

DNA damage leads to a Cyclin A–dependent delay in metaphase-anaphase transition in the *Drosophila* gastrula

Tin Tin Su and Burnley Jaklevic

Background: In response to DNA damage, fission yeast, mammalian cells, and cells of the *Drosophila* gastrula inhibit Cdk1 to delay the entry into mitosis. In contrast, budding yeast delays metaphase-anaphase transition by stabilization of an anaphase inhibitor, Pds1p. A variation of the second response is seen in *Drosophila* cleavage embryos; when nuclei enter mitosis with damaged DNA, centrosomes lose γ -tubulin, spindles lose astral microtubules, chromosomes fail to reach a metaphase configuration, and interphase resumes without an intervening anaphase. The resulting polyploid nuclei are eliminated.

Results: The cells of the *Drosophila* gastrula can also delay metaphase-anaphase transition in response to DNA damage. This delay accompanies the stabilization of Cyclin A, a known inhibitor of sister chromosome separation in *Drosophila*. Unlike in cleavage embryos, γ -tubulin remains at the spindle poles, and anaphase always occurs after the delay. Cyclin A mutants fail to delay metaphase-anaphase transition after irradiation and show an increased frequency of chromosome breakage in the subsequent anaphase.

Conclusions: DNA damage delays metaphase-anaphase transition in *Drosophila* by stabilizing Cyclin A. This delay may normally serve to preserve chromosomal integrity during segregation. To our knowledge this is the first report of a metazoan metaphase-anaphase transition being delayed in response to DNA damage. Though mitotic progression is modulated in response to DNA damage in both cleaving and gastrulating embryos of *Drosophila*, different mechanisms operate. These differences are discussed in the context of differential cell cycle regulation in cleavage and gastrula stages.

Background

Damage to DNA leads to a variety of cellular responses that include activation of the DNA repair machinery and slowing of the cell division cycle, presumably to allow time for repair. Although most cells delay cell division in response to damaged DNA, the exact nature of this delay and the mechanistic basis for it can differ among species. In fission yeast and cultured human cells, a common arrest point occurs prior to the entry into mitosis [1]. Both the inhibition of mitotic Cdk activity and subcellular sequestration of cyclins or Cdk activators contribute mechanistically to this delay [2–6]. Budding yeast, in contrast, do not delay the entry into mitosis in the presence of damaged DNA [1]. Instead, DNA damage delays the transition from metaphase to anaphase. This delay differs mechanistically from the G2/M delay because stabilization of an anaphase inhibitor, Pds1p, and not inhibition of Cdk1, is responsible [7, 8]. There are several indications, however, that more than one step in metazoan mitosis may be modulated in response to DNA damage. Human cells that are unable to delay mitosis in the presence of damaged DNA enter mitosis but fail to complete it or cytokinesis [3, 9, 10]. Likewise, when human cells incur DNA damage

during metaphase, they fail to complete subsequent stages of mitosis or to enter the next cell cycle [11]. These data indicate the presence of additional arrest points during metazoan cell division, at least one of which must operate after metaphase [11], although the exact transition(s) affected remains to be documented. In an analogous manner, spindle defects are thought to arrest mitosis primarily at the metaphase-anaphase transition, but a recent report documented an additional, novel arrest point between prophase and metaphase [12].

To find out if metazoan mitotic progression may be regulated at more than one point in response to damaged DNA, we examined the effect of DNA-damaging agents on mitosis in *Drosophila* embryos. *Drosophila* embryogenesis begins with nuclear division cycles that oscillate between S phase and mitosis and that lack gap phases [13]. These cycles are driven by maternally deposited gene products. Previous work showed that DNA damage in these cycles does not delay the entry into mitosis. Instead, nuclei enter mitosis, chromosomes condense but fail to congress properly on the metaphase plate, anaphase does

Address: MCD Biology, University of Colorado, Boulder, Colorado 80309, USA.

Correspondence: Tin Tin Su
E-mail: tin.su@colorado.edu

Received: 25 September 2000

Revised: 6 November 2000

Accepted: 10 November 2000

Published: 9 January 2001

Current Biology 2001, 11:8–17

0960-9822/01/\$ – see front matter

© 2001 Elsevier Science Ltd. All rights reserved.

not occur, and mitosis is aborted [14, 15]. Interphase nuclei reform after abortion of mitosis but are eliminated and do not contribute to the somatic lineage. This response has been proposed to be an embryonic equivalent of elimination of damaged cells via programmed cell death [37].

After the 13th embryonic division in *Drosophila*, cell cycle regulation shifts from maternal to embryonic control. Embryonic transcription replaces maternally supplied products, embryos cellularize and gastrulate, and a G2 phase is added to subsequent division cycles [13]. We reported previously that, concurrent with the acquisition of G2/M regulation, DNA damage delays the initiation of mitosis 14 (M14; [16]). Similar to the cases of fission yeast and vertebrate cells, this delay relies on inhibitory phosphorylation of Cdk1.

We report here a close examination of mitoses that follow the G2/M delay in irradiated embryos. We found that metaphase-anaphase transition is delayed in these embryos and that this delay accompanies the failure to degrade a mitotic cyclin, Cyclin A. Experimental stabilization of Cyclin A blocks cells in metaphase in *Drosophila* [17, 18]. Therefore, inhibition of Cyclin A proteolysis can mechanistically account for the delay in metaphase-anaphase transition. In support of this idea, we found that Cyclin A mutants failed to prolong metaphase following irradiation.

Results

Cells delay metaphase-anaphase transition following irradiation or in the presence of MMS

We reported previously that DNA damage, caused by X-rays or methyl methane sulfonate (MMS), delays the initiation of mitosis in cellularized embryos of *Drosophila* [16]. At longer times after irradiation, cells recover and enter M14 ([16]; Figure 1). In these we found three kinds of abnormalities, which we report here. In untreated embryos, cells enter mitosis in stereotypical clusters called “mitotic domains” [13, 19]. Cells within a domain initiated mitosis within a few minutes of each other so that each domain consisted of cells of various mitotic stages (Figure 1a). In embryos that were recovering from radiation-induced delay of M14, mitotic domains were enriched in metaphase figures (Figure 1b; quantified below). Moreover, anaphase and telophase subsequently showed lagging chromosomes and chromosomal bridges, which are indicative of persistent DNA defects ([16]; Figure 1e,f; quantified below). We infer that premitotic delay was insufficient to repair all damages caused by irradiation (half-lethal dose of 570 rad used in all experiments kills about 50% of embryos; [16]). Likewise, in embryos incubated in MMS, a DNA-damaging agent, cells delayed M14 [16] but eventually resumed mitosis. Metaphase clusters, lagging chromosomes, and chromosome bridges

were observed in these mitoses (Figure 1c,d,g,h). We infer that irradiated and MMS-laden cells are unable to finish all repairs during premitotic delay but enter mitosis nonetheless. Such “adaptation” to checkpoint delay is well documented in yeast and human cells [20, 21].

While other explanations are possible, we hypothesized that accumulation of metaphase figures reflects a delay in metaphase-anaphase transition following DNA damage. We tested this hypothesis directly in live studies. For these, embryos carrying a histone-GFP construct [22] were irradiated, and the duration of mitotic phases was measured by visualization of chromosomal dynamics (Figure 2). We found that metaphase lengthened by about 3-fold after irradiation, while the duration of anaphase and telophase remained unchanged (Figures 2b and 3b). These data indicate a delay in metaphase-anaphase transition with little or no effect on the duration of subsequent mitotic phases. Collectively, these data suggest that the execution of mitosis in the presence of DNA defects delays metaphase-anaphase transition.

