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## p53 Suppresses Structural Chromosome Instability Following Mitotic Arrest In Human Cells

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### Abstract

The p53 tumor suppressor inhibits the proliferation of cells which undergo prolonged activation of the mitotic checkpoint. However, the function of this antiproliferative response is not well defined. 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. In contrast, cells made polyploid without mitotic arrest exhibited neither significant structural chromosome instability nor p53-dependent cell cycle arrest. We also observed that p53 suppressed both the frequency and structural chromosome instability of spontaneous polyploids in HCT116 cells. Furthermore, time-lapse videomicroscopy revealed that polyploidization of *p53*<sup>-/-</sup> HCT116 cells is frequently accompanied by mitotic arrest. These data suggest that a function of the p53-dependent postmitotic response is 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.

### Keywords

p53; cell cycle arrest; chromosomal instability; DNA damage; mitotic checkpoint; polyploidization

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### Conflict of Interest

None of the authors has any competing financial interests in relation to this work.

## Introduction

The p53 tumor suppressor represents a central defense against human cancer (Vousden and Lane, 2007). Its inactivation is one of the most common alterations in human tumors, and numerous studies have established the tumor suppressing properties of p53 (Toledo and 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 and 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 and Lane, 2007). One example is prolonged activation of the mitotic checkpoint, which elicits a p53-dependent cell cycle arrest (Ganem and Pellman, 2007). 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 and Jacks, 1998; Minn *et al.*, 1996; Rajagopalan *et al.*, 2004). Despite the ubiquity of the postmitotic response, its function is not well defined (Ganem and Pellman, 2007; Stukenberg, 2004).

One clue to the function of the postmitotic response may be that prolonged activation of the mitotic checkpoint has been causally implicated in tumorigenesis (Dalton and Yang, 2009). Indeed, mitosis is frequently prolonged in cancer cells, and several genetic and epigenetic defects which cause mitotic arrest can contribute to cancer (Dalton and Yang, 2009). 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 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 (Ganem and Pellman, 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 changes resulting from double-stranded DNA breaks (Dalton *et al.*, 2007; Quignon *et al.*, 2007; Stevens *et al.*, 2007). Given the role of structural chromosome aberrations in tumorigenesis, 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 this structural chromosome instability. 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.

## Materials and Methods

### Cell lines and treatments

IMR90 HDFs were obtained from the ATCC. *p53<sup>+/+</sup>* and *p53<sup>-/-</sup>* 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  $3 \times 10^4$  cells/cm<sup>2</sup> onto fibronectin-coated dishes or slides 24h prior to experiments. IMR90 cells were cultured in DMEM and also seeded at a density of  $3 \times 10^4$  cells/cm<sup>2</sup> 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).  $\gamma$ -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°C in 70% ethanol. Antibody incubations were 1h at room temperature, and DNA was counterstained with propidium iodide. For  $\gamma$ -H2AX/CC-3 flow cytometry, cells were harvested by trypsinization, fixed with 2% formaldehyde/PBS, permeabilized with methanol, and incubated overnight at 4°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<sub>2a</sub> allotype, and can thus be used simultaneously with IgG<sub>1</sub> antibodies, such as  $\gamma$ -H2AX (Thibodeau and 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- $\gamma$ -H2AX (Upstate), rat anti- $\alpha$ -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 2F).

## Time-lapse imaging

Phase-contrast images of  $p53^{-/-}$  HCT116 cells grown inside a 37°C, 5% CO<sub>2</sub> chamber were automatically obtained at 6 min intervals in multiple locations over 48h using an Olympus IX81 microscope. All images were analyzed with Slidebook.

