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TESIS DOCTORAL 2012

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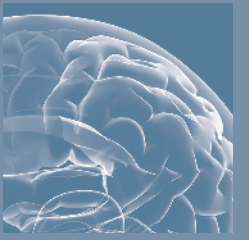

Hyperactivation of the TGF-β signaling pathway in glioblastoma: mechanisms and consequences

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Tesis Doctoral

Barcelona 2012

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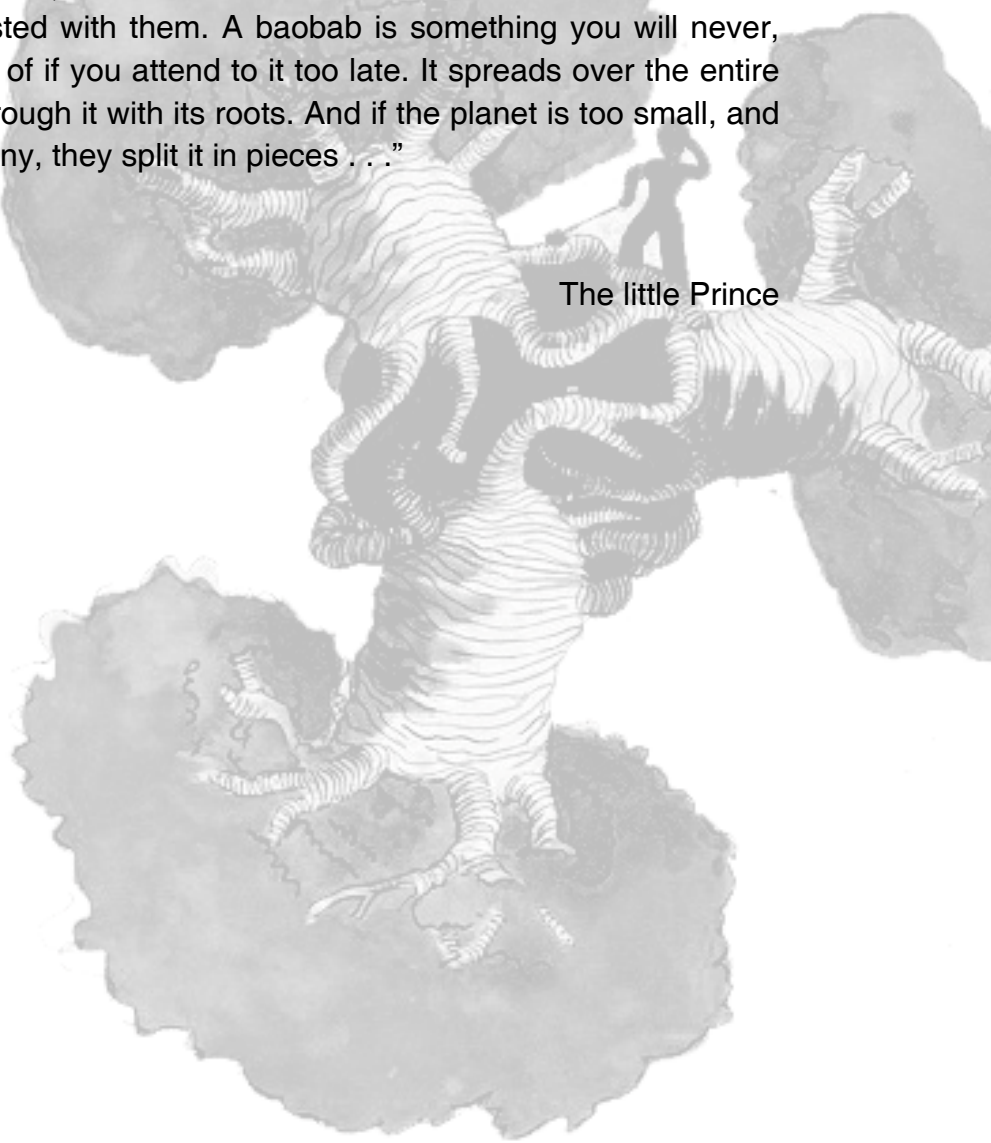
En recuerdo de Daniel Luque Díaz

“Indeed, as I learned, there were on the planet where the little prince lived--as on all planets--good plants and bad plants. In consequence, there were good seeds from good plants, and bad seeds from bad plants. But seeds are invisible. They sleep deep in the heart of the earth's darkness, until some one among them is seized with the desire to awaken. Then this little seed will stretch itself and begin--timidly at first--to push a charming little sprig inoffensively upward toward the sun. If it is only a sprout of radish or the sprig of a rose-bush, one would let it grow wherever it might wish. But when it is a bad plant, one must destroy it as soon as possible, the very first instant that one recognizes it. Now there were some terrible seeds on the planet that was the home of the little prince; and these were the seeds of the baobab. The soil of that planet was infested with them. A baobab is something you will never, never be able to get rid of if you attend to it too late. It spreads over the entire planet. It bores clear through it with its roots. And if the planet is too small, and the baobabs are too many, they split it in pieces . . .”

The little Prince



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Introducción

Los gliomas, incluyendo los astrocitomas, los oligodendrogliomas, los oligoastrocitomas y los glioblastomas (GBM), son los tumores malignos primarios de cerebro más comunes. En función de su agresividad, los gliomas se clasifican en cuatro grados clínicos. La forma más maligna de todos ellos, el GBM (astrocitoma de grado IV), es uno de los tumores humanos más agresivos, no tiene curación y su esperanza de vida media es de tan solo un año.

Un factor de gran relevancia en la progresión del glioma es el factor de crecimiento transformante β (TGF-β). Esta citoquina controla el desarrollo embrionario y regula la homeostasis de los tejidos epiteliales. El TGF-β inhibe la proliferación de células epiteliales y de ciertos tumores de estadío temprano, y es por tanto un factor anti-tumorogénico. Sorprendentemente, en ciertos tumores avanzados, como GBM, el TGF-β actúa como factor inductor de la progresión tumoral.

En GBM, la vía de señalización de TGF-β se encuentra hiperactivada y es factor de mal pronóstico ya que promueve la invasividad, la angiogénesis y la metástasis del tumor, así como la creación de un ambiente local de inmunosupresión. Por otro lado, TGF-β también es capaz de inducir la auto-renovación de una población celular con características de células madre llamada “células iniciadoras de glioma”.

La hiperactivación de la vía de señalización de TGF-β en GBM en parte está causada por un aumento de la secreción por parte de las células tumorales y las células del estroma de los ligandos de TGF-β. En mamíferos, se han descrito 3 isoformas de TGF-β: TGF-β1, TGF-β2 y TGF-β3. De entre ellas, TGF-β2 es la principal responsable de la actividad TGF-β en GBM.

TGF-β activa un complejo heterodimérico formado por el receptor de TGF-β de tipo II (TβR-II) y tipo I (TβR-I) e inicia una cascada de señalización intracelular a través de la fosforilación de los SMADs regulados por receptor (R-SMADs),

SMAD2 y SMAD3. La fosforilación de R-SMADs facilita la unión a SMAD4 y entonces el complejo SMAD se dirige al núcleo donde regula la expresión génica.

Uno de los mecanismos de regulación de la vía de señalización de TGF-β es a través de la ubiquitinación de sus componentes. SMAD7 se une a la enzima ligasa de ubiquitina específica de SMADs 2 (SMURF2) y al complejo receptor de TGF-β para facilitar su ubiquinización. Esto conduce a la degradación del complejo receptor de TGF-β por el proteasoma y a la atenuación de la señalización TGF-β. La ubiquitinación es un proceso reversible, de tal manera que los residuos ubiquitina pueden excindirse de las proteínas mediado por la actividad de las deubiquitinasas. Las deubiquitinasas juegan un papel de gran importancia en cáncer. Sin embargo, se conoce muy poco aún sobre su implicación en la vía de TGF-β y GBM.

Dado su papel oncogénico en GBM, la vía de señalización de TGF-β es una diana terapéutica contra esta enfermedad. Diversos fármacos anti-TGF-β están siendo evaluados en ensayos clínicos y muestran resultados esperanzadores. Por otro lado, los mecanismos precisos que originan la hiperactivación de la vía de señalización de TGF-β en GBM son aún ampliamente desconocidos. Es de gran importancia, pues, estudiar en profundidad los mecanismos que causan esta aberrante activación de TGF-β en GBM con el fin de identificar nuevos marcadores de respuesta a fármacos anti-TGF-β y diseñar nuevas y eficientes estrategias terapéuticas contra esta grave enfermedad.

Objetivos

La vía de señalización de TGF- β se encuentra hiperactivada en GBM y es factor de mal pronóstico. El principal objetivo de esta tesis es descifrar los mecanismos moleculares que generan la hiperactivación de la actividad TGF-β en GBM con el fin de descubrir nuevas dianas terapéuticas contra esta enfermedad.

Para ello, hemos seguido dos aproximaciones. En primer lugar, ya que las deubiquitinasas tienen un papel muy relevante en cáncer y su participación en la regulación de la vía de señalización de TGF-β y GBM es ampliamente desconocida, estudiaremos la implicación de estas enzimas en la actividad TGF-β en este tipo de cáncer y centraremos nuestra atención en aquellas que específicamente activan TGF-β. En segundo lugar, dado que TGF-β2 está sobre-expresado en GBM y juega un papel clave en la hiperactivación de la vía de señalización, estudiaremos los mecanismos moleculares que regulan la expresión de TGF-β2 en GBM.

Resultados

Con el fin de identificar nuevas deubiquitinasas involucradas en la regulación de la vía de señalización de TGF-β, realizamos un cribaje funcional utilizando una librería de vectores con ARN de interferencia contra las diversas deubiquitinasas descritas en el genoma humano. Identificamos la proteasa específica de ubiquitina 15 (USP15) como una deubiquitinasa clave en la regulación de la señalización de TGF-β. USP15 se une al complejo SMAD7-SMURF2 y deubiquitiniza y estabiliza TβR-I. Por tanto, USP15 induce la activación de la vía TGF-β a través de la estabilización de TβR-I. Observamos que algunos GBMs expresan niveles elevados de USP15 como consecuencia de la amplificación del gen y que la amplificación del gen USP15 confiere mal pronóstico a pacientes con GBM. La amplificación de USP15 se da también en otros tipos tumorales como cáncer de mama y de ovario. La reducción de los niveles o la inhibición de la actividad de USP15 en un modelo murino ortotópico derivado de células tumorales de pacientes de GBM disminuye la actividad TGF-β. Así mismo, la depleción de USP15 reduce la capacidad oncogénica de las células iniciadoras de tumores derivadas de pacientes debido a la represión de la señalización de TGF-β. Nuestros resultados, por tanto, muestran que USP15 regula la vía de señalización de TGF-β y es un factor clave en la patogénesis de GBM.

Paralelamente, observamos que TGF-β induce la expresión de TGF-β2 en GBM. TGF-β2 está sobre-expresado en GBM y confiere mal pronóstico. Identificamos la proteína de unión al elemento de respuesta a AMPc (CREB) como un activador crítico en la regulación de este ciclo autocrino de producción de TGF-β2. En respuesta a TGF-β, la proteína fosforilada CREB (p-CREB) y SMAD2 se unen al promotor de TGF-β2 e inducen su transcripción. La activación de CREB, mediada por la vía de transducción de señal de fosfoinositol 3 quinasa (PI3K) a través de la proteína quinasa B (AKT) y la vía de señalización de la quinasa S6 ribosomal (RSK), aumenta la expresión de TGF-β2 en respuesta a TGF-β. Observamos que los niveles de AKT fosforilada (p-AKT), p-CREB y TGF-β2 correlacionan en muestras de pacientes de GBM. La inhibición farmacológica de PI3K y la disminución de los niveles totales de CREB mediante ARN interferente reducen la expresión de TGF-β2 en un modelo murino ortotópico derivado de células tumorales de pacientes de GBM. Así mismo, la depleción de CREB reduce la capacidad oncogénica de las células iniciadoras de tumores derivadas de pacientes. Nuestros resultados, por tanto, muestran que CREB regula la inducción de la expresión de TGF-β2 mediada por TGF-β y es un factor clave en la patogénesis de GBM.

Discusión

GBM es uno de los tumores humanos más agresivos y actualmente es prácticamente incurable.Una de las proteínas clave en el desarrollo de GBM es TGF-β. La vía de señalización de TGF-β se encuentra hiperactivada en GBM y es factor de mal pronóstico. Sin embargo, los mecanismos moleculares que dan lugar a la aberrante activación de TGF-β en GBM son ampliamente desconocidos.

En este trabajo, mediante el uso de líneas celulares y especialmente de muestras derivadas de pacientes de GBM, hemos descrito dos nuevos mecanismos moleculares que dan lugar a una hiperactivación de la actividad TGF-β en GBM, tales como la estabilización de TβR-I debido a la sobre-

expresión de USP15 o la sobre-secreción de TGF-β2 debido a la generación de un ciclo maligno autocrino mediado por CREB.

Nuestros resultados sugieren que tanto USP15 como CREB podrían ser evaluados como dos nuevos marcadores de respuesta a fármacos anti-TGF-β y nuevas dianas terapéuticas contra GBM.

Conclusiones

- USP15 es una deubiquitinasa clave en la regulación de la vía de señalización de TGF-β y la patogénesis de GBM.
- USP15 deubiquitiniza y estabiliza TβR-I, aumentando, así, la actividad de TGF-β y la tumorogénesis mediada por TGF-β.
- El gen USP15 está sobre-expresado y amplificado en GBM y es factor de mal pronóstico.
- TGF-β2 está sobre-expresado en GBM y es factor de mal pronóstico.
- La sobre-expresión de TGF-β2 en GBM en parte está causada por la generación de un ciclo autocrino en el cual TGF-β induce la expresión de TGF-β2 a través de SMAD2 y del factor de transcripción CREB.
- La sobre-expresión de USP15 y TGF-β2 en GBM causa una hiperactivación de la vía de señalización de TGF-β y en consecuencia progresión tumoral.

Abbreviations

ABBREVIATIONS

AKT	v-akt murine thymoma viral oncogene homolog
ANGPTL4	angiopoietin-like 4
AP1	adaptor protein complex 1
APC	adenomatous polyposis coli
ATF1	activating transcription factor 1
ATF2	activating transcription factor 2
ATF3	activating transcription factor 3
BCA	bicinchoninic acid
BCL2	B-cell CLL/lymphoma 2
BMP	bone morphogenetic protein
BMP4	bone morphogenetic protein 4
BSA	bovine serum albumin
bZIP	basic leucine zipper domain
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
CDKN2A	cyclin-dependent kinase inhibitor 2A
CEN12	centromere 12
ChIP	chromatin immunoprecipitation

ABBREVIATIONS

CREB	cAMP responsive element binding protein
CREM	cAMP responsive element modulator
CTGF	connective tissue growth factor
CTXCR4	chemokine (C-X-C motif) receptor 4
CYLD	cylindromatosis
d	days
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DUB	deubiquitinating enzyme
DUSP	domain present in ubiquitin-specific proteases
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EWS	Ewing's sarcoma
FGF	fibroblast growth factor
FISH	fluorescence *in situ* hybridization
FOXO	forkhead box O
FOXO3	forkead box O 3
FRA2	FOS-like antigen 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBM	glioblastoma
GFP	green fluorescent protein

ABBREVIATIONS

GIC	glioma initiating cell
H1047R	mutated form of PI3K (PI3K H1047R) which is constitutively active
HA	hemaglutinin
HEC1	NDC80 kinetochore complex component homolog
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOXA10	homeobox A10
HRP	horseradish peroxidase
ID1	inhibitor of DNA binding 1
IDH1	isocitrate dehydrogenase 1
igG	immunoglobulin G
IKBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL11	interleukin 11
inh	inhibitor
IP	immunoprecipitation
JAMM	Josephins and JAB1/MPN/MOV34 metalloenzymes
JUND	jun d proto oncogene
KID	kinase inducible domain
LIF	leukemia inhibitory factor
LYS	lysine
MCPIP	monocyte chemotactic protein-induced protein
MET	hepatocyte growth factor receptor

ABBREVIATIONS

MHC	major histocompatibility complex
MMP2	matrix metalloprotease 2
MMP9	matrix metalloprotease 9
MRI	magnetic resonance imaging
mRNA	messenger RNA
mutCRE	mutated CREB binding site
MYC	v-myc myelocytomatosis viral oncogene homolog
NaCl	sodium cloryde
NF1	neurofibromin 1
NF2	neurofibromin 2
NFK-B	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NOD-SCID	non obese diabetic- severe combined immunodeficiency
OTU	ovarian tumor proteases
p-	phospho
p15INK4b	cyclin-dependent kinase inhibitor B
p21CIP1	cyclin-dependent kinase inhibitor 1A
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCTC	primary culture tumor cell
PDGF-B	platelet-derived growth factor B

ABBREVIATIONS

PDGFRA	platelet-derived growth factor receptor A
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real time PCR
RB	retinoblastoma
RBX1	ring-box protein 1
REMBRANDT	Repository for Molecular Brain Neoplasia Data
RFX1	regulatory factor X, 1
RNA	ribonucleica cid
RNAi	RNA interference
RSK	ribosomal protein S6 kinase
R-SMAD	receptor regulated SMAD
RTK	receptor tyrosine kinases
s.d.	standard deviation
S6	ribosomal protein S6
SBE	smad binding element
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sh	short hairpin

ABBREVIATIONS

shRNA	short hairpin RNA
si	small interfering
siRNA	small interfering RNA
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
SMURF2	SMAD specific E3 ubiquitin protein ligase 2
SOX2	SRY (sex determining region Y)-box 2
TCGA	The Cancer Genome Atlas
TGF-β	transforming growth factor β
TGF-β1	transforming growth factor β1
TGF-β2	transforming growth factor β2
TGF-β3	transforming growth factor β2
TORC	CREB regulated transcription coactivator
TP53	tumor protein 53
TβR-I	transforming growth factor β receptor I
TβR-I–CA	TβR-I constitutively active
TβR-II	transforming growth factor β receptor II
UB	ubiquitin
UBL	ubiquitin like domain
UCH	ubiquitin C-terminal hydrolases
UCH37	ubiquitin carboxyl-terminal hydrolase L5
UCHL1	ubiquitin carboxyl-terminal esterase L1
USF1	upstream transcription factor 1
USF2	upstream transcription factor 2

ABBREVIATIONS

USP	ubiquitin-specific proteases
USP11	ubiquitin-specific protease 11
USP14	ubiquitin-specific protease 14
USP15	ubiquitin-specific protease 15
USP15C/S	catalytically inactive USP15 mutant C269S
USP4	ubiquitin-specific protease 4
USP8	ubiquitin specific peptidase 8
USP9X	ubiquitin specific peptidase 9, X-linked
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WNT	wingless-type MMTV integration site family

Introduction

1. INTRODUCTION

1.1 Glioma

1.1.1 General aspects of gliomas

Gliomas are primary brain tumors that have morphological and gene-expression characteristics similar to glial cells (astrocytes, oligodendrocytes and ependymal cells). They are the most frequent malignant primary brain tumors, with an incidence of approximately six cases per 100,000 individuals a year (**Fig. 1a**).

The causes that give rise to gliomas are complex and largely unknown. Most gliomas arise sporadically and are not inherited within families suggesting that complex genetic abnormalities combined with unknown environmental factors predispose individuals to glioma. However, a genetic predisposition is known in the setting of rare familial tumor syndromes such as neurofibromatosis, due to *neurofibromin 1* and *2* (*NF1* and *NF2)* mutations, or Li Fraumeni syndrome, due to mutations in *tumor protein 53 (TP53) [1, 2]*.

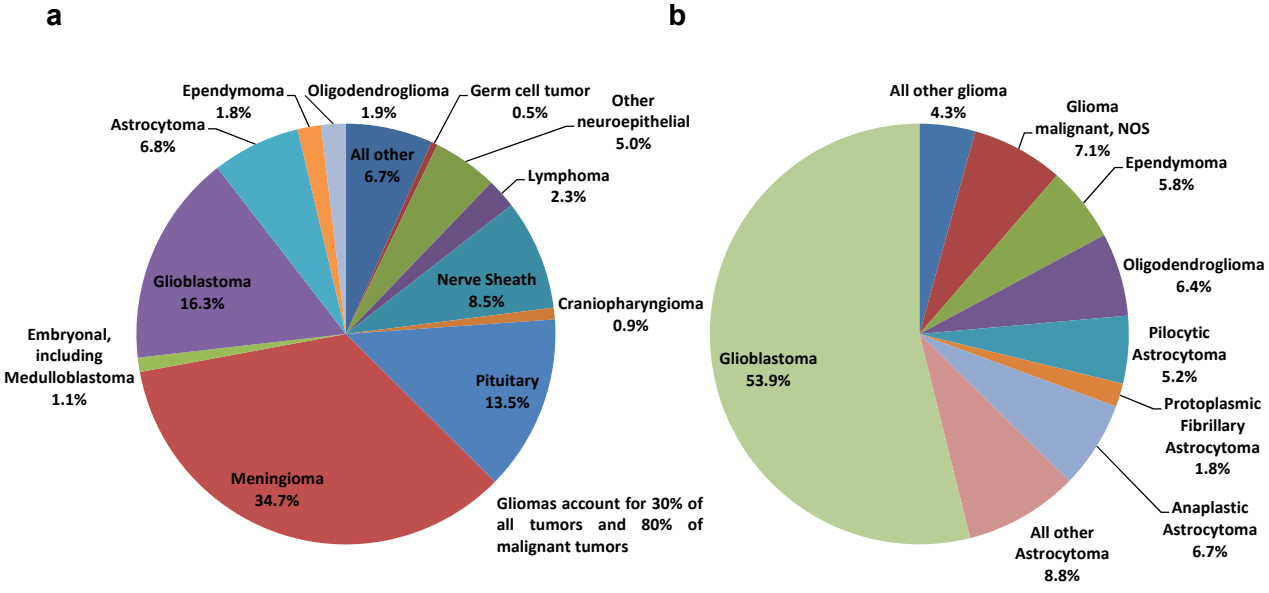


Figure 1. Incidence of the different primary brain and central nervous system tumors types between 2004-2008. Data obtained from the Central Brain Tumor Registry of the United States. (**a**) Distribution of all primary brain and central nervous system tumors by histology. (**b**) Distribution of all primary brain and central nervous system gliomas by histology subtypes.

1.1.2 Classification of gliomas

The common gliomas affecting the cerebral hemispheres of adults are termed "diffuse" gliomas due to their propensity to infiltrate, early and extensively, throughout the parenchyma. These gliomas are classified histologically, immunohistochemically and/or ultrastructurally as astrocytomas, oligodendrogliomas and mixed oligoastrocytomas respectively, relying on similarities between tumor cells and mature normal glial cells.

Tumors are then graded based on a World Health Organization (WHO) consensus-derived scale of I to IV according to tumor differentiation, cellularity, cytonuclear atypia, mitotic activity, microvascular proliferation, necrosis status and genetic alterations. Grade I tumors are biologically benign and can be cured if they can be surgically resected; grade II tumors (diffuse infiltrating low-grade gliomas) are low-grade malignancies that may follow long clinical courses but early diffuse infiltration of the surrounding brain renders them incurable by surgery; grade III tumors (anaplastic gliomas) exhibit increased anaplasia and proliferation and are more rapidly fatal; and finally, grade IV tumors (glioblastomas) exhibit more advanced features of malignancy, including vascular proliferation and necrosis. On the basis of clinical presentation, glioblastomas (GBMs) have been further subdivided into primary or secondary GBM subtypes according to whether they form de novo (primary) or arise from lower-grade gliomas (secondary) [1, 2].

1.1.3 GBM

1.1.3.1 General aspects of GBMs

GBM is an astrocytic tumor grade IV. It is the most frequent and severe subtype of glioma and accounts for about 50% of diffuse gliomas. GBM affects men in a higher proportion than women (3:2) and usually occurs in adults around 60 years of age (**Fig. 1b**).

GBM is defined by an uncontrolled cellular proliferation, diffuse infiltration, propensity for necrosis, robust angiogenesis and rampant genomic instability. Moreover, GBM presents significant intratumoral heterogeneity on the cytopathological, transcriptional and genomic levels. This complexity and an

incomplete atlas of (epi)genetic lesions driving GBM pathogenesis, makes this cancer one of the most difficult to understand and treat.

Standard therapy consists of surgical resection, followed by radiation and temozolomide therapy. Unfortunately, median survival is only 12-15 months [1, 2].

