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Eyes Absent, a key repressor of polar cell fate during *Drosophila* oogenesis

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SUMMARY

Throughout *Drosophila* oogenesis, specialized somatic follicle cells perform crucial functions in egg chamber formation and in signaling between somatic and germline cells. In the ovary, at least three types of somatic follicle cells, polar cells, stalk cells and main body epithelial follicle cells, can be distinguished when egg chambers bud from the germarium. Although specification of these three somatic cell types is important for normal oogenesis and subsequent embryogenesis, the molecular basis for establishment of their cell fates is not completely understood. Our studies reveal the gene *eyes absent* (*eya*) to be a key repressor of polar cell fate. EYA is a nuclear protein that is normally excluded from polar and stalk cells, and the absence of EYA is sufficient to cause epithelial follicle cells to develop as polar cells. Furthermore, ectopic expression of EYA is capable of suppressing normal polar cell fate and compromising the normal functions of polar cells, such as promotion of border cell migration. Finally, we show that ectopic Hedgehog signaling, which is known to cause ectopic polar cell formation, does so by repressing *eya* expression in epithelial follicle cells.

Key words: Eyes absent (EYA), Polar cell, Hedgehog, Oogenesis, *Drosophila melanogaster*

INTRODUCTION

Drosophila oogenesis provides an excellent system with which to study the mechanisms underlying specification of different cell fates. The *Drosophila* ovary is made up of germline cells and somatic follicle cells. Germline and somatic stem cells can be found at the anterior end of the ovary in a structure called the germarium (King, 1970; Spradling, 1993). Germline stem cells divide asymmetrically and produce cystoblasts, which undergo four rounds of incomplete cell division and give rise to 16-cell germline cysts. One of the cyst cells becomes the oocyte and the remaining 15 cells differentiate as nurse cells. In the germarium, somatic follicle cells surround the 16-cell cysts. As the nascent egg chamber buds off from the germarium, at least three types of somatic cells can be distinguished by their morphologies and locations: polar cells, stalk cells and epithelial follicle cells. Polar cells are pairs of specialized follicle cells at each pole of the egg chamber, whereas the five to eight stalk cells separate adjacent egg chambers. Stalk and polar cells may descend from a common precursor (Tworoger et al., 1999). They differentiate and cease division soon after egg chambers form (Margolis and Spradling, 1995; Tworoger et al., 1999). The remaining somatic follicle cells, referred to here as epithelial follicle cells, proliferate until stage 6 of oogenesis and form a continuous epithelium around the sixteen germ cells. Subsequently, further differentiation of epithelial follicle cells occurs. Throughout oogenesis, these specialized somatic follicle cells perform crucial functions in egg chamber morphogenesis, follicle cell patterning, and/or signaling between somatic and germline cells (Dobens and Raftery, 2000). The discrimination of cell types among the somatic follicle cells is crucial not only for oogenesis, but also later for the specification of embryonic polarity; however, the molecular basis for establishment of their cell fates is not completely understood.

Recent studies have demonstrated that the specification of all somatic follicle cells requires the activation of Notch signaling (Deng et al., 2001; Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). Early in oogenesis, Notch is essential for differentiation of the polar cells and stalk cells. In the absence of polar cells that is caused by the loss of Notch (N) function, cyst encapsulation fails and stalk formation does not occur. As a result, neighboring egg chambers fail to separate from each other. This gives rise to compound egg chambers that contain multiple germline cysts in a single follicle epithelium. Notch signaling also plays an essential role later in oogenesis in promoting the differentiation of the epithelial follicle cells from an immature, undifferentiated precursor state. Although Notch is indispensable for polar cell specification, activation of Notch is not sufficient to induce polar cell fate in the epithelial layer (Grammont and Irvine, 2001).

Another signal that affects somatic cell fates is Hedgehog (HH). Overexpression of HH generates ectopic polar cells throughout the follicle epithelium. The HH signaling pathway has been well characterized, and the same pathway is employed in many different tissues (Murone et al., 1999). Upon binding of HH to its receptor, Patched (PTC), the activity of a second transmembrane protein, Smoothened (SMO) is derepressed, ultimately leading to the activation of Cubitus interruptus (CI). CI is a transcription factor that transmits the HH signal from the cytoplasm to the nucleus, where it is primarily responsible

for the activation and repression of HH target genes (Aza-Blanc and Kornberg, 1999). CI exists in at least two forms. In the absence of the HH signal, CI is proteolytically cleaved to a 75 kDa repressor form. In the presence of HH, this cleavage is prevented, and CI instead functions as a transcriptional activator. Consistent with this model, effects of HH signaling are observed only in the presence of CI (Methot and Basler, 2001). The regulation of CI is complex and involves the function of positive as well as negative regulators. The negative regulators required for repression of this pathway include the transmembrane protein PTC (Hooper and Scott, 1989; Nakano et al., 1989), protein kinase A (PKA) (Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995), and a kinesin-related protein, Costal2 (COS2; COS – FlyBase) (Robbins et al., 1997; Sisson et al., 1997). Loss of either *ptc*, *cos2* or *Pka* stimulates intracellular HH signaling, even in the absence of HH. In the ovary, this results in ectopic polar cells, as does overexpression of HH (Forbes et al., 1996; Liu and Montell, 1999; Zhang and Kalderon, 2000). The mechanism by which overexpression of HH signaling promotes ectopic polar cell fate is not known. Nor is it completely clear how polar cell, stalk cell and epithelial follicle cell fates are normally specified.

We have isolated a new allele of the gene *eyes absent* (*eya*), which, when mutated, generates ectopic polar cells throughout the follicular epithelium, a phenotype similar to that caused by ectopic HH signaling. EYA encodes a nuclear protein best known for its essential role in the formation of the adult eye in *Drosophila* (Bonini et al., 1993; Bonini et al., 1998; Boyle et al., 1997). Our results indicate that EYA protein is normally absent from polar and stalk cells, and EYA is a key repressor for polar cell fate. Furthermore, the absence of EYA is sufficient to cause epithelial follicle cells to develop as polar cells. Thus, EYA expression must be repressed in polar and stalk cells in order for these fates to be determined. Finally, we show that the mechanism by which ectopic HH signaling is able to transform other follicle cells into ectopic polar cells is through suppressing EYA expression.

