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DNA Damage during Mitosis in Human Cells Delays the Metaphase/Anaphase Transition via the Spindle-Assembly Checkpoint

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Summary

Background: DNA damage during mitosis triggers an ATM kinase-mediated cell cycle checkpoint pathway in yeast and fly embryos that delays progression through division. Recent data suggest that this is also true for mammals. Here we used laser microsurgery and inhibitors of topoisomerase IIα to break DNA in various mammalian cells after they became committed to mitosis. We then followed the fate of these cells and emphasized the timing of mitotic progression, spindle structure, and chromosome behavior.

Results: We find that DNA breaks generated during late prophase do not impede entry into prometaphase. If the damage is minor, cells complete mitosis on time. However, more significant damage substantially delays exit from mitosis in many cell types. In human (HeLa, CFPAC-1, and hTERT-RPE) cells, this delay occurs during metaphase, after the formation of a bipolar spindle and the destruction of cyclin A, and it is not dependent on a functional p53 pathway. Pretreating cells with ATM kinase inhibitors does not abrogate the metaphase delay due to chromosome damage. Immunofluorescence studies reveal that cells blocked in metaphase by chromosome damage contain one or more Mad2-positive kinetochores, and the block is rapidly overridden when the cells are microinjected with a dominant-negative construct of Mad2 (Mad2ΔC).

Conclusions: We conclude that the delay in mitosis induced by DNA damage is not due to an ATM-mediated DNA damage checkpoint pathway. Rather, the damage leads to defects in kinetochore attachment and function that, in turn, maintain the intrinsic Mad-2-based spindle assembly checkpoint.

Introduction

DNA damage during G_2 activates a checkpoint that delays entry into mitosis by using the ATM kinase to block activation of cyclin B/CDK1 [1]. In vertebrates two signal-transduction pathways that link ATM activation to cyclin B/CDK1 inhibition are normally present during G_2. One delays CDK1 activation by using p53 to induce the formation of P21, a potent CDK1 inhibitor. The other activates the Chk1/Chk2 kinases, which, in turn, prevent the Cdc25C phosphatase from activating CDK1 [2]. Because both pathways rely on the ATM kinase, inhibiting this enzyme with, for example, caffeine [3] or wortmannin [4] overrides the checkpoint and allows cells with damaged DNA to enter mitosis.

In vertebrates the DNA damage checkpoint remains functional during the terminal phases of G_2, which include the early stages of chromosome condensation [5]. However, by late prophase cells are committed to mitosis, even if their chromosomes are subsequently damaged (reviewed in [6]). When whole cells are irradiated with UV light after committing to division, spindle formation is disrupted and mitotic progression is arrested [7, 8]. Under these circumstances the arrest can be attributed to the kinetochore-based Mad2-mediated spindle-assembly checkpoint, which operates whenever spindle microtubule (Mt) assembly is perturbed (reviewed in [9]). In contrast to whole-cell UV irradiation, when rat kangaroo (PtK_1) or newt lung cells are selectively irradiated in the nucleus during late prophase, the chromosomes are damaged but the cells complete mitosis with normal kinetics [5, 10, 11]. These studies imply that the G_2 DNA damage checkpoints stop functioning in vertebrates once the cell is committed to mitosis [6].

Recently, Smits and coworkers [12] reported that damaging the DNA in U2OS cells that are blocked in mitosis with nocodazole significantly delays exit from mitosis upon removal of the block. This suggests that, as in yeast [13, 14], "a DNA damage checkpoint must also be active in mitosis in mammalian cells" [12]. Subsequently, Su and Jaklevic [15] noted that DNA damage in *Drosophila* gastrula delays anaphase onset 3-fold, even though the cells formed normal-looking spindles. A mechanism for this delay was suggested by the finding that it coincides with the stabilization of cyclin A, the destruction of which is required for anaphase onset [16].

The notion that a DNA damage checkpoint exists during mitosis in vertebrate cells conflicts with reports that chromosome fragments do not impede mitotic progression in PtK_1 and newts (see above). One possibility is that these latter cells lack a DNA damage checkpoint during mitosis. In this regard, although damaging DNA during mitosis by inhibiting topoisomerase II delays the onset of anaphase in HeLa cells [17, 18], it does not affect mitotic progression in PtK_1 [19] or CHO cells [17].

In those vertebrate cells (e.g., U2OS, HeLa) in which chromosome damage delays exit from mitosis, the mechanism behind the delay remains vague. One idea is that DNA damage prevents activation of the anaphase-promoting complexes (APCs) by, for example, down-regulating centrosome function [20] and/or polo-kinase (Plk1) activity [12]. Another is that the damage prevents cyclin A degradation (15), which is required for the meta-

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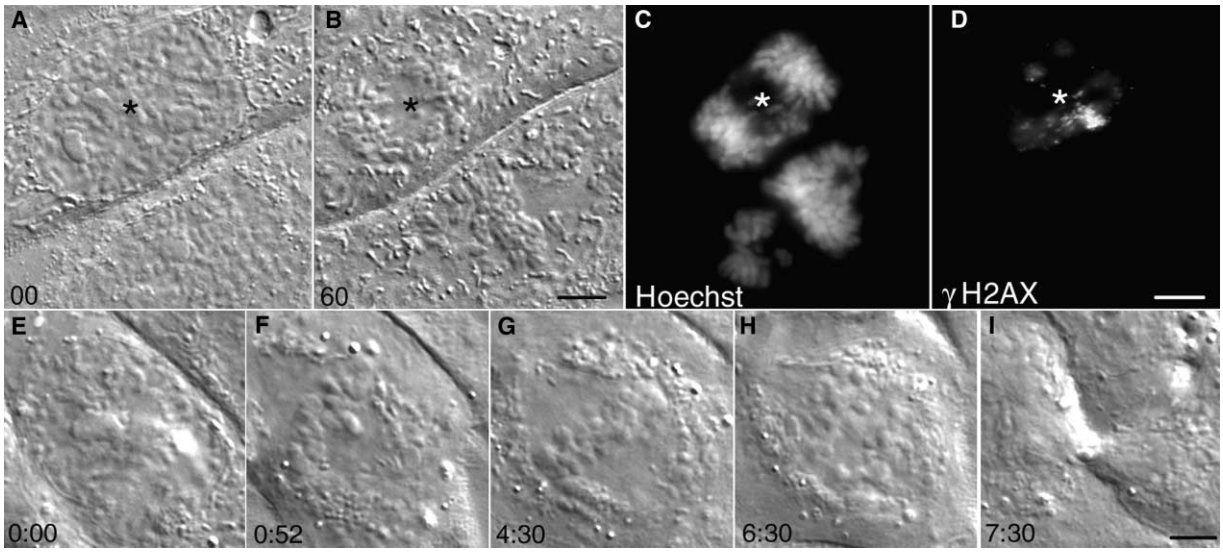

Figure 1. Minor Amounts of DNA Damage Do Not Impede Progression through Mitosis, Whereas Extensive Damage Significantly Delays Anaphase Onset

