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IMPACT OF HUMAN ANEUPLOIDY ON CHROMOSOMAL STABILITY

ANA RITA DE SOUSA CASTRO

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Supervisor: Dr. Elsa Clara Carvalho Logarinho Santos
Assistant Researcher, Instituto de Investigação e Inovação em Saúde, I3S
Group leader of Aging and Aneuploidy Lab, Instituto de Biologia Molecular e Celular (IBMC)

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Abstract

Chromosome segregation fidelity is an issue of major concern during mitosis to guarantee the successful distribution of all the chromosomes to both daughter cells. Lack of chromosome segregation fidelity results in an imbalanced distribution of chromosomes to the daughter cells, a condition known as aneuploidy. Aneuploidy is the most common chromosomal abnormality in humans. The close link between aneuploidy and miscarriages, birth defects, mental retardation and aging in humans is now widely acknowledged. Besides, both aneuploidy and chromosomal instability (CIN) - an increased rate of chromosome mis-segregation - have for long been identified as common tumor phenotypes and found to endow tumor aggressiveness and drug resistance. However, their roles in tumor initiation and evolution are still largely dubious. It is well known that chromosome mis-segregation leads to aneuploidy. However, and despite their widespread prevalence in cancer, the role of aneuploidy on chromosomal instability remains elusive. The complex and variable cancer karyotypes likely explain the difficulties faced by previous. In humans, trisomy 21 (or Down syndrome) is one of the best-known constitutional aneuploidies, characterized by the presence of an extra copy of the chromosome 21. Besides 21, only 13 (or Patau syndrome) and 18 trisomies (or Edwards syndrome) are viable to birth in humans. These human constitutional trisomies hold an untransformed aneuploid karyotype that is highly homogeneous and thus favorable to measure chromosomal instability in the absence of tumor karyotype complexity.

To uncover the causative role between aneuploidy on chromosomal instability, we used a simplified cellular model of human primary cells karyotyped as 13, 18 or 21 trisomies established from surplus pre-natal diagnosis samples. First, our findings support that aneuploidy impairs normal chromosome segregation, leading to CIN and triggering further aneuploidy. We showed that distinct trisomies induce similar rates of chromosome mis-segregation. However, depending on the extra chromosome, the mitotic phenotypes observed in aneuploid cells are distinct, i.e., different mechanisms contribute to chromosome mis-segregation. In addition, even though the overall rates of chromosome mis-segregation are similar in all trisomies, we found karyotype-specific patterns of mis-segregation associated with each trisomy, inclusively a biased chromosome mis-segregation for the trisomic chromosome in the trisomies 13 and 18. Therefore, our data demonstrate that aneuploidy triggers chromosomal instability in untransformed cells and suggest that different chromosomes mis-segregate at distinct rates depending on the aneuploid karyotype.

Keywords: *Mitosis, aneuploidy, chromosomal instability, trisomy, cancer.*

Resumo

A correta distribuição dos cromossomas durante a divisão celular é de fulcral importância para se assegurar a fidelidade da transmissão genética. Erros na distribuição dos cromossomas durante a divisão originam células filhas com um número desigual de cromossomas, uma condição denominada de aneuploidia. Atualmente, a aneuploidia é a anomalia cromossómica mais comum na espécie humana e há muito tem despertado o interesse científico devido à sua associação com abortamentos, defeitos congénitos, atraso mental e envelhecimento. Também as células cancerosas são notoriamente aneuploides e apresentam reiteradamente erros na distribuição dos cromossomas, condição designada de instabilidade cromossómica. Acredita-se que a acumulação sucessiva de anomalias ao longo das divisões está na origem das características de agressividade e resistência terapêutica das células tumorais aneuploides. Contudo, o seu papel na iniciação e evolução tumoral é ainda ambíguo. É também amplamente aceite que erros na distribuição de cromossomas conduz, indubitavelmente, à aneuploidia. Todavia, e mesmo apesar da sua prevalência no cancro, não são ainda totalmente conhecidas as consequências da aneuploidia na instabilidade cromossómica. Uma possível explicação para este fato prende-se com as dificuldades encontradas no estudo de cariótipos tumorais, altamente complexos e variáveis. No humano, existem aneuploidias constitucionais associadas a defeitos do recém-nascido, entre as quais a trissomia 21 (ou síndrome de Down) – caracterizada pela presença de uma cópia extra do cromossoma 21 - é a mais comum e melhor estudada. Para além da trisomia do 21, apenas a trissomia do 13 (sindrome de Patau) e a trissomia do 18 (sindrome de Edwards) resultam num número viável de deficiências à nascença. Estas aneuploidias constitucionais apresentam um cariótipo aneuploide homogéneo que é assim favorável ao estudo da instabilidade cromossómica

De modo a elucidar a complexa relação entre a aneuploidia e a instabilidade cromossómica, foram usadas neste estudo células humanas primárias, cariotipadas como trissomia do 13, 18 e 21, obtidas a partir de amostras recolhidas no diagnóstico pré-natal. Este trabalho demonstrou que a aneuploidia compromete a correta distribuição cromossómica, gerando instabilidade cromossómica e, consequentemente, mais aneuploidia. Contudo, foram observados diferentes fenótipos mitóticos nas distintas aneuploidias, sugerindo a existência de mecanismos cariótipo-específicos na origem da instabilidade cromossómica. Para além disso, este trabalho demonstrou que, mesmo apesar de diferentes trissomias induzirem taxas semelhantes de distribuição errónea de cromossomas, estas originam padrões específicos no que diz respeito à taxa

de distribuição errónea de cada cromossoma. Inclusivamente, existe maior tendência do cromossoma extra na trissomia do 13 ou 18 para sofrer erro de distribuição durante a divisão da célula trissómica. Em resumo, este trabalho demonstra que a aneuploidia gera instabilidade cromossómica e sugere que diferentes cromossomas apresentam distinta propensão a erros na segregação de acordo com o cariótipo aneuploide em questão.

Palavras chave: *Mitose, aneuploidia, instabilidade cromossómica, trissomia, cancro.*

Aos meus Pais, à Fabiana e ao Fábio,

No intelligent idea can gain general acceptance unless some stupidity is mixed in with it.

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Table of Contents

2. MATERIALS AND METHODS

List of figures

List of tables

List of Equations

Abbreviations

aCGH	Array-based comparative genomic hybridization
AO	Anaphase Onset
APC/C	Anaphase-promoting complex/cyclosome
ATP	Adenosine Triphosphate;
Cdks	Cyclin-dependent kinases
CIN	Chromosomal Instability
CHMP1B	Charged multivesicular body protein 1B
CTs	Chromosome Territories
CycB1	Cyclin B1
DMEM	Dulbecco´s Modified Eagle Medium
FISH	Fluorescence *in situ* hybridization
GIN	Genomic instability
GFP	Green fluorescent protein
KT	Kinetochores
MCC	Mitotic checkpoint complex
MT	Microtubule
MSI	micro- and mini-satellite instability
NEB	Nuclear Envelope Breakdown
PCNT	Pericentrin
PCR	Polymerase chain reaction
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type
Plks	Polo-like kinases
SAC	Spindle Assembly Checkpoint
S-CIN	Structural chromosome instability
UPS	Ubiquitin-proteasome system
W-CIN	Whole chromosome instability
SNP	Single nucleotide polymorphism
SPG20	Spastic Paraplegia 20
STLC	S-trityl-L-cysteine

1

INTRODUCTION

1.1 - The Cell cycle: phases and DNA structure checkpoints

Faithful distribution of the genetic information to the daughter cells is a critical process to ensure organism viability. The cell's self-replication process includes several phases, such as cell components duplication, genome duplication and metabolic changes, collectively known as cellular life cycle, or cell cycle. Cell cycle is generally divided into four phases: G_1 phase, corresponding to the interval where cell grows but does not replicate its DNA; S phase, where the genomic information is duplicated; G_2 phase during which cell growth continues and protein synthesis takes place; mitotic phase or M-phase, corresponding to cell division. Together, G_1, S and G_2 phase are referred as interphase. The mitotic phase can be subdivided in two main phases: mitosis, corresponding to the nuclear division, and cytokinesis, when the cytoplasm of a single cell is divided to form two daughter cells (Cooper, 2000).

Cells can also exit cell cycle and enter a quiescent state in response to adverse conditions, such as nutrient or growth factor deprivations, or in response to inhibitory signals from other cells and intracellular damage. This cell state is commonly referred as G_0 and is considered as a special phase that can occur just before G_1 or in cells while in G_1. In the case of nutrient or growth factor starvation, cell cycle exit is reversible. However, some cells remain in G_0 for the entire lifetime of the organism, referred to as terminally differentiated cells (Morgan, 2007).

As evidenced by microscope observations of human cell cycle, the eukaryotic cell cycle takes typically 24h to be completed. Approximately 95% of the cell cycle duration corresponds to interphase, where chromosomes present a decondensed morphology and uniform nuclear distribution. As so, G_1, S, and G_2 cell cycle phases cannot be distinguished by microscope observations. Curiously, the most critical phase during cell division, mitosis, takes approximately only one hour to be completed and can be easily identified under the microscope (Cooper, 2000).

In diploid organisms, there is two of each chromosome type (2N). In humans, a diploid cell exhibits 46 chromosomes organized in 23 pairs, 22 pairs of autosomes (pairs 1-22) and one pair of heterosomes (23 pair), that are duplicated during S-phase and then equally distributed to two daughter cells during mitosis (Alberts et al., 2002).

To ensure the proper cell growth and division, cell must pass through a series of DNA structure checkpoints, complex networks of regulatory protein, which governs cell cycle progression in response to improper or incomplete DNA replication (Cooper, 2000). Therefore, this system is essential in preventing chromosomal imbalances in newly produced daughter cells and in the maintenance of genomic integrity.

1.1.1 – The cell cycle control system

The cell cycle control system is responsible for regulating cell cycle events and progression, blocking cell cycle progression into the next phase when the cell either fails an essential cell-cycle process or meets unfavorable environmental conditions (Hartwell & Weinert, 1989). The progression through cell cycle phases is mainly controlled by three constitutively active checkpoints. The first occurs at G1/S transition, is called 'start' or the G1 checkpoint, and is responsible for arresting cycle progression if environmental or cellular conditions are not favorable. The G2 checkpoint occurs at G2/M transition and checks if DNA was properly replicated so that division can correctly occur. Finally, the third checkpoint is at the anaphase-to-metaphase transition and is the Spindle Assembly Checkpoint (SAC) which checks for correct chromosome-microtubule attachments and proper chromosome alignment at the metaphase plate (Alberts et al., 2002; Morgan, 2007). The SAC will be revised with more detail later on in this chapter.

The basic components of the cell cycle control system are a family of protein kinases known as cyclin-dependent kinases (Cdks). Cdks activity depends on a separate regulatory protein subunit, called cyclin, to become enzymatically active. The fluctuations on the amount of cyclins are responsible for regulating Cdks oscillatory activity throughout the cell cycle (Alberts et al., 2002). Depending on the type of cyclins produced in each cell cycle phase, different cyclin-Cdk complexes will be formed, triggering distinct cell cycle events. This mechanism could be partly explained by the fact that different cyclin-Cdk complexes phosphorylate different sets of substrate proteins (Minshull, Blow, & Hunt, 1989).

In vertebrates, there are four cyclins (A, B, D, and E) involved cell cycle regulation. At early S phase, cyclins D and E must be degraded, and progression through S phase is then controlled by cyclin A-Cdk1 activity. In the beginning of M phase, cyclin A is degraded and cyclin B-Cdk1 is required to the G2/M transition. Finally, the degradation of cyclin B is

necessary for mitotic exit. At mitosis, e.g. an increase in Cdk activity in this phase is responsible for the control of nuclear envelope breakdown and spindle assembly (Alberts et al., 2002; Minshull et al., 1989).

When functioning properly, cell cycle regulatory proteins control cell growth and induce the death of damaged cells. Disruption of the normal functioning of the cell cycle control system can result in catastrophic consequences for the cell, leading to carcinogenesis and tumor development.

1.2 - Mitosis

Mitosis is the most critical phase during cell division. It has been studied since the early 1880s, when Walther Flemming first described the cell division process. Although several decades have passed since then, we now have a detailed, but still incomplete knowledge of mitosis (Rieder & Khodjakov, 2003). In mammals, a set of distinct events organizes mitosis into five different phases: prophase, prometaphase, metaphase, anaphase and telophase. During prophase the chromatin[1] condenses forming discrete chromosomes that will be independently moved by the mitotic spindle apparatus[2]. Chromatin condensation is followed by cytoskeleton reorganization, where the centrosome[3] divides to generate two centriole pairs that start to move to opposite sides of the nucleus by late prophase, to form the two poles of the mitotic spindle. At prometaphase, the nuclear envelope[4] breaks down, leading to further chromatin condensation and allowing the spindle microtubules to interact and align the fully condensed chromosomes at the metaphase plate. In metaphase, the bi-oriented chromosomes are aligned at the spindle equator and the spindle forms a clear bipolar structure. Metaphase chromosomes must have sister kinetochores[5] attached to microtubules emanating from opposite poles. At anaphase, sister chromatids separate synchronously, with kinetochore microtubules pulling daughter chromosomes towards opposite poles. At late anaphase, the spindle poles are already wide apart, as well as the two groups of

1 Chromatin: DNA - protein complex that forms chromosomes within the nucleus of eukaryotic cells.

2 Mitotic spindle apparatus: network of microtubules that forms during mitosis and meiosis of a eukaryotic cell. Some of these microtubules attach to individual chromosomes at their kinetochores and separated toward opposite poles of the dividing cell.

3 Centrosome: the major microtubule-organizing center of the cell consisting of two centrioles surrounded by pericentriolar material. The centrosome is duplicated during the cell cycle and in mitosis the two centrosomes form the poles of the mitotic spindle.

4 Nuclear envelope: structure that surrounds and defines the nucleus and separates it from the cytoplasm. It contains specialized nuclear pore structures that permit communication and transport between the nucleus and cytoplasm.

5 Kinetochore: large protein complexes that assemble at the centromere of each chromosome, and serves to connect the chromosome to microtubules in the mitotic spindle.

chromosomes (Gorbsky, 1992). Finally, by telophase, the daughter nuclei re-form, with the rearrangement of a nuclear envelope around the DNA followed by chromosome de-condensation. By the end of telophase, the spindle disassembles and two nuclei are formed, each with a group of daughter chromosomes and a centrosome (Figure 1). By that time, cytokinesis is almost complete, with the midbody[6] still connecting the two daughter cells (Rieder & Khodjakov, 2003). Abscission (midbody cleavage) is the final stage of cytokinesis, physically dividing a single mitotic cell into two daughter cells (Alberts et al., 2002).

It should be noted that mitotic duration is often considered as the time from nuclear envelope breakdown (NEB) up to anaphase onset (AO) in the current practice, as abscission, the complete separation of two daughter cells at the end of cytokinesis, may actually occur after the beginning of the next cell cycle (Gershony, Pe'er, Noach-Hirsh, Elia, & Tzur, 2014).

A tight regulation of nuclear events during mitosis is crucial for the symmetrical chromosome segregation by the two daughter cells. Such regulation is performed by the cell-cycle control system in two main phases: early mitosis, comprising prophase, prometaphase and metaphase, and late mitosis, including anaphase and telophase.

1.2.1 - Early mitosis

The G2/M transition is triggered by an increase in mitotic cyclin-Cdk complexes, which in turn drives events in early mitosis. In vertebrates, Cdk1 and cyclin B1 (cycB1) form one of the mitotic cyclin-Cdk complexes. This complex is composed by the catalytic subunit Cdk1, which is keep inactive during G2 due to the activity of two Cdk1 inhibitory protein kinases, Myt1 and Wee1. Dephosphorylation of the Cdk1 catalytic subunit by Cdc25 protein phosphatase in late G2 phase activates the CycB1-Cdk1 complex and triggers the initiation of mitosis. Once activated, the mitotic cyclin-Cdk complexes induce chromosome condensation, nuclear envelope breakdown, assembly of the mitotic spindle apparatus, and chromosome alignment at the metaphase plate (Sullivan & Morgan, 2007). Active CycB1-Cdk1 activates the Polo-like kinase 1 (Plk1) that in turn simultaneously inhibits Myt1 and Wee1 and activates Cdc25, generating a positive feedback loop (Abrieu et al., 1998; Mailand et al., 2002).

6 Midbody: microtubule-rich structure extending out of each daughter cell and terminate within the overlap region (polar microtubules).

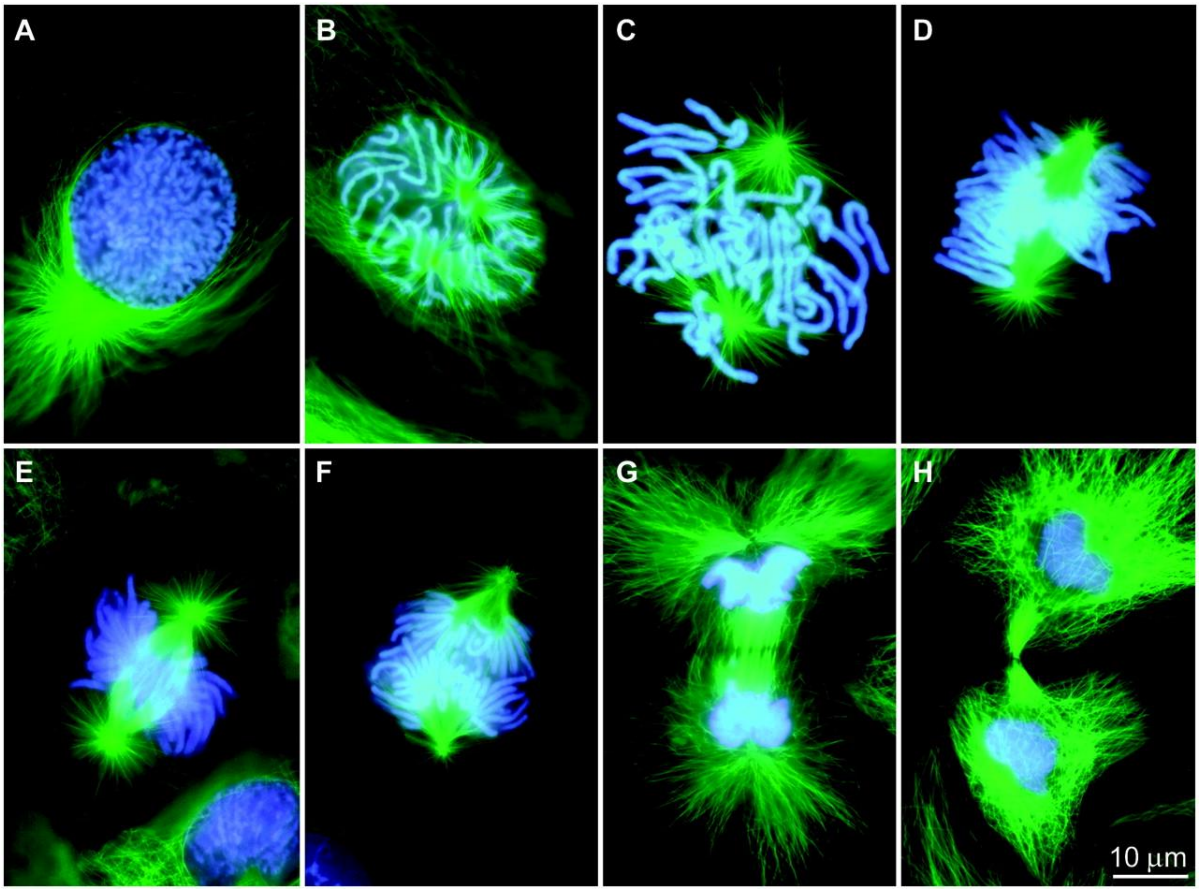

Figure 1.1 | Mitosis. Fluorescence micrographs representing the different stages of mitosis in fixed newt lung cells. Microtubules are show in green and chromosomes in blue. A, early prophase; B-D, prometaphase; E, metaphase; F, early anaphase; G, late anaphase; H, late telophase (Rieder & Khodjakov, 2003).