MATERIALS AND METHODS

Drosophila genetics

Fly culture and crosses were performed according to standard procedures. The mosaic screen for mutations on 2L that affected border cells has been described previously (Bai et al., 2000). *54C2* complemented all 2L deficiency lines available from the Bloomington Stock Center. To map the locus by meiotic recombination, recombinants were scored for *dp* (25A2), *PZ6356* (34C1, 2), ectopic polar cells in mutant egg chambers, the eye phenotype and inter se lethality. A lethal mutation between *dp* and *PZ6356* co-segregated with the mosaic phenotypes. The recombination frequency between *dp* and *54C2* was ~7% (5/72). Combined with the deficiency complementation result, these data place *54C2* locus between 26D and 27A. *54C2* complemented all available P insertions in this region. *eya* is located in this region and mutations generate a similar eye phenotype to that of *54C2*, and can be female sterile (Bonini et al., 1998). Further complementation and phenotypic analysis established that *54C2* was an allele of *eya* (see Results).

Mutant clones were generated by mitotic recombination using the *FLP/FRT* system (Xu and Rubin, 1993) either by an X-chromosome *hs-flp* or by *T155, UAS-flp* (Duffy et al., 1998). *T155, UAS-flp* directs expression of the recombinase *flp* in follicle cell precursors and some imaginal discs, including the eye-antenna, wing and leg discs. Clones were marked using the α*tub84BlacZ* (‘*tublacZ*’) transgenes on 2L and 2R (Harrison et al., 1995) or *ubi-nlsGFP* (Davis et al., 1995) (Bloomington Stock Center). Three to five-day-old female flies were dissected when clones were produced by *T155, UAS-flp*. *hs-flp* induced clones were generated by heat-shocking newly hatched females for 1 hour at 37°C, twice a day, for 3 days in a row. Adult females were dissected 2-8 days after the last heat shock. The following alleles were used:

cli^{E11}, FRT40A and *cli^{D1}, FRT40A* (from Dr Steve DiNardo);

FRT42D, ptc^{S2}; *FRT42D, ptc^{IIW}* (from Dr Gary Struhl);

DCO^{H2}, FRT40A (DCO^{H2} is a *pka* null allele) (Ohlmeyer and Kalderon, 1998);

G13, cos2^{52B7} (Liu and Montell, 1999); and

FRT42D, so^{3} (Pignoni et al., 1997).

ci clones marked by the loss of GFP were generated using flies of the genotype: *hs-flp;FRT42D P[Ci^{+}] P[hsp70-GFP]/FRT42D; ci^{94}/ci^{94}* (Methot and Basler, 1999). Ectopic expression of constitutively activated CI was performed by excision of an FRT-flanked CD2 gene from an *act5C>CD2>GAL4* transgene (Jiang and Struhl, 1998) in the presence of the activated *UAS-Ci5m* transgene from line T5m-s1 (Price and Kalderon, 1999). Overexpression of *eya* in the ovary was performed using either *hs-GAL4* or *C306* (Manseau et al., 1997) or *upd-Gal4* (from Dr Harrison) in animals carrying either *UASeya1* or *UASeya2* (from Dr Nancy Bonini). Female flies were heat shocked for 1 hour twice a day for 3 days and then their ovaries were dissected, stained and examined either immediately or 1-3 days later. Under these conditions, the frequency and severity of the formation of compound egg chambers increased with longer exposure to heat shock. If dissected 3 days after a 3-day heat-shock, ~30% of the ovary was composed of compound egg chambers. Overexpression of activated Notch was performed by using FO64 line (*Act5Cflip-out Gal4, UAS-GFP/CyO; hsFLP,MKRS/TM6B)* (from Dr Y. Hiromi) and *UAS-N^{intro}* (from Dr Norbert Perrimon).

The following fly stains were also used: *w^{1118}* as wild type controls; *93F/TM3* (Ruohola et al., 1991); *A101/TM3* (Bier et al., 1989), *so^{5}-lacZ* (from Dr Pignoni), *eya^{3cs}/CyO; E(P)10/CyO* (Bonini et al., 1998); ub-Cadherin-GFP (from Dr Oda).

Immunohistochemistry and immunofluorescence

Ovary dissections were performed in Grace’s medium plus 10% fetal calf serum. For β-galactosidase staining, whole ovaries were fixed in PBS containing either 0.1% glutaraldehyde or 3.7% formaldehyde for 5 minutes, and rinsed once with PBT (PBS+0.1% Triton X-100) followed by incubation of the ovaries with 0.2% X-gal in staining solution (10 mM phosphate buffer pH7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_4[FeII(CN)_6]$, 3 mM $K_3[FeIII(CN)_6]$, 0.3% Triton X-100) for 10 minutes to overnight. After staining, the ovaries were rinsed twice with PBT and mounted in PBS containing 50% glycerol.

For fluorescence staining, the egg chambers were typically fixed in 3.7% formaldehyde (Polysciences) in 0.1 M phosphate buffer (pH 7.4) containing 0.5% NP40 for 20 minutes and rinsed three times in NP40 wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP40, 1 mg/ml BSA). Egg chambers were then blocked in NP40 wash buffer plus 5% normal goat serum for 30 minutes and were incubated in block solution containing the primary antibodies overnight at 4°C. After four washes for 20 minutes each in NP40 wash buffer, the egg chambers were incubated in block solution containing fluorescence-conjugated secondary antibodies at a dilution of 1:200 for 2 hours at room temperature. For rhodamine-phalloidin (Molecular Probes) and DAPI (Molecular Probes) staining, egg chambers were incubated with rhodamine-phalloidin in NP40 wash buffer at a dilution of 1:200 for 25 minutes followed by adding DAPI to a final concentration 0.5 μg/ml and incubation of egg chambers for another 5 minutes. After three washes for 20 minutes in NP40 wash buffer, the egg chambers were mounted in VECTASHIELD® (Vector Laboratories) or Aqua Poly/Mount (Polysciences). All images were captured either on a

Noran OZ laser confocal microscope or on a LEICA TCSNT laser confocal microscope, or on a Zeiss Axioplan microscope.

The primary antibodies used were mouse anti-FAS3 (1:3), mouse anti-DAC (1:250) (Developmental Studies Hybridoma Bank), mouse anti-EYA (10H6 1:1000) (Bonini et al., 1993), rat anti-full length form of CI (2A1 1:5) (Slusarski et al., 1995), rabbit anti-CI (CIN, which recognizes both the full length and degraded form; 1:250) (Aza-Blanc and Kornberg, 1999), rabbit anti-EY(1:300) (Kurusu et al., 2000), mouse anti-β-galatosidase (1:500) (Promega) and rabbit anti-β-galatosidase (1:2000) (Cappel). The secondary antibodies used were horse fluorescein anti-mouse (Vector Laboratories), Cy™5-conjugated donkey anti-rabbit, Cy™5-conjugated donkey anti-mouse; fluorescein (FITC)-conjugated donkey anti-rabbit, FITC-conjugated donkey anti-rat, rhodamine red™-X-conjugated donkey anti-mouse and rhodamine red™-X-conjugated donkey anti-rabbit (Jackson ImmunoResearch).

