The *Drosophila grapes* gene is related to checkpoint gene *chk1/rad27* and is required for late syncytial division fidelity

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Background: Cell cycle checkpoints maintain the fidelity of the somatic cell cycle by ensuring that one step in the cell cycle is not initiated until a previous step has been completed. The extent to which cell cycle checkpoints play a role in the initial rapid embryonic divisions of higher eukaryotes is unclear. The initial syncytial divisions of *Drosophila* embryogenesis provide an excellent opportunity to address this issue as they are amenable to both genetic and cellular analysis. In order to study the relevance of cell cycle checkpoints in early *Drosophila* embryogenesis, we have characterized the maternal-effect *grapes* (*grp*) mutation, which may affect feedback control during early syncytial divisions.

Results: The *Drosophila grp* gene encodes a predicted serine/threonine kinase and has significant homology to *chk1/rad27,* a gene required for a DNA damage checkpoint in *Schizosaccharomyces pombe*. Relative to normal embryos, embryos derived from *grp*-mutant mothers exhibit elevated levels of DNA damage. During nuclear cycles 12 and 13, alignment of the chromosomes on the metaphase plate was disrupted in *grp*-derived embryos, and the embryos underwent a progression of cytological events that were indistinguishable from those observed in normal syncytial embryos exposed to X-irradiation. The mutant embryos also failed to progress through a regulatory transition in Cdc2 activity that normally occurs during interphase of nuclear cycle 14.

Conclusions: We propose that the primary defect in *grp*-derived embryos is a failure to replicate or repair DNA completely before mitotic entry during the late syncytial divisions. This suggests that wild-type *grp* functions in a developmentally regulated DNA replication/damage checkpoint operating during the late syncytial divisions. These results are discussed with respect to the proposed function of the *chk1/rad27* gene.

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Background

In many higher eukaryotes, the initial embryonic divisions are synchronous and rapid. For example, in *Xenopus* embryos, the early cell cycle alternates between M and S phases without intervening G1 and G2 phases. It appears that the speed of these early divisions may have been achieved in part by sacrificing many of the feedback controls that characterize more conventional somatic cell cycles [1]. For example, mitosis in the early *Xenopus* embryo is not dependent on DNA replication or undamaged DNA, and the initiation of anaphase is not dependent on proper spindle assembly [2,3]. Perhaps most striking is that centrosome duplication occurs in the absence of protein synthesis during the initial divisions [4]. It is likely that relaxation of these dependency relationships in the early *Xenopus* embryos reflects a lack of activated cell cycle checkpoints [5].

Early *Drosophila* development also begins with a series of rapid synchronous divisions [6]. In addition to lacking G1 and G2 stages, these divisions are syncytial — that is, they occur without accompanying cytokinesis. Nuclear cycles 1 through 8 occur in the interior of the embryo. During nuclear cycles 9 and 10, most of the nuclei migrate towards the cortex. Once at the cortex, they undergo four more rounds of synchronous divisions before cellularizing during interphase of nuclear cycle 14. In contrast to other embryonic systems, *Drosophila* embryogenesis maintains a surprising number of dependency relationships: treating syncytial embryos with drugs that disrupt the spindle results in an arrest in metaphase [7]; altered chromosome structure extends metaphase and delays entry into anaphase [8]; and centrosome duplication depends on proper DNA synthesis [9]. These dependency relationships ensure that one event in the syncytial cycle is

completed before the next is initiated and many are likely to be the result of cell cycle checkpoints.

Drosophila syncytial divisions are amenable to genetic analysis, and this provides a means of identifying components responsible for the dependency relationships. There is little zygotic transcription during the syncytial divisions and consequently these divisions are primarily under maternal control. Mutations involved in these early events therefore behave as maternal-effect mutations. Among the many maternal-effect mutations that have been examined, one class is particularly interesting because it disrupts the nuclear divisions only after the nuclei have migrated to the cortex [10]. Many of the genes affected in this class are likely to be involved in the regulation of cortical cytoskeletal dynamics [11]. However, we have shown that the gene affected by one member of this class of mutations, *grapes* (*grp*), appears to be involved in feedback controls of the early syncytial divisions [12].

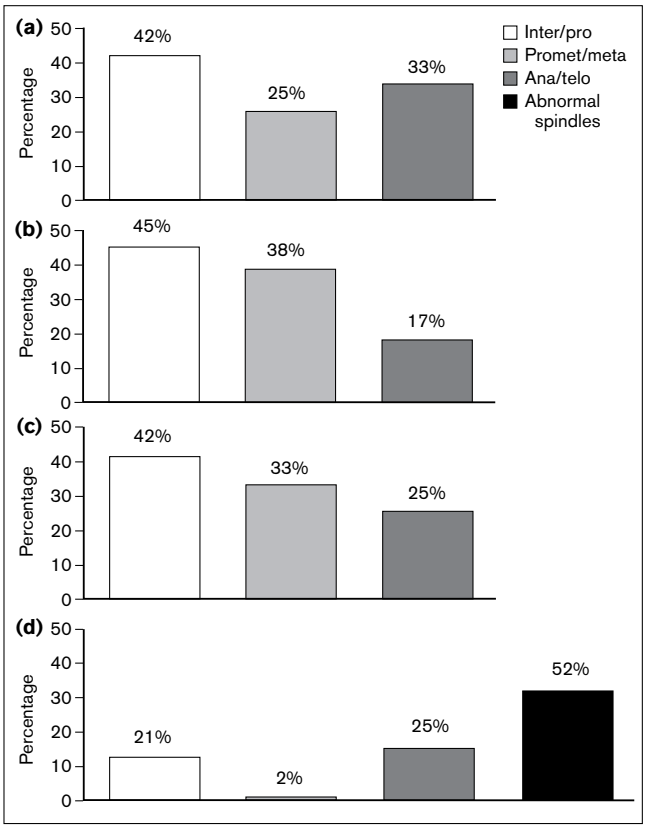
In our previous study [12], we showed that embryos derived from *grp*-mutant mothers develop relatively normally through nuclear cycle 11; however, during nuclear cycle 12, a large percentage of sister telophase nuclei snap back and fuse with one another. This results in tetraploid nuclei, with two centrosomes at each pole, which rapidly progress into an abnormal metaphase and exhibit a terminal phenotype of abnormal grape-like clusters of nuclei encompassed by dense rafts of spindles. X-irradiation analysis of *grp*-derived and normal embryos indicates that the *grp* gene may play a role in feedback controls that monitor the state of chromatin during the syncytial divisions [12]. In this paper, we report a detailed cellular analysis of *grp*-derived and X-irradiated embryos and molecular characterization of the *grp* gene which provide further evidence for this model.

