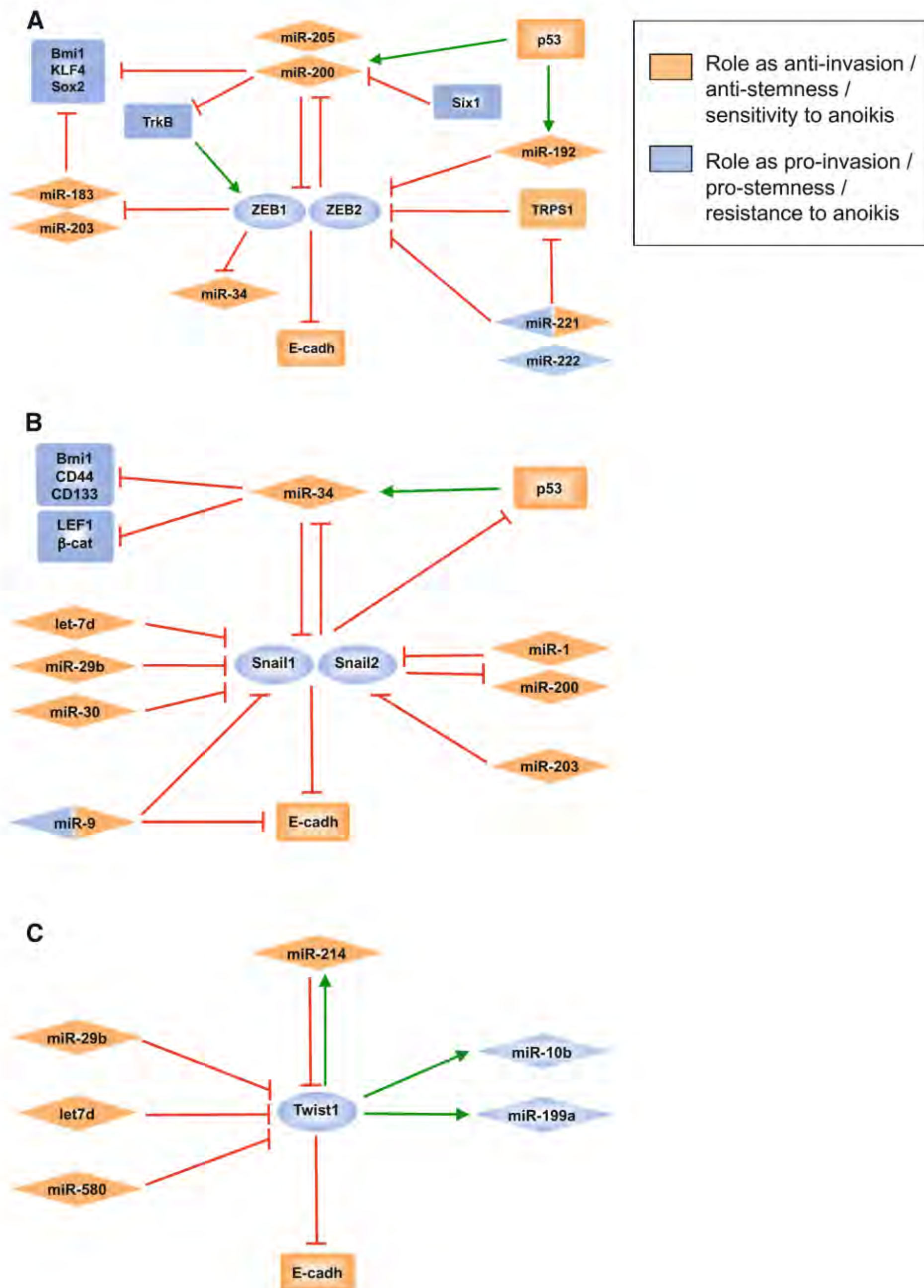

Fig. 2 Regulatory networks between microRNAs and EMT-ATFs of the ZEB (**a**), Snail (**b**) and Twist (**c**) families. For simplification, some relationships have been consolidated or are not shown. *Double color* identification refers to miRs that have been described as having both pro- and anti-invasion/metastatic roles depending on the context and/or cell system analyzed. See text for discussion and references

inhibiting proliferation and invasiveness [136]. p53 also downregulates Snail1 and Snail2 via induction of miR-34a/b/c. Conversely, Snail1 and Snail2 (and ZEB1) transcriptionally repress miR-34a/b/c, thus forming a double-negative loop similar to the one between ZEB factors and miR-200 [137, 138]. As with miR-200, miR34a inhibits stem cell-related factors Bmi1, CD44, and CD133 [137]. Members of the miR-30 family also target Snail1 [139, 140] and, as with miR-200, overexpression of either miR-30 and miR-34 prevents TGFβ-induced EMT. It is worth noting here that miR-34 also targets several Wnt signaling genes like Wnt1, Wnt3, LEF1 and β-catenin, suggesting that loss of p53 tumor suppressor activity could be accompanied by activation of β-catenin/TCF signaling,

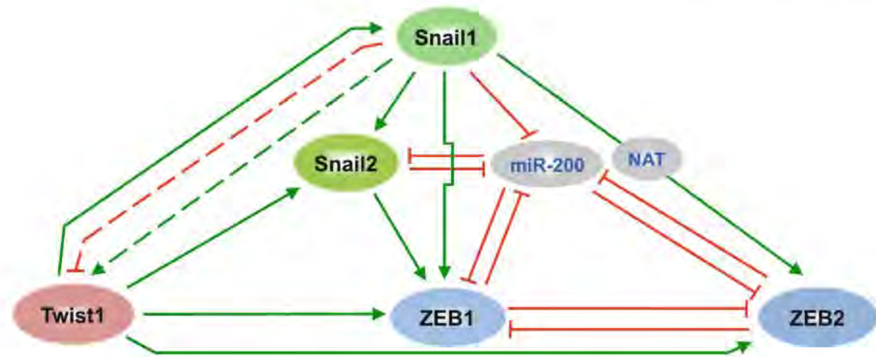

Fig. 3 Cross-regulation among EMT-ATFs. *Dashed lines* indicate that different relationships have been reported depending on the cell system analyzed. See text for discussion and references

which may reinforce EMT and stemness programs [141]. Snail1 is also repressed by let-7d and miR-29b, microRNAs that block EMT and invasiveness in HNSCC and prostate carcinomas, respectively [142, 143].

Snail2 participates in yet another double-negative loop with miRs: miR-1 and miR-200 inhibit Snail2 expression and are repressed by binding of Snail2 to their promoters [144]. Snail2 transcript is also inhibited by miR-203, which is upregulated in primary tumors and hypermethylated and silenced in metastatic cell lines [145].

To date, fewer miRs have been linked to Twist factors. Overexpression of let-7d inhibits Twist1 expression in oral squamous cell carcinomas [142]. Twist1 is also inhibited by miR-29b, miR-580, and miR-214, but it is not clear whether downregulation of Twist1 by miR-29b is mediated by inhibition of Snail1 [117, 143, 146]. In turn, Twist1 activates the expression of miR-10b, miR-199a, and miR-214 genes through direct binding to E-boxes in their promoters [147, 148]. miR-10b is expressed in metastatic breast cancer cells and induces cell motility and migration by indirectly inducing RhoC.

The emergence of all these new regulatory loops between miRs and EMT-ATFs provides evidence of dynamic epigenetic silencing of miR-200 (and likely other miRs) during cancer progression, which endows cancer cells with enhanced plasticity to reversibly switch between EMT and MET during tumor invasiveness and metastasis.

Cross-regulation among EMT-ATFs

Previous sections have highlighted the existence of significant overlap among EMT-ATFs in their regulatory signals, target genes, and mechanisms of action, raising the question of what is the specific contribution of each factor in the regulation of EMT and cancer. Recent evidence indicates that ZEB factors are downstream of the Snail and Twist families in the EMT interactome [149] (Fig. 3). The hierarchical Snail–Twist/ZEB relationship supports earlier evidence of the strongest correlation of ZEB factors, especially ZEB1, with E-cadherin loss and EMT across cancer cell types [150].

Snail1 increases ZEB1 protein levels through both transcriptional and post-transcriptional mechanisms [57, 151, 152] (Fig. 3). Snail1 also enhances the stability of Twist1 and triggers nuclear translocation of Ets1, both directly activating the ZEB1 promoter. Additionally, Snail1 not only represses miR-200, thus derepressing ZEB1 mRNA, but also stabilizes ZEB1 protein through still undefined mechanisms. Snail2 also activates ZEB1 by direct binding to its promoter [308].

ZEB2 expression is also regulated by Snail1 through several mechanisms. Repression of miR-200 by Snail1 upregulates ZEB2 mRNA (Fig. 3). In addition, Snail1 induces a natural antisense transcript (NAT) that overlaps with an internal ribosomal entry site in the 5′ UTR of ZEB2, preventing its splicing and increasing ZEB2 [153].

Twist1 mediates some of its EMT-activating effects through Snail2 by binding to its promoter and activating its transcription [154]. Expression of Twist1 and Snail1 are also mutually dependent although in different directions, depending on the cell system. Knock-down of Snail1 downregulates Twist1 protein and mRNA, while knock-down of Twist1 interferes with TGFβ-mediated induction of Snail1 [152]. Alternatively, it has also been reported that Snail1 transcriptionally represses Twist1, which only increases as Snail1 declines in later stages of EMT [155].

Microarray gene expression signatures of human mammary epithelial cells (HMEC) driven towards EMT have confirmed this hierarchical/network relationship among EMT-ATFs. HMEC that have been either treated with TGFβ or subjected to overexpression of Snail1, Twist1, or Goosecoid, or knock-down for E-cadherin, display an overlapping gene signature characterized by the induction of ZEB1, ZEB2, and FOXC2 [149]. In any case, several of the signaling pathways (e.g., TGFβ, Wnt, Notch, TNFα/IL1β, receptor tyrosine kinase, etc.) inducing Snail and Twist factors also activate ZEB1 and ZEB2 expression directly. There is also considerable cross-talk among many of the upstream signals regulating all three EMT-ATF families. For instance, besides regulation of ZEB factors via TGFβ/Snail1-mediated inhibition of miR-200 [152],

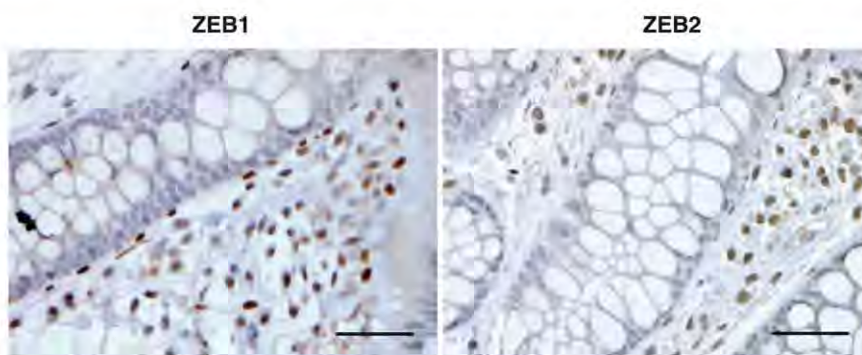

Fig. 4 ZEB1 and ZEB2 are expressed in stromal cells of normal colonic mucosa. Immunohistochemistry of colon samples from normal individuals stained by the 3,3′-diaminobenzidine (DAB) method with ZEB1 (H-102; Santa Cruz Biotechnology) and ZEB2 (H-260; Santa Cruz Biotechnology) as described in [69]. *Scale bars* 25 μm

ZEB1 induction by Notch involves NFκB [71] and growth hormone induces ZEB2 via NAT [75]. TrkB induces EMT through a ZEB1-dependent mechanism that requires the cooperation of Snail1 and Twist1 [63].

Expression and functional redundancy among EMT-ATFs suggests that their specificity could occur at the spatial and temporal level, with Snail1 being required to initiate the EMT and, subsequently, Twist and ZEB factors to consolidate it [57, 152, 155].