RESULTS

Mutation of *eya*, like ectopic HH signaling, causes ectopic polar cells

In wild-type egg chambers, the anterior polar cells recruit four to eight follicle cells to surround them and become migratory border cells at early stage 9 (Fig. 1A). They migrate through the nurse cell cluster during stage 9 (Fig. 1A,H) and arrive at the border between the oocyte and the nurse cells at stage 10 (Fig. 1A,B). Ectopic HH signaling, e.g. caused by loss of *cos2*, results in the formation of ectopic polar cells and recruitment of extra border cells, which frequently migrate (Liu and Montell, 1999) (Fig. 1D,G). In the course of a genetic screen for mutations on the left arm of the second chromosome (2L) that affect border cells in mosaic clones (Bai et al., 2000), one ethyl methanesulfonate (EMS)-induced mutation, *54C2*, was identified that caused extra clusters of border cells to form (Fig. 1C). Border cells were marked by β-galactosidase expression from an enhancer trap line, PZ6356 (Tinker et al., 1998). In wild-type egg chambers, there was only one cluster of border cells (Fig. 1B). By contrast, in egg chambers containing *54C2* mutant clones, either multiple clusters of migrating border cells (Fig. 1C) or a single abnormally large cluster (Fig. 1F) was observed. This phenotype resembled that of egg chambers containing *cos2* (Liu and Montell, 1999) (Fig. 1C,G) or *ptc* mutant clones (Zhang and Kalderon, 2000) (data not shown).

The extra border cell clusters found in *cos2* or *ptc* mutant egg chambers result from overproduction of polar cells (Liu and Montell, 1999; Zhang and Kalderon, 2000) (Fig. 1G,J). Polar cells can be detected by staining with an antibody against Fasciclin III (Ruohola et al., 1991) (FAS3, Fig. 1E-G), or by expression of β-galactosidase from the enhancer trap line A101 (*neuralized-lacZ*) (Bier et al., 1989), which is a marker for mature polar cells (Fig. 1H-J). FAS3 is a homophilic cell adhesion molecule that accumulates to the highest levels in immature follicle cells in the germarium (Fig. 1K) and at the interface between the two polar cells from stage 3 to stage 10A of oogenesis in wild-type egg chambers (Fig. 1K,L). There are two pairs of polar cells in wild-type egg chambers, one pair located at the anterior pole of the egg chamber and another at the posterior (Fig. 1H,L). In *54C2* mutant egg chambers, the extra border cell clusters that formed contained extra polar cells (Fig. 1F,I). In addition, ectopic polar cells were observed in early stage egg chambers (not shown) and were found in many positions throughout the follicle epithelium in egg chambers containing mutant clones (Fig. 1I).

We identified the gene mutated in the *54C2* line based on deficiency and meiotic recombination mapping. *54C2* was found to reside in the 26D-27A region (see Materials and Methods for details). Mutations in one known gene in this region, *eya*, failed to complement the *54C2* mutation with respect to lethality, whereas all other mutations in this region complemented. In addition, two independent *eya* alleles, cli^{E11} and cli^{D1}, caused ovarian phenotypes in mosaic clones that were similar to those of *54C2*, including ectopic polar cells and overproduction of border cells (data not shown). The phenotype of *54C2* in other tissues also resembled that of *eya* (Hazelett et al., 1998) (data not shown). *54C2* clones in the head were characterized by a loss of eye tissue and its replacement with head cuticle containing orbital bristles, whereas we observed no abnormality in either legs or wings. Both the eye and ovarian phenotypes appeared to result from a single mutation, because the two phenotypes co-segregated in recombination experiments. Therefore, we concluded that *54C2* was a new allele of the *eya* gene.

Consistent with these findings, select *eya* allelic combinations are female sterile (Bonini et al., 1998). In addition, in $eya^{3cs}/E(P)10$ mutant females, the polar cell markers FAS3 and A101 were expressed in virtually all of the follicle cells (Fig. 1L,O). These egg chambers usually contained abnormal germline cells (Fig. 1M,P). In extreme examples with this genotype ($eya^{3cs}/E(P)10$), ovarian development ceased in the germarium and the germarium appeared swollen compared with the wild type (Bonini et al., 1998) (Fig. 1K,N). This extreme phenotype was not observed in ovaries containing *eya* mosaic clones, presumably because it resulted from a reduction (but not elimination) of EYA expression in all cells, whereas the mosaic clone phenotype resulted from more focal loss of EYA expression.

EYA protein is normally absent from both polar cells and stalk cells

As loss of *eya* in follicle cells led to ectopic polar cells in the ovary, we postulated that expression of EYA might normally be repressed in the polar cells. Alternatively, EYA might be repressed via a post-translational modification in polar cells. To distinguish between these possibilities, we examined the expression pattern of the EYA protein in the ovary. Egg chambers were double stained with antibodies against EYA (Bonini et al., 1993) and anti-β-galactosidase antibodies in order to identify either polar cells, in the A101 enhancer trap line, or stalk cells in the enhancer trap line 93F (Ruohola et al., 1991) (Fig. 2).

The earliest expression of EYA was observed in follicle cells in region 2b of the germarium (Fig. 2A,B). EYA continued to be expressed in all follicle cells with the exception of polar and stalk cells until late stage 8 (Fig. 2C,D). After stage 8, EYA protein was restricted to the anterior follicle cells, including border cells, squamous cells and centripetal cells (Fig. 2E,F). EYA was not expressed detectably in the germ cells of any stage. Thus, the absence of EYA protein in the polar cells was consistent with a role as a repressor of polar cell fate.

