Aneuploidy impairs protein folding and genome integrity in human cells

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vorgelegt von

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Table of Contents

1. Abbreviations

4-NQO	4-nitroquinoline-N-oxide
17-AAG	17-N-allylamino-17-demethoxygeldanamycin
53BP1	p53 binding protein 1
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AIF	Apoptosis-inducing factor
AMPK	5' adenosine monophosphate-activated protein kinase
APP	Amyloid beta precursor protein
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
CDC6	Cell Division Cycle 6
CDC37	Cell Division Cycle 37
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CENP-E	Centromere-associated protein E
CIN	Chromosomal instability
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DS	Down's syndrome
DSB	Double strand break
DSCR	Down syndrome critical region
DSCR1	Down syndrome critical region gene 1
EGF	Epidermal growth factor

EOAD	Early onset Alzheimer's disease
ESC	Embryonic stem cell
ESR	Environmental stress response
FANCA	Fanconi anemia, complementation group A
FANCD1/BRCA2	breast cancer 2/Fanconi anemia, complementation group D
FISH	Fluorescence in situ hybridization
GDBH	Gene dosage balance hypothesis
HSF1	Heat Shock Factor 1
Hsp104	Heat Shock Protein 104
HSP27	Heat Shock Protein 27
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
HSR	Heat Shock Response
iPSC	Induced pluripotent stem cell
LC3	Microtubule-associated proteins 1A/1B light chain 3A
MCAK	Mitotic centromere-associated kinesin
MCM	Minichromosome maintenance protein complex
MEF	Mouse embryonic fibroblast
MM	Multiple myeloma
MVA	Mosaic variegated aneuploidy
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
ORC1	Origin Recognition Complex, Subunit 1
ORC2	Origin Recognition Complex, Subunit 2
Pol ζ	DNA polymerase ζ

PN	Proteostasis network
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
ROS	Reactive oxygen species
RPA1	Replication Protein A1, 70kDa
RPE-1	Retinal pigment epithelium cells
SAC	Spindle assembly checkpoint
SNP	Single nucleotide polymorphism
TKNEO	thymidine kinase with neomycin phosphotransferase reporter gene
Ubp6	Ubiquitin carboxyl-terminal hydrolase 6
UPR	Unfolded protein response
v-Src	Proto-oncogene tyrosine-protein kinase Src
XIST	X-inactive specific transcript
XRCC1	X-ray repair cross-complementing protein 1
YAC	Yeast artificial chromosome

2. List of publications

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

Passerini V*, Ozeri-Galai E*, de Pagter M, **Donnelly N**, Schmalbrock S, Kloosterman WP, Kerem B, Storchová Z. The presence of extra chromosomes leads to genomic instability. Nat Commun. 2016 Feb 15;7:10754

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3. Declaration of contributions as co-author

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

Neysan Donnelly contributed to this work by designing, planning and performing the experiments depicted in Figure 1, Figure 2 C,D, Figure 3 and Figure 4 and corresponding supplementary figures. In addition, he participated in the creation of figures, the interpretation and discussion of results, as well as in the writing of the paper.

Passerini V*, Ozeri-Galai E*, de Pagter M, **Donnelly N**, Schmalbrock S, Kloosterman WP, Kerem B, Storchová Z. The presence of extra chromosomes leads to genomic instability. Nat Commun. 2016 Feb 15;7:10754

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Neysan Donnelly contributed to this work by planning and performing the experiments shown in Figure 5 E and Figure 6 D-F. He also participated in the interpretation and discussion of results.

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4. Summary

Aneuploidy or imbalanced chromosome content is the cause of pathological conditions such as Down's syndrome and is also a hallmark of cancer where it is linked with malignancy and poor prognosis.

A growing body of evidence has demonstrated that aneuploidy exerts a large number of effects at the cellular level. These effects include an impairment of proliferation, distinct changes to the transcriptome and proteome, as well as a disturbance of cellular proteostasis. However, the molecular mechanisms underlying the impairment of proteostasis and the changes in gene expression are not well understood. Further, the consequences of the altered gene expression in aneuploid cells also remain incompletely characterised. The work described herein was performed to gain insights into the consequences of aneuploidy in human cells.

We have found that human aneuploid cells are impaired in HSP90-mediated protein folding. Further, we demonstrate that aneuploidy hampers induction of the heat shock response suggesting that the activity of the transcription factor HSF1 is compromised in human aneuploid cells. Increasing the levels of HSF1, either by endogenous or exogenous means, counteracts the effects of aneuploidy on HSP90 function, indicating that the defective HSP90 function of aneuploid cells is due to insufficient HSF1 capacity. We also demonstrate that the deficient protein folding capacity is at least partly responsible for the complex changes in gene expression observed in aneuploid cells.

One of the most striking characteristics of the gene expression changes elicited by aneuploidy is the consistent downregulation of factors related to DNA transactions. Thus, the second study described here was undertaken to determine the effects of aneuploidy on DNA replication and genome stability. Our analysis showed that DNA replication is indeed impaired in human aneuploid cells, leading to higher levels of anaphase bridges, ultrafine bridges, chromosome breaks, as well as ultimately, complex chromosomal rearrangements. These defects were shown to stem from lower expression of the MCM2-7 helicase and could be rescued by MCM2-7 overexpression.

The results described here provide mechanistic insight into the causes of the disturbed proteostasis in aneuploids as well as revealing the consequences of impaired protein folding capacity for aneuploid cells. Further, they demonstrate that aneuploidy is by itself capable of destabilising the genome and delineate a molecular mechanism by which this can occur. Taken together, the gleaned insights may have important implications for the role of aneuploidy in pathological conditions.

5. Zusammenfassung

Aneuploidie ist eine numerische Chromosomenaberration, ein Ungleichgewicht der Chromosomenzahl, die Down Syndrom verursacht und zu den Hauptcharakteristiken von Krebs zählt. Bei Tumoren ist Aneuploidie mit Malignität und schlechter Prognose verbunden.

Eine stetig wachsende Evidenzlage zeigt, dass Aneuploidie eine Vielzahl von Effekten auf zellulärer Ebene hat. Unter anderem führt Aneuploidie zu einer Beeinträchtigung der Zellproliferation, zu bestimmten Veränderungen des Transkriptoms und des Proteoms, sowie zu Störungen der zellulären Proteostase. Es ist allerdings unklar, welche Mechanismen die Proteostase und die Genexpression beeinträchtigen. Auch sind die genauen Konsequenzen der veränderten Genexpression noch nicht geklärt. Die in dieser Dissertation beschriebene Forschungsarbeit setzte sich zum Ziel, neue Erkenntnisse zur Beantwortung dieser Fragen beizutragen.

Wir haben herausgefunden, dass menschliche aneuploide Zellen eine Beeinträchtigung in der HSP90-abhängigen Proteinfaltung aufweisen. Darüber hinaus zeigen wir, dass Aneuploidie die Induktion der zellulären Hitzeschockantwort hemmt, was auf eine Störung des Transkriptionsfaktors HSF1 hindeuten könnte. Tatsächlich führt eine Erhöhung der zellulären HSF1 Konzentration, entweder auf endogene oder auf exogene Weise, zu einer Umkehrung des Effekts von Aneuploidie auf die Funktion von HSP90, was die Hypothese stützt, dass die gestörte HSP90 Funktion in aneuploiden Zellen auf eine unzureichende Kapazität von HSF1 zurückzuführen ist. Wir zeigen außerdem, dass die geminderte Proteinfaltungskapazität zumindest teilweise für die komplexen Veränderungen in der Genexpression in aneuploiden Zellen verantwortlich ist.

Eine der auffälligsten Veränderungen der Genexpression in aneuploiden Zellen ist die konstante Repression von Faktoren, die in die DNS-Transaktionen verwickelt sind. Aus diesem Grund setzten wir uns mit der zweiten in dieser Arbeit beschriebenen Studie zum Ziel, die Auswirkungen von Aneuploidie auf DNS-Replikation und auf die genomische Stabilität zu ermitteln. Unsere Analysen beweisen, dass in aneuploiden menschlichen Zellen die DNS-Replikation tatsächlich beeinträchtigt ist, was zu erhöhten Mengen von Anaphase-Brücken, fadenförmigen DNS-Brücken, Chromosombrüchen und letztendlich zu komplexen Umordnungen der Chromosomen führt. Wir zeigen, dass diese Schäden auf eine verringerte Expression der MCM2-7 Helikase zurückzuführen sind und durch Überexpression von MCM2-7 revidierten werden können.

Die hier beschriebenen Ergebnisse liefern neue mechanistische Erkenntnisse zu den Ursachen der gestörten Proteostase in Aneuploidie und der Auswirkungen von beeinträchtigter Proteinfaltungskapazität auf aneuploide Zellen. Sie beweisen, dass Aneuploidie selbst imstande ist, das Genom zu destabilisieren und beschreiben den molekularen Mechanismus, der dazu führt. Zusammengefasst könnten die gewonnen Erkenntnisse wichtige Implikationen für die Rolle von Aneuploidie in Krankheitszuständen haben.

6. Aims of the thesis

The effects of an imbalanced karyotype or aneuploidy on cellular physiology are mediated by the expression of genes encoded on aneuploid chromosomes. While the functions of the specific gene products on these chromosomes are responsible for some of these effects, recent work has shown that many of the consequences of aneuploidy are independent of the exact karyotype. The work described in this thesis was undertaken to gain cellular and molecular insights into the karyotype-independent consequences of aneuploidy in humans.

The starting point for the first study comprising this cumulative thesis was the growing body of evidence that suggests that aneuploidy exerts detrimental effects on proteostasis. Earlier work had shown that aneuploid cells are hypersensitive to conditions that interfere with protein production, folding and degradation (Torres et al. 2007; Tang et al. 2011; Oromendia et al. 2012); that they accumulate protein aggregates (Oromendia et al. 2012; Stingele et al. 2012); and that aneuploidy leads to an upregulation of autophagic degradation (Tang et al. 2011; Stingele et al. 2012). I hypothesised that all these observations may have a common root: compromised protein folding capacity. Further, the general sensitivity of aneuploid cells to chemical inhibition of the HSP90 molecular chaperone (Torres et al. 2007; Tang et al. 2011), indicated that HSP90-dependent protein folding might be particularly affected. Thus, together with my colleagues, I set out to directly determine if protein folding capacity is impaired in human aneuploid cells, if this impairment specifically concerns HSP90, the underlying mechanism for these effects, as well as to determine the consequences of the protein folding defect for aneuploid cells.

The second study was undertaken to determine the effects of aneuploidy on genome stability. Previous work in yeast had shown that aneuploid cells exhibit a higher mutation rate, accrue DNA damage and lose chromosomes at an elevated frequency compared to euploid cells (Sheltzer et al. 2011; Zhu et al. 2012; Blank et al. 2015). Whether aneuploidy also compromised genome stability in metazoan cells was largely unknown. Previous analysis from our laboratory had demonstrated that pathways and proteins associated with DNA transactions are generally downregulated in human aneuploid cells, and revealed a particularly striking reduction in expression of factors involved in DNA replication (Stingele et al. 2012; Durrbaum et al. 2014). Thus, we hypothesised that aneuploid cells may experience problems during DNA replication, an idea which is also supported by the observation that S phase is prolonged in most human aneuploid cells (Stingele et al. 2012). This work aimed to directly establish whether DNA replication is impaired in response to aneuploidy, and to determine the molecular mechanisms as well as the consequences of such impairment for genome stability in aneuploid cells.

7. Introduction

Changes to DNA quantity and thus, gene copy number can be beneficial from an evolutionary perspective as they allow divergence of gene function and the evolution of novel traits (Ohno 1970). However, the immediate consequences of changes to DNA amount are often deleterious for the organism concerned and generally result in decreased fitness. Paradoxically, such changes are also likely to play an important role in the development of cancer. However, while often deleterious, large-scale changes to DNA copy number do occur relatively often in nature, indicating that control mechanisms that function to maintain genome integrity frequently fail. Moreover, the fact that they can also be frequently detected in viable, healthy organisms is a strong indication that under certain conditions changes in DNA copy number can play important direct roles. Taken together, these observations illustrate the importance of understanding how changes in chromosome copy number occur and how they affect organismal and cellular physiology.

Broadly, changes to DNA that affect chromosome number are of two types: polyploidy, which describes a state in which cells possess two or more entire genomes; and aneuploidy, which denotes unbalanced changes to chromosome number that result in increased or decreased numbers of one or more individual chromosomes. While aneuploidy can thus by definition refer to a state of overall chromosome loss, in humans this is only frequently observed in cancer cells with complex karyotypic changes. Although monosomy, a state that describes the loss of one homologue of a chromosome pair, is observed in yeasts where it represents an adaptation to stress (Berman 2016), the condition is very rare in man and is usually not compatible with live birth (Pai et al. 2003).

Although polyploidy and aneuploidy describe distinct states, with very different consequences for cellular physiology, the phenomena are intimately linked. In eukaryotes the inherent instability of the polyploid state (Mayer and Aguilera 1990; Fujiwara et al. 2005; Storchová et al. 2006), means that such cells often rapidly become aneuploid. Indeed, aneuploidy itself is often not a static state of imbalance, but rather a manifestation of a chronic predicament in which chromosomes are continually gained and lost. This ongoing gain and loss of chromosomes is known as chromosomal instability (CIN) ((Vogelstein et al. 1989; Lengauer et al. 1997; Lengauer et al. 1998), and reviewed in (Vogelstein et al. 1989; Lengauer et al. 1997; Lengauer et al. 1998; Potapova et al. 2013; Giam and Rancati 2015; Nicholson and Cimini 2015)). Also, aneuploidy can be whole-chromosomal, or can extend only to portions of chromosomes, in which case it is known as structural aneuploidy (Gordon et al. 2012). At an organismal level, two types of aneuploidy can generally be distinguished: constitutional aneuploidy, which arises

during meiosis, and thus affects the whole organism, and somatic aneuploidy, which is a consequence of errors during mitosis. Delineating the links between CIN, whole-chromosomal and structural aneuploidy will facilitate a better understanding of the role of aneuploidy, and is particularly crucial for apprehending the consequences of aneuploidy in disease conditions (Janssen et al. 2011; Burrell et al. 2013; Russo et al. 2015). With the research presented here I aimed to gain new insights into the consequences of aneuploidy for the physiology of human cells.

7.1 Causes of aneuploidy

Aneuploidy nearly always arises as a result of defective distribution of duplicated chromosomes to daughter cells during cell division (Figure 1), whereas tetraploidy is usually a consequence of a failure in the physical separation of dividing cells, a process known as cytokinesis, or due to mitotic slippage, a phenomenon by which cells escape from a prolonged mitotic arrest and re-enter the cell cycle (Ganem et al. 2007). However, there are important exceptions to this general rule. Tetraploidy can also be an outcome of programmed reduplication of the genome in the absence of cell division, a phenomenon known as endoreplication, and plays important specialised functions in a range of different organisms (Lee et al. 2009). Further, tetraploidy also arises as a consequence of cell fusion, which can be induced in response to viral infection (reviewed in (Duelli and Lazebnik 2007)).

The proper segregation of chromosomes during mitosis and meiosis is governed by the activity of a system known as the spindle assembly checkpoint (SAC), which functions as the major gatekeeper to cell division (Musacchio 2015). The SAC acts as a brake on chromosome segregation by sensing the proper attachment of chromosomes to microtubules emanating from centrosomes on opposing sides of a mitotic cell. At a molecular level, the SAC's sensing of proper chromosome attachment is facilitated by the physical recruitment of SAC proteins to unattached kinetochores during prometaphase, which then signal to inhibit further progression through mitosis (Howell et al. 2004).

Consistent with its critical role in ensuring proper chromosome segregation (Kops et al. 2005), defects in SAC function, and specifically, deleting or reducing the expression of several SAC genes leads to improper chromosome segregation, chromosomal instability and aneuploidy *in vitro* and *in vivo* (e.g., (Dobles et al. 2000; Michel et al. 2001; Baker et al. 2004).

The occurrence of aneuploid embryos that arise from chromosome missegregation in meiosis represents the single biggest cause of spontaneous miscarriages in human pregnancies, and the

individuals that survive experience an array of severe developmental defects (Hassold and Hunt 2001). Thus, there is great interest in understanding the causes of constitutional

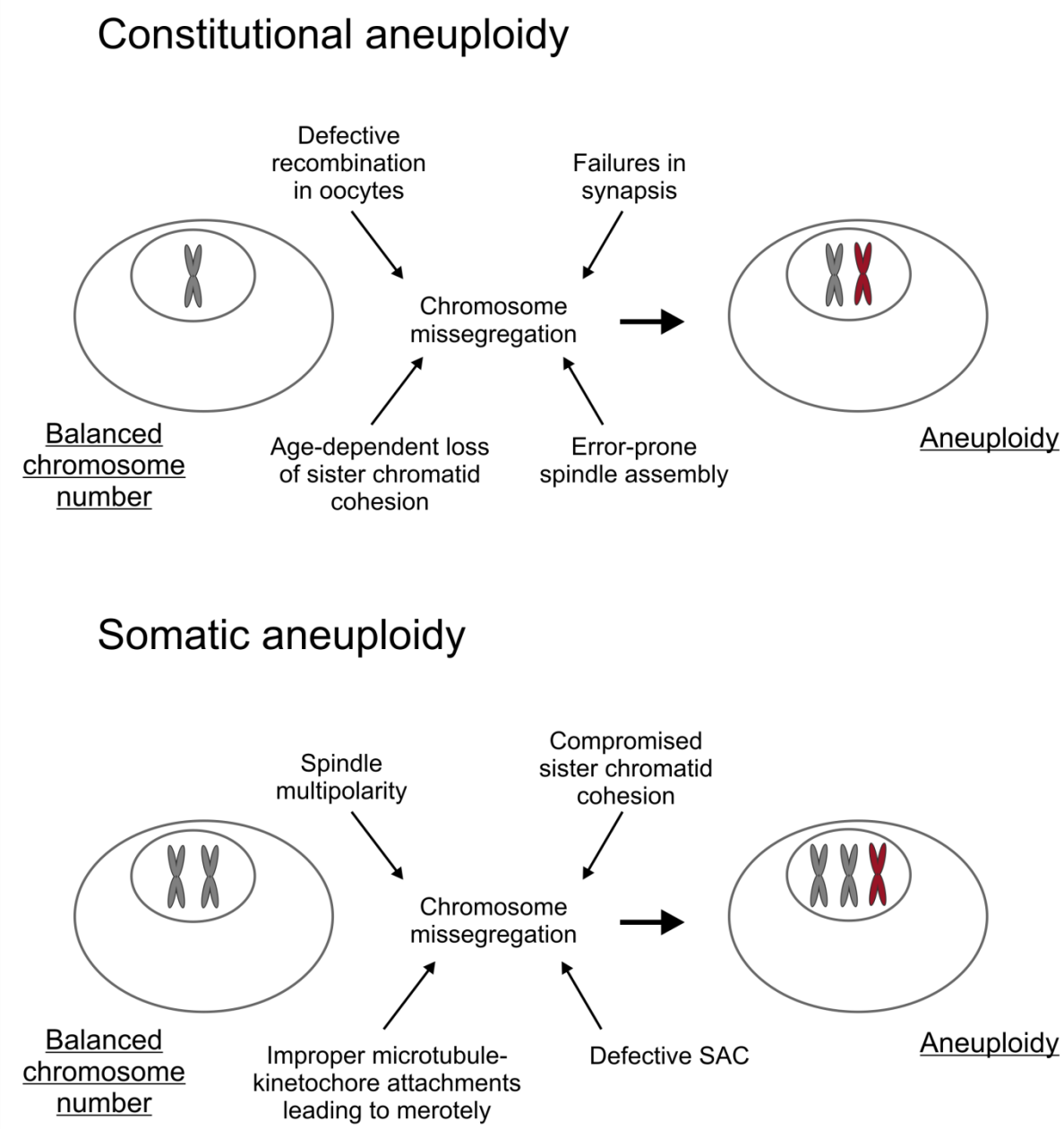

Figure 1: In both germ cells as well as somatic cells aneuploidy arises as a result of circumstances that lead to chromosome mis-segregation, such as spindle multipolarity, defective recombination, compromised sister chromatid cohesion or impaired function of the Spindle Assembly Checkpoint (SAC). An example of chromosome gain is depicted, as this situation is well studied.

aneuploidy in humans. Strikingly, the female's oocytes are almost always the source of aneuploidy in such cases and increasing maternal age is a clear risk factor (Hassold and Hunt

2001). In fact, meiosis in human oocytes appears to be inherently error-prone (Pacchierotti et al. 2007; Templado et al. 2011; Nagaoka et al. 2012; Danylevska et al. 2014). Multiple factors are likely to contribute to these phenomena, including faulty recombination (Hassold et al. 1995; Lamb et al. 1996; Lamb et al. 1997), an elevated tolerance to failures in synapsis (Celeste et al. 2002; Bannister et al. 2007; Kuznetsov et al. 2007), and error-prone spindle assembly (Holubcová et al. 2015). Several mechanisms have also been proposed to be involved in determining the causes of the age-dependent rise in chromosome non-disjunction in oocytes. One hypothesis which has garnered particular attention is the premature loss of sister chromatid cohesion (reviewed in (Jessberger 2012)). In support of this model, in mouse oocytes it has been demonstrated that cohesion is lost from chromosomes as mice age (Chiang et al. 2010; Lister et al. 2010; Tachibana-Konwalski et al. 2010). Sister chromatid cohesion is maintained by a protein complex named cohesin that encircles sister chromatids during both meiosis and mitosis to prevent their premature separation (Brooker and Berkowitz 2014). In meiotic cells it prevents premature disjunction by promoting the proper resolution of cross-overs between pairs of homologous chromosomes and by ensuring that sister chromatids within each pair are kept together until anaphase II (Rankin 2015). A recent study has shown that an additional reason for the maternal age effect in human oocytes might lie in the observation that sister kinetochores are very often split, and behave as separate functional units in the oocytes of women over 30 years of age (Zielinska et al. 2015).

Impairments in sister chromatid cohesion can also lead to aneuploidy in yeast (Guacci et al. 1997; Michaelis et al. 1997), as well as to somatic aneuploidy in human cells (e.g. (Solomon et al. 2011). In fact, mutations in cohesin may partially underlie the chromosomal instability observed in certain forms of cancer (Barber et al. 2008; Solomon et al. 2011; Welch et al. 2012).

A second set of defects responsible for the generation of aneuploidy in somatic cells relates to erroneous microtubule-kinetochore attachments and their consequences (reviewed in (Bastians 2015)). Those which pose the greatest danger for proper chromosome segregation are merotelic attachments (Cimini et al. 2001), a state in which the two kinetochores are attached to microtubules from opposite spindle poles, but where, in addition, one of the kinetochores is bound by further microtubules emanating from one of the two poles. Merotelic attachments are particularly challenging for the cell to resolve as under these conditions, i.e. when both kinetochores are attached to microtubules which emanate from opposite poles, the activity of the SAC is not triggered. Progression into anaphase leads to the generation of lagging sister chromatids, which are not segregated towards either pole and thus remain separate from both chromosome masses (Gregan et al. 2011). Lagging chromatids are strongly associated with chromosome mis-segregation (Cimini 2008; Thompson and Compton 2008). Merotelic

attachments and consequently, lagging chromosomes can arise in response to a number of defects, including those that result in the hyper-stabilization of microtubule-kinetochore attachments (Bakhoum et al. 2009a; Bakhoum et al. 2009b; Kabeche and Compton 2012), and those that affect microtubule dynamics (Ertych et al. 2014). The presence of a supernumerary number of centrosomes is another mechanism that can result in merotely and the generation of aneuploid cells (Ganem et al. 2009; Silkworth et al. 2009), by promoting the formation of transient multipolar spindles during mitosis. Centrosome duplication can arise in a number of ways. For instance, centrosomes themselves can be overduplicated in the absence of p53 (Fukasawa et al. 1996), in response to DNA replication stress (Balczon et al. 1995), or upon DNA damage (Sato et al. 2000). Tetraploidization represents an additional route to centrosome duplication (Fujiwara et al. 2005). This not only establishes a molecular link between tetraploidy and aneuploidy, but is also important for understanding the source of aneuploidy in cancer, as analysis indicates that over 1/3 of cancers undergo tetraploidization (Zack et al. 2013).

Thus, maintaining a correct karyotype is a highly complex undertaking that is sensitive to a wide range of perturbations affecting the machinery that governs chromosome segregation and cell division. Moreover, although it is severely detrimental in humans at the organismal level, constitutional aneuploidy is relatively common in man, due in large part to the error-proneness of chromosome segregation in oocytes. Further, chromosome segregation errors that give rise to somatic aneuploidy, such as is found in cancer, can arise either as a result of defective distribution of duplicated chromosomes to daughter cells or via an unstable tetraploid intermediate.

7.2 Models to study aneuploidy

Theodor Boveri first proposed more than 100 years ago that aneuploidy may play a causative role in cancer development (Boveri 2008). Further, it has been known for more than 50 years that Down's Syndrome (DS) is caused by the presence of a third copy of chromosome 21 (LEJEUNE and TURPIN 1961). However, despite these strong links between aneuploidy and pathology, ascribing specific roles to aneuploidy in cancer has been difficult and similarly, we still lack a complete understanding of how aneuploidy underlies the plethora of developmental defects observed in individuals with trisomy syndromes. One reason for these deficits in understanding was the dearth of suitable model systems in which to study the effects of aneuploidy. Further, as the effects of aneuploidy on cellular physiology are often subtle, there

was also a lack of methods which could detect these effects with sufficient sensitivity. The intimate link between aneuploidy and chromosomal instability represents another challenge to studying aneuploidy, as cells that are aneuploid are often rapidly changing, hampering efforts to separate the effects of aneuploidy *per se* from those of chronic chromosome mis-segregation. In addition, cancers, which represent the best source of aneuploid cells, are characterised by a myriad of other genetic and epigenetic changes that mean that disentangling the effects of aneuploidy from those elicited by other factors is fraught with difficulty. Finally, research on aneuploidy has broadly been hampered by the lack of suitable control cell lines with the correct number of chromosomes.

Despite these challenges, progress has been made in the last decade and two developments have been particularly important in allowing the generation and study of suitable aneuploid model systems. The first is the establishment or application of techniques that allow *de novo* formation of aneuploid cells with diverse karyotypes, which can then be directly compared with the cells from which they were generated (summarised in Table 1). For example, chromosome transfer has been used to generate a range of disomic *Saccharomyces cerevisiae* strains in a haploid genetic background (Torres et al. 2007). The second key innovation is the use of systematic global approaches, namely high-resolution analyses of DNA, RNA and protein levels, which has facilitated quantitative investigation of changes in DNA copy number and the corresponding effects on the transcriptome and proteome.

One way in which aneuploid yeast can be generated is by chromosome transfer. In this technique a wild-type haploid yeast strain is crossed with a donor strain, which is defective in nuclear fusion (Conde and Fink 1976). In addition, both strains carry selection markers on homologous chromosomes. Even though mating is not possible, chromosomes can occasionally be transferred from one nucleus to the other. An aneuploid cell can then be selected for using the selection markers present on each of the homologous chromosomes.

Generation of aneuploid yeast from triploid or pentaploid cells (Pavelka et al. 2010b; St Charles et al. 2010), takes advantage of the fact that during meiosis I of these cells homologous chromosomes are segregated randomly giving rise to two aneuploid progenies. Meiosis II then gives rise to four spores, with those that survive exhibiting highly aneuploid karyotypes.

Conditional centromere inactivation, which is achieved by incorporation of a construct that can be induced to elicit high levels of transcription over the centromeric region, can also be used to induce chromosome mis-segregation in yeast (Reid et al. 2008; Anders et al. 2009). This method can be utilised when the aim is either targeted chromosomal removal or the generation of a specific aneuploid yeast strain.

Finally, low concentrations of the HSP90 inhibitor radicicol have been described to inhibit kinetochore function and in this way to induce variable levels of aneuploidy (Chen et al. 2012). However, it should be noted that not all cells will become aneuploid under these conditions.

A number of techniques have also been developed to study aneuploidy in mammals. One which has been used to generate mouse embryos with different trisomic karyotypes (Gropp et al. 1975; Williams et al. 2008), involves the breeding of mice with different Robertsonian translocations, chromosomal aberrations that are generated by the breakage and subsequent re-joining of non-homologous acrocentric chromosomes (Pai et al. 2003). The techniques relies upon the fact that the segregation of Robertsonian translocations is error-prone during meiosis, and is thus likely to result in the generation of aneuploid progeny in a significant number of cases. Even though the majority of these trisomic embryos die *in utero*, they survive long enough to allow the establishment of mouse embryonic fibroblast (MEF) cell lines (Gropp et al. 1983; Dyban and Baranov 1987; Williams et al. 2008).

Mice have also been used to model DS. The most commonly used model is the Ts65Dn mouse, which phenocopies many of the behavioural and cognitive defects found in human individuals with trisomy 21, although it should be noted that the overlap with genes found on human chromosome 21 is far from complete (reviewed in (Rueda et al. 2012). Further, a large number of mouse models, with over 30 different genes involved in chromosome segregation being targeted thus far, have been established to study the *in vivo* effects of CIN in metazoans (reviewed in (Simon et al. 2015) and (Giam and Rancati 2015)).

Table 1: Different models to study aneuploidy in yeast, mice and humans

Yeast	Description	Advantages	Disadvantages	References
Chromosome transfer	Abortive mating of haploid yeast followed by inter-nucleus chromosome transfer	Allows generation of yeast disomic for nearly all 16 chromosomes	Not all potential yeast disomies possible	(Torres et al. 2007)
Meiosis of triploid or pentaploid cells	Random segregation of yeast chromosomes during meiosis	Allows generation of cells with complex, highly aneuploid karyotypes	Only some of the resulting spores are viable; those that are, often unstable	(Pavelka et al. 2010b); (St Charles et al. 2010)
Centromere inactivation	Elicited by high levels of transcription over centromeres	Allows targeted chromosome removal	-	(Anders et al. 2009); (Reid et al. 2008)
HSP90 inhibition	Chemical or genetic inhibition of HSP90 leading to CIN	Ease of use	Not all treated cells become aneuploid; potential bias in karyotypes	(Chen et al. 2012)

Mouse				
Robertsonian translocations	Relies on inherently error-prone segregation of these translocations	Reveals consequences of aneuploidy in higher eukaryotes	-	(Gropp et al. 1975); (Gropp et al. 1983); (Williams et al. 2008)
DS models	Diverse mouse strains with chromosomes containing mouse homologues of Hsa21-encoded genes	Facilitate an understanding of the pathology of DS and of the consequences of aneuploidy at the organismal level	Hsa21-homologous genes are spread across different mouse chromosomes	Reviewed in (Rueda et al. 2012)
Diverse CIN models	Mice generated to harbour mutations or deletions in genes involved in chromosome segregation	Shed light on the consequences of aneuploidy *in vivo*	-	Reviewed in (Giam and Rancati 2015) and (Simon et al. 2015)
Human				
Embryo-derived ESCs	ESCs derived from embryos discovered to be aneuploid during PGD	Reveal the consequences of aneuploidy in humans at the cellular level	Lack of appropriate control cell lines	(Lavon et al. 2008)
Patient-derived cell lines	Cell lines established from tissue of human trisomic individuals	Reveal the consequences of aneuploidy in humans at the cellular level	Lack of appropriate control cell lines	Available from e.g. Coriell Biorepository
Microcell-mediated chromosome transfer	Transfer of individual chromosomes into control diploid cells	Karyotypically stable; comparison with control reveals aneuploidy-dependent phenotypes	Not suitable for analysis of the immediate effects of aneuploidy	(Upender et al. 2004); (Stingele et al. 2012)
Targeted chromosome removal or silencing	Diverse methods employed to remove or silence chromosome 21	Easy determination of direct effects of aneuploidy	Technically challenging	(Li et al. 2012); (Jiang et al. 2013)
Drug-induced chromosome mis-segregation	Use of mitotic spindle poisons such as nocodazole and monastrol	Allows study of immediate effects of chromosome mis-segregation	Not all cells become aneuploid; no control over identity of mis-segregating chromosomes	(Thompson and Compton 2008); (Elhajouji et al. 1997)
Transient tetraploidization	Block of cytokinesis generating tetraploid cells which rapidly becomes aneuploid	Generates complex aneuploid karyotypes such as those found in cancer	Largely random karyotypes; cells often have high levels of CIN	(Ho et al. 2010); (Vitale et al. 2010); (Kuznetsova et al. 2015)
Chemical or genetic inhibition of the SAC	Knockdown or inhibition of key SAC effectors leading to chromosome mis-segregation	Allows study of immediate effects of chromosome mis-segregation	Not all cells become aneuploid; no control over the identity of the mis-segregating chromosomes	(Hewitt et al. 2010); (Santaguida et al. 2010); (Michel et al. 2001); (Meraldi and Sorger 2005)

Table 1: Different models to study aneuploidy in yeast, mice and humans (cont.)

In humans, embryonic stem cells (ESCs) have been derived from embryos that were diagnosed to be aneuploid during pre-implantation genetic screening (Lavon et al. 2008). Further, primary cell lines from individuals with trisomy syndromes have also been established and are commercially available, e.g, from the Coriell Biorepository (Camden, USA). Although the advantage of using these cells to understand the pathology of trisomy syndromes is obvious, one significant disadvantage is the lack of isogenic controls to which these cell lines can be compared. A few recent studies took advantage of a very rare condition in which twins were simultaneously monozygotic yet discordant for trisomy 21 (Dahoun et al. 2008; Hibaoui et al. 2014; Letourneau et al. 2014).

Targeted chromosome removal or silencing has also been successfully carried out in human cells. In one study, the authors generated induced pluripotent stem cells (iPSCs) from the fibroblasts of individuals with DS and subsequently used gene targeting to introduce a TKNEO fusion gene, which encodes thymidine kinase as well as neomycin resistance, into one of the three copies of chromosome 21. This approach allowed the authors to then select against TKNEO and thus for loss of chromosome 21 (Li et al. 2012). In another study, again targeting chromosome 21, an approach utilising an adapted version of the endogenous silencing of the inactive X chromosome was utilised. Using zinc finger nucleases, one copy of chromosome 21 was engineered to encode *XIST* long non-coding RNA. This resulted in the coating of the chromosome with *XIST* and to heterochromatinization and the silencing of gene expression (Jiang et al. 2013).

Less specific approaches to produce mammalian aneuploid cells include strategies to induce chromosome mis-segregation. Drugs such as nocodazole, which perturbs microtubule polymerisation (De Brabander et al. 1976), and monastrol, which inhibits the mitotic kinesin Eg5 (Mayer et al. 1999), interfere with the organisation of the mitotic spindle and lead to elevated levels of merotelic attachments, lagging chromosomes and ultimately, chromosome missegregation (Elhajouji et al. 1997; Thompson and Compton 2008). Chemical or genetic inhibition of SAC function in human cells using drugs like reversine and AZ3146, which both inhibit the crucial SAC effector kinase, Mps1 (Hewitt et al. 2010; Santaguida et al. 2010), and reduced expression of the mitotic checkpoint complex member Mad2 (Michel et al. 2001), or of the kinase Bub1 (Bernard et al. 1998; Meraldi and Sorger 2005), also leads to the generation of aneuploid cells and, similarly to spindle poisons are suitable for studying the immediate consequences of aneuploidization (recently, for example in (Santaguida and Amon 2015)).

Transient tetraploidization, which can be achieved by inhibition of cytokinesis, rapidly leads to gross defects in chromosome segregation and to the generation of cells with near-tetraploid

karyotypes (Ho et al. 2010; Vitale et al. 2010; Kuznetsova et al. 2015). Aneuploid cells generated in this way are often highly chromosomally instable (Ho et al. 2010; Vitale et al. 2010; Kuznetsova et al. 2015).

A more targeted method for generating aneuploid mammalian cells is known as microcell-mediated chromosome transfer (Fournier and Frelinger 1982; Saxon and Stanbridge 1987; Killary and Lott 1996). In this technique, a donor cell line (usually MEFs) are engineered to carry an extra human chromosome that harbours an antibiotic selection marker. Prolonged mitotic arrest of these donor cells leads to breakdown of the nuclear envelope and to the formation of micronuclei which engulf single or small collections of chromosomes. These micronuclei can then be selectively isolated and introduced to cultures of recipient cells in the presence of polyethylene glycol to induce membrane fusion. The subsequent application of the antibiotic ensures that only those cells that have taken up the extra chromosome survive and continue to proliferate. Because of the selection period, this method is not suitable for studying the acute effects of aneuploidization. However, it holds two important advantages: first, the effects of aneuploidy per se can be easily deduced from comparison with the control cell line that did not receive any chromosomes. Secondly, the aneuploid cells that are generated in this way are relatively chromosomally stable (depending of course on the identity of the recipient cell line), allowing analysis of the long-term effects of an aneuploid karyotype. In the work presented here, my colleagues and I utilised this method to study the effects of the gain of one or two chromosomes in two near-diploid human cell lines: the colorectal cancer cell line HCT116 and the immortalised but untransformed retinal pigment epithelium cell line RPE-1.

The above-described techniques, developed to generate and study aneuploid cells, have greatly facilitated research into the consequences of aneuploidy in different eukaryotic organisms. These techniques have accelerated research in a number of ways: firstly; the possibility, especially in haploid budding yeast, of generating disomic strains carrying an extra copy of each (or most) of the organism's 16 chromosomes has enabled researchers to distinguish chromosome-specific effects of aneuploidy from those that are shared by aneuploid cells of diverse karyotypes; secondly, the fact that these methods are now being applied to unicellular yeast as well as to mammalian cells means that the evolutionarily conserved effects of aneuploidy can be discerned. Finally, as well as generally facilitating research into the effects of imbalanced karyotypes, the ability to easily generate aneuploid cells *de novo* means that the consequences of chromosome mis-segregation, i.e. acute aneuploidization, can be distinguished from sustained aneuploidy. This is important as it is now clear that these phenomena differ in their effects, both qualitatively and quantitatively.

7.3 Consequences of aneuploidy

7.3.1 Immediate effects of chromosome mis-segregation

The immediate effects of chromosome mis-segregation are often drastic and, as first reported in three independent studies, converge on the guardian of the genome, the tumour suppressor p53 (Li et al. 2010; Thompson and Compton 2010; Janssen et al. 2011).

In two of these studies, p53 activation was found to arrest the proliferation of the mis-segregating cells (Thompson and Compton 2010; Janssen et al. 2011), whereas in the other, p53 activation led to apoptosis (Li et al. 2010). This discrepancy may be due to the fact that the degree of p53 activation seems to correlate with the degree of mis-segregation (Li et al. 2010). Interestingly, however, no clear picture has emerged for how exactly p53 is activated under these circumstances at the molecular level. In the first study, cells were induced to mis-segregate their chromosomes by either monastrol washout treatment or by depletion of the centromere-associated kinesin-13 protein MCAK. In both cases, total levels of p53 and its target p21 were elevated in the treated cells and a nuclear accumulation of both proteins could be observed. Based on the lack of any staining for γ-H2AX, a marker of DNA double-strand breaks, the authors concluded that the p53 activation observed in response to chromosome mis-segregation is not due to DNA damage. Instead, the authors found that the stress-activated p38 MAK kinase is involved (Thompson and Compton 2010).

In the second study, in which MEFs were rendered aneuploid by depletion of several SAC components, it was found, through direct measurements of reactive oxygen species (ROS) as well as quantification of oxidative damage to DNA, that aneuploidization leads to oxidative stress. Based on knockdown experiments and the use of ROS scavengers the authors proposed that the p53 activation was due to the generation of these ROS, which then activated the ATM kinase (Li et al. 2010).

In the third study, it was revealed that lagging chromosomes that are mis-segregated in mitosis are often damaged during cytokinesis as a result of forces generated by the cleavage furrow, leading to the activation of ATM, Chk2 and p53 and cell cycle arrest in G1 (Janssen et al. 2011). In addition, the incorporation of lagging chromosomes into micronuclei, an environment in which chromosomes experience problems in properly replicating their DNA and therefore accrue high levels of DNA damage (Crasta et al. 2012), might also contribute to activating p53 upon defective chromosome segregation. Further, a recently published study reported high levels of replication stress and DNA damage upon centromere-associated protein-E (CENP-E)

knockdown-induced chromosome-mis-segregation in SAC-deficient cells concomitant with the activation of p53 (Ohashi et al. 2015).

It seems possible, therefore, that different types of DNA damage might converge on activation of p53 in response to chromosome mis-segregation. In fact, cells that lose chromosomes were described to activate p53 to the same extent as cells that gained an extra chromosome, suggesting that the DNA damage suffered by lagging chromosomes is not required for p53 activation in aneuploid cells (Thompson and Compton 2010). Further, a very recent study has now shown that p53 activation in response to chromosome mis-segregation can occur without any detectable DNA damage (Hinchcliffe et al. 2016). The authors of this study found that during anaphase, misaligned chromosomes undergo phosphorylation at position Ser31 on the histone variant H3.3, which persists into the subsequent G1 phase of the cell cycle. This phosphorylation along the arms of mis-segregated chromosomes signals to activate p53, by a mechanism which remains to be defined (Hinchcliffe et al. 2016). In conclusion, several mechanisms may be responsible for activating p53, and thus blocking the proliferation of cells that have undergone chromosome mis-segregation. Perhaps the key issue to be resolved is the relative importance of these different mechanisms for eliciting cell-cycle arrest upon aneuploidization.

What are the other immediate effects of chromosome mis-segregation? Altered activity of the protein homeostasis (proteostasis) network appears to be a general and evolutionarily conserved feature of aneuploid cells (discussed in detail below). Interestingly, recent studies indicate that proteostasis is affected very soon after chromosome mis-segregation in aneuploid cells (Oromendia et al. 2012; Santaguida and Amon 2015). In yeast, cells that underwent chromosome mis-segregation during mitosis as well as the viable products of triploid meiosis were described to rapidly accumulate foci positive for the heat shock protein Hsp104, a disaggregase and a marker of protein aggregates (Oromendia et al. 2012). Further, it has been reported that a defect in autophagic degradation represents a striking early feature of human cells that have undergone chromosome mis-segregation and become aneuploid (Santaguida and Amon 2015). Using inhibitors of Mps1 as well as siRNA against Bub1 or Mad2 to generate aneuploid human cells, the authors observed a decrease in autophagic degradation and an accumulation of proteins within lysosomes in aneuploid cells two to three cell divisions after the chromosome mis-segregation event. The exact reasons for this are unclear, but seem to stem from an inability to proper upregulate the degradative capacity of the lysosome to match an increased requirement for protein degradation (Santaguida and Amon 2015).

Taken together, the findings discussed above suggest that wild-type cells that undergo chromosome mis-segregation must find a way to both bypass the p53-mediated block in

proliferation, as well as to dampen the detrimental effects of aneuploidy on proteostasis, in order to survive and to continue to divide. What is the fate of aneuploid cells that succeed in overcoming the immediate detrimental effects of an imbalanced karyotype?

7.3.2 Chronic consequences of aneuploidy

While the immediate consequences of aneuploidization are likely to be determined in part by the chromosome mis-segregation event itself, in the long term the effects of aneuploidy on cellular physiology are mediated solely by the expression of genes encoded on aneuploid chromosomes. As a general rule, mRNA and protein levels both scale with DNA copy number in aneuploid cells from various species (e.g., (Torres et al. 2007; Torres et al. 2010; Stingele et al. 2012) exceptions will be discussed below). That, in fact, the gene expression is responsible, has been demonstrated in several studies in yeast harbouring artificial aneuploid chromosomes that do not encode any yeast proteins. Crucially, such chromosomes have only mild effects on cell function (Torres et al. 2007; Sheltzer et al. 2011; Oromendia et al. 2012).

