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# Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in *Drosophila* follicle cells

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## SUMMARY

**In many developmental processes, polyploid cells are generated by a variation of the normal cell cycle called the endocycle in which cells increase their genomic content without dividing. How the transition from the normal mitotic cycle to endocycle is regulated is poorly understood. We show that the transition from mitotic cycle to endocycle in the *Drosophila* follicle cell epithelium is regulated by the Notch pathway. Loss of Notch function in follicle cells or its ligand Delta function in the underlying germline disrupts the normal transition of the follicle cells from mitotic cycle to endocycle, mitotic cycling continues, leading to overproliferation of these cells. The regulation is at the transcriptional level, as Su(H), a downstream transcription factor in the pathway, is also required cell autonomously in follicle cells for proper transitioning to the endocycle. One target of Notch and Su(H) is likely to be the G2/M cell cycle regulator String, a phosphatase that activates Cdc2 by dephosphorylation. String is normally repressed in the follicle cells just before the endocycle transition, but is expressed when Notch is inactivated. Analysis of the activity of String enhancer elements in follicle cells reveals the presence of an element that promotes expression of String until just before the onset of polyploidy in wild-type follicle cells but well beyond this stage in Notch mutant follicle cells. This suggests that it may be the target of the endocycle promoting activity of the Notch pathway. A second element that is insensitive to Notch regulation promotes String expression earlier in follicle cell development, which explains why Notch, while active at both stages, represses String only at the mitotic cycle-endocycle transition.**

Key words: *Drosophila*, Notch, Delta, Cell cycle, Follicle cells

## INTRODUCTION

The Notch signaling pathway is evolutionarily conserved and is widely used in cell fate determination and cell differentiation (Artavanis-Tsakonas et al., 1999; Bray, 1998; Greenwald, 1998). In humans, defects in this pathway are implicated in multiple diseases, including leukemia and heart disease (Joutel and Tournier-Lasserve, 1998; Selkoe, 2000). The basic components of the pathway are the Notch receptor, the two Notch ligands Delta and Serrate (Delta and Jagged in vertebrates), the transcription factor Suppressor of Hairless (Su(H); CBF1/RJBk in mammals) and the bHLH transcription factors encoded by the *Enhancer of Split* complex genes *E(spl)*. In addition to differentiation, the Notch pathway also regulates some aspects of growth control (Kimble and Simpson, 1997; Artavanis-Tsakonas et al., 1999). In the developing *Drosophila* wing, for example, Notch induces cell cycle arrest indirectly through modulating the function of Wingless, a secreted signaling molecule, in the nonproliferative boundary zone (Johnson and Edgar, 1998). Other evidence suggests that the cell cycle function of Notch in the wing is more direct (Baonza and Garcia-Bellido, 2000). No direct molecular link, however, has been made between Notch and cell cycle regulation.

During *Drosophila* oogenesis, the Notch pathway plays multiple roles in specifying follicle cell groups, the terminal follicle cells, dorsal anterior follicle cells and polar cells (Ruohola et al., 1991; Xu et al., 1992; Bender et al., 1992; Larkin et al., 1996; Keller Larkin et al., 1999; Gonzalez-Reyes and St Johnston, 1998; Jordan et al., 2000). Our analyses of Notch function in the ovary have revealed a connection between Notch signaling and regulation of a transition from a mitotic cell cycle to an endoreplication cycle (this study). The endoreplication cycle, or endocycle, is a variation of the normal mitotic cell cycle, in which cells increase their genomic DNA content without dividing. The endocycle is widespread among multicellular organisms and uses much of the same G1/S regulatory machinery as mitotic cycles. The mechanism responsible for skipping over M phase during the endocycle is the subject of much current investigation (Edgar and Orr-Weaver, 2001). The follicle cells in the *Drosophila* ovary offer an excellent system in which to study the mitotic-to-endocycle transition. The *Drosophila* germline is surrounded by an epithelial follicle cell layer. The follicle cells undergo multiple rounds of mitotic divisions during the early stages of oogenesis. At stage 6, these cells switch from the normal mitotic cycle and undergo three rounds of endocycles. After

the endocycles, most DNA is no longer replicated except the chorion genes, which continue to be amplified in a specific, tightly regulated fashion (Royzman and Orr-Weaver, 1998).

String is a *Drosophila* Cdc25-type phosphatase that triggers mitosis by dephosphorylating and thereby activating the Cdk1/Cyclin B kinase (Edgar et al., 1994). Differential expression of *string* regulates mitosis during most stages of *Drosophila* development: loss-of-function mutations in the gene cause G2 arrest in both embryos and imaginal disc cells (Edgar and O'Farrell, 1989; Neufeld et al., 1998), whereas ectopic *string* expression in both embryo and discs drives G2 cells into mitosis (Edgar and O'Farrell, 1990; Neufeld et al., 1998; Johnston and Edgar, 1998). These results suggest that String is rate limiting for mitotic initiation and therefore regulation of *string* expression is a key in controlling mitosis. The abundance of String protein is mainly controlled at the transcriptional level. Moreover, mutations in many patterning genes alter string expression in specific ways, indicating that *string* transcription is controlled by the same network of factors that controls cell fates (Arora and Nüsslein-Volhard, 1992; Edgar et al., 1994; Johnston and Edgar, 1998). The large upstream control region of the *string* gene has been dissected and shown to consist of many modular elements with separable activities (Lehman et al., 1999). These elements pattern mitoses in the embryo and in neural lineages at many stages of development. In some cases *Drosophila* String is functionally redundant with another Cdc25 homolog, Twine (Edgar and Datar, 1999). In addition to transcriptional regulation, String protein turnover and Twine translational levels can affect cell cycle transition (Mata et al., 2000; Seher and Leptin, 2000; Grosshans and Wieschaus, 2000; Maines and Wasserman, 1999).

We show that the transition from mitotic cycle to endocycle in the *Drosophila* follicle cell epithelium is regulated by the Notch pathway. Loss of Notch or its downstream transcription factor Su(H) in follicle cells, or its ligand Delta in the underlying germline, disrupts the normal transition of the follicle cells from mitotic cycle to endocycle, leading to overproliferation of these cells. A target of Notch and Su(H) is likely to be the G2/M cell cycle regulator *string*, as *string* is normally repressed in the follicle cells just before the endocycle transition but is expressed when Notch is inactivated. Analyses of the activity of *string* enhancer elements in follicle cells reveal the presence of an element that promotes expression of *string* until just before the onset of polyploidy in the wild-type follicle cells, but much beyond that in egg chambers that are lacking germline Delta. These results suggest that the *string* gene may be at least one of the targets of the endocycle-promoting activity of the Notch pathway.

