The mitotic-to-endocycle switch in *Drosophila* follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions

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Accepted 16 March 2004

Development 131, 3169-3181
Published by The Company of Biologists 2004
doi:10.1242/dev.01172

Summary

The Notch signaling pathway controls the follicle cell mitotic-to-endocycle transition in *Drosophila* oogenesis by stopping the mitotic cycle and promoting the endocycle. To understand how the Notch pathway coordinates this process, we have identified and performed a functional analysis of genes whose transcription is responsive to the Notch pathway at this transition. These genes include the G2/M regulator Cdc25 phosphatase, String; a regulator of the APC ubiquitination complex Hec/Cdh^{Fzr} and an inhibitor of the CyclinE/CDK complex, Dacapo. Notch activity leads to downregulation of String and Dacapo, and activation of Fzr. All three genes are independently responsive to Notch. In addition, Cdh^{Fzr}, an essential gene for endocycles, is sufficient to stop mitotic cycle and promote precocious endocycles when expressed prematurely during mitotic stages. In contrast, overexpression of the growth controller Myc does not induce premature endocycles but accelerates the kinetics of normal endocycles. We also show that Archipelago (Ago), a SCF-regulator is dispensable for mitosis, but crucial for endocycle progression in follicle epithelium. The results support a model in which Notch activity executes the mitotic-to-endocycle switch by regulating all three major cell cycle transitions. Repression of String blocks the M-phase, activation of Fzr allows G1 progression and repression of Dacapo assures entry into the S-phase. This study provides a comprehensive picture of the logic that external signaling pathways may use to control cell cycle transitions by the coordinated regulation of the cell cycle.

Key words: *Drosophila*, Notch, String, Fizzy related, Dacapo, Cell cycle, Follicle cells

Introduction

Cancer cells often show abnormal cell cycle control in response to external stimuli and this feature has attracted much scientific interest. Many examples now exist in which signaling pathways can induce the G1/S cell cycle transition. Fewer examples exist whereby signaling pathways stop cells from cycling and dividing. Studies on a natural variation of the mitotic cycle, the endocycle, have helped to illuminate this question (Edgar and Orr-Weaver, 2001). The endocycle (as seen for example in cardiomyocytes, trophoblasts, and *Drosophila* nurse and follicle cells) is a variation of the normal cell cycle in which rounds of DNA replication and growth occur without intervening mitoses (Zimmet and Ravid, 2000). A critical characteristic of the endocycle is its capacity to bypass the important controls that regulate normal transitions in mitotic cycles. How this bypass is accomplished is largely unknown; although some of the factors that control the endocycle have been partially characterized (Edgar and Orr-Weaver, 2001). Importantly, a comparative approach that addresses this question might aid our understanding of how cells become cancerous.

In normal mitotic cells cyclin-dependent kinases (Cdks) induce phosphorylation events that control whether the cell enters the M- or S-phase. CycA/Cdk1 and CycB/Cdk1 complexes that can be activated by Cdc25-type phosphatases mediate M-phase control. S-phase is controlled by other complexes: CycE/Cdk2, CycA/Cdk2, and CycD/Cdk4 or Cdk6. Some of these complexes are known to be negatively regulated by several Cdk inhibitors, such as p27Kip1. p27Kip1 inhibits CycE/Cdk2 complexes and thereby arrests cells in the G1-phase (Olashaw and Pledger, 2002).

Cells normally progress through the cell cycle in a series of sequential steps in which each step is dependent on the proper completion of the previous stage. However, endocycling cells are exceptions to this rule because they proceed to S-phase without completing M-phase. How this occurs is not understood. In some endocycling cells, bypassing the mitotic stage correlates with eliminating or reducing the Cdk activity required for M-phase entry. For example in *Drosophila* early embryonic cells and follicle cells, components and regulators of the M-phase can be eliminated at a transcriptional level and/or at a posttranscriptional level (Deng et al., 2001; Sauer et al., 1995; Sigrist and Lehner, 1997). Posttranscriptional modulations are often mediated by ubiquitination via the APC-

complex with degradation of the cyclins and securins (Peters, 2002; Vodermaier, 2001).

The key question in endocycle regulation is how the transition from the mitotic phase to the endocycle is controlled. Two signaling pathways have been identified as regulators of the mitotic-to-endocycle transition: the thrombopoetin pathway, which acts during differentiation of megakaryocytes and the Notch pathway, which acts during *Drosophila* oogenesis and during the differentiation of trophoblasts (Deng et al., 2001; Lopez-Schier and St Johnston, 2001; Nakayama et al., 1997; Wu et al., 2003; Zimmet and Ravid, 2000). Human teratocarcinomas also seem to arise from defects in the mitotic-to-endocycle transition in trophoblasts (Cross, 2000). The key cell-cycle targets of these pathways, however, remain elusive.

In *Drosophila* follicle cells the function of the Notch pathway in the mitotic-to-endocycle transition has been well established (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Specifically, the ligand Delta is secreted by germ line cells and activates Notch in the follicle cells. Subsequently, the cytoplasmic portion of Notch is cleaved by Presenillin and moves to the nucleus where, in combination with a transcription factor, Suppressor of Hairless [Su(H)], it affects the transcription of various target genes. Lack of Notch activity in *Drosophila* follicle cells leads to prolonged mitosis at the expense of endocycles. This has led to the suggestion that Notch functions in this context as a tumor suppressor (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Because very few signaling pathways have been identified that stop the mitotic cell cycle, it is important to understand in detail the relationship between the Notch pathway and known cell cycle regulators.

We have previously shown that one of the cell cycle components that responds to Notch activity at the transcriptional level, String/Cdc25 phosphatase, which is a regulator of the transition between S- and M-phase, is not sufficient, by itself, to keep all the cells in mitotic phase, suggesting that other components are needed for the mitotic-to-endocycle transition (Deng et al., 2001; Schaeffer et al., 2004). One such Notch-regulated component, essential for the mitotic-to-endocycle transition is Hec1/Cdh^{Fzr}, a WD40-domain regulator of the APC-Ubiquitination complex. Hec1/Cdh^{Fzr}, which acts with other cellular components important for M-phase entry, is dispensable for mitosis but essential for the mitotic-to-endocycle transition in follicle cells (Schaeffer et al., 2004). However, cells in *fzr–/–* clones do not prolong mitosis unless accompanied with ectopic *string*. We have now shown that this APC regulator is sufficient to induce, albeit with low penetrance, premature endocycles if precociously expressed in the follicle cell epithelium. We furthermore identify an inhibitor of the CyclinE/CDK complex, Dacapo, that is reduced because of Notch activity in the mitotic-to-endocycle transition as a repressor of endocycles in follicle cells. Notch activity, therefore, executes the mitotic-to-endocycle transition by regulating three cell cycle transitions: repression of String blocks M-phase, activation of Hec1/Cdh^{Fzr} allows G1 progression, and repression of Dacapo assures entry into S-phase.

Materials and methods

Fly stocks

The following fly stocks were used: *FRT82B Dl^{rev10e}* (*Dl^{rev10}* is an amorphic allele of Delta that is produced by excision of the promoter region, transcription start site and first exon) (Heitzler and Simpson, 1991; Zeng et al., 1998); *Su(H) FRT40A* [*Su(H)^{SF8}* is a strong loss-of-function but not a null allele of *Suppressor of hairless*, a gift from S. Blair), whereas *Su(H)^{047} FRT40A* is a null allele] (Li et al., 1998); *fzr^{ie28} FRT101* (Schaeffer et al., 2004); *FRT42B dap^{4}* [*dap^{4}* is a *w$^{–}$* derivative associated with 95%-100% lethality, which was found to represent an imprecise excision that resulted in an intragenic deletion of the *dacapo* gene] (Lane et al., 1996), *ago^{1} FRT80B*, *ago^{3} FRT80B* and *ago^{4} FRT80B* all have similar *archipelago* phenotypes (Moberg et al., 2001). For generating follicle cell clones we used: *hsFLP;Ubi-GFP FRT40A*, *hsFLP;Ubi-GFP FRT42B*, *hsFLP;lacZ FRT42B*, *hsFlp;;Ubi-GFP FRT80B*, *hsFlp;;FRT82B Ubi-GFP*, *yw Ubi-GFP FRT101*, *w$^{–}$;;MKRS P{ry=hsFlp}86E/Tm6BTb*, *hsFlp;;UAS-GFPact<FRT-CD2-FRT<Gal4/TM3* (Pignoni and Zipursky, 1997). For analysis of overexpression patterns the following stocks were used: *UAS-stg^{N4}* and *UAS-stg^{N16}* (Bloomington Stock Center/Bruce Edgar), *UAS-inx2* (Stebbings et al., 2002), *mew$^{\Delta.Scer\UAS}$* (Li et al., 1998), *w$^{–}$;UAS-cycD/CyOGFP;Dichaetae/TM6B*, *UAS-cycE*, *UAS-cycA*, *UAS-dap II.2* and *UAS-dap II.3* (gifts from Bruce Edgar), *UAS-fzr III.2*, *UAS-fzr II.1*, *fzr^{ie28}* (gifts from Christian Lehner) and *w; UAS-dMyc^{132}*, a gift from Robert Eisenman (Johnston et al., 1999). We also used the following p53 lines: *w[1118];P{w[+mC]=GUS-p53}2.1*, dominant negative constructs of p53 *w[1118];P{w[+mC]=GUS-p53.259H}3.1* and *w[1118];P{w[+mC]=GUS-p53.Ct}3.1/TM6B, Tb[1]* (Bloomington Stock Center/Gerry Rubin), and the p53 viable mutants *y[1]w[1118];p53[5A-1-4]* and *y[1]w[1118];p53[11-1B-1]* (Bloomington Stock Center/Kent Golic). *Rbf^{120a}/FM7* and *Rbf^{14}/FM7* were gifts from Giovanni Bosco and Terry Orr-Weaver. In addition, we used the *fzr-lacZ* fusion line *G0326* (Bloomington Stock Center), *6.4-string-lacZ* fusion transgene construct [a gift from Bruce Edgar (Lehman et al., 1999)] and various *dap* transgene constructs [gifts from Christian Lehner (Meyer et al., 2002) and Harold Vaessin (Liu et al., 2002)]. *dap-lacZ* fusion transgene constructs that lacked the 1.5 kb upstream region of the *dap* gene gave no staining, however, most of the *dap*-gm constructs, which included the entire gene as well as different lengths of the promoter region fused to a *myc*-epitope tag, showed clear staining in the follicle cells before stage 6 and a downregulation of expression thereafter.

