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Drosophila follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways

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The specification of polar, main-body and stalk follicle cells in the germarium of the *Drosophila* ovary plays a key role in the formation of the egg chamber and polarisation of its anterior-posterior axis. High levels of Notch pathway activation, resulting from a germline Delta ligand signal, induce polar cells. Here we show that low Notch activation levels, originating from Delta expressed in the polar follicle cells, are required for stalk formation. The metalloprotease Kuzbanian-like, which cleaves and inactivates Delta, reduces the level of Delta signaling between follicle cells, thereby limiting the size of the stalk. We find that Notch activation is required in a continuous fashion to maintain the polar and stalk cell fates. We further demonstrate that mutual antagonism between the Notch and JAK/STAT signaling pathways provides a crucial facet of follicle cell patterning. Notch signaling in polar and main-body follicle cells inhibits JAK/STAT signaling by preventing STAT nuclear translocation, thereby restricting the influence of this pathway to stalk cells. Conversely, signaling by JAK/STAT reduces Notch signaling in the stalk. Thus, variations in the levels of Notch pathway activation, coupled with a continuous balance between the Notch and JAK/STAT pathways, specify the identity of the different follicle cell types and help establish the polarity of the egg chamber.

KEY WORDS: Notch, Delta, JAK/STAT, Unpaired, Follicle cells, Oogenesis, Kuzbanian-like (Kul)

INTRODUCTION

During oogenesis in *Drosophila*, successive rounds of symmetry-breaking events shape the egg chamber, and consequently define the future axes of the embryo. Egg chambers are individual units, comprising a germline cyst and a surrounding layer of somatic follicle cells, which are connected by short 'stalks' and develop within a shared ovariole. Patterning of the egg chamber relies on a continuous crosstalk between somatic and germline cells, which through processing and refinement of positional information serves to drive a diverse series of morphogenetic processes and cell-fate determinations. These include establishment of a stem cell 'niche' for the germline progenitors, definition of specialized cell types within the follicle cell epithelium, specification and positioning of the oocyte, and construction of a specialized cytoskeleton within the oocyte that defines the embryonic axes (Lopez-Schier, 2003; Ohlstein et al., 2004; Van Buskirk and Schupbach, 1999).

Establishment of distinct follicle cell fates at the early stages of oogenesis is crucial for achieving the proper morphology of individual egg chambers. Three distinct follicle cell populations are defined at this stage: polar cells, which serve as key signaling centers, stalk cells, which will form the short bridge that connects neighboring egg chambers, and main-body follicle cells, which form an epithelium overlying the germline cyst (see Fig. 1). Deciphering the mechanisms underlying the assignment of these distinct cell fates is a particularly challenging endeavour, in view of the limited asymmetries existing at this stage.

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Polar and stalk cells are thought to arise from a common precursor population (Lopez-Schier, 2003; Tworoger et al., 1999). Polar cell fate is induced in a restricted subset of this population by the Notch ligand Delta (Dl), which is produced in germline cells, in conjunction with expression of Fringe (Fng) in the follicle cells (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). Polar cells, in turn, express the ligand Unpaired (Upd; Outstretched – Flybase), which activates the JAK/STAT signaling pathway in neighboring polar/stalk precursors, thereby inducing the stalk cell fate (Baksa et al., 2002; Ghiglione et al., 2002; McGregor et al., 2002). JAK/STAT signaling is not sufficient to induce the stalk, however, as the Notch pathway has also been implicated in the establishment of stalk cell fate (Larkin et al., 1996; Torres et al., 2003). The effect of Upd expression in polar cells on follicle cell patterning extends beyond the polar/stalk precursors to the adjacent population of main-body follicle cells. The resulting gradient of JAK/STAT signaling in these cells induces them to adopt terminal fates, but this probably occurs at a later stage of oogenesis, many hours after stalk induction (Beccari et al., 2002; Grammont and Irvine, 2002; Silver and Montell, 2001; Xi et al., 2003).

Notch signaling typically dictates a binary cell-fate choice (reviewed by Lai, 2004). In this study, we report an alternative function of this pathway, in which multiple levels of Notch activation, coupled with antagonistic interactions with the JAK/STAT pathway, define three distinct follicle cell types.

MATERIALS AND METHODS

Drosophila genetics and stocks

For temporal regulation of UAS construct expression, 2- to 3-day-old females carrying various combinations of GAL4 and UAS constructs, as well as P(tubP-GAL80^{ts}) (McGuire et al., 2003), were shifted from 25°C to 29°C for 2-3 days, leading to GAL80 inactivation and promotion of GAL4 activity.

Mutant clones were generated by mitotic recombination using the FLP-FRT technique (Xu and Rubin, 1993), heat shocking newly hatched females for 2 hours at 37°C on 2-3 consecutive days. Flies were kept at 25°C and dissected 2 days after heat shock.

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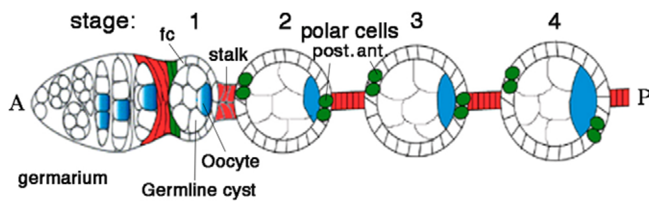

Fig. 1. Early oogenesis in *Drosophila*. A diagram of the anterior portion of a wild-type ovariole, including the germarium and four young egg chambers (stages 1-4). Polar cells (green) and stalk cells (red) are shown both at the precursor stage (near the germarium), and following their differentiation. Oocytes are marked in blue throughout. fc, follicle cells; post., posterior; ant., anterior.

