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Notch-Dependent Fizzy-Related/Hec1/Cdh1 Expression Is Required for the Mitotic-to-Endocycle Transition in *Drosophila* Follicle Cells

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Summary

During *Drosophila* oogenesis, Notch function regulates the transition from mitotic cell cycle to endocycle in follicle cells at stage 6 [1, 2]. Loss of either Notch function or its ligand Delta (Dl) disrupts the normal transition; this disruption causes mitotic cycling to continue and leads to an overproliferation phenotype [1, 2]. In this context, the only known cell cycle component that responds to the Notch pathway is String/Cdc25 (Stg), a G2/M cell cycle regulator [1]. We found that prolonged expression of *string* is not sufficient to keep cells efficiently in mitotic cell cycle past stage 6, suggesting that Notch also regulates other cell cycle components in the transition. By using an expression screen, we found such a component: Fizzy-related/Hec1/Cdh1 (Fzr), a WD40 repeat protein. Fzr regulates the anaphase-promoting complex/cyclosome (APC/C) and is expressed at the mitotic-to-endocycle transition in a Notch-dependent manner. Mutant clones of Fzr revealed that Fzr is dispensable for mitosis but essential for endocycles. Unlike in Notch clones, in Fzr mutant cells mitotic markers are absent past stage 6. Only a combined reduction of Fzr and ectopic Stg expression prolongs mitotic cycles in follicle cells, suggesting that these two cell cycle regulators, Fzr and Stg, are important mediators of the Notch pathway in the mitotic-to-endocycle transition.

Results and Discussion

In *Drosophila,* nurse and follicle cells in the adult ovary endocycle in a regulated manner (Figure 1A; [3]; reviewed in [4, 5]). It has been suggested that endocycling requires the loss of M-phase cyclin-dependent kinase (Cdk) activity and oscillations in the activity of S-phase Cdk [4, 6]. In *Drosophila* follicle cells, the function of the Notch pathway in the mitotic-to-endocycle transition has been well established [1, 2]. Lack of Notch activity in *Drosophila* follicle cells leads to prolonged mitosis at the expense of endocycles, suggesting that Notch functions in this context as a tumor suppressor [1, 7]. Because very few signaling pathways that stop the mitotic cell cycle have been identified, it is important to understand the relationship between the Notch pathway and known cell cycle regulators in more detail.

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Overexpression of *string* Is Not Enough to Induce Efficient Mitosis in Follicle Cells after Stage 6

String encodes the *Drosophila* homolog of the yeast cell cycle regulator Cdc25, a phosphatase whose role is to activate the Cdk-cyclin complex at the G2/M transition by dephosphorylating the inhibitory sites. As a consequence, cells are propelled into mitosis. 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 [1]. The 4.9 kb and 6.4 kb elements found in the 50 kb-long *string* promoter drive *string* expression in follicle cells from germarium to stage 3 and from stage 4 to stage 6, respectively (Figure 1B). The Notch-Delta cascade achieves the tight downregulation of the 6.4 kb element at stage 6, when the mitotic-to-endocycle transition takes place (Figure 1C, red channel; [1]). A *string* rescue construct that contains 15.3 kb of the *string* promoter restores only the early *string* expression pattern between germarium and stage 1–2 egg chambers (because of the 4.9 kb element) but does not contain the control element active between stages 3 and 6 (the 6.4 kb element) (Figure 1B; [1]). Although *stg* clones produce cells arrested in G2, the mutant nuclei were larger than in the wild-type cells when *stg* clones were produced in the background of the 15.3 kb rescue construct (Figures S1A and S1A′ in the Supplemental Data available with this article online; [1]). Furthermore, the mutant clones are half the size of sister clones (Figure S1A; [1]), suggesting that the mutant cells stop division and possibly enter endocycle too early. If downregulating String leads the follicle cells to enter an endocycle rather than to completely arrest, then the sole role of Notch, which downregulates *string* expression at the switch, is to act on *string* to promote endocycling. If this is the case, *string* expression is the only limiting factor in the mitotic-to-endocycle transition. Also, because ectopic expression of *stg* in *Drosophila* embryos and discs is capable of driving cells blocked in G2 into mitosis [8], continuous *string* expression should keep most cells in the mitotic phase.