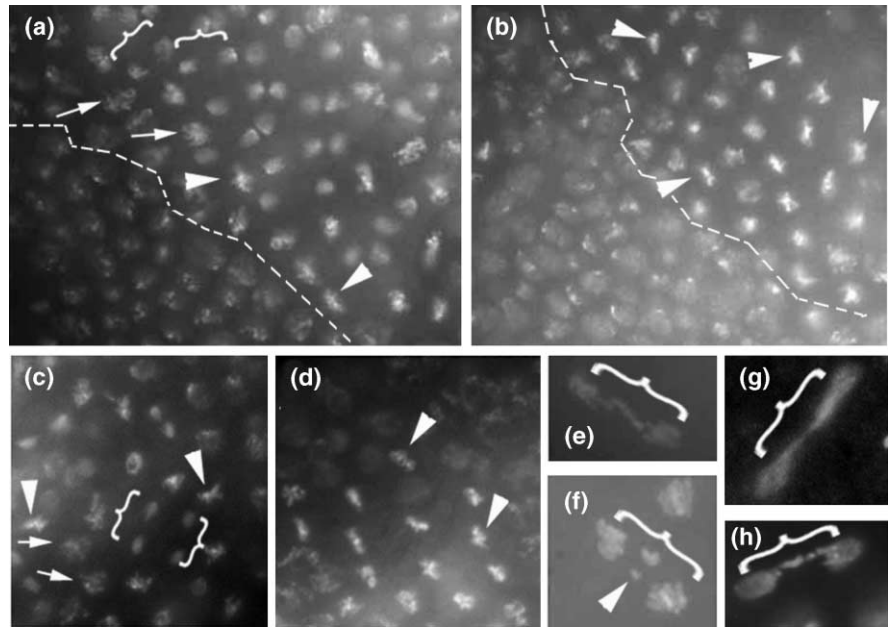
Cyclin A is stabilized after irradiation

In *Drosophila*, as in other species, sequential degradation of proteins is thought to drive mitotic progression [17]. In *Drosophila* cellular embryos, Cyclin A degradation accompanies metaphase-anaphase transition ([17]; Figure 3a). Expression of a stable Cyclin A that lacks targeting sequences for proteolysis blocks chromosome separation and produces metaphase clusters [17, 18]. This indicates that stabilization of Cyclin A is sufficient to block metaphase-anaphase transition, and it suggests that proteolysis of Cyclin A is necessary for this transition. In this regard, Cyclin A is analogous to Pds1p and Cut2p of budding and fission yeast, respectively [23, 24]. Unlike yeast anaphase inhibitors, however, the role of Cyclin A in chromosome separation remains to be understood. In addition to Cyclin A, stabilization of the *pimple* gene product, PIM, can also inhibit sister chromosome separation in *Drosophila* [25]. The relationship between Cyclin A and PIM remains unclear, but PIM may act to regulate sister chromosome separation in response to spindle damage.

To discover if stabilization of Cyclin A is responsible for the observed metaphase delay, we examined Cyclin A levels in mitoses following irradiation. The disappearance of Cyclin A during metaphase is rapid enough that antibody staining can distinguish cells that contain Cyclin A from those that do not ([17, 26, 27]; e.g., Figure 3a). In nonirradiated control embryos, two thirds of metaphase cells ($66 \pm 11\%$; $n = 94$ in eight embryos from three different experiments) contained Cyclin A, while the remainder of metaphase cells and all anaphase and telophase cells lacked this protein. This is consistent with conclusions from earlier works that Cyclin A persists through most of metaphase and becomes degraded prior to meta-

Figure 1

Mitotic abnormalities observed after treatment of *Drosophila* embryos with DNA-damaging agents. Embryos were treated with (a) 0 or (b) 570 R of X-rays, allowed to rest for 45 min, fixed, and stained for DNA. In the control embryo in (a), cells of mitotic domain 1 (above the dotted line) are seen at various stages of M14. Prophase is indicated by arrows, metaphase is indicated by arrowheads, and anaphase is indicated by brackets. In contrast, in the irradiated embryo shown in (b), cells of the same mitotic domain (above the dotted line) are mostly in metaphase (arrowheads) of M14. Fluorescence in situ hybridization with chromosome-specific probes confirmed that accumulated metaphase figures contain unseparated sister chromosomes (data not shown). Similar observations were made for other mitotic domains (not shown). (c,d) Embryos were treated with (c) 0% or (d) 0.1% MMS for 30 min, fixed, and stained for DNA. In the control embryo in (c), various stages of mitosis are visible within mitotic domain 1, as follows: prophase (arrow), metaphase (arrowheads), and anaphase (brackets). In contrast, corresponding cells in (d) are mostly in metaphase (arrowheads). Similar observations were made for other mitotic domains (not shown). (e-h) Chromosome bridges and lagging chromosomes occur during mitosis in cells (e,f) irradiated with 570 R of X-rays



55 min prior to fixation or (g,h) incubated in 0.1% MMS for 1 hr. Embryos were fixed and stained with an antibody to phosphorylated

histone H3 (PH3) to visualize mitotic chromosomes. Brackets indicate pairs of anaphase/telophase figures.

phase-anaphase transition ([17]; schematized in Figure 3b₁). If the timing of Cyclin A degradation occurred with normal kinetics and the prolongation of metaphase occurred independently of this degradation in irradiated cells, we would expect Cyclin A to persist through only the first quarter of metaphase because metaphases are longer (schematized in Figure 3b₂). Accordingly, Cyclin A would be present in only about 25% of metaphase cells in an irradiated embryo. This is clearly not the case. In irradiated embryos, we found that Cyclin A is present in $79 \pm 11\%$ of metaphase cells ($n = 127$ cells in seven embryos from three different experiments). We interpret this result to mean that Cyclin A persists through most of a lengthened metaphase (schematized in Figure 3b₃). We infer from these data that Cyclin A remains stable during the delay in metaphase after irradiation. During normal cell cycle progression, Cyclin B remains stable through metaphase and is degraded during anaphase. Antibody staining showed this to be true for irradiated embryos also (data not shown).

