Aneuploidy triggers a conserved global response and impairs cellular homeostasis

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER FAKULTÄT FÜR BIOLOGIE DER LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

vorgelegt von

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München, den 25.04.2016 Verena Passerini

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Verena Passerini contributed to this work by culturing the cell lines and preparing the samples for the microarray analysis. She also participated in reading and commenting the manuscript.

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Verena Passerini contributed to this work by carrying out the experiments showed in figure 1a, 1b, 5a and 5b. She also participated in discussion and interpretation of the results as well as in reading and commenting the manuscript.

Passerini, V*, Ozeri-Galai E*, de Pagter MS, Donnelly N, Schmalbrock S, Kloosterman WP, Kerem B, Storchová Z **The presence of extra chromosomes leads to genomic instability.** Nat. Commun. 7:10754 doi: 10.1038/ncomms10754 (2016).

Verena Passerini contributed to this work by designing, planning and performing the experiments showed in main and corresponding supplementary figures 1b, 1c, 1d, 2a, 2b, 2c, 3c, 5 and 6. She created the figures and participated in the interpretation and discussion of obtained results as well as in writing the manuscript.

Martinsried, 22.04.16

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1. Abbreviations

17-AAG	17-N-allylamino-17-demethoxygeldanamycin
53BP1	p53 binding protein 1
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
APC/C	anaphase-promoting complex / cyclosome
ARP	aneuploidy response pattern
ATM	ataxia telangiectasia mutated
ATR	ATM- and RAD3-related
BER	base excision repair
BS	bloom’s syndrome
CDK	cyclin-dependent kinase
CIN	chromosomal instability
CNA	copy number aberration
CENP-E	centromere-associated protein E
DDK	Dbf4-dependent protein kinase)
DDR	DNA damage response
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DS	down’s syndrome
DSB	double strand break
ESR	environmental stress response
FA	fanconi anemia
GG-NER	global genome nucleotide excision repair
GIN	genomic instability
HR	homologous recombination
HSF1	Heat shock factor 1
HSP90	heat shock protein 90
HU	Hydroxyurea
KAR1	karyogamy gene 1
ICL	interstrand cross-link
iPSC	inducible pluripotent stem cells
MCM	Minichromosome maintenance protein complex
MEF	Mouse Embryonic Fibroblasts
MMBIR	microhomology-mediated break-induced replication
MMR	mismatch repair
MVA	mosaic variegated aneuploidy
NER	nucleotide excision repair

NGS	next generation sequencing
NHEJ	Non homologous end joining
HVP	Human Papilloma Virus
ORC	origin recognition complex
PEG	Polyethylene glycol
PCNA	proliferation cell nuclear antigen
PIKK	Phosphatidylinositol kinase related kinase
pre-IC	pre-Initiation complex
pre-RC	pre replication complex
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPA	replication protein A
SAC	Spindle assembly checkpoint
SNP	single nucleotide polimorfism
SSB	single strand breaks
ssDNA	single stranded DNA
TC-NER	transcription-coupled nucleotide excision repair
TKNEO	thymidine kinase with neomycin phosphotransferase reporter gene
TLS	translesion synthesis
XIST	X-inactive specific transcript
YAC	yeast artificial chromosome

2. Summary

Aneuploidy, or unbalanced chromosome number, has often detrimental physiological effects in eukaryotic cells. Aneuploidy is associated with congenital trisomy syndromes, e.g. Down syndrome, but it is also linked to several other pathological states such as Alzheimer's disease, schizophrenia and autism. In addition, aneuploidy is often found in cancer cells and high rates of aneuploidy in tumors correlate with poor prognosis and drug resistance. Although it has been proposed that aneuploidy could contribute to tumorigenesis by facilitating genomic instability, whether and how aneuploidy can lead to genomic instability remains elusive.

To study aneuploidy in human cells, we have previously generated cell lines carrying one or two supernumerary chromosomes in an otherwise diploid background by microcell mediated chromosome transfer. Similarly to other aneuploid model systems of earlier studies, our human aneuploid cell lines showed impaired proliferation and a conserved cellular response to the presence of extra chromosomes. Moreover, we found that aneuploidy alters protein homeostasis and impairs induction of heat shock response in human cells. Pathway analysis based on transcriptome and proteome data revealed characteristic gene expression changes called aneuploidy response pattern that is defined, among others, by down-regulation of factors involved in DNA replication and repair. Consistent with these observations we found that aneuploidy increases the frequency of anaphase chromatin bridges, broken chromosomes and ultrafine DNA bridges. Moreover, aneuploid cells accumulate more DNA damage even in unperturbed conditions and display higher sensitivity to replication stress than diploids. Using next generation sequencing we determined that a presence of extra chromosomes elevates frequency of chromosomal rearrangements with a breakpoint junction pattern suggestive of replication defects. Finally, we demonstrated that the observed decreased levels of MCM2-7 contribute to the replication stress and consequent genomic instability detected in aneuploid cells.

Taken together, these results provide a new insight into the possible mechanisms responsible for impaired genomic stability in response to aneuploidy. Our study provides the first evidence that a gain of chromosomes triggers replication defects and accumulation of DNA lesions, thus promoting genomic instability and possibly contributing to tumor development.

3. Zusammenfassung

Aneuploidie oder numerische Chromosomenaberration hat meist einen schädlichen Einfluss auf die Physiologie eukaryotischer Zellen. Aneuploidie wird mit angeborenen Trisomien, wie zum Beispiel Down-Syndrom assoziiert, aber auch mit anderen pathologischen Zuständen wie Alzheimer, Schizophrenie und Autismus. Zusätzlich findet man Aneuploidie häufig in Krebszellen. Komplexe Aneuploidien sind mit schlechter Prognose und Resistenz gegen Krebsmedikamente verbunden. Obwohl hypothetisiert wird, dass Aneuploidie durch genomische Instabilität zur Tumorgenese beiträgt, sind die genauen molekularen Mechanismen wie dies geschieht nicht bekannt.

Um Aneuploidie in menschlichen Zellen zu untersuchen, haben wir mit Hilfe von Mikrozell-vermitteltem Chromosomentransfer Zelllinien generiert, welche ein oder zwei zusätzliche Chromosomen, in einem sonst diploiden Hintergrund, haben. Diese humanen aneuploiden Zellen weisen eine beeinträchtige Zellproliferation und eine uniforme Deregulation zellulärer Signalwege auf. Darüber hinaus haben wir gezeigt, dass Aneuploidie die Proteinhomöostase verändert und die Reaktion humaner Zellen auf Hitzeschock beeinträchtigt. Analyse von Transkriptom- und Proteom-daten, offenbarte charakteristische Änderungen in der Genexpression bestimmter Signalwege. Unter anderem wird dieses wiederkehrende Muster an veränderten Signalwege durch nach unter regulierte Faktoren, die in DNS-Replikation und -Reparatur involviert sind, definiert. Übereinstimmend mit diesen Beobachtungen haben wir herausgefunden, dass Aneuploidie zur Akkumulation von DNS-Brücken, beschädigten Chromosomen und ultrafeinen DNS-Brücken in der Anaphase führt. Ferner akkumulieren aneuploide Zellen mehr DNS-Schäden, sogar unter normalen Bedingungen, und reagieren sensibler auf Replikationsstress als diploide Zellen. Mit Hilfe von Next Generation Sequencing konnten wir zeigen, dass die Präsenz von zusätzlichen Chromosomen die Frequenz chromosomaler Reorganisation erhöht. Das Muster der Chromosomenbrücke weist auf Replikationsdefekte hin. Darüber hinaus demonstrierten wir, dass die erniedrigten MCM2-7 Proteinlevel zur genomischen Instabilität beitragen, wie sie in aneuploiden Zellen entdeckt wurden.

Unsere Ergebnisse ermöglichen neue Einblicke in die molekulare Mechanismen, die für genomische Instabilität in Aneuploidie verantwortlich sind. Unsere Studie zeigt zum ersten Mal, dass zusätzliche Chromosomen Replikationsdefekte und DNS-Läsionen auslösen, wodurch genomische Instabilität gefördert wird und diese Instabilität möglicherweise zur Entstehung von Tumoren beiträgt.

4. Introduction

The hereditary information of each eukaryotic species, coded in the genome, is packed in the nucleus and organized into a defined number of chromosomes. Some organisms, such as fungi and algae, and germ cells have a single set of unpaired chromosomes and they are called haploids (1N). However, the majority of the metazoans cells are diploid, which means that each of their chromosomes is present in two homologues copies, one inherited from the mother and one from the father (2N). A normal human cell contains 46 chromosomes: 22 pairs of autosomes and one pair of sex chromosomes. In every cell division, the genome needs to be accurately duplicated and distributed evenly into the daughter cells. Cells have evolved surveillance mechanisms to ensure that this happens correctly, but sometimes these molecular machineries fail. As a consequence the daughter cells either die or survive with a change in numbers and structure of chromosomes, a scenario also called aneuploidy.

4.1 Aneuploidy

Aneuploidy is a condition where the number of chromosomes in the cell is not a multiple of the haploid set. The term aneuploidy describes very diverse karyotypes that could be classified in two categories: “numerical aneuploidy”, where one or more entire chromosomes are present in abnormal copy number (Figure 1a), and “structural aneuploidy” referring to chromosomal abnormalities that arise from breakage and incorrect rejoining of chromosome segments (Figure 1b) (Storchova, 2012).

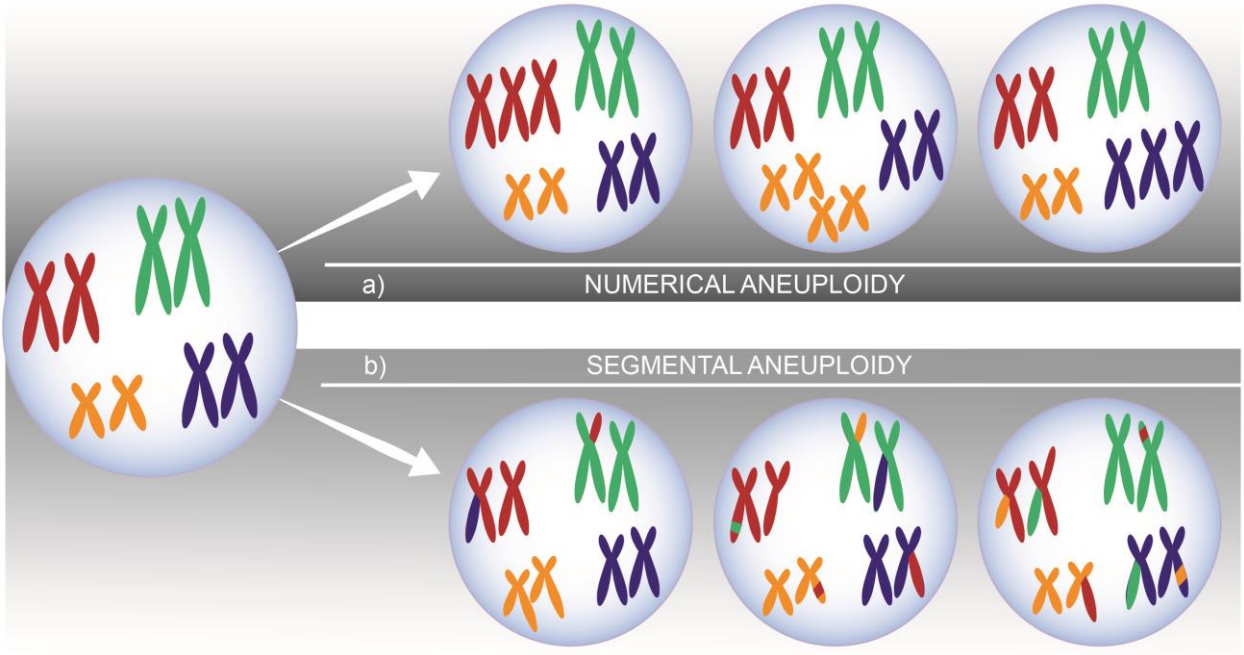

Figure 1: Numerical and segmental aneuploidy. Scheme showing examples of (**a**) whole chromosome gains and losses (numerical aneuploidy) and (**b**) sub-chromosomal gains, losses, inversions and translocations (structural aneuploidy).

More than a century ago the German zoologist Theodor Boveri discovered the adverse effects of aneuploidy on cell and organism development of sea urchin embryos (Boveri, 2007). Similarly, evidences of the deleterious consequences of aneuploidy were found in several other species. Lindsley and colleagues showed that structural aneuploidy is not well tolerated in flies leading to reduced viability and developmental deficiencies (Lindsley *et al*, 1972). Aneuploid mice carrying Robertsonian translocations often do not survive embryogenesis or die shortly after birth (Magnuson *et al*, 1982). Moreover, a recent systematic characterization of disomic yeast strains revealed that aneuploidy hampers cell proliferation (Torres *et al*, 2007).

In humans, one of the best-known aneuploidies is trisomy 21 (Down syndrome), with chromosome 21 present in three copies, leading to severe mental retardation and developmental defects. Importantly, Down syndrome is the only trisomy compatible with survival until adulthood. Other identified aneuploid disorders, although quite rare, are trisomy 8, 13 and 18 that are responsible for life-threatening complications in early life (Agrawal & Agrawal, 2011; Loane *et al*, 2013). Besides these, no other trisomic syndromes are found in humans, indicating that most aneuploidies are lethal at embryonic stages. This hypothesis is supported also by the evidence that aneuploidy is the major cause of spontaneous abortions (Lebedev *et al*, 2004). In addition to trisomic syndrome, somatic aneuploidy is also found during aging of the brain and may contribute to the development of neurodegenerative pathologies such as Alzheimer's disease, schizophrenia and autism (MacIntyre *et al*, 2003; Yurov *et al*, 2014). Finally, possible aneuploid karyotypes do not only involve chromosome gains but also chromosome losses or monosomies. No full monosomies are compatible with survival in humans, but some partial monosomies of chromosome 21 have been described (Toral-Lopez *et al*, 2012). The consequences of partial monosomies are very heterogeneous and variable depending on the size of the chromosomal region that is monosomic (Toral-Lopez *et al*, 2012). Therefore, it is still unknown, whether the consequences of chromosomes gains or losses are the same and unfortunately no model systems to study the effects of monosomies on human cells are available so far.

Surprisingly, there are some cases where aneuploidy is not pathological, but a physiological state. It has been suggested that such physiological aneuploidy may have beneficial effects for cells or organisms. Normal human liver contains 25-50% polyploid and aneuploid hepatocytes (Duncan *et al*, 2010) and it has been estimated that 30-35% of neurons in the fetal brain are aneuploid (Yurov *et al*, 2007). A proposed explanation is that these unusual somatic aneuploidies evolved as an adaptive mechanism in response to injury as it was demonstrated in hepatocytes. In fact, Duncan and colleagues showed that selection of a specific aneuploid karyotype can result in the adaptation of chronic liver injury (Duncan *et al*, 2012). Thus, acquired aneuploidy appears to be a quick way for mammalian cells to adapt to stressful conditions. Similarly, it was shown in yeast that under prolonged stress conditions aneuploidy arises as a transient adaptation to allow cell to survive while developing a long-term stable evolutionary solution (Yona *et al*, 2012). Moreover, aneuploidy is frequently associated with drug resistance in

some pathological species of yeast and fungi such as *Cryptococcus neoformans* and *Candida albicans* (Selmecki *et al*, 2006; Sionov *et al*, 2010). Beyond yeast and fungi, aneuploidy is also often found in plants, where it seems to be well tolerated in the germline cells suggesting that aneuploidy might represent a quick and reversible route to introduce genetic variation (Henry *et al*, 2010).

Interestingly, numerical and structural aneuploidy are also a common feature of human tumors and compelling evidences show that high aneuploidy rate in tumors correlate with poor prognosis and drug resistance (Carter *et al*, 2006; Birkbak *et al*, 2011; Lee *et al*, 2011). These findings suggest that in stressful conditions or extreme environments aneuploidy could confer an adaptive advantage to cancer cells. However, despite the high frequency of chromosome copy number alterations in cancer, it is not yet clear what is the role of aneuploidy in tumorigenesis and in cancer phenotypes.

