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Manon Budzyk

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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

Préparée à l'Institut Curie
UMR144 – Biologie Cellulaire et Cancer

**Identification de facteurs essentiels pour la prolifération
de cellules polyploïdes**

Identification of factors essential for polyploid cell
proliferation

Soutenue par

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Le 12 octobre 2023

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Spécialité

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Thesis outlines

This manuscript resumes experimental findings conducted during the four years of my PhD in the lab of Renata Basto, at the Institut Curie in Paris, France. My work is organized around two main experimental findings, the first regarding mechanisms of DNA damage generation at mitotic entry in asynchronous polyploid cells (**Results – section 1**), and the second on the identification of Gen1 as a factor involved in polyploid cell proliferation (**Results – section 2**).

Both **Chapter 1 – Introduction** and **Chapter 3 – Discussion and Perspectives** of the manuscript are built around these two sections, with the aim to provide a comprehensive view of the literature, in light of the new data reported in this manuscript.

Chapter 2 – Material and methods describes all the techniques used for experimental setup, data analysis, image acquisition and processing.

The data presented in **Results – section 2** will shortly be submitted for publication. New experimental data are being produced by Anthony Simon and Margot Budzyk, both engineers in the lab of Renata Basto. This data will be part, together with the work described in **Results – section 2** of this manuscript, of a publication entitled “GEN1 nuclease is essential for the proliferation of unscheduled polyploid cells” (Budzyk et al., in preparation).

The data provided in **Results-section 1** originate from a project that aimed to investigate the link between polyploidy and genetic instability. Indeed, whole genome duplication (WGD) is a feature of many human tumors, which are characterized by high levels of genetic instability. Previous work conducted by Maddalena Nano, a former PhD student, showed that multinucleated polyploid cells – which carry multiple copies of the whole chromosome set – have an asynchronous cell cycle progression. As a consequence, at mitotic entry, certain nuclei are not ready to enter mitosis and accumulate high levels of DNA damage. However, the mechanisms responsible for DNA damage generation in this context were not identified. I hypothesized that nucleases – molecular scissors involved in DNA metabolism – would access prematurely the delayed-nucleus and generate abnormal DNA damage. To investigate this hypothesis, I performed a screen in an *in vivo* model of unscheduled polyploid cells, *Drosophila* neuroblasts (NBs). Using this system, I assessed the effect of the depletion of each nuclease of the *Drosophila* genome. While conducting this screen, I first observed that a large

number of nucleases are required for polyploid cell proliferation, which led me to the work described in **Results-section 2**. Furthermore, I identified two nucleases which might be responsible for generating DNA damage in the delayed nucleus at mitotic entry in polyploid NBs. These two candidates are Gen and Mre11, two nucleases which were known to be active during mitosis.

In the work described in **Results-section 2**, I focused on the role of GEN1, a nuclease involved in DNA repair by homologous recombination (HR), and its role in the proliferation of cells having whole genome duplication (WGD). Indeed, our lab has previously shown that WGDs lead to high levels of DNA damage in a single S-phase. This led us to investigate the requirement of HR factors for the proliferation of cells after unscheduled duplication of their genome. We found that the HR factor RAD51 is essential, and surprisingly so GEN1, while other proteins involved in HJ resolution are dispensable. Using both *Drosophila* NBs and RPE-1 cells, live imaging approaches, several quantitative methods, and multicolored FISH karyotyping, we showed that depleting GEN1 causes replication stress and increased levels of mitotic errors including multipolarity, ultrafine DNA bridges and generation of micronuclei. Further, we observed that tetraploid cells without GEN1 can undergo mitotic bypass culminating with a permanent cell cycle exit, accompanied by high p53 levels. Eventually, we described that, human tumors with WGD events had increased Gen1 levels when compared with non-WGDs tumors. These findings reveal a requirement for Gen1 in the survival of cancers after WGD.

Overall, the results obtained during my PhD contribute to better understand the molecular mechanisms of DNA damage generation during mitosis in polyploid cells. Further, this work provides new insights on factors essential for the survival of polyploid cells.

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List of abbreviations

a-EJ	Alternative End-Joining
APC/C	Anaphase promoting complex
BER	Base excision repair
BFB	Break-fusion-Bridge
BIR	Break induced replication
CAK	CDK-activating kinase
CDK	Cyclin-dependent kinases
CFS	Common fragile sites
DDR	DNA damage response
dNTP	Deoxyribonucleotide
DSB	Double strand break
GMC	Ganglion mother cell
HJ	Holliday Junction
HR	Homologous recombination
ICL	Interstrand crosslink repair
LOH	Loss of heterozygosity
MCC	Mitotic checkpoint complex
MiDAS	Mitotic DNA synthesis
MMEJ	Microhomology mediated End-joining
MMR	Mismatch repair
MN	Micronucleus
MT	Microtubule
NB	Neuroblast
NE	Nuclear envelope
NEBD	Nuclear envelope break down
NER	Nucleotide excision
NHEJ	Non-homologous end joining
OE	Overexpression
PH3	Phospho histone-H3
RNAi	RNA interference

ROS	Reactive oxygen species
RS	Replication stress
SAC	Spindle assembly checkpoint
SCI	Sister chromatid intertwinings
SPG	Subperineural glia
SSA	Single strand annealing
ssDNA	Single strand DNA
ssDNAB	Single strand DNA break
TGC	Trophoblast giant cells
TRC	Transcription-replication conflict
UFB	Ultrafine DNA bridge
VNC	Ventral nerve cord
WGD	Whole genome duplication
WT	Wild type

Note on *Drosophila* nomenclature. The rules used for the nomenclature are the ones indicated in (Lindsley and Zimm, 1992) and adopted by the *Drosophila* community, as stated in FlyBase (<http://flybase.org/>). *Drosophila melanogaster* gene names are shown in italic. Proteins are always indicated with an uppercase initial letter, in regular font. For a broader outreach, some *Drosophila* gene names were changed in favor of their human orthologs, maintaining the same rules listed above.

Chapter 1 - Introduction

1. THE CELL CYCLE

1.1 Introduction to the cell cycle

In the 19th century, Matthias Schleiden a German botanist and Theodor Schwann, a German physiologist proposed the cell theory. This theory relies on three statements: first, every living organism is composed of one or more cells; second, the cell is the basic unit of life and third, new cells can arise only by the division of pre-existing cells (Schleiden, 1838; Schwann, 1839). To duplicate, cells must first replicate their genetic material and distribute it equally into two daughter cells. This is coordinated by the cell cycle, which is composed of serial events occurring in a precise order leading to the formation of two genetically identical daughter cells. The typical human cell cycle is composed of two main phases, one long phase called interphase -during which the cell grows both in size and DNA content- and a shorter one, mitosis (M), where the cell physically divides and resets the cell cycle back to interphase (**Figure 1**). In this section, I will describe the different phases of the cell cycle, as well as their regulation mechanisms, and introduce the notion of “checkpoints”, which is essential to ensure proper cell division.

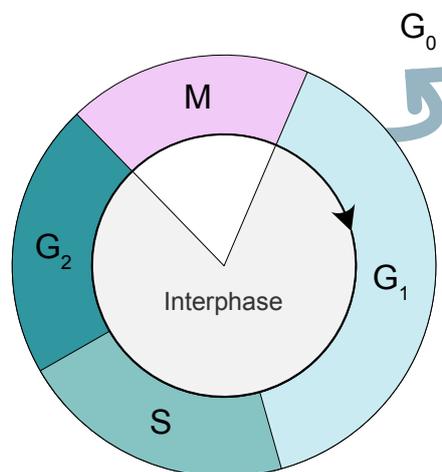


Figure 1: Cell cycle phases relative duration in mammalian cells. The cell cycle is divided in two phases: interphase (in green – G1/S/G2) and mitosis (M- in pink). In most of mammalian cells, G1 phase is the longest phase of the cell cycle, while it is must shorter in fission and budding yeast (Morgan, 2007). Cells can exit the cell cycle and enter a G0 resting phase or quiescence phase. During S phase, cells replicate their genome (described in figure 2), while

the G2 phase consists in preparing the cell for division. Mitosis is further described in the *section 1.1.2*.

1.2 Cell cycle phases

1.2.1 Interphase

Interphase is subdivided into three other phases: two growth phases (called “gap” phases G1 and G2) and a DNA-synthesis phase (S) between the two gap phases (Norbury & Nurse, 1992; Schafer, 1998). The pre-replicative G1 phase has a metabolic role with the synthesis of nutrients, RNA and proteins and to prepare the cell for DNA replication (Pardee, 1989). In addition, G1 is the phase during which cells make the choice to enter a proliferative cycle or to stay in G1. During G1, cells can also exit the cell cycle and enter in a non-proliferative G0 phase, during which they can remain quiescent until their reactivation, or arrest permanently to terminally differentiate into specialized cells having specific functions. Most of the cells in our body are in a G0 resting phase, meaning that they are in a non-growing and non-proliferating state. If these cells want to re-enter the cell cycle, they will have to enter in G1 again before being able to initiate the following S phase. After S phase, there is the initiation of the second gap phase, G2 which role is to increase cell mass and organelle number to prepare cells for chromosome segregation in mitosis (M-phase).

DNA replication

During S-phase, the DNA-double helix opens, and the replication machinery starts to synthesize DNA to obtain an identical copy, forming two sister-chromatids for all chromosomes. Unwinding of the DNA double helix is mediated through the activity of helicases, which generates single strand DNA (ssDNA) (Abdel-Monem et al., 1976). DNA synthesis is performed by DNA polymerases, which catalyze the addition of nucleotides only in the 5' → 3' direction (Friedberg, 2006; A. Kornberg et al., 1956; Lehman, 2003). For this reason, while the leading strand is synthesized continuously from 5' → 3', the lagging strand is synthesized in a discontinuous manner, forming DNA fragments called Okazaki fragments (Burgers, 2009).

These fragments are later re-assembled by a specific machinery containing nucleases and ligases (Zheng & Shen, 2011).

DNA replication begins with the assembly of pre-replication complexes (pre-RCs) at replication origins during G1 (**Figure 2**). DNA replication origins are usually defined as the genomic region at which DNA replication machinery is loaded and DNA replication starts. In the yeast *Saccharomyces cerevisiae*, replication origins have specific DNA sequences (Marahrens & Stillman, 1986), while distinct consensus DNA sequences have not been described in metazoans so far. However, it is known that thousands (approximately 30000 to 50000 in human cells) of replication origins are activated at each cell cycle, and that there are many more origins than needed for each cell cycle. In addition, replication origins are activated in clusters, that can be separated in early- and late-firing origins (Fangman & Brewer, 1992). Replication initiation in eukaryotes occurs in a two-step fashion: recognition of the pre-RCs, also called “licensing”, and activation of DNA synthesis, termed “firing”. Origin licensing involves the recruitment of several proteins, such as the origin recognition complex (ORC1 to ORC6), followed by CDC6 and CDT1, which in turn recruit the mini-chromosome maintenance (MCM) complex. MCM has a helicase activity, which is required to unwind the DNA double-helix (Fragkos et al., 2015; Masai et al., 2010). Then, pre-RCs are converted into a replication fork complex (replisome), by recruiting other factors such as DNA polymerases to synthesize the DNA of both daughter strands, a process also called DNA chain elongation. Further, DNA replication is completed by replication termination, which involves the convergence of replication forks, disassembly of the replisome, and processing of the daughter DNA molecules (Dewar & Walter, 2017).

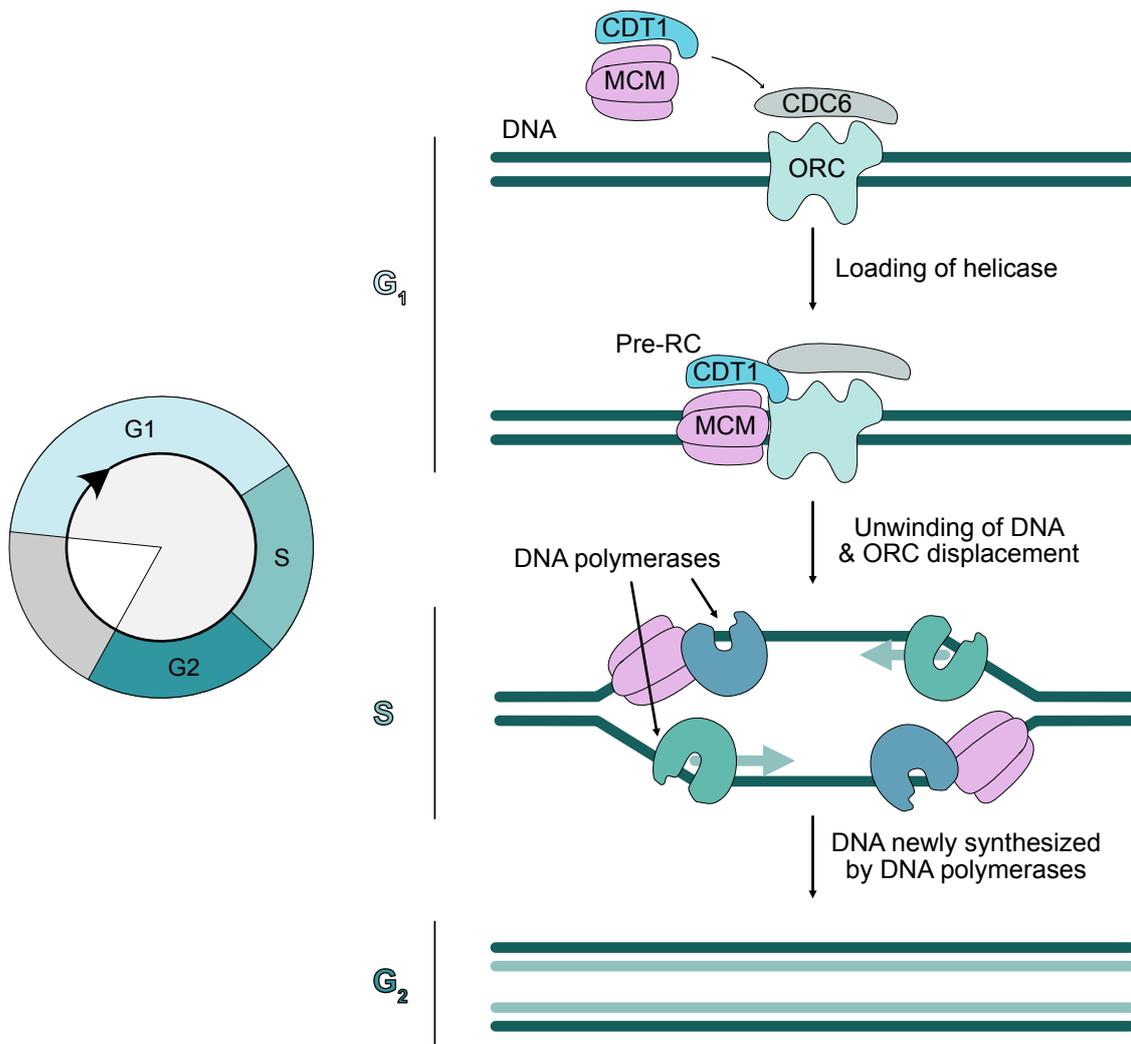


Figure 2. DNA replication initiation in eukaryotes. During G₁, the recruitment of the pre-RC complex (ORC-CDC6-CDT1-MCM) on replication origins triggers their “licensing”. Unwinding of DNA by MCM and recruitment of DNA polymerases generates origin firing, during which DNA starts to be synthesized, marking the beginning of S-phase. After completion of DNA replication, cells enter in G₂.

1.2.2 Mitosis

The process of cell division has first been described by Walther Flemming in 1882. Flemming observed filamentous structures changing size and shape before cell division takes place (Flemming, 1882; Uzbekov & Prigent, 2022). These filaments were later called “chromosomes” by Heinrich Waldeyer (Waldeyer, 1888). Mitotic chromosomes consist of a pair of sister-

chromatids linked together by a region called the centromere, which is the site of recruitment of a large protein complex, the kinetochore. The kinetochore is a complex made of several dozens of proteins that represent the connection point between chromosomes and the mitotic spindle (described below) (Batty & Gerlich, 2019; Cleveland et al., 2003; Walczak et al., 2010). Mitosis is subdivided into different phases, each one having their own function and establishment (**Figure 3**). Mitosis starts with prophase, when DNA condenses and individualises into visible mitotic chromosomes (Flemming, 1882; Sedat & Manuelidis, 1978). At the same time, the two centrosomes start to nucleate microtubules (MTs) and form larger asters (Zhai et al., 1996) in the cytoplasm. Nuclear envelope break down (NEBD) marks the entry in prometaphase and while the two poles of the bipolar mitotic spindle are assembled (Peter et al., 1990; Zhai et al., 1996). In parallel, mitotic chromosomes are captured by MTs and move to align to the equator in a process called chromosome congression (Darlington, 1937). The correct alignment of all chromosomes on a plate corresponds to metaphase. Shortly after, anaphase begins, the cohesion between sister chromatids is removed, chromosomes move poleward and spindle poles separate (Saunders, 1992). In telophase, nuclear envelope reforms around decondensing chromosomes, while the cell physically divides its cytoplasm during a process called cytokinesis, generating two genetically identical daughter cells. Cytokinesis is the very last step of cell division and is crucial for the physical separation of daughter cells. Briefly, it involves the formation of a machinery composed of several elements of the cytoskeleton. In *Drosophila*, many studies have identified molecular components required for cytokinesis (Gatti et al., 2000; Karess et al., 1991; Giansanti et al., 2001 ; Somma et al., 2002; Williams et al., 1995). In mammalian, yeast and fungal cells, filamentous actin, type II myosin and other proteins assemble into a ring in a plane perpendicular to the axis along which the chromosomes are segregated (Cabernard, 2012; Gatti & Baker, 1989; McKenzie & D'Avino, 2016; Schroeder, 1973). Then, constriction of this acto-myosin ring generates the force necessary for the cleavage of the mother cell into two daughter cells. The two sibling cells can have different fate depending on their mode of division. If the division is symmetric, the two cells are similar. In contrast, upon asymmetric division, daughter cells are asymmetric in fate through the inheritance of differential fate determinants and in some cases, as in *Drosophila* neuroblasts (NBs), they are also asymmetric in size (Rhyu et al., 1994; Robert Horvitz & Herskowitz, 1992; Yu et al., 2006) and further described in section 4)

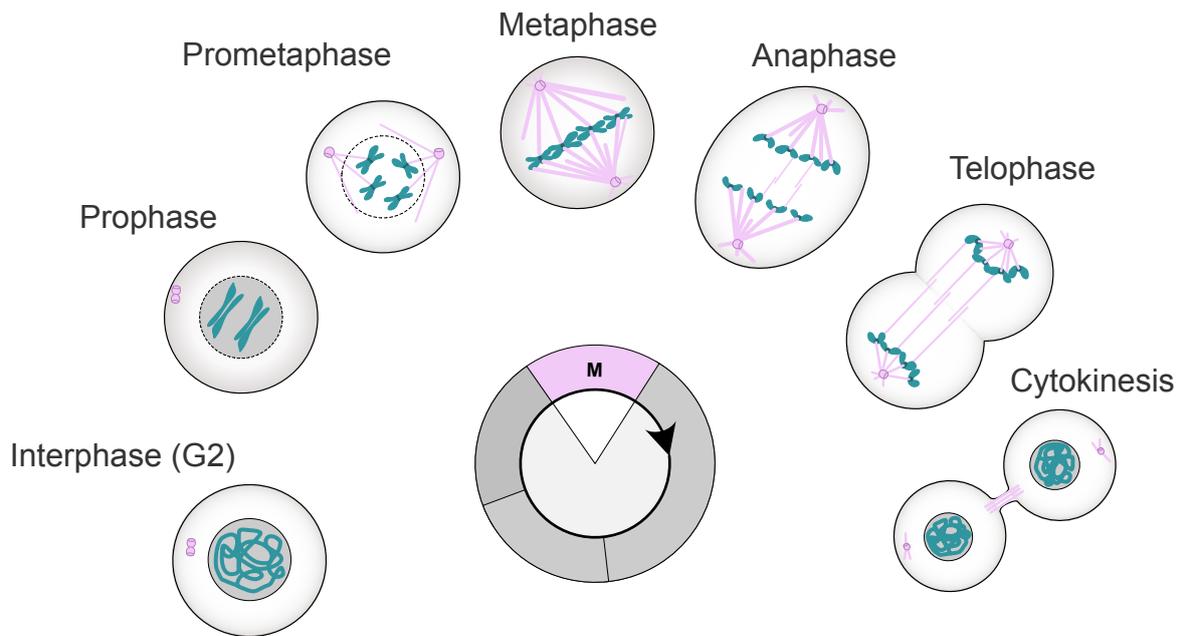


Figure 3. Different phases of mitosis. Mitosis starts with prophase, where DNA condenses, and forms individualized chromosomes (in blue). In prometaphase, the nuclear envelope is ruptured, and MTs are nucleated from the centrosomes (in pink), which start to establish the two poles of the mitotic spindle. Chromosomes are captured by MTs which place them at the equator of the cell. During metaphase, chromosomes are aligned on the equatorial plate of the cell. In anaphase, chromosomes move poleward and the two spindle poles separate. NE is reformed during telophase, and the cell physically divides into two daughter cells during cytokinesis.

Mitotic chromosome formation

The DNA contained in the nucleus is a very long molecule, which measures near to 2 meters in human diploid cell (Ross, 1999). To reduce its size, DNA is organized into nucleosomes, which are composed of DNA wrapped around histone proteins (Kornberg, 1974). Over the cell cycle, the chromatin undergoes numerous changes in its organization. In interphase, the chromatin is compacted around histones but decondensed into a meshwork of chromosomes intertwined with each other inside the nucleus. Long before M phase begins, the cell prepares for mitosis by initiating sister-chromatid cohesion, which is essential to promote correct chromosome segregation (Hopfner, 2003). This process relies on a protein complex called cohesin, which links the duplicated DNA molecule together as they are synthesized during S phase (Nishiyama, 2019). When S phase is completed, the cell contains several sister-chromatid pairs that form a mass of tangled DNA and protein. Therefore, any attempt to

segregate these intertwined chromatids would lead to DNA breakage. To avoid this, entry into mitosis triggers several structural changes in the chromosomes, such as their individualization into compact structures. This compaction is mediated through the recruitment of a ring-shaped complex named condensin, which forms chromosomal loops (Chan et al., 2004; Cuylen & Haering, 2011; Hirano, 2005). Chromosome condensation is essential for chromosome segregation, as inactivation of condensin leads to defect in sister chromatid resolution during mitosis (Hirano, 2005) and mutations in condensins are lethal in yeast and *Drosophila* (Coelho et al., 2003; Steffensen et al., 2001).

1.3 Cell cycle regulation

1.3.1 Cyclin-CDKs

The transition from one phase to another is regulated by a large number of proteins. Key regulators are the cyclins and cyclin-dependent kinases (CDK), a family of serine/threonine protein kinases which require the binding of a regulatory cyclin subunit to ensure their enzymatic activation (Hochegger et al., 2008; Matthews et al., 2022; Norbury & Nurse, 1992; Vermeulen et al., 2003, Murray and Hunt, 1993). CDK protein levels may remain stable during the cell cycle, but their activity rise and fall as the cell progresses through the cell cycle, thanks to waves of accumulation of specific cyclins (Evans et al., 1983; Hunter & Pines, 1991). When they are activated, CDK induce downstream processes by phosphorylating selected proteins (Morgan, 1995; Pines, 1995) (**Figure 4**). On the other hand, cyclins display dramatic changes in concentration during the cell cycle, by changes in cyclin gene expression and/or destruction of cyclin by proteolysis (further described in the *section 1.3.2*). Cyclins can be divided into four classes, based on the timing of their expression and their function in the cell cycle: G1, G1/S, S and M cyclins. The choice of entering the cell cycle in G1 is mediated through the accumulation of the G1 cyclin D, which associates with CDK4/6 (Sherr, 1994). The transition from G1 to S is regulated by the cyclin E-CDK2 complex. The association of cyclin A-CDK2 is required for replication initiation and S-phase progression (Fang & Newport, 1991). Cyclin B catalyzes entry into mitosis, together with CDK1 (Nurse, 1994, 2000).

However, cyclin binding alone is not sufficient to completely activate CDKs involved in cell cycle control, as it also requires phosphorylation of a threonine residue adjacent to the

kinase active site. This phosphorylation is catalyzed by enzymes called CDK-activating kinases (CAKs), which activity is maintained at constant level throughout the cell cycle. In mammalian cells, phosphorylation of CDKs can occur only after cyclin is bound, while it occurs before cyclin binding in budding yeast (Morgan, 1997). Conversely, two inhibitory phosphorylations have an important role in the regulation of CDK activity in animals. The first one is localized at a conserved tyrosine residue found in almost all the CDKs (Tyr 15), and the other occurs at an adjacent threonine residue (Thr 14). The phosphorylation state of these two sites in CDK1 is controlled by a balance of opposing kinases and phosphatase activities, namely WEE1 and CDC25. Therefore, WEE1 kinase inactivates cyclin-CDK complexes, while CDC25 phosphatase leads to its activation (Cheng-Yuan Peng, 1997; Rhind & Russell, 2001; Sanchez et al., 1999).

In G1, an important target of the CDKs is the retinoblastoma tumor suppressor protein (RB) (described in the next paragraph), which is inactivated by CDK4-dependent phosphorylation (Kato et al., 1992). Hypo-phosphorylated RB binds the E2F transcription action and keeps it inactive during G1 (Weintraub et al., 1992). Once CDK4 phosphorylates RB, it releases E2F, resulting in E2F-dependent transcription of factors essential for cell cycle progression and G1/S transition (Bertoli et al., 2013; Matthews et al., 2022; Rubin et al., 2020). In addition, the most important inhibitors of the G1 phase are p16 and p21, which can form stable complexes with the CDK subunit before they bind to cyclins (further described in the section 1.3.3, and figure 2B).

The transition from G2 to M is triggered by the activation of CDK1, which acts in complex with cyclins A and B (Crncec & Hochegger, 2019; Hochegger Helfrid et al., 2008; Gautier et al., 1988; Gavet and Pines, 2010) . More precisely, cyclin B cytoplasmic levels increase during G2 until it binds to CDK1 (Hunter & Pines, 1991). After the activation of cyclin B-CDK1, the complex relocates to the nucleus, which contributes to nuclear envelope breakdown (Gavet & Pines, 2010a, 2010b) Cyclin B-CDK1 complex activity is controlled by rapid change in CDK1 phosphorylation, mediated by WEE1 and CDC25. All along the cell cycle, CDK1 remains inactive thanks to its phosphorylation by WEE1, while CDC25 levels increased just before mitotic entry, allowing CDK1 activity (Heald et al., 1993; MCGowan1 & Russell, 1995; Perry & Kornbluth, 2007).

During mitosis, this complex is also required to activate the anaphase-promoting complex (APC/C), which promotes the destruction of cyclin A and B (Geley et al., 2001). Cyclin B destruction is delayed by the spindle assembly checkpoint (SAC – discussed in the next

section 1.4) until all sister chromatids are aligned on the metaphase plate (Clute & Pines, 1999). Once this is achieved, checkpoint inhibition is relieved and the anaphase-promoting complex (APC/C), a ubiquitin ligase, triggers the proteolytic degradation of cyclin B by the proteasome, promoting mitotic exit (Acquaviva et al., 2004; Lara-Gonzalez et al., 2012; Yamano, 2019).

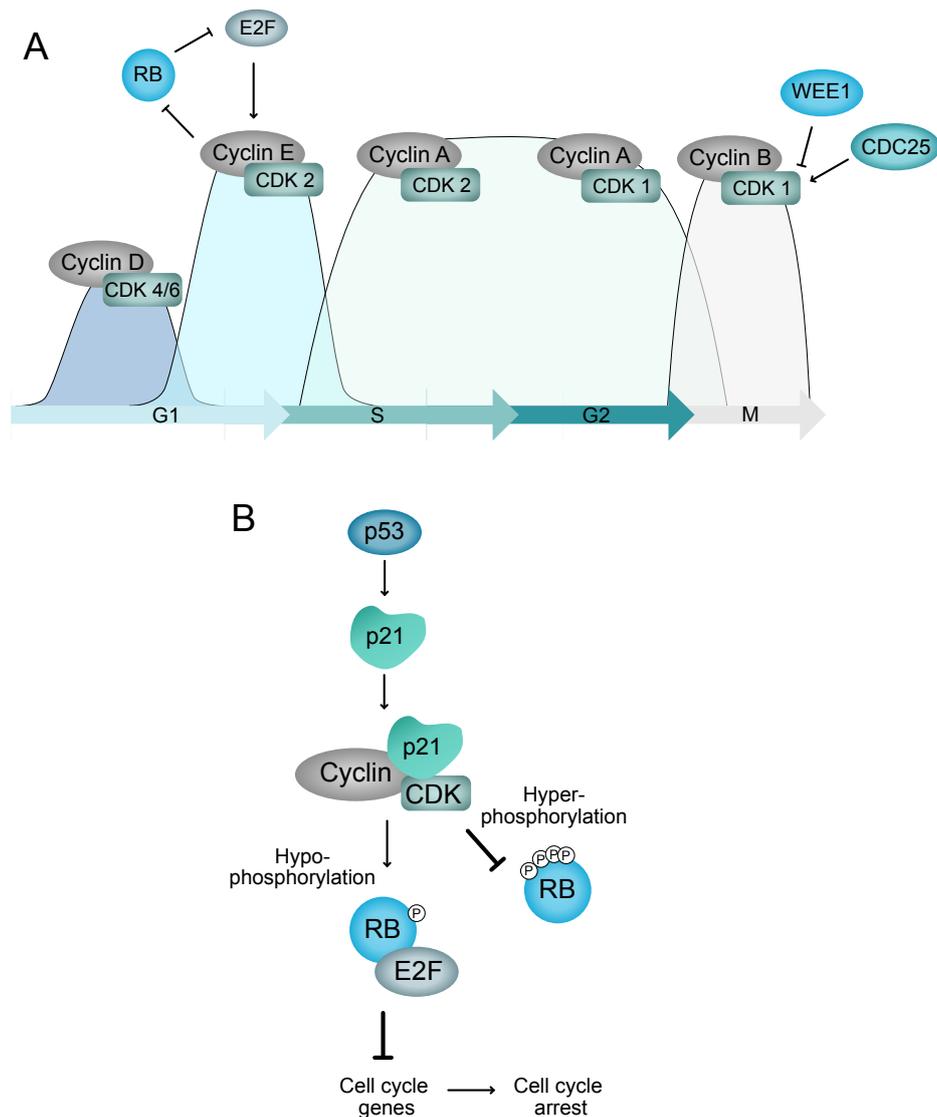


Figure 4: Cell cycle regulation by Cyclin CDK and RB-E2F. A. Cyclin-CDKs association throughout the cell cycle. E2F dependent transcription allows accumulation of Cyclin E and cyclin A to promote initiation of replication. Cyclin association with CDK1 allows G2 entry to prepare mitosis. Then accumulation of cyclin B-CDK1 drives mitotic entry and activates APC/C to allow mitotic exit. **B. Cell cycle regulation by RB.** Under stress conditions, p53 promotes the transcription of p21, which inhibits multiple CDKs, and releases RB from its inhibitory

phosphorylation. Association of RB-E2F downregulates the expression of E2F target genes, leading to cell cycle arrest.

1.3.2 Proteolytic regulation of CDK activity

As described above, the levels of CDK activity oscillate to promote cell cycle progression. These oscillations are regulated by the levels of CDK activators or inhibitors, which can be controlled by gene expression, phosphorylation levels, or proteolysis (King et al., 1995). One of the major pathways required for proteolysis relies on the assembly of a ubiquitin chain on the substrate, which targets it for degradation by the 26S proteasome, a multiprotein complex carrying protease activity (Peters, 1994). The formation of ubiquitin chain is catalyzed by several enzymes, which act sequentially. First, ubiquitin protein gets activated by a ubiquitin-activating enzyme called E1. Then, the ubiquitin molecules get transesterified by a ubiquitin-conjugating enzyme, named E2. Finally, ubiquitin is transferred from the E2 to the target protein to its substrate by a E3 ubiquitin ligase (**Figure 5**). E3 ubiquitin ligase also catalyzed the formation of a polyubiquitin chain on the target, which facilitates efficient recognition of the substrate by the proteasome.

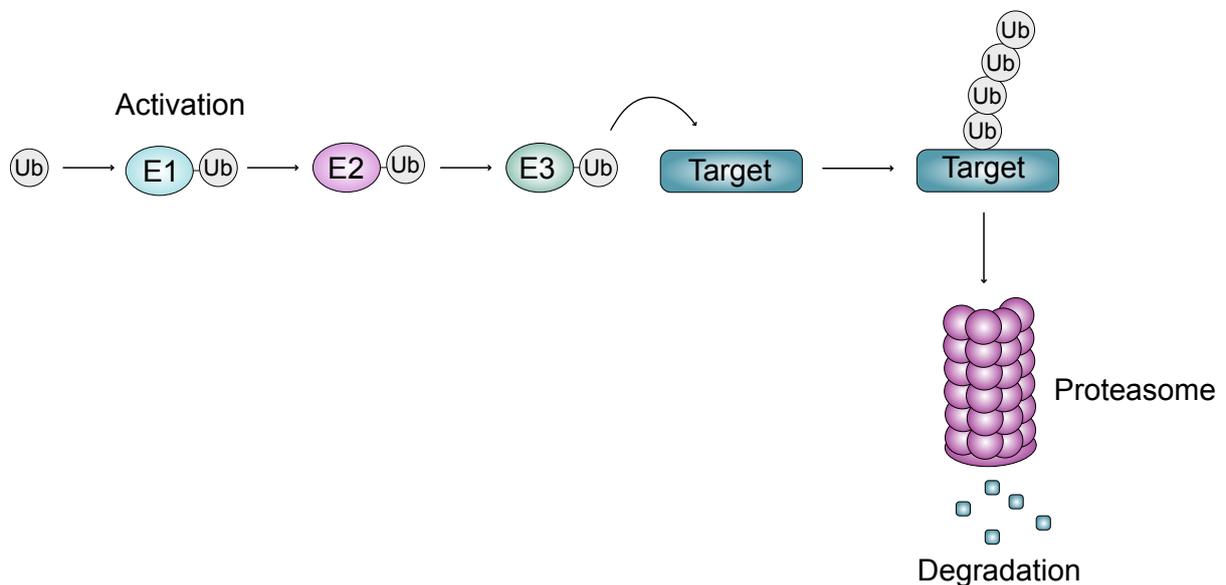


Figure 5: Mechanism of protein targeting to the proteasome by ubiquitination. Ubiquitin protein is first activated by E1 enzyme, conjugated by E2 enzyme, and transferred to its target protein by E3 ubiquitin ligase.

Several cell cycle regulators are targeted by ubiquitin post translational modifications, to tightly regulate the transitions from one phase to the other (Craney & Rape, 2013). The best understood E3 ligases involved in the cell cycle are cullin-RING-ligases (CRLs) and their relative, and the anaphase-promoting complex (APC/C). At the G1/S transition, Cyclin D levels decrease through the destruction of the protein, which is controlled by ubiquitin-mediated proteolysis (Diehl et al., 1997). More precisely, Cyclin D is polyubiquitinated by specific E3 ligases called Cul1 based RING E3 ligases forming a complex called SCF (Zheng et al., 2002). In addition, to be addressed to the proteasome, cyclin D is first phosphorylated threonine 286 by the glycogen synthase kinase 3 β (GSK3 β) (Diehl et al., 1998) This phosphorylation leads to Cyclin D nuclear export and rapid degradation within the cytoplasm (Alt et al., 2000).

Furthermore, the G1/S transition is not the only cell cycle stage to be controlled by proteolysis, as mitotic exit is also controlled by a E3 protein ligase, APC/C. Its role will be further discussed in the section 1.4.3.

1.3.3 Retinoblastoma susceptibility gene (RB)

RB was the first tumor suppressor gene to be identified. It is a central regulator of the cell cycle that acts as a transcriptional co-repressor in a complex with the E2F family of transcription factors (Arroyo & Raychaudhuri, 1992; Dyson, 1998; Weinberg, 1995). RB-E2F complex formation depends on the phosphorylation of RB (Rubin et al., 2020), which binds to E2F when it is hypo-phosphorylated and downregulates the transcription of E2F target genes (Weintraub et al., 1992). These are essentially genes controlling the cell cycle, such as DNA polymerases, Cyclin A and Cyclin E, CDK1, and several factors required for DNA replication licensing (Chicas et al., 2010). When RB is hyperphosphorylated by either cyclin D-CDK4/6, it gets inactivated and released E2F, allowing the expression of E2F targets genes and leading to G1/S transition (Bertoli et al., 2013). RB inactivation compromises cell cycle checkpoint control at the G1/S transition (Burkhart & Sage, 2008; Dyson, 2016). In addition, it was recently described that cell growth during G1 dilutes RB, which in turns releases E2F inhibition and triggers cell division in human cells (Zatulovskiy et al., 2020). This suggests that S phase entry is not only controlled by CDK-dependent phosphorylation of RB, but also by cell size-dependent dilution of RB.

Importantly, CDKs can be inhibited by the tumor suppressor p21 (Sherr & Roberts, 1995). p21 is one of the most well-known p53 targets (discussed in the next section – cell cycle checkpoints), which mediates G1 cell cycle arrest. p21 can bind a to a wide range of CDKs to inhibit their function. Therefore, it can stimulate the formation of RB-E2F complex, leading to downregulation of cell cycle genes (**Figure 4B**). p21 targets, such as CDK4/6, have been addressed by several inhibitors such as Palbociclib or abemaciclib, as a cancer therapy to compensate p21 loss in tumors (O’Leary et al., 2016).

1.4 Cell cycle checkpoints

Besides cell cycle control by Cyclin-CDKs, cells contain cell cycle checkpoints to monitor the quality of the major events of the cell cycle. These include growth to the appropriate cell size, the replication and integrity of the chromosomes, and their accurate segregation at mitosis. In G1, the “restriction point” control sufficient cell growth, while the G1/S checkpoint monitors the absence of DNA damage before commitment to S-phase. (Blagosklonny & Pardee, 2002; Pardee, 1974; Tobey, 1973). This checkpoint relies on RB signaling, as described in the previous section (Bartek et al., 1996). When the cell is engaged to enter the cell cycle can now initiate DNA synthesis during S-phase. During this phase, the intra S-phase checkpoint monitors DNA replication and is activated under perturbed conditions. At the end of G2, another checkpoint controls for correct duplication of DNA and absence of DNA damage before mitotic entry (reviewed in (Cuddihy & O’connell, 2003). Indeed, the presence of DNA damage leads to the activation of the sensor checkpoint protein 1 and 2 (CHK1/2) (discussed in the section 2.3) and the tumor suppressor P53 to induce cell cycle arrest, allowing DNA repair either before DNA replication (G1/S checkpoint), or before mitosis (G2/M) checkpoint. During mitosis, the spindle assembly checkpoint ensures the correct attachment of chromosomes to the mitotic spindle, therefore ensuring faithful chromosome segregation (reviewed in (Musacchio & Salmon, 2007).

1.4.1 The restriction point

The concept of restriction point was described in 1974 by Arhtur Pardee (Pardee, 1974) who assessed cell cycle commitment in response to growth factors in animal cells. By withdrawing

growth factors at different time points during G1, it has been proposed that there is a moment at which cells commit to enter the cell cycle and beyond this point, removal of growth factors will no longer affect the initial commitment. This key step has been called “restriction point”. Later, this point was suggested to coincide with hyperphosphorylation of RB (described in the previous section). More precisely, re-stimulation of starved cells with growth factors led to increased cyclin D, which in complex with CDK4/6, initiate RB phosphorylation (Ezhevsky et al., 1997). Therefore, this liberates E2F, which in returns initiate the transcription of cyclins E and A, which in complex with CDK2 forms a positive feedback loop to trigger the switch from hypo to hyper-phosphorylated RB. This switch is believed to mark cell cycle commitment (Zarkowska & Mitnacht, 1997).

1.4.2 DNA damage checkpoints

DNA damage checkpoints prevent the accumulation of genetic aberrations during cell division (Hartwell & Weinert, 1989). These checkpoints detect errors and act on the cell cycle machinery to slow or stop the progression, allowing the cell to correct these errors. Precisely, they induce cell-cycle delay, help activate DNA repair (described in *section 2.4*), maintain cell-cycle arrest until DNA repair is complete, and re-initiate cell-cycle progression (Bartek & Lukas, 2001; Nyberg et al., 2002). Very important actors of the cell cycle checkpoints are the sensor checkpoint proteins 1 and 2 (Chk1 and Chk2) kinases, which have the capacity to regulate crucial cell cycle regulators, such as CDKs, but also p53 tumor suppressor (further described below).

G1/S checkpoint

In G1, the major checkpoint pathway in response to DNA damage is the Ataxia-telangiectasia mutated (ATM)-p53 signaling, which activates p21 to promote sustained or even permanent G1 arrest (Bartek & Lukas, 2001; Polyak et al., 1996; Schafer, 1998). Precisely, in response to DNA damage, ATM, a kinase involved in DNA damage response (further discussed in *section 2.3.1*), phosphorylates p53 transcription factor (described below), allowing the transcription of genes required to halt cell-cycle progression or trigger programmed cell death. One of these genes encodes p21, a cell cycle inhibitor protein which binds and inhibits the G1 CDKs (El-Deiry et al., 1993; Harper et al., 1993 and **Figure 6**). In addition to the p53 pathway, a more

immediate mechanism has been described, which is independent from p53 (Costanzo et al., 2000; Mailand et al., 1996) This pathway relies on the CDC25A phosphatase, which activity is required to activate CDK2 and allow G1-S transition (Blomberg & Hoffmann, 1999; Donzelli & Draetta, 2003; Hoffmann et al., 1994; Jinno et al., 1994). Precisely, upon UV or IR exposure, CHK1 and CHK2 kinases directly phosphorylate CDC25 which addresses CDC25 to the proteasome for its degradation. Therefore, CDK2 remains inactive and cell cycle cannot progress from G1 to S (Mailand et al., 1996).

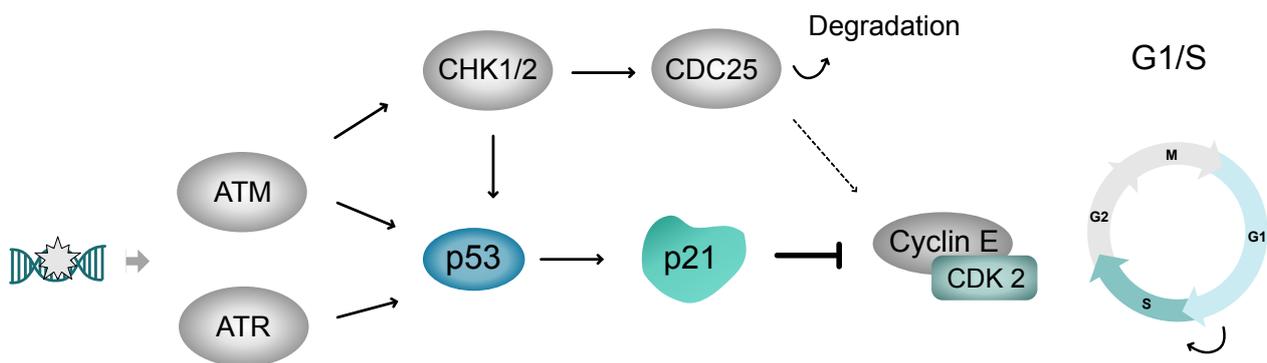


Figure 6. G1/S Checkpoint. Upon DNA damage, ATM and ATR kinases activate downstream effectors in two distinct pathways. One immediate pathway is mediated through Chk1 and Chk2 kinases, which lead to the degradation of Cdc25 phosphatase, a positive regulator of Cyclin E-CDK2. The other pathway is mediated through p53 activation, which allows the transcription of p21, inhibitor of cyclin E-CDK2. This second mechanism is more indirect, because it involves the transcription of effectors, which requires more time.

Intra-S and replication checkpoint

During S-phase, DNA replication takes place from replication origins that are dispersed throughout the genome. Origin firing occurs within a predictable pattern: some origins are fired early while some others much later (Fangman & Brewer, 1992). Different studies have revealed the existence of an intra-S checkpoint, which inhibits the firing of late replication origins under conditions of perturbed DNA replication (Feijoo et al., 2001; Heffernan et al., 2002; Karnani & Dutta, 2011; Katsuhiko Shirahige et al., 1998; Petermann & Caldecott, 2006; Santocanale & Diffley, 1998). Inhibition of origin firing is mediated through the activity of checkpoint kinase 1 (CHK1). When it is activated, CHK1 disrupts the association between component of replication initiation with replication origins (Feijoo et al., 2001; Kelly et al., 2022). Later, the intra-S checkpoint ensures that damaged or stalled replication forks are

stabilized and can restart. In addition, this checkpoint acts as a surveillance mechanism that ensures that DNA is only replicated once, by inhibiting DNA re-replication (Liu et al., 2007). Another mechanism controlling replication has also been described, via E2F-dependent transcription. Indeed, upon S phase entry, E2F dependent transcription is inactivated via a negative feedback loop involving the transcriptional repressor E2F6, which is a E2F target (Bertoli et al., 2013). Under perturbed DNA replication (also called replication stress – RS – discussed in the *section 2.2.1*), E2F-dependent transcription is maintained through Chk1-dependent phosphorylation (Bertoli et al., 2013). This further allows the transcription of key factors involved in checkpoint response, replication fork stabilization and protection, which prevents DNA damage (Bertoli et al., 2016). In addition, E2F-dependent transcription was recently shown to determine replication capacity and S-phase length in mammalian cells (Pennycook et al., 2020).

G2/M checkpoint

The G2/M checkpoint prevents cells to enter mitosis with DNA damage generated either in G2 or unrepaired damage from the previous S or G1 phases (Nyberg et al., 2002; Xu et al., 2001). If DNA damage is detected, the cell stays in G2 until DNA damage either gets repaired or in extreme cases, triggers terminal responses such as senescence or apoptosis. The G2 checkpoint targets principally cyclin B-CDK1 kinase, the key complex promoting mitosis. As described in section 1.3, CDK1 is regulated via its phosphorylation levels, mediated by the WEE1 kinase, which inhibits CDK1 activity, and CDC25B phosphatase. Upon DNA damage, CDC25B can be inhibited by ATM/ATR and sensor checkpoint proteins 1 or 2 (CHK1/CHK2) kinases, which phosphorylate CDC25B to decrease its activity, resulting in CDK1 inactivation. Therefore, upon checkpoint activation, CDC25B remains inactive, resulting in reduction of cyclin B-CDK1 activity (Cuddihy & O'connell, 2003; Donzelli & Draetta, 2003). Moreover, other regulators of CDC25 have been described, which are members of Polo-like kinase family (PLK), such as PLK1. PLK1 protein is a CDC25B activator and promotes mitotic entry (Qian et al., 1998; Smits et al., 2000; S. Xie et al., 2001). Thus, upon DNA damage detection, checkpoint proteins block PLK1 activity, leading to reduction in CDK1-CyclinB levels (Qin et al., 2013; Smits et al., 2000; Tsvetkov & Stern, 2005) (**Figure 7**).

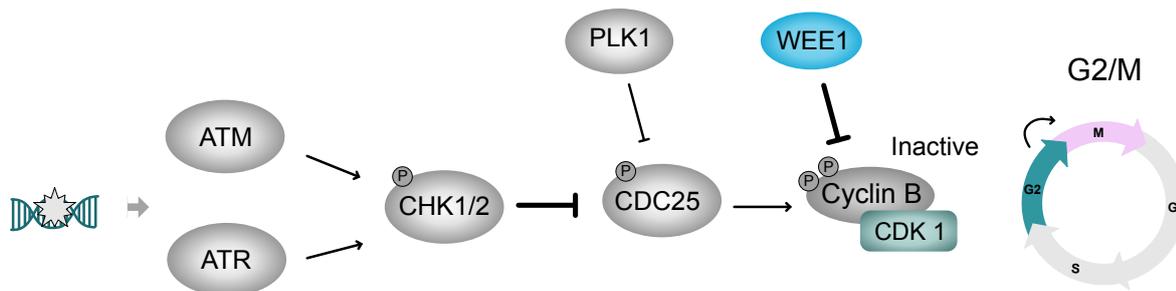


Figure 7. Main pathway for G2 checkpoint. If a cell carries DNA damage in G2, ATM and ATR proteins get activated, leading to the phosphorylation of Chk1 and Chk2 kinases. Further, these kinases negatively regulate CDC25 phosphatase, which is a positive regulator of Cyclin B-CDK1. Alternatively, PLK1 can also regulate CDC25. Therefore, Cyclin B-CDK1 stays inactivated by WEE1 phosphorylations at Tyr25 and Thr14. This leads to a G2 arrest until the inhibition is released.

p53 tumor suppressor

Many of the cell cycle checkpoints described above rely on the tumor suppressor p53 activation. Indeed, p53 has a central role in the response to cellular stresses (reviewed in (Hafner et al., 2019)) and in the DNA damage response (DDR) (discussed in the *section 2 - DNA damage*). In addition, p53 is the most frequently mutated gene in cancer (Baker et al., 1987; Kandoth et al., 2013; Vogelstein et al., 2013). In response to various stresses, such as DNA damage, hypoxia, or oncogene activation, p53 can promote the transcription of multiple genes involved in cell cycle arrest, DNA damage repair, cell death via various pathways and metabolic changes. Specifically, following DNA damage, p53 upregulates the expression of genes involved both in cell cycle arrest and DNA repair, which leads to cell survival, but it can also target genes involved in apoptosis, leading to cell death. In non-stressed conditions, p53 levels are regulated by mouse double minute 2 protein (MDM2), a ubiquitin ligase which promotes p53 degradation (Haupt et al., 1997; Kubbutat et al., 1997). When DNA damage is detected, p53 is phosphorylated, which makes it resistant to MDM2 (Shieh et al., 1997). p53 accumulation culminates with the activation of the transcription of its target genes, such as proapoptotic BAX, PUMA and NOXA (Riley et al., 2008), cell cycle arrest inducer p21 (Mirza et

al., 2003; Polyak et al., 1996), but also its own regulator MDM2 as a feedback loop (Barak et al., 1993).