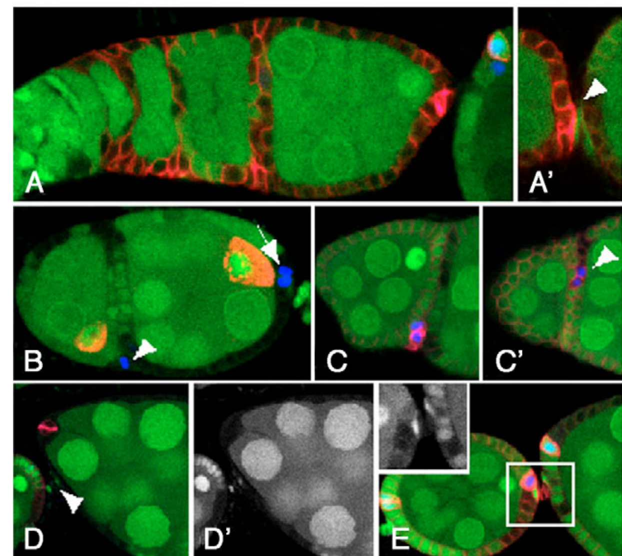

Fig. 2. Delta is required in anterior follicle cells for stalk formation. *Dl* homozygous clones were induced in FRT82B Dl^{M1}/FRT82B GFP females, and are marked by the loss of GFP (green). (**A**,**A′**) Germline cysts completely surrounded by *Dl*-mutant follicle cells fuse with the neighboring anterior cyst, but have a normal posterior stalk (arrowhead in A′). Fas3 (red) marks the follicle cell membranes. (**B**) Both the anterior (arrowhead) and posterior (arrow) pairs of polar cells (marked with PZ80, blue) are specified in *Dl* follicle cell clones. (**C**,**C′**) Two confocal planes of a pair of fused egg chambers show that the anterior polar cells (C′, arrowhead) of the older cyst are mutant for *Dl*. Polar cells are labeled with $neur^{A101}$ (blue) and Fas3 (red). (**D**,**D′**) In rare cases, an anterior stalk (arrowhead in D) forms even when both anterior polar cells (Fas3, red) are mutant for *Dl*. (**E**) *Dl* stalk cell clones appear as wild type. $neur^{A101}$ (blue) and Fas3 (red).

The following *Drosophila* lines were used: N^{55e11}FRT101/FM7 (Couso and Martinez Arias, 1994), FRT82B Dl^{M1}/TM3 (de Celis et al., 1991), FRT82B GFP, FRT101 GFP (Luschnig et al., 2000) and hop^{msvi}/FM7 (Perrimon and Mahowald, 1986); reporter lines: *Gbe+Su(H)*$_{m8}$*-lacZ* (Furriols and Bray, 2001), *m7-lacZ* (gift of S. Bray, University of Cambridge, UK), *2XSTAT92E-GFP* (Bach et al., 2006), PZ80-*lacZ* (Karpen and Spradling, 1992), $neur^{A101}$ (Clark et al., 1994), how^{93F} (Ruohola et al., 1991) and hop^{GA32}*/Dp/C(1:Y)* (gift of D. Harrison, University of Kentucky, KY); GAL4 lines: P(GAL4)how^{24B} (referred to in text as 24B-GAL4) (Brand and Perrimon, 1993), P(GAL4) $neur^{P72}$ (referred to in text as A101-GAL4) (Bellaiche et al., 2001), Upd-GAL4 (gift of D. Harrison) and 109-53-GAL4 (Bloomington); UAS lines: UAS-N^{ECN}, UAS-Dl^{H}-MH1, UAS-mCD8::GFP (Lee and Luo, 1999), UAS-N-$dsRNA^{14E}$ (Presente et al., 2002), UAS-Upd (Chen et al., 2002), UAS-ds*kul* (Sapir et al., 2005) and UAS-p35 (Bloomington).

In situ hybridization and antibody staining procedures

Ovary dissections, in situ hybridization and antibody stainings were performed according to standard procedures (Roth and Schupbach, 1994; Verheyen and Cooley, 1994). Detection of the 500 bp *Kul* RNA antisense probe was performed using a fluorescent alkaline phosphate substrate (SK-500, Vector Laboratories).

Primary antibodies used included the following: mouse anti-Fas3 (gift of T. Volk, Weizmann Institute, Rehovot, Israel; 1:20), rabbit anti-GFP (Cappel, 1:10,000), mouse anti-Dl (DSHB monoclonal C594.9B, 1:100), rabbit anti-β-gal (Cappel, 1:10,000), rabbit anti-STAT (Chen et al., 2002) (1:1000), mouse anti-Eya (DSHB monoclonal 10H6, 1:10), anti-Bib (Larkin et al., 1996) (1:1000), anti-Orb (DSHB 6H4 and 4H8, 1:400), anti-E-Cadherin (Shotgun; 1:100) (Oda et al., 1993) and rabbit anti-BicD (gift of R. Wharton, Duke University Medical Center, Durham, NC; 1:1000).

Secondary antibodies conjugated with Alexa-488, Cy3 or Cy5 (Molecular Probes) were used at 1:200. Samples were mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories).

RESULTS

Delta is required in the anterior polar cells to form the stalk

Stalk formation between adjacent egg chambers is induced by directional signaling from the anterior polar cells of the older (posterior) egg chamber (Torres et al., 2003). Signaling via the JAK/STAT pathway provides an essential component of this process (Baksa et al., 2002; McGregor et al., 2002; Xi et al., 2003), but various indications have suggested a role for the Notch pathway as well (Bender et al., 1993; Keller Larkin et al., 1999; Lopez-Schier and St Johnston, 2001; Ruohola et al., 1991). To verify the requirement for Notch signaling in the induction of stalk cells, we generated follicle cell clones that are mutant for *Dl*, the primary Notch ligand during oogenesis. Despite the proper specification of polar cells, egg chambers containing *Dl* follicle cell clones often failed to form a stalk on their anterior side, and as a result fused to the neighboring egg chamber (Fig. 2A-C). Such clones always encompassed follicle cells at the anterior portion of the egg chamber, indicating that Dl produced by anterior follicle cells is necessary to form an anterior stalk. However, the stalk positioned on the posterior side of these egg chambers was normal, even when the *Dl* clone surrounded the entire germline cyst (Fig. 2A). This is in keeping with our previous report (Torres et al., 2003) that posterior follicle cells do not contribute to stalk formation.

In order to determine which cells of the anterior follicle cell population provide the signal for stalk formation, we analyzed small anterior *Dl*-mutant follicle cell clones. In all cases where *Dl*-mutant clones led to loss of the stalk, the anterior polar cells were included in the mutant clone (*n*=24, Fig. 2B,C), suggesting that these cells are the source of Dl signaling. We did observe, however, a few instances in which an anterior stalk formed even though both polar cells were mutant for Dl (Fig. 2D). Since the polar cell population defined by expression of Fng is initially larger, and is reduced to two cells by programmed cell death (Althauser et al., 2005; Besse and Pret, 2003), this most probably resulted from the presence of wild-type Dl-expressing polar cells that provided the signal prior to their apoptosis. No phenotype was observed when the stalk cells themselves were mutant for *Dl* (Fig. 2E), indicating that Dl production by the stalk cells is not required for stalk specification.

