Chromosome segregational defects: their origin, fate and contribution to genomic instability

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Abstract

Chromosome instability (CIN), a continuous change in the structure or number of chromosomes, is proposed to be a key mechanism driving the genomic changes associated with tumorigenesis. One major cause of CIN in cells is chromosome segregational defects occurring during mitosis. Two such examples are anaphase bridges and multipolar spindles, which are common in most cancer cells and many tumor tissues.

Anaphase bridges are chromatin bridges in between separating chromosome masses during anaphase, which may result in gene amplification or loss when breaking. We have found that cigarette smoke condensate (CSC) induced anaphase bridges in cultured primary human cells, which in a short time led to genomic imbalances. The frequency of the induced bridges within the entire population decreased with time, independent of the p53-mediated apoptotic pathway. We also showed that CSC induced DNA double-stranded breaks (DSBs) in cultured cells as well as purified DNA. The reactive oxygen species (ROS) scavenger, 2' deoxyguanosine 5'-monophosphate (dGMP) prevented CSC-induced DSBs, anaphase bridge formation and genomic imbalances. Therefore, we propose that CSC induces bridges and genomic imbalances via DNA DSBs. Further analysis in live oral cancer cells shows that cells with anaphase bridges mostly

survive and these bridges frequently result in micronuclei formation, indicating that anaphase bridges actively contribute to CIN.

Multipolar spindles (MPS) are aberrant mitotic structures when cells divide with greater than two spindle poles, which may result in uneven chromosome segregation. Multipolarity is strongly linked to centrosomal amplification, the mechanism of which remains controversial. We have examined the origin and fate of cells with MPS in real time. In both human embryonic kidney and oral cancer cells, the vast majority of multipolar cells originated from multinucleated cells. The frequency of cytokinesis failure was similar to the frequency of MPS, and each observed bipolar division that ended in a cytokinesis failure led to MPS formation in the subsequent mitosis. While grossly abnormal, these cells are still capable of dividing, often giving rise to a mixed progeny of multinucleated and mononucleated cells. These observations support the model that failure of cytokinesis may be the most common mechanism by which cells form MPS.

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Chapter I : Introduction

1. Genomic instability

Genomic instability, defined as an abnormal cell state associated with continuous genomic alterations including mutations, chromosome rearrangement, deletions, amplifications and inversions, is one hallmark of tumorigenesis (reviewed in (Jallepalli and Lengauer 2001; Draviam, Xie et al. 2004)). There are at least two sources of genomic instability: (i) microsatellite instability (MIN) and (ii) chromosome instability (CIN). MIN is the destabilization of simple repeat sequences known as microsatellites, occurring in sporadic colon cancers and hereditary non-polyposis colon cancer (HNPCC) (Thibodeau, Bren et al. 1993). MIN has been shown to be due to inactivation of genes involved in post-replicative DNA mismatch repair (Ionov, Peinado et al. 1993). MIN is frequent only in colorectal, endometrial and gastric cancers (10-15%), but not in other cancer types (2% or less) (Peltomaki, Lothe et al. 1993; Risinger, Berchuck et al. 1993; Kobayashi, Sagae et al. 1995; Gleeson, Sloan et al. 1996). MIN colorectal tumors have normal or near normal karyotypes, and MIN does not influence large-scale genomic alterations found in virtually all cancer types (reviewed in (Atkin 2001)).

CIN, defined as a continuous change in chromosome structure and number, is the less well understood of these two processes (Lengauer, Kinzler et al. 1998; Gollin 2004). Aneuploidy, the state of having an altered number of chromosomes, has been found ubiquitously in human cancers for over a century (Hansemann 1890). Moreover, the severity of aneuploidy is typically

correlated with clinical grades of tumor (Cavalli, Danova et al. 1989). In 1914, Boveri postulated that abnormal chromosome numbers are a cause rather than a consequence of the cancerous state (Boveri 1914). However, it still remains unclear whether aneuploidy arises early or late in tumorigenesis, and whether it is a primary cause or simply appears as a general breakdown of cell cycle control or the mechanisms of division. Some experiments argue that CIN is not important for tumorigenesis. Studies in mice have shown that adenomas can develop without changes in karyotypes or obvious genomic instability (Haigis, Caya et al. 2002). However, in other cases, cancer cells often have numerous genetic alterations. For example, there are more than 11,000 genetic alterations human colorectal cancers (Stoler, Chen et al. 1999). For such great many of alteration to occur, an abnormally high rate of chromosome loss and gain is likely. CIN is believed to be responsible for the genome-wide changes. The mutator hypothesis postulates that CIN arises early in tumorigenesis and increases subsequent occurrence of tumor-promoting mutations, genetic lesions and amplifications (Nowell 1976). Cell fusion studies in human colorectal cancer cells also suggest that specific recessive mutations are required for CIN (Lengauer, Kinzler et al. 1997). More recently, CIN has been found in early stages of tumorigenesis (Rabinovitch, Dziadon et al. 1999), and modeling studies have shown that CIN is sufficiently powerful to initiate and drive tumorigenesis (Nowak, Komarova et al. 2002). Therefore, CIN has been proposed to be a key mechanism driving the genomic changes associated with the multi-step tumorigenesis (Saunders, Shuster et al. 2000; Wu and Pandolfi 2001; Pihan, Wallace et al. 2003; Gisselsson, Palsson et al. 2004).

2. Chromosome segregation defects in mitosis

Mitosis is the most dramatic and potentially dangerous step in cell cycle progression, as chromosomes are segregated irreversibly into each of the daughter cells. In order to achieve a successful cell division, the central cell cycle control system, which entails interactions and disassociations of cyclins and cyclin-dependent kinases (CDKs), needs to be able to respond to the environmental stimuli and perturbations of cell cycle progression. One mechanism to achieve this goal is through checkpoint controls, which monitor the cell cycle progression at critical points during the cell cycle and halt the progression if error occurs. There are many checkpoints during the cell cycle (Lukas, Lukas et al. 2004; Nojima 2004; Sancar, Lindsey-Boltz et al. 2004; Stark and Taylor 2004), and one very important checkpoint is the last one in mitosis, the spindle assembly checkpoint that controls anaphase onset. This checkpoint not only monitors the attachment between kinetochores and microtubules and controls anaphase entry, but also plays a role in exit from mitosis (reviewed in (Lew and Burke 2003)). The majority of spindle assembly checkpoint genes are highly conserved in eukaryotes, Mad1, Mad2, BubR1, Bub1, Bub3, Mps1, and Aurora-B. Only two are restricted to metazoans, Rod and Zw10, and Rae1 regulates the checkpoint in mice but not in yeast (Yu 2002; Babu, Jeganathan et al. 2003; Carmena and Earnshaw 2003).

Most types of chromosome missegregation are monitored by DNA damage and spindle assembly checkpoints, and such events are rare in normal cells. Defects in checkpoint controls may lead to failure in the maintenance of fidelity of cell division and allow CIN. Tumor cells with complex genomic changes are often defective in checkpoint controls and invariably have chromosome

segregational defects (Saunders, Shuster et al. 2000; Jallepalli and Lengauer 2001; Gisselsson 2003). However, only a small fraction of CIN cancer cells appear to have mutations in Mad or Bub checkpoint genes (Tighe, Johnson et al. 2001). Temporary inhibition of Bub1 results in CIN and delayed senescence in human cells, but mutant cells are not tumorigenic when reintroduced into mice (Musio, Montagna et al. 2003). This indicates some chromosome segregational defects are able to bypass the spindle assembly checkpoint controls.

The chromosome segregational defects found in various cancer cells include, but are not limited to, lagging chromosomes, anaphase bridges, multipolar spindles and micronuclei. Lagging chromosomes are chromosomes or chromosome fragments that fail to congress to the metaphase plate, or those that lag behind the segregating anaphase chromosomes (Figure 1, left panel). Lagging chromosomes may be caused by a defective kinetochore causing failure to attach to spindle microtubules (Dulout and Olivero 1984), or by merotelic attachment of one kinetochore to both spindles (Cimini, Howell et al. 2001; Cimini, Fioravanti et al. 2002; Cimini, Moree et al. 2003). We have observed that lagging chromosomes can also be the remnants of broken anaphase bridges.

Micronuclei are small fragments of chromatin that remain physically distinct from the main nucleus, but still within the same cytoplasmic compartment (Figure 1, right panel). Micronuclei may originate from lagging chromosomes or broken pieces of chromatin from anaphase bridges (Hoffelder, Luo et al. 2004). They may also come from exclusion of double minutes from prematurely condensed chromosomes or as recombinant products of gene amplification in some

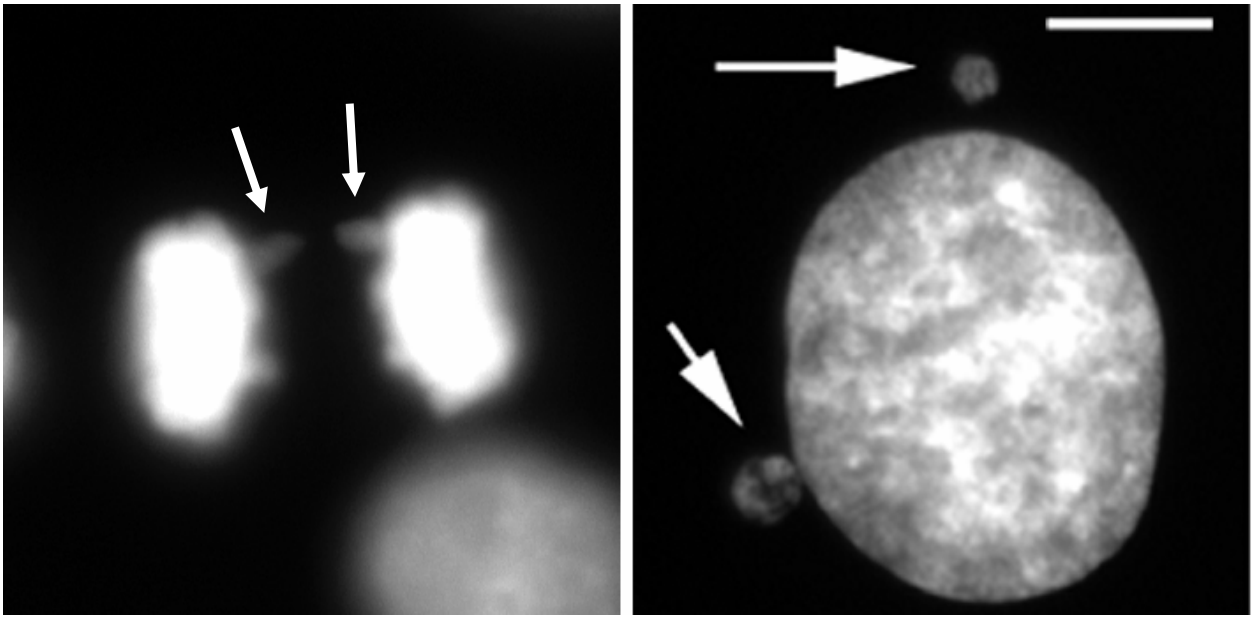


Figure 1

Figure 1. Examples of a cell with lagging chromosomes and one with micronuclei in UPCI:SCC40 cell line. Cells were stained with DAPI for DNA. Arrows indicate lagging chromosomes (left panel) or micronuclei (right panel). Bars are 10μm.

cell lines (Gebhart, Bruderlein et al. 1986; Sen, Hittelman et al. 1989; Shimizu, Shimura et al. 2000).

The most potentially dangerous types of chromosome segregation defects are the ones that may not be recognized by proper checkpoints. Two such examples of missegregation are anaphase bridges and multipolar spindles, which I will address separately in detail.

3. Anaphase bridges

Anaphase bridges are a chromosome segregational defect shown as one or more chromatin bridges between the segregating chromosome masses during anaphase (Figure 2). Anaphase bridges are abundant in cancer cells and have been strongly linked to tumorigenesis in mice (Artandi, Chang et al. 2000). Recently, the presence of bridges has been correlated with CIN in cancer cells (Gisselsson, Pettersson et al. 2000) and tumor tissue (Montgomery, Wilentz et al. 2003).

3.1. Breakage-Fusion-Bridge cycle

Anaphase bridges, first observed in maize by Barbara McClintock (McClintock 1941; McClintock 1942), are thought to follow a classic model referred to as the breakage-fusion-bridge cycle (Gisselsson 2003) (BFB cycle, Figure 3). Initiation of a BFB cycle involves a DNA double-stranded break (DSB), exposing a telomere-free chromosome end. These naked ends are believed to fuse with other broken strands or with the sister chromatid formed after DNA replication. Depending on the cell cycle stage in which this fusion occurs and results in the

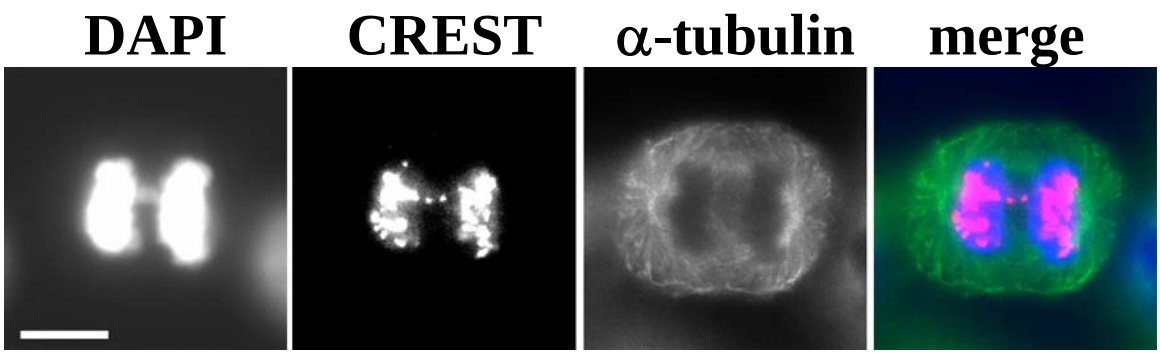


Figure 2

Figure 2. An anaphase cell with a bridge in UPCI:SCC40 cell line. Cells were fixed in cold methanol for 30 min and stained with CREST serum for kinetochores, anti-α-tubulin for microtubule asters, and DAPI for DNA. Bar is 10μm.

formation of a dicentric chromosome, either a "chromatid-type" or a "chromosome-type" bridge will form in anaphase. If DNA replication precedes the fusion of two broken ends of DNA, a dicentric chromosome forms in G2 phase, which will be pulled bi-directionally and eventually break and segregate into each daughter cell. Gene amplification or gene loss may occur if the breakage is asymmetric, and the resulting broken ends may enter this cycling pool again (Saunders, Shuster et al. 2000). This is called a chromatid-type BFB cycle. If DNA replication occurs after end-fusion and dicentric chromosome formation, a double-dicentric chromosome will be produced and may result in either a parallel or a bi-directional segregation in anaphase. A parallel separation of the double-dicentric chromosome will not lead to formation of bridges in anaphase, but the daughter cells will each inherit a dicentric chromosome. A bi-directional segregation forms a double bridge in anaphase, which will break into two telomere-free ends in each daughter cell. These two ends may fuse into a dicentric chromosome and enter the chromosome-type BFB cycle again (McClintock 1941; McClintock 1942).

By entering the BFB cycle, cells may develop CIN by continuous gene loss or gain. Furthermore, there is no evidence that dicentric chromosomes are recognized as abnormal by any cell cycle checkpoints. During metaphase, a dicentric chromosome pulled by both spindle poles is likely to supply the tension needed to prevent activations of the spindle assembly checkpoint. Anaphase bridge formation can thus cause irreversible genomic changes during the process of proliferation.

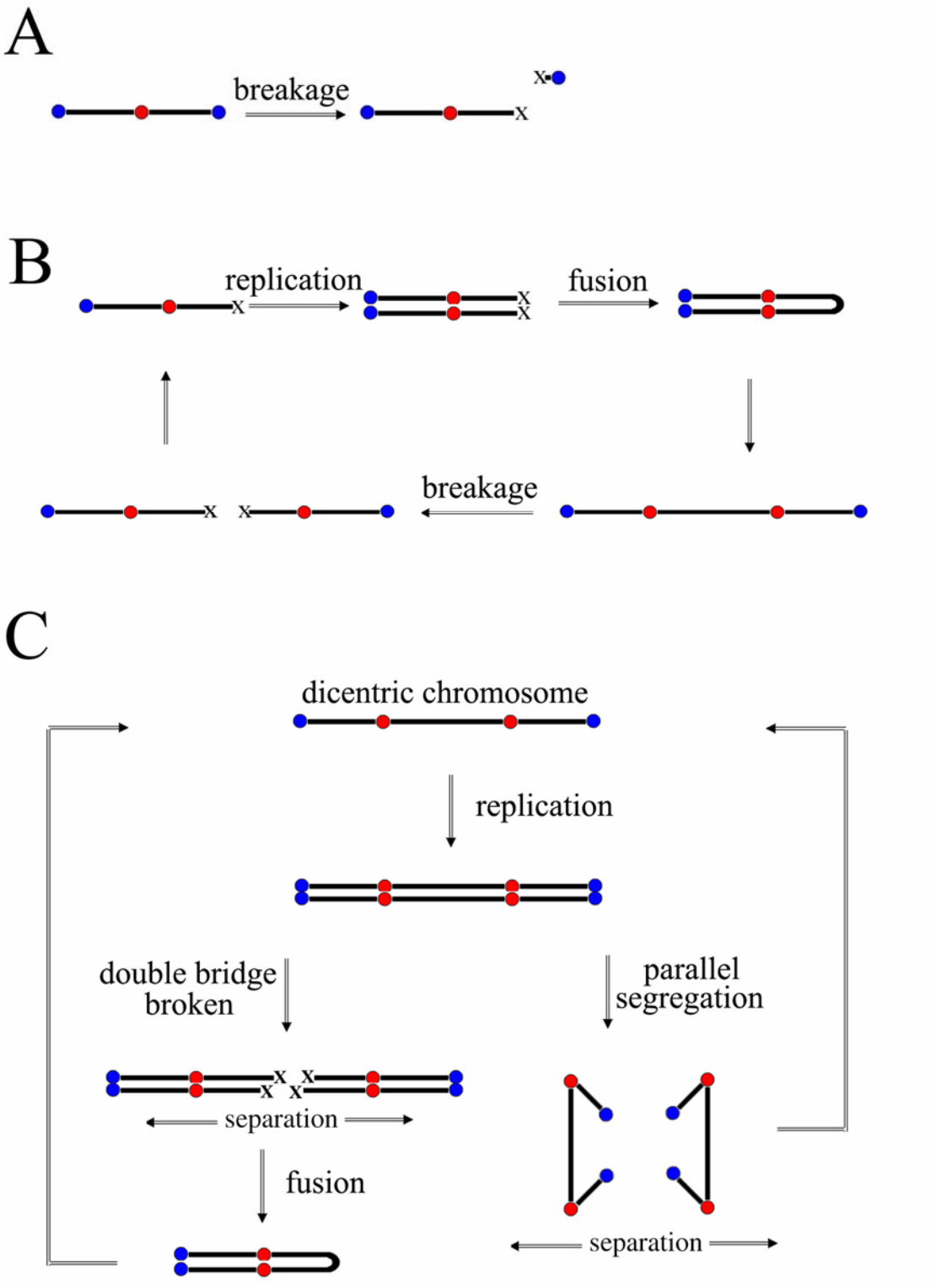

A
breakage
B
replication
fusion
breakage
C
dicentric chromosome
replication
double bridge broken
parallel segregation
separation
fusion
separation

Figure 3

Figure 3. Breakage-Fusion-bridge cycles. (A) DNA DSBs initiate the BFB cycle. (B) Chromatid type BFB cycle. (C) Chromosome type BFB cycle. Centromeres are represented by red dots, telomeres by blue dots. Broken telomere-less ends are indicated by "X".

3.2. DNA DSBs and repair

DSB formation is the most mutagenic DNA damage leading to ongoing genetic instability in the form of micronuclei, translocations and BFB cycles (Ward 1995; Khanna and Jackson 2001). DSBs in cells may either arise endogenously or exogenously. Endogenous processes that produce DSBs include metabolically-derived reactive oxygen species (ROS), DNA replication fork collapse, and meiotic and V(D)J recombination (Klar and Miglio 1986; Haber 1999; Mahadevaiah, Turner et al. 2001; Moshous, Callebaut et al. 2001). Exogenous agents such as ionizing radiation (IR) or chemical agents (e.g., bleomycin) can also induce DSBs (Coquerelle, Weibezahn et al. 1987; Mallya and Sikpi 1999).

When a chromosomal DSB arises, DNA damage sensors are first recruited to the damage site (reviewed in (Thompson and Schild 2002)). Although the sensor proteins that first recognize DSBs are not well understood, the large ATM (ataxia telangiectasia mutated) and ATR (AT and Rad3-related) kinases have been shown to play central roles in detecting DSBs. These kinases regulate more than 20 downstream substrates as DNA damage transducers and effectors, and initiate the subsequent checkpoint activation, DNA repair or apoptosis. Upon activation by inter-molecular autophosphorylation at serine 1981(Bakkenist and Kastan 2003), ATM phosphorylates an early substrate of the DSB repair response pathway, histone H2AX at serine 139 (a modification referred to as γ-H2AX), which within minutes forms nuclear foci over a megabase region of DNA surrounding the DSB (Rogakou, Pilch et al. 1998). Discrete nuclear foci of γ-H2AX are visible by immunofluorescence and appear to be the specific markers of DSBs.

The mechanism behind bridge formation following DNA damage is unclear, but probably involves repair of DNA DSBs. Inhibition of DSB repair is strongly linked to BFB cycles and the formation of dicentric chromosomes (Zhu, Mills et al. 2002). Additionally, chromosomal translocations were observed to result when DSBs are introduced in the cells by exogenous restriction endonucleases or as a result of defects in DSB repair pathways (Bryant 1985; Ferguson, Sekiguchi et al. 2000; Richardson and Jasin 2000). The two major pathways to repair DNA DSBs are referred to as nonhomologous end-joining (NHEJ) and homologous recombination repair (HRR). In mammals, the preferred way to repair DSBs is through the error-prone NHEJ pathway using DNA–PKcs/Ku80 as the key proteins involved in it (reviewed in (Lieber, Ma et al. 2004)). NHEJ repair does not require a homologous template. By comparison, HRR is an error-free mechanism of DSB repair in S and G2 phase, but is not activated in G1 phase at any appreciable frequency (Moynahan and Jasin 1997). HRR needs a template, and its essential components involve Rad51, Rad54 and BRCA1/2 (reviewed in (Wyman, Ristic et al. 2004)). The relationship between formation of anaphase bridges and NHEJ and HRR is currently under investigation (C. Acilan and W. Saunders).

3.3. Telomere fusion and anaphase bridges

Telomeres are specialized nucleoprotein complexes at the ends of eukaryotic chromosomes that protect chromosomes from DNA degradation and end-to-end fusion (reviewed in (Callen and Surralles 2004)). Telomeres consist of a tandemly repeated DNA sequences (TTAGGG in vertebrates) that varies from 5-15kb in humans (Moyzis, Buckingham et al. 1988; de Lange, Shiue et al. 1990), ending in a G-rich 3’ single-strand overhang that folds back to form a “T-

loop" stabilized by several telomeric proteins (van Steensel, Smogorzewska et al. 1998; Griffith, Comeau et al. 1999). Telomeres have been shown to shorten as a function of age *in vitro* and *in vivo* (Harley, Futcher et al. 1990; Lindsey, McGill et al. 1991). When telomeres shorten to the point that they cannot fulfill their normal functions, the resulting genomic instability allows further mutations that promotes cell death by apoptosis (de Lange 2002). Consistent with this, the majority of tumor and immortal cells up-regulate the enzyme telomerase that extends and stabilizes telomeric ends (Vonderheide 2002).

If the initial DNA DSBs are in the telomere region resulting in inactive telomeres, a dicentric chromosome may form from telomere fusion. Many tumors exhibit shortened telomeres, which may trigger telomeric fusions between chromosome arms (Hastie, Dempster et al. 1990; Counter, Hirte et al. 1994). It is known that anaphase bridges can arise from telomeric fusions in cancer cells that have shortened telomere sequences (Gisselsson, Jonson et al. 2001), telomere loss (Fouladi, Sabatier et al. 2000; Lo, Sabatier et al. 2002) or telomerase deficiency (Artandi, Chang et al. 2000; Rudolph, Millard et al. 2001). Telomere dysfunction promotes CIN, including complex non-reciprocal translocation, regional amplification and deletions in p53-mutant mice (Artandi, Chang et al. 2000; O'Hagan, Chang et al. 2002) and an increase in initiated lesions in human colon carcinomas (Rudolph, Millard et al. 2001) that drive carcinogenesis. Bridges can also be induced in cells with normal telomeres by DNA damaging agents such as X-irradiation (Bryant 1984).

4. Multipolar spindles

Multipolar spindles (MPS) are aberrant mitotic structures observed when cells divide with greater than two spindle poles (Figure 4). MPS are found in various carcinomas and tumors, and are typically correlated with supernumerary centrosomes (Lingle and Salisbury 1999; Saunders, Shuster et al. 2000; Gisselsson, Palsson et al. 2004).