Other common hallmarks of cancer that are frequently associated with aneuploidy are chromosomal instability (CIN) and genomic instability (GIN). Often the term GIN is used to describe broadly all the forms of genomic instability, including CIN. However, in this work the term GIN will refer only to a class of genomic alterations such as micro and mini-satellite instability, point mutations, gross chromosomal rearrangements or copy number variations arising as a consequence of defective DNA replication or repair. On the other hand CIN describes whole-cromosomal numerical abnormalities caused by defects in chromosome segregation. While aneuploidy is defined as an abnormal number of chromosomes, CIN refers to increased frequency of chromosome missegregation during cell division. Cells showing CIN are always aneuploid, but this does not mean that aneuploid cells are always chromosomally instable. An example of a stable aneuploidy that occurs without CIN is trisomy 21, as it was shown that there is no additional increase in chromosome gain or loss in cells from Down syndrome patients (Valind *et al*, 2013). Moreover, Lengauer and colleagues showed that aneuploid cells obtained by introducing an extra chromosome into a diploid cell line do not display CIN, suggesting that aneuploidy *per se* does not cause CIN (Lengauer *et al*, 1997). In tumors CIN is often, but not always associated with aneuploidy. Some tumor cells propagate their aneuploid karyotype stably, while others are chromosomally instable leading to population with heterogeneous karyotypes (Lengauer *et al*, 1997). GIN is described as the increased rate of gaining structural chromosomal rearrangements. Current models of tumorigenesis suggest that GIN is critical for cancer development, as it can explain the rapid accumulation of mutations typical of tumors (Beckman & Loeb, 2006). The correlation between GIN and aneuploidy remains unclear. Although there are studies showing that the two phenomena could be related, no evidences of a direct link have been collected so far (Necchi *et al*, 2015; Natarajan, 2015). However, co-existence of GIN and aneuploidy in cancer cells suggest that a direct association between them might exist and uncovering this connection could explain their possible role in tumor onset and development.

4.2 Causes of aneuploidy

Numerical aneuploidy arises due to chromosome missegregation events that can occur spontaneously or as a consequence of defects in crucial genes involved in cell division. In human RPE1 and HCT116 cells cultured *in vitro,* the sporadic chromosome segregation error rate is 0.025% per chromosome and cell division (Thompson & Compton, 2008). The sporadic missegregation error rate is maintained so low by the mitotic checkpoint that delays the onset of mitosis until all the chromosomes are correctly bi-oriented on the microtubule spindle (Figure 2a). Accordingly, inactivation of mitotic checkpoint leads to massive chromosome missegregation. Several mechanisms could lead to defect in mitotic checkpoint function and errors in chromosome partitioning:

1. *Weakened mitotic checkpoint.* The spindle assembly checkpoint (SAC) delays mitotic progression until kinetochores of all chromosomes are properly attached to microtubules. One single unattached kinetochore is sufficient to trigger SAC activation and delay of anaphase onset (Rieder *et al*, 1995). However, due to altered expression or mutation of mitotic checkpoint components the SAC signal could be weakened and the cell divide before the chromosomes are correctly attached to microtubules (Figure 2b).
2. *Defective chromatid cohesion.* After DNA replication, the pairs of sister chromatids are kept physically connected with each other by cohesin rings during G2 and mitosis until the onset of anaphase. Sister chromatid cohesion is essential to oppose the pulling force of microtubules and to allow correct bi-orientation of chromosomes on the mitotic spindle (Tanaka *et al*, 2000). For correct chromosome segregation, cohesin rings are cleaved by separase at anaphase onset once all the chromosomes are properly aligned at the spindle midzone. Defects in proteins involved in chromatid cohesion could lead to lack of cohesion, premature segregation and eventually to aneuploidy as demonstrated by studies in yeast and human cells (Guacci *et al*, 1997; Solomon *et al*, 2011) (Figure 2c).
3. *Centrosome amplification.* Eukaryotic cells contain normally one centrosome that is duplicated in S phase in order to establish two spindle poles during mitosis. Formation of bipolar mitotic spindles is an essential step to faithfully segregate chromosomes into daughter cells. However, cells can acquire multiple centrosomes for example due to cytokinesis failure, cell fusion or centrosome overduplication (Wong & Stearns, 2003). If a cell with more than two centrosomes enters mitosis, it forms sometimes a multipolar spindle, resulting in three or more highly aneuploid and often inviable daughter cells. However, frequently the multipolarity is resolved forming a “pseudo-bipolar” spindle by clustering of centrosomes that still might lead to chromosome missegregation due to merotelic attachment (Quintyne *et al*, 2005) (Figure 2d).

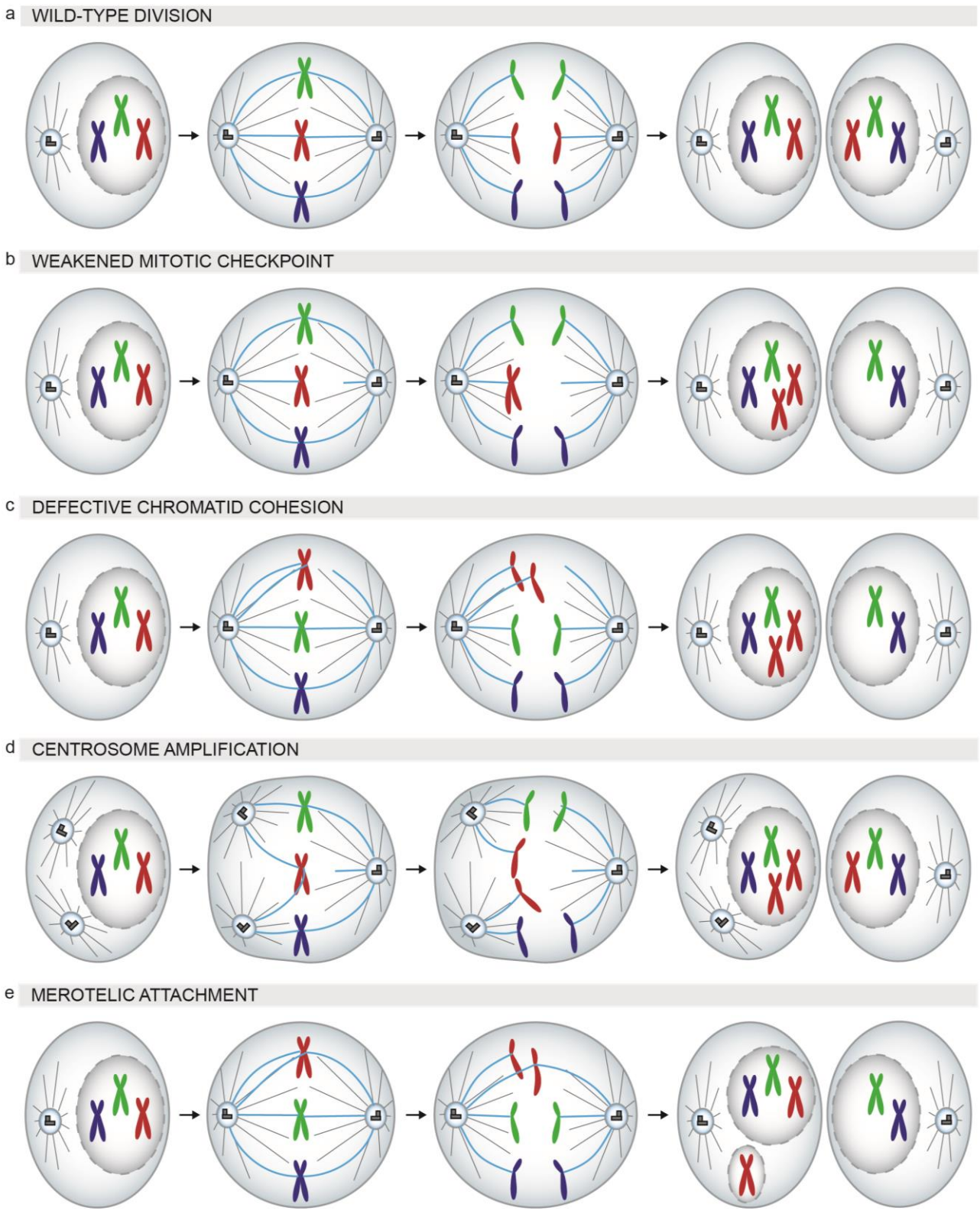

Figure 2: Mechanisms of chromosome missegregation in mitosis. Chromosome missegregation can lead to whole chromosome aneuploidy. At least four mechanisms can lead to chromosome missegregation: Spindle assembly checkpoint defects (**b**), Cohesion defects (**c**), centrosome amplification (**d**) and merotelic attachment (**e**). Adapted from (McGranahan et al, 2012).

4. *Merotelic attachment*. Microtubules from each of the opposing spindle poles must attach to kinetochores on opposite sides of the sister chromatids and create tension. Merotelic attachment occurs when microtubules from opposing spindle poles attach to the same kinetochore (Figure 2e). Frequently this error is corrected in early stage of mitosis, but if persisting until anaphase it leads to lagging chromosomes, micronuclei formation and uneven chromosome numbers in daughter cells (Cimini *et al*, 2003).

To achieve a correct cell division, it is necessary that the newly replicated chromosomes not only segregate equally in the daughter cells, but also segregate intact. In contrast to numerical aneuploidy caused mainly by defect in mitotic spindle function, structural aneuploidy arises from sporadic errors in DNA replication and repair (Asaithamby *et al*, 2011). Therefore, the primary source of chromosomal rearrangements is the presence of single-stranded DNA (ssDNA) gaps or double-strand breaks (DSBs) (Zhang *et al*, 2015a). DNA breaks can result from replication fork stalling and collapse at specific hotspots such as common fragile sites. Interestingly, the breakpoint junctions of chromosomal rearrangement commonly found in tumors do not overlap with the mapped common fragile sites (Beroukhim *et al*, 2010), suggesting that additional factors are involved in DSBs formation in cancer cells. Replication checkpoint dysfunction or defective DNA repair pathways also lead to persistence of DNA breaks through S-phase. In addition, DSBs can also occur on lagging chromosomes. It has been shown that lagging chromosomes can be trapped and broken in the cleavage furrow during cytokinesis and subsequently both daughter cells inherit parts of the damaged chromosome (Janssen *et al*, 2011). Moreover, lagging chromosomes that are left behind might form micronuclei. DNA replication in micronuclei is often defective due to compromised recruitment of replicative factors leading to incomplete replication and consequent chromosome breaks (Crasta *et al*, 2012).

4.3 Experimental model systems to study aneuploidy

Due to its close link with cancers and to the tempting perspective of developing aneuploidy-specific therapeutic strategies, aneuploidy has been intensively investigated in the last decade. Different groups developed several methods to obtain yeast strains, mouse models or mammalian cell lines to study the consequences of aneuploidy on cellular level and their link to tumorigenesis. There are two main categories of approaches to obtain aneuploid cells that are independent of the model system. In the first approach, aneuploid cells are obtained after inducing chromosome missegregation or via an unstable polyploid intermediate (Weaver & Cleveland, 2007; Pavelka *et al*, 2010; Santaguida *et al*, 2015; Ohashi *et al*, 2015). As a result, a population of cells with a random and complex aneuploid karyotypes arises that in one hand better recapitulates the cancer chromosome content, but on the other hand is highly chromosomally instable and it is therefore difficult to dissect whether observed phenotypes are caused by aneuploidy or by chromosomal instability. In the second approach, aneuploidies are generated via specific chromosome transfer

or meiotic nondisjunction (Upender *et al*, 2004; Torres *et al*, 2007; Williams *et al*, 2008; Stingele *et al*, 2012). The resulting cells harbor specific additional chromosomes that carry selectable markers to allow propagation through selection. With this approach, the obtained cell lines with stable low-complexity aneuploidy are appropriate for studies of the long-term effect of aneuploidy on cell physiology. Since the main focus of this work is on whole chromosome aneuploidy, this last approach will be further illustrated below.

Disomic yeast strains with haploid genome and one extra copy of one chromosome have been created by Torres and colleagues using a random chromosome transfer strategy. During mating, the presence of a mating partner carrying a mutation in the karyogamy gene (KAR1) causes defective nuclear fusion (Torres *et al*, 2007). However, it happens at a low frequency that during these abortive matings individual chromosomes are transferred from one nucleus to the other, which can then be selected using various selectable markers (Figure 3a).

To generate trisomic mouse embryonic fibroblasts (MEFs), Williams and colleagues took advantage of male mice heterozygous for two separate Robertsonian translocations (Figure 3b). When these mice were mated with wild type females, trisomic embryos were generated as a result of a meiotic non-disjunction event in the male germline (Williams *et al*, 2008). However, cultivation of MEF cells quickly leads to polyploidization and immortalization therefore aneuploid MEF cells are difficult to use for experiments that require long passaging (Todaro & Green, 1963).

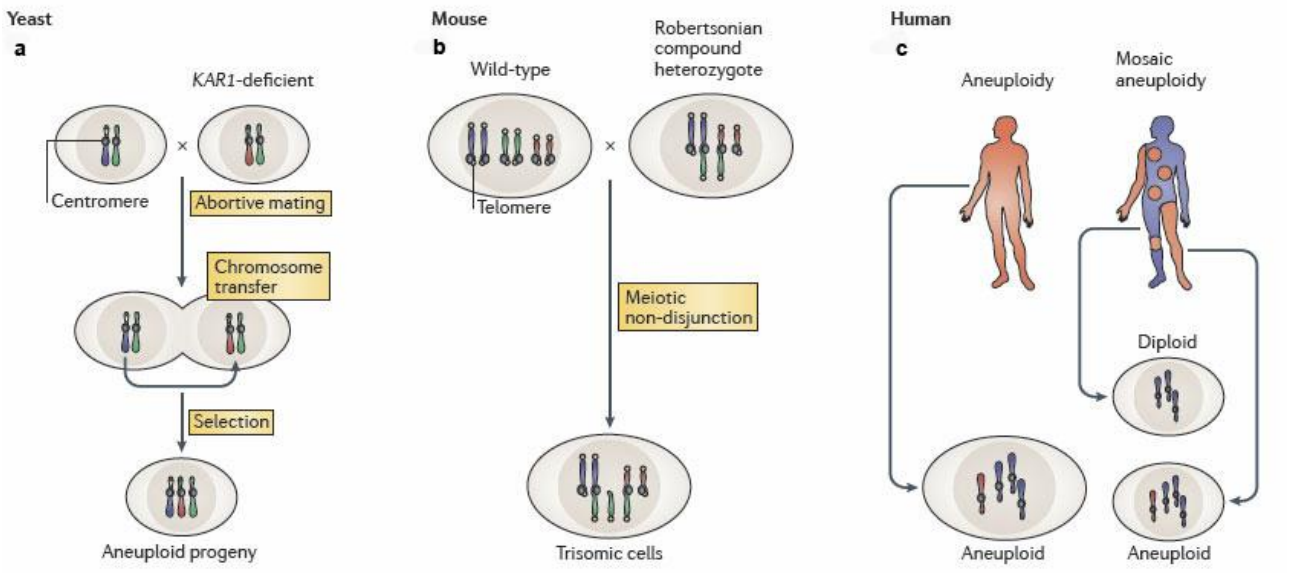

Figure 3: Experimental models of aneuploidy. (a) Chromosome transfer strategy followed by selection to engineer haploid yeast cells with a single extra chromosome. **(b)** Aneuploid mouse embryonic fibroblasts generated by taking advantage of Robertsonian translocations. **(c)** Model human aneuploid cells can be isolated from patients with trisomy 21. Both diploid and aneuploid cells can be obtained from patients with mosaic aneuploidy (adapted form (Gordon *et al*, 2012)).

In humans it is possible to isolate tissue samples and cell lines from patients with trisomy syndromes. Nevertheless, using tissue cells for cellular biology assays which require passaging is difficult as primary aneuploid cells grow poorly and become senescent after few passages (Segal & McCoy, 1974). In addition, one of the critical point in aneuploidy studies is the lack of an

appropriate human model with matching diploid and aneuploid cells. The only possibility would be to obtain sample from an individual with mosaic aneuploidy that present at the same time both aneuploid and diploid cells which are isogenic and could be used for direct correlation (Figure 3c). The problems of poor proliferation and early senescence, however, still remain, thus necessitating development of an alternative model.

Recently, Park and colleagues described a method to successfully derive human inducible Pluripotent Stem (iPS) cells from fibroblast or mesenchymal cells obtained from patients with various pathological conditions, including Down's Syndrome (Park *et al*, 2008). This model has been already employed in studies investigating the silencing of one copy of the chromosome 21 (Li *et al*, 2012; Jiang *et al*, 2013) however the possible trisomies that can be studied are limited to the trisomies viable in humans, which are very few. Therefore, it might be difficult to use this model to investigate the general effects of aneuploidy as the available trisomies could not represent a wide variety of different karyotypes.

