Regulation of mitosis in response to damaged or incompletely replicated DNA require different levels of Grapes (*Drosophila* Chk1)

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Summary

Checkpoints monitor the state of DNA and can delay or arrest the cell cycle at multiple points including G_1-S transition, progress through S phase and G_2-M transition. Regulation of progress through mitosis, specifically at the metaphase-anaphase transition, occurs after exposure to ionizing radiation (IR) in *Drosophila* and budding yeast, but has not been conclusively demonstrated in mammals. Here we report that regulation of metaphase-anaphase transition in *Drosophila* depends on the magnitude of radiation dose and time in the cell cycle at which radiation is applied, which may explain the apparent differences among experimental systems and offer an explanation as to why this regulation has not been seen in mammalian cells. We further document that mutants in *Drosophila* Chk1 (Grapes) that are capable of delaying the progress through mitosis in response to IR are incapable of delaying progress through mitosis when DNA synthesis is blocked by mutations in an essential replication factor encoded by *double park* (*Drosophila* Cdt1). We conclude that DNA damage and replication checkpoints operating in the same cell cycle at the same developmental stage in *Drosophila* can exhibit differential requirements for the Chk1 homolog. The converse situation exists in fission yeast where loss of Chk1 is more detrimental to the DNA damage checkpoint than to the DNA replication checkpoint. It remains to be seen which of these two different uses of Chk1 homologs are conserved in mammals. Finally, our results demonstrate that *Drosophila* provides a unique opportunity to study the regulation of the entry into, and progress through, mitosis by DNA structure checkpoints in metazoa.

Key words: *Drosophila*, Checkpoints, Chk1, *grapes*, Embryo, Mitosis, Metaphase-anaphase transition

Introduction

Eukaryotic cells employ a variety of surveillance mechanisms or checkpoints that detect damage to DNA and act to prevent the propagation of damage to the next generation by slowing cell cycle progress, and activating repair and apoptotic pathways (Zhou and Elledge, 2000). The canonical DNA damage checkpoint is made of four conserved checkpoint kinases that regulate cell cycle progress after DNA damage and incomplete replication. Two phosphatidylinositol 3-kinase (PI3K)-like protein kinases (PI3KKs), ATR and ATM, transduce signals to Chk1 and Chk2, which then act on a number of effectors (Motoyama and Naka, 2004; Rhind and Russell, 2000).

In response to different types of damage, these four checkpoint kinases are used in different combinations that vary among different model organisms (Melo and Toczyski, 2002; Rhind and Russell, 2000). For instance, Chk1 homologs in yeast seem to be mainly required for DNA damage checkpoints, but play redundant roles in DNA replication checkpoints. In *S. cerevisiae*, scChk1 is required for Securin-dependent regulation of metaphase-anaphase transition after DNA damage, but as of yet, has no established role in responding to incomplete DNA replication (Rhind and Russell, 2000; Sanchez et al., 1999). Similarly in *S. pombe*, spChk1 is required for cell cycle arrest after exposure to UV and ionizing radiation (IR), but not upon treatment with hydroxy urea, an inhibitor of DNA replication (Brondello et al., 1999; Walworth and Bernards, 1996). In both yeast systems, Chk1 appears to act downstream of the ATR homologs, Rad3 and Mec1 in checkpoint signaling pathways (Rhind and Russell, 2000). By contrast, the opposite trend is evident in *Xenopus* where Xchk1 is activated after exposure to UV or treatment with aphidicolin, an inhibitor of DNA replication, but not in response to double strand DNA ends (Guo et al., 2000). However, similar to yeast, Xchk1 acts exclusively downstream of Xatr (Guo et al., 2000). In mammals, whether Chk1 acts together with ATM or ATR may depend on the type of damage (Liu et al., 2000; Motoyama and Naka, 2004; Takai et al., 2000); Chk1 is activated in an ATR-dependent manner after UV and HU treatments, whereas ATM is hypothesized to be the upstream activator of Chk1 after IR treatment. In any case, it appears that in mammals, Chk1 is involved in replication and DNA damage checkpoints.

Adding another dimension to this complex picture is the fact that cell cycle regulation by checkpoints changes dramatically during metazoan development. *Drosophila melanogaster* has

Table 1. Comparison of the requirement for *mei-41* and *grp* in checkpoints throughout *Drosophila* development

		Syncytial		Cellular		Larval
Checkpoint	Mutant	M entry	M progress	M entry	M progress	M entry
Incomplete replication	*mei-41*	–	+	–	ND	ND
	grp	–	+	–*	–*	ND
DNA damage	*mei-41*	NA	+	–	–	–
	grp	NA	+	+*	+*	–

Multiple checkpoints operate during *Drosophila* development to control the entry into and progress through mitosis (M entry and M progress, respectively) when replication is incomplete or DNA is damaged. Here we compare the consequence of the loss of *mei-41* or *grp* on each of these checkpoints in three different developmental stages (syncytial, cellular and larval). –, checkpoint is abrogated in a particular mutant; +, checkpoint is intact; ND, not determined. Syncytial stage embryos delay the entry into mitosis following incomplete replication (Sibon et al., 1999); however, when DNA is damaged or replication is incomplete, syncytial embryos delay the progress through mitosis via centrosome inactivation (Sibon et al., 2000; Takada et al., 2003). DNA damage does not affect entry into M (NA). The role of *grp* in regulating progress through M in response to damaged DNA in larvae was published recently (Royou et al., 2005) but not shown in the Table. *Data presented in this paper.

been an ideal model system to document such changes because of the well-characterized cell cycles that occur during development, the genetic tools that allow mutational analysis with both spatial and temporal control and because detailed cell biological characterizations are possible. We know, for example, that regulation of mitosis in response to damaged DNA can differ according to embryonic stage. During rapid cleavage cycles that occur in a common syncytium, so-called 'syncytial cycles', DNA damage results not in a delay of entry into mitosis, but in a Chk2-dependent inactivation of centrosomes and disruption of mitotic chromosome segregation (Sibon et al., 2000). Thirteen syncytial cycles are followed by cellularization of the embryo, onset of general zygotic transcription and gastrulation, and the introduction of the first G_2 phase into cell cycles (Foe et al., 1993). During these cell cycles, DNA damage now results in a checkpoint-dependent delay of entry into mitosis (Su et al., 2000). Thus, regulation of the cell cycle by checkpoints can drastically change during development.

Drosophila Chk1, encoded by *grp*, along with the ATR homolog, *mei-41*, are required to delay the entry into mitosis when DNA replication is incomplete during syncytial cycles (Fogarty et al., 1997; Sibon et al., 1999) (Table 1). Neither *grp* nor *mei-41*, however, is needed for DNA damage responses in syncytial cycles (Sibon et al., 2000) (Table 1). After cellularization, cells of the embryonic ectoderm undergo three additional cell cycles (cycles 14-16) during gastrulation and arrest in G_1 until the embryo hatches into a larva. Both *mei-41* and *grp* are required to delay mitosis in response to damaged DNA in the larva (Brodsky et al., 2000) (Table 1), and the role of *mei-41* has been documented for the DNA damage and replication checkpoint in gastrulating embryos (Table 1). The role of *grp* in either DNA damage or replication checkpoints in the gastrula stage has not been investigated before and forms the focus of this work.

Previously, we have shown that cells of the *Drosophila* gastrula can regulate both the entry into and progress through mitosis in response to either incomplete DNA replication or DNA damage by IR (X-rays) or an alkylating agent (MMS) (Su and Jaklevic, 2001; Su et al., 2000). Here, we use live measurement of mitotic phase duration to establish that regulation of metaphase-anaphase transition (mitotic progress) occurs in response to IR during all three gastrula cell cycles. We find that grp^1 mutants are capable of regulating both the entry into mitosis and the metaphase-anaphase transition in response to IR, but are unable to delay either transition when DNA replication is inhibited (Table 1). These results indicate that DNA damage and DNA replication checkpoints during the same cell cycle at the same stage in development have distinct requirements for a Chk1 homolog.

