

# ***mei-41* and *bub1* block mitosis at two distinct steps in response to incomplete DNA replication in *Drosophila* embryos**

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***Drosophila double park* encodes a homolog of Cdt1 that functions in initiation of DNA replication in fission yeast and *Xenopus* [1–3]. *dup* mutants complete the first 15 embryonic cell cycles, presumably via maternal *dup* products, and show defects in the 16<sup>th</sup> S phase (S16) [1]. Cells carrying *dup*<sup>at</sup> allele forgo S16 altogether but enter mitosis 16 (M16) [1]. We find that the timing of entry into M16 is similar in *dup*<sup>at</sup> and heterozygous or wild-type (wt) controls. In contrast, we find that mutant cells carrying another allele, *dup*<sup>as</sup>, undergo a partial S16 and delay the entry into M16. Thus, initiation of S16 appears necessary for delaying M16. This delay is absent in double mutants of *dup*<sup>as</sup> and *mei-41* (*Drosophila* ATR), indicating that a *mei-41*-dependent checkpoint acts to delay the entry into mitosis in response to incomplete DNA replication. *dup*<sup>as</sup> and *dup*<sup>at</sup> mutant cells that enter M16 become arrested in M16 [1]. We find that mitotic cyclins are stabilized and that a spindle checkpoint protein, Bub1, localizes onto chromosomes during mitotic arrest in *dup* mutants. These features suggest an arrest prior to metaphase-anaphase transition. *dup*<sup>as</sup> *bub1* double mutant cells exit M16, indicating that a *bub1*-mediated checkpoint acts to block mitotic exit in *dup* mutants. To our knowledge, this is the first report of (1) incomplete DNA replication affecting both the entry into and the exit from mitosis in a single cell cycle via different mechanisms and (2) the role of *bub1* in regulating mitotic exit in response to incomplete DNA replication.**

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## **Results and discussion**

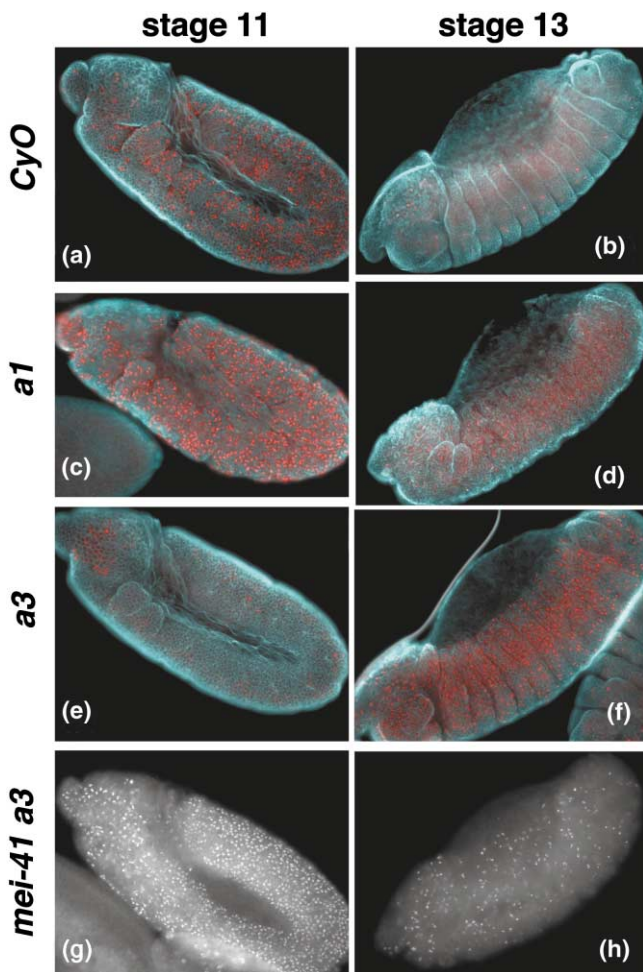
Yeast mutants that cannot complete DNA replication arrest before mitosis, but yeast mutants that fail to initiate