We overexpressed *stg* (either with a heat-shock-inducible promoter or with one or two copies of the *UAS-stg* transgene via the flip-out Gal4 system [9]) to analyze whether String is sufficient to prolong division of follicle cells past stage 6. Overexpression of *string* with a transgene driven by a heat-shock promoter did not show any ectopic Cyclin B or Phospho-Histone 3 (PH3) expression (Figure 1E). With one copy of the UAS *string* transgene, we rarely observed prolonged mitotic divisions in follicle cells past stage 6, except in the posterior region, where 10% (Figure 1E) of the clones that overexpressed *string* showed ectopic Cyclin B or PH3 expression (mitotic markers). When two copies of the *UAS-stg* construct were present, leading to higher *string* expression levels, we noticed a higher incidence of Cyclin B (data not shown) and PH3 (Figures 1D, 1D′, and 1D″) expression in posterior clones (Figure 1E, 35%, $n = 23$ posterior clones beyond stage 6). However, only in a few cases (Figure 1E, 2%, $n = 388$) did the ectopic

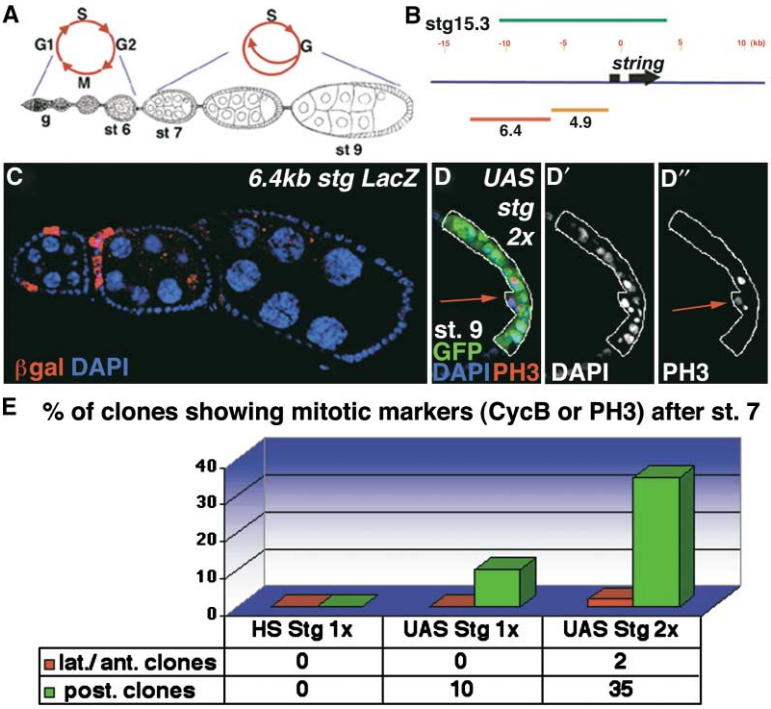

Figure 1. Overexpression of *string* Is Not Enough to Induce Efficient Mitosis in Follicle Cells after Stage 6

(A) Follicle cells in *Drosophila* oogenesis undergo a mitotic-to-endocycle transition at stage 6 of oogenesis. From the germarium (g) to stage (st.) 6, somatically derived follicle cells undergo mitotic cycles, which are not synchronized. At stage 7 they switch to endocycles.

(B) The genomic region around the string gene: the *string* gene is indicated by a thick black arrow (the *string* intron is white). *Stg15.3* (green line) indicates the genomic fragment used for rescue experiments. Fragments of the promoter driving expression in the ovarian follicle cells are shown as orange and red lines. The 4.9 kb element (in orange) supports follicle cells expression in the germarium and stage 1–3 egg chambers [1]. The 6.4kb element (red line) expression pattern is shown in (C).

(C) The 6.4 kb element drives LacZ expression in stage 4–6 egg chambers and is turned off by Notch activation after stage 6, as shown by an anti β-gal staining (red). DAPI staining is shown in blue.

(D–D″) Follicle cells expressing two copies of *UAS-string* construct (*hsFlp; UAS-stg*^{4}/*UASstg*^{16}*;UASGFP act<FRT-CD2-FRT<Gal4)* showed staining for PH3 (red channel) in the posterior region, indicative of mitosis past stage 6 in oogenesis. (D and D′) The green channel shows GFP, and blue is DAPI. Red arrows point to the region with a PH3-positive staining. (D″) Only the red channel, representing the PH3 staining, is shown. (E) A table summarizing mitotic phenotypes obtained in the String overexpression experiments.

expression of *string* in lateral and anterior follicle cells prolong their mitotic state. In addition, in most cells, overexpression of *string* did not affect endocycling (as shown in Figures S1B and S1B′).

Because the String protein is not enough to create extra cell divisions, except in the highly sensitized posterior area [1, 10], we proposed that the mitotic-to-endocycle transition is regulated by a combination of String and other Notch-controlled components yet to be uncovered. Lack of String generally arrests cells in G2, when high levels of mitotic cyclins can be found. Cyclin A and Cdc2 have been implicated in inhibiting the assembly of prereplication complexes in G2. Furthermore, when Cdc2 or Cyclin A activity was eliminated, mutant cells entered endocycles in *Drosophila* [11, 12] and in yeast [13] because the assembly of prereplication complexes was then allowed. Our hypothesis is that the Notch signaling pathway allows cells to bypass this inhibition by activating a specific gene/genes that would allow cells to continue to cycle without undergoing the mitotic phase. Expression of such a gene would be activated after/during the mitotic-to-endocycle transition and possibly act on mitotic cyclin regulation and/or the mitotic cyclin-associated kinase, Cdc2. In order to find these genes, we performed an expression screen for genes differentially expressed before and after the transition.