EMT-ATFs in EMT and tumor invasiveness

ZEB1 and ZEB2

Expression of ZEB factors drives an EMT by repressing and activating epithelial and mesenchymal specification genes, respectively (reviewed in [33, 53, 54]). ZEB1 and ZEB2 both bind to E-box sequences in the E-cadherin promoter but recruit different set of corepresors: CtBP and SWI/SNF in the case of ZEB1, and CtBP and NuRD by ZEB2. ZEB proteins bind and repress the promoters of other epithelial markers such as P- and R-cadherins, cell polarity markers (Crumbs3, Pals1-associated tight junction protein, lethal giant larvae homologue 2), components of tight junctions (occludin, claudin 7, junctional adhesion molecule 1, zonula occludens protein 3), gap junctions (connexins 26 and 31), and desmosomes (desmoplakin, plakophilin 3) (reviewed in [53] and see also [14–16, 20] Conversely, ZEB proteins activate mesenchymal markers such as vimentin and N-cadherin. Although the mechanism of ZEB1- and ZEB2-mediated induction of mesenchymal genes has not been fully elucidated, in some cases (e.g., vimentin) it involves direct binding of ZEB proteins to the promoter regions of mesenchymal genes and transcriptional activation [156, 157]. ZEB1 and ZEB2 also repress epithelial splicing regulatory proteins-1 and -2 (ESRP-1 and -2) that coordinate the epithelial pattern of alternative splicing of FGFR2 and whose overexpression inhibits EMT [158].

Breakdown of the basement membrane, that separates the epithelial compartment from the surrounding stroma, is considered a key step in the progression from in situ to invasive carcinoma. Interestingly, ZEB1 regulates the expression of several components of the epithelial basement membrane (e.g., α3 chain of laminin 5, α2 chain of collagen IV, γ2 chain of laminin 5), although in a tissue-specific manner [69, 159, 160]. In colorectal carcinomas, the γ2 chain of laminin 5 coexpresses with MT1-MMP in cancer cells at the tumor invasive edge and cleavage of the former by the latter promotes cancer cell migration [69, 161].

As an inhibitor of the epithelial phenotype, ZEB1 is not expressed in normal epithelium, although it is found in isolated fibroblasts and immune cells in the interstitial stroma (Fig. 4). ZEB1 expression is also absent at the center of relatively well-differentiated carcinomas expressing E-cadherin [29, 159]. However, ZEB1 is highly expressed in invading dedifferentiated cancer cells of many tumors including colorectal, breast, liver, endometrial, lung, prostate, and pancreatic carcinomas (Table 1). Like ZEB1, ZEB2 is expressed by stroma cells in epithelial tissues (Fig. 4), but, interestingly, is also detected in normal E-cadherin-positive epithelial cells in several organs [162]. Nevertheless, upregulated expression of ZEB2 at the invasive front has been reported in most carcinomas expressing ZEB1, such as colorectal, breast, gastric, bladder, liver, and pancreatic (Table 1). In most of these tumors, there is also an increase in the number of ZEB1- and ZEB2-positive cells (fibroblasts, macrophages, and endothelial cells) in the peritumoral stroma—often at higher levels than in invading cancer cells—representing both tumoral cells that have undergone an EMT and activated stromal cells (Table 1; e.g., [16, 31, 159, 163–166]). It has been postulated that ZEB-dependent paracrine signaling from the stroma could cooperate in E-cadherin repression in other parts of the tumor [163].

Expression of ZEB proteins by both cancer and stromal cells at the invasive front of carcinomas translates into increased tumor invasiveness: ZEB proteins promote

Table 1 Upregulated expression of EMT-ATFs in selected human cancers

EMT-ATF	Human cancer	Reference
ZEB1	Bladder	[164, 284–286]
	Breast	[165, 287]
	Digestive tract (colorectal)	[16, 69, 159]
	Digestive tract (esophagus squamous cell carcinoma)	[288]
	Digestive tract (gastric)	[171][a]
	HNSCC	[289]
	Hepatocarcinoma	[174][a]
	NSCLC	[70, 163]
	Pancreas	[131], [176][a], [273]
	Prostate	[65]
	Uterus (leiomyosarcoma, endometrial adenocarcinoma)	[168, 170]
ZEB2	Bladder	[164][a], [284, 286]
	Breast	[169]
	Digestive tract (colorectal)	[173][a]
	Digestive tract (gastric)	[175][a], [290]
	HNSCC	[291][a], [292]
	Hepatocarcinoma	[166][a], [293]
	Ovarian	[169, 172][a]
	Pancreas	[176][a], [294]
Snail1	Breast	[165], [169][a], [182, 192, 295, 296]
	Digestive tract (colorectal)	[181][a], [184, 297]
	Digestive tract (gastric)	[290]
	HNSCC	[292, 298]
	Hepatocarcinoma	[185], [187, 191][a]
	NSCLC	[254][a]
	Ovarian	[169, 189][a]
	Pancreas	[299]
Snail2	Breast	[182, 192]
	Digestive tract (colorectal)	[189]
	Digestive tract (esophagus)	[188][a]
	Digestive tract (gastric)	[300]
	HNSCC	[292]
	NSCLC	[253][a]
	Ovarian	[169]
	Pancreas	[299]
Snail3	Lung	[301]
	Melanoma	[301]
Twistl	Bladder	[201]
	Breast	[19], [165][a], [192, 248, 255]
	Digestive tract (colorectal)	[248, 302][a]
	Digestive tract (esophagus squamous cell carcinoma)	[200, 207][a], [248]
	Digestive tract (gastric)	[206, 290]
	HNSCC	[199, 292]
	Hepatocarcinoma	[191, 205][a], [303]
	Ovarian	[203, 204][a]
	Pancreas	[304]
	Prostate	[282, 309]
Twist2	Breast	[202]
	Digestive tract (esophagus)	[248]
	HNSCC	[305][a]
	Uterus (cervical intraepithelial neoplasia, cervical squamous cell carcinoma)	[306]

Only some representative publications are included

[a] Studies demonstrating prognostic value of EMT-ATFs expression (correlation with metastasis, recurrence and/or survival)

metastasis in xenograft models [131, 167] and associate with increased aggressiveness and higher metastatic capacity in a wide range of primary human carcinomas (e.g., [65, 70, 131, 164, 168–171]). ZEB factors have been shown to have an independent prognostic value for nodal dissemination, metastasis, response to treatment, and/or survival in multiple carcinomas, inter alia, of the ovary, bladder, colorectal, gastric, pancreas, and hepatocarcinoma [164, 166, 169, 171–176] (see representative entries with superscript letter in Table 1).

Snail1, Snail2 and Snail3

Independently of their inductive effects on ZEB1 and ZEB2, Snail factors also directly regulate epithelial and mesenchymal markers ([21, 177, 178] and references therein). In fact, the emerging consensus in the literature points to Snail1 as the factor responsible for the initiation of EMT in response to inducing signals [152, 155]. In addition to direct binding to the E-cadherin promoter and inhibition of its transcription, Snail factors repress other epithelial markers independently of their effect on E-cadherin—e.g., desmoplakin, adherens junction (claudin-1, -3, -4, -7), tight junctions (occludins), cytokeratins, and Mucin-1. On the other hand, Snail factors activate the expression of mesenchymal-like and pro-invasive genes (e.g., vimentin, fibronectin MMP1, MMP2, MMP7, MT1-MMP) that promote cell migration. While the epithelial and mesenchymal genes controlled by Snail1 and Snail2 overlap to a great extent, there is also evidence of some level of differential regulation in the degree of induction or repression and even in its direction [21]. For some genes, regulation requires cooperation between Snail1 and Snail2 or between Snail1 and Twist1: for example, Snail1 and Snail2 cooperate to transcriptionally repress the vitamin D receptor by which engagement by vitamin D3 promotes cell differentiation which is associated with a good prognosis in colorectal carcinomas [179, 180].

Snail factors are not present in normal epithelial cells but their expression is evidenced, usually higher for Snail1,

in cancer cells at the invasive front of carcinomas of the breast, digestive tract, liver, pancreas, ovary ,and lung among others (Table 1). Characterization of Snail3 lags behind, but ESTs have been identified in melanomas and lung carcinomas. In addition to the tumor front, Snail1 is found at areas of inflammation in colorectal carcinomas [181]. Tumor progression correlates with increasing nuclear expression of Snail1, but Snail1 can also be aberrantly detected in the cytoplasm of cancer cells of several carcinomas. Additionally, strong Snail1 staining is found—often at a higher percentage than in tumor cells, when not even exclusively—among fibroblast-like cells, macrophages, and endothelial cells at the peritumoral stroma [181–184].

Expression of Snail factors correlates with malignancy and less differentiated tumors, lymph node invasion, and metastasis, and Snail1, but not always Snail2, is considered an independent prognostic factor of worst evolution and poorer survival in a large number of carcinomas [182, 185–189] (Table 1). Snail1 promotes the recurrence of Her2/neu-induced primary breast tumors in mice, and recurrent human carcinomas tend to display mesenchymal-like characteristics [186]. Thus, Snail1 is spontaneously induced in recurrent breast carcinomas and high levels of Snail1 are an independent predictor for decreased relapse-free survival in breast cancer patients. At least in head and neck carcinomas, only Snail1 nuclear staining, but not cytoplasmic, is associated with a worse clinical outcome [190]. Interestingly, reactivity for Snail1 in stromal but not in cancer cells correlates with distant metastasis and lower survival in early stage colorectal carcinomas [181].

Up to now, evidence for differential roles of Snail1 and Snail2 in cancer progression is scarce. In hepatocarcinomas, only Snail1 but not Snail2 correlates with E-cadherin loss [191], whereas another study reported that Snail2 but not Snail1 is associated with a poorer outcome in breast carcinoma patients [192].

Twist1 and Twist2

Twist factors induce EMT directly and indirectly through their effect on other EMT-ATFs. Thus, Twist1 has been shown to repress E-cadherin by binding to its promoter [19], but also through induction of Snail1 [193] or Snail2 [154]. In HMEC, Twist1 overexpression yields a similar gene signature than Snail1 with repression of E-cadherin and other epithelial specification genes and induction of mesenchymal markers [149]. Several array studies found that overexpression of Twist1 also upregulates the expression of cytoskeletal and extracellular matrix genes involved in cell motility. However, the extent to which induction of mesenchymal genes by Twist is dependent on E-cadherin repression is less clear [194]. Twist1 induces N-cadherin via directly driving its transcription [108] and/or through post-transcriptional mechanisms [195]. Nevertheless, in glioblastoma cells, Twist1 promotes expression of mesenchymal markers without eliciting an E-cadherin/N-cadherin switch [196]. Likewise, Twist1 induces fibronectin in gastric and ovarian cancer cells [195] but not in breast cancer cells [109]. In ovarian carcinoma cells, Twist1 also activates MMP2 and MT1-MMP, and its knock-down reduces cell adhesion to extracellular matrix components in parallel with a decline in several adhesion molecules (e.g., β1 integrin, CD44) [197].

A role for Twist1 in cancer was first evidenced by its ability to promote greater invasiveness and metastasis in xenograft models [19]. Like other EMT-ATFs, Twist1 and Twist2 are absent in normal epithelium but are induced in a number of human carcinomas, including those of the digestive tract, breast, liver, prostate, endometrium, and ovary (Table 1). Interestingly, the Twist1 gene has also been found to be deleted or amplified in some osteosarcomas [198]. Again, mirroring ZEB and Snail factors, Twist1 and Twist2 are not only upregulated among cancer cells at the invasive front of carcinomas but also in stromal cells. Likewise, upregulation of Twist factors is not limited to the nuclei of cancer cells but, in many cases, also in their cytoplasm (e.g., [199–202]).

Twist factors are not only upregulated in human cancers but their reactivity increases during tumor progression from being mostly negative in benign neoplasias to be highly overexpressed in carcinomas of a wide range of tissue origins (Table 1). Knock-down of Twist1 in breast cancer cells inhibits their ability to metastasize in xenograft models but not the formation of primary tumors [19]. Twist factors also correlate with higher tumor grade, invasiveness, and metastasis, being independent prognostic factors for enhanced tumor aggressiveness, tumor recurrence, and poorer patient survival (e.g., [155, 191, 197, 199, 203–207]). Interestingly, in hepatocarcinomas, expression of either Twist1 or Snail1 is associated with shorter survival, and their simultaneous presence has an additive negative effect, suggesting that they somehow play distinct but collaborative roles in cancer progression [191]. In that line, another study in hepatocarcinomas found that Twist1 associates with increased tumor angiogenesis and metastasis but not with downregulation of E-cadherin [205]. Differential expression of Twist and Snail factors in some studies suggests a division of labor among EMT-ATFs. Breast carcinomas with lymph node involvement and poor clinical outcome display high levels of Twist1 and Snail2 while, contrary to other reports, expression of Snail1 declined [192].