## Results

### Both $p53^{+/+}$ and $p53^{-/-}$ cells acquire $\gamma$ -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 and Maiato, 2004). To determine the extent of DNA damage acquired during this arrest, we measured the formation of  $\gamma$ -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  $\gamma$ -H2AX foci, as compared to prometaphase controls (Figure 1B). Increased  $\gamma$ -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  $\gamma$ -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

$\gamma$ -H2AX foci in mitotic  $p53^{+/+}$  and  $p53^{-/-}$  cells (Figure 1D–F). Thus, both pharmacologic and genetic manipulations which provoke mitotic arrest through distinct mechanisms 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 studies (Castedo *et al.*, 2006; Kim *et al.*, 2004; Vogel *et al.*, 2004),  $p53^{-/-}$  cells exhibited greater polyploidization and reduced apoptosis when compared to  $p53^{+/+}$  cells during 48h nocodazole (Figure 2A–E and Supporting Figure S1). Because the specificity of gene targeting in cancer cells is not always certain (Matoba *et al.*, 2006; Pfliegerhaer *et al.*, 2005), we independently confirmed the p53-dependence of these phenotypes using miRNA (Supporting Figure S2). Moreover, because short-term rates of apoptosis do not always correspond to long-term rates of survival (Bunz *et al.*, 1999), we also determined the clonogenicity of these nocodazole-treated cells. This analysis revealed that colony survival of  $p53^{-/-}$  cells exposed to 48h nocodazole, while low overall, was 5-fold higher than that of  $p53^{+/+}$  cells (Figure 2F). Collectively, these data indicate that p53 inhibits polyploidization, promotes apoptosis, and suppresses clonogenicity 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 exhibit not only greater survival but also 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. Consistent with our previous findings (Dalton *et al.*, 2007), nocodazole-induced polyploid HCT116 cells exhibited multiple types of structural chromosome aberrations (Figure 3). Notably, nocodazole-treated  $p53^{-/-}$  cells exhibited a 50% higher burden of aberrations than  $p53^{+/+}$  cells ( $p < 0.0001$ ) (Figure 3). This result suggests that p53 preferentially inhibits the polyploidization and survival of those cells which suffer the greatest DNA damage during mitotic arrest. In this way, p53 suppresses structural chromosome instability following mitotic arrest in HCT116 cells.

### **Polyploidy is not responsible for structural chromosome instability following mitotic arrest**

To address the possibility that the polyploid state itself might elicit structural chromosome changes (Ganem *et al.*, 2007), 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 and Stearns, 2005). In contrast to nocodazole, blebbistatin was capable of producing polyploidy without significant cell death, cell cycle arrest, or increased chromosome aberrations (Figure 4A–C). Moreover, no difference in these parameters was observed between  $p53^{+/+}$  and

$p53^{-/-}$  cells (Figure 4A–C). This result 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 4C). This pattern was also observed following cytokinesis inhibition using dihydrocytochalasin B (unpublished observations). Thus, while polyploidization itself does not account for the observed structural chromosome instability following prolonged mitotic arrest, it may be that polyploidization, or pharmacologic inhibition of cytokinesis, may induce some degree of DNA damage.

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

To test whether p53 suppresses structural chromosome instability following mitotic arrest in untransformed human cells, we examined the response to nocodazole of human diploid fibroblasts (HDFs) in which p53 was silenced through lentivirus-mediated expression of miRNAs (Figure 5A). In HDFs expressing either p53 or control miRNAs, nocodazole produced an increase in  $\gamma$ -H2AX foci during mitotic arrest (Figure 5B). 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 5C–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 (Supporting Figure S3) (Ikeuchi *et al.*, 1972; Kato and Sandberg, 1967). Nocodazole-induced cell death, however, did not appear to be influenced by p53 knockdown in HDFs (Supporting Figure S4), demonstrating that the role of p53 on apoptosis following prolonged mitotic arrest is cell type-dependent, as previously discussed. These data therefore demonstrate that, by inducing postmitotic cell cycle arrest, p53 suppresses structural chromosome instability following mitotic arrest in untransformed, as well as transformed, 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 6A). 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 6A). Color karyotyping of these spontaneous  $p53^{-/-}$  polyploids ( $>4N$ ) confirmed the presence of cells with chromosome fragments and chromosome rearrangements (Figure 6C). In contrast, spontaneous  $p53^{+/+}$  polyploids did not exhibit an elevated frequency of chromosome aberrations, when compared to  $p53^{+/+}$  diploid cells (Figure 6A). 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 6B), 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.

