The Effects of Chromosome Number on Mitotic Fidelity and Karyotype Stability

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Abstract

The correct number of chromosomes is important for the maintenance of healthy cells and organisms. Maintenance of a correct chromosome number depends on the accurate distribution of chromosomes to the daughter cells during cell division, and errors in chromosome segregation result in abnormal chromosome numbers, or aneuploidy. Aneuploidy is typically associated with deleterious effects on organismal and cellular fitness; however, aneuploidy has also been associated with enhanced cellular growth in certain contexts, such as cancer. Another type of deviation from the normal chromosome number can occur when entire sets of chromosomes are added to the normal (diploid) chromosome number, resulting in polyploidy. Whereas polyploidy is found in certain normal tissues and organisms, tetraploidy (four sets of chromosomes) is associated with a number of precancerous lesions and is believed to promote aneuploidy and tumorigenesis. While it is clear that chromosome mis-segregation causes aneuploidy, the effect of aneuploidy on chromosome segregation is less clear. Similarly, it is unclear whether and how tetraploidy may affect chromosome segregation. The work described here shows that aneuploidy can cause chromosome mis-segregation and induces chromosome-specific phenotypic effects. In contrast, tetraploidy does not *per se* induce chromosome mis-segregation, but enables the accumulation of aneuploidy thanks to a “genetic buffer” effect that allows tetraploid cells to tolerate aneuploidy better than diploid cells.

Dedication

Dedicated to Professor Richard C. Strohman, my grandfather.

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Attribution

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Chapter 1. Introduction and literature review

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&

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Overview

At the crux of carcinogenesis lies complexity. Amongst a single tumor, cell-to-cell variability is manifest in many different properties: karyotype, morphology, enzyme receptors, ability to metastasize, mutations, and drug resistance. While seemingly chaotic per cell, the tumor as a whole is much like a mosaic displaying interdependency and organization. For this reason, it has been called a species (Duesberg and Rasnick, 2000; Huxley, 1956; Klein et al., 2010; Van Valen and Maiorana, 1991; Vincent, 2010), a society of cells (Heppner, 1984), and a complex ecosystem (Heppner, 1984; Merlo et al., 2006; Sachs and Hlatky, 2010). It has long been known that cancer cells are characterized by aneuploid karyotypes (Bayreuther and Klein, 1958; Chu and Giles, 1958; Hauschka and Levan, 1958), and an increasing body of work has unveiled a causal relationship between aneuploidy and tumorigenesis [reviewed in (Cimini, 2008; Pavelka et al., 2010b; Sen, 2000)]. A key role of aneuploidy in tumorigenesis and tumor progression, is also indicated by the fact that measuring DNA indices or ploidy is clinically informative as a prognostic indicator in various cancers (Atkin and Kay, 1979). For instance, ploidy measurements in different types of cancer (Frankfurt et al., 1985; Grote et al., 2001; Jakobsen, 1984; Jakobsen et al., 1988; Kallioniemi et al., 1987; Susini et al., 2011) are as, if not more, accurate when predicting survival than other widely used measures such as the prostate-specific antigen (PSA) test (Stamey, 2004; Vickers et al., 2011).

Karyotypic analysis also indicates that there is wide variability in chromosome number within the same cancer cell population, suggesting that errors in mitotic chromosome

segregation are recurrent in cancer cells. In this chapter, I review the current knowledge about intratumor karyotypic diversity, the effects of aneuploidy on cellular phenotypes, and the mechanisms that promote chromosome mis-segregation in cancer cells. In chapter two, I describe my work on the effects of aneuploidy on the fidelity of cell division. In chapter three, I describe my work on how tetraploidy influences chromosome segregation and karyotype stability. Finally, in chapter four I focus on future directions and unanswered questions.

Pre-neoplastic Aneuploidy

Aneuploidy is ubiquitous in cancer (Gebhart and Liehr, 2000; Mertens et al., 1997; Mitelman et al., 2014b; Weaver and Cleveland, 2006), and was proposed to have a causal role in cancer already over a century ago (Boveri, 1902; Boveri, 1914). Over the years, the idea that aneuploidy plays a causal role in the origin of cancer has been supported by substantial experimental evidence. For example, random aneuploidy appears after carcinogen application but before transformation in Chinese hamster (Duesberg et al., 2000a), mouse (Conti et al., 1986), rat (Sudilovsky and Hei, 1991), and human cells (Hittelman, 2001; Hittelman et al., 1996). Furthermore, although the results were occasionally ambiguous (see below for further discussion), a number of studies in mouse models carrying mutations/deletions that cause aneuploidy, showed a correlation between aneuploidy and tumorigenesis (Babu et al., 2003; Diaz-Rodriguez et al., 2008; Iwanaga et al., 2007; Michel et al., 2001; Sotillo et al., 2007; Weaver et al., 2007). For aneuploidy to play a causal role in tumorigenesis, it would be expected to occur in undifferentiated dividing cells within the organism's tissues. Unfortunately, the rates of chromosome mis-segregation and the frequencies of aneuploidy *in situ* are very difficult to assess due to the inaccessibility of primary tissues. One exception in humans is represented by peripheral blood lymphocytes, which can be easily isolated. Isolated lymphocytes can be either analyzed directly by chromosome- or centromere-specific fluorescence *in situ* hybridization (FISH) or can be used to establish short-term cultures. Cultured lymphocytes can be used for metaphase spread analysis (Minissi et al., 1999), micronucleus assay (Countryman and Heddle, 1976), or cytokinesis-block assay (Fenech, 1993a) to determine spontaneous rates of chromosome mis-segregation and

aneuploidy. Studies using these approaches have yielded a large amount of information, and indicate that the basal frequencies of aneuploidy for individual chromosomes in human lymphocytes range between 0.03% and 10% (Carere et al., 1998; Catalan et al., 2000; Hayes et al., 2000; Minissi et al., 1999; Ramirez et al., 2007; Rehen et al., 2005; Zhang et al., 1998). Furthermore, studies in brain cells have found aneuploidy for individual chromosomes to be ~4% (Kingsbury et al., 2006; Rehen et al., 2005). Such frequencies are surprisingly high. Indeed, if the same aneuploidy rates were occurring in all dividing cells, hundreds of thousands of aneuploid cells would be produced every day in each individual, given that ~2.5 x 10^8 cells are dividing at any given time (Alberts et al., 1994). If such frequencies were realistic, cancer would be expected to occur at much higher rates in the population. So, how can these high frequencies of aneuploidy be reconciled with the relatively low incidence of cancer if indeed aneuploidy is a cause of cancer? One possibility is that the rates of aneuploidy observed in peripheral blood lymphocytes and neurons are not representative of all cell types. However, it is noteworthy that the frequency of aneuploidy in peripheral blood lymphocytes of healthy individuals correlates with cancer risk (Hagmar et al., 1994; Olaharski et al., 2006; Rossner et al., 2005). In addition, aneuploidy in human peripheral blood lymphocytes is increased by various factors typically associated with cancer, such as smoking and age (1990; Hittelman, 2001), suggesting that aneuploidy in lymphocytes may be, if not equivalent, at least proportional to that of other cell types. The other possible explanation for such high frequencies of spontaneous aneuploidy resulting in a relatively low incidence of cancer is that not all aneuploidies are equal and the majority of aneuploid cells die (Li et al., 2009; Zhivotovsky and Kroemer, 2004). This

latter explanation is more likely given that high rates of aneuploidy are found in a number of pre-malignant lesions, such as head and neck amongst others (Hittelman, 2001; Hittelman et al., 1996; Ried et al., 1999).

Chromosome Instability and the Cancer Karyotype – between Order and Disorder

In addition to being aneuploid, cancer cells commonly exhibit high rates of chromosome mis-segregation, a phenomenon termed chromosomal instability (CIN). One consequence of CIN is the generation of karyotypic diversity in cancer cells. Such karyotypic variability within a cancer cell population typically ranges at any one time ±20% around the modal chromosome number (Beheshti et al., 2001; Li et al., 2009; Nicholson and Duesberg, 2009). For example, the modal chromosome number of the breast cancer cell line Ma6 is 83 and can fluctuate within the population by ±13% (Li et al., 2009). In other words, many cells possess 83 chromosomes, but cells with as few as 70, and as many as 96 chromosomes can be found within the population (e.g., one cell with 83 versus another with 72 chromosomes can be observed in the example shown in Fig. 1.1). Polyploidization can also occur causing a 100% change, but these cells only represent a minority of the entire cell population. Moreover, polyploidization may occur sporadically rather than recurrently in the cancer cell population (see below for further discussion). Thus, the majority of karyotypic variations within a cancer cell population generally result from gains or losses of individual chromosomes (Furuya et al., 2000; Haruki et al., 2001a; Li et al., 2009; Miyazaki et al., 1999; Nicholson and Duesberg, 2009; Yoon et al., 2002). Indeed, 30% of cells in Ma6 deviate from the modal copy number of chromosome 13 (n=4) by ±1-2 chromosomes whereas only 5% of cells

deviate from the modal copy number of chromosome 6 by ±1-2 chromosomes (n=3) (Fig. 1.1).

While the karyotypes of cancer are heterogeneous, they are not totally random. Indeed, the karyotypes of cancer cells are, over time (68 cell generations), relatively stable, varying only ±6% despite a variability of ±22% at any one time (Nicholson and Duesberg, 2009). The idea of a characteristic karyotype, termed the "stemline concept," was first described in the analysis of tumor ascites by Winge (Winge, 1930), and later supported with additional cytological evidence by Hauschka (Hauschka, 1953), Levan (Hauschka and Levan, 1958; Levan, 1956), and Makino (Makino, 1956). In short, the stemline concept holds that within each tumor, out of the heterogeneity of cancer cells, is an underlying karyotypic pattern or karyotypic stemline. Analysis of thousands of tumors from different tissues of origin using array comparative genomic hybridization (aCGH) showed these karyotypic patterns to be strongly tissue-dependent (Beroukhim et al., 2010; Gebhart and Liehr, 2000; Mertens et al., 1997) (Table 1.1). A possible explanation for this is that aneuploidies for specific chromosomes undergo negative selection in certain tissues but not others. Karyotypic stemlines in cancers have also been recently identified using multicolor fluorescence in situ hybridization (mFISH), corroborating the initial identification by Winge and leading to the conclusion that the cancer karyotype is "selected for oncogenic function" (Nicholson and Duesberg, 2009) or that "a dynamic equilibrium is maintained" (Heim and Mitelman, 2010). This stemline or equilibrium can be visualized graphically in a karyograph (Fig. 1.1), which plots the copy number for each chromosome in individual cells and in the cell population.

These stemline patterns have led some to propose that a specific karyotype may be the result of selective gain of chromosomes containing activated oncogenes and/or loss of chromosomes carrying tumor-suppressor genes (Lengauer et al., 1997b). In support of this idea, Baker et al. found that whole chromosome mis-segregation results in loss of heterozygosity for the tumor suppressor gene p53 (Baker et al., 2009). However, it is unclear in this study why cancer cells gained an extra copy of the chromosome carrying the mutant gene rather than simply losing the chromosome harboring the wild-type copy of p53. Moreover, the hypothesis of preferential gain/loss of chromosomes carrying oncogenes/tumor suppressor genes predicts that specific karyotypes should be expected because of the specific location of certain gene loci. However, such specific karyotypes have yet to be identified. Accordingly, clones with identical oncogenic mutations display distinct karyotypes (Duelli et al., 2007; Nicholson and Duesberg, 2009) and breast cancers sharing karyotypic patterns (Beroukhim et al., 2010; Gebhart and Liehr, 2000; Mertens et al., 1997) do not share mutations (Wadman, 2011). Finally, both transgenic and transfected oncogenes have been shown to induce cancers that become oncogene independent over time (Gilbert and Harris, 1988; Klein et al., 2010).

An alternative theory explaining why cancer karyotypes are not completely random, proposes that the selection of the karyotype depends on the fact that the karyotype as a whole is responsible for carcinogenesis (Nicholson and Duesberg, 2009). Equivalently, just as a species cannot be defined by a singular gene or a singular chromosome, nor can cancer (Heng, 2009).

Identifying specific contributions of individual chromosomes or individual genes in cancer will be exceedingly difficult given the complexity of aneuploid karyotypes and the continual generation of new karyotypes (CIN). For example, 5^{23} or ~10^{16} different karyotypes are possible if we assume 5 copy number states (monosomy, disomy, trisomy, etc.) per chromosome. Moreover, the difficulty may be exacerbated by the dependence of outcome on the karyotpic contexts. For instance, activated oncogenes of the RAS family have different effects in different karyotypic contexts, ranging from loss of tumorigenicity to enhanced tumorigenicity (Geiser et al., 1989). Additionally, the insertion of specific chromosomes into different cancer cell lines via microcell-mediated chromosome transfer show different effects on tumorigenicity, further demonstrating dependency on the karyotypic context (Cao et al., 2001; Negrini et al., 1994; Rimessi et al., 1994).

In conclusion, although analysis of cancer cell populations reveals some kind of equilibrium (order), the chromosomal instability of cancer cells generates ever-changing karyotypes (disorder). Regardless of how and why cancer karyotypic diversity originates, it yields phenotypic differences per cell, conferring flexibility to the tumor as a whole. Such flexibility is apparent in numerous properties of cancer, wherein CIN is associated with poor patient prognosis (Carter et al., 2006; Sheffer et al., 2009; Walther et al., 2008), and is responsible for cancer drug resistance (Duesberg et al., 2000b; McClelland et al., 2009; Swanton et al., 2009).

The effects of aneuploidy on the transcriptome and proteome

In aneuploid cells large numbers of genes are either lacking or present in excess. This may lead to underproduction or excessive production of both mRNA and proteins. Indeed, numerous studies have found chromosome copy number, including sub-chromosomal derivative chromosomes, to directly affect global transcript levels [Table 1.2; (FitzPatrick et al., 2002; Gao et al., 2007; Hyman et al., 2002; Phillips et al., 2001; Pollack et al., 2002b; Upender et al., 2004; Virtaneva et al., 2001; Williams et al., 2008)]. Moreover, recent studies in aneuploid budding yeast show a clear copy number-transcriptome-proteome relationship (Pavelka et al., 2010c). Despite this connection, the overall relationship between karyotype and phenotype cannot be considered linear. For instance, single trisomies, arising spontaneously or produced artificially, not only raise the gene expression of the trisomic chromosome, but alter that of hundreds of other genes on different chromosomes (FitzPatrick, 2005; FitzPatrick et al., 2002; Saran et al., 2003; Upender et al., 2004). Thus, chromosomal gains or losses exhibit pleiotropic effects across the whole genome. The complex effects of aneuploidy can alter gene expression both negatively and positively (underexpression and overexpression, respectively), and can act either directly or indirectly (Table 1.2 and Fig. 1.2).

Studies on the effects of aneuploidy on the cell phenotype have yielded contrasting results. For instance, experiments with trisomic mouse embryonic fibroblasts show that aneuploidy impairs proliferation, alters metabolic properties, and negatively affects immortalization (Williams et al., 2008). Yet, aneuploidy remains ubiquitous in cancer. A

recent study identified “aneuploidy tolerating” mutations capable of attenuating the changes in intracellular protein composition caused by aneuploidy (Torres et al., 2010). These findings could reconcile the prevalence of aneuploidy in cancer with the detrimental effects caused by it. However, another study found no evidence of benefit from stress response gene enrichment and/or no stress response gene enrichment at all in various aneuploid strains of budding yeast (Pavelka et al., 2010c). Importantly, there is no universal gene expression signature specific to aneuploidy (Pavelka et al., 2010c; Upender et al., 2004). Therefore, it appears that whether aneuploidy promotes or inhibits tumorigenesis largely depends on the context and karyotype.

The adaptive potential of aneuploidy

The large-scale genomic changes caused by aneuploidy, which alters the expression of hundreds to thousands of genes (Gao et al., 2007; Pollack et al., 2002b; Thayer, 1996; Upender et al., 2004), can limit the growth of aneuploid cells under standard environmental conditions (Torres et al., 2007). Under certain circumstances, however, these same changes can confer enhanced fitness (Pavelka et al., 2010c; Tang et al., 2011). For example, in *Candida albicans*, formation of isochromosome 5L causes azole resistance via upregulation of the genes ERG11 and TAC1 (Selmecki et al., 2008). Similarly, in aneuploid *Saccharomyces cerevisiae*, the gain of chromosome XIII confers resistance against the DNA damaging agent 4-NQO (Pavelka et al., 2010c) due to the overexpression of ATR1, a gene on chromosome XIII whose overexpression is sufficient to confer resistance to 4-NQO (Mack et al., 1988; Pavelka et al., 2010c). The adaptive potential of aneuploidy in yeast is well demonstrated for many other exogenous stresses (Pavelka et al., 2010c; Rancati and Pavelka, 2013; Selmecki et al.,

2006; Selmecki et al., 2009; Tang et al., 2011). Aneuploidy has also been proposed as a mechanism that can counteract the accumulation of deleterious mutations, a process termed Muller's Ratchet (Bignold, 2007a; Bignold, 2007b; Duesberg and McCormack, 2013; Muller, 1964; Torres et al., 2008; Vincent, 2011). An elegant study by Rancati et al. supports this idea showing that aneuploidy can rescue deleterious mutations in a conserved cytokinesis motor (Rancati et al., 2008). Similarly, the gain of chromosome VI in yeast is lethal due to the overexpression of the highly dosage-sensitive gene encoding □-tubulin (TUB2) (Anders et al., 2009; Katz et al., 1990; Torres et al., 2007); however, gain of chromosome XIII, which encodes □-tubulin, restores the balance and the double disome VI/XIII is viable (Anders et al., 2009). The findings in yeast extend to many other situations, including naturally occurring chromosomal imbalances, and aneuploidy in cancerous and non-cancerous mammalian cells. For instance, immortalized colon epithelial cells with trisomy 7 out-compete immortalized diploid colon cells in serum-free media (Ly et al., 2011). Likewise, tyrosinemia-induced stress in mice livers can be overcome by the emergence of aneuploid hepatocytes lacking chromosome 16 (Duncan et al., 2012). Chromosome 16 carries the homogentisic acid dioxygenase (HGD) gene and loss of HGD in a heterozygous background causes resistance to tyrosinemia-induced hepatic injury (Duncan et al., 2012). Although the exact mechanisms have been hard to elucidate due to the complexity of cancer karyotypes, the karyotype-phenotype relationship has also been proposed as a mechanism for adaptation in cancer (Duesberg et al., 2007). Indeed, cancer cells that display high rates of CIN, and consequent karyotypic heterogeneity, display intrinsic drug resistance to a wide range of kinase inhibitors (Lee et al., 2011) and other drugs

(Li et al., 2005). There have been attempts to selectively inhibit proliferation of aneuploid cells with specific drugs (Tang et al., 2011), however such drugs have not proven effective in other aneuploid cells, even at the highest tolerated dose (Li et al., 2012). In summary, the effects of aneuploidy on the adaptive potential of cancer cells are related to aneuploidy itself and the degree of chromosome instability. Thus, identifying the defects that lead to aneuploidy is important in understanding cancer.

How mitotic errors contribute to the karyotypic diversity of cancer cells

The goal of mitosis is to accurately segregate the replicated chromosomes into two daughter cells. This is achieved through the interaction between the sister chromatids of each replicated chromosome with microtubules from opposite poles of the mitotic spindle (Fig. 1.3). This interaction occurs at the kinetochore, a specialized protein structure that assembles at the centromeric region of each chromatid [for review see (Cheeseman and Desai, 2008; Maiato et al., 2004; Santaguida and Musacchio, 2009)], and leads to the alignment of chromosomes at the metaphase plate with the two sister chromatids oriented back-to-back (Fig. 1.3). When the cohesion between the two sisters is “dissolved” at anaphase onset, the two chromatids (now chromosomes) are pulled poleward by their associated kinetochore-microtubules (Fig. 1.3). A mitotic checkpoint (also known as the spindle assembly checkpoint or SAC) delays anaphase onset until kinetochore-microtubule attachment has been achieved by all chromosomes [reviewed in (Musacchio and Salmon, 2007)], thus increasing the fidelity of mitosis. It is easy to infer that the chromosome number instability associated with cancer must be a result of inaccurate mitotic chromosome segregation in cancer cells. Indeed, a number of recurrent errors have been observed in cancer cells, and the remaining part of this

section will focus on mitotic errors typically found in cancer cells and how they contribute to cancer karyotypic diversity.

Kinetochore-Microtubule Attachments

Accurate chromosome segregation is ensured by the attachment of the two sister chromatids to microtubules from opposite spindle poles (amphitelic attachment; Fig. 1.4A). However, other types of attachments can and do occur in mitosis. These include monotelic, syntelic, and merotelic attachment (Fig. 1.4B-D).

Monotelic attachment occurs when one of the two sister kinetochores of an individual chromosome is attached to one spindle pole whereas the other sister is unattached (Fig. 1.4B). This type of attachment is very common in early mitosis, and it is known to trigger a checkpoint-dependent mitotic delay (Rieder et al., 1995; Rieder et al., 1994). Thus, if the mitotic checkpoint is functional (see below for a more specific discussion of mitotic checkpoint and cancer), monotelic kinetochore attachment will not contribute to cancer karyotypic diversity.

Syntelic attachment occurs when the two sister kinetochores of an individual chromosome bind microtubules from the same spindle pole (Fig. 1.4C). Syntelic attachments are not commonly observed in untreated cells (Hauf et al., 2003), and there are no reports of increased frequencies of syntelic attachments in cancer cells, suggesting that syntelic attachment does not play an important role in cancer cell chromosome mis-segregation.

Finally, merotelic attachment occurs when a single kinetochore binds microtubules from two poles rather than just one (Cimini et al., 2001a; Ladrach and LaFountain, 1986) (Fig. 1.4D). Merotelic attachments occur frequently in early mitosis (Cimini et al., 2003) and can be corrected via an Aurora B-dependent mechanism prior to anaphase onset (Cimini, 2007; Cimini et al., 2006; DeLuca et al., 2006). However, merotelic attachments do not induce a SAC-dependent mitotic delay (Cimini et al., 2004; Cimini et al., 2002; Khodjakov et al., 1997; Wise and Brinkley, 1997; Yu and Dawe, 2000), and thus they can persist into anaphase, at which time their behavior will depend on the relative size of the microtubule bundles bound to the individual kinetochore [Fig. 1.5; (Cimini et al., 2004); reviewed in (Cimini, 2008; Gregan et al., 2011)]. If the two microtubule bundles attached to a merotelic kinetochore are different in size, during anaphase the chromosome will be shifted to the side attached to the thicker bundle [Fig. 1.5A; (Cimini et al., 2004)]. Conversely, if the two microtubule bundles are comparable in size, the merotelically attached chromosome will lag behind at the spindle equator while all the other chromosomes segregate to opposite spindle poles [Fig. 1.5B; (Cimini et al., 2004; Cimini et al., 2001a)]. Although the experimental observations on the fate of these anaphase lagging chromosomes are relatively limited (Cimini et al., 2002; Utani et al., 2010), they seem to suggest that the lagging chromosome can end up in either one of the two daughter cells, depending on where the cleavage furrow ingresses with respect to the lagging chromosome (Fig. 1.5B, middle and right panels). These experiments (Cimini et al., 2002; Utani et al., 2010) also suggest that anaphase lagging chromosomes form micronuclei upon mitotic exit (Fig. 1.5B, left panels). Interestingly, micronuclei are frequently observed in cancer cells (1990) and represent a biomarker

for increased risk of cancer [(reviewed in (Fenech, 2002; Majer et al., 2001)]. Although some micronuclei may contain chromosome fragments rather than entire chromosomes, it is noteworthy that a number of studies have shown that anaphase lagging chromosomes are frequently observed in cancer cells (Ganem et al., 2009; Gisselsson et al., 2005; Reing et al., 2004; Saunders et al., 2000; Silkworth et al., 2009; Thompson and Compton, 2008b), and possibly represent the most common type of chromosome mis-segregation in CIN cancer cells [reviewed in (Cimini and Degrassi, 2005; Compton, 2011)]. Notably, mutations in (or depletion of) certain genes traditionally classified as oncogenes or tumor suppressor genes cause defects that lead to merotelic attachments and anaphase lagging chromosomes. For example, truncation of the adenomatous polyposis coli (APC) gene or depletion of the APC protein causes anaphase lagging chromosomes (Draviam et al., 2006; Green and Kaplan, 2003; Green et al., 2005). Similarly, a recent series of studies showed that the retinoblastoma protein (pRb) plays an important role in mitosis and its depletion results in aberrant chromosome condensation (Coschi et al., 2010), centromere dysfunction (Manning et al., 2010), chromatid breaks and cohesion defects (van Harn et al., 2010), which can in turn lead to merotelic attachment and anaphase lagging chromosomes (Manning et al., 2010; Sage and Straight, 2010).

