The *Drosophila* ATM homologue Mei-41 has an essential checkpoint function at the midblastula transition

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Background: *Drosophila* embryogenesis is initiated by 13 rapid syncytial mitotic divisions that do not require zygotic gene activity. This maternally directed cleavage phase of development terminates at the midblastula transition (MBT), at which point the cell cycle slows dramatically, membranes surround the cortical nuclei to form a cellular blastoderm, and zygotic gene expression is first required.

Results: We show that embryos lacking Mei-41, a *Drosophila* homologue of the ATM tumor suppressor, proceed through unusually short syncytial mitoses, fail to terminate syncytial division following mitosis 13, and degenerate without forming cells. A similar cleavage-stage arrest is produced by mutations in *grapes*, which encodes a homologue of the Checkpoint-1 kinase. We present biochemical, cytological and genetic data indicating that Mei-41 and Grapes are components of a conserved DNA-replication/damage checkpoint pathway that triggers inhibitory phosphorylation of the Cdc2 kinase and mediates resistance to replication inhibitors and DNA-damaging agents. This pathway is nonessential during postembryonic development, but it is required to terminate the cleavage stage at the MBT. Cyclins are required for Cdc2 kinase activity, and mutations in *cyclin A* and *cyclin B* bypass the requirement for *mei-41* at the MBT. These mutations do not restore wild-type syncytial cell-cycle timing or the embryonic replication checkpoint, however, suggesting that Mei-41-mediated inhibition of Cdc2 has an additional essential function at the MBT.

Conclusions: The *Drosophila* DNA-replication/damage checkpoint pathway can be activated by externally triggered DNA damage or replication defects throughout the life cycle, and under laboratory conditions this inducible function is nonessential. During early embryogenesis, however, this pathway is activated by developmental cues and is required for the transition from maternal to zygotic control of development at the MBT.

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Background

Embryonic development is typically initiated by a cleavage stage characterized by rapid mitotic divisions that are driven by material that is synthesized during oogenesis. There is little or no zygotic transcription during this maternally directed stage of embryogenesis, and transcriptional inhibitors do not affect the mitotic program. At the midblastula transition (MBT), the cell cycle slows, high-level transcription is initiated, and further embryonic development requires zygotic gene activity [1–3]. The molecular mechanisms controlling the MBT are not understood. The timing of this transition is controlled by the ratio of nuclei to cytoplasm (the nucleo-cytoplasmic ratio), however, suggesting that the MBT is triggered when a maternal factor is titrated out by nuclear material, which accumulates exponentially during the cleavage stage [4–9].

In *Drosophila*, maternal genetic control extends through the first 13 embryonic mitoses. These divisions proceed without cytokinesis and thus produce a syncytial embryo (reviewed in [2]). The first 10 divisions are very rapid — 8–9 minutes per division — and the cell cycle begins to slow at the tenth division, as the majority of nuclei complete migration from the interior to the cortex to form a syncytial blastoderm (reviewed in [2]). Transcription is initiated during cell cycles 9 and 10, but it is not required until interphase 14, when zygotic expression of the *bottleneck*, *serendipity*-α and *nullo* genes drives blastoderm cellularization [10–14]. A general increase in zygotic gene expression is also observed during interphase 14, and subsequent development requires zygotic gene activity (reviewed in [2]). Cellularization thus cytologically marks the *Drosophila* equivalent of the MBT.

Embryos derived from females mutant for the *Drosophila grapes* (*grp*) gene arrest development at the cleavage stage. These embryos fail to terminate rapid syncytial division after mitosis 13, do not cellularize, and do not initiate

high-level zygotic gene expression [15]. The *Drosophila grp* gene encodes a Checkpoint-1 (Chk1) kinase homologue [16]. In systems ranging from yeast to man, Chk1 kinases function in checkpoint pathways that delay the cell cycle in response to DNA damage or replication defects [17–22]. We found that the *Drosophila grp* gene is required for the DNA-replication checkpoint control of the early embryonic cell cycle [15]. This observation, combined with the developmental defects produced by the *grp* mutation, led us to propose that replication-checkpoint-dependent cell-cycle delays are required to terminate the cleavage stage and to trigger the MBT [15]. Increases in cell-cycle length during the syncytial blastoderm divisions are required for the production of functional mRNAs from genes with relatively long primary transcription units [23,24]. We therefore speculated that the only essential developmental function for the *grp* checkpoint pathway is to slow the syncytial cell cycle, which in turn allows production of mRNAs encoding transcription factors that act on a second group of target genes. In this simple 'domino' model, checkpoint-dependent cell-cycle delays initiate a transcriptional cascade that drives the switch from maternal to zygotic control of development [15,25].

The *Drosophila mei-41* gene, originally identified in a screen for mutations causing defects in DNA repair or recombination, encodes a homologue of the human ATM tumor suppressor [26,27]. We present evidence that Mei-41 and the Grp Chk1 homologue are components of a replication-checkpoint pathway that is not essential for zygotic development, but which has an essential role during the transition from maternal to zygotic control of embryogenesis at the MBT. This pathway is triggered by developmental cues at the MBT, and can be activated by environmentally induced DNA damage or replication defects throughout the life cycle. Our genetic and cytological analyses also indicate that checkpoint-dependent delays in the embryonic cell cycle alone may not be sufficient to trigger the MBT, and suggest that the checkpoint pathway has an additional essential function at this critical developmental transition.

Results

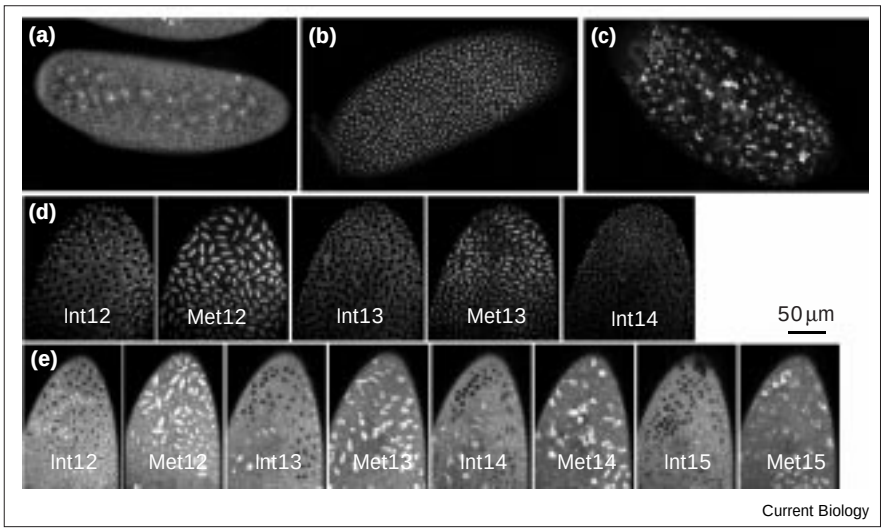
Mutations in *mei-41* lead to cleavage-stage arrest

