

The Cyclin-dependent Kinase Inhibitor Dacapo Promotes Genomic Stability during Premeiotic S Phase

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The proper execution of premeiotic S phase is essential to both the maintenance of genomic integrity and accurate chromosome segregation during the meiotic divisions. However, the regulation of premeiotic S phase remains poorly defined in metazoa. Here, we identify the p21^{Cip1}/p27^{Kip1}/p57^{Kip2}-like cyclin-dependent kinase inhibitor (CKI) Dacapo (Dap) as a key regulator of premeiotic S phase and genomic stability during *Drosophila* oogenesis. In *dap*^{-/-} females, ovarian cysts enter the meiotic cycle with high levels of Cyclin E/cyclin-dependent kinase (Cdk)2 activity and accumulate DNA damage during the premeiotic S phase. High Cyclin E/Cdk2 activity inhibits the accumulation of the replication-licensing factor Doubleparked/Cdt1 (Dup/Cdt1). Accordingly, we find that *dap*^{-/-} ovarian cysts have low levels of Dup/Cdt1. Moreover, mutations in *dup/cdt1* dominantly enhance the *dap*^{-/-} DNA damage phenotype. Importantly, the DNA damage observed in *dap*^{-/-} ovarian cysts is independent of the DNA double-strands breaks that initiate meiotic recombination. Together, our data suggest that the CKI Dap promotes the licensing of DNA replication origins for the premeiotic S phase by restricting Cdk activity in the early meiotic cycle. Finally, we report that *dap*^{-/-} ovarian cysts frequently undergo an extramitotic division before meiotic entry, indicating that Dap influences the timing of the mitotic/meiotic transition.

INTRODUCTION

During the meiotic cycle, germ cells complete two divisions to produce haploid gametes. Before the two meiotic divisions, the germ cells duplicate their genomes during the premeiotic S phase. Events unique to the premeiotic S phase, such as the expression of REC8, a member of the kleisin family of structural maintenance of chromosome proteins, are required for the full execution of the downstream meiotic program (Watanabe and Nurse, 1999; Watanabe *et al.*, 2001; Strich, 2004). How this specialized meiotic S phase is regulated, as well as how similar it is to the mitotic S phase, has long been a question of interest. Studies from yeast indicate that the mitotic cycle and the meiotic cycle use much of the same basic machinery to replicate their genomes (reviewed in Strich, 2004). For example, the minichromosome maintenance complex (MCM2-7), which functions as a DNA replication helicase, is essential for the duplication of the genome during both the mitotic and premeiotic S phase (Murakami and Nurse, 2001; Lindner *et al.*, 2002). Additionally, both the mitotic and premeiotic S phase require the activity of cyclin-dependent kinases (Cdks) (Stuart and Wittenberg, 1998; Bell and Dutta, 2002a; Benjamin *et al.*, 2003). Yet, despite its fundamental importance to both the maintenance of genomic integrity and the downstream events of meiosis, little is known about the regulation of premeiotic S phase metazoa.

Drosophila provides an excellent model to examine the early events of the meiotic cycle, because the entire process

of oogenesis takes place continuously within the adult female. In *Drosophila*, each ovary is composed of 12–16 ovarioles containing linear strings of maturing follicles also called egg chambers. New egg chambers are generated at the anterior of the ovariole in a region called the germarium that contains both germline and somatic stem cells. The germarium is divided into four regions according to the developmental stage of the cyst (Figure 1A). Oogenesis starts in region 1 when a cystoblast, the asymmetric daughter of the germline stem cell, undergoes precisely four rounds of mitosis with incomplete cytokinesis to produce a cyst of 16 interconnected germline cells with an invariant pattern of interconnections (individual cells in the cyst are referred to as cystocytes). Stable actin-rich intercellular bridges called ring canals connect individual cystocytes within the cyst (Robinson and Cooley, 1996). Germline cyst formation is accompanied by the growth of the fusome, a vesicular and membrane skeletal protein-rich organelle that forms a branched structure extending throughout all the cells of the cyst (Figure 1A; de Cuevas *et al.*, 1997; McKearin, 1997). After the completion of the mitotic cyst divisions, all 16 cystocytes complete a long premeiotic S phase in region 2a of the germarium (Carpenter, 1981). Subsequently, the two cystocytes with four ring canals form long synaptonemal complexes (SCs) and begin to condense their chromatin, suggesting that they are in pachytene of meiotic prophase I (Figure 1A; Carpenter, 1975). Several of the cells with three ring canals also assemble short SCs, and even cells with only one or two ring canals are occasionally seen to contain traces of SCs. However, as oogenesis proceeds, the SC is restricted to the two pro-oocytes and finally to the single oocyte in region 2b (Carpenter, 1975; Huynh and St Johnston, 2000). The other 15 cystocytes lose their meiotic characteristics, enter the endocycle and develop as polyploid nurse cells.

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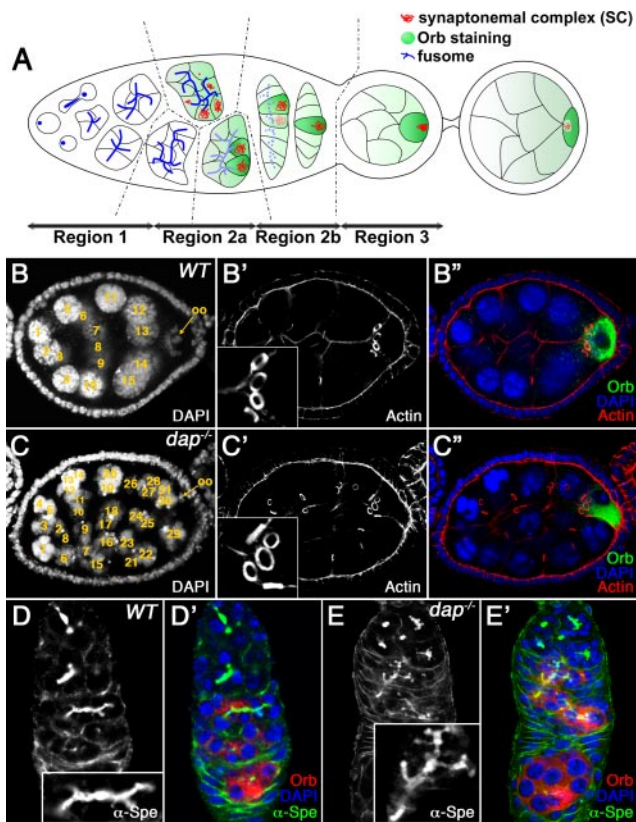


Figure 1. *dap* mutants undergo extraovarian cyst divisions. (A) Schematic representation of the germarium. See text for details. The regions of the germarium (1, 2a, 2b, and 3) are indicated at the bottom. Germ cell cyst formation is accompanied by growth of the fusome (blue dots and lines), which forms a branched structure extending through all the ring canals. In midregion 2a, the SC (red) is assembled in the two pro-oocytes, which progress to pachytene. In region 2a, cytoplasmic proteins, such as Orb (green), accumulate in the germline cyst and are progressively restricted to the oocyte. (B) Wild-type and (C) *dap*^{-/-} mutant egg chambers, stained with DAPI (B, C; B', C', blue), rhodamine-conjugated phalloidin (B', C'; B'', C'', red), and α -Orb (B'', C'', green). The oocyte is indicated by an arrow. Note that the *dap*^{-/-} mutant oocyte in C'', marked by the α -Orb staining, is surrounded by five ring canals. (D) Wild-type and (E) *dap*^{-/-} mutant germlaria, stained with α -Spe (D, E; D', E', green) and α -Orb (D', E', red) antibodies and DAPI (D', E', blue). The inset in D corresponds to a fusome from a 16-cell cyst. Note that the *dap*^{-/-} mutant fusome in E, inset, is larger and more branched and corresponds to a 32-cell cyst. Insets in D and E are shown at the same magnification.