1.4.3. Mitotic checkpoint

The mitotic checkpoint is also known as the spindle assembly checkpoint (SAC). This checkpoint was discovered in 1991 by genetic screens in the yeast *Saccharomyces cerevisiae* which identified key components of the SAC. In these two studies, the authors reported various genes which, when mutated, are able to bypass the mitotic arrest induced by spindle poisons (Hoyt et al., 1991; Li & Murray, 1991). These genes included the *MAD* (mitotic-arrest deficient) genes *MAD1*, *MAD2* and *MAD3* (BubR1 in humans), and the *BUB* (budding inhibited by benzimidazole) gene *BUB1* and *BUB3*. These components are all involved in a pathway active in prometaphase, which prevents the premature separation of sister chromatids. This checkpoint makes sure that all chromosomes are attached to spindle MT through specialized sites known as kinetochores (Cheeseman, 2014). Once all kinetochores are attached, the SAC is satisfied and allows the progression from metaphase to anaphase (Lara-Gonzalez et al., 2012; McAinsh & Kops, 2023; Musacchio & Salmon, 2007). In detail, during prometaphase, unattached kinetochores catalyze the formation of the mitotic checkpoint complex (MCC), composed of BUBR1, BUB3 and MAD2, which concentrate at the kinetochore with CDC20 (a co-factor and activator of the anaphase promoting complex/cyclosome – APC/C) (**Figure 8**). Another factor important is the MPS1 kinase, (Weiss & Winey, 1996), which activity is required to form the MCC complex at the kinetochore (Stucke et al., 2002). Localization of the MCC and CDC20 at the kinetochore leads to the inhibition of the APC/C, a E3 ubiquitin ligase targeting several proteins for their degradation by the proteasome. Once all the chromosomes are aligned on the metaphase plate with their kinetochores attached to the spindle, CDC20 is released, which in turn activates the APC/C, leading to the ubiquitylation and degradation of several substrates required for anaphase and mitotic exit: Securin, a protein involved in chromatid cohesion, and Cyclin B1. Degradation of Securin liberates Separase, which in turn cleaves the Cohesin ring structure. This cleavage opens the Cohesin ring and allows sister chromatids to separate during anaphase. Meanwhile, degradation of Cyclin B1 inactivates CDK1, which is essential to promote mitotic exit (Holloway et al., 1993; Luca et al., 1991). After

CDK1 inactivation, another APC/C regulator, CDH1, is dephosphorylated and binds to APC/C to degrade CDC20. The association APC/C-CDH1 retains cells in the following G1 phase or allows them to enter G0. Therefore, APC/C is also a key regulator of G1/G0 progression (Kernan et al., 2018; Kimata, 2019). Inactivation of CDH1 in cultured human cells shortens G1 phase, resulting in premature S phase (Sigi et al., 2009). In *Drosophila*, CDH1 (also called Fizzy-related) is required for G1 arrest in the embryo, in which it negatively regulates several Cyclins (Sigrist & Lehner, 1997).

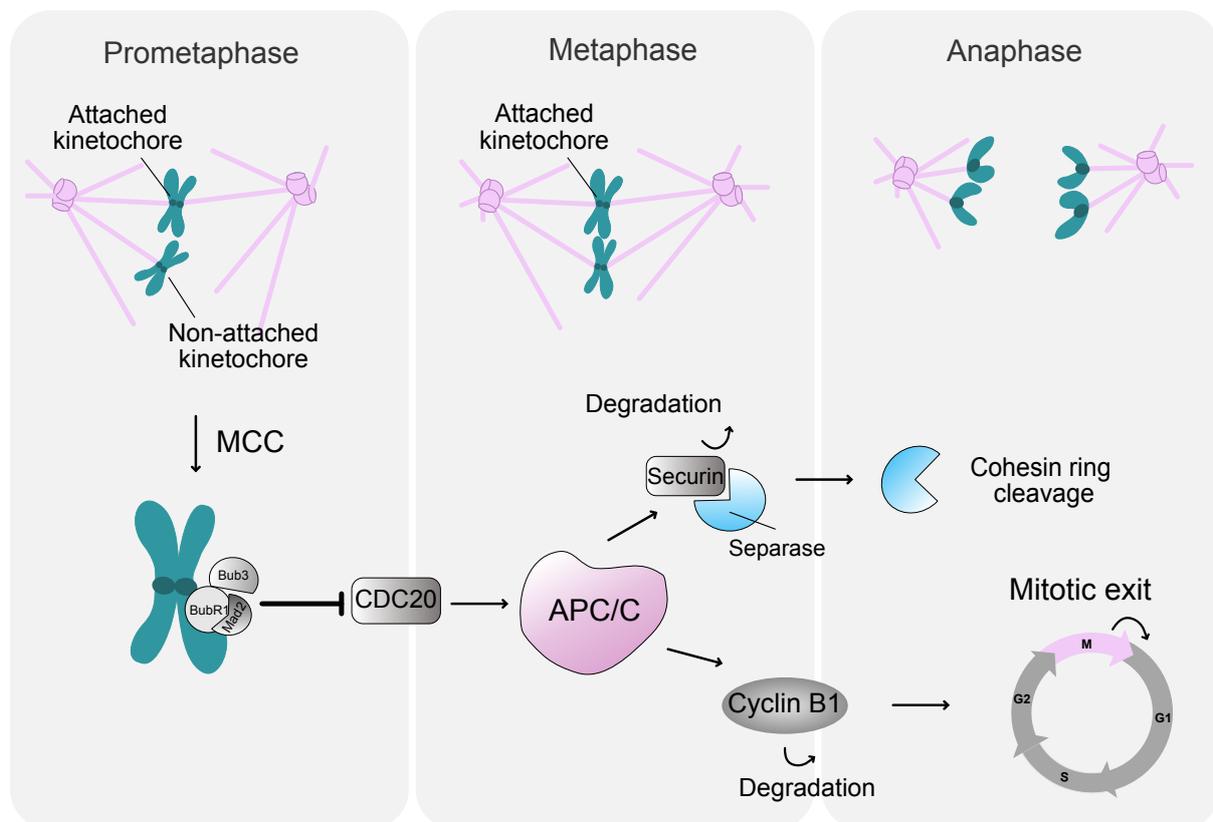


Figure 8. Core principles of the spindle assembly checkpoint (SAC). The SAC monitors the attachment between kinetochore and MTs. When a single unattached is present, the MCC complex (BUBR1, BUB3 MAD2, and CDC20), remains active which inhibits APC/C until all chromosomes are properly attached during metaphase. When the SAC is satisfied, CDC20 is released and APC/C is activated, which leads to the degradation of both Securin, allowing separation of sister chromatids in anaphase, and cyclin B1, to allow mitotic exit.

2. DNA DAMAGE & DNA REPAIR

2.1 Introduction to DNA damage

DNA can be damaged in many endogenous or exogenous situations leading to the perturbation of genetic integrity. Our body is almost constantly exposed to many environmental DNA-damaging agents, such as ultra-violet light, which can induce thousands of lesions per cell, per hour. In addition, energy released by free oxygen radicals, generated either by normal metabolic processes or by exposure to radiation can break the phosphodiester bonds in the backbone of the DNA helix. DNA aberrations can arise via other processes, such as mismatches between nucleotides or bases during DNA replication by DNA polymerases. Moreover, defects in topoisomerases activity can lead to enhanced DNA breaks, depending on which topoisomerase is inhibited and on the phase of the cell cycle (Berti et al., 2013; Promonet et al., 2020; Ray Chaudhuri et al., 2012). As a result, each cell in our body receives tens of thousands of DNA lesions per day (Hoeijmakers, 2009; Lindahl & Barnes, 2000). When two of these DNA breaks are close to each other, but on opposite DNA strands, a double-strand break (DSB) is generated, which is highly cytotoxic.

To respond to these threats, cells have developed various mechanisms that altogether form the DNA Damage Response (DDR) (Ciccia & Elledge, 2010; Jackson & Bartek, 2009 and further described below). Indeed, each type of DNA damage requires a specific set of cellular responses to repair specifically NA lesions, depending if they are localized on the DNA backbone or DNA bases. Moreover, the challenges of DNA repair can vary according to different phases of the cell cycle (Clay & Fox, 2021).

To optimally repair DNA damage, cells must also control other cellular processes before or during the repair, such as DNA replication or mitosis. In this section, I will describe the pathways involved in the DDR, as well as the various ways of repairing DNA damage along the cell cycle.

2.2 Sources of DNA damage

2.2.1 Replication stress (RS)

A large fraction of DNA damage in unperturbed cells is attributed to DNA replication errors or replication stress (RS). RS is a broad term which is defined as the slowing or stalling of replication fork progression (Zeman & Cimprich, 2014), leading to reduced replication fidelity and DNA breaks (Saxena & Zou, 2022; Técher et al., 2017; Zeman & Cimprich, 2014). RS appears to represent an important source of genome instability, and it is a hallmark of cancer cells (Bester et al., 2011; Burrell et al., 2013; Gorgoulis et al., 2005). Several sources of RS have been described (reviewed in (Zeman & Cimprich, 2014)), such as unrepaired DNA lesions or secondary DNA structures, RNA-DNA hybrids forming R-loops (Gan et al., 2011; Hamperl et al., 2017) or even lack of nucleotides (Bester et al., 2011). RS is known to cause DSBs at specific loci in the genome, known as ‘common fragile sites’ (CFS) (Le Tallec et al., 2014; Sutherland & Richards, 1995). These regions are very sensitive to even mild RS and are believed to be essentially the consequence of late-replicating regions entering prematurely in mitosis with un-replicated DNA (further described in the *section 2.2.2.1*). However, it is now established that CFS breakage can occur as a consequence of various stresses, leading to replication fork stalling or slowing. Here, I will describe the different causes and consequences of RS.

2.2.1.1 Replication fork barriers

This category includes any physical obstacle perturbing the progression of replication forks. One of the most recognized replication fork barriers is unrepaired DNA lesions or adducts (Ashour & Mosammaparast, 2021), which can be caused by different sources. These include chemical mutagens, UV radiation, reactive oxygen species (ROS) or byproducts of cellular metabolism (Tubbs & Nussenzweig, 2017). They can lead to roadblocks for DNA polymerases that will bypass the lesions through a process called translesion synthesis (TLS) (Lehmann et al., 2007). In addition, DNA cross-linkers such as cisplatin derivatives or mitomycin C -used in cancer therapy- induce DNA inter-strand crosslinks (ICLs), which prevents separation of the two strands of the DNA double helix (Deans & West, 2011; H. Kim & D’Andrea, 2012). In such conditions, the replisome cannot progress through the replication fork and DNA replication is halted.

Another source of RS results from the collision between the DNA replication and transcription machineries, also called transcription-replication conflict (TRC) (Aguilera & García-Muse, 2012; Kumar et al., 2021). Such collisions can generate abnormal DNA-RNA hybrids called R-loops, which physically impede replication fork progression (Gan et al., 2011; Hamperl et al., 2017; Li & Manley, 2006). R-loops have been observed in many organisms, from bacteria to humans, and can be cleaved by specific nucleases (Chappidi et al., 2020; Sollier et al., 2014) to avoid genomic instability. Precisely, they can either be removed by RNAses or excised by *Xeroderma pigmentosum* complementation group G and F (XPG/XPF) nucleases, as well as the MUS81 nuclease (Chappidi et al., 2020).

2.2.1.2 Nucleotide imbalance

To preserve genome integrity, nucleotide pools must be coordinated with replication dynamics (Anglana et al., 2003). Indeed, small variations in pool size substantially affect fork progression from yeast to mammalian cells (Poli et al., 2012). More precisely, low deoxyribonucleoside triphosphate (dNTPs) levels compromise DNA polymerase activity (Beck et al., 2012; Buisson et al., 2015) and are associated with increased genomic instability and tumorigenesis in mammalian cells (Bester et al., 2011; Chabosseau et al., 2011). Lack of nucleotides can arise from various origins, such as accelerated cell proliferation or inhibition of the ribonucleotide reductase – essential enzyme for the synthesis of dNTPs- due to mutations in genes involved in the biosynthetic pathway, inhibition of ATR, CHK1 or WEE1, which impairs polymerase activity (Beck et al., 2012; Buisson et al., 2015) or by hydroxyurea (HU) treatment, which directly inhibits the ribonucleotide reductase (Singh & Xu, 2016). It has been shown that RS due to lack of nucleotide can be attenuated upon exogenous supply of nucleotides in human fibroblasts and hamster cell lines, as well as in colorectal cancer cell lines (Anglana et al., 2003; Bester et al., 2011; Burrell et al., 2013; Gay et al., 2010; Wilhelm et al., 2014). However, exogenous supply can also be deleterious for cells, as imbalance between the pool of the four different nucleoside triphosphates can have genotoxic effects (Mathews, 2006). In addition, inhibition of dNTP triphosphohydrolase SAMHD1, which degrades dNTPs, leads to the dramatic increase of the dNTP pool, which perturbs cell cycle progression and increase cell sensitivity to DNA damaging agents (Franzolin et al., 2013).

2.2.1.3 Origin firing dysregulation

As previously described in the first section, DNA replication starts at specific loci called replication origins, which are present in exceeding numbers than the ones that will be fired during a replication cycle. It has been described that the “dormant” but licensed origins can be activated if replication forks are stalled, to provide a backup mechanism to complete DNA replication (Cortez, 2015; McIntosh & Blow, 2012). However, genomic regions without enough licensed origins do not have this mechanism, which results in under-replication and therefore expression (breakage) of common fragile sites (CFs) (further described in the *section 2.2.1.4*). In addition, mutations in genes encoding replisome proteins also compromise genome stability by reducing the number, stability or fidelity of replication forks. For instance, mutations in genes encoding members of the helicase MCM complex are associated with a reduction in active forks, leading to increased RS (Alvarez et al., 2015). These mutants also have a decrease in the rate of origin licensing and have been shown to induce cancer in mouse models (Shima et al., 2007). Conversely, firing too many origins can rapidly deplete the nucleotide pool (as described above) and therefore slow replication fork speed (Beck et al., 2012; Buisson et al., 2015), resulting in genomic instability (Thakur et al., 2022).

2.2.1.4 Consequences of RS

As mentioned earlier, RS compromises the fidelity and timely completion of genome duplication. It can lead to DNA breaks in S phase and subsequent stages of the cell cycle, impairing chromosome segregation (discussed in the next *section 2.2.2 – mitotic errors*) and induce genomic rearrangements in the following cell cycle. Locally, obstruction to replication progressions can cause fork stalling, which is one of the major contributors to genomic instability (Gaillard et al., 2015). Indeed, impairment of DNA synthesis can generate DNA damage either on the leading, the lagging or both DNA strands. Depending on which strand is affected, it can lead to single strand DNA (ssDNA) stretches of different lengths (Byun et al., 2005; Pasero & Vindigni, 2017). Such ssDNA is further recognized by members of the DNA damage response (DDR), which will lead to cell cycle arrest and DNA repair (discussed in *section 2.3*). In addition, prolonged stalling of replication fork leads to fork collapse and DSBs (Dungrawala et al., 2015). DSBs can also be generated by structure-specific nucleases at stalled

forks (Hanada et al., 2007; Lemaçon et al., 2017; Pepe & West, 2014). When these breaks are wrongly repaired by the DNA repair machinery, they can cause mutations and several types of chromosomal alterations, such as deletion, duplications, and translocations. In addition, RS is highly linked to structural and numerical chromosomal instability in cancer (Burrell et al., 2013), unreplicated DNA persisting in mitosis leading to mitotic defects and further, aneuploidy and chromosomal rearrangements (Chan et al., 2009; Wilhelm et al., 2020).

Fragile sites

Fragile sites are specific loci that exhibit gaps and breaks on metaphase chromosome of cells grown under RS conditions. This term was used for the first time in 1970, to describe recurrent chromosome breaks on the chromosome 16 which was transmitted in a large family (Magenis et al., 1970). Fragile sites are generally classified into two main categories, based on their frequency: rare fragile sites are observed in less than 5% of individuals and are usually caused by the expansion of nucleotide repeats, while common fragile sites (CFSs) are seen in all individuals and represent the largest class of fragile sites (Durkin & Glover, 2007; Irony-Tur Sinai & Kerem, 2018). For this reason, I will focus on CFSs, their origins and their genomic implications.

The predispositions of CFSs to breakage have been extensively studied and several pieces of evidence suggest that they might co-localize with large genes, which require more time to be both transcribed and replicated. Therefore, under RS, collisions between the transcription and replication occurs, generating DNA breaks (as described in *section 2.2.1.1*) (Helmrich et al., 2011). Another key feature proposed to promote CFS fragility is the presence of AT-dinucleotide rich sequences, which tend to form unusual DNA structures during DNA replication and impair replication fork progression (H. Zhang & Freudenreich, 2007; Zlotorynski et al., 2003). Finally, CFSs loci might be associated with late-replicating regions, as some of them have been shown to extend their replication in G2 upon mild replication stress (Le Beau et al., 1998; Letessier et al., 2011). However, this last possibility is debatable, as a new class of fragile sites composed of early replication fragile sites have been described. These sites are located near early replication origins but have increased fork collapsing under RS (Barlow et al., 2013). In agreement with the late-replicating hypothesis, it has been proposed that condensation of incompletely replicated DNA at mitotic entry triggers completion of CFS replication in early mitosis, in a process called mitotic DNA synthesis (MiDAS), which favor CFS

stability (Minocherhomji et al., 2015). This process has been shown to involve the activity of a structure-specific nuclease, MUS81-EME1, to promote the appearance of the break and the recruitment of DNA synthesis machinery. More recently, another nuclease, GEN1, has also been described to be involved in CFSs resolution, and to promote MiDAS upon replication stress (Benitez et al., 2023).

Moreover, fragile sites loci have been described to be preferential sites for the formation of UFBs after RS, a process called fragile site bridging. More precisely, these studies showed that RS induction give rise to replication intermediates linking sister chromatids at difficult-to-replicate loci, such as fragile sites. They further described that protection and resolution of these sites mostly rely on BLM and Fanconi Anemia proteins (FANCD2), which specifically binds to CFS under RS (Chan et al., 2009; Naim et al., 2013). In addition, structure-specific endonucleases that act on stalled replication forks and joint molecules (XPF-ERCC1 and MUS81-EME), have been shown to process replication intermediates at CFSs and to facilitate their resolution before mitosis to ensure correct mitotic progression (Naim et al., 2013).

2.2.2 Mitotic errors

As mentioned above, DNA damage generated during interphase (mostly as a result of RS) can lead to defects in chromosome segregation in the following mitosis, such as anaphase bridges, or acentric chromosomes. In addition, errors occurring in mitosis, such as defects in chromosome attachment, may in turn lead to the acquisition of DNA damage in the next cell cycle /G1 and contributes to genetic instability (Pedersen et al., 2016). In this section, I will first describe mitotic errors resulting from RS and their consequences on the genome, and then focus on other types errors occurring in mitosis, leading to problems in the next anaphase.

2.2.2.1 Mitotic errors due to defects in the previous interphase

A number of structures that arise during S-phase, if not resolved, can manifest during mitosis and impede correct segregation of genetic material. Among these, unresolved replication intermediates, R-loops (DNA-RNA hybrids formed during transcription), double stranded DNA (ds DNA) catenates or recombination intermediates are suitable examples (Lee et al., 2013; Lucas & Hyrien, 2000; Mankouri et al., 2013 and **Figure 9**).

R-loops containing RNA-DNA hybrids can be eliminated by the action of RNases or by specific helicases. If not, single stranded DNA (ssDNA) will not re-anneal and will remain exposed during mitosis (Skourti-Stathaki & Proudfoot, 2014). However, recent findings are prompting to re-evaluate the contribution of R-loops to genome stability, which appear to be more complex than previously thought (Hamperl et al., 2017; Sebastian & Oberdoerffer, 2017).

Similarly, dsDNA catenates -chromosomal entanglements unwounded by topoisomerases releasing the torsional stress at the sites of replication termination- can contribute to abnormal chromosome behavior in mitosis. Indeed, if mitotic entry precedes topoisomerase-dependent resolution, the two sister chromatids will remain connected and eventually manifest as bridges during anaphase (Martin et al., 2016).

Anaphase bridges

Anaphase bridges are DNA structures that connect the two chromosome masses during anaphase. They can be classified as either bulky chromatin bridges or ultrafine DNA bridges (UFBs) (reviewed in (Finardi et al., 2020)). Chromatin bridges are thick threads of chromatinized DNA packed with histones and can be stained by classical DNA dyes such as DAPI or Hoechst, while UFBs are non-chromatinized bridges and cannot be detected by DNA staining. Instead, they are labelled by PICH (Plk1- interacting checkpoint helicase) (Baumann et al., 2007), Bloom syndrome helicase (BLM) (Chan et al., 2007), and a number of other proteins such as RPA.

More generally, anaphase bridges (chromatinized and non-chromatinized) are a consequence of sister chromatid intertwines (SCIs) taking place naturally during DNA replication to promote sister chromatid cohesion. Three types of intertwines have been described: short regions of unreplicated DNA, recombination intermediates (Mankouri et al., 2013, and further discussed in the section 2.4.1.1) or DNA catenates (**Figure 9**). Such structures are usually resolved before mitosis to promote a correct sister-chromatid disjunction, but under RS conditions, their levels increase, resulting in DNA bridging. Another

way of generating anaphase bridges has been attributed in human cells to telomere attrition, which can generate dicentric chromosomes by chromosome fusion (Maciejowski et al., 2015). Persistent chromatin bridges will inevitably be trapped in the midzone during cytokinesis, and can either lead to cytokinesis delay (Steigemann et al., 2009), or be broken during cytokinesis (Hoffelder et al., 2004; Janssen et al., 2011). This phenomenon has been described 70 years ago by Barbara McClintock, who proposed that chromatin bridges drive chromosome fusions and rearrangements via a so-called breakage-fusion-bridge (BFB) cycle (McClintock, 1941). In agreement with McClintock's observations, this type of bridges has recently been shown to undergo BFB, during which the bridge is initially broken by actomyosin contractility (Umbreit et al., 2020). This breakage further leads to defective DNA replication, and provokes chromothripsis in the next mitosis, which is characterized by a massive chromosomal shattering and rearrangement and contributes to rapid genome evolution (Stephens et al., 2011). In *Drosophila*, chromosome bridges have been reported as a result of under-replicated DNA (Unhavaithaya & Orr-Weaver, 2012) and of BFB cycles of dicentric chromosomes (Bretscher & Fox, 2016; Titen & Golic, 2008). However, not all trapped bridges will undergo breakage, as some of them can persist for hours between two interphase nuclei (Umbreit et al., 2020). In human cells, telomere bridges have been shown to be processed by the TREX1 nuclease, which promotes the recruitment of RPA, leading to bridge resolution (Maciejowski et al., 2015). In *Caenorhabditis elegans*, another nuclease called LEM-3 has been proposed to resolve DNA bridged persistent at the midbody (Hong et al., 2018). More recently, ANKLE-1, the human homolog of LEM-3, have been showed to also localize at the midbody in human cell lines, and act on chromatin bridges to prevent the formation of cytosolic DNA fragments (Jiang et al., 2023).

UFBs can arise from different origins, such as telomeres (t-UFBs), centromeres (c-UFBs), fragile sites (CFS-UFBs) or homologous recombination (HR-UFBs) (reviewed in (Wilhelm et al., 2020). c-UFBs are the most prevalent type of UFBs, and are formed under physiological conditions, as a consequence of late decatenation and disjunction of the repetitive centromeric DNA (Wang et al., 2008, 2010), **Figure 8C**). CFS-UFBs and t-UFBs are mainly derived from under-replicated DNA or late-replication intermediates. In addition, HR-UFBs are derived from persisting recombination intermediates (Chan & West, 2018; Y. Liu et al., 2014). CFS-UFBs originate from under-replicated DNA which persists into mitosis and are marked by FANCD2 and FANCI at their extremities, possibly to orchestrate their resolution by other

factors (Chan et al., 2009; Naim et al., 2013). Furthermore, they are bound by the ssDNA binding protein RPA, suggesting that they are under-replicated regions (Chan et al., 2009). On the other hand, HR-UFBS arise as a consequence of DSB repair and the formation of DNA junctions called Holliday Junctions (further described in section 2.4.1.1), which needs to be processed before DNA segregation.

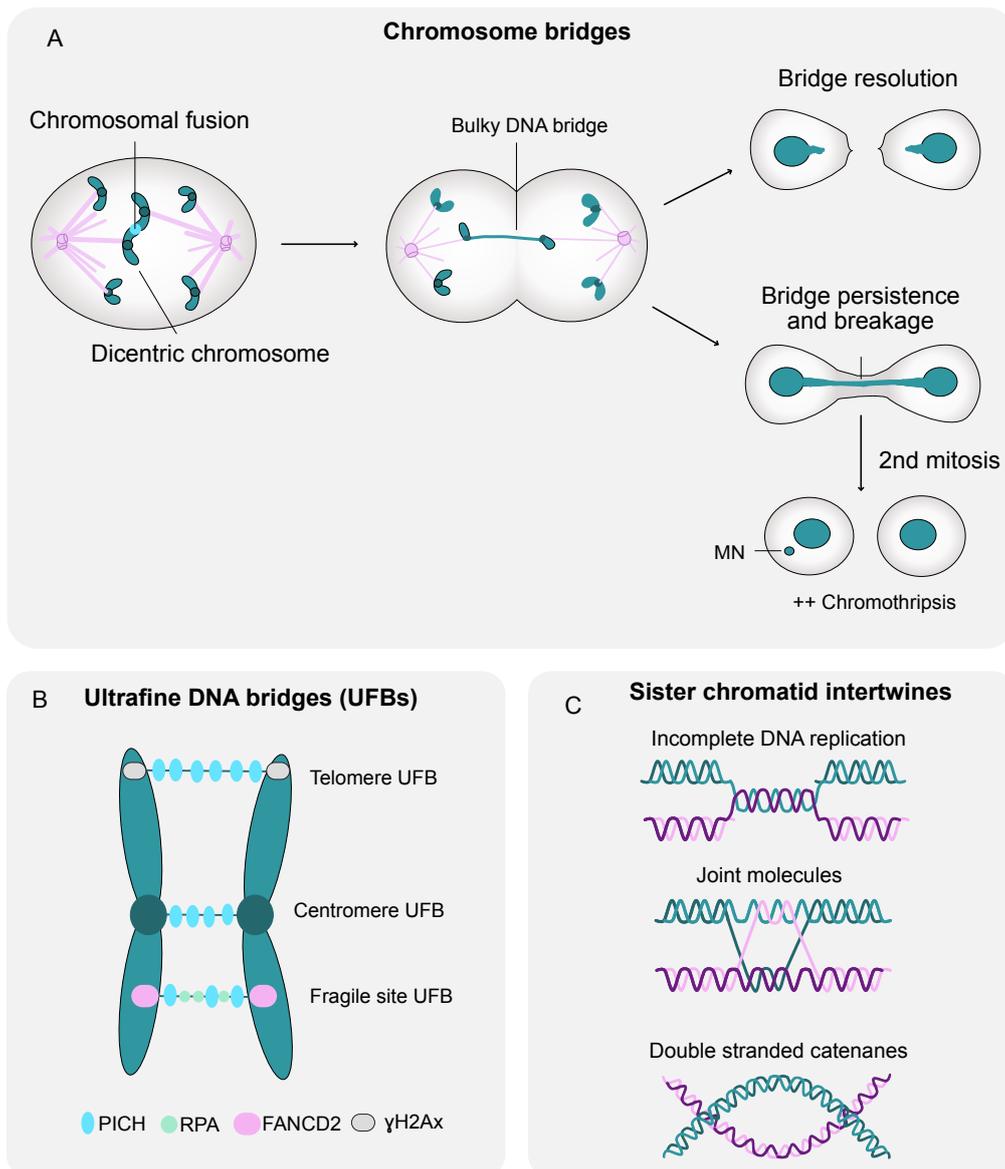


Figure 9: Anaphase DNA bridges. **A.** Chromosome anaphase bridges occurs following the formation of dicentric chromosomes, which can arise from telomeric fusion. During anaphase, they form bulky DNA bridges between the two chromosome pools. These bridges can be resolved in telophase by a mechanism involving the activity of TREX 1 nuclease. Alternatively, they can be broken during cytokinesis, leading to defective DNA replication. In the 2nd mitosis,

this generates chromosome missegregation, and formation of micronuclei (MN) in the daughter cells. **B.** Ultrafine DNA bridges can arise from various origins, such as telomeres (t-UFBs), centromeres (c-UFBs), or fragile sites. UFBs are labelled by PICH, and the ones emanating from fragile sites are supplementary coated by RPA. **C.** Bridges originates from sister chromatid intertwinings, which can arise from incomplete DNA replication, joint molecules occurring after recombination or replication, or double stranded catenates.

Acentric chromosomes

Acentric chromosomes are chromosome fragments that lack centromeres, the site of kinetochore assembly. They arise from a variety of mechanisms, but the most common might be when cells enter mitosis and progress to anaphase with unrepaired DNA double strand break (Fenech et al., 2011). This generates two chromosome fragments: one containing a telomere, a centromere, and a broken end, and the other one carrying one telomere and one broken end (**Figure 10**). Acentrics have been described for the first time by Gordon Carlson in 1938, who observed chromosomal fragment lacking spindle attachment after x-ray treatment in the neuroblasts of the grasshopper (Carlson, 1938). Later, many studies on different organisms have described acentric chromosomes and their transmission in the daughter cells, showing that the transmission efficiency strongly varies across species (Ishii et al., 2008; Royou et al., 2010). However, several mechanisms have been described for acentric transmission (reviewed in (Warecki & Sullivan, 2020)). Studies in fission yeast have showed a direct association of the acentric with normal centric chromosomes (Ishii et al., 2008; Ohno et al., 2016), while other studies described antipolar movement of the fragment, generated by association with microtubules (Karg et al., 2017). Moreover, in *Drosophila* neuroblasts, acentrics have been shown to be able to connect to centric chromosome via long-range tether-based associations. More precisely, in this cell type, acentric chromosomes can be artificially induced through the expression of the I-Crel endonuclease transgene (Royou et al., 2005). I-Crel is an intron-encoded double-strand-specific endonuclease from *Chlamydomonas reinhardtii* (Thompson et al., 1992), which in *Drosophila* recognizes a repetitive sequence within the pericentric heterochromatin of the X chromosome. Induction of I-Crel expression results in the formation of a short and a long acentric fragment in over 80% of third instar larval neuroblast cells (Rong et al., 2002; Royou et al., 2005). As mentioned earlier, despite a characteristic delay, the acentric fragment retains the ability to be segregated correctly (Derive

et al., 2015; Karg et al., 2015; Royou et al., 2010). Separation of the two fragments occurs in three distinct patterns: either by sliding one on the other, by unzipping or by dissociating (Vicars et al., 2021). The correct segregation of acentric chromosomes depends on a chromatin tether that holds the acentric fragment to the broken chromosome and is decorated by BubR1 (Bub1- related kinase, a SAC member), Polo (the *Drosophila* ortholog of Polo like kinase 1), INCENP and Aurora B (a serine-threonine kinase required in different mitotic events). BubR1 and Polo have been shown to play an active role in promoting tether stability and correct acentric chromosome segregation (Royou et al., 2010). In detail, the tether first forms thanks to the recruitment of BubR1 to the chromosome end, which is mediated by its partner, Bub3. In turn, BubR1 sequesters Cdc20 (a component of APC/C, also known as Fizzy in *Drosophila*), inhibiting APC/C activity around the segregating broken chromosome. In addition, a delay in nuclear envelope reformation mediated by Aurora B which can function as a negative regulator of lamin assembly (Ramadan et al., 2007). This will promote the inclusion of the late-segregating acentric, avoiding the formation of micronuclei (Karg et al., 2015). More recently, the recruitment of Polo to the DNA lesion was showed to be dependent on Mre11, an important factor for double strand break detection (Landmann et al., 2020a) (see section 3.2). In mammalian cancer cell lines, such tethering mechanism has also been observed, mediated by MDC1 (Mediator of DNA damage Checkpoint 1) and TOPBP1 (DNA Topoisomerase II Binding Protein 1), which promotes chromosomal stability during mitosis (Bagge et al., 2021; Leimbacher et al., 2019).

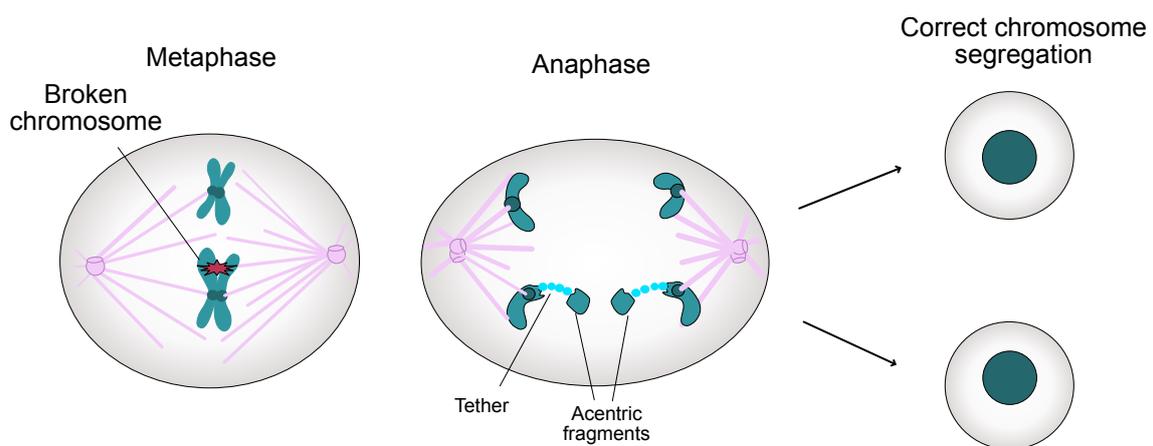


Figure 10: Acentric chromosomes segregation. Acentric fragments results from DSB that results in chromosomal fragment without the centromere. During anaphase, these fragments remain connected to the centric chromosome by tethers composed of Polo and BubR1 in *Drosophila* NBs and MDC1 and TOPBP1 in mammalian cells, allowing correct chromosome segregation.

2.2.2.2 Errors in mitosis leading to DNA damage in interphase

In principle, replication intermediates are normally resolved by MUS81-EME1, or GEN1 activity. However, when this type of resolution fails, the intermediate can still be unwound by BLM but might contain regions of ssDNA that will persist during mitosis (Chan et al. 2007; Chan et al. 2009). It has recently been shown that, in G1, 53BP1 (p53-binding protein 1) is responsible for the protection of replication intermediates that have been transmitted during mitosis (Spies et al., 2019). Moreover, 53BP1 can regulate p53-dependent cell-cycle arrest in response to DNA damage (Cuella-Martin et al. 2016).

Lagging chromosomes

Lagging chromosomes are chromosomes that are delayed during anaphase and remain behind the others while they are heading towards the spindle poles (**Figure 11**) (Cimini et al., 1999), reviewed in Ganem & Pellman, 2012). They mostly originate from error in kinetochore-microtubule attachment called merotelic orientation, which occurs when a single kinetochore from one chromosome is attached to microtubules emanating from two spindle poles (Cimini et al., 2001, 2002; Salmon et al., 2005). This type of attachment is dangerous because it satisfies the spindle assembly checkpoint (SAC) and allows anaphase entry, even if not corrected (Cimini et al., 2001). After anaphase completion, lagging chromosomes can be incorporated in one of the daughter cells main nuclei, thanks to mechanisms that temporarily restrain nuclear envelope reformation (Karg et al., 2015). In this case however, the lagging chromosome can be incorporated in the wrong daughter nucleus and further lead to aneuploidy. Alternatively, laggings can remain trapped in the cytokinetic furrow and break during constriction of the furrow (Hoffelder et al., 2004), leading to DNA damage and chromosomal translocations in daughter cells (Janssen et al., 2011). In addition, lagging

chromosomes can recruit their own nuclear envelope (NE) and form a structure called micronucleus. The existence of micronuclei is known for long (Kato & Sandberg, 1968), but recent efforts have better characterized the life cycle of micronuclei. Using live imaging approaches combined with next generation sequencing techniques, work from the Pellman lab has shown that that micronucleus can undergo chromothripsis. Chromothripsis results from complex set of rearrangements due to DSBs and non-homologous end joining of chromosomes trapped in the micronucleus (Crasta et al., 2012; S. Liu et al., 2018; Tang et al., 2022; C. Z. Zhang et al., 2015). The origins of chromosome breaks can be multiple, such as defects in NE reformation in the micronucleus, leading to catastrophic NE ruptures (Hatch et al., 2013; Liu et al., 2018). Indeed, NE reformation on the lagging chromosome is blocked by spindle microtubules, leading to an improper import of key proteins that are necessary for the integrity of the nuclear envelope and genome (Liu et al., 2018). In addition, disruption of NE exposes DNA to cytoplasm, which is detected by the cytosolic DNA sensor GMP–AMP synthase (cGAS) (Harding et al., 2017; Mackenzie et al., 2017), a member of the innate immune system. cGAS activation leads to the production of cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), which in association with stimulator of interferon genes (STING) promotes the translocation of Interferon Regulatory Factor 3 (IRF3) and nuclear factor-kappa B (NF- κ B). This promotes the expression of several interferons and immunomodulatory proteins.

More recently, micronuclei have been described to accumulate large levels of RNA-DNA hybrids, which rely on a specific endonuclease to be resolved. This process further leads to the formation of DSBs, potentially via DNA replication (Tang et al., 2022). Finally, MN appears to have a global reduction in gene expression and chromatin defects, which remains persistent through cell division, even after-reincorporation into the main nucleus (Agustinus et al., 2023; Papathanasiou et al., 2023). Therefore, the exclusion of chromosomes from the main nucleus into micronuclei can also lead to heritable alteration in transcription and gene expression.

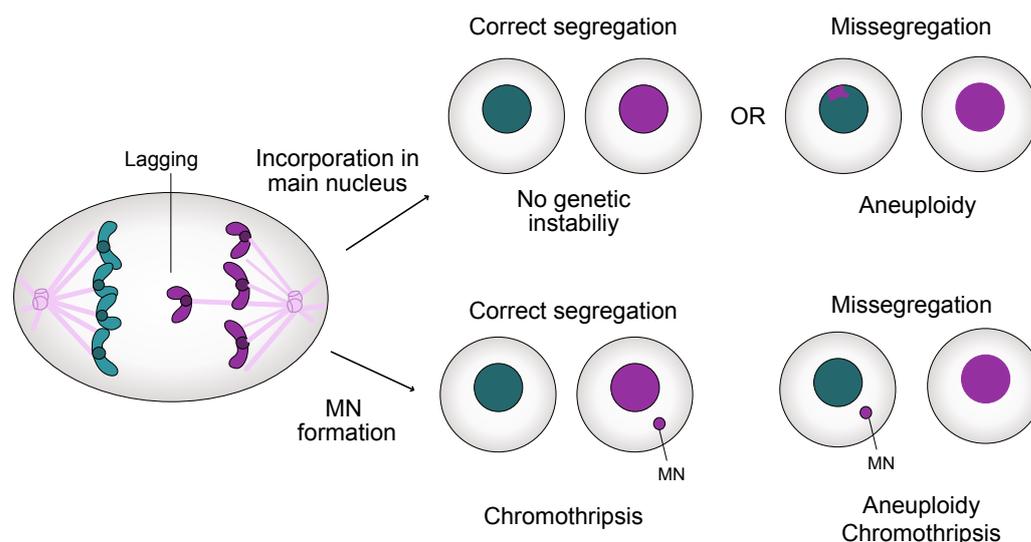


Figure 11: Lagging chromosome segregation and fate of micronuclei. Lagging chromosome is delayed during anaphase and can be either be further incorporated in the main nucleus in the daughter cells or individualize into a micronucleus. In the case of its reincorporation, it can be segregated correctly, which does not induce genetic instability; or missegregated and reincorporated in the wrong daughter nucleus, which leads to aneuploidy. When a MN is formed, it can be localized in the correct daughter cell, but will further induce chromothripsis, or be missegregated, which leads to increased aneuploidy and chromothripsis.

2.3 DNA damage response (DDR)

The DDR is a signal transduction pathway which involves several players organized depending on their position in the signaling cascade. There are DNA damage sensors, DNA damage mediators, downstream kinases, and effectors (Ciccia & Elledge, 2010; D'Adda Di Fagagna, 2008). DNA damage sensors are composed of kinases responsible for signal transduction. Three protein kinases have been described to initiate DDR: ataxia-telangiectasia mutated protein (ATM), ATM-Rad3-related protein (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Blackford & Jackson, 2017; Jackson & Bartek, 2009). ATM and DNA-PKcs are activated by DSBs, whereas ATR is mostly activated by single strand DNA lesions (Blackford & Jackson, 2017; Shiloh, 2003). Once sensors are activated, they phosphorylate DNA damage mediators that amplify the signal, as well as downstream kinases. Two of the best studied downstream targets of ATM/ATR are the protein kinases CHK1 and CHK2, which activate cell cycle checkpoints at the G1/S, intra-S or G2/M transition by reducing CDK activity. This results in a slow down or arrest of the cell cycle, which can be mediated by activation of the p53

transcription factor (Discussed in the first section “cell cycle”). Depending on the type of DNA damage, different repair mechanisms can be initiated, with each pathway leading to a specific DNA repair machinery (**Figure 12**).

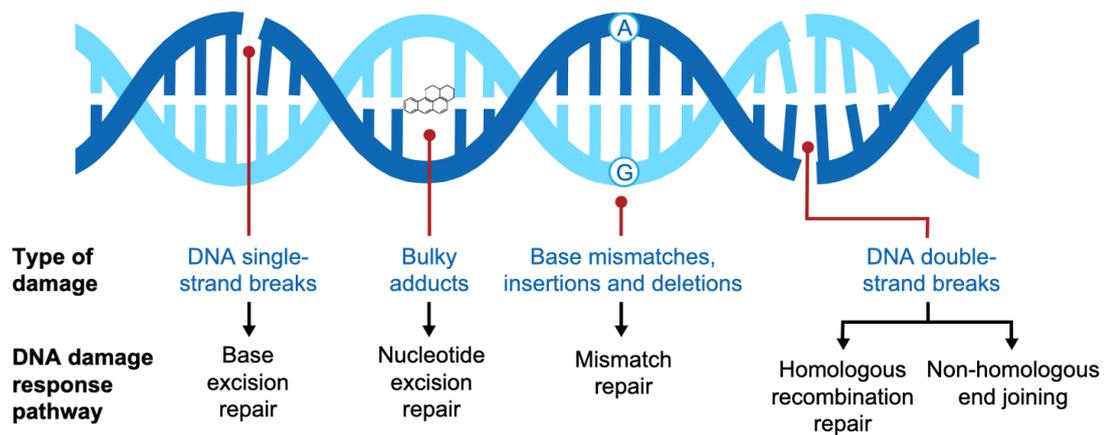


Figure 12: DNA damage response according to the type of DNA damage, From (Cortesi et al., 2021). Each type of DNA damage leads to a different DDR. ssDNA breaks are repaired via base excision repair (BER). Bulky adducts are processed by nucleotide excision repair (NER). Mismatch repair (MMR) is used to repair base mismatches or insertions/deletions. Several pathways are involved in DSB repair, HR or NHEJ.

2.3.1 Detection of double strand breaks (DSBs)

DSBs are extremely toxic lesions for the cell. Indeed, one DSB in an essential gene can lead to cell death, and errors in repairing can lead to gain or loss of genetic material or chromosomal translocations (Christine Richardson & Maria Jasin, 2000), which can eventually lead to tumorigenesis. Once formed, DSBs are sensed by the Mre11-Rad50-Nbs1 (MRN) complex, which recruits ATM to the break site (Lee & Paull, 2005). In parallel, ATM phosphorylates the C-terminus of histone variant H2AX to produce γ -H2AX (γ -H2Av in *Drosophila*), which serves as a mark for DNA breaks and recruits a high numbers of DDR factors. ATM also phosphorylates DDR mediators such as 53BP1 (p53 binding protein 1) and MDC1 (mediator of damage-checkpoint 1), which facilitate the recruitment of ATM to γ -H2AX. ATM phosphorylates Breast Cancer 1 (BRCA1), which promotes the formation of ssDNA via DNA

end resection and repair by Homologous recombination (HR, further described in *section 2.3.1*) (Bunting et al., 2010). In addition, ATM phosphorylates the downstream kinase CHK2 which activates among other targets p53 (Hirao, 2000), leading either to apoptosis or to cell cycle arrest. Interestingly, the resection triggered by ATM generates ssDNA, which holds the potential of activating a different DNA damage response (Shiotani & Zou, 2009; described below). Phosphorylated Mediator of DNA damage Checkpoint 1 (MDC1) then recruits the RING finger (RNF)-containing E3 ubiquitin ligase RNF8, which mediates ubiquitination of proteins at the damage site. Another E3 ubiquitin ligase, RNF168, recognizes RNF8 ubiquitination products and then ubiquitinates additional proteins. The ubiquitination cascade leads to the recruitment of DNA repair proteins.

Alternatively, DSBs can be detected by sensors named Ku, and the signal transduction is mediated by a different apical kinase, DNA-PKcs. In this case, it promotes DNA repair by non-homologous end joining (NHEJ, further described in *section 3.3.1*).

2.3.2 Detection of single strand DNA (ssDNA)

ssDNA can result from RS but also from DNA resection after DSBs. When ssDNA is detected, the single-stranded DNA-binding replication protein A (RPA) is recruited and binds to DNA to protect the strand (Blythe and Wieschaus, 2015; Mitsis, et al., 1993; Zhou & Elledge, 2000), which generates a signal for ATR (Cimprich & Cortez, 2008). ATR recognition of RPA-ssDNA depends on another protein called ATR-interacting protein (ATRIP), which allows the binding between the two complexes (Cortez et al., 2001). ATR kinase activity is then stimulated by the 9-1-1 complex, composed of RAD9, RAD1 and Hus1 proteins. ATR phosphorylates DNA damage mediators such as DNA Topoisomerase II Binding Protein 1 (TOPBP1), but also components of the Fanconi anemia (FANC proteins, described in the “DNA repair” section) and shares targets with ATM. Therefore, DSBs primarily activate ATM, whereas RPA-coated single-stranded DNA generated during RS triggers ATR. In addition, resection of one DNA strand at DSBs can be a substrate for ATR activation, and therefore an opportunity for the engagement of both kinases at the same lesion.

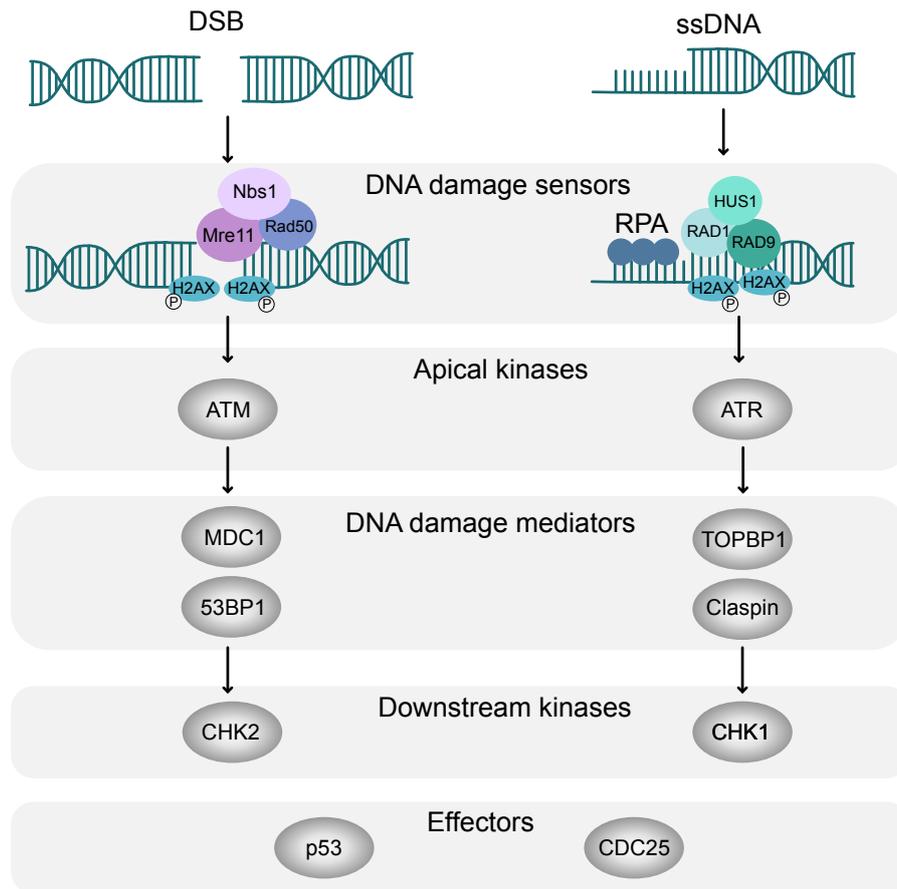


Figure 13: DDR overview. Depending on the nature of the lesion, different sensors are recruited to the damaged site. DSB involve the recruitment of Mre11-Rad50-Nbs1, which activate the apical kinase ATM. The signal gets amplified by MDC1 and 53BP1, that will target cell cycle checkpoint kinase CHK2, resulting in cell cycle arrest through p53. If ssDNA is detected RPA binds to it, and the 9-1-1 machinery (Hus1-Rad1-Rad9) is recruited, leading to the activation of ATR, which mediates the signal through TOPBP1 and Claspin. These activate CHK1, which will arrest the cell cycle by phosphorylating CDC25 to inhibit mitotic entry (Adapted from (D’Adda Di Fagagna, 2008)).

