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W. Brian Dalton Date

DNA Damage During Mitotic Arrest: A Novel, p53-Regulated Source of Structural Chromosome Instability in Human Cells

By

W. Brian Dalton
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Biochemistry, Cell, and Developmental Biology

_____
Vincent W. Yang, M.D., Ph.D.
Advisor

_____
Anita Corbett, Ph.D.
Committee Member

_____
Paul Doetsch, Ph.D.
Committee Member

_____
Dean Jones, Ph.D.
Committee Member

_____
David Pallas, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D. Dean of the Graduate School

_____ Date

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By

W. Brian Dalton
B.A., Dartmouth College, 1997

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Abstract

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By W. Brian Dalton

Mitosis is the process whereby a cell divides to produce two identical copies of itself. One mechanism which ensures that these copies are identical is the mitotic checkpoint. This safeguard works by preventing division until all chromosomes have achieved proper attachment to mitotic spindles. In cancer cells, mitosis—and the mitotic checkpoint—are frequently disturbed. One way this occurs is through prolonged activation of the checkpoint. Increasingly, evidence indicates that prolongation of mitosis may be a cause, rather than just a consequence, of tumorigenesis. At the same time, antimitotic chemotherapeutics are used to deliberately prolong mitosis, which, when severe, can be cytotoxic to cancer cells. Thus, prolongation of mitosis appears to play important roles in the biology of both the formation and treatment of cancer. Despite identification of these roles, our knowledge of the mechanisms underlying them remains limited. In this dissertation, we present evidence that during mitotic arrest, human cells acquire DNA damage which subsequently manifests, in a p53-dependent manner, as structural chromosome aberrations. These findings thus describe a novel source of DNA damage in human cells, suggest that prolonged mitosis may promote tumorigenesis and antimitotic toxicity through infliction of DNA damage, and propose a novel anticancer role for the p53 tumor suppressor.

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Chapter I

General Introduction

W. Brian Dalton[1,2]

[1]Division of Digestive Diseases, Department of Medicine, and
[2]Graduate Program in Biochemistry, Cell and Developmental Biology,
Emory University School of Medicine, Atlanta, GA 30322

Mitosis is frequently abnormal in cancer cells

Mitosis is the process whereby a eukaryotic cell divides to produce two identical copies of itself (Figure 1). Many things must go right for this process to succeed. During prophase, chromosomes must begin to condense and centrosomes must move to opposite poles of the cell. Nuclear envelope disassembly marks the beginning of prometaphase, during which mitotic spindles must capture and congress chromosomes to the equator of the cell. Once this equatorial congression is complete, the cell is said to be in metaphase. In anaphase, sister chromatids must separate and egress to opposite poles of the cell. Finally, in telophase, chromosomes must decondense, the nuclear membrane must reassemble, and the cell must cleave itself into two "daughter" cells. With so much that must go right in mitosis, it follows that many things can also go wrong (Dalton & Yang, 2007; Kops et al, 2005). Indeed, during the century since the German pathologist David von Hansemann first described abnormal mitotic figures in human tumors (von Hansemann, 1890), it has become clear that cancer is a disease in which things frequently go wrong in mitosis (Dalton & Yang, 2007; Kops et al, 2005; Therman & Kuhn, 1989). More recently, studies have indicated that rather than being a mere secondary consequence of the transformation process, abnormal mitosis can play a causative role in tumorigenesis (Ganem et al, 2007; Kops et al, 2005; Weaver & Cleveland, 2006). At the same time, a class of drugs used widely in the chemotherapeutic treatment of cancer—the antimitotics—exert their toxicity by directly disrupting mitosis (Jackson et al, 2007; Rieder & Maiato, 2004; Weaver & Cleveland, 2005). Thus, disruption of mitosis appears to play important roles in the biology of both the formation and treatment of cancer. Despite identification of these roles, our knowledge of the mechanisms underlying them

remains limited (Kops et al, 2005; Rieder & Maiato, 2004; Weaver & Cleveland, 2006). Thus, greater insight into the causes and consequences of abnormal mitosis should contribute to our understanding not only of cell biology, but also of tumorigenesis and chemotherapy.

The mitotic checkpoint

By casting chromosomes loose into the cytoplasm in order to be found, captured, and congressed by mitotic spindles, the prometaphase cell introduces an interesting problem for accurate segregation of chromosomes. Namely, given that searching and capturing of chromosomes by mitotic spindles is a stochastic process that is bound to vary in length from one mitosis to the next, how does the cell know when it has completed this process and is, thereby, ready for anaphase? The answer is that eukaryotic cells have evolved an elegant mechanism which monitors the attachments of chromosomes to mitotic spindles and then communicates the status of these attachments to a protein complex which controls entry into anaphase (Figure 2) (Kops et al, 2005; Rieder et al, 1995; Rieder & Maiato, 2004). This surveillance mechanism is known as the mitotic, or spindle assembly, checkpoint (Kops et al, 2005; Rieder & Maiato, 2004). Since the initial discovery of the Bub and Mad family of genes in yeast genetic screens, nearly a dozen genes involved in mitotic checkpoint function have been identified in a range of different organisms (Kops et al, 2005; Rieder & Maiato, 2004). Some of the protein products of these genes, such as Bub1, Mad2, CENP-E and others, interact directly with the proteinaceous center of the chromosome to which mitotic spindles attach, known as the kinetochore (Figure 2a) (Kops et al, 2005). If the kinetochore is

unattached to mitotic spindles, some of these checkpoint proteins are activated, form a diffusible protein complex, and sequester CDC20 (Figure 2a) (Kops et al, 2005). This sequestration inhibits the anaphase promoting complex (APC), whose activity is required for the degradation of cyclin B and securin (Figure 2a) (Kops et al, 2005; Rieder & Maiato, 2004). Cyclin B is required for the function of the master mitotic regulator CDK1, whose activity, in turn, maintains the prometaphase/metaphase state, and securin prevents activation of separase, whose activity is required for sister chromatid separation at the onset of anaphase (Kops et al, 2005). Thus, by inhibiting APC-mediated degradation of cyclin B and securin, the mitotic checkpoint prevents anaphase onset when chromosomes remain unattached to mitotic spindles. Once all kinetochores make proper attachments to mitotic spindles, this diffusible "wait anaphase" signal is extinguished, the APC is disinhibited, cyclin B and securin are degraded, and the cell proceeds into anaphase (Figure 2b-c) (Kops et al, 2005). In this way, the mitotic checkpoint prevents entry into anaphase before all chromosomes have stably attached to mitotic spindles, thereby ensuring equal segregation of chromosomes into daughter cells.

Weakening of the mitotic checkpoint in cancer cells

Due to the central importance of the mitotic checkpoint in safeguarding the execution of normal mitosis, it is perhaps not surprising that disruption of this mechanism can occur in cancer (Dalton & Yang, 2007; Kops et al, 2005). One such disruption that has received considerable attention is the existence of reduced, or "weakened," checkpoint signaling in cancer cells (Holland & Cleveland, 2008; Yuen & Desai, 2008). Indeed, animal models have provided a compelling proof of principle that mitotic

checkpoint deficiencies can contribute to tumor formation in mammals, as mice expressing reduced levels of the mitotic checkpoint proteins Mad2, Mad1, CENP-E, Bub1, BubR1, and Bub3 exhibit both an impaired ability to delay anaphase in the presence of unattached kinetochores and an increased susceptibility to cancer (Babu et al, 2003; Baker et al, 2004; Dai et al, 2004; Iwanaga et al, 2007; Jeganathan et al, 2007; Michel et al, 2001; Weaver et al, 2007). Although the reasons that such deficiencies encourage tumorigenesis are not fully understood, one causative factor is likely to be the increase in aneuploidy observed in these animals, which may create cancer-promoting genomic imbalances in oncogenes and tumor suppressors (Weaver & Cleveland, 2006; Weaver et al, 2007).

The first evidence that the mitotic checkpoint may be weakened in human cancer came from a 1998 study by Cahill et al, which reported that aneuploid colon cancer cell lines exhibit a defect in their ability to execute mitotic arrest when challenged with antimitotic agents (Cahill et al, 1998). In some of these cell lines, mutations in the mitotic checkpoint genes BubR1 and Bub1 were found, providing a potential explanation for the observed defects (Cahill et al, 1998). Subsequent studies found additional mutations in other mitotic checkpoint genes, including Bub1, BubR1, Mad1, Zw10, and Zwilch, in human tumors (Imai et al, 1999; Nomoto et al, 1999; Sato et al, 2000; Shichiri et al, 2002; Tsukasaki et al, 2001; Wang et al, 2004). Moreover, nonmutational impairment of mitotic checkpoint proteins, including transcriptional repression and inactivating interactions with viral oncoproteins, has also been observed in human tumors (Guardavaccaro et al, 2008; Jeong et al, 2004; Jin et al, 1998; Kim et al, 2008; Shichiri et al, 2002; Wang et al, 2008). Finally, mosaic variegated aneuploidy, a rare human genetic

disease characterized by aneuploidy and cancer predisposition, has been causally linked to germline mutation of the BubR1 mitotic checkpoint gene (Hanks et al, 2004; Matsuura et al, 2006). These studies thus provide evidence that weakened mitotic checkpoint signaling can promote human cancer.

At the same time, there is compelling evidence which disfavors the idea that weakened mitotic checkpoint signaling is a common occurrence in cancer cells. First, although they do exist, mutations in mitotic checkpoint genes are rare in human tumors (Cahill et al, 1999; Jones et al, 2008; Kops et al, 2005; Parsons et al, 2008; Sjoblom et al, 2006; Wang et al, 2008; Wood et al, 2007). Second, data from several studies have now directly challenged the conclusion that the cell lines studied by Cahill et al. actually have impaired mitotic checkpoints (Gascoigne & Taylor, 2008; Kaplan et al, 2001; Kasai et al, 2002; Tao et al, 2007; Thompson & Compton, 2008; Tighe et al, 2001). Indeed, elegant timelapse videomicroscopy studies from Thompson et al. and Gascoigne et al. have shown that aneuploidy in these cell lines is attributable to merotelic chromosome-spindle attachments, and not to weakened mitotic checkpoint signaling (Gascoigne & Taylor, 2008; Thompson & Compton, 2008). Thus, taken together, these studies indicate that a weakened mitotic checkpoint is not a widespread property of cancer cells, although it may occur in some tumors.

Prolonged activation of the mitotic checkpoint in cancer cells

In addition to exhibiting, albeit infrequently, weakened mitotic checkpoint signaling, cancer cells have also been found to possess abnormalities which prolong activation of this checkpoint (Dalton & Yang, 2007; Rieder & Maiato, 2004; Therman &

Kuhn, 1989). Indeed, it has long been recognized by pathologists that tumors not only possess higher mitotic indices than normal tissue, but also a higher ratio of prometaphase/metaphase to prophase cells (Therman & Kuhn, 1989). Because this ratio should reflect the relative durations of these phases of mitosis, such observations suggested that prometaphase/metaphase is prolonged in cancer cells (Therman & Kuhn, 1989). Subsequently, timelapse videomicroscopy studies revealed that mitosis can indeed be lengthened up to 4-fold in transformed cells, as compared to their normal counterparts (Sisken et al, 1982; Sisken et al, 1985). More recently, Yang et al. have confirmed these initial findings and further demonstrated that the prolongation is dependent on mitotic checkpoint signaling (Yang et al, 2008). Thus, cancer cells commonly possess defects which lengthen mitosis by delaying satisfaction of the mitotic checkpoint.

What defects might prolong mitosis in cancer cells? Although knowledge is still relatively limited on this subject, several causes have been described (Figure 3). Inactivation of the Rb tumor suppressor has been shown to transcriptionally induce supranormal expression of the mitotic checkpoint protein Mad2, which in turn causes prolonged prometaphase by directly inhibiting the anaphase promoting complex (Hernando et al, 2004; Sotillo et al, 2007). Analogously, inactivation of the hCDC4 tumor suppressor decreases the degradation of cyclin E, whose abnormal accumulation also lengthens mitosis through inhibition of the APC (Keck et al, 2007; Rajagopalan et al, 2004). Activation of the c-Myc oncogene, too, has been reported to delay prometaphase through supranormal transactivation of the Mad2 and BubR1 mitotic checkpoint proteins (Menssen et al, 2007). Another intriguing example is that of Shwachman-Diamond syndrome, an inherited human disease characterized by leukemia predisposition (Austin

et al, 2008). This disease results from recessive mutation of the SBDS gene, whose inactivation results in destabilization of the mitotic spindle and, thus, prolonged activation of the mitotic checkpoint (Austin et al, 2008). Finally, Yang et al. found that mitotic prolongation can result from the simple presence of extra chromosomes and/or centrosomes, a common feature of cancer cells (Yang et al, 2008). By extension, processes which create supernumerary chromosomes and/or centrosomes, such as polyploidization and centrosome overduplication, can also be thought of as increasing the frequency of prolonged mitosis. Thus, multiple mechanisms can delay timely satisfaction of the mitotic checkpoint in cancer cells.

At this point, it is important to consider whether prolonged mitosis might be a causative factor in, rather than simply a secondary consequence of, tumorigenesis. Although inactivation of the Rb and hCDC4 tumor suppressors, along with activation of oncogenic c-Myc, are common contributors to human cancers (Jones et al, 2008; Parsons et al, 2008; Sjoblom et al, 2006; Wood et al, 2007), prolongation of mitosis is only one of many potentially oncogenic phenotypes created by these gene changes. Likewise, although polyploidization has been shown to promote tumorigenesis (Duelli et al, 2007; Fujiwara et al, 2005; Mazumdar et al, 2006; Roh et al, 2008), the induction of prolonged mitosis through supernumerary chromosomes and/or centrosomes is also only one of many potentially oncogenic properties of polyploid cells. For these reasons, a recent study by Sotillo et al. is of particular importance (Sotillo et al, 2007). In it, the authors show that isolated overexpression of Mad2, the downstream effecter of Rb inactivation responsible for prolonged mitosis, can itself induce dramatic incidences of spontaneous tumors in mice. This result thus provides a compelling proof-of-principle that prolonged

activation of the mitotic checkpoint can directly promote tumorigenesis. Whether this tumorigenicity occurs for other conditions in which mitosis is prolonged remains to be determined.

Why might prolonged activation of the mitotic checkpoint be oncogenic? One explanation is that by disrupting the normal sequence of events in mitosis, prolonged checkpoint activation increases the frequency of chromosome missegregation and cell division failure, leading to aneuploidy and polyploidy, respectively (Pellman, 2007; Sotillo et al, 2007; van Deursen, 2007). Indeed, this occurs in Mad2-overexpressing mice, which develop aneuploidy and polyploidy, as well as cancer (Sotillo et al, 2007). Aneuploidy and polyploidy, as mentioned previously, can themselves promote tumorigenesis (Duelli et al, 2007; Fujiwara et al, 2005; Mazumdar et al, 2006; Roh et al, 2008; Weaver et al, 2007). However, aneuploidy does not always promote cancer (Babu et al, 2003; Baker et al, 2004; Baker et al, 2006; Jeganathan et al, 2006), and in some contexts it can actually suppress tumorigenesis (Weaver et al, 2007). Thus, it is possible that other, or additional, factors may contribute to the tumorigenicity of prolonged mitotic checkpoint activation. Interestingly, mice overexpressing Mad2 exhibited not only aneuploidy, but also evidence of significant structural chromosome alterations, indicating an increase in spontaneous DNA damage (Sotillo et al, 2007). Inactivation of the hCDC4 tumor suppressor, too, produced not only prolonged mitosis and aneuploidy, but also evidence of structural DNA damage (Rajagopalan et al, 2004). A similar effect was seen with activation of the c-Myc oncogene (Menssen et al, 2007). Furthermore, inactivation of the KIF4A mitotic motor protein, while not a phenomenon yet investigated in human cancer, can nonetheless elicit prolonged mitosis, spontaneous tumors, and DNA damage

in a mouse model (Mazumdar et al, 2006; Mazumdar et al, 2004). Finally, Wong and Stearns reported that human cells which had been presynchronized with double-thymidine and nocodazole later possessed foci of γ-H2AX (Wong & Stearns, 2005), the phosphorylated form of histone H2AX which forms around sites of double-stranded DNA breaks (Rogakou et al, 1999). Taken together, these studies raised, in our minds, the intriguing possibility that prolonged activation of the mitotic checkpoint might induce DNA damage. If true, such a phenomenon could provide an additional explanation for why prolonged mitosis might promote cancer, given the well-established role of DNA damage in tumorigenesis (van Gent et al, 2001). We thus endeavored to test this hypothesis, and the study described in Chapter II presents our novel evidence that prolonged activation of the mitotic checkpoint can indeed provoke DNA damage in human cells.

Cell fate after mitotic arrest

Vital to an understanding of the consequences of prolonged mitotic arrest is an understanding of cell fate after the arrest (Rieder & Maiato, 2004). As it turns out, several possible fates can befall cells that endure prolonged activation of the mitotic checkpoint (Figure 4) (Weaver & Cleveland, 2005). First, cells may eventually complete cell division after a mitotic delay. Such divisions are frequently accompanied by missegregation of chromosomes, thereby leading to aneuploidy (Gascoigne & Taylor, 2008; Hernando et al, 2004; Rajagopalan et al, 2004; Sotillo et al, 2007; Thompson & Compton, 2008). Second, cells can undergo "mitotic slippage," a process whereby mitotic cells return to interphase without completing anaphase (Rieder & Maiato, 2004;

Weaver & Cleveland, 2005). Because in this case cell division does not occur, the resulting interphase cells, which are often referred to as "postmitotic," will possess twice the normal number of chromosomes, making them tetraploid (Rieder & Maiato, 2004). After either of these first two fates, cells will then realize one of three additional fates: continuation of the cell cycle, cell death, or cell cycle arrest (Gascoigne & Taylor, 2008; Rieder & Maiato, 2004; Weaver & Cleveland, 2005). Finally, a third fate following prolonged checkpoint activation is mitotic cell death, where cells die directly from the mitotic state (Gascoigne & Taylor, 2008; Rieder & Maiato, 2004; Weaver & Cleveland, 2005).

What determines which of these fates a cell will meet? Gascoigne and Taylor have shown that the answer to this question is surprisingly complex, as both genetic and nongenetic factors create profound variation in the fates of cells following prolonged mitotic arrest (Gascoigne & Taylor, 2008). Amid this complexity, however, these authors provide evidence that the first fate decision point—whether a cell will exit from, or die in, prolonged mitosis—is controlled by stochastic competition between progressive degradation of cyclin B and progressive activation of a caspase-dependent death pathway (Gascoigne & Taylor, 2008; Holland & Cleveland, 2008). If cyclin B is degraded to a critical threshold level first, the mitotic cell will undergo slippage or, if possible, cell division; if activation of caspase-dependent cell death reaches a critical threshold first, the cell will die in mitosis. Although a plausible explanation for the progressive degradation of cyclin B during mitotic arrest is "leaky" activity of the anaphase promoting complex (Brito & Rieder, 2006), the mechanisms responsible for progressive activation of a mitotic cell death pathway are unknown (Gascoigne & Taylor, 2008;

Holland & Cleveland, 2008). Interestingly, Gascoigne and Taylor proposed that the death signal may be influenced by the accumulation of DNA damage in cells undergoing prolonged mitosis (Gascoigne & Taylor, 2008). The results of our study, described in Chapter II, lend support to this proposal.

p53 and the postmitotic response

For those cells which exit from, rather than die in, prolonged mitotic arrest, knowledge of the determinants of their subsequent fates is rather limited (Holland & Cleveland, 2008; Rieder & Maiato, 2004; Weaver & Cleveland, 2005). However, there is one node of regulation which has been characterized in several cell systems: control of the decision between postmitotic cell cycle arrest and continuation of the cell cycle by the tumor suppressor p53 (Figure 4) (Stukenberg, 2004). The initial evidence that p53 regulates the fate of cells following mitotic arrest came from Cross et al., who in 1995 discovered that treatment of p53-/- MEFs with the antimitotic agents nocodazole and colchicine resulted in significant polyploidization, compared to p53+/+ MEFs (Cross et al, 1995). Although initially proposed to result from an early exit from mitotic arrest (Cross et al, 1995), this inhibition of polyploidization was subsequently shown to result from p53-dependent imposition of growth arrest in postmitotic cells which had undergone mitotic slippage (Lanni & Jacks, 1998; Minn et al, 1996). Since these initial reports, additional studies have observed this p53-dependent "postmitotic response" in mouse and human cells cultured from numerous tissues of origin (Andreassen et al, 2001; Borel et al, 2002; Di Leonardo et al, 1997; Khan & Wahl, 1998; Vogel et al, 2004). Moreover, biochemical investigations have revealed that elements of the pathway which

are important for the p53-dependent response to DNA damage, such as transcriptional induction of p21, inhibition of cyclin E/cdk2, and hypophosphorylation of Rb, are also activated and/or required in the postmitotic response (Di Leonardo et al, 1997; Khan & Wahl, 1998; Lanni & Jacks, 1998; Minn et al, 1996; Stewart et al, 1999). Furthermore, studies have presented evidence, albeit indirect, that the postmitotic response is not merely a reaction to the artificial conditions of treating cultured cells with drugs. p53-deficient mice, for example, exhibit an increase in spontaneous aneuploidy and polyploidy, and p53-deficient human cells exhibit an increase in spontaneous, as well as genetically-induced, polyploidy (Bellamy et al, 1997; Bunz et al, 2002; Cross et al, 1995; Fujiwara et al, 2005; Harvey et al, 1993; Meraldi et al, 2002; Pantic et al, 2006; Sphyris & Harrison, 2005). Although the mechanisms underlying these spontaneous defects has not been determined, chromosome missegregation and/or mitotic slippage following prolonged mitosis is a plausible explanation, especially since this process has been found to occur spontaneously at low frequency in both transformed and normal cells in culture (Shi & King, 2005). Thus, the p53-dependent induction of growth arrest following prolonged mitotic arrest, like the p53-dependent DNA damage response, appears to be a ubiquitous cell cycle response in mammalian cells.

Why is p53 activated during prolonged mitotic arrest?. Despite several proposed explanations, the answer to this question has remained elusive (Chan et al, 2008; Ganem & Pellman, 2007; Stukenberg, 2004). Although one initial idea was that cells activate p53 upon sensing the presence of tetraploid genomes (Andreassen et al, 2001; Margolis et al, 2003), subsequent studies have shown that polyploid cells created in the absence of prolonged mitosis are not necessarily growth arrested by p53 (Uetake & Sluder, 2004;

Uetake & Sluder, 2007). Therefore, tetraploidy itself does not appear to be the signal for postmitotic arrest (Ganem & Pellman, 2007; Stukenberg, 2004). Another proposal has been that progressive mitotic accumulation of p53 may serve as a molecular "timer" which measures the duration of mitosis, thereby inducing postmitotic arrest in those cells which spend too long—and thus accumulate too much p53—in mitotic arrest (Blagosklonny, 2006). However, other studies have demonstrated that pharmacologic and genetic interventions which dramatically shorten the length of mitotic arrest, such as inhibition of CDK1 activity or reduction of spindle checkpoint proteins, do not diminish the activation of p53 (Chan et al, 2008; Vogel et al, 2004). Moreover, there is evidence that, at least in mouse cells, p53 is activated after, and not during, mitotic arrest (Minn et al, 1996). Thus, a mitotic timer mechanism is unlikely to explain the postmitotic activity of p53.

As previously mentioned, Wong and Stearns found that human cells presynchronized with double-thymidine and nocodazole later possessed foci of γ-H2AX, which raised the intriguing possibility that mitotic arrest might induce DNA damage (Wong & Stearns, 2005). If true, this phenomenon could provide a potential explanation for the mechanism of postmitotic arrest: DNA damage acquired during mitotic arrest activates a p53-dependent DNA damage response in postmitotic cells. Such a mechanism would also provide an explanation for the existence of the postmitotic response: the need to suppress potentially dangerous structural chromosome instability formed during mitotic arrest. Indeed, the study described in Chapter III provides novel evidence to support this idea. At the same time, it is important to note that other, or additional, stimuli may be responsible for p53 activation. Indeed, p53 has been reported to be activated

following a variety of cellular stresses other than DNA damage (Jimenez et al, 1999). Moreover, recent studies have shown that disrupting the function of the centrosome can elicit a p53-dependent G1 arrest (Mikule et al, 2007; Srsen et al, 2006; Uetake et al, 2007), which may be of relevance to p53 activation following mitotic arrest, as signaling at the centrosome has been suggested to regulate this process (Aylon et al, 2006; Ciciarello et al, 2001). Finally, Uetake et al. have shown that, in some contexts, combinations of different cellular stresses, but not each stress alone, can activate p53-dependent arrest (Uetake et al, 2007).