Fzr Is Expressed from the Mitotic-to-Endocycle Transition on in Response to Notch Signaling

We screened 400 lethal X chromosome P element enhancer trap lines [14] for changes in expression levels at stage 7 by using the β-gal reporter gene. Three interesting functional groups were obtained from this screen; adhesion molecules, transcriptional control proteins, and cell cycle regulators (see note in proof). Premature expression of *fzr* caused formation of enlarged nuclei, a potential indication of precocious endocycles (Figures 2A and 2A′). We therefore analyzed the cell cycle regulator Fzr in more detail in the mitotic-to-endocycle transition.

The lines *fzrG0326* and *fzrG0418* have the *P{lacW}* element inserted in the first intron and at the 5′-end of the *Fizzy-related* gene, respectively, and are hypomorphic alleles of *fzr* [15]. These constructs drive expression of the reporter gene after the transition, from stage 6–7 onward (Figure 2B, red channel and red arrow). This expression is tightly correlated with the end of mitotic cycles; no *fzr* expression is observed in follicle cells that show PH3 staining (Figure 2B, green channel and green arrow). A similar expression pattern of Fzr was observed with the Fzr specific antibody (Figure 2C), and the *fzr* mRNA pattern in follicle cells reflects this pattern as well (data not shown).

Fzr, also known as Retina Aberrant in Pattern, is a conserved WD domain protein that is required during G1 for proteolysis of mitotic regulators such as Aurora-A kinase and Cyclins A, B, and B3 in an APC/C-dependent manner [16–21]. Previous studies have shown that loss of Fzr in *Drosophila* causes cells to progress through an extra division cycle in the epidermis and inhibits endoreduplication in the salivary gland cells, whereas *fzr* overexpression inhibits mitosis and transforms mitotic cycles into endoreduplication cycles [17]. This finding

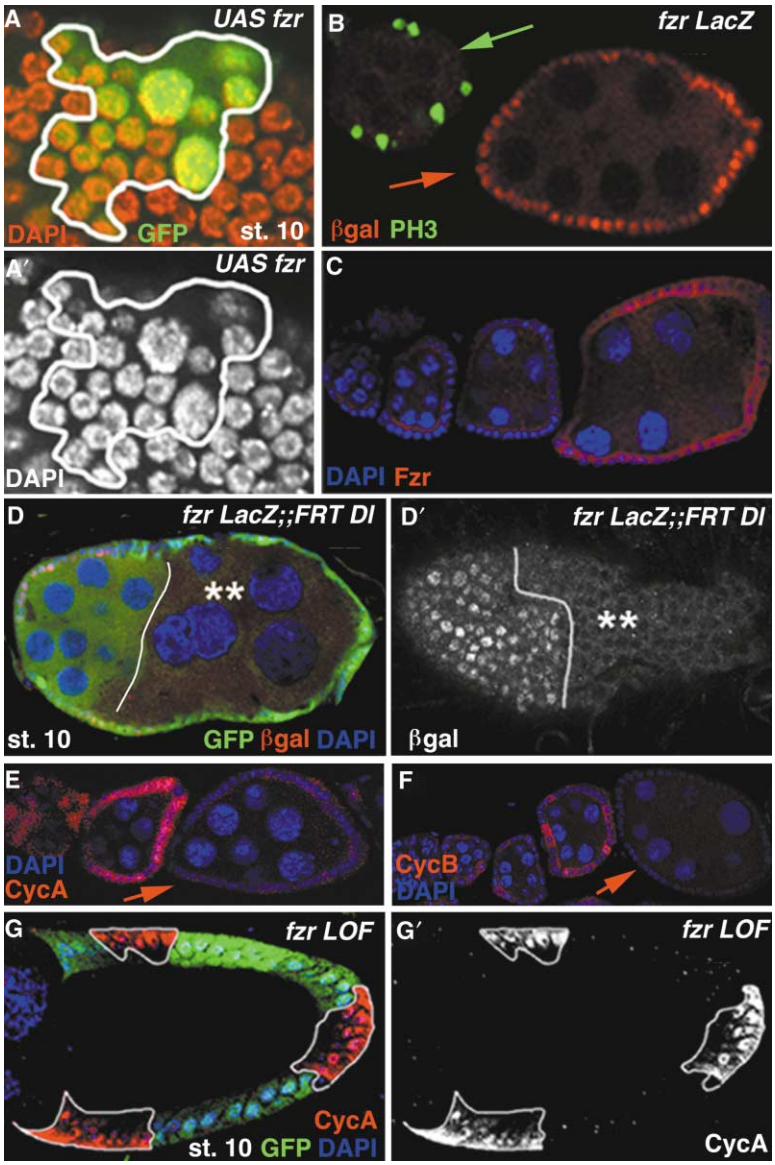

Figure 2. Fzr Is Expressed from the Mitotic-to-Endocycle Transition under the Control of Notch and Induces Mitotic-Cyclin Degradation in Follicle Cells