During both the mitotic cycle and the meiotic cycle, it is essential that the entire genome is duplicated precisely once during the S phase. In the mitotic cycle, the licensing of the DNA occurs when Cdc6 and Cdt1/Double Parked (Dup) load the MCM2-7 complex onto the origin recognition complex (ORC) to form the prereplication complex (preRC) (reviewed in Bell and Dutta, 2002b; DePamphilis *et al.*, 2006). PreRC formation occurs in late mitosis and G1 when Cdk activity is low. At the onset of S phase, Cdk activity increases, and the preRC initiates bidirectional DNA replication. PreRC formation must be suppressed after the initiation of S phase to prevent rereplication and thus ensure that each segment of the DNA is replicated exactly once per cell cycle (Bell and Dutta, 2002b; DePamphilis *et al.*, 2006). Cdks play a critical role in this process by preventing reestablishment of the preRC through multiple redundant mechanisms

(Drury *et al.*, 1997; Fujita *et al.*, 2002; Mendez *et al.*, 2002). Thus, during the mitotic cycle the precise regulation of Cdk activity ensures that each segment of DNA is replicated once, and only once, per cell cycle.

The p21^{cip1}/p27^{kip1}/p57^{kip2}-like cyclin-dependent kinase inhibitor (CKI) Dacapo (Dap) specifically inhibits Cyclin E/Cdk2 complexes (de Nooij *et al.*, 1996; Lane *et al.*, 1996). In *Drosophila*, Cyclin E/Cdk2 activity is required for DNA replication during both mitotic cycles and endocycles (Knoblich *et al.*, 1994; Lilly and Spradling, 1996). Similar to what is observed with CKIs in other animals, Dap functions to coordinate exit from the cell cycle with terminal differentiation (de Nooij *et al.*, 1996; Debec *et al.*, 1996). Indeed, high levels of Dap are observed upon exit from the cell cycle in multiple tissues during both embryonic and larval development (de Nooij *et al.*, 1996; Lane *et al.*, 1996; de Nooij *et al.*, 2000; Liu *et al.*, 2002b). Additionally, in the adult ovary, high levels of Dap prevent oocytes from entering the endocycle with the nurse cells as ovarian cysts exit the germarium in stage 1 of oogenesis (Hong *et al.*, 2003). However, in addition to its well-established developmental function, recent work indicates that during developmentally programmed endocycles Dap facilitates the licensing of DNA replication origins by reinforcing low Cyclin E/Cdk2 kinase activity during the Gap phase (Hong *et al.*, 2007). In *dap*^{-/-} mutants, cells undergoing endocycles have reduced chromatin bound MCM2-7 complex, indicating a reduction in the density of preRCs along the chromatin. Additionally, *dap*^{-/-} cells accumulate high levels of DNA damage due to the inability to complete genomic replication (Hong *et al.*, 2007). Thus, during developmentally programmed endocycles Dap functions to reinforce low Cdk activity during the Gap phase.

Here, we demonstrate that the CKI Dap promotes genomic stability during the premeiotic S phase of the *Drosophila* oocyte. Our data indicate that Dap facilitates the licensing of DNA replication origins for the premeiotic S phase by restricting Cyclin E/Cdk2 activity during the early meiotic cycle. These studies represent the first example of a CKI regulating premeiotic S phase and genomic stability in a multicellular animal. Additionally, we find that Dap influences the timing of the mitotic/meiotic switch in ovarian cysts.

MATERIALS AND METHODS

Fly Stocks

The *FRT42B*, *dap*⁴ stock (lane *et al.*, 1996) was a gift of Iswar Hariharan (University of California, Berkeley, CA). *hs-FLP1*, *w¹¹¹⁸*; *Adv¹/CyO*, *y¹ w¹/Dp(1;Y) y⁺*; *mei-P22^{P22}*; *st^{ts}pa-pol* and *w¹¹¹⁸*; *FRT42B ubi-GFP* were obtained from the Bloomington Stock Center (University of Indiana, Bloomington, IN). *dup⁹¹* (Whittaker *et al.*, 2000b) was a gift of Terry Orr-Weaver (Massachusetts Institute of Technology, Cambridge, MA). The *dap*⁴, *dup⁹¹* chromosomes were generated by meiotic recombination (Hong *et al.*, 2007). Homozygous *dap*⁴ mutant germline cell clones were generated by FLP/FLP recombinase target (FRT)-mediated site-specific recombination (Xu and Rubin, 1993).

Immunocytochemistry

Immunocytochemistry of adult ovary staining was performed as described in McKearin and Ohlstein (1995). For 5-bromo-2'-deoxyuridine labeling, ovaries were dissected and stained according to Calvi and Lilly (2004). 5-Ethynyl-2'-deoxyuridine (EdU) incorporation and labeling were done using the Click-it EdU Imaging kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The following antibodies were used in this study: mouse monoclonal α -MPM2 (1:50; Dako North America, Carpinteria, CA), rabbit polyclonal α -C(3)G (1:3000; Hong *et al.*, 2003), mouse monoclonal α -green fluorescent protein (GFP) (1:200; Roche Diagnostics, Indianapolis, IN), rabbit polyclonal α -GFP (1:500; Invitrogen), mouse monoclonal α -Orb 6H4 (1:50; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse monoclonal α -Spectrin 3A9 (1:20; Developmental Studies Hybridoma Bank), mouse monoclonal α -proliferating cell nuclear antigen (PCNA) PC10 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Guinea pig α -Dup/Cdt1 (1:1000; Whittaker *et al.*, 2000a) was provided by Terry Orr-Weaver. Rabbit

α -Dup/Cdt1 (1:200) was kindly provided by Michael Botchan (University of California, Berkeley, CA). Rabbit polyclonal α - α -Spectrin (1:1000; Byers *et al.*, 1987) was a gift of Ron Dubreuil (University of Illinois, Chicago, IL). Mouse monoclonal α -C(3)G (1:500; Anderson *et al.*, 2005) was provided by R. Scott Hawley (Stowers Institute, Kansas City, MO). Mouse monoclonal α -CycE 8B10 and rat polyclonal α -CycE (1:10 and 1:100, respectively; Richardson *et al.*, 1995) were a gift of H. E. Richardson (Peter McCallum Cancer Centre, Melbourne, Australia). Rabbit polyclonal α - γ -H2Av antibodies (1:3000 and 1:500) were provided by Bob Glaser (Wadsworth Center, Albany, NY) and Kim McKim (Waksman Institute, Piscataway, NJ), respectively (Leach *et al.*, 2000; Mehrotra and McKim, 2006). Numbers shown in Tables 1 and 2 were obtained using the rabbit α - γ -H2Av antibody from Bob Glaser. Fluorescence-conjugated secondary antibodies were purchased from Invitrogen and were used at a 1:800 dilution. Actin was labeled with rhodamine-conjugated phalloidin (Invitrogen) at 0.1 μ g/ml, and DNA was labeled with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO) at 1 μ g/ml. All samples were mounted in cytofluor (University of Kent, Canterbury, Canterbury, United Kingdom). Samples were examined with a FluoView FV1000 microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Data were analyzed using a Student's *t* test. Data were considered statistically significant when *p* was <0.05.