A null allele of *mei-41* (*mei-41^{D3}*, see Materials and methods section) does not affect development to the adult stage, but the resulting homozygous *mei-41^{D3}* females are sterile and produce eggs that do not hatch. To gain insight into the embryonic function of Mei-41, we examined the syncytial mitoses in these embryos. Cytological analysis of fixed embryos suggested that most *mei-41* mutant embryos develop to the syncytial-blastoderm stage and have relatively normal nuclear morphology up to division 12 or 13 (Figure 1a,b). Later embryos showed significant variability in nuclear size and distribution (Figure 1c), however, and embryos undergoing cellularization or gastrulation were never observed (data not shown). To gain a better understanding of the syncytial-blastoderm phenotype, embryos derived from *mei-41^{D3}* homozygous females were microinjected with rhodamine-conjugated tubulin and mitosis was visualized directly using time-lapse confocal microscopy [28]. All of the syncytial-blastoderm-stage embryos examined by this *in vivo* method (nine in total) failed to cellularize following mitosis 13 and proceeded through at least two additional syncytial cycles of spindle assembly and disassembly (15 cycles in total; Figure 1e). One of these embryos continued through 17 syncytial mitoses before the recording was terminated and all of the embryos ultimately appeared to degenerate without forming cells. In these analyses, performed *in vivo*, mitotic errors were observed at a higher frequency than expected from our examination of fixed material (compare Figure 1a–c with 1e). We speculate that the mitotic errors observed in the living embryos are caused by laser-light-induced DNA damage and the absence of a functional damage/replication-checkpoint pathway (see below).

In wild-type embryos, the cell cycle progressively slows during the syncytial-blastoderm divisions that precede cellularization, with most of the increases being due to changes in interphase length (Table 1) [29,30]. In *mei-41* mutant embryos, the lengths of interphase and mitosis are close to wild type until division 11 (Table 1). During divisions 12 and 13, interphase is significantly shorter than in wild type, however (Table 1). Similar defects in the syncytial-division timing were observed in embryos from *grp* mutant females [15] (Table 1). The syncytial divisions in *mei-41* mutant embryos were not as rapid as those observed in *grp* mutants (Table 1). Nonetheless, *mei-41* mutant embryos, like *grp* mutants, were defective in slowing the syncytial-blastoderm cell cycle, failed to terminate syncytial division following mitosis 13, and thus appeared to be arrested at the cleavage stage of embryogenesis.

To obtain further evidence for cleavage-stage arrest, we assayed *mei-41* mutants for the inhibitory changes in the cell-cycle machinery that normally accompany the termination of syncytial divisions at the MBT [30]. In wild-type embryos, the increases in cell-cycle timing at the MBT are accompanied by a decline in the amount of String/Cdc25 phosphatase to essentially undetectable levels and increased Cdc2 phosphorylation (Figure 2) [30]. In embryos at the late syncytial blastoderm stage, derived from *mei-41* mutant females, String levels did not change significantly and only a modest increase in Cdc2 phosphorylation was observed (Figure 2). In *grp* mutants, similar defects in Cdc2 phophorylation were observed (Figure 2) [15]. String levels did decline in the *grp* mutants, but not to the undetectable levels observed in wild type (Figure 2). Thus, both *mei-41* and *grp* are required for complete String degradation and for wild-type levels of Cdc2 phosphorylation at the MBT.

Figure 1



Mitosis in *mei-41* mutant embryos. (a–c) Embryos derived from females homozygous for *mei-41^{D3}* were fixed and stained with the fluorescent DNA-binding dye Oli-green to reveal nuclear organization and distribution. Most (a) premigration-stage and (b) syncytial-blastoderm-stage embryos displayed relatively uniform nuclear distribution and normal chromatin organization. (c) In later embryos, by contrast, nuclear size and distribution were uneven, and none of these embryos appeared to cellularize. To analyze cell-cycle dynamics during the syncytial divisions, embryos were injected with rhodamine–tubulin and imaged by time-lapse confocal microscopy. (d) Interphase 12 to interphase 14 in a wild-type embryo injected with rhodamine–tubulin; cells are formed during interphase 14. Int, interphase; Met, metaphase. (e) Interphase 12 to metaphase 15 in an embryo, derived from a *mei-41* mutant female, in which 15 syncytial mitoses were followed by embryonic degeneration without cell formation. In these *in vivo* imaging studies, the syncytial-blastoderm-stage *mei-41* mutant embryos appeared more disorganized than in fixed preparations. This observation may reflect an accumulation of light-induced DNA damage and the absence of a functional DNA replication/damage checkpoint.

In *mei-41* mutant embryos, the extent of Cdc2 phosphorylation and the length of the syncytial-blastoderm cell cycles were intermediate between those of wild type and *grp* mutants (Table 1, Figure 2). These observations suggest that a Mei-41-independent process triggers low levels of Cdc2 phosphorylation and modest cell-cycle delays. This could reflect the presence of a second *Drosophila* ATM homologue, an idea consistant with the observation that two ATM-related genes are present in systems ranging from yeast to man [31–37]. Embryos from females double-mutant for *grp* and *mei-41* show cell-cycle times that are essentially the same as those observed in *grp* single mutants (data not shown). The Mei-41-independent cell-cycle delays thus appear to act through the Grp/Chk1 kinase.

An additional hallmark of the MBT is a dramatic activation of zygotic transcription. We therefore assayed for zygotic expression of several pair-rule and gap embryonic patterning genes that are normally transcribed at high levels at the MBT (Figure 3). For these studies, embryos

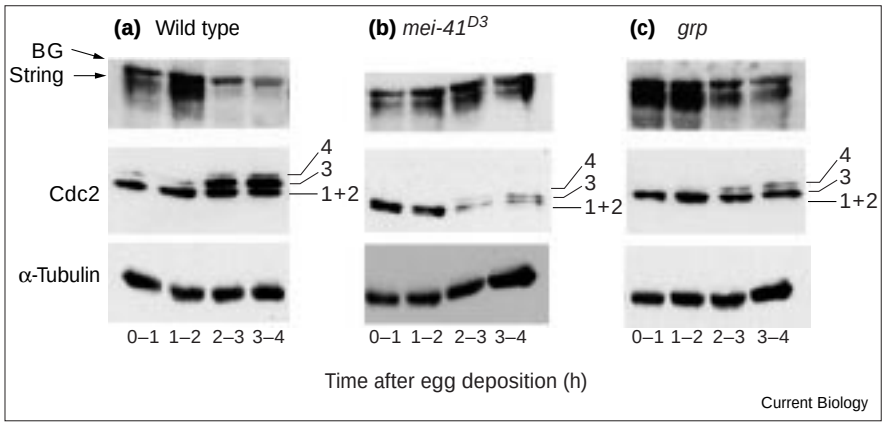
Table 1

Cell-cycle times in checkpoint mutant embryos.

