Chromosomal Instability: Interplay between proteotoxic and metabolic stress

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“There is only one thing that makes a dream impossible to achieve: the fear of failure”

- Paulo Coelho, The Alchemist

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ABSTRACT

Chromosomal Instability (CIN) and associated aneuploidy are salient features of the majority of human solid tumors. In the *Drosophila* epithelial model of CIN, the generation of highly aneuploid karyotypes drive cell delamination and c-Jun N-terminal Kinase (JNK) dependent cell death. Aneuploidy associated generation of Reactive Oxygen Species (ROS) plays a key role in the activation of JNK. When delaminating cells are maintained in the tissue by apoptosis inhibition, aberrant karyotypes promote a cell-autonomous malignant behavior. Here we have dissected the molecular mechanisms underlying aneuploidy-induced ROS production and cell delamination. On one hand, we have shown that aneuploidy associated proteotoxic stress is being sensed and activates the major protein quality control mechanisms in a cell. On the other hand, mitochondria act as signaling centers as well as major sensors of the unbalanced proteome in CIN tissues. Aneuploidy associated proteotoxic stress leads to mitochondrial dysfunction and ROS production, which further drives cell delamination as well as cell JNK activation in CIN tissues.

RESUMEN

La inestabilidad cromosómica y la aneuploidía son características destacadas de la mayoría de los tumores sólidos en humanos. En el modelo epitelial de *Drosophila*, la generación de cariotipos altamente aneuploides promueve la delaminación y la muerte celular dependiente de c-Jun N-terminal Kinase (JNK). La producción de especies reactivas de oxígeno (ROS) juega un papel clave en la activación de JNK bajo dichas condiciones. Cuando las células delaminadas se mantienen en el tejido gracias a la inhibición de la apoptosis, los cariotipos aberrantes promueven un comportamiento maligno tumoral. En esta tesis hemos analizado los mecanismos moleculares subyacentes a la producción de ROS como consecuencia de la aneuploidía. Hemos demostrado que bajo una situación de inestablidad cromosómica se genera un estrés proteotóxico, detectado por la célula que activa los principales mecanismos de control de calidad de las proteínas. Además, dicho estrés, promueve la disfuncionalidad de las mitocondrias, favoreciendo la generación de ROS, que a su vez contribuye a la activación de JNK y a la delaminación celular al afectar el citoesqueleto de actina-miosina en los tejidos CIN.

PROLOGUE

Chromosomal Instability (CIN) - the continuous change in the number of chromosomes or parts of them - and the resulting aneuploidy - an unbalanced set of chromosomes or parts of them - are salient features of the majority of human solid tumors. While CIN promotes the gain of oncogene-carrying chromosomes and the loss of tumor-suppressor-gene-carrying chromosomes in certain cancers, its impact on the physiology of the cell and on the homeostasis of the tissue has not been well elucidated. Our lab has generated an epithelial model of CIN in *Drosophila*, where the generation of highly aneuploid karyotypes drives cell delamination and c-Jun N-terminal Kinase (JNK) dependent cell death. Whereas gene dosage imbalance contributes to aneuploidy-induced cell delamination, the production of Reactive Oxygen Species (ROS) plays a key role in the activation of JNK. When delaminating cells are maintained in the tissue by apoptosis inhibition, aberrant karyotypes promote a cell-autonomous malignant behavior and the entry into a senescence-like state. Cross-feeding interactions between senescent cells and dividing epithelial cells maintain the unlimited growth potential of CIN-induced tumors. Here we have dissected the molecular mechanisms underlying aneuploidy-induced ROS production and cell delamination. We found that proteotoxic stress as a consequence of a general imbalance in the proteome of highly aneuploid cells is being sensed and activates Unfolded Protein Response (UPR) in CIN tissues. Major protein quality control mechanisms are activated in order to dampen the deleterious effects of CIN. The main catabolic process of the cell, autophagy, is activated by many different

regulators in CIN tissues. Although protein quality control mechanisms are activated upon CIN, mitochondrial homeostasis is severely perturbed in CIN tissues. We also identified that aneuploidy-induced cellular stresses limit mitophagy this serves as a major source of ROS in CIN tissues. Here, mitochondria act as major sensors of the unbalanced proteome as well as signaling centers to activate JNK through ROS production. The generation of ROS is a key event to promote tumorigenesis in our model. On one hand, ROS activates JNK through Ask1. On the other hand, ROS activates Src kinase to drive cell delamination by modifying cell adhesion and the actin-myosin cytoskeleton. Ameliorating proteostasis or mitochondrial homeostasis by upregulating protein quality control mechanisms dampens the deleterious effects of CIN. In general, protein quality control mechanisms are vital for the maintenance of cellular physiology and tissue homeostasis upon CIN. A deep understanding of these mechanisms will help us to find the Achilles' heel of CIN and, as a consequence, of most human epithelial tumors.

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INTRODUCTION

1. Chromosomal instability and cancer

1.1 The hallmarks of cancer

As one of the leading causes of death, cancer has a major impact on society worldwide. The Egyptians were the first to describe cancer back in 1600 BC. Later around 400 BC, Hippocrates named it *karkinos*, the Greek word for crab or crayfish. He compared the tumor to a crab, where the body represented the mass of the tumor and the legs the veins around the tumor. Then in 25 BC, Celsus translated *karkinos* to cancer, the Latin word for crab, and that's how the word for this disease came about.

Regardless of the nature and origin of the condition, cancer is a heterogeneous disease with many general well-conserved hallmarks. The main ones are self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, induction of angiogenesis, reprogramming cellular metabolism, evasion of the immune system, tissue invasion, and metastasis **(Figure 1)**. Cancer cells acquire these functional capabilities thanks to two enabling characteristics: the inflammatory state, which is determined by the lesions caused by the sustained activity of the immune system; and genomic instability, which generates random mutations, including chromosomal rearrangements. Genomic instability is the prominent characteristic of the two and it can orchestrate other hallmarks in a cancer cell (Hanahan and Weinberg, 2000, 2011).

Introduction

Genomic instability is an evolving general feature of cancer. Mutations in DNA repair genes drive tumor growth in hereditary cancers, whereas mutations in caretaker genes and oncogene-induced DNA replication stress drive tumor growth in sporadic cancers. The main forms of genomic instability are chromosomal instability (CIN), microsatellite instability, and types of genomic instability that are mapped by elevated rates of base-pair mutations. CIN, which refers to the high frequency at which chromosome structure and number change over time, is the most prominent form of instability (Negrini et al., 2010).

Just over a century ago, David von Hansemann reported the presence of aberrant mitotic figures in carcinoma samples (Hansemann, 1890). Later, Theodor Boveri proposed a causal relationship between cancer and the presence of abnormal karyotypes. In this regard, he hypothesized that chromosomes carrying oncogenes are maintained (Teilungsfoerdernde Chromosomen), while chromosomes carrying tumor suppressors are lost (Teilungshemmende Chromosomen) (Boveri, 1914). Consistent with this notion, Benezra and colleagues found that chromosomes-7, -12 and -20—carrying EGFR, BRAF, SHH, KRAS, CDK4, MDM2, BCL2L1, EZF1, and CDC25B oncogenes—were preferentially gained in human solid tumors. In such tumors, chromosome loss occurs more frequently than chromosome gain. Although the loss of small chromosomes appears to occur more frequently, there is no clear correlation between the tendency to lose a chromosome and its size. It has been speculated that the loss of smaller chromosomes during the onset of tumorigenesis is less likely to affect vital housekeeping functions,

while at the same time allowing for tumor suppressor loss (Duijf et al., 2013). Finally, CIN caused by lack of the spindle assembly checkpoint (SAC) gene Bub1 has been reported to drive tumorigenesis through loss of heterozygosity of tumor suppressor genes Rb and p53 (Li et al., 2010).

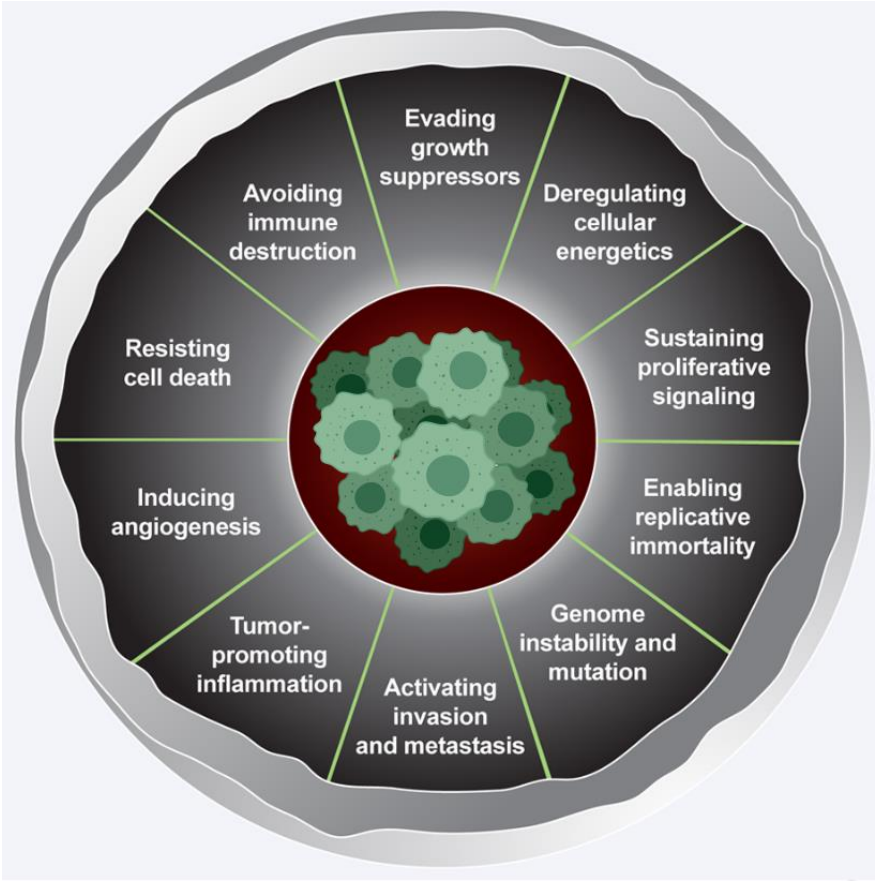

Figure 1. Hallmarks of cancer

1.2 Chromosomal instability as a hallmark of cancer

CIN, which is defined as the dynamic change in chromosome number or structure, is a salient feature of human tumors. CIN often leads to aneuploidy, one of the most widely studied signs of genomic alteration in cancer (Sen, 2000, Rajagopalan and Lengauer, 2004). Aneuploidy is the state of having a karyotype that deviates from an exact multiple of a haploid set of chromosomes. CIN and the associated aneuploidy maintain the heterogeneity of many tumors. Our understanding of cancer genomes and their evolution has been enhanced by recent sequencing studies. These have reinforced the notion that high mutation frequencies, along with evolutionary dynamics, lead to the clonal expansion of tumor cells and corresponding tumor heterogeneity (Hanahan and Weinberg, 2011, Greaves and Maley, 2012). The occurrence of aneuploidy in cancer is pervasive and diverse, with a great heterogeneity within tumors and also between tumor types. For example, 91% of glioblastomas and about 65% of lung tumors are aneuploid, while aneuploidy is rare in prostate cancer (Jaarsveld and Kops, 2016). Careful examination of 43,205 human tumors found that 68% of human solid tumors are aneuploid (Duijf et al., 2013).

CIN-associated chromosomal alterations are classified into two types, namely numerical or structural. The former is defined as a gain or loss of whole chromosomes, while the latter involves amplifications, inversions, deletions, and translocations of chromosomal regions (Burrell et al., 2013, Bayani et al., 2007). As discussed above, gain or loss of chromosomes or structural variations

creates genetically distinct cells. Thus, in the context of cancer, CIN increases intratumoral heterogeneity. Such heterogeneity might enhance a selective growth advantage to tumor cells, in terms of proliferative capacity, metastatic behavior and drug resistance. It is therefore not surprising that CIN is highly correlated with disease relapse and poor patient outcome (Lee et al., 2011, Gerlinger and Swanton, 2010, Bakhoum and Cantley, 2018, Levine and Holland, 2018, Carter et al., 2006, Canovas et al., 2018, Benhra et al., 2018). However, in some rare cases, CIN has been reported to improve diagnosis (Birkbak et al., 2011, Jamal-Hanjani et al., 2015). One possible explanation for this apparent paradox is that, depending on the rate of CIN present, tumors behave differently. In addition, it has been demonstrated that high levels of CIN are not compatible with cell viability, and cells with high CIN are removed by apoptosis. CIN may, therefore, represent a therapeutic vulnerability that can be leveraged to achieve the targeting killing of cancer cells (Heng et al., 2013, McGranahan et al., 2012, Thompson et al., 2017, Janssen et al., 2009, Bakhoum and Compton, 2012).

As stated above, CIN is defined as a dynamic change in chromosome number or structure, while its associated aneuploidy is a static state. Given this observation, single-cell analysis is the best approach through which to study CIN. In fact, traditional techniques, such as comparative genome hybridization, which is widely used to identify aneuploidy or copy number variations, would mask the CIN-induced cell heterogeneity within the sample (Bakker et al., 2016). CIN can be assessed in the following two ways: (1) monitoring chromosome numbers within a cell and its daughters in a timely manner using live-

cell imaging and chromosome markers; and (2) monitoring cell heterogeneity within a population in a quantitative fashion by means of single-cell approaches, such as cytogenetic techniques, single-cell genomics, and high-throughput imaging cytometry. The latter is widely used as it can be easily adapted to traditional endpoint analysis and can be carried out on a wide variety of samples (Geigl et al., 2008).

Understanding CIN and how this condition is translated into tumor growth is of tremendous clinical interest. As mentioned, CIN often results in aneuploidy, the state of having a chromosome number that varies from the haploid set. Importantly, aneuploidy has been observed in most malignant tumors, with a frequency of around 90% in solid tumors and 35-60% in hematopoietic cancer (Chunduri and Storchova, 2019). Therefore, the role of CIN-induced aneuploidy on tumor growth has been widely addressed during recent years.

2. Chromosomal instability-induced aneuploidy

2.1 Spindle assembly checkpoint

Gunnar Täckholm introduced the term “aneuploidy” in 1922. While conducting experiments in rose species, he observed the presence of a chromosome number that differed from that of the haploid set (Täckholm, 1922). The SAC **(Figure 2)**, a mitotic checkpoint and a surveillance mechanism, ensures that chromosomes are properly segregated, and reduces the rate of aneuploidy (Holland and Cleveland, 2009, Pfau and Amon, 2012, Musacchio and Salmon, 2007). The presence of an unbalanced karyotype is highly detrimental, causing miscarriages, mental retardation, and cancer in humans. Constitutional aneuploidy, the state of aneuploidy throughout the organism, is mostly embryonic lethal, except for trisomy 21 (Down Syndrome). Mosaic variegated aneuploidy, a somatic aneuploidy caused by a mutation in BUB1B, a gene required for accurate chromosome segregation, results in growth retardation, microcephaly and childhood cancers. Taken together, whether constitutional or somatic, aneuploidy has a dramatic effect on health. The SAC mechanism safeguards the proper segregation of chromosomes (Santaguida and Amon, 2015), and therefore the presence of aneuploid karyotypes are rare in normal tissues (Knouse et al., 2014).

SAC, an evolutionarily conserved mechanism, prevents the missegregation of chromosomes. Detection of syntelic-attachment (both kinetochores are attached to microtubules emanating from the same centrosome) or monotelic-attachment (only one kinetochore is

attached to microtubules) activates SAC and also inhibits anaphase onset. Furthermore, proper re-attachment of microtubules from both poles or amphitelic-attachment silences the SAC complex and anaphase commences.

The core components of the SAC are the checkpoint kinases Aurora B, BUB1, and Monopolar Spindle Protein 1 (MPS1), and Mitotic Arrest Deficient 1 (MAD1), MAD2, and BUB1-related1 (BUBR1). The core components are recruited upon detection of an incorrect attachment, which further initiates the inhibition of the Anaphase-Promoting Complex (APC, also known as cyclosome-CDC20). APC/C^{CDC20} is an E3 ubiquitin ligase that triggers the metaphase to anaphase transition by ubiquitinating Securing and Cyclin-B for proteasomal degradation. Upon SAC activation, APC/C is inhibited by the incorporation of CDC20, the activator of APC/C, into the mitotic checkpoint complex (MCC), which consists of MAD2, BUBR1, BUB3 and CDC20 itself. Merotelic attachment, a state in which a kinetochore is attached to microtubules that emanate from both spindle poles, is one of the main reasons for aneuploidy in mammalian cells. Aurora B and MPS1 kinases convert this kind of attachment to an amphitelic one by phosphorylating outer core components and thus destabilizing the microtubule-kinetochore interaction (Musacchio and Salmon, 2007, Santaguida and Amon, 2015).

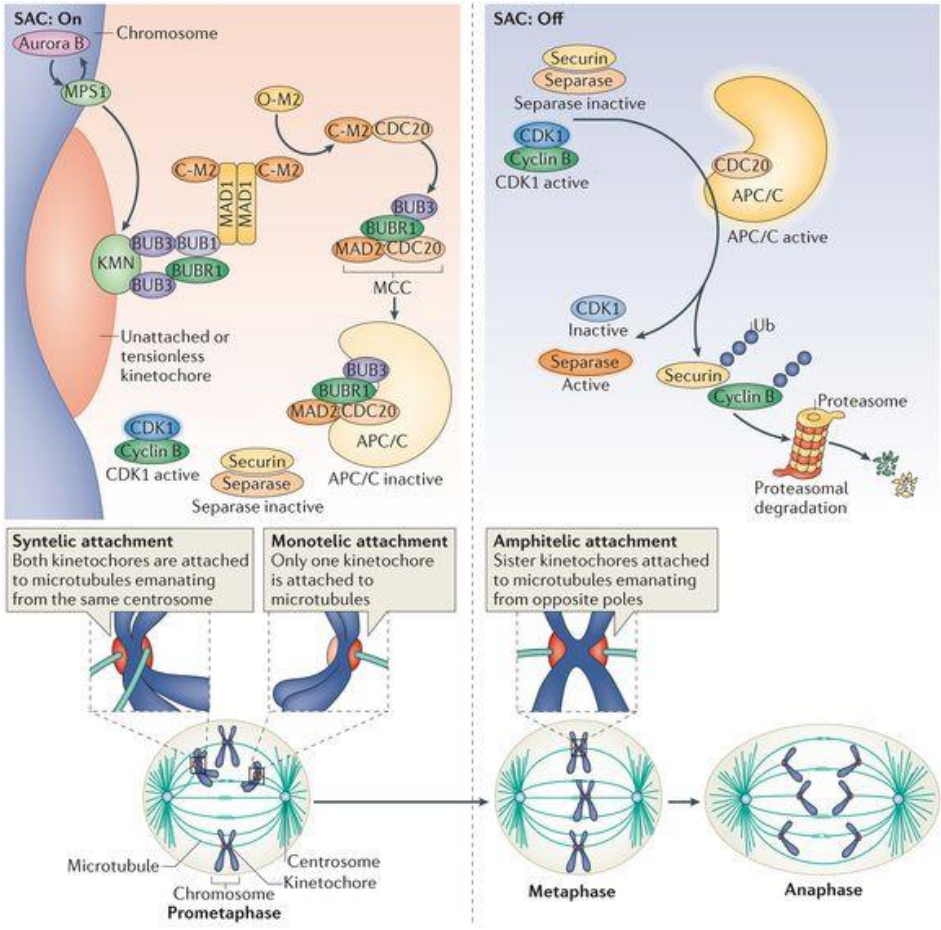

Figure 2. Spindle assembly checkpoint mechanism

(Adapted from (Santaguida and Amon, 2015))

2.2 Aneuploidy-associated cellular stress

Normal cell physiology is well-tuned through the balance of gene products. However, this balance is altered in aneuploidy (Torres et al., 2008). Reduced growth fitness is one of the consequences of aneuploidy. The poor proliferation capacity of aneuploid cells was observed almost 40 years ago, by comparing the growth potential of

normal euploid cells with respect to skin fibroblasts from Down syndrome patients (Segal and Mccoy, 1974). Later, a systemic study using yeast aneuploid strains (Torres et al., 2007), trisomic mouse embryonic fibroblasts (MEFs) (Williams et al., 2008) and human trisomic and tetrasomic cell lines (Stingele et al., 2012) showed that aneuploidy reduces growth rates. Interestingly, upon growth under diverse conditions, some aneuploid cells grow better than euploid ones (Pavelka et al., 2010). This observation could be explained by evolutionary adaptability to conditions of stress (Chen et al., 2012, Yona et al., 2012, Sunshine et al., 2015). Aneuploidy has been associated with multiple cellular stresses, such as mitotic, replication, proteotoxic and metabolic stress **(Figure 3)** (Zhu et al., 2018).

- **Mitotic Stress**

An imbalance in aneuploidy-associated gene dosage may lead to protein imbalance and the production of non-stoichiometric forms of proteins. This protein dysregulation could affect the functioning of the chromosome separation machinery and thus may cause mitotic stress. Aneuploid yeast strains were found to lose chromosomes, suggesting that aneuploidy interferes with chromosome fidelity (Campbell et al., 1981, Shelter et al., 2011, Zhu et al., 2012). In contrast, the impact of aneuploidy on mitotic fidelity is not clear in mammalian cells. The inconsistency regarding the presence of lagging chromosomes and mitotic aberrations in many studies is attributed to differences in the experimental strategies and karyotypes used (Zhu et al., 2018). A recent study followed a more general approach, namely treating RPE-1 cells with MPSI inhibitor to create

aneuploid karyotypes. This approach generated aneuploid cells with high levels of mitotic aberrations, lagging chromosomes, and micronuclei (Santaguida et al., 2017). Thus, aneuploidy leads to mitotic stress and could drive CIN.

- **Replication Stress**

Replication stress refers to the stalling of replication fork progression. DNA replication starts from special places called replication origins, which are many in number and consist of two steps: origin licensing and origin firing. Origin licensing depends on an origin recognition complex, which further recruits the helicase. Aneuploidy-associated gene dosage imbalance leads to the production of non-stoichiometric forms of proteins, and this may impair the functionality of DNA replication complexes. Consistent with this, an imbalance in the production of helicase subunits was observed in the condition of aneuploidy (Passerini et al., 2016), and the presence of increased replication fork stalling (Santaguida et al., 2017). One of the immediate consequences of aneuploidy and associated replication stress is DNA damage and P53 activation (Thompson and Compton, 2010, Janssen et al., 2011, Blank et al., 2015, Santaguida et al., 2017). In addition to replicative stress, aneuploid cells are also characterized by their ability to cause cell-cycle delays, senescence, DNA condensation defects, inappropriate mitotic entry and activation of the immune system (Lamm et al., 2016, Meena et al., 2015, Andriani et al., 2016, Santaguida et al., 2017).

Introduction

- **Proteotoxic Stress**

CIN-induced aneuploidy causes reduced organismal fitness and affects cellular physiology, and one characteristic feature of this type of aneuploidy is proteotoxic stress (Oromendia and Amon, 2014). Since subunits of protein complexes are not encoded in operons in the eukaryotic system, they have evolved in such a way to coordinate the expression of subunits of the same complex (Li et al., 2014, Taggart and Li 2018). However, a condition like aneuploidy alters the gene dosage balance, which can disrupt this coordination and culminate in the production of multi-protein complexes that lack binding partners. The excess of non-stoichiometric proteins can either lead to overburdening of the proteostasis or to protein aggregation, thus generating proteotoxic stress upon aneuploidy.

According to studies in both yeast and human aneuploid cells, most genes are expressed in proportion to their copy number, and the corresponding protein translation is determined by the abundance of mRNA present. Therefore, aneuploidy causes a dramatic alteration of cellular protein levels (Stingele et al., 2012, Pavelka et al., 2010, Torres et al., 2010), which in turn severely impairs normal cell physiology and organismal fitness (Santaguida and Amon, 2015, Zhu et al., 2018). Aneuploidy activates a unique type of stress response across the species, which resembles the environmental stress response (ESR) observed in yeast (Torres et al., 2010). Signatures similar to the ESR were observed in aneuploidy models in arabidopsis, MEFs and human cell lines (Sheltzer et al., 2011). This stress response upregulates genes involved in various functions, such

as lysosome-related pathways, the MHC complex, antigen processing, the ER, the Golgi apparatus, and metabolic pathways (Durrbaum et al., 2014).

Aneuploidy-driven proteotoxic stress overloads the protein quality control mechanisms of a cell **(Figure 4)**. Normal protein homeostasis is widely affected in aneuploid yeast strains and human cell lines, which show features like protein aggregation, misfolding, reduced chaperone activity, altered autophagy and hypersensitivity to conditions that interfere with proteasomal degradation (Torres et al., 2007, Oromendia et al., 2012, Tang et al., 2011, Stingele et al., 2012, Donnelly and Storchova , 2015, Santaguida et al., 2015). Multiple protein homeostasis mechanisms are activated to dampen proteotoxic stress upon aneuploidy. Importantly, lysosome-mediated autophagy macro machinery is also activated in aneuploid human HCT116 and RPE-1 cell lines (Stingele et al., 2012). In fact, upregulation of p62/sequestosome and LC3, the latter an autophagy marker, can be observed. In addition, another study in which HeLa and mammary gland epithelial cells underwent chromosome missegregation reported p62 upregulation and unfolded protein response (Ohashi et al., 2015). Interestingly, a study conducted in human aneuploid RPE-1 cells showed that the autophagic mechanism is activated. However, although the lysosome appears to be functional, lysosomal-mediated clearance is affected, indicating saturation of autophagy degradation (Santaguida et al., 2015). This saturation activates a lysosomal stress response, which further upregulates TFEB, a master transcription factor of lysosomal and autophagic genes. This lysosomal saturation is not an immediate effect of aneuploidy and it requires the

continuous presence of aneuploidy. The precise way in which TFEB is regulated in this situation and why the cargos that accumulate inside the lysosomes are hard to degrade is still unknown.

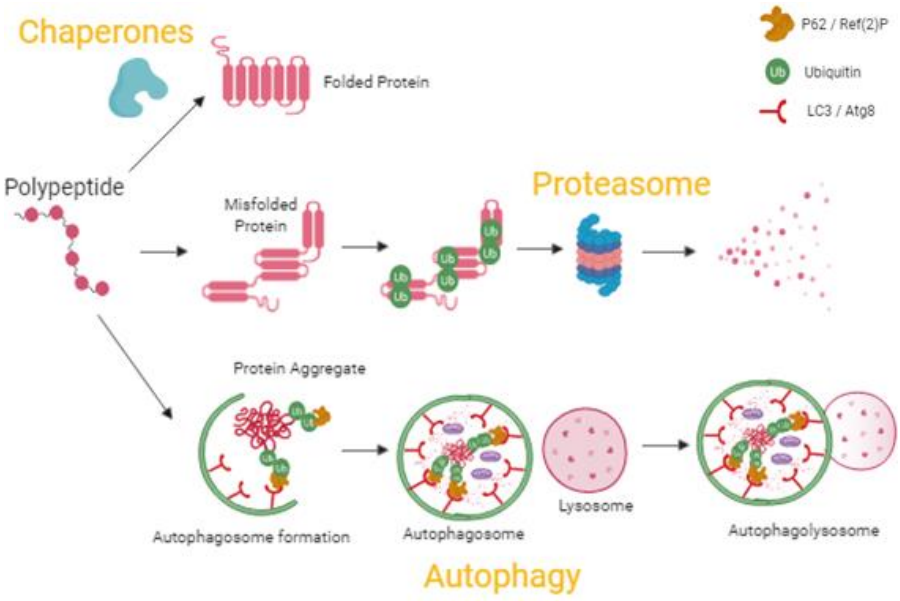

Figure 4. Protein quality control mechanisms

While aneuploidy-associated proteotoxic stress is understood, how eukaryotic cells deal with stoichiometric imbalances and the molecular basis of protein aggregate formation remain to be answered. A recent study (Brennan et al., 2019) using genomic and genetic techniques revealed that sequestration of non-stoichiometric subunits into aggregates is an alternative gene dosage mechanism, apart from the quick degradation of recognized subunits. The exceeding proteins undergo one of these processes, and this decision depends on the half-life of the protein. Analysis focusing on heteromeric complexes suggests that short half-life proteins undergo degradation and long half-life proteins are mostly sequestered.

Ribosomal components were found to be the proteins that most aggregated. This observation is consistent with recent studies suggesting that ribosomal proteins are aggregated upon proteotoxic stress (Pathak et al., 2017, Tye et al., 2019).

In conclusion, aneuploidy-induced proteotoxic stress has been widely described in many model systems, ranging from yeast to human cells. The level of proteotoxic stress present varies greatly between karyotypes. Cells obtaining a chromosome that carries genes related to chaperones or the proteasome system are more tolerant of this stress (Chen et al., 2012, Kalapis et al., 2015). Major protein quality control mechanisms are activated upon aneuploidy, and overwhelming continuous production of proteins seems to saturate these mechanisms, thereby leading to proteotoxic stress. Overexpression of heat shock factor 1 (HSF1), a master regulator of chaperones, can mitigate proteotoxic stress upon aneuploidy in human cell lines (Donnelly et al., 2014). Upregulating the protein homeostasis mechanisms in the context of aneuploidy emerges as a potential approach to tackle the proteotoxic stress associated with this condition. Aneuploid cells largely depend on these protein quality control mechanisms for survival, and the downregulation of any of these core mechanisms, like proteasome-mediated degradation, chaperones, and lysosomal-mediated autophagy, reduces the growth potential of these cells.