(A–D) A late-prophase CFPAC-1 cell was irradiated in the nucleus ([A], asterisk) with 20 laser pulses. It then entered anaphase (B) with kinetics comparable to that of nonirradiated controls, after which it was fixed and processed for the IMF localization of γ-H2AX (D) prior to being stained with Hoechst 33342 (C). Note that the irradiated cell shows significant chromosome damage, whereas the adjacent prometaphase cell shows no γ-H2AX staining. (E–I) This CFPAC-1 cell was irradiated across the nucleus during late prophase with 400 pulses of laser light (E). It then proceeded with normal kinetics into prometaphase, during which it rapidly formed a normal-looking spindle (F), but it was then blocked in metaphase for more than five hours (G) prior to entering anaphase (H) and attempting cytokinesis (I). Time is given in hr:min in the lower left-hand corner of each frame. The scale bars represent 10 μm.

phase/anaphase transition [16]. A third possibility is that chromosome damage does not prolong mitosis via a DNA damage checkpoint, but instead through the spindle assembly checkpoint.

To distinguish between these possibilities, we used pulses of green (532 nm) laser light, or the topoisomerase IIα inhibitors adriamycin or ICRF-193, to induce DNA damage in various types of mammalian cells after they had become committed to mitosis. The results of our live-cell investigations reveal that mitosis is prolonged by extensive—but not by minor—damage to the chromosomes. This delay occurs because kinetochores regulate exit from mitosis, via the Mad2 pathway, and extensive damage to the DNA eventually leads to problems in kinetochore function.

Results

At the level of the specimen, each ns pulse from our laser contains approximately 400 nJ of energy [21] and induces multiple DNA double-stranded breaks (DSBs). The induction of DSBs results in the rapid phosphorylation of serine 139 on histone H2AX. This in turn forms γ-H2AX complexes [22], which are readily detected by antibodies [23]. We therefore used the formation of this complex as a reliable immunofluorescence (IMF) assay for the presence of DSBs. With this assay we found that 10–20 laser pulses placed randomly in a prophase nucleus always produced multiple detectable DSBs and chromosome fragments (Figures 1A–1D).

Extensive Radiation-Induced Chromosome Damage Prolongs Mitosis in Human Cells

When late-prophase PtK_1 cells are irradiated randomly in the nucleus with 400 laser pulses, they enter prometaphase and complete mitosis with normal kinetics ([5]; Table 1). However, PtK_1 cells are derived from a marsupial and may respond differently than placental mammalian cells to DNA damage in mitosis [e.g., 19, 24]. To explore this possibility, we repeated our laser studies on CFPAC-1 (human ductal adenocarcinoma; p53−) cells, which, like PtK_1, remain flat during mitosis. At 34°C, mitosis in CFPAC1 cells takes 60 min (Table 1). When we randomly irradiated multiple regions of late prophase nuclei in CFPAC1, hTERT-RPE (human telomerase immortalized retinal pigment epithelium; P53+), or HeLa (P53−) cells with 10–20 laser pulses, the cells proceeded into and completed mitosis with normal kinetics (Table 1), even though they contained γ-H2AX complexes (DSBs) and broken chromosomes (Figures 1A–1D). By contrast, when we placed 200–400 pulses randomly into the nuclei of late prophase CFPAC-1 cells, the duration of mitosis was prolonged more than 5-fold relative to controls (Table 1; Figures 1E–1I). Interestingly, when late-prophase cells were irradiated with 400 pulses only in the nucleolus, the duration of mitosis was significantly shorter (103 ± 13 min; N = 11) than when the radiation was distributed randomly throughout the nucleoplasm (332 ± 36 min). Control cytoplasmic irradiations (200–400 pulses) during late prophase did not delay entry into, or exit from, mitosis in CFPAC-1 cells (68 ± 7 min, N = 12). Similar results were obtained when late prophase nuclei in HeLa and hTERT-RPE1, and to a lesser extent CV1 (green monkey kidney; P53+) and Cos7 (green monkey kidney; P53−) cells, were randomly irradiated with 200–400 pulses of laser light (Table 1).

From these studies we conclude that extensive photodamage to the chromosomes during late prophase significantly prolongs the division process in many mam-

Table 1. Duration of Mitosis

Cell Type	Control	10–20 Pulses[a]	200–400 Pulses[a]	Adriamycin (1 μM[b])	ICRF-193 (4 μM[b])
PtK1	50 ± 2, N = 7		52 ± 4, N = 15	47 ± 19, N = 4	66 ± 6, N = 9
hTERT-RPE1	45 ± 5, N = 6	50 ± 8, N = 5	273 ± 60, N = 6	38 ± 7, N = 4	68 ±15, N = 5
CFPAC1	60 ± 7, N = 9	53 ± 5, N = 6	332 ± 36, N = 31	58 ± 5, N = 4	180 ±35, N = 10
Hela	46 ± 6, N = 8	64 ± 4, N = 7	342 ± 50, N = 5	55 ± 8, N = 4	198 ± 56, N = 7
U2OS	43 ± 14, N = 5			52 ± 8, N = 4	145 ± 30, N = 5
CV1	43 ± 3, N = 8		73 ± 3, N = 8		
COS7	50 ± 7, N = 7		150 ± 6, N = 7		

Mitosis is defined as nuclear envelope breakdown to anaphase onset.
[a] Into late-prophase nuclei.
[b] Cells recorded 10 min after drug addition.

malian cells. This delay is not seen in response to minor DNA damage, and it is not dependent on an intact P53 pathway.

Inhibiting Topoisomerase II by ICRF-193 Prolongs Mitosis in Some Cells

Adriamycin produces DSBs by stabilizing covalent complexes between topoisomerase II and DNA [25]. When applied to U20S cells (human osteosarcoma; p53+) blocked in mitosis with nocodazole, a 1 hr treatment with 0.5 μM adriamycin causes the cells to retain a 4N DNA and high MPM-2 content for up to 8 hr after the block is released [12]. We repeated this same experiment on U2OS, CFPAC-1, hTERT-RPE, and HeLa cells by using 1.0 μM adriamycin. Surprisingly, we found that the mitotic index of all four cell types fell to control levels within 2 hr (Figure 2A), even though, as seen by γ-H2AX staining, this treatment produces easily detected DSBs (Figure 2B). These data imply that treating mitotic cells with 1 μM adriamycin for 1 hr damages the DNA but does not delay exit from mitosis. To confirm this, we added 1–5 μM of adriamycin to CFPAC-1 cultures, located late prophase cells within the cultures 10–20 min later, and followed their progression through mitosis. Although the separation of sister chromatids during anaphase was incomplete (especially at higher drug concentrations), the duration of mitosis was not different from that of control cells (Table 1).