1.1.3.2 Classification of GBMs

On the basis of clinical presentation, GBMs have been further subdivided into primary or secondary subtypes. Primary GBMs arise *de novo* and exhibit p53 and retinoblastoma (RB) pathway dysfunction, as well as receptor tyrosine kinases (RTK)/RAS/phosphoinositide-3-kinase (PI3K) signaling deregulation, leading to tumors that arise in older patients with a worse prognosis. In contrast, secondary GBMs are preceded by lower-grade II lesions, which progress either through grade III lesions or directly to GBM. These latter tumors generally occur in younger patients and are characterized by a mutant *isocitrate dehydrogenase 1 (IDH1)* genotype that confers a better prognosis.

On the basis of genomic features, GBMs can be divided into 4 transcriptional subclasses termed proneural, neural, mensenchymal and classical. The proneural subtype is dominated by amplifications of *platelet-derived growth factor receptor A* (*PDGFRA)*, cyclin-*dependent kinase 6* and *4* (*CDK6*, *CDK4)* and *hepatocyte growth factor receptor (MET)* and mutations in *IDH1* and *TP53*. The classical subtype is dominated by amplifications of *epidermal growth factor receptor (EGFR)* and loss of *phosphatase and tensin homolog* (*PTEN)* and *cyclin-dependent kinase inhibitor 2A (CDKN2A)* whereas the mesenchymal subtype shows mutations and/or loss of *NF1*,*TP53* and *CDKN2A*. To date, no unique genetic alterations define the neural class from the other classes. The prognostic value of the molecular subclassification has also been evaluated, with several studies suggesting that GBMs with a gene signature associated with neurogenesis (proneural subtype) generally have improved survival. In contrast, GBMs with mesenchymal gene expression (mesenchymal subtype) usually have a poorer outcome [1-4].

1.1.3.3 Core signaling pathways in GBM

In the past two decades, cytogenetic and molecular genetic studies have identified a number of recurrent chromosomal abnormalities and genetic alterations in malignant gliomas, particularly in GBM. In all, three signaling pathways have been identified as the core signaling pathways commonly activated in GBM: the p53 pathway, the RB pathway and the RTK pathway, with *CDKN2A/B*, *TP53*, *EGFR*, *PTEN* and *NF1* the most frequently altered genes in GBM (**Fig.2**). [1, 5, 6]

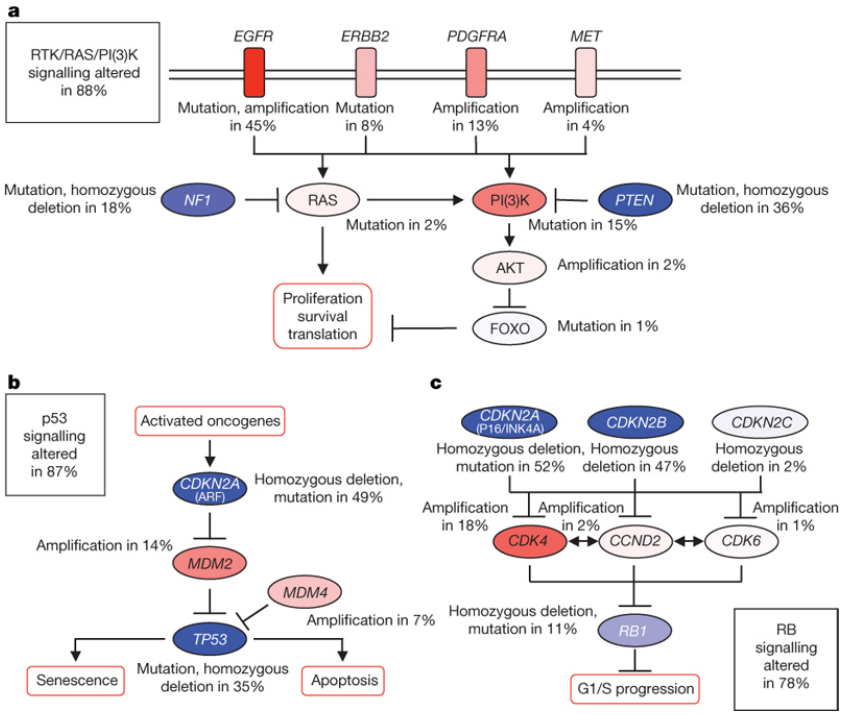


Figure 2. Frequent genetic alterations in GBM. *CDKN2A/B*, *TP53*, *EGFR*, *PTEN* and *NF1* are the most frequently altered genes in GBM (adapted from [6]).

1.1.4 Cells of origin and glioma initiating cells

The cellular origins of GBM continue to be a source of debate. Numerous studies have provided evidence that gliomas arise from the normal reservoir of stem cells and progenitor cells within the brain, and numerous studies in genetically engineered mouse models have supported this idea in that a diverse range of glioma-relevant mutations targeted to neural stem cells in vivo readily produce gliomas with high fidelity and penetrance. However, gliomas may also

arise from terminally differentiated cells, most likely through a process of dedifferentiation. Given the diverse number of histological subtypes, various subsets of molecular patterns and subclasses, and increasingly broad numbers of stem and progenitor cells in the brain, further studies will be required to clarify the origins of GBM and the programs that drive their subsequent evolution [1].

Recently, a subpopulation of tumor cells with stem-cell-like properties have been identified in gliomas. This cell population, known as cancer stem cells, glioma stem cells, brain tumor initiating cells, or glioma initiating cells (GICs), is considered to be responsible for the initiation, propagation, and recurrence of tumors, indicating that more effective therapies will result from approaches aimed at targeting the stem-cell-like compartment of gliomas. GICs are characterized by their highly oncogenic potential, their self-renewal capacity, their multilineage differentiation properties, and their ability to generate detached spherical cellular structures (neurospheres) when cultured in serum-free medium [1, 7].

1.2 TGF-β signaling pathway

1.2.1 The TGF-β pathway

Transforming growth factor β (TGF-β) is a cytokine with a vast array of biological functions including cell proliferation, morphogenesis, migration, extracellular matrix production, cytokine secretion and apoptosis, and plays a critical role in tissue homeostasis, embryonic development and cancer.

Three different TGF-β isoforms are expressed in mammalian tissues: TGF-β1, TGF-β2 and TGF-β3. TGF-β binds and activates a heterodimeric complex formed by the type II TGF-β receptor (TβR-II) and the type I TGF-β receptor (TβR-I), both of which are transmembrane Ser/Thr kinase receptors. Upon TGF-β binding, TβR-II phosphorylates TβR-I, which in turn initiates an intracellular signaling cascade through phosphorylation of the specific receptor regulated SMADs (R-SMADs, SMAD2 and SMAD3) in their C-terminal SXS motif. The phosphorylation of R-SMADs facilitates the binding to SMAD4. The SMAD complex then shuttles to the nucleus, where it interacts with various DNA

binding proteins to regulate gene expression in a cell type and context specific manner. Although SMAD proteins possess DNA-binding activity, the SMAD4-R-SMAD complexes must associate with additional DNA-binding cofactors in order to achieve binding with high affinity and selectivity to specific target genes. These SMAD partners are drawn from various families of transcription factors, such as the forkhead, homeobox, zinc-finger and adaptor protein complex 1 (AP1) families. Through this combinatorial interaction with different transcription factors, a common TGF-β stimulus can activate or repress hundreds of target genes at once [8].

The TGF-β signaling pathway is tightly regulated through protein ubiquitination. SMAD7 acts as a scaffold protein to recruit SMAD specific E3 ubiquitin protein ligase 2 (SMURF2) to the TGF-β receptor complex, thus facilitating its ubiquitination. This leads to proteasome-mediated degradation of TGF-β receptors and attenuation of TGF-β signaling [9-11]. (**Fig.3**)

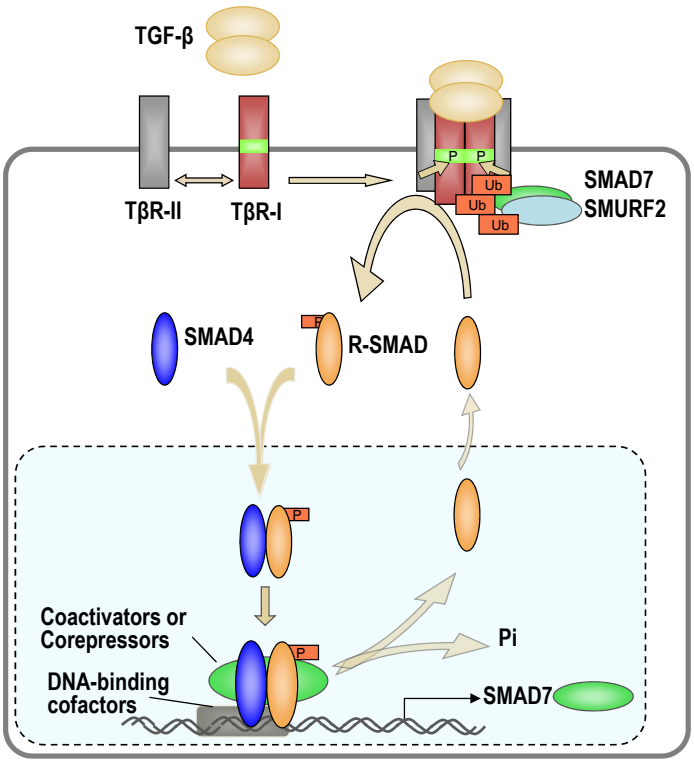


Figure 3. The TGF-β signaling pathway. TGF-β binds and activates a membrane receptor serine/threonine kinase complex, which phosphorylates SMAD2 and SMAD3. Upon phosphorylation, SMADs accumulate in the nucleus, form transcriptional complexes with SMAD4 and other transcription factors, and regulate transcription. SMAD7 recruits the E3 ubiquitin protein ligase SMURF2 to the TGF-β receptor

complex, thus facilitating its ubiquitination, degradation by the proteasome and attenuation of TGF-β signaling.

1.2.2 TGF-β signaling in cancer

Many of the TGF-β cellular responses are critical for tissue homeostasis, and disruption or aberrant regulation of the TGF-β pathway is implicated in several human disorders including cancer.

TGF-β is a potent antiproliferative factor in premalignant cells through the induction of two CDK inhibitors, cyclin-dependent kinase inhibitor B (p15INK4b*)* and cyclin-dependent kinase inhibitor 1A (p21CIP1) and the downregulation of v-myc myelocytomatosis viral oncogene homolog (MYC). Moreover, depending on the cell type, TGF-β can act as an inducer of apoptosis. Due to its anti-proliferative and apoptotic functions, TGF-β is considered to be a tumor suppressor factor.

During tumor progression, tumor cells tend to lose the tumor-suppressive responses to TGF-β. This can occur due to loss of the receptor, as it occurs in a proportion of colorectal cancers, or by silencing of tumor suppressive gene responses, as in breast adenocarcinoma and GBM. Cancer cells that retain TGF-β signaling but lack tumor suppressive responses are free to use the SMAD signaling pathway to their advantage, by allowing it to stimulate genes, such as *interleukin 11* (*IL11)*, *connective tissue growth factor* (*CTGF)*, *chemokine (C-X-C motif) receptor 4* (*CXCR4)*, *angiopoietin-like 4 (ANGPTL4)* and platelet-derived growth factor B (*PDGF-B*), and reprogram phenotypes (e.g., the epithelial-mesenchymal transition) that, in this context, enhance proliferation, angiogenesis, immunosuppression, invasiveness and metastasis .

Work carried out over recent years has begun to uncover several mechanisms involved in the disruption of the TGFβ-induced cell cycle arrest. For example, a highly active PI3K pathway prevents forkhead box O (FoxO) nuclear localisation and inhibits the TGF-β-dependent induction of p21CIP1. High levels of MYC repress the induction of p15INK4b and p21CIP1 by TGF-β through the repressive transcriptional complex MYC-MIZ1. In some tumors, CDK2 and 4 can phosphorylate SMAD3 inhibiting its transcriptional activity and hence preventing its ability to induce p15INK4b and p21CIP1 and down-regulate MYC levels [12, 13].

Due to its oncogenic function, there is growing interest in TGF-β as a therapeutic target. Inhibitors of the TGF-β pathway developed to date include antisense oligonucleotides, anti-TGF-β antibodies, anti-receptor antibodies, TGF-β-trapping receptor ectodomain proteins and small molecule inhibitors that target TGF-β receptor kinases. Members of each class of these inhibitors have entered clinical trials [14, 15].

1.2.2.1 TGF-β in glioma

The TGF-β pathway plays a critical role in glioma [16, 17]. TGF-β is highly active in high grade glioma and elevated TGF-β activity confers poor prognosis in glioma patients (**Fig. 4**) [16].

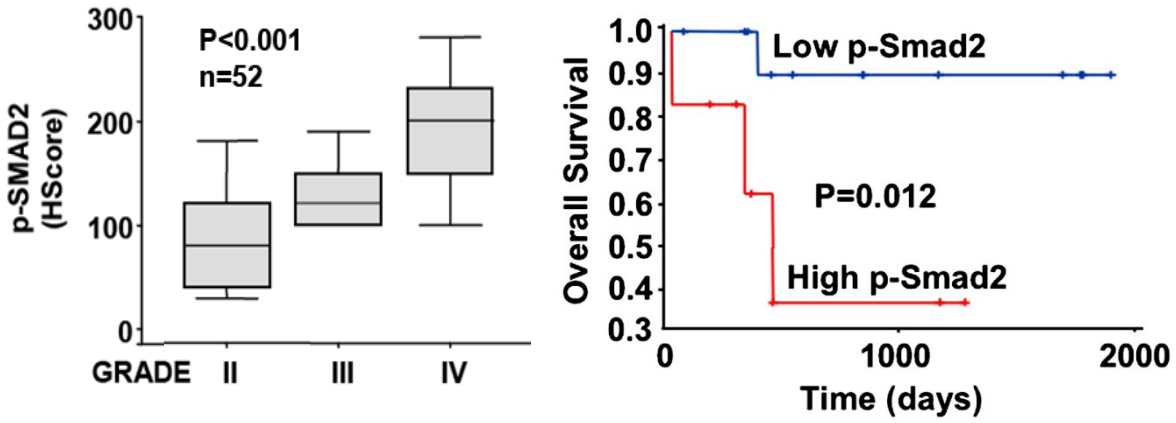


Figure 4. The TGF-β signaling pathway is highly active in high grade glioma and confers poor prognosis in glioma patients (adapted from [16]).

High TGF-β activity can be achieved through diverse mechanisms, including the oversecretion of TGF-β by tumor cells or tumor stroma cells.

In gliomas, the tumor suppressor function of TGF-β is lost owing to PI3K hyperactivation, loss of *p15INK4b*, overexpression of *MYC* or mutational inactivation of *RB*. This allows glioma cells to profit from TGF-β-oncogenic properties. The oncogenic response to TGF-β is pleiotropic and includes the induction of proliferation, invasion, angiogenesis, and immunosuppression.

TGF-β promotes tumor cell proliferation by stimulating the production of autocrine mitogenic factors such as PDGF-B. TGF-β also has an important role in promoting tumor invasion via different mechanisms that include the upregulation of matrix metalloproteases (MMP2 and MMP9), extracellular matrix proteins (collagen, fibronectin, laminin, tenascin, vitronectin and versican) and cell adhesion molecules (*L1*, *α3V3*). Furthermore, TGF-β induces the expression of vascular endothelial growth factor (VEGF) by vascular endothelial cells and glioma cells and triggers endothelial growth and angiogenesis. Immunosupression is achieved mainly by inhibiting the proliferation, differentiation and activation of T-cells, in particular that of CD8-positive cytotoxic T-cells [17]. Of note, TGF-β can increase the self-renewal capacity of GICs through the induction of leukemia inhibitory factor (LIF) or SRY (sex determining region Y)-box 2 (SOX2) [18, 19]. Inhibition of the TGF-β pathway targets a cell population, enriched for GICs, characterized by high expression of CD44 and inhibitor of DNA binding 1 (ID1). The depletion of the $CD44^{high}/ID1^{high}$ GIC population by TGF-β inhibitors prevents tumor initiation and recurrence [20].

The notion that TGF-β is a key mediator of various pathological characteristics of gliomas has resulted in attention being focused on TGF-β as a potencial target in glioma therapy. Currently, there are phase III clinical trials ongoing with Trabedersen, an anti-TGF-β2 oligonucleotide, and phase II clinical trials with LY2157299, a small molecule TβR-I kinase inhibitor [15].

1.2.3 TGF-β ligands

Three different TGF-β isoforms have been identified in mammals, namely TGF-β1, TGF-β2 and TGF-β3 which share about 80% amino acid sequence identity. The three isoforms show a distinct spatial and temporal expression pattern and also display differences regarding processing, activation and receptor interaction.

Throughout embryogenesis all three TGF-β isoforms are expressed. In normal adult tissues, TGF-β1 is by far the predominant isoform, while expression of TGF-β2 and TGF-β3 is much more restricted. All three isoforms are expressed

within the nervous system, in neurons and glial cells. In general, TGF-β2 and TGF-β3 are found ubiquitously and are mostly coexpressed in the central nervous system, whereas TGF-β1 is predominantly found in the choroid plexus and in the meninges; interestingly, this isoform is upregulated following central nervous system injuries or in cases of neurodegeneration. In the last mentioned cases, it is involved in coordinating inflammatory responses and brain repair [21].

The three isoforms of TGF-β are differentially expressed in gliomas. GBM cells preferentially express the TGF-β2 isoform [22, 23].

1.2.3.1 TGF-β2

TGF-β2 is ubiquitiously expressed both during development and in adult tissue in the central nervous system. Remarkably, TGF-β2 is the predominant TGF-β isoform expressed in GBM [22, 23]. Elevated TGF-β2 levels are responsible for the increased activity of the TGF-β pathway in GBM [16] and are associated with advanced disease stage, poor prognosis and loss of tumor immune surveillance [16, 22-25].

Given the high importance of TGF-β2 in GBM, many efforts have been directed towards understanding the molecular mechanisms that drive the expression of TGF-β2 in order to design future strategies that block TGF-β2 secretion. Among them, the wingless-type MMTV integration site family (WNT) signaling pathway induces TGF-β2 expression via activating transcription factor 2 (ATF2) binding to the *TGF-β2* promoter [26]. At the same time, it has been shown that RB binds to ATF2 and JNK/p38 forming a transcriptional complex that activates TGF-β2 expression [27]. cAMP responsive element binding protein (CREB) can also bind to the *TGF-β2* promoter and induce its expression [28]. In different cellular contexts, the PI3K signaling pathway has been shown to both activate and repress TGF-β2. PI3K induces the phosphorylation of TWIST which in turn binds to an E-BOX present in the *TGF-β2* promoter and induces its expression [29]. On the other hand, PI3K activation avoids the binding of forkhead box O 3 (FOXO3) to the *TGF-β2* promoter and inhibits TGF-β2 expression [30]. Other

transcription factors that regulate TGF-β2 are upstream transcription factor 1 and 2 (USF1 and USF2) [28], homeobox A10 (HOXA10) [31] and regulatory factor X, 1 (RFX1) [32].

Due to the unique role of TGF-β2 in glioma, a targeted therapy to modulate TGF-β2 has been developed. The antisense oligonucleotide Trabedersen specifically blocks *TGF-β2* mRNA. In three phase I/II studies and a randomized, active-controlled dose-finding phase IIb study, Trabedersen treatment of high-grade glioma patients with recurrent or refractory tumor disease led to long-lasting tumor responses and promising survival data. On the basis of these data the currently ongoing phase III study SAPHIRRE has been initiated [33].

1.3 Ubiquitination and deubiquitination

1.3.1 General aspects of ubiquitination and deubiquitination processes

Ubiquitin is a key member of a family of structurally conserved proteins that regulate a host of processes in eukaryotic cells. Proteins can be post-translationally modified by binding to ubiquitin moieties, thereby changing their stability, localization or activity. This process is dynamic and reversible and is coordinated by the action of ubiquitinating and deubiquitinating enzymes.

The conjugation of ubiquitin to proteins is catalyzed by the successive action of three enzymes: a ubiquitin-activating enzyme, E1; a ubiquitin-conjugation enzyme, E2; and a ubiquitin ligase that transfers ubiquitin to a lysine residue or to the N-terminus of the target protein, E3 (**Fig.5b**).

Ubiquitin can be conjugated to target proteins either as a monomer or as polyubiquitin chains that vary in length and linkage type. Depending on the lysine residue involved in the formation of the polyubiquitin chain, there are multiple types of ubiquitin linkages with different physiological roles. Thus, Lys63-linked polyubiquitin chains and multiple mono-ubiquitin conjugations are preferentially involved in the lysosomal pathway, whereas Lys11-, Lys29-, and Lys48-linked polyubiquitin chains target proteins for proteasome degradation.

Ubiquitination is a reversible process and ubiquitin moieties can be removed from polypeptides by deubiquitinating enzymes (DUBs). The human genome encodes at least 98 DUBs, subdivided into 6 families, based on sequence and structural similarity: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs) and the recently discovered monocyte chemotactic protein-induced protein (MCPIP) family. All of them are cysteine proteases with the exception of JAMMs, which belong to the catalytic class of metalloproteases (**Fig. 5a**).

DUBs have fundamental roles in both ubiquitin homeostasis and protein stability control through varying enzymatic activities. First, DUBs can contribute to the generation of free ubiquitin moieties through their ability to process ubiquitin precursors. DUBs can also remove ubiquitin chains from post-translationally modified proteins, leading to reversal of ubiquitin signaling or to protein stabilization and rescue from either proteasomal or lysosomal degradation. And finally, DUBs can be used to edit the form of ubiquitin modification by trimming ubiquitin chains (**Fig. 5b**).

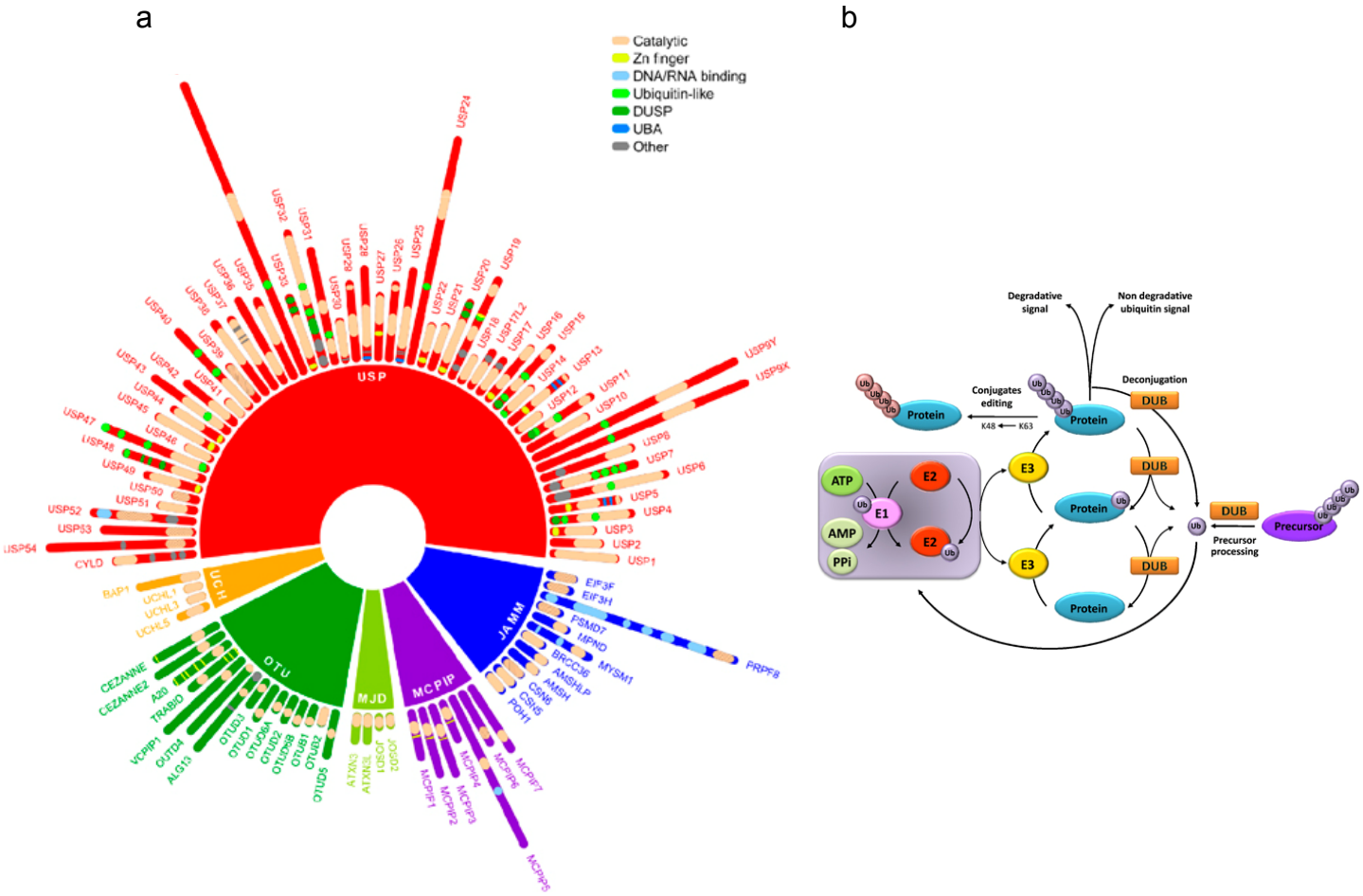


Figure 5. (**a**) Classification of human DUBs. DUBs are divided into 6 families, based on sequence and structural similarity: UCHs, USPs, OTUs, JAMMs and MCPIPs. (**b**) Enzymatic activities of DUBs. Schematic representation of the involvement of DUBs at different steps of the ubiquitination pathway. Ubiquitin is conjugated by the action of three consecutive enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligases E3 (adapted from [34]).