Results

X-irradiation of normal syncytial embryos phenocopies *grp*-associated mitotic defects

Extending our previous observations of X-irradiated embryos [12], we have examined in detail the initial defects observed in normal syncytial embryos exposed to X-irradiation to allow comparison with abnormalities in unirradiated, nuclear cycle 12 and 13, *grp*-derived embryos. The cell cycle states of untreated and X-irradiated (using 300 rads) embryos derived from normal (*grp*/+) females were determined by staining fixed embryos for DNA and microtubules; embryos between nuclear cycles 9 and 11 were identified and scored as described in Figure 1. The increase in the prometaphase/metaphase index 8 minutes after X-irradiation (compare Fig. 1a and 1b) suggested that the initial response to DNA damage was a delay during prometaphase/metaphase. At 16 minutes after X-irradiation, the prometaphase/metaphase index remained high and fused sister telophase nuclei were frequently observed

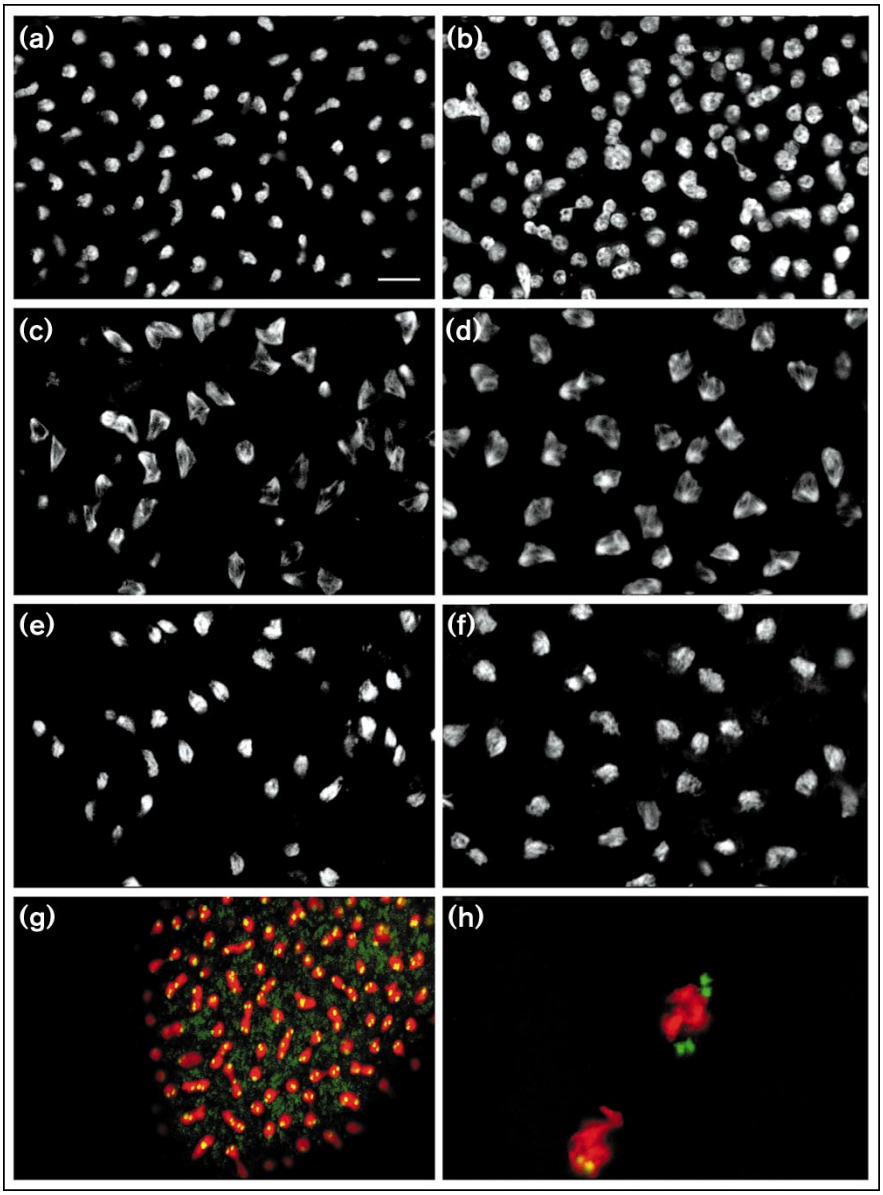
Figure 1



Alterations in the mitotic index in response to X-irradiation. Wild-type embryos, collected between 0 and 2.5 h, were either **(a)** left unirradiated (n = 167) as a control, or irradiated with 300 rads, incubated for **(b)** 8 min, **(c)** 16 min or **(d)** 25 min ($n = 114$, 52 and 52, respectively), and fixed using established protocols [29]. The fixed embryos were then double-stained to visualize microtubules and DNA [29]. Embryos between nuclear cycles 9 and 11 were identified by their nuclear density. As syncytial divisions proceed in a wave [6], the mitotic state of the embryos was scored according to the cell cycle stage of the majority of nuclei. The following criteria were used for scoring: interphase (inter) markers were large diffusely stained nuclei, no spindles, and partially separated centrosomes; prophase (pro) markers were some condensed chromatin and fully separated centrosomes; prometaphase (promet) markers were condensed chromosomes distributed asymmetrically around the metaphase plate, and distinct spindles; metaphase (meta) markers were condensed chromosomes distributed symmetrically on the metaphase plate with a distinct gap between the DNA and the spindle poles; anaphase (ana) markers were sister chromosomes forming a distinct diamond shape, and an elongated spindle; telophase (telo) markers were small round nuclear pairs with a prominent midbody (if the daughter nuclei fuse, the tetraploid product will contain duplicated centrosomes at each pole); embryos whose nuclei did not fit into any of the descriptions and which had abnormal spindles were also scored.

(Figs 1c,2b). At 25 minutes after X-irradiation, the frequency of sister telophase fusion was much higher and a large percentage of the embryos contained decondensed nuclei (Fig. 2f) encompassed by multipolar and malformed spindles (Figs 1d,2d). Many of the nuclei fixed 25 minutes after X-irradiation contained a pair of centrosomes at each

Figure 2