(A and A′) Ectopic expression of *fzr* (*hsFLP; UAS-fzr; UAS GFP, act<FRT-CD2-FRT<Gal4*), marked by GFP expression in clone (delimited area) allows formation of large nuclei (A′, red arrow), suggesting precocious endocycle entry. The green channel shows GFP, and the red channel shows DAPI staining.

(B) The β-gal expression of Fzr enhancer trap line *fzr*G0326, fzr-lacZ (red channel, red arrow) turns on in wild-type follicle cells right after the mitotic stage when PH3 staining (green channel, green arrow) stops.

(C) A similar expression pattern is observed with Fzr-specific antibody (the red channel shows Fzr staining, and the blue channel shows DAPI staining).

(D and D′) A mutant Dl germ line clone (*hsFlp/fzr*G0326*;;FRT82B Dl^{rev10e}/FRT82 Ubi-GFP*) egg chamber after stage 6. Follicle cells surrounding a Dl germ line clone show an absence of Fzr staining. The red channel represents the β-gal expression of the enhancer trap line *fzr*G0326, green is GFP, and blue is DAPI. (D′) A surface view of (D) shows the β-gal expression of Fzr enhancer trap line *fzr*G0326. The clonal egg chamber is indicated by two asterisks.

(E and F) Cyclin A (E) and cyclin B (F) antibody staining (red channel). Both cyclin A and cyclin B are downregulated after the mitotic-to-endocycle transition (red arrows). The blue channel shows DAPI staining.

(G and G′) Fzr loss-of-function (lof) clones (*fzr^{ie28}FRT 101/GFP FRT 101;;MKRS-hsFLP*) show abnormal accumulation of cyclin A. A white line delimits mutant clones, which lack GFP (green channel). The blue channel represents DAPI. Cyclin A staining is shown in red (G) and white (G′).

suggests that, in at least some cell types, the Fzr protein is essential for the mitotic-to-endocycle transition. Because *fzr* expression is upregulated in follicle cells when the Notch cascade is activated, we tested whether the *fzr* expression was responsive to Notch activity by using the *fzrG0326* (fzr-LacZ) enhancer trap line to analyze *fzr* expression levels in follicle cells that surround the Dl germline clones. We observed a clear reduction of *fzr* expression in all Dl germline clones past stage 6 (Figures 2D and 2D′), demonstrating that *fzr* expression is dependent on Notch activity in the mitotic-to-endocycle transition.

Fzr Induces Cyclin A and Cyclin B Degradation in Follicle Cells

Mitotic-cyclin protein levels are downregulated at the mitotic-to-endocycle transition. Cyclin A protein levels are reduced at the end of mitotic cycles in the follicle cells (Figure 2E, red arrow). Similarly, Cyclin B is downregulated at the protein level at the mitotic-to-endocycle transition ([1], Figure 2F, red arrow). In situ hybridization studies indicated that neither gene was regulated at the transcriptional level during or after the transition but showed mRNA expression in the follicle cells throughout oogenesis until stage 10 (data not shown). Thus, both the Cyclin A and the Cyclin B protein levels are regulated posttrancriptionally at the mitotic-to-endocycle transition. This regulation is critical for the mitotic-to-endocycle transition because we observed that continuous expression of *cyclin A* in posterior follicle cells results in small nuclei and a reduced DNA level, indicative of a defect in the transition to endocycles (Figures S1C, and S1C′). This supports previous reports showing that overexpression of *cyclin A* inhibits the progression of endoreplication cycles in *Drosophila* salivary glands [22].

Because the downregulation of Cyclin A and B expression coincides with the upregulation of Fzr and because Fzr is required for proteolysis of Cyclin A and Cyclin B