A final consideration of the role of p53 in cell fate following mitotic arrest concerns its participation in cell death. Although some studies have found that p53 promotes cell death following prolonged activation of the mitotic checkpoint (Galmarini et al, 2001; Kienitz et al, 2005; Wu & El-Diery, 1996; Yamaguchi et al, 2004), others have found that p53 is uninvolved in death signaling (Minn et al, 1996; Strobel et al, 1996; Woods et al, 1995). To complicate matters further, another study has found that p53 promotes cell survival, not death, following mitotic arrest (Wahl et al, 1996). The discrepancies between these studies are most likely attributable to cell-type differences in p53 activity, as tissue of origin has been shown to greatly influence the consequences of p53 inactivation (Bunz et al, 2002). Thus, it can be concluded that while the p53-dependent imposition of growth arrest following mitotic arrest appears to be a ubiquitous phenomenon, the role of p53 in postmitotic cell death appears to depend on the genetic background of the postmitotic cells.

Antimitotic chemotherapy and the mitotic checkpoint

In addition to genetic disruptions which alter elements like the activity of mitotic checkpoint proteins or the number of mitotic spindles, antimitotic drugs can also cause prolonged activation of the mitotic checkpoint (Jackson et al, 2007; Jordan & Wilson, 2004). Drugs like nocodazole, colchicine, and the vinca alkaloids induce mitotic arrest by destabilizing mitotic spindles (Jordan & Wilson, 2004). Others, like taxanes and epothilones, cause arrest by pathologic stabilization of spindles (Jordan & Wilson, 2004). Moreover, a newer class of agents have been developed which provoke arrest through inhibition of mitotic motor proteins known as kinesins (Jackson et al, 2007). Demonstrating the clinical importance of inducing mitotic arrest, many of these agents are used routinely in cancer chemotherapy, and others are in various stages of clinical trials (Jackson et al, 2007; Jordan & Wilson, 2004).

Although our understanding of how these agents exert their cytotoxicity is incomplete, some general insights into their mechanisms of actions have nonetheless been established (Rieder & Maiato, 2004; Weaver & Cleveland, 2005). First, entry into mitosis is necessary for cytotoxicity, as cells cotreated with antimitotics and agents which prevent entry into mitosis, such as the DNA replication inhibitor aphidicolin or the CDK1 inhibitor purvalanol, are spared from the cell death which occurs upon antimitotic treatment alone (Shi et al, 2008). Second, prolonged activation of the mitotic checkpoint promotes cell death, as impairment of mitotic checkpoint signaling can reduce the cytotoxicity of antimitotic agents (Chan et al, 2008; Kienitz et al, 2005; Tao et al, 2005). Third, cell death appears to be a form of caspase-dependent apoptosis, as caspase inhibitors can, in most cases, prevent antimitotic-induced cell death (Dalton et al, 2007;

Gascoigne & Taylor, 2008; Rieder & Maiato, 2004; Shi et al, 2008; Tao et al, 2005). Fourth, as mentioned previously, the first cell fate decision following prolonged mitotic arrest—whether the cell dies in, or exits, mitosis—depends on a stochastic competition between progressive degradation of cyclin B and activation of apoptosis during the arrest (Gascoigne & Taylor, 2008). Finally, activation of p53 following prolonged mitosis can, in some contexts, impose not only cell cycle arrest, but also cell death, as discussed above. However, a central question regarding antimitotic-induced cell death remains: what triggers the caspase-dependent apoptosis during prolonged mitotic arrest? Although several, non-mutually-exclusive, answers to this question are possible, one explanation is DNA damage acquired during mitotic arrest. The studies described in Chapters II and III provide support for this explanation.

Goals of this Dissertation

The work presented in this dissertation sought to investigate two ideas relevant to understanding the consequences of prolonged activation of the mitotic checkpoint. First, we tested the **hypothesis that human cells acquire DNA damage during prolonged mitotic arrest.** To do so, we performed biochemical and cytogenetic measurements of DNA damage after pharmacologic and genetic provocation of mitotic arrest in human cell lines. This investigation revealed that, during mitotic arrest, human cells can indeed acquire DNA damage which occurs independently of cell death, activates a DNA damage response, and subsequently manifests as structural chromosome aberrations. Thus, DNA damage acquired during mitotic arrest represents a novel source of DNA damage in human cells. Moreover, this phenomenon suggests that prolonged mitosis may contribute

to tumorigenesis and/or the toxicity of antimitotic chemotherapy through the induction of DNA damage. This study is the subject of Chapter II.

Due to the importance of p53 in both human cancer and cell fate following prolonged mitotic arrest, we next investigated the role of p53 in determining the consequences of DNA damage acquired during mitotic arrest. Specifically, we tested the **hypothesis that p53 suppresses structural chromosome instability following mitotic arrest.** To do so, we measured the acquisition and propagation of DNA damage incurred during mitotic arrest in human cells which differ only in their p53 status. This investigation revealed that p53 inhibits the propagation, but not the acquisition, of DNA damage incurred during mitotic arrest. This finding indicates that p53 can function to suppress structural chromosome instability following abnormal mitosis. It also provides supportive evidence to the idea that the p53-dependent postmitotic checkpoint may be a response to DNA damage incurred during mitotic arrest. This study is the subject of Chapter III.

Taken together, these studies describe a novel, p53-regulated source of structural chromosome instability in human cells: DNA damage during mitotic arrest.

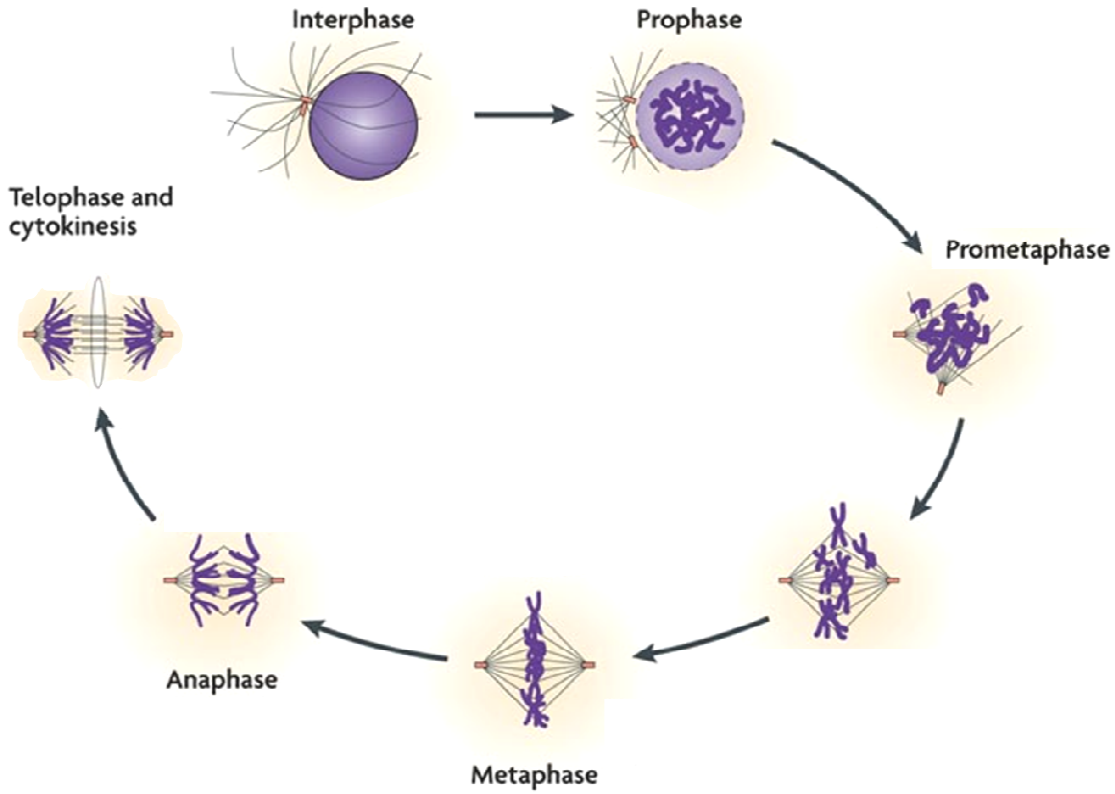

Figure 1. The five phases of mitosis.

When an interphase cells enters prophase, centrosomes move to opposing poles of the cell and chromosomes begin condensing. In prometaphase, chromosomes are cast loose into the cytoplasm, and mitotic spindles capture and congress these chromosomes to the equator of the cell. Once this congression is complete, the cell is in metaphase. In anaphase, sister chromatids separate and are pulled to opposite poles of the cell. Finally, in telophase, chromosomes decondense, the nuclear membrane reassembles, and the cell undergoes cytokinesis. Adapted from Jackson et al (Jackson et al, 2007).

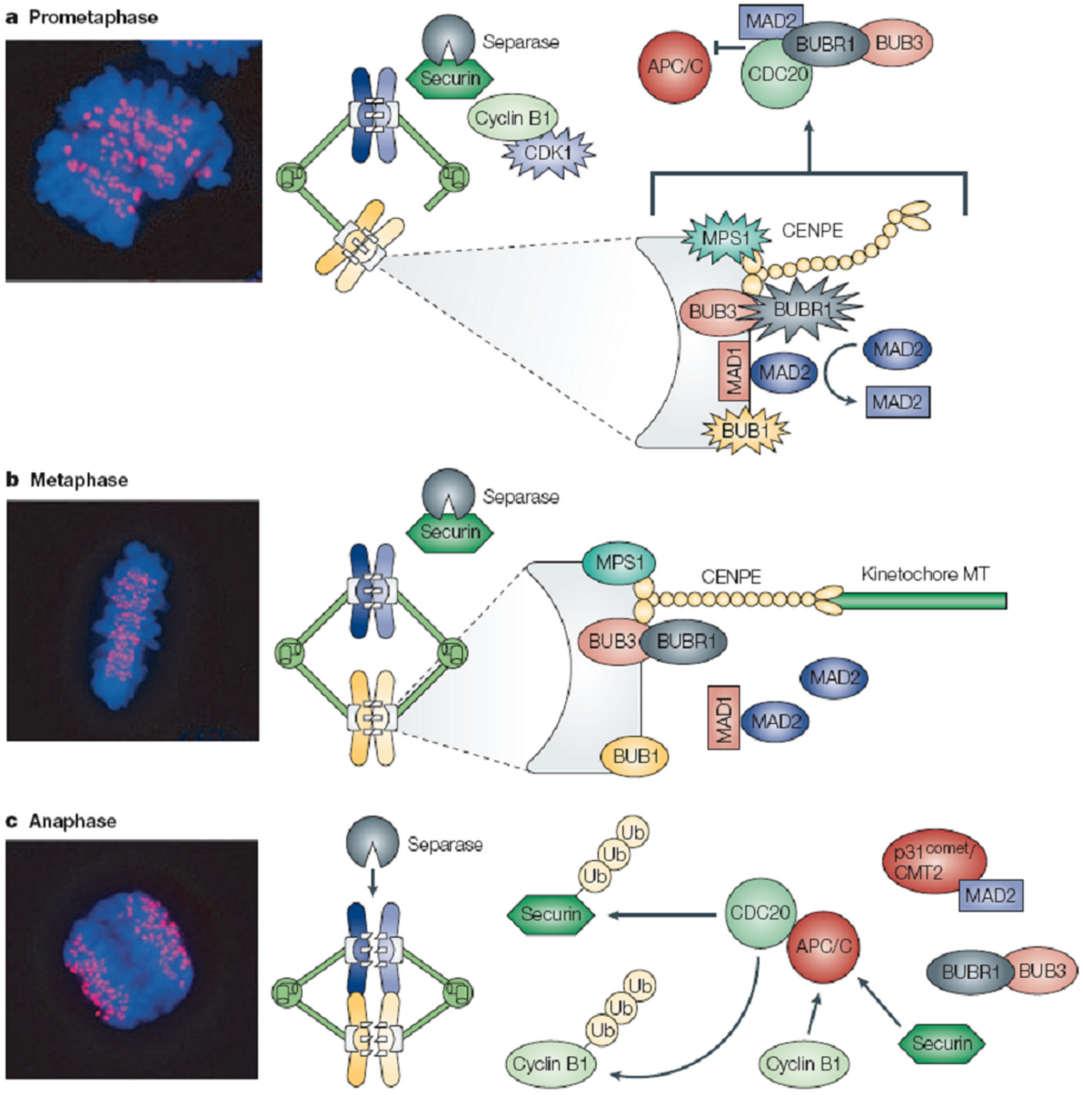

Figure 2. The mitotic checkpoint.

a. In prometaphase, the unattached kinetochore recruits MAD1, MAD2, MPS1, BUB1, BUB3, BUBR1, and CENPE (grey sections, expanded diagram indicated by dashed lines). These components generate the diffusible complex that sequesters CDC20, which is thus prevented from activating the anaphase-promoting complex (APC). Separase, the protease that cleaves the cohesins that hold sister chromatids together, is inhibited by binding to securin. The photograph represents prometaphase in HeLa cells. DNA, stained

with DAPI, is in blue, kinetochores, immunostained with anticentromere antiserum, are in red. **b.** Attachment of mitotic spindles (MT, green) to kinetochores, and the generation of tension by motor proteins, silences the checkpoint signaling complex. The photograph represents metaphase in HeLa cells. **c.** Following silencing of the checkpoint signaling complex, APC/C-mediated ubiquitylation (Ub) of securin and cyclin B1 and subsequent degradation by the proteosome triggers anaphase entry. Turnover of the wait anaphase complex is aided by p31comet/CMT2. The photograph represents anaphase in HeLa cells. Adapted from Kops et al(Kops et al, 2005).

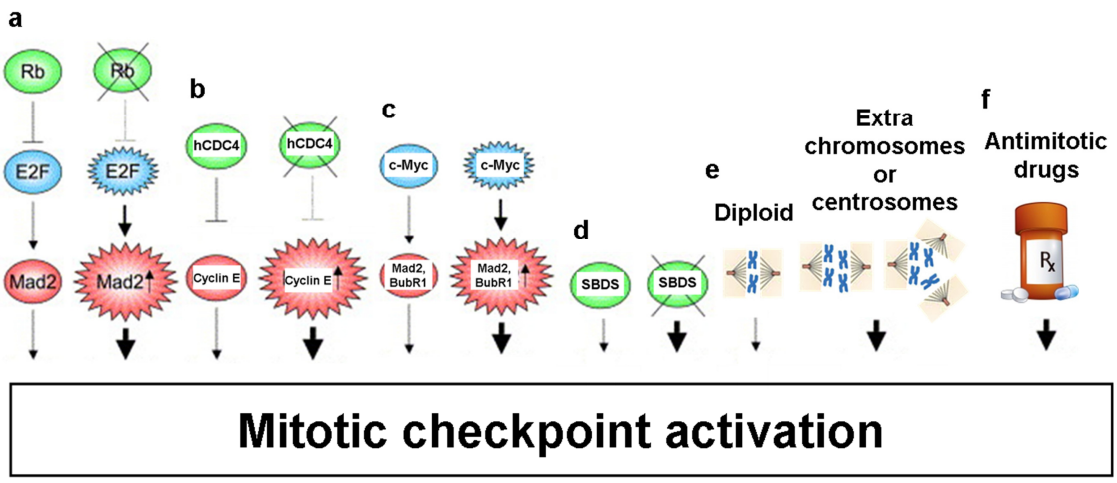

Figure 3. Cancer-associated causes of prolonged mitotic checkpoint activation. **a.** Inactivation of the retinoblastoma (Rb) tumor suppressor causes activation of E2F and elevation of Mad2. **b.** Inactivation of the hCDC4 tumor suppressor leads to elevated levels of cyclin E. **c.** Oncogenic c-Myc upregulates the expression of the mitotic checkpoint proteins Mad2 and BubR1. **d.** Inactivation of the SDBS gene causes destabilization of the mitotic spindle in Schwachman-Diamond syndrome. **e.** Cancer cells frequently contain supernumerary chromosomes and/or centrosomes. **f.** Antimitotic drugs are routinely used in cancer chemotherapy. Adapted from Jackson et al. and van Deursen et al (Jackson et al, 2007; van Deursen, 2007).

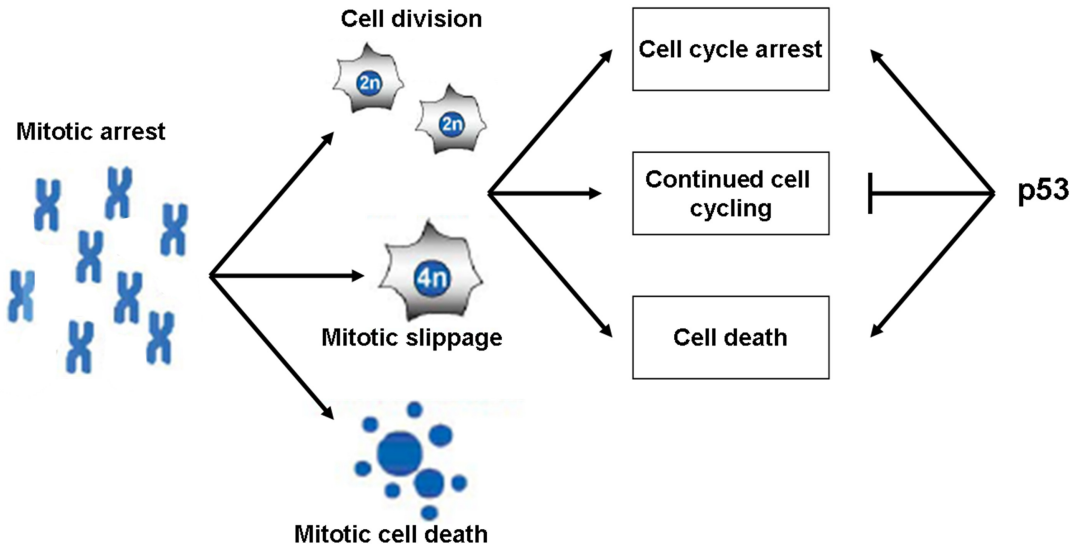

Figure 4. Cell fates following mitotic arrest.

The first fate decision is between cell division, mitotic slippage, or mitotic cell death. For those cells which exit mitosis, a second fate decision is made between cell cycle arrest, continuation of the cell cycle, or cell death. The p53 tumor suppressor inhibits continuation of the cell cycle and promotes cell cycle arrest or, in some contexts, cell death. Adapted from Weaver et al. (Weaver & Cleveland, 2005).

Chapter II

Human Cancer Cells Commonly Acquire DNA Damage During Mitotic Arrest

W. Brian Dalton[1,2], Mandayam O. Nandan[1], Ryan T. Moore[3], and Vincent W. Yang[1,4*]

[1]Division of Digestive Diseases, Department of Medicine,
[2]Biochemistry, Cell and Developmental Biology Graduate Program, and
[4]Winship Cancer Institute,
Emory University School of Medicine,
Atlanta, Georgia 30322, U. S. A.
[3]Institute for Quantitative Social Science,
Harvard University,
Cambridge, Massachusetts 02138, U. S. A.

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Abstract

The mitotic checkpoint is a mechanism which arrests the progression to anaphase until all chromosomes have achieved proper attachment to mitotic spindles. In cancer cells, satisfaction of this checkpoint is frequently delayed or prevented by various defects, some of which have been causally implicated in tumorigenesis. At the same time, deliberate induction of mitotic arrest has proven clinically useful, as antimitotic drugs which interfere with proper chromosome-spindle interactions are effective anticancer agents. However, how mitotic arrest contributes to tumorigenesis or antimitotic drug toxicity is not well defined. Here we report that mitotic chromosomes can acquire DNA breaks during both pharmacologic and genetic induction of mitotic arrest in human cancer cells. These breaks activate a DNA damage response, occur independently of cell death, and subsequently manifest as karyotype alterations. Such breaks can also occur spontaneously, particularly in cancer cells containing mitotic spindle abnormalities. Moreover, we observed evidence of some breakage in primary human cells. Our findings thus describe a novel source of DNA damage in human cells. They also suggest that mitotic arrest may promote tumorigenesis and antimitotic toxicity by provoking DNA damage.

Introduction

Mitosis is often abnormal in cancer cells (Rieder & Maiato, 2004). Among the defects observed is frequent prolongation of prometaphase, which can occur when the spindle checkpoint arrests progression to anaphase (Rieder & Maiato, 2004). Several studies have now identified genetic abnormalities which provoke such arrest (Dalton &

Yang, 2007). Some of these, such as loss of hCDC4 or overexpression of Mad2, have been causally implicated in cancer (Rajagopalan et al, 2004; Sotillo et al, 2007). At the same time, antimitotic chemotherapeutics which induce prolonged mitotic arrest and "mitotic slippage," a process whereby prometaphase cells return to interphase without undergoing anaphase, can also elicit subsequent cell death or growth arrest (Rieder & Maiato, 2004). Therefore, evidence suggests that events occurring during mitotic arrest influence tumorigenesis and the toxicity of antimitotic chemotherapy. However, the nature of these events is not well defined (Rieder & Maiato, 2004). Interestingly, Wong and Stearns have shown that human cells which had been pre-synchronized with double-thymidine and the microtubule depolymerizer nocodazole later possessed foci of γ-H2AX, the phosphorylated form of histone H2AX which forms around sites of DNA breaks (Wong & Stearns, 2005). We thus wondered whether DNA breaks might be generated during mitotic arrest in human cancer cells.

Materials and Methods

Cell lines and treatments

All cell lines were from the ATCC, except BG-1 cells, which were from C. Moreno (Emory University, Atlanta, GA). Cancer cells were cultured in McCoy's and IMR90 cells in DMEM. Cells were seeded at a density of 3×10^4 cells/cm^2 onto fibronectin-coated dishes or slides 24h prior to experiments. Nocodazole, paclitaxel, and monastrol were used at 200nM, 100nM, and 200μM, respectively, the minimum concentrations which inhibited cell division (data not shown). Q-VD-OPh was used at 50μM, the minimum concentration which inhibited apoptosis (data not shown). γ-irradiation was

performed with a Cs-137 Gammacell. Stealth™ Select siRNAs targeted to CENP-E, control siRNA, and Lipofectamine RNAiMax™ were obtained from, and used according to the instructions of, Invitrogen. All analyses were performed 24h following transfection.

Immunodetection

For immunocytochemistry, cells were fixed with 2% formaldehyde/PBS and permeabilized with -20^{o}C methanol. Antibody incubations were 1h at room temperature, and DNA was counterstained with Hoechst. Images were acquired with a Zeiss Axiovert 100M confocal microscope, except images of cells with spontaneous spindle defects, which were acquired with a Zeiss Axioskop 2 Plus microscope. For flow cytometry, cells were harvested by trypsinization and fixed overnight at -20^{o}C in 70% ethanol. Antibody incubations were 1h at room temperature, and DNA was counterstained with propidium iodide. Data were acquired using a FACSCalibur (Becton-Dickinson) and analyzed with CellQuest. Immunoblotting was performed as previously described (Yoon et al, 2005). Antibodies used and image quantification methodology are detailed in Supplementary Methods, and specificity of the mouse anti-γ-H2AX antibody is shown in Supplementary Fig. S5.

Time-lapse imaging

Phase-contrast images of cells grown inside a 37^{o}C, 5% CO_2 chamber were automatically obtained at 6 min intervals in multiple locations using an Olympus IX81 microscope. All images were analyzed with Slidebook.

Cytogenetic analyses

Chromosome spreads were prepared using standard cytogenetic techniques, DNA was stained with DAPI, and images were obtained using a Zeiss Axioskop 2 Plus microscope. Scoring of chromosome aberrations was performed according to the classification of Savage (Savage, 1976).

Results and Discussion

To address whether human cancer cells acquire DNA damage during mitotic arrest, we first examined nocodazole-arrested HCT116 colon cancer cells for the presence of γ-H2AX foci. During 36h of treatment, nocodazole produced a transient rise in mitotic index that peaked at 12h and was followed by mitotic slippage (Supplementary Fig. S1). While control prometaphase cells exhibited few γ-H2AX foci, nocodazole-arrested prometaphase cells showed a time-dependent increase in γ-H2AX foci per cell (Fig. 1*A* and *B*). A similar increase was observed after treatment with the microtubule-stabilizer paclitaxel and the Eg5 mitotic kinesin inhibitor monastrol (Fig. 1*A*), indicating that agents which induce mitotic arrest through different mechanisms also induce γ-H2AX foci. Notably, cells exposed to drug treatment, but not yet in mitosis, displayed no increase in γ-H2AX foci (Supplementary Fig. S2), indicating that only cells which have entered mitosis exhibit the response.

We next determined whether these γ-H2AX foci recruit additional proteins involved in the DNA damage response (DDR). However, it has been shown previously in HeLa cells that while γ-H2AX foci form in irradiated mitotic cells, other DDR proteins

are only recruited once cells return to interphase (Huang et al, 2005). As we also observed this lack of DDR protein recruitment in mitotic HCT116 cells (data not shown), we examined cells after 36h of nocodazole treatment, at which point virtually all cells had undergone mitotic slippage (Supplementary Fig. S1). Indeed, these postmitotic cells had increased γ-H2AX foci (Fig. 1*D*) which recruited the DDR proteins 53BP1 and a phospho-activated form of ATM (pATM) (Fig. 1*C*). Thus, γ-H2AX foci acquired during mitotic arrest, like those produced by γ-irradiation, recruit other DDR proteins once cells have exited mitosis.

