Apoptosis-mediated cell death within the ovarian polar cell lineage of
Drosophila melanogaster

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SUMMARY

Polar cells have been described as pairs of specific follicular cells present at each pole of *Drosophila* egg chambers. They are required at different stages of oogenesis for egg chamber formation and establishment of both the anteroposterior and planar polarities of the follicular epithelium. We show that definition of polar cell pairs is a progressive process since early stage egg chambers contain a cluster of several polar cell marker-expressing cells at each pole, while as of stage 5, they contain invariantly two pairs of such cells. Using cell lineage analysis, we demonstrate that these pre-polar cell clusters have a polyclonal origin and derive specifically from the polar cell lineage, rather than from that giving rise to follicular cells. In addition, selection of two polar cells from groups of pre-polar cells occurs via an apoptosis-dependent mechanism and is required for correct patterning of the anterior follicular epithelium of vitellogenic egg chambers.

Key words: Oogenesis, Polar cells, Cell lineage, Apoptosis, *Drosophila melanogaster*

INTRODUCTION

In *Drosophila*, embryonic anteroposterior (AP) and dorsoventral (DV) axes are already specified in non-fertilized eggs and result from a series of symmetry-breaking steps occurring during oogenesis. Indeed, ovarian follicles, called egg chambers, are highly polarized structures composed of 16 interconnected germline cells surrounded by somatic follicular cells. Specification of distinct cellular types along AP or DV axes, as well as organization of planar polarity within the follicular epithelium, are established during mid-oogenesis and clearly required for final patterning of the egg. Strikingly, the establishment of such cellular asymmetries relies to a great extent on soma-soma and germen-soma intercellular communication involving previously characterized signaling pathways such as Delta/Notch and Gurken (TGF-α)/Torpedo (EGF receptor) (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St Johnston, 1998; Keller Larkin et al., 1999) (for reviews see Ray and Schupbach, 1996; Riechmann and Ephrussi, 2001; van Eeden and St Johnston, 1999).

Both somatic and germline cell populations derive from stem cells present in anterior structures of the ovary called germaria (Lin and Spradling, 1993; Margolis and Spradling, 1995; Wieschaus and Szabad, 1979). Each germarium is connected to a polarized string of maturing egg chambers that constitute an autonomous structural unit termed ovariole (for reviews see King, 1970; Spradling, 1993; Spradling et al., 1997). The assembly of 15-20 ovarioles forms an ovary. The germarium has been divided into three distinct subregions according to morphological criteria. Anterior region 1 contains 2 to 3 germline stem cells (GSCs) and their differentiating daughter cells termed cystoblasts. Each cystoblast undergoes exactly four mitosis with incomplete cytokinesis to produce a syncytium of 16 cystocytes known as a germline cyst. One of these cells develops into the oocyte, while the 15 remaining cells develop into nurse cells. Complete cysts mature through region 2a and become enveloped individually in region 2b by inwardly migrating somatic cells (or prefollicular cells) deriving from approximately 2 somatic stem cells (SSCs) lying at the border between regions 2a and 2b. The prefollicular cell population diverges soon after to give rise to interfollicular cells organized in stalk structures, a pair of polar cells at each extremity of the egg chamber, and follicular cells forming a polarized epithelium around the newly formed egg chamber. Region 3 of the germarium corresponds to a stage 1 egg chamber that will bud off upon completion of stalk formation. The follicular cell epithelium will not remain as a uniform layer as different follicular cell populations distinguishable by specific gene expression patterns and morphogenetic properties are defined along the anteroposterior axis. In particular, the terminal anterior follicular cells become subdivided into 3 distinct follicular cell types at mid-oogenesis [stage 9 according to Spradling (Spradling, 1993)]: (1) border cells that delaminate from the epithelium and migrate between the nurse cells to reach the oocyte, (2) stretched or squamous cells that spread to cover the nurse cells and (3) centripetal cells that migrate between the oocyte and the nurse cells (Spradling, 1993).

Although polarization of the follicular epithelium along the AP axis is not detected before stage 6, the positioning of this

axis can be visualized in egg chambers as early as stage 2, by the presence of terminal polar cells located at each pole of the egg chamber. Polar cells are pairs of particular follicular cells that express specific markers and differ from neighboring follicular epithelial cells in that they have a round morphology and lack apicobasal polarity (Ruohola et al., 1991; Spradling, 1993). They have been shown to assume various functions during oogenesis, including individualization of germline cysts and control of stalk formation in late germarial regions (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001), induction of planar epithelial polarity starting at stage 5 (Frydman and Spradling, 2001) and recruitment of border cells at stage 9 (Liu and Montell, 1999; Silver and Montell, 2001). Polar cell specification has been shown to require critical levels of both Delta/Notch and Jak/Stat signaling pathways. Indeed, removal of either Notch or Su(H) function in somatic cells leads to a complete loss of polar cells (Lopez-Schier and St Johnston, 2001). In addition, overexpression of Unpaired (Upd), a *Drosophila* Jak pathway ligand, frequently results in the disappearance of polar cell marker-expressing cells (McGregor et al., 2002). Once specified, polar cells are in turn assumed to achieve their different functions by producing specific signals, in particular early Delta1/Notch signal to specify stalk cells (Lopez-Schier and St Johnston, 2001), a presently unknown signal to reorganize planar actin filament bundles during mid-oogenesis (Frydman and Spradling, 2001), and Upd-mediated Jak/Stat-activating signal to recruit border cells and pattern anterior follicular cells (Beccari et al., 2002; Silver and Montell, 2001).

Despite the crucial role played by polar cells, little is known about their origin. Previous analysis of marked mitotic clones revealed that polar cells stop dividing in region 2b of the germarium, long before the rest of the follicular epithelial cells (which stop at stage 6), suggesting that they are already determined at this stage (Margolis and Spradling, 1995). A second clonal analysis confirmed this result and further showed that polar cell pairs share common precursors with cells of the adjacent interfollicular stalk, these precursors being distinct from those of follicular cells (Tworoger et al., 1999). Nonetheless, these studies did not describe the lineage relationship between polar cells of a given pair, nor the mechanisms leading to specification of invariantly 2 polar cells at a given extremity. In addition, in these studies, only stage 6 or older egg chambers were examined, while earlier egg chambers are of particular interest since previous reports indicate that polar cell markers can be expressed in more than 2 cells at the extremities of some egg chambers that have just budded (Forbes et al., 1996; Grammont and Irvine, 2001; Ruohola et al., 1991). In the present work, we have thus concentrated on early polar cell development by re-investigating the polar cell lineage in early stage egg chambers. We show that establishment of polar cell pairs is a several step process controlled, in part, by apoptosis.

MATERIALS AND METHODS

Fly strains

The following enhancer-trap lines were used for polar cell staining: fng^{lacRF4} (Grammont and Irvine, 2001), A101(*neuralized-lacZ*)/TM6 (Ruohola et al., 1991) and PZ80(*unpaired-lacZ*)/CyO (McGregor

et al., 2002). *Neuralized*^{P72}-Gal4,UAS-histoneYFP/TM6 (Bellaiche et al., 2001), UAS-p35 (Neufeld et al., 1998) and *reaper-lacZ* (Nordstrom et al., 1996) lines were used to study apoptotic cell death. The fu^{JB3} allele contains an alteration in the N-terminal *fused* kinase domain (Therond et al., 1996).

Egg chamber staining procedure

Immunocytochemistry was performed as previously described (McKearin and Ohlstein, 1995). The following antibodies were used in this study: mouse monoclonal anti-Fasciclin III (1:30; Developmental Studies Hybridoma Bank), rabbit polyclonal anti-β-galactosidase (1:200; Boehringer Mannheim). Fluorescence-conjugated secondary antibodies were purchased either from Jackson Immunoresearch Laboratories or from Molecular Probes. All were used at a 1:200 dilution. Actin was labeled with Alexa-conjugated phalloidin (Molecular Probes) at 0.1 µg/ml for 1 night in PBT1X. All samples were mounted in cytifluor (Kent).