RESULTS

dap^{-/-} Ovarian Cysts Delay Meiotic Entry

Relative to yeast, little is known about how Cdks influence early meiotic progression in multicellular organisms. In *Drosophila*, the levels of the G1 Cyclin, cyclin E, influence the number of mitotic divisions that occur before meiotic entry (Lilly and Spradling, 1996; Doronkin *et al.*, 2003; Ohlmeyer and Schupbach, 2003). In wild-type ovaries, a cystoblast undergoes four synchronous mitotic divisions to produce an ovarian cyst with 16 interconnected cells. However, mutations that decrease Cyclin E levels in the female germline result in a reduced number of mitotic cyst divisions and the production of four- or eight-cell cysts (Lilly and Spradling, 1996). Conversely, mutations that raise the levels of Cyclin E protein increase the number of mitotic divisions resulting in 32-cell cysts (Doronkin *et al.*, 2003; Ohlmeyer and Schupbach, 2003). Thus, in the female germ line, the Cyclin E/Cdk2 activity is precisely regulated as cells exit the mitotic cycle and enter the meiotic cycle.

To define the pathways that control Cyclin E/Cdk2 activity during oogenesis, we examined whether the CKI Dap influences the cell cycle program of premeiotic and/or early meiotic ovarian cysts. Consistent with Dap functioning to inhibit Cyclin E/Cdk activity before meiotic entry, 25% of *dap*^{-/-} ovarian cysts undergo a fifth mitotic cyst division to produce egg chambers with 32 cells (*n* = 102, Figure 1C and Supplemental Figure 1). Importantly, *dap*^{-/-} egg chambers with 32 cells contain only a single oocyte, as indicated by the preferential accumulation of the oocyte marker Orb in a single cell (Figure 1C'). Additionally, in egg chambers with 32 cells, the oocyte always contains five ring canals, demonstrating that the cyst has undergone five divisions (Figure 1C', inset, and C''). Finally, in *dap*^{-/-} mutants the fusome, a germline specific organelle that connects all the cells within an individual cyst, often contains extra branches (Figure 1E, inset). Together, these data confirm that the *dap*^{-/-} egg chambers containing 32 germline cells are the result of an extramitotic division, rather than the fusion of two 16-cell cysts. Thus, mutations in the CKI *dap* phenocopy mutations that increase Cyclin E levels in the female germ line. These data demonstrate that *dap* is a component of the pathway that regulates the transition from the mitotic to the meiotic cycle and further support the role of Cyclin E/Cdk2 activity in determining the number of mitotic cyst divisions that occur before meiotic entry.

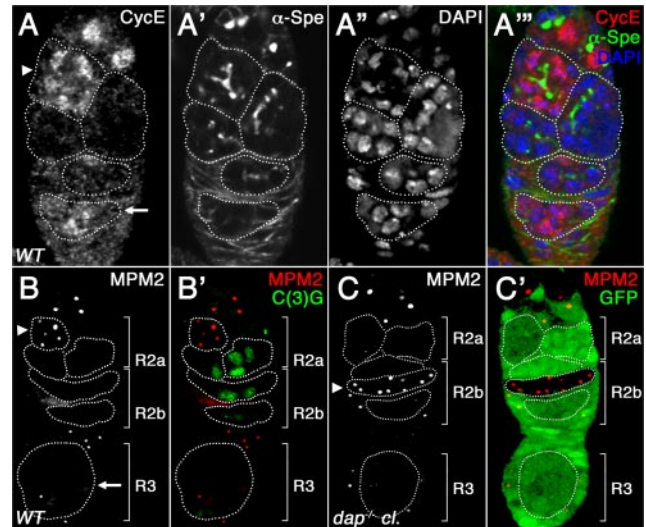


Figure 2. *dap* mutants have increased Cyclin E/Cdk2 activity. (A) Wild-type germarium, labeled with α -Cyclin E (A; A'', red) and α - α -Spe antibodies (A'; A''', green) and DAPI (A''; A''', blue). Cyclin E is expressed in the mitotically active cysts in region 1 and in 16-cell cysts in early region 2a (number of cells in the cyst is determined by the fusome branching pattern, arrowhead), is absent from late region 2a and region 2b, and reappears in region 3 as the nurse cells enter the endocycle (arrow). (B) Wild-type and (C) *dap*^{-/-} mutant clones stained with α -MPM2 (B, C; B', C', red), α -C(3)G (B', green), and α -GFP (C', green) antibodies. *dap*^{-/-} clones are identified by the absence of α -GFP staining. Note that MPM2 and C(3)G are never expressed in the same cysts. (C) In region 2b of *dap*^{-/-} ovarian cysts, all cells have α -MPM2 positive histone loci bodies (arrowhead).

Dap Inhibits Cyclin E/Cdk2 Activity in Early Ovarian Cysts

The increased number of ovarian cyst divisions observed in *dap*^{-/-} mutants is consistent with increased Cyclin E/Cdk2 activity before meiotic entry (Lilly and Spradling, 1996; Lilly *et al.*, 2000; Ohlmeyer and Schupbach, 2003). Therefore, to assess Cyclin E/Cdk2 activity in wild-type versus *dap*^{-/-} ovarian cysts, we used the α -mitotic protein monoclonal-2 (MPM2) antibody (Davis *et al.*, 1983). The α -MPM2 antibody, raised against a phospho-epitope from human mitotic cells, detects Cyclin E/Cdk dependent staining of the histone locus body in *Drosophila* (Calvi *et al.*, 1998; White *et al.*, 2007). During the mitotic cyst divisions, α -MPM2-positive histone loci bodies are observed as small foci in a fraction of dividing cysts, consistent with the oscillation of Cyclin E/Cdk2 activity during the ovarian cyst divisions (Figure 2B, arrowhead). After the completion of the mitotic cyst divisions, all 16 cystocytes enter into premeiotic S phase in early region 2a of the germarium (Carpenter, 1975). We find that ovarian cysts in early region 2a are often Cyclin E positive (Figure 2A, A'', arrowhead) and contain MPM2-positive foci (Supplemental Figure 2, arrowhead). However, when cysts enter prophase of meiosis I and construct an SC in late region 2a and into early region 2b, we observe no MPM2-positive histone loci bodies, suggesting that the level of Cyclin E/Cdk2 activity has fallen (Figure 2B'). Finally, in region 3 of the germarium, coincident with a burst of Cyclin E expression that accompanies the asynchronous entry of the nurse cells into the first endocycle (Figure 2, A and A'', arrow), a fraction of nurse cells again have α -MPM2-positive foci (Figure 2B, arrow). In contrast to the developmentally dynamic α -MPM2 expression observed in wild-type

ovarian cysts, in *dap^{-/-}* mutant germaria all ovarian cysts, at all stages of development, contain α -MPM2-positive foci (Figure 2C, arrowhead; data not shown). These results strongly suggest that in *dap^{-/-}* mutants, the baseline level of Cyclin E/Cdk activity is increased both before and after ovarian cysts enter the meiotic cycle. Additionally, these data suggest that the dynamics of Cyclin E oscillations are altered in the *dap^{-/-}* background or that the low levels of Cyclin E protein present in region 2b and region 3 of the germarium are sufficient to activate Cdk2 in the absence of the inhibitor Dap.