Generation of follicle cell clones

Drosophila melanogaster stocks were raised on standard cornmeal-yeast-agar-medium at 25°C. To obtain follicle cell clones, 1-to-5-day-old flies were heat-shocked as adults for 50-60 minutes at 37°C and put in freshly yeasted vials for 3 or 5 days. To obtain germ line clones, flies were heat-shocked as second and third instar larvae for 2 hours on 2 consecutive days. Once they emerged as adults, they were placed in vials with fresh yeast paste for 1-5 days prior to dissection.

Nuclear preparation and flow cytometric analysis

Nuclear preparation was done essentially as described in Bosco et al. (Bosco et al., 2001) and Calvi and Lilly (Calvi and Lilly, 2004) with minor modifications. Ovaries from 50-100 females were incubated in 1 ml of 5 mg/ml collagenase (Blend type H, Sigma-Aldrich C8051) in 90% Graces insect medium at 4°C for 15 minutes on a Clay-Adams Nutator. Ovaries were then disrupted by pipeting them several times through a P-1000 tip and then pelleted in an Eppendorf centrifuge by a 2-second spin at 1000 ***g***. The pellet was resuspended in 500 μl of buffer A (15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine) plus 0.25 M sucrose and 0.5% NP-40. Next the ovaries were homogenized at 4°C by 10 strokes in a 2 ml Kimble/Kontes glass dounce with a glass B-clearance pestle. The resulting lysate was cleared by serial passage through 150-, 100-, 50- and finally 30-μm Nitex filters (Sefar America, Depew, NY, USA). Nuclei were then pelleted by passage through a sucrose (0.25 M-2.5 M, in 1X Buffer A) density step gradient 15,000 ***g*** for 20

minutes at 22°C in a Beckman TLS-55 rotor and resuspended in 200 μl of buffer A that contained 0.1% NP-40 and 20 μg/ml propidium iodide. Follicle cells nuclear ploidy was determined by fluorescence-activated cell sorting (FACS) analysis using a Becton Dickinson FACScan cytometer by numbering the intensity of propidium fluorescence in stained nuclei. Nuclei were exited with a 488 nm laser, and the emission was monitored through a 585/42 nm band pass filter. Results were analyzed by using CellQuest and Multicycle software.

Staining procedures

Ovaries were dissected in phosphate-buffered saline (PBS) and fixed while shaking on a nutator for 10 minutes in PBS containing 5% Formaldehyde. Next, they were rinsed with PBT (PBS/0.2% Triton X-100) four times (15 minutes, each rinse) and blocked in PBTB (PBT, 0.2% BSA, 5% Normal Goat Serum) for one hour at room temperature. The tissue was incubated with primary antibodies overnight at 4°C. The next day they were rinsed with PBT four times (15 minutes, each rinse) and blocked in PBTB for one hour at room temperature. The ovaries were then incubated in secondary antibodies overnight at 4°C. The next day they were rinsed with PBT (4×15 minutes) and stained with DAPI (1 μg/ml in PBT) for 10 minutes. Finally, they were washed with PBT twice four times (5 minutes, each wash) and dissected onto slides in 70% glycerol, 2% NPG, 1X PBS.

Follicle cells were labeled with BrdU as described previously (Bosco et al., 2001; Calvi and Lilly, 2004; Lilly and Spradling, 1996) with slight modifications. Ovaries were dissected in Grace's insect medium and then incubated with 10 μM BrdU (Boehringer Mannheim) in the same medium for 1.5 hours at room temperature. The ovaries were then fixed in 10.5% formaldehyde for 15 minutes, washed with PBT, then treated with 2N HCl for 45 minutes. Sodium borate (100 mM) was used for neutralization. The tissues were then rinsed with PBT three times (10 minutes, each rinse) and blocked in PBTB for half an hour at room temperature and incubated with mouse anti-BrdU antibodies (Becton Dickinson) overnight at 4°C. The next day, ovaries were rinsed with PBT six times (5-10 minutes, each rinse), blocked in PBTB for 30 minutes at room temperature and incubated with secondary antibodies for 2 hours at room temperature. Thereafter, the ovaries were rinsed with PBT four times (15 minutes, each rinse) and stained with DAPI for 10 minutes. Finally, they were washed with PBT twice (5 minutes, each wash) and dissected onto slides in 70% glycerol, 2% NPG, 1X PBS.

Confocal microscopy, X-gal staining and in situ hybridization were performed as described previously (Keller Larkin et al., 1999; Tworoger et al., 1999). For in situ RNA hybridization studies *stg* cDNA (LD47579) was labeled with fluorescine, whereas *cycD* (LD22957), *cycE* (LD22682), *cycA* (LD44443), *cycB* (LD23613), and *fzr* (LD21270) cDNAs were labeled with digoxigenin (all cDNAs were from the Berkeley Drosophila UniGene Collection). A two-photon laser-scanning confocal microscope (Leica TCS SP/MP) was used in this study.

The following primary antibodies were used at the designated dilutions: mouse anti-Fasciclin III (1:20), mouse anti-CycA (1:20) and mouse anti-CycB (1:20) from Developmental Studies Hybridoma Bank, rabbit anti-CycA [1:100, David Glover (this antibody showed CycA downregulation at stage 6 but some immunoreactive material was later observed at stage 8)], mouse anti-CycE (1:5, a gift from Helena Richardson), guinea pig anti-CycE (1:500, a gift from Terry Orr-Weaver), rabbit anti-PH3 (1:200, Upstate Biotechnology), rabbit anti-Fizzy-related (1:800, a gift from Christian Lehner), mouse or rabbit anti-β-gal (1:5000, Sigma) and mouse anti-c-Myc (1:50, Calbiochem). The following secondary antibodies were used at the designated dilutions: Alexa 488, 568 or 633 goat anti-mouse (1:500), Alexa 488, 568 or 633 goat anti-rabbit (1:500, Molecular Probes), Alexa 488 goat anti-guinea pig (1:500, Molecular Probes).

Studying the Cyclin D role in mitotic-to-endocycle transition

We analyzed the expression pattern of CycD during mitotic-to-endocycle transition, because it has been shown to be critical for cell growth in *Drosophila* (Chen et al., 2003; Datar et al., 2000; Meyer et al., 2000) and the cell growth is a major component of endocycle. Surprisingly, a clear downregulation of *cycD* RNA level was observed at the onset of endocycles, stage 6 (data not shown). The functional relevance of this downregulation is not clear because overexpression of the protein, in combination with its kinase Cdk4, does not dramatically affect entry into endocycles. During these overexpression studies, we observed no difference in the size of nuclei compared with those in wild-type cells and there was no upregulation of CycB or PH3 (data not shown).

Results

The endoreplication cell cycle or endocycle is a variation of the normal mitotic cell cycle, in which cells increase their genomic DNA content without dividing. How the transition from the mitotic cycle to the endocycle is regulated is not well understood. *Drosophila* oogenesis provides an excellent system in which to study this transition (Calvi et al., 1998).

In the *Drosophila* ovary both the germ line and somatic cells arise from stem cell populations located in an anterior ovary structure called the germarium (Fig. 1A). At the posterior end of the germarium, somatic cells encapsulate a 16-cell cyst of germ line cells, a configuration in which the oocyte will eventually develop during a three-day period (this developmental process has been divided into 14 stages). Meanwhile, the somatic follicle cells will undergo three tightly developmentally controlled cell cycle modifications (Calvi et al., 1998). First, these epithelial cells undergo a mitotic division program that gives rise to approximately 1000 follicle cells by stage 7 in oogenesis (Fig. 1A, part I). At this mid-oogenesis point, signaling through the Notch pathway stops the mitotic cycles in the follicle cells and allows them to enter endoreplication to become polyploid (Deng et al., 2001; Lopez-Schier and St Johnston, 2001) (Fig. 1A, part II). After stage 6, the follicle cells then undergo three endocycles to become polyploid. Later in oogenesis at stage 10B in response to unknown developmental signals, four different loci, encoding several different genes two of which are chorion genes, synchronously initiate a gene amplification event, that increase their copy number (Fig. 1A, part III). During this phase all other genomic replication origins remain inactive (Orr-Weaver, 1991; Spradling, 1999). The chorion genes encode the eggshell proteins and amplification of these genes is needed to produce sufficient chorion protein for a normal eggshell. These three replication patterns are readily observed by BrdU analysis (Calvi et al., 1998) (Fig. 1B). In addition, these cell cycle programs can be distinguished by different markers, for example, CycB and PH3 (Deng et al., 2001) (Fig. 1C).