Why is the expression of genes from aneuploid chromosomes detrimental to cellular fitness? A highly influential framework in which to tackle this question has been the gene dosage balance hypothesis (GDBH (Veitia and Potier 2015)). This explanation for the adverse effects of altered gene dosage was first developed by Birchler and Newton (Birchler 1979; Birchler and Newton 1981), and postulates that disturbing the balanced expression of subunits of macromolecular complexes or of proteins involved in signalling networks is harmful, as it can affect the amount of functional product that is synthesised, or in the case of signalling pathways, because of altered activity of the pathway (Birchler and Veitia 2012).

Several lines of evidence support the idea that the imbalanced gene expression of aneuploid cells is the factor that disrupts cellular function. On the most fundamental level, this notion gains credence from the fact that polyploidy is often better tolerated in nature than aneuploidy (Comai 2005). Further, in humans, only three trisomic karyotypes (13, 18 and 21) are compatible with survival and post-embryonic development and only one (trisomy 21) allows individuals to survive past a few months of age (Pai et al. 2003). Strikingly, according to the annotated reference human genome published by the European Bioinformatics Institute (EBI) and Wellcome Trust Sanger Institute, chromosomes 13, 18 and 21 are the most gene-poor autosomal chromosomes in humans, with chromosome 21 the most gene-poor of all. Indeed, in many studies the severity of aneuploid phenotypes tends to scale with the degree of chromosomal imbalance in a given aneuploid cell. This fact is exemplified in two evolutionarily distant species. In aneuploid yeast, the addition of an extra chromosome to a haploid yeast strain (resulting in 100% more of the affected genes) has much stronger effects than addition of

a chromosome to a diploid background (which results in only 50% more of the genes) (Torres et al. 2007; Sheltzer et al. 2011; Oromendia et al. 2012). Similarly, in maize, the addition of an extra chromosome to a diploid plant has much less severe effects on growth than adding a chromosome to a haploid (described in (Birchler and Veitia 2012).

The most obvious effects of aneuploidy stem directly from the increased expression of the specific genes encoded on aneuploid chromosomes, i.e. the identities of the specific gene products present in excess determine the phenotype. Indeed, global analyses of gene expression have revealed that protein as well as RNA levels tend to scale with DNA copy number in aneuploid cells (Upender et al. 2004; Pavelka et al. 2010b; Torres et al. 2010; Stingele et al. 2012; Dephoure et al. 2014; Durrbaum et al. 2014). For instance, the resistance of yeast that are disomic for chromosome XIII to the pro-tumourigenic compound 4-nitroquinoline-N-oxide (4-NQO) could be narrowed down to the enhanced expression of the ATR1 gene (Pavelka et al. 2010b), which is encoded on chromosome XIII and is a protein transporter whose overexpression is described to confer resistance to 4-NQO (Mack et al. 1988). Humans with DS are significantly protected from developing solid tumours (Hasle 2001), while at the same time exhibit a markedly elevated risk of succumbing to Alzheimer's disease (Wiseman et al. 2015). Evidence suggests that the former may be partly because of the enhanced expression of the DSCR1 gene (Baek et al. 2009), which is encoded on chromosome 21 and which is a factor which potently suppresses angiogenesis by inhibiting the calcineurin pathway (Ryeom et al. 2008); the latter is likely to be at least partly because DS individuals harbour an extra copy of the chromosome 21-encoded amyloid precursor gene, *APP* (Goate et al. 1991).

While genes encoded on supernumerary chromosomes are generally expressed at levels that correspond to their copy number, it appears that the expression of certain classes of genes, namely, members of macromolecular complexes (Torres et al. 2010; Stingele et al. 2012; Dephoure et al. 2014), as well as kinases (Stingele et al. 2012), is adjusted towards diploid levels at the protein level. While experiments performed in aneuploid yeast indicate that these proteins are degraded shortly after synthesis in a proteasome- and autophagy-dependent manner (Dephoure et al. 2014), the underlying reasons for these observations are not clear. We have previously proposed that the impaired proteostasis of aneuploid cells (discussed in detail below), may play a role in this phenomenon (Donnelly and Storchova 2014), but this hypothesis has yet to be rigorously tested.

The direct effects that can be exerted by gene products encoded on supernumerary chromosomes also illustrate that aneuploidy is not always detrimental. Aneuploidy may be selectively neutral, as suggested by the fact that many naturally occurring yeast strains are

aneuploid (Kvitek et al. 2008), and can indeed be highly beneficial under specific circumstances. Most examples for the advantageous effects of aneuploidy come from yeasts, where a change in karyotype has been described to mediate resistance to anti-fungal agents (Selmecki et al. 2006; Sionov et al. 2010), a range of cytotoxic drugs (Pavelka et al. 2010b; Chen et al. 2012), as well as hostile environmental conditions (Yona et al. 2012). In many of these instances the beneficial effects of aneuploidy appear to be directly mediated by the altered expression of specific gene products encoded on supernumerary chromosomes (Selmecki et al. 2006; Pavelka et al. 2010b; Sionov et al. 2010). Further, it should be noted that the beneficial effects are generally only evident in response to harsh conditions and do not confer any advantage in the absence of stress. A notable example from other species concerns hepatocytes in both mice and humans (Duncan et al. 2010; Duncan et al. 2012b). Intriguingly, a large proportion of mature hepatocytes in both species have been described to be polyploid or aneuploid and there is evidence to suggest that the altered karyotypes of hepatocytes might play a role in protection against liver injury or the toxic effects of metabolites (Duncan et al. 2012a). The healthy human brain has also been described to harbour appreciable numbers of aneuploid cells (Rehen et al. 2001; Rehen et al. 2005), raising the question of aneuploidy's role during normal development and metabolism (Iourov et al. 2006). It should be noted, however, that the presence of aneuploid cells in healthy human tissue remains controversial (Knouse et al. 2014).

The identity of the gene products encoded on aneuploid chromosomes evidently plays a role in determining the phenotypes of aneuploid cells. However, research on aneuploidy has, over the last decade, demonstrated that many of the important characteristics of aneuploid cells are independent of the exact karyotypic changes found in a given aneuploid cell. The remarkable implication of this discovery is that while the phenotypes of aneuploid cells depend on the expression of genes encoded on aneuploid chromosomes, they are independent of the identity and function of the genes themselves.

What are these changes evoked by aneuploidy? Perhaps the most common feature of aneuploid cells, and one that is conserved in aneuploidy models from yeast to man, is an impairment in proliferation, a defect which appears to stem from problems in progression through both the G1 and S phases of the cell cycle (Torres et al. 2007; Williams et al. 2008; Pavelka et al. 2010b; Stingele et al. 2012). Remarkably, while this defect was one of the first cellular phenotypes to be attributed to aneuploidy (Segal and McCoy 1974), at the molecular level it still remains largely unclear why aneuploid cells proliferate poorly (Thorburn et al. 2013). What appears clear, at least in yeast, is that aneuploidy-induced defects in proliferation are not due to the altered expression of any particular dosage-sensitive genes, but rather reflect the combined action of

many genes together (Bonney et al. 2015). Whether and how this impaired proliferation is linked with other phenotypes of aneuploid cells are questions that await definitive answers.

In recent years, several ground-breaking studies, performed in different species and analysing different aneuploid karyotypes, have demonstrated that aneuploidy affects mRNA and protein expression globally, and not only at the level of those genes whose copy number is altered (Upender et al. 2004; Torres et al. 2007; Pavelka et al. 2010b; Torres et al. 2010; Sheltzer et al. 2012; Stingele et al. 2012; Durrbaum et al. 2014). It should be noted, of course, that a proportion of the gene expression changes occurring in *trans* in aneuploid cells is a direct result of changes that occur in *cis*, e.g., the presence of a transcriptional regulator on an aneuploid chromosome driving the expression of target genes located on other chromosomes (e.g., (Rancati et al. 2008)).

While earlier studies had reported that gene expression was altered genome-wide in response to aneuploidy (Upender et al. 2004), the first systematic analysis of global expression changes in aneuploid cells was undertaken by Torres et al. in 2007 (Torres et al. 2007). They discovered a transcriptional response common to aneuploid yeast of different karyotypes which bore a similarity to the environmental stress response (ESR), a transcriptional response mounted by yeast in response to a range of different stressful conditions (Gasch et al. 2000). Intriguingly, this response was determined in part by the impaired proliferation of aneuploid cells because when disomic yeast were grown in a chemostat under phosphate-limiting conditions to control their rate of division, the ESR was no longer evident (Torres et al. 2007). Since then, a number of other global studies performed in yeast, murine and human aneuploid cells have further characterised the transcriptome and proteome of aneuploid cells and a clearer picture of the effects of aneuploidy on gene expression has now emerged (Pavelka et al. 2010b; Torres et al. 2010; Sheltzer et al. 2012; Stingele et al. 2012; Dephoure et al. 2014; Durrbaum et al. 2014). Strikingly, a broad conservation of the gene expression changes observed in aneuploid cells from yeast to man can be discerned. This conservation is particularly evident in the downregulated pathways at both the transcriptional and proteomic levels and manifests as a downregulation of DNA and RNA metabolism as well as ribosome-related and cell cycle-related pathways (Sheltzer et al. 2012; Stingele et al. 2012; Durrbaum et al. 2014). More variation is evident in the pathways that are commonly found to be upregulated in aneuploid cells. As mentioned above, aneuploid *S. cerevisiae* activate the ESR (Torres et al. 2007), while at the protein level the response is characterised by a prominent enrichment in factors related to the cellular response to oxidative stress (Dephoure et al. 2014). Variation between the response of mouse and human aneuploid cells is also evident. Pathways related to the extracellular region as well as inflammatory and stress responses are upregulated in model aneuploid cells from

both species (Sheltzer et al. 2012; Stingele et al. 2012; Durrbaum et al. 2014), whereas factors related to lysosomes, vacuoles and membrane metabolism have been described to be enriched only in human aneuploids until now (Stingele et al. 2012; Durrbaum et al. 2014).

As mentioned above, impaired proliferation plays a role in determining the transcriptional response to aneuploidy in yeast (Torres et al. 2007; Sheltzer et al. 2012). However, complex human aneuploid cells recovered after transient tetraploidization exhibit no gross defects in proliferation, yet display the same transcriptional signature as slowly-proliferating trisomic and tetrasomic aneuploid cell lines (Durrbaum et al. 2014). Thus, there must be additional triggers for the changes in gene expression in human aneuploid cells. The observation that in human cells aneuploidy elicits upregulation of factors related to lysosomal degradation coupled with the fact that the transcriptional response of aneuploid cells bears a strong resemblance to cells in which autophagy has been inhibited indicates that the effects of aneuploidy on proteostasis may be partially responsible for the gene expression changes in aneuploid cells (Stingele et al. 2012; Durrbaum et al. 2014).

An additional as yet poorly understood effect of aneuploidy on cellular physiology relates to changes in cellular metabolism, in particular those that concern mitochondrial respiration. As in the case of proliferation, the first indications that aneuploidy may affect metabolism came from studies on DS fibroblasts (Kedziora and Bartosz 1988). Many studies have now documented increased levels of reactive oxygen species (ROS) in these cells (reviewed in (Pagano and Castello 2012)), and recent evidence suggests that this observation is not restricted to trisomy of human chromosome 21, as other human aneuploid cells as well as aneuploid yeast also harbour elevated levels of ROS (Li et al. 2010; Dephoure et al. 2014). However, the source of these ROS is not well understood. Further evidence for altered metabolism in aneuploid cells comes from global expression profiling. Aneuploid yeast exhibit up-regulation of pathways related to carbohydrate metabolism (Torres et al. 2007), and analysis of human aneuploid cells revealed a uniform up-regulation of pathways involved in energy metabolism, in particular those related to mitochondrial respiration and carbohydrate metabolism (Stingele et al. 2012). Aneuploid yeast cells exhibit a decreased efficiency of glucose utilisation (Torres et al. 2007), whereas in trisomic MEFs glutamine consumption as well as the production of its breakdown product, ammonium, is increased. Lactate production seems to be also increased in these trisomic cells (Williams et al. 2008). The underlying reason and significance of these effects is unclear. Finally, the fact that these same trisomic MEFs are sensitive to the AMPK inhibitor AICAR represents additional indirect evidence for changes to metabolism in aneuploid cells (Tang et al. 2011).

Thus, while our understanding of the consequences of aneuploidy remains far from complete, some important principles can already be discerned. Firstly, it is clear that aneuploidy, while generally detrimental can, under stressful conditions, play an important cytoprotective role. Secondly, it has become obvious that aneuploidy exerts karyotype-dependent as well as karyotype-independent effects. These karyotype-independent effects include an evolutionarily conserved expression pattern of up- and downregulated pathways in which the most prominent features are a lower expression of factors involved in nucleic acid metabolism and protein synthesis and heightened levels of gene products related to stress responses and protein degradation. In addition, aneuploidy has profound effects on cellular metabolism. The underlying reasons for the karyotype-independent effects of aneuploidy remain poorly understood at the cellular and molecular levels; therefore, elucidating the molecular mechanisms that determine these phenotypes of aneuploid cells must be regarded as one of the main tasks of research on aneuploidy. The research presented here was undertaken with the aim of better understanding the karyotype-independent effects of aneuploidy in human cells.

7.4 Role of aneuploidy in human disease

7.4.1 Trisomy syndromes

The proof that aneuploidy is directly linked to human disease came with the discoveries almost 60 years ago that DS, Edward's syndrome and Patau syndrome are due to third copies of chromosomes 21, 18 and 13, respectively (EDWARDS et al. 1960; PATAU et al. 1960; LEJEUNE and TURPIN 1961). It is now clear that aneuploidy represents the main cause of spontaneous abortions in humans. Further, complete or mosaic trisomy of chromosomes 21, 13 and 18 and aneuploidy of sex chromosomes, are the only aneuploid karyotypes that are compatible with live birth. Individuals with any of three autosomal trisomies suffer from profound developmental defects, which include intellectual deficits as well as a wide range of physical abnormalities (Pai et al. 2003). Research on trisomy syndromes has focused on two broad questions: firstly, scientists have striven towards an understanding of the meiotic defects that lead to aneuploidy in humans, with a particular focus on elucidating the basis for the age-related increase in these errors (reviewed in (Hassold and Hunt 2001; Nagaoka et al. 2012)); secondly, researchers have attempted to understand the pathology of trisomy syndromes, mostly by linking specific aspects of the pathology of trisomy syndromes with the elevated expression of specific genes or chromosome regions (Antonarakis et al. 2004; Lana-Elola et al. 2011).

The majority of efforts with respect to the second question have been concerned with DS as it is the most common human trisomy and the only one which, generally speaking, permits survival beyond the first weeks and months of life. The chromosome 21-centric approach has utilised both rare cases of partial human trisomies as well as several mouse models of DS to identify "dosage-sensitive" genes on chromosome 21 that are likely to make critical contributions to DS phenotypes ((reviewed in (Lana-Elola et al. 2011)), and a DS Critical Region (DSCR), a region on chromosome 21 spanning approximately 5.4 Mb and encompassing 33 genes, has been proposed to account for most, if not all, of the major symptoms in DS individuals (Korenberg et al. 1990; Delabar et al. 1993; Belichenko et al. 2009). However, recent studies have called into question the pre-eminent role of the DSCR in determining the majority of DS phenotypes (Olson et al. 2004; Korbel et al. 2009; Lyle et al. 2009). More generally, it is far from clear whether approaches focused purely on chromosome 21 will allow us to reveal the basis for all of the many symptoms in DS individuals and the reasons for the large variation in DS phenotypes.

7.4.2 Role of aneuploidy in other conditions

As individuals with DS now have much greater life expectancies than previously (e.g., (Englund et al. 2013; Wu and Morris 2013), it has become increasingly apparent that trisomy of 21 is associated with a significantly elevated risk of developing further health complications, in an age-dependent manner (Glasson et al. 2014).

Foremost among the age-associated conditions linked to DS is Alzheimer's disease: individuals with DS exhibit a significantly elevated risk of developing Alzheimer's disease and dementia (Wiseman et al. 2015). In fact, the development of characteristic amyloid plaques appears to exhibit universal penetrance by the age of 40 and two-thirds of DS individuals that live until the age of 60 develop dementia (Zigman et al. 2002; McCarron et al. 2014). These striking phenotypes are likely to be, in large part, due to the fact that chromosome 21 harbours the amyloid precursor gene *APP* (Goate et al. 1991), and, by itself, three copies of *APP* seems to be sufficient to lead to early-onset Alzheimer's disease (e.g., (Rovelet-Lecrux et al. 2006; Sleegers et al. 2006)). However, whether the extra copy of *APP* is the only underlying reason for the increased AD in DS individuals, remains to be elucidated (Oromendia and Amon 2014), as mouse models of DS which lack an extra copy of *APP* also exhibit cognitive defects, as well as hyperphosphorylation of tau, a key characteristic of AD pathology (Shukkur et al. 2006; Roubertoux and Carlier 2010).

7.4.3 Aneuploidy and aging

The short lifespans of individuals with DS together with the fact that aneuploidy causes decreased fitness at the cellular level suggests that aneuploidy may also play a more general role in aging. A growing body of work carried out in cell culture systems as well as in mice indicates that aneuploidy may indeed accelerate the aging process and/or be a hallmark of natural aging. For instance, a recent study has shown that replicative lifespan is decreased in aneuploid baker's yeast (Sunshine et al. 2016). Evidence from mouse models with hypomorphic alleles of the critical SAC proteins Bub3, Rae1 and BubR1 has shown that such mice develop a range of age-associated phenotypes, including short lifespan, cataracts and impaired wound healing (Baker et al. 2004; Baker et al. 2006). Further studies have indicated that aneuploidy-induced aging may particularly affect skin cells in mice (Foijer et al. 2013; Tanaka et al. 2015). Mosaic variegated aneuploidy (MVA) syndrome in humans is a condition, which is often associated with cancer but which also presents with aging-associated phenotypes (Jacquemont et al. 2002; García-Castillo et al. 2008). In addition, aneuploidy may be a hallmark of aging in the mouse (Faggioli et al. 2012; Baker et al. 2013), a phenomenon which is accompanied by a drop in BubR1 levels and which can be blocked by BubR1 overexpression (Baker et al. 2013). Finally, a number of works also report an increase in aneuploidy in the brains of aged humans (reviewed in (Andriani et al. 2016)).

Thus, the research performed up to now appears to implicate aneuploidy in the aging process and suggests that CIN leading to whole chromosomal aneuploidy can contribute to aging. A key open question concerns the exact nature of the relationship between aneuploidy and aging in healthy individuals: is aneuploidy merely a consequence of normal aging or does it actively accelerate the aging process? It is noteworthy that two of the most prominent detrimental effects of aneuploidy, namely impaired abilities to maintain the integrity of the genome and proteome, have been proposed to be hallmarks of aging (Lopez-Otin et al. 2013). Taken together, it seems likely that impaired fidelity of chromosome segregation, due for example, to decreased expression of BubR1, is not only a consequence of the aging process, but may also, through the effects of aneuploidy on cellular physiology, be a mechanism underlying normal aging.

7.4.4 Aneuploidy and cancer - friend or foe?

The relationship between CIN, aneuploidy and carcinogenesis is complex and conflicting. On the one hand, CIN and aneuploidy are very frequent in cancer; many cancers show defects in chromosome segregation and up to 90% of solid tumours and 50% of haematological malignancies exhibit imbalanced karyotypes (Mitelman et al., 2016)(Storchova and Pellman 2004). On the other hand, CIN, as well as the aneuploid state itself, almost always results in

decreased fitness in model systems. It is worth stressing here again that CIN and aneuploidy are not synonymous. While aneuploidy in cancer is generally a result of CIN, not all aneuploid cells are karyotypically instable. Further, as discussed above, the effects of CIN, i.e., the ongoing gain and loss of chromosomes are distinct from those of aneuploidy, which are due to altered gene dosage.

One scenario in which the seemingly contradictory roles of CIN and aneuploidy in cancer could be reconciled would be one in which they exerted differing effects on tumour initiation and progression. This seems to be true to some extent. On the one hand, CIN leading to aneuploidy can contribute to initiation of tumorigenesis *in vivo* (Weaver et al. 2007); on the other hand, chromosome mis-segregation in tumours that are induced by other means, such as loss of tumour suppressors or by chemicals, appears to rather exert suppressive effects (Weaver et al. 2007). The rate of chromosome mis-segregation also appears to be an important determinant. Further exacerbating CIN in cancer-prone mice that are heterozygous for CENP-E results in reduced tumour formation (Silk et al. 2013). In several types of cancer, an intermediate rather than extreme level of CIN results in the poorest prognosis for cancer patients (Birkbak et al. 2011). In addition, high rates of chromosome mis-segregation may suppress cancers in certain tissues while promoting them in others. For example, mice prone to chromosome mis-segregation because of CENP-E heterozygosity are predisposed to develop tumours in the spleen and lungs. However, these same mice develop liver tumours at a reduced rate compared to wild-type mice (Weaver et al. 2007). Further, mice with mutations that compromise the fidelity of chromosome segregation are generally prone to cancers, but not in all organs (reviewed in (Giam and Rancati 2015; Simon et al. 2015). Additional support for this idea comes from individuals with DS. The incidence of solid tumours is greatly reduced in people with DS, while the occurrence of haematological cancers is increased (Nižetić and Groet 2012). Taken together, these observations suggest that there is an optimal timing as well as level of CIN and aneuploidy that promote the initiation and development of cancer. Beyond a certain threshold or in cells that are already malignant, the negative effects of acute chromosome mis-segregation as well as the longer-term detrimental effects of an imbalanced karyotype combine to effectively inhibit tumour progression.

While these considerations can potentially reconcile the detrimental effects of CIN and aneuploidy with their pervasiveness in cancer, they still do not answer the question of how CIN and aneuploidy themselves may actually promote tumourigenesis. The most likely explanation for the role of CIN in promoting cancer is that the aneuploid karyotypes generated as a result of mis-segregation will, on rare occasions, lead to improved proliferative potential as well as the variation necessary for adaptation to hostile micro-environmental conditions and

chemotherapy. But how can aneuploidy *per se* promote malignancy? Indeed, it should be noted that the theory that aneuploidy can contribute to cancer does not enjoy universal acceptance, and it has been argued that aneuploidy is either irrelevant or simply a bystander in tumourigenesis (Hahn et al. 1999; Marx 2002). As discussed above aneuploidy exerts both karyotype-dependent and karyotype-independent effects on cellular physiology. Applying this framework, it is likely that there are two broad ways in which aneuploidy could contribute to carcinogenesis. In the first instance, it is probable that, because of the precise set of genes present, certain karyotypes are more likely to promote tumourigenesis whilst others rather exert tumour-suppressive effects. For instance, gain of chromosome 8, or its long arm is one of the most frequent karyotypic abnormalities in cancer and is thought to be driven by the presence of the *MYC* oncogene on 8q24.3 (Sato et al. 1999; Mahdy et al. 2001; Beroukhim et al. 2010; Jones et al. 2010). Further, specific types of cancer are often characterised by specific recurrent karyotypic abnormalities (Mitelman 2000), and, the loss or gain of specific chromosome pairs is found to exhibit a significant co-occurrence (Ozery-Flato et al. 2011). Moreover, compelling evidence suggests that the distribution of tumour suppressors, oncogenes and essential genes on a given chromosome is a strong predictor of whether that chromosome is likely to be gained or lost in cancer (Davoli et al. 2013). It is also possible that aneuploidy can promote tumourigenesis in a manner which is independent of the exact karyotype. Given that these karyotype-independent effects are largely detrimental, this might seem paradoxical. The most likely scenario in which aneuploidy is likely to contribute to carcinogenesis in this way, is through the effects that it exerts in further destabilizing the genome (discussed in detail below). While such effects on genome stability are detrimental in the majority of cases, the chronically elevated rate of mutations and also additional large-scale structural changes in aneuploid cells are likely, in rare cases, to lead to the emergence of cells with proliferative advantages and other requisites for tumour initiation. While such a route leading from aneuploidy to tumorigenesis has long been postulated (Duesberg et al. 2004; Duesberg et al. 2006), a precise delineation of this path has been hampered by the lack of molecular understanding of how aneuploidy affects genome stability.

7.5 The effects of aneuploidy on the proteostasis network

As mentioned briefly above, aneuploidy exerts profound effects on the maintenance of proteostasis. In fact, it is becoming clear that the detrimental effects of aneuploidy are mediated, in part, by the altered function of proteins and pathways that serve to keep the balance between

protein production and folding, on the one hand, and protein degradation, on the other (Oromendia and Amon 2014).

7.5.1 The proteostasis network

The proteostasis network (PN) regulates all stages of the protein life-cycle from production to proteolysis and thus encompasses all factors involved in protein synthesis, trafficking, stability and degradation (Balch et al. 2008). In eukaryotes, highly elaborate signalling networks respond to and cater for the specialised needs of the proteome across the different compartments of the cell and function to ensure that PN capacity matches cellular requirements (Anckar and Sistonen 2011; Walter and Ron 2011; Haynes et al. 2013). Molecular chaperones are proteins that function to promote the proper folding, stability and function, as well as timely and efficient degradation of other cellular proteins. Thus, they represent key players in the PN and accompany proteins "from the cradle to the grave", i.e., immediately upon exit from the ribosome (Preissler and Deuerling 2012), to degradation (Kettern et al. 2010). Eukaryotic cells have evolved a highly sophisticated battery of chaperones that function in different cellular organelles and that are specialised in the folding of different classes of client proteins. The cytosolic HSP90 molecular chaperones (comprising inducible HSP90α and constitutively expressed HSP90β) are among the most important: they are essential for viability and are among the most abundant proteins in eukaryotic cells (Borkovich et al. 1989). In contrast to the HSP70 chaperones, which are rather promiscuous in their interactions and which interact with proteins immediately upon exit from the ribosome tunnel (Rüdiger et al. 1997; Hundley et al. 2005; Vos et al. 2008; Jaiswal et al. 2011), the HSP90 chaperones are more specialised protein folding machines that, together with over 20 co-chaperones, function later in the folding cycle and act on partially folded substrates received from HSP70 (Jakob et al. 1995; Nathan et al. 1997). Intriguingly, aneuploid cells appear to be critically dependent on the HSP90 machinery.

HSP90 proteins are composed of 3 domains, an N-terminal region that binds ATP, a C-terminal domain that mediates homodimerisation of HSP90 and binding to co-chaperones, and an M-domain between these two that assists in ATP hydrolysis (Ali et al. 2006). The chaperone function of HSP90 homodimers is characterised by widespread conformational dynamics, which are determined by ATP binding and hydrolysis, binding to different co-chaperones as well as client proteins themselves (Shiau et al. 2006; Graf et al. 2009; Hessling et al. 2009; Retzlaff et al. 2010; Street et al. 2011). In the absence of ATP binding, HSP90 dimers exist in an "open" or "V-shaped" conformation. The binding of client proteins, often facilitated by chaperones of the HSP70 family together with co-chaperones and subsequently, ATP binding, leads to a series of conformational changes. These result finally, in a closed conformation of the HSP90 dimer, ATP

hydrolysis and client protein release, and the return to the open state (Ali et al. 2006; Shiau et al. 2006). Post-translational modifications (PTMs; to date, phosphorylation, acetylation, ubiquitination, oxidation and S-nitrosylation of HSP90 have been described) also regulate HSP90 function by affecting binding to co-chaperones and substrates, as well as influencing the HSP90 ATPase cycle (reviewed in (Mollapour and Neckers 2012)).

Early studies on HSP90, based on small-scale co-purification experiments, revealed kinases and steroid hormone receptors as two important classes of clients (e.g., (Brugge et al. 1981; Schuh et al. 1985)). In recent years a number of genome-wide studies have revealed that HSP90 has evolved a large but specific clientele of substrates (Millson et al. 2005; Zhao et al. 2005; Taipale et al. 2012). Most prominently, it chaperones a large number of protein kinases, a specificity which is largely determined by the co-chaperone CDC37, which presents kinase clients to HSP90 (Caplan et al. 2007). It also appears to play an important role in the folding of steroid hormone receptors and ubiquitin ligases (Pratt and Toft 1997; Taipale et al. 2012). HSP90 has also been implicated in the assembly and stability of several multi-subunit protein complexes, such as RNA polymerase II, the 26S proteasome, as well as kinetochores, telomeres and transport-related complexes, indicating that the chaperone might play a broad role in regulating protein complex assembly ((McClellan et al. 2007) and reviewed in (Makhnevych and Houry 2012)).

A consequence of the specialised clientele of HSP90 is that HSP90 chaperoning activity is not randomly required for different processes inside the cell but is rather implicated in a number of specific cellular pathways. Notable examples include protein trafficking, progression through the cell cycle, innate immunity and the DNA damage response (DDR) (McClellan et al. 2007; Sharma et al. 2012). The number of HSP90 clients that function in a given pathway is likely to determine why some processes, such as protein secretion are more sensitive to inhibition of HSP90 than others. A further salient feature of HSP90 function, is that, at least in yeast, under conditions of stress the function of HSP90 undergoes a dramatic shift, from playing a prominent role in protein transport and the secretory pathway, to facilitating progression through the cell cycle and cell division (McClellan et al. 2007).

The transcription factor Heat Shock Factor 1 (HSF1) is the master regulator of inducible chaperone expression in the cytoplasm and functions to ensure that cells can regulate protein folding capacity to match fluctuating requirements. Although HSF1 is best recognised for inducing the transcription of molecular chaperones such as members of the HSP70 and HSP90 chaperone families (Anckar and Sistonen 2011), it should be noted that, particularly in cancer

cells, it also regulates the expression of a large number of other genes, including those with no direct involvement in proteostasis (Mendillo et al. 2012).

An elegant mechanism has evolved to couple HSF1 activation to the cellular protein folding status. Under conditions where the chaperone armamentarium matches or exceeds cellular needs, HSF1 is kept in an inactive state by being bound by HSP70 and HSP90. Misfolded or unfolded proteins are thought to titrate away chaperones from HSF1, which allows HSF1 to be converted into an active homotrimer with high affinity for DNA (Baler et al. 1993; Rabindran et al. 1993; Shi et al. 1998; Zou et al. 1998). Homotrimerization and DNA binding are accompanied by hyperphosphorylation (Guettouche et al. 2005; Batista-Nascimento et al. 2011), which regulates the subcellular localization of HSF1 and its affinity for DNA (Xu et al. 2012). Sumoylation and acetylation confer additional layers of regulation by repressing HSF1 activity in the absence of stress (Hietakangas et al. 2003) and by regulating the duration of HSF1 signalling and HSF1 stability, respectively (Westerheide et al. 2009; Raychaudhuri et al. 2014). HSF1 activates or represses the expression of hundreds of genes, among them many molecular chaperones, which serves to ensure that HSF1 signalling is then curtailed once proteostasis has been re-established.

7.5.2 The role of the PN in aging and disease

HSF1 and the Heat Shock Proteins were so named because of their indispensable roles in protecting cells from the adverse effects of heat stress, which results in widespread protein unfolding, misfolding and aggregation. However, as a large range of factors compromise proper protein folding or lead to an enhanced need for Heat Shock Proteins, the PN is involved in shielding cells from the effects of a wide array of toxic insults that lead to proteotoxic stress.

Given the fundamental task that the PN fulfils in cellular physiology, and the central role that cellular stress plays in a large number of pathologies, it is no surprise that the activity of the PN is altered in many disease conditions (Hipp et al. 2014; Dai and Sampson 2016). Two groups of diseases are worthy of particular mention in this regard: the first are cancers, which are generally characterised by a heightened activity of proteostasis factors and by an increased reliance on these factors to sustain carcinogenesis (Dai and Sampson 2016); the second group are neurodegenerative conditions, the hallmark of which is the accumulation of protein aggregates and a marked decline in the function of the PN (Hipp et al. 2014).

Why do cancers rely so heavily on the PN? Alongside the classic hallmarks of cancer, a series of prerequisites, which are critical for the initiation and progression of tumorigenesis (Hanahan and Weinberg 2000), a common characteristic of many established cancer cells is high levels of

cell stress (Solimini et al. 2007; Luo et al. 2009). These stress phenotypes include high levels of DNA damage and chromosomal instability, metabolic stress and proteotoxic stress, and lead to the phenomenon of "non-oncogene addiction" (Luo et al. 2009). Non-oncogenes describe those genes which are not mutated or otherwise activated to promote tumour progression, but rather represent an essential supporting cast critical for maintaining tumourigenesis (Solimini et al. 2007; Luo et al. 2009). Thus, on a fundamental level the increased requirement for PN function in cancer is likely to stem from the role of PN components in mitigating the effects of cell stress.

Growth in environments with fluctuating levels of oxygen and nutrients imposes a severe stress on solid cancers and it has been demonstrated that chaperones play an important cyto-protective role under such conditions. In addition, tumours often depend, for their proliferation and survival, on oncogenes, which are frequently mutated and unstable and thus critically rely on chaperones such as HSP90 (Neckers 2006). Notable examples include HSP90's chaperoning of the v-Src tyrosine kinase and the mutated EGF receptor (Xu and Lindquist 1993; Shimamura et al. 2005). Chaperones also directly contribute to one of the acknowledged hallmarks of malignant cells: their ability to evade apoptosis (Lanneau et al. 2008). For instance, HSP70 and HSP27 impede programmed cell death by blocking the nuclear import of apoptosis-inducing factor (AIF) and through sequestration of cytochrome c, respectively (Bruey et al. 2000; Ravagnan et al. 2001).

Additionally, cancer cells are often critically dependent on processes of protein degradation, which can be clearly discerned from the fact that a large number of tumour cell lines are sensitive to drugs that inhibit proteasomal or autophagic degradation (Adams 2004) (Amaravadi et al. 2011). What underlies this sensitivity? Intriguingly, although the use of proteasome inhibitors in cancer treatment has been touted for approximately 20 years now, there is still no definitive answer to this question. It is likely that many factors are at play. Most generally, the sensitivity of cancer cells to proteasome inhibition appears to be determined by the fact that cells that proliferating cancer cells are more susceptible to a block in proteasomal degradation than post-mitotic cells (Drexler 1997; Masdehors et al. 1999; Drexler et al. 2000). This is probably partly due to the fact that progression through the cell cycle relies on the timely degradation of many critical factors (Glotzer et al. 1991; Pagano et al. 1995). A further reason is related to the general tendency for pro-apoptotic proteins, such as p53, to be short-lived compared to anti-apoptotic ones (Maki et al. 1996; Haupt et al. 1997; Kubbutat et al. 1997). The specific characteristics of certain cancers also play a role in determining sensitivity to proteasome inhibitors. The most prominent example in this respect is multiple myeloma (MM), a cancer of the blood characterised by the aberrant activation and proliferation of antibody-secreting plasma cells. The high levels of protein production and secretion in these activated

cells leads to a heavy reliance on the endoplasmic reticulum stress-activated Unfolded Protein Response (UPR) (Vincenz et al. 2013). Proteasome inhibition in these cells leads to maladaptive hyperactivation of the UPR and subsequently, apoptosis (Obeng et al. 2006), likely contributing to their exquisite sensitivity to proteasome inhibitors (Hideshima et al. 2001; LeBlanc et al. 2002).

The reliance of tumour cells on autophagic degradation stems partly, as for proteasome activity, from the role of autophagy in protecting solid tumours against inhospitable microenvironments (Degenhardt et al. 2006; Rabinowitz and White 2010). Dependence on autophagy has also been strongly linked to cancers driven by the RAS and BRAF oncogenes (Guo et al. 2011; Lock et al. 2011; Strohecker et al. 2013). Autophagy in these contexts appears to be responsible for disposing of defective mitochondria, and for limiting activation of the p53 pathway (Guo et al. 2011; Lock et al. 2011; Strohecker et al. 2013).

A large number of studies have described proteostasis function and HSF1 activity, in particular, to be reduced with age (Labbadia and Morimoto 2015b). Intriguingly, recent studies carried out in the nematode *C. elegans* have demonstrated that proteostasis decline appears to be a precipitous programmed event which occurs suddenly when organisms reach reproductive maturity (Ben-Zvi et al. 2009; Labbadia and Morimoto 2015a). The impaired HSF1 function in aging cells and tissues seems to be caused by a reduced ability of HSF1 to contact DNA (Fawcett et al. 1994; Locke and Tanguay 1996; Jurivich et al. 1997; Kregel 2002); intriguingly, in *C. elegans* this appears to be due to a remodelling of the chromatin landscape to prevent HSF1 binding (Labbadia and Morimoto 2015a).

Diminished capacity of the PN is also a hallmark of one of the main groups of disorders associated with aging, neurodegenerative diseases (Hipp et al. 2014). These conditions are characterised by the accumulation of protein aggregates within affected neurons, leading to their eventual demise. These protein aggregates place a severe strain on cellular proteostasis, by sequestering molecular chaperones and other PN factors (Yamanaka et al. 2008; Olzscha et al. 2011; Xu et al. 2013; Yu et al. 2014), overwhelming the capacity of degradation pathways (Lam et al. 2000; Holmberg et al. 2004; Hipp et al. 2012), and hindering the activation of stress-responsive transcription factors (Labbadia et al. 2011; Olzscha et al. 2011; Riva et al. 2012). Diminished proteostasis function can also be a cause of the protein misfolding and aggregation in such conditions, meaning that affected cells are locked in a vicious cycle of misfolding, aggregation, and progressive worsening of proteostasis capacity (Hipp et al. 2014).

Thus, in cancer, the activity of the PN is stretched to its limits and inhibition of PN function represents a rational therapeutic strategy (Whitesell and Lindquist 2009; Hetz et al. 2013). In

aging and neurodegenerative conditions, conversely, PN capacity is often severely curtailed, and much effort has been directed at augmenting proteostasis in these conditions (Baranczak and Kelly 2016). In conclusion, alterations in PN capacity and activity can be viewed as a defining feature of many pathological states and play critical roles in disease progression.

7.5.3 Effects of aneuploidy on proteostasis

The first hints that aneuploidy might generally affect the maintenance of proteostasis came from the observed general sensitivity of aneuploid yeast to drugs that interfere with protein synthesis, namely, cycloheximide and rapamycin (Torres et al. 2007; Pavelka et al. 2010b). Further, a general sensitivity to heat stress as well as to HSP90 and proteasome inhibition was also observed, indicating that aneuploid yeast are preferentially affected by conditions that lead to high levels of protein unfolding and misfolding and by treatments that block protein degradation (Torres et al. 2007).

More detailed analyses of the effects of aneuploidy on proteostasis in yeast revealed that aneuploid yeast harbour endogenous protein aggregates and are compromised in their ability to deal with the ectopic expression of toxic aggregation-prone proteins. Intriguingly, this study also showed that the function of the HSP90 molecular chaperone seemed to be impaired in aneuploid yeast (Oromendia et al. 2012).

Initial results from trisomic MEFs indicated the generality of these phenomena in mammalian cells by demonstrating that both the HSP90 inhibitor 17-AAG as well as the inhibitor of autophagic degradation, chloroquine, were preferentially toxic to aneuploid mouse cells (Tang et al. 2011). Further, these cells were found to harbour increased levels of the inducible HSP72 chaperone (but not HSP90) and of LC3 II (the lipidated form of LC3 that is inserted into autophagosomes), indicating protein folding stress and altered levels of autophagy, respectively (Tang et al. 2011). Global analysis of gene expression in human aneuploid cells revealed the elevated expression of pathways related to autophagy and the lysosome. Further, higher levels of p62- and ubiquitin-marked foci were observed in trisomic and tetrasomic cells, which were found to co-localize with autophagosomes, indicating that aneuploid human cells upregulate the p62-dependent degradation of ubiquitinated proteins (Stingele et al. 2012; Stingele et al. 2013).

It should be noted here that autophagic degradation has in fact been described to be inhibited in human aneuploid cells immediately after chromosome mis-segregation and to a lesser extent also in trisomic MEFs (Santaguida and Amon 2015). Putting the data together, it can be supposed that in the acute response to aneuploidy, the high levels of protein misfolding and general stress experienced by cells mean that lysosomal capacity initially fails to keep pace with

demand. However, aneuploid human cells eventually do upregulate lysosomal function to match their needs (Stingele et al. 2012).

Taken together, these results indicate that the imbalanced proteomes of aneuploid cells may elicit widespread protein misfolding and aggregation and lead to the (eventual) upregulation of proteasomal and autophagic activity in order to dispose of the misfolded proteins. However, some important questions remain. Firstly, direct evidence that protein folding is impaired in aneuploid cells is still lacking. Further, the molecular pathways involved in upregulating autophagic and proteasomal degradation in aneuploid cells remain unknown. In the work presented here I aimed to better characterise the effects of aneuploidy on proteostasis by focusing on three main questions: Does aneuploidy generally impair protein folding? If so, what underlies this deficient protein folding capacity? How is the impaired protein folding of aneuploid cells linked with the other characteristic hallmarks of aneuploid cells?

7.6 The effects of aneuploidy on the maintenance of genome stability

A number of studies have documented high levels of DNA damage in cells from DS individuals, implying that aneuploidy may exert detrimental effects on genome stability (Zana et al. 2006; Morawiec et al. 2008; Necchi et al. 2015). Indeed, while it is obvious that aneuploidy may arise as a consequence of CIN, a growing body of work from aneuploidy model systems shows that aneuploidy can itself promote further changes to the genome, although the mechanisms involved remain largely unclear. Elucidating the link between aneuploidy and further genomic instability will be an important step towards understanding the role of aneuploidy in pathological conditions.

7.6.1 Aneuploidy and numerical CIN - a two-way street?

The question of whether or not aneuploidy can be a cause as well as a consequence of numerical chromosomal instability is a controversial one and one which is awaiting a definitive answer. It has been argued, most forcibly by Duesberg and colleagues, that aneuploidy is itself the main cause of the high levels of CIN in cancer cells (Duesberg et al. 1998; Duesberg et al. 2004). This assertion is primarily based on the observation that in human cancer cells the degree of chromosomal instability correlates with the deviation from normal ploidy, i.e., the more aneuploid a cell is, the more prone it is to exhibit further chromosomal instability (Duesberg et al. 1998). Analysis of cancer genomes has also shown that tumours with non-diploid karyotypes are more likely to exhibit numerical CIN than diploid tumours (Storchova and Kuffer 2008). This hypothesis is further supported by experiments in yeast that demonstrated that the closer the

karyotype of an aneuploid cell is to the haploid state, the more chromosomally stable it is. It should be noted, however, that the authors also found that the specific aneuploid karyotype also played a role in determining these effects (Zhu et al. 2012). In addition, a study on disomic haploid yeast reported higher levels of chromosome loss in the majority of strains (Sheltzer et al. 2011). The data from mammalian systems is contradictory. A couple of studies have reported that chromosome mis-segregation rates are elevated in trisomic human cells (Reish et al. 2006; Reish et al. 2011), while other reports have found no evidence of this (Upender et al. 2004; Williams et al. 2008). A recent study tackled this question by utilising the precise method of dual-colour fluorescence in situ hybridization (FISH) to examine the chromosomal instability of human cells from patients with trisomies. The authors unambiguously concluded that aneuploidy was insufficient to lead to levels of chromosomal instability comparable to those seen in chromosomally unstable human cancer cell lines (Valind et al. 2013), although it should be noted that it would be expected that in these experiments cells positive for p53 would arrest upon chromosome mis-segregation and thus might be missed from the analysis. Further, a very recent report revealed that while chromosome mis-segregation rates do appear to be elevated in trisomic cancer as well as primary human cell lines, these still lag behind those observed in CIN cancer cells (Nicholson et al. 2015). In accordance with observations from yeast (Zhu et al. 2012), this study suggested that aneuploidy affects chromosome mis-segregation in a karyotype-specific manner (Nicholson et al. 2015).