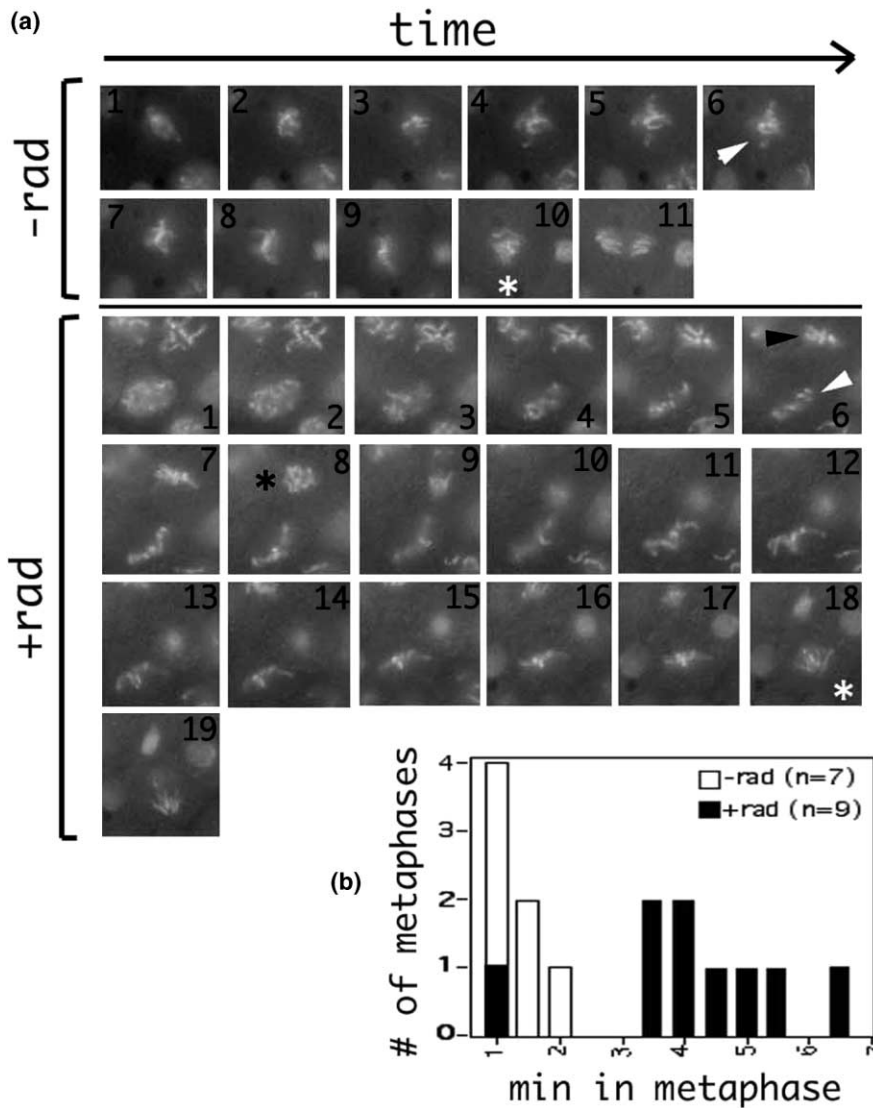
Cyclin A mutants do not show increased metaphase figures after irradiation

If Cyclin A stabilization were responsible for the delay in metaphase-anaphase transition, we would expect that cells that lack Cyclin A would fail to show this response. In *Drosophila*, Cyclin A has an essential mitotic function,

and homozygous mutants die as embryos [26–28]. A maternal supply of Cyclin A from a heterozygous mother, however, is sufficient to allow homozygous mutant embryos to progress through interphase of embryonic cycle 16 and to arrest before mitosis 16. Thus, mitosis 15 is the last mitosis in homozygous Cyclin A mutant embryos. Mitosis 15 occurs in Cyclin B mutants but not in double mutants of cyclins A and B, and these results suggest that entry into this mitosis in Cyclin A mutants is being driven by Cyclin B and residual Cyclin A [28]. Therefore, mitosis 15 provides an ideal situation to test our hypothesis — namely, cells that can still enter mitosis but presumably have diminished amounts of Cyclin A.

In order to determine whether Cyclin A mutant cells show a delay during metaphase of cell cycle 15 after irradiation, we first ensured that wild type-cells in cycle 15 do so. We chose to focus on the dorsal epidermis, which corresponds closely to domain 11 in M14 ([13, 19]; Figure 4a). We chose this region for practical reasons; it is easy to identify, and it comprises a large number of cells that lie relatively flat when embryos are mounted on slides. Quantification of metaphase, anaphase, and telophase figures in M15 shows a relative increase in metaphase cells after irradiation (Figure 4 and Table 1). These data are in good agreement with a 3-fold increase in metaphase duration, without an accompanying change in the duration of ana-

Figure 2



Metaphase delay in irradiated live embryos. (a) GFP-histone fluorescence allows visualization of the progression through mitosis. The frames are approximately 30 s apart (see Materials and methods) and are numbered sequentially. In control embryos ($-rad$), once chromosomes reach maximal compaction on the metaphase plate (arrowhead; scored as beginning of metaphase), chromosome separation (asterisk; scored as end of metaphase) ensues in about 1.5 min. In irradiated samples ($+rad$), many cells linger in metaphase. Of the two cells indicated here, one (black arrowhead) remained in metaphase for about 1 min (until black asterisk) whereas the other (white arrowhead) remained in metaphase for about 6.5 min (until white asterisk). (b) These and other similar data from 3 experiments each ($\pm rad$) are summarized. All data were from mitoses in domains 1, 3, or 5, anterior to the cephalic furrow.

phase and telophase, that was observed in live studies of M14 (Figures 2 and 3).

Importantly, the relative increase in metaphase figures is not seen in homozygous Cyclin A mutant embryos that have been irradiated (Table 1). The simplest explanation for these data is that Cyclin A mutant cells are unable to delay metaphase-anaphase transition after irradiation. Interestingly, embryos that stained for a marker on the balancer chromosome (a 2:1 mixture of Cyclin A heterozygotes and wild type; see legend to Table 1) showed an intermediate phenotype. Thus, the duration of metaphase delay may correlate with Cyclin A gene dosage, although further work is required to strengthen this correlation.

In the above experiments, we found that the fixing of irradiated embryos needed to be delayed by 30 min rela-

tive to that of nonirradiated controls if we wanted to achieve a comparable number of dorsal epidermal cells in mitosis (Materials and methods), even though both samples were from the same timed collection. This was likely due to the presence of a premitosis delay in irradiated embryos [16]. Among irradiated embryos, the fraction of dorsal epidermal cells that were in mitosis was comparable from embryo to embryo regardless of its genotype (supplementary material and data not shown). This suggests that cells of Cyclin A homozygous mutants experienced a similar premitosis delay as did their heterozygous and wild-type siblings.

In an attempt to quantify DNA damage that persists through mitosis, we quantified the fraction of anaphases and telophases that showed lagging and, presumably, bro-

Figure 3

Cyclin A is stabilized after irradiation. (a) Embryos were fixed and stained for Cyclin A (blue) and DNA (red). Cyclin A is present in some metaphases (closed arrowheads), but it is absent in other metaphases (open arrowhead) and all anaphases (bracket) of control embryos. (b) A schematic interpretation of the staining data. (1) Cyclin A profile in nonirradiated controls, as deduced from the finding that 66% of metaphase cells stain for this protein; (2) Cyclin A profile expected if its degradation occurred with normal timing while metaphase lengthened independently. (3) Cyclin A profile seen in irradiated embryos, as deduced from the finding that 79% of metaphase cells stain for this protein. The bars represent, to scale, the lengths of metaphase, anaphase, and telophase in (1) control and (2,3) irradiated embryos; the duration of mitotic phases (in minutes) is compiled from live analyses of cells represented in Figure 2b and is shown above the bars. Anaphase-plus-telophase duration is scored from the time of chromosome separation (asterisk in