We then repeated this live-cell work on the other cell lines, including hTERT-RPE1, Hela, and U2OS. Again, we found that a 10–20 min treatment with 1–5 μM adriamycin did not prolong the duration of mitosis in vivo (Table 1). However, after longer (60 min) exposures to 5 μM adriamycin, U2OS, but not the other cell types, exhibited an unusual behavior. Unlike in the other cell lines, where entry into mitosis was inhibited by the G2 DNA damage checkpoints, cells in U2OS cultures continued to cycle into mitosis after 60 min in 5 μM adriamycin. Few of these cells completed mitosis with normal kinetics. Instead most became arrested in a prophase-like stage for 6–8 hr, prior to undergoing apoptosis. The remainder entered a prolonged prometaphase, after which they exited mitosis without forming a normal spindle.

ICRF-193 is also a potent inhibitor of topoisomerase IIα that, unlike adriamycin, binds to the enzyme and converts it to a form that is unable to bind or rebind DNA [26]. Although ICRF-193 is not thought to directly induce DNA damage [27], we found that DSBs arise in

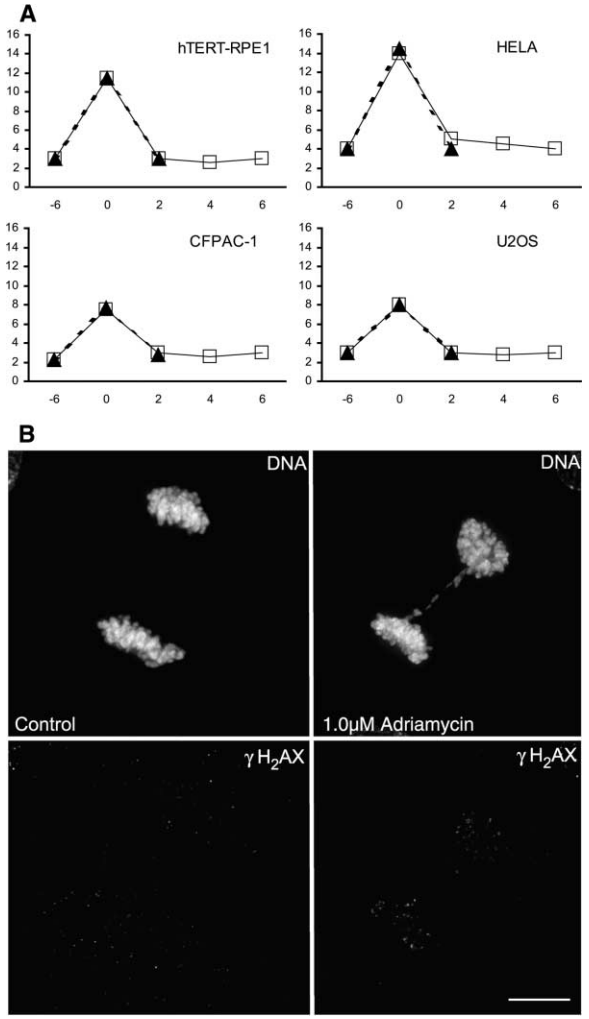

Figure 2. Adriamycin Treatment during Mitosis Does Not Affect the Timing of Mitotic Exit but Causes DNA Damage

(A) hTERT-RPE1, CFPAC-1, HeLa, and U2OS cultures were treated with 10 μM nocodazole for 6–8 hr at 37°C. During the last hour of the nocodazole treatment, they were also treated with 1.0 μM adriamycin prior to washing 3X with fresh medium and re-incubating at 37°C. Solid lines represent the percent of cells in mitosis (*y* axis) versus time (hr) (*x* axis) after release from the drugs (solid lines) or nocodazole alone (dashed lines).

(B) Typical Hoechst 33342 (top) and γ-H2AX (bottom) staining seen in late-anaphase cells from CFPAC-1 cultures recovering from treatment with nocodazole alone (control) or nocodazole + 1.0 μM adriamycin for 1 hr. The scale bar represents 10 μm.

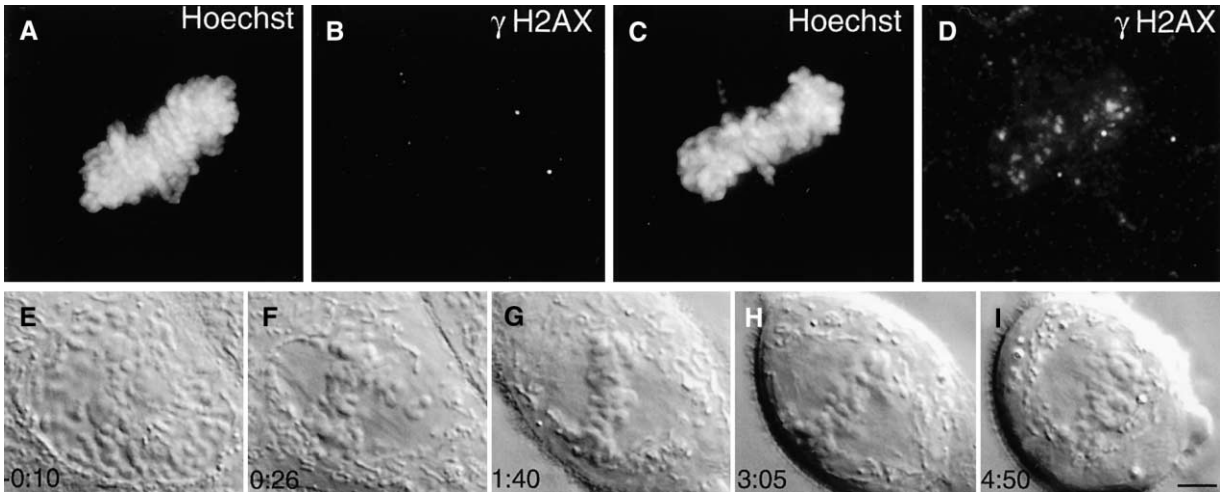

Figure 3. ICRF-193 Induces DNA Double-Stranded Breaks and Prolongs Mitosis in CFPAC-1 Cells

ICRF-193 induces double-stranded DNA breaks (γ-H2Ax foci) during mitosis in CPAC-1 cells (A–D), and anaphase onset is delayed (E–I). Nontreated (A and B) and ICRF-193 treated (C and D) metaphase cells visualized by Hoechst (A and C) and γ-H2AX (B and D) staining. Note the presence of multiple γ-H2Ax foci in the ICRF-193-treated cell. (E–I) A late-prophase cell (E) was located in a culture treated for 20 min with 4 μm ICRF-193. After nuclear envelope breakdown (0:00 time), this cell rapidly formed a normal-looking spindle (F), but it then remained arrested in metaphase for several hours (G–H) prior to entering anaphase (I). Time is given in hr:min in the lower left corner of each frame. The scale bars represent 10 μm.

the presence of the drug as cells enter mitosis (Figures 3A–3D). Based on population studies, others have reported that exit from mitosis is delayed in a dose-dependent manner when HeLa cells are arrested in mitosis with nocodazole and then washed with medium lacking nocodazole but containing ICRF-193 [17, 18]. To investigate this behavior in more detail, we treated PtK_1, HeLa, CFPAC-1, hTERT-RPE, and U2OS cultures with 4 μM ICRF-193 and then located and followed prophase cells by video light microscopy (LM) (10–30 min later (Figures 3E–3I). We found that inhibiting topoisomerase IIα with ICRF-193 prolonged the duration of mitosis approximately 4-fold in HeLa, CFPAC-1, and U2OS cells, but not in PtK_1 or hTERT-RPE cells (Table 1).