Taken together, the available data suggest that aneuploidy probably does exert a certain effect in promoting errors in chromosome segregation in a manner, which is likely to depend on the species, on the degree of aneuploidy, as well as on the exact karyotype.

7.6.2 Structural and whole-chromosomal aneuploidy

Whether whole-chromosomal aneuploidy is also connected with the occurrence of structural aneuploidy is even more of an open question. As in the case of whole chromosomal aneuploidy and CIN, structural and whole-chromosomal aneuploidy are often found side-by-side in tumourigenesis, raising the possibility that the phenomena might be linked (Mitelman et al., 2016). The most direct way in which these occurrences might be related would be through the process of chromosome mis-segregation itself. As discussed above, during mitosis the lagging chromosome is thought to be often subject to DNA damage, either as a result of being caught in the cleavage furrow during cytokinesis or because it is trapped in micronuclei (Janssen et al. 2011; Crasta et al. 2012). The accruing damage, either breaks to DNA or in rare cases, complete shattering and subsequent rejoining of an entire chromosome (chromotripsis; (Zhang et al. 2015)) would then give rise to structural aneuploidy on the mis-segregated chromosome. A

recent study found that replication stress, defined as a slowing or stalling of DNA replication fork progression, might be a unifying mechanism linking structural and whole-chromosomal aneuploidy, at least in certain types of cancer (Burrell et al. 2013). Specifically, the authors identified 3 genes on the long arm of chromosome 18 whose deletion precipitated replication stress, structural aneuploidy and finally, chromosome mis-segregation in colorectal cancer cell lines (Burrell et al. 2013). A couple of small-scale studies have also reported elevated levels of structural aberrations in response to trisomy of chromosome 3 or 8 in cultured cells, respectively (Kost-Alimova et al. 2004; Nawata et al. 2011). Intriguingly, in the former case this was hypothesised to be due to defects in DNA replication and specifically, to incomplete replication within pericentromeric regions leading to a higher level of DNA breaks (Kost-Alimova et al. 2004).

Thus, even though recent reports indicate the existence of a link between structural and whole-chromosomal aneuploidy, the nature of this link is far from being fully characterised. Most pointedly, it remains unclear whether aneuploidy *per se* can affect the accrual of further structural changes to the genome and what the underlying molecular mechanisms might be.

7.6.3 Aneuploidy and single-nucleotide genetic aberrations

There is also strong evidence from yeast aneuploids to suggest that aneuploidy can enhance mutation rates, most likely in part by increasing levels of DNA damage. Disomic yeasts are sensitive to drugs which cause DNA damage and were found to harbour a higher load of complex mutational events, which could be suppressed by deletion of the catalytic subunit of the translesion polymerase, Pol ζ (Sheltzer et al. 2011). Consistent with these observations, a majority of aneuploid yeast are impaired in DNA replication and accumulate DNA double-strand breaks (DSB) in S phase, which then persist into the subsequent mitosis (Blank et al. 2015). The DNA damage in aneuploid yeast also appears to be due to defects in DNA repair as well as DNA recombination. Notably, these phenotypes are not simply due to the fact that aneuploid cells have more DNA to replicate: yeasts harbouring YACs with human DNA do not exhibit elevated levels of genomic instability (Sheltzer et al. 2011; Blank et al. 2015).

Whether or not aneuploid cells in higher eukaryotes also exhibit problems with DNA replication and higher levels of DNA damage is not yet clear, but a recent study described that inducing chromosome mis-segregation in SAC-impaired human cells by inhibiting CENP-E resulted in higher levels of DSBs as well as an apparent gross impairment in progression through the S phase of the cell cycle (Ohashi et al. 2015). Moreover, it is striking that pathways related to DNA transactions are consistently downregulated both at the protein as well as the RNA level in human aneuploid cells (Stingele et al. 2012; Durrbaum et al. 2014).

Although scant, the available data suggest that the imbalanced proteomes of aneuploid cells lead to errors during DNA replication and to the generation of DNA lesions. However, the molecular mechanisms underlying these phenomena, particularly in human cells, remain largely uncharacterised.

In the second paper contributing to this thesis my colleagues and I set to determine the effects of aneuploidy on the stability of the genome, and in particular, to answer the questions of whether aneuploidy *per se* may lead to further changes to the genetic material, and if so, by what means.

8. Results

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

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In this study we used luciferase-based protein folding sensors to test the hypothesis that protein folding capacity is reduced in trisomic and tetrasomic aneuploid human cells. Our results revealed striking defects in the ability of aneuploid cells to re-fold these sensors after heat shock or to fold them in the presence of a chemical inhibitor of HSP90, indicating that HSP90 activity is limiting in human aneuploid cells. This hypothesis was further supported by our finding that the proliferation of aneuploid cells is specifically sensitive to HSP90 inhibition, but not to inhibitors of other chaperones or other inducers of protein misfolding. We observed a general reduction in the levels of HSP90 protein in aneuploid cells as well as those of other molecular chaperones, suggesting that a systematic problem in regulating chaperone expression might be an underlying reason for the impaired protein folding capacity of aneuploids. Consistent with this, expression of HSF1, the master regulator of inducible chaperone expression was also generally reduced in aneuploid cells. Overexpression of HSF1, both in an endogenous manner, by transfer of chromosome 8, which harbours the HSF1 locus, or exogenously, by transient plasmid transfection, rescued the levels of HSP90, improved protein folding capacity and protected aneuploid cells from the anti-proliferative effects of HSP90 inhibition. Our analysis strongly suggests that the impaired HSP90 and HSF1 function of aneuploid cells affects their physiology. As a first step we analysed the HSP90-dependent proteome, taking advantage of a recent study that characterised HSP90 clients in a systematic-genome wide manner (Taipale et al. 2012), as well of the database of HSP90 interactors curated by the Picard laboratory (www.picard.ch). We hypothesised that if HSP90 function is indeed limiting in aneuploid cells then we should see effects on the levels of protein that rely on HSP90 for their stability. Indeed, in two out of four aneuploid cell lines tested we observed a significant trend for clients described to interact strongly with HSP90 to be expressed at lower levels at the protein but not mRNA level. When analysing HSP90 interactors we also observed significantly lower protein levels in two out of the four aneuploid cell lines tested, with a non-significant trend towards lower expression in the other two. We also employed pathway analysis to determine if pathways and processes described to be altered in response to chemical inhibition

of HSP90 in HeLa cells (Sharma et al. 2012), were similarly deregulated in aneuploid cells. We observed a pronounced overlap between pathways downregulated in response to HSP90 inhibition and those downregulated in aneuploid cells. In addition, transcriptome analysis revealed a striking similarity between transcriptional changes observed in aneuploid cells and those elicited upon HSF1 depletion.

Taken together, our results show that protein folding is impaired in human aneuploid cells and that this impairment is characterised by specific defects in HSF1 and HSP90 function, which can be rescued by overexpression of HSF1. Further, our results show that the diminished function of HSF1 and HSP90 partially underlies the complex, genome-wide expression changes in mRNA and protein observed in aneuploid cells.

Article

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

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Abstract

Aneuploidy is a hallmark of cancer and is associated with malignancy and poor prognosis. Recent studies have revealed that aneuploidy inhibits proliferation, causes distinct alterations in the transcriptome and proteome and disturbs cellular proteostasis. However, the molecular mechanisms underlying the changes in gene expression and the impairment of proteostasis are not understood. Here, we report that human aneuploid cells are impaired in HSP90-mediated protein folding. We show that aneuploidy impairs induction of the heat shock response suggesting that the activity of the transcription factor heat shock factor 1 (HSF1) is compromised. Indeed, increased levels of HSF1 counteract the effects of aneuploidy on HSP90 expression and protein folding, identifying HSF1 overexpression as the first aneuploidy-tolerating mutation in human cells. Thus, impaired HSF1 activity emerges as a critical factor underlying the phenotypes linked to aneuploidy. Finally, we demonstrate that deficient protein folding capacity directly shapes gene expression in aneuploid cells. Our study provides mechanistic insight into the causes of the disturbed proteostasis in aneuploids and deepens our understanding of the role of HSF1 in cytoprotection and carcinogenesis.

Keywords aneuploidy; cancer; HSF1; HSP90; protein folding
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Introduction

Aneuploidy is defined by karyotypes that differ from multiples of the haploid chromosome set. Aneuploidy is not well tolerated in higher eukaryotes and represents one of the leading causes of spontaneous abortions in humans, with the rare surviving newborns suffering from multiple defects (Colnaghi *et al*, 2011). Moreover, aneuploidy is prevalent in cancer, where nearly 80% of solid tumors and approximately 60% of hematopoietic cancers show karyotypes differing from normal diploidy (Stankiewicz & Lupski, 2010). At the cellular level, aneuploidy is often associated with global pathway deregulation, impaired proliferation, increased energetic and metabolic demands and altered sensitivity to cytotoxic drugs (Upender *et al*, 2004; Niwa *et al*, 2006; Torres *et al*, 2007; Williams *et al*, 2008; Pavelka *et al*, 2010; Nawata *et al*, 2011; Stingele *et al*, 2012). The cellular response to aneuploidy is highly conserved from yeast to human and remarkably, appears to be largely independent of the exact karyotypic composition (Sheltzer *et al*, 2012; Stingele *et al*, 2012; Durrbaum *et al*, 2014). Experiments in budding yeast have shown that the mere presence of a transcriptionally silent additional chromosome does not lead to any apparent phenotypes; hence, taken together, it is the gene expression of the aneuploid genome that determines the phenotypic changes observed in aneuploids (Torres *et al*, 2007). However, in spite of these important insights, the molecular mechanisms underlying these global cellular changes are not yet fully understood.

Recent progress in understanding the cellular effects of aneuploidy was facilitated by analysis of model aneuploid cells with defined karyotypic changes such as aneuploid budding and fission yeast strains, fruit flies with segmental aneuploidy, mouse embryonic fibroblasts with unbalanced Robertsonian translocations and trisomic and tetrasomic human cell lines (Upender *et al*, 2004; Niwa *et al*, 2006; Torres *et al*, 2007; Williams *et al*, 2008; Stenberg *et al*, 2009; Pavelka *et al*, 2010; Nawata *et al*, 2011; Stingele *et al*, 2012). Analysis of disomic budding yeast revealed increased sensitivity to drugs that interfere with protein synthesis and degradation (Torres *et al*, 2007). Moreover, the proliferation defect of some disomies can be ameliorated by mutation of the Ubp6 protein, a deubiquitinating enzyme that was proposed to negatively regulate protein degradation (Torres *et al*, 2007, 2010). In line with these findings in yeast, aneuploidy in murine and human cells imposes profound changes in protein homeostasis (proteostasis). Human trisomic cells show an accumulation of cytoplasmic foci positive for both ubiquitin and SQSTM1/p62, a marker of selective autophagy (Stingele *et al*, 2012). Further, primary trisomic MEFs are sensitive to chemical inhibition of the chaperone HSP90 as well as to the inhibitor of autophagy chloroquine (Tang *et al*, 2011). In agreement with this finding,

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chromosomally unstable aneuploid cancer cell lines are more sensitive to HSP90 inhibition than chromosomally stable cell lines (Tang *et al*, 2011). A recent study demonstrates that aneuploid budding yeast harbor protein aggregates and that protein folding of HSP90 clients is compromised in these cells (Oromendia *et al*, 2012). These results suggest that the protein expression from supernumerary chromosomes places a burden on cellular proteostasis and that the HSP90 machinery might be particularly affected. However, the status of HSP90 function and the protein folding capacity of aneuploid cells in higher eukaryotes were so far unknown.

Here, we demonstrate for the first time that protein folding is significantly impaired by aneuploidy in human cells. Taking advantage of trisomic and tetrasomic human cells that we constructed using micronuclei-mediated chromosome transfer into the human near-diploid colorectal cancer cell line HCT116 and human immortalized retinal pigment epithelial cell line RPE-1 (Stingele *et al*, 2012), we show that in particular HSP90-mediated protein folding is compromised. Intriguingly, we found that HSF1-dependent activation of the heat shock response (HSR) is impaired, suggesting a mechanism by which aneuploidy impairs protein folding capacity. Importantly, endogenous or exogenous overexpression of HSF1 counteracts the effects of aneuploidy on HSP90-dependent protein folding, thereby identifying enhanced expression of HSF1 as the first aneuploidy-tolerating genetic modification in human cells. Finally, we demonstrate that the functional HSP90 and HSF1 deficiency has marked consequences for protein abundance and shapes the patterns of gene expression observed in aneuploid cells.

Results

Trisomic and tetrasomic human cell lines show defects in protein folding

Using model trisomic and tetrasomic human cell lines, we previously found an increased amount of cytoplasmic ubiquitin-positive foci in aneuploids in comparison to cognate diploids (Stingele *et al*, 2012). The accumulation of ubiquitinated proteins might be either due to a defect in their removal or due to their increased production in aneuploid cells. However, human aneuploid cells activate the catabolic pathway of autophagy (Stingele *et al*, 2012), and proteasome activity is not impaired by aneuploidy (Supplementary Fig S1A–C). This suggests that ubiquitinated proteins accumulate at higher rates, possibly due to an overwhelmed or impaired protein folding capacity, as previously proposed (Oromendia *et al*, 2012; Donnelly & Storchova, 2014). To directly test whether trisomic or tetrasomic human cells display protein folding defects, we employed a set of three Firefly luciferase-based sensor proteins comprising wild-type Firefly luciferase (FlucWT) and single and double mutant luciferase, FlucSM and FlucDM, that are highly sensitive to changes in the protein folding environment (Gupta *et al*, 2011). First, we performed luciferase refolding assays after transiently expressing FlucWT in diploid HCT116 cells and HCT116* cells, which stably express histone H2B-GFP, and their respective aneuploid derivatives. To this end, we subjected transfected cells to heat shock at 43°C for 2 h, which is sufficient to denature > 70% of luciferase, but does not result in toxicity. We then monitored refolding at 37°C over 4 h by measuring luminescence. We observed a significant impairment of FlucWT refolding in cells with trisomy and tetrasomy of chromosome 5 compared to their respective parental cell lines (Fig 1A and B). Next, we examined the effect of aneuploidy on the more sensitive mutants FlucSM and FlucDM. The mutations disrupt the stability of the Fluc protein, but do not affect its enzymatic activity. We thus hypothesized that the effect of aneuploidy on luciferase refolding should be even more pronounced in cells transfected with the destabilized mutant proteins. Indeed, the relative ability to refold both FlucDM (Fig 1C and D) and FlucSM (Supplementary Fig S1E and F) was almost threefold reduced in aneuploid cells in comparison to the parental HCT116. Thus, aneuploidy causes protein folding defects in human cell lines.

The HSP90 chaperone is required for the refolding of heat-denatured luciferase, and the destabilizing mutations in FlucSM and FlucDM lead to an indispensable requirement for HSP90 for folding even in the absence of stress (Schneider *et al*, 1996; Gupta *et al*, 2011). Thus, whereas FlucWT activity is not impaired by HSP90 inhibition, the luminescent signal of FlucSM and FlucDM decreases upon treatment with 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), a derivative of the antibiotic geldanamycin that binds the ATP pocket of the chaperone HSP90 (Supplementary Fig S1D). To specifically investigate HSP90-dependent protein folding capacity in aneuploid cells, we transfected diploid and aneuploid HCT116 cells with the Fluc sensors and measured luminescence in response to treatment with 17-AAG. Strikingly, we observed a consistent and significant decrease in FlucDM activity following treatment with 17-AAG in aneuploid cells compared to the control HCT116 cells (Fig 1E). These findings indicate that HSP90 function is indeed limiting in aneuploids. To exclude the possibility that the aneuploidy-induced protein folding defect is specific for aneuploid derivatives of HCT116 or for cells carrying extra copies of chromosome 5, we analyzed two additional aneuploid cell lines derived from RPE-1 (trisomy 21 and trisomy of chromosomes 5 and 12). Similarly as in HCT116-derived aneuploids, we observed consistently lower relative levels of FlucDM activity in aneuploid cells in contrast to diploid RPE-1 cells after treatment with 17-AAG (Fig 1F). These observations provide direct evidence that impaired protein folding and defective HSP90 function are common features of human aneuploid cells.

Aneuploid cells are more sensitive to inhibition of HSP90 but not to other inducers of protein folding stress

To elucidate in more detail the protein folding defect in human aneuploid cells, we analyzed their sensitivity to drugs that either directly inhibit molecular chaperones or impose a severe strain on the protein folding machinery. To this end, we measured cell viability after treatment with the HSP90 inhibitor 17-AAG, the HSC70/HSP70 inhibitor VER 155008, L-azetidine-2-carboxylic acid (AZC), a toxic L-proline analog that leads to the misfolding of newly synthesized polypeptides (Qian *et al*, 2010), and heat shock (45°C, 15 h). We observed a significant sensitivity of trisomic and tetrasomic HCT116 and RPE-1 cells to 17-AAG as measured by both cell viability as well as colony-forming assays (Fig 2A and B). In contrast, the response to the other compounds was less uniform: While aneuploid RPE-1 cells were more sensitive to HSC70/HSP70 inhibition by VER 155008 than diploids, aneuploid HCT116 cells were slightly more resistant to this treatment than controls (Fig 2C). Cell

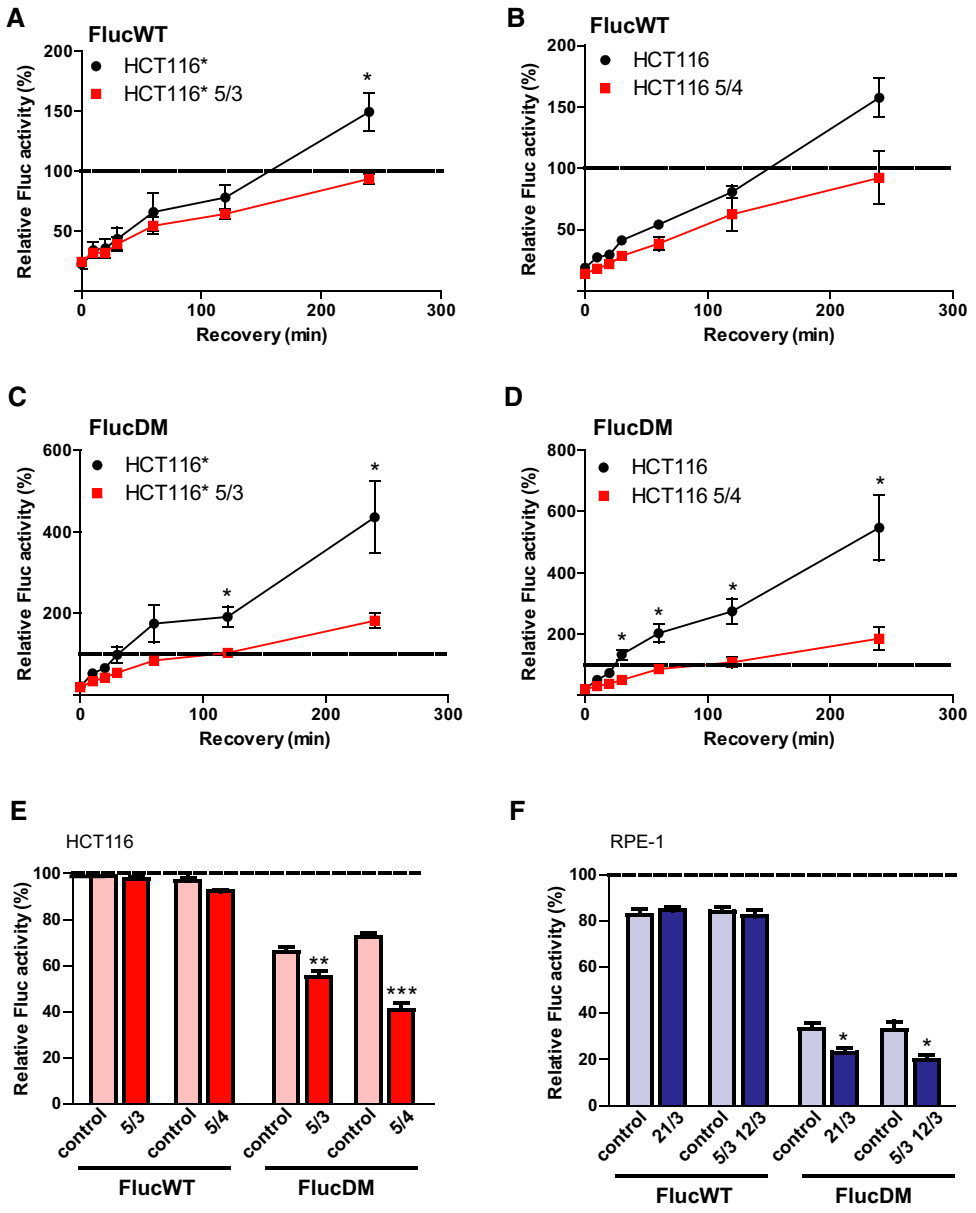


Figure 1. Trisomic and tetrasomic human cell lines show defects in protein folding.

A–D Refolding of the sensor proteins upon heat shock in control cells and in respective aneuploids. HCT116 and HCT116* stably expressing histone H2B-GFP and their aneuploid derivatives were transfected either with FlucWT-mCherry (A, B) or FlucDM-mCherry (C, D) and subjected to heat stress for 2 h at 43°C. Controls were maintained at 37°C. Luminescence readings were taken immediately from heat-stressed cells (0 min) and at indicated time points after recovery at 37°C. The luminescence values of control cells maintained at 37°C were set to 100% (indicated by dotted line).

E, F Refolding of the sensor proteins upon HSP90 inhibition in control cells and in respective aneuploids. FlucWT-mCherry or FlucDM-mCherry was expressed in parental and aneuploid HCT116 or HCT116* (E) and RPE-1 or RPE-1* (F) cell lines for 36 h. Cells were then incubated with either solvent control (DMSO) or 50 nM 17-AAG for 8 h followed by measurement of luminescent activity. The depicted values show the percentage of luminescence in cells treated with 17-AAG relative to DMSO-treated cells (which were set to 100%).

Data information: All plots show the means of at least three independent experiments ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; non-parametric t-test.

line-specific responses were also observed after heat shock: Only HCT116* 5/3 cells were highly sensitive to heat shock, while the other aneuploidies either showed a similar response as controls or even a marked resistance (Fig 2D). Finally, aneuploid cells were not significantly more sensitive to treatment with AZC (Supplementary Fig S2A and B). We conclude that aneuploidy exerts profound effects on cellular proteostasis, but only the increased sensitivity to HSP90 inhibition was common to all aneuploid cells that we tested. Thus, in line with previous observations from aneuploid yeast (Oromendia *et al*, 2012) and murine cells (Tang *et al*, 2011), a specific impairment of HSP90-mediated protein folding represents a general and conserved consequence of aneuploidy.

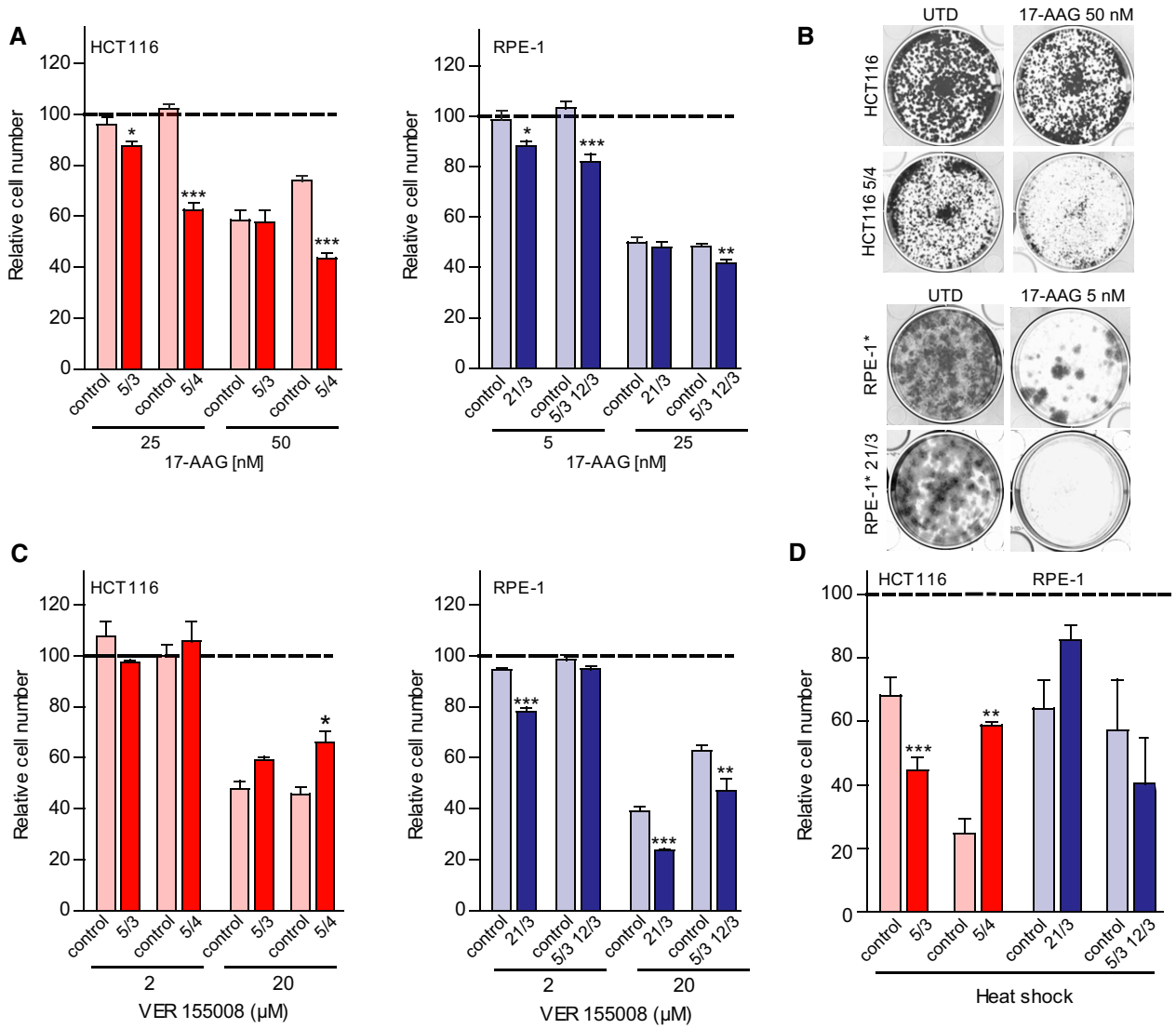


Figure 2. Sensitivity to inhibition of HSP90 but not to other inducers of protein folding stress increases in aneuploid cells.

A Wild-type, trisomic and tetrasomic cells were treated with the indicated concentrations of 17-AAG, and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control.

B Colony formation efficiency of aneuploid and parental RPE-1 treated with either solvent control (DMSO) or 17-AAG at indicated concentrations. Cells were stained with crystal violet after 2 weeks.

C Wild-type, trisomic and tetrasomic cells were treated with the indicated concentration of VER 155008, and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control.

D Wild-type, trisomic and tetrasomic cells were subjected to heat stress for 15 h at 45°C, and cell number was determined. Cell number is shown as the percentage of the untreated control (maintained at 37°C).

Data information: All plots show the means of at least three independent experiments ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; non-parametric *t*-test.

The basal and stress-induced activity of HSF1 is impaired in human aneuploid cells

Our results suggest that aneuploid cells may be compromised in their ability to mount a robust HSR when challenged with stress. Moreover, the impaired ability to refold luciferase evident already at very early time points of recovery (10, 20 min; Fig 1C and D) may point to a reduction in steady-state protein folding capacity. We hypothesized that the HSP90-specific protein folding defect might be due to changes in expression levels of factors involved in the maintenance of cellular proteostasis. Analysis of the expression changes in heat shock protein families (Kampinga *et al*, 2007) from our previously obtained global transcriptome and proteome data (Stingele *et al*, 2012) revealed a small but statistically significant reduction in both mRNA and protein abundance for the HSP90 family across a panel of six different aneuploid cell lines (Supplementary Fig S3A). Protein levels of chaperonins were also slightly decreased, although mRNA levels were not significantly changed

(Supplementary Fig S3A). Analysis by immunoblotting revealed that the levels of HSP27, HSP70, and HSP90 were modestly, but consistently and significantly reduced in the majority of aneuploid cell lines analyzed (Fig 3A and B). The protein folding capacity of molecular chaperones is regulated, in part, by binding to co-chaperones and other co-factors. Phosphorylation at the C-termini of HSP70 and HSP90 enhances binding to the co-chaperone HOP, thereby increasing productive protein folding and cellular proliferation rates (Muller *et al*, 2013). However, the ratios of phosphorylated HSP90 and HSP70 over total levels were unchanged when comparing parental cell lines and HCT116 aneuploid derivatives (Supplementary Fig S3C and D). Our finding that the chaperone expression is impaired in aneuploid cells prompted us to analyze the expression of the heat shock response transcription factor and master regulator of chaperone expression HSF1 as well. The immunoblotting revealed a consistent reduction in protein levels in all four cell lines tested (Fig 3A and B). Notably, the transcription of the *HSF1* gene is not altered in aneuploid cells, as we observed only negligible changes in HSF1 mRNA levels in qPCR experiments (Supplementary Fig S3B).

In light of our observations regarding HSF1 protein levels, we asked whether the induction of HSF1 activity, that is, the ability to mount a HSR, was also impaired in aneuploid cells. To this end, we expressed a HSP70-luc construct that contains the *HSP70* promoter fused to luciferase in diploid and aneuploid cells (Williams *et al*, 1989). We then treated the cells with 17-AAG and the proteasome inhibitor MG132, compounds that induce acute proteotoxic stress and are well-characterized activators of HSF1-dependent transcription (Mathew *et al*, 2001; Sharma *et al*, 2012). We observed that the parental cell lines induced the expression of the HSP70-luc sensor two- to threefold soon after the proteotoxic treatment and up to sevenfold in response to prolonged treatment (Fig 3C and D). In contrast, the ability of HCT116-derived and RPE-1-derived aneuploids to induce HSP70-luc was on average reduced to 50 and 60% of the control at early and later time points, respectively (Fig 3C and D). Interestingly, at the later time point, RPE-1 21/3 had recovered the ability to activate the *HSP70* promoter. This is in line with the relatively mild decrease in HSF1 and chaperone levels in this cell line and with its relatively modest sensitivity to 17-AAG (Figs 2A and 3A and B). These observations might be explained by the small size of chromosome 21; hence, RPE-1 21/3 is burdened with the least amount of extra genetic material of all the aneuploid cell lines analyzed in this study. Consistent with these findings, we also observed an impaired ability to induce HSP70 expression after acute heat shock in both HCT116- and RPE-1-derived aneuploid cells (Supplementary Fig S3E). The decrease in HSF1 expression observed in aneuploid cells is relatively small, and therefore, we asked whether it is sufficient to cause the observed impairment in maintenance of proteostasis and protein folding. To address this concern, we transfected the control cell lines with siRNA to partially deplete HSF1 to 75 and 50%, respectively (Fig 3E). Indeed, consistent with previous results (Chen *et al*, 2013), this partial and transient depletion of HSF1 rendered cells sensitive to treatment with 17-AAG, thus suggesting a striking dosage sensitivity of the cellular response to proteotoxic stress (Fig 3F). Therefore, we conclude that the protein folding defect engendered by aneuploidy may be caused by inhibitory effects on basal and induced HSF1 activity.

Endogenous or exogenous overexpression of HSF1 ameliorates the negative effects of aneuploidy on protein folding

If the reduced protein folding capacity of aneuploid cells is due to a deficiency in HSF1 function, aneuploid cells with increased levels of HSF1 may be protected against this impairment. In fact, increased expression of HSF1 due to gene amplification is frequent in cancer, similarly as gain of chromosome 8 or its long arm where the *HSF1* gene is located on 8q24.3 (Beroukhim *et al*, 2010). Thus, we reasoned that cells that gained chromosome 8 with the resulting increased expression of HSF1 might escape the defects in HSP90 function and protein folding caused by gain of a chromosome without HSF1. To test this possibility, we generated four clonal cell lines derived from individual HCT116 cells upon micronuclei-mediated transfer of chromosome 8. Using chromosome painting, we confirmed the presence of an extra copy of chromosome 8 in all imaged cells from all four clonal cell lines (HCT116 8/3 c1-c4; Supplementary Fig S4A). Analysis of the expression of HSF1 protein revealed an increased abundance according to expected gene copy number, that is, approximately 1.5-fold higher relative to diploid HCT116, in the clonal cell lines c1, c2, and c4. Interestingly, HSF1 levels were not substantially changed in c3 (Fig 4A and B). This was likely due to a loss of the distal region of chromosome 8 where HSF1 is located (Supplementary Fig S4B, our unpublished data). Thus, we reasoned that comparison of c1, c2, and c4 with c3 would enable us to directly test whether increased levels of HSF1 protect cells from the protein folding defects caused by the introduction of the extra copy of chromosome 8. We first analyzed the expression of HSP90. Similarly to other trisomies and tetrasomies that we analyzed (Fig 3A and B, and Supplementary Fig S3A), introduction of chromosome 8 elicited a slight decrease in HSP90 levels in c3 (Fig 4A and B). Strikingly, however, in c1, c2, and c4, we observed no decrease in HSP90 levels relative to control HCT116 (Fig 4A and B). Next, we tested the sensitivity of FlucDM to 17-AAG treatment in the four clones. The luminescent output of FlucDM was significantly lower after treatment with 17-AGG in HCT116 8/3 c3 compared to control HCT116. In contrast, the relative decreases in luminescence in response to 17-AAG treatment were comparable to the control in the trisomic cell lines c1, c2, and c4 (Fig 4C). Moreover, whereas c3 exhibited a sensitivity to 17-AAG that was comparable with the other trisomies, c1, c2, and c4 were as resistant to 17-AAG as the parental HCT116 (Fig 4D). Thus, the increased levels of HSF1 counteract the negative effect of aneuploidy on HSP90 expression and on protein folding.

To directly determine a role for increased HSF1 activity in mitigating the effects of aneuploidy on HSP90 and protein folding, we performed transient transfections with a constitutively active truncated HSF1 allele (ca-HSF1) (Zuo *et al*, 1995). Immunoblotting confirmed efficient expression of ca-HSF1 upon transient transfection in HCT116 8/3 c3 aneuploids as well as in HCT116 5/4 and RPE-1 21/3 and increased the expression levels of its downstream targets (Fig 4E). Importantly, the transient ca-HSF1 expression significantly improved the survival of aneuploid cells in the presence of 17-AAG as well as protected the folding of the FlucDM sensor against its effects (Fig 4F and G). Our observations suggest that cellular sensitivity to 17-AAG is finely tuned to the levels of HSF1. To test the generality of this conclusion, we transfected the control HCT116 and

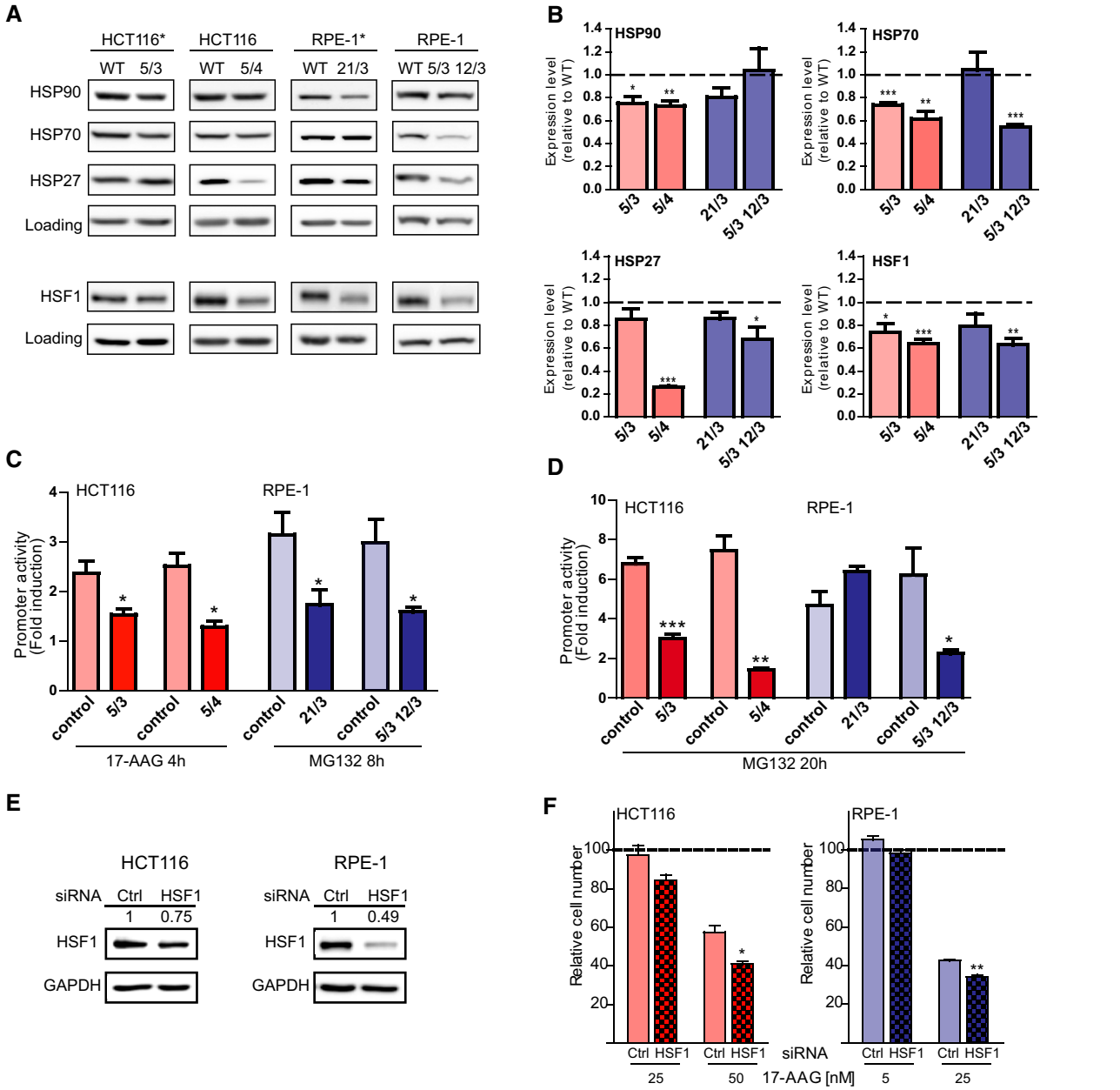


Figure 3. The basal and stress-induced activity of HSF1 is impaired in human aneuploid cells.

A, B Western blot analysis for HSP27, HSP70, HSP90 (the used antibody recognizes both constitutive and inducible forms of HSP90) and HSF1 in parental and aneuploid cell lines (A). Loading control: GAPDH; HSC70 (constitutively expressed chaperone) in RPE-1 5/3 12/3 and corresponding control (note that GAPDH is encoded on chromosome 12). Shown are representative images of at least 3 independent experiments. In panel B the quantification of the signal intensities from the Western blots shown in (A) are depicted, calculated relative to control cells (which were set to 1).

C, D HSP70-luc plasmid was expressed in parental and aneuploid HCT116 and RPE-1 cell lines for 36 h. Cells were then incubated with solvent control (DMSO), 2 μM 17-AAG or 5 μM MG132 for the indicated times. The depicted values show the fold induction in 17-AAG- or MG132-treated cells compared to DMSO-treated cells (which were set to 1).

E HCT116 (left panel) and RPE-1 (right panel) cells were transfected with siRNA targeting HSF1 or the GL2 subunit of luciferase as a control (ctrl). Cell extract was prepared 72 h after transfection and subjected to immunoblotting for HSF1 and GAPDH as a loading control. Quantification of the signal normalized to the loading control is shown above the images.

F HCT116 (left panel) and RPE-1 (right panel) cells transfected with siRNA targeting HSF1 or the GL2 subunit of luciferase as a control (ctrl). Forty-eight hours after transfection cells were incubated with the indicated concentrations of 17-AAG, and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control.

Data information: All data are the mean of at least three independent experiments ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; non-parametric *t*-test.
Source data are available online for this figure.

RPE-1 cells with ca-HSF1 (Fig 4H). In agreement with our hypothesis, the transient overexpression of ca-HSF1 also significantly protected the control cell lines against 17-AAG-associated toxicity (Fig 4I and J). Taken together, these results show that increasing the levels of the HSR master regulator HSF1 is sufficient to counteract the impaired HSP90 function of human aneuploid cells.

Since trisomy in human cells leads to proliferation defects, we asked whether the difference in HSF1 expression is reflected by changes in proliferative capacity. In line with our hypothesis that HSF1 mitigates some of the phenotypes caused by aneuploidy, we observed that HCT116 clones with high HSF1 expression proliferated markedly faster than HCT116 8/3 c3 (Supplementary Fig S4E). This also suggests that the decreased protein folding capacity contributes to the proliferation defects observed in trisomic cells.

Chromosome 8 carries approximately 4,170 open reading frames, among them the *CCNE2* gene encoding cyclin E2 that plays a critical role in the G1 and in the G1-S transition and is often overexpressed in cancers, and *MYC* encoding the c-Myc transcription factor, a critical oncogene which upregulates a large number of genes involved in cell proliferation (Dang, 1999; Hwang & Clurman, 2005). We thus asked whether increased levels of c-Myc or cyclin E2 may be involved in improved proliferation rates in c1, c2 or c4. Western blotting revealed that cyclin E2 levels were increased on average 1.23-fold, and c-Myc levels were either unchanged or slightly reduced in all four HCT116 8/3 cell lines (Supplementary Fig S4C and D), in line with our previous finding that the abundance of some proteins is lower than expected based on the corresponding copy number changes in aneuploid cells (Stingele *et al*, 2012). As expected, based on these observations, Spearman correlation analysis revealed that only HSF1 levels highly correlated with the sensitivity of trisomic cells to HSP90 inhibition, their protein folding capacity as well as with proliferation rate (Supplementary Fig S4F). Taken together, both endogenous and exogenous overexpression of HSF1 ameliorates the adverse effects of aneuploidy on HSP90-dependent protein folding and proliferation in human cells.

Impaired HSP90 function in aneuploid cells affects the abundance of HSP90 client proteins

HSP90 plays a critical role in the folding of a wide variety of client proteins, in particular protein kinases as well as steroid hormone receptors and subunits of macromolecular complexes (Rohl *et al*, 2013). Thus, we asked whether the defect in HSP90 activity in aneuploid cells leads to a decreased abundance of client proteins that rely on HSP90. To this end, we compared recently reported data that elucidate the global HSP90 interactome and that classify interactors based on the strength of their interaction with HSP90 (Taipale *et al*, 2012) to the transcriptome and proteome changes that we observed in human aneuploid cell lines (Stingele *et al*, 2012). Our analysis revealed that the abundance of proteins that strongly interact with HSP90 was significantly lower in two out of the four aneuploid cell lines tested (HCT116* 5/4 and HCT116 5/4). In contrast, the abundance of non-interacting proteins was not affected in any of the analyzed cell lines (Fig 5A, Supplementary Datasets S1 and S2). Additionally, mRNA levels of strong interactors were unchanged, indicating that only the protein levels are affected (Fig 5A). Because this dataset of interactors may not represent a comprehensive list of all HSP90 clients, we also compared protein expression data from aneuploids with respect to another database of HSP90-interacting proteins generated by the Picard laboratory (http://www.picard.ch/Hsp90Int/index.php). Again, we observed a significant reduction in the expression of HSP90 interactors in two out of four aneuploid cells lines (HCT116 5/4 and RPE-1* 21/3; Fig 5B, Supplementary Dataset S3). Taken together, the reduced abundance of HSP90-interacting proteins in three out of four aneuploid cell lines supports the hypothesis that the HSP90 machinery is impaired in aneuploid cells and suggests that this impairment directly contributes to the altered protein composition of aneuploid cells.