Introduction

- **Metabolic Stress**

Metabolic homeostasis of a cell is defined by the precise coordination of metabolic pathways that underlie the proper functioning of many regulators and enzymes. Aneuploidy-associated gene dosage imbalance might disturb this tight coordination. Studies in yeast suggest that aneuploid cells have altered nucleotide metabolism and carbohydrate metabolism, and increased glucose uptake and TCA cycle (Torres et al., 2007, Thorburn et al., 2013). Mammalian aneuploid models also show some metabolic alterations, such as increased glucose uptake (Williams et al., 2008), increased mitochondrial metabolism, and alteration of DNA metabolism (Stingele et al., 2012). Another study in yeast and human cell lines suggests that aneuploidy cells highly depend on sphingolipids for survival (Tang et al., 2017, Hwang et al., 2017).

Proteome imbalance-induced hypo-osmotic stress is a general characteristic of aneuploid cells (Tsai et al., 2019). This study reported that these cells show increased plasma membrane stress, which culminates in impaired endocytosis. Those authors also demonstrate that the intracellular nutrient homeostasis of aneuploidy cells depends on the ubiquitin-mediated endocytic recycling of nutrient transporters.

The production of Reactive oxygen species (ROS) is one of the consequences of metabolic alterations. ROS are a diverse pool of oxygen-containing compounds that have additional electrons, thus making them highly reactive. Mitochondria, the powerhouse of the cell, is the major source of ROS. In this regard, ROS are produced

intracellularly as an outcome of mitochondrial metabolism, peroxisomes, and some cytoplasmic enzymes, and they are quenched with the aid of intracellular antioxidants (Payen et al., 2019). Several findings support the notion that ROS are upregulated upon aneuploidy. In yeast models of aneuploidy, oxidative stress response pathways, which upregulate thioredoxins and oxidoreductases, are upregulated and ROS are produced (Dephoure et al., 2014). MEF models of aneuploidy also show high ROS levels, and the administration of antioxidants partially rescues their proliferative rate (Li et al., 2010). ROS production results in oxidative DNA damage, which would be one of the inputs for DNA damage checkpoint activation in aneuploid cells and could even act as a feedback loop (Degtyareva et al., 2008, Li et al., 2010).

How ROS are generated upon aneuploidy remains unclear. A recent study addressing epithelial CIN-induced tumor model in *Drosophila* reveals that aneuploidy-associated gene dosage imbalance leads to the production of ROS (Clemente-Ruiz et al., 2016), and this further activates the stress response pathway, c-Jun N-terminal kinase (JNK), by activating Apoptosis signal-regulating kinase 1 (ASK1). In the epithelial CIN model, aneuploid cells undergo JNK-dependent apoptosis and they are removed from the tissue, and additional blockade of apoptosis leads to tumor outgrowth (Dekanty et al., 2012). Furthermore, blockade of ASK1-dependent JNK activation reduces the growth potential of aneuploid tumors (Muzzopappa et al., 2017). Genetic overexpression of antioxidants and supplementation of the same rescues JNK-dependent apoptosis (Clemente-Ruiz et al., 2016). However, how ROS are generated remains unexplained.

To sum up, the response to aneuploidy seems to be common across species. Depending on the karyotype and the way in which aneuploidy is induced, there can be a wide variety of responses. However, there are some general characteristics associated with aneuploidy. The interplay between aneuploidy-associated stresses and its impact on cellular physiology and tissue homeostasis is still elusive. Understanding the root cause of these stresses and the corresponding signaling pathways activated will help us to reduce the deleterious effects of aneuploid karyotypes. More interestingly, aneuploidy is a hallmark of cancer. Cancer cells may have mechanisms to dampen these stresses, and targeting these survival mechanisms may provide a way to eliminate these cells from the tissue.

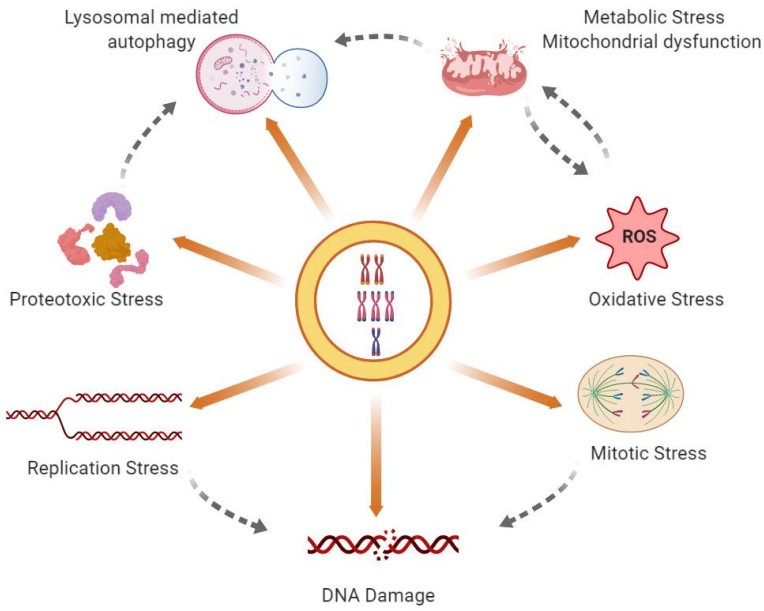

Figure 5. Aneuploidy-associated stresses

3. *Drosophila* as a model to study chromosomal instability

3.1 *Drosophila* as a cancer model

The conservation of core biological mechanisms across the species and universality of the genetic code indicates the appropriateness of using the use of model organisms, to address many complex biological questions. In this research work, I used *Drosophila melanogaster* as a model system. Its short life span, low-cost maintenance, low gene redundancy, and the availability of an enormous genetic tool kit (Kaufman, 2017, Bilder and Irvive, 2017) make *Drosophila* an ideal system for biological studies. Research using *Drosophila* has laid significant foundations for current biology. Indeed, research in *Drosophila* has been acknowledged by ten Nobel prizes for six key discoveries in physiology. Unquestionably, *Drosophila* will endure as a major model system for scientific discoveries and for the advancement of biomedical research.

Development is a highly complex process that needs to be tightly orchestrated in order to give rise to a healthy organism. In cancer, this tight regulation is lost, which results in tissue overgrowth (Hariharan and Bilder, 2006). Many of the hallmarks of cancer are well conserved in *Drosophila* models of the disease, and nearly 75% of disease-associated genes have orthologues in the fly, including 68% of human cancer genes (Reiter et al., 2001, Rubin et al., 2000). As early as 1916, Mary Stark demonstrated the genetic origin of tumors (Villegas, 2019). Many pathways, including Notch, Hedgehog, Hippo, Wingless, and Toll, involved in cancer were also first defined

in flies, and models of tumor development and cell invasion have been effectively established using *Drosophila* (Gonzalez, 2013, Richardson and Portela, 2018). *Drosophila* is also used as a pharmacological screening platform to identify cancer therapeutics and to reveal their mode of action (Dar et al., 2012, Markstein et al., 2014, Willoughby et al., 2013). The tremendous efforts of many scientists have established and confirmed the legitimacy of *Drosophila* in cancer research. However, although *Drosophila* is not considered a direct track to medical breakthroughs, the last two decades of cancer research in this model have demonstrated its value for the advance of biomedical research (Hombria and Serras, 2013, Villegas, 2019).

Drosophila is a holometabolous insect, its life cycle consisting of four developmental stages **(Figure 5A)**: egg, larva, pupa, and adult. The transitions between stages are temporally orchestrated by the steroid hormone Ecdysone. The larval stage is a growth phase, and much of the adult size is dependent on the growth achieved during this phase. The larvae are composed of two types of tissues, namely polyploid endoreplicative ones, which give rise to most of the larval parts, and diploid proliferative imaginal ones. During metamorphosis, most larval tissues undergo apoptosis, while imaginal discs are maintained and later give rise to adult structures.

Imaginal wing discs of the larva symbolize the primordial structure of the adult wing and proliferate exponentially during the larval stage. Furthermore, the epithelial cells of the disc have morphological features that resemble those of mammalian epithelial cells,

presenting basal and apical polarity, as well as adherens and separate junctions. Since most human cancers are of epithelial origin, *Drosophila* wing epithelial tissue is an ideal system in which to study cell proliferation and tissue homeostasis. To achieve gene regulation in a specific tissue of the fly, the epithelial transformation model makes use of the transactivation GAL4/UAS system (Brand and Perrimon, 1993). This system relies on two genetic components, namely GAL4, a transcriptional activator of yeast expressed in a tissue-specific manner, and a transgene under the control of an upstream activator sequence (UAS) that can be bound by GAL4, which then results in the tissue-specific expression of the transgene (Phelps and Brand, 1998).

The wing imaginal disc **(Figure 5B)** is a sac-like structure formed by a continuous epithelial monolayer, which comprises two opposing layers that surround the disc lumen. One side of the imaginal disc is the columnar epithelium, a pseudostratified epithelium, while the other side is the peripordial membrane, a squamous epithelium. The wing disc is partitioned into various regions by the restricted expression of selector genes, which confer specific cell identity (Garcia-Bellido, 1975), and these groups of cells never mix (Anterior, Posterior, Dorsal, Ventral). Specific growth signals like Hedgehog, Notch, Wingless and Decapentaplegic orchestrate the growth and patterning of the wing during development (Garcia-Bellido and Merriam, 1971, Lawrence and Struhl, 1996, Vincent, 1998).

Introduction

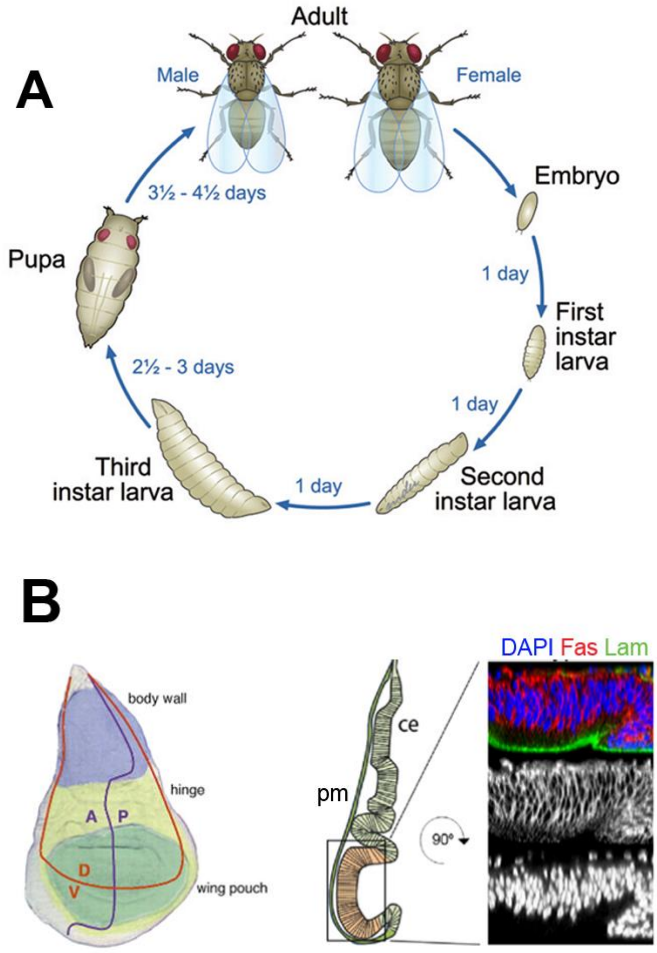

Figure 5. *Drosophila* life cycle and wing imaginal disc

3.2 *Drosophila* model of chromosomal instability-induced tumorigenesis

Aneuploidy is a salient feature of most sporadic cancers, the most predominant one in humans and of epithelial origin (Weaver and Cleveland, 2006). The epithelial cells of *Drosophila* have proven to be an excellent model system to decipher the molecular and cellular mechanisms underlying tumorigenic growth (Brumbhy and Richardson, 2005, Pastor-Pareja and Xu, 2013). In this work, I used *Drosophila* epithelial cells to unravel the molecular mechanisms of deleterious CIN-induced aneuploidy and its impact on cellular physiology and tissue homeostasis.

The genome of *Drosophila* consists of four pairs of chromosomes carrying 15,682 genes, and the fourth one carries a few genes compared to the other three. Considering this, loss or gain of the single chromosome could make a huge difference in the genome. This would make one think that *Drosophila* is not an ideal system in which to study the consequences of aneuploidy. However, interestingly, research in *Drosophila* has contributed to the aneuploidy field (Milan et al., 2014). In 1921, Calvin Bridges demonstrated that chromosome trisomies were lethal while increasing the whole genome three-fold was viable (Bridges, 1921). Subsequently, studies addressing segmental aneuploidies demonstrated a reverse correlation between the size of the fragment and viability (Patterson et al., 1935, Lidsley et al., 1972, Ripoll, 1980). These studies reinforce the idea that aneuploidy-associated reduced viability is not a consequence of the individual effect of

certain genes but rather an additive effect of the genes. More precisely, gene dosage imbalance upon aneuploidy leads to this harmful situation at a cellular and organismal level. Early experiments by Calvin Bridges also demonstrated that trisomies of the major autosomes (second and third chromosomes) are lethal, whereas flies with different copies of X and the fourth chromosome are viable. The chromosome-specific compensation mechanisms, male-specific lethal complex (MSL-C) for the X chromosome in males (Laverty et al., 2010), and Painting of fourth (POF) fuel the expression of the fourth chromosome in a 4/0 situation (Larsson et al.,2001, Johansson et al.,2007). Compensation of the fourth chromosome, an autosome, leads to speculation of the presence of an autosomal compensation mechanism. Later work demonstrated that the POF mechanism is a likely indication of its earlier life as a sex chromosome (Vicoso and Bachtrog, 2013). The loss of POF or MSL-C in males causes lethality, and this observation again reinforces the idea that the deleterious effects of aneuploidy are due to the imbalance of a large set of genes.

Depletion of SAC genes (bub3, rod), chromatin condensation (orc2), cytokinesis (diaphanous(dia)) and genes involved in spindle assembly (abnormal spindle (asp)) induces CIN in *Drosophila* epithelial cells (Dekanty et al., 2012, Dekanty and Milan, 2013). CIN-induced aneuploidy in these cells leads to loss of apical-basal polarity, consequent cell delamination, and apoptosis. Aneuploid cells are removed from the tissue through JNK-dependent caspase-mediated apoptosis **(Figure 6A),** in contrast to the mammalian system, which depends on p53 (Thompson and Compton, 2010). CIN

is not sufficient to drive tumorigenesis in *Drosophila* stem cells (Castellanos et al., 2008), or in SAC mutant mice (Holland and Cleveland, 2009). However, an additional mutation in the caretaker gene p53, along with SAC depletion, drives this process in mice (Li et al., 2010). Consistent with this, blockade of apoptosis at different levels of caspase activation and further maintenance of aneuploid cells in the tissue results in tumorigenesis. The characteristics of this tumorigenic behavior are DE-cadherin delocalization, basement membrane degradation and neoplastic growth (Dekanty et al., 2012). Regarding the *Drosophila* epithelial CIN-induced model, the following two points should be taken into consideration: (1) cell death is not a consequence of delamination; and (2) cell delamination does not depend on JNK activation. In addition to promoting tumorigenesis, blockade of JNK activation prevents aneuploid cells from dying; however, they still delaminate from the main epithelium (Dekanty et al., 2012).

JNK is a stress response pathway in *Drosophila* tissues. CIN drives tumorigenesis in epithelial cells by exploiting a subversive role of JNK in these cells. The original role of JNK activation is to remove aneuploidy cells from tissue through apoptosis, but blockade of apoptosis leads to a JNK-dependent transcriptional program in these cells, which drives tumorigenesis (Dekanty et al., 2012, Clemente-Ruiz et al., 2016, Muzzopappa et al., 2017, Benhra et al., 2018). JNK exerts its tumor-promoting role by inducing the expression of mitogenic molecule Wingless (Wg) (Perez-Garijo et al., 2004, Smith-Bolton et al., 2009, Ryoo et al., 2004) and Matrix Metalloproteinase 1 (MMP1) (Uhlirova and Bohmann, 2006). The

induction of CIN and blockade of apoptosis leads to two distinct cell populations in the tissue, namely the growing epithelial population, with low levels of aneuploidy, and the delaminated population, with higher levels of aneuploidy and arrested in the cell cycle. The latter population has high levels of JNK activation and is highly secretory (MMP1, Wg) and senescent. Mitogens signal the low aneuploid epithelial population and induce tissue overgrowth, and these cells show more errors, become highly aneuploid and delaminate. In summary, the cross-feeding interaction between the low-aneuploid-epithelial-population and highly-aneuploid-delaminated-population drives the uncontrolled growth potential of CIN tumors **(Figure 6B)** (Muzzopappa et al., 2017)

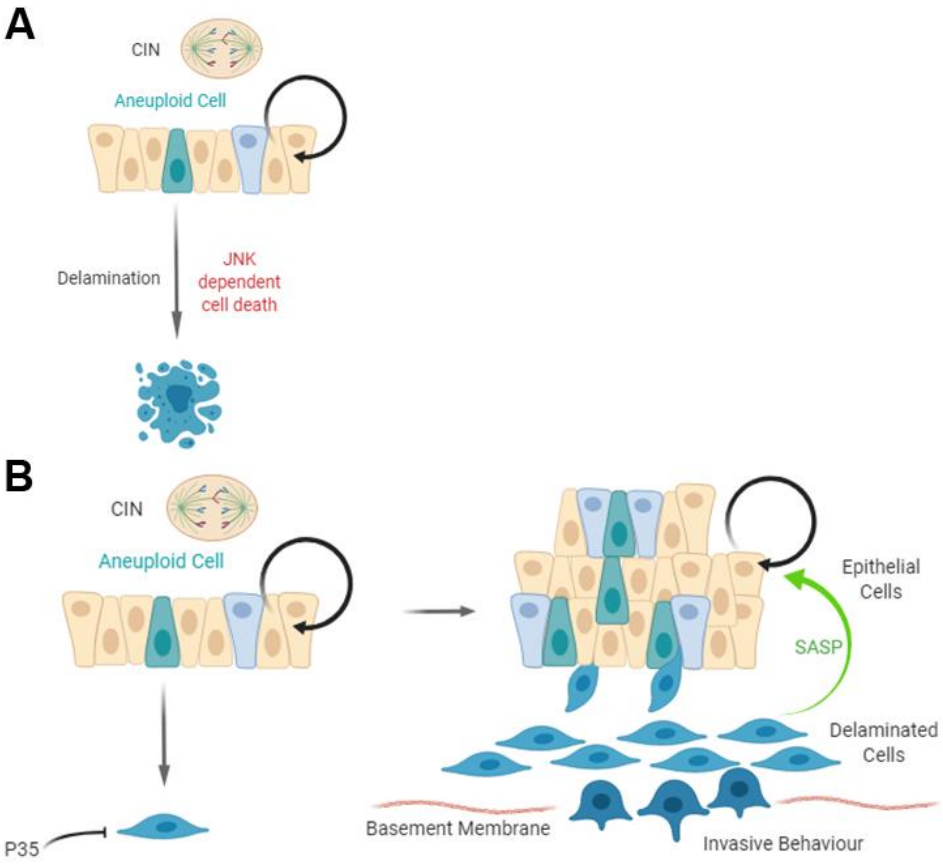

Figure 6. CIN-induced tumorigenesis in the *Drosophila* epithelial model

As mentioned previously, the dosage compensation mechanism present in *Drosophila* buffers the effects of aneuploidy. A recent study (Clemente-Ruiz et al., 2016) exploiting this mechanism demonstrated that chromosome-wide gene dosage imbalance adds to CIN-induced aneuploidy and its pro-tumorigenic role. Gene dosage imbalance leads to ROS production and JNK activation through ASK1 (Sekine et al., 2012, Muzzopappa et al., 2017, Santabarbara-Ruiz et al., 2019). This work also revealed that various cellular and

tissue-wide mechanisms, such as DNA-damage repair, activation of the P38 pathway and cytokine induction, are activated to buffer the deleterious effects of CIN and thus promote compensatory proliferation. Remarkably, from gene set enrichment assays, several metabolic regulators were up-regulated in the highly aneuploid delaminated population, such as lactate dehydrogenase, known as ImpL3 in flies and Insulin Growth Factor Binding Protein (IGFBP), known as Impl2 in flies. Impl3 contributes to a Warburg-like metabolic shift (Hanan and Weinberg, 2011) in tumors. Many antioxidant genes were also upregulated in the delaminated population. Moreover, genetic overexpression of antioxidants or supplementing antioxidants in the food reduced the levels of cell death caused by CIN in epithelial tissues, reinforcing that these mechanisms buffer the deleterious effects of CIN. However, it is not clear how ROS is generated upon CIN nor the identity of the signal for cell delamination. Since ROS-mediated JNK activation is the key event for tumorigenesis, understanding how ROS is generated is highly relevant.

In *Drosophila* brains, CIN does not cause tumorigenesis. Centrosome amplification is a common feature of many cancers (Fukasawa, 2005). A study of centrosome amplification (Basto et al., 2008) in the fly shows that this process results in bipolar rather than multipolar mitosis, and in less aneuploidy. However, tumors developed because of the expansion of neural stem cells caused by impaired asymmetric division. Another study with dysfunctional centrosomes in the fly brain reported in tumor formation, but this abnormal growth was not attributed to aneuploidy (Castellanos et al., 2008). This observation

leads to speculation that neural cells are more resistant to aneuploidy insult. A couple of recent studies suggest that, upon aneuploidy, neuroblasts undergo premature differentiation and apoptosis (Gogendeau et al., 2015, Poulton et al., 2017, Mirkovic et al., 2019). More studies are required to decipher how cell fate is determined upon aneuploidy in *Drosophila* neuroblasts. Understanding how neural stem cells deal with the CIN-associated stresses will shed light in this direction.

Various studies on different species have demonstrated that there is a stress response to aneuploidy. This response is independent of the identity of the genes and it depends mostly on the gene dosage imbalance associated with the aneuploid karyotype. In the *Drosophila* CIN-induced epithelial model, aneuploidy-associated gene dosage imbalance causes ROS production and activation of JNK, a stress response pathway for apoptosis. An additional blockade of apoptosis makes JNK a pro-tumorigenic and drives tissue overgrowth. As I mentioned previously, a key event in JNK activation is the production of ROS. Therefore, how aneuploidy-associated gene dosage imbalance is translated to ROS production is a highly relevant and central question to answer. More precisely, we must unravel how the aneuploidy-associated stresses are resolved in an aneuploid cell. Although there is a deep understanding of mitotic, replicative and proteotoxic stress, little is known about CIN-induced metabolic stress and how it is activated. In addition, the interplay between proteotoxic and metabolic stress is still elusive. A full understanding of these mechanisms will help us to find the Achilles' heel of aneuploid karyotypes and associated tumorigenesis.

Introduction

In this thesis, I attempt to elucidate the interplay between proteotoxic and metabolic stress and the corresponding generation of ROS in CIN-induced epithelial cells of *Drosophila melanogaster*.

OBJECTIVES

Objectives

The three main objectives of this thesis are:

1. To elucidate the links between aneuploidy associated gene dosage imbalance and ROS production in CIN tissues.
2. To understand the role and cause of cell delamination observed in CIN tissues.
3. Find the Achilles´ Heel of CIN tumors.

RESULTS

1. Characterization of the cell populations in CIN tissues

1.1 Generation of CIN tissues

As mentioned previously, I used the *Drosophila* wing epithelium to model CIN. Using basic fly genetics, the UAS/GAL4 system, aneuploid cells were generated by blocking (using RNAi) the SAC gene, *rough deal (rod)*, in the posterior (p) compartment of the wing disc (using *engrailed (en)* driver line). As these aneuploid cells undergo JNK-dependent apoptosis through caspase action, baculovirus P35 (Hay et al., 1994) was expressed to maintain the aneuploid cells in the tissue. Additional mutation of caretaker genes are the ones that drive tumor formation in most of the cases. In the mouse aneuploidy model, depletion of P53 drives tumorigenesis (Li et al., 2010). To thoroughly characterize the cellular behaviors and molecular mechanisms underlying cell delamination and JNK activation, aneuploid cells were maintained in the tissue by blocking cell death. Consequently, maintaining the highly aneuploid cells in the tissue exerts a JNK dependent transcriptional program and tissue overgrowth (Dekanty et al., 2012). The protocol for CIN-induction in epithelial tissues is described in **Figure 7**.

Interestingly, the highly aneuploid cells delaminate from the main epithelium, which creates two populations of cells in the tissue: (1) low-aneuploid-proliferating-cells and (2) highly-aneuploid-delaminated-cells. As highly aneuploid cells activate the JNK pathway, one of its downstream targets, MMP1 (Uhlirova and Bohmann, 2006), is used to mark the delaminated population. In

addition, they possess a loosely attached morphology. Delaminated cells have senescent characters like high secretion (Wg, MMP1) and they are arrested in the cell-cycle (Dekanty et al., 2012, Muzzopappa et al., 2017).

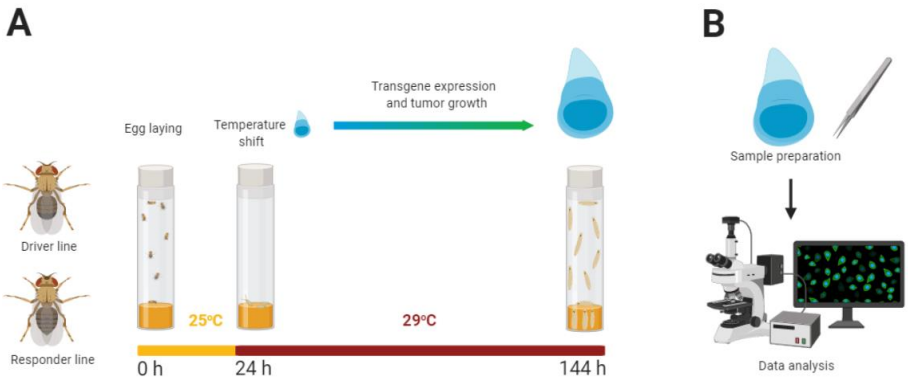

Figure 7. Protocol to induce CIN in the *Drosophila* wing epithelium

(A) Flies carrying constructs for the tissue-specific generation of CIN and additional blockade of the apoptotic cascade were crossed and kept at 25ºC for 24h. Tubes carrying embryos and recently-hatched larvae were transferred to 29ºC for stronger induction of gene expression. Following 120h of CIN induction, imaginal discs were dissected and stained for visualization under the confocal microscope **(B).**

High levels of JNK activation (monitored using MMP1-GFP) were seen in the delaminated cells (**Figure 8**). Boundaries marked with cyan represent the delaminated population from here onwards (**Figures 8 B, C', D**). MMP1 staining and morphological differences (epithelial cells are tightly bounded whereas delaminated cells are loosely packed) were used to mark the delaminated population. MMP1 channels are removed from the rest of the images from here onwards to make it simple.

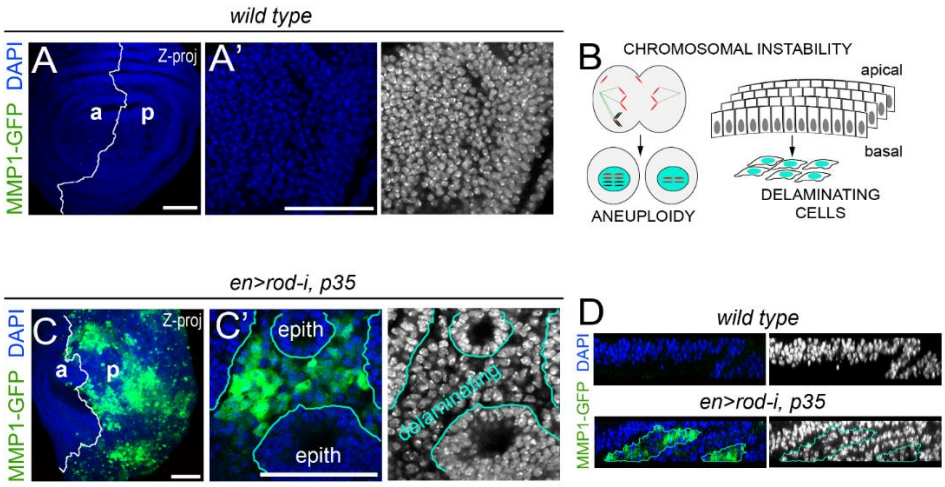

Figure 8. Two cell populations in CIN-induced epithelial tumors

(**A**) Wild type wing disc. **(A')** Zoomed view of the p compartment, showing tightly packed cells. **(B)** CIN creates two populations of cells in the tissue. **(C)** High levels of MMP1 staining were seen in the p compartment upon CIN. **(C')** Zoomed view of the p compartment, showing delaminated cells (cyan boundary) enriched with MMP1 staining (green) and their loose morphology. **(D)** Orthogonal view of the tissue. The boundary between anterior (a) and posterior (p) was marked in all the figures by using the Ci staining (that labels the ´a´ compartment, not shown). CIN was induced in the ´p´ compartment in all figures and the ´a´ compartment serves as the control. DAPI (blue or white) is used to label the nuclei in all figures. (Scale bars, 50 μm).