Hec1/Cdh^{Fzr} is sufficient to stop mitosis and induce premature endocycles

We have previously described the role of Notch signaling in downregulating *string* at stage 6 of oogenesis to allow the cells to transit into the endocycle (Deng et al., 2001) (Fig. 1D). In order to find other cell cycle genes activated by Notch activity after/during the transition from mitotic-to-endocycle, we

performed an expression screen for genes differentially expressed before and after the transition. We screened 400 lethal X-chromosome P-element enhancer trap-lines (Peter et al., 2002) for changes in expression levels at stage 7 using the β-gal reporter gene. Seven genes that fall into three interesting functional groups were obtained from this screen: adhesion molecules, transcriptional control proteins and cell cycle regulators (Table 1). The two cell cycle regulators, Fizzy related (Hec1/Cdh^{Fzr}) and Myc (Bourbon et al., 2002), were analyzed in more detail. The expression of *string* is observed until stage 6, whereas high *fizzy related* (*fzr*) expression marks the start of endocycles (Fig. 1D,E). Importantly, we have shown that Hec1/Cdh^{Fzr} is required for the mitotic-to-endocycle transition in these cells (Schaeffer et al., 2004). Furthermore, we have shown that the combination of reduced Hec1/Cdh^{Fzr} and prolonged Stg expression can keep the follicle cells in the mitotic cycle past stage 6.

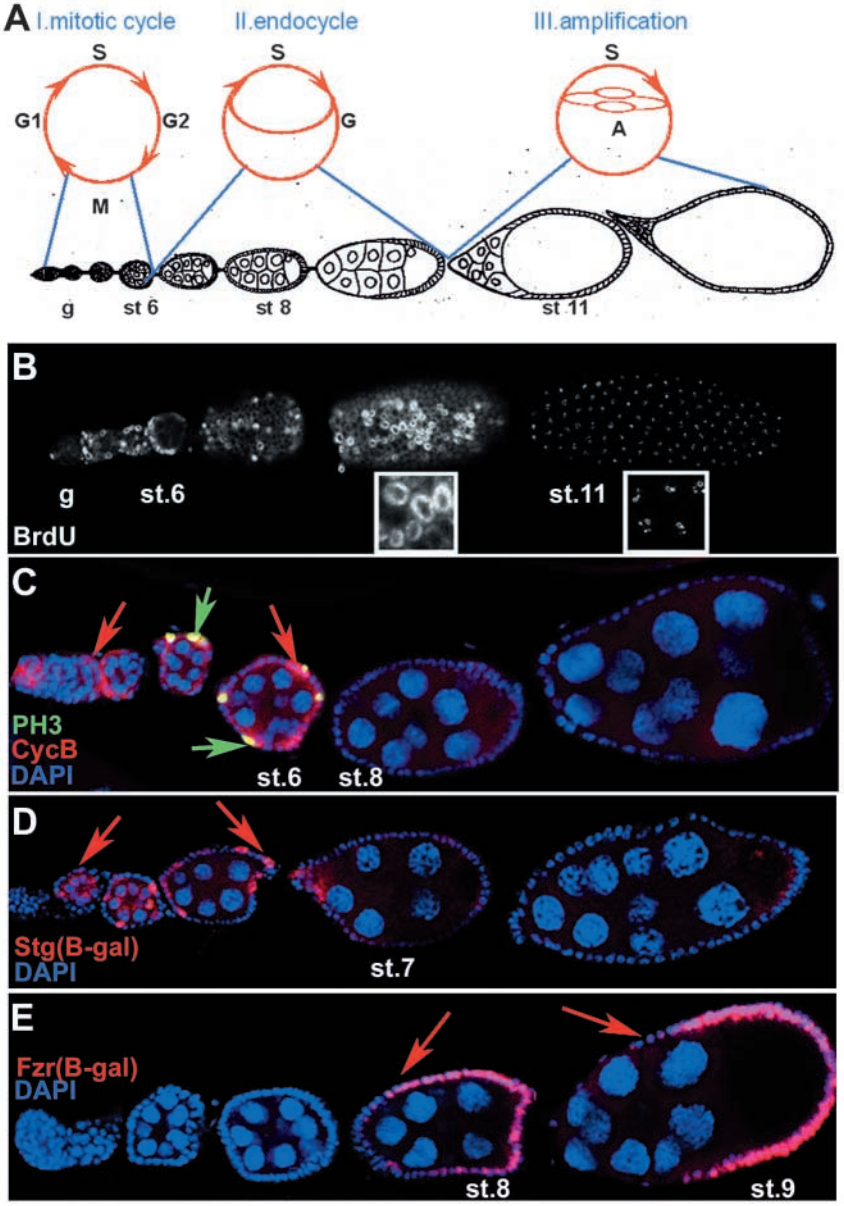

Fig. 1. Cell cycle transitions in epithelial follicle cells. (A) Follicle cells in *Drosophila* oogenesis undergo two cell cycle transitions: from a mitotic cell cycle to an endocycle and from endocycle to amplification (A, parts I-III, drawing of the stages of oogenesis). From the germarium (g) to stage (st) 6, somatically derived follicle cells undergo mitotic cycles, which are not synchronized (A, part I). At stage 7 they switch to endocycles. From stage 7 to stage 10A, these cells undergo three rounds of endoreplication (A, part II) and thereafter switch to the localized replication pattern characteristic of chorion gene amplification (A, part III). These three replication patterns are observed in BrdU incorporation analysis (B). The staining of mitotic markers Cyclin B (CycB) (red arrows), Phospho-Histone 3 (PH3) (green arrows) (C) and *stg* (6.4) promoter construct (D) show expression in mitotic follicle cells, whereas Fzr enhancer trap line, *fzr*G0326 (β-gal expression, red arrow) shows Fzr expression in endocycling cells (E). Green, PH3; red, CycB (C); Anti-β-gal (D,E); blue, DAPI (C-E).

To test what is required to turn mitotic cycles to endocycles prematurely, we over-expressed candidate genes at stages 1-6 and analyzed the effects in follicle cells undergoing mitotic cycles. Premature expression of the adhesion molecules did not alter the timing of the mitotic-to-endocycle transition (Table 1). However, premature expression of the Notch-responsive cell cycle component, Hec1/Cdh^{Fzr}, caused the formation of enlarged nuclei, a potential indication of precocious endocycles (Fig. 2A,B) (Schaeffer et al., 2004). Importantly, analysis of cell cycle markers revealed a premature reduction of cyclins A and B (70% and 91% reduction, respectively; Fig. 2C). Consequently, a halt in mitotic cell cycle (60% reduction in PH3 staining; Fig. 2C) was observed because of the premature expression of Hec1/Cdh^{Fzr}. In contrast, as a control we overexpressed other cell cycle components, such as dMyc and p53 and observed no change in the expression of mitotic markers compared with that seen in wild-type cells (Fig. 2C). These findings suggest that the premature expression of Hec1/Cdh^{Fzr} is sufficient, at least partially, to stop mitosis.

The enlarged nuclei observed upon overexpression of Hec1/Cdh^{Fzr} could be caused via a ploidy-independent mechanism (Gao and Pan, 2001; Tapon et al., 2001) or via increase in ploidy as some cells undergo ectopic endocycles. To distinguish between these possibilities, we determined the exact DNA content in the cells overexpressing *fzr* by FACS on purified ovarian nuclei (Bosco et al., 2001; Calvi et al., 1998; Lilly and Spradling, 1996). Although control nuclei revealed the normal 2n, 4n, 8n and 16n DNA peaks, some of the nuclei from UAS-*fzr* mutants showed DNA content of 32n, comparable with that in *Rbf* mutants, in which follicle cells are known to undergo extra endocycles (Bosco et al., 2001) (Fig. 2H-J). These data suggest that some of the follicle cells overexpressing *fzr* not only stopped mitotic cycles prematurely but also proceeded through extra endocycles increasing the ploidy of the cells.

Table 1. Genes that show a change in expression levels at the mitotic-to-endocycle transition in follicle cells

			Cell cycle phenotypes	
Functional group	Protein	Expression	Loss of function	Ectopic expression
Adhesion	Fasciclin 2	After stage 7-8	N/A	N/A
	Innexin 2	After stage 7-8*	N/A	No phenotypes (this study)
	PS1α (mew)	After stage 7-8	N/A	No phenotypes (this study)
Transcriptional control	BRC	After stage 7-8	Abnormal amplification (Tzolovsky et al., 1999)	Abnormal amplification (Tzolovsky et al., 1999)
	Trf2	After stage 7-8	N/A	N/A
Cell cycle control	Fzr	After stage 7-8	Defective endocycle (Schaeffer et al., 2004)	Precocious endocycle (this study)
	Myc	After stage 7-8	Defective endocycle (Maines et al., 2004)	Accelerated endocycle (this study)

*mRNA expression pattern of *innexin 2* in *Drosophila* follicle cells has been previously described (Stebbings et al., 2002).