The global expression changes in aneuploids resemble the cellular responses to HSP90 and HSF1 deficiency

HSP90 represents a critical hub in cell signaling through its chaperoning of a wide array of kinases and other proteins. Indeed, pharmacological inhibition of HSP90 results in significant alterations in the activity of multiple signaling pathways (Sharma *et al*, 2012). Our previous analysis of the changes in pathway regulation in human aneuploid cells identified a specific set of pathways that are up- or downregulated in response to aneuploidy, and these pathways appear to be conserved (Sheltzer *et al*, 2012; Stingele *et al*, 2012; Durrbaum *et al*, 2014). However, it remains unclear which molecular processes are responsible for these protein expression changes observed in aneuploid cells. Thus, we first asked whether the impairment in HSP90 function contributes to the changes of protein abundance in aneuploids. We compared the quantitative proteome changes in aneuploids cells with the proteome changes occurring upon pharmacological inhibition of HSP90 for 24 h (Sharma *et al*, 2012). The analysis of proteome changes upon HSP90 inhibition was performed in HeLa cells that are extensively aneuploid. Therefore, the proteome of treated cells was normalized to the proteome of untreated HeLa cells. Using 2-dimensional annotation enrichment analysis that enables direct comparison of relative pathway enrichments (Cox & Mann, 2012), we found an overlap between the proteome changes due to aneuploidy and proteome changes due to HSP90 inhibition (Supplementary Dataset S4), in particular among the downregulated pathways, which includes pathways of DNA and RNA metabolism, such as DNA repair and replication and RNA splicing, as well as cell cycle pathways (Fig 5C, Supplementary Fig S5A and B).

HSF1 predominantly regulates the expression of genes involved in proteostasis as part of the heat shock response, but has recently been shown to control the transcription of multiple additional target genes (Mendillo *et al*, 2012). We therefore compared the transcriptional profile of aneuploid cells with the transcriptional profile of a human hepatocellular carcinoma cell line (HCC) in which HSF1 was depleted by RNAi (Chuma *et al*, 2014). Comparison of the pathway enrichment in aneuploid cells and in cells depleted of HSF1 revealed a striking similarity in both downregulated and upregulated pathways (Fig 5D, Supplementary Fig S5C, Supplementary Dataset S4). Similar comparison with cells depleted of c-Myc showed no similarities between the pathways changes. This analysis suggests that the transcriptional activity of c-Myc does not affect the pathways that are deregulated by aneuploidy and supports the notion that the observed effect is specific for HSF1 (Supplementary Fig S5D). These results suggest that functional deficiency in HSF1 is a major determinant of the previously identified transcriptional aneuploidy response

Figure 4. Endogenous or exogenous overexpression of HSF1 ameliorates the negative effects of aneuploidy on protein folding.

A, B Western blot analysis of HSF1 and HSP90 expression in HCT116 8/3* c1-c4 (A). Loading control: GAPDH. Shown are representative images of at least 3 independent experiments. Quantification of the signal intensities from the Western blots (B), calculated relative to control cells (which were set to 1).

C FlucDM-mCherry was expressed in parental HCT116* and HCT116* 8/3 c1-c4 for 36 h. Cells were then incubated with either solvent control (DMSO) or 50 nM 17-AAG for 8 h followed by measurement of luminescent activity. The depicted values show the percentage of luminescence in cells treated with 17-AAG relative to DMSO-treated cells (which were set to 100%).

D Parental HCT116* and HCT116* cells trisomic for chromosome 8 (HCT116 8/3* c1-c4) were treated with the indicated concentrations of 17-AAG, and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control (which was set to 100%).

E Western blot analysis of HSF1 expression and its downstream targets in the indicated aneuploid cells transfected with ca-HSF1. Loading control: GAPDH. Shown are representative images of at least 3 independent experiments. Quantification of the signal intensities normalized to the loading control is shown above the images.

F RPE-1* 21/3 cells were transiently transfected with either pCDNA or ca-HSF1 by electroporation. Forty-eight hours post-transfection cells were incubated with the indicated concentrations of 17-AAG, and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control.

G FlucDM-mCherry was co-expressed with either pCDNA or ca-HSF1 in the indicated cell lines for 36 h. Cells were then incubated with either solvent control (DMSO), 50 nM 17-AAG (HCT116), or 5 nM 17-AAG (RPE-1) for 8 h followed by measurement of luminescent activity. The depicted values show the percentage of luminescence in cells treated with 17-AAG relative to DMSO-treated cells (which were set to 100%).

H Western blot analysis of HSF1 expression and its downstream targets in the indicated aneuploid cells transfected with ca-HSF1 using electroporation. Loading control: GAPDH. Shown are representative images of at least 3 independent experiments. Quantification of the signal intensities normalized to the loading control is shown above the images.

I Control HCT116* cells were transiently transfected with either pCDNA or ca-HSF1 by electroporation. Fourty-eight hours post-transfection cells were incubated with the indicated concentrations of 17-AAG and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control.

J Control RPE-1* cells were transiently transfected with either pCDNA or ca-HSF1 by electroporation. Fourty-eight hours post-transfection cells were incubated with the indicated concentrations of 17-AAG and cell number was determined 72 h thereafter. Cell number is shown as the percentage of the DMSO-treated control.

Data information: The data are the mean of at least three independent experiments ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; non-parametric *t*-test.

pattern (Stingele *et al*, 2012; Durrbaum *et al*, 2014). Taken together, our analyses suggest that the functional HSF1 and HSP90 deficiency caused by aneuploidy determines the global transcriptome and proteome changes in these cells.

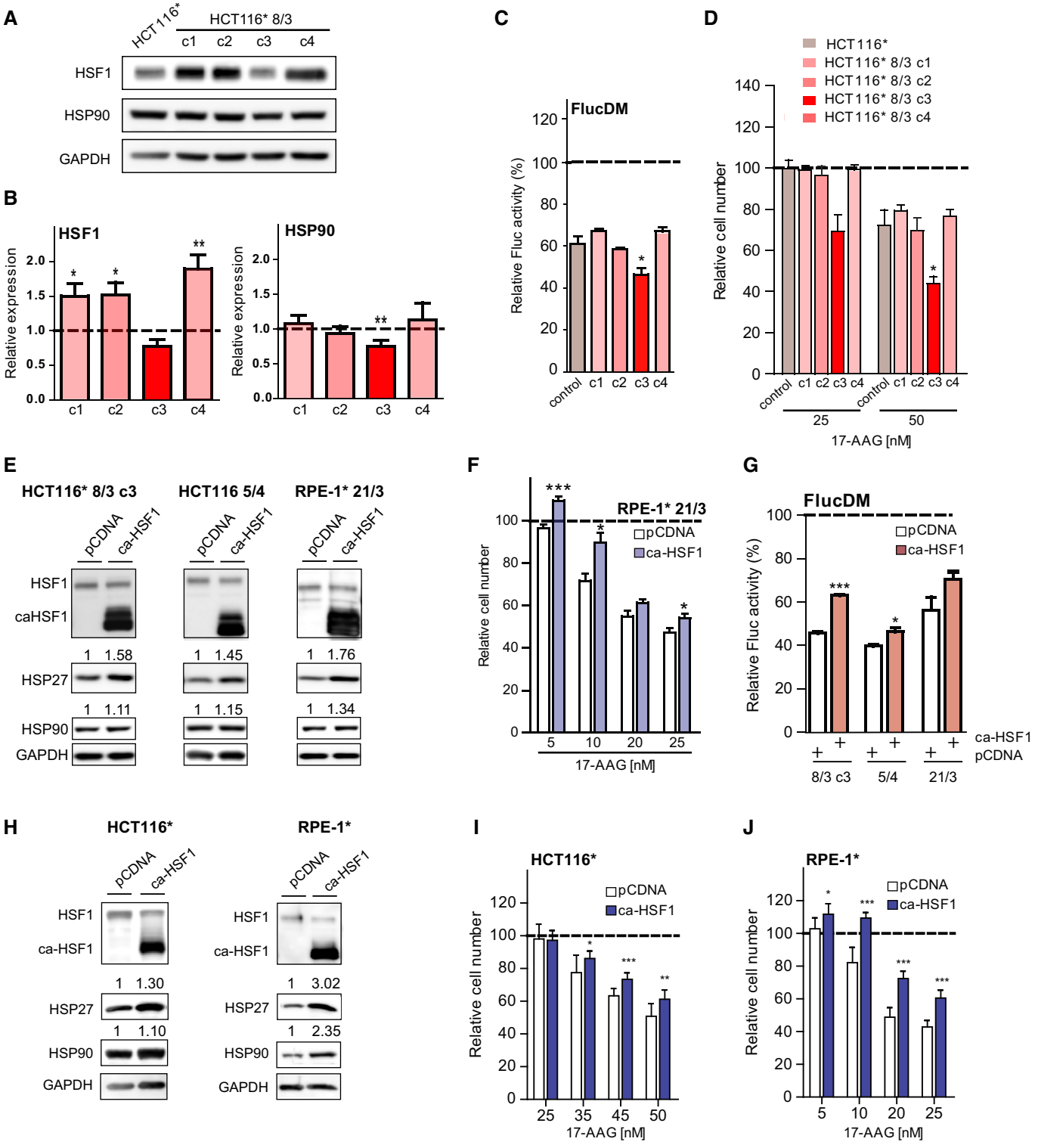
Discussion

Aneuploid cells often suffer from low proliferation rates and exhibit hallmarks of cells undergoing proteotoxic stress as evidenced by their sensitivity to conditions that compromise or overburden protein folding (Torres *et al*, 2007; Tang *et al*, 2011; Oromendia *et al*, 2012; Stingele *et al*, 2012). Here, we directly demonstrate for the first time that human aneuploid cells suffer from a protein folding defect and show that in particular HSP90-dependent protein folding is affected. Additionally, we identify a pronounced impairment in the ability of aneuploids to trigger a full heat shock response, suggesting that the functionality of heat shock-associated factors, in particular, the responsible transcription factor HSF1, is compromised. Importantly, we demonstrate that increasing the gene copy number of *HSF1* counters the effects of aneuploidy on HSP90 expression and protein folding. Finally, our analysis suggests that the observed functional deficiency in HSP90 and HSF1 contributes to the transcriptome and proteome changes observed in aneuploid cells. Thus, we propose that the cellular defects associated with aneuploidy may be direct consequences of impaired protein folding capacity.

Aneuploidy impairs protein folding

Both disomic budding yeast and tri- and tetrasomic human cells accumulate cytoplasmic protein deposits (Oromendia *et al*, 2012; Stingele *et al*, 2012). Previously, it has been proposed that the increased protein expression in aneuploid cells leads to a saturation of protein folding capacity and to low-level but chronic protein misfolding (Oromendia *et al*, 2012; Donnelly & Storchova, 2014). The misfolding, in turn, leads either to the aggregation, or destabilization and degradation of proteins with high or specific demands on the chaperone machinery. Indeed, we show that the presence of even one extra chromosome significantly impairs cellular protein folding in human aneuploid cells (Fig 1). This is mainly due to a defect in HSP90-dependent protein folding, whereas targeting the early steps in protein folding immediately after release from the ribosome, through AZC or HSP70 inhibition, does not preferentially impair the viability of human aneuploid cells (Figs 1 and 2, and Supplementary Fig S2). We hypothesize that the toxicity associated with impairment of proper protein folding at an early stage is determined by how efficiently and quickly cells can dispose of terminally misfolded proteins. Since both proteasome activity and autophagic degradation are elevated in mammalian aneuploid cells (Supplementary Fig S1B and C and Tang *et al*, 2011; Stingele *et al*, 2013), this may explain why they are not more sensitive or even slightly more resistant to such impairment. In contrast, we propose that the sensitivity to HSP90 inhibition observed in all the aneuploids analyzed in this study, regardless of the identity of the supernumerary chromosome(s) or the cell line rather reflects the loss-of-function of HSP90 clients and of HSP90-dependent processes. Therefore, our data together with previous observations in trisomic MEFs and disomic budding yeast (Tang *et al*, 2011; Oromendia *et al*, 2012) make a compelling argument that aneuploidy leads to a specific functional deficiency in HSP90-mediated protein folding.

In seeking to determine an explanation for why the HSP90 chaperone machinery is particularly affected by aneuploidy, we discovered that HSP90 family proteins were downregulated at both the mRNA and protein levels across a panel of six aneuploid cell lines. Intriguingly, this downregulation correlated with a decrease in total HSF1 levels in all four aneuploid cells lines tested and an impaired ability to induce HSF1 activity in response to acute proteotoxic stress (Fig 3). We emphasize, however, that the response to acute proteotoxic stress was not completely abolished, but rather delayed



and diminished. Notably, while a delay and decrease can be detected in aneuploid budding yeast strains, this effect appears to be more modest (Oromendia *et al*, 2012). Interestingly, elevated levels of HSP72, but not HSP90 or other heat shock factors, were identified in aneuploid murine fibroblasts compared to diploid controls (Tang *et al*, 2011). The differential regulation of HSP72 and HSP90 suggests that the activation of HSP72 in murine aneuploids is not due to elevated HSF1-dependent transcription, but rather modulated by other means that are specific to HSP72. Despite this difference, aneuploidy renders murine fibroblasts sensitive to the HSP90 inhibitor 17-AAG similarly as human aneuploids, thus further strengthening the notion that HSP90-mediated protein folding is specifically

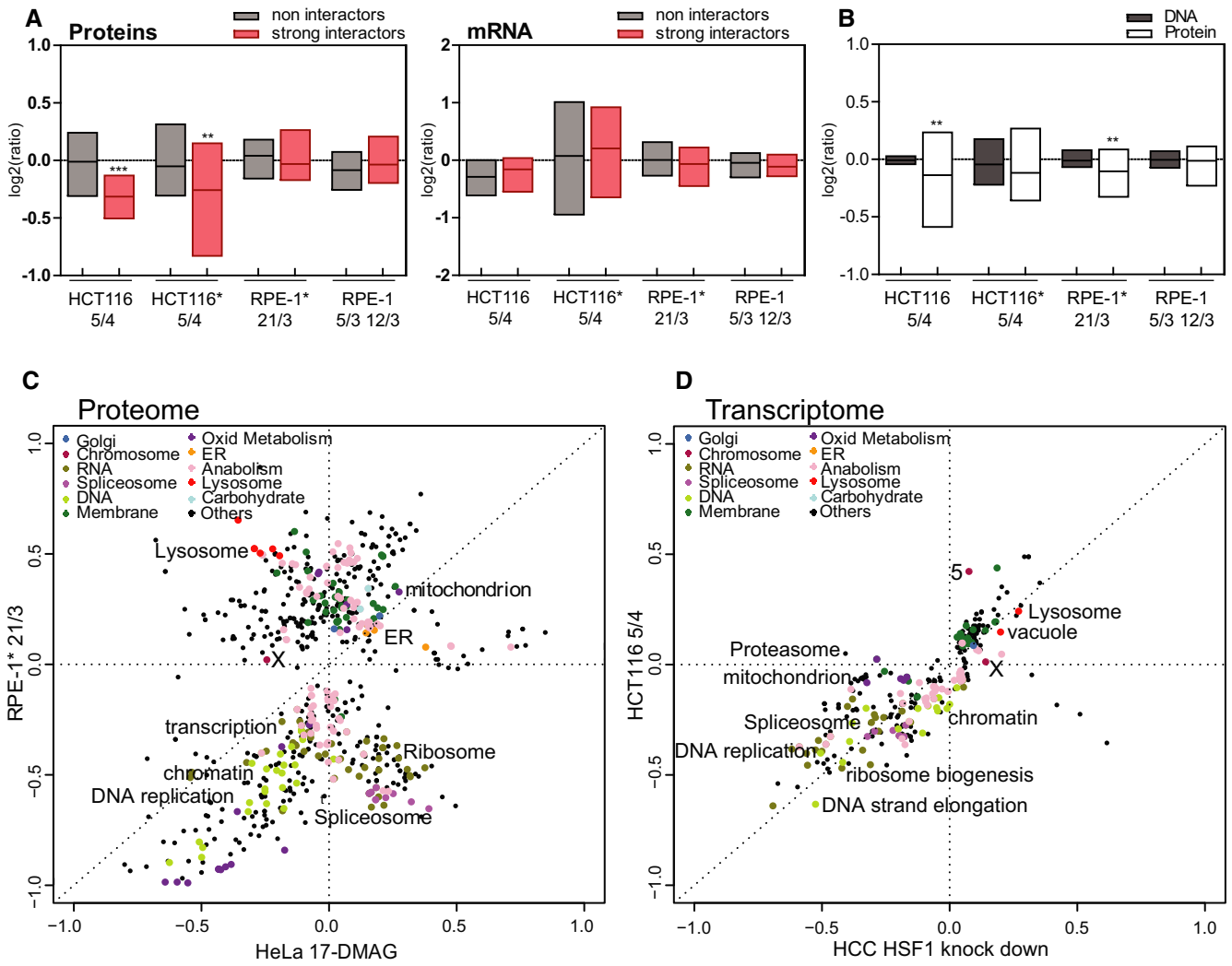


Figure 5. The proteome and transcriptome changes in aneuploid cells resemble the cellular response to protein folding deficiency.

A Relative abundance (calculated aneuploid/diploid ratio) of proteins that were identified as non-interactors and strong interactors of HSP90 in human cells (left panel). Relative abundance (calculated aneuploid/diploid ratio) of corresponding mRNAs (right panel). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Mann–Whitney *U*-test.

B Relative abundance (calculated aneuploid/diploid ratio) of factors that were identified to interact with HSP90. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Mann–Whitney *U*-test.

C Changes in pathways identified in the proteome of cells upon inhibition of HSP90 compared to the proteome changes in trisomic cells using the 2D-annotation enrichment analysis.

D Changes in pathway regulation identified in the transcriptome of HCC cells upon HSF1 knockdown compared to transcriptome changes in trisomic cells using the 2D-annotation enrichment analysis.

limiting for aneuploid cells. In future, it will be important to address by what mechanism aneuploidy impairs HSF1 function and why HSP90-dependent protein folding is particularly affected. We propose two hypothetical mechanisms for how HSF1 function might be impaired by aneuploidy. First, the metastable protein HSF1 may be incorporated into the ubiquitin-positive cytoplasmic deposits in aneuploid cells, and thereby rendered inactive. Alternatively, the HSF1 protein may be subject to post-translational inhibitory regulation that is elevated in aneuploids. Interestingly, overexpression of model β-sheet proteins in human cells also impairs the induction of cellular stress responses (Olzscha *et al*, 2011). Uncovering the similarities and differences in these two models of chronic proteotoxic stress will improve our understanding of the mechanisms involved in the maintenance of protein homeostasis.

We found that increased copy numbers of HSF1 can alleviate the protein folding defect and the impaired response to proteotoxic stress in aneuploid cells. This was confirmed in two different scenarios, by transfer of chromosome 8 and by transient overexpression of transgenic HSF1 (Fig 4). We employed overexpression of the upstream regulator of the heat shock response to ensure integrated and balanced expression of HSP90 and its co-chaperones. This is essential because the concentration of essential co-chaperones is limiting for HSP90 activity (Li *et al*, 2012), and the chaperone-dependent processes often require all components of a given chaperone

system (Rampelt *et al*, 2012). It should be noted that the transformed cell line HCT116 contains segmental aneuploidies, specifically copy gain on the long arms of chromosomes 8, 10, 16, and 17 and a loss of the Y-chromosome. These copy number changes are preserved in all created trisomic and tetrasomic cell lines (Stingele *et al*, 2012). However, our results suggest that an additional increase in copy numbers of HSF1 is necessary to rescue the defects arising in response to another whole chromosomal aneuploidy in *de novo* created trisomic cells so that the activity of HSF1 is sufficient to override the negative effects of aneuploidy on the protein folding machinery. More generally, our results suggest that in the context of the cellular response to severe proteotoxic stress (e.g. HSP90 inhibition), augmentation of HSF1 levels and/or activity fulfills a powerful cytoprotective function.

Previously, it was shown that a loss-of-function mutation in the gene encoding the deubiquitinating enzyme Ubp6 also markedly alleviated the negative effects of aneuploidy including impaired proliferation and accumulation of cytoplasmic protein deposits in budding yeast (Torres *et al*, 2010; Oromendia *et al*, 2012). Here, we have identified the first aneuploidy-tolerating genetic modification in human cells. These results support the interesting possibility that the adverse effects of aneuploidy can be suppressed either by enhancing protein degradation or by increasing cellular protein folding capacity. It is generally accepted that the sensitivity of cancer cells to HSP90 inhibitors stems from their reliance on heat shock proteins to chaperone the high number of overexpressed or mutated oncoproteins, and from the role of chaperones in protecting against general cellular stress associated with tumorigenesis (Dai *et al*, 2007). Our findings together with previous work (Tang *et al*, 2011) may provide an additional rationale for why inhibitors of protein degradation and protein folding emerge as a potentially effective cancer therapy and suggest that levels of HSF1 protein and/or activity may be important determinants of sensitivity to 17-AAG.

Consequences of the protein folding defects for aneuploids

Chemical or genetic impairment of HSP90 leads to the destabilization of multiple protein kinases and other proteins with critical roles in diverse cellular processes (Taipale *et al*, 2012). We found that the proteome of human aneuploid cells resembles the proteome of HeLa cells treated with the HSP90 inhibitor, 17-DMAG. In particular, pathways involved in DNA metabolic processes, chromatin modification, and transcription were downregulated in both conditions, whereas the overlap among upregulated pathways was rather modest. Even more similarities in both upregulated and downregulated pathways were revealed by comparison between transcriptional aneuploidy response patterns and the transcriptome changes in response to HSF1 depletion in the human HCC cell line (Chuma *et al*, 2014). Although HSF1, the major heat shock transcription factor, is most known for its role in regulating the expression of chaperones and proteins involved in the maintenance of proteostasis, recent discoveries have revealed its role in the regulation of a plethora of cellular processes (Mendillo *et al*, 2012). Based on our observations, we propose that the reduced HSP90 activity and the resulting decrease in stability of HSP90 client proteins partially underlies the pathway downregulation observed in aneuploid cells. Simultaneously, the impaired HSF1 activity affects, both directly, through the reduced expression of HSF1 target genes and indirectly, through the reduced protein folding capacity of aneuploid cells, the transcription of many targets. Taken together, we propose that the proteotoxic stress imposed by the presence of extra genetic material is a major determinant of the changes in gene expression in aneuploid cells.

We hypothesize that the HSP90 defect may have additional consequences for aneuploid cells. Two phenomena are worthy of particular mention. First, there is now a large body of evidence to suggest that HSP90 acts as a buffer against phenotypic variation by masking the effects of genetic polymorphisms (Jarosz *et al*, 2010). Second, HSP90 inhibition itself leads to chromosomal instability in budding yeast (Chen *et al*, 2012). Taken together with our results, we propose that aneuploidy is likely to further accelerate both the rate and manifestation of genetic change and our data suggest a general mechanism whereby changes in DNA copy number can lead to further genetic alterations.

Relevance for cancer and other pathologies

Aneuploidy is a hallmark of cancer, where it correlates with malignancy, drug resistance, and poor prognosis. However, trisomy and tetrasomy markedly impair cellular functions including proliferation, suggesting that aneuploidy-tolerating changes might be necessary to facilitate the growth of aneuploid cancer cells. Further, whether a similar impairment of protein folding capacity also occurs in cells of trisomy syndromes is currently not known and should be addressed in future. Intriguingly, somatic trisomy of chromosome 8 is frequently found in myeloid lineage disorders, some lymphomas and solid tumors such as breast and ovarian cancer. Interestingly, 8q24, where the *HSF1* gene is located, is one of the most commonly amplified regions in cancer cells (Beroukhim *et al*, 2010; Davoli *et al*, 2013), and chromosome 8 is the largest somatic chromosome whose trisomy is compatible, although extremely rarely, with postnatal survival (Ganmore *et al*, 2009; Beroukhim *et al*, 2010). Indeed, we show that the presence of *HSF1* on chromosome 8 protects against some of the adverse effects of aneuploidy. HSF1 is a critical facilitator of malignant proliferation, a role which it performs by supporting many important cellular processes (Dai *et al*, 2007). An additional role of HSF1 in promoting carcinogenesis may be to protect cancer cells from the proteotoxic stress induced by aneuploidy. Our results lend strong support to this notion and suggest a causal link between two recurring features of cancer cells: aneuploidy and altered HSF1 activity.

Materials and Methods

Cell lines and culturing conditions

The HCT116- and RPE-1-derived tri- and tetrasomic cell lines have been constructed by micronuclei-mediated chromosome transfer as described previously (Stingele *et al*, 2012). Parental cell line HCT116 (human colon carcinoma cell line): HCT116 3/3 (trisomy 3), HCT116 5/4 (tetrasomy 5) (Haugen *et al*, 2008); parental cell line HCT116* stably expressing histone H2B-GFP: HCT116* 5/3 (trisomy 5), HCT116* 5/4 (tetrasomy 5), (Stingele *et al*, 2013), HCT116 8/3

c1-c4; parental cell line RPE-1 (human retinal pigment epithelial cell line, hTERT immortalized): RPE-1 5/3 12/3 (trisomy 5, 12); parental cell line RPE-1* stably expressing histone H2B-GFP: RPE-1* 21/3 (trisomy 21). Cells were grown in DMEM GlutaMax (Gibco) supplemented with 10% FBS and 5% Pen/Strep under standard conditions.

Transfections and luciferase assays

Cells were transfected with a total of 1 or 1.5 μg of the indicated plasmids in 12-well plates using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol. Cells were trypsinized, counted, and seeded into 96-well plates 24 h after transfection and then allowed to recover for 24 h. Then, cells were treated with either solvent control (DMSO), 5 or 50 nM 17-AAG (8 h), 2 μM 17-AAG (4 h), or 5 μM MG132 (8 or 20 h). 17-AAG and MG132 were purchased from Enzo Life Sciences and Tocris Bioscience, respectively. To measure luminescence, 30 μl of SteadyGlo reagent (Promega) was added directly to the wells of the 96-well plates and the plates were shaken for 10 s to ensure mixing and cell lysis. Luminescence was measured on a Tecan plate reader after 15-min incubation in the dark.

Proteasome activity assay

Cells were seeded at 2×10^4 per well in triplicates in 96-well plates. Forty minutes later, Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay (Promega) was added according to the manufacturer's protocol. Luminescence was detected using a Fluoroskan Ascent FL plate reader operated by the Ascent software. For evaluation the mean with SEM of biological triplicates was calculated.

Western blotting

Exponentially growing cells were harvested and lyzed in RIPA buffer supplemented with protease inhibitors (Roche). 20 μg of protein were then resolved on 10% polyacrylamide gels and transferred to nitrocellulose membranes using the semi-dry technique. After blocking in low fat 5% milk in TBS-T, membranes were incubated with the following primary antibodies: HSP90 (1:1,000; Cell Signaling #4877), HSC70 (1:1,000; Enzo Life Sciences ADI-SPA-815), HSP70 (1:1,000; Enzo Life Sciences ADI-SPA-810), HSP27 (1:1,000; Enzo Life Sciences ADI-SPA-800), HSF1 (1:1,000; Enzo Life Sciences ADI-SPA-901), cyclin E2 (1:1,000; Cell Signaling #4132), c-Myc (1:200, Santa Cruz Biotechnology sc-40), GAPDH (1:2,000; Cell Signaling #2,118). The antibodies against phospho-HSP70 and phospho-HSP90 were a kind gift from Petr Müller, Masaryk University, Brno. After incubation with HRP-conjugated secondary antibodies, HRP substrate was added and luminescent signals were quantified using a LAS 3000 instrument (FujiFilm). Protein bands were quantified using ImageJ software.

Colony formation assays

Cells were seeded at 1,000 per well in 6-well plates 24 h before the treatment. Subsequently, cells were continuously treated with 17-AAG (5 or 25 nM) or DMSO for 10–12 days. Colonies were fixed with methanol:glacial acetic acid (1:1) and stained with 0.02% crystal violet for 15 min before washing with tap water and air-drying.

Cell viability assays

Cells were seeded at 2,000 per well in 96-well plates 24 h before the treatment. Subsequently, cells were treated with the indicated drugs at the indicated concentrations and cell viability was determined after 72 h, unless otherwise stated. Viability was determined using the CellTiterGlo luminescent cell viability assay (Promega) according to the manufacturer's instructions.

Chromosome transfer and preparation of chromosome spreads and paints

Chromosome transfer and the preparation of spreads and paints were performed as previously (Stingele *et al*, 2012).

RNA isolation and qPCR

Total RNA was isolated using the RNeasy kit (Qiagen) and reverse-transcribed into cDNA using the First Strand cDNA synthesis kit (Roche). qPCR was performed with a HSF1 assay from Qiagen (Cat. No. 330001 PPH00164F) on a LightCycler 480 (Roche) instrument using the KAPA SYBR FAST master mix. Absolute quantification was performed with an external standard, and the specificity of the amplicons was confirmed by melting curve analysis. HSF1 mRNA expression was normalized to ribosomal protein L27 (RPL27) as a housekeeping gene (de Jonge *et al*, 2007).

siRNA transfections

Cells were transfected at 50% confluency using 800 pmol siRNA and Oligofectamine according to the manufacturer's instructions. Transfections were conducted in OptiMEM for 4 h. The siRNA sequences used were acquired from Eurofins Genomics and are as follows: HSF1 (5′ CGGAUUCAGGGAAGCAGCUGGUGCA 3′; (Jacobs & Marnett, 2009); GL2 (5′ CGUACGCGGAAUACUUCGATT 3′).

Electroporation

Cells were electroporated using the Amaxa Nucleofector II apparatus and following the manufacturer's instructions and protocols for HCT116 cells and RPE-1 cells, respectively. Briefly, 1 million cells were resuspended in Cell Line Nucleofector Solution V containing 2 μg of either pCDNA or ca-HSF1 plasmid and transferred to cuvettes. HCT116 cells were electroporated using the D-032 Program and for RPE-1 cells the Program was U-017.

Transcriptome and proteome data analysis

The analyses of quantitative transcriptome and proteome data were performed as previously described (Durrbaum *et al*, 2014). The data were retrieved from the Gene Expression Omnibus database with the following accession numbers: knockdown of HSF1 in HCC: GSE47639; knockdown of c-myc in HeLa, BT-474, MCF-7, MDA-MB-231: GSE5823; HCT116- and RPE-1-derived trisomies and tetrasomies: GSE47830 and GSE39768.

Statistical analyses

All data related to viability, Fluc folding and protein expression were analyzed using Student's *t*-test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. All statistically analyzed experiments were performed at least three times.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

ND and VP performed experiments, SS performed initial experiments and analyzed the proteasome activity; MD contributed the bioinformatics analysis; ZS and ND conceived the study and wrote the manuscript, all authors analyzed the data and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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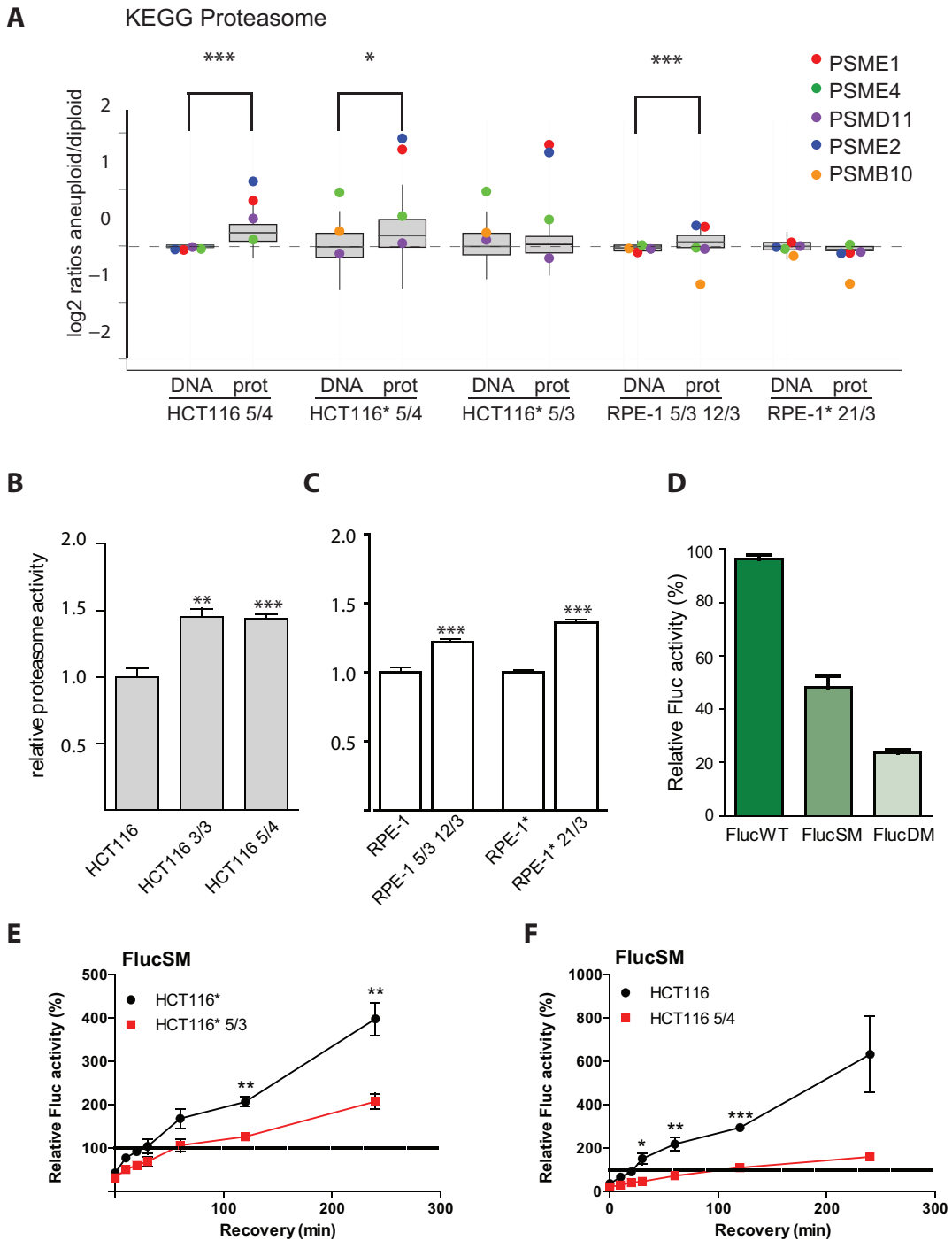

Figure S1 Protein degradation and protein folding in aneuploid cells

A. The expression of proteins involved in proteasomal activity is either unchanged or increased in aneuploid cells. Relative abundance of all proteins assigned to the KEGG pathway Proteasome was calculated using the data from Stingele et al., 2012. B. Relative proteasome activity in HCT116 and its aneuploid derivatives measured using the Proteasome-Glo kit (Promega). Mean of three independent experiments and SD are plotted, t-test. C. Relative proteasome activity in RPE-1 and its aneuploid derivatives. Mean of three independent experiments and SD are plotted, t-test. D. Normalized luminiscence activity of Fluc sensors upon treatment with 17-AAG. HCT116 cells were transiently transfected with the respective vectors, and luminescence activity was measured after 8 hrs treatment with 17-AAG and normalized to the untreated controls. Mean of three independent experiments and SEM are plotted. E, F. HCT116 and its aneuploid derivatives were transfected with FlucSM-mCherry and subjected to heat stress for 2 h at 43 °C. Controls were maintained at 37 °C. Luminescence was measured at indicated timepoints during recovery at 37 °C. The luminescence is normalized to the control cells maintained at 37 °C (set to 100% as indicated by dotted line). All plots show the means of at least three independent experiments ± SEM, t-test.

Donnelly et al, Supplementary Figure 1

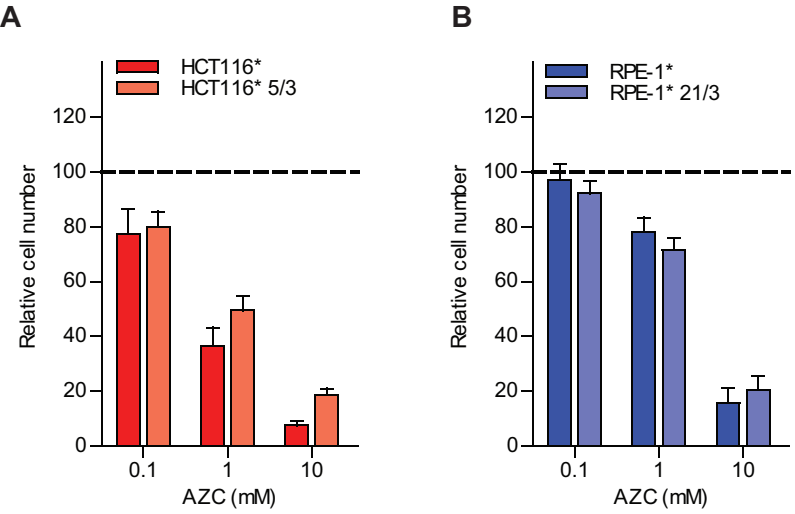

Figure S2 Sensitivity of aneuploid cell lines to L-Azetidine-2-carboxylic acid (AZC)

A. Wild-type and trisomic HCT116 cells were treated with the indicated concentrations of AZC and cell number was determined 72 hours thereafter. Cell number is shown as the percentage of the DMSO-treated control. B. Wild-type and trisomic RPE-1 cells were treated as in A and cell number was determined 72 hours thereafter. Cell number is shown as the percentage of the DMSO-treated control. Mean of three independent experiments with SEM are shown.

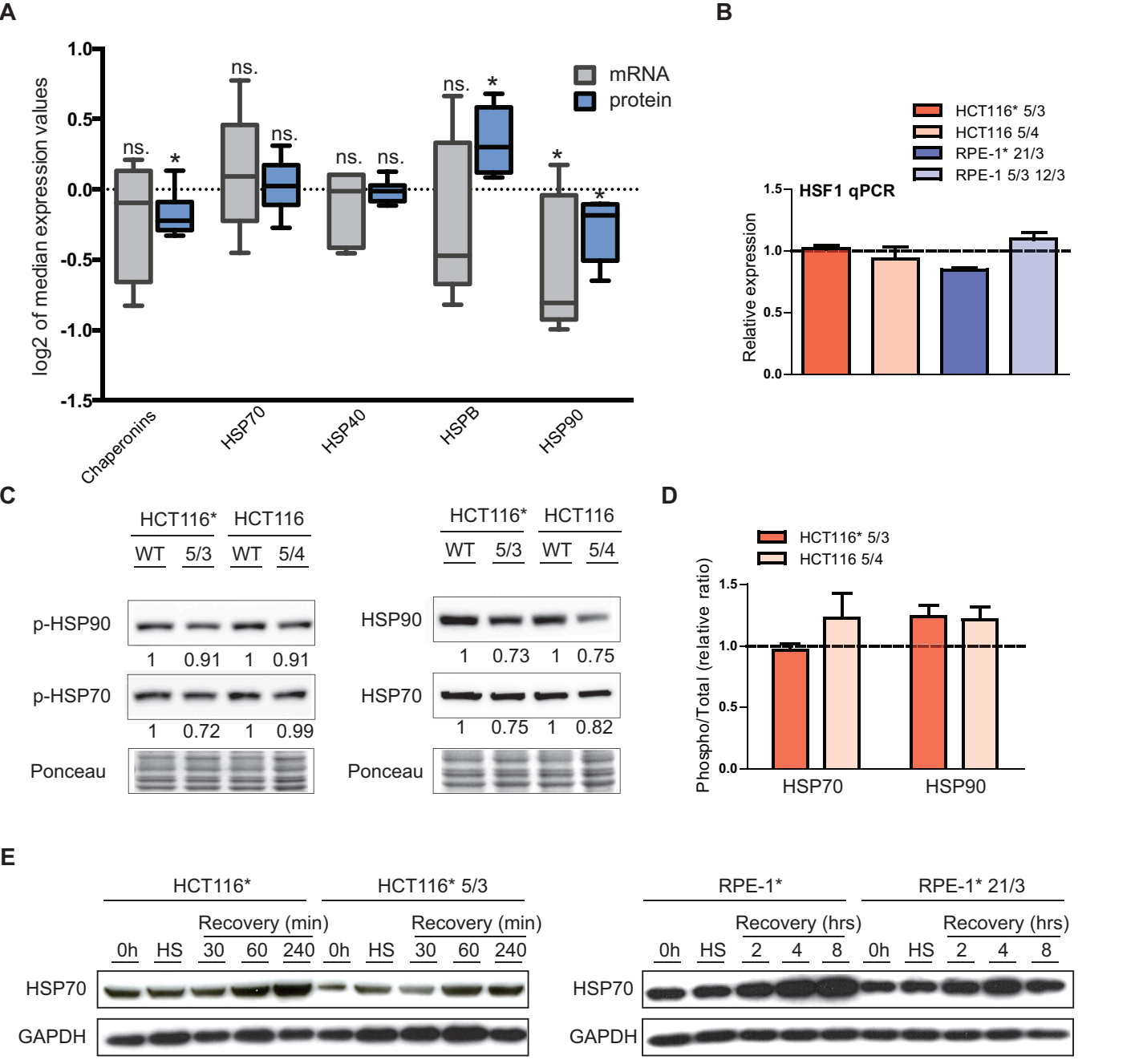

Figure S3 Summary of heat shock protein expression levels in aneuploid cell lines.
A. Expression of heat shock protein families in aneuploid cells. Each box plot shows the log2 median of relative mRNA and protein abundance changes, respectively, in the aneuploid cell lines HCT116 5/4, HCT116 3/3, HCT116* 5/4, HCT116* 5/3, RPE-1 12/3 5/3 and RPE-1* 21/3 compared to corresponding diploid for theindicated HSP gene families. Data from Stingele et al., 2012 and Dürrbaum et al. 2014. Kolmogorov-Smirnov test. B. qPCR analysis of HSF1 mRNA expression in the indicated cell lines. Expression levels are relative to the respective diploid control cell line and normalized to RPL27 expression as housekeeping gene. Mean and standard error of mean is plotted, n=2. C. Phosphorylation of HSP70 and HSP90 in aneuploid cell lines and the parental controls. Expression in the aneuploids is normalized to the respective wild-type (set to 1). Ponceau staining was used as a loading control; n=2. D. Quantification of the ratio of phosphorylated/total protein relative to the respective WT cell line. Mean and standard error of mean is plotted, n=2. E. Western blot analysis of HSP70 induction following heat shock in HCT116- and RPE-1-derived aneuploids. Cells were heat-shocked at 43 °C for 2 hours (HCT116) or 30 minutes (RPE-1) and then allowed to recover at 37 °C for the indicated times. 0h denotes expression in cells at 37 °C at the start of the experiment. Shown are representative blots of experiments performed at least 3 times.

