Development 130, 1235-1242

doi:10.1242/dev.00352

The p27$^{cip/kip}$ ortholog *dacapo* maintains the *Drosophila* oocyte in prophase of meiosis I

Amy Hong, Steven Lee-Kong, Takako Iida, Isamu Sugimura and Mary A. Lilly*

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

*Author for correspondence (e-mail: mlilly@helix.nih.gov)

Accepted 20 December 2002

SUMMARY

Animal oocytes undergo a highly conserved developmental arrest in prophase of meiosis I. Often this marks a period of rapid growth for the oocyte and is necessary to coordinate meiotic progression with the developmental events of oogenesis. In *Drosophila*, the oocyte develops within a 16-cell germline cyst. Throughout much of oogenesis, the oocyte remains in prophase of meiosis I. By contrast, its 15 mitotic sisters enter the endocycle and become polyploid in preparation for their role as nurse cells. How germline cysts establish and maintain these two independent cell cycles is unknown. We demonstrate a role for the p21^{CIP}/p27^{Kip1}/p57^{Kip2}-like cyclin-dependent kinase inhibitor (cki) *dacapo* in the maintenance of the meiotic cycle in *Drosophila* oocytes. Our data indicate that it is through the differential regulation of the cki Dacapo that two modes of cell-cycle regulation are independently maintained within the common cytoplasm of ovarian cysts.

Key words: Oogenesis, Meiosis, *dacapo*, p27, *Drosophila*, Cyclin E, Oocyte differentiation

INTRODUCTION

During metazoan development, cells alter their cell cycles in response to specific developmental cues. Often these signals result in a change in proliferative state, prompting a cell either to divide in response to a growth signal or to exit the cell cycle in order to begin the process of differentiation. Occasionally, however, cells stray from the canonical mitotic cycle, in which DNA synthesis is followed by chromosome segregation, in order to fulfill a specific biological function. Two common examples of this phenomenon occur during *Drosophila* oogenesis when cells within a germline cyst undergo either the reductional divisions of meiosis and produce a functional gamete or enter the endocycle and develop as a highly polyploid nurse cell.

Ovarian cyst formation begins when one, out of a group of two to three germline stem cells, divides asymmetrically to produce a cystoblast. The cystoblast undergoes a series of four synchronous mitotic divisions in which cytokinesis is incomplete (de Cuevas et al., 1997; McKearin, 1997). Individual cells within the cyst, referred to as cystocytes, are connected in an invariant pattern by actin-rich intercellular bridges called ring canals (Fig. 1A). After all 16 cystocytes complete premeiotic S phase, a meiotic gradient develops with the two cells with four ring canals having the most meiotic features as assayed by the presence of mature synaptonemal complexes and recombination nodules (Chandley, 1966; Carpenter, 1975; Carpenter, 1981; Schmekel et al., 1993). Ultimately, the cell cycles of the future nurse cells and oocyte diverge dramatically. The single pro-oocyte arrests in prophase of meiosis I for most of the growth phase of oogenesis, while the 15 nurse cells go on to complete 10-12 endocycles to become highly polyploid. During these endocycles the nurse cells cycle asynchronously.

How one of the 16 cystocytes is selected to differentiate as the oocyte has long been an issue of interest. It has been known for some time that one of the two cells with four ring canals always develops as the oocyte (reviewed by Büning, 1994). This observation indicates that the invariant pattern of cystocyte connections is crucial for later cell fate decisions within the cyst (reviewed by de Cuevas et al., 1997; McKearin, 1997). Studies over the last few years suggest that oocyte differentiation is a two-step process. The first step entails the establishment of an asymmetry between the pro-oocyte and the pro-nurse cells. Exactly when and how this asymmetry is established is unknown, although it may occur as early as the cystoblast division through the unequal distribution of the fusome, a germline-specific organelle (Lin and Spradling, 1995; de Cuevas and Spradling, 1998). The second step involves the directional transport of cellular components, including specific mRNAs and proteins, to the pro-oocyte. Drugs that destabilize microtubules eliminate the accumulation of specific mRNAs in the pro-oocyte and lead to the production of cysts that contain 16 nurse cells and no oocyte (Koch and Spitzer, 1985; Theurkauf et al., 1993). Similarly, recessive mutations in the genes *Bicaudal D* (*BicD*) and *egalitarian* (*egl*) prevent the differential accumulation of specific mRNAs and proteins in the pro-oocyte and result in cysts with 16 nurse cells (Suter et al., 1989; Schupbach and Wieschaus, 1991; Suter and Steward, 1991; Mach and Lehmann, 1997). Although it is clear

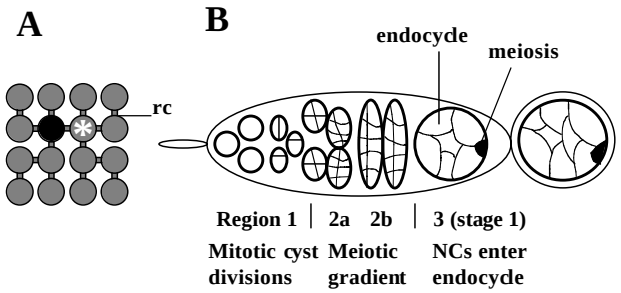

Fig. 1. *Drosophila* ovarian cyst and germarium. (A) The 16 cells of an ovarian cyst are connected in an invariant pattern via actin-rich ring canals (rc, ring canal). The single oocyte (black) always develops from one of the two cells with four ring canals. The other 15 cells in the cyst, including the other cell with four ring canals (asterisk), enter the endocycle and develop as highly polyploid nurse cells. (B) A germarium. The four mitotic cyst divisions take place in region 1 of the germarium. In region 2a, all 16 cells enter premeiotic S phase. In region 2b, a meiotic gradient forms with the two cells with four canals progressing to pachytene. By the time cysts enter region 3 of the germarium (stage 1) only the cell that is destined to become the oocyte (black) remains in the meiotic cycle. By contrast, the 15 mitotic sisters of the oocyte enter the S phase of the first endocycle on their way to becoming highly polyploid nurse cells. NC, nurse cell.

that microtubule-based polarized transport is a critical component of oocyte differentiation, exactly why meiosis proceeds in the oocyte while the adjacent nurse cells enter the endocycle remains undefined.

