

Experimental Paper

# Degradation of ATM-Independent Decatenation Checkpoint Function in Human Cells is Secondary to Inactivation of p53 and Correlated with Chromosomal Destabilization

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\*\*In Memoriam, 1959-2002

Received 02/2002; Accepted 03/25/02

Previously published online as a *Cell Cycle* "Paper In Press" at <http://www.landesbioscience.com/journals/cellcycle/papersinpress/inpress13.html>

## KEY WORDS

Checkpoints, DNA damage, Decatenation, Topoisomerase II, ICRF-193, Radiation

## ABSTRACT

DNA topoisomerase II is required in the cell cycle to decatenate intertwined daughter chromatids prior to mitosis. To study the mechanisms that cells use to accomplish timely chromatid decatenation, the activity of a catenation-responsive checkpoint was monitored in human skin fibroblasts with inherited or acquired defects in the DNA damage G2 checkpoint. G2 delay was quantified shortly after a brief incubation with ICRF-193, which blocks the ability of topoisomerase II to decatenate chromatids, or treatment with ionizing radiation (IR), which damages DNA. Both treatments induced G2 delay in normal human fibroblasts. Ataxia telangiectasia fibroblasts with defective G2 checkpoint response to IR displayed normal G2 delay after treatment with ICRF-193, demonstrating that ATM kinase was not required for signaling when chromatid decatenation was blocked. The G2 delay induced by ICRF-193 was reversed by caffeine, indicating that active checkpoint signaling was involved. ICRF-193-induced G2 delay also was independent of p53 function, being evident in cells expressing HPV16E6 to inactivate p53. However, as fibroblasts expressing HPV16E6 aged in culture, they lost the ability to delay entry to mitosis, both after DNA damage and when decatenation was blocked. This age-related loss of G2 delay in response to ICRF-193 and IR in E6-expressing cells was blocked by induction of telomerase. Expression of telomerase also prevented chromosomal destabilization in aging E6-expressing cells. These observations lead to a new model of genetic instability, in which attenuation of G2 decatenation checkpoint function permits cells to enter mitosis with insufficiently decatenated chromatids, leading to aneuploidy and polyploidy.

## INTRODUCTION

The high fidelity of chromatid duplication and separation during cell division requires checkpoint surveillance systems that monitor the cell cycle and impose delays when conditions are inopportune or inappropriate.<sup>1-4</sup> The checkpoints that regulate mammalian cell entry to mitosis recognize and respond to ionizing radiation (IR)-induced DNA damage,<sup>5-10</sup> solar radiation-induced cellular damage,<sup>11</sup> incompletely replicated DNA,<sup>12</sup> aberrant histone acetylation,<sup>13</sup> and insufficiently decatenated daughter chromatids caused by inhibition of topoisomerase II.<sup>14,15</sup> Of these, the G2 checkpoint response to IR-induced DNA damage is the best characterized. The gene products, ATM, ATR, BRCA1, CtIP, Nbs1, hRad17, Wee1, Myt1, Chk1, Cdc25B/C, Plk-1, and Crm1 all appear to contribute to the IR-induced G2 checkpoint response. These proteins ultimately regulate the location<sup>14,16,17</sup> and activity<sup>18,19</sup> of mitosis-promoting factor (MPF), which is a heterodimer of a cyclin-dependent kinase, Cdk1, and its regulatory subunit, cyclin B1.<sup>20,21</sup> Cells that have incurred supra-lethal levels of DNA damage display additional elements of response mediated primarily by p53 to sustain the G2 delay.<sup>22-24</sup> Thus, the G2 checkpoint system includes immediate and sustained signaling events to prevent cells from entering mitosis with damaged chromatids. Ataxia telangiectasia (AT) cells with inactivating mutations in ATM display a defect in G2 checkpoint function associated with enhanced frequencies of IR-induced chromosomal aberrations.<sup>10</sup> Li-Fraumeni fibroblasts with mutations in p53 spontaneously develop aneuploidy.<sup>25</sup> Attenuation or inactivation of G2 checkpoint function thus may contribute to the genetic instability that characterizes cancer.

Two major effectors of cell cycle checkpoint responses are ATM and ATR, protein kinases that phosphorylate cellular substrates in response to various forms of genetic stress.

Inactivating mutations in *ATM* in patients with AT,<sup>10,19</sup> and enforced overexpression of a kinase-inactive *ATR* allele,<sup>6,8</sup> attenuate the G2 delay response to DNA damage and establish the requirements for ATM and ATR in the G2 checkpoint. Substrates of ATM and ATR that are required for G2 checkpoint function include, hRad17,<sup>5</sup> BRCA1<sup>26-31</sup> and p53<sup>32-34</sup>; these have diverse modes of action. Hrad17 interacts with replication factor C subunits to load the rad9/rad1/hus1 checkpoint sliding-clamp complex onto DNA.<sup>5</sup> Expression of a form of hRad17 that could not be phosphorylated by ATM or ATR prevented IR-induced G2 delay.<sup>5</sup> BRCA1 protein interacts with several repair enzymes in a large complex<sup>35</sup> and transactivates the cyclin-dependent kinase inhibitors, p21<sup>Waf1</sup> and Gadd45.<sup>36,37</sup> Its associated protein CtIP is also an ATM substrate and becomes dissociated after phosphorylation.<sup>29</sup> Mouse embryo fibroblasts that were engineered to express a splice variant form of BRCA1 in which exon 11 was deleted displayed intact G1 checkpoint response to IR but the G2 delay response to IR was fully ablated.<sup>9</sup> P53 transactivates p21<sup>Waf1</sup>, 14-3-3 $\sigma$  and Gadd45,<sup>24</sup> and transrepresses cyclins B1 and B2, Cdk1, Cdc25C, and topoisomerase II $\alpha$ .<sup>38-41</sup> Although p53-defective cells are able to trigger an immediate G2 checkpoint response to low doses of IR (<2 Gy),<sup>7,42</sup> they have difficulty in sustaining G2 delay after high doses (>10 Gy), due in part to reduced expression of 14-3-3 $\sigma$  and p21<sup>Waf1</sup>.<sup>22,23</sup>

Along a different pathway, one element of the G2 checkpoint acts to sustain inhibitory phosphorylation of Cdk1 by activation of Wee1 and sequestration of Cdc25C and/or Cdc25B. The action of ATM and ATR kinases activates the checkpoint effector kinases, hCds1<sup>43-46</sup> and Chk1,<sup>47,48</sup> respectively. In the former case, the activation requires ATM-dependent phosphorylation of the Nbs1 protein, which is defective in the Nijmegen Breakage Syndrome, a radiosensitive genetic disease resembling AT.<sup>49</sup> The effector kinases phosphorylate the mitotically active Cdc25C phosphatase at ser216, causing it to be sequestered by 14-3-3 proteins in the cytoplasm,<sup>50</sup> and the S/G2/mitotically-active Cdc25B phosphatase at the functionally equivalent ser309 and ser361.<sup>11</sup> Chk1 also appears to activate Wee1 kinase which phosphorylates Cdk1 at tyr15.<sup>51</sup> Cdc25B and C phosphatase can remove in vitro inhibitory phosphates that are placed by Wee1 and Myt1 at tyr15 and thr14 in the ATP-binding pocket of Cdk1.<sup>11,18,50</sup> At least one of the phosphatases is involved in the DNA damage G2 checkpoint; microinjection of antibodies to either causes G2 arrest,<sup>52,53</sup> though mice lacking Cdc25C are phenotypically normal, and show normal G2 arrest in cells given IR.<sup>54</sup>