1.2 CIN-induced ROS production in the epithelial tissues

Prior studies from the lab demonstrated that CIN-induced aneuploidy generates ROS and contributes to JNK dependent tumorigenesis (Clemente-Ruiz et al., 2016) and that ROS is the main source of JNK activation in CIN-induced epithelial tumors (Muzzopappa et al., 2017). Here, ROS production is not a consequence of JNK-activation or JNK-dependent apoptosis. ROS was still present even after the

blockade of JNK or apoptosis, suggesting that it´s upstream of JNK (Clemente-Ruiz et al., 2016). Geneset enrichment analysis of epithelial CIN tissues revealed the upregulation of genes required for tumorigenesis and altered metabolism (**Table1**). Genes upregulated from the delaminated population were classified into two classes; (1) JNK and growth (2) Metabolism and ROS (Clemente-Ruiz et al., 2016).

Class 1: JNK and growth

Symbol	Gene Name	Fold change
puc	Puckered (Martin-Blanco et al., 1998)	2.21
Mmp1	Matrix metalloproteinase 1 (Uhlirova and Bohmann, 2006)	9.19
scaf	scarface (Rousset et al., 2010; Sorrosal et al., 2010)	8.38
Pvf1	PDGF- and VEGF-related factor 1	3.95
os	outstretched (Upd1) (Ohsawa et al., 2012)	13.71
upd2	JAK-STAT signaling (Bunker et al., 2015; Ohsawa et al., 2012)	3.24
upd3	JAK-STAT signaling (Bunker et al., 2015; Ohsawa et al., 2012)	5.87
Socs36E	Suppressor of cytokine signaling at 36E	2.22
Wnt4	Wnt oncogene analog 4	5.71
Sulf1	Sulfated (negative regulator Wnt signaling)	4.26
Idgf3	Imaginal disc growth factor 3	4.77
Ilp8	Insulin-like peptide 8 (Colombani et al., 2012; Garelli et al., 2012; Katsuyama et al., 2015)	7.98
ImpL2	dILP-inhibitor (Figueroa-Clarevega and Bilder, 2015; Kwon et al., 2015)	2.93

Class 2: Metabolism & ROS

Symbol	Gene Name	Fold change
AcCoAS	Acetyl Coenzyme A synthase	4.30
ImpL3	Lactate Dehydrogenase	7.04
Men	Malic enzyme	2.93
Tret1-1	Trehalose transporter 1-1	3.74
Cyp18a1	Cytochrome P450-18a1	7.85
Cyp6a17	Cytochrome P450-6a17	2.32
GstD3	Glutathione S transferase D3	2.24
GstD6	Glutathione S transferase D6	3.03
GstE1	Glutathione S transferase E1	3.01
GstE2	Glutathione S transferase E2	2.44
GstE3	Glutathione S transferase E3	2.15
GstE6	Glutathione S transferase E6	2.85

Table 1. Transcriptional profile of delaminated cells vs non-delaminated cells

We used different methods to monitor ROS production in epithelial cells, such as Dihydroethidium (DHE) and gstD-GFP (Sykiotis and Bohmann, 2008). High levels of ROS were detected in the delaminated cells, which are highly aneuploidy (**Figure 9**).

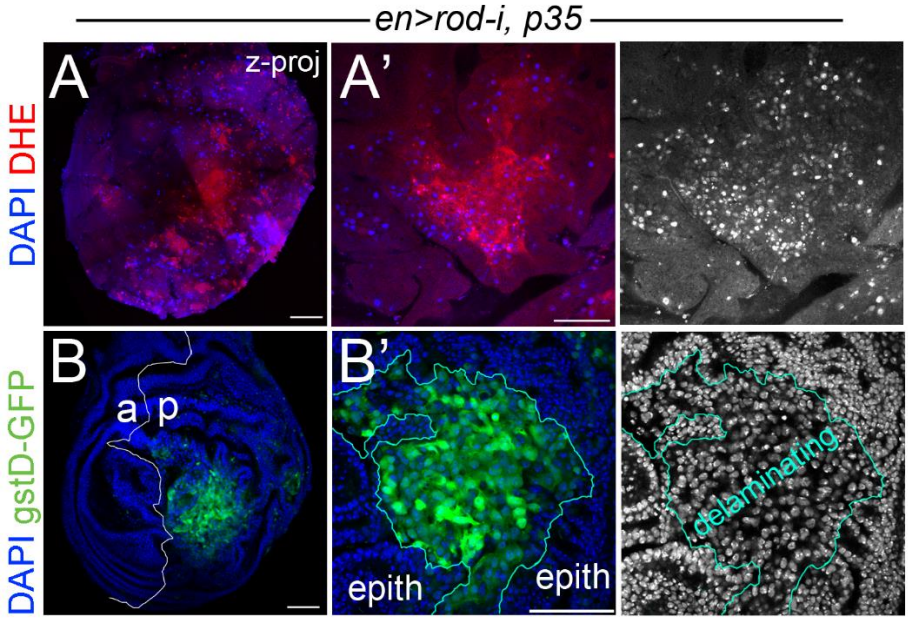

Figure 9. ROS production in CIN-tissues

(A) DHE staining in CIN-induced tumors. High levels were observed in the 'p' compartment. **(A')** High levels of DHE staining were specifically observed in the delaminated cells. (**B)** GstD-GFP in the CIN tissues. (**B'**) High levels of GstD-GFP were specifically observed in the delaminated population. (Scale bars, 50 μm).

How aneuploidy mediated gene dosage imbalance is translated to ROS production is still elusive in *Drosophila* epithelial cells. As previously mentioned, aneuploidy-driven gene dosage imbalance creates a protein imbalance and this could lead to proteotoxic stress. So, I started looking at the proteostasis mechanisms in CIN tissues.

2. Proteatoxic stress in CIN tissues

2.1 Protein aggregation upon CIN

The induction of CIN results in the accumulation of protein aggregates in the tissue. Protein aggregates were monitored using poly-ubiquitin (Ubq) and Ref(2)P staining. The single *Drosophila* homolog of mammalian p62, *refractory to Sigma P* (*ref(2)P*) shares similar functional motifs with p62. It is known that Ref(2)P is a regulator of protein aggregation in flies (Nezis et al., 2008). Protein aggregates were observed only in the posterior compartment whereas the anterior compartment, which is wild type, does not show any protein aggregate accumulation (internal control) (**Figure 10A**). More precisely, protein aggregates were found in delaminated cells. Protein aggregate size was heterogeneous (**Figures 10B, B'**).

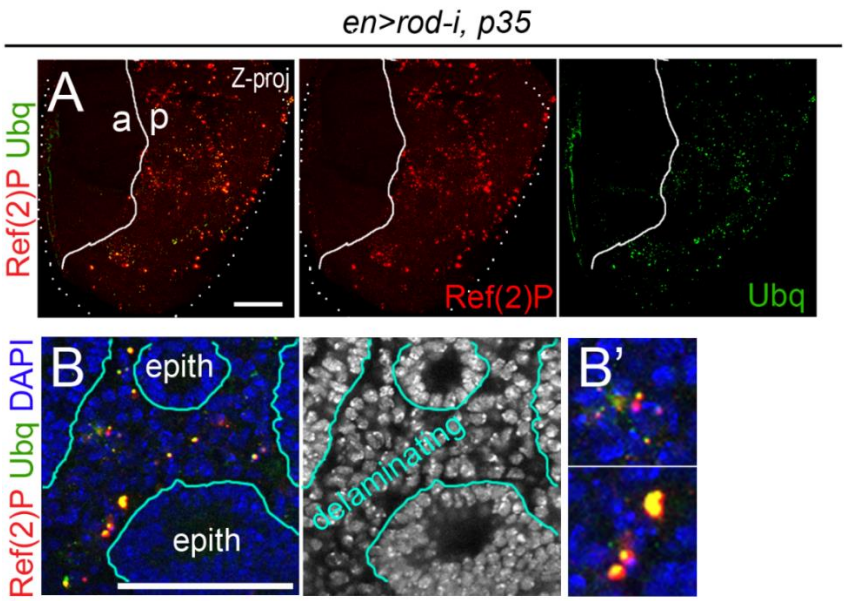

Figure 10. Proteotoxic stress in CIN-tissues

(A) Accumulation of Ref(2)P (red) and ubiquitin (Ubq, green) in the p compartment. **(B)** Protein aggregates were preferentially observed in the delaminated cells (cyan boundary) **(B')** Heterogeneity in the size of the protein aggregates. Note strong co-localization between Ref(2)P and Ubq. (Scale bars, 50 μm).

Protein aggregation was also observed in other models of CIN (**Figure 11**). The depletion of another SAC gene *bub3* resulted in a similar accumulation of protein aggregates. The depletion of *msl-1*, a MSL-C gene, gave similar results in male tissues, indicating that gene-dosage imbalance leads to proteotoxic stress. By contrast, depletion of *mud*, a spindle orientation gene, did not result in protein aggregate formation. These results imply that protein aggregation is an outcome of CIN-induced aneuploidy and the associated gene dosage imbalance, and not a trivial consequence of cell delamination, as interfering with the planar orientation of the mitotic spindle is not sufficient to cause similar effects on the proteome.

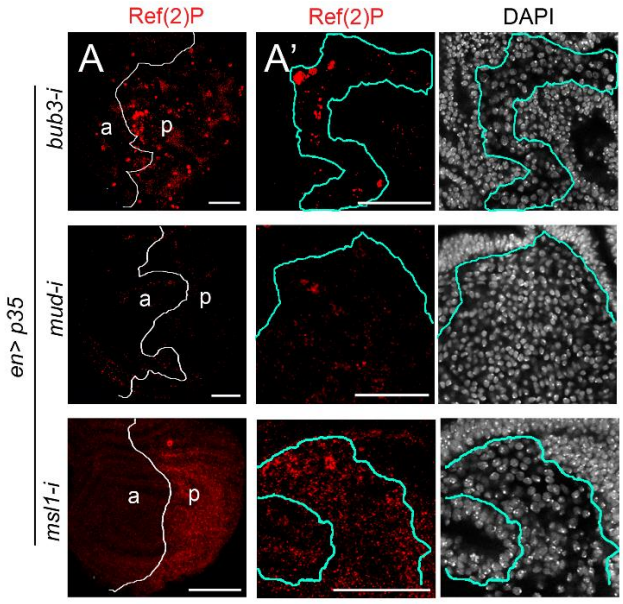

Figure 11. Proteotoxic stress is a consequence of gene dosage imbalance

(A) Accumulation of Ref(2)P in *bub3-i* and *msl1-i*-expressing tissues, but not in *mud-i*-expressing tissues. **(A')** Protein aggregates were specifically enriched in the delaminated cells. (Scale bars, 50 μm).

2.2 The proteasome machinery is saturated in CIN- tissues

The main two pathways responsible for protein catabolism in a cell are the ubiquitin-proteasome system (UPS) and lysosomal-mediated autophagy. These mechanisms are vital for normal cellular proteostasis. The proteasome is a multi-subunit protease complex, comprising of a 20S core particle and a 19S regulatory particle. The 20S holds the structural and catalytic parts whereas the 19S consists of non-ATPase subunits that are responsible for the identification of polyubiquitinated proteins, and ATPase part responsible for the protease activity. Most of the short-lived proteins are degraded by proteasomes and their timely action orchestrates diverse biological processes such as transcription, cell cycle progression, metabolism and differentiation (Wong and Cuervo, 2010).

To understand the in-vivo activity of the proteasome system, a fluorescent reporter of the UPS function was used. CL1-GFP is a fusion protein created by attaching a proteasome degradation signal to a green fluorescent protein (GFP) (Pandey et al., 2007) and this protein is rapidly degraded by the UPS (**Figure 12A**).

CLI-GFP was expressed in the posterior compartment of the tissue. CLI-GFP was rapidly degraded in the control situation and no accumulation was observed, suggesting that the proteasome is fully

functional. By contrast, CLI-GFP was accumulated upon CIN and mostly in delaminating cells, suggesting that the proteasome machinery is saturated in these cells (**Figures 12 C, D**).

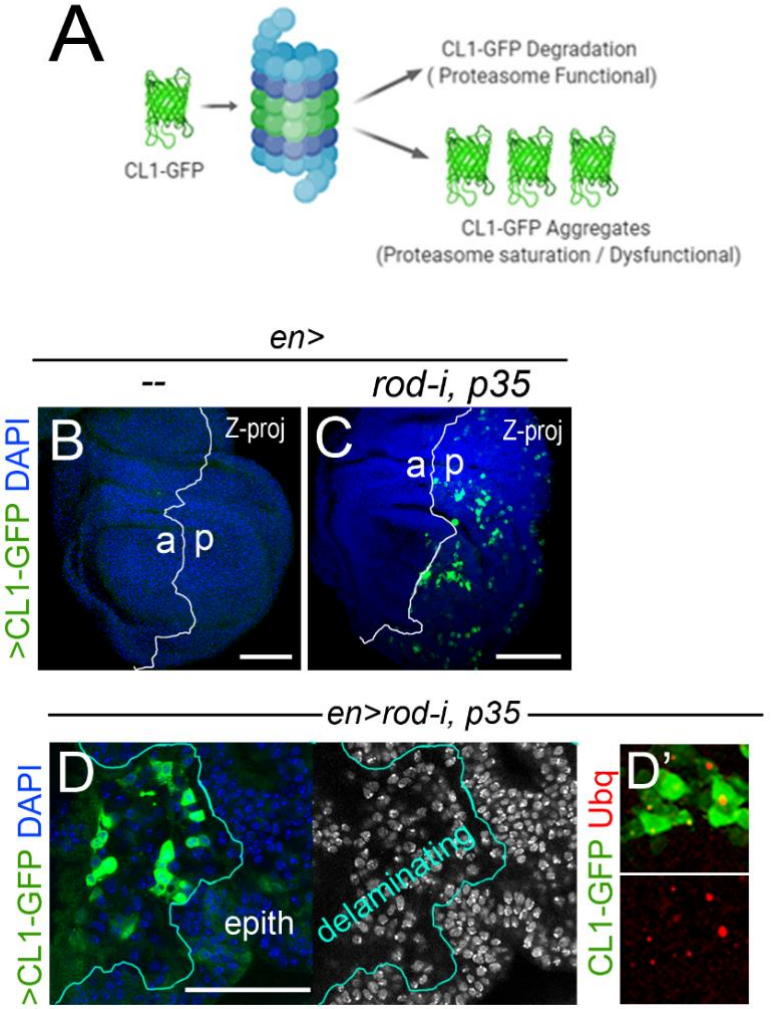

Figure 12. The proteasome machinery is saturated in CIN tissues

(A) Cartoon depicting the CL1-GFP mechanism of action. **(B, C)** CL1-GFP is accumulated in CIN (**C**) but not in wild type **(B**) tissues. **(D)** CL1-GFP aggregates were mainly observed in the delaminated cells (cyan boundary) **(D')** Co-staining of CL1-GFP aggregates with ubiquitin (Ubq). (Scale bars, 50 μm).

3. Autophagy induction in CIN tissues

3.1 Macroautophagy is induced upon CIN

Autophagy is a catabolic process present in a cell. It is well-preserved from yeast to mammals and it is an imperative cellular response to starvation and stress (Klionsky et al., 2003). Autophagy also plays a major role in cell death, development, aging, immunity, and cancer. There are three types of autophagy according to the route being used to reach the lysosome: (1) Macroautophagy – large portions of cytoplasm or organelles are captured by a membrane cistern (phagophore or isolation membrane) into a double-membrane autophagosome, which later fuses with the acidic lysosomal compartment (2) Microautophagy – lysosomes directly engulf small portions of cytoplasm or organelles (3) Chaperone-mediated autophagy – proteins could also reach lysosomal lumen through Lysosome associated membrane protein 2A (Lamp-2A) with the aid of Hsc70. In all situations, the ultimate aim is to degrade the cargo and the recycling of the resultant monomers to the cytosol to fuel biosynthesis and energy production (Mizushima et al., 2008). The main type of autophagy, macroautophagy, will be referred to as autophagy from here onwards.

Initial studies in mice and later in *Drosophila* demonstrated that loss of core autophagy genes results in the accumulation of ubiquitinated protein aggregates in neurons (Hara et al., 2006, Komatsu et al., 2006, Juhasz et al., 2007, Simonsen et al., 2008). The multidomain protein p62 (also called sequestome-1 in mammals and Ref(2)P in *Drosophila*) is the first identified intracellular receptor for the

selective autophagic breakdown of the ubiquitinated proteins (Pankiv et al., 2007). The C-terminal UAB domain of p62 mediates the interaction with ubiquitin and its N-terminal PB1 domain promotes homo-oligomerization. The LC3-interacting region/Atg8 interacting motif (LIC/AIM) of p62 mediates the interaction with Atg8 family proteins (Pankiv et al., 2007). Disruption of the autophagy pathway generally ends up in the buildup of p62 aggregates (Klionsky et al., 2012). Thus, P62 accumulation could be used as a readout for basal autophagy faults.

Ref(2)P was accumulated in the CIN tissues (**Figures 10,11**) and this accumulation was more pronounced in the highly aneuploid delaminated cells (**Figure 10B**). This suggests that autophagy is saturated in the delaminated cells. Atg8a tagged with mCherry (ChAtg8a) under the control of its endogenous promoter was used to study the autophagy induction in epithelial tissue (Katheder et al., 2017). High levels of autophagy induction were observed in CIN tissues, characterized by the accumulation of ChAtg8a puncta (**Figure 13B**). More precisely, delaminated cells show strong activation of autophagy (**Figure 13C**) and autophagic cargo looks quite big in size (**Figure 13C'**). Interestingly, the induction of autophagy was also observed in neighboring cells not subjected to CIN (eg. anterior compartment, **Figures 13B, C**).

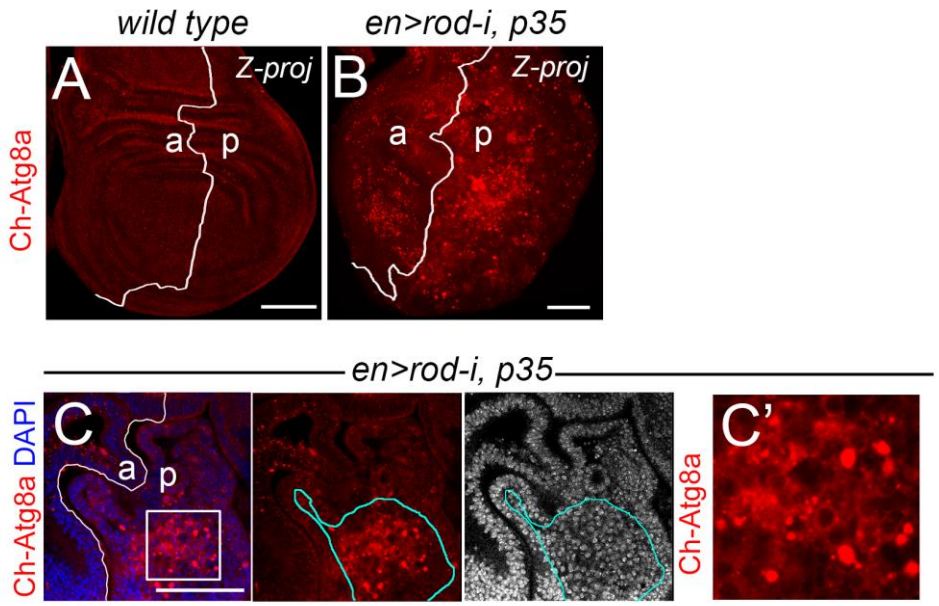

Figure 13. Autophagy induction in CIN tissues

(**A**, **B**) ChAtg8a staining in control **(A)** and CIN (**B**) tissues. ChAtg8a puncta were observed in CIN tissues in both the anterior and posterior compartments. **(C)** The highest levels of ChAtg8a were observed in the delaminated cells. Non-autonomous ChAtg8a was also observed (anterior). **(C')** Heterogeneity in the size of the autophagic cargoes. (Scale bars, 50 μm).

3.2 Autophagic flux is functional in CIN tissues

Autophagy is a multistep process and fusion with lysosomes is a key event for cargo degradation. The tandem tagged mCherry-GFP-Atg8a reporters are widely used to study autophagic flux which works in a similar manner to mammalian RFP-GFP-LC3B (Klionsky et al., 2016, Lorincz et al., 2017). The low lysosomal pH rapidly quenches the GFP signal after fusion, so the corresponding autophagolysosome will be positive for mCherry only. If fusion is not happening, then the dots will be positive for both GFP and

mCherry, hence yellow in color. In CIN tissues, most of the dots were positive for mCherry only (**Figure 14A**). A few big dots positive for both signals were also observed in the delaminating cells. Consistent with the ChAtg8a reporter, most of the dots were observed in the delaminated population (**Figure 14A**). To validate the tool, we blocked lysosomal fusion by depleting Syntaxin17, which is required for lysosomal fusion (Takats et al.,2013). This resulted in the accumulation of dots positive for both signals (**Figure 14B**). Interestingly, autophagy dots were also observed in the epithelial cells too (**Figure 14B'**). This suggests that autophagic flux is very efficient in epithelial cells and difficult to observe dots in the CIN situation (**Figure 14B'**). Moreover, this also explains why protein aggregates were only observed in delaminated cells. To sum up, autophagy flux is working well in CIN tissues. Taking into consideration that protein aggregates and few double-positive dots were observed in delaminated cells, autophagy might be close to saturation in highly aneuploid cells.

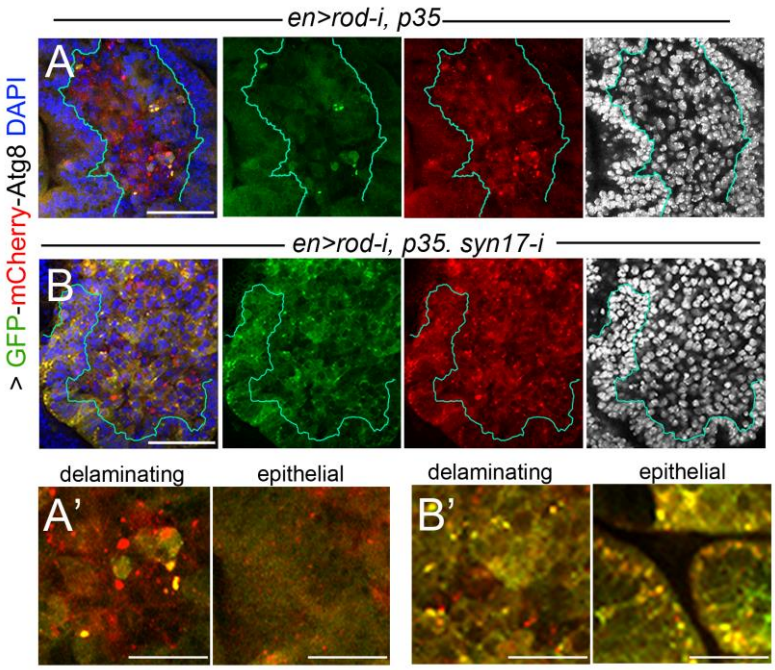

Figure 14. Autophagic flux in CIN tissues

(**A**) GFP-Cherry-Atg8 staining in CIN tissues, where the number of GFP-positive dots is rather small when compared to Cherry-dots. **(B)** The depletion of Syntaxin17 (*syn17*) resulted in the accumulation of yellow dots. High magnification of delaminating and epithelial cells in CIN tissues (**A'**) and in CIN tissues subjected to Syntaxin17 depletion (**B'**). (Scale bars, 50 μm).

4. Regulation of autophagy in CIN tissues

4.1 Cargo independent autophagy induction upon CIN

The autophagy system, as well as its regulation, are evolutionarily conserved (Melendez and Neufeld, 2008, Kamada et al., 2000). The protein kinase Target of Rapamycin (TOR) plays a chief role in nutrient sensing and autophagy regulation. During nutrient availability state, TOR is activated through the Class 1

phosphatidylinositol-3-kinase (PI3K) signaling pathway, and TOR hinders autophagy through direct phosphorylation and repression of Atg1 (Scott et al., 2007, Pattingre et al., 2008). When nutrients are limiting, TOR is inactivated, Atg1 inhibition is relieved, and autophagy is induced.

RNAi-mediated depletion of a core autophagy gene, *atg1*, inhibits the activation of autophagy in CIN tissues, indicating that autophagy induction goes through the canonical pathway (**Figures 15 A, A',** compared with **13 C**). Moreover, it also reinforces ChAtg8a as a bona fide reporter for autophagy analysis. CIN-induced aneuploidy and the associated gene dosage imbalance are hypothesized as a source of proteotoxic stress, and this stress activates the protein quality control mechanisms like autophagy. So, identifying the molecular mechanisms involved in activating the autophagy machinery is key. The autophagy adaptor, Ref(2)P, is an important player in this process. RNAi mediated depletion of *ref(2)P* resulted in low levels of autophagy induction, but still, some autophagy induction was seen (**Figures 15 B, B',** compared with **13 C**). By contrast, RNAi-mediated depletion of *ref(2)P* completely removed the Ref(2)P staining in the CIN compartment. This prompted us to look for cargo-independent ways of autophagy regulation in CIN tissues (He and Klionsky, 2009).

The main pathways potentially involved in the regulation of autophagy in CIN tissues are ER-stress related ways of autophagy induction, proteasome-impairment-mediated activation of autophagy, and nutrient sensing (He and Klionsky, 2009). So, we

next tested whether the pathways related to these above-mentioned processes are activated in our CIN tissues and whether they do play a functional role in activating autophagy upon CIN. We assumed that many pathways could regulate autophagy in a pleiotropic situation like CIN.

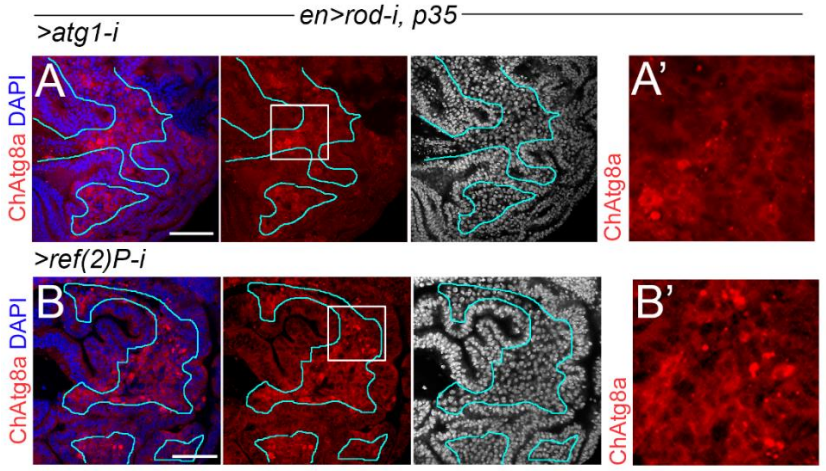

Figure 15. Induction of autophagy relies on Atg1 and cargo detection

(A, B) RNAi-mediated depletion of *atg1* (**A**) or *ref(2)P* **(B)** reduces the overall levels of autophagy induction in CIN tissues, monitored by ChAtg8 in red, as well as the highest levels observed in delaminating cells (**A'** and **B'**). (Scale bars, 50 μm).

4.2 Hypoxia and autophagy induction

The α subunit of hypoxia-inducible transcription factor-1 (HIF-1) is one of the best-characterized substrates of the Ubiquitin-Proteasome System (UPS) (Nys et al., 2011). Under normoxic conditions, HIF-1α is ubiquitinated by the tumor suppressor protein von Hippel-Lindau (VHL), resulting in its proteasomal degradation. During the hypoxic situation, HIF-1α is prevented from degradation and leads to

its stabilization and upregulation of its target genes (**Figure 16**). Hypoxia is known to induce autophagy and the fly ortholog of HIF-1α is called Sima (Short for similar) (Low et al., 2013, He and Klionsky, 2009).

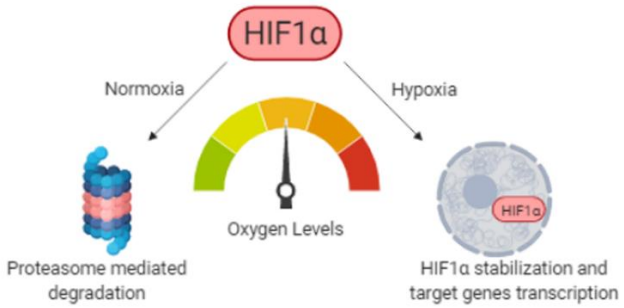

Figure 16. Mechanism of HIF-1α regulation

Warburg effect, defined as a shift from oxidative phosphorylation to aerobic glycolysis, is a metabolic hallmark of aggressive cancers (Hanahan and Weinberg, 2011, Pavlola and Thompson, 2016). Lactate dehydrogenase (LDH) converts the key metabolite pyruvate to lactate during aerobic glycolysis in tumors. The *Drosophila* genome comprises a single gene encrypting LDH (*ImpL3*). The geneset enrichment analysis of the delaminated population in CIN tissues revealed that there is a strong upregulation of *ImpL3* (refer to **Table 1**). LHD is one of the targets of HIF-1α/Sima. GFP tagged to the oxygen-dependent-degradative (ODD-GFP) motif of Sima (Misra et al., 2017) and the MiMIC line of Sima (Sima-GFP) (Nagarkar-Jaiswal et al.,2015) was used to visualize it in in-vivo samples. Consistent with the geneset enrichment analysis, a strong

upregulation of LDH-GFP (Wang et al., 2016) was observed (**Figure 17A**), especially in the delaminated cell population (**Figure 17A'**). Similarly, the upregulation of ODD-GFP and Sima-GFP was observed (**Figures 17B, C**) in the posterior compartment, especially in the delaminated cells of CIN tissues (**Figures 17B', C'**).