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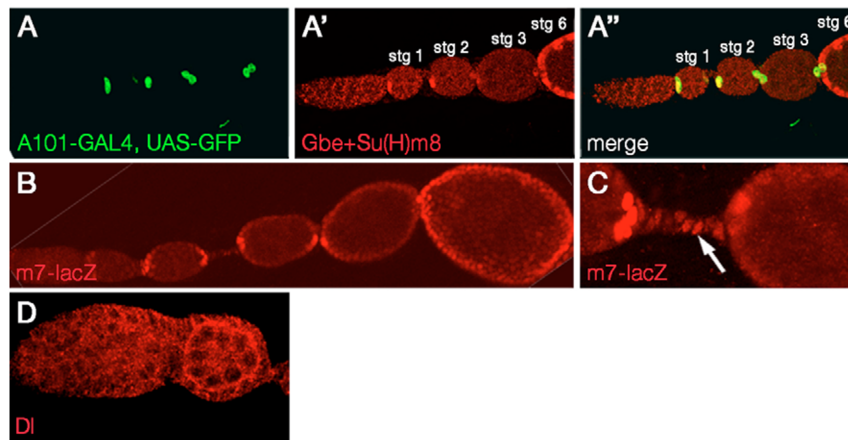

Fig. 3. Activation profile of Notch during early stages of follicle cell patterning. (**A-A″**) During the earliest stages of oogenesis, Notch activation in follicle cells (monitored by anti-β-gal staining of the *Su(H)*$_{m8}$*-lacZ* reporter, red) is restricted to the polar cells (marked by A101-GAL4/UAS-mCD8::GFP, green), and cannot be observed in stalks. The prominent signal in follicle cells of later egg chambers corresponds to the differentiation of main-body follicle cells and their switch from mitosis to endoreplication, following germline Dl induction of Notch signaling at stages 5-6 of oogenesis (Lopez-Schier and St Johnston, 2001; Ruohola et al., 1991). (**B**) Anti-β-gal staining of the Notch reporter *m7-lacZ* identifies essentially the same pattern as the *Su(H)*$_{m8}$*-lacZ* reporter. (**C**) However, with the *m7-lacZ* reporter, low levels of Notch activation could also be observed in the stalk cells at early stages (arrow). (**D**) Immunolocalization of Dl (red) reveals relatively higher levels of Dl protein in the germ line than in the follicle cells in early egg chambers.

Profile of Notch activation in follicle cells during early oogenesis

The results above indicate that Notch signaling is required for at least two processes of follicle cell patterning during early oogenesis: specification of polar cells induced by Dl from the germ line (Lopez-Schier and St Johnston, 2001) and, as shown here, induction of stalk by Dl provided by anterior polar cells. How are these two signals distinguished, and what is the temporal relationship between them?

We used the universal Notch transcriptional reporter *Gbe+Su(H)*$_{m8}$*-lacZ* (Furriols and Bray, 2001) to follow the activation profile of Notch signaling throughout oogenesis (Fig. 3). During stages 2-3 of oogenesis, we observed variations in the strength of Notch pathway activation within different anterior follicle cell types (Fig. 3A). Activation of Notch was observed in the polar cells, but no activation could be detected at this resolution in the stalk cells. These observations indicate that the level of Notch activation in the stalk cells is significantly lower than in the polar cells. Utilization of a second Notch reporter (*m7-lacZ*) identified essentially the same pattern. However, as this reporter appears to be more sensitive than *Gbe+Su(H)*$_{m8}$*-lacZ*, low levels of Notch activation in the stalk cells at early stages could also be observed (Fig. 3B,C).

Expression of Fng specifically in the future polar cells (Grammont and Irvine, 2001), provides a possible basis for the enhanced magnitude of Notch signaling in these cells. Polar cells are also part of the follicle cell population adjacent to the germline nurse cell complex, in which overall levels of Dl protein appear relatively high (Fig. 3D). However, the fraction of Dl localized to the nurse-cell membranes is difficult to quantify, preventing us from attributing with confidence the differences in signaling levels during early oogenesis to this parameter.

To define the temporal sequence of polar and stalk cell induction, we followed the expression of specific markers for each cell type (Fig. 4A,B). Polar and stalk cell markers are first detected in stage 1 egg chambers (region 3 of the germarium). Markers of both cell types could be detected simultaneously in some egg chambers, where they were aligned as broad adjacent bands, with the polar cell marker always positioned towards the posterior. All other egg chambers at this stage displayed expression of the polar cell marker alone. These observations imply that polar cells are induced first, and, in agreement with the genetic evidence, are properly positioned to signal and induce stalk cell formation at the anterior end of the egg chamber.

Taken together, these data suggest that distinctions in both the strength of signaling via the Notch pathway and the temporal sequence of pathway activation contribute to distinct cell-fate outcomes within the population of anterior follicle cells during early *Drosophila* oogenesis.

Delta levels determine stalk size

We previously showed that the metalloprotease Kuzbanian-like (Kul) cleaves Dl in a cell-autonomous manner, leading to its downregulation (Sapir et al., 2005). Modulation of Kul levels therefore provides a sensitive tool for manipulating Dl signaling activity in vivo. We sought to determine whether Kul functions within follicle cells during early oogenesis. The expression pattern of Kul during oogenesis was monitored by fluorescent RNA in situ hybridization. Whereas *Kul* RNA was not detected in the germ line, prominent expression was observed in follicle cells, up to stage 3 (Fig. 4C,D).

Kul levels can be effectively reduced by expression of a specific UAS-dsRNA construct (Sapir et al., 2005). Since expression of *Kul* dsRNA by various GAL4 drivers resulted in lethality, expression of this construct was restricted to adult stages through the use of a temperature-sensitive GAL80 inhibitor system (McGuire et al., 2003). This approach was used throughout the study to enable expression of various UAS-based transgenes during oogenesis. We employed the GAL80^{ts} system in conjunction with the *neur*-GAL4 driver (A101-GAL4) (Bellaiche et al., 2001) to specifically express *Kul* dsRNA in polar cells, and assess the effect of Kul on Notch signaling in early follicle cells. Notch transcriptional reporter activity was examined in these egg chambers, and the position and intensity of staining compared with wild-type egg chambers that