X-irradiation phenocopies *grp*. Sister telophase nuclei snap back and fuse in both **(a)** unirradiated syncytial *grp*-derived embryos and **(b)** normal embryos fixed 16 min after X-irradiation, as revealed by DNA staining. Embryos, either **(c,e)** *grp*-derived or **(d,f)** normal, were fixed 25 min after X-irradiation and double-stained to visualize either (c,d) microtubules or (e,f) DNA. The abnormal multipolar spindles characterizing the terminal phenotype of *grp*-derived embryos (c) were also observed in normal embryos fixed 25 min after X-irradiation (d). **(g)** Fusion of nuclei in an unirradiated *grp*-derived nuclear cycle 12 embryo. The embryo was hybridized with the histone probe (yellow) and nuclei were counterstained with propidium iodide (red). **(h)** The centrosomes (green) and DNA (red) of a normal embryo 25 min after X-irradiation; the embryo is a phenocopy of *grp*-derived embryos in which nuclei have a pair of centrosomes at each pole [12]. (a–f) Magnifications are identical; the scale bar is 10μm.

pole (Fig. 2h). As described previously, this is due to fusion of sister telophase nuclei (centrosome duplication occurs during telophase in the syncytial *Drosophila* embryo [13]). At this point (Fig. 1d), the nuclei accumulated in an abnormal multipolar metaphase.

It is striking that many of the initial defects observed in X-irradiated normal embryos were equivalent to those observed in unirradiated *grp*-derived embryos. In unirradiated *grp*-derived embryos, sister telophase nuclei snap-back and fuse during nuclear cycle 12 ([12]; Fig. 2a,g). The fusion of sister nuclei was readily observed by hybridization with a probe to the histone-gene cluster [14]; rather than the normal two clusters per nucleus, in *grp*-derived embryos many elongated nuclei contained four clusters (Fig. 2g). Consequently, nuclei with a pair of centrosomes at each pole were observed [12]. This configuration was also frequently seen in normal embryos exposed to X-irradiation (Fig. 2h). In addition, late syncytial collections of *grp*-derived embryos were characterized by multipolar spindle complexes encompassing fused nuclei that resemble those seen in the X-irradiated normal embryos (compare Fig. 2c,e with 2d,f). These shared phenotypes suggest that, during the late syncytial divisions, unirradiated *grp*-derived embryos may also be entering mitosis with damaged or incompletely replicated DNA.

Elevated levels of DNA damage in *grp*-derived embryos

To further investigate chromosome and DNA damage in *grp*-derived embryos, the number of single and double stranded breaks in DNA from *grp*-derived and normal embryos was determined using modifications of previously described procedures [15]. Genomic DNA was isolated from embryos collected between 1 and 2 hours, incubated with T4 DNA kinase and [^{32}P]ATP, and the amount of ^{32}P incorporation was determined. This value reflects the number of exposed 5′ phosphate groups in the DNA and thus the number of single stranded and double stranded lesions [16]. Genomic DNA from normal embryos assayed for ^{32}P incorporation gave values of 875 ± 175 cpm ng^{-1}, whereas that from *grp*-derived embryos gave values of 4110 ± 790 cpm ng^{-1}. This dramatic increase in lesions may be a consequence of *grp*-derived embryos progressing through mitosis with damaged or incompletely replicated DNA.

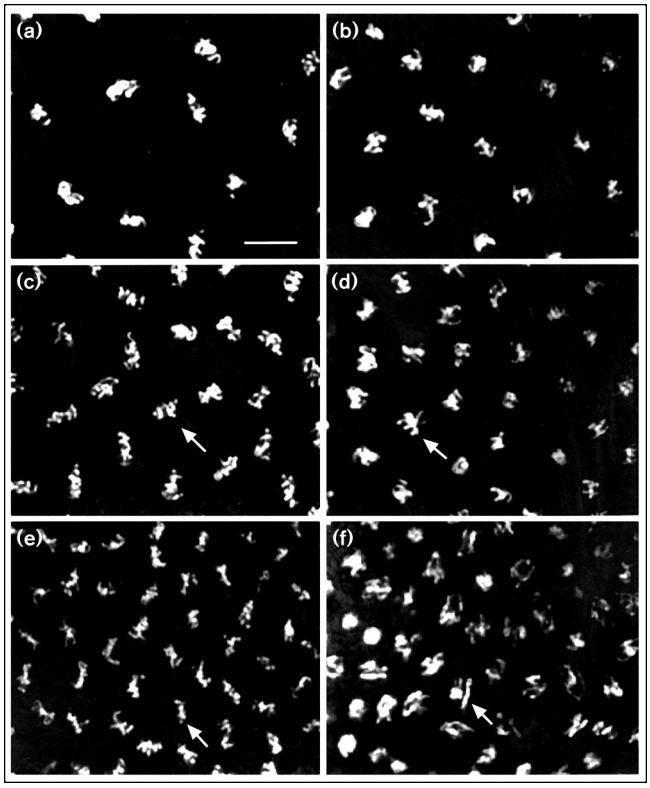
Alignment of chromosomes on the metaphase plate is disrupted in *grp*-derived embryos