The TUNEL (TdT-mediated dUTP nick end labeling) assay was carried out using the In Situ Cell Death Detection Kit (Roche). Ovaries were dissected in PBS1X, then fixed for 20 minutes in 4% paraformaldehyde and rinsed three times in PBT (0.3% Triton X-100). Ovaries were then incubated in the TUNEL reaction mixture (including FITC-conjugated modified nucleotides and terminal deoxynucleotidyl transferase) for 1 hour at 37°C. The samples were rinsed in PBT and stained for other markers using the classical procedure cited above. TUNEL signal was visualized directly under a FITC filter.

Clonal analysis

For cell lineage analysis, females of the following genotypes were analyzed: hsp-*flp*/+; A101(*neu*-lacZ)/Act>CD2>Gal4, UAS-GFP and fu^{JB3}, hsp-*flp*/fu^{JB3}; A101(*neu*-lacZ)/Act>CD2>Gal4, UAS-GFP (Neufeld et al., 1998). Females were heat-shocked upon eclosion for 25-30 minutes at 32°C, placed at 29°C, and dissected either 20 hours (±4 hours) or 44 hours (±4 hours) later.

As described previously (Tworoger et al., 1999), the χ-squared test was applied to the clonal studies to establish cell lineage independence between supernumerary A101$^+$ cells and A101$^-$ follicular epithelial cells. Recovered GFP$^+$ clones were divided into 3 classes: clones containing only A101$^+$ cells, clones containing only A101$^-$ cells and clones containing both A101$^+$ and A101$^-$ cells. A fourth class was defined by chambers with no GFP$^+$ cells. Observed numbers for each category were respectively: 8/110, 55/110, 7/110 and 40/110, while expected numbers were: 10/110, 57/110, 5/110, 38/110. The expected outcome for each category was predicted based on independent induction of clones, i.e. with a frequency of clones containing both A101$^+$ and A101$^-$ epithelial cells equivalent to the product of the frequency of clones containing only A101$^+$ cells and the frequency of clones containing only A101$^-$ cells.

Somatic overexpression of p35 was carried out by first applying a heat-shock to hsp-*flp*/+; UAS-p35/+; Act>CD2>Gal4/+ or fu^{JB3} hsp-*flp*/fu^{JB3}; UAS-p35/+; Act>CD2>Gal4, UAS-GFP/+ mid-pupae for 1 hour at 37°C and dissecting females 5-8 days after eclosion. This allowed generation of large clones encompassing all follicular cells of a given ovariole.

RESULTS

The number of polar cell marker-expressing cells is progressively restricted to 2 at each end of the egg chamber

Close examination of wild-type ovarioles using a polar cell-specific enhancer-trap line (A101/*neuralized-lacZ*) revealed that in early egg chambers, more than 2 cells expressing this marker can be observed at each pole (Fig. 1A), and that this
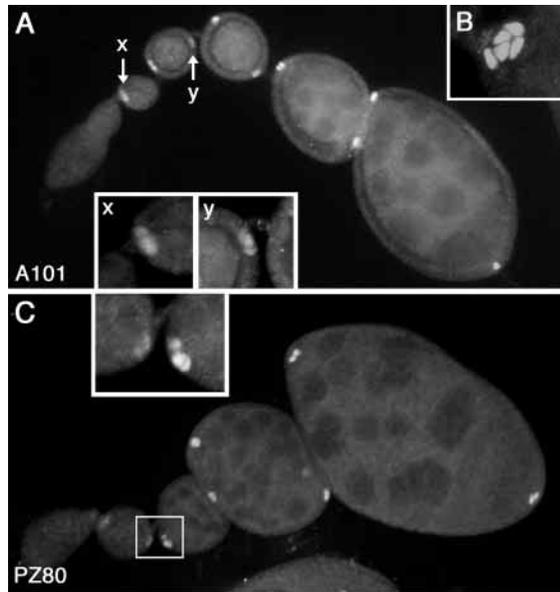
Fig. 1. Progressive definition of polar cell marker expression. A101/TM6 (A,B) and PZ80/Cy0 (C) ovarioles stained with anti-β-galactosidase antibodies. Insets in A and C show magnification of indicated groups of 3 polar cell marker-expressing cells found in stage 2 to 4 egg chambers. B shows a group of 5 A101^{+} cells found in a stage 2 egg chamber.

number is progressively restricted to 2 cells (Table 1). As shown in Fig. 1A and Table 1, A101 staining can be broken down into successive phases, using criteria defined by Spradling (Spradling, 1993) to determine egg chamber stages. (1) From germarial region 2 to the stage 1 egg chamber: A101 staining is not detected except in a minority of late stage 1 egg chambers. (2) Stage 2 and 3 egg chambers: although some egg chambers still show no A101^{+} cells, a significant proportion of egg chambers with groups of 3 and 4 A101^{+} cells are observed in addition to egg chambers with pairs of A101^{+} cells. Rarely, groups of 5 A101^{+} cells can also be observed (Fig. 1B, insert). (3) Stage 4 egg chambers: groups of 3 A101^{+} cells continue to be observed in addition to an increasing number of egg chambers with 2 A101^{+} cells. (4) From stage 5 onwards, A101 staining is restricted to precisely 2 cells at each pole. In addition, although both anterior and posterior polar cells exhibit such a dynamic expression pattern, A101 staining appears, and is restricted, slightly later in posterior cells than in anterior cells (Fig. 1A and Table 1).

This progressive definition of polar-like cells is not specific to the A101 enhancer-trap line since we obtained similar results using other specific polar cell fate markers such as PZ80/*unpaired-lacZ* (Fig. 1C), *fringe-lacZ* and Fasciclin III (data not shown).

Thus, restriction in the number of polar cell marker-expressing cells seems to be a progressive process that is not completed before stage 5 of oogenesis.

Each group of A101^{+} cells originates from several polar cell precursors

Given that groups of 3 to 5 polar cell marker-expressing cells were observed in early stage egg chambers, we aimed at characterizing the lineage relationship between these cells, in particular to determine whether they actually originate from the polar cell lineage. Previous cell lineage studies did not address these groups of A101^{+} cells, which are characterized here for the first time (Margolis and Spradling, 1995; Tworoger et al., 1999). In addition, though the most recent of these studies examined the lineage relationship between mature polar cells and stalk cells, or between stalk cells and neighboring follicular cells, the lineage relationship between cells within a given polar cell pair was not clearly described.

We therefore undertook a cell lineage analysis by generating dominantly marked clones using the flip-out/Gal4 system and a UAS-GFP reporter transgene (see Materials and Methods). Flipase expression was induced by mild heat-shock treatment of adult females in order to minimize the frequency of recombination events. Examination of stage 3 egg chambers 20 hours (±4 hours) after this treatment, or of stage 6 egg chambers 44 hours (±4 hours) after, allowed us to visualize small transient GFP[+] clones whose progenitors were in region 2b at the time of flipase-induced GFP expression, and therefore to avoid observation of large somatic stem cell- or early prefollicular cell-derived clones.

We first analyzed the distribution of GFP^{+} cells within clones in which a recombination event occurred specifically within the population of precursors common to stalk cells and polar cells (i.e. clones containing both GFP[+] stalk and A101^{+} cells). Examination of stage 3 egg chambers containing groups of 3 to 5 A101^{+} cells revealed that among 24 clones recovered, no GFP uniformly-marked groups of A101^{+} cells were found (Fig. 2A,B). This suggests that groups of 3 to 5 A101^{+} cells found in early stage egg chambers have a polyclonal origin. In addition, clones contained either 1 or 2 GFP^{+} stalk cells, coupled with either 1 or 2 GFP[+]/A101^{+} cells (Table 2). Whether 3, 4 or 5 A101^{+} cells were present within these

Table 1. Progressive restriction in the number of A101^{+} cells found at each extremity of egg chambers

	Stage 1 (*n*=29)		Stage 2 (*n*=52)		Stage 3 (*n*=52)		Stage 4 (*n*=48)		Stage 5 (*n*=36)	
	A	P	A	P	A	P	A	P	A	P
0 A101^{+} cells	89.8	96.6	19.2	50	0	0	0	0	0	0
1 A101[+] cell	3.4	0	0	11.5	0	0	2.1	2.1	0	0
2 A101^{+} cells	3.4	0	28.8	30.8	67.3	69.2	93.7	87.5	100	100
3 A101^{+} cells	3.4	3.4	38.5	5.8	32.7	25	4.2	10.4	0	0
4 A101^{+} cells	0	0	13.5	1.9	0	5.8	0	0	0	0

Results are expressed as the percentage of egg chambers containing either 0, 1, 2, 3 or 4 A101^{+} cells at each extremity. *n* represents the total number of egg chambers of a given stage in which anterior (A) and posterior (P) A101[+] clusters were examined. For stage 1 egg chambers, only chambers in the process of being budded from the germarium were scored.