DNA replication fail to arrest; thus, initiation of DNA replication appears necessary to activate a checkpoint that couples mitosis to the completion of S phase [4–6]. This can account for *Drosophila dup* mutants that fail to undergo S16 but enter M16 [1]. It was, however, surprising to find that cells of *dup*<sup>at</sup> and *dup*<sup>as</sup> mutant embryos, previously thought to behave similarly [1], entered M16 at different times (Figure 1). *dup*<sup>at</sup> mutant cells entered M16 approximately concurrent with heterozygous controls, whereas *dup*<sup>as</sup> mutant cells entered M16 after heterozygotes. While the *dup*<sup>at</sup> allele results from a stop codon at 171 (out of a total of 743 amino acids), the *dup*<sup>as</sup> allele results from a stop codon at position 592 [1]. Thus, *dup*<sup>as</sup> mutants may retain partial Dup activity that allows a partial S16 and consequently activates a checkpoint to delay M16. This idea is supported by two pieces of data. First, we detect a partial S16 in *dup*<sup>as</sup> mutants, while confirming the previous report that S16 is absent in *dup*<sup>at</sup> mutants (Figure 2). Second, the delay of M16 seen in *dup*<sup>as</sup> mutants is found to be absent in *mei-41 dup*<sup>as</sup> double mutants (Figure 1, compare 1g to 1e). *mei-41* encodes a homolog of the checkpoint kinase ATR and is required to delay mitosis upon inhibition of DNA synthesis in *Drosophila* syncytial embryos (cycles 11–13; [8]). We conclude that partial DNA synthesis in *dup*<sup>as</sup> mutants delays the entry into M16 via a *Mei-41*-dependent checkpoint.

The effect of *dup* mutations on entry into mitosis is in agreement with findings in yeast and *Xenopus* [4–6, 9]; the effect on exit from M16, however, is contrary to previous results [6]. Budding yeast cells that lack CDC6 skip S phase and enter mitosis (similar to *dup*<sup>at</sup> mutants) but are reported to subsequently exit mitosis with wild-type kinetics to undergo a “reductional” anaphase [6]. In contrast, epidermal cells of both *dup*<sup>as</sup> and *dup*<sup>at</sup> mutants that enter M16, with and without a prior delay, become arrested [1]. *dup*<sup>at</sup> cells are in M16 in stage 11 and mitotic cells are still seen in stage 13 [1]; *dup*<sup>as</sup> cells are in M16 in stage 13 and mitotic cells are still visible at stage 15, if not later (Figure 1f; data not shown). Because the stages are at least 2 hr apart in each case, the arrest in M16 is likely to average at least 2 hr, significantly longer than normal embryonic mitosis of about 10 min.

The apparent difference in the behavior of yeast and *Drosophila* cells that harbor unreplicated chromosomes led us to determine the basis for mitotic arrest in *dup* mutants. To this end, we examined mitotic spindles by staining for  $\alpha$ - and  $\gamma$ -tubulin, and we looked at chromosomes by staining for a mitotic phosphoepitope on histone H3 (PH3; Figure 3). While the spindles are bipolar and appear to

Figure 1



*dup<sup>a3</sup>* mutants delay entry into M16 in a *mei-41*-dependent manner. Embryos were fixed and stained with PH3, an antibody to phosphorylated histone H3 [red in (a)–(f)]; white in (g) and (h)], to identify mitotic cells and with an antibody to  $\alpha$ -tubulin [blue in (a)–(f)] and DNA (not shown) to reveal morphology that aid in embryo staging, as in [7]. *CyO* refers to *dup<sup>a3</sup>/CyO* heterozygotes or *CyO/CyO* that are wt with respect to the *dup* gene, identified by staining for  $\beta$ -galactosidase encoded by the *CyO* balancer chromosome. *a1* indicates *dup<sup>a1</sup>* homozygotes, *a3* indicates *dup<sup>a3</sup>* homozygotes, and *mei-41 a3* indicates homozygous for *dup<sup>a3</sup>* and either homozygous or hemizygous for *mei-41*. *mei-41* mutants were identified by a lack of antibody staining for GFP encoded by the FM7 balancer chromosome. Embryos are shown with anterior to the left and dorsal side up. (a,b) In heterozygous or wt embryos, epidermal cells are in M16 in stage 11, and most have finished M16 by stage 13. (c) *dup<sup>a1</sup>* mutant cells enter M16 concurrently with controls [e.g., (a)]. As previously described, arrest in M16 lasts for at least 2 hr; (d) by stage 13, the number of mitotic cells are reduced. (e) *dup<sup>a3</sup>* mutant cells are delayed in entering M16 such that most are still in interphase and lack PH3 stain in stage 11 embryos. (f) By stage 13, most have entered M16 and are arrested there. (g) The delay in entry into M16 in *dup<sup>a3</sup>* single mutants [e.g., (e)] is abolished in *dup<sup>a3</sup>mei-41* double mutants. (h) Most cells have escaped the arrest by stage 13, similar to *dup<sup>a1</sup>* mutants seen in (d). *dup* and *mei-41<sup>DS</sup>* mutants and antibodies to PH3 (Upstate Biotechnologies),  $\beta$ -galactosidase (Chemicon International), and  $\alpha$ -tubulin (Sigma) have been described before [1, 8, 11]. A rabbit polyclonal antibody to GFP was a gift from J. Kahana and P. Silver. Detailed methods and stock information are in Supplementary materials.