These studies reveal that treatment with ICRF-193 leads to chromosome damage (DSBs) during mitosis and also delays exit from mitosis in some but not all cultured mammalian cells.

Chromosome Damage Does Not Inhibit Centrosome Function

Is the delay in mitosis induced by DNA damage in some mammalian cells due, as in *Drosophila* embryos [20], to centrosome inactivation? The answer to this question is clearly no; when late prophase CFPAC-1 cells were irradiated in the nucleus with 400 laser pulses, they always assembled normal-looking bipolar spindles via a pathway involving the two separating centrosomes (Figure 4). Moreover, these spindles formed with normal kinetics (Figures 1E–1I), even though the cells contained acentric chromosome fragments (our unpublished data; see Figures 1A–1D). In these cells the prolongation of mitosis occurs after the spindle has formed, and during this metaphase "block" the spindle is indistinguishable from that in nonirradiated metaphase cells (Figures 1E–1I and 4D–4F). The same results were obtained when HeLa, hTERT-REP1, and CV1 cells were selectively irradiated in the nucleus during late prophase (our unpublished data).

The mitotic delay seen in response to ICRF-193 treatment also occurs in metaphase (Figures 3E–3I). As others have reported [17], in the presence of 4 μM ICRF-193, chromosome segregation was highly abnormal; during anaphase the chromatids became progressively smeared along the spindle and ultimately formed a single multi-lobed 4N restitution nucleus. Although cytokinesis was always attempted, it was seldom completed. This is not unexpected; chromosome bridges in the spindle midzone lead to furrow regression [28].

These studies reveal that chromosome damage near the onset of mitosis delays the metaphase/anaphase transition in some mammalian cells without producing obvious defects in spindle assembly or chromosome congregation.

The Metaphase Block Induced by Chromosome Damage Is Not Due to the Inhibition of Cyclin A Degradation

Cyclin A degradation is a prerequisite for anaphase onset [16]. To determine if, as in *Drosophila* gastrulae [15, 29], the transient metaphase block induced in mammals by DNA damage correlates with the delayed destruction of cyclin A, we fixed CFPAC-1 cells for IMF analyses 2 hr after irradiating them during late prophase with 400 laser pulses. At the time of fixation, the cells contained spindles that would have spent an additional 4+ hr in metaphase. In all seven cells examined, cyclin A was degraded to levels similar to those of surrounding G_1 and S cells (Figure 5). Thus, the metaphase block induced by extensive chromosome damage during prophase is not correlated with the inability to degrade cyclin A in a timely manner.

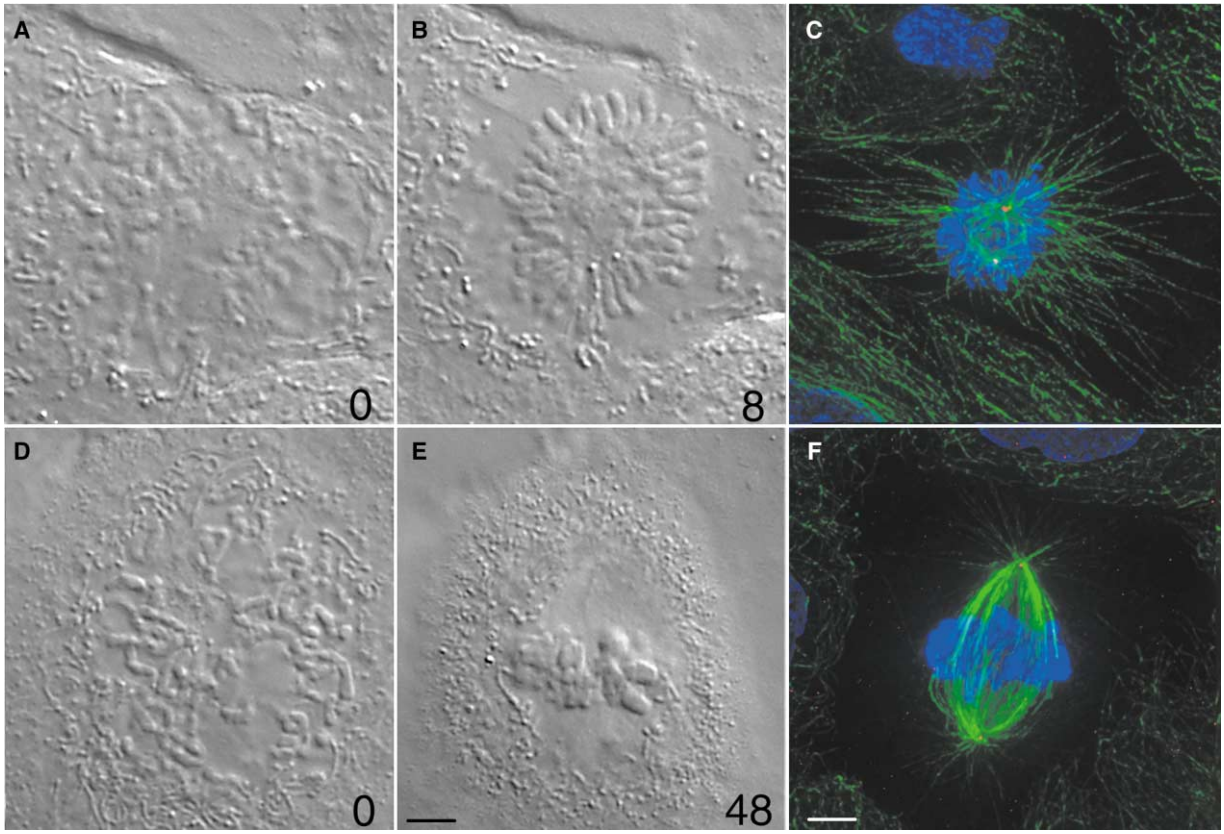

Figure 4. Normal Centrosomal Spindles Form When the Chromosomes Are Severely Damaged by Laser Microsurgery during Late Prophase

(A–C) A CFPAC-1 cell was irradiated in the late prophase nucleus with 400 laser pulses (A) and then fixed during early prometaphase (B) for an IMF localization of microtubules and γ-tubulin (C). Note that a bipolar spindle was forming between the two separating centrosomes (red dots in [C]) and their associated astral microtubule arrays (green). (D–F) Similar to (A–C) except this cell was fixed approximately 1 hr after irradiation. Note that the cell contains a normal-looking bipolar spindle with centrosomes at the poles. The scale bars in (E) and (F) represents 10 μm.