Other transcription factors inducing EMT and tumor invasiveness

Like the ZEB, Snail, and Twist factors, E12/E47 binds directly to E-cadherin promoter [6] and induces an EMT by regulating an overlapping—but still distinct—set of epithelial and mesenchymal markers compared to Snail1 and Snail2 [21]. E12/E47 complexes not only promote tumor invasiveness and growth but injection of Madin–Darby Canine Kidney (MDCK) cells overexpressing E47 into nude mice generates tumors with higher invasiveness, growth, and vascularization than those formed by MDCK cells overexpressing Snail1 [177]. T-box transcription factor Tbx3 also binds directly to the E-cadherin promoter and inversely correlates with E-cadherin in human melanomas [7].

The homeobox factor Goosecoid also induces a full EMT by repressing epithelial markers (including E-cadherin) and activating mesenchymal genes [8]. Goosecoid is induced by TGFβ in adult breast epithelial cells and, compared to normal breast tissue, is significantly overexpressed in atypical ductal hyperplasia and ductal breast carcinomas [8].

FOXC2 also induces a mesenchymal phenotype but, departing from the rest of EMT-inducing factors reviewed here, FOXC2 does not alter E-cadherin mRNA levels but rather delocalizes E-cadherin protein from the plasma membrane to the cytoplasm [208]. FOXC2 is induced by TGFβ, and in response to overexpression of Snai1, Twist1, and Goosecoid, and has been suggested to mediate activation of mesenchymal genes by these other EMT-ATFs. FOXC2 is not expressed in normal epithelium but is overexpressed in basal-like breast and esophageal squamous cell carcinomas where it correlates with adverse prognosis [208, 209].

Other roles of EMT-ATFs: beyond EMT and tumor invasiveness

In addition to promoting tumor invasiveness, over the last few years a number of works have demonstrated that EMT-ATFs are also critical during earlier stages of cancer development, collaborating with oncogenes in malignant transformation, inducing tumor formation, contributing to bypass failsafe programs against cancer, and determining resistance to chemotherapy. While these additional cancer hallmarks are not necessarily dependent on EMT, they are jointly regulated with the EMT by EMT-ATFs. While many of these functions are interconnected, they will be addressed here in turn.

EMT, cancer stem cells, and tumorigenesis

The great level of heterogeneity displayed by most human tumors led to the formulation in the 1990s of a hierarchical model of cancer initiation where a small subpopulation of cells in a tumor—referred to as cancer stem cells (CSCs)—retain the capacity for self-renewal and tumor initiation (tumorigenesis) (reviewed in [28]). CSCs have been identified in a number of human cancers including colon, breast, pancreas, ovary, prostate, and brain tumors.

As indicated earlier, Brabletz and coworkers [27] postulated that invading carcinoma cells that have undergone an EMT could function as "migrating CSCs". Later studies by the Weinberg group and others confirmed that forcing an EMT in non-tumorigenic, immortalized HMECs—by treatment with TGFβ or overexpression of EMT-ATFs—concurrently confers on cells a phenotype similar to breast CSCs (CD44^{high}/CD24^{low}, miR-200^{low}), along with the capacity to form mammospheres, self-renewal, and increasing tumorigenecity in xenotransplants [12, 26, 132, 202]. The ability of miR-200 to suppress colony formation in vitro and tumorigenesis in vivo depends on their repressor effect on Bmi1 and ZEB1 [132].

Signaling pathways that control stem cell homeostasis during embryogenesis and later in adults (e.g., Wnt, Shh, Notch) are active in cancer and trigger—via induction of EMT-ATFs—not only EMT but also stem-like properties [3]. Acquisition and maintenance of stemness by EMT-ATFs could explain the capacity of these factors to induce tumorigenesis and promote recurrence and metastasis.

Isolation of stem-like (CD44^{high}/CD24^{low}) and non-stem-like (CD44^{low}/CD24^{high}) populations from resected primary breast tumors identified high levels of mesenchymal markers (N-cadherin, vimentin, fibronectin) including EMT-ATFs (ZEB2, Snail1, Snail2, Twist1 and Twist2) in the first set but not the second [26]. While ZEB1 did not show up in this study, ZEB1 was found to be uniquely required to maintain the viability and stem-like phenotype of spheres—a property classically linked to stem cells—formed from mouse embryo fibroblasts (MEFs) with targeted deletions of Rb1 and Rb family members [210]. In pancreatic carcinomas, ZEB1 determines stemness in cancer cells by direct repression of several miRs (miR-200, miR-183, miR-203) targeting stemness regulators Bmi1, KLF4, and Sox2 [131]. By contrast, activation of miR-200c by p53 reduces ZEB1 expression and suppresses both EMT and stemness, while downregulation of miR-200c—upon loss of p53 or overexpression of oncogenic p53 mutants—increases ZEB factors and stem cell markers in mammary and pancreatic acinar epithelial cells [128, 130] (Fig. 2).

Similar results have been obtained in other models. Spheres formed out of primary colorectal cancer cells also express high levels of Snail1, whose overexpression upregulates stem cell markers [211]. Another study found that overexpression of Snail1 or Snail2 in ovarian cancer cells derepresses a stemnes gene signature (e.g., Nanog, KLF4, Oct4, Bmi1, Nestin) and increases the number of

CSC-like $CD44^{+}/C117^{+}$ cells, suggesting that CSCs originate from the dedifferentiation of non-stem cancer cells rather than proliferation of existing CSCs [212]. In that line, oncogenic transformation of mammary stem-like cells produces more aggressive tumors than transformation of differentiated mammary epithelial cells and enhances the conversion of non-CSCs into CSCs [213].

The capacity of EMT-ATFs to generate CSCs is important to explain some other EMT-ATFs functions, like enhanced survival, tumor recurrence, and metastasis. The possibility that cancer cells could transition between tumorigenic and non-tumorigenic states [213], and that EMT-ATFs expression could modulate this balance, represents an important avenue in therapy. In the same line, the association between expression of EMT-ATFs by cancer cell lines and primary tumors and resistance to DNA damage and chemotherapy may also be related to EMT-ATFs' capacity to generate CSCs (see below). It remains to be firmly established whether, in line with the migrating CSC model [27], the joint induction by EMT-ATFs of a proinvasive/metastatic phenotype and a tumorigenic/stemness capacity in cell systems and xenograft models also occurs in human tumors.

EMT-ATFs in resistance to apoptosis and anoikis

EMT allows cancer cells to overcome safeguard mechanisms and become more resistant to signals triggering programmed cell death. Aberrant expression of EMT-ATFs by tumors may thus promote tumorigenesis and tumor growth through increased resistance to apoptosis. EMT triggers pro-survival programs in response to multiple apoptotic stimuli through mechanisms closely linked to the cell cycle. For instance, in an hepatocyte cell line TGFβ triggers an EMT when cells are in G1/S but apoptosis during G2/M [214]. Both Twist1 and Twist2 inhibit myc- and p53-dependent apoptosis by repressing $p19^{ARF}$ [215, 216]. Twist1 also inhibits p53 targets by direct interaction and blocking of the DNA binding domain of p53 [217]. Overexpression of Snail1 induces an EMT at the same time as suppressing TGFβ-induced apoptosis of non-transformed hepatocytes and hepatocarcioma cell lines [218]. Snail1 also protects cells against cell death caused by TNFα or growth factor withdrawal by activating MEK and PI3K signaling [219]. Direct repression by Snail factors of the promoters of p53 and pro-apoptotic target genes protects cells against apoptosis induced by DNA damage in response to genotoxic stress (e.g., BID, PIG8, caspase 6, DFF40) [212, 220]. In hematopoietic progenitors, Snail2 is induced by p53 in response to DNA damage and protects against apoptosis by repressing p53-mediated induction of Puma, an inhibitor of Bcl2 [221]. By contrast, mutant p53 in NSCLC cells results in low Mdm2 and stabilization of Snail2 [222]. In neuroblastomas, overexpression of Snail2 induces Bcl2 [223], and cancer cells with mutant K-Ras that have undergone EMT require Snail2 expression for their survival [224]. In different cell systems, ZEB1 directly inhibits pro-apoptotic TAp73 but also anti-apoptotic ΔNp73 and ΔNp63 [225, 226]. The pro-survival effect of ZEB2 is, however, independent of cell cycle arrest and intercellular adhesion and is mediated through inhibition of cleavage of PARP and pro-caspase 3 and phosphorylation of ATM/ATR substrates [164].

As cancer cells detach from the tumor, start migrating across the stroma, and intravasate into the circulation, they lose most (if not all) of their intercellular contacts and adhesion to the original extracellular matrix. Just like migrating progenitors during development, metastatic cancer cells are able to survive in this new environment, a condition that in normal cells triggers a caspase-dependent apoptosis program known as anoikis or anchorage-dependent cell death (recently reviewed in [227]). Acquisition of resistance to anoikis is therefore a critical cancer cell capability during tumor invasion and metastasis. Downregulation of E-cadherin expression is sufficient to determine resistance to anoikis [14, 194, 228]. ARF induces apoptosis and anoikis, and downregulation of ankyrin G—a binding partner of E-cadherin that concentrates it at sites of cell–cell contact—during EMT promotes the formation of NRAGE–Tbx2 complexes that represses ARF [229]. Several EMT-inducing pathways (e.g., receptor tyrosine kinase, oncogenic Ras, hypoxia) trigger resistance to anoikis through activation of PI3K-AKT and ERK signaling and regulation of Bcl2 family members. Likewise, in different cell systems and conditions, expression of ZEB, Snail, and Twist factors induces anchorage-independent cell growth [14, 64, 194, 230]. In a seminal contribution by Frisch's group, the capacity of ZEB1 conferring resistance to anoikis was shown to be dependent on the formation of ZEB1/CtBP E-cadherin repressor complexes, in a process reversed by E1a [14]. Snail1 expression promotes resistance to anoikis by activation of the MAPK and PI3K cascades [219]. The tyrosine kinase receptor TrkB, overexpressed in many human cancers, is a known inducer of both EMT and anoikis resistance [63]. Ligand activation of TrkB activates MAPK signaling leading to direct induction of Snail1, Twist1, and ZEB1, but with ZEB1 as the ultimate effector in TrkB-mediated resistance to anoikis. It is of note that TrkB is also a direct target of miR-200 in endometrial and breast carcinoma cells [231], forming yet another miR-regulated regulatory loop, this time for the control of anoikis resistance (Fig. 2a).

EMT-ATFs in regulation of cell cycle, senescence and transformation

Like apoptosis, cell cycle arrest by senescence represents a crucial safeguard mechanism against cancer. In addition to telomerase shortening during replicative senescence, oncogenic transformation and DNA damage can trigger cell cycle arrest and senescence through induction of the p53 and $p16^{INK4a}$/Rb pathways [232]. Oncogene-induced senescence has been identified in precancerous lesions and needs to be overcome for progression to full tumorigenic status [232, 233]. Evidence accumulated in recent years shows that EMT-ATFs allow cancer cells to bypass senescence thus contributing to the continuous proliferation of immortalized cells. In the same line, an inflammatory environment in the tumor area not only drives cancers cells into an EMT program but also overrides oncogene-induced senescence [234].

ZEB1 transcription, but not ZEB2 or Snail1, is inhibited by the $p16^{INK4a}$/Rb1 tumor suppressor pathway and in turn represses $p15^{INK4b}$, $p19^{ARF}$, and $p21^{CIP/WAF1}$ ([48], [76], [235]; see [236] for a comprehensive review). Loss of Rb1 is not only involved in tumor initiation but has been reported to also induce EMT [237], and may contribute to overexpression of ZEB1 in proliferating cells and in many primary tumors. MEFs from ZEB1 (+/−) and ZEB1 (−/−) mice undergo premature replicative senescence in a dose-dependent manner compared to wild-type MEFs [235]. Likewise, progenitors in the palate, skeleton, and nervous system in the ZEB1 (−/−) mice display decreased proliferation [235]. Oxidative stress induces senescence through miR-200-mediated inhibition of ZEB1 [224]. As indicated earlier, p53 represses ZEB1 expression through induction of miR-200, [128, 129]. However, while ZEB1 represses ΔNp63 and both isoforms of p73, it does not affect TAp63 or p53 [225].

