UNIVERSITAT DE BARCELONA

Understanding chromosomal instability-induced senescence

Celia Santos Tapia

Tesis doctoral
Universidad de Barcelona

Understanding Chromosomal Instability- induced Senescence

Memoria presentada por
Celia Santos Tapia
para optar al grado de Doctora por la Universidad de Barcelona

Programa de Genética

Development and Growth Control Laboratory
Institute for Research in Biomedicine (IRB Barcelona)
Parc Científic de Barcelona

Barcelona Julio 2019

Marco Milán	Celia Santos Tapia	Florenci Serras
(Director)	(Alumna)	(Tutor)

“La aventura tiene valor por sí misma”

Amelia Earhart

A mi yayo Pedro,
porque calzó los pies que me trajeron hasta aquí.

Quiero dedicar esta tesis a mi familia: mi madre Carmen, mi padre Manuel y mi hermana Gracia. A mi madre por enseñarme creer en mi fortaleza y a tener siempre los ojos y el corazón abierto. A mi padre por impulsarme a vivir una vida de acuerdo mis valores.

También quiero dedicar esta tesis a Humberto, quien estuvo a mi lado día a día durante estos años. Gracias por poner tanto amor en tus actos.

Quiero agradecer también a todas las mujeres científicas que se han cruzado en mi camino. Mis compañeras de laboratorio y del centro, porque cada una de ellas representa a su manera la fortaleza y la bondad. Por todos sus cuidados y apoyo sincero. Habéis sido una inspiración para mí. Gracias también a mis compañeros por su apoyo y los buenos momentos.

Quiero agradecer a Barcelona y a todas las personas que he conocido durante estos años por haberme hecho crecer en lo profesional y en lo personal.

Por último, quiero agradecer a Marco la oportunidad de hacer esta tesis y haberme guiado en ella.

Table of contents

Abstract

Aneuploidy, defined as a chromosome number that deviates from a multiple of the haploid set, is a common feature in human cancer, and around 70% of human solid tumours are aneuploid. The resulting metabolic imbalance is proposed to play a fundamental role in the compromised fitness of these cells and lead to malignant transformation by causing proteotoxic stress and affecting cell cycle proliferation and growth. However, most of the molecular pathways and cellular behaviours underlying aneuploid-induced tumorigenesis remain uncharacterized. *Drosophila* larval epidermal primordia have proved useful model systems to demonstrate the contribution of aneuploidy-induced metabolic stress to tumour growth. By depleting different Spindle Assembly Checkpoint (SAC) genes in the epithelial cells, we induce chromosomal instability and generate aneuploidy. When prevented from undergoing programmed cell death (PCD), these cells give rise to a neoplastic overgrowth. Here we propose that CIN-induced aneuploidy in epithelial cells activates low levels of the c-Jun N-terminal kinase (JNK) and the DNA damage response (DDR). This induces a G1 stall that prevents the accumulation of damage. However, when due to CIN these cells become highly aneuploid, they delaminate from the epithelium and acquire a senescent behaviour. This senescent behaviour is dependent on high levels of JNK and DDR signalling, and induce the secretion of wide variety of factors - also known as the senescence-associated secretory phenotype (SASP) -, and a permanent G2 arrest, among other senescent features. In addition, we have identified two target effectors, Tribbles and String, that are miss-regulated and could act downstream JNK and the DDR to induce the G2 arrest. Finally, we were able to explore two different ways to target CIN-aneuploid cells based on their basal levels of replicative stress: genetic (CycE/Dap overexpression) and chemical (Hydroxyurea), which significantly affect tissue growth and impair tumour progression.

Resumen

La aneuploidía, que se define como un número de cromosomas diferente al haploide, es una característica muy común en cáncer ya que se encuentra en alrededor de 68% de los tumores sólidos. Esta resulta en un desequilibrio metabólico que compromete la función celular induciendo estrés proteotóxico y afectando el crecimiento celular, dando lugar a así a una transformación maligna de estas células. Sin embargo, muchos de los mecanismos moleculares detrás de esta transformación generada por la aneuploidía son aún desconocidos. En este trabajo, usamos el epitelio del ala de *Drosophila* como tejido modelo para demostrar la contribución de la aneuploidía al crecimiento tumoral. Mediante la inhibición de genes del *checkpoint* del huso mitótico, inducimos inestabilidad cromosómica y aneuploidía que, tras bloquear la muerte celular, da lugar a un sobrecrecimiento tumoral. Cuando las células no tienen aún altos niveles de aneuploidía, activan bajos niveles de la quinasa c-Jun N-terminal (JNK) y activan la respuesta a daño en el DNA (DDR). Esto induce un arresto temporal en G1 que previene la acumulación de más daño. Sin embargo, debido a la inestabilidad cromosómica, estas células continúan proliferando y acumulan altos niveles de aneuploidía, delaminan del epitelio y adquieren un comportamiento senescente. Este depende de la activación de altos niveles de JNK y la DDR y tiene como consecuencia la secreción de distintos factores (SASP) y un arresto permanente en G2, entre otras características senescentes. Además, hemos podido identificar dos factores involucrados en el arresto, Tribbles and String, que podrían actuar en respuesta a JNK y a la DDR para hacer efectivo el arresto en G2. Finalmente, exploramos dos maneras distintas de afectar los tumores CIN aprovechando que sufren daño en el DNA: genéticamente (mediante la sobreexpresión de CycE/Dap) y químicamente (mediante el tratamiento con hidroxiurea), afectando ambas el crecimiento del tejido e inhibiendo la progresión tumoral.

Introduction

1. Chromosomal instability and cancer

1.1 Chromosomal instability as a hallmark of cancer

Cancer is a heterogeneous disease, but most cancer cells share several molecular, biochemical, and cellular capabilities (Hanahan and Weinberg, 2000), that are acquired in a multistep process that allows them to gain complexity and become malignant. Their acquisition is made possible by the appearance of two enabling characteristics: the inflammatory state and genomic instability. The first is based on the fact that many lesions are made by the sustained activity of the immune system. The second refers to a state that generates random mutations including chromosomal rearrangements; among which are the genetic changes that can orchestrate hallmark capabilities (Hanahan & Weinberg, 2011).

One of the forms of genomic instability is chromosomal instability (CIN) which is defined as a persistently high rate of loss and gain of whole or parts of chromosomes. Its definition and contribution to disease were first proposed by Boveri nearly a century ago: he proposed that a definite combination of chromosomes in a cell is essential for normal development, and tumours might be the consequence of a certain abnormal chromosome constitution (Boveri, 1914). Many years after, Vogelstein's group proposed that CIN was one of the two molecular mechanisms of genomic instability, together with micro-satellite instability (Christoph, Kenneth, & Bert, 1998). Following Vogelstein's research, different reports began to suggest that CIN was an important causative factor in most cancers (Ye et al., 2013) and that it is present in all stages, from precancerous lesions to advanced cancers (Hanahan & Weinberg, 2011).

What is the molecular basis of chromosomal instability? Its origin in hereditary and non-hereditary tumours differs. In hereditary tumours, the presence of CIN has been explained by the mutator hypothesis: mutations in DNA repair genes, such as DNA mismatch and base excision repair genes (Negrini, Gorgoulis, & Halazonetis, 2010) leads to the gain of genomic instability that will allow the acquisition of additional

mutations. The identification of these "primary" mutations in hereditary cancers provides strong support for the mutator hypothesis, confirming that genomic instability is present in precancerous lesions and drives tumour development by increasing the spontaneous mutation rate (Nowell, 1976).

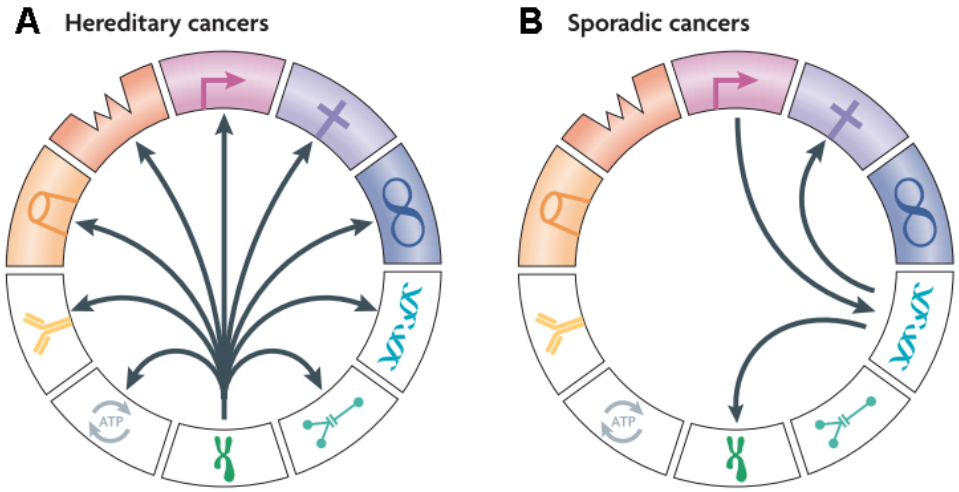

Figure 1. Genomic instability as a hallmark of cancer. (A) The temporal order by which the hallmarks are acquired in hereditary cancers. The establishment of genomic instability is probably the initiating event, which then facilitates the establishment of all the other hallmarks. (B) The temporal order by which the hallmarks are acquired in sporadic (non-hereditary) cancers. Deregulation of growth-regulating genes can be the initiating event. (Hanahan & Weinberg, 2011).

However, in non-hereditary tumours, the way genomic instability and CIN appears and the temporal order by which the hallmarks are acquired is different. According to high-throughput sequencing studies, in sporadic tumours, mutations in these caretaker genes are common but only four genes, such as p53 and other DNA damage checkpoint genes, are altered in more than 20% of cancers (Negrini et al., 2010). These results argue against the mutator hypothesis for sporadic cancers in which mutations in one of these caretaker genes would be responsible for the CIN acquisition. The proposal for these tumours is that the initial event would be a mutation in oncogenes and the consequent induction of DNA replication stress. Specific genomic sites, called common fragile sites, would be particularly sensitive to

this stress and lead to the acquisition of new mutations and appearance of chromosomal instability (Yoder, Vincent, Morgan, & Grush, 1985).

The consequences of CIN are broadly studied. Cells with CIN have longer metaphases that culminate with telomere dysfunction and DNA damage at the end of the chromosomes. Besides, lagging chromosomes that appear in mitosis are often trapped upon cytokinesis, producing double-strand breaks and structural chromosome aberrations (Janssen et al., 2015). However, the most relevant consequence of CIN is aneuploidy: as a result of incorrect segregation in mitosis, daughter cells will inherit a wrong number of chromosomes. Importantly, aneuploidy is found in most malignant tumours, with occurrence depending on the cancer type and ranging up to 90% in solid tumours and 35–60% in hematopoietic cancers, and it also correlates with metastasis, resistance to drugs and disease progression (Chunduri & Storchová, 2019). For that reason, the effects of aneuploidy on CIN-induced tumorigenesis have been largely studied during the last years.

1.2 Chromosomal instability-induced aneuploidy

As mentioned above, the direct consequence of CIN is aneuploidy, a situation in which a cell has a number of chromosomes that deviates from multiples of the haploid set. Although many efforts have been made to understand the effects of aneuploidy in the tissues, the molecular mechanisms underlying CIN-induced tumorigenesis are still largely unknown.

Despite its high prevalence, the role of aneuploidy in tumorigenesis remains unclear and is complicated by the paradoxical observation that aneuploidy can act both as a tumour suppressor and a tumour-promoting factor. Although aneuploidy interferes with the proliferation of untransformed cells, it is also, paradoxically, a hallmark of cancer, a disease defined by increased proliferative potential. On the one hand, aneuploidy has dramatic effects on genome integrity, causing DNA damage and activation of p53 (Santaguida & Amon, 2015). According to this, the prevalence

of aneuploidy in healthy mammalian tissues is very low: single-cell sequencing reveals less than 1% of aneuploid neurons and fibroblast. In mice, none of the full trisomies, except trisomy 19, survive beyond birth (Epstein, 1985) and, in humans, only trisomies in chromosomes 13, 18, 21 and X are viable (Hassold T & Hunt P, 2001). Indeed, embryos often accumulate aneuploid cells due to mitotic errors, however, these cells induce senescence or apoptosis and are clear are cleared by the immune system during embryogenesis (Chunduri & Storchová, 2019). Altogether, these observations suggest that aneuploidy *per se* is highly detrimental and poorly tolerated in mice and human tissues.

On the other hand, the fact that aneuploidy is found in around 68% of solid tumours (Duijf, Schultz, & Benezra, 2013) points out the necessity for acquiring extra mutations in caretaker or apoptotic genes that allow these cells to remain in the tissue and contribute to tumorigenesis. The most common mutations were found in genes that encode growth signalling factors (oncogenes and anti-oncogenes) or in the DNA damage checkpoint proteins such as p53 (Negrini et al., 2010). In this context, loss of p53 function allows evasion from cell death, whereas the genomic instability provides a fertile ground for additional mutations that lead to the establishment of the other hallmarks (Negrini et al., 2010).

What are the direct consequences of aneuploidy in cells? Although the presence of additional or lack of specific chromosomes can drive specific behaviours, analysis of mammalian and yeast aneuploid cells determined a common “aneuploidy stress response” (Dodgson et al., 2016). This implies a downregulation of pathways involved in cell cycle regulation, nucleic acid metabolism and ribosomal biogenesis, upregulation of autophagy, lysosomal pathways, membrane metabolism and glycolysis, and increased protein secretion and cell-wall integrity defects (Dodgson et al., 2016).

The first consequence of aneuploidy is a change in gene expression. In general, it has been shown that most genes that are present in an additional chromosome are expressed at an accordingly increased level in yeast, except for

some proteins forming complexes, such as the ribosome (Santaguida & Amon, 2015). In *Drosophila*, two dosage compensation mechanisms have been identified: the one for the X chromosome and the painting of fourth. The presence or absence of autosome dosage compensation is controversial, however, if existing, this putative buffer never restores wild-type expression levels completely (Stenberg & Larsson, 2011). As a consequence, the presence of extra or lacking chromosomes usually impairs protein homeostasis and stoichiometric balance of macromolecular complexes, leading to overwhelming of the protein-folding machinery, increased requirements for protein degradation pathways or the formation of protein aggregates (Chunduri & Storchová, 2019). This protein accumulation generates lysosomal stress and activates genes to increase the expression of genes needed for autophagy-mediated protein degradation (Santaguida, et al., 2015). In yeast, the proteotoxic stress induces the so-called aneuploidy-associated protein signature (APS), which is characterized by the upregulation of proteins but not transcripts of genes involved in the oxidative-stress response. The strength of this signature correlates with the degree of aneuploidy and the increase in Reactive Oxygen Species (ROS) (Dephoure et al., 2014).

Another relevant effect of aneuploidy is on cell proliferation. Experiments in *S. cereviseae* showed that the effects on cell cycle progression depend on the affected chromosome (Chunduri & Storchová, 2019), with positive, negative or neutral effects. Most probably, these specific effects are due to the presence of specific genes located in the affected chromosomes. However, upon CIN, where aneuploidy affects to more than one chromosome, a general response inducing cell cycle arrest has been proposed (Santaguida et al., 2017). Indeed, many stress conditions present in aneuploid cells, such as proteotoxic or genotoxic stress, has been shown to interfere with G1–S progression (Barr et al., 2017) (Jonas, Liu, Chien, & Laub, 2013).

Aneuploid cells not only suffer from lysosomal or proteotoxic stress but also replicative stress and DNA damage. For example, it has been shown that the stochiometric imbalance of replication factors in CIN-induced aneuploidy or the

replicative stress present in CIN-induced oncogene mutations can also activate the DNA damage response (DDR) and induce cell cycle arrest (Halazonetis, Gorgoulis, & Bartek, 2008). Another alternative previously mentioned is that chromosome bridges, generated by problems in chromosome alignment of extra chromosomes during mitosis, produce DSBs during cytokinesis and DNA damage (Janssen et al., 2015). Altogether, aneuploidy generates different responses to buffer the deleterious effects of an imbalance number of chromosomes, summarized in Figure 2.

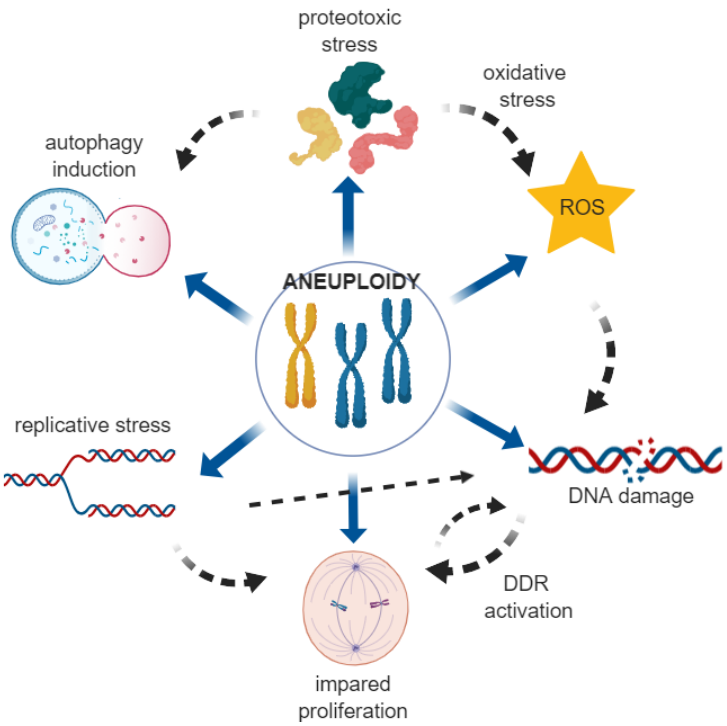

Figure 2. Aneuploidy-associated effects and responses. The aneuploid state elicit several responses. As a consequence of the protein imbalance, cells suffer from proteotoxic an oxidative stress, which lead to ROS production and DNA damage. In response to DNA damage, the DDR signals to prevent cell cycle proliferation and repair the damage. In addition, most probable due to the protein imbalance, the cells suffer from replicative stress which also affects cell cycle progression and generates more DNA damage. Moreover, in order to cope with the protein aggregates, cells induce autophagy, contributing to their survival. Image created with biorender.com.

1.3 *Drosophila* as a model to study CIN

Drosophila has become one of the most powerful animal models to use to understand the genetic bases of many different diseases. During the last century, the assortment of strains containing endogenous mutations has reached a substantial number, and it is relatively easy to generate flies carrying DNA to overexpress or inactivate genes of interest. Moreover, comparisons between the fully sequenced *Drosophila* and human genomes revealed that approximately 75 % of known human disease genes have a recognizable match in the genome of fruit flies consolidating its legitimacy as a model organism for medical research (Jennings, 2011). Thanks to powerful genetic techniques, many signalling pathways involved in tumour development and progression have been first discovered in the flies and found later in humans, such as the NF-kB (Lemaitre, et al., 1996) or the sonic hedgehog pathway (Niisslein-Volhard & Wieschaus, 1980).

In the last few years, different *Drosophila* cancer models have been used to analyse the contribution of CIN to tumorigenesis. Among all the different tissues, the wing imaginal disc has become very advantageous to decipher the molecular mechanisms underlying neoplastic progression. The wing imaginal disc is a larval sac-like structure formed by a pseudostratified epithelial monolayer. This pseudostratified architecture is determinant to follow specific cells within the tissue and to monitor behaviours such as over-proliferation, delamination or apoptosis, and the non-autonomous effects on the neighbouring cells. Since about 90% of human cancers are of epithelial origin (Hanahan and Weinberg, 2000), *Drosophila* wing imaginal discs constitute a popular system to model the onset of epithelial tumour progression.

In order to model CIN-induce tumorigenesis, different mitotic processes can be disrupted, such as the formation of the mitotic spindle or DNA damage repair mechanisms (Liu, Shaukat, Hussain, Khan, & L. Gregory, 2015). However, the most common approach used in epithelial tissues in *Drosophila* is to genetically remove

different spindle assembly checkpoint (SAC) genes. The SAC is crucial for the maintenance of a stable chromosome number because it blocks cell cycle progression until chromosomes are correctly attached to the spindle apparatus (Karess & Glover, n.d.) (Lopes, Sampaio, Williams, Goldberg, & Sunkel, 2004). Indeed, SAC mutants have been shown to induce chromosome rearrangements and aneuploidy (Castellanos, Dominguez, & Gonzalez, 2008), thus recapitulating the genomic defects associated with human cancer.

CIN is a process that drives most cells to aneuploidy and, as mention in section 1.2, aneuploidy is highly detrimental for the cells in both mice and humans. According to that, mutants for the SAC or chromosome replication- checkpoints in the larval brain did not induce tumorigenesis (Castellanos et al., 2008). Similar results were observed in the wing epithelial tissue, where mutations in genes involved in the mitotic assembly checkpoint or chromatin condensation mechanisms lead to an apoptotic response (Dekanty, Barrio, Muzzopappa, Auer, & Milan, 2012).

However, CIN is found in around 70% of solid tumours (Giam & Rancati, 2015) so, as previously mentioned, there must be additional mutations that contribute to the survival of these cells and CIN-induced tumorigenesis. Indeed, in mammalian cells, CIN leads to p53-dependent apoptosis and, mutations in the p53 tumour suppressor gene increase the frequency of spontaneous tumorigenesis (Li et al., 2010). In *Drosophila*, this CIN-dependent apoptosis is not a consequence of Dp53 activity, but it is due to the activation of JNK signalling. When apoptosis is blocked by expression of the baculovirus protein P35, which inhibits the activity of the effector caspases Dcp1 and DrIce (Hay, Wolff, & Rubin, 1994), activation of JNK induces non-autonomous proliferation (Ryoo, Gorenc, & Steller, 2004) (Dekanty et al., 2012) (Pinal, Martín, Medina, & Morata, 2018).

Accordingly, apoptosis blockade in SAC-depleted cells in the wing imaginal epithelium maintains these highly aneuploid cells - that otherwise would be eliminated - in the tissue and induces a transcriptional program of mitogenic molecules, metalloproteinases or inflammatory cytokines (Dekanty et al., 2012) (Clemente-Ruiz

et al., 2016) (Muzzopappa, Murcia, & Milán, 2017). Mitogens signal to the low-aneuploid epithelial population and induce tissue overgrowth, while the metalloproteases degrade the basement membrane and allow the migration and invasion of neighbouring tissues (Dekanty et al., 2012). This non-autonomous and growth-promoting role of delaminating cells increases the chances of chromosome segregation errors and aneuploidy levels in the non-delaminating cell layer and generates more delamination. This feedback loop amplification can explain the unlimited growth potential of these tumours (Muzzopappa et al., 2017) (Figure 3), and deeper characterization of the delaminated population could help to better understand tumour behaviour.

In addition to the transcriptional JNK-dependent regulation of specific molecules, delaminated cells are permanently arrested in the cell cycle according to the lack of mitogenic markers (Muzzopappa et al., 2017). Interestingly, the lack of proliferative capacity and secretion of specific molecules upon high levels of damage or stress are the main features of senescent cells. Indeed, CIN-induced aneuploidy has been shown to cause senescence in mammals (He et al., 2018) (Santaguida et al., 2017). Whether these highly aneuploid and delaminated population is senescent is something that needs to be investigated in more detail.

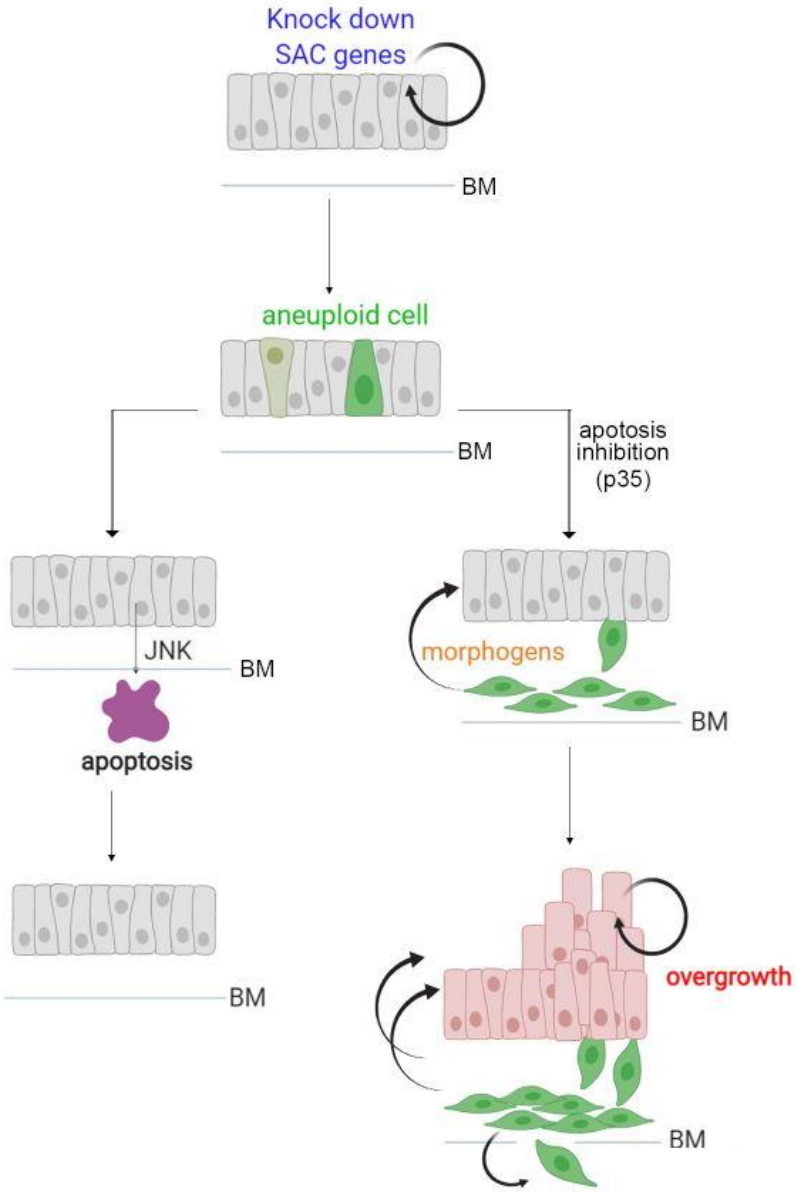

Figure 3. Temporal progression in CIN-induce tumorigenesis. Upon depletion of SAC genes, the proliferative epithelium suffers from CIN and accumulates aneuploidy. High and detrimental levels of aneuploidy induce JNK-dependent apoptosis to maintain tissue homeostasis. However, additional mutations can maintain aneuploid cells in the tissue and promote tumorigenesis by the induction of a specific transcriptional program to express mitogenic molecules that promote the overgrowth of the epithelial population and metalloproteases that degrade the basement membrane and allow the invasion of neighbouring tissues. Image created with Biorender.com.

2. Senescence and cancer

2.1 The senescent program

Living organisms have different ways of preventing unhealthy or damaged cells to accumulate in the tissues and one of them is cellular senescence, which is defined as an irreversible cell cycle arrest induced by different stimuli (Hernandez-Segura, Nehme, & Demaria, 2018).

Senescence is a relevant event in non-pathologic states, for example during mammalian embryonic development. One example is the involution of the mesonephros: just before disappearing during embryogenesis, most of the mesonephric tubules show high levels of SA-β-Gal activity and no proliferation marker, which together constitute a hallmark of senescence (Muñoz-Espín et al., 2013). Another example is natural aging since senescent cells accumulate in some tissues in aged humans, monkeys and mice. In this context, the SASP is essential for the creation of an inflammatory microenvironment that promotes the elimination of the senescent cells by the immune system and the replacement of the removed cells (Muñoz-Espín & Serrano, 2014). Last, but not least, senescence has been shown to promote tissue repair or regeneration in the face of injury. Senescence is strongly induced during the initial stage of damage, but then it is completely cleared by the time the damaged organ is regenerated. This phenomenon of induction and clearance of senescent cells appears to be central to the regeneration process (Yun, Davaapil, & Brockes, 2015). All in all, the central purpose of the senescent program may be to prevent the propagation of damaged cells and promote tissue regeneration and recovery once the damage ceases (Figure 4A).