A second mechanism for inhibiting MPF as part of the G2 checkpoint response to IR involves regulated expression of its components.<sup>55</sup> Cyclin B1 and Cdk1 are transrepressed by p53 via interaction with NF-Y.<sup>38,39,41</sup> One consequence of inactivation of p53 function is failure to repress expression of cyclin B1 and Cdk1,<sup>24</sup> and this may contribute to attenuation of p53-dependent G2 checkpoint function.<sup>38</sup> Cdk1 is also transactivated by E2F1. Consequently, expression of SV40 large T antigen, which inactivates both p53 and pRB, enhances expression of MPF kinase and attenuates G2 checkpoint function.<sup>57</sup>

A third mechanism of G2 checkpoint response involves Crm1-mediated exclusion of MPF from the nucleus. Not only is MPF sequestered in the cytoplasm by interaction with Myt1,<sup>18</sup> it is actively excluded from the nucleus by the Crm1 nuclear exporter.<sup>14,17</sup> Plk-1 phosphorylates cyclin B1 in the nuclear export sequence to inhibit Crm1-mediated nuclear export<sup>58</sup> and expression of a dominant kinase-active Plk-1 allele attenuated G2 checkpoint response to

DNA damage.<sup>59</sup> Inhibition of ATM and ATR kinase activity with caffeine when combined with leptomycin B to inhibit the Crm1 exporter also attenuated G2 delay in response to the topoisomerase II inhibitor, etoposide.<sup>17</sup> Finally ectopic coexpression of a non-repressible Cdk1 allele (Cdk1AF) with cyclin B1 containing a dominant nuclear localization sequence appeared to fully ablate G2 checkpoint response to IR-induced DNA damage.<sup>16</sup>

Examination of the DNA signals that trigger the G2 checkpoint response in diploid human fibroblasts suggested that DNA double-strand breaks (dsb) were efficient inducers of the response but DNA single-strand breaks were not.<sup>60</sup> Effective inducers of G2 delay that produce DNA dsb included IR, bleomycin, which targets oxidative damage to DNA, and the topoisomerase II poison, etoposide, which induces protein-associated DNA dsb. Topoisomerase II inhibitors including etoposide were recently shown to activate the Chk2 kinase<sup>61</sup> that is implicated in checkpoint response to DNA damage. UVC did not induce a G2 checkpoint response after a low fluence (1 J/m<sup>2</sup>), although a severely cytotoxic fluence (10 J/m<sup>2</sup>) did induce G2 delay.<sup>60</sup> The G2 checkpoint response to such high doses of UVC appears to require the p38 mitogen-activated protein kinase to inactivate cytoplasmic Cdc25B<sup>11</sup> and Gadd45 to inhibit MPF.<sup>62</sup> Topoisomerase II catalytic inhibitors, such as ICRF-193 and merbarone, which do not induce topoisomerase-associated DNA dsb,<sup>16,63,64</sup> also arrest mammalian cells in G2.<sup>14,15,60,65</sup> This result suggested that there may be a DNA-damage-independent mechanism for triggering G2 delay in cells with insufficient topoisomerase II activity.

DNA topoisomerase II is required in eukaryotic cells to separate catenated daughter chromatids produced by DNA replication.<sup>66</sup> Type II topoisomerases are homodimeric proteins that form a covalent intermediate with both strands at DNA dsb, via phosphoryl-tyrosine residues.<sup>67</sup> DNA duplexes are passed through the cleaved complex of topoisomerase II and DNA, relieving catenation. ICRF-193 inhibits topoisomerase II by holding the enzyme in the form of a closed clamp which cannot form covalent complexes with DNA, nor pass DNA strands.<sup>63,64,68,69</sup> Incubation of mammalian cells with ICRF-193 was shown to induce cohesive chromatids in prophase, intertwined daughter chromatids in metaphase and chromosome bridges in anaphase.<sup>70-72</sup> Once cells have entered mitosis, topoisomerase II catalytic activity is required to segregate sister chromatids at the metaphase/anaphase transition. Topoisomerase II is also required at this step in yeast.<sup>73</sup> However, mammalian cells exposed to a lower concentration of ICRF-193 prior to mitosis arrest growth in G2.<sup>14,15,74,75</sup> The fact that caffeine could override ICRF-193-induced, but not IR-induced, G2 delay in the DM87 muntjac cell line suggested that IR and ICRF-193 trigger G2 checkpoint response using different signaling pathways.<sup>15</sup>

The studies reported here were designed to characterize genetic requirements for the decatenation checkpoint in human cells and determine whether conditions that lead to inactivation of DNA damage G2 checkpoint function also affect the decatenation checkpoint. Because complete chromatid decatenation may be required for accurate chromatid segregation at anaphase, attenuation of decatenation checkpoint function could contribute to genetic instability during human carcinogenesis.

## MATERIALS AND METHODS

**Cell Lines and Culture Conditions.** Conditions for growth of human cell strains and lines were as described previously.<sup>42</sup> Cells used in this study included NHF4-neo, NHF4-E6, NHF5-neo, NHF5-E6, NHF7-neo, and NHF7-E6, which represent secondary cultures of fibroblasts derived from neonatal foreskin and which had been infected with a replication-defective retrovirus carrying *neo<sup>R</sup>*, with or without *HPV16E6*.<sup>42</sup> The NHF1 foreskin fibroblast strain was also used.<sup>76</sup> AT fibroblasts, SVAT4BI/NE and AT191BE, were obtained from the Coriell Institute Human Genetic Mutant Cell Repository. Neo-expressing fibroblasts were studied at low population doubling levels prior to senescence (PDL<60), while E6-expressing fibroblasts were studied at low PDL (<60, before crisis) and high PDL (>60, during and after crisis). The NHF7-E6 fibroblasts at low PDL were transfected with a linearized plasmid containing the catalytic subunit of telomerase, *hTERT* (in the sense or antisense orientation), and a *puro<sup>R</sup>* gene, then drug-resistant transformants were selected with 0.23 µg/ml puromycin. Drug-resistant cells were expanded and expression of telomerase was confirmed by TRAPeze kit assay (Intergen Inc.) according to manufacturer's instructions. *hTERT* cDNA was provided by Geron Corporation.