Target specificity is dependent on both substrate and ubiquitin chain type. For instance, ubiquitin-specific protease 14 (USP14) processes Lys48-linked chains whereas cylindromatosis (CYLD) only efficiently cleaves Lys63-linkages and linear ubiquitin chains. DUBs are also subject to several layers of regulation modulating their activity and specificity. These include transcriptional control of gene expression, post-translational modifications (phosphorylation, ubiquitination, sumoylation or proteolytic cleavage), changes in subcellular localization and activation mediated by interacting proteins [34-36].

1.3.2 USP15

1.3.2.1 Description of USP15

Ubiquitin specific peptidase 15 (USP15) is a member of the USP family of DUBs. The USPs constitute the largest DUB family described to date, with more than 50 members. All of them have highly conserved USP domains formed by three subdomains resembling the palm, thumb and fingers of a right hand. The catalytic site is located between the first two (palm and thumb) domains, whereas the distal, finger-like domain is responsible for interactions with ubiquitin. Many USPs exhibit additional domains that have important roles in their activity and specificity.

USP15 forms a subfamily with ubiquitin specific peptidase 4 and 11 (USP4 and USP11) called the DU family. The DU family members are characterized by presenting, aside from the USP domains, an N-terminal domain present in ubiquitin specific proteases (DUSP), followed by a predicted ubiquitin like domain (UBL). Neither of these domains has yet been assigned a function (**Fig 5a**).

1.3.2.2 Functions of USP15

USP15 associates with the COP9 signalosome and regulates protein degradation and protein signaling by catalyzing the ubiquitin deconjugation reaction of a discrete number of substrates. USP15 stabilizes adenomatous polyposis coli (APC) and thus promotes β-catenin degradation [37], and also regulates the Ring-box protein RBX1 [38] and influences the stability and activity of caspase-3 during Paclitaxel-induced apoptosis [39]. USP15 has also been implicated in the correct processing of polyubiquitinated substrates bound to p97 [40]. Moreover, USP15 plays a role in the downregulation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκ-B) pathway through NFκ-B inhibitor alpha (IκBα) [41].

1.3.3 DUBs in the TGF-β signaling pathway

The TGF-β pathway, as with many other signaling pathways, is tightly regulated to generate a precise and specific output depending on the cellular context. The fine tuning of the amplitude and intensity of the TGF-β signal is in part achieved through the modulation of the stability of several pathway components, such as SMADs and TGF-β receptors. Reversible post-translational modifications through ubiquination are one of the mechanisms that regulates the expression levels of SMADs and TGF-β receptors [8].

A number of reports have focused on the ubiquitination processes that regulate the TGF-β signaling and several E3 ubiquitin ligases and DUBs that target components of the TGF-β pathway have been identified [9, 10, 42-45]. Notably, SMAD7 acts as a scaffold protein recruiting SMURF2 to the TGF-β receptor complex to facilitate its ubiquitination. This leads to proteasome-mediated degradation of the TGF-β receptors and attenuation of TGF-β signaling [9, 10].

Before the publication of our work, two DUBs had been reported to be involved in the regulation of this pathway: ubiquitin specific peptidase 9, X-linked (USP9X) [42] and ubiquitin carboxyl-terminal hydrolase L5 (UCH37) [44]. USP9X positively regulates TGF-β signaling by deubiquitinating SMAD4 and promoting its association with SMAD2 [42]. UCH37 binds to SMAD7 and antagonizes the action of SMURF2 by specifically deubiquitinating the TβR-I, resulting in its rescue from proteosomal degradation [44].

1.3.4 DUBs in cancer

The wide functional diversity of DUBs has a profound impact on the regulation of multiple biological processes such as cell-cycle control, DNA repair, chromatin remodeling and several signaling pathways that are frequently altered in cancer. DUBs have also been related to different steps of cancer progression, such as epithelial-to-mesenchymal transition, cell migration or apoptosis.

The finding of several mutated DUBs acting as oncogenes or tumor suppressors, together with the frequent changes in the expression levels of

multiple DUB family members in malignant tumors, highlights the relevance of these enzymes in cancer development.

So far, no DUBs have been related to the development of glioma. Nevertheless, some DUBs related to cancer, such as ubiquitin carboxyl-terminal esterase L1 (UCHL1), ubiquitin specific peptidase 8 (USP8) and ubiquitin specific peptidase 11 (USP11), have been found to be mutated in some gliomas [34].

Because of their druggable enzymatic activity, DUBs can be considered therapeutic targets. Recent studies have evaluated different possibilities for targeting DUBs in cancer. The proposed therapies are based on blocking either DUB activity through specific inhibitors or DUB transcription by targeting extracellular factors, signal-transduction pathways or nuclear factors that activate expression of these genes. Interestingly, a selective small-molecule inhibitor of USP14 has been shown to effectively activate the proteasome-mediated degradation of unfolded proteins suggesting that it could be effective against neurodegenerative diseases or myeloma [46].

1.4 CREB transcription factor

1.4.1 General aspects of CREB transcription factor

Recent studies implicate the ATF/CREB family of transcription factors in cancer progression. The ATF/CREB family consists of sixteen cellular-stress-responsive transcription factors, divided into six subgroups, according to their sequence similarity. The common feature that all these proteins share is the bZIP element, through which they dimerize (homo or heterodimerize) and bind to DNA, and their ability to bind to the ATF/CREB consensus TGACGT$^{C}_{A}{}^{G}_{A}$ [47-49].

Subgroup	**Protein Member**
CREB	CREB CREM ATF1
CRE-BP1	ATF2 ATFa ATF-a0 CRE-BPa
ATF3	ATF3 ATF3b JDP2
ATF4	ATF4 ATF5
ATF6	ATF6α CREB-RP
B-ATF	B-ATF JDP1

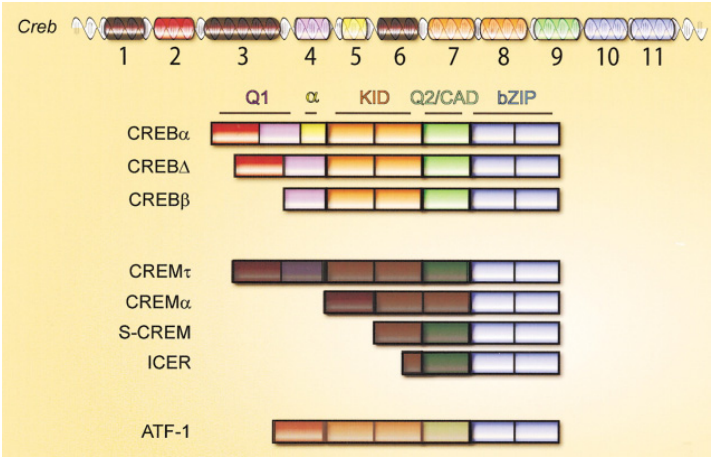


Figure 6. The ATF/CREB family of transcription factors. The ATF/CREB family consists of sixteen transcription factors divided into six subfamilies. CREB, CREM and ATF1 comprise a subgroup referred to as the CREB family (adapted from [50, 51]).

CREB and the closely related factors cAMP responsive element modulator (CREM) and activating transcription factor 1 (ATF1) comprise a subgroup referred to as the CREB family. Like all bZIP transcription factors, CREB family members contain a C-terminal basic domain that mediates DNA binding and a leucine zipper domain that facilitates dimerization. The remaining domains of CREB family members serve to facilitate interactions with coactivators and components of the transcriptional machinery. Interestingly, both CREB and CREM encode transcription factors that, depending on the exon usage, can function as either transcriptional activators or repressors. A truncated CREM bZIP polypeptide, referred to as ICER, is synthesized from an alternative CRE-driven intronic promoter in response to cyclic adenosine monophosphatase (cAMP) stimulation. Consistent with the absence of an activator domain, ICER acts as a potent transcriptional repressor.

The most abundant CREB isoforms, CREBα and CREBΔ, contain the bZIP domain and two glutamine rich domains, referred to as Q1 and Q2/CAD, which

are separated by the kinase inducible domain (KID). These two activators differ only with respect to the presence or absence of the α-domain. Within the KID resides the critical residue, Ser-133, which when phosphorylated in a stimulus-inducible manner makes the KID domain a binding target for the transcriptional coactivator CREB binding protein (CBP). CBP binds to the Ser-133-phosphorylated KID domain via its KIX domain and it is this stimulus-dependent interaction between these two domains that is believed to function as the trigger for inducible gene expression. Q2/CAD interacts with components of the basal transcriptional machinery and is responsible for facilitating stimulus-independent CRE-driven gene expression (**Fig. 6**) [52].

1.4.2 CREB signaling pathway

In the absence of stimulation, CREB is bound to the consensus site TGACGT$^{C}_{A}{}^{G}_{A}$ and, via its Q2/CAD domain, assembles the basal transcriptional machinery. This results in a low level of CRE-driven transcription. Upon stimulation, CREB is phosphorylated in the Ser-133 (p-CREB) located in the KID domain and thus becomes activated. CREB is a substrate for multiple cellular kinases including ribosomal protein S6 kinase (RSK), protein kinase C (PKC), v-akt murine thymoma viral oncogene homolog (AKT), and calcium-calmodulin kinases II and IV. This phosphorylation causes an increased affinity to various transcriptional coactivators, such as CBP, p300 and CREB regulated transcription coactivator (TORC), which lead to the stabilization of the preinitiation complex that forms at the promoter and assembly of transcriptional machinery complexes, thereby increasing CRE- promoter activity in a stimulus-dependent manner (**Fig.7**) [52].

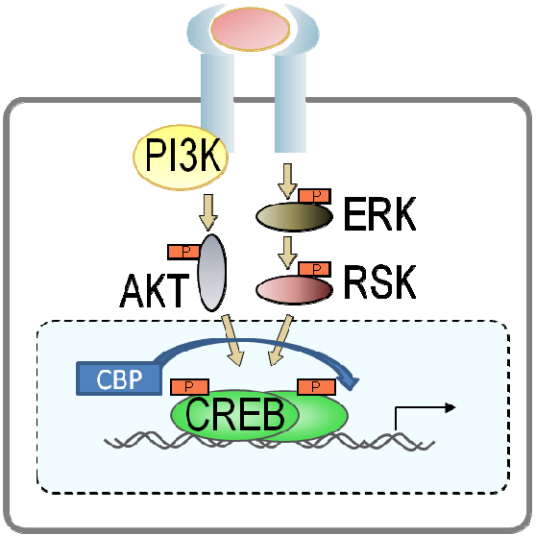


Figure 7. CREB signaling pathway. The activation of different signaling pathways lead to the phosphorylation of CREB which in turn binds to the coactivator CBP and triggers the transcription of multiple genes.

1.4.3 CREB in cancer

A role for CREB-mediated transcription in cancer was first reported through the identification of a chromosomal translocation t(12;22)(q13;q12) in clear cell sarcomas of soft tissue, resulting in a fusion protein denominated Ewing's sarcoma (EWS)-ATF1. This chimaeric protein, consisting of the N-terminal region of EWS fused with the C-terminal DNA-binding domain of the CREB related protein ATF1, generates a constitutively active transcription activator capable of binding to the promoters of CREB target genes. This, in turn, promotes tumor development and growth. More recently, an EWS-CREB fusion protein was discovered in a clear cell sarcoma variant and angiomatoid fibrous histiocytomas.

CREB has been implicated in contributing to the progression of several tumor types other than sarcomas and hystiocytomas. Analysis of prostate tumors from patients has demonstrated that p-CREB expression was restricted to poorly differentiated prostate cancers and bone metastatic tissue but not to non tumor benign prostate glands. Increased mRNA levels of CREB are also a feature of breast cancer tissue compared to non-tumor mammary tissue, and the level of CREB expression is correlated with disease progression and survival. In non-

small-cell lung cancer, the expression levels of CREB and p-CREB are elevated in tumors compared to adjacent normal tissues, and increased CREB expression correlates with poor patient survival. Human ovarian tumors also exhibit increased CREB expression, and ovarian tumor cell lines in which CREB expression is silenced display significantly reduced proliferation. The studies that more strongly implicate CREB in cancer come from evidence showing that CREB has a role in the development of bone marrow malignancies. Finally, CREB also appears to regulate malignant melanoma biology by promoting tumor cell survival and metastasis.

How CREB regulates tumor growth remains unanswered. Several genes known to be directly regulated by CREB (*cyclin D1* and *D2, cyclin A1* and *A2, B-cell CLL/lymphoma 2 (BCL2)* and *NDC80 kinetochore complex component homolog (HEC1*) are implicated in tumorigenesis and uncontrolled proliferation. Increased cyclin D2 transcription regulates the proliferation of lymphocytes via phosphorylation of CREB by PI3K and protein kinase A (PKA). Of note, CD44 induces the proliferation of thyroid cancer cells through the induction of cyclin D1 by CREB. VEGF is transcriptionally regulated by CREB in prostate cancer cells and p-CREB and VEGF levels are elevated in tandem in metastatic prostate cancer, strongly supporting a direct role for CREB in mediating cellular proliferation and possibly metastasis. Thus, data from cell lines, animal models and patient tumor samples, indicate that CREB has a role in promoting and supporting the development of tumors in a variety of cell and tissue types [53-57].

Due to its oncogenic function, there is growing interest in CREB as a therapeutic target. Although still in the very early stages, some work has already been carried out towards discovering compounds that can target CREB that may be useful for antitumor therapies [58].

1.4.3.1 CREB in brain tumors

Various studies using brain tumor cell lines suggest that signaling pathways that involve CREB activation are important for tumor cell growth and differentiation. There is evidence that CREB can be activated by prostaglandin E2 via the PKA pathway to stimulate cell proliferation. Furthermore, CREB is activated in human glioma cell lines and inhibition of CREB leads to reduced survival of glioma cells. Interestingly, immunohistochemical data from the Human Protein Atlas shows that CREB is strongly expressed in 100% of patient glioma tumors. Nevertheless, today there is no direct evidence linking CREB to brain cancer development or progression *in vivo* [53].

1.4.4 CREB and the TGF-β signaling pathway

The CREB/ATF family of transcription factors has been shown to regulate and cooperate with TGF-β in the transcriptional regulation of multiple genes. TGF-β induces the phosphorylation of ATF2 via p38 followed by the formation of a ATF2/SMAD3/SMAD4 transcriptional complex and transcription activation [59]. Similarly, TGF-β induces the expression of ATF3 which binds to SMAD3 and inhibits ID1 expression in epithelial cells [60]. In GICs, given that there is very little expression of ATF3, TGF-β induces the expression of ID1 [20].

CREB, on the other hand, cooperates with BMP-stimulated SMAD signaling to enhance the transcription of SMAD6 [61] and is involved in TGF-β1-mediated induction of TGF-β3 expression [62] and TGF-β3 autoregulation [63]. Furthermore, TGF-β2 expression is induced by the activity of both ATF2 and CREB [26-28].

Objectives

2. OBJECTIVES

The TGF-β signaling pathway is aberrantly activated in glioma and this confers poor prognosis [16]. The main objective of this work is to unravel the mechanisms that govern the hyperactivation of TGF-β in GBM in order to discover new therapeutic targets against this disease.

For this purpose we have followed two different approaches:

1. In the last few years, DUBs have proven to play a very important role in cancer. Since very little is known about their role in the regulation of the TGF-β pathway and GBM, we will evaluate the implication of DUBs in TGF-β signaling, focusing our attention in those that upregulate the TGF-β signaling pathway.
2. TGF-β2 is overexpressed in GBM and plays a key role in the hyperactivation of the TGF-β signaling pathway in GBM. Therefore, we will study the transcriptional regulation of TGF-β2 in GBM to dissect the mechanisms that lead to an oversecretion of TGF-β2.

Results

3. RESULTS

3.1 USP15 stabilizes TGF-β receptor I and promotes oncogenesis through the activation of TGF-β signaling in glioblastoma

3.1.1 A functional screen identifies USP15

To identify DUBs involved in the TGF-β signaling pathway, we performed an RNAi loss-of-function screen using a library of shRNA vectors targeting human *DUB* genes [64]. The DUB library consists of pools of four non-overlapping shRNAs targeting 94 known or putative DUBs [36, 64, 65]. We found that suppression of a number of DUBs markedly repressed or enhanced the activity of a TGF-β–responsive luciferase reporter (CAGA-luc) to TGF-β (**Fig. 8a**). After a thorough validation of the candidate DUBs, we decided to focus our attention on USP15.

First, we observed that shRNA vectors A and B, out of the four total shRNA vectors (A–D) targeting USP15, efficiently decreased TGF-β–induced CAGA-luc activity (**Fig. 8b**) and were able to downregulate ectopically expressed and endogenous USP15 in 293T cells (**Fig. 8c, d**). Moreover, we observed that a well-known direct transcriptional target of TGF-β, SMAD7 [66], was affected by the decrease in USP15 levels (**Fig. 8e**).

Having established that USP15 knockdown diminishes TGF-β signaling, we tested the effect of USP15 gain of function. Overexpression of USP15 led to an increase in CAGA-luc activity in untreated and TGF-β treated 293T cells. In contrast, overexpression of a catalytically inactive USP15 mutant, USP15 C269S (USP15C/S) [67], did not have a major effect on the activity of the TGF-β–responsive reporter (**Fig. 8f**). This suggests that the protease activity of USP15 is required to regulate the TGF-β pathway.

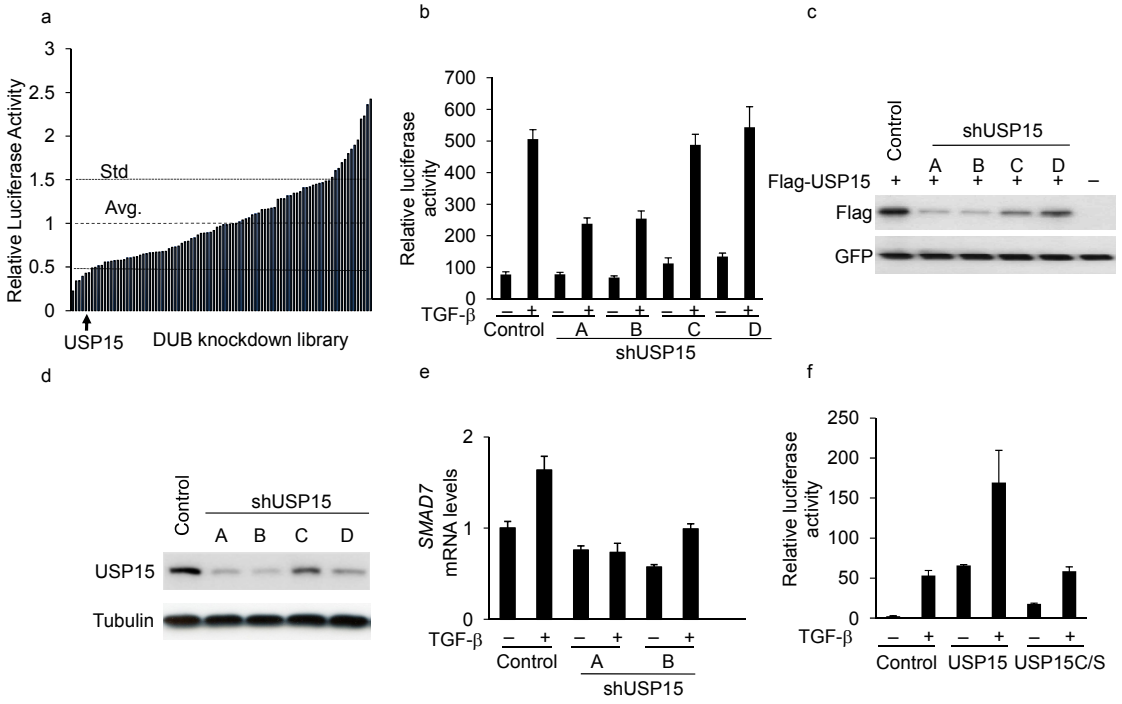


Figure 8. A functional screen identifies USP15. (**a**) Graph representing the luciferase values obtained from the DUB shRNA screen. 293T cells were cotransfected with the CAGA-luc and the individual pools of the DUB shRNA library. Cells were treated with TGF-β for 16 hours and a luciferase assay was performed. (**b**) Luciferase assay in 293T cells cotransfected with the CAGA-luc reporter and the individual shRNA vectors (A-D) targeting USP15 treated with TGF-β for 16 hours. Data are represented as mean ± s.d. (**c**) Immunoblot analysis of the indicated proteins in 293T cells cotransfected with Flag-USP15 and the individual shRNA vectors (A-D) targeting USP15. (**d**) Immunoblot analysis showing the levels of USP15 and tubulin in 293T cells transfected with the individual shRNA vectors (shUSP15 A–D) targeting USP15.(**e**) Quantitative real time PCR (qRT-PCR) of *SMAD7* in 293T cells transfected as indicated and treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. Data are represented as mean ± s.d. (**f**) Luciferase assay in 293T cells transfected with USP15 and USP15C/S and treated with TGF-β for 16 hours. Data are represented as mean ± s.d.

3.1.2 USP15 regulates the amount of phosphorylated SMAD2

To determine the mechanism through which USP15 regulates the TGF-β pathway, we first assessed how USP15 affects the main mediators of the TGF-β signal, SMADs. Knockdown of endogenous USP15 decreased the amount of phosphorylated SMAD2 (p-SMAD2) but did not affect the total levels of SMAD2 or SMAD4 (**Fig. 9a, b**). Conversely, overexpression of USP15 increased the levels of p-SMAD2, whereas USP15C/S slightly decreased its levels (**Fig. 9c**). These results suggest that in this experiment, USP15C/S might act as a

dominant negative, which is in contrast to the effect of USP15C/S that we observed in luciferase reporter assays. Of note, USP15 also regulated the activity of two other members of the TGF-β superfamily, activin and BMP. USP15 knockdown decreased activin-induced p-SMAD2 levels (**Fig. 9d**) and BMP-induced p-SMAD1 levels (**Fig. 9e**).