Spindle Assembly Checkpoint (SAC)

The spindle assembly checkpoint (SAC) is a biochemical pathway that can delay the metaphase to anaphase transition in the presence of unattached kinetochores and possibly lack of tension across sister kinetochores (Li and Nicklas, 1995; Musacchio and Salmon, 2007; Rieder et al., 1995; Rudner and Murray, 1996). The various genes

and corresponding proteins that act within the SAC pathway were initially identified in genetic screens of yeast. These screens revealed 6 genes necessary for SAC function, *MAD1-3* (*M*itotic *a*rrest *d*efective), *BUB1*, *BUB3* (*B*udding *u*inhibited by *b*enomyl), and *MPS1* (*M*ono*p*olar *s*pindles) (Hardwick and Murray, 1995; Hoyt et al., 1991; Li and Murray, 1991; Roberts et al., 1994; Weiss and Winey, 1996; Winey et al., 1991). Subsequent studies have identified homologs in higher eukaryotes, wherein *MAD3* is termed *BUBR1*.

Upon mitotic entry, the SAC is active (Khodjakov and Rieder, 2009), and it is maintained in an active state as long as unattached kinetochores are present. Both Mad1 and Mad2 localize at unattached kinetochores (Howell et al., 2004; Shah et al., 2004; Waters et al., 1998), and the interaction between Mad1 and Mad2 promotes a conformational change in Mad2 (DeAntoni et al., 2005), driving it to bind to and sequester Cdc20 (Fang et al., 1998; Hwang et al., 1998; Kim et al., 1998), an activator of the anaphase promoting complex, or cyclosome (APC/C) (Hwang et al., 1998; Kim et al., 1998; Li et al., 1997). Additionally, Bub1 and BubR1 (MAD3 in yeast) are recruited to tensionless kinetochores (Skoufias et al., 2001), where they further interact with Mad2 forming the mitotic checkpoint complex (Sudakin et al., 2001; Tang et al., 2001), which also inhibits APC/C. APC/C is an E3 ubiquitin ligase that tags specific substrates for degradation by the proteasome. Two key substrates are the proteins Securin and Cyclin B, both necessary for the completion of mitosis (Cohen-Fix et al., 1996; Funabiki et al., 1996; Minshull et al., 1990; Peters, 2006; Whitfield et al., 1990; Zou et al., 1999). Securin inhibits the enzyme Separase, which is necessary to cleave the Cohesin proteins

holding the sister chromatids together (Cohen-Fix et al., 1996; Funabiki et al., 1996; Uhlmann et al., 1999). Thus, degradation of Securin leads to activation of Separase, degradation of Cohesin, and sister chromatid separation, which marks anaphase onset (Cohen-Fix et al., 1996; Funabiki et al., 1996; Zou et al., 1999). Cyclin B degradation will then ensure mitotic exit and completion of cell division.

SAC dysfunction invariably leads to chromosome mis-segregation because it causes cells to enter anaphase before amphitelic attachment can be achieved by all chromosomes either by accelerating mitotic progression or by rendering the cells unable to delay anaphase onset in the presence of unattached kinetochores (Meraldi et al., 2004). As a consequence, precocious anaphase onset is associated with a number of mitotic defects, including anaphase lagging chromosomes and segregation of both sister chromatids to the same daughter cell, that generate aneuploidy in the progeny. Thus, mutations in SAC genes have the potential to play a role in tumorigenesis and cancer cell karyotypic diversity. Indeed, Cahill and co-workers identified a mutation in the SAC gene *BUB1* in a CIN colorectal cell line (Cahill et al., 1998) and postulated that this could explain the chromosomal instability that had been previously observed in colorectal cancers (Lengauer et al., 1997b). Interestingly, *BUB1* and *BUBR1* mutations have been identified in some individuals affected by mosaic variegated aneuploidy (MVA) (Hanks et al., 2004; Suijkerbuijk et al., 2010), a syndrome associated with increased risk of cancer, and in which the patients' somatic cells exhibit high levels of trisomies and monosomies (Kajii et al., 2001; Kajii et al., 1998).

The idea that SAC dysfunction may play a role in tumorigenesis prompted a number of studies in mouse models. Because SAC-null mice are usually embryonic lethal, these studies used mice that were either haploinsufficient or carried hypomorphic mutations in specific SAC genes. The results varied somewhat and did not reveal a clear correlation between mutations in SAC genes, aneuploidy, and tumorigenesis. However, in many cases the haploinsufficient or mutant animals were more prone to develop either spontaneous (Iwanaga et al., 2007; Michel et al., 2001) or carcinogen-induced (Babu et al., 2003; Dai et al., 2004; Iwanaga et al., 2007; Jeganathan et al., 2007) tumors compared to wild type mice. One exception is represented by *BubR1*, whose mutations do not result in increased tumor incidence but rather in senescence phenotypes (Baker et al., 2004). Interestingly, overexpression of various SAC genes are found in cancer cells (Yuan et al., 2006) and functional tests of Mad2 overexpression in mice result in 40-55% aneuploid MEFs (mouse embryonic fibroblasts) and a 50% tumor incidence (Sotillo et al., 2007). These findings suggest that the SAC pathway must be finely balanced to prevent aneuploidy. Alternatively, this effect may be specific to Mad2 overexpression and may be due to other SAC-independent function(s) of the protein (Weaver et al., 2008).

Because of the initial identification of a checkpoint gene mutation in colorectal cancer cells (Cahill et al., 1998) and because of the impact that SAC gene mutations have on tumorigenesis in mouse models, many researchers embarked in a number of mutational analyses of cancer cells of different origin with the idea that most cancers would display mutations in SAC genes. Surprisingly, many of these studies found no mutations in SAC

genes (Myrie et al., 2000; Saeki et al., 2002; Sato et al., 2000; Yamaguchi et al., 1999) and only a few identified mutations in a minor fraction of the cell lines analyzed (Haruki et al., 2001b; Sato et al., 2000), pointing to the idea that mutations in SAC genes may result in rates of chromosome mis-segregation that are too high to be compatible with cell survival. Thus, although SAC mutations can cause aneuploidy and in principle induce tumors, the fact that SAC gene mutations are largely absent in cancer indicates that they do not significantly contribute to the CIN phenotype and the karyotypic diversity of cancer cells.

Multipolarity and Spindle Geometry

It has long been known that centrosome amplification and multipolar mitotic spindles are common features of cancer cells (Ganem et al., 2009; Ghadimi et al., 2000; Gisselsson, 2005; Jin et al., 2007; Kaplan et al., 2001; Lingle et al., 2002; Lingle and Salisbury, 1999; Pihan et al., 1998; Reing et al., 2004; Sato et al., 2001; Saunders et al., 2000; Silkworth et al., 2009; Stewenius et al., 2005; Stewenius et al., 2007). Centrosome amplification can arise through different mechanisms, including cytokinesis failure (Fujiwara et al., 2005b), mitotic slippage (Dai et al., 2004), centriole overduplication (Balczon et al., 1995; Basto et al., 2008; Habedanck et al., 2005), centriole fragmentation (Peloponese et al., 2005), aberrant centriole cohesion (Wang et al., 2008), and cell fusion (Duelli and Lazebnik, 2003), although it is not clear how prevalent these mechanisms are in cancer. The presence of extra centrosomes leads to the assembly of multipolar mitotic spindles (Fig. 1.6A), which may in turn lead to segregation of chromosomes into more than two daughter cells, thus yielding high rates of chromosome mis-segregation and aneuploidy. Nevertheless, whereas multipolar

spindles are frequently observed in prometaphase, they are not very common in anaphase in a majority of cancer cell types. This observation led to the finding that some cancer cell types can cluster the extra centrosomes to produce a bipolar spindle before anaphase onset (Fig. 1.6B) (Brinkley, 2001; Ganem et al., 2009; Kwon et al., 2008; Quintyne et al., 2005; Silkworth et al., 2009). Indeed, this appears to be the case for most cancer cells displaying centrosome amplification and multipolar spindles. One exception is represented by Wilms tumor cells, which frequently exhibit tripolar chromosome segregation followed by cleavage of the cell in two instead of three, so that approximately two thirds of the chromosomes will end up in one daughter cell and one third in the other daughter cell (Gisselsson et al., 2010). In most other cancer cells studied, the initial assembly of a multipolar spindle is followed by centrosome clustering and bipolarization, so that chromosome segregation occurs in a bipolar fashion (Fig. 1.6) (Brinkley, 2001; Ganem et al., 2009; Kwon et al., 2008; Quintyne et al., 2005; Silkworth et al., 2009). In fact, those cells that undergo multipolar division produce daughter cells that exhibit either cell cycle arrest or post-mitotic cell death (Ganem et al., 2009). Despite the ability of multipolar cells to undergo bipolar cell division, the transient assembly of a multipolar spindle was recently shown to be strictly linked to the high rates of chromosome mis-segregation in CIN cancer cells (Ganem et al., 2009; Silkworth et al., 2009). Indeed, the transient multipolar prometaphase promotes the formation of large numbers of kinetochore mis-attachments, particularly merotelic (Fig. 1.6A) (Ganem et al., 2009; Silkworth et al., 2009). Because merotelic attachments do not trigger a SAC-dependent mitotic delay, such high rates of merotelic attachments

result in high rates of anaphase lagging chromosomes (Fig. 1.6C) (Ganem et al., 2009; Silkworth et al., 2009; Thompson and Compton, 2008b).

Interestingly, other factors previously believed to possess strictly intrinsic oncogenic properties, such as proteins from different viruses implicated in cancer, have been shown to affect spindle assembly and geometry. For instance, the protein E7 of human papillomavirus 16 (HPV-16) induces rapid centrosome duplication (Duensing et al., 2000), and transfection of viral genes encoding viral proteins E7 and E6 result in 50% of cells assembling multipolar spindles (Duensing et al., 2000). Similarly, expression of the viral protein HBX of hepatitis B virus also induces centrosome amplification (Fujii et al., 2006; Yun et al., 2004). These observations, plus the fact that systemic infection results in monoclonal cancers may point to viruses as indirect carcinogens that would induce cancer by inducing chromosome mis-segregation and karyotypic evolution. This would explain the long latency between infection by oncogenic viruses and cancer development, as cancer would result from the emergence of a specific karyotype within the infected cell population.

In conclusion, multipolarity and aberrant spindle geometry in cancer cells can be caused by a number of different mechanisms, which make it a wide-spread feature of cancer cells and a major mechanism of chromosomal instability. The extent to which abnormal spindle geometry and multipolarity contribute to cancer progression is likely a function of the variability they produce. Indeed, centrosomal abnormalities parallel chromosomal

instability in numerous cancers (D'Assoro et al., 2002; Ghadimi et al., 2000; Pihan et al., 2001; Sato et al., 2001; Skyldberg et al., 2001).

Microtubule Dynamics

As described above, merotelic kinetochore attachment can persist into anaphase and induce chromosome mis-segregation in the form of lagging chromosomes. Most cancer cells analyzed so far exhibit significantly higher rates of merotelically attached anaphase lagging chromosomes than non-tumor cells (Ganem et al., 2009; Silkworth et al., 2009; Thompson and Compton, 2008b). Increased rates of merotelic attachments can result from either increased rates of merotelic kinetochore formation, or from decreased rates of merotelic attachment correction. Recent studies have shown that cancer cells incur both these problems. Indeed, correction of kinetochore mis-attachments relies on the ability of incorrectly attached kinetochore microtubules to detach from their attachment site and leave an empty site to which microtubules from the correct spindle pole can attach. This is possible thanks to the intrinsic properties of kinetochore microtubules, which turn over with half-lives on the order of a few minutes during the early stages of mitosis (Zhai et al., 1995) to allow for such correction to occur (Cimini et al., 2006; DeLuca et al., 2006). A recent study showed that microtubule dynamics must be finely controlled, and that experimentally-induced minor reductions in kinetochore microtubule turnover are associated with high rates of chromosome mis-segregation resulting from the inability to correct merotelic attachments (Bakhoum et al., 2009b). Interestingly, many different cancer cell lines were found to exhibit kinetochore microtubule turnover rates falling within those ranges known to prevent efficient correction of kinetochore mis-attachments (Bakhoum et al., 2009a). Therefore, chromosome mis-segregation in

cancer cells appears to depend on both increased rates of formation (see previous section) and decreased rates of correction of merotelic (and possibly other) kinetochore mis-attachments.

Polyploidization

Polyploidy is the condition of a cell or organism possessing extra haploid sets of chromosomes. Although species with up to eight sets of chromosomes (octoploidy) are known in plants, the most typical polyploidies observed in humans are triploidy and tetraploidy (Comai, 2005; Leitch and Leitch, 2008). The addition of a complete set of chromosomes, while seemingly a large genetic perturbation, is better tolerated than certain trisomies in humans, with reported cases of both triploid and tetraploid live births surviving up to 1 year (Golbus et al., 1976; Pitt et al., 1981; Russell et al., 1981; Scarbrough et al., 1984; Sherard et al., 1986). Moreover, polyploid cells are found in some normal tissues, including the liver and aorta (Barrett et al., 1983; Biesterfeld et al., 1994; Celton-Morizur and Desdouets, 2010; Goldberg et al., 1984). Such tolerance of polyploidy has been suggested to depend on the balance in genomic content being maintained with polyploidy as opposed to aneuploidy (Papp et al., 2003; Tal, 1979; Torres et al., 2008; Veitia, 2002). Indeed, in a comparison between mouse hepatocytes with different polyploidies the gene transcriptome was largely unchanged (Lu et al., 2007).

While the karyotypes of most cancer cells are not perfect multiples of the haploid karyotype many are found to be near-triploid or near-tetraploid with chromosome number frequently ranging between ~60 and ~100 (Mitelman et al., 2014b).

Polyploidization can be caused by several different mechanisms, including cytokinesis failure, mitotic slippage, and endoreduplication. Cytokinesis failure can occur due to a failure of the actin-myosin cytokinetic ring to assemble or to initial furrow ingression followed by cleavage furrow regression. Typically, this would produce a single binucleate daughter cell. If such a cell progressed through the cell cycle, the following mitosis would be characterized by a tetraploid karyotype and four centrosomes. This could lead to high rates of chromosome mis-segregation due to transient multipolarity (Fig. 1.6) or multipolar cell division (see earlier section for a detailed discussion on multipolarity). Cytokinesis failure is unlikely to be recurrent in cancer cells because this would lead to an ever-increasing chromosome number. However, it is possible that occasional failure of cytokinesis may be a source of karyotype diversity in cancer. Moreover, it is possible that polyploidization may represent an early step in carcinogenesis (Boveri and Harris, 2008; Fujiwara et al., 2005b; Ganem et al., 2007). Indeed, tetraploid cells are found in a number of premalignant lesions, including those of the cervix and esophagus (Galipeau et al., 1996; Olaharski et al., 2006). In addition, experimentally-induced cytokinesis failure can promote tumorigenesis when the tetraploid cells are injected in nude mice (Fujiwara et al., 2005b). Interestingly, the carcinogenic effect of asbestos fibers has been associated with their ability to physically block cytokinesis and induce furrow regression (Jensen et al., 1996). Similarly, chromatin bridges spanning the spindle midzone can induce furrow regression (Mullins and Biesele, 1977; Steigemann et al., 2009).

Polyploidization can also occur by mitotic slippage, a phenomenon by which cells exit mitosis in the absence of chromosome segregation. Mitotic slippage typically occurs when cells are kept in the presence of microtubule poisons for prolonged periods of time (Brito and Rieder, 2006; Di Leonardo et al., 1997; Rieder and Palazzo, 1992). This adaptation has also been observed *in vivo* with the polyploidization of liver cells, a mechanism proposed to confer resistance to different cellular stresses (Duncan et al., 2010). Whereas mitotic slippage has not been documented as a common phenomenon in cancer cells, it may occur during chemotherapeutic treatment (e.g., paclitaxel treatment), and may play a role in development of drug resistance.

Finally, polyploidization can be generated by endoreduplication, a phenomenon by which the DNA replicates without intervening cell division (Edgar and Orr-Weaver, 2001). Although endoreduplication has been suggested to be associated with tumorigenesis (Lee et al., 2009; Storchova and Pellman, 2004), to date, there are no reports of experimental data linking endoreduplication to tumorigenesis or CIN.

Other mechanisms capable of inducing polydploidization are cellular fusion and entosis, both generating polyploid cells starting from two different nuclei. In the process of cellular fusion two cells fuse to become one (Duelli and Lazebnik, 2003; Lu and Kang, 2009). Cellular fusion can arise spontaneously or as an effect of different viruses (Duelli and Lazebnik, 2003). For example, otherwise harmless viruses contribute to CIN independently of the expression of viral proteins by inducing cell fusion (Duelli et al., 2007), which has been shown to induce destabilization of the genome (possibly due to

the presence of extra centrosomes) and cause global gene expression changes (Duelli and Lazebnik, 2007). In the process of entosis one cell envelopes an entire separate cell (Krajcovic et al., 2011).

Knowledge gaps and open questions

Many mechanisms and causes of CIN have been identified in cancer cells, including transient spindle geometry defects (Ganem et al., 2009; Silkworth and Cimini, 2012; Silkworth et al., 2009), impaired microtubule dynamics (Bakhoum et al., 2009a; Bakhoum et al., 2009b), polyploidization (Fujiwara et al., 2005b), and, rarely, a dysfunctional mitotic checkpoint (Cahill et al., 1998; Haruki et al., 2001b; Sato et al., 2000), although the mitotic checkpoint is functional in most cancer cells (Tighe et al., 2001). Some investigators have argued that a potential cause of CIN is aneuploidy itself (Biron-Shental et al., 2015a; Duesberg et al., 1998b; Sheltzer et al., 2011; Zhu et al., 2012), although there has been disagreement on whether this is really the case (Duesberg, 2014; Heng, 2014; Valind and Gisselsson, 2014a; Valind et al., 2013), with a number of reports concluding that CIN is an aneuploidy-independent trait (Lengauer et al., 1997b; Storchova and Kuffer, 2008; Zasadil et al., 2013). In my work (chapter two), I sought to clarify whether or not aneuploidy *per se* can cause chromosome mis-segregation.

Additionally, tetraploidy is acknowledged to play a role in carcinogenesis (Ganem et al., 2007) and to preceed aneuploidy (Galipeau et al., 1996; Shackney et al., 1989). Tetraploidy can be caused by several different mechanisms, including cytokinesis failure (Fujiwara et al., 2005b), mitotic slippage (Azeddine et al., 1998), and

endoreduplication (Edgar and Orr-Weaver, 2001). Typically, this would produce a single binucleate daughter cell. If such a cell progressed through the cell cycle, the following mitosis would be characterized by a tetraploid karyotype and four centrosomes.Both cytokinesis failure and mitotic slippage would produce daughter cells with twice the number of chromosomes (tetraploidy) and twice the number of centrosomes. The accepted view has been that upon mitotic entry these cells would form multipolar spindles due to the presence of extra centrosomes, and this would lead to high rates of chromosome mis-segregation due to transient multipolarity (Fig. 1.6) or multipolar cell division (see above for a detailed discussion on multipolarity). A recent study has also suggested that tetraploidy may promote the accumulation of aneuploidy by increasing the tolerance for chromosome gains and losses. In chapter three I analyze mitotic defects in experimentally induced tetraploid cells to study how tetraploidy contributes to the accumulation of aneuploidy and the generation of karyotypic heterogeneity.

Table 1.1 Recurrent aneuploidies in cancers from different tissues

Cancer type	Recurrent aneuploidies		Cases
	Gains	Losses	
Acute Lymphoblastic Leukemia	21	-	533
Breast Cancer	**1q**, **8q**, 17q, **20**	**8p**, 16q, **17p**	2108
Cervical Cancer	**3q**	NA	526
Colorectal Cancer	**7**, **8q**, 13, **20**	**8p**, **17p**, 18	989
Esophageal Cancer	**1q**, **3q**, 5p, **7**, **8q**, 12p, **20**	**3p**, 4, 5q, **8p**, 13, 18q, 19	402
Gastric Cancer	**8q**, **20**	-	777
Glioma	**7**	1p, 19q	591
Head & Neck Cancer	**3q**, **8q**, 11q	**3p**, **8p**	714
Hepatic Cancer	**1q**, **8q**	4q, **8p**, 13, 16q, **17p**	903
Medulloblastoma	**7**, 17q	**17p**	1153
Pancreatic cancer	**1q**, **3q**, 5p, 7p, **8q**, 20q	**3p**, 4q, 6, **8p**, 9p, **17p**, 18, 22	327

Cancers display common recurrent aneuploidies (indicated in bold), as well as recurrent aneuploidies that are tumor- and tissue/organ of origin-specific. Data are adapted from the arrayMap website [www.arraymap.com; (Cai et al., 2012)] and represent gains or losses that occur in at least 25% of cases analyzed.

Table 1.2 The Effect of Aneuploidy on Global Gene Expression

Cells	Aneusomy	Genes on chr.	Over-expressed genes		Under-expressed genes		Method	Reference
			# (on chr.)	Fold (or %) increase	# (on chr.)	Fold (or %) decrease		
DLD1	Trisomy 3	1924	81 (17 on chr3)	>1.7-fold	67 (0 on chr3)	>1.7-fold	Micro-cell mediated chr. transfer	Upender et al., 2004
	Trisomy 7	1880	155 (32 on chr7)	>1.7-fold	47 (3 on ch7)	>1.7-fold		
	Trisomy 13	703	92 (10 on chr13)	>1.7-fold	72(0 on chr13)	>1.7-fold		
HME	Trisomy 3	1924	91 (21 on chr3)	>1.7-fold	49 (2 on chr3)	>1.7-fold	Same as above	
Amniocyte	Trisomy 21	449	54 (3 on chr21)	>1.7-fold	54 (0 on chr21)	>1.7-fold	Spontaneous trisomy	Fitzpatrick et al., 2002
	Trisomy 13	703	273 (7 on chr13)	>1.7-fold	86 (0 on chr13)	>1.7-fold		
MEF	Trisomy 19	1037	10(2); 64(10)	2-fold; 1.5-fold	8(0); 75(0)	2-fold; 1.5-fold	Selective breeding	Williams et al., 2008
	Trisomy 1	1944	18(4); 139(29)	2-fold; 1.5-fold	33(0); 144(2)	2-fold; 1.5-fold		
	Trisomy 13	1497	6(3); 76(10)	2-fold; 1.5-fold	6(0); 118(1)	2-fold; 1.5-fold		
	Trisomy 16	1025	2(1); 50(11)	2-fold; 1.5-fold	4(0); 45(1)	2-fold; 1.5-fold		
DBA-2/DB-P	No change (avg)	NA	NA	Log_2 ratio (0.02)	NA	NA	Subclone of established glioblastoma cell line versus parental gliobastoma line	Gao et al., 2007
	Chr2 (4somy/3somy)	2346	NA	Log_2 ratio (0.18)	NA	NA		
	Chr7 (6somy/5somy)	1880	NA	Log_2 ratio (0.16)	NA	NA		
	Chr8 (4somy/3somy)	1317	NA	Log_2 ratio (0.30)	NA	NA		
	Chr14 (2somy/3somy)	1526	NA	NA	NA	Log_2 ratio (-0.33)		
	Chr15 (4somy/5somy)	1272	NA	NA	NA	Log_2 ratio (-0.25)		
	Chr20 (4somy/5somy)	884	NA	NA	NA	Log_2 ratio (-0.32)		
	Chr21 (4somy/7somy)	449	NA	NA	NA	Log_2 ratio (-0.54)		
AML	Trisomy 8	1317	NA	32% up compared to other chr.	NA	NA	Natural	Virtaneva et al., 2001
Prostate Cancers	Various	NA	Up-regulated genes on chr. with increased copy number	42%	Down-regulated genes on chr. with decreased copy number	51%	Natural	Phillips et al., 2001

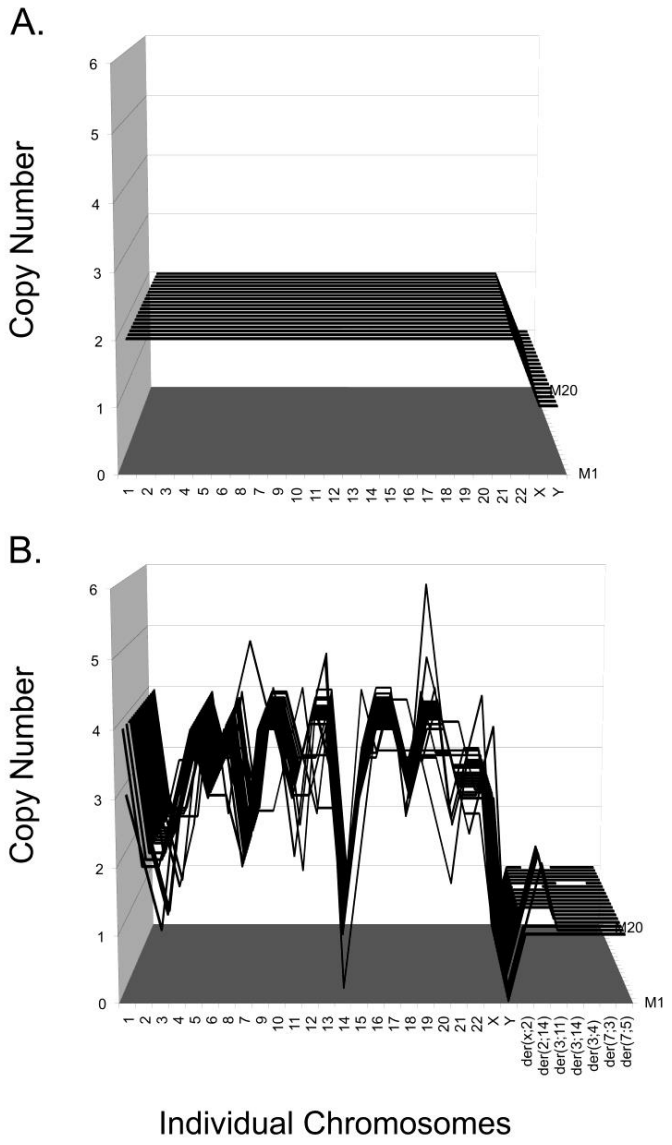


Figure 1.1 Visualization of the karyotypic stemline in a karyograph

The 3-dimensional graphs (karyographs) show the copy number of individual chromosomes per cell for a population of cells. **(A)** Karyograph of normal male diploid cells. **(B)** Karyograph of the transformed mammary epithelial cell line Ma6 (Kendall et al., 2005). Each karyograph shows the results obtained by M-FISH analysis of 20 different cells. "M" denotes metaphase; M1-M20 are displayed along the y-axis. Note, both the karyotypic stemline and the karyotypic heterogeneity of the cancer cell population (**B**) are immediately apparent when the karyotypes are displayed in a karyograph.