Materials and Methods

Fly stocks

All mutant alleles used here, $mei\text{-}41^{D12}$, grp^1, $bubR1^{KO3110}$, dup^{a1} and dup^{a3}, have been described before (Fogarty et al., 1997; Laurencon et al., 2003; Whittaker et al., 2000). Fly stocks carrying Histone H2Av-GFP transgene has also been described previously (Clarkson and Saint, 1999). The chromosomal deficiency uncovering *grp*, Df(2L)H2O, was obtained from Bloomington stock center as was the deficiency uncovering *dup*, Df(2R)Jp1. Double mutants were generated by standard *Drosophila* techniques.

Antibodies

Polyclonal antibodies to Grp were generated at a commercial facility (Cocalico Biologicals Inc., Reamstown, PA). The C-terminus of Grp (amino acid 261 to the C-terminus) was expressed in bacteria and used as antigen. Antisera were purified against recombinant antigen immobilized onto nitrocellulose. Detailed purification protocol is available upon request.

Western blotting

To prepare extracts for western blotting, embryos were dechorinated with 50% bleach, washed extensively in double-distilled water, and homogenized in extract buffer (25 mM HEPES pH 7.6, 0.1 M EDTA, 12.5 mM MgCl2, 150 mM KCl, 10% glycerol, 150 mM KCl, 2 mM Na_3VO_4, 1 mM sodium fluoride, 10 mM β-glycerol-phosphate, 1 mM sodium metabisulfite, 1 mM benzamidine, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin, 1.5 mM DTT). An equal volume of 2×SDS sample buffer was added to the sample and boiled for 10 minutes before separation by SDS-PAGE and western blotting. The blot was blocked with PBT (0.2%Tween-20 + PBS, 140 mM NaCl, 10 mM Na_2HPO_4, 2.6 mM KCl, 1.8 mM KH_2PO4) containing 5% milk, probed with affinity-purified anti-Grp antibody diluted 1:200 in PBT, washed in blocking solution, probed with HRP-conjugated secondary antibody (Amersham) diluted 1:2000 in PBT, and visualized by ECL (Supersignal; Pierce, Rockford IL). Homozygous grp^1 mutant embryos from heterozygous mothers were identified by the lack of GFP signal encoded by the balancer chromosome and processed for western blotting as above.

Immunofluoresence

To examine Grapes localization, wild type (Sevelen) embryos were dechorinated in 50% bleach, washed extensively in double-distilled water and fixed for 20 minutes at room temperature in a two-phase mixture of heptane and PBS containing 10% formaldehyde. Embryos were devitallinized in methanol, washed with PBT, and blocked in PBT containing 10% normal goat serum (NGS). Embryos were stained with affinity-purified anti-Grp antibody diluted 1:50 in blocking solution. HRP-conjugated secondary antibody was used at 1:500 dilution in blocking solution and was visualized by tyramide amplification according to manufacturer's instructions (Perkin-Elmer Life Sciences, Boston MA). The embryos were also stained with Texas-Red-conjugated wheat germ agglutinin (WGA) to visualize the nuclear envelope and with Hoechst33258, at 10 μg ml^{-1} in PBT, to visualize the DNA.

To compare immunofluoresence signals for Grp, embryos were collected from wild-type parents carrying a GFP transgene or grp^1 homozygyous parents and fixed as described above. The grp^1 mutant (GFP-negative) and wild-type (GFP-positive) embryos were mixed together prior to fixing and processed for Grp immunofluoresence in the same tube. This excludes sample-to-sample variations in antibody staining and allows a direct comparison of wild-type and mutant embryos on the same microscope slide.

For analysis of *dup* and *grp dup* double mutants, embryos were collected for 30 minutes and aged for various amounts of time from 4.5 to 10.5 hours, dechorinated, and fixed as above with 3% formaldehyde in PBS. Fixed embryos were blocked in PBT containing 10% NGS and stained with a rabbit antibody to phosphorylated histone H3 (Upstate Biologicals), PH3, to visualize mitotic cells and a mouse anti-βgal antibody (Roche) to identify homozygous mutants that lack β-gal encoded by the balancer chromosome. PH3 was visualized by staining with a Rhodamine-conjugated secondary antibody diluted from 1:50 to 1:150 (Jackson). β-Gal was identified by staining with an HRP-conjugated secondary antibody diluted 1:2000, followed by Tyramide-FITC amplification. Embryos were also counterstained with the DNA dye, Hoechst33258.

Live analysis of cell cycle duration

For embryos irradiated in early interphase: for live analysis of metaphase length, embryos were collected for 30 minutes and aged for 330 minutes to reach interphase of cycle 16 (Foe et al., 1993). Embryos were irradiated with 8.3 Gy of X-rays in a TORREX X-ray generator (Astrophysics Research) set at 5 mA and 115 kV, and then allowed to rest for 30 minutes. Embryos were dechorionated as described above and transferred to a glass coverslip with a soft brush. A thin layer of halocarbon 700-oil (Sigma) was placed over embryos and the coverslip was inverted and placed over a hole in the center of a microscope slide such that oil-covered embryos were otherwise exposed to air. Live analysis began as close to 40 minutes after IR as possible. Time-lapsed images were taken with 100× objective lens on a Leica microscope using spinning-disc confocal illumination and Ultraview software for image analysis (PerkinElmer). Images were taken every 30 seconds up to 10 minutes at 150 milliseconds exposure.

For embryos irradiated in late interphase/early mitosis, embryos were collected and aged as above. However, embryos were transferred to a coverslip and prepared for visualization prior to irradiation to minimize the time between irradiation and analysis (approximately ~2-8 minutes).

Homozygous embryos from heterozygous mothers were identified by lack of immuno-staining for β-gal encoded by the balancer chromosome. After live analysis, the halocarbon oil was removed by rolling the embryo on a glass coverslip. Each embryo was fixed separately and blocked in PBT containing 10% normal goat serum, stained for 60 minutes with the primary antibody against β-gal diluted 1:300 in blocking solution, washed in blocking solution and stained with FITC-conjugated secondary antibody diluted 1:50 in blocking solution. Embryos were also counterstained with the DNA dye, Hoechst33258.

Analysis of mitotic and metaphase index

For mitotic index, embryos were collected for 10 minutes, aged for 325 minutes at 25°C to reach interphase of embryonic cycle 16 (Foe et al., 1993), irradiated, and allowed to rest for 20 minutes before fixing in 37% formaldehyde for 5 minutes. For metaphase index, embryos were collected for 60 minutes and aged for 330 minutes at 25°C before fixing (for non-irradiated controls) or aged for 310 minutes, irradiated, and rested for 40 minutes to allow cells to enter mitosis before fixing. Embryos were irradiated with 8.3 Gy of X-rays. Fixed embryos were blocked in PBT containing 3%NGS and stained with a rabbit anti-PH3 to visualize mitotic cells and a mouse anti-βgal antibody to identify homozygous mutants. Embryos were also counterstained with Hoechst33258 as above.

Statistical analysis of data

P-values were generated using the standard Student's *t*-test available with Microsoft Excel.

Results

Live analyses show a delay in the metaphase-anaphase transition in response to DNA damage in all three cellular cycles. *Drosophila* embryogeneis commences with 13 nuclear division cycles that consists of S and M phases only. These are followed by cellularization of the embryo and three additional cell cycles (cycles 14-16) that consist of S, G_2 and M phases. Most cells of the embryonic ectoderm then arrest in G_1 until the embryo hatches into a larva. We reported previously that DNA damage by X-rays or MMS affects two cell cycle transitions in cellularized *Drosophila* embryos. The first affects the entry into mitosis, whereas the second affects the metaphase-anaphase transition and requires Cyclin A, a known inhibitor of the metaphase-anaphase transition (Su and Jaklevic, 2001; Su et al., 2000). The delay in metaphase-anaphase transition was expressed as an increase in metaphase index of fixed embryos, which is defined as the ratio of metaphase cells to those in anaphase and telophase (M/A+T). This response was documented in the first two cell cycles of the cellularized embryo, cycles 14 and 15, and has been corroborated in direct measurement of metaphase length during cycle 14 in live embryos expressing a GFP-tagged Histone H2Av transgene (Su and Jaklevic, 2001). Here, we first confirmed the regulation of metaphase-anaphase transition in cycle 15 using live analysis and, second, extended it to cycle 16 (Fig. 1A,B). All three cellular cycles of the embryonic ectoderm now appear to show this response, allowing us to analyze mutants in the latest cellular cycle of embryogenesis. This is especially useful for studies of homozygous embryos from heterozygous mothers that deposit wild-type gene products into the embryo; we can, thereby, maximize the depletion of deposited maternal product.