2.4 DNA damage repair

In addition to signaling pathways for DNA damage detection and response, cells have established DNA repair strategies. Several DNA repair pathways have been described and can be initiated via different types of lesions. For instance, mis-incorporation of nucleotides can be corrected almost instantly by the exonuclease activity of DNA polymerases (Alberts et al.,

2015) that can remove and replace nucleotides. Alternatively, cells possess other pathways to correct bases or nucleotides that do not match with their opposite partners, such as base excision repair (BER) (Krokan & Bjørås, 2013; Zharkov, 2008) or nucleotide excision repair (NER) (Hoeijmakers, 2009), or mismatch repair (Fishel, 2015; Li, 2008). Each of these mechanisms involves different enzymes which act upon specific kind of lesions, with the same pattern: first, DNA damage is excised, then the DNA sequence is restored by a high-fidelity DNA polymerase using the undamaged strand as a template, and the break eventually gets sealed by a ligase (Alberts et al., Vol 7 Chap 5). When cells encounter DSBs, two main DNA repair pathways have been described, homologous recombination (HR) and non-homologous end-joining (NHEJ) (Scully et al., 2019). The pathway choice appears to be cell-cycle dependent, as NHEJ is active all along interphase, while HR can occur only after DNA replication, therefore in S/G2 phases.

2.4.1 Double strand break repair

Homologous recombination

DNA repair by homologous recombination involves the use of a homologous DNA sequence, such as sister chromatid or homologous chromosome, as a template to repair DSBs. This is an error-free mechanism, which results in the maintenance of the complete DNA sequence that was damaged (Heyer et al., 2010; X. Li & Heyer, 2008). The choice of HR pathway for DSB repair is mediated by MDC1 protein via H2Ax phosphorylation (Xie et al., 2007). Because of requirement of a homologue template for the repair provided by sister chromatids, HR is largely restricted to S and G2 phases of the cell cycle, when DNA has been replicated (Takata et al., 1998). After detection of a DSB and recruitment of the MRN (see *section 3.2.1*), HR starts with the resection of one DNA strand by both the endo- and exonuclease activities of Mre11 (Cannavo & Cejka, 2014; Garcia et al., 2011; Sartori et al., 2007). This leads to ssDNA that is further coated by RPA protein to protect the strand from pairing with another ssDNA. RPA is then removed by recombination “mediators” (Rad52 in yeast and BRCA2 in vertebrates) (Jensen et al., 2010; Thorslund et al., 2010), allowing the recruitment of Rad51 recombinase on the strand. Loading of Rad51 on ssDNA is mediated by BRCA1 protein and forms a

nucleoprotein filament that mediates homology search by invading the double strand of the undamaged template. This forms a synaptic complex containing 3-stranded DNA intermediates, composed of the invading strand, the complementary strand, and the invaded template (**Figure 13**). When homology is detected, base pairing takes place and stabilizes the whole synapse, with the help of Rad51 (van der Heijden et al., 2008). A displacement loop (D-loop) is formed, leading to the disassembly of Rad51 filaments (Morrical, 2015; van der Heijden et al., 2008) and the recruitment of DNA polymerases to extend the invading strand using the invaded donor as a template. When the nascent strand is fully synthesized, the D-loop can be disassembled through a process called synthesis-dependent strand annealing or lead to the formation of four-stranded DNA structure called Holliday Junction (HJ) (Holliday, 1964).

HJs need to be resolved by specific pathways. There are three main ways of processing HJs. The first one involves a dissolution complex composed of the BLM helicase, topoisomerase III and RMI1/2 called the BTR complex (Bizard and Hickson, 2014). This complex decatenates the junction and generate non-crossover products, thus limiting sister-chromatid exchanges and avoiding loss of heterozygosity. The second and third mechanism are “resolving” the junction through the activity of two distinct complexes comprising either several nucleases SLX1–SLX4–MUS81–EME1 (SLX–MUS complex) (Wyatt et al., 2013; Wyatt & West, 2014) or just one - GEN1 (Garner et al., 2013; Punatar et al., 2017; Rass et al., 2010). These mechanisms can either lead to crossover or non-crossovers production depending on the process utilized. Interestingly, these nucleolytic pathways have been showed to be essential in the absence of BLM (Andersen et al., 2011), suggesting that both processing complexes can compensate for the loss of the other. Moreover, the choice of the HJ processing pathway appears to be cell cycle-dependent, as cells in S/G2 would rather use the BTR pathway, while HJs occurring later during the cell cycle mostly rely on the resolution complexes (Chan & West, 2014; Matos & West, 2014) .

It is important to mention that DNA repair by HR is not restricted to DSBs only. Indeed, cells can use HR as a support for replication fork stalling, but also for the repair of DNA interstrand crosslinks (ICLs) (see *section 3.1.1 and 3.3.3*). In the case of replication forks, lesions interfering with the progress of the fork can lead to its arrest, therefore producing DNA gaps or one-sided DSBs (Li & Heyer, 2008) that are processed by HR (Scully et al., 2021). Regarding the repair of ICLs, which are covalent links between the two strands of DNA double

helix, they can also lead to replication fork blockage and have been shown to be targeted by several members of the HR pathway (Deans & West, 2011). Furthermore, HR has a critical role in cancer. Indeed, it is well established that germline mutations in BRCA1 and BRCA2 genes - important regulators of HR- are associated with inherited breast or ovarian cancers (Paul & Paul, 2014). Therefore, patients carrying BRCA1/2 mutations have frequently deregulated DNA repair pathways and tumors are more vulnerable to DNA damaging agents. This susceptibility has been exploited for the development of cancer therapies, with the use of inhibitors of Poly ADP-ribose polymerase (PARP), involved in the repair of ssDNA breaks, in combination with DNA damaging agents (Cortesi et al., 2021; Lord & Ashworth, 2017; Slade, 2020).

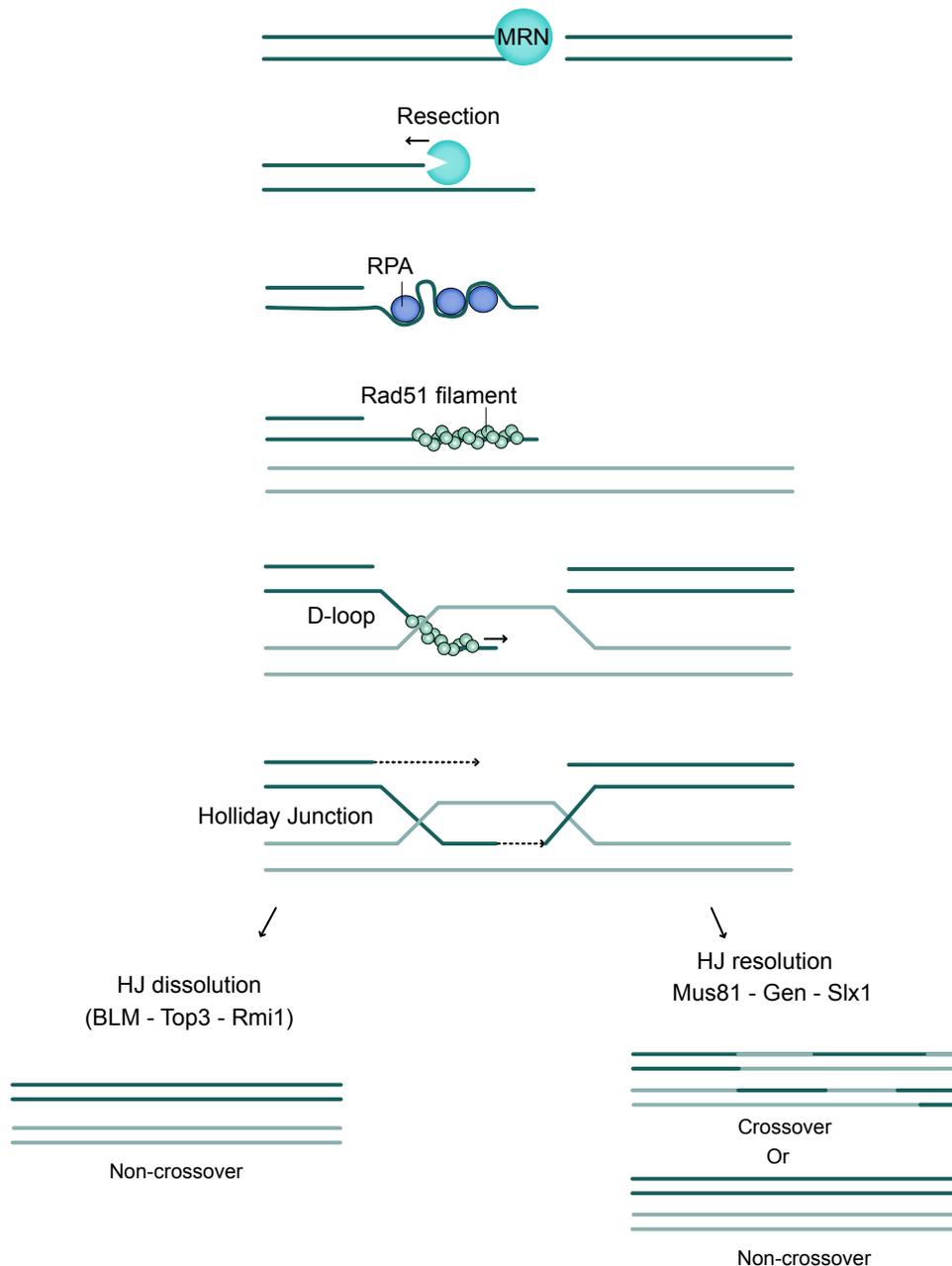


Figure 14: DNA repair by Homologous recombination (HR). HR starts with the resection of the break by MRE11 nuclease activity. RPA binds to the ssDNA and is further displaced by the recruitment of Rad51 filament with the help of BRCA1/2. Rad51 catalyzes the formation of a D-loop, allowing the recruitment of DNA synthesis machinery. HJs that remain need to be processed, either by the dissolution BTR pathway, leading to non-crossover products, or by nucleolytic pathway, that can either generate crossover or non-crossover products.

Break induced replication (BIR)

In the large majority of events of HR repair, the two broken DNA ends work together to invade a single homologous DNA sequence, synthesize new DNA and ligate the ends (**Figure 15**).

However, during BIR, only one DNA end of the DSB participates in this process. Such mechanism can occur typically when the DSB manifests as a “one-ended break”, for instance after replication fork collapse (Anand et al., 2013; Llorente et al., 2008; Sakofsky & Malkova, 2017). Similar to other HR pathways, BIR starts by DNA resection to generate ssDNA strand that is bound by RPA, which is further removed by the formation of a Rad51 nucleoprotein filament and D loop formation. Then, DNA synthesis is initiated using the homologous chromosome as a template. While DNA is synthesized, the BIR replication bubble migrates, which generates a long ssDNA tail behind the bubble. Eventually, BIR is completed on the lagging strand, which uses the leading strand as template, to resulting in conservative inheritance of the newly synthesized DNA (Donnianni & Symington, 2013). The molecular mechanisms driving BIR progression, and more precisely the helicases or polymerases involved in the process, remain poorly understood. However, such a conservative mechanism has been described to confer an elevated risk of genetic instabilities, as the mismatch repair which corrects DNA synthesis errors is less efficient during BIR (Deem et al., 2011).

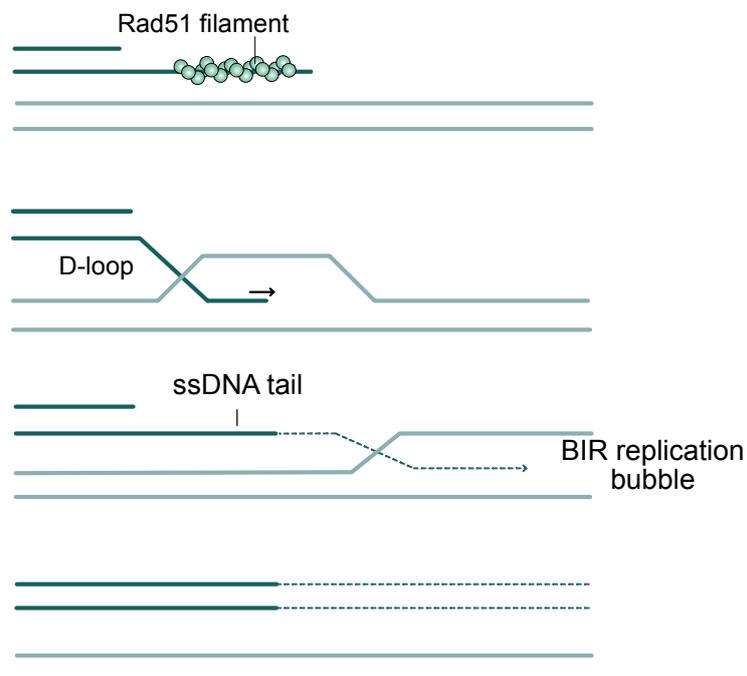


Figure 15: DSB repair by BIR. BIR starts with the same molecular mechanism than HR. However, only one DNA end invades and forms a BIR replication bubble, while creating a ssDNA tail. Eventually, DNA synthesis is completed on the lagging strand, using the new synthesized strand as a template.

Non-homologous End-joining

NHEJ consists in the ligation of the broken ends of the DSBs and rely on DNA-PKc apical kinase instead of ATM (Chang et al., 2017; Scully et al., 2019; Shibata & Jeggo, 2020). In vertebrates, the repair of conventional DSB by NHEJ is promoted by 53BP1, which suppress DNA end resection required for HR (Bunting et al., 2010; Xie et al., 2007b; Zimmermann & De Lange, 2014). Classical NHEJ is initiated by the binding of Ku70-Ku80 heterodimers, which function as sensors of the DSB ends (McElhinny et al., 2000). Ku was originally discovered because it was a target of autoantibodies in patients with autoimmune disease, and the name 'Ku' derives from the surname of the prototype patient (Mimori et al., 1981). Ku proteins prevent extensive DNA-end resection (Mimitou & Symington, 2010), and allow the recruitment of DNA-PKcs, which tethers DNA broken ends and activate downstream effectors required for NHEJ. Such factors are required for ligation of free DNA ends, and are composed of Artemis, an endonuclease which process DNA ends, DNA ligase IV, and the scaffolding factors XRCC4 and XRCC4-like factor (XLF) (Ahnesorg et al., 2006). Single molecule analysis on NHEJ showed that this process occurs in a two-step manner, mediated by the formation of a synapse between the two ends of DSBs (Graham et al., 2016). First, Ku heterodimers establish a long-range synapse with DNA-PKc. Then, the two DNA ends become closely aligned in a process requiring XLF, non-catalytic functions of XRCC4-LIG4 and the kinase activity of DNA-PKc (**Figure 16**).

In addition to classical NHEJ, cells possess alternative end-joining pathways (Alt-EJ), which rely on using micro-homology between DNA ends. When NHEJ is compromised, the activity of these other end-joining pathways become apparent. Typically, they also begin with DNA-end resection, but this resection is much more extensive, to reveal a sequence homology and then allow a more efficient joining and ligation (Lieber, 2010). Therefore, alternative pathways require the activity of the MRN complex, such as in the first steps of DNA repair by HR (Truong et al., 2013). The alternative end-joining pathway (a-EJ - also known as microhomology-mediated end-joining (MMEJ)) requires microhomology that ranges between 2 and 20 base pairs (bp). This pathway involves Pol θ activity, which is able to stabilize the annealing of two ssDNA overhangs with only 2 bp of homology and extend the DNA end by using the annealing partner as a template (Wyatt et al., 2016). The other alternative pathway is called single strand annealing (SSA) and require > 20 base pair of homology (Chang et al.,

2017). Therefore, SSA requires more extensive resection, which is performed by the nuclease EXO1, BLM, or replication helicase/nuclease 2, which generates longer ssDNA tails. These tails are bound by RPA, as described for DNA repair by HR, but instead of relying on Rad51 filament formation, they will be annealed to any suitable complementary strand, via the action of Rad52 (Chang et al., 2017).

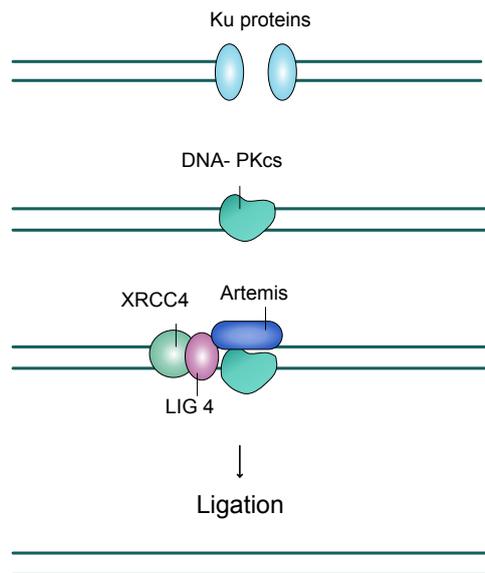


Figure 16: DNA repair by non-homologous end-joining (NHEJ). NHEJ is initiated by Ku proteins, which are recruited on the DSB and allow the recruitment of DNA-PKCs. DNA PKCs activates the formation of a complex composed of Artemis, XRCC4 and LIG4 to ligate the two DNA ends.

2.4.2 Repair of ssDNA breaks (SSBs)

ssDNA breaks (ssDNABs) can occur from oxidative stress, irradiation or errors in topoisomerase activity (Wang, 2002). In addition, ssDNABs can arise indirectly during DNA base excision repair, which corrects bases that are damaged or altered (Dempfle & Demott, 2002), or due to erroneous incorporation of ribonucleotides into DNA (Williams & Kunkel, 2014). Unresolved ssDNA breaks can impact cell fate in multiple ways. First, it can lead to blockage or collapse of the replication fork during S phase, leading to DSBs (Kuzminov, 2001). Alternatively, ssDNABs can also block the transcription machinery, and lead to altered

transcription profiles (Bendixen et al., 1990; Zhou et al., 1995; Zhou & Doetsch, 1993). The major actor involved in SSBs detection and repair is poly (ADP-ribose) polymerase-1 (PARP1), which activate a cascade of other ADP-ribose unit to transmit the signal (Caldecott, 2014; Fisher et al., 2007; Woodhouse et al., 2008). In addition, PARP1 promotes the recruitment of XRCC1, a critical scaffold protein required for the recruitment of SSB repair factors (Caldecott, 2003). Then, DNA repair occurs in a three-step manner. First, DNA ends get processed by restoring their conventional hydroxyl configuration. The pathway for end-processing appears to be lesion-dependent (Caldecott, 2014). Then gap filling can occur, during which either a single nucleotide is inserted (short patch repair – most frequent category of SSB repair), or multiple nucleotides are incorporated (long-patch repair). Long patch repair involves the activity of the FEN1 nuclease for removing the displaced nucleotides. The last step of ssDNA repair DNA ligation, mediated through Ligase1 and Ligase 3 DNA ligases (Caldecott, 2007).

One important aspect concerning DNA repair pathways is that these are not mutually exclusive. Indeed, as described above, ssDNA breaks can lead to DSBs and vice versa. For instance, upon RS leading to fork collapsing or stalling, several pathways can take over, such as translesion synthesis, template switch by fork regression or HR. Translesion synthesis involves the recruitment of a polymerase which “bypass” the lesion (Prakash & Prakash, 2002). During template switching by fork regression, the nascent strand serves as a template for the blocked leading strand through the formation of a Holliday junction, which is later processed. Finally, if fork stalling is associated with DSB, this will be repaired by HR.

2.4.3 Repair of DNA interstrand crosslinks (ICLs)

Another source of DNA damage is DNA interstrand crosslink (ICLs), which are covalent linkage between two bases on opposing strands (reviewed in (Deans & West, 2011)). ICLs can be induced by several crosslinking agents, such as mitomycin C or cisplatin, which are commonly used chemotherapeutic agents. Such lesions are extremely toxic, because they prevent the separation of the two DNA strands, which is essential during DNA replication or transcription. Depending on the cell cycle stage at which the ICLs are generated, different repair mechanisms are required. In G0/G1, ICLs are removed by nucleotide excision repair (NER) (Sarkar et al., 2006). Briefly, this involves two incisions to remove the nucleotide, and the gap is filled by translesion DNA polymerases (Sarkar et al., 2006). In S phase, ICLs perturb replication fork

progression, leading to fork collapse and further DSB. In this case, DSBs are repaired by HR, and the ICL is excised by MUS81 nuclease. In certain cases, stalled replication forks form a specific structure called “chicken foot”, which generates a free dsDNA end and the recruitment of the members of the Fanconi Anemia Complementation group (FANCM, FANCD2, FANCN, FANCP). These proteins stabilize the replication fork and allow it to restart via a pathway based on HR (Legerski, 2010).

2.4.4 DNA repair in mitosis

For a long time, it was thought that there is no DDR in mitosis. Indeed, once cells have entered mitosis, there is no DNA damage checkpoint until the daughter cells re-enter in G1 (Rieder & Cole, 1998). However, it has now emerged that there is at least a partial DNA damage response in mitotic cells. After DSB, mitotic cells are able to recruit the MRN complex, and ATM, DNA-PK, γ -H2AX and MDC1 are activated (Giunta et al., 2010). Nonetheless, cells progress into anaphase in the presence of DNA breaks, suggesting that the upstream parts of DDR are functional during mitosis, but they are disconnected from the downstream effectors controlling cell cycle progression. In addition, treatment of mitotic HeLa cells with topol inhibitors led to accumulation of DSBs and dicentric chromosome, and a small proportion of these DSBs were repaired in mitosis (Terasawa et al., 2014). Furthermore, certain mechanisms that negatively regulate DDR seem to be activated. Indeed, the mitotic kinase CDK1 inhibits 53BP1 which prevents downstream DNA damage signaling. PLK1 also phosphorylates the cell cycle kinase CHK2 to inhibit its activity (Van Vugt et al., 2010). It has been shown that aberrant CHK2 activity during mitosis can result in stabilization of kinetochore-microtubule attachment, leading to an increase in lagging chromosomes frequency during anaphase (Bakhoun et al., 2014).

In addition, several pieces of evidence suggest that HR is inactive during mitosis, as no resection has been observed in mitosis and HR factors such as BRCA1 or RAD51 are not recruited to DSB sites (Giunta et al., 2010). However, even if studies suggested that DSBs are not repaired by NHEJ until the next G1 (Spies et al., 2019), other studies have shown that laser induced DSB generated in mitosis can be repaired by NHEJ, but they also observed recruitment of several HR factors (Godinez et al., 2020). Therefore, it is still unclear to what extent the DNA

repair machinery is inhibited during mitosis. Moreover, as described in the *section 2.2.2.1*, mitotic cells are able to segregate correctly broken chromosomes, through tethering mechanism that joins the broken chromosome ends until they can be repaired in the next G1 (Bagge et al., 2021; Landmann et al., 2020a; Leimbacher et al., 2019; Royou et al., 2005).

Furthermore, recent studies in *Drosophila* have identified another mechanism for DNA repair in mitosis, through Alternative End joining mediated through Pol θ , also called microhomology-mediated end-joining (MMEJ). Precisely, they described that Pol θ links the acentric fragment resulting from DSB to a broken chromosome carrying a centromere, to prevent micronuclei formation (Clay et al., 2021).

2.4.5 Nucleases: molecular scissors required for DNA repair

For my thesis, I investigated the role of a specific population of DNA repair factors called nucleases, in cells having whole genome duplication, also called polyploid cells (described in the *section-3* below). Nucleases are molecular scissors that cut the phosphodiester bond between two nucleotides, and are involved in many biological processes, such as DNA replication, DNA repair and recombination (reviewed in (Nishino & Morikawa, 2002; Yang, 2011). They contain conserved minimal motifs, which usually consist of acidic and basic residues forming the active site, that collaborates with several cations and metals as co-factors. Nucleases can be separated into two subtypes, depending on the location of their cleavage reaction: endonucleases, that can cleave within DNA in a sequence of structure-specific manner, such as four-way DNA junctions, 5' - or 3' flap and fork structure; and exonucleases, that can digest DNA from its termini or nicks or breaks (**Figure 17**). Nucleases can also be classified depending on their substrate-preference. However, many of them exhibit both endo and exonuclease activity, and are able to cleave several substrates. For this reason, it has been challenging to generate an accurate classification of nucleases. In addition, the role of nucleases and their mode of action have been mostly studied *in vitro*, with very few studies *in vivo* and in *Drosophila*. In this section, I will introduce several nucleases which have been described in *Drosophila melanogaster* and their human homologues and focus specifically on their role in DNA repair. They are also listed in the table 1.

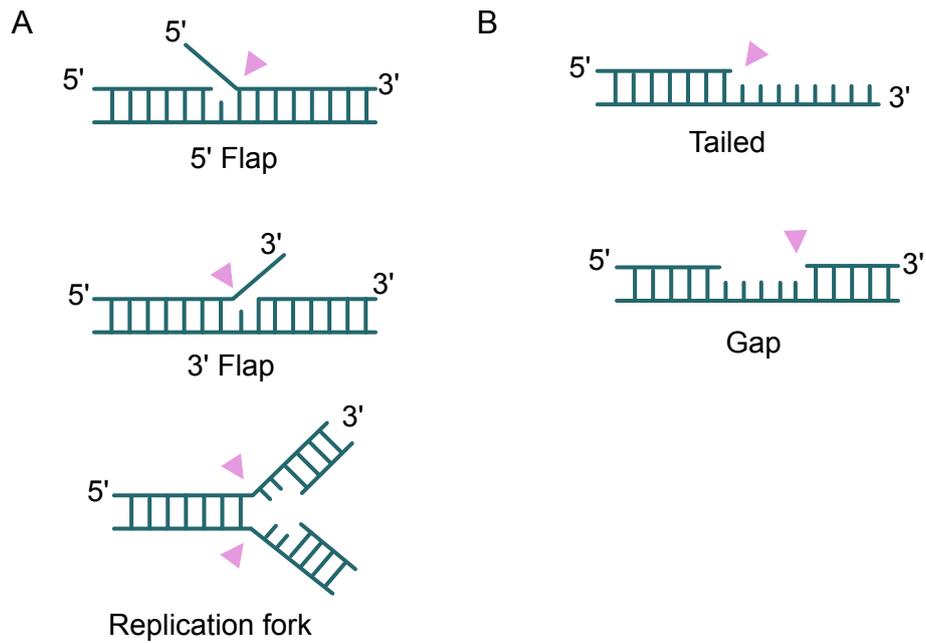


Figure 17: Examples of structures recognized by endo and exo-nucleases **A.** Example of different structures recognized by endonucleases. Endonucleases can cut 5' or 3' flap, replication forks, but also 4 ways DNA junctions such a HJ (not depicted here, but in figure 13) **B.** Exonucleases digest DNA from its end, which generates ssDNA tails. They can also digest from ends localized in DNA gaps.

Holliday Junction resolvases

As described in the HR section, HJs are intermediates structures that are formed during recombination events. Processing of these junctions relies on two main different pathways: one “dissolution” pathway mediated by the BLM complex, and one “resolution” pathway involving structure-selective nucleases either MUS81-EME1 and SLX1-SLX4, or GEN1 (Sarbjana et al., 2014).

MUS81

MUS81 is a structure-selective endonuclease involved in the processing of recombination intermediates, which interacts with its non-catalytic partner, EME1 in human cells and Mms4 in budding yeast (García-Luis & Machín, 2014; Garner et al., 2013; Naim et al., 2013; Wyatt et al., 2013). In these two systems, MUS81 activity appears to be controlled during the cell cycle, mostly by phospho-regulation of its partner (reviewed in (Pfander & Matos, 2017)). Besides

resolving HJs, MUS81 has been described to cleave persistent replication intermediates at late cell cycle stage (Naim et al., 2013), promoting mitotic DNA synthesis (Minocherhomji et al., 2015). Depletion of MUS81 in human fibroblasts leads to accumulation of ssDNA and slowing of replication forks, showing that MUS81 activity is required for the maintenance of replication fork integrity (Xing et al., 2015). Depletion of MUS81 *in vivo* leads to more defects than GEN1 after treatment with DNA damaging agents, suggesting that in general cells use MUS81 preferentially (Blanco et al., 2010; Boddy et al., 2000; Dendouga et al., 2005; Ho et al., 2010; Tay & Wu, 2010). However, in unperturbed cells, loss of MUS81 does not confer a strong DNA repair-deficient phenotype, suggesting an overlapping between the function of structure-selective endonucleases (Fernandez et al., 2022; Sarbajna et al., 2014). In *Drosophila*, Mus81 interacts with its partner Mms4 for HJ resolution (Trowbridge et al., 2007), and these complexes become essential in the absence of the BLM helicase, similarly to what has been described in *Saccharomyces cerevisiae* (Andersen et al., 2011; Mullen et al., 2001). Indeed, the absence of both MUS81 and BLM is synthetic lethal and associated with elevated apoptosis in proliferating tissues (Trowbridge et al., 2007).

SLX1-SLX4

Slx1 is the catalytic subunit of SLX1-SLX4 endonuclease and is able to cleave in a symmetric or asymmetric manner. The name “SLX” originates from a synthetic-lethal screen in *Saccharomyces cerevisiae*, which identified proteins which function is redundant with an helicase called Sgs1 (Mullen et al., 2001). SLX1-SLX4 acts in coordination with MUS81-EME1 to resolve HJs in human cells during G2/M phases (Ciccina et al., 2008; Wyatt et al., 2013). As for MUS81 and GEN1, SLX1-SLX4 is also required for the repair of ICLs and cells lacking these proteins have increased sensitivity to cross linking agents (Fekairi et al., 2009; Muñoz et al., 2009). In *Drosophila*, very little is known about Slx1, but it has been described to be essential in the absence of BLM helicase (Andersen et al., 2011) and to generate mitotic crossover in the absence of the FANCM helicase (Kuo et al., 2014)

SLX4 (Mus312 in *Drosophila*) is not a nuclease per se, but instead provides a scaffold for the binding of several other nucleases, such as Mei-9 (human XPF) and Mus81 (Fekairi et al., 2009; Muñoz et al., 2009).

GEN

Gen is the *Drosophila* homolog of the mammalian structure-specific endonuclease GEN1, which is involved in the resolution of recombination intermediates. Biochemical *in vitro* studies and nuclease assays showed that GEN1 cuts symmetrically across HJs (Ip et al., 2008; Punatar et al., 2017). Interestingly, human GEN1 is regulated during the cell cycle via a nuclear export sequence (NES) and a large fraction is only able to access DNA after the nuclear envelope breakdown (Chan & West, 2014). In yeast, Yen1 exhibits low activity in S phase, because Cdk promotes its phosphorylation, leading to nuclear exclusion and inhibition of catalytic activity by reducing its affinity for DNA. Later in the cell cycle, Yen1 is dephosphorylated by Cdc14, allowing nuclear localization and DNA binding (Blanco et al., 2014; Matos et al., 2011). Recently, GEN1 has been described to resolve replication intermediates in human cell lines, promoting common fragile site expression (also called breakage) following replication stress (Benitez et al., 2023). *In vivo*, both GEN1 and Yen1 act secondarily to MUS81, as loss of MUS81 results in hypersensitivity to a large range of DNA damaging agents, while mutations in GEN1/Yen1 do not cause DNA repair defect on their own but increased the severity of MUS81 loss (Blanco et al., 2010; Fernandez et al., 2022; Ho et al., 2010; Tay & Wu, 2010). However, this hierarchical relationship appears to be reversed in *Drosophila*, as Gen (the fly ortholog of Gen1) single mutants are more severely hypersensitive to DNA damaging agents than Mus81 single mutants (Bellendir et al., 2017). In addition, *in vitro* studies showed that *Drosophila* Gen has the ability to cleave replication fork substrates, suggesting that it can also cut replication intermediates in *Drosophila* (Kanai et al., 2007).

Other structure-specific nucleases

MRE11

Meiotic recombination 11 (MRE11) is both an endo and exonuclease, which acts in a complex with RAD50 and NBS1 as a sensor of DSB. In the presence of a DSB, the main role of MRE11 is to digest dsDNA from its free ends, in a process called DNA resection, which generates ssDNA and allow the recruitment of proteins involved in DSB repair (Cannavo & Cejka, 2014; Garcia et al., 2011; Sartori et al., 2007). The same complex regulates the mammalian DNA damage response (Stracker & Petrini, 2011). In *Drosophila*, Mre11 has been shown to be required to cap and protect telomeres to prevent telomeric fusion and chromosome breakage (Bi et al.,

2004; Ciapponi et al., 2004; Gao et al., 2009). In addition, Mre11 is essential for the G2/M checkpoint induced by low-dose irradiation in the *Drosophila* wing disc (Bi et al., 2005). More recently, Mre11 complex was described to be recruited to DNA lesions induced during mitosis, to promote the tethering of chromosomal fragments in *Drosophila* NBs and rectal papillar cells (Clay et al., 2021; Landmann et al., 2020).

MEI-9 & MUS201

Mei-9 is the ortholog of human XPF endonuclease, involved in nucleotide excision repair (NER) pathway but also in HR repair. In *Drosophila*, Mei-9 is required for both NER and meiotic recombination. Characterization of *mei-9* mutants showed that they have increased sensitivity to ionizing radiation and crosslinking agents, suggesting a role for repair of ICLs (Baker et al., 1987; Mason et al., 1981). Mei-9 has been proposed to function as a HJ resolvase during meiotic recombination, by interacting with Mus312 scaffold (Slx4 homologue) (Sekelsky et al., 1995, 1998; Yildiz et al., 2002, 2004).

Mus201 is the *Drosophila* ortholog of mammalian XPG nuclease, the other endonuclease involved in the NER pathway (Houle & Friedberg, 1999; Pal et al., 2022; Sekelsky, Brodsky, et al., 2000). Therefore, mutations in *mus201* increases the sensitivity to UV-induced DNA lesions, nitrogen mustard and X-rays.

WRNEXO

WRNexo is the ortholog of the exonuclease component of the WRN human protein. In human cells, WRN plays an essential role at stalled replication forks to counteract replication stress, thanks to both its helicase and nuclease activity (Ammazzalorso et al., 2010; Franchitto et al., 2008; Palermo et al., 2016). In addition, human WRN is thought to play a significant role in DNA repair by base excision repair (Brosh et al., 2006). Interestingly, the helicase domain of WRN, is encoded by another locus in *Drosophila*. It is involved in the resolution of recombinational intermediates that arise from replication arrest due to either DNA damage or replication fork collapse (Boubriak et al., 2009; Saunders et al., 2008). *Drosophila* mutants for WRNexo have increased deficiency in DNA repair, which was shown to be dependent from its nuclease activity (Bolterstein et al., 2014; Epiney et al., 2021) and display phenotypes consistent with accelerated aging, such as shortened lifespan and increased tumor incidence (Cassidy et al., 2019).

FEN1

Flap endonuclease 1 (FEN1) has both endo and exonuclease activity. It is a core protein in the base excision repair (BER) pathway and participates in Okazaki fragment maturation during DNA replication (Zheng & Shen, 2011). Defects in FEN1 activity leads to failure in the maturation of Okazaki fragments, resulting in DSBs, and reduced BER efficiency, leading to unconnected DNA intermediates (Sun et al., 2017). In *Drosophila*, mutations in Fen1 shorten lifespan and generates apoptosis in NBs due to defects in DNA replication (Barclay et al., 2014; Kuang et al., 2014).

TOSCA

Tosca is believed to be the ortholog of EXO1 nuclease although very few studies demonstrated its nuclease function in *Drosophila*. During its identification, Tosca was showed to be specifically expressed in the female germline (Digilio et al., 1996). In humans, the Tosca homologue is called EXO1 and its nuclease activity contributes to DNA replication. It is also involved in several DNA repair processes, such as mismatch repair, NER and HR (reviewed in (Keijzers et al., 2019). Recently, EXO1 was also described to participate to NHEJ and to contribute to drug resistance in ovarian cancer (He et al., 2020).

Non-structure specific nucleases

Among nucleases which have been described in *Drosophila*, certain did not have a structure-specificity but are normally associated with stress conditions: DNaseII, Endonuclease G (EndoG) and Stress induced DNase (Sid). Both DNase II and Sid play an important role in the innate response to bacterial infection. More precisely, these genes have increased expression after bacterial infection in flies (Evans et al., 2002; Seong et al., 2014; Seong et al., 2006) and mutations in both genes decreased fly viability in response to viral infection. In addition, both nucleases belong to a family of nucleases whose main function is to degrade DNA within phagolysosomes (Evans et al., 2002).

Concerning EndoG, it is a mitochondrial nuclease believed to be released during apoptosis for the degradation of nuclear DNA in budding yeast, although this role remains controversial in mice (Büttner, Carmona-Gutierrez, et al., 2007; Büttner, Eisenberg, et al., 2007; Irvine et al.,

2005; Zhang et al., 2003). In *C. elegans*, EndoG promotes autophagy and activates the DNA damage response (Wang et al., 2021). In *Drosophila*, very little is known about EndoG, but *in vitro* assays showed that its catalytic domain is highly conserved (Loll et al., 2009). In addition, the fly genome encodes for an inhibitor of EndoG (Temme et al., 2009).

<i>Drosophila</i>	Human	Pathway
Gen	GEN1	HJ resolution & replication intermediates resolution
Mus81	MUS81	HJ resolution and replication intermediate removal
Slx1	SLX1	HJ resolution
Mus312	SLX4	Scaffold for Mus81 Slx1 and Mei-9
Mre11	MRE11	DNA damage response & telomere protection
Mei-9	XPF	DNA repair by NER
WRNexo	WRN	Stalled replication forks and BER
Fen1	FEN1	DNA replication - removal of Okazaki fragments
Mus201	XPG	DNA repair by NER
Tosca	EXO1	DNA repair (NER, HR, NHEJ)
Dnase II	DNASEII	Clearance of bacterial DNA
Sid	DNASEII	Clearance of bacterial DNA
EndoG	ENDOG	Mitochondrial nuclease induced during apoptosis

Table1: List of nucleases described in the *Drosophila* genome with their human homolog and corresponding pathways. Nucleases involved in HJ resolution are highlighted in blue. Other nucleases involved in DNA repair are depicted in pink and non-specific and stress-induced nucleases are highlighted in green.

3. POLYPLOIDY

3.1 Introduction to polyploidy

Genome content can be defined as a C-value, indicating chromatin amount or DNA content, as a multiple of the haploid genome (Brodsky et al., 1985). When chromosomes can be visualized, ploidy can be expressed as n values, where n is the haploid number of chromosomes (Orr-Weaver, 2015). Since most eukaryotic cells have a diploid genome, it could be represented as $2n$ DNA content. Therefore, polyploid cells can be defined as having $3n$ DNA content or more before DNA replication (Øvrebø & Edgar, 2018). It was believed for long that every cell in an organism carries the same genomic content, which would be associated with genomic stability. However, there are many examples showing variation in the diploid DNA content, within eukaryotic organisms, from plants to mammals. Usually, two types of variations have been described: aneuploidy -characterized by the gain or loss of at least one chromosome or a chromosome arm (Holland & Cleveland, 2009; Taylor et al., 2018; Weaver & Cleveland, 2006) – and polyploidy, the multiplication of the whole chromosome set. For this manuscript, I will focus on polyploidy and its implications in both physiological and non-physiological conditions.

Polyploidy was first described more than a hundred years ago by the German biologist Eduard Strasburger who taught at the University of Jena and conducted extensive research on the process of cell division in plants. During his studies, Strasburger discovered cells with abnormal chromosome numbers, which he termed "polyploid cells" (Strasburger, 1910). However, in 1907 other botanists such as A. Lutz were already describing variations in genome content in various plant species, such as *O. lamarckiana* (**Figure 18** and Lutz, 1907).

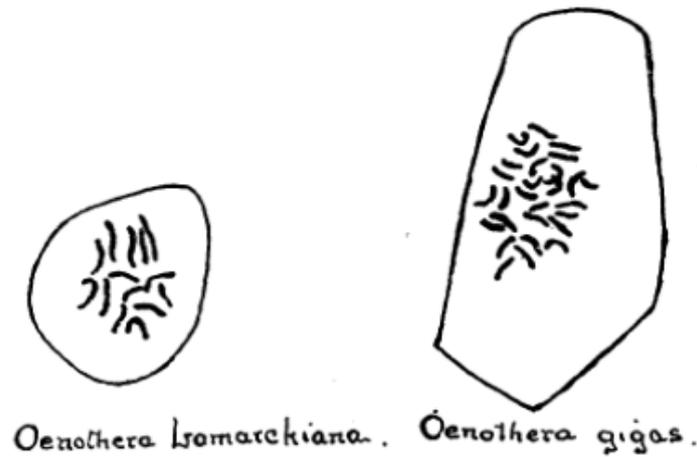


Figure 18: Chromosome content in “parental” *Oenothera lamarckiana* and a “mutant” form called *Oenothera gigas*. Picture from Lutz, 1907.

The main purpose of polyploidization is to generate large cells, as larger cells have functional advantages compared to an increased number of smaller cells, over a similar total mass. For instance, large cells can act as tissue envelope, and they are often more metabolically active (Frawley & Orr-Weaver, 2015; Gentric & Desdouets, 2014; Orr-Weaver, 2015; Unhavaithaya & Orr-Weaver, 2012). In *Drosophila*, most of the differentiated larval tissues are polyploid (Almeida Machado Costa et al., 2022), and several insects use polyploidy to increase their organ size. Many examples addressing the use polyploidy during development are reviewed in Orr-Weaver, 2015.

Polyploidy can be achieved by a variety of processes, such as cell fusion, endocycle, or endomitosis. Cell fusion occurs during the formation of muscle fibers (Chen & Olson, 2004; Rochlin et al., 2010), while most of the *Drosophila* tissues become polyploid by endocycling (Øvrebø & Edgar, 2018), and megakaryocytes undergo endomitosis (Ravid et al., 2002). In this section, I will first describe the different way of generating a polyploid cell in the *section 3.2*, then discuss the role of polyploid in physiology in the *section 3.3*, but also what happens when it occurs in an unscheduled manner in the *section 3.4*.

3.2 Strategies to generate a polyploid cell

Cell fusion

Cell fusion is a biological process in which two or more cells merge into a single cell. This is a cell-cycle independent mechanism that can give rise to mono or multinucleated cells (**Figure 19A**). Cell fusion occurs during skeletal muscle formation, where myoblasts fuse to form multinucleated muscle fibers (Chen & Olson, 2004; Rochlin et al., 2010) or the fusion of cytotrophoblasts during pregnancy for the formation of syncytiotrophoblast in the mammalian placenta (Zybina & Zybina, 2020). The molecular mechanisms of cell fusion involve a series of complex and coordinated events, such as cell recognition and adhesion, membrane rearrangement, formation of a fusion pore and mixing of cytoplasmic content (reviewed in Brukman et al., 2019). Fusion has also been described among cancer cells and between malignant and non-malignant cells to contribute to initiation and progression of cancer (Clawson, 2013; Goldenberg et al., 1974; Platt & Cascalho, 2019). Moreover, it is possible to experimentally induce cell-cell fusion, using specific molecules, electroporation, or viral infections (Duelli & Lazebnik, 2007; Radomska & Eckhardt, 1995).

Endocycle

Endocycle is defined as the uncoupling of DNA replication from cell division. This mechanism is cell-cycle dependent and is found in many groups of plants or animals (reviewed in (Øvrebø & Edgar, 2018)). During the endocycle, cells replicate their DNA without dividing afterwards, resulting in successive phases of growth with gene expression (G-phase) and DNA synthesis (S-phase) (**Figure 19B**). To switch from the regular cell cycle (described in the “*Cell cycle regulation*” section 1.3) to an endocycle, the mitotic machinery is suppressed through the inhibition of CDK1, and the cell cycle is therefore regulated through CDK2-Cyclin E oscillations (Broek et al., 1991; Chen et al., 2016). The endocycle is considered as the main driver of polyploidization in animals. One very striking example is the fruit fly *Drosophila melanogaster*, in which most of differentiated larval tissues develop through endocycling, such as cells of the salivary glands, fat body, midgut, trachea and epidermis (Almeida Machado Costa et al., 2022; Nandakumar et al., 2020; Smith & Orr-Weaver, 1991; Unhavaithaya & Orr-Weaver, 2012). *Drosophila* salivary glands are a well-studied example of endocycling tissues. In this organ, cells can reach up to 2048C via multiple rounds of endocycles (Rudkin, 1972; Smith & Orr-Weaver, 1991; Urata et al., 1995). Salivary glands exhibit polytene chromosomes, which are formed when DNA is replicated but chromosomes do not separate and form very peculiar chromosome arrangements with banded patterns (Bridges, 1935; Ashburner, 1970;

Lefevre, 1976; Spradling, 2017). Another feature of these endocycling cells is that several genomic regions remain under-replicated, mostly late-replicated heterochromatin regions (Edgar et al., 2014; Hannibal et al., 2014; J. Nordman et al., 2011; Nordman et al., 2014). Recent studies using genome-wide copy number profiling revealed that under-replication in *Drosophila* is mediated through Rap1-interacting factor 1 (Rif1) protein. Precisely, Rif1 inhibits replication fork progression to control DNA copy number in salivary glands, but also in ovarian follicle cells which is another example of polyploid tissue in the fruit fly (Munden et al., 2018, Armstrong 2020).

Endomitosis

Another mechanism generating polyploid cells is endomitosis, in which cells enter M-phase but do not complete it and so the daughter nuclei are kept in the same cell. According to how far the cell had progressed in the mitotic phase, endomitosis can generate either mono- or multinucleated polyploid daughter cells. Cells can prematurely halt mitosis – a phenomenon also called mitotic slippage- before chromosome segregation due to premature Cyclin B1 degradation (Hégarat et al., 2020; Ravid et al., 2002; Y. Zhang et al., 1998). In this case, a mononucleated polyploid cell is generated (**Figure 19C left panel**). Mitotic slippage is used by megakaryocytes, hematopoietic cells responsible for platelet production, as a strategy to become polyploid (Ravid et al., 2002). Alternatively, cells can also exit mitosis after chromosome segregation during cytokinesis. Failure of cytokinesis usually generates a binucleated cell (**Figure 19C right panel**) and can be observed upon several conditions. For instance, increased expression of Aurora A, a Ser/Thr kinase essential for the formation and function of the mitotic spindle, lead to defective cytokinesis (Meraldi et al., 2002; Zhang et al., 2004; Zhou et al., 1998). In addition, defects in the distribution of Anillin, an actin binding protein localizing at the cleavage furrow, can also promote cytokinesis failure (Oegema et al., 2000). In *Drosophila*, cytokinesis failure can be observed in perturbations of *spaghetti squash* (*sqh* - encoding or the regulatory light chain of myosin II), *pavarotti* (encoding for kinesin-like protein), but also other genes encoding for molecules involved in cytokinesis (Adams et al., 1998; Karess et al., 1991; Somma et al., 2002).

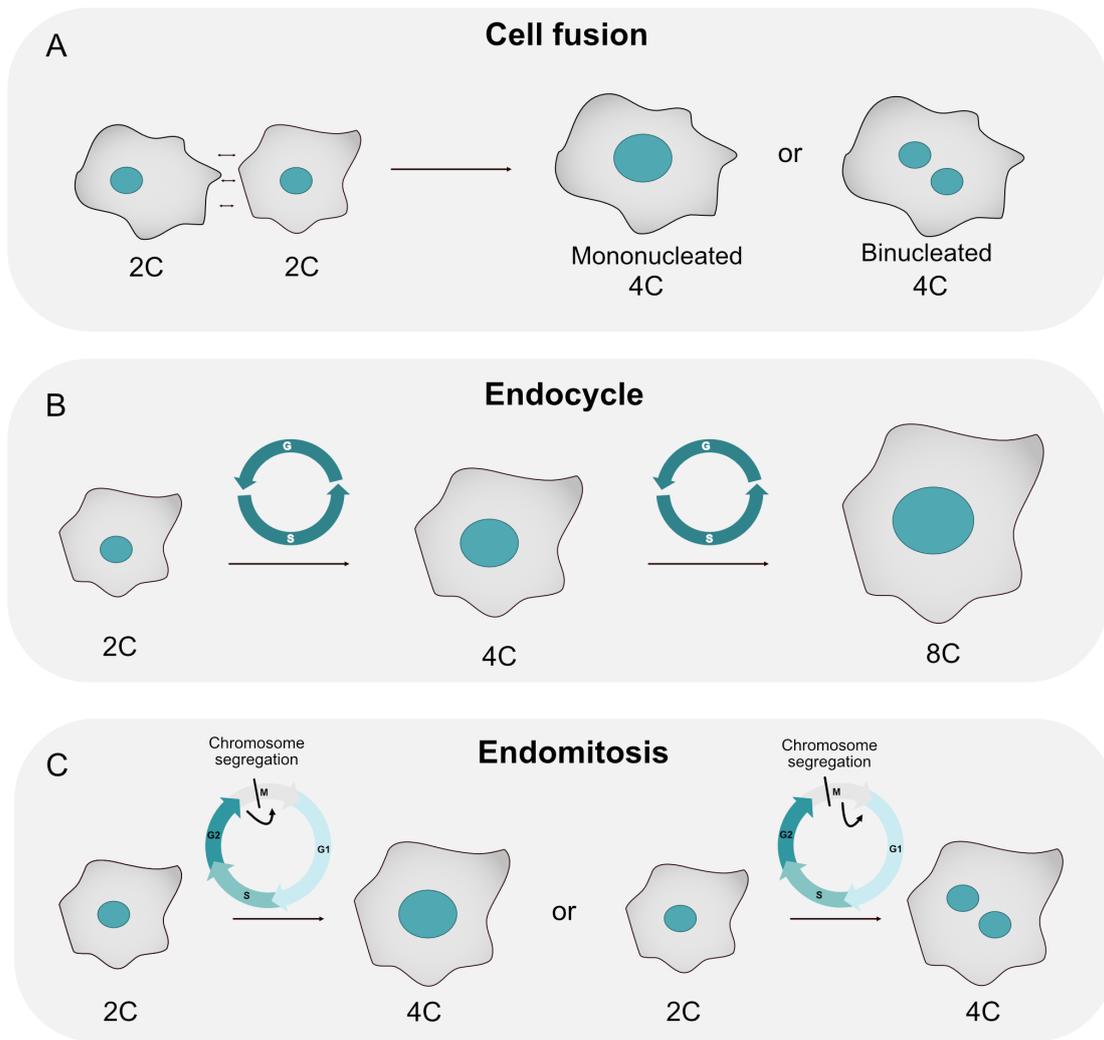


Figure 19: Several strategies to generate a polyploid cell. A. Cell fusion can occur between two diploid cells. B. Endocycles consists in a succession of growth (G) and DNA synthesis (S) without cell division, generating a mononucleated cell. C. Endomitosis occurs when cells enter mitosis but do not complete it. If mitotic exit occurs before chromosome segregation (on the left) it generates a mononucleated cell. If the cell exits mitosis after chromosome segregation, a binucleated cell is generated.