dap^{-/-} Ovarian Cysts Have Increased γ -H2Av Staining

The extramitotic cyst division, as well as the dramatically increased number of ovarian cysts with α -MPM2-positive histone loci bodies, suggest that *dap^{-/-}* ovarian cysts have inappropriately high cyclin E/Cdk2 activity as they enter meiosis. We wanted to determine whether the increased Cdk activity observed in *dap^{-/-}* ovarian cysts, influences progression through the premeiotic S phase. In mammals and yeast, deregulated Cdk activity during the mitotic cycle inhibits the formation of preRCs, often resulting in genomic instability (Spruck *et al.*, 1999; Lengronne and Schwob, 2002; Tanaka and Diffley, 2002; Ekholm-Reed *et al.*, 2004). This genomic instability stems from inappropriately high Cdk activity during G1, reducing the number of licensed origins assembled along the chromatin before S phase. Ultimately, the low density of DNA replication origins leads to intraorigin distances that are too large to be transversed by DNA polymerase during a single S phase. This causes DNA replication forks to stall and ultimately collapse, resulting in DNA damage and the production of double-stranded breaks (DSBs) (Spruck *et al.*, 1999; Tanaka and Diffley, 2002; Ekholm-Reed *et al.*, 2004).

To determine whether the increased Cyclin E/Cdk2 activity observed in *dap^{-/-}* ovarian cysts results in DNA damage during the premeiotic S phase, we used an anti-phosphoprotein antibody specific for phosphorylated H2Av (γ -H2Av) (Madigan *et al.*, 2002). One of the earliest responses to DNA damage is the phosphorylation of H2A histone variants near the sites of DSBs (Modesti and Kanaar, 2001; Madigan *et al.*, 2002). In the *Drosophila* ovary, meiotic DSBs are generated after the initiation of SC formation in late region 2a of the germarium (Carpenter, 1975; Jang *et al.*, 2003; Mehrotra and McKim, 2006). Accordingly, γ -H2Av nuclear foci are first observed in the two pro-oocytes, which are in early pachytene, in late region 2a (Jang *et al.*, 2003; Mehrotra and McKim, 2006 and Figure 3A, arrowhead). Additionally, a small number of DSB are also observed in the pronurse cells cysts in late region 2a (Table 2). As meiosis proceeds and the DSBs are repaired, γ -H2Av-positive foci are reduced and ultimately disappear in late region 2b (Jang *et al.*, 2003; Figure 3A, arrow). By germarial region 3, there is no detectable γ -H2Av signal in the oocyte (Figure 3A), whereas γ -H2Av staining is again observed in nurse cells as they begin to endoreplicate their DNA and become polyploid (Mehrotra and McKim, 2006; Hong *et al.*, 2007; Figure 3A, asterisk).

Beginning in region 2a of the germarium, which marks the onset of meiosis, we find that in *dap^{-/-}* ovarian cysts, the two pro-oocytes have at least 3 times as many γ -H2Av foci as observed in similarly staged wild-type cysts (Table 1). Additionally, in *dap^{-/-}* mutants the γ -H2Av foci persist into late region 2b and region 3 (Figure 3, B and B'). In wild-type cysts, the majority of DSBs are repaired by region 3 of the germarium, with only an occasional focus observed in wild-type oocytes (0.2 γ -H2Av foci observed, Table 1; Mehrotra

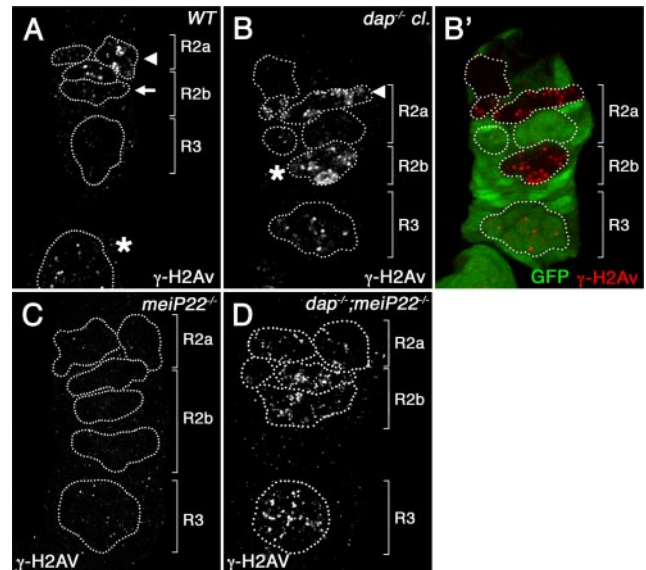


Figure 3. γ -H2Av foci accumulate in *dap^{-/-}* ovarian cysts and are independent of meiotic DSBs. Wild-type (A), *dap^{-/-}* mutant clones (B), *mei-P22^{P22/P22}* (C), and *dap^{-/-}; mei-P22^{-/-}* double mutant germaria (D), stained with α - γ -H2Av (A, B, C, D; B', red) and α -GFP (B', green) antibodies. *dap^{-/-}* clones are identified by the absence of α -GFP staining. (A) In wild-type, γ -H2Av foci are restricted to region 2a of the germarium, where meiotic DSBs form, whereas in B, *dap^{-/-}* mutant cysts they increase in number (B, arrowhead) and persist into region 2b (B, asterisk). (D) The DNA damage induced in *dap^{-/-}* cysts is not suppressed in a *mei-P22^{-/-}* mutant background.

and McKim, 2006). In contrast, in *dap^{-/-}* mutants, oocytes retain ~ 30 γ -H2Av foci in region 2b and region 3 (Figure 3B, asterisk, and Table 1). The number of DSBs, as measured by γ -H2Av staining, is also increased in the pronurse cells in *dap^{-/-}* mutants. In wild-type ovaries, there is less than one γ -H2Av foci per pronurse cell in cysts from region 2a and region 2b of the germarium (Table 2; Mehrotra and McKim, 2006). However, in *dap^{-/-}* cysts this number increases greater than 10-fold, with ~ 12 and 14 γ -H2Av foci found in pronurse cells from region 2b and region 3, respectively (Table 2 and Figure 3B, asterisk). Thus, in *dap^{-/-}* germaria, ovarian cysts have an increased number of γ -H2Av foci and therefore increased levels of DNA damage in both the pro-oocyte and pronurse cells.