Previously we found that *mei-41* (*Drosophila* ATR) is required to delay metaphase-anaphase transition after DNA damage in cellularized embryos (Laurencon et al., 2003) and Table 1). In those studies, $mei\text{-}41^{D12}$ mutants, in contrast to wild type, failed to show a robust increase in metaphase index in embryos fixed after DNA damage. The $mei\text{-}41^{D12}$ allele is a hypomorphic loss of function allele that can be maintained as

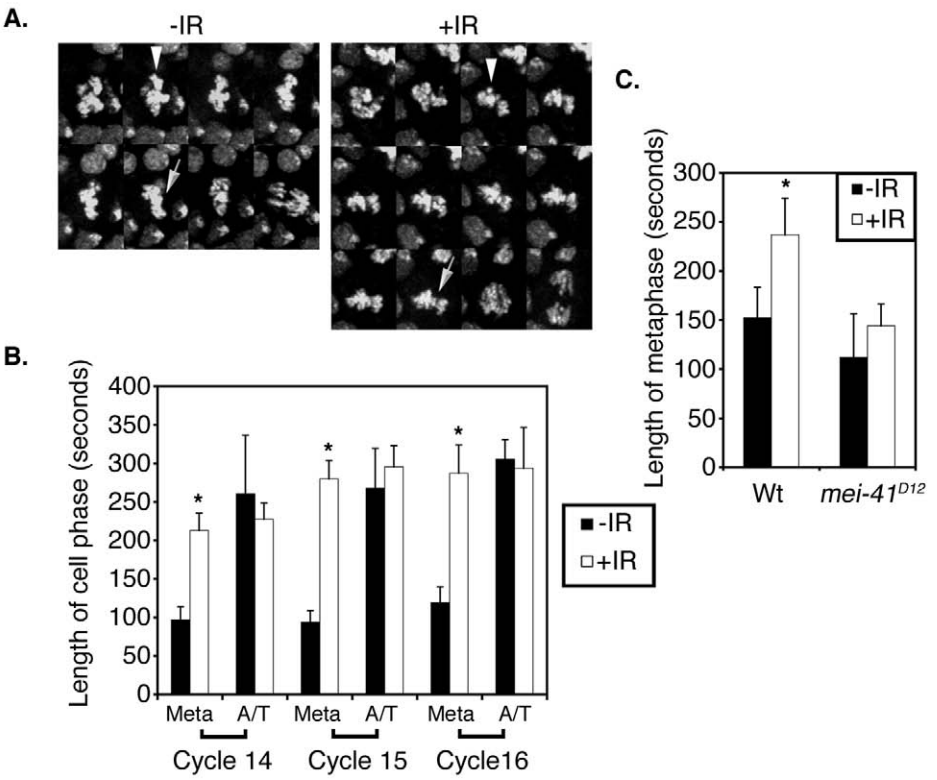

Fig. 1. After exposure to X-rays, cellularized embryos delay metaphase-anaphase transition by a *mei-41*-dependent mechanism. (A) Irradiation delayed the metaphase-anaphase transition. Montages of two mitotic cells from the dorsal ectoderm of embryos expressing a GFP-Histone H2Av transgene are shown. Embryos were irradiated with 0 (–IR) or 8.3 Gy (+IR) of X-rays and irradiated embryos were rested for 40 minutes to allow cells to enter mitosis, prior to analysis. Frames were taken every 30 seconds for up to 10 minutes. Arrowheads mark the beginning of metaphase and arrows mark the end of metaphase. (B) The length of metaphase specifically lengthens following irradiation in the 14th, 15th and 16th cellular cycles. Embryos were collected for 30 minutes and aged 130 minutes to reach cycle 14, 240 minutes to reach cycle 15 or 330 minutes to reach cycle 16. Embryos were irradiated with 0 (–IR) or 8.3 Gy (+IR) of X-rays and analyzed 0 minutes (–IR) or 40-60 minutes (+IR) later. For each time point, at least seven nuclei from at least three different embryos were analyzed. Asterisks denote statistically significant data with $P<0.0001$. *P* values were generated from the comparison with the non-irradiated control for each cycle (i.e. Cyc14 +IR versus Cyc14 –IR). (C) *mei-41* is required to delay the metaphase-anaphase transition after irradiation. Wild type (Wt) and *mei-41*^{D12} mutant embryos in cycle 16 (330-360 minutes after egg deposition, AED) were irradiated with 0 (–IR) or 8.3Gy (+IR) of X-rays and analyzed 0 minutes (–IR) or 40 minutes (+IR) later. For each time point at least 10 nuclei from four different embryos were analyzed. Asterisks denote statistically significant data with $P<0.0001$.

a homozygous stock. The *mei-41* null mutants are maternal-effect lethal; embryos from homozygous null mutant mothers die during syncytial nuclear divisions and do not reach cellular stages. By contrast, embryos from *mei-41*^{D12} homozygous mothers progress to cellular stages where metaphase-anaphase delay has been documented. Here we use live analysis to corroborate our conclusions from analysis of fixed embryos. Cells of the dorso-lateral ectoderm (e.g. enclosed in brackets in Fig. 3A) are analyzed in these studies because of their tendency to lie flat on a coverslip. The increase in length of metaphase in these cells after DNA damage is significantly shorter in *mei-41*^{D12} mutants than in wild type, confirming that *mei-41* is required to delay the metaphase-anaphase transition after DNA damage (Fig. 1C and Table 1).

The importance of cell cycle phase at time of irradiation

Most mammalian cells do not show a delay in metaphase-anaphase transition following DNA damage, and this has led to the suggestion that the delay observed in *Drosophila* embryos is specific to *Drosophila* (Mikhailov et al., 2002; Smits et al., 2000). We address here the alternate explanation that these differences are instead due to differences in the radiation dose and the cell cycle phase at the time of irradiation. In our experiments, we irradiated 310-370-minute-old embryos in which lateral ectodermal cells are in cell cycle 16, with 8.3 Gy, which represents a repairable dose since around 20% of the treated embryos survive (our unpublished data) and will be called 'LD_{80}'. We observed the most reproducibly robust increase in metaphase index at 40 minutes after irradiation (Fig. 2A). These data suggested that embryos must be irradiated during a specific phase of the cell cycle that occurs approximately 40 minutes prior to mitosis, to produce a subsequent delay in mitotic progress. Cells of the lateral ectoderm divide asynchronously, but the length of each cell cycle appears to be similar among cells and from embryo to embryo (Foe et al., 1993). The 15th and 16th cell cycles are composed of M phase (10 minutes), directly followed by S phase (approximately 35-45 minutes) and a well-defined G_2 phase (approximately 10 minutes) (Foe et al., 1993) (Fig. 2B). Following irradiation, interphase (G_2+S) increases by about 10 minutes; whether this is due to a slowing of S phase or increase in G_2 length is unknown (Su et al., 2000). This information and our data from analysis of fixed embryos (Fig. 2A) led us to hypothesize that cells must be 40 minutes prior to mitosis, i.e. in S phase, at the time of irradiation to produce a subsequent delay in metaphase-anaphase transition. To address this more directly in live analysis, embryos were irradiated and allowed to rest for 40 minutes prior to analysis of metaphase length (to examine those in S phase at the time of irradiation) or analyzed without a rest and after only the time needed for processing, which averaged about 5 minutes (to examine those in late G_2/early M at the time of irradiation). We find that the first