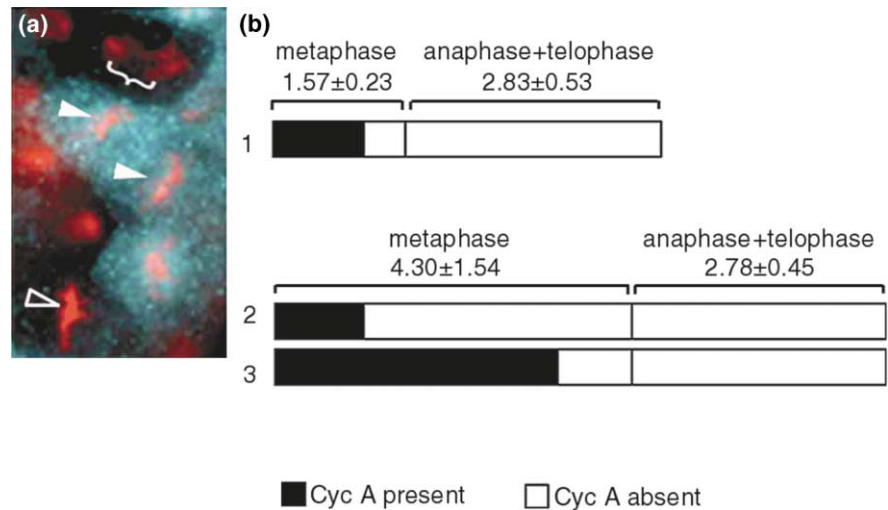


Figure 2a) to when separated nuclei reach the fully rounded state of the next interphase. All data were from mitoses in domains 1, 3, or 5, anterior to the cephalic furrow.

ken chromosomes (e.g., arrowhead in Figure 1f; see Table 1 legend). In irradiated wild-type embryos, 16% of anaphases and telophases in M15 showed lagging chromosomes (at LD₅₀ of 570R used in all our experiments), whereas this number increased to 25% in irradiated Cyclin A homozygotes (Table 1). The increase in lagging chromosomes in Cyclin A mutants suggests that the delay in metaphase-anaphase transition, a delay that is absent in Cyclin A mutants, normally serves to reduce chromosome breakage in subsequent anaphase and telophase.

Metaphase delay in cellular embryos differs in key aspects from how cleavage stage nuclei respond to DNA damage

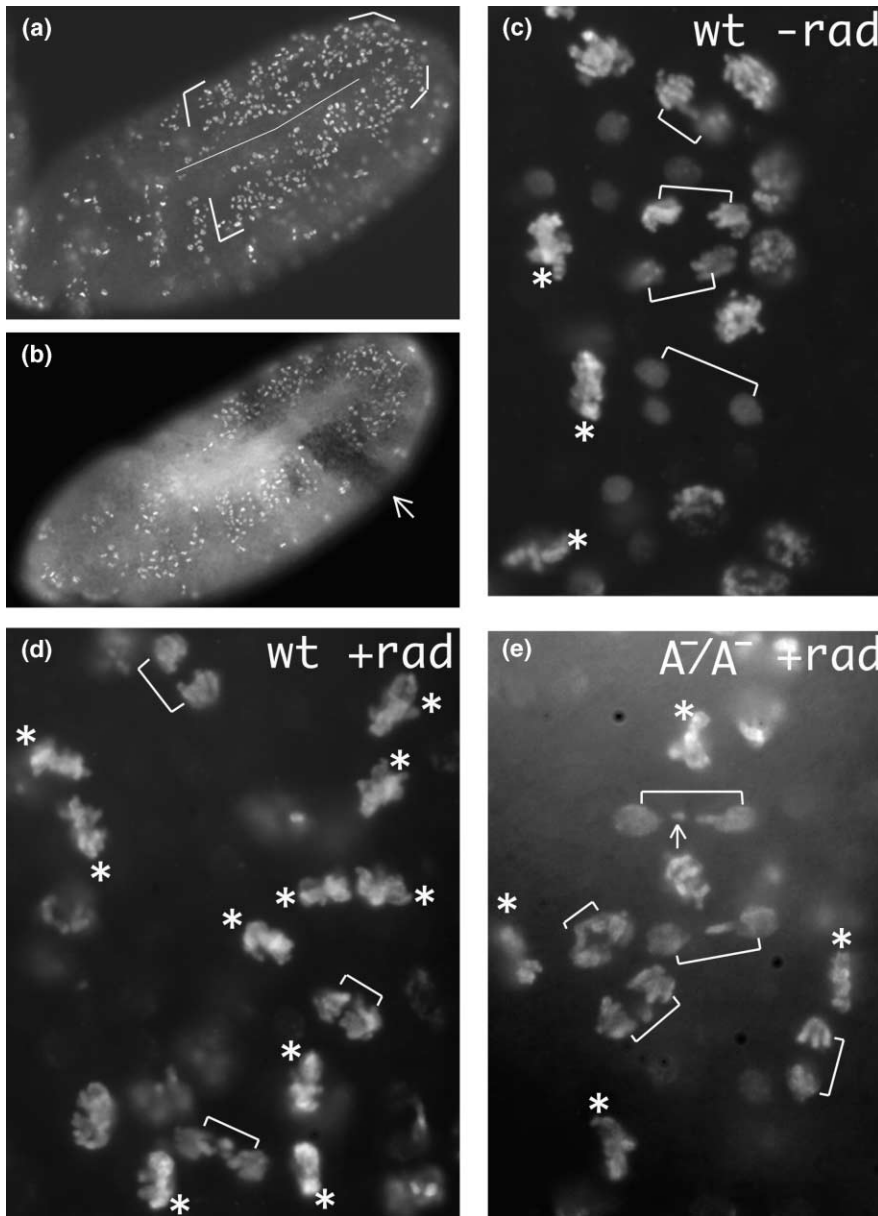
Previous work showed that DNA damage in precellular, cleavage stage embryos results in the failure of chromosomes to congress onto the metaphase plate, the failure to execute anaphase, and the subsequent abortion of mitosis [14, 15]. A recent study found that in such mitoses centrosomes lose components of the γ -tubulin ring complex and that the resulting spindles are anastral and broad-poled [15]. Impaired centrosomes and anastral spindles were proposed to cause the failure to align and segregate chromosomes after DNA damage in cleavage embryos. The spindles we observed in irradiated cellularized embryos, in contrast, appeared focused, and γ -tubulin remained at the poles (Figure 5b,c). These observations suggest that centrosomes retain at least some organizing capability. These spindles also appeared to be functional; unlike what was observed in precellular embryos, chromosomes in irradiated cellular embryos compacted on the metaphase plate just as well as did those in nonirradiated controls, and anaphase occurred, albeit with a delay (Figures 2a and 5b). We did, however, observe a diminishment

of astral microtubules during M14 in irradiated embryos, although the extent of loss was variable from experiment to experiment (Figure 5b represents the worst cases). Interestingly, in M15 the difference in the abundance of astral microtubules in irradiated and control cells was less pronounced than in M14 (compare Figure 5e,f to 5a,b). More importantly, the spindles in the irradiated wild type and in irradiated Cyclin A mutants were indistinguishable (compare Figure 5f with 5h) even though cells in the former, and not the latter, delayed metaphase-anaphase transition. We conclude that the loss of astral microtubules may be one consequence of DNA damage but is insufficient to prolong metaphase in cellular embryos.