Although Cdks are main regulators of mitosis, Polo-like kinases (Plks) and Aurora kinases are mitotic kinases phosphorylated by CycB1-Cdk1 equally important for mitotic progression. Plks are involved in spindle assembly, kinetochore function and cytokinesis. Aurora A is involved in spindle assembly and centrosome function, whereas Aurora B has a role on chromosome condensation, spindle assembly, kinetochore-microtubule attachments, sister-chromatid segregation and cytokinesis. Cell cycle progression is driven by Cdk activity as far as metaphase. However, when all the chromosomes are properly attached to spindle microtubules, the mitotic Cdk complexes activate the anaphase-promoting complex/cyclosome (APC/C)[7] (Morgan, 2007). APC/C is a regulatory component responsible for targeting anaphase inhibitors for proteasome degradation (Sullivan & Morgan, 2007).

7 APC/C: A E3 ubiquitin ligase, downstream target of the SAC, ligase that targets several proteins for proteolytic degradation, including mitotic cyclins.

1.2.2 - The Spindle Assembly Checkpoint

The spindle assembly checkpoint (also referred as mitotic or M-phase checkpoint) is the key division control mechanism that protects against chromosome mis-segregation events and thereafter the emergence of aneuploid cells (Musacchio & Salmon, 2007). The SAC acts to delay the metaphase-to-anaphase transition through inhibition of the APC/C until all chromosomes are correctly attached to the microtubule (MT) spindle apparatus via their kinetochores (KT) and properly bi-oriented at the metaphase plate (Andrea Musacchio & Hardwick, 2002; Musacchio & Salmon, 2007). Ideally, when all KTs are properly attached to the spindle MTs in metaphase, SAC is "off" and Cdc20[8] is free to activate the APC/C. APC/C activity then targets cyclin B1 and securin for degradation. Securin is an inhibitor of separase, a protease that cleaves the kleisin subunit of the cohesin ring structure. Therefore, securin degradation allows separase to catalyse sister chromatid separation, whereas cycB1 degradation inhibits Cdk1 activity promoting mitotic exit. However, in the presence of unattached KTs at prometaphase, SAC is "on" and catalyses the formation of the mitotic checkpoint complex (MCC). MCC (composed by BubR1, Bub3, Mad2 and Cdc20) acts to sequester Cdc20 preventing APC/C activation. Because APC/C is inactive, CycB1 and Securin are not degraded and, consequently, cell cycle progression is blocked (Lara-Gonzalez, Westhorpe, & Taylor, 2012; Logarinho & Bousbaa, 2008). Importantly, components of the SAC signalling pathway are strategically positioned at the kinetochores to monitorize the KT-MT attachments and regulate the anaphase onset.

1.2.3 - Late mitosis

In a regulatory point of view, late mitosis comprises the events starting in anaphase, when the mitotic spindle segregates the duplicated chromosomes, until the mitotic exit, when there is a complete disassemble of the spindle and chromosome decondensation and packaging into two daughter nuclei. These final stages of mitosis are mainly regulated by two mechanisms: dephosphorylation of Cdk substrates and ubiquitination of APC/C substrates.
For anaphase onset and mitotic exit, Cdk1 activity is shutdown by CycB1 proteasome-mediated degradation. The proteolysis of CycB1 is thought to ensure the uni-directionality of the M/G1 phase transition and it triggers spindle disassembly and cytokinesis (McCollum & Gould, 2001; Sullivan & Morgan, 2007). While a lot was been suggested about the regulatory mechanisms involved in mitotic entry, quite few is known about the mechanisms controlling mitotic exit and cytokinesis.

8 Cdc20: regulatory subunit, APC/C co-activator protein.

1.3 - Chromosomal abnormalities: contextualizing aneuploidy

As mentioned, the regulatory mechanisms acting during mitosis are crucial for the generation of genetically identical daughter cells. However, if these mechanisms eventually fail, aneuploid daughter cells will be generated by gain or loss of genetic material (Rieder et al., 1995; Sheltzer & Amon, 2011).

Aneuploidy, an abnormal number of chromosomes in a cell that is not an exact multiple of the haploid[9] number, leads to a karyotype[10] characterized by extra or missing whole chromosomes (whole chromosome aneuploidy) or chromosome segments (segmental aneuploidy). It is crucial to understand that aneuploidy and polyploidy are not synonymous. Polyploidy is a condition characterized by a chromosome number that is more than two multiples of the haploid number. Polyploidy arises due to severe errors on mitosis or meiosis which lead to the formation of cells or gametes that have a complete set of duplicate chromosomes (Comai, 2005).

Polyploidy is differentially viable among species. It is commonly seen in plants and amphibians and most of them are well adapted to their environment. It appears that the presence of balanced number of each chromosome ensures a stable karyotype (Comai, 2005). Nonetheless, it is not very common in higher vertebrates, which report little tolerance to it (polyploid zygotes are believed to be the cause of nearly 10% of spontaneous abortions in humans). Intriguingly, polyploidy is present in a few types of somatic cells (for example hepatocytes) but its fallout is still unknown (Duncan et al., 2010; Otto & Whitton, 2000). Conversely, the biological consequences of aneuploidy are sharply different. An unbalanced copy of chromosomes translates into a gain or considerable loss of genes, which seriously impact cellular fitness compromising the maintenance of subsequent healthy cell and organism's generations (Williams et al., 2008). Besides, organisms proved to be less tolerable to loss of genetic material associated with monosomies when compared to the gain of genetic material due to trisomies (Torres, Williams, & Amon, 2008).

Acquired aneuploidy[11] leads to a huge genomic variability between somatic cells. In fact, whole chromosome aneuploidy was been reported as the major cause of miscarriages (Hassold, Hall, & Hunt, 2007), congenital birth defects, lethality (Hassold & Hunt, 2001), sterility (Martin, 2006), cancer (Williams & Amon, 2009) and neurological pathologies (i.e. schizophrenia and Alzheimer disease) (Iourov, Vorsanova, Liehr, & Yurov, 2009).

9 Haploid: describes cells that contain a single set of chromosomes.
10 Karyotype: an individual organism's complete set chromosomes.
11 Acquired aneuploidy: aneuploid state that affects differentiated somatic cells.

Controversially, a selective advantage to naturally occurring aneuploid cells was reported in certain organs (liver and brain) (Iourov, Vorsanova, & Yurov, 2010; Knouse, Wu, Whittaker, & Amon, 2014). Both human and murine hepatocytes are aneuploid, as well as a fraction of human neuroblasts. Additionally, some studies claim that aneuploidy can even improves cell proliferation and fitness (Sheltzer & Amon, 2011). In this context, aneuploidy has been shown to confer drug resistance in cancer cells (Duesberg et al., 2007), and helps budding yeast and *candida* to overcome unfavorable intrinsic and extrinsic stress conditions (Pavelka, Rancati, Zhu, et al., 2010; Selmecki, Forche, & Berman, 2006).

The aneuploid state may be an organism-wide feature if due to meiotic chromosome mis-segregation. This aneuploid state arises during gametes formation and is commonly referred as constitutional aneuploidy[12]. On the contrary, when aneuploidy is only detected in a fraction of organism's cells, it is named as mosaic aneuploidy[13]. Mosaic aneuploidy can be then subclassified in acquired aneuploidy, autosomal aneuploidy (involving autosomes: chromosomes 1-22) and sex chromosome aneuploidy (X or Y chromosomes are affected) (Jackson-Cook, 2011).

1.4 - Mechanisms of aneuploidy

Whole-chromosome aneuploidy results mainly from sporadic chromosome segregation errors during mitosis. Therefore, whole-chromosome aneuploidy will end up with two daughter cells that have gained or lost one or more individual chromosomes. Under unperturbed conditions, chromosome mis-segregation events are indeed very rare. It has been reported a range of 1/1000 to 1/10000 for human cells (Storchova, 2012).

There are multiple different mechanisms that may lead to aneuploidy (Figure 1.2). The most obvious cause of aneuploidy is a compromised SAC activity that allows chromosome segregation to occur even in the presence of unattached or incorrectly attached chromosomes to the spindle. If SAC signalling is weakened, but not completely suppressed, cells will pursue to anaphase even though not all chromosomes are properly bi-oriented (with sister KTs attached to MTs emanated from opposite spindle poles), leading to chromosome mis-segregation. As a result, one daughter cell will acquire both copies of the same chromosome(s) (Holland & Cleveland, 2009). Mutation or faulty expression of checkpoint

12 Constitutional aneuploidy: aneuploid state that arises during gametes formation/ embryonic cells.
13 Mosaic aneuploidy: aneuploid state that arises by mitotic chromosome mis-segregation.

components is accepted to be the cause of checkpoint weakness (Cahill et al., 1998). On the other hand, complete loss of this mitotic checkpoint is lethal in vertebrates due to massive chromosome mis-segregation (Tao, 2005).

Defects in kinetochore composition, microtubule dynamics or centrosome function can also increase the frequency of chromosome mis-segregation events and aneuploidy. Faulty sister chromatids cohesion and defective KT-MT connection may also result in an aneuploid state. Faulty sister chromatids cohesion is often associated with overexpression of key regulators of chromatids cohesion (i.e separase). Premature loss of chromatid cohesion can result in a random segregation of single chromatids (Nasmyth & Haering, 2009; Zhang et al., 2008).

An example of a defective KT-MT connection is the merotelic attachment, when a KT is attached to MTs coming from opposite spindle poles. Merotelically attached KTs are kept under tension and do not activate the SAC (Gregan, Polakova, Zhang, Tolić-Nørrelykke, & Cimini, 2011). Consequently, this condition often triggers anaphase lagging chromosomes, as well as sister chromatids mis-segregation to the same pole (Cimini, Fioravanti, Salmon, & Degrassi, 2002). Also, anaphase lagging chromosomes are often left out of the daughter's cell nuclei and form micronuclei (Cimini, Cameron, & Salmon, 2004).

Lastly, multipolar spindles can also escape the SAC and give rise to three or more aneuploid daughter cells. Multipolar spindles are commonly associated with supernumerary centrosomes, which in turn may arise from centriole overduplication, cytokinesis failure, or mitotic slippage (Kops, Weaver, & Cleveland, 2005; Maiato & Logarinho, 2014).

Interestingly, tetraploid cells (cells containing two complete sets of chromosomes) may also be an intermediate state in an alternative route to aneuploidy. Tetraploidy can arise as a result of an abnormal cell division, by mitotic slippage or cytokinesis failure, or due to cell fusion events (Zuzana Storchova & Pellman, 2004). Mitotic slippage occurs when kinetochores at metaphase persistently make errors attaching to the spindle. Following chronic activation of the SAC and prolonged mitotic delay, cells can scape SAC arrest without completing division, becoming tetraploid with a single nucleus and two centrosomes. Further division of tetraploid cells can foster high rates of chromosome mis-segregation due to multipolar spindle formation (King, 2008).

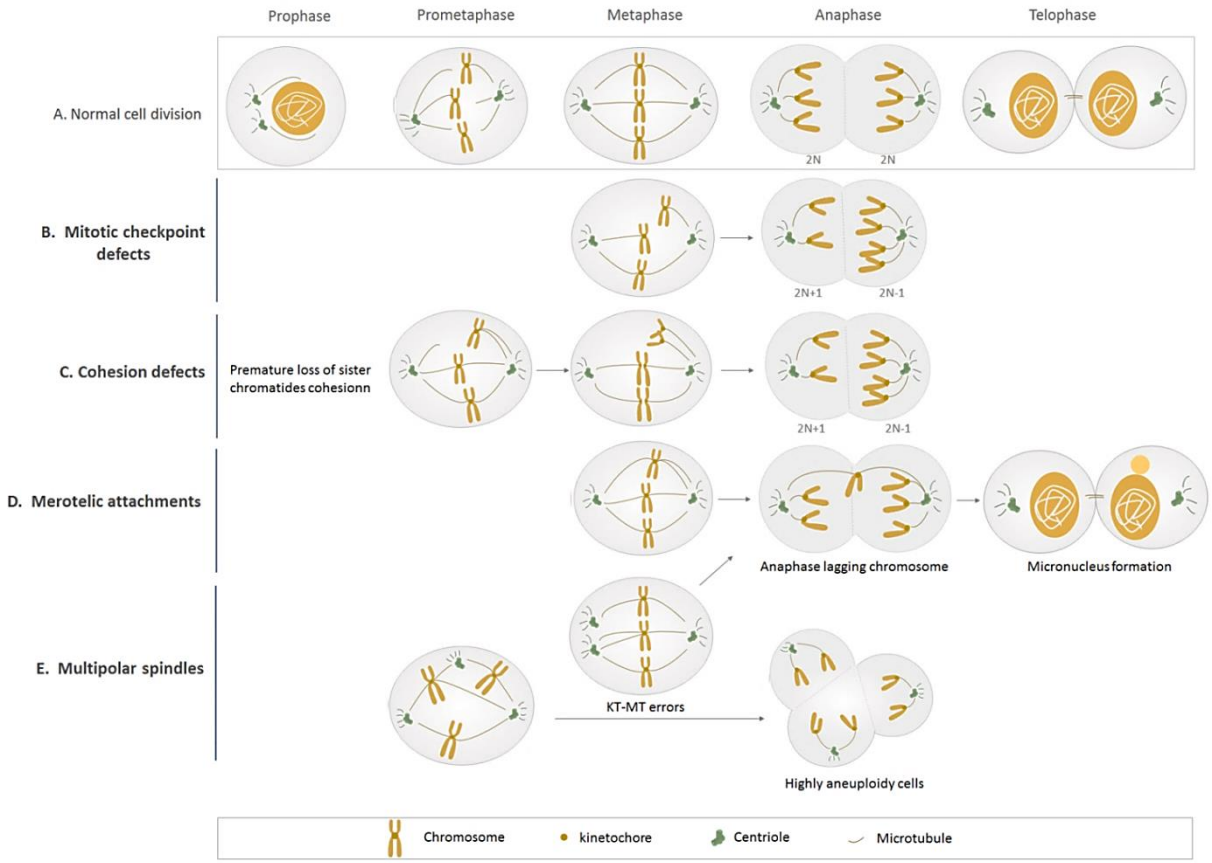

Figure 1.2 | Mechanisms in the origin of aneuploidy. **A** - Normal cell division. **B** - When the mitotic checkpoint signaling is weakened, cells will be able to start anaphase even though not all chromosomes are properly attached or bi-oriented, leading to chromosome mis-segregation. As a result, one daughter cell will acquire both copies of the same chromosome(s). **C** - Faulty sister chromatids cohesion can occur either by premature loss of sister chromatid cohesion as represented or its persistency during anaphase, resulting in chromosome mis-segregation. **D -** Merotelic attachments may trigger anaphase lagging chromosomes and sister chromatids mis-segregation to the same pole. Anaphase lagging chromosomes are often left out of the daughter's cell nuclei and form micronuclei. **E** - Multipolar spindles commonly arise in cells possessing more than two centrosomes. Centrosomes can cluster at the cell poles to allow bipolar anaphase, however this will induce formation of merotelic attachments. If multiple centrosomes do not cluster at the cell pole, highly aneuploid and unviable daughter cells will result. The diploid chromosome number is here represented by 2N.

1.5 - Aneuploidy effects on cell physiology

Several studies have proven that aneuploidy is detrimental to cell physiology and fitness of eukaryotic cells (Torres et al., 2007). Actually, their consequences on cellular fitness directly correlate with the magnitude of aneuploidy (Tang & Amon, 2013). However, it is still not completely understood whether changes in chromosome numbers trigger common

physiological effects on cells, or if additionally such effects are related to a specific karyotype combination, depending on the altered chromosome.

1.5.1 - Global effects of aneuploidy

There is a common set of phenotypes accompanying aneuploid cells, which are independent from chromosome or cell types. All aneuploid cells display slow growth, proteotoxic stress (Oromendia, Dodgson, & Amon, 2012; Stingele et al., 2012), metabolic stress (Dürrbaum et al., 2014), cell cycle arrest (Silvia Stingele et al., 2012) and increased genomic instability (Sheltzer et al., 2011). These set of phenotypes are commonly referred as aneuploidy stress response (Figure 1.3).

Aneuploid cells with extra chromosomes exhibit impaired cell grow and prolonged G1 and S phases when compared to their disomic counterparts (Torres et al., 2007). Accordingly, aneuploid cells show a down-regulation in DNA and RNA metabolism while simultaneously up-regulate energy metabolism, lysosome function and membrane biosynthesis pathways (Stingele et al., 2012). The reported changes in the energy metabolism of aneuploid cells seem to be a result of an increased energy required for translation and degradation of proteins encoded on the extra chromosome (Williams et al., 2008).

The most widely accepted explanation for the observed phenotypes is the gene dosage hypothesis, which states that the observed phenotypes are a direct consequence of the cumulative effect of the imbalance of several hundreds of individual genes. Genomic and transcriptional analyses conducted on aneuploid cells proved that gene expression directly correlates with gene copy number. Furthermore, protein translation also reflects a direct correlation with abundance of mRNA (Stingele et al., 2012), meaning that aneuploid cells will be adversely affected either by an excess or deficit of genes as a consequence of an altered chromosome number (Sheltzer, Torres, Dunham, & Amon, 2012). However, the correlation between gene copy number and gene transcription levels does not seem to be universal. Sex chromosomes have evolved dosage compensation mechanisms at the transcriptional level so that equilibrium between the two sexes could be reached. Those mechanisms are also able, at least in some extent, to compensate gene expression for allosome[14] related aneuploidy (Straub & Becker, 2007).

14 Allosome: one of the chromosomes differing in appearance or behavior from the autosomes and sometimes unequally distributed among the germ cells.

1.5.2 - Proteotoxic stress in aneuploid cells

The overexpression of several genes present in a human chromosome might burst protein homeostasis in a cell by the accumulation of useless proteins that may eventually lead to compromised cell development and disease.

The presence of an imbalanced protein composition may compromise normal cell physiology in multiple ways. First, the altered concentration of certain proteins can directly affect the cellular function of those proteins. Second, the excess of proteins can lead to the saturation of quality control mechanisms, such as protein chaperones, autophagy and the ubiquitin-proteasome system (UPS), which in turn can trigger protein misfolding and aggregation, compromising protein homeostasis (Oromendia & Amon, 2014; Stingele et al., 2012). The excess of proteins present in an aneuploid cell will lead to the depletion of available chaperones, preventing them from assisting normal cell needs. Additionally, in normal cell physiology, UPS is responsible for tagging substrates with ubiquitin, marking them for degradation by the 26S proteasome (Hicke & Dunn, 2003). UPS overwhelming can prevent misfolded proteins from being degraded, resulting in intracellular accumulation of cytotoxic protein aggregates. Third, the excess of protein-complex subunits may modify the formation of stoichiometric complexes. An unequal ratio of protein-complex subunits results in the accumulation of unstable free protein subunits or partly assembled protein complexes in the cell. Consequently, defective protein conformations will congregate in aneuploid cells, culminating in cellular proteotoxicity and stress response (Oromendia & Amon, 2014; Sheltzer et al., 2012). Attempting to achieve protein homeostasis, cells have evolved adaptive protein stress response machineries, such as UPS and autophagy, responsible for removing misfolded protein aggregates (Ciechanover, 2005). Autophagy is a lysosomal recycling pathway where dysfunctional cellular protein aggregates and organelles are triggered to degradation. Accordingly, this mechanism was found to be overactive in aneuploid cells (Mizushima, 2005). The upregulation of lysosome-mediated degradation and p62-dependent autophagy is considered as a way to counterbalance proteotoxicity caused by aneuploidy (Stingele, Stoehr, & Storchova, 2013).