4.1. Mitotic spindle assembly

For a cell to divide correctly into two identical daughter cells, not only should genomic DNA replicate once and only once, but also chromosomes should segregate evenly into two sets. The mitotic spindle is the machinery to align the chromosomes at the metaphase plate in a bipolar fashion, confirm the attachment of kinetochores of each chromosome, and pull them into each daughter cell. The mitotic spindle is a bipolar apparatus composed primarily of microtubules organized around the microtubule organizing center (MTOC). In metazoans this MTOC is called the centrosome and is crucial to a series of important cellular processes, as will be addressed later. There are three types of microtubules in a fully formed spindle: polar microtubules, kinetochore microtubules and astral microtubules. Polar microtubules extend toward the middle of the spindle, overlap with polar microtubules from the opposite pole, and serve as tracks for motor proteins to generate outwardly-directed forces to push the two half spindles apart. Polar microtubules may become kinetochore microtubules if their plus ends attach to the centromere regions of chromosomes. Kinetochore microtubules are responsible for coupling microtubule dynamics with chromosome movement and pulling chromosomes toward each pole. The rest of

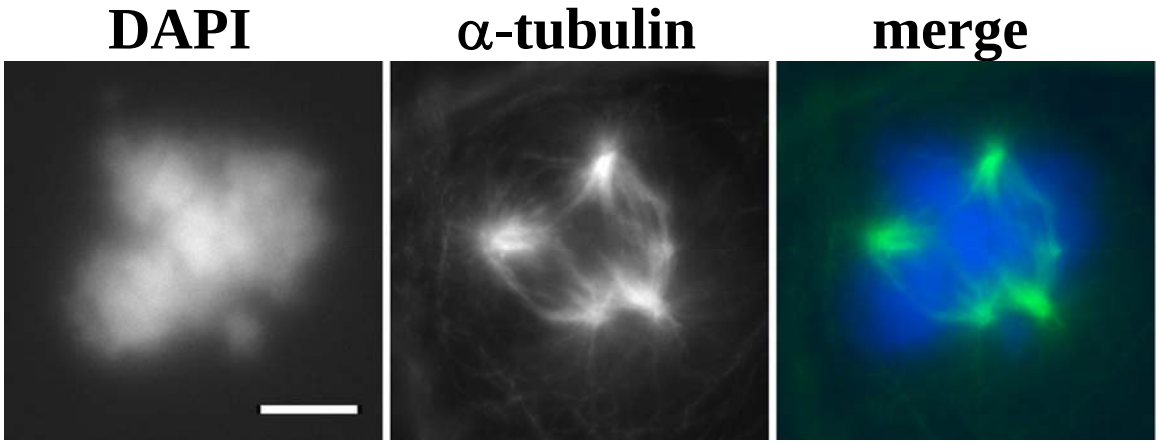


Figure 4

Figure 4. A mitotic cell with a multipolar spindle in UPCI:SCC103 cell line. Cells were fixed in cold methanol and stained with antibody to α-tubulin (green) for microtubule asters and DAPI (blue) for DNA. Images are courtesy of Dr. Nick Quintyne. Bar is 10μm.

microtubules are astral microtubules that may extend and interact with the cell cortex and associated proteins, which generate a pulling force, contributing to spindle positioning and elongation.

4.2. Centrosome functions and structure

Centrosomes were first identified by Boveri and van Beneden over a hundred years ago as a structure from which fibers extended from the center of cells. This structure was seen to duplicate prior to mitosis and become the mitotic spindle poles. The morphology of the centrosome is broadly defined as a central pair of microtubule barrels called centrioles, surrounded by a large variety of protein complexes called pericentriolar material (PCM). Centrioles are cylindrical structures that are made up of nine triplet microtubules, but they are unequal in that only one (maternal) carries appendages close to its distal end (Figure 5). Not all PCM components have been identified, but prominent among them are γ-tubulin ring complexes, which act as a template for microtubule nucleation (Zheng, Wong et al. 1995). There are also several structural components, protein kinases, phosphatases, components of the ubiquitin-dependent proteolytic machinery and microtubule-dependent motors associated permanently or transiently with centrosomes (reviewed in (Nigg 2002)).

It is known that microtubules can polymerize *in vitro* without other factors (Wiche and Cole 1976). Also plants do not possess centrosome-like organelles and build their spindles from the cortical arrays associated with γ-tubulin small complex (Schmit 2002). However, in most other eukaryotes, microtubule dynamics appears to be regulated and controlled at the centrosome *in vivo*. The major centrosome functions include microtubule nucleation, mitotic spindle formation

and microtubule organization and microtubule anchoring (Quintyne, Gill et al. 1999; Bornens 2002). Centrosomes are not essential in spindle formation, as bipolar spindles can also assemble in *Xenopus* extract and mammalian cells lacking centrosomes (Heald, Tournebize et al. 1996; Khodjakov, Cole et al. 2000). It is unclear if cells always have a centrosome-independent pathway to assemble the bipolar spindle merely based on the interaction between microtubule-based motors and chromatin, or it is a cellular response to loss of centrosomes. Regardless, the spindle organization is considerably facilitated by the presence of centrosomes. Centrosomes also control the release of central spindles from the midbody and the completion of cytokinesis in animal cells (Khodjakov and Rieder 2001; Piel, Nordberg et al. 2001). Somatic cells with microsurgically removed centrosomes fail to regenerate centrioles and enter S-phase (Hinchcliffe, Miller et al. 2001). In addition to its role in cytokinesis and S phase entry, the centrosome is also involved in cell motility (Bornens 2002), polarity and shape (Niu, Mills et al. 1997). In *S. cerevisiae*, the functional equivalent of centrosomes, the spindle pole bodies, play a key role in regulating mitotic exit (reviewed in (Pereira and Schiebel 2001)). Thus, centrosomes appear to be a central component of the cell regulatory machinery (Doxsey 2001; Hinchcliffe and Sluder 2001; Rieder, Faruki et al. 2001; Stearns 2001; Lange 2002).

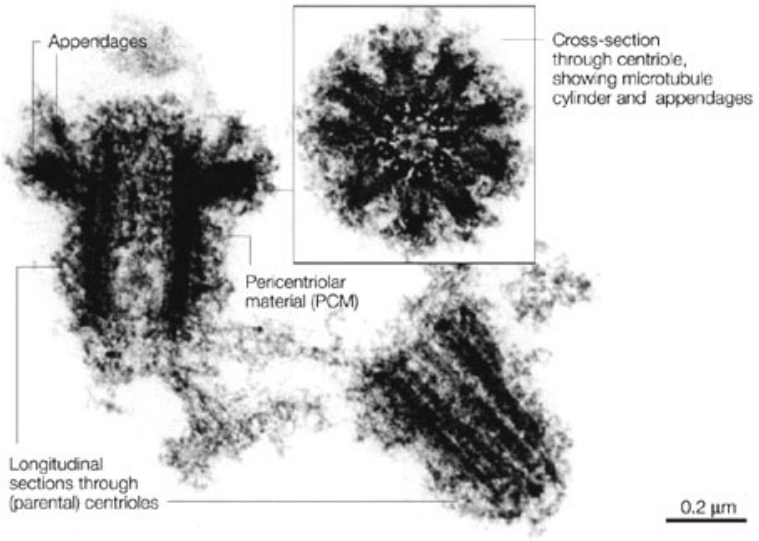


Figure 5

Figure 5. Centrosome structure. Figure is from (Nigg 2002) .

4.3. Centrosome replication

For chromosomes to segregate accurately, each daughter cell must receive only one centrosome, and this centrosome must only replicate once per cell cycle. Centrosome replication, a semi-conservative process, usually begins near the G1/S boundary of the cell cycle and is completed by G2 (Robbins, Jentzsch et al. 1968; Brinkley, Cox et al. 1981; Ring, Hubble et al. 1982). The exact timing may vary in different cell types. Replication begins by the separation of the centriolar pair and accumulation of PCM and centrosome-associated proteins such as γ-tubulin and pericentrin (Dictenberg, Zimmerman et al. 1998). Mother and daughter centrioles are asymmetric as both centrioles nucleate microtubules while only mother centrioles anchor them (Piel, Meyer et al. 2000). Centriole separation begins by G1/S phase of the cell cycle, and each centriole duplicates by growth of a procentriole perpendicular to the parent in S phase. Centriole duplication requires hyperphosphorylation of the retinoblastoma (RB) protein and the activation of Cdk2, which are also key events of DNA replication (Hinchcliffe, Li et al. 1999; Lacey, Jackson et al. 1999; Meraldi, Lukas et al. 1999; Matsumoto and Maller 2004). The predominant binding partner of Cdk2 in *Xenopus* is cyclin E, in mammalian cells it appears to be cyclin A (Hinchcliffe, Li et al. 1999; Meraldi, Lukas et al. 1999). The downstream targets of Cdk2 kinase include the centrosome kinase Mps1 and the putative chaperone nucleophosmin/B23 (Okuda, Horn et al. 2000; Fisk and Winey 2001). Mps1 has been shown to be involved in spindle assembly checkpoint and centrosome duplication in yeast, mice and humans (Fisk and Winey 2001; Winey and O'Toole 2001; Fisk, Mattison et al. 2003). Phosphorylation and disassociation of nucleophosmin from centrosome are also required for centrosome duplication (Okuda, Horn et al. 2000). Centriole duplication is also controlled by other kinases of Aurora/Ipl1, Polo-like and Nek/NIMA families (Fry, Meraldi et al. 1998; Hinchcliffe, Li et al. 1999; Lacey, Jackson et al.

1999; Mayor, Meraldi et al. 1999; Mussman, Horn et al. 2000; Meraldi and Nigg 2001). Aurora kinases are often found amplified in human cancer cells (Bischoff, Anderson et al. 1998). Work in *C. elegans* and *D. melanogaster* (Hannak, Kirkham et al. 2001; Berdnik and Knoblich 2002) shows that knockdown of Aurora A reduces γ-tubulin accumulation required for centrosome maturation and prevents centriolar separation during replication. In addition, calcium, calmodulin and the calmodulin-dependent protein kinase II are essential in initiation of centrosomal replication in *Xenopous* and SPB duplication in *S. cerevisiae* (Matsumoto and Maller 2002). In yeast, mitotic cyclins also function to both promote replication and inhibit re-replication of the spindle pole body (Haase, Winey et al. 2001). In *C. elegans*, the Zyg-1 kinase is required for pro-centriole formation (O'Connell, Caron et al. 2001). Many of these kinases can be overexpressed or hyperactive in tumor tissues and are associated with centrosome over-replication (Bischoff, Anderson et al. 1998; Lingle, Lutz et al. 1998).

Shortly before mitosis, centrosome maturation occurs, recruiting a series of proteins to the centrosomes, particularly γ-tubulin ring complexes. This process is controlled by centrosome associated kinases Aurora A, Plk1, Nek2 and PP4 (reviewed in (Palazzo, Vogel et al. 2000)). The complete maturation process for a centriole takes 1.5 cell cycles.

Centrosome separation occurs at the G2/M transition, which includes disrupting cohesion between the two parental centrioles and separating centrosomes through the action of microtubule-dependent motor proteins. Parental centrioles are tethered by the coiled-coil protein C-Nap1 (also known as Cep250) (Mayor, Stierhof et al. 2000; Meraldi and Nigg 2001). When C-Nap1 is phosphorylated by Nek2 kinase, the cohesion between two centrosomes is lost, and the

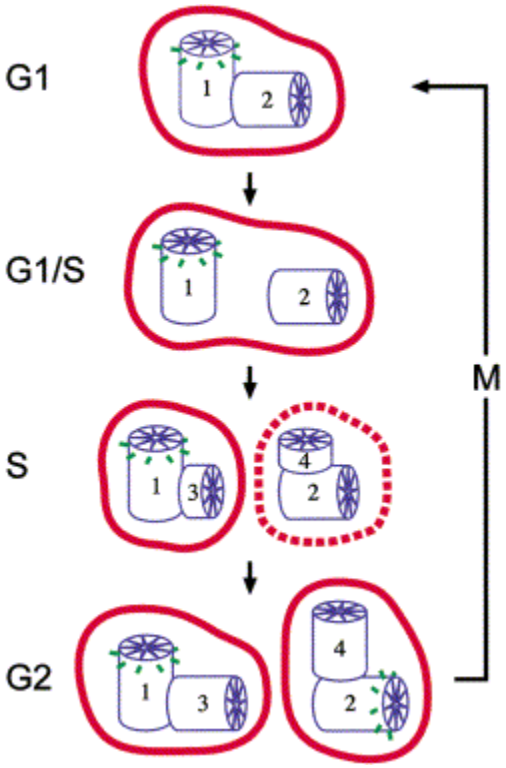


Figure 6

Figure 6. Centrosome duplication. Centrioles are indicated as blue cylinders, surrounded by PCM (red). Distal appendages are shown in green on centrioles 1 and 2 (G2/M only). Centriole 1 is the mother centriole (which has at least undergone two cell cycles), centriole 2 is the daughter created in the prior S phase and matures in G2/M. Centriole 3 and 4 are newly-born immature daughter centrioles. Dotted line indicates the recently duplicated centrosome. Figure is from (Stearns 2001)

centrosomes are available for separation through microtubule-dependent motors (Blangy, Lane et al. 1995).

Besides phosphorylation, ubiquitin-dependent proteolysis also plays a role in regulating the centrosome cycle. Inhibitors of protein degradation block centrosome duplication in *Xenopus* (Freed, Lacey et al. 1999), and mutations in SCF (Skp1-cullin-F-box) ubiqitin ligase *Slimb* in *Drosophila* cause centrosome amplification (Robinson, Wojcik et al. 1999). Furthermore, certain components of the SCF complex are localized to centrosomes (Freed, Lacey et al. 1999). Recently, studies in breast cancer cells showed that γ-tubulin is ubiquitinated by BRCA1/BARD1, and transient inhibition of BRCA1 results in centrosome amplification and fragmentation (Starita, Machida et al. 2004). The role of proteolysis in centrosome cycle regulation in still unclear, but it has been suggested to be required to degrade certain proteins to allow centrosome disorientation required before centriole duplication (Meraldi and Nigg 2002).

4.4. Centrosome amplification

Centrosomal amplification is a common source of divisional errors in cancer cells and has been suggested to play a role in tumor formation first by Boveri over a century ago (Boveri 1901). Indeed, supernumerary centrosomes have been reported in a variety of carcinomas including breast, gall bladder, lung, bone, pancreas, colorectal and prostate (Lingle, Lutz et al. 1998; Carroll, Okuda et al. 1999; Kuo, Sato et al. 2000; Pihan, Purohit et al. 2001). Centrosomal amplification is also seen as part of many cancer-related mutant phenotypes. For example, loss of the tumor suppressors p53 and Rb (Tarapore and Fukasawa 2002), defects in many genes involved in DNA repair pathways including BRCA1, BRCA2, ATM, and ATR (Smith, Liu et al.

1998; Tutt, Gabriel et al. 1999), or expression of human papillomavirus or adenovirus oncoproteins (Duensing and Munger 2002; Schaeffer, Nguyen et al. 2004), all lead to an abnormally high number of centrosomes in the cell. Thus, centrosomal amplification is part of many oncogenic pathways. The main impact of supernumerary centrosomes appears to be to increase the chances that the microtubule spindle formed in the subsequent mitosis will be multipolar and that the chromosomes will be unequally distributed to multiple daughter cells (Brinkley 2001; Nigg 2002; Sluder and Nordberg 2004). Consistent with this conclusion, centrosomal changes, including amplification, size and hyperactivity, are strongly linked to aneuploidy and chromosomal instability in numerous studies (Lingle, Lutz et al. 1998; Ghadimi, Sackett et al. 2000; Lingle, Barrett et al. 2002; Pihan, Wallace et al. 2003).

There are currently three major models to explain how centrosomes become amplified (Fukasawa 2002; Nigg 2002; Sluder and Nordberg 2004) as shown in Figure 7. In the first model, centrosomes over-replicate, independently of DNA replication. Centrosome amplification can occur without additional DNA replication when Rad51 deficient cells enter prolonged G2/S phase after γ-irradiation, and this process involves ATM (Dodson, Bourke et al. 2004). Cell lines lacking normal cell cycle checkpoints continue to duplicate their centrosomes when treated with the DNA synthesis inhibitor hydroxyurea, even though the cell cycle is blocked (D'Assoro, Busby et al. 2004). Inactivation of the transcription factor E2F3 results in upregulation of cyclin E kinase activity, defects in nucleophosmin B association with centrosomes, and premature centriole separation and duplication (Saavedra, Maiti et al. 2003). Similarly, overexpression of the cdk2/cyclin E kinase in a p53 -/- background, or the human papillomavirus oncoprotein E7 (which elevates cyclin E levels), the cdk2 substrate hMps1, or the polo-like kinase-2, all induce

additional rounds of centrosomal duplication (Mussman, Horn et al. 2000; Duensing and Munger 2002; Fisk, Mattison et al. 2003; Kawamura, Izumi et al. 2004; Warnke, Kemmler et al. 2004). The second model predicts that centrosomal amplification results from a failure of cytokinesis. If the contractile ring fails to partition the duplicated centrosomes, a single cell can inherit more than one, even though centrosomal replication is normal. A failure of cytokinesis has been reported previously in enucleated sea urchin eggs with amplified centrosomes (Sluder, Miller et al. 1986) and anecdotally in p53 -/- mouse embryonic fibroblasts with multipolar spindles (Sluder and Nordberg 2004). It is also known that inhibiting cytokinesis, either chemically or via overexpression of Aurora A, leads to both multinucleated cells and MPS in the absence of centrosome replication defects (Meraldi, Honda et al. 2002; Sluder and Nordberg 2004; Uetake and Sluder 2004). Furthermore, binucleated cells in sea urchin zygotes, and PtK1 cells, form MPS as shown by live cell microscopy (Sluder, Thompson et al. 1997). Similarly, expression of the human papilloma virus E6 oncoprotein leads to both centrosomal amplification and multinucleation, consistent with a cytokinesis defect causing the increase in centrosome number (Duensing and Munger 2002). A third scenario is that centrosome amplification may occur by cell fusion. Fusion-induced centrosome amplification has been observed following exposure to by X-rays or UV treatment (Kura, Sasaki et al. 1978; Brathen, Banrud et al. 2000), or ectopic expression of the RAD6 ubiquitin-conjugating enzyme in human breast epithelial cells (Shekhar, Lyakhovich et al. 2002). In the latter two models, the resulting G1 cell will contain not only twice the centrosome numbers, but also twice the genetic information of such a cell. While centrosomal amplification can be induced experimentally by any of these three mechanisms, it is currently unknown which pathway is most relevant for MPS formation in cancer cells.

The fate of such cells with centrosome amplification, whether they will arrest in G1 phase, apoptose or replicate their DNA and enter the subsequent mitosis, is suggested to be dependent upon the activation of a functional G1/S checkpoint pathway, namely the p53 checkpoint pathway. However, there have been controversies as to whether the p53 checkpoint pathway is triggered following polyploidy (for review see (Stukenberg 2004)). Work in primary rat embryo fibroblast cells showed that cytochalasin-treated (actin inhibitor) cells arrested indefinitely with 4n DNA, but could overcome this arrest when transformed with the simian virus-40 large T-antigen to inhibit p53, later resulting in severe aneuploidy (Andreassen, Lohez et al. 2001). Loss of p53 activity is thus suggested to allow cells with polyploidy to proliferate (Minn, Boise et al. 1996; Khan and Wahl 1998; Casenghi, Mangiacasale et al. 1999; Andreassen, Lohez et al. 2001). Human colorectal cancer cell lines with p53 mutations also have an increased tendency towards polyploidy (Bunz, Fauth et al. 2002). Recently however, Uetake and Sluder found that transient treatment with very low concentrations of cytochalasin D can block cytokinesis and generate binucleated cells, but they did not arrest in G1 (Uetake and Sluder 2004). The same cells arrested at higher concentrations of cytochalasin treatment. Similarly, Wong and Stern fused human foreskin fibroblasts and showed the resulting binucleated hybridomas entered S-phase without a prolonged arrest (Stukenberg 2004). These findings argue strongly that p53-dependent arrest is not triggered by multinucleation, polyploidy, supernumerary centrosomes or failure of cytokinesis.

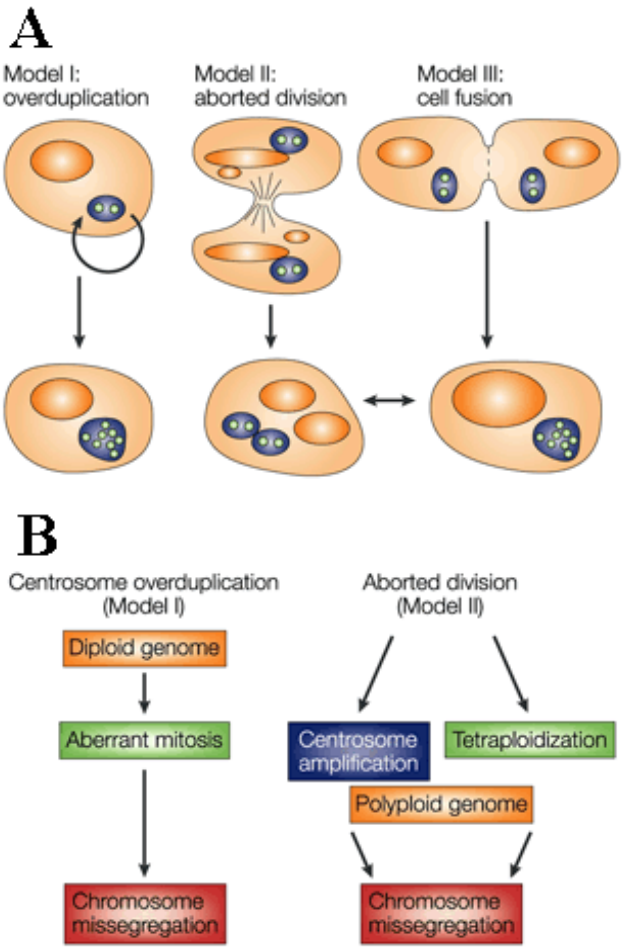


Figure 7

Figure 7. Models for centrosome amplification. (A). Three models for centrosome amplification. Centrosomes are shown as clustered centrioles with other centrosomal components. (B). Centrosome amplification results in chromosome missegregation. Figure adapted from (Nigg 2002).

Regardless of the mechanisms, cells with amplified centrosomes, when entering mitosis, most likely will form multipolar spindles and missegregate their chromosomes. However, cells with a polyploid genome have the likelihood of generating more viable and potentially more harmful progeny than those with a diploid genome.

4.5. MPS formation

The main impact of centrosome amplification in cancer cells is most likely MPS formation. Multipolar spindles and supernumerary centrosomes are usually found concomitantly in tumors (Pihan, Purohit et al. 2001; Gisselsson, Palsson et al. 2004), suggesting MPS are intimately linked to centrosome amplification. However, cells with amplified centrosomes do not always lead to formation of multipolar spindles. A classic example of one such cell line is N1E-115 cells (Ring, Hubble et al. 1982) in which extra centrosomes are clustered into two spindle poles and segregate the chromosomes in a bipolar fashion. We have observed the similar clustering effect in UPCI:SCC114 cells (N. Quintyne and W. Saunders), an oral squamous cell carcinoma cell line. In addition, BSC-1 cells with extra centrosomes induced by cytochalasin B still had a bipolar division in 30-50% of cases (Sluder and Nordberg 2004). Also, a normal fibroblast cell line did not form MPS by inhibiting cytokinesis using microtubule poison colcemid (Gisselsson, Palsson et al. 2004), suggesting a mechanism behind the bipolar spindle formation in the presence of extra centrosomes. In 2001, Brinkley (Brinkley 2001) suggested a centrosome coalescence mechanism that couples more than one centrosome together and prevents the formation of MPS. More recently, Quintyne and Saunders proposed a two-step model of multipolar spindle formation. In this model, centrosome amplification is the first step to supply multiple potential MTOCs. The second step of forming more than two spindle poles involves

inhibition of dynein, a minus-end directed microtubule motor on the spindle. Quintyne et al. discovered the lack of dynein immunostaining on multipolar spindle poles in various cancer cell lines, and when dynein was restored at the spindle, cells coalesced their extra centrosomes and divided with a bipolar spindle. The involvement of dynein in centrosome coalescence suggests that this process may be an active mechanism triggered by the presence of supernumerary centrosomes. However, the exact nature of centrosome coalescence remains to be explored.

As I discussed above, centrosome amplification is not sufficient to form MPS. On the other hand, MPS can also form in the absence of centrosome amplification. Centrosome splitting and MPS can be induced by overexpression of Nek-2 kinase (Fry, Meraldi et al. 1998) or DNA damaging conditions, for example ionizing radiation (C. Acilan and W. Saunders, unpublished observation). Treatment with tumor promoter 12-0-tetradecanoylphorbol-13-acetate (Schliwa, Pryzwansky et al. 1983), or 2,4-dinitrophenol (DNP) in sea urchin eggs (Kojima and Czihak 1990), or cold treatment in Drosophila embryos (Callaini and Marchini 1989) also results in centrosome splitting and MPS formation. However, fragmented centrosomes are rare in various cultured cancer cells (N. Quintyne and W. Saunders, unpublished observation). Whether centrosome splitting accounts for MPS formation in cancer generally is yet to be explored.

Cells dividing with MPS apparently lose the fidelity of chromosome segregation. Moreover, a careful study of the timing of anaphase onset in cells with MPS showed that cells have no checkpoint control for monitoring more than a bipolar spindle (Sluder, Thompson et al. 1997). The slight delay in multipolar divisions was caused by the spindle assembly checkpoint that

controls kinetochore attachment of lagging chromosomes. Thus MPS formation is another hazardous route that may lead to aneuploidy.

4.6. Cytokinesis

Cytokinesis is the process that divides one cell into two. It occurs at the end of mitosis after the chromosomes have been segregated into two separated nuclei. Although conceptually simple, cytokinesis is mediated by a complex and dynamic interplay between the microtubules of the mitotic spindle, the actomyosin cytoskeleton and membrane fusion events. Much of the mechanisms required to sever this intercellular connection between post-mitotic cells remains a mystery, as reviewed recently (Glotzer 2001; Guertin, Trautmann et al. 2002; Glotzer 2003). The onset of cytokinesis is usually marked by the ingression of cleavage furrow, typically a few minutes after anaphase. The newly divided cells may remain physically connected for a significant amount of time, up to 4 hours in cultured mammalian cells (Sanger, Pochapin et al. 1985). The cleavage furrow is created by an actomyosin-based structure called the contractile ring that assembles under the plasma membrane. During anaphase, when chromosomes move polewards on kinetochore microtubules, non-kinetochore microtubules become bundled in an antiparallel configuration and the assembly of the central spindle occurs (Mishima, Pavicic et al. 2004). The central spindle has an essential role in completion of cytokinesis, and in some cells the central spindle is also critical for furrow ingression. The furrow ingresses until it comes into contact with and compresses the central spindle, and this process is accompanied by the fusion of membrane vesicles. This new membrane may be required to accommodate the surface area increase necessary for the plasma membrane to surround the two daughter cells. At a late stage of cytokinesis, a narrow cytoplasmic bridge called the midbody connects the two daughter cells

(Breckler and Burnside 1994). The central spindle is a prominent component of the midbody. This bridge is ultimately resolved by a process called abscission, and the two cells separate, completing cytokinesis.