Are the increased levels of DNA damage found in *dap^{-/-}* ovarian cysts simply due to the inability of the mutants to repair the DSBs that initiate meiotic recombination? To address this question, we examined γ -H2Av staining in *dap^{-/-}; mei-P22^{P22}* double-mutant females. *mei-P22* is required for DSB formation during meiosis (Liu *et al.*, 2002a). As demonstrated previously, we find that γ -H2Av-positive foci are not observed in *mei-P22* ovarian cysts (Liu *et al.*, 2002a; Klattenhoff *et al.*, 2007; Figure 3C). In contrast, *dap^{-/-}; mei-P22^{P22}* double-mutant ovarian cysts retain large numbers of γ -H2Av foci (Figure 3D). These results demonstrate that the DNA damage observed in *dap^{-/-}* ovarian cysts is independent of the production of the DSBs that initiate meiotic recombination.

dap^{-/-} Ovarian Cysts Accumulate DNA Damage during Premeiotic S Phase

The observation that *dap^{-/-}* cysts accumulate DNA damage independently of meiotic DSB formation, as well as the more uniform accumulation of γ -H2Av staining throughout the

Table 1. Average number of γ -H2Av foci in the two pro-oocytes (region 2a) and oocyte (region 2b)

	Region 2a	Early region 2b	Late region 2b	Region 3
<i>CantonS</i>	1.5 \pm 1.5	13.5 \pm 2.0	5.5 \pm 4.7	0.2 \pm 0.4
<i>dap⁴/dap⁴</i>	22.1 \pm 5.1 [†]	30.1 \pm 8.2 [†]	28.7 \pm 5.2 [†]	35.0 \pm 4.2 [†]
<i>dap⁴, dup^{o1}/dap⁴/+</i>	26.1 \pm 6.2 [‡]	40.9 \pm 10.6 [‡]	47.3 \pm 9.1 [‡]	41.5 \pm 6.1

For each sample, n is >25.

[†] p < 0.05 in comparison with wild type, [‡] p < 0.05 in comparison with *dap⁴/dap⁴*.

cyst, suggest that in *dap^{-/-}* ovaries DNA damage may be induced during the premeiotic S phase. In *Drosophila*, the production of meiotic DSBs occurs after the assembly of the mature SC in prophase of meiosis I (Carpenter, 1975; Jang *et al.*, 2003; Mehrotra and McKim, 2006). Therefore, to establish the timing of DSB formation in *dap^{-/-}* ovarian cysts, we double stained wild-type and *dap^{-/-}* ovaries with antibodies against the SC component C(3)G and γ -H2Av (Page and Hawley, 2001). In wild-type ovaries, γ -H2Av foci are absent in the earliest region 2a cysts that contain at most small patches of SC staining (zygotene stage, Figure 4, A–A₁). Indeed, γ -H2Av foci are not observed until SC formation seems complete in the two pro-oocytes (pachytene stage, Figure 4, A–A₂) as visualized by C(3)G staining (McKim *et al.*, 1998; Jang *et al.*, 2003; Mehrotra and McKim, 2006). In contrast, in *dap^{-/-}* germaria, α - γ -H2Av staining is often observed in the earliest cysts in region 2a, well before the appearance of α -C(3)G staining (Figure 4, B–B₁). Importantly, in both wild-type and mutant ovaries, γ -H2Av foci are not observed during the mitotic cyst divisions in region 1 of the germarium (Figure 3, A and B). Thus, in *dap^{-/-}* ovarian cysts α - γ -H2Av staining is first observed after the completion of the mitotic cyst divisions but before the construction of the mature SC. These data are consistent with *dap^{-/-}* mutants incurring DNA damage during the premeiotic S phase.

To determine directly whether DNA damage occurs during premeiotic S phase, we colabeled wild-type and mutant ovaries with antibodies against γ -H2Av and the nucleotide analogue EdU. As reported previously, in wild-type females meiotic DSBs are generated after the initiation of SC formation in late region 2a of the germarium (Mehrotra and McKim, 2006). Accordingly, we find that in wild-type germaria, EdU incorporation always occurs in 16-cell cysts before the appearance of γ -H2Av foci. Therefore, in wild-type ovaries, we do not observe ovarian cysts that are positive for both EdU and γ -H2Av (Figure 5, A–A", arrowhead). In contrast, in *dap^{-/-}* ovaries γ -H2Av foci accumulate in region 2a before the completion of the premeiotic S phase. Thus, in *dap^{-/-}* mutants a fraction of ovarian cysts in region 2 of the germarium colabel with EdU and γ -H2Av (Figure 5, B–B", arrowhead). To confirm that DNA damage accumulates dur-

ing the premeiotic S phase, we used an antibody against another S phase marker, PCNA. The eukaryotic DNA polymerase processivity factor PCNA is an essential component of the DNA replication and repair machinery and can be used to label cells in the S phase (Celis and Celis, 1985; Eissenberg *et al.*, 1997; Maga and Hubscher, 2003; Kisielewska *et al.*, 2005). As is observed with EdU incorporation, in wild-type ovaries PCNA is always expressed in 16-cell cysts before the appearance of γ -H2Av foci (n = 23; Supplemental Figure 3, A–A"). In contrast, 16% of *dap^{-/-}* cysts in early region 2a are colabeled with antibodies against both PCNA and γ -H2Av (n = 25; Supplemental Figure 3, B–B", arrowhead). These observations confirm that *dap^{-/-}* ovarian cysts accumulate DNA damage during the premeiotic S phase.

Consistent with a lengthening of the premeiotic S phase, in *dap^{-/-}* mutants an increased proportion of germaria contain at least one EdU-positive 16-cell cyst. Specifically, although 24% (n = 50) of control germaria contain an EdU-positive 16-cell cyst, in *dap^{-/-}* ovaries this number is increased to 62% of germaria (n = 31). Additionally, in *dap^{-/-}* germaria, EdU-positive 16-cell cysts are often present in late region 2a and in region 2b (Figure 5B). In contrast, in wild type germaria, EdU-positive 16-cell cysts are restricted to early region 2a. These data are consistent with *dap^{-/-}* ovarian cysts having a prolonged premeiotic S phase that extends into the later stages of development. Importantly, we recognize that because 25% of *dap^{-/-}* ovarian cysts undergo a fifth mitotic division to produce 32-cell cysts, one would predict an increase in the number of 16-cell cysts that incorporate EdU. However, the 25% of ovarian cysts that undergo a fifth mitotic division are unlikely to account for the greater than twofold increase in the number of 16-cell cysts that incorporate EdU in *dap^{-/-}* germaria. Thus, we believe the simplest explanation for this data are that in *dap^{-/-}* ovaries the premeiotic S phase is extended.

