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The *Drosophila* Grp/Chk1 DNA Damage Checkpoint Controls Entry into Anaphase

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Summary

It is well established that DNA damage induces checkpoint-mediated interphase arrest in higher eukaryotes, but recent studies demonstrate that DNA damage delays entry into anaphase as well. Damaged DNA in syncytial and gastrulating *Drosophila* embryos delays the metaphase/anaphase transition [1–6]. In human cultured cells, DNA damage also induces a delay in mitosis [7–9]. However, the mechanism by which DNA damage delays the anaphase onset is controversial. Some studies implicate a DNA damage checkpoint [6, 7, 10], whereas other studies invoke a spindle checkpoint [8]. To resolve this issue, we compared the effects of random DNA breaks induced by X-irradiation to site-specific I-CreI endonuclease-induced chromosome breaks on cell-cycle progression in wild-type and checkpoint-defective *Drosophila* neuroblasts. We found that both the BubR1 spindle checkpoint pathway and the Grp/Chk1 DNA damage checkpoint pathway are involved in delaying the metaphase/anaphase transition after extensive X-irradiation-induced DNA damage, whereas Grp/Chk1, but not BubR1, is required to delay anaphase onset in the presence of I-CreI-induced double-strand breaks. On the basis of these results, we propose that DNA damage in nonkinetochore regions produces a Grp/Chk1 DNA-damage-checkpoint-mediated delay in the metaphase/anaphase transition.

Results and Discussion

To resolve the issue of whether the DNA damage checkpoint controls the metaphase/anaphase transition, we take advantage of whole-organism in vivo approaches and mutational analysis available in *Drosophila*. Specifically, we analyzed the effects of DNA damage on cell-cycle progression in wild-type and checkpoint-compromised third-instar *Drosophila* larval brains. At this stage, the larval brain contains two types of dividing cells: the neuroblast that divides asymmetrically to give rise to another neuroblast and a glial mother cell. We did not make any distinction between the two cell types when the brains were scored; therefore, we will refer to all the dividing cells scored in these experiments as neuroblasts.

To explore the in vivo response of cells to DNA damage, we exposed late-third-instar larvae to X-irradiation. Different doses of X-irradiation were employed: a low dose (340 Rads [R]) that does not affect the survival of the wild-type and *grp* (Grp is the *Drosophila* homolog of the Chk1 DNA damage checkpoint kinase); a moderate dose (680 R) that generates 32.2% and 34.5% X-irradiation-induced lethality in the wild-type or *grp* mutants, respectively; and a high dose (1360 R) that leads to 100% lethality of both genotypes (Figure 1A).

Untreated wild-type neuroblasts yielded a mitotic index of 3.0 (Figure 1C; see the Supplemental Experimental Procedures available with this article online for details), similar to *grp* neuroblasts (3.1). As wild-type larvae were exposed to increasing doses of X-irradiation, the mitotic index dropped progressively to a value of 1.1 for the highest dose (1360 R), reflecting a significant delay in initiating mitosis ($p < 10^{-6}$; see Table S1 for comprehensive p values analysis). No decrease in the mitotic index was observed in *grp* mutants, even after exposure to 1360 R (3.0). These results confirm the importance of Grp/Chk1 in preventing entry into mitosis in the presence of damaged DNA and are in agreement with previous studies of larval imaginal disc cells [11, 12].

To determine whether this interphase delay is specifically mediated by the DNA damage checkpoint, we analyzed mitotic indices in neuroblasts of *bubR1* mutant individuals, which have a defective spindle checkpoint [13, 14]. In untreated *bubR1* brains, the mitotic index is significantly lower than in untreated wild-type and *grp* neuroblasts (1.8 versus 3.0, $p < 0.001$). A low mitotic index in *bubR1* mutant larval brain was also observed by Basu et al. [13], who suggested that this difference is due to a more rapid progression through mitosis in the *bubR1* mutant. In any case, it is clear that in the *bubR1* mutant, the DNA damage checkpoint is intact because exposure to 680 R produces almost a 75% reduction in the mitotic index. As expected, in the *grp bubR1* double mutant, the mitotic index for the equivalent dose of X-irradiation is significantly higher than in the *bubR1* single mutant (1.1 versus 0.5, $p < 0.01$).