5. Correlation between anaphase bridges and MPS

Anaphase bridges and multipolar spindles are correlated in several ways. These two chromosome segregation defects usually co-exist in tumor cell lines, though in rare cases each can be present without another (Gisselsson, Palsson et al. 2004). In head and neck as well as in bone and soft tissue tumors that have both defects, immunofluorescence studies exhibited a positive correlation in between the occurrence of anaphase bridges and MPS (Gisselsson, Jonson et al. 2002; Gisselsson, Palsson et al. 2004). Cancer cells with MPS often enter anaphase with chromatin bridges between each set of chromosomes (Figure 8), showing that the two defects are not exclusive of each other. Ionizing radiation, which causes DNA damage and centrosome overduplication, induces both anaphase bridges and MPS (Sato, Mizumoto et al. 2000; Gisselsson, Bjork et al. 2001). X-ray exposure induces both defects, with the frequency of bridges 4 times that of MPS (Scott and Zampetti-Bosseler 1980). Loss of the tumor suppressor p53 induces centrosome amplification (Carroll, Okuda et al. 1999; Tarapore and Fukasawa 2002) and promotes anaphase bridges in a telomerase-deficient or a NHEJ-deficient background (Artandi, Chang et al. 2000; Zhu, Mills et al. 2002). Expression of human papillomavirus oncoproteins E6, which degrades p53, and E7 which inhibits Rb (Duensing and Munger 2002; Schaeffer, Nguyen et al. 2004), also lead to both missegregations in the cell. In addition, both anaphase bridges and MPS are influenced by DNA repair pathways. For example, overexpression of DNA damage sensor ATM or ATR induces centrosome amplification (Smith,

Liu et al. 1998), while deficiency in ATM enhances anaphase bridge formation in telomere-null mice (Qi, Strong et al. 2003).

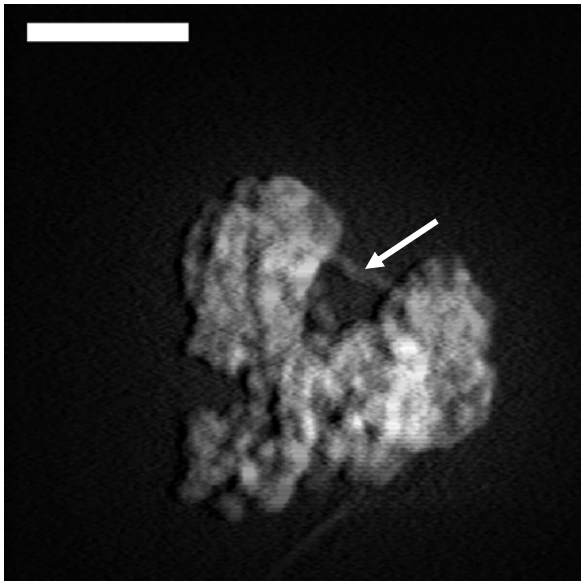


Figure 8

Figure 8. A multipolar anaphase cell in UPCI:SCC103 cell line. Cells were labeled with GFP-histone H2B and image was a 3D reconstruction of a Z-series at various focal planes of the mitotic cell dividing in a tetrapolar fashion. Arrow points to the chromatin bridge in between chromosome sets. Bar is 10μm.

Chapter II : Cigarette smoke exposure induces anaphase bridges and genomic imbalances in primary cells

1. Introduction

1.1. Cigarette smoke and cancer

Cancer is associated with numerous environmental risk factors and one of the most common is exposure to tobacco. Cigarette smoking has been correlated with cancer of the oral cavity, larynx, lung, bladder and esophagus in many epidemiological studies (Wynder and Hoffmann 1976; Kanda, Sullivan et al. 1998; Giovino 2002) as well as coronary artery disease (Czernin and Waldherr 2003). Tobacco exposure is the most important risk factor in the occurrence of oral squamous cell carcinomas (Moreno-Lopez, Esparza-Gomez et al. 2000), and approximately 95% of cases of oral and pharyngeal cancer in the USA have been attributed to smoking (Reichart 2001). The mutagenic ability of cigarette smoke has been well documented (Vineis and Caporaso 1995; Pfeifer, Denissenko et al. 2002), but the influence of smoke exposure on CIN has not yet been shown.

Work in onion root-tips first showed that CSC induces chromosome aberrations including lagging chromosomes and acentric fragments in 1959 (Venema 1959). Since then, work in humans and mice has revealed that cigarette smoke exposure causes other multiple alterations to cells and tissues, including the formation of DNA adducts, single strand breaks in the DNA, sister chromosome exchanges, formation of micronuclei and ring chromosomes, and cell cycle arrest (Hopkin and Evans 1979; Nakayama, Kaneko et al. 1985; Bender, Preston et al. 1988;

Hsu, Cherry et al. 1991; Gu, Whong et al. 1992; Leanderson and Tagesson 1992; Lohani, Dopp et al. 2002; De Flora, Balansky et al. 2003). Cigarette smoke induces cell transformation in organ cultures and cell cultures (Lasnitzki 1958; Inui and Takayama 1971; Inui and Takayama 1971), and the transformed cells can cause tumors in hamsters and mice (Inui and Takayama 1971; Inui and Takayama 1971). It is known that cigarette smoke causes cytogenetic alterations in rat cells, including chromosomal deletions (Rithidech, Chen et al. 1989). CSC also induces mitotic gene conversion, reverse mutation and reciprocal mitotic recombination in yeast (Gairola 1982). However, cigarette smoke exposure has not yet been shown to directly cause DNA DSBs, which are thought to be the most mutagenic type of DNA damage (Ward 1995).

Cigarette smoke is composed of more than 5,000 organic compounds in aqueous and gaseous phases, including many tumorigenic or carcinogenic chemicals such as polycyclic aromatic hydrocarbons, polyphenols and tobacco-specific nitrosamines (Guerin, Jenkins et al. 1992). Major DNA damaging reagents in cigarette smoke and their main pathways are summarized in Table 1.

1.2. reactive oxygen species (ROS)

Many compounds in cigarette smoke have oxidant capacities that produce stable reactive oxygen species (ROS) (Pryor and Stone 1993). Gas phase cigarette smoke contains extremely unstable oxidants such as nitrogen oxide and is thought to only cause damage in the buccal region and upper respiratory tract (Eiserich, Vossen et al. 1994). Cigarette smoke is thought to cause DNA damage through formation of ROS such as superoxide (O2•), hydroxyl radicals, (OH•), and singlet oxygen (^{1}O2) (Nakayama, Kaneko et al. 1985; Kodama, Kaneko et al. 1997; Pryor 1997).

Table 1 Representative DNA damaging reagents in cigarette smoke

families	feature chemicals	descriptions	references
polycyclic aromatic hydrocarbons (PAH)	benzo[a]pyrene (BaP)	forms DNA adducts with deoxyguanosine, SSBs	(Autrup, Seremet et al. 1985; Cosma, Jamasbi et al. 1988)
N-nitrosamines	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	causes α-hydroxylation, DNA methylation and pyridyloxobutylation, SSBs, DSBs	(Weitberg and Corvese 1993; Hecht 1999)
polyphenols	hydroquinone	SSBs	(Walles 1992)
miscellaneous organic compounds	1,3-Butadiene (BD)	forms N7-guanine adducts	(Blair, Oe et al. 2000)
inorganic compounds	Nickel, Chromium	catalysts for Fenton reaction to cause SSBs	(Hassoun and Stohs 1995; Lloyd and Phillips 1999)

One report indicates that $5x10^4$ hydroxyl radicals are generated with each inhalation from a cigarette (Kiyosawa, Suko et al. 1990). The aqueous extract of cigarette smoke contains a quinone-hydroquinone-semiquinone system that can reduce oxygen to produce superoxide and hence hydrogen peroxide and hydroxyl radicals (Schumacher, Green et al. 1977; Nakayama, Kodama et al. 1984; Nakayama, Kaneko et al. 1985; Pryor 1997), via the Fenton reaction: H_2O_2+M(n-1)-->HO•+HO-+M(n)(Avunduk, Yardimci et al. 1997; Kodama, Kaneko et al. 1997). "M" indicates a requirement for metal ions, usually iron or copper, both abundant in cellular fluids. These cigarette-tar derived radicals can penetrate viable cells and bind to DNA, causing nicks (Nakayama, Kaneko et al. 1985).

ROS can cause DNA damage via indirect or direct mechanisms (Pryor and Stone 1993; Pryor 1997). Indirect damage includes altering the activity of key enzymes, such as those involved in DNA synthesis or repair. Direct DNA damage includes DNA strand breaks, creation of abasic sites, and base adduct formation, such as 8-hydroxy-2-deoxyguanosine (Joenje 1989). Since oxidants play such a pronounced role in smoke-induced damage, it is not surprising that antioxidant micronutrients provide protection to smokers against smoking-related diseases. *In vivo* studies have shown that vitamin C prevents cigarette smoke-induced oxidative damage (Panda, Chattopadhyay et al. 2000). Vitamin E and β-carotene can also provide significant protection against DNA SSBs induced by tobacco-specific nitrosamines in cultured human lung cells (Weitberg and Corvese 1997). Other ROS scavengers such as catalase, thiol N-acetyl-L-cysteine (NAC), dimethylthiourea, and o-phenantroline have been shown to prevent the CSC-induced SSBs and oxidative DNA adducts in human and rat cell cultures (Nakayama, Kaneko et al. 1985; Izzotti, Balansky et al. 1992; Leanderson and Tagesson 1992).

1.3. Anaphase bridges

Anaphase bridges are a chromosome segregation defect which are abundant in cancer cells and have been strongly linked to tumorigenesis in mice (Artandi, Chang et al. 2000). Recently, the presence of bridges has been correlated with CIN in cancer cells (Gisselsson, Pettersson et al. 2000) and tumor tissue (Montgomery, Wilentz et al. 2003). Anaphase bridges, first observed in maize by Barbara McClintock (McClintock 1941; McClintock 1942), are thought to undergo the repeated occurrence of BFB cycle (Gisselsson 2003). It is known that anaphase bridges can arise from both telomeric fusions (Artandi, Chang et al. 2000; Fouladi, Sabatier et al. 2000; Gisselsson, Jonson et al. 2001; Rudolph, Millard et al. 2001; Lo, Sabatier et al. 2002; O'Hagan, Chang et al. 2002) and in cells with normal telomeres by DNA damaging agents (Bryant 1984). The mechanism behind bridge formation following DNA damage is unclear, but probably involves repair of DNA DSBs, as inhibition of DSB repair is strongly linked to BFB cycles, the formation of dicentric chromosomes and chromosomal translocatons (Bryant 1985; Ferguson, Sekiguchi et al. 2000; Richardson and Jasin 2000; Zhu, Mills et al. 2002).

In this chapter we show for the first time that cigarette smoke condensate exposure leads to anaphase bridge formation and genomic imbalances in untransformed human diploid cells. The frequency of induced bridges diminishes after several cell cycles, and this decrease in bridge formation is not dependent on p53-mediated apoptosis. While the frequency of CSC-induced bridges decreases with time in culture, the bridges lead to significant chromosomal rearrangements in the untransformed cells. Additionally, CSC treatment results in DNA DSBs in both purified viral DNA and in cultured cells. The anaphase bridges, DSBs, and genomic

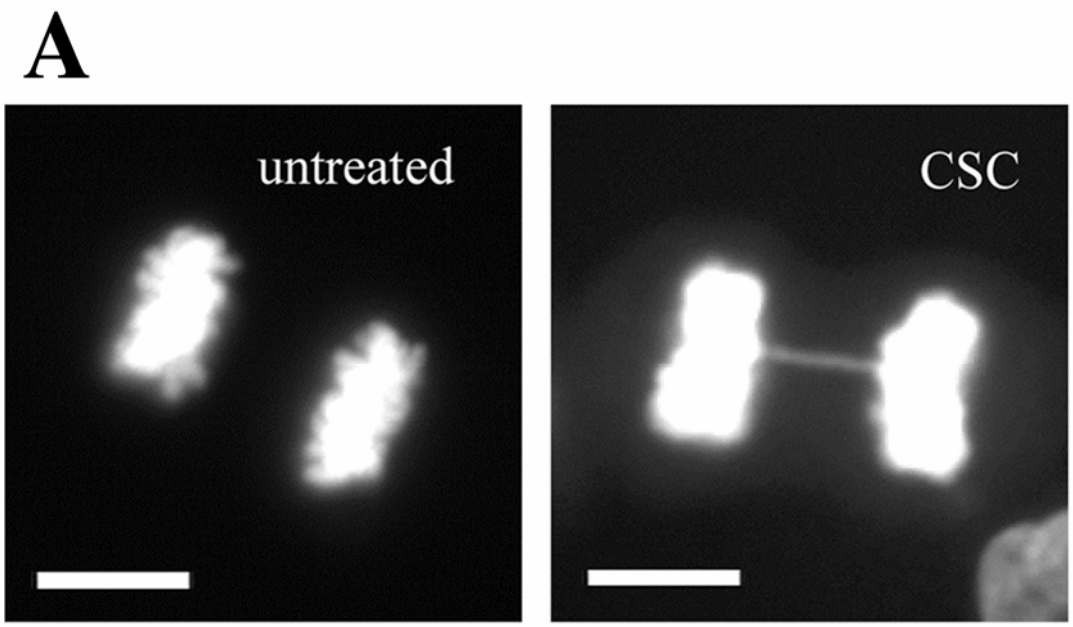
imbalances could be prevented by the antioxidant 2'deoxyguanosine 5'-monophosphate (dGMP) (O'Neill 1983; Ma, Cao et al. 1999) suggesting that they occur through a common mechanism involving ROS.

2. Results

2.1. CSC induces anaphase bridges in untransformed human diploid fibroblasts

To examine the linkage between anaphase bridges and cigarette smoke exposure, we exposed an untransformed diploid human fibroblast cell line, GM03349B to increasing concentrations of CSC for 2 hours, and scored chromosomal segregation defects after one to two cell cycles had been completed. To increase the frequency of mitotic cells, we synchronized cultures with nocodazole overnight, allowed them to recover for 1 hour and fixed the cells. We scored anaphase defects after staining the cells with DAPI. Examples of a normal cell in anaphase and a cell with an anaphase bridge are shown in Figure 9A. The CSC-treated fibroblasts expressed a dose-dependent increase in the frequency of anaphase bridges up to about 9% of the total anaphase cells (Fig. 9B). By comparison, we observed that cells treated with DMSO alone showed a similar level of anaphase bridges (~2%) as nocodazole only (untreated in Figure 9B). Similarly, CSC-treated normal human oral keratinocytes (UP3 cells) also showed an induction of anaphase bridges (from 1% in untreated cells to 5% after CSC treatment, n=200). This indicates that in untransformed diploid human cells, a single exposure to CSC can induce anaphase bridges in subsequent cell divisions.

In nocodazole treated cells, we also saw an induction of lagging chromosomes (data not shown). This is consistent with previous studies (Cimini, Howell et al. 2001; Cimini, Fioravanti et al. 2002; Cimini, Moree et al. 2003) in PtK1 cells, in which they saw merotelic chromosomes in between chromosome masses in anaphase, when microtubules re-polymerized after nocodazole release. We thus discounted lagging chromosomes and only scored for anaphase cells with one


A
untreated
CSC

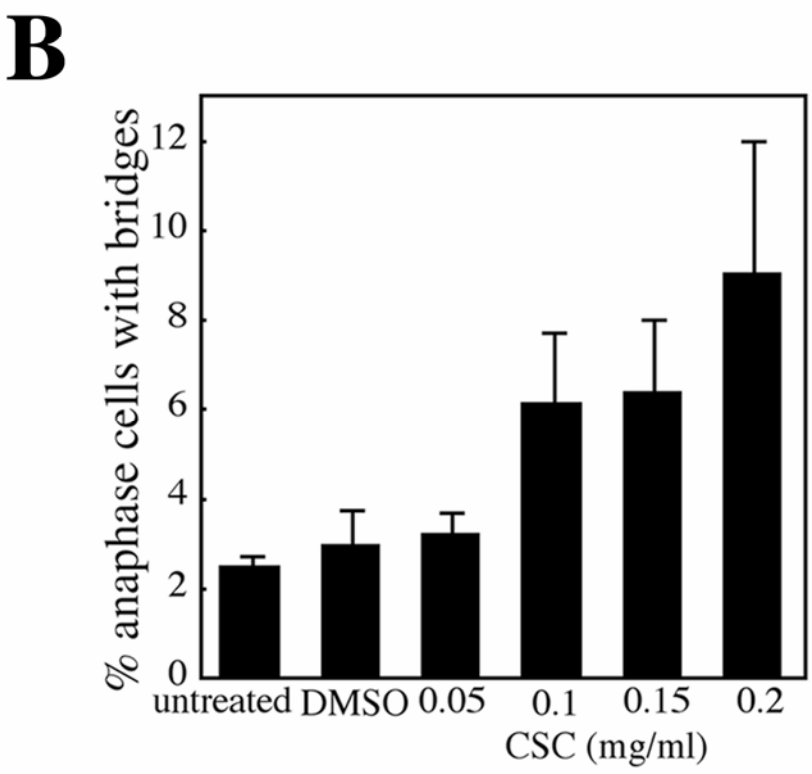

B
% anaphase cells with bridges
0
2
4
6
8
10
12
untreated
DMSO
0.05
0.1
0.15
0.2
CSC (mg/ml)

Figure 9

Figure 9. Induction of anaphase bridges with CSC in diploid human fibroblasts. Cells were grown on glass cover slips and treated with increasing concentrations of CSC in DMSO or with DMSO alone. (A) A normal anaphase in an untreated control cell (left) and an anaphase cell with chromatin bridges from a culture treated with 0.2 mg/ml CSC (right). Cells were stained with DAPI. Bars are 10μm. (B) Quantification of induced anaphase bridges scored as a percentage of the total anaphase population. The results are combined from 5 independent experiments with >200 anaphase cells scored for each sample. Bars indicate S.D.

or more continuous bridges. Since some cells exhibiting a broken bridge could be interpreted as one with lagging chromosomes and thus excluded, the actual frequency of cells with anaphase bridges was most likely under-represented.

Treatment of the fibroblasts with CSC at a concentration greater than 0.2 mg/ml or for a period longer than 2 hr resulted in frequent cell death and substantial decrease in the mitotic index. To examine the level of apoptosis induced by CSC, we stained cells with antibodies to cleaved caspase 3, which is specific for apoptotic cells. Representative images of untreated, CSC-treated and staurosporine-treated (positive control) are shown in Figure 10A. Note that cells with positive anti-cleaved caspase 3 staining often have condensed nuclei. Quantification shows that CSC at the highest concentration used (0.2 mg/ml) produced a similar level of apoptosis (1.8%, n=500) to the untreated control (1.9%, n=500). Consistently, these cells have low levels of condensed nuclei or nuclear blebbing (1-2%, n=500) or cell death, as judged by trypan blue exclusion (2%, n=200), compared to untreated control (<1% in each case).

2.2. CSC also induces anaphase bridges in cancer cells

We also observed a similar increase in the frequency of anaphase bridges induced by CSC in human cervical cancer HeLa cells (Figure 11). We noticed that untreated levels of anaphase bridges are usually higher in cancer cells. The results in another human colon cancer cell line, HCT116 cells were also consistent (data not shown). Thus, CSC induces anaphase bridges in both human untransformed and cancer cells.

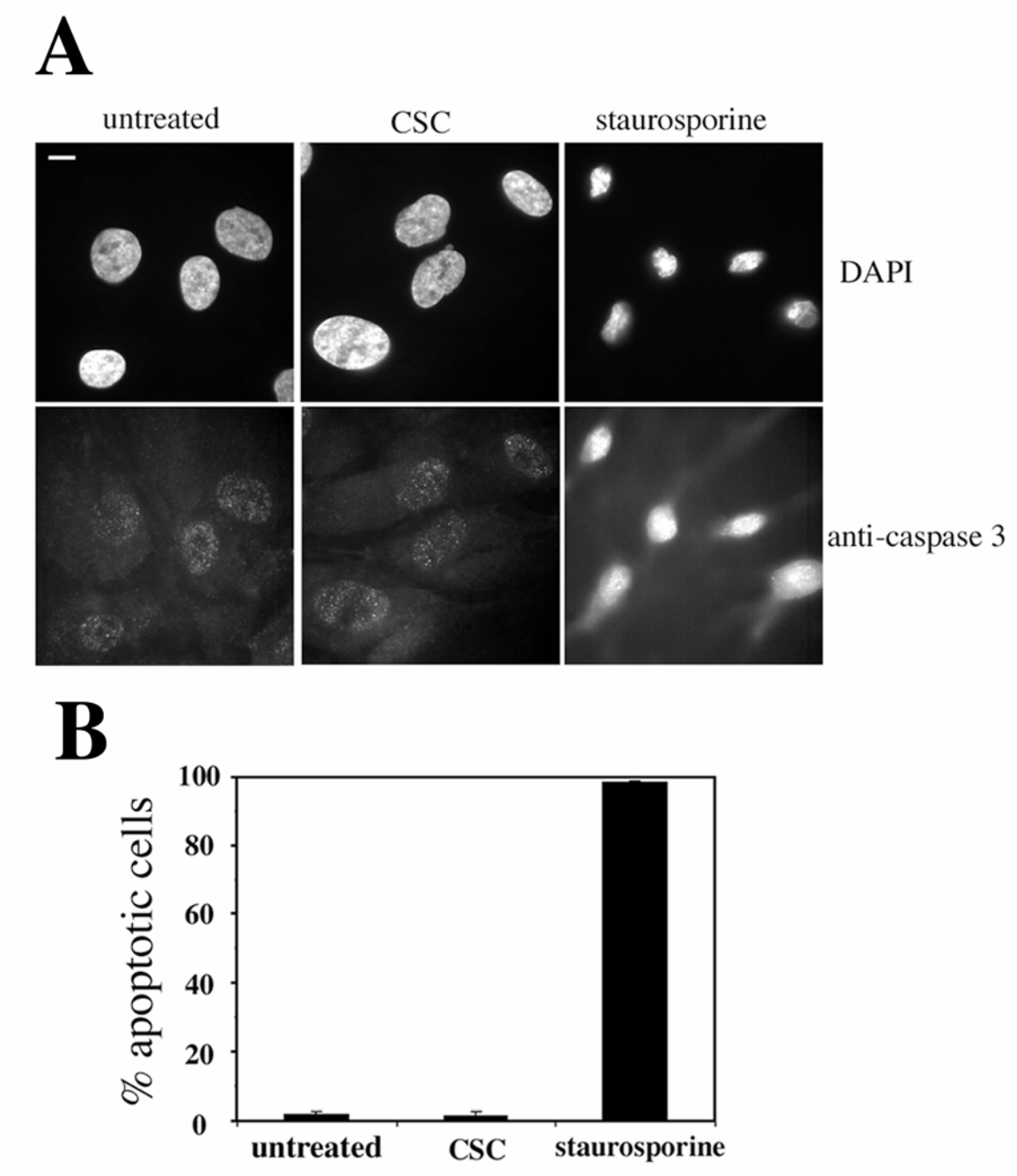

A
untreated
CSC
staurosporine
DAPI
anti-caspase 3
B
% apoptotic cells
100
80
60
40
20
0
untreated
CSC
staurosporine

Figure 10

Figure 10. CSC does not induce apoptosis at the standard concentration used. Fibroblasts were treated with or without 0.2mg/ml CSC as described in Figure 9. For a positive control of apoptosis, cells were serum-starved overnight and treated with 1 μM staurosporine for 4.5 h before fixation. (A) Representative images of untreated, CSC-treated and staurosporine-treated fibroblast cells stained with anti-cleaved caspase 3 antibody and DAPI. Bar is 10 μm. (B) Quantification of cells with positive caspase 3 staining. Error bars are S.D. from 3 experiments with 500 cells per experiment.

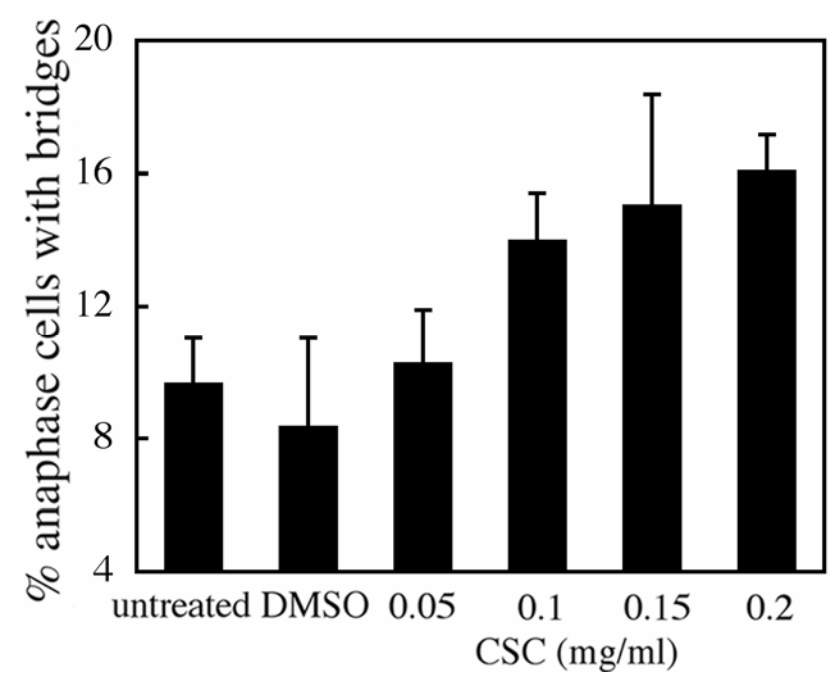


Figure 11

Figure 11. Induction of anaphase bridges with CSC in HeLa cells. HeLa cells were treated with CSC identically as fibroblasts but were not subsequently arrested with nocodazole. The results are combined from 3 independent experiments with >200 anaphase cells per experiment. Bars indicate S.D.

2.3. CSC induces DNA DSBs in cell culture

Previous analysis has shown that CSC produces DNA single strand breaks via the Fenton reaction (Nakayama, Kaneko et al. 1985). These DNA single strand breaks may be repaired or replicated during S phase leading to the formation of genetic destabilizing DSBs. To determine if CSC can produce DNA DSBs in cells, we examined CSC-treated fibroblasts for γ-phosphorylated histone H2AX foci, a cellular DSB marker (Rogakou, Pilch et al. 1998; Rothkamm and Lobrich 2003). Immunofluorescence analysis showed that γ-H2AX foci appeared in the cells within 3 min of CSC exposure (Fig. 12A), similar to those found in cells exposed to ionizing radiation (Rogakou, Pilch et al. 1998). Some of the increase in γ-H2AX staining could be due to DNA replication, as γ-H2AX foci increase up to 3 fold in S/G2 as compared to G1 phase of the cell cycle (Rothkamm and Lobrich 2003). To rule out this possibility, we co-stained cells with anti-PCNA (proliferating cell nuclear antigen) which exhibits a pronounced nuclear localization in S/G2 phase. These cells were discounted, and we scored the remaining G1/early S cells for H2AX foci. The percentage of cells with H2AX foci increased from 14.3% in untreated to 21.7% in treated fibroblasts, independent of cell passage number (Fig. 12B). These results indicate that CSC increases the frequency of DSBs in untransformed cells.