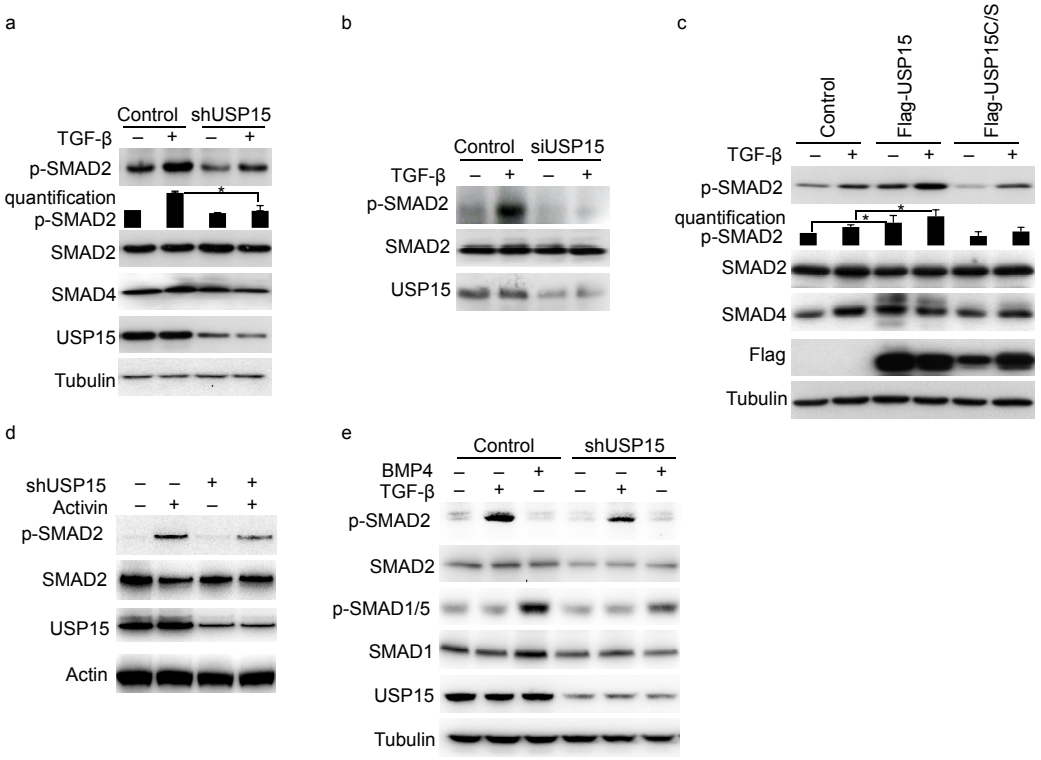


Figure 9. USP15 regulates the amount of phosphorylated SMAD2. (**a**) Immunoblot analysis in 293T cells expressing shRNA targeting USP15 treated with TGF-β for 16 hours. Also shown are the band intensities of p-SMAD2 from three independent experiments. **P* < 0.05 using Student's t test. (**b**) Immunoblot analysis of the indicated proteins in 293T cells transfected with siRNAs targeting USP15 and treated with TGF-β for 16 hours. (**c**) Immunoblot analysis in 293T cells expressing Flag-tagged USP15 (Flag-USP15) or Flag-USP15C/S and treated with TGF-β for 16 hours. Also shown are the band intensities of p-SMAD2. **P* < 0.05 using Student's t test. (**d**) Immunoblot analysis of the indicated proteins in 293T cells expressing a shRNA targeting USP15 and treated with Activin for 16 hours. (**e**) Immunoblot analysis of the indicated proteins in 293T cells expressing a shRNA targeting USP15 and treated with TGF-β or BMP4 for 16 hours.

Next, we performed coimmunoprecipitation assays and found that USP15 bound with a higher affinity to SMAD7 than to SMAD1, SMAD2, SMAD3, SMAD4 or SMAD6 (**Fig. 10a, b**). To identify the USP15 protein domain that is involved in the interaction with SMAD7, we generated three different deletion

constructs of USP15 and found that the N-terminus construct containing the ubiquitin-specific protease domain of USP15 coimmunoprecipitated with SMAD7 (**Fig. 10c**). To assess whether the interaction between USP15 and SMAD7 was direct, we obtained recombinant purified histidine (His)-tagged USP15 (His-USP15) and GST-tagged SMAD7 (GST-SMAD7) and found that SMAD7 pulldown precipitated USP15 *in vitro (***Fig. 10d**). In addition, USP15 interacted with SMAD7 in physiological conditions, as seen by endogenous SMAD7 coimmunoprecipitating with endogenous USP15 (**Fig. 10e**).

TGF-β treatment of 293T cells decreased the binding of USP15 to SMAD7. Furthermore, coimmunoprecipitation assays showed a TGF-β dose-dependent interaction between USP15 and SMAD7. At low doses of TGF-β, USP15 was bound to SMAD7, whereas at high doses of TGF-β, USP15 was dissociated from the complex (**Fig. 10f**). Hence, the formation of the USP15-SMAD7 complex is finely regulated by the intensity of the TGF-β signal.

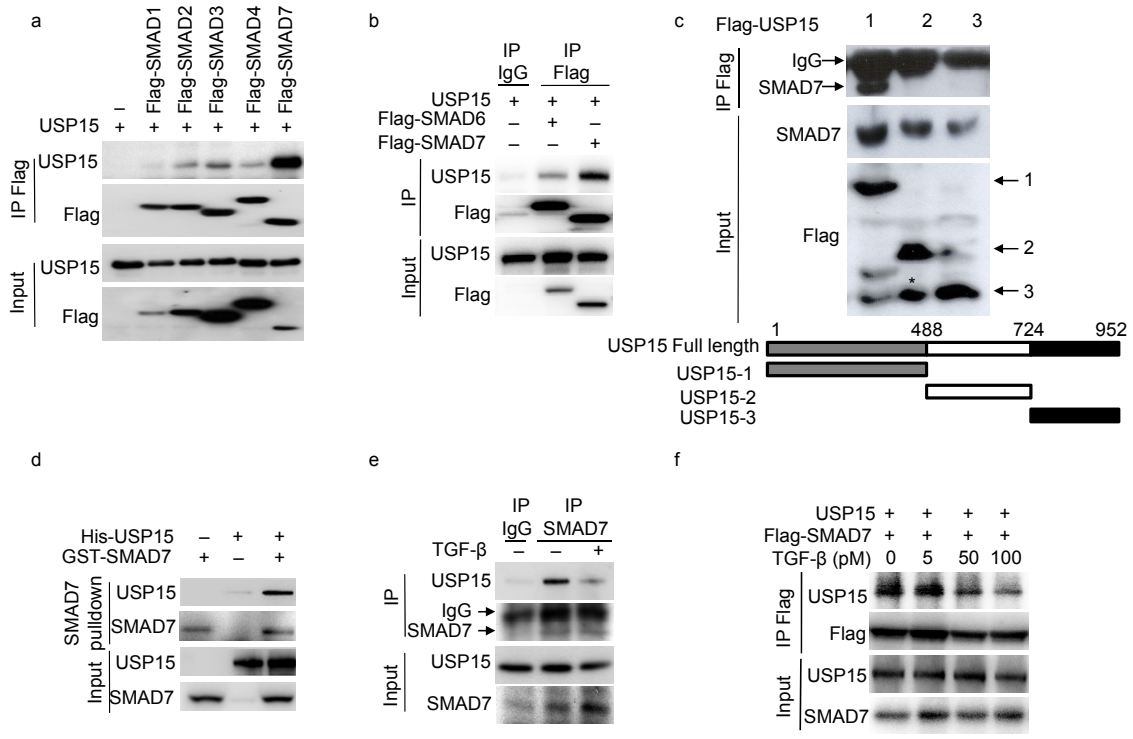


Figure 10. USP15 binds to SMAD7. (**a**) Immunoblot analysis in 293T cells expressing USP15 and Flag-tagged SMAD1, SMAD2, SMAD3, SMAD4 and SMAD7 and immunoprecipitated (IP) with anti-Flag resin. (**b**) Immunoprecipitation with an anti-Flag resin in 293T cells overexpressing Flag-SMAD6, Flag-SMAD7 and USP15 and immunoblot analysis of the indicated proteins. (**c**) Immunoprecipitation with an anti-Flag

resin in 293T transfected with three different Flag-USP15 fragments and immunoblot analysis of the indicated proteins. * indicates a band corresponding to a degradation product. (**d**) An immunoblot analysis of USP15 and SMAD7 after coincubation of recombinant His-USP15 and GST-SMAD7 and pulldown of SMAD7 using antibodies to SMAD7. (**e**) Immunoprecipitation of SMAD7 in 293T cells treated with TGF-β for 16 hours and an immunoblot analysis of the indicated proteins. (**f**) Immunoprecipitation with anti-Flag resin of 293T cells overexpressing USP15 and Flag-SMAD7 that were treated with increasing concentrations of TGF-β for 16 hours; an immunoblot analysis of the indicated proteins is shown.

3.1.3 USP15 forms a complex with SMAD7 and SMURF2

Several reports have shown that SMAD7 binds to the E3 ubiquitin ligase SMURF2 [9, 10, 43, 68]. Because USP15 binds to SMAD7, we assessed whether USP15 and SMURF2 were both present in the same complex with SMAD7. Indeed, ectopically expressed SMURF2, as well as endogenous SMURF2, coimmunoprecipitated with endogenous USP15 (**Fig. 11a,b**). However, USP15 did not interact with SMURF1 (**Fig. 11c**). We found that SMAD7 was present in the same complex with USP15 and SMURF2, and moreover, that SMAD7 was mediating the interaction between USP15 and SMURF2, as expression of a shRNA targeting SMAD7 markedly decreased the ability of USP15 to bind to SMURF2 (**Fig. 11d**). These results indicate that SMAD7 acts as a scaffolding protein, facilitating the interaction of USP15 with SMURF2. Knockdown of USP15 did not affect the ability of SMURF2 to form a complex with SMAD7 (**Fig. 11e**).

Previous work has shown that the SMAD7-SMURF2 complex is recruited to the TGF-β receptor complex [9]. Hence, we hypothesized that SMAD7 could be mediating the interaction of USP15 with the TGF-β receptors. Indeed, USP15 interacted with TβR-I, and this interaction was decreased when we knocked down SMAD7, indicating that USP15 was recruited to the TβR-I complex by SMAD7 (**Fig. 11f**).

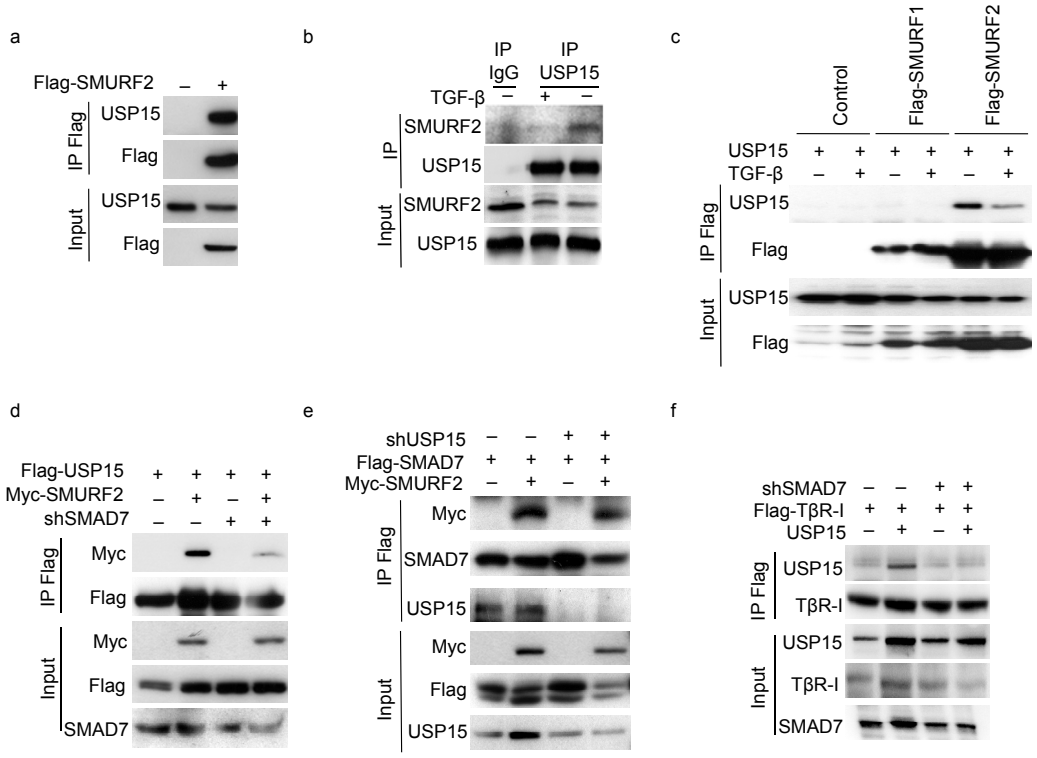


Figure 11. USP15 forms a complex with SMAD7 and SMURF2. (**a**) Immunoprecipitation with an anti-Flag resin in 293T cells transfected with Flag-SMURF2 and immunoblot analysis of the indicated proteins. (**b**) Immunoprecipitation of USP15 in 293T cells treated with TGF-β for 16 hours; an immunoblot analysis of the indicated proteins is shown. IgG, immunoglobulin G. (**c**) Immunoprecipitation with an anti-Flag resin in 293T cells overexpressing Flag-SMURF1, Flag-SMURF2 and USP15, treated with TGF-β for 16 hours and immunoblot analysis of the indicated proteins. (**d**) Immunoprecipitation with anti-Flag resin in 293T cells overexpressing Myc-tagged SMURF2 (Myc-SMURF2), Flag-USP15 and shRNAs against SMAD7; an immunoblot analysis of the indicated proteins is shown. (**e**) Immunoprecipitation with an anti-Flag resin in 293T cells transfected with Myc-SMURF2, Flag-SMAD7, and shRNAs against USP15 and immunoblot analysis of the indicated proteins. (**f**) Immunoprecipitation with anti-Flag resin in 293T cells overexpressing Flag–TβR-I, USP15 and shRNA targeting SMAD7; an immunoblot analysis of the indicated proteins is shown.

3.1.4 USP15 regulates the ubiquitination of TβR-I

Because USP15 is present in the SMAD7-SMURF2 complex, we reasoned that USP15 could be affecting the ubiquitination of TβR-I. Overexpression of USP15 in 293T cells caused a reduction in TβR-I ubiquitination, whereas USP15C/S, acting as a dominant-negative, increased TβR-I ubiquitination (**Fig. 12a**). Consistently, knockdown of USP15 increased the incorporation of ubiquitin into TβR-I (**Fig. 12b**). USP15 overexpression did not further decrease TβR-I ubiquitination in cells expressing shRNA against SMAD7 (**Fig. 12c**). This result

confirmed that in the absence of SMAD7, USP15 is unable to interact with the TGF-β receptor complex and deubiquitinate TβR-I. As previously described, TGF-β signaling promoted the ubiquitination of TβR-I [9]. Interestingly, overexpression of USP15 in 293T cells decreased the ubiquitination of TβR-I even in the presence of TGF-β, indicating that high levels of USP15 expression can in part protect against the TGF-β–induced ubiquitination of TβR-I (**Fig. 12d**). Of note, overexpression of USP15 or USP15C/S did not substantially alter the ubiquitination of SMAD7 (**Fig. 12e**).

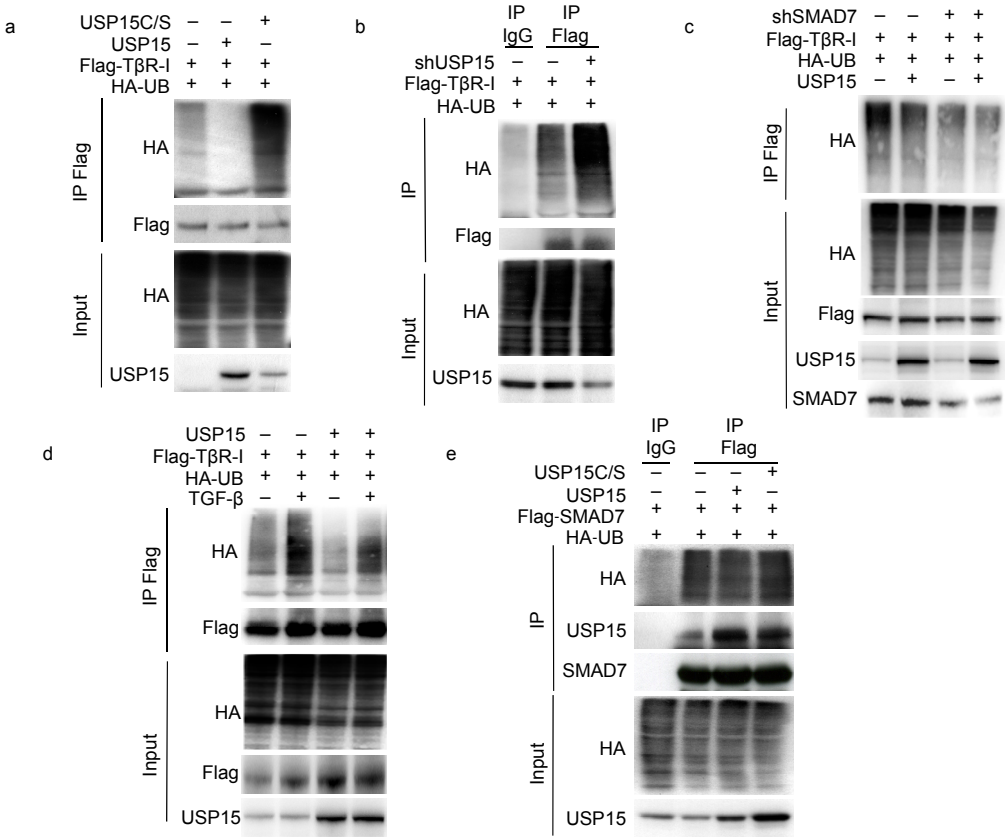


Figure 12. USP15 regulates the ubiquitination of TβR-I. (**a**) Immunoprecipitation with anti-Flag resin in 293T cells overexpressing Flag–TβR-I, USP15, USP15C/S and hemagglutinin (HA)-tagged ubiquitin (HA-UB); an immunoblot analysis of the indicated proteins is shown. (**b**) Immunoprecipitation with anti-Flag resin in 293T cells overexpressing Flag–TβR-I, shRNA against USP15 and HA-ubiquitin; an immunoblot analysis of the indicated proteins is shown. (**c**) Immunoprecipitation with an anti-Flag resin in 293T cells overexpressing Flag-TβR-I, USP15, a shRNA targeting SMAD7 and HA-Ubiquitin and immunoblot analysis of the indicated proteins. (**d**) Immunoprecipitation with anti-Flag resin in 293T cells overexpressing Flag–TβR-I, USP15 and HA-ubiquitin treated with or without TGF-β for 16 hours; an immunoblot analysis of the indicated proteins is shown. (**e**) Immunoprecipitation with anti-Flag resin

in 293T cells overexpressing Flag-SMAD7, USP15, USP15C/S and HA-ubiquitin; an immunoblot analysis of the indicated proteins is shown.

3.1.5 USP15 regulates the TGF-β pathway by stabilizing TβR-I

Consistent with the results on ubiquitination, overexpression of USP15, but not USP15C/S, markedly increased the expression of TβR-I (**Fig. 13a**). In addition, knockdown of USP15 expression diminished the levels of both overexpressed and endogenous TβR-I (**Fig. 13b, c**). We also observed that knockdown of USP15 decreased the protein levels of TβR-II (**Fig. 13d**), indicating that USP15 regulates the stability of the TGF-β receptor complex. The effect of USP15 on TβR-I appears to be mediated by the proteasome complex, as addition of the proteasome inhibitor MG132 prevented the downregulation of TβR-I by USP15 (**Fig. 13e**).

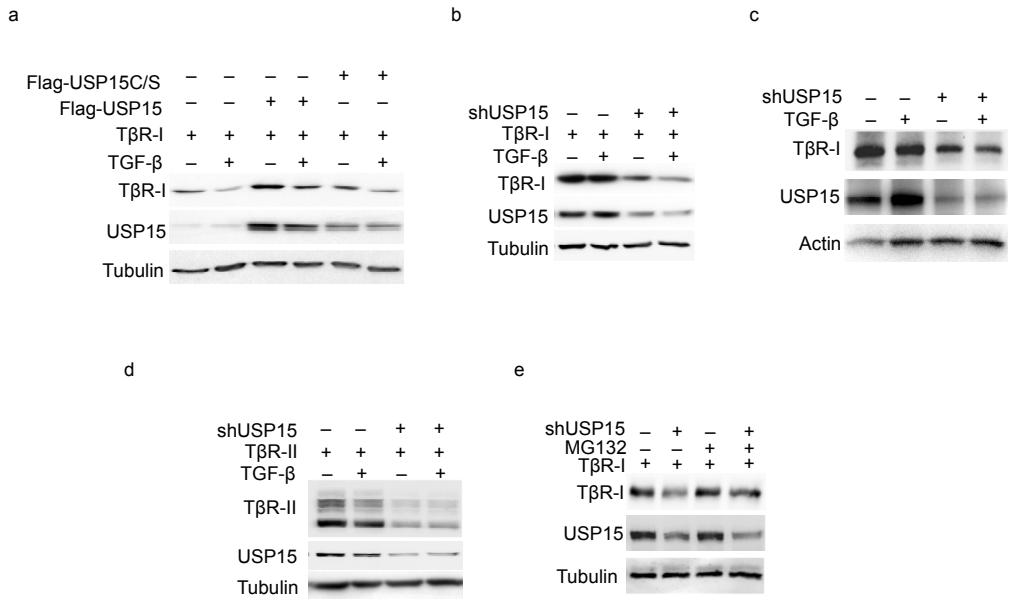


Figure 13. USP15 stabilizes TβR-I. (**a**) Immunoblot analysis in 293T cells expressing Flag-USP15, Flag-USP15C/S and TβR-I treated with TGF-β for 16 hours. (**b**) Immunoblot analysis in 293T cells overexpressing TβR-I and shRNA against USP15 treated with TGF-β for 16 hours. (**c**) Immunoblot analysis in 293T cells expressing shRNA targeting USP15 treated with TGF-β for 16 hours. (**d**) Immunoblot analysis in 293T cells overexpressing TβR-II and shRNA against USP15 treated wit h TGF-β for 16 hours. (**e**) Immunoblot analysis in 293T cells overexpressing TβR-I and shRNA against USP15 treated with TGF-β for 16 hours in the presence or absence of MG132.

Taken together, our results indicate that USP15 sustains the activation of the TGF-β pathway by regulating the ubiquitination and stability of TβR-I and suggest that USP15 could counteract the effect of SMURF2 on the TGF-β receptors. Indeed, increasing amounts of USP15 prevented SMURF2-dependent degradation of TβR-I in a dose-dependent manner (**Fig. 14a**). Moreover, overexpression of SMURF2 inhibited the activation of the TGF-β–responsive reporter by a constitutively active version of TβR-I or by TGF-β. USP15 attenuated the blockage of the TGF-β transcriptional activity by SMURF2 (**Fig. 14b, c**). In addition, USP15 also prevented the inhibitory effect of SMURF2 on p-SMAD2 levels, as shown by USP15 overexpression attenuating the SMURF2-mediated decrease of p-SMAD2 levels, whereas USP15C/S slightly enhanced the SMURF2-dependent inhibition of p-SMAD2 levels (**Fig. 14d**). Together, our results indicate that there is a balance between USP15 and SMURF2 to control TβR-I stability and, hence, the activity of the TGF-β pathway.

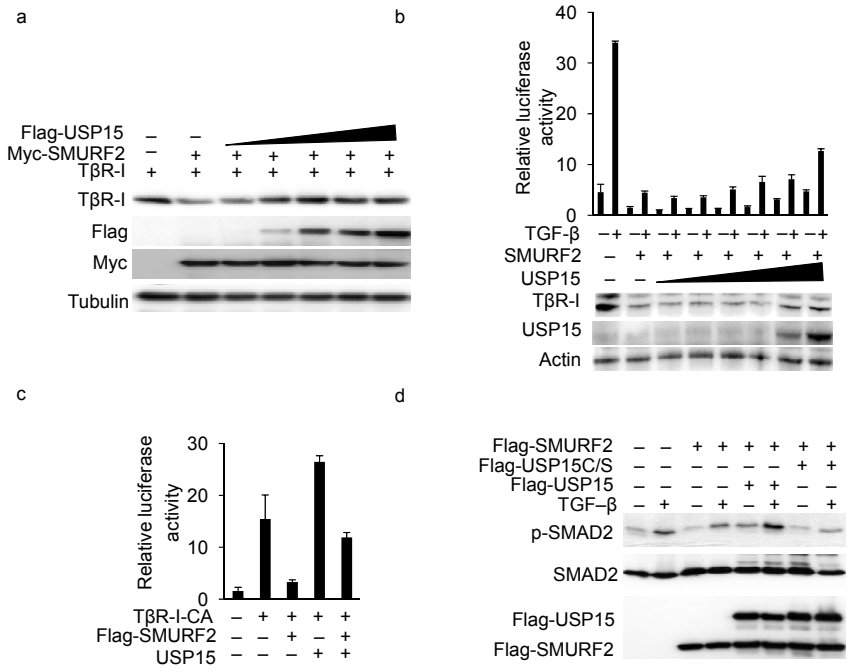


Figure 14. USP15 regulates TβR-I and counteracts SMURF2 activity. (**a**) Immunoblot analysis in 293T cells overexpressing Myc-SMURF2, TβR-I and increasing amounts of Flag-USP15. (**b**) Luciferase assay in 293T cells transfected with the CAGA-luc reporter, SMURF2 and increasing concentrations of USP15 and treated with TGF-β for 16 hours. Protein levels determined by immunobloting in lysates from TGF-β–treated samples (bottom). Data are represented as mean ± s.d. (**c**) Luciferase assay in 293T cells expressing CAGA-luc, Flag-SMURF2, a constitutively active version of TβR-I

(TβR-I–CA) and USP15. Data are represented as mean ± s.d. (**d**) Immunoblot analysis in 293T cells expressing Flag-SMURF2, Flag-USP15 and Flag-USP15C/S treated with TGF-β for 16 hours.