3.3 The role of polyploidy in physiology

Polyploidy for barrier function

Several tissues are composed of polyploid cells to form a physiological barrier. This is the case of the glia, placenta and epidermis. Indeed, growing by polyploidization allows cells to maintain the integrity of intercellular junctions, which are usually removed when cells undergo classical proliferation through division.

One striking example is the sub-perineurial glia (SPG) found in the *Drosophila* nervous system (Unhavaithaya & Orr-Weaver, 2012). The SPG is composed of flat cells attached through septate junctions, which constitute the blood-brain barrier in the fly (Limmer et al., 2014; Stork et al., 2008). These junctions must remain very tight all along development, while the nervous system increases in size. Indeed, during larval stages, the animal grows remarkably, but the number of SPG cells remains the same and the blood-brain barrier remains intact (Stork et al., 2008). Therefore, SPG cells increase in size through endocycle or endomitosis, leading to a mixed population of mono- and multinucleated cells. Ploidy levels have recently been shown to be controlled by the Öbek protein in SPG cells (Zülbahar et al., 2018). Polyploidy is essential in these cells, as inhibiting polyploidization disrupts cell-cell junctions during brain growth, therefore disrupting the blood brain barrier (Unhavaithaya & Orr-Weaver, 2012). More recently, polyploidy has been described in many cell types of the adult *Drosophila* brain, specifically in neurons and glia that re-enter the cell cycle. This polyploidization appears to occur in the ageing fly in response to DNA damage and is also believed to compensate for cell loss during brain ageing (Nandakumar et al., 2020).

Another example of polyploidization as a strategy to provide barrier function is the trophoblast giant cells (TGC) found in the mammalian placenta, which provide a barrier between the maternal and fetal blood. In rodents, these cells can reach a ploidy level of thousands C by endocycle. Other type of polyploid cells compose the placenta, such as the syncytiotrophoblasts, which are produced by cell fusion (Barlow & Sherman, 1974; Zybina & Zybina, 2020). Recently, many other polyploid cell types have been described in the placenta, and their polyploidization appears to be driven by the activity of c-myc (Singh et al., 2023). Polyploidy in TGC is believed to protect cells from DNA damage and mutagenesis, as well as protecting the maternal and fetal organisms from mutual immunological attacks (Zybina & Zybina, 2020).

In addition, polyploid cells might also have a barrier function in the mammalian skin. Indeed, it has been shown that during differentiation, keratinocytes - epithelial cells composing the epidermis- become polyploidy either by endoreplication or mitotic slippage (Gandarillas et al., 2019; Gandarillas & Freije, 2014; Zanet et al., 2010). It was proposed that due to their large size, polyploid cells increase their mechanical resistance by covering a larger surface area in the skin. Moreover, polyploidy may protect from mutations or chromosome

aberrations induced by DNA-damaging agents, such as UV light exposure (Gandarillas & Freije, 2014).

Polyploidy for increased metabolic activity

In addition to the role of polyploid cells for barrier function, polyploidy can be required in some cell types to increase their metabolic capacity. Very good examples are mammalian megakaryocytes, giant cells derived from the hematopoietic lineage which produce anucleated platelets from their cytoplasm. During their maturation, in response to thrombopoietin, megakaryocytes undergo endomitosis by skipping late anaphase and cytokinesis (Geddis et al., 2007; Nagata et al., 1997; Ravid et al., 2002; Trakala et al., 2015)). They can reach up to 128C and polyploidy is essential for they function as reduction in ploidy leads to defects in platelet production (Trakala et al., 2015).

Another example of polyploidization to enhance cell function are hepatocytes, which represent 70% of the liver content. Among hepatocytes, up to 90% have been described to be polyploid in mice and around 40% in humans (Epstein, 1966; Gentric & Desdouets, 2014). Despite being polyploid, hepatocytes are one of the rarest type of cells that retain de ability to divide (as *Drosophila* glial and rectal papillar cells), while polyploid cells are usually terminally differentiated. In the liver, polyploidization occurs mostly through cytokinesis failure, leading to a binucleated cell (Margall-Ducos et al., 2007). Later, bipolar cell division generates mainly tetraploid and octoploid hepatocytes that can be mono- or binucleated (Guidotti et al., 2003). It has been proposed that polyploidy in hepatocytes increases the metabolic capacity of the liver (Donne et al., 2020). Indeed, an attractive model would be that having twice the number of genes would produce twice as many proteins, although it was shown that it is not exactly the case (Hannibal et al., 2014; Nordman et al., 2011). However, it was described that highly polyploid livers produce energy via anaerobic pathway using ATP from carbohydrates instead of fatty acids, suggesting a link between polyploidy and energy demands (Anatskaya & Vinogradov, 2007). In addition, whole-genome analysis comparing liver tissues suggested a link between polyploidy and gene controlling DNA damage, cell survival, hypoxia and oxidative stress (Anatskaya & Vinogradov, 2007).

Polyploidy for organ regeneration and wound healing

In the examples described above, polyploidy was essentially discussed as being part of the developmental program of the cell. However, it can also be induced in response to stress or an injury. Hepatocytes, despite their role in enhancing the liver function, are a striking example of polyploidy induction as a stress response. Indeed, in response to partial hepatectomy, hepatocytes undergo hypertrophic growth via polyploidization (Miyaoaka et al., 2012). Thus, an increase in size rather than in number is at the origin of liver regeneration. Later during regeneration, hepatocytes undergo mitotic cell division, which results in ploidy reduction (Duncan et al., 2010). This role for polyploidy in the liver has been controversial, as recent studies on ploidy reduction has shown that it can initiate tumorigenesis in the liver (Lin et al., 2020; Matsumoto et al., 2021).

The use of polyploidy for wound healing has also been described in *Drosophila* tissues. After wound induction in the *Drosophila* abdominal epithelium, this tissue is repaired by polyploidization. Precisely, large multinucleated cells are formed by cell fusion as well as mono-nucleated cells via endoreplication to re-establish the epithelial barrier and restore tissue mass (Bailey et al., 2021; Besen-McNally et al., 2021; Losick et al., 2013). The endocycle compensates cell loss by providing epithelial synthetic capacity while cell fusion speeds the closure of the wound. Polyploidization in this tissue is dependent on the Yorkie transcription factor, which activates specific genes involved in cell cycle and growth: Myc, E2f1 and CycE (Grendler et al., 2019). In this cell type, it has been proposed that polyploidization enables tissue repair when cell division is detrimental, as forcing mitosis in an injured epithelium seems to induce DNA damage (Grendler et al., 2019).

3.4 Polyploidy in pathologies as a source of genetic instability

Even if polyploidy can be programmed and be an advantage for some tissues, increase in genome content is found in many pathologies and highly contributes to genetic instability. However, when related with pathology, polyploidy occurs in an unscheduled manner. In addition, a majority of programmed polyploid cells are terminally differentiated, meaning that they do not divide anymore. Whole genome duplications (WGD) have been described in various cancers, as more than 40% of human tumors are near tetraploid and it is now well established that tetraploidization is very common at the early stages of tumorigenesis (Davoli

& De Lange, 2011; Zack et al., 2013). Indeed, having extra copies of genetic material can compensate the effects of chromosome loss or lethal mutations (Storchova, 2014), but also confer resistance to anti-cancer treatment and proliferative advantage to cells that are genetically unstable. Therefore, polyploidy has the potential to be at the origin of tumor development and metastasis formation (Dewhurst et al., 2015; Gerlinger et al., 2012; Kuznetsova et al., 2015).

Conversely, certain studies have shown that errors in chromosome segregation can also lead to polyploidization. For instance, diploid cells with telomere dysfunction have structural and numerical chromosome aberration, leading to cytokinesis failure and accumulation of polyploid cells (Pampalona et al., 2012). It is also the case for cells with lagging chromosomes or acentric fragments, which can persist in the midzone during cytokinesis, causing regression of the cleavage furrow and cytokinesis failure (Mullins & Biesele, 1977; Shi & King, 2005). Such mechanism has also been observed upon kinetochore protein depletion in the moss *Physcomitrella patens*, in which lagging chromosomes led to cytokinesis failure (Kozgunova et al., 2019). In this sub-section, I will describe several mechanisms by which polyploidy contributes to genetic instability.

3.4.1 Polyploidy leads to aneuploidy

One of the well-known models for the contribution of polyploidy to tumorigenesis is the idea that tetraploid cells are a transient intermediate before becoming aneuploid (Andreassen et al., 1996; Ganem et al., 2007; Storchova & Pellman, 2004). This model originates from Theodor Boveri's theory more than 100 years ago, who described abnormal multipolar mitosis when he double fertilized sea urchin eggs - (Boveri, 1912). Indeed, it is now established that cycling polyploid cells have the tendency to generate aneuploid daughters. This is the case for physiological polyploid cells, such as hepatocytes which can generate several daughter cells with chromosome number variations after multipolar mitoses (Duncan et al., 2010), but also *Drosophila* rectal papillar cells that undergo endocycle before dividing multipolarly (Schoenfelder et al., 2014). This was also observed in unscheduled polyploid cells, as in many human tumors, cells with tetraploid DNA content arise as an early step in tumorigenesis and precede the formation of aneuploid cells (Andreassen et al., 1996; Dewhurst et al., 2015; Fujiwara et al., 2005; Kuznetsova et al., 2015; Storchova & Pellman, 2004; Zack et al., 2013).

In *Saccharomyces cerevisiae*, polyploid strains have been reported to have increased chromosome loss (Andalis et al., 2004; Mayer & Aguilera, 1990; Storchová et al., 2006). In most of the cases, this phenomenon relies on abnormal mitoses. Indeed, polyploid cells do not carry only extra DNA, but also extra centrosomes, which have been described to be at the origin of multipolar mitotic spindle poles and chromosome missegregation (Ganem et al., 2009, Duncan et al., 2010; Fox et al., 2010). It was recently shown that in polyploid cells, the high number of chromosomes acts as a barrier during mitotic spindle formation, which blocks spindle pole coalescence and leads to multipolar mitoses (Goupil et al., 2020).

3.4.2 Replication stress and DNA damage in polyploid cells

In addition to the link between polyploidy and aneuploidy, polyploid cells have under-replicated regions in their genome, which can favor genetic instability. Under-replication occurs in physiological polyploid cells, such as salivary gland cells or even trophoblast cells (Edgar et al., 2014; Hannibal et al., 2014). It has been proposed that under-replicated regions in polyploid cells contributes to generate heterogeneity between different cells of the same tissue. It is also a strategy for decreasing gene expression in endocycling cells (Hannibal et al., 2014; J. Nordman et al., 2011). Thus, programmed polyploidy can be used as driver of genomic regulation.

However, under-replicated regions are also present in unscheduled tetraploid cells and have been described to contribute to genetic instability. Studies in tetraploid budding yeast revealed that even if the kinetics of DNA replication in tetraploids are identical than those in diploids, tetraploids have double the amount of DNA damage during S phase (Storchová et al., 2006). In human fibroblasts, tetraploidization increase the sensitivity to DNA damaging agent with increased DSBs (Hau et al., 2006). In addition, gene expression profiling in tetraploid cancer cells revealed an enrichment in transcripts involved in cell cycle and DNA replication. This enrichment was accompanied by increased levels of RS (Wangsa et al., 2018). Recently, new evidence revealed that such genetic instability is induced in the first interphase following tetraploidization. In human osteosarcoma cell lines, cytokinesis failure can precipitate replication stress already during the subsequent S phase (Pedersen et al., 2016). Moreover, recent work from our lab described that newly born tetraploid cells do not scale DNA

replication factors with their genome content, which perturbs DNA replication dynamics and generates DNA damage. This lack of scaling up can be rescued by overexpressing E2F1, which positively regulates many DNA replication factors, and therefore decreases DNA damage (Gemble et al., 2022).

Yet, the mechanisms linking polyploidy to DNA damage remain controversial and are debated. Indeed, several studies failed to demonstrate DNA damage after polyploidization. As an example, polyploidy generated following Eg5 inhibition did not generate DNA damage in HeLa cells (Ohashi et al., 2015). In addition, DNA damage was not detected in a number of studies that induced tetraploidization in mouse mammary epithelial cells and in several human transformed and non-transformed cell lines (Fujiwara et al., 2005; Ganem et al., 2014; Krzywicka-Racka & Sluder, 2011). Tetraploid HCT116 and RKO cells are more resistant to DNA damaging agents than their diploid counterparts (Castedo et al., 2006). Moreover, polyploid tumor cells have altered DNA damage response pathways, and triggers ssDNA break repair, and NHEJ pathways to increase DNA repair activity in a Fen1 mutant background (Zheng et al., 2012). In agreement with this, it has been shown that tetraploid budding yeast exhibit increased dependency on HR pathway and tetraploid human cell lines have increased levels of HR members compared to diploids (Gemble et al., 2022; Storchová et al., 2006).

3.4.3 Cell cycle asynchrony

Among the sources of DNA damage in polyploid cells, one concerns specifically multinucleated polyploid cells. Indeed, diploid (thus mononucleated) cells rely on a timely regulation of the cell cycle to ensure genetic stability, through the action of various regulators localized in their cytoplasm (further details in the “*cell cycle*” section 1). As multinucleated cells contain several nuclei within the same cytoplasm, it is likely that each nucleus receive different signals for cell cycle progression. Such asynchrony has been described in physiology, and one striking example is a filamentous fungus, *Ashbya gossypii*. In this polyploid multinucleated organism, neighboring nuclei divide asynchronously while sharing a common cytoplasm. This asynchrony is necessary for fungal development, as depleting regulators of the asynchrony leads to mitotic defects (Gladfelter, 2006).

Cell cycle asynchrony has also been described in non-programmed polyploid cells and is associated with DNA damage and genetic instability. One of the most famous experiments

concerning asynchronous cells is the classical cell fusion experiment performed by Rao and Johnson in 1970 (Johnson & Rao, 1970) In their study, they generated multinucleated cells using viral approaches to fuse HeLa cells at different cell cycle stages together. They observed that depending on the cell cycle stage at which the cells are at the moment of fusion, the resulting polyploid cell will have a different fate. Precisely, fusing a mitotic cell with a cell in interphase results in premature chromosome condensation of the interphase nucleus, in which the DNA gets “pulverized”. This raised the idea that there are components in the mitotic cytoplasm that can force mitotic entry, but also that the DNA has to be “ready” and most likely condensed before being exposed to a mitotic cytoplasm to prevent its “pulverization”(Johnson & Rao, 1970 ; Rao & Johnson, 1972).

Our lab recently described cell cycle asynchrony in *Drosophila* polyploid NBs induced after multiple rounds of cytokinesis failure. These cells provide an *in vivo* model system to study the consequences of non-physiological polyploidy as they continue to proliferate (Gatti & Baker, 1989; Goupil et al., 2020; Nano et al., 2019; Karess et al., 1991; Reed and Orr-Weaver; 1997), reaching high levels of multinucleation. Within a single polyploid NB, our lab found that nuclei progress asynchronously in the cell cycle. Consequently, at mitotic entry, nuclei that were not ready to enter mitosis but were forced to do so, transiently accumulate markers of DNA damage. This phenomenon was also observed after polyploidy induction in mouse neural stem cells and human cancer cells (Nano et al., 2019).

Both experiments described above raise the idea that DNA must be “ready” for mitotic entry. Therefore, asynchronous multinucleated cells might be exposed to another layer of genomic instability, that occurs specifically during mitosis.

3.4.4 Ploidy as an adaptation to genetic instability

Another way by which polyploidy can lead to tumorigenesis is that tetraploid cells are better at buffer the negative effects of deleterious mutations or chromosomal instability (Dewhurst et al., 2015; López et al., 2020) In addition, cells that underwent WGD have been showed to adapt to their tetraploid status, notably by several changes in gene expression (Quinton et al., 2021). Among these, enrichment in mutations in p53 and PPP2R1A have been observed (Quinton et al., 2021). Since p53 is a barrier for cell proliferation, inactivating it would confer an advantage for tetraploid cells, even if this remains controversial (see next *section 3.5*). In

addition, PP2R1A promotes centrosome clustering and is therefore required to prevent multipolar mitoses, which can lead to cell death. To discuss about this type of studies, it is important to mention the notion of ploidy-specific lethal (PSL) genes (Storchová et al., 2006), which was introduced to describe a gene deletion non-lethal in diploid or haploid, which becomes lethal in tetraploid or triploid budding yeasts. In a screen for these PSL genes, Storchova et al. have identified 39 genes essential for polyploid yeast. Among these genes, they found factors involved in homologous recombination and sister chromatid cohesions. Later, with the purpose of identifying specific vulnerabilities in tumors with WGD, Quinton et al. found that tumors that undergo WGD are more dependent on the spindle assembly checkpoint and specifically on KIF18A kinesin. Thus, there are now growing data suggesting that polyploid cells are able to adapt to their genomic status to survive.

3.5 Polyploidy and cell cycle checkpoint activation

As described above in this section, polyploidy and the cell cycle are highly linked to each other. Indeed, among the ways to generate a polyploid cells, some involve an alternative regulation of the cell cycle, such as endoreplication (mentioned in *section 3.2*), in which CDK1 is completely inhibited to allow the generation of a polyploid cell. Moreover, defective cell cycle checkpoints can lead to the generation of tetraploid cells, which are not necessary eliminated by the system and further lead to abnormal karyotypes (see *section 3.4*). In addition, understanding how cells having multiple copies of the genome deal with the canonical cell cycle is also a fascinating question, as it is known that polyploid cells have under replicated regions, which can be considered as a result from replication stress. Indeed, even if programmed polyploid cells have adapted their cell cycle regulators, unscheduled polyploidy requires adjustments for polyploid cell surviving. This is particularly true in the first cell cycle after WGD, during which tetraploid cells experience RS that can be rescued by increasing G1 duration (Gemble et al., 2022). In addition, several studies reported that under stress conditions, certain cells “escape” the checkpoint through a mitotic bypass which leads to tetraploidization (Davoli et al., 2010; Johmura et al., 2014; Zeng et al., 2023). Precisely, it was showed that tetraploidization by mitotic bypass can occur in response to DNA damage and the loss of telomere protection (Davoli et al., 2010). This was also described in human fibroblasts during senescence, permanent cell cycle exit (Johmura et al., 2014). More recently,

induction of replication stress through overexpression of Cyclin E induces WGD through mitotic bypass (Zeng et al., 2023). Surprisingly, tetraploidization in this context was dependent on p53. In theory, to ensure genomic stability, tissues possess mechanisms to limit the proliferation of cells with an unbalanced chromosome content. A “tetraploid” checkpoint has been described 20 years ago, which triggers cell cycle arrest in cell with an unscheduled tetraploid DNA content (Andreassen et al., 2001). However, this idea has been disputed later, as both human fibroblasts and RPE-1 cells treated with drugs blocking cytokinesis do not undergo cell cycle arrest, and tetraploid cells generated through cell fusion can proliferate (Uetake & Sluder, 2004; Wong & Stearns, 2005). In addition, hepatocytes are often polyploid and can perform mitosis with high chromosome content (Guidotti et al., 2003).

Rather than via a “tetraploid” checkpoint, WGD seem to have the capacity to activate cell-cycle checkpoints in an indirect manner. For instance, abnormal centrosome numbers occurring upon tetraploidization are detected by the PIDDosome pathway, which is activated by supernumerary centrosomes to induce p53 activation (Fava et al., 2017; Sladky et al., 2020). In addition, damage occurring on the mitotic spindle can activate p53 through the translocation of a tumor suppressor kinase Lats2, prior to mitotic slippage, suggesting that it is the MT damage *per se* which activates p53, and not WGD (Aylon et al., 2006). Finally, it is important to mention that, even if such checkpoints exist in non-transformed cells, cancer cells usually have shut down checkpoint mechanisms, and keep proliferating despite having genomic aberration or DNA damage. In agreement with this, tetraploid has been shown to promote tumorigenesis in p53 -null mouse mammary epithelial cells (Fujiwara et al., 2005). In addition, it was recently shown that tetraploid clones derived from human colon carcinoma cell lines have increased cyclin D levels, which was suggested to be an early event allowing a rapid adaptation to WGD (Crockford et al., 2017).

4. DROSOPHILA AS A MODEL SYSTEM

Drosophila melanogaster, also known as the fruit fly, is one of the most frequent model organisms used during in the past 100 years to study genetics, development, and animal behavior. *Drosophila* was introduced as a model system for biological research in 1906 by William Castle (Castle, 1906). In the beginning of the 20th century, almost all the significant work on *Drosophila* as a model organism has been carried out by Thomas Hunt Morgan in Columbia University, who established a number of main principles of classical genetics by studying genes, chromosomes and the inheritance of genetic information (Morgan, 1910). Later in the century, various molecular, developmental, and biological techniques started to be applied to *Drosophila*, such as gene cloning, hybridization, P-element based transformation and clonal analyses (Yamaguchi & Yoshida, 2018), which allowed scientists to perform analytical studies. Many studies carried out on the *Drosophila* genome revealed high homology between the *Drosophila* and human genome, with nearly 75% of human disease-related genes having functional orthologues in *Drosophila* (Pandey & Nichols, 2011; Yamamoto et al., 2014), and the overall nucleotide sequence having 40% identity. Thus, *Drosophila* is still one of the first model system to study modern biology, in which new discoveries are often achieved and then generalized to other organisms including humans.

One of the many advantages of this model organism is their relatively rapid life cycle, as a single mating can produce hundreds of adult offspring in approximately 10 days. *Drosophila* has several developmental stages (**Figure 20**): embryo, larval, pupal and adult (Pandey & Nichols, 2011). After fertilization, the embryo undergoes synchronized nuclear division cycles, which forms a multinuclear syncytium. At this stage, embryos are transcriptionally silent and rely on maternal gene products to develop (Laver et al., 2015). The larva, and most precisely the third instar larva, is commonly used to study developmental processes. At this stage, several structures called imaginal discs are formed, which will later produce the future adult external structures. During my thesis, I mostly performed experiments during the larval stage. In the pupal stage, *Drosophila* undergoes metamorphosis, and imaginal discs cells proliferate and differentiate to form various adult external structures, while most larval tissue undergo programmed cell death (Aguila et al., 2007). In this section, I will briefly introduce *Drosophila* genetics and genetic tools I used during my thesis, as well as

the use of *Drosophila* to study polyploidy *in vivo*, and more precisely focus on NBs as a model to study the consequences of unscheduled polyploidy.

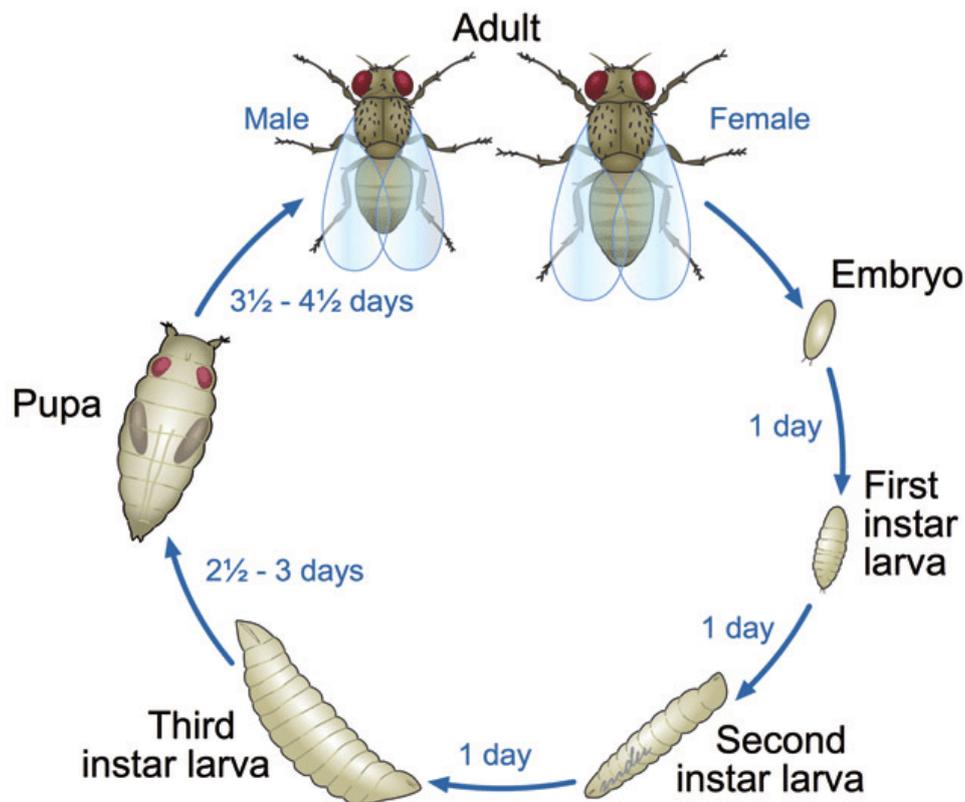


Figure 20: *Drosophila* life cycle. After fertilization, the egg rapidly develops to form an embryo. After 24h, the embryo reaches the first larval stage, which lasts around 3 days. In my thesis, I used the 3rd instar larvae as a model. Then, larvae enter the pupal stage, during which they will undergo metamorphosis until becoming an adult fly. The whole *Drosophila* life cycle last around 10 days, at 25 °C. Images from Ong et al., 2014.

4.1 Introduction to *Drosophila* genetic tools

***Drosophila* genome**

The haploid *Drosophila* genome is composed of 4 chromosomes: one sexual pair (XX or XY) and three autosomes. Among the autosomes, the chromosome 4 is the smallest and is mainly composed of heterochromatin. The large chromosomes 2 and 3 are metacentric, with the centromere located near the center of two left and right arms, named 2L, 3L and 2R, 3R, respectively (Deng et al., 2007; Metz, 1914). The X chromosome is also referred to as the first

chromosome. However, cytogenetic mapping of *Drosophila* genome has been strongly improved with the discovery of polytene chromosomes, which develop from successive duplication of each chromatid of a chromosome without their segregation. This leads to very long chromosomal structures which can be 100 times longer than typical metaphase chromosome. Every polytene chromosome shows a unique banding pattern caused by the differential condensation of chromatin to form darkly stained bands and less stained interbands. Based on the banded pattern of polytene chromosomes, Bridges (Bridges, 1935) codified these regions and created the first map of *Drosophila* chromosomes. In this map, still used to date, each chromosome arm is divided in 20 units (X = 1–20; 2L = 21–40; 2R = 41–60; 3L = 61–80; 3R = 81–100; and the small fourth 4R = 101–102), in turn subdivided into 6 lettered segments (A to F) composed of numbered bands (Bridges, 1935). This mapping gained in resolution and precision with the improvement of microscopy (Lefevre, 1976; Saura et al., 1999). In 2000, the annotated molecular mapping emerged with WGS (Adams et al., 2000). The estimated size of the *Drosophila* genome is ~180Mb (Bosco et al., 2007) and its annotation identified ~17,700 genes (reviewed in (Kaufman, 2017)).

UAS-GAL4 system

A commonly used approach to express or knock-down specific genes in *Drosophila* is the UAS-GAL4 targeted expression system (Brand & Perrimon, 1993). GAL4 is a yeast transcription factor that is used to control the spatial and temporal expression of target genes (Fischer et al., 1988; Giniger et al., 1985; Kakidani & Ptashne, 1988), which consequently directs gene activity at a specific developmental stage and specific cells and tissues. In one parental strain, promoter regions for a particular gene are designed to drive the expression of GAL4 in some tissues. In another strain, the GAL4-binding upstream-activating sequence (UAS) is placed in front of the transgene. When these two strains are genetically crossed, their progenies express the transgene in specific tissues driven by the GAL4-UAS system. In combination with RNA interference (RNAi), it is also possible to knockdown specific genes by expressing double-stranded RNAs targeted to specific mRNAs using the GAL4-UAS system.

4.2 Using *Drosophila* larval tissue to study polyploidy

Drosophila is a well-established model to study polyploidy, as polyploid cells are found across a variety of organs and tissues throughout the four stages of the life cycle (reviewed in (Almeida Machado Costa et al., 2022)). In the embryo, DNA is endoreplicated via endocycle between 8 and 15 hours after fertilization, at specific times in the salivary gland, Malpighian tubules and gut (Smith & Orr-Weaver, 1991). During the larval stage, both salivary gland and Malpighian tubules remain polyploid, and polytene cells are also found in the fat body, epidermis, trachea, brain, and prothoracic glands (Mirth et al., 2005; Richards, 1980; Smith & Orr-Weaver, 1991). Later, adult *Drosophila* exhibit polyploid cells in the ovary, which contains endoreplicated follicle and nurse cells and, the female spermatheca, composed of polyploid secretory cells and in Malpighian tubules. In addition, the adult gut contains polyploid enterocytes (Ohlstein & Spradling, 2006) , while *Drosophila* brain carries polyploid glial cells (Nandakumar et al., 2020).

In contrast with the developmentally programmed polyploidy found in many of its tissues, *Drosophila* larvae has also been used as a model for polyploid tumors. For instance, ectopic expression of oncogenes, in combination with tumor suppressor inhibition can transform the wing disc epithelia into neoplasms containing polyploid giant tumor cells. These neoplasms exhibit features that recapitulates certain hallmarks of cancer (Cong et al., 2018). Neoplastic tumorigenesis can also be induced through Notch hyperactivation in imaginal ring polyploid cells (Wang et al., 2021; Yang et al., 2019). In addition, polyploidy can be induced in some larval tissues such as NBs, which, under certain mutant conditions, can lead to large multinucleated cells (further described in the next section).

4.3 *Drosophila* NBs

Drosophila NBs are the stem cells of the developing fly brain. They are a well-established model for neural stem cell biology, for studying asymmetric cell division, but also tumor formation (Homem & Knoblich, 2012). These highly proliferative cells divide asymmetrically to self-renew, giving rise to another NB and to a smaller ganglion mother cell (GMC) (Reichert, 2011). GMCs divide once more to produce neurons and/or glial cells. There are two waves of neurogenesis in *Drosophila*. The first one during embryogenesis and a second one during larval stages. My studies have been performed on NBs that are active in the second wave of neurogenesis. At this stage, several types of NBs are present in the brain and have different

characteristics depending on their position and lineage. In the ventral nerve cord (VNC), we can distinguish abdominal and thoracic NBs, while the brain lobes contain type I, II, mushroom body and optic lobe NBs (**Figure 21A**). Type I NBs are found in both anterior and posterior sides of the brain, and they constitute the majority of central brain NBs. For this reason, I will mostly consider type I NBs while referring to NBs.

Drosophila NBs have been developed as a model to study polyploidy and its consequences (Gemble et al., 2022; Goupil et al., 2020; Nano et al., 2019). Indeed, upon certain mutant conditions, they can accumulate high numbers of chromosomes (Gatti & Baker, 1989; Karess et al., 1991) more refs as mentioned above while keeping proliferating, unlike other *Drosophila* tissues, such as the wing imaginal disc (Gerlach et al., 2018; Nano et al., 2019). As mentioned above, they can tolerate multiple rounds of cytokinesis failure, which leads to large multinucleated cells. Because of their tolerance to polyploidy, they are a very relevant model to study the consequences of unscheduled polyploidy *in vivo*. For this reason, I used this model system for most of the work described in this manuscript (**Figure 21B**).

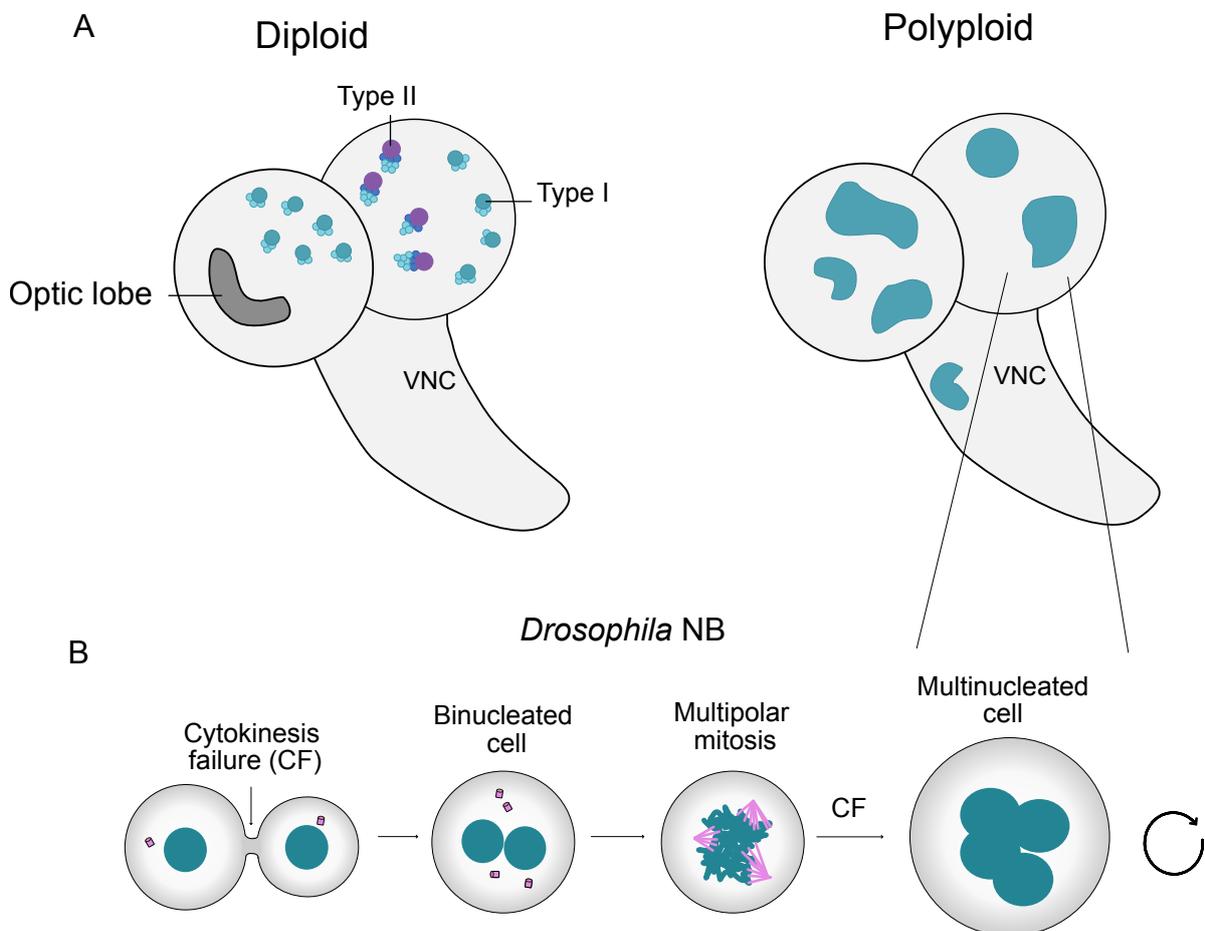


Figure 21: *Drosophila* NBs as a model to study polyploidy. **A.** *Drosophila* NBs are found in the larval brain. Typical polyploid larval brain is depicted on the left. Type I and II NBs divide asymmetrically to give GMCs (in light blue below NBs). Upon genetic conditions, such as mutations in genes involved in cytokinesis, NBs can become highly polyploid and multinucleated, and can be detected both in the central brain and in the VNC. In this condition, it is quite difficult to distinguish between NB types, as they fail to divide properly and generate GMCs. VNC = ventral nerve cord. **B.** RPE-1 cells can be used as an *in vitro* model of unscheduled polyploidy. **B.** Polyploidy is achieved in *Drosophila* NBs via multiple rounds of cytokinesis failure (CF). On the first round, CF generates a binucleated cells. On the next cell divisions, polyploid NB undergo multipolar mitoses and keep failing cytokinesis, which leads very large and highly multinucleated cells.

Chapter 2 – Material and Methods

During my thesis, I used two different model systems of unscheduled polyploidy to identify factors involved in polyploid cell proliferation and genetic. The first one is an *in vivo* model, *Drosophila* neural stem cells or neuroblasts (NBs), which are tolerant to polyploidy. As an *in vitro* model, I also used human Retinal Pigment Epithelial cells (RPE-1), which have been immortalized. These cells have a stable near-diploid content and can be used as a model to study the outcome of WGD. Most of the work described in the results section was performed in *Drosophila*, and a part of the section 2 of results was performed in RPE-1 cells. In this section, I will describe the experimental procedures used first for *Drosophila* experiments, and secondly for experiments conducted in RPE-1 cells.

DROSOPHILA EXPERIMENTS

1. Fly husbandry

1.1 Fly culture

Flies were raised on cornmeal medium (0.75% agar, 3.5% organic wheat flour, 5.0% yeast, 5.5% sugar, 2.5% nipagin, 1.0% penicillin-streptomycin and 0.4% propionic acid). Fly stocks were maintained at 18°C. Crosses were carried out in plastic vials and maintained at 25°C unless differently specified. Stocks were maintained using balancer inverted chromosomes to prevent recombination. In all experiments, larvae were staged to obtain comparable stages of development. Egg collection was performed at 25 °C for 24 h. After 5 days of development at 25 °C, third instar larvae were used for brain dissection.

1.2 Fly stocks

Control flies used in this study vary depending on the experiment. For experiments performed in diploid brain lobes, controls were *wild-type* (WT) flies *w*¹¹¹⁸ (BL 3605), or flies carrying Worniu-Gal4 driver and the insertion site (BL 9752) without the UAS transgene (for experiments conducted in UAS-Gen-mCh, UAS-Mre11-mCh, UAS-Mus312-mCh and UAS-Slx1-mCh). For experiments performed in polyploid brain lobes, controls were *sqh*¹ (Karess et al., 1991), or *sqh*¹, Worniu-Gal4 without the UAS target gene (for experiments conducted in UAS-Gen-mCh, UAS-Mre11-mCh, UAS-Mus312-mCh and UAS-Slx1-mCh).

Table 1: Fly stocks used in this study.

Stock name	Source	Chromosome	Reference
Mutant lines			
<i>sqh</i> ¹		X	Karess, 1991
<i>spn-A</i> ⁰⁹³	McVey, M lab	III	Staeva-Viera et al., 2003
<i>spn-A</i> ⁰⁵⁷	McVey, M lab	III	McVey et al., 2004
<i>gen</i> ^{z597}	Sekelsky, J lab	III	Andersen et al., 2011
<i>mre11</i> ^{58s}	Sekelsky, J lab	II	Gao et al., 2009
<i>slx1</i> ^{F93I}	Sekelsky, J lab	III	Andersen et al., 2011
<i>mus312</i> ^{z-1973}	Sekelsky, J lab	III	Yildiz et al., 2002
RNAi lines			
Gen ^{RNAi}	BL42787	III	Perkins et al., 2015
Mre11 ^{RNAi}	BL 50628	III	Perkins et al., 2015
Mus81 ^{RNAi}	BL65012	II	Perkins et al., 2015
Mus312 ^{RNAi}	BL34873	III	Ni et al., 2011
Slx1 ^{RNAi}	BL34949	III	Ni et al., 2011
Fen1 ^{RNAi}	BL35764	III	Ni et al., 2011
Mei-9 ^{RNAi}	BL55313	II	Perkins et al., 2015
Mus201 ^{RNAi}	BL31052	III	Perkins et al., 2015
Tosca ^{RNAi}	BL35603	II	Perkins et al., 2015
WRNexo ^{RNAi}	BL38297	II	Perkins et al., 2015
Dnasell ^{RNAi}	BL63635	II	Perkins et al., 2015
EndoG ^{RNAi}	BL55228	III	Perkins et al., 2015
Sid ^{RNAi}	BL62346	II	Perkins et al., 2015
Rad51 ^{RNAi}	BL 51926	II	Perkins et al., 2015
Blm ^{RNAi}	BL31330	III	Perkins et al., 2015
Fluorescent proteins			
UAS-Gen-mCh	This study	II	N.A
UAS-Mre11-mCh	This study	III	N.A
UAS-Mus312-mCh	This study	II	N.A
UAS-SLx1-mCh	This study	II	N.A
UAS-Gen ND -mCh	This study	II	N.A
H2Av-mRFP	BL 23651	II	BDSC
hisH2Av-EGFP	BL24163	II	BDSC
Ubq-Gen-NeG	This study	II	N.A
Driver & deficiencies			
worniu-Gal4	Basto lab	II	
Df (3L)Exe16103	BL7582	III	Andersen et al., 2011

2. Molecular Biology

2.1 Targeted insertion

PhiC31 integrase-mediated transgenesis was used to insert UAS-Gen-mCh, UAS-Mre11-mCh, UAS-Mus312-mCh, UAS-Slx1-mCh, Ubq-Gen-NeG and UAS-GenND-mCh transgenes in a precise site of the *Drosophila* genome. This system is based on the use of an integrase from the PhiC31 bacteriophage that mediates sequence-directed integration between attachment (att) sites of 34 base pairs (Bischof et al. 2007; Fish et al. 2007). The transgene was cloned in a P[acman] plasmid containing the attB site (from bacterial genome) and injections were performed by BestGene (BestGene Inc, CA, USA). The recipient *Drosophila* stock for injection ($\gamma[1] w[1118]; PBac\{\gamma[+]-attP-3B\}VK00037$), containing attP sites (from the PhiC31 bacteriophage genome), was obtained from Bloomington Drosophila Stock Center (BDSC, #9752), Indiana University, IN, USA. Integration was targeted to Chromosome II at chromosomal location 2L:1582820.

2.2 Genomic DNA extraction and PCR

Drosophila genomic DNA was extracted from 40 *wild type* (*w1118*, BL3605) adult flies using the following protocol: flies were homogenized on ice in 250 μ L Tris HCl 0.1 M pH 9, EDTA SDS 1%, and incubated for 30 min at 70°C. 35 μ L KAc 8M pH 7.8 was added and incubated on ice for 30 min, centrifuged for 15min and 250 μ L phenol-chloroform was added to the supernatant. Sample was centrifuged for 5 min, and 150 μ L isopropanol was added to the supernatant and centrifuged for 5 min to precipitate DNA. DNA pellet was washed with 70% EtOH, centrifuged for 5 min and dried, and resuspended in Tris EDTA.

For amplification of *Drosophila* Gen, Mre11, Mus312 and Slx1, 30 cycles of PCR were performed in 50 μ L volume using 100 ng purified DNA of template, 1 μ L dNTP mix (10mM), 1 μ L of each primer nucleotides (25mM), annealing was performed for 30 seconds between 55-65°C depending on the sequence to be amplified, and elongation at 72°C for 30 seconds/kb of the template length. Each PCR product was analyzed by agarose gel electrophoresis and purified from an agarose gel slice using a gel purification kit (28704, QIAGEN). Primers used for PCR are listed in the table 2.

2.3 Molecular cloning

Plasmid constructions were designed and produced with the help of Anthony Simon, the lab manager of our lab (Institut Curie, Paris). 1 µg of Gen, Mre11, Mus312 or Slx1 fragments amplified from *Drosophila* genomic DNA and carrying restriction sites for EcoRV and SpeI were digested for 1h at 37°C with 5U EcoRV and 5U of SpeI, with 5 µL Cutsmart Buffer (New England Biolabs) in 50 µL total volume. pBlueScript II SK (+) (PBSK+) vector, containing an Ascl restriction site at the 5' of a UAS-mCherry (GenScript) and a NotI restriction site at the 3' was also digested using the same protocol. Digestion products were purified using a PCR purification kit (QIAGEN 28104). Each fragment was inserted in PBSK-UAS-mCh using a 1:3 ratio of vector:insert diluted in a total volume of 2,5 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer, and 2,5 µL of ligation mix (Takara, 6023), for 30 min at 16 °C. Ligation mixture was directly used for transformation with 50 µl *E. coli* competent cells (Dh5α, New England Biolabs, C2987), and several positive colonies were collected and purified by Miniprep (17106, QIAGEN). Constructs were first verified using restriction profile, and one positive clone was chosen for each construct to be sent to Eurofins Genomics for Sanger sequencing. Then, UAS-Gen-mCh, UAS-Mre11-mCh, UAS-Mus312-mCh or UAS-Slx1-mCh were digested with Ascl and NotI restriction enzymes (New England Biolabs) for 1 hour at 37°C, purified and ligated with a P[acman] plasmid for 30 min at 16°C. Ligation product was used to transform TransforMax EPI300 *E. coli* (C300C105, Epicentre). P[acman] was purified from several clones and length of the insert was tested by restriction enzyme digestion with Ascl and NotI enzymes. One clone was used to generate transgenic flies by PhiC31 integrase-mediated transgenesis.

2.4 Generation of UAS-GenND-mCh

To generate a nuclease-dead construct for Gen, a sequence containing modified nucleotides to replace the EGVA residues in the first XPG domain, and the EAEA in the second XPG domain by AAAA, was neo-synthesized by GenScript. This sequence carry a EcoRV in 5' site and a HindIII site in 3', and was digested with EcoRV and HindIII for 1h at 37°C was cloned into the UAS-Gen-mCh to replace the 5' sequence of Gen.

Table 2: List of oligonucleotides used in this study.

List of oligonucleotides
Gen
Primers for cloning Gen in PBSK-UAS-mCh
CGCG <u>GATATC</u> ATGGGCGTCAAGGAATTATGGGG
CGCG <u>ACTAGT</u> ATCACTAATCACTACCAGGTCATCC
Primers for sequencing Gen
CGCG GGGAGCAAGTTACTCCGGTTTTTCG
CGCG GGAGCCGTTCTGTCTATCG
CGCGGCCTTGGAGATCCGAGTGGACG
CGCG CGGACATCTCTTGCAAGTGGCC
CGCG GCGTATCCCGATTTAGTAGCGGCC
CGCG GGACATAGTTAAAGGCATTATCTCG
CGCG GCCCTTGGATAACATAAGTTAC
Mre11
Primers for cloning Mre11 in PBSK-UAS-mCh
CGCG <u>GATATC</u> ATGAATGGCACCACGACAGCAGAGC
CGCG <u>ACTAGT</u> ATCGGAATCATCCGA
Primers for sequencing Mre11
CGCG GCCCAGCCAGAATGCGTTGC
CGCG CCCCCTCCTGATGCGCAAGGG
CGCG CGAGCCAGAGGAGAATGCC
CGCG CCTAAGCAGCCAACGCTACCGC
CGCG GGCTCTTGCAAGATGACCT
CGCG GCCACAGCTGCCACACGTGG
Mus312
Primers for cloning Mus312 in PBSK-UAS-mCh
CGCG <u>AGTACT</u> ATGGATCGCAAGACGCGGCGAGCC
CGCG <u>ACTAGT</u> CTTCTTTTAGAAGGCTTTTTTTGTG
Primers for sequencing Mus312
CGCG GTTCGAAAACCTGGGCC
CGCGGAGCTGAGGGTGAATCTCG
CGCG GAAATAGAAATTGTGTCCAGC
CGCG CAGTTTCAAAGTCCG
CGCG GCACCAAGGAAGCCGCT
CGCG GCCACTGCACATCGCC
Slx1
primers for cloning Slx1 in PBSK-UAS-mCh
CGCG <u>GATATC</u> ATGAACTCCTACGACCCACAGG
CGCG <u>ACTAGT</u> ATCGCTGAGCTCCGGGGTATC
Primers for sequencing Slx1
CGCG GGAAAACCAAGTCGCAAAGGGCC
CGCGGGTTTTCCATTAGTGCCTCACAACG

3. Immunohistochemistry

3.1 Whole-mount tissue preparation and imaging of *Drosophila* brains

Brains from third instar larvae were dissected in PBS and fixed for 30 min in 4% paraformaldehyde in PBS. They were washed 3 times in PBST 0.3% (PBS, 0.3% Triton X-100 (Sigma T9284), 10 min for each wash) and incubated for several hours in agitation at room temperature and overnight at 4 °C with primary antibodies at the appropriate dilution in PBST 0.3% (a list of antibodies used in this study is reported in table 3). Tissues were washed three times in PBST 0.3% (10 min for each wash) and incubated overnight at 4 °C with secondary antibodies diluted in PBST 0.3%. Brains were then washed 2 times in PBST 0.3% (30 min for each wash), rinsed in PBS and incubated with 3 µg ml⁻¹ DAPI (4',6-diamidino-2-phenylindole; Sigma Aldrich D8417) at room temperature for 30 min. Tissues were then washed in PBST 0.3% at room temperature for 30 min and mounted on mounting media. A standard mounting medium was prepared with 1.25% n-propyl gallate (Sigma P3130), 75% glycerol (bidistilled, 99.5%, VWR 24388-295), 23.75% H₂O). Images were acquired with 40x oil objective (NA 1.4) on two a wide-field Inverted SpinningDisk Confocal Gattaca/Nikon (a Yokagawa CSU-W1 spinning head mounted on a Nikon Ti-E inverted microscope equipped with a camera complementary metal-oxide semiconductor 1,200× 1,200 Prime 95B; Photometrics), controlled by Metamorph software. Interval for z-stacks acquisitions was set up from 0.5µm to 1 µm.

Table 3: List of antibodies used for *Drosophila* experiments.

Antibody	Working dilution	Source	Identifier
Rabbit polyclonal anti- χ H2Av	1:500	Rockland	600-401-914
Mouse monoclonal anti-Phospho histone H3 (Ser10)	1:500	Cell signaling Technologies	CST 9706
Guinea pig anti-Deadpan	1:1000	This laboratory	N/A
Rat monoclonal anti-RFP	1:500	Chromotek	5F8
Mouse monoclonal anti-mNeonGreen	1:500	Chromotek	32F6

Mouse monoclonal anti-lamin	1:500	Hybridomabank	ADL101
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific	A11008
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1:250	Thermo Fisher Scientific	A11030
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1:250	Thermo Fisher Scientific	A11081
Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor	1:250	Thermo Fisher Scientific	A11073
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific	A11001
Alexa Fluor® 647 Phalloidin	1:100	Thermo Fisher Scientific	A22287

4. Live imaging of *Drosophila* brains

Mid third-instar larval (L3) brains expressing fluorescent protein (See table 1 for *Drosophila* stocks) were dissected in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Penicillin-Streptomycin 15140, Gibco). Several brains were placed on a glass-bottom 35 mm dish (P35G-1.5-14-C, MatTek Corporation) with approximately 10 µL of medium, covered with a permeable membrane (Standard YSI), and sealed around the membrane borders with oil 10 S Voltalef (VWR BDH Prolabo). Images were acquired with 60x oil objective (NA 1.4) on two wide-field Inverted SpinningDisk Confocal Gattaca/Nikon (a Yokagawa CSU-W1 spinninghead mounted on a Nikon Ti-E inverted microscope equipped with a camera complementary metal-oxide semiconductor 1,200× 1,200 Prime 95B; Photometrics), controlled by Metamorph software. Images were acquired at time intervals spanning from 1 min (diploid conditions) to 10 min (polyploid conditions) and 30 to 50 Z-stacks of 1–1.5 µm.