To determine if X-irradiation also delays progression through mitosis, as well as mitotic entry, we used a cytological assay to calculate the fraction of cells in anaphase in relation to the total number of cells in mitosis (prometaphase/metaphase and anaphase). Although X-irradiation produced numerous chromosome fragments and bridges (data not shown), anaphase is readily scored by observing separated sister chromatids. We assumed that the anaphase rate is the same in all experiments for a given genotype. In untreated larvae, regardless of genotype, about 30% of mitotic cells were in anaphase (Figure 1D). Exposure to a high dose of X-irradiation (680 R) markedly reduced the frequency of anaphase in the wild-type (18.5) and *grp* (21.1) and *bubR1* mutants (14.1). Exposure to the highest dose of X-irradiation (1360 R) produced a further reduction in the anaphase index in wild-type and *grp* mutant neuroblasts (Figure 1D). We interpret the reduction in the anaphase index as resulting from a delay of the cells in previous mitotic phases (prometaphase/metaphase). Importantly, no sig-

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A

Genotype	X-irradiation (Rads)	Number of Larvae	Survival (%)	Lethality due to X-irradiation (%)
WT	None	115	47.8*	0
	340	115	48.7	0
	680	105	32.4	32.2
	1,360	50	0	100
grp	None	88	45.5*	0
	340	94	46.8	0
	680	94	29.8	34.5
	1,360	35	0	100

*The climbing larval stage we are using for our experiments are very sensitive to manipulation

B

	Genotype	WT	grp	bubR1	grp, bubR1
	X-irradiation (Rads)				
Number of Brains	None	20	20	13	8
	340	30	25	13	9
	680	11	15	8	5
	1,360	13	15		
Number of Fields	None	2,043	2,401	1,066	956
	340	2,337	2,047	1,724	1,123
	680	1,201	1,375	353	372
	1,360	1,383	1,308		

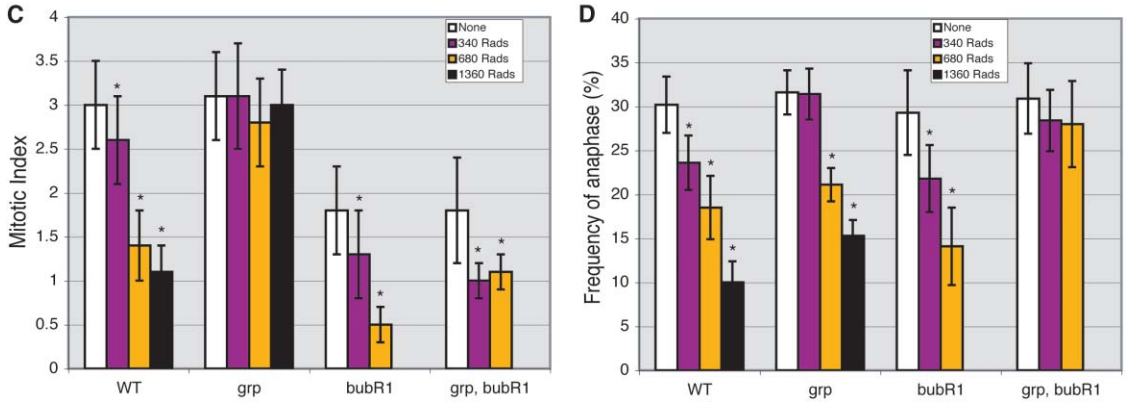


Figure 1. X-Irradiation-Induced DNA Damage Produces a Grp/Chk1-Dependent Delay in Interphase as Well as Mitosis

(A) Survival test after increasing doses of X-irradiation.