During meiosis, the integrity of the genome must be maintained through recombination and two rounds of cell division. Animals in which the oocyte develops within the context of a cyst, such as *Drosophila*, are faced with an additional set of challenges. Intercellular bridges physically connect the *Drosophila* oocyte to nurse cells that are undergoing repeated rounds of DNA replication. However, to produce a functional gamete, it is essential that the oocyte does not fire a single DNA replication origin. How does the germline cyst maintain this extreme cell cycle dichotomy? One potential mechanism involves the spatial regulation of cyclinE-Cdk2 activity (Lilly and Spradling, 1996). In *Drosophila*, cyclinE-Cdk2 activity is required for S phase (Knoblich et al., 1994). *dacapo* (*dap*) is a vital gene that encodes a $p21^{CIP}/p27^{KIP1}/p57^{KIP2}$-like cki that specifically inhibits the activity of cyclinE-Cdk2 complexes (de Nooij et al., 1996; Lane et al., 1996). Throughout much of the growth phase of *Drosophila* oogenesis, the levels of the cki Dap oscillate in the 15-polyploid nurse cells but remain persistently high in the single oocyte (de Nooij et al., 2000). We reasoned that the differential regulation of Dap might provide a mechanism to maintain the oocyte in prophase of meiosis I, while allowing the endocycle to proceed in the adjacent nurse cells.

MATERIALS AND METHODS

Drosophila strains and cultures

The FRT42B, dap^4 (Lane et al., 1996) stock was a gift of Iswar Hariharan. The *P[mini-w; arm–LacZ]* (Lecuit and Cohen, 1997) stock was provided by Steve Cohen. The *cycE*^{01672} mutation was recovered from a single P-element mutagenesis (Karpen and Spradling, 1992). All additional stocks were obtained from the Bloomington Stock Center. The *cycE*^{01672}, *dap*^{4} chromosome was generated by meiotic recombination.

Generation of *dap*^{4} germline clones

Germline clones were generated using the FLP:FRT system (Chou and Perrimon, 1996). Specifically, *y, w, FLP12; FRT42B arm-lacZ* females were crossed to *w*; *FRT42B, dap*^{4}/CyO males. Eggs produced from this cross were heat-shocked for 1 hour at 37°C in a circulating water bath 96 hours after egg deposition. A second identical heat shock was administered 24 hours later. After eclosion *y, w, FLP12; FRT 42B arm-LacZ/FRT42B, dap*^{4} adult females were collected and heat-shocked at least once for 1 hour at 37°C. Females were allowed to recover for 8 days at 25°C. Clones were negatively marked by the absence of β-galactosidase protein as determined by immunocytochemistry.

Immunohistochemistry and BrdU labeling

Ovaries were dissected, immunologically stained and mounted as described previously (Lin et al., 1992). Dap monoclonal antibody (de Nooij et al., 2000) was provided by Iswar Hariharan. Dap rabbit polyclonal antibody was provided by Christian Lehner. Orb monoclonal antibody (Lantz et al., 1994) was provided by the Developmental Studies Hybridoma Bank. BicD monoclonal antibody (Junyoung and Steward, 2001) was provided by Ruth Steward. Ovaries were fixed in methanol/EGTA for tubulin staining. α-Tubulin monoclonal antibody DM1a (Sigma) was used at 1:500. BrdU staining was essentially as described by Avedisov et al. (Avedisov et al., 2000). C(3)G antibody was generated from 301 amino acid C-terminal fragment in rabbits using standard techniques. The C(3)G antibody was used at 1:3000 dilution.

RESULTS

Dap is differentially expressed in nurse cells and oocytes

In order to determine the precise onset of the differential regulation of Dap in the nurse cells versus the oocyte, we stained wild-type ovaries with an antibody against Dap (de Nooij et al., 2000). Dap levels oscillate during the four mitotic cyst divisions in region 1 of the germarium (Fig. 2A). In region 2, a meiotic gradient forms that emanates from the pro-oocytes, the two cells with four ring canals (Carpenter, 1975; Carpenter, 1979; Huynh and St Johnston, 2000; Page and Hawley, 2001). Although there is a clear gradient with respect to meiosis, this is not reflected in the distribution of the Dap protein. In young post-mitotic cysts, in region 2a and early 2b, Dap is at uniformly low levels throughout the cyst. However, just prior to when the nurse cells enter the endocycle, in late region 2b/early region 3 (stage 1), Dap levels rise dramatically throughout the cyst (Fig. 2A,B). Thus, uniformly high levels of Dap are observed in all cyst nuclei in early stage 1 egg chambers, which contain a single oocyte in late prophase of meiosis I and 15 nurse cells that are poised to enter the first endocycle S phase.