Discussion

We describe here a novel regulatory point in metazoan mitosis that occurs in response to damaged DNA. In studies of fixed and live *Drosophila* embryos we found that ionizing radiation and a DNA damaging drug cause a delay in metaphase-anaphase transition. This delay occurs in addition to a delay in the entry into mitosis [16], which remains the best characterized regulatory point for metazoan mitosis in response to DNA damage [1, 29]. Delaying the entry into mitosis requires the inhibition of mitotic kinase Cdk1 [16]. We report here that, first, delaying metaphase-anaphase transition accompanies the stabilization of Cyclin A and that, second, it does not occur in Cyclin A mutants. Stabilization of Cyclin A is sufficient to block cells in metaphase in cellular embryos. We therefore suggest that the primary mechanism for the delay in metaphase-anaphase transition after irradiation is the stabilization of a mitotic cyclin, and hence its associated Cdk1 activity. Thus, the ability to arrest the mitotic phase of a

Figure 4



Cyclin A mutants do not prolong metaphase after irradiation. Wild-type embryos or those from heterozygous Cyclin A mutant parents were treated with 570 R of X-rays, allowed to rest for 1 hr, and fixed. Staining embryos for PH3 allowed visualization of mitotic chromosomes. **(a)** A wild-type embryo. The dorsal epidermis flanks the midline (white line) at this stage and is shown enclosed by brackets. **(b)** Staining Cyclin A mutant embryos with an antibody against β -galactosidase distinguished those with the balancer chromosomes (positive for β -galactosidase) from homozygous mutant embryos that lack the balancer (no β -galactosidase). An embryo positive for β -galactosidase (histochemical stain indicated by the arrow) is shown here. The embryos are shown with the anterior end to the left and the ventral side down. **(c–e)** Cells of the dorsal epidermis from (c) wild-type control (“wt”), (d) irradiated wild-type, and (e) irradiated homozygous Cyclin A mutant (“A⁻/A⁻”) embryos are shown magnified. Each cell that was scored as metaphase is indicated by an asterisk. The state of maximal compaction at metaphase was judged by physical compaction of chromosomes as well as by maximal PH3 immunofluorescence. Brackets indicate cells in anaphase and telophase, but not all such cells are bracketed for the sake of visual clarity. Note a fragmented chromosome in (e) (arrow).

single cell cycle at two distinct points, before initiation and during chromosome segregation, relies on mechanisms that have opposite effects on Cdk1 activity.

It is possible that stabilization of Cyclin A is a secondary consequence of a delay in metaphase rather than a cause for it. We do not favor this possibility for two reasons. First, Cyclin A is unstable during a spindle checkpoint arrest in metaphase in both *Drosophila* and other systems [34, 35]. Thus, a delay in metaphase alone cannot delay Cyclin A proteolysis. Second, Cyclin A appears to be necessary for the metaphase delay after irradiation (this work) and to be sufficient for a metaphase delay in general

[17, 18]. Thus, our proposal that stabilization of Cyclin A mediates a metaphase delay after irradiation is the simplest one that fits the data. Note also that the metaphase delay reported here is mechanistically distinct from the metaphase delay that occurs in response to spindle defects. This is because Cyclin A is stable during the first response but unstable during the second.

In budding yeast, the only other cell type known to delay metaphase-anaphase transition in response to DNA damage, this delay occurs by the stabilization of Pds1p [7, 8, 30, 31]. Thus, the stabilization of molecules that normally inhibit metaphase-anaphase transition appears to be a re-

Table 1

Quantification of mitotic phases and broken chromosomes in the wild type and in Cyclin A mutants

Genotype	Radiation	m/(a + t)	n	Percent with lagging [†] chromosomes	n
WT	–rad	0.82 ± 0.23	559	0 ± 0	103
	+rad	2.35 ± 1.16*	465	16 ± 10	178
<i>cycA</i> [–] / <i>CyO</i> [†] or <i>CyO</i> / <i>CyO</i>	–rad	0.77 ± 0.29	403	0 ± 0	94
	+rad	1.20 ± 0.53	423	18 ± 6	146
<i>cycA</i> [–] / <i>cycA</i> [–]	–rad	0.78 ± 0.21	671	0 ± 0	112
	+rad	0.88 ± 0.27	362	25 ± 6	145

* This large standard deviation is consistent with the wide range of metaphase duration observed in live studies (Figure 2b). Here the data ranged from 1.25 to 4.40.

[†] Both Cyclin A heterozygotes (*cycA*[–]/*CyO*) and their siblings that carry only the balancer (*CyO*/*CyO*) would have shown β-galactosidase staining and could not be distinguished in these experiments. However, the former should outnumber the latter by 2-fold. Additionally, because the histochemical deposit quenched immunofluorescence, we examined embryos with weaker β-galactosidase staining, which were likely to be heterozygotes with a single copy of *CyO*. Therefore, we believe this particular sample to be biased toward heterozygotes.

[‡] Lagging chromosomes are defined as those that are physically detached from the main pair of separating chromatin masses. To clearly discern the detachment it was necessary to visualize PH3-stained chromosomes through a 100× objective lens. We chose to count lagging chromosomes rather than chromosome bridges because the former is a clear indicator of chromosomal breakage whereas the latter may reflect ongoing attempt at resolution that may or may not result in breakage later.

WT = wild type, n = the number of cells counted. For each data point cells from 9 to 13 embryos, from three separate experiments, were counted.

response to damaged DNA that is in common between *Drosophila* and budding yeast. In addition to the delay in metaphase-anaphase transition, budding yeast can also delay the exit from mitosis (i.e., the progression beyond anaphase) in response to DNA damage [8, 31]. Recent data suggest the existence of a similar delay in human cells [11]. Our finding that the combined length of anaphase and telophase remained unchanged after irradiation (Figure 3b), despite the presence of DNA defects through these phases (Figure 1e–h and Table 1), suggests that these phases are not affected by irradiation in *Drosophila* cellularized embryos under our experimental conditions.

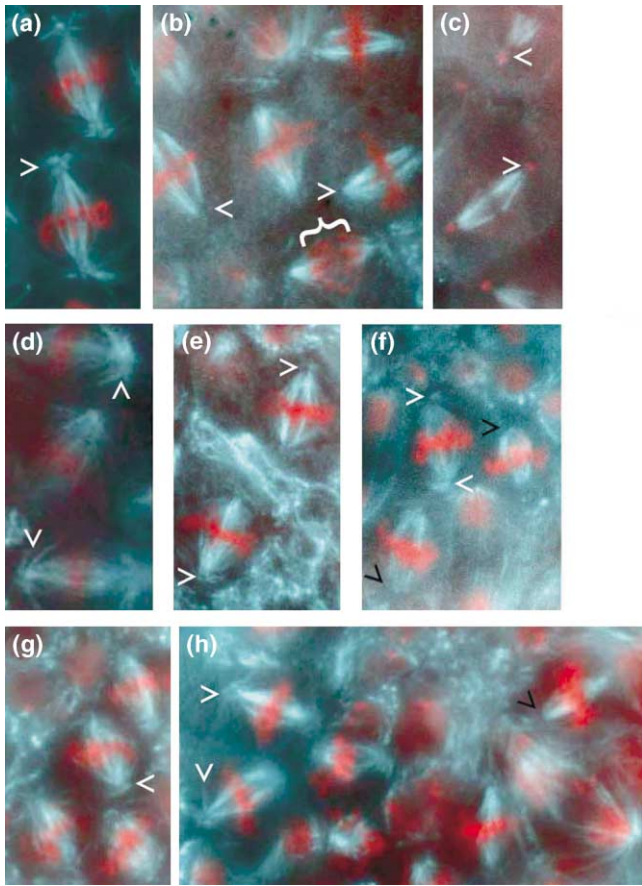
In *S. cerevisiae*, stabilization of Pds1p inhibits the proteolysis of B-type cyclins by APC and thereby ensures that exit from mitosis and cytokinesis are prevented during a delay in metaphase. We noted above that Cyclin B, which normally becomes degraded after metaphase-anaphase transition [17], persists during prolonged metaphases that follow irradiation (data not shown). Thus, DNA damage in *Drosophila* may also lead to stabilization of B-type cyclins during the delay in metaphase, much like in budding yeast. What role Cyclin A plays, if any, in the stabilization of Cyclin B after DNA damage remains to be examined.