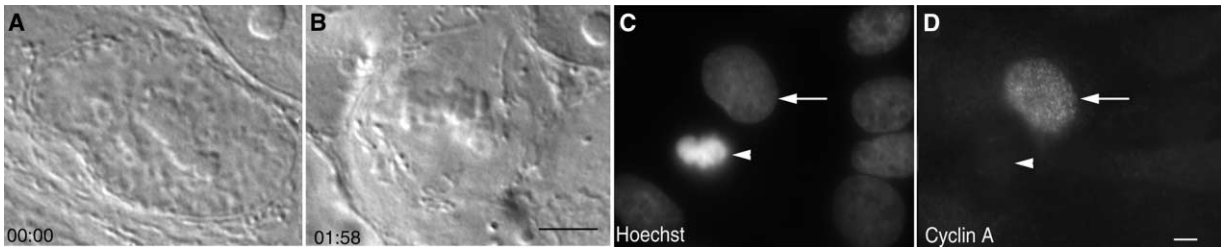

Figure 5. Cyclin A Is Degraded in Cells Blocked in Metaphase by Chromosome Damage

A late-prophase CFPAC-1 cell was irradiated in the nucleus with 400 laser pulses (A) and then fixed 2.5 hr later, after it had formed a normal metaphase spindle (B). It was then stained with Hoechst for the fluorescent localization of chromatin (C) and also for the IMF localization of cyclin A (D). Note that the cyclin A level in this cell ([C and D], arrowhead) is barely detectable, whereas that of an adjacent G_2 cell ([C and D], arrow) remains high. The scale bars (B and D) represent 10 μm.

The Metaphase Block in Response to Chromosome Damage Is Not Due to an ATM-Mediated Checkpoint

All DNA damage checkpoints work through the ATM/ATR kinase pathway and are inhibited by caffeine or wortmannin (see Introduction). To determine if the transient metaphase block caused by DNA damage requires ATM kinase activity, we treated cultures of CFPAC-1 cells with 2 mM caffeine 12 hr prior to damaging the chromosomes with the laser during early mitosis. By itself, caffeine does not significantly alter the duration of mitosis in CFPAC-1 cells (71 ± 6 min; N = 9). We found that, when caffeine-treated cells were randomly irradiated in the nucleus during late prophase with 200 pulses of laser light, anaphase onset was still greatly delayed (360 ± 90 min; N = 11).

Next, we treated growing cultures with caffeine (2–5 mM; 5–18 hr) or wortmannin (4 μM; 4 hr) prior to adding

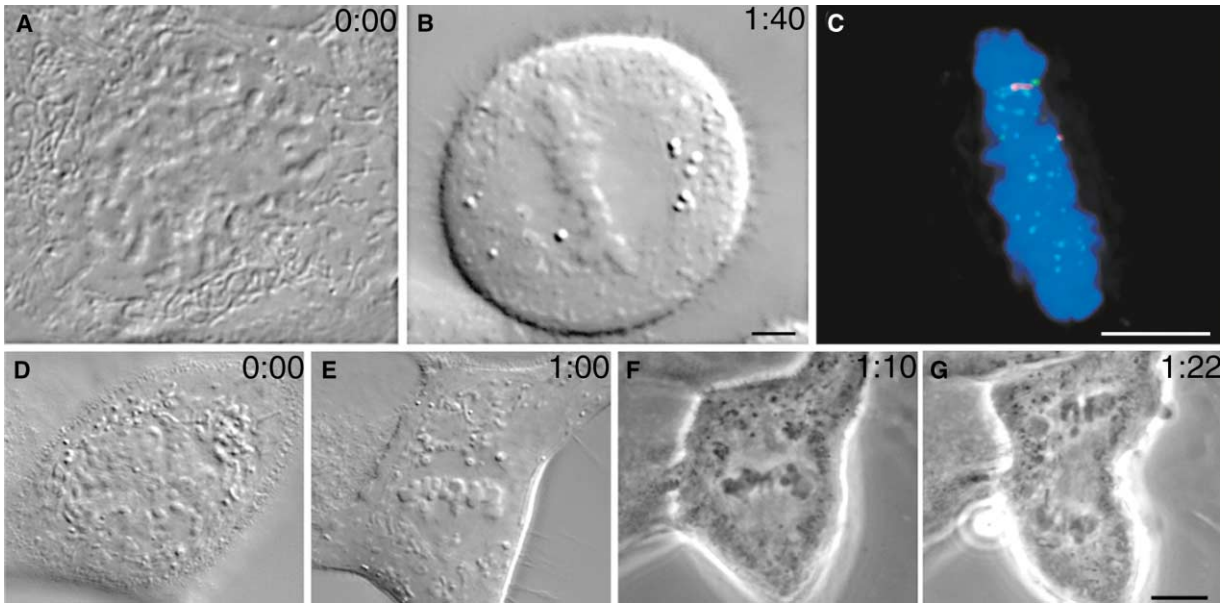

Figure 6. Cells Blocked in Metaphase by Laser-Induced Chromosome Damage Contain at Least One Mad2-Positive Kinetochore and Rapidly Exit Mitosis When Microinjected with Mad2ΔC

(A–C) A HeLa cell was irradiated in the prophase nucleus with 400 laser pulses (A) and fixed 100 min later during metaphase (B). It was then stained for kinetochores (CREST serum; green), Mad2 (red), and chromatin (Hoechst; blue). Even after 100 min in mitosis, several kinetochores stain positive for Mad2 (yellow and red staining encircling green in [C]). (D–G) A CFPAC-1 cell irradiated in the prophase nucleus entered a prolonged metaphase (E), during which it was microinjected with Mad2ΔC (F) 70 min into the block. Shortly thereafter it entered anaphase (G). Time is given in hr:min in the upper right-hand corner. The scale bar in (B) and (C) represents 4 μm; G = 5 μm.

4 μM ICRF-193. We found that neither of these pretreatments significantly shortened the duration of mitosis in CFPAC-1 cells exposed to ICRF-193 (caffeine = 181 ± 46 min, N = 4; wortmannin = 178 ± 96, N = 4). Finally, we repeated the caffeine pretreatment study on U2OS cells and found that mitotic progression was still substantially delayed in the presence of ICRF-193 (169 ± 69 min; N = 11). These results are consistent with the report [17] that caffeine does not prevent the ICRF-193-induced prolongation of mitosis in HeLa cells.

Together, these studies reveal that the ATM/ATR kinase pathway does not mediate the metaphase block induced by chromosome damage.