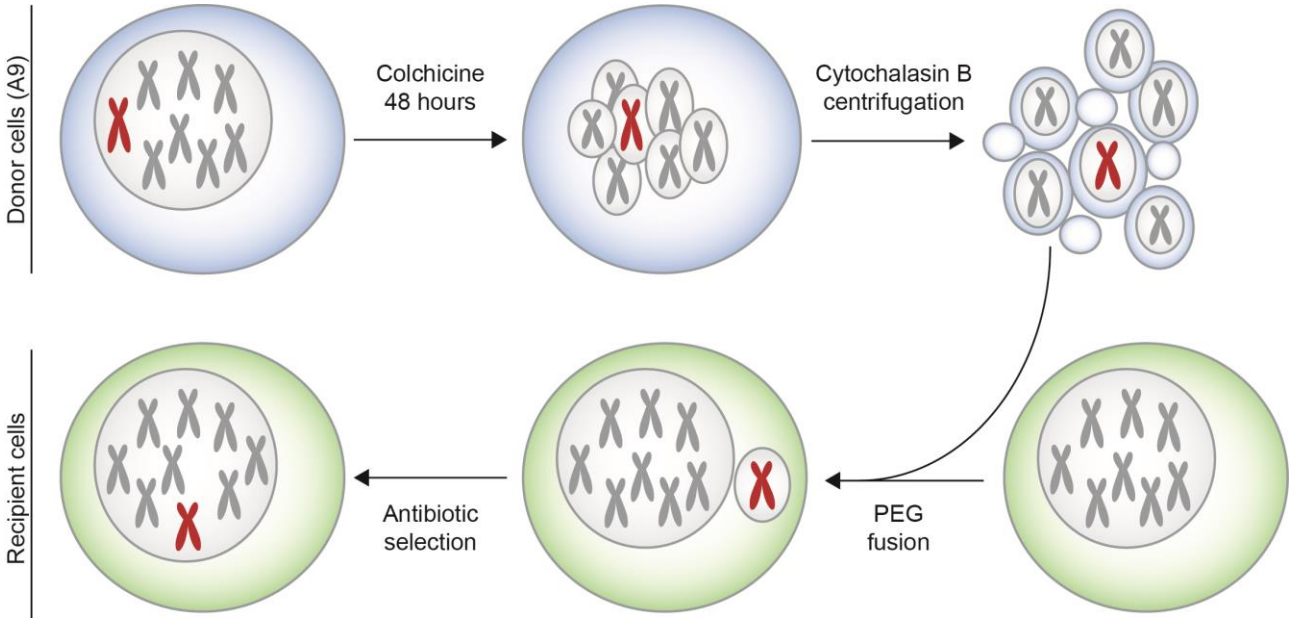

Figure 4: Microcell mediated chromosome transfer. Microcells with micronuclei generated from a mouse donor cell line carrying a specific human chromosome (depicted in red) are transferred into a human acceptor cell line. Acceptor cells are subsequently selected for the presence of the transferred human chromosome and stably propagated in antibiotics.

To circumvent this limitation and to investigate the effect of chromosome copy number alterations in genetically identical cells, human aneuploid cell lines can be created using the microcell-mediated chromosome transfer methodology (Upender *et al*, 2004; Nawata *et al*, 2011; Stingele *et al*, 2012). With this technique a defined human chromosome is introduced into karyotypically diploid human cells (Figure 4). A9 mouse cell lines, each containing one specific human chromosome with a selectable marker, are used as donor cells. Prolonged Colchicine treatment inhibits microtubules polymerization, which prevents the formation of a functional spindle during mitosis and thus induces micronucleation. Enucleation of micronucleated cells by

centrifugation in the presence of Cytochalasin B allows isolation of microcells. Each microcell consists of an intact plasma membrane, a small portion of cytoplasm and a single micronucleus containing from one to five chromosomes (Killary & Lott, 1996). Microcells are then purified by filtration to avoid donor cell contamination and fused to recipient diploid cells using polyethylene glycol (PEG). Recipient cells containing the human chromosome can be selected and stably propagated in medium with appropriate antibiotics (Killary & Lott, 1996). Thus, this technique allows generation of cell lines, which differs from the control only for the added chromosome, are relatively stable over time and easy to cultivate, therefore overcoming frequent problems of the previous models and facilitating the study of aneuploidy in humans.

4.4 Consequences of aneuploidy

Aneuploidy usually has adverse effects on the physiology of organisms and the individual cells. Systematic analyses of aneuploid yeast, murine and human cells suggested that the cellular response to aneuploidy is conserved among species and does not depend on the identity of the extra chromosome. This is quite surprising as it would be obvious to imagine that the altered phenotypes observed in aneuploid cells are genes- and therefore chromosome-specific. Changes in the levels of some specific genes could have indeed dramatic effects on cell viability and are sometimes linked to severe toxicity. One example is overexpression of the β-tubulin in yeast: having one extra copy of this gene is lethal. β-tubulin is coded on chromosome 6 and accordingly Torres and colleagues failed to isolate disomic yeast strains with an extra copy of chromosome 6 (Torres *et al*, 2007). Moreover, it has been observed that duplication of the *APP* gene is linked with a familiar form of early onset of Alzheimer's disease. Interestingly, *APP* is located on chromosome 21 and early onset of Alzheimer's disease is a common trait of Down's Syndrome (Shi *et al*, 2012). Similarly, duplication of *PMP22* gene on chromosome 17 in human leads to Charcot-Marie-Tooth 1A neuropathy (Hanemann & Müller, 1998). In this case we cannot confirm that trisomy of chromosome 17 causes the same phenotypes because trisomy 17 is not viable.

Despite these few exceptions, it has been shown that the effects of aneuploidy are mainly not chromosome-specific, but rather arise as a general consequence of harboring an unbalanced karyotype. These phenotypes include altered transcriptome and proteome (Torres *et al*, 2007; Sheltzer *et al*, 2012; Stingele *et al*, 2012; Sheltzer, 2013; Dürrbaum *et al*, 2014), proteotoxicity (Torres *et al*, 2007; Tang *et al*, 2011; Oromendia *et al*, 2012; Stingele *et al*, 2012; Donnelly *et al*, 2014; Ohashi *et al*, 2015), proliferation impairment and cell cycle delay in G1 and S phases (Torres *et al*, 2007; Stingele *et al*, 2012; Thorburn *et al*, 2013), metabolic alterations (Williams *et al*, 2008; Tang *et al*, 2011; Torres *et al*, 2007) and genomic instability (Sheltzer *et al*, 2011; Zhu *et al*, 2012; Necchi *et al*, 2015). These phenotypes are described in detail below.

4.4.1 Global gene expression changes in response to aneuploidy

Genomic, transcriptomic and proteomic profiles of human aneuploid cells revealed that

aneuploidy leads to common alterations in several pathways conserved between species (Sheltzer *et al*, 2012). Notably, quantification of aneuploidy-associated changes of transcriptome and proteome is technically challenging. In fact, the presence of one extra chromosome results only in 0.5-fold increase in the expression levels of genes coded on the aneuploid chromosome, a rather mild difference. However, it has been shown that aneuploidy does not only affect the expression of the genes located on the extra chromosomes, but also of multiple other genes across the entire genome (Upender *et al*, 2004; Torres *et al*, 2007; Sheltzer *et al*, 2012; Stingele *et al*, 2012). A straightforward explanation for these global changes is that some transcriptional regulators are coded on the aneuploid chromosome. In this case the aneuploidy-associate response would be strictly dependent on the identity of the extra chromosome. However, the same categories of pathways were consistently up or downregulated in different aneuploidies independently of the specific chromosomal aberration (Torres *et al*, 2007; Stingele *et al*, 2012; Dürrbaum *et al*, 2014). Moreover, a similar pattern of changes in gene expression is also observed in cells with complex aneuploidy (Dürrbaum *et al*, 2014) and in cultured amniocytes derived from trisomic pregnancies (Sheltzer *et al*, 2012).

Analysis of transcriptome to identify significantly altered pathways in murine and human aneuploid cells revealed consistent downregulation of DNA and RNA metabolisms, which include processes involved in DNA replication and repair, cell cycle progression, splicing and ribosome biogenesis (Sheltzer *et al*, 2012; Stingele *et al*, 2012; Dürrbaum *et al*, 2014). Upregulated categories seem to be more species-dependent. Upregulation of inflammatory and stress responses are common in all mammalian models, while pathways required for lipid and membrane biogenesis, Golgi vesicles, endoplasmic reticulum and lysosome functions are specific only for human cells (Sheltzer *et al*, 2012; Stingele *et al*, 2012; Dürrbaum *et al*, 2014). Interestingly, analysis of proteome changes leads to identification of the same altered pathways (Stingele *et al*, 2012; Dephoure *et al*, 2014). Similarly, human samples and mouse models of Down syndrome revealed upregulation of oxidative stress response and down regulation of proliferative related genes (Contestabile *et al*, 2009) (Slonim *et al*, 2009). In budding and fission yeast the aneuploidy-associated transcriptome changes resemble the environmental stress response (ESR), a transcriptional signature with upregulation of genes associated with oxidative stress and response to heat and downregulation of genes associated with ribosome biogenesis and nucleolus (Sheltzer *et al*, 2012).

Despite the conserved pathway deregulation in different aneuploidies, it has not been possible to determine a list of individual genes that are commonly downregulated. The individual genes associated with altered pathways show variable expression levels in different cell lines probably as a consequence of the different genetic background. Apparently each cell line adapts differently to aneuploidy leading, however, to the same physiological defects (Dürrbaum *et al*, 2014).

One main question that has not been answered yet is why aneuploidy triggers a global

response that is not dependent on the identity of the extra chromosome. In yeast the similarity between the transcriptional pattern of aneuploidy response and ESR suggests that the presence of extra DNA might induce a stress response due to for example increased energy requirements (Torres *et al*, 2007) (Williams *et al*, 2008), production of reactive oxygen species (Li & Marbán, 2010) or saturation of folding machineries by overexpressed proteins and formation of cytotoxic aggregates (Oromendia *et al*, 2012). However, it was shown that the ESR is not only a common transcriptional response to different stresses, but also a result of lower proliferation rate (Regenberg *et al*, 2006) suggesting that, similarly, the aneuploidy response could be a consequence of the slower proliferation.

This possibility can be excluded in humans as cells with complex aneuploidy proliferate comparably to diploid cell, but still exhibit the transcriptional aneuploidy response pattern (Dürrbaum *et al*, 2014). In human, a unique ESR transcriptional signature has not been described so far. However, the transcriptional changes caused by several stress-inducing conditions have been compared with the aneuploidy response (Dürrbaum *et al*, 2014). Specifically, the transcriptional profiles of HCT116 cultured in the presence of low or high glucose, hypotoxic conditions, hydrogen peroxide, nitric oxide, hydroxyurea, actinomycin D or bafilomycin A1 were analyzed. A similarity has only been observed when comparing the transcriptional response triggered by aneuploidy and the transcriptional response triggered by treatment with actinomycin D or bafilomycin A1. These evidences suggest that the response to aneuploidy differs from the response to common stress stimuli, but there is a partial overlap with conditions that interferes with autophagy (bafilomycin A1) or transcription (actinomycin D). Moreover, the transcriptome and proteome changes induced by aneuploidy partially resemble the cellular response to protein folding deficiency (Donnelly *et al*, 2014). Comparison between transcriptome of aneuploid cells and transcriptome of hepatocellular carcinoma cells after HSF1 depletion display strong similarity in both the upregulated and downregulated pathways. A partial overlap was observed also when comparing the proteome changes of aneuploid cells and proteome changes of HeLa cells treated with Hsp90 inhibitors for 24 hours. In this case, a stronger similarity was found in the downregulated pathways, including DNA and RNA metabolism and cell cycle progression, whereas the upregulated pathways did not show strong similarity. Taken together, a presence of extra DNA in human cells induces a stress response that resemble the one observed upon interference with protein degradation and folding, thus suggesting that the conserved global deregulation is partially caused by an aneuploidy-dependent proteotoxic stress.

4.4.2 Gene-dosage imbalance

An important aspect to consider when investigating the consequences of aneuploidy on gene expression is whether the physiological alterations induced by aneuploidy are due to the mere presence of extra DNA or due to the expression of the genes present on the extra chromosome. It has been shown in budding yeast that only the introduction of yeast DNA that

leads to production of extra proteins triggers phenotypic changes associated with aneuploidy. On the contrary, the presence of one artificial chromosome containing mammalian DNA that cannot be transcribed and translated in yeast has little effect on the fitness of yeast strains (Torres *et al*, 2007). Thus, presence of extra DNA is detrimental only when the genes on the additional chromosome are transcribed and translated.

Nevertheless, the presence of transcribed extra DNA is not always equally detrimental, because the severity of the consequences depends on the relative copy number changes of individual genes. The concept of gene dosage balance was first described in the works of Blakeslee and Bridges (Blakeslee *et al*, 1920) (Bridges, 1925). They demonstrated in *Datura stramonium* and *Drosophila melanogaster* respectively, that the addition of a single chromosome to a genotype was highly detrimental or lethal, whereas the addition of a complete genome that creates a polyploid karyotype was viable and resulted in milder effects on the phenotype. Similarly, in budding yeast aneuploidy associated phenotype are markedly alleviated when an extra chromosome is added to diploid cells in comparison to a chromosome gain in haploid cells (Torres *et al*, 2007; Oromendia *et al*, 2012). Moreover, the three human trisomies that survive until birth are those involving chromosome 13, 18 and 21, which are the smallest chromosomes with respect to the number of transcripts encoded. Therefore, whole chromosomal aneuploidy is extremely detrimental and poor tolerated as a result of the dosage imbalance of the several hundreds of genes located on the affected chromosomes.

These findings provoke the question whether the genes coded on the extra chromosome are expressed according to the gene copy number or whether the increased abundance is balanced by compensatory mechanisms similar to those responsible for a sex-chromosome inactivation. Recent studies on the impact of aneuploidy on gene expression have showed that genes coded on the aneuploid chromosome are mainly transcribed accordingly to their gene copy number in various organisms such as in yeast cells with an extra chromosome (Torres *et al*, 2007), in trisomic mouse cells (Kahlem *et al*, 2004), in human cells with trisomy 21 (Mao *et al*, 2003) and in aneuploid human cell lines created by microcell-mediated chromosome transfer (Upender *et al*, 2004) (Stingele *et al*, 2012). The only exceptions are found in Drosophila and some plants where mechanisms of dosage compensation similar to those described for sex chromosomes can also function for autosomes (Guo & Birchler, 1994; Stenberg *et al*, 2009).

Consistently with these observations, the silencing or loss of the extra chromosome should attenuate the defects induced by aneuploidy. This was shown to be true in two recent studies in Down's syndrome patient's pluripotent stem cells. In the first work, Li and colleagues generated induced pluripotent stem cells (iPSCs) from Down's syndrome fibroblasts and removed the extra copy of chromosome 21 by introducing a TKNEO (thymidine kinase with neomycin phosphotransferase reporter gene) transgene in one copy of chromosome 21. Subsequent culture of cells first under positive and then under negative selection resulted in loss of chromosome 21. Interestingly, the arising diploid iPSCs proliferate significantly faster compared to the trisomic

iPSCs (Li *et al*, 2012). In the second study, the natural mechanism responsible for chromosome X silencing in females was employed to silence one of the chromosomes 21. After a single inducible XIST transgene was integrated into one copy of chromosome 21, complete transcriptional silencing of the genes coded on the extra chromosome was observed upon XIST induction. Consistent with previous findings, analysis of parallel cultures of identical cells with or without XIST induction revealed rescue of proliferation impairment in trisomic cells (Jiang *et al*, 2013).

Thus, genes coded on the extra chromosome are expressed according to their gene copy number and the consequent gene-dosage imbalance is mainly responsible for the phenotypes associated with aneuploidy.

4.4.3 Protein homeostasis

Recent developments in proteomics facilitate the analysis of the effects of whole chromosome aneuploidy on the proteome. It has been demonstrated in aneuploid yeast and human cells that the protein levels strongly correlate with the DNA copy number changes, similarly to the increased mRNA levels, (Torres *et al*, 2010) (Stingele *et al*, 2012). However, while majority of the proteins coded on the extra chromosome are expressed according to the gene copy number, in some cases their levels are lower than expected. This adjustment of protein abundance to diploid levels affects specifically certain protein categories. In particular, subunits of multimolecular complexes tend to maintain the stoichiometric abundance levels despite increased mRNA level (Torres *et al*, 2010) (Stingele *et al*, 2012). Accordingly, it was shown previously that the relative abundance of members of multimolecular complexes is tightly regulated and monomers in excess can undergo preferential protein degradation (Veitia *et al*, 2008). Stingele et al. also identified kinases as another class of proteins subjected to compensation at protein level. Similarly to protein complexes, rigorous regulation of protein abundance is also required in signaling network to ensure the balance between proteins with opposite enzymatic function (Veitia *et al*, 2008). Overall, this proteomic data suggest that although some protein classes are expressed at significantly lower levels, there is no general gene-dosage compensation mechanism as most proteins are expressed according to their gene copy number.