### **Mitotic slippage following prolonged mitosis occurs spontaneously in p53<sup>-/-</sup> HCT116 cells**

While the origin of spontaneously damaged p53<sup>-/-</sup> polyploids is unknown, one possibility is that they 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 time-lapse videomicroscopy. This analysis revealed that 4% (24/592) of mitoses resulted in spontaneous cell division failure in p53<sup>-/-</sup> 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) (Figure 7). Indeed, some cells spent up to 10h in spontaneous mitotic arrest, before undergoing mitotic slippage (Supporting Videos S1–S3). 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<sup>-/-</sup> 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 and Yang, 2009), (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 and 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 p53-dependent postmitotic response 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).

Previous studies have shown that the role of p53 in the regulation of spontaneous structural chromosome integrity is context-dependent. For example, increased structural aberrations have been observed in bone marrow cells of p53<sup>-/-</sup> mice (Bouffler *et al.*, 1995) and fibroblasts from patients with germline p53 mutations (Bischoff *et al.*, 1990), but not in lymphoblastoid cells from patients with germline p53 mutations (Lalle *et al.*, 1995) or isogenic human cancer cell lines (Bunz *et al.*, 2002) (see Supporting Info for a discussion of the last example). Similar context-dependence has been observed in cytogenetic and comparative genomic hybridization studies of human tumor tissue, as p53 mutations correlate with structural chromosome instability in several case series (De Angelis *et al.*, 1999; Jong *et al.*, 2004; Kleivi *et al.*, 2005), but not in others (Curtis *et al.*, 2000; Eshleman *et al.*, 1998; Westra *et al.*, 2005). Interestingly, some of this context-dependence may be due to differences in the types of p53 mutations which occur, as there is evidence that gain-of-function p53 mutations are more likely than null p53 mutations to promote structural chromosome instability (Song *et al.*, 2007). Conversely, much of the complexity may result from the different genetic backgrounds of tissue types and individual tumors in which p53 is mutated. Indeed, several studies have shown that while p53 mutation alone may have minimal effects on structural chromosome instability in certain mouse models, its mutation can greatly exacerbate structural chromosome instability produced by mutations in other genes, such as H2AX, 53BP1, telomerase, and others (Artandi *et al.*, 2000; Bassing *et al.*, 2003; Ward *et al.*, 2005). In this way, p53 appears to facilitate the underlying tendencies to structural chromosome instability initiated by other defects. From this view, our data suggest that prolonged activation of the mitotic checkpoint may be one such instigator of structural chromosome instability that is facilitated by p53 mutation. Of note, this situation resembles the complexity recently discovered in numerical chromosome instability, where errors in chromosome missegregation require additional “aneuploidy-tolerating” defects in order to produce stable numerical changes (Thompson and Compton, 2008).

Our data also have implications for understanding the cellular responses to antimetabolic chemotherapeutics. The p53-regulated survival of nocodazole-treated HCT116 cells suggests that p53 can be a determinant of antimetabolic 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 antimetabolic 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. This context-dependence is likely influenced by the same factors enumerated



above for the role of p53 in structural chromosome instability, as tissue of origin and p53 mutation type have previously been implicated in p53-dependent sensitivity to other chemotherapeutics (Lu and El-Deiry, 2009). Thus, a conservative interpretation of these findings is that p53 may be a determinant of antimitotic sensitivity in a subset of human tumors which also share other modulators of drug sensitivity. Identification of such modulators will be an interesting area for future work, and might include investigations into DNA repair pathways (Swanton *et al.*, 2009), as well as other p53 family members, such as p63 and p73, both of which are known to influence chemosensitivity (Muller *et al.*, 2006). Finally, with the ongoing development of pharmaceuticals which partially restore p53 activity in cancer cells (Lu and El-Deiry, 2009), it may be worthwhile to determine whether such agents might work in synergy with antimitotics by restoring p53-dependent apoptotic and growth arrest signaling following prolonged mitotic arrest.