Pharmacologic induction of mitotic arrest is accompanied by some degree of cell death in most cell lines, and γ-H2AX formation can participate in apoptosis (Lu et al, 2006; Rieder & Maiato, 2004). We thus investigated whether γ-H2AX formed during mitotic arrest represents ongoing cell death. Live-cell videomicroscopy indicated that <1% of cells died in a 30h period after release from a 6h nocodazole arrest (Fig. 2*B*) whereas 25% of cells died if continuously treated with the drug (Fig. 2*B*). Given that the average γ-H2AX per cell is increased 2.5-fold after 6 h of nocodazole (Fig. 1*B*), but 99% of these cells survive following drug washout, acquisition of γ-H2AX foci must precede any eventual commitment to cell death. Consistent with this, only cells with apoptotic nuclear morphology, which comprised <10% of the total after 18h nocodazole, exhibited caspase 3 cleavage, cytoplasmic localization of cytochrome c, or activation of Bax, whereas mitotic cells with normal nuclear morphology and abundant γ-H2AX foci displayed none of these events (Fig. 2*A*). Moreover, in contrast to the γ-H2AX foci seen in mitotic cells, apoptotic cells exhibited a distinct γ-H2AX staining pattern at the periphery of apoptotic chromatin (Fig. 2*A*). Finally, co-treatment of nocodazole-arrested

cells with the caspase inhibitor Q-VD-OPh (Caserta et al, 2003) produced a 98% inhibition of apoptotic cells, but no inhibition of γ-H2AX foci (Fig. 2*C*). Taken together, these data indicate that while death indeed follows mitotic arrest in a fraction of HCT116 cells, such death is neither the cause of, nor the obligatory conclusion to, γ-H2AX foci acquired during mitotic arrest.

To explore the downstream consequences of this DNA damage, we examined cells for the presence of acquired chromosome aberrations. While untreated cells harbored few spontaneous aberrations (Fig. 3*A*), cells which were grown in drug-free medium for 20h following release from an 18h nocodazole arrest exhibited a 6-fold increase in the frequency of chromosome breaks (Supplementary Table). The majority were of the chromosome-type, such as double-minutes, acentric fragments, and dicentrics (Fig. 3*B*, lower inset and Fig. 3*C*, all insets), consistent with γ-H2AX data indicating that breaks arise during mitotic arrest, and thus before subsequent DNA replication. However, some chromatid-type aberrations were also observed (Fig. 3*B*, upper inset), raising the possibility that additional DNA lesions, such as single-stranded breaks, base damages, or post-replication double-strand breaks, might be acquired by the cells (Savage, 1976; Zhuanzi et al, 2007). Notably, aberrations were observed not only in tetraploid cells produced through mitotic slippage (Fig. 3*C*) but also in near-diploid cells generated from cell division following drug washout (Fig. 3*B*), indicating that breaks occur even in cells which recover from transient arrest. Thus, DNA breaks incurred during mitotic arrest manifest as structural karyotype changes in subsequent cell cycles.

We next asked whether a genetic induction of mitotic arrest might also provoke DNA damage. As depletion of the kinetochore protein CENP-E disrupts chromosome-

spindle interactions and induces transient mitotic arrest in human cancer cells (Schaar et al, 1997; Tanudji et al, 2004; Yao et al, 2000), we examined HCT116 cells which had been transfected with siRNAs against this protein. Consistent with previous reports, CENP-E siRNAs produced an elevated mitotic index (Fig. 4*B*) and aberrant mitotic figures (Fig. 4*C, left*), as compared to control siRNA, indicating the induction of mitotic arrest. Moreover, CENP-E-depleted prometaphase cells exhibited an increase in γ-H2AX foci (Fig. 4*C*). Thus, genetic induction of mitotic arrest, like that elicited by antimitotic agents, provokes γ-H2AX foci in HCT116 cells.

Interestingly, while performing γ-H2AX immunocytochemistry, we noticed that occasional untreated mitotic HCT116 cells bore characteristics of prolonged mitotic arrest: a prometaphase arrangement of chromosomes and abundant γ-H2AX foci. We thus wondered whether these cells might contain spontaneous spindle defects, which can disrupt mitotic progression (Sluder et al, 1997; Stewenius et al, 2005). Out of 4,000 mitotic cells examined, prometaphase cells with marked elevation of γ-H2AX foci comprised 2.2% of the total, and 69% of these contained either monopolar or multipolar spindles (Fig 4*D*). In contrast, only 5.3% of prometaphase cells with minimal γ-H2AX foci contained mitotic spindle abnormalities. Thus, the frequency of prometaphase γ-H2AX elevation was increased 13-fold in cells with spontaneous spindle defects (Chi square $p<0.0001$). Of note, prophase cells with nascent monopolar or multipolar spindles did not possess elevated γ-H2AX foci (Supplementary Fig. S3), suggesting that γ-H2AX-intense mitotic cells acquire their foci during, and not before, prometaphase. These data indicate that prometaphase acquisition of γ-H2AX foci can occur spontaneously in HCT116 cells, and may arise during mitotic arrest provoked by spindle abnormalities.

Finally, we explored the extent to which this phenomenon occurs in other human cells. Out of eight colorectal cancer lines (including HCT116) exposed to nocodazole, six exhibited a γ-H2AX increase in some or most mitotic cells (Supplementary Fig. S4). Increased γ-H2AX was also observed in HeLa cervical cancer cells and BG-1 ovarian cancer cells (Supplementary Fig. S4). Thus, DNA damage during mitotic arrest is a common occurrence in human cancer cells. Additionally, we observed a modest γ-H2AX increase in IMR90 primary human fibroblasts (Supplementary Fig. S4), suggesting that even nontransformed human cells can acquire some DNA damage during mitotic arrest. However, the degree of γ-H2AX induction in primary cells was lower than in the majority of cancer lines, suggesting that malignant cells may be more prone to this damage.

Our study demonstrates that many human cells acquire DNA damage during mitotic arrest. While the mechanism responsible for this damage remains an area for future work, our results have several important implications. First, although unscheduled DNA breaks are known to derive from sources such as replication, oxidative stress, and exogenous mutagens (van Gent et al, 2001), it was hitherto unknown that such breaks could accumulate during mitotic arrest. As such, our findings describe a novel source of DNA damage. Second, because cancer cells frequently contain mitotic defects which induce arrest (Rieder & Maiato, 2004; Therman & Kuhn, 1989) — and because DNA breaks can promote tumorigenesis (van Gent et al, 2001) — our findings suggest that one way mitotic arrest may promote tumorigenesis is through DNA damage. Along these lines, it is interesting to note that inactivation of the tumor suppressor hCDC4 and oncogenic overexpression of Mad2 are accompanied not only by prolongation of mitosis

and aneuploidy, but also by evidence of DNA damage (Rajagopalan et al, 2004; Sotillo et al, 2007). Our results suggest that breaks acquired during mitotic arrest could contribute to the damage observed in these systems. In this way, mitotic arrest may be a source of structural, as well as numerical, chromosomal instability in human cells.

At the same time, this and other studies indicate that cells which endure prolonged or complete inhibition of mitosis exhibit reduced reproductive capacity (Rieder & Maiato, 2004). Indeed, although cell death was not the compulsory fate of HCT116 cells which acquired damage during mitotic arrest, we did observe subsequent death in a fraction of cells. Given that γ-H2AX foci increased with time of mitotic arrest — and that the cytotoxicity of DNA damage is dose-dependent — a third implication of our findings is that extensive DNA damage imposed by prolonged or complete inhibition of mitosis may promote cell death or growth arrest. In this way, our data support and extend the idea, offered by Wong and Stearns (Wong & Stearns, 2005), that the growth arrest which follows pharmacologic induction of mitotic arrest (formerly known as the "tetraploidy checkpoint,") may, at least in some contexts, be a response to DNA damage. Moreover, this consequence may have clinical implications: given the variable degree of γ-H2AX formed upon drug-induced arrest in different cancer cell lines, it is plausible that a tumor's sensitivity to antimitotic chemotherapy could be influenced by its propensity to sustain DNA damage during mitotic arrest. By extension, efforts to identify markers of this propensity might aid attempts to tailor antimitotic chemotherapy to susceptible tumors, as is currently being tried with markers of numerical chromosomal instability (Swanton et al, 2007).

Lastly, our findings do not support a recent claim that microtubule destabilizing agents elicit DNA breaks during G_1 phase, and not mitosis (Quignon et al, 2007). Although we do corroborate these authors' finding that such agents induce breaks, our data indicate that these breaks arise on mitotic chromosomes. This explains why even cells which divide following mitotic arrest possess DNA damage, and it indicates that some yet undefined property of mitosis itself commonly confers vulnerability to DNA damage in human cancer cells.

ACKNOWLEDGEMENTS

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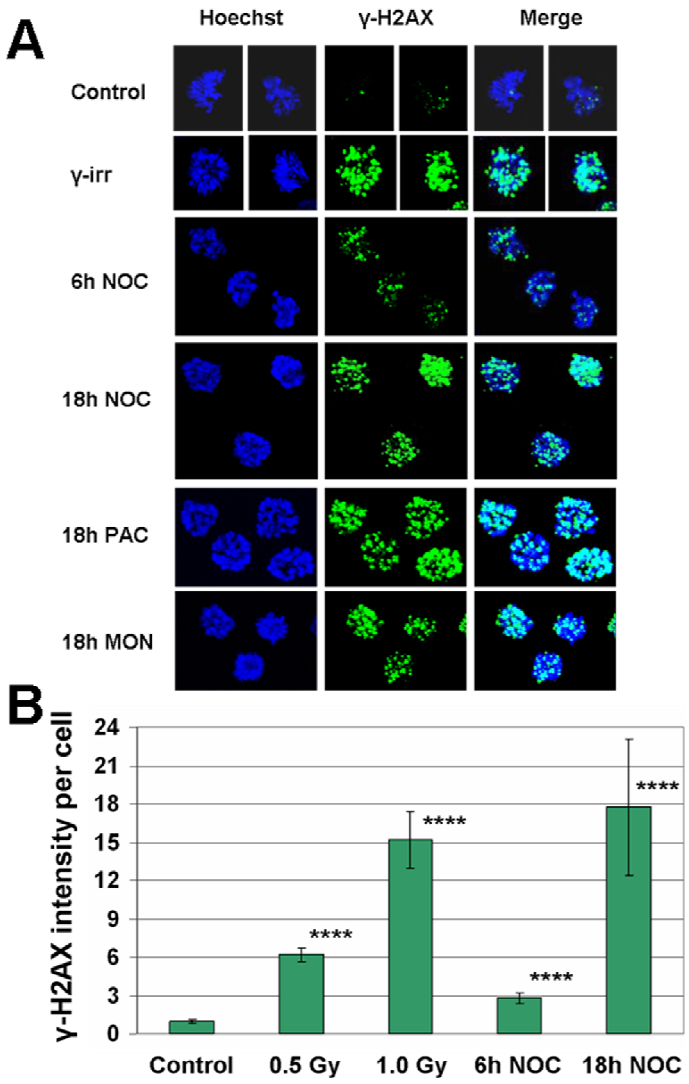


Figure 1. γ-H2AX foci accumulate during drug-induced mitotic arrest in HCT116 cells. *A*, Maximum projection images of stacks of prometaphase cells stained for γ-H2AX. γ-irr = 30 min after 2 Gy γ-irradiation. NOC = nocodazole. PAC = paclitaxel. MON = monastrol. *B*, Quantification of γ-H2AX pixel intensity per prometaphase cell. Means and SEMs are from at least 60 cells per sample taken from two independent experiments. **** = $p<0.0001$ for t-tests on log-transformed values, which were normally distributed, as compared to control.

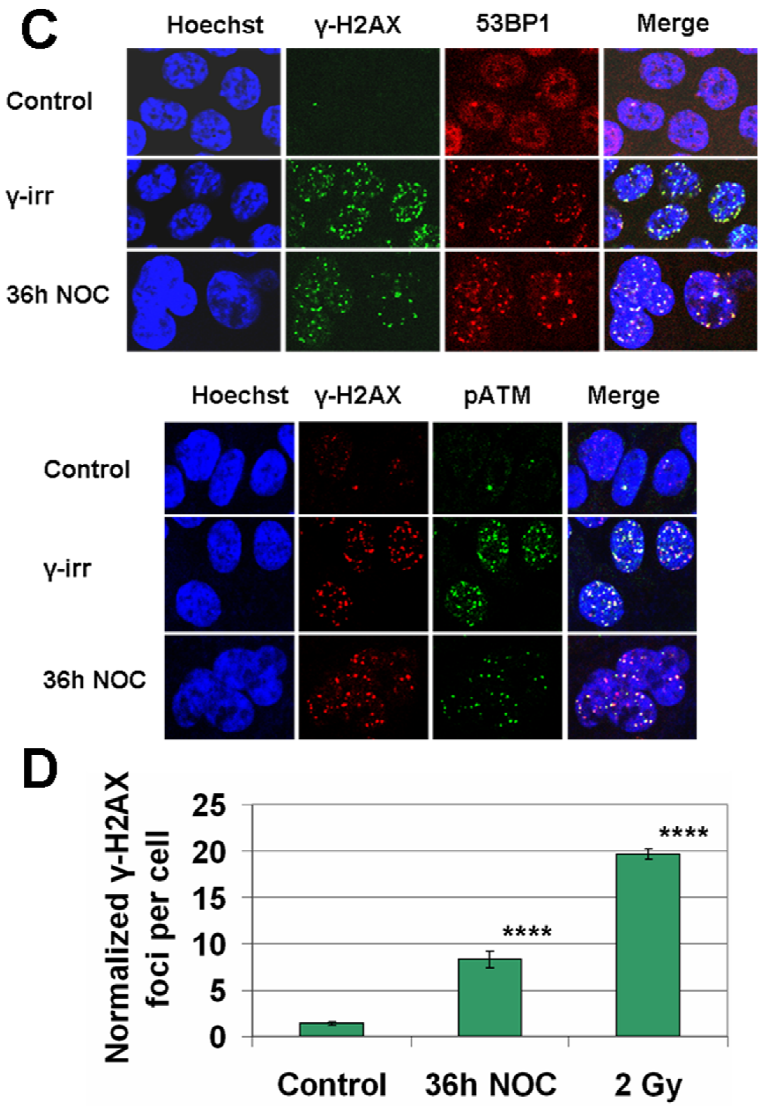


Figure 1C-D.

C, Single focal planes of interphase cells costained for γ-H2AX and either 53BP1 or pATM. *D*, Quantification of γ-H2AX foci per interphase cell. To adjust for cell-cycle differences in DNA content, foci number was normalized to the DNA content of a 2N cell using the Hoechst signal. Means and SEMs are from at least 145 cells per sample, taken from three independent experiments. **** = $p<0.0001$, for Mann-Whitney tests, as compared to control.

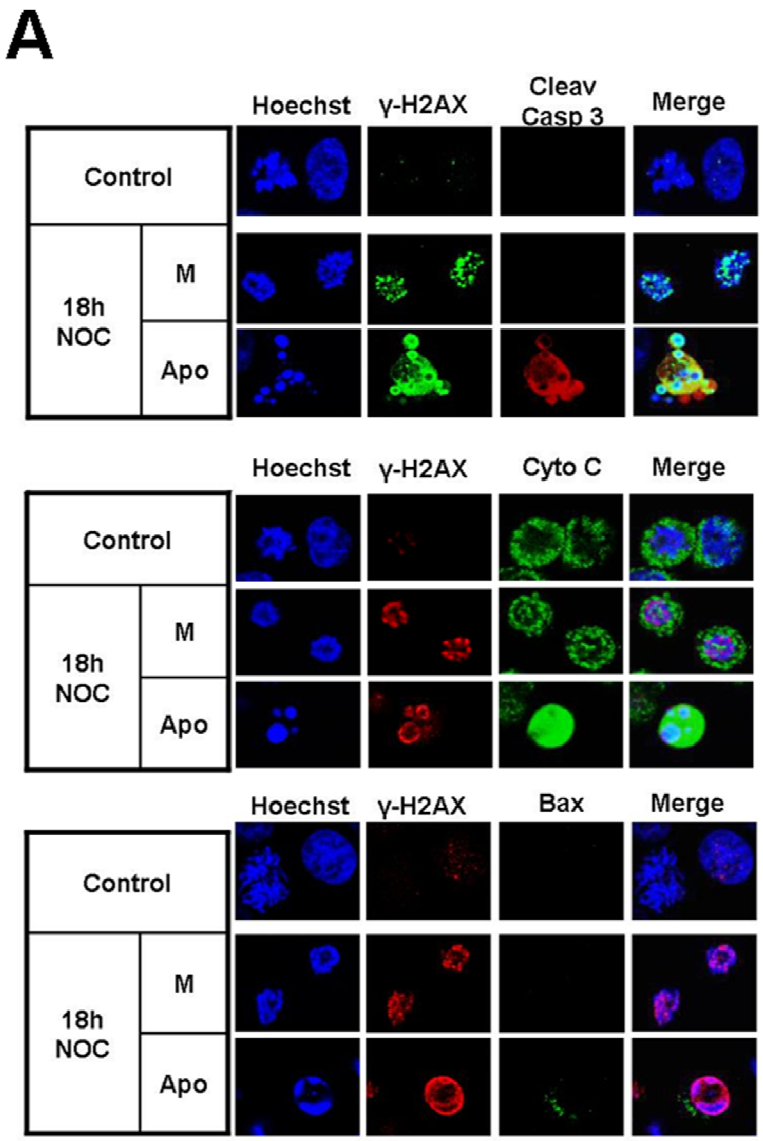


Figure 2. γ-H2AX foci acquired during mitotic arrest arise independently of cell death in HCT116 cells.

A, Single focal planes of cells stained for γ-H2AX and either cleaved caspase 3 (*top*), cytochrome c (*middle*), or activated Bax (*bottom*). M = mitotic cells. Apo = apoptotic cells. NOC = nocodazole. Cleav Casp 3 = cleaved caspase 3. Cyto C = cytochrome c.

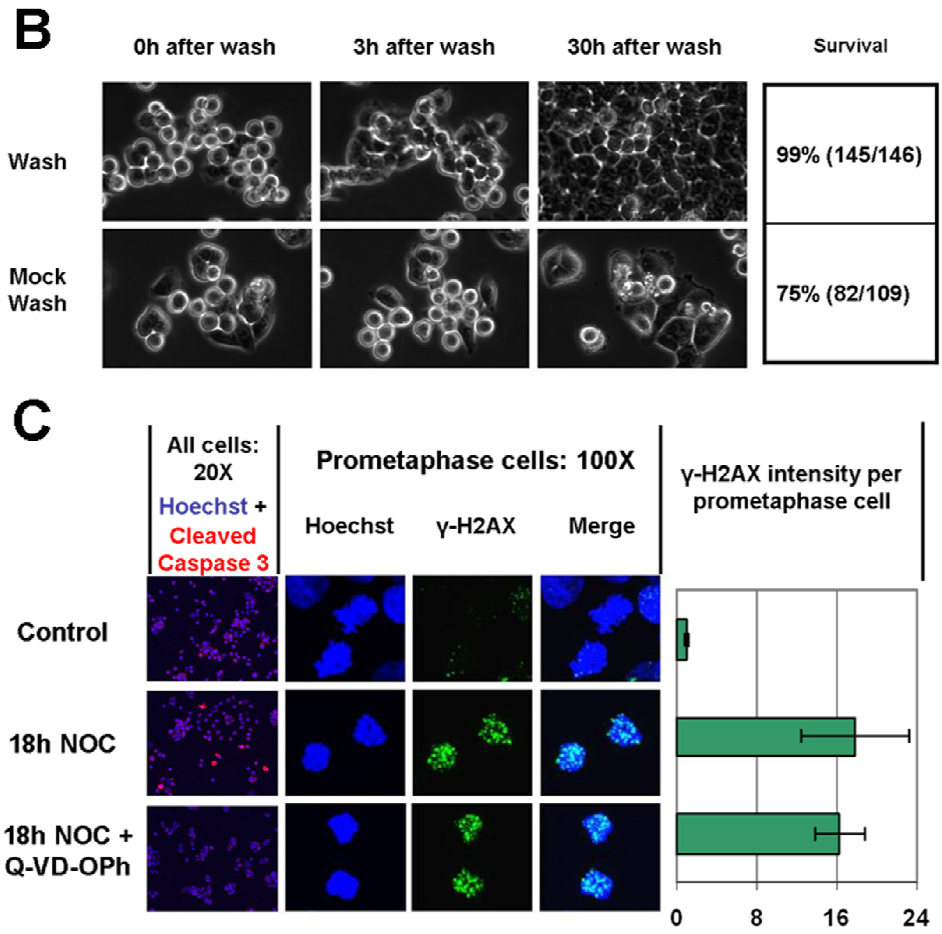


Figure 2B-C.

B, Cells were treated with nocodazole for 6h, washed either with drug-free or drug-containing medium, and returned to the chamber for an additional 30h of filming. Shown are timelapse images of cell fate at increasing times after either drug washout (Wash) or mock washout and continued culture in the drug (Mock wash). *C*, Q-VD-OPh inhibits cleaved caspase 3-positive apoptotic cells (20X column) but not γ-H2AX foci in prometaphase cells (100X column and chart). Means and SEMs are from at least 60 cells per sample taken from two independent experiments.

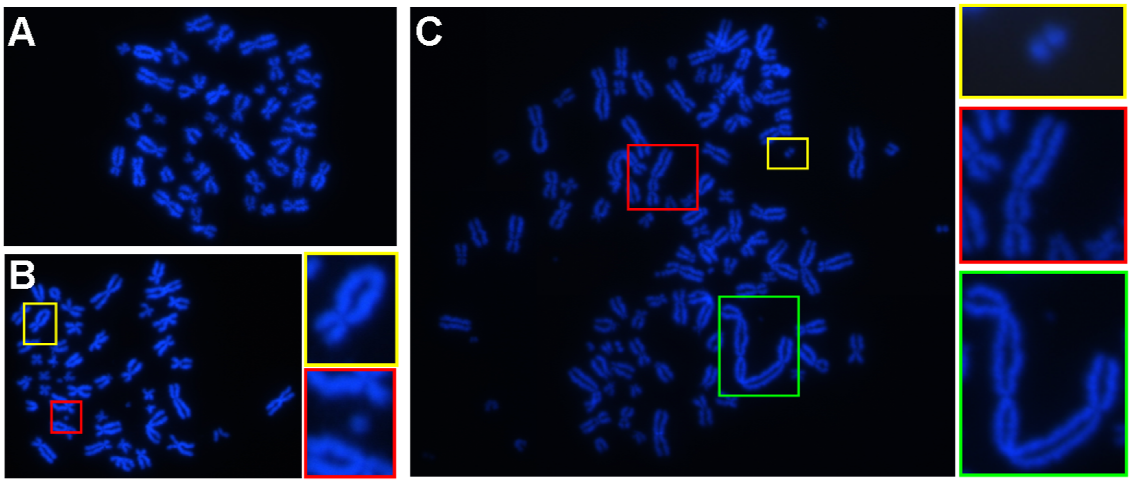


Figure 3. HCT116 cells released from mitotic arrest harbor chromosome aberrations. Treated cells were exposed to 18h nocodazole, washed, and released into drug-free medium for 19h. Control cells were incubated in drug-free medium for 19h. All cells were then exposed to nocodazole for 1h to disassemble spindles for spreading, and chromosome spreads were prepared. *A*, A representative control HCT116 cell. *B*, A representative near-diploid cell produced through cell division following drug washout. Insets show a chromatid exchange (*top*) and a chromosome fragment (*bottom*). *C*, A representative tetraploid cell produced through mitotic slippage. Insets show a double-minute chromosome (*top*) and two dicentric chromosomes (*middle and bottom*).