Accumulation of proteins coded on the extra chromosome might challenge the cellular ability of maintaining proteostasis, therefore leading to elevated protein misfolding and aggregation. Normally, a complex network of cellular processes guarantees the maintenance of protein homeostasis. These processes include chaperone-mediated protein folding pathway that guarantees the folding of proteins into their fully active form and the degradation pathways that ensure maintenance of adequate protein levels or elimination of damaged proteins. The chaperone-mediated folding pathway includes several different heat shock proteins and their regulators that assist in *de novo* folding or refolding (McClellan *et al*, 2007)}. Protein degradation is carried out mainly by ubiquitin-proteasome system (UPS) and by autophagy and these two pathways are important for maintenance of physiological protein levels and timely removal of

irreversibly misfolded and aggregated proteins. Protein folding and protein degradation are tightly coordinated to avoid deficiencies in proteostasis that have been shown to facilitate the onset of several diseases, including neurodegeneration and dementia, cystic fibrosis, cancer and cardiovascular disease (Morimoto, 2008).

Evidences from studies in various aneuploid model systems show that the aneuploidy-induced proteomic changes might compromise or overwhelm the activity of the proteostasis network. Several hallmarks of proteotoxic stress have been identified in aneuploid yeast, mouse and human cells. Aneuploid yeast strains show higher sensitivity to chemical compounds that impair proteasome degradation, translation and protein folding (via HSP90 inhibition) compared to the control (Torres *et al*, 2007), suggesting that the protein imbalance and the consequent proteotoxic stress lead the aneuploid cells to rely more heavily on their protein quality-control machinery. Indeed, analysis of aneuploidy-induced transcriptome changes revealed upregulation of the protein chaperone HSP104 in murine trisomies (Sheltzer *et al*, 2012), lysosome-mediated degradation and p62-dependent autophagy (Tang *et al*, 2011; Stingele *et al*, 2012). In addition, aneuploid yeast strains and human tri- and tetrasomic cells accumulate cytoplasmic protein inclusions, and showed reduced HSP90 folding activity and lysosomal saturation (Oromendia *et al*, 2012) (Stingele *et al*, 2012) (Donnelly *et al*, 2014) Ohashi *et al*, 2015; (Santaguida *et al*, 2015; Ohashi *et al*, 2015). Similarly, trisomic MEFs and human aneuploidies are more sensitive to the HSP90 inhibitor 17-AAG compared to control cells (Tang *et al*, 2011; Donnelly *et al*, 2014), suggesting a saturation of the chaperone capacity by the increased protein expression, which lead to accumulation of misfolded protein that aggregate in the cytoplasm. Indeed, even a 0.1% increase in misfolded proteins saturates the protein quality-control systems of the yeast cells resulting in reduced cellular fitness (Geiler-Samerotte *et al*, 2011).

The available evidence suggests that aneuploidy triggers a specific functional impairment of HSP90-dependent protein folding. Our group showed that the reason might be a downregulation of HSP90 family protein levels due to a deficient HSF1-dependent activation of heat shock response (HSR) in human aneuploid cells. Consistently, endogenous or exogenous overexpression of HSF1 rescues the compromised folding (Donnelly *et al*, 2014). Interestingly, it was previously observed that mutations in UBP6 improved the fitness of several different disomic strains (Torres *et al*, 2010). UBP6 is a gene encoding a deubiquitinase that antagonizes proteasome function and its inactivation results in increased protein turnover rates, possibly allowing faster degradation of the overexpressed proteins coded on the extra chromosome. Thus, enhanced protein degradation or elevated protein folding capacity can restore protein balance and partially suppress the adverse effects of aneuploidy. Notably, pathways involved in protein folding and degradation are often found up-regulated in cancer to confer stress tolerance and allow survival of cancer cells (Kang *et al*, 2008; Rouschop *et al*, 2009; Kon *et al*, 2011). Accordingly, drug interfering with protein quality-control processes such as inhibitor of proteasome, autophagy and chaperones are currently investigated in pre-clinical research for treatment of several kinds of tumors (Kraus *et al*, 2015;

Lazenby *et al*, 2015; Ishitsuka *et al*, 2015; Spreafico *et al*, 2015; Rangwala *et al*, 2014).

The observed defect in HSP90-dependent protein folding can have severe consequences on several HSP90 clients that rely strictly on HSP90 function to adopt and maintain their correct conformation. Although many molecular chaperones have little specificity, HSP90 is a highly conserved chaperone shown to have a specific subset of protein clients, most of which are kinases and signal transduction proteins (Caplan *et al*, 2007; Sharma *et al*, 2012; Taipale *et al*, 2014). Moreover, emerging evidences suggest that functional HSP90 is important also for protein complexes assembly (Makhnevych & Houry, 2012; Gopinath *et al*, 2014). Insufficient protein folding capacity is deleterious for HSP90 clients that might fail to be folded and therefore undergo degradation. Consistently, kinases and members of multimolecular complexes coded on the extra chromosome show a deviation of protein abundance from expected levels (Torres *et al*, 2010; Stingele *et al*, 2012; Dephoure *et al*, 2014), and the reduction of protein complexes subunits is mediated by proteasome and autophagy (Dephoure *et al*, 2014). Moreover, analysis of proteome data revealed that the protein level of previously identified HSP90 clients was significantly decreased in majority of aneuploid cell lines tested (Donnelly *et al*, 2014). Therefore, the apparent dosage compensation of some particular classes of proteins may be a consequence of the compromised folding capacity elicited by aneuploidy.

4.4.4 Proliferation impairment

Among the key phenotypes shared by aneuploid cells, the most prominent is their slower proliferation and reduced viability relative to those of diploid cells. The aneuploidy-associated growth defect was first observed in cultured fibroblasts isolated from patients with Down syndrome when compared to diploid fibroblasts (Segal & McCoy, 1974) and later severe growth defects were observed in multiple other aneuploid organisms. Torres and colleagues demonstrated that aneuploid yeast strains exhibit defects in cell cycle progression with a specific G1-delay. The delay appeared to be more severe in strains carrying an extra copy of a large chromosome or several extra chromosomes, which suggests that the phenotype scales with the amount of additional yeast DNA (Torres *et al*, 2007; Thorburn *et al*, 2013). Similarly, reduced growth was observed in mouse embryonic fibroblasts (MEFs) isolated from trisomic mice and MEFs harboring mutations in the spindle-checkpoint component BubR1 that frequently carry one or two extra chromosomes (Torres *et al*, 2008; Baker *et al*, 2004). In addition to budding yeast and mice, it has been showed that aneuploidy negatively affects cell proliferation also in fission yeast (Niwa *et al*, 2006), fruit fly (Lindsley *et al*, 1972) and worms (Hodgkin *et al*, 1979). Analysis of proliferation in human aneuploid cell lines obtained via micronuclei mediated chromosome transfer revealed that cells with extra chromosomes display significant growth impairment. Consistently with previous results, the proliferation defect seems to scale with the amount of extra DNA with tetrasomic cells growing significantly slower than trisomic cells. Interestingly, the delay does not affect to the same extent each cell cycle phase. In fact, Stingele and colleagues demonstrated that aneuploid cells progress

slowly through G1 and S while the progression through G2 and mitosis is not affected (Stingele *et al*, 2012).

An important question about the growth defects of aneuploid cells is whether they are the consequence of overexpression of a few detrimental genes that affect pathways essential for cell survival or of simultaneous copy number changes of genes that are not harmful when deregulated individually. A recent genome-wide screen developed to measure the limit of gene overexpression in yeast, identified 55 dosage-sensitive genes that are lethal at more than 5 copies per haploid genome (Makanae *et al*, 2013). However, it has been shown that introducing an additional copy of these dosage-sensitive genes cannot explain the proliferation defects observed in yeast cells carrying an additional copy of the chromosome on which the genes are located (Bonney *et al*, 2015). Moreover, recent studies on trisomy 21 iPSCs demonstrated that after silencing one of the three copies of chromosomes 21 the obtained disomic cells proliferate faster (Li *et al*, 2012; Jiang *et al*, 2013). This evidence suggests that the proliferation impairment of aneuploid cells are likely caused by cumulative effect of copy number changes of many genes that are not harmful when overexpressed independently but the underlying molecular mechanismis remain unknown. Importantly it is not the presence of extra DNA *per se* to affect cellular growth. In fact introduction of one artificial chromosome containing mammalian DNA that cannot be transcribed and translated in yeast has no effect on proliferation (Torres *et al*, 2007). Finally, cancer cells usually proliferate faster than non-transformed cells despite being aneuploid, suggesting that there are additional adaptive changes can compensate the aneuploidy-dependent growth delay.

4.4.5 Metabolic alterations

Cancer cells are characterized by marked changes in energy metabolism, particularly in the glycolytic pathway. It has been observed that glucose concentration in tumor tissues is significantly lower compared to non transformed tissues (Hirayama *et al*, 2009). Consistently, cancer cells are more sensitive to glucose limitation and glycine consumption correlates with proliferation rate (Jain *et al*, 2012; Birsoy *et al*, 2014). Interestingly, there are some preliminary evidences that aneuploidy leads to an altered energy metabolism as well. Torres and colleagues observed that aneuploid yeast produces less biomass per internalized glucose compared to wild-type yeast. Interestingly, the production of biomass seemed to be inversely proportional to the proliferation defect and to the amount of extra DNA. In addition, aneuploid cells showed an increase in the glucose uptake. Consistently, amplification of genes loci of the two glucose transporters Hxt6 and Hxt7 and gene expression changes in the carbohydrate metabolism suggested that aneuploid yeast needs more glucose to survive and proliferate (Torres *et al*, 2007). Studies on aneuploid MEFs revealed slightly different metabolic changes. Aneuploid cells showed no significant increase in glucose uptake, but increase in glutamine consumption as well as lactate and ammonium production was observed in all trisomic MEFs when compared to control (Williams *et al*, 2008). Moreover, aneuploid MEFs heavily rely on energy metabolism as they showed increased sensitivity to AICAR, a drug that

induces energy stress (Tang *et al*, 2011). Upregulation of energy metabolism pathways such as mitochondrial respiratory metabolism and carbohydrate metabolism was also observed in model human aneuploid cell lines (Stingele *et al*, 2012).

So far, no further studies are available that investigate the metabolic changes induced by aneuploidy and no general conclusions could be drawn based on the existing data. A very ventured yet possible speculation inspired by the similarity of the metabolic changes in aneuploid and cancer cells is that the metabolic alterations observed in tumors are due to the aneuploid state of the tumor cells. Based on existing findings we can only hypothesize that transcription, translation, folding and degradation of factors encoded on the extra chromosome require more energy, which leads to an increased need for energy metabolites. The altered metabolism and the increased energy requirements could be in turn some of the factors limiting the proliferation in aneuploid, cells but further studies are necessary to test this possibility.

4.4.6 Genomic instability

The term GIN describes an unstable genome that can be manifested on a broad scale from single nucleotides variations to structural and numerical chromosomal abnormalities. It has been proposed that genomic instability plays a crucial role in cancer initiation and progression facilitating the accumulation of growth-promoting mutations (Beckman & Loeb, 2006). Its major source is the presence and persistence of unrepaired DNA lesions caused by endogenous or exogenous stress (Hakim *et al*, 2012; Roberts *et al*, 2012). Interestingly, recent studies in yeast suggest that genomic instability is a widespread consequence of imbalanced karyotype. Haploid budding yeast strains carrying one more copy of one of the yeast chromosome exhibit increased levels of chromosome loss rates, mutation rates or mitotic recombination compared to euploid control strains. Interestingly, it was observed that different aneuploidies trigger different combinations of these phenotypes, but all of them display elevated levels of Rad52 foci, a marker for DNA damage and homologous recombination (Sheltzer *et al*, 2011). Also in fission yeast aneuploidy leads to genomic instability as demonstrated by increased sensitivity do DNA damaging agent and accumulation of Rad22 foci (Rad52 homologue) over time (Sheltzer *et al*, 2011).

What is the cause of the genomic instability in aneuploid yeast is not yet understood, but it might arise as a consequence of aneuploidy-induced stoichiometric imbalance. Presence of extra chromosome and its replication *per se* is not sufficient to induce genomic instability since yeast strains harboring yeast artificial chromosomes (YACs) containing human DNA do not show accumulation of DNA lesions or sensitivity to genotoxic agents. Moreover, the relative protein imbalance is greater in haploid yeast strain carrying a single extra chromosome (disomic strains) compared to diploid yeast strain carrying a single extra chromosome (trisomic strains). In trisomies the protein imbalance is partially mitigated because there is only a 50% relative increase in the abundance of proteins coded on the extra chromosome. In disomies the effect are more drastic as addition of one extra chromosome leads to doubling the protein production. Accordingly, trisomic

strains were more resistant to genotoxic agents compared to their isogenic disomies (Sheltzer *et al*, 2011). Thus, genomic instability, similarly to many other aneuploidy-associated-phenotypes is likely caused by a relative protein imbalance and not by additional DNA to be replicated, although the exact molecular mechanisms underlying this effect remains enigmatic. Further analysis using the same aneuploid yeast strains demonstrate that Rad52 foci form during S phase suggesting that they arise from replication defects. Accordingly, they observed that DNA replication initiation and elongation are impaired in several disomic yeast strains. Moreover, many disomic yeast strains enter mitosis in the presence of DNA damage, although no defects in DNA damage checkpoint were observed (Blank *et al*, 2015). Persistence of low levels of DNA breaks through mitosis could provide the substrate for chromosomal rearrangements such as translocations or deletions.

Similar experiments have not been performed in human cells so far. Evidence of the effects of whole chromosomal aneuploidy on genomic stability in humans is available only from the limited studies of Down Syndrome's (DS) patient cells. In the past several research groups addressed the issue of genomic instability in DS, drawing the general conclusion of increased genomic instability. It was proven that DS lymphocytes exhibited increased chromosomal aberrations in response to different types of DNA damaging agents, compared with those of healthy individuals (Natarajan, 2015). Moreover, DS lymphocyte accumulate significantly higher levels of endogenous DNA damage and are not able to repair DNA damage induced by certain genotoxic conditions (Morawiec *et al*, 2008). Similarly, DS skin fibroblasts show DNA damage checkpoint activation in basal growth conditions, suggesting that the checkpoint was induced by an endogenous source of stress. Indeed, accumulation of DNA damaged and deficiency in DNA repair was observed in DS fibroblasts when compared with control cells (Necchi *et al*, 2015). However, research in cells from DS patients is not always reliable as only small population is available. Moreover, contrasting results were obtained occasionally, likely due to inter-individual variability and a lack of proper controls. Therefore, additional investigations are required to confirm the generality of the hypothesis that whole chromosomal aneuploidy triggers genomic instability in humans and to understand the molecular basis for the observed increased accumulation of endogenous DNA damage.

4.5 Causes of genomic instability

GIN is described as the increased rate of gaining genetic alteration and matematical models suggest that GIN is critical for cancer development as it can explain the rapid accumulation of mutations typical of tumors (Beckman & Loeb, 2006). Different forms of GIN have been described depending on the mechanisms involved. GIN include micro and mini-satellite instability, point mutations, gross chromosomal rearrangements or copy number variations as a result of erroneous DNA synthesis, defective DNA repair or persistence of DNA breaks due to deregulation of checkpoints (Hakim *et al*, 2012; Roberts *et al*, 2012; Waddell *et al*, 2015).

Maintenance of a stable genome is an essential cellular task that requires a perfect coordination between cellular processes. Although the ideally perfect situation is an absolutely stable genome, this would not allow any evolutionary change to occur. On the other hand, a very unstable genome could result in cancer development and eventually cell death. Despite the variety of possible mutations that can cause alteration of DNA, somatic gene mutation is a rare event, occurring with a frequency of 10^{-10} per cell generation (Balin & Cascalho, 2010). This suggests that cells have mechanisms of defense against the accumulation of DNA damage, thereby maintaining genomic stability and ensuring the transmission of the appropriate genetic information from one cell to another. The stability of a genome is a dynamic mechanism determined by the balance between DNA damage and repair (Figure 5). Mainly three processes contribute to the maintenance of this balance: a correct DNA replication, a functional DNA repair and an efficient activation of checkpoint response to DNA damage and replication fork stalling.

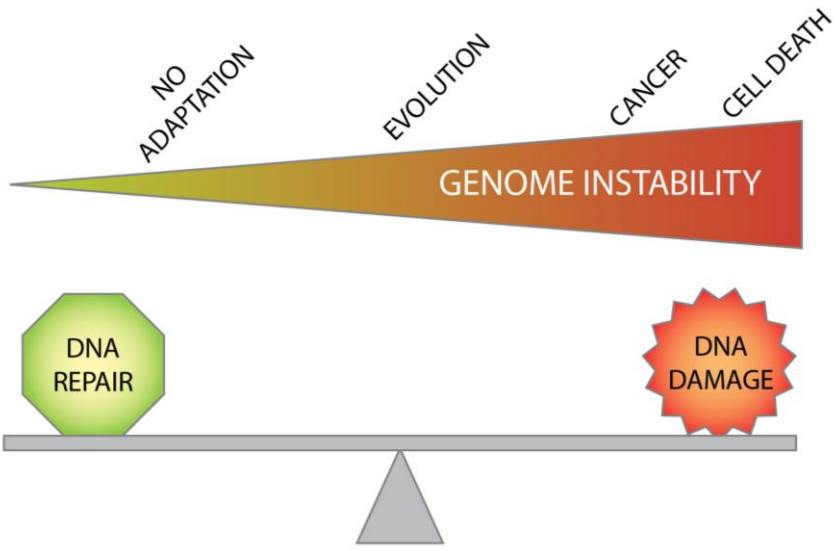

Figure 5: The stability of the genome is determined by the balance of DNA damage and DNA repair: The stability of the genome should be high enough to allow faithful transmission of genomic information of an organism but at the same time still allow evolutionary modifications to occur (adapted from (Bohlander & Kakadia, 2015)).