Cycle	Wild type*	*grp$^{fs(A)4}$/grp$^{fs(A)4}$**	*mei-41^{D3}/mei-41^{D3}*	*mei-41^{D3};+/cycA;+/cycB*
Mitosis 10	5.5 ± 0.7(8)	5.6 ± 0.7(6)	ND	ND
Interphase 11	6.1 ± 1.1(9)	4.7 ± 1.0(8)	6.9 ± 0.3(7)	6.4 ± 0.4(6)
Mitosis 11	5.3 ± 0.8(10)	5.4 ± 0.7(10)	4.5 ± 0.8(7)	4.3 ± 0.5(6)
Interphase 12	8.7 ± 0.9(10)	6.1 ± 1.1(11)	7.9 ± 1.1(8)	8.8 ± 1.2(9)
Mitosis 12	5.9 ± 1.0(10)	6.1 ± 1.1(12)	4.8 ± 0.8(9)	4.2 ± 0.6(8)
Interphase 13	14.0 ± 1.3(9)	7.3 ± 1.6(12)	9.6 ± 1.2(9)	10.4 ± 2.5(7)
Mitosis 13	6.2 ± 1.0(9)	10.4 ± 2.3(10)	5.7 ± 1.1(8)	5.4 ± 1.2(7)
Interphase 14	70.3 ± 3.8(3)	11.8 ± 3.9(8)	15.1 ± 2.6(8)	15.7 ± 5.1(6)
Mitosis 14	ND	19.4 ± 5.9(3)	6.3 ± 4.3(3)	8.6 ± 4.4(3)

The length of interphase and mitosis was measured, as described in the Materials and methods section. Embryos derived from females homozygous for *mei-41^{D3}* or *grp$^{fs(A)4}$*, or homozygous for *mei-41^{D3}* and heterozygous for both *cyclin (cyc) A* and *cyclin B*, were analyzed. Average times with standard deviations (in minutes) are indicated. The number of individual embryos scored is given in brackets. *Data from [15]. ND, not determined.

Figure 2

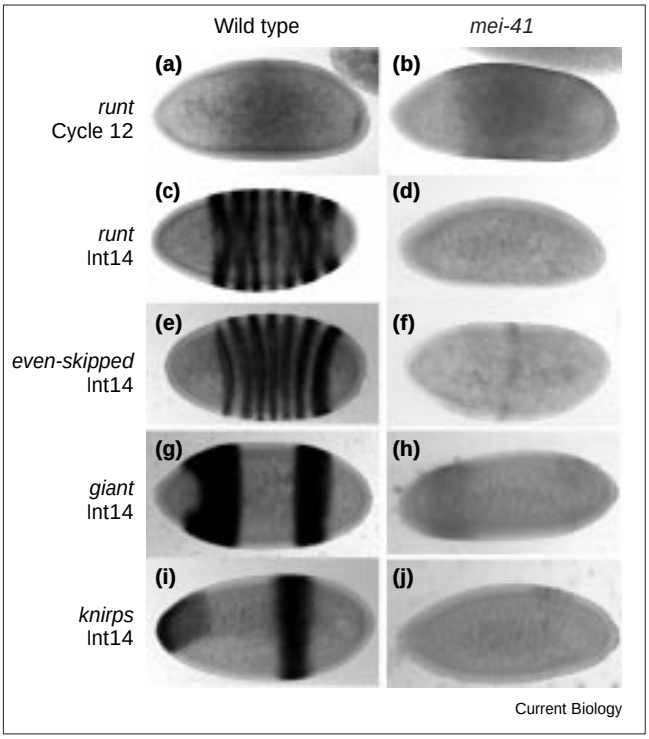


Mei-41 is required for the down-regulation of String/Cdc25 and for the inhibitory tyrosine-phosphorylation of Cdc2 at the MBT. Western blots of extracts prepared from (a) wild-type, (b) *mei-41^{D3}* mutant and (c) *grp* mutant embryos were probed for the Cdc25 homologue String and for Cdc2. A background band (BG) is observed on the blots probed for String. The String signal is indicated, and is absent in String mutant embryos [30]. (a) In wild-type embryos, levels of String/Cdc25 decrease as the cell cycle slows at the MBT (between 2 and 3 h after egg deposition). This decrease in String level is accompanied by an increase in inhibitory phosphorylation of Cdc2 (forms 3 and 4; compare 0–1 h lane with 2–3 h lane). The different phosphorylated forms of Cdc2 are indicated by numbers on the right-hand side of each blot. (b) In *mei-41^{D3}* mutant embryos, this decline in String levels and increase in inhibitory phosphorylation of Cdc2 is not observed. (c) In *grp* mutant embryos, String levels decline slightly and a modest increase in Cdc2 phosphorylation is observed. As a loading control, the western blots were probed for α-tubulin.

were counter-stained with the fluorescent DNA dye Oligreen, which allowed precise correlation of gene expression with cell-cycle number and phase. During wild-type syncytial-blastoderm divisions, the genes we examined were expressed at low levels over relatively broad regions of the embryo, or were not expressed (Figure 3a). Within a few minutes of completing mitosis 13, however, all of these genes were strongly transcribed in characteristic spatially restricted patterns (Figure 3c,e,g,i). In embryos derived from *mei-41* mutant females, these genes showed the expected expression patterns during syncytial divisions 12 and 13 (Figure 3b). Thus, the *mei-41* mutation does not prevent the initiation of transcription prior to the MBT. On completion of mitosis 13, however, the expected high-level, spatially restricted expression of these genes was not initiated (Figure 3d,f,h,j). Mutations in *mei-41* thus prevent the onset of the high-level, patterned gene expression that characterizes the transition from maternal to zygotic control of development.