1.5.3 - Genomic instability

Genomic instability (GIN) refers to a cellular state characterized by an increased frequency of accumulating genetic alterations. GIN can be divided into two main classes, nucleotide instability (including micro- and mini-satellite instability - MSI) and CIN. MSI is caused by mutations and epigenetic changes in the mismatch-repair genes, consequently resulting in a

dramatic increase of point mutations. In contrast, CIN is caused either by failure in cell cycle progression and checkpoint control or by improper chromosome segregation in mitosis, frequently leading to changes in chromosome copy number – aneuploidy (Aguilera & Gomez-Gonzalez, 2008).

Aneuploidy and genomic instability seem to be closely intertwined. It is accepted that the presence of abnormal chromosome content may result in increased genomic instability (Nicholson & Cimini, 2013; Potapova, Zhu, & Li, 2013). It has been suggested that aneuploidy increases genomic instability by several routes: I) directly increasing double-strand breaks in the lagging chromosome trapped in the cleavage furrow[15] during cytokinesis (Janssen, van der Burg, Szuhai, Kops, & Medema, 2011); II) causing imbalance in the levels of proteins required for DNA replication, repair and mitosis (Stingele et al., 2012); or even III) by inadequate replication in micronuclei, which are vulnerable to DNA damage and extensive DNA pulverization due to defects in DNA replication (Crasta et al., 2012). Zhang et al reported that micronucleus formation can indeed generate a spectrum of genomic rearrangements due to the premature condensation of DNA in the micronuclei (chromothripsis). Those genomic rearrangements appear to be restricted to the mis-segregated chromosome and to occur within one cell division (Zhang et al., 2015).

Quite recently, it was also suggested that the addition of single chromosomes to human cells stimulates genomic instability by increasing DNA damage and sensitivity to replication stress (Passerini et al., 2016).

On the other hand, genomic instability has been proposed to drive tumorigenesis by generating phenotypic diversity, which may confer adaptability to cancer cells. Importantly, it increases cell-to-cell variability that may facilitate the adaptation of cancer cell populations during metastasis (Pfau & Amon, 2012). Accordingly, genomic instability has been strongly associated with poor prognosis for certain types of cancer (Ferguson et al., 2015).

Currently, there is very limited evidence whether genomic instability is a consequence of aneuploidy or if aneuploidy arises from ongoing genomic instability. Some authors suggest that they both can trigger each other reciprocally in a vicious cycle, where an increased CIN in cancer is proportional to the degree of aneuploidy and an increase in CIN triggers further karyotype diversity (Pavelka, Rancati, Zhu, et al., 2010; Potapova et al., 2013).

15 Cleavage furrow: actin rich structure that forms a contractile ring that shrinks at the equator of the cell, pinching the plasma membrane inward.

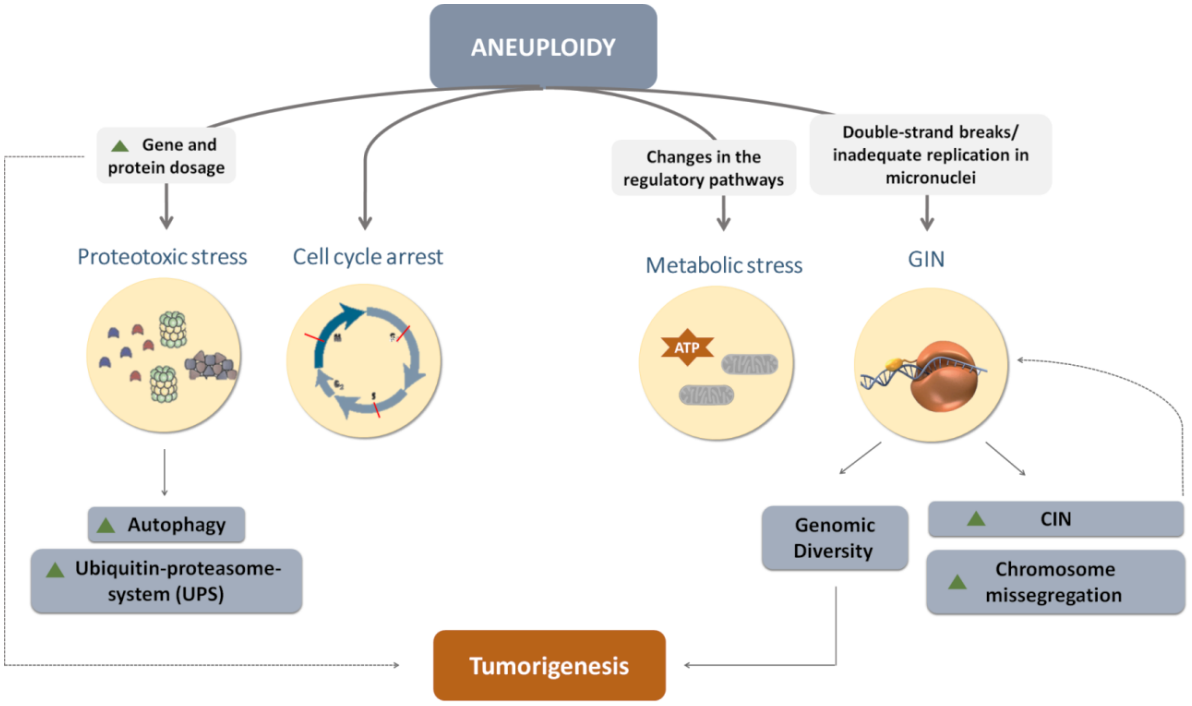

Figure 1.3 | Global effects of aneuploidy in cellular physiology. Proteotoxic stress, cell cycle arrest, metabolic stress and genomic instability are the main cellular outcomes of aneuploidy. Although aneuploidy can trigger cell death by cell cycle arrest, it is also responsible for a set of deleterious phenotypes that can ultimately lead to tumorigenesis. See text for more detail. Abbreviations: ATP –Adenosine Triphosphate; CIN – Chromosomal Instability; GIN – Genomic Instability.

1.5.4 - Karyotype-specific effects

Some studies revealed that aneuploid phenotypes differ widely and cannot be explained by general consequences of aneuploidy. Hence, it appears that aneuploid phenotypes are dependent on the gained or lost chromosome, the cell type, global karyotype and cell environment (Dodgson et al., 2016; Nicholson et al., 2015). Karyotype-specific effects of aneuploidy are caused by amplification of individual genes on the aneuploid chromosome, which may explain the paradoxical consequences on cellular fitness within aneuploid karyotypes (Pavelka, Rancati, & Li, 2010; Upender et al., 2004).

Studies on the transcriptional profile of human trisomic cell lines revealed overexpression of genes localized in the aneuploid chromosome (Stingele et al., 2012). Supporting this idea, significant changes in expression levels between chromosome 3 (comprising ~1100 genes) and chromosome 13 (comprising ~400 genes) were reported, reflecting changes dependent on the gene copy number of these chromosomes (Nicholson et al., 2015). This may explain

why only three human trisomies are viable to birth (13 trisomy or Patau syndrome; 18 trisomy or Edwards syndrome; 21 trisomy or Down syndrome). Human chromosomes 13, 18 and 21 reveal the fewest protein-coding genes on human karyotype, and hence lead to the lowest protein dosage imbalances (Torres et al., 2008). Intriguingly, 20 and 22 human chromosomes originate unviable aneuploidies, even though they also have a small number of genes. Thus, not only the number of genes, but also the identity of genes can have impact on the aneuploid cell transcriptome. An increase in the copy number of regulator genes or coding genes for a transcription factor might affect transcription levels of genes chromosome-wide (Rancati et al., 2008). Still, some studies reported aneuploidy dependent changes on DNA methylation profiles (Davidsson, Veerla, & Johansson, 2013) and chromosome replication and condensation (Kost-Alimova, Fedorova, Yang, Klein, & Imreh, 2004).

Cell type is another factor that may constrain the expression patterns. Different cell types are characterized by different expression patterns during development, which may trigger distinct cell responses to different aneuploidies. This may explain why Down syndrome patients tend to acquire haematological tumors, but have a reduced risk to develop solid tumors (Rabin & Whitlock, 2009).

1.6 - Constitutional aneuploid syndromes

Only a few aneuploidies are compatible with human life. The Patau, Edwards and Down syndromes (constitutional trisomies of chromosomes 13, 18 and 21, respectively) are the unique human aneuploidies that result in live birth.

Considering the existence of meiotic divisions underlying gametes formation, errors in human conception are quite common. Most human trisomies are incompatible with fetal development and lead to miscarriage very early in the pregnancy. The lethality associated with human trisomies reflects the deleterious effect of an abnormal chromosome number in organism development (Richards, 2010). Because chromosomes 13, 18, and 21 carry a rather small number of genes, it might explain why those trisomies are still compatible with life. Even though Edwards and Patau trisomics survive to birth, normally they die soon after birth due to severe cerebral and cardiovascular defects (Hsu & Hou, 2007). Hence, human trisomy 21 is the most-studied and better-known aneuploidy in vertebrates so far (Siegel & Amon, 2012).

As happens for other vertebrates and human trisomies, individuals with Down syndrome frequently exhibit stunted growth, mental retardation, decreased fertility, reduced life expectancy and increased rates of specific diseases (Glasson et al., 2002). Sex chromosome trisomies, such as Klinefelter's (XXY) and Triple X (XXX) syndromes, can equally succeed pregnancy and commonly result in less severe development impairments. Such is likely due to the epigenetic silencing of the X chromosome that seems to mitigate copy-number imbalances derived outcomes (Berletch, Yang, Xu, Carrel, & Disteche, 2011).

1.7 - Aneuploidy and Chromosomal Instability

CIN was initially used to describe the chromosome number variability experimented in cancer cells (Lengauer, Kinzler, & Vogelstein, 1998). Nonetheless, CIN denomination is still rather ambiguous and imprecise.

Chromosomal instability can be classified as structural and numerical. Structural CIN (S-CIN) comprises structural chromosome rearrangements (i.e. translocations, deletions and duplications of large parts of chromosomes). Numerical or whole chromosome instability (W-CIN) defines the inability to precisely segregate whole chromosomes (Ricke & van Deursen, 2013). However, CIN commonly refers to numerical instability.

1.7.1 - Chromosomal instability and cancer

CIN and aneuploidy have for long been considered as tumor hallmarks (Holland & Cleveland, 2009). 75 % of haematopoietic and 90% of solid tumors contain abnormal chromosome numbers (Storchova & Kuffer, 2008). Theodor Boveri was the first to find out a connection between aneuploidy and solid tumors, suggesting that a single aneuploid cell may lead to cancer (Boveri, 1902). Although aneuploidy has since then been hypothesized to trigger tumor formation, it remains unclear how chromosomal changes contribute to the observed malignant phenotypes. Karyotype variations in cancer cell populations are mostly caused by gains or losses of individual chromosomes. Besides, aneuploid cancer cells commonly exhibit high rates of CIN, which is the main mechanism contributing to genetic heterogeneity in cancer cells (Li et al., 2009; Nicholson & Duesberg, 2009). Such heterogeneity might promote enhanced fitness and phenotypic variability amongst cancer cells, helping on tumor adaptation and increasing drug resistance (Li, Hehlman, Sachs, & Duesberg, 2005).

Intriguingly, this opens the question: how can cancer cells have fairly stable karyotypes despite mis-segregating chromosomes at high rates? It seems that cancer karyotypes reach

a strange equilibrium between the negative effects of aneuploidy and the selection for tumor evolution. One possibility is that cancer-specific microenvironments might generate selective pressure that accelerates karyotype evolution (Nicholson & Cimini, 2013). Furthermore, aneuploid tumor cells might be able to mitigate aneuploidy negative effects resulting from chromosomal imbalances, so that they can reach high proliferation levels. Aneuploid tumor cells can do so by accumulating additional mutations, changing protein post-translation levels or becoming near tetraploid (Torres et al., 2010). Some researchers argue that aneuploidy and CIN facilitate the gain of extra copies of oncogenes and/or the loss of tumor-suppressor genes (Silk et al., 2013).

Although aneuploidy and CIN have been suggested as a driving force to cancer, some studies indicate that they might be disadvantageous for tumors in certain circumstances. High CIN rates lead to excessive aneuploidy and cell death, suppressing tumorigenesis in the presence of additional genetic damage (Kops, Foltz, & Cleveland, 2004; Sussan, Yang, Li, Ostrowski, & Reeves, 2008; Weaver, Silk, Montagna, Verdier-Pinard, & Cleveland, 2007). Other studies alternatively suggest that aneuploidy and CIN have later effects on tumorigenesis, caused by the inactivation of p53 pathway (Bunz et al., 2002; Li et al., 2010). Finally, cancer genomes contain additional mutations besides chromosome copy number changes, leading to highly complex genomes in which remains controversial the positive effects versus negative effects of CIN in tumorigenesis.

The highly complex karyotypes founded in clinically similar tumors may explain, at least partially, the faced difficulties in studying the role of aneuploidy in cancer.

1.7.2 - Does aneuploidy lead to chromosome instability?

Aneuploidy has been linked to an increased incidence of structural defects, possibly leading to condensation and chromosome replication errors (Kost-Alimova et al., 2004). It was shown that aneuploidy disturbs the normal synchronicity of allelic pair during replication. Curiously, such asynchrony was not only observed in aneuploid chromosomes, but it was been shown to be a chromosome-wide effect (Kost-Alimova et al., 2004; Loupart, Krause, & Heck, 2000).

Furthermore, studies in budding and fission yeast proposed that aneuploidy also impairs normal chromosome segregation, suggesting that aneuploidy correlates with increased CIN (Zhu, Pavelka, Bradford, Rancati, & Li, 2012). However, these authors proposed that the accuracy of the mitotic system to accurately segregate chromosomes does not directly scale with an increase in the number of chromosomes, but rather it may depend on the addition of a specific set of chromosomes (Zhu et al., 2012). Accordingly, different degrees of CIN were

observed in cells with distinct aneuploid karyotypes, suggesting that CIN may depend on the specific aneuploid karyotype (Zasadil, Britigan, & Weaver, 2013). Recently, the ability of aneuploidy to induce CIN was directly tested using human trisomies 7 and 13. In this study it was shown that the aneuploid cells studied have increased rates of anaphase chromosome mis-segregation (Nicholson et al., 2015).

Comparisons between CIN and non-CIN cells have disclosed merotelic kinetochores as the main cause of CIN (Thompson & Compton, 2008). In this context, cells with higher levels of aneuploidy were found to display an increased frequency of lagging chromosomes (Nicholson & Cimini, 2013). In turn, lagging chromosomes can promote genomic instability either by breakage during cytokinesis (Janssen et al., 2011) or due to the formation of micronuclei and chromothripsis (Crasta et al., 2012).

Reversely, CIN can lead to aneuploidy via increased chromosome mis-segregation, potentially increasing genomic instability. Indeed, some studies reveal that the degree of CIN directly correlates with karyotype stability (Nicholson & Cimini, 2013).

Overall, aneuploidy and CIN shape a vicious cycle in which chromosome imbalances impair faithful chromosome segregation, prompting further aneuploidization. On this basis, aneuploidy is the cause or a by-product of CIN. Nevertheless, this close relation between chromosomal stability and aneuploidy is not always straightforward. Chromosomally unstable cells are always aneuploid (e.g., cancer cell). However, aneuploid cells are not necessarily chromosomally unstable and some cells can remain in a stable aneuploid status for multiple cell divisions. Taking Down syndrome patients as an example, a condition associated with a permanent aneuploidy state, usually show stable karyotypes (Holland & Cleveland, 2009).

Finally, there are also some researchers arguing that CIN is an aneuploidy-independent phenomenon (Lengauer, Kinzler, & Vogelstein, 1997). The presented disparities can result from the quite ambiguous definition of CIN, as well as the widely diverse approaches used to assess CIN.

1.8 - Chromosomal instability detection methods

There are a wide variety of approaches now used to assess chromosomal aberrations. However, it remains unclear the line between aneuploidy and CIN because part of those tools are unable to discriminate between aneuploidy and CIN.

Because aneuploidy is characterized by an abnormal number of chromosomes and CIN by an increased rate of chromosome mis-segregation, different approaches are needed to

investigate each condition. Aneuploidy can be easily detected by any method capable of quantifying chromosome numbers. Karyotype analysis, FISH, spectral karyotyping, or array-based comparative genomic hybridization (aCGH) are some of the methods regularly used to study aneuploidy. Although they seem useful to quantify aneuploidy, they are not sufficiently robust to measure CIN. Discrepancies reported in the literature for the CIN term are partially due to the lack of resilient methods to assess it (Geigl, Obenauf, Schwarzbraun, & Speicher, 2008). Approaches capable of monitoring the rate of chromosomal changes (i.e by chromosome mis-segregation events), as well as, cell-cell variability (both numerical and structural abnormalities) are needed to understand the emergency and evolution of this condition (McGranahan, Burrell, Endesfelder, Novelli, & Swanton, 2012). Current CIN analysis methods can be divided in both, single-cell or multi-cell approaches. Interphase FISH, karyotyping, array-CGH and micronuclei counting are single-cell approaches. Multi-cell approaches encompass conventional array-CGH, polymerase chain reaction (PCR), single nucleotide polymorphism (SNP) and flow cytometry (Geigl et al., 2008).

FISH is probably the most used single-cell approach for CIN studies. Interphase FISH enables a fast detection of chromosomal copy numbers or chromosomal segment variations in hundreds of cells using either chromosome- or centromere-specific probes (Speicher & Carter, 2005). It is a relatively low cost method, has the ability to examine hundreds of individual cells and infer about CIN state and cell-to-cell chromosome number variation. However, only a limited number of probes can be imaged simultaneously, increasing the risk of missing chromosomal copy number variations. Besides, it will only detect mis-segregation events that generate cells able to survive, hiding mis-segregation events that result in cell death (Fiegler et al., 2007). Contrarily to FISH, multi-cell approaches are amenable to high-throughput analysis and are less expensive. However, CIN dynamics cannot be captured by multi-cell approaches that have only the ability to capture states (Speicher & Carter, 2005). Globally, multi-cell approaches provide means to indirectly estimate CIN levels from a population of cells, missing the ability to capture intrinsic cells genetic variability and CIN dynamics (Gerlinger & Swanton, 2010). On the other hand, single-cell methods allow a more accurate measure of CIN. Still, they are labor-intensive, require the use of fixed tissue and are still limited in assessing the frequency of anaphase segregation errors. Overall, all methods have their own advantages and limitations, introducing different technical artefacts and none is able to capture both the state and rate of CIN, which is essential to truly measure instability. Therefore, there is a need for further experimental developments to assess CIN by accurately measuring chromosomal mis-segregation rates in cell populations. Apparently, an optimal approach to detect CIN (through the measure of chromosome mis-

segregation rates) may be achieved by coupling different methods, including a staining one. Recently, our group proposed the combined use of FISH karyotyping techniques with high-resolution live cell imaging (Nicholson et al., 2015). This approach allows an accurately measure of the frequency of chromosome mis-segregation events and is one of the few approaches able to measure the dynamic nature of CIN.