An important but unanswered question is what triggers p53 during this process (Chan *et al.*, 2008; Ganem and 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 and Sluder, 2004; Uetake and Sluder, 2007; Wong and 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 data showing 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 and Lu, 2002), it is tempting to speculate that postmitotic activation of p53 is induced, or 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 and Pellman, 2007). Indeed, we previously observed significant cell-type variation in nocodazole-induced  $\gamma$ -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  $\gamma$ -H2AX in nocodazole-treated U2OS cells has been reported (Aylon *et al.*, 2006; Chan *et*

*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 and Stearns, 2005). Furthermore, it is important to note that a lack of increased  $\gamma$ -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  $\gamma$ -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 p53 functions to inhibit the potentially dangerous consequences of DNA damage acquired during mitotic arrest.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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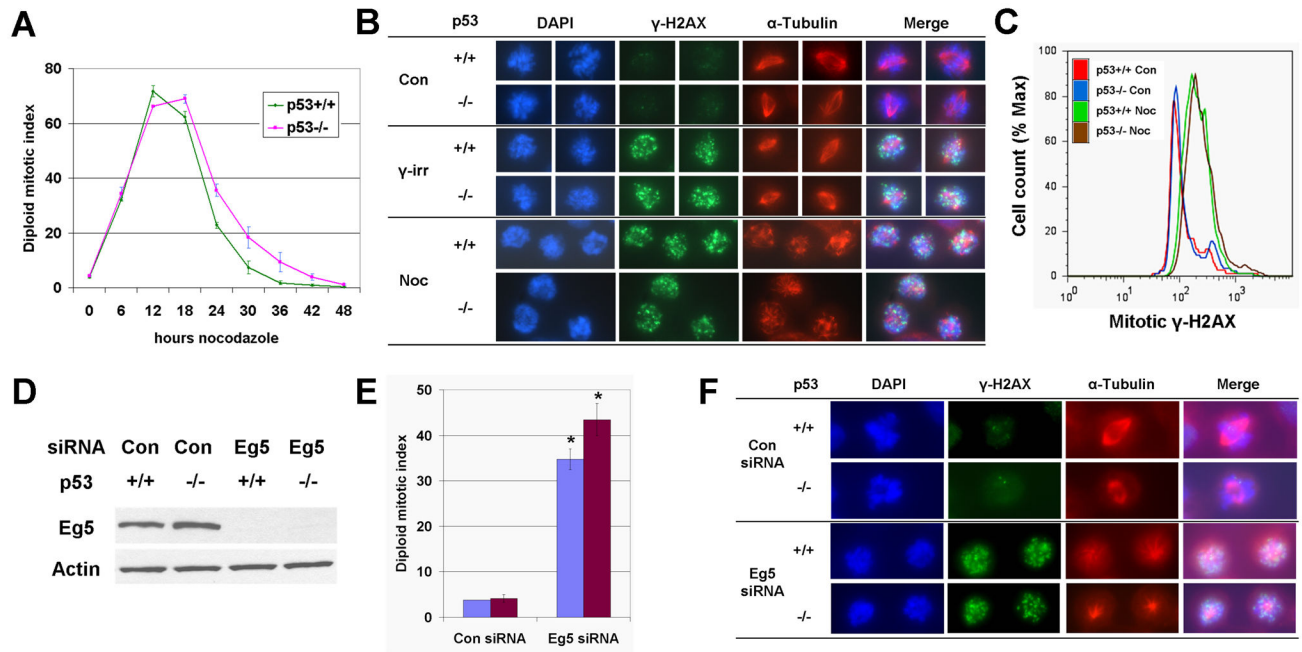