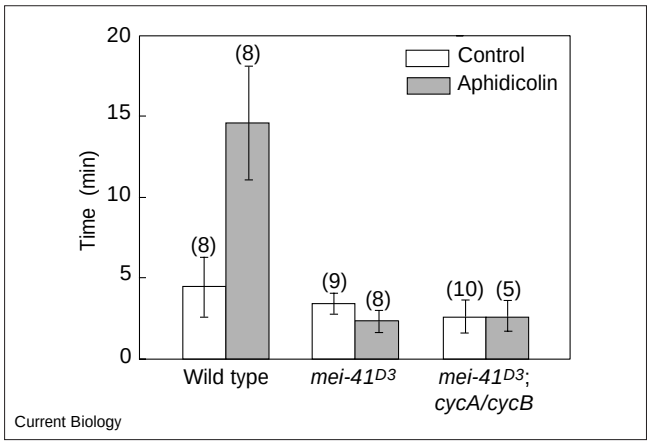
The absence of maternal Mei-41 leads to mitotic defects during the later syncytial mitoses (Figure 1), raising the possibility that the gene expression defects in later embryos are a secondary consequence of these mitotic errors. But a number of other maternal-effect lethal mutations produce dramatic mitotic defects during the syncytial-blastoderm stage, and do not block transcriptional activation at the MBT. For example, the *dal* mutation produces mitotic errors that lead to loss of approximately one half of the syncytial nuclei during divisions 10 to 13.

Figure 3



Zygotic gene activation is impaired in *mei-41^{D3}* null mutant embryos. Embryos derived from wild-type females or from *mei-41^{D3}* homozygous mutant females were assayed for expression of early zygotic transcripts by whole-mount *in situ* hybridization. To allow determination of cell-cycle division number, the embryos were counter-stained with Oligreen (data not shown). (a,b) During the early syncytial blastoderm mitoses, both wild-type and *mei-41^{D3}* embryos express the *runt* gene over a broad central domain. (c) During interphase of cycle 14, wild-type embryos show the seven-stripe-pair-rule pattern typical of *runt* expression. (d) This pattern is never established in embryos derived from *mei-41^{D3}* mutant females. Similarly, (e) wild-type cycle 14 embryos express the pair-rule gene *even-skipped* and the gap genes (g) *giant* and (i) *knirps* in distinct patterns, whereas high-level expression of these genes is not observed at the same stage in embryos from *mei-41^{D3}* mutant females (f,h,j).

Figure 4



Expression of *mei-41* is required to delay the cell cycle in response to the DNA-replication inhibitor aphidicolin. The graph shows the effect of aphidicolin on the cell-cycle times for wild-type and *mei-41^{D3}* homozygous mutant embryos, and for *mei-41^{D3}* homozygous mutant embryos in which the maternally supplied levels of Cyclin (cyc) A and Cyclin B have been reduced. In wild-type embryos, the onset of mitosis is significantly delayed in response to aphidicolin. By contrast, embryos derived from *mei-41^{D3}* homozygous mutant females show no such delay when injected with aphidicolin, but proceed immediately into mitosis. Reducing the maternally supplied levels of Cyclin A and Cyclin B partially rescues the *mei-41^{D3}* embryonic lethality (see below); however, reducing maternal Cyclin levels in a *mei-41^{D3}* mutant background does not restore the aphidicolin-induced replication checkpoint. The height of the bars reflects the mean time to NEB, with the standard deviation indicated. The number of live recordings scored is given in brackets.

Nonetheless, this mutation does not prevent the onset of zygotic gene expression or progression through the MBT [38]. Similarly, we have found that the *scrambled* mutation, which leads to severe mitotic defects during divisions 11 to 13, does not block high-level, spatially restricted gap and pair-rule gene expression (V. Stevenson, O.C.M.S. and W.E.T., unpublished observations). We cannot rigorously rule out the possibility that mitotic defects affect the activation of zygotic gene expression in the *mei-41* mutant embryos, but mitotic errors alone do not appear to be sufficient to block transcription at the MBT; we therefore favor a model in which *mei-41* has a specific role in zygotic gene activation (see below).

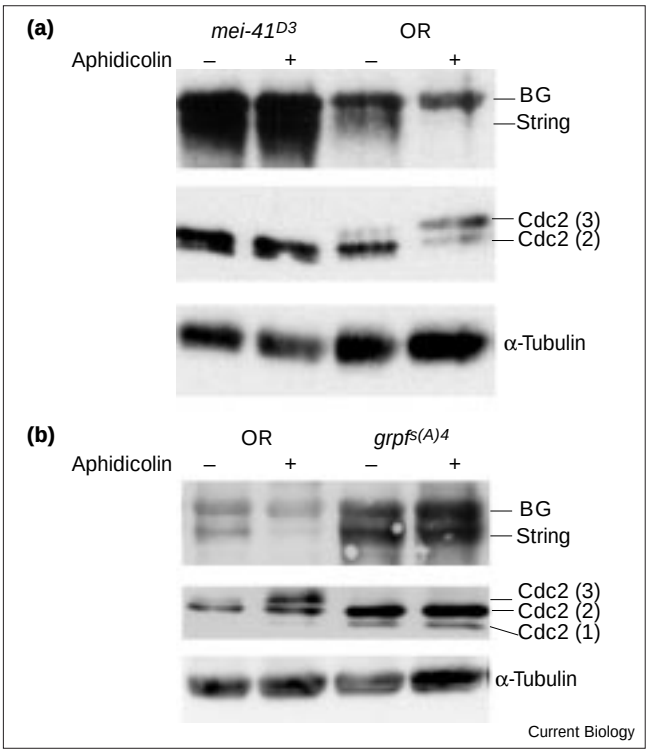
Both *mei-41* and *grp* are required for embryonic replication-checkpoint function

Our earlier studies demonstrated that the *grp* gene is required for an embryonic DNA-replication-checkpoint pathway [15]. To determine whether *mei-41* is also required for embryonic cell-cycle control mediated by the DNA-replication checkpoint, we assayed mutant embryos for the ability to delay mitosis in response to the DNA-synthesis inhibitor aphidicolin [15]. For these studies, embryos were injected with rhodamine-conjugated tubulin alone, or with a mixture of rhodamine-conjugated tubulin and aphidicolin at a level sufficient to inhibit DNA replication by greater than 95% [39]. Cell-cycle progression was then assayed by time-lapse confocal microscopy, using centrosome migration as an interphase marker and nuclear envelope breakdown (NEB) and spindle assembly as cytological markers for mitosis [15] (see Materials and methods section). In control embryos at cell cycles 11 and 12, NEB was triggered approximately 5 minutes after the completion of centrosome migration (Figure 4). When aphidicolin was included in the injection buffer, however, NEB was delayed by an average of 15 minutes (Figure 4). By contrast, *mei-41* mutant embryos did not delay NEB in response to aphidicolin treatment (Figure 4). Embryos derived from *grp* mutant females also fail to delay mitosis in response to aphidicolin [15]. Thus, *mei-41* and *grp* both appear to be required for DNA-replication checkpoint control of the embryonic cell cycle.