5. Quantitative analysis of DNA damage, cell area and mitotic index

5.1 Imaging for quantitative analysis

Staged 3rd instar larval brains were dissected, stained, and imaged using the procedures described in Material and Methods 3.1. For DNA damage quantification, γ -H2Av primary antibody was preferentially detected using a secondary antibody conjugated Alexa Fluor 488, which was found to give the best signal to noise ratio. Imaging was performed using a 40X NA 1.3 oil objectives in Metamorph software with z-stacks of 1 μ m. Excitation parameter for the green channel were maintained constant. For cell area detection, Phalloidin conjugated Alexa Fluor 647 was used and incubated with secondary antibodies.

5.2 Image analysis for DNA Damage and cell area

Image analysis was performed using Fiji. Images were imported as Z-stack of 4 channels (PH3-red, γ -H2Av – green, DAPI – blue, Phalloidin – far red). Individual cells were manually cropped from the original image, as Z-stacks of 5 Z each. For DNA damage and cell area analysis, a Fiji macro was developed by Anne-Sophie Macé, (Institut Curie, UMR144) to automatize the signal quantification. For each image of individualized cell, cell area was manually segmented from a projection of the 5 Z on the far-red channel using the freehand selection tool. Similarly, nuclei were manually segmented from a projection of the 5 Z on the blue channel, using the freehand selection tool. These two segmentations were automatically saved as regions of interest (ROIs), and the green channel was used to separate positive pixels from negative pixels by a thresholding operation. We assigned a constant threshold value of 300 for DNA damage quantification, and 1000 for detecting PH3+ nuclei. The macro generates an output for each cropped images, indicating for each: cell area in pixel², nuclear area in pixel², the average intensity of the green channel in the ROI (named *DNA damage fluorescence intensity*), the area of positive pixels of the green channel in the ROI (named *DNA damage area*), the area of positive pixels of the red channel in the ROI (name *mitotic area*). In addition, the macro generates a montage, which is a RGB image providing an overview of the measured ROIs. Once the output obtained, cell area was adjusted in μ m², based on the pixel value given by the microscope and objective. To obtain the γ -H2Av index, DNA damage area was divided by the nuclear area, which provides the DNA damage coverage. DNA damage coverage was expressed as a percentage of coverage, and multiplied by the DNA damage fluorescence

intensity, to give the γ -H2Av index in arbitrary units (A.U). Statistical analysis was performed with GraphPad Prism (RRID SCR 002798) version 9.00 for Mac (GraphPad Software), using the tests mentioned in the figure legends. Image processing was made using Image J V2.1.0/1.53c software and mounted using Affinity Designer.

5.3 Image analysis for mitotic index

For mitotic index, images of diploid brain lobes were imported as Z-stack of 4 channels (DPN-red, γ -H2Av – green, DAPI – blue, Ph3 – far red). DPN+ (green) NBs were manually counted using Fiji Software, to get the total number of NBs per lobe. Among these, PH3+ (far red) NBs were manually counted for each lobe. The number of PH3+ (DPN+) NBs was divided by the total number of NBs (DPN+) and expressed as a percentage. Statistical analysis was performed with GraphPad Prism (RRID SCR 002798) version 9.00 for Mac (GraphPad Software), using the tests mentioned in the figure legends. Image processing was made using Image J V2.1.0/1.53c software and mounted using Affinity Designer.

HUMAN CELL LINE EXPERIMENTS

1. Cell culture and generation of human cell lines

1.1 RPE-1 cells

hTERT RPE-1 cells are retinal pigment epithelial cells from a female origin. This cell line has been immortalized by transfecting RPE-40 cells (Matsunaga et al., 1999; Kuznetsova et al., 2014) with a plasmid expressing the human telomerase reverse transcriptase subunit (hTERT) (Bodnar et al., 1998). The RPE-1 cell line is near-diploid, with a stable karyotype. For this reason, it is a relevant model to study the effects of unscheduled polyploidy. In Gemble et al., several treatments have been used in RPE-1 cells to induce WGD using mitotic slippage, cytokinesis failure or endoreplication, and each of them resulted in various degrees of tetraploidization.

1.2 Cell culture

Cells were maintained at 37 °C in a 5% CO₂ atmosphere. hTERT RPE-1 cells (ATCC cat. no. CRL-4000, RRID:CVCL 4388) were grown in Dulbecco's modified medium (DMEM) F12 (11320-033 from Gibco) containing 10% fetal bovine serum (GE Healthcare), 100 U ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin (15140-122 from Gibco). Cells were routinely checked for mycoplasma infection and were negative for mycoplasma infection.

1.3 Generation of RPE-1 shGen1 and shCtrl stable cell lines

Generation of RPE-1 shGen1 stable cell lines was performed by Simon Gemble a post-doctoral researcher in the lab. To produce lentiviral particles, HEK 293 cells were transfected with 4 µg pLKO.1-puro shRNA GEN1 (Sigma-Aldrich TRCN0000051878) + 4 µg pMD2.G (Addgene 12259) + 4 µg psPAX2 (Addgene 12260) using a FuGENE HD Transfection Reagent (Promega E2311) in OptiMEM medium (ThermoFisher 51985034). Cells were incubated at 37 °C in a 5% CO₂ atmosphere for 16 h and then growth media were removed and replaced by 6 ml fresh OptiMEM. The following day, viral particles were isolated by filtering the medium containing them through a 0.45-µm filter (Sartorius Stedim Biotech 16537). Then, RPE-1 cells were

incubated with viral particles in the presence of $8 \mu\text{g}\cdot\text{ml}^{-1}$ polybrene (Santa Cruz sc-134220) at 37°C in a 5% CO_2 atmosphere for 24 h.

1.4 Generation of RPE1 shCtrl or shGen1 FUCCI or H2B-GFP stable cell line

Generation of RPE-1 shCtrl and shGen1 FUCCI or H2B-GFP cell lines was performed by Simon Gemble, a post-doctoral researcher in the lab at Institut Curie. To produce lentiviral particles, HEK 293 cells were transfected with $4 \mu\text{g}$ pBOB-EF1-FastFUCCI-Puro (Addgene 86849) or pHR_dSV40-H2B-GFP (Addgene 67928) + $4 \mu\text{g}$ pMD2.G (Addgene 12259) + $4 \mu\text{g}$ psPAX2 (Addgene 12260) using a FuGENE HD Transfection Reagent (Promega E2311) in OptiMEM medium (ThermoFisher 51985034). Cells were incubated at 37°C in a 5% CO_2 atmosphere for 16 h and then growth media were removed and replaced by 6 ml fresh OptiMEM. The following day, viral particles were isolated by filtering the medium containing them through a $0.45\text{-}\mu\text{m}$ filter (Sartorius Stedim Biotech 16537). Then, RPE-1 shCtrl or shGen1 cells were incubated with viral particles in the presence of $8 \mu\text{g}\cdot\text{ml}^{-1}$ polybrene (Santa Cruz sc-134220) at 37°C in a 5% CO_2 atmosphere for 24 h. RPE-1 GFP and RFP-positive cells were then collected using Sony SH800 FACS (BD FACSDiva Software Version 8.0.1). RPE-1 shCtrl or shGen1 clones expressing FUCCI or H2B-GFP were selected, and the cell lines were established from several clones.

1.5 Tetraploidy induction in human RPE-1 cells

To induce mitotic slippage, cells were incubated with DMSO (D8418 from Sigma Aldrich) or with $50 \mu\text{M}$ monastrol (S8439 from Selleck- chem) + $1 \mu\text{M}$ MPI-0479605 (S7488 from Selleckchem) for at least 20h. This treatment was used for all immunostainings and for live imaging analyses. Alternatively, cells were treated for $50 \mu\text{M}$ monastrol alone for 18h, and then $1 \mu\text{M}$ MPI-0479605 was added for at least 2h. This treatment was mostly used for Western Blot analysis and for the mFISH experiments, to allow a brief synchronization of tetraploid cells.

2. Immunohistochemistry

2.1 Immunostaining and imaging of RPE-1 cells

Cells were plated on cover slips in 12-well plates and treated with the indicated drugs. To label cells, they were fixed using 4% of paraformaldehyde (Electron Microscopy Sciences 15710) + Triton X-100 (2000-C from Euromedex) 0.1% in PBS (20 min at 4 °C). Then, cells were washed three times using PBS-T (PBS + 0.1% Triton X-100 + 0.02% Sodium Azide) and incubated with PBS-T + BSA (Euromedex 04-100-812-C) 1% for 30 min at room temperature. After 3 washes with PBS-T + BSA, primary and secondary antibodies were incubated in PBS-T + BSA 1% for 1 h and 30 min at room temperature, respectively. (List of antibodies is available in table 4) After 2 washes with PBS, cells were incubated with 3 $\mu\text{g ml}^{-1}$ DAPI (Sigma Aldrich D8417) for 15 min at room temperature. After two washes with PBS, slides were mounted using 1.25% n-propyl gallate (Sigma P3130), 75% glycerol (bidistilled, 99.5%, VWR 24388-295), 23.75% H₂O. Images were acquired on an upright widefield microscope (DM6B, Leica Systems, Germany) equipped with a motorized xy stage and a 40 \times objective (HCX PL APO 40 \times /1.40–0.70 Oil from Leica). Acquisitions were performed using Metamorph 7.10.1 software (Molecular Devices) and a sCMOS camera (Flash 4V2, Hamamatsu). Stacks of conventional fluorescence images were collected automatically at a z-distance of 0.3 μm (Metamorph 7.10.1 software; Molecular Devices, SCR 002368). Images are presented as maximum intensity projections generated with ImageJ software (SCR 002285).

Table 4: List of antibodies used for human RPE-1 cells experiments.

Name	Working dilution	Source	Identifier
Immunostaining			
Mouse monoclonal anti- γ -H2Ax	1:500	Abcam	ab22551
Rat monoclonal anti-RPA32	1:500	Cell signaling Technologies	CST 2208
Rabbit polyclonal anti-Fancd2	1:250	Novus	Novus 100-182
Mouse monoclonal anti-LaminA/C	1:500	Sigma	ab200236
Rabbit monoclonal anti-Rad51	1:500	Abcam	ab133534

Mouse monoclonal anti-53BP1	1:500	Millipore	MAB3802
Rabbit polyclonal anti-PICH	1:150	Cell signaling Technologies	CST 8881
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific	A11008
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1:250	Thermo Fisher Scientific	A11030
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1:250	Thermo Fisher Scientific	A11081
Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor	1:250	Thermo Fisher Scientific	A11073
Western blotting			
Rabbit polyclonal anti-GEN1	1:500	Gift from West Lab	N/A
Mouse monoclonal anti-p53	1:200	Santa Cruz	sc126
Mouse monoclonal anti-p21	1:200	Millipore	OP-64
Rabbit polyclonal anti-GAPDH	1:5000	Sigma-Aldrich	G9545
anti-H2B	1:2000	Santa Cruz	sc-515808

3. Western Blotting

3.1 Western blotting for scaling up experiment

Scaling up experiments were conducted by Simon Gemble. For scaling up detection, RPE-1 cells were treated with the indicated drugs to induce mitotic slippage, and then treated with 1 μ M Palbociclib (Selleckchem S1579) to synchronize cells at G1/S transition, as described in Gemble et al., 2022. For a whole-cell extract, cells were lysed in 8 M urea, 50 mM Tris HCl, pH 7.5 and 150 mM β -mercaptoethanol (Bio-Rad 161-0710), sonicated and heated at 95 °C for 10

min. The same number of cells (see 'FACS sorting of diploid and tetraploid cells') were loaded for diploid and tetraploid conditions, allowing us to compare one diploid cell with, allowing us to compare one diploid cell with one tetraploid cell. Protein fractions from the gel were electrophoretically transferred to PVDF membranes (PVDF transfer membrane; GE Healthcare RPN303F). After 1 h saturation in PBS containing 5% dry non-fat milk and 0.5% Tween 20, the membranes were incubated for 1 h with a primary antibody diluted in PBS containing 5% dry non-fat milk and 0.5% Tween 20. After three 10-min washes with PBS containing 0.5% Tween 20, the membranes were incubated for 45 min with a 1:2,500 dilution of peroxidase-conjugated antibody. Membranes were then washed three times with PBS containing 0.5% Tween 20, and the reaction was developed according to the manufacturer's specifications using ECL reagent (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific 34080). The background-adjusted volume intensity was calculated and normalized using a H2B signal (H2B was used as a readout of DNA content) for each protein, using Image Lab software version 6.0.1, Bio-Rad Laboratories.

3.2 Western blotting for other experiments

RPE-1 cells were treated with the indicated drugs to induce mitotic slippage. Cells were lysed in RIPA (150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50mM Tris, pH8.0) complemented with protease (Sigma-Aldrich #11697498001) and phosphatase (Sigma-Aldrich #4906845001) inhibitors. Samples were then dosed using a BiCinchoninic acid Assay (Pierce BCA protein assay, ThermoFisher Scientific # 23227). Samples were diluted in RIPA with 4X LDS sampling buffer (ThermoFisher Scientific #NP0007), and heated at 80C for 10min. 20ug of protein was then loaded in NuPage Bolt 4-12% Bis-Tris precast gels, and subjected to electrophoresis in Bolt MOPS SDS running buffer (ThermoFisher Scientific # B0001). The gels were then transferred to PVDF membranes (PVDF transfer membrane; GE Healthcare RPN303F) using transfer buffer (25mM Tris, 192mM Glycine, 20% Methanol) for 90min at 4C. Membranes were stained in primary or horse-radish peroxidase coupled secondary antibodies diluted in PBS or TBS + 0,5% Tween 20 + 0,5% BSA or non-fat milk according to providers instructions. Membranes were first stained using Ponceau, before saturating for 1h at room temperature in 5% non-fat dry milk or 5% BSA in PBS or TBS + 0,5% Tween20. Membranes were then incubated overnight in primary antibodies, washed 5 times

in PBS or TBS + 0,5% Tween 20, then incubated for 1hr at room temperature in secondary antibodies. Membranes were then washed again 5 times in PBS or TBS +0,5% Tween 20. Horse-radish Peroxidase reaction was developed using SuperSignal Plus Chemiluminescent substrates (Thermo Fisher Scientific # 34580 and #34094) and imaged (BioRad ChemiDoc MP). The Image Lab software (BioRad version 6.0.1) was used to measure background-adjusted volume intensity, which was normalized using GAPDH signal. Antibodies used are listed in **table 4**.

3.3 FACS of diploid and tetraploid

FACs sorting of diploid and tetraploid cell was performed by Simon Gemble. Mix of diploid and tetraploid cells were incubated with 2 $\mu\text{g ml}^{-1}$ Hoescht 33342 (Sigma Aldrich 94403) for 1 h at 37 °C, 5% CO₂. Then, a single cell suspension was generated. Cells were washed using PBS, the supernatant was removed, and cells were resuspended in a cold cell culture medium at 1 $\times 10^7$ cell per ml and kept at 4 °C during all the experiments. Fluorescence-activated cell sorting (FACS) was performed using Sony SH800 FACS (BD FACSDiva Software Version 8.0.1). Compensation was performed using the appropriate negative control samples. Experimental samples were then recorded and sorted using gating tools to select the populations of interest. RFP+GFP⁻ cells (G1 cells) were first selected. Then, in this population, DNA content was used to segregate diploid (2n) and tetraploid (4n) G1 cells. Once gates have been determined, the same number of diploid and tetraploid G1 cells were sorted into external collection tubes. The number of cells was then checked using a cell counter and the same number of diploid and tetraploid cells were collected for western blot analysis.

4. Time lapse microscopy

RPE-1 cells were plated on a dish (627870 from Dutscher) and treated with the indicated drugs. After 20h, drugs were washout and replaced by fresh culture medium. Images were then acquired every 2 min for H2B-HGFP, and every 15 min for Fucci on a spinning disc microscope (Gataca Systems). Based on a CSU-W1 (Yokogawa), the spinning head was mounted on an inverted Eclipse Ti2 microscope equipped with a motorized xy stage (Nikon). Images were acquired through a 40 \times NA 1.3 oil objective with a sCMOS camera (Prime95B,

Photometrics). Optical sectioning was achieved using a piezo stage (Nano-z series, Mad City Lab). Gataca Systems' laser bench was equipped with 405-, 491- and 561-nm laser diodes, delivering 150 mW each, coupled to the spinning disk head through a single mode fibre. Laser power was chosen to obtain the best ratio of signal/background while avoiding phototoxicity. Multi-dimensional acquisitions were performed using Metamorph 7.10.1 software (Molecular Devices). Stacks of conventional fluorescence images were collected automatically at a z-distance of 0.5 μm (Metamorph 7.10.1 software; Molecular Devices, RRID SCR 002368). Images are presented as maximum intensity projections generated with ImageJ software (RRID SCR 002285), from stacks deconvolved with an extension of Metamorph 7.10.1 software.

5. Quantitative analysis of DNA damage

To assess DNA damage in human cells, an ImageJ software-based plugin developed by QUANTACELL was used, where individualized nuclei were automatically segmented based on the DAPI channel, and FANCD2, $\gamma\text{-H2Ax}$, Rad51 and 53BP1 signals were measured using z-projection stacks after thresholding. Nuclear size, DAPI intensity, the number of $\gamma\text{-H2Ax}$ foci, $\gamma\text{-H2Ax}$ fluorescence intensity and the percentage of nuclear coverage by $\gamma\text{-H2Ax}$ signal were obtained for each nucleus. Tetraploid nuclei were distinguished from diploid based on their size.

6. Clonogenic assay

For clonogenic assays, diploid and tetraploid cells were FACS-sorted as described in 3.3, and 500, 1000, and 2000 cells were plated in 6 wells plates. Cells were maintained at 37 °C in a 5% CO₂ atmosphere for 8 days (for diploid) or 13 days (for tetraploid). Cells were washed with PBS and fixed for 10 min with methanol, washed with PBS and stained for 10 min with crystal violet (1% diluted in EtOH 20%), and rinsed multiple times with PBS and dried. Colonies were counted manually in each well using Fiji software.

7. Chromosome spreads and mFISH

Chromosome spreads and mFISH was performed by Marie Dumont, research engineer in Daniele Fachinetti lab, Institut Curie. Cells were grown to 80% confluency in 10 cm petri dish and were treated with colcemid for 3h. Cells were trypsinized and centrifuged at 1000 rpm for 10 min. Cell pellets were resuspended in 75 mM KCl and incubated for 15 min in a 37°C waterbath. 1/10 volume of 3:1 methanol/acetic acid was added to cells followed by centrifugation at 1000 rpm for 15 min. Cells were then fixed by resuspension in 3:1 methanol/acetic acid solution, incubated for 30 min at room temperature, centrifuged at 1200 rpm for 5 min and finally washed once more with fixative. Cells were resuspended in a small volume of fixative, dropped onto clean glass slides, and left to air dry. Multicolor FISH (mFISH) staining was performed following manufacturer's instructions (Meta-Systems). The Metafer imaging platform (MetaSystems) and the Isis software (MetaSystems, version 5.5) were used for automated acquisition of the chromosome spread and mFISH image analysis.

8. Gene expression analysis in WGD vs non-WGD tumors

Gene expression analysis was performed by Carolin Shauer, post-doctoral researcher in the lab of Isidro Cortes-Ciriano, at the European Molecular Biology Laboratory, European Bioinformatics Institute, Wellcome Genome Campus, Hinxton, UK. WGD was determined using two approaches: 1.) Using the same threshold as applied to the human cancer cell line dataset, in which samples with a ploidy > 2.7 were considered as WGD and 2.) Utilizing WGD estimates from Drews et al, 2022 where WGD status of a tumour sample is determined based on a sample's ploidy and the fraction of the genome affected by loss of heterozygosity (LOH). Gene expression data (RNAseq V2) from TCGA (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) were downloaded using Firebrowse (<http://firebrowse.org/>). Gene expression as transcripts per million (TPM) were used. To account for normal/stromal contamination which will impact gene expression levels measured by bulk RNAseq, tumours with purities $\geq 75\%$ were considered, which included a total of $n=2444$ tumours across 33 cancer types.

Chapter 3 – Results

Results- Section 1

*Identification of factors responsible for DNA damage at mitotic entry
in asynchronous polyploid cells*

Introduction

Polyploidy, the multiplication of the whole genome, is commonly found in many tumors and is a driver of genetic instability. It is now well established that whole genome duplications (WGD) lead to genome reshuffling via different mechanisms, such as replication stress, errors in chromosome segregation, or even transcriptomic changes (Dewhurst et al., 2015; Fujiwara et al., 2005; Gemble et al., 2022; Kuznetsova et al., 2015; Storchová et al., 2006). However, certain molecular mechanisms linking polyploidy to genetic instability are still not completely understood. A recent study from our lab has identified a new way of generating DNA damage in multinucleated polyploid cells during mitosis. This work described that upon unscheduled polyploidy, several nuclei of a given multinucleated polyploid cell can undergo cell cycle progression in an asynchronous manner. Consequently, at mitotic entry, nuclei that were not ready to enter mitosis, but were forced to do so, transiently accumulate markers of DNA damage (Nano et al., 2019). These observations were made in *Drosophila* NBs, mouse embryonic neural stem cells, and human cancer cell lines after polyploidy induction. However, the mechanisms generating DNA damage, specifically in the delayed nuclei have not been described yet. I hypothesized that nucleases present in the cytoplasm may generate DNA damage in the nucleus containing uncondensed chromatin, that experiences premature mitotic entry (Nano et al., 2019).

Nucleases are molecular scissors that cut the phosphodiester bond between two nucleotides, and are involved in many biological processes, such as DNA damage repair and replication (Nishino & Morikawa, 2002). Considering their ability to cut DNA sequences, they are attractive candidates for the generation of DNA damage observed in mitotic polyploid cells. Importantly, several nucleases have been shown to be required during mitosis to cleave DNA intermediates and to avoid defects in chromosome segregation (Chan & West, 2014; Duda et al., 2016; Matos et al., 2011; Naim et al., 2013). It is then tempting to hypothesize that when a polyploid cell enters mitosis in an asynchronous manner, mitotically active nucleases are recruited and targeted the nucleus (or nuclei) that are not ready for mitosis. However, the nucleases responsible for this and the mechanisms by which they generate damage specifically in the delayed nucleus have not been identified yet.

To investigate the role of nucleases in generating DNA damage in the mitotically delayed nucleus in polyploid cells, we used as a model *Drosophila* NBs in which we induced

polyploidy through cytokinesis failure. In this system, we performed an *in vivo* RNA interfering (RNAi) screen, by depleting each of the 13 nucleases of the fly genome. We found that several nucleases are involved in the proliferation of polyploid NBs, in agreement with their major role in several DNA repair processes. By measuring DNA damage in asynchronous polyploid cells upon nuclease depletion, we were able to identify 4 candidates that might be responsible for the generation of DNA damage at mitotic entry. We later confirmed these candidates using genetic mutations. Eventually, we established tagged versions of these candidates, and assessed both their localisation and the effect of their overexpression in polyploid NBs upon mitotic entry. This allowed us to identify 2 potential nucleases, Gen and Mre11, that would be involved in generating DNA damage in mitotically delayed nucleus of asynchronous polyploid cells.

Results

A certain number of nucleases are required for polyploid NB proliferation

To identify nucleases responsible for generating DNA damage at mitotic entry in polyploid cells, we took advantage of the low redundancy of *Drosophila* genome and performed an RNA interfering (RNAi) screen in *Drosophila* NBs after polyploidy induction. As described in more details in the results section-2, multinucleated NBs can be obtained via the hypomorphic mutation of *spaghetti squash* (*sqh*¹) (Gatti & Baker, 1989; Gemble et al., 2022; Karess et al., 1991; Nano et al., 2019). To perform such a screen, we gathered several *Drosophila* lines expressing UAS-RNAi for each of the 13 nucleases described in the fly genome (In *introduction section* - 3.4.3). Among them, some were known to be active during mitosis, such as Gen (homolog of mammalian Gen1), Mus81, Slx1, Mus312 and Mre11 (the homolog of Slx4 in mammals) ((Blanco et al., 2014; Y. W. Chan & West, 2014a; Ciccio et al., 2008; Gao et al., 2009; García-Luis & Machín, 2014; Garner et al., 2013; Landmann et al., 2020b; Minocherhomji et al., 2015; H. D. M. Wyatt et al., 2013). The majority of the remaining candidates were described as being involved in several DNA repair pathways or DNA replication (Boubriak et al., 2009; J. Sekelsky, 2017; J. J. Sekelsky, Brodsky, et al., 2000; J. J. Sekelsky, Hollis, et al., 2000; Yildiz et al., 2002, 2004). Importantly, Mus312 does not contain nuclease activity *per se*, but it is rather a scaffold protein which has been described to interact with Mei-9, Slx1 and Mus81

(Andersen et al., 2009; Fekairi et al., 2009). In addition, three of them, EndoG, Dnasell and Sid were previously described as non-structure specific nucleases, normally associated with stress responses (Schäfer et al., 2004; C. S. Seong et al., 2014; C.-S. Seong et al., 2006).

To deplete each candidate specifically in polyploid NBs, I used the UAS-GAL4 system to express specific RNAi against each nuclease (Fig 1A). Precisely, I used the Worniu-Gal4 driver, which allows the expression of its target genes only in NBs. Therefore, I generated fly lines carrying both *sqh^{mut}* and Worniu-G4 (Ashraf et al., 2004; Lai et al., 2012). By crossing this line with each UAS-RNAi line individually, I was able to obtain *Drosophila* larvae expressing nuclease-RNAi specifically in polyploid NBs (Fig 1A).

In the results section 2, we described that homologous recombination factors are required for polyploid cell proliferation. Therefore, I first assessed if any of the nucleases was involved in polyploid cell proliferation by measuring polyploid cell area as a readout of polyploid cell proliferation (as described in *results - section 2 figure S1*). I noticed that the depletion of some candidates was strongly reducing polyploid cell area, suggesting that they are required for polyploid cell proliferation. This is the case for Gen, Mus312, Slx1, Fen1, Mus201 and EndoG (Fig 1B and D).

In agreement with their role in DNA repair, depletion of the large majority of these factors led to increased DNA damage in interphase in polyploid NBs (Fig 2A), suggesting that they might be required for DNA repair in polyploid NBs. However, with the exception of Fen1, none of these factors appeared to be essential in *Drosophila* NBs, as their viability was not affected when they were depleted in diploids. Concerning Fen1, its depletion led to very high levels of DNA damage already in diploid NBs, and we were barely able to detect any polyploid cell in this condition (N= 16 cells out of 28 brain lobes, with a mean cell area of 300 μm^2 , while polyploid ctrl had a mean cell area of 1142 μm^2) (Figure 2B-D). This data is consistent with previous work showing that Fen1 is essential in *Drosophila*, and that its depletion leads to apoptosis in NBs, because of defects in DNA replication (Barclay et al., 2014; Kuang et al., 2014). I focus the follow up studies on the remaining 12 other nucleases.

Altogether, this data suggests that most of the nucleases encoded in the fly genome influence proliferation and most likely DNA repair in polyploid NBs.

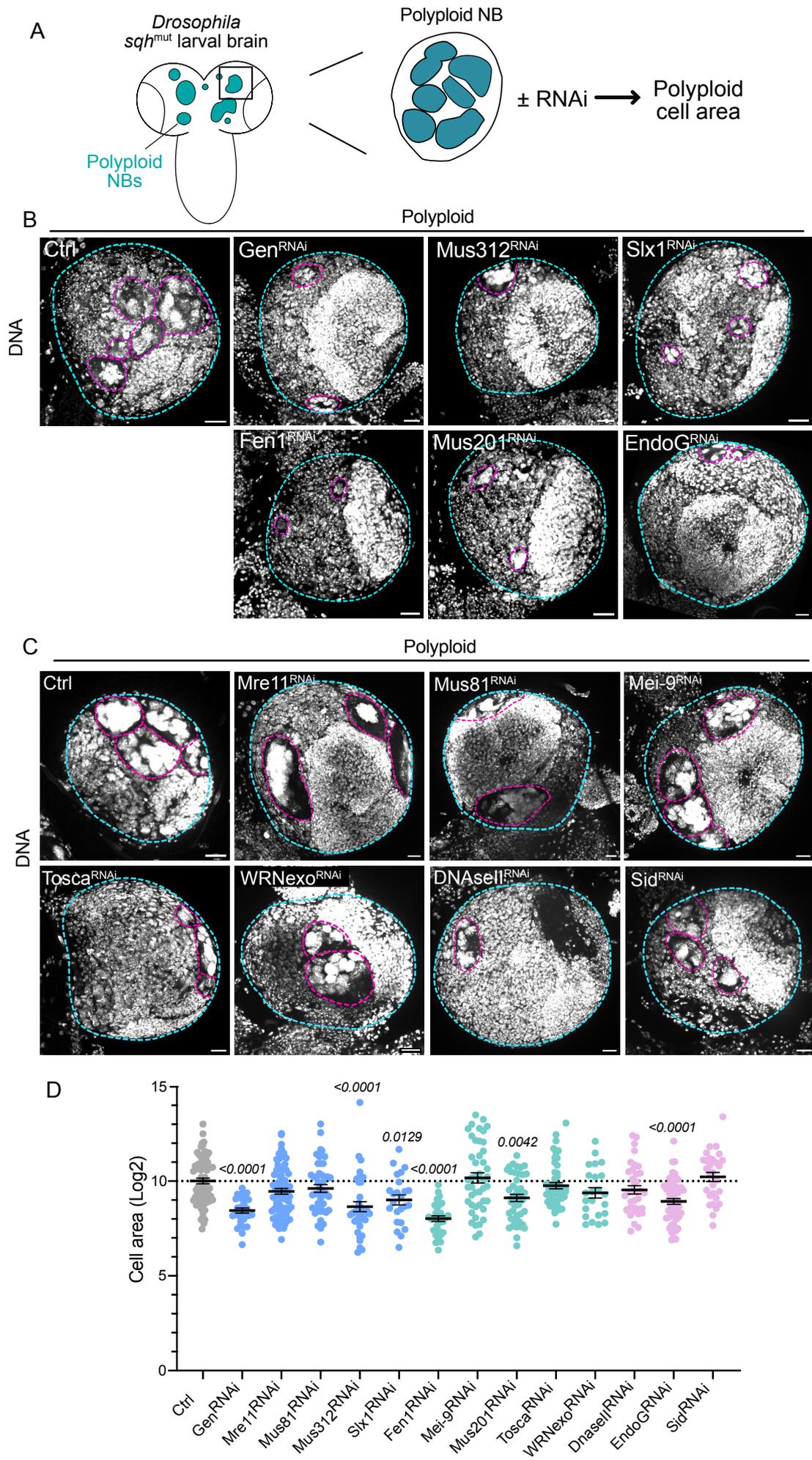


Figure 1. The majority of *Drosophila* nucleases are required for polyploid cell proliferation

(A) Diagram of experimental procedure for depletion of nuclease candidates in *Drosophila* polyploid NBs. *sqh*^{mut} larval brains carry polyploid NBs (in blue) resulting from repeated cytokinesis failure (as described in **Introduction section 4.3 - Figure 21**). RNAi targeting each candidate were expressed specifically in NBs using a Worniu-G4 driver. Polyploid cell area was measured as a readout of polyploid cell proliferation. **(B)** Images of *Drosophila* polyploid brain lobes labelled with DAPI to reveal nuclear staining (in grey) upon the expression of the indicated RNAi, leading to a significant decrease in polyploid cell area. Ctrl brains correspond to *sqh*^{mut} polyploid brains and the remaining conditions *sqh*^{mut}, nuclease^{RNAi} polyploid brains. **(C)** Images of *Drosophila* polyploid brain lobes labelled with DAPI to reveal nuclear staining (in grey) upon the expression of the indicated RNAi, leading to no difference in polyploid cell area. **(D)** Dot plot graph showing polyploid NBs area (in μm) measurement for each of the indicated genotypes. Results are expressed as a log2 of the area measured in μm^2 area. Cell area was measured using Phalloidin to label the cell membrane (not shown). For statistical analysis, Kruskal Wallis test was performed on the rank of each sample. Non-significant p-values are not depicted on the graph. On the images, scalebars = 20 μm .

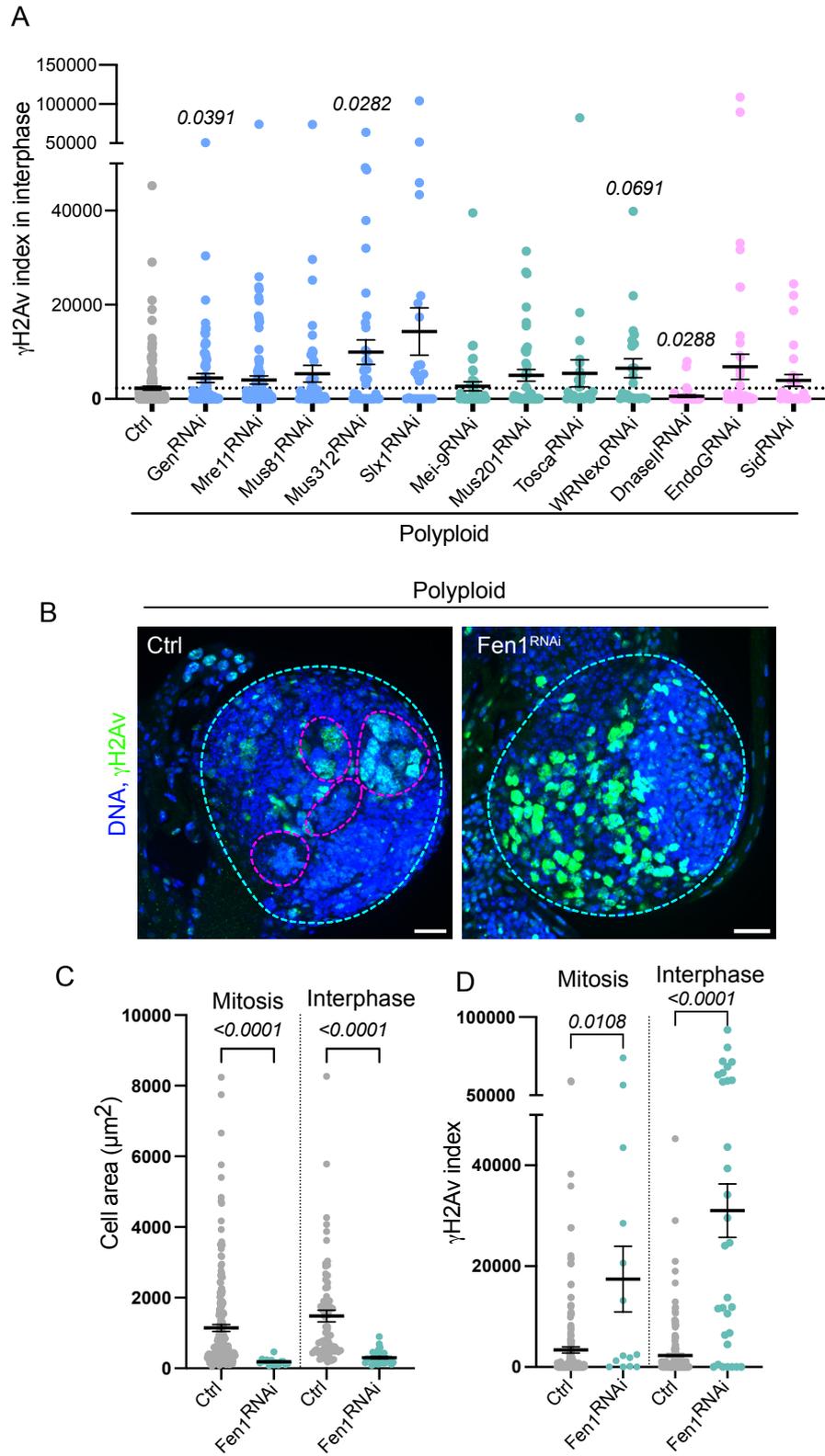


Figure 2: Depletion of certain nucleases increases DNA damage levels in polypliod cells

(A) Dot plot graph showing γ -H2Av index (A.U., measured as the γ -H2Av signal coverage multiplied by γ -H2Av signal intensity) in interphase in polypliod NBs of the indicated genotypes. For each condition, except *SLx1*^{RNAi} and *WRNexo*^{RNAi} (n=25 and n=23 cells,

respectively) more than 40 cells were analysed. **(B)** Images of *Drosophila* polyploid brain lobe upon the depletion of Fen1, labelled with DAPI to reveal nuclear staining (in blue) and γ H2Av to reveal DNA damage (in green). Ctrl brain correspond to *sqh*^{mut} polyploid brain, and Fen1^{RNAi} corresponds to *sqh*^{mut} Fen1^{RNAi} **(C)** Dot plot showing polyploid NB area (in μm^2) in mitosis or in interphase upon Fen1 depletion **(D)** Dot plot showing γ -H2Av index (A.U) measured in polyploid NBs carrying Fen1^{RNAi} in interphase and mitosis. (For **(C)** and **(D)** n= 170 cells for Ctrl mitosis, n=14 cells for Fen1^{RNAi} mitosis, n= 72 cells for Ctrl interphase, n= 30 cells for Fen1^{RNAi} interphase). For statistical analysis, Kruskal-Wallis was performed on the rank of each condition. Non-significant p-values are not depicted on the graphs.

Screening for nucleases generating DNA damage at mitotic entry in polyploid cells

In our model system, the asynchrony at mitotic entry would make the delayed-nucleus vulnerable to the activity of a nuclease (Fig 3C). To first assess if any of the 12 candidates is responsible for DNA damage occurring at mitotic entry, we measured the γ -H2Av index in each mitotic multinucleated polyploid NBs. For this, we depleted each nuclease as described previously and performed co-immunostaining of phospho-histone H3, an early mitotic marker (Beroukhim et al., 2011; Prigent & Dimitrov, 2003), and γ -H2Av to assess DNA damage. We then selected each polyploid NBs positive for Ph3 (Ph3+) and measured the overall coverage and intensity of γ H2Av. By measuring the global γ -H2Av index in mitosis, we were not able to detect any decrease in DNA damage in mitotic cells upon nuclease depletion (Fig 3D). Therefore, we decided to have a qualitative rather than quantitative approach: we selected mitotic polyploid NBs in which we could detect asynchrony between the nuclei, and manually assessed if this cell had DNA damage only in the delayed nucleus. In this case, we refer to mitotically asynchronous when a given multinucleated cell contains a mix of Ph3+ and Ph3- nuclei (Figure 3 A-C). In control polyploid NBs, we found that 23% of asynchronous mitotic cells had DNA damage restricted to the delayed nucleus. This percentage remained similar upon the depletion of Mus81, Tosca and EndoG, suggesting that they are not responsible for DNA damage in asynchronous cells (Fig 3F). However, the depletion of Gen, Slx1, WRNexo and Sid led to a decrease in the percentage of polyploid cells carrying DNA damage in the delayed nucleus compared to controls, suggesting that they might have a role in the generation of DNA damage. Most importantly, the depletion of Mre11, Mus312, Mus201 and DNAsell completely suppressed the damage in the mitotically delayed nucleus. However, it is important to

mention that for both Mus201 and Dnasell RNAi, we could detect only 2 or 3 asynchronous polyploid cells among more than 30 polyploid brain lobes analyzed (Fig 3B). In such conditions, only a few multinucleated cells could be detected, as nuclease depletion seemed to affect polyploid cell proliferation. Thus, even if we observed a decrease in DNA damage in mitotically- delayed nuclei, it is possibly explained as an overall decrease in cell proliferation and therefore we could not distinguish between these two effects. For this reason, we kept as candidate nucleases that both decreased DNA damage in delayed nucleus, and for which we had at least 15 multinucleated cells analysed (Fig 3B and 3F). This led to a final list of 7 candidates: Gen, Mre11, Slx1, Mus312, Mei-9, WRNexo, and Sid. Eventually, among these 7 nucleases, I decided to focus first on the ones that have been shown to be active during mitosis, which are Gen, Mre11, Slx1 and Mus312.

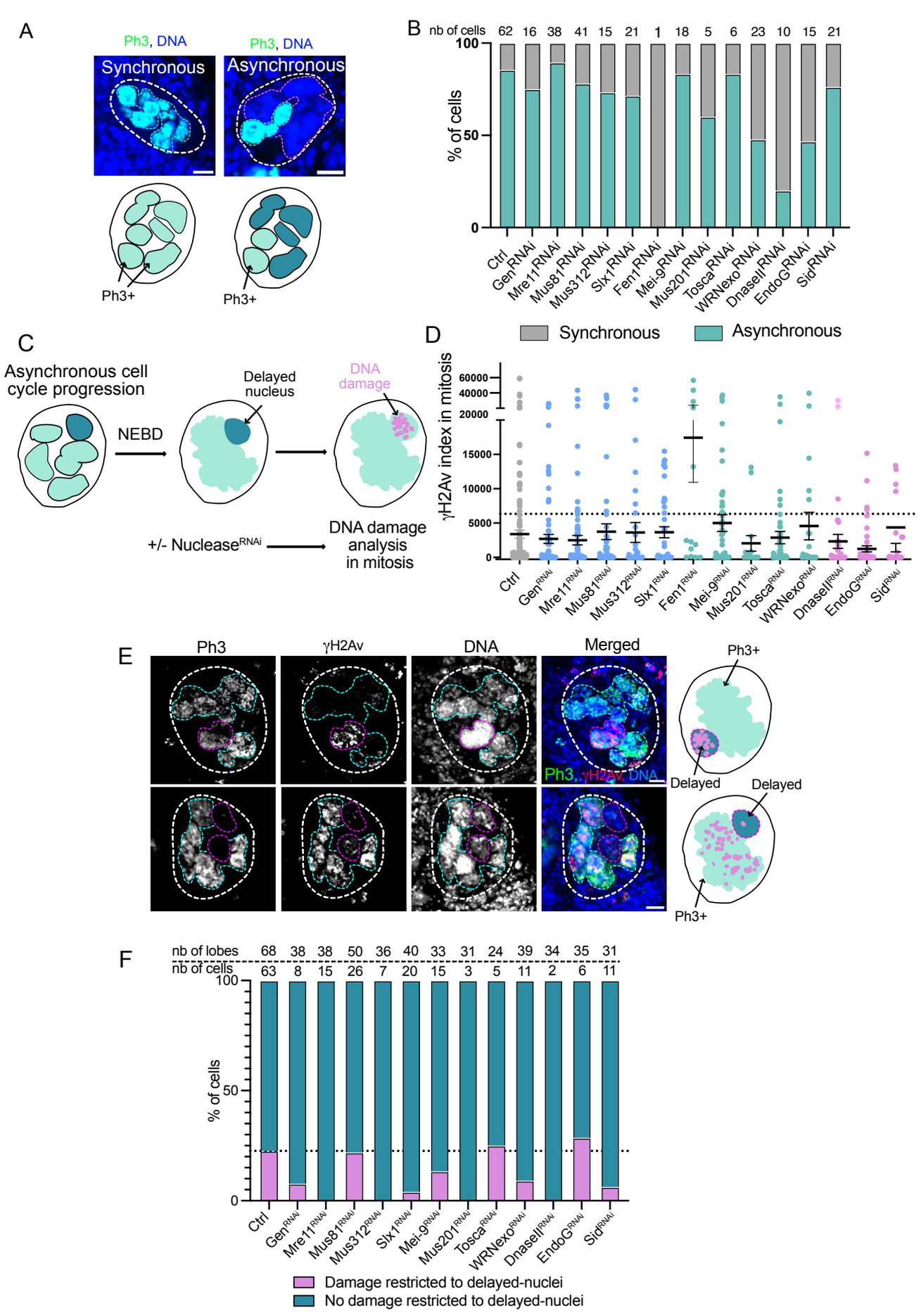


Figure 3. Screening for nucleases generating DNA damage at mitotic entry in asynchronous polyploid cells

(A) Example of a synchronous (on the left) and asynchronous (on the right) polyploid cells in mitosis, labelled with Ph3 antibody to reveal mitotic nuclei and DAPI to reveal nuclear staining (in blue). Ph3+ nuclei are depicted with a blue dashed line. Mitotically delayed nuclei (Ph3-) are depicted with a pink dashed line. **(B)** % of asynchronous polyploid NBs among all mitotic polyploid cells in the indicated genotypes. Total number of cells analysed for each condition are annotated on the graph. **(C)** Hypothesis for DNA damage induction in asynchronous polyploid cell. At mitotic entry, the nucleus that is mitotically delayed gets damaged through the action of a nuclease. In the screen, nuclease^{RNAi} was expressed specifically in NBs, and DNA damage was further quantified in mitotic cells. **(D)** Dot plot showing γ -H2Av index measured in mitotic polyploid NBs upon nuclease depletion. For each condition, except Fen1^{RNAi}, Mus201^{RNAi} and WRNexo^{RNAi} (n=14, n=15 and n=23 cells, respectively), more than 40 cells were analyzed. **(E)** Examples of asynchronous cells labelled using Ph3 antibody to reveal mitotic nuclei (in green), γ -H2Av antibody to reveal DNA damage (in red) and DAPI to reveal nuclear staining (in blue). The cell on the top is a typical example of an asynchronous cell exhibiting DNA damage in the delayed-nucleus, while at the cell at the bottom illustrates DNA damage present in all nuclei, even with low levels in the delayed-nucleus. Mitotically- delayed nuclei are depicted with a pink dashed line, while Ph3+ nuclei are delimited with the blue dashed line. Scalebars = 10 μ m **(F)**. Graph bar showing the percentage of asynchronous mitotic cells displaying DNA damage specifically in the delayed nucleus upon the depletion of each candidate. Number of brain lobes and asynchronous cells per conditions are annotated on the graphs. For statistical analysis, Kruskal-Wallis was performed on the rank of each condition. Non-significant p-values are not depicted on the graphs.

Gen, Mre11, Mus312 and Slx1 as candidates for generating DNA damage in mitosis

Since the RNAi screen revealed a decrease in cells having DNA damage in the mitotically delayed nucleus upon the depletion of Gen, Mre11, Mus312 and Slx1, I wanted to confirm this result using genetic mutants for each nuclease. Therefore, we obtained several mutant lines for each gene. *Gen*^{z-1997} carries a frameshift mutation leading to a non-functional Gen protein and will be further described in the results - section2 (Andersen et al., 2011). I will refer to this mutant as *gen*^{mut}. Several mutant lines have been described for Mre11 and one of them, *mre11* ^{Δ 35K1} is a genetically null, which is lethal before pupal stages, suggesting that Mre11 is essential in *Drosophila*. Therefore, I used an hypomorphic mutant, *mre11*^{58s}, which carries a missense mutation leading to aminoacid replacement H230Y (Gao et al., 2009). This mutant

is viable, although it leads to sterility in both male and female *Drosophila*, and I will refer to it as *mre11^{mut}*. *mus312²³⁹⁹⁷* carries a nonsense mutation at Q689 which creates a premature stop codon (Yildiz et al., 2002). I will refer to this mutant as *mus312^{mut}*. *Slx1^{F93I}* missense mutation causes an aminoacid replacement of F93I (Andersen et al., 2011). In our hands, this mutation appears to be lethal, and we could not get any homozygous larvae for the mutation. Therefore, we generated *Drosophila* lines with combined *sqh^{mut} gen^{mut}*, *sqh^{mut} mre11^{mut}* and *sqh^{mut} mus312^{mut}*.

To confirm the screen data, I first measured polyploid cell area for each of the double mutants, to assess whether cell proliferation was affected. As described both in the screen and in later in the result section 2, mutations in Gen strongly reduced polyploid cell area, suggesting a role for this nuclease in polyploid cell proliferation (Fig 4A and B). However, neither *sqh^{mut} mre11^{mut}* and *sqh^{mut} mus312^{mut}* had reduced polyploid NBs area, suggesting that they are not implicated in polyploid cell proliferation (Fig 4 A-B). Then, I measured DNA damage signals in mitosis in the polyploid double mutants, by immunostaining for Ph3 and γ -H2Av. Surprisingly, we found that γ -H2Av index was strongly reduced in mitotic polyploid NBs in all conditions (Fig 4C), conversely to the results found in the RNAi screen (Fig 3D). Such differences between the RNAi screen data and the genetic mutants can be explained by the fact that genetic mutants would lead to a much stronger decrease of the protein levels than the RNAi. Thus, using genetic mutants might reveal a reduction in γ -H2Av signal that could not be described using RNAis.

I then thought about assessing DNA damage specifically in the mitotically- delayed nucleus, using the qualitative approach described above. I focused specifically on cells exhibiting asynchrony during mitosis (Fig 4D). I found that among 28 polyploid NBs detected in *sqh^{mut} gen^{mut}*, only one NB exhibited cell cycle asynchrony, while *sqh^{mut} mre11^{mut}* and *sqh^{mut} mus312^{mut}* NBs showed 40 % of asynchronous cells among 96 and 100 cells, respectively (Fig 4E). I will describe in the results-2 section that Gen is essential for polyploid cell proliferation, with *sqh^{mut} gen^{mut}* exhibiting very small polyploid cells. I suspected that I could no longer detect the asynchrony because cells were not proliferating efficiently enough. For this reason, I analysed DNA damage specifically in the delayed nucleus in *sqh^{mut} mre11^{mut}* and *sqh^{mut} mus312^{mut}*. Interestingly, both double mutants no longer had DNA damage in the mitotically-delayed nucleus, confirming them as candidates for generating DNA damage in asynchronous polyploid cells (Fig 4F). However, it remains important to mention that, since Mus312 is a

scaffold protein for Mei-9, Mus81, and Slx1 (Yildiz et al., 2002, Kondo and Perrimon, 2011), the consequences of *mus312^{mut}* might reflect the activity of one or several of these three nucleases. Nonetheless, the RNAi screen revealed either no or very little effect of the depletion of Mus81 and Mei-9 on the proportion of asynchronous cells having DNA damage in their delayed-nuclei (Fig 3F). It is therefore likely that *mus312^{mut}* phenotype reflects the activity of its other binding protein - Slx1. Alternatively, another hypothesis would be that the three partners have the capacity to induce DNA damage in the delayed nucleus and depleting only one is compensated by the two others.