## MATERIALS AND METHODS

### Fly stocks

The following fly-stocks were used: *$N^{55e11}$FRT101* (*$N^{55e1}$* is an amorphic allele of *Notch*, gift from the laboratory of Y. N. Jan), *FRT82B $Dl^{rev10}$ e* (*$Dl^{rev10}$* 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), *FRT82B Df(3R)$gro^{b32.2}$P[gro$^+$]E8* (this combination removes all seven bHLH-encoding genes of E(spl)-C but provides a wild-type copy of *groucho*) (Heitzler et al., 1996), *$fng^{13}$ FRT 80B* (*$fng^{13}$* is a strong loss-of-function allele of *fringe*) (Irvine and Wieschaus, 1994), *$lgl^4$ FRT40A* (*$lgl^4$* is a null allele) (Mechler et al., 1985; Woods and Bryant, 1991), *FRT82B $stg^{7B,}$ STG-15.3; FRT82B $stg^{7B}$* (*$stg^{7B}$* is a strong loss-of-function allele of *string*) (Lehmann et al., 1999), *$Su(H)^{SF8}$ FRT40A (Su(H)$^{SF8}$* is a strong loss-of-function (but not a null) allele of *suppressor of hairless*, a gift from S. Blair), *hs FLP; +/+; Ubi-GFP FRT 80B*, *hs FLP; +/+; FRT 82B Ubi-GFP* and *yw Ubi-GFP FRT 101; MKRS P{ry=hs FLP}86E / TM6B Tb.* String 15.3 kb and 31.6 kb transgenes were tested for function in follicle cells using the following flies: *hs-FLP122/+; P[w$^+$STG-15.3]/+; FRT82B $stg^{7B}$/FRT82B Ubi-GFP* and *hs-FLP122/+; P[w$^+$STG-31.6]/+; FRT82B $stg^{7B}$ /FRT82B Ubi-GFP.* The *string-lacZ*-fusion (Table 2 and Fig. 6A) and *string*-rescue transgene constructs were a kind gift from the laboratory of B. Edgar (Lehmann et al., 1999). Multiple insertion lines were analyzed when available.

### 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 with new males for 2 or 3 days. To obtain germline clones, flies were heat shocked as second and third instar larvae for 2 hours on 2 consecutive days. Once they emerged as adults, females were placed in vials with fresh yeast paste for 1-5 days with young males in preparation for dissection.

### Staining procedures

Ovaries were dissected in 1× phosphate-buffered saline (PBS) and fixed shaking for 20 minutes in 200 µl of 4% paraformaldehyde in PMES (0.1 M Pipes pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA) with 0.5% NP40 and 600 µl of Heptane. They were rinsed for 5 minutes with NP40 wash (50 mM Tris 7.4, 150 mM NaCl, 0.5% NP40, 1 mg/ml bovine serum albumin (BSA), 0.2% sodium azide) and blocked in 20% goat serum (in NP40 wash) for 2 hours at room temperature. The tissue was incubated with primary antibodies overnight at 4°C. The next day they were rinsed with NP40 wash four times, for 20 minutes each. They were then incubated with a secondary antibody for 2 hour at room temperature and stained with DAPI (1 µg/ml in NP40 wash), washed three times with NP40 wash (20 minutes each) and dissected onto slides in 70% glycerol, 2%NPG, 1×PBS. Confocal microscopy, X-gal staining and in situ hybridization was performed as described previously (Keller Larkin et al., 1999; Tworoger et al., 1999). *stg* cDNA (a gift from B. Edgar) was labeled with digoxigenin. A two-photon laser-scanning microscope (Leica TCS SP/MP) was used in this study.

The following antibodies were used: mouse anti-Armadillo (1:20, Developmental Studies Hybridoma Bank), mouse anti-FasciclinIII (1:20, Developmental Studies Hybridoma Bank), mouse anti-Cyclin B (1:20, Developmental Studies Hybridoma Bank), mouse anti-Notch intracellular domain (1:20, Developmental Studies Hybridoma Bank), mouse or rabbit anti-β-gal (1:5000, Sigma), mouse anti-Broad-Complex Z1 (1:50, a gift from G. Guild), mouse anti-Delta (1:100) (Bender et al., 1993), rabbit anti-PH3 (1:200, Upstate Biotechnology), Alexa 488, 568 or 633 goat anti-mouse (1:500) and Alexa 568 or 633 goat anti-rabbit (1:500) (Molecular Probes).

## RESULTS

### Follicle cell cycle

Follicle cells are descendents of somatic stem cells located in region 2 of the germarium (Fig. 1A) (Margolis and Spradling, 1995). In region 3 of the germarium, the follicle cells encapsulate the germline cyst, which contains 15 nurse cells and an oocyte to form a stage 1 egg chamber that then exits from the germarium (stage 1). The follicle cells carry out a mitotic division program from stage 2 to 6 (~30 hours), giving rise to approximately 1000 follicle cells. At stage 6, follicle cells cease

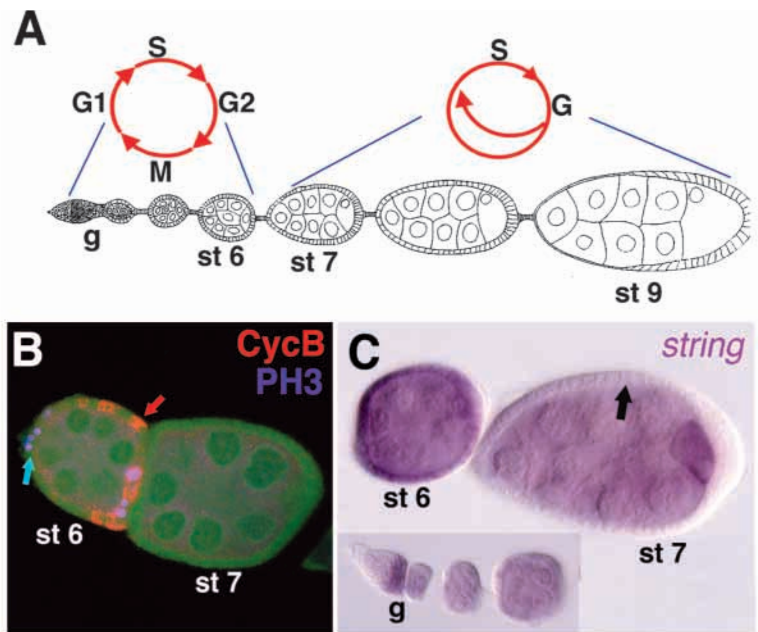


**Fig. 1.** Follicle cells in *Drosophila* oogenesis undergo a transition from a mitotic cell cycle to an endocycle. (A) Drawing of the stages of oogenesis. From the germarium (g) to stage (st) 6, somatically derived follicle cells undergo mitotic cell cycle. At stage 7 they switch to endocycles. From stage 7 to stage 10A, these cells undergo three rounds of endoreplication. (B) Staining of mitotic markers Cyclin B (CycB, red, red arrow) and Phospho-Histone 3 (PH3, blue, blue arrow) of two egg chambers at stages 6 and 7, showing the transition from mitotic cell cycle to endocycle at these two stages. (C) Whole-mount in situ hybridization shows that *string*, which encodes a Cdc25 phophatase, is expressed in follicle cells from the germarium to stage 6. *string* expression in follicle cells is turned off at stage 7 (arrow), suggesting that the transition from mitotic cell cycle to endocycle is regulated at the transcriptional level.