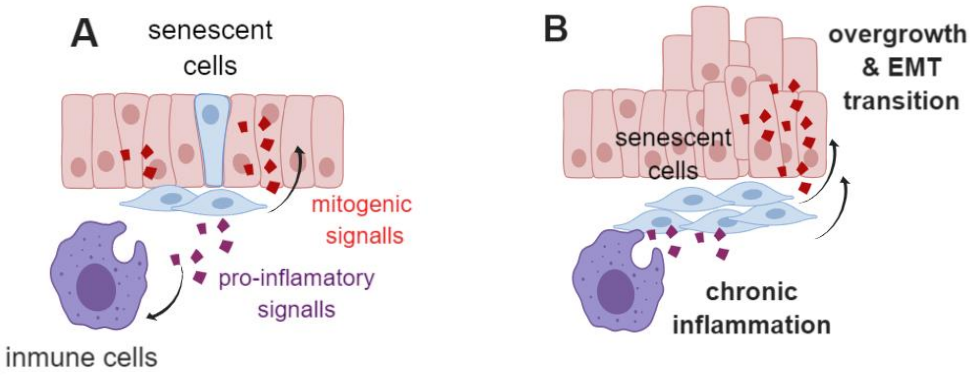

Figure 4. Pro-tumorigenic and anti-tumorigenic roles of senescence. Cartoon showing the behavior of senescent cells (A) In normal development or regeneration. Senescent cells secrete specific molecules to promote tissue regeneration (mitogens) and pro-inflammatory signals to promote their clearance by the immune system. (B) In disease, or sustained damage, these cells keep signaling generating a chronic inflammation and the overgrowth and EMT of the neighboring cells. Created with biorender.com

However, senescence has also a dark side. Chronic senescence induction has been shown to promote tumorigenesis and age-related disorders due to the secreted pro-inflammatory signals and their ability to modify the tissue microenvironment. Accumulation of senescent cells in mammals appears to be linked to the age-related decline in regenerative capability and may lead to some age-related disorders (Konstantinov, Ye, & Fricke, 2017). On the other hand, it has been shown that chemotherapy-induced senescent cells can promote some treatment side effects and their elimination can reduce the toxicity of anticancer treatments (Demaria et al., 2017). Moreover, chronic secretion of pro-inflammatory SASP molecules might support carcinogenesis by promoting epithelial to mesenchymal transition and invasion, tumour vascularization and abnormal cell morphology (Salama, Sadaie, Hoare, & Narita, 2014) (Figure 4B).

According to the previous data, increasing evidence indicates that both pro-senescent therapies and anti- senescent therapies can be beneficial. In cancer, pro-senescent therapies contribute to minimizing the damage by limiting proliferation. On the contrary, anti-senescent therapies may help to eliminate accumulated senescent cells and to recover tissue function. (Muñoz-Espín & Serrano, 2014)

2.2 Main features of senescent cells

Although the senescent state is highly heterogeneous and dynamic, there are several markers and morphological changes that are considered to be present in all senescent cells. These features include, as mentioned before, the absence of proliferative markers but also significant metabolic and morphological changes.

Senescence associated secretory phenotype (SASP)

One of the main features of senescent cells is the activation of a transcriptional program to produce and secrete specific proteins, the so-called Senescent Associated Secretory Phenotype (SASP), that are able to modify the surrounding environment and neighbouring cells (Kuilman & Peeper, 2009). Although the SASP composition can differ depending on the different senescence programs, there are common and relevant proteins that are present in all the different contexts (Hernandez-Segura et al., 2018). For example, cells usually secrete pro-inflammatory cytokines to modulate the immune system response, extracellular matrix (ECM)-degrading proteins that induce tissue remodelling and promote metastasis, or mitogenic molecules that induce the proliferation of the neighbouring cells.

In mammals, different SASP factors have been shown to be regulated by two pathways: NF-κB (Chien et al., 2011) and JNK signalling (Byun et al., 2006) (Cheung, Leung, & Wong, 2006). In *Drosophila*, the SASP is controlled by JNK signalling that has been shown to regulate the expression of cytokines like unpaired (upd) ligands (Santabárbara-Ruiz et al., 2015) or remodelling proteins such as the metalloproteinase MMP1 (Uhlirova & Bohmann, 2006).

Permanent cell cycle arrest

Another relevant feature of senescent cells is the permanent cell cycle arrest, which differs with other non-proliferative cells (such as quiescent cells) in that they do not respond to mitogenic molecules or growth factor stimuli (Herranz, Gil, Herranz, & Gil, 2018). The cell cycle arrest is often triggered by a persistent DNA damage

response (DDR) (d'Adda di Fagagna, 2008) in response to the different stress situations. In mammals, the canonical pathway of the senescent cell cycle arrest is through p53/p21 (described in detail in the following lines (See section 3.3 Cell cycle arrest in senescence)).

Metabolic changes

Another feature of senescence is the presence of high senescence-associated β-galactosidase (SA-β-Gal) activity, which is attributed to the high lysosomal content (Demaria, 2019). The increased lysosomal content could be attributed to increased autophagy, that has been proposed to be essential for the production of SASP factors and the rapid protein turnover to facilitate the translation of proteins highly necessary for the senescent state (Young et al., 2009).

In addition, senescent cells also show an increased number of old and dysfunctional mitochondria, most probably due to mitophagy and autophagy saturation (Hernandez-Segura et al., 2018). Mitochondrial dysfunction has been shown to be one of the main sources of Reactive Oxygen Species (ROS) and oxidative stress (Mailloux & Harper, 2011), which is the driving force for stress-induced senescence (Tai et al., 2017). Indeed, restoring autophagy, that has been shown to revitalize both mitochondrial and lysosomal functions, retards senescence (Tai et al., 2017).

Last, senescent cells have an increased Unfolded Protein Response (UPR) to cope with the Endoplasmic Reticulum (ER) stress. The accumulation of aggregated and misfolded proteins that accumulate due to the increased protein synthesis demanded by the SASP could be the main reason for this stress and the activation of the ER stress. In addition, activation of the ER stress response has also been shown to modulate the SA-β-Gal activity (Druelle et al., 2016).

Morphological changes

A common mark of senescent cells is the loss of LaminB1, a structural protein of the nuclear lamina. This destabilization results in other nuclear changes such as

the loss of condensation of constitutive heterochromatin (Hernandez-Segura et al., 2018). The upstream pathways that eliminate LaminB1 from senescent cells remain unclear, although it is likely to be orchestrated by multiple pathways (Wang, Ong, Chojnowski, Clavel, & Dreesen, 2017). In addition, senescent cells show an enlarged and irregular shaped cell body. It has been shown that the UPR can control the size of the ER and the changes in cell shape during senescence (Druelle et al., 2016).

2.3 Tumoral senescence models in *Drosophila*

During the last years, *Drosophila* has been also used as a model to understand the molecular mechanisms underlying oncogene or DNA damage-induced senescence and the role of the senescent cell cycle arrest. It has been proposed that Ras^{v12} oncogene activation in combination with mitochondrial dysfunction can induce cellular senescence and JNK/CycE-dependent G1 cell cycle arrest (Nakamura, Ohsawa, & Igaki, 2014). In this context, the G1 arrest may be crucial for the JNK-induced senescent phenotype, since CycE overexpression (that promotes G1/S transition, see section 3) rescues the SASP and the non-autonomous overgrowth. However, recent evidence contradicts the G1 arrest contribution in the senescent phenotype. According to his new data, upon tissue injury, cells activate JNK signaling and induce G2 transient stalling. This temporal arrest is crucial to induce the appearance of the SASP that induce non-autonomous proliferation (Cosolo et al., 2019). Indeed, it has been shown that upon CIN, JNK-dependent G2 lengthening promotes cell survival, proposing this mechanism as a timely and effective response to DNA damage (Wong, Shaukat, Wang, Saint, & Gregory, 2014) (Dekanty, Barrio, & Milán, 2015). As a summary, inconclusive data has been published about the role of the cell cycle arrest in the senescence phenotype in *Drosophila*. Thus, how this arrest modulates the SASP and other senescent features, and the contribution of G1 or G2 arrest to the senescent state need to be elucidated.

3. Cell cycle arrest in senescence

3.1 Cell cycle regulation

Actively dividing eukaryote cells pass through a series of stages known as the cell cycle: two gap phases (G1 and G2); an S (for synthesis) phase, in which the genetic material is duplicated; and an M phase, in which mitosis partitions the genetic material and the cell divides.

As in yeast and mammalian cells, G1-S and G2-M transitions are driven by Cyclin/CDK complexes. Cyclin-dependent kinases (CDKs) are heterodimeric enzymes with a protein kinase subunit and a Cyclin (Cyc) subunit that are regulated by various chemical changes (Hochegger, A, & Hunt, 2008). The expression of these CDKs is constant - in most of the cases – during the cell cycle and their association with proteins called Cyclins controls their activity, acting as regulatory subunits of the kinase complex (Follette & O'Farrell, 1997).

In *Drosophila,* the rate-limiting G1–S regulator is cyclin E (CycE), which acts with Cdk2, and the rate-limiting G2–M regulator is cdc25/string, an activator of cyclin B (CycB)/Cdk1 complexes (Thompson, 2010). Thus, overexpression of Stg or CycE induces premature entry into mitosis or S-phase, respectively, inducing a shortening in the length of the corresponding gap phases, G2 and G1 (Milan, 1998).

G1 phase

After mitosis, cells need to grow and synthesize proteins to prepare for DNA replication. During this phase, the APC/C together with his partner Fizzy-related (Fzr)/Cdh1 actively degrade all the cyclins (Sigrist & Lehner, 1997). This degradation has been proved to be important since ectopic expression of some cyclins during G1 can drive premature G1/S transition and generate problems in pre-replication complexes organization (Sigrist & Lehner, 1997). That is also the case in *Drosophila*, where premature G1/S transition induced by CycE overexpression (Jtirgen A.

Knoblich, Sauer, Helena Richardson, Saint, & Lehner, 1994) generates specific defects in loading pre-replication complexes (pre-RC) and drives genome instability (Ekholm-Reed et al., 2004a). As previously mentioned, the regulated expression of Fzr and the APC/C complex is crucial to maintain low cyclin levels and to promote the integrity of the cells.

In *Drosophila* cells, replication initiation requires the formation of the pre-RC during G1 phase. First, the origins of replication are bound by the origin recognition complex (ORC) (composed of the six proteins ORC1–6). The replication initiation factor cell division cycle 6 (Cdc6) is then recruited to the origin to form a complex with ORC. ORC and Cdc6 work cooperatively to recruit the initiation factor Cdt1 [Double Parked (DUP) in *Drosophila*] and the six-membered Minichromosome Maintenance (MCM) 2–7 replicative helicase complex (Hua & Orr-Weaver, 2017).

As previously mentioned, G1/S transition is driven by CycE (Jtirgen A. Knoblich et al., 1994). The oscillatory expression of CycE is mediated by oscillations in the levels of the transcription factor E2F1, which reaches high levels during G1 phase and is degraded at the beginning of S phase (Norman Zielke et al., 2011). CycE triggers the recruitment of Cdc45 to the pre-RC, which allows the binding of DNA polymerase (Quinn, Herr, McGarry, & Richardson, 2001).

Another important protein in G1-S transition is the *Drosophila* Cip/Kip Homolog *dacapo (dap),* a cyclin-dependent kinase inhibitor (CKI). Its function is to inhibit the activity of cyclin-dependent kinases (CDK's), most probably CDK2 which mediate G1-S transition (de Nooij, Graber, & Hariharan, 2000). Premature *dacapo* expression in transgenic embryos results in a precocious G1 arrest and its expression needs to be tightly regulated by integrating the information from the proliferative status as well as the differentiation program of these cells in order to ensure a correct G1-S transition (Lane et al., 1996). Interestingly, Dap seems to be positively regulated by CycE, since its expression in the posterior compartment of the wing imaginal disc increases both the expression of *dap* mRNA and protein but only in a permissive

window in cells that are about to exit from the mitotic cycle or cells that have recently complete mitotic exit (Lane et al., 1996).

S phase

Once origins fire and DNA replication commences, cells need to balance accuracy, speed, and the consumption of relevant resources such as nucleotides and replication factors to complete replication in an efficient manner (Greenberg et al., 2014).

During replication, due to the induced degradation of the APC/C complex at the end of G1, mitotic cyclins Cyclin A (CycA) and Cyclin B (CycB) start accumulating. Although the role of CycA in G1/S transition in mammals is quite clear, its role in *Drosophila* replication is still controversial (Salle, Campbell, Gho, & Audibert, 2012) and the only well-known function is in G2-M transition (Jtirgen A. Knoblich et al., 1994). Another consequence of APC/C complex degradation is the expression of Geminin, a protein that prevents re-replication by preventing binding of MCMs to chromatin during S phase (Quinn et al., 2001).

At the beginning of S phase, degradation of E2F is followed by ubiquitin-dependent degradation of Cyclin E via the E3 ubiquitin ligase CRL1-Ago (Moberg et al., 2001). Degradation of Cyclin E allows for the completion of S phase and the relicensing of replication origins in the subsequent G phase.

G2 phase

In the G2 phase, the cell recovers its energy stores and synthesizes proteins necessary for chromosome segregation. Some cell organelles are duplicated, and the cytoskeleton is dismantled to provide resources for the mitotic phase (Avissar et al., 2013). During this phase, the accumulation of CycB/CycA – CDK1 complexes is indispensable to allow progression to metaphase, since double mutant embryos that express neither cyclin A nor cyclin B zygotically, cell cycle progression is blocked (J A Knoblich & Lehner, 1993). However, they not only need to be present but also active, and for that, the phosphatase Cdc25/string plays a fundamental role. String (stg)

activates CDKs by removing phosphate from residues in their active site (Donzelli & Draetta, 2003). This activation allows the cell to target and phosphorylate specific targets involved in chromosome segregation and exit from mitosis (Enserink & Kolodner, 2010).

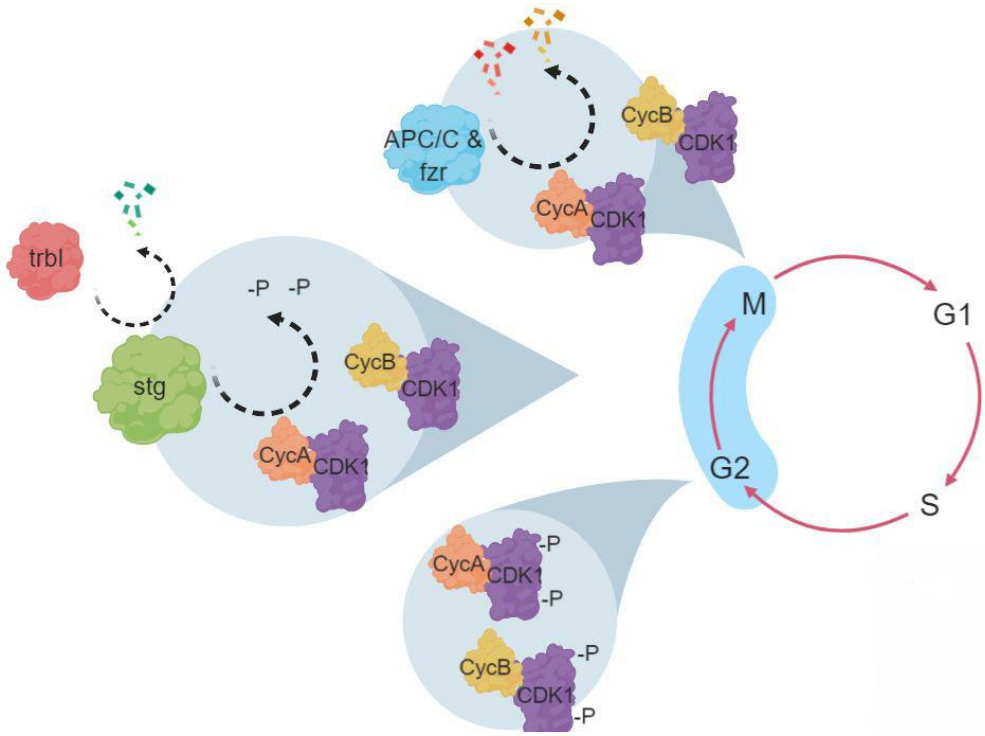

Figure 5. Relevant events in G2-M cell cycle progression. Cartoon summarizing the main events that control G2-M cell cycle progression. During G2, Cyc-CDK complexes accumulates in an inactive (phosphorylated) state. At the end of G2, CDK dephosphorylation is crucial to allow progression to mitosis. The phosphatase Stg is responsible for this event and Trbl can induce Stg degradation thought the APC/C complex. In metaphase, the active CycA-CDK complexes need to be degraded by the APC/C complex together with its target adaptor Fzr/Cdh1 in order to reach G1 phase. Created with BioRender.com

Once cells reach metaphase, the APC/C mediates the destruction of mitotic cyclins and the inhibitor of chromo some segregation securin, thereby allowing the transition through mitosis into G1 (B. R. Thornton & Toczyski, 2003). The APC/C is transiently associated with Cdc20 (Fizzy) and Cdh1 (Fizzy-related) which are "activating" subunits that interact with target substrates. Fizzy functions up to the metaphase–anaphase, and Fizzy-related continues to facilitate APC-mediated

ubiquitination once cyclin and separase degradation starts (McLean, Chaix, Ohi, & Gould, 2016) (Figure 5). When reaching G1, cells maintain APC/C complex activity to sustain mitotic cyclins degradation and prepare for DNA replication and start the cell cycle again.

3.2 DDR and replicative stress

DNA replication must be tightly regulated to ensure the correct duplication of the genetic material, a process that happens normally in healthy cells. However, in chronic stress scenarios or after the loss of key pathways which help to deal with this stress, a range of deleterious events can occur. For example, in tumorigenic situations, activating mutations in oncogenes induce aberrant proliferation by enhancing the activity of the CDKs and generate DNA replication stress. This stress leads to the collapse of DNA replication forks, that is, dissociation of the replication proteins from the DNA (Hills & Diffley, 2014). This situation leads to microdeletions and chromosome rearrangements that cause the formation of DNA single and double- strand brakes.

To prevent this damage to be transmitted during mitosis, cells have specific checkpoints to stop cell cycle progression and activate repair pathways to remove the breaks. These checkpoints activate the DNA damage response pathway (DDR), a signalling cascade that starts with the identification and labelling of the damaged DNA and generates a downstream activation of different kinases that end up with the repair or elimination of the damaged cell (Figure 6).

In mammals, upon a double DNA break or appearance of single-stranded DNA (DBS, ssDNA), the MRN complex (Mre11, Rad50 and Nbs1) and RPA (Replication protein A) are recruited respectively to the damage and activate the downstream kinases. Ataxia-Telangiectasia Mutated (ATM) responds to DSB and phosphorylates Chk2 that induces a second wave of phosphorylation. On the other

hand, ssDNA activates Ataxia telangiectasia and Rad3-related protein (ATR) that phosphorylates Chk1. In both cases, the responses can go from DNA repair activation and cell cycle arrest to apoptosis or senescence the case on non-reparable damage (Elledge & Zou, 2003).

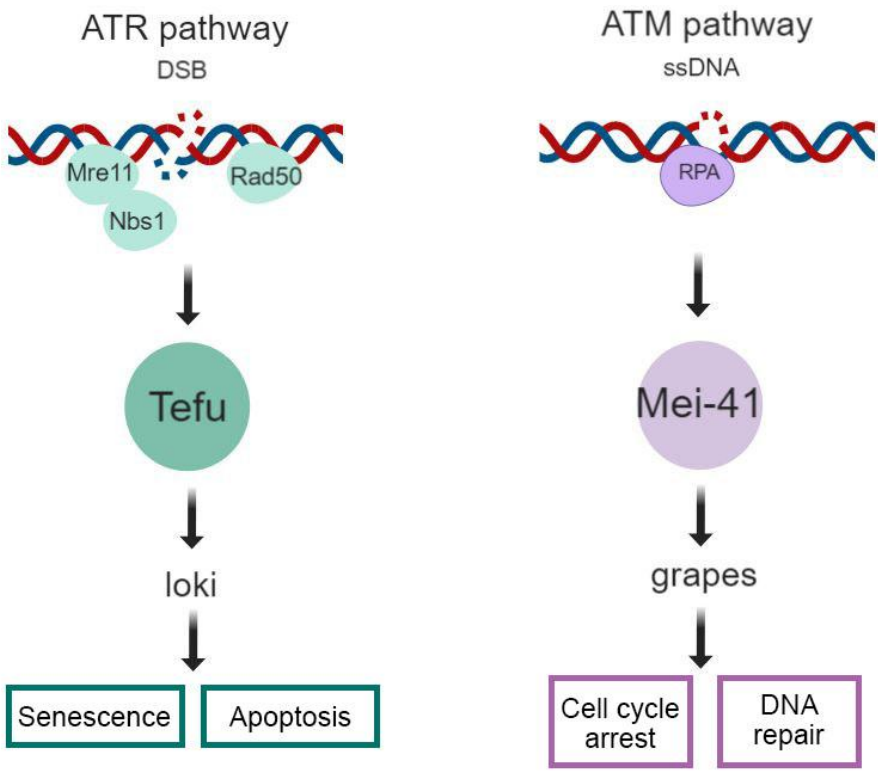

Figure 6. DNA damage response pathways in *Drosophila*. Cartoon showing the two different DDR pathways. (A) In response to DSB the MRN complex binds and activates Tefu/ATM that phosphorylates Loki/Chk2 to induce apoptosis or senescence (B) The ATR/Mei-41 pathway responds to RPA activation when bind to ssDNA. Its phosphorylation activates grapes/Chk1 that activates cell cycle arrest and DNA repair. Created with BioRender.com

In *Drosophila*, the two pathways have been shown to work independently and induce separate responses. Upon DSB, Tefu/ATR is activated and phosphorylates Loki/Chk2 to induce apoptosis or senescence. On the other hand,

ssDNA activates Mei-41/ATR that phosphorylates grapes/Chk1 to induce DNA repair and cell cycle arrest (Song, 2005).

3.3 Cell cycle arrest in senescence

As previously mentioned, the different senescent-inducing stressors that persistently activate the DNA damage response (DDR) (d'Adda di Fagagna, 2008) can induce cell cycle arrest. This arrest was originally defined to happen in G1 phase (Gire & Dulic, 2015b). and controlled by activation of the p53/p21 pathway (Herranz et al., 2018). However, already some years ago, p21 was shown to have an additional role in DNA damage-dependent G2 arrest (Agarwal, Agarwal, Taylor, & Stark, 1995). Indeed, the decision to enter in a quiescence state has been shown to occur at late cell cycle stages and to be dependent on the levels of CDK2, controlled by p21 (Spencer et al., 2013). The role of p21 may be to directly inhibit mitotic cyclin–CDK complexes, and by phosphorylating pocket proteins of the pRb (retinoblastoma) family (Gire, Baus, Fisher, & Piette, 2001). Besides, p21/p53 was shown to premature activate the APC complex (APC/C) upon sustained DNA damage and promote degradation of mitotic cyclins, such as CycB (Wiebusch & Hagemeier, 2010).

Although the role of G2 arrest upon DNA damage is gaining more relevance in mammals, it is mostly transient. Cells arrested in G2 eventually progress to G1 without chromosome segregation and permanently arrest in this phase (Toettchera, et al., 2012). Indeed, the phenotype of G2 arrested cells is similar to that of cells arrested in G1, that is, the absence of G2/M cyclins and accumulation of G1 cyclins (Gire & Dulic, 2015a). According to this, G1 arrest seems to be more robust in mammals most probably because of a strong p21 activation (Gire & Dulic, 2015a). However, this does not seem to be the case in *Drosophila*, where G2 plays a crucial role in DNA damage or stress. For example, the tumorigenic response of wing imaginal epithelium to ionizing radiation (IR) is enhanced by depletion of Grapes/Chk1 or Mei-41/ATR and lengthening of G2 had a positive impact on the dynamics of DNA repair and suppressed the tumorigenic response of the tissue to IR (Dekanty et al.,

2015). Besides, it has been shown that temporal G2 arrest is crucial to reduce the number of apoptotic cells upon JNK activation in response to CIN (Wong et al., 2014) and tissue injury (Cosolo et al., 2019). Altogether, these results highlight a relevant contribution of the G2 arrest in response to different stresses, such as DNA damage, but its contribution to cell cycle arrest-induced senescent still needs to be elucidated.

Objectives

Brief background

In the wing imaginal disc in *Drosophila*, CIN induces tumorigenesis upon PCD blockade. The high aneuploid cells drive tumorigenesis by promoting the over-proliferation of the low-aneuploid cells. Some of their features described so far made us think that this population could be senescent. Moreover, the molecular mechanism by which these cells induce cell cycle arrest to enter senescence is still controversial.

In addition, the highly proliferative and low-aneuploid cells, which contribute to tumour overgrowth, are largely uncharacterized. Specific features to target these cells and impair tumour growth need to be found.

Objectives

1. To look for senescent features in the highly aneuploid/delaminated population in CIN tissues.

2. To unravel the molecular mechanisms underlying the senescent-induced cell cycle arrest in CIN tissues.

3. To unravel the molecular mechanism controlling cell cycle progression in the low-aneuploid epithelial population in CIN tissues.

4. To identify genetic or chemical ways to selectively target CIN tissues.

Results

1. Characterization of the highly aneuploid population in CIN tissues

1.1 Generation of CIN tumours in the wing imaginal epithelium

As described in the introduction, upon CIN induction in the wing imaginal epithelium and additional blockade of the apoptotic machinery, cells proliferate and accumulate different levels of aneuploidy. The highly aneuploid population delaminate and secrete specific mitogens, metalloproteases and other factors that induce the over-proliferation of the low aneuploid ones. In addition to this secretory phenotype, these high aneuploid cells do not show mitotic markers, suggesting that they could be arrested in the cell cycle. Based on this secretory phenotype and the lack of proliferation, we wanted to investigate if these cells were senescent.

With that purpose, we induced CIN tumours in the wing imaginal epithelium by using the UAS- GAL4 system, a system for targeted gene expression that allows the selective activation of any cloned gene. The system is based on the expression of the GAL4 protein under the control of tissue-specific enhancers (kept in one fly), and the upstream activation sequence (UAS) driving the expression of genes of interest (kept in another). When flies of these two lines undergo crossing, the GAL4 protein will bind to the UAS and activate the gene at the tissue that the promoter is specific for (Brand & Perrimon, 1993).

We depleted the SAC by means of GAL4-mediated expression of *bub3* or *rod* RNAis together with UAS-p35 to block apoptosis (see introduction (see introduction- section 1.3). The protocol for CIN induction in the wing imaginal epithelium is detailed in Figure 7A. Depletion of SAC genes (rod in Figure 7B) and blocked apoptosis generates CIN- induced aneuploidy in the posterior compartment. Low levels of aneuploidy allow the cells to remain in the tissue and contribute to a neoplastic overgrowth. However, those cells that accumulate more aneuploidy delaminate and activate a JNK-transcriptional pathway. Among the JNK-target

proteins, we will use MMP1 to label the delaminated population from now on. Delaminated population also show a poorly bounded organization that can be used as a feature for identification (Dekanty et al., 2012), (Figure 7B).

