Notch Signaling and Developmental Cell-Cycle Arrest in *Drosophila* Polar Follicle Cells

Li-Fang Shyu,^{*†} Jianjun Sun,^{*†‡} Hui-Min Chung,[§] Yi-Chun Huang,^{*}
and Wu-Min Deng^{*}

^{*}Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295; and [§]Department of Biology, University of West Florida, Pensacola, FL 32514

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Temporal and spatial regulation of cell division is critical for proper development of multicellular organisms. An important aspect of this regulation is cell-cycle arrest, which in many cell types is coupled with differentiated status. Here we report that the polar cells—a group of follicle cells differentiated early during *Drosophila* oogenesis—are arrested at G2 phase and can serve as a model cell type for investigation of developmental regulation of cell-cycle arrest. On examining the effects of String, a mitosis-promoting phosphatase Cdc25 homolog, and Notch signaling in polar cells, we found that misexpression of String can trigger mitosis in existing polar cells to induce extra polar cells. Normally, differentiation of the polar cells requires Notch signaling. We found that the Notch-induced extra polar cells arise through recruitment of the neighboring cells rather than promotion of proliferation, and they are also arrested at G2 phase. Notch signaling is probably involved in down-regulating String in polar cells, thus inducing the G2 cell-cycle arrest.

INTRODUCTION

Regulation of the cell cycle is critical to many aspects of cellular behavior during animal development. A prominent aspect of this regulation is control of cell-cycle exit: cells are arrested at a particular cell-cycle phase and become nonproliferative. Many differentiated cells undergo cell-cycle arrest as part of their differentiation process. During development, cell-cycle arrest is critical to controlling cell number, proliferation rate, and organ size. In the early *Xenopus* gastrula, a G2/M arrest is necessary for bottle-cell formation (Kurth, 2005). During *Caenorhabditis elegans* development, the CDC-14 phosphatase controls developmental cell-cycle arrest and is required for the quiescent state of specific precursor cells (Saito *et al.*, 2004). The *Drosophila* sensory-organ precursor (SOP) cells in the peripheral nervous system are arrested in G2 phase of the mitotic cycle (Nègre *et al.*, 2002; Chang *et al.*, 2008). Despite the significance of cell-cycle arrest, the developmental signals that cause cells to exit the cell cycle are poorly understood.

During *Drosophila* oogenesis, somatically derived follicle cells form a monolayer to cover the germ-line cells and form an egg chamber. Polar cells, which are aligned at the termini of the developing egg chamber, are among the earliest differentiated follicle cells. The polar cells are derived from a pool of precursor cells of the polar cell/stalk cell lineage (Tworoger *et al.*, 1999); the anterior polar cells are specified in germarium region 3 and the posterior polar cells during

stage 2 of oogenesis (Torres *et al.*, 2003). Once differentiated, polar cells cease to proliferate and remain unproliferative throughout oogenesis (Margolis and Spradling, 1995). By stage 5, the number of polar cells is reduced through apoptosis to two pairs, one pair each at the anterior and the posterior poles of the egg chamber (Besse and Pret, 2003). Polar cells can be visualized by means of an array of specific markers. For example, *neuralized-lacZ* (*A101*; Ruohola *et al.*, 1991) and *PZ80* (Karpen and Spradling, 1992) are expressed specifically in the polar cells, whereas expression of Fasciclin III (*FasIII*) is up-regulated during stages 1–5 and is restricted to the polar cells at stage 6 (Ruohola *et al.*, 1991). On the other hand, Eyes absent (*Eya*), a transcription factor and a phosphatase, is expressed in all but polar and stalk cells during early stages of oogenesis. Loss of *eya* in early follicle cells has been reported to induce polar-cell differentiation (Bai and Montell, 2002).

Functionally, polar cells act as organizers that direct differentiation of surrounding follicle cells into a multitude of subtypes. Around stage 1 of oogenesis, they control the formation of stalk cells, which are responsible for bridging adjacent egg chambers (Baksa *et al.*, 2002; McGregor *et al.*, 2002; Torres *et al.*, 2003), whereas during stages 7–9, they induce differentiation of the anterior follicle cells, through the Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, into several subtypes, including border cells, stretched cells, and centripetal cells (Silver and Montell, 2001; Beccari *et al.*, 2002; Ghiglione *et al.*, 2002; Grammont and Irvine, 2002; Xi *et al.*, 2003). Differentiation of polar cells is regulated by Notch signaling (Ruohola *et al.*, 1991; Grammont and Irvine, 2001). Loss of *Delta*, which encodes a Notch ligand, in the germ line or of *Notch* in follicle cells results in fused egg chambers that lack polar cells (Torres *et al.*, 2003). Loss of *fringe* (*fng*), a Notch pathway modulator, also results in missing polar cells (Grammont and Irvine, 2001, 2002). Expression of an active

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[†] These authors contributed equally to this work.