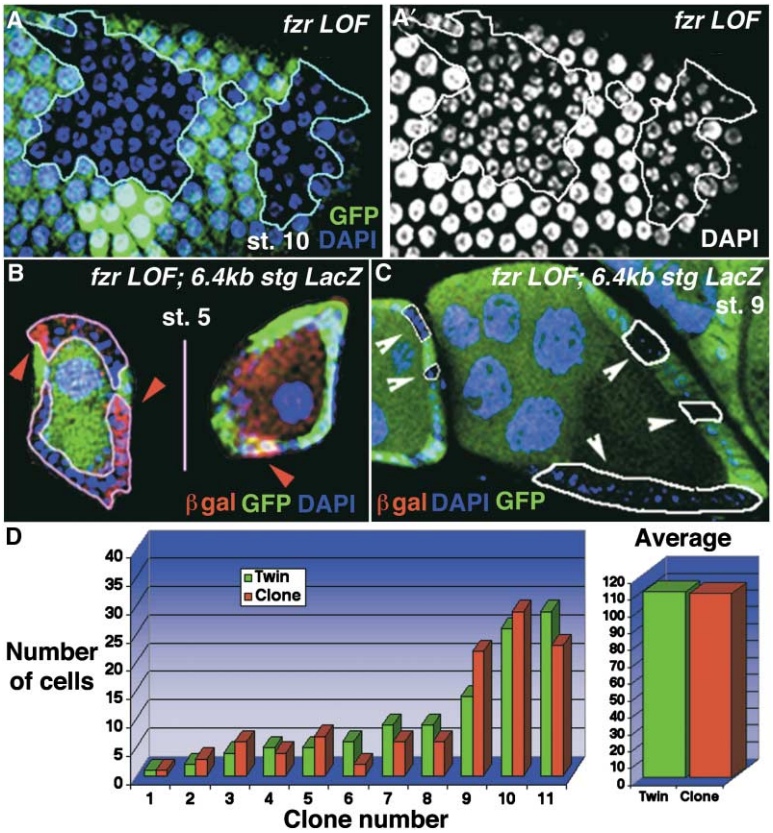

Figure 3. Fzr Is Required for the Transition to Endocycle but Is Dispensable for Mitosis

(A and A′) Loss of *fzr* (loss-of-function mutant clones lack GFP and are delimited by a green [A] or white [A′] line) does not allow the follicle cells to enter endocycles; nuclei size is smaller in fzr^{-} clones than in their neighbors, as shown by DAPI staining (blue channel in [A] and white in [A′]).

(B and C) Fzr clones (*fzr^{ie28} FRT101/GFP FRT 101; 6.4 kb stg LacZ;MKRS-hsFLP*) show normal regulation of the *6.4 kb stg LacZ* reporter at stage 6. The green channel shows GFP, the red channel represents β-gal staining, and the blue channel is DAPI. Clonal areas are marked by a white line. (B) Loss of *fzr* function does not disrupt the normal stage 5 *6.4 kb stg LacZ* expression (left side of the picture, red arrows), nor does loss of one copy of Fzr (right side, red arrow). (C) Loss of *fzr* function does not prolong *6.4 kb stg LacZ* expression after the mitotic-to-endocycle transition (white arrows). The green channel is GFP, the red channel represents β-gal staining, and the blue channel is DAPI.

(D) Quantification of the number of nuclei in the *fzr* mutant clones (red bars) compared to their associated sister clones (green bars). The x axis represents the clone number, and the y axis represents the number of cells per clone or sister. On average, the number of nuclei in the clonal area is the same as the number of nuclei in the associated sister clone.

in embryonic epidermal cells, we generated clones for a *fzr* null allele (*fzr^{ie28}*) [15] to test whether Fzr might be responsible for the mitotic-to-endocycle transition by downregulating the mitotic cyclin levels. First, we immunostained the ovaries with antibodies against Cyclin B. The clonal cells lacking Fzr function showed a limited but consistent increase of Cyclin B level after stage 6 (Figures S1D and S1D′), when Cyclin B is normally absent. In a similar manner, *fzr^{ie28}* mutant cells strongly upregulated Cyclin A after the mitotic-to-endocycle transition (Figures 2G and 2G′). We therefore conclude that, as seen in other systems, Fzr function in follicle cells is to degrade mitotic cyclins.

Fzr Is Required for the Transition to the Endocycle but Is Dispensable for Mitosis

Upregulation of the mitotic Cyclin A during endocycles has been shown to inhibit endoreplication ([22], Figures S1C and S1C′). We stained the ovaries bearing *fzr^{ie28}* mutant cells with DAPI to observe nuclei size and shape. We found that, in addition showing a failure of Cyclin A and B removal, the *fzr^{ie28}* mutant cells showed phenotypes that indicated endocycle inhibition. The small nuclei size and reduced DNA level seen in the *fzr^{ie28}* mutant cells (Figures 3A and 3A′) are reminiscent of the Notch phenotype [1] and thereby show that Fzr is required for the mitotic-to-endocycle transition. Unlike the Notch clones in which Cyclin B and PH3 expression were detected after stage 6 [1], the *fzr^{ie28}* mutant cells do not shown signs of overproliferation. No PH3 staining is ever observed in mutant clones after the transition (data not shown), suggesting that the *fzr^{ie28}* mutant cells do not continue to divide past stage 6. The *6.4 kb Stg-LacZ* transgene, abruptly downregulated by Notch at the mitotic-to-endocycle transition (Figure 1C; [1]), did not show any prolonged expression in *fzr^{ie28}* mutant cells after stage 6 (Figure 3C, white arrows), indicating that cells are not in a mitotic phase. We also counted the number of cells in mutant and sister clones (two copies of GFP). The same number of cells was observed in mutant clones as in the associated twin spot (Figure 3D; the ratio varied from 0.64 to 3, with a mean of 1.01). All these clues lead us to think that despite mitotic cyclins' upregulation in *$fzr^{-/-}$* mutant cells, those cells do not divide past stage 6.