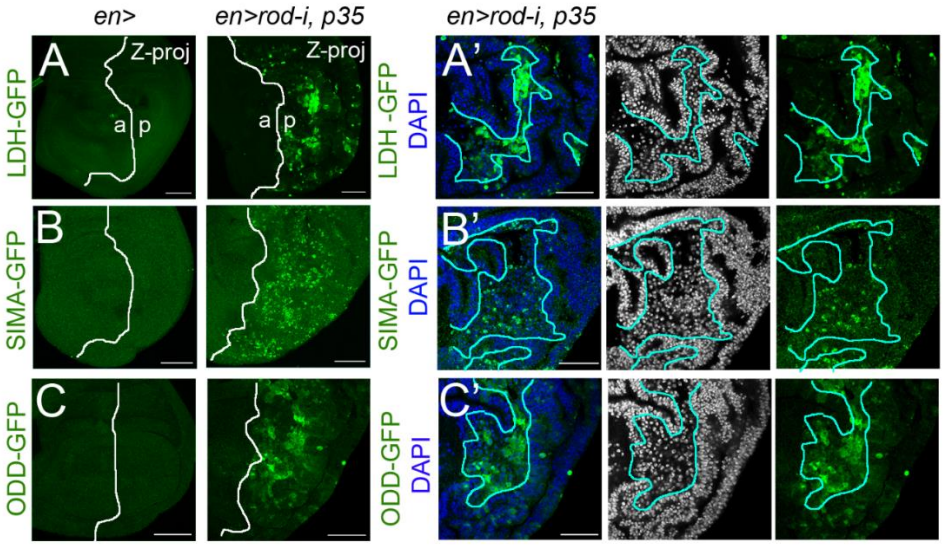

Figure 17. Sima/HIF activation in CIN tissues

(A, B, C) LDH-GFP, Sima-GFP, and ODD-GFP were upregulated in CIN tissues and this upregulation is highest in the delaminating cell population **(A', B', C')**. (Scale bars, 50 μm).

Since hypoxia is one of the mechanisms by which HIF-1α can be stabilized, we checked whether CIN tissues are hypoxic or not. A DsRed-FT (Timer) reporter was used for this (Lidsky et al., 2018). Oxygen concentration determines the maturation route of DsRed-FT. High oxygen levels favor a red-fluorescent isoform, whereas low oxygen levels lead to a green-fluorescent isoform. CIN tissues were

not hypoxic as per the reporter and it looks almost like the wild type situation (**Figure 18**). I can then conclude that the stabilization of HIF-1α in CIN tissues was not a consequence of low oxygen levels. Based on my previous observation that the proteasome is saturated in CIN-induced delaminating cells, I would then like to propose that HIF-1α is stabilized as a consequence of proteasome impairment (Low et al., 2013).

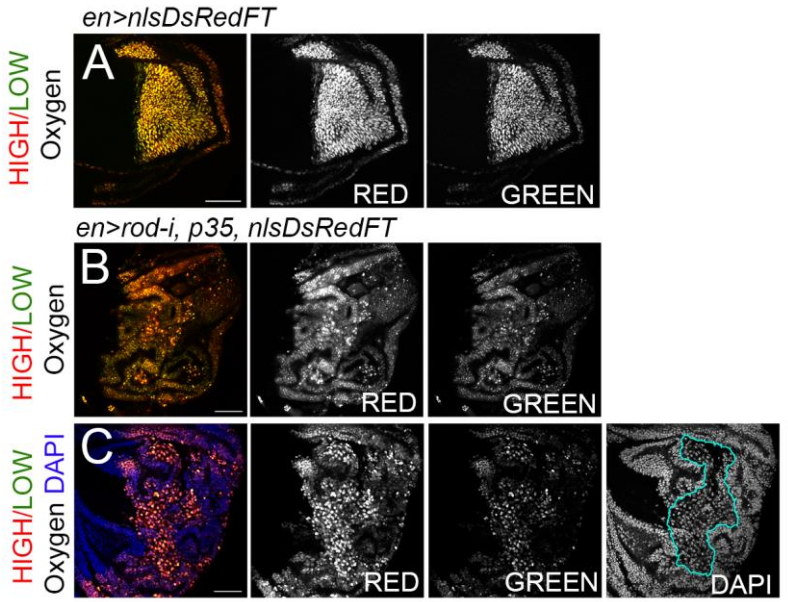

Figure 18. CIN tissues are not hypoxic

(A-C) The DsRed-FTreporter (Timer) is used in control (**A**) and CIN tissues (**B, C**) to monitor the levels of oxygen. Independently of whether the tissue is fixed (**C**) or not fixed (**B**), the ratio between low (green) and high (red) levels of oxygen is very similar in control and CIN tissues. (Scale bars, 50 μm).

As previously mentioned, HIF-1α stabilization is known to activate autophagy. RNAi mediated depletion of *sima* in CIN-tissues reduced the levels of autophagy induction (**Figure 19**). This indicates that

Sima stabilization in CIN tissues, most probably as a consequence of proteasome impairment, plays a key role in the induction of autophagy.

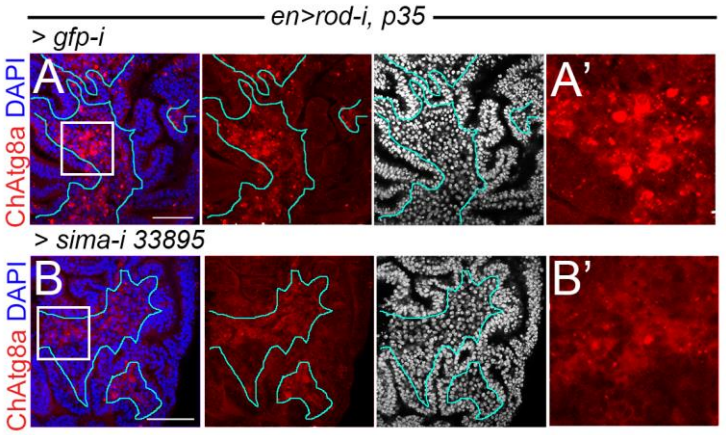

Figure 19. Sima stabilization regulates autophagy in CIN tissues

(A, B) RNAi-mediated depletion of *sima* (**B**) reduces the overall levels of autophagy induction in CIN tissues, monitored by ChAtg8 in red, as well as the highest levels observed in delaminating cells (**B'**) when compared to CIN-tissues expressing an RNAi form against *gfp* (**A**, **A'**). (Scale bars, 50 μm).

4.3 ER-stress and autophagy induction

Cellular homeostasis is a key process and endoplasmic reticulum (ER) is a chief organelle that makes sure that processes like Ca^{2+} storage, lipid and carbohydrate metabolism, and protein folding are well orchestrated in a cell. Any perturbation to these processes will lead to an ER stress response intended at either re-establishing cellular homeostasis or committing to cell death (Hetz, 2012). One of the major molecular mechanisms activated by ER stress and involved in a coordinated intra-cellular response is the Unfolded Protein

Response (UPR) which is activated upon the buildup of potentially toxic misfolded proteins (**Figure 20**).

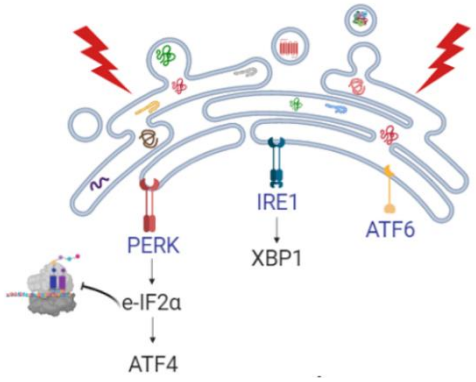

Figure 20. ER stress and the UPR

ER stress activates three ER membrane-embedded sensors of UPR: PERK (protein kinase RNA-like endoplasmic reticulum kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol-requiring enzyme 1 (IRE1). They further activate dedicated transcriptional programs intermediated by distinct transducers: cleaved ATF6, spliced XBP1 (X-box binding protein 1), and ATF4 (activating transcription factor 4) through phosphorylated eIF2α (eukaryotic Initiation Factor 2). This leads to pathways that activate chaperones, cellular metabolism, inhibition of translation, autophagy and cell death (Senft and Ronai, 2015).

UPR activation was observed in CIN tissues (**Figure 19**). Levels of p-eIF2α, a readout of PERK branch and XBP1-GFP (Sone et al., 2013), a readout of the IRE1 branch, were both upregulated in CIN tissues, especially in the highly aneuploid delaminated cell population.

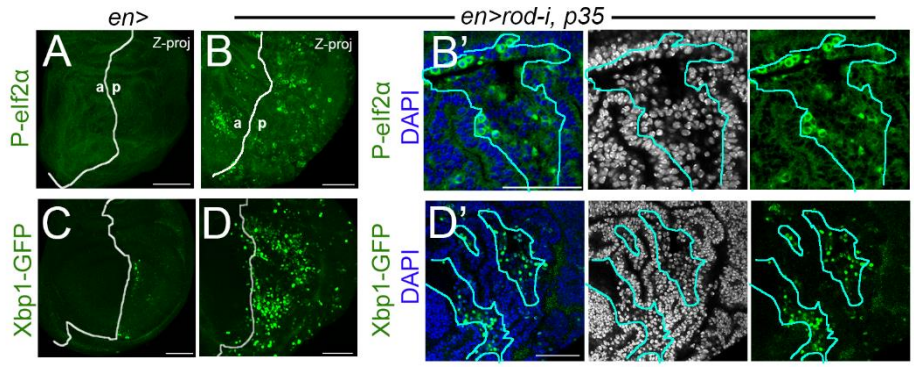

Figure 21. UPR is activated in CIN tissues

(**A-D**)UPR activation was observed in CIN tissues **(B, D)** and not in wild type tissues **(A, C)**. Upregulation is highest in the delaminating cell population **(B', D')**. Non-autonomous staining of P-elf2α (green) was observed in ´a´ compartment of CIN tissues due to cell death (**B**). (Scale bars, 50 μm).

Accordingly, high levels of heat shock factor (HSF), a master transcription factor of chaperones, was observed (**Figure 22**). HSF-GFP was predominantly high in the CIN compartment. Upregulation was observed in both delaminated and epithelial cells. This also suggests that ER stress could be an early event of CIN.

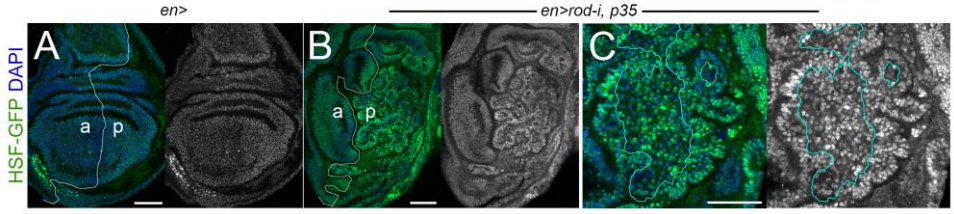

Figure 22. HSF levels are increased in CIN tissues

(A) Basal levels of HSF-GFP in wild type tissue **(B)** Higher level of HSF-GFP was observed in the CIN compartment **(C)** Similar levels of HSF-GFP were observed in delaminating as well as proliferating cells. Grey channel is HSF-GFP. (Scale bars, 50 μm).

PERK and IRE1 branches of UPR are known to activate autophagy in many different situations. (He and Klionsky, 2009, Senft and Ronai, 2015). RNAi mediated depletion of *perk, ire1, and xbp1* lowers the levels of autophagy induction in CIN epithelial tissues (**Figure 23**). This indicates that ER stress associated UPR activates autophagy in this situation.

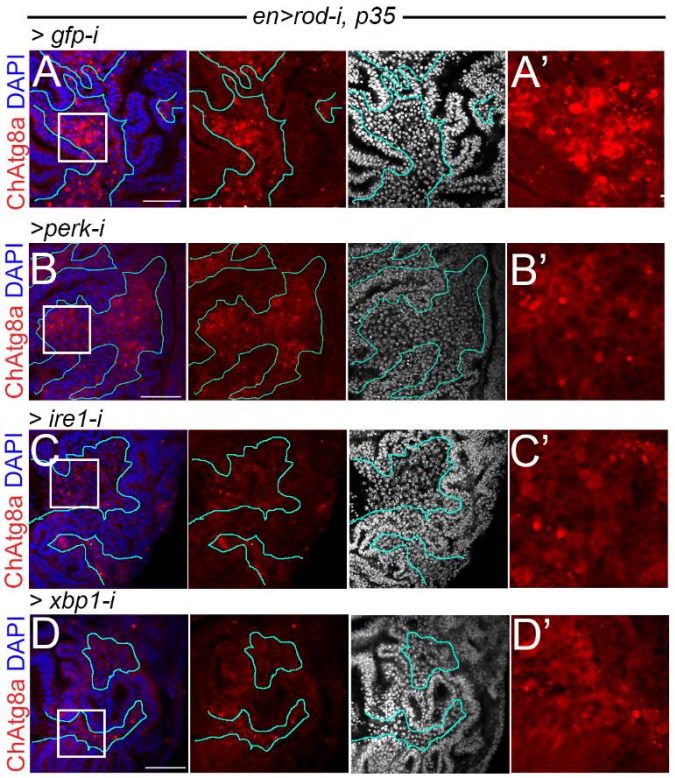

Figure 23. UPR regulates autophagy in CIN tissues

(A-D) RNAi mediated depletion of *perk, ire1, xbp1* in CIN tissues reduces the overall levels of autophagy induction **(B, C, D)**, monitored by ChAtg8 in red, as well as the highest levels observed in delaminating cells (**B', C', D'**), when compared to CIN-tissues expressing an RNAi form against *gfp* (**A**, **A'**). (Scale bars, 50 μm).

4.4 TOR and autophagy induction

The protein kinase Target of Rapamycin (TOR) plays a vital role in nutrient sensing and autophagy regulation. During nutrient availability state, TOR is activated through the Class 1 phosphatidylinositol-3-kinase (PI3K) signaling pathway, and TOR hinders autophagy through direct phosphorylation and repression of Atg1 (Scott et al., 2007, Pattingre et al., 2008). When nutrients are limited, TOR is inactivated, inhibition of Atg1 is relieved, and autophagy is induced. Another key pathway that is also known to activate the TOR complex is the amino acid-sensing Rag complex (Sancak et al., 2008). So the main two pathways that regulate TOR are the insulin pathway and amino acid sensing pathway (**Figure 24**).

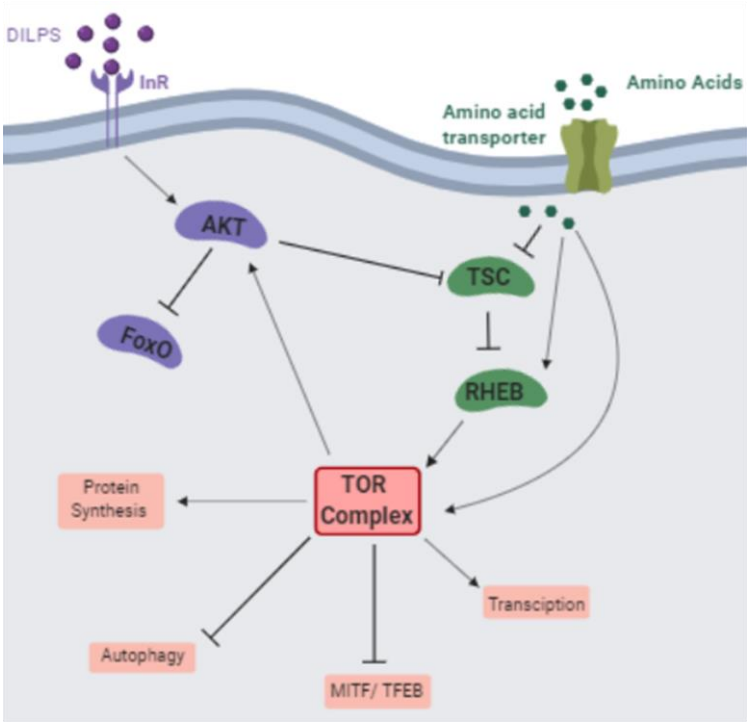

Figure 24. Nutrient sensing and TOR activation

Transcriptional stress response activated downstream of TORC1 inhibition is known to be mediated by REPTOR (Tiebe et al., 2015), similar to the role of FOXO downstream of AKT. TORC1 active state inhibits nuclear localization of REPTOR through phosphorylation. REPTOR-GFP (Tiebe et al., 2015) was used to observe TOR levels in-vivo. Levels of REPTOR-GFP was high in CIN tissues (**Figure 25B)**, especially in the delaminated population (**Figure 25B')**. This suggests that TOR is downregulated in highly aneuploid cells.

Amino acid pools are maintained by proteasome-dependent degradation. Proteasome inhibition is known to activate autophagy as a compensatory mechanism to replenish the amino acid pool (Suraweera et al., 2012). Proteasome saturation or impairment upon CIN could create an obstacle for amino acid pool maintenance and this could also be a potential way of downregulating TOR in delaminating cells. As TOR is known to inhibit autophagy, we checked whether the upregulation of the TOR pathway could reduce the levels of autophagy induction in the CIN tissues. Consistent with this assumption, overexpression of *Rheb* lowers the level of autophagy induction (**Figures 25 D, D')**.

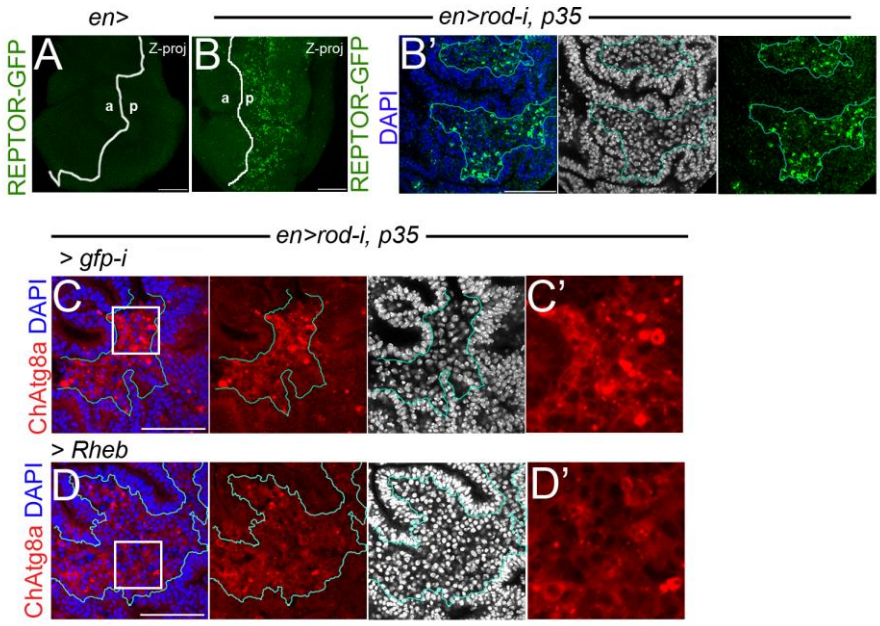

Figure 25. Nutrient sensing and TOR activation

(A-B) Reptor-GFP was observed in CIN tissues (**B**), and not in wild type tissues **(A)**. High levels of Reptor-GFP was observed in the delaminating cells of CIN tissues **(B')**. **(C-D)** Overexpression of *rheb* in CIN tissues reduces the overall levels of autophagy induction **(D)**, monitored by ChAtg8 in red, as well as the highest levels observed in delaminating cells (**D'**) when compared to CIN-tissues expressing an RNAi form against *gfp* (**C**, **C'**). (Scale bars, 50 μm).

5. Lysosome biogenesis in CIN tissues

The lysosomal-mediated autophagy pathway is a chief mechanism to maintain cellular homeostasis. The lysosome is a vital component of this system, which provides the acidic environment and hydrolases for autophagosomal cargo degradation after autophagolysosome formation (Klionsky and Emr, 2000). The basic helix-loop-helix leucine zipper (bHLH-Zip), transcription factor EB (TFEB), functions as a master regulator of lysosomal biogenesis and autophagy in mammals (Sardiello et al., 2009, Settembre et al.,

2011). TFEB orchestrates the expression of genes belonging to the coordinated-lysosomal-expression-and-regulation (CLEAR) network, through binding to specific CLEAR-box sequences situated close to their promoters (Palmieri et al., 2011). In *Drosophila*, Mitf, the single ortholog of TFEB and MITF (microphthalmia-associated transcription factor), controls the expression of vacuolar-type H^+-ATPase (V-ATPase) pump subunits and coordinate the lysosome biogenesis (Zhang et al., 2015, Tognon et al., 2016, Bouche et al., 2016). The Mitf$^{2.2}$-GFP reporter, which contains a 2.2 kb upstream DNA and 5´UTP driving nuclear GFP (Zhang et al., 2015), showed higher levels in CIN tissues (**Figures 26 B, B'**) compared to wild type. A synthetic Mitf target, 4Mbox-GFP (Zhang et al., 2015), an activity reporter, also showed higher levels in CIN tissues (**Figures 26 D, D'**).

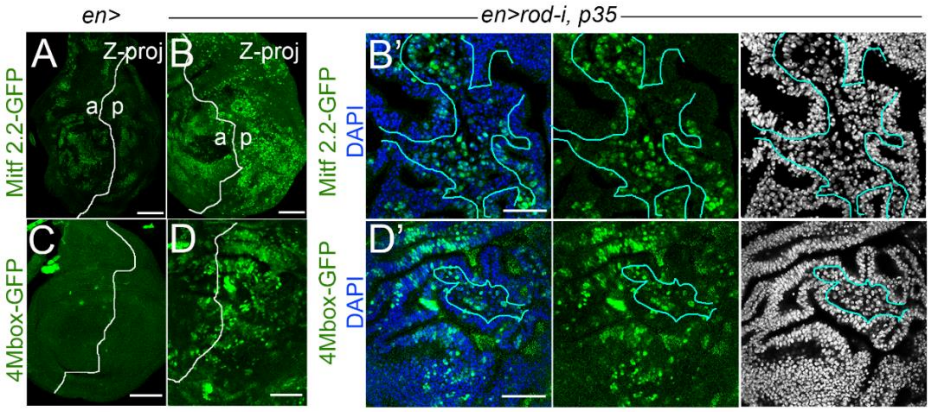

Figure 26. Mitf activation in CIN tissues

(A-B) Mitf$^{2.2}$-GFP expression in wild type (**A**). High levels of *Mitf$^{2.2}$-GFP* expression was observed in the CIN tissues **(B)**, especially in the delaminated cells**(B')**. **(C-D)** 4Mbox-GFP expression in wild type (**C**). High levels of 4Mbox-GFP expression were observed in the CIN tissues **(D)**, in both epithelial and delaminated cells **(D').** (Scale bars, 50 μm).

Results

We next tried to look at the lysosome biogenesis. To mark the lysosomes we used the acidophilic dye Lysotracker Red and tagged versions of Lysosomal-associated membrane protein 1 (LAMP1). Increased levels of Lysotracker (**Figure 27 B**), as well as LAMPI staining (**Figures 27 D, F**), were observed in CIN tissues, especially in the delaminated population (**Figures 27 B', D', F'**).

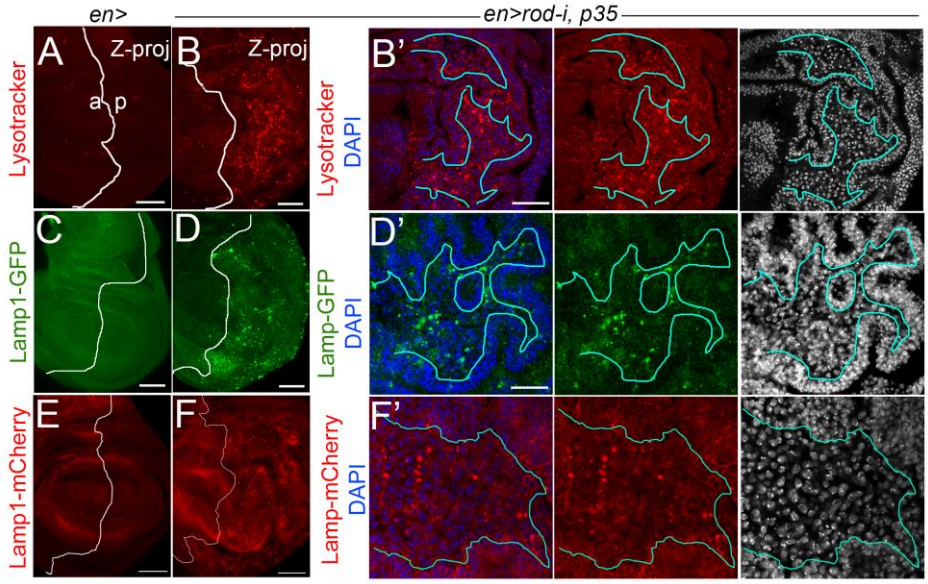

Figure 27. Increased lysosomal biogenesis in CIN tissues

(A, B, C, D, E, F) Increased levels of Lysotracker and Lamp1 was observed in the CIN, especially in the delaminated cells **(B', D', E')**. (Scale bars, 50 μm).

TFEB is known to regulate autophagy too (Settembre et al., 2011). Mitf regulates very few autophagy-related genes in *Drosophila* (Bouche et al., 2016), and it is not a very strong inducer of autophagy in the wing-disc (Tognon et al., 2016). Autophagy induction was still

observed in CIN tissues upon Mitf depletion (**Figure 28**), indicating that Mitf does not play a major role in the induction of autophagy.

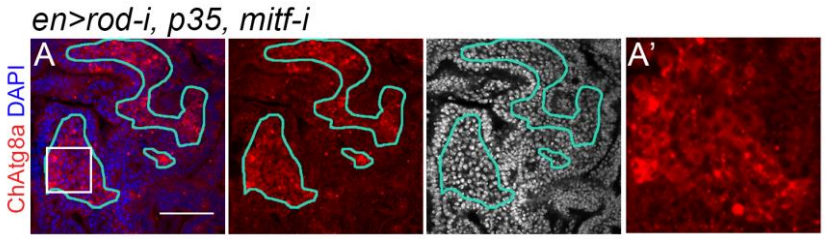

Figure 28. Mitf is not a major regulator of autophagy in CIN tissues

(A) RNAi mediated depletion of *mitf* doesn't reduce much the overall activation of autophagy, monitored by ChAtg8 in red (compared with **25 C**). Elevated levels of autophagy induction were observed in delaminated cells (**A'** compared with **25 C'**). (Scale bars, 50 μm).

6. Lysosome-mediated autophagy dampens proteotoxic stress and ROS production in CIN tissues

The lysosomal dependent degradative pathway is activated by multiple ways in CIN tissues. Although ubiquitinated cargoes are found in these tissues, autophagy flux is closed to saturation but functional. The main aim of these systems is to maintain proteostasis and cellular homeostasis. So, we next analyzed the functional relevance of these mechanisms in CIN tissues.