It is formally possible that the delay in metaphase-anaphase transition is simply due to a direct physical hindrance to chromosome separation imposed by aberrant DNA structures. We do not favor this explanation for three reasons. First, aberrant DNA structures cannot easily account for the observed inhibition of cyclin proteolysis. In addition, in *Drosophila pim* mutants sister chromosomes do not separate, but mitotic progression is otherwise normal. This indicates that a hindrance to chromosome separation alone cannot modulate other mitotic

steps [25]. Second, our observation of chromosome bridges indicates that spindles *can* segregate unresolved sister chromosomes, although not always to completion. Third, irradiated cells of Cyclin A mutants failed to delay metaphase-anaphase transition even though these cells presumably suffered as much DNA damage as wild-type controls that were irradiated simultaneously. Thus, damaged DNA alone cannot prolong metaphase. Any delay in chromosome segregation, therefore, is more likely to be due to regulatory control rather than to physical hindrance.

The delay in metaphase-anaphase transition in cellular embryos that we report here might seem similar to the failure in chromosome segregation seen in response to damaged DNA in precellular-stage embryos [15]. Important phenomenological and mechanistic differences, however, distinguish these responses. First, in precellular embryos that initiate mitosis in the presence of damaged DNA, chromosomes fail to compact and reach a recognizable metaphase configuration, mitosis is aborted, and interphase is resumed without an intervening anaphase. In the irradiated cellular embryos that we observed here, chromosomes clearly condensed and compacted onto metaphase plates, and anaphase always followed; in live analyses we never observed an abortion of mitosis under our experimental conditions. Second, irradiation in precellular embryos results in the loss of γ-tubulin ring components from the centrosome and the loss of astral microtubules. These have been proposed to account for the failure to compact and segregate chromosomes in precellular embryos. In contrast, in irradiated cellular embryos γ-tubulin remains at the spindle poles, and the spindles appear functional. Although we did observe a diminishment of astral microtubules, it is unclear what role this plays in prolonging metaphase (see above). Instead, our data iden-

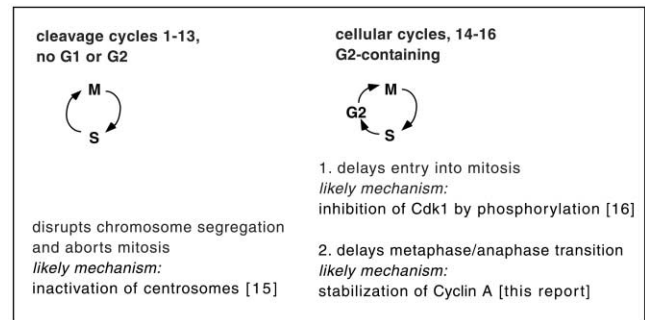
Figure 5



Spindles and centrosomes after irradiation. **(a–d)** Spindles and centrosomes in M14. (a) Control embryos show metaphase figures (DNA in red) enclosed in bipolar spindles (α -tubulin in blue). Astral microtubules (arrowheads) are apparent. (b) In mitoses that follow irradiation, astral microtubules are diminished (arrowheads), but chromosomes compact and segregate (bracket). (c) Anastral spindles, such as those in (b), still retain γ -tubulin (red) at the poles (arrowheads). (d) Spindles in cells arrested in metaphase with stable versions of Cyclin A (not shown) or cyclins A and B together (shown here) exhibit asters (arrowheads), which indicates that stabilization of cyclins alone is not sufficient to diminish astral microtubules. **(e–h)** Spindles (α -tubulin in blue) and centrosomes (DNA in red) in cells of the dorsal epidermis in M15. (e) Nonirradiated wild type; (f) irradiated wild type; (g) nonirradiated Cyclin A homozygote; (h) irradiated Cyclin A homozygote. Astral microtubules appear to be less abundant in M15 than in M14 in nonirradiated controls (compare [e] and [g] to [a]). In (f) irradiated wild-type cells and (h) irradiated Cyclin A mutants the loss of astral microtubules is variable (white arrowheads show some astral microtubules, while black arrowheads show a near-complete loss). Importantly, we see little difference between the wild-type and Cyclin A mutants.

tify the stabilization of Cyclin A as a likely mechanism for the metaphase delay in cellular embryos. Finally, the delay in chromosome segregation in precellular embryos seems to target these nuclei for elimination. In cellular embryos, on the other hand, prolonging metaphase seems

Figure 6



A summary of how mitosis is regulated in response to DNA damage in early *Drosophila* embryos. Data from this and previous work are summarized [15, 16]. See text for details.

to promote successful anaphase because cells that cannot delay metaphase-anaphase transition are more likely to show lagging chromosomes in anaphase.

We suggest that the above-described differences seen in cleavage and cellular embryos reflect a developmental change in how chromosome segregation is regulated in response to DNA damage in *Drosophila* (summarized in Figure 6). Prior to cellularization, nuclei share a common cytoplasm. The loss of a few nuclei at these stages may be of little consequence to the embryo. In addition, nuclear division cycles are extremely rapid (approximately 10 min per cycle; [13]). Speed, rather than fidelity, may be the goal in these cycles, which eliminate damaged nuclei rather than pausing to fix them. Mechanisms therefore exist to selectively “cull” damaged nuclei, which subsequently “fall into” the embryo interior and become incorporated into yolk [14, 15, 32, 33, 37]. The centrosomes are closely associated with individual nuclei and are thought to aid in attaching nuclei to the embryo cortex [13, 37]. Compromising centrosomal function in precellular stages, therefore, may be a way both to abort mitosis at a single nucleus and to eliminate it. In cellular stages “falling in” is no longer an option because a cell membrane encloses each nucleus. Therefore, a delay in metaphase-anaphase transition may allow time to resolve DNA defects and increase the chance of a successful anaphase. Note also that stabilization of an anaphase inhibitor may be an unsuitable mechanism for precellular embryos in which nuclei share a common cytoplasm; stabilization of molecules near one nucleus may affect its neighbors.

The finding that cells that can delay metaphase-anaphase transition after irradiation show a lower incidence of chromosome breakage in anaphase suggests that repair mechanisms operate during cell division. Although there is no direct evidence for this in mitosis, there is precedence for

DNA repair on condensed chromosomes, namely during meiotic crossing over.