mitosis and undergo endocycles (also called endoreplication or endoreduplication cycle) in which the mitotic stage of the cell cycle is bypassed (Fig. 1A). This cell cycle transition can be visualized with anti Phospho-Histone3 (PH3) (Bradbury, 1992; Handzel et al., 1997) and anti-Cyclin B (CycB, a G2 cyclin) antibodies (Fig. 1B). Until stage 6, phosphorylation of Histone3 (indicative of mitosis) and expression of CycB is observed in the follicle cells, while no obvious staining is detected after stage 6 (Fig. 1B). The expression of CycB is observed in groups of 5-10 follicle cells (Fig. 1B), suggesting that mitosis in the follicle cell layer is controlled in small domains, reminiscent of mitotic domains observed in early embryos. However, it is not clear yet how synchronized these domains are, what controls their size and whether the positions of the domains are sporadic or developmentally controlled. Data obtained by flow sorting of follicle cell nuclei show that follicle cells undergo three rounds of endocycles, giving rise to 16 copies of genomic DNA (Lilly and Spradling, 1996). BrdU-labeling of stage 7-9 egg chambers demonstrates that the timing of endocycles is not synchronized among follicle cells within an egg chamber (Calvi et al., 1998). By the end of stage 10A, all follicle cells complete their overall endoreplication program but continue chorion gene amplification (Spradling, 1993; Royzman and Orr-Weaver, 1998; Bosco et al., 2001).

The abrupt and relatively synchronous transition from mitotic cycle to endocycle makes the follicle cell epithelium an excellent model system to study this cell cycle control process. In other cell types, the mitotic-to-endoreplication switch involves transcriptional repression of G2/M regulators such as *CycA, CycB* or *string*, which encodes a CDC25 phosphatase (Edgar and Orr-Weaver, 2001). We therefore analyzed transcription of *string* in oogenesis using whole-mount in situ hybridization. *string* expression is detected in a patchy manner in mitotic follicle cells (Fig. 1C). Downregulation of *string* expression in follicle cells was observed at stage 7 (Fig. 1C, arrow), suggesting that the transition from mitotic cell cycle to endocycle is regulated in part by repression of *string* at the transcriptional level.

### Delta from the germline is required for the mitotic to endocycle transition in follicle cells

To investigate the possibility that the Notch pathway is involved in the regulation of the mitotic cycle-to-endocycle transition, we first analyzed the expression of the Notch ligand, Delta, in oogenesis. Weak Delta expression is observed in follicle cells and germline from the germarium to stage 5 in oogenesis. At around stage 6, a dramatic upregulation of Delta expression is observed in the germline cells reaching the highest level at stage 7 (Fig. 2A) (Bender et al., 1993; Keller Larkin et al., 1999). This upregulation of Delta in the germline coincides with the transition from mitotic cell cycle to endocycle in the follicle cells (Fig. 2A, note the lack of PH3 in the egg chamber with high germ line Delta at stage 7).

To determine if Delta plays a role in regulating the mitotic-to-endocycle-transition, we undertook a mosaic analysis. We employed mitotic recombination (Xu and Rubin, 1993) to create clones of cells lacking Delta function, then analyzed cell cycle stage by examining PH3 and CycB. The mutant cell clones were marked by lack of green fluorescence protein (GFP) expression, while the wild-type cells were labeled by GFP. As wild-type follicle cells lack PH3 and CycB after stage 6, owing to the transition into the endocycle, we analyzed the expression of these markers in follicle cells adjacent to Delta germline clones after stage 6. This analysis revealed defects in cell cycle regulation in follicle cells surrounding the Delta germline clones. In almost all cases (99%, $n$=68), follicle cells surrounding the Delta germline clones expressed PH3 and CycB beyond stage 7, suggesting that these cells remained in the mitotic cell cycle program (Fig. 2C,D; Table 1). Although dividing, these follicle cells appeared to maintain their apical-basal polarity, as shown by normal apical localization of Armadillo, a β-catenin homolog in *Drosophila* (Fig. 2C) (Peifer et al., 1993) (some abnormalities were observed in later stages). In Delta follicle cell clones, no PH3 and CycB staining was detected in follicle cells after stage 6 (Fig. 2B; Table 1 and data not shown), suggesting that germline but not follicle cell contribution of Delta is essential for the follicle cell mitotic

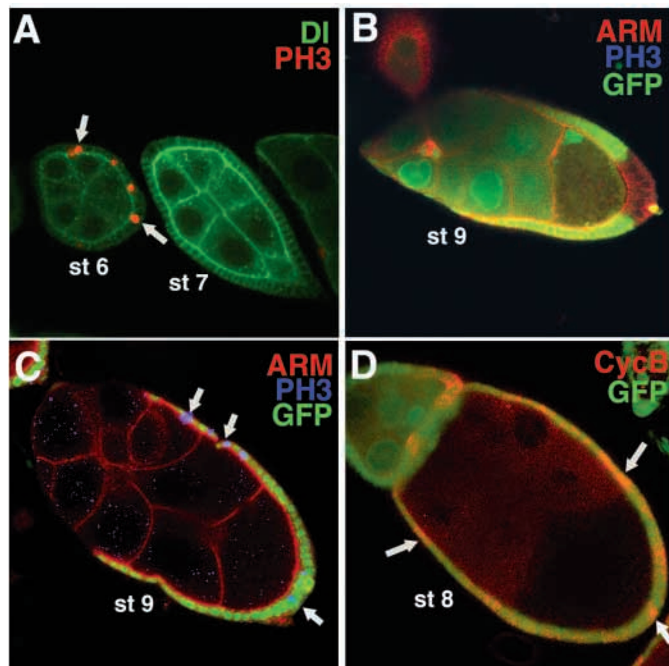


**Fig. 2.** Delta signals from the germline cells to regulate the transition from mitotic cycle to endocycle in follicle cells. (A) *Delta* expression is upregulated at stage 6 and reaches its highest at stage 7 in the germline cells (green). This upregulation is coincident with the cell cycle transition (Phospho-Histone 3 staining in red is observed in stage 6 (arrows), but not in stage 7 egg chambers). (B) Delta is not required in the follicle cells for the transition, as no extra cell divisions were observed in $Dl^{rev10}$ follicle cell clones (PH3, blue in B). (C,D) Delta is required in the germline, as Phospho-Histone 3 (PH3, blue in C, arrows) and Cyclin B (Cyc B, red in D, arrows) are observed in follicle cells of stage 8 and 9 egg chambers carrying germline clones of *Delta*. Green in B-D shows GFP, which marks the wild-type cells, and cells that lack GFP expression are mutant clones. Epithelial polarity is normal in these egg chambers based on normal Armadillo localization (red in B,C). In later egg chambers, some epithelial defects were observed (data not shown).

cycle transition into the endocycle program. These results differ from earlier studies (Ruohola et al., 1991), which failed to detect a role for Delta in the germline in regulating follicle cell division, because germline clones were made with a weaker allele and/or the extent of proliferation of the follicle cells was not analyzed because of a lack of suitable markers.