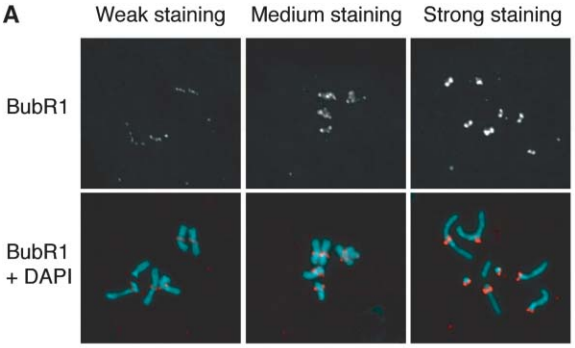
(B–D) Frequency of mitotic (C) and anaphase figures (D) of brains from third-instar larvae untreated or exposed to increasing doses of X-irradiation. In our analysis, mitosis is defined as prometaphase/metaphase (condensed chromosomes) and anaphase (separation of sister chromatids). (C) The mitotic index is defined as the number of cells in mitosis divided by the number of optical fields scored (approximately 100–200 cells/field), as previously described [15]. Because the mitotic index depends on the position of the cells in the brain, all fields scored were located in the brain periphery (a region containing mostly neuroblasts and glial mother cells, the two dividing cell types in the third-instar larvae brain). (D) The anaphase index is defined as the number of cells in anaphase divided by the total number of cells in mitosis. For each brain, we established a mitotic and anaphase index. Each column of graphs C and D represents the average of the mitotic index and the frequency of anaphase, respectively, of all brains scored for each genotype—WT (wild-type), grp (*grp/grp*), bubR1 (*bubR1/bubR1*), and grp,bubR1 (*grp,bubR1/grp,bubR1*)—presented in (B). The Mann and Whitney test was used for statistic analysis. Error bars are ± the standard deviation; * denotes significant differences from treated to untreated larvae within the same genotype (for comprehensive p value analysis, see Table S1).

nificant delay in the mitotic progression was observed in *grp bubR1* double mutants after extensive irradiation (28.0 versus 30.9). These observations suggest that the DNA damage and spindle-checkpoint components act in parallel pathways to mediate a mitotic delay after extensive X-irradiation.

To determine if Grp/Chk1 is involved in monitoring the proper microtubules-kinetochore attachment, we used colchicine to disrupt this association and activate the spindle checkpoint. Specifically, we used previously described assays to determine the frequency of anaphase and/or sister chromatid separation in wild-type, *grp*, and *bubR1* neuroblasts after colchicine treatment (Table S2) [15, 16]. In wild-type as well as *grp* mutant neuroblasts incubated with colchicine for 30–45 min, the frequency of sister chromatid separation dropped significantly, reflecting the activation of the spindle checkpoint (Table S2). These results indicate that Grp/Chk1 is not part of a checkpoint that maintains sister chromatid cohesion when kinetochores are not properly attached to the spindle microtubules.

To determine the relative roles of the DNA damage and spindle checkpoints in delaying the metaphase/anaphase transition in response to DNA damage, we scored the frequency of anaphase after exposure to a low dose of X-irradiation (340 R). This treatment induces a slight but significant reduction in the frequency of anaphase in both the wild-type (23.6 versus 30.2, $p < 10^{-7}$) and *bubR1* mutants (21.8 versus 29.3, $p < 0.001$) compared to untreated neuroblasts. In contrast, no difference in the anaphase index was observed in *grp* mutants and *grp bubR1* double mutants. Taken together, these studies suggest that Grp/Chk1 and BubR1 are involved in delaying the metaphase/anaphase transition in response to high doses of X-irradiation, but only Grp/Chk1 is involved in delaying this transition in response to low doses of X-irradiation (Figure 1D).