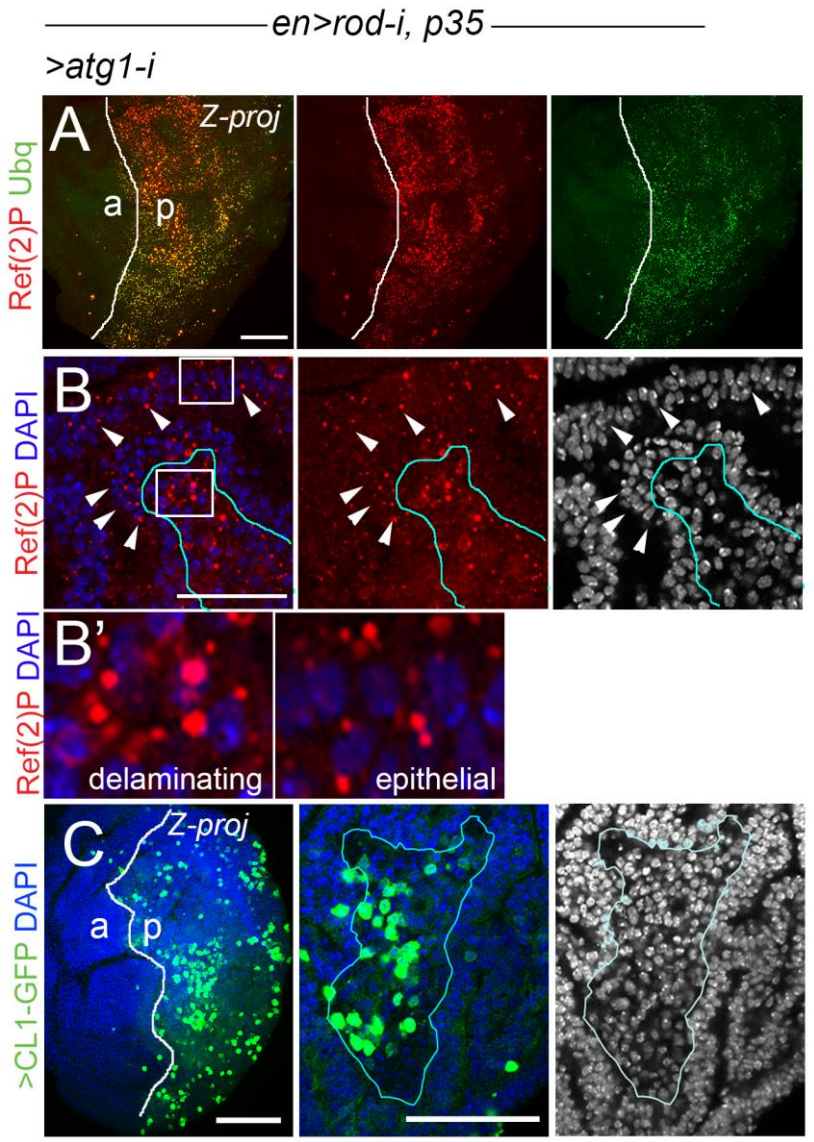

Figure 29. Autophagy dampens proteotoxic stress in CIN tissues

(**A-B**) RNAi mediated depletion of *atg1* enhances the accumulation of Ref(2)P and ubiquitin (Ubq) accumulation. Enhanced levels were observed in both epithelial and delaminated cells (**B, B'**). Similarly, depletion of *atg1* enhances the accumulation of CL1-GFP, especially in the delaminated cells (**C**). Arrowheads mark epithelial cells. (Scale bars, 50 μm).

RNAi-mediated inhibition of the core autophagy gene, *atg1*, resulted in elevated levels of Ref(2)P and ubiquitin accumulation in CIN-tissues (**Figure 29 A**, compare with **Figure 10A**). Moreover, Ref(2)P was accumulated in both epithelial and delaminated cells (**Figures 29 B, B'**, compared with **Figure 10B**). This points out that autophagy induction dampens the proteotoxic stress associated with CIN in the epithelial cells.

As shown previously, the in-vivo activity tracker of the proteasome, CL1-GFP, was accumulated in CIN tissues (**Figure 12 C**). Depletion of autophagy enhanced the accumulation of CL1-GFP in the tissue (**Figure 29 C,** compared with **12 C, D**). This also indicates that autophagy is being used as a compensatory mechanism to degrade the proteins directed to the proteasome and that this helps cells to deal with proteotoxicity upon proteasome saturation or impairment.

As previously mentioned, the main driver of tissue-overgrowth upon CIN in epithelial tissue is the stress response pathway, JNK, which is activated by ROS. The depletion of autophagy or lysosomal production enhanced the levels of ROS production and tissue growth (**Figure 30**). As previously mentioned, gstD-GFP was used to quantify ROS production. Posterior to anterior (P/A) ratio was used to measure the tumor growth.

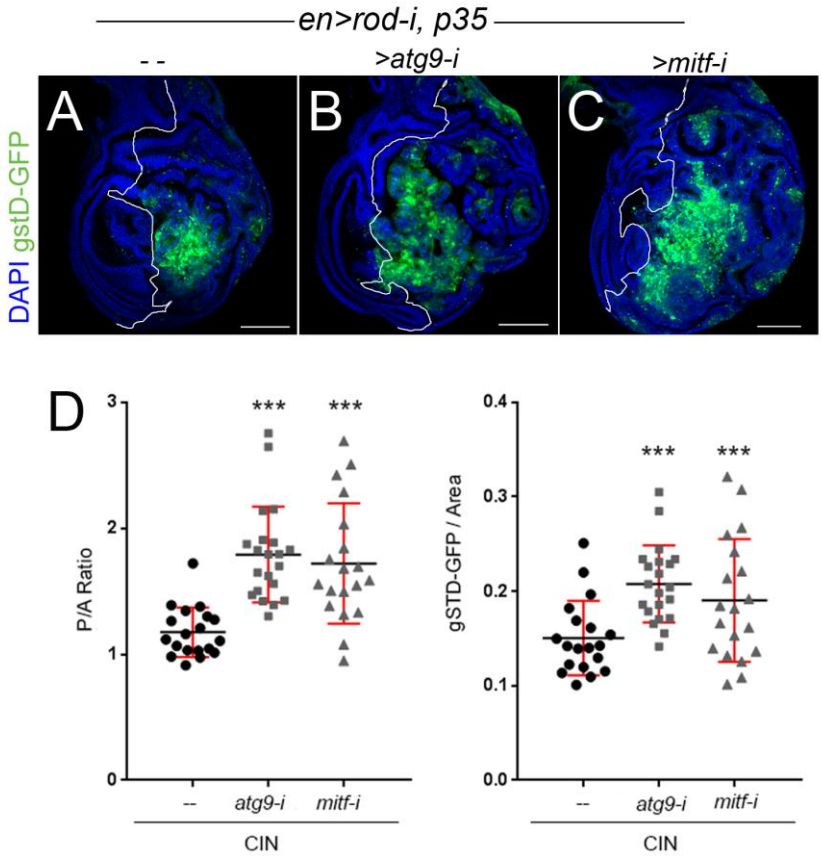

Figure 30. Depletion of lysosomal-mediated autophagy enhances ROS production and tissue-overgrowth in CIN tumors

(**A-C**) RNAi mediated depletion of *atg9* or *mitf* enhances the gstD-GFP levels and promotes tissue-overgrowth. (**D**) Quantification of tissue overgrowth and gstD-GFP levels. (Scale bars, 50 μm). Error bars indicate SD (*** P<0.001)

7. Mitochondrial homeostasis is affected in CIN tissues

7.1 Mitochondrial dynamics in CIN tissues

Mitochondria are very dynamic organelles and their accurate function is vital for the maintenance of cellular homeostasis. Two major mechanisms that maintain mitochondrial homeostasis are mitochondrial biogenesis and mitophagy. Cellular adaptation to many stressful situations is maintained by the tight control of these two major mechanisms. Mitochondrial dynamics, defined as a balance between mitochondrial fission and fusion, and its alteration has been associated with many disease conditions. Moreover, dysfunctional regulation of mitochondrial dynamics is one of the main sources of mitochondrial impairment, which leads to oxidative stress and cell death in many situations (Seo et al., 2010)

Mitochondrial dynamics were altered in CIN tissues, as observed with MitoGFP, a mitochondrial reporter (**Figure 31**). Mitochondria were accumulated in the delaminated cell population (**Figure 31C**) and a fragmented mitochondrial pool was observed (compare **Figures 31 B** and **D**). This suggests that more mitochondrial biogenesis is happening upon CIN and mitochondrial fission is highly favored.

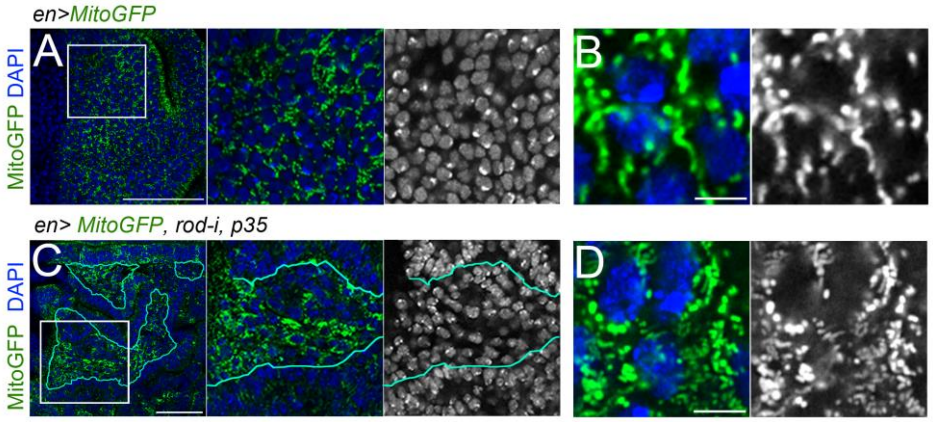

Figure 31. Mitochondrial dynamics are altered in CIN tissues

(**A-B**) MitoGFP in wild type situations showing a homogenous pattern (**A**). A higher magnification image shows that most of the mitochondria are in the elongated form (**B**). (**C-D**) MitoGFP in the CIN situation showing a heterogeneous pattern. More mitochondrial accumulation was observed in delaminated cells (**C**). A higher magnification image shows that most of the mitochondria are in the elongated form (**D**). (Scale bars, 50 μm (A,C), 5 μm (B,D)).

7.2 Presence of oxidized mitochondria in CIN tissues

Mitochondria are defined as the powerhouse of the cell and major cellular metabolism happens here. One of the end products of metabolism is superoxide (O_2^-), which is created by one-electron reduction of oxygen (O_2). ROS are molecules derived from O_2 that can freely oxidize additional molecules. Eight sites in mitochondria could generate O_2^- and deposit it in the mitochondrial matrix (Brand, 2010). However, only complex III and glycerol 3-phosphate dehydrogenase can discharge O_2^- to intermembrane space and could easily escape to the cytosol (Muller et al., 2004). Mitochondrial ROS (mtROS) is tightly regulated in a healthy cell. Superoxide dismutases (SODs) convert O_2^- to hydrogen peroxide (H_2O_2) and further

converted to water (H_2O) by peroxiredoxins (PRXs), glutathione peroxidases (GPXs) and catalases (CATs). mtROS are known to determine many cell fates apart from oxidative damage (Sena and Chandel, 2012).

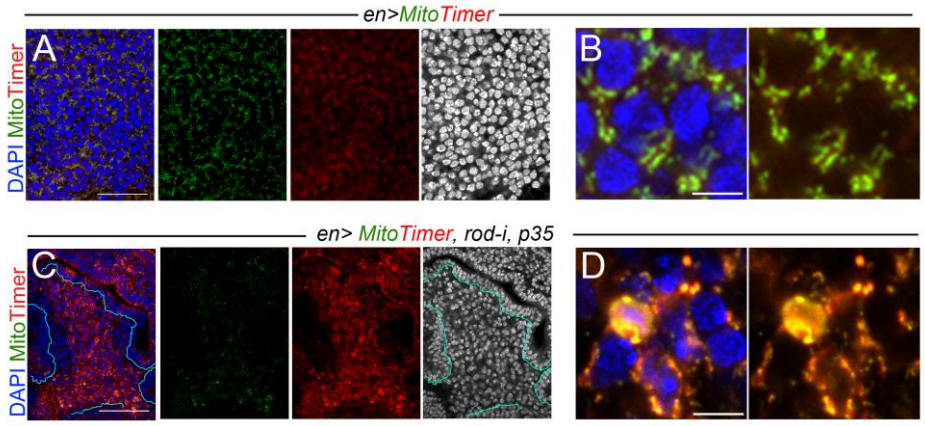

Figure 32. Mitochondrial dynamics are altered in CIN tissues

(**A**) MitoTimer in wild type situation. Fluorescence in both channels could be seen and predominantly in the green channel. (**B**) A higher magnification image of wild type situations showing mostly healthy mitochondria. (**C**) MitoTimer in CIN situation. Fluorescence was mostly observed in the red channel suggesting the oxidative state of mitochondria. (**D**) A higher magnification image of CIN tissue showing mostly fragmented and oxidized mitochondrial pool. (Scale bars, 50 μm (A,C), 5 μm (B,D)).

An in-vivo mitochondrial reporter, MitoTimer (Laker et al.,2014), was used to analyze the healthy state of mitochondria. MitoTimer encrypts a mitochondria-targeted green fluorescent protein that shifts to red upon oxidation. In detail, the mitochondria-targeting sequence of the cytochrome c oxidase subunit is attached to the N-terminus of fluorescent Timer protein (DsRED1-E5). Following oxidation (dehydrogenation) of the Tyr-67 residue, Timer shifts its

fluorescence. MitoTimer is used to analyze mitochondria content, structure, stress, and damage. Compared to wild type, mitochondria in CIN tissues were more oxidized (**Figure 32**). Consistent with Mito-GFP data, mitochondria were accumulated and highly fragmented in CIN-induced delaminated tissues (compare **Figure 32D** with **32B**).

7.3 Mitophagy is saturated in CIN tissues

The presence of fragmented and oxidized mitochondria in CIN tissues prompted us to look for mitochondria positive for autophagy markers. Fragmented and accumulated mitochondria present in the tissue were positive for the autophagy adaptor Ref(2)P (**Figures 33A, A'**). Interestingly, super-resolution microscopy analysis revealed that the large autophagic cargoes present in the tissue were actually filled with fragmented mitochondria (**Figures 33B, B'**).

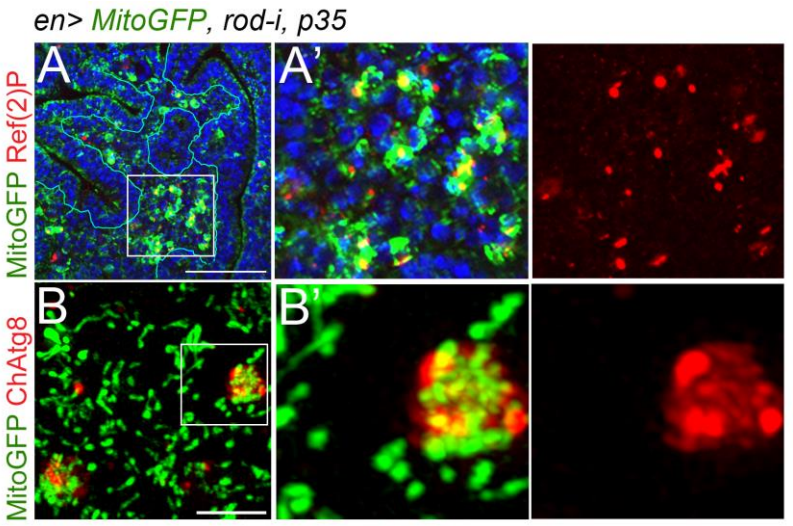

Figure 33. Fragmented mitochondria were positive for autophagy markers

(**A**) Accumulated and fragmented mitochondria present in the delaminated population of CIN tissues were positive for autophagy marker P62/Ref(2)P. (**A'**) A magnification showing colocalization of Mito-GFP with Ref(2)P. (**B**) Accumulated and fragmented mitochondria were colocalizing with autophagy marker ChAtg8a. (**B'**) A higher magnification showing the accumulation of fragmented mitochondrial pool in bulky autophagy cargo. (Scale bars, 50 μm (A), 5 μm (B)).

Damaged mitochondria are removed by selective autophagy, commonly known as mitophagy. PINK1/Parkin-mediated mitophagy has been widely studied and the mechanisms are well described (Pickrell and Youle, 2015, Ashrafi and Schwarz, 2013). Parkin, a cytosolic ubiquitin E3 ligase, and PINK1, a mitochondria-directed kinase, play the key roles here. Loss of mitochondria potential leads to the accumulation of PINK1 on the outer mitochondria membrane and further phosphorylate ubiquitin and parkin. This stimulates parkin´s E3-ligase activity, thus placing more ubiquitin for successive phosphorylation. Later, mitochondria labeled with phospho-ubiquitin-chains will be recognized by the autophagic adaptor which results in autophagosome formation.

Results

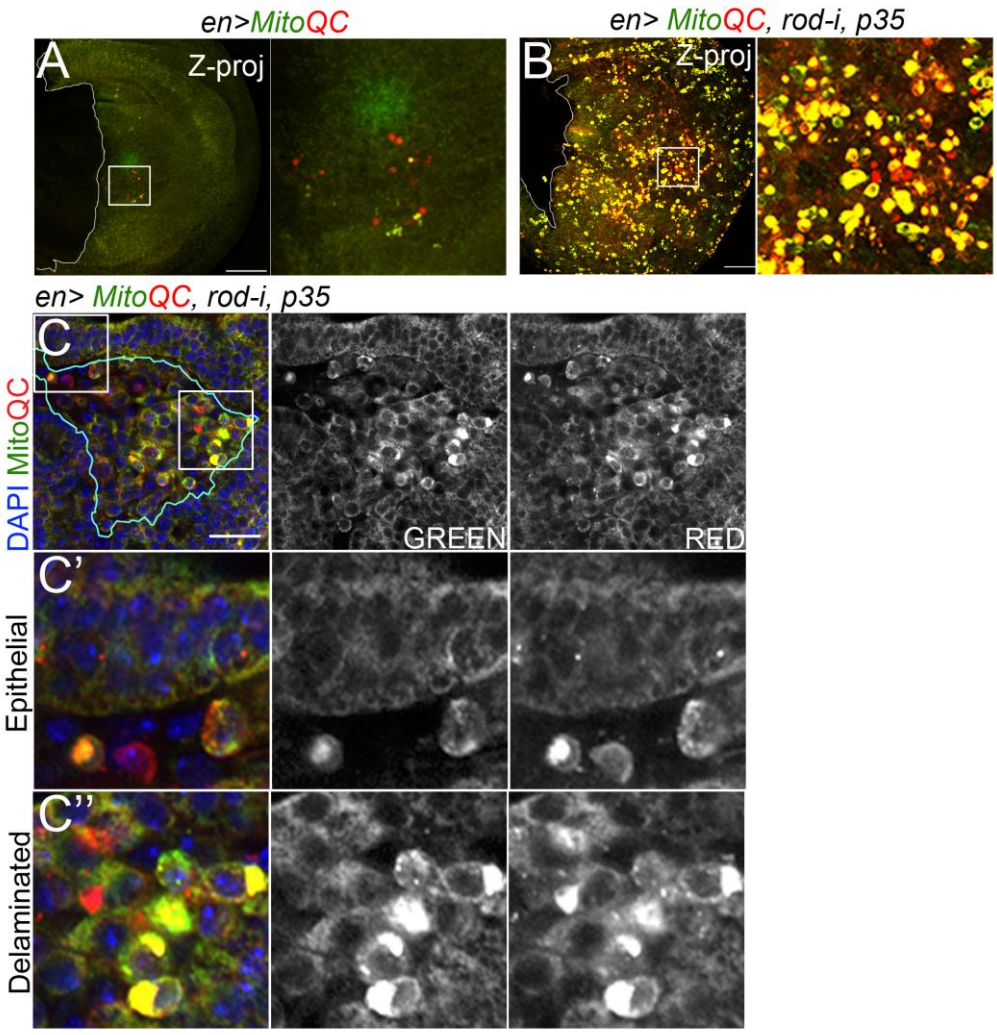

Figure 34. Mitophagy is saturated in CIN tissues

(**A**) MitoQC in wild type situation, showing a few mitophagy inductions in wing discs (**B**) MitoQC in CIN situation, showing enhanced levels of mitophagy induction, as well as mitophagy saturation, observed by yellow dots. (**C**) Yellow big dots were observed in delaminated cells, suggesting mitophagy saturation. (**C'**) A magnification image showing that mitophagy is also induced in epithelial cells and flux looks normal. (**C''**) A magnification image showing bulky and saturated mitophagy cargoes in the delaminated cells. (Scale bars, 50 μm).

An in-vivo mitophagy reporter, called Mito-QC (Lee et al.,2018) was used to analyze mitophagy in CIN tissues. This reporter exploits pH-sensitive characteristics of GFP, where a tandem GFP-mCherry fusion protein is targeted to the outer mitochondria membrane. So, red-only puncta marks the mitolysosomes. Enhanced levels of mitophagy induction were observed in CIN tissues (**Figure 34B**), compared to the wild type situation (**Figure 34A**). Moreover, large mitophagy vesicles positive for both colors were observed, which suggests that mitophagy is saturated upon CIN (**Figure 34B, C, C''**). Few ´red-only´ dots were observed in the epithelial cells (**Figure 34C'**). In the delaminated population, big mitophagy cargoes that are not fused with lysosomes were mostly observed. Interestingly, some ´red-only´ puncta of small and big sizes were also observed in the delaminated cells (**Figures 34C, C''**). All these results suggest mitophagy is, to a certain extent, saturated in CIN tissues.

8. ROS drives cell delamination in CIN tissues

8.1 Mitochondrial ROS and calcium

Prior results suggest that the mitochondria population in CIN tissues is fragmented as well as oxidized. The turnover of mitochondria is affected due to mitophagy saturation. This could result in the maintenance of damaged mitochondria in the cell and might be a source of ROS in CIN tissues. Mitochondria fragmentation or dysfunction seems to be a key event in the process. Fragmented or damaged mitochondria positive for Ref(2)P, which is a characteristic feature of delaminated cells, were observed in some epithelial cells.

This suggests that it could be an event prior to delamination initiation (**Figure 35**).

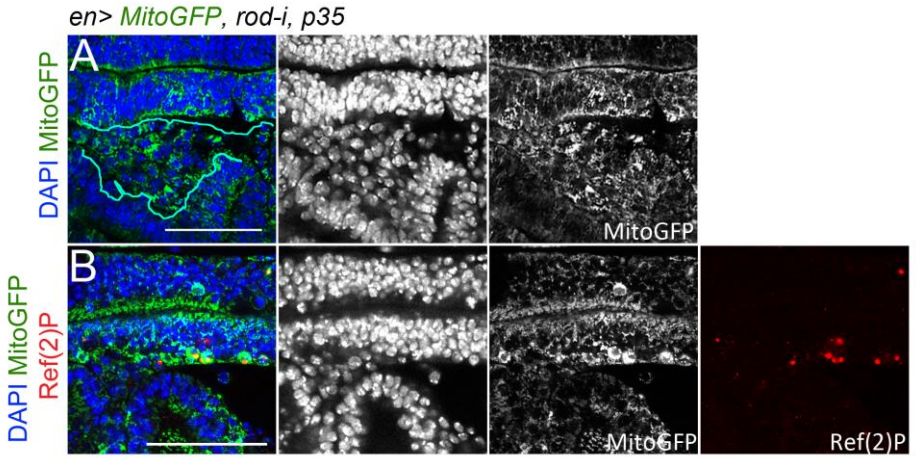

Figure 35. Mitochondrial fragmentation seems to be an early event in CIN tissues

(**A-B**) The fragmented or accumulated mitochondrial population was observed in epithelial cells (**A**), and they were positive for Ref(2)P (**B**). (Scale bars, 50 μm).

Prior results also demonstrated that ER stress is a salient feature of CIN tissues (**Figure 21**). ER is also a major storehouse of calcium (Ca^{2+}), a second messenger for many signaling pathways. ER has close contact with mitochondria, and are also known to store and receive calcium from ER for physiological processes (Clapham, 2007). ER stress is known to release Ca^{2+}, and it could be highly possible that adjacent organelles like mitochondria will experience a heavy Ca^{2+} influx. Moreover, mitochondrial Ca^{2+} overload is known to generate ROS and apoptosis (Giorgi et al., 2012, Gorlach et al., 2015). Genetically encoded calcium indicators (GECIs) are versatile tools for calcium-sensing (Tian et al., 2009). GCaMP is a GECI,

created from the fusion of GFP, Calmodulin, and M13, a peptide sequence from myosin light chain kinase. Cytoplasmic-GCaMP reporter revealed that there is an increased Ca^{2+} in CIN tissues, and this increase was observed in the two cell populations (**Figures 36 B, B'**). This suggests that ER stress could be an early event. We also looked at the levels of Ca^{2+} in mitochondria using Mito-GCaMP. Ca^{2+} levels were high in mitochondria on both epithelial as well as delaminated cells (**Figures 36 D, D', D'', D'''**).

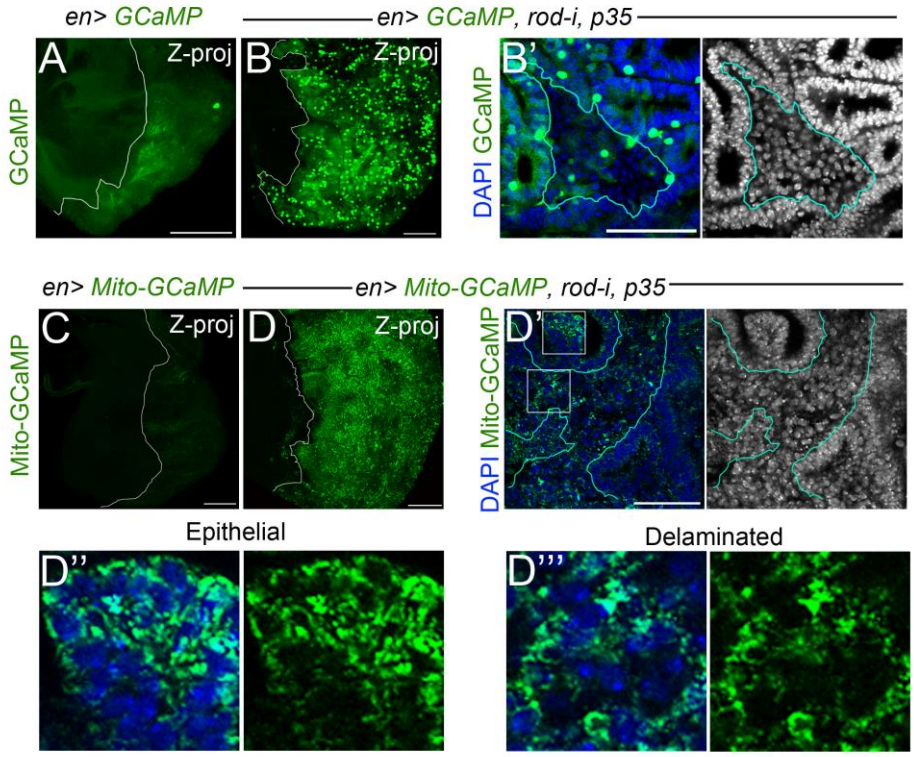

Figure 36. Altered Ca^{2+} levels in CIN tissues

(**A**) GCaMP levels in wild type. (**B**) High GCaMP levels were observed in CIN tissues. (**B'**) A higher magnification image showing that GCaMP levels were high in epithelial cells. (**C**) Mito-GCaMP levels in wild type. (**D**) High Mito-GCaMP levels were observed in CIN tissues. (**D'**) A higher magnification image showing

that high Mito-GCaMP levels were observed in both epithelial (**D''**) as well as delaminated cells (**D'''**). (Scale bars, 50 μm).

8.2 ROS and Src activation

Cell delamination in CIN tissues is characterized by loss of apical-basal polarity and delocalization of DE-cadherin (Dekanty et al., 2012). The blockade of the JNK pathway in CIN tissues does not rescue cell delamination, suggesting that cell delamination is an upstream event (Dekanty et al., 2012). ROS is known to activate Src tyrosine kinases (Gianonni et al., 2005). Src family kinases are active in a wide range of cancer types and promote metastasis (Irby and Yeatman, 2000, Yeatman, 2004). Src is also known to promote tumor growth in *Drosophila* tissues, and mostly through the activation of the JNK pathway (Stewart et al., 2003, Vidal et al., 2006, Enomoto and Igaki, 2012, Poon et al., 2018). In *Drosophila*, Src is known to activate the JNK pathway to regulate cell shape change and Src affects the F-actin organization (Tateno et al., 2000). A recent study in a Drosophila model of wound healing (Hunter et al., 2018) demonstrates that Src is activated by ROS and that activated Src rearranges cell junctions and the cytoskeleton. Increased levels of mitochondrial Ca^{2+} influx were the cause of mtROS production in the model (Xu and Chisholm, 2014, Hunter et al., 2018), where ROS-dependent Src activation contributes to E-cadherin and myosin polarization around the wounds.

Drosophila has two Src family homologs, Src42A and Src64B. In CIN tissues, mislocalized expression of Src and E-cadherin was observed, especially in the delaminated cells, like dots (**Figures 37**

B, B', C). Interestingly, RNAi mediated depletion of both Src42A and Src64B rescued the cell delamination in CIN tissues (**Figures 37 C, D**). Moreover, JNK activation observed in the CIN tissues was also rescued (**Figures 38 A, B)**.