### Notch is required in follicle cells in a cell-autonomous manner for the mitotic-to-endocycle transition

As Delta signal from the germline is required for the mitotic-to-endocycle transition in follicle cells, we tested whether Notch is the receptor for the signal in the follicle cells. Notch is predominantly localized to the apical side of the follicle cell epithelium (facing the germline cells; Fig. 3A) (Xu et al., 1992), making it a possible candidate to receive Delta signal from the germline. We generated Notch mutant follicle cell clones to analyze Notch function in the transition by inducing mutant clones in the adult flies and allowing development to proceed at 25°C for 2 days. Many smaller nuclei were observed in Notch follicle cell clones (Fig. 3B) compared with the

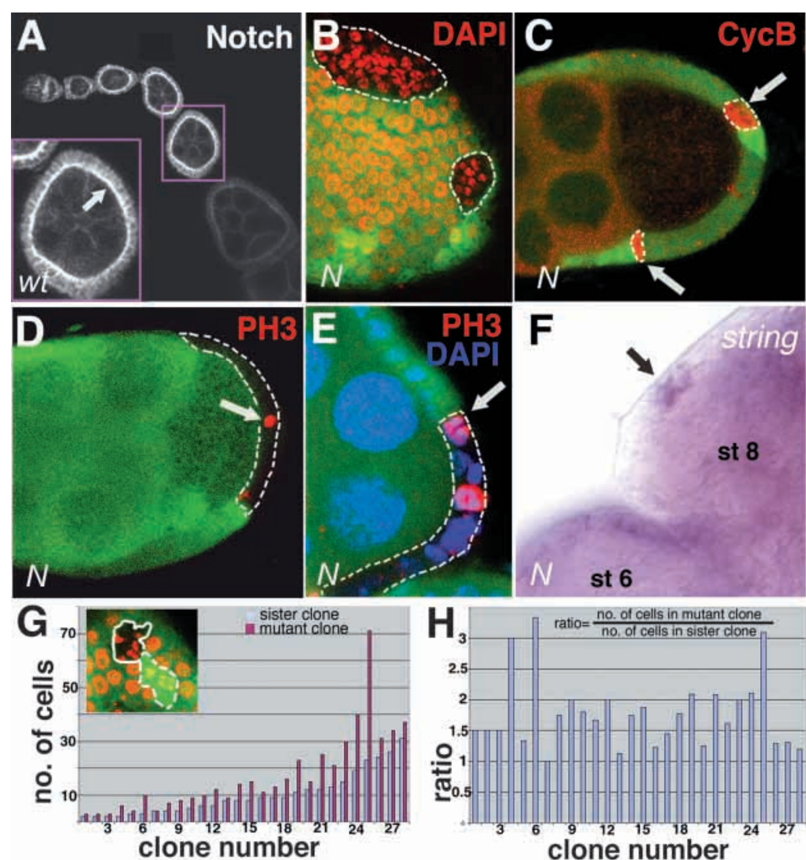


**Fig. 3.** Notch is required in the follicle cells for the mitotic-to-endocycle transition. (A) Notch protein is observed in the apical surface of the follicle cell layer in wild-type egg chambers. (B) Cells in Notch clones (black, outlined by broken white line) have smaller nuclei than their wild-type neighbors. This most probably indicates lack of endocycles in the mutant clones. (C-E) Mitotic markers (PH3, CycB) are observed in cells in Notch mutant clones at stage 8-9, indicating a failure to enter the endocycle. (F) *string* expression can still be observed at stage 8 in egg chambers with *Notch* mutant clones. In this case, the mutant clone is unmarked. (G) Quantitation of the number of cells in *Notch* mutant clones (dark purple bar, the black clone in the figure is marked with an unbroken white line) compared with the sister clones (light purple bar, bright green in the figure marked with broken white line). The results are plotted as a function of sister clone size. (H) Ratio of the size of mutant clone per the size of sister clone in G plotted as a function of sister clone size. More cells are observed in the mutant than in sister clones (mean ratio=1.8). Importantly, the ratio does not change as a function of clone size, suggesting that the defect in *Notch* clones only occurred after the normal mitotic stage. Notch clones marked with broken lines in B-D and with an unbroken line in G.

**Table 1. A defect in the Notch pathway results in follicle cell overproliferation**

| Mutant | Follicle cell clones* (CycB- or PH3-positive clones/clones counted) | Germline clones* (CycB- or PH3-positive clones/clones counted) |
|---|---|---|
| $Dl^{rev10}$ | 0/34 (0%) | 67/68 (99%) |
| $N^{55e11}$ | 61/65 (94%) | NA |
| $fng^{13}$ | 0/78 (0%) | 0/25 (0%) |
| $Su(H)^{SF8}$ | 35/46 (76%) | NA |
| $E(spl)^{b32.2}$ | 0/65 (0%) | 0/28 (0%) |

*Stage 8-9 egg chambers were used in this analysis.
NA, not analyzed.

polyploid wild-type neighbors. Furthermore, PH3 and CycB expression were detected in Notch follicle cell clones in a cell autonomous manner after stage 6 (94%, *n*=65), suggesting that these cells remain in a mitotic cell cycle (Fig. 3C-E; Table 1). These results show that Notch acts cell autonomously to regulate the transition from mitotic cycle to endocycle in follicle cells.

If the follicle cells in Notch mutant clones proceed through full mitotic cell cycles, more cells should be observed in mutant clones than in twin spots carrying wild-type copies of the *Notch* gene (sister clone). We tested this prediction by counting the number of cells in mutant and sister clones. More cells were observed in the mutant clones than in the associated twin spots (Fig. 3B,G,H, the ratio varied from 1 to 3.3, mean=1.8), suggesting that the mutant cells have undergone more mitotic cycles than their endocycling sister clones. In addition, as expected, the DNA content was higher in sister clones than in mutant clones, based on DAPI staining, and the wild-type cells were larger on average than the mutant cells (Fig. 3B). Importantly, the ratio between the cell number in mutant and sister clones did not change as a function of the clone size (Fig. 3G,H; G is plotted as a function of increasing sister clone size, H is the mutant:sister ratio of G as a function of increasing sister clone size), suggesting that there are no defects in division in the Notch mutant before the transition to the endocycle. Therefore the cells in Notch mutant clones divide at a normal rate before the endocycle switch.