Donnelly et al, Supplementary Figure 3

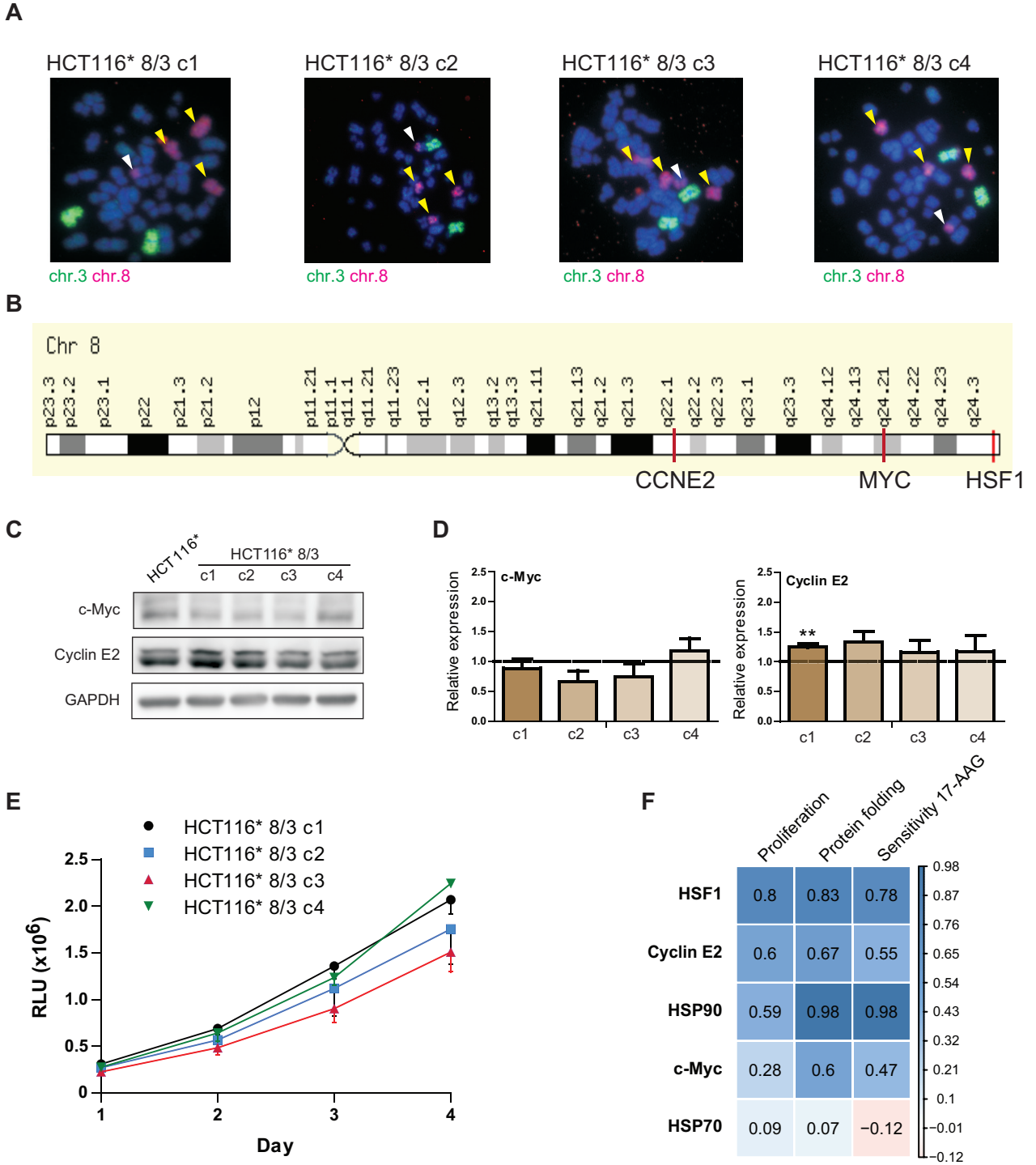

Figure S4 HCT116 with trisomy of chromosome 8

A. Examples of chromosome paints of HCT116* 8/3 clones. HCT116* cell lines containing an additional copy of chromosome 8 were generated using microcell fusion and chromosome spreads were prepared and stained with specific probes for chromosome 8 and chromosome 3 as a control, followed by counterstaining with DAPI. Yellow arrows mark the chromosome 8, white arrows mark the fusion of a chromosome 8 fragment to chromosome 16, a chromosomal imbalance that is characteristic for HCT116. B. Schematic representation of human chromosome 8 showing the location of the *CCNE2*, *MYC*, and *HSF1* genes. C. Western blot analysis of cyclin E2 and c-Myc expression in aneuploid cell lines. D. Quantification of the relative expression changes in c-Myc and cyclin E2. Mean and SEM of three independent experiments is shown. E. Growth curves of HCT116* 8/3 clones. Each point represents the mean with SEM of three independent experiments. F. Spearman correlations between the expression levels of the indicated proteins and proliferation, protein folding (FlucDM sensitivity) and sensitivity to 17-AAG in HCT116* 8/3 clones.

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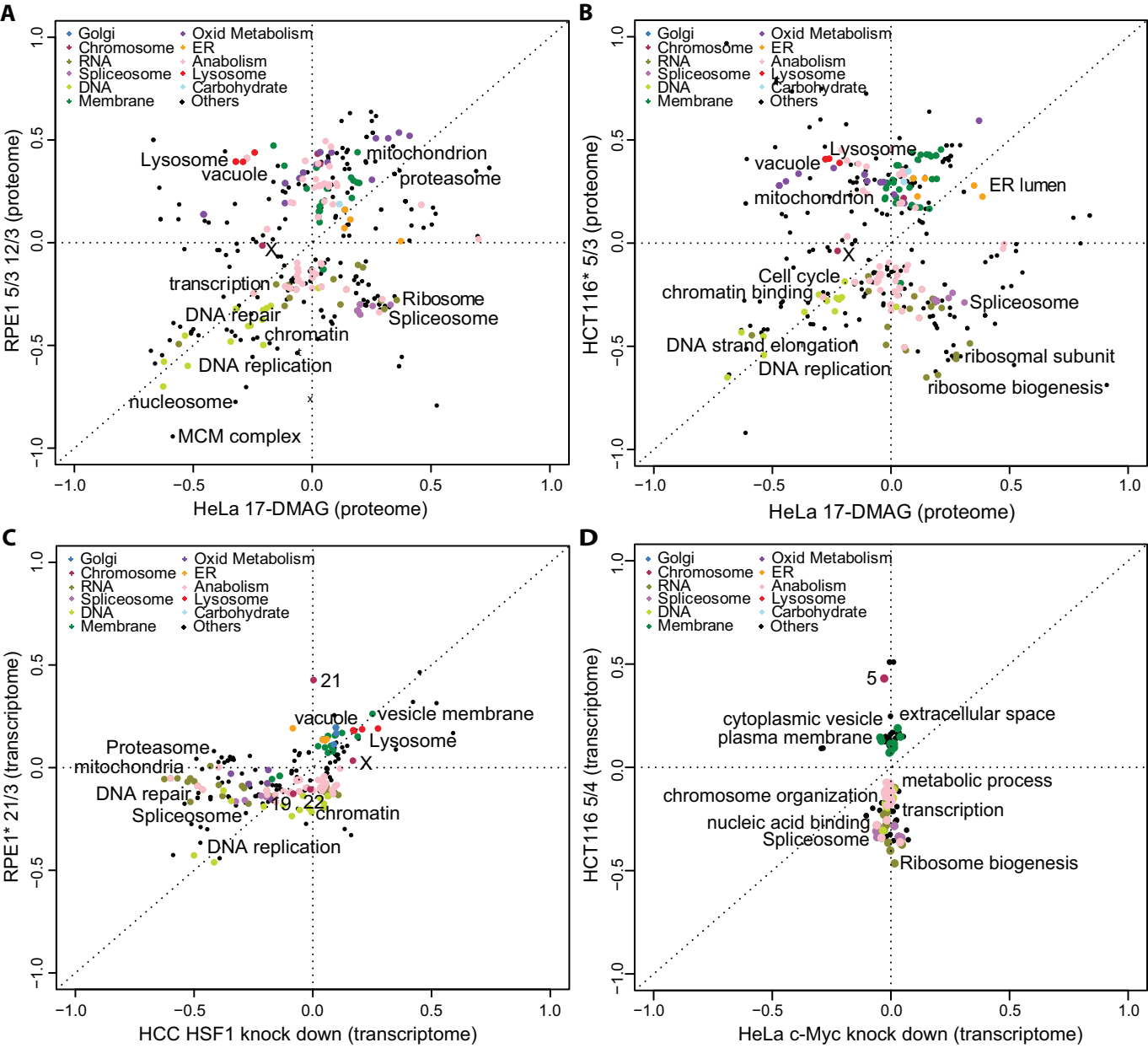

Figure S5 Comparison of proteome and transcriptome

A. Changes in pathways identified in the proteome of cells upon inhibition of HSP90 compared to the proteome changes in RPE1 5/3 12/3. B. Changes in pathways identified in the proteome of cells upon inhibition of HSP90 compared to the proteome changes in HCT116* 5/3. C. Changes in pathway regulation identified in the transcriptome of cells upon HSF1 knockdown compared to transcriptome changes in RPE1* 21/3. D. Changes in pathway regulation identified in the transcriptome of cells upon c-Myc knockdown compared to transcriptome changes in HCT116 5/4.

8.2 The presence of extra chromosomes leads to genomic instability

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In this study we addressed the question of whether aneuploidy, specifically gain of one or two chromosomes, is sufficient to increase genomic instability.

Using high-resolution microscopy, we observed that trisomic and tetrasomic cells showed increased levels of markers of DNA damage as well as anaphase bridges and ultrafine bridges during mitosis. Further, a combination of next-generation sequencing and SNP-array analysis revealed that aneuploid cells accumulated chromosomal rearrangements with break point junction patterns that suggested that the rearrangements arose as a result of defects in replication. Indeed, direct analysis of replication dynamics revealed slower replication of DNA in aneuploid cells and a heightened sensitivity to exogenous replication stress. We found that the impaired DNA replication and higher levels of genomic instability in human aneuploid cells can be explained by a general reduction in the expression of replicative factors. Aneuploid cells exhibited a strikingly consistent downregulation of the subunits of the replicative helicase MCM2-7, suggesting that lower levels of this complex may be a critical factor contributing to the genomic instability of aneuploid cells. In fact, boosting levels of chromatin-bound MCM helicase partially rescued the genomic instability phenotypes.

In conclusion, this paper shows that the gain of one or two extra chromosomes causes replication stress, which in turn leads to genomic instability, and provides a molecular link between numerical and structural changes to the genome.

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The presence of extra chromosomes leads to genomic instability

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Aneuploidy is a hallmark of cancer and underlies genetic disorders characterized by severe developmental defects, yet the molecular mechanisms explaining its effects on cellular physiology remain elusive. Here we show, using a series of human cells with defined aneuploid karyotypes, that gain of a single chromosome increases genomic instability. Next-generation sequencing and SNP-array analysis reveal accumulation of chromosomal rearrangements in aneuploids, with break point junction patterns suggestive of replication defects. Trisomic and tetrasomic cells also show increased DNA damage and sensitivity to replication stress. Strikingly, we find that aneuploidy-induced genomic instability can be explained by the reduced expression of the replicative helicase MCM2-7. Accordingly, restoring near-wild-type levels of chromatin-bound MCM helicase partly rescues the genomic instability phenotypes. Thus, gain of chromosomes triggers replication stress, thereby promoting genomic instability and possibly contributing to tumorigenesis.

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Most eukaryotic organisms are diploid as they contain two sets of chromosomes. A deviation from the normal chromosome number often markedly affects their physiology. Whole-chromosome gains or losses—called numerical aneuploidy—have particularly detrimental effects in Metazoans, as cells often suffer from impaired proliferation, increased sensitivity to proteotoxic stress and altered gene expression[1–4]. Structural aneuploidy, characterized by sub-chromosomal unbalanced rearrangements, that is, duplications or deletions of large genomic regions, also affects cellular functions. Both numerical and structural aneuploidy are found in nearly 75% of malignant tumours[5], where they are often associated with ongoing whole-chromosome gains and losses, a process known as chromosomal instability (CIN), and with additional structural rearrangements due to genomic instability (GIN). Both CIN and GIN phenotypes promote cancer and contribute to tumour heterogeneity and drug resistance[6,7].

Experiments in mouse models carrying mutations that impair faithful chromosome segregation revealed that the resulting CIN is sufficient to trigger tumour formation in some tissues, although it should be noted that CIN and aneuploidy also exert tumour-suppressive effects (reviewed in ref. 8). Mounting evidence shows that GIN and replication stress occur early in tumorigenesis, and many known oncogenes and oncogenic mutations act by triggering replication stress and subsequently genomic instability[9–11]. The occurrence of both structural and numerical aneuploidy in early stages of cancer provokes the question of whether there is a link between these two features. It was recently shown that lagging chromosomes suffer from breakage during cytokinesis[12]. Missegregated chromosomes are often contained in micronuclei in daughter cells, where they experience DNA damage likely due to aberrant replication[13]. Chromosomally unstable colorectal cancer cells show elevated DNA replication stress that was proposed to further promote chromosome missegregation[14]. Aneuploidy also correlates with chromosomal and genomic instability in transformed Chinese hamster embryo cells[15]. Yet, delineating the functional relationship between numerical aneuploidy and genomic instability, and the molecular mechanisms involved has remained challenging.

To understand the relationship between CIN and GIN, it is crucial to determine whether and how numerical aneuploidy by itself affects genome stability. Recently established model cells with defined aneuploid karyotypes have facilitated the analysis of the immediate consequences of aneuploidy *per se*[1,4,16–19]. Direct comparison of cognate euploid and aneuploid cells revealed that aneuploidy triggers distinct and conserved changes in gene expression[20,21]. Addition of even a single extra chromosome causes profound defects in the maintenance of proteostasis as aneuploid cells are more sensitive to inhibitors of protein folding, protein translation and degradation, activate protein degradation pathways and show marked protein folding defects[3–5,18,22,23]. Observations in budding and fission yeasts suggested that aneuploidy impairs the fidelity of chromosome segregation, and increases mutation and recombination rates, although the underlying mechanisms remain enigmatic[17,24–26]. Analysis of trisomy of chromosome 7 or 13 in the p53-deficient cancer cell line DLD1 revealed increased chromosome missegregation of the extra chromosome and a frequent cytokinesis failure in trisomy 13 (ref. 27). Yet, no systematic analysis of the effects of aneuploidy on the maintenance of genome stability has been performed in higher eukaryotes so far.

Here we use a series of trisomic and tetrasomic human cells with defined karyotypes derived from near-diploid and chromosomally stable parental cell lines. By direct comparison with the parental cell lines, we found that addition of even a single chromosome is associated with accumulation of replication-related DNA damage and chromosomal rearrangements. Strikingly, we found profound abundance changes of proteins involved in DNA replication in response to the presence of extra chromosomes, in particular, consistently low levels of the replicative helicase MCM2-7. Our data provide the first evidence that the presence of even a single extra chromosome in human cells triggers genomic instability by impairing DNA replication, thus establishing a new link between numerical and structural aneuploidy that might play a role in cancer.

Results

Accumulation of pre-mitotic DNA errors in aneuploid cells. To determine whether the presence of one or two extra chromosomes induces genomic instability, we created a panel of trisomic and tetrasomic human cell lines using micronuclei-mediated chromosome transfer (MMCT; see Supplementary Information for further details). We analysed five different cell lines that were derived from the chromosomally stable colorectal cell line HCT116 and five cell lines originating from the primary human cell line RPE1 (retinal pigment epithelium) immortalized by the overexpression of human telomerase. Each of these cell lines contained an extra copy of either chromosome 3, 5, 8, 12 or 21. The chromosomal content was verified by single-nucleotide polymorphism (SNP) array analysis, and the cell lines were named according to the identity of the extra chromosome and its copy number, for example, HCT116 8/3 for trisomy of chromosome 8 in the HCT116 cell line (Fig. 1a). Evaluation of microscopic images of anaphases revealed that all 10 analysed trisomic and tetrasomic cells contained anaphase bridges at higher frequency than the parental cell lines (Fig. 1b–d). This finding was further confirmed using high-resolution imaging combined with staining with a centromere-specific CREST antibody. On average, 46% of HCT116 3/3, 25% of HCT116 5/3 and 28% of HCT116 5/4 anaphase cells contained anaphase bridges and broken chromosome fragments devoid of centromeric staining that are indicative of pre-mitotic errors, whereas only 10% of HCT116 anaphase cells showed similar defects (Fig. 1e,f). A similar trend was observed in RPE1-derived cell lines, with an increase from 2.5% in control to 12.7% in RPE1 5/3 12/3 and 9% in RPE1 21/3 (Fig. 1f). In contrast, the analysed cell lines showed a mild, but insignificant increase in the frequency of lagging chromosomes in comparison with the parental HCT116 or RPE1 cells (Supplementary Fig. 1a–e).

Next, we analysed the occurrence of ultrafine anaphase DNA bridges (UFBs), thread-like DNA structures that associate with the Bloom's syndrome helicase (BLM) protein and link the dividing DNA masses[28]. UFBs originate either from DNA catenanes or from replication or recombination intermediates, and their occurrence is suggestive of un-replicated or abnormal DNA structures[28]. Staining with an antibody against Bloom helicase (BLM) revealed increased frequency of UFBs by up to 60% in the trisomic and tetrasomic cells compared with controls (Fig. 1g,h). Collectively, these findings suggest that the presence of even a single extra chromosome increases the frequency of pre-mitotic errors, while chromosome segregation is not significantly impaired.

DNA damage and chromosome breaks increase with aneuploidy. In order to determine whether trisomy and tetrasomy lead to increased levels of DNA damage, we determined the occurrence of 53BP1-containing nuclear bodies in cyclin A-negative G1 cells. Such 53BP1 foci have been suggested to mark unrepaired replication-induced DNA lesions that persist through mitosis[29]. We determined the number of 53BP1 foci in G1 cells using automatized image acquisition and analysis (Fig. 2a). The

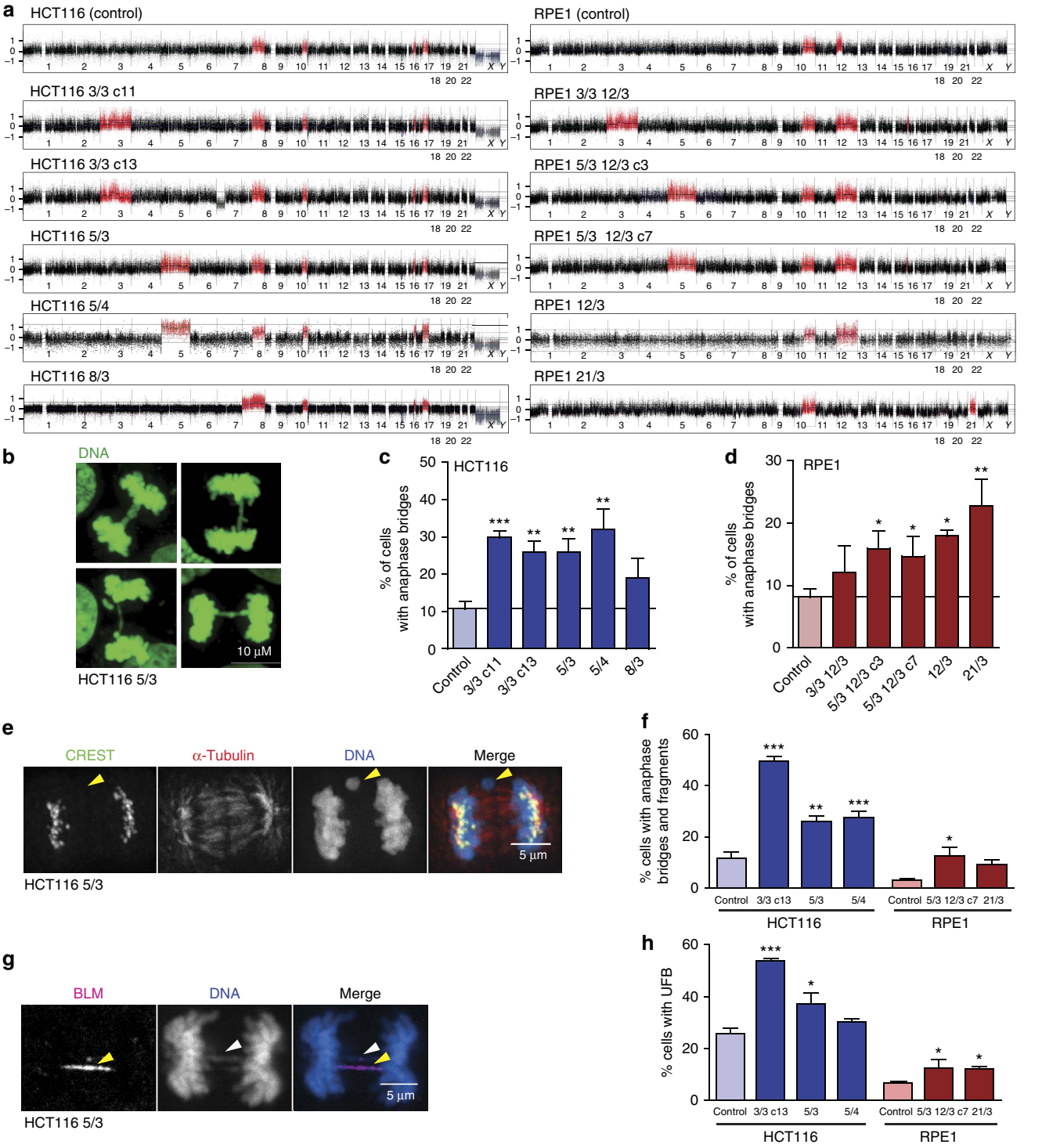

Figure 1 | Trisomy and tetrasomy elevates the frequency of pre-mitotic errors. (**a**) Chromosome copy number changes in the parental HCT116, RPE1 and the respective trisomic and tetrasomic cell lines. Chromosome gains are marked in red and chromosome losses in grey. Note that both HCT116 and RPE1 contain several previously documented structural copy number variations that remained largely unchanged in the trisomic and tetrasomic derivatives. The identity of the extra chromosome and the number of copies were used for identification of each cell line, for example, HCT116 3/3 contain three copies of chromosome 3. Two cell lines with identical trisomies, but originating from different single cells, were selected for HCT116 3/3: clone 11 and clone 13 (c11 and c13) and for RPE 5/3 12/3 (c3 and c7) to determine the effect of clonal differences. (**b**) Representative images of a HCT116 5/3 anaphase cell with anaphase bridges. (**c**,**d**) Quantification of anaphase bridges in controls HCT116 and RPE1, and the respective trisomic and tetrasomic derivatives. (**e**) Representative images of an HCT116 5/3 anaphase cell stained with DAPI, anti-centromere antibody CREST and anti-α-tubulin. Arrowhead marks an acentric chromosome fragment. (**f**) Quantification of acentric chromosomal fragments and anaphase bridges. Bridges extend fully between DNA masses; acentric fragments were distinguished from whole-lagging chromosomes by absence of the CREST signal. (**g**) Examples of HCT116 5/3 anaphase cells stained with DAPI and antibodies against BLM (yellow arrowheads), which bind to UFBs. White arrowhead marks an anaphase bridge. (**h**) Quantification of UFBs. Plots (**c**,**d**,**f** and **h**) show mean ± s.e.m. of three independent experiments. At least 100 anaphases were scored in each experiment in **c**,**d**,**f** and **h**; in RPE1 21/3, only 68 (**d**), 51 (**f**) and 54 (**h**) anaphase cells were scored in each experiment. Nonparametric *t*-test; $*P<0.05$, $**P<0.01$ and $***P<0.001$.

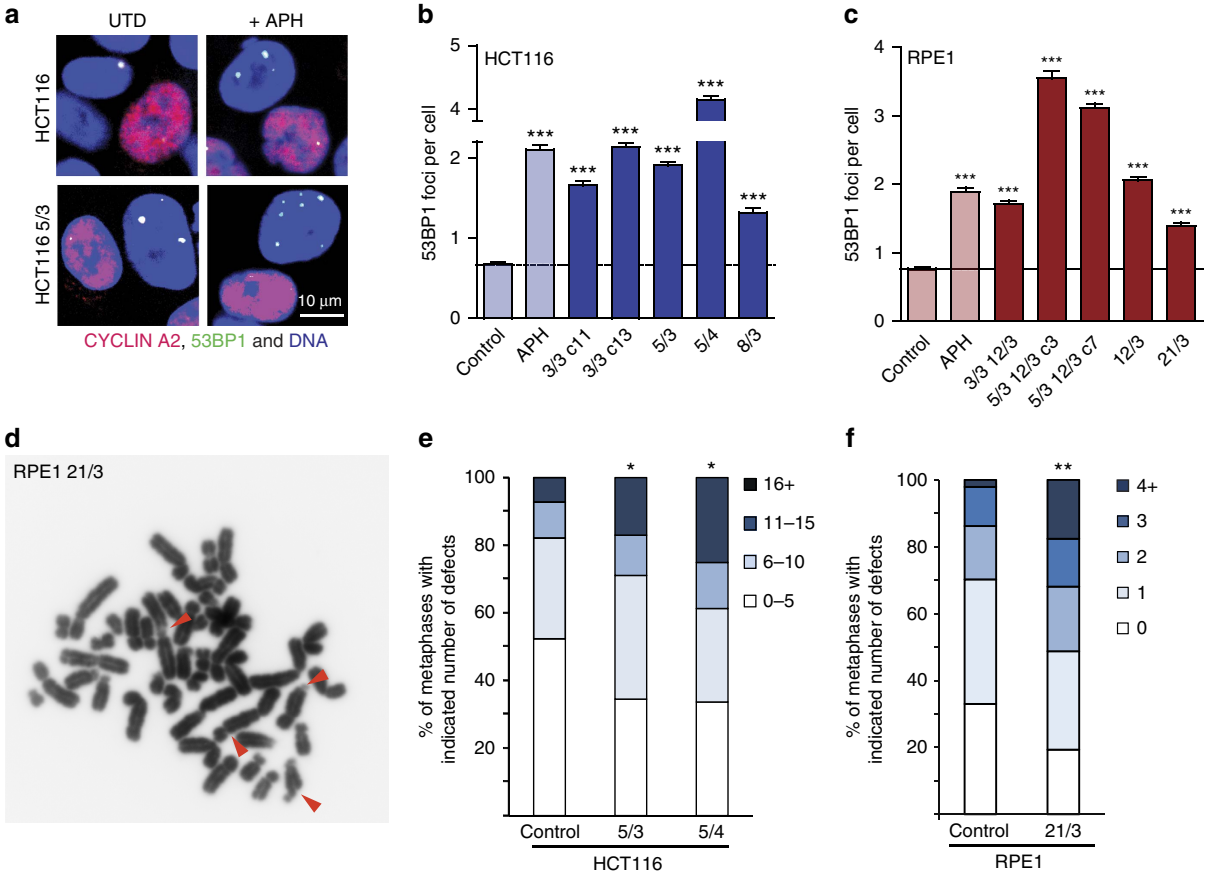

Figure 2 | Trisomy and tetrasomy elevates DNA damage. (**a**) Example of control and aneuploid cells stained with DAPI and antibody against 53BP1 and cyclin A2 to distinguish G1 cells (cyclin A2 negative). (**b**) Average number of 53BP1 foci per G1-phase cell counted in untreated HCT116 (control), upon treatment with 0.25 µM aphidicolin (APH) and in the untreated aneuploid derivatives. (**c**) Average number of 53BP1 foci per G1-phase cell counted in untreated RPE1 (control), upon exposure to 0.25 µM aphidicolin (APH) and in the untreated aneuploid derivatives. All plots show mean ± s.e.m. of three independent experiments, at least 500 cyclin A-negative cells were scored in each experiment. Nonparametric *t*-test; ***$P<0.0001$. (**d**) Structural aberrations, such as gaps and constrictions, on metaphase chromosomes from RPE1 21/3 cells grown under replication stress conditions (0.1 µM aphidicolin and 0.73 mM caffeine). Representative metaphase spread; aberrations are indicated by red arrowheads. (**e**) Quantification of chromosomal aberrations detected in metaphase spreads of HCT116 cells exposed to 0.3 µM aphidicolin for 24 h. Fisher's exact test, *$P<0.05$, $n=94$ (HCT116), 93 (5/3) and 95 (5/4) metaphases analysed in two independent experiments. (**f**) Distribution of metaphases according to the number of gaps and constrictions in RPE1, and its trisomic derivative under treatment with 0.1 µM aphidicolin and 0.73 mM caffeine for 24 h. Fisher's exact test, **$P<0.01$, $n=94$ (RPE1) and 119 (21/3) metaphases analysed in two independent experiments.

presence of extra chromosomes caused a significant two- to fourfold increase in the average number of 53BP1 foci per cell as well as a higher percentage of cells with >3 53BP1 foci in all trisomic and tetrasomic cell lines (Fig. 2b,c and Supplementary Fig. 2a,b). Of note, the numbers of 53BP1 foci tend to correlate with the amount of excessive DNA (Supplementary Fig. 2c). Treatment with the replication inhibitor aphidicolin further increased the number of 53BP1 foci in the tested cell lines (Supplementary Fig. 2d,e).

The higher levels of DNA damage may indicate perturbed DNA replication and/or repair at genomic regions sensitive to replication stress defined as fragile sites[30] that can be observed as structural aberrations on metaphase chromosomes. Our analysis of metaphase spreads from cells grown under normal conditions showed a small, but insignificant trend indicative of higher fragility in response to extra chromosomes (Supplementary Fig. 2f,g). Aphidicolin treatment evoked significantly higher numbers of structural aberrations in trisomic and tetrasomic cells than in control HCT116 and RPE1 cells (Fig. 2d–f). While ~50% of the control HCT116 cells contained >5 breaks, gaps and constrictions, a similar level of aberrations was observed in almost 70% of the metaphases in the trisomic HCT116 5/3 (Fig. 2e). In HCT116, the effect scales with aneuploidy as 25% of metaphases showed very high fragility (>16 gaps and breaks) in tetrasomic cells compared with only 7% of the control diploid cells (Fig. 2e). Increased levels of structural aberrations were also found in trisomic RPE1 21/3 cells compared with the control cells (Fig. 2f). Taken together, the addition of an extra chromosome leads to increased DNA damage and significantly higher levels of chromosome fragility under replication stress.

Extra chromosomes increase sensitivity to replication stress. Replication stress conditions alter the cell cycle profile by slowing the progression of the cells through S-phase and/or by arresting the cells at the S-phase or G2/M checkpoints. Cell cycle analysis demonstrated that whereas under normal conditions both aneuploid HCT116 5/3 and 5/4, and their cognate controls showed nearly identical cell cycle profiles, there was a marked difference upon replication stress (Fig. 3a). Treatment with a low aphidicolin concentration led to an accumulation in late S-phase and G2/M in control cells, and this accumulation was further

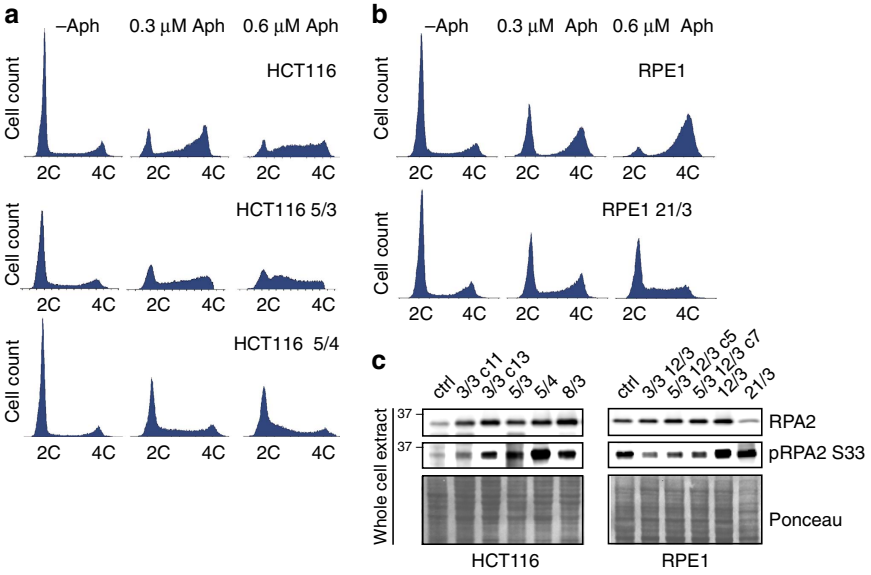

Figure 3 | Altered replication dynamics in trisomic and tetrasomic cells. (**a**) Cell cycle profiles of HCT116, HCT116 5/3 and HCT116 5/4, and (**b**) control RPE1 and trisomic RPE1 21/3 under normal conditions, and also upon treatment with the replication inhibitor aphidicolin. (**c**) Levels of total and phosphorylated RPA2 in the parental cell lines and the trisomic and tetrasomic cell lines.

increased upon stronger replication stress (Fig. 3a). In contrast, trisomic HCT116 5/3 cells accumulated in S-phase following treatment with aphidicolin in a dose-dependent manner (Fig. 3a). The tetrasomic HCT116 5/4 cells were even more sensitive than trisomic cells, as a large fraction accumulated early in S-phase even in the low aphidicolin concentration (Fig. 3a), suggesting that the defects scale with the size of the extra chromosome. Similar results were found in RPE1-derived cells, in which aneuploidy caused arrest earlier in the cell cycle compared with the control cell line for each aphidicolin concentration analysed (Fig. 3b). These results suggest changes in progression through replication in cells with extra chromosomes. Quantifying the average incorporation of a thymidine analogue 5-ethynyl-2′-deoxyuridine (EdU) followed by its visualization by copper-catalyzed azide alkyne cycloaddition (Click reaction) showed that all trisomic and tetrasomic cells replicated their DNA slower than the control cells (Supplementary Fig. 3a–c). In addition, most cell lines with extra chromosome activated the replication checkpoint as we observed increased phosphorylation of RPA2 even under non-perturbed conditions (Fig. 3c). These findings are in agreement with our previous observations that the progression through S-phase is impaired by the presence of extra chromosomes[4].

Aneuploidy causes genomic rearrangements. We hypothesized that the replication stress caused by the presence of extra chromosomes might induce stably inherited genomic rearrangements in human cells. To determine whether this is the case, we performed mate-pair next-generation sequencing (NGS) of the parental cell line HCT116 and cognate trisomic and tetrasomic cell (HCT116 5/3 and HCT116 5/4). Comparison with the control cell line revealed two unique chromosomal rearrangements in the trisomic line and four in the tetrasomic line on five different chromosomes (Fig. 4a). Verification by PCR using primers designed to amplify the break point junctions confirmed that five of the six rearrangements have occurred *de novo* in the aneuploid cells (Supplementary Fig. 4a). The rearrangements involved three head-to-tail tandem duplications, one tail-to-head deletion and one tail-to-tail inversion (Fig. 4b,c). Sanger sequencing of the break point junctions revealed microhomologies in all cases, indicative of replication-mediated rearrangement formation (Fig. 4c and Supplementary Fig. 4b)[31].

To determine the rate of *de novo* chromosomal rearrangements in aneuploids and in parental cells, we generated two sets of 12 clonal cell lines, each originating either from a single HCT116 5/3 cell or from a single HCT116 cell. After 30 cell doublings, DNA was isolated from each clonal cell line and subjected to SNP-array analysis (Supplementary Fig. 4c). Eight out of the 24 trisomic clonal lines contained *de novo* copy number aberrations (CNAs), whereas none were detected in the 24 clonal cell lines derived from the parental HCT116 cells (Fig. 4d). In total, we identified 12 CNAs on 7 different chromosomes that include a gain of chromosome 7, four duplications and seven deletions ranging from 105 kb to several megabases (Fig. 4e, Supplementary Fig. 5 and Supplementary Table 2). Permutation testing confirmed that the accumulation of *de novo* CNAs among HCT116 5/3 as compared with HCT116 cells was unlikely to have occurred by chance (P value = 0.0021). By comparison of the identified break point junctions with previously documented common fragile sites, we found that one site overlapped with the fragile sites mapped in the HCT116 cell line after treatment with the replication inhibitor aphidicolin[32], and 8 out of the 12 CNAs were mapped to fragile sites identified in lymphocytes[33] (Fig. 4e and Supplementary Table 2). Taken together, the presence of extra chromosomes significantly increases the occurrence of CNAs in human cells. The break point sequences, the types of identified chromosomal rearrangements and their frequent overlap with common fragile sites indicate that they arose due to defects during DNA replication.

Reduced MCM2-7 levels contribute to genomic instability. The presence of extra chromosomes triggers global and highly conserved expression changes that affect a large number of cellular pathways[20,21]. We asked whether these expression changes might explain the phenotypes observed in aneuploid cells. To this end, we analysed protein expression data previously generated using quantitative mass spectrometry[4]. Unsupervised hierarchical clustering revealed downregulation of several replication factors in aneuploid cells (Fig. 5a). Strikingly,

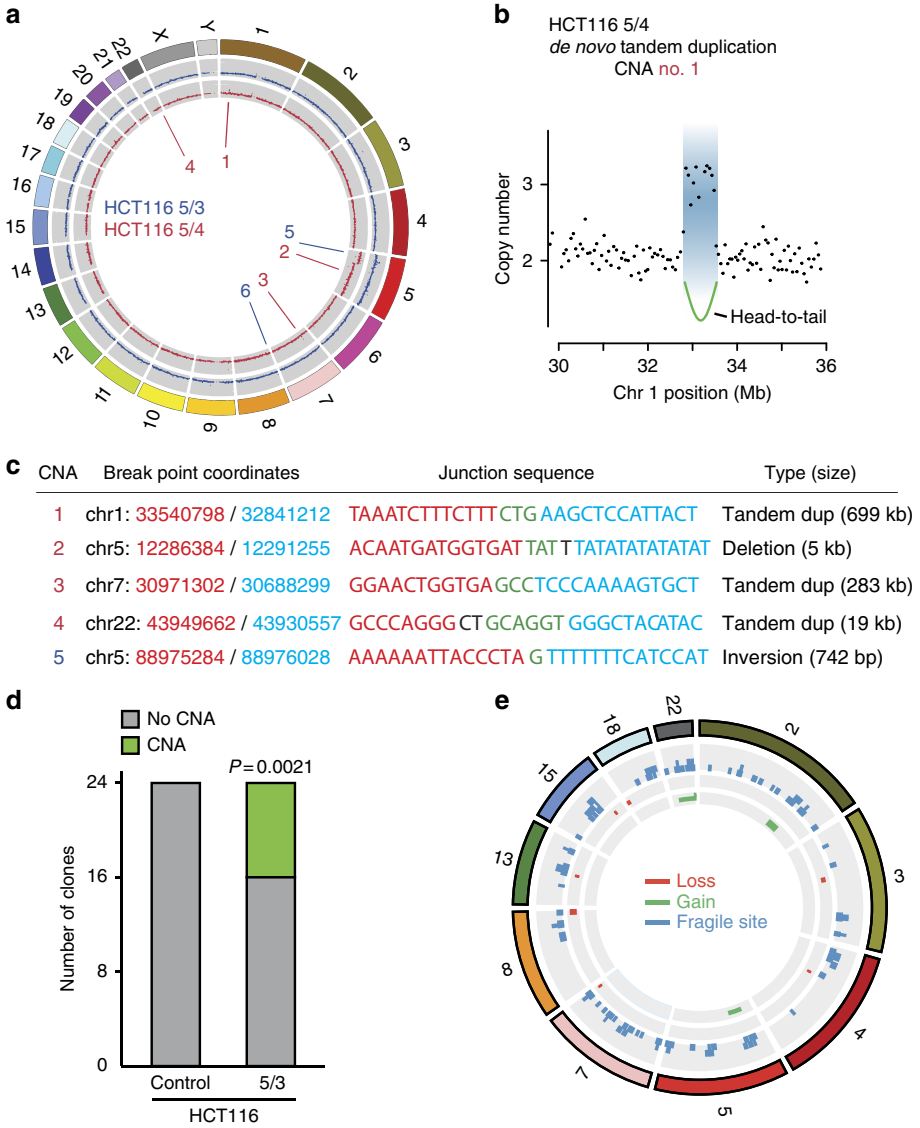

Figure 4 | Trisomic and tetrasomic cells acquire *de novo* chromosomal rearrangements. (**a**) Circos plot displaying *de novo* chromosomal rearrangements in HCT116 5/3 (blue) and HCT116 5/4 (red) cells. The outer ring shows all chromosomes. Both grey rings highlight the copy number profile based on mate-pair sequencing data of HCT116 5/3 (blue) and HCT116 5/4 (red) relative to the parental control. *De novo* CNA break point junctions derived from mate-pair sequencing are indicated with red and blue lines in the middle of the Circos plot. CNA No. 6 was found also in the parental cell line. (**b**) Example of a *de novo* 699 kb tandem duplication identified in HCT116 5/4 cells (CNA no. 1). (**c**) Break point junction sequences obtained by Sanger sequencing of *de novo* chromosomal rearrangement break points from **a**. The two chromosomal loci joined together are indicated in red and blue. Microhomology at the junction sequences is highlighted in green. For full results, see Supplementary Fig. 4b. (**d**) Bar plot depicting the number of clonal lines with and without *de novo* CNAs identified by SNP-array profiling. Twenty-four clonal lines were derived from HCT116 5/3 and parental control, respectively. For full results, see Supplementary Figure 5. (**e**) Circos plot depicting the CNAs identified in the clonal trisomic lines. Only affected chromosomes are visualized. Copy number losses are marked in red, gains in green, and known fragile sites in blue.

expression of all six subunits of the replicative helicase MCM2-7 (hereafter MCM) was consistently and significantly decreased in all analysed cells. MCM is a heterohexameric DNA helicase required for licensing of replication origins and for replication progression. Insufficient licensing owing to low levels of MCM helicase impairs the activation of dormant origins under replication stress conditions[34,35]. Immunoblotting confirmed a general 20–50% decrease in all subunits of MCM helicase in HCT116 5/3 and RPE1 21/3 compared with control cells (Fig. 5b). Strikingly, a decrease of MCM2, MCM3 and MCM7 abundance was observed in 9 out of 10 HCT116- and RPE1-derived aneuploidies, regardless of the specific karyotype (Fig. 5c,d). Only chromatin-bound MCM contributes to the activation of replication origins; immunoblotting of MCM2, MCM3 and MCM7 from the chromatin fraction in both asynchronous and synchronized cells confirmed that trisomic and tetrasomic cells load significantly less helicase on DNA than cognate controls (Fig. 5e,f and Supplementary Fig. 6). It should be noted that immunoblotting confirmed downregulation of additional replication proteins, but none of them as consistently as the subunits of the helicase MCM (Fig. 5c,d).