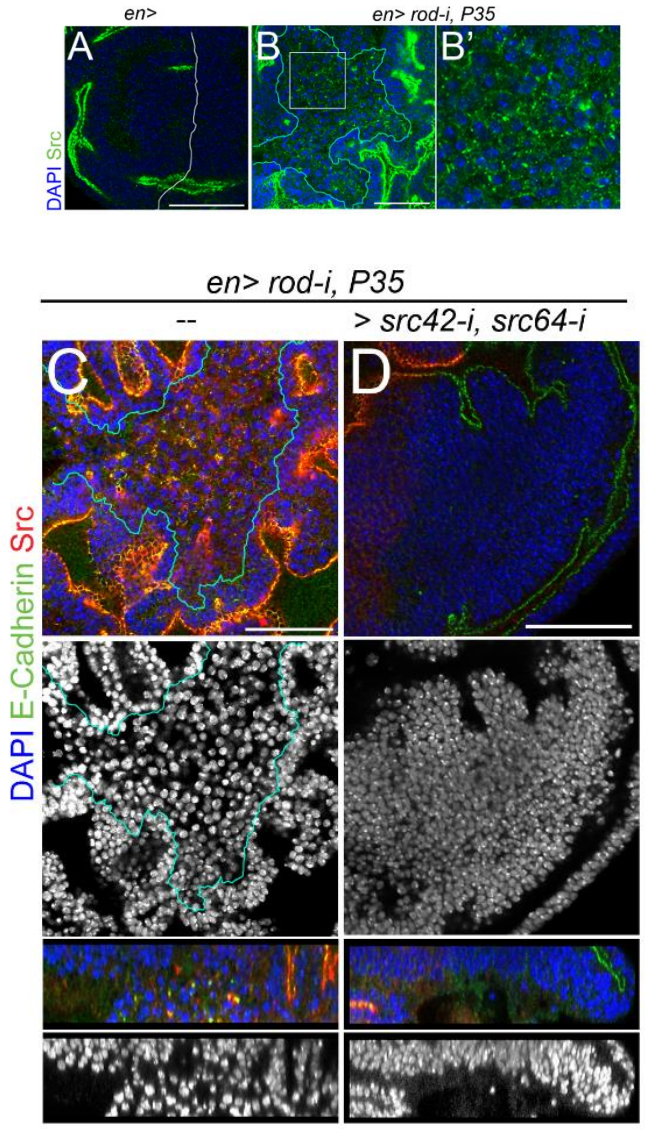

Figure 37. Depletion of Src rescues cell delamination in CIN tissues

(**A**) Src staining in wild type. (**B- B'**) Mislocalized Src staining was observed in delaminated cells. (**C**) Colocalization of E-Cadherin and Src was observed in CIN tissues. Orthogonal session showing the cell delamination upon CIN. (**D**) RNAi mediated depletion of *src42* and *src64* rescues cell delamination and loose cell morphology observed in CIN tissues. (Scale bars, 50 μm).

A recent study from our lab has demonstrated the JNK dependent migratory behavior of CIN cells (Benhra et al., 2018). A key feature was the upregulation of the actin-myosin cytoskeleton. Inhibition of the JNK pathway rescued the migratory phenotype and enhanced levels of myosin, but not the levels of actin. Consistent with the already known functions of Src, RNAi mediated depletion of both Src42A and Src64B rescued the enhanced levels of F-actin in the CIN tissues (**Figures 38 C, D**).

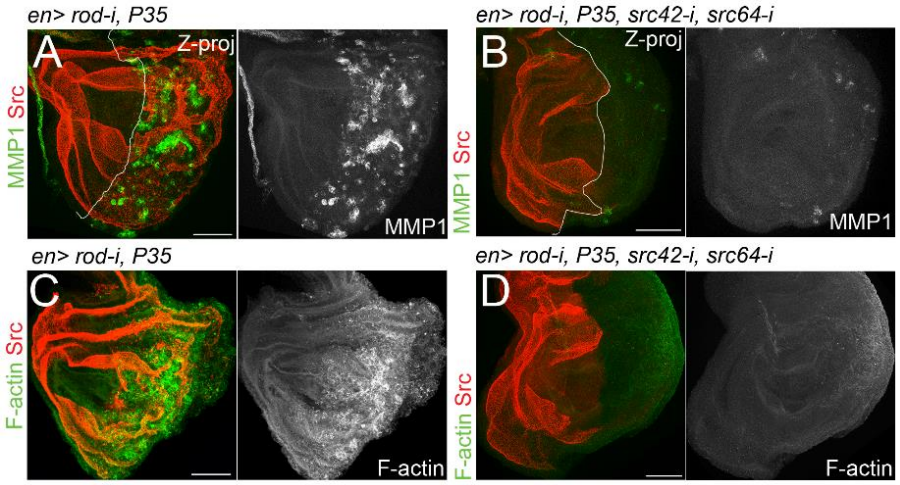

Figure 38. Depletion of Src rescues JNK activation as well as F-actin levels

(**A, C**) Levels of JNK (monitored using MMP1) and F-actin in CIN tissues. (**B, D**) RNAi mediated depletion of *src42* and *src64* rescues both JNK and F-actin levels in CIN tissues. (Scale bars, 50 μm).

9. Tissue homeostasis in CIN tissues

So far, we have utilized the wing epithelium of Drosophila to demonstrate that CIN-induced aneuploid cells undergo Src-dependent cell delamination and JNK activation and that these two events are a consequence of the production of ROS. We have also delineated the pathway from the generation of aneuploidy karyotypes to the production of ROS, where we have identified the major protein-quality control mechanism dampening aneuploidy-induced proteotoxic stress and propose the mitochondria as the major source of ROS. As stated at the beginning of the results section, all these mechanisms were molecularly dissected in a setting where apoptosis was blocked by the expression of the Caspase inhibitor p35. In order to functionally validate the roles of these protein quality control mechanisms in dampening aneuploidy-induced proteotoxic stress, reinforce our proposal that mitochondria are the major source of ROS and identify the Achilles heel of CIN-tissues, we used another CIN-induced experimental setting in which apoptosis was not blocked. For this purpose, we used the eye epithelial primordium to induce CIN and score the easy-to-score adult eyes to genetically test our candidate genes. Even though depletion of the SAC gene *bub3* results in a dramatic response of the tissue in terms of cell death (Dekanty et al, 2012), the impact on the size of the resulting adult eyes was rather weak (**Figure 39**), most probably as a consequence of the activation of stress-response pathways driving compensatory proliferation and dampening the deleterious effects of CIN-induced aneuploid karyotypes. Due to the random nature of CIN, the eye phenotype

could be divided into two groups: (1) weak phenotype (~ 60-80%), and (2) strong phenotype (~ 20-40%).

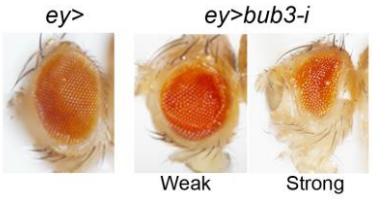

Figure 39. Induction of CIN in eye epithelial tissues

RNAi-mediated depletion of the main protein quality mechanisms like proteasome, chaperones or autophagy in the wild type situation did not give any phenotype in the eyes. By contrast, the depletion of the same genes in CIN eyes gave a strongly reduced eye phenotype (**Figure 40**).

Depletion of various regulatory particle non-ATPase (*rpn*) of proteasome system as well as septin interacting protein 3 (*sip3*), which encodes a transmembrane protein in the ER membrane with a ubiquitin ligase activity resulted in strong eye phenotype. The depletion of core particles of the proteasome was lethal in wild type situation (data not shown). Similarly, depletion of subunits of chaperones (*hsp70, hsc70*) also resulted in strong eye phenotypes.

As previously mentioned, macroautophagy is a multistep process and depletion of autophagy genes belonging to various steps such as induction (*atg1, atg13*), nucleation (*atg6, atg18, atg2*), elongation (*atg8*), completion (*atg12*), and fusion to the lysosome (*syn17*) resulted in a strong eye phenotype. Moreover, the depletion of autophagy adaptor (*ref(2)P*), the master regulator of lysosomal

biogenesis (*mitf*) and mediator of mitophagy (*park*) also resulted in strong eye phenotypes.

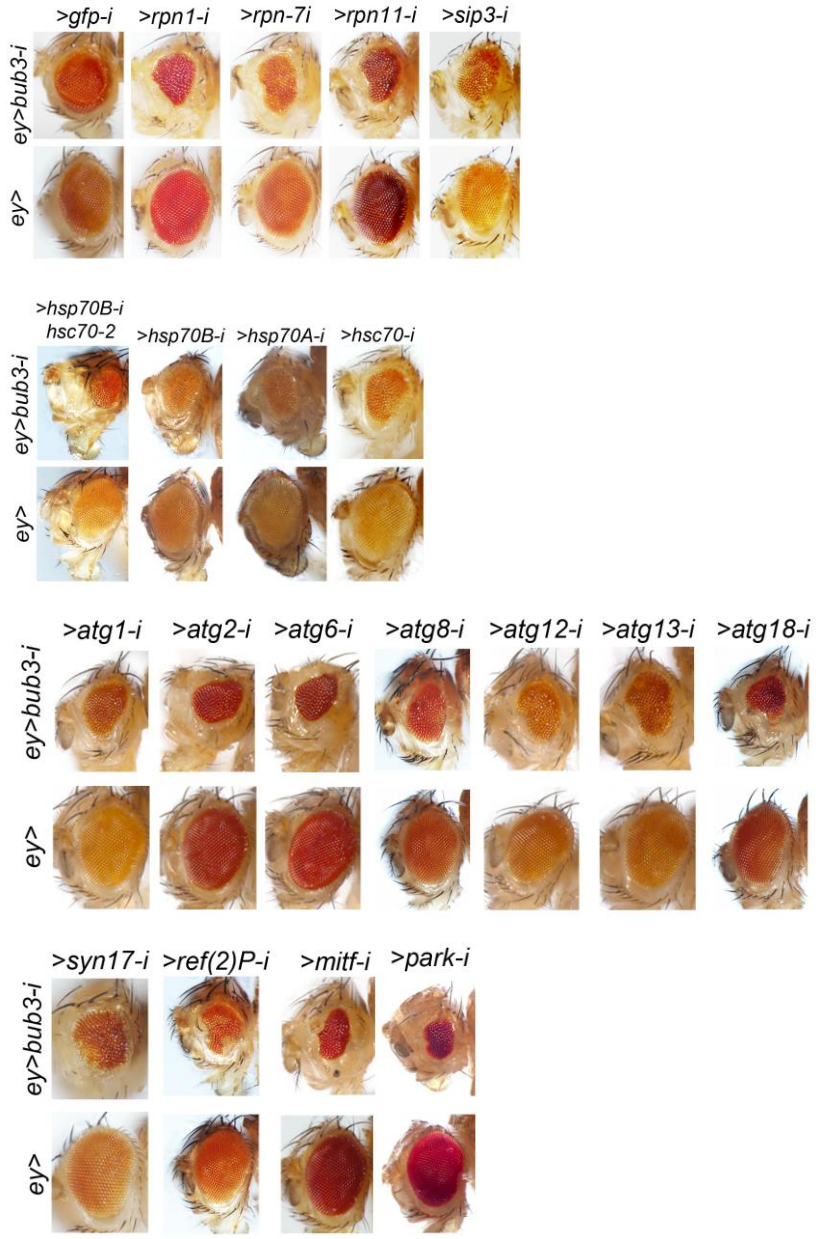

Figure 40. Protein quality mechanisms maintain tissue homeostasis upon CIN

Results

Consistent with the previous results, blockade of autophagy, lysosomal biogenesis or mitophagy in CIN tissues enhanced the levels of cell death, which was monitored using cleaved-Dcp1 staining (effector caspase) in the wing epithelium (**Figure 41**). Here, CIN was induced in the dorsal compartment using the *apterous* driver (marked with MyrTomato). Similarly, overexpression of the TOR pathway (*rheb* overexpression), which is known to inhibit autophagy induction, enhances the levels of cell death. On the other hand, depletion of TOR (*rheb-i* and *tor-i)*, as well as upregulation of mitophagy (*park* overexpression), rescued the levels of cell death. Not much cell death was observed in the control situation (data not shown).

Proteostasis is a major issue in the CIN situation. The depletion of protein quality control mechanisms in CIN tissues largely affected the tissue homeostasis. On the other hand, the enhancement of these mechanisms seems to improve tissue homeostasis. Consistent with this notion, overexpression of the ER chaperone (*hsc70*) or overexpression of *ref(2)P* in order to enhance autophagy rescued the eye phenotypes (**Figure 42**).

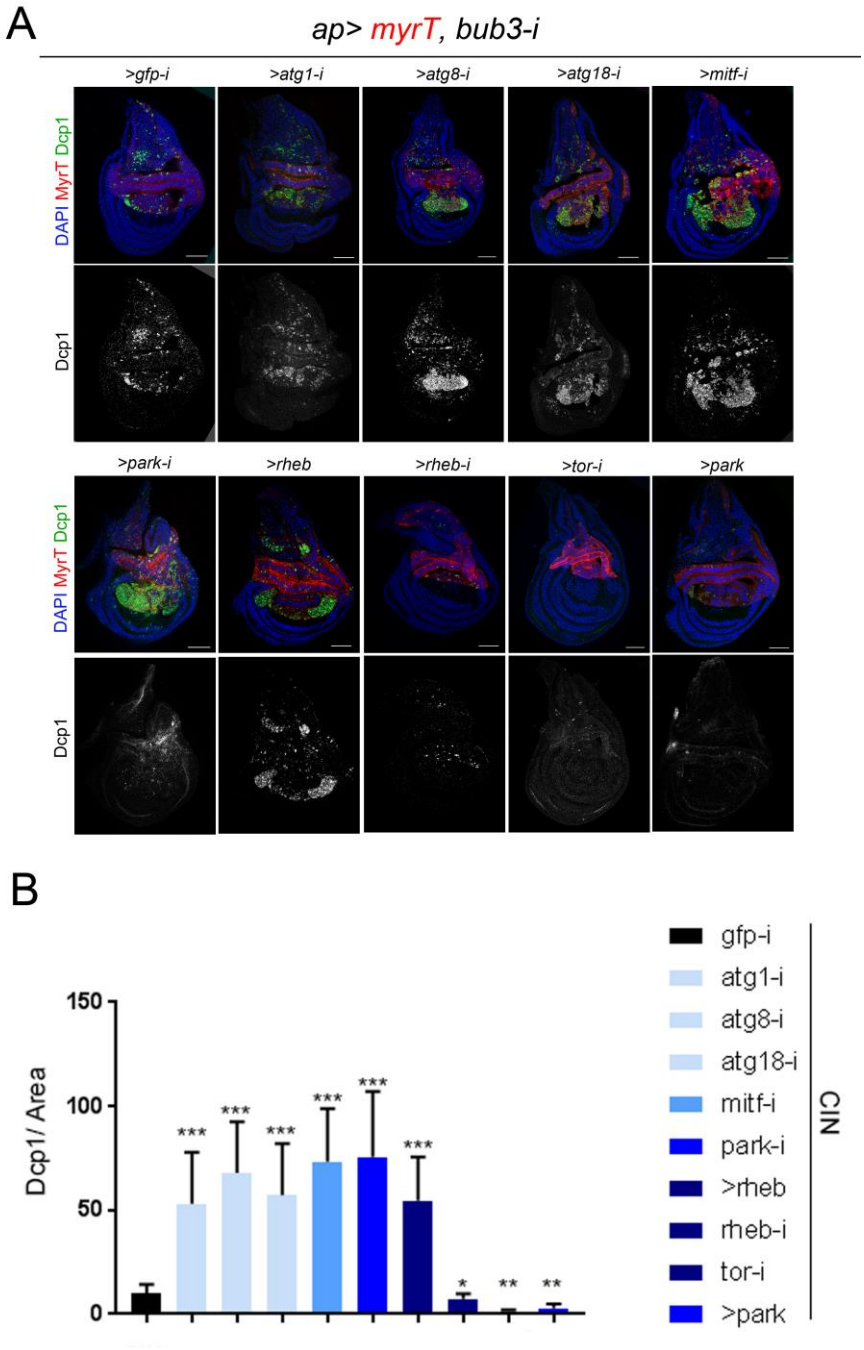

Figure 41. Autophagy inhibition enhances cell death in CIN tissues

Results

(A)RNAi mediated depletion of autophagy, lysosomal biogenesis, mitophagy or overexpression of the TOR pathway enhanced the levels of cell death in CIN tissues. Blocking the TOR pathway or upregulating mitophagy rescues cell death observed in CIN tissues. (Scale bars, 50 μm). Error bars indicate SD, p values were less than 0.001 (***), 0.01 (**), or 0.05 (*).

Mitochondria was the organelle that seems to be largely affected by CIN. Quenching of the mtROS by overexpressing the superoxide dismutases (*sod2*) and glutathione peroxidase (*GTPx-1*) rescued the eye phenotype (**Figure 42**). We next checked whether mitochondria act as a sensing and signaling center in CIN tissues. More precisely, whether mitochondria per se could sense the proteotoxic stress. In that situation, there should be activation of mitochondrial UPR (mtUPR) in the tissues. A recent study in *Drosophila* demonstrated that Phosphoglycerate Mutase-5 (PJAM5) is a mediator of mtUPR (Jensen et al., 2017). The depletion of *pjam5* resulted in a strong eye phenotype. On the other hand, the upregulation of mitochondrial chaperones (*hsp60, hsp60c*) rescued the eye phenotypes (**Figure 42**). Mitochondrial homeostasis seems to be a key thing in the CIN tissues. The inhibition of the deubiquitinating enzyme USP14 is known to increase proteasomal function and promote mitophagy (Chakraborty et al., 2018). Consistent with this, the depletion of *usp14* also rescues the eye phenotype (**Figure 42**).

Mitochondrial homeostasis, as well as proteostasis, are central for maintaining cellular physiology and tissue homeostasis in CIN tissues. Boosting the levels of protein quality control mechanisms ameliorates the deleterious effects of CIN.

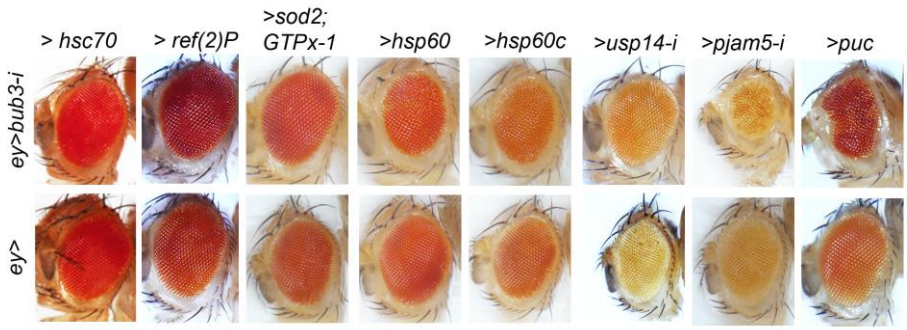

Figure 42. Mitochondrial homeostasis is critical for tissue homeostasis upon CIN

DISCUSSION

1. CIN epithelial model in the context of tumorigenesis

CIN is a salient feature of the majority of human solid tumors. While CIN promotes the gain of oncogene-carrying chromosomes and the loss of tumor-suppressor-gene-carrying chromosomes in certain cancers, its impact on the physiology of the cell and on the homeostasis of the tissue has not been well elucidated. In this work, I characterized the link between aneuploidy-associated gene dosage imbalance and activation of the stress response pathway, JNK, in *Drosophila* epithelial tissues subjected to CIN. Prior studies from the lab have demonstrated that JNK dependent tumorigenesis occurs in CIN tissues (Dekanty et al., 2012, Clemente-Ruiz et al., 2016, Muzzopappa et al., 2017, Benhra et al., 2018), and JNK activation was ROS dependent (Clemente-Ruiz et al., 2016, Muzzopappa et al., 2017). However, links between aneuploidy and ROS production were missing. This work unravels the complex pathways underlying the CIN-induced tumorigenesis, especially how gene dosage imbalance is translated to ROS production in *Drosophila* epithelial tissue.

The availability of splendid genetic tools makes *Drosophila* epithelial tissue a wonderful model to study CIN-induced tumorigenesis. Compartment specific creation of CIN situation helps us to study the autonomous and non-autonomous effects in the tissue quite easily. In the present study, most of the experiments were carried out in the posterior compartment and

the anterior compartment serves as an internal control. Depletion of SAC genes and maintenance of aneuploid cells by blocking apoptosis makes two populations of cells in the same tissue: (1) low-aneuploid-epithelial cells and (2) highly-aneuploid-delaminated cells. This mosaic situation in the tissue helps us to track many markers in a spatio-temporal manner and to characterize the cellular behaviors of aneuploid cells in a tissue context.

Consistent with observations in various other aneuploidy models, this work also demonstrates that aneuploidy-associated gene dosage imbalance and the corresponding protein imbalance lead to proteotoxic stress in *Drosophila* CIN tissues (**Figure 10**). This proteotoxic stress situation activates multiple protein quality control mechanisms to dampen the stress situation. The continuous and overwhelming production of proteins results in proteasome saturation in CIN tissues (**Figure 12**). The catabolic pathway of the cell, autophagy, is induced in CIN tissues (**Figure 13**) and multiple pathways seem to regulate its activation in this context. Although protein quality systems are working and necessary protein homeostasis mechanisms to deal with the proteotoxic stress are initiated, ROS production was still an issue.

Mitochondrial homeostasis was largely affected in CIN tissues, especially the turn-over of damaged mitochondria. This work suggests that mitochondrial homeostasis impairment is the key reason for ROS production in CIN tissues (**Figures 32, 34**). A central player for CIN-induced tumorigenesis is the ROS

generated in CIN tissues. ROS in one hand drive the delamination of highly aneuploid cells through activation of Src kinase and on the other hand also activate the JNK pathway through both Src and Ask1. Delamination creates the mosaic situation in CIN tissues and cross-feeding interactions between the two cell populations result in the uncontrolled growth of tissues. More studies are required to understand why specifically mitophagy is largely affected in CIN tissues.

This work also suggests that Ca^{2+} levels are altered in CIN tissues. ER stress due to aneuploidy could be the primary reason for this. Leakage of Ca^{2+} from the ER and influx into adjacent mitochondria could be a possible explanation for mitochondrial dysfunction and the presence of high mitochondrial calcium. Additionally, it could be possible that mitochondria could directly sense and be affected by proteotoxic stress, which could also lead to mitochondrial dysfunction. Exhaustive epistatic studies about calcium signaling and mtUPR are required to clearly understand their role in CIN context.

The beauty of the epithelial CIN model is that one could study both cellular behaviors and tissue homeostasis. Epithelial tissue is also an ideal model to study regeneration (Worley et al., 2012). Although highly aneuploid cells are removed from the epithelial cells by JNK dependent apoptosis, compensatory proliferation is activated in the tissue for tissue homeostasis (Clemente-Ruiz et al., 2016). The depletion of protein quality control mechanisms

massively affected the tissue homeostasis in CIN tissues, pointing out that they are vital for tissue maintenance.

Using a variety of genetic tools and characterizing the cellular behavior of CIN tissues, this work elucidated the interplay between proteotoxic and metabolic stress in CIN tissues (**Figure 43**). Moreover, the requirement of the underlying mechanisms activated in CIN tissues was also validated in the context of tissue homeostasis.

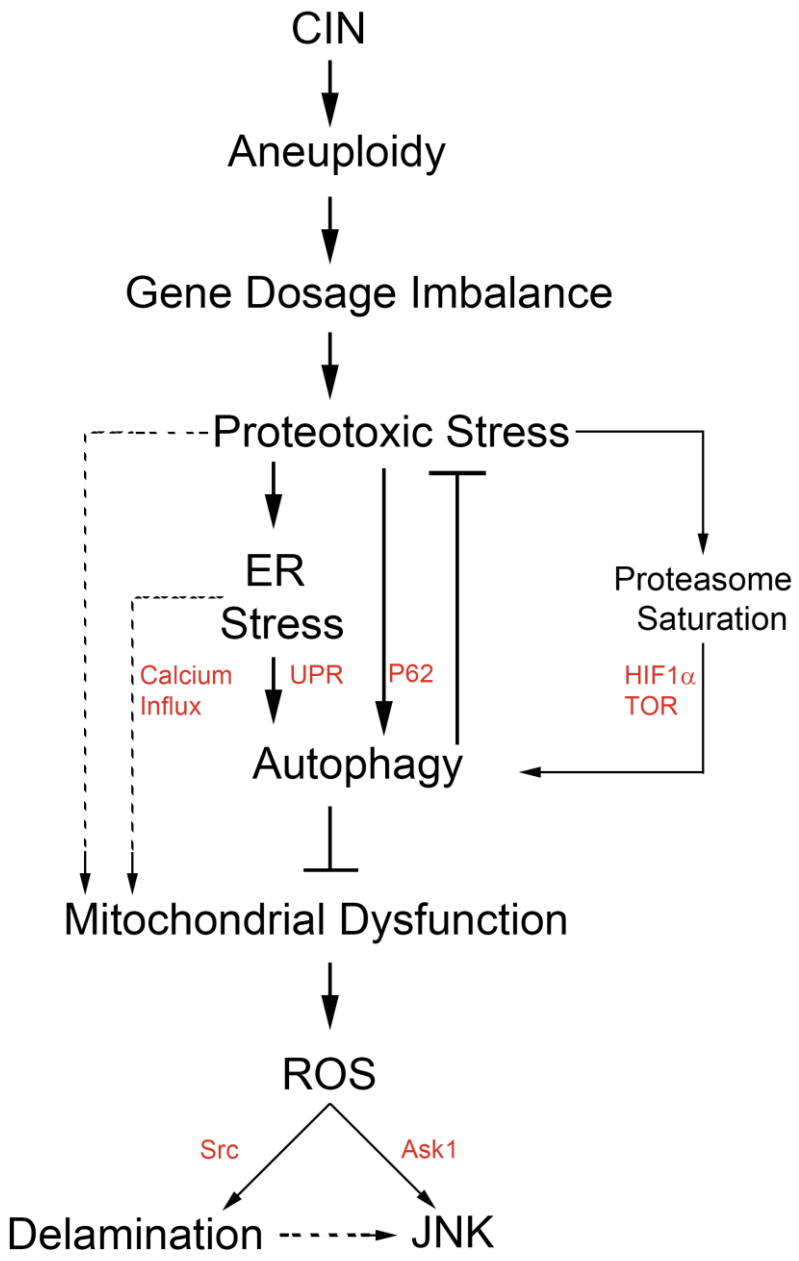

Figure 43. *Drosophila* epithelial CIN model

2. A central role of autophagy in antagonizing proteotoxic stress

Catabolic pathway autophagy is activated in CIN tissues to deal with the proteotoxic stress. Autophagy could be regulated by multiple pathways in the CIN context. Apart from the regular cargo-dependent and P62-mediated autophagy, HIF1α, the TOR pathway, and UPR were regulating autophagy in CIN tissues (**Figures 15, 19, 23, 25**). Accumulation of unfolded proteins in the ER and corresponding activation of UPR is a trivial response to a proteotoxic stress situation. PERK and IRE1 branch of UPR were activated in response to ER stress (**Figure 21**). Apart from the autophagy regulation by UPR activation, PERK-dependent phosphorylation of eIF2α activation is also an important antagonizing mechanism. Activation of eIF2α shuts down protein synthesis, which is a quite favorable scenario in the CIN situation to deal with proteotoxic stress.

HIF1α stabilization is not because of the fact that tissue is hypoxic (**Figure 18**). Rather, proteasomal saturation and corresponding stabilization of HIF1α could be one way to activate the hypoxic response in the CIN tissues. ROS dependent activation could also be an alternative way to stabilize HIF1α in CIN tissues. More studies are required to resolve this. HIF1α stabilization to activate LDH could be a strategic move for the tumor growth to meet its energy demands. Even in aerobic situations, tumors are known to favor metabolism by glycolysis rather than the much proficient oxidative phosphorylation, which is widely known as the Warburg effect.

Regarding the TOR pathway, Reptor-GFP upregulation suggests that TOR is inhibited in delaminated cells (**Figure 25**). Two main pathways that regulate TOR are insulin pathway and amino acid pathway. Amino acid pools are replenished by proteasomal dependent degradation and proteasomal saturation in CIN situation could be a reason for TOR inhibition. Another possibility is that the insulin pathway is downregulated in delaminated cells. One of the upregulated genes in the array was ImpL2 (an ortholog to human IGFBP7 (Insulin Growth Factor Binding Protein 7), refer **Table 1**). ImpL2 is known to inhibit insulin signaling in both flies and mammals (Honeger et al., 2008, Evdokimova et al., 2012). In a study from flies, it has been demonstrated that mitochondrial perturbation in muscles results in increased levels of ImpL2 in flies, but the molecular pathway regulating this remains elusive (Owusu-Ansah et al., 2013). A similar regulation might be happening in the CIN situation. More studies are required to demonstrate the link between mitochondrial dysfunction and ImpL2 production.

TOR inhibition might be advantageous in the CIN context. There are three main reasons to support this: (1) TOR inhibition activates autophagy induction, (2) TOR inhibition activates TFEB/MITF induction to promote lysosome biogenesis, (3) protein synthesis will be reduced upon TOR inhibition. The three outcomes are quite favorable to dampen the deleterious effects of CIN.

The regulation of TFEB in the CIN context needs more clarification. Calcium is also known as a strong inducer of TFEB. Whether TFEB is regulated by TOR or calcium requires more epistatic studies. Work

from aneuploidy model in mammals demonstrated that there are autophagic saturation and activation of TFEB mediated stress response (Santaguida et al., 2015). The depletion of TFEB reduced the growth potential of aneuploid cells in this context. The upregulation of autophagy genes observed in this aneuploidy model was dependent on TFEB. In our model, TFEB does not play a major role in autophagy induction (**Figure 28**). However, it is still a master regulator of lysosomal biogenesis. The depletion of TFEB resulted in increased ROS production and enhanced tumor growth similar to the autophagy depletion condition (**Figure 30**).