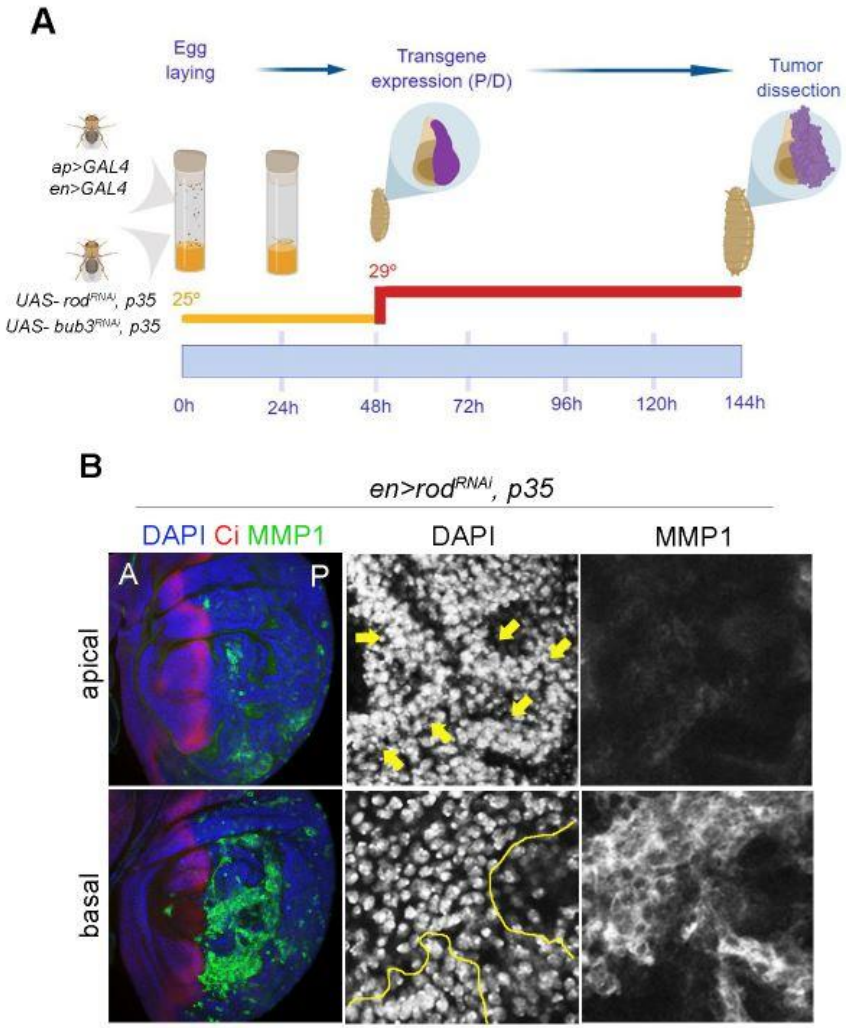

Figure 7. Protocol to induce CIN tumours in the larval wing imaginal disc. (A) Flies carrying the corresponding constructs are crossed. Females carrying GAL4 under control of tissue-specific enhancers: engrailed (en) to drive expression in the posterior compartment and apterous (ap) to drive expression in the dorsal compartment. Males carrying the genes of interest under control of the UAS sequence. The larvae are two days at 25º and four days at 29º. The expression of the transgenes happens throughout the entire development, although it is stronger at 29º. After 144h, the wing imaginal discs from the larvae are dissected and stained for the specific proteins. (B) Wing imaginal disc depleted for rod and blocked for apoptosis in the posterior compartment under the *en>gal4* driver control. Anterior compartment is labelled with Ci (red) and is oriented to the left, while posterior compartment is oriented to the right. In the apical part of the epithelium tissue morphology is maintained and

folded due to the neoplastic overgrowth (yellow arrows). Delaminated cells are located in the basal part, expressing MMP and poorly bounded (yellow line).

1.2 Identification of senescent features in the delaminated population in CIN tissues

Senescence Associated Secretory Phenotype (SASP)

One of the main features of senescent cells is their ability to secrete specific factors that modify the microenvironment and neighbouring cells. This specific group of secreted proteins has been called Senescence Associated Secretory Phenotype (SASP). To further characterize these cells and identify a possible SASP program, we analysed the transcriptional profile of highly aneuploid delaminating cells and look for the expression of different secreted molecules. For that, we carefully analysed a microarray previously performed in the lab (Clemente-Ruiz et al., 2016) and selected up-regulated secreted genes in delaminating versus non-delaminated cells. We could find a collection of secreted proteins, such as upd1-3, wnt4 (involved in growth control) or PGRP-SA (involved in the immune system response) (Table 1).

Gene	Function	Fold Change Delaminated vs Non delaminated
upd1	Immune System Response	13,70
mmp1	Matrix metalloproteinase	9,18
scaf	Polarization	8,37
dILP8	Growth control	7,98
ly6	Immune System Response	7,56
Impl3	Metabolism	7,03
moody	Behaviour response	6,37
wnt4	Growth control	5,71
mtg	Synapse organization	5,46
PGRP-SA	Immune System Response	5,33
idgf3	Growth control	4,76
gbp3	Immune System Response	4,73
upd3	Growth control	3,94
pvf1	Growth control	3,94
upd2	Growth control	3,24
bnl	Growth control	3,03
Impl2	Metabolism	2,92

Table 1. Top upregulated secreted genes in the transcriptional profile of CIN delaminated cells compared to CIN epithelial cells. The fold-change represents the different expression of these genes in the delaminated population versus the epithelial population.

According to an enrichment of secreted proteins in the delaminated population, this should lead to an increased secretory activity compared to the epithelial or wild-type population. After translocation into the Endoplasmic Reticulum, secretory cargo is collected and then loaded into membrane vesicles that transfer the cargo to the Golgi compartment (Bannykh, Rowe, & Balch, 1996). For that reason, we decided to check the amount of Golgi reticulum in these cells as an indirect measurement of increased trafficking and sorting of secretory proteins. Moreover, the retrograde endosome transport at the trans-Golgi network has also been shown to increase in highly secretory cells (Hernandez-Segura et al., 2017). In order to monitor both Golgi and endosomes, we took advantage of the antibody toolkit to study membrane trafficking in *Drosophila* (Riedel, Gillingham, Rosa-Ferreira, Galindo, & Munro, 2016) (Riedel et al., 2016) and stained CIN tissues with Golgin - a cis-face Golgi protein – and Rab7- a protein that controls transport to endosomes and fundamental for lysosomal biogenesis and trafficking (Guerra & Bucci, 2016).

First, we induced CIN following the detailed protocol (Figure 7) and identified the delaminated population according to its basal localization. In the wild-type compartment (anterior), basal expression of both Golgin and Rab7 was detected. However, expression of both proteins increased in both the epithelium and the delaminated population of CIN tissues, but more significantly in the delaminated one (Figure 8A and 8B). According to the increased expression of proteins involved in the secretory pathway, delaminated cells might have an increase secretory capacity, most probably due to the expression of the SASP.

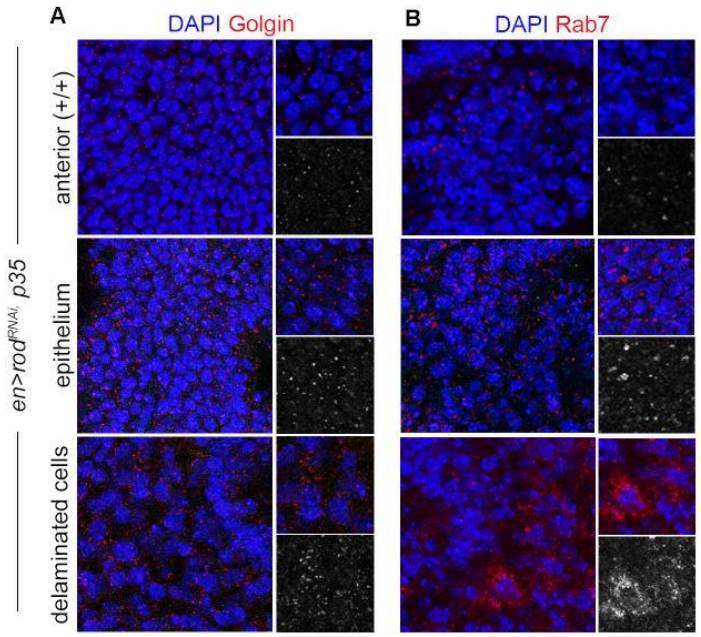

Figure 8. Expression of Golgin and Rab7 in CIN tissues. (A, B) Magnification of a larval wing primordia expressing the indicated transgenes stained for DAPI (blue) to label the nucleus and (A) Golgin (red and grey) and (B) Rab7 (red and grey) in anterior (wild-type), posterior - epithelial population and posterior - delaminated population.

Lysosomal activity

The senescence state is characterized by the upregulation of many lysosomal proteins (such as the β-galactosidase) and increased lysosomal content (Cho & Hwang, 2012). In order to monitor the expression of the resident β-Galactosidase (β-gal), we used a GFP fusion protein construct.

Although we could detect a few epithelial cells with perinuclear Ect3 accumulation, the number of cells with Ect3 accumulation increased in the delaminated population (Figure 9A), suggesting that the lysosomal content was increased in delaminated cells.

To validate this result and monitor lysosomal dynamics, we measured β-gal activity with the β-galactosidase assay. The basis for this assay is that senescent cells, as a consequence of lysosome accumulation, over-accumulate β-gal protein,

and its activity can be detected at a suboptimal pH of 6.0 (Hernandez-Segura et al., 2018). By performing this assay, we localized positive cells in the basal part of the tissue where delaminated cells are located (Figure 9B) in most of the CIN tissues analysed.

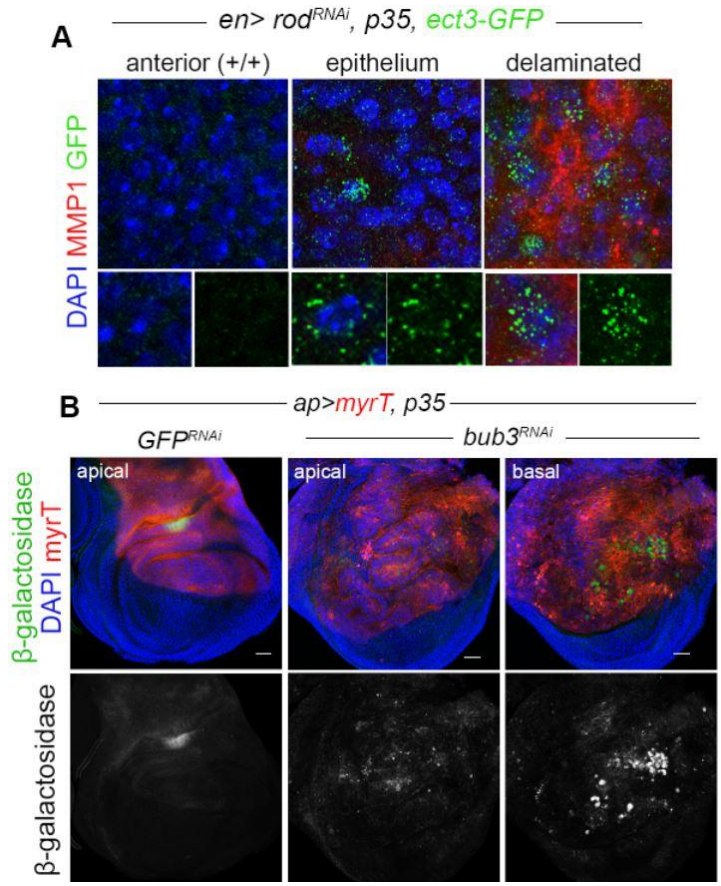

Figure 9. β-Galactosidase (β-gal) activity and ect3 expression in CIN tissues. Larval wing primordia expressing the indicated transgenes showing (B) expression of the resident β -galactosidase and stained for anti-GPF (green), MMP1 (red) and DAPI (blue) (C) the β-gal activity (green and grey) of delaminated cells located in the basal part of the tissue.

Changes in cellular morphology

To follow with the characterization, we analysed different nuclear and cellular changes. First, we analysed the nuclear lamina, which has been shown to be lost in senescent cells. Lamin, the major structural component of the nuclear lamina, is

maintained in epithelial cells. However, delaminated cells labelled with strong levels of GFP showed no Lamin nuclear localization, suggesting that these cells lose the lamina (Figure 10A). Consistent with this, senescent cells show an enlarged and bigger nucleus than epithelial cells (Figure 10B).

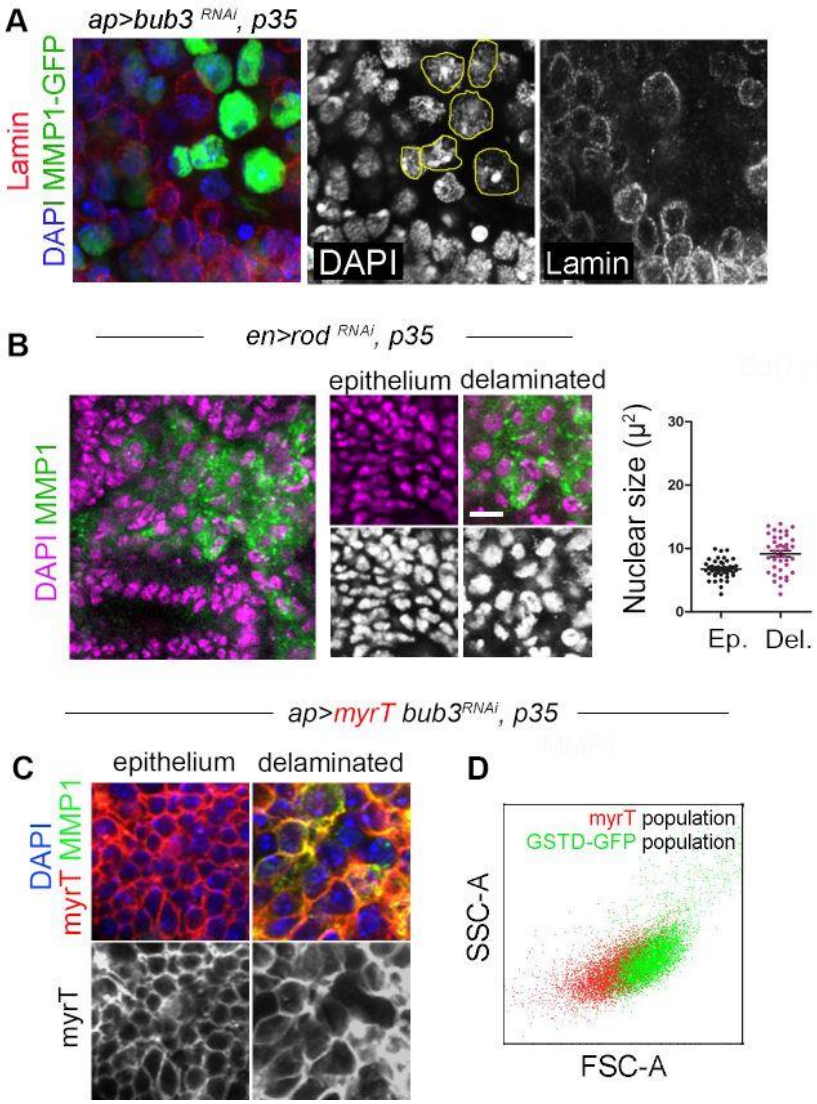

Figure 10. Changes in nuclear and cell morphology in CIN tissues. (A-C) Larval wing primordia expressing the indicated transgenes stained for (A) GFP (green) that labels the delaminated cells, Lamin C (red and grey) and DAPI (blue and grey) to monitor the absence of the nuclear lamina, (B) MMP1 (green) to label the delaminated cells and DAPI (magenta and grey) to show the nuclear size with the corresponding quantification. Error bars indicate SD (*** P< 0.001). (C) DAPI (blue), myrT (red and grey), MMP1 (green), showing epithelium

and delaminated population. (D) Scatterplot representing cellular size (FSC) versus cellular complexity (SSC) of epithelial CIN cells (myrT -positive in red) and delaminated CIN cells (GSTD-GFP-positive in green) obtained by FACs analysis.

Senescent cells usually show an enlarged and irregular shaped cell body. In order to compare the size of the delaminated population with the epithelial one, we performed a Fluorescence-Activated Cell Sorting (FACS) to separate and measure the size of the two populations. We drove the expression of the membrane protein myrT in the hole CIN tissue and separated the highly aneuploid/delaminated population according to the presence of ROS by using GstD-GFP reporter (Sykiotis & Bohmann, 1982). The GstD1 gene encodes for glutathione-S- transferase, whose regulatory sequences contain consensus binding motifs for the stress-responsive transcription factors Nrf2 and FoxoA. In this construct, these regulatory sequences are fused to GFP as a way to monitor ROS production. As represented in the scatterplot representing cellular size vs cellular complexity (Figure 10D), the highly aneuploid and delaminated population (GstD-GFP positive) had in general larger body size that the epithelial ones (GstD-GFP negative). We validated this result by analysing cell size according to the expression the membrane Tomato Myristoylated-Tomato (myrT) and identifying the delaminated cells by the expression of MMP1 (Figure 10C).

DNA damage

CIN epithelial cells are highly proliferative. However, their mitosis might be aberrant according to the presence of lagging chromosomes (Clemente-Ruiz et al., 2016), which usually lead to DNA breaks. Moreover, gain of chromosomes has been shown to trigger replication stress and promote genome instability contributing to tumorigenesis (Passerini et al., 2016). We then asked if CIN tissues could suffer DNA damage and breaks that could lead to replicative stress, especially in the senescent-like population.

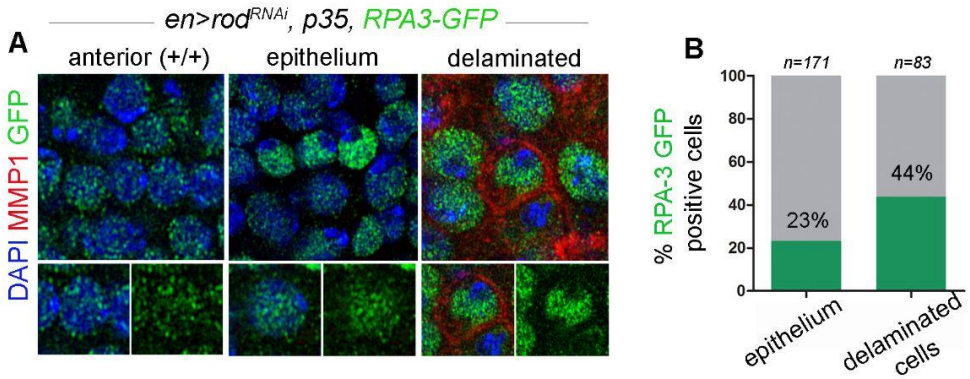

Figure 11. Replicative stress in CIN tissues. (A) Magnification of larval wing primordia expressing the indicated transgenes in the posterior compartment and stained for DAPI (blue), MMP1 (red) and GFP (green, gray). (B) Histogram plotting the percentage of RPA3-GFP positive cells in CIN epithelial and delaminated cells.

The persistence of ssDNA upon replication stress or DNA damage triggers the binding of replication protein A (RPA), a protein that protects the DNA and generates a signal for activation of the stress response (Greenberg et al., 2014) and DNA repair pathways. In *Drosophila*, Rpa complex consists of three subunits, RpA-70, Rpa2 and Rpa3 (Elledge & Zou, 2003). In order to visualize ssDNA in CIN tissues, we monitored the protein localization of Rpa3 by using a RPA3-GFP reporter. Compared with anterior (+/+) cells, we detected a significant number of cells with localized foci of RPA3-GFP in CIN epithelium, and this number was even higher in the delaminated population (Figure 11 A and B). Interestingly, although the number of cells with RPA foci is higher in the delaminated population, both epithelial and delaminated cells had ssDNA.

Stalled DNA replication forks or increased topological stress during replication may cause DNA damage (Greenberg et al., 2014). We first measure the amount of DNA damage present in the tissue by using Rad50-RFP reporter. Rad50 is a member of the MRN complex and a central player in most aspects of the cellular response to DNA double-strand breaks (Assenmacher & Hopfner, 2004). We could detect a dense accumulation of foci in a significant percentage of epithelial cells and this number was increased in the delaminated population (Figure 12).

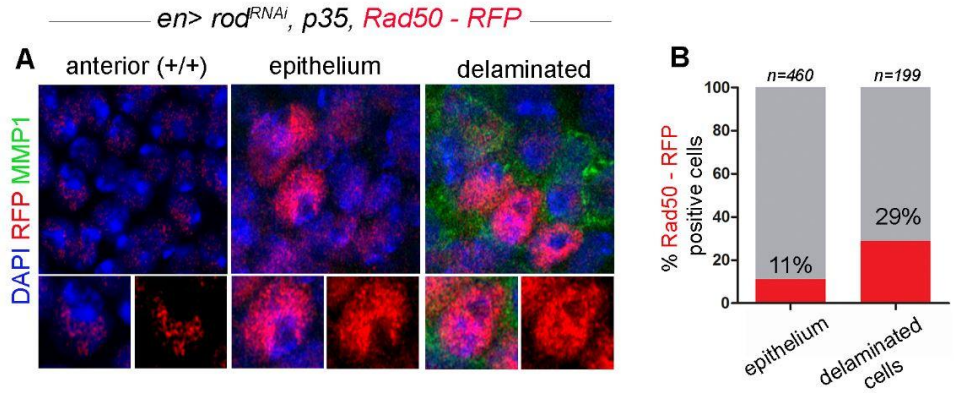

Figure 12. Double strand breaks in CIN tissues. (A) Magnification of larval wing primordia expressing the indicated transgenes in the posterior compartment and stained for DAPI (blue), RFP (red and grey) and MMP1 (green) (B) Histogram plotting the percentage of Rad50-RFP positive cells in CIN epithelial and delaminated cells.

To confirm the presence of DNA damage we performed a comet assay (single-cell gel electrophoresis), a simple method for measuring deoxyribonucleic acid (DNA) strand breaks. We detected a significant increase of DNA breaks in CIN tissues compared to the control cells (Figure 13). Altogether, our results indicate that CIN tissues present ssDNA and DNA breaks that could be a consequence of replicative stress.

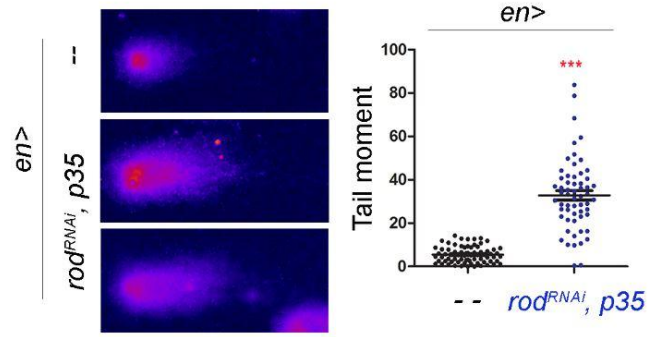

Figure 13. Double and single strand breaks in CIN tissues. (A) Images of wing disc single cells subjected to comet assay in control and CIN tissues. (B) Histogram plotting the tail length for each cell in control and CIN tissues. Error bars indicate SD (*** P< 0.001).

Cell cycle arrest

One of the most important characteristic of senescence cells is cell cycle arrest. Thus, we decided to monitor mitotic activity by the presence of phosphorylation of the nuclear core histone protein H3 (pH3) that occurs from late G2 to early telophase. We could not see any cell positive for pH3 in the delaminated population (Figure 14A) and, on the contrary, we did see pH3 incorporation in highly proliferative epithelial cells.

We then checked if these cells were able to replicate the DNA. For that, we incubated the CIN tissue for 15 min with 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analogue of thymidine which is incorporated into DNA during active DNA synthesis. Interestingly, we could not detect any EdU incorporation in the delaminated population, only cells from the epithelial tissue were labelled (Figure 14B). This result suggest that the delaminated cells have also problems to properly proceed through S phase, contrary to epithelial cells.

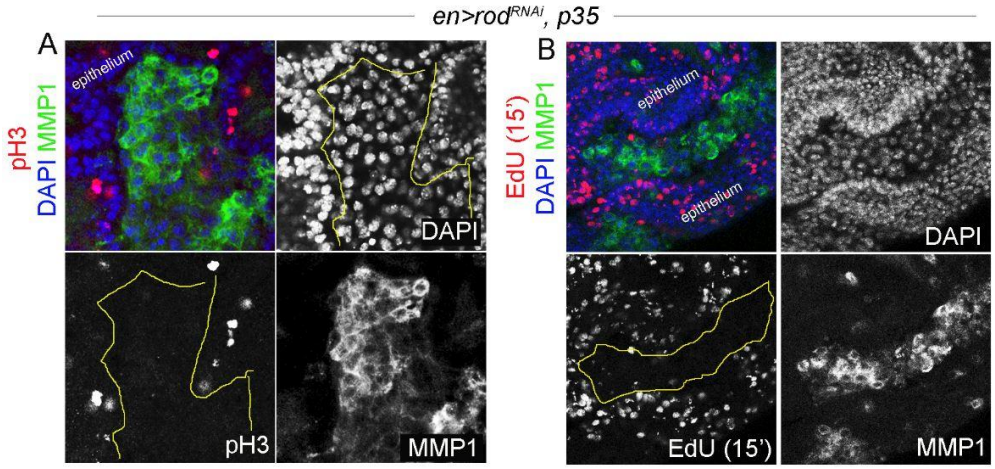

Figure 14. Proliferation analysis in the delaminated population. (A-B) Larval wing primordia expressing the indicated transgenes (A) stained for MMP1 (green), pH3 (red) and DAPI (blue and grey) (B) stained for MMP1 (green and grey), EdU (red and grey) and DAPI (blue and grey). The delaminated area was bounded by a yellow line.

To further characterize the cell cycle arrest, we used the Fly- FUCCI system: Fly-FUCCI relies on fluorochrome-tagged degrons from the Cyclin B and E2F1 proteins, which are degraded by the ubiquitin E3-ligases APC/C and CRL4- Cdt2

(Norman Zielke et al., 2014). By expressing the Fly-FUCCI system in the CIN tissues we were able to analyse the number of cells in each part of the cell cycle (Figure 15D). In a wild-type epithelium, the percentage of cells in G1, S phase and G2 is 19%, 38% and 43% respectively. However, in the delaminated population of a 6-days CIN epithelium, the number of cells in G2 increases to 53% and this number goes up to 71% in an 8-days CIN tissue (Figure 15B). These results suggest that delaminated cells accumulate in G2. According to the lack of mitotic activity (Figure 14) this G2 arrest seem to be permanent.

Interestingly, we were also able to detect some cells in S phase (Figure 15A, red). This suggests that delaminated cells are able to progress through S phase, but probably slower than the epithelium due to higher levels of aneuploidy. Indeed, many stress conditions, such as proteotoxic or genotoxic stress, identified in aneuploid cells can interfere with S phase progression (Chunduri & Storchová, 2019). According to this, we were not able to detect any labelled cell with 15 minutes of EdU incubation (Figure 14B), most probably due to problems in replication. To verify that delaminated cells were progressing - although slower - through S phase, we incubated the CIN tissue for 1 hour with (EdU) and then we performed a Fluorescence-Activated Cell Sorting (FACS analysis to separate the epithelial versus de delaminated population. For that we used myrT to label the whole CIN tissue and expressed GstD-GFP to label the delaminated population.

Around 30% of cells were in S phase according to the EdU incorporation profile in control tissues. Similarly, around 30% of delaminated cells were replicating DNA at 7 days tumour growth, however this percentage decreased down to a 20% after 9 days (Figure 15 B & C). This decrease in the number of replicating cells with time supports the fact that cells are being arrested and accumulate in G2.

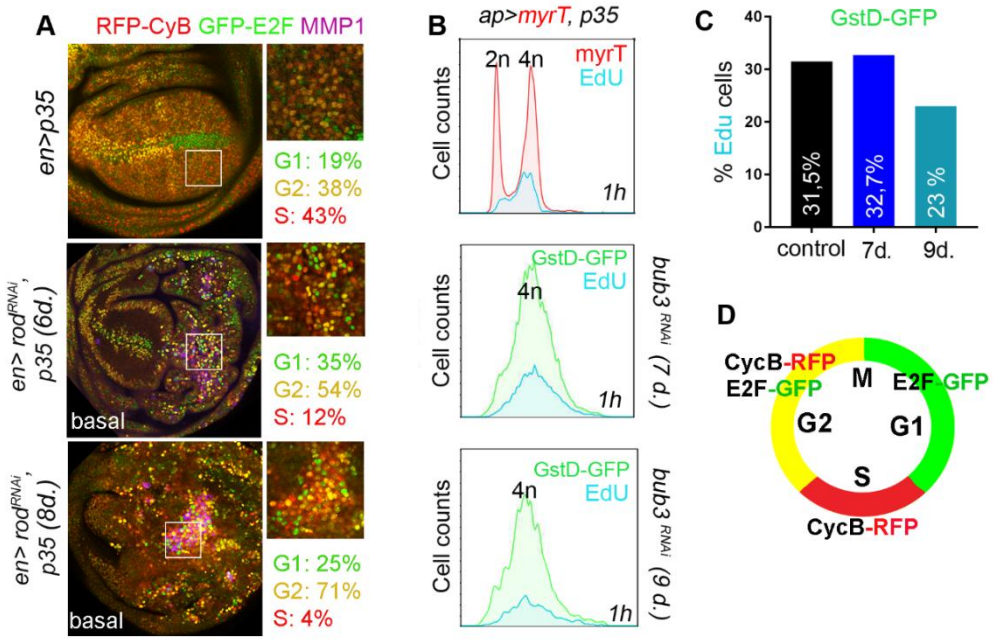

Figure 15. Cell cycle profile of CIN delaminated cells. (A) Larval wing primordia expressing the indicated transgenes stained and expressing the Fly-FUCCI system. Delaminated cells are labelled with MMP1 (magenta). (B) DNA content profile of larval wing primordia expressing the indicated transgenes in the dorsal compartment under the control of the ap>Gal4 driver at 7 and 9 days. The epithelium was labelled with myrT (red) and the delaminated population with Gstd-GFP (green) (C) Histogram plotting the percentage of cells incorporating EdU in the delaminated population (GstD-GFP positive) at 7 and 9 days. (D) The Fly-FUCCI system labels cells in G1 in green, cells in S phase in red and cells in G2 in yellow.