2.4. CSC induces DNA DSBs in solution via ROS

The DSBs observed in cells could be a result of direct damage to the DNA or be induced by cellular events. It is currently unknown whether cigarette smoke can directly produce DSBs in DNA. To test this, we incubated ϕX174 phage DNA with CSC for 30 min at 37°C. DSBs are

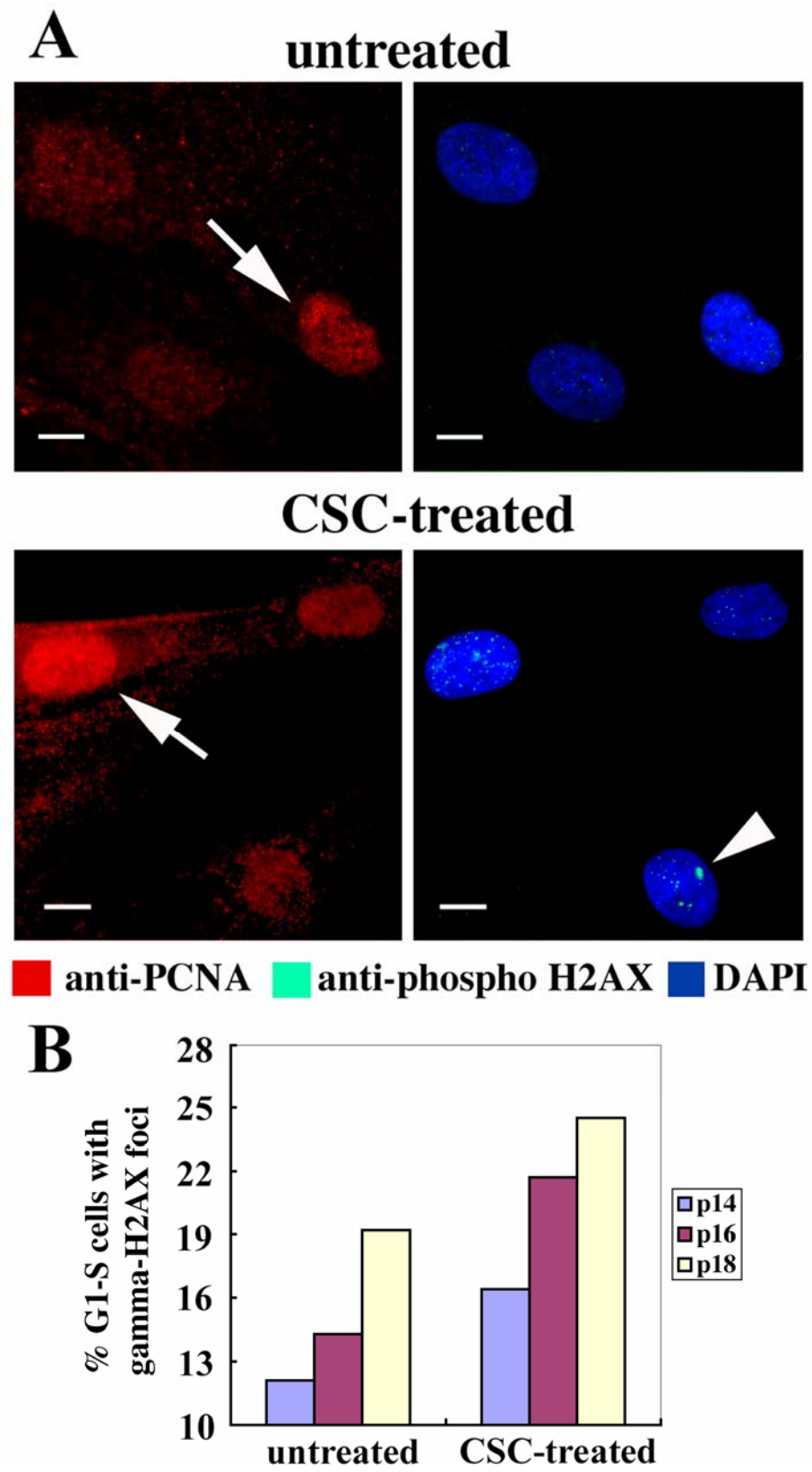

A
untreated
CSC-treated
anti-PCNA
anti-phospho H2AX
DAPI
B
% G1-S cells with gamma-H2AX foci
28
25
22
19
16
13
10
untreated
CSC-treated
p14
p16
p18

Figure 12

Figure 12. DSBs are induced by CSC in cell culture. (A) CSC induces DSBs in fibroblasts. Cells were exposed to 0.2 mg/ml CSC for 15 min and fixed 3 min after CSC treatment. Cells were stained with DAPI for DNA, anti-PCNA for cells in S/G2 phase and anti-γ-H2AX for DSB repair foci. Bar is 10μm. The arrows point to the PCNA positive cells and the arrowhead points to a γ–H2AX positive cell. (B) Quantification of cells that were PCNA negative and γ–H2AX positive for immunostaining. Experiments were performed in 3 independent passages of >300 cells per experiment. p14-p18, passage 14-18.

demonstrated by the formation of the linear form of this plasmid (Figure 13, lane 1). To determine whether the DSBs we observed were a result of the Fenton reaction and the formation of ROS, we tested for the requirement for a metal catalyst. Neither CSC alone, nor Cu^{2+} alone, was sufficient to induce DSBs. But when CSC and Cu^{2+} were added together, nearly all of the supercoiled circular plasmid was converted into nicked circular or linear DNA (Figure 13, lane 6). Other metal ions such as Zn^{2+} were less effective at promoting CSC-induced DSBs (data not shown). In all subsequent experiments with CSC, Cu^{2+} was added to the reaction. Furthermore, these DSBs were reduced substantially in a concentation dependent manner by the antioxidant dGMP, a known scavenger of ROS in solution (Figure 13, lane 7, 8).

ROS include superoxide anions, singlet oxygens and hydroxyl radicals, and they are commonly interchangeable *in vivo* (Cross, Halliwell et al. 1987). Besides dGMP (a singlet oxygen scavenger), inhibition of ROS by catalase (also named hydrogen peroxidase, an enzyme that specifically degrades hydrogen peroxide), histidine (quenches singlet oxygen) and Tris (reduces hydroxyl radicals, data not shown) can also prevent CSC-induced DSBs (Figure 14A, B). In addition, a dietary antioxidant from red wine, resveratrol can also prevent CSC-induced DNA DSBs (Figure 14C). Note that all the antioxidants were added with CSC simultaneously to DNA. These results suggest that CSC produces the DNA DSBs via the Fenton reaction and the formation of ROS.

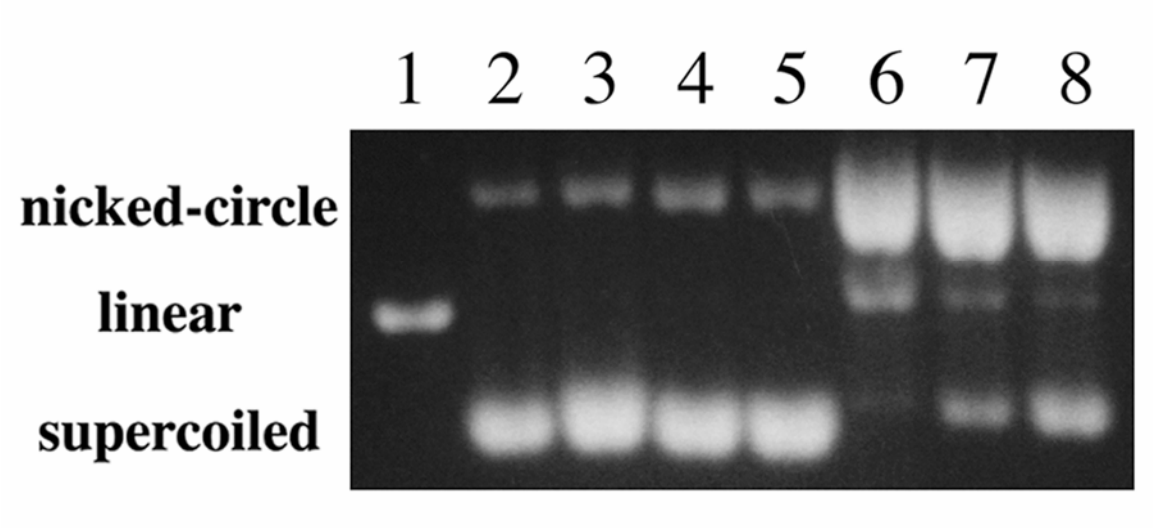


Figure 13

Figure 13. CSC causes DNA DSBs *in vitro* in the presence of copper (II). Lanes: 1. DNA digested with the restriction enzyme XhoI, 2. untreated ϕX174 phage DNA only, 3. DNA and CSC, 4. DNA and copper (II), 5. DNA and 13.3mM dGMP, 6. DNA and CSC and copper (II), 7. DNA, CSC, Copper (II) and 6.7mM dGMP, 8. DNA, CSC, Copper (II) and 13.3mM dGMP. The final concentration for DNA is 0.036mg/ml, $CuSO_4$ is 3.3mM, CSC is 2 mg/ml.

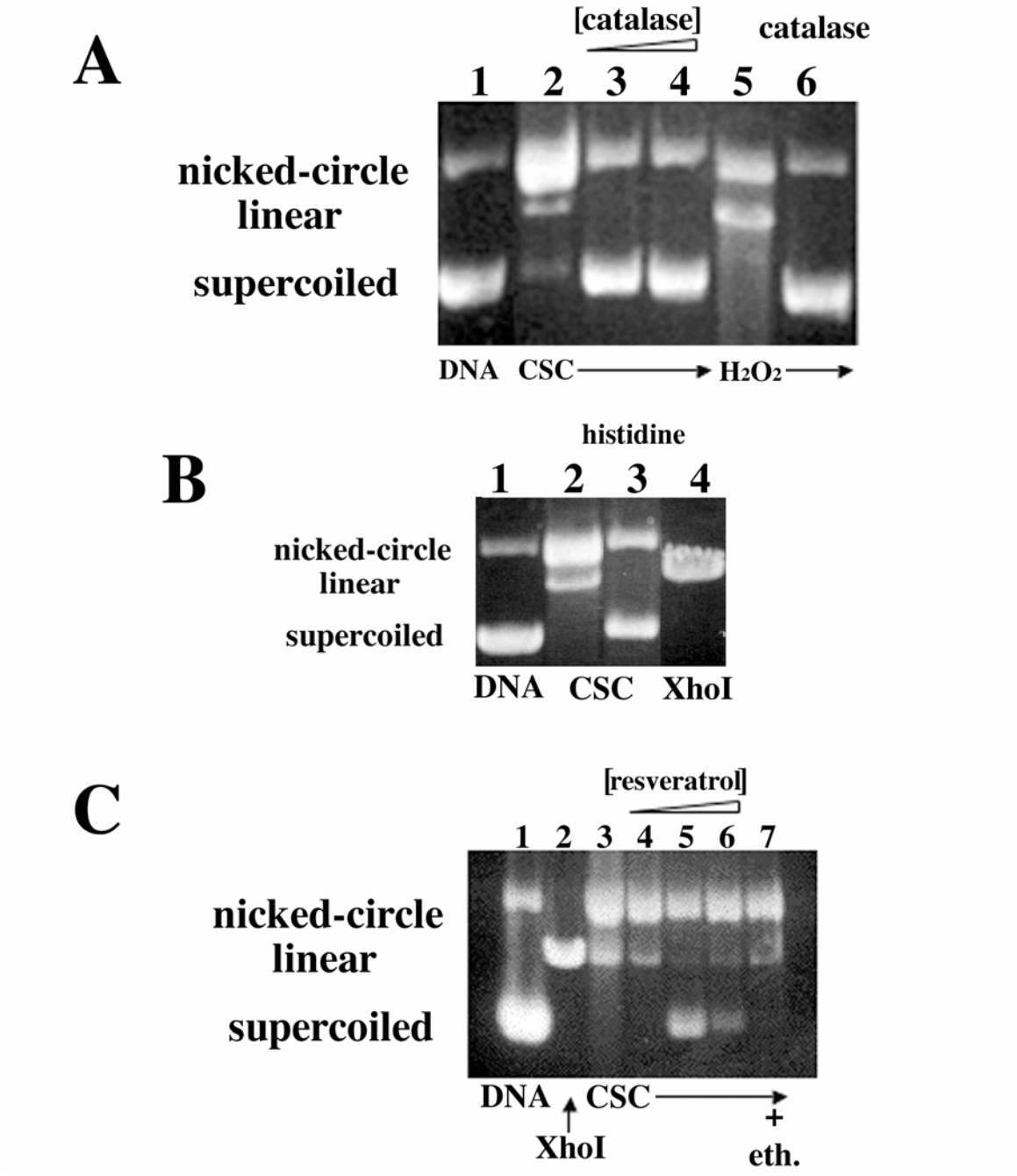

A
[catalase]
catalase
1 2 3 4 5 6
nicked-circle
linear
supercoiled
DNA CSC
H2O2
B
histidine
1 2 3 4
nicked-circle
linear
supercoiled
DNA CSC XhoI
C
[resveratrol]
1 2 3 4 5 6 7
nicked-circle
linear
supercoiled
DNA CSC
XhoI
+
eth.

Figure 14

Figure 14. DSBs from CSC can be prevented by cellular and dietary antioxidants. (A) Lanes: 1. DNA alone, 2. DNA with CSC ($+Cu^{2+}$ in this and all subsequent uses of CSC), 3-4. DNA with CSC and catalase at 7,650 units/ml and 30,600 units/ml, 5. DNA with 0.02 μg/ml hydrogen peroxide, 6. DNA with hydrogen peroxide and 204 units/ml catalase. (B) Lanes: 1. DNA alone, 2. DNA with CSC, 3. DNA with CSC and 200mM histidine, 4. DNA cut with XhoI. (C) Lanes: 1. DNA alone, 2. DNA cut with XhoI, 3. DNA with CSC, 4-7. DNA with CSC and ethanol and resveratrol added at 0.7, 1.3, 2.7 and 0 mg/ml. Resveratrol was dissolved in ethanol (highest concentration = 27%, last two lanes) before being mixed with DNA.

2.5. Correlation between CSC-induced DNA DSBs and SSBs

A DNA DSB could be generated by a single cut to both strands of the DNA, or by two SSBs that are adjacent to each other on opposite strands. To examine if CSC-induced DNA DSBs are due to the accumulation of SSBs, we tested the time and dose dependence of CSC activity (Figure 15A, B). Induction of SSBs preceded that of DSBs with time or with CSC concentration in a dose-dependent manner, consistent with the interpretation that CSC-induced DSBs *in vitro* are due to an accumulation of SSBs. Indeed, increasing concentration of DNase I (generates DNA SSBs) caused a similar dose-dependent induction of SSBs and eventually DSBs (Figure 15C). Although we cannot exclude the possibility that these DNA DSBs were caused by an independent mechanism from SSBs, the similar generation of SSBs and DSBs by CSC and by DNase I, and the fact that both breaks can be prevented by antioxidants suggest that DSBs induced by CSC may be caused by accumulation of SSBs.

Next, we tested the sensitivity of CSC-induced DNA DSBs to DNA length. If the amount of DSBs generated by CSC is dependent on the length of DNA *in vitro*, fewer DSBs would be expected in cell culture for the full length genome. We thus compared the concentration dependence of CSC on a 5.4kb plasmid DNA versus a 22.8kb cosmid DNA (Figure 16A). The ratios of CSC-induced DSBs were similar regardless of DNA length (Figure 16B). This indicates that CSC induces DNA DSBs independent of the length of DNA.

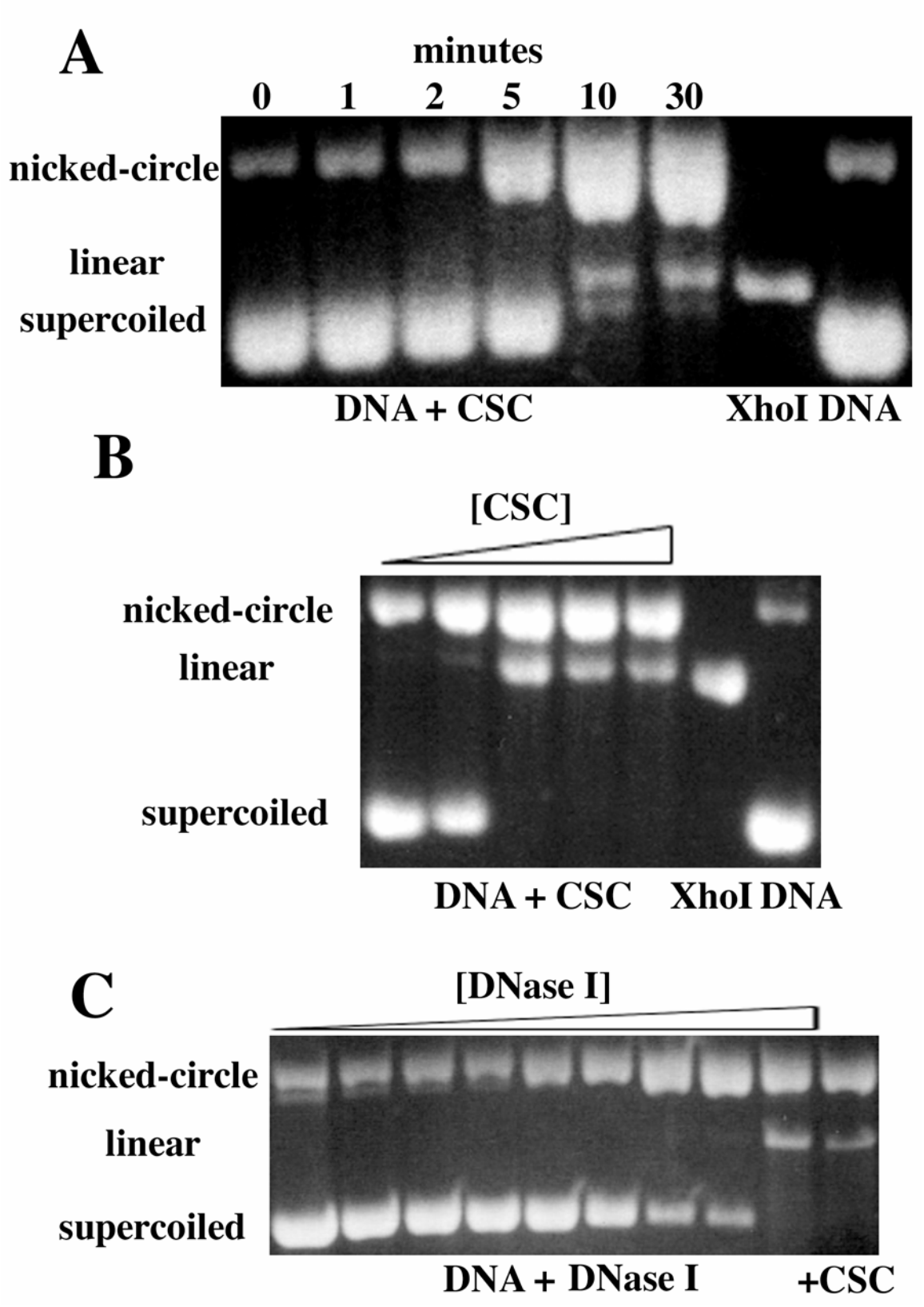

A
minutes
0
1
2
5
10
30
nicked-circle
linear
supercoiled
DNA + CSC
XhoI DNA
B
[CSC]
nicked-circle
linear
supercoiled
DNA + CSC
XhoI DNA
C
[DNase I]
nicked-circle
linear
supercoiled
DNA + DNase I
+CSC

Figure 15

Figure 15. CSC-induced SSBs precede DSBs. (A) Increasing time of exposure to CSC in minutes. (B) Increasing CSC concentrations of 16.7, 33.3 ug/ml, 0.7, 2.0, and 3.3 mg/ml. (C) Increasing concentrations of DNase I are 6.7, 26.7, 53.2, 66.7, 133, 263, 526, 670, 1250 units/ml. CSC is 0.2 mg/ml. Note the position of the linear DNA relative to the other forms of the plasmid varied according to when ethidium bromide was added to the gel and the use of TBE or TAE buffer.

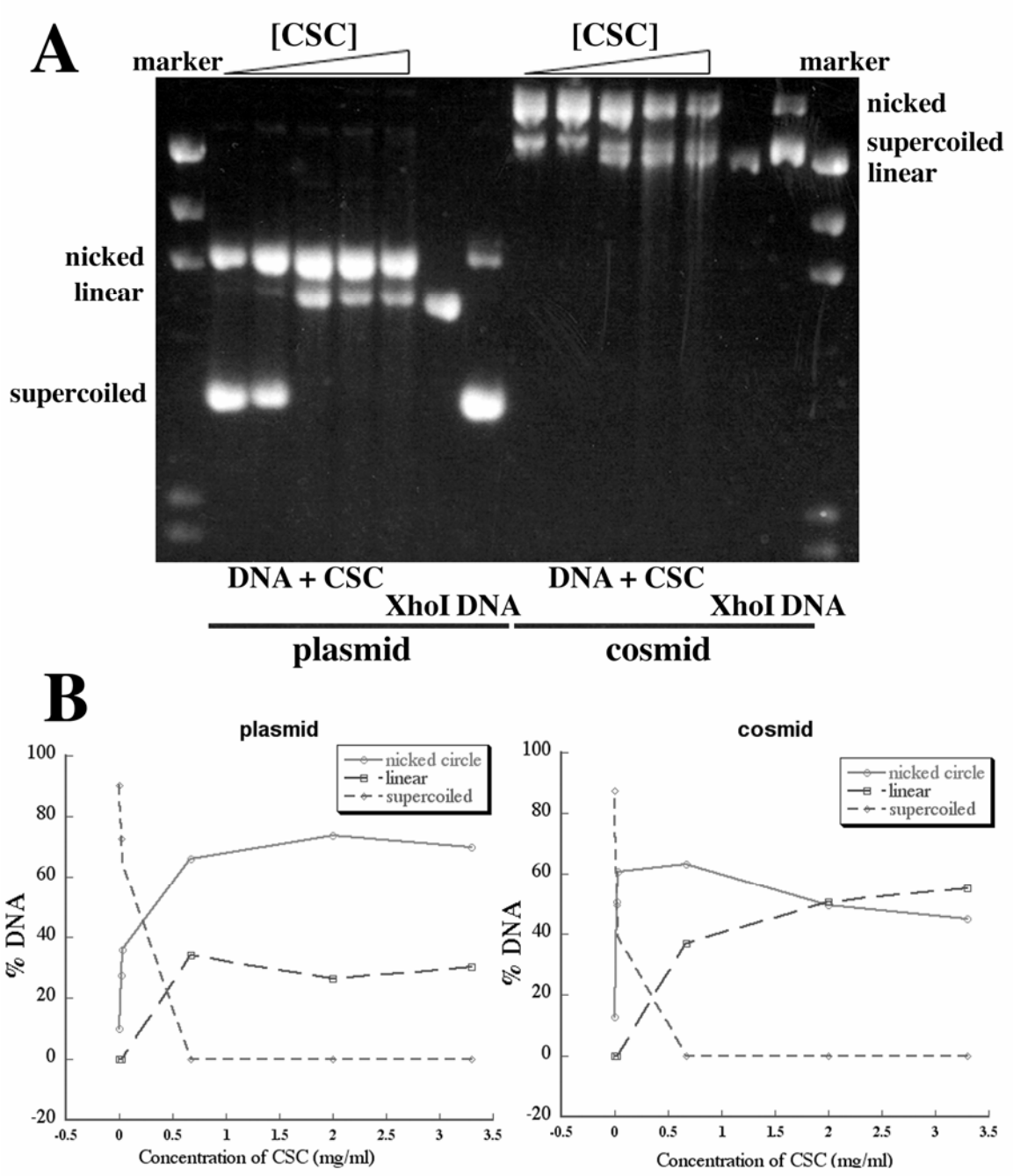

A
marker
[CSC]
[CSC]
marker
nicked
supercoiled
linear
nicked
linear
supercoiled
DNA + CSC
XhoI DNA
DNA + CSC
XhoI DNA
plasmid
cosmid
B
plasmid
cosmid
nicked circle
linear
supercoiled
% DNA
Concentration of CSC (mg/ml)

Figure 16

Figure 16. CSC-induced DSBs induction is independent of DNA length. (A) Increasing concentrations of CSC with plasmid DNA and cosmid DNA. The left 2-8 lanes are identical to Figure 15B, and the lanes on the right were the same experiment except using the cosmid PB64. Note the change in the relative migration of supercoiled versus linear form of this cosmid DNA. Markers were λ DNA/ Hind III fragments (from top to the bottom: 23.1kb, 9.4kb, 6.6kb, 4.3kb, 2.3kb). (B) Quantification of nicked-circle, linear and supercoiled DNA. Percentages calculated by measuring the band intensity.

2.6. CSC-induced anaphase bridges are not due to bulk telomere shortening

Telomeric association has been previously suggested to cause dicentric chromosomes and anaphase bridges (Artandi, Chang et al. 2000; Fouladi, Sabatier et al. 2000; Gisselsson, Jonson et al. 2001; Rudolph, Millard et al. 2001; Lo, Sabatier et al. 2002). To investigate if CSC-induced anaphase bridges were caused by telomeric association, we examined the presence of telomeres on CSC-induced anaphase bridges in fibroblasts. The diffused pattern of TRF proteins in mitosis made it technically difficult to identify telomeres by immunofluorescence. To solve this problem, we hybridized CY3-labeled telomere $(CCCTAA)_3$ probes in situ on CSC-treated cells. Anaphase cells usually do not persist through the harsh treatment required for PAN-telomere FISH, but within a small sample that did (n=9 total), 4 of these cells with anaphase bridges had detectable levels of telomere repeats (Figure 17), and 5 did not. The presence of telomeres on anaphase bridges are most likely telomeric associations, which are usually a result of telomere shortening. To test if CSC causes a general telomere shortening event, we performed a Southern Blot assay to compare the length of telomeres in CSC-treated and untreated fibroblasts. The cells were harvested for genomic DNA extraction. Intact genomic DNA was digested with HinfI and RsaI and examined on a 0.7% agarose gel (data not shown). The telomere terminal restriction fragments (TRFs) were then transferred onto a Nylon membrane, hybridized with telomere probe TAGGG conjugated with DIG and identified by anti-DIG-AP (alkaline phosphatase). The presence of anti-DIG-AP was then recognized by chemiluminescence substrate showing the migration of TRFs. As shown in Figure 18, CSC did not induce bulk telomere shortening under the condition by which it induced anaphase bridges. However, individual telomere shortening that may have caused the formation of anaphase bridges cannot be ruled out by this assay.