contain functional centrosomes, i.e., they contain  $\gamma$ -tubulin and nucleate aster microtubules (see Supplementary material available with this article online), chromosomes fail to align normally (Figure 3a–f). Most chromosomes lie within the bipolar spindle but are scattered and not compacted into a metaphase plate. Severe alignment defects are readily visible in 84% ( $\pm 11\%$ ) of mitotic cells in *dup<sup>a1</sup>* mutants and 80% ( $\pm 7\%$ ) of mitotic cells in *dup<sup>a3</sup>* mutants; it is probable that higher resolution analyses may reveal higher incidences of defective alignment. Because chromosome configuration in *dup* mutants deviates from normal configurations, we examined other markers of mitotic progression to determine at which stage in mitosis *dup* mutant cells arrest.

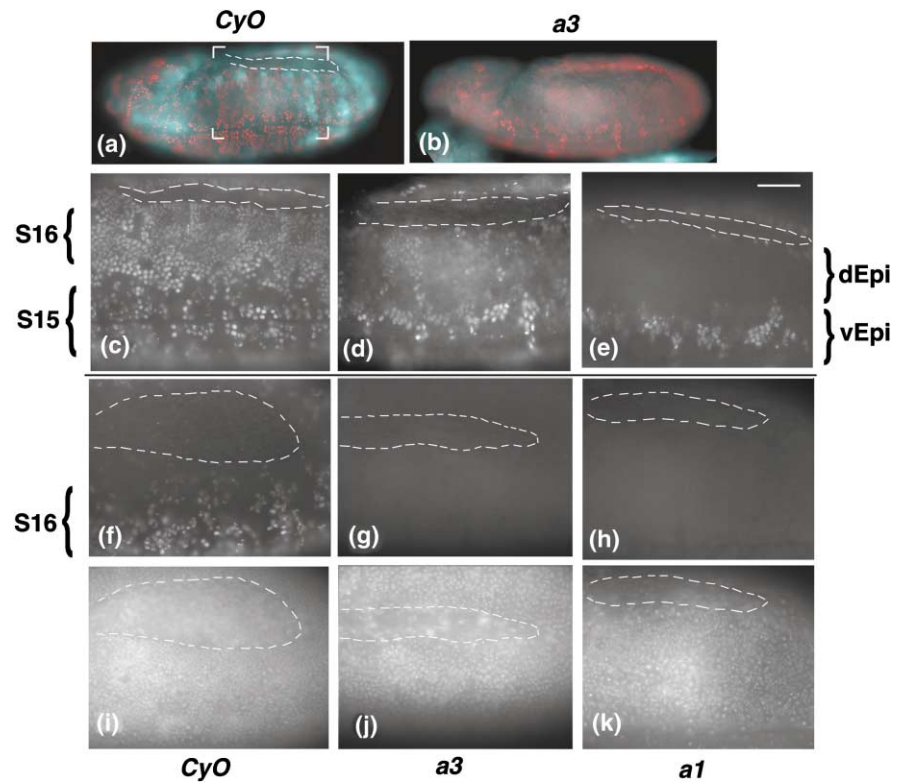
In normal mitosis, Cyclin A degradation concludes in metaphase while Cyclin B degradation concludes in early anaphase [12]. Spindle checkpoint proteins such as Bub1 that bind kinetochores upon spindle damage localize to kinetochores during unperturbed mitosis in metazoa, indicating that the spindle checkpoint is active through earlier parts of mitosis [13–15]. *Drosophila* Bub1 localizes on kinetochores during prometaphase and dissociates during metaphase ([16]; Figure 3g). In *dup* mutants, both cyclins persist during mitotic arrest (Figure 3j,k; see Figure S1 in Supplementary material), and Bub1 is present on discrete sites on chromosomes, presumably at kinetochores (Figure 3h,i). These data suggest that *dup* mutant cells arrest prior to metaphase-anaphase transition.