Fig. 2. Polyclonal origin of A101^{+} cells. hsp-*flp*/+; A101/Act>CD2>Gal4, UAS-GFP ovarioles stained with anti-β-galactosidase (red/A101, A,B,D) or anti-Fas III antibodies (red, C), GFP (green, A-D), and DAPI (white, A-D). In A-D, yellow marks the superimposition of A101 or Fas III staining and GFP staining. (a1-d1) Magnifications of the boxed regions in A-D showing A101 (a1,b1,d1, red) or Fas III (c1, red) staining and GFP staining (green). (a2-d2) Magnifications of the boxed regions in A-D showing A101 (a2,b2,d2) or Fas III (c2) staining. As shown in c2, Fas III accumulates most strongly at the contact surface between the two polar cells of a given pair in late egg chambers. F III denotes Fas III.
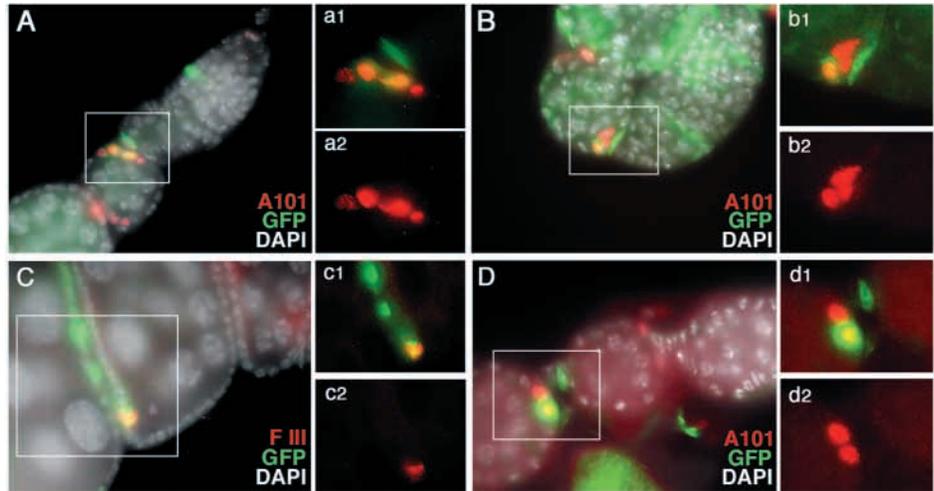
Table 2. Composition of recovered GFP[+] clones containing both stalk cells and A101^{+} cells

No. of stalk cells	3 A101[+]		4 A101[+]		5 A101[+]	
	1 GFP[+]	2 GFP[+]	1 GFP[+]	2 GFP[+]	1 GFP[+]	2 GFP[+]
1 GFP[+]	13	2	3	0	0	2
2 GFP[+]	1	1	0	2	0	0

The number of clones containing either 1 or 2 GFP^{+} stalk cells coupled with either 1 or 2 GFP[+] A101^{+} cells is given for egg chambers containing clusters of 3 to 5 A101^{+} cells at a given extremity. Late stage 2 and stage 3 egg chambers were examined 20 hours (±4 hours) after induction of flipase expression.

clusters, no more than 2 GFP[+]/A101^{+} cells were ever observed. Since clones containing up to eight follicular cells could be found under these conditions (data not shown), this shows that A101^{+} cells and their direct precursors stop dividing earlier than follicular cells.

Interestingly, as described for stalk cells (Tworoger et al., 1999), no clustering of sister cells was observed. Indeed, GFP^{+} cells separated by unmarked (GFP^{−}) cells could be found (data not shown). In addition, GFP^{+} cells were found either in the middle (Fig. 2A), or at the border (Fig. 2B) of groups of 3-5 A101^{+} cells.

Since we show that early groups of 3 to 4 A101^{+} cells derive from several precursors, it is possible that some of these cells do not derive exclusively from the polar cell lineage, but could be recruited from the follicular cell lineage, their proximity to the poles being responsible for the induction of polar cell markers. In order to address this point, we compared the relative proportions of egg chambers with GFP[+] clones containing only A101^{+} cells, only A101[−] epithelial follicle cells, or both A101[+] and A101^{−} cells. If some of the A101^{+} cells belong to the follicular cell lineage, we would expect a high frequency of egg chambers with GFP[+] groups containing both A101^{+} cells and A101[−] follicular epithelial cells in a given egg chamber extremity. In contrast, we found a low frequency of this type of clone-containing egg chamber (6.36%, *n*=110), statistically consistent with the frequency expected as a result of independent flip-out events (α=0.71, see Materials and Methods). All precursors of A101^{+} cells have thus already diverged from the follicular cell lineage in germarial region 2b.

Given that early groups of A101^{+} cells derive from several polar cell precursors, we wondered how the final pairs of polar cells are selected, and whether the mature polar cells always derive from the same direct precursor. We therefore examined GFP[+] clones containing both stalk and polar cells, specifically in stage 3 and stage 6 egg chambers with only 2 polar cells at a given extremity. Among 10 clones recovered, clones containing 1 polar cell/1 stalk cell (6/10); 1 polar cell/2 stalk cells (1/10); 2 polar cells/1 stalk cell (2/10), or 2 polar cells/2 stalk cells (1/10) were found. The fact that polar cell pairs containing either 2 GFP^{+} cells (Fig. 2C), or 1 GFP[+] and 1 GFP[−] cell (Fig. 2D) were observed suggests that polar cells of a given pair do not exclusively derive from the same precursor, and therefore that selection of mature polar cells does not result from a strictly lineage-based mechanism.

Altogether, our results indicate that early groups of A101^{+} cells (that we name pre-polar cells) derive from at least 2 different precursor cells, and that these precursors belong specifically to the polar cell lineage. In addition, our results also imply that a stochastic selection of 2 polar cells takes place from among the pre-polar cells produced in the germarium.