Evidence for ZEB2 points in both directions—promoting and reverting senescence—perhaps reflecting cellular background differences. ZEB2 induces a G1 arrest in epidermoid and bladder carcinoma cell lines by direct transcriptional repression of cyclin D1 [164, 238]. Overexpression of cyclin D1 uncouples ZEB2-mediated cell cycle arrest from EMT [238]. Contrary to other EMT-ATFs, ZEB2 induces senescence in hepatocarcinoma cells by inhibiting hTERT [239]. However, overexpression of ZEB2 in lung epithelial cells raises the concentration of TGFβ needed to trigger growth arrest [48], and conditional targeted deletion of ZEB2 (−/−) in the developing cerebral cortex decreases proliferation of neural precursor cells [240]. Expression of both ZEB1 and ZEB2 has been shown to abrogate EGFR-induced senescence while their knock-down induces $p15^{INK4b}$ and $p16^{INK4a}$, thus reactivating the senescence program [241]. In this direction, miR-200 induces senescence in endothelial cells by inhibiting ZEB1 [224]. The regulatory loop between p53/miR-200 and ZEB1/ZEB2 therefore seems to also be involved in control of senescence (Fig. 2a). The ability of ZEB factors to override senescence is closely tied to their activation of EMT: triggering of senescence by knock-down of ZEB1 and ZEB2 (or p53) makes cells insensitive to the EMT-driving effects of TGFβ [241].

In the developing embryo, Snail1 inversely correlates with areas of proliferation and cyclin D2 expression, results confirmed in canine kidney epithelial cells where Snail1 transcriptionally represses cyclin D2 and associates to high levels of $p21^{CIP/WAF1}$ and G1 arrest [219, 242]. In this line, knock-down of Snail1 drives prostate cancer cells into senescence [243]. Snail2 is also excluded from areas of proliferation in the neural tube of the developing chick [219], and its overexpression in prostate cancer cells represses cyclin D1 [244]. However, other groups have reported opposite results, suggesting that, as in the case of ZEB2, the role of EMT-ATFs in cell cycle arrest and senescence may be cell type-dependent. Thus, in osteosarcoma cells, Snail1 inhibits E12/E47 transcriptional activation of the $p21^{CIP/WAF1}$ promoter, cooperating in this function with Twist1 [245]. Likewise, in breast cancer cells, Snail2 upregulates cyclin D1 and fosters proliferation by forming a complex with CtBP and transcriptionally repressing UbcH5c, an ubiquitin that targets cyclin D1 [246].

Growth arrest and senescence is also achieved by knocking down Twist1 in immortalized non-malignant prostate cells, while its overexpression inhibits p53-dependent senescence via inhibition of ARF [247]. Repression of $p14^{ARF}$ also results in inhibition of Chk1/2 phosphorylation in response to DNA damage [247]. Twist1 and Twist2 override Ras^{V12}-induced senescence of cell lines, and of breast cancer cells in MMTV-Erb2/Neu transgenic mice, by inhibition of $p16^{INK4a}$ and $p21^{CIP/WAF1}$ [248]. This study also found that Twist factors cooperate with Ras^{V12} in the transformation of MEFs, an effect that was abolished when Ras-induced senescence was inhibited. Twist1 cooperates with N-myc in the transformation of wild-type but not INK4a/ARF (−/−) MEFs, confirming that Twist activity involves inhibition of the ARF/p53 pathway [216]. As in the case of ZEB proteins, Twist1/2-mediated override of senescence is linked to their ability to induce an EMT and promote tumor invasion.

EMT-ATFs and angiogenesis

Snail1 is required for proper vascular development during embryogenesis, and its overexpression in embryonic stem cells induces the formation of VEGFR-2-positive endothelial cells through downregulation of miR-200 [249].

The growth of a tumor from an avascular hyperplasia into a larger mass requires the formation of new vessels through a process known as the angiogenic switch, which involves the production of angiogenic factors and proteases by tumor and stromal cells. Loss of E-cadherin is sufficient to trigger this angiogenic switch. Conditional knock-down of E-cadherin in mouse models of NSCLC promotes the development of tumor vasculature and growth through upregulation of vascular growth factors VEGF-A and VEGF-C and its receptor Flt-4 [250]. Angiogenesis is classically triggered by inflammation and hypoxia, conditions that are also known inducers of EMT [116]. Hypoxic conditions also increase the population of cancer cells with stem-like phenotype [251]. Stabilization of HIF-1α in response to hypoxia or its overexpression promotes invasion and metastasis by directly activating Twist1 and inducing an EMT [116]. Likewise, knock-down of HIF-1α or overexpression of the VHL gene—whose product targets HIF-1α for ubiquitin-mediated degradation—downregulates expression of ZEB1, ZEB2, and Snail1 [60, 252]. Additionally, HIF-1α feeds into upstream EMT-inducing signals (e.g., Notch, NFκB) [104]. Twist1-mediated induction of EMT by HIF-1α is not redundant with Snail1, suggesting that both factors have distinct but complementary roles in the pro-invasive and pro-angiogenic response to hypoxia. In that line, joint expression of HIF-1α, Twist1, and Snail1 in primary HNSCC associates with poorer prognosis [116].

Most EMT-ATFs promote tumor angiogenesis in vivo. ZEB, Snail, and Twist factors are often overexpressed by endothelial cells in the peritumoral stroma. Expression of Twist1 also associates with enhanced tumor microvessel vasculature and VEGF expression in hepatocarcinomas [205]. In mice, injection of lung adenocarcinoma cell lines overexpressing Snail1 or Snail2 generated tumors with enhanced vasculature compared to control cells [177, 253, 254], that in the case of Snail1 is accompanied by higher levels of proangiogenic factors CXCL5 and CXCL8 [254]. Likewise, compared to control cells, xenotransplant of breast cancer cell lines overexpressing Twist1 generate tumors with higher tumor angiogenesis and upregulated expression of several key vascular growth factors and receptors (e.g., VEGF, VEGFR2/KDR, Angiotensin-2, chemokine GRO-α, and CD31) [255, 256]. ZEB2 also promotes angiogenesis by direct transcriptional repression of the anti-angiogenic homeobox GAX factor [134]. However, and contrary to what would be expected from its induction by HIF-1α and its role as promoter of tumor progression, ZEB1 can also function as a negative regulator of angiogenesis in vivo. Xenotransplanted melanoma cells develop larger tumors with more developed vascularization in mice with a haploinsufficient ZEB1 background [257].

EMT-ATFs in oncogenic addiction and resistance to therapy

Activation of oncogenic pathways (or loss/inactivation of tumor suppressor signals) induces pro-survival and pro-growth signals on which tumors can become dependent. This dependency of cancer cells on oncogenes—referred as "oncogenic addiction"—has been exploited in the development of new chemotherapy drugs [258]. The success of antibodies and drugs targeting specific oncogenes in the treatment of a number of solid and hematologic cancers in mice models and humans has helped to reinforce the oncogenic addiction concept.

EMT and EMT-ATFs allow cancer cells to overcome their dependency on the oncogenic signals originally involved in their transformation. Thus, the epithelial status of pancreatic and lung cancer cells with activating mutations of K-Ras determines their dependency on K-Ras for their growth and survival [259]. Cancer cells that are dependent on K-Ras exhibit epithelial characteristics, while those independent of K-Ras have a mesenchymal phenotype. Induction of EMT by TGFβ or ZEB1 overrides K-Ras addiction protecting cancer cells from apoptosis following K-Ras knock-down. Conversely, elimination of ZEB1 in K-Ras-independent cancer cells restores K-Ras dependency [261]. Snail2 is also required for the survival of colorectal cancer cells with mutant K-Ras [260]. These results support EMT as a mechanism for cancer cells to escape from oncogenic addiction and highlight the potential of EMT-ATFs as therapeutic targets.

In the same line, and in parallel with the anti-apoptotic role of EMT described earlier, a wealth of articles have shown an association between EMT and chemotherapy resistance. Cancer cell lines expressing E-cadherin are more sensitive to chemotherapy drugs compared to those displaying a mesenchymal phenotype [261–264]. Resistance to chemotherapy and hormone therapy in patients with breast carcinomas correlates with tumoral expression of stem and mesenchymal markers. Poor response of HNSCC and NSCLC to EGFR inhibitors gefitinib and erlotinib in cell lines, human tumors, and xenograft mice models is associated with expression of EMT and stem-like cell markers [265–268]. Acquisition of resistance to oxaliplatin in colorectal carcinoma cells is also accompanied by expression of mesenchymal markers [261]. Responsiveness to cetuximab (a chimeric mouse–human antibody against EGFR) in Ras wild-type colorectal cancer cells depends on high expression of epithelial markers and low levels of ZEB1, Snail1, and Snail2, and of stem-like phenotype [269]. It is worth noting that the EMT-inducing effect of some drugs is cell-cycle dependent: e.g., in breast cancer cells, doxorubicin induces a mesenchymal phenotype during G_1/S and apoptosis in G_2/M [270]. The

Table 2 EMT-ATFs confer resistance to chemotherapy and radiotherapy

	Evidence	Resistance	Reference
ZEB1	Breast carcinoma cell lines	Doxorubicin	271
	Head and neck squamous carcinoma cell lines	Ertotinib	272
	Non-small lung carcinoma cell lines	Gefitinib	262
	Pancreatic carcinoma cell lines	Gemcitabine, 5-Fluorouracil, Cisplatin	71,131,273
ZEB2	Bladder and squamous carcinoma cell lines	Cisplatin, UV radiation	164
	Primary transitional cell carcinomas of the bladder	Radiotherapy	164
Snail1	Breast cancer cell line	5-Fluorouracil	276
	Lung carcinoma cell lines	Cisplatin	275
	Ovarian adenocarcinoma cell lines and primary tumors	Cisplatin	278
Snail2	Malignant mesothelioma	Doxorubicin, Paclitaxel, Vincristine	279
	Non-small cell lung carcinoma cell lines and Primary lung adenocarcinoma	Gefitinib	277
	Ovarian adenocarcinoma cell lines and primary tumors	Cisplatin	278
Twist1	Breast cancer cell lines	Doxorubicin	270
	Breast cancer cell lines	Paclitaxel	109
	Nasopharyngeal carcinoma cell lines	Paclitaxel	283
	Prostate carcinoma cell lines	Daunirubicin, cisplatin	280
	Prostate carcinoma cell lines	Paclitaxel, Cisplatin	247, 282
	Various carcinoma cell lines (bladder, nasopharyngeal, ovarian, prostate)	Paclitaxel, Vincristine	281
Twist2	Prostate adenocarcinoma cell lines	Daunirubicin, Cisplatin	280

Only some representative publications are included. See text for discussion

peritumoral stroma also plays an important role in chemoresistance to EGFR inhibitors. Interestingly, in a xenograft model of EGFR-resistant NSCLC, cancer-associated fibroblasts derived out of tumor cells that have undergone an EMT are not only EGFR-resistant but also tumorigenic [268].

The association between EMT and drug resistance is mediated, at least in part, by EMT-ATFs (Table 2). Expression of different EMT-ATFs by cancer cell lines and primary tumors confers tumor cells resistance to chemotherapy and radiotherapy. Resistance to doxorubicin in breast carcinoma cell lines correlates with higher expression of ZEB1 and SIRT1 [271]. In NSCLC cell lines, expression of ZEB1—but not of ZEB2, Snail1, or Snail2—correlates with higher resistance to gefitinib [262]. ZEB1 is also associated with resistance to erlotinib in HNSCC cell lines, and its knock-down increases drug sensitivity with the induction of E-cadherin expression [272]. Interestingly, simultaneous knock-down of ZEB1 and E-cadherin cancels out sensitization to erlotinib by ZEB1 elimination, suggesting that sensitivity to this EGFR inhibitor requires E-cadherin expression. Depletion of ZEB1 also sensitizes pancreatic cancer cell lines to gemcitabine, 5-fluorouracil, and cisplatin, and gemcitabine-resistant clones express higher levels not only of ZEB1 but also of Snail1 and Snail2 [71, 131, 273] (Table 2).