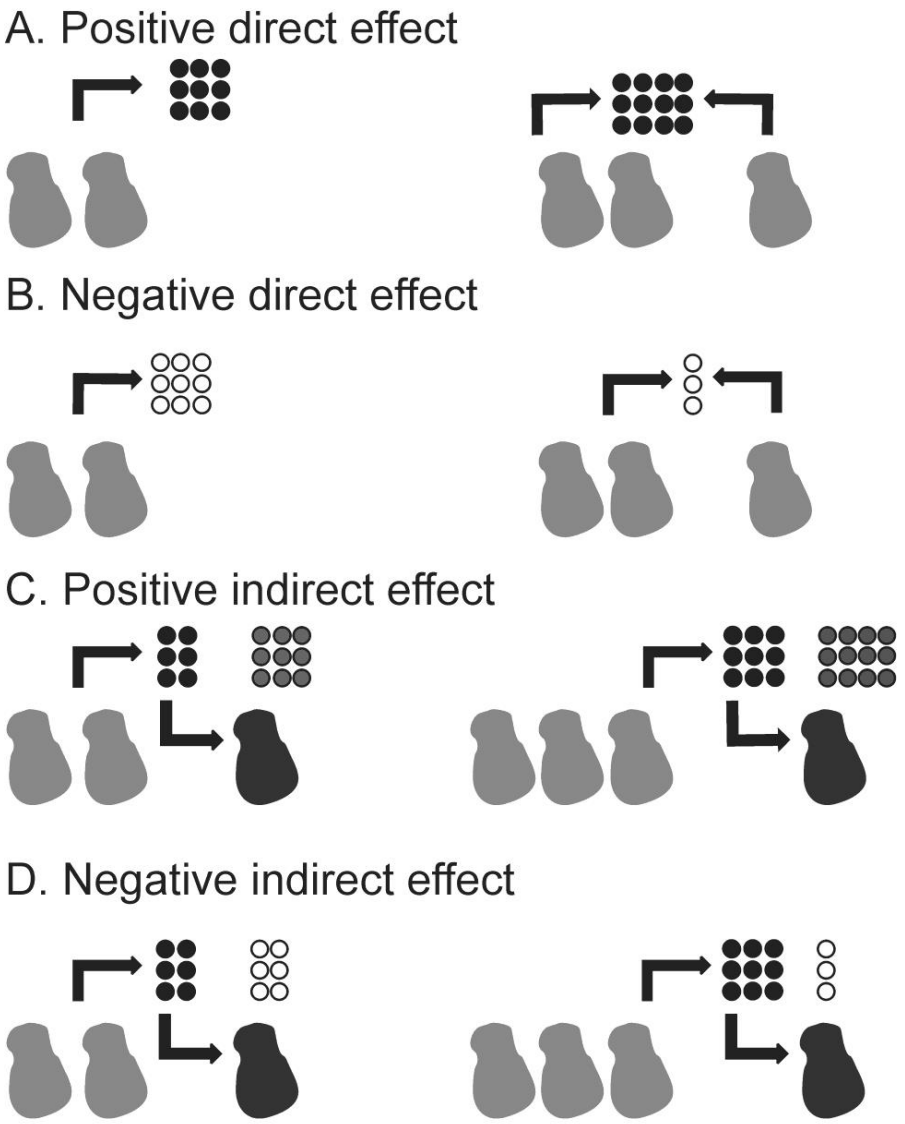


Figure 1.2 The pleiotropic effects of aneuploidy

Diagrammatic representation of the effects of trisomy on gene expression. The small circles in the figure represent gene products. The diagrams on the left show gene regulation under disomic conditions, whereas the diagrams on the right show possible effects of trisomy on gene regulation. Trisomy of an individual chromosome produces a 1.5-fold increase in gene copy number of genes on that chromosome. This would be expected to induce a 1.5-fold increase in the amount of gene products. However, the effect seen is often more complex and pleiotropic. For example, an individual trisomic chromosome may increase (**A**) or decrease (**B**) the expression of various genes on that chromosome. Additionally, a trisomic chromosome may also increase or decrease (**C** and **D**, respectively) the gene expression on one or more other chromosomes

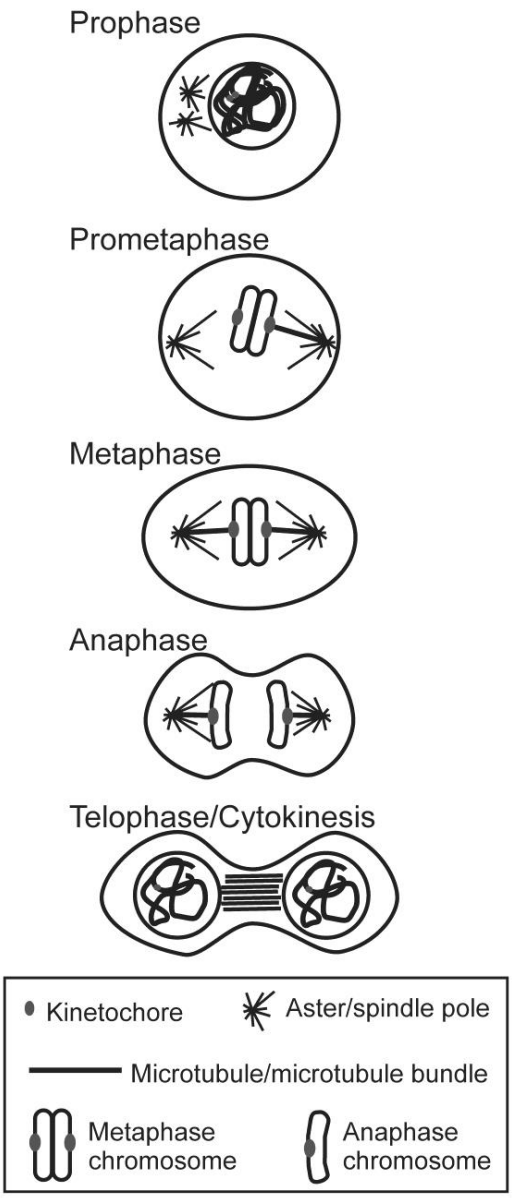


Figure 1.3 Diagrammatic representation of the stages of mitosis

A simplified view of the five stages of mitosis is illustrated in this cartoon. For simplicity, only one chromosome is diagrammed. Upon mitotic entry, each chromosome is constituted of two sister chromatids, which represent the products of DNA replication from the preceding S-phase. **Prophase** is characterized by chromosome condensation and separation of the duplicated centrosomes (spindle poles). Nuclear envelope breakdown marks the beginning of **prometaphase**. During prometaphase, microtubules emanating from the spindle poles establish attachment with the kinetochores. Typically, one sister kinetochore binds microtubules first (monotelic attachment). When the other sister kinetochore binds microtubules, the chromosome congresses to the spindle equator. When all the chromosomes are aligned at the spindle equator, the cell is said to be in **metaphase**. During metaphase, the aligned chromosomes, forming the so-called metaphase plate, undergo oscillatory movements about the spindle equator. Once all the kinetochores are bound to microtubules and all chromosomes are aligned at the metaphase plate, the sister chromatids separate, marking the metaphase-to-anaphase transition. During **anaphase**, chromosome segregation is ensured by two mechanisms; first, the sister chromatids are pulled apart toward opposite spindle poles (anaphase A). Second, the spindle poles move apart causing the spindle to elongate (anaphase B, not depicted here). When chromosome segregation is complete, the cell is said to be in **telophase**, at which time the nuclear envelope reforms around the two groups of chromosomes. During the latest stages of mitosis, an actin-myosin ring assembles at the cell equator and pinches the cell into two in a process termed cytokinesis, which ensures cytoplasmic division and formation of two individual daughter cells.

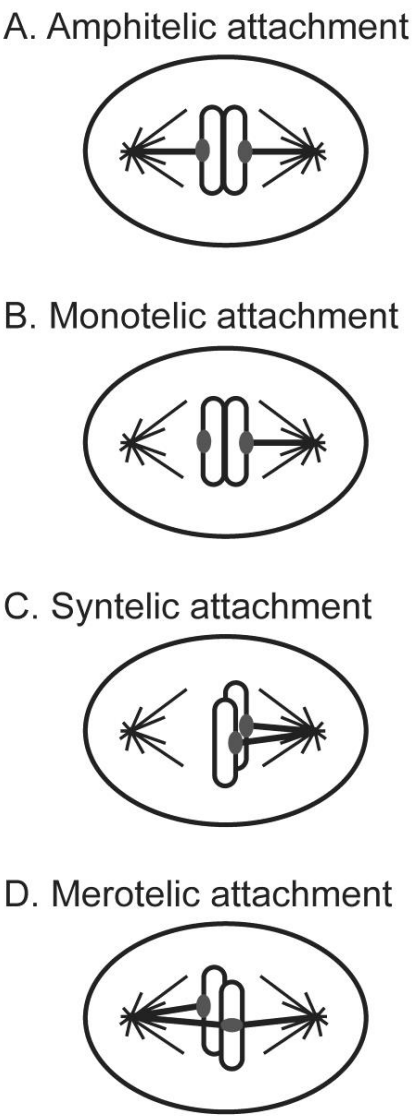


Figure 1.4 Types of kinetochore-microtubule attachments

Four different types of attachments can be formed during the early stages of mitosis. These include amphitelic, monotelic, syntelic, and merotelic attachments. **(A)** Amphitelic attachments are defined by the attachment of the two sister kinetochores to microtubules from opposite spindle poles. This type of attachment is necessary for accurate chromosome segregation. **(B)** Monotelic attachment occurs when one of the two sister kinetochores of an individual chromosome is attached to one spindle pole whereas the other sister is unattached. **(C)** Syntelic attachment occurs when the two sister kinetochores of an individual chromosome bind microtubules from the same spindle pole. **(D)** Merotelic attachment occurs when a single kinetochore binds microtubules from two poles rather than just one. See text for further details.

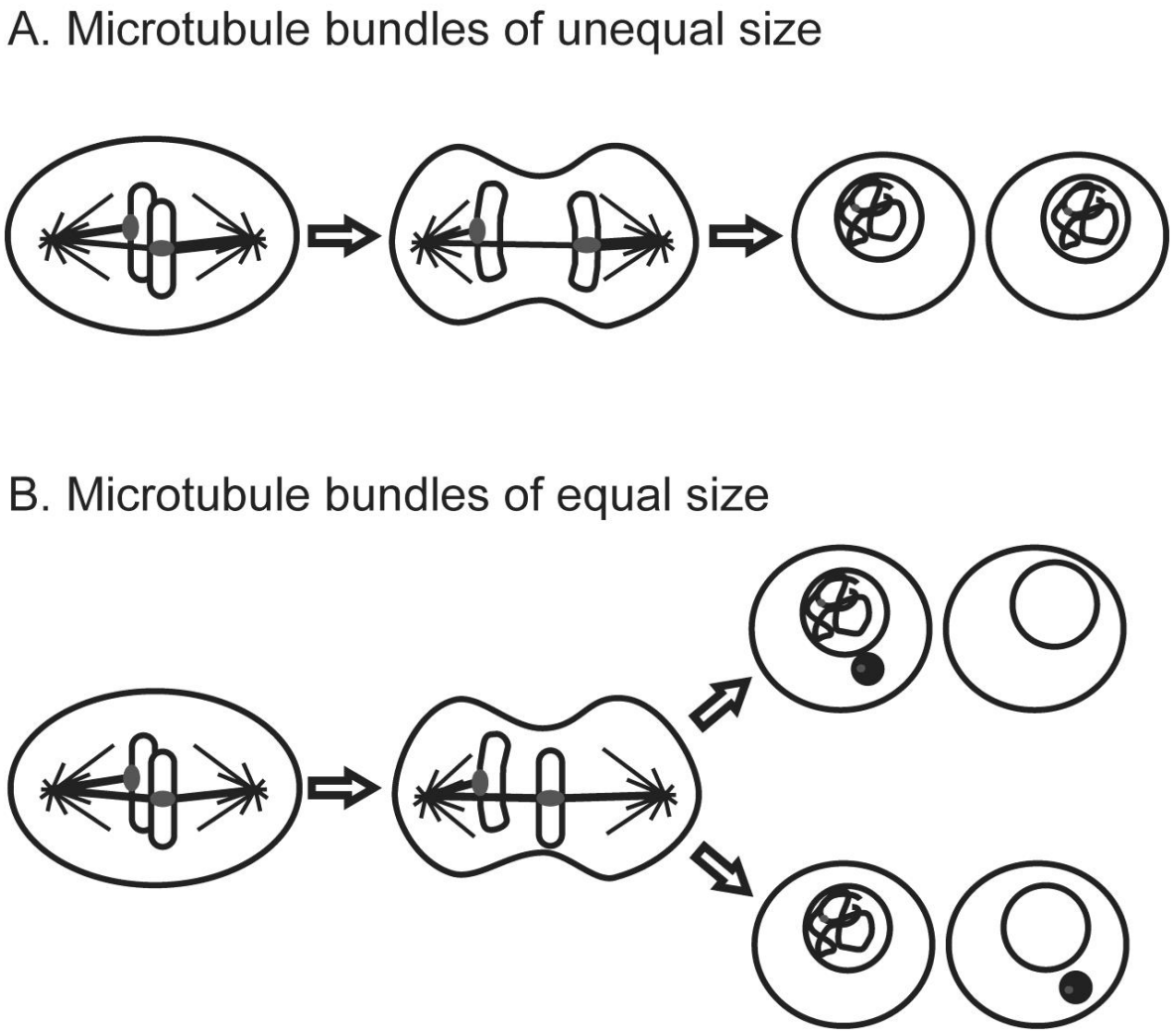


Figure 1.5 Possible fates of merotellically attached anaphase lagging chromosomes

Merotelic attachments do not induce a SAC-dependent mitotic delay, and thus they can persist into anaphase, at which time their behavior will depend on the relative size of the microtubule bundles bound to the individual kinetochore. If the two microtubule bundles attached to a merotelic kinetochore are different in size (**A**), during anaphase the chromosome will be shifted to the side attached to the thicker bundle. Conversely, if the two microtubule bundles are comparable in size (**B**), the merotelically attached chromosome will lag behind at the spindle equator while all the other chromosomes segregate to opposite spindle poles. Upon mitotic exit, the lagging chromosome forms a micronucleus. See text for further details.

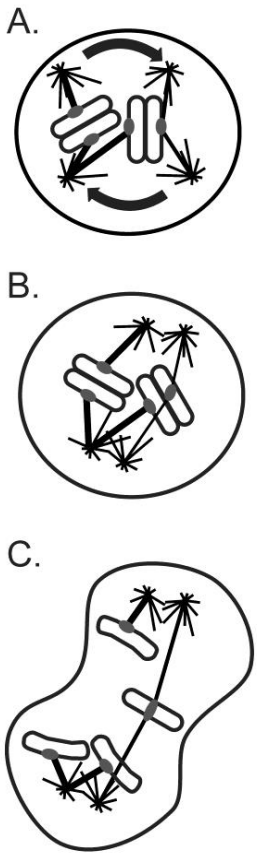


Figure 1.6 Multipolarity, centrosome clustering and chromosome mis-segregation

Within a multipolar spindle, a single kinetochore is more likely to face and establish attachment with two spindle poles than it would be in a bipolar spindle **(A)**. Most cancer cells possessing extra centrosomes undergo spindle bi-polarization by centrosome clustering before anaphase onset **(B)**, but not all merotelic attachments are corrected before anaphase onset. Those that persist into anaphase can give rise to a lagging chromosome **(C)**.

Chapter 2. Chromosome mis-segregation and cytokinesis failure in trisomic human cells.

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Abstract

Cancer cells display aneuploid karyotypes and typically mis-segregate chromosomes at high rates, a phenotype referred to as *c*hromosomal *in*stability (CIN). To test the effects of aneuploidy on chromosome segregation and other mitotic phenotypes we used the colorectal cancer cell line DLD1 (2n=46) and two variants with trisomy 7 or 13 (DLD1+7 and DLD1+13), as well as euploid and trisomy 13 amniocytes (AF and AF+13). We found that trisomic cells displayed higher rates of chromosome mis-segregation compared to their euploid counterparts. Furthermore, cells with trisomy 13 displayed a distinctive cytokinesis failure phenotype. We showed that up-regulation of SPG20 expression, brought about by trisomy 13 in DLD1+13 and AF+13 cells, is both required and sufficient for the cytokinesis failure phenotype. Overall, our study shows that aneuploidy can induce chromosome mis-segregation. Moreover, we identified a trisomy 13-specific mitotic phenotype that is driven by up-regulation of a gene encoded on the aneuploid chromosome.

Introduction

Aneuploidy, an abnormal number of chromosomes, is a leading cause of mis-carriage and birth defects in humans (Nagaoka et al., 2012). In the vast majority of cases this is due to errors occurring in the oocyte (Nagaoka et al., 2012). However, aneuploidy can also arise in somatic cells, and a number of studies have reported age-dependent increases in aneuploidy in human peripheral blood lymphocytes (Carere et al., 1999; Leopardi et al., 2002; Nowinski et al., 1990). Moreover, aneuploidy was recognized as a common feature of cancer cells already a century ago (Boveri, 1914; Boveri, 2008), and a causal role of aneuploidy in carcinogenesis is currently largely acknowledged [reviewed in (Nicholson and Cimini, 2011; Pavelka et al., 2010a)]. In addition to being aneuploid, cancer cells typically display high rates of chromosome mis-segregation, a phenomenon termed *c*hromosomal *in*stability (CIN) (Bakhoum et al., 2014; Lengauer et al., 1997a). The observation that even mosaic aneuploidy can cause severe physical and cognitive developmental defects (Biesecker and Spinner, 2013) indicates that aneuploidy has pleiotropic deleterious effects. This idea is further supported by a number of experimental observations: first, knocking down spindle assembly checkpoint genes, which results in high rates chromosome mis-segregation and high levels of aneuploidy, invariably causes embryonic lethality in mouse models (Foijer et al., 2008). Second, aneuploid yeast strains were shown to exhibit defects in cell cycle progression and metabolism (Torres et al., 2007). Third, MEFs derived from mice carrying specific trisomies were found to display cell proliferation defects and metabolic

alterations (Williams et al., 2008). Finally, genes involved in stress response were shown to be upregulated in aneuploid yeast and human cells (Sheltzer et al., 2012; Stingele et al., 2012). But in the context of cancer, aneuploidy and CIN strongly correlate with drug resistance (Lee et al., 2011) and poor patient prognosis (Bardi et al., 2004; Carter et al., 2006; Sheffer et al., 2009; Walther et al., 2008), indicating that aneuploidy and CIN may confer a proliferative advantage to cancer cells. In support of this idea, certain aneuploidies were found to confer drug resistance in aneuploid *Saccharomyces cerevisiae* (Pavelka et al., 2010c) and *Candida albicans* (Selmecki et al., 2006; Selmecki et al., 2009). These studies suggest that aneuploidy may confer adaptability by inducing chromosome-specific phenotypic changes, despite general negative effects on cell physiology. However, this problem remains to be investigated in human cells. Recent work in aneuploid budding yeast also showed that aneuploidy is sufficient to cause CIN (Sheltzer et al., 2011; Zhu et al., 2012), but whether this is true in human cells is still a matter of debate (Duesberg, 2014; Heng, 2014; Valind and Gisselsson, 2014a; Valind and Gisselsson, 2014b). In fact, this question has been difficult to address in cancer cells due to the complexity of cancer karyotypes (Gisselsson, 2011; Mitelman et al., 2014a), and previous studies in human cancer and non-cancer cells have reached discrepant conclusions (Duesberg et al., 1998a; Lengauer et al., 1997a; Miyazaki et al., 1999; Valind et al., 2013). To determine the effect of aneuploidy on chromosome segregation and cell division in human cells, we utilized a number of diploid human cell types and trisomic counterparts, including: colorectal cancer cell line

DLD1 (2n=46) and trisomic counterparts carrying extra copies of chromosomes 7 or 13 (DLD1+7 and DLD1+13, respectively); diploid amniotic fibroblasts (AF) and amniotic fibroblasts with trisomy 13 (AF+13). These different cell types constitute a good model for our study for two main reasons: first, their karyotypes are aneuploid, but not as complex as typically found in tumors and cancer cell lines; second, they represent different cellular models (transformed and untransformed) of aneuploidy.

Results

DLD1+7 and DLD1+13 cell lines were previously generated by micro-cell mediated chromosome transfer (Upender et al., 2004), whereas AF and AF+13 cells (three cases each; see Table 1) were collected upon amniocentesis. The presence of the additional chromosome was confirmed by fluorescence *in situ* hybridization (FISH) with locus-specific probes (Figure 2.1A-B). Analysis of DLD1+7 cells previously showed that a large fraction (87%) of the population was trisomic (Upender et al., 2004). However, the DLD1+13 cell population was shown to rapidly accumulate disomic (by loss of one copy of chromosome 13) and tetraploid cell populations (Upender et al., 2004). Thus, for this study we sub-cloned DLD1+13 cells in order to select a more homogenous cell population. When we analyzed the clone selected for this study at early passages (P. 3-4) by chromosome 13 painting, we found that 83.5% of the cells in the population carried the trisomy 13 (Figure 2.1C). Similarly, analysis of AF+13 interphase nuclei (passage 1-2) FISH-stained with probes specific for chromosomes 13 and 21 showed that the cell populations used in this study were highly homogenous ($88.1 \pm 6.5\%$) for the trisomic karyotype (Figure 2.1C). Furthermore, we performed array comparative genomic hybridization (aCGH) of all three DLD1 cell lines (Figure 2.1-figure supplement 1A, B, E). In all DLD1 cell lines, we found amplification of regions on the p arm of chromosomes 2 and 11 and a deletion of a region on the p arm of chromosome 6, which are known to be recurrently found in DLD1 cells. In addition to these common copy number variations (CNVs), the DLD1+7 cell line (analyzed at passage 4) carried a partial

trisomy 7 including most of the q arm (Figure 2.1-figure supplement 1B-C). FISH staining with a probe specific to the centromere of chromosome 7 confirmed that the extra chromosome included a centromere (Figure 2.1-figure supplement 1D). aCGH of DLD1+13 cells (at passage 11) showed that in addition to the CNVs identified in all three DLD1 cell lines, there was an extra copy of the entire chromosome 13 (Figure 2.1-figure supplement 1E-F). The experiments described hereafter were performed at passage number 7-25 for DLD1+7 cells and 13-25 for DLD1+13 cells to limit evolution of the karyotypes and passage number 1-3 for amniocytes, whose proliferation was limited to few passages.