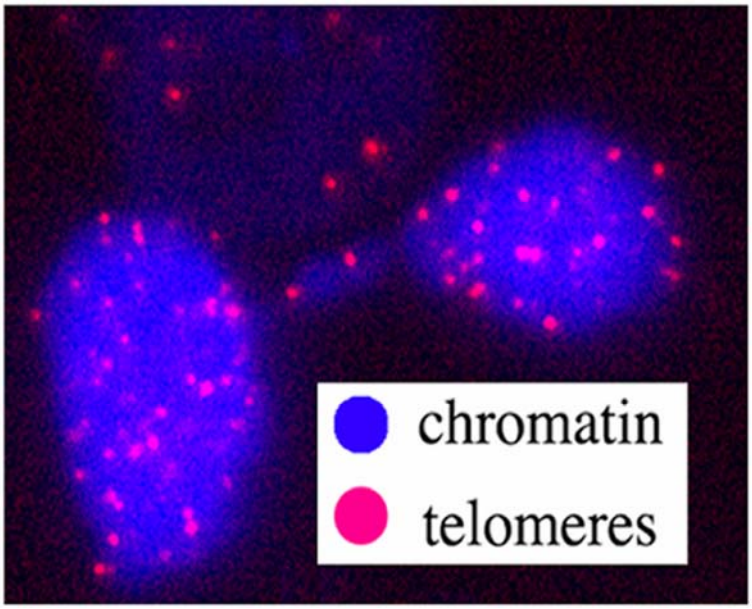


Figure 17

Figure 17. Identification of telomeres on CSC-induced anaphase bridges. A PAN-telomere FISH assay was performed (with adapted protocol, see Materials and Methods) on CSC-treated fibroblasts, demonstrated by telomeres in red and chromatin in blue. An example of anaphase cell is shown with telomeres on its chromatin bridge.

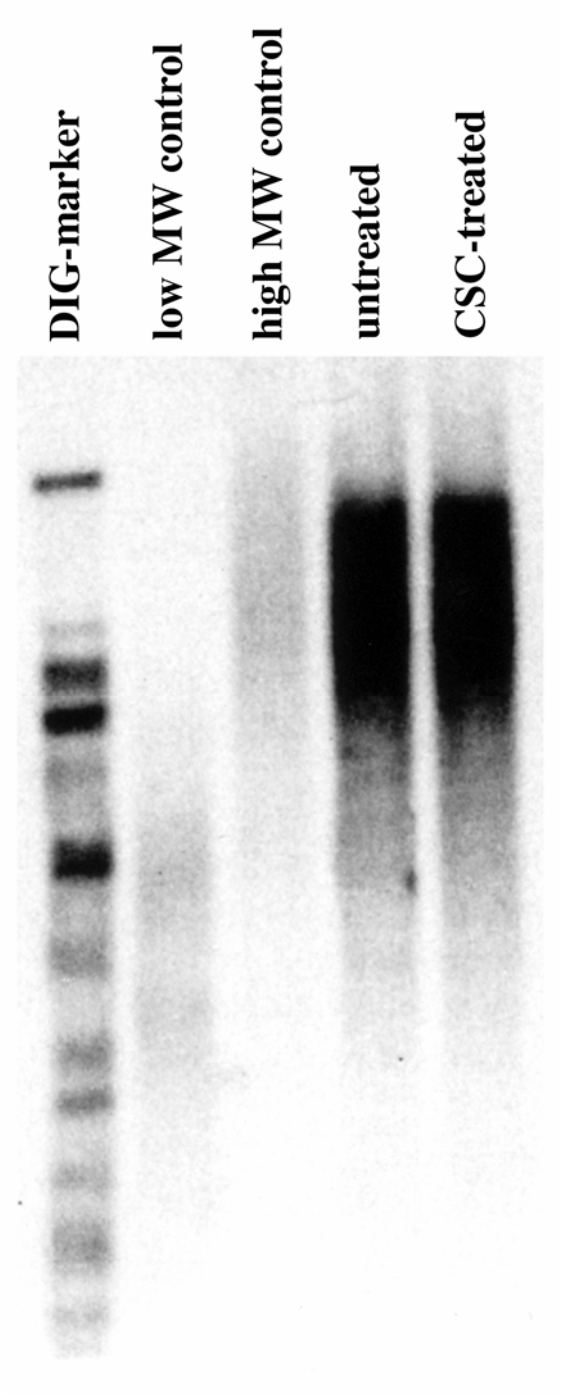

DIG-marker
low MW control
high MW control
untreated
CSC-treated

Figure 18

Figure 18. CSC does not induce general telomere shortening in human diploid fibroblasts. GM03349B cells at passage 14 were treated as before (Figure 9) and harvested for genomic DNA extraction. Intact genomic DNA was digested with *HinfI* and *RsaI* and examined on a 0.7% agarose gel. The telomere terminal restriction fragments were then transferred onto a Nylon membrane, hybridized with telomere probe TAGGG conjugated with DIG, and identified by anti-DIG-AP and chemiluminescence substrate. Control DNAs (2 μg each) contained either low (3.9kb) or high (10.2kb) molecular weight telomeres. Lanes with DNA from untreated or CSC-treated fibroblasts were loaded with 5 μg each.

2.7. The frequency of cells with anaphase bridges decreases with time in normal cells and p53 mutants

To determine whether the CSC-induced bridges led to CIN in the cells, we first examined whether the bridges persisted after CSC exposure (Fig. 19A). In fibroblasts, the frequency of bridges returned to the pretreated level in ~ 4 days. One possibility for the decrease in the frequency of anaphase bridges was that affected cells were undergoing p53-mediated apoptosis during the course of the experiment. To address this possibility, the experiment was performed in p53-knockout HCT116 human colorectal cancer cells (Bunz, Dutriaux et al. 1998) compared to wild-type HCT116 cells (Fig. 19B). In p53-/- cells, the frequency of the induced, as well as the pre-existing bridges, was much higher than observed in control HCT116 cells, indicating that p53 plays a role in the prevention of bridges. However, even in the p53-/- cells, the frequency of bridges diminished with time and returned to the pretreated level after ~10 days. Consistently, the epithelial kidney cells from p53 knockout mouse (Chiba, Okuda et al. 2000) showed a similar trend of the initial induction and the subsequent drop in the frequency of anaphase bridges after CSC treatment (Figure 20A). These results indicate that the reduction of anaphase bridges is not a consequence of p53-mediated apoptosis.

If CSC is inducing anaphase bridges through ROS, ROS exposure to cells would cause an induction and then a decrease in the frequency of anaphase bridges. Indeed, hydrogen peroxide induced anaphase bridges in HeLa cells, which diminished to baseline levels in ~15 days (Fig. 20B). This further supports the interpretation that CSC is inducing anaphase bridges through ROS.

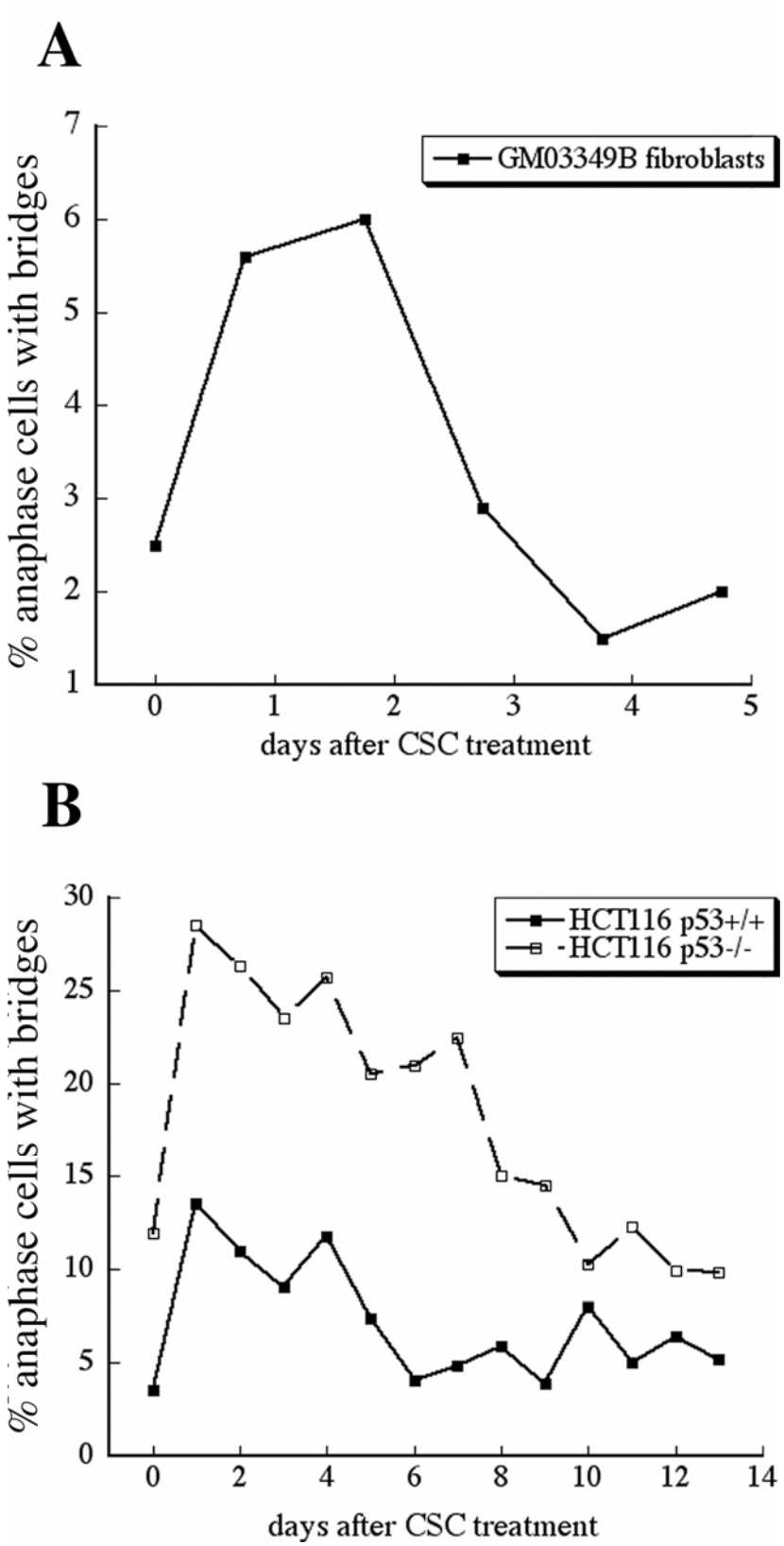

A
% anaphase cells with bridges
GM03349B fibroblasts
days after CSC treatment
B
% anaphase cells with bridges
HCT116 p53+/+
HCT116 p53-/-
days after CSC treatment

Figure 19

Figure 19. Quantification of anaphase bridges over time in (A) fibroblasts and (B) HCT116 (p53+/+ and p53-/-) human colorectal cancer cells. Cells were treated with CSC at 0.2 mg/ml for 2 hr, fixed and stained at 24-hr intervals after the removal of CSC. Fibroblasts were arrested with nocodazole and released for 1 hr before fixation.

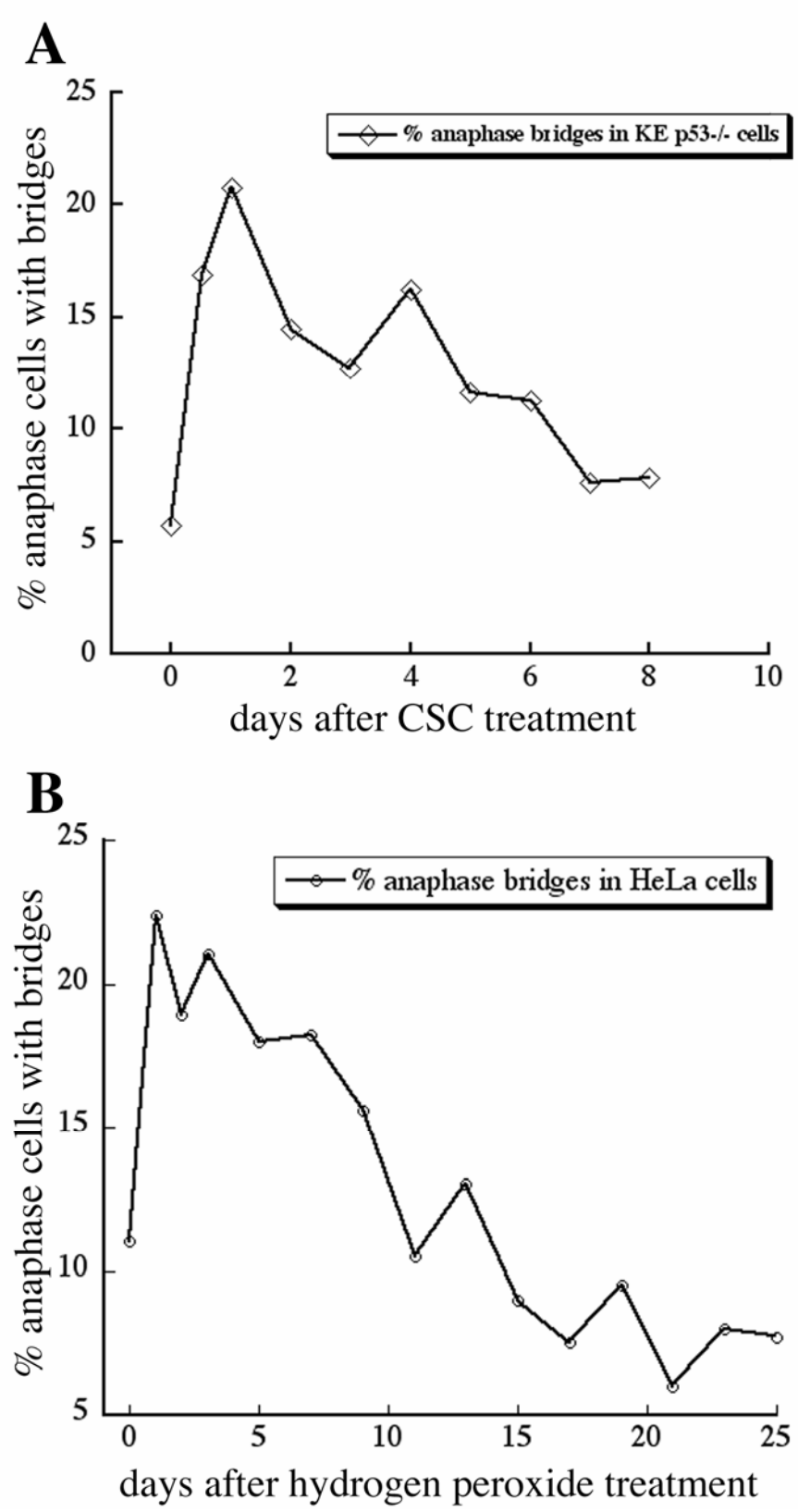

A
% anaphase bridges in KE p53-/- cells
% anaphase cells with bridges
days after CSC treatment
B
% anaphase bridges in HeLa cells
% anaphase cells with bridges
days after hydrogen peroxide treatment

Figure 20

Figure 20. Quantification of anaphase bridges over time in (A) p53-knockout KE mouse kidney cells treated with 0.15 mg/ml CSC and (B) HeLa cells treated with 1 μg/ml hydrogen peroxide. Cells were treated with CSC for 2 hr or hydrogen peroxide for 30 min, fixed and stained at 24-hr intervals after the removal of CSC or hydrogen peroxide.

2.8. Anaphase bridges lead to micronuclei formation in oral cancer cells

To image how anaphase bridges resolve, we transfected the oral squamous cell carcinoma line UPCI:SCC040 with a plasmid expressing GFP-tagged histone H2B (Kanda, Sullivan et al. 1998). This specific cell line was chosen for its high frequency of anaphase bridges (~60%) and transfection rate (~30%). The presence of the tagged histone did not affect the population doubling time, mitotic frequency, or the occurrence of segregational defects (Diane Hoffelder, personal communication).

To determine the fate of bridges, we identified and imaged labeled metaphase cells at 2-20 minute intervals, for up to 48 hrs. We observed anaphase bridges in 46% of the anaphase cells. This compares with 58% of cells with anaphase bridges in fixed non-GFP-expressing cells stained with DAPI. Among cells that formed chromatin bridges during anaphase, 45 could be followed for sufficient time in frame with clear focus to determine the fate of the bridges. In all 45 cases, the anaphase cells with bridges were able to exit mitosis and enter the subsequent interphase. In 40 out of 45 cases, the bridge quickly broke during anaphase into at least two major fragments. This is consistent with the previous analysis of bridge resolution in plants (Bajer 1963). In none of the 45 cells containing an anaphase bridge was the bridge observed to be pulled intact into one of the daughter cells. The cells that formed a bridge also did not undergo cell cycle arrest while under observation. In 5/45 cells with an anaphase bridge, the bridge persisted into interphase as has been observed previously (Bajer 1963). In addition, a micronucleus formed in 70% of the cells with broken anaphase bridges (Figure 21 and

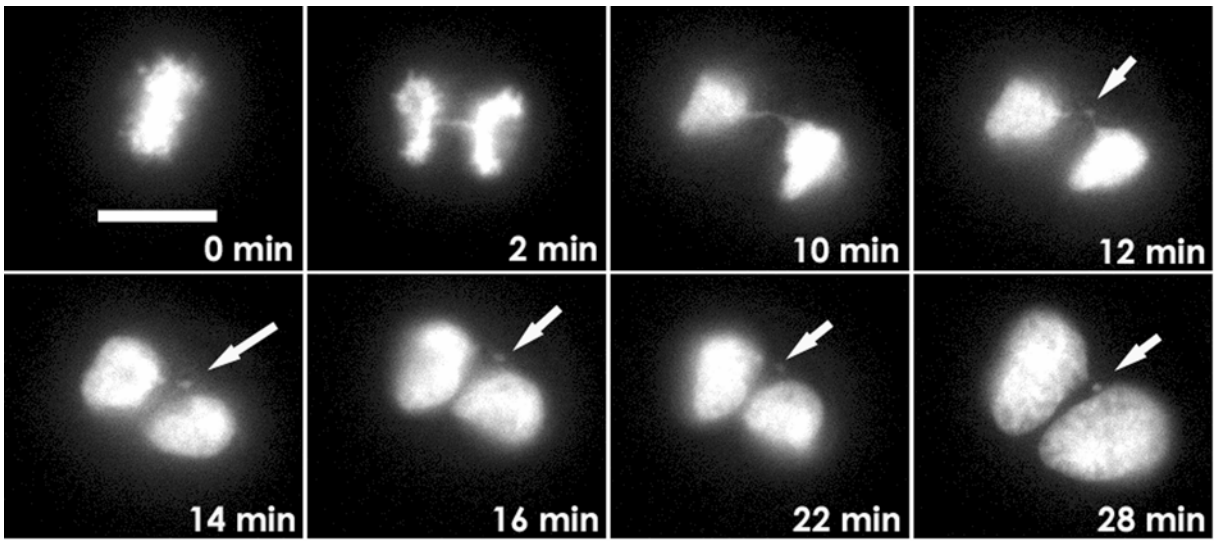


Figure 21

Figure 21. Anaphase bridges lead to micronuclei formation. A time-lapse series of a UPCI:SCC40 cell expressing GFP-tagged histone H2B showing micronucleus formation from a single broken bridge is shown. The sample chosen is one where the micronucleus fragment (arrows) remains visible throughout mitosis and into interphase. Time in minutes from an arbitrary start is indicated. Bar is 10 μm.

Supplemental movie 1). In ~40% of the cases, two or more micronuclei were observed to form from a single bridge. The formation of multiple large nuclear fragments (>4) was rarely observed and was assumed to result from apoptosis or injury, thus not counted as micronucleation. When two micronuclei were observed after bridge resolution, there was typically one in each daughter cell. These results show that cells with anaphase bridges typically survive and often give rise to micronuclei, thus actively contribute to CIN in the UPCI:SCC40 cell line.

2.9. CSC induces genomic imbalances in human diploid fibroblasts

The reduced frequency of bridges in fibroblasts after CSC treatment suggested that they might not have any long-term consequences to the cells. To test this, we examined the chromosome constitution in CSC-treated fibroblasts by in situ hybridization with fluorescently labeled DNA probes to the unique sub-telomeric sequences for both arms of individual chromosomes. Interphase cells were scored as normal if they contained two red and two green dots or aneuploid if they contained greater or fewer than that number (Figure 22). Examples of closely paired dots of the same color were counted as a single telomere, as cells in late S phase or G2 phase would have duplicated and adjacent telomeres. Note that cells with balanced translocations of chromosome arms could not be distinguished by this method, thus the values shown may under-represent the total amount of genomic imbalances. CSC-treated fibroblasts showed a statistically significant ($p=0.0001$ by student-T test) increase in telomere loss or gain, with 29.4% of treated cells observed to possess an abnormal number of telomeres compared to 16.7% in control cells. Neither treatment with 2% DMSO alone (% cells with telomere loss and gain = 16.4%, n=658) nor cell culturing in the absence of CSC was able to induce increased genomic imbalances

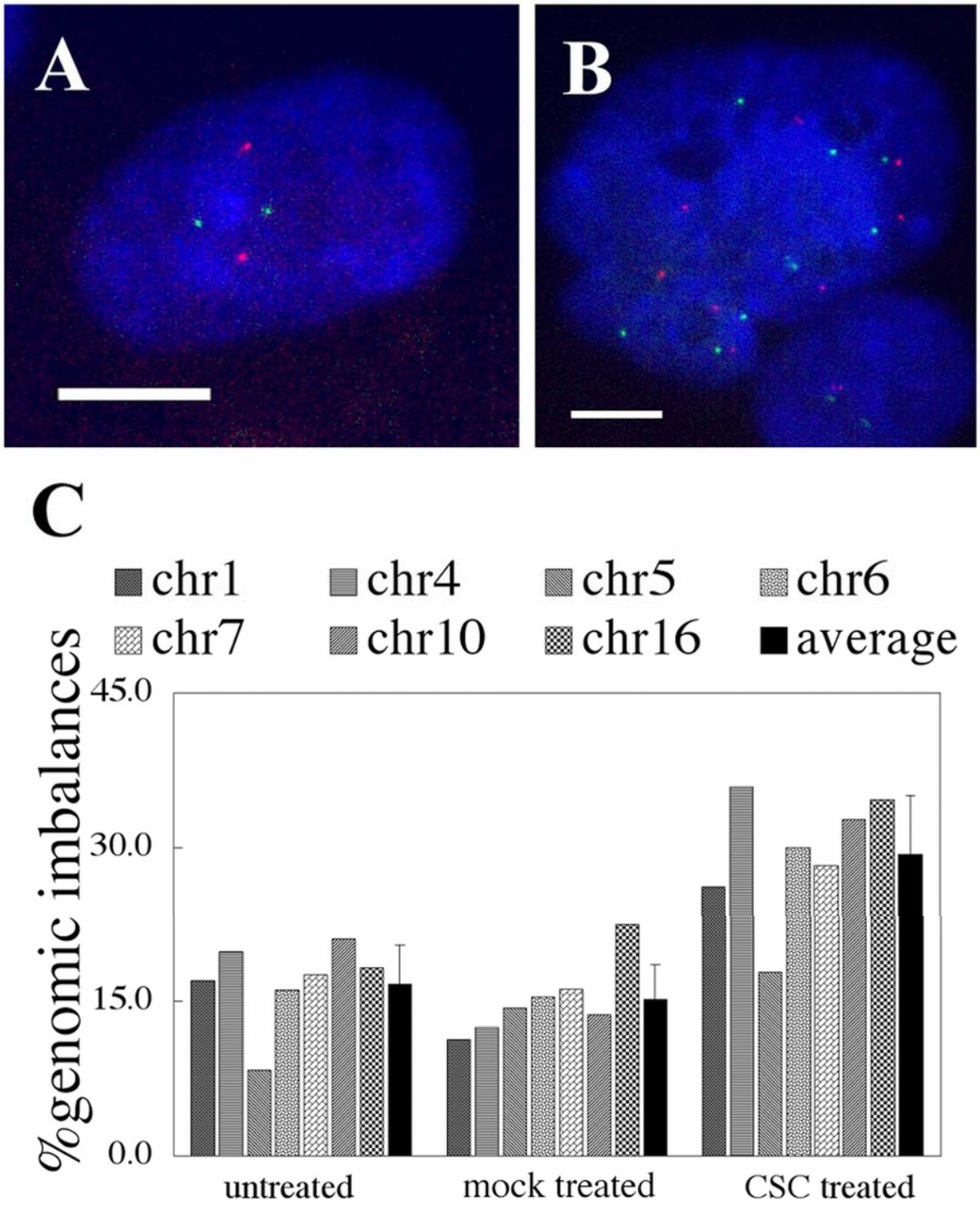

A
B
C
chr1
chr4
chr5
chr6
chr7
chr10
chr16
average
%genomic imbalances
45.0
30.0
15.0
0.0
untreated
mock treated
CSC treated

Figure 22

Figure 22. FISH analysis of CSC-treated diploid human fibroblasts. Cells were treated with 3 cycles of 0.2 mg/ml CSC for 2 hr at 4-day intervals. Cells were harvested after three passages and hybridized with DNA probes containing unique sequences from the p-arm (FITC-labeled in green) or q-arm (Texas Red-labeled in red) of individual chromosomes. (A) A cell probed for the telomere sequences of chromosome 5 showing the normal number of telomere signals. (B) An aneuploid cell stained with telomere probes for chromosome 16. Nuclei were counterstained with DAPI shown in blue. Bars are 10μm. (C) Quantification of aneuploid cells showing each of the tested chromosomes (chr). "Untreated" refers to cells fixed and stained prior to any treatment. "Mock treated" refers to untreated cells that were passaged three times parallel to the treated cells. Standard deviations were calculated from the average for all chromosomes. Between 1300-1400 cells total (including all chosen chromosomes) were counted for each the untreated, mock-treated and CSC-treated conditions.