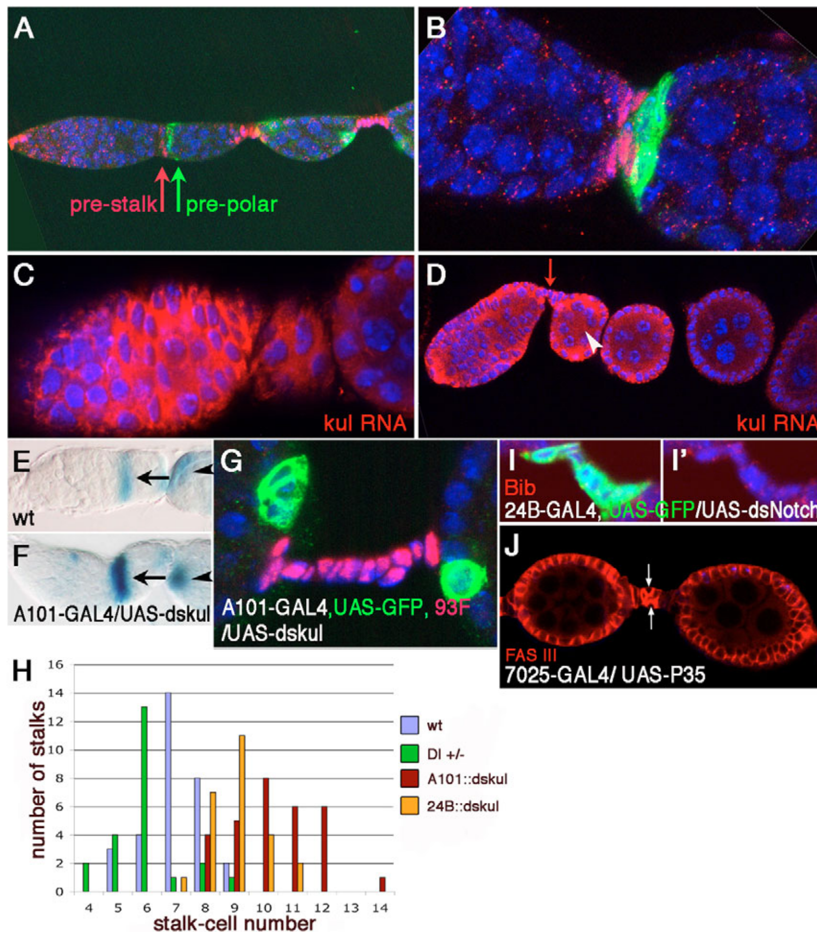

Fig. 4. The levels of Dl from follicle cells determine stalk size. (**A**,**B**) Precursor-stage polar cells (green, marked by A101-GAL4/UAS-mCD8::GFP) and stalk cells (red, marked by the *how*^{93F} reporter) initially align as broad neighboring bands in region 3 of the germarium. The polar cell precursors are always positioned posteriorly. Nuclei are stained with DAPI (blue). (**C**,**D**) In situ hybridization using a fluorescent *Kul* RNA probe. In surface (C) and cross-section (D) views, *Kul* RNA (red) is detected in all follicle cells at early stages, including the stalk (arrow), but hardly in the germ line itself (arrowhead). (**E**,**F**) Notch activation during early oogenesis, monitored by X-Gal staining of the *Su(H)*$_{m8}$-*lacZ* reporter. Arrows point to the germarium and arrowheads to stage 2 polar cells. Notch activation in A101-GAL4/UAS-ds*kul*- ovarioles (F) is elevated as compared with wild type (E). (**G**) Expression of UAS-ds*kul* in polar cells by A101-GAL4 (green) leads to an increase in stalk-cell number. The stalk is marked by the 93F *lacZ* reporter (red). (**H**) Quantification of stalk size in various genetic backgrounds. Stalk-cell numbers are increased following expression of ds*kul* in either polar (dark red columns) or stalk cells (orange columns), and reduced in *Dl* heterozygous ovaries (green columns). (**I**,**I'**) Expression of *Notch* dsRNA in the stalk cells (green) using the 24B-GAL4 driver leads to loss of the stalk marker Bib (red). (**J**) Expression of p35 in both polar and stalk cells using the 7025-GAL4 driver results in an abnormal stalk containing an excess of cells (arrows).

were processed under identical conditions (Fig. 4E,F). Following expression of ds*kul* in polar cells, Notch reporter levels were significantly elevated, both in the germarium and in stage 1-3 egg chambers. These observations indicate that Kul acts as an attenuator of Dl signaling in early-stage follicle cells. Interference with Kul function in this fashion thus provides a means to address the significance of follicle cell Dl levels for proper stalk cell induction. Indeed, expression of ds*kul* in the polar cells led to a significant increase in stalk-cell number, from an average of 7.0 to 10.3 cells per stalk (Fig. 4G,H).

These results indicate that the size of the stalk is highly sensitive to the amount of Dl signaling between follicle cells. This is in agreement with previous experiments, in which the size of the stalk was dramatically increased following a mild hyperactivation of Notch (Larkin et al., 1996). Consistent with these data, ovaries from heterozygous *Dl* females have a reduced number of stalk cells (averaging 6.0, Fig. 4H), underscoring the sensitivity of the system to levels of Dl signaling.

To determine whether stalk cells remain sensitive to Notch pathway signaling following their differentiation, we first expressed ds*kul* in the stalk cells themselves, using the 24B-GAL4 stalk cell-specific driver, and observed an increase in the number of stalk cells to an average of 9.0 (Fig. 4H). Kul thus attenuates Dl levels even after the stalk is formed, implying that stalk-cell number is regulated by Dl signaling from both polar cells and the stalk cells themselves. In a converse experiment, Notch signaling was reduced or eliminated from the stalk cells. Expression of ds*Notch* (Fig. 4I), or of a dominant-negative Notch construct (not shown), by the 24B-GAL4 stalk cell-specific driver led to the disappearance of the stalk marker Big brain (Bib) (Larkin et al., 1996). Thus, persistent, low level activation of Notch is required to maintain stalk cell fate. The low levels of Dl employed for this purpose are presented initially at the polar cell-stalk cell boundary, but as the stalk becomes elongated they might be displayed by neighboring stalk cells.

We have shown above that Dl is required for establishment and maintenance of the stalk cell fate (Fig. 2). The sensitivity of stalk size to the levels of Dl provided by the stalk cells themselves (Fig. 4) suggests that Dl also affects stalk cell proliferation or survival. To examine this possibility, the anti-apoptotic protein p35 was expressed in both polar and stalk cells using the 109-53-GAL4 driver. We observed a greater abundance of cells not properly arranged into a one-cell-wide stalk (Fig. 4J). This suggests that excess stalk cells are normally eliminated by apoptosis, and would support a model in which Dl is required for stalk cell survival, as well as stalk differentiation.