Increased chromosome mis-segregation in cells with trisomy 7 or 13. To investigate the effect of aneuploidy on chromosome segregation, we analyzed anaphase lagging chromosomes, a common cause of aneuploidy in normal and cancer cells (Cimini et al., 2001b; Thompson and Compton, 2008a). By analyzing fixed cells with immunostained kinetochores and microtubules, we found that DLD1+7 and DLD1+13 cells displayed significantly higher frequencies of anaphase lagging chromosomes compared to the parental DLD1 cell line (Figure 2.2A-B). We found no evidence of aneuploidy-dependent increases in other mitotic defects, such as multipolar mitoses and anaphase chromosome bridges (Figure 2.2-figure supplement 1). Frequencies of anaphase lagging chromosomes in AF and AF+13 cells could not be analyzed in fixed samples due to low mitotic indices. However, we optimized live-cell imaging of AF and AF+13 cells expressing H2B-GFP/RFP-tubulin (Figure 2.2C) and found higher

frequencies of anaphase lagging chromosomes in AF+13 compared to AF cells (Figure 2.2D). Anaphase chromosome bridges or multipolarity were never observed in AF or AF+13 cells.

As an additional method to measure chromosome mis-segregation and to account for events in which two sister chromatids co-segregate to the same spindle pole/daughter cell, we combined the cytokinesis-block assay (Fenech, 1993b) with FISH staining using locus-specific probes for chromosomes 3, 7, 11, and 13 in DLD1 cell lines, and probes specific for chromosomes 7, 11, 12, 13, 18, and 19 in amniocytes. Using this approach, which allows analysis of the reciprocal distribution of chromosomes between the daughter nuclei of a single mitotic division (Figure 2.3A, Figure 2.3-figure supplement 1), we found a significant increase in chromosome mis-segregation in DLD1+7, DLD1+13, and AF+13 compared to the corresponding diploid cell cultures (Figure 2.3B-C). However, the higher mis-segregation rates were specific to certain chromosomes. Namely, mis-segregation appeared to be increased for chromosome 7 in the DLD1+7 cell line, chromosomes 7 and 13 in DLD1+13 cells, and chromosome 13 in AF+13 cells as compared to their diploid counterparts. We further investigated whether the observed increases in chromosome mis-segregation rates impacted chromosome number variability (or karyotypic heterogeneity) in the trisomic cells compared to the diploid counterparts. To this end, we performed chromosome counts in metaphase spreads and found increased karyotypic heterogeneity in both DLD1+7 and

DLD1+13 compared to DLD1 cells. We also found higher karyotypic heterogeneity in AF+13 vs. AF cells around the modal chromosome number of 47 (Figure 2.3D). Finally, our chromosome counts revealed the presence of a tetraploid/near-tetraploid sub-population in DLD1+13 cells (Figure 2.3D), confirming previous findings (Upender et al., 2004). Because chromosome counts did not reveal a significant difference in tetraploid cells between AF and AF+13 cell populations, we decided to further characterize chromosome number variability in these cells by performing FISH analysis with locus-specific probes for chromosomes 7, 12, and 18 on interphase nuclei (Figure 2.3E), which allowed for larger numbers of cells to be examined. This analysis confirmed higher degrees of aneuploidy in AF+13 compared to AF cells (Figure 2.3F). Furthermore, it revealed the presence of a tetraploid sub-population in AF+13 cells (Figure 2.3F). The difference between numbers of AF+13 tetraploid interphase cells and tetraploid chromosome spreads (compare Figure 2.3D and Figure 2.3F) may be due to the inability of tetraploid AF+13 cells to re-enter mitosis (see also Discussion section). Taken together, these experiments (Figures 2.2-2.3) show that trisomies 7 and 13 cause chromosome mis-segregation, that mis-segregation affects certain chromosomes more than others, and that such increases in mis-segregation rates are associated with karyotypic heterogeneity within the cell population. However, because only trisomies 7 and 13 were examined, and a limited number of chromosomes analyzed in our FISH experiments, it remains elusive whether chromosome mis-segregation is a karyotype-specific or a general effect of aneuploidy in human

cells.

Trisomy 13 promotes cytokinesis failure due to the overexpression of SPG20. Our chromosome counts (Figure 2.3D) showed that DLD1+13 cells displayed a near-tetraploid sub-population, which was also evident in our FISH experiments in which nuclei with four or more signals per chromosome were more frequent in DLD1+13 compared to DLD1 cells (24.5% vs. 16.6%, χ^2, $p<0.0001$). Similarly, a tetraploid sub-population was evident in AF+13 cells (4.5% vs. 0.3% in AF, χ^2, $p<0.0001$) analyzed by interphase FISH, which revealed the presence of nuclei with four signals per chromosome (Figure 2.3E-F). These observations suggested a possible causal link between trisomy 13 and tetraploidy. Acknowledged mechanisms of tetraploidy induction include mitotic slippage (Rieder and Maiato, 2004), cytokinesis failure (Normand and King, 2010), and cell fusion (Duelli and Lazebnik, 2003). To determine which of these mechanisms causes tetraploidy in DLD1+13 and AF+13 cells, we performed phase contrast time-lapse microscopy and found no evidence of mitotic slippage or cell fusion. Instead, we found that both DLD1+13 and AF+13 cells failed cytokinesis (Figure 2.4A-B) at significantly higher rates than their diploid counterparts (Figure 2.4C). To identify the molecular mechanism that causes cytokinesis failure in cells with trisomy 13, we referred to microarray data available for DLD1+13 cells (Upender et al., 2004). Interestingly, located on chromosome 13q13.3 is the gene *SPG20*, which encodes for the protein Spartin, previously suggested to act as a regulator of cytokinesis (Lind et al., 2011;

Renvoise et al., 2010), and shown to be overexpressed in DLD1+13 compared to DLD1 cells (Upender et al., 2004). None of the other mis-expressed genes (Upender et al., 2004) was found to have any link with cytokinesis based on published data. We confirmed SPG20 overexpression by western blot in both DLD1+13 and AF+13 cells (Figure 2.4D-F). Importantly, neither DLD1+7 (Figure 2.4E) nor other trisomic AF cells (Figure 2.4-figure supplement 1) overexpressed spartin, indicating that high levels of spartin are specifically associated with trisomy 13.

To test whether *SPG20* overexpression could explain the cytokinesis-failure phenotype, we transfected the parental cell line DLD1 with YFP-SPG20 (DLD1-YFP-SPG20; Figure 2.5A), and found that high levels of Spartin (Figure 2.5B) induced high rates of cytokinesis failure (Figure 2.5C). Moreover, we could rescue the cytokinesis failure phenotype in both DLD1+13 and AF+13 cells by siRNA-mediated Spartin knockdown (Figure 2.5D-G). Thus, we conclude that the aneuploidy-dependent overexpression of Spartin in DLD1+13 and AF+13 cells induces cytokinesis failure, a karyotype-dependent phenotype.

How does Spartin overexpression induce cytokinesis failure? To determine how Spartin overexpression may lead to cytokinesis failure, we analyzed the amount and localization of Spartin in fixed DLD1 cells (Figures 2.6A-B, Figure 2.6-supplement 1). Spartin localized to the centrosomes throughout mitosis and to some extent along the microtubules of the mitotic spindle (Figure 2.6-

supplement 1), and localized to the midbody during cytokinesis (Figure 2.6A), as previously described (Lind et al., 2011). We quantified the total intracellular amount of Spartin by measuring total Spartin fluorescence intensity in interphase cells, and found that it was significantly higher in DLD1+13 cells compared to DLD1 cells (Figure 2.6B; see also Figure 2.4D). However, we did not observe any difference in Spartin localization between the two cell lines during mitosis (Figures 2.6A, Figure 2.6-figure supplement 1), although there was clearly a large amount of Spartin in the cytoplasm, away from the mitotic spindle, in DLD1+13, but not in the other cell lines (Figure 2.6-figure supplement 1). Thus, although Spartin overexpression induces cytokinesis failure (Figure 2.5A-C), the mechanism by which this happens is not simply mis-localization of Spartin at the midbody (Figure 2.6A).

Spartin is recruited to the midbody by binding hIST1 (Renvoise et al., 2010), a component of the ESCRTIII complex, which binds various proteins involved in cytokinesis, including the microtubule severing protein Spastin (Renvoise et al., 2010), whose depletion was shown to cause cytokinesis failure (Bajorek et al., 2009). Both Spartin and Spastin bind hIST1 through their MIT (Microtubule Interacting and Trafficking) domains, which show considerable structural homology (Figure 2.6C) and comparable binding affinities [Spartin, K_d=10.4±0.3μM, Spastin, K_d=4.6±0.1μM, respectively (Renvoise et al., 2010)]. Therefore, we postulated that Spartin overexpression might act in a dominant negative manner by preventing Spastin from binding to hIST1. To test this, we

analyzed Spastin localization at the midbody. Consistent with our hypothesis, DLD1+13 and AF+13 cells frequently lacked Spastin at the midbody (Figure 2.6D-G). Moreover, by knocking down Spartin we could rescue Spastin mis-localization fully in DLD1+13 (Figure 2.6E) and partially in AF+13 cells (Figure 2.6G).

In summary, we showed that overexpression of SPG20, a gene on chromosome 13 encoding the protein spartin, can cause cytokinesis failure in cells with trisomy 13 (Figures 2.4-2.5). Although spartin overexpression may cause cytokinesis failure by interfering with multiple pathways, here we provide evidence of interference with a pathway responsible for spastin localization at the midbody (Figure 2.6D-G).

Discussion

Chromosomal instability in trisomic cells. We show here that cells with trisomy 7 or trisomy 13 display rates of anaphase lagging chromosomes that are significantly higher than the rates observed in euploid counterparts. Anaphase lagging chromosomes are a major source of aneuploidy in normal vertebrate cells (Cimini et al., 2001b) and the main type of chromosome segregation defect observed in CIN cancer cells (Bakhoum et al., 2014; Thompson and Compton, 2008a). Previous studies have shown that anaphase lagging chromosomes can be caused by transient spindle multipolarity in CIN cancer cells (Ganem et al., 2009; Silkworth and Cimini, 2012; Silkworth et al., 2009). However, we can exclude transient multipolarity as a cause of anaphase lagging chromosomes in our experimental systems, given that we did not find differences in the frequencies of multipolar mitoses in DLD1+7 and DLD1+13 compared to DLD1 cells and we did not observe transient spindle multipolarity in live AF+13. This finding may seem surprising, particularly considering that we found trisomy 13 to be associated with cytokinesis failure, which is believed to result in extra centrosomes and multipolar mitoses (Fujiwara et al., 2005a; Storchova and Pellman, 2004). However, this finding is in agreement with recent findings showing that experimental inhibition of cytokinesis can produce tetraploid cells with normal centrosome number (Godinho et al., 2014a). Our finding that aneuploidy is associated with increased rates of anaphase lagging chromosomes, known to arise from errors in mitosis (Bakhoum et al., 2014), but not with multipolarity or chromosome bridges, known to arise from errors in

centrosome duplication or DNA metabolism (occurring prior to mitosis), suggests that compared to events occurring during other cell cycle stages, mitotic events may be more sensitive to the gene imbalance brought about by the aneuploidy. Although abnormal chromosome number was reported by others as not being sufficient to cause CIN (Lengauer et al., 1997a; Valind et al., 2013), such difference may be due to the different methods used to evaluate CIN. For example, whereas we examined chromosome mis-segregation in mitosis (anaphase lagging chromosomes) or at a post-mitotic interphase (BN cell analysis), Lengauer and colleagues determined the degree of aneuploidy in the overall population after 25 serial passages (Lengauer et al., 1997a). This kind of analysis may produce a biased result because selective pressure against arising aneuploid cells may mask the ability of aneuploidy to induce chromosome mis-segregation at each cell cycle. Importantly, we were able to perform high-resolution live cell imaging of individual human amniocytes with constitutional trisomy 13 at passage number 2-3 and directly measure how frequently chromosome mis-segregation events occur. The frequency of lagging chromosomes found in mitotic cells was considerably higher compared to the frequency of chromosome mis-segregation measured by FISH analysis both in interphase and post-mitotic fixed cells. This shows how different results are generated from distinct methods and might also explain the divergence between our data and those previously reported by Valind et al. (2013) using interphase FISH analysis. It should also be noted that, although we do observe an increase in the rates of anaphase lagging chromosomes in trisomic cells, such rates are

lower than those reported for most CIN cancer cell lines (Bakhoum et al., 2014; Nicholson and Cimini, 2013; Thompson and Compton, 2008a), in agreement with findings by Valind et al. (2013). This suggests that, although single chromosome gains may result in only modest increases in chromosome mis-segregation rates, the high degrees of aneuploidy typical of most cancer cells may have a cumulative effect and could thus explain the high rates of CIN displayed by many cancer cells [e.g., 20-75% anaphase lagging chromosomes in cells with modal chromosome number 66-78; (Ganem et al., 2009; Lengauer et al., 1997a; Nicholson and Cimini, 2011; Nicholson and Cimini, 2013; Silkworth et al., 2009; Thompson and Compton, 2008a)]. Finally, and perhaps most importantly, the discrepancies in the conclusions reached in different studies may depend on the specific chromosomes analyzed. Indeed, we find that the degree and type of CIN elicited by aneuploidy depend on the aneuploid chromosome (see Figure 2.3D). This is an agreement with findings in aneuploid budding yeast strains, in which different aneuploidies were found to result in different rates of chromosome loss/CIN (Sheltzer et al., 2011; Zhu et al., 2012). Similarly, Valind and colleagues reported increased rates of CIN for certain chromosomes in specific aneuploid contexts (e.g., increased CIN for chromosome 17 in cells with trisomy 18), but not in others (Valind et al., 2013). In our study, we specifically found that high rates of mis-segregation for chromosome 7 were observed both in DLD1+7 and DLD1+13 cells, whereas high mis-segregation for chromosome 13 was observed in DLD1+13 and AF+13 cells. The observation that the trisomic chromosome displayed the highest rates of mis-segregation across the trisomies

studied (Figure 2.3B-C) suggests that the aneuploid chromosomes may undergo changes that affect their mitotic behavior and segregation, such as delayed replication and/or delayed condensation timing (DRT and DCT, respectively). Indeed, aneuploidy was shown to correlate with DRT and DCT (Grinberg-Rashi et al., 2010) and previous studies in trisomic cells showed that one of the chromosomes of the trisomic set displays DRT (Kost-Alimova et al., 2004). On the other hand, the finding that chromosome 7 mis-segregated in DLD1+13, but not in AF+13 cells raises the question as to whether types and rates of mis-segregation may vary in a cell type-dependent manner and whether copy number variations in the DLD1-derived cell lines may account for chromosome 7 mis-segregation. Given that gain of chromosome 7 and chromosome 13 are commonly found in colon cancer (Ried et al., 2012), a cell type-specific effect is plausible.

Although our cytokinesis-block assay data (Figure 2.3B-C) show the higher mis-segregation rates to be limited to certain chromosomes, the chromosome count data show extensive karyotypic heterogeneity in the trisomic cell populations (Figure 2.3D-F), thus suggesting that chromosome mis-segregation is more widespread than the cytokinesis-block assay reveals. One explanation is that the limited number of cells and chromosomes analyzed in the cytokinesis-block assay might only reveal differences in mis-segregation rates when such differences are large, but other methods, such as anaphase lagging chromosomes may be a better indicator of general mis-segregation rates.

Moreover, some mis-segregation events may result in cell cycle arrest or cell death. Cases of chromosome mis-segregation leading to cell death would only be accounted for when examining cells undergoing mitosis, but not interphase cells. On the other hand, chromosome mis-segregation events leading to cell cycle arrest may only be appreciated when examining interphase nuclei, but not BN or mitotic cells (Figure 2.3D-F). These considerations argue for the use of multiple assays in studies aimed at dissecting the link between aneuploidy and CIN (Nicholson and Cimini, 2015). We would also like to point out that chromosome mis-segregation events causing cell death or cell cycle arrest under tissue culture conditions, may not do so in the context of the tumor environment, suggesting that low level aneuploidy could be enough to drive CIN in cancer. Indeed, our data show that the complex aneuploidies observed in cancer cells are not a requirement for increased rates of lagging chromosomes and that at least some low grade and constitutional aneuploidies are sufficient to induce such an effect. This would be in agreement with previous observations showing that haploid budding yeast strains carrying disomies displayed increased genomic instability, and strains with different degrees of aneuploidy displayed variable degrees of chromosomal instability (Sheltzer et al., 2011; Zhu et al., 2012). Similarly, previous reports have shown that lymphocytes of congenitally trisomic individuals displayed aneuploidies for chromosomes other than the congenitally trisomic ones (Reish et al., 2006; Reish et al., 2011). Finally, random aneuploidy and senescent phenotypes were recently reported in aneuploid amniocytes (Biron-Shental et al., 2015b). Nonetheless, we do not

exclude the possibility that certain aneuploidies may not be sufficient to induce chromosome mis-segregation.

Aneuploidy confers karyotype-dependent phenotypes. Our finding that trisomy 13 caused a specific cytokinesis failure phenotype (Figures 2.4-5) clearly indicates that different karyotypes can be associated with distinct phenotypic changes. It is important to note that, although we observed cytokinesis failure in AF+13, the frequency of tetraploid metaphase spreads in these cells was very low, as opposed to the DLD1+13 cells (Figure 2.3D). This may be due to activation of a post-mitotic tetraploidy checkpoint in the AF+13 cells, but not in DLD1+13. Indeed, previous studies have shown a cell cycle arrest following cleavage failure in untransformed human cells (Andreassen et al., 2001b; Krzywicka-Racka and Sluder, 2011), whereas transformed cells continue cycling (Duelli et al., 2007; Panopoulos et al., 2014), but the triggering of a p53-dependent arrest in tetraploid cells still remains a matter of debate (Andreassen et al., 2001b; Fujiwara et al., 2005a; Stukenberg, 2004; Uetake and Sluder, 2004a; Wong and Stearns, 2005).

The observation that overexpression of a gene mapping on chromosome 13 is specifically linked to the cytokinesis failure phenotype in DLD1+13 and AF+13 cells demonstrates that there is a direct causal relationship between aneuploidy, overexpression of genes on the aneuploid chromosome, and phenotypic changes caused by the consequent proteomic imbalance. These findings

support previous studies showing that aneuploidy directly affects transcript and protein levels in various systems in a karyotype-dependent manner (Gao et al., 2007; Gemoll et al., 2013; Pavelka et al., 2010c; Pollack et al., 2002a; Ried et al., 2012; Stingele et al., 2012; Upender et al., 2004). Previous studies in budding yeast also showed that aneuploid strains displayed karyotype-specific phenotypic variations that conferred resistance to a variety of drugs (Pavelka et al., 2010c). However, such phenotypic variations in aneuploid yeast strains were only revealed when cells were grown under specific selective conditions (Pavelka et al., 2010c), whereas we show here that such karyotype-dependent phenotypic changes can be intrinsic to the aneuploid cells. Although some studies have shown that aneuploidy can have overall deleterious effects on cell fitness (Torres et al., 2007; Williams et al., 2008), we provide strong evidence that specific aneuploidies can also induce specific phenotypes, which could, under certain conditions, provide a selective advantage. This particular concept was recently exploited to demonstrate that in fungi, certain drug treatments can lead to the evolution of populations with defined aneuploid karyotypes (Chen et al., 2015). In the particular case observed in our study, one could also envision how the increase in tolerance to aneuploidy that tetraploidy was recently shown to confer (Dewhurst et al., 2014a) could enable the evolution of specific aneuploid karyotypes that may allow cells to overcome the detrimental impact of aneuploidy on cellular fitness (Gordon et al., 2012). Karyotype-specific phenotypic changes such as those observed in our study can also explain the recurrent aneuploidies that are found in tumors from certain anatomical sites [e.g., gain of chromosome

13 and loss of chromosome 18 in colon cancer (Nicholson and Cimini, 2013; Ried et al., 2012; Ried et al., 1996)]. Indeed, aneuploidies for certain chromosomes may result in phenotypes that confer a selective advantage at a certain site (e.g., breast), but not at a different one (e.g., colon). And this could explain why, despite the high degrees of aneuploidy and the extensive karyotypic heterogeneity, the distribution of aneuploidies in different cancers is not completely random (Nicholson and Cimini, 2011; Ried et al., 2012).

Materials and Methods

Cell lines and culture conditions. The DLD1 cell line was obtained from American Type Culture Collection (ATCC), DLD1+7 and DLD1+13 cell lines were created previously by microcell-mediated chromosome transfer as described in (Upender et al., 2004), and DLD1+13 cells were sub-cloned for this study as described in the results section. All cell lines were maintained in RPMI 1640 (ATCC) supplemented with 10% FBS (Gibco), penicillin, streptomycin, and amphotericin B (antimycotic). Passage 1-3 fibroblast cultures were established from surplus amniocentesis samples used in pre-natal diagnosis. Three cases of constitutional trisomy 13 and three diploid controls were used in our study (Table 1). The study acknowledged the ethics guidelines under national rules and according to the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of Hospital de S. João-Porto (dispatch 14 Nov 2012). Informed consent forms with detailed information were provided to all patients. The study did not imply collection of extra material from the healthy female donors (only surplus cells/tissues were used); the study did not bring any direct benefits to the volunteers; there were no risks or costs for the volunteers; there was no access to patient clinical data (samples were obtained in anonymous form from the Hospital Genetics Department); participation was volunteer and free to be interrupted at any moment; there are no ethical impacts predicted; there will be no commercial interests. Amniotic fibroblasts were grown in EMEM (Lonza) supplemented with 15% FBS, 2.5 mM glutamine and 1x antibiotic-antimycotic solution (all from Gibco). All cells were kept in a humidified incubator

at 37°C with 5% CO_2.

Immunostaining. For immunostaining, DLD1 cells were grown on sterilized glass coverslips inside 35 mm Petri dishes, whereas AF cells were grown on sterilized glass coverslips coated with fibronectin (Sigma Aldrich). For analysis of anaphase lagging chromosomes in the DLD1 lines, cells were fixed in freshly prepared 4% paraformaldehyde in PHEM (60mM Pipes, 25mM HEPES, 10mM EGTA, 2mM $MgSO_4$, pH 7.0) for 20 minutes at room temperature and then permeabilized for 10 minutes at room temperature in PHEM buffer containing 0.5% Triton-X 100. Following fixation and permeabilization, cells were washed with PBS three times and then blocked with 10% boiled goat serum (BGS) for 1 hour at room temperature. Cells were then incubated at 4°C overnight with primary antibodies diluted in 5% BGS. Cells were washed in PBS-T (PBS with 0.05% Tween 20) three times, and incubated at room temperature for 45 minutes with secondary antibodies diluted in 5% BGS. Cells were finally washed, stained with DAPI for 5 minutes, and coverslips were mounted on microscope slides in an antifade solution containing 90% glycerol and 0.5% N-propyl gallate. For analysis of proteins at the midbody, cells were fixed in ice cold methanol for 4 minutes, washed with PBS three times, and blocked in 10% BGS with 0.5% Triton-X for one hour. The rest of the procedure was the same as described above, except that primary antibodies were diluted in 1% BGS. Primary antibodies were diluted as follows: ACA (human anti-centromere protein, Antibodies Inc.), 1:100; mouse anti-□-tubulin (DM1A, Sigma Aldrich), 1:500;

rabbit anti-SPG20 (Protein Tech Group Inc.), 1:300; mouse anti-Spastin (SP 3G11/1, Abcam), 1:150. Secondary antibodies were diluted as follows: Rhodamine Red-X goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc.), 1:100; Rhodamine Red-X goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.), 1:100; Alexa 488 goat anti-human (Invitrogen), 1:200; Alexa 488 goat anti-mouse (Molecular Probes), 1:200.

Metaphase spreads. Cell cultures were incubated in 50ng/ml Colcemid (Karyomax, Invitrogen) at 37°C for 3-4 hours to enrich in mitotically arrested cells. The cells were then collected by trypsinization and centrifuged at 800 rpm for 8 minutes. Hypotonic solution (0.075M KCl) was added drop-wise to the cell pellet and incubated for 10 minutes at room temperature. Cells were fixed with an ice-cold 3:1 methanol:acetic acid solution for 5 minutes and then centrifuged at 800 rpm for 8 minutes. This last step was repeated two more times and fixed cells were finally dropped on microscope slides.