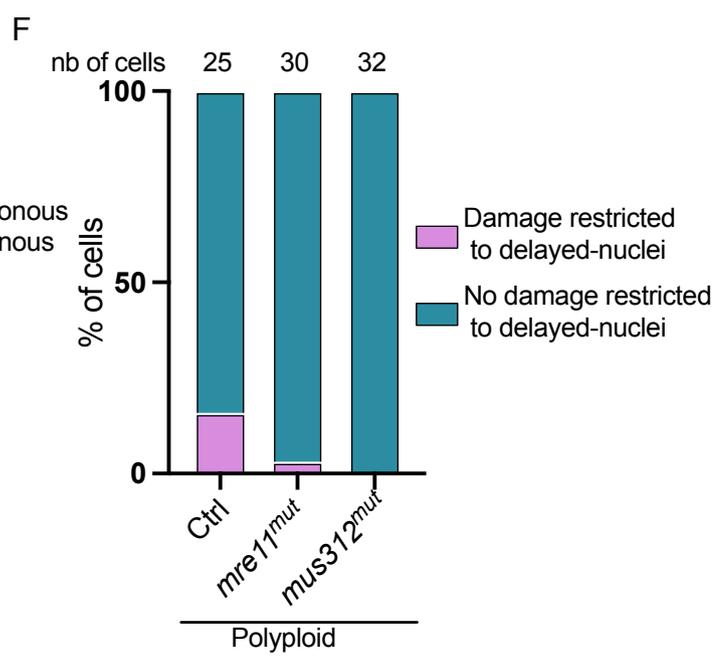
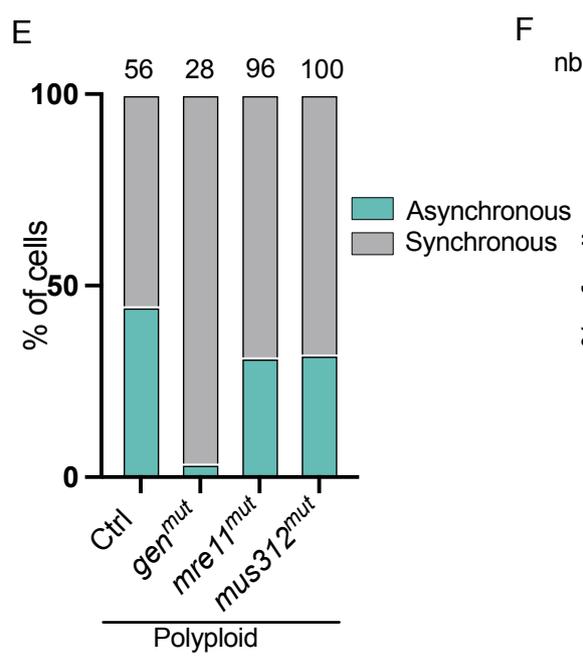
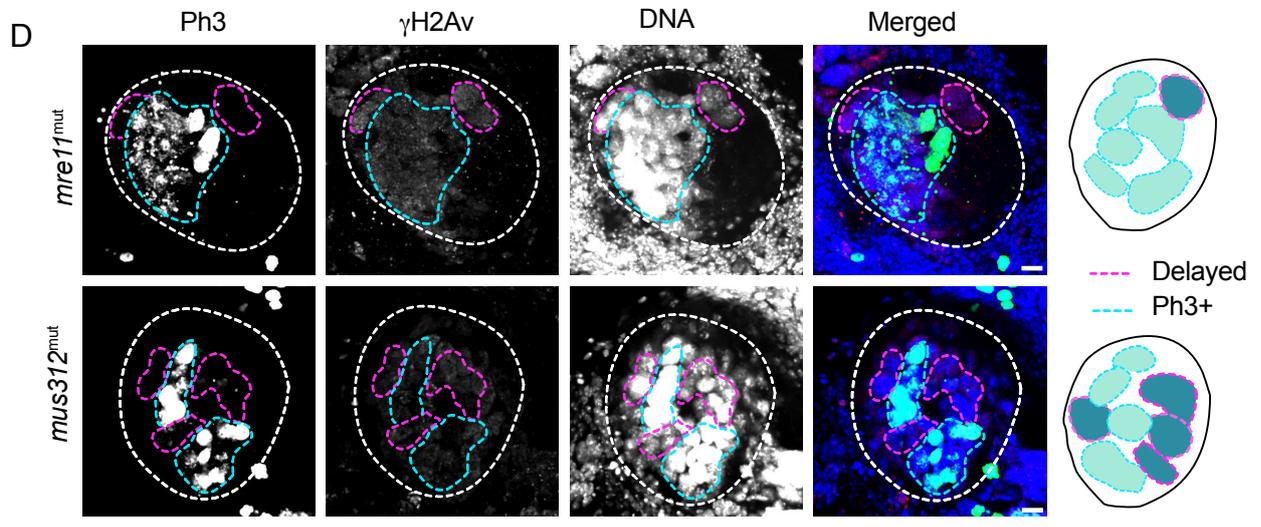
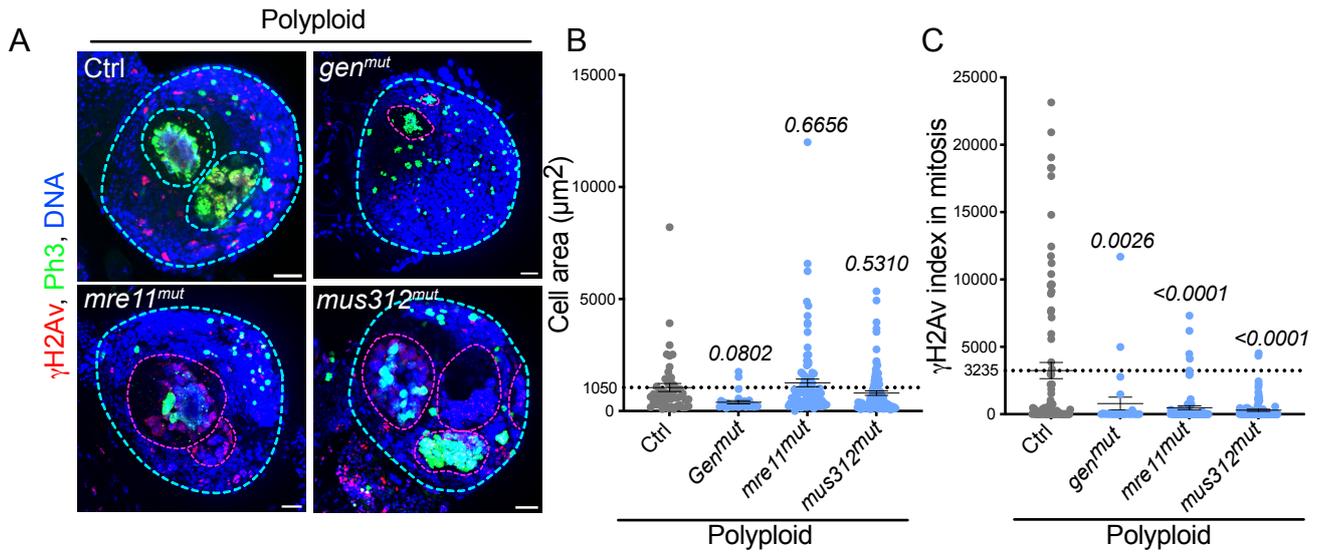


Figure 4. Genetic mutations of Mre11 and Mus312 decrease DNA damage in the mitotically-delayed nucleus

(A) Images of *Drosophila* polyploid brain lobes labelled with γ -H2Av antibody (in red), Ph3 antibody (in green) and DAPI to reveal nuclear staining (in blue), in the combined mutations of *sqh^{mut} gen^{mut}*, *sqh^{mut} mre11^{mut}* or *sqh^{mut} mus312^{mut}* **(B)** Dot plot showing polyploid NB area (in μm^2) in the indicated genotypes. Cell area was measured using Phalloidin to label the cell membrane (not shown) (n= 56 cells for Ctrl, n= 29 cells for *gen^{mut}* n= 100 cells for *mre11^{mut}*, n=101 cells for *mus312^{mut}*). **(C)** Dot plot graph showing the γ -H2Av index (A.U) in mitosis in the indicated genotypes (n= 95 for Ctrl, n=28 cells for *gen^{mut}*, n=80 for *mre11^{mut}*, n=101 cells for *mus312^{mut}*). **(D)** Pictures of asynchronous polyploid NBs at mitotic entry for *sqh^{mut} mre11^{mut}* (top panel) or *sqh^{mut} mus312^{mut}* (bottom panel), labelled using Ph3 antibody to reveal mitotic nuclei (in green), γ -H2Av to reveal DNA damage (in red) and DAPI to reveal stained nuclei (in blue). Mitotically delayed nuclei (Ph3-) are depicted with a pink dashed line, while Ph3+ nuclei are delimited with the blue dashed line. **(E)** Percentage of synchronous and asynchronous mitotic cells for each condition. Number of mitotic cells analysed are depicted on the graph. **(F)** Bar plot showing the percentage of asynchronous mitotic cells exhibiting or not DNA damage specifically in the delayed nucleus upon the depletion of each candidate. Number asynchronous cells per conditions are annotated on the graphs. For statistical analysis of panel (B) and (C), Kruskal-Wallis test was performed on the rank of each condition. Non-significant p-values are not depicted on the graphs.

Gen and Mus312 are not essential in *Drosophila* NBs

Since these mutants have essentially been described under DNA damaging agent conditions before (Andersen et al., 2011; Kenny Kuo et al., 2014; Kondo & Perrimon, 2008; Trowbridge et al., 2007), I wanted to characterize their role in diploid NBs without any other perturbation. As mentioned earlier, *mre11^{Δ35K1}* and *Slx1^{F93I}* mutants are lethal in *Drosophila*. However, *gen^{mut}* and *mus312^{mut}* are viable and did not induce sterility, suggesting that these are not essential genes for *Drosophila*.

To investigate whether these mutations could affect diploid NB proliferation, I first measured the mitotic index in NBs (defined by the percentage of Ph3+ NBs among the overall number of NBs for each lobe) (Fig 5 A-B). I did not detect a significant difference in the mitotic index in *gen^{mut}* and *mre11^{mut}*, while *mus312^{mut}* had a slight increased mitotic index (Fig 5B). This suggests that mutations in *mus312* might affect the cell cycle and the completion of mitosis even in diploid cells. Therefore, I assessed errors in anaphase for each mutant and

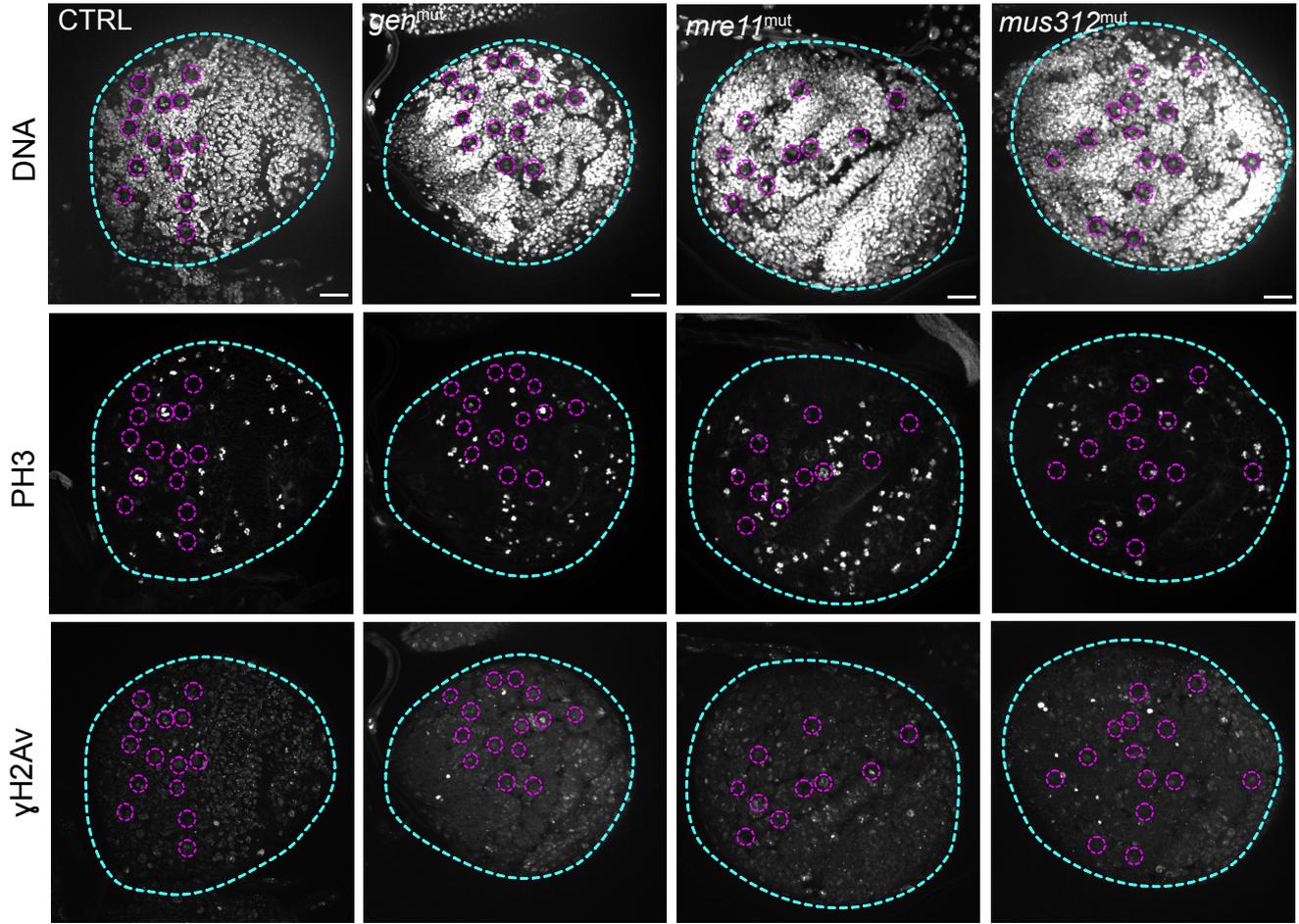
measured the percentage of anaphase bridges and/or lagging chromosomes. As expected by the increased mitotic index, *mus312^{mut}* diploid NBs exhibited increased mitotic errors, reaching a total of 10% of errors in anaphase (Fig 5C). Both *mre11^{mut}* and *gen^{mut}* also has increased mitotic errors in anaphase compared to Ctrl diploid NBs even if the levels were lower than in *mus312^{mut}*.

In addition, I quantified the levels of DNA damage in NBs by measuring the γ -H2Av index in diploid NBs for *gen^{mut}*, *mre11^{mut}*, and *mus312^{mut}*. I observed an enrichment in NBs with a higher γ -H2Av index (Fig 5D), but this was not significant. This suggests that mutations in any of these nucleases does not result in high increase in the levels of DNA damage in diploid NBs, which can be explain either through redundancy or the hypomorphic nature of the alleles.

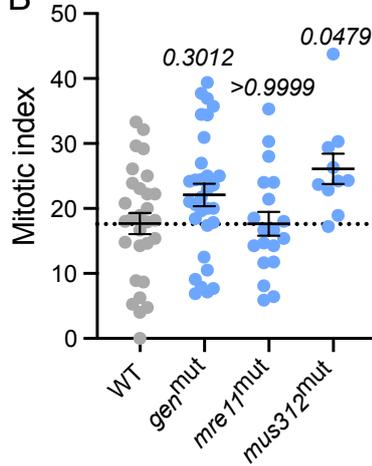
Altogether, analysis of *gen^{mut}*, *mre11^{mut}*, and *mus312^{mut}* revealed that, while Mre11 appears to be essential in *Drosophila*, this is not the case of Gen and Mus312. Moreover, even if *mus312^{mut}* lead to increased mitotic index in diploid NBs, with increased in anaphase errors, it does not seem to correlate with higher levels of DNA damage in these cells. Overall, these mutations are tolerated and do not generate major genetic instability in NBs.

Diploid

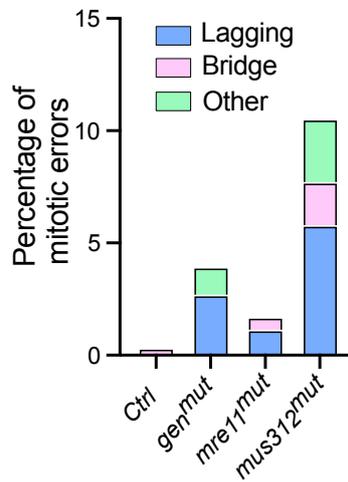
A



B



C



D

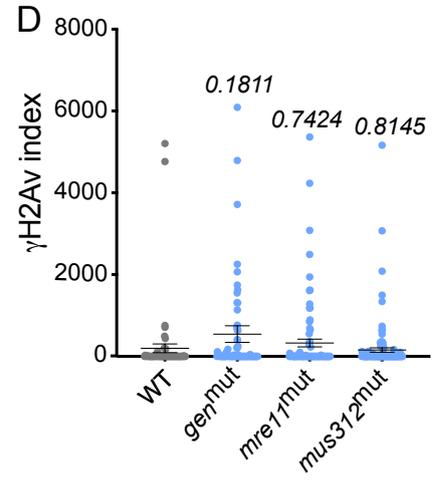


Figure 5. Characterization of *gen^{mut}*, *mre11^{mut}* and *mus312^{mut}* in diploid NBs

(A) Images of *Drosophila* diploid brain lobes labelled with DAPI to reveal nuclear staining (in grey) in *gen^{mut}*, *mre11^{mut}* or *mus312^{mut}* labeled with DAPI to reveal nuclear staining (in grey, on top), Ph3 antibody to reveal mitotic nuclei (in grey, in the middle), and γ -H2Av to reveal DNA damage (in grey, on the bottom). NBs are depicted by the pink dashed lines. Scalebars = 20 μ m (B) Dot plot showing the mitotic index measured as the percentage of NBs Ph3+ among

the total number of NBs per lobe (n= 29 lobes for controls, n=32 lobes for *gen*^{mut}, n=19 lobes for *mre11*^{mut}, n= 10 lobes for *mus312*^{mut}). **(C)** Bar plot showing the percentage of mitotic errors quantified in *gen*^{mut}, *mre11*^{mut} and *mus312*^{mut}. **(D)** Dot plot showing the γ -H2Av index (A.U.) measured in diploid NBs in *gen*^{mut}, *mre11*^{mut} or *mus312*^{mut} (n=63 cells for Ctrl, n=85 cells for *gen*^{mut}, n=89 cells for *mre11*^{mut}, n=133 cells for *mus312*^{mut}). For statistical analysis, Kruskal-Wallis was performed on the rank of each condition. p-values depicted on the graphs correspond to the analysis for the mutant condition compared to the WT.

Gen, Mre11, Mus312 and Slx1 overexpression induces DNA damage in diploid NBs

After confirming Mre11 and Mus312 as candidates for generating damage in mitotically delayed nucleus, I generated constructs expressing a fluorescent tag, so that I could assess their localisation in asynchronous polyploid cells. In addition to Mre11 and Mus312, I also chose to generate Gen and Slx1 tagged constructs for several reasons. First, Gen is the only nuclease which localises mostly in the cytoplasm and has been described to get access to DNA during mitosis in both yeast and mammalian cells (Blanco et al., 2014; Chan & West, 2014). Therefore, we hypothesized that a premature mitotic entry could allow Gen to get access to the delayed-nucleus prematurely and generates DNA damage. Secondly, since Mus312 is a scaffold for Slx1, I questioned if the decrease in DNA damage observed in *mus312*^{mut} would reflect the inability of Slx1 to bind to DNA.

To generate tagged constructs, I benefit from the help of Anthony Simon, the lab manager of the lab. The constructs were designed so that each gene was under the control of the UAS promoter, which would allow a strong overexpression in a tissue-specific manner and a mCherry tag. Since these nucleases had the catalytic domain at the N-terminus, I chose to add the mCherry tag at the C-terminus (Fig 6A). To express each construct specifically in NBs, I used a Worniu-Gal4 driver as described earlier.

I analysed the localisation and behaviour of each protein in wild type (WT) NBs to verify that they were not inducing toxicity, proliferation defects or even DNA damage (Fig 6B). As further described in the results 2 section, UAS-Gen-mCh localises mostly to the cytoplasm. UAS-Mre11-mCh localised both in the nucleus and cytoplasm. UAS-Mus312-mCh appeared to localise preferentially in the nucleus, however, its signal was very weak compared to the other nucleases, suggesting that either it is very weakly expressed or highly regulated/degraded.

Finally, Slx1 localized both in the nucleus and the cytoplasm, although the signal was more enriched in the nucleus. I noticed that some cells showed a weak signal of Slx1 in the nucleus, while others showed very strong signals (Fig 6B, right). When measuring the mitotic index, I found that all nuclease over-expressing conditions displayed an increased mitotic index (Fig 6 C-D). I quantified the γ -H2Av index in mitosis and found that increased DNA damage signals in mitosis, when compared to controls (Fig 6E).

Overall, this data show that overexpressing Gen, Mre11, Mus312 and Slx1 nucleases in diploid WT NBs leads to mitotic errors and increased DNA damage levels, suggesting that their levels must be tightly regulated to avoid this type of errors. Interestingly however, the over-expression in the NB compartment did not affect fly viability.

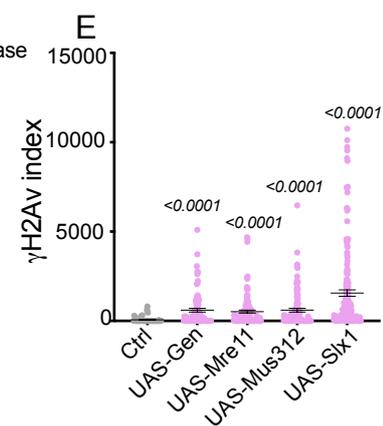
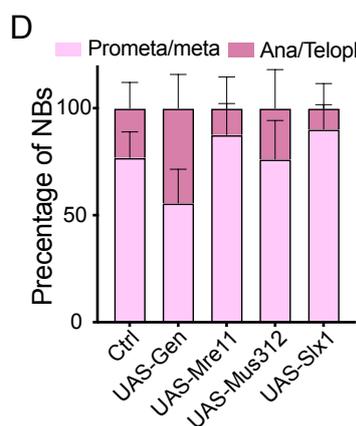
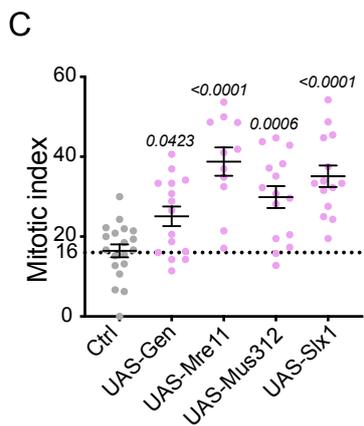
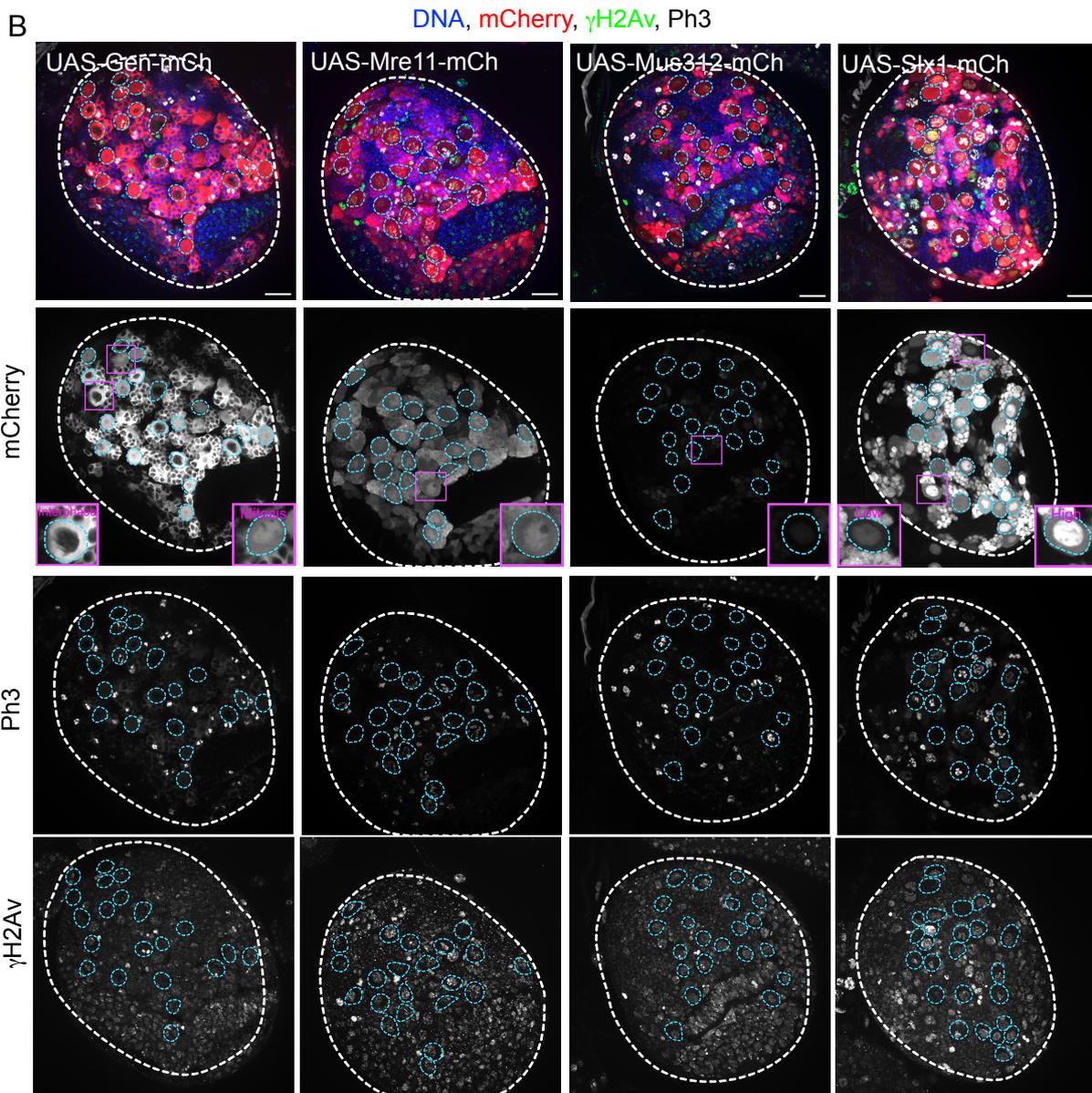
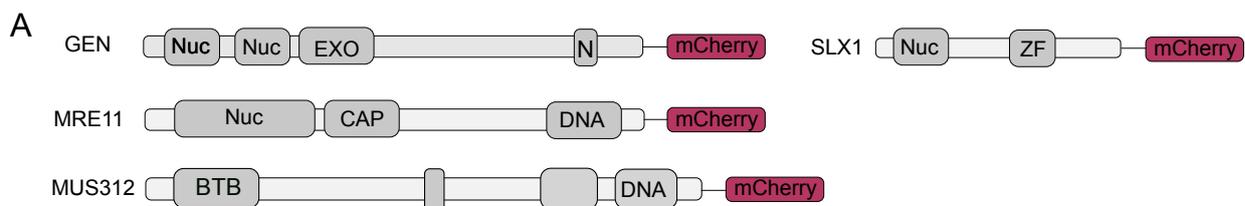


Figure 6. Gen, Mre11, Mus312 and Slx1 overexpression induces DNA damage in diploid NBs.

(A) Schemes of the constructs for tagging Gen, Mre11, Slx1 and Mus312 with mCherry. Gen protein contains 2 catalytic domains and DNA binding domain (N). Mre11 contains one nuclease domain, one telomere capping domain and one DNA binding domain. Mus312 contains a Broad Complex Tramtrack (BTB) domain (Stogios et al., 2005), binding domains for its partners, and a DNA binding domain. Slx1 contains a nuclease domain in C-ter, and a Zinc-finger domain, which is believed to be involved in protein-protein interaction. **(B)** Images of *Drosophila* diploid brain lobes upon the overexpression of UAS-Gen-mCh, UAS-Mre11-mCh, UAS-Mus312-mCh and UAS-Slx1-mCh, labelled using mCherry antibody to reveal each construct (in red) Ph3 antibody to reveal mitotic nuclei (in grey), γ -H2Av antibody to reveal DNA damage (in green), and DAPI to reveal DNA (in blue). Individual channels are shown in grey, below the merged images. **(C)** Dot plot showing the mitotic index measured as the percentage of NBs Ph3+ among the total number of NBs per lobe, for the indicated genotypes (n= 20 lobes for controls, n=15 lobes for UAS-Gen-mCh, n=11 lobes for UAS-Mre11-mCh, n= 15 lobes for UAS-Mus312-mCh, n=14 lobes for UAS-Slx1-mCh). **(D)** Bar plots showing the percentage of mitotic phases upon the overexpression of the indicated constructs. **(E)** Dot plot showing the γ -H2Av index measured in diploid NBs upon the overexpression of each candidate (n=63 cells for Ctrl, n=85 cells for UAS-Gen-mCh, n=109 cells for UAS-Mre11-mCh, n=98 cells for UAS-Mus312-mCh, n=165 cells for UAS-Slx1-mCh). For statistical analysis, Kruskal-Wallis was performed on the rank of each condition. p-values depicted on the graphs correspond to the analysis for the mutant condition compared to the WT.

Nuclease overexpression in polyploid NBs increases DNA damage in mitosis

After describing the localisation of each candidate in diploid NBs, I characterized their behaviour in polyploid NBs. For this, I crossed each UAS-nuclease line with *sqh^{mut}* Worniu-G4, to overexpress the constructs in polyploid NBs in a specific manner (Fig 7A) and performed immunostaining for Ph3 and γ -H2Av. I measured polyploid NB area and, as described in the results 2 section, UAS-Gen led to increased polyploid NB area, suggesting that increased Gen levels enhances polyploid cell proliferation (Fig 7A-B). UAS-Mre11 did not have any significant effect on polyploid cell area, while overexpression of both Mus312 and Slx1 resulted in decreased polyploid cell area (Fig 7A-B). This suggests that increasing the levels of these two nucleases impairs polyploid cell proliferation.

To decipher whether one of these candidates was able to induce DNA damage in mitotic polyploid cells, I quantified the γ H2Av index in polyploid NBs in mitosis (Fig 7A and 7 C). In both UAS-Gen and UAS-Mre11, there was a significant increase in γ -H2Av index in

mitotic polyploid cells. This was not the case for UAS-Mus312 and UAS-Slx1, for which I could only analyse 8 and 18 mitotic cells respectively, as overexpression of these two constructs impairs polyploid cell proliferation. For this reason, I decided to focus exclusively on the role of Gen and Mre11 in asynchronous cells. Indeed, while searching for asynchronous cells among mitotic polyploid NBs, I found that around 40% of polyploid controls exhibited asynchrony. This level remained the same upon UAS-Mre11 expression, while UAS-Gen led to more than 50% of asynchronous cells (Fig 7D). In agreement with their effect on polyploid cell proliferation, I was not able to detect any asynchronous cells in UAS-Mus312 and rarely (2 out of 18 cells) in UAS-Slx1 (Fig 7D). Moreover, I quantified DNA damage levels in asynchronous polyploid cells over-expressing Mre11 and Gen and observed an increase in the overall DNA damage in these cells (Fig 7E).

Altogether, these results show that increasing the levels of both Gen and Mre11 nuclease can lead to increased DNA damage levels in mitotic polyploid cells, both in synchronous and asynchronous cells.

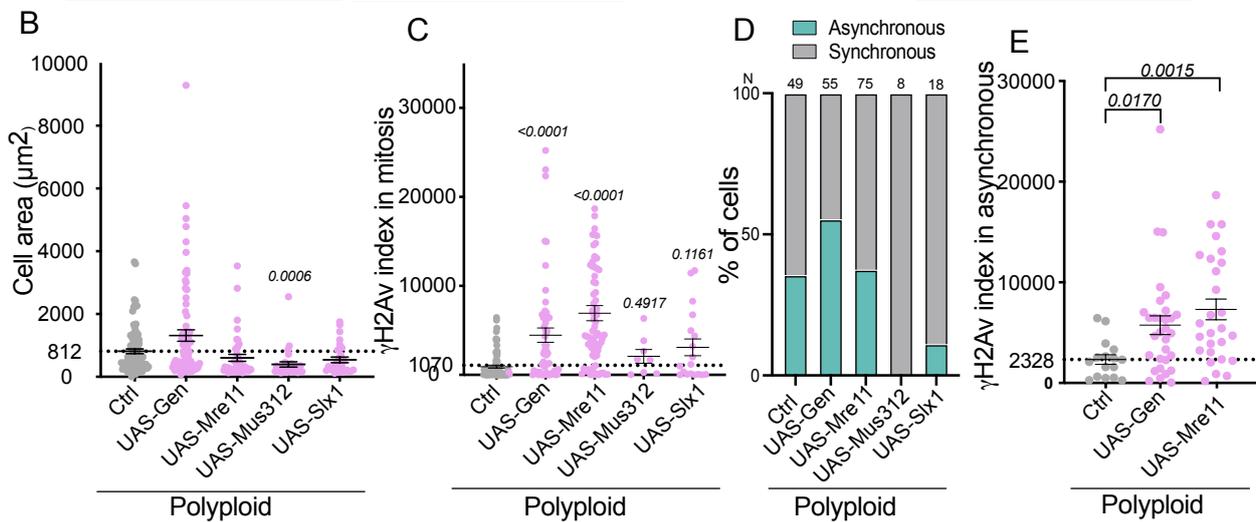
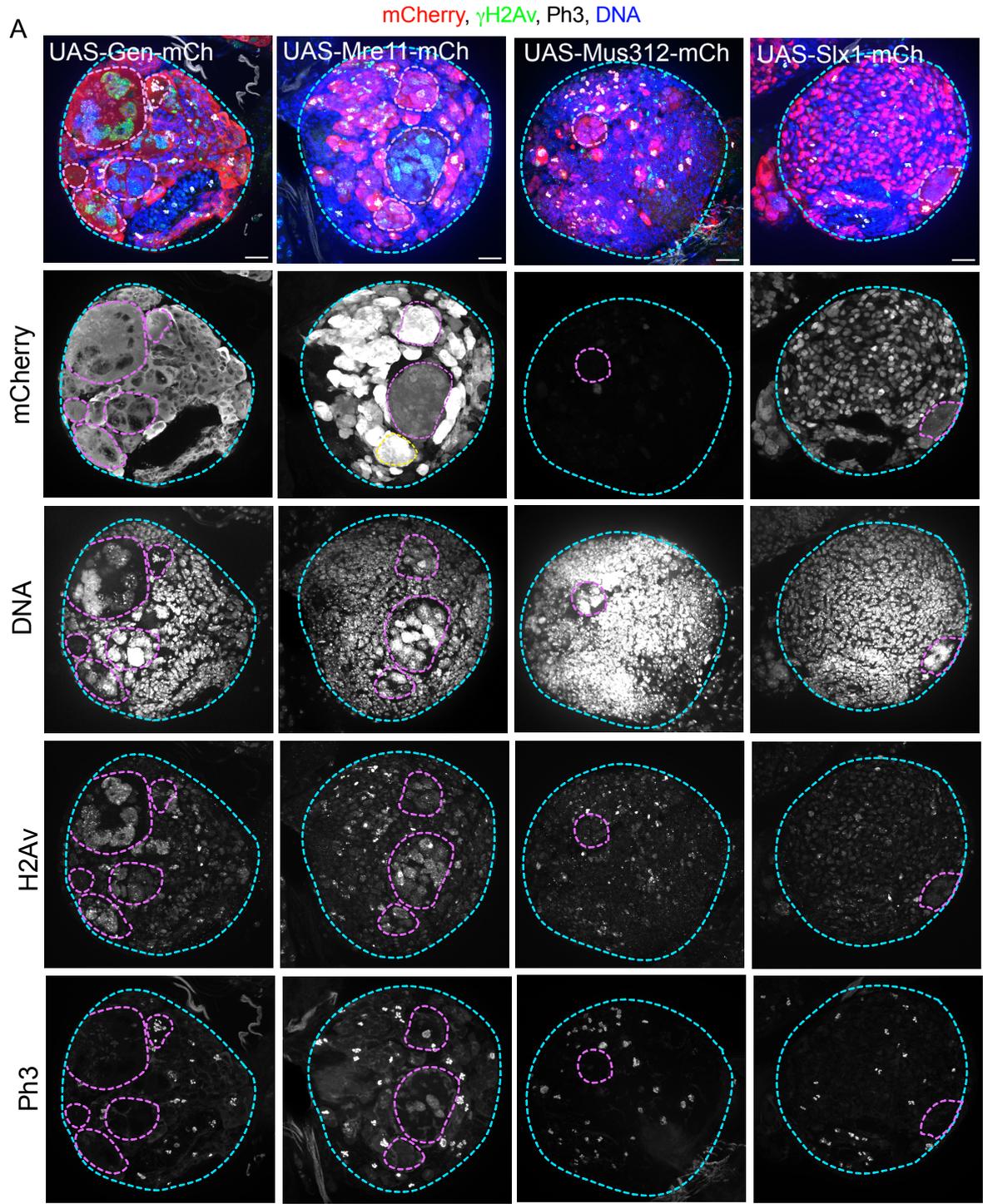


Figure 7: UAS-Gen-mCh and UAS-Mre11-mCh increase DNA damage in mitotic polyploid NBs

A. Images of *Drosophila* polyploid brain lobes labelled using mCherry antibody to reveal each construct (in red) Ph3 antibody to reveal mitotic nuclei (in grey), γ -H2Av antibody to reveal DNA damage (in green), and DAPI to reveal nuclear staining (in blue), in *sqh^{mut}* UAS-Gen-mCh, *sqh^{mut}* UAS-Mre11-mCh, *sqh^{mut}* UAS-Mus312-mCh and *sqh^{mut}* UAS-Slx1-mCh. Individual channels are shown in grey, below the merged images. Blue dashed line delimitates brain lobes, and pink dashed line delimitates polyploid cells. **B.** Dot plot showing polyploid NB area (in μm^2) in the indicated genotypes. Cell area was measured using Phalloidin to label the cell membrane (not shown). (n= 96 cells for Ctrl, n=76 cells for *sqh^{mut}* UAS-Gen-mCh, n=42 cells for *sqh^{mut}* UAS-Mre11-mCh, n=30 for *sqh^{mut}* UAS-Mus312-mCh, n=28 cells for *sqh^{mut}* UAS-Slx1-mCh). **(C)** Dot plot showing the γ -H2Av index (A.U) measured in mitotic polyploid NBs upon the overexpression of each candidate. (n=49 cells for Ctrl, n=55 cells for *sqh^{mut}* UAS-Gen-mCh, n=75 cells for *sqh^{mut}* UAS-Mre11-mCh, n= 8 cells for *sqh^{mut}* UAS-Mus312-mCh, n= 18 cells for *sqh^{mut}* UAS-Slx1-mCh). **(D)** Graph bar showing the percentage of synchronous and asynchronous polyploid NBs, in the indicated conditions. Numbers of cells analysed per conditions are indicated on the graphs. **(E)** Dot plot showing the γ H2Av index (A.U) measured in asynchronous polyploid cells in *sqh^{mut}*, *sqh^{mut}* UAS-Gen-mCh and *sqh^{mut}* UAS-Mre11-mCh. (n=16 cells for *sqh^{mut}* Ctrl, n= 31 cells for *sqh^{mut}* UAS-Gen-mCh, N=27 cells for *sqh^{mut}* UAS-Mre11-mCh). For statistical analysis, Kruskal-Wallis was performed on the rank of each condition. p-values depicted on the graphs correspond to the analysis for the mutant condition compared to the Ctrl (*sqh^{mut}*).

Gen and Mre11 as candidates for DNA damage generation in polyploid NBs

To go further and try to better understand the potential role of Gen and Mre11 in asynchronous polyploid cells, I characterized the localisation of DNA damage and the nuclease-tag signal in asynchronous polyploid cells. I hypothesized that if the nuclease is responsible for DNA damage in the mitotically- delayed nuclei, I would observe a colocalization of the nuclease with γ -H2Av foci. Unfortunately, since UAS-Gen signal diffused in the cytoplasm and UAS-Mre11 diffused throughout the cell, I could not detect such colocalized signal at sites of DNA damage (Fig 8 A-B).

While analysing the signals in UAS-Gen, I found that after NEBD, the delayed nucleus seemed to contain the nuclear envelope, as most of the UAS-Gen-mCh signal was excluded from it. However, I could detect several regions of strong UAS-Gen aggregates in the delayed-nucleus (Fig 8A). Interestingly, the delayed nucleus also displayed strong γ -H2Av signals in this

case. This suggests that upon mitotic entry in asynchronous polyploid cells, Gen gets access to the delayed nucleus and may generate DNA damage (Fig 8C).

Interestingly, in polyploid cells over-expressing Mre11, I found that the majority of asynchronous cells no longer displayed DNA damage specifically in a nuclear region, but instead the γ -H2Av signal covered the entire nuclear surface independently of the Ph3 status (Fig 8B). This confirms that Mre11 over-expression in polyploid cells can generate more DNA damage during mitosis. I hypothesize that in polyploid conditions, Mre11 normally gets activated at mitotic entry and its activity is restricted to the delayed nucleus (Fig 8D). However, when higher Mre11 are present, ectopic DNA damage is generated in both the delayed and non-delayed nucleus.

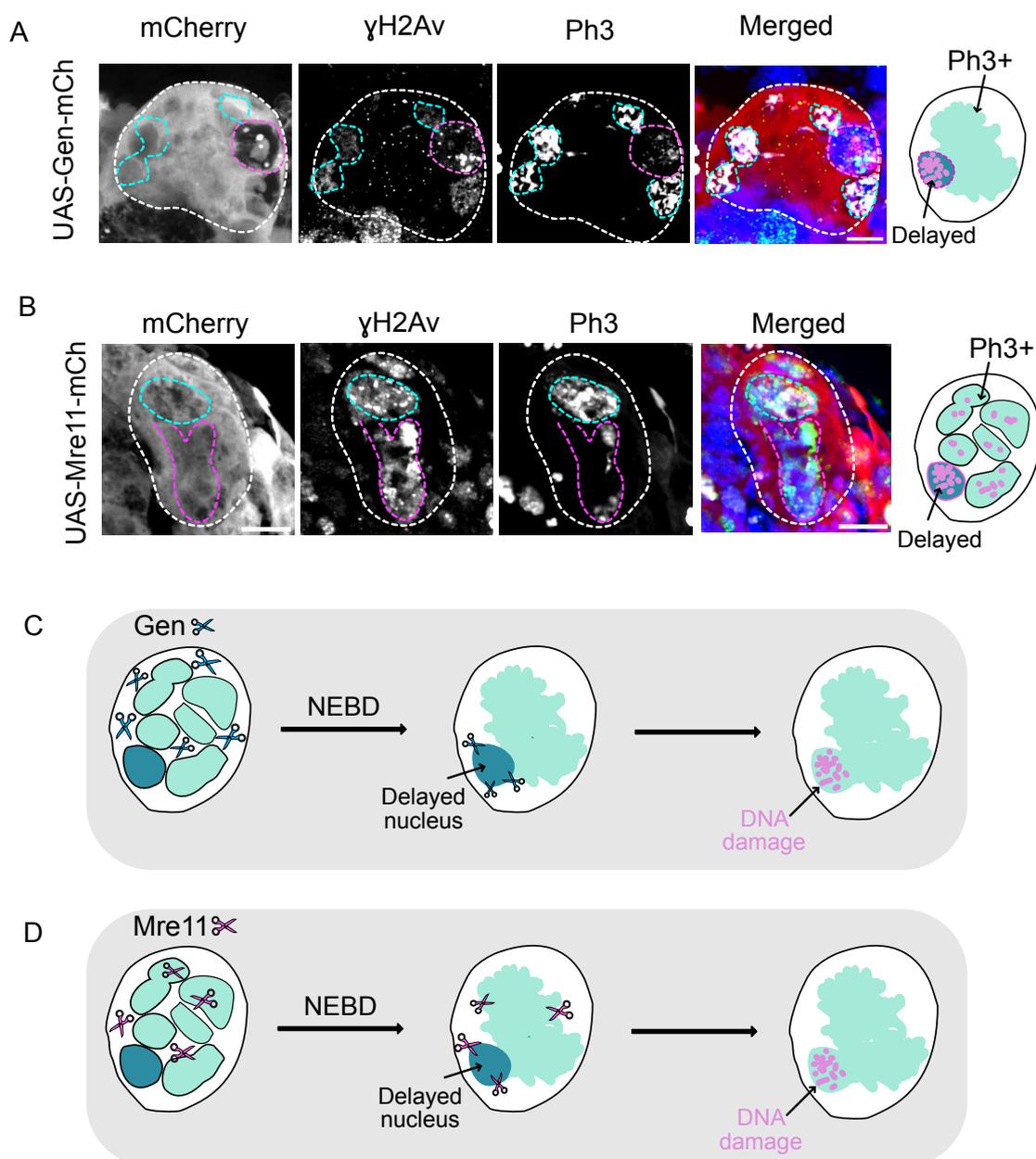


Figure 8. Mre11 and Gen localization in asynchronous cells polyploid cells

(A) Picture of an asynchronous polyploid NB carrying the UAS-Gen-mCh construct, labelled using mCherry antibody to reveal UAS-Gen-mCh (in red), Ph3 antibody to reveal mitotic nuclei (in grey), γ -H2Av antibody to reveal DNA damage (in green), and DAPI to reveal nuclear staining (in blue). Individual channels are shown in grey, before the merged images. Blue dashed line delimitates Ph3+ nuclei, and pink dashed line the mitotically- delayed nucleus.

(B) Picture of an asynchronous polyploid NB carrying the UAS-Mre11-mCh construct, labelled using mCherry antibody to reveal UAS-Mre11-mCh (in red), Ph3 antibody to reveal mitotic nuclei (in grey), γ -H2Av antibody to reveal DNA damage (in green) and DAPI to label chromosomes (in blue). Individual channels are shown in grey, before the merged images. Blue dashed line delimitates Ph3+ nuclei, and pink dashed line the mitotically- delayed nucleus

(C) Model for DNA damage generation by Gen nuclease in asynchronous polyploid cell. Gen localizes in the cytoplasm of the cell. Upon mitotic entry it gets access to the nucleus which is not ready to enter mitosis (in dark green), generating DNA damage (in pink).

(D) Model for DNA damage generation by Mre11 nuclease in asynchronous polyploid cell. Mre11 localises in the whole cell. Upon mitotic entry, Mre11 gets activated, during which it will recognize the delayed nucleus (in dark green) and generates DNA damage.

Results- Section 2

GEN1 nuclease is essential for the proliferation of unscheduled polyploid cells

GEN1 nuclease is essential for the proliferation of unscheduled polyploid cells

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Abstract

Whole genome duplication (WGD) is a feature of many human tumors, which are characterized by high levels of genetic instability. We have previously shown that WGDs lead to high levels of DNA damage in a single S-phase and so we have investigated the requirement of homologous recombination (HR) factors for the proliferation of cells after unscheduled duplication of their genome. We found that the HR factor Rad51 is absolutely essential, and surprisingly so is the Holliday Junction resolvase Gen1, while other proteins involved in HJ resolution are dispensable. Using both *Drosophila* neural stem cells and RPE-1 cells, live imaging approaches, several quantitative methods and multicolored FISH karyotyping, we show that depleting GEN1 causes replication stress and increased levels of mitotic errors including multipolarity, ultrafine DNA bridges and generation of micronuclei. Further, tetraploid cells without GEN1 can undergo mitotic bypass culminating with a permanent cell cycle exit, accompanied by high p53 levels. Importantly, human tumors with WGD events show increased Gen1 levels when compared with non-WGDs tumors. These findings reveal a requirement for Gen1 in the survival of cancers after WGD with obvious therapeutic opportunities.

Introduction

Polyploidy – the presence of multiple sets of all chromosomes– is commonly found in many physiological processes, as it promotes cell size increase increased metabolic capacity, barrier function or regeneration. Examples of physiological polyploid cells are megakaryocytes, which produce large amounts of proteins and mRNAs that will form the basis of anucleated platelet production, or *Drosophila* glia, which composed the blood-brain barrier protecting the central nervous system (Bailey et al., 2021; Orr-Weaver, 2015; Øvrebø & Edgar, 2018; Unhavaithaya & Orr-Weaver, 2012). In contrast, whole genome duplications (WGDs) are commonly found in human tumors (Davoli & De Lange, 2011; Zack et al., 2013). It has been suggested that WGDs generate genetic instability, promoting the rapid evolution of cancer genomes (Storchova & Pellman, 2004). We recently showed that newly born human tetraploid cells accumulate DNA damage in the first S-phase in a DNA replication dependent manner due to sub-scaling of DNA replication factors (Gemble et al., 2022). This leads to the generation of cells containing abnormal karyotypes even before entering mitosis. Importantly, an increased number of Rad51 -an essential protein in homologous recombination (HR)- foci was detected in these cells, suggesting that cell cycle progression relies on HR induced DNA repair. In addition, genome wide analysis looking for mutations affecting genomic stability in polyploid yeast revealed that several HR factors are “ploidy-specific lethal” (Storchová et al., 2006), advocating for an increased requirement of HR in tetraploids.