2. Analysis of the cell cycle arrest driving event in delaminated cells

The mitotic spindle is oriented by the dynein/dynactin motor complex, whose cortical localization depends on Mud/NUMA. Mud depletion has been shown to affect spindle orientation in the wing epithelium (Nakajima, et al., 2013).–Interestingly, blocking the death of cells depleted for Mud was sufficient to drive tumour-like overgrowth tissues with delaminated cells expressing MMP1 and Wg and that drive the over-proliferation of epithelial cells (Muzzopappa et al., 2017). This suggests that delamination per se might be able to induce the SASP, so we decided to verify that these cells just failed to orient the mitotic spindle and they do not have aneuploidies. For that, we performed a FACs analysis and isolate the two different populations by

their expression of myrT alone (epithelial population) or myrT and mmp1-GFP (delaminated). We confirmed that both the epithelial and delaminated cells are mostly in G1 and, more importantly, they do not have aneuploidies (Figure 16B). However, according to the comet assay, these cells show DNA breaks (Figure 16C), suggesting that they may induce DNA damage when fail to orient the mitotic spindle.

Then, we wondered whether mitotic spindle miss-orientation is also sufficient to induce the permanent cell cycle arrest in the delaminated population. To check that, we depleted Mud and check for mitotic activity in the delaminated cells. Contrary to what happens in CIN tissues, we could detect mitotic cells (Figure 16A). This suggests that the cell cycle arrest might be dependent on the aneuploidy levels and not a simple consequence of delamination. To check this idea, we took advantage of the dosage compensation mechanism in *Drosophila*. The existence of a single X chromosome in *Drosophila* males creates an imbalance between X-linked and autosomal genes. The male-specific lethal complex (MSLc) targets X chromosomes, leading to decompaction of the chromatin fibre and increases gene transcription to a similar level to that in autosomal chromosomes.

Depletion of Msl1 in males generates gene dosage imbalance and induces JNK activation, and together with p35 promotes epithelial overgrowth (Clemente-Ruiz et al., 2016). To check if the cell cycle arrest was dependent on the levels of aneuploidy, we induced gene dosage imbalance by depleting Msl1 in males and checked for mitotic activity. Interestingly, we could not see any mitotic cell in the delaminated population (Figure 16D), suggesting that the cell cycle arrest is indeed aneuploidy dependent.

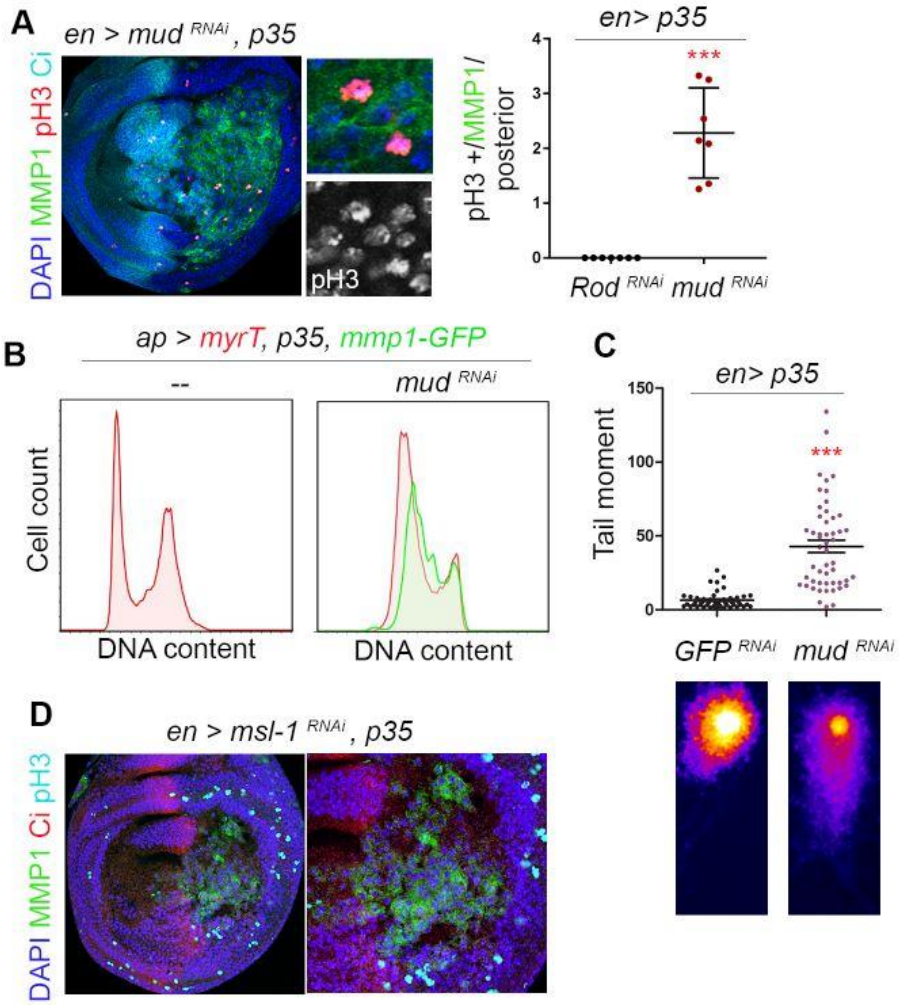

Figure 16. Mitotic activity of delaminated cells in Mud and Msl-1 depleted tissues. (A -D) Larval wing primordia expressing the indicated transgenes showing (A) stained for MMP1 (green), ph3 (red), Ci (cyan) and DAPI (blue) + Histogram plotting the number of mitotic cells in the delaminated population. Error bars indicate SD (*** P< 0.001) and (D) stained for MMP1 (green), pH3 (cyan), Ci (red) and DAPI (blue). (B) DNA content profile of larval wing primordia expressing the indicated transgenes in the dorsal compartment under the control of the ap>Gal4 driver. The epithelial population is labelled with myrT (red) and the delaminated population with mmp1-GFP (green) (C) Images of wing disc single cells subjected to comet assay in control and Mud depleted tissues + Histogram plotting the tail length for each cell in control and Mud depleted tissues. Error bars indicate SD (*** P< 0.001).

3. Analysis of signalling pathways responsible for the cell cycle arrest in the senescent population

3.1 Contribution of the DDR to the cell cycle arrest in the senescent population

It has been shown that the DDR kinases are activated during replicative senescence (reviewed in Muñoz-Espín & Serrano, 2014). Upon appearance of ssDNA the DDR pathway is activated in order to induce cell cycle arrest and repair the damage (Song, 2005). To check a possible role of the DDR in the arrest of senescent cells, we blocked the expression of the main effectors of the ATM pathway in *Drosophila*, grapes/check1 and mei-41/ATM in CIN tissues and stained for the mitotic marker pH3.

We could detect a significant number of mitotic cells within the delaminated population with both RNAis (Figure 17 A and B), suggesting a possible contribution of the DDR in the cell cycle arrest of highly aneuploid cells.

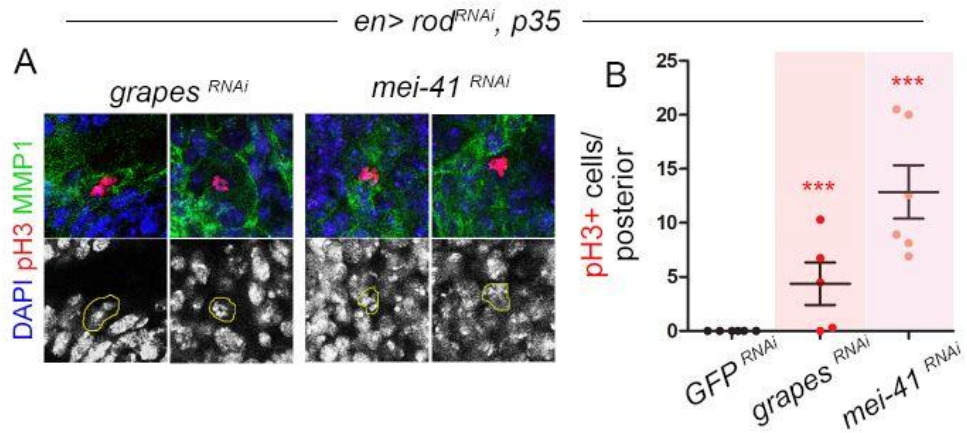

Figure 17. Proliferation of CIN delaminated cells upon DRR depletion. (A-B) Magnification of larval wing primordia expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue and grey), pH3 (red) and MMP1 (green). (B) Histogram plotting the ratio between the number of mitotic cells expressing MMP1 and the size of the CIN compartment (posterior) (C) Error bars indicate SD (*** P< 0.001).

3.2 Contribution of JNK to the cell cycle arrest in the delaminated population

Given that JNK was activated specifically in the delaminated population and is responsible for the SASP (Dekanty et al., 2012), we decided to check if it also contributes to the cell cycle arrest. Interestingly, depletion of JNK signalling in CIN tissues by expressing puckered (puc) – an inhibitor of the *Drosophila* JNK - significantly rescued the mitotic activity in the delaminated cells, localized in the basal part of the tissue (Figure 19 A & B). Since JNK inhibition rescues the SASP, we identified the delaminated population not by the expression of MMP1 or other secreted proteins but by their basal localization.

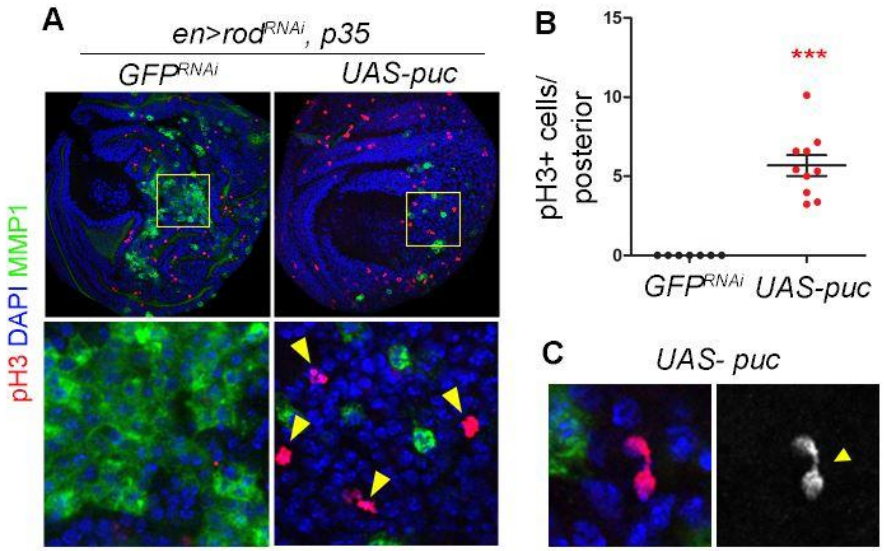

Figure 18. Mitosis in CIN tissues blocking JNK signalling. (A) Larval wing primordia expressing the indicated transgenes and stained for DAPI (blue), MMP1 (green) and Ph3 (red). Magnification of the delaminated population in the basal part of the figure. (B) Histogram plotting the ratio between the number of mitotic cells and the size of the CIN compartment. Error bars indicate SD (*** P< 0.001). (C) Magnification of cells in the CIN compartment showing the presence of chromosome bridges (pH3).

These mitoses were aberrant according to the presence of chromosome bridges (Figure 18C). The lack of epithelial adhesion and the highly aneuploid state most probably affects the correct chromosome segregation during mitosis.

If delaminated cells are now able to go through mitosis, there might be a higher percentage of cells in G1. To check that, we performed a FACs analysis of the whole CIN tissue upon JNK inhibition. We were not able to separate the two populations based on GstD- GFP expression - that previously allowed us to isolate the delaminated population - because puc expression rescued also the activity levels of this reporter (Figure 19C). For that reason, we analysed the complete CIN tissue and quantified the number of cells in G1 and G2. Compared to the control tissues, where the number of cells in G2 did not change upon JNK signalling inhibition (Figure 19A), CIN tissues showed a significant increase in the G1 population (Figure 19B). In accordance to FACs analysis, quantification of the Fly-FUCCI system confirmed a decrease in the percentage of cells in G2 from 60% to 36% and a consequent increase in the number of cells in G1 and S phase upon JNK signalling blockade in the delaminated cell population.

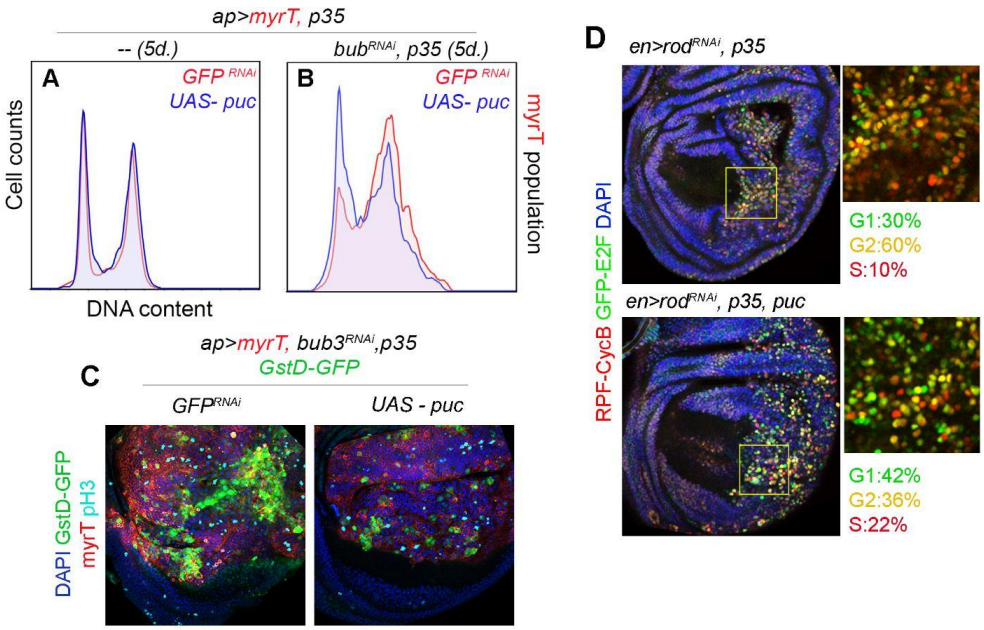

Figure 19. Cell cycle analysis in CIN tissues without JNK signalling. Larval wing primordia expressing the indicated transgenes (A, B, C). (A-B) DNA content profile of (A) wild-

type discs with (red) and without (blue) JNK signalling and (B) CIN tissues with (red) and without (blue) JNK signalling. (C) with and without JNK signalling stained for DAPI (blue), GSTD-GFP (green).

To summarize, depletion of JNK signalling in delaminated cells rescued the mitotic capacity. However, according to the fly-FUCCI, JNK blockade not only recued the number of cells in G2 but also increased the number of S phase cells. To validate this result, we performed an EdU incorporation assay and monitored the number of cells in S phase upon puc expression. CIN delaminated cells were unable to incorporate EdU in a short incubation of 15 minutes (Figure 20). However, upon JNK signalling depletion, these population showed an increased proportion of replicating cells. These suggests two different explanations: the increased number of replicating cells can be a direct consequence of the increased number of mitotic cells or JNK signalling could also play a role in G1-S transition upon high levels of aneuploidy.

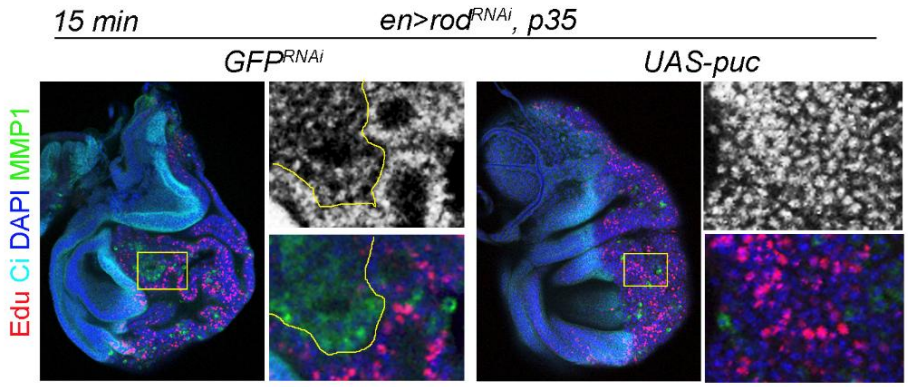

Figure 20. S phase analysis in CIN tissues without JNK signalling. Larval wing primordia expressing the indicated transgenes and stained for EdU (red), Ci (cyan), MMP1 (green) and DAPI (blue). The delaminated population in CIN was delimited according to MMP1 expression and surrounded by a yellow line.

3.4 Analysis of downstream effectors of JNK and the DDR

Our next step was to find cell cycle regulators that could be controlled by JNK and the DDR in the senescent population to induce the cell cycle arrest in G2. Both in mammals and *Drosophila*, mitotic cyclins degradation occurs in a physiological fashion due to the APC/C-Cdh1/Fizzy-related activity in metaphase. However, they

can be ectopically degraded upon DNA damage to induce G2 arrest and promote DNA repair directly through Cdh1/Fizzy-related activation (Qiao, Zhang, Gamper, Fujita, & Wan, 2010). Interestingly, we have shown that CIN- induced -delaminated cells are highly aneuploid and suffer DNA damage (Figure 11, 12 & 13), so we wanted to analyse if a premature mitotic cyclin degradation (CycA and CycB) in these cells could be the cause of the cell cycle arrest.

Interestingly, most of the delaminated cells showed no CycB (Figure 21A) or CycA (Figure 21B) expression. According to the already demonstrated role of JNK in the cell cycle arrest in these cells (Figure 18), we wondered if JNK signalling inhibition could restore CycA expression. Interestingly, upon JNK blockade. delaminated cells showed a more heterogeneous CycA profile (Figure 21B), similar to that present in wild type epithelial cells (Figure 21C).

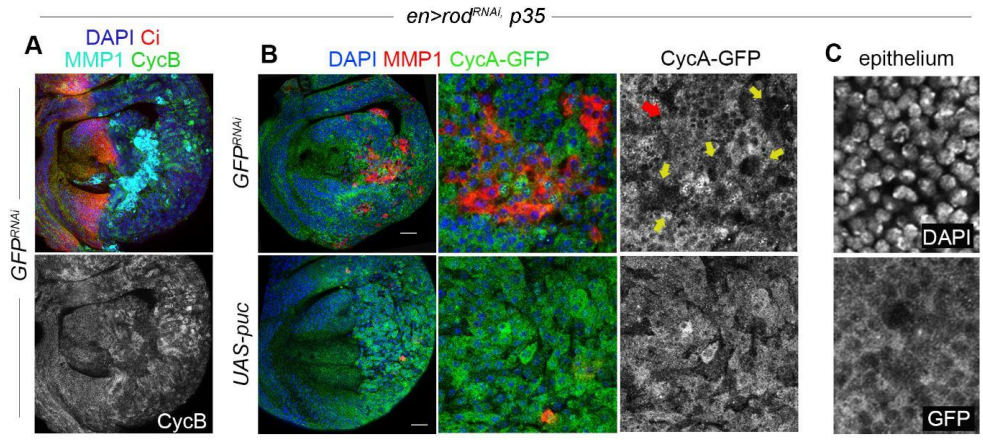

Figure 21. Expression of mitotic cyclins in CIN delaminated cells. Larval wing primordia expressing the indicated transgenes stained for (A) Ci (red), MMP1 (cyan), CycB (green) and DAPI (blue) (B) MMP1 (red), CycA- GFP (green) and DAPI (blue). Delaminated cells lacking CycA are pointed with a yellow arrow. Delaminated cells expressing CycA are marked with a red arrow.

However, not all the delaminated cells lacked CycA and CycB (Figure 21B, red arrow), so there must be additional mechanisms to prevent mitotic progression in this population. In *Drosophila*, Cdc25/ string (stg) has been shown to be responsible for the dephosphorylation and activation of the Cyclin-CDK mitotic complexes (Farrell

et al., 2017). According to previous evidences, JNK can control the expansion of cells with cytokinesis failure by repressing stg in a context of stg overexpression (Gerlach, Eichenlaub, & Herranz, 2018). By using a stg-LacZ enhancer trap, we detected a downregulation of stg levels in the delaminated population when compared with the epithelium in CIN tissues (Figure 22B). Downregulation of its transcriptional reporter suggests that Stg could be regulated at the transcriptional level in highly aneuploid cells. However, additional mechanisms could be playing a role, for example, at the level of post-transcriptional regulation. Tribbles (Trbl), a protein involved in Stg degradation through the proteasome (Seher & Leptin, 2000), is upregulated by JNK in damaged cells (Cosolo et al., 2019), and this upregulation could be contributing to the cell cycle arrest through Stg repression. Interestingly, CIN tissues showed increased Trbl protein levels compared to the epithelial wild type tissue (Figure 22B), suggesting that Trbl could also be contributing to the cell cycle arrest through Stg degradation.

Given Trbl and Stg differential expression in the delaminated population of CIN tissues compared to epithelial cells or to wild-type cells (Figure 22 A and B), we decided to check if this differential expression had any role in the cell cycle arrest. When we overexpressed Stg in CIN tissues, the mitotic index was significantly increased in the delaminated population (Figure 22 C and D), compared to CIN tissues where delaminated cells never go through mitosis (Figure 8). Then, by depleting Trbl we could also detect an increased mitotic activity in the delaminated population (Figure 22 C and D). Interestingly, the presence of chromosome bridges in these cells (Figure 22 C, pH3 in grey) suggests that these mitoses are highly aberrant.

Altogether, we have been able to identify three different mechanisms involved in G2 arrest in CIN-induced senescent cells: transcriptional inhibition of mitotic cyclins and Stg/Trbl regulation to prevent CDK-CyC activity.

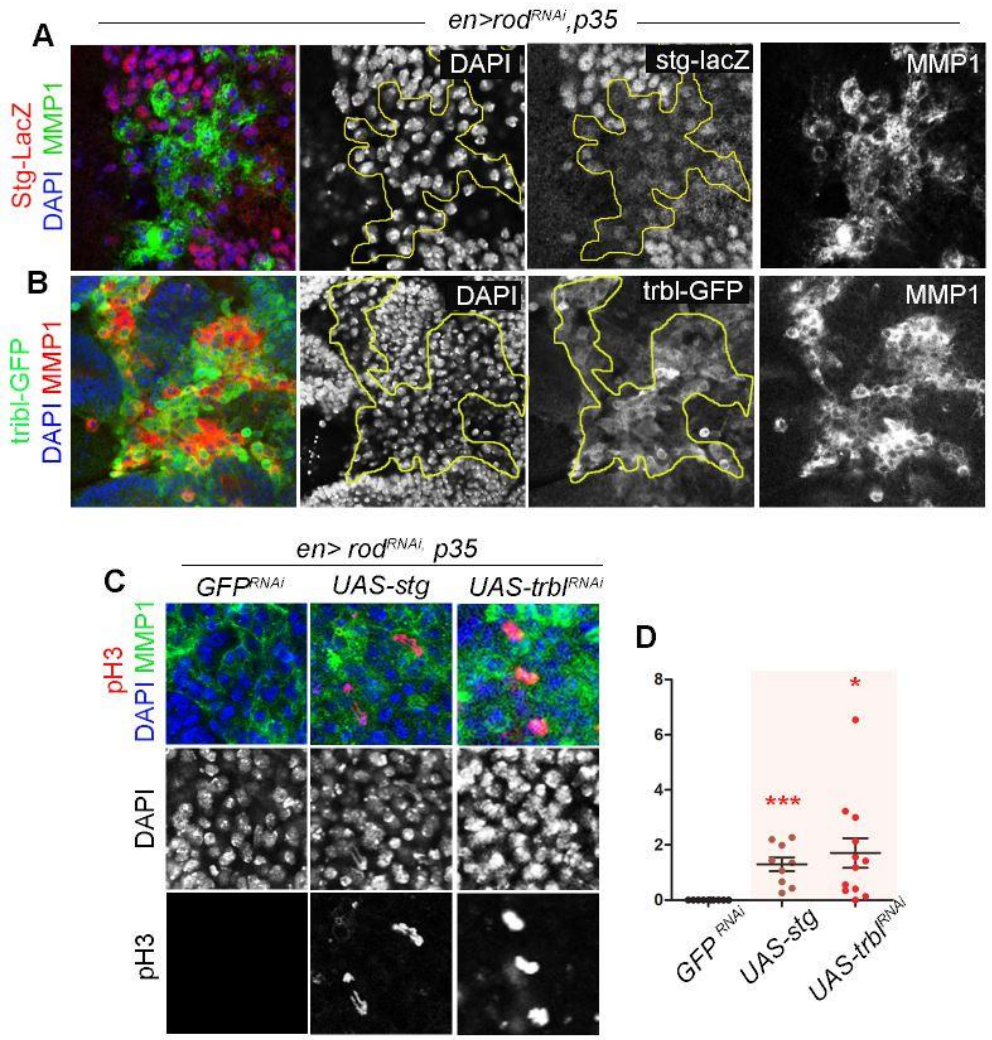

Figure 22. Stg and Trbl expression and function in CIN epithelial tumours. (A-B) Magnification of CIN epitheliums stained (A) for DAPI (blue), MMP1 (green) and anti β-Gal (red), (B) DAPI (blue). MMP1 (green) and anti GFP (green). Delaminated population was localized according to MMP1 expression (B) Magnification of larval wing primordia expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue), pH3 (red) and MMP1 (green) (C) Histogram plotting the ration between the number of mitotic cells expressing MMP1 and the size of the CIN compartment (posterior). Error bars indicate SD (*** P< 0.001**).**

3.6 Biological relevance for the cell cycle arrest in senescent cells

According to the literature, senescent cells lunch a stable proliferative arrest in order to prevent the propagation of damage (Muñoz-Espín & Serrano, 2014). We checked if this was the case by monitoring the amount of damage present in CIN tissues upon JNK signalling inhibition.

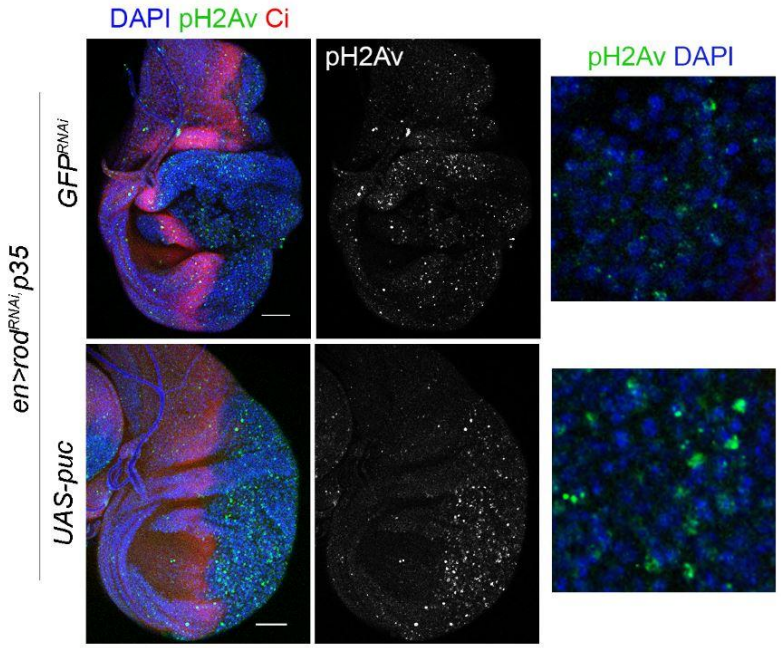

Figure 23. DNA damage in JNK signalling depleted CIN tissues. (A) Larval wing primordia expressing the indicated transgenes and stained for DAPI (blue), Ci (red) and pH2Av (green and grey).

For that we stained the tissues with anti–phosphorylated histone H2Av, that becomes phosphorylated at sites of DSBs by DNA damage recognizing factors (Madigan, Chotkowski, & Glaser, 2002). Depletion of JNK signalling and the consequent restored mitotic capacity increased the amount of pH2Av in CIN delaminated cells (Figure 23). Interestingly, this increase was not pan-nuclear but localized in foci, a label of increased DNA damage (Toledo et al., 2011). Thus, JNK

could be inducing G2 arrest in delaminated cells to avoid propagation of damaged cells within the tissue.