In order to define when the EYA protein was first lost in the polar/stalk cell lineage in the germarium, we examined

germaria for cells that did not express EYA. In this experiment, Cadherin-GFP expression driven by the ubiquitin promoter was used to outline all cells in the germarium, and the cells were double stained with antibodies for EYA. We found that EYA protein was absent from a group of cells along the border between regions 2b and 3 of the germarium (Fig. 2A). This was

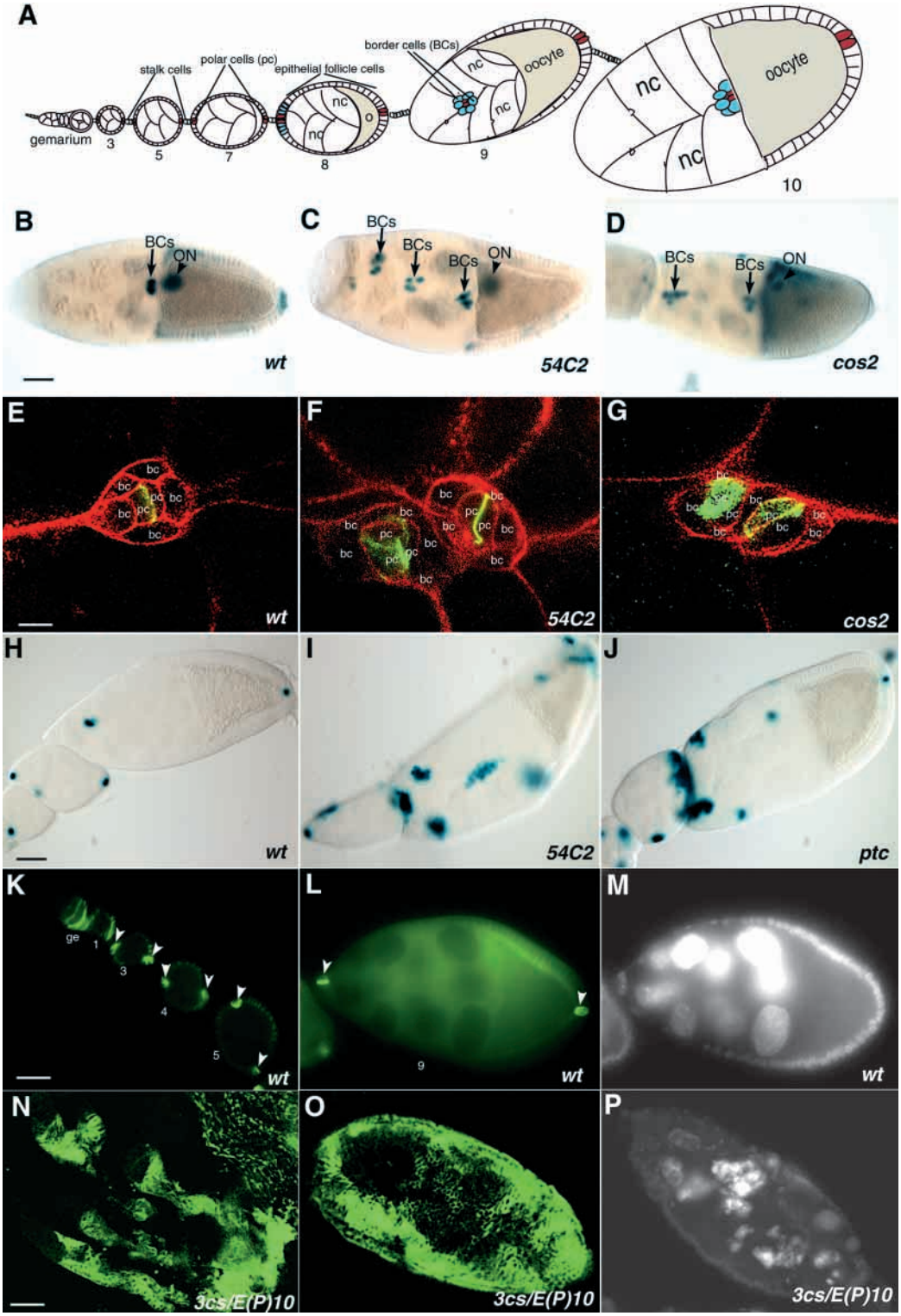

Fig. 1. Egg chamber development in wild-type and mutant ovaries. (A) A *Drosophila* ovariole showing the germarium and egg chambers from stages 3-10 (anterior towards the left). Each egg chamber contains 16 germ cells, including15 nurse cells (nc) and one oocyte (o). Polar cells are shown in red. Stalk cells separate adjacent egg chambers. Border cells are shown in blue. (B-D) Normarski optics images of egg chambers from enhancer trap line PZ6356, stained for β-galactosidase, which is expressed at highest levels in the border cells (arrows) and the oocyte nucleus (arrowheads). (B) A wild-type, stage 10 egg chamber with one cluster of border cells (BCs) at the nurse cell/oocyte border (ON, oocyte nucleus). (C) A stage 10 mosaic egg chamber containing *54C2* mutant cells and three clusters of border cells. (D) A stage 10 mosaic egg chamber containing *cos2* mutant cells and two clusters of border cells. (E-G) Fluorescence micrographs of a border cell cluster double stained with anti-FAS3 (green) and rhodamine phalloidin (red). In wild-type egg (E), one pair of anterior polar cells stains. The polar cells (pc) are surrounded by border cells (bc). In both *54C2* (F) and *cos 2 (*G) mosaic egg chambers, extra border cells surround extra polar cells. (H-J) Normarski optics images of egg chambers stained for β-galactosidase from enhancer trap line A101 (*neu-lacZ*), which is a marker for mature polar cells. (H) In wild-type, polar cells are located at the anterior and posterior poles. In egg chambers containing *54C2* (I) or *ptc* (J) mosaic chambers, polar cells are found at many locations. (K-P) Fluorescence micrographs of egg chambers stained with anti-FAS3 (K,L,N,O) or DAPI (M,P). (K) A series of wild-type egg chambers up to stage 5. (L) A wild-type, stage 9 egg chamber. In the germarium and stage 1 egg chamber, all follicle cells are FAS3 positive. Polar cells are indicated by white arrowheads. (M) A wild-type, stage 9 egg chamber stained with DAPI. (N-P) Egg chambers from $eya^{3cs}/eya^{E(P)10}$ females. Egg chambers are either arrested early in oogenesis (N) or are covered with FAS3-positive cells (O). In the egg chamber in P, which is the same one as in O, the germ cells are degenerating. Scale bars: in B, 50 μm for B-D; in E, 10 μm for E-G; in H, 50 μm for H-J; in K and N, 25 μm for K-M and N-P, respectively.

more obvious in the cells between region 2 of the germarium and the stage 1 egg chamber (Fig. 2B).

Loss of *eya* is sufficient to transform epithelial follicle cells into polar cells

To examine the autonomy of the effects of *eya* in repressing polar cell fate, we examined egg chambers carrying marked mosaic clones. In this experiment, wild-type follicle cells expressed GFP, whereas *eya* mutant cells lacked GFP (Fig. 3). EYA protein was only detected in wild-type follicle cells, and not in the mutant clones, indicating that eya^{54C2} was a protein null allele (Fig. 3A-C). In addition, both polar cell markers, Fasciclin III (Fig. 3D-I) and A101 (Fig. 3G-I), were ectopically expressed in all *eya* mutant follicle cells. Thus, loss of *eya* is sufficient to cause ectopic expression of polar cell markers in the follicle epithelium in a cell autonomous fashion.