The differential behavior of Dap in the nurse cells versus the oocyte is first observed in late stage 1 egg chambers. Although high levels of Dap persist in the oocyte, the levels of Dap begin to oscillate in the nurse cells as they asynchronously enter the endocycle (Fig. 2C,D) (de Nooij et al., 2000). Dap levels continue to oscillate in the nurse cells until stage 10 of oogenesis when the nurse cells stop replicating their DNA (Fig. 2F). By contrast, Dap levels remain high in the oocyte until

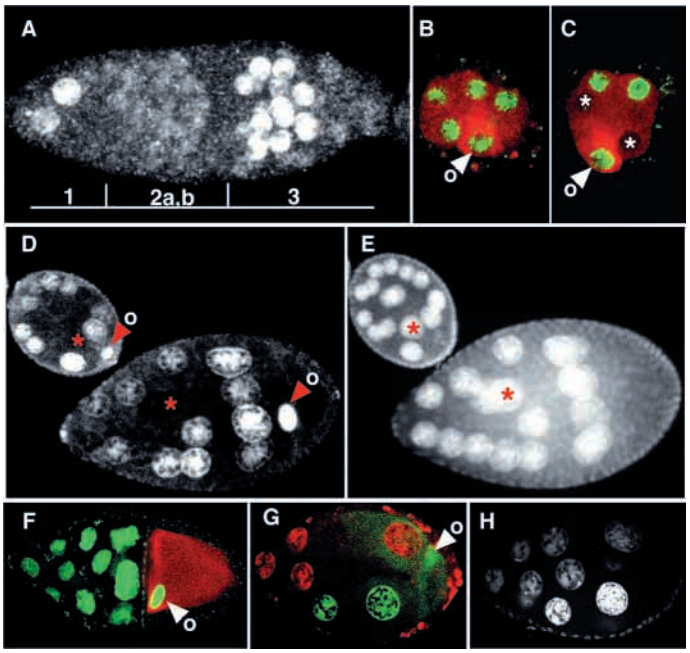

Fig. 2. Dap is differentially regulated in oocytes and nurse cells. (A) A germarium stained with αDap antibody. Regions of the germarium are noted below. (B,C) Confocal sections of (B) an early stage 1 egg chamber and (C) a late stage 1 egg chamber stained for Dap (green) and Orb (red) to highlight the oocyte. Note that Dap is at high levels in all cyst nuclei in B, but has begun to cycle in C, as evidenced by the absence of Dap in some nuclei (asterisks). (D,E) Egg chambers stained with (D) αDap antibody and (E) DAPI to highlight nuclei. Consistent with the cycling of the Dap protein, nurse cells with high, medium and low levels of Dap are observed. Asterisks mark nuclei that are negative for Dap protein. (F) A stage-10B egg chamber stained for Dap (green) and Orb (red). Note that the nurse cells, which have exited the endocycle, contain uniformly high levels of Dap protein. (G) Double labeling for Dap (green) and BrdU (red) indicates that S phase takes place when Dap levels are low. (H) Corresponding DAPI staining. o, oocyte.

well after the nurse cells exit the endocycle. Dap inhibits DNA replication in both mitotic and endocycling cells (de Nooij et al., 1996; Lane et al., 1996; Calvi et al., 1998). Thus, it is likely that the cycling of Dap protein in the nurse cells allows cyclinE-Cdk2 kinase activity to rise high enough to trigger the endocycle S phases (de Nooij et al., 2000; Edgar and Orr-Weaver, 2001). Consistent with this proposal, S phase in the nurse cells occurs when Dap levels are low (Fig. 2G,H). Therefore, as the nurse cells enter the endocycle in stage 1 there is a clear correlation between the differential regulation of Dap and the distinct cell cycles of the nurse cells and oocyte.

dap oocytes enter the endocycle

Does the cki Dap prevent the oocyte from entering the endocycle with the nurse cells and thus preserve the prophase I meiotic arrest? To answer this question, we generated homozygous germline clones of the *dap* null allele dap^4 (Lane et al., 1996). dap^4 contains a deletion of the conserved CDK-binding domain and acts as a complete loss-of-function allele (Lane et al., 1996). In greater than 80% of the egg chambers (n>250) that contain *dap* germline clones, the oocyte enters the endocycle and becomes polyploid (Fig. 3). The extent of

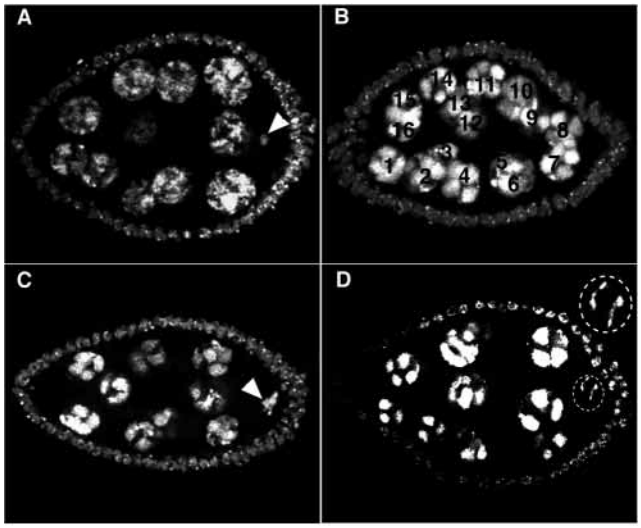

Fig. 3. *dap* oocytes enter the endocycle. (A) Wild-type egg chamber stained with DAPI to highlight nuclei, note the large size of the nurse cell DNA mass relative to the compact oocyte karyosome (arrowhead). (B-D) In *dap* germline clones, oocytes achieve varying degrees of polyploidy. Egg chambers containing *dap* germline clones in which (B) there are 16-polyploid nurse cell nuclei and no oocyte, (C) the oocyte is partially polyploid (arrowhead) and (D) the oocyte DNA has abnormal morphology but is not obviously polyploid. The broken circle outlines a magnification of the oocyte defect.