Maternal Mei-41 and Grp are required for wild-type cell-cycle delays during the later syncytial-blastoderm divisions; these developmentally controlled cell-cycle delays are associated with a loss of String and phosphorylation of Cdc2 (Table 1, Figure 2) [15]. To determine whether similar inhibitory changes in the cell-cycle machinery are triggered during the aphidicolin response, we assayed Cdc2 phosphorylation and String levels after aphidicolin treatment (Figure 5). In wild-type embryos, aphidicolin caused a shift in Cdc2 to the tyrosine- and threonine-phosphorylated forms — which migrate with a low mobility in SDS–PAGE — and a dramatic decrease in String levels (Figure 5). The control levels of String and Cdc2 phosphorylation in *mei-41* and *grp* mutants differ somewhat from wild type. Nonetheless, in both of these mutant backgrounds, Cdc2 phosphorylation and String levels do not change in response to aphidicolin. Thus, the products of *mei-41* and *grp* are required for inhibitory modifications of the cell-cycle machinery in response to aphidicolin and at the MBT. These observations provide strong support for the hypothesis that the *mei-41* and *grp* genes are components of the same checkpoint pathway.

To test genetically the hypothesis that *mei-41* and *grp* are components of a single pathway, we assayed for interactions between mutations at these loci. If *mei-41* and *grp* encode components of the same pathway, animals homozygous for null alleles of both genes should be phenotypically similar to either single-null mutant. By contrast, if the two genes function in distinct pathways, the double mutant combination should produce a more severe phenotype than either of the single mutations. Animals homozygous for null alleles of *mei-41* and *grp* developed to the adult stage, were female-sterile, and produced embryos with normal external morphology that were arrested in the cleavage stage of embryogenesis (data not shown). By these criteria, the double mutants

Figure 5



Aphidicolin induces *mei-41*- and *grp*-dependent modifications of String and Cdc2. Biochemical changes in String and Cdc2 were examined by western blotting whole embryo preparations from 1–2 h embryos incubated with aphidicolin (+) or carrier solution (–). A background band (BG) is observed on the blots probed for String; the phosphorylated forms of Cdc2 are indicated, as in Figure 2. In wild-type embryos (Oregon R strain; OR), aphidicolin induces a decrease in String levels and accumulation of inhibitory phosphorylated forms of Cdc2. In embryos derived from *mei-41*^{D3} and *grp*$^{fs(A)4}$ mutant females, by contrast, aphidicolin treatment does not induce detectable changes in String levels or Cdc2 phosphorylation state.

are indistinguishable from either single mutant, supporting the hypothesis that the two genes function in the same pathway.

The single-pathway hypothesis also predicts that the phenotype produced by a partial loss of function in one component will be enhanced by mutations in a second component that further compromise checkpoint function *in vivo*. We therefore assayed for genetic interactions between a weaker *mei-41* allelic combination (*mei-41*^{D3}/*mei-41*^{D5}) and a *grp* null (Table 2). At 25°C, approximately 4% of the embryos derived from *mei-41*^{D3}/*mei-41*^{D5} females hatched. At 20°C, the hatch rate increased to approximately 45%. By contrast, *mei-41*^{D3}/*mei-41*^{D5} females that are also heterozygous for a *grp* null produced embryos with a 1% hatch rate at 25°C, and a 23% hatch rate at 20°C (Table 2). Therefore, the *grp* null allele is a dominant enhancer of the *mei-41* embryonic lethality, further supporting the hypothesis that

Table 2

A *grp* null mutation is a dominant enhancer of the *mei-41* embryonic lethality.

Temperature	Hatch rate (%)	
	mei-41^{D3}/*mei-41*^{D5}; *Sp*/*SM6a*	*mei-41*^{D3}/*mei-41*^{D5}; *grp*$^{fs(A)4}$/*SM6a*
25°C	4.4 (1547)	1.0 (1641)
20°C	44.7 (693)	23.3 (707)

Hatching rates were determined for embryos derived from females carrying a semi-sterile allelic *mei-41* combination (*mei-41*^{D3}/*mei-41*^{D5}; *Sp*/*SM6a*) and for embryos derived from females carrying this combination and heterozygous for a null allele of *grp* (*mei-41*/*mei-41*; *grp*$^{fs(A)4}$/*SM6a*). *Sp* designates a second chromosome carrying the dominant *Sp* mutation, and *SM6a* is a second chromosome balancer [48]. The total number of flies scored is indicated in brackets.

Mei-41 and Grapes function in the same pathway during early embryogenesis.

Evidence for *mei-41*/*grp* function during zygotic development

In single-celled organisms and cultured cells grown under laboratory conditions, cell-cycle checkpoints are triggered only in response to environmentally induced cellular damage and are generally nonessential [31,40,41]. Mutations in *grp* and *mei-41*, which encode checkpoint pathway homologues, lead to embryonic lethality and a failure to undergo the MBT, however ([15] and this paper). These observations raise two possibilities: first, a *mei-41*- and *grp*-independent pathway provides nonessential checkpoint control throughout the life cycle; or second, a single replication-checkpoint pathway has an essential function at the MBT and mediates the nonessential checkpoint response to cellular damage throughout development. Supporting the latter hypothesis, cytological studies indicate that *mei-41* is required for DNA-damage checkpoint function in larval neuroblasts [26] and eye imaginal discs (A.L, M.H. Brodsky and R.S.H., unpublished observations).

To further test for zygotic checkpoint function in *grp* and *mei-41* mutants, we assayed for sensitivity to hydroxyurea (HU) and methylmethane sulfonate (MMS) during post-embryonic development. For these studies, flies heterozygous for *mei-41* or *grp* were crossed and their progeny were raised on food supplemented with low levels of HU or MMS. Control animals were raised on food supplemented with carrier solution alone. In this assay, sensitivity is indicated by a preferential loss of homozygous offspring in the presence of MMS or HU. Previous studies indicated that *mei-41* mutations lead to zygotic sensitivity to HU and MMS [42–45]. We confirmed these observations, and found that *grp* mutants were also sensitive to low levels of HU and MMS (Table 3). Thus, both *grp* and

mei-41 are required for zygotic resistance to agents that modify DNA structure or metabolism.