Supernumerary pre-polar cells are eliminated in an apoptosis-dependent process

As described above, each pair of polar cells seems to be selected from a group of several pre-polar cells. The next question addressed was how supernumerary pre-polar cells are eliminated. We considered 2 possibilities: first supernumerary pre-polar cells could loose their specific properties (including marker expression and lack of cell polarity), thus becoming indistinguishable from neighboring follicular cells, second, supernumerary pre-polar cells could be physically eliminated. The second possibility was tested by looking for apoptosis events using the TUNEL assay. Analysis of ovaries from A101/TM6 females revealed that, from early stage 2 to early stage 5 egg chambers, some groups of 3 or more A101^{+} cells contain 1 TUNEL-positive cell (Fig. 3A,a2 arrow). This cell could be located at any position within the group (data not shown). In addition, it was typically smaller and rounder than other A101^{+} neighboring cells (Fig. 3a3, arrow) and also contained fragmented nuclei visible after DAPI staining (Fig. 3a1, arrow). Detection of apoptosis events affecting pre-polar
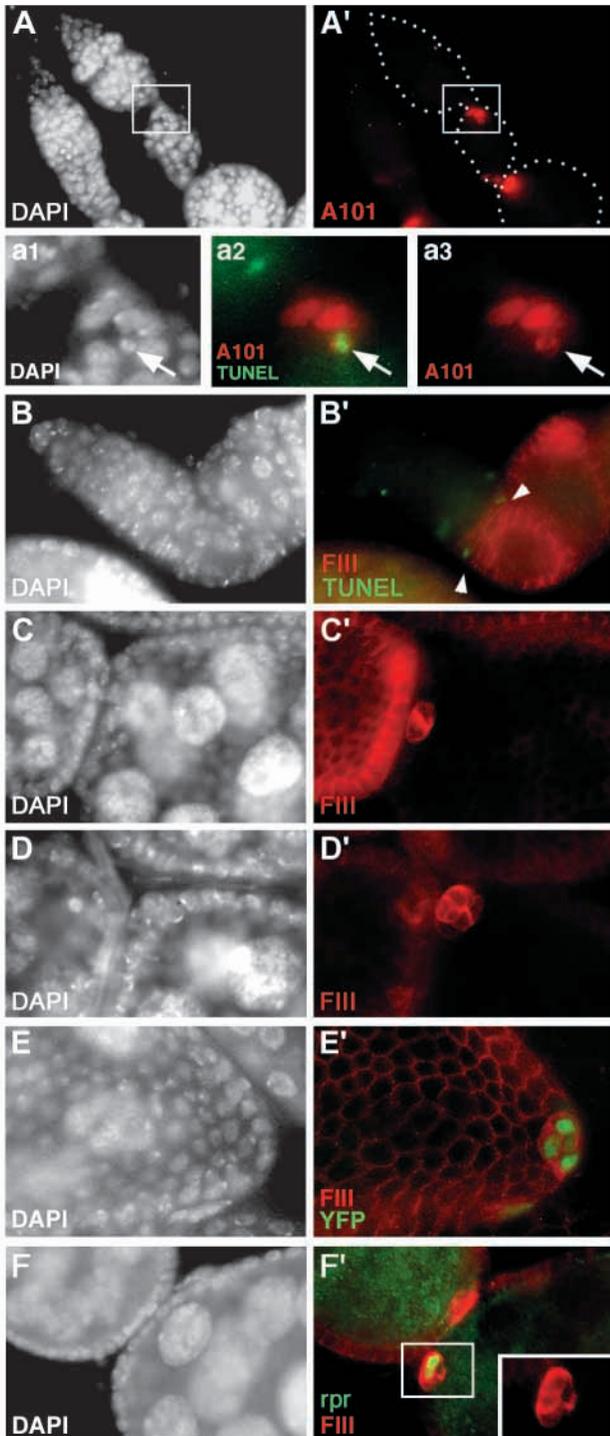
Fig. 3. Apoptosis-dependent elimination of supernumerary A101^{+} cells. (A) A101/TM6 ovariole triple-stained with DAPI (A, a1), anti-β-galactosidase antibodies (red, A′,a2,a3) and TUNEL (green, a2). (a1-a3) Magnification of the boxed region in A, A′. Arrows point to the dying A101[+]/TUNEL[+] cell. (B) Wild-type ovariole triple-stained with DAPI (B), anti-Fas III antibodies (red, B′) and TUNEL (green, B′). Arrowheads point to early germarial region 2b/3 Fas III^{+} somatic cells undergoing apoptosis. (C,D) hsp-*flp*/+; +/SM6; Act>CD2>Gal4, UAS-GFP/TM6 (C) and hsp-*flp*/+; +/UAS-p35; Act>CD2>Gal4, UAS-GFP/+ (D) stage 5-6 egg chambers double-stained for DAPI (C,D) and anti-Fas III antibodies (C′,D′). (C′) In a wild-type egg chamber, Fas III is expressed at a basal level in follicular cells, but is strongly expressed in pairs of polar cells. Six anterior Fas III^{+} polar cells are present in D′. All follicular cells of the ovarioles in C and D exhibited GFP staining (data not shown). (E,E′) UAS-p35/*neu*[P72]-Gal4; UAS-HistoneYFP/+ stage 6 egg chamber triple-stained with DAPI (E), anti-Fas III antibodies (red, E′) and YFP (green, E′). Four Fas III^{+} polar cells are present posteriorly in E′. (F,F′) UAS-p35/+; *neu*[P72]-Gal4/*rpr-lacZ* egg chamber triple-stained with DAPI (F), anti-Fas III (red, F′) and anti-β-galactosidase (green, F′) antibodies. Among the 4 Fas III^{+} cells present anteriorly (F′, boxed area and close up in inset), 2 also exhibit *reaper-lacZ* expression (boxed area). FIII denotes Fas III and rpr denotes *reaper-lacZ*.

cells and thereby to produce supernumerary polar cells. We thus overexpressed the bacculoviral p35 caspase inhibitor protein in somatic cells of the ovary using the flip-out/Gal4 system (Hay et al., 1994). Induction of large somatic clones covering entire ovarioles resulted in a significant increase in the number of cells strongly expressing Fas III at the poles of p35-overexpressing egg chambers compared to control egg chambers (3.47 versus 2.13 respectively, $n=178$, $P<0.001$). Groups of 3, 4, 5 and even 6 Fas III^{+} cells were found at the poles of stage 5 and later stage clonal egg chambers (Fig. 3D,D′, compare with Fig. 3C,C′, and data not shown). Furthermore, restriction of p35 overexpression to the polar cell lineage using a *neuralized*-Gal4 transgene (Bellaiche et al., 2001) also produced supernumerary polar cells in advanced egg chambers (Fig. 3E,E′) and groups of up to 6 Fas III^{+} cells could be found (data not shown). Interestingly, in the clusters of polar cells produced under these conditions, consistently, 2 cells do not express a *reaper-lacZ* construct while the supernumerary cells do (Fig. 3F,F′). The *reaper* gene encodes a pro-apoptotic protein that has been shown to function upstream of caspases to induce cell death (White et al., 1996). In addition, expression of this gene is confined to cells destined to die. In wild-type ovarioles, however, we did not detect expression of *reaper-lacZ* in the germarium or in early groups of pre-polar cells, possibly because of transient expression of *reaper* and rapid elimination of apoptotic cells. In contrast, in ovarioles overexpressing p35 within the polar cell lineage, the terminal block in the cell death program, downstream of *reaper* induction, probably leads to the accumulation of a high level of β-galactosidase (Fig. 3F,F′). Such a phenomenon has already been described in midglial cells of the embryonic nerve cord where *reaper* expression is difficult to detect by in situ hybridization in wild-type embryos, but easily detectable after p35 overexpression (Zhou et al., 1997).

Altogether, these results strongly suggest that apoptosis-mediated cell loss occurs within the polar cell lineage in wild-type ovarioles to allow selection of 2 mature polar cells from a pool of pre-polar cells.
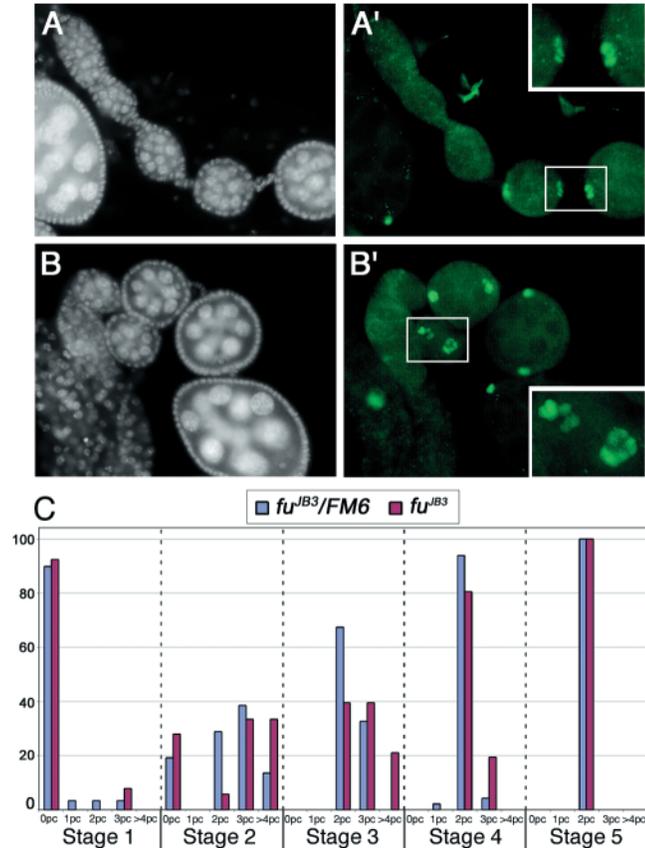
cells earlier was largely hindered by the lack of an early polar cell marker. However, using anti-Fasciclin III (Fas III) antibodies, we stained region 2b/3 prefollicular cells and found Fas III^{+} cells also positively stained with the TUNEL reagent (Fig. 3B′, arrowheads). Such cells could thus also correspond to pre-polar cells in the process of being eliminated. Altogether, these results suggest that the restriction in the number of polar cells occurs via apoptosis-induced cell death.