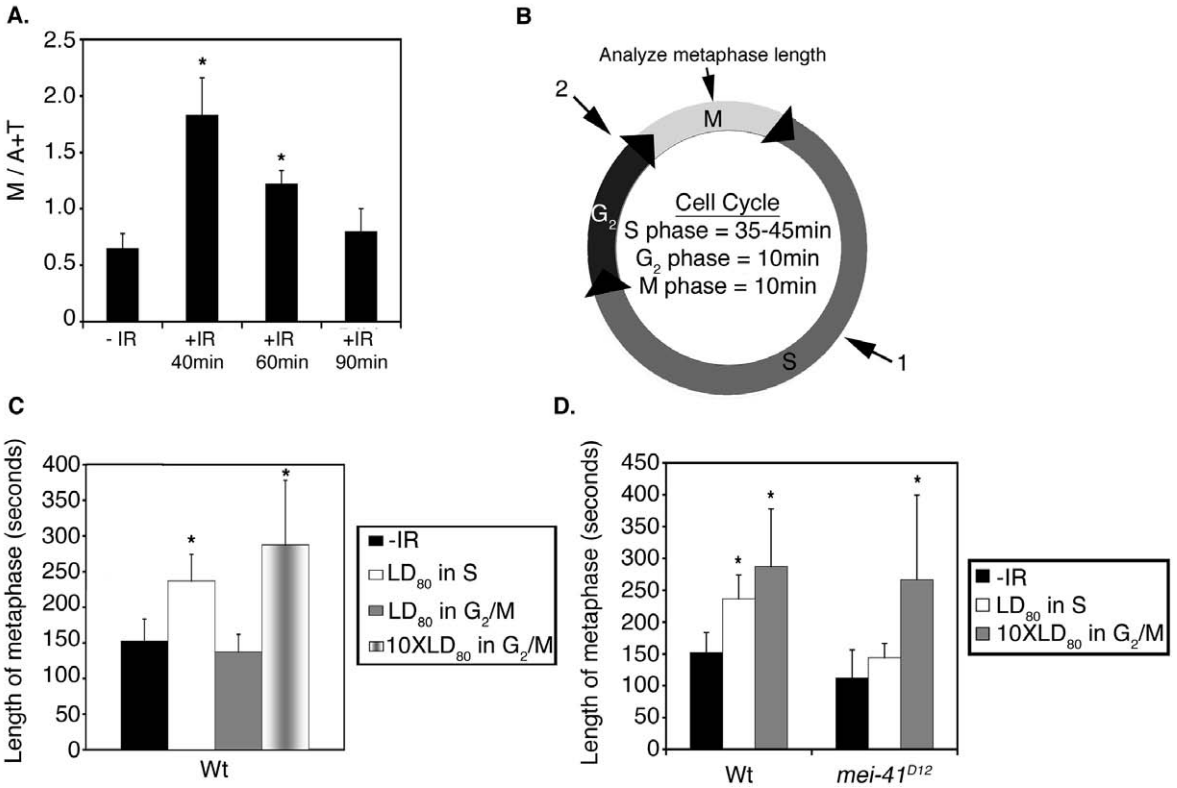

Fig. 2. Delay in metaphase-anaphase transition requires prior irradiation at specific parts of the cell cycle. (A) Metaphase index, the ratio of cells in metaphase to those in anaphase and telophase (M/A+T), is maximal at 40 minutes after irradiation. Wild-type embryos in cycle 16 (330-390 minutes AED for non-irradiated controls and 310-370 minutes AED for irradiated embryos) were treated with 8.3 Gy of X-rays (LD_{80} dose) and fixed 40, 60 or 90 minutes later. Embryos were stained with an antibody to phosphorylated histone H3 (PH3), a mitotic marker, and Hoechst33258 to visualize DNA. The number of metaphases, anaphases and telophases within the dorsal ectoderm was quantified and the metaphase index calculated. Each bar represents approximately 1000 cells from at least 10 embryos. (B) Irradiation with an LD_{80} dose during S phase, but not G_2/M, delayed metaphase-anaphase transition. Schematic of the 15th-16th mitotic cell divisions within the lateral ectoderm. The approximately 60 minutes mitotic cell cycle is separated into M phase (10 minutes), followed directly by S (35-45 minutes) and G_2 phase (10 minutes). Numbers 1 and 2 mark the approximate place within the cell cycle in which cells were irradiated (see below). (C) The ability to delay metaphase-anaphase transition is dependent upon the dose of irradiation and cell cycle phase. Wild-type embryos with ventral ectoderm in cycle 15 and dorsal ectoderm in cycle 16 (330-360 minutes AED) were irradiated with 0, 8.3 Gy (LD_{80}) or 83 Gy ($10\times LD_{80}$) of X-rays. Those irradiated with LD_{80} were allowed to recover for 40 minutes before analysis. Therefore, based on embryonic age, mitotic cells analyzed were in S phase at the time of irradiation (point 1 in B). For irradiation in G_2/M, embryos were irradiated and immediately analyzed without an intervening recovery period (point 2 in B approximately 2-10 minutes after IR). Each bar represents at least eight nuclei from six embryos. (D) The *mei-41*^{D12} mutants can regulate the metaphase-anaphase transition after exposure to $10\times LD_{80}$. Wild-type and *mei-41*^{D12} mutant embryos were irradiated with a LD_{80} dose and allowed to recover for 40 minutes before analysis (data reproduced from Fig. 1C for direct comparison), or irradiated with a $10\times LD_{80}$ dose and analyzed immediately. $10\times LD_{80}$ sample includes mitotic cells in the ventral ectoderm (cycle 15) and the dorsal ectoderm (cycle 16). For $10\times LD_{80}$, at least eight nuclei from seven different embryos were analyzed. As in Fig. 1, asterisks denote statistically significant data with $P<0.0001$. All values were compared with non-irradiated values.

set of embryos, which were irradiated in S phase, showed a delay in metaphase-anaphase transition in mitosis whereas the second set that were irradiated in late G_2/early M, did not (Fig. 2C). These data suggest that damage incurred in late G_2/early M cannot cause a delay in metaphase-anaphase transition, whereas the same level of radiation applied earlier in the cell cycle can. These observations are in agreement with results from mammalian cells in which doses of radiation that readily produced a G_2/M delay if applied in interphase had no effect on mitotic progress if applied in early M (Rieder and Cole, 1998). It is possible that damage caused by radiation at G_2/M is not recognized by the checkpoint or that the damage is sensed but that cells are unable to activate the checkpoint at such a late point in the cell cycle.

Dose-dependence of mitotic regulation

In the majority of mammalian cells, irradiation in late G_2/early M with extremely high doses (approximately 8-16-fold over what is needed to elicit a G_2/M delay if applied earlier) does induce a subsequent delay in mitotic progress (Mikhailov et al., 2002). This delay is independent of the DNA checkpoint functions ATM and ATR, and appears to be mediated by damaged centromeres/kinetochores because it requires a functional spindle checkpoint. To determine if we could induce a similar delay in mitotic progress in *Drosophila* simply by increasing the dose of radiation, we irradiated wild-type embryos in late G_2/early M with a 10-fold higher dose of X-rays ($10\times LD_{80}$). In contrast to cells irradiated in late G_2/early M with a $10\times LD_{80}$ dose, cells irradiated with $10\times LD_{80}$

lingered in mitosis (Fig. 2C). Furthermore, the delay in mitotic progress caused by $10\times LD_{80}$ still occurred in *mei-41*^{D12} embryos that are unable to delay metaphase-anaphase transition after irradiation in S phase with LD_{80} (Fig. 2D). We conclude that the regulation of mitotic progress after irradiation in late G_2/early M is not dependent on a *mei-41*-mediated checkpoint as the regulation in response to irradiation earlier in the cell cycle is. We used mutants in *bubR1* to address the to role of the spindle checkpoint in the mitotic delay caused by $10\times LD_{80}$. Unfortunately, *bubR1* mutants have significant pre-existing defects in mitotic progress without irradiation treatment, thus precluding unambiguous interpretation of the data.

grp^{1} mutants can regulate mitosis after irradiation

To further analyze the *mei-41*-dependent checkpoint that regulates mitotic progress after DNA damage, we examined mutants in the Chk1 homolog, *grp*. Grp is required during early embryogenesis and larval cycles to delay cell cycle progress: *grp* acts in the same pathway as *mei-41* in responding to incomplete replication in syncytial embryos (Sibon et al., 1999) (Table 1). Like *mei-41*, *grp* is also needed to regulate the entry into mitosis in response to ionizing radiation in the larvae (Brodsky et al., 2000; Jaklevic and Su, 2004) (Table 1). To address the role of Grp in the DNA damage checkpoint in cellular stage embryos, we quantified mitotic index (for regulation of mitotic entry) and metaphase length (for regulation of mitotic progress) in *grp*^{1} mutants after irradiation.