4.5.1 DNA replication as a source of genomic instability

Defective DNA replication is one of the major endogenous source of spontaneously arising DNA damage (Zeman & Cimprich, 2014). It is crucial that DNA is entirely replicated before each cell division to preserve genomic stability. The DNA replication is one of the most vulnerable cellular processes. In eukaryotic cells it is complicated by the large size of genomic DNA and its organization into chromatin structure, therefore the process must be strictly regulated. Due to the large amount of DNA that has to be duplicated in a limited time, in many eukaryotes DNA replication is initiated simultaneously at several replication origins that are organized into clusters, each composed of two to five adjacent origins. The complete genome duplication is achieved by sequential activation of different clusters (Guilbaud *et al*, 2011). To ensure that no DNA sections are left unreplicated and no DNA sections are replicated twice, origin must be activated only once per cell cycle. Cells achieve precise DNA duplication by dividing the initiation of replication in two non-overlapping phases: origin licensing and origin firing (Yeeles *et al*, 2015).

Origin licensing takes place in G1-phase, before DNA replication begins, with assembly of pre-replication complexes (pre-RCs) at replication origins. The pre-RC complex is composed by several proteins that sequentially bind the DNA, starting with recruiting of the origin recognition complex (ORC) to the replication origin. Independent loading of CDC6 and CDT1 follows ORC binding to chromatin. Only when ORC, CDC6 and CDT1 are loaded, the mini-chromosome maintenance (MCM) complex is recruited (Méndez & Stillman, 2000; Remus *et al*, 2009). Importantly, the loading of the pre-RC component on DNA is only allowed when there is no or low CDK activity. Low CDK activity ensures that MCM2-7 proteins remain enzymatically inactive in G1 and after their activation at the beginning of S phase no more MCM2-7 complex can be loaded on chromatin (Yeeles *et al*, 2015).

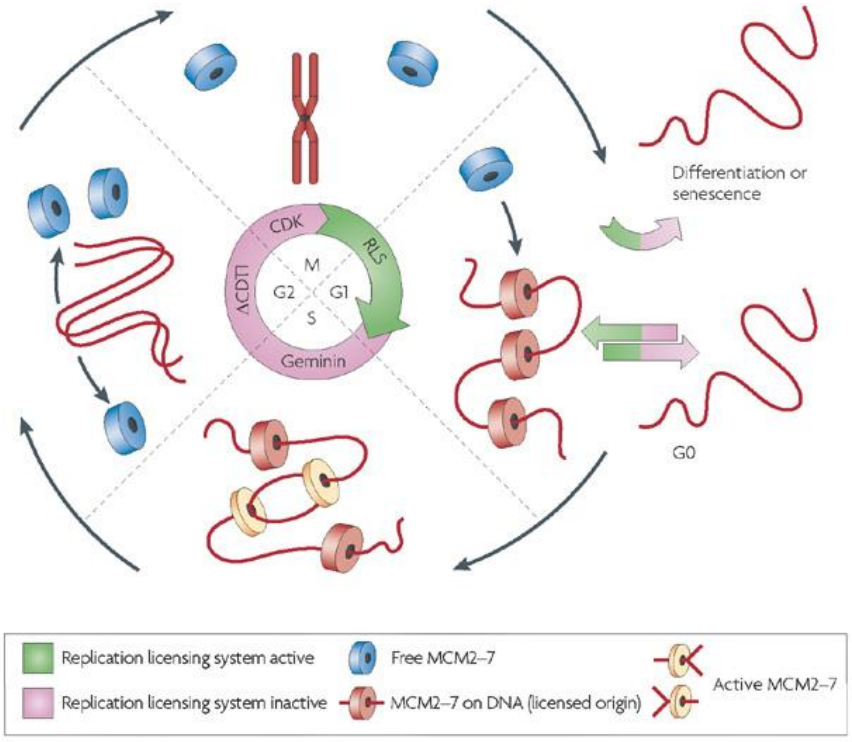

Figure 4: **Temporal separation of origin licensing and activation.** MCM2–7 complexes (depicted as red/blue rings) are loaded on DNA during G1 when the licensing system is active. Before entry into S phase, the licensing system is inactivate and no more MCM2–7 can be loaded. This occurs as a consequence of geminin activation and CDT1 degradation during S and G2 and a high cyclin-dependent kinase (CDK) activity during mitosis. During S phase, MCM2–7 complexes at licensed origins are activated to form part of the replication fork. When replication forks terminate, MCM2–7 is displaced from the DNA. (from (Blow & Gillespie, 2008))

To prevent pre-RC assembly outside of G1 phase, cells employ redundant mechanisms including targeted protein synthesis of pre-RC components during G1 and inactivation of these proteins following replication initiation via protein degradation and nuclear export. In addition, one common mechanism in metazoans is the stabilization of the protein Geminin that binds to CDT1 and inhibits MCM2-7 recruitment after S-phase onset. Geminin is then degraded by anaphase-promoting-complex (APC) mediated ubiquitination during mitosis to allow new pre-RC formation in following G1 (McGarry & Kirschner, 1998). Multiple studies showed that overexpression of CDT1 triggers re-replication in several cell systems (Zhong *et al*, 2003) (Thomer *et al*, 2004), thus emphasizing the importance of the separation of origin licensing and origin activation to prevent over-replication and maintain genomic stability.

After the pre-RCs are loaded, chromatin becomes competent for replication, but several other proteins must associate with pre-RC to successfully initiate DNA synthesis (Yeeles *et al*, 2015). The mechanism of origin activation is not completely understood yet, but it is carefully controlled by the activity of the S-phase promoting kinases. As the cells enter S-phase, Cdk2/CyclinE and Cdc7/Dbf4 catalyze the activation of the pre-RC to pre-initiation complex (pre-IC), which results in recruiting several additional replication factors. Although S-phase CDK and DDK activity is high after entry in S-phase, not all origins of the genome are activated immediately. Some regions start replicating at the beginning of S-phase, others more towards the end (Guilbaud *et al*, 2011). Finally, not all origins are necessarily activated. There is a high excess of licensed origins that remains dormant and function only in case of replicative stress (Ge *et al*, 2007) (Woodward *et al*, 2006).

In some cases internal or external stress stimuli can interfere with fork progression causing inefficient DNA replication. This scenario called replication stress leads to slowing or stalling the replication fork progression and it is the main source of genomic instability. Replication stress has been identified to be involved in the very early steps of tumorigenesis and aging. In fact, it has been shown that oncogene activation induces DNA replication stress and can lead to DNA double strand break formation in precancerous lesions (Gorgoulis *et al*, 2005). Several factors can hamper DNA replication, thus causing fork stalling: replication of telomeres and repetitive sequences, DNA lesions, secondary DNA structures, depletion of dNTPs pool, common fragile sites or down-regulation of limiting replicative factors (Zeman & Cimprich, 2014). To overcome this problem, cells load MCM2-7 complexes in excess on the chromatin in G1-phase. It was indeed demonstrated that only 10% of the total amount of MCM2-7 complexes loaded on DNA are activated in unperturbed S-phase (Lei *et al*, 1996). These backup origins are called dormant origins meaning that under normal condition they usually stay dormant and are passively replicated, but can be activated depending on the circumstances (Woodward *et al*, 2006; Ge *et al*, 2007) (Ibarra *et al*, 2008). Unfortunately, unambiguous markers to safely detect replication stress have not been described yet. Replication stress is not always coupled with DNA double strand breaks (Ray Chaudhuri *et al*, 2012), but often the sources of replication stress cause the formation of single stranded DNA (ssDNA) regions as a result of the unwinding of DNA after replication fork stalling (Pacek & Walter, 2004).

Independent studies demonstrated that reduction of chromatin-bound MCM complex up to 90% in human cultured cells does not affect DNA replication and cell proliferation under normal conditions (Ge *et al*, 2007; Ibarra *et al*, 2008). However, cells display increased DNA damage and micronucleus formation after several days of proliferation in limited licensing conditions (Ibarra *et al*, 2008). Moreover, when challenged with replication inhibitors, cells were unable to maintain high rates of DNA synthesis and the long-term survival was significantly compromised (Ibarra *et al*, 2008) (Ge *et al*, 2007). Accordingly, proliferation defects were observed after partial knockdown of MCM2-7 and treatment with proliferation inhibitors also in *Xenopus laevis* egg extracts and

Caenorhabditis elegans (Woodward *et al*, 2006). Additional evidences collected using mice models with hypomorphic mutant alleles of MCM2 and MCM4 confirmed the essential role of MCM2-7 in dormant origin licensing. In both models the amount of MCM2-7 loaded on the chromatin is decreased, which results in a reduction in the number of dormant origins, increased fork stalling and elevated DNA damage even in unperturbed conditions. Moreover, this mice experience a dramatic increase in tumors development suggesting that dormant origins play a critical role as tumor suppressors (Shima *et al*, 2007) (Kunnev *et al*, 2010; Klotz-Noack & Blow, 2011). Thus, the presence of dormant origins is absolutely required to achieve completion of DNA replication under replication stress conditions and for maintenance of genome stability.

4.5.2 Defective DNA repair

The replication machinery is quite robust and well equipped to overcome obstacles and resume DNA synthesis. In some cases, the lesion is repaired or the barrier removed. In other cases, the lesion is bypassed in an error-prone or error-free manner and left to be repaired later. Cells have evolved a variety of repair pathways according to the type of DNA damage. Under normal physiological conditions six major DNA repair pathways can be activated: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), nonhomologous end joining (NHEJ) homologous recombination (HR) and translesion DNA synthesis (TLS). The different DNA repair pathways are composed of a series of biochemical events that work in coordination with the cell cycle checkpoint regulators to allow time for the DNA repair to prevent the damage from becoming permanent in the cell (Tian *et al*, 2015).

The BER pathway is activated in response single strand breaks (SSB) mainly generated by increased levels of reactive oxygen species (ROS) (Trivedi *et al*, 2005). The SSB can be repaired by short-patch BER when only a single nucleotide is exchanged or by long-patch BER when several nucleotides are replaced. BER is particularly important for removing damaged bases that could lead to fork stalling during replication (Fortini *et al*, 1999). The MMR pathway repairs post-replicative errors that have escaped the 3'- 5' exonucleolytic proofreading activity of replicative DNA polymerases. Moreover, it can also remove DNA adducts caused by chemotherapeutic agents (Young *et al*, 2004). NER pathway counteracts mainly the pyrymidine dimers induced by UV and it includes two distinct sub-pathways. Global genome NER (GG-NER) slowly and randomly inspect the entire genome for damage, while transcription-coupled NER (TC-NER) repairs damages that leads to defective progression of RNA polymerase II (Tian *et al*, 2015). HR and NHEJ are both involved in the repair of double strand breaks (DSB). NHEJ can repair DSB at any cell cycle stage. It is simpler and faster than HR as it does not need a homologous template. On the other hand it is also less accurate, frequently leading to generation of mutations, deletions and chromosomal rearrangements. HR repairs DSB with high fidelity but it requires a homologous template therefore it occurs mainly in late S and G2 phase (Chapman *et al*, 2012). In addition to these pathways, cells have evolved another system to survive lesions that could lead to replication

fork blockage. This last mechanism is TLS and, although it is a part of the DNA repair pathways, it results in lesion bypass or damage tolerance (Yamashita *et al*, 2002). Although the mechanisms of various repair pathways are sometimes similar, the proteins recruited by each pathway are different and the final decision of which DNA repair pathway is used depends on the type of DNA lesion and on the cell cycle phase. Recent studies have shown a contribution of the Fanconi Anemia (FA) pathway to DNA repair and maintenance of genome stability. In particular this pathway is essential to protect the genome from DNA interstrand cross-link (ICLs). ICLs are deleterious DNA lesions that can be caused by endogenous or exogenous sources and result in blockage of DNA replication and transcription (Liu *et al*, 2010). All the described pathways involved in DNA repair are not mutually exclusive and there is a frequent crosstalk between them.

Mutations in DNA repair genes are associated with several forms of hereditary cancers. The well documented hereditary non-polyposis colon cancer is linked with mutations in MMR genes (Papadopoulos *et al*, 1994). Additionally, mutations in BER genes (*MYH*) and HR (*BRCA1, BRCA2*) predispose to the development of various tumors (King *et al*, 2003; Kim *et al*, 2004). Although rare, one additional example is the autosomal recessive disorder xeroderma pigmentosum (XP). Mutations in XP genes result in enhanced photosensitivity and increased skin cancer rates (Bradford *et al*, 2011). Finally, patients with the genetic disorder Fanconi Anemia (FA) also exhibit increased cancer susceptibility (Rosenberg *et al*, 2003). Thus, a functional DNA repair is important for maintaining genome stability and preventing the development of cancer.

4.5.3 Cell cycle checkpoints

To monitor cell cycle progression and to ensure that late events start only upon completion of early events, cells have evolved elaborate mechanisms known as checkpoints. DNA checkpoints have been developed to sense and respond to DNA lesions and to ensure proper transmission of genetic information to the next generation of cells. There are three major checkpoints whose function is essential for maintaining genome stability: DNA damage checkpoint, DNA replication checkpoint and licensing checkpoint (Kastan & Bartek, 2004).

A first checkpoint playing an important role in the maintenance of genome stability is the DNA damage checkpoint. In the presence of DNA damage, activation of the cell cycle checkpoints is accomplished by the DNA damage response (DDR) signaling pathway (Hirao *et al*, 2000). Central players of DDR are the phosphatidylinositol-kinase related protein kinases (PIKKs), ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related). ATM and ATR sense DNA lesions and activate downstream effectors and other kinases. Once activated, the downstream kinases mediate DNA repair and delay cell cycle progression (Matsuoka *et al*, 2007). As the genome is constantly challenged by endogenous and exogenous sources of stress, defects in the checkpoint or other components of the DNA damage response leads to accumulation of DNA damage and increased genomic instability. Interestingly, precancerous lesions frequently show activation of DNA damage responses. Therefore, checkpoint response to DNA damage forms a

barrier to cancer development and act as tumor suppressor (Bartkova *et al*, 2006). The DNA damage checkpoint might be one constrain, therefore tumor cells with impaired checkpoints might have an advantage and become preferentially selected. This can explain the high prevalence of p53 mutations in cancer cells. It was shown in p53-deficinet mice that the transition from pre-cancerous lesion to cancer was accelerated (Bartkova *et al*, 2005; Gorgoulis *et al*, 2005). Moreover, mutations in both alleles of ATM result in development of the disorder ataxia-telangiectasia characterized by breast cancer predisposition (Renwick *et al*, 2006) .

The DNA replication checkpoint is an intra S-phase checkpoint activated in response to replication fork stalling. Replication fork can stall due to an encounter with physical impediments, depletion of dNTP pool, direct inhibition of DNA synthesis by drug treatments, or downregulation of replicative factors (Branzei & Foiani, 2010). Central to this checkpoint signaling pathway is the kinase ATR that is activated by its association with RPA-coated ssDNA resulting from the replication fork stalling (Zou & Elledge, 2003). However, it is not completely clear yet how the replication stress is sensed. Once activated, the replication checkpoint kinases trigger different signaling pathways that block cell cycle progression, downregulate late origin firing, stabilize stalled replication forks, and facilitate the restart of collapsed forks. It was shown that upon ATR inhibition early replicons fail to complete DNA replication in presence of aphidicolin, PCNA (proliferating cell nuclear antigen) and RPA (replication protein A) redistribute from early to late replicating regions and MCM2 is released from chromatin (Dimitrova & Gilbert, 2000). Thus, DNA replication checkpoint is crucial for maintaining genomic stability by prevent collapsing of stalled replication forks and subsequent double strand breaks.