As EYA is also normally excluded from stalk cells (Fig. 2D), we wondered whether loss of *eya* could lead to ectopic stalk cells. We stained *eya* mosaic egg chambers with the stalk cell marker 93F (Ruohola et al., 1991). We never observed ectopic 93F expression in *eya* mutant cells (data not shown). Nor did we observe any long stalks between egg chambers in *eya* mosaic ovaries. Therefore, loss of *eya* only leads to ectopic polar cells but not stalk cells.

EYA is downstream of *ptc, pka and cos 2* in regulating polar cell fate

Ectopic polar cells are produced either by overexpression of HH or by loss of negative regulators of the HH pathway, such as *ptc*, *Pka* and *cos2* (Forbes et al., 1996; Liu and Montell, 1999; Zhang and Kalderon, 2000). To test whether *eya* expression was under the control of HH signaling, we examined the expression of EYA in follicle cell clones lacking *ptc*, *Pka* or *cos2*. When *Pka* mosaic egg chambers were double stained for EYA and for the polar cell marker A101, EYA protein was missing from all of the ectopic polar cells found in mutant clones (Fig. 4D-F). The same was true of *ptc* and *cos2* mosaic egg chambers (data not shown). Thus, EYA expression is influenced by ectopic HH signaling, and *eya* is downstream of *ptc*, *cos2* and *Pka* in terms of polar cell fate specification. However, not all *Pka* mutant follicle cells expressed FAS3 and not all of the FAS3-positive cells lacked EYA (Fig. 4A-C). FAS3 is expressed not only by polar cells, but also by immature or undifferentiated follicle cells, whereas A101 labels only mature polar cells (Grammont and Irvine, 2001). Thus, the cells that expressed FAS3 and EYA and lacked A101 may have been cells in which differentiation had been delayed or prevented. Besides ectopic polar cells and undifferentiated, immature follicle cells, there were also EYA-positive, FasIII-negative cells, a combination normally found in differentiated epithelial follicle cells (Fig. 4C).

Mutual repression of EYA and CI^{AC}

The effects of HH signaling require the function of CI (Methot and Basler, 2001). In the absence of HH, CI is processed to produce a transcriptional repressor, a short form referred to as CI^{R}. In the presence of HH, the processing is prevented and the full-length form of CI, CI^{AC}, activates transcription. Polar cell fate might either be repressed by CI^{R} or promoted by CI^{AC}, or both. To test the possibility that the CI^{R} represses polar cell fate, egg chambers containing clones homozygous for a null mutation in *ci* were analyzed. If polar cell fate were repressed by CI^{R}, we would expect loss of both forms of CI to lead to ectopic polar cells. However, ectopic polar cells were not observed in clones lacking CI, and EYA expression was normal (Fig. 5A). These results suggest that loss of CI^{R} is not sufficient to cause ectopic polar cells and CI activity is not normally required for polar cell fate specification.

To test the possibility that CI^{AC} promotes polar cell fate, we induced the expression of a constitutively active derivative of CI (Price and Kalderon, 1999) (see Materials and Methods). Three days after induction of constitutively active CI by heat shock, recovered egg chambers were double stained with anti-β-galactosidase and antibodies against EYA. In such egg chambers, β-galactosidase marked cells expressing constitutively active CI. Similar to the situation for PKA

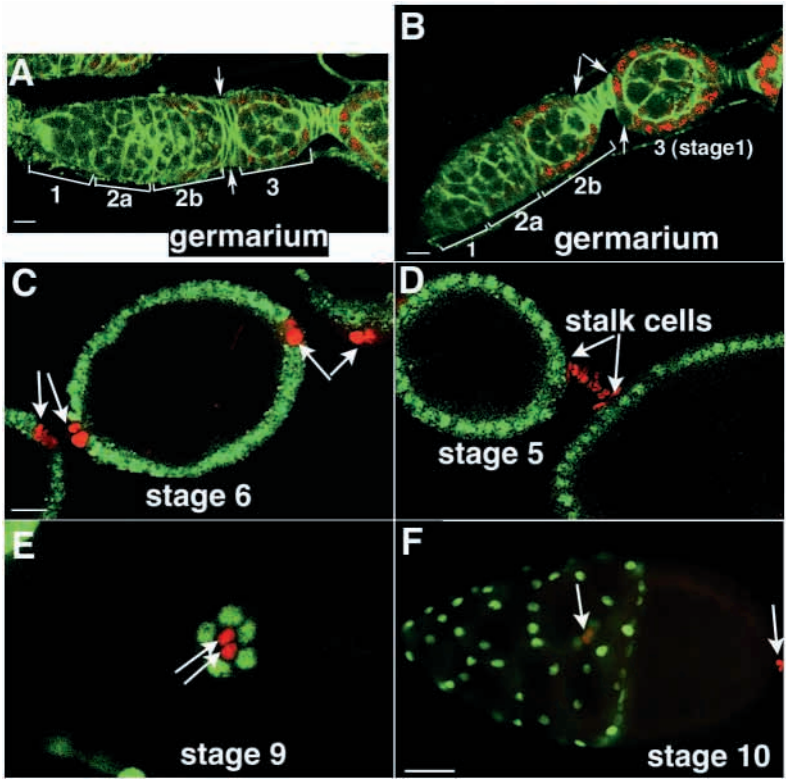

Fig. 2. EYA protein expression during *Drosophila* oogenesis. Fluorescence confocal micrographs of wild-type egg chambers stained for GFP (ub-Cadherin-GFP) (green) and EYA (red) (A,B), or for EYA (green) and β-galactosidase (red) (C-F). In C,E,F, expression of β-galactosidase (red) from enhancer trap line A101 labels polar cells. In D, β-galactosidase expression from enhancer trap line 93F is shown, which labels stalk cells. EYA protein is expressed in the germarium (A) and in all follicle cells except polar cells (A-C) and stalk cells (D) up to stage 8. (E) A stage 9 egg chamber in which EYA is excluded from the polar cells (arrows), but is expressed in migrating border cells. (F) EYA expression in a stage 10 egg chamber. Scale bars: in A, 10 μm for A; in B, 10 μm for B; in C, 10 μm for C-E; in F, 50 μm for F. Arrows in A,B indicate regions lacking EYA expression; arrows in C,E,F indicate polar cells.

mutant clones, there were three types of cells in the CI^{AC} flip-out clones. The phenotypes were very similar to *pka* mutant clones. Those β-galactosidase-positive cells that lacked EYA expression (Fig. 5D) ectopically expressed the polar cell marker FAS3 (not shown). This result suggests that EYA expression can be repressed, and polar cell fate promoted, by CI^{AC}.