The *grp*^{1} allele is the strongest extant allele of grp. Homozygous or hemizygous *grp*^{1} animals from heterozygous parents survive to adulthood, presumably by using maternally supplied gene products. These females, however, are sterile; they produce embryos that fail to complete syncytial cycles and do not progress into the cellular stage (Sibon et al., 1997). The *grp*^{1} is reported to be a genetic null allele because it lacks detectable levels of mRNA and because hemizygotes behave similarly to homozygotes; both mutants exhibit the same degree of nuclear abnormalities in the early syncytial cycles (Fogarty et al., 1994).

We performed our studies of checkpoints during the cellular cycles in *grp*^{1} homozygous mutant embryos from heterozygous mothers. To analyze mitotic entry we quantified the mitotic index in embryos fixed 20 minutes after irradiation – at which time cells of irradiated wild-type embryos are still delayed before M16. A decrease in mitotic index reveals a delay in mitotic entry and activation of the mitotic entry checkpoint. The assay for metaphase-anaphase transition is performed by fixed and live measurement at 40 minutes after irradiation, at which time cells have recovered from the pre-mitotic delay and have progressed into M16 (see Fig. 1). We find that *grp*^{1} mutants can regulate the entry into M16; mitotic index decreased in mutant embryos at 20 minutes after irradiation similar to wild type (Fig. 3A,B). Similarly, metaphase index in fixed embryos and metaphase length in live embryos indicate that *grp*^{1} mutants are as capable of regulating metaphase-anaphase transition as wild type after DNA damage (Fig. 3C-E). Similar results were obtained from hemizygous mutant embryos (Df/*grp*^{1}) generated from a cross of *grp*^{1} heterozygotes with heterozygotes for a chromosomal deficiency that includes the *grp* gene (not shown). Furthermore, embryos homozygous for this deficiency (thus completely devoid of the *grp* gene and is a true genetic null) were also capable of delaying the metaphase-anaphase transition (Fig. 3D). Thus, these data suggest that mutations in *grp* do not affect the ability to regulate the metaphase-anaphase transition in cellular stage embryos when DNA is damaged.

The appearance of mitotic chromosomes in irradiated *grp* mutants is similar to those in irradiated *mei-41* mutants (not shown), even though the delay in metaphase-anaphase transition is present in the former but not the latter. In other words, gross chromosome anomalies, that might otherwise explain the delay in metaphase-anaphase transition, are not observed in *grp* mutants.

grp^{1} mutants cannot regulate mitosis when DNA replication is incomplete

We have documented above that *grp*^{1} mutant embryos are able to regulate both the entry into mitosis and mitotic progress after irradiation during cellular stages. Chk1 homologs in *Xenopus* and mammalian systems are involved in replication checkpoints and as mentioned above, Grp is also implicated in a replication checkpoint in syncytial cycles that occur earlier in embryogenesis. Therefore, we asked if the DNA replication checkpoint is functional in *grp*^{1} mutants.

We blocked DNA replication using two alleles of *double park*, a homolog of an essential replication factor, Cdt1. Mutants carrying either allele complete the first 15 embryonic cell cycles, presumably using the maternally supplied Dup. The *dup*^{a3} mutants initiate but fail to complete S16 and show a *mei-41*-dependent delay in the entry into M16 (Garner et al., 2001; Whittaker et al., 2000) (Table 1). Conversely, *dup*^{a1} mutants show no detectable DNA synthesis in cycle16 and do not significantly delay the entry into M16, consistent with the findings from other systems that initiation of DNA synthesis is necessary to activate a checkpoint that blocks mitosis (D'Urso et al., 1995; Piatti et al., 1995). Additionally, both *dup*^{a1} and *dup*^{a3} mutants subsequently arrest during M16 (Whittaker et al., 2000).

The *dup* alleles allow us to determine the requirement for *grp* in blocking the entry into mitosis in response to incomplete replication (in *dup*^{a3}) and in regulating mitotic progress in response to the same signal (in both alleles). To determine the role of *grp* in regulating mitotic entry we compared M16 in *dup*^{a3} and *grp*^{1} *dup*^{a3} mutants. At 7-7.5 hours after egg deposition (AED), cells of the lateral ectoderm of *dup*^{a3} are delayed in entering M16 as shown by the near absence of PH3 staining, in agreement with previous reports (Garner et al., 2001; Whittaker et al., 2000) (Fig. 4A). By contrast, the corresponding cells of age-matched *grp*^{1} *dup*^{a3} mutants have entered M16 and show robust PH3 staining (Fig. 4A). We conclude that *grp* is needed to delay the entry into mitosis when DNA replication is incomplete as in *dup*^{a3} mutants, and that the failure to execute this delay is apparent in *grp*^{1} mutants (Table 1). Similarly, previous work with *mei-41*^{D3} *dup*^{a3} mutants showed that *mei-41* is required to delay mitotic entry after incomplete replication (Garner et al., 2001) (Table 1).

Because *grp* affected the time of entry into M16 in *dup*^{a3} mutants, we chose not to examine the role of *grp* in regulating mitotic progress in this allele of *dup*. We reasoned that the