Finally, a growing body of evidence is supporting the presence of an additional checkpoint critical for genome stability. As explained previously, no new origins can be licensed after entry into S-phase to prevent DNA over-replication, which means that cells rely on additional backup origins loaded in the previous G1-phase. Therefore, the entry into S phase needs to be delayed until sufficient excess of origin are licensed. To achieve this, it was demonstrated that several cell types employ a “licensing checkpoint” (Shreeram *et al*, 2002). Reduced loading of MCM2-7 on the DNA by depletion of several component of pre-RC complex leads indeed to a delayed entry in S-phase (Shreeram *et al*, 2002; Machida *et al*, 2005a; Nevis *et al*, 2009). Together with a prolonged G1-phase a reduction in cyclin E/CDK2 activity, Cyclin D/CDK4-6 activity and Rb hypophosphorylation has been observed (Liu *et al*, 2009; Machida *et al*, 2005a; Nevis *et al*, 2009). This suggests that the activation of licensing checkpoint results in repression of E2F that prevents S-phase gene transcription. However, it is not yet understood how the assembly of pre-RC complex is monitored and how the signal is transduced to downstream factors. Interestingly, many cancer cells can enter S phase despite a severe reduction in the number of licensed origins suggesting a defective licensing checkpoint (Shreeram *et al*, 2002; Feng *et al*, 2003; Liu *et al*, 2009; Nevis *et al*, 2009). This is not surprising since this defect can contribute to the genomic instability that is one of the most prominent hallmarks of cancer.

Although many different causes of genomic instability have been identified and current studies are providing new evidence in this direction, deciphering how and when the instability arises remains an open-ended question. Importance of genomic instability is underscored by its recurrent presence in pre-cancerous lesions. Moreover, compelling evidences are fueling the possibility that instable genome is a driving force in tumor growth. Thus, unraveling novel molecular mechanisms that lead to genomic instability would shed new lights on our understanding of the onset and development of cancer.

5. Aims of this study

Numerical aneuploidy causes genetic disorders such as Down's or Edwards syndrome that are characterized by severe developmental defects. In addition, aneuploidy is often found in cancer cells and high rates of aneuploidy in tumors correlate with poor prognosis and drug resistance. However, despite the striking association of aneuploidy with nearly 75 % of malignant tumors, it has been disputed whether aneuploidy directly and causatively contributes to cancer development. One current hypothesis is that genomic instability can occur as the initiating event of tumorigenesis allowing the acquisition of growth-promoting mutations (Ferguson *et al*, 2015). Recently, it has been proposed that aneuploidy may play a role in tumorigenesis by triggering genomic instability. Accordingly, several studies in yeast demonstrated that aneuploidy leads to errors in chromosome segregation, sensitivity to genotoxic agents and accumulation of DNA lesions (Sheltzer *et al*, 2011); (Zhu *et al*, 2012); (Blank *et al*, 2015). A few studies in human Down Syndrome's cells suggest that aneuploidy might lead to impaired DNA replication and genomic instability in human as well (Morawiec *et al*, 2008) (Necchi *et al*, 2015). Yet, molecular mechanisms explaining possible effects of aneuploidy on genome stability have remained elusive.

To shed light into the role of anepuloidy in tumorigenesis, it is crucial to unveil whether and how numerical aneuploidy by itself affects genome stability. Trisomic and tetrasomic human cell lines previously created in our laboratory by microcell mediated chromosome transfer facilitate the analysis of the consequences of aneuploidy *per se*. Direct comparison of such aneuploid cells with isogenic control cells revealed that addition of even a single extra chromosome causes transcriptome and proteome changes and profound defects in cell cycle progression, similarly as observed in previous studies (Torres *et al*, 2007; Stingele *et al*, 2012; Sheltzer, 2013).

Using this trisomic and tetrasomic model cell lines as well as cells with complex aneuploidy we asked whether aneuploidy triggered the same transcriptional pattern independently of the type and origin of karyotype imbalance. Moreover, by comparing the transcriptome changes in aneuploid cells with the transcriptional responses to various stress stimuli we planned to uncover the causative factor(s) of the global response to aneuploidy.

Second, we addressed the question whether our model aneuploid cells are sensitive to inhibition of protein folding similar to what was previously showed in yeast and MEFs (Torres *et al*, 2007; Tang *et al*, 2011). We also investigated the causes underlying this increased sensitivity by analyzing the ability to induce a functional heat shock response upon proteotoxic stress in presence of extra chromosome(s). In addition, we asked whether aneuploidy *per se* causes proteotoxic stress and whether this might affect the gene expression pattern.

Third, because the previous pathway analyses revealed that some of the most consistently downregulated pathways in aneuploid cells are those involved in DNA replication and repair, we addressed the fundamental question whether the presence of extra chromosome(s) triggers genomic instability by affecting DNA metabolism. To achieve this goal, we planned to analyze

several phenotypes associated with genomic instability, such as the presence of anaphase and ultrafine bridges as well as the accumulation of DNA lesions. Moreover, thanks to the recent progress in the field of next generation sequencing we addressed the question whether aneuploidy elicits *de novo* chromosomal rearrangements. Furthermore, we wanted to identify how aneuploidy triggers genomic instability. As one of the major causes of genomic instability is replication stress, we investigated whether aneuploid cells show evidence of fork stalling or sensitivity to replication inhibition. These results combined with our previous transcriptome and proteome data suggest that downregulation of replication factor might be responsible for the previously observed phenotypes. Finally, we identified MCM2-7 as the factors whose abundance is most affected by aneuploidy. By depleting MCM2-7 subunits or overexpressing functional or mutant alleles of various replicative factors we determined what is their contribution to the maintenance of genome stability in aneuploid cells. Deciphering the precise mechanisms and pathways that are involved in regulating these processes in aneuploid cells may have a direct relevance for several pathological conditions.

6. Results

6.1 Unique features of the transcriptional response to model aneuploidy in human cells.

Dürrbaum M, Kuznetsova AY, Passerini V, Stingele S, Stoehr G, Storchová Z. **Unique features of the transcriptional response to model aneuploidy in human cells.** BMC Genomics 2014 Feb 18;15:139.

http://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-15-139

This work addresses the question whether there is a uniform cellular response to the presence of extra DNA in human cells independently of the types and origins of aneuploidy. The cause of the physiological effects of aneuploidy remains unclear, but it has been previously shown that they arise due to the expression of the extra genes on the supernumerary chromosome.

By analyzing the transcriptional changes in eleven different trisomic and tetrasomic cell lines and two cell lines with complex aneuploid karyotypes, we identified a characteristic aneuploidy response pattern (ARP). The ARP is characterized by upregulation of genes linked to endoplasmic reticulum, Golgi apparatus and lysosomes, MHC protein complex and antigen processing and by downregulation of DNA replication, transcription and ribosomes related pathways. Interestingly, the response pattern of aneuploid cell lines that carry either functional or mutant p53 suggests that the ARP is not mediated by a p53-dependent stress response. Strikingly, we demonstrated for the first time that complex aneuploidy, which is often found in cancer, elicits the same transcriptional changes as the gain of a single chromosomes. This is of particular interest, because it implies that studies of the trisomic cell lines might give us useful understandings of the role of aneuploidy in tumors. Notably, despite the conserved ARP only 23 genes whose expression is significantly altered in all aneuploids were identified. It is on one hand surprising that only a few genes are consistently altered, but on the other hand they could represent valuable biomarkers to detect aneuploidy in tumors. Finally, to uncover what triggers the conserved aneuploid response, we compared the aneuploidy transcriptional profile with transcription changes in diploid cell lines that were subjected to various stress conditions. Interestingly, we found a striking overlap with the response to a treatment with the autophagy inhibitor bafilomycin A1. Inhibition of autophagy leads to accumulation of autophagic vacuoles and proteotoxic stress. Notably, it was proposed previously that aneuploidy also causes proteotoxic stress (Oromendia *et al*, 2012). Therefore the strong overlap between the ARP and the transcriptional profiles after bafilomycin A1 treatment could indicate that the pathway alterations in aneuploid cells might be elicited by proteotoxic stress.

To summarize, this study showed that (1) there is an uniform transcriptional response to aneuploidy reflected by deregulation of specific pathways, although (2) only few genes have been

identified as consistently and uniformly altered in response to aneuploidy and that (3) the conserved aneuploid response might be triggered by proteotoxic stress.

6.2 HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells.

Donnelly N, Passerini V, Durrbaum M, Stingele S and Storchova Z. **HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells**. The EMBO journal. 2014 Oct 16; 33(20):2374-87

http://emboj.embopress.org/content/33/20/2374.long

In this study we investigated whether aneuploidy leads to proteotoxic stress in humans and which of the pathways that respond to proteotoxic stress are most affected. It was previously demonstrated in our group that human trisomic cells show an accumulation of ubiquitin and p62 positive cytoplasmic foci, a marker of selective autophagy (Stingele *et al*, 2012). Moreover, aneuploid cancer cell lines are more sensitive to HSP90 inhibition suggesting that the HSP90 machinery might be particularly affected by the increased protein expression (Tang *et al*, 2011).

We demonstrated for the first time that human aneuploid cells suffer from a protein folding defect that is mostly due to an impairment of HSP90-dependent protein folding. Moreover, we observed a significant deficiency in the ability of aneuploid cell lines to trigger a fully functional heat shock response. We therefore suggested that the activity of heat shock-associated factors is compromised. Analysis of mRNA and protein levels revealed that HSP90 family proteins were consistently downregulated in all aneuploid cell lines considered. In addition, the responsible transcription factor HSF1 was also downregulated and unable to induce a full heat shock response upon acute proteotoxic stress. Consistently, we showed that exogenous and endogenous overexpression of HSF1 alleviates the detrimental consequences of aneuploidy on HSP90 expression and protein folding. Finally, we demonstrate that the observed functional deficiency in HSP90 and HSF1 directly shapes gene expression in aneuploid cells. Specifically, we show that the protein folding deficiency contributes both to proteome and transcriptome alterations. We first compared the quantitative proteome changes in aneuploid cells with the proteome changes occurring upon pharmacological inhibition of HSP90 for 24 h. Additionally we compared transcriptional profile of aneuploid cells with the transcriptome changes of an HSF1-depleted human cell line. In both cases we observed a strong overlap of the downregulated pathways, which includes pathways of DNA repair and replication and RNA splicing, as well as the cell cycle pathways. The similarity with upregulated pathways was only partial, and more evident in comparison of the aneuploids with the HSF1-depleted cell lines.

Taken together, in this work we found that aneuploidy leads to a proteotoxic stress and this, in turn, can shape the pattern of gene expression. Moreover we demonstrated that increased expression of HSF1 might represent the first aneuploidy-tolerating genetic modification in human cells.

6.3 The presence of extra chromosomes leads to genomic instability

Passerini, V, Ozeri-Galai E, de Pagter MS, Donnelly N, Schmalbrock S, Kloosterman WP, Kerem B, Storchová Z **The presence of extra chromosomes leads to genomic instability.** Nat. Commun. 7:10754 doi: 10.1038/ncomms10754 (2016).

http://www.nature.com/ncomms/2016/160215/ncomms10754/full/ncomms10754.html

Despite the striking association of aneuploidy with nearly 75 % of malignant tumors, whether and how aneuploidy contributes to tumorigenesis remain open questions. Previously, it has been proposed that aneuploidy may play a role in tumorigenesis by triggering genomic instability. Genomic instability is proposed to occur as the initiating event of tumorigenesis and might favor the acquisition of growth-promoting mutations. In this study we used a series of human cells with defined trisomies and tetrasomies to address the questions whether gain of a single chromosome increases genomic instability and what are the underlying molecular mechanisms.

We demonstrated that aneuploid cells show accumulation of DNA damage, sensitivity to replication stress and abnormal DNA replication. By taking advantage of next-generation sequencing and SNP-array analysis we observed accumulation of chromosomal rearrangements in trisomic and tetrasomic cells, suggesting that a presence of DNA damage and altered DNA replication compromise genome stability. It was previously shown by us and others that pathways involved in DNA metabolisms are consistently down-regulated as a consequence of aneuploidy. Accurate analysis of proteome data, with particular focus on DNA replicative factors, revealed that expression levels of all six subunits of the replicative helicase MCM2-7 were decreased in all aneuploid cells analyzed. Strikingly, we found that aneuploidy-induced genomic instability can be explained by the reduced expression of MCM2-7 subunits and that MCM2-7 are the limiting factors for replication in cells with extra chromosome(s). Accordingly, rescuing the levels of chromatin-bound MCM2-7 by exogenous overexpression partially alleviated the genomic instability phenotypes.

Taken together, these results provide a new insight into the possible mechanisms responsible for impaired genomic stability in response to aneuploidy. As replication stress and consequent genomic instability have been frequently observed in pre-cancerous lesions, our findings might be the first step in understanding how random missegregation of a single chromosome can contribute to early events in tumorigenesis.

7. Discussion

Unbalanced karyotype, called aneuploidy, is not well tolerated in higher eukaryotes. Aneuploidy represents one of the main causes of spontaneous abortions in humans and the surviving children suffer from severe developmental disabilities (Colnaghi *et al*, 2011). Furthermore, aneuploidy is found in 90% of solid tumors and increased rates of aneuploidy in cancers correlate with poor prognosis and drug resistance (Birkbak *et al*, 2011; Lee *et al*, 2011). Although the phenotypes caused by altered chromosome numbers are widely described, the underlying mechanisms of the physiological consequences of aneuploidy remain unclear. We demonstrated that human cells with unbalanced karyotype show a conserved gene expression changes that we call aneuploidy response pattern (ARP) similar to the previously described pathway alterations in yeast and murine aneuploid cells. Moreover, comparison of ARP with transcriptome data of human cells subjected to proteotoxic stress revealed a partial overlap. Therefore, we investigated whether aneuploidy causes proteotoxic stress. Indeed, we demonstrated that aneuploid cells fail to trigger a fully functional heat shock response and show a specific defect in HSP90-dependent protein folding. Finally, because the ARP is characterized by downregulation of DNA metabolism and it was shown that protein involved in DNA replication are often HSP90 clients we asked whether and how aneuploidy affects replication. We observed that defective DNA replication caused by downregulation of replicative factors leads to additional genomic instability in aneuploid cells.

7.1 Aneuploidy triggers a conserved gene-expression pattern

Aneuploidy leads not only to an increased expression level of majority of the genes encoded on the extra chromosome, but also triggers broader transcriptional changes that affect multiple genes coded on other chromosomes. We showed that there is a uniform transcriptional response to aneuploidy reflected by deregulation of specific pathways in eleven different trisomic and tetrasomic cell lines and two cell lines with complex aneuploid karyotypes. This specific aneuploidy response pattern (ARP) is characterized by upregulation of pathways linked to endoplasmic reticulum, Golgi apparatus and lysosomes, MHC protein complex and antigen processing and by downregulation of DNA replication, transcription and ribosome-related pathways. Despite the conserved ARP we identified only a few genes that were consistently and uniformly altered in response to aneuploidy. Finally, we compared the aneuploidy transcriptional profile with transcriptional changes in human cells subjected to various stress conditions. Interestingly we found a striking overlap with the gene expression changes upon treatment with the autophagy inhibitor bafilomycin A1 suggesting that the conserved aneuploid response might be triggered by proteotoxic stress.

It was previously shown that in different organisms the same pathway categories that we identified were consistently up- or downregulated independently of the specific karyotypic

alterations (Torres *et al*, 2007; Stingele *et al*, 2012; Sheltzer *et al*, 2012). Interestingly, while the same downregulated pathways have been identified both in murine and human aneuploid cells, upregulated processes appeared to be more species-dependent (Sheltzer *et al*, 2012; Stingele *et al*, 2012). The reason for the difference in the upregulated pathways is not yet understood. We can speculate that while the pathway downregulation is a direct consequence of the stresses arising due to the altered karyotype, the upregulation of different processes depends on how cells adapt to aneuploidy and might be species or cell line specific. This hypothesis is also underscored by the evidence that only a few genes have been identified as consistently changed in all analyzed aneuploid cell lines, despite the similarity of pathways alteration. Therefore, different species and cell lines may adapt to aneuploidy through diverse mechanisms to aneuploidy, but the physiological effects of aneuploidy are conserved.

Notably, we also showed that complex aneuploidy, which is often found in cancer, elicits the same transcriptional changes as whole chromosomal aneuploidies. Similarly, chromosomally instable cancer cell lines share some transcriptional changes with aneuploid cells (Sheltzer, 2013). This is particularly important, because it implies that studies of cell lines with a simple and defined karyotype could be used to gain precious understandings of the role of aneuploidy in tumors. Moreover, based on these findings we can also exclude that the ARP is a consequence of the slow proliferation rate that is typical of aneuploid cells. It is a tempting possibility that DNA metabolism is consistently downregulated and replicative factors are less abundant because of the reduced growth capacity. However, both the aneuploid cells with complex karyotype and the chromosomally unstable cancer cells do not show any growth impairment but demonstrate the same pathway alterations. In yeast, a similarity was observed between the aneuploidy response pattern and the environmental stress response (ESR) (Sheltzer *et al*, 2012). However, the ESR is triggered not only by several environmental stresses, but occurs also a consequence of a diminished growth rate (Regenberg *et al*, 2006). Therefore in yeast the slower proliferation might partially contribute to the gene expression changes caused by aneuploidy.