1.9 - Aneuploidy model systems: yeast, mouse and human studies

A huge variety of yeast, mouse and human models have been emerging to investigate the consequences of aneuploidy and CIN on cells. Aneuploid karyotypes can be established either by engineered mutations that cause increased chromosome mis-segregation, generating random aneuploidies, through chromosome transfer or induced chromosome non-disjunction in meiosis resulting in defined aneuploidies (Pfau & Amon, 2012). Budding yeast is a simple and useful model to study the effects of aneuploidy on cellular physiology. The simplicity of this unicellular organism is due to its small genome and its high tolerance to aneuploidy (Mulla, Zhu, & Li, 2014). However, multicellular organisms may respond differently to aneuploidy and the physiological consequences of all organisms' aneuploidies may significantly diverge from single cell effects verified in unicellular organisms. For instance, it was suggested that the proliferation of aneuploid mouse and human cells could be limited through the p53 pathway, which is absent in yeast (Thompson & Compton, 2010).
Aneuploidy mouse models allow scientists to infer about abnormal gene dosage effects on the molecular, cellular, physiological and behavioral level, helping to understand the relationships between genotype and phenotype in chromosome or segmental aneuploidy defects. Several chromosome engineering approaches (e.g. gene knockouts and Cre–loxP technology) have been routinely used to create engineered mice that help to identify individual dosage-sensitive genes that more closely recapitulate human chromosomal disorders (Sheppard, Wiseman, Ruparelia, Tybulewicz, & Fisher, 2012). However, such chromosome engineering approaches packs other types of defects, making it difficult to separate the effect of aneuploidy from other genetic aberrations and to generate simple cause-and-effect associations (Pfau & Amon, 2012). Similarly, human cancer cells (that typically mis-segregate chromosomes at high level) do not seem to be a good model to study aneuploidy and CIN. Again, the karyotype complexity (i.e. numerous point mutations and a huge variety of other chromosomal abnormalities) hamper the association between genotype

and phenotype, particularly important to determine the casual role of aneuploidy on chromosome segregation and cell division in human cells (Gisselsson, 2011).
Altogether, the mentioned challenges highlight the need for new study models combined with more efficient detection techniques to put forward our knowledge about cellular and organism aneuploidy. Recently, we pioneered the use of human diploid primary cells and their trisomic counterparts to address the impact of constitutional aneuploidy on mitotic fidelity.

1.10 - Nuclear chromosome architecture and its functional implications

Chromosomes are non-randomly arranged in the interphase cell nucleus, occupying distinct locations within the nuclear space, commonly called chromosome territories (CTs). The term chromosome territory was first suggested by Theodor Boveri in 1909 (Boveri, 1909). Since then, there has been extensive research to find the mechanisms responsible for the non-random chromatin assemblies, as well as, their functional implications. Numerous studies came up reporting a functional implication of the nuclear position in gene expression and silencing, splicing and processing, DNA replication and DNA repair, and genome stability (Andrulis, Neiman, Zappulla, & Sternglanz, 1998; Boyle et al., 2001).
Accumulating evidence point out the nuclear architecture and the spatial organization of the genome as major factors in gene expression regulation (Francastel, Schubeler, Martin, & Groudine, 2000). It was suggested that chromosomes are placed in a radial pattern according to gene density with the most gene-dense human chromosomes (such as chromosome 19) preferentially positioned in the nucleus interior and the ones with the lowest gene density (such as chromosome 18) preferentially positioned towards the nucleus periphery, in lymphocytes (Croft et al., 1999). However, this correlation between increased gene density and nuclear position is not only true for these chromosomes and has shown to be evolutionarily conserved (Tanabe et al., 2002). Besides lymphoblastoid cells, the same correlation can be found for proliferative human fibroblasts (Bridger, Boyle, Kill, & Bickmore, 2000; Mehta, Figgitt, Clements, Kill, & Bridger, 2007). By contrast, chromosome size seems to have a role in chromosome positioning in non-proliferating cells with large chromosomes positioned towards the periphery and small chromosomes closer to the nucleus interior (Sun, Shen, & Yokota, 2000) .
Chromosome positioning within nuclear compartments is mainly constrained by its anchoration to certain cellular structure: the nuclear lamina, nucleolus and the nucleoskeleton. Ribosomal RNAs are synthesized and processed in the nucleolus. In

humans, acrocentric chromosomes containing the ribosomal repeat genes and tRNA are located in the nucleoli (Boisvert, van Koningsbruggen, Navascues, & Lamond, 2007). These finding corroborate the idea that perinuclear chromosome localization helps in the transcriptional silence of chromatin (Andrulis et al., 1998).

Disruption of the nuclear architecture can result in severe alteration in normal chromosome regulation, and consequently cell viability. It has been increasing the number of reports correlating chromosome mis-localization and diseases, such as cancer and the premature aging syndrome called Hutchinson–Gilford progeria syndrome (Mehta, Eskiw, Arican, Kill, & Bridger, 2011). Interestingly, very few studies came up addressing the impact of chromosome organization in an aneuploid situation. Studies performed using Edward syndrome cells 18 did not observe any repositioning of an extra chromosome 18. Corroborating this finding, Koutna et al concluded that the location of the extra copy of a specific locus does not significantly alter the organization of chromosome territories (Koutna et al., 2000). More recently, another study artificially introduce chromosomes 7, 18, or 19 in immortalized or cancer cell lines verified a shift in positioning for chromosomes 18 and 19, but not for chromosome 7 (Sengupta et al., 2007). Finally, specific chromosomes positioning inside the nucleus was suggested to have a role in homologues alignment during meiosis (Zickler & Kleckner, 1999).

So far, there is no clue about a possible impact of specific chromosomes positioning in the mitotic cell division and chromosomal stability.

1.11 - Aneuploidy as therapeutic target

Over the past decades, our understanding about the mechanisms behind aneuploidy and CIN, as well as, our awareness of its consequences on cellular physiology have grown considerably. Still, the aneuploidy paradigm is far from being solved. Aneuploidy has detrimental effects at the cellular level and organism level, as proliferation defects, death and disease. However, aneuploidy has been proven to promote tumorigenesis despite its anti-proliferative effects.

Attractively, the aneuploid state of cancer cells could be explored in cancer therapy. Two different approaches could be considered: 1) using strategies that exacerbate the adverse effects of aneuploidy; and 2) interfering with pathways that are essential to aneuploid cell survival (Tang, Williams, Siegel, & Amon).

Studies in yeast and mouse fibroblasts suggested that cells carrying extra chromosomes display increased sensitivity to compounds that interfere with protein folding and turnover, such as AICAR and 17-AAG (Torres et al., 2007; Williams et al., 2008).

Drugs that exacerbate chromosome alignment defects or that disrupt the mitotic checkpoint have been largely explored to kill cancer cells. Chromosome misalignment can be induced using microtubule poisons (such as paclitaxel and vinblastine). Their use potentiates SAC activation thereby arresting cells in mitosis (Manchado & Malumbres, 2011). On the other hand, disruption of the mitotic checkpoint can be achieved by the use of BubR1 or Mps1 kinase inhibitors, a checkpoint component and a kinase required for chromosome bi-orientation, respectively. Depletion of these kinases has been reported to cause spindle checkpoint defects not compatible with cellular survival (Kops et al., 2004; Tighe, Staples, & Taylor, 2008).

Unfortunately, not all tumors are susceptible to these drugs. Due to the diversity and different characteristics of aneuploid tumor cells, distinct strategies might be needed to treat them. Possibly, combined strategies using proteotoxic stress inducers, microtubule poisons or mitotic checkpoint inhibitors, might enhance their overall effect (Torres et al., 2007).

Aim of research

So far little is known about the causal role between aneuploidy and chromosomal instability. Gaining insights on the effects of aneuploidy on chromosomal stability will certainly be paramount to the understanding of their cellular and organismal outcomes. Indeed, this knowledge could even be the breaking point to the development of new clinical strategies and drugs to treat aneuploidy-associated diseases.

In a previous study, we found that aneuploidy induces CIN through karyotype-specific phenotypes (Nicholson et al., 2015). First, we observed a specific cytokinesis failure phenotype associated with trisomy 13 caused by the overexpression of a single-gene. Second, FISH analyses indicated an increased frequency of chromosome mis-segregation in trisomic cells analysed, whose extent varies with the number and type of the extra chromosome(s) involved. Finally, it seems that different chromosomes mis-segregate at distinct rates (Nicholson et al., 2015). However, the molecular mechanisms by which different aneuploidies might trigger distinct mis-segregation rates remain unknown.

The aim of this work was to further dissect how aneuploidy might induce CIN and evaluate the existence of trisomic-specific mitotic phenotypes. To accomplish this, we used cellular models of human constitutional trisomies 18 and 21 consisting of primary cell cultures derived from prenatal diagnosis (chorionic villus sampling and amniocentesis). By combining FISH analysis with centromere/locus-specific probes and high resolution live cell imaging we were able to i) perform a quantitative analysis of chromosome mis-segregation events in karyotype-specific aneuploidies vs. euploid controls; ii) ascertain if distinct trisomies induce different rates of chromosome mis-segregation; and iii) address whether the extra chromosome in a trisomic cell exhibits higher mis-segregation rate than the remaining chromosomes in the cell, and finally iv) observe mechanisms driving aneuploidy, as well as aneuploidy-driven mitotic phenotypes.

The privileged access to human primary cell samples and the group's expertise in advanced light microscopy in these cellular models ensured the scientific impact and feasibility of the work, which findings are a step further on the understanding of aneuploidy-induced chromosomal instability.

2

MATERIALS AND METHODS

2.1 - Cell culture

2.1.1 - Primary cells

Fibroblast cultures were established from surplus amniocentesis samples used in pre-natal diagnosis. From those cells, passages 1-5 were established. The casuistic used in this study included 11 constitutional trisomies 18, 13 constitutional trisomies 21 and 14 diploid controls (Tables 2.1, 2.2 and 2.3). The study was approved by the Ethics Committee of Hospital de S. João-Porto (dispatch 14 Nov 2012). All the patients signed a consent form with detailed information about these studies; there was no access to patient clinical data and the participation was volunteer. Primary fibroblasts were cultivated in MEM supplemented with 15% FBS, 1% L-Glutamine and 1% antibiotic-antimycotic solution (all from Gibco®; Thermo Scientific, USA). Cells were cultured under optimal conditions, at 37°C and humidified atmosphere with 5% CO_2, using ventilated T25 and T75 flasks (SARSTEDT®, Germany).

2.1.2 - Cell lines

To monitor chromosome motion, individual loci were tagged with arrays of *lacO* sequences. Green fluorescence protein (GFP) was fused to the lac repressor, allowing the tagged loci to be visualized as fluorescent spots. lacO integrant cell lines stably express the GFP-lacO repressor fusion protein. In our study, we used cell lines with two different integration sites: B49.2.7 (GFP-lacO at 1q11) and B49.12.8 (GFP-lacO at 13q22). The 1q11 array is found in the nuclear interior, in a satellite-rich region associated with nucleoli. On the other hand, 13q22 array is enriched at the nuclear periphery. lacO arrays were proved to behave like endogenous loci, not interfering with normal nuclear compartments architecture (Chubb, Boyle, Perry, & Bickmore, 2002). These cell lines were a kind gift of Wendy A. Bickmore (Medical Research Council, United Kingdom). In order to visualize DNA in these cell lines, pc-DNA3 H2B-mcherry plasmid (Addgene, UK) was transfected accordingly to EndoFectin™

Max manufacturer's instructions (Tebu-bio, GeneCopoeia, USA) (see transfection protocol in section 2.9). The cell lines were cultivated in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% antibiotic-antimycotic and 1% L-Glutamine (all from Gibco®, Thermo Scientific, USA). Cells were cultured under optimal conditions, at 37°C and humidified atmosphere with 5% CO_2, using ventilated T25 and T75 flasks (SARSTEDT®, Germany).

2.1.3 - Sub-culturing and passaging

Cells were grown as described, to a confluence of ~80%. Then, cells were washed with 1x PBS and trypsin was added to the cells and incubated for 2 min at 37°C in 5% CO_2 atmosphere. Cells were then resuspended in supplemented medium as described for cultivation and diluted 1:5-1:10 to new T25 or T75 cell culture flasks. Fresh medium was then added according to the flask size.

2.1.4 - Cryopreservation and defrosting

Cells lines were grown to a confluence of ~80%. Cells were then detached with trypsine and carefully centrifuged at 1500 rpm for 5 min. The supernatant was removed and cells were resuspended in FBS + 10% DMSO. 1 mL of suspension was added to cryotubes and frozen at -80°C using a freezing box. To recover cells from frozen stocks, cryotubes were rapidly defrosted in a few minutes and cell suspension was pipetted into a well in 6-well plate with 1mL of pre-warmed cell culture medium.

2.2 - Cytokinesis-block assay

Primary fibroblasts were grown on SuperFrost plus slides (Menzel, Thermo Scientific, USA) in quadriperm plates (SARSTEDT®, Germany). In order to generate binucleated cells for FISH analysis (see section 2.5), cultures were incubated with 1µM dihydrocytochalasin D (Sigma-Aldrich; USA) for 24h to block cytokinesis. Prior to fixation, cells were incubated in 3 mL hypotonic solution, 0.03 M sodium citrate (Sigma-Aldrich, USA) at 37ºC for 30 min. Then, 3 mL of freshly prepared carnoy fixative ice-cold 3:1 methanol:acetic acid solution was added drop-wise and left fixating for 5 min. This last step was repeated two more times.

2.3 - Interphase FISH

Primary fibroblasts were grown on SuperFrost plus slides (Menzel, Thermo Scientific, USA) in quadriperm plates (SARSTEDT®, Germany). Cells were fixed 48h after plating. Prior to fixation, cells were given a hypotonic shock in 0.03 M sodium citrate solution (Sigma-Aldrich, USA) at 37ºC for 30 min. Then, 3 mL of freshly prepared ice-cold 3:1 methanol:acetic acid solution was added drop-wise and left fixating for 5 min. This last step was repeated two more times.

Table 2.1 | **Fluorescence *in situ* hybridization in interphase and post-mitotic cells treated with cytochalasin D**

Identification	Type of sample	Diagnosis	Karyotype	Gestational age (WG)	Passage
A1	AF	Control	46,XX	16	2
A2	AF	Control	46,XX	16	2
A3	CV	Control	46,XX	13	2
A4	CV	Control	46,XX	12	2
B1	AF	Trisomy 13	47,XY,+13	16	2
B2	AF	Trisomy 13	47,XX,+13	16	2
B3	AF	Trisomy 13	47,XX,+13	16	2
B4	AF	Trisomy 13	47,XX,+13	31	3
C1	AF	Trisomy 18	47,XX,+18	24	2
C2	AF	Trisomy 18	47,XX,+18	15	2
C3	CV	Trisomy 18	47,XX,+18	14	3
C4	CV	Trisomy 18	47,XY,+18	13	3
D1	AF	Trisomy 21	47,XY,+21	18	2
D2	AF	Trisomy 21	47,XX,+21	16	2
D3	CF	Trisomy 21	47,XX,+21	12	2
D4	CF	Trisomy 21	47,XX,+21	14	2

AF, amniotic fluid; CV, chorionic villus; WG, Weeks of gestation

2.4 - Artificial induction of chromosome mis-segregation

Primary fibroblasts were grown on SuperFrost plus slides (Menzel, Thermo Scientific, USA) in quadriperm plates (SARSTEDT®, Germany). Cells were then treated with 2.5 µM of the Eg5 inibitor,S-Trityl-L-cysteine (STLC) (Tocris, United Kingdom) for 7h. Following mitotic cell enrichment, STLC was washed out and cells rinsed 5 times with 5% FBS supplemented media. Next, cells were incubated with 0.5 µM of Aurora B inhibitor (ZM447439) (Sigma-Aldrich, USA) for 2h. Cells were then fixed as described in the previous section.

Table 2.2 | Fluorescence *in situ* hybridization in post-mitotic cells treated with STLC+ZM inhibitors to increase chromosome mis-segregation

Identification	Type of sample	Diagnosis	Karyotype	Gestational age (WG)	Passage
A1	CV	Control	46,XX	16	3
A2	AF	Control	46,XY	16	1
A3	CV	Control	46,XX	16	2
A4	AF	Control	46,XY	16	1
B1	AF	Trisomy 18	47,XX,+18	23	3
B2	AF	Trisomy 18	47,XY,+18	16	3
B3	AF	Trisomy 18	47,XX,+18	14	1
B4	CV	Trisomy 18	47,XX,+18	16	2
C1	CV	Trisomy 21	47,XY,+21	12	3
C2	CV	Trisomy 21	47,XY,+21	12	4
C3	CV	Trisomy 21	47,XX,+21	12	1
C4	AF	Trisomy 21	47,XX,+21	14	3

AF, amniotic fluid; CV, chorionic villus; WG, Weeks of gestation

2.5 - Fluorescence *in situ* hybridization

Chromosomes 13 and 21 were labeled with the commercially available locus-specific FISH probe XA13/21 spectrum green/orange (MetaSystems, Germany). Chromosomes 7, 12 and 18 were labeled with the commercially available chromosome-specific centromeric FISH probes, CEP7 Spectrum Blue, CEP12 Spectrum green and CEP18 Spectrum Orange (Abbott Laboratories, USA), accordingly to the manufacturer's instructions.

2.6 - Microscopy and Image analysis of FISH-stained cells

FISH samples were analyzed either in the IN Cell Analyzer 2000 (GE Healthcare, UK) or in the Zeiss AxioImager Z1 (Carl Zeiss, Oberkochen, Germany). IN Cell Analyzer 2000 is equipped with a Photometrics CoolSNAP K4 camera and a Nikon 20x/0.45 NA Plan Fluor objective. The GE IN Cell Analyzer 2000 5.2 software was used to acquire the images. Zeiss AxioImager Z1 is equipped with an Axiocam MR and a 40x/1.30 NA EC-Plan-Neofluor objective. The Zeiss Axiovision 4.7 software was used to acquire images. Images were subsequently deconvoluted with autoquant X2 (Media Cybernetics).
For FISH staining quantitative analysis, 15 to 150 images were taken from areas of optimal cell density avoiding areas with cellular clumps or overlapping cells. All the FISH-stained samples were analyzed blindly, using open source Fiji/ImageJ (http://rsb.info.nih.gov/ij/).

2.7 - Viral production and cell infection

The bicistronic construct H2B-GFP-T2A-Cherry-tubulinA, cloned in a retroviral vector, generously provided by Floris Foijer (ERIBA, Groningen), was subcloned into the lentiviral vector pLVX-Tight-Puro Vector (Clontech®, CA, USA) by Joana Macedo (PhD in our group). Lentiviruses were produced in Hek 293 T cells. 5-6 x 10^6 Hek 293 T cells were plated on 10 cm dishes (60 cm^2) in DMEM supplemented with 10% of Tet System Approved FBS (Clontech, CA). 24h after, the culture medium was changed to DMEM only. In starvation conditions, Hek 293 T cells were transfected with a plasmid DNA mix (5.6 µg VCV-G/pMd2.G, 16.6 µg Pax2 and 7 µg of pLVX-Tight-H2B-tubulin) or (5.6 µg VCV-G/pMd2.G, 16.6 µg Pax2 and 7 µg of pLVX-Tet-On Advanced). Plasmids were incubated with lipofectamine 2000 (Invitrogen, Thermo Scientific, USA) for 30 min at RT to allow nanoparticle complexes to form. 3 mL of this solution was added carefully to the cells drop-wise. Cells were incubated at 37ºC overnight. Afterwards, the transfection medium was replaced with fresh DMEM + 10% of Tet System Approved FBS. Media supernatants containing infectious lentiviruses were collected at 24h, 48h and 72h time points, centrifuged at 1200 rpm for 10 min, and filtered through 0.45 µm (cellulose acetate) to eliminate cell debris. Viral particles were aliquoted and stored at -80ºC until further use.

For lentiviral infection, $1x10^5$ primary fibroblasts (50-60% confluence) were seeded in sterilized glass-bottom 35mm u-dishes (Ibidi GmbH, Germany) coated with fibronectin (Sigma-Aldrich®, USA) and filled with 1.5 mL of MEM supplemented with 5% FBS and without antibiotics. Afterwards, a mix of 250 µl of pLVX-Tight-H2B-tubulin viral particles, 125 µl of pLVX-rTTA viral particles, and 8 µg/mL of polybrene, was added drop-wise to the cells and incubated overnight. In the following day, the media were replaced by complete medium supplemented with 0.75 µg/mL doxycycline to start transduction. All cultures used in live cell imaging were transduced between 48-72h.