4. Characterization of mild levels of aneuploidy in CIN tissues

4.1 Cell cycle regulation in low aneuploid cells

Highly aneuploid cells, when accumulate a detrimental amount of aneuploidy and DNA damage, delaminate, arrest the cell cycle in G2 (Figure 14 and 15) and induce a senescence program (Figure 8-15) in order to prevent the propagation of the damaged population (Figure 23). However, the proliferating cells that remain in the epithelium also showed certain levels of aneuploidy (Dekanty et al., 2012) (Clemente-Ruiz et al., 2016) (Muzzopappa et al., 2017) and DNA damage (Figures 11, 12 and 13). These cells are the ones that proliferate and contribute to tumour overgrowth but, according to EdU incorporation and FACs analysis, the percentage of proliferating cells in the epithelium decreases with time (Figure 24 A and B). This suggests that lower levels of aneuploidy also have an impact on cell proliferation.

In order to investigate in which part of the cell cycle these cells were accumulated or delayed, we decided to use again the Fly-FUCCI system. In early time points (6 days) of tumour development, most of the cells were accumulated in G1, but the cells were equally distributed in G1 and G2 at late time points (8 days). This suggests that two different mechanisms could be playing a role in low aneuploid cells, one in G1 during early tumorigenesis and other in G2 in response to increased levels of aneuploidy (Figure 24C).

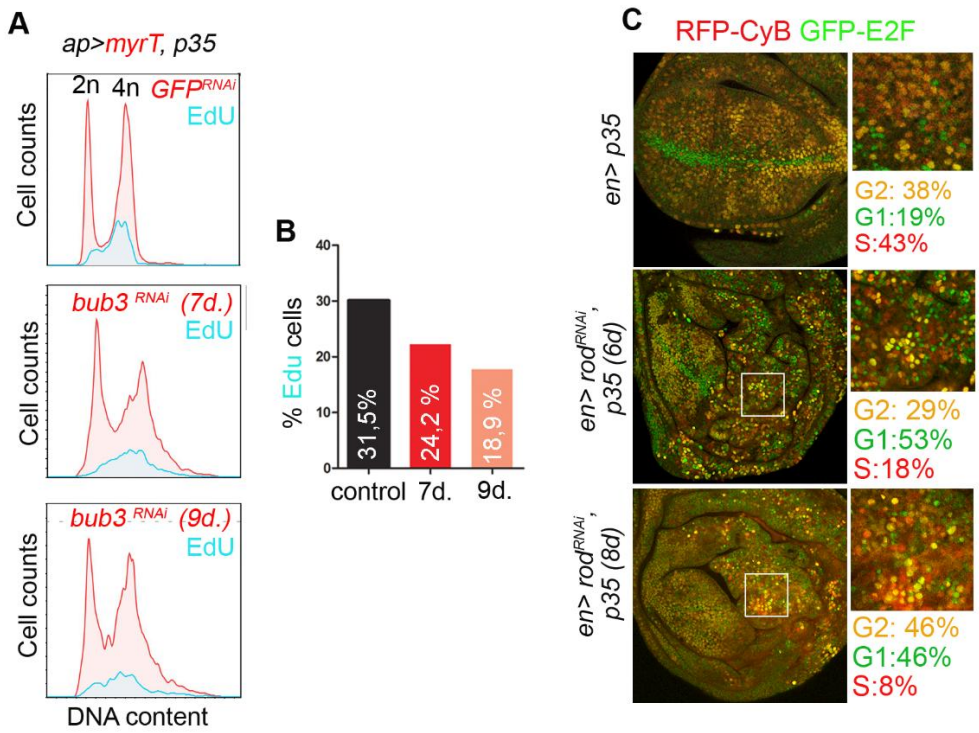

Figure 24. Cell cycle profile and replication in the epithelial population of CIN tumours. A) DNA content profile of larval wing primordia expressing the indicated transgenes in the dorsal compartment under the control of the ap>Gal4 driver. The epithelial population was labelled and isolated by the expression of myrT. B) Histogram plotting percentage of cells that incorporate EdU in the myrT population in control, 7 days and 9 days of induction. C) Larval wing primordia expressing the indicated transgenes in the posterior compartment under the control of the en>Gal4 driver and expressing the Fly-FUCCI system for 6 days and 8 days and stained for E2F-GRF (green) and CycB-RFP (red).

4.2 Role of the JNK signalling in the cell cycle arrest in CIN epithelial cells

In order to investigate if JNK was responsible for the activation of any of the checkpoints in G1 or G2 in low-aneuploid epithelial, we decided to block JNK signalling by overexpressing puc and analyse the cell cycle with the Fly-FUCCI system. Upon depletion of JNK signalling, the number of cells in S phase increased greatly (from 17% to 29%) (Figure 25A), suggesting a possible role of JNK signalling preventing G1-S transition in response to very low levels of aneuploidy.

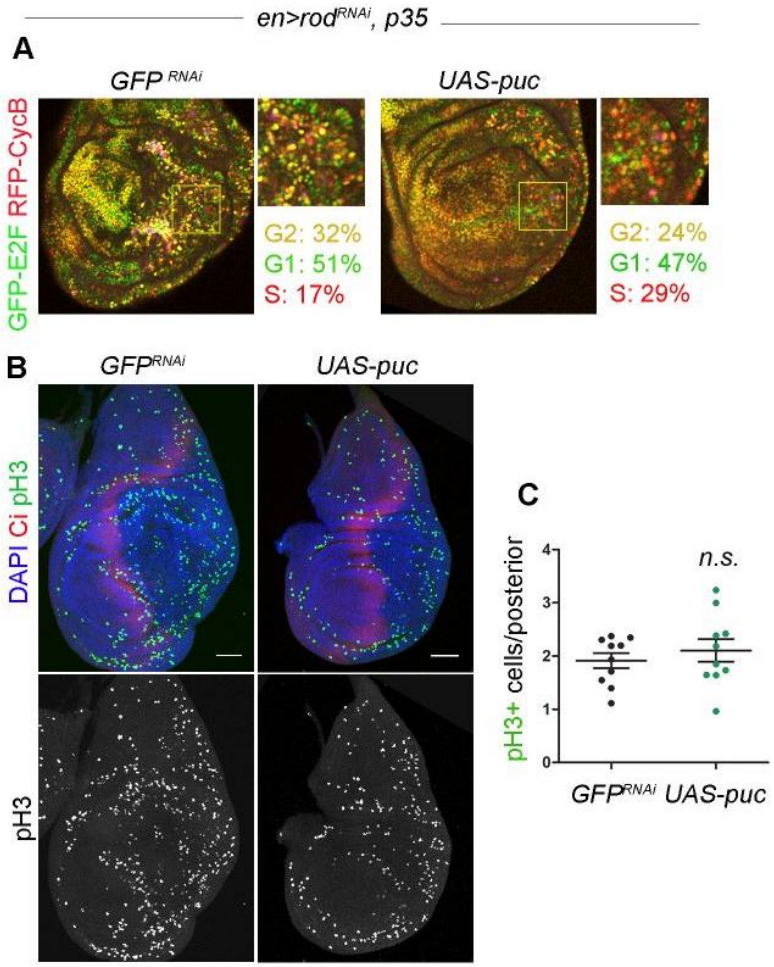

Figure 25. Fly-FUCCI analysis and mitotic activity in epithelial CIN cells upon JNK signalling inhibition. Larval wing primordia expressing the indicated transgenes in the posterior compartment (A) expressing the Fly-FUCCI system stained for MMP1 (magenta), E2F-GFP (green) and CycB-RFP (red) with the corresponding quantifications for each genotype. (B) Stained for DAPI (blue), MMP1 (green) and Ci (red and grey). (C) Histogram plotting the ratio between the number of mitotic cells and the size of the CIN compartment**.**

In addition, the percentage of cells in G2 also decreased (Figure 25 A), suggesting that JNK might have an additional role in G2-M transition in epithelial cells. To test that, we quantified the number of mitotic cells in CIN and CIN tissues without JNK signalling. No changes in mitotic activity were detected according to pH3 staining (Figure 25 B and C). To sum up, JNK seem to play a role in G1-S transition in epithelial cells in response to low levels of aneuploidy, but its role in G2-M seem to be specific of highly aneuploidy cells.

4.3 Role of the DDR in the cell cycle arrest in CIN epithelial cells

We have shown that the DDR has a contribution in the cell cycle arrest of CIN-dependent senescence. In order to check a possible contribution in the G1 stall in epithelial cells, we depleted Mei-41 and analyse the fly-FUCCI profile. According to previous results, at short time points CIN tissues showed an increased number of cells in G1 (53%), but this number decreased upon depletion of the DDR (35%) and, consequently, the percentage of G2 cells increased (from 37% to 51%) (Figure 26 D).

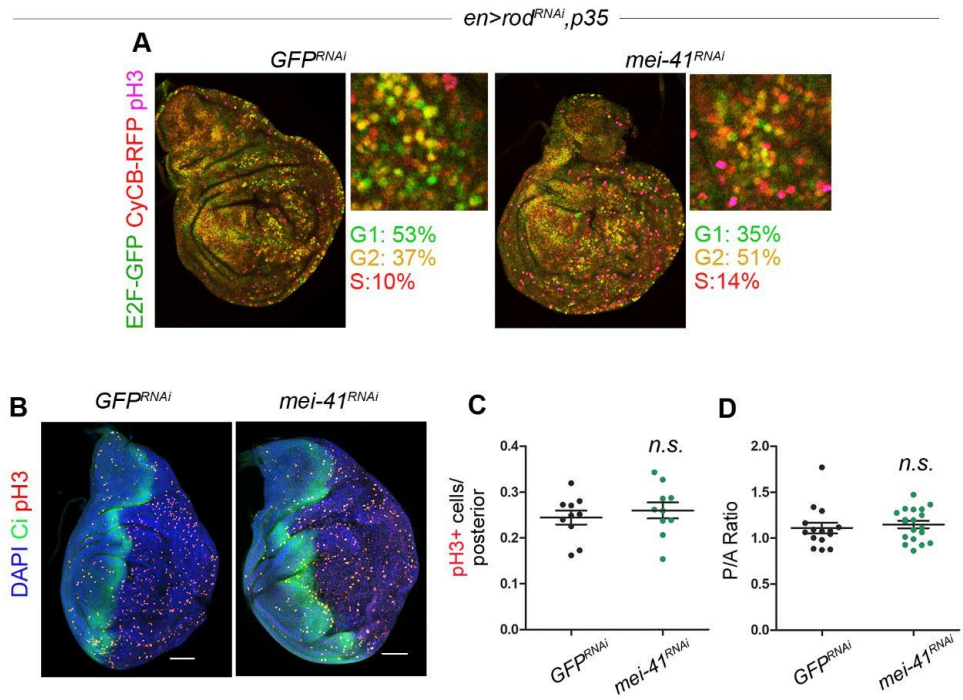

Figure 26. Mitotic activity in epithelial CIN cells blocking the DDR signalling. Larval wing primordia expressing the indicated transgenes (A) stained for pH3 (magenta), E2F-GFP (green) and CycB-RFP (red). Quantifications are shown in the basal part of each genotype, (B) stained for DAPI (blue), MMP1 (green) and pH3 (red). (C-D) Histogram plotting the ratio between (C) the number of mitotic cells and the size of the CIN compartment with and without the DDR and (D) the ratio between the size of the CIN compartment and the anterior compartment with and without the DDR.

To test if the DDR has a role in G2-M transition, we depleted Mei-41 and analyse mitotic activity. No changes in tissue size of pH3 staining were detected in

DDR depleted CIN tissues (Figure 26 A and B). All together, these results suggest that at low levels of aneuploidy both the DDR and JNK induce G1-S stall.

5. Selectively killing CIN tissues

5.1 Genetic inhibition of tumour overgrowth

So far, we have demonstrated that the over-proliferative and low aneuploid population of CIN tissues suffer DNA damage and chromosome breaks that might induce replicative and oxidative stress (Figures 11, 12 and 13). JNK signalling and the DDR seems to temporally arrest these cells in G1 in order to help with tissue repair and prevent more damage accumulation (Figure 25 and 26).

Based on these levels of DNA damage and stress, we wonder if we could exploit this feature to affect tumour growth by genetically manipulating the tumour to induce more damage. For that, we decided to over-express CycE, which has been proved to prematurely induce G1-S transition [69] and generate replicative stress and genomic instability (Ekholm-Reed et al., 2004b).

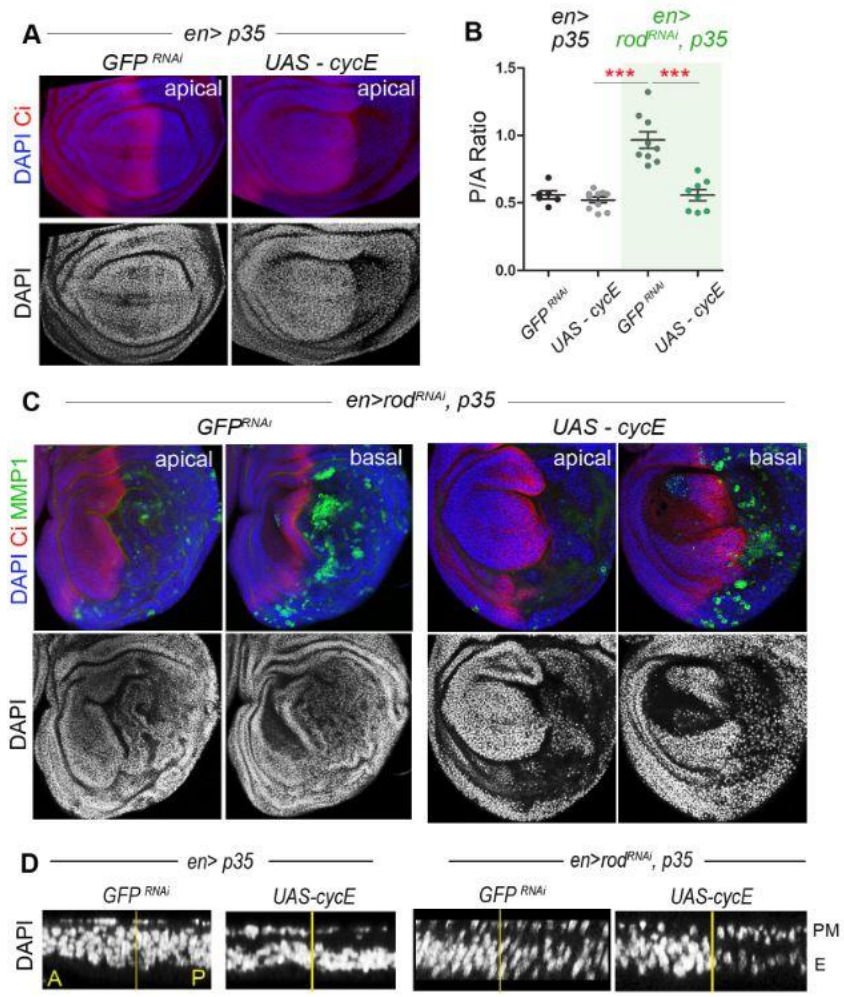

Figure 27. Proliferation of CIN epithelial cells upon CycE overexpression. (A and C) Larval wing primordia expressing the indicated transgenes in the posterior compartment stained with DAPI (blue), Ci (red) and MMP1 (green). (B) Histogram plotting the ration between the CIN compartment (posterior) and the wild-type compartment (anterior) when expressing the indicated transgenes. Error bars indicate SD (*** P< 0.001). (D) Cross-sections of the control and CIN tissue without and with expression of CycE. Separations between the anterior (A) and posterior (P) are shown. PM= peripodial membrane, E= epithelium.

Expression of CycE in control wing discs did not affect tissue size upon apoptosis blockade, according to the P/A ratio quantification (Figure 27 A and B). However, in CIN tumours, tissue overgrowth was significantly affected (Figure 27 C and B). Interestingly, the tissue was not only smaller, but the epithelium presented a des-compacted organization (Figure 27 D). However, this loss of epithelial

organization did not induce more delamination and activation of JNK, since MMP1 and Wg levels were equal compared to control tumours (Figure 28 A and B).

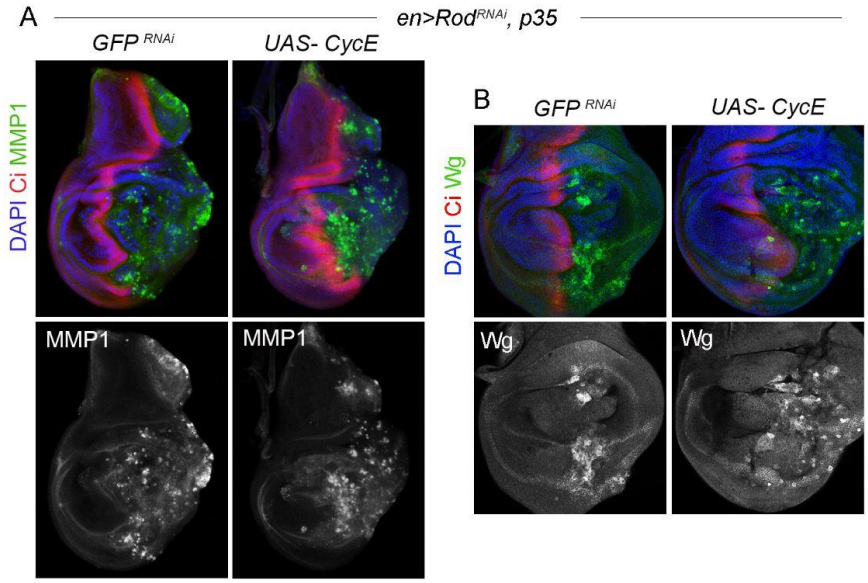

Figure 28. MMP1 and Wg expression in CIN epithelial cells upon CycE overexpression. (A-B) Larval wing primordia expressing the indicated transgenes in the posterior compartment stained with DAPI (blue), Ci (red) and a) MMP1 (green and grey) and Wg (green and grey).

We next asked if the reduced tissue size was a consequence of a reduced proliferative capacity of the tissue. We quantified the number of mitotic cells in both conditions by analysing pH3, and CIN tissues with CycE overexpression presented significantly reduced number of mitotic cells compared with CIN tissues (Figure 29 B and C), which did not happen in control discs overexpressing CycE (Figure 29 A). Moreover, the number of cells in S phase, according to the EdU incorporation profile was also significantly reduced in CycE overexpressing CIN tissues (Figure 29 C and E).

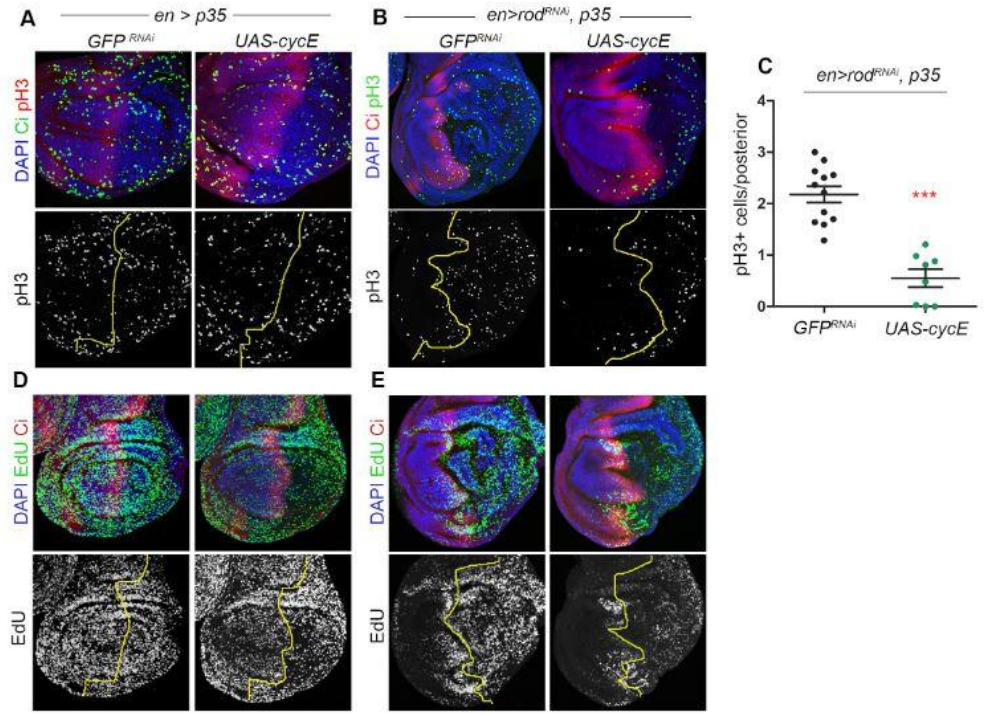

Figure 29. Proliferation of CIN epithelial cells upon CycE overexpression. (A-B) Larval wing primordia expressing the indicated transgenes in the posterior compartment under the control of the en>Gal4 driver and stained with DAPI (blue), Ci (green) and (A) EdU (green and grey) and (B) pH3 (green and grey) (C) Histogram plotting the ration between the number of mitotic cells in the epithelium and the size of the CIN compartment (posterior). Error bars indicate SD (*** P< 0.001).

These results suggest that CycE is somehow affecting the proliferation capacity of cells subjected to CIN. In order to better characterize this phenotype, we performed a FACs analysis and analysed the cell cycle profile of the whole CIN tissue, labelled with myrT expression. According to the DNA content, most of the cells in CIN tissues were in G2 and presented high levels of aneuploidy. However, overexpression of CycE induced accumulation of cells in G1 (Figure 30A). This suggests a specific sensitivity of CIN tissues to CycE overexpression, since CycE expression in wild-type tissues with p35 induced G1-S transition as previously described (Jtirgen A. Knoblich et al., 1994) (Figure 30A). Interestingly, due to this lack of proliferation, CIN cells overexpressing CycE had a significantly reduced amount of double/single strand breaks (Figure 30C). Since CycE overexpression induced an

unexpected G1 arrest in CIN cells, we wanted to investigate the molecular mechanism underlaying this phenotype.

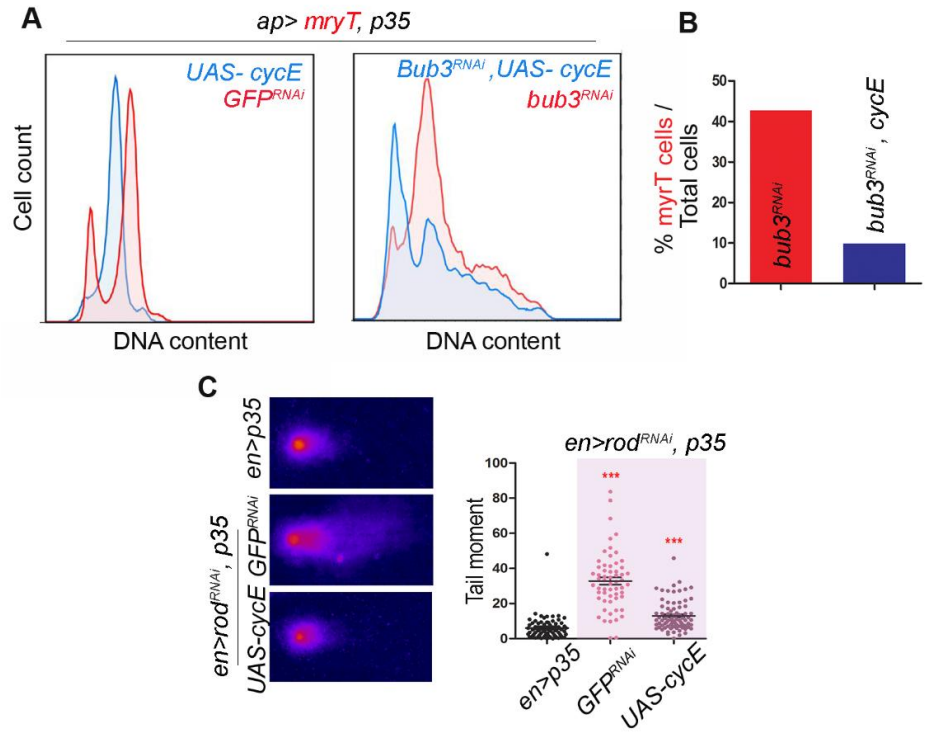

Figure 30. Cell cycle analysis in CIN tissues overexpressing CycE. Larval wing primordia expressing the indicated transgenes (A-B) DNA content profile of (A) control wild-type without (red) and with CycE overexpression (blue) and (B) CIN tissues without (red) and with CycE overexpression (blue). (C) Images of wing disc single cells subjected to comet assay in control. CIN tissues, and CIN tissues overexpressing CycE + Histogram plotting the tail length for each cell in the different genotypes. Error bars indicate SD (*** P< 0.001).

Dap inhibits the activity of cyclin-dependent kinases (CDK's), most probably CDK2, which mediate G1-S transition (de Nooij et al., 2000). CycE overexpression has been proved to positively regulate Dap expression only in a permissive window: in cells that are about to exit from the mitotic cycle or cells that have recently complete mitotic exit, resulting in a precocious G1 arrest (Lane et al., 1996). We wondered whether the reduced tissue size and lack of proliferation in CIN cells upon CycE overexpression was dependent on Dap.

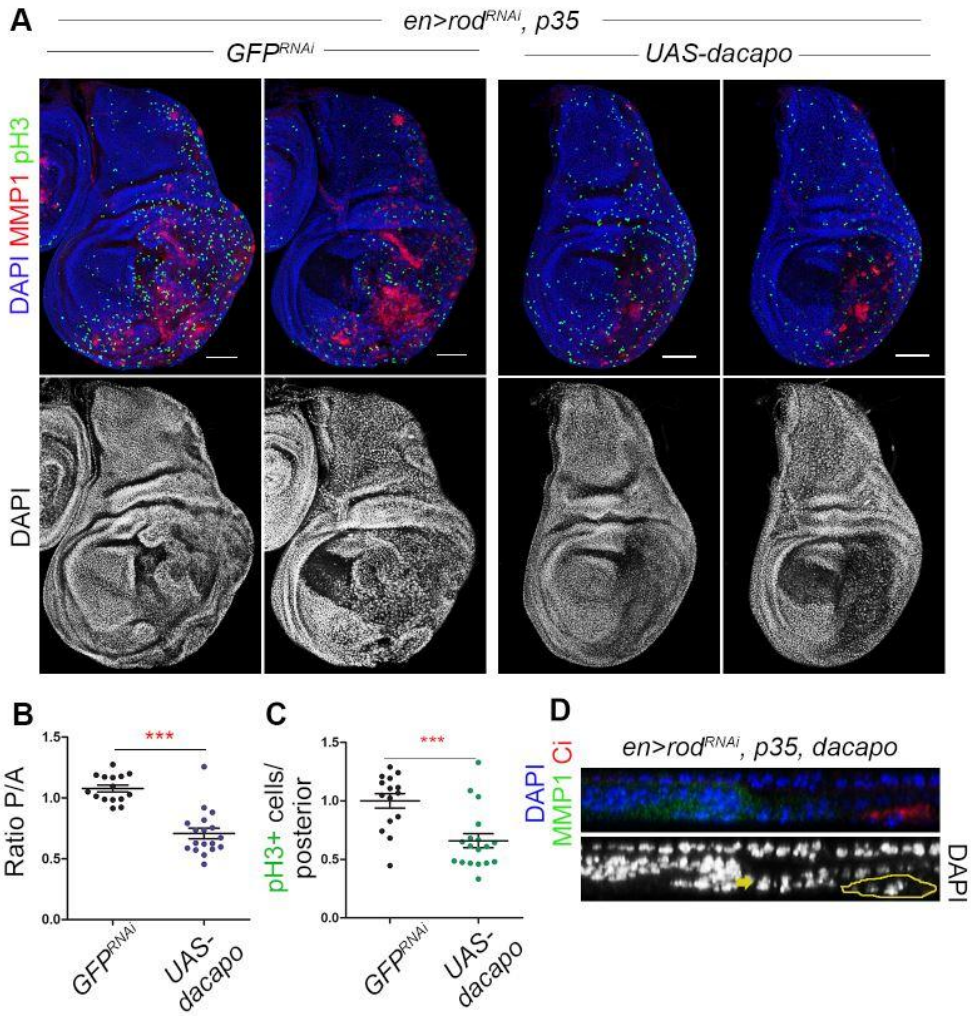

Figure 31. Proliferation of CIN epithelial cells upon Dacapo overexpression. A-B) Larval wing primordia expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue), Ci (green) and (A) EdU (green and grey) and (B) pH3 (green and grey) (C) Histogram plotting the ration between the number of mitotic cells in the epithelium and the size of the CIN compartment (posterior). Error bars indicate SD (*** P< 0.001). (D) Z-stack of CIN tissues depleted for Dacapo and stained for DAPI (blue and grey), MMP1 (green) and Ci (red). Delaminated cells are surrounded by a yellow line and the epithelium is marked by a yellow arrow.