Cytokinesis-block assay and fluorescence *in situ* hybridization (FISH). For FISH on binucleate cells, amniocytes were grown in superfrost ultra plus slides (Thermo Scientific), whereas DLD1 cells were grown on coverslips. For the cytokinesis block assay, cells were treated with 3 □g/ml dihydrocytochalasin B (Sigma-Aldrich, MO, USA) for 24 hours before fixation, and the experiment was repeated at least three independent times. Prior to being processed for FISH staining, cytokinesis-blocked cells were fixed according to previously published

protocols: a standard FISH protocol (Nicholson and Duesberg, 2009) was used for all cells; an alternative (3-D FISH) protocol (Cremer et al., 2008) was used in some experiments with DLD1, DLD1+7, and DLD1+13 cells. Bacterial artificial chromosome (BAC) contigs using three to six BAC sequences specific to each region were made for the following four probes: *CDX2* on chromosome 13q12, *MET* on chromosome 7q31, *CHEK1* on chromosome 11q24, and *TERC* on chromosome 3q26. The BAC clone contigs were labeled by nick translation with Spectrum Orange-dUTP (Abbott Laboratories; IL, USA) for CDX2, Dy-505-dUTP (Dyomics; Jena, Germany) for MET, Spectrum Orange-dUTP (Abbott Laboratories; IL, USA) for *CHEK1*, and Dy-505-dUTP (Dyomics; Jena, Germany) for *TERC*. Dual color human whole chromosome paint probes were generated in-house using PCR labeling techniques. Chromosome 7 was labeled with Spectrum Orange (Abbott Laboratories; Chicago, IL) and chromosome 13 was labeled with Dy505 (Dyomics; Jena, Germany). Additionally, commercial locus-specific FISH probes for chromosomes 7, 11, 19 (red 5-ROX dUTP) and 12, 13, 18 (green 5-fluorescein dUTP) were also used (Empire Genomics; Buffalo, New York). The probe mixtures were co-denatured with the coverslips at 72°C for five minutes before being placed in a moist chamber at 37°C for two nights. After two nights, the coverslips were washed in 2XSSC for five minutes and then mounted on microscope slides with mounting media (Vectashield; CA, USA) and DAPI. FISH on DLD1 metaphase spreads was performed with the commercial FISH probes listed above. Additionally, centromeric FISH probe against chromosome 7 (FITC) (Cytocell) was used in DLD1+7 cells to confirm the presence of

centromeric DNA. FISH on metaphase spreads of AF and AF+13 was performed with the XA 13/21 probe (MetaSystems, Germany) according to the manufacturer's instructions. Interphase FISH was performed with the Vysis centromeric probes CEP7 Spectrum Aqua, CEP12 Spectrum Green, and CEP18 Spectrum Orange (Abbott Molecular, USA) according to the manufacturer's instructions. All the FISH-stained samples were analyzed blindly.

SPG20 transfection and SPG20 siRNA. DLD1 cells, grown on sterilized coversips inside 35 mm Petri dishes, were transiently transfected with either P-EYFP-N1 or P-EYFP-N1-SPG20 (a kind gift from J. Bakowska) using Fugene HD (Roche) according to the manufacturer's protocol. For SPG20 knockdown, cells grown in glass-bottom 35 mm u-dishes (Ibidi) or on sterilized coverlips inside 35 mm Petri dishes, were transfected with Silencer Select siRNA specific to SPG20 (S23057, Ambion/Invitrogen) using Oligofectamine according to the manufacturer's instructions (Invitrogen). Silencer Select Negative control siRNA (Invitrogen) was used as a negative control and was also transfected into cells with Oligofectamine (Invitrogen). Cells were observed 48-72 hours after transfection.

Western blot analysis. Whole-cell extracts were separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked 1hr at room temperature with 5% milk in tris-buffered saline and then incubated over night with primary antibodies at 4°C. Antibodies were diluted as follows: rabbit anti-

Spartin (Protein Tech Group Inc.), 1:1000; rabbit anti-□-actin (Abcam), 1:500. Blots were detected using goat anti-rabbit secondary antibodies conjugated to horse radish peroxidase and visualized with SuperSignal West Femto (Thermo Scientific). A GS-800 calibrated densitometer, or alternatively a ChemiDoc XRS system, was used for quantitative analysis of protein levels with the help of ImageLab 4.1 software (BioRad).

Confocal microscopy of immunostained cells and image analysis.

Immunofluorescently labeled DLD1 cells were imaged with a swept field confocal system (Prairie Technologies) on a Nikon Eclipse TE2000-U inverted microscope (Nikon Instruments Inc.) equipped with a 100x/1.4 NA Plan-Apochromatic objective and an automated ProScan stage (Prior Scientific). The confocal head was accessorized with multiband pass filter set for illumination at 405, 488, 561, and 640 nm, and illumination was obtained through an Agilent monolithic laser combiner (MLC400) controlled by a four channel acousto-optic tunable filter. Digital images were acquired with a HQ2 CCD camera (Photometrics). Acquisition time, Z-axis position, laser line power, and confocal system were all controlled by NIS Elements AR software (Nikon Instruments Inc.) on a PC computer (Dell). Both anaphase lagging chromosomes and Spartin localization were analyzed by acquiring Z-sections of cells at 0.6 □m steps. The frequencies of anaphase lagging chromosomes were determined from 3 independent experiments performed in duplicate. Spartin localization at the midbody was determined from three independent experiments. Image acquisition of Spastin

immunostaining in AFs was carried out on a Zeiss AxioImager Z1 equipped with an Axiocam MR and using a Plan-Apochromat 63x/1.4 NA objective. Images of telophase cells from three independent experiments and/or samples were acquired as Z-stacks with 0.3 □M steps and scored for the presence or absence of Spastin staining at the midbody. All data were analyzed blindly.

Microscopy and image analysis of FISH-stained binucleate cells. FISH samples were viewed and imaged either with a Leica DM-RXA fluorescence microscope (Leica; Wetzlar, Germany) or with a swept field confocal system (Prairie Technologies) on a Nikon Eclipse TE2000-U inverted microscope (Nikon Instruments Inc.). The Leica DM-RXA fluorescence microscope was equipped with custom optical filters and a 63x/1.3 NA objective. The Leica CW 4000 FISH software was used to acquire multifocal images for each fluorescence channel. Fifteen to 25 images were taken in areas of optimal cell density with minimal cellular clumps and overlapping cells. The Nikon Eclipse TE2000-U inverted microscope was equipped with a 100x/1.4 NA Plan-Apochromatic objective and an automated ProScan stage (Prior Scientific). The confocal head was accessorized with a multiband pass filter set for illumination at 405, 488, 561, and 640 nm and illumination was obtained through an Agilent monolithic laser combiner (MLC400) controlled by a four channel acousto-optic tunable filter. Digital images were acquired with a HQ2 CCD camera (Photometrics). Exposure time, Z-axis position, laser line power, and confocal system were all controlled by NIS Elements AR software (Nikon Instruments Inc.) on a PC computer (Dell).

FISH-stained samples were analyzed blindly and only cases with a total even number of signals were included in the analysis.

Phase contrast live cell imaging. DLD1, DLD1+7, and DLD1+13 cells were grown on sterilized coverslips inside 35 mm Petri dishes. Coverslips at 60-70% confluency were mounted in Rose chambers filled with L-15 medium supplemented with 4.5 g/l glucose. Images were acquired on a Nikon Eclipse Ti inverted microscope (Nikon Instruments Inc.) equipped with phase-contrast transillumination, transmitted light shutter, ProScan automated stage (Prior Scientific), and a HQ2 CCD camera (Photometrics). Cells were maintained at ~36 °C using an air stream stage incubator (Nevtek). For analysis of cytokinesis in untransfected or siRNA-transfected cells, 10-15 different fields of cells were imaged every 2 minutes for 6-8 hours using a 60x/1.4 NA Plan-Apochromatic phase contrast objective controlled by Nikon Perfect Focus (Nikon Instruments Inc.). For P-EYFP-N1 and P-EYFP-N1-SPG20 transfection experiments, 10-15 different fields of cells were imaged by fluorescence and phase contrast using a 20x or 60x objective every 4 minutes for 8 hours. The time-lapse movies were analyzed using NIS Elements AR (Nikon Instruments Inc.) software on a PC computer (Dell).

Amniocytes were grown in glass-bottom 35 mm u-dishes (Ibidi) coated with fibronectin and filled with phenol red-free EMEM complete medium. Images were acquired on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Germany) equipped with a CoolSnap camera (Roper), XY motorized stage and

NanoPiezo Z stage, under controlled temperature, atmosphere and humidity. 20-25 neighbor fields were imaged every 2.5 min for 1-2 days using a 20x/0.3 NA A-Plan objective. Grids of neighboring fields were generated using the plugin Stitch Grid (Stephan Preibisch) from open source Fiji/Image J (http://rsb.info.nih.gov/ij/).

Spinning-disk confocal live cell imaging. Amniotic cells, glass-bottom 35 mm u-dishes (Ibidi) coated with fibronectin, were co-transfected with H2B-GFP and pmRFP-tubulin expression plasmids (Addgene) using Lipofectamine 3000 transfection reagent and according to the manufacturer's instructions. Live cell imaging was performed 48h following transfection under a spinning-disk confocal system Andor Revolution XD (Andor Technology, UK) coupled to an Olympus IX81 inverted microscope (Olympus, UK) equipped with an electron-multiplying CCD iXonEM Camera and a Yokogawa CSU-22 unit based on an Olympus IX81 inverted microscope. Two laser lines at 488 and 561 nm were used for the excitation of GFP and pmRFP and the system was driven by IQ software (Andor). Z-stacks (0.8-1.0 □m) covering the entire volume of the mitotic cells were collected every 1.5 min with a PLANAPO 60x/1.4 NA objective. ImageJ was used to process all the movies.

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Table 2.1. Euploid and trisomic amniocytes used in this study.

Name	Karyotype	Gestational age (week)
AF 1	46,XX	17
AF 2	46,XY	20
AF 3	46,XX	21
AF+13 1	47,XX,+13	16
AF+13 2	47,XX,+13	21
AF+13 3	47,XX,+13	21
AF+18	47,XX,+18	17
AF+21	47,XX,+21	17

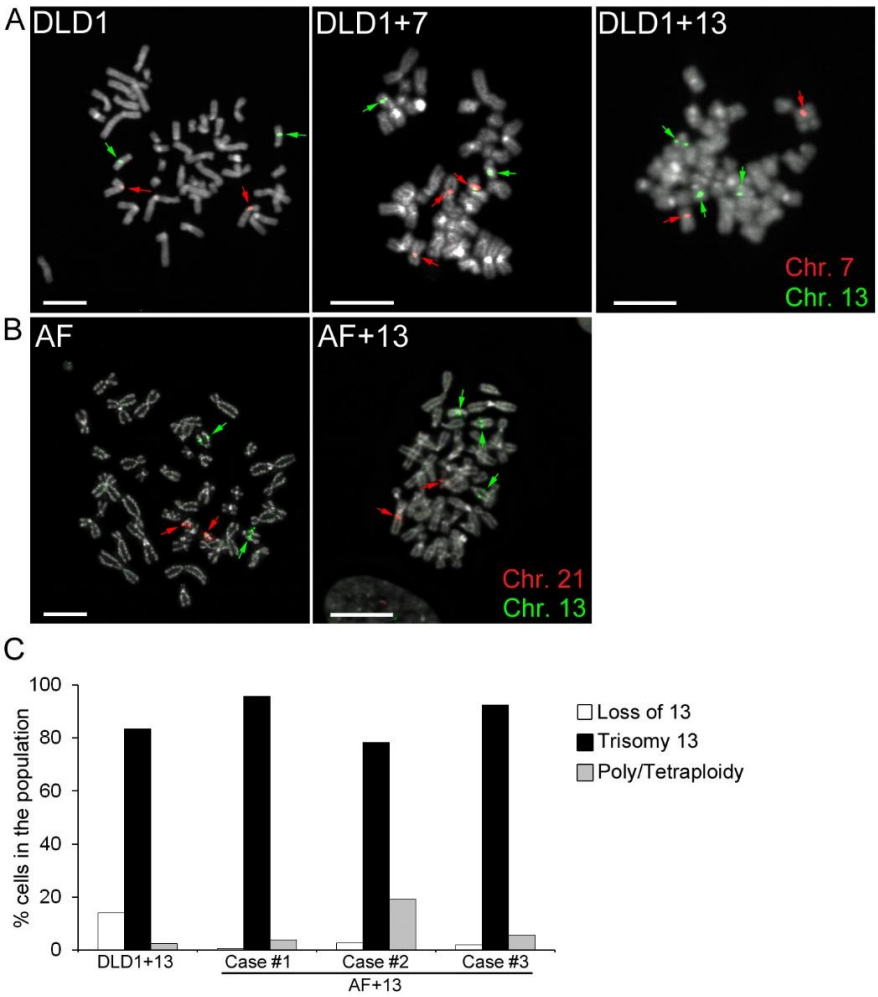


Figure 2.1. Trisomy 7 and 13 in DLD1 and AF cells.

(A-B) Chromosome-specific FISH staining for chromosomes 7 (red),13 (green), and 21 (red) in metaphase spreads confirms trisomy 7 in DLD1+7 and trisomy 13 in DLD1+13 and AF+13 cells. DNA is shown in grey. Arrows point to FISH signals. Scale bars, 5 □m. (C) Characterization of cells with trisomy 13 used in this study. The DLD1+13 cells were subcloned and the clone used for this study was analyzed by chromosome painting of metaphase spreads with chromosome 13-specific probes. Cells were classified as having lost chromosome 13, trisomic for chromosome 13, or poly/tetraploid when they carried six copies of chromosome 13 and ~88 other chromosomes. The AF+13 cells were analyzed by interphase FISH at passage 1-2 with probes specific to chromosomes 13 and 21. Cells were classified has having lost/gained a copy of chromosome 13 (two or four nuclear signals for chr. 13, two signals for chr. 21), displaying trisomy 13 (three signals for chr. 13 and 2 signals for chr. 21), or displaying poly/tetraploidy (six signals for chr. 13 and four signals for chr. 21). A small fraction (1.9%) of the AF+13 Case #3 displayed loss/gain of chr. 21.

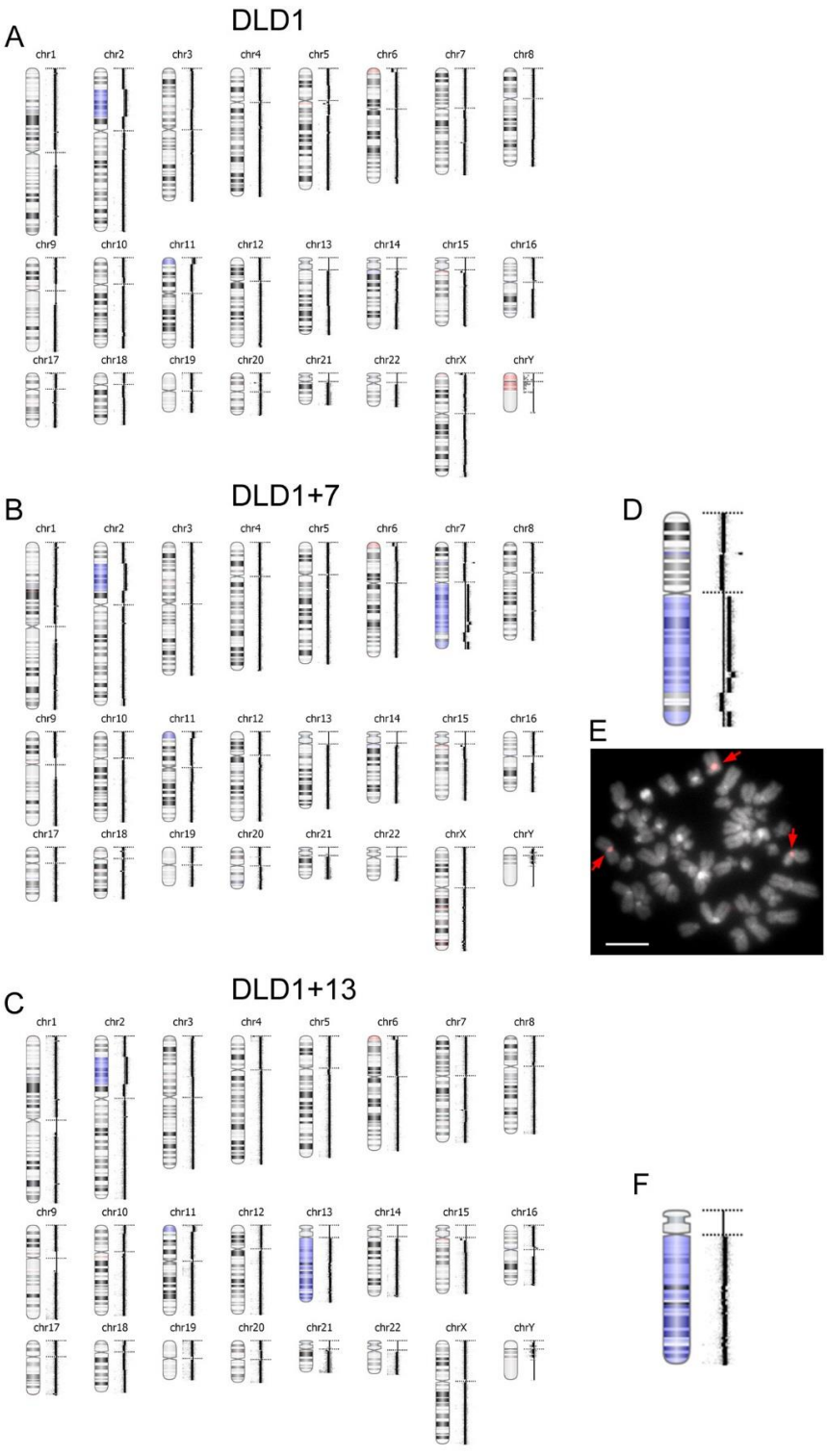

A
DLD1
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chr20
chr21
chr22
chrX
chrY
B
DLD1+7
C
DLD1+13
D
E
F

Figure 2.1-figure supplement 1. aCGH in DLD1, DLD1+7, and DLD1+13 cells.

(A-C) The images show the results of aCGH in the three cell lines, with regions shaded in blue corresponding to gains and regions shaded in red corresponding to losses. The data show that the DLD1+7 and DLD1+13 cell lines share CNVs, such as duplication of a portion of the short arm of chromosome 2, gain of a short portion of the p arm of chromosome 11, and loss of a short region of the p arm of chromosome 6, with the parental cell line. Moreover, the data highlight the gain of most of the q arm of chromosome 7 in the DLD1+7 cell line and gain of chromosome 13 in the DLD1+13 cell line. (D) 2X enlargement of the aCGH image of chr. 7 from the DLD1+7 cell line. (E) FISH staining of DLD1+7 metaphase spread using a probe (red) specific to the centromeric DNA of chromosome 7. The image shows that the trisomic chromosome 7q maintains a centromere. DNA is shown in grey. Arrows point to FISH signals. (F) 2X enlargement of the aCGH image of chr. 13 from the DLD1+13 cell line.

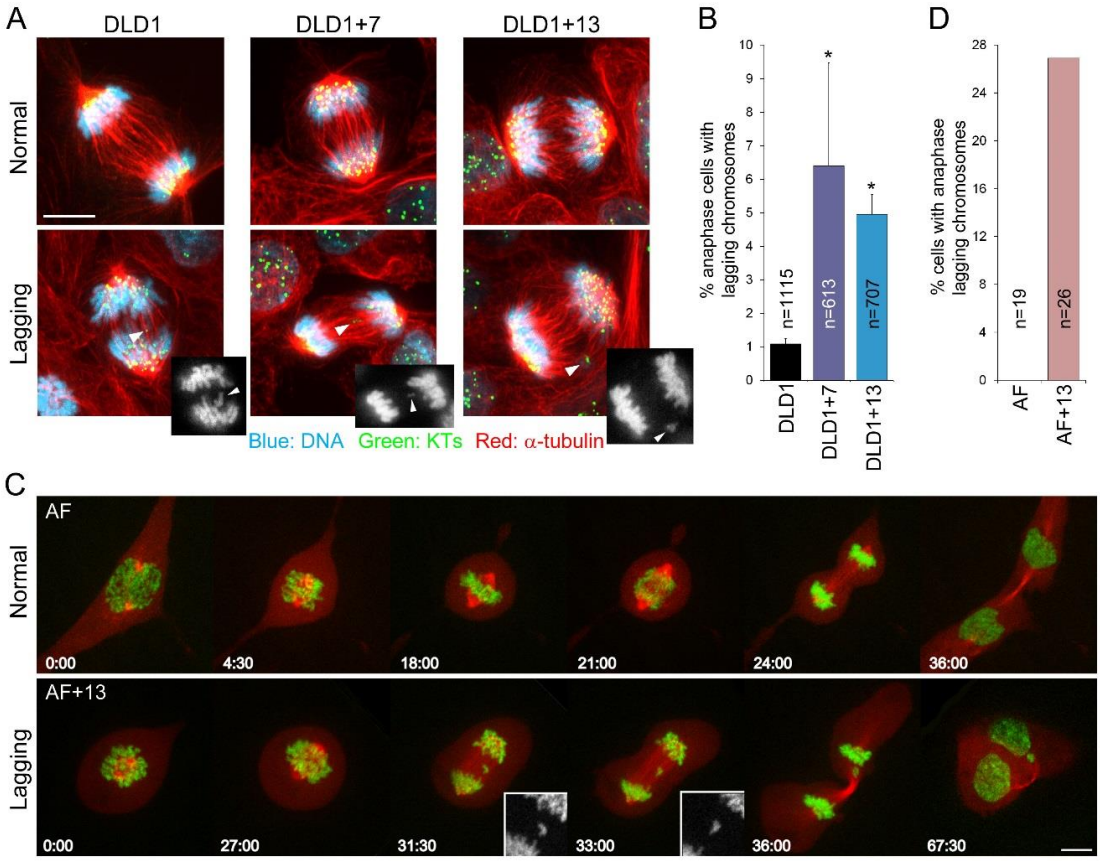


Figure 2.2 Increased rates of anaphase lagging chromosomes in cells with trisomy 7 or 13.

(A) Examples of normal anaphases (top row) and anaphase cells with lagging chromosomes (bottom row). Cells were immunostained for microtubules (red) and kinetochores (green). DNA is shown in blue. Images represent maximum intensity projections of Z-stacks. Arrowheads point at anaphase lagging chromosomes. Grey scale images at the bottom right corners of the images in the bottom row are single focal planes of DAPI-stained chromosomes shown for easier visualization of the lagging chromosomes. (B) Frequencies of anaphase lagging chromosomes were significantly higher (*χ^2 test, P<0.0001) in both DLD1+7 and DLD1+13 compared to DLD1 cells. Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 613-1115 anaphases were analyzed. (C) Time-lapse microscopy of AF and AF+13 cells undergoing mitosis. An example of AF undergoing normal mitosis is shown in the top row and an example of AF+13 displaying an anaphase lagging chromosome is shown in the bottom row. DNA is shown in green (H2B-GFP) and microtubules in red (RFP-tubulin). Images are maximum intensity projections of Z-stacks. Insets in the bottom row display enlarged views of the DNA alone (in grey scale) in the region around the lagging chromosome.

(D) None of the 19 AF cells (from cases #1 and #2) imaged displayed anaphase lagging chromosomes, whereas 7 out of 26 AF+13 cells (5 out of 18 in case #1 and 3 out of 8 in case #2) displayed anaphase lagging chromosomes. Time stamps indicate elapsed time in min:sec. Scale bars, 5 μm.

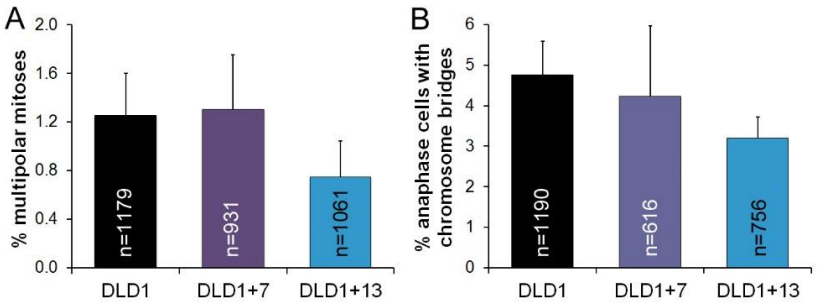


Figure 2.2-figure supplement 1. Similar frequencies of multipolar mitoses (A) and anaphase chromosome bridges (B) in diploid vs. aneuploid DLD1 cells.