ZEB2 determines resistance to treatment independently of ZEB1, protecting bladder and squamous carcinoma cell lines against DNA damage-inducing agents such as cisplatin or UV radiation [164]. Importantly, patients with ZEB2-negative bladder carcinomas also exhibit better response to radiotherapy [164].

Snail1 is upregulated in NSCLC xenotransplanted tumors resistant to EGFR inhibitors [268] and determines resistance to cisplatin in HNSCC primary tumors and HNSCC and NSCLC cell lines [274, 275] (Table 2). Snail1-related resistance to cisplatin in HNSCC cell lines is mediated by activation of the DNA excision repair protein ERCC1 [275]. Snail1 expression also confers resistance to 5-fluorouracil in breast carcinoma cell lines [276]. In contrast, other studies have found that resistance to gefitinib displayed by some NSCLC cell lines and developed by primary lung adenocarcinomas associated with overexpressed Snail2—but not Snail1, Twist1, or ZEB1—being reversed by Snail2 knock-down through a mechanism involving upregulation of Bim and activation of caspase 9 [277]. In breast carcinoma cell lines refractory to doxorubicin, knock-down of Snail1 and Snail2 have a synergistic effect inducing drug sensitiveness, suggesting that Snail1 and Snail2 determine resistance through non-overlapping mechanisms. Chemotherapy resistance induced by EMT-ATFs is not only tightly linked to the induction of mesenchymal markers but also of stemness. Knock-down of Snail1 and Snail2 in ovarian cancer cell lines increases their sensitivity to cisplatin [278]. As noted earlier, Snail1- and Snail2-induced resistance to paclitaxel and radiation in

Table 3 EMT-ATFs regulate multiple hallmarks of cancer

Hallmarks of cancer	ZEB1	ZEB2	Snail1	Snail2	Twist1	Twist2
Activating invasion and metastasis	+ [167]	+ [15]	+ [177]	+ [253]	+ [19]	
Inducing angiogenesis	− [257]	+ [134]	+ [177]	+ [253]	+ [255]	
Sustaining proliferative signaling	+ [235]	− [238]			+ [215, 248, 307]	+ [248]
Evading growth suppressors					+ [215, 217, 248]	+ [248]
Resisting cell death	+ [230]	+ [164]	+ [219]	+ [219]	+ [215]	+ [215]
Enabling replicative immortality	+ [235, 241]	+ [241] − [239]	+ [243]	+ [244]	+ [247, 248]	+ [248]

Hallmarks of cancer refers to Ref. [1]. Only representative publication(s) were included. (+) represents activation of the cancer hallmark by the EMT-ATF. (-) represents repression of the cancer hallmark by the EMT-ATF. See text for discussion and other references

ovarian cancer cells relates to the repression of pro-apoptotic p53 targets and activation of stemness markers [212]. In malignant mesotheliomas, activation of SCF/c-kit signaling by Snail2 induces multidrug resistance [279].

Twist proteins are also involved in drug resistance. In breast carcinoma cells, Twist1 mediates resistance to paclitaxel via direct transcriptional activation of AKT2 [109]. Twist1 knock-down increases sensitivity of breast cancer cells to doxorubicin—a drug that triggers a p53-dependent DNA damage response—by disrupting p53-Mdm2 association [270]. Independently of p53 and $p19^{ARF}$ pathways, Twist1 and Twist2 block breast cancer cells death by daunorubicin by suppressing daunorubicin-induced phosphorylation of Bcl-2 [280]. In nasopharyngeal, bladder, ovarian, and prostate cancer cell lines, Twist1 increases resistance to paclitaxel and/or vincristine by upregulating Bcl2 and lowering Bax and Bak [281–283].

Concluding remarks

In the span of just a few years, EMT-ATFs have evolved from simple repressors of E-cadherin to inducers of most of the traits that cancer cells need to acquire for successful tumor progresssion (Table 3). Cancer cells can acquire these capabilities through overexpression, gain-of-function mutations, or amplification of oncogenes and/or repression, mutation or deletion of tumor suppressors [1], and mounting evidence indicates that EMT-ATFs critically regulate these cancer hallmarks. From participating only at late stages in cancer (promoting EMT and metastasis), EMT-ATFs are now known to be also involved in the initial phases of tumor development. First, EMT and metastasis may be a much earlier event in cancer progression than previously thought, playing an important role in tumor formation itself [31]. But, more importantly, ZEB, Snail and Twist factors regulate the acquisition of key early cancer hallmarks: EMT-ATFs contribute to overriding cancer safeguard programs (apoptosis, senescence), promoting tumor angiogenesis, cooperating with (or mediating) oncogenic signals (e.g., Ras), and antagonizing tumor suppressor pathways (e.g., p53) (Table 3). In turn, classical tumor repressors like Rb or p53 have recently been shown to regulate EMT and tumor invasiveness [128, 237].

The attention of molecular oncologists is moving from an early focus on the identification of irreversible mechanisms of cancer initiation and progression (e.g., mutations/deletions, amplifications) to the discovery of the dynamics involved in reversible programs of gene regulation in cancer cells (e.g., epigenetic/transcriptional, translational). Human tumors display a great level of heterogeneity, not only among patients but also within different areas and stages in a given tumor. Dynamic regulated expression of miRs and EMT-ATFs grants cancer cells with a great level of functional plasticity [54]. These transcriptional and translational regulatory networks allow cancer cells to reversibly transition between different states as they adapt to the environment during tumor progression, not only between epithelial and mesenchymal phenotypes but also between stemness and differentiation or between proliferation and growth arrest.

The data reviewed here attest to a significant level of overlapping among EMT-ATFs in their pattern of

expression, mechanisms of action, target genes and regulation of hallmarks of cancer. Increasing specificity and temporal and spatial hierarchies among EMT-ATFs are progressively being revealed with the identification of an expanding set of upstream regulatory miRs and translational regulators [149, 152, 155]. Similarly, the discovery of new cofactors and chromatin remodeling complexes used by EMT-ATFs to transcriptionally regulate their targets also points to some divergence; even if several EMT-ATFs are coexpressed, availability of these cofactors may dictate the functional capabilities of the EMT-ATFs.

Codification of the hallmarks of cancer by Hanahan and Weinberg [1] has helped researchers not only to systematize their findings and current understanding of cancer but has also contributed to guiding new therapy strategies. Compared to the irreversible effects of mutations and deletions, the dynamic regulation of miRs and EMT-ATFs renders them attractive targets for personalized oncology treatment. Incorporating the analysis of EMT-ATF expression in primary tumors into routine pathology diagnosis could help to prospectively identify resistance to particular chemotherapy. Since chemotherapies targeting a single oncogenic signal or cancer cell trait are not failsafe against resistance and tumor recurrence, a simultaneous approach to several signaling pathways and cancer hallmarks could be more successful. The highly modular structure and complex transcriptional activities of EMT-ATFs and their simultaneous regulation of multiple cancer hallmarks makes them attractive therapeutic targets for translational researchers.

On the other hand, reversibility of the EMT/MET balance and plasticity in its regulation represent adaptive mechanisms developed by cancer cells. Reverting the mesenchymal phenotype of invading cells by blocking the expression and/or function of EMT-ATFs may attenuate the ability of cancer cells to invade and increase their sensitivity to chemotherapy. However, as metastatic cancer cells need to regain an epithelial phenotype to grow into a macroscopic tumor at the site of distant colonization, reverting the mesenchymal status of tumor cells may actually foster metastasis. This is illustrated by the somewhat paradoxical metastatic-enhancing effect of miR-200 [125, 313]. In this regard, if reversible and dynamic regulation of miR and EMT-ATFs during cancer progression offers new avenues for therapy, it also complicates their manipulation. More information of the upstream regulatory networks and mechanisms of transcriptional regulation by EMT-ATFs is therefore needed before interference of their expression/function can be considered in cancer treatment. As far as we know, induction of EMT by EMT-ATFs occurs along with their regulation of other hallmarks (e.g., apoptosis, senescence). It remains to be ascertained how regulation of these hallmarks overlaps at the mechanistic level. To the extent that separate mechanisms are discovered, therapeutic approaches could be more precisely targeted.

In sum, as our understanding on the expanding roles of EMT-ATFs improves, these factors are likely to become not only prognostic and predictive personalized biomarkers in cancer but also important therapeutic targets in the near future.

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Review Article

Expanding roles of ZEB factors in tumorigenesis and tumor progression

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Abstract: The ZEB family of transcription factors regulates key factors during embryonic development and cell differentiation but their role in cancer biology has only more recently begun to be recognized. Early evidence showed that ZEB proteins induce an epithelial-to-mesenchymal transition linking their expression with increased aggressiveness and metastasis in mice models and a wide range of primary human carcinomas. Reports over the last few years have found that ZEB proteins also play critical roles in the maintenance of cancer cell stemness, control of replicative senescence, tumor angiogenesis, overcoming of oncogenic addiction and resistance to chemotherapy. These expanding roles in tumorigenesis and tumor progression set ZEB proteins as potential diagnostic, prognostic and therapeutic targets.

Keywords: Cancer, cancer stem cells, chemotherapy resistance, E-cadherin, EMT, transcription, tumor invasiveness, ZEB1, ZEB2

Introduction

Cancer is a multistep process where normal cells evolve to cancer cells through the acquisition of a number of properties, referred as the hallmarks of cancer [1, 2] that include: self-sufficient proliferative signaling, insensitivity to tumor suppression, replicative immortality, resistance to cell death, induction of angiogenesis and invasion and metastasis. Attainment of these cancer traits could occur through overexpression, gain-of-function mutations or amplification of oncogenes and/or epigenetic/transcriptional repression, mutation or deletion of tumor suppressors. In addition to systematize our current understanding of cancer, the hallmarks of cancer have helped guiding efforts to design therapeutic targets.

One of the areas in tumor biology that has seen greater developments in recent years has been the study of how transcriptional and epigenetic alterations contribute to the acquisition of the cancer hallmark capabilities. This article reviews the role of the ZEB family of transcription factors in cancer. Early evidence about the promotion of tumor metastasis by ZEB factors has been greatly expanded in recent years by a wealth of reports involving ZEB proteins in the regulation of several other hallmarks of cancer.

Invasion of an *in situ* carcinoma into normal surrounding tissue requires that cancer epithelial cells lose their cell-cell adhesion and polarity characteristics in favor of a more motile fibroblast-like phenotype as part of a transdifferentiation process known as the epithelial-to-mesenchymal transition (EMT) [3]. Originally described during embryogenesis, the phenotypic and functional reprogramming associated to the EMT also takes place during the invasion of carcinoma cells from a primary tumor into normal tissues. A key initial step in the EMT is the downregulation of the E-cadherin intercellular adhesion protein, which expression could be regulated at genetic, epigenetic, transcriptional

and post-translational levels [3,4]. Loss of E-cadherin often occurs through transcriptional repression, mediated by the binding of a small set of transcription factors (E-cadherin transcriptional repressors, EcTRs) to its promoter region. The EcTRs described so far include the ZEB family (ZEB1 and ZEB2, although identified under different names, see below), Snail1 (Snail), Snail2 (Slug), Twist1, Twist2 and E12/E47. Expression of EcTRs associates with EMT and more mesenchymal and invasive properties in cancer cell lines and increased metastasis and poorer clinical prognosis in primary carcinomas [4,5]. In addition to overlapping roles and mutual regulation among EcTRs, evidence indicates that ZEB1—and, to a lesser extent, ZEB2 and Snail2—has the strongest correlation with EMT across cancer tissue origins [6,7].

A rapidly growing literature has involved ZEB1 and ZEB2 in the regulation of a large number of physiological and pathological processes [8,9]. Both ZEB proteins have recently gained special relevance in the field of molecular oncology for their roles in tumorigenesis, tumor invasiveness and metastasis, and resistance to chemotherapy drugs. The rest of this article is organized as follows. Next section reviews the structural organization of ZEB proteins, their interaction with other factors and transcriptional activities. Section three outlines the roles of ZEB proteins in tumor invasiveness, tumorigenesis, cell proliferation and senescence, and resistance to chemotherapy.