Surprisingly, Dap overexpression completely phenocopied CycE overexpression, according to the reduced tumour size, loss of epithelial organization (Figure 31 A and B) and reduced mitotic activity (Figure 31 A and C). To sum up, CycE and Dap overexpression induce G1 arrest and prevent CIN epithelial cells from

proliferation. This effect is specific for CIN cells, since CycE overexpression in wild-type tissues induced G1-S transition (Figure 30A) as it has been previously reported.

5.2 Chemical inhibition of tumour overgrowth

Based on the previous results, we wanted to look for alternative ways to induce replicative stress in these tumours. For that, we treated CIN tissues with Hydroxyurea (HU), a ribonucleotide reductase inhibitor that affects the metabolism of deoxyribonucleotide triphosphates (dNTPs) and blocks the replication fork progression (Koç, Wheeler, Mathews, & Merrill, 2004), thus inducing replicative stress. Then, we measured the impact on tissue size (P/A Ratio) and proliferation (pH3).

In control discs expressing p35 (blocked apoptosis), the hydroxyurea treatment induced overgrowth. This goes according to the results showing that DNA damage can elicit a tumorigenic behaviour in cells unable to activate the apoptotic program (Dekanty et al., 2015). Thus, this confirms that the amount of hydroxyurea used increased the amount of damage in the tissues (Figure 32 A and B). Interestingly, the response in CIN tissues was completely the opposite. Tumour size (posterior) was significantly reduced compared to the wild-type part (anterior) when treated with hydroxyurea (Figure 32 B and C). Moreover, the mitotic capacity of these cells was significantly affected comparing with an untreated tumour (Figure 32 D). On the other hand, tissue morphology was not affected as in CycE or Dacapo overexpression (Figure 32 E), suggesting that tissue morphology affection might be a consequence of the G1 stall. Altogether, these results show that CIN tissues are highly sensitive to replicative stress most probably due to the amount of damage that they already suffered.

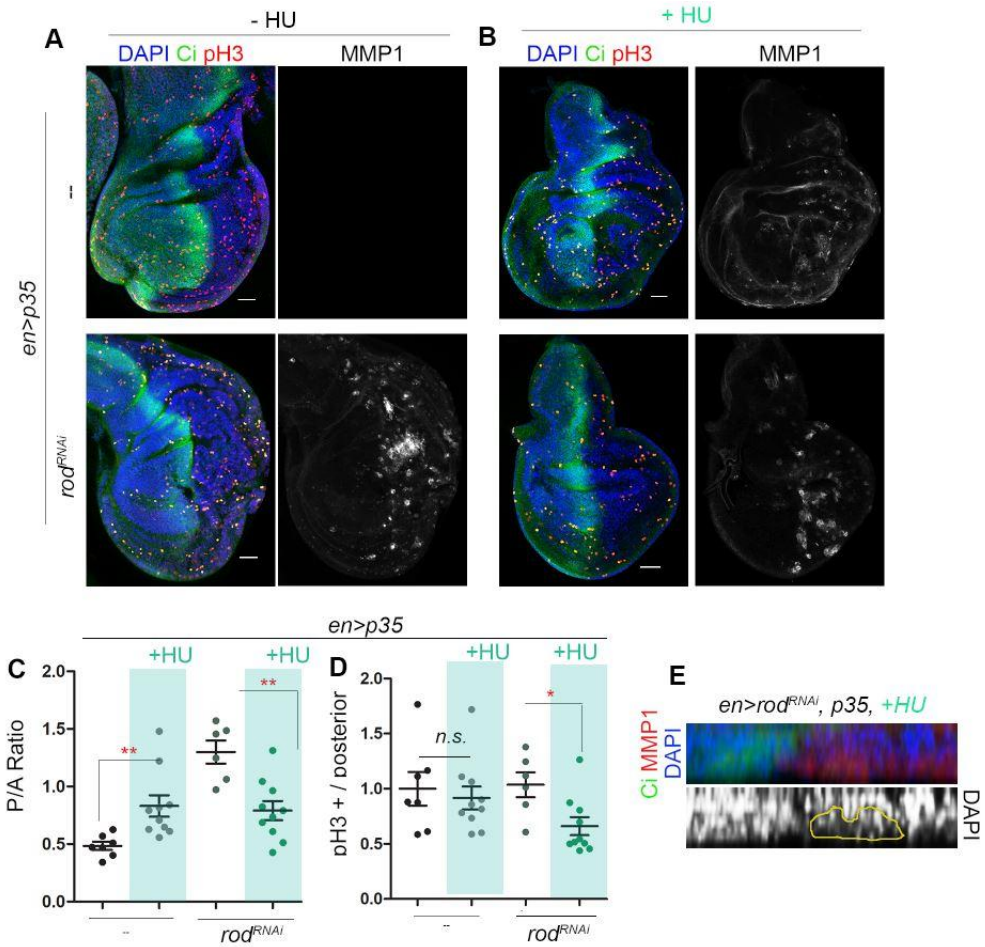

Figure 32. Effects of HU treatment on CIN tissues. (A-D) Larval wing primordia expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue), pH3 (red) and MMP1 (green). (A-B) Control epithelium without (A) and with (B) hydroxyurea treatment. (C) Histogram plotting the ratio between the CIN epithelium size (posterior) and the wild-type size (anterior) of control and CIN epithelium. (D) Histogram plotting the ratio between the number of mitotic cells in the epithelium and the size of the CIN compartment (posterior) of control and CIN epithelium. Error bars indicate SD (* P< 0.05, ** P< 0,005). (E) Z-stack of CIN tissues treated with HU and stained for DAPI (blue and grey) Ci (green) and MMP1(red). Delaminated cells are surrounded by a yellow line

Discussion

1. Epithelial cells become senescent in response to high levels of aneuploidy

During normal development, the clearance and regeneration of damaged cells is a process that needs to be completed. In aged or unhealthy tissues, this process is disrupted, promoting the accumulation of senescent cells, which appears to be the case of CIN tissues. The chronic instability generates the accumulation of highly-aneuploid secretory cells that induce the over-proliferation of the low-aneuploid epithelial ones (Dekanty et al., 2012). According to our investigation, these delaminated and highly-aneuploid cells are senescent. The secretion of specific proteins, such as morphogens or cytokines - known as the SASP- contributes to modifying neighbouring cells and the microenvironment of the tissue. Moreover, these cells show other senescent features, such as lysosomal accumulation and higher β-galactosidase activity, morphological changes and permanent cell cycle arrest. Here we describe a CIN-induced senescence model that could be used to dissect the molecular mechanisms underlying different senescent features and exploit these features to find possible therapy approaches and prevent CIN induced tumour progression (Figure 33).

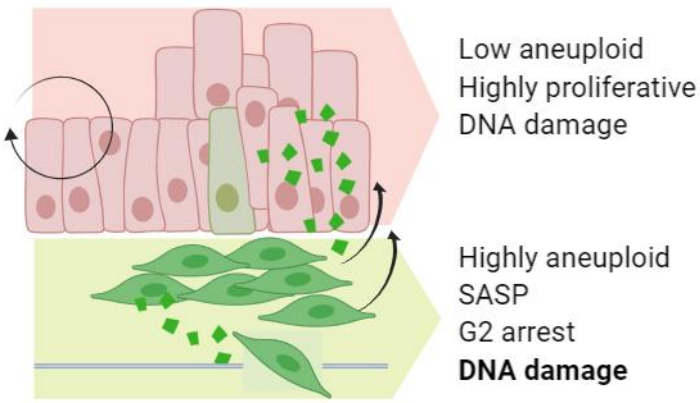

Figure 33. Chromosomal Instability induced senescence model. In the CIN tissue, there are two populations different in nature. On the one hand, the highly proliferative cells that remain in the epithelium and have tolerable levels of aneuploidy and damage that contribute to tumour overgrowth. On the other hand, the senescent population, which is highly aneuploid

and with high levels of damage and contributes to the overgrowth of the low aneuploid population by secreting specific molecules (SASP). This population is permanently arrested.

1.1 Transcriptional and post-trancriptional regulation of String/Cdc25 to induce a G2 arrest in CIN-induced senescent cells

Epithelial cells subjected to CIN undergo many stresses such as mitotic problems, replicative and metabolic stress. Given the highly proliferative nature of these cells, they end up accumulating high levels of damage and segregation problems that give rise to higher levels of aneuploidy. Upon a certain amount of damage, cells delaminate, activate JNK and arrest in G2. In this work, we demonstrated that the G2 arrest in the delaminated population is dependent on high levels of JNK signalling (Figure 18) and the DDR (Figure 17), and we elucidated some factors responsible for this G2 arrest.

Senescence has been defined as an irreversible cell cycle arrest. According to this, cells might use different mechanisms to achieve a robust stop. We propose a cooperative mechanism in CIN-induced senescent cells. First, delaminated cells showed low levels of CycA and CycB (Figure 21). This lack of mitotic cyclins is not due to post-transcriptional regulation since the Fly-FUCCI system showed a lack of active protein degradation (Figure 15) in most of the delaminated cells. The absence of cyclins could be due to transcriptional inhibition that might depend on JNK or the DDR signalling. However, more experiments are required to demonstrate this idea, for example by using transcriptional reporters or quantifying their expression by qRT-PCR in the presence/absence of the two signalling pathways. On the other hand, the absence of cyclins is not absolute, and some cells show basal levels of CycA or CycB that could allow them to progress through mitosis. For that reason, cells could mechanistically regulate the activity of the CDK-CyC complexes by repressing Stg, which in normal conditions dephosphorylates and activates these complexes. This regulation could happen at two different levels. On the one hand, JNK is known to repress Stg at a transcriptional level to prevent G2-M transition in a tumoral context

(Gerlach et al., 2018). This suggests a possible way for JNK-dependent downregulation of Stg in delaminated cells, which is reinforced by the downregulation of its transcriptional reporter (Figure 22). On the other hand, delaminated cells showed higher levels of Trbl, whose regulation through JNK has been also reported (Cosolo et al., 2019) and that could ensure a post-transcriptional regulation of Stg. by targeting it to proteasome-mediated degradation More epistatic analysis should be done to demonstrate the possible regulation of these two proteins by JNK in a CIN context and its possible cooperation with the DDR.

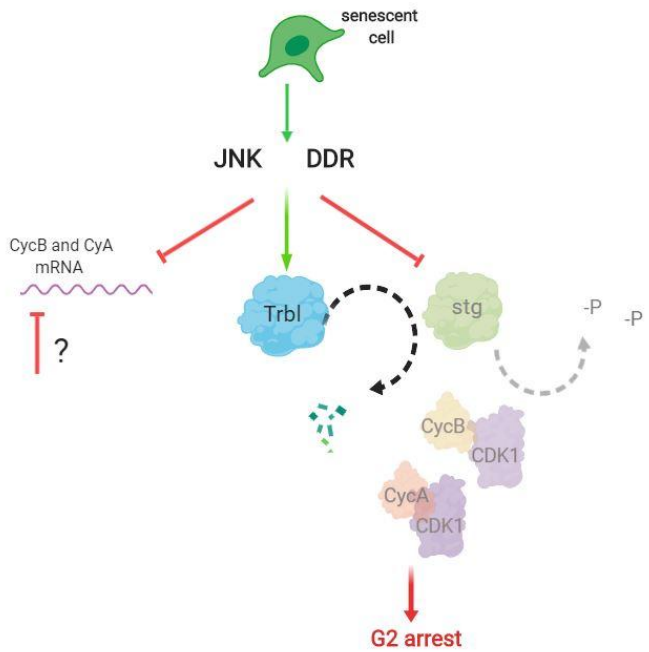

Figure 34. Working model to explain the cell cycle arrest in senescent cells. Upon high levels of aneuploidy and damage, these cells activate JNK signalling and the DDR. This might inhibit Stg, which prevents the dephosphorylation and activation of the CDK-Cyc complexes and promote G2 arrest. On the other hand, Trbl is also overexpressed and promotes Stg degradation. Besides, CycA and CycB are also repressed, most probably at a transcriptional level. These cooperative mechanisms would ensure the irreversible arrest of senescent cells in G2.

Altogether, these different mechanisms could ensure the proper inactivation of mitotic complexes that would induce an irreversible G2 arrest (Figure 34).

On the other hand, in cells that remain in the epithelium with low levels of aneuploidy, JNK signalling and DDR seem to prevent G1-S transition, according to the increased number of cells in S or G1 phase in the epithelium upon their signalling inhibition (Figure 26, 27 and 29). Interestingly, this G1 accumulation correlates with an upregulation of dacapo expression (Figure 35), which is known to induce G1 arrest (Lane et al., 1996). The biological relevance of the transient G1 stall needs to be further understood. On the one hand, this seems to happen only in early tumours: while at early time points there is a significant number of cells in G1, the percentage decreases in favour of G2 cells (Figure 24). G1 arrest could be an early response to certain levels of aneuploidy and damage. Surprisingly, based on our preliminary results, inhibition of the DDR prevents the G1 stall in the epithelium (Figure 26) and its depletion causes more damage (Clemente-Ruiz et al., 2016) but does not have a major impact on tumour growth (Figure 25 & 26), suggesting that these pathways probably perform a cooperative but not decisive role in epithelial cells in response to low levels of aneuploidy.

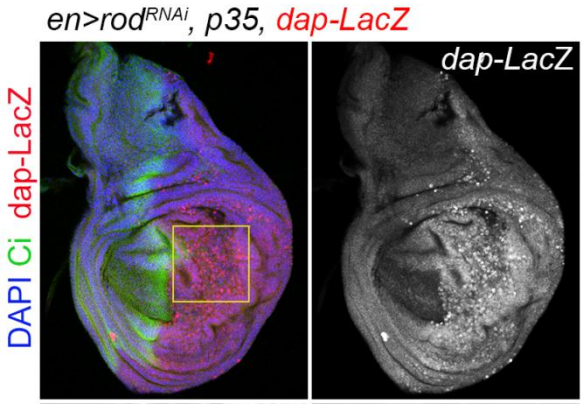

Figure 35. Upregulation of dacapo in CIN tissues. Larval wing primordia expressing the indicated transgenes in the posterior compartment and stained with DAPI (blue), Ci (green) and β-gal (red and grey).

However, other stress kinases could be playing a role in the epithelium. Low aneuploid epithelial cells express high levels of p38 (Clemente-Ruiz et al., 2016). This expression prevents the activation of JNK signalling (Heinrichsdorff, et al., 2008) (Heinrichsdorff et al., 2008) and could be relevant for preventing the initiation of the

senescent state. One possibility is that cells in the epithelium activate p38 in response to damage and this activation could induce a transient G1 stall through the activation of p21/Dacapo (T. M. Thornton & Rincon, 2009) to promote a sustained proliferation and prevent higher levels of damage and JNK activity. Upon high levels of aneuploidy, p38 is inactivated before delamination and allows JNK signalling to promote G2 arrest and the senescent state. Indeed, depletion of p38 in CIN epithelial cells in an apoptotic mutant background increases the number of senescent cells and enhances tumour overgrowth (Clemente-Ruiz et al., 2016). Thus, a deeper analysis of the contribution of p38 in epithelial cells in response to low levels of aneuploidy could help us to find signalling pathways to specifically target the highly proliferative population in CIN tissues.

1.2 Impact of the cell cycle arrest in senescence-induced tumorigenesis

The main purpose of senescence is to prevent the accumulation of damaged cells during development and disease. This is based on the sequence of events triggered by senescent cells that starts with the induction of the senescent state and ends with the clearance of the senescent cell. During this process, cell cycle arrest is crucial to avoid the propagation of these cells (Muñoz-Espín & Serrano, 2014). In this thesis, we demonstrate that CIN- induced delaminated cells that are forced to proliferate (by means of JNK inhibition) accumulate more levels of damage (Figure 25). This damage might have a negative impact on tissue proliferation and tumour growth. However, upon JNK inhibition the SASP is also rescued, thus inhibiting the systemic delay observed in larvae carrying CIN tissues (Dekanty et al., 2012) and reducing the time to measure the long-term impact of the accumulated damage in CIN tissues. An alternative to monitoring long-term effects could be to target the downstream effectors (Stg, Trbl) to specifically rescue the mitotic activity (but maintaining the SASP) and use allograph models. In these models, a small piece of the CIN tissue can regenerate a solid tumour in the abdomen of female adult hosts

after several days (Dekanty et al., 2012) (Muzzopappa et al., 2017). This way, the long-term effects of this recovered mitotic capacity in the delaminated population can be properly measured and analysed, both at the tissue and systemic level.

In addition to its role in preventing the amplification of the DNA damage levels, the impact of the cell cycle arrest on other senescent features is also under investigation. In mammals, activation of the DDR by different stimuli can induce and maintain the senescent state, both by regulating the cell cycle arrest and the production of the SASP. However, downstream of the DDR, the different senescent features are independently regulated. The senescence growth arrest is governed mainly by two pathways, p53 and p16/p21. By contrast, the SASP is cooperatively regulated by three main pathways: NF-kB, C/EBPb (Salama et al., 2014) and JNK (Byun et al., 2006). Depending on the stage of the senescence progression, the affected cell type or the nature of the inducing stressor(s), cells activate one or several of the previously mentioned pathways that will determine the secretome composition (Herranz et al., 2018). This is not the case for CIN tissues, where both the cell cycle arrest and the SASP seem to be regulated by JNK/DDR although the downstream effectors are independent. On the one hand, Stg overexpression and Tribl depletion restored the mitotic capacity of delaminated cells without affecting the SASP, according to the expression of MMP1 in these cells (Figure 22). In any case, their regulation by JNK needs to be demonstrated in the CIN context. On the other hand, JNK directly regulates Wg, a mitogen part of the SASP, at the transcriptional level (Dekanty et al., 2012). Thus, both senescent features seem to depend on JNK and the DDR.

1.3 G2 phase, a good place to consolidate senescence

According to our results, most of the delaminated cells are arrested in G2. Many researchers have suggested that the phase of the cell cycle is decisive for the establishment of the senescent state (Cosolo et al., 2019) (Nakamura et al., 2014). However, the proposals are not conclusive. On the one hand, although most of CIN-

induced senescent cells are arrested in G2, we can detect some of them in G1 or S phase (Figure 15). On the other hand, expression of Stg forced delaminated cells to cycle but did not affect MMP1 expression. Interestingly, Cosolo and colleagues proposed that cells in G2 could have a well-established and stronger expression of JNK and that this expression could determine the presence of some senescent features. In this regard, different experiments could be done to understand the relationship between the cell cycle and JNK signalling. For example, the use of activity reporters to analyse JNK expression levels by FACS or in combination with the Fly-FUCCI system could be useful to determine if cells in G2 show higher levels of JNK. Also, other SASP factors or different senescent features should be analyzed to understand a possible relation with the cell cycle phase of the cell, for example, if the secretory capacity of these cells is affected upon cell cycle forced progression.

2. Aneuploidy is the driving force for the cell cycle arrest in CIN-induced senescence

2.1 Aneuploidy as the driving force for the cell cycle arrest

During the last years, many studies have been carried out to understand the specific consequences of aneuploidy and chromosomal instability. Chromosomal instability in the *Drosophila* wing epithelium generates aneuploidy that induces delamination, activation of a JNK dependent transcriptional program and the acquisition of migratory and invasive capacity (Dekanty et al., 2012) (Benhra, Barrio, Muzzopappa, & Milán, 2018) of highly aneuploid cells. However, further investigations need to be done to understand which ones of the CIN-dependent phenotypes are a direct consequence of the generation of aneuploidy karyotypes.

Some progress has been done in this direction, using aneuploidy-specific models. Depletion of the X-chromosome compensatory mechanism induce gene dosage imbalance and, together with apoptosis inhibition, recapitulates most of the features of CIN-induced tumorigenesis, such as delamination, activation of the JNK-

dependent transcription program and the acquisition of migratory capacity (Clemente-Ruiz et al., 2016). Regarding other features, we have demonstrated that these cells are also arrested in the cell cycle (Figure 16), but these larvae do not suffer developmental delay (Clemente-Ruiz et al., 2016), suggesting that some of the SASP components in cells subjected to CIN could be gene dosage imbalance-independent and a direct consequence of aneuploidy.

Another aneuploidy-specific model, FLP/FRT-Induced terminal deletion to generate a loss of chromosome ends, has been shown to drive cell delamination and JNK activation in delaminating cells, inducing Wg and MMP1 (Dekanty et al., 2012). However, telomere loss per se can induce senescence independently on the aneuploid state (Gire & Dulic, 2015b) and other models are needed to unravel the direct consequences of the aneuploidy-induced gene dosage imbalance. For example, the CRISPR-Cas9 technique allows us to induce specific chromosome deletions (or portions of chromosomes) in a specific time window and limited to some cells. Moreover, the FLP/FRT-based strategy can also be designed to create genetic mosaics (Griffin, Binari, & Perrimon, 2014) and maintain the telomeres in the chromosomes after recombination and induction of chromosome deletion. The observed effects in this model would be due to the original deletion event, and not due to a persistent source of instability (as is the case in the CIN model) and the aneuploid specific consequences could be analysed.

The process of delamination also needs to be considered. Indeed, blocking the death of cells depleted for Mud, a protein involved in the mitotic spindle orientation, was sufficient to drive tumour-like overgrowth tissues with delaminated cells expressing MMP1 and Wg to promote the over-proliferation of epithelial cells (Muzzopappa et al., 2017). However, Mud depletion did not induce a cell cycle arrest (Figure 16), confirming that the lack of mitotic capacity is a feature of aneuploidy-induced senescence and not a simple consequence of delamination.

2.2 JNK drives different features depending on the stressor

As discussed before, different stressors can lead to different phenotypes. The conserved JNK pathway integrates signals from a diverse range of stimuli to elicit a convenient response. Upstream of JNK signalling, three different kinases have been identified in *Drosophila*, which are Tak1, Ask1, and Wallenda (Wnd). Ask1 which is directly regulated by ROS through its binding to thioredoxin (Saitoh et al., 1998), is the one responsible for JNK activation in CIN tissues (Muzzopappa et al., 2017). On the contrary, in Scrib/Ras^{V12} tumours, Wnd is the main kinase responsible for JNK activation. Different stressors activate different kinases, and the different outputs could be explained by different sensibilities and efficiencies to phosphorylate Hep, the upstream effector of the JNK pathway. In this way, different levels of JNK activation could lead to different phenotypes. For example, JNK levels in Mud depleted delaminated cells might be lower than in cells subjected to CIN. Thus, low levels of JNK may induce the SASP, however, only high levels of JNK might induce cell cycle arrest. This hypothesis needs to be carefully analysed by comparing JNK levels of both situations and, by epistatic analysis, to describe the mechanism responsible for JNK activation upon different stressors.

3. DNA damage contributes to CIN-induced tumorigenesis

Aneuploidy can lead to replicative and mitotic stress (J. Zhu, Tsai, Gordon, & Li, 2018), that can end up generating DNA damage and chromosome breaks. We show that CIN tissues have chromosome breaks and DNA damage, according to different markers, such as localization of RPA (Figure 11) and Rad50 foci in the nuclei (Figure 12) and the comet assay (Figure 13). We could detect an increased number of damage foci mainly in the delaminated population, although they were also present in the epithelium. Thus, the amount of damage seems to correlate with the levels of aneuploidy present in the cell.

The fact that not all cells in CIN tissues show damage markers can be explained by different levels of aneuploidy. Aneuploid cells are highly heterogeneous due to CIN, and this heterogeneity could be translated into different levels of damage or even to a different capacity to repair this damage. On the one hand, CIN can induce aneuploidy due to chromosome missegregation and DNA damage due to breaks in lagging chromosomes (Janssen et al., 2015). Moreover, aneuploidy can also induce chromosomal instability and DNA damage due to umbalance of replicative complexes (Passerini et al., 2016). All this situations can be present at different levels in cells subjeted to CIN and could explain the diffent levels of damage that we detect. Mutations in distinct DNA repair systems or even loss of chromosome regions where these genes are located, due to CIN, might elevate the susceptibility to damage and even inhibit the cell capacity to repair it. Indeed, it has been shown that the DDR pathway buffers the deleterious effects of CIN and counteract its pro-tumorigenic effect in the epithelial cells (Clemente-Ruiz et al., 2016). Consequently, we show that the DDR has an impact on cell cycle progression both in the epithelium (by inducing G1 stall) (Figure 26) and the senescent population (by preventing mitosis) (Figure 17). Although cells subjected to CIN could suffer different levels of DNA damage due to their genetic diversity, this damage seems to be sensed by the DDR both in the epithelium and the delaminated population.

4. Increasing DNA damage to target CIN tumours

Most anticancer drugs used today were selected based on their ability to rapidly kill dividing cancer cells. However, when given to patients, many of these drugs also injure rapidly dividing normal cells, such as bone marrow haematopoietic precursors and gastrointestinal mucosal epithelial cells (Kaelin, 2005). According to this, one of the main aims of cancer science today is to find concrete drugs that specifically target cancer cells without harming healthy cells.

In tumours, drugs that induce a loss-of-function phenotype and affect processes that are more relevant in cancer cells, such as DNA repair or replication

processes, are starting to be used to target tumour growth. Indeed, there is increased availability of chemical and genetic tools for perturbing gene function in cancer cells (Kaelin, 2005). In this project, we used two different approaches to target tumour progression based on the levels of DNA damage present in CIN tissues.

4.1 CycE overexpression collapses tissues subjected to CIN

We have shown that CIN tissues suffer DNA damage (Figure 11, 12 and 13) , most probably as a consequence of replicative and oxidative stress. Based on that, additional sources of stress could increase the amount of damage and affect tumour overgrowth. Overexpression of CycE, which has been proved to prematurely induce G1-S transition (Jtirgen A. Knoblich et al., 1994) (Jtirgen A. Knoblich et al., 1994) and generate replicative stress and genomic instability (Ekholm-Reed et al., 2004b), significantly affected tissue growth and organization (Figure 30). However, we show that cells are not able to progress through S phase and get arrested in G1, according to the FACS results (Figure 30) and the lack of EdU incorporation (Figure 29). We propose a different hypothesis for CIN-tumour cells behaviour in response to CycE overexpression. On the one hand, it is possible that CycE induces G1-S transition in these cells, but they get arrested at the beginning of S phase due to the high levels of damage that they suffer. They might not be able to progress through S phase and might be stalled before the replication starts. Indeed, it has been shown that overexpression of CycE in human cells can generate an S phase collapse without even initiating the nucleotide incorporation (Ekholm-Reed et al., 2004b). Fly-FUCCI analysis could be useful to determine if these cells are in G1 or early S phase.

Another alternative is that these cells are stalled in G1 by inducing Dap expression, a Cip/Kip CKI in *Drosophila* that causes G1 arrest. CycE overexpression has been proved to positively regulate Dap mainly post-transcriptionally in *Drosophila* nurse cells and induce premature G1 arrest (Lane et al., 1996). This could explain why CycE overexpression in cells subjected to CIN did not induce more JNK activation (Figure 31), which could be expected in situations of increased stress.

Indeed, the lack of proliferation seem to avoid the accumulation of double and single-strand breaks (Figure 30) and thus preventing tumour growth. Interestingly, tissue morphology is significantly affected (Figure 27), although cells do no lose their apicobasal polarity (data not shown). A prolonged G1 stall due to Dap/CycE expression could be generating a quiescent-like state, defined as a temporary and reversible lack of proliferation (Daignan-Fornier & Sagot, 2011). This hypothesis is strongly suported by the fact that dacapo overexpression completly phenocopies CycE overexpression (Figure 31). In this context, this stall might prevent the overproliferation of epithelial cells even in the presence of the highly secretory population and block tumour growth.

It has been recently proposed that Ras^{V12} cells with mitochondrial defects induce G1 arrest and promote SASP. In this context, G1 arrest is proposed to be essential for the SAPS establishment, since overexpression of CycE (based on the expected role in G1/S transition) counters the function of p21/Dap and force the cells to reach G2 phase, blocking SASP production inhibiting and the non- autonomous effects (Nakamura et al., 2014). Based on our results, we interpret these results differently: these cells might not progress through S phase upon CycE overexpression and may be stalled in G1- early S phase. According to this, the SASP would be rescued not because G1 arrest is required for its induction, but because the proliferative and tumorigenic capacity of these cells is inhibited, and tumour growth is prevented.