Chromosome Damage Delays the Metaphase/Anaphase Transition via the Mad2-Spindle Assembly Checkpoint

Extensive chromosome damage could deleteriously affect one or more kinetochores, the proper function of which depends on the structure of the underlying centromere. Under this condition normal bipolar spindles would form, but one weakly attached kinetochore could delay anaphase onset via the Mad2 checkpoint pathway. To evaluate this idea, we irradiated prophase Hela cells with 400 pulses of laser light and then fixed them 100 min later for a subsequent IMF analysis of Mad2 distribution. We used Hela because our antibody produced a much cleaner kinetochore-staining pattern than in CFPAC-1 cells. As for CFPAC-1, random laser-induced damage to the prophase chromosomes blocks HeLa cells at metaphase for approximately 5 hr (Table 1). Our IMF analysis revealed that Mad2 was present on at least one kinetochore in every cell analyzed (N = 5/5; Figures 6A–6C). This suggests that photodamage to the chromosomes invariably compromises the function of one or more kinetochores.

Next, we repeated this experiment on CFPAC-1 cells. However, instead of fixing them for IMF, we microinjected each with a dominant-negative mutant of Mad2. This Mad2ΔC construct induces anaphase onset within 20 min when injected into cells arrested in mitosis by nocodazole ([30]; unpublished data). When we injected Mad2ΔC into CFPAC-1 cells blocked in metaphase by laser-induced chromosome damage, all (N = 5) entered anaphase 4–18 min later (Figures 6D–6G).

To determine if the metaphase block induced by ICRF-193 also involves Mad2, we treated HeLa cultures with ICRF-193 and followed prophase cells into mitosis. These were then fixed approximately 100 min after NEB and stained for the IMF localization of Mad2. All four cells examined contained 1–3 kinetochores that were Mad2-positive (Figures 7A–7C). Finally, we microinjected Mad2ΔC into CFPAC-1 cells 30–60 min after they were blocked in metaphase by ICRF-193 treatment. In all cases (N = 5), the cells exited mitosis 11–23 min after the microinjection (Figures 7D–7F).

Together, these studies reveal that the transient metaphase block seen in response to severe chromosome damage is mediated by the spindle assembly checkpoint.

Discussion

Chromatin damage during G_2 invariably prohibits entry into mitosis. Therefore, to determine how chromosome damage affects mitotic progression in mammals, previ-

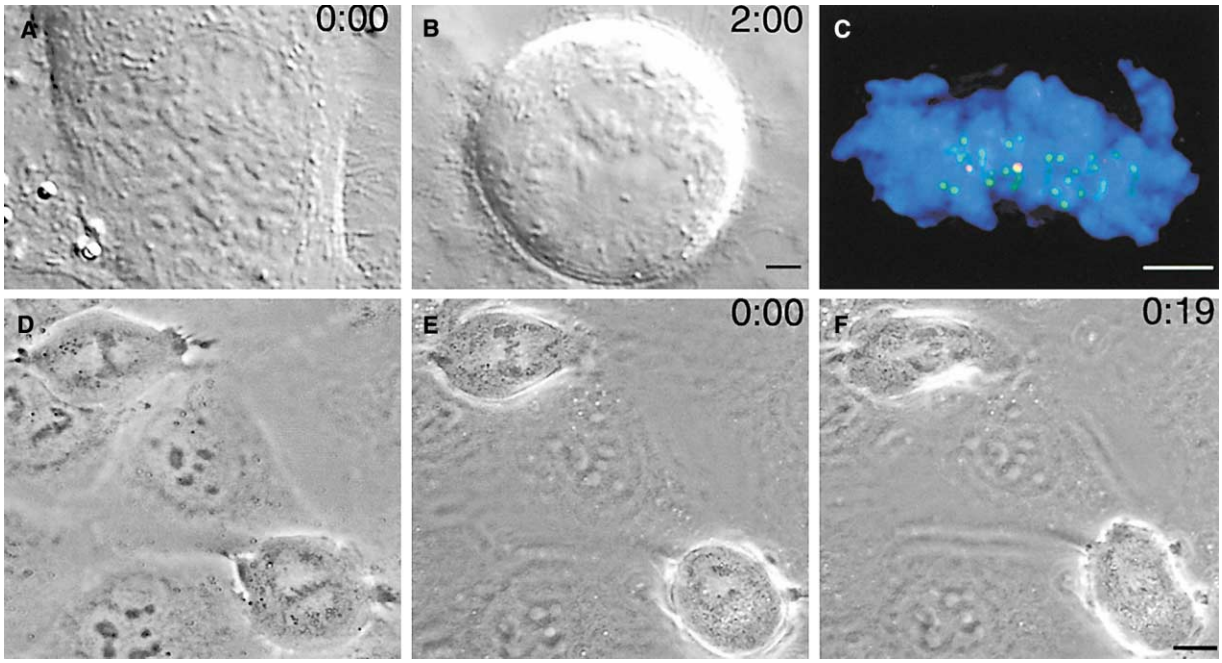

Figure 7. Cells Delayed in Metaphase by a Treatment with 4 μM ICRF-193 Contain at Least One Mad2-Positive Kinetochore and Rapidly Enter Anaphase When Microinjected with Mad2ΔC

(A–C) A late-prophase HeLa cell (A) entered mitosis in the presence of ICRF-193. Two hours later this cell was fixed and processed for the IMF distribution of CREST antigens (green), Mad2 (red), and DNA (blue; Hoechst 33342). Two kinetochores (yellow overlap and a red crescent around crest staining in [C]) still contained Mad2.

(D–F) One hour after a CFPAC-1 culture was treated with 4 μM ICRF-193, many cells are blocked in metaphase (D). When these are microinjected with Mad2ΔC (E), they enter anaphase within 19 min (F). The scale bar (B and C) represents 4 μm.

ous workers arrested cells in mitosis with Mt inhibitors before treating them with DNA-damaging agents. They then assayed the effect of the damage indirectly by determining changes in the percentage of mitotic or 4N cells at various times after washing out the Mt inhibitor. Unfortunately, this approach provides little information about whether the division was actually completed or whether spindle assembly was disrupted. To directly define how DNA damage affects mitosis in mammals, we took advantage of the fact that cells become committed to division during late prophase, as the nucleoli fade [5, 6]. By using video LM to follow living cells, we could then determine how damaging DNA during terminal G_2 subsequently affects the duration of mitosis.

In our initial studies we used a laser microbeam to randomly damage the chromosomes in late prophase nuclei. We found that minor damage did not significantly affect progression through mitosis in the human cell lines we used, even though it produced multiple detectable DSBs. However, more substantial damage prolonged mitosis approximately 5-fold by a mechanism that was not sensitive to caffeine. Here it is noteworthy that, as for other cell-cycle checkpoints, those that monitor DNA damage are thought to contain an amplification step that converts minor damage into a full arrest response [1]. The fact that we see a delay only in response to major, but not minor, chromosome damage is therefore not in accord with how checkpoints are envisioned to work.

Unlike adriamycin, inhibiting topoisomerase IIα with ICRF-193 is not thought to induce DSBs [26, 27]. However, we found significant amounts of γ-H2AX staining, indicative of DSBs, on chromosomes in ICRF-193-treated cells. These breaks may be produced as forces generated by the spindle stretch the nondecatenated centromere regions during mitosis, and/or they may be due to the ICRF-193-induced cross-linking of topoisomerase IIβ to DNA [31]. Regardless of their origin, this drug transiently blocks some (HeLa, CFPAC-1, U2OS), but not all (hTERT-RPE, our study; PtK_1 [19]; CHO [18]), cell types that have fully formed spindles in metaphase. However, as with radiation-induced chromosome damage, we found no evidence that the transient mitotic block in response to ICRF-193 treatment is mediated by a DNA damage checkpoint; it could not be overridden with caffeine or wortmannin.