The persistence of Bub1 on chromosomes and stabilization of Cyclin B occurs when the spindle checkpoint is active; therefore, mitotic arrest in *dup* mutants may be mediated by the spindle checkpoint. To test this directly, we examined double mutants of *dup<sup>a3</sup>* and *bub1*. We found that *dup<sup>a3</sup>bub1* double mutants have fewer mitotic cells when compared to *dup<sup>a3</sup>* single mutant embryos of similar stage (Figure 4a,b). Two types of additional evidence indicate that this difference is due to *dup<sup>a3</sup>bub1* double mutants exiting M16 (rather than reverting to previous interphase). First, approximately 10 times more cells are seen in the act of exiting mitosis (i.e., in telophase) in the double mutants (brackets in Figure 4d; quantified in figure legend). Most telophase cells show chromosome bridges, consistent with the failure to complete DNA replication in these mutants (Figure 4e, arrows). Second, nuclear density is higher in *dup<sup>a3</sup>bub1* double mutants than in *dup<sup>a3</sup>* single mutants, and it approaches that of heterozygotes or wt controls that complete M16 (See Figure S2 in Supplementary Materials). Collectively, these data indicate that a significant number of *dup<sup>a3</sup>bub1* mutant cells exited M16 and suggest that *bub1* is required for mitotic arrest in *dup* mutants.

During mitotic arrest by the spindle checkpoint, Cyclin A is degraded while Cyclin B remains stable [17, 18].

**Figure 2**

S phase in *dup* mutants as revealed by incorporation of BrdU. Genotype denotations are as in Figure 1. Embryos were pulsed with BrdU for 13 min and fixed, and incorporation was detected by immunostaining as described before [21] [red in (a) and (b) and white in (c)–(h)]. Embryos were also stained for (a,b)  $\beta$ -galactosidase and for (i–k) DNA. Detailed methods are in Supplementary materials. Amnioserosa in each case is enclosed by broken lines. At these stages, the dorsal epidermis lies adjacent to the amnioserosa on both sides, followed by the ventral epidermis. The scale bar equals 32  $\mu$ m in (c)–(k). (a,b) Whole embryos illustrate  $\beta$ -galactosidase stain [cyan stripes present in (a) and absent in (b)]. Brackets depict an area similar to those magnified and shown in (c)–(h). (c–e) In late stage 10/early stage 11 embryos, cells of the dorsal epidermis initiate cycle 16 while those in the ventral epidermis are still in cycle 15 (all staging as in [7]). *dup* mutants complete cycle 15 [1]. Therefore, ventral epidermis (vEpi) incorporates BrdU in all genotypes and serves as a positive control. In these embryos, dorsal epidermal cells (dEpi) of heterozygous or wt embryos in S16 and (c) show robust BrdU signal. This signal (d) is diminished in the corresponding cells of *dup<sup>ts3</sup>* mutants and (e) is absent in the corresponding cells of *dup<sup>ts1</sup>* mutants. (f) In stage 11 embryos that are presumably older, ventral epidermis is finishing S16 in controls. (g,h) At similar stages, little or no BrdU incorporation is detected throughout *dup<sup>ts1</sup>*



and *dup<sup>ts3</sup>* mutant embryos. Thus, *dup<sup>ts1</sup>* mutants forgo S16 as reported previously [1], whereas *dup<sup>ts3</sup>* mutants initiate but do not

complete S16 (summarized in Figure 4f). (i), (j), and (k) show DNA signal corresponding to (f), (g), and (h), respectively.