polyploidy in these oocytes is variable. 53±9% of *dap* cysts have 16-polyploid nurse cells and no oocyte (Fig. 3B). In this phenotypic class, all cells in the cyst have similar DNA contents, indicating all 16 cells have undergone approximately the same number of endocycles. These data suggest that in greater than 50% of the egg chambers with *dap* germline clones, the oocyte enters the endocycle at the same time as the nurse cells. In 28±7% of the germline clones the oocyte is polyploid, but can be distinguished from the adjacent nurse cells by its lower DNA content and posterior position (Fig. 3C). In wild-type egg chambers the oocyte DNA condenses into a compact karyosome in stage 3 of oogenesis (Fig. 3A). In the 10±6% of *dap* clones in which the oocyte is not obviously polyploid, karyosome formation is often aberrant. For example, the oocyte DNA is present in one or more elongated masses within the oocyte nucleus (Fig. 3D) or in a thin rim near the nuclear envelope (data not shown). The ~9% of *dap* clones that contained greater than 16 nurse cells were not included in this analysis. These data indicate that *dap* regulates entry into or is required for the maintenance of the meiotic cycle in the oocyte.

What accounts for the observed variability of the *dap* phenotype? Because we examined the clonal progeny from germline stem cell clones that had undergone numerous divisions, we do not believe the observed phenotypic variability results from the perdurance of the Dap protein. In support of this conclusion, immunocytochemistry using an antibody against Dap indicated that clonal mutant egg chambers contain no Dap protein (data not shown). In addition, a similar phenotypic distribution was observed when examining the ovaries from dap^4 homozygous escapers. Finally, *dap* germline cysts surrounded by dap^4 (n=88 egg chambers) and dap^+ (n=104 egg chambers) follicle cells had

similarly variable phenotypes, indicating that the genotype of the follicle cells is not the source of variability. Protecting the oocyte from inappropriate entry into the endocycle is critical to the production of a functional gamete. Therefore, we predict that additional factors act in concert with Dap to inhibit DNA replication during meiosis.

dap is not required for oocyte specification or entry into meiosis

Mutations that disrupt cyst polarity and oocyte specification, such as *BicD* and *egl*, result in the failure to localize oocyte specific markers and the production of egg chambers with 16 nurse cells and no oocyte (Suter et al., 1989; Suter and Steward, 1991; Christerson and McKearin, 1994; Lantz et al., 1994). We wanted to determine if mutations in *dap* indirectly influence the oocyte cell cycle by disrupting cyst polarity. In wild-type cysts, the Orb and BicD proteins preferentially accumulate in the cytoplasm of the pro-oocyte, beginning in region 2a of the germarium (Suter and Steward, 1991; Lantz et al., 1994). With few exceptions, the distribution of the Orb (Fig. 4A) and BicD (data not shown) proteins in *dap* cysts is indistinguishable from wild type in region 2 of the germarium (Fig. 4A). As shown in Fig. 4, Orb protein preferentially accumulates in a single centrally located cystocyte in both wild-type (arrow) and *dap* (arrowhead) cysts. These data indicate that unlike what is observed in *BicD* and *egl* mutants, the polyploidization of *dap* oocytes is not the consequence of a failure to specify an oocyte or a general disruption in cyst polarity. However, it should be noted that in *egl* mutants, Dap protein does not preferentially accumulate within a single cell in post-germarial egg chambers, but instead appears to oscillate in all 16-cyst cells as they undergo repeated endocycles (data not shown). Thus, although *dap* is not required for oocyte specification, the specification of the oocyte is required to establish the two modes of Dap regulation within ovarian cysts. These data indicate that oocyte specification is upstream of the effects of Dap on the meiotic cycle.

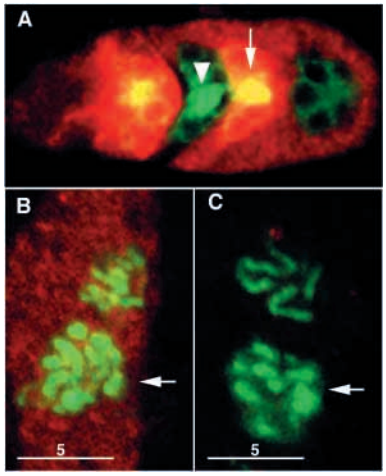

Fig. 4. *dap* oocytes accumulate Orb protein and progress to pachytene. (A) A *dap* cyst (arrowhead) in region 2b of the germarium accumulates Orb protein (green) in a manner indistinguishable from an adjacent wild-type cyst (arrow). In both (B) wild type and (C) *dap* clones, in region 2b of the germarium the two pro-oocytes contain thread-like C(3)G staining (green) indicative of cells in pachytene. In both panels, the other 14 cells in the cyst that are negative for C(3)G are not shown. In B,C the cell with the stronger C(3)G staining is the true oocyte (arrow). In A,C *dap* clones were identified by the absence of β-gal staining (red). Scale bar: 5 μm.

In the absence of Dap the majority of oocytes do not undergo the two meiotic divisions but instead enter the endocycle and replicate their DNA. Next, we wanted to examine if Dap is required for the initiation and/or the maintenance of meiosis? In order to determine if *dap* oocytes enter meiosis, we assayed meiotic progression using an antibody directed against the synaptonemal complex (SC) component C(3)G (Page and Hawley, 2001). In wild-type cysts, up to four cells construct SC with the two pro-oocytes entering pachytene (Carpenter, 1975; Carpenter, 1979; Huynh and St Johnston, 2000; Page and Hawley, 2001) (Fig. 4B). By late region 2b, the meiotic gradient sharpens such that C(3)G staining is primarily concentrated in the oocyte. *dap* oocytes progress to pachytene as assayed by the production of continuous SC along bivalents (Fig. 4C). These data indicate that *dap* is not necessary to initiate the meiotic cycle nor is it necessary for progression to pachytene. Therefore, the first disruption to the meiotic cycle observed in *dap* oocytes is when they inappropriately enter the endocycle in stage 1. These data are consistent with Dap acting to inhibit DNA replication specifically in the oocyte as the adjacent nurse cells enter the endocycle.