Given these results, blocking the endogenous apoptosis program would be expected to prevent elimination of pre-polar

Fig. 4. Polar cell differentiation is delayed in *fused* mutant females. (A,B) fu^{JB3}; A101/+ ovarioles double stained with DAPI (A,B) and anti-β-galactosidase antibodies (A′,B′). Higher magnification of two groups of three A101^{+} cells (inset A′) and two groups of 5 and 6 A101^{+} cells (left and right, respectively, inset B′) are shown. The A101 enhancer-trap line was used to visualize polar cells since introduction of this insertion in a *fu* mutant context does not modify *fu* ovarian phenotypes. (C) Percentage of fu^{JB3}/FM6 (blue) and fu^{JB3} (pink) stage 1 to 5 egg chambers containing 0, 1, 2, 3 or 4 and more polar cells ($n \geq 23$ for each stage). Note that reported data are of anterior polar cells.

Mutations in the Hedgehog signal transducer *fused* result in a delay in the polar cell differentiation program and allow visualization of putative pre-polar cells

The *fused* (*fu*) gene encodes a serine/threonine kinase identified as a positive effector of the Hedgehog signal transduction pathway in *Drosophila* embryo and imaginal disc development (Alves et al., 1998; Ingham, 1993; Limbourg-Bouchon et al., 1991; Sanchez-Herrero et al., 1996). In the ovary, Hedgehog signal transduction has been shown to control somatic stem cell (SSC) proliferation. Indeed, SSC self-renewing properties are not maintained in the absence of Hh signaling, whereas excessive Hh signaling produces supernumerary stem cells and leads to the accumulation of poorly differentiated somatic cells between egg chambers (Forbes et al., 1996; Zhang and Kalderon, 2000; Zhang and Kalderon, 2001). Our previous analysis of *fu* mutations had indicated that *fu* function is not involved in this process. Rather, we showed that *fu*-dependent Hedgehog signal transduction is necessary for somatic

prefollicular cell differentiation and morphogenesis (Besse et al., 2002). In particular, *fu* function seems to be required for correct timing of the polar cell differentiation program. Indeed, *fu* mutant females exhibit a global shift in the dynamics of A101 staining, as visualized after anti-β-galactosidase staining of fu^{JB3}/fu^{JB3}; A101/+ females (Fig. 4A′, compare with Fig. 1A). First, the appearance of A101 staining is delayed, as 28% (10/36) of stage 2 *fu* egg chambers do not exhibit any marked anterior cells compared to 19% (10/52) in heterozygous sisters (Fig. 4C). Second, restriction of A101 staining to 2 polar cells is also delayed, as 60% (26/43) of stage 3 and 19% (7/36) of stage 4 *fu* egg chambers contained 3 or more stained anterior cells compared to 33% (17/52) and 4% (2/48), respectively, for fu^+ egg chambers (Fig. 4C). Strikingly, 100% of stage 5 *fu* mutant egg chambers exhibit only 2 A101^{+} cells (Fig. 4C), indicating that restriction in the number of polar cells does eventually occur as in wild-type ovarioles. Altogether, these results suggest that *fu* mutations lead to a delay in the polar cell differentiation program.

Close examination of *fu* ovarioles revealed that a higher proportion of groups containing 4 A101^{+} cells (17/156), 5 A101^{+} cells (8/156), and even 6 A101^{+} cells (1/156) can be found in stage 2 as well as stage 3 *fu* mutant egg chambers compared to the wild-type situation (Fig. 4B′, inset, C). We reasoned that the presence of such groups of cells could result either from abnormally slow apoptosis-dependent elimination of pre-polar cells, or from an overproduction of pre-polar cells or their precursors. We could not directly test the first hypothesis because the relatively low number of TUNEL-positive cells found in both wild-type and *fused* females (possibly due to rapid elimination of apoptotic cells) made it impossible to compare quantitatively the dynamics of polar cell apoptotic cell death between these two contexts. We therefore tested the second hypothesis and looked for defects in polar and pre-polar cell proliferation, or in the number of polar cell precursor cells. First, polar cell proliferative properties do not seem to be altered in the vitellarium of *fu* ovarioles since (1) no increase in the size of A101[+] terminal clusters is observed with increasing age of *fu* egg chambers from stage 2 to 5 (Fig. 4C), and (2) no prolongation beyond stage 6 of somatic cell mitotic activity is observed in *fu* ovarioles (data not shown). Second, using the dominantly marked clone approach described above, we showed that early clusters of 4-6 A101^{+} cells found in *fu* females never contain more than 2 GFP^{+} cells (*n*=35, data not shown), and therefore that they do not result from extra divisions of precursor cells within the germarium. Third, we reasoned that preventing apoptosis in the polar cell lineage in a *fu* mutant context should give us an indication about the number of polar cell precursors present in these flies. If the number of such precursors is greater in *fu* females than in wild-type females, then blocking apoptosis should result in a greater number of "rescued" cells in polar cell clusters than in a wild-type context (that is more than 6 cells). Therefore, we used the flp-out/Gal4 system to generate large somatic clones of p35 overexpressing cells in a *fu* mutant context. The number of Fas III^{+} cells found in terminal clusters of fu^{JB3} hsp-*flp*/fu^{JB3}; UAS-p35/+; Act>CD2>Gal4 UAS-GFP/+ females was scored and compared to that of control fu^{JB3} hsp-*flp*/fu^{JB3}; Sp/+; Act>CD2>Gal4 UAS-GFP/+ sisters. Although an increase in the average size of the terminal Fas III[+] cell cluster was
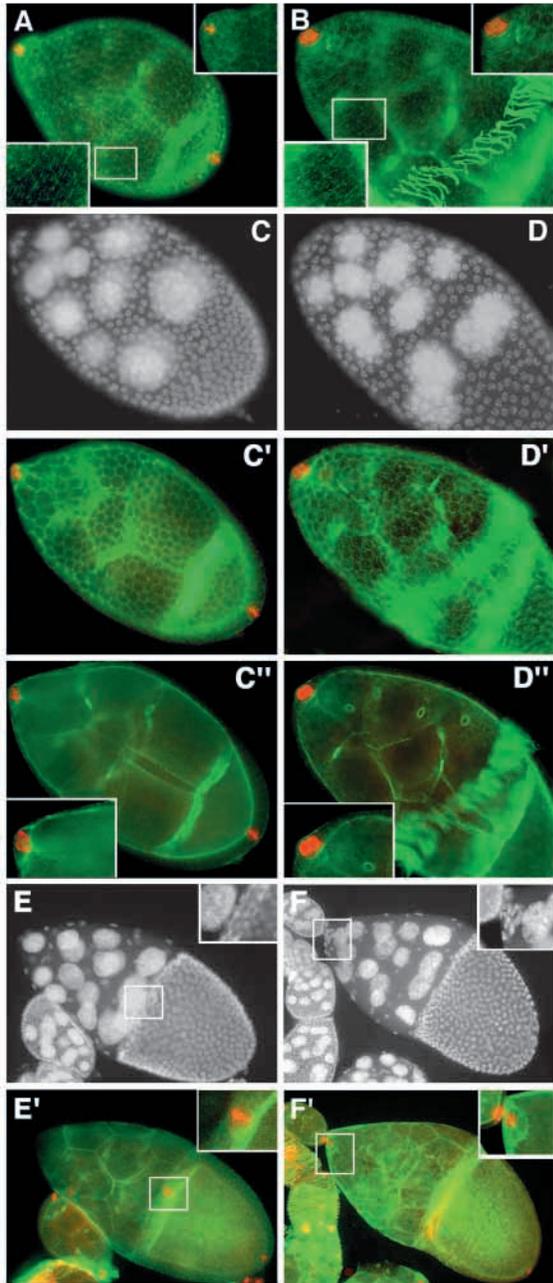
mediated by apoptosis since TUNEL-positive A101^{+} cells are found in *fused* females (data not shown).