4.2 Hydroxyurea treatment impairs CIN tumour growth

Based on the already existing DNA damage in CIN tissues, we explored other ways to increase stress and impair tumour growth. HU blocks the replication fork progression (Koç et al., 2004), thus inducing replicative stress. In this thesis, we demonstrated that, based on the highly proliferative nature of CIN epithelial cells and the already existing levels of DNA damage, these cells are especially sensitive to hydroxyurea, affecting their proliferative capacity and tumour growth (Figure 33).

CIN-induced DNA damage is a hallmark of cancer. In that sense, genes involved in DNA repair or DNA damage detection genes have been classified as tumour suppressors. Increasing evidence indicates that numerous tumours with high levels of replicative stress (RS) are particularly vulnerable to the loss of ATR, the DDR pathway that responds to RS and ssDNA. For that reason, and given that RS is a major source of genomic instability in cancer, targeting ATR has emerged as a promising alternative (Lecona & Fernandez-Capetillo, 2018). We have previously shown that low aneuploid CIN cells in the epithelium activate the DDR to repair the basal levels of damage present in the epithelium (Clemente-Ruiz et al., 2016) and to induce G1 stall without affecting the mitotic capacity (Figure 26). However, when the amount of damage is increased with hydroxyurea, the mitotic capacity of these cells suffers a significant reduction (Figure 33). We wonder if, as in the case of delaminated cells, high levels of DNA damage would induce a G2 arrest of hydroxyurea treated cells in response to ATR/JNK. In this context, CIN epithelial cells might become "addicted" to the DDR/JNK pathway to maintain tissue homeostasis upon hydroxyurea treatment. Thus, one future perspective could be to check if inhibition of the DDR or JNK increases CIN-tumours sensitivity to hydroxyurea treatment and enhances its effect on tumour growth inhibition.

Upon Hydroxyurea treatment and DDR blockade in CIN tissues, the increased damage might induce more JNK activation and cell death, which in a background mutant for p35, can lead to the accumulation of more senescent cells. Although the induction of senescence to prevent damaged cells proliferation has emerged as therapy during the last years (Nardella, Clohessy, Alimonti, & Pandolfi, 2011), senescence induction can end up being detrimental. This is especially true in the context of tumorigenesis, where senescent cells promote the non-autonomous overgrowth of neighbouring cells or induce tumour-beneficial systemic effects as it happens in CIN tissues. Thus, a desirable pro-senescent therapy should be combined with a complementary treatment to eliminate the senescent cells, thereby impairing the possible pro-tumorigenic effect of the SASP. In this direction, different approaches have been used to find the Achilles' heel of these cells: drugs that

specifically inhibit the SASP or senolytics that block pro-survival pathways active in senescent cells leading to apoptosis.

On the one hand, anti-SASP drugs have been developed to manly disable activation of the NF-kB pathway, by inhibiting its transcriptional activity, preventing its translocation to the nucleus or downregulating it upstream activators. Other independent NF-kB targets have also been used, such as the JAK/STAT pathway, that activates the transcription factor C/EBPβ and drives the expression of IL-6 or IL-8 (Soto-gamez & Demaria, 2017). On the other hand, the use of senolytics is becoming more popular, and its aim is to eliminate senescent cells based on their dependence on anti-apoptotic pathways to be maintained in the tissue. For example, Bcl-2/ Bcl-xL proteins, which are the main inhibitors for mitochondrial apoptosis, are the main targets to drive apoptosis in senescent cells (Y. Zhu et al., 2015). In addition to the previous features, other particularities can be used to selectively eliminate senescent cells. For instance, it is known that they sustain high levels of proteotoxicity, which require a high lysosomal activity, a situation that makes them particularly sensitive to chemical inhibitors of lysosomal ATPases (Dörr et al., 2013). In addition, therapies that boost the immune system by increasing the number of immune cells or their activity against senescent cells can help to eliminate senescent cells, especially in age-related diseases. The search for more features to specifically target senescent cells is one of the main objectives of cancer science today (Figure 36).

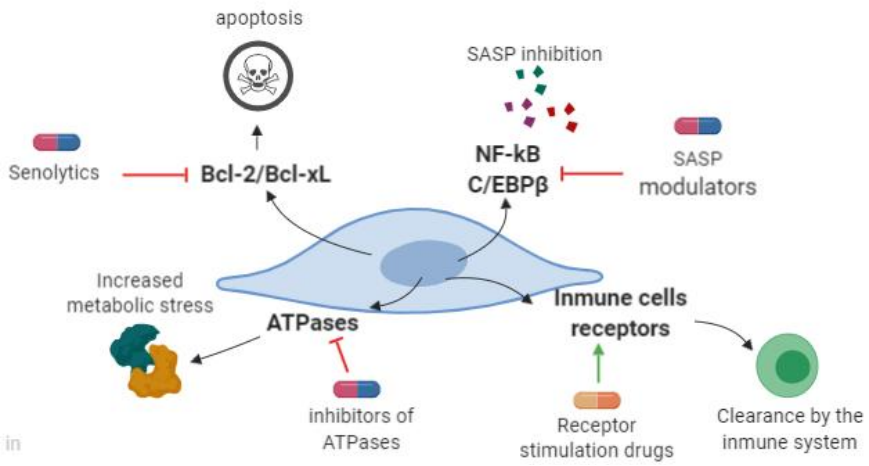

Figure 36. Therapeutic interventions againts celullar senescence. Cartoon depicting different targets to selectively kill senescent cells. Inhibition of NF-kB and C/EBPβ, the main pathways driving the SASP, stimulation of immune receptors recognized by Natural Killers or T cells, inhibition of lysosomal ATPases to increase the metabolic stress and the use of senolytics to force apoptosis.

5. Future perspectives for the CIN-induced senescence model

We have shown that CIN tissues accumulate high levels of aneuploidy and damage that induce a senescent program in the most aneuploid cells. Both populations are relevant for tumour progression. The senescent population is critical because it drives the overgrowth of the epithelium, and the epithelial one contributes to tumour overgrowth. In this thesis, we have discussed many of the features present in these two populations. This section aims to propose future perspectives for the CIN model in the context of cancer and senescence research.

5.1 Use tools to independently affect the two populations

So far, we have demonstrated the presence of two populations different in nature in CIN tissues. One which is highly proliferative and low-aneuploid, mostly stalled in G1 and with low levels of DNA damage. The other is senescent in nature, highly aneuploid and damaged and gets permanently arrested in G2. To properly study the molecular mechanisms underlying the different behaviours of the two populations, it will be important to use tools that allow us to manipulate them independently. For that, we could make use of the LexA-LexO system (Yagi, Mayer, & Basler, 2010) which follows the same logic that the UAS-Gal4 system and allow us to drive gene expression in two cell populations independently at the same time by using specific enhancers. Thus, we could specifically target signalling pathways in the senescent population and study the impact on tumour growth without affecting the proliferating tissue.

5.2 Exploiting replicative stress and damage as a therapeutic opportunity

The dependence of highly proliferative and damaged tissues to repair pathways is being use in cancer treatment to increase genomic instability and selectively target these tissues (Lecona & Fernandez-Capetillo, 2018). CIN epithelial tissues suffer DNA damage that might cause replicative stress, however, although depletion of the DDR prevented the G1 stall (Figure 26) and induced more DNA damage (Clemente-Ruiz et al., 2016), it did not increased the amount of highly aneuploid cells and did not promoted tumour growth. On the other hand, JNK depletion in the epithelium also prevented cells from being stalled in G1 but did not affect the mitotic capacity of these cells (Figure 25). For that reason, extra sources of damage could increase CIN tissues dependence to the DDR or JNK signalling. We demonstrate that CIN tissues are highly sensitive to hydroxyurea and, when treated, the mitotic capacity of these cells is significantly affected (Figure 32). In the future, we would like to combine hydroxyurea treatments with DDR or JNK inhibition to enhance their sensitivity and induce tumour collapse.

Although the combine hydroxyurea plus DDR inhibition treatment could affect the mitotic capacity, it could also increase the amount of damage in these cells that will delaminate and become senescent. Interestingly, JNK inhibition prevents the SASP in this population, avoiding the possible detrimental effects of an increased amount of senescent cells upon treatment with hydroxyurea and co-depletion of the repair pathways.

5.3 CIN model to find senescence therapies

One of the main features of senescence is apoptosis resistance. In the CIN model, this apoptosis resistance is given by the expression of the anti-apoptotic baculovirus protein p35. This genetic background mimics some tumoral conditions in

which malignant cells inherit mutations in caretaker genes that prevent them from dying and make them choose the senescent state as an alternative to prevent proliferation. However, the base for treatment with senolytics relies on the capacity of these drugs to inhibit the function of anti-apoptotic proteins. In the case of CIN tissues, the apoptotic blockade with p35 impedes a forced apoptotic response. Thus, more physiological ways to prevent apoptosis, such as the expression of RNAis against different apoptotic genes (such as Dronc) to partially block apoptosis and allow us to monitor the effects of senolytics on CIN-induced senescent cells.

In this context, CIN-induced senescent tumoral cells could be useful to find the Achilles' heel of the senescent state. Different genetic tools (such as RNAis or the CRISPR-Cas9 technique) could be used to selectively target a wide variety of genes in forward genetic screenings to find specific pathways that prevent the survival of senescent cells. CIN epithelial cells can be easily labelled with different fluorescent proteins that allow us to follow tumour growth without dissection, just monitoring fluorescent intensity during larval development. In addition, there are many groups trying to identify new anti-senescent drugs that work *in vivo* (Y. Zhu et al., 2015) (Campisi et al., 2019), and *Drosophila* could be used as a good model organism to screen and monitor the effect of these drugs before their use in more expensive animal models or humans.

5.4 Combined therapy to selectively target CIN tissues

According to the previous lines, CIN tissues are quite heterogeneous with two populations that are different in nature and require very specific therapies. The highly proliferative epithelium accumulates high levels of DNA damage that can induce replicative stress. This features makes the tissue more sensitive to increased levels of damage caused by hydroxyurea, which affects the mitotic capacity of the tissue (Figure 32). However, combined inhibition of the pathways involved in DNA repair and cell cycle in the epithelium, such as JNK and the DDR (Figure 25 and 26), could increase this sensitivity and enhance the effect of the hydroxyurea on tumour growth.

On the other hand, this increased damage can enhance the amount of senescent cells, a population that could end up enhance tumour-beneficial effects. To prevent this situation, a combined treatment with anti-senescent drugs could help to eliminate them and to prevent tissue relapse. This combined treatment could be a promising approach to fight cancer progression in the future (Figure 37).

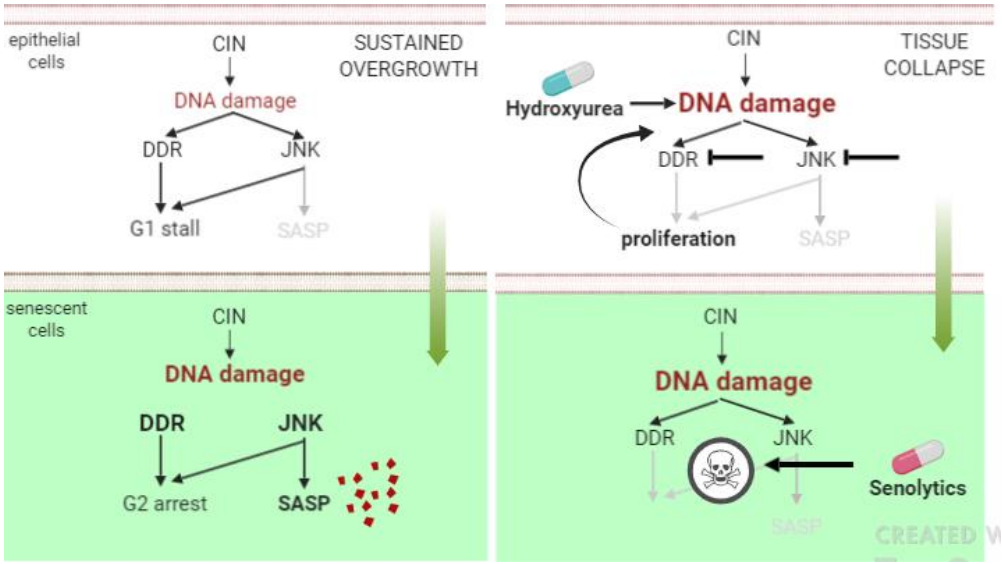

Figure 37. Combined pro and anti-senescence therapies to target CIN tissues. Cartoon depicting effects of Hydroxyurea and senolytic treatments on CIN tissues. In untreated CIN tissues, DNA damage would induce G1 stall in the epithelium to maintain a sustained proliferation, when these cells become highly aneuploid, they delaminate, activate JNK, become senescent and arrest in G2. Elevate levels of replicative stress induced with Hydroxyurea might activate the DDR/JNK in G2 also in epithelial cells and prevent cell proliferation. However, additional block of the DDR/JNK might allow these cells to progress thought mitosis increasing the amount of damage and inducing tissue collapse. The addition of anti-senescence drugs to avoid the tumour-beneficial effects generated by senescent cells would have a positive impact on tumour elimination. Image created with biorender.com.

Conclusions

1. CIN-induced highly aneuploid cells become senescent and reproduce most of the features find in human senescent cells, such as permanent cell cycle arrest, higher lysosomal activity, different morphological changes and the presence of a SASP.

2. JNK signalling and the DDR are responsible for the G2 cell cycle arrest in the CIN-induced senescent program.

3. CIN-induced senescent cells downregulate the mitotic cyclins.

4. Stg and Trbl are differentially expressed in senescent cells and contribute to the G2 cell cycle arrest.

5. JNK-dependent arrest in senescent cells prevents the proliferation of highly aneuploid cells to avoid damage propagation within the tissue.

6. Low-aneuploid epithelial cells activate low levels of JNK and the DDR and induce a G1 stall.

7. In CIN tissues, both the epithelium and the senescent population suffer from DNA damage, but this damage is higher in the senescent population.

8. CIN epithelial cells are highly sensitive to CycE and Dap overexpression that induces a cell cycle stall and prevents tumour growth.

9. CIN epithelial cells are highly sensitive to increased levels of replicative stress caused by hydroxyurea treatment which impairs cell proliferation and tumour overgrowth.

Materials and methods

Drosophila Strains

The following strains were provided by the following sources: (1) Bloomington *Drosophila* Stock Center (BDSC): UAS-bskK53R (UAS-bsk^{DN} in the text), UAS-msl-1^{RNAi} (BDSC 9239), UAS-myristoylated-Tomato (UAS-myrT, BL32222); UAS-p35 (BL5073); UAS- dap^{RNAi} (BL64026), dap-LacZ (BL10406), CycA-GFP (BL53857), Tribl-GPF (BL35056), UAS- mud^{RNAi} (BL28074), UAS-CycE (BL4781); UAS-GFP^{RNAi} (BL9330); (2) Vienna *Drosophila* RNAi Center (VDRC): UAS-rod^{RNAi} (GD19152), UAS-bub3^{RNAi} (GD21037), UAS–mei-41^{RNAi} (GD 11251); UAS–grp^{RNAi} (GD 12680); UAS–dp53^{RNAi} (GD 38235), UAS-fzr^{RNAi} (GD25550) ; (3) FlyORF: UAS-Dap (F001262) (4) kind gifts from Bruce A. Edgar: ub-EGFP-E2F11-230, ub-RFP-CycB1-266 [Fly-fucci in the text, (N Zielke et al., 2014), BL 55123 & 55124]; and Anne Royou: tub-RFP-Rad50; tub-GFP-RPA3. ap-gal4, and en-gal4 are described in Flybase, MMP1-GFP (Uhlirova & Bohmann, 2006), GstD-GFP (Sykiotis & Bohmann, 1982).

Protocol for tumour induction

Flies were allowed to lay eggs on fly food for 24 h at 25°C. Larvae were maintained at 25°C for 24h, switched to 29ºC for 4 days before dissection. The fly food standard medium contains 4% glucose, 55 g/L yeast, 0,65% agar, 28g/L wheat flour, 4 m/L propionic acid and 1,1 g/L nipagin. In Hydroxyurea (HU) experiments, 200µL of HU (0.0486 g/mL) was added to the fly food after 2 days at 29ºC and 2 days before dissection.

Immunofluoresncence and confocal imaging

Wing imaginal discs from third instar larvae were dissected in cold PBS, fixed in 4% formaldehyde for 20 minutes, washed 3 times in PBT for 15 minutes each time (PBS + 0.1% Triton), blocked for 1 hour in BBT (PBS + 0.3% BSA, 0.2% Triton + 250mM NaCl) and incubated with primary antibodies overnight (O/N). Discs were then washed 3 times with BBT and incubated with secondary antibodies for 2 hours.

Finally, discs were rinsed with PBT 3 times and kept on mounting media (80mL glycerol + 10mL PBS 10x + 0,8 ml N-propyl-gallate 50%) at 4ºC.

The primary antibodies used are the following: mouse anti-MMP1 (1:20) (14A3D2, Developmental Studies Hybridoma Bank, DSHB); rabbit anti-PH3 (1:1000; Merk Millipore); rabbit anti-P-H2Av (1:500; S137, Rockland); rat anti-Ci (1:10; 2A1, DSHB); mouse anti-CycA (1:50) (A12, DSHB); mouse anti-CycB (1:50; F2F4, DSHB); anti-Rab7 (1:50; Rab7-s, DSHB); anti-Golgin (1:50Golgin84 12-1, DSHB); anti-Lamin (Dmo) (1:50; ADL84.12, DSHB), anti-Wg (1:10; 4D4, DSHB) and rabbit anti-CycE (1:100; sc-481, Santa Cruz Biotechnology). Secondary antibodies Cy2, Cy3, Cy5 and Alexa 647 (1:400) were obtained from Jackson ImmunoResearch and diluted (1:400). Zeiss LSM780 and Zeiss LSM 880 with airyscan confocal microscopes were used to obtain high resolution images.

DNA Synthesis

Click-iT™ Plus EdU Alexa Fluor™ 647 Imaging Kit from Invitrogen (C10640) was used to measure DNA synthesis (S phase) in proliferating cells, following the manufacturer's indications. EdU (5-ethynyl-2´-deoxyuridine) provided in the kit is a nucleoside analogue of thymidine.

Senescence labelling

CellEvent™ senescence green detection kit from Invitrogen (C10850) was used to detect senescence-associated β-galactosidase (SA-β-gal) activity following the manufacturer's instructions. Wing discs were fixed in 4% paraformaldehyde for 20 minutes, washed (1% BSA in PBS) and then incubated in working solution for 2h at 37ºC. Discs were then washed in PBS and mounted for confocal imaging.

Image processing and analysis

Fiji [National Institute of Health (NIH) Bethesda, MD] was used to measure the size of the Anterior (A) compartment (based on Ci), or the whole wing discs (based on DAPI staining), and to manually count mitotic cells (PH3 positive cells) or DDR active cells (pH2Av positive cells). Image stacks were obtained using a Zeiss LSM780 confocal microscope, 25X glycerol immersion objective with 1,5 or 0,5 µm per optical section to cover the entire thickness of each disc. Control wing discs were grown in parallel and subjected to the same experimental conditions (temperature and time of transgene induction) as the experimental wing discs. Samples were always quantified in parallel. At least 10 wing discs per genotype were scored.

Flow cytometry analysis

Third instar larvae of each genotype were dissected in cold PBS. To label S-phase cells, we used the Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (C10634), following the manufacturer´s indications with some modifications. After incubating larvae with 10 µM EdU in PBS for 5 min, tissues were trypsinized at 32ºC for 45 min, as described in Neufeld et al., 1998. Once the Click-iT™ reaction was complete, permeabilized cells were stained with DAPI solution (10ml Triton 0.1%-X100; 10µL DAPi; 20µL RNase 10mg/ml) and incubated for 1h at Room Temperature (RT). Fluorescence was determined by flow cytometry using a FacsAria I SORP sorter (Beckton Dickinson, San Jose, California). Excitation with the blue line of the laser (488 nm) permits the acquisition of scatter parameters, green (530 nm) fluorescence from GFP, and red (635 nm) fluorescence from Tomato. A UV laser (350nm) was used for DAPI excitation. Doublets were discriminated using an integral/peak dotplot of DAPI fluorescence. Optical alignment was performed on the optimized signal from 10 µm fluorescent beads (Flowcheck, Coulter Corporation, Miami, FL). DNA analysis (cell cycle) on single fluorescence histograms was done using FlowJo, LLC Software.

Fly-FUCCI quantification

Images were taken using a Zeiss LSM780 confocal microscope with a 40X oil immersion objective. Apical and basal sections of the wing discs were imaged under identical confocal settings. A region of interest was delimited within the asynchronously dividing cells of the wing pouch area, carefully avoiding the zone of non-proliferating cells (ZNC). Red, green or red and green cells were manually quantified. All the different genetic conditions were grown in parallel with the proper control and subjected to the same experimental conditions. At least 10 wing discs per genotype were scored.

Hydroxyurea treatment

Flies were allowed to lay eggs on fly food for 24 h at 25°C. Larvae were maintained at 25°C for 24h and switched to 29ºC for 4 days. Hydroxyurea (24,33 mg/mL final concentration) was added to the fly food vials after 2 days at 29ºC and vials were kept at 29ºC for 2 more days before dissection. 200µL of HU (Sigma, Catalog #H8627-56) (0.0486 g/mL) was added to vials at a final concentration of 24.33 mg/mL

Comet assay

The Comet Assay Kit (Trevigen, Catalog #4250-050K) was performed as described in Rimkus and Wassarman, 2018 to detect Double Strand Breaks (DSBs) in DNA at a single cell level. Cells embedded in agarose on a microscope slide are lysed to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis results in structures resembling comets because loops containing a break lose their supercoiling conformation and become free to extend toward the anode. Then, the intensity of the comet tail relative to the head reflects the number of DNA breaks (Collins, 2004). Briefly, 6 wing imaginal discs were dissected and place in 1,5 mL Eppendorf tube with 100 µL of cold PBS 1X. Samples were

homogenized with plastic pestles for 5 sec, and 10 µL of homogenate was added to 100 µL of LMA-agarose that had been boiled and cooled to 37ºC in a heat block. Comets were imaged using an ECLIPSE E800 + OLYMPUS DP72 upright microscope with a 10X objective. For each sample, >200 comets were imaged and analysed using CaspLab Software. Damage was quantified as the comet tail moment, which is defined as the product of the tail length and the fraction of total DNA in the tail (Tail moment=tail length x % of DNA in the tail). Unpaired equal-variance two-tail t-test was performed using GraphPad Prism 7 Project to compare comet tail moment between pairs of samples.

Statistical Analysis

Statistical analysis was generally performed by a two tailed unpaired t-test assuming equal variances, carried out in Microsoft Excel. Differences were considered significant when p values were less than 0.001 (***), 0.01 (**), or 0.05 (*). All genotypes included in each histogram were analysed in parallel. All data points were graphed in Prism 7.0 (Graphpad) statistical software.

Expression array

The data showed in Table 1 was obtained by the analysis of the microarray previously performed in the lab. All the protocols and procedures followed to perform the microarray can be checked in Clemente-Ruiz et al., 2016. Selected up-regulated genes in delaminating versus non-delaminated cells were selected based on their secreted nature. Each column contains the following information: gene name, function and fold change. Microarray datasets were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE71242.

Bibliography

Agarwal, M. L., Agarwal, A., Taylor, W. R., & Stark, G. R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts (p21/WAF1/Li-Fraumeni cells/tetracycline/mimosine/cyclin-cyclin-dependent kinase). *Cell Biology*, *92*(August), 8493–8497. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC41183/pdf/pnas01496-0413.pdf

Assenmacher, N., & Hopfner, K. P. (2004). MRE11/RAD50/NBS1: Complex activities. *Chromosoma*, *113*(4), 157–166. https://doi.org/10.1007/s00412-004-0306-4

Avissar, Y., Choi, J., DeSaix, J., Jurukovski, V., Rye, C., & Wise, R. L. (2013). *Biology*.

Bannykh, S. I., Rowe, T., & Balch, W. E. (1996). The organization of endoplasmic reticulum export complexes. *Journal of Cell Biology*, *135*(1), 19–35. https://doi.org/10.1083/jcb.135.1.19

Barr, A. R., Cooper, S., Heldt, F. S., Butera, F., Stoy, H., Mansfeld, J., … Bakal, C. (2017). DNA damage during S-phase mediates the proliferation-quiescence decision in the subsequent G1 via p21 expression. *Nature Communications*, *8*. https://doi.org/10.1038/ncomms14728

Benhra, N., Barrio, L., Muzzopappa, M., & Milán, M. (2018). Chromosomal Instability Induces Cellular Invasion in Epithelial Tissues. *Developmental Cell*, 161–174. https://doi.org/10.1016/j.devcel.2018.08.021

Boveri, T. (1914). Theodor Boveri - - the origin of malignant tumours. *Trends in Cell Biology*, *5*(October), 384–387.

Bowman, S. K., Neumüller, R. A., Novatchkova, M., Du, Q., & Knoblich, J. A. (2006). The Drosophila NuMA Homolog Mud Regulates Spindle Orientation in Asymmetric Cell Division. *Developmental Cell*, *10*(6), 731–742. https://doi.org/10.1016/j.devcel.2006.05.005

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Cambridge, England)*, *118*(2), 401–415. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8223268

Byun, H. J., Hong, I. K., Kim, E., Jin, Y. J., Jeoung, D. Il, Hahn, J. H., … Lee, H. (2006). A splice variant of CD99 increases motility and MMP-9 expression of human breast cancer cells through the AKT-, ERK-, and JNK-dependent AP-1 activation signaling pathways. *Journal of Biological Chemistry*, *281*(46), 34833–34847. https://doi.org/10.1074/jbc.M605483200

Campisi, J., Kapahi, P., Lithgow, G. J., Melov, S., Newman, J. C., & Verdin, E. (2019). From discoveries in ageing research to therapeutics for healthy ageing. *Nature*, *571*(7764), 183–192. https://doi.org/10.1038/s41586-019-1365-2

Castellanos, E., Dominguez, P., & Gonzalez, C. (2008). Centrosome Dysfunction in Drosophila Neural Stem Cells Causes Tumors that Are Not Due to Genome Instability. *Current Biology*, *18*(16), 1209–1214. https://doi.org/10.1016/j.cub.2008.07.029

Cheung, L. W. T., Leung, P. C. K., & Wong, A. S. T. (2006). Gonadotropin-releasing hormone promotes ovarian cancer cell invasiveness through c-Jun NH2-terminal kinase-mediated activation of matrix

metalloproteinase (MMP)-2 and MMP-9. *Cancer Research*, *66*(22), 10902–10910. https://doi.org/10.1158/0008-5472.CAN-06-2217

Chien, Y., Scuoppo, C., Wang, X., Fang, X., Balgley, B., Bolden, J. E., … Lowe, S. W. (2011). Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemosensitivity. *Genes and Development*, *25*(20), 2125–2136. https://doi.org/10.1101/gad.17276711

Cho, S., & Hwang, E. S. (2012). Status of mTOR activity may phenotypically differentiate senescence and quiescence. *Molecules and Cells*, *33*(6), 597–604. https://doi.org/10.1007/s10059-012-0042-1

Christoph, L., Kenneth, W. K., & Bert, V. (1998). Genetic instabilities in human cancers. *Nature*, *396*(1), 643–649. https://doi.org/10.1016/0346-251X(96)81094-3

Chunduri, N. K., & Storchová, Z. (2019). The diverse consequences of aneuploidy. *Nature Cell Biology*, *21*(1), 54–62. https://doi.org/10.1038/s41556-018-0243-8

Clemente-Ruiz, M., Murillo-Maldonado, J. M., Benhra, N., Barrio, L., Pérez, L., Quiroga, G., … Milán, M. (2016). Gene Dosage Imbalance Contributes to Chromosomal Instability-Induced Tumorigenesis. *Developmental Cell*, *36*(3), 290–302. https://doi.org/10.1016/j.devcel.2016.01.008

Collins, A. R. (2004). The comet assay for DNA damage and repair. *Molecular Biotechnology*, *26*, 249–261. https://doi.org/10.1385/MB:26:3:249

Cosolo, A., Jaiswal, J., Csordás, G., Grass, I., Uhlirova, M., & Classen, A.-K. (2019). JNK-dependent cell cycle stalling in G2 promotes survival and

senescence-like phenotypes in tissue stress. *ELife*, *8*, 1–27. https://doi.org/10.7554/elife.41036

d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. *Nature Reviews. Cancer*, *8*(7), 512–522. https://doi.org/10.1038/nrc2440

Daignan-Fornier, B., & Sagot, I. (2011). Proliferation/quiescence: The controversial "aller-retour." *Cell Division*, *6*(1), 10. https://doi.org/10.1186/1747-1028-6-10

de Nooij, J. C., Graber, K. H., & Hariharan, I. K. (2000). Expression of the cyclin-dependent kinase inhibitor Dacapo is regulated by Cyclin E. *Mechanisms of Development*, *97*(1–2), 73–83. https://doi.org/10.1016/S0925-4773(00)00435-4

Dekanty, A., Barrio, L., & Milán, M. (2015). Contributions of DNA repair, cell cycle checkpoints and cell death to suppressing the DNA damage-induced tumorigenic behavior of Drosophila epithelial cells. *Oncogene*, *34*(8), 978–985. https://doi.org/10.1038/onc.2014.42

Dekanty, A., Barrio, L., Muzzopappa, M., Auer, H., & Milan, M. (2012). Aneuploidy-induced delaminating cells drive tumorigenesis in Drosophila epithelia. *Proceedings of the National Academy of Sciences*, *109*(50), 20549–20554. https://doi.org/10.1073/pnas.1206675109

Demaria, M. (2019). *Cellular senescence Exeter-Discussion*. 229.