In *dap* germline cysts there is a delay in the restriction of SC to a single cell such that cysts in region 2b and 3 occasionally have 2 or more cells with strong C(3)G staining (data not shown). We believe it is unlikely that the retention of SC in the adjacent nurse cells is responsible for the subsequent polyploidization of the oocyte observed in *dap* mutants. The *spindle* mutants, which activate the meiotic checkpoint (Ghabrial et al., 1998), show a very similar delay in the restriction of SC to the oocyte (Huynh and St Johnston, 2000). Importantly, mutations in the *spindle* genes do not result in the polyploidization of the oocyte. We believe the delay in the restriction of SC to a single cell may represent an earlier function of *dap*, perhaps during the mitotic cyst divisions or in the regulation of premeiotic S phase.

dap is required to maintain oocyte differentiation

In stage 1 egg chambers the cell-cycle environment within the cyst changes dramatically as the nurse cells asynchronously enter the S phase of the first endocycle. In wild-type stage 1 egg chambers, the oocyte remains safely arrested in prophase of meiosis I. However, in the majority of *dap* mutant cysts, the oocyte enters the endocycle with the nurse cells. Our data indicate that inappropriate entry into the endocycle disrupts oocyte differentiation (Fig. 5). Beginning at stage 1, we observe a decrease, relative to similarly aged wild-type egg chambers, in the preferential accumulation of the BicD and Orb proteins in *dap* oocytes. As oogenesis progresses, there is a strong inverse correlation between the degree of polyploidization in *dap* oocytes and the preferential accumulation of BicD and Orb. *dap* cysts with highly polyploid oocytes have little to no preferential accumulation of BicD (Fig. 5C,D) or Orb (data not shown). By contrast, *dap* cysts in which the oocyte has undergone limited polyploidization frequently have BicD and Orb levels indistinguishable from wild type (Fig. 5E,F). As is observed in other mutants that disrupt oocyte differentiation, egg chambers that contain *dap*

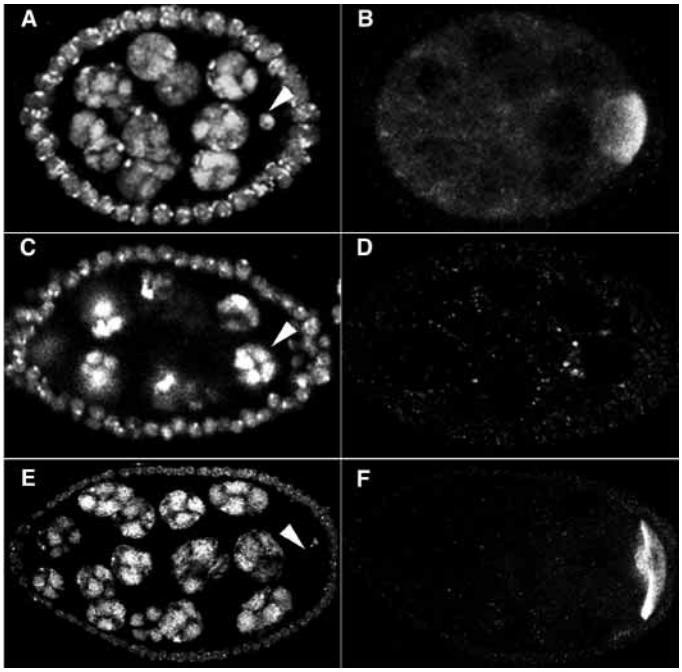

Fig. 5. *dap* is required to maintain oocyte fate. (A,B) Wild-type and (C-F) *dap*^{4} clones double labeled with DAPI (A,C,E) to highlight nuclei and αBicD antibody (B,D,F). Note that in D, where the *dap*^{4} oocyte has become highly polyploid, there is little preferential accumulation of the BicD protein. By contrast, in F, where the *dap*^{4} oocyte has undergone little to no DNA replication, BicD accumulates to levels similar to those observed in wild-type oocytes.

clones rarely develop beyond stage 6 (Suter et al., 1989; Clark and McKearin, 1996; Mach and Lehmann, 1997; Huynh et al., 2001). In *Drosophila* vitellogenesis begins in stage 7 when the oocyte begins to take up large quantities of yolk. The small percentage of *dap* oocytes that progress far enough to take up yolk, invariably have undergone little to no polyploidization (data not shown). These data demonstrate that *dap* is required for oocyte differentiation. In addition, they indicate that the loss of oocyte identity observed in *dap* clones is a direct consequence of the oocyte entering the endocycle.

To explore further the apparent loss of oocyte identity that accompanies inappropriate entry into the endocycle, we examined the distribution of microtubules in *dap* cysts. The preferential accumulation of Orb and BicD in the oocyte is dependent on a polarized network of microtubules that directs these, and other oocyte-specific factors, from the nurse cells to the oocyte (Theurkauf et al., 1993). The disruption of this network by microtubule depolymerizing agents leads to the production of egg chambers with 16 polyploid nurse cells (Koch and Spitzer, 1985; Theurkauf et al., 1993; Huynh and St Johnston, 2000). In wild-type cysts, the asymmetric distribution of microtubules within the germline cyst can be visualized as a preferential accumulation of α-tubulin in the single oocyte, which contains the microtubule-organizing center (Theurkauf et al., 1992). In *dap* cysts, this focus of α-tubulin staining is present in nonpolyploid oocytes but absent in polyploid oocytes (Fig. 6). These data suggest that entry into the endocycle may disrupt the polarized microtubule network, which in turn blocks oocyte differentiation. However, the exact