Restriction in the number of polar cells is required for late egg chamber development

Restriction in the number of polar cells, though progressive, seems to be tightly controlled, as 100% of wild-type stage 5 egg chambers contain exactly 2 pairs of polar cells (Table 1). In order to determine the significance of this process, we looked for possible defects associated with the production of extra polar cells upon p35 overexpression. No obvious defects in early development of egg chambers, for example in individualization of germline cysts or formation of stalk structures, were observed (data not shown). Given that polar cells are assumed to produce a signal required for planar polarization of follicular cell actin filament bundles (Frydman and Spradling, 2001), we then examined actin organization in somatic epithelial cells of p35-overexpressing ovarioles. In wild-type ovarioles, actin filament bundles are present basally and orient themselves perpendicularly to the AP axis (Fig. 5A, insets). Polarization has been shown to arise gradually, beginning at stage 5, and proceeding from the poles toward the center (Frydman and Spradling, 2001). No perturbation, in either establishment or maintenance of actin filament orientation was detected in egg chambers containing extra polar cells (Fig. 5B and data not shown). However, two aspects of anterior somatic cell morphogenesis seem to be compromised in such ovarioles, whether p35 overexpression is ubiquitous or restricted to the polar cell lineage. Normally, starting at stage 9, anterior-most somatic follicular cells (or squamous cells) begin to stretch over nurse cells, which results in both the elongation of these cells (Fig. 5C′) and thinning of the epithelium (Fig. 5C″, inset) as visualized after phalloidin staining of actin in these cells. Other consequences of these morphogenetic changes are a reduction in the density of nuclei at the anterior pole and the development of a pointed shape at the anterior end of the egg chamber, as observed upon DAPI and phalloidin staining (Fig. 5C,C′). In p35-overexpressing ovarioles, anterior somatic cell stretching is perturbed as about 80% (35/44) of stage 9 egg chambers with multiple polar cells exhibited round anterior follicular cells (Fig. 5D′), a constant thickness of epithelial cells along the AP axis (Fig. 5D″, insert) and a homogeneous distribution of anterior follicular cell nuclei (Fig. 5D). Nonetheless, stage 10 egg chambers with extra polar cells were produced, but 91% (21/23) of these

observed after p35 overexpression (a mean number of 3.64 cells per cluster was observed in egg chambers from stage 3 to 7 compared to 2.45 in control females, $n \geq 426$, $P < 0.001$), only groups containing 2 to 6 cells were recovered. This indicates that *fused* females contain the same number of polar cell precursors as wild-type females.

Therefore, we interpret the supernumerary polar cells in both wild-type and *fused* mutant contexts to represent pre-polar cells, and propose that slower apoptosis-mediated reduction in the number of these cells in a *fused* context allows easier visualization of these cells. Thus, *fused* mutations, by delaying the somatic cell differentiation program, confirm the existence of pre-polar cell clusters and allow detection of up to 6 pre-polar cells. However, restriction in the final number of polar cells is achieved by stage 5 (Fig. 4C) and is probably also

presented defects in delamination and migration of border cells. In wild-type ovarioles, groups of 6-8 border cells detach from the anterior epithelium and migrate through the nurse cells during stage 9, and reach the oocyte at stage 10. Anterior polar cells, though not by themselves migratory, are dragged along by outer border cells and therefore can be used to mark the migratory pathway (Fig. 5E,E′, insets). In stage 10 egg chambers with extra polar cells, clusters of border cells were either still stuck at the anterior pole (Fig. 5F,F′, insets) or only partway between the anterior pole and the oocyte (data not shown). In addition, these clusters were often observed to contain supernumerary border cells, as a mean of 11 border cells was found in extra polar cell-containing stage 10 egg chambers (compared to 7.2 in control females, $n{\geq}26$). Interestingly, clusters of 5 or 6 polar cells recruited a higher number of border cells than those containing only 3 or 4 (data not shown).

Thus, prevention of pre-polar cell death leads to defects in both squamous and border cell morphogenetic properties, showing that polar cells act in a non cell-autonomous fashion to define anterior follicular cell identity.

DISCUSSION

Refining the polar cell lineage

In the literature, polar cells are described as pairs of cells found at both egg chamber termini and characterized by their round morphology and specific pattern of gene expression. In addition, using clonal analysis, previous studies demonstrated that polar cells stop dividing as early as germarial region 2b, unlike epithelial follicular cells that continue to divide until stage 6 of oogenesis (Margolis and Spradling, 1995; Tworoger et al., 1999). These results indicate that polar cell precursors have already diverged from follicular cell precursors in this region of the germarium. In the present study, we examined early egg chambers and found that polar cell number is only progressively restricted to 2, since stage 2 to 4 egg chambers contain groups of 3 to 5 cells strongly expressing polar cell markers. Using flp-out/Gal4 based clonal analysis, we were able to show, first, that these clusters have a polyclonal origin, and, second, that the extra polar cells belong to the polar/stalk cell lineage and not to the follicular cell lineage. In addition, several lines of evidence suggest that for each polar cell pair, a pool of up to 6 pre-polar cells is generated in the germarium: (1) groups of up to 5 pre-polar cells can be observed in wild-type females; (2) blocking apoptosis, in an otherwise wild-type context, leads to the accumulation of up to 6 pre-polar cells (see below); and (3) *fused* mutations, by delaying the prefollicular cell differentiation program, allow visualization of up to 6 pre-polar cells. Finally, we showed that polar cells of a given pair can derive either from the same direct precursor cell, or from two different precursors. Therefore, we propose that each mature polar cell pair is selected from among pre-polar cells in a stochastic manner.

In addition, Tworoger and co-workers showed that polar and stalk cells derive from a common population of precursor cells and together form a lineage distinct from that of main body follicular cells (Tworoger et al., 1999). Based on this study, the authors proposed that all polar cells and stalk cells separating two consecutive egg chambers derive from common precursors

and therefore form a so-called 'polar cells-stalk cells-polar cells' unit. However, since neither their work, nor our work indicates a lineage relationship between polar cells at the extremities of a given unit, we prefer to consider half of such a unit in the following working model. As shown in Fig. 6, we propose that mature polar cells and stalk cells originate from a several step process. First, polar cell and stalk cell precursors (white circles, first row) would divide at least twice to generate groups of cells within which pre-stalk and pre-polar cells are specified. Divergence between stalk cell (blue circles) and polar cell (pink circles) fate could occur either through a lineage-based process (arrow 1), or after completion of divisions (arrow 2), through cell-cell interactions between stalk and polar cell precursors (lateral inhibition-type mechanism) or between stalk/polar cell precursors and other neighboring cell types. A subsequent step would then be final differentiation of stalk cells and polar cells from groups of pre-stalk and pre-polar cells. Although the number and fate of pre-stalk cells remains to be determined, our results indicate that polar cell pairs are generated through apoptosis-mediated elimination of supernumerary pre-polar cells (see below).

Apoptosis as a way to refine cell lineage

Selective removal of cells by programmed cell death is an evolutionarily conserved and widely used mechanism that plays a fundamental role in pattern formation and morphogenesis during embryonic and adult development of multicellular organisms (Rusconi et al., 2000; Vaux and Korsmeyer, 1999). Apoptosis, the most well studied form of programmed cell death, is also involved in precise elimination of either unnecessary or deleterious supernumerary cells within specific cell lineages, as exemplified during *C. elegans* hermaphrodite development during which exactly 131 somatic cells are invariantly fated to die (Ellis and Horvitz, 1986).