(p=0.2). A separate analysis confirmed that anaphase bridges increased from 2.4% to 16% during this experiment. These results show that while the frequency of CSC-induced bridges in cultures decreased over time, CSC exposure was sufficient to lead to structural changes in the chromosomes of many of the cells. The level of genomic imbalances in the untreated cells was high at ~17% of total cells. It is not clear how much of this irregularity was due to intrinsic defects in the interphase fibroblasts cells or represents error within our assay. However, in either case, the level of aneuploid cells significantly increased due to triple exposure to CSC.

2.10. Antioxidant dGMP prevents CSC-induced anaphase bridges and genomic imbalances

We have previously shown that the antioxidant dGMP reduces CSC-induced DNA DSBs in solution (Figure 13). To determine if the chromosome rearrangements were due to ROS, dGMP was added to the cell culture during CSC exposure. Anaphase bridges were then scored as in Figure 9. dGMP nearly prevented the CSC-induced anaphase bridge formation (Fig. 23), suggesting that bridges result from the formation of ROS. Similar results were found in HCT116 cells using both dGMP and another antioxidant, histidine (Table 2). To determine if dGMP could also prevent the CSC-induced genomic imbalances, the telomere FISH analysis was performed with dGMP added during the CSC treatment. As shown in Figure 24A, after three cycles of CSC and dGMP treatment, the genomic imbalances were significantly reduced compared to CSC treatment alone (21.9% vs. 30.6%, p=0.003). The genomic imbalances for the untreated control were 16.2%. A separate analysis confirmed that dGMP prevented CSC-induced anaphase bridges (Figure 24B). These results show that CSC–induced genomic imbalances also most likely result from the formation of ROS.

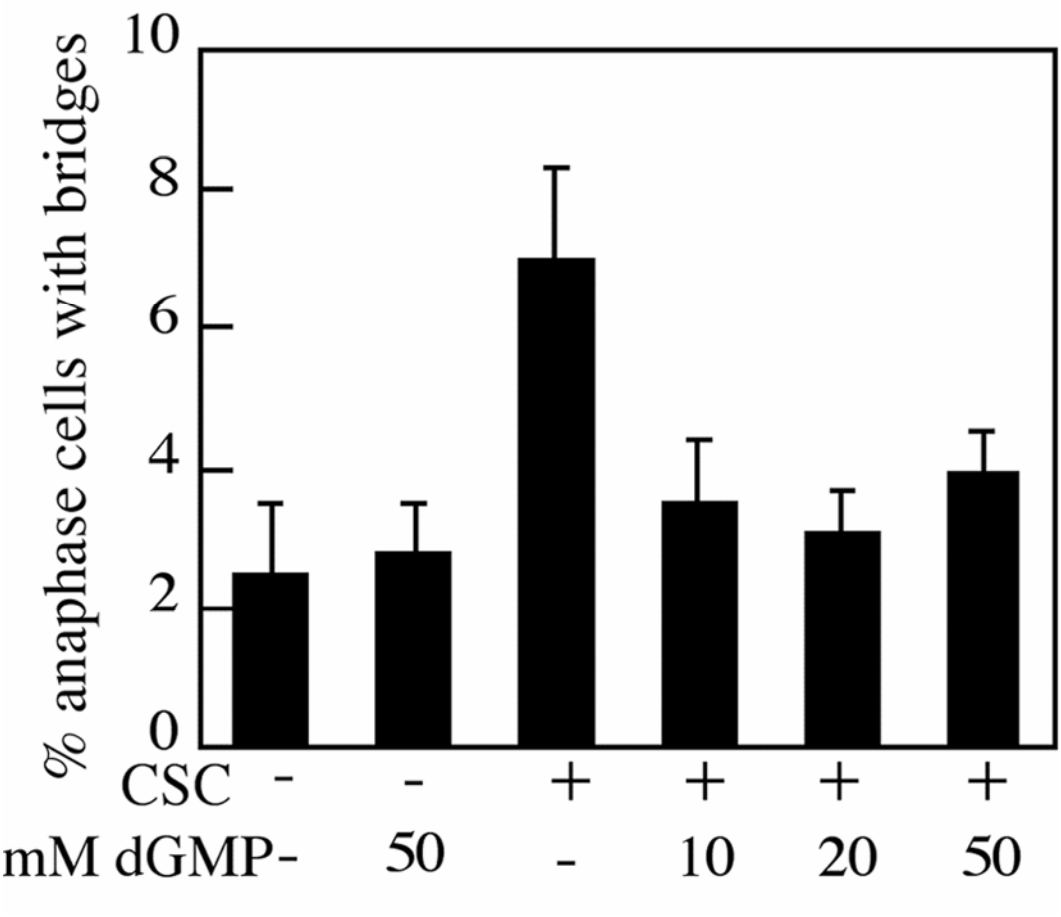


Figure 23

Figure 23. dGMP prevents anaphase bridges in fibroblasts induced by CSC. The analysis was performed as indicated in Figure 1 but increasing concentrations of the antioxidant dGMP were added in addition to 0.2mg/ml CSC.

Table 2 Antioxidants prevent CSC-induced anaphase bridges in human colorectal cancer HCT116 cells.

% anaphase cells with bridges	un-treated	DMSO control	anti-oxidant control	CSC with antioxidants					
				0 mM	2 mM	5 mM	10 mM	20 mM	50 mM
with dGMP	6.1	N.D.	6.2	10.9	11.5	10.5	9.0	5.1	5.6
with histidine	8.5	5.9	6.8	13.8	N.D.	N.D.	6.2	6.2	N.D.

dGMP and histidine prevents CSC-induced anaphase bridges in HCT116 cells. Various concentrations of antioxidants were added with CSC (as indicated in the table) or without CSC (50mM for dGMP, 20mM for histidine). N.D., not determined.

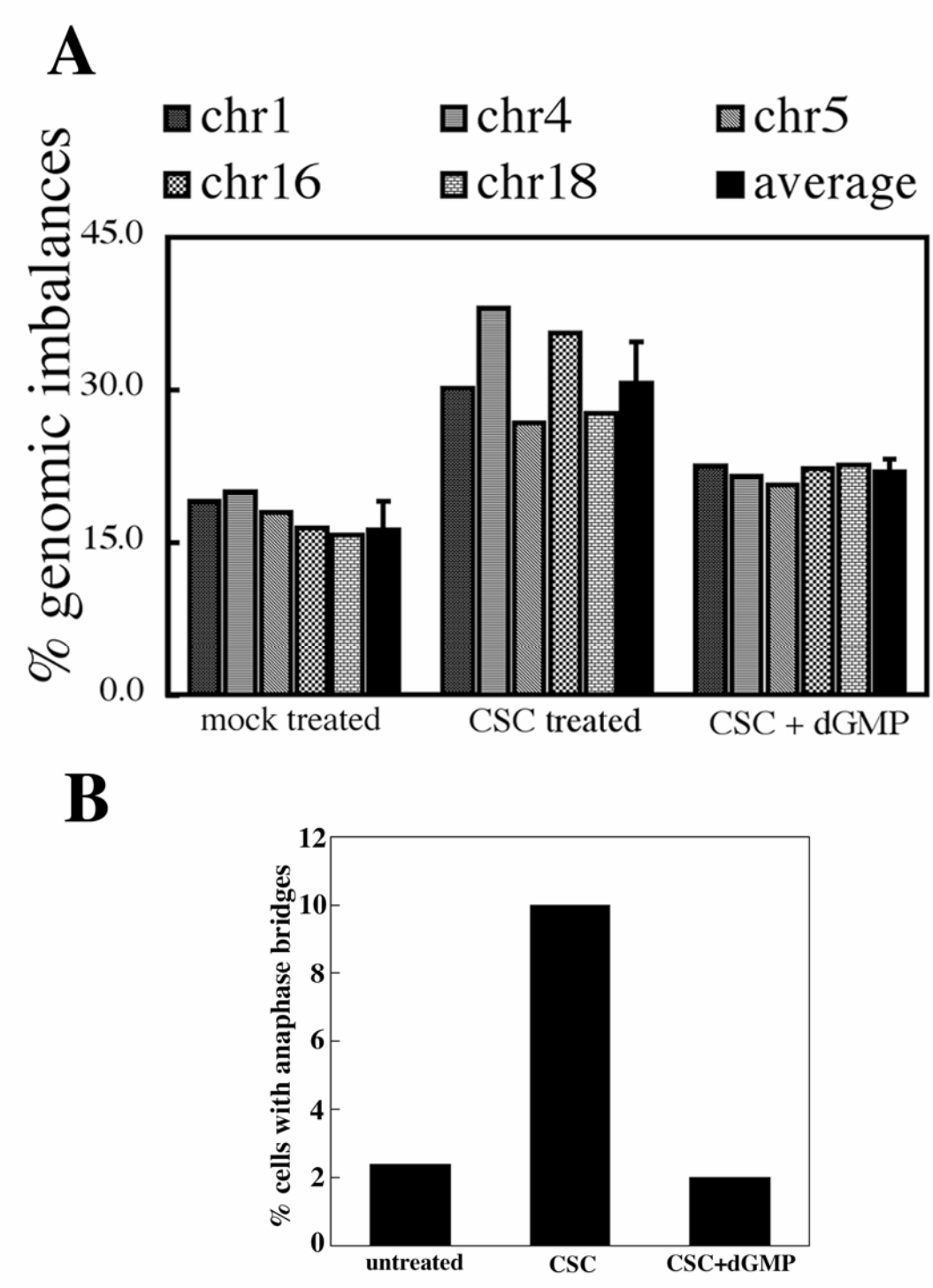

A
chr1
chr4
chr5
chr16
chr18
average
% genomic imbalances
45.0
30.0
15.0
0.0
mock treated
CSC treated
CSC + dGMP
B
% cells with anaphase bridges
12
10
8
6
4
2
0
untreated
CSC
CSC+dGMP

Figure 24

Figure 24. dGMP prevents CSC-induced genomic imbalances. (A) FISH analysis of dGMP and CSC treated diploid human fibroblasts. Experiment was performed as in Figure 22 but with 20 mM dGMP added with the 0.2 mg/ml CSC. "Mock treated" refers to untreated cells that were passaged three times in parallel to the treated samples. Between 600-800 cells total (including all chromosomes) were counted for each condition. (B) dGMP prevents CSC-anaphase bridges concurrently with genomic imbalances. Experiment was performed as in Figure 23 but in tandem to the first CSC cycle of the samples scored in (A).

3. Discussion

We have shown for the first time that CSC can induce DSBs, anaphase bridges and genomic imbalances in primary cells, and that each of these is prevented by the antioxidant dGMP. Thus, we propose that failure to accurately repair the CSC-generated DSBs via ROS leads to anaphase bridge formation and genomic imbalances.

Exposure to cigarette smoke has long been linked to carcinogenesis, but most of our understanding about its role is limited to mutational changes in the DNA sequence (Vineis and Caporaso 1995; Pfeifer, Denissenko et al. 2002). Our results suggest an additional consequence of cigarette smoke exposure in generating anaphase bridges and genomic imbalances. As chromosomal changes have become appreciated as an important feature of tumorigenesis, this new aspect of cigarette smoke damage is significant. The amount of cigarette smoke condensate used in these experiments equals ~ 3/1000 of a cigarette for the plasmid cleavage experiment and ~1/25 of a cigarette for the cells grown in culture. Thus, even low levels of exposure to CSC, much less than that generated from a single cigarette, can lead to DSBs in DNA and chromatin with accompanying anaphase bridges and genomic imbalances *in vitro*. Our results show an important new role for cigarette smoke exposure as a mitotic destabilizing agent and these results suggest a mechanism to explain the correlation between cigarette smoking and genomic imbalances in humans (Robbins, Vine et al. 1997; Rubes, Lowe et al. 1998; Shi, Ko et al. 2001). Future analysis will be required to determine if those chromosomal damages in humans are also due to CSC-induced DSBs. Our results are also consistent with previous observations that cigarette smoking is linked to fragile site expression (Stein, Glover et al. 2002), since fragile sites

have been shown to be correlated with the onset of breakage-fusion-bridge cycles (Coquelle, Pipiras et al. 1997).

The enhanced frequency of bridges from CSC exposure in p53 null cells most likely reflects the known roles of p53 in inducing cellular arrest and DNA repair or apoptosis in response to DNA damage (Khanna and Jackson 2001). The higher thresholds of initial anaphase bridges in cancer cells compared to primary cells may also be due to the complex modifications of DNA repair pathways and checkpoint controls in cancer cells. For example, HCT116 cells are defective in DNA mismatch repair (Branch, Hampson et al. 1995).

Besides the partial prevention of anaphase bridge formation, p53 could also play a role in elimination of CSC-induced anaphase bridges, possibly in cooperation/ redundancy with other mechanisms that have caused the reduction of anaphase bridge frequency. This was demonstrated by ~2 fold difference in the recovery rates of CSC-induced anaphase bridges decreasing over time in both p53 mutant and wild-type cells. However, both p53 mutants and wild-type cells have the same trend in bridge frequencies over time, showing that p53 is not vital for the decrease in anaphase bridges.

It is unclear why we observe the subsequent decline in anaphase bridges after exposure. Similar observations were made in a previous study following γ-irradiation of malignant and normal cells (Gisselsson, Pettersson et al. 2000), in which anaphase bridges returned to pretreated frequencies after approximately three passages in fibroblasts. There are three likely possibilities as to why this decrease occurs. One possibility is that cells are able to heal or eliminate the damaged

chromosomes independent of the p53-mediated apoptosis pathway. In this case, nascent DSBs can be repaired through both homologous recombination and nonhomologous end-joining pathways (van Gent, Hoeijmakers et al. 2001) or by the addition of telomere sequences (Melek and Shippen 1996). A dicentric chromosome may also behave as a normal one if it has one inactive centromere (Therman, Trunca et al. 1986). Another possibility is that the decrease in the frequency of bridges may occur through selection if they confer a growth disadvantage to the cells. The cells with anaphase bridges may arrest due to DNA damage or other problems associated with a chromatin bridge, and fail to progress through the cell cycle or do so at a much slower rate, therefore resulting in the gross decrease in frequency of bridges. A third possibility is that the CSC-induced bridges are chromosome-type bridges that do not have a 100% reformation rate in the subsequent cell cycle (Figure 3). Nascent DSBs of telomere-less ends may be repaired in the G1 phase of the cell cycle to form a dicentric chromosome. This dicentric chromosome is replicated in S phase, and in anaphase may either separate in a parallel or bi-directional fashion. In the former case, we will not observe bridges despite the presence of intact dicentrics in either daughter cell. In the latter, we will observe adjacent double bridges, which may not be distinguishable from single anaphase bridges by immunofluorescence. The ratio of the occurrence of these two events is unknown, but almost certainly the frequency of anaphase bridges in following divisions is diluted out in the entire population. Dicentric chromosomes however, will remain a constant frequency. Indeed, cells with anaphase bridges do not always give rise to progeny with bridges by live cell analysis on bridge resolution in oral cancer cells (Diane Hoffelder, unpublished data and my data not shown).

We have shown by live cell analysis that in oral cancer cells, cells with anaphase bridges survive, and the majority of them break their bridges in anaphase, often resulting in formation of micronuclei. This is consistent with the previous study describing a linear relationship between micronuclei and anaphase bridges in tumors (Gisselsson, Bjork et al. 2001). The vast majority of these micronuclei are transcriptionally inactive, according to lack of RNA polymerase II immunofluorescence staining and failure to incorporate fluorescently-labeled dUTP (Hoffelder, Luo et al. 2004). Hoffelder et al. revealed that these micronuclei have defective nuclear pore complexes and nuclear import failures. Hence, anaphase bridges in these cells actively contribute to CIN by breaking and giving rise to transcriptionally inactive micronuclei. The fact that a bridge often has multiple break points implies a more complicated mechanism than mere mechanical forces pulling and breaking it. Fragile sites, DNA binding proteins or cytokinesis machinery may all play a role in the outcome of bridge breakage. This warrants further investigation.

Our results have revealed for the first time that CSC can induce DNA DSBs, and this process requires a metal catalyst and hydrogen peroxide *in vitro*. It has been shown that cigarette smoke can induce SSBs in DNA both *in vitro* and in cultured cells via the Fenton reaction-induced formation of ROS (Cosgrove, Borish et al. 1985; Nakayama, Kaneko et al. 1985), but DSBs were not observed in these earlier experiments. Thus, both SSBs and DSBs from the CSC result from oxidative damage. In cells, SSBs can be replicated to form DSBs, but the direct relationship, if any, between these two types of DNA damage is less clear. Radiation-induced hydroxyl radicals can form on a linear track created by the ionizing particle, thus frequently producing SSBs on adjacent DNA strands (Lomax, Gulston et al. 2002). If these two SSB sites are within 6-10 base

pairs of each other, a DSB can be created (Hanai, Yazu et al. 1998). We have shown that CSC induces DNA DSBs *in vitro* in a similar manner as DNase I, and both DSBs and SSBs were prevented by inhibiting ROS, suggesting that CSC-induced DNA DSBs are due to accumulation of SSBs. However, we cannot exclude the possibility that these DSBs may represent a single molecular event instead of adjacent SSBs. Regardless of how DSBs form in solution, CSC induces DSBs in cells immediately (within 18 min after the addition of CSC), not relying on replication of SSBs. In addition, DSBs and SSBs are induced by CSC independently of DNA length. We thus suggest the same mechanism of DSB induction in cell culture as in solution.

Our *in vitro* data are consistent with the conclusion that DNA breaks from cigarette smoke exposure are the result of formation of ROS. Both the gaseous and particulate phases of smoke can give rise to active oxygen derivatives. The ROS from CSC are generated mostly from polyphenols, such as catechols and catechol-methyl derivatives, as well as a hydroquinone-semiquinone-quinone redox system to form hydrogen peroxide (Pryor and Stone 1993). Both cellular fluids and the cigarette tar itself contain metal ions that can catalyze the production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction: H_2O_2+M(n-1)-->HO•+HO-+M(n)(Avunduk, Yardimci et al. 1997; Kodama, Kaneko et al. 1997). Fenton reaction-generated free radicals are known to be capable of inducing DSBs (Chevion 1988). The prevention of ROS by various antioxidants eliminates CSC-induced DSBs. Therefore, we propose that CSC is inducing the formation of DSBs via ROS.

Antioxidants that we tested for prevention of CSC-induced DSBs include both chemical compounds and a dietary antioxidant. dGMP is known to quench ROS and is oxidized into 8-

OH-dG (O'Neill 1983; Kasprzak, North et al. 1992; Henle, Luo et al. 1996). Histidine is a free amino acid and is antioxidative by acting as a chelator of metal ions. It removes transition metals from their reactive site resulting in a markedly decreased rate of ·OH formation (Nagy and Floyd 1984; Chevion 1988; Kukreja, Loesser et al. 1993). Both dGMP and histidine prevent CSC-induced DSBs and anaphase bridges, showing that CSC is inducing both DSBs and bridges via ROS.

A dietary antioxidant we found able to prevent DSBs from CSC exposure was resveratrol, a naturally occurring phenolic compound which is present in various food sources including wine and peanuts. This compound was shown previously to prevent CSC-induced DNA SSBs by the comet assay (Sgambato, Ardito et al. 2001). We show here that both SSBs and DSBs are strongly prevented by this reagent. Resveratrol has been effective in inhibiting development of mammary gland preneoplastic lesions and epithelial tumor formation in mice (Jang, Cai et al. 1997). Resveratrol did not prevent lung tumor multiplicity in A/J mice exposed to the tobacco carcinogen 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)(Hecht, Kenney et al. 1999). However, this mouse strain is predisposed to adenocarcinomas indicating some of the early mutagenic changes required for tumor formation may be inherited. Our results support further analysis of resveratrol as a chemopreventive agent for smoke-induced genomic damage.

The mechanism by which resveratrol prevents DNA damage is still being investigated. It is known to be an inhibitor of cytochrome oxidase P450 (Ciolino, Daschner et al. 1998) and some carcinogens present in cigarette smoke are converted into DNA-damaging metabolites by hepatic cytochrome P450-related enzymes (Bartsch and Hietanen 1996). Thus, one mechanism that

could explain the chemoprotective activity of resveratrol is by blocking P450-mediated chemical conversion (Villard, Seree et al. 1998). However, our data also show that resveratrol can also have a direct role in preventing DNA damage in the absence of other cellular factors, suggesting more than one mechanism is involved in its antioxidative activity.

Anaphase bridge formation is likely to result from DSBs induced by the CSC exposure. DSBs are strongly linked to breakage-fusion-bridge cycles and the formation of dicentric chromosomes (Zhu, Mills et al. 2002). Chromosomal translocations have been observed to result when two DSBs were introduced in the cell by exogenous restriction endonucleases (Richardson and Jasin 2000) or as a result of defects in DSB repair pathways (Ferguson, Sekiguchi et al. 2000). If such a translocation produced a dicentric chromosome, an anaphase bridge may form in the next mitosis. Telomeric association may result from fusions of chromatin following DSBs that occur at the telomere region, which appears as telomeres on bridges in anaphase. On the other hand, DSBs occurring proximal to centromeres beyond sub-telomere regions may produce telomere-less anaphase bridges. Both types of anaphase bridges induced by CSC were observed by *in situ* hybridization, suggesting DSBs may occur either within or beyond telomere regions. The sample size for this data is very small and the experiment needs to be redone with a more reliable technique. However, our preliminary results suggest that the frequency of DSBs in telomere regions may be higher than expected by chance. This high occurrence of DSBs within telomere regions may be due to the preferential accumulation of single-stranded regions in telomeres that are more susceptible to ROS (Petersen, Saretzki et al. 1998).

One other possibility for how anaphase bridges form has been difficult to test. Instead of the covalent bonds of DNA fusion, anaphase bridges might also form from unresolved proteins binding to DNA or to each other, most likely DNA repair proteins or a late-resolving cohesion complex. Most DNA repair proteins, for example Ku80, have diffused immunostaining in mitosis and are technically difficult to test (data not shown). On the other hand, two components of mammalian cohesion complex, hSmc1 and hRad21 have negative immunostaining on anaphase bridges in both UPCI:SCC172 cells and CSC-treated fibroblasts (data not shown), suggesting that anaphase bridges may not be a result of cohesion defects in these cells.

Chapter III : Multipolar spindles arise primarily in multinucleated cells

1. Introduction

Chromosome instability, defined as a continuous change in the structure or number of chromosomes, is proposed to be a key mechanism driving the genomic changes associated with tumorigenesis (Saunders, Shuster et al. 2000; Pihan, Wallace et al. 2003; Gisselsson, Palsson et al. 2004). A major cause of chromosomal instability in cells appears to be segregational defects during mitosis. One source of these divisional errors in cancer cells is centrosome amplification, which has been suggested to play a role in tumor formation for over a century (Wunderlich 2002). Supernumerary centrosomes have been reported in a variety of carcinomas including breast, gall bladder, lung, bone, pancreas, colorectal and prostate (Lingle, Lutz et al. 1998; Carroll, Okuda et al. 1999; Kuo, Sato et al. 2000; Pihan, Purohit et al. 2001). Centrosomal amplification is also seen as part of many cancer-related mutant phenotypes. For example, loss of the tumor suppressors p53 and Rb (Tarapore and Fukasawa 2002), defects in many DNA-damage control genes including BRCA1, BRCA2, ATM, and ATR (Smith, Liu et al. 1998; Tutt, Gabriel et al. 1999), or expression of human papillomavirus or adenovirus oncoproteins (Duensing and Munger 2002; Schaeffer, Nguyen et al. 2004), all lead to abnormally high numbers of centrosomes in the cell. Thus, centrosomal amplification is part of many oncogenic pathways. The main impact of supernumerary centrosomes appears to be to increase the chances that the microtubule spindle formed in the subsequent mitosis will be multipolar and that the chromosomes will be unequally distributed to multiple daughter cells (Brinkley 2001; Nigg 2002; Sluder and Nordberg 2004). Consistent with this conclusion, centrosomal changes, including amplification, are strongly linked to aneuploidy and chromosomal instability in

numerous studies (Lingle, Lutz et al. 1998; Ghadimi, Sackett et al. 2000; Lingle, Barrett et al. 2002; Pihan, Wallace et al. 2003).

Centrosome amplification may occur from centrosome over-replication, cell fusion or failure of cytokinesis (reviewed in (Fukasawa 2002; Nigg 2002; Sluder and Nordberg 2004)). While centrosomal amplification can be induced experimentally by any of these three mechanisms, it is currently unknown which pathway is most relevant for MPS formation in cancer cells.

Division with MPS is suspected to lead to inviable or apoptotic progeny due to the loss of chromosomes from multipolar division (Brinkley 2001; Nigg 2002; Sluder and Nordberg 2004). Paradoxically however, centrosome numbers actually increase during tumor progression (Pihan, Purohit et al. 1998; Lingle and Salisbury 1999; Ried, Heselmeyer-Haddad et al. 1999). How do cancer cells prevent the presumed mitotic catastrophe associated with MPS in order to allow survival and even expansion of the cells with amplified centrosomes? One proposed model is that tumor cells may cluster centrosomes together to allow bipolar division, thus at least temporarily stabilizing the genome (Salisbury, Whitehead et al. 1999; Brinkley 2001; Nigg 2002). But it remains unclear how often MPS lead to loss of cell viability and whether compensatory mechanisms are necessary.

We have investigated MPS formation in human embryonic kidney cells and oral cancer cells using a histone-GFP marker for chromosomes and a farnesylated-GFP marker for the plasma membrane (Kanda, Sullivan et al. 1998; Haigo, Hildebrand et al. 2003). In both cell lines, nearly all the MPS arose from cells that were multinucleated in interphase, indicating that a failure of

cytokinesis is associated with multipolarity in these cells. The corollary was also true, failure of cytokinesis always gave rise to multinucleation, while multinucleation was rarely seen in the absence of cytokinesis defects. Unexpectedly, many cells with MPS not only survived mitosis, but divided again, indicating that the division with MPS did not necessarily severely reduce the chromosome number. This paradox may be explained by our observation that cells with MPS nearly always had an incomplete cytokinesis, combining different segregated chromosome sets into the same cell. We suggest a model whereby failure of cytokinesis gives rise to a self-perpetuating population of cells that contain MPS and multiple nuclei. This pool of cells is proposed to serve as a testing ground for generating viable aneuploid cells that serve as the basis for clonal selection during tumorigenesis.