3.1.6 USP15 is targeted for amplification in cancer

We recently showed that TGF-β is highly active in high-grade glioma and that elevated TGF-β activity confers poor prognosis in individuals with glioma [16]. Given the role of USP15 in the regulation of TGF-β activity, we investigated the possibility that USP15 might be a relevant factor in GBM. First, downregulation of USP15 by RNAi in the GBM cell line T98G resulted in the inhibition of SMAD2 phosphorylation (**Fig. 15a**), a decrease in the endogenous levels of TβR-I (**Fig. 15b**) and an overall downregulation of the TGF-β target genes *SMAD7* and *PAI-1* (also known as *SERPINE1*) (**Fig. 15c**). Moreover, endogenous USP15 interacted with endogenous SMAD7 in T98G cells (**Fig. 15d**), and the ubiquitination of TβR-I decreased when USP15 was overexpressed in T98G cells (**Fig. 15e**).

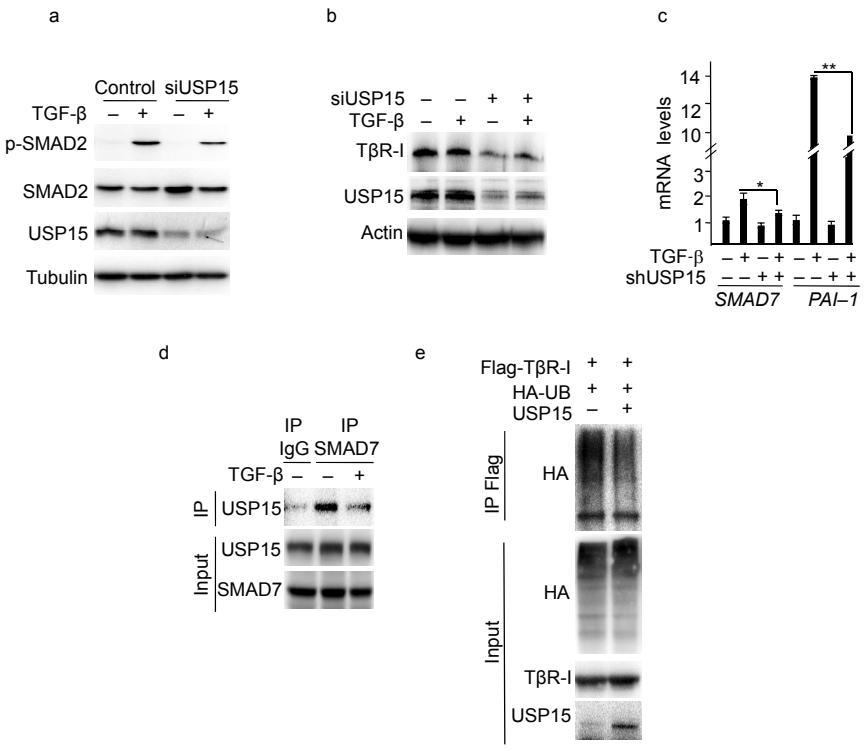


Figure 15. USP15 regulates the TGF-β signaling pathway in GBM cell lines. (**a,b**) Immunoblot analysis in T98G cells expressing siRNAs targeting USP15 treated with TGF-β for 16 hours. (**c**) qRT-PCR of *SMAD7* and *PAI-1* in T98G cells expressing shRNAs targeting USP15 treated with TGF-β for 3 hours. *GAPDH* mRNA levels were

used as an internal normalization control. **P* < 0.05, ***P* < 0.01 using Student's t test. Data are represented as mean ± s.d. (**d**) Immunoprecipitation of SMAD7 in T98G cells treated with TGF-β for 16 hours; an immunoblot analysis of the indicated proteins is shown. (**e**) Immunoblot analysis in T98G cells overexpressing Flag–TβR-I, USP15 and HA-ubiquitin immunoprecipitated with anti-Flag resin.

Because the characteristics of cell lines tend to diverge from the characteristics of real tumors as a consequence of prolonged artificial culture conditions, we confirmed our results using short-term primary cultured tumor cells (PCTCs) obtained from patient-derived GBM specimens. The knockdown of USP15 in PCTCs inhibited SMAD2 phosphorylation (**Fig. 16a**), decreased the levels of endogenous TβR-I (**Fig. 16b**) and blunted the induction of *SMAD7* and *PAI-1* transcripts by TGF-β (**Fig. 16c**). In addition, endogenous USP15 bound endogenous SMAD7 in PCTCs (**Fig. 16d**).

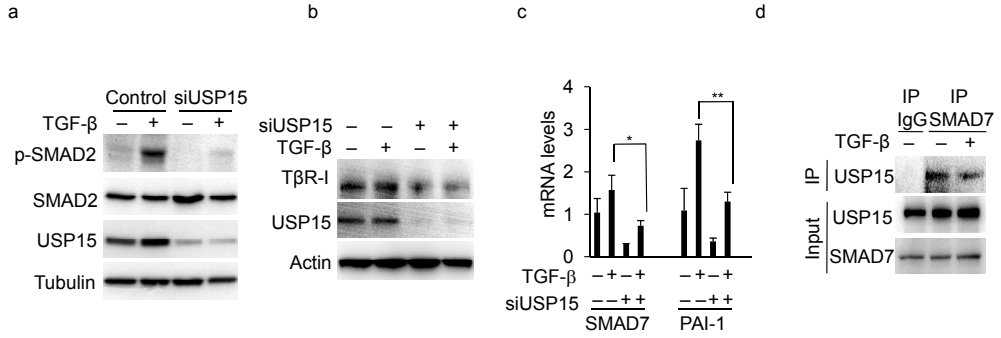


Figure 16. USP15 regulates the TGF-β signaling pathway in PCTCs from patient-derived GBM specimens. (**a,b**) Immunoblot analysis in PCTCs expressing siRNAs targeting USP15 and treated with TGF-β for 16 hours. (**c**) qRT-PCR of *SMAD7* and *PAI-1* in PCTCs expressing siRNAs targeting USP15 treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. **P* < 0.05, ***P* < 0.01 using Student's t test. Data are represented as mean ± s.d. (**d**) Immunoprecipitation of SMAD7 in PCTCs treated with TGF-β for 16 hours; an immunoblot analysis of the indicated proteins is shown.

In light of our observations, we sought to determine whether USP15, SMAD7 and SMURF2 expression was altered in a collection of 31 patient-derived samples of glioma obtained from surgical resections. We observed that 5 out of these 31 samples presented remarkably high USP15 levels (**Fig. 17**). Moreover, an immunohistochemical analysis of a tissue microarray containing samples

from 41 individuals with GBM showed a statistically significant correlation between the levels of p-SMAD2 and TβR-I with that of USP15 (**Fig. 18a**). This finding confirmed our hypothesis that USP15 regulates TGF-β activity in GBM.

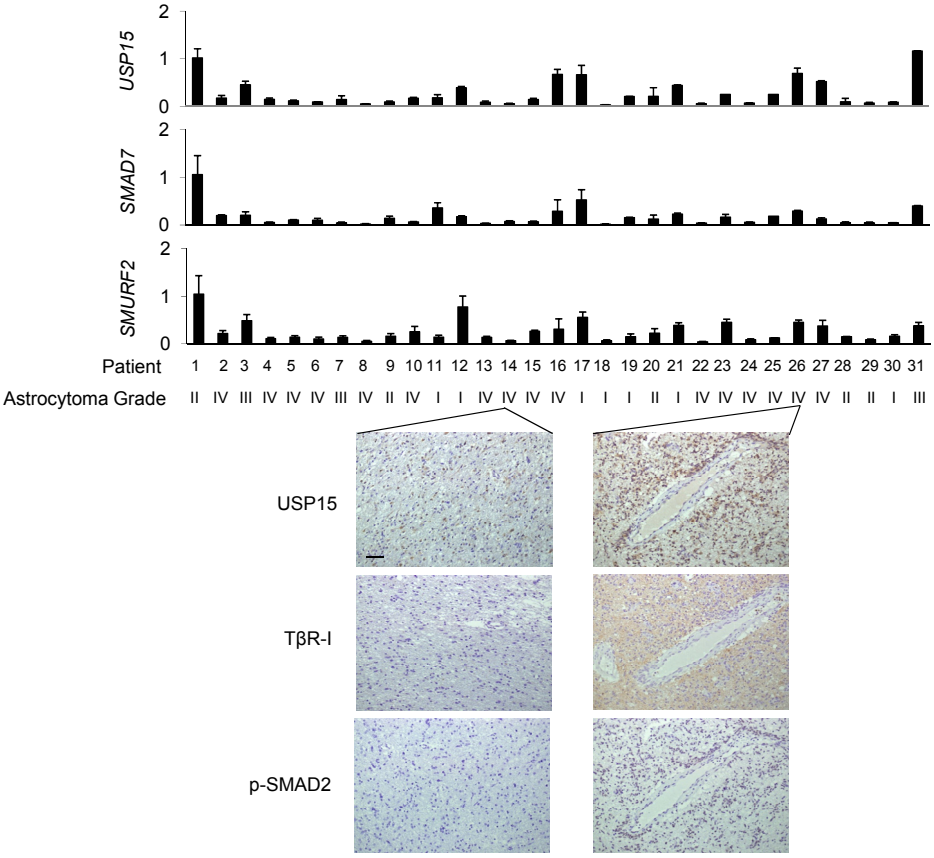


Figure 17. Expression of *USP15*, *SMAD7* and *SMURF2* in glioma. (**a**) qRT-PCR of *USP15*, *SMAD7* and *SMURF2* in 31 human glioma patient-derived samples. Immunohistochemistry of USP15, TβR-I and p-SMAD2 was performed. Scale bar, 50μm. *GAPDH* mRNA levels were used as an internal normalization control. Data are represented as mean ± s.d.

We analyzed the samples that had aberrantly high levels of USP15 to detect possible *USP15* gene amplification using fluorescence *in situ* hybridization (FISH). One of the subjects who presented with high levels of USP15 (patient 26 in Fig. 17) also showed an amplification of the *USP15* gene (**Fig. 18b**). Using the TCGA database [6], we observed that individuals with GBM carrying more than 2.5 copies of *USP15* had a shorter life expectancy than those with GBM and two or less copies of the gene (**Fig. 18c**). Hence, the amplification of *USP15* in GBM confers a poor prognosis.

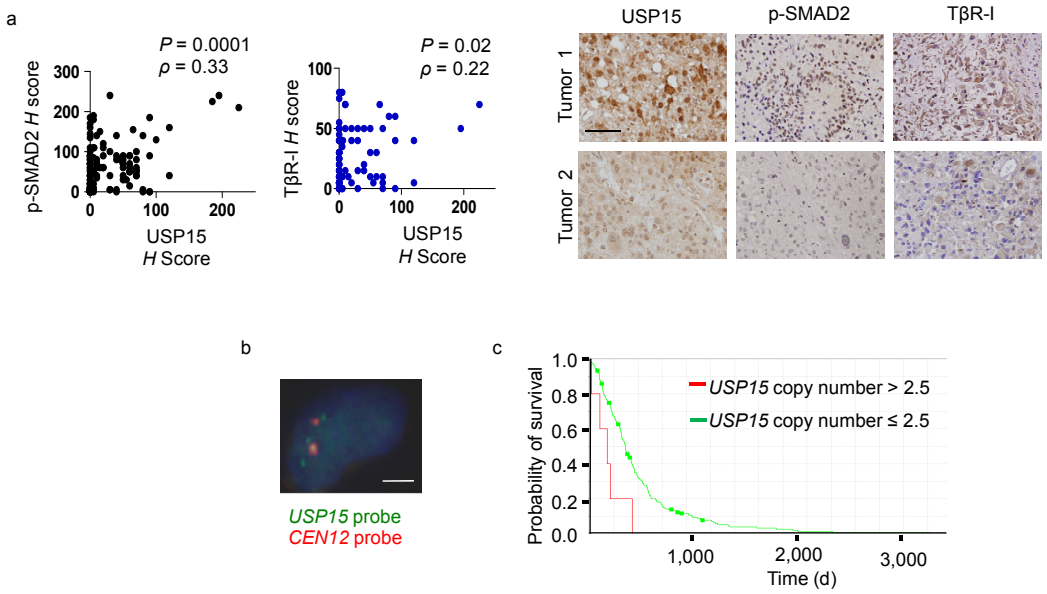


Figure 18. USP15 is targeted for amplification in GBM and confers poor prognosis. (**a**) A graph showing the correlation between USP15 levels and those of p-SMAD2 or TβR-I in tissue microarrays from human GBM samples. Not all spots were evaluable in all stainings. A Spearmann's test was used, and the correlation coefficient (ρ) and the two-tailed significance are shown. Representative images from the tissue microarrays are shown (scale bar, 50 μm). (**b**) Representative FISH image of an individual with GBM using a probe encompassing the amplified genomic region containing the *USP15* locus labeled with SpectrumGreen and a chromosome 12 reference probe labeled with SpectrumOrange. Scale bar, 5 μm. (**c**) Kaplan-Meier curves showing that the overall survival of individuals with GBM having a *USP15* gene copy number greater than 2.5 is significantly lower than those with a copy number of 2.5 or below ($P = 0.01$) by log-rank test. Data were obtained from the TCGA database of the National Cancer Institute.

In addition, we further analyzed the TCGA database looking for *USP15* gene amplifications in tumor types other than GBM. Indeed, *USP15* is targeted for amplification in around 1.5–2% of ovarian [69] and breast tumors (**Fig. 19a**). An analysis of a tissue microarray of 23 breast tumors showed that USP15 protein levels correlated with the levels of p-SMAD2 and TβR-I (**Fig. 19b**), indicating that USP15 also has a major role in the control of TGF-β activity in breast cancer.

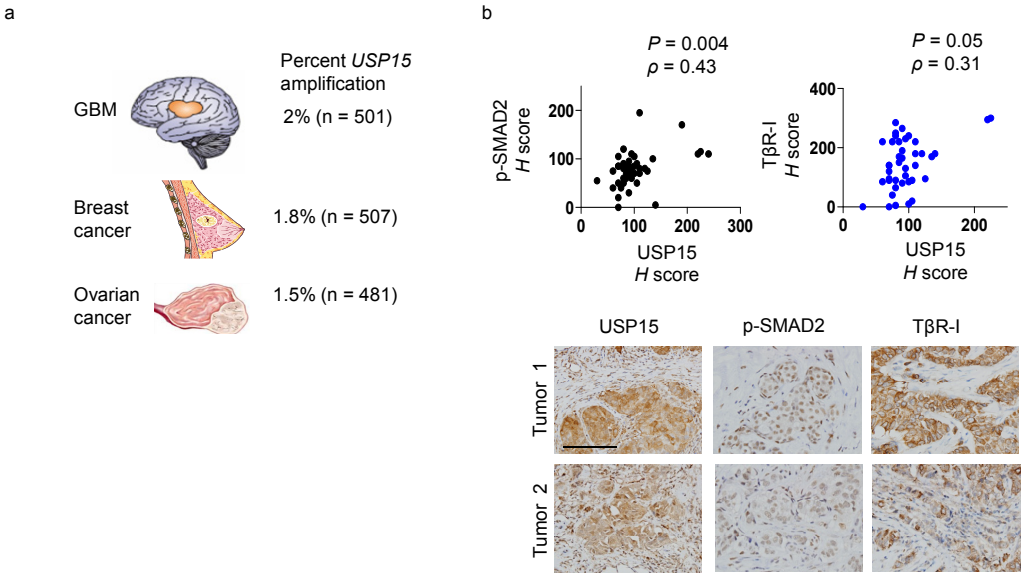


Figure 19. USP15 regulates the TGF-β signaling pathway in breast cancer and is targeted for amplification in breast and ovarian cancer. (**a**) Graph showing the number of *USP15* amplifications in different tumor types. Data were obtained from the TCGA database. (**b**) Graph showing the correlation between USP15 levels and those of p-SMAD2 or TβR-I in tissue microarrays from 23 samples from individuals with breast cancer. Not all spots were evaluable in all stainings. A Spearmann's test was used, and the correlation coefficient (ρ) and the two-tailed significance are shown. Representative images of USP15, p-SMAD2 and TβR-I immunohistochemistry are shown at the bottom (scale bar, 50 μm).

3.1.7 USP15 regulates oncogenesis in GBM

Because of the involvement of USP15 in the regulation of the TGF-β pathway and the key role of TGF-β in oncogenesis, we decided to address the effect of USP15 on tumorigenesis using a mouse model based on the inoculation of patient-derived GICs into the brains of immunocompromised mice [18]. GICs inoculated into the brains of mice generate tumors with the same histopathological characteristics and oncogenic mutations as the tumor of the human from which the GICs were derived [18].

We identified two individuals that showed elevated expression of USP15 in their tumors. Neurospheres from both of these tumors were infected with *USP15* shRNA lentivirus, leading to a decrease of USP15 levels and TGF-β activity, as evidenced by diminished p-SMAD2 and TβR-I levels (**Fig. 20a**), and a

decreased expression of the TGF-β target genes *SMAD7*, *PAI-1* and *LIF* (**Fig. 20b**).

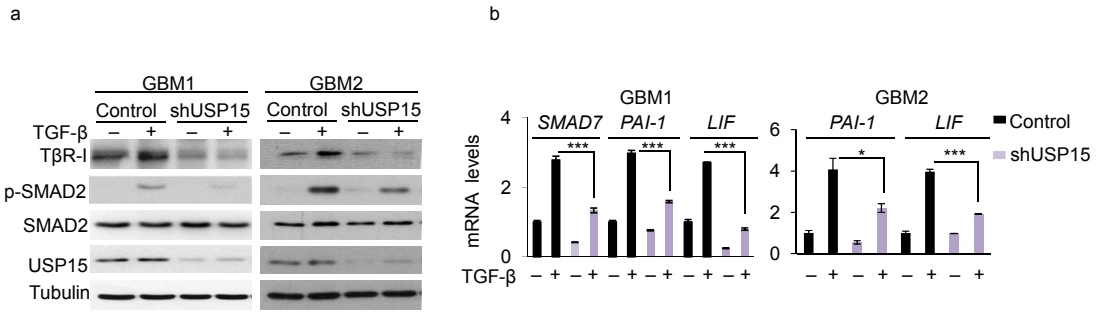
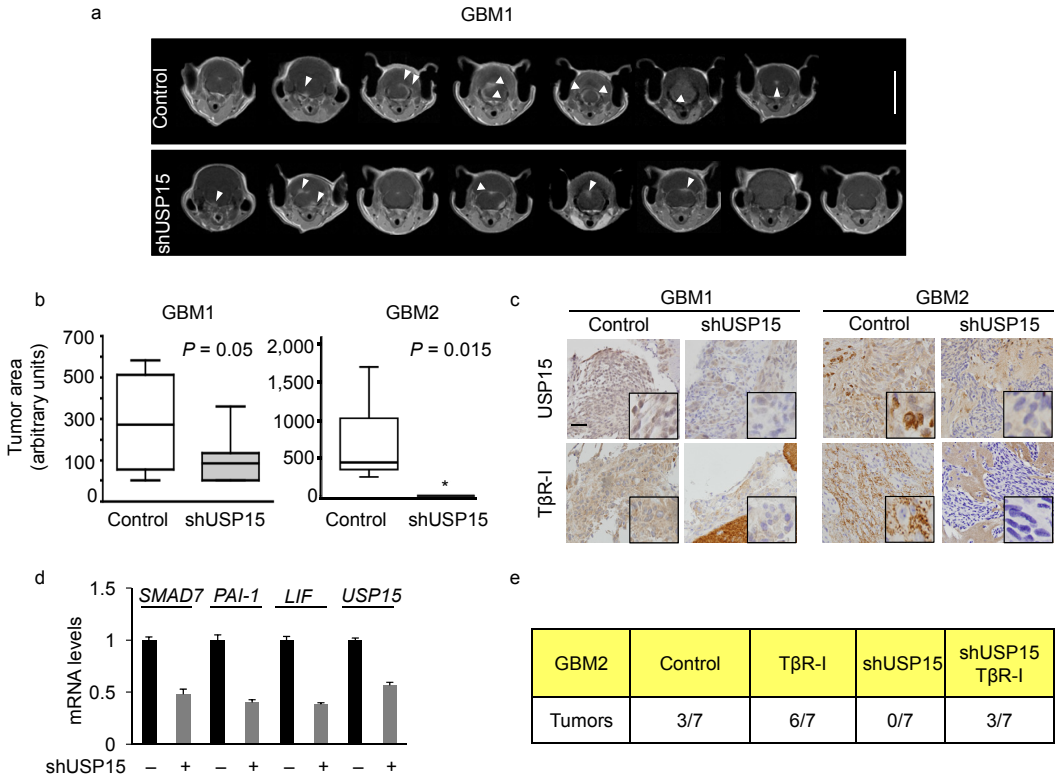


Figure 20. Knock down of USP15 inhibits TGF-β activity of patient-derived GBM neurospheres. (**a**) Immunoblot analysis in neurospheres derived from two different individuals with GBM (GBM1 and GBM2) infected with lentiviral vectors expressing shRNAs targeting USP15 and treated with TGF-β for 16 hous. (**b**) qRT-PCR of *SMAD7*, *PAI-1* and *LIF* in neurospheres derived from GBM1 and GBM2 expressing shRNAs targeting USP15 and treated with TGF-β for 5 hours. *GAPDH* mRNA levels were used as an internal normalization control. **P* < 0.05, ****P* < 0.005 using Student's t test. Data are represented as mean ± s.d.

After being inoculated into immunocompromised mice, neurospheres expressing shRNAs targeting USP15 generated smaller and fewer tumors than neurospheres expressing control shRNAs (**Fig. 21a,b**). This finding is in agreement with our previous work, in which we showed that TGF-β regulates the ability of neurospheres to initiate tumors [18]. In addition, the few small tumors generated from neurospheres expressing shRNAs against USP15 showed decreased levels of USP15 and TβR-I (**Fig. 21c**) and decreased expression of the TGF-β targets *SMAD7*, *PAI-1* and *LIF* (**Fig. 21d**) .

Our results show that USP15 regulates oncogenesis in GBM. However, we wanted to address how much of the effect of USP15 was mediated by the regulation of the TGF-β pathway. To investigate this issue, we overexpressed TβR-I in cells depleted of USP15. Indeed, cells overexpressing TβR-I and depleted of USP15 generated the same number of tumors than cells expressing control shRNAs (**Fig. 21e**). Hence, TβR-I overexpression rescued the anti-tumoral effect of the knockdown of USP15, showing that the main oncogenic effect of USP15 is mediated by the regulation of TGF-β activity.



GBM2	Control	TβR-I	shUSP15	shUSP15 TβR-I
Tumors	3/7	6/7	0/7	3/7

Figure 21. USP15 regulates oncogenesis in GBM via the regulation of TGF-β signaling pathway. (**a,b**) Entire-brain magnetic resonance imaging (MRI) from mouse brains 40 days after inoculation with neurospheres from GBM1 expressing shRNAs targeting USP15 and control shRNAs (scale bar, 1 cm) (**b**) and quantification of the resulting tumor volumes (GBM1, *P* = 0.05 and GBM2, *P* = 0.015 by two-tailed t test comparing control with shUSP15-expressing tumors). (**c**) The tumors generated in a and b were analyzed by inmunohistochemistry of USP15 and TβR-I (scale bar, 50 μm; insets 4× magnification). (**d**) qRT-PCR of the indicated genes in tumors generated from GBM neurospheres infected with lentiviral vectors expressing shRNAs targeting USP15. (**e**) Number of mice with or without tumors counted one month after inoculation of patient-derived neurospheres (GBM2) expressing shRNAs targeting USP15 and overexpressing TβR-I.

DUBs were recently shown to be targetable enzymes and, although there is currently no specific inhibitor compound for USP15, several broad-spectrum DUB inhibitors, such as PR-619, are available. We treated T98G cells with PR-619 (**Fig. 22a**) and, corroborating our previous results, pharmacological inhibition of USP15 decreased TβR-I and p-SMAD2 levels in these cells (**Fig. 22b**). To determine how much of the DUB inhibitor's effect was mediated by the inhibition of USP15, we repeated the experiment in cells depleted of USP15. There was no further downregulation of TβR-I by PR-619 in USP15 knockdown cells in addition to that resulting from the USP15 knockdown itself, indicating

that the effect of the DUB inhibitor on TβR-I is mediated by USP15 (**Fig. 22c**). Unfortunately, PR-619 is toxic *in vivo* and has unfavorable pharmacodynamic characteristics. However, to establish a proof of concept for the therapeutic implication of USP15 inhibition, we inoculated PR-619 directly into tumors in the brains of mice using stereotaxis, thereby avoiding the issue of poor pharmacodynamics. The treatment was acute to prevent toxicity. Tumors treated with the DUB inhibitor had lower levels of TβR-I and p-SMAD2 than tumors treated with vehicle (**Fig. 22d**).