**Assay of Mitotic Delay.** Mitotic delay was enumerated by quantification of mitotic index in replicate cultures of treated and control cells.<sup>7,14,15</sup> For treatment with  $\gamma$ -rays, cells were irradiated in growth medium with a <sup>137</sup>Cs source at a dose-rate of 1 Gy per min (Gammacell 40). Sham-treated controls were subjected to the same movements in and out of incubators as irradiated cells. For treatment with ICRF-193 or etoposide, a stock solution of drug was prepared in DMSO. Cells in monolayer culture generally were incubated for 15 min in Hank's balanced salt solution with 0.5% DMSO and various concentrations of drug.<sup>60</sup> After incubation with drug, fresh media was replaced and cells were incubated for various intervals before fixation for enumeration of mitotic index. After fixation cell nuclei were stained with an intercalating fluorescent dye and mitotic figures were counted under a fluorescence microscope.<sup>42</sup>

## RESULTS

**G2 Checkpoint Response in Normal Human Fibroblasts.** G2 checkpoint response was quantified in human cells using two biological paradigms that monitor cellular movement from G2 into mitosis. In one, cells were treated with IR to damage DNA, or with ICRF-193 to block chromatid decatenation, and mitotic index was determined various times later. As shown in Figure 1A, IR produced a dose-dependent inhibition of mitosis in diploid human fibroblasts. Irradiation blocked cellular entry to mitosis without affecting exit, thereby emptying the mitotic compartment. When measured at 2 h post-treatment, irradiation with 1.5 Gy inhibited mitosis by greater than 99%. The 1.5 Gy dose of IR inactivates colony formation by diploid human fibroblasts by 63%.<sup>77</sup> A lower dose of 0.5 Gy induced a 70% inhibition of mitosis within 2 h (Figure 1B). After this lower dose the mitotic index recovered to control levels by 6 h after irradiation. Treatment of diploid human fibroblasts with ICRF-193 for 15 min also produced a concentration-dependent inhibition of mitosis seen 2 h after treatment, indicative of G2 delay (Fig. 1C). Low concentrations of 0.2-0.3 µM produced 70% inhibition of mitosis while 2 µM ICRF-193 induced >90% inhibition. The inhibition of mitosis by ICRF-193 treatment was reversible. While mitosis was inhibited by 80-90% at 2 h after treatment with 1 µM ICRF-193, by 6 h after drug treatment the mitotic index had recovered to or exceeded the control (Fig. 1D). The inhibition of mitosis seen in IR-treated human fibroblasts was associated with inhibition of MPF kinase activity.<sup>19,42</sup> Although an initial study suggested that ICRF-193 inhibited MPF kinase,<sup>60</sup> a more extensive analysis demonstrated that the ICRF-193-induced inhibition of mitosis was associated with exclusion of cyclin B1/Cdk1 complexes from the nucleus by the Crm1 nuclear exporter.<sup>14</sup>

A second method for assay of G2 checkpoint function used addition to culture medium of the spindle poisons, colcemid or nocodazole, to depolymerize microtubules and arrest mitotic cells at metaphase. The rate of accumulation of mitotic cells during incubation with a spindle poison was used as an index

of G2 checkpoint response. When tested with the NHF6 fibroblast strain, colcemid induced an accumulation of mitotic cells at an apparent rate of 2.5% per h (Fig. 2). Irradiation with 1.5 Gy immediately before addition of colcemid completely blocked the accumulation of mitotic cells. This dose of IR produced a complete emptying of the mitotic compartment 2-6 h post-irradiation in cells that were not incubated with colcemid (Fig. 2). These methods for quantification of mitotic delay were used to test the roles of ATM and p53 in the G2 delay induced by ICRF-193.

**ATM is Not Required for ICRF-193-Induced G2 Delay.** ATM is required for the G2 checkpoint response to IR-induced DNA damage,<sup>10,14,19</sup> apparently at an early step as ATM kinase is activated within 30 min after damaging DNA.<sup>32,33</sup> Figure 3 shows that incubation with nocodazole produced a continuous increase in mitotic SVAT4BI cells in the first five hours after adding drug. AT cells that had been irradiated with 4 Gy of IR displayed no reduction in accumulation in mitosis during incubation with the spindle poison, (Fig. 3A), consistent with their defect in G2 checkpoint function. Caffeine has long been known to override checkpoint-associated cell cycle delays (e.g., see ref 78), including ICRF-193-induced G2 delay,<sup>15</sup> and recent studies have demonstrated that the checkpoint kinases ATM and ATR are inhibited by millimolar concentrations of the drug.<sup>79</sup> Incubation of irradiated and unirradiated AT fibroblasts with 2 mM caffeine did not affect the rate of accumulation of mitotic cells in nocodazole (not shown). In contrast to their lack of response to IR, AT cells arrested growth in G2 when incubated with ICRF-193. Addition of 6 µM ICRF-193 with nocodazole blocked the accumulation of mitotic cells (Fig. 3B) and addition of 2 mM caffeine reversed the inhibition caused by ICRF-193.

A second set of experiments monitored the inhibition of mitosis 2 h after treatment with IR or ICRF-193 as done in Figure 1. The AT fibroblast strain AT191JE displayed the expected attenuation of response to IR, with 1 Gy inducing only 33-45% inhibition of mitosis. This dose of IR typically induced >90% inhibition of mitosis in normal human fibroblasts (Fig. 1). Treatment with 2 or 6 µM ICRF-193 produced an inhibition of mitosis in the AT cells (92-94%) that was equivalent to that seen in the NHF1 normal fibroblast strain run in parallel (87-100%). ICRF-193-induced mitotic inhibition in NHF1 and AT191JE cells was also reversed by post-treatment incubation with caffeine. Thus, mutations in ATM that interfere with DNA damage-induced G2 checkpoint function did not affect the G2 delay that was induced by blocking decatenation. This delay nevertheless was susceptible to override by caffeine.

**p53 is Not Required for ICRF-193-Induced G2 Delay But the Response is Attenuated as E6-Expressing Cells Age.** The role of p53 in ICRF-193-induced G2 delay was tested using diploid fibroblast strains that had been transduced with HPV16E6 to inactivate p53.<sup>42,80</sup> As shown in Figure 4A, NHF4-neo fibroblasts displayed inhibition and recovery of mitosis 2-6 h after 0.5 Gy of IR. NHF4-E6 fibroblasts expressing HPV16E6 to inactivate p53 also displayed inhibition and recovery of mitosis after  $\gamma$ -irradiation when at low population doubling levels (PDL<20). However, as E6-expressing NHF4 fibroblasts were aged through their *in vitro* proliferative lifespan, G2 damage-related checkpoint function was attenuated and lost. When tested at PDL 46, the NHF4-E6 cells failed to delay mitosis after irradiation with 0.5 Gy (Figure 4A). This biological model system, in which human fibroblasts expressing HPV16E6 spontaneously lose G2 checkpoint function, was employed to study the contribution of p53 to the G2 delay that occurs after inhibition of chromatid decatenation by ICRF-193.