Live analysis of *grp*-derived embryos revealed chromosomal abnormalities at mitosis of nuclear cycle 12, a stage prior to the mitotic defects and DNA damage described above. Congression of the chromosomes to the metaphase plate during nuclear cycles 12 and 13 was defective in *grp*-derived embryos. To observe and quantify this phenotype, normal and *grp*-derived embryos were injected with fluorescently-labeled histones, and the chromosome dynamics were followed during nuclear cycles 10–13 (Fig. 3). Analysis of the recordings enabled us to determine the point of maximum compaction of the chromosomes on the metaphase plate for each of these cycles. Cycle 11 and 12 nuclei were considered properly aligned on the metaphase plate if they fit within a 6.9×4.4 μm rectangle at the point of maximum compaction, whereas the smaller cycle 13 nuclei were considered properly aligned if they fit within a 5.8×3.3 μm rectangle. By these criteria, 95% ($n = 21$), 88% ($n = 98$), and 91% ($n = 53$) of the nuclei in normal cycle 11, 12 and 13 embryos, respectively, exhibited chromosomes properly aligned on the metaphase plate. In *grp*-derived embryos, although 87% ($n = 46$) of the nuclei exhibited properly aligned chromosomes during metaphase of nuclear cycle 11, the values for metaphase of nuclear cycles 12 and 13 were only 54% ($n = 142$) and 5% ($n = 311$), respectively.

Failure of *grp*-derived embryos to undergo the normal transition in Cdc2 regulation that follows mitosis 13

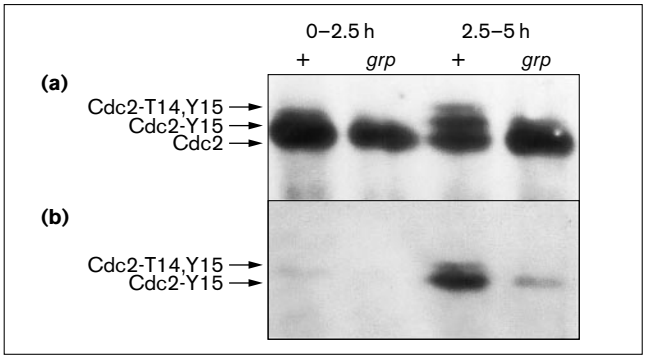
Because of the possible involvement of *grp* in a DNA-replication-dependent or repair-dependent cell cycle checkpoint, we analyzed the state of Cdc2 phosphorylation in *grp*-derived and control embryos. Both control and *grp*-derived embryos, collected between 0 and 2.5 hours, predominantly exhibited two fast migrating forms of Cdc2 (Fig. 4a); these correspond to the unmodified and the active Thr161-phosphorylated phosphoisoforms [17]. In control embryos collected between 2.5 and 5 hours (which include a significant fraction of nuclear cycle 14 embryos), two slower migrating Cdc2 isoforms are also present (Fig. 4a). Previous studies have shown that these represent two inactive phosphoisoforms of Cdc2 (phosphorylated at Tyr15 and Thr14, or Tyr15 only) that accumulate to high levels during interphase of nuclear cycle 14 [17]. Traces of the Tyr15-phosphorylated inactive Cdc2 isoform seen in the control 0–2.5 hour sample (Fig. 4b) can be accounted for by a small proportion of older embryos present in these collections. Intriguingly, the slowest migrating inhibitory phosphoisoform of Cdc2 (phosphorylated at Thr14 and Tyr15) never appears in *grp*-derived embryos collected between 2.5 and 5 hours, and the accumulation of the Tyr15-phosphorylated inactive phosphoisoform is greatly reduced (Fig. 4b). These observations verify our previous studies indicating that *grp*-derived embryos do not progress into a normal cycle 14 interphase during development [12].

Figure 3



Images of chromosomes at maximum metaphase compaction for nuclear cycles **(a,b)** 11, **(c,d)** 12 and **(e,f)** 13 from (a,c,e) wild-type and (b,d,f) *grp*-derived living embryos injected with fluorescently labeled histones [28]. During nuclear cycles 12 and 13, chromosomes in *grp*-derived embryos do not compact on the metaphase plate as tightly as those in normal embryos. Arrows indicate maximally compacted nuclei in normal and *grp*-derived embryos. The scale bar is 10 μm.