Dap Promotes the Accumulation of Dup/Cdt1 before Premeiotic S Phase

Why might mutations in the CKI *dap* result in DNA damage during the premeiotic S phase? PreRCs are built by sequential binding at the DNA origins of ORC, Cdt1/Dup and Cdc6, and the MCM2-7 complex (Whittaker *et al.*, 2000b; Bell

Table 2. Average number of γ -H2Av foci per cystocyte (excluding the pro-oocytes and oocytes)

	Region 2a	Early region 2b	Late region 2b	Region 3
<i>CantonS</i>	0.6 \pm 0.7	0.5 \pm 0.6	0.9 \pm 0.8	0.2 \pm 0.5
<i>dap⁴/dap⁴</i>	5.8 \pm 1.2 [†]	11.3 \pm 1.2 [†]	11.7 \pm 2.8 [†]	14.3 \pm 1.5 [†]
<i>dap⁴, dup^{o1}/dap⁴/+</i>	8.7 \pm 1.7 [‡]	13.2 \pm 3.6 [‡]	18.1 \pm 3.4 [‡]	22.5 \pm 6.7 [‡]

For each sample, n is >8.

[†] p < 0.05 in comparison with wild type, [‡] p < 0.05 in comparison with *dap⁴/dap⁴*.

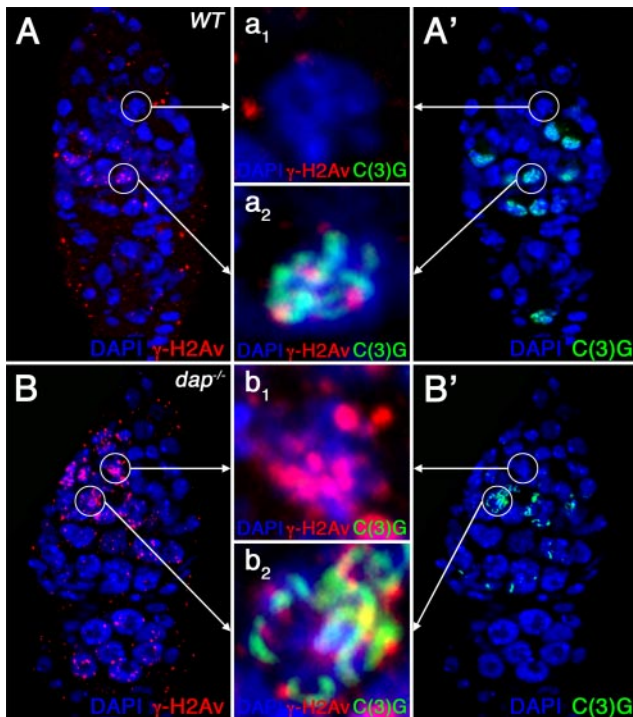


Figure 4. DNA damage occurs before SC formation in *dap*^{-/-} ovarian cysts. Wild-type (A) and *dap*^{-/-} mutant germlaria (B) labeled with α - γ -H2Av (A, B, red) and α -C(3)G (A', B', green) antibodies and DAPI (A–B', blue). The magnified images in the middle (a₁–b₂) show C(3)G protein (green) and γ -H2Av foci (red) (a₁ and b₁) in one cell of a 16-cell cyst in early region 2a before the appearance of α -C(3)G staining and in one of the two pro-oocytes in region 2a after the appearance of α -C(3)G staining (a₂ and b₂). Note that whereas no α - γ -H2Av staining is seen in a₁, wild-type 16-cell cysts of region 2a before the appearance of C(3)G, (b₁) *dap*^{-/-} mutant cells of the same region show several γ -H2Av foci. In meiotic region 2a, when C(3)G protein is expressed, an increased number of γ -H2Av foci are observed in *dap*^{-/-} mutant pro-oocytes (b₂) compared with wild type (a₂).

and Dutta, 2002a). The regulation of preRC assembly is tightly controlled and is an important mechanism by which DNA replication is restricted to a single round per cell cycle (Blow and Dutta, 2005; Machida and Dutta, 2005). Deregulated Cdk activity inhibits adequate preRC formation and resulting in DNA damage and genomic instability (Spruck *et al.*, 1999; Lengronne and Schwob, 2002; Tanaka and Diffley, 2002; Ekholm-Reed *et al.*, 2004). Thus, one model to explain the DNA damage observed in *dap*^{-/-} ovarian cysts is that inappropriately high Cdk activity inhibits preRC assembly before premeiotic S phase.

In *Drosophila*, Cyclin E/Cdk2 activity inhibits the accumulation of the preRC component Dup/Cdt1 (Thomer *et al.*, 2004). During the mitotic cycle in somatic cells, Dup/Cdt1 is expressed during the G₂, M, and G₁ phases and then is rapidly destroyed at the G₁/S transition (Whittaker *et al.*, 2000a; Thomer *et al.*, 2004; May *et al.*, 2005). Consistent with these observations, we find that Dup/Cdt1 levels oscillate in mitotically active germline stem cells, cystoblasts, and dividing ovarian cysts. As ovarian cysts enter the meiotic cycle, Dup/Cdt1 accumulates in 16-cell cysts in early region 2a before the initiation of SC formation, shown by C3G expression (Supplemental Figure 4, A and A', arrowhead). As noted previously, early region 2a of the germlarium corresponds to when ovarian cysts enter premeiotic S phase, as

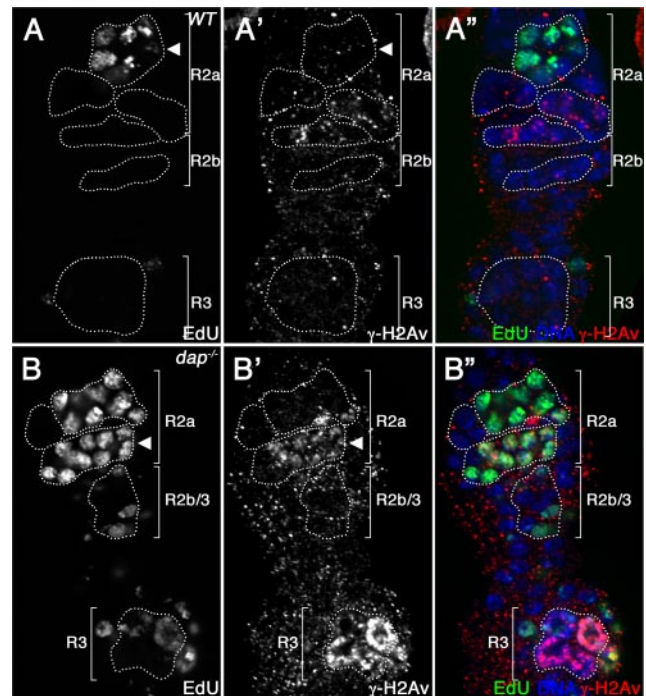


Figure 5. DNA damage accumulates during the premeiotic S phase in *dap*^{-/-} cysts. Wild-type (A) and *dap*^{-/-} mutant germlaria (B) stained with EdU (A, B; A'', B'', green) and α - γ -H2Av (A', B'; A'', B'', red) antibodies and DAPI (A'', B'', blue). Note that in A, wild-type, 16-cell cysts in region 2a of the germlarium never colabel with antibodies against both EdU and γ -H2Av. In contrast, a fraction of B *dap*^{-/-} cysts in region 2a incorporate EdU and contain γ -H2Av foci (B, B', arrowhead).

indicated by nucleotide incorporation and PCNA expression (Carpenter, 1981; Figure 5A, arrowhead, and Supplemental Figure 3A, arrowhead). Subsequently, in late region 2a and early region 2b, the levels of Dup/Cdt1 protein fall below the level of detection as the cysts enter prophase of meiosis I and progress through the early steps of meiotic recombination (Supplemental Figure 4A, arrows). Thus, Dup/Cdt1 accumulates in 16-cell cysts just before and/or during the premeiotic S phase in early region 2a of the germlarium. A possible role for Dup/Cdt1 in licensing DNA replication origins for the premeiotic S phase are consistent with many recent studies indicating that much of the machinery responsible for initiating DNA replication is conserved between the mitotic cycle and the meiotic cycle (Murakami and Nurse, 2001; Lemaitre *et al.*, 2002; Lindner *et al.*, 2002; Ofir *et al.*, 2004).