The above observations suggest that different levels of Notch signaling determine the final fate of cells from within the polar/stalk precursor population – a strong germline signal induces the polar cell fate, whereas a weaker follicle cell signal induces the stalk. As an additional test of this model, we examined the effects of strongly elevating the Notch follicle cell signal, by overexpression of Dl specifically in polar cells. Overexpression of Dl using polar cell-specific GAL4 drivers had dramatic effects on anterior follicle cell fate and tissue morphology (Fig. 5A-C). Significantly, this alteration in Notch signaling resulted in an excess of polar cells. Supernumerary polar cells formed primarily at the expense of stalk

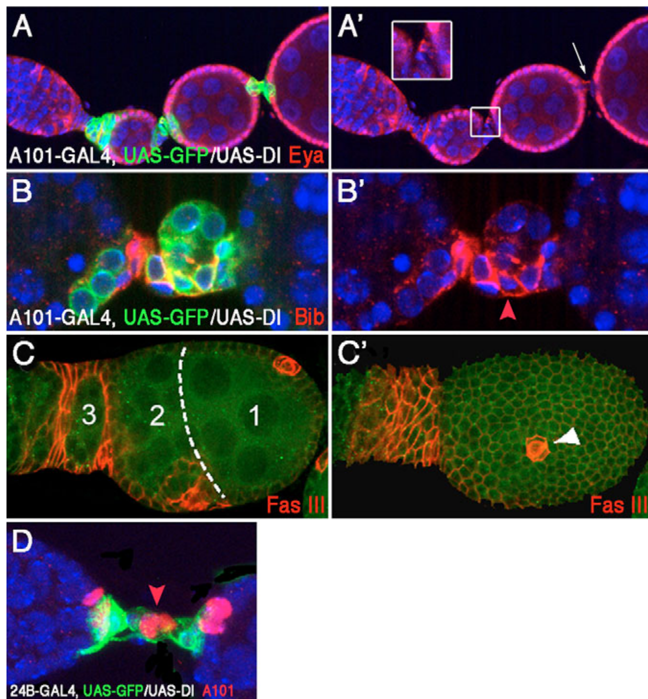

Fig. 5. Overexpression of Delta alters follicle cell fates and antagonizes Unpaired signaling. (**A-B′**) Dl overexpression using the A101-GAL4 polar cell-specific driver induces extra polar cells (green). Some of these extra cells express the main-body follicle cell marker Eya (arrow, red in A,A′) or the stalk marker Bib (arrowhead, red in B,B′). (**C**,**C′**) Dl overexpression using the Upd-GAL4 polar cell-specific driver results in loss of the stalk and fusions between neighboring cysts. Two confocal planes of three germline cysts from a single ovariole are shown. Fas3 (red) marks polar cells in the oldest egg chamber (designated #1, arrowhead). (**D**) Dl overexpression using the 24B-GAL4 stalk cell-specific driver induces the expression of the polar cell marker, $neur^{A101}$ (arrowhead, red).

cells, as evidenced by their expression of both polar and stalk cell markers (Fig. 5B), and as fusions between adjacent egg chambers (Fig. 5C). Some of the excess polar cells expressed the main-body follicle cell marker Eya (Bai and Montell, 2002) (Fig. 5A), suggesting that the elevated Dl signal was capable of recruiting polar cells from this neighboring population as well. Furthermore, overexpression of Dl within the stalk cells themselves, using the 24B-GAL4 driver, induced the expression of a polar cell marker within the stalk (Fig. 5D).

Notch activation antagonizes JAK/STAT signaling by blocking the nuclear localization of STAT

The JAK/STAT ligand Upd is expressed in polar cells, and like Dl is required for induction of the stalk (McGregor et al., 2002). The binding of Upd to its receptor, Domeless, activates the JAK kinase Hopscotch, which then phosphorylates STAT (Stat92E) to induce its translocation into the nucleus, where it regulates transcription (Arbouzova and Zeidler, 2006). The observed shift from stalk to polar cell fate upon overexpression of Dl implies that Notch activation has the capacity to antagonize JAK/STAT signaling. To explore this issue further, we used the Notch *m7-lacZ* and the *STAT92E-GFP* transcriptional reporters to simultaneously monitor Notch and JAK/STAT signaling in the ovary (Fig. 6A,B). We observed two distinct distributions of transcriptional activation.

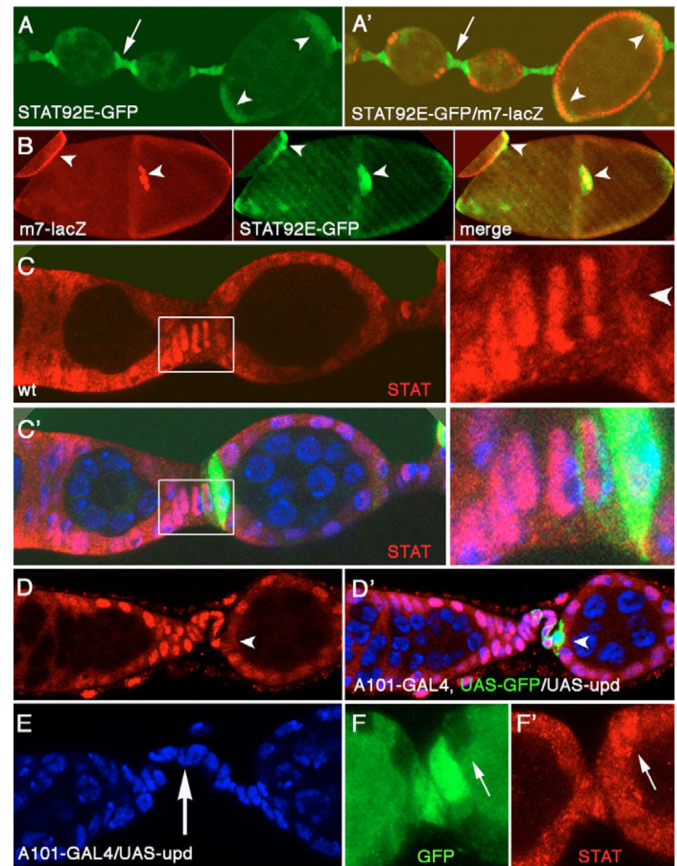

Fig. 6. Unpaired signaling from polar cells is limited in range. (**A-B**) Anti-GFP staining (green), monitoring JAK/STAT activation using the *2XSTAT92E-GFP* reporter. Anti-β-gal staining (A′, red) of the Notch reporter *m7-lacZ.* During early stages of oogenesis, Upd signaling from the polar cells is capable of inducing strong STAT activation in stalk cells (arrows in A,A′), but fails to elicit activation in either the polar cells themselves, or in the neighboring main-body follicle cells. At later stages, follicle cell populations, including main-body and border cells, exhibit concomitant Notch and STAT activation (arrowheads in A,A′,B). (**C**,**C′**) Localization of STAT in early follicle cells. Ovarioles in which polar cells were visualized by A101-GAL4/UAS-GFP (green), were stained for STAT (red) and the nuclear marker DAPI (blue). Nuclear localization of STAT staining (boxes) shows the limited range of JAK/STAT activation by Upd emanating from polar cells. STAT staining alone (C) demonstrates the non-responsiveness of the polar cells to the Upd signal they produce, as STAT does not localize to polar cell nuclei (arrowhead in C, enlarged view). (**D**,**D′**) Overexpression of Upd using the A101-GAL4 polar cell-specific driver. The range of JAK/STAT activation (monitored by nuclear localization of STAT, red) broadens exclusively towards the anterior. Polar cells remain refractory to the signal (arrowhead). (**E**) DAPI staining of egg chambers overexpressing Upd in polar cells reveals an abnormally long stalk (arrow). (**F**,**F′**) Main-body follicle cell clones homozygous for N^{55e11} (marked by loss of GFP), display nuclear localization of STAT (arrow), demonstrating that Notch activation in these cells antagonizes the JAK/STAT pathway. Clones that were further than four cell-diameters from the polar cells did not display nuclear STAT, owing to the restricted diffusion of Upd (not shown).