HR is an error-free DNA repair mechanism that uses the homologous chromosome as a template to repair double strand breaks. HR is initiated by DNA-end resection through the action of the MRE11-RAD50-NBS (MRN) complex. This leads to a 3' ssDNA tail that will be coated by replication protein A (RPA), a single strand binding protein (Sung & Klein, 2006; X. Wang & Haber, 2004; Wolner et al., 2003). Then, Rad51 recombinase is recruited and mediates the strand exchange with the help of BRCA2 and BRCA1 proteins (Scully et al., 2019). During HR, four-stranded DNA intermediates called Holliday Junctions (HJs) are formed and these need to be processed, to ensure proper chromosome segregation during mitosis (Holliday, 1964; Wyatt & West, 2014). Three distinct processes have been described to resolve HJs. The first one involves the dissolution of the junctions by the Bloom helicase (BLM)-topoisomerase III α - RecQ-mediated genome instability protein 1 and 2 (RMI1 -RMI2) (BTR) complex. The second and third mechanism involves the activity of two different complexes comprising

either several nucleases SLX1–SLX4–MUS81–EME1 (SLX–MUS complex) (Wyatt et al., 2013) or just one - GEN1 (Punatar et al., 2017; Rass et al., 2010). The processing pathway choice appears to be cell cycle dependent with BTR preferentially used early during S-phase, while SLX-MUS complex is active mainly in G2 and GEN1 in late G2 and mitosis (Matos & West, 2014). Indeed, GEN1 has a nuclear export sequence and localizes preferentially to the cytoplasm, which suggested that it mostly cleaves replication intermediates after nuclear envelope breakdown (Chan & West, 2014). In addition, it has been recently described that GEN1 is required to cleave under-replicated regions at specific loci called common fragile sites (Benitez et al., 2023).

Here we have investigated the requirement of HR in the proliferation of non-programmed polyploid cells *in vivo* and *in vitro*. Using the *in vivo* non programmed *Drosophila* polyploid neural stem cells, also known as neuroblasts (NBs), we screened for cell proliferation defects. While mutations in RAD51 impaired polyploid cell proliferation, we found that BLM, Mus81 and Slx1 were dispensable for this process. Strikingly, Gen1 was absolutely required, as mutations or overexpression in *Drosophila* impacted polyploid NB proliferation. Further, by investigating the role of Gen1 in human tetraploid RPE-1 cells, we found that Gen1 depletion causes increased replication stress, multipolar mitoses, p53 activation and mitotic bypass followed by a permanent cell cycle arrest. Furthermore, bioinformatic analysis of tumors enriched in WGDs revealed that tetraploid tumors had increased Gen1 expression compared to non-WGD tumors. This work described a novel role for Gen1 as an essential factor for polyploid cell proliferation, in which Gen1 seems to be essential for genetic stability in polyploid cells.

Polyploid neuroblasts rely on homologous recombination to proliferate

To investigate the role of HR in polyploid cell proliferation, we used *Drosophila* NBs as a model system. Polyploidy can be obtained in NBs using the hypomorphic mutation of spaghetti squash (*sqh*¹). *sqh* encodes the myosin regulatory light chain II, which is essential during cytokinesis (Karess et al., 1991). Therefore, in *sqh*¹ brain lobes, NBs undergo multiple rounds of cytokinesis failure, which generates cells of different sizes and ploidies (Fig S1A and 1A). These cells also accumulate high levels of DNA damage (Gemble et al., 2022; Nano et al., 2019 and Fig S1B), while proliferating. In this study, we refer to cell proliferation as an increase in

cell area and DNA content, rather than the classical process describing increase in cell number through cell division. Precisely, we assessed cell area, as nuclear area and cell area are highly correlated (Fig S1B).

To identify HR factors essential for polyploid NB proliferation, we performed an *in vivo* screen in *Drosophila* polyploid NBs. For this, we used inducible RNA interference (RNAi) against members of the HR pathway: Rad51 - spnA in flies -, BLM, Mus81, Gen and Slx1, which are recruited downstream in the pathway, to dissolve and/or resolve HJ (Fig 1C-D). To deplete each factor only in polyploid NBs, we took advantage of the UAS-GAL4 system, which allows tissue specific expression of any gene under the control of the UAS promoter (Brand & Perrimon, 1993). Using a Gal4 driver under the control of the promoter of *Worniu* (Worniu-Gal4), a transcription factor continuously expressed in neuroblasts, each UAS-RNAi was expressed exclusively in NBs throughout development.

As expected, Rad51 RNAi strongly decreased polyploid cell area, suggesting an essential role for HR in promoting the proliferation of polyploid NBs. Interestingly, depletion of BLM, Slx1, or Mus81, did not cause obvious proliferation defects as the cell area remain comparable to control polyploid cells (Fig 1C and S1C). However, depletion of Gen resulted in even smaller polyploid NBs, comparable to the effect of Rad51 RNAi on polyploid NBs (Fig 1C and 1D).

To assess if these factors were essential for polyploid cells only, we characterized mutations in Rad51 and Gen in diploid NBs. For Rad51 analysis, we used a trans heterozygous combination between Rad51⁰⁵⁷ and Rad51⁰⁹³ mutants. Rad51⁰⁵⁷ has a missense mutation that behaves as a genetically null allele, while Rad51⁰⁹³ carries an early stop codon at amino acid 70, which eliminates detectable Rad51 protein (McVey et al., 2004; Staeva-Vieira et al., 2003). We refer to this combination as Rad51^{mut}. *gen*^{mut} is hemizygous for *Gen*^{z-5997} (carrying a frameshift mutation at codons 374-5 in the conserved nuclease domain (Andersen et al., 2011), and the Df(3L)Exel6103 deficiency, which deletes 19 genes in 64C4-64C8, including Gen. Several mutations in *Drosophila Rad51* have been described and are known to cause defects in the germline -therefore sterility in adults-, but they do not lead to lethality in somatic tissues (Khan et al., 2021). As expected, Rad51^{mut} did not exhibit noticeable developmental defects, as individuals reached adult stage even if they were non fertile. Brains contained the same NB number than wild type (WT) brains and obvious proliferation defects were not detected (Fig S1D). *gen*^{mut} were viable and fertile, with no developmental defect

either (Fig S1D). These data first suggest that decreased levels of HR factors through hypomorphic mutations are not sufficient to affect the proliferation of diploid NBs, while they strongly affect polyploid cell proliferation. However, among HJ resolution factors, Gen was the only one essential in polyploid NBs. For this reason, we focused our analysis on Gen with the aim to decipher its role in polyploid cells.

Gen is an essential factor for polyploid NB proliferation

We first confirmed the RNAi screen data by combining *sqh^{mut}* with *gen^{mut}* and analyzed proliferation levels. As for the RNAi data, polyploid cell area was reduced in polyploid *gen^{mut}*, confirming the essential role of Gen in the proliferation of polyploid NBs (Fig 1D-E).

We next generated transgenes expressing mCherry (mCh) or NeonGreen (NeG) tagged Gen under the control of a UAS or Ubq promoter, to characterize the behavior of Gen by live imaging in tissues. Since Gen transgenes have not been generated before, we reasoned that we should fused the fluorescent tag to the C-terminus of Gen, to avoid affecting the XPG catalytic domains located at the N-terminus of the protein (Fig S2A and S3H). In flies, it is well established that UAS-driven expression results in stronger over-expression levels than Ubq-driven over-expression, which results only in moderate but ubiquitous expression of a specific gene. Importantly, Ubq-Gen-NeG was sufficient to rescue the increase in mitotic index observed in *gen^{mut}*, validating the function of the new transgenic lines (Fig S3B and S3H-I).

Since little is known about the localization of Gen in NBs, we decided to use time-lapse microscopy to follow Gen dynamics along the cell cycle. During interphase, Gen localized in the cytoplasm, as described for several human cell lines and *Drosophila* early embryos and S2 cells using immunostaining (Bellendir et al., 2017; Chan & West, 2014 and Fig S2). As the nuclear envelope broke down (NEBD) during mitosis, Gen signals diffused towards the chromosome region. With the reformation of the nuclear envelope, most fluorescent signal was excluded from the nucleus. In polyploid NBs, Gen behaved in a similar manner. During interphase, it was mainly retained in the cytoplasm and as NEBD took place, Gen-mCh covered the chromosome region (Fig S2D). Interestingly, careful analysis of Gen signals in interphase revealed that even if the highest fluorescent Gen-mCh levels are present in the cytoplasm, a weaker signal was also detected in the nuclei in both diploid and polyploid NBs (Fig S2C-E). These results show that, as described in human cells, the largest fraction of *Drosophila* Gen is

retained in the cytoplasm during interphase, but a smaller fraction is detected inside the nucleus (Benitez et al., 2023; Chan & West, 2014). Strikingly, while filming polyploid brain lobes expressing GenOE, we noticed that certain cells were very large. We measured cell area and found that indeed, polyploid GenOE display larger areas than control polyploid NBs (Fig 1H and 1I). Altogether, this data shows that in the absence of Gen, polyploid NBs display a proliferation disadvantage, while GenOE promotes proliferation.

Gen levels must be tightly controlled to prevent increase in DNA damage in polyploid NBs

Since Gen1 is a nuclease with known roles in DNA damage repair of HR byproducts, we investigated the levels of DNA damage in diploid *gen^{mut}* and polyploid, *gen^{mut}* NBs. We measured the fluorescence intensity levels of phosphorylated γ -H2Av, which is an early marker of double strands breaks (Cleaver et al., 2011; Kinner et al., 2008). Diploid *gen^{mut}* did not show increased DNA damage compared to WT (Fig S3G), while polyploid, *gen^{mut}* displayed increased DNA damage when compared to polyploid NBs (Fig 2A and 2B). In agreement, characterization of γ -H2Av levels in diploid *Rad51^{mut}* and polyploid, *Rad51^{mut}* NBs also revealed increased DNA damage levels in polyploid, while there was no increase in DNA damage in diploid NBs (Fig 2C-D and S3J). Altogether, these results suggest a link between increased DNA damage and loss of proliferative capacity.

A way of testing this hypothesis is to assess γ -H2Av levels in polyploid GenOE brains, which continue to proliferate and display larger NB sizes when compared to polyploid NBs. Surprising, however, polyploid GenOE NBs displayed increased γ -H2Av levels, when compared to polyploid NBs (Fig 2E-F). To ascertain if the increased DNA damage levels in polyploid GenOE brains were explained by nuclease activity, we generated a catalytic nuclease-dead Gen by replacing the EGVA residues in the first XPG domain and the EAEA in the second XPG domain by four alanine residues, and placed it under the UAS-promoter, fused to mCherry (Fig S2A). We refer to this construct as GenOEND. In this case, the endogenous Gen protein is still present, as we failed, from a genetic point of view to obtain the combination of GenOEND in a *gen^{mut}* background. Interestingly, polyploid GenOEND NBs displayed increased cell area when compared to polyploid NBs, but similar size when compared with polyploid, GenOE NBs (Fig 2E-F). Importantly, DNA damage levels were much higher values than polyploid Gen^{OE} (Fig 2F).

These results confirm on one hand that polyploid NBs proliferation takes place despite having very high levels of DNA damage, and on the other hand they suggest a novel role for Gen in promoting polyploid cell proliferation. Consequently, decrease in polyploid cell area observed in *gen^{mut}* cannot be explained by increased DNA damage in these cells.

Deviations in Gen levels impact mitotic progression even in diploid NBs

While analyzing diploid WT *gen^{mut}* brain lobes, we noticed that even if overall brain development was similar to controls, the mitotic index – which is defined by the number of NBs positive for Ph3 signal (PH3+) divided by the total number of NBs- was increased when compared to WT diploid brains, with a strong distribution of cells in prometaphase (Fig S3B-E). This increase was also noticed in GenOE brains, suggesting that mitotic progression is affected when Gen levels are altered (Fig S3B and 3C). We characterized mitotic errors and described that, while *gen^{mut}* anaphases showed chromosome bridges, lagging chromosomes and misaligned anaphases, GenOE NBs contained acentric chromosomes. Importantly, the frequency of any of these defects was much higher in either *gen^{mut}* or GenOE than in WT brains. These findings suggest that in NBs, optimal Gen levels are required to prevent chromosome bridges and furthermore to prevent ectopic double strands break generating acentric chromosomes. Interestingly, these mitotic errors do not seem to be sufficient to increase the overall DNA damage level or to hinder diploid brain development. To assess the origin of these mitotic errors, we performed time-lapse microscopy in *gen^{mut}* and GenOE expressing H2B-GFP or H2B-RFP. We found that *gen^{mut}* had a slight increase in mitotic duration, with a mean of 15 min compared to 13 min for Ctrl, while GenOE had faster mitoses, with a mean of 7 min (Fig S4 A-D). Precisely, *gen^{mut}* NBs spent longer time to align chromosomes in metaphase, suggesting that removing Gen affected their alignment capacity (Fig S4B). In addition, we assessed the overall cell cycle duration in diploid NBs, measured from the first NEBD to the next NEBD, and found that *gen^{mut}* NBs had longer cell cycles, while GenOE had shorter cell cycle than diploid ctrl (Fig S4 F-G). However, neither increased cell cycle duration, nor increased mitotic errors seemed to affect the overall development of *Drosophila* larvae, since both *gen^{mut}* and GenOE flies were viable and fertile.

Since *Drosophila* larvae also contains physiological polyploid tissues, we wondered whether Gen is required for polyploid cell proliferation in general. To assess this, we analyzed

nuclear area in physiological polyploid cells, that can be found in the salivary gland of the developing larvae. Nuclear area was similar in *gen^{mut}* compared to control, suggesting that Gen is not required for physiological polyploid cells, but only when we induce polyploidy in cells that were not programmed for it. (Fig S1 G-H)

Altogether, this data shows that the impact of Gen on cell proliferation can be detected in diploid NBs, but only becomes essential in unscheduled polyploid cells.

Human Gen1 depletion causes mitotic errors and chromosome loss in tetraploid RPE-1 cells

Gen1, the mammalian homolog of Gen, has been studied in several human cell lines, and was recently described as a factor required for fragile site expression under conditions of replicative stress (Benitez et al., 2023). We have established the use of stable diploid RPE-1 cell lines in which whole genome duplication (WGD) can be induced through mitotic slippage, generating a tetraploid mononucleated cell (Gemble et al., 2022; Ohashi, 2016; Tsuda et al., 2017). This approach has the advantage of allowing to study the initial consequences of tetraploidy just after birth, and so to avoid the accumulation of defects through abnormal mitosis or other events.

Considering our results obtained in flies, we next tested whether Gen1 is also required to sustain proliferation of human tetraploid cells. We generated RPE-1 cell lines stably expressing short hairpins (sh) Gen1 RNAs, leading to an overall reduction of 40%, when compared to shCtrl cells (Fig 3B). In shGen1 tetraploid cells, quantification of γ -H2Ax, 53BP1 or Rad51 foci revealed comparable levels to shCtrl tetraploid cells (Fig S5A-F). These results suggest that reduction of Gen1 does not impact DNA damage repair in newly born tetraploid cells. Striking, however, a strong increase in FANCD2 foci in shGen1 tetraploid cells was noticed. Interesting, and in agreement with Benitez et al., diploid ShGen1 cells also showed increased FANCD2 foci even if lower than tetraploid cells (Fig3 C and 3D).

FANCD2 is known to form foci in regions of replication stress such as common fragile sites, which often generate ultrafine DNA bridges (UFBs) visible in the subsequent mitosis during anaphase (Chan et al., 2009). We thus characterized the first mitosis of tetraploid ShGen1 cells expressing H2B-RFP to follow chromosome behavior. Unexpectedly, very few cells entered mitosis, when compared to shCtrl tetraploid cells. Nevertheless, out of 7 movies comprising more than 150 cells, we succeeded to follow 14 cells. Interestingly, while only 20%

of RPE1 ShCtrl tetraploid cells divided in a multipolar manner, which is considerably lower than what we have found in cancer cells after whole genome doubling (Goupil et al., 2020), this percentage reached 60% in ShGen1 tetraploid cells. One prediction would be that shGen1 cells have increased DNA structures, which would act as a barrier for centrosome clustering, leading to increased multipolarity, as described in Goupil et al. Thus, we assessed the levels of ultrafine DNA bridges (UFBs) using PICH as a marker. We found that 50% of shCtrl tetraploid had UFBs, while 94% of shGen1 tetraploid cells contained PICH-labeled UFBs (Fig 3G-H). This data is consistent with the increased levels of FANCD2 described in shGen1 and might explain the increased in multipolar anaphases.

The frequency of other defects such as lagging chromosomes and chromatinized DNA bridge formation were comparable between tetraploid shCtrl and shGen1 (Fig S4I). However, we noticed that shGen1 tetraploid had increased micronuclei (MN) formation (Fig3 I-J). To assess the chromosomal consequences of shGen1 in tetraploid cells, we performed multicolor FISH (mFISH) in tetraploid shCtrl and shGen1 cells (Fig 3K-L). We found that, while shCtrl tetraploid had 4 copies of almost each chromosome - except for chromosome 12 (6 copies), which has been described to be commonly gained with passages in RPE1 cells (Potapova et al., 2016), shGen1 tetraploid cells exhibited fewer copies of many chromosomes (Fig 3K-L), with a mean total number of 72 (Fig S5J). We also analyzed diploid cells, as the consequences of Gen depletion in RPE-1 cells were not described so far. Interestingly, ShGen1 diploid RPE1 cells showed increased frequency of anaphase errors than shCtrl, most specifically increased UFBs levels (Fig 3G-H). Unlike *Drosophila* NBs, mitotic timing was not affected in either diploid or tetraploid ShGen1 cells, even if tetraploid cells displayed lengthier mitosis (Fig S4, S5H).

Together, the characterization of the first cell cycle in newly born tetraploid revealed that Gen1 depletion leads to increased replication stress features, such as FANCD2 foci, UFBs in anaphase and increased MN. This suggests that cells with WGD rely on Gen1 to keep proliferating, not only through its role in HJ resolution, but also via its role in replication stress and probably for fragile site expression.

Gen1 depletion causes mitotic bypass and cell cycle arrest in tetraploid cells

The low number of tetraploid shGen1 cells undergoing mitosis suggested defects in cell cycle progression. To characterize the first cell cycle of newly born tetraploid cells, we performed

time-lapse microscopy in cells expressing the fluorescence ubiquitination cell cycle indicator (FUCCI) (Sakaue-Sawano et al., 2008). Tetraploid cells were distinguished from diploid cells based on their cell and nuclear size. Interestingly, tetraploidy induction *per se* did not seem to affect the proliferation their proliferation in the 1st cell cycle, as shCtrl tetraploid cells had the same mean cell cycle length (approximately 18h) after mitotic slippage (Fig S6A). However, tetraploid shCtrl had increased G1 duration, while they exhibited no significant difference in S/G2 duration compared to diploid shCtrl (Fig 4B and 4F-G). Moreover, while diploid shCtrl cells had a mean of 2 cell divisions during 40h time lapse movies, tetraploid shCtrl cells had a slight reduction in the mean number of cell division (Fig 4B-E). This suggests that, even if tetraploid shCtrl cells kept proliferating, they might have a slower rate of cell division compared to diploid shCtrl cells.

Interestingly, Gen1 depletion in diploid cells did not seem to affect the cell cycle, as shGen1 diploid cells had a similar G1 duration, G2 duration, number of cell division per lineage (Fig 4E), and overall cell cycle length than shCtrl diploid cells (Fig 4 E-G and S6A). However, a large number of shGen1 tetraploid cells spent the whole movie in G1 (n=41), suggesting a cell cycle arrest (Fig 4B). In addition, shGen1 tetraploid cells spent more time in S-G2 than shCtrl tetraploid cells, consistent with higher FANCD2 levels (Fig 4G). Moreover, while shCtrl tetraploid S/G2 cells always entered mitosis, visible by the diffusion of GFP signal into the cell as NEBD occurred, a high number of ShGen1 tetraploid cells (n= 18 out of 95 cells) went directly from S/G2 into G1 and remained arrested in G1 up to 38 hours. The mitotic bypass found in tetraploid shGen1 cells, was never noticed in shCtrl cells, and very rarely in ShGen1 diploid cells (3 out of 94 cells), suggesting that this only occurs in tetraploid cells with lower Gen1 levels and presumably with high FANCD2 levels, likely revealing the presence of unresolved structures generated during DNA replication.

Interestingly, it was recently shown that mitotic bypass can occur in a p53 dependent manner due to increasing levels of cell cycle proteins (Zeng et al., 2023). We thus tested whether p53 and p21 were upregulated in ShGen1 tetraploid cells. p53 is a tumor suppressor, that prevents cell cycle progression when genome integrity is lost, and p21 is a CDK inhibitor, that controls cell cycle arrest, mainly in response to p53 activation (El-Deiry et al., 1993; Harper et al., 1993; Sherr & Roberts, 1995). As expected, p53 and p21 levels presented a 8 and 20 fold increases respectively, in ShGen1 tetraploid cells when compared to ShCtrl tetraploid cells (Fig 4I). Because of their role in cell cycle regulation and arrest, p53 and p21

upregulation levels correlate with lower proliferative potential (El-Deiry et al., 1994; El-Deiry et al., 1993; Harper et al., 1993; Karimian et al., 2016; Xiong et al., 1993). We next tested the proliferative capacity of shGen1 cells, using clonogenic assays. We found that shGen1 diploid presented lower proliferation capacity than the controls and reach more than 75 colonies after 8 days, while controls reached more than 100 colonies (Fig S6B). However, tetraploid clones were even more affected by the depletion of Gen1, as they were only able to make less than 20 colonies after 13 days, while controls reached 75 colonies (Fig4 J-K).

Altogether, this confirmed that Gen1 is an essential factor for human tetraploid cell proliferation, and that this role seems to be conserved from *Drosophila* to human cells.

Gen1 is overexpressed in WGD-enriched tumors

We have previously reported a sub-scaling of DNA replication factors in newly born tetraploid RPE1 cells (Gemble et al., 2022). Since our finding show that Gen1 is essential for polyploid/tetraploid cell proliferation, we tested whether Gen levels scale with ploidy increase in the first cell cycle. We found an almost perfect scaling relationship between Gen1 levels and DNA content (Fig 5A-C), reinforcing the idea that tetraploid cells might rely on Gen1 to keep proliferating.

These findings beg the obvious question related with WGDs in human cancers. A prediction from our findings is that in tumors displaying WGDs, GEN1 levels must at least scale with DNA content to prevent cell cycle withdraw or the generation of catastrophic mitosis. To investigate this possible relationship, we compared gene expression levels in TCGA tumors with or without whole genome duplication (WGD). We first determined the ploidy levels of the samples using the latest dataset generated by Drews et al., 2022. In this study, absolute copy number fits were obtained using allele-specific copy number analysis of tumors (ASCAT) in collaboration with the Peter Van Loo group. We used two different approaches: 1.) we set up a threshold for which we considered samples with a ploidy > 2.7 as whole genome duplicated; and 2.) we used WGD estimated from Drews et al, where the WGD status of a tumour sample is determined based on a sample's ploidy and the fraction of the genome affected by loss of heterozygosity (LOH) (Drews et al., 2022). Both approaches showed that tumors with WGD had increased expression of Gen1 compared to diploid samples. When we

searched into different tumor subtypes, we found that some subtypes were overexpressing Gen1 more than others, such as lung adenocarcinoma (LUAD) (Fig 5 D-E, left panel, and 5F)

Moreover, we found that tetraploid tumors had increased Rad51 levels (Fig 5D-E right panel, and 5G), advocating for a role of homologous recombination in tetraploid cells, as suggested by our experiments in polyploid *Drosophila* NBs (Fig1). In agreement with polyploid NBs data, our analysis revealed that tetraploid tumors did not have increased Mus81 levels (Fig 5H). These results were somehow unexpected, as Mus81 was previously showed to be required to resolve aberrant mitotic DNA structures in mammals (Naim et al., 2013; Wyatt et al., 2013), and loss of Mus81 leads to increased sensitivity to DNA damaging agents in yeast and mice. (Blanco et al., 2010; Boddy et al., 2000; Dendouga et al., 2005; Ho et al., 2010; Tay & Wu, 2010)

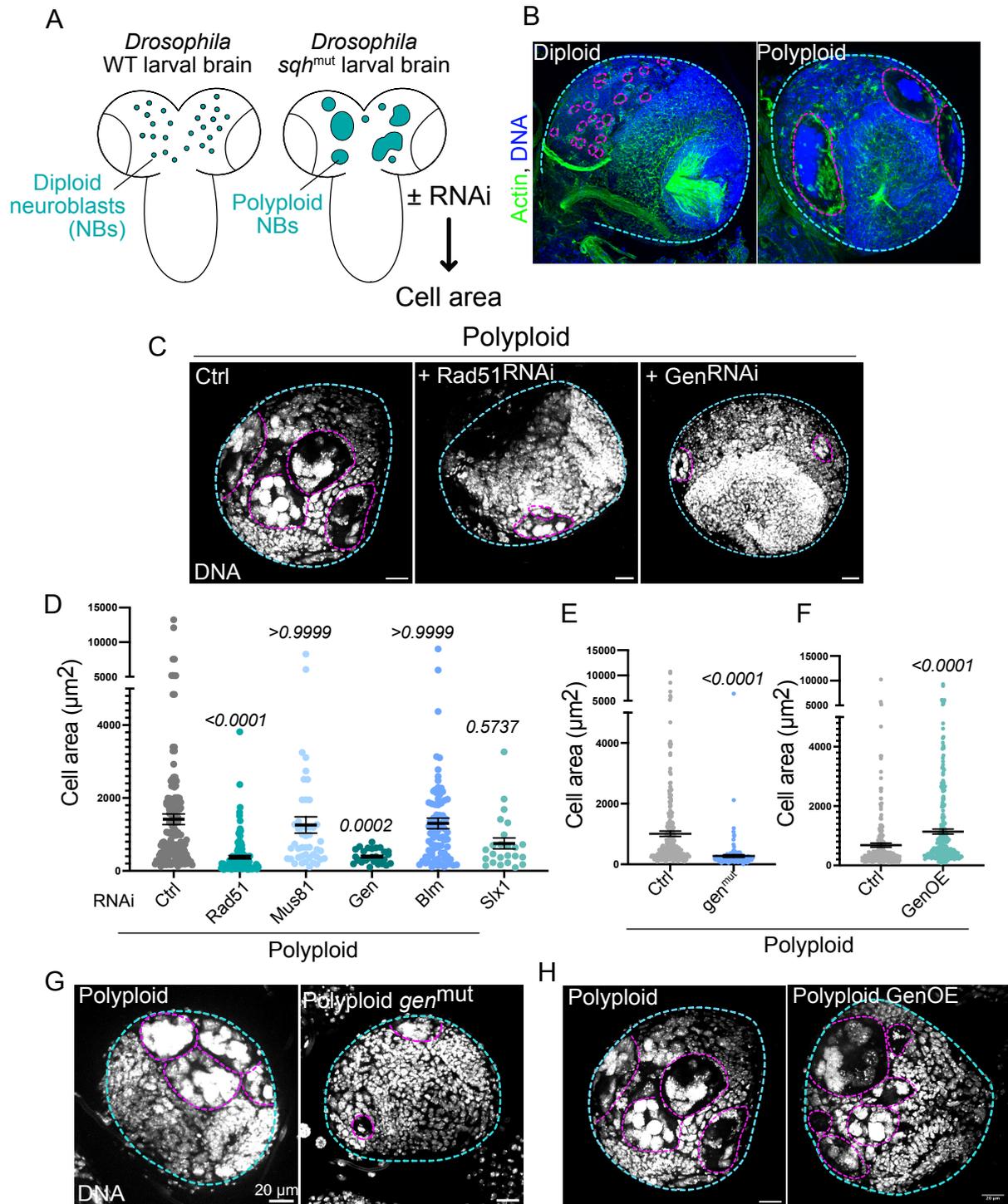
Overall, these data suggests that Gen1 is essential for the proliferation for tumors with high levels of WGDs, but not in other tumors.

Results – section 2

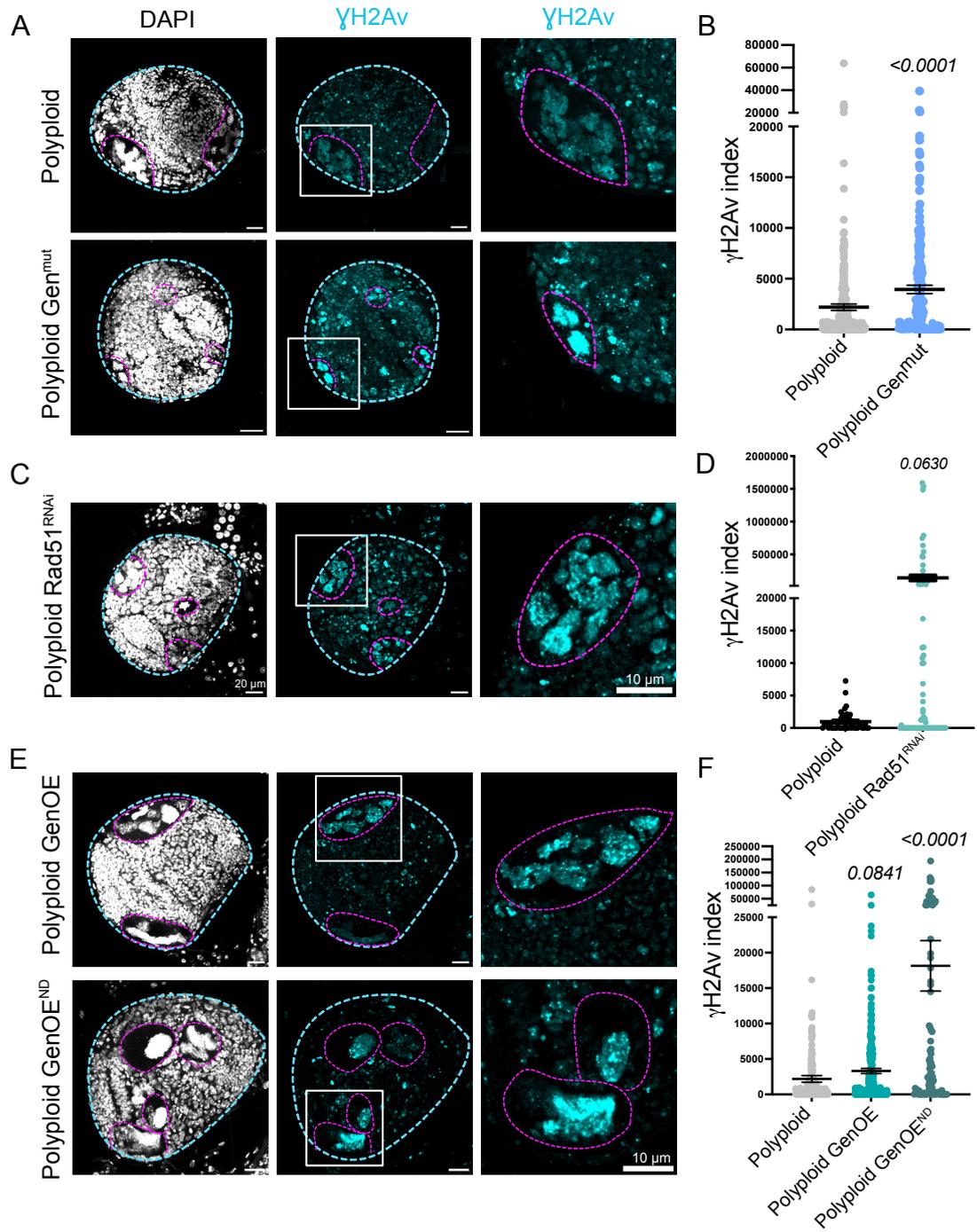
Figures

This section is composed of 5 figures and 6 supplemental figures.

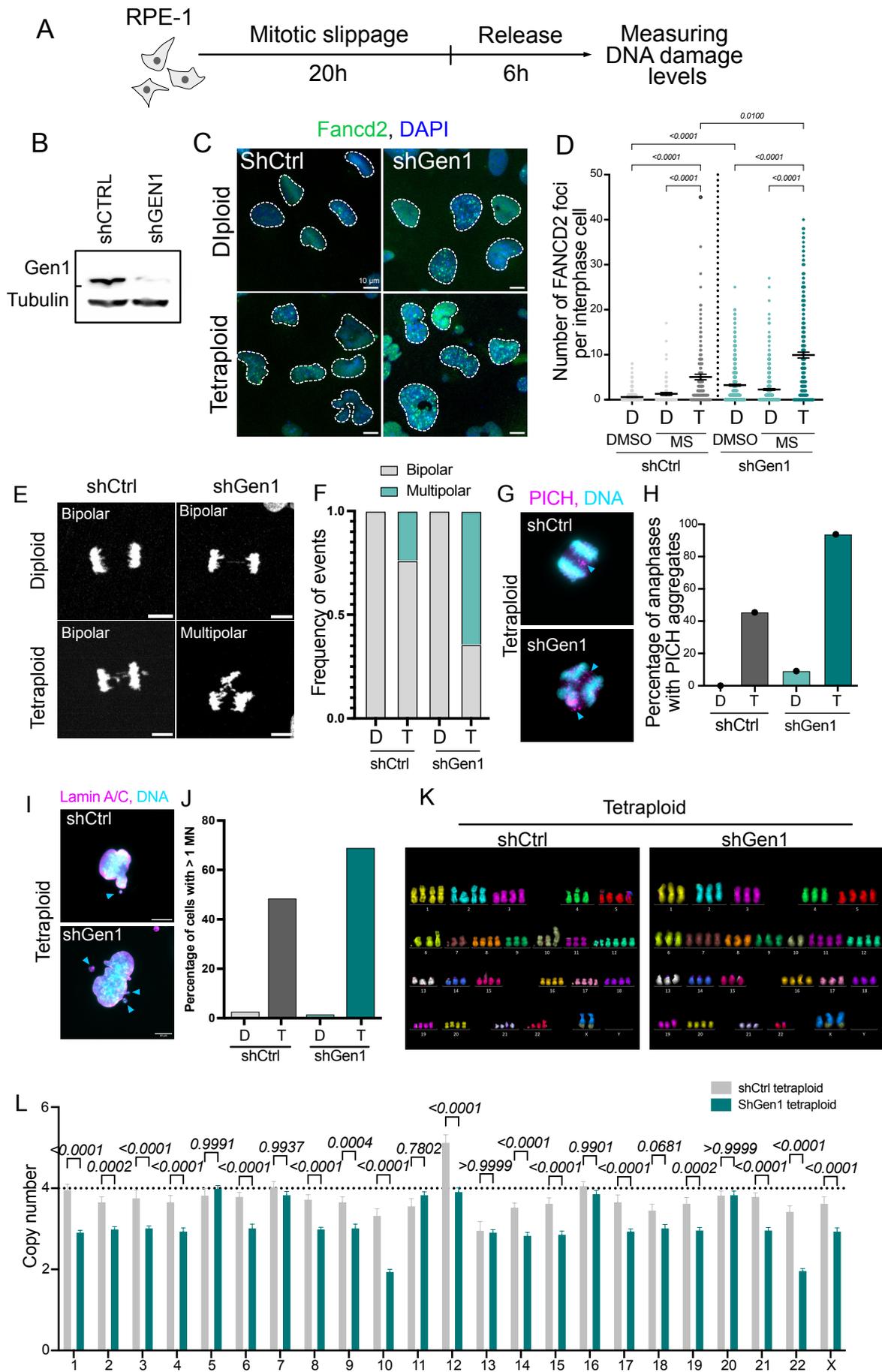
Budzyk, et al. Figure 1



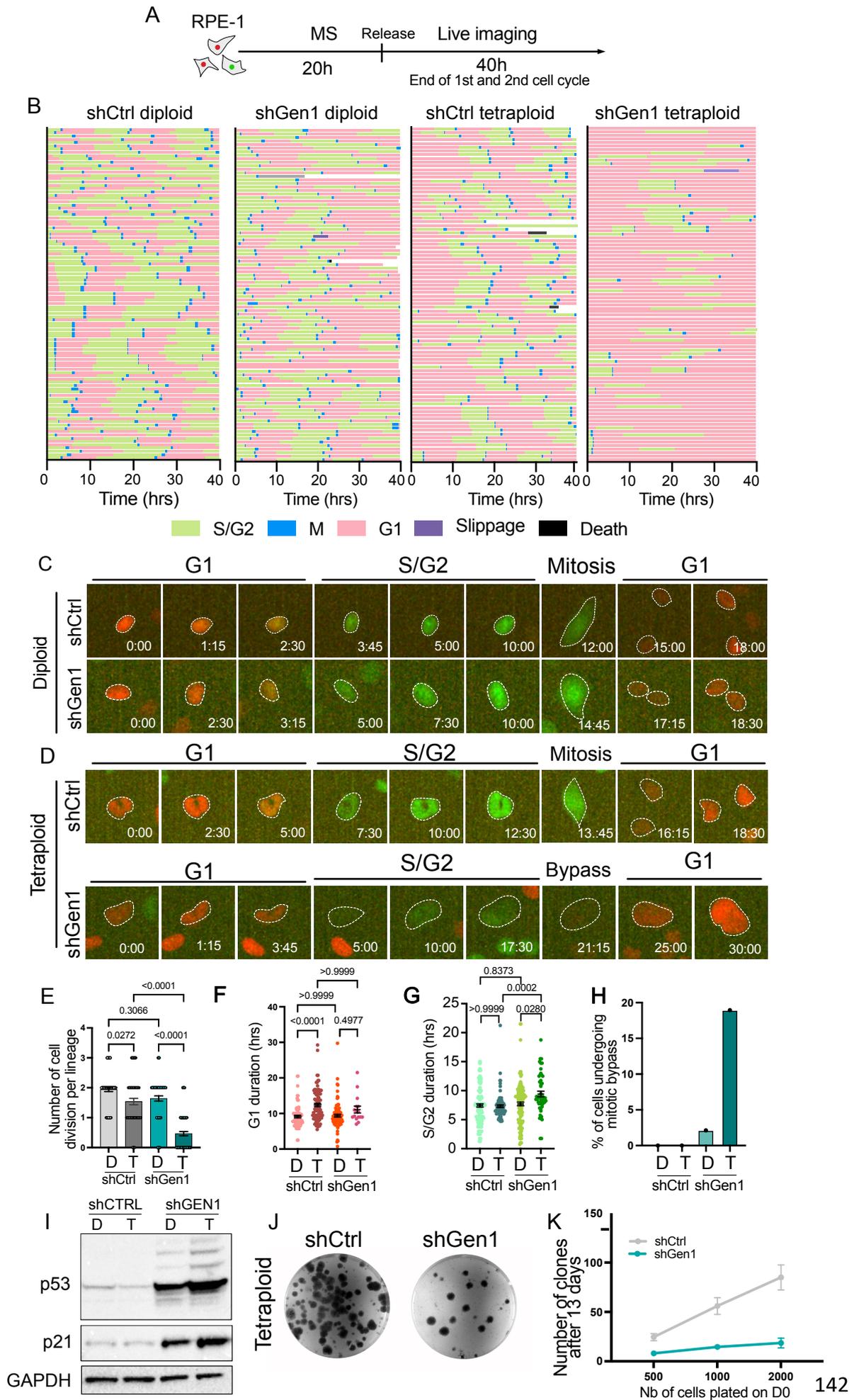
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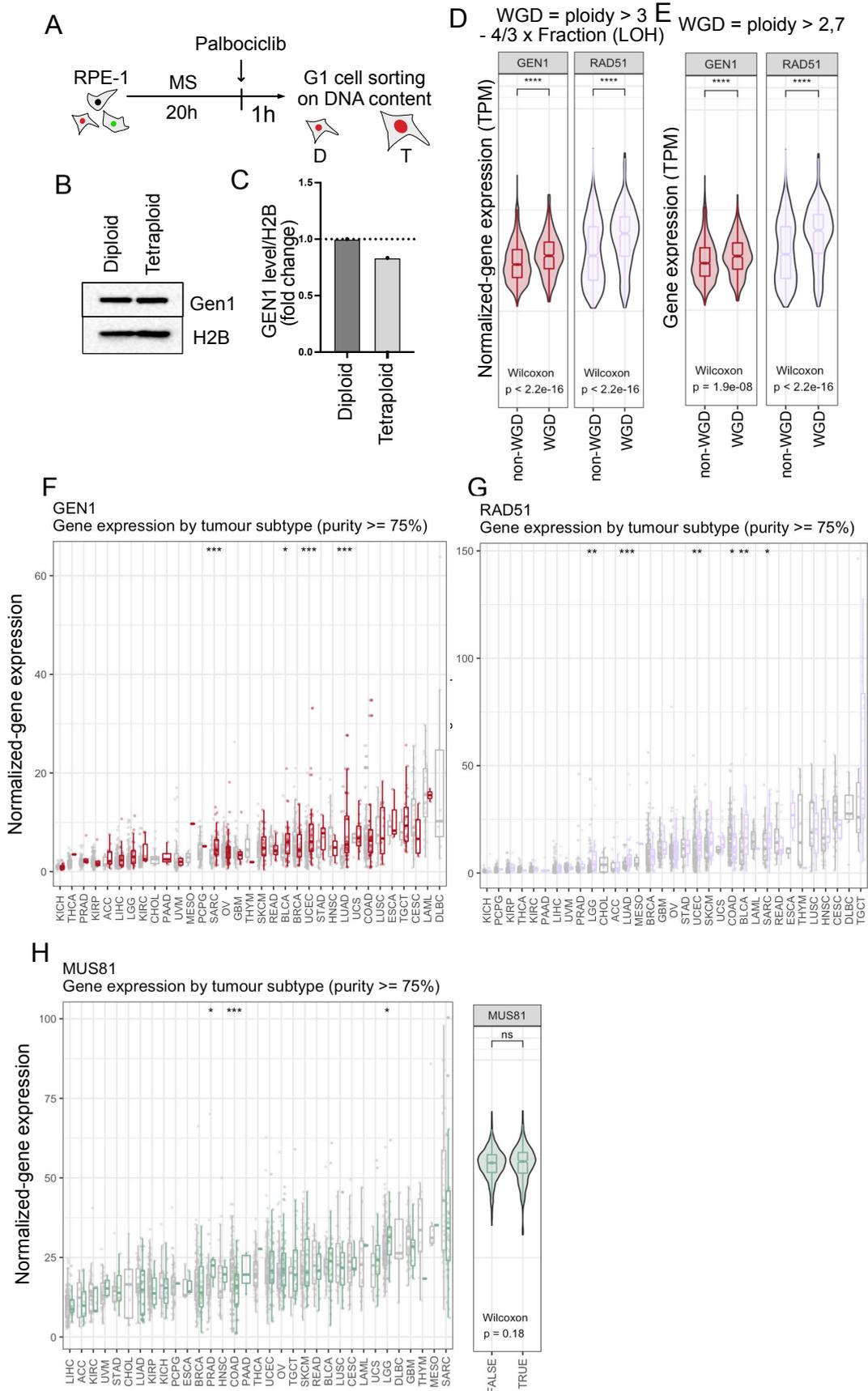
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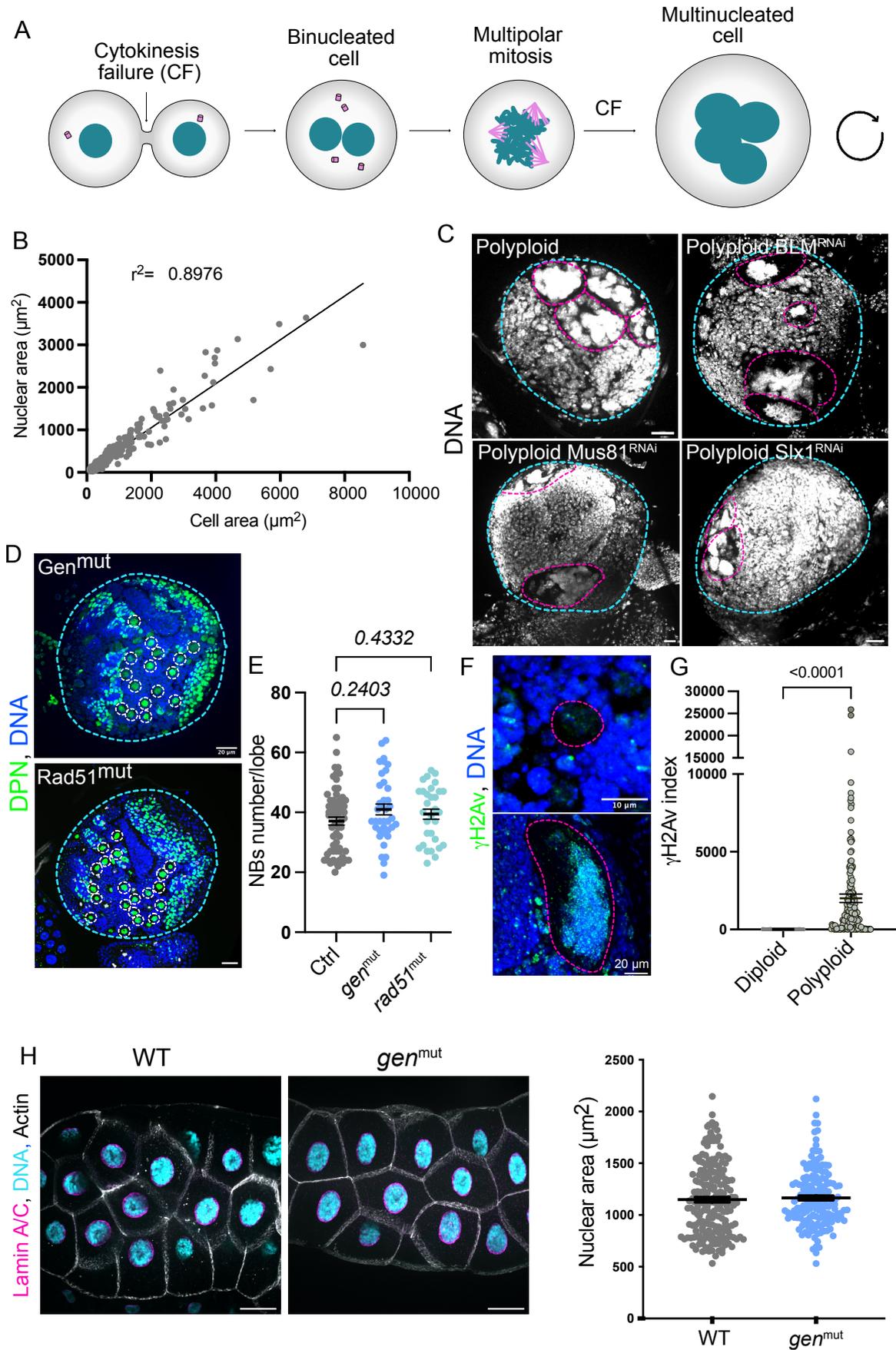
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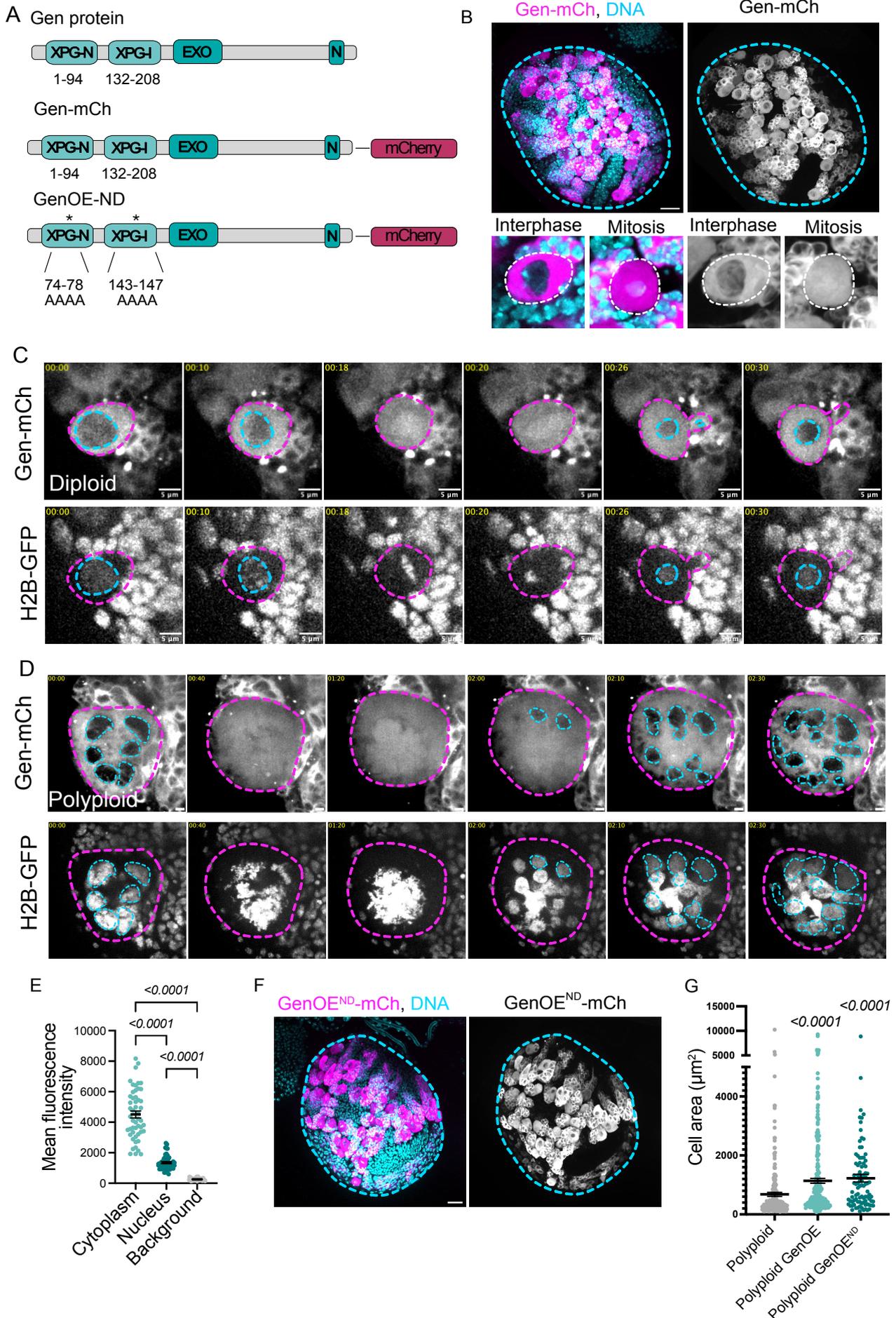
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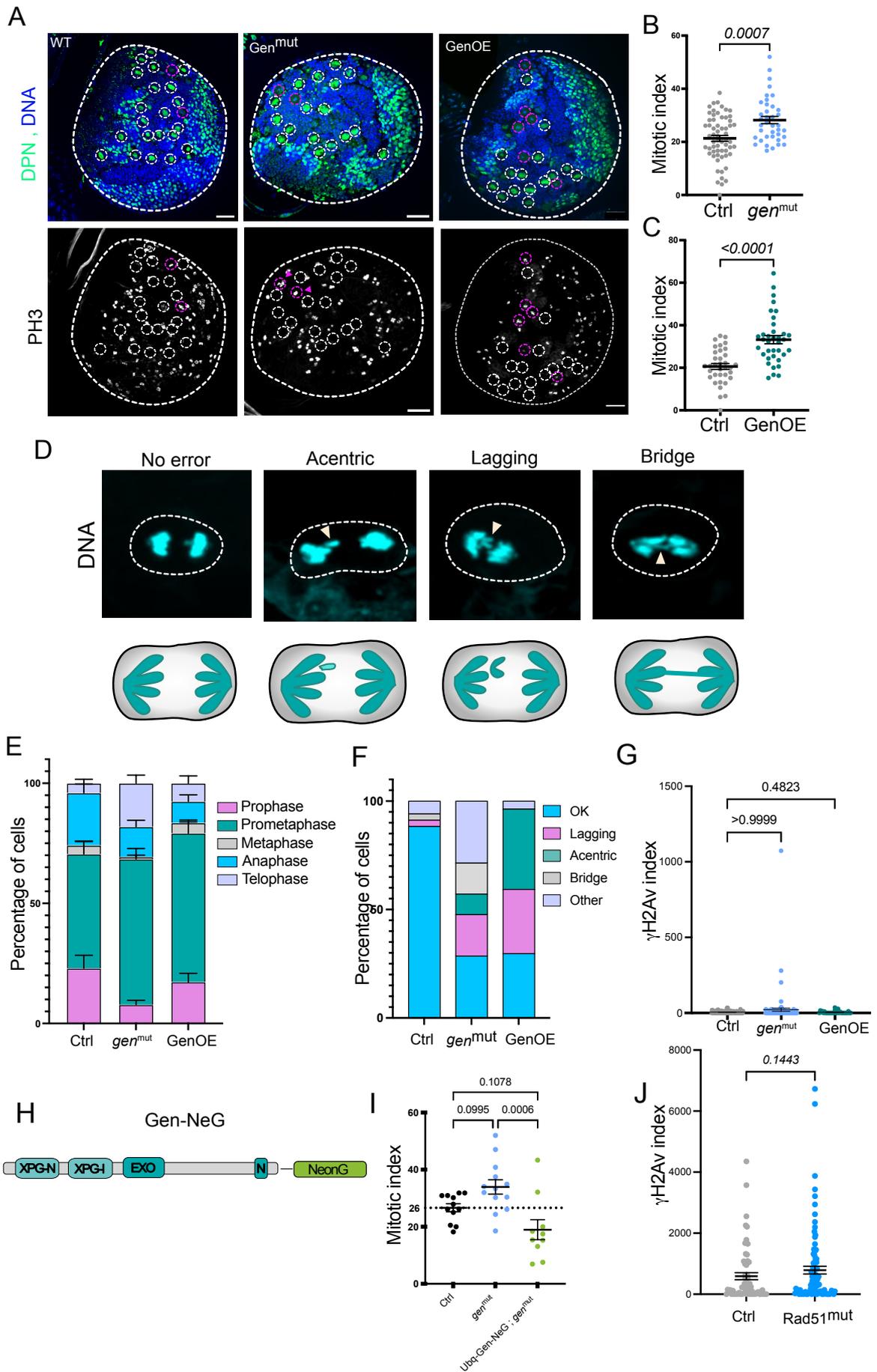
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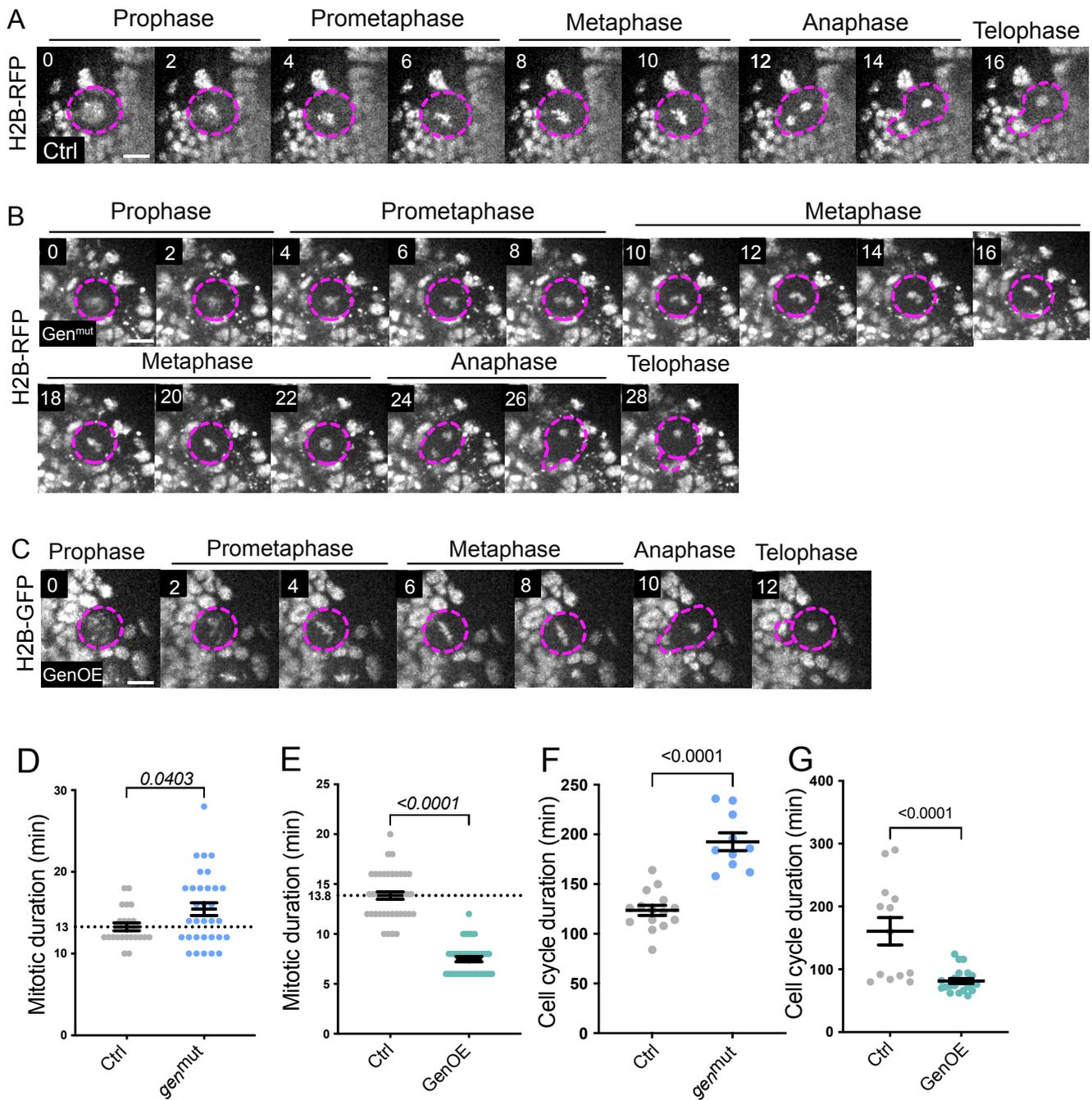
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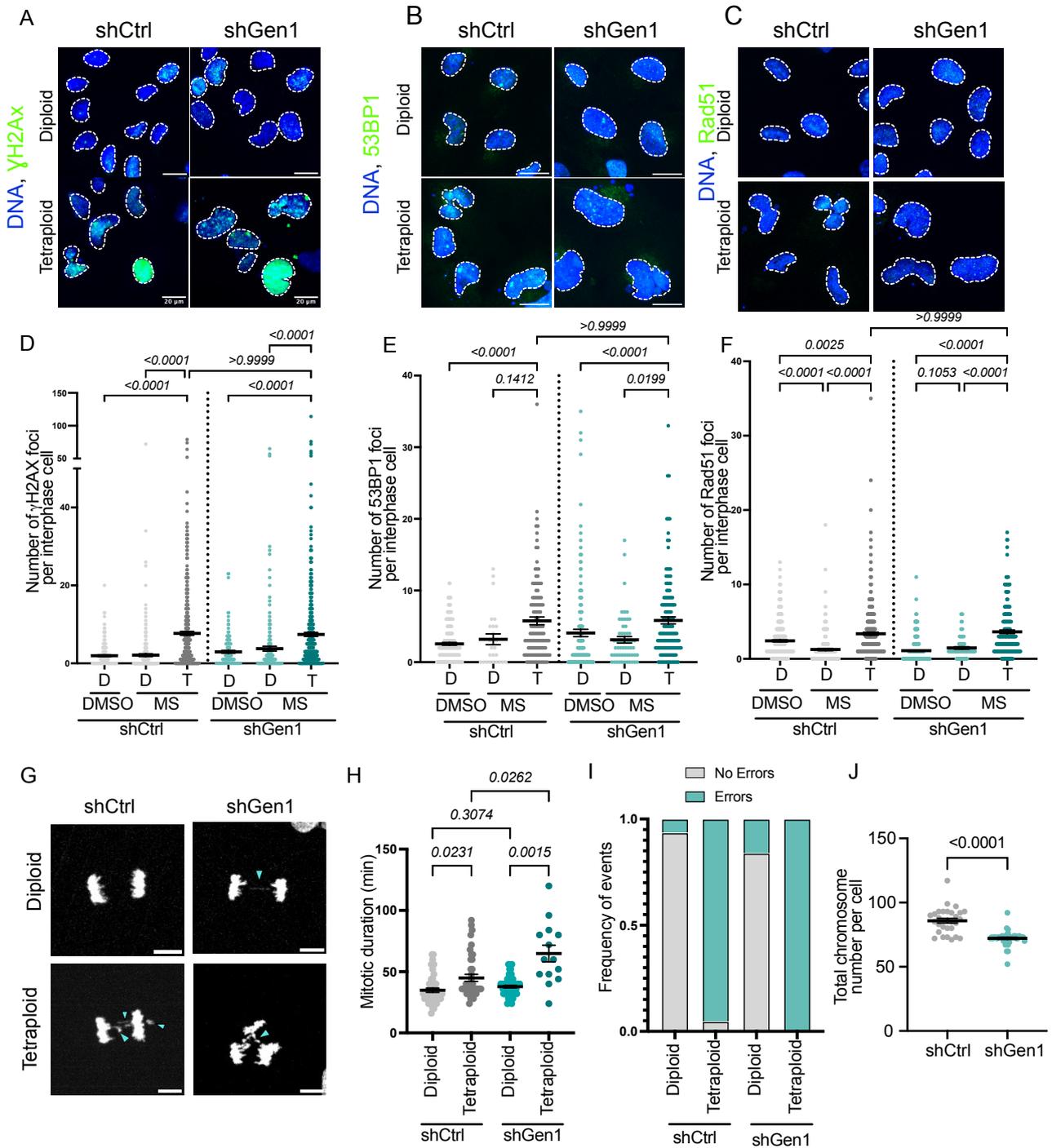
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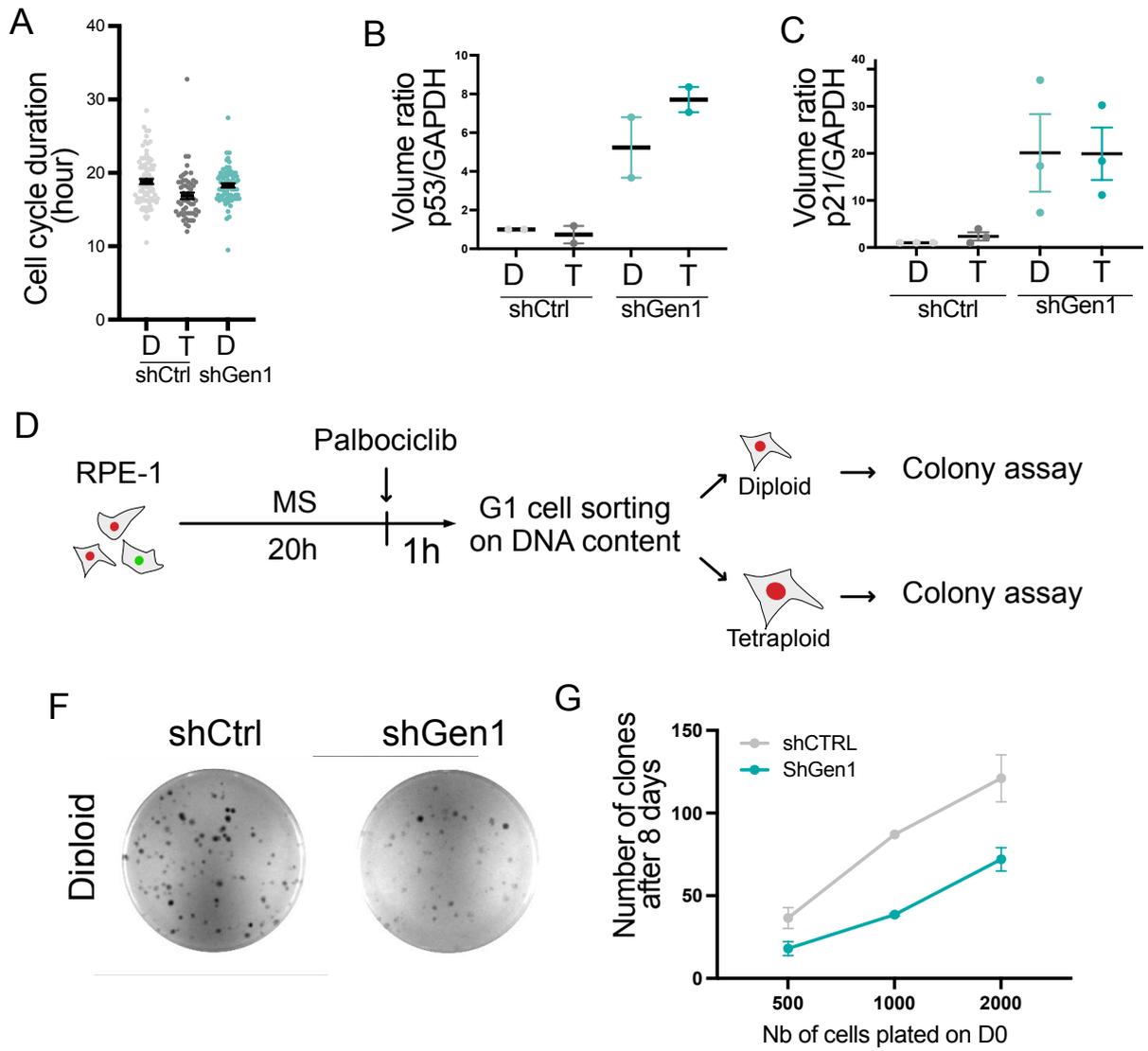
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Budzyk et al. Figure S6