The data are reported as mean ± S.E.M. and represent the average of three experiments in which a total of 931-1179 mitotic cells (A) or 616-1190 anaphase cells (B) were analyzed.

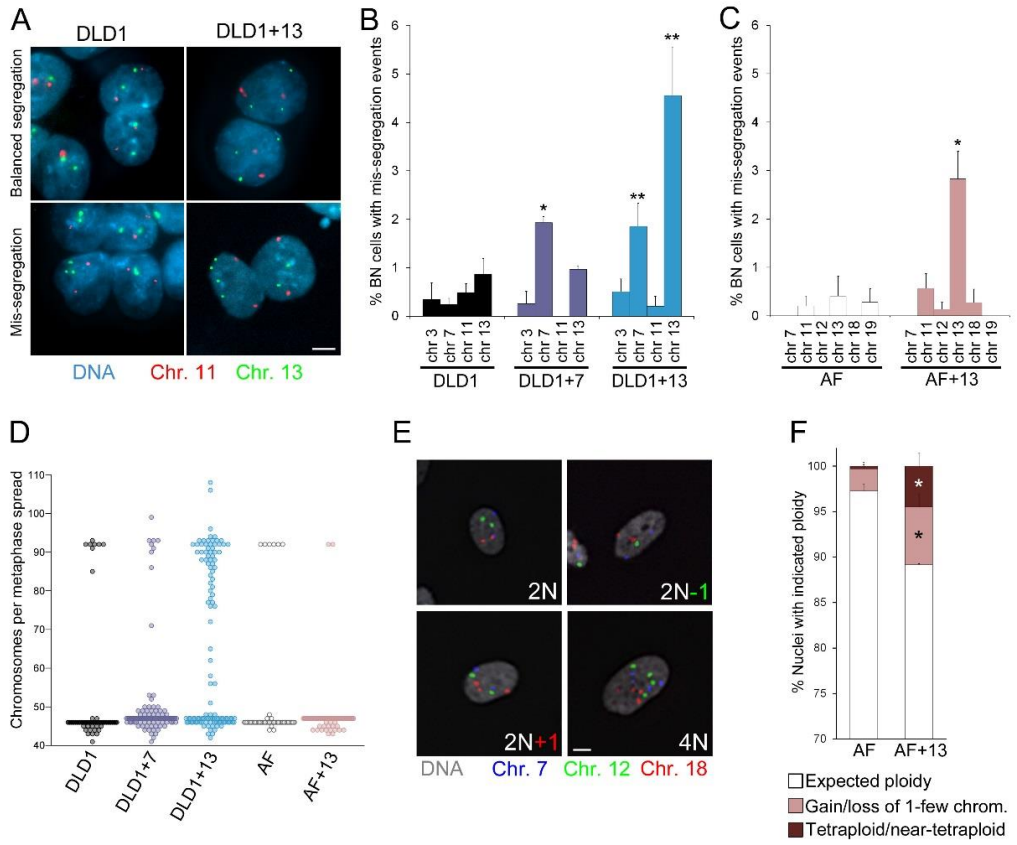


Figure 2.3. Increased chromosome mis-segregation rates and karyotypic heterogeneity in cells with trisomy 7 or 13.

(A-C) Combination of cytokinesis-block assay and FISH staining with chromosome-specific probes shows higher chromosome mis-segregation rates in DLD1+7 and DLD1+13 compared to DLD1 cells and AF+13 compared to AF cells. (A) Examples of FISH-stained binucleate (BN) DLD1 and DLD1+13 cells. (B-C) Frequencies of BN cells displaying mis-segregation events. *two-tailed χ^2 test, P<0.005; **two-tailed χ^2 test, P<0.0001, when compared to mis-segregation of the same chromosome in the euploid cell line. Data are presented as mean ± S.E.M. and represent the average of at least three independent experiments/samples in which a total of 460-1229 BN cells were analyzed. Scale bar, 5μm. (D) Beeswarm plot displaying data from chromosome counts in metaphase spreads from the five cell lines. DLD1+7, DLD1+13, and AF+13 (modal chromosome number 47, shown by the high concentration of sampled points) displayed increased karyotypic heterogeneity compared to DLD1 and AF cells (modal chromosome number 46, shown by the high concentration of sampled points), respectively. In addition, DLD1+13 cells displayed a large sub-population of near-tetraploid cells (modal chromosome number 92). Chromosome counts were performed on 89-303 metaphase spreads. (E-F)

FISH staining with chromosome-specific probes in interphase nuclei reveals higher rates of aneuploidy and tetraploidy in AF+13 vs. AF cells. (E) FISH staining in interphase AF and AF+13 cells with probes specific for chromosomes 7 (blue), 12 (green), and 18 (red). Scale bar, 5μm. (F) Quantification of interphase FISH data shown in (E). Cells were classified as having gained or lost 1-few chromosomes or as tetraploid (4 signals for each of the three chromosomes analyzed). The data show a larger fraction of cells with gain/loss of 1-few chromosomes in the AF+13 population compared to AF cells (*χ^2 test, $p<0.0001$). Additionally, AF+13 cells displayed a larger sub-population of tetraploid cells (*χ^2 test, $p<0.0001$). Data are presented as mean + S.E.M. and represent the average of three different samples in which a total of 2,117-2,410 cells were analyzed.

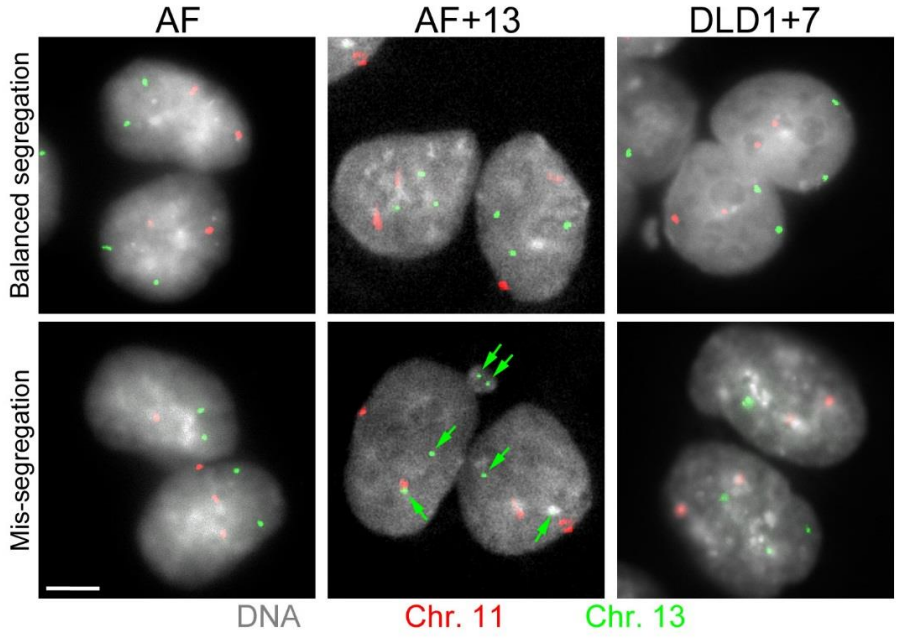


Figure 2.3-figure supplement 1. Combined cytokinesis-block assay and FISH staining with chromosome-specific probes.

The images show examples of binucleate AF, AF+13, and DLD1+7 cells hybridized with FISH probes specific to chromosomes 11 and 13. Green arrows in image of mis-segregation in AF+13 cell point at signals for chromosome 13. Scale bar, 5 μm.

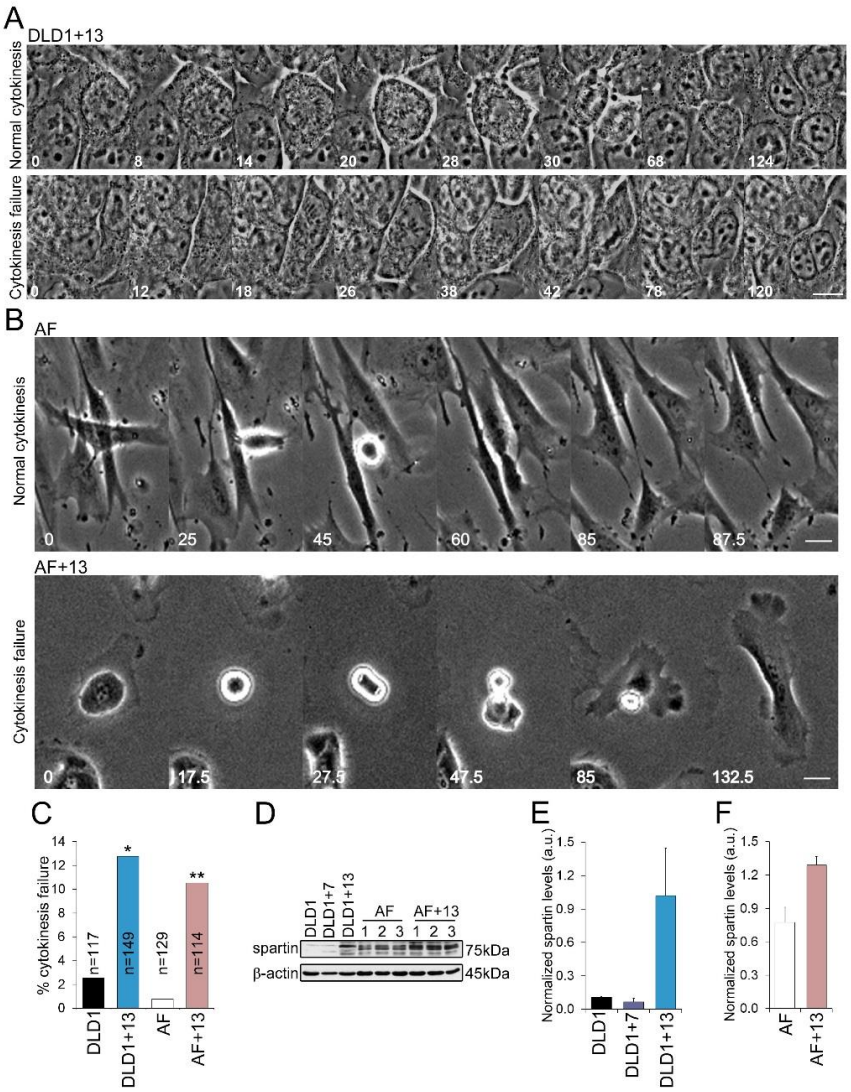


Figure 2.4 DLD1+13 and AF+13 cells overexpress SPG20 and fail cytokinesis at high rates.

(A-B) Time-lapse microscopy indicates that cells with trisomy 13 frequently fail cytokinesis. Time stamps indicate elapsed time in minutes. Scale bars, 10 μm. (A) Still images from time-lapse phase contrast movies of DLD1+13 cells undergoing mitosis and completing (top row) or failing (bottom row) cytokinesis. (B) Still images from time-lapse phase contrast movies of AF (top row) and AF+13 (bottom row) cells undergoing mitosis and completing (AF, top row) or failing (AF+13, bottom row) cytokinesis. (C) Quantification of cytokinesis failure from phase-contrast time lapse movies showing that the rates of cytokinesis failure in DLD1+13 and AF+13 cells are significantly higher than those observed in their diploid counterparts, DLD1 and AF cells (*χ^2 test, $p<0.01$; ** χ^2 test, $p<0.001$). (D) Western blot analysis of Spartin across DLD1 cell lines, three

samples of AF and three samples of AF+13 cells. □-actin was used as a loading control. (E) Quantification of spartin levels (normalized to □-actin) in DLD1, DLD1+7, and DLD1+13 cells. The data reported are the average of three independent experiments and are displayed as mean + S.E.M. (F) Quantification of spartin levels (normalized to □-actin) in AF and AF+13 cells. The data reported are the average of the three AF and AF+13 samples shown in (E) and are displayed as mean + S.E.M.

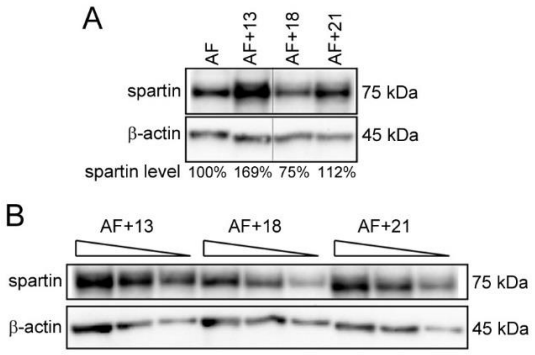


Figure 2.4-figure supplement 1. Trisomies other than 13 are not associated with spartin overexpression.

(A) Western blot analysis of Spartin levels in AF control and AF+13, AF+18 and AF+21 trisomic cells. Spartin levels were normalized to □-actin and are shown as percentage of the amount in the euploid control. (B) Western blot with decreasing amounts (30, 20 and 10 μg) of total extracts from the trisomies used in (A) performed for titration of the western blot conditions.

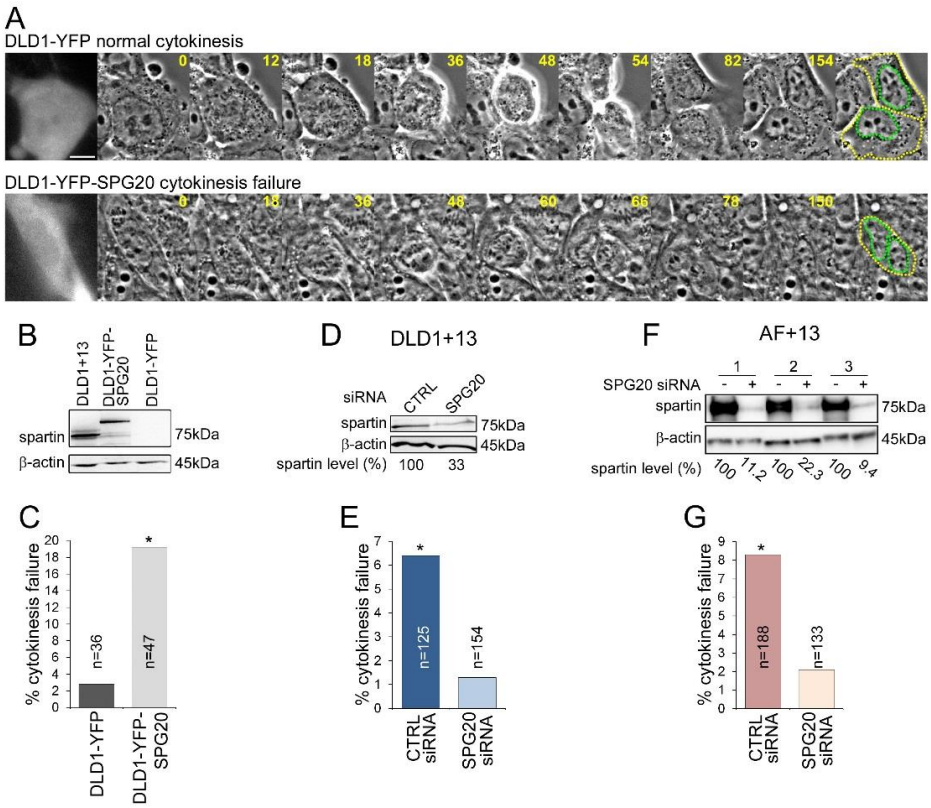


Figure 2.5. Spartin overexpression induces cytokinesis failure.

(A) Time-lapse microscopy of DLD1 cells transiently transfected with a YFP-N1 vector (DLD1-YFP, top row) or a YFP-SPG20-N1 vector (DLD1-YFP-SPG20, bottom row). Representative still images of time-lapse movies show a DLD1-YFP cell undergoing mitosis and completing cytokinesis (top row) and a DLD1-YFP-SPG20 cell undergoing mitosis and failing cytokinesis (bottom row). YFP expression was verified by fluorescence imaging and it is shown in the first panel for each time-lapse series. A copy of the last frame was added at the end of the sequence to highlight cell (yellow) and nuclear (green) outlines. Scale bar, 10 □m. (B) Western blot analysis of Spartin levels in DLD1+13, DLD1-YFP, and DLD1-YFP-SPG20 cells shows that the levels of YFP-Spartin in DLD1-YFP-SPG20 cells (center lane) are much higher than the levels of Spartin in untransfected DLD1 cells. WB of Spartin in DLD1+13 cells is shown for comparison. (C) Quantification of cytokinesis failure rates in DLD1-YFP and DLD1-YFP-SPG20 showing that overexpression of SPG20 leads to increased rates of cytokinesis failure (*χ^2 test, $p<0.01$ when comparing DLD1-YFP-SPG20 to DLD1-YFP cells). (D) Western blot analysis of Spartin levels in DLD1+13 cells treated with a SPG20 siRNA or with a control siRNA. (E) Reducing the levels of Spartin by SPG20 siRNA significantly reduces the rate of cytokinesis failure in DLD1+13 cells (*χ^2 test, $p<0.02$ when comparing cells treated with a control siRNA to cells treated with SPG20 siRNA). (F) Western blot analysis of Spartin levels in AF+13 cells treated with a SPG20 siRNA or with a control siRNA. (G)

Reducing the levels of Spartin by SPG20 siRNA significantly reduces the rates of cytokinesis failure in AF+13 cells (*χ^2 test, $p<0.02$ when comparing cells treated with a control siRNA to cells treated with SPG20 siRNA). Average values from three independent experiments; variability between AF+13 samples was not significant.

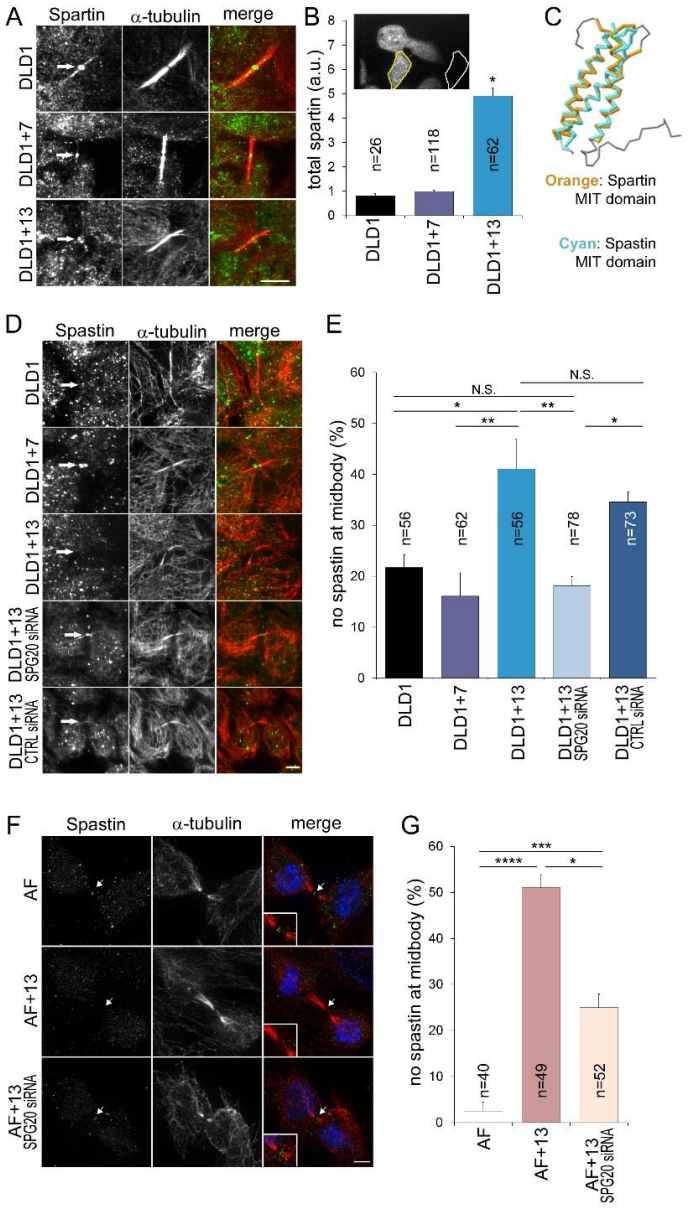


Figure 2.6. Spartin overexpression impairs Spastin localization to the midbody.

(A-B) Spartin localization at the midbody is not affected by the high levels of intracellular Spartin in DLD1+13. (A) Images showing Spartin localization (arrows) at the midbody of the three DLD1 cell lines. (B) The image shows interphase DLD1+13 cells immunostained for Spartin and the yellow and white outline indicate the regions of interest (ROI) selected for measurements of total intracellular fluorescence (yellow ROI) and background fluorescence (white ROI). The data in the graph report the total intracellular Spartin fluorescence intensity

after background subtraction in randomly sampled interphase cells and are represented as mean ± S.E.M (*t-test, $p<0.0001$ when comparing DLD1+13 to either DLD1 or DLD1+7). (C) Superimposition of the MIT domains of Spartin (orange) and Spastin (cyan) illustrates the considerable degree of structural homology between the two. PDB ID #2DL1 (Suetake et al., 2009) and #3EAB (Yang et al., 2008) for Spartin and Spastin, respectively. (D-E) Spastin localization at the midbody is impaired in DLD1+13 cells, and is rescued by SPG20 siRNA. (D) Images of midbodies of cells immunostained for Spastin (green) and microtubules (red). Arrows point at sites of Spastin localization or lack thereof. (E) Frequencies of cells lacking Spastin at the midbody. Higher frequencies of cells lacking Spastin at the midbody can be found in DLD1+13 cells compared to either DLD1 or DLD1+7 cells. Spastin localization could be rescued by knocking down Spartin levels in DLD1+13 cells via SPG20 siRNA. Statistical significance was calculated using a χ^2 test (*$p<0.05$; **$p<0.01$; N.S. = not significant). Data are presented as mean ± S.E.M. and represent the average of three independent experiments. (F-G) Spastin localization at the midbody is impaired in AF+13 cells, and is partially rescued by SPG20 siRNA. (F) Images of midbodies of cells immunostained for Spastin (green) and microtubules (red). DNA is shown in blue in the merged images. Arrows point at sites of Spastin localization or lack thereof. The insets show 3X magnifications of the midbody region. (G) Frequencies of cells lacking Spastin at the midbody are higher in AF+13 compared to AF cells. Spastin localization was partially rescued by SPG20 siRNA-mediated Spartin knock down in AF+13 cells. Statistical significance was calculated using a χ^2 test (*$p<0.05$; ***$p<0.001$; ****$p<0.0001$). Data are presented as mean ± S.E.M. and represent the average of three independent experiments/samples. Scale bars, 5 μm.

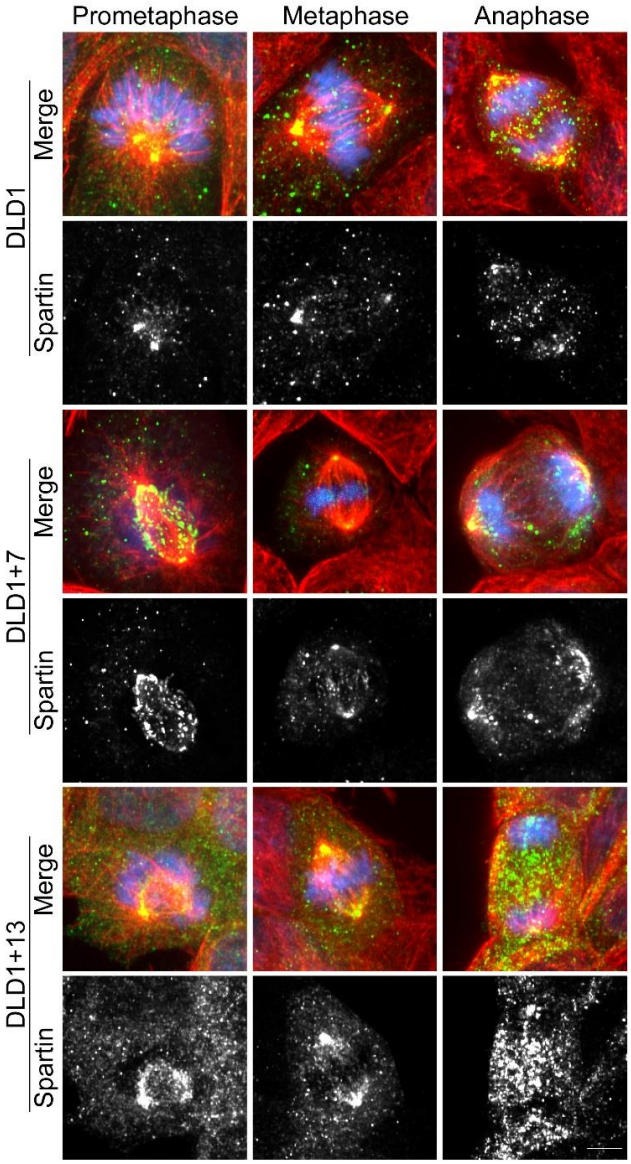


Figure 2.6-figure supplement 1. Spartin localization during mitosis.