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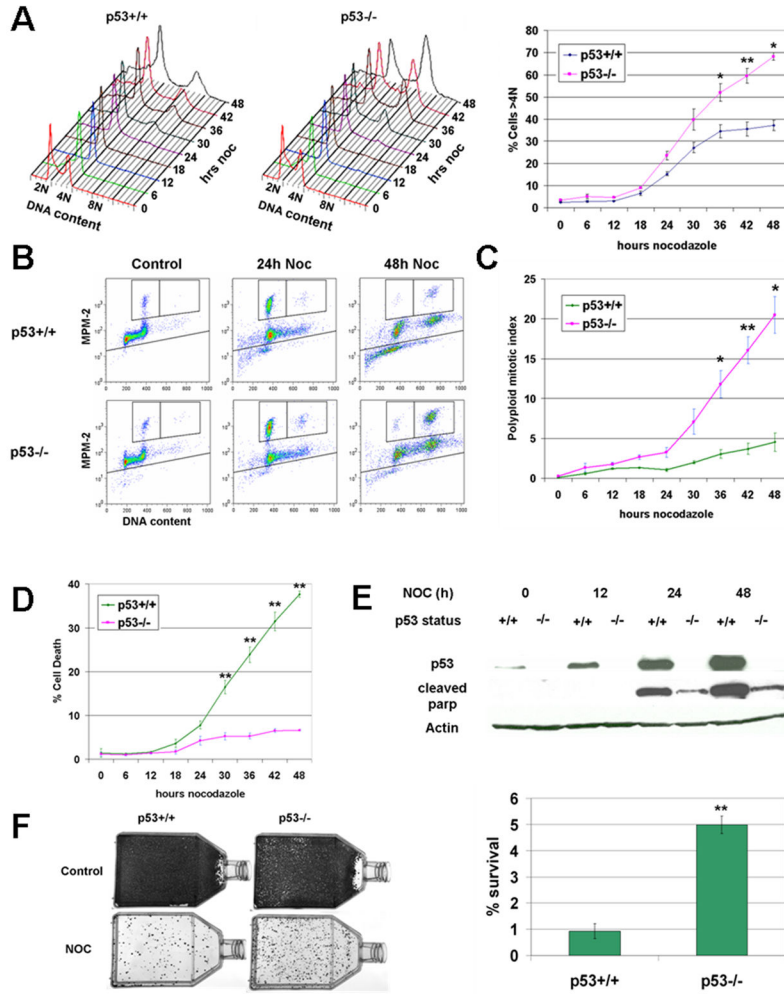
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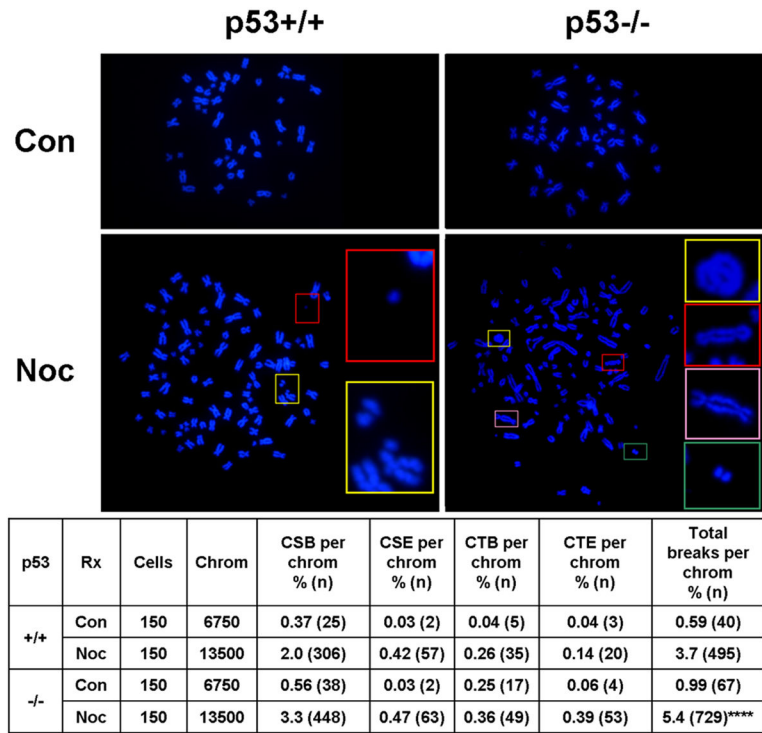
**Figure 1. Both  $p53^{+/+}$  and  $p53^{-/-}$  HCT116 cells acquire DNA damage during mitotic arrest**  
 A. Diploid mitotic index of HCT116 cells during 48h nocodazole, as determined by MPM-2 flow cytometry. Means and SEMs are from 2–3 independent experiments. B. Images of prometaphase cells stained for  $\gamma$ -H2AX and  $\alpha$ -tubulin. Nuclei were counterstained with DAPI. Noc = 18h nocodazole.  $\gamma$ -irr = 30 min after 2 Gy  $\gamma$ -irradiation. C. Flow cytometric analysis of mitotic  $\gamma$ -H2AX. Cells were treated with or without 18h nocodazole and stained for CC-3 and  $\gamma$ -H2AX. CC-3-positive cells, which are mitotic (Thibodeau and Vincent, 1991), were gated, and  $\gamma$ -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. \* =  $p < 0.05$ , for t-tests, as compared to control. F. Images of prometaphase cells stained for  $\gamma$ -H2AX and  $\alpha$ -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**