[‡] Present address: Department of Embryology, Carnegie Institution of Science, Baltimore, MD 21218.

Address correspondence to: Wu-Min Deng (wumin@bio.fsu.edu).

Figure 1. Staining of cell-cycle markers demonstrates that polar cells are arrested in the G2 phase. In all figures, the anterior end of the egg chamber is to the left, and the blue channel indicates DAPI staining, which marks cell nuclei. Primes indicate a magnification of the outlined area and highlight of the corresponding staining. β -Gal staining in A101 and PZ80 (red) highlights the positions of all polar cells. (A) CycB (green) can be seen in main-body follicle cells undergoing mitosis but is absent from G2-arrested polar cells in the wild type. (B) The G2/M-phase marker CycA (green) is shown in wild-type polar cells. (C) Wild-type polar cells do not express mitosis marker PH3 (green). (D) BrdU incorporation is not detected in wild-type polar cells, as revealed by the absence of BrdU (green) in cells expressing A101 (red).

form of Notch produces increased polar-cell numbers and long stalks between the egg chambers (Larkin *et al.*, 1996).

Although the mechanisms that regulate differentiation and apoptosis of the polar cells have been investigated, the molecular mechanism by which polar cells cease to proliferate and the functional significance of this event have not been addressed.

MATERIALS AND METHODS

Fly Stocks

The following fly stocks were used: *UAS-stg* (*P[UAS-stg.N]16*), *UAS-EGFP* (*P[UAS-2XEGFP/AX]*) (Bloomington Stock Center), *UAS-fzr* (gift from C. F. Lehner), *UAS-NICD* (an active form of Notch; Doherty *et al.*, 1996), *yw122*; *185.14/CyO* (*185.14* is a *UAS>CD2*, *y+>Su(H)-VP16* construct), and *E(Spl)mβ-CD2* (a Notch-activity reporter; gift from Sarah Bray, University of Cambridge, Cambridge, United Kingdom). To generate misexpression phenotypes and mutant clones, we used *hsFLP*; *UAS-GFP*, *act<cd2>Gal4/TM3, w[*]*; *noc-[Scal]/CyO*; *P[w/+mC] = tubP-GAL80[ts]7* used in expression of *UAS-NICD*, *upd-Gal4* (*Gal-E132*, a gift from Douglas Harrison, University of Kentucky, Lexington, KY), and *c306-Gal4* (Bloomington Stock Center). *A101/TM3* (*neuritized-lacZ*) and *c306:PZ80/CyO* were used as polar-cell fate markers (Larkin *et al.*, 1996; Adam and Montell, 2004). The *GBE-lacZ* (Uv *et al.*, 1997), *string-lacZ* (*psgβ-R6.4*; Lehman *et al.*, 1999), *fzr-lacZ* (*fzrG0326*, a *P[lacW]* enhancer trap line, Sun and Deng, 2005), and *domeless-lacZ* (Xi *et al.*, 2003) were used to detect expression of Notch, Stg, Fzr, and Domeless in wild-type and misexpression cells, respectively.

Immunocytochemistry and Bromodeoxyuridine Labeling

Immunocytochemistry was carried out as previously described (Deng *et al.*, 2001). The following antibodies were used: mouse anti-FasIII 1:15, mouse anti-CycB 1:5, mouse anti-Eya 1:10, mouse anti-Gurken, 1:20 (Developmental Studies Hybridoma Bank; DSHB), mouse anti-bromodeoxyuridine (BrdU) 1:50 (BD Bioscience), rabbit anti-PH3 1:200 (Upstate Biotechnology, NY), rabbit anti-CycA 1:500 (a gift from C. Lehner, University of Zurich, Zurich, Switzerland), rabbit anti- β -galactosidase (β -Gal) 1:5000 (Sigma), rabbit anti-Stau, 1:5000 (St. Johnston *et al.*, 1991), rabbit anti-cleaved caspase-3 (Asp 175) 1:100 (Cell Signaling Technology), and rabbit anti-VP16 (Abcam, Holmberg *et al.*, 2008).