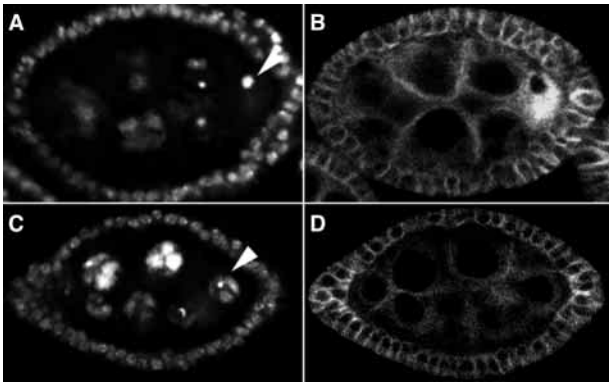

Fig. 6. Entry into the endocycle disrupts the asymmetric distribution of α-tubulin in *dap* cysts. (A-D) *dap*^{4} cysts double labeled with DAPI (A,C) and α-tubulin antibody (B,D). In C, where the oocyte has become polyploid (arrowhead), there is no concentration of α-tubulin staining (D) in the oocyte (compare with B).

relationship between entry into the endocycle and the disruption of the microtubule network remains undefined.

dap dominantly suppresses the two oocyte phenotype of a cycE hypomorph

In females homozygous for the hypomorphic mutation *cycE*^{01672}, a fraction of egg chambers contain two cells that have oocyte-like nuclear features, such as low ploidy values, an endobody and a small DNA mass in a very large nucleus (Lilly and Spradling, 1996). Egg chambers that contain two oocyte nuclei have only 14 polyploid nurse cells, indicating that a cell that was destined to develop as a nurse cell has been partially transformed towards the oocyte fate. The extra oocyte nucleus, which can be distinguished from the true oocyte by its presence in a small cell that lacked signs of cytoplasmic oocyte differentiation, almost invariably is the other four-ring canal cell in the cyst (Lilly and Spradling, 1996). Interestingly, these transformed nuclei accumulate persistently high levels of Dap protein in a manner similar to the true oocyte (Fig. 7A,B). Cells with persistently high levels of Dap have low ploidy values, indicating they have either not entered the endocycle or have prematurely exited the cycle. By contrast, in wild-type egg chambers the other four-ring canal cell develops as a highly polyploid posterior nurse cell in which Dap levels oscillate. Thus, mutations in *dap* result in germline cysts in which all 16 cells enter the endocycle and develop as nurse cells, while a mutation in *cycE* has the opposite effect, resulting in two or more cells that have persistently high levels of Dap that cannot enter and/or maintain the endocycle.

To examine the relationship between *cycE* and *dap* in the regulation of the cell-cycle program of ovarian cysts, we examined if mutations in *dap* could dominantly modify the *cycE*^{01672} two oocyte phenotype. We found that reducing the dose of the *dap* gene by half, resulted in ~2.5 fold suppression of the *cycE*^{01672} two oocyte phenotype. In wild-type egg chambers the four posterior nurse cells, which are connected to the oocyte via ring canals, have the highest ploidy values in the cyst (Fig. 7C). In *cycE*^{01672} females 37±7% (n>200) of egg chambers contain a cell adjacent to the true oocyte with inappropriately low ploidy values (Fig. 7D,E). When a single copy of the null allele *dap*^{4} was placed in the *cycE*^{01672}

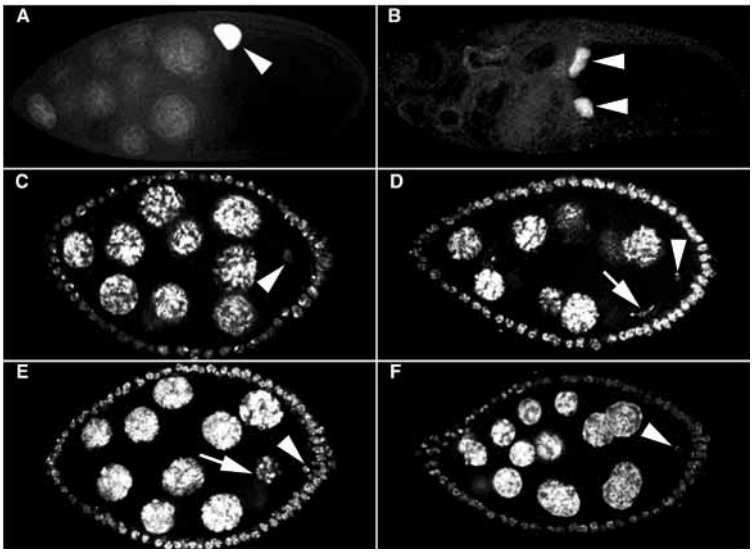

Fig. 7. A *dap* null allele dominantly suppresses the two oocyte phenotype of $cycE^{01672}$ egg chambers. (A) Wild-type and (B) $cycE^{01672}$ stage 10 egg chambers stained with αDap antibody. Note that Dap accumulates to high levels in two cells, the oocyte and an adjacent cyst cell, in the $cycE^{01672}$ egg chamber. (C) In wild-type egg chambers, the posterior nurse cells have the highest DNA contents in the cyst. By contrast, in $cycE^{01672}$ egg chambers(D,E), a cell that is normally specified to become a posterior nurse cell frequently undergoes a reduced number of endocycles and has an inappropriately low DNA content (arrow). This phenotype is variable and can range from egg chambers in which the second four-ring canal cell undergoes little to no DNA replication (D) to a relatively small reduction in DNA content (E). This reduction in DNA content is suppressed by ~2.5 fold when a single copy of the null allele dap^4 is placed in the $cycE^{01672}$ background. (F) An egg chamber from a $cycE^{01672}$,dap^4/+ female in which the posterior nurse cells have wild-type ploidy values (compare C with F). (C-F) Egg chambers stained with DAPI to highlight nuclei.

background, fewer than 14±7% (n>200) of egg chambers had a posterior nurse cell with a reduced DNA content. These data indicate that whether a cyst cell enters and/or maintains the endocycle is at least partially determined by the balance of CycE and Dap. In addition, they strongly suggest that, as is observed during embryogenesis, the primary target of Dap in the ovary is the CycE/Cdk2 complex.