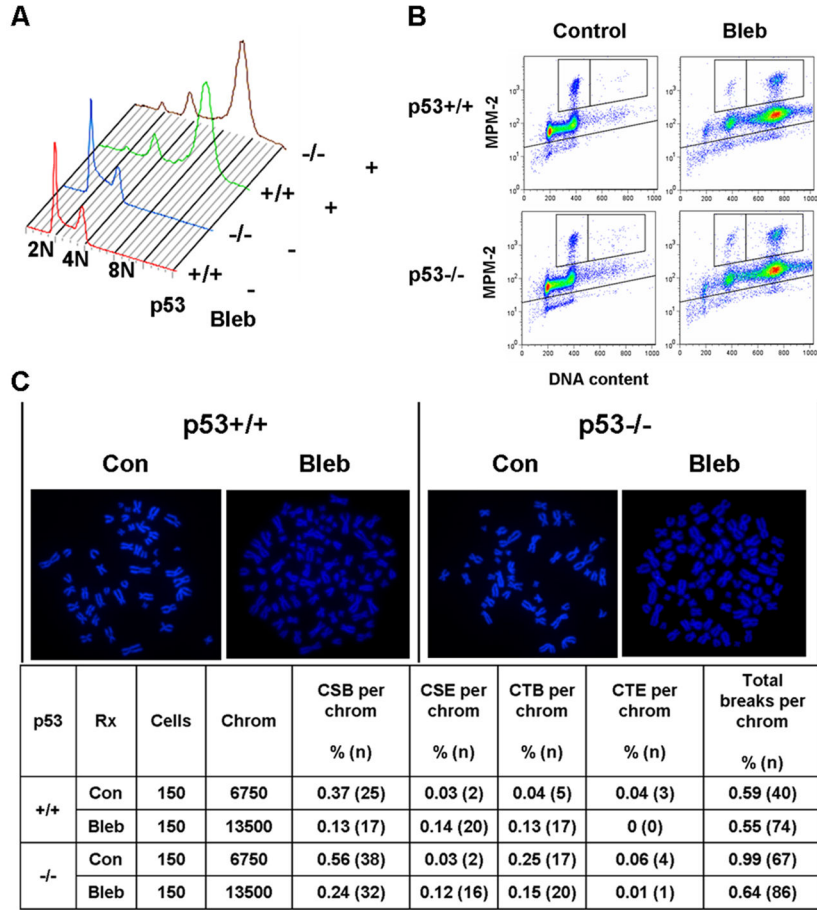
A. Example of cell ploidy (left panel) and quantification of polyploidy (right panel) in nocodazole-treated cells. Means and SEMs are from 2–3 independent experiments. \* =  $p < 0.05$ , \*\* =  $p < 0.01$  for t-tests. B. Dot plots of cells stained for MPM-2 and DNA content after 24h and 48h nocodazole 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. D. Quantification of cell death (lower gate in Figure 2B) during 48h nocodazole treatment. Cells that were entirely negative for the MPM-2 phosphoepitope were found, through fluorescent microscopy, to be apoptotic (Supporting Figure S1). 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.