A 15 min incubation with ICRF-193 produced a strong mitotic inhibition in fibroblasts with normal G2 checkpoint function. Treatment with 2 µM ICRF-193 produced 98% inhibition of mitosis in the NHF5-neo line (Table 1). Inactivation of p53 with HPV16E6 did not influence ICRF-193-induced G2 delay when cells were at low PDLs. Mitosis was inhibited 95% by ICRF-193 in NHF5-E6 cells at low PDLs, when DNA damage G2 checkpoint function was also intact. However, after NHF5-E6 cells had aged *in vitro* and attenuated DNA damage G2 checkpoint function, they also became resistant to ICRF-193-induced G2 delay. Indeed, mitotic indices in ICRF-193-treated NHF5-E6 cells at high PDL exceeded the indices seen in DMSO-treated control cells. This was likely due to the drug treatment slowing movement through mitosis of cells that were unable to

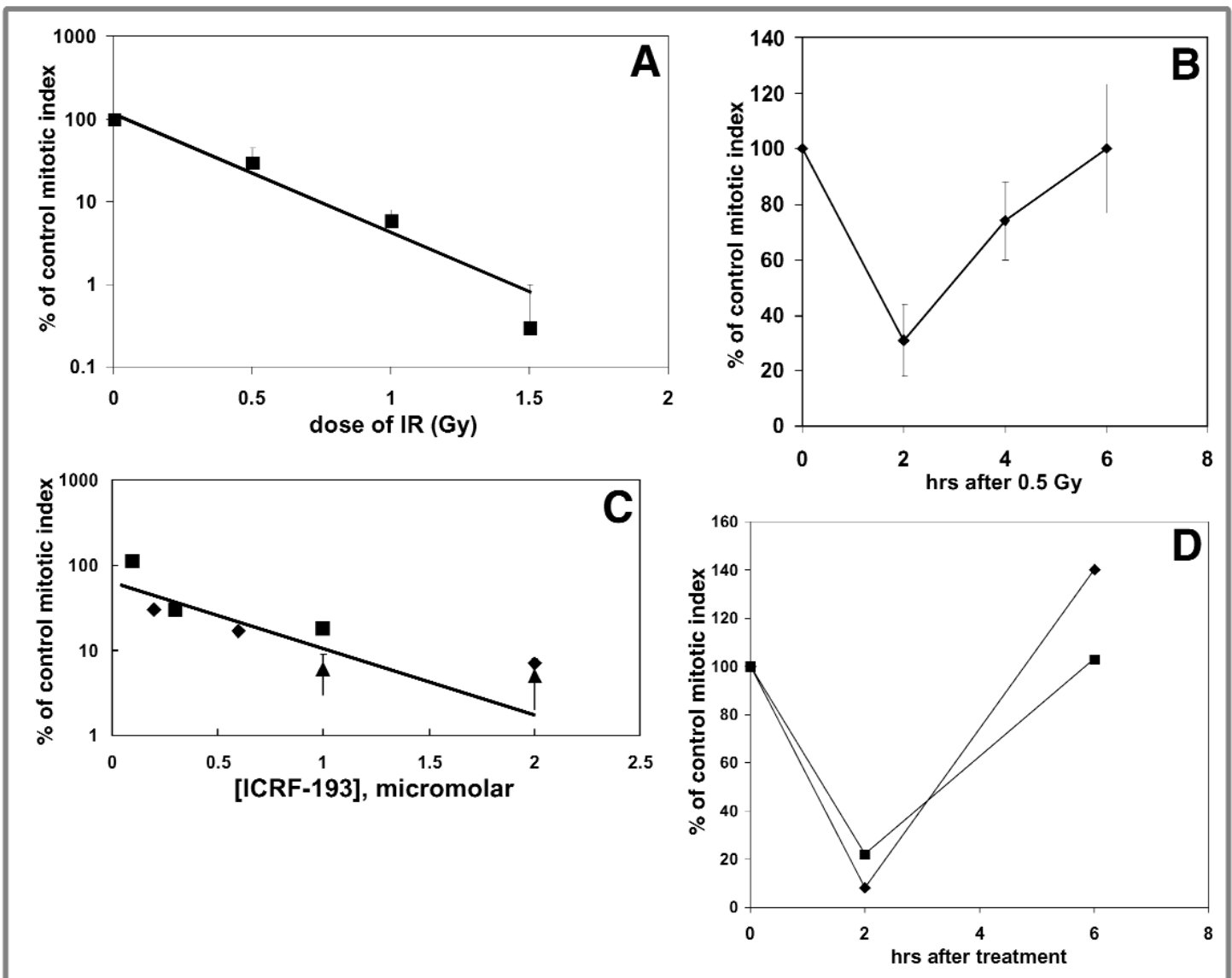


Figure 1. IR and ICRF-193 induce reversible G2 delay in diploid human fibroblasts. A. Replicate cultures of NHF1 foreskin fibroblasts were sham-treated or irradiated with various doses of IR. Mitotic indices were determined 2 h after irradiation or sham-treatment and expressed as a percentage of the sham-treated control. (mean±sd, n=5-8). B. Mitotic inhibition in NHF1 fibroblasts at various times after treatment with 0.5 Gy (mean±sd, n=3). C. Various foreskin fibroblast cultures were treated for 15 min with various concentrations of ICRF-193, then incubated for 2 h before determination of mitotic index. Mitotic indices in drug-treated cells were expressed as a percentage of the solvent (DMSO)-treated control (symbols depict results obtained with different fibroblast strains (diamonds, NHF4-neo; squares, NHF5-neo; triangles, NHF7-neo with error bars enclosing one sd of the mean, n=3). D. NHF7-neo (diamonds) and NHF7-E6 (squares) fibroblasts at population doubling levels <10 were treated for 15 min with 1  $\mu$ M ICRF-193 or DMSO solvent, then incubated for 2 or 6 h before determination of mitotic index in 2000 cells.

delay in G2.<sup>70</sup> A longer residence time in mitosis will increase mitotic index. Essentially the same results were obtained with the NHF7 line of fibroblasts although in these cells a lower concentration of 1  $\mu$ M ICRF-193 was used. This concentration induced 95% and 81% inhibition of mitosis, respectively, in NHF7-neo and NHF7-E6 cells at low PDL, but only 26% inhibition in NHF7-E6 cells at high PDL. The NHF4-E6 line was tested against a range of concentrations of ICRF-193 at high PDL when IR-induced G2 checkpoint response was attenuated (Fig. 4A). Exposure to 0.2-2  $\mu$ M ICRF-193 had no effect on mitotic index under conditions in which mitosis in NHF4-neo cells was inhibited by 70-90% (Fig. 4B). In three different lines of E6-expressing skin fibroblasts, attenuation of DNA damage G2 checkpoint function acquired during cellular aging was associated with attenuation of the G2 delay that occurs when chromatid decatenation is blocked with ICRF-193.

The loss of responsiveness to ICRF-193 in E6-expressing cells at high PDLs could be due to inactivation of a checkpoint signaling pathway other

than the p53 and ATM pathways, or it could be a trivial consequence of reduced drug uptake or loss of topoisomerase II function. To test for expression of topoisomerase in the E6-expressing fibroblasts at high PDLs, NHF5-E6 cells were treated with etoposide to freeze topoisomerase-II-cleaved complexes and produce DNA dsb.<sup>60,63</sup> Cytogenetic analysis 2 h after treatment revealed the presence of a high level of chromatid damage in etoposide-treated cells (1.9 chromatid breaks per metaphase, n=23). Solvent-treated controls displayed 0.2 chromatid breaks per metaphase (n=75). The NHF5-E6 fibroblasts at high PDL therefore expressed topoisomerase II in G2, indicating that the resistance to ICRF-193-induced G2 delay was not due to loss of topoisomerase II. Moreover, cytogenetic analysis of ICRF-193-treated E6-expressing cells demonstrated the presence of improperly condensed, entangled chromatids in metaphase spreads (not shown) as commonly observed in ICRF-193-treated mammalian cells.<sup>15,70,75</sup> Evidently, ICRF-193 reached its nuclear target, topoisomerase II, and