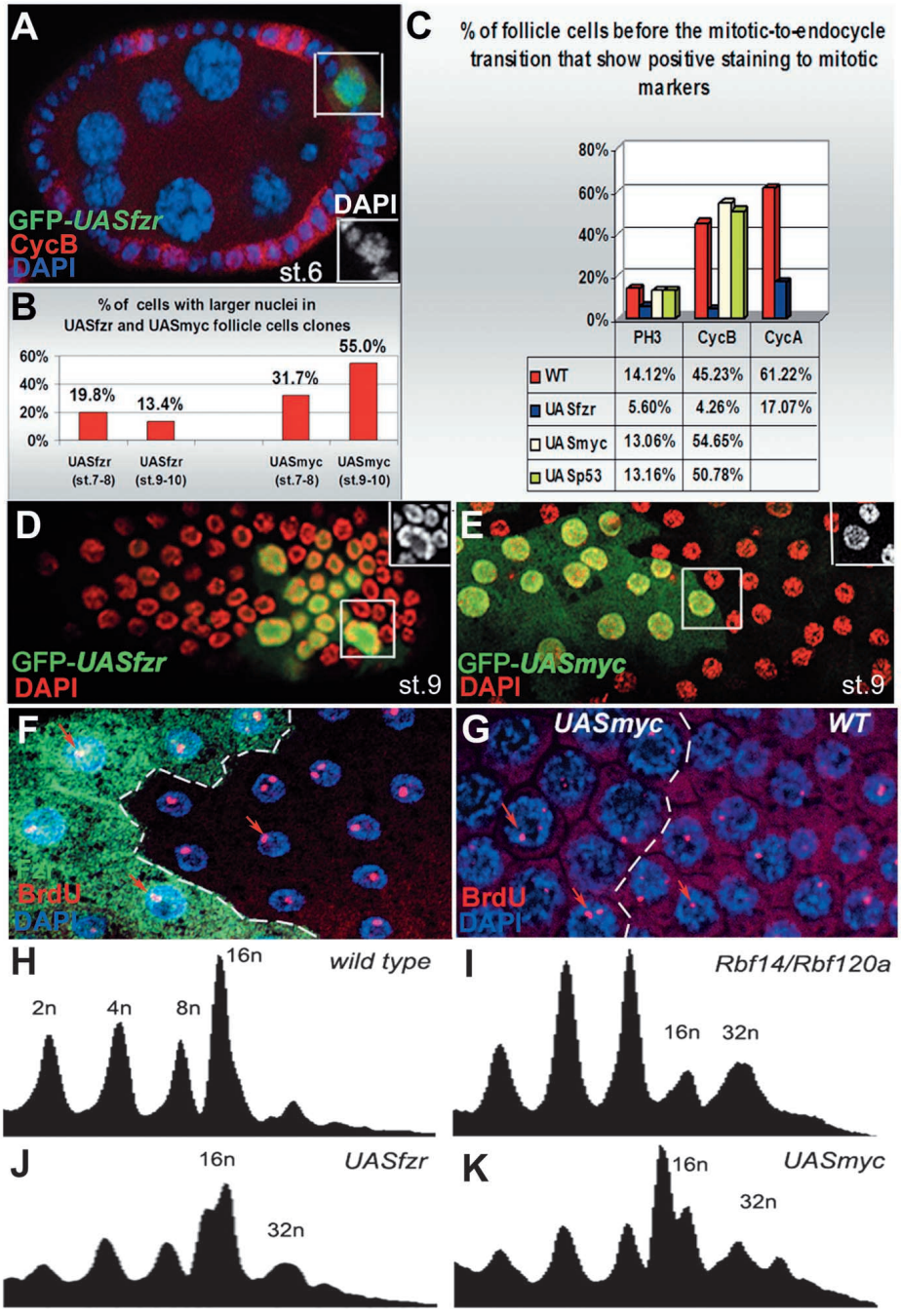

Fig. 2. Fzr is partially sufficient to induce precocious endocycles, dMyc to accelerate endocycles. Ectopic expression of *fzr* (*hsFlp; UAS-fzr; UASGFP act<FRT-CD2-FRT<Gal4*) reduces CycA and B levels, stops mitosis (A,B) and allows the formation of large nuclei (B) indicative of precocious endocycles prior to stage 6 in oogenesis. The diagram in C presents the percentage of the wild-type and mutant follicle cells that show positive staining to mitotic markers during the mitotic stage. *fzr* (D,F,J) and *myc* (E,G,K) overexpression (*hsFlp; UAS-fzr or UAS-myc; UASGFP act<FRT-CD2-FRT<Gal4*) generate enlarged nuclei, which is indicative of abnormal endocycles but do not affect the amplification stage because normal BrdU-incorporation pattern at stage 10B-12 (red arrows) is observed in cells overexpressing *fzr* (F) and *myc* (G). (H-K) FACS profiles of DNA content in *UAS-fzr* (J) and *UAS-myc* (K) mutant nuclei compared with these in WT nuclei (H) show the appearance of a cell population that has 32n DNA, also observed in *Rbf* mutants (I) (Bosco et al., 2001). Green, GFP (A,D,E), Fzr Ab (F); red, CycB (A), DAPI (D,E), BrdU (F,G); blue, DAPI (A,F,G).

Ectopic Hec1/Cdh^{Fzr} affects mitotic-to-endocycle but not endocycle-to-amplification transition

After three rounds of endocycles, the follicle cells synchronously initiate a chorion gene amplification event that continues to increase the copy number of four different loci. The amplification occurs by the initiation of repeated rounds of DNA replication and fork movement to produce a gradient of amplified DNA extending ~100 kb (Calvi and Spradling, 1999; Orr-Weaver, 1991). How the onset of the endocycle-to-amplification transition is regulated is not understood.

To further analyze whether the larger nuclei observed upon ectopic *fzr* expression was because of precocious endocycling during the mitotic phase and/or prolonged endocycling during the amplification phase, we analyzed whether the amplification stage was defective or delayed in cells that overexpressed *fzr*. BrdU incorporation revealed normal amplification patterns in control and *fzr*-overexpressing cells (Fig. 2F), suggesting that the extra endoreplication observed upon *fzr* overexpression was not caused by defects in the endocycle-to-amplification transition. We therefore conclude that the larger nuclei arose because of a premature switch in the timing of the mitotic-to-endocycle transition.

Myc in the mitotic-to-endocycle transition

Premature expression of Fzr is sufficient to halt mitotic cell cycles 60% of the time, however, only one-third of these cells will enter premature endocycles (Fig. 2B,C). One possible explanation for these results is that components at G1/S transition, such as G1 cyclins, further restrict from entering the premature endocycles by blocking entry into the S-phase in mitotic cells.

In other systems G1/S transition is controlled in part by factors that regulate cell growth such as CycD or Myc (Frei and Edgar, 2004). Curiously, CycD does not seem to be critical for endocycles in follicle cells (see Materials and methods). This presents a paradox because CycD is known to be critical for cellular growth in *Drosophila* (Datar et al., 2000; Meyer et al., 2000) and a key feature of all endocycles is an increase in cell size. Interestingly, the expression screen described previously (Table 1) (Bourbon et al., 2002) might provide an answer to this apparent paradox, because this screen demonstrated that Myc, another component required for growth, is transcriptionally upregulated at the transition.

dMyc is the *Drosophila* homologue of the BHLHZ Myc-oncogene family of transcription factors. Recent studies have shown that dMyc has a function in cellular growth (Iritani and Eisenman, 1999; Johnston and Gallant, 2002; Johnston et al., 1999). To test whether dMyc can influence the mitotic-to-endocycle transition, we overexpressed *dmyc* in developing follicle cells and analyzed cell cycle markers in both cell cycle programs. No obvious change was observed during the mitotic stage upon ectopic expression of *dmyc* (Fig. 2C); however during the endocycling stage, cells overexpressing *dmyc* were larger than the neighboring wild-type cells (Fig. 2E). In particular, larger nuclei were observed in 32% and 55% of the cells at stage 7-9 and 12 (respectively) after overexpressing *dmyc* (Fig. 2B). FACS analysis revealed that these large nuclei were indicative of extra endocycles because cells with 32n ploidy were observed among these follicle cells (Fig. 2K). Similar to the case of *fzr* overexpression, BrdU incorporation revealed normal amplification patterns during stage 12 in oogenesis in cells overexpressing *myc* (Fig. 2G), suggesting that the extra endoreplication observed during *myc* overexpression was not caused by defects in the endocycle-to-amplification transition. Because the higher ploidy accompanies no change in the timing of mitotic-to-endocycle or endocycle-to-amplification transition, we conclude that this defect was caused by faster endocycle kinetics than in wild-type follicle cells. Thus, these data suggest that Myc-dependent growth can regulate endocycle kinetics in follicle epithelial cells.

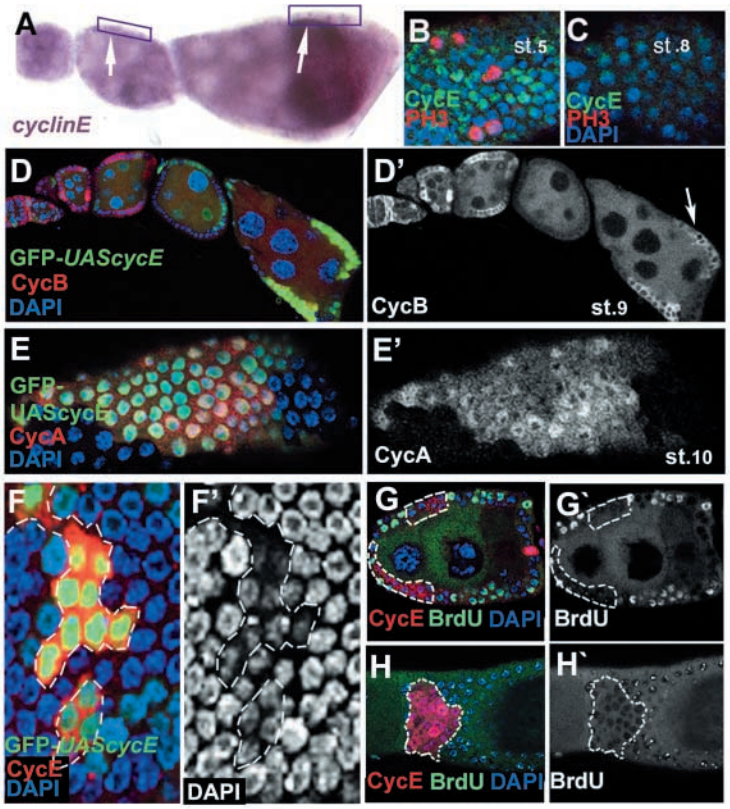

Fig. 3. The Cyclin E levels are dynamic during cell cycle transitions; constant CycE levels halt endocycles and amplification. (A) Wholemount in situ hybridization indicates equal level of *cyclin E* mRNA in the follicle cells throughout oogenesis (arrows). (B,C) Cyclin E antibody staining pattern (green) between stages 5 (B) and 8 (C) shows a clear downregulation of the protein after the transition to the endocycle, when cells stop expressing PH3 (red). Cells overexpressing CycE (*hsFlp;;UAS-cycE/UASGFP act<FRT-CD2-FRT<Gal4*) express mitotic cyclins B and A past stage 6 in oogenesis (D-E, arrow), have smaller nuclei (F,F′) and show highly reduced BrdU incorporation during endocycling (G,G′) and amplification (H,H′) stages, suggesting that cells ectopically expressing *cyclin E* fail to undergo S-phase and are halted at G2. Green, CycE (B,C), GFP (D-F), BrdU (G,H); red, PH3 (B,C), CycB (D), CycA (E), CycE (F-H); blue, DAPI.