2.8 - Spinning-disk confocal live cell imaging

35 mm glass bottom u-dishes were placed in the stage of an inverted microscope under controlled atmosphere, humidity and temperature. Images were acquired with a PLANAPO 60x/1.4 NA objective under a spinning-disk confocal system, Andor Revolution XD (Andor Technology, Belfast, UK) coupled to an Olympus IX81 inverted microscope (Olympus, Southend-on-Sea, UK) equipped with an electron-multiplying CCD iXonEM Camera and a Yokogawa CSU-22 unit. Two laser lines at 488 and 561nm were used for the excitation of GFP and mCherry and the system was driven by IQ software (Andor Technology, Belfast, UK). Z-stacks (0.9 µm interval) covering the entire volume of the mitotic cells were collected. For primary cells, 1.5 min acquisition intervals were used, with live imaging starting 48-72h after the beginning of transductions. For cell lines, 2 min time lapse imaging was performed, starting 1h-12h after addition of the GSK small molecule inhibitor. Later image processing was conducted using open source Fiji/ImageJ software (http://rsb.info.nih.gov/ij/).

2.9 - Transfection using EndoFectin™ Max

Cell lines were seeded at 60-70% confluence in a 6-well plate format. For transfection, 10-16h later, the medium supplemented with 10% FBS was exchanged to 1.5 mL DMEM supplemented with 5% FBS and without antibiotics. Two mixes were prepared: 1) 500 µl OptiMEM with 1µg pc-DNA3 H2B-mcherry, and 2) 500 µl OptiMEM + 6 µl EndoFectin™ Max (Tebu-bio, GeneCopoeia, USA). Mixes were first incubated for 5 min and then brought together and incubated for further 30 min at room temperature. Then the mixture was added

drop-wise to the cells and incubated overnight. In the following day, the media was exchanged to DMEM supplemented with 10% FBS.

To select B49.2.7 and B49.12.8 cells that were transfected with pc-DNA3 H2B-mcherry, 0.4 ug/mL G418 was added to the medium. Cells stably expressing H2B-mcherry were then grown in media with 100 µg/mL of hygromycin B (Sigma-Aldrich®, USA) and 5 µg/mL basticidin (Sigma-Aldrich®, USA) to keep the selection for GFP-lacO and *LacR* transgenes. Antibiotics were omitted just before any experiment to avoid possible side effects on the experimental outcome.

Table 2.3 | Euploid and trisomic amniocytes used for live cell imaging

Identification	Type of sample	Diagnosis	Karyotype	Gestational age (WG)	Passage
A1	AF	Control	46,XY	16	3
A2	CV	Control	46,XX	13	4
A3	AF	Control	46,XX	16	1
A4	AF	Control	46,XY	16	2
A5	AF	Control	46,XY	16	1
A6	AF	Control	46,XY	16	1
A7	AF	Control	46,XY	16	2
A8	AF	Control	46,XX	16	3
A9	AF	Control	46,XY	16	2
A10	AF	Control	46,XX	16	1
B1	AF	Trisomy 18	47,XX,+18	23	3
B2	AF	Trisomy 18	47,XX,+18	23	5
B3	AF	Trisomy 18	47,XX,+18	24	4
B4	CV	Trisomy 18	47,XY,+18	13	4
B5	AF	Trisomy 18	47,XY,+18	16	2
C1	CV	Trisomy 21	47,XX,+21	12	2
C2	CV	Trisomy 21	47,XY,+21	12	3
C3	CV	Trisomy 21	47,XY,+21	12	2
C4	CV	Trisomy 21	47,XX,+21	14	2
C5	AF	Trisomy 21	47,XX,+21	15	1
C6	CV	Trisomy 21	47,XX,+21	12	2
C7	CV	Trisomy 21	47,XX,+21	14	2
C8	CV	Trisomy 21	47,XY,+21	13	1

AF, amniotic fluid; CV, chorionic villus; WG, Weeks of gestation

2.10 - CENP-E inhibition assay

Stable transfected cell lines were seeded at 60-70% confluence in glass-bottom 35 mm u-dishes (Ibidi GmbH, Germany) coated with fibronectin (Sigma-Aldrich®, USA) and filled with DMEM medium supplemented as already described. The cell lines were then incubated overnight in a humidified incubator at 37°C with 5% CO2. 1h before live cell imaging, the medium was exchanged to 1.5mL DMEM supplemented with 10% FBS but without G418, blasticidin and hygromicin antibiotics. Then, CENP-E inhibitor (GSK-923295, Selleckchem, USA) was added to a final concentration of 4 nM.

2.11 - Statistical Analysis

Statistical analyses were performed with Prism version 6.00, GraphPad Software®, (La Jolla California USA, www.graphpad.com). After testing for normality, Mann-Whitney U test (Figure 3.4A), student's unpaired t-test (Figure 3.9D) two-tailed $\chi 2$ test test (Figure 3.2B, 3.3B, 3.5B, 3.5D, 3.6B, 3.7C, 3.8B and 3.8C), Spearman correlation test (Figure 3.4 C and E) and D'Agostini-Pearson omnibus normality test (Figure 3.4C and E) were used for non-parametric data to determine whether differences between the groups were statistically significant ($p \leq 0.05$). The data represented in figure 3.2, 3.8 and 3.9 illustrates the average of at least three independent experiments and are displayed as mean + S.E.M.

3

RESULTS AND DISCUSSION

Recent research from our lab showed that human trisomy 13 cells displayed higher rates of chromosome mis-segregation compared to their euploid counterparts and a specific cytokinesis failure phenotype (Nicholson et al., 2015). Whereas the molecular mechanism behind the cytokinesis failure phenotype was shown to be karyotype-specific caused by overexpression of a gene located in chromosome 13, the molecular mechanism that leads to an increased chromosome mis-segregation rate remains unknown. Moreover, chromosome 13 was found to exhibit an increased mis-segregation rate in trisomy 13 in comparison to other chromosomes, and the reason behind this also remains unidentified. Therefore, two important questions were raised from these previous findings in the lab: i) do other aneuploidies lead to increased chromosome mis-segregation rates, i.e., is this phenotype an aneuploidy-induced global effect or instead a karyotype-specific effect? and ii) do different chromosomes mis-segregate at distinct rates?

To test the effects of aneuploidy on chromosome segregation and other mitotic phenotypes, human primary cells, both disomic (2N) and trisomic for chromosomes 13, 18 and 21 (hereafter referred to as Ts13, Ts18 and Ts21, respectively) (see Tables 2.1, 2.2 and 2.3), were established from surplus pre-natal diagnosis samples. The presence of the additional chromosome was confirmed by FISH with locus-specific probes for chromosome 13 and 21 and centromere-specific probe for chromosome 18 (Figure 3.1). The experiments described were performed at a low passage number (1-5) to discard any detrimental effects arising from *in vitro* culture and due to the limited proliferation rates of amniocytes and chorionic villi cells.

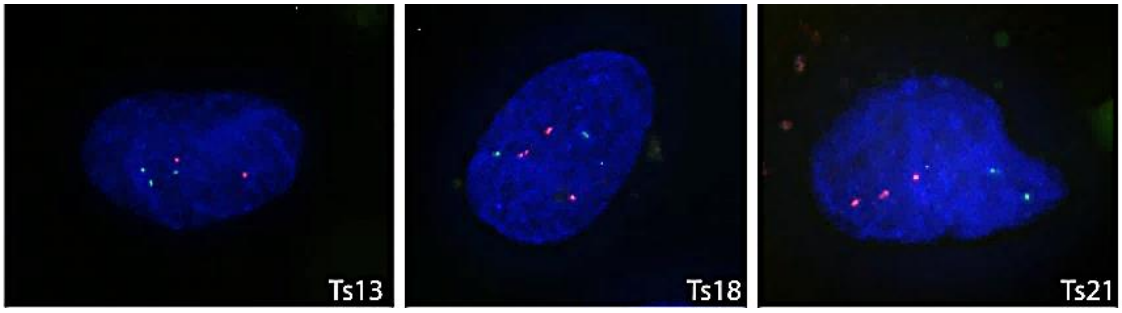

Figure 3.1 | FISH karyotyping of human trisomic cells for chromosomes 13, 18 and 21. The presence of an additional chromosome 13, 18 or 21 was confirmed by FISH analysis using chromosome-specific probes for chromosomes 13 (green), 18 (red) and 21 (red). DNA is shown in blue.

3.1 - Increased chromosome mis-segregation rates in human 13, 18 and 21 trisomies

To determine the effect of aneuploidy on chromosome segregation in human cells we first measured aneuploidy indexes in interphase fixed nuclei (Figure 3.2A). Recently, the ability of aneuploidy to induce CIN was directly tested using human trisomies 7 and 13. It was shown that aneuploid cells displayed higher rates of chromosome mis-segregation compared to their euploid counterparts (Nicholson et al., 2015). Additionally, the same study showed that trisomy 7 or trisomy 13 display increased rates of anaphase lagging chromosomes (Nicholson et al., 2015). Interestingly, anaphase lagging chromosomes are a major source of aneuploidy in normal vertebrate cells and the most common chromosome segregation defect in CIN cancer cells (Cimini et al., 2001; Compton, 2011). What is still not known is whether other human trisomies have increased rates of chromosome mis-segregation and if distinct trisomies induce different rates of chromosome mis-segregation.

We started by performing interphase nuclei FISH analysis for three different chromosome pairs, as this is a fast detection method of copy number or segmental variations of a chromosome fraction in hundreds of cells (Speicher & Carter, 2005). We found that human trisomic 13, 18 and 21 cells displayed significantly higher frequencies of chromosome mis-segregation (Ts13, 0.70%; Ts18, 1.0%, Ts21, 1.1%) compared to the diploid control (0.13%, two- tailed test ***$p \leq 0.001$, ****$p \leq 0.0001$) (Figure 3.2 B). These data further corroborated our previous findings showing that aneuploidy triggers chromosome mis-segregation events (Nicholson & Cimini, 2013; Nicholson et al., 2015). Because similar rates of chromosome mis-segregation were found for all the human trisomies analysed, there is no evidence that distinct trisomies might induce different rates.

The higher levels of chromosome mis-segregation found by this approach for trisomies 18 and 21 led us to ask whether these are due to an increased frequency of anaphase lagging chromosomes, as previous shown for trisomy 13.

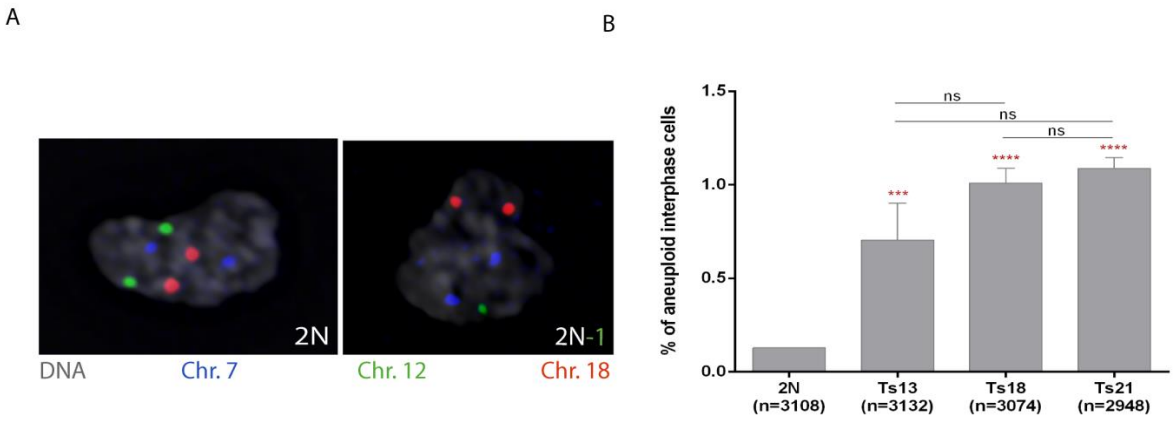

Figure 3.2 | Increased rates of chromosome mis-segregation in human trisomies. A - Chromosome-specific FISH staining of interphase nuclei from cells retrieved upon pre-natal diagnosis with specific probes for chromosomes 7 (blue), 12 (green), 18 (red). **B -** Percentage of interphase cells exhibiting gain or loss of one or more of the three chromosomes tested. Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 2948-3132 cells were analyzed for each condition. Two-tailed $\chi 2$ test, ns $p > 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$; n = total interphase cell counts.

3.2 - Anaphase lagging chromosomes are not an aneuploidy-induced global phenotype

Frequencies of anaphase lagging chromosomes could not be analyzed in fixed experiments due to technique inherent constrains, such as the loss of mitotic cells during the fixation step (mitotic cells are poorly adherent and therefore are often washed out) and the low percentage of anaphase cells in the mitotic cell population (anaphase is a very rapid stage of mitosis taking 2-3 min). Therefore, we optimized live cell imaging of individual human amniocytes with constitutional trisomy 18 and 21 expressing a bicistronic construct H2B-GFP-T2A-Cherry-tubulinA (Figure 3.3A). This approach allowed us to accurately measure not only how frequent chromosome mis-segregation events are, but also any alternative mechanisms driving aneuploidy in these cells, as well as aneuploidy-driven mitotic phenotypes. Surprisingly, we found that trisomic cells of the 18 and 21 did not display significantly higher rates of chromosome mis-segregation compared to their euploid counterparts (2N, 14.29%; Ts18, 17.39%; Ts21, 14.3%; two-tailed χ^2 test, ns $p > 0.05$), even considering anaphase lagging chromosomes and chromatin bridges events (Figure 3.3B).

Actually this finding was quite unexpected, particularly considering what was found for trisomy 13, in which anaphase lagging chromosomes were significantly increased. However, we found an aneuploidy-dependent increase in other mitotic defects, as described below.

3.3 - Mitotic duration is not significantly increased in aneuploid cells

From the live cell imaging analysis, we calculated mitotic duration as the time, in minutes, between nuclear envelope breakdown and anaphase onset. We found no significant mitotic delay in the trisomic cells analysed (Ts18, 33.94 min; Ts21, 31.64 min) compared to diploid controls (30.38 min; Mann-Whitney test; ns $p > 0.05$) (Figure 3.4A). This suggests that either i) trisomies 18 and 21 generate mitotic defects unable to activate the mitotic checkpoint, or ii) trisomic 18 and 21 cells have compromised mitotic checkpoint function enabling them to delay anaphase onset in the presence of defective kinetochore-microtubule attachments. We tend to exclude this later hypothesis, given that we did observe trisomic cells spending longer time in prometaphase (data not shown), meaning that the mitotic checkpoint is functional. Therefore, we believe that trisomies 18 and 21 most likely induce mitotic defects that are undetectable to the mitotic checkpoint, thereby leading to aneuploidy. These mitotic defects might include merotelic kinetochore-microtubule attachments, chromatin bridging due to replication stress, tetraploidy, aberrant centrosome number, cell size, or cytokinesis failure (Uetake & Sluder, 2004; Wong & Stearns, 2005). Although not significant, the slight increase observed in mitotic duration for trisomy 18 could be correlated with an increase in the nuclear DNA content/tetraploidy. To address this question, we plotted nuclear volume versus mitotic duration (Figure 3.4C). It is known that the nuclei of most cells are either round or oval. (Webster, Witkin, & Cohen-Fix, 2009).

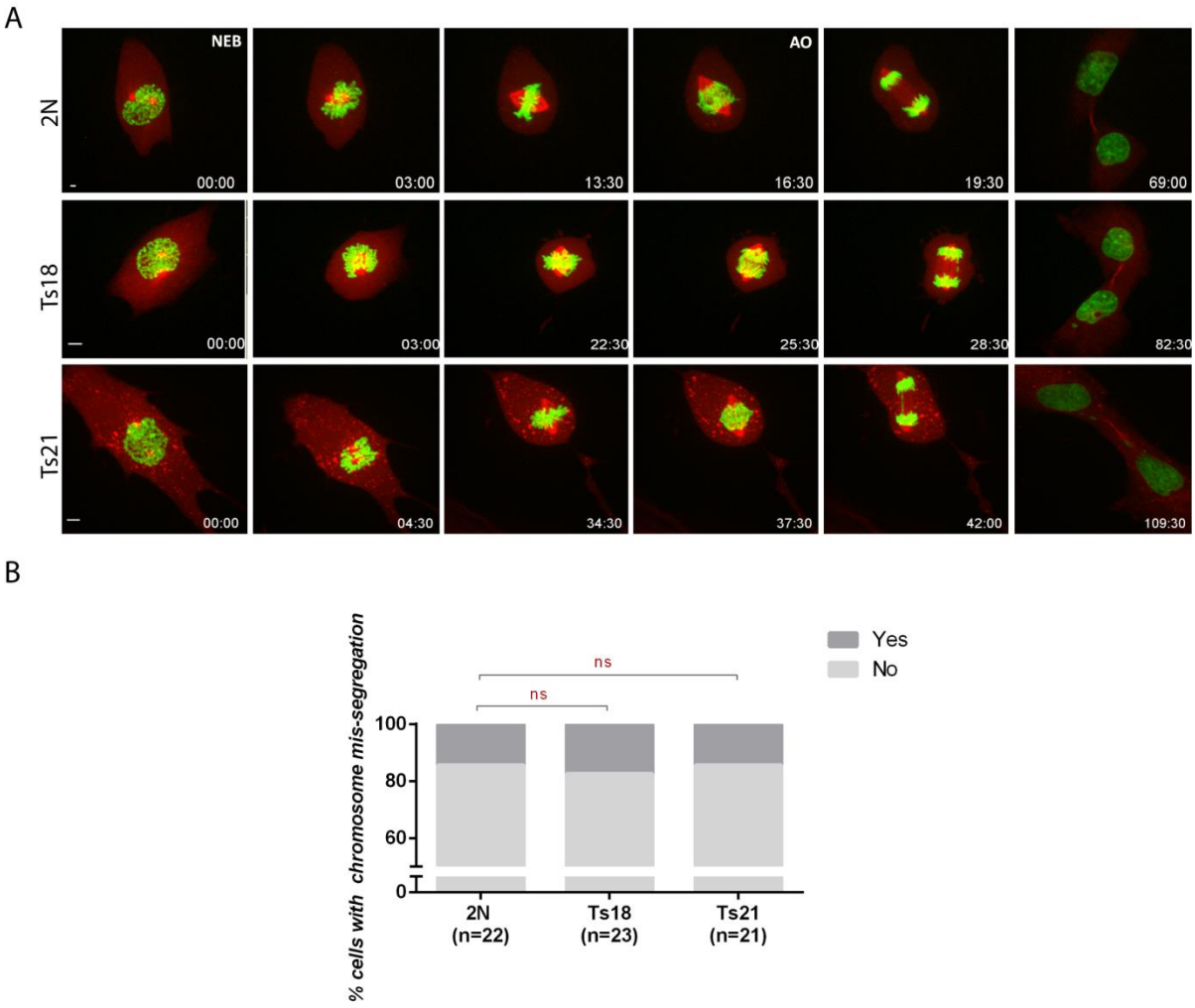

Figure 3.3 | Live cell imaging analysis of chromosome mis-segregation events in trisomies 18 and 21. A - Time-lapse microscopy of 2N, Ts18 and Ts21 cells undergoing mitosis. Representative movie frames of normal mitosis are shown in the top; in the middle row is an example of Ts18 displaying an anaphase lagging chromosome and in the bottom row is represented an example of Ts21 displaying a chromatin bridge. DNA is shown in green (H2B-GFP) and microtubules in red (Cherry-tubulin). Images are maximum intensity projections of Z-stacks. NEB, nuclear envelope breakdown. AO, anaphase onset. Time stamps indicate elapsed time in min:sec. Scale bars, 5 μm; **B** - Percentage of cells exhibiting chromosome mis-segregation (anaphase chromosome laggings or chromatin bridges). Data represent the average of at least three independent experiments in which total 21-23 cells were analyzed for each condition. Statistical significance was calculated using a two-tailed $\chi 2$ test, ns $p > 0.05$; n = number of cells analyzed.