Results – Section 2
Figure legends

Figure 1. Gen is required for *Drosophila* polyploid neuroblast proliferation

(A) Description of polyploidy induction in *Drosophila* NBs, and screening protocol for identification of factors essential for polyploid cell proliferation. **(B)** Image of a *Drosophila* polyploid (*sqh^{mut}*) brain lobes labelled with DAPI (in blue) to reveal nuclear staining and Phalloidin to reveal cell membrane (in green) (scalebar = 20 μm). **(C)** Images of *Drosophila* polyploid brain lobes labelled with DAPI (in grey) to reveal nuclear staining, upon the induction of Rad51 and Gen RNAi. **(D)** Dot plot showing polyploid cell area, after induction of the indicated RNAis (N= 158 for Ctrl, N= 30 cells for Gen^{RNAi}, N= 119 cells for Rad51^{RNAi}, N= 82 cells for BLM^{RNAi}, N=46 cells for Mus81^{RNAi}, N= 24 cells for Slx1^{RNAi}). **(E)** Dot plot showing polyploid NBs area (in μm^2) in *sqh^{mut} gen^{mut}* brain lobes (N=270 cells for polyploid Ctrl, N=154 cells for polyploid Gen^{mut}). **(F)** Dot plot showing polyploid NBs area quantification in *sqh¹* GenOE brain lobes (N=226 cells for polyploid ctrl, N=285 cells for polyploid GenOE). **(G)** Images of *Drosophila* polyploid brain lobes labelled with DAPI (in grey) to reveal nuclear staining, in *sqh^{mut} gen^{mut}*. **(H)** Images of *Drosophila* polyploid brain lobes labelled with DAPI (in grey) to reveal nuclear staining, after GenOE. GenOE construct description is available in Fig S2.

For each cell area measurement, cell outline was delimited using Phalloidin to label the cell membrane, as illustrated in figure S1A. On the pictures, single polyploid cells are delimited by pink dashed lines, and brain lobes are delimited by blue dashed lines. For statistical analysis, Kruskal-Wallis test was performed for panel C by comparing the rank of each condition with the ctrl, and student t test was performed for panel E and F.

Figure 2. Gen levels must be controlled to prevent increase in DNA damage in polyploid NBs

(A) Images of *Drosophila* polyploid brain lobes labelled with DAPI to reveal nuclear staining (in grey) and using γH2Av antibody to reveal DNA damage (in cyan), in *sqh^{mut} gen^{mut}* brain lobes. **(B)** Dot plot showing γH2Av index (A.U) in *sqh^{mut} gen^{mut}* cells. γH2Av index was measured by multiplying the coverage by the intensity of γH2Av signal, as described in Nano et al., 2019 (N=279 cells for polyploid Ctrl, N=175 cells for polyploid Gen^{mut}). **(C)** Images of *Drosophila* polyploid brain lobes labelled using γH2Av antibody (in cyan), to reveal DNA damage in *sqh^{mut} rad51^{RNAi}* brain lobes. **(D)** Dot plot showing γH2Av index (A.U) analysis in *sqh^{mut} rad51^{RNAi}* *Drosophila* brain lobes (N= 36 cells for polyploid Ctrl, N= 56 cells for polyploid Rad51^{RNAi}). **(E)** Images of *Drosophila* polyploid brain lobes labelled using γH2Av antibody (in cyan), and DAPI

to reveal nuclear staining (in grey), in *sqh^{mut}* GenOE and *sqh^{mut}* GenOEND brain lobes. GenOEND construct description is available in Fig S2. **(F)** Dot plot showing γ -H2Av index (A.U.) measured in *sqh¹* Gen^{OE} and *sqh* Gen^{OE-ND} cells (N= 302 cells for polyplod Ctrl, N= 179 cells for polyplod GenOE, N=91 cells for polyplod GenOEND) For statistical analysis, Mann-Whitney test was performed between the two samples in each graph.

Figure 3. Gen1 depletion causes mitotic errors and chromosome loss in tetraploid cells

(A) Scheme of the experimental procedure for tetraploidy induction in human RPE-1 cells. **(B)** Immunoblot for Gen1 levels in RPE1 cells shCtrl and shGen1. **(C)** Images of RPE1 cells labelled using FANCD2 antibody (in green), and DAPI to reveal nuclear staining (in blue), for shCtrl and shGen1 cells diploid or tetraploid. **(D)** Quantification of Fancd2 foci in interphase in diploid (D) and tetraploid (mitotic slippage = MS) shCtrl or shGen1 (N= 385 D shCtrl cells; N=86 D MS shCtrl cells, N= 134 T MS shCtrl cells; N=498 D shGen1 cells; N=434 D MS shGen1 cells; N=259 T MS shGen1 cells). **(E)** Image of anaphase figures and errors observed in RPE-1 shCtrl and shGen1 diploid and tetraploid, labelled using H2B-GFP construct. **(F)** Frequency of bipolar and multipolar division in the indicates genotypes (N= 47 cells for shCtrl diploid, N= 42 cells for shCtrl MS; N= 50 cells for shGen1 diploid; N= 14 cells for shGen1 MS). **(G)** Images of tetraploid RPE-1 in anaphase, labelled using PICH antibody (in magenta), DAPI to reveal DNA staining (in cyan), in the indicated genotypes. **(H)** Bar plot showing the % of anaphases with PICH aggregate as illustrated in panel G. **(I)** Picture of tetraploid RPE1-1 cells labelled using lamin A/C antibody (in magenta) and DAPI (in cyan), in shCtrl and shGen1 cells. Blue arrows indicate micronuclei. **(J)** Bar plot showing the % of cells with more than one micronucleus in the indicated genotypes. **(K)** Karyotypes of tetraploid shCtrl and shGen1 cells. **(L)** Bar plot showing the chromosome number in shCtrl and shGen1 tetraploid cells. (N= 30 cells for shCtrl and N= 39 cells for shGen1). For statistical analysis, Kruskal-Wallis test was performed and the associated p-values are shown. (D= diploid, T= tetraploid, MS= mitotic slippage treatment). Scalebars = 10 μ m

Figure 4. Gen1 depletion causes mitotic bypass and cell cycle exit in tetraploid

(A) Schematic of the experimental procedure for cell cycle analysis in tetraploid RPE-1 cells. **(B)** Bar plots of single cell tracking of FUCCI RPE-1 cells filmed for 40 hours with the annotated genotypes and treatments. Each bar indicates a single cell followed during 40h, with each

color corresponding to cell cycle phase (N=95 cells per condition). **(C)** Still images of time-lapse showing a single RPE-1 diploid cell carrying a FUCCI reporter, for shCtrl and shGen1. **(D)** Still images of time-lapse showing a single RPE-1 tetraploid cell carrying a FUCCI reporter, for shCtrl and shGen1. The panel below shows an example of a mitotic bypass occurring in tetraploid shGen1 cell. **(E)** Bar plot showing the mean number of cell division per lineage, in the indicated genotypes (N=86 cells for shCtrl diploid, N=66 cells for shCtrl tetraploid, N=85 cells for shGen1 diploid, N= 93 cells for shGen1 tetraploid). **(F)** G1 duration for the indicated genotypes and treatment, measured from single cell live-imaging of FUCCI cells (N= 80 cells for shCtrl diploid, N=97 cells for shCtrl tetraploid, N= 95 cells for shGen1 diploid, N= 16 for shGen1 tetraploid). **(G)** S/G2 duration for the indicated genotypes and treatment, measured from single cell live-imaging of FUCCI cells (N= 130 cells for shCtrl diploid, N=94 cells for shCtrl tetraploid, N=136 cells for shGen1 diploid and N= 50 cells for shGen1 tetraploid). Each dot represents a single cell. **(H)** Bar plot showing the % of cells undergoing a mitotic bypass in the indicated genotypes. **(I)** Western blot for p53, p21 and GAPDH in the indicated genotypes and treatments. **(J)** Pictures of colony assay of tetraploid shCtrl and shGen1, after 13 days. **(K)** Graph showing the number of clones after 13 days of proliferation, according to the number of cells plated on day 0.

Figure 5. Gen1 is overexpressed in tetraploid tumors

(A) Schematic showing the experimental procedure for tetraploidy induction in human RPE-1 cells, cell cycle synchronization using palbociclib, and immunoblots. **(B)** Immunoblot for Gen1 and H2B after tetraploidy induction in RPE-1 cells. **(C)** Bar plot showing the Gen1 levels in tetraploid cells, measured as a ratio to H2B volumes. **(D)** Violin plot showing normalized Gen1 and Rad51 expression levels in WGD vs non-WGD tumor samples, in which WGD = ploidy > 3 - 4/3 x Fraction (LOH), as described in Drews et al., 2022. **(E)** Violin plot showing normalized Gen1 and Rad51 expression levels in WGD vs non WGD tumor samples, in which WGD = ploidy >2,7 from TCGA samples (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) **(F)** Box & whiskers showing Gen1 expression levels per tumor samples, from TCGA **(G)** Box & whiskers showing Rad51 gene expression levels per tumor samples, from TCGA. **(H)** Box & whiskers showing Mus81 gene expression levels per tumor samples, from TCGA (on the left). On the right, violin plot showing

normalized gene expression of Mus81 in which in WGD vs non WGD tumor samples, in which WGD = ploidy >2,7 from TCGA samples. For statistical analysis, Wilcoxon test were performed.

Figure S1. Supplementary data for Figure 1

(A) Schematic representing polyploidy induction by repeated cytokinesis failure in *Drosophila* NBs. **(B)** Dot plot showing the correlation between polyploid NBs area and nuclear area. **(C)** Images of *Drosophila* polyploid brain lobes labelled with DAPI (in grey) to reveal nuclear staining, upon the induction of BLM^{RNAi}, Mus81^{RNAi} or Slx1^{RNAi}. **(D)** Images of *Drosophila* diploid brain lobes labelled with DAPI (in blue) to reveal nuclear staining and using Deadpan antibody (in green), to reveal NBs, in Gen^{mut} and Rad51^{mut}. **(E)** Dot plot showing the total NB per lobe in *gen*^{mut} and *rad51*^{mut} brain lobes (N=64 WT brains lobes, N= 39 *gen*^{mut} brain lobes, N=33 *rad51*^{mut} brain lobes). **(F)** Images of a *Drosophila* diploid and polyploid NBs, labelled with γ -H2Av antibody (in green) to reveal DNA damage and stained with DAPI (in blue) to reveal nuclear staining. **(G)** Dot plot showing the γ H2AV index in diploid and polyploid NBs (N= 93 diploid NBs and N=166 polyploid NBs). **(H)** Images of *Drosophila* WT and *gen*^{mut} salivary gland (SG) stained with DAPI (in cyan) to reveal nuclear staining, using lamin A/C antibody (in magenta) to reveal nuclear envelope and Phalloidin (in grey) to reveal the cell membrane. **(I)** Dot plot showing the nuclear area (in μm^2) for WT and *gen*^{mut} SG (N= 202 cells for WT, N=180 cells for *gen*^{mut}). Scalebars = 20 μm . For statistical analysis, Kruskal-Wallis test was performed and the associated p-values are shown for panel (E).

Figure S2. Constructs for GenOE and GenOE-ND

(A) Scheme of Gen protein domains. Gen contains two catalytic domains, XPG-N and XPG-I, a 3'-5' exonuclease (EXO) domain and a DNA binding domain (N). Gen-mCh construct carries a mCherry tag in C-terminus. GenOEND has substitutions in both XPG nuclease domains, in which the conserved nucleotides were replaced by AAAA. **(B)** Image of a *Drosophila* diploid brain lobe expressing WorG4 UAS-Gen-mCh construct (GenOE-mCh), labelled with DAPI (in cyan) to reveal nuclear staining, and using a RFP antibody to increase GenOE signal (in magenta). **(C)** Stills showing time lapse imaging of a diploid NB carrying UAS-Gen-mCh undergoing mitosis. Gen-mCh channel is depicted on top, and H2B-GFP channel on the bottom **(D)** Stills showing time lapse imaging of a polyploid NB carrying UAS-Gen-mCh construct undergoing mitosis. Gen-mCh channel on top and H2B-GFP on the bottom. Nucleus delimited by nuclear envelope

are depicted in blue. **(E)** Dot plot showing the fluorescence intensity of UAS-Gen-mCh in diploid NBs in cytoplasm or nucleus compartment (N= 51 NBs). **(F)** Image of a *Drosophila* diploid brain lobe expressing WorG4 UAS-GenND-mCh (GenOEND) construct, labelled with DAPI (in cyan) to reveal nuclear staining, and using a RFP antibody to increase GenOE signal (in magenta) **(G)** Dot plot showing polyploid cell area quantification in *sqh*^{mut} GenOE and *sqh*¹ GenOEND brain lobes (N= 223 cells for Ctrl, N= 285 for GenOE, N=91 cells for GenOEND). For statistical analysis, Kruskal-Wallis test was performed and the associated p-values are shown.

Figure S3. Gen is not essential for diploid NBs but generates mitotic errors

(A) Images of *Drosophila* diploid *gen*^{mut} or GenOE brain lobes labelled with DAPI (in blue) to reveal nuclear staining, using Deadpan (DPN) -a pan-NBs marker- antibody (in green), and using PH3 antibody (in grey, bottom) or mitotic figures. **(B)** Dot plot showing the mitotic index (measured as the % of Ph3+ NBs among the total number of NBs) in *Drosophila* diploid brain lobes *gen*^{mut}. **(C)** Dot plot showing the mitotic index in *Drosophila* diploid brain lobes in GenOE. **(D)** Pictures showing typical errors observed in anaphase in diploid NBs, labelled with DAPI to reveal DNA staining. Errors are indicated by yellow arrows. **(E)** Bar plot showing the proportion of each mitotic phases among the Ph3+ NBs in *gen*^{mut} and GenOE. **(F)** Bar plot showing the proportion of the listed errors in anaphase in diploid *gen*^{mut} and GenOE. **(G)** Dot plot showing the γ -H2Av index (A.U.) measured in diploid NBs *gen*^{mut} or GenOE. **(H)** Schematic of the construct used for tagging Gen with NeonGreen, under the expression of a Ubq promoter. **(I)** Dot plot showing the mitotic index (measured as the % of Ph3+ NBs among the total number of NBs) in the indicated genotypes. **(J)** Dot plot showing the γ -H2Av index (A.U) measured in *Drosophila* diploid brain lobes carrying *rad51*^{mut}.

Figure S4. Gen impact diploid cell cycle duration

(A) Stills showing time-lapse imaging of a mitosis in a control diploid NB expressing H2B-RFP. **(B)** Stills showing a mitosis in a diploid *gen*^{mut} NB expressing H2B-RFP. **(C)** Stills showing time-lapse imaging of a mitosis in a diploid GenOE NB expressing H2B-GFP. Pink dashed lines are depicting NB cell membrane. **(D)** Dot plot showing the mitotic duration in diploid NBs for control and *gen*^{mut} expressing H2B-RFP as showed in panel A and B (N=22 NBs for ctrl, N=32 NBs for *gen*^{mut}). **(E)** Dot plot showing the mitotic duration in diploid control and GenOE NBs expressing H2B-GFP, as showed in panel C (N=40 NBs for ctrl, N=38 for GenOE). **(F)** Dot plot

showing cell cycle duration (from NEBD to NEBD) in diploid control and *gen^{mut}* NB expressing H2B-RFP (N= 15 NBs for Ctrl, N=10NBs for *gen^{mut}*). **(G)** Dot plot showing cell cycle duration (from NEBD to NEBD) in diploid ctrl and GenOE expressing H2B-GFP (N=13 NBs for ctrl, N=22 NBs for GenOE). In graphs, each dot represents one NB. For statistical analysis, unpaired t-test was performed on the mean of each sample.

Figure S5. Gen1 depletion does not increase DNA damage in the 1st cell cycle after WGD

(A) Pictures of RPE-1 shCtrl and shGen1 diploid and tetraploid cells, labelled using γ H2Ax antibody (in green) and DAPI. **(B)** Pictures of RPE-1 shCtrl and shGen1 diploid and tetraploid cells, labelled using 53BP1 (in green) and DAPI to reveal nuclear staining **(C)** Pictures of RPE-1 shCtrl and shGen1 diploid and tetraploid cells, labelled using Rad51 (in green), and DAPI to reveal nuclear staining. **(D)** Dot plot showing the number of γ -H2Ax foci per cell in interphase in the indicated conditions. **(E)** Dot plot showing the number of 53BP1 foci per cell in interphase in the indicated conditions. **(F)** Dot plot showing the number of Rad51 foci per cell in interphase in the indicated conditions. **(G)** Images showing example of errors in anaphase observed in H2B-GFP RPE-1 cells. Arrow in shGen1 diploid indicates a DNA bridge. Arrows in shCtrl tetraploid indicate bridges, lagging, polar and acentric chromosomes. **(H)** Dot plot showing the mitotic duration (in min) in RPE-1 cells carrying H2B-GFP reporter, in the indicated conditions (N= 46 cells for shCtrl diploid, N= 50 cells for shGen1 diploid, N= 42 cells for shCtrl tetraploid, N= 14 cells for shGen1 tetraploid). **(I)** Bar plot showing the frequency of errors in anaphase observed in each condition. (D=diploid, T=tetraploid, MS=mitotic slippage treatment).

Figure S6. Additional information for figure 4

(A) Dot plot showing the cell cycle duration (from NEBD to NEBD) in RPE-1 cells in the indicated genotypes. **(B)** Graph showing the volume ratio of p53 and p21 levels relative to GAPDH levels and normalized to controls in shCtrl and shGen1 diploid and tetraploid RPE-1 cells, from 2 (p53) or 3 (p21) independent experiments. **(C)** Schematic showing the experimental procedure for sorting cells for clonogenic assay. **(D)** Clonogenic assay for diploid shCtrl and shGen1 cells, grown after 8 days.

Chapter 4 – Discussion

1. On polyploidy and its contribution to genetic instability

In the results section 1, I identified a potential role for two nucleases – Mre11 and Gen - in the generation of DNA damage at mitotic entry in unscheduled polyploid NBs. Indeed, even if the mechanisms by which polyploidy contributes to genetic instability are being more and more described (Davoli & De Lange, 2011; Ganem et al., 2007; Schoenfelder & Fox, 2015; Storchova & Kuffer, 2008; Storchova & Pellman, 2004a), the role of cell cycle asynchrony remains poorly explored. Since such asynchrony has also been found in mouse neural stem cells and ovarian cancer cell line after induction of cytokinesis failure (Nano et al., 2019), it is likely that DNA damage occurring at mitotic entry might contribute to genetic instability in other cell types.

In the model described in the results - section 1, I propose that Gen, which is enriched in the cytoplasm, gets access to the mitotically delayed nucleus because of premature NEBD, leading to DNA damage. Further time-lapse imaging of Gen-mCh combined with a DNA damage sensor, such as RPA (Nano et al., 2019) would be required to assess the temporality of DNA damage generation with Gen localization at mitotic entry in polyploid NBs. In the case of Mre11, which is found both in the nucleus and the cytoplasm, one could hypothesize that it gets activated at mitotic entry, and target specifically the non-mitotic nucleus. Both nucleases have been previously described to be active during mitosis, with Gen1 resolving recombination intermediates in yeast and mammals (Y. W. Chan & West, 2014a; Garner et al., 2013; J. H. Kim et al., 2022; Matos & West, 2014), and Mre11 in the tethering of broken chromosomes in *Drosophila* (Landmann et al., 2020), and DNA resection in mitosis (Peterson et al., 2011). It is also important to mention that, since I found that many nucleases are important for polyploid cell proliferation, the RNAi screen did not allow me to investigate their role in asynchronous polyploid cells. Thus, it remains plausible that other nucleases could have a role in DNA damage generation in mitotically delayed nuclei. This could be assessed easily in the other model systems described above, as cytokinesis failure in these system leads to cells that are mostly binucleated and never reach the multinucleation levels of *Drosophila* NBs that keep proliferating (Nano et al., 2019).

Additionally, since Mre11 is a major factor involved in the DNA damage response and in the detection of DSB (Garcia et al., 2011; Hopfner, 2023; Lee & Paull, 2005; Shibata et al., 2014; Stracker & Petrini, 2011), one possibility would be that decrease of DNA damage upon Mre11

depletion reflects a decrease in global DNA damage signaling rather than a real decrease in DNA damage generation.

Importantly, the reasons of the vulnerability of the mitotically delayed nucleus remain to be understood. Since Gen and Mre11 are both structure-specific nucleases, one could suggest that the delayed-nucleus carries specific structures that are recognized by nucleases, such as stalled replication forks (Chappidi et al., 2020; Coquel et al., 2018; Kanai et al., 2007; Pasero & Vindigni, 2017; Pepe & West, 2014; Scully et al., 2021). This would be an attractive hypothesis, as MRE11 has been described to be involved in replication fork stability (Kolinjivadi et al., 2017; Lemaçon et al., 2017; Stracker & Petrini, 2011), and GEN1 to be able to cleave replication fork in vitro (Rass et al., 2010; Garner et al., 2013). However, EdU incorporation experiments conducted in polyploid NBs revealed that polyploid NBs in mitosis are not synthesizing DNA (Nano et al., 2019). Therefore, it remains unlikely that mitotically-delayed nuclei carry stalled replication forks. However, another possibility would be that there are still replication or recombination intermediates, such as HJs, which are remaining in the delayed nuclei, and are prematurely resolved by nucleases, which would lead to DSBs. Another hypothesis would be that delayed nuclei are less condensed, and so the chromatin is more accessible for the nucleases, compared to the highly condensed chromatin in the neighbour mitotic DNA. It would be interesting to monitor chromatin compaction in the delayed nucleus and assess DNA damage levels.

Interestingly, such cycle asynchrony has also been observed in cells containing micronuclei. More precisely, it has been described that micronuclei undergo asynchronous DNA replication, resulting in DNA damage and fragmentation of the chromosome in the micronucleus (Crasta et al., 2012; Zhang et al., 2016). In this case, it has been proposed that premature chromosome condensation (PCC) is responsible for chromosome shattering, as described in cell fusion experiments (Johnson and Rao., 1970). Indeed, forcing mitotic entry of an S phase nucleus leads to chromosome breaks and fragmentation. Once again, this mechanism requires the delayed nucleus to be in S-phase to be shattered, as forcing mitotic entry of a G1 or G2 nucleus does not lead to chromosome breakage (Pantelias et al., 2019; Terzoudi et al., 2015). Recently, micronuclei were shown to accumulate large amount of RNA-DNA hybrids, which are further cleaved by a mechanism involving the BER apurinic/apyrimidinic endonuclease (APE1), leading to chromosome breakage in micronuclei

(Tang et al., 2022). Thus, it would be interesting to assess if such RNA-DNA hybrids are present in the delayed-nuclei of asynchronous polyploid cells, and are targeted by endonucleases.

Furthermore, the mechanisms explaining asynchronous cell cycle progression in polyploid cells remain to be identified. One could suggest that each nucleus would have individual cell cycle regulation. In agreement with this hypothesis, it has been shown that in newly fertilized binucleated sea urchin embryos, the two pronuclei enter mitosis asynchronously if one nucleus is arrested due to DNA damage (Hinchcliffe et al., 1999). In addition, multinucleated polyploid yeast only activate DNA damage checkpoint in the nucleus with DNA damage, and do not spread to the other nuclei (Demeter et al., 2000). Therefore, it is tempting to suggest that during interphase, cell cycle asynchrony between nuclei is due to the availability of cell cycle components and checkpoint activation. According to this view, at mitotic entry, one could also hypothesize that activation of a “mitotic catalyzer”, such as CDK1-CyclinB, in a region of a multinucleated cell would spread to other region of the cell and triggers mitotic entry of the delayed-nuclei.

Eventually, since DNA damage resulting from cell cycle asynchrony has been described in mouse neural stem cells and ovarian cancer cell line in culture upon unscheduled polyploidization, the role of these two candidates remains to be described in these models.

Overall, if Gen and Mre11 appear to be attractive candidates for DNA damage generation in asynchronous polyploid cells, the mechanism by which they induce this damage still need further explorations. However, regardless of the role of nucleases in DNA damage generation, the consequences of such DNA damage in asynchronous cells at mitotic entry are still not understood and remain a potential source of genetic instability in human tumors.

2. On factors essential for polyploid cell proliferation

In the results section 2, we described a novel role for Gen1 nuclease after whole genome duplication. Interestingly, Gen1 appears to be dispensable in diploid cells, which is consistent with previous studies showing that *Drosophila* Gen only becomes essential in the absence of BLM (Andersen et al., 2011). A compensation mechanism between structure-selective nucleases (Mus81, Slx1, Gen1) has also been described, suggesting that loss of Gen1 can be overcome by other resolvases in diploid cells (Andersen et al., 2011; Garner et al., 2013;

Fernandez., 2022). In addition, both Gen1 and Mus81 have been shown to have complementary function in mouse B lymphocytes (Fernandez et al., 2022). However here, WGD status seemed to reveal a specific requirement for Gen1, while other nucleases appear to be dispensable. In addition, Gen1 was recently described to promote fragile site expression after replication stress induction (Benitez et al., 2023). Since tetraploid cells have high levels of replication stress (Gemble et al., 2022), one would hypothesize that they consequently have increased fragile site expression, and thus rely more on Gen1 activity. In agreement with this study, *Drosophila gen^{mut}* have been described to be hypersensitive to DNA damaging agents such as hydroxyurea (HU) (Bellendir et al., 2017), which generates replication stress. Surprisingly however, this phenomenon does not involve Mus81 nuclease (at least for *Drosophila* polyploid NBs), which has also been described to resolve late replication intermediates in mitosis (Minocherhomji et al., 2015; Naim et al., 2013). Studies have shown that Gen mutants have more severe defects than Mus81 mutants in *Drosophila* after DNA damage induction, advocating for a more important role for Gen in *Drosophila* compared to yeast and mammals (Bellendir et al., 2017). In addition, our work suggests that BLM helicase is not involved and the proliferation of polyploid cells either, although it was described to have an important role in response to DNA damaging agents (Andersen et al., 2011; Trowbridge et al., 2007; Adams et al., 2003).

Moreover, this work provides a novel *in vivo* characterization of Gen nuclease in *Drosophila*, not only for the proliferation of polyploid NBs, but also in terms of cell cycle and genetic stability in diploid cells. Indeed, depletion of Gen delays mitosis in diploid NBs, but also impacts the overall cell cycle duration, while adding more Gen generates faster cell cycles. Moreover, changing the levels of Gen increases mitotic errors in diploid NBs. However, neither modifications in cell cycle duration, nor increased mitotic errors seemed to affect the overall *Drosophila* development, as *gen^{mut}* flies are viable and fertile. Indeed, Gen appears to be essential only after treatment with DNA damaging agents (Bellendir et al., 2017).

Since overexpressing a nuclease-dead construct had even a more drastic effect than a complete Gen OE in polyploid cells, which increased DNA damage and large cells, it raises the hypothesis that the levels of Gen *per se* can impact cell cycle and cell proliferation. For instance, one suggestion would be that a certain amount of Gen is sufficient to signal the cell to keep proliferating. However, several studies indicate that GEN1 is a monomeric protein in solution and requires dimerization to cleave HJs, thus, we can also make the assumption that

GenOEN^D dimerizes with the endogenous Gen which would decrease its overall activity. One could also imagine that GenOEN^D would behave as a dominant negative of the endogenous form, by binding preferentially to its target site, and not allowing the binding of endogenous Gen. To distinguish between these possibilities, we would need to generate a GenOEN^D in a *gen* null background, which we failed from a genetic point of view.

Characterization of Gen1 in tetraploid RPE-1 cells revealed that shGen1 cells had increased levels of Fancd2 foci, which could be the consequence of unresolved replication intermediates occurring during replication stress. In agreement with this, Aphidicolin treatment of Gen1 KO cells lead to increased 53BP1 nuclear bodies, and micronuclei (Benitez et al., 2023). These results were also accompanied by a decrease of mitotic DNA synthesis (MiDAS), characteristic of the response to replication stress. It would be interesting to investigate whether tetraploid cells have increased MiDAS, and therefore rely on Gen1 for duplicating DNA in mitosis.

Importantly, cell cycle analysis showed that Gen1 depleted cells strongly upregulate p53. However, we detected a mitotic bypass only tetraploid shGen1 cells and not in their diploid counterparts. Since the overall DNA damage level is much higher in tetraploid cells, one could think that the p53 response might be different between diploids and tetraploids. On one hand, tetraploid cells would be oriented towards a cell cycle exit via p53, while diploid cells only activate DNA repair pathways to compensate for Gen1 loss, and therefore keep proliferating. Thus, it remains important to further study the role of p53 in shGen1 and investigate whether the mitotic bypass is p53-dependent, as described in Zeng et al. (Zeng et al., 2023). In addition, tetraploid shGen1 displayed higher MN and increased multipolar mitoses compared to ctrl tetraploids. This observation might explain why only shGen1 had p53 activation, as tetraploidy has been showed to not be able to activate p53 per se (Uetake & Sluder, 2004; Wong & Stearns, 2005), while supernumerary centrosome does (Fava et al., 2017, Sladky et al., 2020). However, the mechanisms by which Gen1 depletion generates more multipolar mitoses and MN need to be further explored.

Importantly, whole-transcriptomics analysis revealed an increased Rad51 expression in tumors that underwent WGD. This is consistent with previous genome-wide analysis in budding yeast, showing that mutations in HR factors are “ploidy-specific lethal (Storchova et al., 2006). This was later confirmed in WGD tumors, which have been showed to be more dependent on Rad51 than non-WGD tumors (Quinton et al., 2021). Surprisingly enough, this

analysis did not show any increased expression of Mus81. Even if this result correlates with our data obtained in *Drosophila*, in which Mus81 depletion did not affect polyploid NBs proliferation, the hierarchy between Mus81 and Gen1 is believed to be inverted in mammals compared to *Drosophila* (Bellendir et al., 2017; . Thus, one could hypothesize that Mus81 would have a more important role the proliferation of tetraploid RPE-1 cells than in *Drosophila* polyploid NBs. Therefore, it remains important to confirm that the depletion of Mus81 do not affect tetraploid RPE-1 proliferation.

Overall, this study supports a model by which Gen1 is involved in the survival of tetraploid cells, presumably through at least two mechanisms. The first one might be via its role in the resolution of recombination intermediates, as HR is believed to be important for polyploid cell proliferation (Storchova et al., 2006; Quinton et al., 2021). Secondly, we propose that Gen1 might be essential to resolve replication intermediates in response to replication stress occurring in tetraploid cells.

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RÉSUMÉ

La polyplœidie - la présence de plusieurs copies de chaque paire de chromosome - se retrouve couramment dans de nombreux processus physiologiques, notamment car elle favorise l'augmentation de la taille des cellules, l'augmentation de la capacité métabolique, ou encore la régénération des tissus. En revanche, les duplications du génome sont souvent retrouvées dans de nombreuses tumeurs et génèrent une instabilité génétique, ce qui contribue à une évolution rapide des génomes des cellules cancéreuses. Cependant, les mécanismes moléculaires liant la polyplœidie à l'instabilité génétique sont encore mal compris.

Au cours de ma thèse, j'ai étudié les voies qui deviennent essentielles lors de la prolifération des cellules polyplœides. Pour cela, notre laboratoire a développé un modèle *in vivo* de polyplœidie non programmée, en utilisant des cellules souches neurales de drosophile, également appelées neuroblastes (NBs). Après l'induction de la polyplœidie, ces cellules peuvent continuer à proliférer et atteindre des niveaux élevés de plœidie, tout en accumulant des dommages à l'ADN.

Afin d'identifier les facteurs essentiels à la prolifération des cellules polyplœides, j'ai orienté mes recherches vers plusieurs membres des voies de réparation de l'ADN. J'ai identifié certains facteurs qui affectent la prolifération des NBs polyplœides. Parmi eux, j'ai découvert que Gen/GEN1, une nucléase impliquée dans la résolution des intermédiaires de recombinaison et de réplication, est absolument nécessaire à la prolifération des cellules polyplœides, alors qu'elle est dispensable dans les cellules diploïdes.

Afin de mieux caractériser le mécanisme par lequel Gen favorise la prolifération des cellules polyplœides, j'ai étudié son rôle dans les lignées cellulaires RPE1 humaines après induction de la tétraploïdie. J'ai constaté que, dans leur premier cycle cellulaire après duplication du génome, les cellules tétraploïdes dans lesquelles GEN1 a été déplété ont une accumulation de marqueurs de stress replicatif, suggérant que GEN1 est nécessaire pour la résolution des régions sous-répliquées dans les cellules tétraploïdes. À l'aide de rapporteurs du cycle cellulaire, j'ai également décrit que la suppression de GEN1 dans les cellules tétraploïdes génère un « bypass mitotique » suivi d'une sortie du cycle cellulaire, accompagné d'une forte activation de p53, tandis que les cellules tétraploïdes continuent de se diviser sans activation de point de contrôle. Enfin, grâce à l'analyse bioinformatique, nous avons découvert que les tumeurs avec des niveaux élevés de cellules tétraploïdes ont une augmentation de l'expression de GEN1, suggérant un besoin de Gen1 pour la survie de ces tumeurs. Ces travaux ont permis de révéler un nouveau rôle pour la nucléase Gen1 dans la prolifération de cellules polyplœides non programmées.

MOTS CLÉS

Polyplœidie / Instabilité génétique / Prolifération / Cancer / Damage à l'ADN

ABSTRACT

Polyploidy – the presence of multiple sets of all chromosomes– is commonly found in many physiological processes, as it is known to promote cell size increase, increased metabolic capacity, barrier function or even tissue regeneration. In contrast, whole genome duplications (WGD) are found in many human tumors and generate genetic instability, which contributes to a rapid evolution of cancer genomes. However, the molecular mechanisms linking polyploidy to genetic instability are still poorly understood. Our lab recently established that in the first S-phase following unscheduled WGD, cells accumulate DNA damage as they fail to efficiently replicate their genome, because of a sub-scaling of DNA replication factors. Here, I investigated the pathways becoming essential during polyploid cell proliferation.

To do so, we developed an in vivo model of WGD, using *Drosophila* neural stem cells, also called neuroblasts (NBs). After polyploidy induction, these cells can continue to proliferate and reach high levels of ploidy, while accumulating DNA damage. To identify essential factors for polyploid cell proliferation, I screened for several members of DNA repair pathways. I identified some factors that significantly affected the proliferation of polyploid NBs. Most importantly, among them, I found that Gen/GEN1, a nuclease involved in the resolution of recombination and replication intermediates, is absolutely required for polyploid cell proliferation, while it is dispensable in diploid cells. Strikingly, depletion of Gen in polyploid NBs slows down the cell cycle, while Gen overexpression results in accelerated cell cycles.

To better characterize the mechanism by which Gen1 promotes polyploid cell proliferation, I investigated its role in human RPE1 cell lines after inducing tetraploidization. I found that, in their first cell cycle after WGD, tetraploid cells depleted from Gen1 have increased levels of Fancd2 foci. This suggests that Gen1 is required for the resolution of under replicated regions in tetraploid cells. Using cell cycle reporters, I also described that removing Gen1 in tetraploid cells generate a “mitotic bypass” followed by a cell cycle exit, accompanied by a strong p53 activation, while tetraploid cells kept dividing without checkpoint activation.

Finally, through bioinformatics analysis, we found that tumors with high levels of tetraploid cells have increased levels of Gen1, suggesting a requirement for Gen1 for their survival. Altogether, this worked revealed a new role for the Gen1 nuclease in the proliferation of unscheduled polyploid cells.

KEYWORDS

Polyploidy / Genetic instability / Proliferation / Cancer / DNA damage