The glycosyltransferase Fringe is a modifier of the Delta-Notch interaction in imaginal discs (Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000; Blair, 2000), and in oogenesis of earlier Notch-dependent processes that allow pinching off to occur in the germarium and define the number of polar cells (Jordan et al., 2000; Zhao et al., 2000; C. A., W.-M. D., K. Jordan and H. R.-B., unpublished). When Fringe is present in Notch-expressing cells, Notch can be activated by low levels of Delta expression. Because Delta expression is upregulated in stage 6 germline cells, we suspected that Fringe would not be required for Delta-Notch signaling in this later case. Indeed, no CycB or PH3 staining was detected after stage 6 of oogenesis in *fringe* mutant clones (Table 1), suggesting that Fringe is not essential in the Delta-Notch interaction that regulates the transition from mitotic cycle to endocycle in follicle cells.

## Su(H) but not E(spl) is required for cell cycle control

Does Notch exert its effect on the mitosis-endocycle transition through transcriptional regulation? Suppressor of Hairless (Su(H)) is a transcription factor that interacts with the cleaved-Notch-intracellular domain after Delta binds to the Notch extracellular domain. This interaction leads to transcription of downstream target genes, including *Enhancer of Split* (*Espl*) complex genes. We made follicle cell clones of a *Su(H)* hypomorphic allele and *E(spl)* null allele, then probed the egg chambers with anti-PH3 and CycB antibodies. As in *Notch* follicle cell clones, small nuclei, PH3 and CycB were observed in *Su(H)* clones (Fig. 4A-C; Table 1; 76%, *n*=46). No extra cell division was detected, however, in follicle cell clones mutant for *E(spl)* (Fig. 4D; Table 1). These results suggest that Notch and Su(H) regulate the follicle cell mitotic-to-endocycle transition but do not act through the *E(spl)* genes.

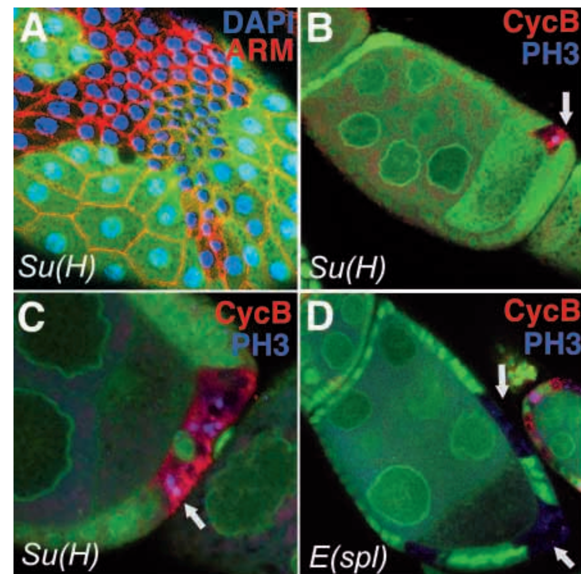


**Fig. 4.** Supressor of Hairless (Su(H)) but not E(spl) is required in the follicle cells for exit from the mitotic cycle. (A) In this stage 12 egg chamber, $Su(H)^{SF8}$ mutant cells (no green GFP) are smaller compared with the wild-type cells marked by nuclear GFP (green). Armadillo staining (red) outlines the cell boundaries while DAPI, blue, labels all cells. (B,C) In these stage 8 egg chambers, CycB (red) and PH3 (blue), indicate additional mitoses in $Su(H)^{SF8}$ clones (arrows). (D) The *Enhancer of Split* (*E(spl)*) gene complex is not required in follicle cells to regulate the mitotic-to-endocycle transition, as no CycB or PH3 staining was observed in *E(spl)* mutant clones after stage 8 (mutant clones are black, arrows).

## Polarity and differentiation in the *Notch* mutant follicle cells

In *Drosophila*, mutations in three neoplastic genes, *discs large* (*dlg*), *lethal(2)giant larvae* (*lgl*) and *scribble* (*scrib*), cause loss of apical-basal polarity accompanied by hyperproliferation (Jacob et al., 1987; Woods and Bryant, 1991; Bilder and Perrimon, 2000; Bilder et al., 2000). It is not understood how loss of apical-basal polarity results in hyperproliferation in these mutants. One attractive hypothesis, however, is that overproliferation is caused by mislocalization of critical plasma membrane proteins that are involved in cell cycle control. We found that the Notch protein was indeed mislocalized throughout *lgl* mutant cells (Fig. 5D); this defect could reduce responsiveness to Delta from the germline and thus lead to overproliferation. In addition, many *lgl* mutant follicle cells existed in multilayered structures where they were