With this in mind, nuclear volume was calculated assuming a near oval shape of the nucleus in G2 (Figure 3.4B) and carefully estimated assuming the simplistic formula of a prolate spheroid:

$$V = \frac{4}{3}\pi a^2 b \quad (3.1)$$

Where: *a* is the length of the minor axis; *b* the length of the major axis.

We found no direct correlation between nuclear volume and mitotic duration in 2N, Ts18 and Ts21 (2N, Spearman correlation, r=0.03188, ns p > 0.05; Ts21, D'Agostini-Pearson omnibus normality test; r=0.3671, ns p > 0.05). However, we could observe a slight trend for trisomy 18: higher nuclear volumes correlating with higher mitotic duration (D'Agostini-Pearson omnibus normality test; r=0.6590, **p ≤ 0.01). This suggests a link between trisomy 18 and tetraploidy. Interestingly however, when we plotted mitotic duration versus cell volume (Figure 3.4E), no correlation was found between nuclear volume and mitotic duration for 2N (Spearman correlation; r=0.05389, ns p > 0.05), Ts21 (D'Agostini-Pearson correlation; r=0.02214, ns p > 0.05), nor for Ts18 (Spearman correlation; r=0.2979, ns p > 0.05). In this assay, cell volume was calculated assuming the round shape of a metaphase cell (Figure 3.4D) and using the simplistic formula of a sphere:

$$V = \frac{4}{3}\pi r^3 \quad (3.2)$$

Where: *r* is the radius of the cell.

These results do not support previous speculations that SAC strength is proportional to cell size, as we observe that cells with bigger volumes do not have higher mitotic durations (due to decreased SAC strength) (Galli & Morgan).

Considering our speculation for a possible link between trisomy 18 and tetraploidy, we do see a higher increase in the tetraploid cell population for Ts18 by interphase FISH data analysis (Figure 3.5D). Thereby, it is possible that the slight increase in mitotic duration observed for this population results from the presence of a tetraploid cell population.

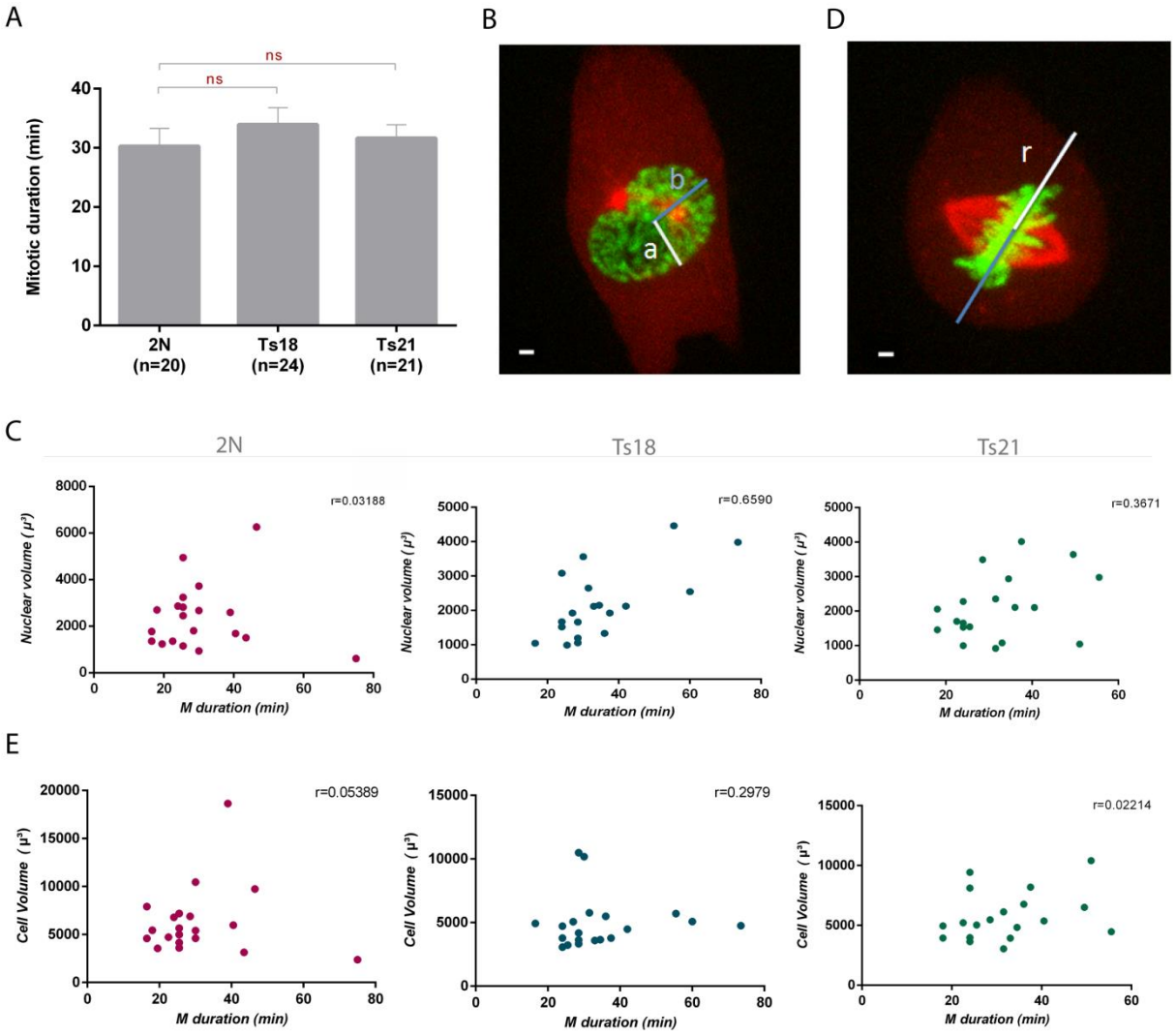

Figure 3.4 | Correlation between nuclear or cell volumes and mitotic duration in trisomic cells. A - Quantification of live cell imaging data showed that there is no significant difference in mitotic duration between 2N cells (30.4 min) and trisomic cells analyzed (Ts18, 33.9 min; Ts21, 31.6 min; Mann-Whitney test, ns $p > 0.05$). Data are reported as mean+S.E.M and represent the average of at least three independent experiments in which a total of 20-24 cells were analyzed for each condition; n= number of cells analyzed; **B** - Schematic representation of a G2 cell nucleus, where *a* is the length of the minor axis and *b* the length of the major axis of a prolate spheroid; Scale bars 5μm; **C** - XY plot showing the relation between nuclear volume and mitotic duration in 2N, Ts18 and Ts21. The graph shows regression values (r). Although there is a slight trend in Ts18, higher nuclear volumes correlating with higher mitotic duration (D'Agostini-Pearson omnibus normality test; r=0.6590, $^{**}p \leq 0.01$), there is no correlation between nuclear volume and mitotic duration for both 2N (Spearman correlation; r=0.03188, ns $p > 0.05$) and Ts21 (D'Agostini-Pearson omnibus normality test; r=0.3671, ns $p > 0.05$); **D** - Schematic representation of a metaphase cell, where *r* is the radius of a sphere; Scale bars 5μm; **E** - XY plot showing the relation between cell volume and mitotic duration in 2N, Ts18 and Ts21 (a, b and c, respectively). The graph shows regression values (r). No correlation was found between cell volume and mitotic duration for 2N (Spearman correlation; r=0.05389, ns, $p>0.05$), Ts18 (Spearman correlation; r=0.2979, ns $p > 0.05$) and Ts21 (D'Agostini-Pearson correlation; r=0.02214, ns $p > 0.05$).

3.4 - Defects in the late mitotic midbody structure in trisomies 13, 18 and 21

Live cell imaging analysis of dividing cells with trisomy 18 or trisomy 21 revealed the presence of distinctive defects in the midbody structure assembled in late mitosis (Figure 3.5B). Disorganization of the midbody matrix has been closely implicated in cytokinesis failure (Matuliene & Kuriyama, 2002). Our lab has recently demonstrated that overexpression of the *SPG20* gene localized in chromosome 13 and encoding for the spartin protein, which has a functional role in cytokinesis, accounts for a specific cytokinesis failure phenotype observed in trisomy 13 cells. This phenotype was shown to arise from a karyotype-specific aneuploidy effect, as downregulation of SPG20 in trisomy 13 cells was able to rescue cytokinesis failure (Nicholson et al., 2015). Nevertheless, we found diverse morphological alterations in the midbody structure for human trisomies 18 and 21, such as a thin and distorted bridging structure and an incorrect position of the abscission site, which result in the asymmetrical separation of the two daughter cells (Figure 3.5A). These defects were significantly increased (Ts18, 71.43%, two-tailed χ^2 test, ***$p \leq 0.001$; Ts21, 55.56%, *$p \leq 0.05$) in comparison to 2N control (14.29%). However, we could not determine whether the midbody defects in trisomies 18 and 21 ended up leading to cytokinesis failure as the live cell imaging records were often interrupted before abscission. Nevertheless, it is expectable that this impaired midbody structure might result in cytokinesis failure, which would in turn give rise to tetraploid cells (Fujiwara et al., 2005). Indeed, in our interphase FISH (Figure 3.5C) experiments we observed a significant increase in the tetraploid cell population for all trisomies analysed (Figure 3.5D) compared to diploid control cells (Ts13, 4.50%; Ts18, 5.69%; Ts21, 3.22% vs. 2N, 1.58%, ****$p \leq 0.0001$). Therefore, our data corroborates previous findings for trisomy 13 and additionally raises the question: what is the molecular mechanism that is causing cytokinesis defects in trisomic 18 and 21 cells? We identified the mechanism behind cytokinesis failure in trisomy 13 and we excluded SPG20 overexpression as a causal mechanism in trisomies 18 and 21 (Nicholson et al., 2015). One possibility is that there are genes involved in cytokinesis located in chromosomes 18 and 21, whose overexpression in trisomies 18 and 21 could lead to karyotype-specific cytokinesis phenotypes.

It should also be noted that tetraploidy can be caused not only by cytokinesis failure (Fujiwara et al., 2005) but also mitotic slippage (Elhajouji, Cunha, & Kirsch-Volders, 1998) and endoreduplication (Edgar & Orr-Weaver, 2001). In our work, mitotic slippage and endoreplication were not investigated. However, it would be interesting to address in future work.

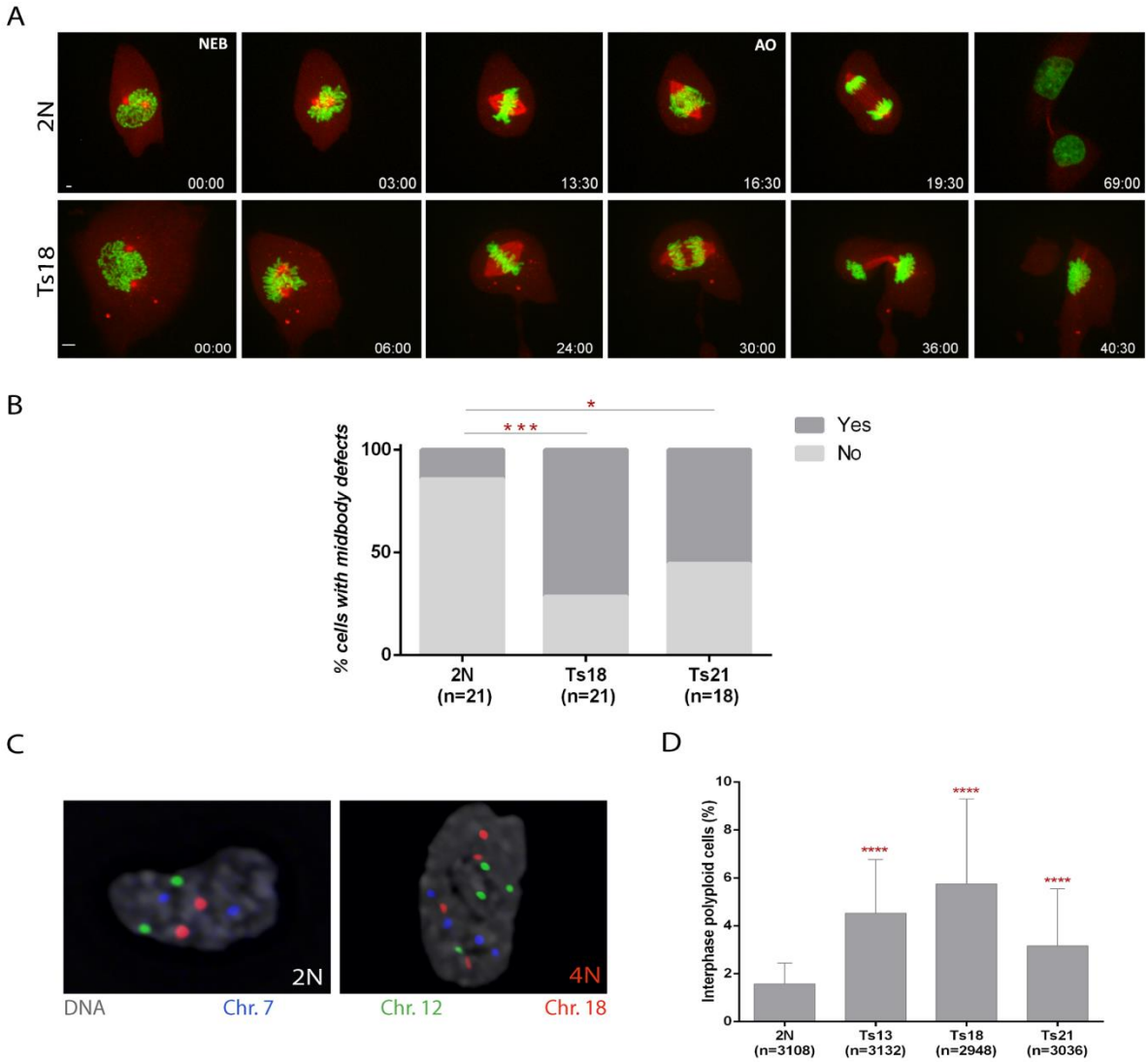

Figure 3.5 | Midbody defects in trisomies 18 and 21. A - Live cell imaging of 2N and Ts18 cells undergoing mitosis. Representative movie frames of normal mitosis are shown in the top row; in the lower row is an example of Ts18 displaying midbody defects. DNA is shown in green (H2B-GFP) and microtubules in red (Cherry-tubulin). Images are maximum intensity projections of Z-stacks. Time stamps indicate elapsed time in min:sec. Scale bars, 5 µm; **B** - Quantification of live cell imaging data shows that midbody defects are significantly increased in trisomic cells (Ts18, 71.43%, two-tailed $\chi 2$ test, ***$p \leq 0.001$; Ts21, 55.56%, two-tailed $\chi 2$ test, *$p \leq 0.05$) compared to 2N control (14.29%). Data are reported as mean and represent the average of at least three independent experiments in which a total of 18-21 cells were analyzed for each condition; n = number of cells analyzed; **C** - Chromosome-specific FISH staining of interphase nuclei from cells retrieved upon pre-natal diagnosis with specific probes for chromosomes 7 (blue), 12 (green), 18 (red). **D** - Quantification of interphase FISH data shows significantly increased frequencies of polyploidy in Ts13 (4.50%), Ts18 (5.69%) and Ts21 (3.22%) compared to 2N cells (1.58%; two-tailed $\chi 2$ test, ****$p \leq 0.0001$). Tetraploid cells were considered as having 4 signals for each of the three chromosomes analyzed, 7, 12 and 18. A total of 2948-3132 cells were analyzed for each condition; n= total polyploid cell counts.

3.5 - Trisomy 21 leads to spindle mispositioning

Our analysis of live cell imaging data revealed spindle mispositioning in relation to growth surface as the most distinctive feature of trisomy 21 cells (Figure 3.6B). Accurate positioning of the mitotic spindle is critical to ensure a correct distribution of chromosomes during cell division (Bird, Heald, & Weis, 2013). Spindle orientation was reported to have a role in the placement of daughter cells within a tissue and spindle misorientation has been implicated in tumor development (Pease & Tirnauer, 2011). Additionally, spindle positioning has been reported to affect the ability of the contractile ring to assemble (McNally, 2013). Interestingly, we observed a significant increase in spindle positioning defects in trisomic 21 cells compared to control 2N cells (59.09% vs. 5.56%; two-tailed χ^2 test, *$p \leq 0.05$). By spindle mispositioning we refer to mitotic spindle rotation unparallel to substratum, causing asymmetric adherence of daughter cells in telophase (Figure 3.6A). As mitotic spindle orientation dictates the position of the cleavage furrow, recent studies suggest that cytokinesis failure may occur in cells in which spindle elongation or spindle positioning is perturbed (Normand & King, 2010; Zuzana Storchova & Pellman, 2004). In light of this, our observations could suggest that the increased rates in spindle mispositioning for trisomy 21 cells justify an increase in cytokinesis failure. Consequently, cytokinesis failure will contribute to tetraploidization in this cell population, which is in line with the increased rates of polyploidy observed for trisomic 21 cells by interphase fixed cell experiments (Figure 3.5D).

3.6 - Karyotype-specific mitotic phenotypes: different routes to aneuploidy

The use of live cell imaging enabled us to identify aneuploidy-induced karyotype-specific mitotic phenotypes, as well as phenotypes common to distinct aneuploidies (Figure 3.7). It was previously reported that Ts13 cells often exhibit anaphase lagging chromosomes that were shown, in this study, to be absent in Ts18 and Ts21, thus suggesting this is a Ts13-specific phenotype. Moreover, we found spindle mispositioning as a unique feature of Ts21 cells. Regarding phenotypes globally present in distinct aneuploidies, we found acentric chromosome fragments, even though occurring very rarely. Acentric chromosome fragments and anaphase chromatin bridges normally arise from pre-mitotic DNA damage and replication stress, respectively (Burrell et al., 2013). In addition, midbody defects were commonly detected in all trisomies 13, 18 and 21, even though in trisomy 13 we know this is a karyotype-specific phenotype.