Proper CycE regulation is required for the mitotic-to-endocycle transition

Another G1/S-regulator that might play a role in the proper

Table 2. Proper Cyclin E regulation is crucial for endocycles: increased Cyclin E activity blocks the cells in G2, reduced Cyclin E in G1

	Cell cycle phenotypes at stage 7-12 follicle cells*						
	Small nuclei	Prolonged staining of mitotic markers			BrdU incorporation		
Genotype	(lack of endocycles)	(CycA,B)	(CycE)	PH3	Endocycles†	Amplification	Cell cycle arrest
hsFlp;; UAS-cycE/UASGFP act <FRT-CD2-FRT<Gal4	+ 63%	+ 44%	+	–	–	–	G2
hsFlp: ago FRT80B/Ubi-GFP FRT80B	+ 92%	+ 37%	+ 91%	–	–	–	G2
hsFlp; UAS-dap;UASGFP act <FRT-CD2-FRT<Gal4	+ 71%	–	+ activity (–)	–	–	–	G1
Wild type	–	–	–	–	+	+	No arrest

*$n \geqslant 100$ cells for each experiment
†Although in wild type, 38% of all endocycling follicle cells are BrdU positive, in mutant clones only 6-7% of the cells showed BrdU staining.

mitotic-to-endocycle transition in follicle cells is Cyclin E (Calvi et al., 1998). *Drosophila* Cyclin E forms a complex with the DmCdc2c/Cdk2 kinase and controls the progression through the S phase; its downregulation limits embryonic proliferation and its oscillation is required for endocycling (Follette et al., 1998; Knoblich et al., 1994; Weiss et al., 1998). The *cycE* mRNA expression pattern in the follicle cells during oogenesis reflects a continuous requirement both in mitotic and endocycles without downregulation at the mitotic-to-endocycle transition; but rather, an equal level of 'patchy' *cycE* mRNA levels within the follicle cells throughout oogenesis (Fig. 3A). The same 'patchy' pattern of CycE protein level is seen in mitotic and endocycling follicle cells, indicating the cell cycle-dependent oscillation of CycE protein. However, we observed a clear, but not complete, downregulation of CycE protein levels at approximately stage 7, just after the follicle cells stopped expressing CycB and PH3 (mitotic markers) (Fig. 3B,C). In follicle cell clones mutant for a transcription factor in the Notch pathway, Su(H), the CycE levels were somewhat abnormally regulated during endocycles, which suggested a role for the Notch signaling pathway in this posttranscriptional regulation (data not shown).

The observed change of CycE level in the mitotic-to-endocycle transition is critical because continuous overexpression of CycE leads to abnormalities in the mitotic-to-endocycle transition; the cells of clones overexpressing *cycE* exhibit prolonged expression of CycA and CycB and have smaller nuclei than their neighbours (44% and 63%, respectively; n=214) (Table 2, Fig. 3D-F) (Lilly and Spradling, 1996). However, no PH3 staining is observed among these mutant cells after stage 6, suggesting that they do not undergo prolonged mitosis. Likewise, BrdU incorporation is inhibited in these cells after stage 6 (including the amplification stage as shown before by Calvi et al. (Calvi et al., 1998), implying that they also fail to undergo S-phase (Fig. 3G,H). Taking into account these findings and the upregulation of CycB and CycA, but lack of PH3 staining, we hypothesize that the cells overexpressing CycE are halted at the G2 stage of the cell cycle in the mitotic-to-endocycle transition.

These and other data (Calvi et al., 1998; Lilly and Spradling, 1996; Sauer et al., 1995) suggest that correct CycE protein levels are critical for the proper mitotic-to-endocycle transitions. We therefore turned our investigation to the role of two regulators of CycE protein: Dacapo, a CIP/KIP homologous protein found to inhibit CycE activity (de Nooij et al., 2000; Lane et al., 1996) and Archipelago, a recently characterized F-box, WD repeat protein found to target CycE for SCF-dependent degradation (Moberg et al., 2001; Strohmaier et al., 2001).

Dacapo downregulation by Notch signaling at mitotic-to-endocycle transition is required for proper endocycling

The Cip/Kip families of cyclin-dependent kinase inhibitors (CKI) are the main effectors linking developmental programs and cell cycle progression (Liu et al., 2002). *Drosophila dacapo* (*dap*) encodes an inhibitor of cyclin E/cdk2 complexes with similarity to the vertebrate Cip/Kip inhibitors (Lane et al., 1996; Liu et al., 2002; Meyer et al., 2002) and thereby inhibits entry into the S-phase. In accordance with this role, mammalian family members are upregulated during checkpoint activation and are expressed in terminally differentiated tissues. Dacapo contains a conserved amino-terminal domain that binds to and inhibits all kinases involved in G1/S transition. This inhibition is achieved by a conformational change in the cyclin/Cdk complex (Pavletich, 1999).

In follicle cells, the in situ mRNA hybridization pattern for *dacapo* indicates that its transcriptional level is downregulated at stage 6-7 **(**Fig. 4A), suggesting a potential regulation by the Notch pathway. Because *dacapo* has previously been shown to be developmentally controlled by an extensive promoter region, we used different *dap*-constructs (Liu et al., 2002; Meyer et al., 2002) and studied their expression patterns in the follicle cells during oogenesis. The smallest constructs, that faithfully represent *dacapo* mRNA pattern (dap5gm and dap6gm), include the entire gene as well as 1.8 kb and 2 kb of the promoter region fused with a *myc*-epitope tag. These transgenic lines showed clear myc-epitope staining in the follicle cells before stage 6 and a downregulation of expression thereafter (Fig. 4B). One of these constructs, dap5gm, was used as a marker for Dacapo expression in the following studies.

In order to determine whether Notch signaling controls the downregulation of *dacapo* at stage 6, we analyzed Dacapo expression in follicle cells surrounding Delta mutant germ line clones in a fly that contained the *dap*5gm construct. In the Dl germ line clones, we clearly observed a prolonged expression of *dap*5gm in stages later than 6 (Fig. 4C), suggesting that the transcriptional downregulation of *dacapo* is dependent on Notch activity.

To analyze whether the Notch-dependent repression of *dacapo* was important for the mitotic-to-endocycle transition, we tested the functional consequence of *dacapo* loss-of-function and prolonged expression for follicle cell cycle control. *Dacapo* loss-of-function clones revealed no obvious phenotype; nuclei sizes were normal and endocycle did not appear to be inhibited. Overexpression of *dacapo* has previously been shown to inhibit entry into late amplification (Calvi et al., 1998) and, in the salivary gland, overexpression of *dacapo* inhibits endoreplication (Weiss et al., 1998). We likewise observed cell cycle defects because of the prolonged expression of *dap*: smaller nuclei and a failure to incorporate BrdU, indicating a lack of S-phases (Fig. 4D-F). CycA and CycB, although upregulated in cells overexpressing CycE past stage 6, were not upregulated in cells overexpressing *dap* (Table 2). These results suggest that Notch-based downregulation of *dacapo* is critical for endocycling because overexpression of *dacapo*, a CIP/KIP-type cyclin-dependent kinase inhibitor of Cdk2/CycE complexes, can halt the follicle cell endocycles at the apparent G1/S transition. Also, one possibility is that Dacapo in mitotic cells aborts premature attempts to enter endocycles and precocious expression of Hec1/Cdh^{Fzr} (Fig. 2A,C).