We hypothesized that the low levels of MCM2-7 contribute to the replication defects observed in aneuploid cells. To test this hypothesis, we partially depleted MCM2 by short interfering RNA (siRNA) in parental HCT116 and RPE1. The abundance of another subunit, MCM7, conjointly decreased, indicating a

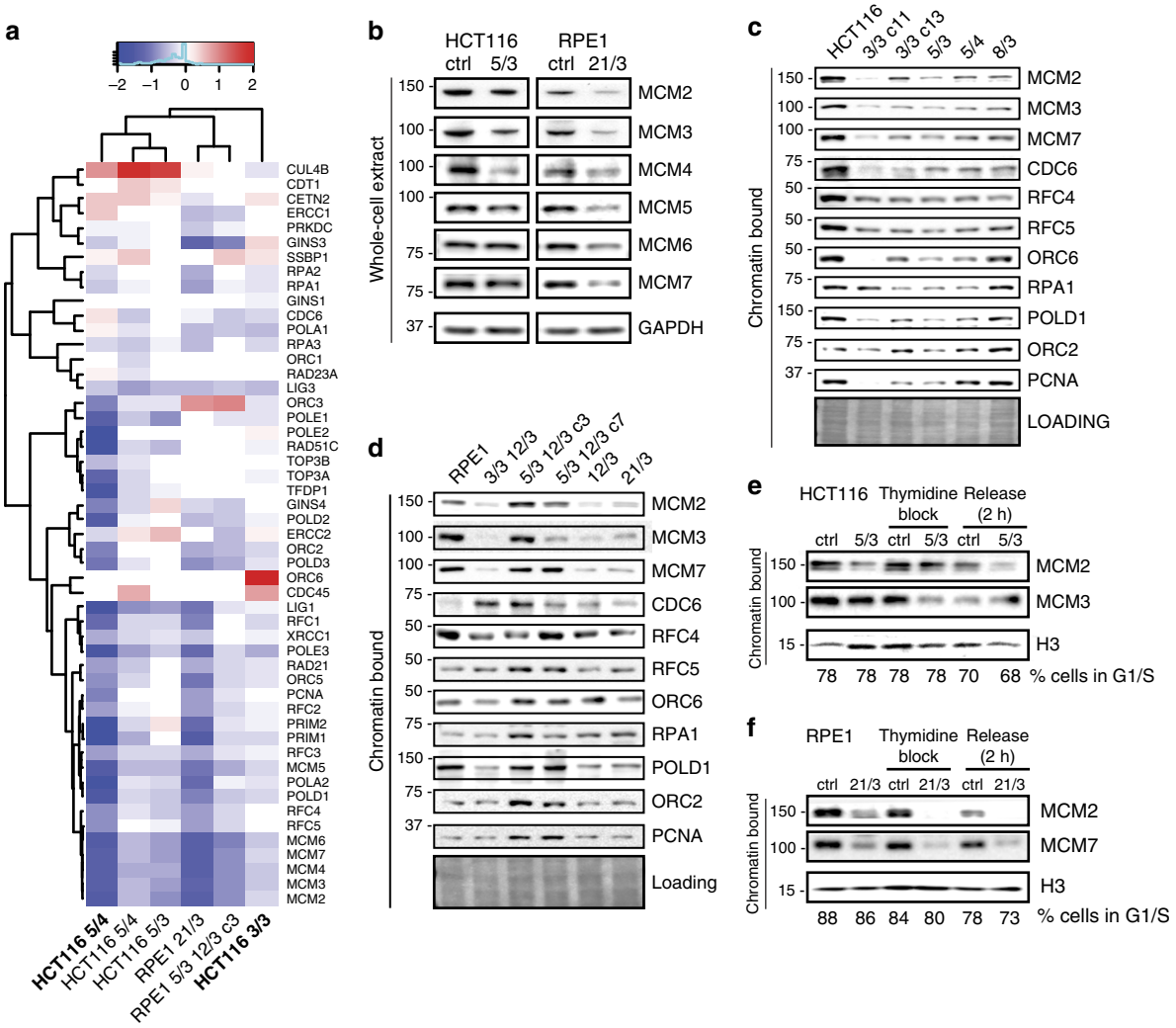

Figure 5 | The abundance of replication factors is decreased in response to extra chromosomes. (**a**) Unsupervised hierarchical clustering of the protein abundance fold changes (calculated log2 aneuploid-to-diploid ratio) of factors assigned to KEGG (Kyoto Encyclopedia of Genes and Genomes)-defined term replication; manually curated. HCT116 5/4 and HCT116 3/3 marked in the figure in bold are cell lines previously constructed by Minoru Koi; these cell lines were used only for the global proteome analysis. See Methods for more details. (**b**) Immunoblotting of subunits of the replicative helicase MCM2-7 in whole-cell extracts from trisomic cell lines and their respective controls. (**c**,**d**) Levels of chromatin-bound replication proteins in the parental cell lines and the trisomic and tetrasomic cell lines. Note that MCM2, 3 and 7 are downregulated in all cell lines with extra chromosomes except for RPE1 5/3 12/3 c3. Ponceau staining was used as loading control. (**e**,**f**) Levels of chromatin-bound subunits of the MCM2-7 helicase in asynchronous and synchronized cells in HCT116 5/3 and RPE1 21/3, and respective controls.

decrease of the entire complex (Fig. 6a). The partial depletion of MCM2 was sufficient to trigger accumulation of 53BP1 foci and an increased frequency of anaphase bridges in the presence or absence of aphidicolin in HCT116 (Fig. 6b,c) as well as in RPE1 (Supplementary Fig. 7a–c). Other replication factors such as CDC6, ORC2 and RPA1 were also partially downregulated in some cell lines with extra chromosomes; however, downregulation of CDC6 and ORC2 in control cells did not trigger accumulation of 53BP1 foci and anaphase bridges, whereas partial downregulation of RPA1 caused high levels of DNA damage (Supplementary Fig. 7d–i). To further test which of the downregulated replicative factors are limiting in cells with extra chromosomes, we transfected both diploid cells and respective trisomic and tetrasomic cell lines with plasmids carrying either functional or mutant alleles of MCM2, ORC1 and RPA1. The mutant protein MCM2-457A cannot be phosphorylated by Dbf4-dependent kinase (DDK) kinase, thereby rendering replication firing inefficient[36]. ORC1ΔBAH shows decreased binding to DNA[37], and RPA1 L221P impairs DNA replication and leads to accumulation of DNA damage[38]. None of the mutant alleles exert detrimental effects in the presence of the endogenous wild-type allele; however, they are toxic for a cell in the absence of the wild-type protein. We found that overexpression of the MCM2-457 A allele was highly toxic in trisomic and tetrasomic cell lines, but showed no effect in the control cell lines (Fig. 6d). In contrast, overexpression of ORC1ΔBAH and RPA1 L221P mutants did not impair the proliferation of any of the aneuploid cell lines significantly more than overexpression of the wild-type allele (Fig. 6e,f). We conclude that the MCM helicase is the limiting factor for replication in cells with extra chromosomes.

Finally, we asked whether increasing the levels of MCM2-7 can rescue the accumulation of DNA damage in aneuploid cells. To this end, we transiently transfected HCT116 5/3 and 5/4 with either empty pcDNA vector or with a version carrying pCMV-MCM7. The levels of the chromatin-bound MCM7 as well as MCM2 increased in cells transfected with pCMV-MCM7

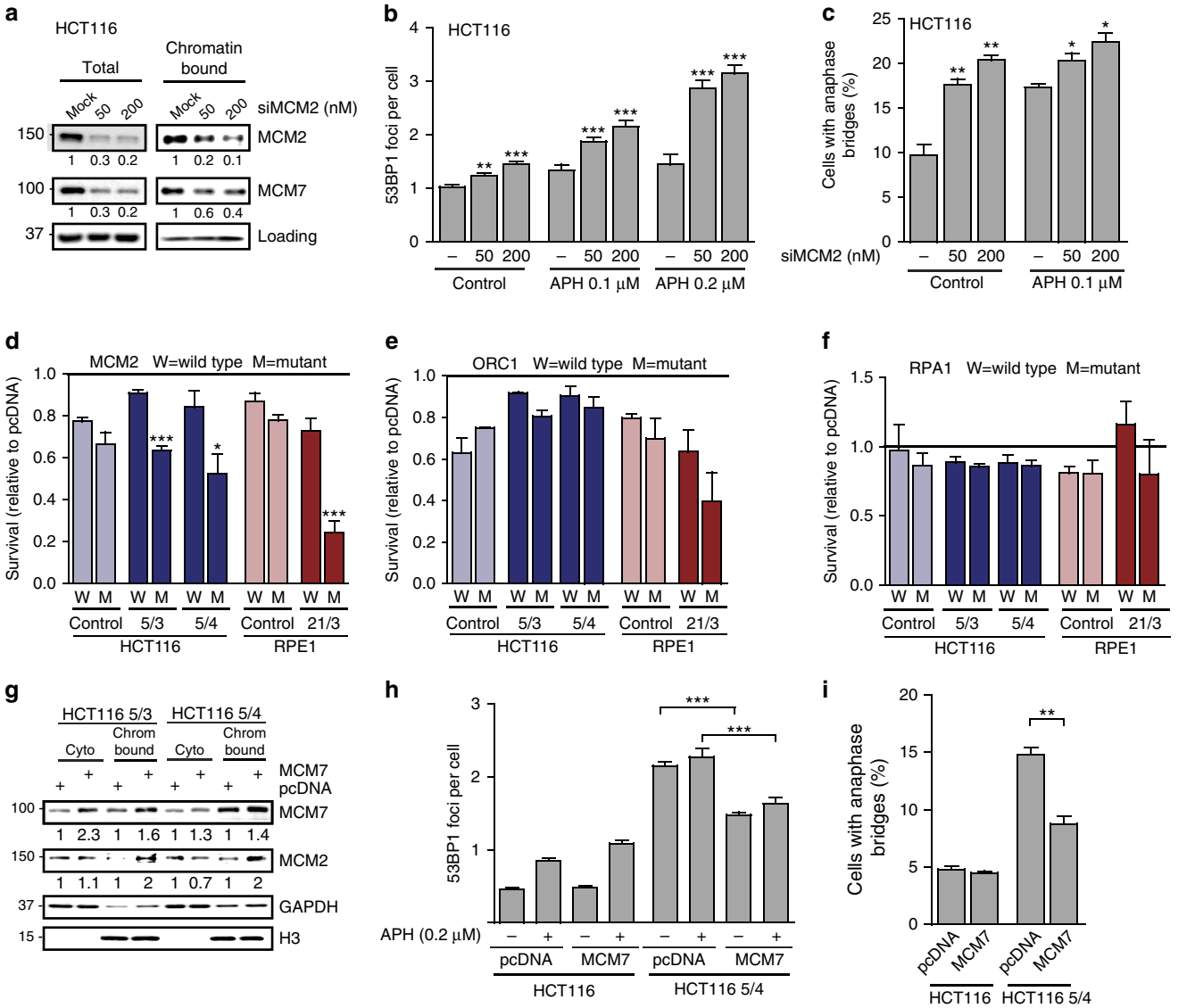

Figure 6 | The accumulation of DNA damage is sensitive to abundance changes of MCM2-7 subunits. (**a**) Levels of total and chromatin-bound MCM2 and MCM7 in parental HCT116 upon siRNA-mediated depletion of MCM2. (**b**) Accumulation of 53BP1 and (**c**) anaphase bridges in HCT116 upon depletion of MCM2 with and without replication stress. All plots show mean ± s.e.m. of three independent experiments, at least 500 cyclin A-negative cells or 50 anaphases were scored in each experiment. (**d**) Survival rates of HCT116, RPE1 and their trisomic and tetrasomic derivatives upon overexpression of wild-type and mutant alleles of MCM2 (MCM-457A). (**e**) Survival rates of HCT116, RPE1 and the trisomic and tetrasomic derivatives upon overexpression of wild-type and mutant alleles of ORC1 (ORC1ΔBAH). (**f**) Survival rates of HCT116, RPE1 and the trisomic and tetrasomic derivatives upon overexpression of wild-type and mutant alleles of RPA1 (RPA1 L221P). Survival rates were normalized to the control (pcDNA transfected sample). All plots show mean + s.e.m. of three independent experiments; nonparametric *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**g**) Levels of total and chromatin-bound MCM2 and MCM7 in HCT116 5/3 upon transient overexpression of MCM7. (**h**) Formation of 53BP1 foci and (**i**) accumulation of anaphase bridges in HCT116 and HCT116 5/4 upon transient overexpression of MCM7. One representative plot of three independent experiments is shown. Nonparametric *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

in comparison with control cells (Fig. 6g). Importantly, the increase in the MCM2-7 abundance resulted in a significant decrease in 53BP1 foci formation as well as lower occurrence of anaphase bridges in four different cell lines with extra chromosomes (HCT116 3/3, 5/3, 5/4 and RPE1 21/3), but did not affect the control HCT116 or RPE1 (Fig. 6h,i and Supplementary Fig. 8a,b). Overexpression of other replication factors (PolD1,4, RPA1,2,3 and CDC6) or control renilla luciferase did not affect the accumulation of 53BP1 foci (Supplementary Fig. 8c,d). Supplementing the medium with a high concentration of DNA synthesis precursors showed a mild rescue of the 53BP1 foci formation in only one cell line and no rescue of the EdU incorporation (Supplementary Fig. 8e–g). Altogether, our findings strongly suggest that the presence of extra chromosomes causes replication stress and genomic instability in human cells owing to downregulation of components of the MCM2-7 helicase.

Discussion

Here we demonstrate for the first time that the addition of even a single chromosome to human cells promotes genomic instability by increasing DNA damage and sensitivity to replication stress. Replication stress is emerging as one of the primary causes of

genomic instability during early stages of tumorigenesis[9–11]. Our findings provide a rationale for how random missegregation of a single chromosome might contribute to genomic instability and potentially to early events in tumorigenesis.

To systematically analyse the effect of trisomy and tetrasomy on genomic instability in human cells, we analysed five different trisomies and tetrasomies derived from HCT116 and five RPE1-derived cell lines. Remarkably, we found strikingly similar phenotypes regardless of the identity of extra chromosomes. Indeed, the presence of extra chromosomes leads to (1) accumulation of DNA damage likely originating from replication errors (Figs 1 and 2); (2) increased sensitivity to additional replication stress (Fig. 3a,b); and (3) abnormal DNA replication (Supplementary Fig. 3 and Fig. 3c). The uniformity of the phenotypes and the fact that the degree of some defects tends to scale with the amount of the added DNA suggest that the presence of an additional chromosome alone is the culprit, which causes the genetic instability in these cells.

The altered DNA replication and increased DNA damage affects genomic stability in trisomic and tetrasomic cell lines, as documented by the increased occurrence of *de novo* CNAs. These structural rearrangements were caused by the presence of extra chromosomes, as parental HCT116 did not show any CNAs after the same number of generations (Fig. 4a,d). The CNAs affected all chromosomes evenly, and no enrichment on the extra chromosome was observed. The pattern of identified CNAs, showing a bias towards deletions and tandem duplications, and the occurrence of microhomology at the break point junctions further support the idea that the addition of extra chromosomes specifically impairs DNA replication, thus inducing replication-induced breaks at stalled/arrested replication forks[31].

What causes the genetic instability in response to extra chromosomes? It has been shown that trisomy and tetrasomy cause genome-wide gene expression changes; one of the most prominent features is a strong downregulation of factors related to DNA replication[20,21]. In particular, the subunits of the replicative helicase MCM2-7 are significantly downregulated in all aneuploid cell lines that we analysed (Fig. 5). Loading of the MCM helicase together with ORC2-5 proteins, CDT1 and CDC6, is essential for licensing of origins of replication, and MCM is also required for fork progression. Replication dynamics are unaffected by partial depletion of MCM under normal growth conditions as the number of MCM complexes loaded onto the DNA is greater than the number of the actual active origins[34,35]. Under replication stress, however, a reduction in the level of MCM proteins leads to a decreased licensing of dormant origins that are needed to allow completion of the DNA replication. Accordingly, cells are markedly sensitive to MCM dosage changes. Several lines of evidence suggest that the observed decrease of 20–50% of MCM2-7 abundance indeed contributes to the genomic instability observed in cells with extra chromosomes. First, depletion of MCM in control cells to levels equivalent to those observed in tri- and tetrasomes results in comparable increase in DNA damage foci, whereas partial depletion of ORC2, RPA1 and CDC6 did not cause similar phenotypes (Fig. 6a–c and Supplementary Fig. 7a–i). Second, depletion of MCM3 was previously shown to lead to an increase in fragile site instability[39], similarly to cells with extra chromosomes that show increased levels of fragile sites on metaphase spreads under replication stress conditions. Moreover, fragile site instability may originate from origin paucity and/or from a failure in dormant origin activation[39,40]. Third, mouse models homozygous for an MCM4^{chaos} mutation that impairs the stability of the MCM4 subunit, or an MCM2$^{IRES\text{-}CreERT2}$ mutation that reduces the levels of MCM2, suffer from impaired proliferation, slow replication rates and genomic instability[41–43], comparable to trisomic and tetrasomic cell lines in this study. Fourth, overexpression of mutant MCM2 protein is toxic in cells with extra chromosomes but not in control cell lines, whereas mutant RPA1 and ORC1 do not affect the proliferation of aneuploid cell lines more than overexpression of the wild-type allele, suggesting that indeed the MCM complex is the limiting DNA replication factor in trisomic and tetrasomic cells. Finally, we show that elevating the levels of MCM2-7 by exogenous overexpression alleviates the replication-induced defects and DNA damage in aneuploid cells (Fig. 6g,h). The effect of MCM overexpression was stronger in trisomies and tetrasomies with a relatively high levels of genomic instability, such as HCT116 5/4 or RPE1 21/3, than in HCT116 5/3 with a rather mild defect. This is in agreement with the idea that high levels of MCM2-7 helicase become critical in cells with elevated replication stress, and also suggest that other factors play a role in genomic instability of aneuploids as well.

Alternative explanations for the observed phenotypes are possible. For example, overexpression of a specific gene located on a supernumerary chromosome might impair maintenance of genome stability. However, the fact that the trisomies and tetrasomies of five different chromosomes (3, 5, 8, 12 and 21) lead to strikingly similar phenotypes does not support this explanation. Another possibility is that the extra DNA simply titrates away a limiting factor, thus slowing down replication. This hypothesis implies that additional DNA and its replication alone should trigger the same phenotypes. However, transcriptional silencing of chromosome 21 using XIST expression in cells with trisomy 21 results in improved proliferation[44], similarly as the full removal of chromosome 21 by counter-selection against TKNeo[45]. Moreover, haploid yeast with extra chromosomes show genetic instability, but the phenotype is not observed in cells that contain a yeast artificial chromosome that can be replicated, but not transcribed by these cells[24]. Thus, we propose that it is not the need to replicate additional DNA, but rather the pathway deregulation and stoichiometric imbalances caused by aneuploidy that promote the observed genetic instability.

Why the replication factors are downregulated in response to aneuploidy remains enigmatic. This question goes together with the observation that aneuploidy causes global expression changes, with DNA replication being among the most consistently downregulated pathways across several analysed cell lines and species[20,23]. One possibility is that some pathways are deregulated due to the proteotoxic stress and protein folding defect in trisomic and tetrasomic human cells[23]. Accordingly, several factors involved in DNA repair and replication are well-characterized clients of molecular chaperones[46]. Alternatively, activity of E2F transcription factors that control expression of MCM subunits or DNA polymerases[47] might be altered in response to trisomy and tetrasomy. Interestingly, recent results revealed that ageing haematopoietic stem cells in mice suffer from replication stress, cell cycle defects and chromosomal aberrations owing to a decreased expression of MCM helicase[48]. The reasons for the downregulation of MCM in ageing haematopoietic stem cells remain unclear. Together with our observations in trisomic and tetrasomic cells, we suggest that the expression levels of MCM helicase are a highly sensitive measure of cellular stress.

In summary, our results show that addition of even a single chromosome renders human cells sensitive to replication stress and elevates the occurrence of CNAs in human cells, thereby suggesting a novel mechanism for how chromosome segregation errors may fuel genomic instability. The observed phenotypes may provide new insights into the causes of developmental defects associated with congenital trisomies and might help address the question of why patients with Down's syndrome show altered spectra of malignant diseases with an increase in haematological malignancies and a decrease in solid tumours [49].

Markedly, cancer cells often express DNA replication factors including the MCM helicase at high levels, and MCM2 and MCM7 are considered as biomarkers and potential therapeutic targets for cervical, colorectal and other tumours (for example, refs 50–52). Identification of the mechanisms that allow cancer cells to adapt to aneuploidy by elevating the expression of replication factors might illuminate novel opportunities for cancer therapy.

Methods

Cell lines. The cell line RPE1 hTERT (redeferred to as RPE1) and RPE1 hTERT H2B-GFP were a kind gift from Stephen Taylor (University of Manchester, UK). HCT116 H2B-GFP was generated by lipofection (FugeneHD, Roche) of HCT116 (American Type Culture Collection no. CCL-247) with pBOS-H2B-GFP (BD Pharmingen) according to manufacturer's protocols. Trisomic and tetrasomic cell lines were generated by microcell-mediated chromosome transfer as described below. The donor mouse cell lines A9(Neo3), A9(Neo5), A9(Neo8) and A9(Neo21) were purchased from the Health Science Research Resources Bank (HSRRB), Osaka 590-0535, Japan. All cell lines were maintained at 37 °C with 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 U penicillin and 100 U streptomycin. The cell lines HCT116 3/3, HCT116 H2B-GFP 5/3, HCT116 H2B-GFP 5/4, RPE1 5/3 12/3, RPE1 H2B-GFP 21/3 and A9(Neo5) were grown supplemented with 400 μg ml^{-1} G418. The cell line HCT116 5/4 as well as the cell lines stably transfected with H2B-GFP were grown in media supplemented with 6 μg ml^{-1} blasticidin S. Before each experiment, aneuploid cells were grown one passage in medium without the antibiotic selection. All the cell lines were tested for mycoplasma contamination. The cell lines HCT116, HCT116 5/4 and HCT116 3/3 were kindly provided by Minoru Koi, Baylor University Medical Centre, Dallas, TX, USA. These cell lines were used only for the global proteome analysis (Fig. 5a). For nucleoside incorporation analysis, the culture medium was supplemented with adenosine (Sigma A9251), cytidine (Sigma C4654), uridine (Sigma U3750) and guanosine (Sigma G6752) to a final concentration of 50μM for 48 h. In order to synchronize cells in S-phase, HCT116- and RPE1-derived cells were cultured for 30 h in 2 mM thymidine, washed three times in PBS and were released into the standard DMEM medium.

Microcell-mediated chromosome transfer. To generate aneuploid HCT116 and RPE1 containing an additional chromosome 3, 5, 8 or 21, microcell fusion (Supplementary Fig. 1a) was performed as follows[53]. In brief, murine donor cells containing additional human chromosome with a resistance gene were treated for 48 h with colchicine (final concentration 60 ng ml^{-1}). Donor cells were trypsinized and seeded on plastic bullets. After the cells were attached to the surface, bullets were centrifuged at 27,000*g* for 30 min at 30–34 °C in DMEM supplemented with 10 μg ml^{-1} cytochalasin B. Cell pellets were resuspended in serum-free DMEM and filtered to clear suspension from mouse cells. Filtered microcells were mixed with phytohaemagglutinin (PHA-P) and added to the recipient cell line HCT116 H2B-GFP, RPE1 or RPE1 H2B-GFP. Fusion of microcells with the recipient cells was facilitated by polyethylene glycol 1500 treatment. Cells containing the additional human chromosome were selected in medium supplemented with 400 μg ml^{-1} G418 or with hygromycin. The chromosome 12 in trisomic and tetrasomic cell lines derived from RPE1 carries no gene coding for resistance. The cell lines were obtained because of a spontaneously occurring aberration. Parental cell line HCT116 stably expressing histone H2B-GFP was used for cell lines marked HCT116 5/3 (trisomy 5) and HCT116 5/4 (tetrasomy 5); parental cell line RPE1 (human retinal pigment epithelial cell line, hTERT immortalized) was used for RPE1 5/3 12/3 (trisomy 5, 12); parental cell line RPE1 stably expressing histone H2B-GFP was used for RPE1 21/3 (trisomy 21). Cells were grown in DMEM GlutaMax (Gibco) supplemented with 10% fetal bovine serum and 5% penicillin–streptomycin under standard conditions. Clonal populations arising from single cell after the MMCTs were isolated and further expanded. Subsequently, chromosome spreads combined with chromosome painting were performed (see below). Clonal populations that gained the expected extra chromosomes were further expanded for three passages and at least 15 vials were frozen in liquid nitrogene. Simultaneously, a sample was subjected to SNP-array analysis or array comparative genomic hybridization (aCGH). Only cells with fully analysed karyotypes were used for the experiments. All experiments were always performed from the same passage vial to minimize clonal effects, and the cells were kept in culture for maximum three passages, unless otherwise stated. No selection for the extra chromosome was applied for 24 h before each experiment. For further details, see Supplementary Table 1.

Preparation of chromosome spreads. Cells were grown to 70–80% confluency, treated with 50 ng ml^{-1} colchicine for 3–5 h, collected by trypsinization and centrifuged at 250*g* for 10 min. Pellets were resuspended in 75 mM KCl and incubated for 10–15 min at 37 °C. After centrifugation at 150*g* for 10 min, cell pellets were resuspended in 3:1 methanol/acetic acid to fix the cells. Cell pellets were washed several times in 3:1 methanol/acetic acid, spread on a wet glass slide and air dried at 42 °C for 5 min.

Chromosome painting. Chromosome spreads were prepared as described above. Each sample was labelled with probes for two different chromosomes: a transferred chromosome and a control chromosome. Probes (Chrombios GmbH, Raubling, Germany) for chromosomes 2, 3, 5 and 21 were tagged with TAMRA, FITC, Cy-5 and TAMRA, respectively. The chromosomes were labelled according to the manufacturer's instructions and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were obtained by a fully automated Zeiss inverted microscope.

Array comparative genomic hybridization. Genomic DNA for aCGH analysis was extracted using the Qiagen Gentra Puregene Kit according to manufacturer's instructions. The aCGH analysis was performed by IMGM Laboratories, Martinsried, Germany. Commercially available human genomic DNA (Promega) was used as a reference sample for all 4 × 44K array-based analyses (HCT116, HCT116 3/3, HCT116 5/4, HCT116 H2B-GFP 5/3, HCT116 H2B-GFP 5/4, RPE1 and RPE1 5/3 12/3). RPE1 H2B-GFP 21/3 was analysed by SurePrint 4 × 180K G3 Human CGH Microarray. Genomic DNA extracted from HCT116 was used as a reference for the high-density CGH analysis of HCT116 5/4 by the 2 × 400K array. gDNA concentration and DNA absorbance ratio (260 nm/280 nm) were measured by NanoDrop ND-1000 ultraviolet–visible spectrophotometer (PeqLab). An amount of 1 μg of gDNA was used for each reaction. gDNA integrity was tested on an 1.0% agarose gel stained with ethidium bromide. A measure of 1.0 μg gDNA of each sample was subjected to restriction digestion with a combination of Alu I and Rsa I restriction enzymes. The digested gDNA samples were directly labelled with exo-Klenow fragments and random primers by incorporation of Cy-5 dUTP (dUTP = 2′-deoxyuridine 5′-triphosphate) for the experimental samples and Cy-3 dUTP for the reference samples (Genomic DNA Enzymatic Labeling Kit, Agilent Technologies). After purification, each experimental sample was combined with its respective reference sample and hybridized to respective arrays. All microarrays have been washed with increasing stringency using Oligo aCGH Wash Buffers (Agilent Technologies) followed by drying with acetonitrile (Sigma-Aldrich). Fluorescent signal intensities for both dyes were detected with Scan Control 8.4.1 Software (Agilent Technologies) on the Agilent DNA Microarray Scanner and extracted from the images using Feature Extraction 10.5.1.1 Software (Agilent Technologies). The software Feature Extraction 10.5.1.1 as well as the software Genomic Workbench 5.0.14 was used for quality control, statistical data analysis and visualization. Raw microarray data were normalized. ADM-2 aberration algorithm was applied together with centralization algorithm. Aberrations for all samples were filtered from the whole-genome data and analysed based on a threshold of log2 $\geq$0.39 for amplifications and log2 ≤ -0.30 for deletions with at least five consecutive aberrant probes.

Mate-pair NGS. Whole-genome mate-pair sequencing of HCT116 5/3 and HCT116 5/4, and the respective parental control was performed as described[54]. In brief, DNA was sheared to 3 kb fragments, followed by mate-pair library preparation according to the SOLiD 5500 Long Mate-Pair procedure (Life Technologies). Each library was sequenced in 2 × 50 bp configuration on a single lane of a SOLiD 5500 instrument. Colour space reads were mapped to the human genome (GRCh37) using BWA version 0.5.0. Discordant mate pairs were detected and clustered for all samples together as described[54]. Unique clusters of discordant mate-pair reads were identified by filtering out clusters supported by read pairs in >1 sample. Based on discordant mate-pair clusters, we designed primers for break point junction amplifications using Primer3 software. PCR amplification was performed using Amplitaq Gold polymerase (Life Technologies) under standard conditions. Sanger sequencing was performed to identify break point junction sequences at nucleotide resolution.

SNP-array profiling. Human CytoSNP-12 bead chip arrays (Illumina) for detecting CNAs were used in clonal aneuploid and control cell lines. Array hybridization was performed according to the manufacturer's recommendations. CNAs were called using Nexus software (version 7.5.1) with standard settings. To identify unique CNAs in clonal cell lines, we used the Nexus call coordinates and removed all calls of the same type with a reciprocal overlap of at least 60%. Unique CNA calls were manually curated based on copy number and allele frequency profiles. Permutation testing was performed to calculate the significance of the difference in the distribution of unique CNAs among HCT116 (controls) and HCT116 5/3 (cases) clonal lines. We generated 1,000,000 permutations of the case/control labels per set and determined the *P* value as the proportion of randomizations where all 12 unique CNAs were found in HCT116 5/3 clonal lines.

Mapping the fragile sites. Genomic positions of common and rare fragile sites were obtained from refs 32,33. The coordinates matching the chromosomal bands with fragile sites were overlapped with CNA break points using BEDTools[55].

Immunofluorescence labelling. Cells were seeded in glass-bottomed black well 96-well plates (Greiner Bio-One, Frickenhausen, Germany), and when necessary treated with aphidicolin (Sigma, 2 μM for 24 h). At the time of evaluation (2 days after plating), the cells were typically 80% confluent. For 53BP1 foci quantification,

cells were fixed with 3.7% buffered formaldehyde (12 min at room temperature), permeabilized in 0.2% Triton X-100 in PBS (5 min) and co-immunostained with 53BP1 (1:1,000; Santa Cruz sc-22760) and cyclin A2 (1:200; Abcam ab16726) overnight at 4 °C, followed by a secondary anti-rabbit antibody (Alexa Fluor 647 1:1,000; Jackson ImmunoResearch 711-605-152) and anti-mouse antibody (Alexa Fluor 594 1:1,000; Jackson ImmunoResearch 715-858-150) for 1 h at room temperature. To detect UFBs, the cells were fixed with 4% paraformaldehyde (15 min at − 20 °C), permeabilized in 0.5% Triton X-100 in PBS (20 min on ice), blocked (10% FCS and 0.1% Triton X-100) for 45 min at room temperature and immunostained with antibodies against BLM (1:200; Santa Cruz sc-7790) overnight at 4 °C, followed by the secondary anti-goat antibody (Alexa Fluor 647 1:1000; Jackson ImmunoResearch 711-605-152) for 1 h at room temperature. For mitotic error analysis, mitotic cells were collected by mitotic shake-off, fixed in glass-bottomed black well 96-well plates with 100% ice-cold methanol for 10 min and blocked (10% fetal calf serum and 0.1% Triton X-100) for 45 min at room temperature. Cells were co-immunostained at 4 °C overnight with antibodies for α-tubulin (1:500; Sigma T6199) and CREST (1:1,000; Immunovision HCT-0100) followed by a secondary anti-human antibody (Alexa Fluor 647 1:1,000 and Molecular Probes A-21445) and anti-mouse antibody (Alexa Fluor 594 1:1,000 and Jackson ImmunoResearch 715-858-150) for 1 h at room temperature. The DNA was counterstained when necessary by SYTOX Green (1:5,000, Invitrogen S7020).

Microscopy and image analysis. Confocal microscopy was performed using a fully automated Zeiss inverted microscope (AxioObserver Z1) equipped with a MS-2000 stage (Applied Scientific Instrumentation, Eugene, OR), the CSU-X1 spinning disk confocal head (Yokogawa) and LaserStack Launch with selectable laser lines (Intelligent Imaging Innovations, Denver, CO). Image acquisition was randomized and at least 12 non-overlapping fields were captured for each well using a CoolSnap HQ camera (Roper Scientific) and a × 40 air objective (Plan Neofluar × 40/0.75) under the control of the Slidebook software (version 5.0; Intelligent Imaging Innovations). The numbers of 53BP1 foci per nucleus were determined by an automated pipeline using the public domain, free software CellProfiler (http://www.cellprofiler.org/). Image processing was performed for the whole data set using standard CellProfiler modules for illumination correction, segmentation, masking and thresholding. The nuclei were detected using the 473 channel. Out-of-focus images were manually excluded from the data set. Within the detected cyclin A2-negative nuclei, 53BP1 foci number was measured using the Alexa 647 fluorescent signal. The average number of 53BP1 foci per nucleus was determined for each cell line. All experiments were performed in at least three biological replicates, and a minimum of 500 cells were analysed in each replicate. The statistical analysis of the data was performed using Prism (GraphPad Software, Inc.) and significance was calculated by nonparametric two-tailed *t*-test or χ^2-test.

Metaphase preparation for chromosomal fragility analysis. Cells were harvested after a 40-min treatment with 100 ng ml^{-1} colchicine followed by a 30-min incubation in 0.4% KCl at 37 °C and multiple changes of 3:1 methanol:acetic acid fixative. Cells were then dropped onto slides and stained with propidium iodide. Gaps and constrictions on metaphase chromosomes were analysed using a Nikon fluorescent microscope. At least 50 metaphases for each condition were analysed. The significance was analysed by Fisher's exact test with defined classes of cells with ≤5 errors and cells with >5 errors for HCT116; two errors were used as a cut off for RPE1.

Cell cycle analysis. Following treatment with aphidicolin, the cells were harvested and washed with cold PBS. Cells were fixed in − 20 °C 100% methanol and incubated in − 20 °C overnight. For FACS analysis, fixed cells were resuspended in PBS containing 0.2 μg μl^{-1} RNase for 30 min, followed by staining with 50 μg ml^{-1} propidium iodide. DNA content was analysed by flow cytometry (Becton Dickinson FACS calibur).

Global rate of DNA synthesis by EdU incorporation. Two days after plating, cells were treated for 4 h with aphidicolin or hydroxyurea at a final concentration of 0.1 μM and 0.2 mM, respectively. During the last 2 h of treatment, EdU (10 μM) was added to cells. For EdU detection, cells were fixed for 15 min with 3.7% paraformaldehyde, permeabilized for 15 min with 0.1% Triton X-100 in PBS and incubated with EdU Click-iT cocktail (Invitrogen) following the manufacturer's instructions. The DNA was counter-stained with DAPI. All samples were imaged by automatized fluorescence microscopy, and EdU-positive cells were quantified by CellProfiler. The analysis was performed in three biological replicates and at least 1,000 cells were imaged in each replicate.

Subcellular fractionation and western blotting. Cytoplasmic and chromatin-bound fractions were extracted using a subcellular protein fractionation kit (Thermo Scientific, Rockford, IL) following the manufacturer's instructions. Whole-cell lysates were obtained using RIPA buffer supplemented with protease inhibitors (Roche). An amount of 20 μg of protein was then resolved on 10% polyacrylamide gels and transferred to nitrocellulose membranes using the semi-dry technique. Ponceau staining was performed by incubating the membrane for 5 min in Ponceau S solution (0.2 (w/v) in 1% (v/v) acetic acid). After blocking in low fat, 5% BSA in Tris-Buffered Saline with Tween 20 for 1 h at room temperature, membranes were incubated overnight at 4 °C with the following primary antibodies: MCM2 (1:2,000; Abcam ab4461), MCM3 (1:1,000; Cell Signaling no. 4012), MCM4 (1:1,000; Biorbyt orb32710), MCM5 (1:1,000; Biorbyt orb128349), MCM6(1:1,000; Biorbyt orb48451), MCM7 (1:1,000 Santa Cruz sc-9966), FANCD2 (1:1,000; Santa Cruz sc-20022), POLD1 (1:1,000; Bethyl A304-007A), POLD3 (1:1,000; Bethyl A301-244A), RPA1 (1:1,000; Abcam ab79398), CDC6 (1:1,000; Santa Cruz sc-9964), ORC6 (1:1,000, Santa Cruz sc-32735), ORC2 (1:1,000, Santa Cruz sc-32734), RFC4 (1:1,000; Santa Cruz sc-20996), RFC5 (1:1,000; Bethyl A300-146A), PCNA (1:1,000; Chromotek 16d10) and RPA2 (1:1,000; Abcam ab2175). pRPA2 S33 (1:1,000; Bethyl A300-246A), GAPDH (1:2,000; Cell Signaling no. 2118) and H3 (1:1,000; Millipore 05-499). After incubation with horseradish peroxidase-conjugated secondary antibodies, horseradish peroxidase substrate was added and luminescent signals were quantified using a LAS 3000 instrument (FujiFilm). Protein bands were quantified using ImageJ software. All experiments were performed in at least two biological replicates. Uncropped blots are shown in Supplementary Figs 9 and 10.

Electroporation. Cells were electroporated using the Amaxa Nucleofector II apparatus according to the manufacturer's instructions for HCT116 and RPE1 cells, respectively. In brief, 1 million cells were resuspended in Cell Line Nucleofector Solution V containing 2 μg of respective plasmids and transferred to cuvettes. For siRNA, cells were resuspended in Cell Line Nucleofector Solution V containing the indicated concentration of siRNA. The previously published sequences were acquired from Eurofins Genomics and are as follows: MCM2 (5′-GGAGCUCAUUGGAGAUGGCAUGGAA-3′); GL2 (5′-CGUACGCGGA AUACUUCGATT-3′), ORC2 (5′-GAUCAGCUAGACUGGAUAGUA-3′), CDC6 (5′-ACUAGAACCAACAAAUGUC-3′) and RPA1 (5′-AACUGGUUGACGAA AGUGGUG-3′). HCT116 cells were electroporated using the D-032 program, and for RPE1 cells the program wags U-017. Co-transfection with a GFP overexpressing vector revealed that ∼50–70% of the cells are transfected in each experiment. All cells were scored for 53BP1 foci and anaphase bridges quantification after transfection.

Statistical analysis. Statistical analyses were performed using a two-tailed *t*-test, two-tailed Fisher's exact test or χ^2-test as indicated in the corresponding figure legend (*t*-test; $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$). Values are shown as the mean ± s.e.m. of multiple independent experiments. The quantifications were software based (CellProfiler); in other cases, the investigators were blinded to allocation during outcome assessment.

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Author contributions

Z.S. conceived the study; Z.S. and V.P. designed the experiments with input from B.K.; V.P., E.O.-G., N.D. and S.S. performed the experiments; and M.S. de P. and W.P.K. performed the next-generation sequencing and SNP-array analysis. All authors analysed and discussed the results; and Z.S. and B.K. wrote the manuscript.

Additional information

Accession codes: The break point junction sequencing data was uploaded to NCBI GenBank under the accession codes KU587988, KU587989, KU587990, KU587991, KU587992. The mate-pair sequencing data is available from the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena/) under the accession number PRJEB7596. SNP array data were uploaded to NCBI Gene Expression Omnibus upon the accession number GSE71978.

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Competing financial interests: The authors declare no competing financial interests.