Cells were immunostained for Spartin (green) and microtubules (red) and counterstained with DAPI for DNA (blue). For each cell line, the merged images (top) and Spartin alone (bottom) are shown. Images are maximum intensity projections of Z-stacks. Spartin localizes to the spindle poles during prometaphase, metaphase, and anaphase in DLD1, DLD1+7 and DLD1+13. An eccess of cytoplasmic spartin (away from the mitotic spindle) can be noticed in DLD1+13 cells, but not in the other two cell lines. Scale bar, 5 μm.

Chapter 3. Polyploidy as a buffer for aneuploidy in human cells

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Abstract

Tetraploidy, the state of having four sets of chromosomes, has been identified as a precursor of aneuploidy and carcinogenesis. Some have suggested that tetraploid cells may undergo multipolar mitoses, which would result in severe chromosome mis-segregation and high degrees of aneuploidy, which in turn may lead to carcinogenesis. Alternatively, tetraploid cells may undergo bipolar mitosis after a transient multipolar stage, a phenomenon linked to chromosomal instability in cancer cells. However, how tetraploidy affects chromosome segregation is not clear. To investigate the effect of tetraploidy on karyotype stability we generated tetraploid clones from DLD1 colorectal cancer cells (2N=46) and noncancerous hTERT-RPE1 cells (2N=46). Using immunofluorescence staining we found no significant differences in the rates of anaphase lagging chromosomes between diploid and tetraploid cell lines, suggesting that tetraploidy per se does not induce chromosome mis-segregation. Nevertheless, chromosome counts in metaphase spreads showed that tetraploid cells accumulate higher degrees of aneuploidy, supporting recent results that tetraploidy acts as a genetic buffer against the negative effects of aneuploidy. Additionally, we found that established tetraploid cell lines contained normal centrosome numbers and assembled bipolar spindles. However, about half of all early un-established tetraploid cells (ie the first cell division after cytokinesis failure) underwent multipolar mitoses. Cells that underwent two consecutive multipolar cell divisions were more likely to die or arrest than those that underwent one or two bipolar cell divisions.

Introduction

Tetraploidy, the state of having four sets of chromosomes, is found at high frequency in a number of healthy organs, including the liver and the aorta (Barrett et al., 1983; Biesterfeld et al., 1994; Celton-Morizur and Desdouets, 2010; Goldberg et al., 1984). The existence of tetraploid cells in normal healthy tissues suggests that tetraploidy may be well tolerated or even beneficial in certain contexts. Nevertheless, in mammalian cells, tetraploidy is most commonly associated with carcinogenesis (Ganem et al., 2007) and was first proposed to be involved in the development and progression of cancer over 100 years ago (Boveri, 1914). Indeed, tetraploidy is frequently observed in the precancerous lesion Barrett's Esophagus (Barrett et al., 2003; Reid et al., 1992) and in pre-neoplastic cervical cells (Olaharski et al., 2006). Systematic analysis of thousands of cancers suggested that an estimated ~37% of cancers have undergone a tetraploidization event at some point in their life cycle (Zack et al., 2013), and tetraploidy was shown to be a prognostic marker for disease progression in a variety of cancers (Shackney et al., 1995; Sudbo et al., 2001). A number of experimental and theoretical studies have provided evidence that tetraploidy may act as an intermediate step to aneuploidy (Levine et al., 1991; Olaharski et al., 2006), a recognized cause of cancer initiation and progression. Based on experimental evidence, tetraploidy has been proposed to promote aneuploidy in two possible ways. One way this could happen would be via an increased rate of multipolar mitoses and chromosome mis-segregation (Storchova and Pellman, 2004). This idea is based on the fact that tetraploidy

can arise as a result of cytokinesis failure, mitotic slippage, or cell fusion, all of which would result in daughter cells with double chromosome and centrosome content (Storchova and Pellman, 2004). An alternative way in which tetraploidy could promote aneuploidy is by acting as a genetic buffer against the detrimental effects of aneuploidy, wherein aneuploid cells arising in a tetraploid (as compared to diploid) background will continue to propagate after chromosome mis-segregation (Dewhurst et al., 2014b). To investigate the effects of tetraploidy on chromosome segregation and karyotype stability, we generated tetraploid clones from cancerous (DLD1, 2n=46) and non-cancerous (hTERT-RPE1, 2n=46; hereafter RPE1) diploid cell lines. In addition to gaining insight on the link between tetraploidy and aneuploidy, we gained novel insights into the patterns of cell division ensuing tetraploid cell formation.

Results

We generated tetraploid cell lines by experimentally inhibiting cytokinesis according to previously published protocols (Fujiwara et al., 2005b). We successfully generated tetraploid clones of both DLD1 and RPE1 cells (DLD1-Tet and RPE1-Tet, respectively). DLD1 is a colorectal cancer cell line characterized by microsateillite instability (MIN) with a modal chromosome number of 46 (ATCC) harboring an inactivating p53 mutation (Sur et al., 2009). p53 has been proposed to limit the growth and survival of tetraploid cells via a so-called "tetraploidy checkpoint" (Andreassen et al., 2001a; Margolis et al., 2003), although this has previously been disputed by several groups (Ohshima and Seyama, 2013; Uetake and Sluder, 2004b; Wong and Stearns, 2005). RPE1 is a non-cancerous cell line derived from retinal pigment epithelial cells stably expressing the active subunit of telomerase (hTERT). These cells have a diploid karyotype and a wild-type p53 (Jiang et al., 1999). The successful generation of tetraploid clones supports previous reports showing that tetraploid cell lines can be established and maintained from normal, healthy cells (Ohshima and Seyama, 2013).

Tetraploid cells display higher rates of aneuploidy, but similar rates of chromosome mis-segregation compared to diploid cells. To investigate the link between tetraploidy and aneuploidy, we performed karyotype analysis by chromosome counts in metaphase spreads (Figure 3.1A). Both parental cell lines displayed the expected modal chromosome number of 46, whereas the

DLD1-Tet and RPE1-Tet cells displayed modal chromosome numbers of 91 and 92, respectively (Figure 3.1B). Importantly, the tetraploid cell lines showed a higher karyotypic diversity compared to the diploid counterparts, with 77% of DLD1-Tet cells possessing karyotypes that deviated from the modal chromosome number as opposed to 21% of DLD1 cells (Figure 3.1C). Similarly, but less pronounced than in DLD1 cells, 58% of RPE1-Tet cells possessed karyotypes deviating from the modal chromosome number, as opposed to 55% in RPE1 cells (Figure 3.1C). Tetraploid cells are expected to possess extra centrosomes, which have been proposed to promote chromosome mis-segregation and aneuploidy by causing multipolar cell division (Storchova and Pellman, 2004). To assess whether this mechanism was responsible for the karyotypic diversity found in tetraploid cells, we quantified the rates of multipolar anaphase cells, but found no differences between diploid and tetraploid cell lines (Figure 3.2A-B). Another mechanism by which extra centrosomes may lead to chromosome mis-segregation and aneuploidy is by promoting multipolar spindle assembly, followed by centrosome clustering. This pathway was shown to be associated with high rates of merotelic kinetochore attachments and anaphase lagging chromosomes in cancer cells with supernumerary centrosomes (Ganem et al., 2009; Silkworth et al., 2009). To test this possibility we measured the rates of multipolar prometaphases and the rates of lagging chromosomes in bipolar anaphases in the four cell lines and found no significant differences between diploid and tetraploid cells for either of these phenotypes (Figure 3.1C-F). The scarcity of multipolar spindles and the low rates of anaphase lagging

chromosomes in the tetraploid cell lines were surprising, but could be explained by centrosome clustering occurring at very early stages of mitosis. To determine the actual centrosome number in the tetraploid cells, we analyzed centrin content in G1 cells. Centrin is a centriole marker required for centriole duplication in mammalian cells (Salisbury et al., 2002). Each centrosome includes one pair of centrioles, and a single pair is expected in normal, diploid cells before centrosome duplication, which occurs at the G1/S transition (Hinchcliffe and Sluder, 2001). We identified G1 cells by co-staining for the G1 marker cyclin D1 (Figure 3.2G) and found that the vast majority of both diploid and tetraploid cells contained only 2 centrin-positive dots (i.e., two centrioles) per cell in G1 (Figure 3.2H). This finding indicates that the low number of multipolar mitoses we found in tetraploid cells is not due to centrosome clustering, but to the actual number of centrosomes. The normal centrosome number also explains the similar rates of anaphase lagging chromosomes found in diploid and tetraploid cells. If the increase in karyotypic heterogeneity observed in tetraploid cells cannot be explained by an increase in chromosome mis-segregation via transient or persistent multipolar mitoses, then a different explanation must be invoked. A recent study showed that tetraploid cells are more likely than diploid cells to keep proliferating after a chromosome mis-segregation event, suggesting that tetraploid cells have an increased tolerance to chromosome mis-segregation (Dewhurst et al., 2014b). Our results provide further support to the conclusion that tetraploidy acts as a buffer against the deleterious effects of chromosome mis-segregation and aneuploidy. Indeed, despite no difference in chromosome

mis-segregation rates, tetraploid cells have a larger population of aneuploid cells, indicating they are able to keep proliferating after mis-segregation compared to diploid cells.

Tetraploidy *per se* is not sufficient to cause chromosome structural defects. A previous study showed that tetraploidy was also associated with chromosome structural defects, such as rearrangements and fragments, in addition to chromosome numerical instability (Fujiwara et al., 2005b). To examine a possible role of tetraploidy in generating chromosome structural defects, we quantified the rates of chromosome bridges and chromosome fragments in diploid and tetraploid anaphase cells (Figure 3.3). Both fragments and bridges are typically produced as a result of DNA damage, specifically double strand breaks (Sofueva et al., 2011). Acrocentric DNA fragments were rare in both diploid and tetraploid cells (Figure 3.3B). DNA bridges were also infrequent, and the only observed difference was that between DLD1 and DLD1-Tet cells, with the diploid cells displaying higher rates (Figure 3.3C). Thus, our data indicate that tetraploidy *per se* is not sufficient to cause chromosome structural defects.

Multipolar mitoses occur immediately immediately after cytokinesis failure Mutlipolar mitoses and supernumerary centrosomes are frequently observed in cancer cells (Brinkley and Goepfert, 1998; Lingle and Salisbury, 1999) and cytokinesis failure, which leads to tetraploidization, has been proposed as a source of extra centrosomes (Ganem et al., 2007; Levine et al., 1991). Thus, our

finding that established tetraploid cell clones did not display multipolar mitoses or extra centrosomes was quite surprising. To investigate how clones with tetraploid karyotypes, but no extra centrosomes may arise, we induced cytokinesis failure in diploid DLD1 and RPE1 cells following the same protocol used for establishing the tetraploid clones and then performed long-term live-cell imaging experiments to examine binucleate cells as they underwent cell division. A significant number of RPE-1 and DLD-1 cells undergo multipolar mitoses during their first division following cytokinesis failure (52% and 79%, respectively) (Figure 3.4). In contrast, our established tetraploid cell lines almost exclusively divide in a bipolar fashion (Figure 3.2A), as has been previously observed in other established tetraploid cell lines (Godinho et al., 2014b). To determine if our established tetraploid cell lines actively suppress supernumerary centrosome replication or if supernumerary centrosomes were lost spontaneously, we compared the frequency of multipolar mitoses in binucleate cells formed from diploid cells versus those formed from tetraploid DLD1 cells. We found that both the majority of binucleate cells formed from diploid cells and established tetraploid cells divided in a multipolar fashion (Figure 3.4). This suggests that no active mechanism is present in our tetraploid cells limiting the number of multipolar mitoses, rather that multipolar mitoses may be inherently self-limiting due to the massive genetic changes they cause. Consistent with this, we found that RPE1 and DLD1 binucleate cells formed after cytokinesis failure are more likely to arrest or die after undergoing multipolar mitosis than cells where bipolar mitosis has occurred (Figure 3.5). Thus, our data suggest that when undergoing bipolar

cell division, tetraploid cells are more likely to keep proliferating then if they were undergoing multipolar cell division. Overall, this suggests that cells that can control their centrosome number, spontaneously lose extra centrosomes, or cluster their spindle poles effectively help ensure the viability of a cell population as a whole. The observation that both of our stable tetraploid cell lines contain a normal centrosome complement suggests that these cells were able to somehow reduce their centrosome number to a normal level following cytokinesis failure, and by avoiding multipolar mitoses increase their fitness.

Discussion

We show that despite no increase in chromosome mis-segregation in our established tetraploid cell lines, there is an increase in karyotypic heterogeneity, supporting recent findings that tetraploidy can buffer the negative effects of chromosome mis-segregation and aneuploidy (Dewhurst et al., 2014b). Wherein it has largely been thought that tetraploidy strictly acts as a mechanism to increase chromosome mis-segregation, we show that it can instead act as a mechanism to better tolerate it. The increased tolerance to aneuploidy in a tetraploid background is likely one reason tetraploidy has been associated with adaptability, as tetraploid cells are able (more than diploid cells) to gain or lose chromosomes that may confer selective advantages to cells without the risk of cell death (Selmecki et al., 2015). Indeed, it would be extremely difficult, if not impossible, to get the levels of aneuploidy seen in cancer without the buffering effect of tetraploidy.

Our results also show that tetraploidy does initially induce multipolar mitoses. However, both of our established tetraploid cell lines displayed a normal centrosome number and as a consequence almost exclusively completed mitosis in a bipolar fashion. This observation is in agreement with similar studies (Godinho et al., 2014b) and raises the question: if many cancer cells display multipolar mitoses, where do the supernumerary centrosomes come from. Our findings indicate that tetraploidy does lead to supernumerary centrosomes initially, however clones undergoing repeated rounds of multipolar mitoses are

likely to arrest or die due to massive genomic loss. Thus, we hypothesize that if tetraploidy is present at early stages of tumorigenesis, this may be in the absence of extra centrosomes. The genetic buffer conferred by tetraploidy would then allow for accumulation of aneuploidy. Finally, supernumerary centrosomes and multipolar mitoses may arise as a result of deregulated centrosome replication caused by the genetic imbalance brought about by the high levels of accumulated aneuploidy.

Materials and Methods

Cell Lines and Culture Conditions. DLD1 and hTERT-RPE1 cell lines were obtained from American Type Culture Collection (ATCC). DLD1 and derivative cell line were maintained in RPMI 1640 (ATCC) supplemented with 10% FBS (Gibco), penicillin, streptomycin, amphotericin B (antimycotic), and plasmocin (anti-mycoplasma). hTERT-RPE1 and derivative cell line were maintained in DMEM/F12 (ATCC) supplemented with 10% FBS (Gibco), penicillin, streptomycin, amphotericin B (anti-mycotic), and plasmocin (anti-mycoplasma). All cells were kept in a humidified incubator at 37°C with 5% CO_2. For experiments, cells were grown on sterilized glass coverslips inside 35 mm Petri dishes.

Generation of tetraploid cell lines. DLD1 and RPE1 cells were grown in T-25 flasks and treated with 3μg/ml dihydrocytochalasin B (Sigma-Aldrich, MO, USA) for 24 hours. Cells were washed, trypsinized, and serially diluted into a 96 well plate and single cell colonies were identified and allowed to grow for 1 week. Cells were expanded into two T-25 flasks and metaphase spread analysis was performed (see below) to identify and confirm the successful generation of tetraploid cell lines.

Metaphase Spreads. Cell cultures were incubated in 50 ng/ml Colcemid (Karyomax, Invitrogen) at 37°C for 3-4 hours to enrich in mitotically arrested cells. The cells were then collected and centrifuged at 800 rpm for 8 minutes.

Hypotonic solution (0.075M KCl) was added drop-wise to the cell pellet and incubated for 10 minutes at room temperature. Cells were fixed with an ice-cold 3:1 methanol:acetic acid solution for 5 minutes and then centrifuged at 800 rpm for 8 minutes. This last step was repeated two more times and fixed cells were finally dropped on microscope slides.

Immunostaining. For analysis of mitotic defects, cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature and then permeabilized for 10 minutes at room temperature in PHEM buffer containing 0.5% Tritron-X 100. Following fixation and permeabilization, cells were washed with PBS three times and then blocked with 10% boiled goat serum (BGS) for 1 hour at room temperature. Cells were then incubated at 4°C overnight with primary antibodies diluted in 5% BGS. Cells were then washed in PBS-T (PBS with 0.05% Tween 20) three times, and incubated at room temperature for 45 minutes with secondary antibodies diluted in 5% BGS. Cells were counterstained with DAPI for 5 minutes, and coverslips were mounted on microscope slides in an antifade solution of 90% glycerol and 0.5% N-propyl gallate. For analysis of centrin, cells were briefly washed in PBS with 5mM EGTA and then incubated for 4 minutes in 1x PHEM with 0.5% or 0.1% Triton-X 100 (lysis) for DLD1 and RPE1 cells, respectively. Cells were prefixed in 95% methanol with 5mM EGTA for 5 minutes at room temperature and again in the same solution for 30 minutes at -20°C. Following this, cells were handled in the same way as described above. Primary antibodies were diluted as follows: ACA (human anti-centromere protein,

Antibodies Inc.), diluted 1:100; mouse anti-α-tubulin (DM1A, Sigma Aldrich), diluted 1:500; rabbit anti-centrin (Santa Cruz Biotechnology, Inc.), diluted 1:200; mouse anti-cyclin D1 (Abcam) diluted 1:100. Secondary antibodies were diluted as follows: Rhodamine Red-X goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc.), diluted 1:100; Rhodamine Red-X goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.), diluted 1:100; Alexa 488 goat anti-human (Invitrogen), diluted 1:200; Alexa 488 goat anti-mouse (Molecular Probes), diluted 1:200.

Confocal Microscopy and Image Analysis. Immunofluorescently labeled cells were imaged with a swept field confocal system (Prairie Technologies) on a Nikon Eclipse TE2000-U inverted microscope (Nikon Instruments Inc.) equipped with a 100x/1.4 NA Plan-Apochromatic objective and an automated ProScan stage (Prior Scientific). The confocal head was accessorized with filters for illumination at 405, 488, 561, and 640 nm through an Agilent monolithic laser combiner (MLC400) controlled by a four channel acousto-optic tunable filter. Digital images were acquired with a HQ2 CCD camera (Photometrics). Acquisition time, Z-axis position, laser line power, and confocal system were all controlled by NIS Elements AR software (Nikon Instruments Inc.) on a PC computer (Dell). Spindle structure, anaphase lagging chromosomes, bridges, and acentric fragments, and centrin content were analyzed by acquiring Z-sections of cells at 0.6 μm steps.

Phase contrast live cell imaging. For binucleate cell division analysis, DLD1 and RPE1 cells were grown in 35 mm glass-bottom Petri dishes and treated with 3 μg/ml dihydrocytochalasin B (Sigma-Aldrich, MO, USA) for 24 hour to induce cytokinesis failure. Cells were then washed 4 times with drug-free media and placed in L-15 medium supplemented with 4.5 g/l glucose. Images were acquired on a Nikon Eclipse Ti inverted microscope (Nikon Instruments Inc.) equipped with phase-contrast transillumination, transmitted light shutter, ProScan automated stage (Prior Scientific), and a HQ2 CCD camera (Photometrics). Cells were maintained at ~36 °C using a stage top incubator (Tokai Hit). 10-15 different fields of cells were imaged every 6 minutes for 40-75 hours using a 20x/0.4 NA phase contrast objective (Nikon Instruments Inc.). The time-lapse movies were analyzed using NIS Elements AR (Nikon Instruments Inc.) software on a PC computer (Dell).

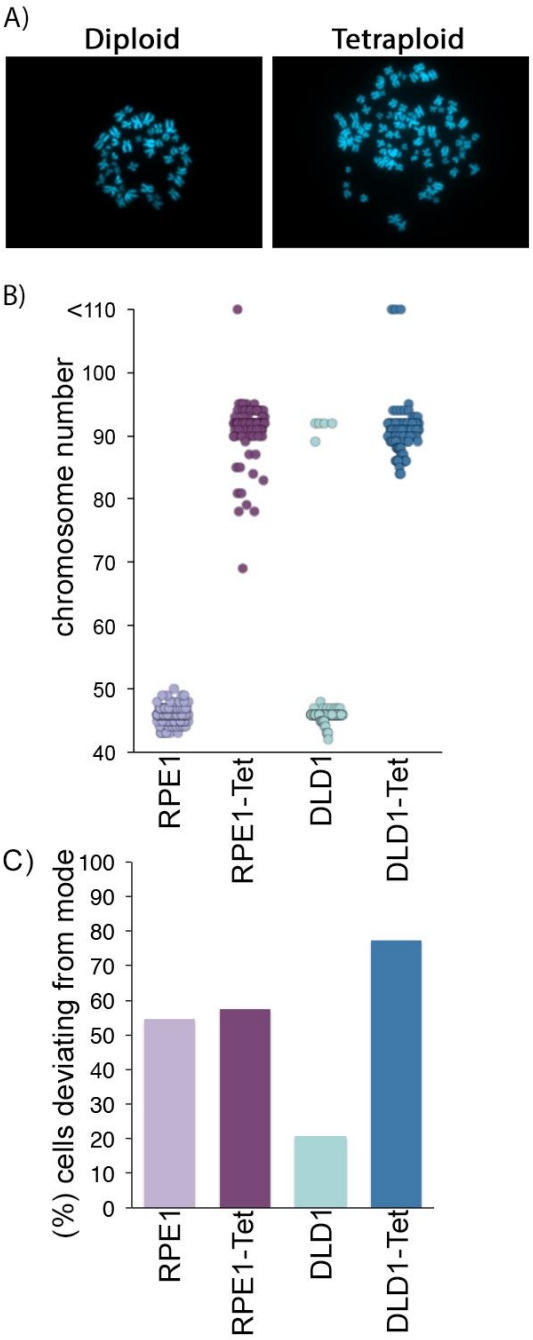


Figure 3.1 Tetraploidy induces karyotypic heterogeneity without an increase in chromosome mis-segregation.

(A) Representative metaphase spreads from diploid and tetraploid cell lines. RPE1-Tet and DLD1-Tet displayed increased karyotypic heterogeneity compared to RPE1 and DLD1 cells, respectively. (B) Beeswarm plot displaying data from chromosome counts in (A). Chromosome counts were performed on 88-109 metaphase spreads. (C) The number of cells deviating from the modal chromosome number was increased in tetraploid cell lines.