Construction of Active *Su(H)*

UAS-Su(H)VP16 encodes a fusion protein in which the DNA-binding domain of *Drosophila* *Su(H)* is fused C-terminally with the activator domain of viral transcription factor protein VP16. *A>CD2*, *y+>flp-out* cassette (Zecca *et al.*, 1995) was inserted into *UAS-Su(H)VP16* to generate *UAS>CD2*, *y+>Su(H)VP16* transgene, which permits generation of *UAS>Su(H)VP16* in various developmental stages. The *>CD2*, *y+>flp-out* cassette of the *UAS>CD2*, *y+>Su(H)VP16* transgene can be flipped out in the germ-line cells, producing the *UAS-Su(H)VP16* transgene in the next generation.

Imaging

Images were taken with a Zeiss LSM 510 confocal microscope (Thornwood, NY) and assembled in Adobe Photoshop (San Jose, CA).

RESULTS

Polar Cells Show Sustained Cyclin A Expression

To determine the cell-cycle status of the polar cells, we stained wild-type egg chambers with different cell-cycle markers, including cyclin A (CycA) for G2 and M phases, BrdU for detection of S phase, and cyclin B (CycB) and phosphohistone-3 (PH3) for M phase. The polar cells were identified by staining of polar-cell markers PZ80 (Figure 1A) or A101 (Figure 1B). A sustained expression of CycA in the polar cells throughout oogenesis (Figure 1B) indicates these cells are arrested at either G2 phase or M phase. Further examination revealed lack of CycB and PH3 expression in polar cells (Figure 1, A and C), so these cells could not be at M phase. In addition, no S phase-specific BrdU incorporation was detected in polar cells (Figure 1D). Overall, these results suggest that the polar cells are arrested at G2 phase.

Expression of Fizzy-related Can Drive Polar Cells into an Endoreplication-like Phase

Cell-cycle-marker staining reveals that wild-type polar cells have continuous CycA expression and are arrested in G2 phase. To investigate this G2 arrest further, we looked at Fizzy-related (*Fzr*), which is a negative regulator of mitotic cyclins and induces cell-cycle switches. More specifically, *Fzr* is known to degrade CycA in main-body follicle cells to drive entry into the endoreplication cycle. Loss of *fzr* also prevents endoreplication in salivary-gland cells and follicle cells (Sigris *et al.*, 1995; Sigris and Lehner, 1997; Schaeffer *et al.*, 2004; Sun and Deng, 2005). In wild-type egg chambers, polar cells do not have *Fzr* expression (Figure 2A), nor do they undergo endoreplication. To determine whether *Fzr* could affect a cell-cycle switch in polar cells as it does in main-body follicle cells, we expressed *Fzr* in polar cells using *upd-Gal4* and detected BrdU incorporation (Figure 2B), as well as what appeared from DAPI staining, to be a size increase in the nuclei (Figure 2C). In the wild type from stages 3–8, none of the egg chambers examined were positive for BrdU incorporation in polar cells, whereas in the *Fzr*-up-regulated egg chambers, 23% (260 polar-cell pairs counted) were positive for BrdU incorporation. To determine whether CycA was still present, we stained with CycA antibody and found that these cells lacked CycA staining (Figure 2D), consistent with *Fzr*-mediated degradation. These data further support the idea that polar cells are initially arrested in G2 phase.

Figure 2. Expression of Fzr in polar cells results in transition from arrest to an endo-cycle-like phase. (A) Fzr (marked by *fzf-lacZ*, green) is not expressed in wild-type polar cells (arrow). (B) BrdU incorporation (green) is detected in the polar cells (red, marked by A101) with *fzf* expression (driven by *upd-Gal4*), indicating they are in S phase. (C) DAPI staining (blue) reveals that the polar-cell (red, marked by A101) nuclei with *fzf* expression are larger than those of the wild type. (D) CycA staining (green) is not detected in polar cells (red) with *fzf* expression, indicating loss of the normal G2 arrest.