Structure and transcriptional activities of ZEB factors

Domain structure and interacting proteins of ZEB factors

In upper vertebrates, the ZEB family comprises two proteins, ZEB1 and ZEB2, known under multiple alternative names. Thus, ZEB1 was also identified as δEF1, AREB6, BZP, MEB1, Nil-2-a, TCF8, ZEB, ZEB-1, Zfhep1 and Zfhx1a [10-17]. In turn, ZEB2 is also referred as KIA0569, SIP1, SMADIP-1, ZEB-2 and Zfhx1b [18-20]. In *Drosophila melanogaster, Caenorhabditis Elegans* and zebrafish a single orthologue has been described, namely Zfh-1, Zag-1, and Kheper, respectively [21-23].

Structurally, ZEB proteins are highly modular with independent regions mediating their binding to DNA, to other transcription factors and to a number of cofactors—proteins with activator or repressor transcriptional activity but lacking a DNA binding domain on their own. All ZEB family members contain two zinc finger clusters (ZFC) located towards the N- and C-terminal ends of the protein (Nt-ZFC and Ct-ZFC, respectively) that bind to ZEB boxes (E-box and E-box-like DNA sequences) in the regulatory regions of target genes (**Figure 1**) [24-26]. Towards the center of ZEB proteins there is an extra zinc finger (mid-ZF, missing in human ZEB1) and a POU-like homeodomain, which has also been involved in binding to DNA [27]. Human and rodent ZEB1 and ZEB2 share a high degree of amino acid similarity in their ZFC and homeodomain, but much less elsewhere (**Figure 1**) [18,19].

ZEB1 and ZEB2 interact with other transcription factors. Downstream of the Nt-ZFC, both proteins contain a Smad Interacting Domain (SID) for binding to phosphorylated receptor-activated Smads (R-Smads), transcription factors that regulate downstream target genes in the TGFβ/BMP signaling pathway [18, 28,29] (**Figure 1**). The ZFCs of ZEB1 and ZEB2 also mediate binding to transcription factors. Thus, the Nt-ZFC and Ct-ZFC of human ZEB1 have been shown to interact with SRF [30] while the mid-ZF/homeodomain region of rat ZEB1 binds to Oct-1 [31]. Meantime, the Nt-ZFCs and Ct-ZFCs of ZEB2 interact directly with the polycomb factor Pc2 [32].

Nevertheless, most of the transcriptional activities of ZEB1 and ZEB2 are mediated through their recruitment of several corepressors and coactivators (in red and green, respectively, in **Figures 1** and **2**). Upon activation of the TGFβ/BMP signaling pathway and binding of ZEB1 to R-Smads, the region N-terminal to the Nt-ZFC of human ZEB1 binds to histone acetyltransferases p300 and p/CAF [28]. This interaction has also been reported for mouse and Xenopus ZEB2 although in this case independently of binding to R-Smads and TGFβ/BMP signaling [33]. The N-terminal half of human ZEB1, but not of ZEB2, also binds to another histone acetyltransferase, the Tat-interacting protein Tip60 [8,34]. The region N-terminal to the Nt-ZFC in both ZEB proteins recruits nucleosome remodeling factors. ZEB1 interacts with BRG1, one of the two ATPases of the SWI/SNF chromatin remodeling complex [35] while ZEB2 binds to the NuRD remodeling and deacetylase complex [36]. Between the homeodomain and the Ct-ZFC

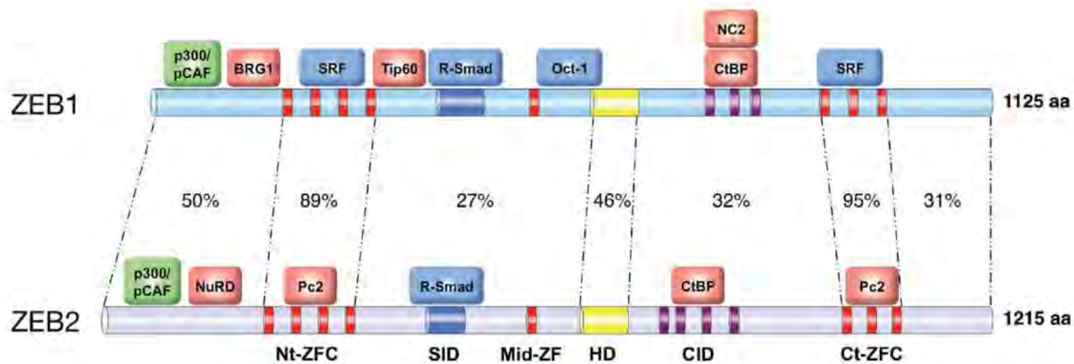


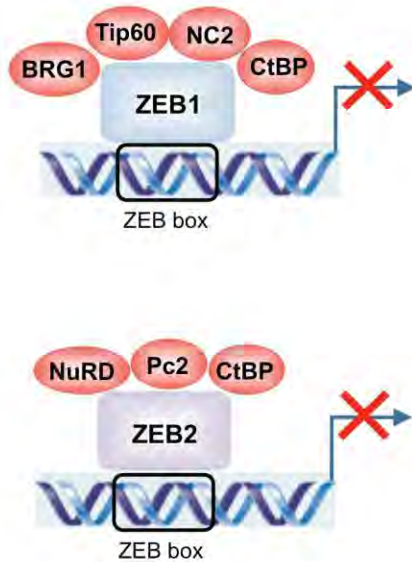
Figure 1. Scheme of the domain structure and main binding proteins of human ZEB1 and ZEB2. Percentages indicate identity at the amino acid level (GenBank accession numbers U12170 and AB011141, respectively). Proteins labeled in green represent coactivators, in red corepressors and in blue other transcription factors. Nt-ZFC: N-terminal zinc finger cluster. SID: Smad-interacting domain; Mid-ZFC: mid zinc finger cluster; HD: Homeodomain; CID: CtBP-interacting domain; Ct-ZFC: C-terminal zinc finger cluster. High degree of amino acid identity between ZEB1 and ZEB2 is mostly restricted to the Nt-ZFC and C-ZFC (89% and 95%, respectively), the SID (42%) and the homeodomain (46%).

vertebrate ZEB1 and ZEB2 have a CtBP interacting domain (CID), containing multiple binding sequences for CtBP cofactors [19, 37-41], that in turn complex with histone deacetylases and methyltransferases, polycomb proteins and coREST [41-43]. In addition, it has been shown that CtBP1 could interact with the bromodomain of p300 blocking its ability to acetylate histones and activate transcription [44]. Zfh-1 and Zag-1 also interact with CtBP although their CID contains a single CtBP binding site [45,46]. Around the same region, human ZEB1 has been reported to interact with NC2α/NC2β (also referred as DRAP1/Dr1), a repressor of RNA polymerase II and III transcription [47].

Molecular mechanisms of transcriptional regulation by ZEB proteins

Such complexity in the number and nature of interacting factors anticipates the multiple transcriptional activities displayed by ZEB proteins. Although ZEB1 and ZEB2 were initially identified as transcriptional repressors and array screenings indicate that this is indeed their main transcriptional function (6 and below), both factors could activate or repress transcription depending on the target gene and tissue. Transcriptional repression by ZEB1 and ZEB2 could take place through either passive or active mechanisms. In the first case, ZEB proteins compete and displace transcriptional activators from their binding to E-box sequences in the DNA. Nevertheless, in some systems, the affinity of ZEB1 for DNA seems to be lower than that of other E-box binding proteins [17, 48-51]. In any case, the predominant mechanism by which ZEB1 and ZEB2 repress gene expression is through active transcriptional repression [19,45,47,51-54]. For instance, when the full-length cDNAs of ZEB1 and ZEB2 are fused to the DNA binding domain of yeast Gal4 and tested in a Gal4/UAS system, both heterologous proteins act as potent active transcriptional repressors [19,51,52]. This approach has allowed the identification of several repressor domains within the N-terminal and central regions of ZEB1 and ZEB2, each with distinct transcriptional repressor specificity, as well as an activation domain near the C-terminal end [19,45, 47,51,52,54].

It is assumed that ZEB proteins selectively recruit their corepressors in a promoter-specific manner. However, the identity of the precise corepressor(s) involved is only known for a few ZEB target genes. Tip60 mediates ZEB1 repression of CD4 [34]. A ZEB1/CtBP repressor complex regulates the growth hormone [42], interleukin-2 [55] and Bcl-6 [56]. Repression of E-cadherin by ZEB1 involves the summative activities of CtBP and BRG1 [35,40] and of CtBP and NuRD in the case of ZEB2 [36,40]. It is of note that the ATPase Mi2β/CHD4 of NuRD could also



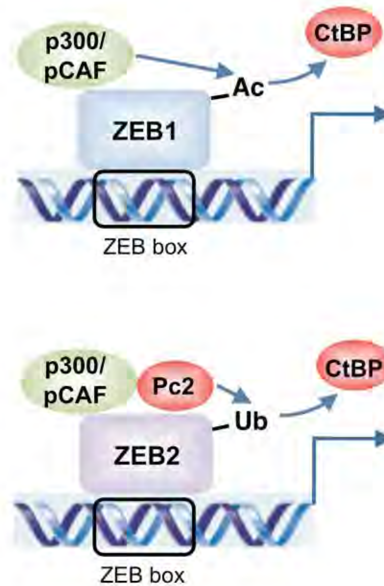


Figure 2. ZEB1 and ZEB2 could act as either transcriptional repressors or activators depending on the target gene and tissue. Transcriptional repression and activation is achieved through differential recruitment of cofactors. Post-transcriptional modifications of ZEB1 and ZEB2 alter the set of coactivators and corepressors bound and switch both proteins from transcriptional repressors to activators.

associate with BRG1 [57].

Activation of transcription by ZEB1 and ZEB2 also involves promoter-dependent recruitment of coactivators. Upon TGFβ/BMP stimulation, ZEB1 binds to R-Smads, synergizing with them in the activation of TGFβ/BMP-dependent genes through binding to p300 and p/CAF [28,29]. Binding to these histone acetylases is also assumed to mediate activation of other target genes positively regulated by ZEB1 and ZEB2. In that line, correlation between ZEB1 and Vitamin D Receptor—a gene directly activated by ZEB1—in primary colorectal carcinomas is stronger the higher the levels of p300 [58]. Although ZEB2 also binds to p/CAF and p300 [33], it remains unknown under what circumstances this mechanism is used to directly activate gene expression and for which genes, as ZEB2 inhibits R-Smad-mediated activation of TGFβ/BMP-dependent genes [28,29] (**Figure 2**).

The interplay among ZEB corepressors and coactivators remains to be fully defined (**Figure 2**). Post-translational modifications of ZEB proteins seem to contribute to switching ZEB1 and ZEB2 from repressors to activators. At least in the activation of TGFβ/BMP-dependent genes, interaction of ZEB1 with p/CAF following TGFβ/BMP stimulation acetylates lysine residues flanking the CID, partly displacing CtBP from its binding to ZEB1 [29]. However, addition of FGF2 over TGFβ restores ZEB1-CtBP interaction [59]. Recruitment of p/CAF also blocks CtBP binding to ZEB2 [33] although, as indicated earlier, it remains to be determined what triggers p/CAF binding to ZEB2 in the first place. SUMOylation of ZEB2 by Pc2 also disrupts ZEB2 binding to CtBP and partially relieves E-cadherin repression but does not affect repression or activation of other ZEB2 target genes [32]. Independently of the binding of Pc2 to the Nt-ZFCs and Ct-ZFCs of ZEB2, CtBP also binds and is sumoylated by