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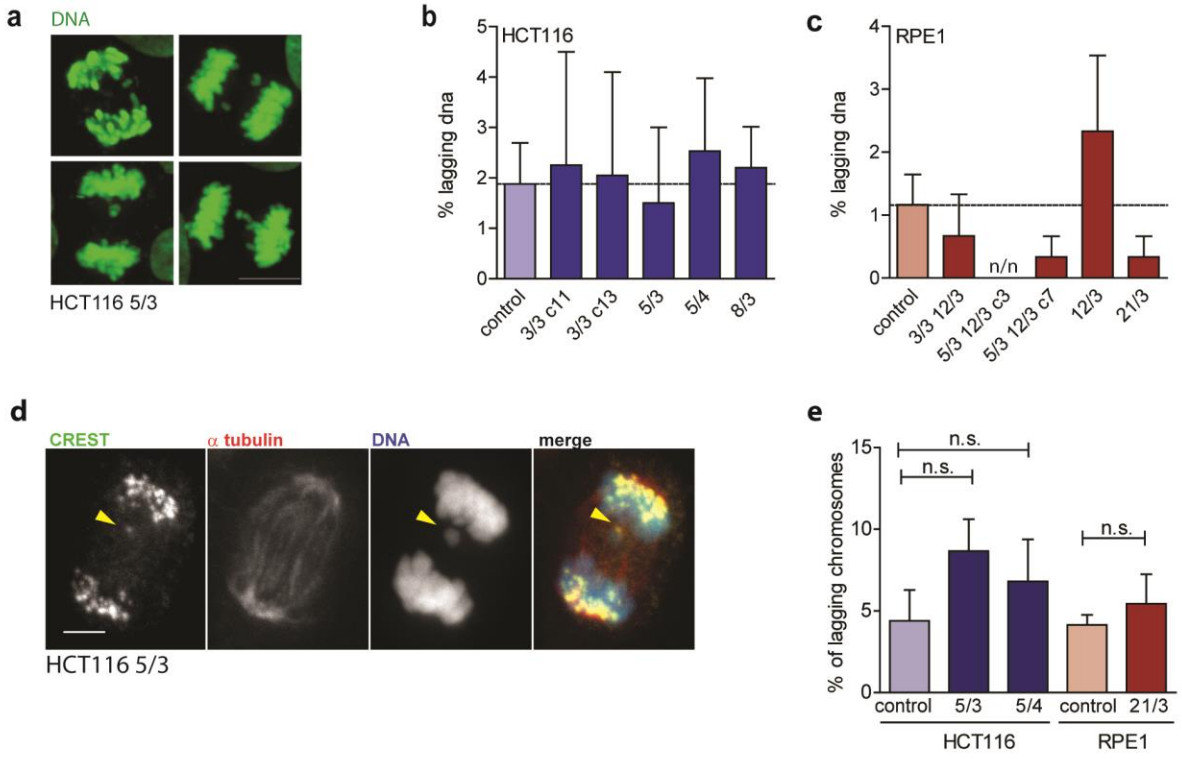
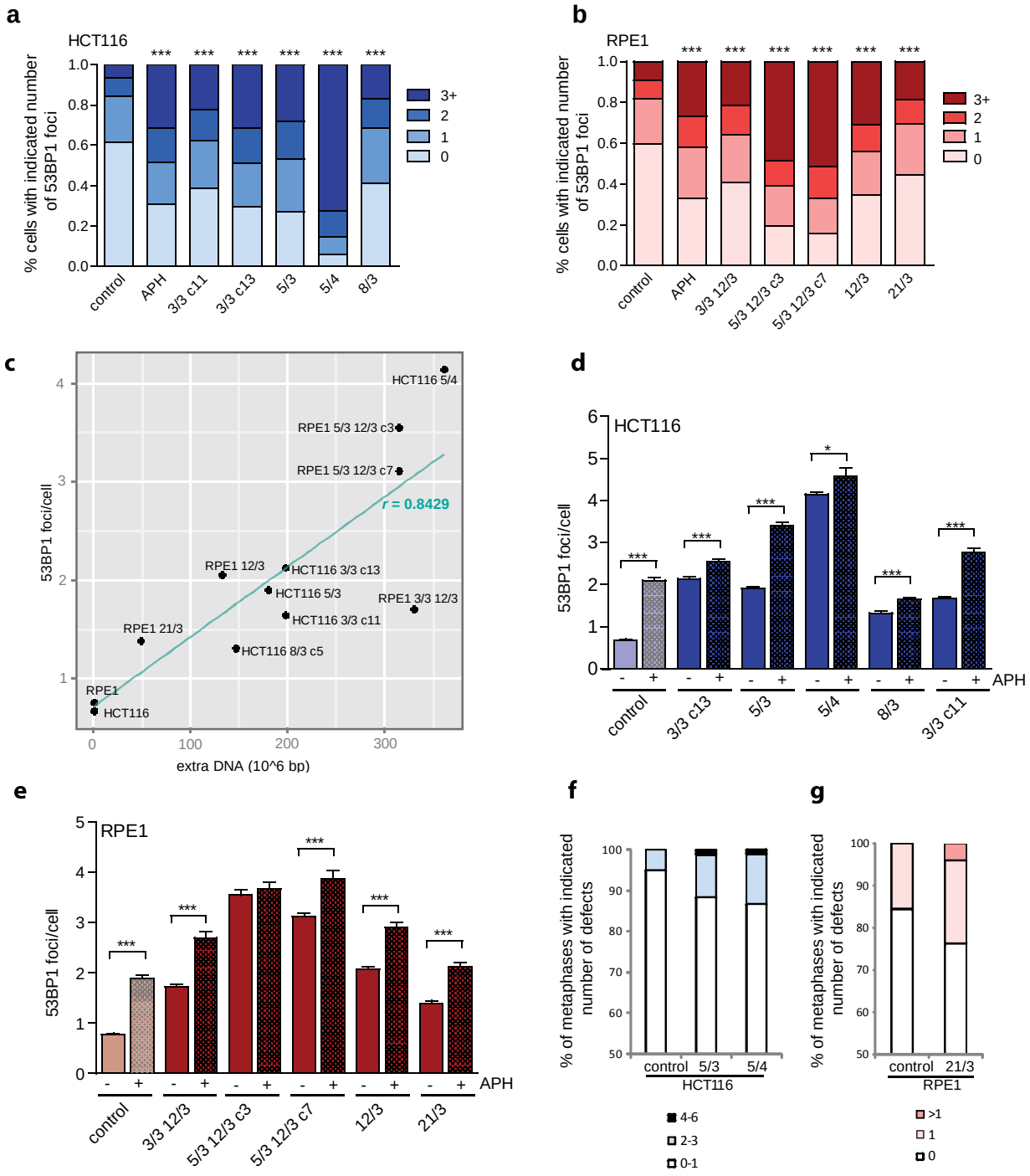


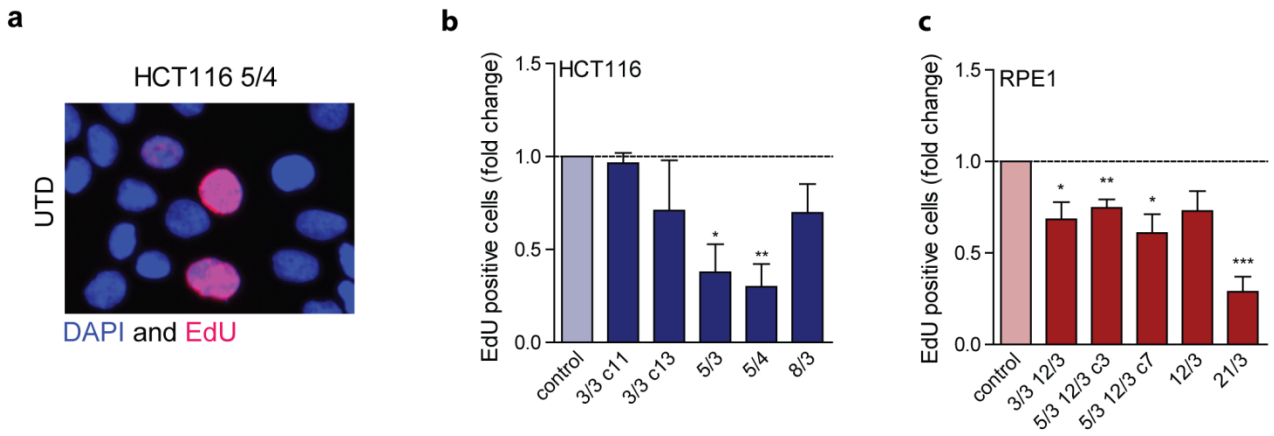
Supplementary Figure 1 Chromosome missegregation in trisomic and tetrasomic cells
(**a**) Example of anaphase cell with a lagging chromosome. Bar = 10 µm (**b**) (**c**) Quantification of lagging chromosomes in diploid controls and the respective trisomic and tetrasomic derivatives. Plots show mean ± SEM of three independent experiments. At least 100 anaphases were scored in each experiment. (**d**) Example of anaphase cell with a lagging chromosome. Bar = 5 µm. (**e**) Percentage of cells with lagging chromosomes scored as DNA mass positive for CREST staining. Plots show mean + SEM of three independent experiments. Non-parametric T-test.



Supplementary Figure 2 Trisomy and tetrasomy elevates DNA damage

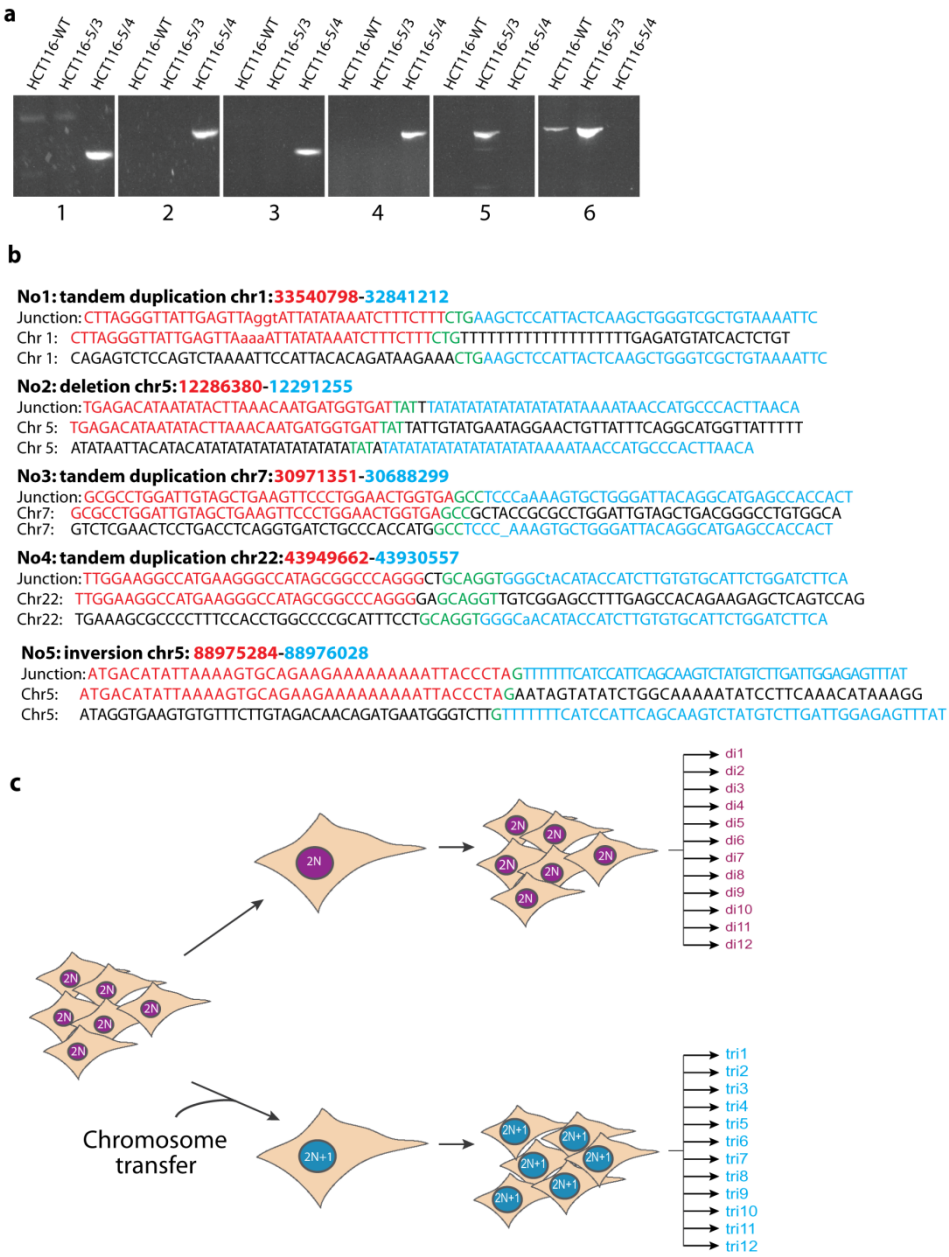
(**a**) (**b**) Quantification of % of cells with specific numbers of 53BP1 foci in HCT116 and the trisomic and tetrasomic derivatives (**a**) and RPE1 and the trisomic and tetrasomic derivatives (**b**). Control - parental cell line. APH -parental cell line treated with aphidicolin. Contingency tables were created from 3 independent experiments (n>500) and chi-square test was calculated comparing the number of cells with less than 3 foci or 3 and more foci in control and each trisomic and tetrasomic derivative. (**c**) The number of 53BP1 foci per cell scales with the amount of additional DNA. Note the similarity of the

independent clonal cells lines with the same extra chromosome - HCT116 3/3 c11 and c13 and RPE1 5/3 12/3 c3 and c7. (**d**)(**e**) 53BP1 foci in parental and derived cell lines upon treatment with aphidicolin. Plots show mean ± SEM of three independent experiments, at least 1000 cyclin A-negative cells were scored in each experiment. Non-parametric T test; ***: P<0.001. (**f**) (**g**) Occurence of metaphases with chromosome breaks and other abnormalities in untreated cells. N = 80,78,90,110,76 metaphases for HCT116, 5/3, 5/4, RPE1 and 21/3, respectively, obtained in two independent experiments.



Supplementary Figure 3 Sensitivity of aneuploid cells to replication stress

(**a**) Representative images of HCT116 5/4 cells stained with DAPI and EdU. Cells were grown in the presence of EdU for two hours. (**b**)(**c**) Quantification of EdU-positive cells in control HCT116 and aneuploid derivatives (**b**) and control RPE1 cells and aneuploid derivatives (**c**). All plots show mean ± SEM of three independent experiments, at least 1000 cells were scored in each experiment. Non-parametric T test; * p<=0.05, ** p<=0.01, *** p<=0.001



Supplementary figure 4 Mate-pair sequencing and single nucleotide polymorphism arrays to identify chromosomal rearrangements in aneuploid cell lines

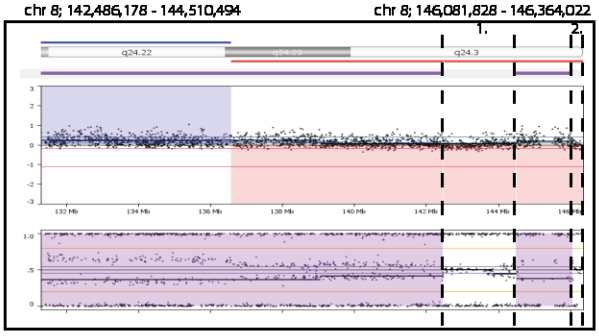
(**a**) Analysis of breakpoint junction by PCR for each of the six predicted de novo chromosomal rearrangements. Note that rearrangement 6 was also identified in the parental HCT116 indicating that this rearrangement did not occur de novo. (**b**) Breakpoint junction sequences of five de novo CNAs identified in aneuploid cells.In each case, the upper sequence denotes the breakpoint junction sequence and the two lower sequences denote the genomic regions where both flanks (red and blue) map to. Microhomologies are indicated in green. (**c**) Schematic depiction of the generation of clones derived from single cells that were used for the single nucleotide polymorphism (SNO) profiling. di1 - 12 clones derived from parental cell line HCT116; tri1 -12 clones derived from trisomic cell line HCT116 5/3. Two sets of these experiments were performed, 2x12 clonal cell populations were analyzed for each cell line.

a

HCT116 5/3 clone 5 — Chromosome 8

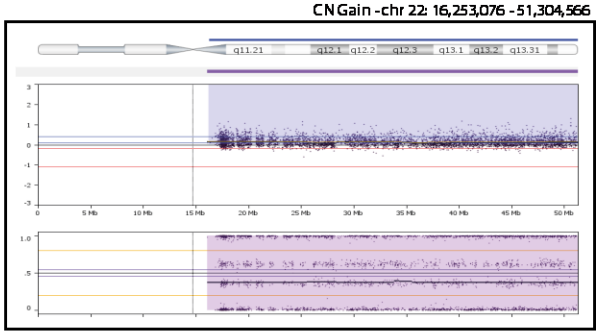
1. CN Loss
chr 8; 142,486,178 - 144,510,494

2. CN Loss
chr 8; 146,081,828 - 146,364,022



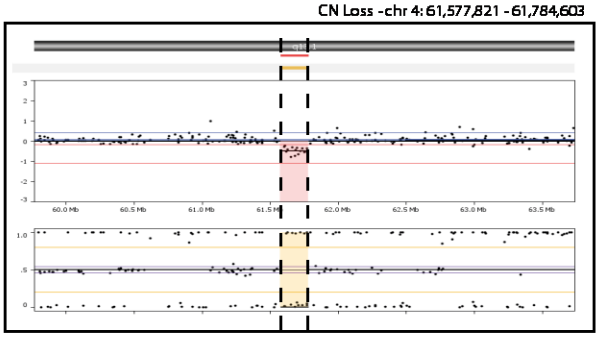
HCT116 5/3 clone 5 — Chromosome 22

CN Gain - chr 22: 16,253,076 - 51,304,566



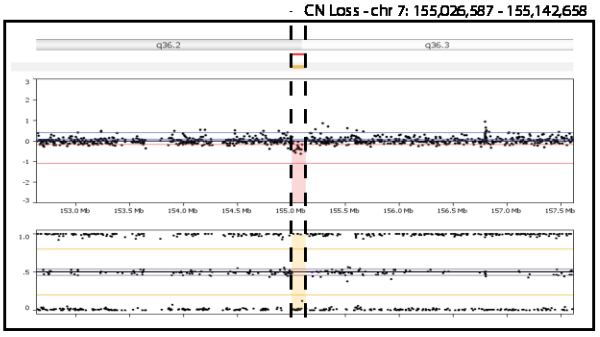
HCT116 5/3 clone 6 — Chromosome 4

CN Loss - chr 4: 61,577,821 - 61,784,603



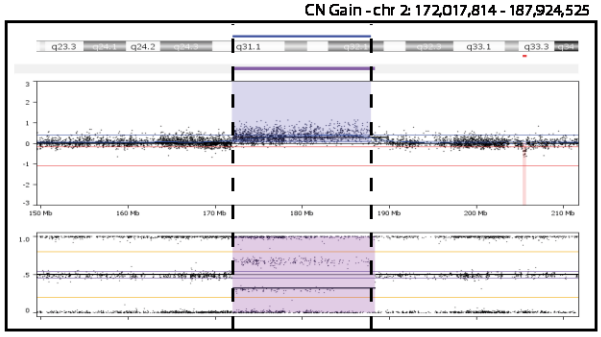
HCT116 5/3 clone 6 — Chromosome 7

CN Loss - chr 7: 155,026,587 - 155,142,658



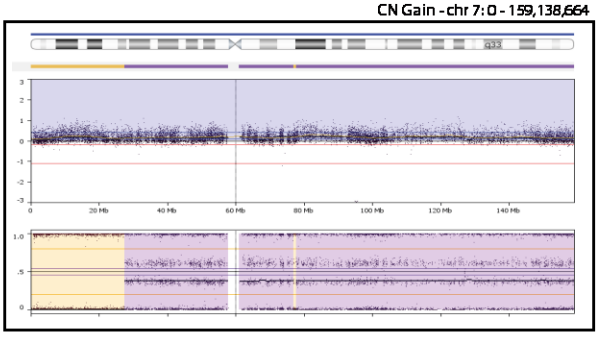
HCT116 5/3 clone 7 — Chromosome 2

CN Gain - chr 2: 172,017,814 - 187,924,525



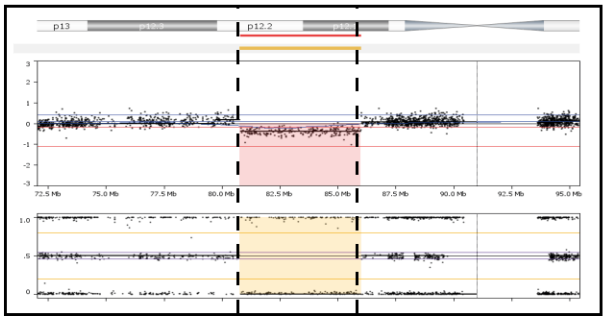
HCT116 5/3 clone 7 — Chromosome 7

CN Gain - chr 7: 0 - 159,138,664

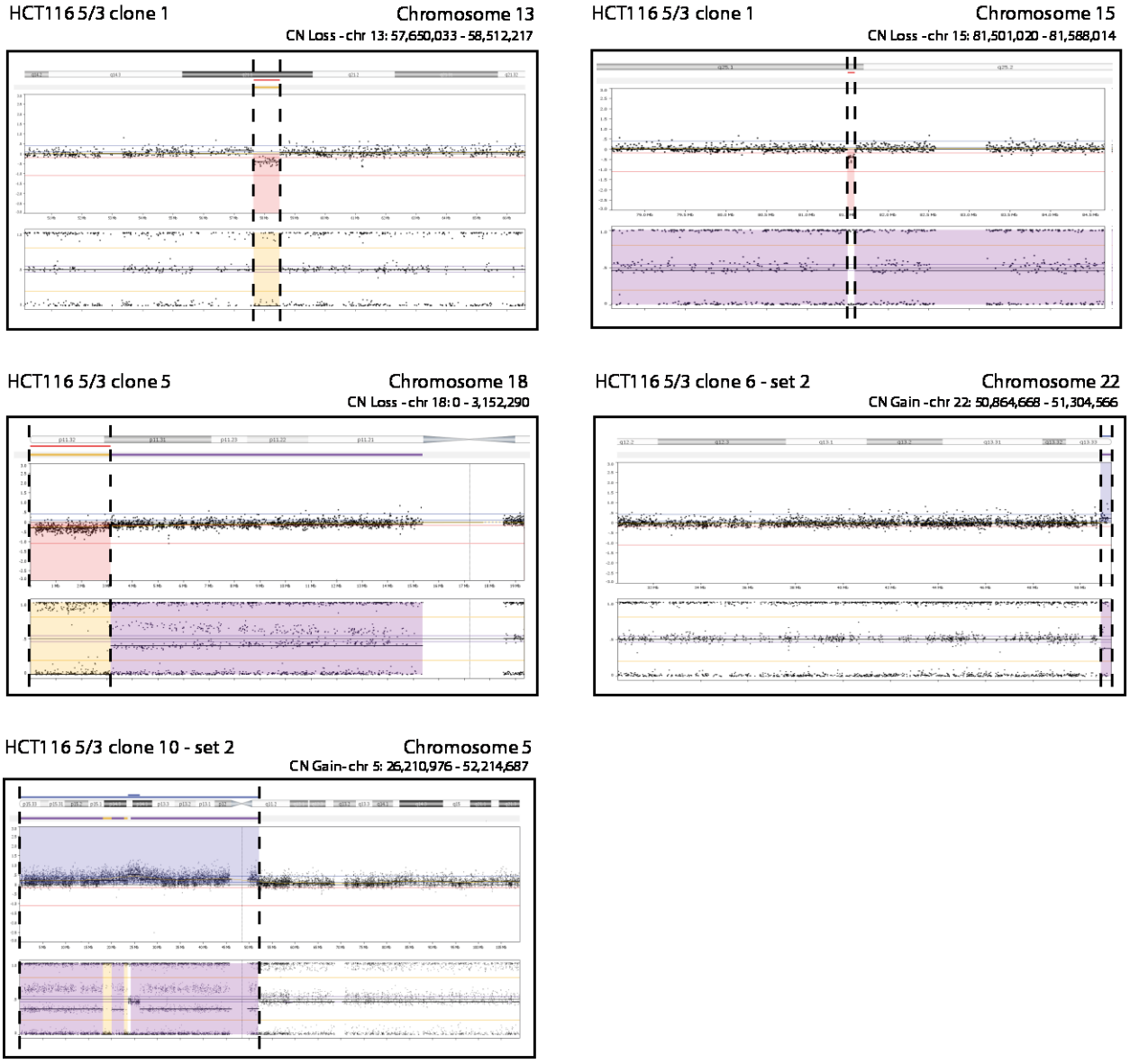


HCT116 5/3 clone 8 — Chromosome 3

CN Loss - chr 3: 80,776,725 - 86,007,923

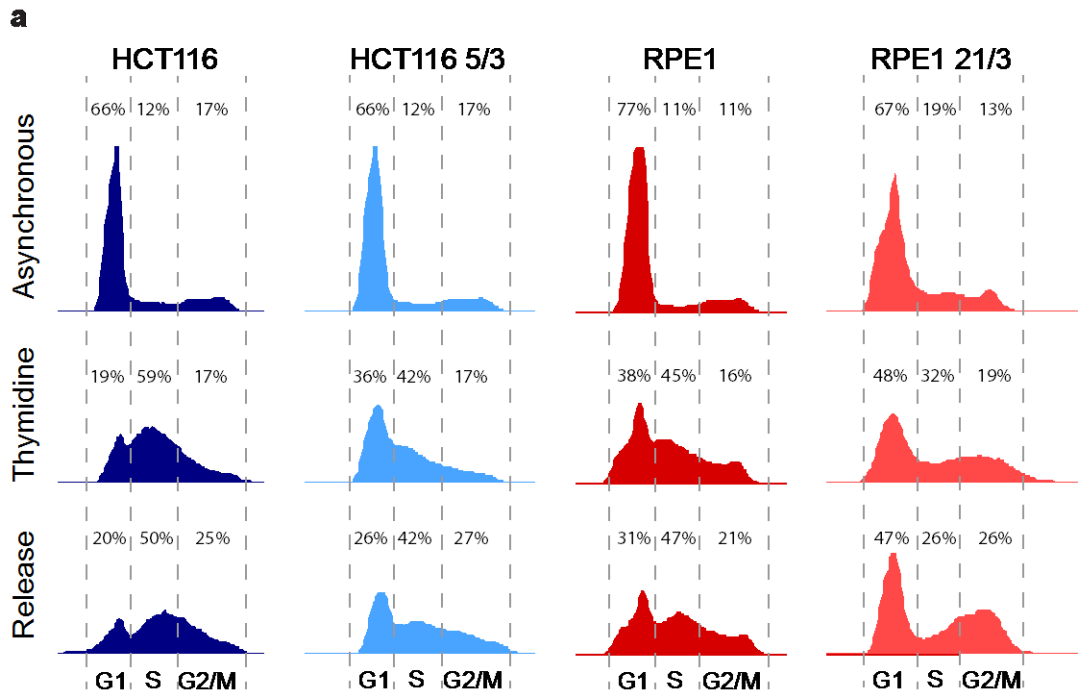


b

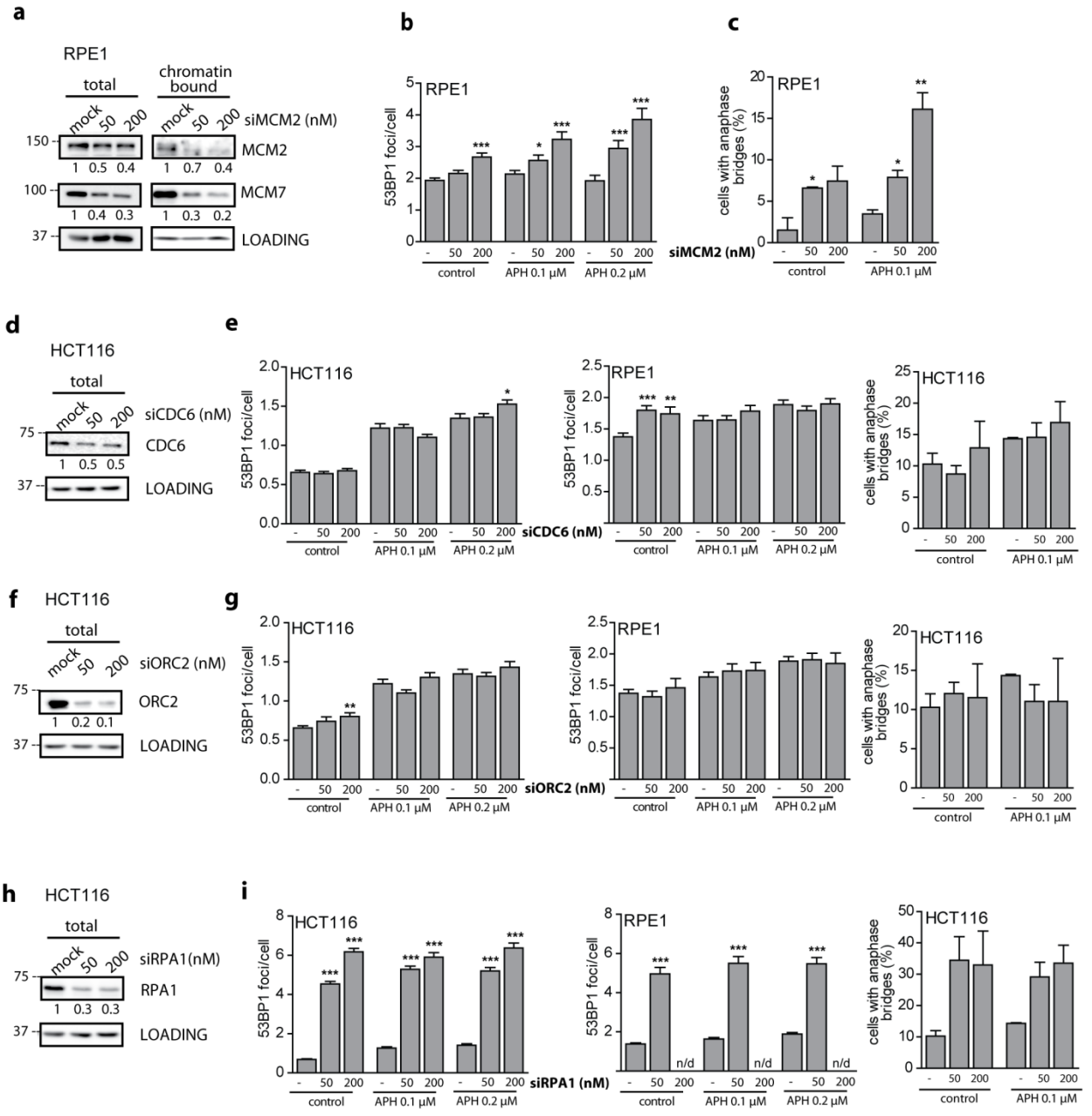


Supplementary Figure 5 Copy number alterations in trisomic cells

Unique *de novo* copy number alterations determinated in the individual clones derived from single aneuploid cell lines. Two independent sets of 12 single-cell clones were analyzed. (**a**) shows the CNAs from set 1; (**b**) set 2. Blue - copy number gain, red - copy number loss. Yellow and magenta denote the alleles A and B, respectively.

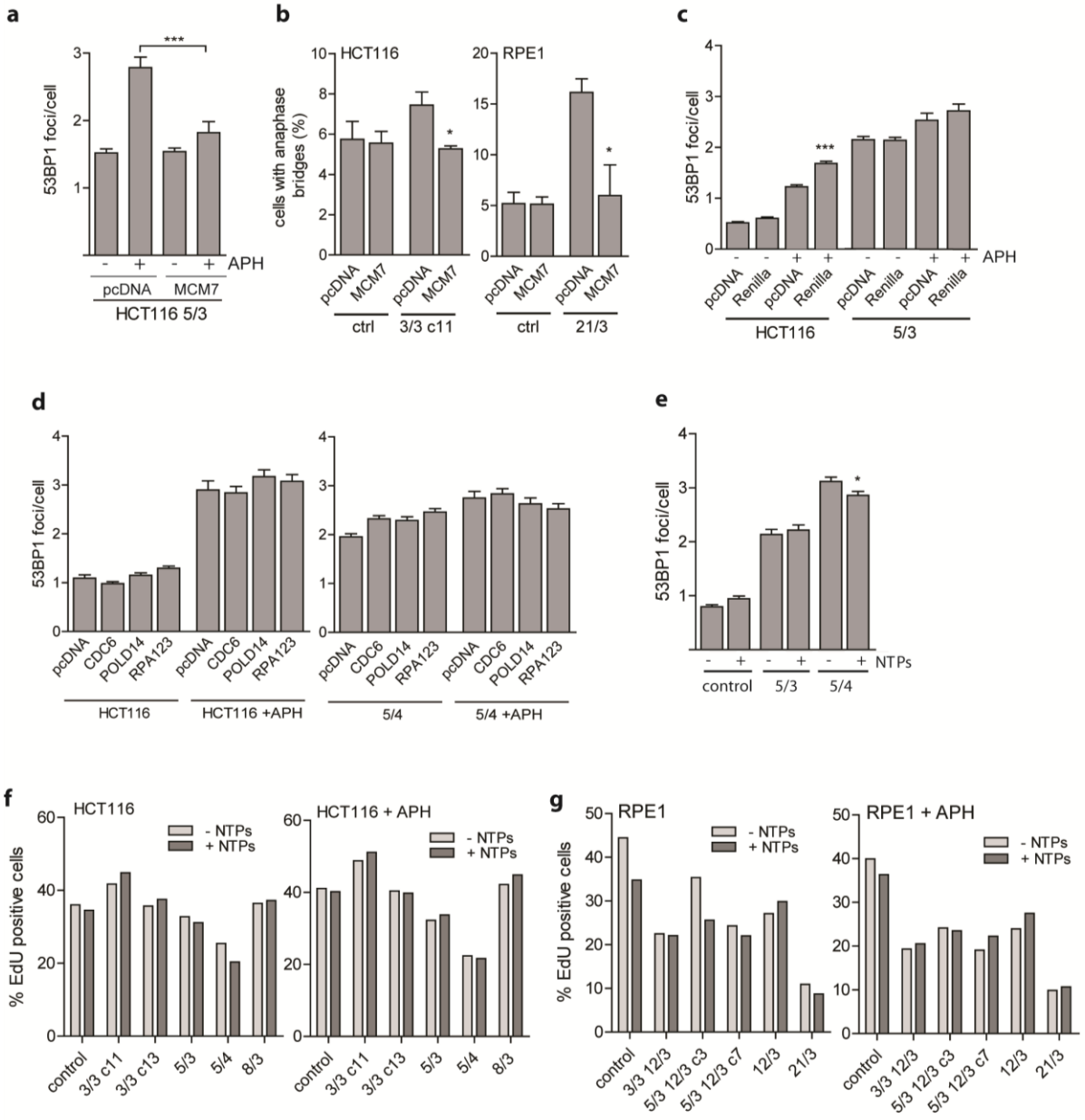


Supplementary Figure 6 Cell cycle profiles after thymidine synchronization and release
(**a**) Cell cycle profile of HCT116, HCT116 5/3, RPE1 and RPE1 21/3 under normal conditions (asynchronous), 30 hours after thymidine addition (thymidine) and 2 hours after thymidine washout (release).



Supplementary Figure 7 Levels of replication proteins in aneuploid cells and the effects of downregulation of replication proteins in control cells

(**a**) Immunoblotting of whole cell lysates and chromatin bound fractions upon partial depletion of MCM2 by siRNA in the parental RPE1 cell line. Note the coordinate decrease in MCM7 abundance. (**b**) Average number of 53BP1 foci and (**c**) average number of anaphase bridges in cells depleted for MCM2. Partial depletion of CDC6 (**d**), ORC2 (**f**) and RPA1 (**h**) by siRNA in the parental HCT116 and RPE1 cell lines. Average number of 53BP1 foci and % of cells with anaphase bridges in cells depleted for CDC6 (**e**), ORC2 (**g**) and RPA1 (**i**). At least two independent experiments were performed and at least 500 cyclin A2-negative or 50 anaphases were scored for 53BP1 foci or anaphase bridge quantification, respectively. All plots show mean ± SEM; non-parametric T-test; * p<=0.05, ** p<=0.01, *** p<=0.001.



Supplementary Figure 8 The effects of NTP supplementation on EdU incorporation and the occurence of 53BP1 foci in aneuploid cells

(**a**) Accumulation of 53BP1 foci and (b) anaphase bridges upon transient overexpression of MCM7. One representative plot of three independent experiments (a) or mean ± SEM of three independent experiments (**b**) is shown . Non-parametric two-sided T test; *: P<0.05, **: P<0.01, ***: P<0.001.

(**c**) Accumulation of 53BP1 foci in HCT116 and in HCT116 5/3 upon overexpression of Renilla luciferase. (**d**) Accumulation of 53BP1 foci in HCT116 and in HCT116 5/4 upon overexpression of replication factors. (**e**) 53BP1 foci formation in HCT116 and its aneuploid derivatives in the presence or absence of nucleoside supplement. Plot shows the average number of 53BP1 foci of at least 500 cyclin A2-negative cells collected in one experiment. Non-parametric t-test. * p<=0.05% EdU positivity in control HCT116 and aneuploid derivatives (**f**) and RPE1 and aneuploid derivatives (**g**) with or without nucleoside supplement (NTPs).

Fig 3c

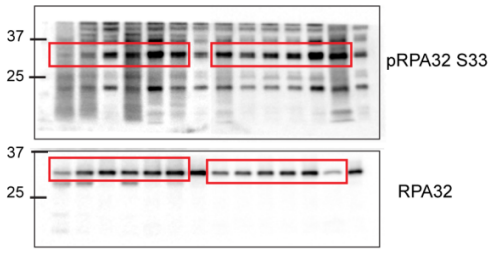


Fig 5b

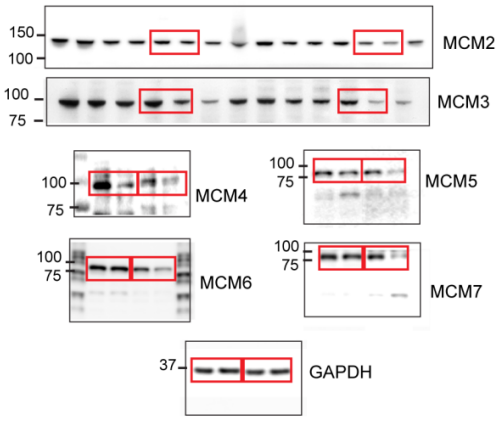


Fig6a

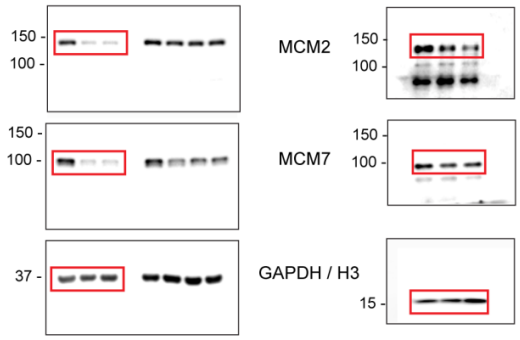


Fig6g

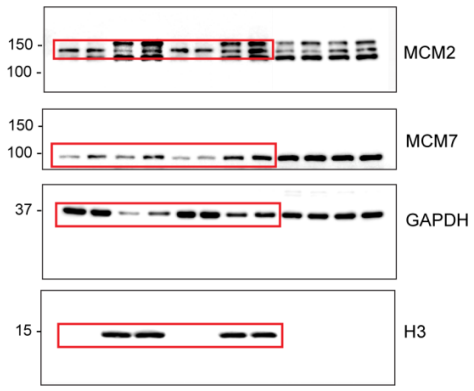


Fig 5c

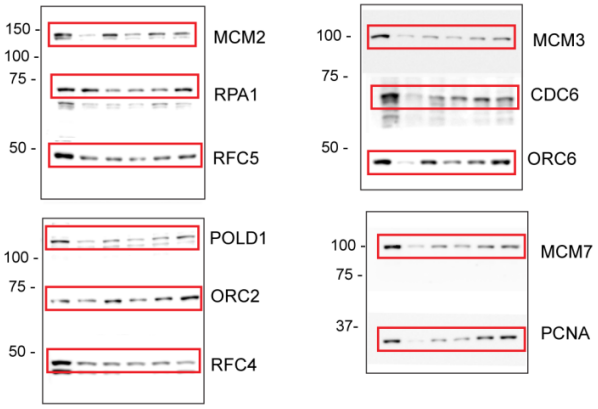


Fig 5d

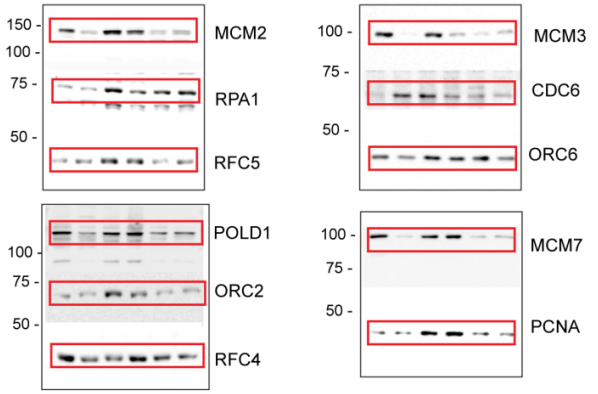
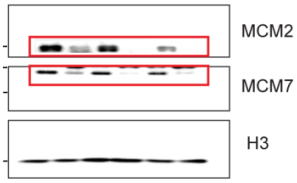


Fig 5e

Fig 5f



Supplementary Figure 9 Uncropped blots of main figures Frames specify shown bands in indicated main figures.

Fig S4a

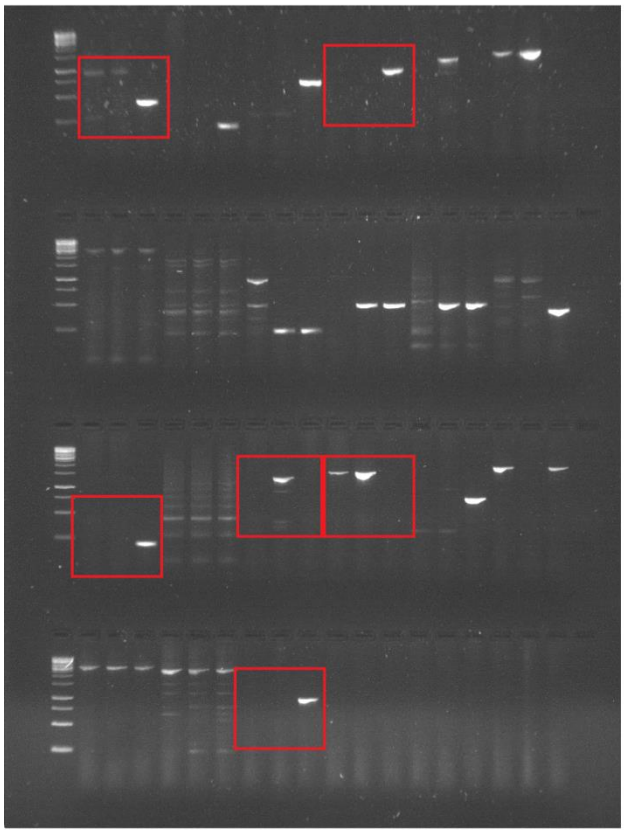


Fig S7a

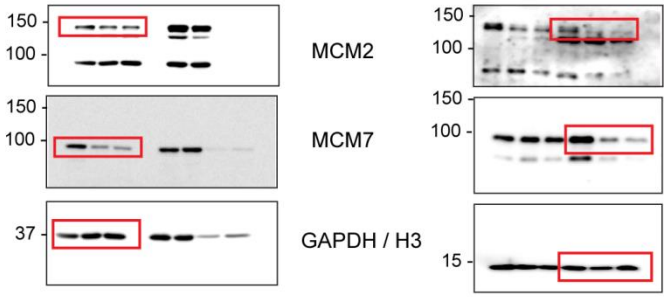


Fig S7d

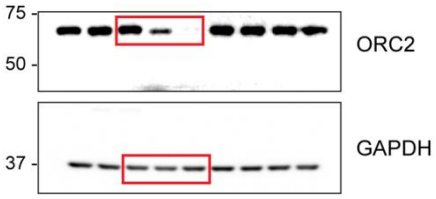


Fig S7f

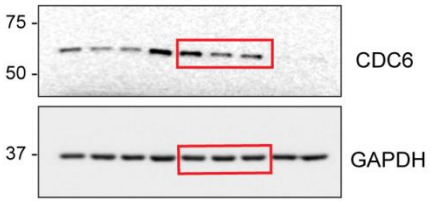
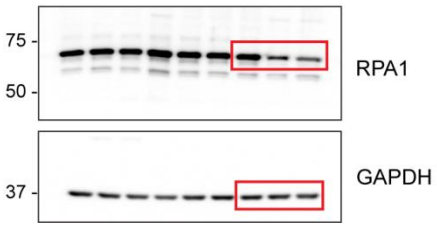


Fig S7g



Supplementary Figure 10 Uncropped blots of supplementary figures Frames specify shown bands in indicated supplementary figures.

Supplementary Table 1 List of all cell lines used in the analysis. % of cells with trisomy/tetrasomy was determined by chromosome painting. Note that the cell lines from Koi laboratory were used only for the analysis of the global proteome changes (Figure 5a).

Cell line name used in the text	Origin	Full cell line name	Analysis	% of trisomy or tetrasomy	Remarks
HCT116	HCT116 from AATC introduction H2B-GFP	HCT116 H2B-GFP	SNParrays CGH	-	Kuffer et al, 2013
HCT116 3/3 c11	MMTC into HCT116 H2B-GFP	HCT116 H2B-GFP 3/3 clone 11	SNParrays CGH	92 %	This work
HCT116 3/3 c13	MMTC into HCT116 H2B-GFP	HCT116 H2B-GFP 3/3 clone 13	SNParrays CGH	85 %	This work
HCT116 5/3	MMTC into HCT116 H2B-GFP	HCT116 H2B-GFP 5/3 clone 6	SNParrays CGH	92 %	Stingele et al, 2012
HCT116 5/4	MMTC into HCT116 H2B-GFP	HCT116 H2B-GFP 5/4 clone 4	SNParrays CGH	83 %	Stingele et al, 2012
HCT116 8/3	MMTC into HCT116 H2B-GFP	HCT116 H2B-GFP 8/3 clone 1	SNParrays CGH	78 %	Donnelly et al, 2014
RPE1	Taylor laboratory	RPE1 hTERT	SNParrays CGH	-	Kindly provided by Steven Taylor
RPE1 3/3 12/3	MMTC into RPE1		SNParrays CGH	100 %	This work Spontaneous gain of chromosome 12
RPE1 5/3 12/3 c3	MMTC into RPE1	RPE1 5/3 12/3 clone 3	SNParrays CGH	95 %	Stingele et al, 2012 Spontaneous gain of chromosome 12
RPE1 5/3 12/3 c7	MMTC into RPE1	RPE1 5/3 12/3 clone 7	SNParrays CGH	95 %	This work Spontaneous gain of chromosome 12
RPE1 12/3	Spontaneously arising trisomy of chromosome 12		CGH	100 %	This work Spontaneous gain of chromosome 12
RPE1	Taylor laboratory	RPE1 H2B-GFP hTERT	SNParrays CGH	-	Kindly provided by Steven Taylor
RPE1 21/3	MMTC into RPE1 H2B-GFP	RPE1 H2B-GFP 21/3	SNParrays CGH	90 %	Stingele et al, 2012
HCT116	Koi laboratory		SNParrays CGH	-	Kindly provided by Minoru Koi Haugen et al, 2008
HCT116 5/4	Koi laboratory		SNParrays CGH	88 %	Kindly provided by Minoru Koi Haugen et al, 2008
HCT116 3/3	Koi laboratory		CGH	82 %	Kindly provided by Minoru Koi Haugen et al, 2008

Supplementary Table 2 Overview of the identified copy number aberrations in HCT116 5/3.