It is not yet understood what triggers the aneuploidy-associated transcriptome changes, proved that it is not the reduced proliferation. When we compared the aneuploidy transcriptional profile with transcription changes in human cells subjected to various stress conditions, we observed a strong overlap between the ARP and the resposse to bafilomycin A1 treatment. Bafilomycin A1 leads to inhibition of autophagy dependent degradation by preventing a fusion between autophagosomes and lysosomes, which leads to proteotoxic stress (Yamamoto *et al*, 1998). It was proposed previously that aneuploidy suffers from impaired protein homeostasis since murine and yeast aneuploid cells are more sensitive to treatment with proteotoxic stress-inducing compounds (Torres *et al*, 2007; Tang *et al*, 2011). The increased sensitivity suggests that these drugs affect pathways that are essential for the survival of aneuploid cells. One of the compounds used in a previous study was the autophagy-inhibitor chloroquine. Consistently, it was demonstrated that autophagy is activated in response to aneuploidy (Tang *et al*, 2011; Stingele *et*

al, 2012) and our data show upregulation of lysosome-associated pathways highlighting the importance of autophagy in aneuploid cells. However, aneuploid MEFs also showed increased sensitivity to the HSP90-inhibitor 17-AAG (Tang *et al*, 2011) suggesting that it might be not only autophagy but the whole protein homeostasis regulatory network on that aneuploid cells heavily rely.

The presence of a global response triggered by aneuploidy in a broad range of human cell lines with different karyotypes might open new horizons in the development of novel cancer therapies targeting conserved altered pathways. Moreover, further investigation on maintenance of protein homeostasis in aneuploid could uncover what exactly causes the ARP.

7.2 Whole chromosomal aneuploidy leads to proteotoxic stress

Aneuploidy affects several quality control pathways important for cellular protein homeostasis. Aneuploid yeast and MEFs showed increased sensitivity to conditions that compromise protein folding or degradation and aneuploid human cells activate autophagy and exhibit cytoplasmic protein inclusions (Torres *et al*, 2007; Tang *et al*, 2011; Stingele *et al*, 2012). We provide the first evidence that protein folding is significantly impaired in trisomic and tetrasomic human cell lines. Specifically we showed that human aneuploid cells suffer from a HSP90-dependent protein folding defect. Analysis of mRNA and protein levels revealed that HSP90 family proteins and the responsible transcription factor HSF1 were downregulated in all aneuploid cell lines analyzed. Moreover, the ability to induce a full heat shock response upon acute proteotoxic stress was compromised. Consistently, we found that exogenous and endogenous overexpression of HSF1 alleviates the detrimental consequences of aneuploidy on HSP90 expression and protein folding. Finally, we showed that the protein folding deficiency contributes both to proteome and transcriptome alterations and partially shapes the characteristic aneuploidy response pattern (ARP).

We showed that aneuploid human cells are specifically sensitive to inhibition of HSP90 but not to other inducer of protein folding stress such as heat shock and inhibition of HSP70. Consistently with our findings, previous data support the idea that particularly HSP90 is compromised in several aneuploid cells. In fact, aneuploidy leads to increased sensitivity to inhibition of HSP90-dependent protein folding in yeast and the HSP90 chaperones capacity is reduced in many aneuploid yeast strains (Torres *et al*, 2007; Oromendia *et al*, 2012). Similarly it was shown that aneuploid MEFs are more sensitive to treatment with 17-AAG, a chemical inhibitor of HSP90 (Tang *et al*, 2011), thus supporting the argument that aneuploidy leads to a specific HSP90-mediated protein folding deficiency.

Notably, restoring the HSF1 levels by exogenous or endogenous overexpression alleviates the negative effects of aneuploidy on protein folding. Enhanced expression of HSF1 represents the first aneuploidy-tolerating genetic modification in human cells. Interestingly, chromosome 8, where HSF1 is coded, is also the largest somatic chromosome whose trisomy is not embryonic lethal

(Agrawal & Agrawal, 2011). Moreover, trisomy or tetrasomy of chromosome 8 is one of the most frequent numerical aberration in tumors (Mitelman *et al, 2016*). Thus, increased expression of HSF1 can alleviate some of the aneuploidy related defects. Another mutation that improves the fitness of aneuploid yeast strains was described by Torres and colleagues. They observed that inactivation of the gene encoding the deubiquitinating enzyme Ubp6 suppress the proliferation defect and attenuated the aneuploidy-dependent alterations in cellular protein composition (Torres *et al*, 2010). In both cases, increased activity of one of the pathways involved in the quality control network is beneficial for aneuploid cells suggesting that aneuploid cells greatly depend on the maintenance of protein homeostasis.

The existence of mutation that could alleviate aneuploidy-associated physiological defects suggests once more that the general response to aneuploidy might be a consequence of proteotoxic stress. Interestingly defects in protein-folding induced by chemical inhibition of HSP90 or knockdown of HSF1 in diploid cells trigger transcriptome and proteome changes similar to those observed in aneuploid cells (Sharma *et al*, 2012; Chuma *et al*, 2014) In both cases we observed a strong overlap of the downregulated pathways, which includes pathways of DNA repair and replication and RNA splicing, as well as cell cycle pathways. The similarity with upregulated pathways was only partial, and more apparent when comparing aneuploid cells with the HSF1-depleted cell lines. This might be due to the fact that HSF1 is involved in regulation of a broad range of additional cellular processes distinct form heat shock response (Mendillo *et al*, 2012). Thus, the HSF1 deficiency caused by aneuploidy contributes to the conserved aneuploidy response pattern.

One more remarkable finding of our work is that the protein levels of previously identified HSP90 clients were significantly decreased in majority of aneuploid cell lines tested. In yeast and human cells, changes in gene copy number lead to a consequent change in the level of mRNAs (Torres *et al*, 2010; 2007; Pavelka *et al*, 2010) (Stingele *et al*, 2012). However, while majority of the proteins coded on the extra chromosome are expressed according to the gene copy number, in some cases their levels are lower than expected. This adjustment of protein abundance to diploid levels affects specifically certain protein categories. In particular, subunits of multimolecular complexes and kinases tend to maintain the stoichiometric abundance levels despite increased mRNA level (Torres *et al*, 2010) (Stingele *et al*, 2012). This specific “compensation” was previously justified by the fact that the relative abundance of these classes of proteins is tightly regulated to ensure the balance between single subunits of the multimolecular complexes and between protein kinases with opposite enzymatic function (Veitia *et al*, 2008). Interestingly, kinases and subunits of multimolecular complexes are also described clients of HSP90 (Caplan *et al*, 2007; Sharma *et al*, 2012; Makhnevych & Houry, 2012; Gopinath *et al*, 2014; Taipale *et al*, 2014). Insufficient protein folding capacity is harmful for HSP90 clients that might fail to be folded and therefore aggregate or undergo degradation. Consistently, kinases and members of multimolecular complexes coded on the extra chromosome show decreased protein levels compared to the expected abundance.

Degradation of subunits of multimolecular complexes is mediated by proteasome and autophagy (Torres *et al*, 2010; Stingele *et al*, 2012; Dephoure *et al*, 2014).

The causes of the observed protein folding impairment and the decreased levels of HSP90 family chaperones and their transcription factor remain unclear. A former study suggests that the proteotoxic stress experienced by aneuploid cells results from the increased expression of proteins coded on the extra chromosome. In fact, yeast strains containing transcriptionally silent yeast artificial chromosome (YAC) do not exhibit increased protein aggregation (Oromendia *et al*, 2012). It was shown in yeast that as little as 0.1% increase in misfolded proteins saturates the protein quality-control systems resulting in reduced cellular fitness and induction of unfolded protein response (Geiler-Samerotte *et al*, 2011). Similarly in aneuploid cells, accumulation of proteins coded on the extra chromosome might place a burden on the cellular ability of maintaining proteostasis, leading to accumulation of misfolded proteins and aggregates resulting in chronic proteotoxic stress.

7.3 Whole chromosomal aneuploidy triggers genomic instability in human

In this study we demonstrated for the first time that the presence of even a single extra chromosome triggers genomic instability by impairing DNA replication. Here we used a human model system that is considerably different from previous studies where aneuploidy was induced by defective mitotic checkpoint. Mutations in mitotic checkpoint components lead to high aneuploidy rate and highly chromosomally instable cells. Oppositely, we used defined tri- and tetrasomic cells that differ from the control only for the added chromosome(s) and do not display any significantly increased chromosome instability. This mimics the situation after random missegregation of a single chromosome and therefore we could uncover how such event contributes to genomic instability and possibly to tumorigenesis. We used a panel of five cell lines derived from HCT116 and five derived from RPE1 carrying extra chromosome 3, 5, 8, 12 or 21 and their combinations. Analysis of both transformed and non-transformed cell lines containing a variety of different chromosome was essential to ensure that the observed phenotypes are not cell line or chromosome-specific. We show that aneuploidy consistently increases the frequency of pre-mitotic errors even in unperturbed conditions and sensitizes cells to replication stress. Moreover presence of extra chromosomes elevates frequency of chromosomal rearrangements with a breakpoint junction pattern suggestive of replication defects. In fact, DNA replication is abnormal in aneuploid cells as demonstrated by the EdU incorporation assay and by levels of RPA32 phosphorylation. Finally, we demonstrated that the limiting factor for replication and genomic instability is the observed decreased levels of MCM2-7 and restoring near wild-type levels of MCM2-7 partially alleviates the defects.

Our experiments provided an answer for a question whether a presence of one extra chromosome is sufficient to trigger additional genomic instability in human cells. We demonstrated that aneuploid cell lines show several phenotypes suggestive of ongoing genomic instability such

as increased frequency of anaphase and ultrafine bridges, higher frequency of structural chromosomal aberration, accumulation of *de novo* chromosomal rearrangement and increased amount of DNA lesions that persist through mitosis. Studies in yeast showed that aneuploidy leads to mutagenesis, sensitivity to genotoxic stress and progression through mitosis in presence of DNA damage which are common hallmarks of genomic instability (Sheltzer *et al*, 2011; Blank *et al*, 2015). In human cells derived from Down Syndrome's patients, aneuploidy enhanced sensitivity to DNA damaging agents and increased accumulation of DNA damage (Morawiec *et al*, 2008; Necchi *et al*, 2015). Thus, these results together strongly suggest that changes in number of chromosomes further destabilizes genome and triggers chromosomal rearrangements.

Notably, we did not detect any significant increase in the frequency of lagging chromosome in aneuploid cells suggesting that a presence of extra chromosome(s) *per se* does not trigger CIN. However, earlier studies are not always in agreement with our results. In yeast the majority of aneuploid strains obtained by sporulation of triploid or pentaploid yeast are chromosomally unstable (Pavelka *et al*, 2010; St Charles *et al*, 2010; Zhu *et al*, 2012). Similarly, haploid yeast strains that carry additional copies of single yeast chromosomes show increased rate of chromosomes missegregation suggesting that CIN is a conserved phenotype of aneuploid yeast (Sheltzer *et al*, 2011). Studies on aneuploid mammalian cells came to conflicting conclusions. On one hand it has been shown that whole chromosomal aneuploidy obtained by introducing an extra chromosome into a diploid cell line do not cause CIN, similar to what we observed (Lengauer *et al*, 1997; Valind *et al*, 2013). On the other hand, Nicholson and colleagues showed that trisomy 7 or 13 lead to chromosome missegregation and that the segregation defect affects some chromosomes more than others. They suggested that CIN might be a karyotype-dependent phenotype because they found that overexpression of a gene coded on chromosome 13 leads to cytokinesis failure (Nicholson *et al*, 2015). As we did not have any cell line with extra chromosome 13 in our study, we were not able to test this possibility. Thus, it is well possible that CIN is a karyotype-dependent phenotype rather than a general consequence of aneuploidy in human cells.

Our observation that aneuploidy causes increased rate of anaphase and ultra fine bridges, but not of lagging chromosomes suggests that presence of extra chromosome affects DNA replication and/or repair rather than chromosome segregation machinery. GIN is caused by defective DNA replication, DNA repair or their incorrect coordination, while CIN arises from defects in one of the numerous processes controlling chromosome segregation and cell division, and is therefore mostly independent of DNA damage. However, It was shown recently that defects in DNA replication affect proper transmission of chromosomes to daughter cells (Burrell *et al*, 2013). Moreover, the phenomenon of DNA damage-induced centrosome amplification represents ad additional evidence that DNA lesions can in certain cases trigger defects in chromosome segregation processes (Löffler *et al*, 2013). Nevertheless, it is still not clear whether impaired DNA replication causes chromosome segregation errors and whether this link is only observed in cancer cells.

Next generation sequencing analyses revealed that the chromosomal rearrangement accumulated *de novo* in aneuploid cells show microhomology at break point junctions and overlap with fragile sites suggesting that the chromosome breaks have a replication stress-dependent origin. This is consistent with previous findings in yeast that replication stress in early S-phase leads to chromosomal rearrangements (Sabatinos *et al*, 2015). Replication stress is one of the major sources of genomic instability in early stages of tumorigenesis (Gorgoulis *et al*, 2005). It can arise due to mutations in genes crucial for DNA replication, repair or checkpoint control, or it can arise as a consequence of activation of oncogenes such as c-Myc and cyclin E. For example overexpression of cyclin E impairs MCM2–7 binding to chromatin during G1, leading to a reduced number of licensed origins after entering S-phase (Ekholm-Reed *et al*, 2004). The contribution of replication stress in tumorigenesis is further strengthened by the findings that replication stress induced by aphidicolin-mediated DNA polymerase inhibition results in high frequency of tumor-like micro-deletions (Durkin *et al*, 2008). Moreover, hydroxyurea-induced dNTP depletion resulting in replication stress and DNA damage, promotes leukaemogenesis in mice (Bilousova *et al*, 2005). However, despite the accumulating evidences supporting a connection between replication stress and tumorigenesis, the molecular processes that lead to fork stalling and impaired replication in the early stages of tumorigenesis remain elusive.

In order to understand the underlying mechanisms, we asked what causes the defective DNA replication in aneuploid cells. Previous analysis of the pathway deregulation in aneuploids revealed that processes involved in DNA replication and repair are consistently down-regulated in different species (Sheltzer *et al*, 2012; Stingele *et al*, 2012; Dürrbaum *et al*, 2014). A closer look at the proteome data revealed a strong downregulation of DNA replicative factors, particularly the subunits of the replicative helicase MCM2-7. MCM2-7 is essential for correct replication and its donwregulation leads to insufficient loading of dormant origin with consequent hypersensitivity to replication stress. MCM2-7 is an essential component of the pre-RC complex together with ORC, CDC6 and CDT1 (Remus *et al*, 2009). It was shown in several studies that excess of MCM2-7 is required for loading of dormant origins and survival of replication stress (Woodward *et al*, 2006; Ge *et al*, 2007; Ibarra *et al*, 2008). Notably, partial depletion of MCM2-7 under normal condition is not detrimental for cells as they do not need to activate dormant origins (Ge *et al*, 2007; Ibarra *et al*, 2008). However, reduced MCM2-7 levels and therefore reduced origin licensing becomes a problem when DNA replication is challenged, as there are no additional origins to be fired and rescue the DNA replication (Ge *et al*, 2007; Ibarra *et al*, 2008). By ensuring timely DNA replication in case of replication stress, the excess MCM2-7 in cells also maintains the genome stability. Mice with mutations in MCM4 show accumulation of double strand breaks, micronuclei and predisposition to mammary tumor development (Shima *et al*, 2007). In humans, a recessive syndrome caused by an N-terminal truncation of MCM4 leads to increased chromosomal fragility in patient's lymphocytes and dermal fibroblasts (Gineau *et al*, 2012; Hughes *et al*, 2012). Accordingly, we observed increased occurrence of DNA breaks and gaps in aneuploid cell lines when treated

with aphidicolin. In addition, it was shown that old hematopoietic stem cells (HSCs) in mice suffer from cell cycle defects, replication stress and chromosome gaps and breaks due to decreased MCM2-7 expression (Flach *et al*, 2014).

Because the presence of an adequate number of dormant origins is essential to prevent genomic instability, it has been proposed that cells monitor the licensing of sufficient dormant origin before onset of S-phase via a so called “licensing checkpoint”. Although the molecular mechanisms of this checkpoint is still under investigation, the evidence suggests that the G1-phase is prolonged by down-regulating G1/S Cdk2 activity until a proper number of origins are licensed (Liu *et al*, 2009; Machida *et al*, 2005b; Nevis *et al*, 2009; Teer *et al*, 2006). Surprisingly, the aneuploid cells we analyzed enter S-phase despite the limited abundance of MCM2-7 proteins suggesting that either the licensing checkpoint is defective, similar to some transformed cells (Shreeram *et al*, 2002), or they can somehow bypass the checkpoint or the defect is not big enough to activate the checkpoint. In the first case aneuploid cells would not show any G1 phase prolongation. On the contrary, we have previously shown that the cell cycle of trisomic and tetrasomic cells is altered compared to those of parental diploid cells and this alteration is reflected in prolongation of G1 and S phases (Stingele *et al*, 2012). Therefore we can speculate that the cells delay the entrance into the S-phase to allow licensing of sufficient dormant origins. However, cells will eventually bypass this arrest and start DNA replication. This change in licensing checkpoint falls outside the aim of this work, however it will be an interesting topic of future investigation. In particular it is appealing because so far the bypass of the G1 arrest after origin licensing failure has only been achieved by depletion of p53 or expression of the HPV E7 oncoprotein (Liu *et al*, 2009; Nevis *et al*, 2009). Here, we can provide a model where, despite the insufficient loading of dormant origins, the licensing checkpoint is overcome.