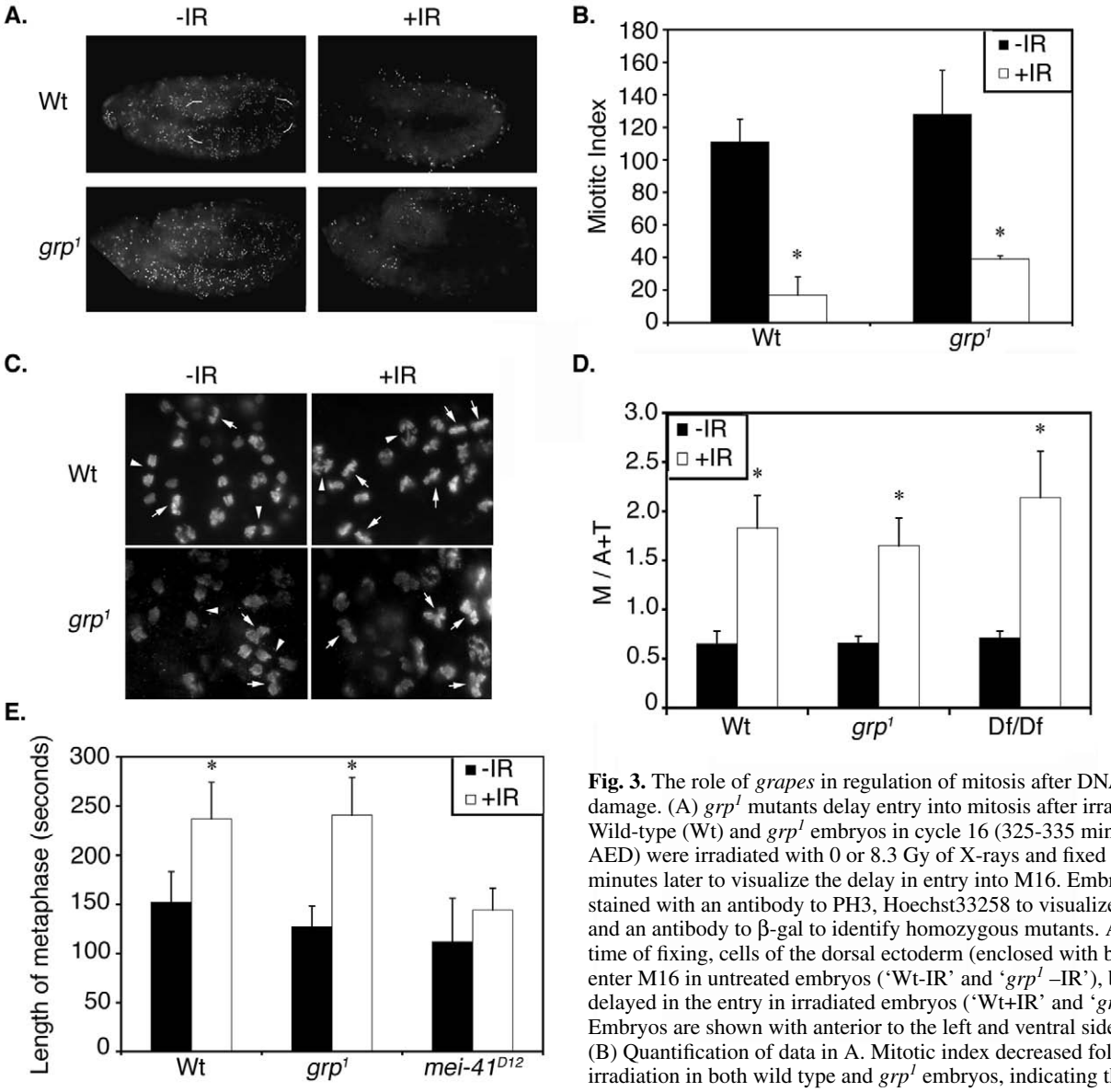

Fig. 3. The role of *grapes* in regulation of mitosis after DNA damage. (A) *grp*^{1} mutants delay entry into mitosis after irradiation. Wild-type (Wt) and *grp*^{1} embryos in cycle 16 (325-335 minutes AED) were irradiated with 0 or 8.3 Gy of X-rays and fixed 20 minutes later to visualize the delay in entry into M16. Embryos were stained with an antibody to PH3, Hoechst33258 to visualize DNA, and an antibody to β-gal to identify homozygous mutants. At the time of fixing, cells of the dorsal ectoderm (enclosed with brackets) enter M16 in untreated embryos ('Wt-IR' and '*grp*^{1} –IR'), but were delayed in the entry in irradiated embryos ('Wt+IR' and '*grp*^{1} +IR'). Embryos are shown with anterior to the left and ventral side down. (B) Quantification of data in A. Mitotic index decreased following irradiation in both wild type and *grp*^{1} embryos, indicating that both genotypes could regulate the entry into mitosis in response to damaged DNA. Mitotic index was quantified within the dorsal ectoderm for at least 10 embryos per genotype. (C,D) *grp*^{1} mutants show an increase in metaphase index after irradiation. Wild type and *grp*^{1} and mutant embryos in cycle 16 (330-360 minutes AED for non-irradiated controls and 310-370 minutes AED for irradiated embryos) were irradiated with 0 or 8.3 Gy of X-rays and fixed 0 (–IR) or 40 minutes (+IR) later. Embryos homozygous for a deficiency (Df/Df) that removes *grapes* [Df(2)H20] also showed a robust increase in metaphase index after irradiation. Metaphase index in the dorsal ectoderm was quantified as in Fig. 2 for at least 29 embryos per treatment for each genotype (D). PH3 staining of a representative sample is shown in C where arrowheads mark anaphase and telophase nuclei and arrows mark metaphase nuclei. (E) Live analysis confirmed that *grp*^{1} mutants delay metaphase-anaphase transition after irradiation as indicated by analysis of fixed embryos in A-D. Wild type and *grp*^{1} embryos in cycle 16 (330-360 minutes AED) were irradiated with 0 or 8.3 Gy of X-rays and analyzed 0 (–IR) or 40 minutes (+IR) later. Each bar represents at least 10 nuclei from four different embryos. The data for *mei-41*^{D12} mutants is reproduced from Fig. 1 for comparison. As in Fig. 1, asterisks denote statistically significant data with $P<0.0001$. All values were compared with non-irradiated values.

effects we see on mitotic progress might be secondary consequences of altered timing in mitotic entry. Instead, we used *dup*^{a1} to determine the role of *grp* in mitotic progress. Previous work has shown that *dup*^{a1} mutant embryos enter M16 on time but become delayed in M16; mitotic cells are readily visible at least until embryonic stages 13 and 14, about 3-4 hours later when mitotic activity has ceased in wild type or heterozygous siblings (Whittaker et al., 2000) (Fig. 4B). In *grp*^{1} *dup*^{a1} double mutant embryos, we find that PH3 staining is reduced at stages when mutants hemizygous for *dup*^{a1} and a deficiency that removes *dup* (*dup*^{a1}*/Df(2R)Jp1*) are still arrested in M16 (8.5-9-hour-old embryos are shown in Fig. 4B). That is, *grp*^{1} mutation rescued the elevation of mitotic index seen in *dup*^{a1} mutants.

The above finding could be because either *grp*^{1} *dup*^{a1} double mutants are progressing through M16 and exiting mitosis

prematurely (indicating a role for *grp* in regulating mitotic progress) or because they never entered M16. To distinguish between the above possibilities, we compared nuclear densities in each of the three thorasic segments in grp^1 dup^{a1} double mutants and dup^{a1} single mutants. We find that nuclear density in dup^{a1} single mutants is about 60% of that in heterozygous controls (consistent with a delay in M16) while the nuclear density in grp^1 dup^{a1} double mutants increased to levels seen in controls (Fig. 4C). In other words, cells of grp^1 dup^{a1} embryos completed M16 and show nuclear density that is normal for the stage in embryogenesis. These results support the first possibility, that double mutant cells have exited M16 while the dup^{a1} cells are still arrested in M16. We therefore infer that *grp* is needed to delay the progress through mitosis when DNA replication is incomplete (Table 1).

Grp protein is reduced in grp^1 mutants

Previous work has shown that homozygous grp^1 mutants from heterozygous mothers (the mutants used in our experiments) lack detectable *grp* mRNA. However, since females deposit both mRNA and protein into early embryos, it is possible that wild-type maternal Grp protein persists in grp^1 mutants. To address this possibility, we raised polyclonal antibodies to Grp and compared protein levels in wild type and grp^1 mutants.

Affinity purified Grp antibodies recognize a band at the predicted MW of ~55 kDa. Grp protein, similar to transcript expression, is present in newly deposited embryos and is at the highest levels during 2-8 hours AED, which loosely corresponds to cellular cycles 14-16 (Fogarty et al., 1997) (Fig. 5A). Grp levels decrease as most embryonic cells exit the cell cycle into G_1 of 17th cellular cycle and are almost absent at 12 hours AED through the rest of embryogenesis. The Grp signal is severely reduced in homozygous grp^1 mutants from heterozygous mothers, attesting to the specificity of the antibody (Fig. 5B). We find a similar level of persistent Grp protein in embryos homozygous for a deficiency that removes the entire *grp* coding region and grp^1 embryos from heterozygous mutant females of approximately 3% of Grp found in wild-type embryos of similar age (Fig. 5C and data not shown). We conclude that either Grp is not required to delay mitotic progress after irradiation or only very low levels of Grp are sufficient to do so. We cannot, however, rule out the possibility that the remaining signal in these mutants comes from a cross-reacting protein that is of exactly the same molecular mass as Grp. We found that 0-2-hour-old embryos