In order to better understand the role of BubR1 in delaying the anaphase onset in response to extensive DNA damage, we monitored the BubR1 accumulation at the kinetochore by immunostaining with polyclonal antibodies in *grp* neuroblasts untreated or exposed to low (340 R) or high (1360 R) doses of X-irradiation (using the *grp* mutation increased the frequency of cells in mitosis, making the analysis more efficient). Six brains per treatment were scored for the percentage of pro-



B

% of cells with BubR1 staining:	Weak	Medium	Strong
Control (n=542)	29.1 ± 10.4	59.6 ± 6.5	11.3 ± 6.9
340 Rads (n=852)	23.8 ± 2.6	63.2 ± 3.4	12.9 ± 2.5
1360 Rads (n=1187)	16.2 ± 5.2	64.7 ± 6.2	19.1 ± 4.1

n = number of cells scored

Figure 2. Neuroblasts Exposed to High Doses of X-Irradiation Exhibit a Greater Number of Cells with Strong BubR1 Kinetochore Staining

(A) Untreated third-instar larvae *grp* mutant neuroblasts were stained with BubR1 antibody (top and bottom panels, red) and DAPI (bottom panels, blue). In prometaphase/metaphase cells, weak (left panels), medium (middle panels), and strong (right panels) BubR1 staining is observed at the kinetochores.

(B) BubR1 kinetochore signal intensity was scored in cells at prometaphase/metaphase in untreated *grp* neuroblasts and *grp* neuroblasts exposed to 340 R or 1360 R of X-irradiation. Six brains per experiment were scored. The table presents the average of the percentage of cells with weak, medium, or strong BubR1 level at the kinetochore. n is the total number of prometaphase/metaphase cells scored for a BubR1 signal at the kinetochore.

metaphase/metaphase cells exhibiting a weak, medium, or strong BubR1 staining (Figures 2A and 2B). In untreated neuroblasts, 11.3% of prometaphase/metaphase cells exhibited a strong BubR1 staining at kinetochores. No significant difference was observed in the amount of cells with strong BubR1 signal in neuroblasts exposed to low doses of X-irradiation when compared to control neuroblasts (12.9 versus 11.3, respectively). However, in neuroblasts exposed to high doses of X-irradiation, a significant increase in the percentage of cells with strong BubR1 staining was detected when compared to untreated neuroblasts (19.1 versus 11.3, $p < 0.05$) and neuroblasts exposed to 340 R (19.1 versus 12.9, $p < 0.01$). Although it was previously demonstrated that chromosomes that have not yet been properly attached to the spindle show a strong accumulation of BubR1 at their kinetochores [13, 14], we do not fully understand the functional significance of the variations in the intensity of the BubR1 kinetochore signal in our context. But taken together with our genetic results, these variations support a model in which BubR1 is involved in delaying mitotic progression specifically in response to high doses of X-irradiation.

An explanation for these results is based on work demonstrating that the spindle checkpoint also responds to DNA damage and DNA replication defects by arresting the cell prior to anaphase independently of the DNA damage checkpoint in budding yeast and *Drosophila* [17, 18]. It may be that extensive DNA damage from high doses of X-irradiation activates the spindle checkpoint independently of its usual role in sensing the proper kinetochore attachment to the spindle. An alternative interpretation, proposed by Mikhailov et al. ([8]), is that an exposure to high doses, but not low doses, of X-irradiation damages kinetochores and activates the spindle checkpoint.