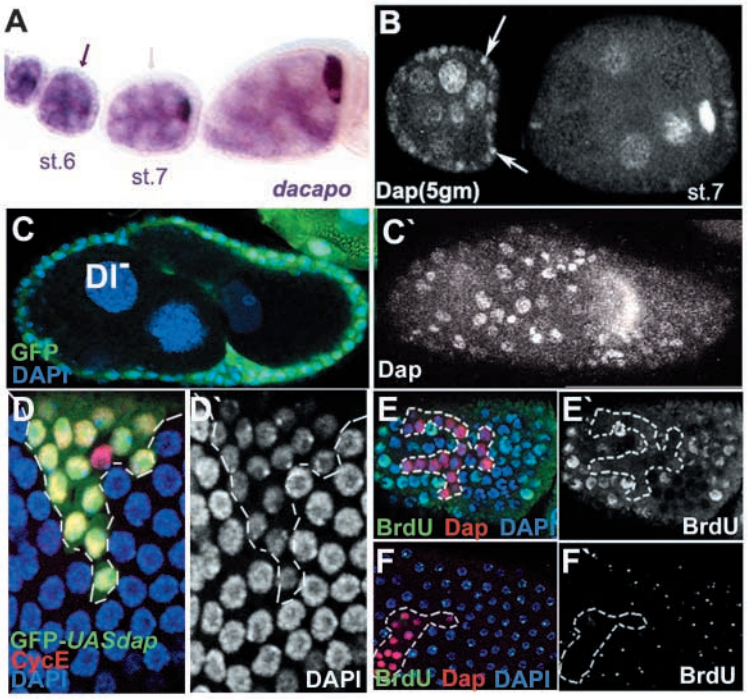

Fig. 4. Notch-dependent repression of the Cyclin E inhibitor Dacapo is required for mitotic-to-endocycle transition. (A) The *dacapo* mRNA in situ pattern indicates transcriptional downregulation after transition to endocycles (light arrow, st. 7). (B) A similar expression pattern was observed in a *dap*5gm construct, which includes the entire gene and a part of the promoter region fused with *myc*-epitope tag (arrow indicates follicle cells staining prior to st. 7). (C,C′) Prolonged follicle cell expression of *dap*5gm in st. 9 Delta germ line clone shows that *dap* is controlled by Notch signaling. (D) Overexpression of *dap* (*hsFlp; UAS-dap; UASGFP act<FRT-CD2-FRT<Gal4*) inhibits endoreplication: follicle cells overexpressing *dacapo* have smaller nuclei (D′) than the neighboring wild-type cells and they fail to incorporate BrdU during both the endocycling (E,E′) and the amplification (F,F′) stages, indicating that these cells could not undergo the S-phase and probably are blocked at the G1/S transition. Green, GFP (C,D), BrdU (E,F); red, CycE (D), Dap (E,F); blue, DAPI.

Ago is essential for endocycles but dispensable for mitotic cycles

Because *dacapo* is downregulated after the mitotic-to-endocycle transition, we investigated whether *archipelago*, a second regulator of CycE protein level was required for proper oscillations of CycE during endocycles. Archipelago is an F-box protein with seven tandem WD repeats that recognizes auto-phosphorylated CycE. Ago protein binds directly to Cyclin E and targets it for ubiquitin-mediated SCF-dependent degradation (Moberg et al., 2001; Strohmaier et al., 2001). The in situ hybridization pattern of *archipelago* reveals continuous mRNA expression both in the mitotic and endocycling stages (Fig. 5A). To investigate the role of Archipelago in these cells we made follicle cell clones with three different *ago* alleles (Moberg et al., 2001). As expected, we saw an increase in CycE protein in most mutant follicle cells at all stages during oogenesis in all three mutants (Fig. 5B). We then analyzed the effect these mutations had on both mitotic and endocycling cells. Based on phenotypic analysis in the *Drosophila* eye, we might expect overproliferation of cells mutant for *ago* (Moberg et al., 2001). Yet, we observed no obvious defects in the control of mitotic divisions in *ago* clones: the ratio between the number of cells in sister clone versus mutant clone was 1:0.9 (n=14) and the BrdU incorporation and CycA and CycB levels during mitotic divisions were normal (Fig. 5D and data not shown). In addition, the expression patterns of PH3 and String in *ago* clones were indicative of a normal halt in mitotic cycles at stage 6 (Fig. 5C).

However, lack of Ago activity seemed to be detrimental for entering the endocycle: *ago* clones showed much reduced or no BrdU incorporation and small nuclei size after stage 7 (Fig. 5F,G, Table 2). In addition, CycB expression is prolonged in 37% (n=200) of *ago*-mutant follicle cells past stage 6 (Fig. 5E**)**. We also analyzed CycA, a marker of the S and G2 phase, and observed an elevation in *ago* clones (data not shown). At the later stages no amplification was observed and some of the mutant cells were seemingly apoptotic (Fig. 5H and data not shown). The phenotypes in *ago*-clones (elevation of CycA and CycB levels, small nuclear size, lack of endocycle and amplification and some cell death at late stages) are also observed upon overexpression of CycE and were indicative of a G2 block (Fig. 3D-H, Table 2). Because overexpression of CycE is observed in most cells mutant for *ago*, we conclude that the phenotypes observed during the mitotic-to-endocycle transition in *ago* clones could be because of the high level of CycE activity. These data suggest that an Archipelago/SFC-dependent regulation of CycE protein levels is dispensable for mitotic divisions but essential for endocycles in follicle epithelium.

Notch controls independently String, Hec1/Cdh^{Fzr} and Dacapo expression

Notch activity affects the expression of Stg (Deng et al., 2001), Fzr (Schaeffer et al., 2004) and Dacapo (this

study) at the mitotic-to-endocycle transition in follicle cells. Because cell cycle regulators can control each other (Futcher, 2002), we tested whether changes in these three Notch-dependent cell cycle regulators lead to changes in each other's expression levels. In this scenario one of the targets might be the primary responder, whereas the others would be downstream components of the pathway.

In particular, because Hec1/Cdh^{Fzr} loss-of-function phenotype is similar to the phenotype observed upon altering Cyclin E activity (Schaeffer et al., 2004) (Figs 3, 4, 5), we tested whether defects in Cyclin E levels can result in downregulation of Hec1/Cdh^{Fzr}. Overactivation of Cyclin E (because of lack of *ago* function or overproduction of CycE) blocks the endocycling cells at G2, whereas inactivation of Cyclin E (because of overproduction of Dacapo) blocks them in G1 (Table 2). However, importantly, neither of these alterations result in repressed Fzr expression at the mitotic-to-endocycle transition: cells that do not endocycle because of overexpression of *dacapo* or *cyclin E* show normal Hec1/Cdh^{Fzr} levels at stages 7-9 (Fig. 6A,B). Based on these data, block of endocycles by CycE or Dacapo overexpression does not repress the expression of the endocycle regulator, Hec1/Cdh^{Fzr}. However accumulation of CycA and CycB are observed upon Cyclin E overexpression (Table 2, Fig. 3D,E), suggesting that even though Hec1/Cdh^{Fzr} is present it might not be in an active form.

Premature expression of Hec1/Cdh^{Fzr} blocks mitotic cycles and induces premature endocycles (Fig. 2A-E). Lack of *string* at stage 4-6 shows similar phenotypes (Deng et al., 2001; Schaeffer et al., 2004). We therefore analyzed whether overproduction of Hec1/Cdh^{Fzr} caused a premature repression of String levels. However, no effect on *string* (or *dacapo*) expression was observed in cells that prematurely expressed *fzr* and entered endocycles (Fig. 6C,D).

Based on these findings, no obvious feedback regulation was observed between these cell cycle regulators, disturbed CycE levels did not block endocycles by repressing Hec1/Cdh^{Fzr} expression levels and premature Fzr does not block mitosis by affecting String levels. Therefore, these data suggest that the Notch-dependent regulation of these targets is independent of each other (Fig. 7A).