Misexpression of String in Polar Cells Results in Polar-Cell Proliferation

In a previous study, misexpression of String (Stg), a *Drosophila* Cdc25-type phosphatase that triggers mitosis in G2-arrested cells by dephosphorylating Cdk1/cyclin B kinase (Edgar *et al.*, 1994), was shown to force G2-arrested SOP cells to enter mitosis during wing development (Nègre *et al.*, 2002). Conceivably, suppression of Stg expression is used to arrest cells in the developing eye and wing (Kylsten and

Saint, 1997; Johnston and Edgar, 1998; Neufeld *et al.*, 1998). Consistent with this idea, wild-type polar cells arrested in G2 phase do not express Stg (Figure 3A). To determine whether ectopic Stg is sufficient to drive polar cells to reenter the mitotic cycle, we applied the Gal4/UAS technique to drive expression of Stg in polar cells (Brand and Perrimon, 1993). First, we used *c306-Gal4*, which is expressed in follicle cells as early as the germarium and is later restricted to the polar cells and neighboring terminal follicle cells (Figure 3B,

Figure 3. Misexpression of String results in proliferation of polar cells. Polar-cell markers PZ80, A101, and FasIII (red) highlight the positions of polar cells. Because of difficulties inherent in producing a 2D image of a 3D egg chamber, not all the extra polar cells present in the egg chambers pictured here could be captured in the same confocal section. (A) Wild-type polar cells marked by FasIII (red) do not exhibit Stg expression (green). (B) The *c306-Gal4* expression pattern demonstrated with enhanced green fluorescent protein (EGFP, green) as a marker in wild-type egg chambers. (C) Chart of average numbers of polar cells observed at both poles from stage 5 to stage 10 for wild-type, *upd::UAS-Stg*, and *c306::UAS-Stg* egg chambers. (D) PH3 (green) expression in a fraction of extra polar cells (red) induced by Stg misexpression, indicating mitosis. (E) In egg chambers with Stg misexpression driven by *c306-Gal4*, extra polar cells are produced by mitosis, as revealed by colocalization of PZ80 (red) and CycB (green). (F) CycA (green) expression in a fraction of extra polar cells (red) induced by Stg misexpression, indicating mitosis. (G) EGFP expression (green) demonstrates the expression pattern of *upd-Gal4*, which is exclusively in the polar cells, indicated by A101 expression (red). (H) CycB (green) detected in polar cells (red) induced by Stg misexpression suggests that they are in mitosis. (I) Extra polar cells are found in *upd::UAS-Stg* egg chambers. Colocalization of polar-cell marker FasIII (green) with A101 (red) confirms the polar-cell fate.

expression pattern for *c306-Gal4*). In *c306::UAS-stg* egg chambers from stages 5–10, we found an average of four to five polar cells at each pole (Figure 3C; 1036 polar cell clusters counted), in contrast to two polar cells at each pole of the wild-type egg chamber. In addition, M phase markers CycB and PH3 were readily observed in these polar cells, suggesting that these cells are undergoing mitosis (Figure 3, D and E). Quantitatively CycA, CycB, and PH3 were expressed in 17.8, 19.4, and 2.34% of polar cells, respectively (Table 1). Furthermore, by careful examination, we found that CycB and PH3 were observed in polar cells with ectopic Stg as early as late stage 1, indicating that ectopic polar cells are already dividing at that point. Main-body follicle cells typically undergo mitosis until stage 6/7, at which point they cease mitosis and switch to the endocycle (Deng *et al.*, 2001; Lopez-Schier and St. Johnston, 2001). In all of the 184 stage 7–10 egg chambers with *c306::UAS-stg* we observed, PH3 staining was always in the polar cells but not in adja-

cent terminal follicle cells with Stg expression. This result suggests that the normal cell-cycle switch that occurs at

Table 1. Cell-cycle marker expression in polar cells in egg chambers with String misexpression

	CycA, %	CycB, %	PH3, %
<i>c306::UAS-stg</i>	17.8 (624)	19.04 (1029)	2.34 (683)
<i>upd::UAS-stg</i>	17.1 (321)	18.7 (315)	3.12 (416)

Each percentage represents the fraction of polar cells in either *c306-Gal4::UAS-stg* or *upd-Gal4::UAS-stg* egg chambers that express the cell-cycle markers CycA, CycB, and PH3. Values in parentheses represent the total number of polar cells examined for the listed cell-cycle marker. Egg chambers from stage 2 up to stage 9 were used.

Figure 4. The behavior of String-misexpression-induced extra polar cells. (A) Cleaved caspase-3 staining (green) detected in such polar cells in a stage 7 egg chamber, demonstrating that apoptosis is not suppressed. (B) Extra polar cells due to Stg misexpression induce extra border cells marked by *dome-lacZ* expression (green). Polar cells are marked by FasIII (red). (C) Grk (green) is properly localized to the anterior-dorsal corner of the oocyte in egg chambers with extra polar cells (red, marked by PZ80) induced by Stg misexpression in a stage 8 egg chamber. (D) Stau (green) is correctly localized at the oocyte posterior in a stage 9 egg chamber with extra polar cells (red) induced by Stg misexpression.

stage 6/7 of follicle cells is not being overridden by Stg misexpression and that continued mitotic proliferation is confined to polar cells.