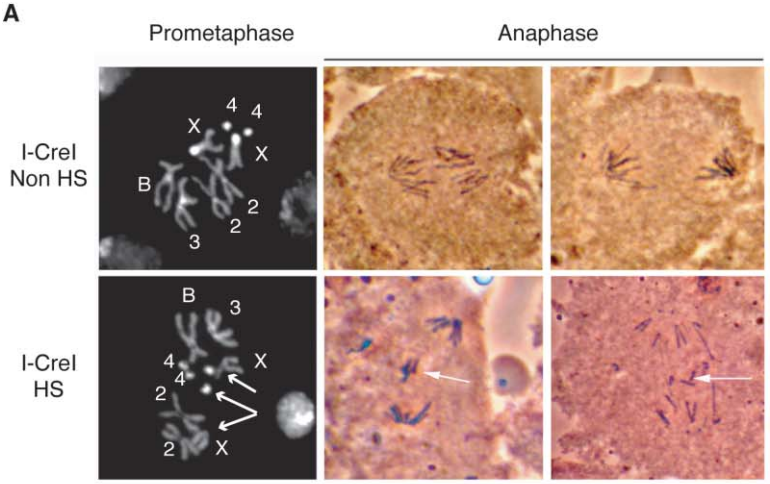
To distinguish between these alternatives, we analyzed the response of normal and checkpoint-compromised neuroblasts to DNA damage unassociated with any kinetochores. To accomplish this we used transgenic lines bearing the I-CreI restriction enzyme regulated by a heat-shock promoter [19]. I-CreI fortuitously recognizes a 20 nucleotide sequence present in the *Drosophila* 18S rDNA localized in the X chromosomes. Seventeen of twenty nucleotides match the normal I-CreI site, and this has been shown to be sufficient to generate I-CreI-induced double-strand breaks [19, 20]. Thus, heat-shock-induced I-CreI provides a means of efficiently generating double-strand breaks without damaging the kinetochore.

One hour induction of I-CreI produced a high frequency of abnormal anaphase (85%; Figure 3B), in which chromosome fragments remained at the metaphase plate (Figure 3A, arrows). Besides an overall reduction in the mitotic index (data not shown), the frequency of anaphase cells dropped significantly after I-CreI induction in comparison to uninduced I-CreI cells (I-CreI non-HS) and wild-type heat-shock cells (from 33 and 26.6, respectively, to 17, $p < 10^{-4}$), suggesting that the double-strand breaks generated by I-CreI delay the metaphase/anaphase transition (Figure 3C). To determine whether this delay depends on the spindle checkpoint, we induced the expression of I-CreI in *bubR1* mutants. The drop in anaphase frequency was essentially the same as in the wild-type ($p < 0.01$). In contrast, induction of I-CreI in *grp* mutant neuroblasts did not significantly decrease the anaphase frequency (Figure 3C). Taken together, these results suggest that DNA damage unassociated with kinetochores delays the metaphase/anaphase transition via the Grp/Chk1-based DNA damage checkpoint but independently of the BubR1 spindle checkpoint (Figure 4).

Previous studies in mammalian cultured cells have come to different conclusions regarding the action of the DNA damage checkpoint at mitosis. Mikhailov et al. ([8]) conclude that this checkpoint is not active in mitosis, whereas Chow et al. ([9]) propose that the DNA damage checkpoint acts to return cells into G2. Studies in *Drosophila* embryos, however, suggest that the checkpoint protein Mei-41 (ATR) delays mitotic exit in response to X-irradiation-induced DNA damage [6]. Because the latter two works analyzed cells artificially arrested in metaphase through the activation of the spindle checkpoint and/or used broadly acting DNA damaging agents, the results are open to alternative interpretations.

In fact, Mikhailov et al. analyzed single cells committed to mitosis in order to avoid these issues. They conclude

A



B

Genotype	Number of Brains	Number of Fields	% of Abnormal Anaphase
I-CreI (non HS)	8	1,072	0.3 ± 0.6
WT	13	2,295	1.1 ± 1.9
I-CreI	30	3,679	85.9 ± 7.6
grp	10	1,121	0.2 ± 0.5
grp; I-CreI	22	3,144	86.8 ± 7.3
bubR1	7	648	58.5 ± 14.4
bubR1; I-CreI	11	1,208	90.6 ± 6.9