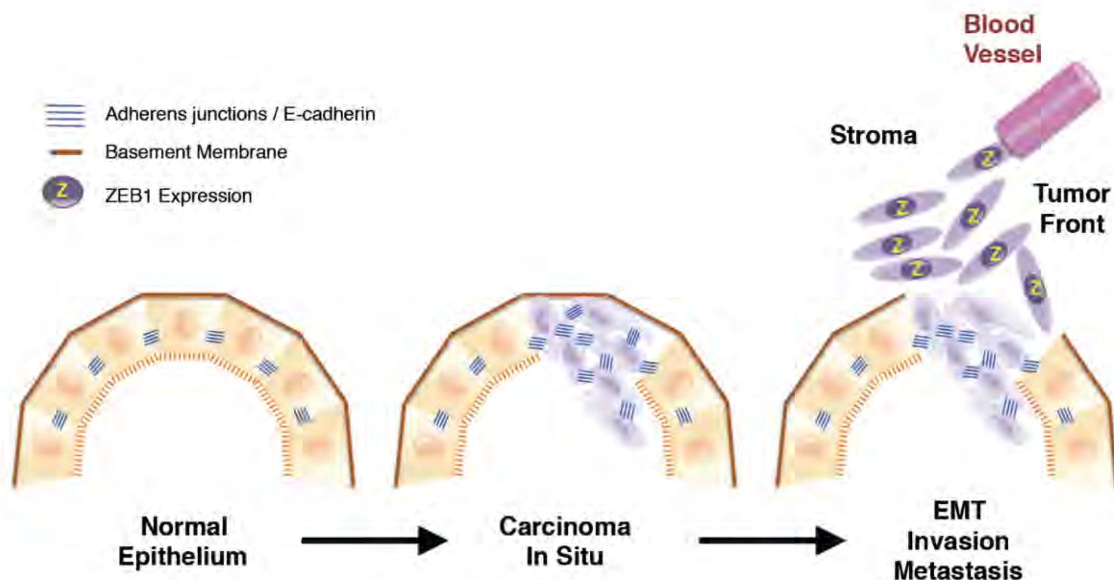


Figure 3. Schematic representation of the evolution of a carcinoma *in situ* into an invasive carcinoma. Invasion of cancer cells into the surrounding stroma involves a complex process by which they lose their epithelial characteristics and acquire instead a mesenchymal phenotype as part of the EMT. ZEB1 is not present in normal epithelial cells or at the center of well-differentiated carcinomas but is expressed by invasive cancer stem cells undergoing an active EMT at the tumor front of several types of carcinomas. ZEB1 also regulates several components of the basement membrane, which breakdown is required for cancer cells to invade the surrounding stroma.

polycomb Pc2 [60]. Although its transcriptional significance remains unclear, phosphorylation of ZEB1 varies widely among cell types [61]. Finally, ZEB1 and ZEB2 functions are also regulated by means of their intracellular localization. Both ZEB proteins are exported to the cytoplasm in a temporal- and/or tissue-specific manner during embryonic development and in adult tissues and tumors [15, 62-64].

ZEB proteins and cancer

ZEB proteins as inducers of EMT and tumor metastasis

Since ZEB1 was originally identified in the early 1990s, several dozen target genes—including key regulatory factors during embryogenesis and cell differentiation—have been reported to be repressed or activated by ZEB proteins, mostly through direct binding to their regulatory regions [8]. But it was their ability to repress the E-cadherin and induce an EMT that set ZEB1 and ZEB2 as important regulators of tumor progression [41, 66-69].

In epithelial cells, E-cadherin locates at adherens junctions and mediates homotypic intercellular adhesion, while interacts intracellularly with catenins and the actin cytoskeleton. Intercellular adhesion via adherens junctions is critical for maintenance of epithelial cell polarity and phenotype. Consequently, loss of E-cadherin is considered a critical initial event not only in EMT, but also in tumor progression and metastasis [3,70] (**Figure 3**). During the EMT loss of E-cadherin is accompanied by reorganization of other intercellular complexes and synthesis of some extracellular matrix components (e.g. fibronectin, collagen I and III) and metalloproteases (MMPs), normally produced by mesenchymal stromal cells [71]. In addition, the EMT is associated with the acquisition of a stem cell phenotype and increased resistance to apoptosis [70,72,73 and see below). Consequently, the EMT endows tumor cells with greater motility, self-renewal capacity and resistance to drugs, at the same time that degradation of the extracellular matrix facilitates their invasion into the surrounding stroma and eventual metastasis to distant organs.

Table 1. Selected targets of ZEB1 and ZEB2 during EMT*

	ZEB1	ZEB2
Direct Transcriptional Targets	**Genes downregulated**	**Genes downregulated**
	E-cadherin [41,66,68,69]	E-cadherin [67]
	Crumbs3, HUGL2, PATJ [6]	P-cadherin [75]
	Paklophilin 3 [74]	Claudin 4 [75]
	MMP1 [102]	Connexin 26 [75]
	miR-200 family [88, 89]	miR-200 family [89]
		Genes upregulated
		Vimentin [76]
Indirect Targets or Unknown Mechanism	**Genes downregulated**	**Genes downregulated**
	P- & R-Cadherin [6]	ZO-3 [75]
	Occludin & JAM1 [6]	Plakophilin 2 & Desmoplakin 1/2 [75]
	Desmocollin-2 [6]	Connexin 31 [75]
	Gap junction protein β2 & β3 [6]	Gap junction protein β2 & β3 [75]
	Epiplakin & Periplakin [6]	EpCAM [65]
	Mucin [6]	ESRP1/2 & ST14 [65]
	EpCAM [6,65]	**Genes upregulated**
	ESRP1/2 & ST14 [65]	Fibronectin (77)
	Genes upregulated	MMP1, MMP2, MMP14 [77]
	Vimentin [68,69]	

*Note that being a direct transcriptional target of ZEB1 and/or ZEB2 does not exclude indirect regulation as well. Genes under the label indirect target/unknown mechanism refer to those that are either known to be regulated by ZEB1 and/or ZEB2 exclusively through indirect mechanisms or where direct binding of ZEB1 and/or ZEB2 to their regulatory regions has not been demonstrated yet. Numbers in brackets refer to the references where these targets were identified.

Overexpression of ZEB1 or ZEB2 in epithelial cells induces a full EMT. ZEB1 and ZEB2 not only repress E-cadherin but also P- and R-cadherins and other epithelial markers involved in cell polarity (e.g. CRB3, HUGL2, PATJ), components of tight junctions (e.g. occludin, claudin 7, JAM1, ZO3), gap junctions (e.g. connexins 26 and 31) and desmosomes (e.g. desmoplakin, plakophilin 3) [6,74,75] (**Table 1**). In turn, ZEB1 and ZEB2 activate the expression of mesenchymal genes such as vimentin and N-cadherin [69,75,76]. Overexpression of ZEB2 has been shown to induce several MMPs, namely MMP1, MMP2 and MMP14 [77]. In colorectal carcinomas, ZEB1 also regulates components of the epithelial basement membrane, which disruption is a key step in tumor invasiveness—e.g., knock down of ZEB1 upregulates the α3 chain of laminin 5 (LAMA3) and the α2 chain of collagen IV (COL4A2) while reduces the levels of the γ2 chain of laminin 5 (LAMC2) [78].

ZEB1 and ZEB2 mediate the EMT triggered by key signaling cascades such as TGFβ/BMP, NFκB, Ras-ERK2, and HIF-1, often activated in tumors [79-82]. On the other hand, ZEB1 and ZEB2 are repressed by non-coding microRNAs of the miR-200 family, which are important in maintaining an epithelial phenotype and preventing an EMT [83-87]. Interestingly, miR-200 members are, in turn, transcriptionally repressed by ZEB1 and ZEB2, thus forming regulatory loops that maintain cells in either an epithelial or mesenchymal state [9,88,89].

Large areas of many carcinomas, including colorectal, are relatively well-differentiated, with tumor cells maintaining their polarity and E-cadherin associated at the membrane with β-catenin [90,91]. By contrast, at their invasive edge, tumor cells undergo an active EMT with loss of E-cadherin and nuclear translocation of β-catenin [90]. These dedifferentiated, fibro-

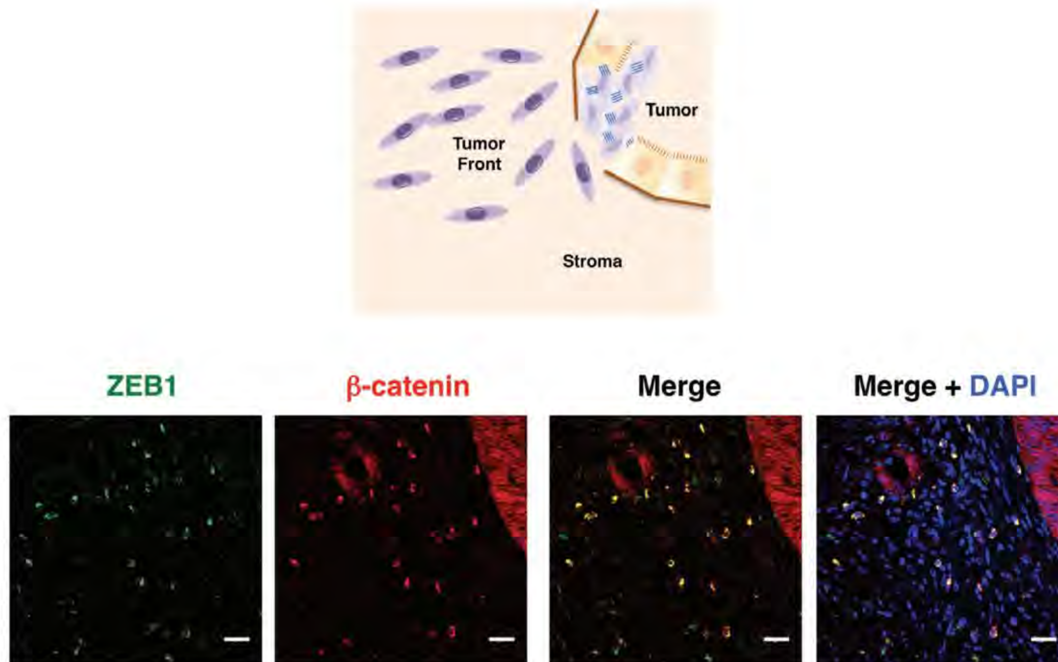


Figure 4. ZEB1 and β-catenin colocalize at the nucleus of invasive cancer cells at the tumor front of colorectal carcinomas. While in epithelial cells of well-differentiated areas of colorectal carcinomas β-catenin has a membranous/ cytoplasmic distribution it becomes mostly nuclear in these invasive cancer cells. ZEB1 (green, E-20 antibody, Santa Cruz Biotechnology) and β-catenin (red, Ab6032 antibody, Abcam) colocalize (yellow, merge panel) in invasive cancer cells of a sporadic colorectal carcinoma [35,78]. Labeling with 4',6-diamidino-2-phenylindole (DAPI) is also shown. Scale bar represents 25 μm.

blastic-like cells at the tumor front have been referred as "migrating cancer stem cells" because of their stem cell-like phenotype [78,91]. As inhibitor of E-cadherin and epithelial phenotype, ZEB1 is not expressed in normal epithelium but is found in isolated cells at the stroma. ZEB1 is neither expressed by tumor cells in well-differentiated areas of carcinomas, but is expressed at high levels in invading tumor cells of endometrial, colorectal, lung, breast, prostate, gallbladder, and pancreatic carcinomas among others [35,78,92-97] (**Figures 3** and **4**). Increased numbers of ZEB1-positive cells in the stroma are found in colorectal, breast, lung and bladder carcinomas [6,65,98] and it has been suggested that ZEB1-dependent paracrine signaling from the stroma could mediate E-cadherin repression in other parts of the tumor [65].

Although stronger than for most other EcTRs, inverse correlation between ZEB2 and E-cadherin across cancer cell lines is not as evident as in the case of ZEB1 [7]. Interestingly, ZEB2 is detected at high levels in the cytoplasm of normal E-cadherin-positive epithelial cells of several tissues (e.g. esophagus, stomach, colon and rectum, hepatocytes, renal tubules), but is downregulated when these epithelia evolve towards carcinomas [63,64]. Since ZEB2 is only known as a transcription factor, the functional significance of its cytoplasmic expression in epithelial cells remains to be elucidated.

Expression of ZEB proteins at the invasive front of carcinomas translates into increased tumor metastasis in ZEB1-positive tumors. Thus, in mouse xenograft models, expression of ZEB1 promotes metastasis of colorectal carcinoma cells [99]. Over the last few years, a wealth of reports have linked ZEB1 and/or ZEB2 expression to increased aggressiveness and higher metastatic capacity in a wide range of primary human carcinomas, including ovarian, breast, endometrium, lung, prostate, colon, gallbladder, pancreas and bladder [92-98,100].

The transition from an avascular hyperplasia into a larger hypervascularized tumor mass requires the formation of new vessels, the so-called "angiogenic switch", which results from the production by the tumor of angiogenic factors and proteases, including MMPs [101]. Evidence in the literature indicates that ZEB1 and ZEB2 play opposing roles in this regard. On the one hand, and contrary to its tumor progression role discussed so far, ZEB1 inhibits tumor angiogenesis *in vivo*. Subcutaneous injection of melanoma cells leads to larger tumors with more tumor vessels—and higher ZEB1 endothelial expression—in heterozygous ZEB1 -/+ mice than in wild type animals [102]. On the other hand, ZEB2 has a pro-angiogenic effect with ZEB2 -/- embryos displaying defective vessel maturation [103].