The *c306-Gal4* driver is expressed in both polar cells and neighboring follicle cells. To exclude the possibility that the extra polar cells resulted from transformation of neighboring nonpolar cells by Stg misexpression, we used the polar-cell-specific Gal4 driver, *upd-Gal4* (Figure 3G, expression pattern), to express Stg. The *upd-Gal4* driver begins expression around late stage 1 or early stage 2. Like the expression of Stg driven by *c306-Gal4*, *upd-Gal4*-driven expression of Stg results in extra polar cells that also have CycA, CycB, and PH3 in a fraction of the cells—17.1, 18.7, and 3.12% respectively (Table 1)—suggesting that these cells enter the mitotic cycle (Figure 3H, CycB; Supplemental Figure S1, A and B, PH3 and CycA). In Stg-misexpressing egg chambers beyond stage 5, we also detected an average of four to five polar cells at each pole (1046 polar cell clusters counted), as revealed by FasIII and A101 (Figure 3, C and I). In some extreme cases, as many as 11 polar cells were detected in a cluster. The observed proliferation is probably not the result of induction of nonpolar cells into the polar-cell fate, because the average number of polar cells seen in both anterior and posterior when Stg was driven by *c306-Gal4* was nearly the same as the number observed when we expressed Stg in just the polar cells using *upd-Gal4*. Instead, Stg misexpression appears to drive existing polar cells to proliferate mitotically and to have no observable effect on the neighboring follicle cells. Taken together, our results suggest that polar cells are arrested in G2 phase and can be driven into proliferation by misexpression of the G2/M-phase modulator Stg.

Apoptosis Is Not Suppressed in Extra Polar Cells Induced by Stg Misexpression

In wild-type egg chambers, a starting pool of five to six polar cells is reduced by apoptosis until one pair remains at each

pole; when apoptosis is suppressed, clusters of up to six polar cells can be detected after stage 5 (Besse and Pret, 2003). To determine whether misexpression of Stg in polar cells affects apoptosis, we applied an antibody against cleaved caspase-3, the cysteine protease that appears in cells undergoing apoptosis, in *c306::UAS-stg* and wild-type egg chambers. In the wild type, apoptosis appears randomly in the polar cells during early oogenesis and ceases at stage 5, when only one pair of polar cells remains at each pole (Besse and Pret, 2003). In polar cells with Stg misexpression, the random appearance of caspase-3 staining was indistinguishable from that of the wild type up until stage 5, but thereafter these extra polar cells continued to show random caspase-3 staining (Figure 4A), suggesting that apoptotic mechanisms not only are still operating despite Stg misexpression but are actively trying to restrict the number of polar cells even after stage 5. Despite apoptosis, the average number of polar cells in Stg misexpressing egg chambers continued to grow from stages 5–8 and reached a plateau at stages 9–10 (Figure 3C). The continued presence of excess polar cells, despite active apoptosis, may result from an inability of the normal apoptotic mechanism to handle the large increase in polar cells that results from their continued mitosis. Nonetheless, these results suggest that apoptosis is still active and not suppressed by misexpression of Stg in polar cells.

Extra Polar Cells Induced by String Misexpression Allow Follicle-Cell Differentiation and Do Not Affect Oocyte Polarity

Differentiation of multiple subtypes of follicle cells is critical for proper egg-chamber development. In the anterior terminus of the egg chamber, polar cells play a critical role in initiating the follicle-cell differentiation process. They do so by releasing the Unpaired (Upd) protein, which in turn activates the JAK/STAT pathway in the neighboring follicle

cells to differentiate them further into border cells, which can be marked by *Dome-lacZ* expression (Brown *et al.*, 2001; Silver and Montell, 2001; Chen *et al.*, 2002; Ghiglione *et al.*, 2002; McGregor *et al.*, 2002; Xi *et al.*, 2003). If these excess polar cells were functional, they would be capable of inducing differentiation of surrounding follicle cells into border cells. In fact, we observed as many as 12–14 border cells, marked by *Dome-lacZ*, by stage 9–10 (Figure 4B) in Stg-misexpressing egg chambers, whereas normally, only four to eight are detected by stage 9–10 in the wild type (Silver and Montell, 2001). These extra border cells tend to form clusters that cover a larger radius than the wild-type polar cells. The ability of the extra polar cells to induce differentiation of surrounding follicle cells suggests that they can function like normal polar cells.