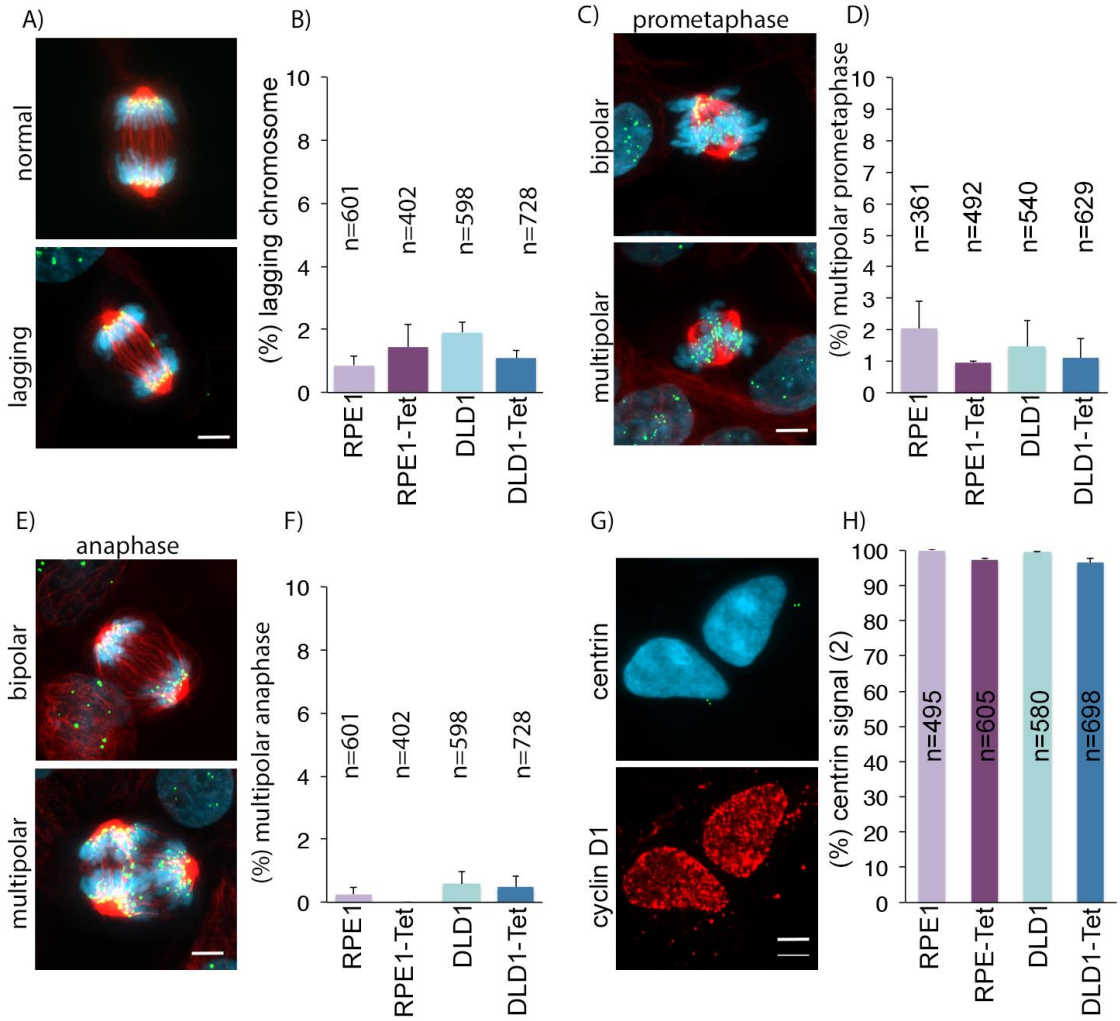


Figure 3.2 Similar frequencies of lagging chromosomes (A-B), multipolar mitoses (C-F), and centrin content (G,H) in diploid vs. tetraploid cells.

(A) Examples of a normal anaphase (top row) and an anaphase cell with a lagging chromosome (bottom row). Cells were immunostained for microtubules (red) and kinetochores (green). DNA is shown in blue. Images represent maximum intensity projections of Z-stacks. (B) Frequencies of anaphase lagging chromosomes were not significantly different (*χ^2 test, P>0.05) between respective diploid and tetraploid cell lines. Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 601-728 anaphases were analyzed. (C) Examples of bipolar prometaphase (top row) multipolar prometaphase cells. Cells were immunostained for microtubules (red) and kinetochores (green). DNA is shown in blue. Images represent maximum intensity projections of Z-stacks. (D) Frequencies of bipolar versus multipolar prometaphase cells were not significantly different (*χ^2 test, P>0.05) between respective diploid and tetraploid cell lines. Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a

total of 361-629 prometaphase cells were analyzed. (E) Examples of normal anaphase (top row) and multipolar anaphase cells with lagging chromosomes (bottom row). Cells were immunostained for microtubules (red) and kinetochores (green). DNA is shown in blue. Images represent maximum intensity projections of Z-stacks. (F) Frequencies of multipolar anaphases were not significantly different (*χ^2 test, P>0.05) between respective diploid and tetraploid cell lines. Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 601-728 anaphases were analyzed. (G) Examples of centrin content in interphase cells. Cells were immunostained for centrin (green) and cyclin D1 (red). DNA is shown in blue. (H) Frequencies of normal centrin content (2 signals) were not significantly different (*χ^2 test, P>0.05 between respective diploid and tetraploid cell lines. Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 495-698 anaphases were analyzed.

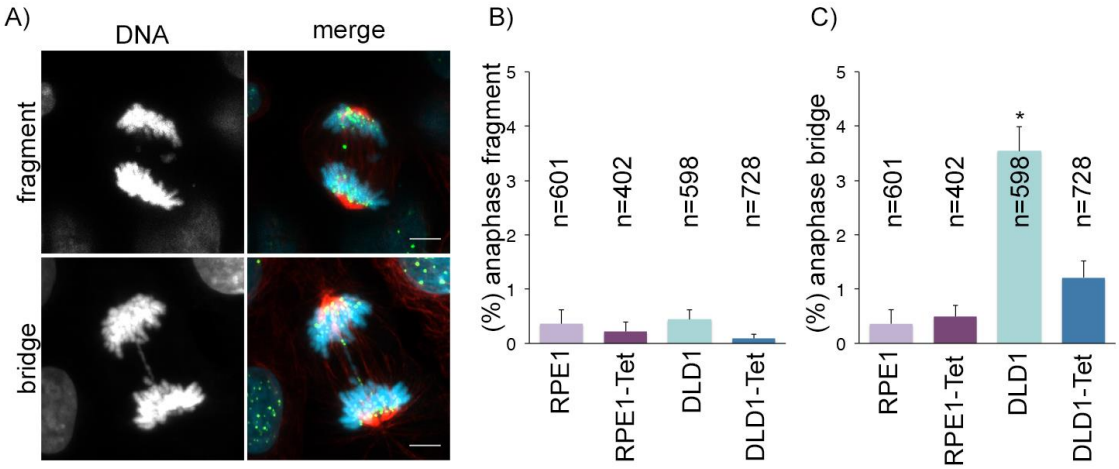


Figure 3.3 Tetraploidy does not induce structural defects

(A) Examples of an anaphase fragment (top row) and an anaphase bridge (bottom row). Cells were immunostained for microtubules (red) and kinetochores (green). DNA is shown in blue. Images represent maximum intensity projections of Z-stacks. (C) Frequencies of anaphase fragments were not significantly different (*χ^2 test, P>0.05) between respective diploid and tetraploid cell lines. (D) Frequencies of anaphase bridges were not significantly different (*χ^2 test, P>0.05) between RPE1 diploid and tetraploid cell lines. However, there were significantly more anaphase bridges in diploid DLD1 cells than tetraploid cells (*χ^2 test, P<0.02) Data are reported as mean ± S.E.M and represent the average of three independent experiments in which a total of 601-728 prometaphase cells were analyzed

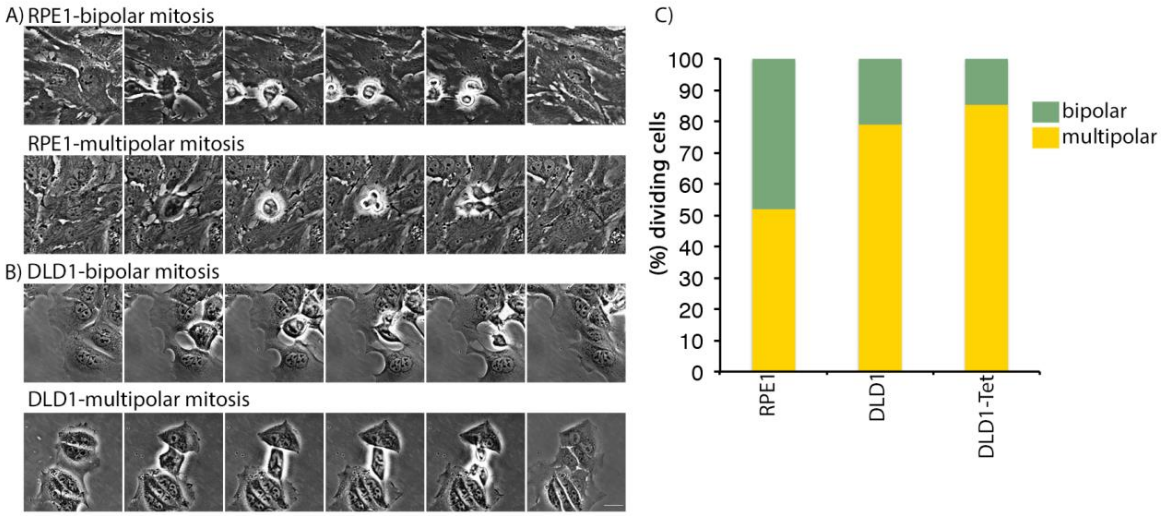


Figure 3.4 Multipolar mitoses occur at high frequencies in early tetraploid cells.

Time-lapse microscopy indicates high frequencies of multipolar mitoses in the first cell division after cytokinesis failure. Scale bars, 5 μm. (A) Still images from time-lapse phase contrast movies of RPE1 cells undergoing mitosis and completing bipolar (top rows) or multipolar (bottom rows) mitoses. (B) Still images from time-lapse phase contrast movies of DLD1 cells undergoing mitosis and completing bipolar (top rows) or multipolar (bottom rows) mitoses (C) Quantification of bipolar and multipolar mitoses from phase-contrast time lapse movies shows that a large number of cells undergo multipolar mitosis. DLD1-tet cells also display high levels of multipolar mitoses indicating there is no active mechanism limiting multipolar mitosis in established tetraploid cells. For analysis 27-301 cells were tracked.

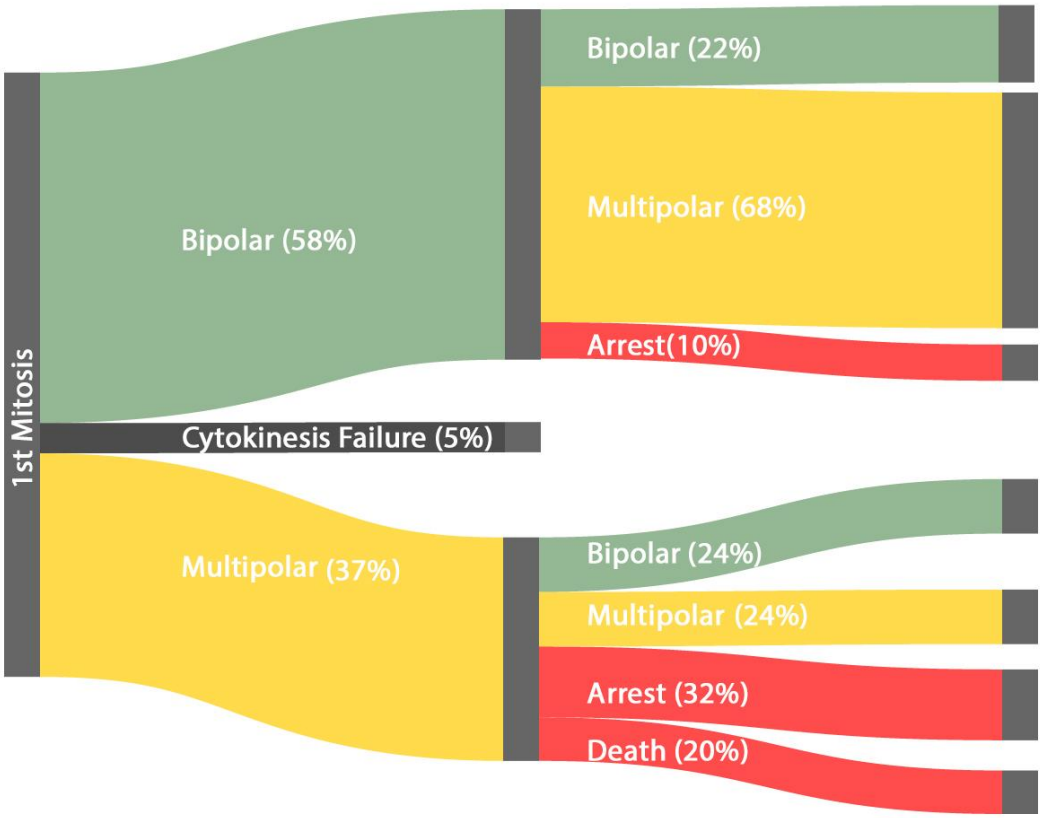


Figure 3.5 Multipolar mitoses lead to cellular arrest/death

Sankey diagram shows the fate of binucleate DLD1 cells undergoing bipolar or multipolar mitoses. Cells that undergo multipolar mitosis are more likely to arrest/die than those undergoing bipolar mitosis.

Chapter 4. Conclusions and perspectives

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Link between Aneuploidy and Chromosome Instability.
Nicholson, J.M., and Cimini, D. *International Review of Cell and Molecular Biology*, edited by W. Jeon Kwang. 2015. 299-317. Academic Press.

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Cancer karyotypes: survival of the fittest.
Nicholson, J.M., and D. Cimini. 2013. Frontiers in oncology. 3:148.

Conclusions and Perspectives

The studies and research presented here support the conclusion that aneuploidy can impair chromosome segregation and thus cause CIN. This is in agreement with recent findings in budding yeast (Sheltzer et al., 2011; Zhu et al., 2012) and previous studies in cancer cells (Duesberg et al., 1998b), but in disagreement with other studies (Lengauer et al., 1997b; Valind et al., 2013). These disagreements are likely to stem from inconsistencies in the way CIN is defined or in the methods used to evaluate CIN. CIN has been loosely defined as an elevated rate of chromosome mis-segregation (Lengauer et al., 1997b), yet how elevated and compared to what is often unclear (Geigl et al., 2008). Geigl et al. suggested that CIN can be defined as a significant increase in the rate of chromosome mis-segregation compared to an appropriate control cell population (Geigl et al., 2008). Further, appropriate statistical tests must be employed (Geigl et al., 2008). Given this definition, many of the reports identifying stable aneuploidies can be reinterpreted. Studies that use data available in the Mitelman database of cancer karyotypes (Mitelman et al., 2014b; Storchova and Kuffer, 2008; Zasadil et al., 2013) often rely on karyotypic analysis of small numbers (5-20) of cells per cancer, thus masking small rates of CIN that may be present (Adeyinka et al., 1998; Bridge et al., 2004). Other studies lack appropriate statistical analysis (Lengauer et al., 1997b). Finally, stable is often used in relative terms. For instance, Roschke et al. defined aneuploid cancer cells with elevated chromosome mis-segregation rates as stable (Roschke et al., 2002). The authors considered these cells to be stable because modal

chromosome numbers did not grossly deviate over time, although they observed deviations per chromosome of up to 20% at a given time (Roschke et al., 2002). Discrepancies in the literature may also depend on the method by which CIN is evaluated/measured in different studies. Many studies measure CIN by one of two methods: (i) performing karyotypic analysis (sometimes simply by chromosome count) at some point in time and measuring what fraction of the cell population possesses a chromosome number that deviates from the mode; (ii) performing FISH staining on interphase nuclei with chromosome-specific probes for 2-3 chromosomes and again evaluating what fraction of the cell population possesses a number of copies for those chromosomes that deviates from the mode. Neither of these methods really measures chromosome mis-segregation directly and both of them are very likely to underestimate the rates of chromosome mis-segregation occurring at each round of cell division. Because the gain or loss of a single chromosome represents a dramatic genetic change, whether a mis-segregation event can become evident as CIN using one of the methods described above will depend on a number of selective factors, including the specific chromosome that is lost or gained, the specific cell type studied, and the context (e.g., current karyotype, presence/absence of certain environmental conditions, etc.) in which the loss/gain occurs. In other words, cells that mis-segregate chromosomes may or may not survive, and therefore analysis of the karyotype in metaphase spreads or chromosome number in interphase nuclei may reveal a stable karyotype even in the presence of CIN defined as an increased rate of chromosome mis-segregation (Bakhoum et al., 2014). A more

accurate way to measure CIN is by analyzing chromosome segregation in mitotic cells. Many labs have used this approach in recent years and found that CIN cells display higher rates of anaphase lagging chromosomes (chromosomes that lag behind at the cell equator while all other chromosomes segregate to the spindle poles, Fig. 1.5) compared to non-CIN cells (Bakhoum et al., 2009a; Ganem et al., 2009; Silkworth et al., 2009; Thompson and Compton, 2008b). Anaphase lagging chromosomes, even when segregated to the correct daughter cell, still represent a mis-segregation event as they typically form micronuclei in the daughter cell (Cimini et al., 2002). Micronuclei have been shown to lead to both numerical and structural defects, including more anaphase lagging chromosomes (Crasta et al., 2012; He et al., 2012). Whereas the analysis of anaphase lagging chromosomes may be a better way to measure CIN, it may still be insufficient to determine the real rates of chromosome mis-segregation, as cases in which two sister chromatids segregate to the same spindle pole would go undetected. A good alternative approach to measure CIN would require the combination of more than one of the methods outlined above, such as, for instance, anaphase lagging chromosomes and interphase FISH or anaphase lagging chromosomes and karyotypic analysis. Alternatively, the analysis of anaphase lagging chromosomes (for all chromosomes) could be combined with analysis of chromosome segregation by FISH with chromosome-specific probes on anaphase cells, on binucleate cells in a cytokinesis-block assay (Cimini et al., 1999), and/or in the interphase ensuing cell division (Thompson and Compton, 2008b).

Despite the significant difficulties of defining and measuring CIN, evidence supporting aneuploidy as a cause of chromosome mis-segregation was first suggested by correlations between the degree of aneuploidy and the degree of CIN in transformed Chinese hamster embryo cells and in colorectal cancer cell lines (Duesberg et al., 1998b). Moreover, studies in haploid yeast strains carrying specific disomies (aneuploidies) showed that aneuploidy induced high rates of chromosome mis-segregation (Sheltzer et al., 2011; Zhu et al., 2012), although not in all disomies tested (70%, 9/13) (Sheltzer et al., 2011), and to varying degrees depending on the specific disomy (Zhu et al., 2012). Finally, some studies have reported elevated rates of aneuploidy in somatic cells of individuals affected by congenital trisomies (Reish et al., 2006; Reish et al., 2011). Thus, together with these previous studies, the research described here strongly argues in favor of a CIN-promoting role of aneuploidy. What remains unclear is *how* aneuploidy promotes chromosome mis-segregation. To gain insight into this question, we have plotted the rates of anaphase lagging chromosomes reported by several laboratories versus the modal chromosome number of the cell lines analyzed and found a significant correlation (Figure 4.1), indicating that a higher degree of aneuploidy (i.e., modal chromosome number considerably above 46) correlates with higher frequencies of chromosome mis-segregation (i.e., CIN). This, suggests that aneuploidy can induce chromosome mis-segregation due to an imbalance in the gene dosage (which may include many mitotic genes), such that a higher degree of aneuploidy will result in a more

severe imbalance. Accordingly, I have also shown that specific karyotypes can induce specific phenotypic effects in human cells, by inducing specific gene expression changes. Indeed, trisomy 13 was shown to induce cytokinesis failure in both cancerous and non-cancerous cells by overexpression of SPG20, a gene involved in cytokinesis regulation that resides on chromosome 13 (Figure 2.5). Further, trisomy 7 caused chromosome mis-segregation to a different extent than trisomy 13, likely an effect of different gene expression changes previously observed (Upender et al., 2004). Thus, my work strongly argues against aneuploidy as a random passenger event in carcinogenesis. Rather, by generating phenotypic variability, aneuploidy can confer an evolutionary advantage to cancer cells that must grow and adapt under numerous different conditions, including chemotherapy.

Lastly, my results also show that aneuploidy may arise in human cells, not necessarily as a result of an increase in chromosome mis-segregation rates but an increase in the tolerance to aneuploidy. Indeed, we found that tetraploidy caused an increase in karyotypic heterogeneity in the cell population despite no significant difference in chromosome mis-segregation rates or multipolar mitoses (Figure 3.1). This finding fits with recently published work showing that tetraploid cells are more likely to continue to divide after chromosome mis-segregation than diploid cells (Dewhurst et al., 2014b). Further, it helps explain why tetraploidy is frequently observed in “pre-cancerous” cells and often times precedes aneuploidy (Olaharski et al., 2006). Interestingly, we observed that our tetraploid

cell lines had normal centrosome numbers. This finding is surprising as it was previously believed that tetraploidy results in centrosome amplification and an increase in multipolar mitoses (Levine et al., 1991).

Future work to be performed around these results would be the identification of the mechanism that ties aneuploidy to chromosome mis-segregation. It is likely that this is an effect of gross gene expression changes, however alternative possibilities exist and should be explored, including asynchronous/delayed replication timing and/or defects in chromosome condensation. Further, identifying exactly how tetraploidy confers a tolerance to aneuploidy is an important area that needs to be explored further. Presumably, tetraploidy may buffer the effects of aneuploidy as numerical chromosome changes in a tetraploid background would have an impact that is 50% the impact it would have in a diploid background. Last, identifying how centrosome number is tied to ploidy will be important to understand. Are there active mechanisms that limit supernumerary cells or are cells with supernumerary centrosomes less likely to survive due to an increase in multipolar mitoses?

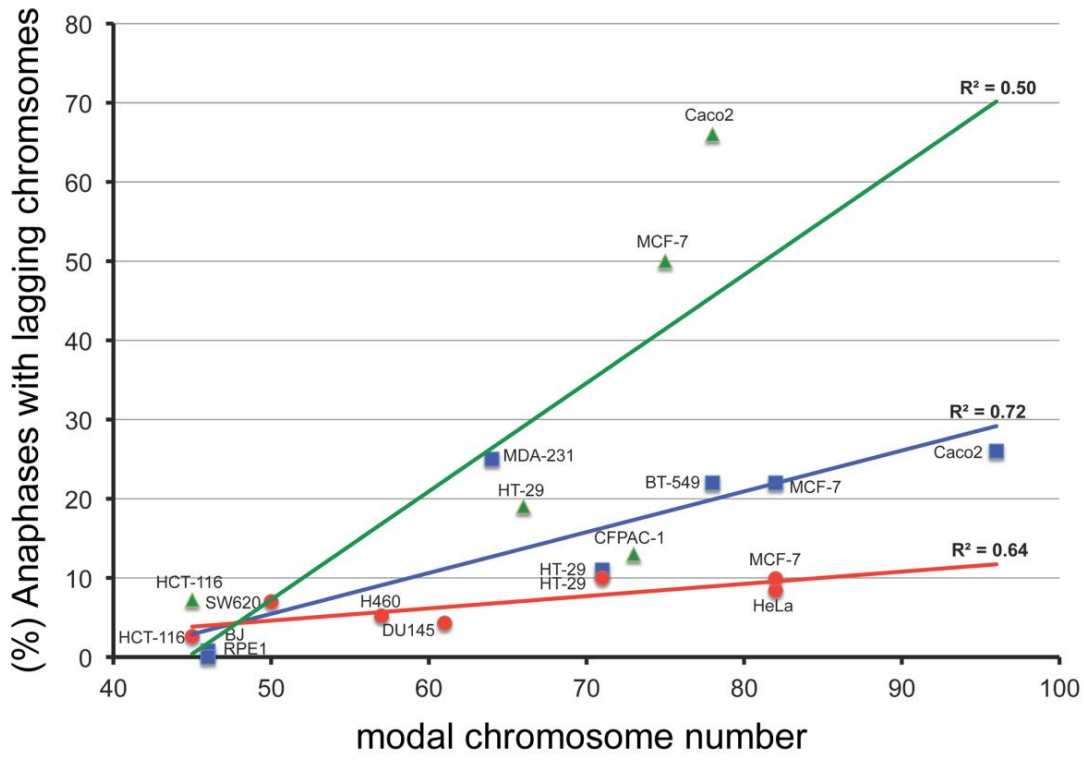


Figure 4.1 The degree of aneuploidy directly correlates with CIN

The degree of aneuploidy directly correlates with CIN, as measured by analysis of anaphase lagging chromosomes. XY plot showing the relation between anaphase lagging chromosomes and modal chromosome number in various cell lines. The graph also shows linear fits and regression values (R^2). The three colors refer to data sets from different labs: red is for data from the Cimini lab [(Silkworth et al., 2009) and (Silkworth, Nardi, and Cimini, unpublished)]; blue is for data from the Pellman lab (Ganem et al., 2009); green is for data from the Compton lab (Thompson and Compton, 2008b). Karyotype information for cell lines from the Cimini lab and the Pellman lab was obtained from The American Type Cell Culture website (ATCC). Karyotype information for cell lines from the Compton Lab is that reported in (Thompson and Compton, 2008b). Although there is a general trend in which higher chromosome modal number correlates with higher rates of anaphase lagging chromosomes, there is a certain degree of variability between different labs. Correlation analysis showed significant correlation between aneuploidy and CIN for the cell lines in blue (Pearson $R=0.85$, $P<0.05$) and those in red (Pearson $R=0.80$, $P<0.05$), but no significant correlation for the data shown in green ($R=0.71$, $P>0.05$).

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