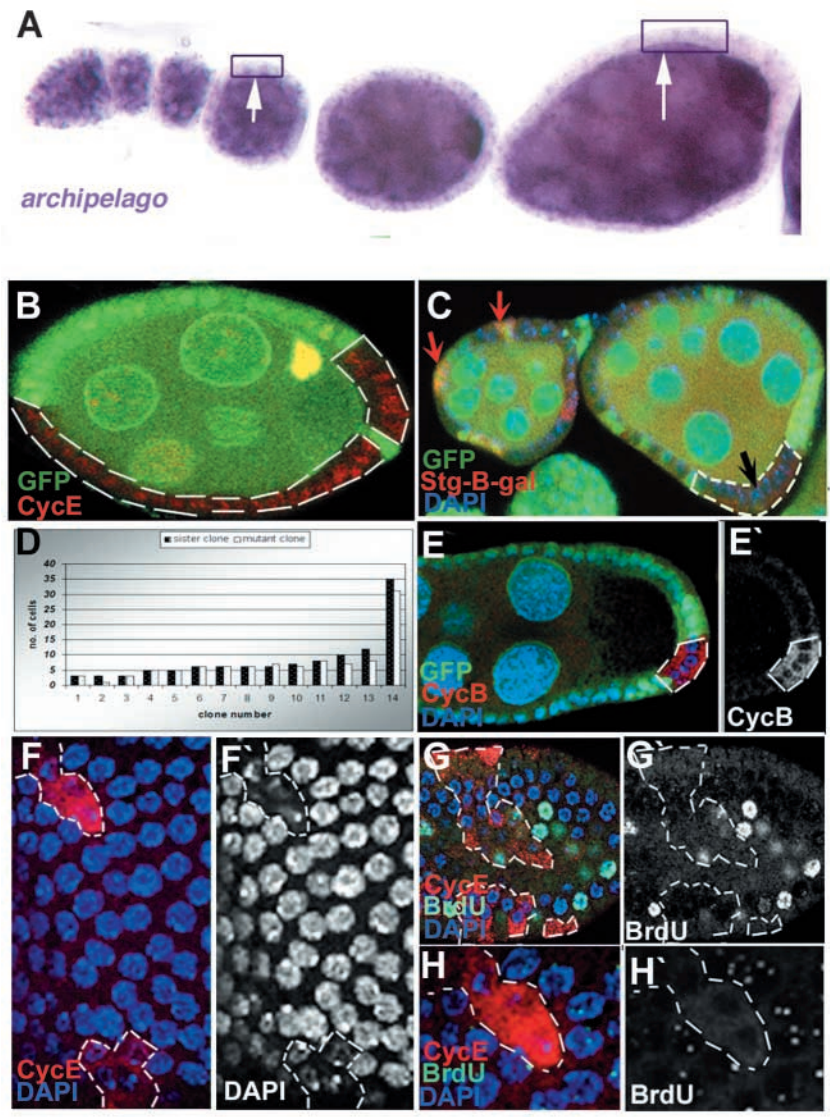

Fig. 5. Ago is dispensable for mitosis but is required for endocycles in follicle cell epithelium. (A) The *ago* mRNA is expressed in both mitotic and endocycling follicle cells (arrows). (B) Ago is responsible for CycE degradation; follicle cells in *ago* (*hsFlp;; ago FRT80B/Ubi-GFP FRT80B*) clones show increased level of CycE protein at all stages during oogenesis. *Ago* loss-of-function does not affect the mitotic cycles in follicle cells: *ago* clones showed normal expression pattern of *stg* (C; expression prior to st. 6, red arrow, but not past st. 6, black arrow) and the ratio between the number of cells in sister versus mutant clone was 1:0.9 (D). However, *ago* clones halt the transition to endocycles: prolonged CycB expression (E,E′), small nuclei size (F,F′; clones marked with elevation of CycE levels) and highly reduced BrdU incorporation during both endocycles (G,G′) and amplification (H,H′) are observed in *ago* clones. Green, GFP (B,C,E), BrdU (G,H); red, CycE (B, CycE level marks the clones in F-H), β-gal (C), CycB (E); blue, DAPI.

Discussion

To unravel how the Notch pathway allows follicle cells to progress through the mitotic-to-endocycle transition we previously identified Cdc25 Phosphatase/String as a transcriptional responder to Notch activation. However, overexpression of *string* is not sufficient to induce the ectopic mitosis in all follicle cells past stage 6; it can only partly prolong mitotic cycles in posterior follicle cells (Schaeffer et al., 2004). This suggests that other critical factors are also regulated in the transition. Interestingly, the regulator of APC-ubiquitination complex, Hec1/Cdh^{Fzr}, and the G1 cyclin CycE inhibitor Dacapo are also regulated at the mitotic-to-endocycle transition. Hec1/Cdh^{Fzr} shows transcriptional activation, whereas Dacapo is reduced at the transcriptional level. We show that the Notch-dependent cell cycle regulator

Hec1/Cdh^{Fzr}, that is required for endocycles, is sufficient to block mitosis and initiate precocious endocycling when expressed prematurely. However, although the efficiency of this process is 60%, only 20% of the cells enter premature endocycles (Fig. 2C), which suggests that other components in the G1-S transition might play a role. We also show that a critical factor in the mitotic-to-endocycle transition, Cyclin E level, is controlled by the posttranslational regulators: the WD40-protein, Ago, and the Notch-responsive CDKI, Dacapo. Lack of Ago activity or ectopic expression of *dacapo* lead to a halt in cell cycle progression in the transition. Of these two CycE regulators, only Dacapo is transcriptionally downregulated at the transition by activation of the Notch pathway. Therefore, Notch allows the follicle epithelial cells to bypass the G1-S transition by downregulating the critical checkpoint component p21/Dacapo. However, we have shown that CycE oscillation remains critical for endocycling; continuous high level of CycE expression blocks the cell cycle in G2. The regulation of CycE levels is achieved by the function of F-box/WD40-domain protein Ago/hCdc4 that presumably binds to auto-phosphorylated CycE and directs it to SCF-complex degradation: high levels of CycE and no endocycling is observed in *ago*-clones. Interestingly, the function of another G1 cyclin, CycD in growth regulation might be compensated by Myc in endocycles, because we have shown that Myc can affect the kinetics of endocycles. These data show that Notch activity executes the mitotic-to-endocycle transition by regulating independently all three mitotic regulators: downregulating the G2 phosphatase Cdc25/String and the G1 CKI p21CIP/Dacapo and upregulating the APC activator Hec1/Cdh^{Fzr} (Fig. 7B). Repression of String blocks the M-phase, activation of Fzr allows G1 progression and downregulation of Dacapo assures entry into S-phase (Fig. 7A).

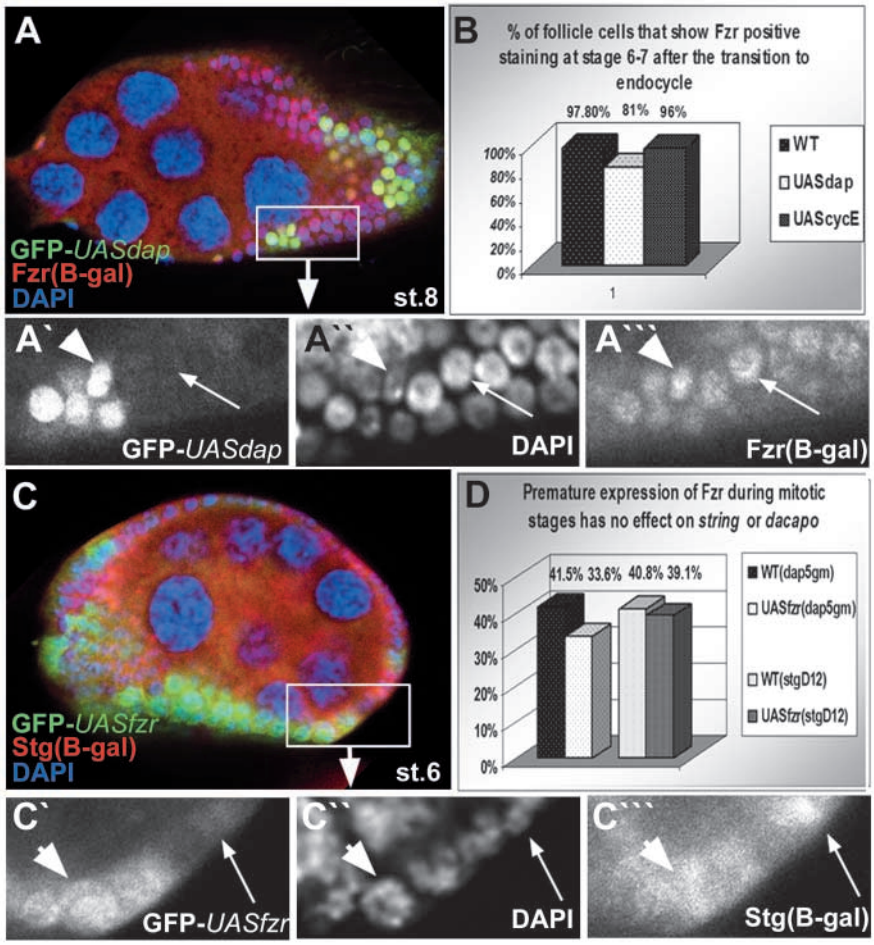

Fig. 6. The Notch pathway regulates Fzr independently from String and Dacapo. (A) Although cells ectopicly expressing *dacapo* (arrowhead) do not undergo endocycles and have smaller nuclei (arrowhead in A″), they express normal levels of Fzr (arrowhead in A‴) compared with wild-type cells (arrow) (*hsFlp/fzrG0326; UAS-dap; UASGFP act<FRT-CD2-FRT<Gal4*). (B) The normal Fzr expression is observed in most cells overexpressing *dacapo* (*hsFlp/fzrG0326; UAS-dap; UASGFP act<FRT-CD2-FRT<Gal4*) or *cyclinE* (*hsFlp/fzrG0326; UAS-cycE/UASGFP act<FRT-CD2-FRT<Gal4*). (C) Premature expression of *fzr* (arrowhead in C′) causes precocious endocycles (large nuclei, arrowhead in C″) but does not affect *string* expression (C‴, arrowhead indicates *UAS-fzr* cells, arrow – wild-type cells). (D) No effect on *string* or *dacapo* expression was observed upon premature expression of *fzr* (the diagram shows that *UAS-fzr* and wild-type follicle cells show close to equal percentage of cells with Dacapo and String staining, *hsFlp; UAS-fzr/UASGFP act<FRT-CD2-FRT<Gal4;6.4-string-lacZ* or *dap5gm*). Green, GFP; red, β-gal; blue, DAPI.

Hec1/Cdh^{Fzr}

The exit from mitosis and/or progression through G1 requires the inactivation of cyclin-dependent kinases, mediated by the APC/C-dependent destruction of cyclins (Sigrist et al., 1995; Sorensen et al., 2001). APC/C is regulated by multiple mechanisms, such as phosphorylation and by spindle checkpoints. Key factors for APC/C function and regulation are the WD proteins Cdc20 and Hec1/Cdh. These proteins seem to bind directly to substrates and recruit them to the APC/C core complex. Importantly, Cdc20 and Hec1/Cdh bind and activate APC/C in a sequential manner during mitosis. APC/C-Cdc20 is activated at the metaphase/anaphase transition, and gets replaced by APC/C-Hec1/Cdh in telophase. This second complex remains active in the subsequent G1 phase.

In *Drosophila* the homologue of Hec1/Cdh, Fzr, also induces the APC/C-complex-dependent proteolysis of CycA and B and is required for the G1-phase progression (Jacobs et al., 2002; Sigrist and Lehner, 1997). Fzr is required for cyclin removal during G1 when the embryonic epidermal cell or follicle epithelial proliferation stops and the cells enter endocycles (Schaeffer et al., 2004; Sigrist and Lehner, 1997).