Posterior polar cells are involved in the differentiation of the posterior follicle cells, whose differentiation plays a key role in the establishment of oocyte polarity (Poulton and Deng, 2007a,b). We examined oocyte polarity using the posterior marker Staufen (Stau; St. Johnston *et al.*, 1991) and dorsal anterior marker Gurken (Grk) in egg chambers containing Stg-induced extra polar cells. Stau is an RNA-binding protein that normally localizes in the oocyte posterior by stage 9 (St. Johnston *et al.*, 1991), whereas *grk* mRNA normally localizes to the dorsal anterior of the oocyte (Neuman-Silberberg and Schüpbach, 1993; Gonzales-Reyes *et al.*, 1995; Roth *et al.*, 1995). The staining patterns of these two markers in *c306::UAS-stg* egg chambers are similar to those in the wild type (Figure 4, C and D), indicating that excess polar cells due to Stg misexpression do not affect oocyte polarity.

Ectopic Notch Activity Results in Extra Polar Cells through Recruitment of Neighboring Follicle Cells

Previous studies show that expression of a constitutively active form of Notch (Notch intracellular domain [NICD]) can result in excessive numbers of polar cells (Larkin *et al.*, 1996; Grammont and Irvine, 2002). We wondered whether NICD-mediated extra polar cells are a result of cell proliferation, much like that seen in misexpression of Stg, or whether a different mechanism was involved. To determine the answer, we counted the polar cells in egg chambers that express *UAS-NICD* or *UAS-Su(H)-VP16* with expression driven by either *upd-Gal4*, which drives expression in the polar cells, or *c306-Gal4*, which drives expression in the terminal regions. Because expression of either NICD or Su(H)-VP16 results in active Notch signaling (Supplemental Figure S1C, Notch activation in Su(H)-VP16 as revealed by Notch reporter *GBE-lacZ*), these two UAS constructs produce similar results. We found that, in contrast to expression of Stg in polar cells, *upd-Gal4*-driven expression of NICD or Su(H)-VP16 did not result in extra polar cells (Su(H)-VP16 expression in Figure 5A, NICD expression not shown); the number of polar cells at each end of the egg chamber remained the same as that in the wild type. No expression of M-phase markers (PH3 or CycB) was detected in the polar cells (data not shown). In contrast to those with *upd-Gal4*-driven expression, egg chambers with *c306-Gal4*-driven expression of NICD or Su(H)-VP16 did have extra polar cells (Figure 5, B–E). The polar-cell status of these cells was confirmed by lack of Eya expression (Bai and Montell, 2002; Figure 5B), but unlike the polar cells generated by Stg misexpression, extra polar cells resulting from Notch activation did not express mitotic markers such as CycB or PH3. Instead, they showed continued expression of CycA (Figure 5, C–E), suggesting that they are arrested in G2 phase and not in M phase. In addition, these extra polar cells showed other noticeable differences from those of the Stg-misexpression

phenotype. The Notch-induced extra polar cells tend to be spread out rather than tightly clustered at the two ends of the egg chambers. Also, these cells were sometimes excluded from the follicle-cell epithelium covering the germ-line cells and instead formed a cluster located between two egg chambers, obviously unassociated with germ-line cells. The contrast in phenotypes for these two drivers is probably due to their regions of expression; *upd-Gal4* is restricted to the polar cells, whereas *c306-Gal4* is expressed in the majority of early follicle cells at the terminal ends. Therefore, induction of extra polar cells by *c306-Gal4* but not *upd-Gal4*-driven Notch activation suggests that the extra polar cells generated by Notch signaling are not the result of extra mitotic divisions of the existing polar cells. Instead, Notch activation probably induces transformation of terminal follicle cells into the polar-cell fate. That is, unlike Stg, Notch signaling does not enable existing polar cells to proliferate mitotically but instead induces a change of cell fate, directing follicle cells that would not normally become polar cells into the polar-cell fate. The lack of a distinctive phenotype in *upd-Gal4*-driven Notch activation is not surprising, because Notch is already active in wild-type polar cells.