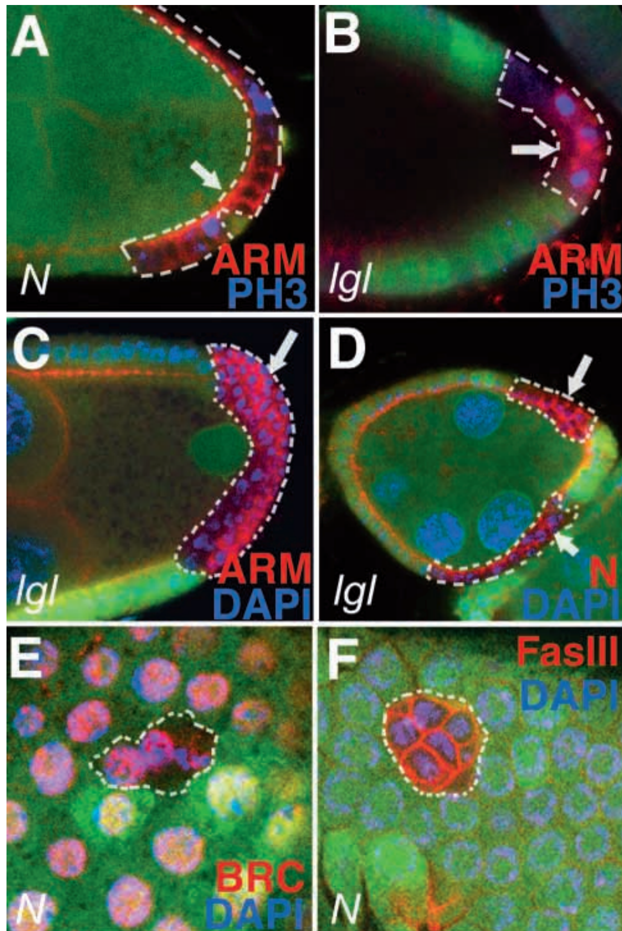


**Fig. 5.** Comparison of the phenotypes of *Notch* and *lgl*, a tumor-suppressor gene. (A) In *Notch* mutant clones, follicle cell apical-basal polarity appears to be normal, as shown by localization of the apical marker, Armadillo, a *Drosophila* homolog of β-catenin (red). PH3 (blue) indicates additional mitosis. (B) In a *lgl* mutant clone, additional cell division is also observed after stage 6 (PH3, blue), but is accompanied by loss of follicle cell apical-basal polarity, as shown by mislocalization of Armadillo in the clone (red). (C) *lgl* mutant clones frequently lead to the formation of multiple layers of follicle cells. (D) Notch protein (red) localization in the apical side of the follicle cells is disrupted in *lgl* clones (arrows). (E) In small *Notch* clones, *Notch* mutant cells can still differentiate, as shown by expression of Broad-Complex proteins, normally observed in all follicle cells beginning at around stage 6. In *Notch* clones, BR-C expression persists, as in the wild-type neighbors. (F) In small Notch clones, several markers, including Cadherin, Armadillo, Disks-lost and Fasciclin III (F; FasIII, red) are upregulated. In all panels, clones are outlined with a broken white line.

less likely to receive the Delta signal because of lack of direct contact with the germline (Fig. 5C, arrow). By contrast, in *Notch* mutant follicle cells, the cell cycle defect was observed before any obvious polarity defect; normal Armadillo localization was observed in *Notch* but not in *lgl* clones (Fig. 5A,B). Additional factors probably also contributed to the overproliferation in *lgl* mutant clones because significantly more proliferation was observed in *lgl* than in *Notch* clones (compare Fig. 5A with B,C).

Notch is known to act on follicle cell differentiation multiple times during oogenesis. To test whether Notch acts primarily on follicle cell differentiation at stage 6, we used a marker that begins to be expressed in all follicle cells at around stage 6, the Broad-Complex (BR-C) (Deng and Bownes, 1997; Tzolovsky et al., 1999). BR-C was expressed at similar levels in *Notch* mutant follicle cell clones and in wild-type follicle cells (Fig. 5E), suggesting that at least on this level differentiation at stage 6 occurred in a normal manner. However, some markers showed elevated expression in the mutant clones compared with the wild-type cells neighboring the mutant cells. One marker, Fasciclin III, is normally expressed in all cells at early stages of oogenesis and then later is restricted to the polar cells (Ruohola et al., 1991). We observed elevated levels of FasIII (Fas3 – FlyBase)in a cell-autonomous fashion in *Notch* clones (Fig. 5F). Although elevated expression of FasIII may indicate potential differentiation defects in *Notch* mutant clones, there is also an alternative explanation. Normally, high FasIII expression occurs up until stage 1 in oogenesis, yet we observed elevated levels of FasIII in two-cell clones that were generated at stage 4-5 in oogenesis. In addition, many other markers that have no general early expression were elevated in the clones. One possible explanation for these expression abnormalities is that the highly mitotically active cells in *Notch* clones show elevated translational activity compared with the neighboring wild-type cells. More experiments are required to distinguish between these possibilities.

## Notch acts on the cell cycle transition through String

Mitosis in most *Drosophila* cells is triggered by brief bursts of transcription of *string*, which encodes a Cdc25-type phosphatase that activates the mitotic kinase, Cdk1 (Cdc2). Transcriptional control is an important mode of regulation for the activity of this G2/M controller. Fig. 1C shows that *string* is expressed in a patchy fashion when follicle cells are in the mitotic cell cycle but repressed when the follicle cells are in the endocycle. This repression requires Notch activity, as *string* mRNA is observed in egg chambers with Notch clones beyond the normal stage 6 in oogenesis (Fig. 3F).

To test whether String is essential for follicle cell mitotic cycles, we generated *string* mutant clones. The mutant clones had far fewer cells than sister clones (zero to two cells in mutant clones, 3-15 in sister clones, $n$=24, mutant:sister=0.1; Fig. 6F,H) and showed no expression of G2/M stage markers, suggesting that String is required for the follicle cell mitotic cycle. In control experiments, the mutant:sister ratio was 1.0 ($n$=10).

Previous studies have revealed that many *cis*-acting elements distributed over >30 kb upstream of *string* that control *string* transcription in different cells and tissue types (Lehman et al., 1999). To map the transcriptional control elements that regulate *string* in follicle cells, we took advantage of gene-fusion constructs in which the *string* locus was dissected into fragments and fused to *lacZ* reporter genes containing the basal 0.7 kb *string* promotor (Lehman et al., 1999). Of 11 different gene fusions tested, two showed specific expression in follicle cells. These constructs define the regions of the *string* promoter that are required for *string* expression and therefore for controlling the cell cycle in follicle cells (Table 2). Interestingly, R4.9 (4.9 kb region) exhibited expression in follicle cells of the germarium to around stage 2 in oogenesis, while R6.4 (6.4 kb region) showed expression beginning in

stage 3 and abruptly terminating at the mitotic-to-endocycle transition in stage 6 (Fig. 6B-E; Table 2).

If the two enhancer elements (4.9 and 6.4; Fig.6A) are required for controlling two different bursts of cell divisions in follicle cells, then a rescue construct that covers only the first element should only partially rescue the division defects in *string* clones. That is exactly what we observed: when *string* clones were produced in the background of the 15.3 kb rescue construct (see Fig. 6A), mutant clones of half the size of sister clones were detected (one to six cells in mutant clones, 2-15 in sister clones, $n$=21, mutant:sister=0.49). This result suggests that the 4.9 kb element completely contained within the 15.3 kb rescue construct supports only the early expression and rescues the first but not the second burst of cell divisions in the follicle cell layer (Fig. 6G,I). Interestingly, the size of most of the nuclei in the clones was twice that of the nuclei typically observed in the sister clones or in the neighboring cells (Fig. 6G; 77%; wild-type nuclear volume/mutant nuclear volume=2.2), possibly suggesting an extra endoreplication cycle. After the action provided by the first *string* promoter element, the cells may have stopped dividing and as a default prematurely entered the endocycle program. However, we cannot rule out the possibility that the abnormal size of nuclei is a result of abnormal condensation of the DNA. We also produced *string* clones in the background of 31.6 kb rescue construct (Fig. 6A) and, as expected, observed mutant clones the size of sister clones and a comparable size of nuclei in each group.