In a previous report, a *Drosophila* checkpoint activity encoded by *grapes* (a homolog of Chk1) was shown to mediate the degradation of Cyclin A during cleavage cycles [38]. Here we find that DNA damage stabilizes Cyclin A. These results appear to be contradictory, but only if *grp* is also mediating the metaphase delay we see here, a possibility we are currently addressing. It is worth noting, however, that the abortion of mitosis and centrosome inactivation that occur in response to DNA damage in syncytial cycles do not require *grp* [15].

During *Drosophila* embryogenesis, the dual-arrest response to DNA damage, namely a delay in G2/M [16] and a delay in metaphase-anaphase transition [this report], is first observed in cellular cycles, prior to which abortion of mitosis occurs in response to damaged DNA [14, 15; Figure 6]. Interestingly, classic studies document a sharp increase in the radiation resistance of embryos at the time of cellular cycles 14–16 [36]. It is therefore possible that the ability to arrest mitosis at two points contributes to increased radiation resistance along with other properties, such as the onset of embryonic transcription. As noted above, arrest points during cell division, in addition to the G2/M arrest point, operate in human cells in response to DNA damage [9–11]. Which mitotic transitions these additional arrest points affect in human cells and what role proteolysis plays are important questions that remain. Understanding the whole range of cellular responses to checkpoint activation, we hope, will help us to design better treatments for diseases that result from a lack of checkpoint proteins such as ATM and p53.

Conclusions

Our observations indicate that DNA damage delays metaphase-anaphase transition in *Drosophila* cellularized embryos. This delay accompanies the stabilization of Cyclin A and requires Cyclin A. We propose that DNA damage leads to inhibition of Cyclin A degradation in a manner analogous to the inhibition of APC-dependent Pds1p degradation in budding yeast that occurs in response to damaged DNA. The delay in metaphase-anaphase transition we observe may normally serve to preserve chromosomal integrity during anaphase because cells that are unable to effect this delay exhibit a higher incidence of chromosomal breakage. We believe this to be the first report of metaphase-anaphase transition being delayed in response to DNA damage in a metazoan cell cycle.

Materials and methods

Fly stocks

Fly stocks that carry GFP-histone transgenes [22] and heat-inducible transgenes for stable versions of *Drosophila* cyclins A and B [17, 18] have been described before. Stable versions of cyclins lack the N-termini that target the proteins for ubiquitin-mediated proteolysis. The stock carrying stable Cyclin A also contained a heat-inducible Cdk1 transgene

[16]. All transgenic stocks are homozygous for the transgene insert. The Cyclin A^{CBLR1} used in this work is a complete-loss-of-function allele [28]. It results in lethality when homozygous and is carried over a *CyO* balancer that contains the Ubx-LacZ transgene.

Irradiation and heatshock

For fixed studies, embryos were collected for 2 hr on grape agar plates at 25°C and aged for 2 hr to reach cycle 14. In some experiments embryos were collected for 3 hr and aged for 1 hr. In such broad collections of embryos, those in cycle 14 were identified by morphogenetic markers such as germ-band extension, which is not influenced by irradiation [16]. Embryos were irradiated at 2.2 rad/sec in a TOR-REX120D X-ray generator (Astrophysics Research) by the placement of agar plates facing up on shelf 6. The generator was set at 5 mA and 115 kV. For MMS treatment, embryos were dechorionated with 50% bleach for 2 min, washed extensively with ddH₂O, and permeabilized by incubation in octane for 4 min. Permeabilized embryos were immersed in Schneider culture medium containing 0 or 0.1% (v/v) methyl methane-sulfonate (Sigma). Embryos were fixed as described below. For the induction of stable cyclins, embryos were collected for 2 hr and aged for 2 hr at 25°C, incubated at 37°C for 30 min to induce transgenes, and allowed to recover for 2 hr at 25°C before fixing.

For analyses in cycle 15, embryos were collected for 40 min and aged for 3.5 hr at 25°C before irradiation at 570 R. Control (nonirradiated) embryos were fixed at 30 min after the end of irradiation, while irradiated embryos were fixed 1 hr after the end of irradiation. This protocol compensated for a delay in entry into mitosis 15 in irradiated embryos and thus allowed dorsal epidermal cells to be in M15 in both control and irradiated embryos.

For live studies, 2 hr, 10 min–2 hr, 40 minute–old embryos (in S phase of cycle 14 [13]) were irradiated with 570 R, allowed to rest for 1 hr, dechorionated, rinsed with ddH₂O, and placed on a glass cover slip by the use of a soft brush. Excess water was removed, and a thin layer of halocarbon 700 oil (Sigma) was placed on the embryos. The cover slip was inverted and placed over a hole in the center of a microscope slide such that oil-covered embryos were otherwise exposed to air. Time-lapsed pictures were taken with a 100× objective lens on a Leica DMR scope and a Sencicam CCD camera. Exposures of 20 ms were made at the lowest illumination setting. The exposures were initiated manually at 0.5 min intervals, and the exact time, as recorded by Slidebook software (Intelligent Imaging Innovations), of each was used in calculations of mitotic duration.

Fixing and staining

Embryos were dechorionated with 50% bleach and fixed either for 5 min in a bilayer of heptane:37% formaldehyde (for tubulin staining) or for 20 min in a bilayer of heptane:PBS + 10% formaldehyde (for all other antibodies). Fixed embryos were blocked in PBT + 3% normal goat serum for at least 1 hr before they were stained with antibodies. PBT is PBS (140 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.4]) + 0.2% Tween. Primary antibodies were used at the following dilutions: affinity purified polyclonal anti-Cyclin A antibody [17], 1:100; monoclonal anti- α -tubulin (Sigma), 1:100; affinity-purified rabbit polyclonal anti- γ -tubulin (Sigma), 1:200; affinity-purified rabbit polyclonal anti-phospho-histone H3 (Upstate Biotechnologies), 1:1000. Rabbit polyclonal anti- β -Gal antibody (5prime-3prime Inc.) was preabsorbed against embryos at 1:10 dilution prior to its use at a final dilution of 1:250. Staining primary antibodies with FITC- or rhodamine-conjugated secondary antibodies (Jackson) diluted at 1:500 allowed for their detection. Anti- β -Gal antibody was detected with an HRP-conjugated secondary antibody (Amersham) used at 1:200 dilution followed by histochemical staining. Embryos were also stained with 10 μ g/ml bizbenzimidazole (Hoechst33258) in PBT for 10 min to allow visualization of DNA.

Quantification of mitotic phases and lagging chromosomes

To count metaphase, anaphase, and telophase figures, we photographed embryos through a 40× objective lens on a Leica DMR microscope by

using a CCD camera (Sensicam) and Slidebook software (Intelligent Imaging Innovations). Mitotic phases were counted by the visual inspection of PH3-positive chromatin on the computer screen. "Metaphase" is defined as the state of maximal chromosome compaction into a rectangular shape (see examples in Figures 1, 2, and 4); "anaphase" is considered to be when two distinct masses of condensed chromosomes are discernable; and "telophase" consists of pairs of chromatin masses that retain PH3 staining. Each pair of anaphase/telophase figures is counted as one. We found it hard to define "prophase" clearly because acquisition of PH3 staining is gradual and highly heterogeneous within a nucleus.

Supplementary material

A figure showing three representative irradiated embryos of each phenotype (homozygous *A/A* mutants and either heterozygous *A/CyO* or wild-type *CyO/CyO*) can be found with the electronic version of this article at <http://current-biology.supmat.com>.