Our study of the *Drosophila* ovarian polar cell lineage reveals that each pair of mature polar cells derives from a pool of precursor pre-polar cells within which supernumerary cells are eliminated via an apoptosis-dependent mechanism. This mechanism probably requires both caspase activity and the 'death' gene *reaper* since it is inhibited by ectopic expression of the bacculoviral p35 protein and is associated with specific induction of *reaper* expression (Hay et al., 1994; Nordstrom et al., 1996). However, whereas the self-death machinery appears to be evolutionary conserved (Meier et al., 2000; Song and Steller, 1999), a wide range of distinct signaling mechanisms can be used to elicit apoptosis. Interestingly, a stochastic cell loss phenomenon has been documented in the *Drosophila* embryonic nerve cord where a subset of midline glial cells undergo apoptosis-mediated reduction in cell number between stages 13 and 16, after these cells have facilitated commissure tract morphogenesis (Sonnenfeld and Jacobs, 1995). In this system, induction of midline expression of p35 prevents the normal reduction of midline glial cell number from 6 to, on average, 3.2, resulting in a subsequent disruption of the central nervous system (CNS) midline (Zhou et al., 1997). At least two cell-cell signaling pathways, the EGF and Notch pathways, are thought to mediate cell-death in the ventral midline. However, although modulation of EGF receptor activity clearly alters the pattern of midline cell death, the role of the Notch signal transduction pathway is less clear, in part because the latter is also required for cell fate

Fig. 6. Proposed polar cell lineage. Given that polar cells and stalk cells have been shown to have common precursors, we propose the existence of at least 3 stalk and polar cell precursors (white circles, first row) for each polar cell pair and neighboring stalk cells. The stalk cells considered are not necessarily those immediately adjacent to the polar cell pair since intercalation events disperse the cells giving rise to an interfollicular stalk. Such precursors would first generate groups of pre-polar (pink circles) and pre-stalk (blue circles) cells. Divergence between pre-stalk and pre-polar cells could occur either in the course of stalk cell/polar cell precursor divisions (1, arrow), or after completion of divisions (2, arrow). A subsequent step involving apotosis-mediated selection of the final polar cell pair (red) would then take place among groups of up to 6 pre-polar cells (indicated by Xs in boxed area). Although we do not have any evidence yet for the existence of a selection process among pre-stalk cells, such a mechanism could also be part of the stalk cell maturation program (X ? in boxed area).
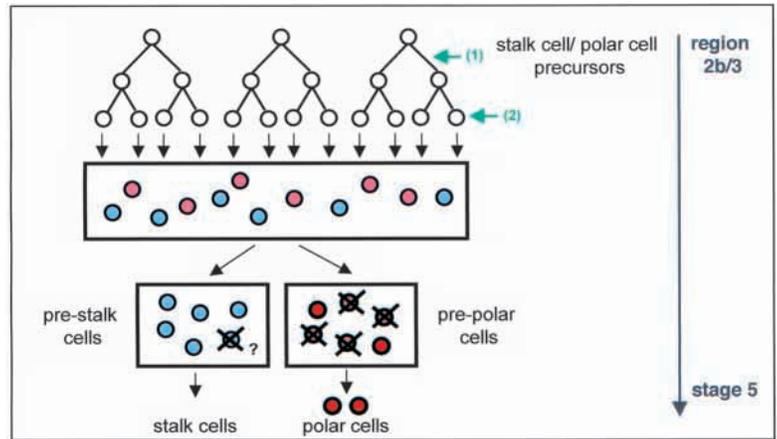
specification (Lanoue and Jacobs, 1999; Menne and Klambt, 1994; Stemerdink and Jacobs, 1997). In the ovarian system, cellular interactions within or without the pre-polar cell cluster may also be crucial for regulation of the selective pre-polar cell loss. In the present study, we were not able to make any correlation between pre-polar cell position and cell removal, at least for apoptosis events occurring after egg chamber budding. It would be interesting nonetheless to examine Notch signaling as a survival factor in this system. Indeed, induction of Notch loss-of-function clones in prefollicular cells is associated with absence of polar cells (Lopez-Schier and St Johnston, 2001). Conversely, egg chambers with terminal clones expressing an activated form of Notch contain up to 6 polar cell marker-positive cells (Grammont and Irvine, 2001). Such phenotypes, interpreted as reflecting a role for Notch signaling in polar cell specification, could also correspond to a Notch-dependent control of apoptosis within the pre-polar cell lineage.

Considering both the embryonic and oogenesis systems, the question can be asked as to the biological significance of creating supernumerary cells to remove them afterwards. One proposed explanation for what is observed in the embryonic CNS is that a differential number of glial cells may be required depending on the developmental stage. By analogy, at least 6 pre-polar cells may be required in the ovary to assume early germarial functions, whereas only 2 polar cells may be needed during later egg chamber maturation. Another possibility is that the process of polar cell production corresponds to an evolutionarily conserved mechanism to which removal of deleterious cells would have been added later. Further studies are now needed to determine whether the pre-polar cell clusters have a functional role in early oogenesis.

Function of polar cells in the patterning of anterior follicular somatic cells

Polar cells are involved in several important signaling processes during oogenesis, including posterior positioning of the oocyte (Tworoger et al., 1999; Zhang and Kalderon, 2000), induction of stalk cells in the germarium (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001), organization of follicular cell epithelial planar polarity

during mid-oogenesis (Frydman and Spradling, 2001) and anteroposterior patterning of follicular epithelial cells at stage 9 (Beccari et al., 2002).

Strikingly, polarization of basal actin filament bundles in the follicular epithelium arises progressively, starting at stage 5 and proceeding from the poles, suggesting the existence of a diffusible signal produced by polar cells. Consistent with this, ectopic polar cells generated upon *hedgehog* overexpression have the capacity to reorganize actin bundles locally (Frydman and Spradling, 2001). However, orientation of planar actin filaments probably does not require a precise level of polarizing signal since we have shown here that an increase in polar cell number does not affect the establishment or maintenance of planar polarity.

In contrast, we find that the restriction in the number of polar cells seems to be required for correct anterior patterning of follicular epithelial cells. Indeed, preventing the elimination of supernumerary pre-polar cells results in morphogenetic defects affecting both stretching of anterior squamous cells and migration of border cell clusters. The latter is also accompanied by an increase in the number of recruited border cells. Production of ectopic polar cells has been described to induce ectopic and poorly migrating border cells at stage 9 (Liu and Montell, 1999; Zhang and Kalderon, 2000). Yet, this phenomenon was observed upon ectopic activation of *hedgehog* signal transduction (*patched* and *Costal 2* mutant contexts). Therefore it is not clear in these cases whether migration defects are a direct consequence of extra border cell number or whether they reflect an additional effect of the Hedgehog signaling pathway on border cell migration and/or specification. In our study, we were able to observe defective border cell migration after having prevented cell death specifically within *neuralized*-expressing pre-polar and polar cells. This suggests, first, that rescued pre-polar cells are not inert and, second, that final polar cell number per se is critical for both border cell recruitment and migration. Interestingly, polar cells show specific expression of Unpaired (Upd), an extracellular ligand described to activate the conserved JAK/Stat signaling pathway (Beccari et al., 2002; McGregor et al., 2002; Silver and Montell, 2001). In the absence of positive effectors of this pathway, such as Unpaired, Hopscotch or STAT92E, defects in both recruitment and

migration of border cells and sometimes also in stretching of squamous cells are observed. In addition, ectopic expression of Upd in a subset of anterior somatic cells is sufficient to induce expression of border cell markers in adjacent squamous cells (Beccari et al., 2002; Silver and Montell, 2001). Interestingly, high levels of Upd result in the formation of egg chambers in which both normal and supernumerary border cells frequently fail to migrate. Altogether, this suggests that Upd could act as a morphogen produced by polar cells and necessary for establishing anteroposterior patterning of the follicular epithelium. In the present study, prevention of pre-polar cell death and subsequent generation of supernumerary polar cells may lead to production of an excess of signaling molecules, such as Upd, and alteration of endogenous morphogen gradients, which could explain why both squamous cells and border cells exhibit aberrant behavior. Our results therefore provide further evidence for a non cell-autonomous role for anterior polar cells in patterning of the follicular epithelium.