Previous studies indicated that Notch signaling causes follicle cells to exit the mitotic cycle and enter the endoreplication cycle through down-regulation of Stg during stage 6/7 of oogenesis (Deng *et al.*, 2001; Lopez-Schier and St. Johnston, 2001). Notch may have a similar role in regulation of Stg in the polar cells to induce cell-cycle arrest. Because loss of Notch signaling in follicle-cell precursors in the gerarium results in a total loss of polar cells (Grammont and Irvine, 2001, 2002; Torres *et al.*, 2003), the loss-of-function approach of generating Notch mutant-cell clones cannot be used to study the polar-cell cell cycle. We therefore analyzed Stg expression by examining a *stg-lacZ* reporter, *pstgβ-R6.4* (Lehman *et al.*, 1999; Deng *et al.*, 2001), in the ectopic polar cells induced by Notch activation. Although wild-type polar cells typically do not express Stg (Figure 3A), the follicle cells neighboring them normally do so until stages 6/7 (Deng *et al.*, 2001), but when the neighboring follicle cells are directed into the polar-cell fate by Notch activation, they appear to lose Stg expression (Figure 6A). Of the 301 polar cells counted in egg chambers with *c306-Gal4*-induced Notch activation, none was found to have Stg expression (Figure 6A), suggesting that Notch activation in the polar cells causes Stg down-regulation.

Given that Notch signaling could induce extra G2-arrested polar cells, we wondered whether Notch signaling induces this cell-cycle arrest by suppressing Stg. If so, misexpressing activated Notch and Stg simultaneously by means of the driver line *c306-Gal4* would enable the Notch-induced extra polar cells to enter mitosis. When ectopic expression of both NICD and Stg was driven by *c306-Gal4*, we observed that the M-phase marker PH3 was detected randomly in the extra polar cells, indicating that these extra polar cells were indeed in mitosis (Figure 6, B and C). This situation is in contrast to Notch activation alone; no PH3 staining or any other mitosis marker was detected in Notch-activated cells. The ability of Stg to induce mitosis when expressed with ectopic Notch activation indicates that it may operate downstream of Notch signaling. Given that Notch has already been shown to direct mitotic follicle cells into an arrested polar-cell state and that Stg expression can reverse this arrest in ectopic polar cells generated by Notch, Stg is a likely target of suppression causing G2 arrest in Notch-induced polar cells. Because Notch signaling is critically required for follicle-cell fate determination during oogenesis (Ruohola *et al.*, 1991; Grammont and Irvine, 2001), Notch signaling may

Figure 5. Misexpression of active Notch induces extra polar cells through recruitment of neighboring follicle cells. (A) Misexpression Su(H)VP16 in just the polar cells (driven by *upd-Gal4*) produces a phenotype similar to that of the wild type. (B) Ectopic expression (driven by *c306-Gal4*) of Su(H)VP16 induces extra polar cells (outlined) as revealed by polar-cell marker PZ80 (red) and lack of Eya (green, normally absent from polar cells but present in main-body follicle cells). (C–E) Extra polar cells (polar cells marked by PZ80, red) induced by misexpression of active Notch do not have CycB (green in C) or PH3 (green in D) expression but show continuous expression of CycA (green in E).

play its role, in part, by ensuring proper differentiation of follicle cells into polar cells and by suppressing Stg expression, so that the differentiated polar cells are arrested in G2 phase and undergo no further proliferation. The events of polar-cell fate specification and G2 arrest may be coupled in response to Notch activation.

DISCUSSION

During *Drosophila* oogenesis, proliferation and differentiation of follicle cells are critical for regulation of growth and polarity of the egg chamber. An important aspect of these processes is

the regulation of the cell cycle through cell-cycle transition or cell-cycle arrest. Previous studies showed that Notch signaling is required for the mitotic cycle/endocycle switch and differentiation in *Drosophila* follicle cells (Deng *et al.*, 2001; Lopez-Schier and St. Johnston, 2001). Notch regulates this switch by down-regulating Stg expression, thus preventing the main-body follicle cells from entering M phase (Deng *et al.*, 2001). Notch signaling is also critical for regulating polar-cell numbers. Normally, the number of polar cells at each end of the egg chamber is reduced to two by stage 5 of oogenesis. Loss of Notch signaling in follicle-cell precursors in the germarium results in a total loss of polar cells, whereas ectopic expression

Figure 6. String and Notch expression in extra polar cells. (A) Extra polar cells (red, marked by FasIII) induced by *c306-Gal4*-driven Su(H)VP16 do not show *stg-lacZ* (green). (B and C) Coexpression of active Notch and Stg in anterior (B) and posterior (C) terminal follicle cells (driven by *c306-Gal4*) results in extra polar cells (red, marked by PZ80) that are in active mitosis, as revealed by PH3 staining (green).

of activated Notch can give rise to excessive numbers of polar cells (Larkin *et al.*, 1996; Grammont and Irvine, 2002). Conceivably, the restriction of polar-cell number is regulated by cell-cycle arrest through Notch signaling and perhaps by Stg as well.