Acknowledgements

We thank Bill Sullivan, Smruti Vidwans, Mark Winey, and Natalie Ahn for comments on the manuscript; Paul DiGregorio and Pat O'Farrell for Cyclin A antibodies; the O'Farrell Lab for fly stocks; and Rob Saint for the histone-GFP stock. This work was supported by a Research Project Grant from the American Cancer Society to T. T. S. and by a National Institutes of Health predoctoral training grant to B. J.

References

- Elledge SJ: **Cell cycle checkpoints: preventing an identity crisis.** *Science* 1996, **274**:1664-1672.
- Jin P, Gu Y, Morgan DO: **Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells.** *J Cell Biol* 1996, **134**:963-970.
- Jin P, Hardy S, Morgan DO: **Nuclear localization of Cyclin B1 controls mitotic entry after DNA damage.** *J Cell Biol* 1998, **141**:875-885.
- Rhind N, Furnari B, Russell P: **Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast.** *Genes Dev* 1997, **11**:504-511.
- Rhind N, Russell P: **Tyrosine phosphorylation of cdc2 is required for the replication checkpoint in *Schizosaccharomyces pombe*.** *Mol Cell Biol* 1998, **18**:3782-3787.
- Toyoshima F, Moriguchi T, Wada A, Fukuda M, Nishida E: **Nuclear export of Cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint.** *EMBO J* 1998, **17**:2728-2735.
- Cohen-Fix O, Koshland D: **The anaphase inhibitor of *Saccharomyces cerevisiae* Pds1p is a target of the DNA damage checkpoint pathway.** *Proc Natl Acad Sci USA* 1997, **94**:14361-14366.
- Tinker-Kulberg RL, Morgan DO: **Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage.** *Genes Dev* 1999, **13**:1936-1949.
- Bunz F et al.: **Requirement for p53 and p21 to sustain G2 arrest after DNA damage.** *Science* 1998, **282**:1497-1501.
- Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B: **14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage.** *Nature* 1999, **401**:616-620.
- Smits VAJ, Klompmaaker R, Arnaud L, Rijksen G, Nigg EA, Medema RH: **Polo-like kinase-1 is a target of the DNA damage checkpoint.** *Nat Cell Biol* 2000, **2**:672-676.
- Scolnick DM, Halazonetis TD: **Chfr defines a mitotic stress checkpoint that delays entry into metaphase.** *Nature* 2000, **406**:430-435.
- Foe VE, Odell GM, Edgar BA: **Mitosis and morphogenesis in the *Drosophila* embryo.** In *The Development of Drosophila melanogaster*. Edited by Bate M and Martinez Arias A. Cold Spring Harbor, New York: CSHL Press; 1993:149-300.
- Fogarty P, et al.: **The *Drosophila* grapes gene is related to checkpoint gene *chk1/rad27* and is required for late syncytial division fidelity.** *Curr Biol* 1997, **7**:418-426.
- Sibon OC, Kelkar A, Lemstra W, Theurkauf WE: **DNA-replication/DNA-damage-dependent centrosome inactivation in *Drosophila* embryos.** *Nat Cell Biol* 2000, **2**:90-95.
- Su TT, Walker J, Stumpff J: **Activating the DNA damage checkpoint in a developmental context.** *Curr Biol* 2000, **10**:119-126.
- Sigrist S, Jacobs H, Stratmann R, Lehner CF: **Exit from mitosis is regulated by *Drosophila* fizzy and the sequential destruction of cyclins A, B and B3.** *EMBO J* 1995, **14**:4827-4838.
- Su TT, O'Farrell PH: **Chromosome association of minichromosome maintenance proteins in *Drosophila* mitotic cycles.** *J Cell Biol* 1997, **139**:13-21.
- Foe VE: **Mitotic domains reveal early commitment of cells in *Drosophila* embryos.** *Development* 1989, **107**:1-22.
- Toczyski DP, Galgoczy DJ, Hartwell LH: **CDC5 and CKII control adaptation to the yeast DNA damage checkpoint.** *Cell* 1997, **90**:1097-1106.
- Lanni JS, Jacks T: **Characterization of the p53-dependent postmitotic checkpoint following spindle disruption.** *Mol Cell Biol* 1998, **18**:1055-1064.
- Clarkson M, Saint R: **A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for *Drosophila* chromosome behavior.** *DNA Cell Biol* 1999, **18**:457-462.
- Yamamoto A, Guacci V, Koshland D: **Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s).** *J Cell Biol* 1996, **133**:99-110.
- Funabiki H, et al.: **Fission yeast Cut2 required for anaphase has two destruction boxes.** *EMBO J* 1997, **16**:5977-5987.
- Leismann O, Herzig A, Heidmann S, Lehner CF: **Degradation of *Drosophila* PIM regulates sister chromatid separation during mitosis.** *Genes Dev* 2000, **14**:2192-2105.
- Lehner CF, O'Farrell PH: **Expression and function of *Drosophila* Cyclin A during embryonic cell cycle progression.** *Cell* 1989, **56**:957-968.
- Lehner CF, O'Farrell PH: **The roles of *Drosophila* cyclins A and B in mitotic control.** *Cell* 1990, **61**:535-547.
- Knoblich JA, Lehner CF: **Synergistic action of *Drosophila* cyclins A and B during the G2-M transition.** *EMBO J* 1993, **12**:65-74.
- Rhind N, Russell P: **Mitotic DNA damage and replication checkpoints in yeast.** *Curr Opin Cell Biol* 1998, **10**:749-758.
- Gardner R, Putnam CW, Weinert T: **RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast.** *EMBO J* 1999, **18**:3173-3185.
- Sanchez Y, et al.: **Control of the DNA damage checkpoint by *chk1* and *rad53* protein kinases through distinct mechanisms.** *Science* 1999, **286**:1166-1171.
- Fogarty P, Kalpin RF, Sullivan W: **The *Drosophila* maternal-effect mutation grapes causes a metaphase arrest at nuclear cycle 13.** *Development* 1994, **120**:2131-2142.
- Sibon OC, Laurencon A, Hawley R, Theurkauf WE: **The *Drosophila* ATM homologue Mei-41 has an essential checkpoint function at the midblastula transition.** *Curr Biol* 1999, **9**:302-312.
- Whitfield WG, Gonzalez C, Maldonado-Codina G, Glover DM: **The A- and B-type cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition.** *EMBO J* 1990, **9**:2563-2572.
- Hunt T, Luca FC, Ruderman JV: **The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo.** *J Cell Biol* 1992, **116**:707-724.
- Wurgler FE, Ulrich H: **Radiosensitivity of embryonic stages.** In *The Genetics and Biology of Drosophila*. Edited by Ashburner M. and Novitski E. London: Academic Press; 1976:11273.
- Debec A, Kalpin RF, Daily DR, McCallum PD, Rothwell WF, Sullivan W: **Live analysis of free centrosomes in normal and aphidicolin-treated *Drosophila* embryos.** *J Cell Biol* 1996, **134**:103-115.
- Su TT, Campbell SD, O'Farrell PH: ***Drosophila* grapes/CHK1 mutants are defective in cyclin proteolysis and coordination of mitotic events.** *Curr Biol* 1999, **9**:919-922.