During early stages of oogenesis, Upd signaling from the polar cells is capable of inducing strong STAT activation in stalk cells, but fails to elicit activation in either the polar cells themselves, or in the neighboring main-body follicle cells (Fig. 6A,A′). At later stages, however, follicle cell populations, including main-body and border

cells, exhibited concomitant Notch and STAT activation (Fig. 6A′,B). This analysis highlights a continuous requirement for both the Notch and JAK/STAT signaling pathways during follicle cell differentiation, throughout oogenesis. As predicted, Notch signaling can antagonize STAT activation in follicle cells, but this capacity is spatially and temporally restricted.

We further pursued the antagonistic effect of Notch signaling in early egg chambers by following nuclear localization of STAT as an assay for JAK/STAT pathway activity. Nuclear STAT staining was pronounced throughout the stalk separating the germarium from the polar cells of the adjacent, posterior egg chamber in wild-type ovaries (Fig. 6C,C′). Consistent with the *STAT92E-GFP* reporter pattern, the anterior polar cells did not exhibit nuclear localization of STAT, indicating that although they produce the Upd ligand, they themselves are refractory to this signal. STAT also remained cytoplasmic in the main-body follicle cells adjacent to the polar cells.

When Upd was overexpressed using a polar cell-specific driver, the anterior range of nuclear STAT localization was significantly increased (Fig. 6D,D′). Consistent with this enhanced activation of JAK/STAT signaling, longer stalk-like structures were observed (Fig. 6E). In spite of the higher levels of Upd, nuclear STAT was still only seen in cells anterior to the source, including the future stalk and posterior polar cells of the adjacent younger egg chamber (Fig. 6E). By contrast, JAK/STAT signaling in the anterior polar cells themselves, and in the neighboring main-body follicle cells, was not activated.

In light of our suggestion of an antagonistic relationship between Notch and JAK/STAT signaling, one possible explanation for failure of the polar and main-body follicle cells to respond to Upd is the higher level of Notch activation in these cells. To test this hypothesis, we generated *Notch*-mutant clones in the main-body follicle cells, and monitored the nuclear localization of STAT. Elimination of Notch in these cells led to nuclear accumulation of STAT in mutant cells situated within four cell-diameters of the polar cells (Fig. 6F,F′). No nuclear localization was detected in *Notch*-mutant cells situated further away (not shown), presumably owing to restricted diffusion of Upd from the polar cells.

These results indicate that moderate to high levels of Notch activation inhibit JAK/STAT signaling, and that this inhibition acts before the nuclear translocation of activated STAT. Furthermore, our results demonstrate that correct specification of the polar, main-body and stalk follicle cells depends on crosstalk between distinct levels of Notch activity and the JAK/STAT pathway. High Notch activation induces polar cell fate, including expression of Upd, and antagonizes JAK/STAT signaling. Intermediate levels of Notch activation in the main-body follicle cells antagonize JAK/STAT signaling, without inducing expression of Upd. Finally, low levels of Notch activation synergize with Upd signaling to induce stalk cell fate and to regulate the size of the stalk.

Regulation of Notch signaling by JAK/STAT

Maintaining the moderate level of Notch signaling that is induced by Dl expressed in the follicle cells, is essential for producing a stalk with the correct cell number, and we have shown that this is achieved at least in part by the activity of Kul in the signal-sending cells. We examined the possibility that Notch signaling is also attenuated in the signal-receiving cells by the activity of JAK/STAT, by monitoring oogenesis in *hopscotch* (*hop*) hypomorphs, in which JAK/STAT signaling is compromised. Stalks formed at early stages of oogenesis in $hop^{mv1/GA32}$ females, and the oocyte moved to the posterior of the egg chamber as in wild type (Fig. 7A). However,

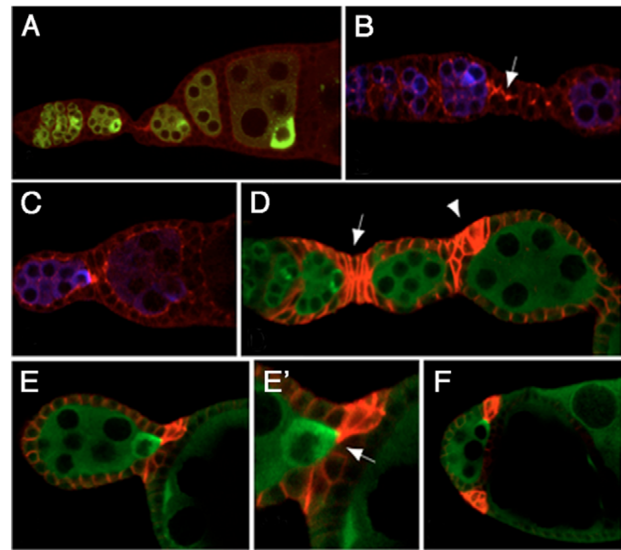

Fig. 7. Extra polar cells are formed in a *hopscotch* hypomorphic mutant. (**A**) At early stages of oogenesis, $hop^{mv1/GA32}$ hypomorphs form a stalk, and the oocyte (marked by anti-BicD, green) moves to the posterior of the egg chamber as in wild type. At later stages, fusions occur and stalks are never seen. (**B**) Stalk cells migrate between two cysts to form a stalk with two layers connected by adherens junctions (labeled with E-Cadherin in red, arrow), but they never intercalate (B and D, arrow). (**C**) The stalk (labeled with E-Cadherin, red) collapses from stage 4/5. The oocyte is stained with anti-Orb (blue). (**D**) When the stalk collapses, a cluster of cells expressing high levels of Fas3 (red) accumulates adjacent to the older egg chamber (arrowhead). (**E-F**) The oocyte (labeled with anti-BicD, green) remains attached to the cells that express high levels of Fas3 (red, arrow in E′), which are identified as polar cells at later stages.

stalk cells failed to intercalate, and the stalk consisted of two rows of cells linked by adherens junctions (Fig. 7B). At later stages, the stalk collapsed and, as was observed for strong *hop* alleles (McGregor et al., 2002), the stalk cells reverted to the polar cell fate. These cells now clustered at the anterior corners of the older cyst, whilst remaining in contact with the oocyte of the younger egg chamber (Fig. 7C-F).