Sample	chr	start	end	event	Size (bp)	Chr. band	Mosaic	Fragile site overlap
tr_13_set_2_clone_1	13	57650033	58512217	CN Loss	862185	q21.1	no	FRA13B
tr_13_set_2_clone_1	15	81501020	81588014	CN Loss	86995	q25.1	no	FRA2B/FRA22A
tr_17_set_2_clone_5	18	0	3152290	CN Loss	3152291	p11.32 - p11.31	no	
tr_18_set_2_clone_6	22	50864668	51304566	CN Gain	439899	q13.33	no	
tr_22_set_2_clone_10	5	26210976	52214687	CN Gain	26003712	p14.1 - q11.2	no	FRA5A/FRA5E
tr_5_set_1_clone_5	8	142486178	144510494	CN Loss	2024317	q24.3	no	FRA8D
tr_5_set_1_clone_5	8	146081828	146364022	CN Loss	282195	q24.3	no	FRA8D
tr_5_set_1_clone_5	22	16253076	51304566	CN Gain	35051491	q11.1 - q13.33	no	FRA2A/FRA2B/FRA22A/FRA22B
tr_6_set_1_clone_6	4	61577821	61784603	CN Loss	206783	q13.1	no	
tr_6_set_1_clone_6	7	155026587	155142658	CN Loss	116072	q36.2 - q36.3	no	FRA7I
tr_7_set_1_clone_7	2	172017814	187925525	CN Gain	15907712	q31.1 - q32.1	no	FRA2G/FRA2H
tr_8_set_1_clone_8	3	80776725	86007923	CN Loss	5231199	p12.2 - p12.1	no	

9. Discussion

The work described in this thesis was undertaken to gain insights into the consequences of an aberrant karyotype in human cells, and in particular, to study the effects of aneuploidy on proteostasis and genome stability. Our findings that aneuploidy disturbs protein folding and promotes genomic instability enhance our understanding of how aneuploidy can be detrimental at the cellular and molecular levels. Further, they may have important ramifications for the role of aneuploidy in pathological conditions and for the treatment of conditions characterised by the presence of aneuploid karyotypes.

9.1 Aneuploidy undermines cellular proteostasis by impairing protein folding

Accumulating evidence has suggested that aneuploidy exerts negative effects on cellular proteostasis. However, the molecular mechanisms underlying these effects as well as the consequences for aneuploid cells have remained unclear. The investigations described in this thesis have provided insights into both of these questions.

Aneuploid cells from yeast and mice exhibit sensitivity to a range of conditions and drugs that inhibit protein production and folding (Torres et al. 2007; Pavelka et al. 2010b; Tang et al. 2011). Further, aneuploid cells in yeast and humans accumulate protein aggregates and are sensitive to exogenous expression of aggregation-prone and difficult-to-fold proteins (Oromendia et al. 2012; Stingele et al. 2012). In addition, aneuploidy appears to lead to an enhanced requirement for proteasomal and autophagic degradation (Torres et al. 2007; Tang et al. 2011; Stingele et al. 2012; Santaguida and Amon 2015).

We hypothesised that a common denominator underlying all of these previous observations might be a defect in protein folding. Based on earlier indications from aneuploid yeast (Oromendia et al. 2012), and on the seemingly general sensitivity of aneuploid cells to chemical inhibition of the HSP90 molecular chaperone (Torres et al. 2007; Tang et al. 2011), we hypothesised that this defect might specifically concern HSP90-dependent protein folding. Indeed, the significant impairment in the ability of aneuploid cells to fold and re-fold luciferase-based HSP90 folding sensors (Figure 1 in (Donnelly et al. 2014)), together with the observed general sensitivity of human aneuploid cells to chemical inhibition of HSP90, but not to other chaperone inhibitors or other inducers of protein misfolding (Figure 2 in (Donnelly et al.

2014)), suggest that human aneuploid cells experience a specific defect in HSP90-dependent protein folding.

What underlies the impaired HSP90 function of aneuploid cells? The general downregulation of molecular chaperones in aneuploid cells, as well as their pronounced impairment in Heat Shock Response (HSR) induction was suggestive of a defect in HSF1, the master regulator of inducible chaperone expression (Figure 3 in (Donnelly et al. 2014)). Strikingly, overexpression of HSF1, either endogenously by transfer of chromosome 8 where *HSF1* is located, or exogenously, by plasmid transfection, was sufficient to correct the HSP90-dependent protein folding defect of aneuploid cells (Figure 4 in (Donnelly et al. 2014)). Thus, our data suggest that impaired HSF1 function in human aneuploid cells leads to a specific defect in HSP90 (Figure 2).

Our analysis also revealed that the impaired HSF1 and HSP90 function of human aneuploid cells partly determines the complex changes in gene expression observed in response to aneuploidy (Figure 2): the transcriptional response of cells in which HSF1 was knocked down bore a striking resemblance to the transcriptional pattern of aneuploid cells; further, HSP90 clients and HSP90-dependent pathways tend to exhibit lower levels and activity in aneuploid cells (Figure 5 in (Donnelly et al. 2014)).

It remains unclear precisely why HSF1 activity is diminished in human aneuploid cells (Figure 2). While it was claimed that aneuploid yeast cells exhibit no impairment in activation of the HSR, careful perusal of the data reveal a slight delay in the induction and repression of Heat Shock Response-associated genes in the majority of disomes (Oromendia et al. 2012). The fact that the levels of HSF1 are reduced by approximately 20-25% in the aneuploid cells analysed in this study, taken together with the rescue of HSF1 function observed upon overexpression, might suggest that the lower levels of HSF1 are the main cause of this impairment. However, experiments using the promoter of the *HSP70* gene fused to luciferase revealed that induction in diploid cells was up to 4-fold higher than in aneuploid cells in response to acute proteotoxic stress. These observations raise the possibility that lower expression of HSF1 might not be the only cause of the defective HSR in human aneuploid cells.

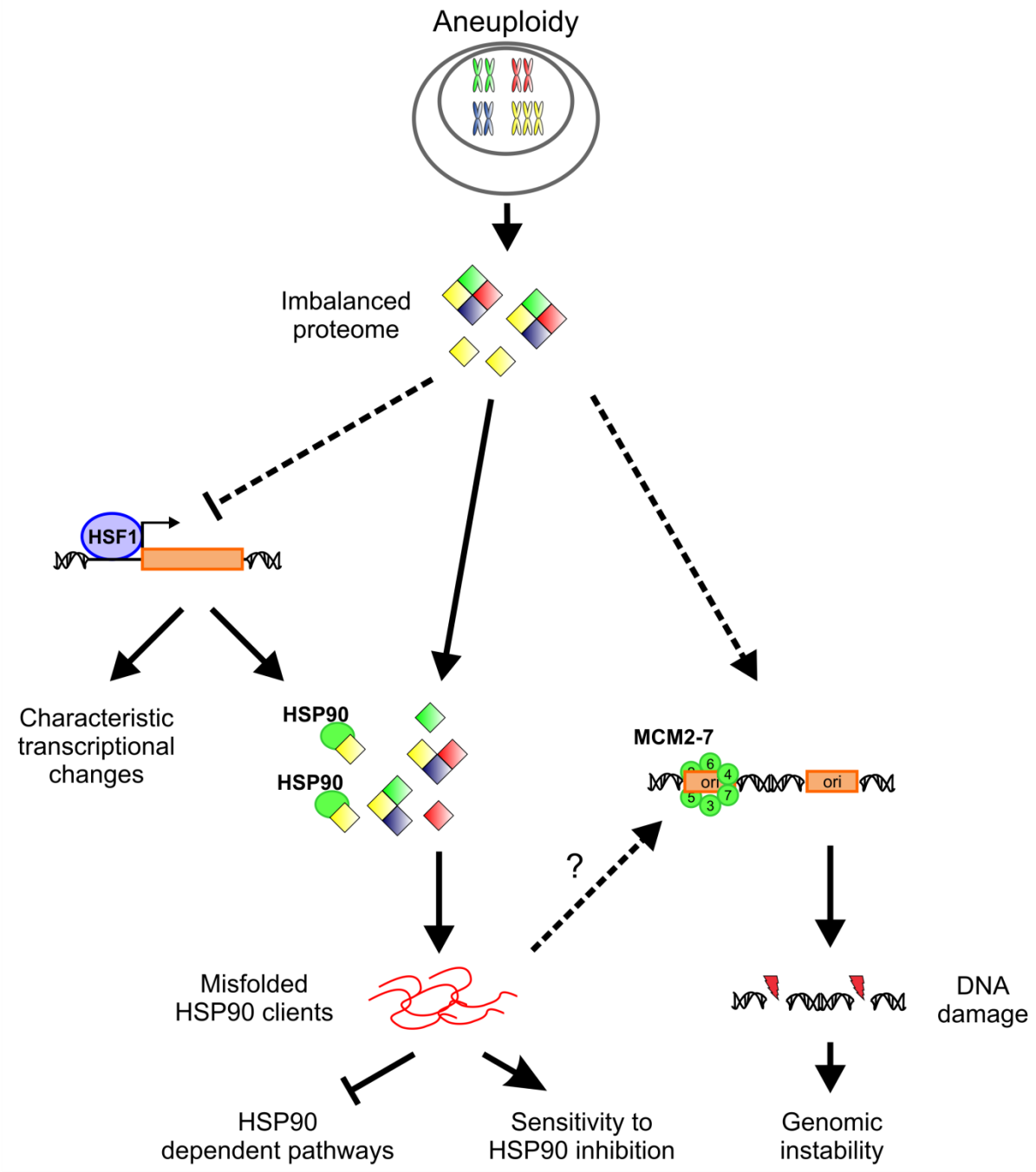


Figure 2: Model depicting the main findings described in this thesis and the potential link between them. The imbalanced proteome of aneuploid cells inhibits HSF1 function by an as yet undefined mechanism leading to a characteristic transcriptional profile as well as lower levels of the HSP90 chaperone. The lower levels of HSF1 together with the imbalanced production of cellular proteins, particularly members of multi-subunit complexes, lead to an exhaustion of HSP90 capacity and to misfolding of HSP90 client proteins. This in turn leads to the impaired function of HSP90-dependent processes and to sensitivity to HSP90 inhibition. Aneuploidy also elicits lower expression of the MCM2-7 complex, resulting in impaired DNA replication, increased levels of DNA damage, and ultimately to structural rearrangements of the genome. I propose that the impaired HSP90 function of aneuploid cells may contribute to the lower levels of MCM2-7. ori; origin of replication.

To determine what else may underlie the reduced activity of HSF1, I have systematically interrogated the different steps in the HSF1 activation cycle. My so far unpublished investigations suggest that there is no defect in nuclear import of the HSF1 protein or in the ability of HSF1 to be converted into a DNA binding-competent, hyperphosphorylated trimer. Further, it appears that there is no obvious impairment of HSF1's ability to contact its binding sites *in vivo* or to recruit RNA polymerase II. Finally, polymerase processivity also appears to be grossly unaffected. Remaining possibilities include a reduced stability of HSF1 target mRNAs and/or proteins or a decreased translation of HSF1 targets. Indeed, the observation that transcriptional downregulation of ribosomal genes is a recurrent feature of human aneuploid cells (Durrbaum et al. 2014), is an indirect indication that translation may be diminished in response to aneuploidy. It also remains possible that multiple minor defects in the HSF1 pathway, not easily discernible by themselves, might combine to result in a profound defect in HSF1 activity.

There is also no definitive answer to the question of why HSP90-dependent protein folding appears to be particularly affected in response to aneuploidy. Our observations that levels of HSP90 as well as other chaperones are generally reduced in aneuploid cells, coupled with the rescue in both HSP90 levels and protein folding that is observed upon HSF1 overexpression, seem to offer a relatively straightforward solution to this problem. However, unanswered questions remain. Firstly, earlier work performed in MEFs had shown that the levels of the inducible HSP72 chaperone were actually elevated in trisomic cells, although the mechanism was not elucidated (Tang et al. 2011). Secondly, it is not clear why a defect in HSF1 function will preferentially affect HSP90. It is conceivable that this may be due to differences in the relative affinity of HSF1 for the promoters of its target genes. In fact, in experiments where a constitutively active HSF1 was transiently overexpressed from a plasmid in aneuploid cells, I observed that the induction of Heat Shock Protein 27 (HSP27) expression was greatest, followed by that of HSP70, and finally HSP90. This is an interesting observation that warrants further investigation. Finally, it is widely accepted that HSP90, as one of the most abundant proteins in eukaryotes (Borkovich et al. 1989), is present in the cell in sizeable excess. Indeed, under normal conditions yeast cells can survive and grow with only 5% of their normal levels of HSP90 (Borkovich et al. 1989). Therefore, I suggest that the aneuploid state imposes a strict requirement for HSP90 function and that aneuploid cells can even be said to be "addicted" to this chaperone.

Aneuploidy in general is detrimental because of the expression of genes from aneuploid chromosomes. This expression leads to an imbalanced proteome, a state which has particularly pronounced repercussions for the members of multi-subunit complexes. According to the GDBH

explanation for the detrimental effects of aneuploidy, such an imbalanced expression of multi-subunit complex members will be harmful for the cell, because it will affect the amount of functional complexes that are finally synthesised (Birchler and Veitia 2012; Veitia and Potier 2015).

Our results suggest that an imbalanced proteome is not only harmful because of the effects that it has on the proteins that are directly affected by this unevenness. Uneven expression of proteins likely leads to an enhanced engagement of chaperones, which try to ensure that proteins remained folded and soluble. Such conditions of imbalanced stoichiometry might be particularly taxing for HSP90, a chaperone that acts late in the folding cycle and which has an emerging role in the assembly of multi-subunit protein complexes (McClellan et al. 2007; Makhnevych and Houry 2012). Imbalanced expression of protein complex members is likely to complicate and prolong the time needed for the proper assembly of protein complexes, which is likely to increase the requirement for chaperone surveillance. This in turn would titrate HSP90 from other important cellular functions, leading to a global impairment in HSP90-dependent processes (Figure 2).

9.1.1 Consequences of the protein folding defect in aneuploid cells

Owing to its specialised clientele and to the specific set of critical cellular pathways that depend on it, HSP90 is regarded as a hub of cellular physiology (Taipale et al. 2010). Our findings that the HSP90-dependent proteome and HSP90-dependent pathways and processes tend to exhibit lower levels and activity strongly suggest that the HSP90 defect of aneuploid cells has far-reaching general consequences for cell function. In addition, although primarily recognised for its role in regulating proteostasis-related genes, in recent years it has become clear that HSF1 also targets a large number of other genes with roles in diverse cellular processes (Page et al. 2006; Mendillo et al. 2012), which also indicates that the impaired HSF1 function in aneuploid cells may have pleiotropic effects on a range of different cellular processes (Figure 2).

It is particularly tempting to speculate about how the impaired proteostasis of aneuploid cells may be linked with some of their other cardinal features. A potentially link would be with the dosage compensation in aneuploid cells. Intriguingly, as mentioned in the introduction, proteins that are dosage-compensated in aneuploid cells are enriched for protein complex members and kinases, two classes of proteins that may be particularly reliant on HSP90 function (Caplan et al. 2007; McClellan et al. 2007; Makhnevych and Houry 2012). I suggest that the enhanced and/or specific chaperone requirements of these classes of proteins may be a cause of the limiting protein folding capacity in aneuploid cells. On the other hand, however, the lower levels of proteins of these classes in aneuploid cells may be a consequence of the impaired protein

folding capacity that they themselves elicit. It will be important to determine if aneuploid cells that have no apparent protein folding defects, e.g., those harbouring an extra copy of chromosome 8 with *HSF1*, also exhibit dosage compensation of proteins encoded on their extra chromosomes.

I also hypothesise that the impaired protein folding capacity of aneuploid cells may contribute to their impaired proliferation. HSF1 and HSP90 play important roles in promoting cellular proliferation and progression through the cell cycle, particularly in cancer cells. In fact, levels of HSP90 are elevated at the border between the G1 and S phases of the cell cycle in a HSF1-dependent manner (Nakai and Ishikawa 2001), suggesting that both proteins are involved in regulating G1 to S transition. Furthermore, HSP90 regulates either directly or indirectly a large number of proteins with important roles in promoting G1 to S progression, most prominently the cyclin-dependent kinases (CDKs), CDK2 (Prince et al. 2005), CDK4 (Stepanova et al. 1996), and CDK6 (Mahony et al. 1998), as well as Cyclin D (Münster et al. 2001), and Cyclin E (Bedin et al. 2004). Consistent with these observations, chemical inhibition of HSP90 often causes a cell cycle arrest at G1/S (Burrows et al. 2004). My observation that cells that gained chromosome 8 with an extra copy of *HSF1* proliferated faster than cells that gained chromosome 8 without *HSF1* suggest that, indeed, protein folding capacity may also be an important determinant of proliferative capacity in human aneuploid cells.

HSP90 plays a critical role in buffering phenotypic change by masking the effects of genetic polymorphisms (Jarosz et al. 2010). It is well described that the altered expression of genes on aneuploid chromosomes plays an important role in promoting adaptive evolution (e.g., (Rancati et al. 2008; Kaya et al. 2015), and reviewed in (Pavelka et al. 2010a)). My findings suggest the possibility that aneuploidy may, through its inhibitory effects on HSP90 function, also promote phenotypic diversity in a less overt manner, by exhausting HSP90's buffering capacity. While intriguing, testing this idea will be far from trivial.

The detrimental effects that aneuploidy frequently has on cellular physiology imply that aneuploid cells require additional mutations or non-genetic changes in order to overcome the negative effects of an imbalanced karyotype. It was previously shown that in yeast, deletion of the deubiquitinating enzyme Ubp6 enhanced the proteasomal degradation of proteins encoded on aneuploid chromosomes, provided protection against the deleterious effects of aneuploidy on proteostasis and alleviated the proliferative defects of aneuploid cells (Torres et al. 2010; Oromendia et al. 2012). My results delineate an alternative route to coping with an imbalanced karyotype: blocking aneuploidy's detrimental effect on HSP90-dependent protein folding through overexpression of HSF1. It is noteworthy in this regard that the 8q24 chromosomal

region, where the *HSF1* gene is located, is frequently amplified in cancer (Beroukhim et al. 2010; Davoli et al. 2013). Further, chromosome 8 is the most common genetic abnormality in the myeloid leukaemias developed by DS individuals (Ganmore et al. 2009), suggesting that genes on this chromosome may be important for overcoming the intrinsic tumour-suppressive effects of aneuploidy in DS individuals.

More generally, my results, when taken together with earlier observations (Torres et al. 2010; Oromendia et al. 2012), suggest that cells have two options when confronted with the impaired PN elicited by aneuploidy: on the one hand, either an upregulation of protein folding capacity to prevent protein degradation and aggregation, or, perhaps, to deal with the consequences of increased levels of proteolysis; or, on the other hand, to further elevate levels of protein degradation to ensure that misfolded proteins are efficiently disposed of and do not accumulate within the cell (Figure 3).

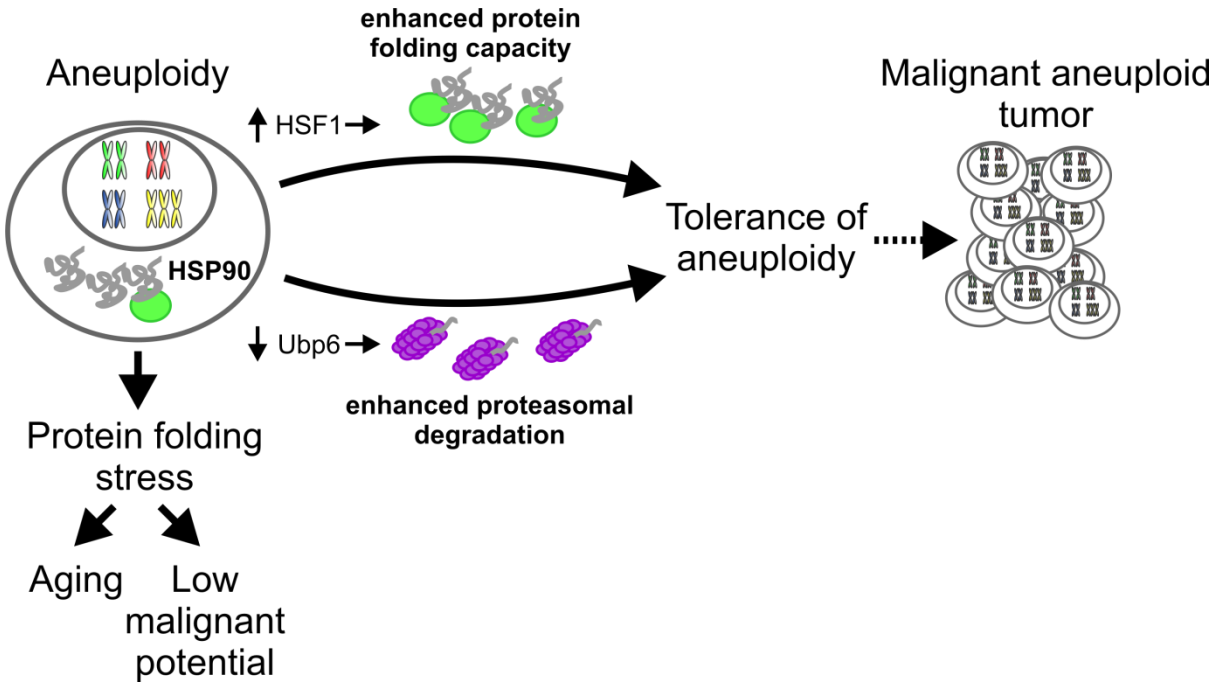


Figure 3: Mechanisms that allow cells to cope with the detrimental effects of aneuploidy on proteostasis. The impaired protein folding capacity of aneuploid cells is a barrier to tumourigenesis and may contribute to cellular aging. Cells can overcome these detrimental effects either by augmenting their protein folding capacity or by enhancing protein degradation, thereby elevating their proliferative capacity and potentially giving rise to malignant aneuploid tumours.

9.1.2 Implications of impaired proteostasis for cancer and trisomy syndromes

The results described herein have further implications for our understanding of aneuploidy in disease. Proteotoxic stress is a recurring feature of cancer cells and leads to a heavy reliance on

proteostasis factors in general, and on HSF1 and HSP90, in particular. Previously, several underlying mechanisms were proposed to explain the proteotoxic stress of cancer cells and their "addiction" to chaperones. Increased levels of protein synthesis and of ribosomes are key facets of the pro-tumorigenic programme and chaperones play critical roles in ensuring proteome integrity under these conditions (Silvera et al. 2010; Dai et al. 2012). In addition, the oncogenes which drive cancer progression are frequently mutated and unstable and thus critically rely on chaperones such as HSP90 (Neckers 2006).

It has also been proposed that the proteotoxic stress of cancer cells stems from their aneuploid karyotypes and their resulting imbalanced proteomes (Luo et al. 2009). However, this hypothesis was lacking experimental proof in human cells as well as a molecular explanation. The results presented here strongly support the idea that the proteotoxic stress experienced by cancer cells is partly due to the fact that they are frequently aneuploid. The stress phenotypes of malignant cells have been identified as constituting an important therapeutic window for the treatment of cancer (Solimini et al. 2007; Luo et al. 2009). The data presented here proffer a novel additional explanation for why cancer cells are so reliant on both HSF1 and HSP90 activity and suggest the existence of a further rationale for the efficacy of drugs that target proteostasis in cancer cells.

My finding that impaired protein folding capacity is a hallmark of human aneuploid cells may also have important implications for our understanding of underlying pathological mechanisms in trisomy syndromes. As discussed in the introduction, the current approach to understanding the aetiology of DS and its associated complications is almost exclusively focused on the role of specific genes encoded on chromosome 21. Our evolving understanding of the karyotype-independent phenotypes of aneuploid cells, however, indicates that the detrimental effects of trisomy in humans are not necessarily mediated solely by chromosome 21-encoded genes. Rather, it is possible that the general features shared by aneuploid cells regardless of karyotype also play a role.

The generally diminished protein folding capacity identified in model human aneuploid cells raises the possibility that the symptoms and complications characteristic of DS individuals may be partially due to an inability to maintain the integrity of their proteomes. Conversely, my observations also suggest that the reason that certain trisomies are compatible with life in humans is because the effects that they exert on PN function are comparatively mild. Indeed, all viable human trisomies, i.e. chromosomes 13, 18, 21, are of chromosomes that are relatively gene-poor and thus likely to encode fewer genes that are members of protein complexes. In any case, in the future it will be important to determine whether the function of molecular

chaperones is also compromised in cells from human trisomies. It is already tempting to speculate that this could contribute to the early onset Alzheimer's disease (EOAD) in DS individuals, a disease characterised by a severe curtailment in proteostasis function and high levels of protein aggregation. In fact, while the extra copy of *APP* seems to be required for the EOAD in DS, it is not by itself sufficient to lead to manifestation of all the phenotypes (Lana-Elola et al. 2011). Thus, my findings raise interesting possibilities regarding potential links between impaired proteostasis and the detrimental effects of aneuploidy in humans. It is to be hoped that future work will integrate findings from general aneuploidy models as well as from specific trisomies in an effort to better elucidate the effects of constitutional aneuploidy in humans.

9.2 Effects of aneuploidy on genome stability

Reports from yeast have demonstrated that aneuploidy can promote genomic instability (Sheltzer et al. 2011; Zhu et al. 2012; Blank et al. 2015). However, it has been unclear whether or not aneuploidy *per se* can also promote further alterations to the genome in metazoans. The data described in this thesis not only demonstrate that aneuploidy can lead to genomic instability in human cells, but also delineate a molecular mechanism by which this can occur.

A previous report on chromosomally unstable colon cancer cells reported that DNA replication stress can lead to chromosome mis-segregation via structural aneuploidy (Burrell et al. 2013). Further, analysis of human cancer cells had suggested that genomic instability increases in proportion to the degree of aneuploidy (Duesberg et al. 1998; Storchova and Kuffer 2008). Our data now show that whole chromosomal aneuploidy leads to replication stress and subsequently, to DNA damage and chromosomal rearrangements in human cells. Taken together, the data illustrate how perturbations to the genome can set off complex chains of events that serve to further exacerbate genomic instability.

The general downregulation of DNA replication-related factors in human aneuploid cells led us to hypothesise that aneuploidy might interfere with replication (Dürrbaum et al. 2014). Indeed, direct measurement of replication dynamics in human aneuploid cells demonstrated that DNA replication was slower in aneuploids than in isogenic controls and that treatment with the replication inhibitor aphidicolin arrested aneuploid cells earlier in the cell cycle than diploids (Figure 3 in (Passerini et al. 2016)). This impairment in DNA replication has severe consequences for genome stability in aneuploid cells (Figure 2). Human aneuploid cells showed enhanced levels of anaphase and ultrafine bridges (Figure 1 in (Passerini et al. 2016)), and exhibited higher levels of DNA damage as well as chromosome breaks (Figure 2 in (Passerini et

al. 2016)). Finally, aneuploid human cells accumulated complex genomic rearrangements, the nature of which are strongly indicative of errors during DNA replication (Figure 4 in (Passerini et al. 2016)).

The broad reduction in replication-associated factors in aneuploid cells means that it is reasonable to assume that aneuploidy interferes with replication at several distinct steps (Figure 5 in (Passerini et al. 2016)). However, the data from this study as well as observations from other researchers, suggest that the downregulation of MCM2-7 helicase is particularly critical for human aneuploid cells. Firstly, for reasons that are unknown (potential explanations are discussed below) components of the MCM complex exhibit the most consistent downregulation in aneuploid cells (Figure 5 in (Passerini et al. 2016)). Further, depletion of MCM levels in control diploid cells to levels comparable to what is observed in aneuploids recapitulates two of the main phenotypes of aneuploid cells, i.e., an accumulation of 53BP1 foci and elevated levels of anaphase bridges. Depletion of the pre-replicative factors CDC6 and ORC2, as well as the single-stranded DNA binding protein, RPA1, did not phenocopy the DNA damage of aneuploid cells (Figure 6 in (Passerini et al. 2016)). Similarly, disrupting MCM function by overexpression of a mutant phospho-resistant MCM2 allele, which compromises origin firing, was toxic to aneuploid cells, but not to controls, whereas overexpression of mutant versions of ORC1 and RPA1 did not reveal any sensitivity (Figure 6 in (Passerini et al. 2016)). Indeed, several earlier studies have documented that decreasing the levels of functional MCM complex leads to impaired DNA replication and genomic instability (Pruitt et al. 2007; Shima et al. 2007; Chuang et al. 2010). The final line of evidence that reduced levels of MCM2-7 are responsible for the replication stress and genomic instability in aneuploid cells comes from the rescue experiments in which exogenous overexpression was found to mitigate the effects of aneuploidy on DNA damage and mitotic errors (Figure 6 in (Passerini et al. 2016)).

Conceivably, the observed phenotypes could be simply due to the presence of extra DNA. However, it should be noted that the trisomic and tetrasomic cell lines analysed in our study harbour only roughly 1.5% (1 extra copy of chromosome 21) - 12% (two extra copies of chromosome 5) more DNA than the diploid controls, while the levels of errors and DNA damage were often two-fold higher. Of course, this does not completely rule out the possibility that additional DNA might be responsible and that extra DNA might lead to disproportionately higher levels of DNA damage. However, previous analysis in yeast aneuploids (Sheltzer et al. 2011), which indicated that the higher levels of DNA damage observed in these cells was dependent on the gene expression from aneuploid chromosomes argues against this possibility.

It is also improbable that the phenotypes are due to chromosome- or cell line-specific effects, as we observe them in both HCT116 and RPE-1 cell lines with different aneuploid karyotypes. Instead, our data strongly suggest that the genome-wide changes in gene expression elicited by aneuploidy are to blame, specifically the observed lower expression of factors involved in DNA homeostasis.

What could underlie the downregulation of DNA-related factors in human aneuploid cells? One potential explanation would be if this was linked with their slower rate of cell division, which is characterised by a seeming impairment in progressing from the G1 to S phases of the cell cycle (Stingele et al. 2012). The protracted G1 phase of human aneuploid cells might indicate a diminished activity of the gene expression program that governs the G1/S transition. This program is largely dependent on the E2F family of transcription factors, which upregulate a large battery of genes involved in DNA replication (Ishida et al. 2001; Polager et al. 2002; Stanelle et al. 2002). Further, in yeast, the transcriptional response to aneuploidy is at least partially determined by their impaired proliferation (Torres et al. 2007). However, complex human aneuploid cells that arise upon cytokinesis block-mediated tetraploidization, and which display no gross impairment in proliferation exhibit the same transcriptional pattern as trisomic cells, including the characteristic downregulation of DNA-associated pathways (Durrbaum et al. 2014). Thus, other mechanisms are likely to contribute to the downregulation of DNA-related factors in human aneuploid cells.

9.3 A link between impaired proteostasis and genomic instability in aneuploid cells?

A further possible explanation for the downregulation of DNA-related pathways in human aneuploid cells would be if this was linked to their impaired proteostasis (Figure 2). Several studies have demonstrated that chemical inhibition of HSP90 leads to a downregulation of genes involved in DNA transactions at both the transcriptome and proteome levels (Proia et al. 2011; Sharma et al. 2012; Che et al. 2013). Indeed, HSP90 counts among its direct clients a significant number of proteins with critical roles in maintaining genome integrity (Kaplan and Li 2012). HSP90 is a crucial regulator of DNA polymerase ζ during error-free translesion synthesis (Sekimoto et al. 2010), as well as of XRCC1 during base-excision repair (Fang et al. 2014), promotes the stability and proper localization of the repair factors Fanconi anemia, complementation group A (FANCA) and breast cancer 2/Fanconi anemia, complementation group D (FANCD1/BRCA2) (Stecklein et al. 2012), and is required for stabilisation of Mis12

complexes at kinetochores, thus contributing to proper microtubule-kinetochore attachments (Davies and Kaplan 2010). Consistent with these observations, inhibition of HSP90 itself leads to increased sensitivity to agents which cause DNA damage and causes aneuploidy in yeast (Chen et al. 2012).

Interestingly, MCM proteins have also been identified to be among those genes targeted by HSP90 inhibitors (Proia et al. 2011; Sharma et al. 2012; Che et al. 2013). Why MCM proteins are particularly affected in aneuploid cells, however, remains an open question. Intriguingly, expression analysis reveals that MCM genes are downregulated modestly at the transcriptional level and more strikingly at the protein level in aneuploid cells. These observations indicate that at least two distinct mechanisms might be responsible for the lower levels of MCM proteins in aneuploids, one acting at the level of mRNA and another at the protein level. The best characterised regulators of MCM expression are the E2F transcription factors, which upregulate the MCM complex members among of a large number of other genes involved in the progression from G1 to S phase (Ishida et al. 2001; Polager et al. 2002; Stanelle et al. 2002). However, as mentioned above, complex aneuploids with no apparent proliferative defect also exhibit a downregulation of DNA replication-associated genes. Thus, this explanation appears unlikely.

It is tempting to speculate that HSP90 may play a direct role in assembling the MCM2-7 complex, but there are, as yet, no indications that this is the case. Nevertheless, it is possible that aneuploid cells are compromised in their ability to properly assemble MCM2-7, leading to instability of the individual complex members. In fact, the MCM2-7 complex appears to be extremely sensitive to improper stoichiometry as documented by the frequent observation that downregulation of individual complex members leads to corresponding decreases in the remaining subunits (e.g., (Ge et al. 2007; Pruitt et al. 2007; Shima et al. 2007; Ibarra et al. 2008)). Additional possibilities include an impaired loading of MCM2-7 hexamers onto DNA or a reduced stability after they are loaded. Both scenarios could potentially lead to the lower levels of MCM complex members in aneuploid cells. Future work will determine the underlying mechanism(s) for the reduced expression of MCM2-7 in human aneuploid cells.

9.4 Implications of aneuploidy-induced genomic instability for disease

Replication stress is emerging as a hallmark of cancer and as a major source of genomic instability in malignant cells (Macheret and Halazonetis 2015; Boyer et al. 2016). So far, several mechanisms have been described to account for the heightened levels of replication stress in

cancer, such as the activation of oncogenes leading to hyper-replication of the genome (Di Micco et al. 2006), a deficiency in nucleotides early during tumourigenesis (Bester et al. 2011), as well as the recurring loss of specific CIN-inhibiting genes (Burrell et al. 2013). Therefore, as well as demonstrating the potential of aneuploidy to cause further changes to the genome, the results described here identify another mechanism whereby replication stress can arise during cancer development.

Our results also contribute to our understanding of how aneuploidy may both inhibit and promote carcinogenesis. Through its effects in promoting DNA damage and activating the DNA damage response (DDR), leading to cell cycle arrest, replication stress is widely seen as a barrier to tumourigenesis and could even be said to constitute one of the stress phenotypes of cancer cells. On the other hand, replication stress and genomic instability can also be powerful drivers of tumourigenesis, in particular, when DDR activity is dampened (Bartkova et al. 2006; Di Micco et al. 2006).

The finding that aneuploidy can promote DNA damage through increasing levels of replication stress may also have important implications for trisomy syndromes. Several studies have documented higher levels of DNA damage in DS cells (Zana et al. 2006; Morawiec et al. 2008; Necchi et al. 2015). The higher levels of oxidative stress in DS cells (Busciglio and Yankner 1995), have been described to be one reason for these observations (Zana et al. 2006; Valenti et al. 2011). Defects in DNA repair appear to also play a role (Athanasiou et al. 1980; Druzhyna et al. 1998; Raji and Rao 1998). Our results suggest that impaired DNA replication may represent another source of DNA damage in human trisomies.

9.5 Conclusions and perspectives

Aneuploidy is extremely common in cancer and there is sufficient evidence to warrant the assertion that aneuploidy can play important roles in tumorigenesis. Thus, theories of cancer development and progression must take aneuploidy into account. What general implications do the results described herein have for the role of aneuploidy in cancer? Perhaps the most basic conclusion to be drawn is that aneuploidy, by itself, is highly unlikely to give rise to cancer, inasmuch as the impaired protein folding capacity and decreased genome stability of aneuploid cells are likely to represent strong barriers to malignancy. Thus, additional changes, such as mutations that would lead to elevated levels of HSF1, those that curtail the activity of the DDR, or those that accelerate progression through G1 and S phases of the cell cycle, are likely to be required to allow carcinogenesis of aneuploid cells. It is noteworthy that such changes are

indeed characteristic of established tumours. Moreover, our finding that aneuploidy destabilises the genome suggests that the paths leading to these changes might be shorter in aneuploid cells than in isogenic diploids. Thus, on the one hand aneuploidy has detrimental effects on cellular fitness and thereby imposes a strong selection pressure on cells to overcome these detrimental effects; on the other hand, it also increases the possibility that cells can evolve genetic changes that allow them to surmount these barriers.

With respect to fundamental research on aneuploidy, it is to be anticipated that yet additional karyotype-independent effects of aneuploidy will come to light as researchers attempt to further our understanding of how aneuploidy affects cell function. One major future challenge will be to gain more detailed molecular insights into the phenotypes of aneuploid cells, e.g. why exactly is protein folding capacity diminished in response to aneuploidy? What is the mechanism responsible for the downregulation of the MCM complex in aneuploid cells? In parallel to this, it is to be hoped that the growing body of research into aneuploidy will soon allow the formulation of overarching theories for how the different phenotypes of aneuploid cells are linked together. For example, is the impaired proteostasis of aneuploid cells really responsible for their increased levels of genomic instability? How is the impaired proliferative potential of aneuploid cells linked to their other phenotypes?

From the translational aspect, two major questions await answers. Firstly, to what extent can findings from model aneuploid cells be extrapolated to human trisomy conditions? Secondly, and following on from the first question, can insights from aneuploidy models be exploited for the treatment of these conditions? The resolution of these questions will require a bridging of the gap between two research areas that are present quite separate, the field of research on aneuploidy models and the field of research into human trisomies, particularly DS.

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12. Curriculum vitae

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National University of Ireland Galway, Ireland

2004 – 2009

BSc Honours Degree Molecular Biology with Industrial Placement

University of Aberdeen, Scotland, UK

Graduated with 1st class honours (top 5%)

Publications

Passerini V, Ozeri-Galai E, de Pagter M, **Donnelly N**, Schmalbrock S, Kloosterman WP, Kerem B, Storchová Z (2016): The presence of extra chromosomes leads to genomic instability. Nat Commun. Feb 15;7:10754 (Research Article)

Donnelly N, Storchová Z (2015): Causes and consequences of protein folding stress in aneuploid cells. Cell Cycle. 14(4), 495-501 (Invited "Extra View")

Donnelly N, Storchová Z (2015): Aneuploidy and proteotoxic stress in cancer. Mol Cell Oncol. 2:2 e976491 (Invited "Author's View")

Donnelly N, Passerini V, Dürrbaum M, Stingele S, Storchová Z (2014): HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells. EMBO J. Oct 16;33(20), 2374-87 (Research Article)

Donnelly N, Storchová Z (2014): Dynamic karyotype, dynamic proteome: buffering the effects of aneuploidy. Biochim Biophys Acta.1843, 473-81 (Invited Review)

Donnelly N, Gorman AM, Gupta S, Samali A (2013): The eIF2α kinases: their structures and functions. Cell Mol Life Sci. 70, 3493-511 (Review)

Gupta S, Giricz Z, Natoni A, **Donnelly N**, Deegan S, Szegezdi E, Samali A (2012): NOXA contributes to the sensitivity of PERK-deficient cells to ER stress. FEBS Lett. 586, 4023-30 (Research Article)

Pribylova R, Kralik P, **Donnelly N**, Matiasovic J, Pavlik I (2011): Mycobacterium Avium Subsp Paratuberculosis and the Expression of Selected Virulence and Pathogenesis Genes in Response to 6 Degrees C, 65 Degrees C and Ph 2.0. Brazilian Journal of Microbiology 42, 807-817 (Research Article)

Shitaye JE, Horvathova A, Bartosova L, Moravkova M, Kaevska M, **Donnelly N**, Pavlik I (2009): Distribution of Non-Tuberculosis Mycobacteria in Environmental Samples from a Slaughterhouse and in Raw and Processed Meats. Czech Journal of Food Sciences 27, 194-202 (Research Article)

Research Experience

2012 - present
Doctoral research on the consequences of aneuploidy in human cells, Max Planck Institute of Biochemistry, Martinsried, Germany
Supervisor: Zuzana Storchová

2009 - 2011
MSc research on the PKR-like ER kinase (PERK) and the Unfolded Protein Response (UPR), Department of Biochemistry, National University of Ireland Galway, Ireland
Supervisor: Prof. Afshin Samali

2009 (8 weeks)

BSc Honours research project on osmotic stress in Caenorhabditis elegans, Institute of Medical Sciences, University of Aberdeen, Scotland

Supervisor: Prof. Anne Glover

2007 – 2008 (10 months)

Industrial placement studying transcriptional response of the animal pathogen Mycobacterium avium subsp. paratuberculosis (MAP) to stresses commonly encountered in the food processing industry, Veterinary Research Institute, Brno, Czech Republic

Supervisor: Prof. Ivo Pavlik

Awards and Scholarships

Thomas Crawford Hayes Trust Fund Award, National University of Ireland, Galway, 2011

Brenda Page Memorial Prize in Genetics, University of Aberdeen, 2009 (http://www.abdn.ac.uk/registry/prizelist09.shtml)

Poster Presentations at International Conferences

August 2014

"HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells", FASEB Protein folding in the Cell, Saxtons River, VT, USA

June 2013

"Upregulation of selective autophagy is a hallmark of human aneuploid cells", Abcam Ubiquitin and Autophagy Conference, Amsterdam, Netherlands

September 2011

"Loss of PERK sensitises to ER stress-induced apoptosis through upregulation of NOXA", 19th Euroconference on Apoptosis, ECDO Meeting, Stockholm, Sweden

Teaching and Mentoring Experience

June – August 2013 (10 weeks) and May – August 2014 (10 weeks)
Supervision of two undergraduate research students as part of the RISE DAAD academic exchange program

2009-2011 (5 semesters)
Teaching weekly undergraduate practical laboratory classes, Department of Biochemistry, National University of Galway, Ireland

Extracurricular and Voluntary Work

2014 – present
Organisation and hosting of distinguished guest lecturers, MPIB, Martinsried, Germany

2003 – 2004
Year of Community Service, Ohrid, Republic of Macedonia

Languages

English	Native speaker
Bulgarian	Business fluent oral and written
German	Business fluent oral and written
Macedonian	Fluent oral and written
Persian	Fluent oral
Czech	Basic knowledge

Advanced training workshops

Poster Presentations by Dr. Ruth Willmott, BioScript International, 2014

Scientific Writing by Dr. Ruth Willmott, BioScript International, 2014