Figure 4



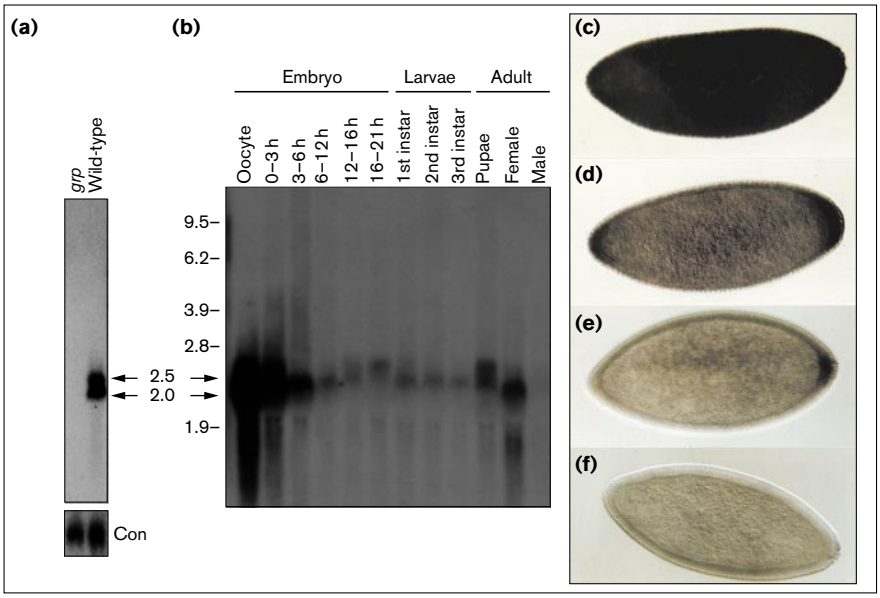
Cdc2 phosphorylation states in normal (+) and *grp*-derived (*grp*) embryos collected between 0 and 2.5 h and between 2.5 and 5 h. Cdc2 was immunoprecipitated from cytoplasmic extracts, resolved by gel electrophoresis and electroblotted. The membrane was stained with either **(a)** an antibody directed against the PSTAIRE motif of Cdc2 or **(b)** an anti-phosphotyrosine antibody. In normal embryos, slower migrating Cdc2 isoforms, phosphorylated at Thr14 and Tyr15 or Tyr15 only, as indicated, appear in the 2.5–5 h collections. These correspond to the inactive phosphoisoforms of Cdc2 which normally accumulate during interphase of nuclear cycle 14. These isoforms do not appear in similar collections of *grp*-derived embryos.

The maternally supplied *grp* message is rapidly degraded during nuclear cycle 14

Mapping and reversion studies demonstrate that the *grp*^{1} mutation is caused by an insertion of a modified transposable P-element [10]. This facilitated our isolation, by plasmid rescue, of a 9 kilobase (kb) *Xba*I fragment of genomic DNA flanking the P-element insertion. Northern analysis using this fragment as a probe detected both 2.0 kb and 2.5 kb transcripts in total RNA derived from 0–2 hour normal embryos; these transcripts were absent in total RNA from 0–2 hour *grp*-derived embryos (Fig. 5a), indicating that the transcripts were derived from the *grp* gene. Developmental northern analysis revealed that the transcripts were abundant in oocytes and in 0–3 hour old embryos (Fig. 5b). Message levels dramatically decreased in embryos that were 3–6 hours old, and were present at reduced levels later in embryogenesis and during larval development (Fig. 5b). The higher transcript levels observed during pupariation were coincident with, and probably the result of, ovarian differentiation, as they were abundant in adult females but not in males (Fig. 5b). Germline transformants containing *grp* cDNA rescued the *grp* maternally-induced lethality (data not shown).

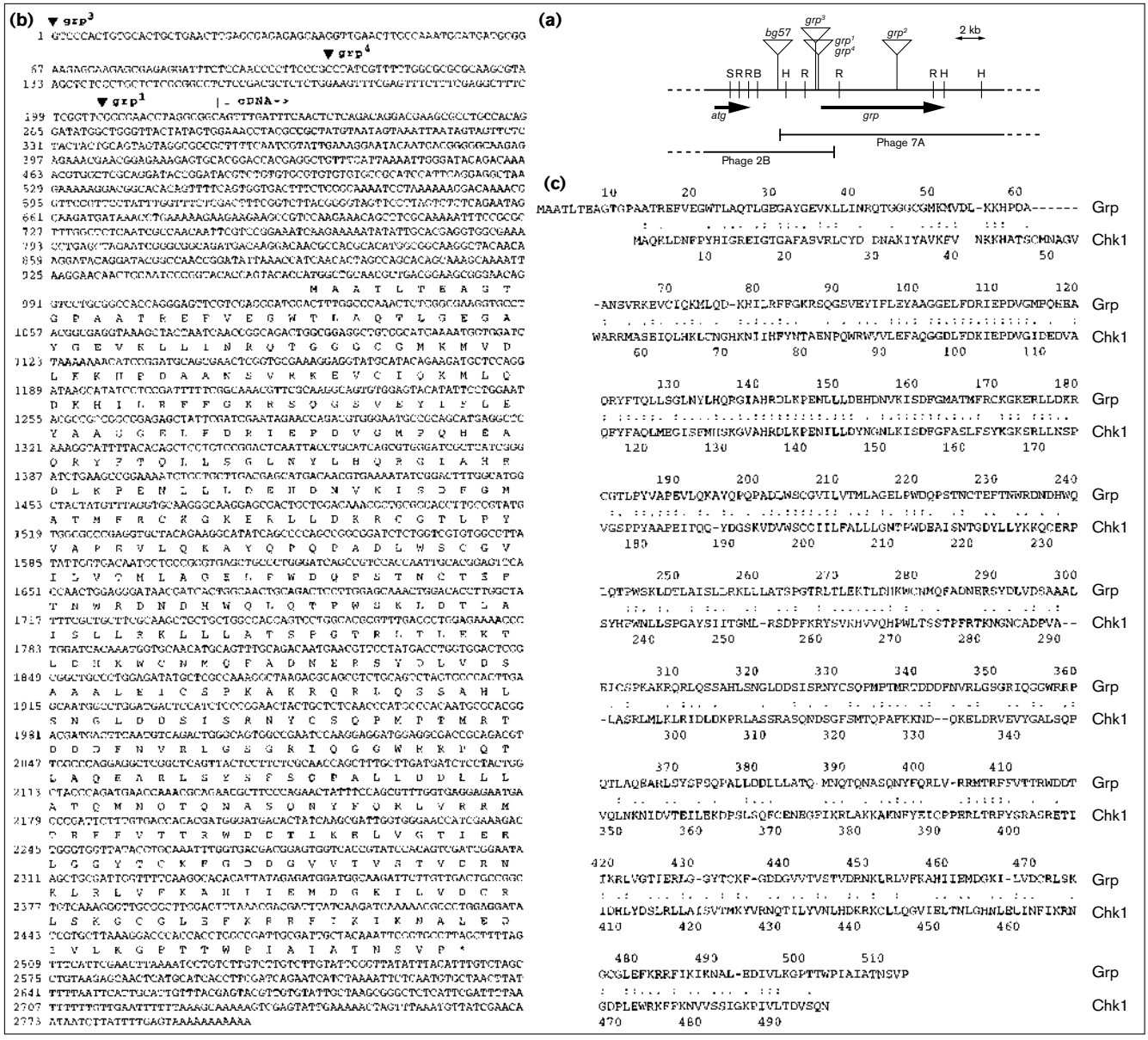
Whole-mount *in situ* analysis using a digoxygenin-labeled 2.0 kb *grp* cDNA demonstrated that, throughout the first 12 nuclear cycles, maternally provided *grp* mRNA was abundant and homogeneously distributed (data not shown). As an embryo progresses from nuclear cycle 12 (Fig. 5c) to 13 (Fig. 5d), extensive degradation of the maternal *grp* mRNA occurred, with the remaining transcripts assuming a pronounced anterior and posterior localization. Whether this pattern reflects a spatial difference in the kinetics of degradation or a difference in the initial abundance of *grp* mRNA is not known. From early interphase of nuclear cycle 14 (Fig. 5e) through cellularization (Fig. 5f), the remaining *grp* mRNA was degraded. The timing of maternal *grp* mRNA degradation was thus similar to that observed for many maternal messages that are destroyed at the maternal–zygotic transition [6].