REFERENCES

Alves, G., Limbourg, B. B., Tricoire, H., Brissard, Z. J., Lamour, I. C. and Busson, D. (1998). Modulation of Hedgehog target gene expression by the Fused serine- threonine kinase in wing imaginal discs. *Mech. Dev.* **78**, 17-31.

Beccari, S., Teixeira, L. and Rorth, P. (2002). The JAK/STAT pathway is required for border cell migration during Drosophila oogenesis. *Mech. Dev.* **111**, 115-123.

Bellaiche, Y., Gho, M., Kaltschmidt, J. A., Brand, A. H. and Schweisguth, F. (2001). Frizzled regulates localization of cell-fate determinants and mitotic spindle rotation during asymmetric cell division. *Nat. Cell Biol.* **3**, 50-57.

Besse, F., Busson, D. and Pret, A. M. (2002). Fused-dependent Hedgehog signal transduction is required for somatic cell differentiation during *Drosophila* egg chamber formation. *Development* **129**, 4111-4124.

Ellis, H. M. and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell* **44**, 817-829.

Forbes, A. J., Lin, H., Ingham, P. W. and Spradling, A. C. (1996). hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. *Development* **122**, 1125-1135.

Frydman, H. M. and Spradling, A. C. (2001). The receptor-like tyrosine phosphatase lar is required for epithelial planar polarity and for axis determination within drosophila ovarian follicles. *Development* **128**, 3209-3220.

Gonzalez-Reyes, A., Elliott, H. and St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature* **375**, 654-658.

Gonzalez-Reyes, A. and St Johnston, D. (1998). Patterning of the follicle cell epithelium along the anterior-posterior axis during Drosophila oogenesis. *Development* **125**, 2837-2846.

Grammont, M. and Irvine, K. D. (2001). fringe and Notch specify polar cell fate during Drosophila oogenesis. *Development* **128**, 2243-2253.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-2129.

Ingham, P. W. (1993). Localized hedgehog activity controls spatial limits of wingless transcription in the Drosophila embryo. *Nature* **366**, 560-562.

Keller Larkin, M., Deng, W. M., Holder, K., Tworoger, M., Clegg, N. and Ruohola-Baker, H. (1999). Role of the Notch pathway in terminal follicle cell differentiation during Drosophila oogenesis. *Dev. Genes Evol.* **209**, 301-311.

King, R. C. (1970). *Ovarian Development in* Drosophila melanogaster. New York: Academic Press.

Lanoue, B. R. and Jacobs, J. R. (1999). Rhomboid function in the midline of the Drosophila CNS. *Dev. Genet.* **25**, 321-330.

Limbourg-Bouchon, B., Busson, D. and Lamour-Isnard, C. (1991). Interactions between fused, a segment-polarity gene in Drosophila, and other segmentation genes. *Development* **112**, 417-429.

Lin, H. and Spradling, A. C. (1993). Germline stem cell division and egg chamber development in transplanted Drosophila germaria. *Dev. Biol.* **159**, 140-152.

Liu, Y. and Montell, D. J. (1999). Identification of mutations that cause cell migration defects in mosaic clones. *Development* **126**, 1869-1878.

Lopez-Schier, H. and St Johnston, D. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. *Genes Dev.* **15**, 1393-1405.

Margolis, J. and Spradling, A. (1995). Identification and behavior of epithelial stem cells in the Drosophila ovary. *Development* **121**, 3797-3807.

McGregor, J. R., Xi, R. and Harrison, D. A. (2002). JAK signaling is somatically required for follicle cell differentiation in Drosophila. *Development* **129**, 705-717.

McKearin, D. and Ohlstein, B. (1995). A role for the *Drosophila* bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121**, 2937-2947.

Meier, P., Finch, A. and Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.

Menne, T. V. and Klambt, C. (1994). The formation of commissures in the Drosophila CNS depends on the midline cells and on the Notch gene. *Development* **120**, 123-133.

Neufeld, T. P., de la Cruz, A. F., Johnston, L. A. and Edgar, B. A. (1998). Coordination of growth and cell division in the Drosophila wing. *Cell* **93**, 1183-1193.

Nordstrom, W., Chen, P., Steller, H. and Abrams, J. M. (1996). Activation of the reaper gene during ectopic cell killing in Drosophila. *Dev. Biol.* **180**, 213-226.

Ray, R. P. and Schupbach, T. (1996). Intercellular signaling and the polarization of body axes during Drosophila oogenesis. *Genes Dev.* **10**, 1711-1723.

Riechmann, V. and Ephrussi, A. (2001). Axis formation during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **11**, 374-383.

Ruohola, H., Bremer, K. A., Baker, D., Swedlow, J. R., Jan, L. Y. and Jan, Y. N. (1991). Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in Drosophila. *Cell* **66**, 433-449.

Rusconi, J. C., Hays, R. and Cagan, R. L. (2000). Programmed cell death and patterning in Drosophila. *Cell Death Differ.* **7**, 1063-1070.

Sanchez-Herrero, E., Couso, J. P., Capdevila, J. and Guerrero, I. (1996). The fu gene discriminates between pathways to control dpp expression in Drosophila imaginal discs. *Mech. Dev.* **55**, 159-170.

Silver, D. L. and Montell, D. J. (2001). Paracrine signaling through the JAK/STAT pathway activates invasive behavior of ovarian epithelial cells in Drosophila. *Cell* **107**, 831-841.

Song, Z. and Steller, H. (1999). Death by design: mechanism and control of apoptosis. *Trends Cell Biol.* **9**, M49-52.

Sonnenfeld, M. J. and Jacobs, J. R. (1995). Apoptosis of the midline glia during Drosophila embryogenesis: a correlation with axon contact. *Development* **121**, 569-578.

Spradling, A. (1993). Developmental genetics of oogenesis. In *The Development of* Drosophila melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 1-70. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Spradling, A. C., de Cuevas, M., Drummond, B. D., Keyes, L., Lilly, M., Pepling, M. and Xie, T. (1997). The Drosophila germarium: stem cells, germ line cysts, and oocytes. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 25-34.

Stemerdink, C. and Jacobs, J. R. (1997). Argos and Spitz group genes function to regulate midline glial cell number in Drosophila embryos. *Development* **124**, 3787-3796.

Therond, P., Alves, G., Limbourg, B. B., Tricoire, H., Guillemet, E., Brissard-Zahraoui, J., Lamour, I. C. and Busson, D. (1996). Functional

domains of fused, a serine-threonine kinase required for signaling in Drosophila. *Genetics* **142**, 1181-1198.

Tworoger, M., Larkin, M. K., Bryant, Z. and Ruohola, B. H. (1999). Mosaic analysis in the drosophila ovary reveals a common hedgehog- inducible precursor stage for stalk and polar cells. *Genetics* **151**, 739-748.

van Eeden, F. and St Johnston, D. (1999). The polarisation of the anterior-posterior and dorsal-ventral axes during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **9**, 396-404.

Vaux, D. L. and Korsmeyer, S. J. (1999). Cell death in development. *Cell* **96**, 245-254.

White, K., Tahaoglu, E. and Steller, H. (1996). Cell killing by the Drosophila gene reaper. *Science* **271**, 805-807.

Wieschaus, E. and Szabad, J. (1979). The development and function of the female germ line in Drosophila melanogaster: a cell lineage study. *Dev. Biol.* **68**, 29-46.

Zhang, Y. and Kalderon, D. (2000). Regulation of cell proliferation and patterning in Drosophila oogenesis by Hedgehog signaling. *Development* **127**, 2165-2176.

Zhang, Y. and Kalderon, D. (2001). Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. *Nature* **410**, 599-604.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L. M., Steller, H. and Nambu, J. R. (1997). Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. *Proc. Natl. Acad. Sci. USA* **94**, 5131-5136.