We also assayed for genetic interactions between *mei-41* and *grp* during the larval response to MMS. We first established a genetic cross that produced both *grp* heterozygotes and *grp*;*mei-41* double heterozygotes. Under control conditions, 52% of the progeny from this cross were double heterozygous (Table 4b). When 0.05% MMS was present in the food, however, only 34.6% of the surviving animals were heterozygous for *mei-41* and *grp*, indicating that this class of progeny was preferentially lost during larval and/or pupal development. We then compared the MMS sensitivity of animals heterozygous for *mei-41* with animals doubly heterozygous for *mei-41* and *grp* . Control experiments again produced approximately equal numbers of *mei-41* heterozygotes and *mei-41*;*grp* double heterozygotes. In the presence of 0.05% MMS, by contrast, only 15.9% of the adults recovered were double heterozygous for *grp* and *mei-41* (Table 4a). Thus, *mei-41* and *grp* show dominant interactions during the larval response to MMS; these dominant genetic interactions suggest that *mei-41* and *grp* function in the same pathway during zygotic development.

Defects in DNA repair, DNA replication, or checkpoint control of the cell cycle could lead to HU and MMS sensitivity during zygotic development, and the genetic assays described above cannot distinguish between these alternatives. Our preliminary studies indicate that the imaginal disks from larvae mutant for either *grp* or *mei-41* are defective in delaying mitosis in response to X-ray damage (A.L. and R.S.H., unpublished observations), however, and both the *grp* and *mei-41* mutations disrupt replication checkpoint function during early embryogenesis [15] (Figure 4). We therefore believe that the sensitivity to MMS and HU of mutant larvae is at least in part due to defects in zygotic checkpoint function.

Table 3

Sensitivity of *mei-41* and *grp* mutants to hydroxyurea or methylmethane sulfonate.

	$grp^{fs(A)4}/grp^{fs(A)4}$: $+/grp^{fs(A)4}$ (%)	$mei\text{-}41^{D3}/mei\text{-}41^{D3}$: $+/mei\text{-}41^{D3}$ (%)
Experiment 1		
Control	30 (128)	44 (115)
20 mM HU	0 (148)	0 (61)
Experiment 2		
Control	32 (590)	65 (214)
0.025% MMS	23 (1421)	0 (238)
0.05% MMS	2 (319)	0 (140)

Zygotic sensitivity to HU and MMS was assayed, as described in the Materials and methods section. The ratio of homozygous to heterozygous adults is reported as a percentage, with the total number of adult flies scored indicated in brackets. The results from two independent experiments are shown.

Table 4

Sensitivity of *mei-41–grp* mutant combinations to methylmethane sulfonate.

	(a) $mei\text{-}41^{D3}/+;grp^{fs(A)4}/+$: $grp^{fs(A)4}/+$ (%)	(b) $mei\text{-}41^{D3}/+;grp^{fs(A)4}/+$: $mei\text{-}41^{D3}/+$ (%)
Control	52.9 (856)	51.6 (151)
0.025% MMS	49.3 (1000)	45.4 (361)
0.05% MMS	15.9 (564)	34.6 (78)

(a) A cross producing animals doubly heterozygous for *mei-41* and *grp* ($mei\text{-}41^{D3}/+;grp^{fs(A)4}/+$) and animals heterozygous for *grp* alone ($grp^{fs(A)4}/+$) was established. Similarly, a cross producing animals doubly heterozygous for *mei-41* and *grp* and animals heterozygous for *mei-41* alone ($mei\text{-}41^{D3}/+$) was established. The resulting offspring were raised on control food or food supplemented with the indicated concentration of MMS. The ratio of doubly heterozygous to singly heterozygous adults recovered is reported as a percentage, with the total number of adults scored indicated in brackets.

Cyclin mutations bypass the embryonic requirement for *mei-41*

The *grp*/*mei-41* replication-checkpoint pathway triggers inhibitory changes in the Cdc2 kinase. We therefore speculated that the essential embryonic requirement for this pathway could be bypassed if checkpoint-independent inhibition of Cdc2 kinase could be induced. Embryos from females heterozygous for *cyclin A* and *cyclin B* null mutations proceed through syncytial-blastoderm cell cycles that are significantly longer than normal, suggesting that Cyclin becomes rate limiting for Cdc2 activation during these divisions [30]. In an attempt to inhibit embryonic Cdc2 kinase in checkpoint-mutant embryos, we generated females homozygous for either *grp* or *mei-41*, and heterozygous for null alleles of *cyclin A*, *cyclin B*, or both *cyclin A* and *cyclin B*; we then analyzed the embryos derived from these females. Embryos from females homozygous for *grp* and heterozygous for the *cyclin* mutations failed to hatch and showed no signs of phenotypic rescue (Table 5 and data not shown). Zygotic gene activation and partial cellularization was observed in a significant fraction of the embryos derived from females homozygous for $mei\text{-}41^{D3}$ and heterozygous for both *cyclin A* and *cyclin B* mutations, however (Figure 6). In addition, approximately 4% of these embryos hatched and developed to the adult stage (Table 1). More striking rescue was observed in the $mei\text{-}41^{D3}/mei\text{-}41^{D5}$ mutant background. Only 3.3% of the embryos derived from $mei\text{-}41^{D3}/mei\text{-}41^{D5}$ females hatched, and a 67.4% embryonic hatch rate was observed when females were $mei\text{-}41^{D3}/mei\text{-}41^{D5}$ and heterozygous for the *cyclin* null mutations (Table 5). We also found that *cyclin A* or *cyclin B* alone produced intermediate levels of rescue (Table 5), as did a deficiency covering the *cyclin B* locus (data not shown).

Table 5

Mutations in *cyclin* genes suppress the *mei-41* embryonic lethality.

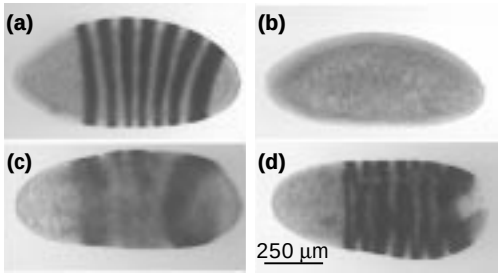
Genotype	Embryonic viability (%)
mei-41^{D5}/mei-41^{D3}; +/+; +/+	3.3 (840)
mei-41^{D5}/mei-41^{D3}; +/+; *cycA*/+	37.4 (1168)
mei-41^{D5}/mei-41^{D3}; *cycB*/+; +/+	46.7 (1138)
mei-41^{D5}/mei-41^{D3}; *cycB*/+; *cycA*/+	67.4 (1874)

Reducing maternal Cyclin levels rescues the *mei-41*-associated embryonic lethality. The percentage hatch rate of embryos derived from females with the indicated genotypes is given, with the total number of embryos scored in brackets. The null alleles of *cyclin B* or *cyclin A* are described in the Materials and methods section.