Although defects in DNA replication are the major endogenous source of DNA damage leading to genomic instability, many other pathways are also involved such as DNA repair and checkpoint response. In a recent study it was shown that reduction in MCM2 levels leads to a corresponding reduction in recruitment of Chk1 on chromatin and consequent reduced phosphorylation by ATR (Han *et al*, 2014). Similarly, MCM7 depletion leads to dysfunction of the DNA replication checkpoint and a subsequent failure to inhibit further DNA replication after fork stalling (Cortez *et al*, 2004). This suggests that proper levels of MCM2-7 are essential for genome stability not only to license sufficient dormant origin, but also because they might have an independent function in the intra S-phase checkpoint signaling. Therefore, it will be interesting in the future to check how low levels of MCM2-7 affect checkpoint responses in aneuploid cells and whether this also has consequences for the maintenance of genomic stability.

An unanswered question is what triggers the global response and in particular the down-regulation of replicative factors in aneuploid cells. The most immediate speculation is that aneuploidy-associated protein deregulation could be linked to the impaired proliferation. However, whether the slow growth of aneuploid cells is a cause or a consequence of protein deregulation is

difficult to say. Two scenarios are possible: aneuploid cell lines may grow slower as a consequence of deregulated expression of specific proliferative proteins, or aneuploidy triggers a cell cycle delay, which results in down-regulation of proteins involved in DNA replication and cell cycle. Interestingly, downregulation of pathways involved in DNA metabolism has also been observed in cells with complex aneuploid karyotype that do not show impaired proliferation (Dürrbaum *et al*, 2014). Therefore, the link between downregulation of replicative factors and proliferation rate might be either indirect or the two phenotyphes might be independent.

One alternative explanation for the reduced levels of replicative factors might reside in the defective folding capacity of aneuploid cells. Accordingly, several factors involved in DNA repair and replication are well-characterized clients of molecular chaperones (Taipale *et al*, 2014). Moreover, the comparison of the proteome changes in HeLa cells upon HSP90 inhibition with the proteome changes in untreated aneuploid cell lines revealed a partial overlap especially among the downregulated pathways (Donnelly *et al*, 2014). The common downregulated pathways include pathways of DNA and RNA metabolism, such as DNA repair and replication and RNA splicing, as well as the cell cycle pathways. The overlap was even greater when the comparison was made between aneuploid cell lines and HSF1-depleted human cell lines (Donnelly *et al*, 2014). Consistent with this hypothesis it was previously demonstrated that HSP90 inhibition itself leads to chromosomal instability in yeast (Chen *et al*, 2012). A straightforward speculation might be that because of the folding impairment, chaperones fail to correctly fold their clients, which include also replicative factors. Unfolded proteins that are not functional can aggregate or be targeted for degradation. This could explain the decrease in replicative factors level. However, there is no direct evidence that MCM2-7 proteins are HSP90 clients. In addition, down-regulation of the pathways involved in DNA metabolism are observed in proteome analysis as well as in transcriptome, although to a lesser extent (Dürrbaum *et al*, 2014). Therefore, HSP90 impairment might contribute to the down-regulation of replicative factors, but it is probably not the only cause.

We should also consider the possibility that down-regulation of DNA metabolism is due to an altered E2F activity. In fact, the E2F transcription factor family controls the expression of many genes involved in DNA replication and cell cycle progression (Dyson, 1998). This would explain the decrease in mRNA levels of several transcripts involved in DNA replication and cell cycle as well as the proliferation defect of aneuploid cells. A decreased E2F activity could be a consequence of limited HSP90 function of aneuploid cells. It has been demonstrated, indeed, that HSP90 inhibition leads to dowregulation of E2F-1 and corresponding target genes (Nagaraju *et al*, 2014).

Finally, one additional hypothesis is that the reduced mRNA levels of MCM2-7 occur due to post-trancriptional modifications. It was recently shown in MEFs carrying a hypomorphic allele of MCM4 that the consequent reduction of other MCM2-7 components occurs due to the destabilization of the hexamer, but also due to an mRNA pan-down-regulation (Chuang *et al*, 2012). This suggests an existence of a mechanism that regulates the MCM2-7 mRNA abundance in response to certain stress stimuli. Chuang and colleagues explored the hypothesis that a

process of active mRNA degradation was responsible for the post-transcriptional modification of MCM2-7 mRNAs. Indeed they observed that the RNAi machinery is involved in regulating MCM2-7 mRNA levels and decrease of MCM2-7 mRNA and protein levels correlated with increase of *miR-34a-c* (Chuang *et al*, 2012; Bai *et al*, 2016). The co-existence of such a negative feedback together with the protein folding defect in aneuploid cells could explain how the MCM2-7 mRNA is down-regulated as a consequence of a lower abundance of MCM2-7 proteins. Obviously this mechanism would cause a vicious cycle where the already decreased protein level is further lowered due to decreased mRNA. Other possibilities to explain the decrease of MCM levels and further studies will be required to clarify the mechanisms underlying the decreased abundance of replicative factors.

7.4 Aneuploidy as a road to cancer

The phenomenon of unbalanced karyotype was first described by Theodor Boveri as a recurrent defect in cancer. Because of this evidence he proposed for the first time that aneuploidy might play a role in cancerous transformation (Holland & Cleveland, 2009). Since then, the long-standing question whether aneuploidy is a cause or a byproduct of cancer has been extensively discussed. The debate is further fueled by the evidence that 90% of solid tumors and 50% of blood cancers are aneuploid (Beroukhim *et al*, 2010). Therefore, several hypotheses have been put forward in the recent years to rationalize this “chicken/egg” dilemma.

Some scientists proposed that aneuploidy is not necessary for transformation, as introduction of three artificially mutated genes is sufficient to transform normal human fibroblasts in cancer cells. Moreover, the obtained tumor cell clones do not show abnormal chromosome number (Zimonjic *et al*, 2001). However, there are limited data supporting these facts and later independent analysis of the obtained transformed clonal cell line with different methods revealed that at least 70% of the cells have a non-diploid karyotype (Li *et al*, 2002).

An additional hypothesis is that aneuploidy is only a consequence of tumor transformation. This assumption is supported by the evidence that aneuploidy has adverse consequences on cell growth while cancer cells usually proliferate faster than non-transformed cells. Moreover, it has been shown that many tumor suppressor genes are components of cell cycle checkpoint (Bric *et al*, 2009). Therefore, tumor suppressor loss causes not only cancerous transformation but also checkpoint disfunction leading to the formation of aneuploid cells (Manning *et al*, 2010). From this point of view, aneuploidy is therefore rather a side effect of the tumor suppressor inactivation. Yet, although it is not involved in tumor initiation, it might contribute to tumor development.

Finally, the third theory is that aneuploidy plays a crucial role in tumorigenesis and precedes cancer formation. The understanding of tumor initiation would be crucial, not only to identify specific therapeutic targets but also to develop prognostic markers for detection of precursor lesions and early-stage malignancies. It has been proposed that activation of oncogenes and inactivation of tumor suppressors occur as consequences of structural and numerical

chromosomal rearrangements proposing aneuploidy as a trigger of tumorigenesis (Mitelman *et al*, 2007).

Unfortunately studies on Down's syndrome do not really help our understanding of the role played by aneuploidy in cancer, as the evidences available are difficult to interpret and to explain. Multiple reports have shown that children with Down's syndrome have significant increased incidence of leukemia, while the risk of most solid tumors is reduced (Nižetić & Groet, 2012). The reason is still not clear, but it has been proposed that it depends on specific genes expressed in a "critical region" of chromosome 21 that have tumor-supressing effects (Sussan *et al*, 2008). In particular there are genes within this region whose overexpression inhibits angiogenesis. As the development of solid tumors depends on efficient vascularization, it might partially explain the reduction in risk of solid tumor compared to leukemia (Nižetić & Groet, 2012). Therefore, Down's syndrome might show a particular behavior due to a chromosome-specific effect. Conversely, analysis of data collected from patient with trisomy 8 mosaicism, trisomy 13, trisomy 18 and Klinefelter's syndrome (47XXY) revealed that these disorders are all associated with a higher susceptibility to developing solid cancer (Ganmore *et al*, 2009). Thus, to date it is still not clear whether aneuploidy *per se* could contribute to tumor initiation.

The major challenge in deciphering the role of aneuploidy in cancer is represented on one hand by the high heterogeneity of tumor karyotypes and on the other hand by the difficulties to test the consequence of aneuploidy *per se* in absence of other defects (Wood *et al*, 2007). In fact, the majority of drugs used to induce aneuploidy lead also to an increase in DNA damage, which has also been postulated as first step of carcinogenesis (Thompson & Compton, 2008). Moreover, alternative strategies to induce aneuploidy consist of mutation or inhibition of checkpoint components that lead to increased CIN. Notably, the role of CIN in tumor initiation has not been clarified. Large scale sequencing of human cancer cells revealed that mutation in spindle checkpoint genes are very rare in tumors (Greenman *et al*, 2007; Wood *et al*, 2007). Moreover, heterozygous knockout of several spindle assembly checkpoint (SAC) genes in CIN mouse models not always lead to spontaneous tumor formation (Dai *et al*, 2004; Iwanaga *et al*, 2007; Jeganathan *et al*, 2007). In addition, overexpression of SAC genes has sometimes cancer-inducing effects and other times protective effects (Baker *et al*, 2013; Ricke *et al*, 2011). Finally, Weaver and colleagues observed that aneuploidy induced by CIN-promoting mutations contributes to tumor transformation both *in vitro* and *in vivo,* but at the same time it inhibits tumorigenesis in tissues prone to tumor development (Weaver *et al*, 2007). To explain how CIN-induced aneuploidy could both trigger and suppress tumorigenesis they proposed a model where the effects of unbalanced karyotype are similar to those of DNA damage. Low levels of DNA damage results in low levels of genomic instability and could promote tumor initiation and development. Oppositely, high levels of DNA damage and genomic instability comparably to the effects of chemotherapy lead to cell death and consequent tumor suppression (Weaver & Cleveland, 2007). Similarly, studies on chromosome missegregation in bacteria revealed that low levels of CIN result in growth advantage while high

levels of CIN lead to cell death or so-called “mutational meltdown” (Lynch *et al*, 1993). Therefore, it is difficult to draw general conclusions from these results, as it is impossible to dissect which are the distinct contribution of CIN and aneuploidy on tumorigenesis. Recently, it has been shown that addition of single extra chromosome in an otherwise diploid and stable karyotype is insufficient to induce neoplastic phenotype and it acts as tumor suppressor in oncogene-transduced populations (Sheltzer *et al*, 2016). Therefore, in the absence of CIN, aneuploidy seems to prevent tumorigenesis, consistently with the previously described proliferation defect of aneuploid cells. Studies supporting the hypothesis that stable aneuploidy *per se* could promote tumor initiation are still missing. Our work provides a first mechanistic view on how aneuploidy might contribute to tumorigenesis in absence of CIN by triggering genomic instability as a consequence of impaired DNA replication.

7.5 A role for genomic instability in tumorigenesis

The contribution of genomic instability to carcinogenesis is supported by several lines of evidence. First, to undergo tumor transformation and to acquire growth advantage cells need to accumulate a certain number of genetic alterations of oncogenes and tumor suppressor genes in a limited amount of time (Beckman & Loeb, 2006). The normal mutation rate is probably insufficient to account for the multiple mutations required for tumor initiation while genomic instability would explain how cells gain in short time enough genetic variations to develop the malignant phenotype. Second, it is well known that humans and animals with germline mutations in genes that are involved in maintenance of genomic stability and DNA integrity are prone to develop tumors (Edelmann *et al*, 1997; Prolla *et al*, 1998; Varley, 2003; Zhang *et al*, 2015b). Third, somatic mutations leading to defects in pathways that contribute to the maintenance of genome integrity have been found in some tumors (Haugen *et al*, 2008; Shlien *et al*, 2015). Finally, karyotypic heterogeneity of most solid tumors is a hallmark of cancer cell and GIN can explain the high rate of continuous generation of new genetic variants during tumor growth (Patel *et al*, 2014). Therefore, GIN is considered one of the key driving forces of cancer development as it can accelerate accumulation of other cancer hallmarks.

A very universal condition of cancer cells is deregulated cell cycle proliferation, therefore suggesting that changes that stimulate proliferation could promote cancer. A still unresolved paradox is how aneuploidy could trigger tumorigenesis, a condition characterized by uncontrolled proliferation, when aneuploidy itself impairs growth. This remains an enigmatic question, and to find the answer it would be first essential to understand what causes the proliferation defects in aneuploid cells. One possibility is that aneuploid cells might be able to “evolve” and improve the proliferation rate in the long-term period. It cannot be excluded that such “evolution” could result in the clonal expansion of a cell population with proliferative advantage. Weaver and colleagues observed that aneuploidy-induced transformation is a slow process requiring at least 30 passages *in vitro* (Weaver *et al*, 2007). We always performed our experiment within a limited number of

passages (not more than 6), because we wanted to avoid any evolution due to ongoing genomic instability. However, this could be an attractive point to investigate in the future. It would be interesting, for example, to test whether there is any difference in proliferation rate between the clones we derived from aneuploid HCT116 cells that did not accumulate any chromosomal rearrangement and those that did accumulate CNAs.

Interestingly, aneuploidy is not the only scenario characterized by decreased cell cycle progression that is associated with tumors. For example, loss of a subclass of transcriptional activators E2F leads to defects in cell cycle progression, proliferation and development (Wu *et al*, 2001). This is not surprising as their major role is promoting the transcription of a variety of genes involved in cell cycle progression. What is surprising is that E2F1/E2F2 mutant mice exhibit higher predisposition to tumor development (Zhu *et al*, 2001). Bilousova and colleagues explained this paradox with the “Poor competition model”. They demonstrated that conditions that impair DNA replication (mutation of E2F1/E2F2) of hematopoietic progenitors significantly enhanced the proliferative advantage after oncogenes expression or p53 mutation while for healthy replicating progenitors cells oncogenic mutations are disadvantageous (Bilousova *et al*, 2005). The competitive advantage allows mutant progenitors cells to outcompete wild type cells in the same niche, promoting leukemogenesis. Thus, in a replication-impaired scenario acquisition of oncogenic mutations might provide a relatively larger selective advantage. Oppositely, oncogenic mutations are mainly disadvantageous and usually are selected against in the wild type population (Bilousova *et al*, 2005). Recently it has been shown that E2F1 and E2F2 play a role in response to DNA damage and maintenance of genome stability in neuronal cells suggesting that this might also contribute to the increased leukemogenesis (Castillo *et al*, 2015).

Something similar might be true for aneuploid cells. Despite the impaired proliferation, genomic instability of aneuploid cells represents a source of genetic variation allowing cells to adapt under strong selective forces. Notably, chronic conditions such as inflammation or environmental stresses have been linked with tumor onset (Coussens & Werb, 2002). In such stressful circumstances or in highly hostile environments wild type cells might die while aneuploid cells might favor accumulation of genetic alterations that will eventually lead to tumorigenesis. Consistently with this hypothesis it has been recently shown that aneuploidy confers a selective advantage when cells are cultured in non-standard conditions (Rutledge *et al*, 2016). Moreover, genomic instability might also provide a way for aneuploid cells to accumulate aneuploidy-tolerating mutations and overcome the physiological defects caused by the presence of extra chromosome(s).

To summarize, our findings can offer a mechanistic view on how a random single chromosome segregation error can contribute to cancer development. We showed that simple addition of an extra chromosome leads to an increased accumulation of CNAs in human cells as a consequence of increased sensitivity to replication stress. Considering that the rate of stochastic chromosome

missegregation is five orders of magnitude higher than the point mutation rate (Thompson & Compton, 2008; Balin & Cascalho, 2010), there is certainly a higher probability that a single chromosome segregation error triggers simultaneous accumulation of several tumor-promoting mutations rather than all single mutations happen independently. Therefore, increasing genomic instability in response to random chromosome segregation errors might represent a new route leading to tumor initiation and chemotherapy resistance.

8. References

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Education

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Publications

Dürrbaum M, Kuznetsova AY, Passerini V, Stingele S, Stoehr G, Storchová Z. **Unique features of the transcriptional response to model aneuploidy in human cells.** BMC Genomics 2014 Feb 18;15:Pa139.

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