Demaria, M., O'Leary, M. N., Chang, J., Shao, L., Liu, S., Alimirah, F., … Campisi, J. (2017). Cellular senescence promotes adverse effects of chemotherapy and cancer relapse. *Cancer Discovery*, *7*(2), 165–176. https://doi.org/10.1158/2159-8290.CD-16-0241

Dephoure, N., Hwang, S., O'Sullivan, C., Dodgson, S. E., Gygi, S. P., Amon, A., & Torres, E. M. (2014). Quantitative proteomic analysis reveals posttranslational responses to aneuploidy in yeast. *ELife*, *3*, 1–27. https://doi.org/10.7554/elife.03023

Dodgson, S. E., Kim, S., Costanzo, M., Baryshnikova, A., Morse, D. L., Kaiser, C. A., … Amon, A. (2016). Chromosome-specific and global effects of aneuploidy in Saccharomyces cerevisiae. *Genetics*, *202*(4), 1395–1409. https://doi.org/10.1534/genetics.115.185660

Donzelli, M., & Draetta, G. F. (2003). Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Reports*, *4*(7), 671–677. https://doi.org/10.1038/sj.embor.embor887

Dörr, J. R., Yu, Y., Milanovic, M., Beuster, G., Zasada, C., Däbritz, J. H. M., … Schmitt, C. A. (2013). Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. *Nature*, *501*(7467), 421–425. https://doi.org/10.1038/nature12437

Druelle, C., Drullion, C., Deslé, J., Martin, N., Saas, L., Cormenier, J., … Pluquet, O. (2016). ATF6& regulates morphological changes associated with senescence in human fibroblasts. *Oncotarget*, *7*(42). https://doi.org/10.18632/oncotarget.11505

Duijf, P. H. G., Schultz, N., & Benezra, R. (2013). Cancer cells preferentially lose small chromosomes. *International Journal of Cancer*, *132*(10), 2316–2326. https://doi.org/10.1002/ijc.27924

Ekholm-Reed, S., Méndez, J., Tedesco, D., Zetterberg, A., Stillman, B., & Reed, S. I. (2004a). Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *Journal of Cell Biology*, *165*(6), 789–800. https://doi.org/10.1083/jcb.200404092

Ekholm-Reed, S., Méndez, J., Tedesco, D., Zetterberg, A., Stillman, B., & Reed, S. I. (2004b). Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *Journal of Cell Biology*, *165*(6), 789–800. https://doi.org/10.1083/jcb.200404092

Elledge, S., & Zou, L. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*, *300*(June), 1542–1548.

Enserink, J. M., & Kolodner, R. D. (2010). An overview of Cdk1-controlled targets and processes. *Cell Division*, *5*, 11. https://doi.org/10.1186/1747-1028-5-11

Epstein, C. J. (1985). *Mouse monosomies and trisomies as experimental mammalian aneuploidy*. (May).

Farrell, P. H. O., Edgar, B. A., Lakich, D., Lehner, C. F., Farrell, P. H. O., Edgar, B. A., … Lehner, C. F. (2017). *Directing Cell Division During Development*. *246*(4930), 635–640.

Follette, P. J., & O'Farrell, P. H. (1997). Cdks and the Drosophila cell cycle. *Current Opinion in Genetics and Development*, *7*(1), 17–22. https://doi.org/10.1016/S0959-437X(97)80104-9

Gerlach, S. U., Eichenlaub, T., & Herranz, H. (2018). Yorkie and JNK Control Tumorigenesis in Drosophila Cells with Cytokinesis Failure. *Cell Reports*, *23*(5), 1491–1503. https://doi.org/10.1016/j.celrep.2018.04.006

Giam, M., & Rancati, G. (2015). Aneuploidy and chromosomal instability in cancer: A jackpot to chaos. *Cell Division*, *10*(1), 1–12. https://doi.org/10.1186/s13008-015-0009-7

Gire, V., Baus, F., Fisher, D., & Piette, J. (2001). *Permanent cell cycle exit in*

G2 phase after DNA damage in normal human fibroblasts. *22*(15).

Gire, V., & Dulic, V. (2015a). Senescence from G2 arrest, revisited. *Cell Cycle*. https://doi.org/10.1080/15384101.2014.1000134

Gire, V., & Dulic, V. (2015b, February 1). Senescence from G2 arrest, revisited. *Cell Cycle*, Vol. 14, pp. 297–304. https://doi.org/10.1080/15384101.2014.1000134

Greenberg et al., N. (2014). Causes and Consequences of Stress. *Integrative and Comparative Biology*, *42*(3), 508–516. https://doi.org/10.1093/icb/42.3.508

Griffin, R., Binari, R., & Perrimon, N. (2014). *Genetic odyssey to generate marked clones in Drosophila mosaics*. *111*(13). https://doi.org/10.1073/pnas.1403218111

Guerra, F., & Bucci, C. (2016). Multiple Roles of the Small GTPase Rab7. *Cells*, *5*(3), 34. https://doi.org/10.3390/cells5030034

Halazonetis, T. D., Gorgoulis, V. G., & Bartek, J. (2008). An Oncogene-Induced DNA Damage Model for Cancer Development. *Science*, *319*(March), 1352–1355. https://doi.org/10.1126/science.1140735

Hanahan and Weinberg. (2000). The Hallmarks of Cancer. *Netherlands Heart Journal*, *100*(9), 399. https://doi.org/10.1007/BF03091804

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hassold T, & Hunt P. (2001). To err (meiotically) is human: the genesis of human aneuploidy. *Nature Reviews. Genetics*, *2*(4), 280–291.

Hay, B. A., Wolff, T., & Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development (Cambridge, England)*, *120*(8), 2121–2129. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7925015

He, Q., Au, B., Kulkarni, M., Shen, Y., Lim, K. J., Maimaiti, J., … Crasta, K. C. (2018). Chromosomal instability-induced senescence potentiates cell non-autonomous tumourigenic effects. *Oncogenesis*, *7*(8). https://doi.org/10.1038/s41389-018-0072-4

Heinrichsdorff, J., Luedde, T., Perdiguero, E., Nebreda, A. R., & Pasparakis, M. (2008). p38α MAPK inhibits JNK activation and collaborates with IκB kinase 2 to prevent endotoxin-induced liver failure. *EMBO Reports*, *9*(10), 1048–1054. https://doi.org/10.1038/embor.2008.149

Hernandez-Segura, A., de Jong, T. V., Melov, S., Guryev, V., Campisi, J., & Demaria, M. (2017). Unmasking Transcriptional Heterogeneity in Senescent Cells. *Current Biology*, *27*(17), 2652-2660.e4. https://doi.org/10.1016/j.cub.2017.07.033

Hernandez-Segura, A., Nehme, J., & Demaria, M. (2018). Hallmarks of Cellular Senescence. *Trends in Cell Biology*, *28*(6), 436–453. https://doi.org/10.1016/j.tcb.2018.02.001

Herranz, N., Gil, J., Herranz, N., & Gil, J. (2018). Mechanisms and functions of cellular senescence Find the latest version : Mechanisms and functions of cellular senescence. *JCI The Jornal of Clinical Investigation*, *128*(4), 1238–1246. https://doi.org/10.1172/JCI95148

Hills, S. A., & Diffley, J. F. X. (2014). DNA Replication and Oncogene-Induced Replicative Stress. *Current Biology*, *24*(10), R435–R444. https://doi.org/10.1016/J.CUB.2014.04.012

Hochegger, H., A, T., & Hunt, T. (2008). PersPectives Cyclin-dependent kinases and cell-cycle transitions : does one fit all ? *Nature*, *9*(NOvEmBER), 911–916. https://doi.org/10.1038/nrm2510

Hua, B. L., & Orr-Weaver, T. L. (2017). DNA replication control during drosophila development: Insights into the onset of S phase, replication initiation, and fork progression. *Genetics*. https://doi.org/10.1534/genetics.115.186627

Janssen, A., Van Der Burg, M., Szuhai, K., Kops, G. J. P. L., Medema, R. H., Burg, M. Van Der, … Kops, G. J. P. L. (2015). Chromosome segregation errors as a cause of DNA damage and structural chromosome. *Science*, *333*(SEPTEMBER 2011), 1895–1898. https://doi.org/10.1126/science.1210214

Jennings, B. H. (2011). Drosophila-a versatile model in biology & medicine. *Materials Today*, *14*(5), 190–195. https://doi.org/10.1016/S1369-7021(11)70113-4

Jonas, K., Liu, J., Chien, P., & Laub, M. T. (2013). Proteotoxic stress induces a cell-cycle arrest by stimulating lon to degrade the replication initiator DnaA. *Cell*, *154*(3), 623–636. https://doi.org/10.1016/j.cell.2013.06.034

Kaelin, W. G. (2005). The concept of synthetic lethality in the context of anticancer therapy. *Nature Reviews Cancer*, *5*(9), 689–698. https://doi.org/10.1038/nrc1691

Karess, R. E., & Glover, D. M. (n.d.). rough deal: A Gene Required for Proper Mitotic Segregation in Drosophila. *1989*, *109*(6), 2951–2961.

Knoblich, J A, & Lehner, C. F. (1993). Synergistic action of Drosophila cyclins A and B during the G2-M transition. *Embo J*, *12*(1), 65–74.

Knoblich, Jtirgen A., Sauer, K., Helena Richardson, Saint, R., & Lehner, and C. F. (1994). Cyclin E Controls S Phase Progression and Its Down-Regulation during Drosophila Embtyogenesis Is Required for the Arrest of Cell Proliferation. *Journal of Affective Disorders*, *85*(1–2), 77–83. https://doi.org/10.1016/S0165-0327(03)00101-0

Koç, A., Wheeler, L. J., Mathews, C. K., & Merrill, G. F. (2004). Hydroxyurea Arrests DNA Replication by a Mechanism that Preserves Basal dNTP Pools. *Journal of Biological Chemistry*, *279*(1), 223–230. https://doi.org/10.1074/jbc.M303952200

Konstantinov, I. E., Ye, X. T., & Fricke, T. A. (2017). From cellular senescence to regeneration: A quest for the holy grail for the next generation of surgeons? *Journal of Thoracic and Cardiovascular Surgery*, *154*(3), 953–954. https://doi.org/10.1016/j.jtcvs.2017.05.036

Kuilman, T., & Peeper, D. S. (2009). Senescence-messaging secretome: SMS-ing cellular stress. *Nature Reviews Cancer*, *9*(2), 81–94. https://doi.org/10.1038/nrc2560

Lane, M. E., Sauer, K., Wallace, K., Jan, Y. N., Lehner, C. F., & Vaessin, H. (1996). Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. *Cell*, *87*(7), 1225–1235. https://doi.org/10.1016/S0092-8674(00)81818-8

Lecona, E., & Fernandez-Capetillo, O. (2018). Targeting ATR in cancer. *Nature Reviews Cancer*, *18*(9), 586–595. https://doi.org/10.1038/s41568-018-0034-3

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J., & Hoffmann, J. A. (1996). The Dorsoventral Regulatory Gene Cassette spatzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults Bruno. *Cell*, *86*(imd), 973–983.

Li, M., Fang, X., Baker, D. J., Guo, L., Gao, X., Wei, Z., … Zhang, P. (2010). The ATM-p53 pathway suppresses aneuploidy-induced tumorigenesis. *Proceedings of the National Academy of Sciences*, *107*(32), 14188–14193. https://doi.org/10.1073/pnas.1005960107

Liu, D., Shaukat, Z., Hussain, R., Khan, M., & L. Gregory, S. (2015). Drosophila as a model for chromosomal instability. *AIMS Genetics*, *2*(1), 1–12. https://doi.org/10.3934/genet.2015.1.1

Lopes, C. S., Sampaio, P., Williams, B., Goldberg, M., & Sunkel, C. E. (2004). The Drosophila Bub3 protein is required for the mitotic checkpoint and for normal accumulation of cyclins during G2 and early stages of mitosis . *Journal of Cell Science*, *118*(1), 187–198. https://doi.org/10.1242/jcs.01602

Madigan, J. P., Chotkowski, H. L., & Glaser, R. L. (2002). DNA double-strand break-induced phosphorylation of Drosophila histone variant H2Av helps prevent radiation-induced apoptosis. *Nucleic Acids Research*, *30*(17), 3698–3705. https://doi.org/10.1093/nar/gkf496

Mailloux, R. J., & Harper, M. E. (2011). Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radical Biology and Medicine*, *51*(6), 1106–1115. https://doi.org/10.1016/j.freeradbiomed.2011.06.022

McLean, J. R., Chaix, D., Ohi, M. D., & Gould, K. L. (2016). *State of the APC/C: Organization, function, and structure*. *25*(3), 289–313. https://doi.org/10.1007/s11065-015-9294-9.Functional

Milan, M. (1998). Cell cycle control in the Drosophila wing. *Bioessays*, *20*(12), 969-71. https://doi.org/10.1002/(SICI)1521-1878(199812)20:12<969::AID-BIES1>3.0.CO;2-L

Moberg, K. H., Bell, D. W., Wahrer, D. C. R., Haber, D. A., Hariharan, I. K., Haber, D., … Sears, R. (2001). Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines. *Nature*, *413*(September), 311–316. https://doi.org/10.1038/35095068

Muñoz-Espín, D., Cañamero, M., Maraver, A., Gómez-López, G., Contreras, J., Murillo-Cuesta, S., … Serrano, M. (2013). Programmed cell senescence during mammalian embryonic development. *Cell*, *155*(5), 1104. https://doi.org/10.1016/j.cell.2013.10.019

Muñoz-Espín, D., & Serrano, M. (2014). Cellular senescence: From physiology to pathology. *Nature Reviews Molecular Cell Biology*, *15*(7), 482–496. https://doi.org/10.1038/nrm3823

Muzzopappa, M., Murcia, L., & Milán, M. (2017). Feedback amplification loop drives malignant growth in epithelial tissues. *Proceedings of the National Academy of Sciences*, *114*(35), E7291–E7300. https://doi.org/10.1073/pnas.1701791114

Nakajima, Y. I., Meyer, E. J., Kroesen, A., McKinney, S. A., & Gibson, M. C. (2013). Epithelial junctions maintain tissue architecture by directing planar spindle orientation. *Nature*, *500*(7462), 359–362. https://doi.org/10.1038/nature12335

Nakamura, M., Ohsawa, S., & Igaki, T. (2014). Mitochondrial defects trigger proliferation of neighbouring cells via a senescence-associated secretory phenotype in Drosophila. *Nature Communications*. https://doi.org/10.1038/ncomms6264

Nardella, C., Clohessy, J. G., Alimonti, A., & Pandolfi, P. P. (2011). Pro-senescence therapy for cancer treatment. *Nature Reviews Cancer*, *11*(7), 503–511. https://doi.org/10.1038/nrc3057

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010, March). Genomic instability an evolving hallmark of cancer. *Nature Reviews Molecular Cell Biology*, Vol. 11, pp. 220–228. https://doi.org/10.1038/nrm2858

Niisslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Geochim. Cosmochim. Acta (in the Press); Meteoritics*, *287*(2), 787–790. Retrieved from https://www.nature.com/articles/287795a0.pdf

Nowell, P. C. (1976). The Clonal Evolution of Tumor Cell Populations. *Science*, *194*(4260), 23–28. https://doi.org/10.1098/rstb.2004.1522

Passerini, V., Ozeri-Galai, E., De Pagter, M. S., Donnelly, N., Schmalbrock, S., Kloosterman, W. P., … Storchová, Z. (2016). The presence of extra chromosomes leads to genomic instability. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms10754

Pinal, N., Martín, M., Medina, I., & Morata, G. (2018). Short-term activation of the Jun N-terminal kinase pathway in apoptosis-deficient cells of Drosophila induces tumorigenesis. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-04000-6

Qiao, X., Zhang, L., Gamper, A. M., Fujita, T., & Wan, Y. (2010). APC/C-Cdh1: From cell cycle to cellular differentiation and genomic integrity. *Cell Cycle*, *9*(19), 3904–3912. https://doi.org/10.4161/cc.9.19.13585

Quinn, L. M., Herr, A., McGarry, T. J., & Richardson, H. (2001). The Drosophila Geminin homolog: Roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. *Genes and Development*. https://doi.org/10.1101/gad.916201

Riedel, F., Gillingham, A. K., Rosa-Ferreira, C., Galindo, A., & Munro, S.

(2016). An antibody toolkit for the study of membrane traffic in Drosophila melanogaster . *Biology Open*, *5*(7), 987–992. https://doi.org/10.1242/bio.018937

Ryoo, H. D., Gorenc, T., & Steller, H. (2004). *Apoptotic Cells Can Induce Compensatory Cell Proliferation through the JNK and the Wingless Signaling Pathways*. *7*, 491–501. Retrieved from http://www.developmentalcell.com/cgi/

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., … Ichijo, H. (1998). *Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1*. *17*(9), 2596–2606.

Salama, R., Sadaie, M., Hoare, M., & Narita, M. (2014). *Cellular senescence and its effector programs*. 99–114. https://doi.org/10.1101/gad.235184.113.gular

Salle, J., Campbell, S. D., Gho, M., & Audibert, A. (2012). CycA is involved in the control of endoreplication dynamics in the Drosophila bristle lineage. *Development*, *139*(3), 547–557. https://doi.org/10.1242/dev.069823

Santabárbara-Ruiz, P., López-Santillán, M., Martínez-Rodríguez, I., Binagui-Casas, A., Pérez, L., Milán, M., … Serras, F. (2015). ROS-Induced JNK and p38 Signaling Is Required for Unpaired Cytokine Activation during Drosophila Regeneration. *PLoS Genetics*, *11*(10), 1–26. https://doi.org/10.1371/journal.pgen.1005595

Santaguida, S., & Amon, A. (2015). Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nature Reviews Molecular Cell Biology*, *16*(8), 473–485. https://doi.org/10.1038/nrm4025

Santaguida, S., Richardson, A., Iyer, D. R., M'Saad, O., Zasadil, L., Knouse, K. A., … Amon, A. (2017). Chromosome Mis-segregation Generates Cell-Cycle-Arrested Cells with Complex Karyotypes that Are Eliminated by the Immune System. *Developmental Cell*, *41*(6), 638-651.e5. https://doi.org/10.1016/j.devcel.2017.05.022

Santaguida, S., Vasile, E., White, E., & Amon, A. (2015). Aneuploidy-induced cellular stresses limit autophagic degradation. *Genes and Development*, *29*(19), 2010–2021. https://doi.org/10.1101/gad.269118.115

Seher, T. C., & Leptin, M. (2000). Tribbles, a cell-cycle brake that coordinates proliferation and morphogenesis during Drosophila gastrulation. *Current Biology*, *10*(11), 623–629. https://doi.org/10.1016/S0960-9822(00)00502-9

Sigrist, S. J., & Lehner, C. F. (1997). Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell*. https://doi.org/10.1016/S0092-8674(00)80528-0

Song, Y.-H. (2005). Drosophila melanogaster: a model for the study of DNA damage checkpoint response. *Molecules and Cells*. https://doi.org/828 [pii]

Soto-gamez, A., & Demaria, M. (2017). Therapeutic interventions for aging : the case of cellular senescence. *Drug Discovery Today*, *22*(5), 786–795. https://doi.org/10.1016/j.drudis.2017.01.004

Spencer, S. L., Cappell, S. D., Tsai, F. C., Overton, K. W., Wang, C. L., & Meyer, T. (2013). XThe proliferation-quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit. *Cell*, *155*(2), 369. https://doi.org/10.1016/j.cell.2013.08.062

Stenberg, P., & Larsson, J. (2011). Buffering and the evolution of chromosome-wide gene regulation. *Chromosoma*, *120*(3), 213–225. https://doi.org/10.1007/s00412-011-0319-8

Sykiotis, G. P., & Bohmann, D. (1982). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Journal of Experimental and Clinical Cancer Research*, *1*(1), 89–91. https://doi.org/10.1016/j.devcel.2007.12.002.Keap1/Nrf2

Tai, H., Wang, Z., Gong, H., Han, X., Zhou, J., Wang, X., … Xiao, H. (2017). Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence. *Autophagy*, *13*(1), 99–113. https://doi.org/10.1080/15548627.2016.1247143

Thompson, B. J. (2010). Developmental control of cell growth and division in Drosophila. *Current Opinion in Cell Biology*, *22*(6), 788–794. https://doi.org/10.1016/j.ceb.2010.08.018

Thornton, B. R., & Toczyski, D. P. (2003). Securin and B-cyclin/CDK are the only essential targets of the APC. *Nature Cell Biology*, *5*(12), 1090–1094. https://doi.org/10.1038/ncb1066

Thornton, T. M., & Rincon, M. (2009). *Non-Classical P38 Map Kinase Functions : Cell Cycle Checkpoints and Sur- vival*. *5*(1).

Toettchera, J. E., Loewer, A., Ostheimer, G. J., Yaffe, M. B., & Tidor, B. (2012). Distinct mechanisms act in concert to mediate cell cycle arrest. *Journal of Cell Science*, *125*(24), 5974–5983. https://doi.org/10.1242/jcs.108886

Toledo, L. I., Murga, M., Zur, R., Soria, R., Rodriguez, A., Martinez, S., … Fernandez-Capetillo, O. (2011). A cell-based screen identifies ATR

inhibitors with synthetic lethal properties for cancer-associated mutations. *Nature Structural and Molecular Biology*, *18*(6), 721–727. https://doi.org/10.1038/nsmb.2076

Uhlirova, M., & Bohmann, D. (2006). JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. *EMBO Journal*, *25*(22), 5294–5304. https://doi.org/10.1038/sj.emboj.7601401

Wang, A. S., Ong, P. F., Chojnowski, A., Clavel, C., & Dreesen, O. (2017). Loss of lamin B1 is a biomarker to quantify cellular senescence in photoaged skin. *Scientific Reports*, *7*(1), 1–8. https://doi.org/10.1038/s41598-017-15901-9

Wiebusch, L., & Hagemeier, C. (2010). P53-and p21-dependent premature APC/C-Cdh1 activation in G2 is part of the long-term response to genotoxic stress. *Oncogene*, *29*(24), 3477–3489. https://doi.org/10.1038/onc.2010.99

Wong, H. W.-S., Shaukat, Z., Wang, J., Saint, R., & Gregory, S. L. (2014). JNK signaling is needed to tolerate chromosomal instability. *Cell Cycle (Georgetown, Tex.)*. https://doi.org/10.4161/cc.27484

Yagi, R., Mayer, F., & Basler, K. (2010). *Refined LexA transactivators and their use in combination with the Drosophila Gal4 system*. *2010*. https://doi.org/10.1073/pnas.1005957107/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1005957107

Ye, C. J., Abdallah, B. Y., Stevens, J. B., Heng, H. H., Bremer, S. W., Ye, K. J., … Horne, S. D. (2013). Chromosomal instability (CIN): what it is and why it is crucial to cancer evolution. *Cancer and Metastasis Reviews*, *32*(3–4), 325–340. https://doi.org/10.1007/s10555-013-9427-7

Yoder, F. E., Vincent, R. A., Morgan, S. K., & Grush, O. C. (1985). Chromosome fragile sites. *Cancer Genetics and Cytogenetics*, *14*(3–4), 369. https://doi.org/10.1016/0165-4608(85)90202-X

Young, A. R. J., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J. F. J., … Watt, F. M. (2009). Autophagy mediates the mitotic senescence transition. *Genes & Development*, 798–803. https://doi.org/10.1101/gad.519709

Yun, M. H., Davaapil, H., & Brockes, J. P. (2015). Recurrent turnover of senescent cells during regeneration of a complex structure. *ELife*, *4*, 1–16. https://doi.org/10.7554/elife.05505

Zhu, J., Tsai, H. J., Gordon, M. R., & Li, R. (2018). Cellular Stress Associated with Aneuploidy. *Developmental Cell*, *44*(4), 420–431. https://doi.org/10.1016/j.devcel.2018.02.002

Zhu, Y., Tchkonia, T., Pirtskhalava, T., Gower, A. C., Ding, H., Giorgadze, N., … Kirkland, J. L. (2015). *The Achilles' heel of senescent cells: from transcriptome to senolytic drugs*. (March), 644–658. https://doi.org/10.1111/acel.12344

Zielke, N, Korzelius, J., van Straaten, M., Bender, K., Schuhknecht, G. F., Dutta, D., … Edgar, B. A. (2014). Fly-FUCCI: A versatile tool for studying cell proliferation in complex tissues. *Cell Rep*, *7*(2), 588–598. https://doi.org/10.1016/j.celrep.2014.03.020

Zielke, Norman, Kim, K. J., Tran, V., Shibutani, S. T., Bravo, M. J., Nagarajan, S., … Edgar, B. A. (2011). Control of Drosophila endocycles by E2F and CRL4 CDT2. *Nature*, *480*(7375), 123–127. https://doi.org/10.1038/nature10579

Zielke, Norman, Korzelius, J., vanStraaten, M., Bender, K., Schuhknecht, G.

F. P., Dutta, D., … Edgar, B. A. (2014). Fly-FUCCI: A Versatile Tool for Studying Cell Proliferation in Complex Tissues. *Cell Reports*, *7*(2), 588–598. https://doi.org/10.1016/j.celrep.2014.03.020

Abbreviations

APC/C: Anaphase-promoting complex

APS: Aneuploidy-associated protein signature

ATM: Ataxia telangiectasia-mutated

ATR: Ataxia telangiectasia and Rad3 related

ASK: Apoptosis signal-regulating kinase

CDC: Cell division cycle

CDK: Cyclin-dependent kinase

CIN: Chromosomal instability

CKI: Cyclin-dependent kinase inhibitors

CyC: Cyclin

Dap: Dacapo

DDR: DNA damage Response

dNTP: Deoxynucleoside triphosphate

DSBs: Double Strand Breaks

ECM: Extracellular matrix

EdU: 5-ethynyl-2′-deoxyuridine

ER: Endoplasmic Reticulum

FACs: Fluorescence-Activated Cell Sorting

FLP: Recombinase Flippase

FRT: Flippase recognition target

FZR: Fizzy-related

IR: Ionizing radiation

JNK: c-Jun N-terminal kinase

MCM: Minichromosome maintenance

MMP1: Matrix metalloproteinase – 1

MSLc: Male-Specific Lethal complex

MyrT: Myristoylated-Tomato

ORC: Origin Replication Complex

PCD: Programmed cell death

pH2AV: Phosphorylated histone H2Av

Pre-RC: Pre-replication complex

RAS: Rat sarcoma

RBF: Retinoblastoma family protein

ROS: Reactive Oxygen Species

RPA: Replication protein A

RS: Replicative stress

SAC: Spindle Assembly Checkpoint

SASP: Senescence associated secretory phenotype

SA-β- gal: Senescence associated beta galactosidase

SCRB: Scribble

ssDNA: Single stranded DNA

Stg: String

TAK: Transforming growth factor- β activated kinase

Trbl: Tribbles

UPR: Unfolded Protein Response

Wg: Wingless

β-GAL: Beta-galactosidase

Figure Index