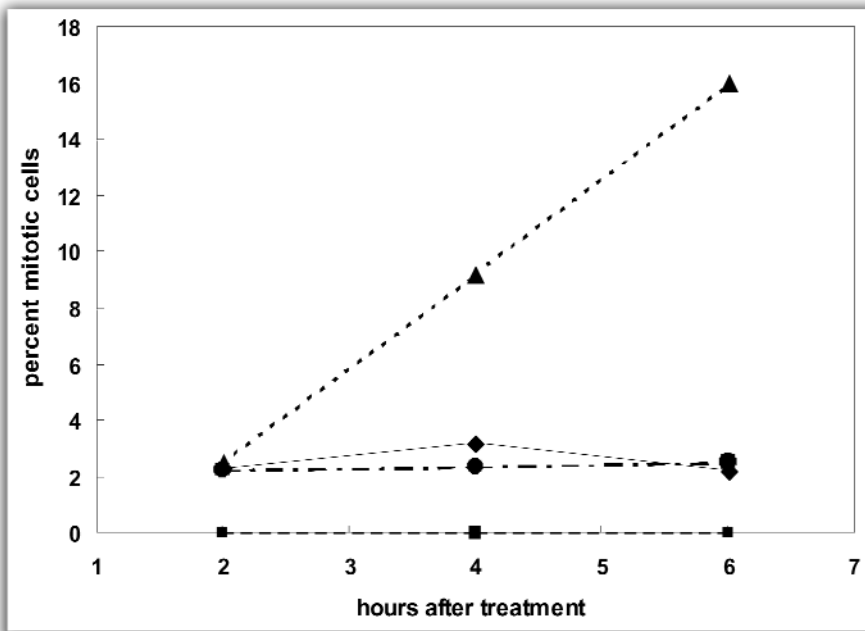


Figure 2. IR-induced G2 delay is associated with reduced entry to mitosis. Replicate cultures of NHF6 fibroblasts were sham-treated (diamonds) or irradiated with 1.5 Gy IR (squares) at various times before harvest to demonstrate IR-induced mitotic inhibition. Additional replicate cultures were incubated with 100 ng/ml of colcemid for various intervals beginning immediately after sham-treatment (triangles) or irradiation with 1.5 Gy (circles).

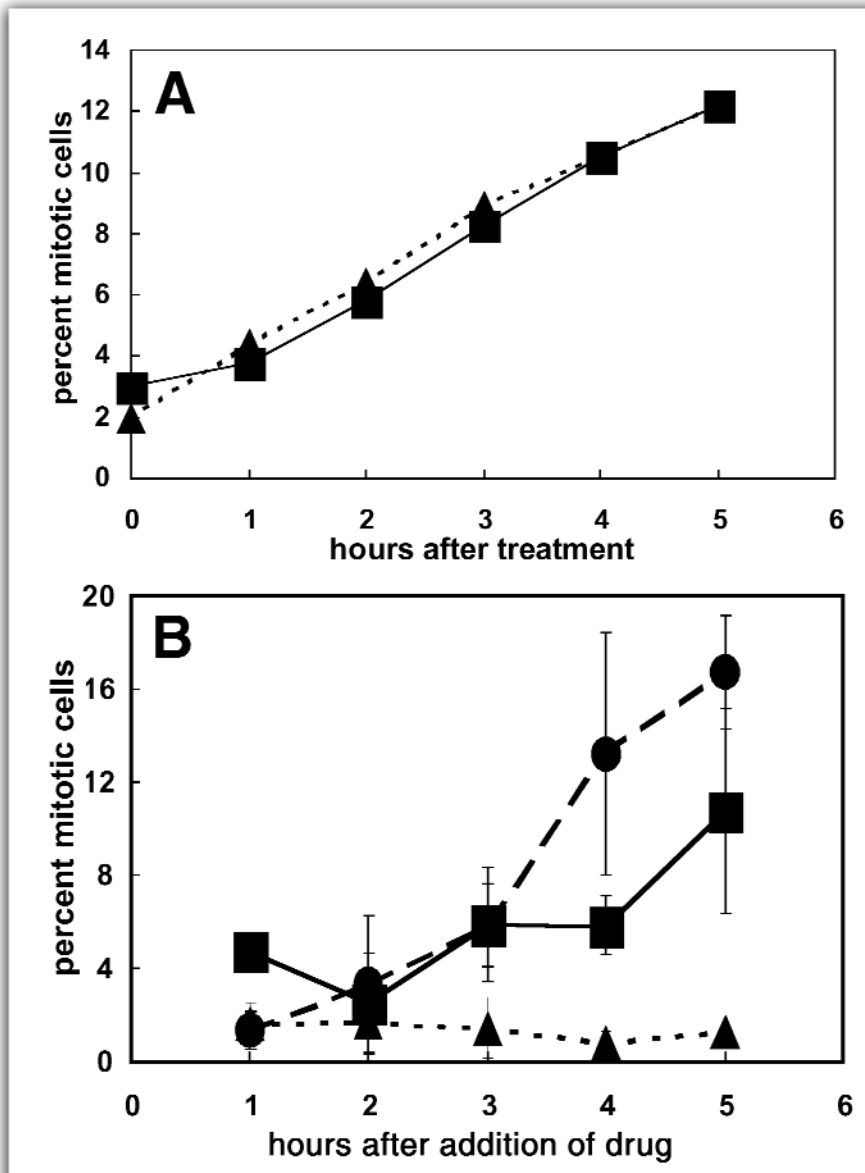


Figure 3. AT cells express an ICRF-193-induced G2 delay that is suppressed by caffeine. A. Mitotic indices were determined in cultures of SVAT4BI fibroblasts that were incubated for 1-5 h with 40 ng/ml nocodazole immediately after sham treatment (squares) or exposure to 4 Gy IR (triangles). B. SVAT4BI fibroblasts were incubated with 40 ng/ml nocodazole alone for 1-5 h (squares), nocodazole plus 6  $\mu$ M ICRF-193 (triangles), or nocodazole plus ICRF-193 and 2 mM caffeine (circles). Cells were fixed and mitotic index determined (mean+sd, n=5).

blocked chromatid decatenation in the E6-expressing cells at high PDL.

**Induction of Telomerase Sustains G2 Checkpoint Function and Preserves Chromosomal Stability in Aging E6-Expressing Fibroblasts.** Inactivation of G2 checkpoint response to IR in the E6-expressing fibroblasts at high PDL has been correlated with telomere erosion. To suppress this erosion the human telomerase reverse transcriptase gene (*hTERT*) was transfected into the NHF7-E6 line at PDL 20 to induce telomerase activity (Fig 4C). Controls were transfected with the *hTERT* cDNA in the antisense orientation. Telomerase-positive NHF7-E6 fibroblasts lacked G1 checkpoint function due to inactivation of p53 (not shown) but retained the stringent G2 delay response to IR and ICRF-193 after outgrowth to high PDL (Fig. 4D). The telomerase-negative NHF7-E6 fibroblasts at high PDL displayed severely attenuated response to IR and ICRF-193, as expected. Thus, the aging-related loss of G2 checkpoint response to IR in E6-expressing fibroblasts was associated with loss of G2 delay in response to ICRF-193, and expression of telomerase in E6-expressing cells preserved G2 checkpoint response both to DNA damage and to blocked decatenation.