C

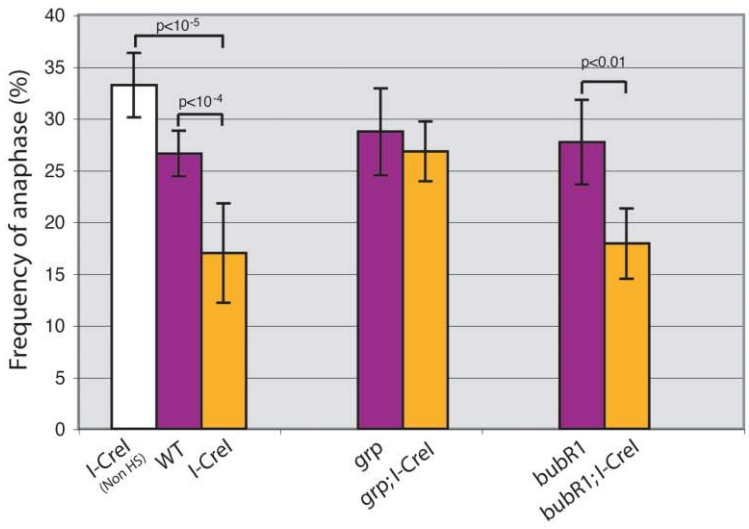


Figure 3. I-CreI Endonuclease Induction Produces Chromosome Breaks and Lagging Chromatides and Provokes a Grp/Chk1-Dependent Delay of the Anaphase Onset

(A) The I-CreI-bearing transgenic larvae are non-heat-shocked (top panels) or heat shocked for 1 hr at 37°C (bottom panels). One hour after heat shock, the brains are dissected, fixed, and stained either with DAPI (prometaphase) or Orcein (anaphase). No cytological abnormalities are observed in neuroblasts from uninduced (non-heat-shock) I-CreI-bearing transgenic larvae (top panels). Similarly, no abnormalities are observed in equivalent preparations from heat-shocked wild-type larvae (data not shown). In contrast, frequent breaks in the X chromosomes and lagging chromatid fragments are detected during prometaphase/metaphase and anaphase, respectively, in heat-shocked I-CreI-bearing transgenic larvae (bottom panels, arrows). Unlike the case of X-irradiation, few anaphase chromosome bridges are observed, indicating that I-CreI efficiently produces double-strand breaks (data not shown).

(B) Frequency of abnormal anaphase figures (lagging chromatid fragments) in third-instar larval neuroblasts after I-CreI-induced double-strand breaks. Preparations of third-instar larval brains were made 1–2 hr after 1 hr of 37°C heat-shock induction. All genotypes have been exposed to 1 hr heat shock unless indicated. We first compare the I-CreI transgenic line, non-heat-shocked (I-CreI non-HS) and heat shocked (I-CreI), and wild-type line, heat shocked (WT), not bearing the I-CreI transgene, for the frequency of anaphase (C) and abnormal anaphase (B). Because the heat-shock treatment is affecting the cell-cycle rate (WT heat shocked [C] has a lower frequency of anaphase than WT non-heat-shocked [Figure 1D]), we chose to compare heat-shocked grp (*grp/grp*) and bubR1 (*bubR1/bubR1*) larvae in the presence or absence of the I-CreI transgene for the same indices.

(C) Frequency of anaphase figures in third-instar larvae neuroblasts after I-CreI-induced double-strand breaks. Each column represents the average of all brains scored for each genotype presented in (B) (error bars are ± the standard deviation). The Mann and Whitney test was used for statistical analysis.

that the X-irradiation-induced delay of the metaphase/anaphase transition is due to damaged kinetochores' activating the spindle checkpoint. Although our studies confirm that high doses of X-irradiation activate the spindle checkpoint, presumably because of damaged kinetochores, we also find evidence for activation of the DNA damage checkpoint at metaphase. Although we cannot fully explain these different results, a key difference between the two experimental protocols is that Mikhailov et al. specifically activate the checkpoint late in prophase, well after the cells have committed to mitosis. In contrast, we are examining mitotic cells in which the DNA damage checkpoint has been previously activated in G2. It may be that once the DNA damage checkpoint has been activated in G2, it is more readily reactivated in mitosis.