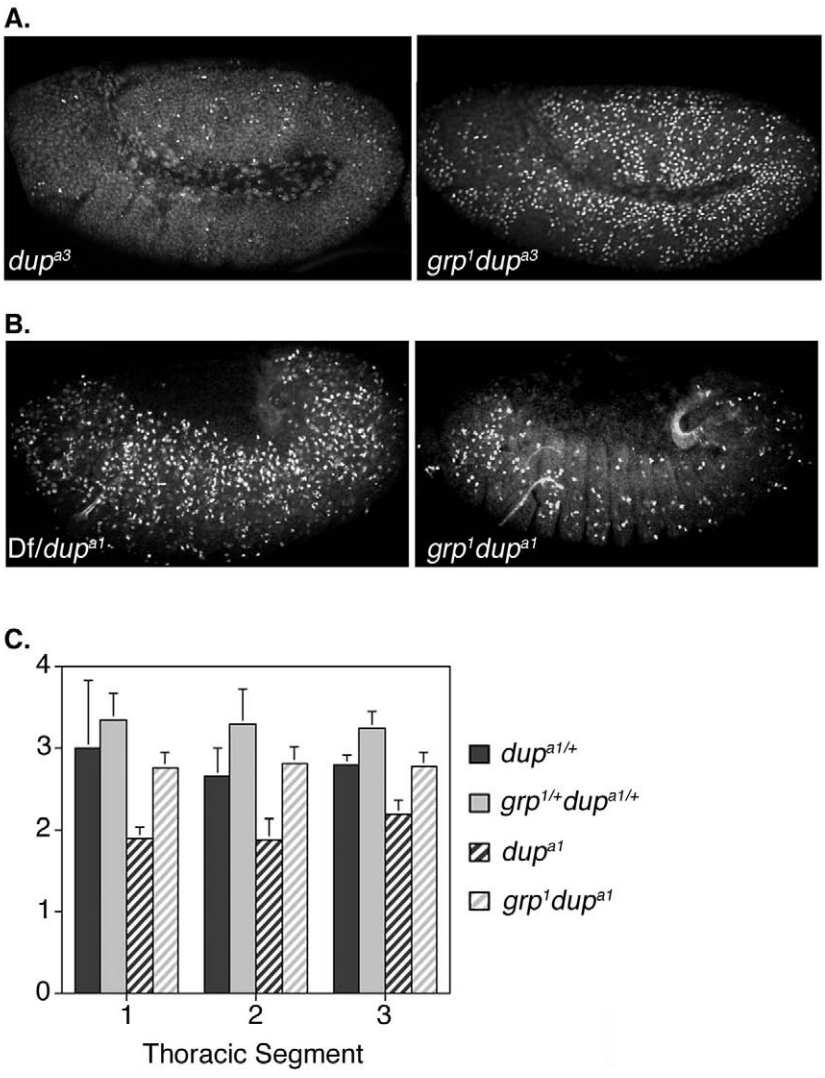

Fig. 4. grp^1 mutants fail to regulate mitosis following incomplete replication. (A) grp^1 dup^{a3} double mutants cannot delay the entry into M16 while dup^{a3} mutants can. 7-7.5-hours-old embryos were fixed and stained to visualize PH3 and DNA. Ectodermal cells in the dup^{a3} mutants are still arrested before M16 as indicated by the near absence of PH3-positive cells. By contrast, the corresponding region of grp^1 dup^{a3} mutant embryos show robust PH3 signal, indicating that double mutants are unable to delay the entry into M16. (B) grp^1 dup^{a1} double mutants fail to delay the progress through and eventual exit from M16 in contrast to Df/dup^{a1}. 8.5-9-hour-old embryos were fixed and stained with an antibody to PH3 and with Hoechst33258 to visualize DNA. Hemizygous Df/dup^{a1} mutants are arrested in M16 and show a robust number of mitotic cells in the lateral ectoderm consistent with published results. By contrast, grp^1 dup^{a1} mutants show a reduced number of mitotic cells. Hemizygous mutants (Df/dup^{a1}) were used due to decreased viability associated with dup^{a1} chromosome. (C) grp^1 mutation restores nuclear density of dup^{a1} mutants to wild-type levels. Nuclear density in Stage 12 embryos was quantified in thoracic segments 1, 2 and 3 and expressed in arbitrary units. Nuclear density is reduced in dup^{a1} mutants; this is expected, as they have not completed M16. Nuclear density in grp^1 dup^{a1} mutants resembles that of heterozygous controls, indicating that cells of the double mutant have completed cycle 16. Homozygous mutants were identified by the lack of β-gal encoded by the balancer chromosome (not shown).

(before the onset of zygotic gene expression) from homozygous *grp*^{1} mothers have significantly reduced (~5%) but not abolished levels of Grp (Fig. 5D). This could be either because *grp*^{1}, caused by a transposon insertion in the non-coding region, is a severe loss-of-function mutation and not a null, or because the cross-reacting protein is also present in pre-cellular stage embryos. We cannot distinguish between these possibilities at this point.

Immuno-staining of fixed embryos demonstrates that Grp localizes to the nucleus in interphase (Fig. 6A). Grp protein is present in syncytial embryos and is enriched in the interphase nucleus during cortical syncytial cycles (embryonic divisions 10-13). Upon nuclear envelope breakdown, Grp signal disperses into the cytoplasm for the duration of mitosis. Only after the reformation of the nuclear envelope in late telophase, does Grp re-localize to the nucleus. This is in contrast to MCMs, a family of chromatin-binding proteins, which localizes to the nucleus in interphase, disperse during mitosis, but re-accumulate onto chromosomes in anaphase prior to the reformation of the nuclear membrane (Su and O'Farrell, 1997). The kinetics of Grp nuclear localization are different and more consistent with nuclear localization via import rather than by association with chromosomes. Similar localization of Grp is observed in cycles 14-16 (not shown).

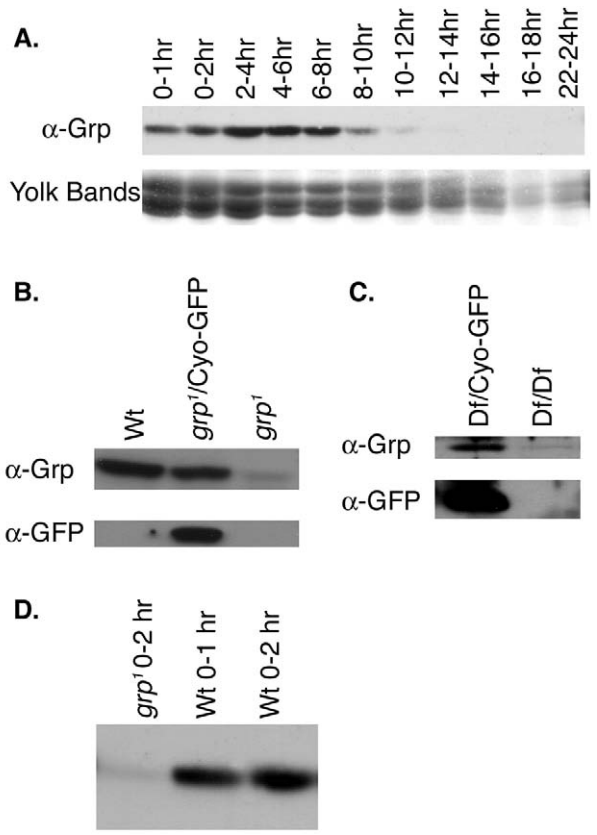

Fig. 5. Grp protein is reduced in *grp*^{1} mutants. (A) Developmental profile of Grp protein levels during embryogenesis. Embryo extracts from different times AED (indicated above lanes in A) were blotted for Grp as in experimental procedures. Approximately 50 embryos were loaded per lane. The yolk proteins, visualized by Ponceau staining, confirm equal loading for the initial part of embryogenesis, but become depleted as embryogenesis progresses. (B) The *grp*^{1} mutants have severely reduced levels of Grp protein. 6-7-hour-old *grp*^{1} homozygous or heterozygous mutant embryos from heterozygous parents were genotyped by the absence or the presence of GFP encoded by the balancer chromosome Cyo-GFP (see experimental procedures). Extracts were prepared and blotted for Grp and for GFP (to confirm genotype). Each lane contained extracts from 25 embryos. Both wild type and *grp*^{1}/Cyo-GFP heterozygotes have comparable Grp levels, while in *grp*^{1} mutants, Grp levels are reduced. (C) Grp protein persists in mutants homozygous for a deficiency that removes *grp*. 7.5-8.5-hour-old Df/Df homozygous or heterozygous mutant embryos from heterozygous parents were genotyped by the absence or presence of GFP encoded by the balancer chromosome Cyo-GFP. Extracts were prepared and blotted for Grp and for GFP. Each lane contained extracts from 10 embryos. The difference in GFP signal between B and C is due to different antibody dilutions of anti-GFP. (D) Extracts of embryos from *grp*^{1} homozygous mothers or wild type (Wt) mothers were analyzed by western blotting. Extracts from approximately 50-75 embryos were loaded in each lane. Maternal genotypes and embryo ages are shown above each lane.