2. Results

2.1. MPS primarily arise in cells with more than one nucleus

In order to investigate the origin and impact of MPS, both tumor and non-tumor cells grown in culture were examined by live-cell microscopy. Human embryonic kidney cells (HEK-293) were transiently transfected with a plasmid expressing GFP-histone H2B (Kanda, Sullivan et al. 1998), and MPS in metaphase cells were identified by irregular chromosome alignment; typically a "Y" or "T" shape for tripolar and an "X" shape for tetrapolar spindles. Immunofluorescence studies of fixed cells confirmed that these abnormal alignments are only observed in cells with MPS ((Saunders, Shuster et al. 2000) and data not shown). Strikingly, the vast majority of MPS arose in multinucleated cells, defined here as cells with two or more nuclei. As shown in Figure 25 (and Supplemental movie 2), a binucleated cell entered mitosis with chromosomes from both nuclei undergoing simultaneous condensation and chromosomes aligned together on a single MPS. Out of a total of 36 cells observed to form MPS, 34 arose from multinucleated cells, and only two from mononucleated cells. Similar results were found in the oral squamous cancer cell line UPCI:SCC103 transfected with GFP-histone H2B, where 25 out of 27 (93%) MPS arose from multinucleated cells. These results show that MPS arise primarily in multinucleated cells in these two cell lines.

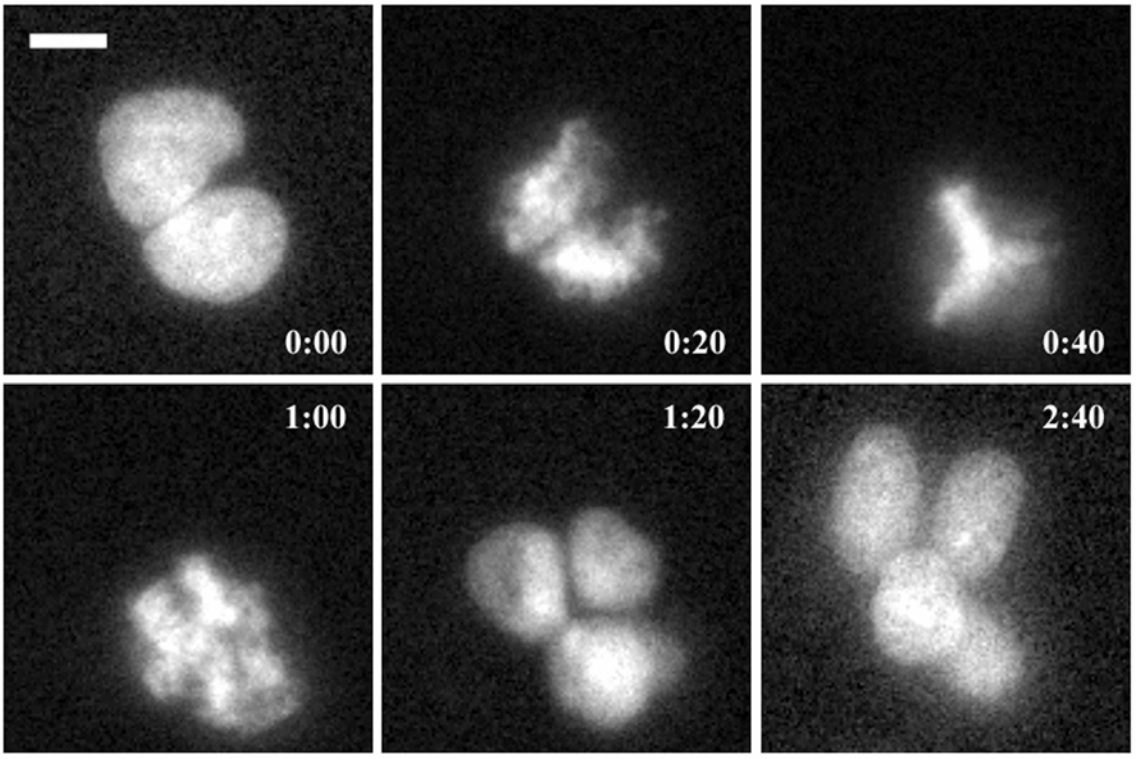


Figure 25

Figure 25. MPS arise primarily in multinucleated cells. HEK-293 cells were transfected with a plasmid expressing GFP-histone H2B and viewed at 20-min intervals by live cell epifluorescence microscopy. In this and subsequent figures, only selected time points are shown. A binucleated cell in interphase (0 min) began chromosome condensation (20 min), aligned its chromosomes on a single MPS (40 min), and divided in multiple directions into a tetranucleated cell (2 hr, 40 min). Bar is 10 μm, and time is in hours:minutes.

2.2. Failure of cytokinesis suffices for formation of MPS

Multinucleated cells usually result from a failure of cytokinesis (Fournier and Pardee 1975; Rigby and Papadimitriou 1984). Multinucleation can also occur by fusion of two or more cells, for example following exposure to X-rays or UV treatment (Kura, Sasaki et al. 1978; Brathen, Banrud et al. 2000). To investigate whether multinucleated cells result from cytokinesis defects in these cells, we transfected HEK-293 and UPCI:SCC103 cells with two plasmids, one expressing GFP-histone H2B and a second one encoding a membrane-GFP marker (Haigo, Hildebrand et al. 2003) to identify the position of the plasma membrane. The great majority of multinucleations observed to form in these cell lines were due to failure of cytokinesis (19 out of 20 in HEK-293 cells and 15 out of 16 in UPCI:SCC103 cells). The rest resulted from cell-to-cell fusion. The frequency of cytokinesis failure was ~ 10% of mononuclear cells with a bipolar division (10 out of 98 total) in HEK-293 cells. Examples of a successful cytokinesis are shown in Figure 26 and an aborted cytokinesis in Figure 27 (Supplemental movie 3 and 4). Similarly, 10.3% (8 out of 78) of the bipolar UPCI:SCC103 cells failed cytokinesis as determined by GFP fluorescence of live cells. This compares with published frequencies of ~5% in both BSC1 monkey kidney cells (Piel, Nordberg et al. 2001) and p53 -/- mouse embryonic fibroblasts (Sluder and Nordberg 2004).

To rule out that these results were influenced by transfection conditions, GFP expression or UV exposure, we also measured the frequency of cytokinesis failure by differential interference contrast (DIC) microscopy. Examples of successful and aborted cytokinesis following bipolar division in UPCI:SCC103 cells are shown in Figure 28 and 29 (and Supplemental movie 5 and 6). The HEK-293 cells did not grow flat enough in culture to be visualized by DIC. The

frequency of cytokinesis failure in UPCI:SCC103 determined by DIC was 10.1% (7 out of 69 total divisions), consistent with that observed by fluorescence. Is this frequency sufficient to account for the multipolarity observed in the cancer cells? The frequency of MPS in fixed samples stained with antibodies to γ-tubulin were ~ 15% in UPCI:SCC103 and ~ 10% in HEK-293 cells. However, we found the likelihood that a cell would divide with MPS was less than this. The chance that an individual cell would divide with a MPS was determined by live cell imaging, at 9.9% (19 out of 192 total divisions) in HEK-293 cells, and 8.7% (10 out of 115) in UPCI:SCC103. Thus, the frequency of failure of cytokinesis is sufficient to account for all of the spindle multipolarity observed in both cell lines.

Cultured cells grow in an environment lacking the normal tissue architecture found *in situ,* and late-stage events in cytokinesis are influenced by tension generated through interactions of the dividing cell with its extracellular environment (Burton and Taylor 1997). To confirm that the observed cytokinesis failures in these cells were not an artifact of the culture conditions, we also examined normal uvulopalatopharyngoplasty specimens (UP3 cells) from surgical samples of human tissue grown and viewed under similar conditions (Rubin Grandis, Zeng et al. 1996). The tissue samples are a mixture of cell types including fibroblasts and keratinocytes. We examined 47 mitotic divisions in real time by DIC in these untransformed cells and each completed cytokinesis normally. Consistent with the absence of cytokinesis defects, no multipolar divisions were observed in real time. In addition, only 1.8% of the metaphase spindles were multipolar (n=56) and 2.6% of the interphase cells were multinucleated (n = 507) in fixed samples viewed by immunofluorescence. While we cannot rule out that long term growth in culture may affect the frequency of cytokinesis failure, the cytokinesis defects we observed were

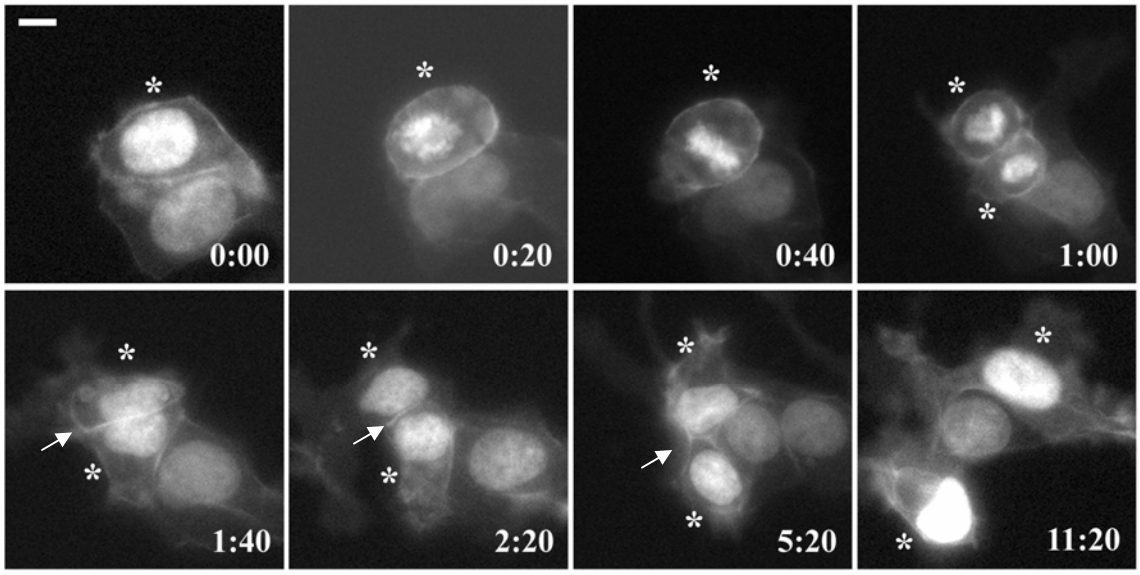


Figure 26

Figure 26. An example of successful cytokinesis in HEK-293 cells imaged by immunofluorescence. The example cell and its progeny are marked with asterisks. The cell was in metaphase 40 min after the start of the viewing period and a cleavage furrow was observed at 1 hr (arrow). By 11 hr and 20 min, the completely divided cells had migrated apart. Images were taken every 20 min.

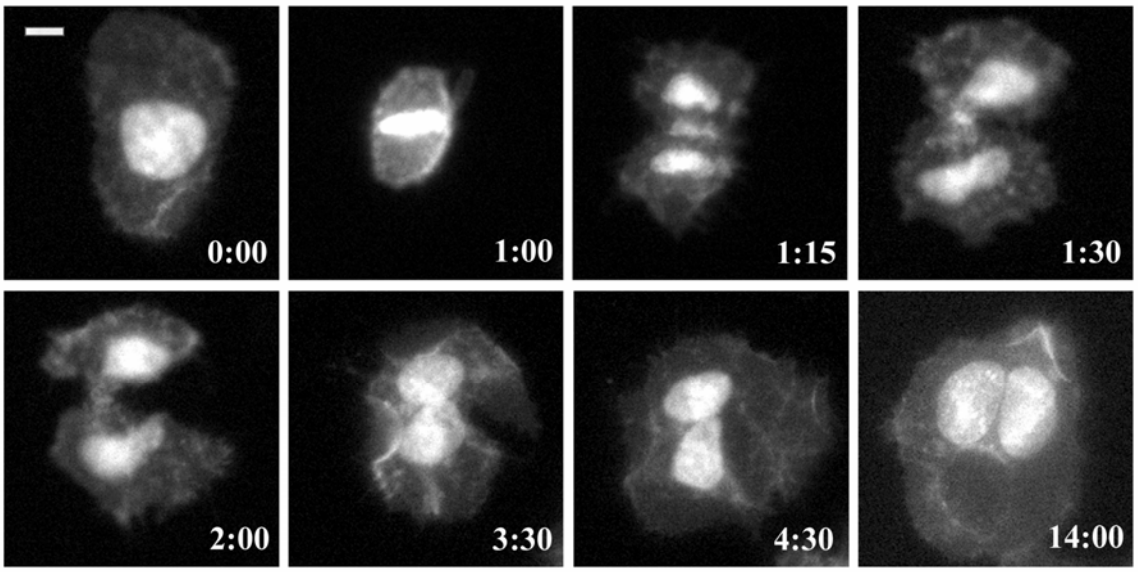


Figure 27

Figure 27. An example of an aborted cytokinesis in HEK-293 cells imaged by immunofluorescence. The cell was in metaphase by 1 hr and began cytokinesis by 1 hr and 15 min, as shown by constriction of the membrane GFP marker. The cleavage furrow has regressed by 3 hr 30 min, and by 4 hr 30 min a binucleated cell formed. The nuclei stay associated together in the same cell ≥13 hr after mitosis. Images were taken every 15 min.

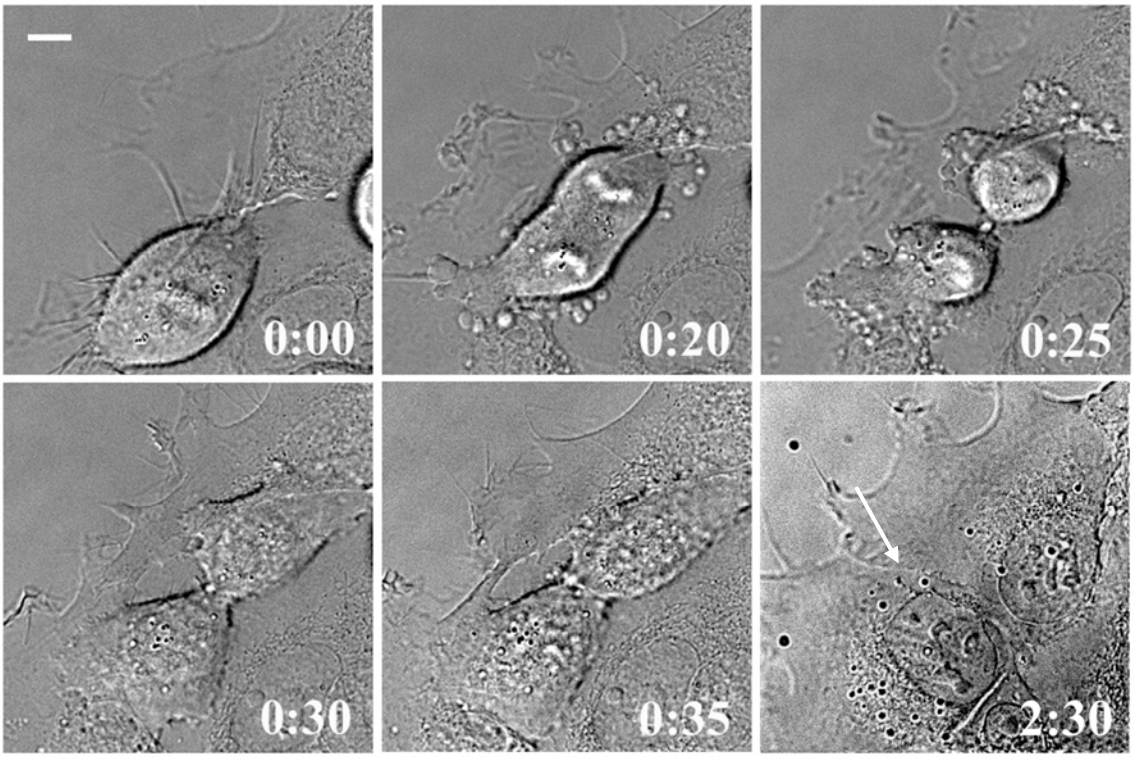


Figure 28

Figure 28. An example of a successful cytokinesis in UPCI:SCC103 cells imaged by DIC microscopy. The cell is in anaphase at 20 min and a contractile ring is observed at 25 min. By 2 hr and 30 min a distinct plasma membrane cleavage is visible between daughter cells (arrow) and the nuclei are separated. Cells imaged every 5 min.

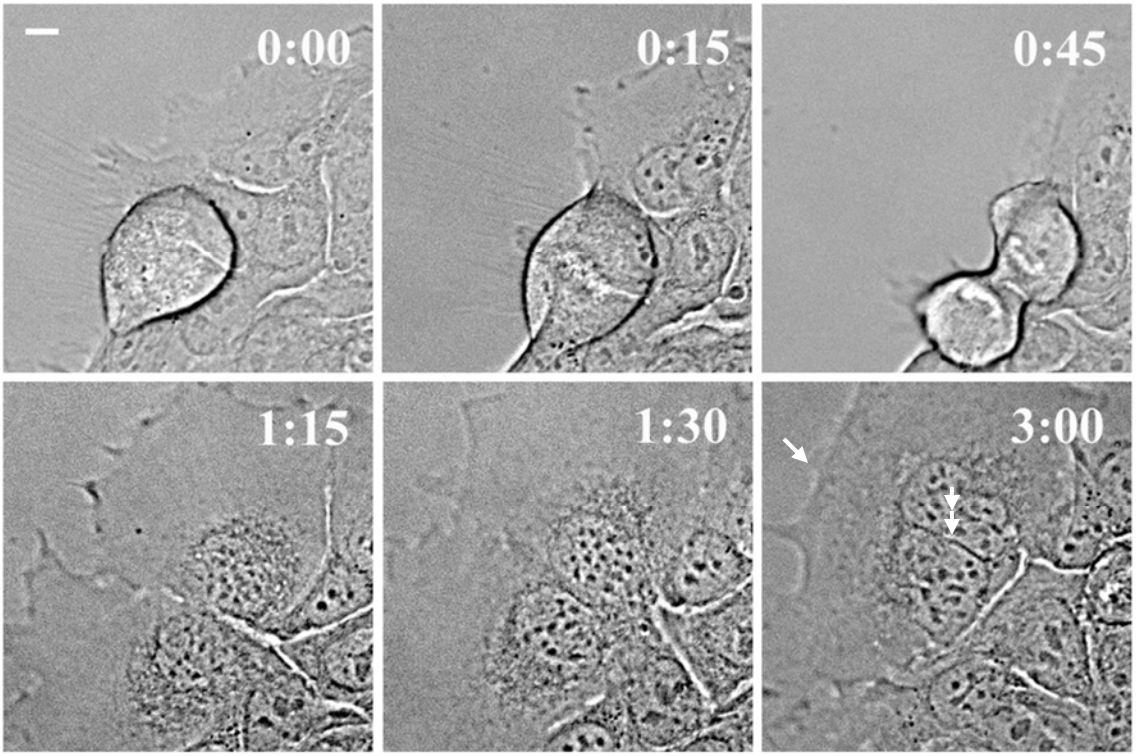


Figure 29

Figure 29. An example of an aborted cytokinesis in UPCI:SCC103 cells imaged by DIC microscopy. The cell initiates contraction at 45 min and a cleavage furrow forms at 1 hr 15 min. But by 1 hr 30 min the furrow has begun to regress, and at 3 hr a binucleated cell has formed. Note the continuous plasma membrane at 3 hr (arrow) and the close proximity of the two nuclei in the binucleated cell (double arrows).

apparently not a consequence of the immediate *in vitro* culturing or microscopic imaging conditions.

2.3. Failure of cytokinesis result in subsequent formation of MPS

In addition to the similarity between the frequencies of cytokinesis failure and MPS that we have found, we performed a more direct analysis of their correlation. When cytokinesis failed, a MPS was invariably seen in the next cell division (Figure 30 and Supplemental movie 7). Nine cases of cytokinesis failure were imaged through a second division in GFP-histone-labeled HEK-293 cells, and all of them formed MPS in the following mitosis. Due to the difficulty in imaging two successive divisions in a minority of cells, this sample size is low. None of 34 cells with complete cytokinesis divided with MPS in the next division. These observations suggest that a failure of cytokinesis usually leads to MPS formation in the subsequent division.

2.4. Cells with MPS mostly survive but result in cytokinesis failure

We next examined the ability of cells with MPS to complete a normal mitosis and divide again. In HEK-293 cells, those with a bipolar division completed anaphase and exited mitosis 100% of the time (n= 93). By comparison, 88% (n= 40) of cells with MPS completed anaphase and exited mitosis, as determined by chromosome decondensation and reformation of the nucleus. The multipolar cells that were unable to proceed usually arrested in metaphase for various periods of time before apparently apoptosing, as determined by the appearance of nuclear blebbing. Similarly, when we examined UPCI:SCC103 cultures we saw 72% (n= 46) of cells with MPS were able to complete anaphase and exit mitosis. Thus, cells with multipolar spindles are typically able to complete mitosis, consistent with previous observations indicating the lack

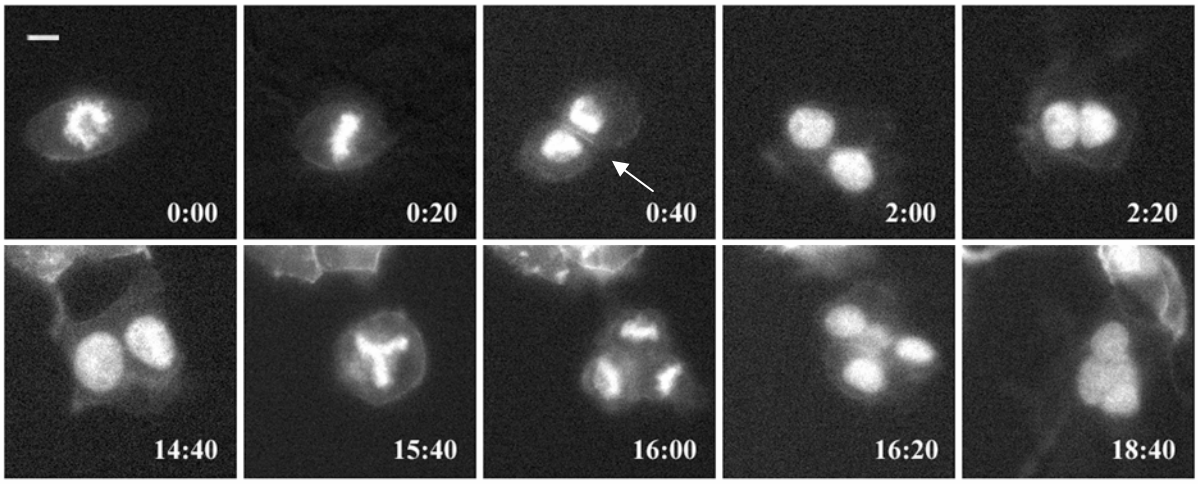


Figure 30

Figure 30. Failure of cytokinesis results in the formation of MPS. HEK-293 cells were transfected with a plasmid expressing GFP-histone H2B and a membrane-GFP marker. The cell first divided with a bipolar division (20 min) and an initial cleavage furrow forms at 40 min, seen as the thin fluorescent line between nuclei (arrow). The cleavage furrow regressed and by 2 hr and 20 min a binucleated cell had formed. At 14 hr the nuclei remained surrounded by a single plasma membrane, and at 15 hr and 40 min chromosomes from both nuclei condensed synchronously and aligned on a single spindle, all indicative of a single cell with two nuclei. In the second division, the cell formed a MPS that segregated the chromosomes into what appeared to be a tetranucleated cell (18 hr 40 min). Images were taken every 20 min.

of a mitotic checkpoint to block division in cells with spindle polarity defects (Sluder, Thompson et al. 1997). Starting with dividing cells may bias the sample towards more viable members of the cell population. To avoid this bias, we also measured the frequency at which multinucleated cells (identified in interphase) divide. In the UPCI:OSCC103 cell culture, 45% (n=22) of multinucleated cells divided within 18 hr (average cell cycle=15 hr, n=9), while 83% (n=30) of the HEK-293 cells divided (average cell cycle=16.5 hr, n=34). While these numbers are lower than the frequency of MPS exiting mitosis, they indicate that many of the multinucleated cells remain capable of further division.

In a related assay, we also investigated whether cells that divide with MPS were viable enough to divide again. The average HEK-293 cell cycle is 16.5 hours in duration as determined by live cell imaging, and the maximum viewing time of the cells under the conditions used is ~ 30 hours. We began the imaging process of cells in interphase. Of the cells that divided with a bipolar division, >90% (n=34) of the daughters divided again within 27 hours. Of the cells that divided with a multipolar division, at least one or more of the daughters from 50% (n=8) of the divisions entered mitosis again within 26.5 hours. Again, this sample size is inevitably low, but there appears to be a substantial loss in viability from multipolar division. However, some of the cells are able to survive and divide again, therefore potentially contributing to future generations of the cell population.

Although most cells with MPS were able to exit mitosis, and in some cases divide again, we found that they consistently had difficulties in completing cytokinesis. In all 14 cases of HEK-293 cells with MPS that were clearly imaged throughout mitosis, either a partial or a complete

failure of cytokinesis was observed. Similarly, 10 out of 13 UPCI:SCC103 cells with MPS did not complete cytokinesis. In some cases, cytokinesis was completely blocked. But in many cases, a partial failure of cytokinesis was observed (12/14 divisions of HEK-293 and 7/13 divisions of UPCI:SCC103), giving rise to a mixture of mononucleated and multinucleated cells (Figure 31A and Supplemental movie 8). In some cases, mononuclear formation included two sets of chromosomes being enclosed in the same nucleus (Figure 31B and Supplemental movie 9). Thus, the frequency of cytokinesis failure jumps to ~ 80% of the cells with MPS, so that when the cells divide with MPS they may regain some or even all of the chromosomes that would have been lost by the multipolar division.