Figure 5



Expression of the *grp* transcript. A 9 kb probe was obtained from genomic sequences immediately flanking the P-element insertion responsible for the *grp*^{1} mutation. **(a)** This probe hybridized to transcripts of 2.0 kb and 2.5 kb that were present in normal embryos but not in *grp*-derived embryos. A 1 kb probe complementary to the maternally supplied *nuf* transcript [10] served as a loading control (con). **(b)** A 2.0 kb *grp* cDNA probe was isolated from a 0–2 h embryonic cDNA library and used for developmental northern analysis of total RNA; each lane contains 10 μg RNA, and both 2.0 kb and 2.5 kb transcripts are indicated with an arrow. To analyze the distribution of *grp* transcripts during syncytial nuclear cycles **(c)** 12 and **(d)** 13, and in **(e)** early and **(f)** late interphase 14 embryos, RNA *in situ* hybridization was performed using dioxygenin-labeled 2.0 kb *grp* cDNA as a probe.

Figure 6



Molecular characterization of the *grp* locus. **(a)** Structure of the *grp* region (located in bands 36A10–14 on chromosome 2L). The transcription units for *grp* and the nearby *atg* (*adjacent-to-grapes*) and the overlapping genomic cDNA clones used to isolate them are indicated below the gene; the positions of the exons and introns in these genes have not been determined in detail. The mapped positions of the P-elements responsible for the *grp* mutations and *bg57*, a maternal effect mutation that incompletely complements *grp* mutations, are indicated above the gene, as are restriction enzyme sites (*Sal*I (S), *Eco*RV (R), *Bam*HI (B) and *Hin*dIII (H)). **(b)** Nucleic acid and predicted amino acid sequences of *grp*. The 5′ extent of the longest (2.5 kb) *grp* cDNA isolated (which begins at nucleotide 222) is shown. Sequences downstream of this position were obtained from this cDNA clone; genomic DNA upstream of this position was analyzed by sequencing from the P-element ends of clones obtained from grp^1, grp^3, and grp^4 by plasmid rescue. **(c)** Amino acid sequence homology between Grp and Chk1. Double dots between the sequences indicate identity, single dots indicate similarity. Grp amino acids 97–193 within the kinase domain exhibit 59% amino acid sequence identity to Chk1 sequences. Grp amino acids 244–267, 397–410, 429–436, 444–454 and 476–497 are regions outside of the kinase domain with greater than 30% amino acid sequence identity to Chk1 sequences.

The *grp* gene encodes a serine/threonine kinase and is homologous to the *chk1/rad27* gene of *S. pombe*

We have mapped a number of P-elements that disrupt the function of the *grp* gene. The P-elements responsible for the grp^1, grp^3 and grp^4 alleles map 5′ of the start of transcription, whereas the P-element responsible for the grp^2 allele maps well downstream of the transcription start site, apparently in an intron (Fig. 6a and data not shown).

Sequence analysis of *grp* cDNA clones revealed a putative open reading frame of 513 amino acids (Fig. 6b) with extensive structural similarity to the catalytic domain of the serine/threonine family of protein kinases (Fig. 6c). One member of this family, the Chk1/Rad27 DNA damage checkpoint kinase of *S. pombe* [18,19], has significant homology to the predicted Grp protein, extending beyond the kinase domain. Over a contiguous 97 amino acid region within the kinase domain, the two proteins exhibit 59% identity and 82% conservation (Fig. 6c). Over the entire protein sequence, the two proteins exhibit 26% amino acid identity. Outside the kinase domain, five regions, ranging in length from 9 to 24 amino acids, exhibit a greater than 30% amino acid sequence identity (see legend to Fig. 6). Sequencing of additional *grp* cDNAs revealed differences in the 5′ untranslated regions (data not shown). These differences are presumably due to alternative splicing or alternative promoter usage at the *grp* locus, but do not affect the translation product (data not shown).