Because the number of cells in the mutant follicle cell clones and the associated twin spots are the same (Figure 3D) and because the expression of the *6.4 kb stg-LacZ* transgene is normal prior to the switch to endocycling (Figure 3B, red arrows), we conclude that, even though Fzr is required for endocycles, it is dispensable for the mitotic stages in the *Drosophila* ovary. Similarly, Jacobs et al. [15] demonstrated that completion of mitosis does not require Fzr in embryos.

Overexpression of *string* in a *fzr* Heterozygous Background Leads to Extra PH3 Stainings

As shown, although *fzr^{ie28}* mutant cells show upregulation of the mitotic cyclins (Figures 2G, 2G′, S1D, and S1D′), these cells do not divide. In all dividing cells, the G2/M transition depends on String to activate the kinase activity of the mitotic cyclin-Cdc2 complexes. Because

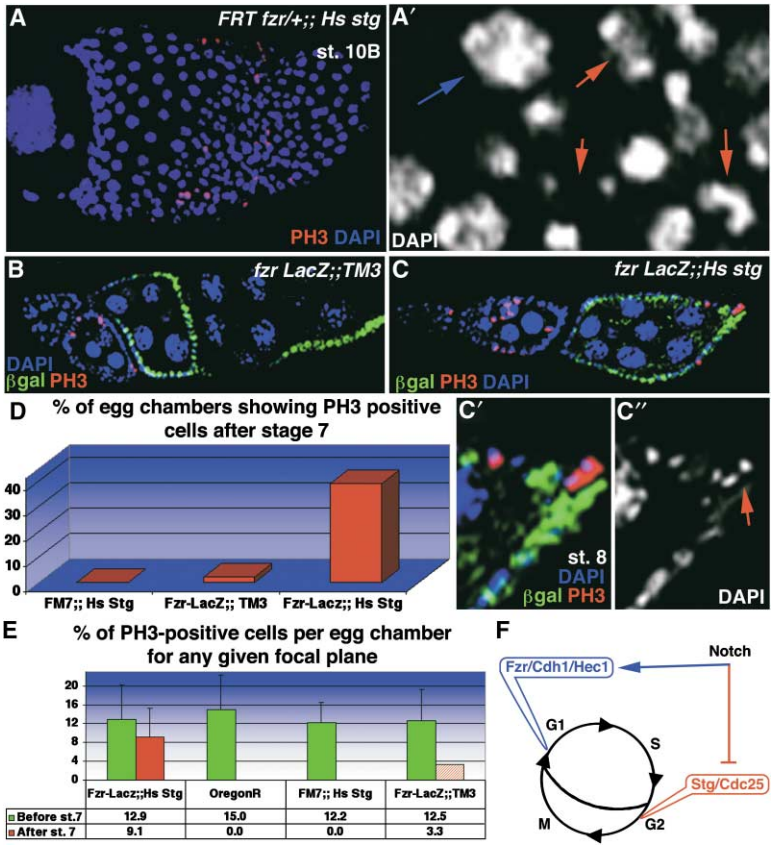

Figure 4. Overexpression of *string* in a *fzr* Heterozygous Background Leads to Extra PH3 Stainings

(A and A′) Overexpression of *string* with a heat-shock promoter in a *fzr* lof background (*fzr*^{ie28} *FRT101/GFP FRT 101; hs Stg, MKRS-hsFLP*). The area shown is nonclonal; cells bear only one copy of Fzr. (A) Around 8.5% of the cells show PH3 stainings (red channel) at day 4, suggesting that cells are entering an extra round of mitosis. DAPI is blue. (A′) Mitotic figures can be observed (red arrows); compared to an endocycling nuclei (blue arrow). DAPI is white.

(B) No PH3-positive cells are seen after stage 7, 2 hr after heat shock, in *fzr*G0326 (Fzr-lacZ);;TM3 control ovaries. PH3 is red, DAPI is blue, and βgal is green.

(C–C″) Several PH3-positive cells are found in egg chambers older than stage 7 when Fzr-lacZ has been upregulated. PH3 is red, DAPI is blue, and βgal is green. (C′ and C″) A mitotic figure is shown (red arrow) from a stage 8 egg chamber.

(D) Quantification of the percentage of ovarioles showing extra PH3 staining after stage 7. Of stage 7–8 Fzr-LacZ;;Hs Stg egg chambers, 38% show extra PH3 staining.