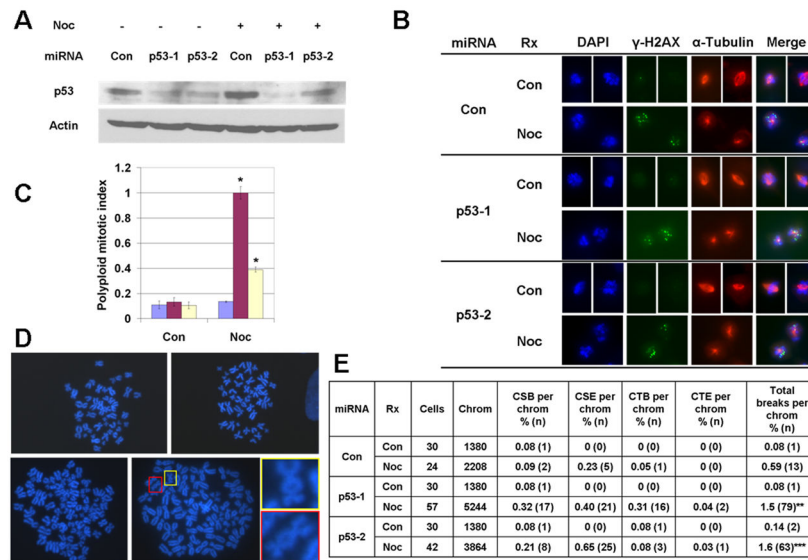
**Figure 3. p53 suppresses structural chromosome instability following mitotic arrest in HCT116 cells**

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.



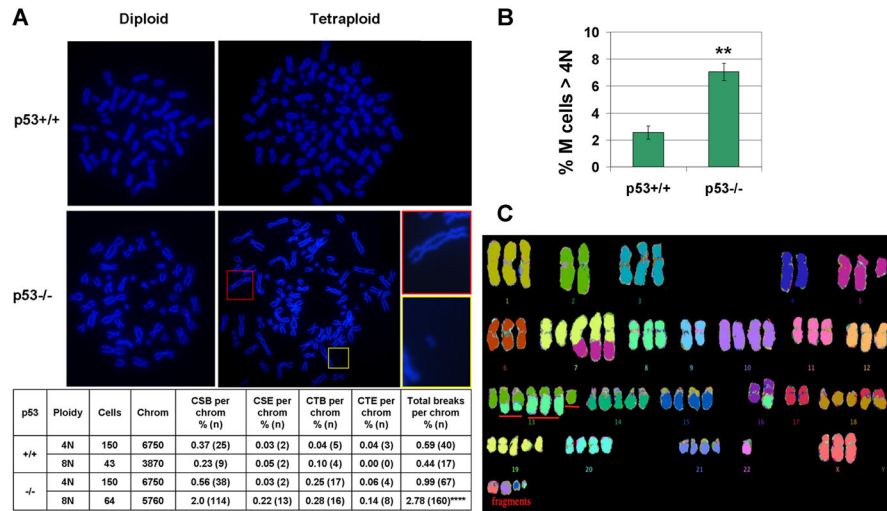
**Figure 4. Polyploidization through inhibition of cytokinesis does not elicit significant DNA damage, postmitotic arrest, or cell death in HCT116 cells**

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.