To examine whether Dap influences the status of Dup/Cdt1 before and during the premeiotic S phase, we compared the expression of Dup/Cdt1 in the first two cysts of region 2a in wild-type versus *dap*^{-/-} ovaries. From these experiments, we determined that *dap*^{-/-} ovaries had a four-fold reduction (2.4%; n = 270) in the percentage of early 2a cysts that expressed Dup/Cdt1 relative to wild-type ovaries (10.4%; n = 247; p = 1.8 × 10⁻⁴). It is important to note that the relatively low percentage of wild-type ovarian cysts that express Dup/Cdt1 is consistent with the fact that a single germlarium rarely contains cysts representing all stages of cyst development. In contrast to meiotic ovarian cysts, the levels of Dup/Cdt1 are not notably altered in the *dap*^{-/-} background during the mitotic cyst divisions with 20.7% (n = 87) of wild-type ovarian cysts expressing Dup/Cdt1

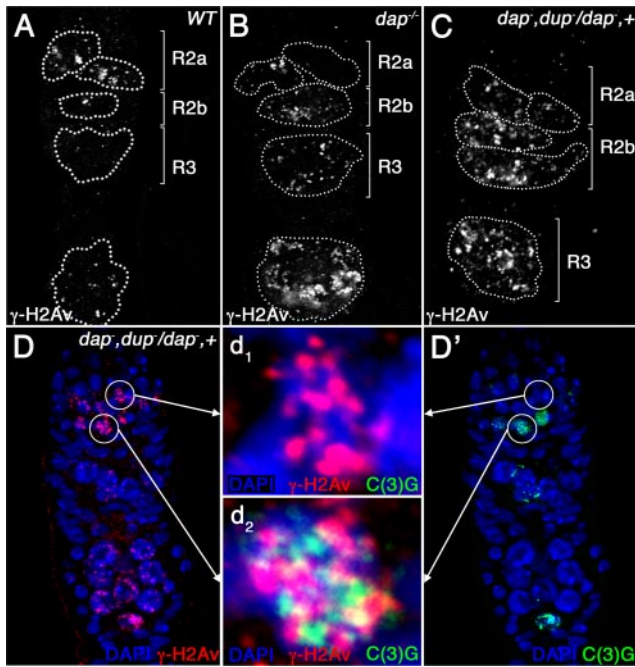


Figure 6. *dup/cdt1* dominantly enhances the DNA damage phenotype in *dap*^{-/-} ovarian cysts. Wild-type (A), *dap*^{-/-} mutant (B), and *dap*⁻, *dup*^{Δ1}/*dap*⁻, + mutant germlaria (C and D) stained with α - γ -H2Av (A–C; D, red) and C(3)G (D', green) antibodies. The magnified images in the middle (d₁ and d₂) show C(3)G protein (green) and γ -H2Av foci (red) in one cell of a 16-cell cyst in early region 2a before the appearance of α -C(3)G staining (d₁) and in one of the two pro-oocytes in region 2a after the appearance of α -C(3)G staining (d₂). Note that γ -H2Av foci are increased in *dap*⁻, *dup*^{Δ1}/*dap*⁻, + cysts (C and D) compared with wild-type (A) and *dap*^{-/-} (B) cysts.

versus 17.9% (n = 86) of *dap*^{-/-} mutant cysts. Thus, the levels of Dup/Cdt1 present before the meiotic S phase are specifically reduced in the *dap*^{-/-} background. Together, our results indicate that Dap promotes the accumulation of the licensing factor Dup/Cdt1 before and/or during the premeiotic S phase in region 2a.

Dup/Cdt1 Is Limiting in dap^{-/-} Ovarian Cysts

We have shown that *dap*^{-/-} ovarian cysts have low levels of the replication-licensing factor Dup/Cdt1. However, it was unclear whether the low levels of Dup/Cdt1 contribute to the observed DNA damage phenotype. To determine whether Dup/Cdt1 levels are limiting during the premeiotic S phase, we removed one copy of *dup/cdt1* and compared the number of γ -H2Av foci in *dap*^{-/-} versus *dap*⁻, *dup*^{Δ1}/*dap*⁻, + ovarian cysts. From these studies, we found an increased number of γ -H2Av foci in *dap*⁻, *dup*^{Δ1}/*dap*⁻, + double mutant ovarian cysts compared with *dap*^{-/-} single mutant ovarian cysts (Figure 6 and Tables 1 and 2). Thus, *dup/cdt1* dominantly enhances the DNA damage phenotype observed in early ovarian cysts. These data support the model that the accumulation of DSBs in *dap*^{-/-} ovarian cysts, during and soon after the premeiotic S phase, is the result of the inadequate preRC formation due to low levels of Dup/Cdt1. Intriguingly, removing one copy of *dup/cdt1* in the *dap*^{-/-} background does not enhance the cyst division phenotype, with *dap*^{-/-} (31 ± 6.4%) and *dap*⁻, *dup*^{Δ1}/*dap*⁻, + (24 ± 4.2%) ovaries containing similar percentages of 32 cell cysts. These data strongly suggest that the extramitotic cyst division is

not a direct result of reducing the number of preRCs assembled before premeiotic S phase.

DISCUSSION

The production of a mature gamete requires the precise duplication of the genome during the premeiotic S phase. However, the regulation of premeiotic S phase remains poorly understood in metazoa. Here, we demonstrate that the regulation of Cyclin E/Cdk activity by the CKI Dap is an important factor influencing both meiotic entry and the execution of the premeiotic S phase in *Drosophila* ovarian cysts.

Cells in the mitotic cycle and the meiotic cycle face a similar challenge. To maintain the integrity of the genome, they must replicate their DNA once, and only once, during the S phase. In mitotic cells, this goal is accomplished, at least in part, through the precise regulation of Cdk activity throughout the cell cycle (Bell and Dutta, 2002b; DePamphilis *et al.*, 2006). During the mitotic cycle, Cdk activity inhibits preRC formation (Bell and Dutta, 2002b; DePamphilis *et al.*, 2006). This inhibitory relationship, restricts the assembly of preRCs to a short window from late mitosis to G1, when Cdk activity is low, and provides an important mechanism by which mitotic cells prevent DNA rereplication. However, the inhibitory effect of Cdk activity on preRC assembly necessitates that cells have a strictly defined period of low Cdk activity before S phase, to assemble preRCs for the next round of DNA replication. In mammals and yeast, compromising this period of low Cdk activity by overexpression G₁ cyclins results in decreased replication licensing and genomic instability (Spruck *et al.*, 1999; Lengronne and Schwob, 2002; Tanaka and Diffley, 2002; Ekholm-Reed *et al.*, 2004).