(E) Quantification of the number of PH3-positive cells per egg chamber for any given focal plan before and after stage 7 (green and red bars, respectively). Only the PH3-positive egg chambers were included in the count after stage 7. The experimental group (Fzr-LacZ;;Hs Stg) is compared to three controls. The control groups Fzr-LacZ;;TM3 and FM7;;Hs Stg, as well as the experimental group, have been dissected 2 hr after 1 hr of heat shock (see Experimental Procedures). The wild-type flies (Oregon R) have not been heat shocked and are shown as a general control. The control group Fzr-LacZ;;TM3 showed two PH3-positive cells only in one egg chamber (n = 47, as shown in Figure [D]) and is therefore shown in light red.

(F) Model representing how Notch acts on String and Fzr to promote the mitotic-to-endocycle transition. Notch shuts down String transcription [1] to ensure that mitosis does not happen and activates Fzr to prevent accumulations of mitotic cyclins, thus allowing endocycle.

the *6.4 kb stg-LacZ* transgene is downregulated by the Notch pathway after the transition, the *fzr*^{ie28} mutant cells might require Stg to prolong mitosis. To test this, we overexpressed *string* by using a heat shock promoter in the *fzr*^{ie28} follicle cell clones. We heat shocked the flies twice, once to promote the formation of *fzr*^{ie28} mutant clones and again, 12 hr prior to dissection, in order to induce *stg* expression. In a wild-type *fzr* background, this low level of *string* expression alone is insufficient for prolonging mitosis past the mitotic-to-endocycle transition (no upregulation of Cyclin B and PH3 markers). However, this prolonged *stg* expression is enough to push the *fzr*^{ie28} mutant cells into mitosis, as shown by the PH3 staining and mitotic figures in egg chambers at stage 9 (Figures S1E and S1E′). More strikingly, PH3-positive cells as well as mitotic figures were seen in nonclonal areas heterozygous for *fzr*^{ie28} (Figures 4A and 4A′, red arrows), which prompted us to test whether reducing the level of Fzr to one copy while overexpressing *stg* by heat shock was sufficient to produce the PH3-positive cells. In order to demonstrate a direct *stg* effect, we then looked at the flies 2 hr after heat shock. We also used the *fzrG0326* enhancer trap line (fzr LacZ) to reduce the level of Fzr and to mark the stages precisely. We found that 39% of ovarioles of the experimental group *fzrG0326*;;Hs Stg (Figure 4D) displayed PH3-positive cells (Figures 4C and 4C′) and mitotic figures (Figure 4C″) at stage 7–8, whereas 0%–2% did so in the control groups FM7;;Hs Stg (Figure 4D) and *fzrG0326*;;TM3 (Figures 4B and 4D), respectively. In order to further determine whether the ratios of cells in a mitotic stage in mutant and control situations were similar, we quantified the number of PH3-positive cells observed in a single focal plan before and at stage 7–8, in the experimental group *fzrG0326*;;Hs Stg as well as in three control groups (Figure 4E). On average, 12%–15% of the cells showed PH3 staining at mitotic stages before the transition (before stage 7), but none did so after the transition (stage 7–8). In contrast, ovaries with reduced *fzr* and prolonged *stg* expression showed PH3 staining after stage 7 (Figures 4C and 4E, mean = 9%), whereas the control groups did not. In comparison, 8.5% of cells in the egg chamber shown in Figure 4A did exhibit PH3 staining. It is possible that the percentage of mitotic cells observed in mutant egg chambers past the transition was somewhat lower than the percentage of mitotic cells observed in wild-type egg chambers before the transition (9% versus 12%–15%, respectively) because of the low level of *string* expression given by the heat shock construct or subtle effects of yet-unraveled components in the transition. However, these data strongly suggest that reducing the Fzr level in combination with prolonged

stg expression can prolong the mitotic stage in follicle cells.

In *Drosophila*, loss of Fzr causes progression through an extra division cycle in the epidermis and inhibition of endoreplication in the salivary glands, in addition to the upregulation of mitotic cyclins [17]. We now show that in follicle cells loss of Fzr causes an inhibition of endoreplication as well as an upregulation of the mitotic cyclins, particularly Cyclin A, but no prolonged mitosis. This difference might be due to the lack of String in follicle cells. It is possible that in the epidermis, residual String might dephosphorylate and therefore activate the mitotic cyclin/Cdk complexes and allow an extra mitosis to proceed, whereas in follicle cells the absence of String might result in G2-arrest. This is supported by the fact that overexpressing a *string* transgene under the control of a heat shock promoter rescues cell division in a *fzr* mutant (Figures 4C, 4C′, and 4C″).