DISCUSSION

The cki Dap inhibits DNA replication in the oocyte

In order to fulfill their specific biological functions, the nurse cells and the oocyte of *Drosophila* ovarian cysts maintain very different cell cycles throughout much of oogenesis. The oocyte faithfully executes the meiotic cycle in order to produce the genetic material for the egg. By contrast, the biosynthetically active nurse cells enter the endocycle and become highly polyploid. During oogenesis, these apparently incompatible cell cycles must co-exist for several days within the shared cytoplasm of the cyst. The studies presented here address how DNA replication is inhibited in the meiotic oocyte, while simultaneously promoted in the adjacent nurse cells. We demonstrate that this is accomplished through the differential regulation of the cki *dap*. Specifically, *dap* is required to maintain the oocyte in prophase of meiosis I during the growth phase of *Drosophila* oogenesis. In *dap* mutants, the majority of oocytes enter the endocycle with the nurse cells. Furthermore, our data indicate that inappropriate entry into the endocycle inhibits oocyte differentiation and promotes the nurse cell developmental pathway.

A model for the maintenance of the meiotic cycle in *Drosophila* oocytes

Our observations suggest a model for how the meiotic cycle and the endocycle are independently maintained within *Drosophila* ovarian cysts (Fig. 8A). We propose that the presence of high levels of the cki Dap in the oocyte, throughout the time the nurse cells are in the endocycle, persistently inhibits cyclinE-Cdk2 kinase activity and prevents inappropriate DNA replication during meiosis. Without the inhibition of cyclinE-Cdk2 kinase activity provided by high levels of Dap, the majority of *dap* mutant oocytes abandon the meiotic cycle and enter the endocycle with the nurse cells. Importantly, these data indicate that, as has recently been observed in mice, oocytes in prophase

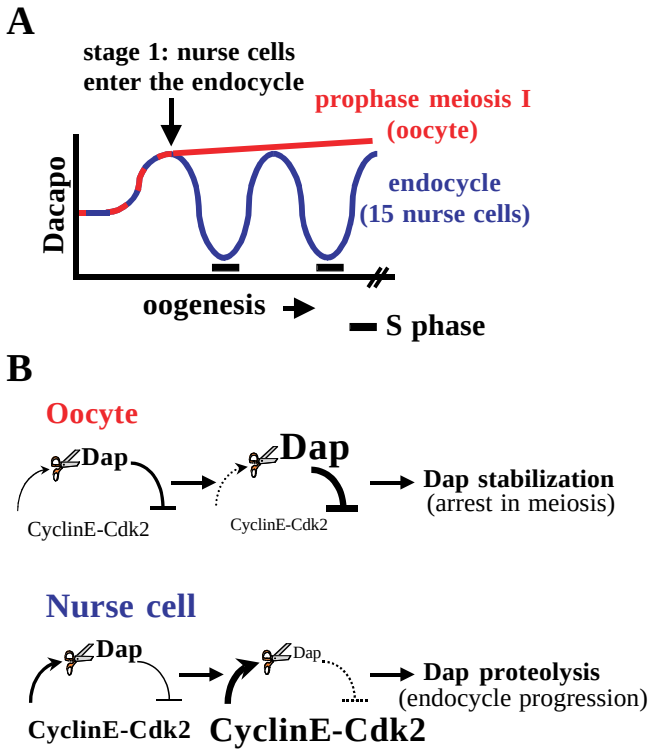

Fig. 8. A model for the maintenance of the two independent cell cycles of ovarian germline cysts. (A) High levels of Dap help maintain the oocyte in prophase of meiosis I (red) by inhibiting inappropriate DNA replication. In the nurse cells the cycling of Dap allows the periodic initiation of DNA replication during the endocycle (blue). (B) The balance of cyclinE-Cdk2 and its inhibitor Dap determine if a cyst cell enters the endocycle or arrests in meiosis (see text for details). Scissors indicate Dap proteolysis.

of meiosis I are competent to replicate their DNA (Czolowska and Borsuk, 2000). In the mouse oocyte, the inhibition of DNA replication during prophase of meiosis I may be accomplished through the downregulation of the G1 cyclins and Cdk2 (Moore et al., 1996; Czolowska and Borsuk, 2000). In the *Drosophila* oocyte, the inhibition of cyclinE-Cdk2 activity by Dap achieves the same aim. In contrast to the oocyte, the nurse cells require a period when Dap levels are low to allow cyclinE-Cdk2 kinase activity to rise high enough to trigger each endocycle S phase. These low points occur during the oscillations of the Dap protein. Our data indicate that it is through the differential regulation of Dap that two apparently incompatible cell cycles are stably maintained within the common cytoplasm of the ovarian cyst.