Autophagy flux is functional in CIN tissues. Protein aggregates were not observed in epithelial cells, suggesting that protein quality control mechanisms are well functioning in these cells (**Figure 10**). Consistent with this observation, the depletion of autophagy resulted in the accumulation of protein aggregates in epithelial cells (**Figure 29**). Moreover, autophagy could be tracked in epithelial cells in a situation where lysosomal fusion was blocked (**Figure 14**). This also supports that autophagy is functional in CIN tissues, and the inability to track much autophagy in epithelial cells could be because of its rapid turnover. Autophagy could handle the proteotoxic stress in low-aneuploid-epithelial-cells. Although flux is functional, some big cargoes that were not fused with lysosomes were observed in delaminated cells, which are highly aneuploidy (**Figure 14**). This could be the reason for accumulated protein aggregates in the CIN tissues. A high level of aneuploidy might be saturating autophagy mechanism in delaminated cells.

Autophagy induction was also observed in the neighboring compartment (**Figure 13**). Autophagy induction in the tumor microenvironment as well as systemically in distant places is known to promote tumor growth (Katheder et al., 2017). Non-autonomous, as well as systemic autophagy, promotes tumor growth by providing nutrients (Katheder et al., 2017). A similar scenario might be working in the CIN tissues. It could be possible that any one of the secreted proteins from the tumor upregulates autophagy in neighboring as well as distant places. An interesting candidate from the group is Upd (Unpaired, IL-6 like cytokine, refer **Table1**). In CIN tissues, JNK activates the JAK-STAT pathway through Upd for compensatory proliferation (Clemente-Ruiz et al., 2016). It is known that the JAK-STAT pathway regulates autophagy (You et al.,2015) and downregulation of the same rescued the non-autonomous autophagy induction as well as tissue growth in our model (data not shown). Characterizing the necessity of non-autonomous as well as systemic autophagy induction observed in CIN tissue bearing hosts will allow us to understand how tumor talks with host tissues and change the overall metabolism in the organism. In addition, ImpL2 is a well-known regulator of cachexia phenotype (Kwon et al., 2015, Figueroa-Clarevega and Bilder, 2015). Cancer cachexia is a metabolic disorder characterized by progressive tissue wasting, mainly the adipose and muscle tissue. It might be interesting to investigate the cachexia phenotype in CIN tissue bearing hosts to unravel the complex inter-organ communications in the CIN tumor context.

Overall, autophagy is a central mechanism upregulated in CIN tissues to deal with proteotoxic stress. There are many different ways in

which autophagy could be regulated in CIN tissues. Downregulation of autophagy enhances the proteotoxic stress, ROS production, and tissue overgrowth of CIN tissues. In a situation where cell death is not blocked, autophagy is vital for tissue homeostasis (**Figures 40**,**41**). The depletion of any autophagic genes resulted in more cell death. On the other hand, the upregulation of autophagy by overexpressing P62 or blocking the TOR pathway reduced the cell death observed in the tissues and promoted tissue homeostasis (**Figures 41, 42**). Genetic overexpression of core autophagic genes was quite strong and induced apoptosis (data not shown). Another interesting experiment is to validate how the system works upon a more stressful situation, such as starvation. Starvation will further inhibit the TOR pathway and might be beneficial for the CIN tissue to reduce the proteotoxic stress. As a therapeutical strategy, it might be also interesting to try some small molecules or drugs to activate autophagy in CIN tissues to dampen the proteotoxic stress. Also, blocking the TOR pathway in CIN tissues by various means will be beneficial to reduce the deleterious effects of CIN.

As the CIN situation is highly pleiotropic, it is not surprising that autophagy is regulated in many different ways. CIN tissues massively depend on autophagy to reduce proteotoxic stress and maintain tissue homeostasis.

3. Mitochondria as a sensing and signaling center in CIN tissues

Mitochondria have predominantly been seen as bioenergetic and biosynthetic organelles that independently co-exist within the cell. Research in the last two decades has provided evidence that mitochondria function as signaling organelles, persistently communicating with the cytosol to initiate biological processes under homeostatic and stress conditions. In general, mitochondria have three main functions to maintain the cellular homeostasis: (1) bioenergetics (2) biosynthesis (3) signaling (Chandel et al., 2014). In 1953, Hans Krebs awarded the Nobel Prize for his discovery of the citric acid cycle, widely known as the TCA cycle. The TCA cycle provides both energy and intermediates to the cell. Reducing equivalents (NADH and FADH2) produced by the TCA cycle is used by electron transport chain (ETC), to maintain an electrochemical gradient across the mitochondrial inner membrane which is required for ATP production as well as mitochondrial protein transport. Maintenance of a high ATP/ADP ratio is vital to drive many biochemical reactions in a cell. Moreover, mitochondria are also involved in the production of heme and porphyrin moieties containing proteins. Precursors for the biosynthesis of many macromolecules like lipid, carbohydrates, proteins, and nucleotides are generated by the TCA cycle. Mitochondria regulate signaling pathways generally through the release of cytochrome c to initiate caspase-dependent cell death, ROS to mediate gene expression as well as cell fate decision, and using their outer membrane as a platform for various signaling complexes. Tight regulation of

mitochondrial membrane potential is vital for cell survival, and any perturbation such as loss of ETC function, mitochondrial proteostasis impairment could decrease the membrane potential. The depletion of mitochondrial potential will end up in initiating mitophagy, a selective autophagy mechanism to clear defective mitochondria from the cell. Mitochondrial dynamics (fission/fusion and motility) is also known to activate many biological processes. In addition, mitochondria have close contact with the ER through specific subdomains, known as mitochondria-associated membranes (MAMs), which are evolving as key regulators of signal transduction.

In CIN tissues, dysfunctional mitochondria were found, especially in the highly-aneuploid- delaminated-cells (**Figures 32, 34**). One of the key observations was that mitochondria get accumulated as well as fragmented upon CIN (**Figure 31**). Either mitochondrial dynamics is extremely altered and favors fission over the fusion or massive de-novo mitochondrial biogenesis is happening. The presence of fragmented mitochondria suggests that they are dysfunctional and undergo mitophagy. Consistent with this colocalization of autophagy markers with fragmented mitochondria was observed (**Figure 33**). In addition, the mitochondrial population was highly oxidized in the CIN tissues suggesting that they are dysfunctional (**Figure 32**). Accordingly, strong mitophagy induction was observed in CIN tissues, but lysosomal degradation was saturated in CIN tissues (**Figure 34**). These observations suggest that the presence of dysfunctional mitochondria is a key source of ROS in the CIN tissues. The ultimate reason for mitophagy saturation is still elusive.

The presence of fragmented and dysfunctional mitochondria in the low-aneuploid-epithelial-cells suggests that it could be an early consequence of CIN. Mitochondria are located in close vicinity of ER Ca^{2+} release channels, and these contact sites called MAMs, are microdomains of high Ca^{2+} concentration. Mitochondrial Ca^{2+} uniporter (MCU) regulates the entry of Ca^{2+} through the inner mitochondrial membrane. Levels of mitochondrial Ca^{2+} are known to regulate cell fate (Rizzuto et al., 2012). Moderate levels of calcium accumulation promote ATP synthesis by modulating the action of the enzymes of the TCA cycle. Low levels of Ca^{2+} in mitochondria reduce ATP production, which activates autophagy as a pro-survival mechanism. High levels of Ca^{2+} levels trigger apoptosis or necrosis by opening the permeability transition pore (PTP) (Hom et al., 2007).

High levels of cytoplasmic calcium were observed in CIN tissues, especially in the low-aneuploid-epithelial-cells (**Figure 36**). This suggests that ER stress could be an early event. Interestingly, mitochondrial calcium levels were high in both delaminated and epithelial cells (**Figure 36**). This supports the idea that ER stress is an early event and there is corresponding Ca^{2+} leakage from the ER and a strong influx of Ca^{2+} to mitochondria might be one reason for massive mitochondrial damage as well as fragmentation. High levels of Ca^{2+} could also be a reason for the increased mitochondrial population observed in CIN tissues (Wright et al., 2007, Dominy and Puigserver et al., 2013). Further epistatic analysis in CIN tissues is

required to delineate the functions of Ca^{2+} signaling in the context of tumorigenesis and tissue homeostasis.

Another possible explanation for increased mitochondrial biogenesis will be the activation of AMPK (AMP-Activated Protein Kinase). AMPK is a cellular sensor that is activated by high cellular demands. Low ATP levels are known to activate AMPK, which in turn activates many genes by phosphorylation. Mainly, catabolic pathways are activated by AMPK and anabolic pathways are inhibited (Kahn et al., 2005). AMPK is an interesting candidate in CIN tissues, as it is known to inhibit the TOR pathway and activate autophagy (Alers et al.,2012). Moreover, AMPK is known to activate mitochondrial biogenesis by regulating PGC-1α (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1α) (Jager et al., 2007, Canto et al., 2009). More studies are required to understand the role of AMPK in CIN tissues and whether it regulates autophagy induction as well as the TOR inhibition in CIN tissues.

Apart from the high influx of Ca^{2+}, it could be also possible that mitochondria per se are affected by proteotoxic stress. Mitochondria contain more than 1000 proteins that are imported from cytoplasm through the TOM complex (translocase of the outer mitochondrial membrane) (Endo et al., 2011, Gebert et al., 2011). Mitochondrial genome encodes 13 oxidative phosphorylation (OXPHOS) proteins, tRNAs and ribosomal RNAs for their synthesis. Multiple forms of mitochondrial dysfunction activate a transcriptional response known as mtUPR, to enhance the repair and recovery of the mitochondrial

network. Various defects like OXPHOS perturbation, excessive ROS, impaired complex assembly and the accumulation of misfolded proteins impair mitochondrial protein import efficiency and activate mtUPR (Shpilka and Haynes, 2017).

Similar to the role of ATFS-1 in worms and ATF5 in mice in mediating mtUPR response, PJAM5 mediates the mtUPR in Drosophila by activating JNK through Ask1 and upregulating FoxO to enhance the transcription of mitochondrial chaperones to promote longevity (Jensen et al., 2017). Another study in flies where ETC complex 1 component was knocked down extended the life span by activating mtUPR through JNK dependent FoxO signaling (Owusu-Ansah et al., 2013). Mitochondrial perturbation resulted in high ImpL2 secretion in this case, similar to the CIN tissues. The depletion of PJAM5 resulted in a strong eye phenotype in CIN tissues, suggesting that it's required for the tissue homeostasis (**Figure 42**). Consistent with this, overexpression of mitochondrial chaperones rescued the eye phenotype observed in CIN tissues (**Figure 42**). More studies are required to characterize the role of mtUPR in CIN tissues.

Overall, in CIN tissues mitochondria act as a hub for various signaling. It could sense the perturbations in the cellular physiology and many signaling are activated from mitochondria to restore the cellular physiology as well as tissue homeostasis.

4. In a search for Achilles´ Heel of CIN

CIN is a major hallmark of cancer. Characterizing how CIN induce tumorigenesis will aid us to improve the therapeutical strategies to eliminate tumor cells and restore the tissue homeostasis. In *Drosophila* epithelial tissues, ROS is activated upon CIN to activate JNK. Activation of JNK removes aneuploid cells from the tissues and in a context where aneuploid cells are maintained by apoptosis blockade results in JNK dependent tumor growth. Two major events that promote tumorigenesis in epithelial CIN tissues are cell delamination and JNK activation. ROS plays a key role in both events. On one hand it activates Src kinase to drive cell delamination and on the other hand, it activates JNK through Ask1. Blockade of Src rescues both delamination as well as JNK activation, suggesting that delamination could also contribute to JNK activation.

Activation of the JNK pathway is key for tumorigenesis in epithelial tissues. JNK activation remodels the actomyosin cytoskeleton in tissue to promote tumorigenesis and aneuploid cells possess migratory behavior (Benhra et al., 2018). The depletion of Src kinase rescues the actomyosin upregulation observed in CIN tissues. It will be really interesting to try some drugs to block Src kinase in CIN tissues and to check the tumor growth.

The genetic depletion of the JNK pathway reduced the growth potential of CIN tissues (Dekanty et al., 2012). Although tissue overgrowth was rescued, the primary insult was still there (presence of aneuploid cells in the tissue). In the eye model of CIN, where cell

death is not inhibited, additional blockade of JNK results in strong eye phenotype (**Figure 42**), suggesting the same. Consistent with this notion, the upregulation of protein quality mechanisms by overexpressing P62 or overexpressing ER chaperones rescued the eye phenotype in the CIN model (**Figure 42**). So, for tissue homeostasis, primary insult should be ameliorated or removed.

The main reason for ROS production in CIN tissues is mitochondrial dysfunction. Quenching of the mitochondrial ROS by overexpressing mitochondrial superoxide dismutases and glutathione peroxidase rescued the eye phenotype (**Figure 42**). Similarly, overexpression of mitochondrial chaperones also rescued the eye phenotypes and restored the tissue homeostasis (**Figure 42**). So, dampening the proteotoxic stress or boosting mitochondrial health promoted tissue homeostasis in CIN tissues. It will be worth trying some chemical molecules to quench mitochondrial ROS in CIN tissues.

Protein quality control pathways play a central role in CIN tissues to maintain tissue homeostasis. Apart from general protein degradation, the proteasome is associated with the regulation of mitophagy, the TOR pathway, and hypoxia. The regulation of autophagy is quite complex and there are many inputs for the same in CIN tissues. An interesting strategy will be to find a way to boost both mechanisms at the same time. Removal of damaged mitochondria by mitophagy requires both the ubiquitin-proteasome system as well as mitophagy. Genetic, as well as pharmacological depletion of the deubiquitinating enzyme USP14, ameliorated Parkinson's Disease phenotype in an in-

vivo model (Chakraborty et al., 2018). Consistent with this knockdown of USP14 in the CIN model rescued eye phenotypes and maintained better tissue homeostasis (**Figure 42**).

A recent study states that proteasomal dysfunction could lead to proteome instability and impairment in mitochondrial homeostasis, and enhancement of mitophagy or mitochondrial fusion could ameliorate the cellular defects associated with it (Tsakiri et al., 2019). Another study also suggests that overexpression of autophagic adaptor protein P62 prolongs the life span by improving proteostasis and mitophagy (Aparicio et al., 2019). Consistent with this, in our model overexpression of autophagy (P62, TOR inhibition) rescued the eye phenotypes and cell death observed in CIN tissues (**Figures 41, 42**).

To sum up, protein quality control mechanisms are vital for the tissue homeostasis upon CIN. Ameliorating the proteotoxic stress or corresponding mitochondrial dysfunction or even ROS production could enhance tissue homeostasis. The implementation of genetic and pharmacological ways to enhance protein quality control mechanisms will be an ideal way to dampen the deleterious effects of CIN.

CONCLUSIONS

Conclusions

- CIN leads to proteotoxic stress in *Drosophila* epithelial tissues.
- The proteasome is saturated in highly-aneuploid-delaminated-cells of CIN tissues.
- Aneuploidy associated proteotoxic stress activates UPR to dampen the deleterious effects of CIN in epithelial tissues.
- Autophagy is induced by several regulators in CIN tissues.
- Autophagy protects low-aneuploid-epithelial-cells from proteotoxic stress. Although autophagy flux is working, high levels of aneuploidy might be saturating the autophagy in delaminating cells.
- Depletion of lysosomal-mediated autophagy enhances proteotoxic stress, ROS production, and tumorigenesis in CIN tissues.
- TOR pathway is blocked in highly-aneuploid-delaminated cells.
- Mitochondrial homeostasis is severely perturbed in CIN tissues.
- Aneuploidy-induced cellular stresses limit mitophagic autophagic degradation in CIN tissues.
- High levels of calcium were observed in CIN tissues and could be a possible reason for severe mitochondrial dysfunction and ROS production.
- Mitochondria act as both sensing and signaling centers in epithelial CIN tissues.

Conclusions

- ROS drives cell delamination through the activation of Src kinase in the epithelial tissues.
- Depletion of Src kinase rescues cell delamination and JNK activation in CIN tissues.
- The depletion of protein quality control mechanisms in CIN tissues severely affects the tissue homeostasis.
- Enhancement of protein quality control mechanisms or boosting mitochondrial homeostasis ameliorates the effects of CIN and maintained better tissue homeostasis.

MATERIALS AND METHODS

Fly husbandry

Tumor induction: Flies were allowed to lay eggs on standard fly food for 24 hours at 25°C, and switched to 29ºC and maintained for 5 days before dissection.

Cell death experiments: Flies were allowed to lay eggs on standard fly food for 24 hours at 25°C and kept at 25°C for another 24 hours, and switched to 29ºC and maintained for 3 days before dissection.

CIN eye model experiments: Flies were allowed to lay eggs on standard fly food for 24 hours at 25°C and kept at 25°C till they eclose.

Immunostaining

Wing imaginal discs of third instar larvae were dissected in cold PBS, fixed with formaldehyde 4% for 20 minutes, rinsed three times in PBT (PBS + 0.1% Triton) and blocked for 1 hour in BBT (PBS + 0.1% Triton+ 0,3% BSA + 250mM NaCl). Then discs were incubated with primary antibodies overnight, rinsed with BBT and incubated with secondary antibodies for 2 hours. After 3 PBT washes, discs were kept on mounting media (80ml glycerol + 10ml PBS 10x + 0,8 ml N-propyl-gallate 50%).

Leica SP2, Leica SP5, Zeiss LSM780, and Zeiss LSM880-with-airyscan confocal microscopes were used.

A most representative image is shown in all experiments. At least 10-15 wing discs per genotype were imaged.

Materials and methods

Drosophila Strains

The following strains were provided by the Bloomington Drosophila Stock Center (BDSC) or the Vienna Drosophila RNAi Center (VDRC) and various reseach groups:

UAS-rodRNAi (VDRC 19152); UAS-bub3RNAi (VDRC 21037); UAS-msl-1RNAi (BDSC 9239); UAS-gfpRNAi (BDSC 35786); en-Gal4 (BDSC 1973); ap-Gal4 (BDSC 3041); ey-Gal4 (BDSC 5535); UAS-p35 (BDSC 5072 and 5073); UAS-sod2 (BDSC 24494) ; Ubiquitin-GFP (Ubi-GFP, BDSC 1681); UAS-myristoylated-Tomato (UAS-myrT, BDSC 32221 and 32222) ; UAS-mudRNAi (BDSC, 28074) ; UAS-src64BRNAi (BDSC 51772) ; UAS-src42ARNAi (BDSC 55868) ; UAS-syn17RNAi (BDSC 36595) ; UAS-sip3RNAi (VDRC 6870) ; UAS-hsp70 (BDSC 32997, 33948, 42639) ; UAS-hsc70RNAi (BDSC 32402) ; UAS-hsc70 (BDSC 5841, 5842) ; UAS-torRNAi (BDSC 33951) ; UAS-park (BDSC 34746) ; UAS-parkRNAi (BDSC 38333) ; UAS-atg8aRNAi (VDRC 43097) ; UAS-atg18aRNAi (VDRC 22643) ; UAS-atg12RNAi (VDRC 29791) ; UAS-atg13RNAi (VDRC 27955) ; UAS-atg6RNAi (VDRC 110197) ; UAS-atg2RNAi(BDSC 34719) ; UAS-ref(2)P(G. Juhász) ; UAS-pjam5RNAi (VDRC 51657) ; UAS-sod2-GTPx1 and UAS-hsp60-60c (Owusu-Ansah et al., 2013) ; UAS-usp14RNAi (66956) ; UAS-syn17RNAi (BDSC 36595) ;UAS- puckered (Dekanty et al., 2012) ; UAS-mitfRNAi(VDRC 108519) ; UAS-atg9RNAi (VDRC 10045) ; UAS-mitoGFP (BDSC 8442) ; UAS-mitotimer (BDSC 57323) ; UAS-mitoQC (Lee et al., 2018) ; UAS-

GCamP (BDSC 42748) ; UAS-mitoGCaMP (Alex Whitworth) ; LDH-GFP (Wang et al.,2016) ; Sima-MiMIC (BDSC 60222) ; ODD-GFP (Misra et al., 2017) ; UAS-nlsDsREDFT (Lidsky et al., 2018) ; UAS-simaRNAi (BDSC 33895) ; Xbp1-GFP (H. Stellar) ; HSF-GFP (BDSC 66741) ; UAS-perkRNAi (BDSC 42499) ; UAS-ire1RNAi (BDSC 62156) ; UAS-xbp1RNAi (36755) ; REPTOR-GFP (Tiebe et al., 2015) ; UAS-rheb, Mitf$^{2.2}$ GFP and 4Mbox-GFP (Zhang et al., 2015) ; Tub-Lamp1-GFP (Krammer Lab) ; 3xCh-Lamp1 (G. Juhász) ;UAS-CL1-GFP (Eric H Baehrecke) ; 3xCh-atg8a (G. Juhász) ; UAS-GFP-mh-Atg8a (BDSC 37749) ; UAS-atg1RNAi (VDRC 16133) ; UAS-ref(2)P RNAi (BDSC 36111) ; MMP1-GFP (Uhlirova and Bohmann, 2006) ; gstD-GFP (Sykiotis and Bohmann, 2008).

Immunohistochemistry

The following antibodies were used at the indicated dilutions:
Mouse anti-MMP1 (1:20; 14A3D2, DSHB); rabbit anti-cleaved-Dcp1(1:100; 9578 S, CST); rat anti-Ci (1:10; 2A1, DSHB); rat anti-E-cadherin (1:50, DCAD2, DSHB); rabbit anti-Ref(2)P (1:5000, Tor Erik) ; mouse anti-Ubiquitin (1:100, Enzo FK2) ; rabbit anti-Src (1:100. Invitrogen PY418) ; rabbit anti-peIF2α (1:100, 3597, CST)
Secondary antibodies Cy2, Cy3, Cy5 and Alexa 647 were obtained from Jackson Immunoresearch.

Materials and methods

Quantification of tissue growth

Size of the Anterior (A), Posterior (P) and Dorsal (D) compartments in the wing primordia were measured using Fiji software [National Institutes of Health (NIH) Bethesda, MD]. The average P/A or D/Total ratios and the corresponding SDs were calculated and t-test analysis was carried out. All genotypes included in each histogram were analyzed in parallel. A 5% level was chosen as a significance threshold.

Quantification of cell death

Cell death was monitored by the use of an antibody that detects the activated form of Dcp-1. Images from basal planes were considered for the determination of the area positively labeled by the number of Dcp-1 positive cells, and these numbers were normalized to the area of the transgene expressing domain. At least 10 wing discs per genotype were scored, the corresponding SDs were calculated and t-test analysis was carried out. All genotypes included in each histogram were analyzed in parallel.

Quantification of ROS.

ROS was monitored by the use of gstD-GFP. Images from basal planes were considered for the determination of the area positively labeled by the gstD-GFP positive cells, and these numbers were normalized to the area of the transgene expressing domain. At least

10 wing discs per genotype were scored, the corresponding SDs were calculated and t-test analysis was carried out. All genotypes included in each histogram were analyzed in parallel.

Detection of ROS

For the detection of Superoxide radicals, imaginal discs were dissected out from third instar larvae in Schneider's medium followed by incubation in 0.3mM DHE (Molecular Probes, Cat# D11347) in Schneider's medium for 5 minutes at RT in dark. After washing the tissues with 1X PBS buffer (pH7.2), brief fixation was done with 8% paraformaldehyde for 10 minutes at RT. Tissues were mounted in vectashield and imaged in Laser Scanning Confocal Microscope (LSM 780, Carl Zeiss).

Statistical Analysis

Statistical analysis was generally performed by Student's t-test. Differences were considered significant if p values were less than 0.001 (***), 0.01 (**), or 0.05 (*). All genotypes included in each histogram were analyzed in parallel. A 5% level was chosen as a significance threshold. All data points were graphed in Prism 7.0 (Graphpad) statistical software.

BIBLIOGRAPHY

Bibliography

1. Alers, S., Löffler, A. S., Wesselborg, S. & Stork, B. Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Mol. Cell. Biol.* **32**, 2–11 (2012).
2. Andriani, G. A. *et al.* Whole Chromosome Instability induces senescence and promotes SASP. *Scientific Reports* **6**, 35218 (2016).
3. Aparicio, R., Rana, A. & Walker, D. W. Upregulation of the Autophagy Adaptor p62/SQSTM1 Prolongs Health and Lifespan in Middle-Aged Drosophila. *Cell Rep* **28**, 1029-1040.e5 (2019).
4. Ashrafi, G. & Schwarz, T. L. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ*. **20**, 31–42 (2013).
5. Bakhoum, S. F. & Cantley, L. C. The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment. *Cell* **174**, 1347–1360 (2018).
6. Bakhoum, S. F. & Compton, D. A. Chromosomal instability and cancer: a complex relationship with therapeutic potential. *J Clin Invest* **122**, 1138–1143 (2012).
7. Bakker, B. *et al.* Single-cell sequencing reveals karyotype heterogeneity in murine and human malignancies. *Genome Biology* **17**, 115 (2016).
8. Basto, R. *et al.* Centrosome Amplification Can Initiate Tumorigenesis in Flies. *Cell* **133**, 1032–1042 (2008).
9. Bayani, J. *et al.* Genomic instability and copy-number heterogeneity of chromosome 19q, including the kallikrein locus, in ovarian carcinomas. *Molecular Oncology* **5**, 48–60 (2011).
10. Benhra, N., Barrio, L., Muzzopappa, M. & Milán, M. Chromosomal Instability Induces Cellular Invasion in Epithelial Tissues. *Developmental Cell* **47**, 161-174.e4 (2018).
11. Bilder, D. & Irvine, K. D. Taking Stock of the Drosophila Research Ecosystem. *Genetics* **206**, 1227–1236 (2017).
12. Birkbak, N. J. *et al.* Paradoxical Relationship between

Chromosomal Instability and Survival Outcome in Cancer. *Cancer Res* **71**, 3447–3452 (2011).

13. Blank, H. M., Sheltzer, J. M., Meehl, C. M. & Amon, A. Mitotic entry in the presence of DNA damage is a widespread property of aneuploidy in yeast. *Mol. Biol. Cell* **26**, 1440–1451 (2015).
14. Borch Jensen, M., Qi, Y., Riley, R., Rabkina, L. & Jasper, H. PGAM5 promotes lasting FoxO activation after developmental mitochondrial stress and extends lifespan in Drosophila. *eLife* **6**, e26952 (2017).
15. Bouché, V. *et al.* Drosophila Mitf regulates the V-ATPase and the lysosomal-autophagic pathway. *Autophagy* **12**, 484–498 (2016).
16. Boveri, T. *Zur Frage der Entstehung maligner Tumoren*. (G. Fischer, 1914).
17. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415 (1993).
18. Brand, M. D. The sites and topology of mitochondrial superoxide production. *Exp. Gerontol.* **45**, 466–472 (2010).
19. Brennan, C. M. *et al.* Protein aggregation mediates stoichiometry of protein complexes in aneuploid cells. *Genes Dev.* (2019). doi:10.1101/gad.327494.119
20. Bridges, C. B. Current Maps of the Location of the Mutant Genes of Drosophila Melanogaster. *Proc Natl Acad Sci U S A* **7**, 127–132 (1921).
21. Brumby, A. M. & Richardson, H. E. Using Drosophila melanogaster to map human cancer pathways. *Nat. Rev. Cancer* **5**, 626–639 (2005).
22. Burrell, R. A., McGranahan, N., Bartek, J. & Swanton, C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature* **501**, 338–345 (2013).
23. Campbell, D., Doctor, J. S., Feuersanger, J. H. & Doolittle, M. M. Differential Mitotic Stability of Yeast Disomes Derived from Triploid Meiosis. *Genetics* **98**, 239–255 (1981).

24. Cánovas, B. *et al.* Targeting p38α Increases DNA Damage, Chromosome Instability, and the Anti-tumoral Response to Taxanes in Breast Cancer Cells. *Cancer Cell* **33**, 1094-1110.e8 (2018).
25. Cantó, C. *et al.* AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458**, 1056–1060 (2009).
26. Carter, S. L., Eklund, A. C., Kohane, I. S., Harris, L. N. & Szallasi, Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat Genet* **38**, 1043–1048 (2006).
27. Castellanos, E., Dominguez, P. & Gonzalez, C. Centrosome Dysfunction in Drosophila Neural Stem Cells Causes Tumors that Are Not Due to Genome Instability. *Current Biology* **18**, 1209–1214 (2008).
28. Chakraborty, J. *et al.* USP14 inhibition corrects an in vivo model of impaired mitophagy. *EMBO Mol Med* **10**, (2018).
29. Chandel, N. S. Mitochondria as signaling organelles. *BMC Biol.* **12**, 34 (2014).
30. Chen, Y., Partow, S., Scalcinati, G., Siewers, V. & Nielsen, J. Enhancing the copy number of episomal plasmids in Saccharomyces cerevisiae for improved protein production. *FEMS Yeast Res* **12**, 598–607 (2012).
31. Chunduri, N. K. & Storchová, Z. The diverse consequences of aneuploidy. *Nat Cell Biol* **21**, 54–62 (2019).
32. Clapham, D. E. Calcium signaling. *Cell* **131**, 1047–1058 (2007).
33. Clemente-Ruiz, M. *et al.* Gene Dosage Imbalance Contributes to Chromosomal Instability-Induced Tumorigenesis. *Dev. Cell* **36**, 290–302 (2016).
34. Dar, A. C., Das, T. K., Shokat, K. M. & Cagan, R. L. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature* **486**, 80–84 (2012).
35. Degtyareva, N. P., Chen, L., Mieczkowski, P., Petes, T. D. & Doetsch, P. W. Chronic oxidative DNA damage due to DNA

repair defects causes chromosomal instability in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **28**, 5432–5445 (2008).