Expression of *ptc* is regulated directly by CI^{AC} in response to HH signaling in all tissues examined, thus *ptc-lacZ* is a reporter for strong activation of the HH pathway (Aza-Blanc and Kornberg, 1999). We found one *ptc-lacZ* line that shows expression in one cell of the polar cell pair (Fig. 5B). If *eya* were strictly downstream of CI in polar cell fate determination, we would not expect *ptc-lacZ* expression to be affected in *eya* mosaic clones. However, 10% of the ectopic polar cells produced in *eya* mutant clones (n>50) expressed *ptc-lacZ* (Fig. 5C). To examine this apparent activation of CI further in *eya* mutant mosaic clones, we stained mutant egg chambers with 2A1, an antibody that recognizes only the full-length, activated form CI^{AC}. In wild-type egg chambers, CI^{AC} is expressed at equal levels in all follicle cells (Forbes et al., 1996). By contrast, CI^{AC} protein was upregulated in *eya* mutant follicle cells, compared with the surrounding heterozygous cells (Fig. 5G-I). The upregulation level of CI^{AC} in *eya* mutant cells was not quite as great as that in *pka* mutant cells (Fig. 5E,F,H,I), but nevertheless different from wild type. Thus, EYA and CI^{AC} exhibited mutual repression.

Upregulation of full-length CI in *eya* mutant clones could result either from an increase in transcription of the *ci* gene, or from inhibition of processing of CI protein from the full-length CI^{AC} to the shorter CI^{R}. To examine the regulation of *ci* transcription in the ovary, we used a *ci-lacZ* enhancer trap line, which is expressed in the same pattern as the endogenous gene (Eaton and Kornberg, 1990). We found no significant increase in *ci-lacZ* expression in *eya* mutant follicle cells (data not shown). We also stained *eya* mosaic egg chambers with an antibody CIN, which recognizes all forms of CI, to monitor total CI protein (Aza-Blanc and Kornberg, 1999). There was no detectable increase in CIN staining in *eya* mutant cells (Fig. 5G). Thus, total CI levels remained constant in *eya* mutant clones, whereas CI^{AC} levels rose, suggesting that processing of full-length CI to CI^{R} was inhibited in the *eya* mutant cells.

Ectopic overexpression of *eya* is sufficient to suppress stalk/polar cell fates

As *eya* loss of function caused ectopic polar cell differentiation, and EYA is normally absent from both polar cells and stalk cells, we wanted to know if over- or mis-expression of *eya* would suppress these cell fates. We used flies containing *hs-Gal4* and *UAS-eya* transgenes to examine the effects of ectopic *eya* expression in the ovary. Ectopic expression of *eya* resulted in compound egg chambers with more than 16 germ cells, which were not separated by interfollicular stalks and were not always completely enveloped by the somatic epithelial layer (Fig. 6A,C,E). The stalk cell marker 93F was missing from these compound egg chambers, although some pairs of FAS3-positive cells were clearly observed (Fig. 6B,F,G), indicating that the formation of compound egg chambers might result from loss of stalk cells. The frequency and severity of the compound egg chamber phenotype varied with heat-shock conditions (see Materials and Methods). To rule out the possibility that formation of compound egg chambers could result from extra germline cell divisions, we examined mutant ovaries with rhodamine-conjugated phalloidin to visualize actin. We found that no germ cells were connected to their neighbors by more than four ring canals (data not shown). In addition, within compound egg chambers, individual sets of germ cells were recognizable as groups of 15 similarly sized nurse cells and a single oocyte nucleus. Therefore, overexpression of *eya* suppressed stalk cell fate.

Because loss of EYA promoted polar cell differentiation, over- or mis-expression would be expected to interfere with polar cell fate. Indeed, when a polar cell specific GAL4 line, *upd*-GAL4, was used to force EYA expression exclusively in the polar cells, one-third of the egg chambers (n=99) were found to be missing polar cells from one end (Fig. 6D).

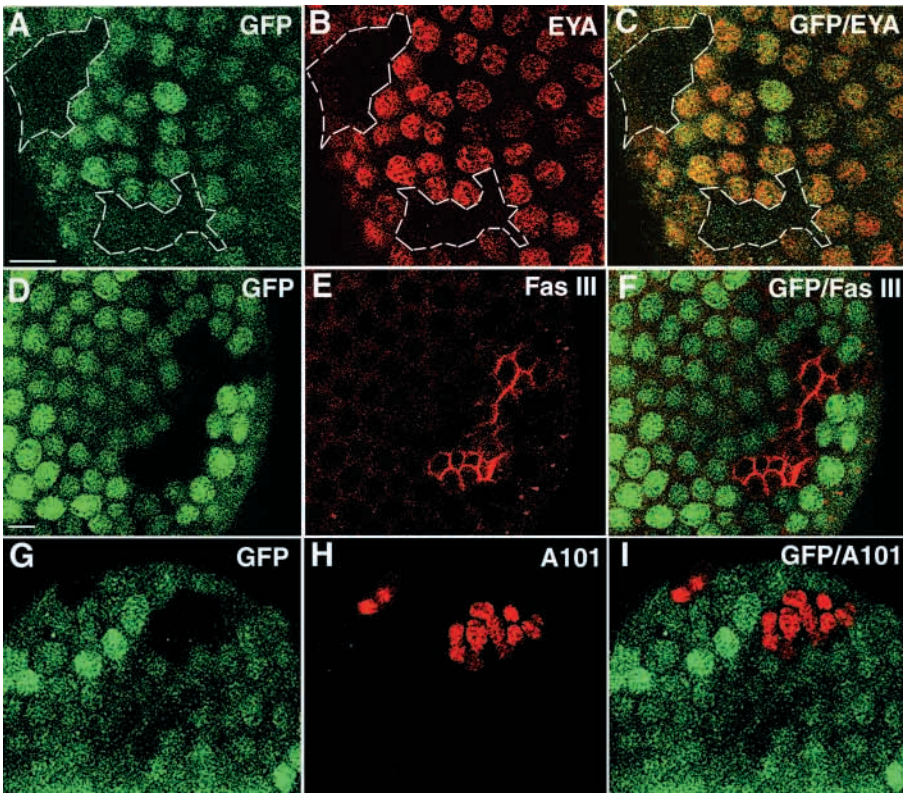

Fig. 3. Loss of *eya* leads to ectopic polar cells in a cell autonomous fashion. Fluorescence confocal micrographs of epithelial follicle cells stained for GFP (green) (A-C) and for EYA (red in B), FAS3 (red in E) or β-galactosidase from A101 (red in H,I). Co-localization appears yellow. GFP-positive cells are wild type, whereas the cells lacking GFP are *eya* mutant cells and are outlined in A-C. Scale bars: in A, 10 μm for A-C; in D, 10 μm for D-I.