Discussion

Molecular analysis of the *Drosophila grp* gene shows that it encodes a putative serine/threonine kinase with extensive homology to the *S. pombe* Chk1 protein, product of a checkpoint gene required for inducing a transient G2 delay in response to DNA damage, so providing time for repair before entering mitosis [18,19]. In normal *Drosophila* embryos, interphase becomes progressively longer during the late syncytial divisions, but time-lapse microscopy has shown that this lengthening fails to occur in *grp*-derived embryos [20]. These two observations support a model in which wild-type *grp* functions in a developmentally regulated interphase checkpoint during the late syncytial divisions of *Drosophila*.

Several observations are consistent with the hypothesis that Chk1 mediates its checkpoint function by inhibiting Cdc2 activity in response to DNA damage. During a G2 arrest elicited by ultraviolet or gamma irradiation of *S. pombe* cells, Cdc2 is maintained in a form inactivated by tyrosine phosphorylation [21,22]. Recent work suggests that Chk1, acting through Wee1, is responsible for maintaining Cdc2 in an inactive form. Overexpression of Chk1 in undamaged cells produces a G2 arrest with Cdc2 in the tyrosine-phosphorylated form. In addition, overexpression of Chk1 has no effect in cells lacking Wee1 kinase activity. Further evidence that Wee1 is a target of the Chk1 kinase comes from studies demonstrating that Chk1 phosphorylates Wee1 *in vitro* and that Wee1 is hyperphosphorylated in cells delayed in G2 by overexpression of Chk1 or irradiation using ultraviolet light [22]. Furthermore, inhibition of the Cdc25 tyrosine phosphatase has been implicated as another likely target for Chk1 kinase activity [21]. Taken together, these studies strongly implicate regulation of Cdc2 tyrosine phosphorylation in the Chk1 mediated DNA damage checkpoint.

Given the evidence for the mechanism of Chk1 kinase function in *S. pombe*, Grp may induce an interphase delay during the late syncytial divisions of *Drosophila* by influencing levels of inhibitory tyrosine phosphorylation on Cdc2. Previous studies have failed to detect tyrosine-phosphorylated Cdc2 in whole embryo extracts during nuclear cycles 1–13, although the possibility of a small nuclear-localized pool of the inhibited isoform has not been excluded [17]. Maternally supplied Cdc25 phosphatases, encoded by *twine* and *string*, and a Wee1 kinase homolog encoded by *Dwee1* are all present during the late syncytial cycles [23,24], again suggesting the possibility of localized tyrosine phosphorylation of Cdc2 that could be a target for Grp control. We have shown that *grp*-derived embryos fail to accumulate high levels of inhibited Cdc2 at a time corresponding to the maternal–zygotic transition that follows mitosis 13 in wild-type embryos [17]. The inhibited Cdc2 isoform can be detected in these late collections of *grp*-derived embryos, however, indicating that its inhibitory kinase is expressed. Our observations could reflect a failure of the embryos to activate the Cdc2-inhibiting kinase completely, or an abnormal perdurance of the tyrosine phosphatases encoded by *string* and *twine*, possibilities that we will be investigating in the future.

Rather than regulating tyrosine phosphorylation of Cdc2, Grp might instead regulate Cdc2 activity by influencing mitotic cyclin levels. During syncytial cycles 10–13, cyclin A and B levels oscillate with increasing amplitude, and there is evidence that mitotic degradation of cyclins contributes to a progressive lengthening of interphase [17]. Grp may thus be influencing the rate or timing of cyclin degradation. Oscillations in accumulation of the activated phosphoisoform of Cdc2 also occur during the late syncytial divisions, suggesting another possible target for the Grp kinase checkpoint function [17].

The observation that X-irradiation of normal syncytial embryos phenocopies the *grp* mitotic defect suggests that nuclei in both types of embryo progress into mitosis with damaged or incompletely replicated chromosomes. This idea is supported by the fact that *grp*-derived embryos exhibit a five-fold excess in damaged DNA relative to similarly staged collections of normal embryos (as assayed by the presence of free 5′ ends). We believe that the delay in metaphase observed in both *grp*-derived and X-irradiated normal embryos is due to the activation of a still operative mitotic checkpoint that is sensitive to the presence of damaged or unreplicated DNA. This mitotic checkpoint is likely to correspond to a previously described system that can detect the presence of defective mitotic spindles or abnormalities in the alignment of chromosomes on the metaphase plate [8]. The spindle checkpoint may be elicited by the improper chromosome congression we observed in *grp*-derived embryos during nuclear cycles 12 and 13.

If the function of wild-type *grp* is to prevent nuclei with damaged or incompletely replicated DNA from entering mitosis, then why does X-irradiation of normal embryos phenocopy the defects observed in *grp*-derived embryos? Syncytial embryos are extremely sensitive to ionizing radiation [25] and it is likely that 300 rads delivered at this stage overwhelm whatever DNA damage response mechanisms may exist. That is, even though the lesions have not been repaired, the nuclei inappropriately progress into metaphase (as observed in yeast [26]). This interpretation is supported by the observation that the nuclei do not delay during interphase/prophase in X-irradiated syncytial embryos (Fig. 1). Alternatively, Grp may be specifically involved in a DNA replication, rather than a DNA damage, checkpoint and the sensitivity of syncytial embryos to X-irradiation may be a direct consequence of the fact that these embryos do not contain effective DNA damage checkpoints.

The involvement of Grp in a DNA replication checkpoint would readily account for the observation that *grp*-derived embryos are disrupted primarily during nuclear cycle 12. The increase in interphase length observed during the late syncytial divisions in normal embryos is thought to reflect an increase in the time required to complete DNA replication, as maternally supplied replication factors are progressively depleted by the exponentially increasing population of nuclei. Nuclear cycle 12 may be the first cycle at which DNA replication cannot be completed before entry into mitosis without operation of the Grp checkpoint. We suggest that wild-type *grp* function is not required in the earlier syncytial divisions because, before cycle 12, the time needed to complete DNA synthesis is less than the time required for nuclei to progress into a mitotic state.