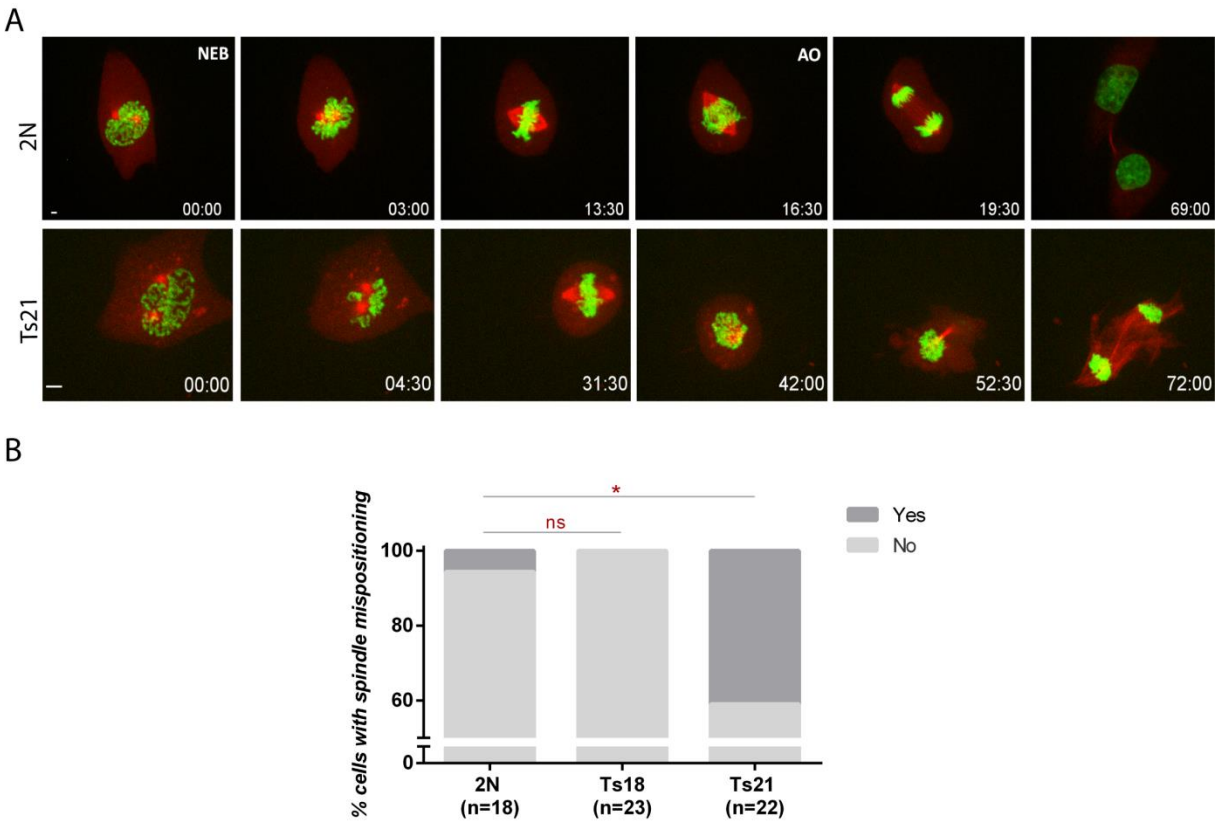

Figure 3.6 | Spindle mispositioning in trisomy 21. A - Live cell imaging of 2N and Ts21 cells undergoing mitosis. Representative movie frames of mitosis are shown in the top row; in the lower row is an example of Ts21 displaying spindle mispositioning. DNA is shown in green (H2B-GFP) and microtubules in red (Cherry-tubulin). Images are maximum intensity projections of Z-stacks. Time stamps indicate elapsed time in min:sec. Scale bars, 5 μm; **B** - There is a significant increase in cells exhibiting spindle mispositioning in trisomy 21 compared to control 2N (59.09% vs 5.56%; two-tailed $\chi 2$ test, *$p \leq 0.05$). No cases of spindle mispositioning were observed for trisomy 18. Data are average of at least three independent experiments in which a total of 18-23 cells were analyzed for each condition; n = number of cells analyzed.

One question from our data is: how can the increase in chromosome mis-segregation detected by interphase FISH be explained for trisomies 18 and 21, if we did not observe an higher frequency of anaphase lagging chromosomes? One possibility is that chromatin pre-mitotic lesions contribute to aneuploidy as previously reported (Passerini et al., 2016). However, unlike the anaphase laggings, anaphase chromatin bridges are often ultrafine and thus hardly detected.

Overall, our live cell imaging data show the different outcomes of aneuploidy for cell division: either by directly compromising mitosis, evidenced by the presence of anaphase lagging chromosomes, or by generating pre-mitotic lesions, evidenced by anaphase chromatin bridges and acentric chromosome fragments.

Alternatively, aneuploidy might be an indirect outcome of other mitotic faults, such as spindle mispositioning and midbody defects, which, by leading to tetraploidy can promote chromosome mis-segregation and aneuploidy due to multipolar spindle formation (King, 2008).

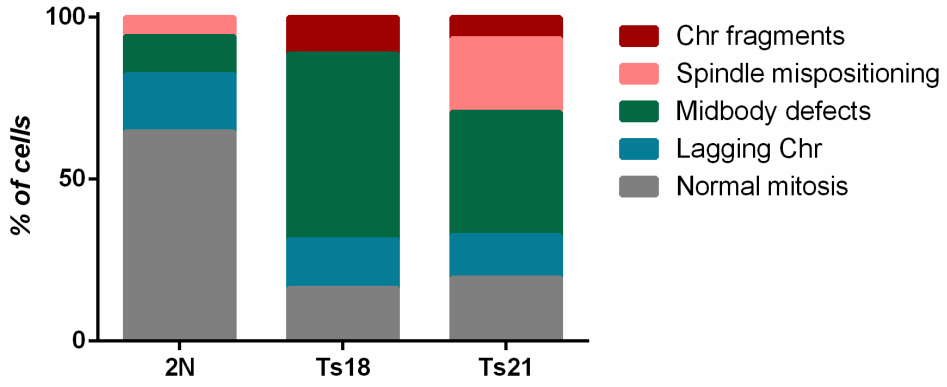

Figure 3.7 | Karyotype-specific mitotic phenotypes: different routes to aneuploidy. Distribution of mitotic phenotypes in human trisomic cells observed under time-lapse microscopy. Acentric chromosome fragments: segment of a chromosome that lacks a centromere and does not align in the metaphase plate; Spindle mispositioning: mitotic spindle rotation unparallel to substratum; Midbody defects: alteration to normal midbody architecture, possibly resulting in failed cytokinesis. Chr stands for chromosome.

3.7 - Specific increase of mis-segregation of the trisomic chromosome in human trisomies 13 and 21

Because mitotic defects that result in post-mitotic cell death are lost by interphase FISH analysis, we decided to further characterize chromosome number variability by performing post-mitotic FISH analysis with chromosome-specific probes for chromosomes 7, 12, 13, 18 and 21 (Figure 3.8A). Post-mitotic FISH analysis consists in the scoring of balanced chromosome mis-segregation events between two daughter cells. The asynchronous cell population is treated during 24h with the cytokinesis inhibitor Cytochalasin D, which leads to accumulation of post-mitotic binucleated cells. Aneuploidy index can then be measured only in these cells thus preventing any bias arising in interphase FISH from the different adaptive potential of distinct aneuploidy karyotypes (Fenech & Morley, 1985).

Our previous studies demonstrated an increased mis-segregation rate of chromosome 7 in the DLD1+7 cell line and of chromosome 13 in DLD1+13 compared to their diploid counterparts (Nicholson et al., 2015). An increased mis-segregation rate of chromosome 13

in comparison to other chromosomes was also found in Ts13 primary fibroblasts (Nicholson et al., 2015). This led us to ask whether chromosome mis-segregation in trisomic cells is normally biased for the extra chromosome. To address if the higher chromosome mis-segregation rates in human trisomies were specifically linked to the trisomic chromosome, we measured chromosome number variations using FISH analysis in cytochalasin D-treated cells. As mentioned, treatment with cytochalasin-D, an inhibitor of the mitotic spindle that prevents cytokinesis, allowed us to recognize cells that had completed one nuclear division by their binucleated appearance (Fenech & Morley, 1985). Using this approach we found a significant increase in chromosome mis-segregation for all the trisomic cells analysed compared to diploid control (Figure 3.8B), corroborating our interphase experiments data (Ts13, 1.65%; Ts18, 2.03%; Ts21, 2.11%; two-tailed χ^2 test, *p ≤ 0.05; ***p ≤ 0.001). Looking to individual chromosome mis-segregation rates (Figure 3.8C), we found chromosome 7 to mis-segregate more often in all the trisomies analysed than in the diploid control (2N, 0.0%; Ts13, 0.62%; Ts18, 0.44%; Ts21, 0.63%; two-tailed χ^2 test, *p ≤ 0.05, **p ≤ 0.01), whereas chromosome 12 behaves similarly in both trisomies and control (2N, 0.26%: Ts13, 0.31%; Ts18, 0.36%; Ts21, 0.70%, two-tailed $\chi 2$ test, ns p > 0.05). When looking to chromosome 13, we found that it has a significantly higher chromosome mis-segregation rate in Ts13 than the corresponding chromosome in diploid cell cultures (2N, 0.0%; Ts13, 1.23%; two-tailed χ^2 test, ***p ≤ 0.001), in agreement with our previous findings. Also, we found chromosome 21 to exhibit higher mis-segregation rate in Ts21 compared to 2N control (2N, 0.19%; Ts21, 0.95%; two-tailed χ^2 test, *p ≤ 0.05) and chromosome 18 to exhibit higher mis-segregation rate in Ts18 compared to 2N control (2N, 0.25%; Ts18, 0.95%; two-tailed χ^2 test, *p ≤ 0.05).

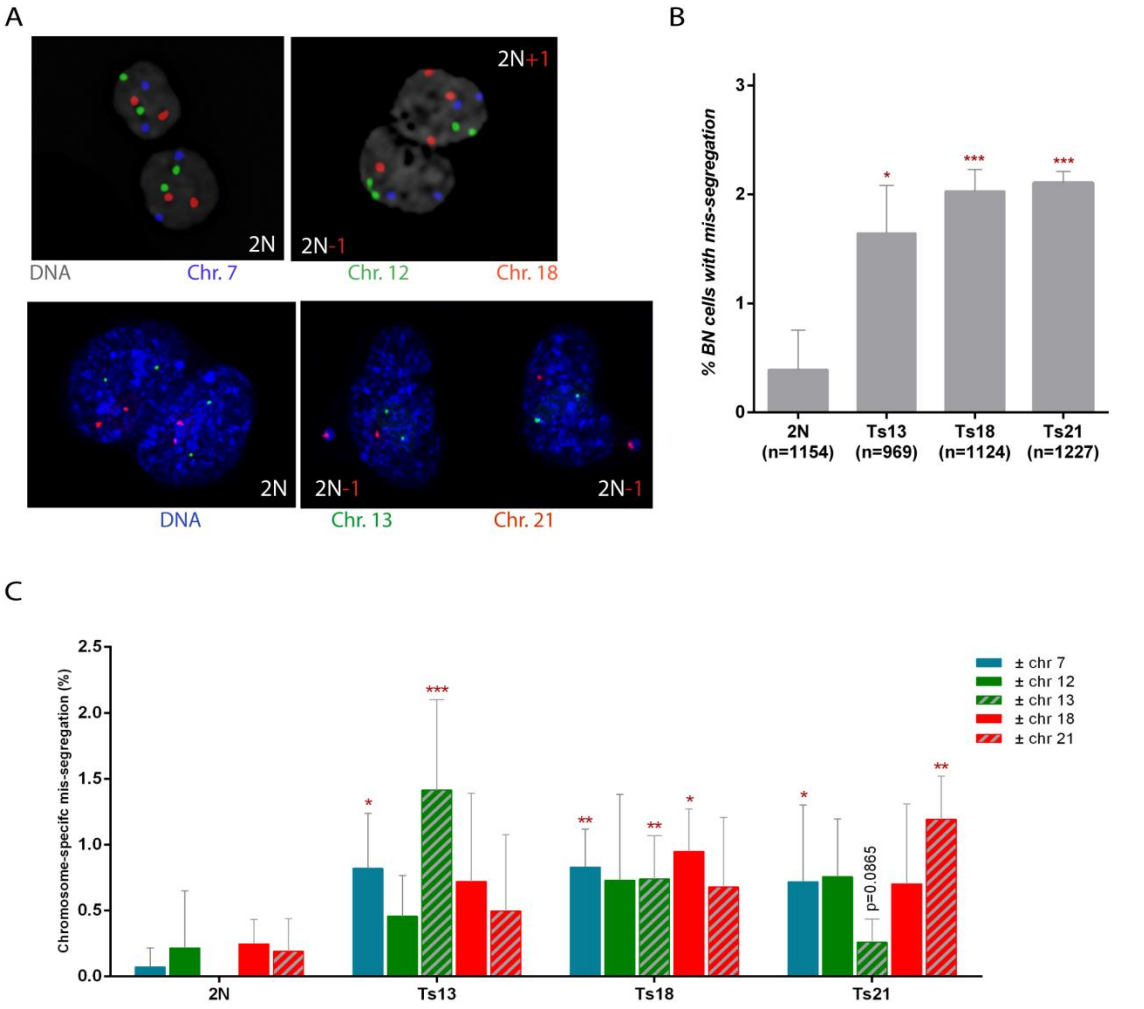

Figure 3.8 | Increased rates of chromosome mis-segregation in post-mitotic cells of human trisomies. A - Examples of FISH-stained binucleated (BN) cells with locus-specific probes for chromosomes 13 (green) and centromere-specific probes for chromosomes 7(blue), 12(green) and 18 (red); **B** - Frequencies of chromosome mis-segregation were significantly higher in Ts13 (1.65%), Ts18 (2.03%) and Ts21 (2.11%) compared to 2N cells (0.39%; two-tailed $\chi 2$ test, *p ≤ 0.05, ***p ≤ 0.001). Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 969-1227 cells were analyzed for each condition; n= total binucleated cell counts; **C** - Quantification of individual chromosome mis-segregation rates in BN cells. Mis-segregation of chromosome 7 is increased for all the trisomies analyzed compared to diploid control (2N, 0.07%; Ts13, 0.82%; Ts18, 0.83%; Ts21, 0.72%; two-tailed $\chi 2$ test, *p ≤ 0.05, **p ≤ 0.01); mis-segregation of chromosome 13 is higher in trisomy 13 (2N, 0.0%; Ts13, 1.41%; two-tailed $\chi 2$ test, ***p ≤ 0.001), as well as in trisomy 18 (0,74%; two-tailed $\chi 2$ test, *p ≤ 0.05); mis-segregation of chromosome 18 is increased in trisomy 18 (2N, 0.25%; Ts18, 0.95%; two-tailed $\chi 2$ test, *p ≤ 0.05); mis-segregation of chromosome 21 is increased in trisomy 21 (2N, 0.19%; Ts21, 1.19%; two-tailed $\chi 2$ test, *p ≤ 0.05); For all the other cases represented, there is no significant differences compared with the corresponding chromosome in diploid control (two-tailed $\chi 2$ test, ns p>0.05). BN stands for binucleated.

Overall, these results uncovered two important observations. First, different chromosomes mis-segregate at distinct rates as evidenced by the behaviour of chromosome 7 vs.

chromosome 12. Chromosome 7 was found to mis-segregate at higher rates in all trisomies compared to control, while chromosome 12 mis-segregation rate remains unchanged in aneuploid cells when compared to control. Second, chromosome mis-segregation seems biased for the trisomic chromosome.

Nevertheless, we must call the attention for the limited number of mis-segregation events scored for each chromosome in this type of analysis (post-mitotic FISH) where the total number of binucleated cells scored is also limiting. To increase the robustness of the results, we should increase the sample size, which is demanding for primary cell cultures. Alternatively, we used a protocol to artificially increase the number of chromosome mis-segregation events in our cell cultures (see description below).

3.8 - Induction of chromosome mis-segregation events in aneuploid cells reveals chromosome-specific rates

To increase the number of chromosome mis-segregation events in 2N, Ts18 and Ts21 cells in order to more accurately measure individual chromosome rates, we used an assay that is able to induce chromosome mis-segregation. Ts13 was not included in this assay due to lack of available samples. This assay consists in a combined treatment of mitotic cells with an Eg5 inibitor (S-trityl-L-cysteine, STLC) followed by an Aurora B inhibitor (ZM447439). The Eg5 inhibitor allows mitotic cell enrichment as it prevents the assembly of a bipolar spindle (cells with monopolar spindle will remain arrested in prometaphase) (Skoufias et al., 2006). Cells with monoastral spindles often establish erroneous syntelic and merotelic kinetochore-microtubule attachments (Cimini & Degrassi, 2005). Following the removal of S-trityl-L-cysteine, the use of an Aurora B inhibitor will prevent the correction of wrong attachments leading to increased chromosome mis-segregation (Ditchfield et al., 2003). We reasoned that this experimental set-up would allow us to more accurately identify any bias for chromosome mis-segregation due to a considerable increase in the number of chromosome mis-segregation events analysed. Chromosome mis-segregation events were quantified in anaphase-telophase mitotic cells following STLC washout and reestablishment of spindle bipolarity in the presence of ZM447439 (Figure 3.9A). As expected, the frequency of chromosome mis-segregation found in anaphase-telophase mitotic cells was considerably higher than previously measured by interphase and post-mitotic fixed experiments (3-9 fold increase), showing that this experiment is working as expected (Figure 3.9B). In agreement with our previous data, we found that trisomic cells have a significant increase in

chromosome mis-segregation compared to diploid control cells (2N, 3.56%; Ts18, 6.56%; Ts21, 6.12%; two-tailed χ^2 test, *p ≤ 0.05; ***p ≤ 0.001; ****p ≤ 0.0001), demonstrating that aneuploidy leads to chromosomal instability and karyotype heterogeneity.

Looking to individual chromosome mis-segregation rates (Figure 3.9C), again we found chromosome 7 to mis-segregate more often in all the trisomies analysed compared to diploid control (2N, 0.90%; Ts18, 2.10%; Ts21, 2.38%; two-tailed $\chi 2$ test, *p ≤ 0.05, **p ≤ 0.01), whereas chromosome 12 behaves similarly in both trisomies and control (2N, 2.56%; Ts18, 2.46%; Ts21, 2.46%, two-tailed $\chi 2$ test, ns p > 0.05). Also, chromosome 21 mis-segregate more often in trisomy 21 compared to diploid control (2N, 0.73%; Ts21, 2.40%, two-tailed $\chi 2$ test, **p ≤ 0.01). Interestingly, we found chromosome 18 to exhibit significantly higher mis-segregation rate in Ts18 and Ts21 compared to 2N control (2N, 1.20%; Ts18, 4.70%; Ts21, 2.84%; two-tailed $\chi 2$ test, **p ≤ 0.01, ****p ≤ 0.0001).

The mis-segregation rates of individual chromosomes were determined by calculating the mean distance to the ratio *observed/expected* values of chromosome mis-segregation for each condition. As *expected* values we considered the number of mis-segregation events assuming that all chromosomes mis-segregated at the same rate: e.g. if we analyse 24 mis-segregation events in fixed experiments using probes for 3 different chromosomes (in a given sample), then we expect to observe 8 (24/3) events for each chromosome. As *observed* values we used the exact number of mis-segregation found for each chromosome and condition. Finally, we calculate the distances to the *observed/expected* ratio (that is equal to 1 when the nº of mis-segregation events *observed* is equal to the nº of mis-segregation events *expected* in that condition). The plot represents the distances calculated to the *observed/expected* ratio and normalized so that a ratio equal to 1 is plotted as y=0. This kind of representation will allow us to easily perceive specific chromosome mis-segregation within each case. When we plotted these distances (Figure 3.9D) we found different patterns of mis-segregation comparing to those inferred from post-mitotic FISH experiments performed in the absence of drug treatments (Figure 3.8C). Using this assay, all chromosomes seem to mis-segregate as much frequently. Chromosome 7 seem to mis-segregate less in trisomic 18 cells than expected for this chromosome (0.30 times less; unpaired t test; **p ≤ 0.01). On the other hand, chromosome 18 show significantly increased in chromosome mis-segregation compared to the other chromosomes analysed (0.53 times more than expected for this chromosome; unpaired t-test; **p ≤ 0.005). Also, chromosome 12 seems to mis-segregate more than any other chromosome in diploid control (1.37 times more than expected for this chromosome; unpaired t-test; ns, p > 0.05). Surprisingly,

chromosome 21 no longer seems to mis-segregation rate more in Ts21 (0.15 times more than expected for this chromosome; unpaired t-test; ns, $p > 0.05$).

These observations suggest that the low number of events quantified in post-mitotic FISH experiments in the absence of STLC/ZM might lead to misinterpretations when chromosome-specific mis-segregation events are considered. Nevertheless, chromosome 18 was consistently found to exhibit biased mis-segregation in trisomy 18, both in untreated and drug-treated experimental conditions used for FISH analysis. Alternatively, we should acknowledge that the STLC/ZM assay might interfere with the natural specific mechanisms contributing to distinct chromosome mis-segregation rates in each karyotype. This assay leads to an artificial increase of chromosome mis-segregation based on the establishment of wrong attachments, which might affect chromosomes that normally would not mis-segregate in the absence of drugs treatment.