Therefore, persistence of Cyclin A during mitotic arrest in *dup* mutants suggests that additional control(s), besides the spindle checkpoint, operate to stabilize Cyclin A. DNA damage leads to stabilization of Cyclin A in *Drosophila* [11]. Possibly, the presence of incompletely replicated DNA during mitosis also leads to stabilization of Cyclin A. *bub1*-mediated controls, however, appear more consequential because *dup<sup>ts3</sup>bub1* double mutants exited mitosis. This would be consistent with the finding that Cyclin A at endogenous levels cannot block mitotic exit in *Drosophila* [18, 19].

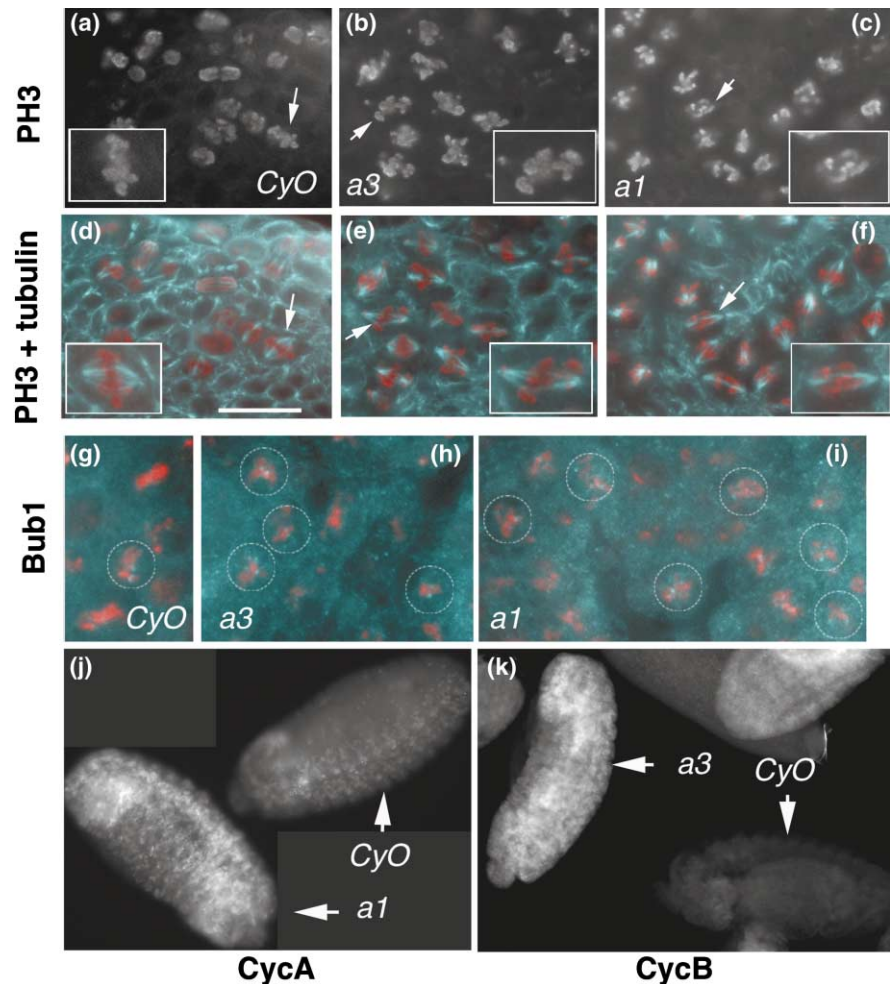
As stated above, CDC6-deficient cells are reported to exit mitosis with normal kinetics [6]. A recent examination of these cells, however, revealed a mitotic arrest that requires MAD2 [23]. Thus, incomplete DNA replication in both yeast and *Drosophila* results in mitotic arrest mediated by members of the spindle checkpoint. Why might this be? A complete or partial absence of sister chromosomes would lead to a complete or partial absence of sister chromatid cohesion. The failure to duplicate centromeres, likely in *dup<sup>ts3</sup>* mutants and certainly in *dup<sup>ts1</sup>* mutants, would preclude the formation of kinetochore pairs. Either

deficiency would preclude stable bipolar attachment of chromosomes to the spindle and thereby activate the spindle checkpoint.