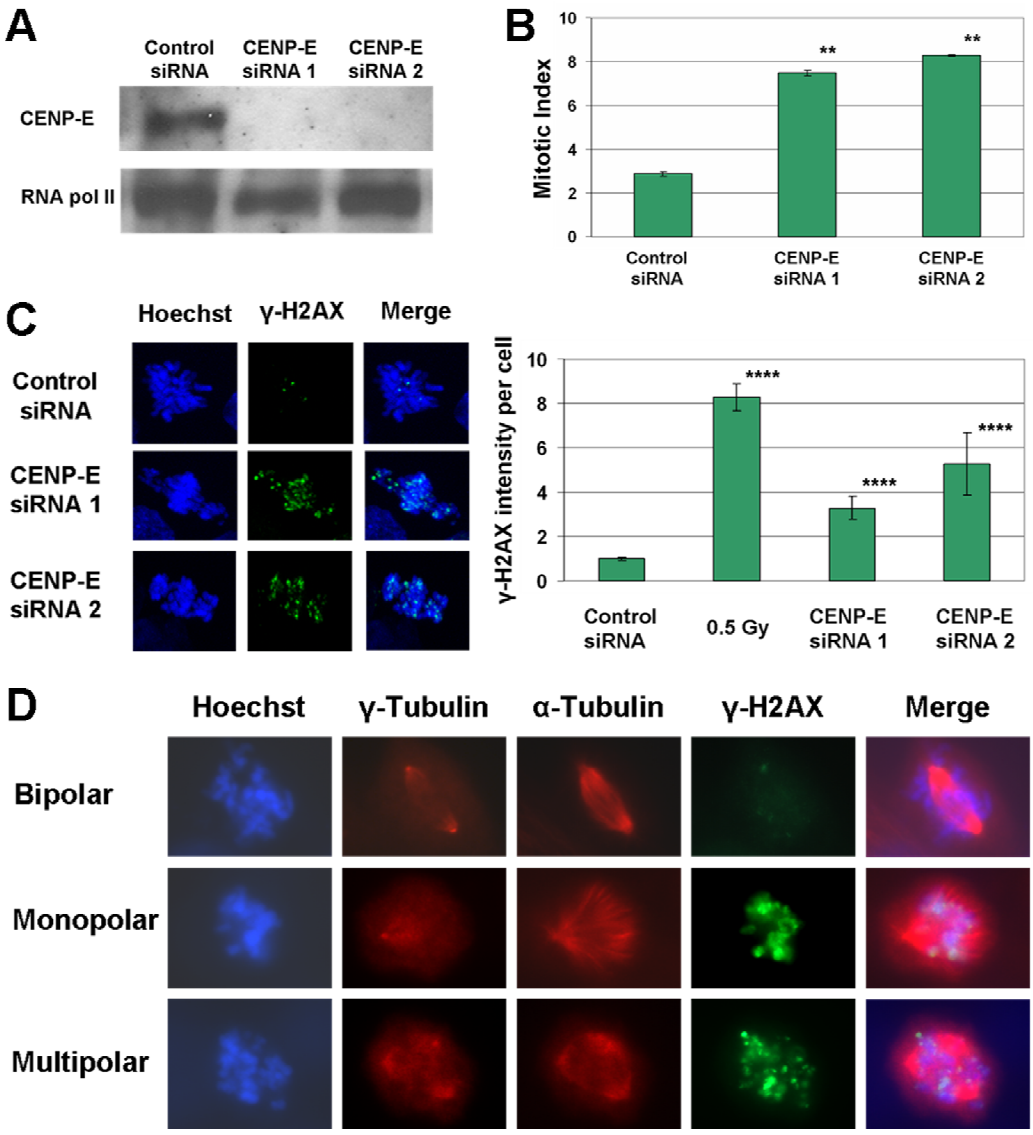


Figure 4. Prometaphase elevation of γ-H2AX in HCT116 cells occurs both upon knockdown of CENP-E and in untreated cells with spontaneous spindle defects. *A*, CENP-E protein levels in cells transfected with two different CENP-E siRNAs, or with control siRNA. RNA polymerase II was used as a loading control. Data are representative of two independent experiments. *B*, Mitotic indices of siRNA-transfected cells. Means and SEMs are from two independent experiments. ** = $p<0.01$, for t-tests,

as compared to control. *C*, Maximum projection images of stacks of siRNA-transfected prometaphase cells stained for γ-H2AX (*left*). Quantification of γ-H2AX pixel intensity per prometaphase cell after siRNA transfection (*right)*. Means and SEMs are from at least 70 cells per sample taken from two independent experiments. **** = $p<0.0001$ for t-tests on log-transformed values, which were normally distributed, as compared to control. *D*, Single focal planes of untreated prometaphase cells stained for γ-H2AX, γ-tubulin, and α-tubulin.

Supplementary Methods

Antibodies

For immunocytochemistry, the following antibodies were used: mouse anti-γ-H2AX (Upstate), rabbit anti-γ-H2AX (Abcam), rabbit anti-53BP1 (Novus), mouse anti-ATM-phosphoserine1981 (Upstate), rabbit anti-cleaved caspase 3 (Cell Signaling), mouse anti-cytochrome c (Cell Signaling), mouse anti-activated-Bax (Cell Signaling), rabbit anti-γ-tubulin (Sigma), and rat anti-α-tubulin (Chemicon). For flow cytometry, mouse anti-MPM-2 (Upstate) and mouse anti-γ-H2AX-FITC (Upstate) antibodies were used. All fluorescent secondary antibodies were Alexa-conjugates (Molecular Probes). For immunoblotting, rabbit anti-CENP-E (Sigma), rabbit anti-RNA Polymerase II (Santa Cruz), mouse anti-γ-H2AX (Upstate), and rabbit anti-actin (Sigma) antibodies were used. To confirm specificity of the mouse anti-γ-H2AX (Upstate) antibody, immunoblotting was performed on control and nocodazole-treated cells (Supplementary Fig. S5).

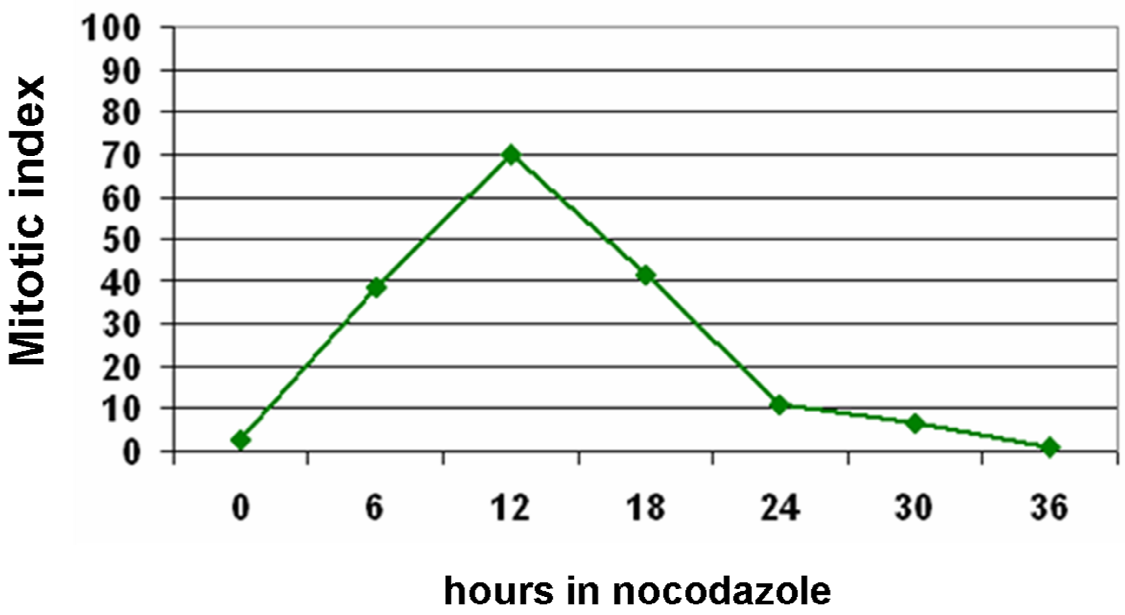
Image Quantification of γ-H2AX

Confocal stacks of individual nuclei stained with mouse anti-γ-H2AX antibody and Hoechst were obtained at 1-micron intervals. For interphase nuclei, maximum projection images were created, and γ-H2AX foci number was quantified using Metamorph (Molecular Devices). For prometaphase nuclei, total γ-H2AX intensity was quantified by summation of pixel intensity of each plane, as mitotic compaction of chromatin produced too much foci overlap for discrimination of foci number. Raw intensity values were then divided by the mean intensity value of control cells, to set the baseline intensity value at 1.

	Cells	Chrom	Chromosome-type aberrations		Chromatid-type aberrations		Total breaks per chrom % (n)	Cells with at least 1 break % (n)
			CSB per chrom % (n)	CSE per chrom % (n)	CTB per chrom % (n)	CTE per chrom % (n)		
con	124	5593	0.43 (24)	0.018 (1)	0.14 (8)	0.036 (2)	0.68 (38)	17 (21)
γ-irr	135	6121	1.8 (108)	0.28 (17)	0.36 (22)	0.41 (22)	3.5 (214)****	59 (80)****
noc	106	8075	2.4 (193)	0.26 (21)	0.50 (40)	0.35 (28)	4.1 (331)****	66 (70)****

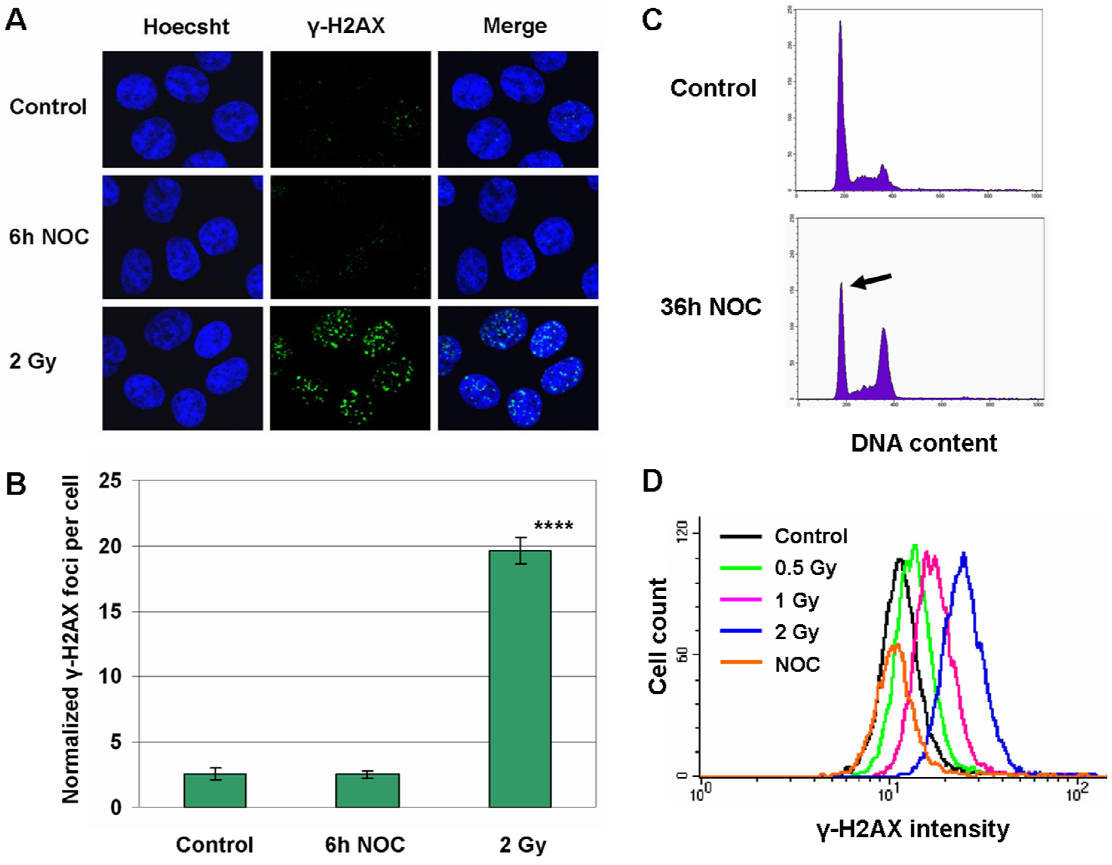
Supplementary Table. Quantification of chromosome aberrations.

Treated cells were exposed to 18h nocodazole, washed, and released into drug-free medium for 19h. Positive control cells were exposed to 2 Gy of γ-irradiation and incubated for 19h, and negative control cells were incubated in drug-free medium for 19h. All cells were then exposed to nocodazole for 1h to disassemble spindles for spreading, and chromosome spreads were prepared. Con = control. γ-irr = γ-irradiated. Noc = pooling of both the tetraploid (70%) and near-diploid (30%) chromosome spreads produced through drug washout. Cells = number of cells analyzed from two independent experiments. Chrom = total number of chromosomes contained in analyzed cells. CSB = chromosome break. CSE = chromosome exchange. CTB = chromatid break. CTE = chromatid exchange. Total breaks = CSB + CTB + 2(CSE + CTE), as two breaks produce exchanges. **** = $p<0.0001$, for chi square tests, as compared to control.



Supplementary Figure S1. Nocodazole induces transient mitotic arrest in HCT116 cells.

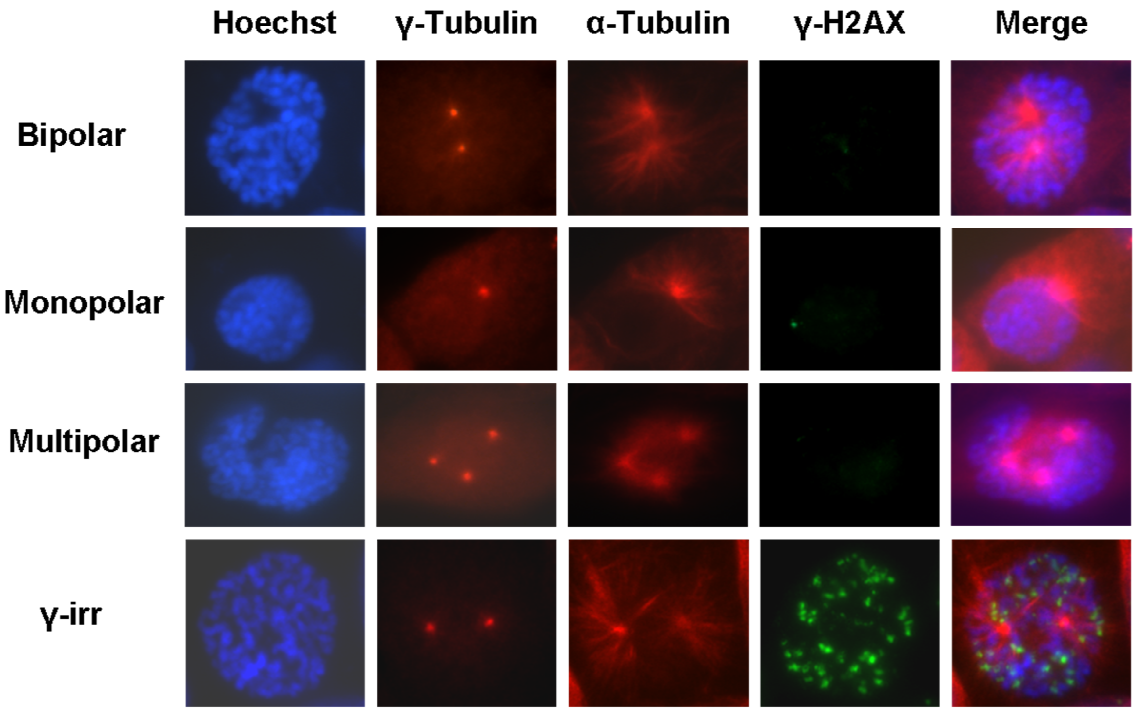
Mitotic index was measured using flow cytometric analysis of cells stained for the mitotic phosphoepitope MPM-2. DNA was counterstained with propidium iodide.



Supplementary Figure S2. Nocodazole does not induce γ-H2AX foci in premitotic HCT116 cells.

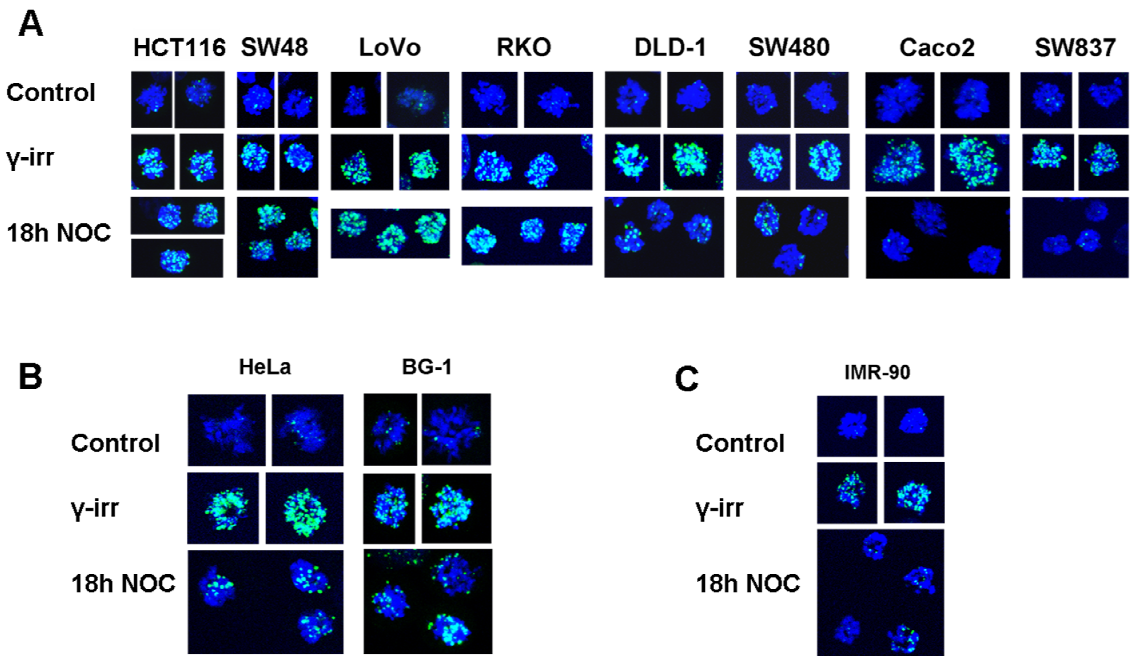
A, Maximum projection images of stacks of interphase cells stained for γ-H2AX after 6h nocodazole, at which point all interphase cells are premitotic. *B*, Quantification of γ-H2AX foci per interphase cell after 6h nocodazole, normalized to DNA content of a 2N cell. Means and SEMs are from at least 98 cells per sample, taken from two independent experiments. **** = $p<0.0001$, for Mann-Whitney tests, as compared to control. *C*, Cells were contact-inhibited by growing to confluency over 6 days, exposed to either 36h nocodazole or 36h drug-free medium, and stained with propidium iodide. The cell cycle distribution at the end of treatment is shown, and arrows indicate cells which remain in

G_1 due to contact inhibition despite nocodazole treatment *D*, γ-H2AX intensity was then measured by gating on the contact-inhibited G_1 cells, which had also been stained for γ-H2AX. Positive control cells were irradiated 30 min before the end of the final 36h incubation in drug-free medium. Data are representative of two independent experiments.



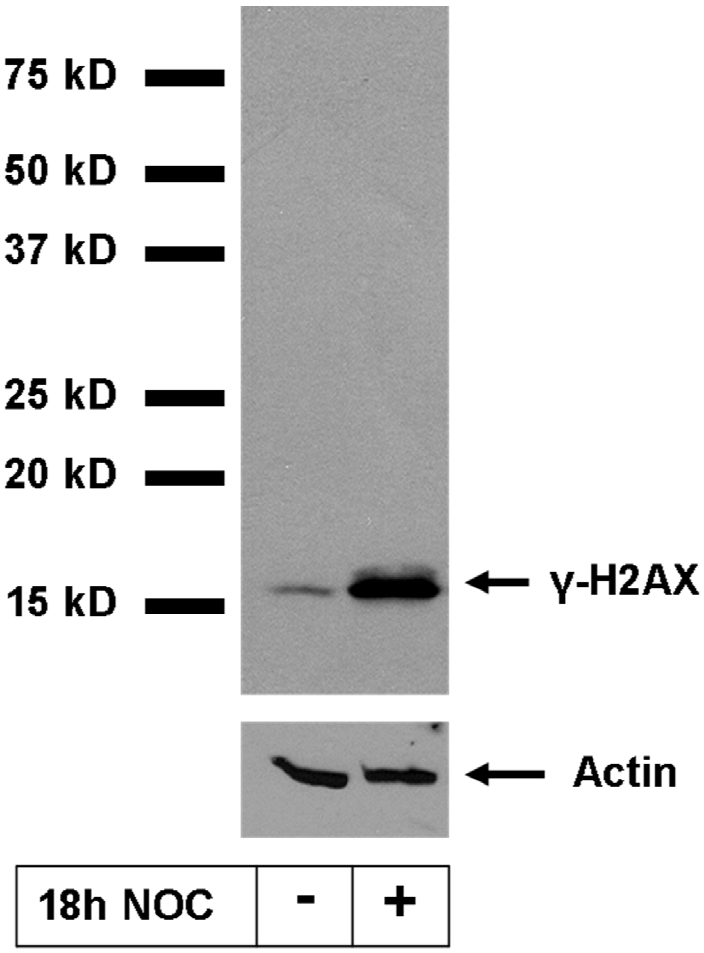
Supplementary Figure S3. Prophase HCT116 cells with spindle abnormalities do not contain elevated γ-H2AX.

Single focal planes of prophase cells stained for γ-H2AX, γ-tubulin, and α-tubulin, indicating that monopolar or multipolar cells do not have elevated γ-H2AX. γ-irr = 30 min after 2 Gy γ-irradiation.



Supplementary Figure S4. Acquisition of γ-H2AX foci during mitotic arrest is a common occurrence in human cell lines.

Maximum projection images of stacks of prometaphase cells stained with Hoechst (blue) and anti-γ-H2AX (green) after 18h nocodazole. γ-irr = 30 min after 2 Gy γ-irradiation. *A*, Colorectal cancer cell lines. *B*, HeLa cervical cancer cells and BG-1 ovarian cancer cells. *C*, IMR90 primary human fibroblasts. Acquisition settings were constant between treatments within a given cell line, but settings were optimized for each cell line to account for differences in background staining.



Supplementary Figure S5. Anti-γ-H2AX antibody recognizes a single protein in HCT116 cells.

Cells treated with or without nocodazole for 18h were subjected to immunoblotting with mouse anti-γ-H2AX antibody (Upstate). Control and nocodazole-arrested cells were also co-treated with the Q-VD-OPh caspase inhibitor to prevent apoptotic γ-H2AX from diluting the signal of non-apoptotic cells. Actin was used as a loading control.

Chapter III

p53 suppresses structural chromosome instability following mitotic arrest in human cells

W. Brian Dalton[1,2], Bing Yu[1,2], and Vincent W. Yang[1,3*]

[1]Division of Digestive Diseases, Department of Medicine,
[2]Biochemistry, Cell and Developmental Biology Graduate Program, and
[3]Winship Cancer Institute,
Emory University School of Medicine,
Atlanta, Georgia 30322, U. S. A.

Abstract

The p53 tumor suppressor inhibits the proliferation of cells which have undergone prolonged activation of the mitotic checkpoint. However, neither the function of this response, nor the mechanism by which p53 is activated during prolonged mitosis, is well characterized. Here we report that p53 suppresses structural chromosome instability following mitotic arrest in human cells. In both HCT116 colon cancer cells and normal human fibroblasts, DNA breaks occurred during mitotic arrest in a p53-independent manner, but p53 was required to suppress the proliferation and structural chromosome instability of the resulting polyploid cells. Moreover, we observed that p53 suppressed both the frequency and structural chromosome instability of spontaneous polyploids in HCT116 cells. These data suggest that a function of the p53-dependent postmitotic response may be the prevention of structural chromosome instability following prolonged activation of the mitotic checkpoint. Accordingly, our study suggests a novel mechanism of tumor suppression for p53, as well as a potential role for p53 in the outcome of antimitotic chemotherapy.

Introduction

The p53 tumor suppressor represents a central defense against the development of human cancer (Vousden & Lane, 2007). Its inactivation is one of the most common alterations in human tumors, and numerous functional studies have established the tumor suppressing properties of p53 (Toledo & Wahl, 2006). A principal mechanism of this tumor suppression is the induction of growth arrest and/or apoptosis in cells which suffer DNA damage (Vousden & Lu, 2002). In this way, p53 inhibits the propagation of cells which harbor potentially oncogenic DNA alterations. In addition, other forms of stress have been shown to activate p53-dependent responses (Vousden & Lane, 2007). One example is prolonged activation of the mitotic checkpoint, which elicits a p53-dependent cell cycle arrest (Cross et al, 1995; Lanni & Jacks, 1998; Minn et al, 1996). This "postmitotic" response, so named because growth arrest is actually imposed on cells which have exited from prolonged mitosis, has been observed in numerous cell systems (Andreassen et al, 2001; Chan et al, 2008; Cross et al, 1995; Di Leonardo et al, 1997; Lanni & Jacks, 1998; Minn et al, 1996; Vogel et al, 2004). Despite the ubiquity of this phenomenon, neither the mechanism by which p53 is activated by prolonged mitotic arrest, nor the function of the postmitotic response, is well defined (Ganem & Pellman, 2007; Stukenberg, 2004).