Generally, we provide strong evidence that specific aneuploidies can induce specific patterns of chromosome mis-segregation. The observation that trisomic chromosomes appear to display higher mis-segregation rates than disomic chromosomes in human trisomies, led us to question whether disturbance of nuclear architecture due to the presence of the extra chromosome might compromise its efficient segregation.

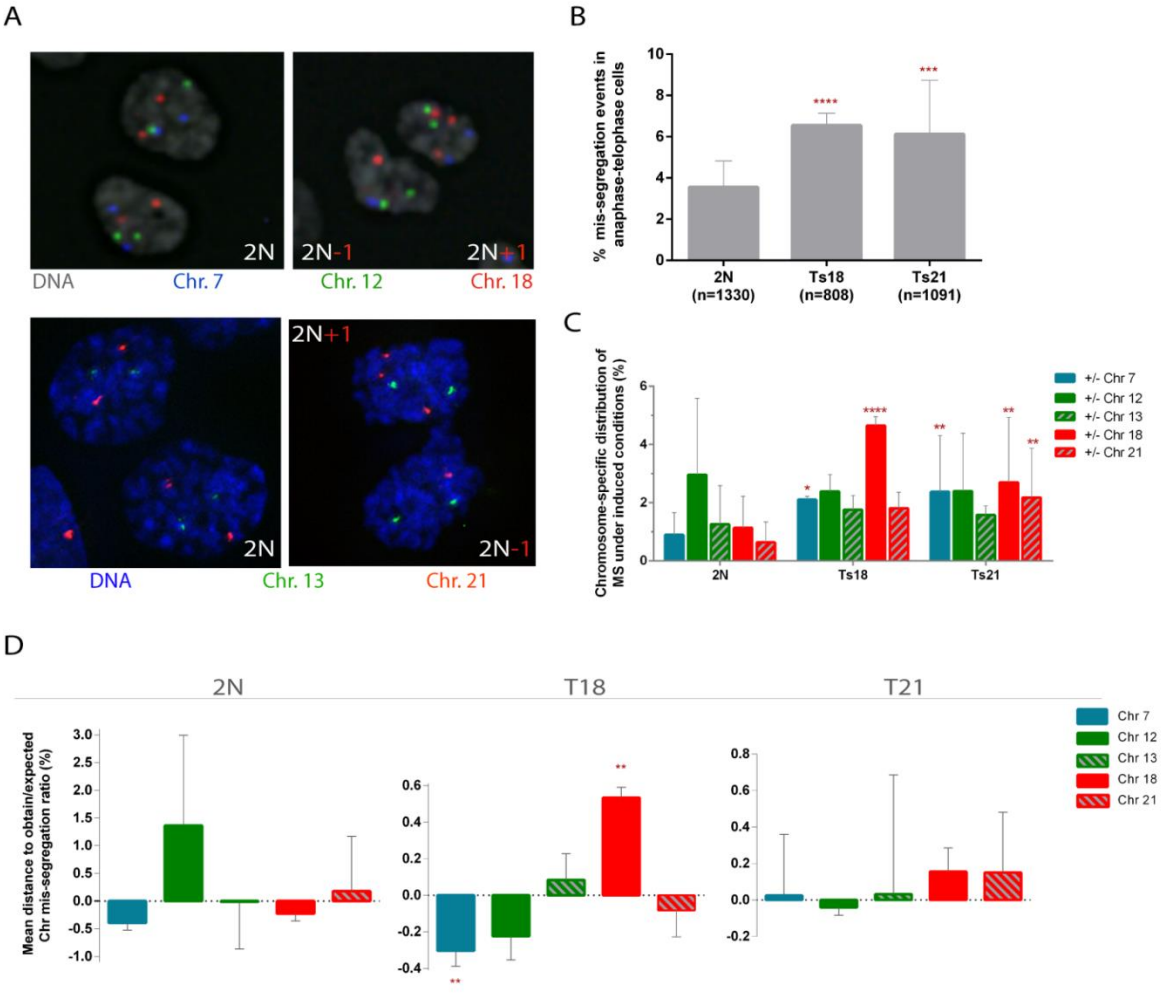

Figure 3.9 | Induced rates of chromosome mis-segregation in anaphase-telophase mitotic cells. A - Examples of FISH-stained anaphase-telophase mitotic cells with chromosome-specific probes for chromosomes 7 (blue), 12 (green), 13(green), 18 (red) and 21 (red); **B** - Frequencies of chromosome mis-segregation events were significantly higher in Ts18 (6.56%) and Ts21 (6.12%) compared to 2N cells (3.56%; two-tailed $\chi 2$ test, ***$p \leq 0.0005$, ****$p \leq 0.0001$). Data are reported as mean ± S.E.M and represent the average of at least three independent experiments in which a total of 808-1330 cells were analyzed for each condition**; C** - Quantification of individual chromosome mis-segregation rates in STLC-ZM treated cells. Mis-segregation of chromosome 7 is increased for all the trisomies analyzed compared to diploid control 2N, 0.90%; Ts18, 2.10%; Ts21, 2.38%; two-tailed $\chi 2$ test, *$p \leq 0.05$, **$p \leq 0.005$); Chromosome 12 behaves similarly in both trisomies and control (2N, 2.56%; Ts18, 2.46%; Ts21, 2.46%, two-tailed $\chi 2$ test, ns $p>0.05$); Mis-segregation of chromosome 18 is increased in Ts18 and Ts21 compared to diploid control (2N, 1.20%; Ts18, 4.70%; Ts21, 2.84%; two-tailed $\chi 2$ test, **$p \leq 0.01$, ****$p \leq 0.0001$). For all the other cases represented, there is no significant differences compared with the corresponding chromosome in diploid control (two-tailed $\chi 2$ test, ns $p > 0.05$); n= total anaphase-telophase cell counts; **D** - Chromosome-specific distance to the ratio between observed and expected mis-segregation rates in anaphase-telophase mitotic cells. No significant differences were observed for each chromosome when compared to the expected value for both, diploid controls (chr 7, -0.3925; chr 12; 1.365; chr 13, -0.01; chr 18, -0.225; chr 21, 0.1775; Unpaired t-test, ns $p>0.05$), and in Ts21 (chr 7, 0.025; chr 12, -0.04; chr 13, 0.0325; chr 18, 0.155; chr 21, 0.15; Unpaired t-test, ns $p>0.05$). Chromosome 18 was found to have significantly higher mis-segregation than expected and chromosome 7 was found to have significantly lower mis-segregation than expected (chr 7, -0.303; chr 12, -0.223; chr 13, 0.0833; chr 18, 0.533; chr 21, -0.0833; Unpaired t-test test, **$p \leq$

0.01) Data are reported as mean ± S.E.M and represent the average of at least three independent experiments in which a total of 808-1330 cells were analyzed for each condition. Chr stands for chromosome.

3.9 - Does nuclear chromosome architecture dictate chromosome specific failure in mitosis?

We raised the hypothesis that the increased mis-segregation rate observed for certain chromosomes under standard growth conditions could be due to their distinctive positioning inside cell nucleus. So far, several studies have correlated physical chromosome properties (such as size) (Hui Bin Sun, Shen, & Yokota, 2000) and their specific positioning inside the nucleus with alignment defects (Zickler & Kleckner, 1999). Therefore we asked whether the wide positional variation of chromosomes inside the nucleus, and considering that the segregation of chromosomes is, at least partially, a process mediated by microtubules, could result in easiness of certain chromosomes to attach to microtubules during mitosis. If this is the case, then we expect that chromosomes positioning near the nuclear periphery may have more trouble to attach to spindle microtubules than inner positioned chromosomes, resulting in higher mis-segregation rates of these chromosomes. To examine this hypothesis, chromosome positioning was monitored using arrays of GFP fused to the *lacO* sequences allowing the tagged loci to be visualized as fluorescent spots during live cell imaging. Live cell imaging provides us a powerful tool for tracking the specific chromosome location and motion in G2 and during mitosis. In our study, we used cell lines with GFP-*lacO* at the long arm of chromosome 1 (B49.2.7) and at the long arm of chromosome 13 (B49.12.8). Chromosome 13 is estimated to be located near the nuclear periphery, in contrast to chromosome 1 that is estimated to position at the nucleus core (Chubb et al., 2002). To address if the most peripheral chromosomes tend to mis-segregate more than inner chromosomes, we treated B49.2.7 and B49.12.8 cell lines with the CENP-E small molecule inhibitor, GSK923295. CENP-E drives the congression of peripheral polar chromosomes and facilitates the attachment of chromosomes to spindle microtubules (Barisic, Aguiar, Geley, & Maiato, 2014). When CENP-E is inhibited, while the majority of chromosomes align at the cell's equator, a small number will cluster near the spindle poles (Bennett et al., 2015). With this in mind, and in accordance with our theory, we expect to observe a higher distribution of peripheral chromosomes towards the spindle poles compared to inner chromosomes.

Although chromosome positioning could be visualized in G2 cells, further analysis of chromosome motion was hampered by a decrease in GFP-lacO array expression levels during mitotic progression (Figure 3.10). Therefore, no conclusion could be reached about a possible causal role between nuclear chromosome positioning and biased chromosome

mis-segregation during mitosis. In line with these observations, a new approach must be developed to better track chromosome motion during mitosis.

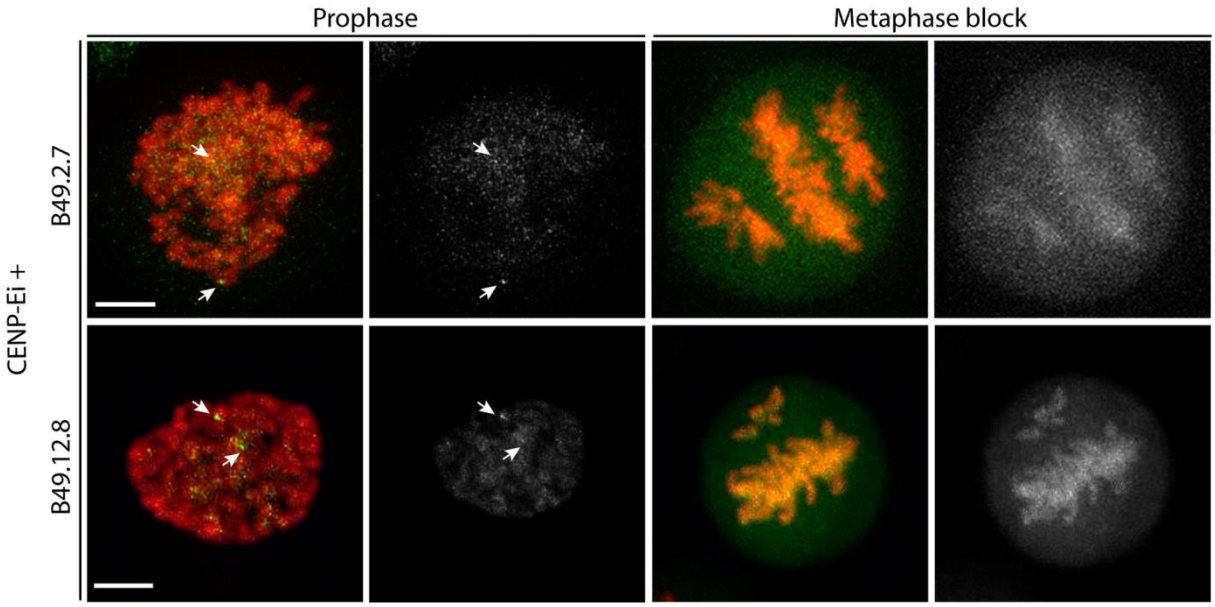

Figure 3.10 | Nuclear chromosome positioning of chromosome 1 and 13 during mitosis in GSK923295 treated cell line. Representative movie frames of live cell imaging of cell lines expressing GFP-tagged chromosome loci - B49.2.7 (GFP-lacO at 1q11) at the top row and B49.12.8 (GFP-lacO at 13q22) at the bottom row, after GSK923295 treatment (CENP-E inhibition). Arrows point to the GFP dots on prophase. Metaphase blocked cells show a small number of chromosomes clustering near the spindle poles and the GFP dots could not be recognized.

4

CONCLUDING REMARKS AND PERSPECTIVES

Previous attempts to address if aneuploidy can trigger chromosomal instability have been limited by i) the use of cancer cell line models that exhibit both complex aneuploidy and chromosomal instability, and ii) the intrinsic variability between the different methodologies used to evaluate chromosome-misegregation. Thus, we went on a strategy that not only used primary cell models of constitutional aneuploidy, but also combined several different approaches to measure chromosome mis-segregation, namely FISH staining with chromosome-specific probes in interphase nuclei and post-mitotic cells and spinning-disk confocal live cell imaging. The latter is actually the best, able to directly measure chromosome mis-segregation through the observation of anaphase lagging chromosomes, balancing possible miscalculations given by the previous methods. Also, this technique enables us to visualize, in real time, mitotic phenotypes arising from a specific aneuploidy condition.

First, our work demonstrates that human constitutional aneuploidies, namely trisomies 13, 18 and 21, induce chromosome mis-segregation and karyotype heterogeneity. This is in agreement with our previous findings for human trisomies 7 and 13 (Nicholson et al., 2015), as well as studies in budding yeast (Sheltzer et al., 2011). Also, we suggest that distinct trisomies induce similar rates of chromosome mis-segregation. However, depending on the extra chromosome, distinct mitotic phenotypes were observed in the aneuploid cells studied. In fact, this suggests that different mechanisms are contributing to chromosome mis-segregation. Our group previously reported that anaphase lagging chromosomes explain the increased CIN in trisomic 13 cells (Nicholson et al., 2015). However, we carefully recorded trisomic 18 and 21 dividing cells by live cell imaging and we didn't found anaphase lagging chromosomes in these trisomies. Because anaphase lagging chromosomes were not significantly more frequent in trisomies 18 and 21, pre-mitotic lesions, as anaphase chromatin bridges and acentric chromosome fragments are likely contributing to aneuploidy, in agreement to what was previously reported (Passerini et al., 2016). However, acentric chromosome fragments and anaphase chromatin bridges were also found to occur very

rarely in the trisomies 18 and 21, even though we should acknowledge that chromatin bridges are often ultrafine and thus hardly detected.

Instead, we found other mitotic phenotypes that seem to be karyotype-specific and might represent alternative mechanisms leading to chromosome mis-segregation. This is the case of the spindle mispositioning phenotype observed as a unique feature of Ts21 cells, and the midbody defects that despite being commonly detected in all trisomies 13, 18 and 21, likely arise due to altered expression of specific cytokinesis genes present in chromosomes 13, 18 and 21. This is the case of the SPG20 gene in chromosome 13, previously shown to account for the cytokinesis failure phenotype in trisomy 13 (see also below).

Our study thus raised the question that we would like to address in a future work. What are the molecular mechanisms causing late mitotic defects in spindle positioning and midbody structure defects in trisomies 18 and 21? Our group previously identified the mechanism behind cytokinesis failure in trisomy 13 and excluded SPG20 overexpression as a causal mechanism in trisomies 18 and 21 (Nicholson et al., 2015). We hypothesised that there are genes involved in cytokinesis located in chromosomes 18 and 21, whose overexpression in trisomies 18 and 21 could lead to karyotype-specific cytokinesis phenotypes. Using the NCBI gene database (http://www.ncbi.nlm.nih.gov/gene) we searched for putative target genes. There are two genes in chromosome 18 that could explain the midbody/cytokinesis defects: charged multivesicular body protein 1B (CHMP1B) and phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3). CHMP1B is located on 18p11.21 and encodes for a protein involved in recruiting VPS4A and/or VPS4B and SPAST to the midbody of dividing cells. Both, VPS4A/B and SPAST have been suggested to act as regulators of cytokinesis: VPS4A/B is required for normal midbody endosomal trafficking and SPAST is involved in abscission step of cytokinesis and in the nuclear envelope reassembly during anaphase (Morita et al., 2010; Renvoise et al., 2010). PIK3C3 is located on 18q12.3 and encodes for a protein involved in regulation of degradative endocytic trafficking and required for the abscission step in cytokinesis (Sagona et al., 2010). Regarding the spindle mispositioning phenotype in trisomy 21, we found one possible target gene, pericentrin (PCNT). PCNT is located on 21q22.3 and encodes for a protein important to normal functioning of the centrosomes, cytoskeleton, and cell-cycle progression. Significant evidence supports a role of PCNT disruption in microtubule nucleation, centrosome and spindle orientation defects (Delaval & Doxsey, 2010).

Lastly, to address for the biased chromosome mis-segregation of the trisomic chromosome vs. the remaining chromosomes in the cell, we performed individual chromosome counts under normal and artificially-induced higher mis-segregation rates. Under untreated conditions, we found a significant increase in the mis-segregation rate of chromosome 21 in Ts21, similarly to our previous finding regarding chromosome 13 in Ts13. However, no significant increase was found for the mis-segregation rate of chromosome 18 in Ts18. Because the number of total mis-segregation events detected in normal/untreated conditions is so low, it is difficult to infer robust data regarding the rates for each individual chromosome. Therefore, we artificially induced higher mis-segregation rates through an experimental treatment with mitotic drugs that promotes the incidence of mis-segregation events. We measured the mis-segregation rate of each individual chromosome out of a small group (5 pairs) in anaphase-telophase mitotic cells and found that specific aneuploidies can induce specific patterns of chromosome mis-segregation and a biased chromosome mis-segregation for the trisomic chromosome in the trisomies 13 and 18. In the case of the extra 21, however, there was not a consistent increase in the mis-segregation rate in untreated and drug-treated experimental conditions. However, we should consider that the STLC/ZM assay might interfere with the natural specific mechanisms contributing to distinct chromosome mis-segregation rates in each karyotype. Therefore, mis-segregation rates should be measured and compared between different conditions. Another assay to artificially increase chromosome mis-segregation would be the combined treatment of mitotic cells with CENP-E and Mps1 inhibitors. In contrast to STLC-treated cells, cells treated with the CENP-E inhibitor are able to assemble bipolar spindles and to align most of their chromosomes. However, a small number of chromosomes remain clustered near the spindle poles, leading to prometaphase delay (Wood et al., 2010). Then, adding an inhibitor targeting the spindle checkpoint kinase Mps1, cells are driven into anaphase and mis-segregate the polar chromosomes originating, on average, two mis-segregation events per division (Bennett et al., 2015). Therefore, this approach could be used as a supplementary experiment to STLC/ZM assay, as it generates congression problems instead merotelic attachments. As so, the combination of two different mis-segregation stimuli could help us to discern whether chromosomes are differently sensitive to treatments that interfere with distinct mechanisms leading to mis-segregation. Importantly, such finding would be extremely important in the context of cell-drug response mechanism, especially concerning cancer drug resistance studies.

Even though the increased frequency of anaphase lagging chromosomes in trisomy 13 was shown as the main mechanism causing CIN and karyotype heterogeneity, it is not known yet

whether the lagging chromosomes in trisomic 13 anaphases are often one chromosome 13. However, this question can only be addressed if more accurate measurements of chromosome specific mis-segregation are achieved by combining live cell imaging and chromosome-specific staining to track chromosome motion during mitosis. The use of an innovative system using CRISPR/Cas9 for precise genome editing of living cells will certainly be an option. In our study, we tried to use one cell line already available (gently provided by Wendy Bickmore) which has a specific integration of a GFP-lacO array in chromosome 13. Nevertheless, we faced several troubleshooting problems, mainly related to unexpected changes in GFP-lacO expression levels during mitotic progression. Once we manage to establish a CRISPR/Cas9-based editing to track chromosome 13, we propose to test whether disturbance of the nuclear architecture due to the presence of an extra chromosome might compromise its efficient segregation in trisomic cells.

5

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