Notch mutant cells are mitotic: in those cells, Stg is upregulated (the *6.4kb stg-LacZ* transgene is expressed after the transition [1]), and Fzr is not activated (Figures 2D and 2D′). Here we show that those two events (upregulation of String and downregulation of Fzr) are able to keep the cells in mitotic cycle in 39% of stage 7–8 egg chambers. It is therefore possible that Notch controls the mitotic to endocycle transition by repressing String to block mitosis and by activating Fzr to allow endocycle progression (Figure 4F).

Based on earlier studies, it has been proposed that endocycle is induced by lack of M-phase Cdk activity. However, the regulation and exact manifestation of this task has not been previously uncovered. We have now shown that in *Drosophila* follicle cells the Notch pathway executes the task by first freezing the mitotic cyclin/Cdk complex in an inactive, phosphorylated form and thereafter inducing the degradation of the mitotic cyclins to allow progression to S phase. Further studies will reveal whether Notch action is also required for G1-to-S-phase transition or whether these two alterations, lack of String, and expression of Fzr are sufficient to transform mitotic cells to endocycling cells.

Experimental Procedures

Fly Stocks

The following fly stocks were used: *FRT82B* Dl^{rev10e} (Dl^{rev10} is an amorphic allele) [23, 24]), and fzr^{ie28} *FRT101* ([15] and this study). For generating follicle cell clones, we used *hsFlp;;FRT82B Ubi-GFP, yw Ubi-GFP FRT101*, w^{-}*;;MKRS P{ry=hsFlp}86E/Tm6BTb*, and *hsFlp;;UAS-GFPact<FRT-CD2-FRT<Gal4/TM3* [9]. For analysis of overexpression pattern, the following stocks were used: *UAS-*stg^{N4} and *UAS-*stg^{N16} (Bloomington Stock Center/Bruce Edgar) and *UAS-cyclin A*, *UAS-fzr III.2*, and *UAS-fzr II.1* (gifts from Christian Lehner). The two fzr-LacZ lines *fzrG0326* (Bloomington Stock Center #12241 w67c23 P{w+mC=lacW}rapG0326/FM7c) and *fzrG0418* (Bloomington Stock center #12297 w67c23 P{w+mC=lacW}rapG0418/FM7c) are described in [14] and have a *P{lacW}* element inserted in the first intron and at the 5′-end of the *Fizzy-related* gene, respectively [15]. The *6.4 kb string-lacZ* fusion (Figures 1B and 1C), the *15.3 kb string* rescue (Figures 1B and 1D), and the *Hs Stg* transgenic lines were a kind gift from B. Edgar and are described in [1, 8].

Generation of Follicle Cell and Germline 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 min at 37°C and put in vials with fresh yeast paste for 3 or 5 days. For the *hs stg;fzr* loss-of-function experiment, flies were heat shocked at days 1 and 3.5, dissected, and stained at day 4; alternatively, they were heat-shocked for 1 hr, dissected, and stained after a 2 hr recovery period. To obtain germline clones, we heat shocked flies as second- and third-instar larvae for 2 hr on two consecutive days. Once they emerged as adults, they were placed in vials with fresh yeast paste for 1–5 days for dissection.

Staining Procedures

Ovaries were dissected in phosphate-buffered saline (PBS) and fixed on a nutator for 10 min in 5% formaldehyde in PBS. They were rinsed four times for 15 min in PBT (PBS/0.2% Triton X-100) and blocked in PBTB (PBT, 0.2% BSA, 5% normal goat serum) for 1 hr at room temperature. The tissue was incubated with primary antibodies overnight at 4°C. The next day, they were rinsed with PBT four times for 15 min and blocked in PBTB for 1 hr at room temperature. The ovaries were then incubated in secondary antibodies overnight at 4°C. The next day they were rinsed with PBT four times for 15 min and stained with DAPI (1 μg/ml in PBT) for 10 min. Finally, they were washed twice for 5 min with PBT. They were then dissected onto slides in 70% glycerol, 2% NPG, and 1× PBS.

Confocal microscopy and in situ hybridization were performed as described previously in [25, 26]. *stg* cDNA (LD47579) was labeled with fluorescein, and *cyclin A* (LD44443), *cyclin B* (LD23613), and *fzr* (LD21270) were labeled with digoxigenin (all cDNAs were from the Berkeley *Drosophila* UniGene Collection). A two-photon laser-scanning microscope (Leica TCS SP/MP) was used for imaging.

The following antibodies were used: mouse anti-Cyclin A (1:20, Developmental Studies Hybridoma Bank), mouse anti-Cyclin B (1:20, Developmental Studies Hybridoma Bank), rabbit anti-Cyclin A (1:100, David Glover), 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), Alexa 568 or 633 goat anti-mouse antibody (1:500), and Alexa 568 or 633 goat anti-rabbit antibody (1:500, Molecular Probes).

Supplemental Data

A supplemental figure is available with this article online at http://www.current-biology.com/cgi.content/full/14/7/630/DC1/.

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