ZEB proteins in stemness maintenance and tumorigenesis

In the classic stochastic model of tumorigenesis, all cells are homogenous and have the same potential to initiate a tumor in response to intrinsic or extrinsic factors. However, oncologists have been puzzled for decades by the great level of heterogeneity displayed by tumors—both hematologic and solid—in terms of morphology, surface markers, genetic mutations and sensitivity to treatment [104,105]. Evidence built in the last two decades have revived an alternative model of tumorigenesis, the "cancer stem cell" (CSC) model [105,106]. Seminal studies in the 1990s demonstrated that some variants of acute myeloid leukemia originate from a subset of cells, which phenotype resembles that of normal hematopoietic stem cells—thus coined as "leukemia stem cells"—and that retain the capacity to reproduce leukemias in xenotransplanted recipient mice [105]. Using similar approaches researchers have also found CSCs in colon, pancreas, breast and brain cancers. Contrary to cells in the bulk of the tumor, CSCs have the capacity for self-renewal, differentiation and initiation of tumorigenesis [104,106]. A recent report has showed that oncogenic transformation enhances the conversion of non-stem cancer cells into cancer stem cells. Oncogenic transformation of mammary stem-like cells also produces more aggressive tumors that transformation of differentiated mammary epithelial cells [107].

Induction of EMT by TGFβ or overexpression of EcTRs—is able to reprogram differentiated populations of normal mammary epithelial cells and breast carcinoma cells into undifferentiated cells with stem cell-like phenotype and functional properties (e.g. generation of spheres in culture, increased turmorigenecity in xenotransplants) [72]. A subpopulation of cancer cells within breast tumors display a phenotype similar to normal stem cells, including lower levels of miR-200c-141, miR-200b-200a-429, and miR-183-96-182 [108]. Expression of miR-200c in normal and cancerous breast stem cells reduces the expression of self-renewal factor Bmi1 and these cells' ability to form mammary ducts and tumors, respectively.

The salience of ZEB proteins in cancer biology was further enhanced by work from Brabletz's group elegantly showing that ZEB1 maintains an stemness phenotype in pancreatic cancer cells and increases their tumorigenic capacity in nude mice [97]. Regulation of stemness by ZEB1 occurs through transcriptional repression of miR-200, miR-183 and miR-203, which in turn repress Bmi1, Sox2 and KLF4. In this line, breast stem cells express higher levels of both ZEB1 and ZEB2 than differentiated cells [108]. Formation of spheres in mouse embryo fibroblasts (MEFs) from Rb family and Rb1 -/- mice is accompanied by the generation of stem-like cells, which phenotype and viability requires of ZEB1 expression [109]. Recent reports have showed that p53 suppresses EMT, stemness and reduces ZEB1 levels through direct transcriptional activation of miR-200c [110]. Conversely, loss of p53—or overexpression of oncogenic p53 mutants—induces a downregulation of miR-200c and increased expression of ZEB1 and stem cells markers (including Bmi1 and KLF4) in mammary and pancreatic acinar epithelial cells and associates with higher tumor grading in breast carcinomas [110, 111]. The connection between ZEB proteins, EMT and stemness may play an important role not only during tumorigenesis but also in embryonic development [9,112].

ZEB proteins in the regulation of cell cycle and senescence

The involvement of ZEB proteins in the regulation of cell cycle and proliferation varies depending on the cell type and model used. Overexpression of either ZEB1 or ZEB2 in lung epithelial cells does not have by itself any effect in cell cycle. However, when combined with low doses of TGFβ—suboptimal to induce a full

growth arrest—ZEB1 increases the percentage of cells in G1 phase [28]. This synergistic effect between ZEB1 and TGFβ depends on the direct interaction of ZEB1 with R-Smads. By contrast, ZEB1 -/- mice display decreased proliferation in tissues affected by developmental defects (e.g. palate, skeleton, nervous system) and MEFs from ZEB1 -/+ and ZEB1 -/- mice arrest at much earlier passage than wild type MEFs [69].

Overexpression of ZEB2 in lung epithelial cells raises the concentration of TGFβ needed to trigger growth arrest [28]. Likewise, specific targeted deletion of ZEB2 in the mice developing cerebral cortex results in decreased proliferation of precursor cells in the hippocampus and dentate gyrus [113]. However, in epidermoid and bladder carcinoma cell lines the reverse is observed, overexpression of ZEB2 induces a G1 arrest by direct transcriptional repression of cyclin D1 [98,114].

ZEB1, but not ZEB2 or Snail1, is repressed by the p16INK4a/Rb1 tumor suppressor pathway. Rb1/E2F1-HDAC1 repressor complexes regulate ZEB1 transcription by direct binding to its promoter and, compared to MEFs from wild type mice, Rb1 -/- and E2F1 -/- MEFs display higher levels of ZEB1 [115]. Loss of Rb1 has been involved in both tumor initiation and progression and may contribute to explain overexpression of ZEB1 in proliferating cells and many primary tumors.

Replicative senescence is considered an important tumor-suppressor mechanism and, for instance, knock down of Rb1 inhibits the ability of cells to undergo oncogene-induced senescence. ZEB1 helps cancer cells to overcome replicative senescence and its elimination (e.g. ZEB1 -/+ and ZEB1 -/- MEFs) triggers premature senescence in a dose-dependent manner [69]. Circumvention of senescence by ZEB1 occurs independent of p16INK4a but rather through direct transcriptional repression of p15INK4b and p21CDKN1a [28,69]. By contrast, ZEB2 induces senescence arrest in hepatic and breast carcinoma cells through repression of hTERT [116].

ZEB proteins in oncogenic addiction and resistance to chemotherapy and radiotherapy

Some tumors require the expression of one or more genes for the maintenance of their malignant phenotype and viability, in what is known as "oncogenic addiction" [117,118]. The success of antibodies and drugs targeting specific oncogenes (e.g. K-Ras, Her2/Neu, Bcr/Abl, EGFR, c-kit) in the treatment of a number of solid and hematologic cancers in mice models and humans has helped to reinforce the oncogenic addiction concept. Given the potential therapeutic applications, the identification of the specific oncogene(s) on which particular types of human cancers are dependent on has attracted a great deal of interest in recent years.

A survey of K-ras mutant lung and pancreatic cancer cell lines found that their epithelial status determines their dependency on K-ras for survival [70,73]. Thus, cancer cells that are independent on K-Ras for their growth and viability have a mesenchymal phenotype while K-Ras-dependent cell lines exhibit epithelial characteristics [73]. Furthermore, induction of EMT protects lung cancer cells from apoptosis following K-ras knock down. The same study found that ZEB1 expression overrides K-ras oncogenic addiction while its elimination reverses K-Ras dependency. In the same line, the phenotype and histology of tumors formed from ZEB1-dependent CSCs in Rb1 -/- MEFs are similar to those observed by expression of activated Ras [109]. The presence of ZEB1 within the "K-ras dependency gene signature" highlights its role as a potential therapeutic target. Other EcTRs seem to play parallel roles—Twist1 and Twist2 override ErbB2-dependent senescence and cooperate with Ras to induce malignant transformation, EMT and metastasis [119]. It has been therefore suggested that the induction of ZEB1 and other EcTRs during tumor progression—and the parallel acquisition of a dedifferentiated stem cell phenotype—may substitute for constitutive Ras activation [120].

EMT is also accompanied by increased resistance to apoptosis by DNA damaging drugs [70,73], and mounting evidence indicates that expression of different EcTRs by cancer cell lines and primary tumors confers tumor cells resistance to chemotherapy and radiotherapy. In lung cancer cell lines, expression of E-cadherin has been associated to higher sensitivity to the EGFR inhibitor gefitinib while expression of ZEB1—but not of ZEB2, Snail1 and Snail2—correlates to higher resistance [121] (**Table 2**). Conversely, knock down of ZEB1 increases the sensitivity of colorectal and pancreatic cancer cell lines to gemcitabine, fluorouracil

Table 2. ZEB1 and ZEB2 confer resistance to chemotherapy and radiotherapy

	Evidence	Resistance	Reference
ZEB1	Pancreatic carcinoma cell lines	Gemcitabine 5-Fluorouracil Cisplatin	[97, 120, 121]
ZEB1	Breast carcinoma cell lines	Doxorubicin	[123]
ZEB1	Non-small cell lung carcinoma cell lines	Gefitinib	[119]
ZEB1	Head and neck squamous carcinoma cell lines	Ertotinib	[122]
ZEB2	Primary transitional cell carcinomas of the bladder (clinical evidence)	Radiotherapy	[98]
ZEB2	Bladder carcinoma cell lines	Cisplatin UV radiation	[98]
ZEB2	Squamous carcinoma cell lines	Cisplatin UV radiation	[98]

and cisplatin while gemcitabine-resistant clones of these cell lines exhibit high levels of ZEB1 [97,122,123]. Likewise, elimination of ZEB1 in non-small cell lung and head and neck cancer squamous carcinoma cell lines reduced cell death in response to the EGFR inhibitor erlotinib in an E-cadherin-dependent manner [124]. Interestingly, simultaneous knockdown of ZEB1 and E-cadherin reverses the sensitization to erlotinib induced by ZEB1 knockdown, suggesting that sensitivity to this EGFR inhibitor requires E-cadherin re-expression. Levels of ZEB1, but not ZEB2, also inversely correlate with sensitivity of breast cancer cells to doxorubicin [125].

Parallelly, ZEB2 has been shown to have an independent prognostic value in transitional cell carcinomas of the bladder [98]. Irrespective of ZEB1 levels, patients with ZEB2-negative bladder carcinomas exhibit better survival outcomes and response to radiotherapy [98]. In addition, expression of ZEB2 protects bladder and squamous carcinoma cell lines against DNA damage-induced cell death (e.g. UV radiation, cisplatin).

The mechanisms involved in ZEB1- and ZEB2-mediated drug resistance are still being investigated. In different cell systems, ZEB1 has been shown to directly inhibit TAp73, which triggers apoptosis, but also ΔNp73 and ΔNp63, that function as anti-apoptotic factors [48, 126, 127]. The pro-survival effect of ZEB2 is independent of cell cycle arrest and intercellular adhesion and is mediated through inhibition of cleavage of PARP and pro-caspase 3 and phosphorylation of ATM/ATR substrates [98].

Interestingly, other EcTRs such as Snail and Twist proteins also protect cells from apoptosis and contribute to drug resistance in cancer cells, in part through regulation of p53/p63/p73 family members. Snail-mediated resistance to cell death is also critical for migration of embryonic cells during development [128]. Snail1 and Snail2 confer resistance to chemotherapy and radiotherapy through two mechanisms: repression of genes involved in p53-mediated apoptosis (e.g. ATM, PUMA, PTEN) and derepression of genes associated to stemness (e.g. Nanog, claudin 3, KFL4) [129]. Snail1 inhibition of ΔNp63α induces increased invasiveness in human squamous cell carcinomas independent of its effect over E-cadherin [130]. Meantime, Twist1 inhibits p53-mediated apoptosis by direct interaction with the DNA binding domain of p53 [131,132].

Concluding remarks

The literature here reviewed involves ZEB1 and/or ZEB2 in the control of several cancer cell capabilities, namely, cell proliferation, senescence, apoptosis, angiogenesis, resistance to chemotherapy and radiotherapy and tumor invasiveness and metastasis. Targeting of a single cancer cell trait often provides limited success in cancer treatment and, thus, simultaneous approach to several of them may be a more appropriate strategy. The contribution of ZEB1 and ZEB2 to multiple cancer hallmarks, along with their highly modular structure and complex transcriptional activities, offers translational

researchers with an attractive therapeutic target. Examination of the levels of ZEB1 and ZEB2 expression (or lack of) in primary tumors may help to prospectively identify their resistance (or sensitivity) to particular chemotherapy treatments. In addition, the inhibition of ZEB1, ZEB2 or its cofactors could be used to reverse drug resistance in cancer patients. Altogether, ZEB1 and ZEB2 are thus poised to become important diagnostic, prognostic and/or therapeutic cancer targets in the near future.

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