The conversion of stalk cells to polar cells when the level of JAK/STAT signaling was compromised suggests that Notch signaling in the stalk cells is normally attenuated by the JAK/STAT pathway. When this inhibition is relieved in *hop* hypomorphs, the increase in the level of Notch signaling leads to their conversion to polar cells. Since the entire polar/stalk precursor cell population expresses Fng (Grammont and Irvine, 2001), even activation by the lower levels of Dl produced by these cells may be sufficient to give rise to polar cells, in the absence of repression by JAK/STAT.

DISCUSSION

Regulation of Notch activation levels patterns the follicular epithelium

Our results show how a few simple cues in early oogenesis can generate a complex pattern of follicle cells through the regulation of Notch signaling. Global patterning of the follicle is driven by two starting conditions. First, the follicle cells are subdivided by an unknown mechanism into two populations: the stalk/polar cell precursors that express Fng, and the main-body follicle cells that do not (Grammont and Irvine, 2001). Second, spatial organization of

follicle cells splits the stalk/polar precursor population into cells that contact the germ line and those that do not. The subsequent cell-fate decisions are then based on how these starting conditions establish different levels of Notch signaling (see Fig. 8A).

The future polar cells show the highest level of Notch activation, as readily demonstrated in our study by the expression pattern of various transcriptional reporters. The correspondence between high Notch activation levels and the polar cell fate is further supported by the observation that ectopic expression of high Dl levels in follicle cells leads to induction of extra polar cells. One obvious reason for enhanced Notch activation is the specific expression of the facilitating factor Fng in polar cells. Although speculative at present, close proximity to a relatively strong germline Dl signal may provide a separate contribution. Dl protein levels appear to be elevated in the germ line (Fig. 3D), whereas the levels of Kul, which acts to attenuate Dl signaling, are particularly high in the follicle cell epithelium.

Main-body follicle cells and stalk cells both display relatively low levels of Notch activation, and these cannot be distinguished from each other by monitoring Notch transcriptional reporters. The main-body cells, however, exhibit Notch-dependent suppression of JAK/STAT activity, whereas stalk cells transduce the JAK/STAT signal, implying that stalk cells experience a particularly low level of Notch activation. Although both cell types express Fng, the differences in Notch signaling may reflect the separation of stalk cells from a germline Dl ligand source.

The low levels of Notch activation are essential for the formation of the stalk, as *Dl*-mutant anterior polar cells do not form a stalk. Notch activation in the stalk cells is likely to begin at stage 1 of oogenesis, when a broad band of polar cells lies adjacent to the future stalk cells. This activation may have a general effect on cell viability or competence, hence defining the size of the precursor population from which stalk cells can be induced. The increase in the size of the stalk-cell population following expression of the anti-apoptotic protein p35 indeed demonstrates that controlled cell survival plays a role in determining the size of this population, suggesting that excess stalk cells are eliminated by apoptosis, as is the case for the polar cells (Besse and Pret, 2003).

This situation is very different from the usual role of Notch signaling, where a binary output dictates the choice between two fates (reviewed by Lai, 2004). Notch signaling is activated by membrane-anchored ligands (as opposed to activation by secreted ligands that form a concentration gradient). Modifications in the signaling level of Notch must therefore occur either in the ligand-presenting cells by controlling the amount of activating ligand, or in the signal-receiving cells by modulating the capacity of the receptor to respond to the signal (reviewed by Le Borgne et al., 2005). In the early egg chamber, the level of Notch signaling is regulated by multiple mechanisms acting in both the ligand-presenting and the ligand-responding cells. The levels of Dl signaling are modulated by a reduction in the Dl levels presented by the follicle cells, through cleavage by Kul. In the responding cells, the amount of Notch activation induced by Dl is controlled by the differential expression of Fng, which enhances the response, and by antagonism by JAK/STAT signaling (see below).

Antagonism between Notch and JAK/STAT signaling

The induction of the polar cell fate leads to prominent expression of Upd, which triggers the JAK/STAT pathway in neighboring cells. By following the nuclear localization of STAT we showed that the Upd-expressing polar cells, as well as the main-body follicle cells

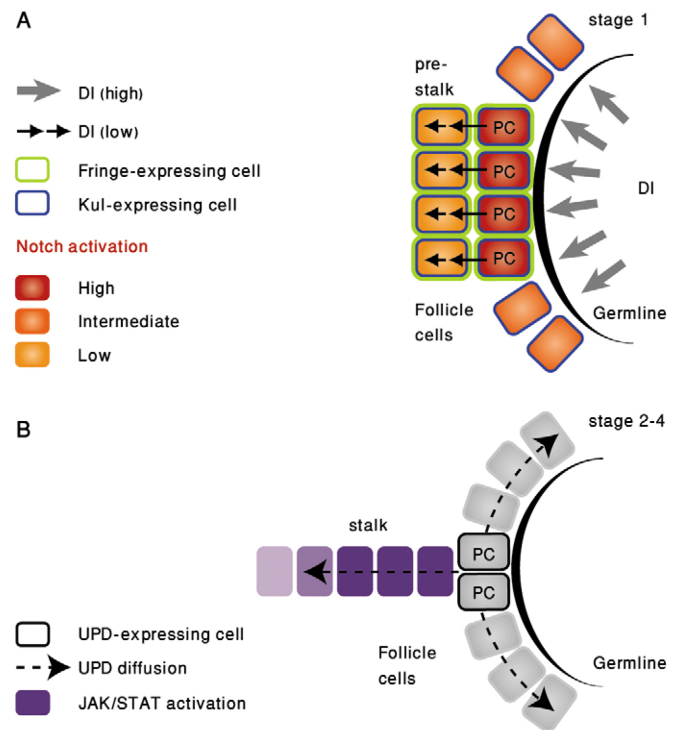

Fig. 8. Scheme for the spacial and temporal order of determination of follicle cell fates by the Notch and JAK/STAT pathways. (**A**) At stage 1 of oogenesis, follicle cells encounter high levels of Dl from the germ line. Expression of Fng in the anterior polar/stalk precursors enhances Notch signaling, leading to the induction of polar cells (PC). The relatively lower levels of Dl expressed by follicle cells are further reduced by the metalloprotease Kul, thus leading to low levels of Notch activation in those follicle cells that do not contact the germ line. This low level of activation promotes cell viability and contributes to induction of the stalk cell fate. (**B**) The polar cells express the Domeless ligand Upd, which diffuses to neighboring follicle cells. The main-body follicle cells, which experience high or intermediate levels of Notch activation, are refractory to JAK/STAT signaling, and repress STAT nuclear localization. By contrast, the pre-stalk cells, which are subject to low levels of Notch activation, undergo JAK/STAT activation. The combined activity of the two signaling pathways thereby facilitates proper stalk cell-fate induction.