These data suggest that Notch pathway activity will impinge in (6.4 kb region)-enhancer element. To test this, we crossed both the R6.4-*lacZ* and R4.9-*lacZ* gene fusions to the line mutant for Delta and analyzed the expression of these elements in egg chambers with germline mutant for Delta. In controls, the expression of R6.4 element was observed in stage 5 but not in stage 9 egg chambers (Fig. 7A-B). However, in Delta germ line clones, expression of R6.4 element was observed in domains of 5-20 cells through the follicle cell layer past stage 6 (Fig. 7C-D, $n$=36). By contrast, when R4.9-element was analyzed in Delta germ line clones, no expression beyond the normal early

**Table 2. Expression of the *string-lacZ* fusion constructs during oogenesis**

| Reporter gene | Expression |
|---|---|
| *pstgβ* | Weak, early germline |
| *pstgβ-B3.2* | NS |
| *pstgβ-R2.2* | NS |
| *pstgβ-R4.9* | Early follicle cells (germarium and stage 1-2) |
| *pstgβ-R6.4* | Follicle cells (stage 3-6) |
| *pstgβ-R2.6* | Weak stalk cells, weak germline |
| *pstgβ-R5.3* | Muscle |
| *pstgβ-R6.7* | Weak germ line |
| *pstgβ k-R2.5* | NS |
| *pstgβ k-R4.3* | NS |

NS, no staining detected.
The locations in genomic DNA are indicated in Fig. 6A.

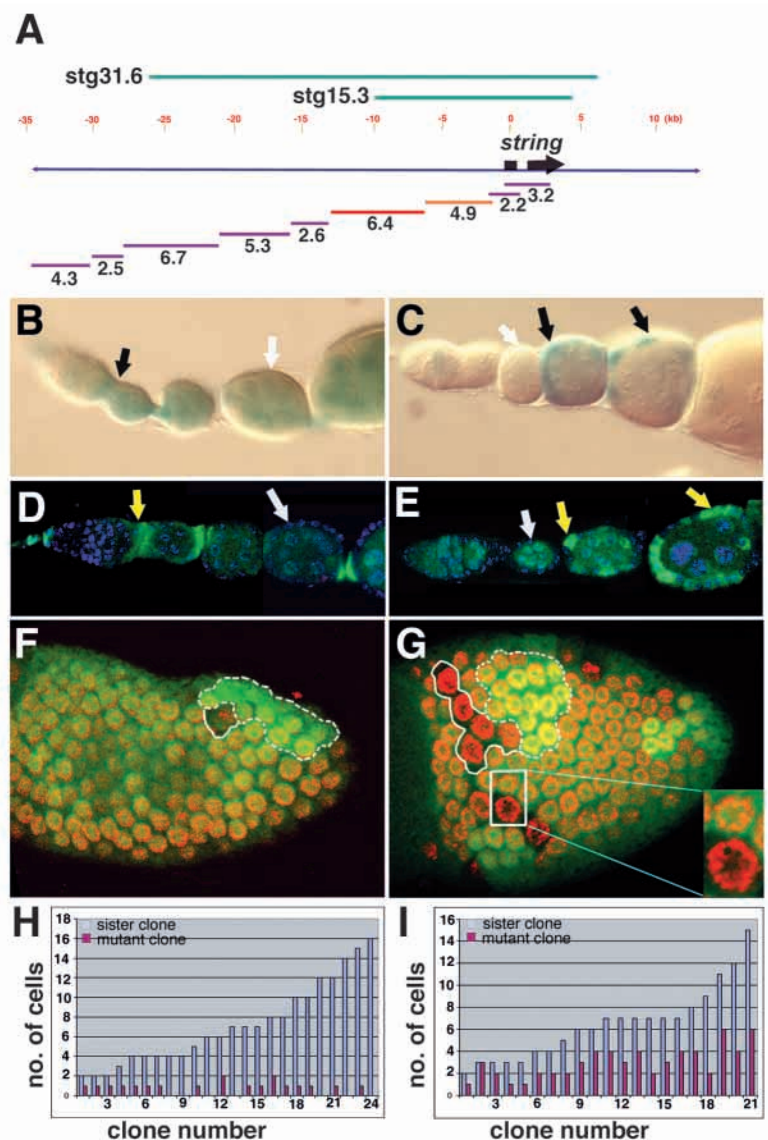


**Fig. 6.** *string* expression in follicle cells. (A) The ~50 kb genomic region surrounding string is indicated. A thick black arrow indicates the *string* gene; the *string* intron is white. Stg15.3 and stg31.6 indicate the genomic fragments tested for rescue. Fragments used to drive *lacZ* expression in transgenic animals are shown below the genomic region. Cell types in ovary in which expression is driven by these fragments are described in Table 2. (B,D) The 4.9 kb element (D10) supports follicle cell expression in the germarium and stage 1-2 egg chambers. (C,E) The 6.4 kb element (D12) supports expression in stage 4-6 egg chambers. (B,C) X-gal staining, black arrows indicate the follicle cells with X-Gal staining, white arrows indicate lack of staining. (D,E) Anti-β-gal (green). Yellow arrows indicate the follicle cells with staining, white arrows indicate lack of staining. (F) String is required for follicle cell mitotic divisions, as only 0-2 cell *string* mutant clones (black) were observed with 2-16 cell sister clones (bright green). On average, the mutant clone is one tenth the size of the sister clone. (H) Quantitation of the size of the *string* mutant (red bar) and sister (blue bar) clones. (G) Stg15.3 rescue construct shows partial rescue for the clone size (on average, the black mutant clone is half the size of the bright green sister clone). (I) Quantitation of the sizes of *string* clones with Stg15.3 rescue (red bar) and sister clones (blue bar). DAPI is blue in D,E and red in F,G. GFP is green in F,G. Mutant clones are indicated with unbroken white lines, sister clones with broken white lines. hhhhhfh

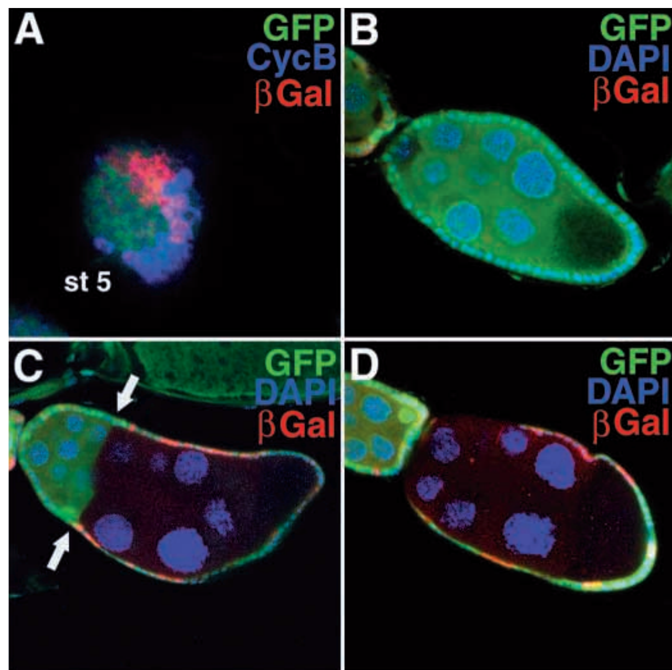


**Fig. 7.** 6.4 kb *string* promoter region is precociously active in *Delta* germline clones. β-Gal expression from *6.4-LacZ string* promoter fusion (red) is observed in follicle cells of stage 5 (A) but not stage 9 (B) control egg chambers. Some overlapping expression is observed between CycB (blue) and the *6.4-LacZ string* promoter fusion construct (red) at stage 5 egg chambers (A). In contrast to control egg chambers, in *Delta* germline clones (black germ line) the *6.4-LacZ string* promoter fusion construct is still observed in the follicle cells at stage 8-9 egg chambers (C,D). Green in A-D shows GFP, which marks the wild-type cells, and cells that lack GFP expression are mutant clones (C is a fused egg chamber, the border between wild-type cyst and *Delta* germline clone is marked with arrows). Red in A-D shows β-gal. Blue is CycB in A and DAPI in B-D.

expression was observed (data not shown). These data suggest that Notch pathway activity does control String at the 6.4 kb element but not at the 4.9 kb element.