One clue to the function of the postmitotic response is that prolonged activation of the mitotic checkpoint has been causally implicated in tumorigenesis (Dalton & Yang, 2007; Rajagopalan et al, 2004; Sotillo et al, 2007). Indeed, mitosis is frequently prolonged in cancer cells, and several genetic and epigenetic defects which cause mitotic arrest can contribute to cancer (Fujiwara et al, 2005; Hernando et al, 2004; Keck et al,

2007; Menssen et al, 2007; Rajagopalan et al, 2004; Sotillo et al, 2007; Yang et al, 2008). For some of these defects, such as inactivation of Rb and hCDC4, oncogenic activation of c-Myc, and the presence of supernumerary chromosomes and/or centrosomes, prolonged mitosis is one of many cellular effects which may or may not be oncogenic (Fujiwara et al, 2005; Hernando et al, 2004; Rajagopalan et al, 2004; Yang et al, 2008). However, mitotic arrest and spontaneous cancer also develop in mice overexpressing Mad2, a protein principally involved in mitotic checkpoint signaling, providing strong evidence that prolonged mitotic checkpoint activation can directly promote tumorigenesis (Sotillo et al, 2007). Accordingly, the p53-dependent postmitotic response may serve to inhibit the propagation of cells which acquire oncogenic properties during prolonged activation of the mitotic checkpoint.

What aspects of mitotic arrest might be oncogenic? Certainly, one candidate is aneuploidy and/or tetraploidy resulting from the chromosome missegregation and/or cytokinesis failure which can follow prolonged activation of the mitotic checkpoint (Pellman, 2007; Rajagopalan et al, 2004; Sotillo et al, 2007; van Deursen, 2007). Indeed, in some contexts, aneuploidy and tetraploidy have themselves been causally implicated in tumorigenesis (Fujiwara et al, 2005; Weaver et al, 2007). At the same time, we and others recently found that mitotic arrest can induce structural chromosome aberrations resulting from double-stranded DNA breaks (Dalton et al, 2007; Quignon et al, 2007; Stevens et al, 2007). Given the causative role of structural chromosome aberrations in cancer, these observations suggest that one way prolonged mitosis could promote cancer is through introduction of DNA breaks. By extension, one function of the p53-dependent postmitotic response may be to prevent the structural chromosome instability which can

result from DNA damage acquired during mitotic arrest. To investigate this possibility, we have measured structural chromosome instability resulting from mitotic arrest in human colon cancer cells and normal fibroblasts which differ only in their p53 status. Our results demonstrate that, by imposing growth arrest and/or apoptosis in cells whose DNA is damaged during mitotic arrest, p53 suppresses structural chromosome instability following prolonged mitotic checkpoint activation in human cells.

Results

Both *p53*$^{+/+}$ and *p53*$^{-/-}$ cells acquire γ-H2AX foci during mitotic arrest

To examine the role of p53 in structural chromosome instability following prolonged mitosis, we first asked whether p53 influences the acquisition of DNA damage during pharmacologic induction of mitotic arrest in isogenic *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells (Bunz et al, 1998). Upon treatment with the microtubule-depolymerizing agent nocodazole, both *p53*$^{+/+}$ and *p53*$^{-/-}$ cells exhibited a transient rise in mitotic index which peaked at 12-18h (Figure 1A). This was followed by "mitotic slippage," a process whereby mitotically-arrested cells return to interphase without undergoing cell division (Figure 1A) (Rieder & Maiato, 2004). To determine the extent of DNA damage acquired during this arrest, we measured the formation of γ-H2AX, the phosphorylated form of histone H2AX which forms around sites of DNA breaks (Rogakou et al, 1999). Similar to our previous findings (Dalton et al, 2007), nocodazole-arrested prometaphase *p53*$^{+/+}$ cells showed an increase in γ-H2AX foci, as compared to prometaphase controls (Figure 1B). Increased γ-H2AX foci were also observed in *p53*$^{-/-}$ cells (Figure 1B), and flow cytometric analysis demonstrated that the magnitude of this increase was comparable to

p53$^{+/+}$ cells (Figure 1C). To determine whether increased γ-H2AX was indeed the result of mitotic arrest, and not some other effects of nocodazole, we performed the assay after siRNA-mediated knockdown of the Eg5 mitotic kinesin protein, whose inactivation prevents centrosome separation, produces a monopolar spindle, and thereby induces mitotic arrest (Koller et al, 2006). Indeed, knockdown of Eg5 produced an elevated mitotic index, monopolar spindles, and increased γ-H2AX foci in mitotic *p53*$^{+/+}$ and *p53*$^{-/-}$ cells (Figure 1D-F). Thus, pharmacologic and genetic manipulations which provoke mitotic arrest through distinct mechanisms both produce evidence of DNA breaks in *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells.

p53 inhibits the polyploidization and survival of postmitotic cells

Having observed that p53 does not influence the acquisition of DNA damage during mitotic arrest, we next investigated whether the cellular consequences of this damage are dependent on p53. We first determined the fates of *p53*$^{+/+}$ and *p53*$^{-/-}$ cells after nocodazole treatment. Similar to results from previous results (Castedo et al, 2006; Kim et al, 2004; Vogel et al, 2004), we found that nocodazole-treated *p53*$^{-/-}$ cells exhibited greater polyploidization when compared to *p53*$^{+/+}$ cells (Figure 2A). Moreover, among those cells which became polyploid (DNA content > 4N), a greater proportion of *p53*$^{-/-}$ cells reached mitosis, as compared to *p53*$^{+/+}$ cells (Figure 2B and 2C), supporting the idea that p53-dependent cell cycle arrest may occur at both G1 and G2 in drug-induced polyploid cells (Vogel et al, 2004).

In addition to increased polyploidization, *p53*$^{-/-}$ cells also exhibited a reduction in

cell death following nocodazole treatment (Figure 2B, D-F). This was evidenced first by the fact that, in comparison to $p53^{+/+}$ cells, $p53^{-/-}$ cells showed a reduced accumulation of cells that were entirely negative for the MPM-2 phosphoepitope (Figure 2B and D), a property which was found, through fluorescent microscopy, to be a marker of apoptotic cells (Supplementary Figure S1). Second, PARP cleavage, a caspase-dependent apoptotic event, was markedly reduced in nocodazole-treated $p53^{-/-}$ cells (Figure 2E). Third, long-term colony survival of $p53^{-/-}$ cells exposed to 48h nocodazole, while low overall, was 5-fold higher than that of $p53^{+/+}$ cells (Figure 2F). Taken together, these data indicate that p53 inhibits not only polyploidization, but also survival, following pharmacologic induction of mitotic arrest in HCT116 cells.

Although p53-dependent growth arrest following prolonged mitotic arrest has been consistently detected in numerous cell systems, as mentioned previously, the observance of p53-dependent cell death following mitotic arrest is variable and cell type-specific (Castedo et al, 2006; Galmarini et al, 2001; Gascoigne & Taylor, 2008; Kienitz et al, 2005; Tao et al, 2007; Wahl et al, 1996; Woods et al, 1995; Yamaguchi et al, 2004). Considering the fact that only a single $p53^{-/-}$ HCT116 clone was isolated from the gene targeting process (Bunz et al, 1998), the differential sensitivity to cell death in $p53^{-/-}$ HCT116 cells might be a consequence of other genetic differences acquired during the drug selection procedure required for gene targeting, as discussed elsewhere (Matoba et al, 2006; Pfleghaar et al, 2005). To address this possibility, we tested the response to nocodazole of HCT116 cells in which p53 was silenced through lentivirus-mediated stable expression of miRNAs. Compared to cells expressing a control miRNA, two cell lines expressing independent miRNAs against p53 exhibited increased polyploidy and

reduced cell death upon nocodazole treatment (Figure 3A-E), phenocopying the behavior of *p53*$^{-/-}$ cells. These data thus provide robust confirmation that p53 inhibits both polyploidization and cell death following pharmacologic induction of mitotic arrest in HCT116 cells.

p53 inhibits structural chromosome instability in postmitotic cells

By inducing growth arrest and apoptosis in cells which have undergone prolonged mitotic arrest, p53 could act to suppress the structural chromosome changes which result from DNA damage acquired during prolonged mitosis. If true, *p53*$^{-/-}$ cells made polyploid following prolonged mitosis might be expected to harbor more structural chromosome aberrations than their *p53*$^{+/+}$ counterparts. To test this hypothesis, we examined *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells for the presence of chromosome aberrations after 48h of nocodazole treatment. Consistent with our previous findings (Dalton et al, 2007), nocodazole-induced polyploid HCT116 cells exhibited multiple types of chromosome aberrations, including double-minutes, fragments, dicentric chromosomes, and chromatid exchanges (Figure 4). Notably, nocodazole-treated *p53*$^{-/-}$ cells exhibited a 1.5-fold higher burden of aberrations than *p53*$^{+/+}$ cells ($p < 0.0001$) (Figure 4). Because p53 does not appear to influence the magnitude of DNA damage acquisition during mitotic arrest (Figure 1), this result suggests that p53 preferentially inhibits the polyploidization and survival of those cells which suffer the most DNA damage during mitotic arrest. In this way, p53 suppresses structural chromosome instability following mitotic arrest in HCT116 cells.

Inhibition of cytokinesis does not elicit significant structural chromosome instability

Taken together, our γ-H2AX and cytogenetic data indicate that the observed p53-dependent structural chromosome instability is a consequence of DNA damage incurred during prolonged mitotic arrest. However, it is also possible that the polyploid state itself might elicit structural chromosome changes, for example, through DNA damage incurred during a disturbed S-phase (Ganem et al, 2007). To explore this possibility, we determined the cell fate and genomic stability of $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells treated with the myosin inhibitor blebbistatin, which creates polyploid cells through inhibition of cytokinesis without provoking mitotic arrest (Wong & Stearns, 2005). In contrast to nocodazole, blebbistatin was capable of producing polyploidy without significant cell death, cell cycle arrest, or increased chromosome aberrations (Figure 5A-C). Moreover, no difference in these parameters was observed between $p53^{+/+}$ and $p53^{-/-}$ cells (Figure 5A-C). This result thus indicates that it is prolonged mitotic arrest, and not polyploidy per se, that elicits significant DNA damage and p53-dependent suppression of structural chromosome instability in nocodazole-treated HCT116 cells. Interestingly, although the total number of breaks per chromosome was not increased by blebbistatin treatment, the frequencies of certain aberrations did change (Figure 5C). For example, chromosome exchanges (CSE column, Figure 5C) were somewhat elevated, whereas chromosome fragments (CSB column, Figure 5C) were reduced, in blebbistatin-treated cells. This pattern was also observed following cytokinesis inhibition using dihydrocytochalasin B (unpublished observations). Thus, while these data demonstrate that polyploidization itself does not account for the structural chromosome instability following prolonged

mitotic arrest, they suggest that polyploidization, or at least pharmacologic inhibition of cytokinesis, may induce some degree of DNA damage.

p53 suppresses structural chromosome instability following mitotic arrest in normal human fibroblasts

We next tested whether p53-dependent suppression of structural chromosome instability following mitotic arrest occurs in untransformed human cells. To do so, we examined the response to nocodazole of human diploid fibroblasts (HDFs) in which p53 was silenced through lentivirus-mediated expression of miRNAs (Figure 6A). In HDFs expressing either p53 or control miRNAs, nocodazole produced an increase in γ-H2AX foci during mitotic arrest (Figure 6B). Thus, as in HCT116 cells, p53 does not influence the acquisition of DNA damage during mitotic arrest in normal human cells. Also similar to HCT116 cells, HDFs expressing p53 miRNA exhibited an increase in polyploidization (3.5– to 7.5-fold, $p<0.05$) and structural chromosome instability (2.6- to 2.8-fold, $p<0.01$) following nocodazole treatment, as compared to control miRNA (Figure 6C-E). In addition to harboring chromosome aberrations like fragments and dicentrics, a minority of HDFs also exhibited evidence of chromosome pulverization, which can occur when micronuclei enter mitosis prematurely (Supplementary Figure S2) (Ikeuchi et al, 1972; Kato & Sandberg, 1967). Nocodazole-induced cell death, however, did not appear to be influenced by p53 knockdown in HDFs (Supplementary Figure S3), supporting the notion that the effect of p53 on apoptosis following prolonged mitotic arrest is cell type-dependent, as previously discussed(Wahl et al, 1996; Woods et al, 1995; Wu & El-Diery, 1996; Yamaguchi et al, 2004). These data therefore demonstrate that, by inducing

postmitotic cell cycle arrest, p53 suppresses structural chromosome instability following mitotic arrest in normal human cells.

p53 suppresses structural chromosome instability in spontaneous polyploids

Interestingly, while performing cytogenetic analysis of HCT116 cells, we noticed that many spontaneous polyploid *p53*$^{-/-}$ cells contained chromosome aberrations, including chromosome fragments, dicentrics, and chromatid breaks (Figure 7A). Indeed, quantification revealed a 2.8-fold increase in the number of breaks per chromosome in polyploid (8N) vs. diploid (4N) *p53*$^{-/-}$ cells ($p<0.0001$) (Figure 7A). Color karyotyping of these spontaneous *p53*$^{-/-}$ polyploids (>4N) confirmed the presence of cells with chromosome fragments and chromosome rearrangements (Figure 7C). In contrast, spontaneous *p53*$^{+/+}$ polyploids did not exhibit an elevated frequency of chromosome aberrations, when compared to *p53*$^{+/+}$ diploid cells (Figure 7A). Moreover, we found that, while of low overall abundance, spontaneous polyploid mitotic cells were 3-fold more frequent in *p53*$^{-/-}$, as compared to *p53*$^{+/+}$, cells (Figure 7B), consistent with previous studies (Bunz et al, 2002; Pantic et al, 2006). These findings thus indicate that, in HCT116 cells, p53 suppresses both the frequency and the structural chromosome instability of spontaneous polyploids.

While the origin of these spontaneously damaged *p53*$^{-/-}$ polyploids is unknown, one possibility is that they may arise from cells which undergo mitotic slippage following mitotic arrest, for example due to the appearance of spontaneous spindle defects, which we previously observed at low frequency in HCT116 cells (Dalton et al, 2007). To examine this possibility, we examined mitotic progression using timelapse

videomicroscopy. This analysis revealed that 4% (24/592) of mitoses resulted in spontaneous cell division failure in *p53*$^{-/-}$ HCT116 cells. Moreover, the average length of mitosis in these cells was significantly longer than that of cells which completed a normal, bipolar mitosis (229 vs. 36 minutes, $p<0.0001$) (Supplementary Figure S4). Indeed, some cells spent up to 10h in spontaneous mitotic arrest, before undergoing mitotic slippage (data not shown). These data thus indicate that mitotic slippage following prolonged mitosis can occur spontaneously, and are consistent with the possibility that this mechanism of polyploidization may be responsible for the increased structural chromosome instability in polyploid *p53*$^{-/-}$ HCT116 cells.

Discussion

Our study demonstrates that in both human colon cancer cells and normal human fibroblasts, p53 suppresses structural chromosome instability following prolonged activation of the mitotic checkpoint. This finding has several important implications. Because (1) certain genetic and epigenetic alterations which elicit prolonged mitotic checkpoint activation are causally implicated in tumorigenesis (Dalton & Yang, 2007; Rajagopalan et al, 2004; Sotillo et al, 2007), (2) prolonged mitosis can provoke DNA breaks (Dalton et al, 2007; Quignon et al, 2007; Stevens et al, 2007), (3) DNA breaks can promote tumorigenesis (van Gent et al, 2001) and (4) inactivation of p53 is one of the most common oncogenic events in human cancer (Vousden & Lane, 2007), our findings suggest that suppression of structural chromosome instability following prolonged mitosis may represent a novel mechanism of tumor suppression for p53. Thus, similar to other sources of DNA damage such as replication stress and radiation, prolonged mitotic

checkpoint activation may produce potentially oncogenic DNA damage which necessitates the antitumor activities of p53.

By extension, our data suggest that one function of the long-observed p53-dependent postmitotic response (Cross et al, 1995; Ganem & Pellman, 2007; Lanni & Jacks, 1998; Minn et al, 1996; Stukenberg, 2004) may be the suppression of structural chromosome instability. Accordingly, if polyploid cells generated through mitotic slippage evade this p53-dependent response, they may bear an increased risk of promoting cancer, due to genetic alterations acquired during their formation. What is more, polyploid cells may experience further DNA damage during subsequent mitosis, as the presence of supernumerary chromosomes and/or centrosomes itself prolongs activation of the mitotic checkpoint (Yang et al, 2008). Consistent with this idea, we previously observed evidence that spontaneous multipolar HCT116 cells, many of which are likely to be polyploid (Stewenius et al, 2005), may acquire DNA damage during a spontaneously prolonged mitosis (Dalton et al, 2007). Indeed, inactivation of p53 increased the frequency of these damaged multipolar cells (data not shown). Thus, p53-dependent postmitotic arrest may suppress a "vicious cycle" of structural chromosome instability occurring during the formation—and propagation—of polyploid cells. This may help explain why polyploid cells created through cytokinesis inhibition—and thus without prior mitotic arrest—have increased tumorigenicity in a p53-deficient background (Fujiwara et al, 2005).

Notably, Bunz et al. have previously reported that, in human cells, inactivation of p53 increases the frequency of spontaneous polyploidy, but not near-diploid aneuploidy nor structural aberrations (Bunz et al, 2002). Our findings are in general agreement with

this study, as p53 inactivation increased spontaneous polyploidy (Figure 7B), but not near-diploid aneuploidy (unpublished observations), in HCT116 cells. We did observe an increase in spontaneous structural aberrations in diploid $p53^{-/-}$, as compared to $p53^{+/+}$, HCT116 cells (Figure 7), although the small magnitude of this difference still supports the conclusion of Bunz et al. that inactivation of p53 does not elicit the degree of structural chromosome changes observed in cancer cells which exhibit chromosomal instability (CIN) (Bunz et al, 2002). However, in contrast to diploid cells, spontaneous polyploid HCT116 cells did harbor significantly increased structural chromosome aberrations in the absence of p53 (Figure 7A and 7C), and some of these spontaneous polyploids also exhibited numerical instability (Figure 7C). These discrepancies may be due to the larger number of cells in our analysis and/or differences in the aberration detection methods. Indeed, although spectral karyotyping is more sensitive than traditional chromosome staining for the detection of certain aberrations, it can actually be less sensitive for others (Szeles et al, 2006). Thus, we conclude that although inactivation of p53 in human cells is not sufficient to produce near-diploid CIN, it may nonetheless promote genetic instability in polyploid cells.

Our data also have implications for understanding the cellular responses to antimitotic chemotherapeutics. The p53-regulated survival of nocodazole-treated HCT116 cells suggests that p53 can be a determinant of antimitotic chemosensitivity. Indeed, we have also observed that p53 knockdown partially attenuated nocodazole-induced apoptosis in RKO colon cancer cells (unpublished observations). These findings are consistent with previous reports in HCT116 and MCF-7 cells (Castedo et al, 2006; Galmarini et al, 2001; Kienitz et al, 2005; Yamaguchi et al, 2004; Zhang et al, 2002). At

the same time, studies in other cell systems have found that p53 inactivation confers no change—or even an increase—in antimitotic sensitivity, demonstrating the cell type-specificity of this effect (Minn et al, 1996; Tao et al, 2007; Wahl et al, 1996; Woods et al, 1995). Our own observation that p53 knockdown did not significantly affect nocodazole-induced apoptosis in HDFs is consistent with this idea. Thus, a conservative interpretation of these findings is that p53 may be a determinant of antimitotic sensitivity in a subset of human tumors. In addition, our cytogenetic data suggest that cells which survive exposure to antimitotic drugs may be at increased risk of further structural chromosome changes, and that such risk may be exacerbated by loss of p53. In this way, inactivation of p53 may not only increase resistance to initial treatment with antimitotics, but may also facilitate the evolution of further tumor aggressiveness caused by the treatment itself.

An important but unanswered question is what triggers p53 during this process (Chan et al, 2008; Ganem & Pellman, 2007; Stukenberg, 2004). Although an initial proposal was that p53 is activated by a mechanism which senses, or counts, the presence of a tetraploid genome (Andreassen et al, 2001), subsequent studies demonstrated that tetraploid cells produced in the absence of prolonged mitosis do not necessarily undergo p53-dependent growth arrest (Uetake & Sluder, 2004; Uetake & Sluder, 2007; Wong & Stearns, 2005). Indeed, the results of our blebbistatin experiment (Figure 5) support the conclusions of these later studies. Another proposal has been that the transcriptional repression which occurs during mitosis leads to inhibition of p53 degradation, which in turn leads to progressive accumulation of p53 protein during prolonged mitotic arrest (Blagosklonny, 2006). However, this “mitotic timer” model for p53 is not supported by

studies showing that conditions which dramatically shorten mitotic arrest do not diminish the accumulation of p53 (Chan et al, 2008; Vogel et al, 2004), nor by the report that p53 accumulation occurs after, and not during, mitotic arrest (Minn et al, 1996).

Because DNA damage is a well-established activator of p53 (Vousden & Lu, 2002), it is tempting to speculate that postmitotic activation of p53 is induced, or at least influenced, by DNA damage acquired during mitotic arrest. Indeed, our finding that loss of p53 increases the burden of chromosome aberrations in nocodazole-induced polyploids—but does not affect the initial acquisition of DNA damage during mitotic arrest—suggests that the p53-dependent postmitotic response preferentially inhibits the most damaged cells. This, in turn, suggests that the extent of DNA damage acquired during prolonged mitosis influences cell fate. In this way, the postmitotic activation of p53 by DNA damage would be analogous to p53 activation following blockage of nucleotide biosynthesis, which while initially believed to be a non-genotoxic inducer of p53 (Linke et al, 1996), has recently been shown, with more sensitive assays, to cause DNA damage (Hastak et al, 2008).

At the same time, it remains possible that DNA damage is one, but not the only, determinant of p53 activation following prolonged mitotic arrest (Ganem & Pellman, 2007). Indeed, we previously observed significant cell-type variation in nocodazole-induced γ-H2AX foci (Dalton et al, 2007), which raises the question of whether DNA damage could account for p53 activation following mitotic arrest in all cell types. Along these lines, a lack of increased γ-H2AX in nocodazole-treated U20S cells has been reported (Aylon et al, 2006; Chan et al, 2008). However, multiple studies have now reported evidence that DNA damage accompanies treatments which induce mitotic arrest

in a variety of cell lines (Dalton et al, 2007; Quignon et al, 2007; Shi et al, 2008; Stevens et al, 2007; Tighe et al, 2004; Wong & Stearns, 2005). Furthermore, it is important to note that a lack of increased γ-H2AX does not rule out the presence of DNA damage, as there are DNA lesions, such as single-stranded breaks and base alterations, which do not induce γ-H2AX (Rogakou et al, 1998). In fact, some of the chromatid-type aberrations we observed after prolonged mitotic arrest could result from lesions other than double-stranded breaks (Zhuanzi et al, 2007). Clearly, future studies into the mechanisms responsible for DNA damage during mitotic arrest will be needed to determine its role in postmitotic p53 activation. Nonetheless, we believe our data demonstrate that, whatever its mechanism of activation, p53 functions to inhibit the potentially dangerous consequences of DNA damage acquired during mitotic arrest.

Materials and methods

Cell lines and treatments

IMR90 HDFs were obtained from the ATCC. *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells were kindly provided by B.Vogelstein (Johns Hopkins Medical Institution, Baltimore, MD). HCT116 cells were cultured in McCoy's and seeded at a density of 3 x 10^4 cells/cm^2 onto fibronectin-coated dishes or slides 24h prior to experiments. IMR90 cells were cultured in DMEM and also seeded at a density of 3 x 10^4 cells/cm^2 24h prior to experiments. Nocodazole and blebbistatin (Sigma) were used at 200nM and 150μM, respectively, the minimum concentrations which completely inhibited cell division in HCT116 cells (data not shown). γ-irradiation was performed with a Cs-137 Gammacell. Stealth™ Select siRNAs targeted to Eg5, control siRNA, and Lipofectamine RNAiMax™ were obtained from, and used according to the instructions of, Invitrogen. miRNAs targeting p53, or a nonspecific control, were also obtained from Invitrogen, initially as DNA oligos. These oligos were then cloned into the pLenti6-GW/EmGFP-miR lentiviral expression vector (Invitrogen), and these vectors were transfected into 293FT cells along with the pLP/VSVG, pLP1, and pLP2 plasmids (Invitrogen) to produce miRNA-containing lentivirus. HCT116 and IMR90 were transduced with high-titer virus, and pools of hundreds of blasticidin-resistant colonies were expanded to produce stable knockdown cell lines.

Immunodetection

For immunocytochemistry, cells were fixed with 2% formaldehyde/PBS and permeabilized with 0.2% Triton-X 100. Antibody incubations were 1h at room

temperature, and DNA was counterstained with DAPI. Images were acquired with a Zeiss Axioskop 2 Plus microscope. For MPM-2 flow cytometry, cells were harvested by trypsinization and fixed overnight at -20^{o}C in 70% ethanol. Antibody incubations were 1h at room temperature, and DNA was counterstained with propidium iodide. For γ-H2AX/CC-3 flow cytometry, cells were harvested by trypsinization, fixed with 2% formaldehyde/PBS, permeabilized with methanol, and incubated overnight at 4^{o}C with primary antibodies, followed the next day by 1h incubations with secondary antibodies. Like MPM-2, CC-3 is an antibody which specifically stains mitotic cells, but is an IgG_{2a} allotype, and can thus be used simultaneously with IgG_1 antibodies, such as γ-H2AX (Thibodeau & Vincent, 1991). Data were acquired using a FACSCalibur (Becton-Dickinson) and analyzed with Flowjo. Immunoblotting was performed as previously described (Yoon et al, 2005).