Together, our results imply that the metaphase delay seen when chromosomes are severely damaged is not mediated by a DNA damage checkpoint but by some other pathway. This conclusion conflicts with that of Smits et al. [12], who treated U2OS cells with adriamycin during a mitotic block and found that they were substantially delayed from exiting mitosis when the block was removed. To resolve this conflict, we repeated their protocol on several cell lines, including U2OS, by using 1 μM adriamycin. We found that even though adriamycin induced DSBs during mitosis, it did not prolong the duration of mitosis in any of these cell lines, as evidenced by a rapid decline in the mitotic index after the nocodazole washout.

To confirm this directly, we treated cultures with 1–5 μM adriamycin, located prophase cells 10–20 min later,

and followed them through mitosis. Again, we found no effect on mitotic progression. We did note, however, that U2OS cells exhibited an unusual behavior after 60 min in 5 μM adriamycin; cells entering mitosis at this time either stalled for many hours in a prophase-like state, before undergoing apoptosis, or they arrested in prometaphase before ultimately decondensing their chromosomes without forming a normal spindle. This behavior appears specific to U2OS cells and to adriamycin treatment because ICRF-193 does not prevent spindle formation and only transiently delays normal mitotic progression in U2OS. In this regard, it may be relevant that adriamycin directly produces DSBs, whereas ICRF-193 induces DNA damage indirectly during mitosis (see above). Also, in addition to interfering with topoisomerase II, adriamycin has other pleiotropic effects in that it leads, for example, to the inhibition of DNA synthesis, to the generation of free radicals, to DNA covalent binding/interstrand cross linking, and to apoptosis [32]. Thus, it is possible that DSBs or other cellular damage produced in U2OS cells during G_2 by high concentrations of adriamycin (5 and not 0.5 μM for 1 hr) do not prevent entry into mitosis. Instead, they could trigger a P53-mediated apoptotic pathway involving the ATM and Plk1 kinases [33, 34], and this pathway might subsequently stall and kill the cells in mitosis. If this is the case, then the mitotic cells that Smits et al. [12] observed 6–8 hr after they removed the nocodazole block may have been in G_2 during the adriamycin treatment and may have subsequently cycled into a prolonged mitosis.

Our observation that cells with damaged chromosomes remain stalled in metaphase with normal spindles, after degrading their cyclin A, is reminiscent of the metaphase block induced by low concentrations of taxol [35, 36]. Under this condition, the block can be attributed to the Mad2-mediated spindle assembly checkpoint, which is based in the kinetochores [9, 36]. With regards to this checkpoint, it is easy to envisage how extensive chromatin (DNA) damage during mitosis could compromise the function of one or more kinetochores. This idea is supported by our initial finding that irradiating just the nucleolus during prophase, when it is expected to be relatively free of kinetochores, induces much less of a delay in anaphase onset than does randomly irradiating the whole nucleus.

To evaluate the idea that extensive chromosome damage compromises kinetochore function, we exposed cells entering mitosis to either laser microsurgery or ICRF-193 and then fixed them while they were blocked in metaphase for a subsequent IMF analysis of Mad2 distribution. We invariably found that one or more kinetochores in these cells possessed significant Mad2 staining, which, as in taxol-treated cells (see above), could explain the arrest. To directly test this possibility, we then injected cells blocked in metaphase by chromosome damage with Mad-2ΔC, which is known to rapidly override the Mad-2 spindle assembly checkpoint [30]. In all cases, this dominant-negative construct rapidly induced anaphase onset and exit from mitosis. These data clearly demonstrate that the transient metaphase block produced in response to extensive DNA damage results from the inability of the cell to overcome the spindle assembly checkpoint because of problems in kinetochore attachment.

In their study, Smits and colleagues [12] hypothesized that DNA damage during mitosis triggers an ATM-dependent checkpoint pathway that ultimately inhibits centrosome maturation and APC activation by downregulating Plk1. Our data demonstrate directly that chromosome damage does not compromise centrosome function during mitosis in mammals. In yeast, Plk activity is required for both chromatid disjunction and cyclin B degradation, presumably because it is required for APC activation (see [37]). In our study, we were able to activate the APCs and induce chromatid disjunction and exit from mitosis in cells blocked in metaphase by chromosome damage. We did this by simply overriding the Mad2 checkpoint pathway. This result implies either that Plk is active in our cells or that its activity is not needed for APC activation.

Our major novel finding is that physically or chemically damaging the DNA delays anaphase onset in mammals because it leads to problems in kinetochore attachment. In turn, this stalls mitotic progression by retarding satisfaction of the intrinsic Mad2-based spindle assembly checkpoint. This same conclusion may also apply to yeast (*S. cerevisiae*), for which the idea first arose that DNA damage checkpoints exist during mitosis. For example, Garber and Rine [38] recently found that chemicals that induce structural changes in the chromatin of *S. cerevisiae* arrest cells in "preanaphase" principally through the spindle assembly and not DNA damage checkpoint pathways. (In yeast, clearly defining when mitosis starts is problematic, which can make determining when G2 checkpoints stop functioning ambiguous.) There is also evidence that the related finding, that nonreplicated chromosomes block entry into anaphase in both yeast and *Drosophila embryos,* can similarly be attributed to the spindle assembly checkpoint and not to one that monitors DNA replication or damage [38, 39].

PtK_1 is the only cell that we have encountered in which extensive chromosome damage by laser microsurgery does not delay progression through mitosis. The reason for this is unknown. PtK_1 contain few (12–13) chromosomes within a large nuclear volume. Because randomly moving the cell through the stationary laser beam produces highly localized lesions on only a few chromosomes, it is possible that damaging a kinetochore is a rare event. On the other hand, PtK_1 (and hTERT-RPE1) also progress through mitosis normally when exposed continuously to ICRF-193, a treatment that is expected to globally affect all of the chromosomes. The key question is why ICRF-193 does not compromise the function of kinetochores in PtK_1 and hTERT-RPE1 cells, as it does in other cell types. In this regard it is possible that the centromere regions in cells that are refractory to ICRF-193 treatment, which include PtK_1, CHO, and hTERT-RPE, are somehow different than those in cells in which the drug prolongs metaphase. In mammals, topoisomerase IIα is concentrated in the centromere and, although it is conserved within the kinetochore domain, its pattern of distribution is species specific [40]. We find that ICRF-193 treatment has no effect on the distribution of topoisomerase II in the kinetochore regions of PtK_1 or hTERT-RPE chromosomes, whereas this treatment strips this enzyme from the kinetochores in HeLa and CFPAC-1 cells (A.M. and C.L.R., unpub-

lished data). Thus, it is possible that ICRF-193 affects kinetochores differently in various cell types and that, in those cells in which it depletes topoisomerase IIα from the kinetochore region, the kinetochores have a more difficult time stably attaching to the spindle.