Anterior polar cells appeared to be more affected than those at the posterior. We did not observe egg chambers lacking polar cells from both ends, and compound egg chambers were rare in this genotype. In the few egg chambers from hs-GAL4;UAS-*eya* females that were well separated by stalk cells, polar cells were also often missing from one end as shown by absence of FAS3 and A101 expression (not shown). In both hs-GAL4;UAS-*eya* and *upd*-GAL4;UAS-*eya*, a border cell migration defect was observed in >50% of stage 10 egg chambers (Fig. 6H), suggesting that the normal function of polar cells in promoting border cell migration had been compromised.

The effect of activated Notch on EYA expression

One signal that is known to be essential for polar cell formation is Notch (N). To investigate the relationship between Notch and EYA, we examined EYA expression in N mutant clones. Loss of Notch leads to the disappearance of polar cells (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). Consistent with this, we found that all N mutant cells expressed

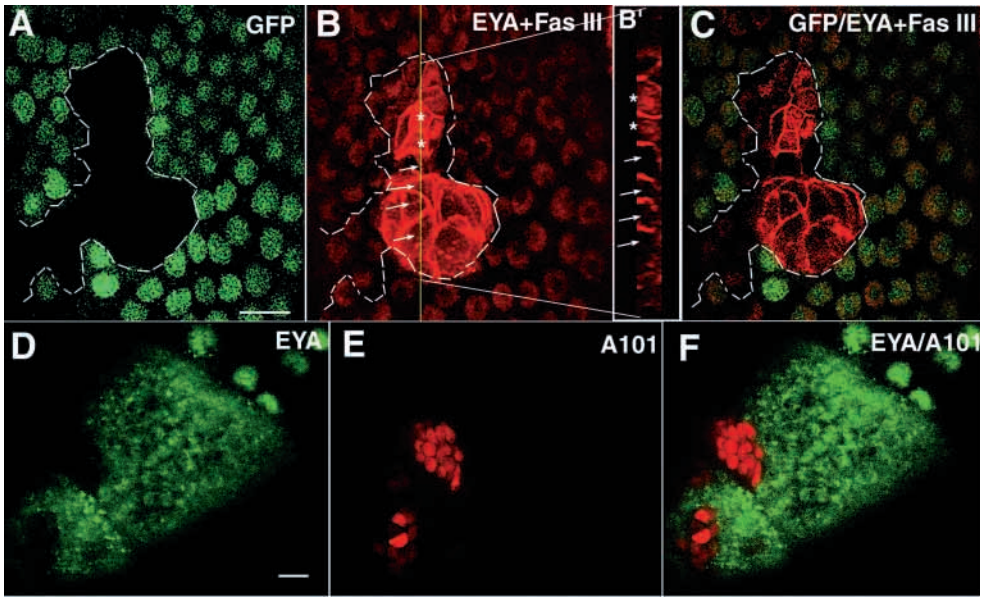

Fig. 4. The relationship between EYA and HH pathway genes. (A-C) Fluorescence confocal micrographs of a *Pka* mosaic egg chamber. GFP expression is shown in green. Both EYA (nuclear) and FAS3 (membrane) are shown in red. The *Pka* mutant cells lack GFP and are outlined by broken lines in A-C. (B) A composite image of a *z*-series of adjacent confocal sections showing the expression of EYA and FAS3 to demonstrate that some FAS3-positive cells have EYA expression, whereas others do not. The YZ plane corresponding to a slice through the Z series of the clone shown in B is shown in (B′). Of the FAS3-positive cells which have red membrane staining in (B), two cells (*) are EYA positive and four cells (arrows) are EYA negative and lack nuclear staining. (C) Merged picture of GFP, *eya* and FAS3 in a single section from A. (D-F) Fluorescence micrographs of a *Pka* mosaic egg chamber stained for EYA (green) and β-galactosidase (red) from the enhancer trap line A101. Scale bars: in A, 5 μm for B′ and 10 μm for A-C; in D, 10 μm for D-F.