The following antibodies were used: mouse anti-γ-H2AX (Upstate), rat anti-α-tubulin (Chemicon), mouse MPM-2 (Upstate), mouse CC-3 (a gift from M. Vincent, Université Laval, Québec, Qc, Canada), rabbit anti-Eg5 (Abcam), goat anti-p53 (Santa Cruz), rabbit anti-cleaved-PARP (Cell Signaling), and mouse anti-actin (Sigma). All fluorescent secondary antibodies were Alexa-conjugates (Molecular Probes).

Cytogenetic analyses

Chromosome spreads were prepared using standard cytogenetic techniques, DNA was stained with DAPI, and images were obtained using a Zeiss Axioskop 2 Plus microscope. Scoring of chromosome aberrations was performed according to the classification system of Savage (Savage, 1976). Furthermore, all scoring was performed,

where possible, in a blinded fashion. For color karyotyping of spontaneous polyploid *p53*$^{-/-}$ cells, analysis was performed by Chrombios GmbH (Munich, Germany).

Colony survival

Following 48h nocodazole treatment, HCT116 cells were harvested by trypsinization and seeded into T75 flasks. After 9 days, colonies were stained with methylene blue, photographs of the flasks were taken, and colony number was quantified using Metamorph imaging software. For quantification of untreated control colonies, 1/100 the number of cells used in nocodazole-treated samples were seeded into T75 flasks and colony number was normalized accordingly, as use of the same number of cells as nocodazole-treated samples produced colonies which were too dense to be quantified (Figure 2E).

Time-lapse imaging

Phase-contrast images of *p53*$^{-/-}$ HCT116 cells grown inside a 37^{o}C, 5% CO_2 chamber were automatically obtained at 6 min intervals in multiple locations over 48h using an Olympus IX81 microscope. All images were analyzed with Slidebook.

Acknowledgements

We thank D. Pallas, P. Doetsch, D. Jones, A. Corbett, G. Davis, and M. Wiltenburg for discussion and support, and B. Vogelstein for providing cell lines. This work was supported in part by grants from the National Institutes of Health to V.W.Y. (DK52230, DK64399, and CA84197) and to W.B.D. (5T32GM008367-18).

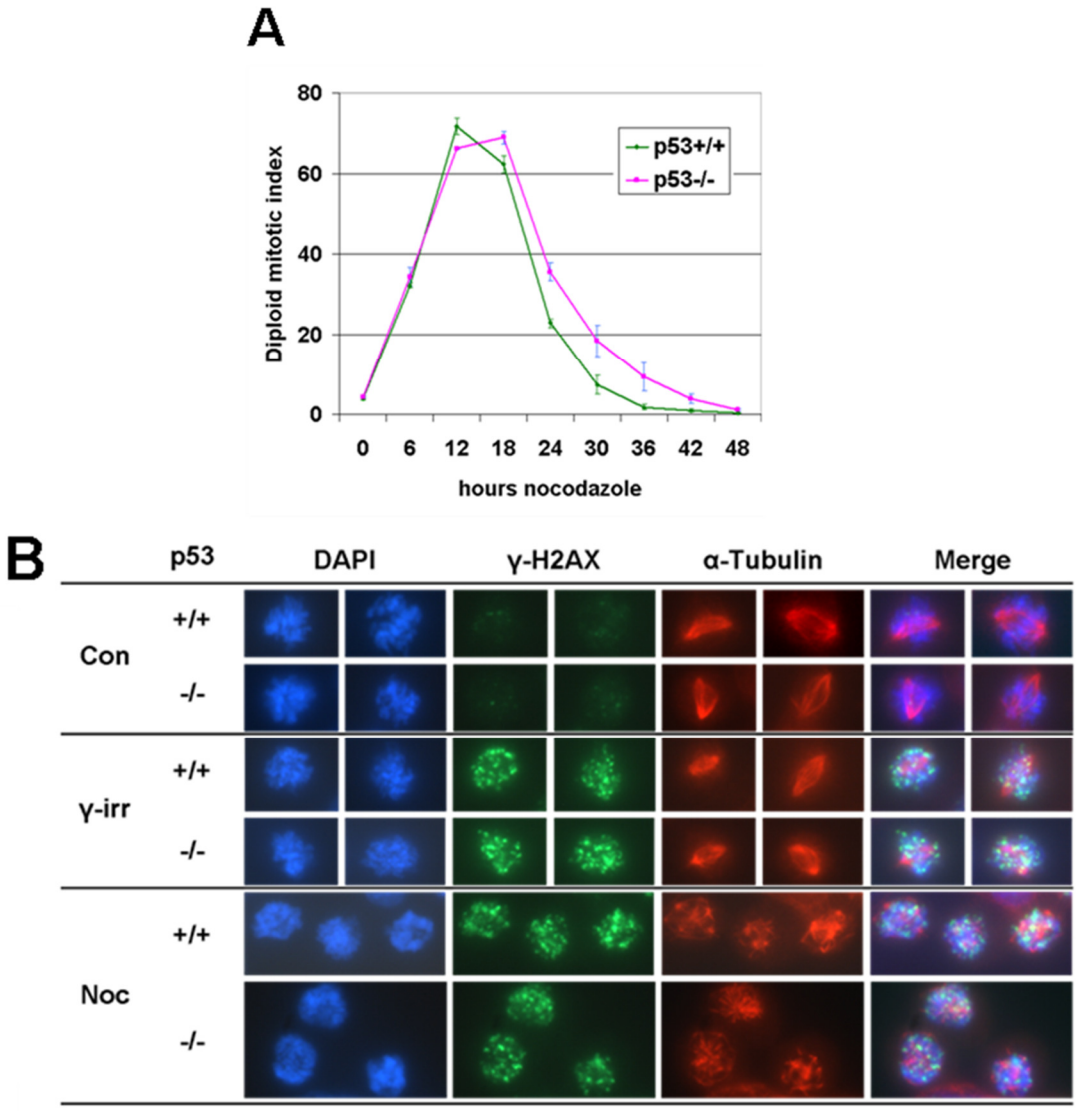


Figure 1 Both p53+/+ and p53-/- HCT116 cells acquire DNA damage during mitotic arrest.

(**A**) Diploid mitotic index of HCT116 cells during 48h nocodazole, as determined by MPM-2 flow cytometry. Means and SEMs are from 2-3 independent experiments. (**B**) Images of prometaphase cells stained for γ-H2AX and α-tubulin. Nuclei were counterstained with DAPI. Noc = 18h nocodazole. γ-irr = 30 min after 2 Gy γ-irradiation.

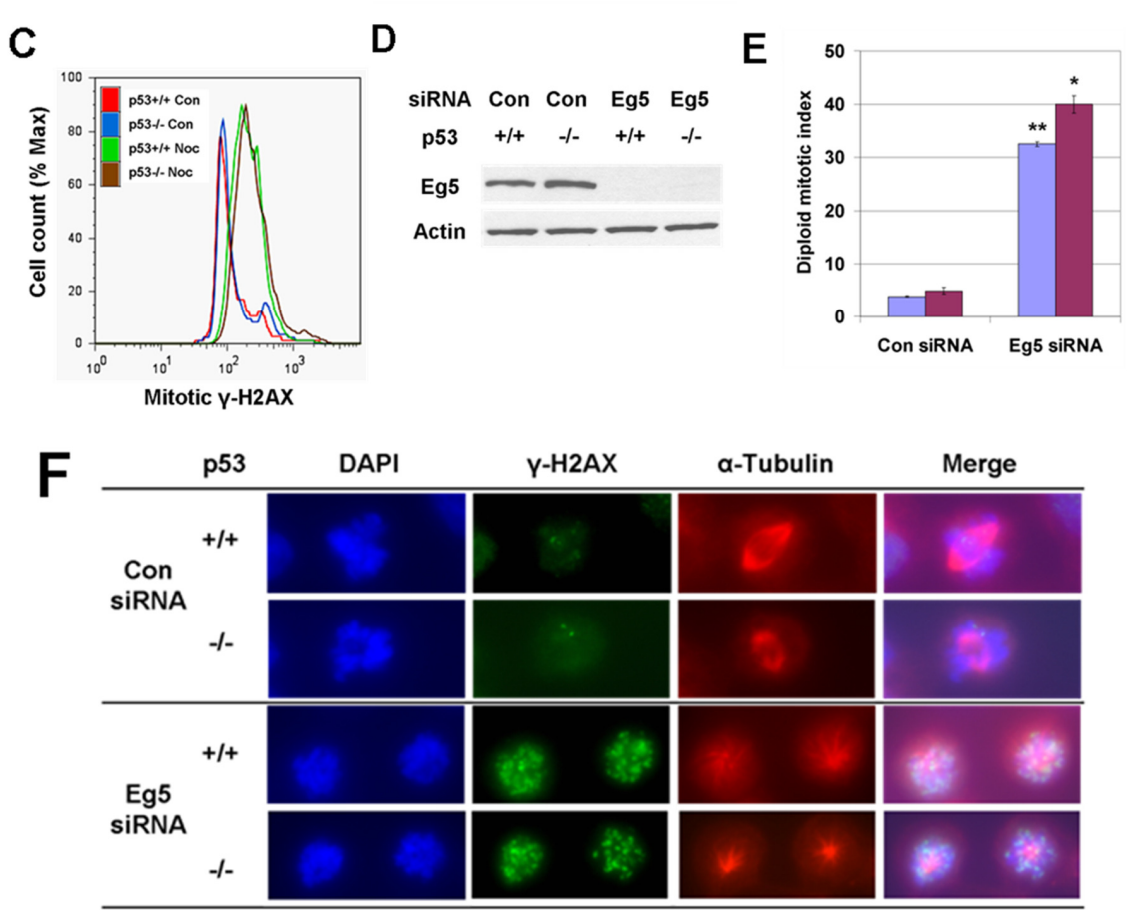


Figure 1C-F.

(**C**) Flow cytometric analysis of mitotic γ-H2AX. Cells were treated with or without 18h nocodazole and stained for CC-3 and γ-H2AX. CC-3-positive cells, which are mitotic (Thibodeau & Vincent, 1991), were gated, and γ-H2AX signals of the gated cells are shown. Data are representative of two independent experiments. (**D**) Western blot analysis of Eg5 in HCT116 cells 24h after transfection with Eg5-specific, or control, siRNA. Actin was used as a loading control. (**E**) Diploid mitotic index of HCT116 cells 24h after transfection with Eg5-specific, or control, siRNA, as determined by MPM-2 flow cytometry. Means and SEMs are from two independent experiments. * = $p<0.05$, **

= $p<0.01$ for t-tests, as compared to control. (**F**) Images of prometaphase cells stained for γ-H2AX and α-tubulin 24h after transfection with Eg5-specific, or control, siRNA.

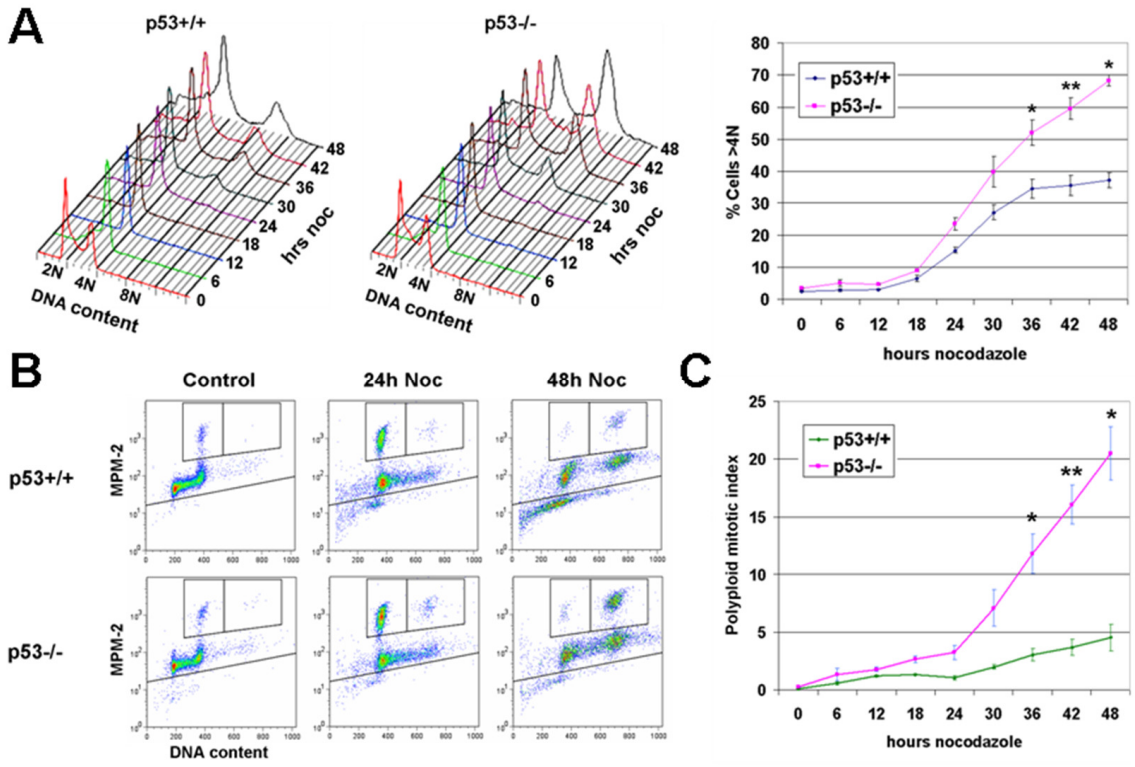


Figure 2 p53 inhibits the polyploidization and survival of HCT116 cells following prolonged mitotic arrest.

(**A**) Example of cell ploidy (left panel) and quantification of polyploidy (right panel) in nocodazole-treated cells. Means and SEMs are from 2-3 independent experiments. * = $p<0.05$, ** = $p<0.01$ for t-tests. (**B**) Dot plots of cells stained for MPM-2 and DNA content after 24h and 48h nocodazole treatment. (**C**) Quantification of polyploid (> 4N) mitotic index (upper right gate in Figure 2B) after 48h nocodazole. Means and SEMs are from 2-3 independent experiments. * = $p<0.05$, ** = $p<0.01$ for t-tests.

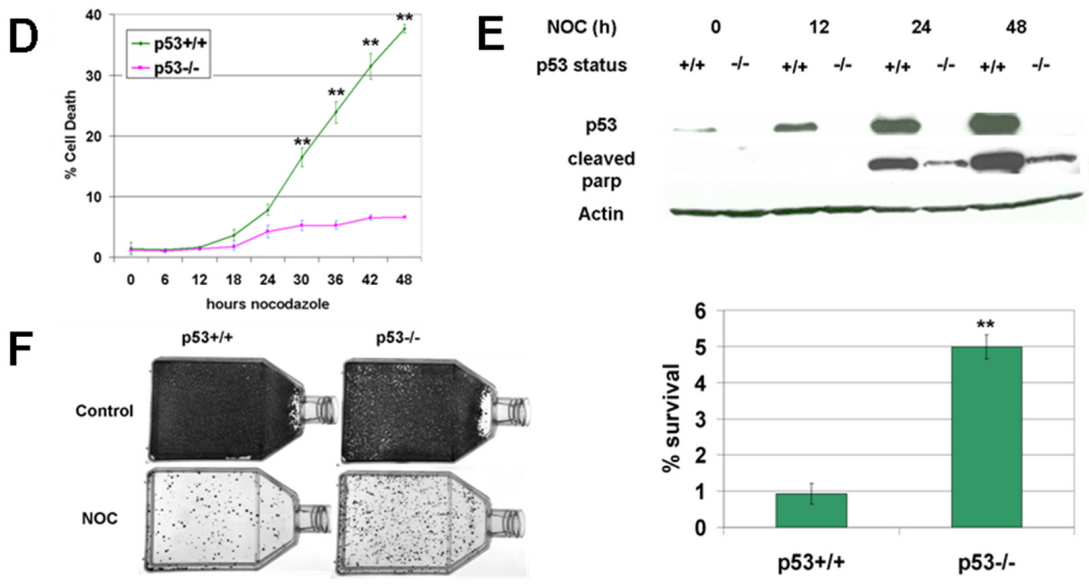


Figure 2D-F

(**D**) Quantification of cell death (lower gate in Figure 2B) during 48h nocodazole treatment. Means and SEMs are from 2-3 independent experiments. * = $p<0.05$, ** = $p<0.01$ for t-tests. (**E**) Western blot analysis of p53 and cleaved PARP levels during 48h nocodazole. Actin was used as a loading control. (**F**) Example of colony survival after 48h nocodazole (left panel) and its quantification (right panel). % survival is the number of nocodazole-treated colonies divided by the number of control colonies, although more sparse control flasks were used for quantification (see Materials and methods). Means and SEMs are from three independent experiments. ** = $p<0.01$ for t-test.

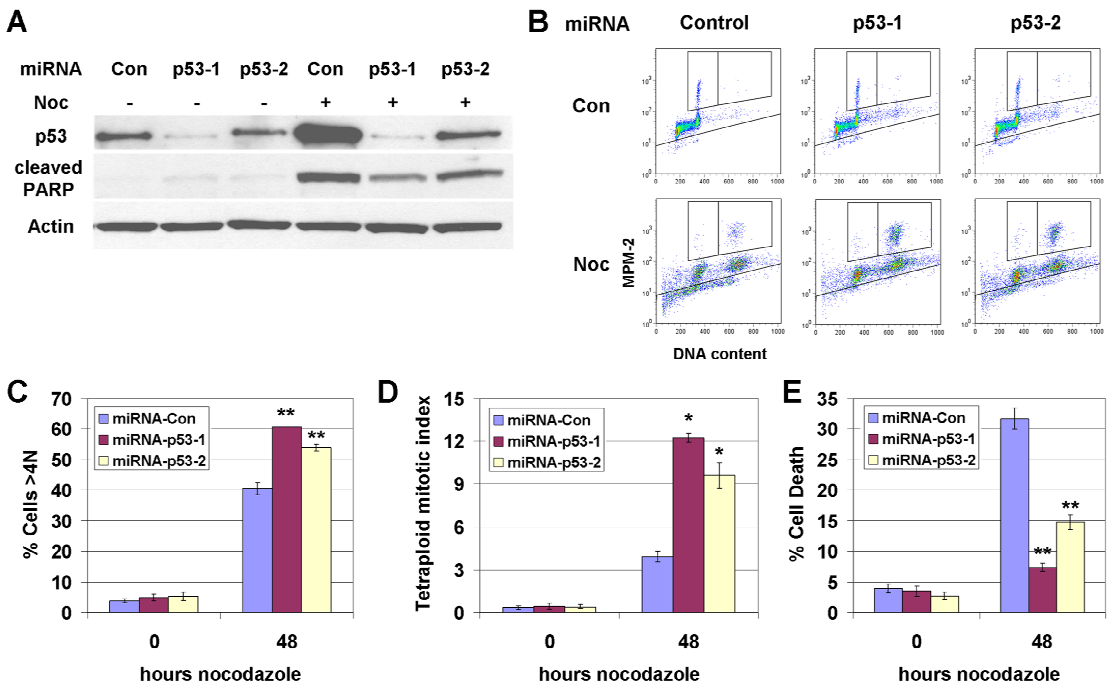
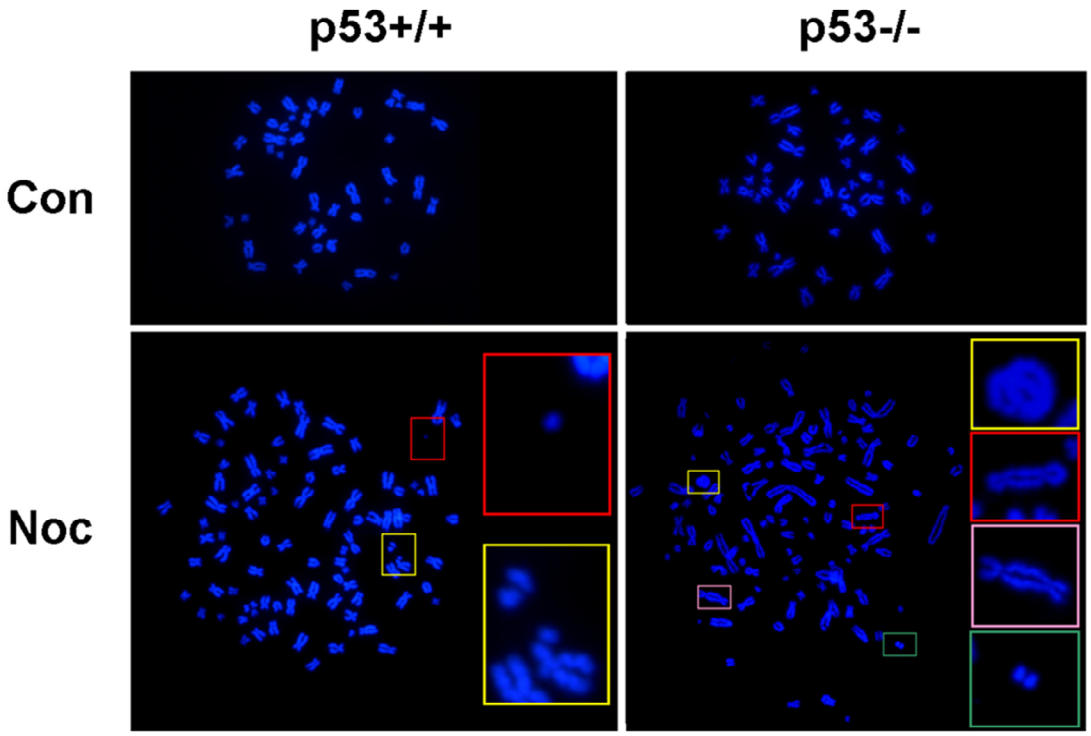


Figure 3 p53 knockdown phenocopies the response of p53-/- cells to nocodazole. (**A**) Western blot analysis of p53 and cleaved PARP levels after 48h nocodazole treatment in HCT116 cells stably expressing either control or two independent p53 miRNAs. Actin was used as a loading control. (**B**) Dot plots of miRNA-expressing cells stained for MPM-2 and DNA content after 48h nocodazole. (**C**) Quantification of polyploidy in miRNA-expressing cells after 48h nocodazole. Means and SEMs are from 3 independent experiments. ** = $p<0.01$ for t-tests, as compared to miRNA-Con cells treated with 48h nocodazole. (**D**) Quantification of polyploid mitotic index (upper right gate in Figure 3B) in miRNA-expressing cells after 48h nocodazole. Means and SEMs are from 3 independent experiments. * = $p<0.05$ for t-tests, as compared to miRNA-Con cells treated with 48h nocodazole. (**E**) Quantification of cell death (lower gate in Figure 3B) in miRNA-expressing cells after 48h nocodazole. Means and SEMs are from 3

independent experiments. ** = $p<0.01$ for t-tests, as compared to miRNA-Con cells treated with 48h nocodazole.

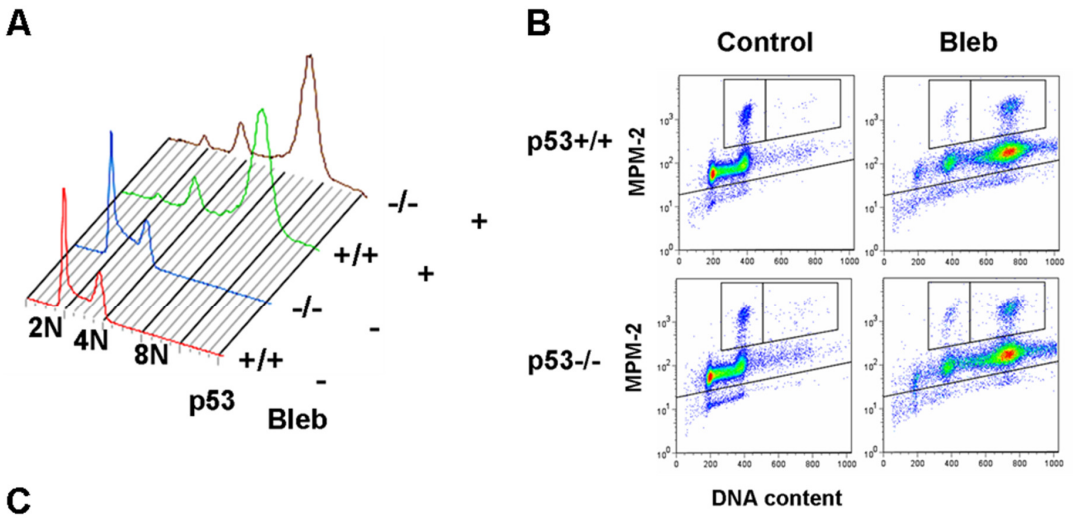


p53	Rx	Cells	Chrom	CSB per chrom % (n)	CSE per chrom % (n)	CTB per chrom % (n)	CTE per chrom % (n)	Total breaks per chrom % (n)
+/+	Con	150	6750	0.37 (25)	0.03 (2)	0.04 (5)	0.04 (3)	0.59 (40)
	Noc	150	13500	2.0 (306)	0.42 (57)	0.26 (35)	0.14 (20)	3.7 (495)
-/-	Con	150	6750	0.56 (38)	0.03 (2)	0.25 (17)	0.06 (4)	0.99 (67)
	Noc	150	13500	3.3 (448)	0.47 (63)	0.36 (49)	0.39 (53)	5.4 (729)****

Figure 4 p53 suppresses structural chromosome instability following mitotic arrest in HCT116 cells.