36. Dekanty, A., Barrio, L., Muzzopappa, M., Auer, H. & Milán, M. Aneuploidy-induced delaminating cells drive tumorigenesis in Drosophila epithelia. *PNAS* **109**, 20549–20554 (2012).
37. Dekanty, A. & Milán, M. Aneuploidy, cell delamination and tumorigenesis in Drosophila epithelia. *Cell Cycle* **12**, 728–731 (2013).
38. Dephoure, N. *et al.* Quantitative proteomic analysis reveals posttranslational responses to aneuploidy in yeast. *Elife* **3**, e03023 (2014).
39. Dominy, J. E. & Puigserver, P. Mitochondrial Biogenesis through Activation of Nuclear Signaling Proteins. *Cold Spring Harb Perspect Biol* **5**, a015008 (2013).
40. Donnelly, N., Passerini, V., Dürrbaum, M., Stingele, S. & Storchová, Z. HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells. *EMBO J.* **33**, 2374–2387 (2014).
41. Donnelly, N. & Storchová, Z. Causes and consequences of protein folding stress in aneuploid cells. *Cell Cycle* **14**, 495–501 (2015).
42. Duijf, P. H. G., Schultz, N. & Benezra, R. Cancer cells preferentially lose small chromosomes. *International Journal of Cancer* **132**, 2316–2326 (2013).
43. Dürrbaum, M. *et al.* Unique features of the transcriptional response to model aneuploidy in human cells. *BMC Genomics* **15**, 139 (2014).
44. Endo, T., Yamano, K. & Kawano, S. Structural insight into the mitochondrial protein import system. *Biochim. Biophys. Acta* **1808**, 955–970 (2011).
45. Enomoto, M. & Igaki, T. Src controls tumorigenesis via JNK-dependent regulation of the Hippo pathway in Drosophila. *EMBO Rep.* **14**, 65–72 (2013).

46. Evdokimova, V. *et al.* IGFBP7 binds to the IGF-1 receptor and blocks its activation by insulin-like growth factors. *Sci Signal* **5**, ra92 (2012).
47. Figueroa-Clarevega, A. & Bilder, D. Malignant Drosophila tumors interrupt insulin signaling to induce cachexia-like wasting. *Dev. Cell* **33**, 47–55 (2015).
48. Fukasawa, K. Centrosome amplification, chromosome instability and cancer development. *Cancer Letters* **230**, 6–19 (2005).
49. García-Bellido, A. Genetic control of wing disc development in Drosophila. *Ciba Found. Symp.* **0**, 161–182 (1975).
50. Garcia-Bellido, A. & Merriam, J. R. Parameters of the wing imaginal disc development of Drosophila melanogaster. *Dev. Biol.* **24**, 61–87 (1971).
51. Gebert, N., Ryan, M. T., Pfanner, N., Wiedemann, N. & Stojanovski, D. Mitochondrial protein import machineries and lipids: a functional connection. *Biochim. Biophys. Acta* **1808**, 1002–1011 (2011).
52. Geigl, J. B., Obenauf, A. C., Schwarzbraun, T. & Speicher, M. R. Defining 'chromosomal instability'. *Trends in Genetics* **24**, 64–69 (2008).
53. Gerlinger, M. & Swanton, C. How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine. *Br J Cancer* **103**, 1139–1143 (2010).
54. Giannoni, E., Buricchi, F., Raugei, G., Ramponi, G. & Chiarugi, P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Mol. Cell. Biol.* **25**, 6391–6403 (2005).
55. Giorgi, C. *et al.* Mitochondrial Ca2+ and apoptosis. *Cell Calcium* **52**, 36–43 (2012).
56. Gogendeau, D. *et al.* Aneuploidy causes premature differentiation of neural and intestinal stem cells. *Nat Commun* **6**, 1–15 (2015).
57. Gonzalez, C. Drosophila melanogaster: a model and a tool to investigate malignancy and identify new therapeutics. *Nat.*

Rev. Cancer **13**, 172–183 (2013).

58. Görlach, A., Bertram, K., Hudecova, S. & Krizanova, O. Calcium and ROS: A mutual interplay. *Redox Biol* **6**, 260–271 (2015).
59. Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306–313 (2012).
60. Hanahan, D. & Weinberg, R. A. The Hallmarks of Cancer. *Cell* **100**, 57–70 (2000).
61. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674 (2011).
62. Hansemann, D. Ueber asymmetrische Zelltheilung in Epithelkrebsen und deren biologische Bedeutung. *Archiv f. pathol. Anat.* **119**, 299–326 (1890).
63. Hara, T. *et al.* Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* **441**, 885–889 (2006).
64. Hariharan, I. K. & Bilder, D. Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu. Rev. Genet.* **40**, 335–361 (2006).
65. Hay, B. A., Wolff, T. & Rubin, G. M. Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121–2129 (1994).
66. He, C. & Klionsky, D. J. Regulation mechanisms and signaling pathways of autophagy. *Annu. Rev. Genet.* **43**, 67–93 (2009).
67. Heng, H. H. *et al.* Chromosomal instability (CIN): what it is and why it is crucial to cancer evolution. *Cancer Metastasis Rev* **32**, 325–340 (2013).
68. Hetz, C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* **13**, 89–102 (2012).
69. Holland, A. J. & Cleveland, D. W. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nature Reviews Molecular Cell Biology* **10**, 478–487 (2009).
70. Hom, J. R., Gewandter, J. S., Michael, L., Sheu, S.-S. & Yoon,

Y. Thapsigargin induces biphasic fragmentation of mitochondria through calcium-mediated mitochondrial fission and apoptosis. *J. Cell. Physiol.* **212**, 498–508 (2007).

71. Hombría, J. C.-G. & Serras, F. Why should we care about fly tumors? *JAK-STAT* **2**, e23203 (2013).
72. Honegger, B. *et al.* Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in Drosophila and is essential for starvation resistance. *J. Biol.* **7**, 10 (2008).
73. Hunter, M. V., Willoughby, P. M., Bruce, A. E. E. & Fernandez-Gonzalez, R. Oxidative Stress Orchestrates Cell Polarity to Promote Embryonic Wound Healing. *Developmental Cell* **47**, 377-387.e4 (2018).
74. Hwang, S. *et al.* Serine-dependent Sphingolipid Synthesis Is a Metabolic Liability of Aneuploid Cells. *Cell Rep* **21**, 3807–3818 (2017).
75. Irby, R. B. & Yeatman, T. J. Role of Src expression and activation in human cancer. *Oncogene* **19**, 5636–5642 (2000).
76. Jaarsveld, R. H. van & Kops, G. J. P. L. Difference Makers: Chromosomal Instability versus Aneuploidy in Cancer. *Trends in Cancer* **2**, 561–571 (2016).
77. Jäger, S., Handschin, C., St-Pierre, J. & Spiegelman, B. M. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 12017–12022 (2007).
78. Jamal-Hanjani, M., Quezada, S. A., Larkin, J. & Swanton, C. Translational Implications of Tumor Heterogeneity. *Clin Cancer Res* **21**, 1258–1266 (2015).
79. Janssen, A., Kops, G. J. P. L. & Medema, R. H. Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. *PNAS* **106**, 19108–19113 (2009).
80. Janssen, A., van der Burg, M., Szuhai, K., Kops, G. J. P. L. & Medema, R. H. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. *Science* **333**, 1895–1898 (2011).

81. Johansson, A.-M., Stenberg, P., Bernhardsson, C. & Larsson, J. Painting of fourth and chromosome-wide regulation of the 4th chromosome in Drosophila melanogaster. *The EMBO Journal* **26**, 2307–2316 (2007).
82. Juhász, G., Érdi, B., Sass, M. & Neufeld, T. P. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes Dev* **21**, 3061–3066 (2007).
83. Kahn, B. B., Alquier, T., Carling, D. & Hardie, D. G. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* **1**, 15–25 (2005).
84. Kalapis, D. *et al.* Evolution of Robustness to Protein Mistranslation by Accelerated Protein Turnover. *PLOS Biology* **13**, e1002291 (2015).
85. Kamada, Y. *et al.* Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* **150**, 1507–1513 (2000).
86. Katheder, N. S. *et al.* Microenvironmental autophagy promotes tumour growth. *Nature* **541**, 417–420 (2017).
87. Kaufman, T. C. A Short History and Description of Drosophila melanogaster Classical Genetics: Chromosome Aberrations, Forward Genetic Screens, and the Nature of Mutations. *Genetics* **206**, 665–689 (2017).
88. Klionsky, D. J. & Emr, S. D. Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717–1721 (2000).
89. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8**, 445–544 (2012).
90. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12**, 1–222 (2016).
91. Klionsky, D. J. *et al.* A unified nomenclature for yeast autophagy-related genes. *Dev. Cell* **5**, 539–545 (2003).

92. Knouse, K. A., Wu, J., Whittaker, C. A. & Amon, A. Single cell sequencing reveals low levels of aneuploidy across mammalian tissues. *PNAS* **111**, 13409–13414 (2014).
93. Komatsu, M. *et al.* Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* **441**, 880–884 (2006).
94. Kwon, Y. *et al.* Systemic Organ Wasting Induced by Localized Expression of the Secreted Insulin/IGF Antagonist ImpL2. *Developmental Cell* **33**, 36–46 (2015).
95. Laker, R. C. *et al.* A novel MitoTimer reporter gene for mitochondrial content, structure, stress, and damage in vivo. *J. Biol. Chem.* **289**, 12005–12015 (2014).
96. Lamm, N. & Kerem, B. Continuous chromosomal instability in human pluripotent stem cells – the role of DNA replication. *Molecular & Cellular Oncology* **3**, e1183743 (2016).
97. Larsson, J., Chen, J. D., Rasheva, V., Rasmuson-Lestander, Å. & Pirrotta, V. Painting of fourth, a chromosome-specific protein in Drosophila. *PNAS* **98**, 6273–6278 (2001).
98. Laverty, C., Lucci, J. & Akhtar, A. The MSL complex: X chromosome and beyond. *Current Opinion in Genetics & Development* **20**, 171–178 (2010).
99. Lawrence, P. A. & Struhl, G. Morphogens, compartments, and pattern: lessons from drosophila? *Cell* **85**, 951–961 (1996).
100. Lee, A. J. X. *et al.* Chromosomal Instability Confers Intrinsic Multidrug Resistance. *Cancer Res* **71**, 1858 (2011).
101. Lee, J. J. *et al.* Basal mitophagy is widespread in Drosophila but minimally affected by loss of Pink1 or parkin. *J Cell Biol* **217**, 1613–1622 (2018).
102. Levine, M. S. & Holland, A. J. The impact of mitotic errors on cell proliferation and tumorigenesis. *Genes Dev.* **32**, 620–638 (2018).
103. Li, G.-W., Burkhardt, D., Gross, C. & Weissman, J. S. Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell* **157**, 624–635 (2014).

104. Li, M. *et al.* The ATM–p53 pathway suppresses aneuploidy-induced tumorigenesis. *PNAS* **107**, 14188–14193 (2010).
105. Lidsky, P. V. *et al.* A genetically encoded fluorescent probe for imaging of oxygenation gradients in living Drosophila. *Development* **145**, dev156257 (2018).
106. Lindsley, D. L. *et al.* Segmental aneuploidy and the genetic gross structure of the Drosophila genome. *Genetics* **71**, 157–184 (1972).
107. Lőw, P. *et al.* Impaired proteasomal degradation enhances autophagy via hypoxia signaling in Drosophila. *BMC Cell Biology* **14**, 29 (2013).
108. Markstein, M. *et al.* Systematic screen of chemotherapeutics in Drosophila stem cell tumors. *PNAS* **111**, 4530–4535 (2014).
109. McGranahan, N., Burrell, R. A., Endesfelder, D., Novelli, M. R. & Swanton, C. Cancer chromosomal instability: therapeutic and diagnostic challenges. *EMBO reports* **13**, 528–538 (2012).
110. Meena, J. K. *et al.* Telomerase abrogates aneuploidy-induced telomere replication stress, senescence and cell depletion. *EMBO J.* **34**, 1371–1384 (2015).
111. Meléndez, A. & Neufeld, T. P. The cell biology of autophagy in metazoans: a developing story. *Development* **135**, 2347–2360 (2008).
112. Milán, M., Clemente-Ruiz, M., Dekanty, A. & Muzzopappa, M. Aneuploidy and tumorigenesis in Drosophila. *Semin. Cell Dev. Biol.* **28**, 110–115 (2014).
113. Mirkovic, M., Guilgur, L. G., Tavares, A., Passagem-Santos, D. & Oliveira, R. A. Induced aneuploidy in neural stem cells triggers a delayed stress response and impairs adult life span in flies. *PLOS Biology* **17**, e3000016 (2019).
114. Misra, T. *et al.* A genetically encoded biosensor for visualising hypoxia responses in vivo. *Biol Open* **6**, 296–304 (2017).
115. Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature* **451**, 1069–1075 (2008).

116. Musacchio, A. & Salmon, E. D. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* **8**, 379–393 (2007).
117. Muzzopappa, M., Murcia, L. & Milán, M. Feedback amplification loop drives malignant growth in epithelial tissues. *PNAS* **114**, E7291–E7300 (2017).
118. Nagarkar-Jaiswal, S. *et al.* A library of MiMICs allows tagging of genes and reversible, spatial and temporal knockdown of proteins in Drosophila. *eLife* **4**, e05338 (2015).
119. Negrini, S., Gorgoulis, V. G. & Halazonetis, T. D. Genomic instability — an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* **11**, 220–228 (2010).
120. Nezis, I. P. *et al.* Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. *J. Cell Biol.* **180**, 1065–1071 (2008).
121. Nys, K., Maes, H., Dudek, A. M. & Agostinis, P. Uncovering the role of hypoxia inducible factor-1α in skin carcinogenesis. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1816**, 1–12 (2011).
122. Ohashi, A. *et al.* Aneuploidy generates proteotoxic stress and DNA damage concurrently with p53-mediated post-mitotic apoptosis in SAC-impaired cells. *Nat Commun* **6**, (2015).
123. Oromendia, A. B. & Amon, A. Aneuploidy: implications for protein homeostasis and disease. *Dis Model Mech* **7**, 15–20 (2014).
124. Oromendia, A. B., Dodgson, S. E. & Amon, A. Aneuploidy causes proteotoxic stress in yeast. *Genes Dev* **26**, 2696–2708 (2012).
125. Owusu-Ansah, E., Song, W. & Perrimon, N. Muscle Mitohormesis Promotes Longevity via Systemic Repression of Insulin Signaling. *Cell* **155**, (2013).
126. Palmieri, M. *et al.* Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. *Hum. Mol. Genet.* **20**, 3852–3866 (2011).

Bibliography

127. Pandey, U. B. *et al.* HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. *Nature* **447**, 860–864 (2007).
128. Pankiv, S. *et al.* p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282**, 24131–24145 (2007).
129. Passerini, V. *et al.* The presence of extra chromosomes leads to genomic instability. *Nat Commun* **7**, 1–12 (2016).
130. Pastor-Pareja, J. C. & Xu, T. Dissecting social cell biology and tumors using Drosophila genetics. *Annu. Rev. Genet.* **47**, 51–74 (2013).
131. Pathak, B. K., Mondal, S., Banerjee, S., Ghosh, A. N. & Barat, C. Sequestration of Ribosome during Protein Aggregate Formation: Contribution of ribosomal RNA. *Scientific Reports* **7**, 42017 (2017).
132. Patterson, J. T., Stone, W. & Bedichek, S. The Genetics of X-Hyperploid Females. *Genetics* **20**, 259–279 (1935).
133. Pattingre, S., Espert, L., Biard-Piechaczyk, M. & Codogno, P. Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie* **90**, 313–323 (2008).
134. Pavelka, N. *et al.* Aneuploidy confers quantitative proteome changes and phenotypic variation in budding yeast. *Nature* **468**, 321–325 (2010).
135. Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab.* **23**, 27–47 (2016).
136. Payen, V. L., Zampieri, L. X., Porporato, P. E. & Sonveaux, P. Pro- and antitumor effects of mitochondrial reactive oxygen species. *Cancer Metastasis Rev.* **38**, 189–203 (2019).
137. Pérez-Garijo, A., Martín, F. A. & Morata, G. Caspase inhibition during apoptosis causes abnormal signalling and developmental aberrations in Drosophila. *Development* **131**, 5591–5598 (2004).
138. Pfau, S. J. & Amon, A. Chromosomal instability and aneuploidy in cancer: from yeast to man. *EMBO reports* **13**, 515–527 (2012).

139. Phelps, C. B. & Brand, A. H. Ectopic gene expression in Drosophila using GAL4 system. *Methods* **14**, 367–379 (1998).
140. Pickrell, A. M. & Youle, R. J. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* **85**, 257–273 (2015).
141. Poon, C. L. C., Brumby, A. M. & Richardson, H. E. Src Cooperates with Oncogenic Ras in Tumourigenesis via the JNK and PI3K Pathways in Drosophila epithelial Tissue. *Int J Mol Sci* **19**, (2018).
142. Poulton, J. S., Cuningham, J. C. & Peifer, M. Centrosome and spindle assembly checkpoint loss leads to neural apoptosis and reduced brain size. *J Cell Biol* **216**, 1255–1265 (2017).
143. Rajagopalan, H. & Lengauer, C. Aneuploidy and cancer. *Nature* **432**, 338–341 (2004).
144. Reiter, L. T., Potocki, L., Chien, S., Gribskov, M. & Bier, E. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res.* **11**, 1114–1125 (2001).
145. Richardson, H. E. & Portela, M. Modelling Cooperative Tumorigenesis in Drosophila. *BioMed Research International* (2018). doi:10.1155/2018/4258387
146. Ripoll, P. Effect of Terminal Aneuploidy on Epidermal Cell Viability in Drosophila Melanogaster. *Genetics* **94**, 135–152 (1980).
147. Rizzuto, R., De Stefani, D., Raffaello, A. & Mammucari, C. Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 566–578 (2012).
148. Rubin, G. M. *et al.* Comparative genomics of the eukaryotes. *Science* **287**, 2204–2215 (2000).
149. Ryoo, H. D., Gorenc, T. & Steller, H. Apoptotic Cells Can Induce Compensatory Cell Proliferation through the JNK and the Wingless Signaling Pathways. *Developmental Cell* **7**, 491–501 (2004).
150. Sancak, Y. *et al.* The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320**, 1496–1501

(2008).
151. Santabárbara-Ruiz, P. *et al.* Ask1 and Akt act synergistically to promote ROS-dependent regeneration in Drosophila. *PLOS Genetics* **15**, e1007926 (2019).
152. Santaguida, S. & Amon, A. Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nature Reviews Molecular Cell Biology* **16**, 473–485 (2015).
153. Santaguida, S. *et al.* Chromosome Mis-segregation Generates Cell-Cycle-Arrested Cells with Complex Karyotypes that Are Eliminated by the Immune System. *Dev. Cell* **41**, 638-651.e5 (2017).
154. Santaguida, S., Vasile, E., White, E. & Amon, A. Aneuploidy-induced cellular stresses limit autophagic degradation. *Genes Dev.* **29**, 2010–2021 (2015).
155. Sardiello, M. *et al.* A gene network regulating lysosomal biogenesis and function. *Science* **325**, 473–477 (2009).
156. Scott, R. C., Juhász, G. & Neufeld, T. P. Direct Induction of Autophagy by Atg1 Inhibits Cell Growth and Induces Apoptotic Cell Death. *Current Biology* **17**, 1–11 (2007).
157. Segal, D. J. & McCoy, E. E. Studies on Down's syndrome in tissue culture. I. Growth rates protein contents of fibroblast cultures. *Journal of Cellular Physiology* **83**, 85–90 (1974).
158. Sekine, Y. *et al.* The Kelch Repeat Protein KLHDC10 Regulates Oxidative Stress-Induced ASK1 Activation by Suppressing PP5. *Molecular Cell* **48**, 692–704 (2012).
159. Sen, S. Aneuploidy and cancer. *Current Opinion in Oncology* **12**, 82–88 (2000).
160. Sena, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* **48**, 158–167 (2012).
161. Senft, D. & Ronai, Z. A. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem. Sci.* **40**, 141–148 (2015).
162. Seo, A. Y. *et al.* New insights into the role of mitochondria in aging: mitochondrial dynamics and more. *J. Cell. Sci.* **123**,

2533–2542 (2010).
163. Settembre, C. *et al.* TFEB links autophagy to lysosomal biogenesis. *Science* **332**, 1429–1433 (2011).
164. Sheltzer, J. M. & Amon, A. The aneuploidy paradox: costs and benefits of an incorrect karyotype. *Trends in genetics : TIG* **27**, 446–453 (2011).
165. Shpilka, T. & Haynes, C. M. The mitochondrial UPR: mechanisms, physiological functions and implications in ageing. *Nat. Rev. Mol. Cell Biol.* **19**, 109–120 (2018).
166. Simonsen, A. *et al.* Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy* **4**, 176–184 (2008).
167. Smith-Bolton, R. K., Worley, M. I., Kanda, H. & Hariharan, I. K. Regenerative Growth in Drosophila Imaginal Discs Is Regulated by Wingless and Myc. *Developmental Cell* **16**, 797–809 (2009).
168. Sone, M., Zeng, X., Larese, J. & Ryoo, H. D. A modified UPR stress sensing system reveals a novel tissue distribution of IRE1/XBP1 activity during normal Drosophila development. *Cell Stress Chaperones* **18**, 307–319 (2013).
169. Stewart, R. A., Li, D.-M., Huang, H. & Xu, T. A genetic screen for modifiers of the lats tumor suppressor gene identifies C-terminal Src kinase as a regulator of cell proliferation in Drosophila. *Oncogene* **22**, 6436–6444 (2003).
170. Stingele, S. *et al.* Global analysis of genome, transcriptome and proteome reveals the response to aneuploidy in human cells. *Molecular Systems Biology* **8**, 608 (2012).
171. Sunshine, A. B. *et al.* The Fitness Consequences of Aneuploidy Are Driven by Condition-Dependent Gene Effects. *PLOS Biology* **13**, e1002155 (2015).
172. Suraweera, A., Münch, C., Hanssum, A. & Bertolotti, A. Failure of Amino Acid Homeostasis Causes Cell Death following Proteasome Inhibition. *Molecular Cell* **48**, 242–253 (2012).
173. Sykiotis, G. P. & Bohmann, D. Keap1/Nrf2 signaling regulates

oxidative stress tolerance and lifespan in Drosophila. *Dev. Cell* **14**, 76–85 (2008).

174. TACKHOLM, G. Zytologische Studien uber die Gattung Rosa. *Acta Hort. Bergiani* **7**, 97–381 (1922).
175. Taggart, J. C. & Li, G.-W. Production of Protein-Complex Components Is Stoichiometric and Lacks General Feedback Regulation in Eukaryotes. *Cell Syst* **7**, 580-589.e4 (2018).
176. Takáts, S. *et al.* Autophagosomal Syntaxin17-dependent lysosomal degradation maintains neuronal function in Drosophila. *J Cell Biol* **201**, 531–539 (2013).
177. Tang, Y.-C., Williams, B. R., Siegel, J. J. & Amon, A. Identification of aneuploidy-selective antiproliferation compounds. *Cell* **144**, 499–512 (2011).
178. Tang, Y.-C. *et al.* Aneuploid Cell Survival Relies upon Sphingolipid Homeostasis. *Cancer Res.* **77**, 5272–5286 (2017).
179. Tateno, M., Nishida, Y. & Adachi-Yamada, T. Regulation of JNK by Src During Drosophila Development. *Science* **287**, 324–327 (2000).
180. Thompson, L. L., Jeusset, L. M.-P., Lepage, C. C. & McManus, K. J. Evolving Therapeutic Strategies to Exploit Chromosome Instability in Cancer. *Cancers (Basel)* **9**, (2017).
181. Thompson, S. L. & Compton, D. A. Proliferation of aneuploid human cells is limited by a p53-dependent mechanism. *The Journal of Cell Biology* **188**, 369–381 (2010).
182. Thorburn, R. R. *et al.* Aneuploid yeast strains exhibit defects in cell growth and passage through START. *Mol. Biol. Cell* **24**, 1274–1289 (2013).
183. Tian, L. *et al.* Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* **6**, 875–881 (2009).
184. Tiebe, M. *et al.* REPTOR and REPTOR-BP Regulate Organismal Metabolism and Transcription Downstream of TORC1. *Dev. Cell* **33**, 272–284 (2015).
185. Tognon, E. *et al.* Control of lysosomal biogenesis and

Notch-dependent tissue patterning by components of the TFEB-V-ATPase axis in Drosophila melanogaster. *Autophagy* **12**, 499–514 (2016).

186. Torres, E. M. *et al.* Identification of aneuploidy-tolerating mutations. *Cell* **143**, 71–83 (2010).
187. Torres, E. M. *et al.* Effects of Aneuploidy on Cellular Physiology and Cell Division in Haploid Yeast. *Science* **317**, 916–924 (2007).
188. Torres, E. M., Williams, B. R. & Amon, A. Aneuploidy: Cells Losing Their Balance. *Genetics* **179**, 737–746 (2008).
189. Tsai, H.-J. *et al.* Hypo-osmotic-like stress underlies general cellular defects of aneuploidy. *Nature* **570**, 117–121 (2019).
190. Tsakiri, E. N. *et al.* Proteasome dysfunction induces excessive proteome instability and loss of mitostasis that can be mitigated by enhancing mitochondrial fusion or autophagy. *Autophagy* **15**, 1757–1773 (2019).
191. Tye, B. W. *et al.* Proteotoxicity from aberrant ribosome biogenesis compromises cell fitness. *eLife* **8**, e43002 (2019).
192. Uhlirova, M. & Bohmann, D. JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. *EMBO J.* **25**, 5294–5304 (2006).
193. Vicoso, B. & Bachtrog, D. Reversal of an ancient sex chromosome to an autosome in *Drosophila* . *Nature* **499**, 332–335 (2013).
194. Vidal, M., Larson, D. E. & Cagan, R. L. Csk-Deficient Boundary Cells Are Eliminated from Normal Drosophila Epithelia by Exclusion, Migration, and Apoptosis. *Developmental Cell* **10**, 33–44 (2006).
195. Villegas, S. N. One hundred years of Drosophila cancer research: no longer in solitude. *Disease Models & Mechanisms* **12**, dmm039032 (2019).
196. Vincent, J. P. Compartment boundaries: where, why and how? *Int. J. Dev. Biol.* **42**, 311–315 (1998).
197. Wang, C.-W., Purkayastha, A., Jones, K. T., Thaker, S. K. & Banerjee, U. In vivo genetic dissection of tumor growth and

the Warburg effect. *eLife* **5**,

198. Weaver, B. A. A. & Cleveland, D. W. Does aneuploidy cause cancer? *Curr. Opin. Cell Biol.* **18**, 658–667 (2006).
199. Williams, B. R. *et al.* Aneuploidy Affects Proliferation and Spontaneous Immortalization in Mammalian Cells. *Science* **322**, 703–709 (2008).
200. Willoughby, L. F. *et al.* An in vivo large-scale chemical screening platform using Drosophila for anti-cancer drug discovery. *Dis Model Mech* **6**, 521–529 (2013).
201. Wong, E. & Cuervo, A. M. Integration of clearance mechanisms: the proteasome and autophagy. *Cold Spring Harb Perspect Biol* **2**, a006734 (2010).
202. Worley, M. I., Setiawan, L. & Hariharan, I. K. Regeneration and Transdetermination in Drosophila Imaginal Discs. *Annu. Rev. Genet.* **46**, 289–310 (2012).
203. Wright, D. C. *et al.* Exercise-induced Mitochondrial Biogenesis Begins before the Increase in Muscle PGC-1α Expression. *J. Biol. Chem.* **282**, 194–199 (2007).
204. Xu, S. & Chisholm, A. D. C. elegans epidermal wounding induces a mitochondrial ROS burst that promotes wound repair. *Dev. Cell* **31**, 48–60 (2014).
205. Yeatman, T. J. A renaissance for SRC. *Nat. Rev. Cancer* **4**, 470–480 (2004).
206. Yona, A. H. *et al.* Chromosomal duplication is a transient evolutionary solution to stress. *PNAS* **109**, 21010–21015 (2012).
207. You, L. *et al.* The role of STAT3 in autophagy. *Autophagy* **11**, 729–739 (2015).
208. Zhang, T. *et al.* Mitf is a master regulator of the v-ATPase, forming a control module for cellular homeostasis with v-ATPase and TORC1. *J Cell Sci* **128**, 2938–2950 (2015).
209. Zhu, J., Pavelka, N., Bradford, W. D., Rancati, G. & Li, R. Karyotypic Determinants of Chromosome Instability in Aneuploid Budding Yeast. *PLOS Genetics* **8**, e1002719 (2012).

210. Zhu, J., Tsai, H.-J., Gordon, M. R. & Li, R. Cellular Stress Associated with Aneuploidy. *Developmental Cell* **44**, 420–431 (2018).