Thus, rescue of the *mei-41* embryonic lethality is not restricted to a specific *cyclin* mutant chromosome or *mei-41* allele. We conclude that these dramatic genetic interactions are not due to background mutations.

In wild-type embryos, cellularization takes place following a syncytial interphase 13 of approximately 14 minutes, and syncytial cell-cycle times close to wild type are thought to be essential to cell formation and the MBT [15,23,24]. We therefore speculated that the *cyclin* mutations would restore wild-type cell-cycle timing to the *mei-41* mutants. Surprisingly, time-lapse confocal analyses demonstrated that the *cyclin* null mutations did not significantly affect cell-cycle timing in the *mei-41^{D3}* mutant background (Table 1). The length of interphase 13 in the 'rescued' embryos showed significant variability, however, and was occasionally close to 14 minutes (Table 1). It was therefore possible that only embryos that proceeded through longer-than-average syncytial divisions formed cells and hatched. Re-evaluation of our time-lapse data demonstrated that cellularization sometimes followed a syncytial interphase of as little as 8 minutes, and a syncytial interphase of close to 14 minutes was followed by additional syncytial mitoses (data not shown). We then assayed embryos from females homozygous for *mei-41^{D3}* and heterozygous for the *cyclin* mutations for replication checkpoint function: these embryos did not delay mitosis in response to aphidicolin (Figure 4). Therefore, reducing maternal Cyclin levels does not restore wild-type cell-cycle delays or the embryonic replication checkpoint, but appears to bypass a distinct and essential developmental function for *mei-41*.

Figure 6



Reducing maternal Cyclin levels rescues the *mei-41*-associated defects in zygotic gene activation. Whole-mount *in situ* hybridization was performed on embryos derived from *mei-41^{D3}* homozygous females and on embryos from females homozygous for *mei-41^{D3}* and hemizygous for *cyclin A* and *cyclin B*. (a) During interphase 14, at the transition to zygotic control of development, wild-type embryos show the typical seven-striped pattern of *runt* expression. (b) In embryos derived from *mei-41^{D3}* mutant females, stripes of *runt* expression do not develop. (c,d) In embryos derived from females homozygous mutant for *mei-41^{D3}* and heterozygous for *cyclin A* and *cyclin B*, the seven-striped *runt* expression pattern is either partially (c) or fully (d) restored.

Discussion

Essential and nonessential functions for the *Drosophila* replication-checkpoint pathway

Checkpoints are generally defined as nonessential pathways that delay the cell cycle in response to environmentally induced defects in the mitotic spindle or DNA [30,39–41]. Our earlier studies showed that mutations in *grp*, which encodes a *Drosophila* homologue of the Chk1 cell-cycle kinase [16], lead to embryonic lethality due to defects at the MBT, and block replication-checkpoint function during early embryogenesis [15]. These observations raised the possibility that a single checkpoint pathway provided both a nonessential function during the cellular response to DNA damage and an essential developmental role during the transition from maternal to zygotic control of embryogenesis at the MBT. Alternatively, the *grp* gene could have encoded a checkpoint kinase isoform with a specific developmental function, with a *grp*-independent pathway providing nonessential checkpoint functions during the remainder of the life cycle.

To distinguish between these possibilities, we analyzed the embryonic function of a second conserved DNA replication/damage checkpoint gene, and examined the zygotic function of the *grp* gene. The *Drosophila mei-41* gene encodes a structural homologue of the ATM protein, and ATM-related proteins are required for checkpoint function in systems ranging from yeast to man [33–37]. We showed that embryos derived from *mei-41* mutant females fail to terminate syncytial division following mitosis 13, are defective in initiating zygotic transcription, and do not cellularize. We also showed that Mei-41 is required for replication checkpoint function during early embryogenesis. Thus, a second conserved checkpoint gene has an essential developmental function during early embryogenesis. We also showed that *grp* mutants are sensitive to HU and MMS during postembryonic development, and observed dominant genetic interactions between *grp* and *mei-41*

mutations during the zygotic response to MMS (Table 4). Finally, we showed that both developmentally controlled cell-cycle delays at the MBT and aphidicolin-stimulated cell-cycle delays in earlier embryos are accompanied by String degradation and increased Cdc2 phosphorylation (Figure 5) [24]. In both of these contexts, these biochemical changes required *mei-41* and *grp*. Based on these observations, we propose that *mei-41* and *grp* are components of a replication-checkpoint pathway that has both a nonessential function during the cellular response to environmentally induced DNA damage or replication defects and an essential developmental function at the MBT.

A revised model for checkpoint function at the MBT

Zygotic transcription is initiated during syncytial divisions 9 and 10, but the production of functional mRNAs from genes with relatively long primary transcripts depends on the wild-type increases in cell-cycle length that take place during the subsequent four syncytial blastoderm divisions [23,24]. This observation, combined with the cell-cycle defects in *grp* mutant embryos, led us to propose that checkpoint-dependent delays in the embryonic cell cycle allow production of full-length primary transcripts encoding key transcription regulators. These regulators then activate expression of a second wave of transcription factors that drive the MBT [15,25]. In this simple domino model, the only essential function for the checkpoint pathway is to delay the syncytial-blastoderm-stage divisions. The observations reported here suggest that cell-cycle delays alone are not sufficient to trigger the MBT, however. Reducing maternal Cyclin dosage, in an otherwise wild-type background, leads to increases in syncytial-blastoderm cell-cycle times [30]. We found that genetically reducing Cyclin levels rescues the embryonic lethality of *mei-41* mutations, but does not restore wild-type syncytial-blastoderm cell-cycle timing (Table 1). Thus, the truncated cell cycles in *mei-41* mutant embryos appear to be long enough to allow production of any early zygotic factors that might be required at the MBT. This observation also implies that the *cyclin* mutations bypass a requirement for *mei-41* that is distinct from the role of this gene in delaying the syncytial-blastoderm cell cycles.

Cdc2-dependent phosphorylation of the transcription factor TFIIH inhibits pol-II-dependent transcription (see [46] and references therein). This raises the possibility that *mei-41*-dependent inhibition of Cdc2 kinase activity is essential to both cell-cycle delays and activation of high-level zygotic gene expression at the MBT. Consistent with this model, the onset of high-level zygotic transcription normally follows mitosis 13, which is when Cyclin levels first decline to baseline [29]. In addition, during the syncytial-blastoderm divisions, cycloheximide triggers both interphase arrest and premature activation of zygotic gene expression [3] (O.C.M.S. and W.E.T., unpublished observations). The cell-cycle arrest is almost certainly due to loss of Cyclin and reduced Cdc2 activity. Cycloheximide-associated increases in transcription may also be a consequence of reduced Cdc2 kinase activity, which in turn allows activation of the basal transcription machinery.