Examples of chromosome spreads in cells treated with or without nocodazole (Noc) for 48h are shown in upper panels. Insets show chromosome fragments (upper left, lower left, lower right), a chromatid break (lower left), a ring chromosome (upper right), and dicentric chromosomes (upper-middle right, lower-middle right). Quantification of chromosome aberrations is shown in table. Con = control cells. Noc = 48h nocodazole.

Cells = number of cells analyzed from three independent experiments. Chrom = number of chromosomes, obtained by multiplying cell number by either 45 chromosomes/cell for control diploids (4N), or 90 chromosome/cell for nocodazole-induced polyploids (8N). CSB = chromosome break. CSE = chromosome exchange. CTB = chromatid break. CTE = chromatid exchange. Total breaks = CSB + CTB + 2(CSE + CTE), as two breaks produce exchanges. **** = $p<0.0001$, for chi square test, as compared to nocodazole-treated $p53^{+/+}$ cells.

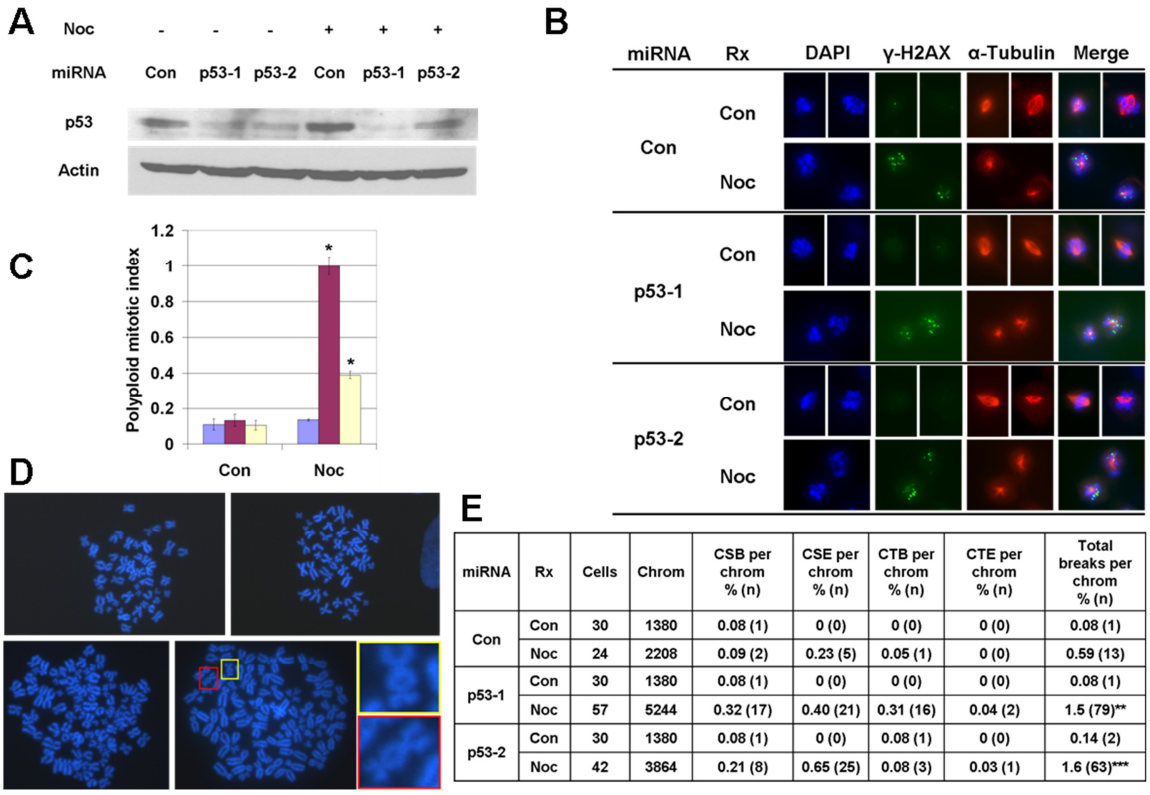


p53	Rx	Cells	Chrom	CSB per chrom % (n)	CSE per chrom % (n)	CTB per chrom % (n)	CTE per chrom % (n)	Total breaks per chrom % (n)
+/+	Con	150	6750	0.37 (25)	0.03 (2)	0.04 (5)	0.04 (3)	0.59 (40)
	Bleb	150	13500	0.13 (17)	0.14 (20)	0.13 (17)	0 (0)	0.55 (74)
-/-	Con	150	6750	0.56 (38)	0.03 (2)	0.25 (17)	0.06 (4)	0.99 (67)
	Bleb	150	13500	0.24 (32)	0.12 (16)	0.15 (20)	0.01 (1)	0.64 (86)

Figure 5 Polyploidization through inhibition of cytokinesis does not elicit significant DNA damage or p53-dependent postmitotic response.

(**A**) Cell ploidy after 24h blebbistatin. (**B**) Dot plot of cells stained for MPM-2 and DNA content after 24h blebbistatin. (**C**) Examples of chromosome spreads (images) and quantification of chromosome aberrations (table) after 24h blebbistatin. Con = control

cells. Bleb = 24h blebbistatin. Cells = number of cells analyzed. Chrom = number of cells multiplied by either 45 chromosomes/cell for control diploids (4N), or 90 chromosomes/cell for blebbistatin-induced polyploids (8N). CSB = chromosome break. CSE = chromosome exchange. CTB = chromatid break. CTE = chromatid exchange. Total breaks = CSB + CTB + 2(CSE + CTE), as two breaks produce exchanges.

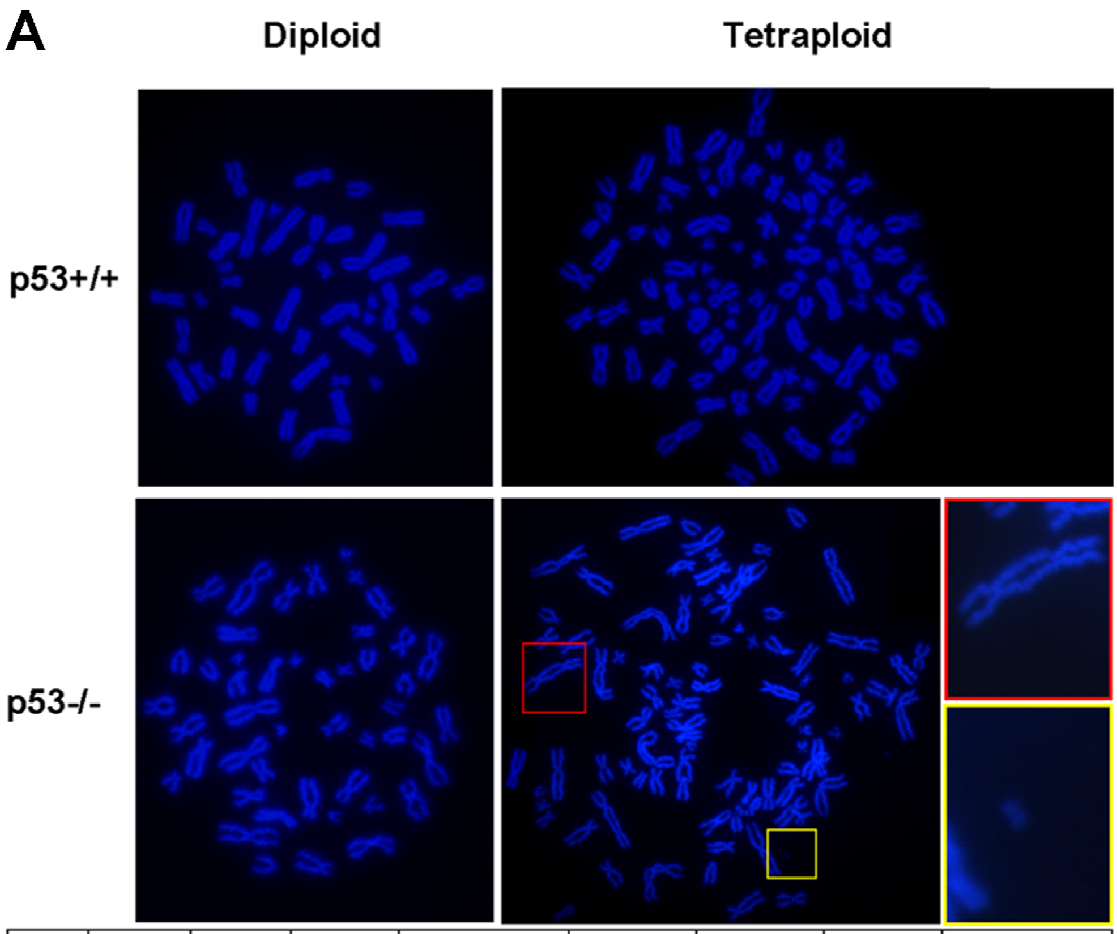


miRNA	Rx	Cells	Chrom	CSB per chrom % (n)	CSE per chrom % (n)	CTB per chrom % (n)	CTE per chrom % (n)	Total breaks per chrom % (n)
Con	Con	30	1380	0.08 (1)	0 (0)	0 (0)	0 (0)	0.08 (1)
	Noc	24	2208	0.09 (2)	0.23 (5)	0.05 (1)	0 (0)	0.59 (13)
p53-1	Con	30	1380	0.08 (1)	0 (0)	0 (0)	0 (0)	0.08 (1)
	Noc	57	5244	0.32 (17)	0.40 (21)	0.31 (16)	0.04 (2)	1.5 (79)**
p53-2	Con	30	1380	0.08 (1)	0 (0)	0.08 (1)	0 (0)	0.14 (2)
	Noc	42	3864	0.21 (8)	0.65 (25)	0.08 (3)	0.03 (1)	1.6 (63)***

Figure 6 p53 suppresses polyploidization and structural chromosome instability following mitotic arrest in IMR90 human diploid fibroblasts (HDFs).

(**A**) Western blot analysis of p53 after 72h nocodazole in cells stably expressing either control or two independent p53 miRNAs. Actin was used as a loading control. (**B**) Images of prometaphase cells stained for γ-H2AX and α-tubulin. Con = control. Noc = 18h nocodazole. (**C**) Quantification of polyploid mitotic index in miRNA-expressing cells after 72h nocodazole, as determined by MPM-2 flow cytometry. Means and SEMs are from two independent experiments. * = $p<0.05$ for t-tests, as compared to miRNA-Con cells treated with 48h nocodazole. (**D**) Examples of chromosome spreads in cells treated with or without 72h nocodazole. Untreated (upper) or nocodazole-treated (lower) cells expressing either control (left) or p53-1 (right) miRNAs. Insets show dicentric

chromosomes. (**E**) Quantification of chromosome aberrations. Con = control cells. Noc = 72h nocodazole. Cells = number of cells analyzed from two independent experiments. Chrom = number of chromosomes, obtained by multiplying cell number by either 46 chromosomes/cell for control diploids (2N), or 92 chromosomes/cell for nocodazole-induced polyploids (8N). CSB = chromosome break. CSE = chromosome exchange. CTB = chromatid break. CTE = chromatid exchange. Total breaks = CSB + CTB + 2(CSE + CTE), as two breaks produce exchanges. ** = $p<0.01$, *** = $p<0.001$, for chi square tests, as compared to nocodazole-treated cells expressing control miRNA.

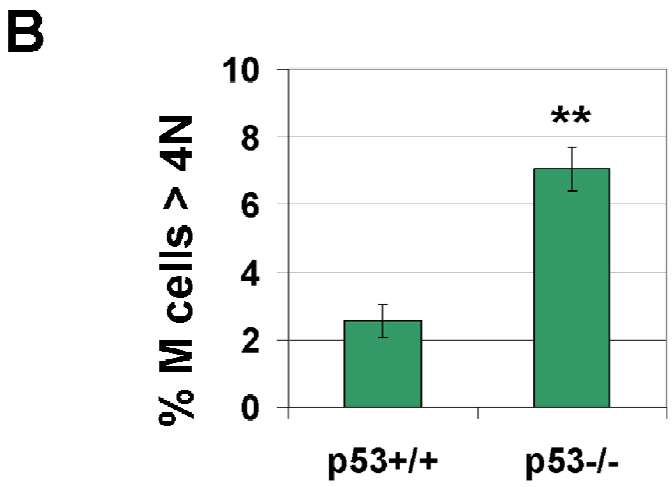


p53	Ploidy	Cells	Chrom	CSB per chrom % (n)	CSE per chrom % (n)	CTB per chrom % (n)	CTE per chrom % (n)	Total breaks per chrom % (n)
+/+	4N	150	6750	0.37 (25)	0.03 (2)	0.04 (5)	0.04 (3)	0.59 (40)
	8N	43	3870	0.23 (9)	0.05 (2)	0.10 (4)	0.00 (0)	0.44 (17)
-/-	4N	150	6750	0.56 (38)	0.03 (2)	0.25 (17)	0.06 (4)	0.99 (67)
	8N	64	5760	2.0 (114)	0.22 (13)	0.28 (16)	0.14 (8)	2.78 (160)****

Figure 7 p53 suppresses the frequency of, and structural chromosome instability in, spontaneous polyploid HCT116 cells.

(**A**) Examples of chromosome spreads in spontaneous diploid (4N) or polyploid (8N) cells are shown in upper panels. Insets show a dicentric chromosome (red rectangle) and a chromosome fragment (yellow rectangle). Quantification of chromosome aberrations is

shown in table. Con = control cells. Cells = number of cells analyzed from three independent experiments. Chrom = number of chromosomes, obtained by multiplying cell number by either 45 chromosomes/cell for diploid (4N), or 90 chromosomes/cell, for polyploid (8N), chromosomes/cell. CSB = chromosome break. CSE = chromosome exchange. CTB = chromatid break. CTE = chromatid exchange. Total breaks = CSB + CTB + 2(CSE + CTE), as two breaks produce exchanges. **** = $p<0.0001$, for chi square tests, as compared to diploid $p53^{-/-}$, diploid $p53^{+/+}$, or polyploid $p53^{+/+}$ cells.



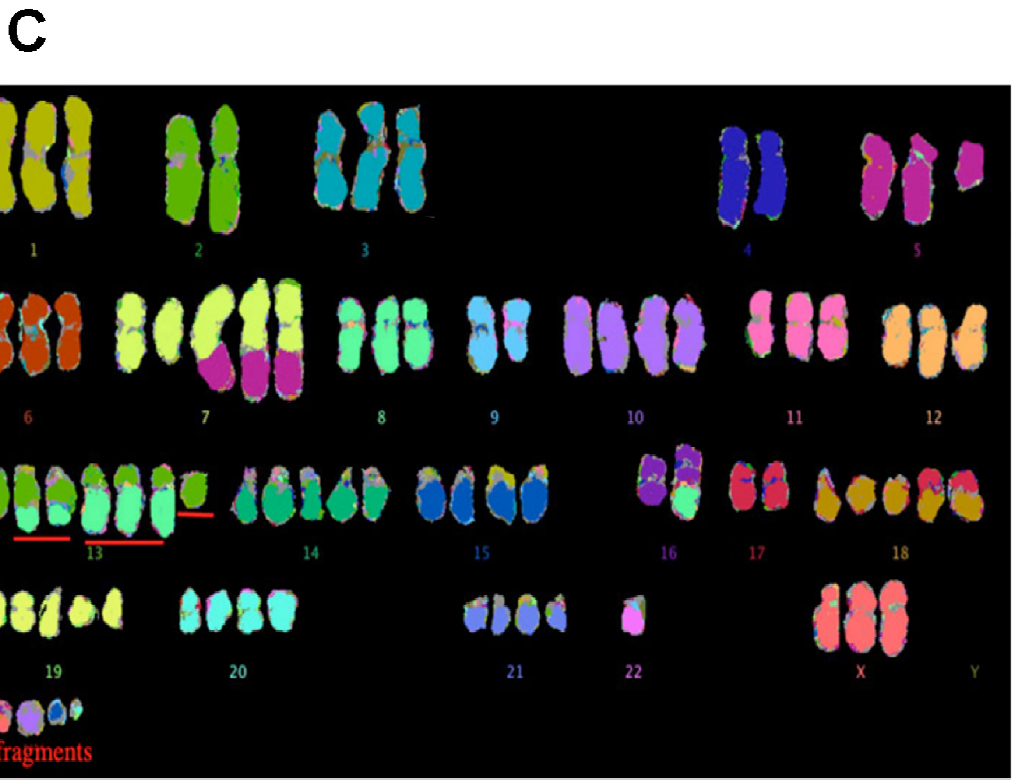
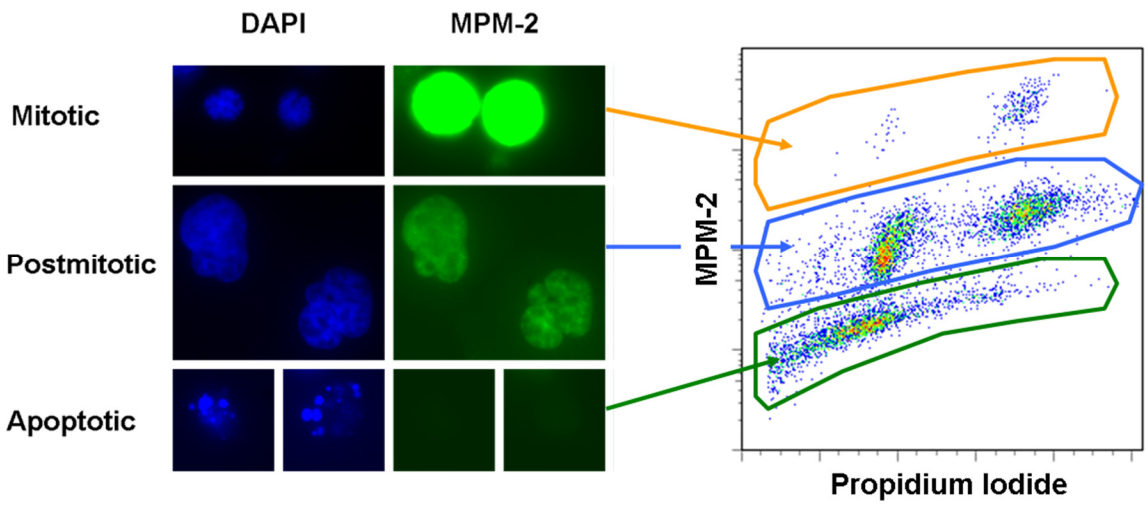


Figure 7B-C.

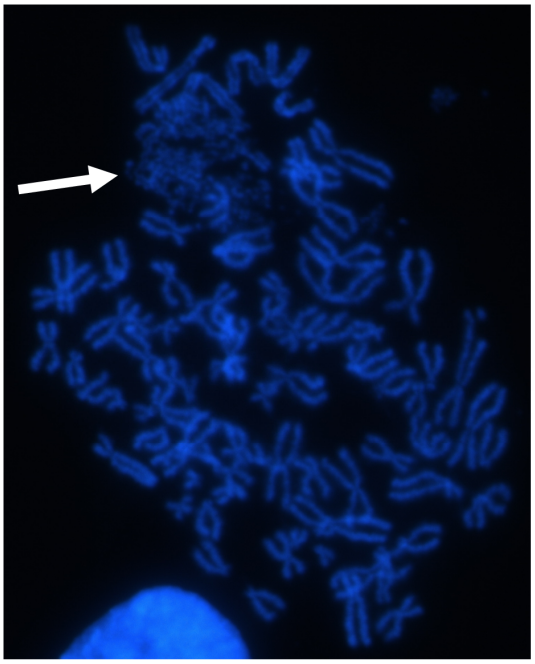
(**B**) Quantification of mitotic cells with polyploid DNA content, as determined by scoring of chromosome spreads. Means and SEMs are from 3 independent experiments. ** = $p<0.01$ for t-test. (**C**) Example of chromosome aberrations in a polyploid *p53*$^{-/-}$ cell, as

determined by color karyotyping. The red underlining indicates an aberration not present in the diploid *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 karyotype (Bunz et al, 2002). Chromosome fragments are also underlined in the lower left.



Supplementary Figure S1 Cells staining entirely negative for MPM-2 have apoptotic nuclear morphology.

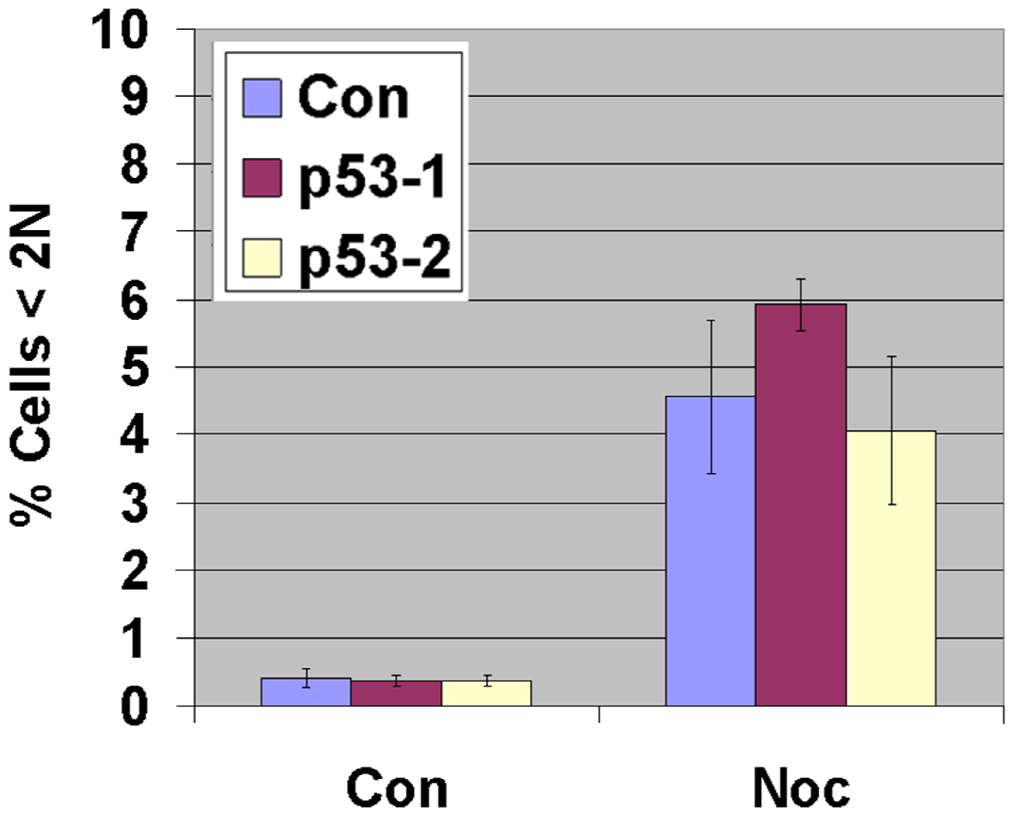
p53$^{+/+}$ HCT116 cells were stained for DNA and MPM-2 after 48h nocodazole and examined by microscopy (left panel) or flow cytometry (right panel). Levels of MPM-2 staining progressively decreased from mitotic to interphase to apoptotic cells.



miRNA	Rx	Cells with pulverization % (n)
miCON	Con	0 (0/30)
	Noc	12.5 (3/24)
p53-1	Con	0 (0/30)
	Noc	28.1 (16/57)
p53-2	Con	0 (0/30)
	Noc	14.3 (6/42)

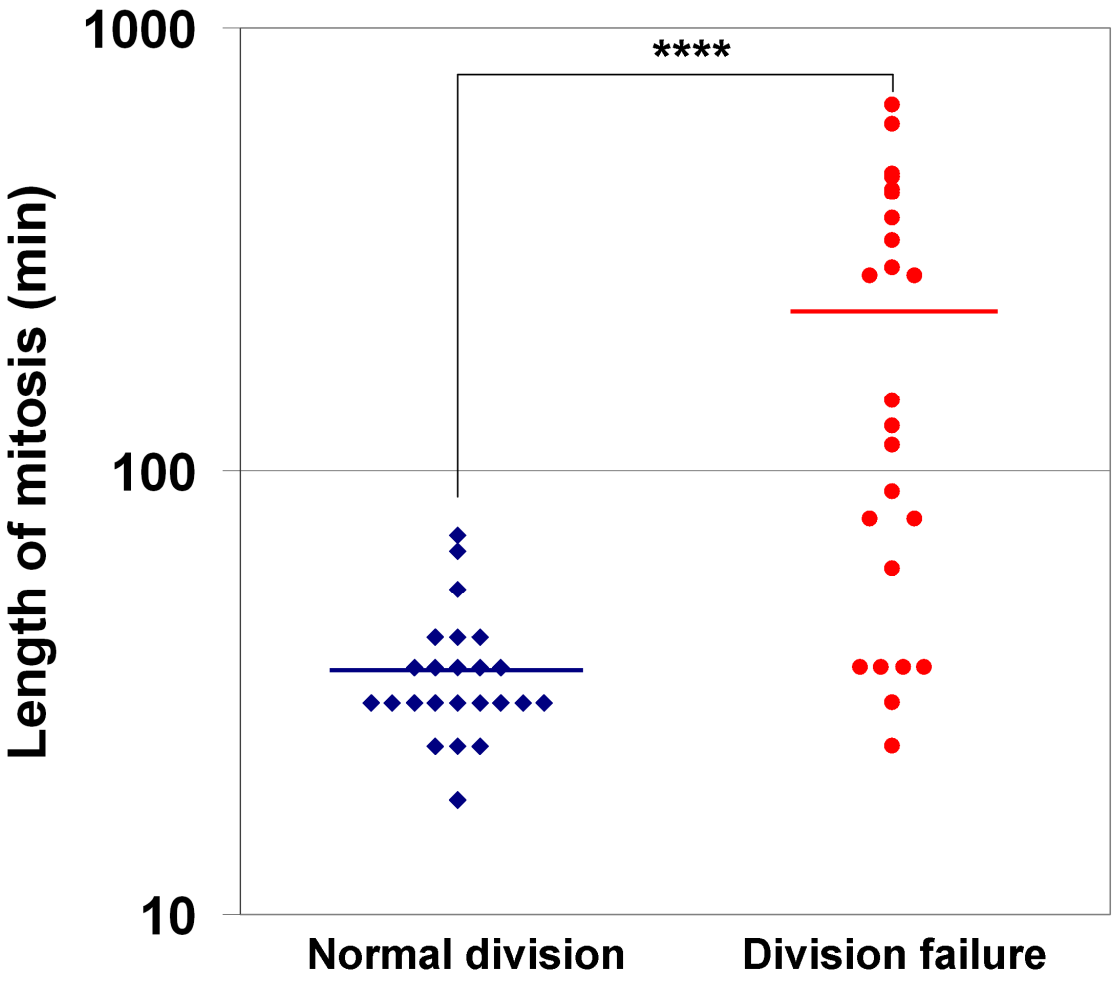
Supplementary Figure S2 Chromosome pulverization occurs in a fraction of nocodazole-treated HDFs.