The regulatory relationship between cyclinE-Cdk2 activity and the Dap ortholog p27 suggest a feedback loop that may account for the long-term stabilization of Dap in post germarial oocytes. In mammalian cells, phosphorylation by cyclinE-Cdk2 targets the p27 protein for destruction by the proteasome (Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997; Montagnoli et al., 1999). Similarly, the Dap protein contains a CDK phosphorylation consensus site (Ser^{205}) and can be phosphorylated by mammalian cyclinE-Cdk2 in vitro (de Nooij et al., 1996). We propose that in early stage 1 egg chambers, the balance of cyclinE-Cdk2 activity and Dap protein is slightly different in the 15-nurse cells versus the single oocyte (Fig. 8B). In the oocyte, the balance is tipped towards the inhibitor Dap, resulting in diminished cyclinE-Cdk2 activity. Lower cyclinE-Cdk2 activity leads to a reduced rate of Dap phosphorylation and proteolysis, thereby increasing the concentration of the Dap protein. The stabilization of the Dap protein ultimately results in the permanent inhibition of cyclinE-Cdk2 activity in the oocyte. In contrast to the oocyte, in stage 1 nurse cells cyclinE-Cdk2 kinase activity reaches high enough levels to trigger the phosphorylation and subsequent destruction of the Dap protein, thus allowing endocycle progression. The above model predicts that additional proteins that are targeted for destruction by cyclinE-Cdk2 phosphorylation should be stabilized in the oocyte but not in the nurse cells. Like p27, the proteolytic destruction of CycE itself is dependent on phosphorylation by the cyclinE-Cdk2 complex (Clurman et al., 1996; Won and Reed, 1996). As predicted by the model, CycE is stabilized in the oocyte and accumulates to high levels as oogenesis progresses (Lilly and Spradling, 1996). This model allows a slight difference in the balance of cyclinE-Cdk2 activity and Dap early in oogenesis to be amplified, resulting in the two cell types of the germline cyst permanently adopting dramatically different cell cycles.

Consistent with the above model, removing one copy of *dap* dominantly inhibits the two oocyte phenotype observed in the $cycE^{01672}$ hypomorph. In $cycE^{01672}$ females, Dap protein frequently accumulates to high levels in one or more cyst nuclei in addition to the true oocyte. The cells that inappropriately stabilize Dap have low DNA contents and are connected to the true oocyte through a ring canal. In effect, it appears that in the $cycE^{01672}$ mutants, the stabilization of Dap and the accompanying inhibition of the endocycle that is normally restricted to the oocyte is allowed to spread outwards to the adjacent nurse cells. Thus, reducing CycE levels favors the stabilization of Dap, which leads to the inappropriate inhibition of the endocycle in cells connected to the true oocyte. Importantly, we demonstrate that this phenotype is suppressed by removing a single copy of the *dap* gene from the $cycE^{01672}$ background. Thus, by bringing CycE and Dap levels back into balance, the nurse cells near the oocyte are no longer inappropriately drawn into the feedback loop that inhibits the endocycle.

However, an important question remains. Beyond the specification of the oocyte, what molecular pathway accounts for the proposed difference in the balance of cyclinE-Cdk2 activity and Dap in nurse cells relative to the oocyte in early stage 1 egg chambers? There are at least three possible mechanisms, not mutually exclusive, that may explain this initial asymmetry. Intriguingly, *dap* mRNA is transported to the oocyte beginning in region 2 of the germarium and remains at high levels in the oocyte throughout oogenesis (de Nooij et al., 2000). Thus, the oocyte may simply be able to translate more Dap protein and therefore keep Dap levels slightly higher in the oocyte. This slight difference may be below the resolution of immunocytochemistry, thus explaining why Dap appears to be evenly distributed in early stage 1 egg chambers. Alternatively, cyclinE-Cdk2 activity may be partially inhibited in stage 1 oocytes via a Dap-independent mechanism. This possibility is supported by the observation that even in the complete absence of Dap, not all oocytes enter the endocycle, indicating that oocytes have an alternative mechanism to inhibit inappropriate DNA replication. Finally, Dap proteolysis may be less efficient in the oocyte for reasons independent of the level of cyclinE-Cdk2 activity, such as a general inhibition of the proteasome. The identification of additional genes that influence the maintenance of the meiotic cycle during oogenesis will help distinguish between the above possibilities.

Cell-cycle regulation and cellular differentiation in ovarian germline cysts

Studies over the last few years indicate that the differentiation of both the nurse cells and oocyte are strongly influenced by cell-cycle events within the germline cyst (Lilly and Spradling, 1996; Ghabrial et al., 1998; Ghabrial and Schupbach, 1999; Myster et al., 2000; Page et al., 2000). Our data support the conclusion that cell-cycle regulation and oocyte differentiation are closely coupled. Specifically, we find that inappropriate entry into the endocycle disrupts oocyte differentiation in *dap* germline cysts and results in the presumptive oocyte developing like a nurse cell. The more polyploid the oocyte, the greater the disruption in oocyte differentiation as measured by the oocyte-specific accumulation of the proteins BicD and Orb, as well as yolk uptake. In mutants like *dap*, where the oocyte is specified but ultimately enters the endocycle, cause and effect can be difficult to determine. Does the oocyte enter the endocycle because of the inability to maintain the oocyte fate or alternatively does entry into the endocycle disrupt the ability to maintain the oocyte identity? Considering the known role of *dap* in cell-cycle regulation, we believe that the phenotype observed in *dap* mutants reflects the second scenario. Our data indicate that entry into the endocycle is incompatible with many aspects of the oocyte developmental program and can serve as the primary cause of the loss of oocyte identity. The identification of additional genes that influence the maintenance of both the nurse cell and oocyte identities will help clarify the exact role of cell-cycle programming in the nurse cell/oocyte fate decision of *Drosophila* ovarian cysts.

We thank Iswar Hariharan, Christian Lehner, Steve Cohen, Scott Hawley, Dennis McKearin and Ruth Steward for providing stocks and antibodies. We thank Nicole Greider, Maggie de Cuevas, Brian Calvi, Andrew Golden and Lori Pile for critical reading of the manuscript.

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