E6-expressing fibroblasts at low PDL displayed a diploid genome without structural damage to chromosomes (Table 2). However, during in vitro aging these cells acquired both numerical and structural chromosomal aberrations. By PDL 58, the NHF7-E6 line displayed significantly increased frequencies of dicentrics/rings,

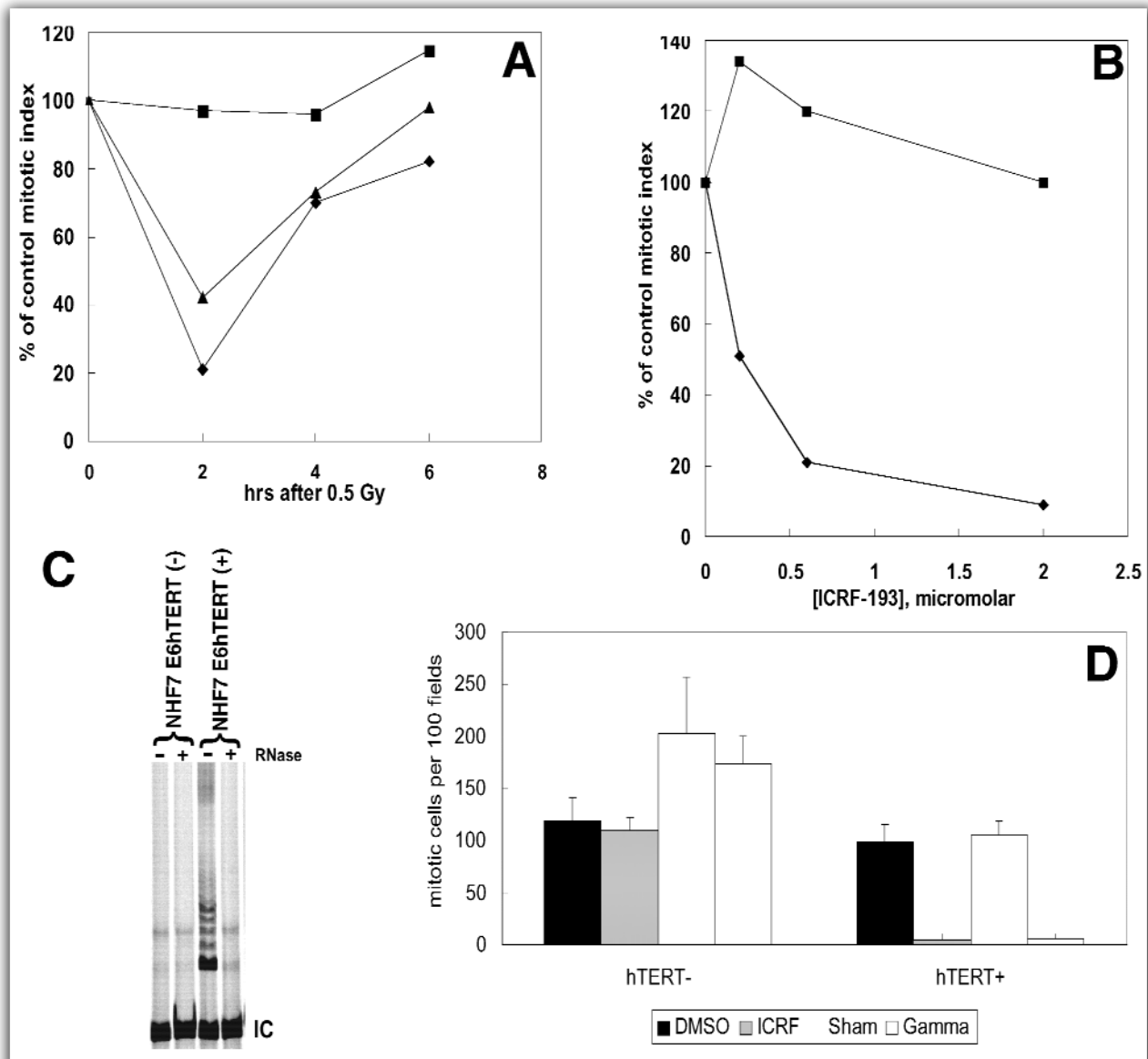


Figure 4. Attenuation of ICRF-193-induced G2 delay in E6-expressing fibroblasts is suppressed by hTERT. A. IR-induced G2 delay in E6-expressing fibroblasts at low and high PDLs. Fibroblasts were irradiated with 0.5 Gy or sham-treated, then harvested for determination of mitotic index 2-6 h later. Results are expressed as a percentage of the sham-treated control. diamonds, NHF4-neo fibroblasts at PDL<20; triangles, NHF4-E6 fibroblasts at PDL<20; squares, NHF4-E6 fibroblasts at PDL 46. B. NHF4-neo (diamonds) and NHF4-E6 (squares) fibroblasts at PDL >50 were treated for 15 min with DMSO solvent or various concentrations of ICRF-193, then incubated for 2 h before determination of mitotic index. C. The NHF7-E6 fibroblast line (Table 1) was transduced with hTERT cDNA in sense (hTERT+) and antisense (hTERT-) orientation at PDL 20. After selection for puromycin-resistant transformants, cells were aged to PDL 60 when they were assayed for expression of telomerase by the telomere repeat amplification protocol. The polymerase chain reaction internal control is designated IC. D. NHF7-E6 fibroblasts with hTERT cDNA in sense (hTERT+) and antisense (hTERT-) orientation were assayed for IR- and ICRF-193-induced mitotic inhibition when at PDL 58. ICRF-193 was tested at 2  $\mu$ M. Results depict the number of mitotic cells in 100 high-power fields (mean+sd, n=3). DMSO (black bars), ICRF-193 (gray bars), sham (checked bars), 1.5 Gy IR (white bars).

chromatid breaks/exchanges, and polyploidy. Transfection of *hTERT* into the NHF7-E6 fibroblast line at PDL 20 preserved chromosomal stability through PDL 58. E6-expressing fibroblasts transfected with the antisense *hTERT* construct displayed severe chromosomal destabilization after the same number of population doublings. Expression of telomerase not only sustained G2 checkpoint function in E6-expressing fibroblasts, it prevented the severe chromosomal destabilization associated with cellular aging.

## DISCUSSION

**ICRF-193-Induced G2 Delay is a Checkpoint Response to Blocked Decatenation.** Human cells actively suppress movement from G2 to mitosis when chromatids are insufficiently decatenated. A brief 15 min interval of inhibition of topoisomerase II was sufficient to induce 95% inhibition of mitosis seen 2 h later. Evidence that ICRF-193-induced G2 delay is an active response includes attenuation of ICRF-193-induced mitotic delay with caffeine (Fig. 3), and with the serine/threonine kinase inhibitor

**Table 1 ICRF-193-INDUCED G2 DELAY IS ATTENUATED IN E6-EXPRESSING CELLS WITH DEFECTIVE DNA DAMAGE G2 CHECKPOINT FUNCTION**

		DNA damage-IR/Sham <sup>a</sup>		Decatenation- ICRF/DMSO <sup>b</sup>	
		Neo	E6	Neo	E6
<b>NHF5</b>	Low PDL <sup>c</sup>	17/140	34/247	2/82	7/129
		12%	14%	2%	5%
	High PDL <sup>c</sup>	senesced	63/155*	senesced	187/105*
			41%		178%
<b>NHF7</b>	Low PDL	70/1000	500/2988	26/517	448/2403
		7%	17%	5%	19%
	High PDL	senesced	118/144*	senesced	38/51*
			82%		74%

<sup>a</sup>DNA damage-induced G2 checkpoint function was assayed by quantifying mitotic cells 2 h after sham treatment or irradiation with 1.5 Gy (IR). The numbers of mitotic cells seen in equal numbers of cells or microscope fields are expressed as a ratio (IR/Sham) which represents the percentage (%) of G2 cells evading DNA damage-induced G2 delay

<sup>b</sup>Decatenation-associated mitotic delay was assayed by quantifying mitotic cells 2 h after treatment with DMSO solvent or ICRF-193. NHF5 cells were treated with 2 μM ICRF-193, NHF7 cells were treated with 1 mM ICRF-193. The ratio of mitotic counts (ICRF/DMSO) is a measure of the percentage of G2 cells evading drug-induced G2 delay.