Conclusions

The syncytial nuclear cycles of *Drosophila* embryogenesis exhibit a number of dependency relationships. It is likely that many of these are the result of cell cycle checkpoints. The studies presented here suggest that Grp may be involved in a checkpoint that delays entry into mitosis in response to damaged or unreplicated DNA. Grp encodes a putative serine/threonine kinase with significant homology to the Chk1 kinase of *S. pombe*. These studies raise the possibility that similar components and mechanisms may be involved in maintaining the fidelity of the somatic cell cycle and the specialized syncytial cycles of early *Drosophila* embryogenesis.

Materials and methods

Drosophila *stocks*

The *grp* stocks used in this study were as described previously [12]. Unless otherwise indicated, all studies were performed using the grp^1 allele. The stocks were maintained on a standard cornmeal and molasses medium at 25°C. Control experiments were performed with grp^1/Cy heterozygous females: *Cy* is a second chromosome balancer containing the normal *grp* allele; in this manuscript we designate it as '+', and embryos derived from grp^1/Cy females as 'normal'.

Fixation and immunofluorence

Propidium iodide (Sigma) and an anti-α-tubulin antibody were used to visualize the nuclei and microtubules, respectively, as described previously [12]. An antibody to the centrosomal protein *cnn* (kindly provided by K. Li and T. Kaufman) was used to visualize the centrosomes [27]. The ploidy of the nuclei in *grp*-derived embryos was visualized by *in situ* hybridization to a DNA histone probe as described previously [14]. Microscopy was performed using an Olympus IMT2 inverted photoscope equipped with a Biorad 600 laser confocal imaging system. The lens used was the Olympus S plan Apo 60×, Oil. The cortical nuclear cycle was determined using the Biorad imaging software to estimate the surface nuclear density. Live analysis was performed using rhodamine-labeled histones as described previously [28].

X-irradiation studies and DNA damage assay

Normal embryos, collected between 0 and 2.0 h, were irradiated with 300 rads using a Torrex 120D X-ray generator (Astrophysics Research), aged and fixed by established protocols [29]. Similarly staged unirradiated embryos served as controls. The fixed embryos were then stained with propidium iodide and an anti-α-tubulin antibody to visualize DNA and microtubules, respectively.

In order to assess DNA damage, genomic DNA was isolated from 1–2 h collections of methanol-fixed normal and *grp*-derived embryos using slight modifications of standard protocols (after NaOH lysis and incubation at 65°C for 20 min, the embryos were gently ground and treated with proteinase K for 12 h at 55°C, followed by RNAse A treatment and standard phenol based extraction protocols [30]). Each sample of DNA (40 ng) was incubated at 37°C for 20 min in 25 μl of a reaction mixture containing 50 mM imidazole pH 6.4, 12 mM $MgCl_2$, 1 mM β-mercaptoethanol, 70 μM ADP, 10 units T4 DNA Kinase (Gibco BRL) and 2.5 μl of 3000 Cl $mmol^{-1}$ [^{32}P]ATP (Amersham). Unincorporated labeled nucleotides were removed through two passages of the reaction mixture through G-50 quick spin columns (Boehringer Mannheim). The level of ^{32}P incorporated into the genomic DNA was measured by liquid scintillation counting. Over 1000 embryos were used to generate the DNA for each trial and each data point represents the average of three or more trials.

Molecular analysis of the grp *gene*

Isolation and northern analysis of total RNA were performed as described previously [31]. Previous studies demonstrated that the *grp* mutation was induced by a P-element insertion [10]. A 9 kb genomic fragment was isolated using plasmid rescue [32] and used as a probe to isolate cDNAs. Full length *grp* cDNA was isolated from a 0–2 h embryonic cDNA library [33] generously provided by T. Hays, and was used to probe the developmental northern blot (Fig. 5b). The distribution of the *grp* transcript in syncytial embryos was revealed by *in situ* hybridization using a 2.0 kb *grp* digoxygenin-labeled cDNA probe (Boehringer Mannheim). Alkaline phosphatase staining [34] was used to visualize the message distribution. Sequencing was accomplished using the Amersham/USB sequenase kit, the Erase a Base kit and a series of 17 bp oligonucleotide primers. Sequences related to *grp* were identified using the BLAST electronic mail server with blastn and blastp programs [35,36]. Chk1 and Grp amino acid sequence identities and similarities were determined using the LALIGN program. Germline rescue of the *grp* mutation was performed using a 2.2 kb *grp* cDNA inserted into the Germ 10 vector (kindly provided by B. Cohen) and established transformation procedures [37].

Western blot analysis

Extracts were prepared by homogenizing the embryos in nuclear isolation buffer (50 mM Tris, pH 7.5, 60 mM KCl, 15 mM NaCl, 250 mM sucrose, 1 mM EDTA, 0.1 mM EGTA) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 2 mM benzamidine-HCl). Particulate matter was removed by a low

speed spin and an aliquot of supernatant containing 1 mg total protein was adjusted to 80 mM glycerophosphate, 1 mM sodium orthovanadate and 1 mM NaF. Crude anti-Cdc2 antibodies (2 μl) were added to each sample and incubated at 4°C for several hours, then precipitated by the addition of Protein-A-agarose beads (Gibco-BRL), followed by 3 washes in the same buffer. The immunoprecipitates were resuspended in SDS-loading buffer and boiled. Samples were resolved on an 11% polyacrylamide gel (22 cm long) run at 200 V for 18 h, then transferred to Immobilon-P (Amersham) following manufacturer's instructions.

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