## DISCUSSION

We have shown that the mitotic-to-endocycle transition in *Drosophila* follicle cells is controlled by the Notch pathway. Notch at the apical surface of the follicle cells is activated by Delta from the germline, which is expressed at high levels just before the switch. Disruption of the Notch pathway results in follicle cell over-proliferation but does not primarily affect their apical-basal polarity. This process does not require Fringe, probably because Delta levels are high enough to activate the Notch pathway in its absence. Notch activation leads through Su(H) to repression of the transcription of *string*, a regulator of the G2/M transition. Analysis of the activity of *string* enhancer elements in wild-type and Notch mutant follicle cells reveals the presence of an element that promotes expression of *string* until just before the onset of polyploidy in wild-type follicle cells but well beyond this stage in follicle cells defective for Notch pathway, suggesting that it may be a target of the endocycle promoting activity of the Notch pathway. A second element that is insensitive to Notch regulation promotes *string* expression earlier in follicle cell development, explaining why Notch only represses *string* at the mitotic cycle-endocycle transition and not in the germarium, where the Notch pathway is also active.

### Control of the mitotic to endocycle transition

Endocycles are widespread in plants and animals including humans and *Drosophila*. In human placenta, subgroups of trophoblast cells enter the endocycle and terminally differentiate to become trophoblast giant cells. It is known that over-proliferation of the trophoblast cells causes choriocarcinoma, placental cancer, but the signal that triggers the endocycle transition is not known. Another example of a mammalian cell type that becomes polyploid by endocycling is the megakaryocyte, a blood cell that is specialized to produce platelets (Zimmet and Ravid, 2000). These cells undergo endomitosis, and the transition from mitotic to endomitotic cycle is influenced by the secreted signal thrombopoietin. Thrombopoietin upregulates Cyclin D3 protein and Cyclin D3 overexpression has been found to increase megakaryocyte ploidy in transgenic mice (Zimmet et al., 1997). In addition, recent data has shown that constitutively active form of Notch can induce *CycD* expression and that CSL (stands for CBF1, Su(H), and Lag-1) binding site is identified in the *CycD* promoter (Ronchini and Capobianco, 2001).

In *Drosophila*, cells adopt the endocycle program during both embryonic and adult stages. Embryonic endocycling cells are found in the gut, fat body, malpighian tubules and salivary glands (Smith and Orr-Weaver, 1991), and in adult tissues, endoreplicating cells are found in the ovary (nurse cells and follicle cells) and in the wing (sensory neurons). The transition from mitotic to endocycle during embryogenesis coincides with transcriptional repression of the mitotic regulators Cdk1, Cyclin A, Cyclin B, Cyclin B3 and Cdc25/string (Sauer et al., 1995). Control of this transcriptional repression is not yet understood. In addition to transcriptional level control, embryonic cells control their mitotic machinery at the protein level to ensure the transition to endocycles (Sigrist and Lehner, 1997).

It is plausible that Notch also regulates other components of the cell cycle in addition to String, because the transition to the endocycle requires not only the repression of a mitosis promoter (*string*) but the upregulation during G2 of an S phase promoter, such as *CycE* and/or *CycD* (for comparison, during early embryogenesis cells that lack String pause in G2 rather than undergoing endocycles). However, when *string* was restored to clones under the control of the 15.3 kb rescue construct, which contains the control element active in the germarium and stage 1 but not the control element active between stage 3 and stage 6, some cell divisions occurred before premature stopping of the mitotic cycle, and interestingly, the nuclei were 2.2 fold bigger in the mutant clones than in the sister clones or in the neighboring wild type cells (Fig. 6G) suggesting that the mutant cells that stopped dividing too early also entered the endocycle too early. Thus, in contrast to embryonic cells, cessation of *string* expression may lead the follicle cells to endocycle rather than simply arrest, and thus Notch might act solely on *string* to stop mitosis and the subsequent transition to the endocycle could be due to other factors constitutively present in follicle cells. However, we cannot rule out the possibility that Notch also acts on

other cell cycle components, and based on the example of megakaryocytes, in which thrombopoietin promotes the mitotic-endocycle switch and also elevates the levels of CyclinD, it will be interesting to examine the role of Cyclin D in the follicle cell transition.

### Notch pathway controls cell division?

We have shown that in *Drosophila* follicle cells *string* mRNA expression coincides with the mitotic cycle of follicle cells. Furthermore, we have shown that two separate controlling elements are found in the promoter region of *string* for early follicle cell expression, one of which is shut off just at the mitotic cycle-endocycle transition and is likely to be the target of regulation by the Notch pathway. Previous work suggests that *string* transcription is altered in embryos mutant for axis, gap, pair rule, segment polarity, homeotic, neurogenic and proneural genes (Arora and Nüsslein-Volhard, 1992; Edgar et al., 1994), suggesting that the upstream regulatory elements of *string* is a sophisticated integrator of patterning signals. It will be interesting to determine how Su(H) functions to repress *string* expression and leads to the transition to the endocycle.

### Germline follicle cell signaling

Signaling between the somatic follicle cells and the underlying germline plays a crucial role in coordinating the complex process of oogenesis. Previous work has shown important roles for epidermal growth factor receptor signaling from the oocyte to the follicle cells at two stages of oogenesis, and revealed that the follicle cells signal back to the oocyte to help define the oocyte anteroposterior axis later in oogenesis (Schupbach, 1987; Gonzalez-Reyes et al., 1995; Roth et al., 1995; Anderson, 1995; Ruohola et al., 1991; Deng and Ruohola-Baker, 2000; Ray and Schüpbach, 1996; Rongo and Lehmann, 1996). The new oocyte-germline signaling event from Delta in the germline to Notch in the surrounding follicle cells defined in this paper adds a new twist to our understanding of germline-follicle cell signaling during oogenesis and plays an important role in coordinating the division of follicle cells with crucial steps in the maturation of the oocyte. The next challenge in this area is to understand what regulates the key regulatory step in the process, the elevation of Delta protein level in the germline.

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