(Left) Example of a chromosome spread from a p53 miRNA-expressing cell after 72h nocodazole. Arrow points to chromosome pulverization. (Right) Tabulation of the frequency of cells exhibiting pulverization. Con = control. Noc = 72h nocodazole.



Supplementary Figure S3 p53 knockdown does not influence cell death following mitotic arrest in HDFs.

Quantification of subdiploidy (< 2N) in control or p53 miRNA-expressing IMR90 cells after 72h nocodazole, as determined by MPM-2 flow cytometry. Means and SEMs are from at least two independent experiments.



Supplementary Figure S4 Mitosis is spontaneously prolonged in *p53*$^{-/-}$ HCT116 cells which fail to divide.

Dot plot of mitotic length, defined as the interval between the start of mitotic rounding up and anaphase (or mitotic slippage), in cells which underwent normal, bipolar cell division or which failed to divide. 24 cells in each category were scored, and the mean lengths of mitosis are represented by horizontal lines. **** = $p<0.0001$ for ttest.

Chapter IV

Discussion

W. Brian Dalton[1,2]

[1]Division of Digestive Diseases, Department of Medicine,
[2]Biochemistry, Cell and Developmental Biology Graduate Program,
Emory University School of Medicine, Atlanta, GA 30322

Conclusions

Because there is evidence that events occurring during and after prolonged mitotic arrest affect both tumorigenesis and the action of antimitotic chemotherapeutics, knowledge of the mechanisms responsible for these effects should expand our understanding of both cancer biology and chemotherapy (Dalton & Yang, 2007; Rieder & Maiato, 2004; Weaver & Cleveland, 2005). In an effort to contribute to this understanding, the work described in this dissertation characterized a novel phenomenon in human cells: the acquisition of DNA damage during, and p53-regulated development of structural chromosome instability after, mitotic arrest (Figure 1). This finding represents an original contribution to the field by describing a novel source of DNA damage in human cells, providing evidence that mitotic arrest may promote tumorigenesis and antimitotic toxicity through infliction of DNA damage, and suggesting a novel anticancer role for the p53 tumor suppressor.

Unscheduled DNA breaks are known to derive from sources such as collapsed replication forks, oxidative stress, radiation, radiomimetic chemicals, telomere dysfunction, and severance of anaphase bridges (Stewenius et al, 2005; van Gent et al, 2001). The data presented in Chapters II and III demonstrate that prolonged mitosis represents an additional source of DNA damage in human cells. Indeed, these breaks were observed following mitotic arrest induced by the microtubule-destabilizing agent nocodazole, the microtubule-stabilizing agent paclitaxel, the Eg5 mitotic kinesin inhibitor monastrol, knockdown of CENP-E, knockdown of Eg5, and spontaneously-arising defects of spindle pole number. We observed evidence for these breaks in multiple colorectal cancer cells, cervical cancer cells, ovarian cancer cells, and normal human

fibroblasts. Around the time of our discovery of this phenomenon, two other groups reported similar findings (Quignon et al, 2007; Stevens et al, 2007). Additionally, Shi et al. recently reported evidence of DNA damage induced by prolonged mitotic arrest in multiple human cancer cell lines (Shi et al, 2008). Taken together, these studies further support our finding that mitotic arrest, induced through multiple mechanisms, can provoke DNA damage in a variety of human and mouse cells.

The work described in this dissertation also suggests that one mechanism by which mitotic arrest may promote tumorigenesis and the toxicity of antimitotic chemotherapeutics is through the infliction of DNA damage. Thus, genetic defects which are both causally implicated in cancer and elicit prolonged mitotic arrest, such as inactivation of Rb, inactivation of hCDC4, overexpression of Mad2, overexpression of cyclin E, inactivation of SBDS, activation of oncogenic c-Myc, acquisition of extra chromosomes, and acquisition of extra centrosomes, may exert their tumorigenicity, at least in part, through introduction of structural chromosome changes during mitotic arrest (Austin et al, 2008; Hernando et al, 2004; Keck et al, 2007; Menssen et al, 2007; Rajagopalan et al, 2004; Sotillo et al, 2007; Yang et al, 2008). Moreover, cancer-associated genetic changes which increase polyploidization or centrosome instability, such as inactivation of p53, inactivation of APC, or amplification of Aurora A, may indirectly introduce DNA damage during mitotic arrest by generating cells with extra chromosomes and/or centrosomes (Bunz et al, 2002; Meraldi et al, 2002; Rusan & Peifer, 2008). Furthermore, our data suggest that the cytotoxicity of antimitotic chemotherapeutics, such as the vinca alkaloids, the taxanes, and the newer mitotic kinesin inhibitors, may depend, at least in part, on their ability to provoke DNA damage during

mitotic arrest. This finding may have important clinical implications, because (1) tumors which are particularly susceptible to DNA damage during mitotic arrest may also be particularly sensitive to antimitotic agents, (2) strategies to maximize infliction of DNA damage during mitotic arrest may improve the efficacy of antimitotic therapy, and (3) induction of structural chromosome instability by antimitotic agents may affect the evolution of tumor aggressiveness in those cells which survive treatment.

Finally, our data describe a novel role for the p53 tumor suppressor: inhibition of structural chromosome instability following mitotic arrest. Because there is, as discussed previously, considerable evidence that mitosis is frequently prolonged in human cancer cells (Therman & Kuhn, 1989; Yang et al, 2008), the DNA damage that may be acquired during such prolonged mitosis runs the risk of introducing potentially oncogenic structural chromosome instability into the cell genome. As such, a safeguard which evolved to prevent the propagation of this instability could be a valuable mechanism of tumor suppression. Our observation that p53 suppresses the propagation of structural chromosome instability following mitotic arrest suggests that p53 may indeed provide this safeguard.

Future Directions

Due to the novelty of this subject, there are many interesting and important questions which remained unanswered about the causes and consequences of DNA damage during mitotic arrest (Dalton et al, 2007; Quignon et al, 2007; Stevens et al, 2007). Although some experiments in our laboratory have begun to explore these questions, future work is needed. We anticipate that such work will focus on four main

areas: the mechanism responsible for DNA damage during mitotic arrest, whether DNA damage contributes to p53 activation following prolonged mitosis, the role of DNA damage in cancer-associated gene changes which provoke mitotic arrest, and the role of DNA damage in sensitivity to antimitotic chemotherapeutics.

What is the mechanism responsible for DNA damage during mitotic arrest?

As mentioned, multiple studies have now reported that prolonged mitotic arrest can elicit DNA breaks (Dalton et al, 2007; Quignon et al, 2007; Shi et al, 2008; Stevens et al, 2007). A question naturally arises from these studies: what mechanism creates double-stranded breaks during mitotic arrest? Neither our own preliminary data, nor published studies, has yet provided an answer to this question, so further investigation is needed. We envision four broad mechanistic areas which could be responsible, either singly or in combination, for DNA damage during mitotic arrest: hypercondensation, enzymatic DNA cleavage, oxidative stress, and decreased DNA repair.

Cytogeneticists have long-observed that mitotic chromosomes exhibit "hypercondensation" when exposed to lengthy treatments with antimitotic agents (Barch MJ, 1997). Although noted by these practitioners mainly because it can hinder the quality of karyotype analysis, chromosome hypercondensation has more recently become a subject of genuine biological interest. Indeed, disruption of many genes important for normal condensation of mitotic chromosomes, such as the condensins, cohesins, and chromokinesins, can produce chromosome hypercondensation in cultured cells and animal models (Belmont, 2006). For example, inactivation of the KIF4 chromokinesin protein induces chromosome hypercondensation, prolonged mitosis, DNA damage, and

cancer susceptibility (Mazumdar et al, 2006; Mazumdar et al, 2004). Interestingly, the hypercondensation appears to be a primary effect of KIF4 inactivation, rather than a secondary consequence of prolonged mitosis, as it occurs in late prophase/early prometaphase (Mazumdar et al, 2004). These data led the authors to speculate that hypercondensation created through KIF4 depletion might directly induce DNA damage by imposing torsional strain on the DNA present in mitotic chromosomes (Mazumdar & Misteli, 2005). If true, such a mechanism of DNA damage induction might also be active during the hypercondensation which occurs during prolonged mitotic arrest. As such, it would be interesting to test whether prevention of hypercondensation during mitotic arrest would also prevent acquisition of DNA damage.

Mammalian cells possess many nucleases which produce DNA double-stranded breaks (Samejima & Earnshaw, 2005). Such nucleases are involved in a variety of cellular processes, including apoptotic DNA degradation, gene transcription, chromosome condensation, chromosome decatenation, DNA replication, DNA repair, and DNA recombination (Ju et al, 2006; Samejima & Earnshaw, 2005). Consequently, there are, in theory, many potential candidates for nucleases which might elicit DNA breaks during mitotic arrest. Topoisomerase II, for example, is an enzyme which transiently creates—and then religates—DNA double-stranded breaks, and its enzymatic activity is required for chromosome decatenation and condensation during all stages of mitosis (Kellner et al, 2002). Moreover, an association between topoisomerase II activity and the toxicity of prolonged mitotic arrest has been observed (Skladanowski et al, 2005; Vogel et al, 2005). Could mitotic arrest induce dysfunction of topoisomerase II in such a way as to interfere with religation, thereby producing DNA breaks? Preliminary

experiments in our laboratory indicated that siRNA-mediated knockdown of topoisomerase IIα did not reduce γ-H2AX activation in nocodazole-arrested mitotic cells (data not shown). While this experiment is by no means an exhaustive test of this hypothesis, these data nonetheless argue against the role of topoisomerase II in the production of DNA damage during mitotic arrest.

Other classes of nucleases could also be considered. For example, while data presented in Chapter II indicate that DNA damage during mitotic arrest occurs independently of the commitment to apoptosis, there are nonetheless reports of reversible activation of apoptosis (Vaughan et al, 2002). Considered together with recent data from Gascoigne and Taylor, which suggest that a caspase-dependent apoptotic process may progressively compete with slow degradation of cyclin B during mitotic arrest (Gascoigne & Taylor, 2008), it is theoretically possible that progressive activation of a reversible apoptotic process—and an apoptotic nuclease—during mitotic arrest could produce DNA damage. If so, this putative nuclease would likely be caspase-independent, as we and others have found that DNA damage during mitotic arrest occurs independently of caspase activity (Dalton et al, 2007; Quignon et al, 2007; Stevens et al, 2007). Interestingly, gene therapy researchers have found evidence that efficient delivery of exogenous DNA to cell nuclei appears to be impeded by active nucleolytic activity in the cytoplasm (Pollard et al, 2001). Presumably, such nucleases would ordinarily be inhibited from damaging mitotic chromosomes, but could prolonged mitotic arrest lead to eventual deinhibition of this activity? Of course, many other scenarios in which nuclease activity could be responsible for DNA damage during mitotic arrest are also possible.

A third potential source of DNA damage during mitotic arrest is oxidative stress. Produced constantly by normal metabolism of the cell, reactive oxygen species (ROS) can inflict various kinds of damage to DNA, including base damages, single-strand breaks, and double-strand breaks (Lombard et al, 2005). There are three general ways in which increased DNA damage during mitotic arrest could result from oxidative stress. First, levels of reactive oxygen species could be elevated during mitotic arrest, thereby inflicting more damage to DNA than occurs during interphase or normal mitosis. Preliminary experiments testing this possibility yielded no measureable differences in ROS levels between mitotically-arrested and control cells (data not shown), arguing against this explanation. Second, mitotic chromosomes may undergo chromatin changes during mitotic arrest which increase their vulnerability to attack by ROS. Indeed, the nature of chromatin structure surrounding DNA can influence its vulnerability to damage by sources such as irradiation (Stenerlow et al, 2003). Thus, one interesting possibility would be to determine whether significant changes in either the chromatin structure or chromatin mass occur during prolonged mitotic arrest. A third way in which ROS might account for increased DNA damage during mitotic arrest is that, while the amount of ROS attack may not be not elevated, the repair of these "normal" levels of damage may be compromised. Indeed, as we shall see next, some evidence suggests that DNA repair may be impaired during mitosis.

Regardless of the initial source of DNA damage, the functionality of DNA repair is an obvious determinant of the consequences of the inflicted damage. Unfortunately, there is no consensus as to whether—or what—DNA repair pathways are active during mitosis. Some studies have reported inactive, or inefficient, DNA repair activity during

mitosis, while other studies have found normal levels of repair (Giulotto et al, 1978; Kato et al, 2008; Morrison & Rieder, 2004; Oleinick et al, 1984; Ross, 1989). However, unpublished data from our laboratory and others have shown that the activity of many DNA repair proteins is altered in a way that suggests their inactivation, as damage response and repair proteins which are normally recruited to sites of DNA damage in interphase fail to be similarly recruited in mitosis (data not shown and (Huang et al, 2005)). Thus, it would be interesting, first, to rigorously test whether—and what—DNA repair pathways are active during mitosis. Then, if repair is indeed inactive—or less active—during mitosis, could this explain the increase in DNA damage observed during mitotic arrest? Alternatively, if DNA repair does occur during mitosis, is it less accurate than in interphase? And could this help explain the increase in structural chromosome instability observed after mitotic arrest?

Is DNA damage, or DNA damage signaling, the stimulus for p53 activation during mitotic arrest?

Another important question arising from these studies is whether—or to what extent—DNA damage directly contributes to the activation of p53 following mitotic arrest. While DNA damage is certainly a well-established trigger for p53 activation, clear evidence of DNA damage has not been found in all cells which nonetheless activate p53 after mitotic arrest (Aylon et al, 2006; Chan et al, 2008; Dalton et al, 2007). While these negative results do not rule out the presence of DNA damage, they do underscore the need to directly test the hypothesis that DNA damage is responsible for—or contributes to—p53 activation following mitotic arrest. It seems to us that there are two general

strategies to test this idea. First, one could attempt to selectively inhibit acquisition of DNA damage but not mitotic arrest, and then measure the resulting activation of p53. While this would certainly be the most definitive test of this hypothesis, it (1) necessitates identifying the mechanism responsible for the damage and (2) requires that the damage can actually be selectively inhibited. If, for instance, DNA damage acquired during mitotic is the result of normal ROS levels imposed on an intrinsically repair-deficient mitotic background, could this repair deficiency actually be reversed? Or, if the damage is due to intrinsic chromatin alterations which take place during mitotic arrest, could these alterations be reversed? Thus, the success of this strategy would depend largely on the nature of the DNA damage mechanism.

A second, more indirect test of this hypothesis would be to dissect the signaling pathway which activates p53. Is activation of p53 following mitotic arrest dependent on the p38 stress pathway, as has been suggested (Ganem & Pellman, 2007)? A recent report demonstrating p38-dependence of p53 activation in Chk1-depleted tetraploid HCT116 cells lends some support to this idea (Vitale et al, 2008). Or, are more canonical elements of the DNA damage response pathways responsible for the activation of p53? Is ATM required, as has been suggested (Tritarelli et al, 2004)? Or are multiple pathways involved? An especially interesting possibility is that inhibition of γ-H2AX alone might dampen, or prevent p53 activation. Although inactivation of H2AX does not diminish DNA damage-induced p53 activation in interphase cells (Kang et al, 2005), presumably due to the redundant recognition of DNA damage by other factors, it might in mitotic cells, as our own unpublished studies have yet to find DNA damage response factors which are recruited to mitotic γ-H2AX foci. In conclusion, though experimentally

challenging, testing the hypothesis of whether DNA damage is involved in p53 activation following mitotic arrest should lend important understanding to the nature of the p53-dependent postmitotic arrest.

Do cancer-associated causes of prolonged mitosis elicit DNA damage during mitotic arrest?

An important question concerning DNA damage during mitotic arrest is the physiologic—and pathophysiologic—relevance of this phenomenon. Do the sorts of defects which have been shown to provoke prolonged mitosis in human cancers, such as overexpression of Mad2, inactivation of Rb, inactivation of hCDC4, and supernumerary chromosomes and/or centrosomes, also provoke DNA damage during mitotic arrest (Hernando et al, 2004; Rajagopalan et al, 2004; Sotillo et al, 2007; Yang et al, 2008)? The study described in Chapters II provides evidence, albeit indirect, that spontaneous spindle defects can indeed provoke prolonged mitotic arrest and concurrent DNA damage. However, a definitive test of this possibility would be to track live cells containing reporters for DNA damage, spindle structure, and ploidy with timelapse videomicroscopy. Likewise, we have preliminary data that inactivation of hCDC4 does indeed cause elevated γ-H2AX in mitotic cells (data not shown), although further experiments would need to determine whether this increase in γ-H2AX actually occurs during mitosis in hCDC4-/- cells. A similar approach could be applied to other cancer-associated changes such as overexpression of Mad2. Finally, it would be useful to determine whether this phenomenon occurs—and can be measured—in vivo. Does human tumor tissue contain abnormal mitotic figures with spontaneous elevation of γ-

H2AX? Does in vivo administration of antimitotic treatment induce γ-H2AX in mitotically-arrested cells? Do mitotically-arrested cells in Mad2-overexpressing mice exhibit increased γ-H2AX? Investigation of these questions would help to determine the extent of physiologic relevance of this phenomenon.

Does DNA damage during mitotic arrest influence sensitivity to antimitotic chemotherapy?

Antimitotic drugs are a widely-used, and often successful, class of chemotherapeutic agents, but tumor development of drug resistance frequently hinders their efficacy (Jackson et al, 2007). Therefore, identification of tumor-specific factors which determine sensitivity should aid in the rational use of antimitotics, as well as help generate approaches to combination therapies of maximal efficacy. Because we have observed significant variation among cancer cell lines in the degree of DNA damage acquired during mitotic arrest, it would be interesting to determine whether the extent of this damage correlates to cellular sensitivity to antimitotic agents in different cell lines. Moreover, would the addition of a classical DNA damaging agent, such as irradiation, doxorubicin, or cisplatin, provide synergistic toxicity in cells which already acquire high amounts of DNA damage during mitotic arrest? Or would synergism instead be achieved in cells which otherwise acquire low amounts of DNA damage during mitotic arrest?

As is the case for the pursuance of other future directions discussed above, investigation of the role of DNA damage in sensitivity to antimitotics would be greatly facilitated by identification of the cause of DNA damage during mitotic arrest. If upon doing so, it appeared possible to inhibit this damage without inhibiting mitotic arrest,

then a direct test of the role of DNA damage in sensitivity to antimitotic chemotherapy might be possible. Moreover, mechanistic understanding of the cause of damage might also suggest ways to maximize its toxicity. For example, would inactivation of certain repair pathways, such as homologous recombination or nonhomologous end joining repair of double-stranded breaks, increase sensitivity to antimitotics? Indeed, this investigation could be carried out even without identification of the damage mechanism, although its identification might suggest more targeted approaches for manipulation.

Finally, given the results presented in Chapter III, it is worth asking whether—and in what circumstances—p53 status may affect the response to antimitotic agents. Although significant variation in the outcomes of the existing studies investigating this question might suggest a degree of unmanageable complexity, a rigorous in vivo test of the role of p53 in sensitivity to antimitotic agents has not been performed (Galmarini et al, 2001; Kienitz et al, 2005; Wahl et al, 1996; Wu & El-Diery, 1996; Yamaguchi et al, 2004). Thus, carefully-controlled studies investigating the role of p53 in antimitotic sensitivity of tumors with different genetic backgrounds should help identify in what circumstances, if any, p53 may be a useful predictor of treatment response.

In conclusion, numerous interesting and important questions concerning the causes and consequences of DNA damage during mitotic arrest remain. No doubt others will arise as more knowledge is gained. We believe that investigating these questions has the potential not only to expand our knowledge of this novel phenomenon, but also our understanding of aspects of cell biology, tumorigenesis, and cancer chemotherapy.

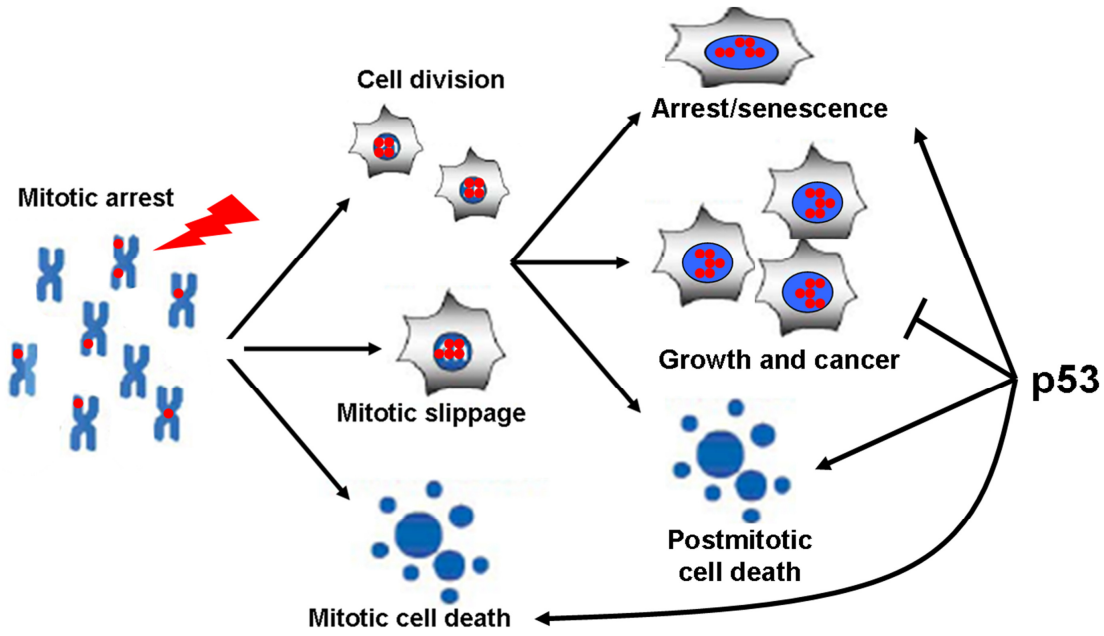


Figure 1. Working model for the consequences of DNA damage during mitotic arrest. Cells which experience mitotic arrest acquire DNA damage (red) which can manifest as potentially oncogenic structural chromosome instability in those cells which evade the p53-dependent postmitotic response. Adapted from Weaver et al. (Weaver & Cleveland, 2005).

Chapter V

References

W. Brian Dalton[1,2]

[1]Division of Digestive Diseases, Department of Medicine,
[2]Biochemistry, Cell and Developmental Biology Graduate Program,
Emory University School of Medicine, Atlanta, GA 30322

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