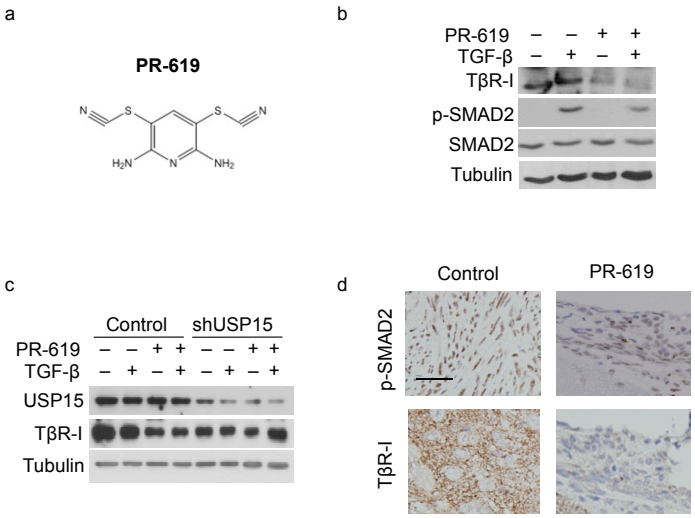


Figure 22. Pharmacological inhibition of USP15 inhibits TGF-β signaling. (**a**) Molecular structure of PR-619. (**b**) Immunoblot analysis in T98G cells treated concomitantly with PR-619 for 5 hours and TGF-β for 3 hours. (**c**) Immunoblot analysis in 293T cells expressing a shRNA targeting USP15 treated with TGF-β and/or PR-619 for 5 hours and TGF-β for 3 hours. (**d**) Immunohistochemistry of p-SMAD2 and TβR-I in tumors generated in mice from GBM neurospheres (Tumor 1) after inoculation with phosphate buffered saline (PBS) or PR-619 10 μM for 16 hours using stereotaxis. Scale bar, 50μm.

*This work was done in collaboration with Pieter Eichhorn and resulted in the following publication:

Eichhorn PJ, Rodón L, Gonzàlez-Juncà A, et al. *USP15 stabilizes TGF-β receptor I and promotes oncogenesis through the activation of TGF-β signaling in glioblastoma*. Nat Med, 2012. **18**(3) :p. 429-435.

3.2 CREB regulates the autocrine induction of TGF-β2 by TGF-β in glioblastoma

3.2.1 TGF-β2 is overexpressed in GBM and is a bad prognosis factor

The TGF-β signaling pathway is highly active in GBM and elevated TGF-β activity confers poor prognosis [16]. Out of the three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), we have previously demonstrated that TGF-β2 is mainly responsible for the observed TGF-β activity in gliomas [16]. To further study the relevance of TGF-β2 in GBM, we assessed the expression of TGF-β2 in healthy brain tissue and GBM (data obtained from the Oncomine Database); we observed that TGF-β2 is highly expressed in GBM compared to healthy brain tissue (**Fig. 23a**). We then analyzed the levels of expression of TGF-β2 by immunohistochemistry using a tissue microarray of low grade (grade II) and high grade gliomas (grade III and IV). The results obtained confirmed that TGF-β2 is overexpressed in GBM (**Fig. 23b**). Using the Repository for Molecular Brain Neoplasia Data (REMBRANDT) database, we observed that individuals with GBM tumors expressing high levels of TGF-β2 had a significantly shorter overall survival as compared to the rest of the patients (**Fig. 23c**). Together, our results indicate that TGF-β2 is overexpressed in GBM and confers bad prognosis.

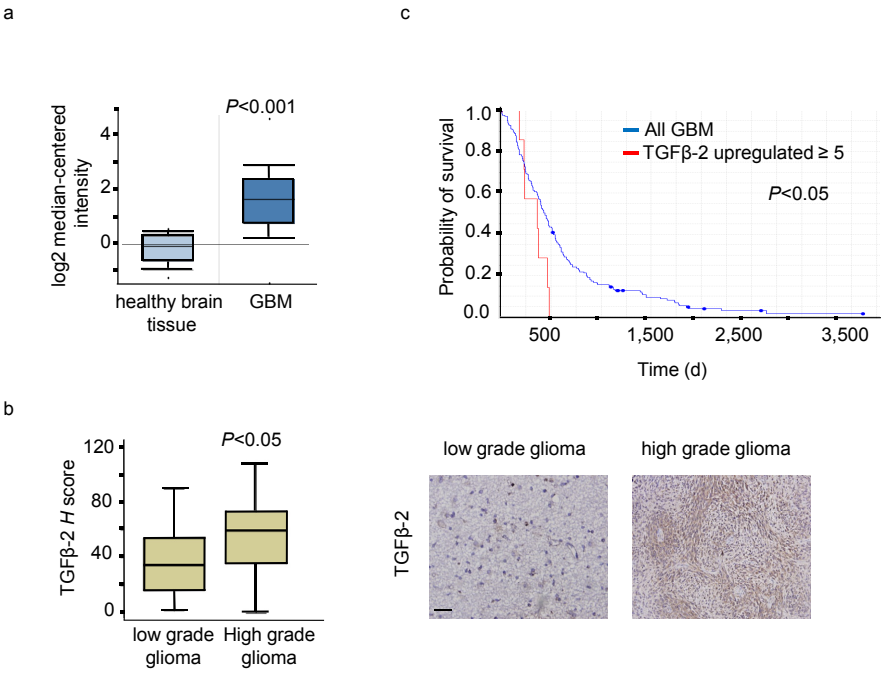


Figure 23. TGF-β2 is overexpressed in GBM and is a bad prognosis factor. (**a**) A graph showing TGF-β2 mRNA expression in human healthy brain tissue and GBM. *P*<0.001 by Student's *t* test. Data were obtained from the Oncomine database. (**b**) A graph showing TGF-β2 levels in tissue microarrays from human low grade and high grade gliomas. *P*<0.05 by Student's *t* test. Representative images of TGF-β2 immunohistochemistry are shown. Scale bar, 50μm (**c**) Kaplan-Meyer curves showing that the overall survival of individuals with GBM expressing TGF-β2 mRNA levels ≥ 5 times higher than baseline is significantly lower than the rest of the patients by log-rank test (*P*<0.05). Data were obtained from the REMBRANDT database.

3.2.2 TGF-β induces TGF-β2 expression in human GBM cells

We then decided to deepen our understanding of the molecular mechanisms responsible for the high levels of TGF-β2 in GBM. We found that TGF-β specifically induced the expression of the *TGF-β2* transcript but not that of *TGF-β1* nor *TGF-β3* in the human GBM cells LN229 and U373 (**Fig. 24a**). Moreover, the three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) were able to induce the expression of the *TGF-β2* transcript (**Fig. 24b**). As expected, the induction of the *TGF-β2* transcript by TGF-β resulted in an increase in TGF-β2 protein secretion when measured by ELISA in conditioned medium from GBM cells (**Fig. 24c**).

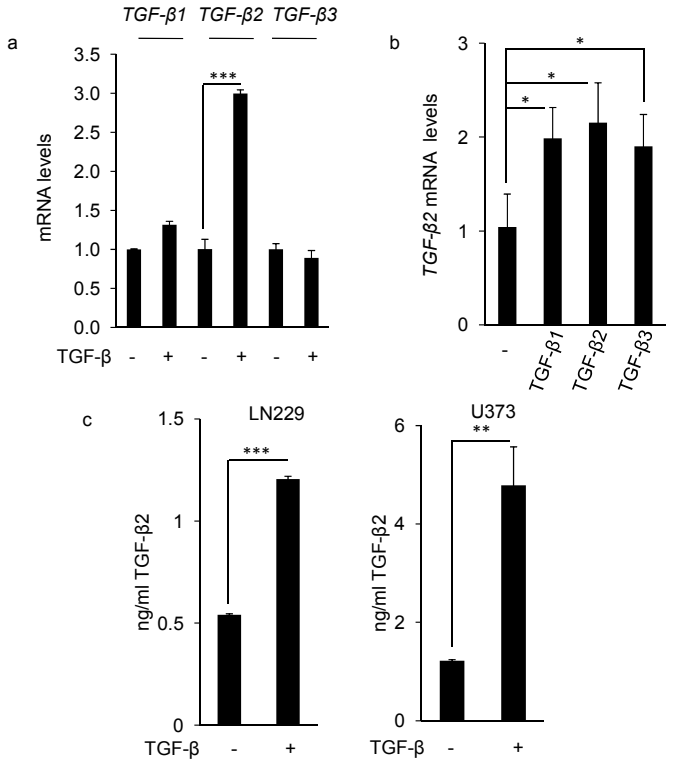


Figure 24. TGF-β induces TGF-β2 expression in human GBM cells. (**a**) qRT-PCR of *TGF-β1*, *TGF-β2* and *TGF-β3* in LN229 cells treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***$P < 0.005$ using Student's t test. Data are represented as mean ± s.d. (**b**) qRT-PCR of *TGF-β2* in U373 cells treated with TGF-β1, TGF-β2 and TGF-β3 for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. *$P < 0.05$ using Student's t test. Data are represented as mean ± s.d. (**c**) Secreted TGF-β2 protein levels determined by ELISA in culture supernatant from LN229 and U373 cell lines treated with TGF-β for 72 hours. ***$P < 0.005$, **$P<0.01$ using Student's t test.

3.2.3 CREB regulates the autocrine induction of TGF-β2 by TGF-β

To determine the molecular mechanisms responsible for TGF-β-mediated induction of TGF-β2 expression, we performed an analysis of the TGF-β2 promoter region. Interestingly, we found a CREB binding site adjacent to a Smad Binding Element (SBE) in the TGF-β2 promoter (**Fig. 25**).

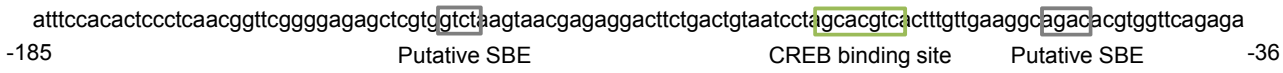


Figure 25. Nucleotide sequence of the proximal region of the TGF-β2 promoter. Nucleotide sequence positions are indicated relative to the transcription start site. The SBE (grey boxes) and CREB site (green box) are indicated.

Previous work has shown that SMADs collaborate with the ATF/CREB family of transcription factors in the induction of many TGF-β target genes [59-62]. Hence, we hypothesized that CREB could be regulating the autocrine induction of TGF-β2 by TGF-β via the transcriptional collaboration between SMAD/CREB proteins.

CREB is a transcription factor that is activated by phosphorylation in Ser-133 by many different kinases including RSK and AKT [52]. We sought then to assess whether the activation of CREB via these signaling pathways regulated the induction of TGF-β2 by TGF-β. To address this issue, we generated stable cell lines that overexpress a mutated form of PI3K (PI3K H1047R) which is constitutively active and commonly found in many different tumor types including glioma ([70-73]). We found that in cells with hyperactivated PI3K signaling, CREB was phosphorylated (**Fig. 26b and Fig. 27b**) and that phosphorylation correlated with an increased transcriptional induction of TGF-β2 in response to TGF-β (**Fig. 26a and Fig.27a**). Moreover, the phosphorylation of CREB and the induction of TGF-β2 by TGF-β was prevented by the presence of a specific pharmacological inhibitor of PI3K (**Fig. 26c, d and Fig 27c, d**). These results were further confirmed when we analyzed TGF-β2 protein secretion by ELISA in GBM cells conditioned medium (**Fig. 26e and Fig 27e**).

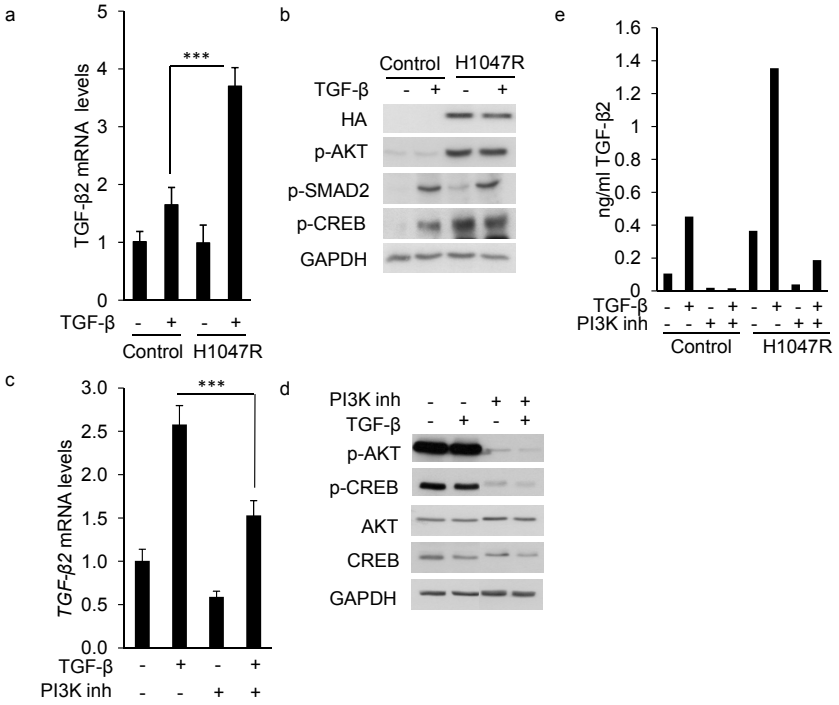


Figure 26. PI3K signaling regulates TGF-β-mediated induction of TGF-β2. (**a**) qRT-PCR of *TGF-β2* in LN229 expressing HA-tagged PI3K H1047R and treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***$P < 0.005$ using Student's t test. Data are represented as mean ± s.d. (**b**) Immunoblot analysis in LN229 cells expressing HA-tagged PI3K H1047R and treated with TGF-β for 3 hours. (**c**) qRT-PCR of *TGF-β2* in LN229 expressing HA-tagged PI3K H1047R and treated with TGF-β and/or PI3K inhibitor for 24 hours and TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***$P < 0.005$ using Student's t test. Data are represented as mean ± s.d. (**d**) Immunoblot analysis in LN229 cells expressing HA-tagged PI3K H1047R and treated with TGF-β and/or PI3K inhibitor for 24 hours and TGF-β for 3 hours. (**e**) Secreted TGF-β2 protein levels determined by ELISA in LN229 cells expressing HA-tagged PI3K H1047R treated with TGF-β and/or PI3K inhibitor for 72 hours and TGF-β for 48 hours.

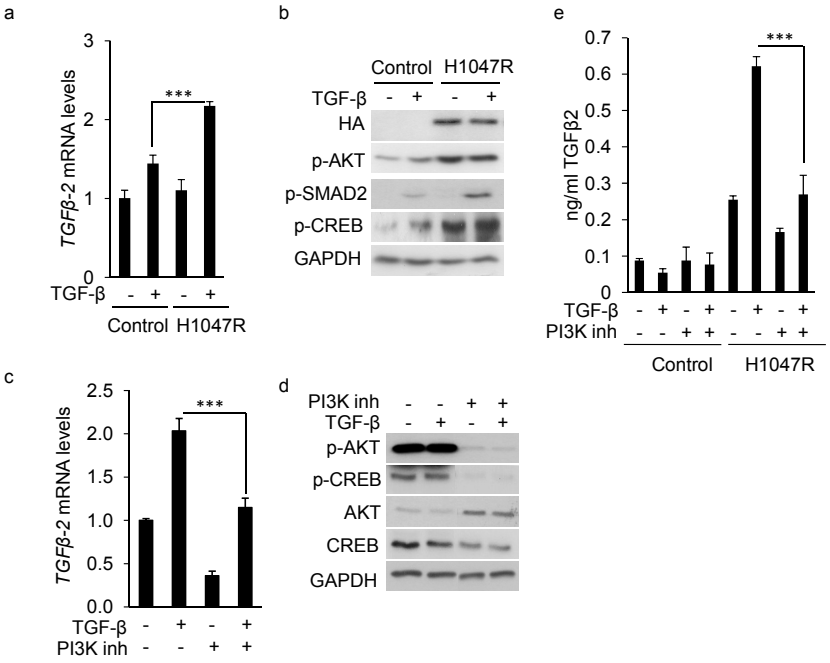


Figure 27. PI3K signaling regulates TGF-β-mediated induction of TGF-β2. (**a**) qRT-PCR of *TGF-β2* in U373 expressing HA-tagged PI3K H1047R and treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***$P < 0.005$ using Student's t test .Data are represented as mean ± s.d. (**b**) Immunoblot analysis in U373 cells expressing HA-tagged PI3K H1047R and treated with TGF-β for 3 hours. (**c**) qRT-PCR of *TGF-β2* in U373 expressing HA-tagged PI3K H1047R treated with TGF-β and/or PI3K inhibitor for 24 hours and TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***$P < 0.005$ using Student's t test. Data are represented as mean ± s.d. (**d**) Immunoblot analysis in U373 cells expressing HA-tagged PI3K H1047R and treated with TGF-β and/or PI3K inhibitor for 24 hours and TGF-β for 3 hours. (**e**) Secreted TGF-β2 protein levels determined by ELISA in U373 cells expressing HA-tagged PI3K H1047R treated with TGF-β and/or PI3K inhibitor for 72 hours and TGF-β for 48 hours. ***$P < 0.005$ using Student's t test .Data are represented as mean ± s.d.

In light of our observations, we sought to determine whether PI3K activation was inducing TGF-β2 expression via CREB. We overexpressed ICER, an endogenous repressor of CREB transcriptional function, in cells with hyperactivated PI3K signaling (transfected with PI3K H1047R). We found that overexpression of ICER (**Fig. 28b, e**) inhibited PI3K-mediated induction of TGF-β2 in response to TGF-β at the RNA (**Fig. 28a, d**) and protein level (**Fig. 28c, f**).

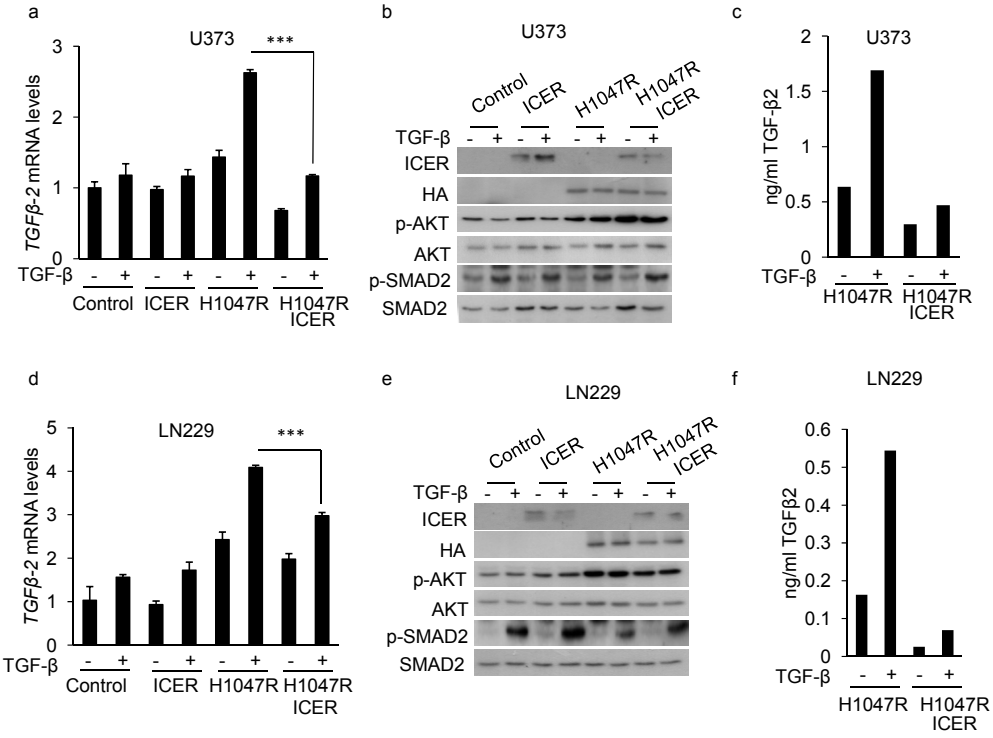


Figure 28. PI3K regulates TGF-β2 expression through CREB. (**a**) qRT-PCR of *TGF-β2* in U373 cells expressing HA-tagged PI3K H1047R and ICER treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***P < 0.005 using Student's t test .Data are represented as mean ± s.d. (**b**) Immunoblot analysis in U373 cells expressing HA-tagged PI3K H1047R and ICER treated with TGF-β for 3 hours. (**c**) Secreted TGF-β2 protein levels determined by ELISA in U373 cells expressing HA-tagged PI3K H1047R and ICER treated with TGF-β for 72 hours. (**d**) qRT-PCR of *TGF-β2* in LN229 cells expressing HA-tagged PI3K H1047R and ICER treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***P < 0.005 using Student's t test. Data are represented as mean ± s.d. (**e**) Immunoblot analysis in LN229 cells expressing HA-tagged PI3K H1047R and ICER treated with TGF-β for 3 hours. (**f**) Secreted TGF-β2 protein levels determined by ELISA in LN229 cells expressing HA-tagged PI3K H1047R and ICER treated with TGF-β for 72 hours.

In line with these results, knockdown of CREB using RNA interference (**Fig. 29b**) attenuated the PI3K-mediated increase of TGF-β2 mRNA (**Fig. 29a**) and secreted protein (**Fig. 29c**).

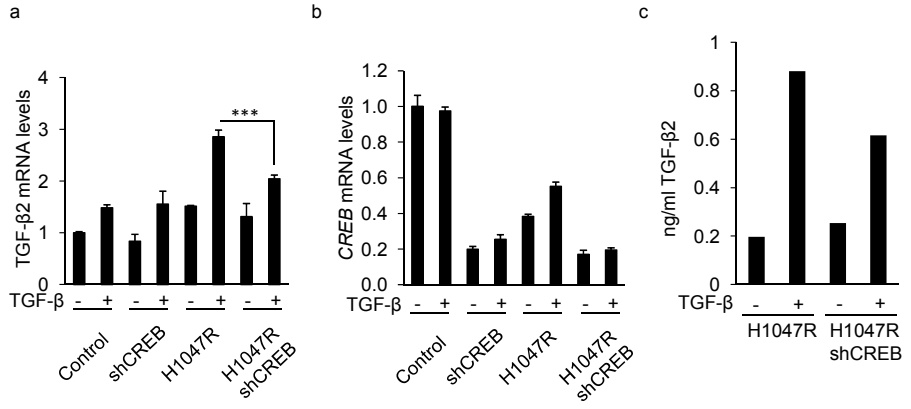


Figure 29. PI3K regulates TGF-β2 expression through CREB. (**a,b**) qRT-PCR of (**a**)*TGF-β2* and (**b**) *CREB* in LN229 cells expressing HA-tagged PI3K H1047R and shRNA targeting CREB treated with TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. ***$P < 0.005$ using Student's t test. Data are represented as mean ± s.d. (**c**) Secreted TGF-β2 protein levels determined by ELISA in U373 cells expressing HA-tagged PI3K H1047R and shCREB treated with TGF-β for 72 hours.

CREB can be activated by the action of different kinases other than AKT, such as RSK [55]. Hence, we reasoned that RSK could also regulate the TGF-β2 autocrine loop via CREB. Indeed, treatment of cells with a pharmacological inhibitor of RSK prevented CREB phosphorylation (**Fig. 30b**) and TGF-β-mediated induction of TGF-β2 both at the level of mRNA (**Fig. 30a**) and protein secretion (**Fig. 30c**). These results indicate that the PI3K and RSK signaling pathways regulate TGF-β-mediated induction of TGF-β2 via CREB.