One means by which cells inhibit Cdk activity is the expression of CKIs (Harper, 1997). In the mitotic cycle of budding yeast, the deletion of the CKI Sic1, which contains a Cdk inhibitor domain that is structurally conserved with the inhibitor domain present in the *dap* homologue p27^{Kip1}, results in inadequate replication licensing and genomic instability due to the precocious activation of Cdks in G1 (Nugroho and Mendenhall, 1994; Schneider *et al.*, 1996; Lengronne and Schwob, 2002). Our data strongly suggest that Dap plays a similar role in defining a critical period of low Cdk activity during the early meiotic cycle in *Drosophila* females.

Based on our results, we propose that the Dap facilitates the licensing of DNA replication origins in ovarian cysts by restricting the inhibitory effects of Cyclin E/Cdk2 kinase activity on preRCs formation before premeiotic S phase. Our data support the model that in the absence of Dap, ovarian cysts enter premeiotic S phase with a reduced number of licensed origins and thus fail to complete genomic replication. This hypothesis is supported by several observations. First, relative to wild-type, *dap*^{-/-} ovarian cysts spend an increased proportion of their time in premeiotic S phase, as evidenced by the increased proportion of 16-cell cysts that incorporated EdU. The lengthening of premeiotic S phase is in line with the hypothesis that *dap*^{-/-} ovarian cysts initiate DNA replication from a reduced number of licensed origins. Second, *dap*^{-/-} ovarian cysts accumulate DNA damage during the premeiotic S phase. The accumulation of DNA damage during the premeiotic S phase is consistent with decreased preRC assembly resulting in intraorigin distances that are too large to be negotiated by DNA polymerase during a single S phase. Third, *dap*^{-/-} meiotic cysts have decreased levels of the preRC component Dup/Cdt1. Moreover, our genetic analysis indicates that Dup/Cdt1 levels are

indeed limiting for premeiotic S phase in the *dap*^{-/-} background. Specifically, we find that reducing the dose of *dup/cdt1* dramatically increases the levels of DNA damage observed in *dap*^{-/-} ovarian cysts in region 2a and 2b of the germarium. In *Drosophila*, the levels of Dup/Cdt1 are negatively regulated by Cyclin E/Cdk2 activity (Harper, 1997).

The use of the CKI Dap to restrict Cdk activity and thus promote the formation of preRCs before S phase is observed in multiple cell types beyond the oocyte. In previous work, we found that in *dap*^{-/-} mutants, cells in developmentally programmed endocycles also accumulate DNA damage and have dramatically reduced levels of Dup/Cdt1 (Hong *et al.*, 2007). Thus, Dap functions to promote the accumulation of Dup/Cdt1 in multiple developmental and cell cycle contexts in *Drosophila*. Indeed, in select mitotic cycles removing one copy of *dup/cdt1* in a *dap*^{-/-} background results in DNA damage and cell death. However, in most mitotic cycles the requirement for Dap is redundant with other mechanisms that restrict Cyclin E/Cdk2 activity (Hong *et al.*, 2007).

Why Dap is required for preRC assembly in some cell types but not others remains unclear. However, it is interesting to note that DNA replication that occurs outside the confines of the canonical mitotic cycle, during the meiotic S phase and the S phase of developmentally programmed endocycles, is most dependent on Dap function (Hong *et al.*, 2007). Thus, the increased reliance on the CKI Dap to establish a period of low Cdk activity before the onset of DNA replication may be explained by the absence of cell cycle programs that are specific to the mitotic cycle. For example, the tight transcriptional control of S phase regulators during the mitotic cycle may make the presence of Dap unnecessary for proper S phase execution. Alternatively, there may be differential regulation of the machinery that controls the regulated destruction of cyclins in the archetypical mitotic cycle versus the variant cell cycles of meiosis and the endocycle (Narbonne-Reveau *et al.*, 2008; Zielke *et al.*, 2008). In the future, determining why Dap plays a nonredundant role in the regulation of DNA replication during the meiotic cycle, but not the mitotic cycle, will be an important avenue of study.

In addition to its role in the regulation of premeiotic S phase, we find that *dap* influences the number of mitotic cyst divisions that occur before meiotic entry. In *dap*^{-/-} mutants, ~25% of ovarian cysts complete a fifth mitotic division to produce ovarian cysts with 32 cells. Similarly, mutations that compromise the degradation of the Cyclin E protein also result in production of 32-cell cysts (Doronkin *et al.*, 2003; Ohlmeyer and Schupbach, 2003). In line with these observations, females with reduced levels of Cyclin E produce ovarian cysts that undergo only three mitotic divisions and thus contain eight cells (Lilly and Spradling, 1996). Why Cyclin E/Cdk2 activity influences the timing of meiotic entry is not fully understood. However, our data suggest that the cyst division phenotype is not a direct result of reducing the number of preRCs assembled for the premeiotic S phase. Specifically, we find that in *dap*^{-/-} females reducing the dose of *dup/cdt1* does not increase the number of ovarian cysts that undergo an extra division. In contrast, reducing the dose of *dup/cdt1* in *dap*^{-/-} females significantly enhances the meiotic DNA damage phenotype. These data strongly suggest that the extramitotic cyst division observed in *dap*^{-/-} ovarian cyst is not the direct result of high CyclinE/Cdk2 activity inhibiting preRC formation.

Intriguingly, Cdk2 is not the only Cdk that influences the number of ovarian cyst divisions in *Drosophila* females. Surprisingly, increasing the activity of the mitotic kinase Cdk1 results in the production of egg chambers with eight-cell

cysts (Mata *et al.*, 2000; Sugimura and Lilly, 2006). Moreover, decreased Cdk1 activity results in ovarian cysts undergoing five mitotic divisions to produce egg chambers with 32 cells. Thus, Cdk1 and Cdk2 seem to have opposing roles in the regulation of the ovarian cyst divisions and/or meiotic entry. One of several possible explanations for these data, is that the number of ovarian cyst divisions is influenced by the amount of time cystocytes spend in a particular phase (G1, S, G2, and M) of the cell cycle (Mata *et al.*, 2000). In the mitotic cycle of the *Drosophila* wing, there is a compensatory mechanism that ensures that changes in the length of one phase of the cell cycle result in alterations in the other phases of the cell cycle to ensure normal division rates (Reis and Edgar, 2004). This compensatory mechanism is likely to be operating in multiple cell types and may account for why Cdk1 and Cdk2 activity have opposite effects on the number of ovarian cyst divisions. Alternatively, Cdk1 and Cdk2 may act on truly independent pathways that have opposing roles in regulating the number of mitotic cyst divisions and/or the timing of meiotic entry. Ultimately, why Cdk1 and Cdk2 activity have opposite effects on the number of ovarian cyst divisions that occur before meiotic entry awaits the identification of essential downstream targets of these kinases.

In summary, we have defined two novel functions for a p21^{Cip}/p27^{Kip1}/p57^{Kip2}-like CKI during the meiotic cycle, the regulation of the mitotic/meiotic transition and the maintenance of genomic stability during the premeiotic S phase.

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