The timing of the MBT is controlled by the nucleocytoplasmic ratio, suggesting that this transition is triggered as a limiting maternal factor is titrated out by DNA or chromatin during the later cleavage divisions [5–7,30]. On the basis of our observations, we favor a model in which a maternal component of the DNA replication machinery serves as the titrated maternal factor that regulates the timing of the MBT [15,47]. In this model, the replication machinery is in excess and the time required to complete the synthesis (S) phase of the cell cycle is constant until division 10, when at least one replication factor becomes limiting. After this point, the length of time required for chromosome replication progressively increases, and the checkpoint pathway is therefore required to delay mitosis to allow S-phase completion. This model explains the progressive increase in S-phase length during the syncytial-blastoderm divisions, and the requirement for replication-checkpoint function for these increases.

Cell-cycle delays during the later syncytial divisions are essential to ensure the full-length transcription of genes with relatively long transcription units [23,24]. The embryonic checkpoint, which mediates these delays, is therefore required for the production of a subset of early zygotic gene products that include transcription factors. These observations support a model in which checkpoint-dependent syncytial-blastoderm-stage cell-cycle delays are sufficient to allow the production of transcription factors that trigger the switch to zygotic control of development at the MBT [15,23,24]. The observations reported here suggest that cell-cycle delays alone are not sufficient to trigger the MBT, however, and that checkpoint-mediated inhibition of Cdc2 may also be required for the general increase in pol-II-dependent transcription that follows mitosis 13 and accompanies the MBT. Although many aspects of this model are speculative, it makes clear and testable predictions and should serve as a useful framework for the molecular and genetic analyses of the *Drosophila* replication checkpoint and the function of this pathway at the MBT.

Materials and methods

Drosophila *mutants*

This study employs two alleles of *mei-41*, *mei-41^{D3}* and *mei-41^{D5}*. The *mei-41^{D3}* allele is a null mutation based on three criteria: first, homozygous *mei-41^{D3}* females are fully sterile, whereas all other alleles produce females that are at least partially fertile; second, Mei-41 protein is not detectable by western blot analyses in *mei-41^{D3}* mutants; and third, sequence analysis of the *mei-41* gene on the *mei-41^{D3}* mutant chromosome reveals a nonsense mutation at codon 42, resulting in a stop six codons later (A.L. and R.S.H., unpublished observations). The *mei-41^{D5}* allele, by contrast, appears to be a partial loss-of-function mutation. Homozygous *mei-41^{D5}* females are partially

fertile, and a weak Mei-41 band can be detected on western blots from *mei-41^{D3}* mutants. The *grp* allele used in this study (*grp$^{fs(A)4}$*) prevents the production of detectable transcript and appears to be a functional null mutation [15]. Null alleles of *cyclin B* were generously provided by Christian Lehner. The *cycB^{3}* and *cycB^{2}* alleles were generated by imprecise excision of the P element in the *cycB^{1}* allele. Both of these null alleles contain deletions in the 'cyclin box'. Df(2R)59AB, which removes the *cyclin B* gene, was also used in the rescue analyses. Balancer chromosomes are described in [48].

In vivo *analysis of cell-cycle progression*

Embryos were collected and injected with rhodamine-conjugated tubulin (Molecular Probes), as described elsewhere [15,49]. Mutant females were mated to wild-type Oregon R males. For quantitative analysis of S-phase and M-phase length, interphase was defined by the presence of a nuclear envelope that excluded the injected fluorescent protein conjugates, and metaphase was defined by the absence of a nuclear envelope and the presence of spindle structures [15].

Embryonic replication checkpoint assay

To assay for replication checkpoint function during the syncytial-blastoderm stage, living embryos were injected either with a 1:1 mixture of rhodamine-conjugated tubulin (Molecular Probes) and 2% dimethyl sulfoxide (DMSO) in distilled H_2O, or with a 1:1 mixture of rhodamine-conjugated tubulin and 200 μg/ml aphidicolin in 2% DMSO in distilled H_2O. Injection of aphidicolin to a final concentration of 100 μg/ml inhibits DNA synthesis by greater than 95% [39]. The time from maximal interphase centrosome separation to nuclear envelope breakdown (NEB) was then determined by time-lapse confocal microscopy [15]. During the syncytial-blastoderm divisions, centrosome separation and migration along the nuclear envelope is initiated very early in interphase, and maximal separation is achieved approximately 1.5 min after mitosis is completed.

Aphidicolin-stimulated changes in Cdc2 and String

To assay for changes in Cdc2 phosphorylation and String levels on aphidicolin treatment, 1–2 h old embryos were bleach dechorionated, rinsed in distilled H_2O, and transferred to a mixture of octane and Robb's buffer [50], with or without 40 μg/ml aphidicolin. After incubating for 20 min at room temperature, the embryos were collected and assayed for Cdc2 and String by western blotting [30]. Phosphorylated forms of Cdc2 are indicated as described by Edgar *et al.* [30].

Female fertility

Female fertility was determined by mating virgin mutant females (10 per vial) to wild-type Canton-S males. The flies were transferred to fresh food every 12 h, and vials containing the resulting embryos were then maintained at 25°C for one day or at 20°C for two days. The number of eggs produced and the number that hatched into larvae were then scored by visual inspection.

MMS and HU sensitivity

Sensitivity to methylmethane sulfonate (MMS) and hydroxyurea (HU) was quantified as follows: five to ten groups, each containing five females and five males, were crossed and the resulting embryos were collected for 24 or 48 hr. The adult flies were then removed. After 24 h at 25°C, 500 μl of drug, dissolved in distilled H_2O at the indicated concentration, was added to the food. After 2 weeks, all classes of adult progeny were scored. MMS/HU sensitivity is indicated by the preferential loss of a specific genotypic class relative to controls.

Other procedures

For western blot analyses, total protein from staged embryos was subjected to SDS–PAGE, transferred to membranes, and probed for Cdc2 and String, as described elsewhere [5]. As a loading control, blots were probed for tubulin with a monoclonal anti-α-tubulin antibody (DM1α, Sigma). *In situ* hybridization with probes for *runt*, *even-skipped*, *giant* and *knirps* was performed as described [51]. Identical probes, hybridization conditions and color development times were used for wild-type and mutant embryos.

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