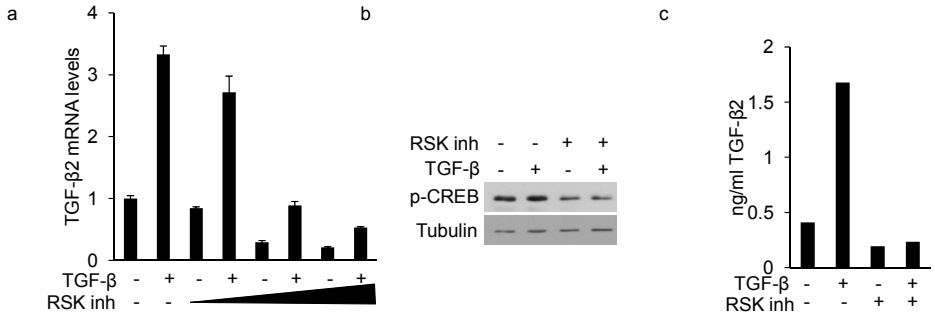


Figure 30. RSK signaling regulates TGF-β-mediated induction of TGF-β2. (**a**) qRT-PCR of *TGF-β2* in LN229 cells treated with increasing amounts of RSK inhibitor for 24 hours in the presence or absence of TGF-β for 3 hours. *GAPDH* mRNA levels were used as an internal normalization control. Data are represented as mean ± s.d. (**b**) Immunoblot analysis in LN229 cells treated with increasing amounts of RSK inhibitor for 24 hours in the presence or absence of TGF-β for 3 hours. (**c**) Secreted TGF-β2 protein levels determined by ELISA in LN229 cells treated with RSK inhibitor for 72 hours in the presence or absence of TGF-β for 48 hours.

3.2.4 CREB binds to the *TGF-β2* promoter along with SMAD2 and is required for TGF-β induction of TGF-β2

Having observed that there is a CREB binding site adjacent to a SBE in the *TGF-β2* promoter, we hypothesized that CREB and SMADs might regulate TGF-β2 expression in a direct fashion. We performed chromatin immunoprecipitation (ChIP) assays and observed that endogenous SMAD2 bound to the proximal region of the *TGF-β2* promoter in response to TGF-β. p-CREB was shown to bind the promoter in basal conditions and binding was enhanced upon TGF-β treatment (**Fig. 31 a**).

Indeed, TGF-β was able to transactivate a reporter construct containing the -77/+64 regions of the *TGF-β2* promoter; this region contains the CREB binding site and an SBE. Consistently, mutation of the CREB binding site (*mutCRE*) inhibited the TGF-β response (**Fig. 31c**). These results indicate that CREB binds to the *TGF-β2* promoter along with SMAD2 in response to TGF-β and is required for TGF-β induction of TGF-β2.

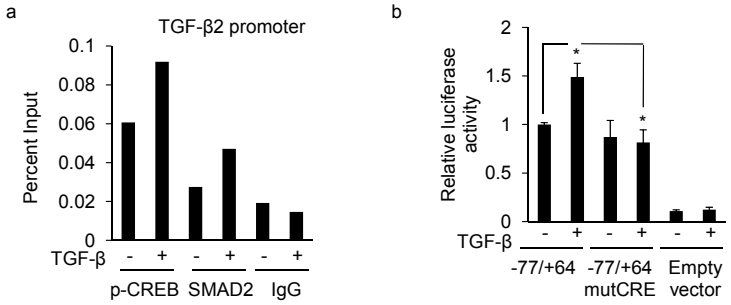


Figure 31. CREB binds to the *TGF-β2* promoter along with SMAD2 and is required for TGF-β induction of TGF-β2. (**a**) ChIP of p-CREB and SMAD2 in LN229 cells treated with TGF-β for 1 hour. A qRT-PCR is shown with specific primers for *TGF-β2* promoter. (**b**) Luciferase assay in LN229 expressing HA-tagged PI3K H1047R transfected with the (*-77/+64*) or *(-77/+64*) *mutCRE TGF-β2* luciferase reporter constructs and treated with TGF-β for 32 hours. ****P* < 0.05 using Student's t test. Data are represented as mean ± s.d.

3.2.5 CREB regulates TGF-β2 expression and oncogenesis in human GBM

An immunohistochemical analysis of a tissue microarray containing samples from individuals with GBM showed a statistically significant correlation between the levels of p-CREB and TGF-β2 (**Fig. 32a, b**). Furthermore, p-AKT levels correlated with p-CREB and TGF-β2 levels (**Fig. 32c, d**).

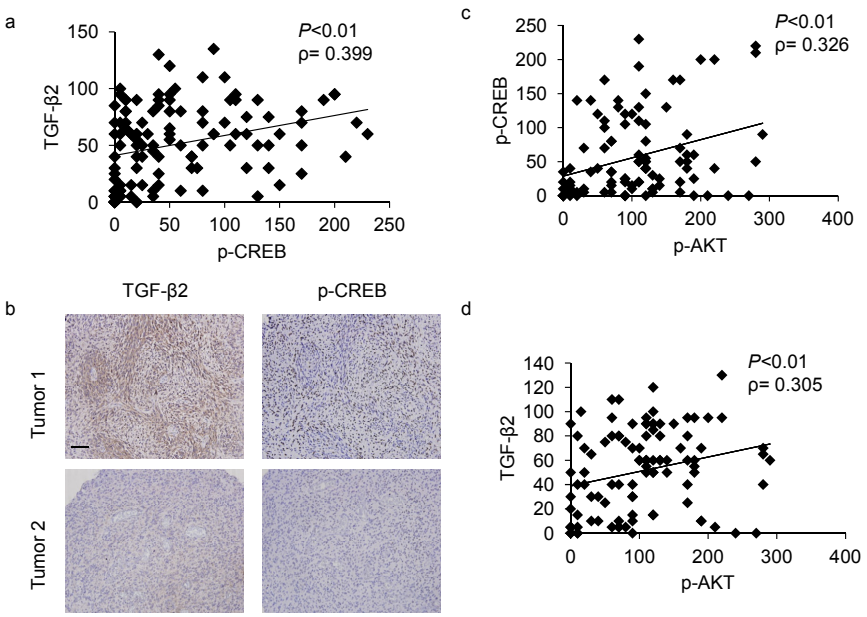


Figure 32. p-AKT, p-CREB and TGF-β2 levels correlate in human GBM tissue microarrays. (**a,b,c,d**) Graphs showing the correlation between p-AKT, p-CREB and TGF-β2 in tissue microarrays from human GBM samples. Not all spots were evaluable in all stainings. A Spearmann's test was used, and the correlation coefficient (ρ) and the two-tailed significance are shown. (**b**) Representative images from the tissue microarrays are shown. Scale bar, 50μm.

Because of the involvement of CREB in the regulation of the TGF-β pathway and the key role of TGF-β in oncogenesis, we decided to address the effect of CREB on tumorigenesis using a model based on the inoculation of patient-derived GICs into the brains of immunocompromised mice [18]. GICs inoculated into the brains of mice generate tumors with the same histopathological characteristics and oncogenic mutations as the tumor of the human from which they were derived [18]. We identified a tumor showing very high levels of expression of p-CREB; neurospheres from this tumor were infected with CREB shRNA lentivirus, leading to a decreased expression of CREB and TGF-β2 (**Fig. 33a**). Mice inoculated with the neurospheres expressing shRNA targeting CREB generated tumors with lower levels of p-CREB and TGF-β2 (**Fig. 33b**) and had a longer overall survival (**Fig. 33c**) than mice inoculated with neurospheres expressing control shRNA.

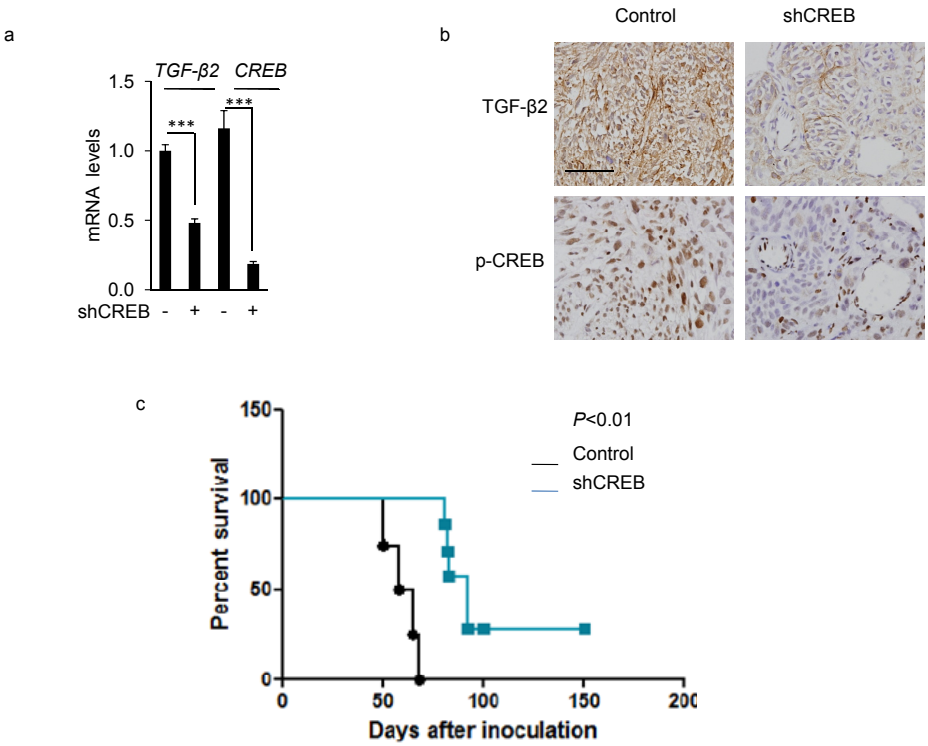


Figure 33. Knockdown of CREB inhibits TGF-β2 expression and prolongs survival of mice inoculated with patient-derived GBM neurospheres.(**a**) qRT-PCR of *TGF-β2* and *CREB* in patient-derived GBM neurospheres expressing shRNA targeting CREB. ***$P < 0.05$ using Student's t test. Data are represented as mean ± s.d.(**b**) Immunohistochemistry of p-CREB and TGF-β2 from mouse tumors 60 days after inoculation with neurospheres expressing shRNA targeting CREB and control shRNAs. Scale bar, 50μm (**c**) Kaplan Meyer survival curves from mice in (**b**)

In addition, acute treatment of mice bearing neurosphere-derived GBMs with a PI3K inhibitor decreased p-CREB and TGF-β2 expression in tumors (**Fig. 34**). These findings confirm our hypothesis that CREB regulates TGF-β2 expression and oncogenesis in GBM.

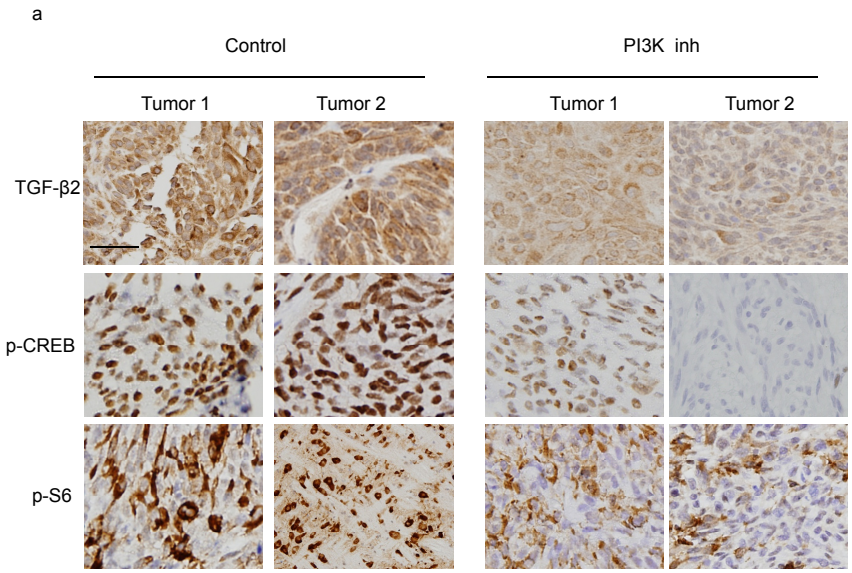


Figure 34. Treatment with a PI3K inhibitor decreases TGF-β2 expression *in vivo*. Patient-derived neurospheres were inoculated in the brain of immunocompromised mice. 54 days after surgery mice were orally treated once a day with 35 mg/Kg of PI3K inhibitor for 17 days. Immunohistochemistry of p-S6, p-CREB and TGF-β2 from mouse tumors is shown. Scale bar, 50μm.

Discussion

4. DISCUSSION

The TGF-β pathway is currently considered a therapeutic target in advanced tumors, including GBM, and several anti-TGFβ agents are in clinical trials and have shown promising results [14, 15, 74, 75]. A thorough understanding of the molecular mechanisms involved in the protumorigenic function of TGF-β will facilitate the identification of markers of response that can be used to stratify patients to be treated with anti-TGFβ compounds and, moreover, will allow for the discovery of new therapeutic agents.

TGF-β is highly active in high-grade glioma, and elevated TGF-β activity confers poor prognosis [16]. Aberrant activation of TGF-β is caused in part by enhanced secretion of the TGF-β ligands by tumor cells and tumor stroma cells. However, the precise mechanisms that lead to the hyperactivation of the TGF-β pathway are not yet fully understood. It is important then to study the mechanisms responsible for the abnormal activity of TGF-β in GBM in order to design effective therapeutic strategies against this disease.

In this work, we identified USP15 as a DUB that regulates the TGF-β signaling pathway and showed that in GBM there is a fraction of patients that presents high levels of USP15 and hence, increased activity of TGF-β. At the same time, we found that TGF-β generates an autocrine loop in collaboration with other signaling pathways (PI3K and RSK) via the transcription factor CREB that leads to an accumulation of TGF-β2 in the tumor. Overall, we have identified two novel mechanisms governed by USP15 and CREB that cause a hyperactivation of TGF-β signaling and ultimately tumor progression.

Through a functional screen, we identified USP15 as a DUB that controls the TGF-β pathway. We found that USP15 forms a complex with SMAD7 and SMURF2 and is recruited to the TGF-β receptor complex, where it deubiquitinates and stabilizes TβR-I. Thus, USP15 induces the activation of the TGF-β pathway through the stabilization of TβR-I. USP15 and SMURF2, two enzymes with opposing activities (a DUB and an E3 ubiquitin ligase, respectively), are present in the same complex with SMAD7. Thus, the balance

between the opposing activities of USP15 and SMURF2 determines the ultimate output of the TGF-β receptor complex **(Fig. 35)**.

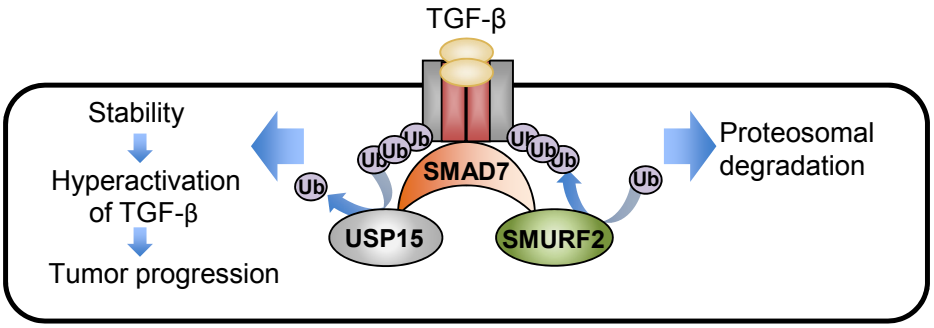


Figure 35. Regulation of TβR-I by USP15. SMAD7 recruits USP15 and SMURF2 to the TGF-β receptor complex. USP15 deubiquitinates and SMURF2 ubiquitinates the TGF-β receptor complex. The balance between USP15 and SMURF2 activities determines the stability of the TGF-β receptor complex. High USP15 concentrations promote TβR-I stability, high TGF-β activity and tumor progression in advanced cancer.

Interestingly, TGF-β induces the dissociation of USP15 from the SMAD7-SMURF2 complex. In an exquisite manner, the intensity of the TGF-β signal regulates the binding of USP15 to SMAD7, and, hence, TGF-β regulates the access of USP15 to the TGF-β receptor complex through SMAD7. Weak TGF-β signals cause USP15 to be recruited to the SMAD7-SMURF2–TβR-I complex to protect the TGF-β output signal, whereas strong TGF-β signals promote the release of USP15 from the SMAD7-SMURF2–TβR-I complex, facilitating the degradation of the complex and promoting the blockade of the TGF-β signal. This generates a rheostat that prevents hyperactivation of the TGF-β signal. However, when USP15 is aberrantly overexpressed, the USP15-SMURF2 balance is tilted toward USP15, and the output of the TGF-β signal is increased, leading to hyperactive TGF-β signaling.

We observed that some GBM tumors express aberrantly high levels of USP15 as a result of *USP15* gene amplification and that *USP15* amplification confers poor prognosis in individuals with GBM. Importantly, *USP15* gene amplification is also present in breast and ovarian cancers, indicating that the oncogenic role of USP15 is not circumscribed to GBM. Moreover, TβR-I and p-SMAD2 levels

correlate with USP15 levels in GBM and breast cancer, further corroborating that USP15 plays a crucial role in the control of TGF-β activity.

Because TGF-β is highly active and is a well-known oncogenic factor in GBM, our results indicate that USP15 could induce oncogenesis in GBM through the upregulation of the TGF-β pathway. To further support this, we used a patient-derived *in vivo* model of GBM. GBM neurospheres enriched for GICs expressing shRNA against USP15 showed a decreased tumor-initiating capacity and generated tumors with diminished TGF-β activity. Furthermore, the anti-tumoral effect resulting from USP15 knockdown was primarily mediated by inhibition of the TGF-β pathway, as restoration of TβR-I expression in cells depleted of USP15 rescued the oncogenic capacity of GBM neurospheres.

Very recently USP15 has also been reported to target monoubiquitinated R-SMADs for deubiquitination. Monoubiquitination was proposed to interfere with SMAD binding to DNA [76]. Overall, USP15 seems to activate the TGF-β pathway by two different means: on the one hand, USP15 deubiquitinates R-SMADs enhancing their transcriptional activity, but it also deubiquitinates and stabilizes the TβR-I. Soon after our work was published, USP11 [77] and USP4 [78] were also described to deubiquitinate and stabilize TβR-I, suggesting a possible functional overlap between the three members of the DU family of DUBs. Interestingly, USP4 activity is regulated through phosphorylation. Upon PI3K activation, AKT phosphorylates USP4 which in turn binds the TβR-I and reverses its ubiquitination [78].

We demonstrated that USP15 is amplified in a subset of cancer types including GBMs and that this confers poor prognosis. The role and contribution of USP4 and USP11 in cancer, however, needs further assessment. Indeed, we have observed that the USP4 gene but not USP11 is amplified in GBM and breast cancer, suggesting that USP4 might also play a role in GBM (data not shown).

It is important to remark that although the incidence of USP15 gene amplification is low (around 2%), we have observed a large proportion of tumors exhibiting high levels of USP15 indicating that the incidence of high USP15 expression is greater than the incidence of gene amplification. These results suggest that USP15 expression in GBM is regulated at many other different

levels. Moreover, USP15 activity can be modified post-transcriptionally: USP15 is subjected to acetylation, methylation, phosphorylation and ubiquitination although the biological roles of these modifications have not been described yet (data from Phosphosite database). Having demonstrated a major role for USP15 in the regulation of TGF-β activity in GBM, it is of great importance to address the mechanisms that regulate USP15 function in GBM and other types of cancer.

Our results show that USP15 could be considered as a marker of response to anti–TGF-β molecules and also that it could be considered as a therapeutic target because USP15 downregulation or inhibition leads to a decrease in TGF-β activity and oncogenesis. DUBs have been shown to be targetable through small organic molecules [46], opening new avenues for therapeutic intervention in GBM and other aggressive tumors.

In addition, we found that TGF-β induces the expression of TGF-β2 in GBM. TGF-β2 secretion is enhanced in GBM and high levels of TGF-β2 constitute a poor prognosis factor. Hence, these results suggest that in GBM, TGF-β generates a malignant autocrine loop that leads to an accumulation of TGF-β2 in the tumor environment and finally to hyperactivation of the TGF-β pathway. Given the oncogenic role of TGF-β in GBM, the aberrant activation of TGF-β ultimately causes tumor progression.

We have now identified CREB as a critical activator of this autocrine loop. Our results show that, in response to TGF-β, p-CREB and SMAD2 bind in the proximal region of the TGF-β2 promoter and activate its transcription. In our cell models, p-CREB is constitutively bound to the CREB binding site while SMAD2 only binds to the promoter after TGF-β stimulation. However, TGF-β induces the phosphorylation of CREB (**Fig. 26b, 27b** and [79]) increasing the amount of p-CREB bound to the TGF-β2 promoter. The binding of both transcription factors in response to TGF-β then triggers TGF-β2 transcription. We further show that CREB is required for TGF-β-mediated activation of TGF-β2 transcription given that knockdown of CREB , expression of an endogenous transcriptional repressor (ICER) or mutation of the CREB binding site in the *TGF-β2* promoter, ablate TGF-β2 expression in response to TGF-β. Because

SMAD transcription factors have low affinity for DNA [8], CREB could be acting as a DNA-binding cofactor of SMADs to help them achieve binding with higher affinity and activate the transcription of *TGF-β2*.

Importantly, p-CREB and TGF-β2 levels correlate in GBM further corroborating that CREB has a crucial role in the regulation of TGF-β2 expression in this type of cancer. To support this, we used a patient-derived *in vivo* model of GBM [18]. As shown in the Results section, GBM neurospheres enriched for GICs expressing shRNA against CREB generated tumors with diminished levels of TGF-β2. Remarkably, mice inoculated with shCREB neurospheres had a longer overall survival than control mice suggesting that CREB is an important oncogenic factor.

Interestingly, CREB is required for TGF-β1-mediated induction of TGF-β3 [62] and TGF-β3 autoregulation [63] in epithelial cells. Moreover, TGF-β3 induces TGF-β1 expression via the AP1 transcription factors JUND and FRA2 [80]. In contrast to epithelial cells, in the GBM cell lines LN229 and U373, TGF-β1 induces the expression of TGF-β2 but not that of TGF-β1 nor TGF-β3. At the same time, the three TGF-β isoforms are able to induce the expression of TGF-β2. Together, these results suggest that TGF-β regulates a common gene program consisting on the CREB family of transcription factors and the closely related AP1 factors that controls the availability of TGF-β ligands. The expression of one isoform or other depends on the cellular context.

We have also found that PI3K and RSK enhance TGF-β-mediated induction of TGF-β2 by activating CREB via phosphorylation in Ser-133. These results indicate that the PI3K and RSK signaling pathways crosstalk and collaborate with TGF-β in the induction of TGF-β2 expression. This is of high relevance given that the vast majority of GBMs (88%) present genomic activation of the RTK/RAS/PI3K signaling pathways [6]. This suggests that TGF-β2 expression will thus be enhanced in a great proportion of cases of GBM. All in all, our results show that TGF-β generates an autocrine loop in collaboration with other signaling pathways (PI3K and RSK) via CREB that leads to an accumulation of TGF-β2 in the tumor (**Fig. 36**).

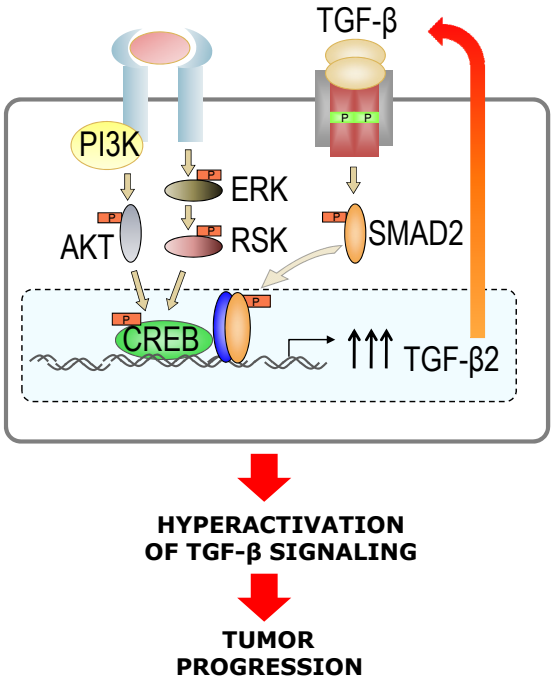


Figure 36. The TGF-β malignant autocrine loop. In GBM, TGF-β generates an autocrine loop in collaboration with other signaling pathways (PI3K and RSK) via CREB that leads to an accumulation of TGF-β2 in the tumor, hyperactivation of TGF-β signaling and tumor progression.

We should bear in mind, though, that GBMs are highly affected by their tumor microenvironment. GBMs are infiltrated by numerous microglial cells that release factors, such as TGF-β , which are important for tumor progression and invasion [81]. TGF-β is then not only secreted by glioma cells but also by cells of the tumor niche.The mechanisms that lead to enhanced secretion of TGF-β by microglial cells in GBM and whether the TGF-β autocrine loop does also occur in cells from the tumor niche should be addressed in the future in order to design effective therapeutic strategies against GBM.