<sup>c</sup>Low PDL, <60; high PDL, >60. \*Significantly different from E6 cells at low PDL (P<0.05, X<sup>2</sup>).

6-dimethylaminopurine and the protein phosphatase inhibitor okadaic acid. The attenuation of the G2 delay response to ICRF-193 in E6-expressing fibroblasts at high PDL (Fig. 4) also suggests that deterioration of this regulatory pathway may contribute to chromosomal instability (Table 2). ATM and p53 do not appear to be required for the immediate G2 delay when chromatid decatenation is blocked.

ATM is required for IR-induced DNA damage checkpoints acting in G1, S and G2. In the G1 checkpoint signaling pathway ATM phosphorylates and activates Cds1 and p53 leading to synthesis of p21<sup>Waf1</sup> and inhibition of G1 cyclin/Cdk's.<sup>81</sup> The targets of ATM at the S and G2 checkpoints are not fully known although Chk1, Cds1, and Plk-1 are likely effectors of S and G2 checkpoint response acting through phosphorylation of Nbs1,<sup>49</sup> hRad17,<sup>5</sup> BRCA1<sup>28,31</sup> and Cdc25C.<sup>82</sup> ATM, but not p53, is also required for the rapid inhibition of cyclin A/Cdk2 or cyclin B1/Cdk1 kinase activities in cells damaged by IR.<sup>19,83</sup>

AT cells displayed normal sensitivity to ICRF-193-induced G2 delay, implying that ATM does not contribute to signaling when

chromatid decatenation is blocked. The reversal by caffeine of the ICRF-193-induced G2 delay in AT cells suggests that another caffeine-sensitive checkpoint effector is required for signaling from sites of catenated chromatids. The ATM-related kinase, DNA-PK, which is required for repair of DNA dsb does not appear to be involved in the response to ICRF-193<sup>75</sup> and it is resistant to caffeine.<sup>79</sup> The ATM- and rad3-related caffeine-sensitive kinase, ATR, may enforce the catenation-sensitive G2 checkpoint. Expression of dominant kinase-inactive *ATR* alleles has been shown to disrupt cell cycle delays in response to DNA damage<sup>6,8</sup> thereby indicating that ATR can interact with checkpoint signaling pathways. Expression of the same kinase-inactive *ATR* allele ablated the G2 delay response to ICRF-193.<sup>14</sup> Moreover, breast cancer cells with inactivating mutations in *BRCA1* displayed little G2 delay when treated with ICRF-193, while restoration of expression of wildtype *BRCA1* restored the G2 delay response to ICRF-193. Thus, *ATR* and *BRCA1* appear to enforce the decatenation

checkpoint, acting at the level of cyclin B1 exclusion. Plant cells also delay entry to mitosis when treated with ICRF-193, apparently by the same mechanism of nuclear exclusion of cyclin B.<sup>84</sup>

The inactivation of G2 checkpoint function that accompanies cellular aging in the HPV16E6-expressing fibroblasts appears to be a response to chromatid damage associated with telomere erosion and formation of unstable dicentric chromosomes.<sup>80,85</sup> Cells lacking G2 checkpoint function gain a growth advantage by being able to progress into mitosis with damaged chromatids. Induction of telomerase before E6-expressing cells acquired chromosomal instability preserved G2 checkpoint function and stabilized chromosomes (Table 2). The severe inhibition of mitosis seen in ICRF-193- and IR-treated hTERT+ fibroblasts that were expressing HPV16E6 emphasizes the dominance of p53-independent elements of the DNA damage and decatenation G2 checkpoints.<sup>24,86</sup>

**Attenuation of Decatenation Checkpoint Function in E6-Expressing Fibroblasts May Contribute to Chromosome Instability.** Expression of HPV16E6 oncoprotein in diploid human fibroblasts ablates p53 function but does not induce polyploidy.<sup>42,80</sup>

**Table 2 AGING-ASSOCIATED CHROMOSOMAL ABERRATIONS, POLYPOIDY, AND INACTIVATION OF G2 CHECKPOINT FUNCTION ARE SUPPRESSED IN E6-EXPRESSING FIBROBLASTS BY EXPRESSION OF TELOMERASE**

Cell Strain	PDL	Aberrations per 50 cells		Percentage of cells	
		Dicentrics/Ring	Breaks/Exchanges	Polyploidy	Evading G2 Delay
NHF7-neo	10	0	2	8	7 ± 5 (n = 3)
	30	0	2	4	0, 2
	10	0	1	2	17 ± 5 (n = 3)
NHF7-E6	30	19	6	4	22, 48
	58	25	38	33	133 ± 66 (n = 3)
NHF7-E6-hTERT(-)	58	40	18	36	88 ± 11 (n = 4)
NHF7-E6-hTERT(+)	58	0	2	3	9 ± 7 (n = 4)

Cells in logarithmic growth phase were incubated with 50 ng/ml colcemid for 30 min before cell harvest and standard processing for cytogenetic analysis. Giemsa-stained metaphase spreads (n = 50) were examined for structural aberrations (dicentrics, rings, breaks and exchanges) and polyploidy (>77 chromosomes). G2 checkpoint function was assayed by quantifying the number of cells in mitosis 2 h after 1.5 Gy of IR, expressed as a percentage of the sham-treated control. At least 2000 cells were counted per determination. Mean ± s.d. (n = number of determinations)