Polar-Cell Cycle Arrest and Cell Number

Previous studies suggest that two mechanisms are involved in controlling the number of polar cells: First, a group of three to five polar cells becomes nonproliferative; second, excess polar cells undergo apoptosis until the number reaches two (Margolis and Spradling, 1995; Tworoger *et al.*, 1999; Besse and Pret, 2003). Our results further reveal that the nonproliferative polar cells are actually arrested in a G2-like phase as revealed by cell-cycle phase markers like CycA CycB, PH3, *fzr-lacZ*, and BrdU incorporation.

We also conclude, on the basis of the following observations, that variation in polar-cell numbers can be tolerated during oogenesis, probably because of some type of inherent plasticity. We found that G2-arrested polar cells can be brought into mitosis through ectopic expression of Stg, resulting in excess numbers of polar cells and consequently extra border cells, without affecting oocyte polarity. Therefore, the number of polar cells does not significantly affect egg-chamber development or their role in directing neighboring follicle cells to differentiate. The flies derived from both the *c306::UAS-stg* and *upd::UAS-stg* egg chambers carrying additional anterior or posterior polar cells developed into adults free of obvious phenotypic defects. This phenomenon is analogous to what occurs in eye development, where misexpression of Stg in G2-arrested cells of eye discs induces proliferation but does not result in obvious phenotypic defects in the eyes of adult flies (Kylsten and Saint, 1997).

Coupling Cell-Cycle Arrest and Polar-Cell Differentiation

Notch signaling is critical for regulating polar-cell numbers (Grammont and Irvine, 2001, 2002; Torres *et al.*, 2003) and is, conceivably, also coupled with cell-cycle arrest. We found that, like ectopic Stg expression, ectopic activation of Notch signaling can result in excess polar cells, but the Notch-induced extra polar cells are derived from follicle cells adjacent to the original polar cells and are arrested in G2 phase, whereas Stg-induced extra polar cells are the result of further cell proliferation of the original G2-arrested polar cells. The role that Notch signaling plays in controlling polar-cell number seems therefore to differ from that of Stg. Previous studies indicate that Notch plays a role in inducing a mitotic-to-endocycle switch at stages 6/7 during oogenesis by suppressing Stg expression (Deng *et al.*, 2001). Similarly, therefore, Notch signaling might arrest polar cells in G2 phase by suppressing Stg expression when regulating polar-cell differentiation. Our results, which show that extra polar cells induced by simultaneous expression of activated Notch and Stg can exit from G2 phase and enter M phase, support the idea that Stg expression might be down-regulated by Notch signaling to arrest the polar cells and restrict their numbers during *Drosophila* oogenesis. Whether Stg suppression is necessary for other functions beyond preventing continued proliferation is currently unknown.

The different roles of Notch and Stg also explain why the extra polar cells they induce have different arrangements in the follicle-cell epithelium. Because Stg induces proliferation of existing polar cells, any extra cells produced will remain in the immediately adjacent area, whereas Notch, driven by *c306-Gal4*, is expressed in the majority of early follicle cells at the terminal ends, including the stalk cells, thus inducing polar cell fate over a wide area.

Why does Notch cause the main-body follicle cells to undergo endoreplication, whereas the polar cells undergo cell-cycle arrest? One of the obvious differences between the two cell types is in whether Cut, a homeodomain protein, is expressed. We previously showed that Cut down-regulation by Notch at stage 6/7 plays a critical role in causing those cells to enter the endocycle. Forced expression of Cut with Stg induces additional mitotic division (Sun and Deng, 2005). In contrast, the polar cells continue to express Cut (Jackson and Blochlinger, 1997; Sun and Deng, 2005), so only expression of Stg is needed for additional mitotic cycles in these cells. A role of Cut in main-body follicle cells is to suppress the expression of Fzr; Cut down-regulation in main-body follicle cells at stage 7 allows accumulation of Fzr and degradation of CycA (Sun and Deng, 2005). In polar cells, because of the consistent presence of Cut, Fzr is repressed to allow CycA expression, and the cells therefore do not undergo endoreplication. Consistently, expression of Fzr in polar cells degrades CycA and drives polar cells into an endocycle-like status.

Our work shows that polar cells, as a model for study, can provide insight into cell-cycle progression and developmental cell-cycle arrest and can allow us a greater understanding of cell proliferation and how developmental signals regulate the arrest.

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