In agreement with our results, it has recently been reported that PI3K induces TGF-β2 expression in breast cancer. In this case, the authors claim that activation of the PI3K signaling pathway leads to AKT-mediated phosphorylation of TWIST, which in turn binds to the *TGF-β2* promoter and induces its transcription [29]. It should be further explored whether TWIST does also regulate TGF-β2 expression in glioma. This could explain why in some

experiments we observed an increased expression of basal TGF-β2 expression when PI3K signaling was hyperactivated. On the other hand, one could hypothesize that under basal conditions there is still a very low activity of TGF-β signaling that could trigger TGF-β2 expression when CREB is phosphorylated by AKT. Although the authors do not find TGF-β2 mediated regulation of TGF-β ligands (TGF-β1 and TGF-β3), we observed that TGF-β induces TGF-β2 in two different breast cancer cell lines. Moreover, treatment with a PI3K inhibitor blocks this effect indicating that PI3K signaling does also regulate the TGF-β autocrine loop in breast cancer (data not shown). It would be of great interest then, to study the relative contribution of TWIST and CREB in the TGF-β autocrine loop in breast cancer and GBM.

PI3K and RSK inhibitory compounds are currently being tested as anti-cancer therapeutic agents. Indeed, several PI3K inhibitors are being evaluated in clinical trials for GBM. Unfortunately, bioavailavility of current RSK inhibitors has not been reported yet and they only have been tested so far in the context of *in vitro* experiments. To address the mechanisms of action of PI3K inhibitors in GBM, we inoculated mice with patient-derived neurospheres and, once they had developed tumors, we treated them with a PI3K inhibitor for seventeen days. The tumors generated exhibited lower levels of p-CREB and TGF-β2 than control tumors. Our results suggest that treatment with PI3K inhibitors would decrease TGF-β activity in GBM, through TGF-β2 downregulation, and thus improve patient outcome.

CREB could be considered as a marker of response to anti-PI3K and anti-RSK molecules and also as a therapeutic target, since CREB downregulation or inhibition leads to a decrease in TGF-β2 expression and oncogenesis. CREB could be targetable through the inhibition of upstream components of the CREB pathway, such as PI3K or RSK. Moreover, new small molecules targeting CREB phosphorylation, CREB-DNA, or CREB-CBP interaction are being developed [58] opening new avenues for therapeutic intervention in GBM .

We have demonstrated then that the TGF-β signaling pathway can be hyperactivated in GBM by different means including the stabilization of the TβR-I by overexpression of USP15 or the enhanced secretion of TGF-β2 by the

generation of a malignant autocrine loop via CREB. In this project, we identified USP15 and CREB as two novel regulators of TGF-β activity. USP15 and CREB could be considered as markers of response to anti–TGF-β molecules and potential therapeutic targets against GBM.

Conclusions

5. CONCLUSIONS

- USP15 is a key regulator of the TGF-β signaling pathway in GBM.
- USP15 binds to the SMAD7/SMURF2 complex and deubiquitinates and stabilizes TβR-I, leading to an enhanced TGF-β signal.
- TGF-β activity is regulated by a rheostat that prevents hyperactivation of the TGF-β signal. When TGF-β signaling is weak, USP15 binds to the SMAD7-SMURF2-TβR-I complex, deubiquitinates the receptor and increases TGF-β signaling. When TGF-β signaling is strong, USP15 is released, the TβR complex is degraded and the intensity of the TGF-β signal lowered. When USP15 is overexpressed, this balance is broken and TGF-β signaling is hyperactivated.
- USP15 is overexpressed and targeted for amplification in GBM, breast and ovarian cancer.
- USP15 amplification confers poor prognosis in individuals with GBM.
- USP15 regulates TGF-β-dependent oncogenesis in GBM.
- TGF-β2 is overexpressed in GBM and is a bad prognosis factor.
- Enhanced secretion of TGF-β2 in GBM is due in part to the generation of an autocrine loop where TGF-β induces the expression of TGF-β2 via the transcriptional collaboration between SMAD2 and CREB.
- PI3K and RSK signaling enhance the TGF-β autocrine loop via activation of CREB.
- CREB regulates oncogenesis in GBM.
- Overexpression of USP15 and enhanced secretion of TGF-β2 in GBM cause a hyperactivation of TGF-β signaling and tumor promotion.

Materials and Methods

6. MATERIALS AND METHODS

6.1 Plasmids

The DUB knockdown library vectors were generated by annealing the individual oligonucleotide primer pairs and cloning them into pSUPER vectors as described in [64]. The bacterial colonies of each DUB hairpin were then pooled and used for plasmid preparation. The *USP15* short hairpin sequences used were (A): 5′-CTTTAACAGAAATTGTCTC-3′ and (B): 5′-GGAACACCTTATTGATGAA-3′, and the siRNAs sequences used were 5′-GATGATACCAGGCATATAA-3′, 5′-GGTATTGTCCGAATTGTAA-3′, 5′-CCAAACCTATGCAGTACAA-3′ and 5′-GAGTATTTCCTCAATGATA-3′. The lentiviral knockdown sequences targeting USP15 (Open Biosystems) used were 5′-CCGTAATCAATGTGGGCCTAT-3′ and 5′-GCTCACCAAGTGAAATGGAAA-3′. The *SMAD7* short hairpin sequence used was 5′-GAGGCTGTGTTGCTGTGAA-3′. The *CREB* short hairpin sequence used was (A): 5’- GAGAGAGGTCCGTCTAATG-3’ and the lentiviral knockdown sequence targeting CREB used was 5’-ACCAACAAATGACAGTTCA-3’ (Open Biosystems). The catalytically inactive USP15 mutant (USP15 C269S) was generated by site-directed mutagenesis. Flag-SMURF1, Flag-SMURF2, Myc-SMURF2, Flag-SMAD6, GST-SMAD7 and HA-ubiquitin, were purchased from Addgene. Purified His-tagged full-length USP15 was purchased from BIOMOL. The USP15 fragments (USP15 N-terminal domain (1–488 amino acids (aa)), USP15 link (488–724 aa) and USP15 C-terminal domain (724–952 aa)) were cloned into a pCMV5-Flag vector. The *TGF-β2* promoter constructs comprise a genomic DNA fragment spanning bases -*77* to +*63* of *TGF-β2* (relative to the transcriptional start codon) cloned into pGL3-basic vector with or without a CREB binding site mutation (CGTCAC to TGGCAC) [26].

6.2 Antibodies and reagents

Specific antibodies against USP15 and SMAD2 (Abcam); p-SMAD2, SMAD2, p-AKT, p-SMAD1/5/8, p-S6, p-CREB and CREB (Cell Signaling); SMAD4, SMAD7, HA, GFP, Myc, TβR-I, TβR-II, TGF-β2 and CREM (Santa Cruz Biotechnology); GAPDH (Trevigen); SMAD1 [82]; Tubulin, Actin and Flag (Sigma) were used for immunoblot and immunohistochemistry. Antibodies against SMAD2 (Cell Signaling), ChIPAb+™ Phospho-CREB (Ser133) (Millopore) and rabbit IgG (Upstate) were used for ChIP.

The treatments used were as follows: TGF-β1, TGF-β2, TGF-β3 (100 pM; R&D), MG132 (5 μM; Sigma), PR-619 (2.5 μM; LifeSensors), BMP4 (200 μM; R&D); Activin (20 ng/ml; R&D); PI3K inhibitor NVP-BEZ235 (250 nM; Novartis); RSK inhibitor BI-D1870 (5 μM; Axon Medchem BV).

6.3 Cell culture

PCTCs and GBM neurospheres were generated as described previously [16, 83]. Briefly, tumor samples were processed within 30 minutes after surgical resection. Minced pieces of human GBM samples were digested with 200U/ml collagenase I (Sigma) and 500 U/ml DNase I (Sigma) in PBS for 2 hours at 37°C with constant vigorous agitation. The single-cell suspension was filtered through a 70 mm cell strainer (BD Falcon) and washed with PBS. Finally, cells were resuspended and subsequently cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (for PCTC culture) or in neurosphere medium (for GBM neurospheres). The neurosphere medium consisted of Neurobasal medium (GIBCO) supplemented with B27 (GIBCO), L-glutamine (GIBCO), penicillin/streptomycin, and growth factors (20 ng/ml EGF and 20 ng/ml FGF2; PeproTech). Human GBM specimens were obtained from the Vall d'Hebron Hospital. The clinical protocol was approved by the Vall d'Hebron Institutional Review Board, with informed consent obtained from all subjects. 293T, T98G, LN229 and U373 cells were cultured in DMEM supplemented with 10% FBS. Transient transfections were carried out using either the the calcium phosphate transfection method or Lipofectamine 2000 (Invitrogen). Lysates were collected 48-72 hours post-transfection. GBM neurospheres were disaggregated with accutase (Sigma) and $5x10^5$ cells were

plated out in 6 cm plates and incubated for 36 hours. TGF-β was added and cells were incubated for a further 16 hours.

6.4 RNA extraction, retrotranscription and Quantitative Real Time PCR

Cells were seeded in 60mm plates at 70% confluency. After the described treatments, cultured cells were disrupted in lysis buffer from RNeasy Mini Kit (Qiagen) and mRNA was purified following manufacturer's instructions. mRNA was quantified and 300-800 ng of mRNA from each sample were retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied biosystems) following the product indications. After cDNA synthesis, qRT-PCR was performed. All qRT-PCR were performed using Taqman probes from Applied Biosystems, according to the manufacturer's recommendations. Reactions were carried out in an ABI 7900 sequence detector (Perkin Elmer) and results were expressed as fold change calculated by the Ct method relative to the control sample. *GAPDH* was used as internal normalization control.

6.5 Western Blot

Cells were lysed in RIPA buffer supplemented with protease inhibitors (Roche). Whole cell extracts were quantified using the BCA protein assay kit (Pierce) and were then separated on 7% - 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk and probed with specific antibodies. Blots were then incubated with an HRP-linked second antibody and developed with chemiluminescence. Band intensities were quantified using the ImageJ software.

6.6 ELISA

For the quantitative determination of TGF-β2 protein levels secreted to the media, we used the Human TGF-β2 Quantikine ELISA kit (R&D systems) following manufacturer's specifications. Supernatant from serum-starved cells was collected 72 hours after the start of the indicated treatments.

6.7 Retroviral infections

293T Phoenix-Ampho cells were transfected using the calcium phosphate transfection method with the retroviral vectors pJP1520, pJP1520-PI3K H1047R, pLNCX2, pLNCX2-ICER, pRetroSuper, pRetroSuper-shCREB. After 16 hours, medium was replaced and recombinant retrovirus was harvested for additional 24 hours. For infection, medium containing recombinant retrovirus was added to LN229 and U373 cell lines. Polybrene (Sigma) was added at a concentration of 8 mg/ml. Phoenix-Ampho cells were incubated in fresh medium for additional 8 hours; after this time, the LN229 and U373 cells were washed and the medium containing recombinant retrovirus from Phoenix- Ampho cells was added. This process of infection was repeated two more times every 8 hours. Two days later, antibiotic selection was added to the medium (puromycin (1µg/ul); neomycin (0.5 µg/ul); higromycin (100 µg/ml)).

6.8 Lentiviral infections

293T cells were transfected using the calcium phosphate transfection method with pMD2.G enveloping plasmid, psPAX.2 packaging plasmid and either pLKO, pLKO-shUSP15 , pEV833, pEV833-TβR-I, pGIZ or pGIZ-shCREB . After 16 hours, medium was replaced by neurosphere medium with 5mM Sodium Butyrate and recombinant lentivirus was harvested for additional 24 hours. For infection, medium containing recombinant lentivirus was added to previously dissociated neurospheres. Polybrene (Sigma) was added at a concentration of 8 mg/ml. Following 16 hours of incubation, the neurospheres were washed and incubated in fresh neurosphere medium. 293T cells were incubated in fresh neurosphere medium containing 5mM Sodium Butyrate for 24 hours and a second round of neurosphere infection was repeated as previously described.

6.9 Luciferase assays

We transfected the CAGA-luc reporter, the pRL-TK Renilla luciferase plasmid (Promega) as a normalization control, and the corresponding plasmids into 293T cells using the calcium phosphate system, and after 72 hours, TGF-β was

added for 16 hours. For LN229 H1047R, cells were transfected with different TGF-β2 promoter reporter constructs and pRL-TK Renilla luciferase plasmid using Lipofectamine 2000. After a 16 hour incubation at 37°C, cells were treated with TGF-β for a further 32 hours. Luciferase counts were measured using a Sirius Luminometer (Berthold).

The *firefly* luciferase activity was normalized with *renilla* luciferase activity. Data are represented as relative activity (compared with basal promoter activity) and are expressed as the mean +/- s.d. of triplicates from a representative experiment.

6.10 Immunoprecipitation and the *in vivo* deubiquitination assay

Cells were lysed in ELB buffer (0.25 M NaCl, 0.1% NP-40 and 50 mM HEPES pH 7.3) supplemented with proteasome inhibitors. Cell lysates were incubated for 2 hours with 2 µg of the indicated antibodies conjugated to protein A or protein G sepharose beads (GE Healthcare), washed in ELB and separated on SDS-PAGE gels. When indicated, cell lysates were immunoprecipitated with anti-Flag M2 affinity resin (Sigma). For the ubiquitination assays, cells were treated with MG132 (5 µM; Sigma) 12 hours before collection.

6.11 FISH

The probes for USP15 (Human BAC clone RP11-169M9, SpectrumGreen) were purchased from the Genome Resources Facility, Hospital for Sick Children, Toronto, Canada. The chromosome 12 control probe (CEP12 SpectrumOrange) was purchased from Abbott Molecular. FISH 5-µm–thick slides were placed on silane-coated slides, deparaffinized, pretreated (with pH 6 citrate buffer and autoclaving at 115 °C for 4 minutes) and digested with proteinase K for 10 minutes. The samples were then fixed in 10% buffered formalin, dehydrated and hybridized at 80 °C and then kept at 37 °C overnight. Signals from USP15 compared to those from chromosome 12 were analyzed in at least ten cells.

6.12 Tissue microarrays and immunohistochemical staining

For tissue microarray generation, three 0.6 mm cores were taken from separate areas, and each one was arrayed into recipient blocks in a 1 mm-spaced grid. Formalin-fixed, paraffin embedded tissue sections were deparaffinized and hydrated. Antigen retrieval was performed using pH 6 Citrate Antigen Retrieval Solution (DAKO) for p-SMAD2, TGF-β2, p-CREB and p-S6 and pH9 Antigen Retrieval Solution (DAKO) for USP15 and TβR-I antibodies. Peroxidase blocking was done with 3% H_2O_2 at room temperature for 10 minutes. For TGF-β2 and p-S6, slides were incubated with a blocking solution (10% normal goat serum, 2% BSA) for 1 hour at room temperature. The slides were incubated with the primary antibody for 2 hours in the case of TGF-β2 and p-CREB and overnight for p-S6, p-SMAD2, USP15 and TβR-I at 4 ºC. p-SMAD2 antibody (Cell Signalling 3108) was used at a 1:250 dilution; USP15 antibody (Abcam 97533) and TβR-I (Santa Cruz sc402) were used at 1:200 dilution; TGF-β2 (Santa Cruz sc-90) was used at a 1:500 dilution; p-CREB (Cell Signaling 9198) was used at a 1:100 dilution; and p-S6 (Cell signaling) was used at a 1:25 dilution. As a detection system, EnVision FLEX+ was used (DAKO) following the manufacturer's instructions and developed with freshly prepared 0.05% 3′,3-diaminobenzidine tetrahydrochloride. Finally, the slides were counterstained with hematoxylin, dehydrated and mounted. Positive and negative controls were performed in each run. The quantification of the staining was expressed as *H* score. The *H* score was determined by the formula: 3 x percentage of strong staining + 2 x percentage of moderate staining + percentage of weak staining, giving a range of 0 to 300 for the *H* scores.

6.13 Generation of recombinant proteins and *in vitro* pulldown assays

Purified His-tagged full-length USP15 was purchased from BIOMOL. The GST-SMAD7 fusion protein (Addgene) was expressed in BL21 (DE3pLysS) cells and purified with the Bulk and RediPack GST Purification Kit (GE Healthcare). Twenty-five micrograms of the proteins were incubated for 6 hours in dialysis buffer (50mM HEPES, 0.1% NP40, 20% glycerol, 1mM EDTA, 1mM PMSF, 1mM DTT, 0.02% BSA, 100nM NaCl), supplemented with proteasome inhibitors

(Roche) at 4 °C with a Slide-A-Lyzer MINI Dialysis Device 3.5k MWCO (Thermo Scientific). Then, the protein mixture was incubated for an additional 2 hours with 2 µg of antibodies to SMAD7 (Santa Cruz) conjugated to protein G sepharose beads. The slurry was then washed in dialysis buffer, and the proteins were separated on SDS-PAGE gels.

6.14 Chromatin immunoprecipitation

LN229 cells were grown to 70% confluence, serum starved for 16 hours and cultured in the presence or absence of TGF-β for 1 hour. Cells were trypsinized and crosslinked in 1% formaldehyde for 10 minutes at room temperature. Crosslinking was quenched with a 0.125 M glycine solution for 5 minutes in formaldehyde, and cells were washed twice with PBS. Pelleted cells were lysed in 1 ml ChIP buffer (1 volume of SDS buffer with 0.5 volumes of Triton buffer), and sonicated in a Bioruptor (Diagenode). Soluble material was quantified by Bradford assays and p-CREB and SMAD2 were immunoprecipitated from 1000 µg of protein. Antibodies were incubated overnight with the chromatin in a 500 µl volume. Immunocomplexes were recovered with 30 µl of a protein A (for p-CREB and IgG) or G (for SMAD2) bead slurry. Immunoprecipitated material was washed three times with a low-salt buffer and once with a high-salt buffer. DNA complexes were de-crosslinked in 100 µl decrosslink buffer (1% SDS and 100 mM $NaHCO_3$) at 65 °C for 3 hours, and DNA was then eluted in 100 µl of water using a PCR purification kit (Qiagen). DNA (2 µl) was used for each qRT-PCR reaction with SYBR green (Roche). Antibodies against SMAD2 (Cell Signaling 3103), ChIPAb+™ Phospho-CREB (Ser133) (Millipore) and rabbit IgG (Upstate) were used for ChIP. The proximal *TGF-β2* promoter primer set utilized spans *-84* to *-18* relative to the transcriptional start site.

6.15 Intracranial tumor assay

All mouse experiments were approved by and performed according to the guidelines of the Institutional Animal Care Committee of the Vall d'Hebron Research Institute in agreement with the EU and national directives. The cells

were stereotactically inoculated into the corpus striatum of the right brain hemisphere (1 mm anterior and 1.8 mm lateral to the bregma; 2.5 mm intraparenchymal) of 9 week-old NOD-SCID mice (Charles River Laboratories). Mice were euthanized when they presented neurological symptoms or a significant loss of weight. An MRI analysis was performed in mice injected intraperitoneally with gadolinium diethylenetriamine penta-acetic acid at a dose of 0.25 mmol gadolinium per kg of body weight. T1-weighted magnetic resonance images were acquired in a 9.4-T vertical bore magnet interfaced to an AVANCE 400 system (Bruker).

6.16 Isolation of human cells from orthotopic xenografts in mouse brains

Brains from mice inoculated with neurospheres were dissociated and stained with the pan-MHC-I-specific monoclonal antibody (mAb) HP-1F7 (Santa Cruz Biotechnology), followed by secondary PE-conjugated mAb (Dako Cytomation) and subsequent cell sorting of human MHC-I-positive cells (MoFlo-DAKO). Cells obtained were washed and immediately used in subsequent experiments.

6.17 Statistical analyses

Student's *t* tests were performed for statistical analyses. Data in all graphs are represented as means ± s.d. A Spearman correlation test was used to analyze the relationships between USP15, p-SMAD2 and TβR-I and p-AKT, p-CREB and TGF-β2.

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Acknowledgements

8. ACKNOWLEDGEMENTS

Buff, y los sentimientos y los recuerdos se atropellan en mi mente mientras pienso en todo lo que he vivido estos años de doctorado. Han sido unos años muy duros, tanto el plano personal como laboral, dos ámbitos de mi vida que no son más que uno, mi vida. Y recuerdo cuando empecé, recién salida de la carrera, pensando que esto iba a ser coser y cantar, que me iba a comer el mundo…Y me veo ahora, con unos cuantos años de más y unas cuantas experiencias más vividas y me río de la persona que era entonces y me enorgullezco de la persona que soy ahora. Con mucho que vivir todavía y mucho que aprender. Una persona soñadora y con la misma ilusión que cuando tenía 3 años pero más consciente de las dificultades y del esfuerzo e integridad que requiere ir superándolas. Agradecida a la vida por haberme dado tanto y haberme hecho sentir la persona más afortunada del mundo. Agradecida a todas las personas que han andado a mi lado todos estos años y de las que he aprendido siempre algo y han dejado una parte de sí en mí. A todos tengo algo que agradecer, que ha sido enriquecer mi vida.

A Joan, por haberme guiado y ayudado a desarrollar mi potencial científico. Por haber sido modelo de esfuerzo y perseverancia.

A los miembros de mi comisión de seguimiento, Montserrat Jaumot, Francesc Vinyals y Antonio García de Herreros, por haber seguido mi trabajo durante estos años y haberme ayudado a enriquecerlo con vuestra crítica siempre tan constructiva.

A mis compañeros de laboratorio, porque habéis sido mi segunda familia. A Eva, Sandra, Isa, Sara y Rosa, porque sois la columna vertebral de este grupo, porque sin vuestro trabajo ni la mitad de los logros de este laboratorio hubiesen sido posibles. A Álex, Silvia, Andrea, Mari, Isa Huber, Anna, Maria del Mar y Rudy, esos postdocs que no solo me han dado una gran lección de ciencia sino de saber hacer. Muchísimas gracias también Maria del Mar y Mari por revisar la tesis y haberme ayudado a mejorarla. A Rosa Prieto y Judit, porque habéis sido mis “hermanas mayores” en el laboratorio. A Joana, por esos ChIPs. A Piet y al resto de autores, por el Nature Medicine que nos sacamos entre pecho y

espalda; un ole por nosotros. A mis compañeros Gerard, Paco, Ada, Carol y Alba, porque habéis compartido conmigo este camino tan arduo y poco agradecido, por vuestro apoyo y compañerismo, por haberos sabido reir conmigo de todos los problemas que nos hemos ido encontrando ya fuera al son de una canción, de una cerveza o de un helado. A mis irlandeses o "irlandeses postizos" preferidos, Dermot y Vanesa, my father once told me that irish people are a bit nuts…well…yeah…he wasn't that wrong! Thanks a lot for revising my thesis, I owe you one.

Durante estos años de doctorado, el laboratorio es, sin duda, el lugar donde más horas he pasado, y a todos vosotros os debo muchísimo, os debo mucha ciencia y sobre todo mucho cariño.

A mis amigas de la universidad, Mireia, Laia, Mar y Nuri…els sopars…les converses…els caps de semana que hem passat plegades…els viatges..quins viatges! uns records per tota la vida. Esta va por vosotras: Asuncióoon Asuncióon….;) A mamá, Jessica, Sergi y Carles, por vuestra amistad, que es mucha, y por vuestros sabios consejos en el diseño de la tesis. Muchísimas gracias especialmente a Marisol Escalera por esa portada tan fantástica que diseñaste. Es maravilloso estar rodeado de gente con gran talento y ganas de echarte un cable y estrujarse la cabeza con ideas y proyectos. A tu Carles també per les nostres converses, perquè en tu vaig trobar la persona amb la qui parlar a cor obert dels meus sentiments i les meves pors en un moment molt dur de les nostres vides. A Kazuko, por el tai chi! Porque el tai chi ha sido mi “yin” perfecto estos años, esa fuerza de equilibrio que mi vida necesitaba durante el doctorado. A mis amigas del alma Berta y Cris. A Berta, por tu empatía y tu risa contagiosa. ¡Cuánta alegría le has dado a mi vida!! A Cris, por tantísimos años de amistad, por todo lo que hemos vivido juntas, por haber estado siempre ahí. A ti Dani, por cuánto has aportado a mi vida, porque hoy sería una persona muy distinta si no te hubiera conocido. Te debo innumerables alegrías y penas, experiencias y recuerdos; te debo mi madurez.

Y dejar para el final a los grandes, a los mejores. A Jordi, porque por muchos años que pasen, sigues tomándote tan en serio tu papel de hermano mayor. Ya te lo dije un día, eres mi ángel de la guarda. Y porque tengo la inmensa suerte

de tener cerca a un gran modelo profesional a seguir, con ambición pero ante todo con ética y humanidad. Y por supuesto a vosotros, mamá y papá, porque me lo habéis dado todo, y todo…es TODO.