In summary, *dup* mutants demonstrate two ways in which mitosis is regulated in response to incomplete duplication of the genome (Figure 4g). First, entry into mitosis is delayed via *mei-41*, *Drosophila* ATR. Second, exit from mitosis is blocked via a spindle checkpoint function, *bub1*. In *Drosophila* syncytial cycles, nuclei delay the entry into mitosis upon inhibition of DNA synthesis, but exit from mitosis is not blocked. Instead, chromosome separation fails during the exit from mitosis, resulting in polyploid nuclei that are subsequently eliminated [8, 10]. In other systems, either the entry into mitosis (in fission yeast and vertebrate cells) or the exit from mitosis (in budding yeast) is blocked in response to incomplete DNA synthesis [20]. Therefore, this is the first report of mitosis in a single cell cycle being regulated at two points via two different mechanisms in response to incomplete DNA replication. Identification of these responses in *Drosophila*, a genetically tractable organism with superb cytology, should en-

**Figure 3**

Mitosis 16 in *dup* mutants. Embryos were fixed and stained with various antibodies and for DNA: in (a)–(c) and red in (d)–(f) for PH3, cyan in (d)–(f) for  $\alpha$ -tubulin, cyan in (g)–(i) for Bub1, cyan in (j) for Cyclin A, cyan in (k) for Cyclin B, and red in (g)–(i) for DNA. Genotype denotations are as in Figure 1. Antibodies to cyclins have been described before [22]. A polyclonal anti-*Drosophila* Bub1 antibody was a gift from C. Sunkel. Detailed methods are in Supplementary materials. The scale bar equals 11  $\mu$ m in (a)–(j). (a), (b), and (c) show PH3 stain corresponding to (d), (e), and (f), respectively. Chromosomes compact into metaphase plates midway between spindle poles in controls [arrow in (a) and (d)]; magnified 2 $\times$  in insets. In *dup* mutants (b,c,e,f), chromosomes are spread along the spindle axis, often as far as the spindle poles (e.g., arrows; magnified 2 $\times$  in insets). (g–i) Bub1 (cyan), may be white when present on red background) is present on chromosomes (red) in a fraction of metaphase cells in heterozygotes or wt, consistent with previous data [16]. One of three metaphase figures in (g) show Bub1 staining and is circled. In *dup* mutants, Bub1 signal is found on chromosomes of most mitotic cells (h,i; those with Bub1 foci clearly in this plane of focus are circled). (j) Two embryos indicated by arrowheads are of similar age (stage 13). In the control (*CyO*), most epidermal cells have exited M16 and lack Cyclin A, while CNS and PNS cells that remain proliferative retain this protein. Cyclin A persists throughout *dup<sup>a1</sup>* mutant embryos (*a1*), although the staining intensity varies from cell to cell (e.g., Figure S1 in Supplementary material). Similar data were obtained with *dup<sup>a3</sup>* mutants (Figure S1 in Supplementary materials). (k) Two embryos indicated by arrowheads are of similar age (stage 14); Cyclin B levels are higher in the *dup<sup>a3</sup>* mutant. Similar data were obtained for *dup<sup>a1</sup>* mutants (Figure S1 in Supplementary materials).



able testing of candidate checkpoint genes and searching for new genes that function at each regulatory point.

#### Supplementary material

Supplementary material including additional Results and discussion and figures is available at <http://images.cellpress.com/supmat/supmatin.htm>.