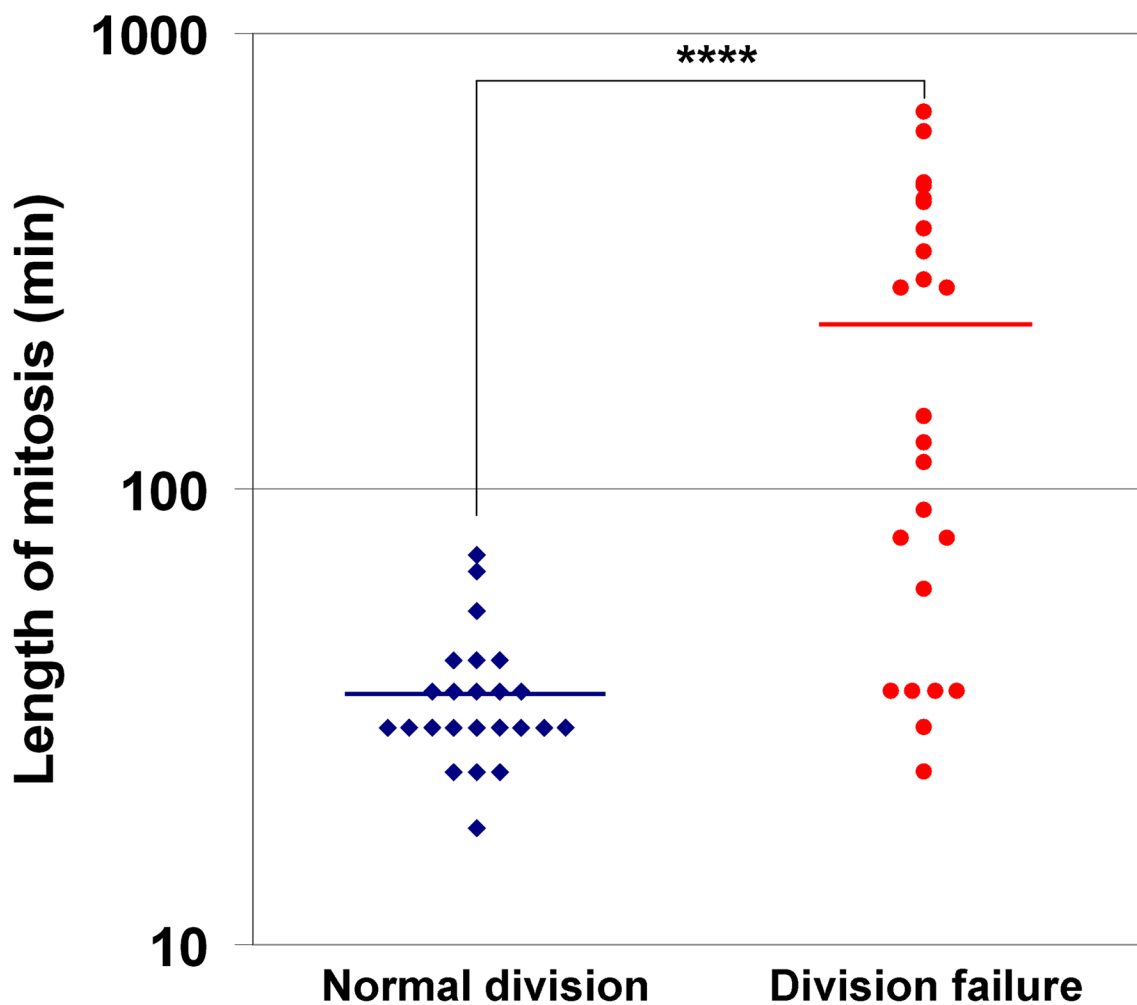
**Figure 5. p53 suppresses structural chromosome instability following mitotic arrest in IMR90 human diploid fibroblasts (HDFs)**

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  $\gamma$ -H2AX and  $\alpha$ -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.



**Figure 6. p53 suppresses the frequency and structural chromosome instability in spontaneous polyploid HCT116 cells**

A. Examples of chromosome spreads in spontaneous diploid (4N) or polyplod (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 polyplod (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 polyplod  $p53^{+/+}$  cells. B. Quantification of mitotic cells with polyplod 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 polyplod  $p53^{-/-}$  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.



**Figure 7. 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.