Previous studies have shown that Grp/Chk1 acts during S and G2 to delay progression into mitosis in response to unreplicated and damaged DNA. Work from a number of systems shows that this is achieved through

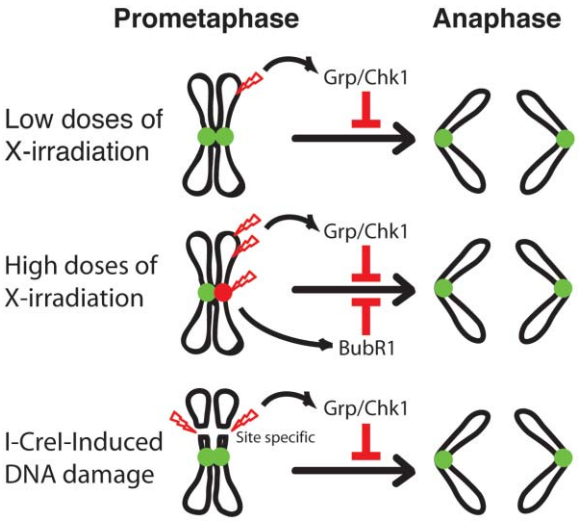


Figure 4. Activation of DNA Damage and Spindle Checkpoints during Metaphase

Mutational analysis demonstrates that high doses of X-irradiation activate both the BubR1 spindle checkpoint and the Grp/Chk1 DNA damage checkpoint at metaphase to prevent entry into anaphase. In contrast, low doses of X-irradiation only activate the Grp/Chk1 checkpoint at metaphase. As suggested by Mikhailov et al. ([8]), high doses of X-irradiation are more likely to damage the kinetochore as well as the DNA. Site-specific I-CreI-induced DNA damage unassociated with the kinetochore produces a Grp/Chk1-dependent, but not a BubR1-dependent, delay in entering anaphase.

Grp/Chk1-dependent inhibition of Cdc25, a phosphatase that activates Cdk1 [21–23]. Our observations that Grp/Chk1 delays anaphase onset, at a time in which Cdk1 activity is high, suggest that Grp/Chk1 must achieve this through a mechanism distinct from its inhibition of Cdc25 during interphase. Van Vugt et al. suggest one possibility by demonstrating that DNA damage checkpoint ATM/ATR inhibits Polo kinase activity in mammalian cells [10]. Moreover, they observe that DNA damage induces a mitotic arrest concomitant with the inhibition of Polo kinase [7]. Polo is an attractive target for Grp/Chk1 because Polo is thought to activate Cdc25 [24, 25], which triggers entry into mitosis, and it is also thought to activate the APC/C [26], which in turn triggers anaphase by promoting Securin and Cyclin B degradation [27, 28]. Alternatively, studies in the *Drosophila* gastrula indicate that DNA damage delays the metaphase/anaphase transition via the stabilization of Cyclin A [5]. Grp might control the anaphase onset by preventing Cyclin A degradation.

A third possibility is based on observations in budding yeast, in which DNA damage induces mitotic arrest and inhibition of Cyclin destruction by Chk1-dependent stabilization of Pds1 [29–31]. If this pathway persisted in metazoa, Pim, the *Drosophila* homolog of Pds1, is also a potential target for Grp/Chk1 in mitosis.

Conclusion

Previous studies have demonstrated that the checkpoint protein Grp/Chk1 acts in interphase to delay entry into mitosis in response to damaged DNA [11, 12]. Here, we provide evidence that upon DNA damage, Grp/Chk1 acts also in mitosis to delay entry into anaphase. A functional DNA damage checkpoint at mitosis would be beneficial for cells entering mitosis with chromosome breaks because it provides a final opportunity for repair. Our results imply that in higher eukaryotes, the spindle and DNA damage checkpoints temporally overlap. This suggests new strategies for killing cancer cells. For example, cancer cells with compromised DNA damage checkpoints may be particularly vulnerable to agents that compromise the spindle checkpoint.

Supplemental Data

Detailed Experimental Procedures and two supplemental tables are available at http://www.current-biology.com/cgi/content/full/15/4/334/DC1/.

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