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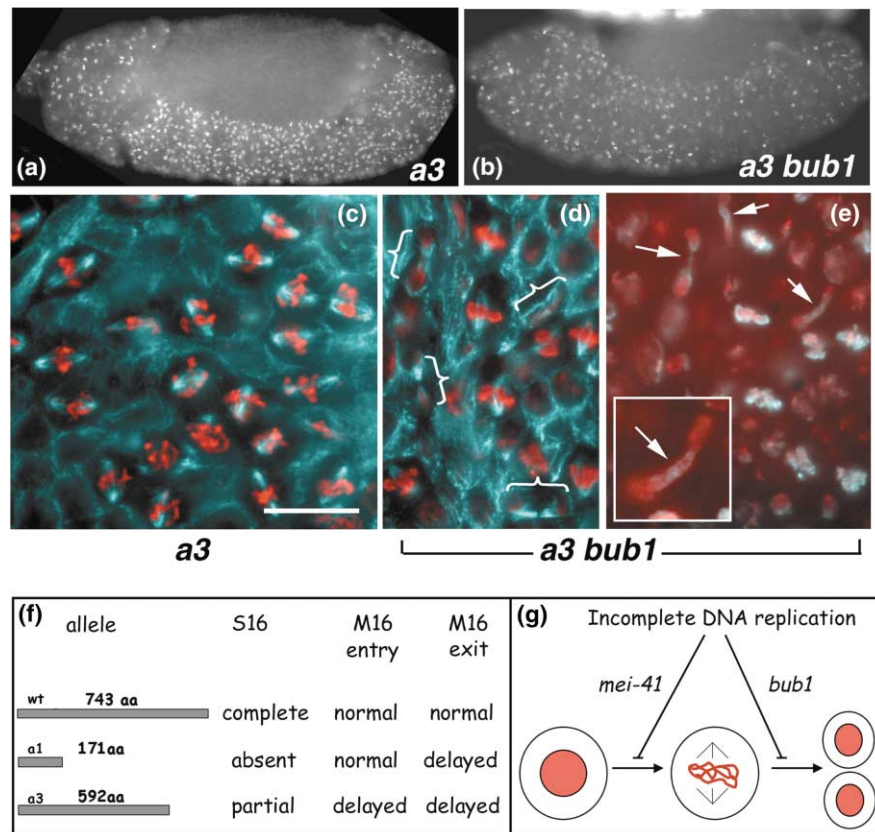
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Figure 4

*dup<sup>a3</sup>bub1* double mutants are unable to sustain arrest in M16. Embryos were fixed and stained for [(a,b), red in (c,d), cyan in (e)] PH3, [cyan in (c,d)]  $\alpha$ -tubulin, and [red in (e)] DNA. *a3 bub1* are double homozygotes of *dup<sup>a3</sup>bub1*. Other genotype denotations are as in legend to Figure 1. *bub1<sup>K03113</sup>* mutants have been described before [16]. Detailed methods and stock information are in Supplementary materials. The scale bar equals 32  $\mu$ m in (c)–(e). (a,b) *dup<sup>a3</sup>bub1* double mutants have fewer mitotic cells than *dup<sup>a3</sup>* mutants. Stage 13 embryos are shown with dorsal up and anterior to the left. The dorsal side of the embryo in (a) is tilted toward the viewer; consequently, amnioserosa (region without mitosis in the middle of the embryo) appears larger in (a) than in (b). (c–e) *dup<sup>a3</sup>bub1* mutants show more telophase cells [brackets in (d)] than *dup<sup>a3</sup>* mutants (c). Epidermal cells of stage 12 embryos are shown. In *dup<sup>a3</sup>* mutants in embryonic stages 12 and 13, the ratio of telophase cells to cells arrested with bipolar spindles is 0.03 ( $\pm 0.01$ ) to 1; for every 33 cells arrested in M16, one was found to be exiting mitosis. The corresponding ratio is 0.35 ( $\pm 0.28$ ) to 1 in *dup<sup>a3</sup>bub1* double mutants. The variation in this number is likely due to the fact that anaphase and telophase are transient states, and the fraction of cells caught in these states at the time of fixing may vary from embryo to embryo. (e) Separating nuclei in *dup<sup>a3</sup>bub1* double mutants show chromosome bridges (arrows; magnified 2 $\times$  in inset), which retain PH3, consistent with incomplete DNA replication. (f) A summary of *dup* phenotypes. Bars represent the predicted length of Dup protein encoded by each allele. (g) A model for regulation of mitosis in the presence of incompletely replicated DNA, such as in *dup<sup>a3</sup>*



mutants. *mei-41* and *bub1* inhibit the entry into and the exit from mitosis, respectively. The inhibition, although depicted as a single step, is likely to be indirect and to involve many steps and molecules. The inhibition of mitotic exit is seen also in *dup<sup>a1</sup>* mutants. Therefore, this

regulation may also occur when cells fail to initiate DNA replication, as well as when cells fail to complete DNA replication. We caution, however, that *dup<sup>a1</sup>* mutants may synthesize a small amount of DNA that is below the level of detection in this study.

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