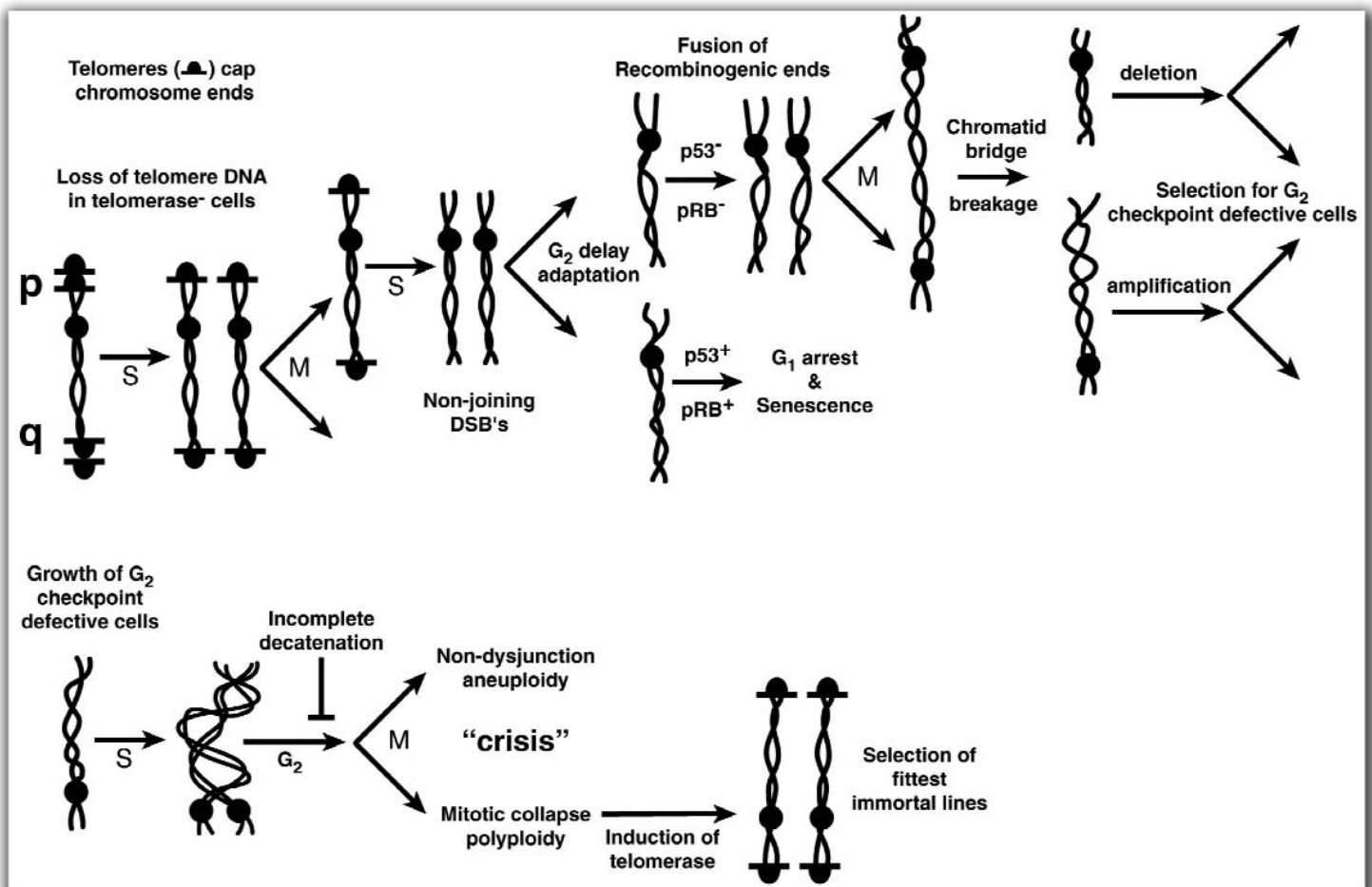


Figure 5. A model depicting steps of chromosomal destabilization in E6-expressing human fibroblasts. As cells that lack expression of telomerase divide, a bit of telomeric DNA is lost every division cycle due to the end-replication problem. Telomere erosion ultimately reaches a limit when the p53-dependent G1 checkpoint is activated to induce replicative senescence. Cells that have lost the function of p53 due to expression of HPV16E6 are unable to arrest growth and display a phenotype of extended proliferative lifespan. If cell division continues, telomeres erode further, producing unstable chromatid termini that may associate and fuse to produce dicentric chromosomes. Dicentric chromosomes are unstable and may be broken at anaphase. As the G2 checkpoint appears to block the entry to mitosis of cells with broken chromosomes, cells with extended lifespan and reduced G2 checkpoint function have a growth advantage. Cells that have lost G1 and G2 checkpoint functions are permitted to divide with broken and unstable chromosomes. These cells also are less responsive to the state of chromatid catenation and enter mitosis before chromatids have been adequately decatenated. This causes nondisjunction errors or mitotic failure producing aneuploid and polyploid cells, respectively. The severe chromosomal destabilization seen in E6-expressing cells at crisis probably accounts for their death. A rare clone emerges from crisis that has stabilized telomeres usually by expression of telomerase. Expression of telomerase to stabilize telomeres breaks the cycle of chromatid fusion and breakage, and selective pressures favor outgrowth of clones with stabilized genomes. In this model the G2 checkpoint is seen as a barrier to carcinogenesis that cooperates with the p53-dependent G1 checkpoint to suppress growth and stabilize the genome.

Although the E6-expressing cells are susceptible to endoreduplication when stressed with the spindle poisons colcemid and nocodazole<sup>80,87</sup> they spontaneously acquire polyploidy only after in vitro aging associated with attenuation and inactivation of DNA damage-responsive G2 checkpoint function.<sup>42,80</sup> This result suggested that reduced G2 checkpoint function in E6-expressing cells might produce stress on the mitotic spindle and thereby stimulate endoreduplication. The results described above suggested a model that explains how G2 checkpoint function limits stress on the mitotic spindle (Fig. 5). Inactivation of G2 checkpoint function in E6-expressing cells is posited to be the result of a selective process, whereby damaged chromosomes resulting from telomere crisis<sup>80,85</sup> cause cells with intact G2 checkpoint function to slow growth. Cells with reduced G2 checkpoint function have a growth advantage and are permitted to enter mitosis with damaged chromosomes.<sup>19,42</sup> These cells with reduced G2 checkpoint function also will tend to enter mitosis before chromatids have been sufficiently decatenated. If one

chromatid pair is affected, the excess of chromatid linkages may cause nondisjunction, if the spindle breaks, or chromatid breakage. Both of these effects have been observed in immortalized human Msu1.1 fibroblasts treated with very low concentrations of ICRF-193.<sup>88</sup> If many chromatids are affected, mitosis may collapse altogether with restitution of interphase nuclear structure without chromatid segregation.<sup>70,72</sup> E6-expressing cells that lack p53 are permitted to initiate DNA synthesis from this restitution G1, thereby undergoing polyploidization. It is noticeable that this work shows ATM not to be needed for a functional decatenation checkpoint. Cells defective in ATM have abnormally high numbers of chromosome aberrations<sup>89</sup> but do not form polyploid nuclei. As the G2 checkpoint system appears to monitor both catenated and damaged chromatids, its attenuation in E6-expressing fibroblasts may enhance instabilities of chromosome structure and number.

The biology of the chromatid decatenation checkpoint thus suggests an additional mechanism for genetic instability in cancer

and its precursors. Attenuation or inactivation of the G2 checkpoint reduces cellular sensitivity to the state of decatenation following DNA replication and prior to mitosis. We propose that one consequence of reduced decatenation checkpoint function is entry to mitosis before chromatids have achieved sufficient decatenation. Defects in chromatid catenation G2 checkpoint function may contribute to the aneuploidy and polyploidy that typify p53-defective human fibroblasts,<sup>42,80,90</sup> especially in the phase of crisis prior to induction of telomerase.

#### Acknowledgements

This work was supported by PHS grants CA42765, CA81343 and P30-ES10126 (WKK), training grants T32-ES07126 (DAS) and T32-ES07017 (DAS,PBD), and by the Ulster Cancer Foundation. We are grateful to John Hurt and Paula Kies who also contributed to these studies, and to Drs. Geoffrey Wahl and Marila Cordeiro-Stone for critical reading of the manuscript.

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