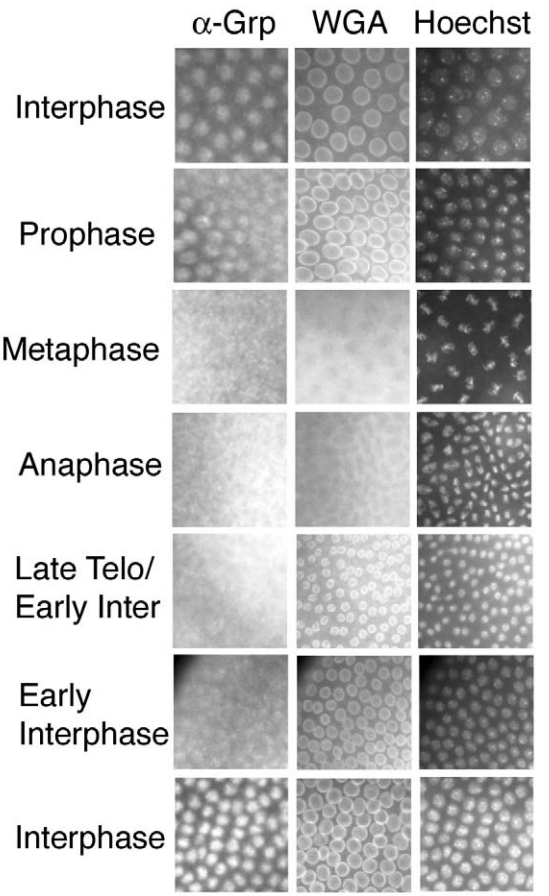

Fig. 6. Subcellular localization of Grp during embryonic cell cycles. Grp localization during syncytial divisions. 1.5-2.5-hour-old wild-type embryos were fixed and stained with an antibody to Grp (α-Grp), wheat germ agglutinnin (WGA) to visualize nuclear envelopes and Hoechst33258 to visualize DNA. Grp is nuclear during interphase and disperses from the nucleus as the nuclear envelope breaks down in mitosis. Grp re-accumulates in the nucleus after the nuclear envelope reforms in the next interphase.

Discussion

Metaphase-anaphase checkpoint

We have used live measurements of metaphase length to document the regulation of metaphase-anaphase transition in all three cell cycles of the cellularized *Drosophila* embryos. Many features of cell cycle responses to radiation in *Drosophila* appear to be conserved in mammalian cells. In both systems, irradiation in late G_2/early M does not cause a delay in mitotic progress, even though similar doses applied earlier in the cell cycle cause a checkpoint-dependent delay in entry into mitosis. In addition, in *Drosophila* embryos, irradiation during S results in a delay in the subsequent metaphase-anaphase transition. In both systems, 10-fold or higher doses of radiation applied in late G_2/early M did cause a delay in mitotic progress but this delay occurs independently of a DNA structure checkpoint. We speculate that additional cellular structures besides DNA are damaged to produce this delay. These results can account for previously reported apparent discrepancies between the behavior of *Drosophila* and mammalian cells, and indicate instead that the cells of the two systems behave similarly. Furthermore, it leaves open the possibility that irradiation, if applied earlier in the cell cycle such as during S phase, may induce a metaphase-anaphase delay in mammalian cells also.

What might be the purpose of delaying metaphase-anaphase transition? The delay is modest, lengthening metaphase by about 50%. Cell cycle delay by checkpoints are thought to allow time for DNA repair, but it is unclear if efficient repair can occur in such a short time on condensed chromosomes. It is possible that the delay acts as a signal to target damaged cells to death. It is in the interest of an organism to cull damaged cells and the presence of broken DNA during mitosis and this checkpoint may act to fulfill that purpose. If so, further work will be needed to address the mechanism that links a *mei-41*-dependent mitotic delay to cell death.

Role of *grp* in regulating mitosis after DNA damage and incomplete replication

We present evidence that grp^1 mutants can delay the entry into mitosis and the metaphase-anaphase transition following IR, but are unable to delay these cell cycle transitions when replication is incomplete. We conclude that *grp* is essential for the DNA replication checkpoints in cellular stage embryos, whereas the DNA damage checkpoint requires little to no Grp. This conclusion agrees well with roles for Grp earlier in embryogenesis; Grp is required for a replication checkpoint in syncytial stage embryos, but not when DNA is damaged by irradiation (Fogarty et al., 1997; Sibon et al., 1997; Takada et al., 2003). Grp plays a redundant role with Chk2 to regulate the entry into mitosis after DNA damage checkpoint in the larvae (Brodsky et al., 2004). Thus it is possible that Grp plays a redundant role with Chk2 in regulation of metaphase-anaphase transition in the embryo. Analysis of *grp chk2* double mutants would be informative in this regard.

A recently published report documents the regulation of metaphase-anaphase transition by checkpoints in response to DNA double-strand breaks in *Drosophila* larvae (Royou et al., 2005). This checkpoint, first described in embryos, is not limited to the embryo. This checkpoint in larvae, however, is abolished in grp^1 mutants, the same allele used in this study, indicating that the larval checkpoint requires a higher level of Grp than what is present in grp^1 mutants and is able to support a similar checkpoint in the embryo.

Other checkpoints safeguarding the M/A transition

A *bubR1*-dependent spindle checkpoint has been shown to contribute to the mitotic arrest in *dup* mutants (Garner et al, 2001). Incomplete replication of centromeres is proposed to interfere with kinetochore function in these mutants, thereby activating a spindle checkpoint. Our current findings in grp^1 dup^{a1} mutants suggest that a *grp*-dependent DNA structure checkpoint also contributes to this arrest. The involvement of both the spindle and DNA checkpoints in mitotic arrest upon incomplete DNA replication has precedence. In budding yeast, both MAD2, a spindle checkpoint protein, and Mec1, an ATR homolog, contribute to mitotic arrest in response to incomplete DNA replication (Garber and Rine, 2002; Krishnan et al., 2004).

ATR and Chk1 homologs act in the same checkpoint pathways in yeast and frogs, but can act together or in separate pathways in mammals depending on the type of damage. In *Drosophila*, Mei-41 and Grp appear to function together in most responses to DNA defects (Table 1). Both are required for a DNA damage checkpoint in larvae and to delay entry into mitosis in response to incomplete replication in syncytial embryos (Fogarty et al., 1997; Sibon et al., 1997). Delaying entry into mitosis in gastrula cycles in response to incomplete DNA replication in *dup* mutants requires *mei-41* (Garner et al., 2001) and *grp* (this report). Grp is also needed for cells of dup^{a1} mutants to remain in mitosis (this report). We have not been able to conclusively determine the role of *mei-41* in the latter response because nuclei of dup^{a1} *mei-41* double mutant embryos are severely fragmented, making determination of nuclear density as in Fig. 4 unreliable.

Chk1 homologs exist in all eukaryotes examined and contribute to the regulation of mitosis in response both to DNA damage by ionizing radiation and to incomplete DNA replication. The differential importance of a Chk1 homolog to these two different responses has been compared only in fission yeast previously. In fission yeast, Chk1 is essential for delaying the entry into mitosis in response to IR damage, but has a redundant, non-essential role in delaying the entry into mitosis in response to incomplete DNA synthesis (Boddy et al., 1998; Brondello et al., 1999; Walworth and Bernards, 1996). Our results suggest a reverse situation in fly embryos; the regulation of mitotic progress after irradiation still occurs when levels of Grp are too low to support the regulation of mitosis in the presence of incomplete DNA replication. It would be of interest to see which of these intricate Chk1 requirements, that of yeast or that of *Drosophila*, are conserved in mammals.

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