Conclusions

Minor damage to the chromosomes during mitosis does not prolong mitosis in mammals. However, extensive damage transiently delays the metaphase/anaphase transition in many cell types. This delay is not due to the disruption of spindle formation or to an ATM/ATR-mediated checkpoint that monitors DNA/chromosome integrity. Instead, it occurs in the presence of a fully formed spindle and involves the Mad2 checkpoint control pathway. A model for block that is consistent with our data is that extensive chromosome damage invariably produces one or more "wounded" kinetochores; these then have a difficult time maintaining a proper (stable) attachment to the spindle.

Experimental Procedures

Cell Culture

PtK_1, Hela, CF-PAC1, CV1, and Cos7 cells were obtained from the American Type Culture Collection (Manassas, VA; ATCC numbers CCL-2, CRL-1918, CRL-6493, CCL-70, and CRL-1651, respectively). U2OS cells were obtained from Dr. John Gierthy (Wadsworth Center, Albany, NY). The telomerase-immortalized human cell line, hTERT-RPE1, was purchased from Clontech (BD Biosciences Clontech, Palo Alto, CA). All lines were maintained in T-flasks and, 1–2 days prior to experiments, they were subcultured into Petri dishes containing 25 mm square coverslips. Stocks of PtK_1 and hTERT-RPE1 cells were maintained in Ham's medium supplemented with 15% FBS. Hela, CV1, U2OS, and Cos7 stocks were grown in DMEM with 5% (U2OS) or 15% (HeLa, CV1, Cos7) FBS. CFPAC1 stocks were maintained in IMEM with 15% FBS. During experiments all cells were maintained within Rose chambers [41] containing L-15 media supplemented with 15% FBS.

Laser Irradiation and Live-Cell Video LM

We conducted laser irradiation and subsequent cell imaging on our microsurgery workstation [21]. This fully shuttered DIC LM is based on a Nikon Diaphot 200 (Nikon, Melville, NY) and employs a Roper Micromax (Roper Scientific, Trenton, NJ) camera and a pulsed Nd:YAG laser. We randomly irradiated the nuclei of selected cells by moving the cell through the fixed laser beam with a Ludl motorized stage. Cultures were maintained on the microscope stage at 34°C with a Rose chamber heater.

For ICRF-193 and adriamycin experiments, selected cells were followed by DIC or phase contrast LM on a Nikon Diaphot 300 equipped with an Orca ER camera (Hamamatsu Corporation, Bridgewater, NJ) and a Narishige microinjection system. The video parameters used included shuttered 546 nm light obtained from a 100 W tungsten source, a framing rate of 2/min, and an exposure of 200–300 ms/frame. All images were processed by Image Pro Plus (Media Cybernetics, Silver Spring, MD) and stored on the computer hard drive.

Microinjection

His-tagged Mad2ΔC (from Dr. E.D. Salmon, Chapel Hill, NC) was purified on a Novagen His-Bind Quick Column, concentrated to 10 μg/μl, and stored at −90°C. CFPAC-1 cells, arrested in metaphase by 400 laser pulses, were injected with purified Mad2ΔC containing rhodamine-dextran 3000 (Molecular Probes, Eugene, OR) at 0.25 μg/μl. CFPAC cells, delayed in metaphase by treatment with ICRF193 at 4 μg/μl, were similarly injected with Mad2ΔC and then followed by phase-contrast LM through anaphase onset and exit from mitosis.

Drug Treatments and Mitotic Index Studies

Coverslip cultures of growing CFPAC-1, HELA, and hTERT-RPE1 cells were treated with 10 μM nocodazole for 6 hr. Some of these were left in nocodazole while others were treated with nocodazole and adriamycin (0.5–2 μM; Sigma-Aldrich, St. Louis, MO). One hour later, all of the cultures were washed with fresh medium lacking adriamycin and nocodazole. They were then incubated at 37°C until fixation 2, 4, or 6 hr later. At each time point, two control (no adriamycin) and two adriamycin-treated coverslip cultures were removed from the incubator, fixed in 1% glutaraldehyde in PHEM buffer, and stained with Hoechst 33342. Mitotic cells were then scored and counted, and the numbers of mitotic cells, as well as their stage in mitosis, were determined per 5,000 cells on each coverslip. Two coverslips (5,000 cells each) were used for each time point, and the results were averaged prior to plotting.

For live-cell studies, Rose chamber cultures were pretreated for 10–20 min with 1–20 μM adriamycin or with 4 μM ICRF-193 (from Dr. A. Creighton, St. Bartholomew's Hospital College, London, UK). We then located late-prophase cells within these cultures and followed them by video LM (at 34°C) to determine the duration of mitosis. In some cases cultures were pretreated with caffeine (2–5 mM; 5–18 hr; Sigma-Aldrich, St. Louis MO) or wortmannin (4 μM; 4 hr; A.G. Scientific, San Diego, CA) prior to laser irradiation or ICRF-193 treatment.

Indirect Immunofluorescence Microscopy

Hu-anti-CREST (from Dr. B.R. Brinkley, Houston, TX), Rb-anti-MAD2 (from Dr. E.D. Salmon, Chapel Hill, NC), and anti-phosphorylated-γH2AX (Trevigen, Inc.) were used as primary antibodies for Indirect Immunofluorescence Microscopy (IMF) studies. Cells were rinsed in PHEM buffer, permeabilized with 0.5% Triton in PHEM, fixed in 4% paraformaldehyde in PHEM (15 min), rinsed, and incubated in PBS with 0.1% Tween-20 (PBST). Cultures stained for γ-H2AX were also post-fixed with −20°C methanol for 2 min. All cultures were then blocked in 10% normal donkey serum in PBST and stained with the primary antibody at various dilutions (1:600, 1:50, 1:100), followed by the appropriate Alexa-conjugated secondary antibody (Molecular Probes, Eugene, OR). After a final rinse, the coverslip cultures were stained with Hoechst 33342 and mounted on microscope slides in a 50:50 mixture of PBS and glycerol containing 1 mg/ml p-phenylenediamine.

To study the distribution of Mad2 after chromosome damage, we either treated HeLa cells with ICRF193 or irradiated them with the microbeam as described above. The cells were then followed by DIC or phase contrast LM for 1–2 hr before fixation and staining for IMF. These cells were then examined, photographed, and deconvolved on a Delta Vision image restoration system (Applied Precision, Issaquah, WA) with a 60× 1.4 NA Planapo objective.

Image Manipulation and Photography

Selected DIC and epi-fluorescent digital images were exported from Image Pro Plus into Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA). After contrast manipulation, masking, and montaging, the final plates were printed on a Tektronix Phaser 450 (Tektronix Inc, Gaithersburg, MD) dye sublimation printer.

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