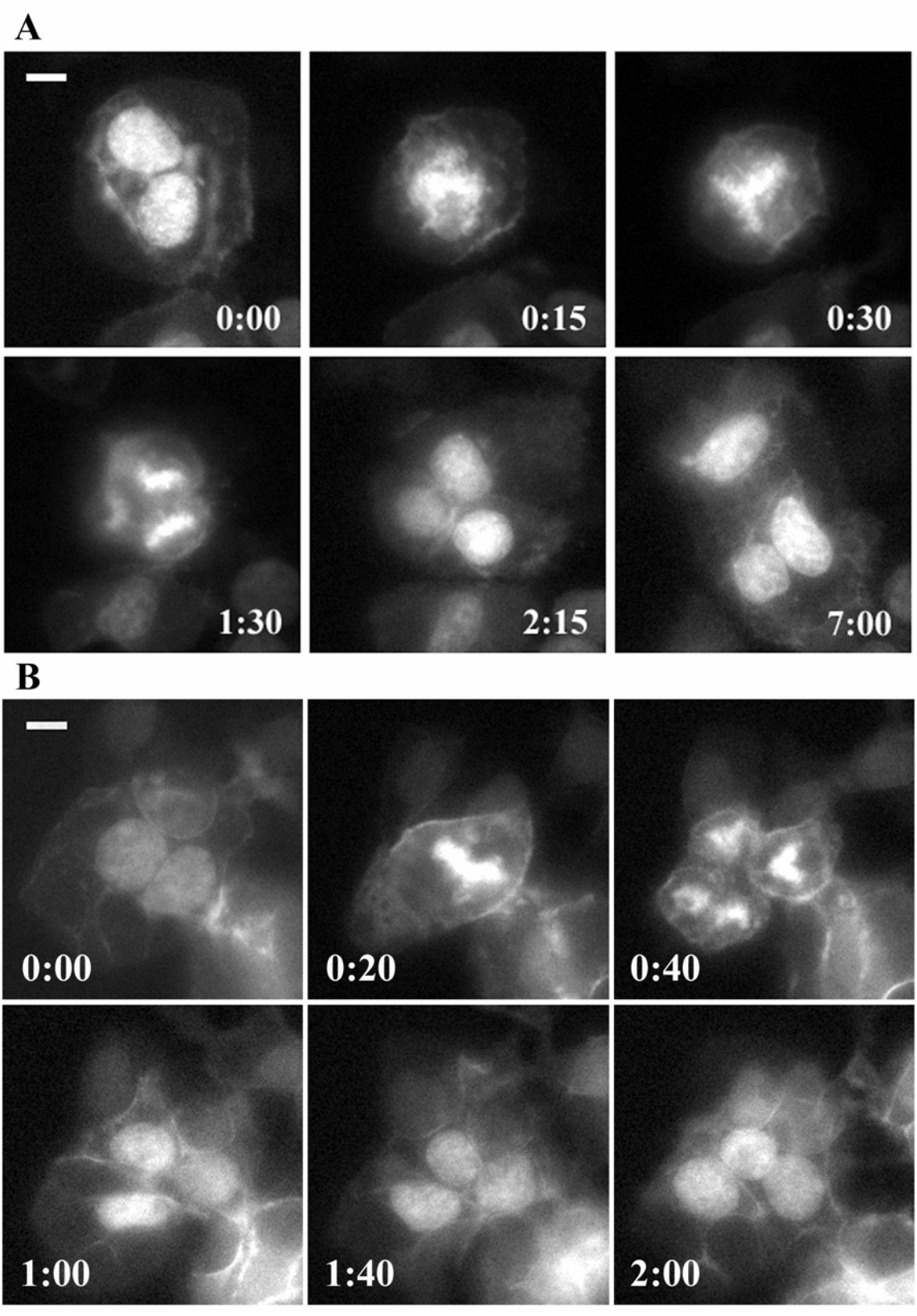


Figure 31

Figure 31. Multinucleated cells fail at cytokinesis. HEK-293 cells were transfected with histone H2B and plasma membrane GFP markers. Two examples of the partial failure of cytokinesis are shown. (A) A binucleated cell was imaged (0 min) and entered mitosis 15 min later. Two hours and fifteen minutes later the cell had exited mitosis. (Note that mitosis was sometimes delayed in multipolar cells and the mechanism is currently under investigation.) Cytokinesis was incomplete, and mononuclear (asterisk) and binuclear cells were formed. Arrows mark the two plasma membranes separating the two cells. (B) In this cell a single nucleus formed around two sets of anaphase chromosomes. Anaphase is shown at 40 minutes and asterisks mark the two sets of chromosomes. At 1 hr a single nucleus formed around both chromosome masses (double asterisks).

3. Discussion

We have shown that MPS most frequently arise in multinucleated cells and only rarely in mononucleated cells in the two cell lines tested. Multinucleated cells were observed to arise from a failure of cytokinesis, and the frequency of aborted cytokinesis was similar to the frequency of MPS. Therefore, a failure of cytokinesis, not centrosome over-replication, is most likely the source of MPS formation in these cells. A further analysis of cytokinesis in other cell types will be required to generalize these observations. Based on our current results, we cannot rule out a role for centrosomal over-replication in MPS formation. However, if centrosomal amplification results from over-replication, then an additional change leading to failure of cytokinesis must also occur in the cells tested. Since centrosomes play a role in cytokinesis (Piel, Nordberg et al. 2001), it is possible that the cytokinesis defects are a secondary phenotype of the over-replication of the centrosomes. However, there is no evidence to support this model, and the simplest interpretation of the data is that the cytokinesis defect is sufficient in itself to account for both the observed multinucleation and spindle multipolarity.

Our results address an important question about the presumed viability of cells with multipolar spindles. Since division into more than two sets of chromosomes seems to invariably lead to chromosome loss, how can cells with MPS remain viable and contribute to genomic instability in the tumor? Since MPS were always associated with, in fact we believe caused by, cytokinesis failure, we suggest that multipolar division does not necessarily lead to a loss of chromosomes or a reduction of cell viability. Indeed, ~50% of the cells with MPS gave rise to at least one daughter capable of further division. We interpret our data to indicate that the chromosomal

complement the daughter of a MPS cell receives is determined not only by the outcome of spindle segregation, but also by whether a cleavage furrow forms between adjacent chromosome sets. Since this often fails, many daughter cells appear to receive more than the one set of segregated chromosomes, apparently enough genetic material to divide again. These results can explain the viability of daughters of multipolar division and reveal for the first time the interplay between the resolution of spindle multipolarity and the completion of cytokinesis.

Previous observations have shown that tetraploidization often precedes aneuploidy in solid tumors (Shackney, Smith et al. 1989; Levine, Sanchez et al. 1991; Galipeau, Cowan et al. 1996; Southern, Evans et al. 1997; Lengauer, Kinzler et al. 1998). However, later stage aneuploid tumor cells typically contain irregular, near triploid numbers of chromosomes (Gollin 2004). How does the early tetraploid population evolve to the aneuploid population of cells? We suggest that a failure of cytokinesis results in an initial tetraploidization of the cell genome, and at the same time amplifies centrosome number, thereby inducing a series of multipolar divisions. The irregular divisions that follow centrosomal amplification may transform the initial tetraploid chromosome complement into the variably aneuploid cells that serve as the basis for clonal evolution and selection during tumorigenesis.

We suggest that the cells with MPS exist as a cycling pool of multinucleated cells (Figure 32). The population is sustained by cytokinesis defects, both in preexisting multinucleated cells, and also arising *de novo* in mononucleated cells. And we observe that some multinucleated cells are lost from the pool by cell death or arrest. But our observation that a significant portion is viable enough for multiple divisions implies that there is an opportunity for selection to favor the most

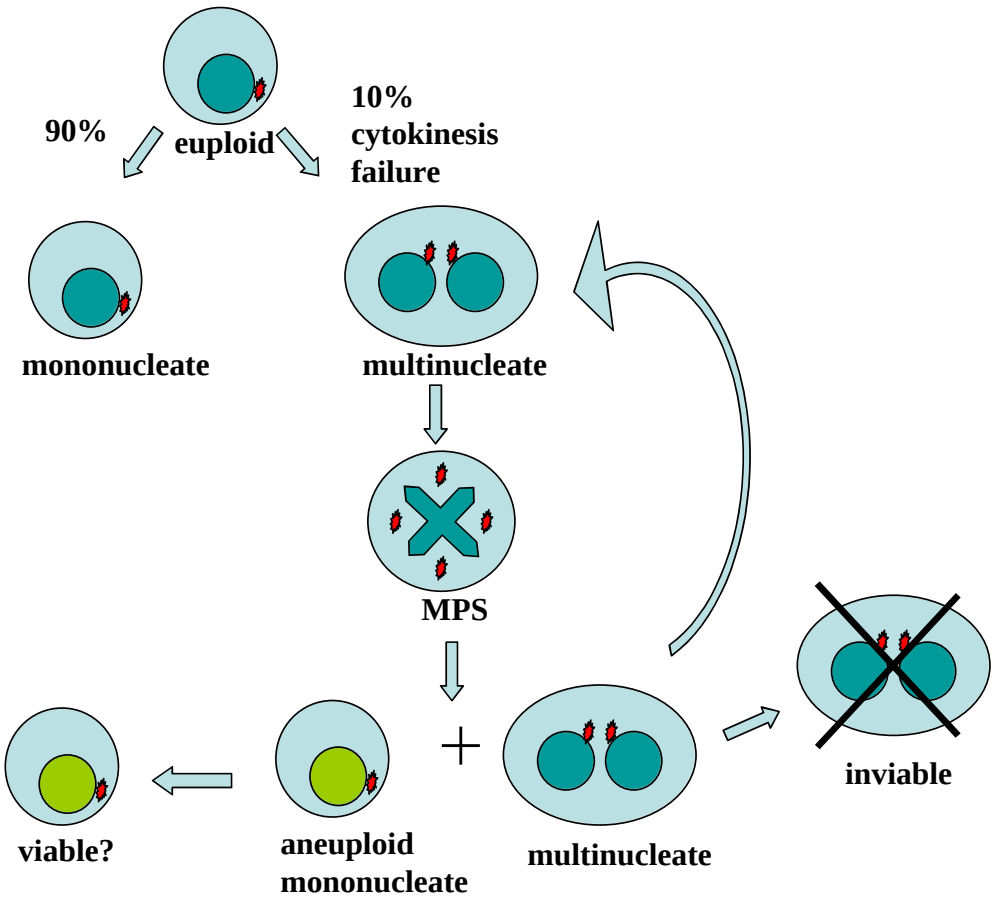


Figure 32

Figure 32. A model is shown to describe the maintenance of a pool of multinucleated and multipolar cells. Cells enter the pool following failure of cytokinesis, which in the populations examined occurred ~ 10% of the time. Multinucleated cells then divide with MPS due to the presence of amplified centrosomes, but usually fail to complete cytokinesis giving rise to multinucleated daughters. Some of these multinucleated cells apoptose or arrest, but others are able to divide giving rise to another generation of multipolar and multinucleated daughters. Occasional mononucleated cells are derived from the multipolar division (chartreuse). We speculate that some of these may give rise to stable mononucleated but aneuploid clonal lines, however, this requires further testing.

adapted. Thus, we propose that this pool of multinucleated cells is a nursery for the creation of the viable aneuploid cells associated with tumor formation. Importantly, some mononucleated cells appear to arise from the multipolar divisions. It will be interesting, in future experiments, to determine if these mononuclear cells can exit the proposed MPS pool and exist as a stable line of bipolar, but aneuploid, clonal lines.

Chapter IV : Summary and speculations

Chromosome segregational defects are invariant features in cancer cells. Many of these dramatic changes of cell morphology have been used as biomarkers for tumors and carcinogenesis. However, how they arise, resolve, and are inherited in cells mostly remain a mystery. Focusing on two chromosome segregational defects, anaphase bridges and multipolar spindles, we have explored their origin, fate and contribution to genomic instability in primary and cancer cells.

Anaphase bridges arise within two cell cycles after a single genetic insult (i.e. exposure to cigarette smoke) in cultured primary cells, suggesting that this chromosome segregational defect may also occur early *in vivo* without any pre-requisites, such as loss of checkpoint control. The subsequent decrease in frequency implies that the induced bridges may be selected against across the entire population. It is not clear whether this decrease is due to a healing mechanism, for example addition of nascent telomeres that stabilizes dicentric chromosomes, or by an elimination mechanism, for example, an apoptotic pathway other than the p53-mediated one, or simply a cell cycle arrest in affected cells. The bridges always maintain a certain baseline level, dependent on cell lines, and this level does not increase an appreciable amount during cell culturing. This is not likely due to a general limit of bridge capacities, since cell lines with 100% anaphase bridges do exist (Blazak, Stewart et al. 1986). Why, then, are new bridges not tolerated compared to the original ones? One explanation is that there is more than one type of bridge, each of which results in a different fate. For example, chromatid-type and chromosome-type bridges have different ratios of bridge inheritance (Zheng, Roseman et al. 1999). Or it may be

that the induced bridges have to fulfill some conditions to be stable, such as gain or loss of specific genes.

Most anaphase bridges inevitably break, and often into multiple fragments in cancer cells. Why cells with bridges have multiple breakage sites remains unknown, but it may relate to chromosome fragile sites. In some cases (less than 10%), anaphase bridges persist into interphase and apparently cause failure of cytokinesis in a bipolar division.

Anaphase bridges are sufficient to cause a sub-population of cells to have irreversible chromosome reconstitution in interphase primary cells. These cells may have sporadic genomic imbalances produced by CSC-induced ROS, as there is no statistical bias towards any individual chromosome when examining chromosome rearrangement events. Most likely they will encounter cell cycle checkpoints and many may not divide. However, such a pool of genomicly-imbalanced cells may generate viable aneuploid cells under favorable circumstances. These cells may gain growth advantages over other undamaged cells and eventually contribute to generating a tumorigenic population.

Multipolar spindles arise primarily in multinucleated cells in the two cell lines tested. Thus, the origin of MPS in such cells is centrosome amplification associated with failed cytokinesis. Why is this type of centrosome amplification, but not centrosome over-replication the primary source of MPS formation? One hypothesis is that both euploid and polyploidy cells with extra centrosomes may divide in a multipolar fashion, but polyploid cells are advantageous to generating viable progeny, as their daughter cells may acquire more genetic information.

Moreover, such progeny contains a mixed population of multinucleated and mononucleated cells, as cells with MPS have a high frequency of failure of cytokinesis. The multinucleated cells may serve as a cycling pool through MPS formation to generate stable clonal aneuploid cells. In this way, cells not only maintain their occurrence of supernumerary centrosomes and MPS, but also continuously produce aneuploid mononucleated cells. These aneuploid cells may contribute eventually to tumor progression.

As the vast majority of multinucleation observed were a consequence of cytokinesis failure, this defect seems to be heritable. A simple explanation is that a single mechanism is driving both the initial failure of cytokinesis in a bipolar division and subsequent failure in a multipolar division. It is also possible that cytokinesis defects in a multipolar division are due to a limited amount of protein or membrane sources insufficient to cleave all chromosome sets (Sluder and Nordberg 2004). Preliminary data show that failure of cytokinesis in these cells occurs at an early stage around furrow ingression and central spindle formation (Q. Wu and W. Saunders, unpublished data). To reveal what is causing such failure of cytokinesis in cancer will further help understanding the initiation of chromosome segregational defects such as MPS, and CIN.

Carcinogenesis is a complicated process, often involving increased expression of oncogenes and decreased expression of tumor suppressor genes. The mutational changes required for carcinogenesis may result from either mutations in specific genes on a relatively small scale or gross changes in chromosome structure and numbers. Chromosome segregational defects, which we show can arise directly following genetic insults or cell division errors, can account for the latter. Whether sporadic local mutational changes or large-scale chromosome alterations are the

major source to generate the required conditions for cancer is unknown. Global chromosome changes presumably produce more dramatic and faster perturbation to the genome, but cells with gain or loss of chromosomes or chromosome fragments instead of point mutations may also undergo an accelerated selection process. Future analysis is required to test if the progeny arising from primary cells with nascent chromosome segregational defects can indeed generate a subset of stable clonal aneuploid cell lines. In summary, our work has made a new step in classifying the role of chromosome segregational defects in initiation and maintenance of CIN.

Chapter V : Materials and Methods

1. Cell Culture and Treatment

Human diploid fibroblast cells originated from primary non-fetal tissue, GM03349B, were obtained from Coriell Cell Repositories (Camden, New Jersey). The fibroblasts were grown in MEM supplemented with 15% fetal bovine serum (FBS) and 2mM L-glutamine, and trypsinized using 0.25% Trypsin, 1mM EDTA (all cell culture liquid supplies were purchased from Invitrogen Corp., Grand Island, NY). HCT116 human colon cancer cell lines (p53+/+ and p53-/-) , previously described by Bunz et al. (Bunz, Dutriaux et al. 1998), were gifts from Dr. Lin Zhang (Department of Pharmacology, University of Pittsburgh Cancer Institute). They were maintained in McCoy's 5A media supplemented with 10% FBS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin, and trypsinized in 0.05% Trypsin, 0.053mM EDTA. Normal oral keratinocytes (UP3 cells) were obtained from the primary uvulopalatopharyngoplasty cultures, grown in KGM2 medium and trypsin kit (Clonetics, San Diego, CA). UPCI:SCC cell lines are heterogeneous populations of keratinocytes grown from oral squamous cell carcinoma tumor tissue (Saunders, Shuster et al. 2000). Patients were not treated with chemotherapy or radiation before surgery. The oral cancer cells were grown in minimal essential medium (MEM) supplemented with 10% FBS, 2 mM L-Glutamine, 0.05 mg/ml Gentamycin, and 1% MEM non-essential amino acids and trypsinized in 0.05% Trypsin, 0.053mM EDTA. HEK-293s cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), cultured in MEM supplemented with 10% FBS, 2mM L-glutamine, Earle's BSS and 1.5g/l sodium bicarbonate, 0.1mM non-essential amino acids and 1.0 mM sodium pyruvate, and tryspinized in 0.05%

Trypsin, 0.053mM EDTA. KE p53-/- were mouse primary epithelial cells prepared from embryonic tissues derived from p53-null mice (MEEs). The cells were a gift from Dr. Fukasawa (University of Cincinnati College of Medicine, Cincinnati) and cultured as described (Chiba, Okuda et al. 2000). HeLa cells were purchased from ATCC, cultured in D-MEM with 10% FBS and trypsinized with 0.05% Trypsin, 0.053mM EDTA. All cultures were grown at 37°C with 5% CO_2.

Cells were grown on 22mm x22mm sterile glass cover slips (Corning, Corning, NY) in 35mm Petri dishes or 6-well plates for immunofluorescence or DAPI staining. All of the experiments on GM03349B fibroblasts were performed between passage 10 and 18. CSC was obtained from the RJ Reynolds Tobacco Company (Winston-Salem, NC) and was derived from 1R4F Kentucky Reference cigarettes from the University of Kentucky Tobacco and Health Research Institute (Lexington, KY). CSC 1R4F contains 10.80 mg total particulate matter/cigarette solubilized in 1 ml of dimethylsulfoxide (DMSO) and was stored at -70°C until use. 2'-deoxyguanosine 5'-monophosphate (dGMP), histidine (Sigma, St. Louis, MO) and CSC at the indicated concentrations were diluted in medium and sterile filtered before addition to cells. Nocodazole (Sigma, St. Louis, MO) was added at 0.6 μg/ml in medium for 16-18 h, and the cells were released in supplemented MEM for 1 h before fixation. For positive control of apoptotic cells, cells were serum-starved overnight followed by 1μM staurosporine treatment at 37°C for 4.5 h before fixation.

2. Trypan blue exclusion

CSC-treated fibroblast cells were trypsinized, spun down, and resuspended in 1ml serum-free medium, then 1ml (1:1 ratio) of 0.4% trypan blue solution was added to the cell suspension. The unstained (viable) and stained (dead) cells were counted by bright field microscopy. % dead cells=No. of dead cells/ total No. of cells x 100.

3. Analysis of isolated DNA

ϕX174 phage DNA plasmid (5.4kb) at 0.036 mg/ml, 3.3 mM $CuSO_4$, and 2 mg/ml CSC were mixed with indicated amount of dGMP, histidine or resveratrol (all from Sigma, St. Louis, MO) in PBS at 37°C for 30 min. Cosmid PB64 (22.8kb) was a gift from Dr. Karen Arnt (Department of Biological Sciences, University of Pittsburgh). The restriction endonuclease XhoI (New England Biolabs, Beverly, MA) was used following manufacturer's instructions. DNA was analyzed by agarose gel electrophoresis (0.8% or 1% agarose in TAE or TBE). Ethidium bromide was added before or after electrophoresis.

4. Antibodies

The following primary antibodies and dilutions were used in this study: anti-α-tubulin undiluted (Shea and Walsh 1987) (gift from Dr. Charles Walsh, University of Pittsburgh), anti-phosphorylated histone H2AX at 1:800 (Upstate Cell Signaling Solutions, Lake Placid, NY), CREST human auto-immune serum at 1:500 (gift from Dr. Carol Feghali, University of

Pittsburgh), anti-cleaved caspase 3 (Asp175) rabbit polyclonal antibody at 1:200 (Cell Signaling Technology, Beverly, MA), anti-PCNA at 1:200 (BD Transduction Laboratories, San Diego, CA). Secondary antibodies and stains included: Alexa Fluor488 and Alexa Fluor568 at 1:250 (Molecular Probes, Eugene, OR), anti-Human Cy3 at 1:250 and 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/ml (Sigma, St. Louis, MO). Antibodies were diluted in 1% BSA/PBS and DAPI in ddH_2O.

5. Immunofluorescence Staining

For anaphase bridge quantification, cells were fixed in methanol at -20°C for 10-30 min and air-dried. Cover slips were either processed within 1 h or stored at -20°C. Rehydration in PBS was performed before DAPI staining for 3-5 min. For caspase 3 staining, cells were fixed in 4% paraformaldehyde in PBS at room temperature for 5 min, followed by methanol fixation at -20°C for 5 min, then blocked in 1%BSA/PBS for 1 hr. Cells were stained with anti-cleaved caspase 3 overnight at 4°C, then with Alexa 568 goat anti-rabbit IgG for 1 hr followed by DAPI staining. For H2AX and PCNA double-staining, cells were fixed in 2% paraformaldehyde in PBS at room temperature for 30 min, rinsed in PBS and permeabilized in 1% Triton/PBS at 4°C for 5 min and blocked in 4% goat serum/PBS in 37°C for 1 hr. Cells were stained with anti-phospho H2AX for 2 hr, anti-PCNA for 1 hr, followed with Alexa 488 goat anti-rabbit IgG and Alexa 568 goat anti-mouse IgG for 1 hr, and stained with DAPI. The samples were then mounted with anti-fade solution (1 mg/ml p-phenylenediamine, 0.1x PBS, pH adjusted to 9.0 with KOH, in 90% glycerol, and stored in the dark at -20°C) onto slides. PBS is 0.14 M NaCl, 2.7 mM KCl, 6.5 mM

Na_2HPO_4, and 1.5 mM KH_2PO_4. Cells were incubated at 37°C unless specified, and cover slips were washed twice for 5 min with PBS in between each step.

Slides were viewed under an Olympus BX60 epifluorescence microscope using 100x or 50x oil immersion objectives. Digital images were captured with a Hamamatsu Argus-20 CCD camera. Anaphase cells were scored with a sample size of at least 200 in each case. Interphase cells were scored with a sample size of at least 300 in each case. Each experiment with quantification was repeated at least three times.

6. PAN-telomere FISH

Fibroblasts on cover slips were treated with CSC as described in 5.1. The cells were then fixed in 3:1 methanol:acetic acid for 100 min, washed with 70% acetic acid five times and air-dried. Cells were subsequently incubated in 2xSSC for 30 min, pre-warmed to 37°C, and dehydrated in 70%, 85% and 100% ethanol for 2 min each. The cover slips were then incubated with 0.1 mg/ml RNase in 2xSSC at 37°C for 30 min, and washed in 2xSSC three times for 5 min each. The cells were treated with 0.03 mg/ml pepsin in 37°C 0.01 M HCl for 10 min and washed in PBS for 5 min. The cells were then treated with 1% formaldehyde, 0.05 M $MgCl_2$ in PBS for 7 min, washed in PBS for 5 min, dehydrated in 70%, 85%, 100% ethanol for 2 min each and air dried. The Cy3-labeled peptide nucleic acid (PNA) telomere probe $(CCCTAA)_3$ (Applied Biosystems, Foster City, CA) was then applied to the cover slips and the subsequent steps were followed as described in the manufacturer's directions.

7. Telomere length assay

Genomic DNA was extracted from CSC-treated cells using standard methods as described in *Current Protocols in Molecular Biology*. A non-radioactive TeloTAGGG Telomere Length Assay kit was purchased from Roche (Indianapolis IN). Genomic DNA was digested with *HinfI* and *RsaI* and their terminal restriction fragments analyzed by DNA agarose gel and southern blotting following instructions.

8. Telomere-FISH

Fluorescence in situ hybridization (FISH) was performed with the Chromoprobe Multiprobe-T system (Cytocell, Cambridge, United Kingdom). Fibroblasts were treated with 0.2 mg/ml CSC with or without 20mM dGMP for three 2-hr cycles at 4-day intervals. Cells were then trypsinized in 0.25% trypsin, 1mM EDTA and mixed with an equal volume of supplemented MEM. Cells were centrifuged in an IEC Centra-CL2 rotor 215 at 224 x g for 5 min, and resuspended in 5ml 0.75mM KCl hypotonic solutions at 37°C for 22 min. 0.3 ml of fresh 3:1 methanol: acetic acid was added to fix the cells in suspension. The cells were centrifuged at 155 x g for 5 min and resuspended in 5 ml 3:1 methanol: acetic acid solution. The samples were stored at -20°C until use. The *in situ* hybridization protocol was as described by Cytocell. Only the telomere probes with the strongest fluorescent signals and without cross-hybridization to other chromosomes were used for quantification.

9. Transfections

Cells were seeded at a density of $2x10^5$ cells on 35mm glass-bottom Petri dishes (MatTek Corp., Ashland, MA) in serum-free medium. After 24 hours in culture, cells were transfected with the pBOS-H2BGFP vector (BD Pharmingen, San Diego, CA) using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis IN) at a 1μg DNA: 3μl lipid ratio. Membrane-GFP plasmid was a gift from Dr. Jeffrey D. Hildebrand (Department of Biological Sciences, University of Pittsburgh) (Haigo, Hildebrand et al. 2003). Double-plasmid transfections were performed identically as single transfections except that a total of 2 μg mixed DNA was used. All cells were transfected for 22 hr, allowed to recover in fresh medium for 8 hr and then viewed on the inverted microscope.

10. Live cell imaging

Live cell imaging was performed on a Nikon TE2000-U inverted microscope (Nikon Inc., Melville, NY) with a Coolsnap HQ digital camera (Roper Scientific Photometries, Tucson AZ). Cells were maintained at 37°C with a heated chamber (Life Images Services, Reinach Switzerland), continuously supplemented with moisturized 100% CO_2 to maintain a pH of between 7.0 and 8.0. Time-lapse images were collected and analyzed using Metamorph imaging software (Universal Imaging Corp., Downingtown, PA). Images from stacks were imported into

Photoshop (Adobe, San Jose, CA) for figure assembly. Movies were created from stacks using Metamorph.

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