We now show that premature Hec1/Cdh^{Fzr} transcription in follicle cells is sufficient to block mitosis and initiate precocious endocycling. This suggests that Fzr is a powerful player in the mitotic-to-endocycle switch, yet regulation of other

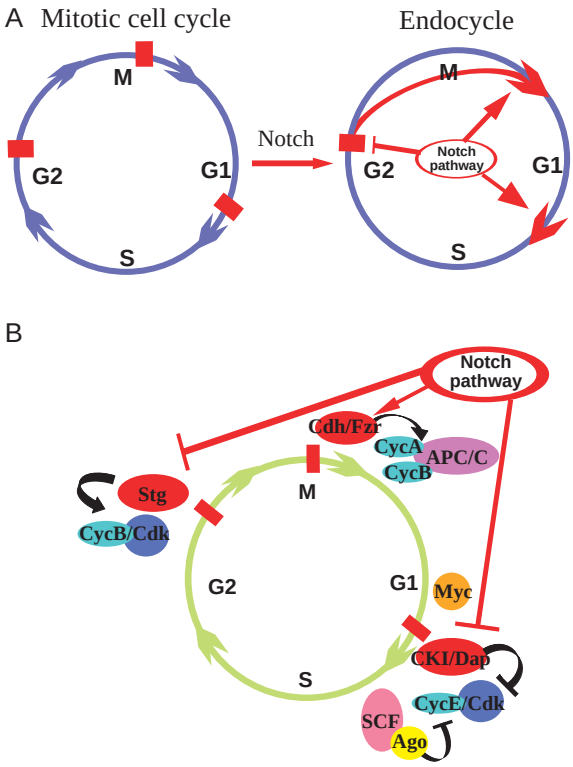

Fig. 7. The mitotic-to-endocycle transition in *Drosophila* follicle cells is executed by Notch-dependent control of cell cycle regulators. (A) A proposed scheme showing that the Notch pathway induces a switch from the mitotic-to-endocycle by controlling the activity of key cell-cycle regulators; cyclin-dependent kinases that control M- and S-phase entry and the APC/ubiquitination complex that regulates degradation of cyclins. Notch activity controls these regulators by downregulating String and Dacapo, and activating Hec1/Cdh^{Fzr}. (B) The molecular model in which Notch activity executes the mitotic-to-endocycle switch by regulating all three major cell cycle transitions. Repression of String blocks M-phase, activation of Hec1/Cdh^{Fzr} allows G1 progression and repression of Dacapo assures entry into S-phase.

components is also required for the efficiency of this process. Regulators of G1-S transition, such as Dacapo/CIP/KIP, which also turns out to be a Notch-regulated component, possibly abort premature attempts by follicle cells to enter the endocycle.

CycD or Myc in growth control

Our data suggest that a component regulating growth and thereby the kinetics of G1/S transition in follicle cell endocycles is the Myc oncogene instead and independent of CycD. In mammals c-Myc controls the decision to divide or not to divide and thereby functions as a crucial mediator of signals that determine organ and body size (Levens, 2003; Trumpp et al., 2001). Interestingly, overexpression of *dmyc* in follicle cells does not affect the mitotic cycles but induces, instead, extra endocycles. Because the timing for entering and exit from the endocycles has not changed, however, increased ploidy is observed, we suggest that the rate of endocycles is increased because of the overexpression of Myc. This finding is in accordance with recent loss-of-function analysis on *myc* in follicle cells, suggesting that *myc* mutant follicle cells can make the transition from mitosis to the endocycle, but that they can only very inefficiently support the endocycle (Maines et al., 2004). Therefore, both loss-of-function and overexpression experiments suggest that Myc is an essential component for the proper rate of endocycles in follicle cells.

Cyclin E in endocycles

In addition to Myc and Cyclin D, Cyclin E also plays an important role in the regulation of the G1/S-transition. Cyclin E binds to and activates the cyclin-dependent kinase Cdk2, and thereby promotes the transition from G1 to S (Knoblich et al., 1994). Oscillation of Cyclin E activity is a mechanism responsible for the timely inactivation of this G1 cyclin/Cdk complex and an arrest in cell proliferation. The oscillation of Cyclin E level is controlled partly by a SCF-ubiquitin-dependent proteolysis (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001; Won and Reed, 1996). Fluctuations of Cyclin E are critical for multiple rounds of endocycles (Follette et al., 1998; Weiss et al., 1998).

Cyclin E is critical for endocycles in follicle cells as well, and our analysis shows that the CycE level is controlled by an SCF-regulator, F-box protein, Ago/hCdc4/Fbw7. Fbw7 (Ago) associates specifically with phosphorylated Cyclin E, and catalyzes Cyclin E ubiquitination in vitro (Koepp et al., 2001). Depletion of Ago leads to accumulation and stabilization of Cyclin E in vivo in human and *D. melanogaster*. This leads to increased mitosis in certain mammalian and *Drosophila* cell types. In addition, *ago* loss-of-function clones in the germ line will cause extra mitotic divisions or, in contrast, cell cycle arrest and polyploidy (Doronkin et al., 2003). However, we have shown that increased Cyclin E levels observed in *ago* loss-of-function mutant clones do not affect the mitotic cycles in follicle cells but do halt the transition to endocycles that normally occurs at stage 6.

Why is the function of Ago/hCdc4/Fbw7 critical to endocycles but not to mitotic cycles in follicle epithelial cells? A potential answer might reside in Dacapo, a CIP/KIP-type inhibitor of Cyclin E/Cdk2 complexes that is regulated in the mitotic to endocycle transition by activation of Notch pathway. We have shown that *dacapo* is downregulated at mitotic-to-endocycle transition because of Notch activation and ectopic expression of *dacapo* represses endocycle progression. It is plausible that during mitotic phases Ago and Dacapo share a redundant role for regulating the Cyclin E activity level, however, *dacapo* is downregulated by Notch pathway at the time of mitotic-to-endocycle transition and at that point Ago gains the critical role of sole regulator of Cyclin E protein activity level. However, downregulation of Dacapo does not readily explain the reduction of CycE levels observed in mitotic-to-endocycle transition (Fig. 3C). We detected elevation of CycE protein level in response to Dacapo overexpression, pointing out that this CKI may stabilize CycE in an inactive form. One possibility therefore is that less CycE protein is observed after the Dacapo downregulation because Dacapo is no longer stabilizing it.

Why is Dacapo downregulated at the time of endocycle transition? Expression of Dacapo is important for proper cell cycle regulation. For example, during vertebrate development, members of the CIP/KIP family of CKIs are often upregulated

as cells exit the mitotic cycle and begin to terminally differentiate. Also, reduced expression of p27Kip1 was frequently shown to correlate with a poor prognosis in various cancers (Fredersdorf et al., 1997; Geisen et al., 2003), and in the absence of p21, DNA-damaged cells arrest in a G2-like state, but then undergo additional S-phases without intervening normal mitoses. They thereby acquire grossly deformed, polyploid nuclei and subsequently die through apoptosis (Waldman et al., 1996). Also, p21 elimination causes centriole overduplication and polyploidy in human hematopoietic cells (Mantel et al., 1999). In the *Drosophila* germ line Dap is differentially regulated in the nurse cells versus the oocyte. High Dap levels in the oocyte are critical to the maintenance of the prophase I meiotic arrest and ultimately to later events of oocyte differentiation, and in the nurse cells the oscillations of Dap drive the endocycle (Hong et al., 2003). In contrast to all these examples, in endocycling follicle cells reduction of p21/Dacapo is a requirement for normal endocycle progression. Similarly, in a megakaryocytic cell line, differentiation is correlated with a downregulation of p27 (Fredersdorf et al., 1997). We propose that the downregulation of Dacapo is a reasonable strategy to bypass the G1/S transition and to enter endocycling when mitosis is not completed, however, how these endocycling cells escape possible centrosome amplification and apoptosis that could be consequences of the lack of Dacapo/p21-activity is not clear. This diversity in the processes, that allow cells to exit from mitotic cell cycle, is generating or representing regulatory multiplicity that might be reflected in the ways eukaryotic cells acquire tumor formation capacity.

Notch as a tumor suppressor

Recent findings by Rangarajan et al. and Nicolas et al., (Nicolas et al., 2003; Rangarajan et al., 2001) have shown that Notch acts as a tumor suppressor in mouse skin epithelium. Ablation of Notch results in epidermal and corneal hyperplasia followed by the development of skin tumors and facilitated chemical-induced skin carcinogenesis. In these cell types Notch1 deficiency results in increased and sustained expression of Gli2 and derepression of beta-catenin. Therefore the authors have suggested, that in mouse skin epithelium Notch pathway represses the activity of Hedgehog- and Wingless-signaling pathways. It remains to be seen whether Notch activity in *Drosophila* follicle cells impinges directly on the transcriptional regulation of *string*, *dacapo* and *fzr* or whether Notch acts through another signaling pathway.

Our studies of follicle cell cycle programs in the ovary are important because they provide a comparison with other cell cycle programs in *Drosophila* development. Furthermore, the transition from mitotic cycles to endocycles is a universal phenomena; understanding how molecular events bring about this transition in follicle cells will shed light on such transitions in general.

We thank Floyd Kregenow and Brian Kennedy for helpful comments on the manuscript. We also thank Andrew Berger for assistance with FACS analysis (Fred Hutchinson Cancer Research Center, Flow Cytometry Facility), and B. Edgar, M. Lily, E. Giniger, C. Lehner, T. Orr-Weaver, G. Bosco, R. Eisenman, H. Vaessin, S. Blair, the Bloomington Stock Center, and Developmental Studies Hybridoma Bank for fly-lines and antibodies. This work was supported by grants from the National Institutes of Health, American Heart Association, American Cancer Society and Pew Memorial Trust and Pew Scholarship in the Biomedical Sciences to H.R.-B.

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