that are posterior to the polar cells, do not exhibit JAK/STAT activation, even under conditions of Upd overexpression in the polar cells (Fig. 6). Assuming that the Upd ligand spreads uniformly, these observations imply that those follicle cells that are in contact with the germ line do not respond to JAK/STAT signaling. The biological activity of Upd is therefore restricted to the anterior presumptive stalk cells, owing to inhibition by the Notch pathway in the polar and main-body follicle cells. Accordingly, overexpressing Dl eliminates the cell fates induced by JAK/STAT (stalk and border cells).

When distinct cell types communicate over extended time periods, the paradigm of eliminating the response to signal by the cells that produce it, allows the generation of stable boundaries. This has been observed in a variety of instances in the developing *Drosophila* wing disc. Posterior compartment cells producing Hedgehog (Hh) do not respond to the Hh signal themselves (reviewed by Tabata and Takei, 2004). Similarly, in the cells producing Dpp, expression of the receptor Thickveins is eliminated

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by Hh signaling (Funakoshi et al., 2001). In the dorsoventral axis, the cells producing high levels of Dl and Serrate (Ser) are refractory to Notch signaling, and the adjacent cells producing Wingless are refractory to the ligand they produce owing to induction of the transcriptional repressor Cut by Notch (de Celis and Bray, 1997; Micchelli et al., 1997). The antagonism between Notch and JAK/STAT could function in a similar way to ensure that the signaling cells producing Upd maintain their fate during this critical developmental stage.

The mechanistic basis for the capacity of Notch signaling to block STAT nuclear translocation is intriguing. The Upd receptor Domeless is expressed in all follicle cells (Ghiglione et al., 2002). However, induction of a protein blocking STAT nuclear localization by Notch signaling may be envisaged. Such an induction may be transient and restricted to a particular stage or cell type, as the antagonism between Notch signaling and STAT activation is not observed at later stages, when activation of both pathways can be detected in the same follicle cells (Fig. 6A′,B). The repression of JAK/STAT signaling by high and intermediate levels of Notch signaling, leading to vectorial signaling by Upd, is presented in Fig. 8B.

Interestingly, JAK/STAT has a reciprocal inhibitory effect on Notch signaling in the stalk cells. In this case, however, the inhibition is only partial and serves a modulating role, as both Notch and JAK/STAT signaling are required for stalk cell differentiation. Similar to Dl overexpression, *hop* hypomorphic mutants lose the stalk and display extra polar cells (Fig. 7). Conversely, Upd overexpression produces longer stalks and missing polar cells (McGregor et al., 2002). Attenuation of Notch signaling in the cells where JAK/STAT is activated is essential, as these cells express Fng at early stages (Grammont and Irvine, 2001), and are therefore particularly sensitive to Notch activation. A dual mechanism thus functions to reduce the level of Notch activation in the stalk cells: the activity of Kul in the Dl-producing cells reduces the level of the signal, while the JAK/STAT pathway compromises the competence of the cells to respond.

Plasticity of stalk and polar cell fate

Several lines of evidence indicate that the specification of the stalk and polar cells is reversible. For example, overexpression of Dl by the 24B-GAL4 driver, which is only expressed after the stalk has been specified, converts stalk cells into polar cells. Similarly, when the levels of Upd signaling are reduced in *hop* hypomorphs, the stalk cells initially develop normally before reverting to a polar cell fate. Conversely, overexpression of Upd converts polar cells into stalk cells. Thus, the fate of these cells is relatively plastic and continuously depends on the balance between Notch and JAK/STAT signaling.

In this context, it is interesting to note that the allocation of stalk and polar cells appears to be buffered in wild type by a negative feed-back loop: increases in Notch signaling will produce more polar cells, which will express the stalk-inducer Upd, whereas increases in JAK/STAT signaling will produce more stalk cells at the expense of polar cells, thereby limiting the supply of Upd.

Patterning of the follicle cells

In addition to revealing how the interplay between the Notch and JAK/STAT pathways specifies the three follicle cell fates, our results refine the model for the origin of the first anterior-posterior asymmetries in the egg chamber. First, the antagonism of the JAK/STAT pathway by high or intermediate levels of Notch activation explains why Upd signaling from the polar cells induces the stalk in a vectorial manner. The moderate to high levels of Notch signaling in the polar and the main-body follicle cells prevent them from responding to Upd, resulting in a gradient of JAK/STAT signaling that extends anteriorly. As a result, the stalk forms from the anterior of the egg chamber towards the posterior of the adjacent younger cyst, forming an essential part of the relay that positions the oocyte in the younger cyst to establish the anterior-posterior axis (Torres et al., 2003).

Although both the anterior and posterior pairs of polar cells express Upd, the posterior cells play no role in the induction of the stalk because they differentiate about 12 hours after their anterior counterparts. Our results suggest an explanation for this delay, based on the antagonism of the Notch pathway by JAK/STAT signaling. Because the future posterior polar cells are linked by the stalk to the anterior polar cells of the adjacent older cyst, they are exposed to Upd emanating from the latter before they are exposed to Dl from the germ line. Indeed, STAT can be detected in the nuclei of these presumptive posterior polar cells as the younger cyst begins to exit the germarium. This presumably inhibits their response to germline Dl when it is expressed at stage 1, resulting in the observed delay in their expression of polar cell markers. Thus, the posterior polar cells probably first differentiate as stalk cells, and then switch to the polar cell fate when Notch activity out-competes the JAK/STAT pathway.

The nuclear localisation of STAT in the posterior 'polar' cells coincides with the time that these cells upregulate expression of DE-Cadherin, adhere to the oocyte and position it at the posterior of the egg chamber (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). The transient stalk-like fate of these cells causes them to upregulate E-Cadherin, and therefore preferentially targets the adhesive interactions of the oocyte to the future posterior polar cells, to generate a reproducible anterior-posterior polarity. Thus, the differential responses to distinct levels of Notch activation, coupled to reciprocal inhibitory interactions between the Notch and JAK/STAT pathways, might serve not only to determine the correct number of polar and stalk cells, but also to specify the anterior-posterior axis.

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