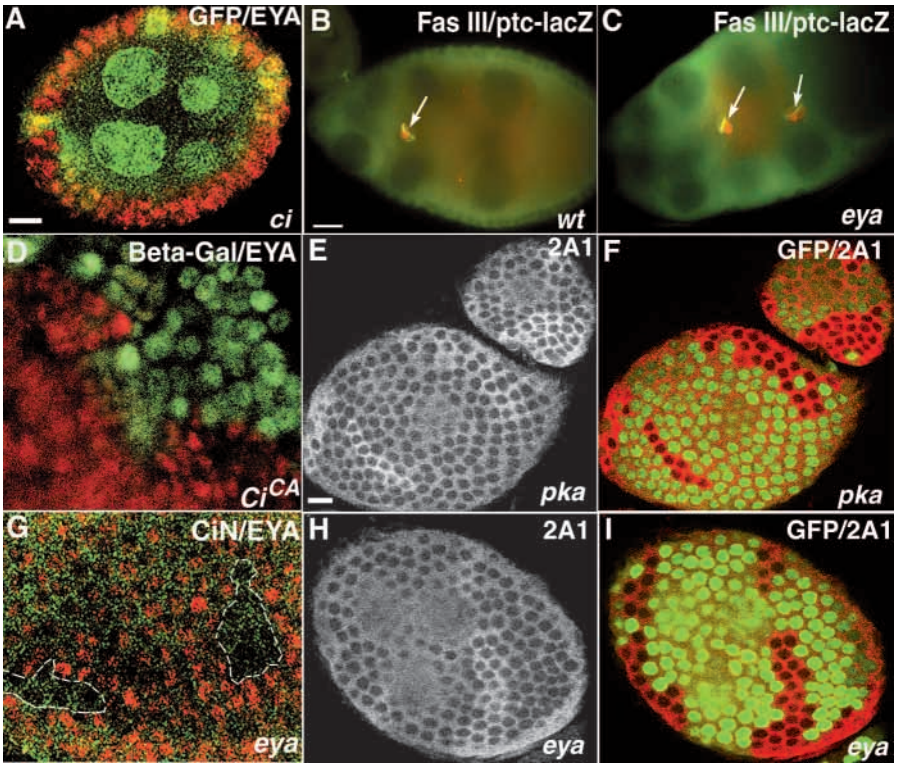

Fig. 5. The relationship between *ci* and *eya*. (A) Fluorescence micrographs of a *ci* mosaic egg chamber showing GFP expression in green and EYA expression in red. Cells that lack GFP are *ci* mutant cells. Co-localization of GFP and EYA appears yellow. (B,C) Fluorescence images of egg chambers double stained for FAS3 (green) and *ptc-lacZ* (red). (B) A wild-type egg chamber in which anterior polar cells (arrow) stain. (C) An *eya* mosaic egg chamber with two pairs of anterior polar cells (arrows), both of which have *ptc-lacZ* expression. (D) Fluorescence images of an egg chamber expressing the constitutively active form of CI (CI^{AC}) (see Materials and Methods). Cells with β-galactosidase expression (green) express CI^{AC}. Expression of EYA is shown in red. (E,F) Fluorescence images of a *Pka* mosaic egg chamber double stained for CI (gray in E, red in F) and GFP in green. (G) Fluorescence micrographs of an *eya* mosaic egg chamber double stained for EYA (red) and CIN (green), which recognizes both CI^{AC} and CI^{R} (*eya* mutant clones are outlined) (H,I) Fluorescence images of an *eya* mosaic egg chamber double stained for CI (gray in H, red in I) and GFP in green. Scale bars: in A, 10 μm for A,D,G; in B, 25 μm for B,C; in E, 10 μm for E,F,H,I.

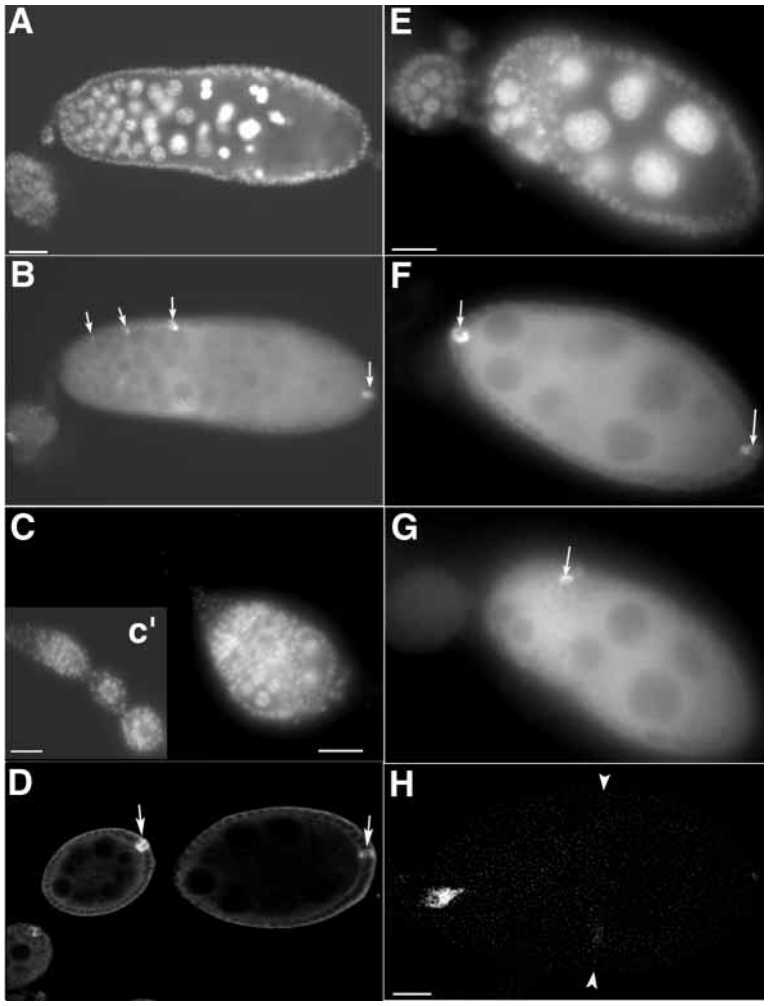

Fig. 6. The phenotype of overexpression of *eya* in the ovary. Egg chambers stained with DAPI (A,C,C′,E) to label nuclei or with anti-FAS3 (B,D,F,G) to label the polar cells. (A) A compound egg chamber produced by overexpression of *eya* in the ovary. (B) The same egg chamber as in A, which has four pairs of polar cells (arrows). (C) Compound egg chambers that never leave the germarium. (C′) A wild-type germarium and the first two egg chambers for comparison. (D) A stage 8 egg chamber in which posterior polar cells express FAS3. Anterior polar cells appear to be missing. (E) A fused egg chamber. (F,G) Two different focal planes of the same egg chamber as in E. There are three pairs of polar cells (arrows) in this compound egg chamber. (H) A stage 10 egg chamber in which border cells express GFP and overexpress EYA. Migration is delayed. Arrowheads indicate the centripetal follicle cells and the normal extent of border cell migration at this stage. Scale bars: in A, 50 μm for A,B; in C, 25 μm for C,D; in C′, 25 μm for C′; in E, 50 μm for E-G; in H, 50 μm for H.

EYA (not shown). To test whether activated N is sufficient to repress EYA expression, we made flip-out clones of UAS-N^{intra} in somatic follicle cells, and triple stained the egg chambers for GFP, A101 and EYA. All A101-positive cells were EYA negative, and all EYA-negative cells expressed A101 (Fig. 7). However, not all GFP-positive cells were A101 positive. Consistent with other reports, we found that ectopic, A101-positive, polar cells only formed near the two poles of egg chambers. Surprisingly, activated Notch was able to cause ectopic polar cells to form non-cell-autonomously (Fig. 7).

Function of eye development genes in *Drosophila* oogenesis

The *eya* gene is essential for *Drosophila* eye development where it functions as part of a cascade of regulatory genes including *eyeless* (*ey*) (Quiring et al., 1994), *twin of eyeless* (*toy*) (Czerny et al., 1999), *sine oculis* (*so*) (Cheyette et al., 1994) and *dachshund* (*dac*) (Mardon et al., 1994). To determine whether this group of genes functions together to specify follicle cell fates, we examined the expression of each gene in the ovary. Expression of EY is found in the nuclei of all follicle cells as assessed by antibody staining (data not shown). We examined the expression of SO in the ovary using a reporter, so^5-*lacZ*. The so^5 reporter was expressed in stalk cells, but not in polar cells (Fig. 8A). Consistent with this expression pattern, stalk cells were missing in *so* mutant egg chambers, whereas the correct number of polar cells was present (Fig. 8B-D). Neither *toy* nor *dac* was expressed in the ovary (data not shown). These results indicate that although some of the eye specification genes function in the ovary, the regulatory network is not the same in this tissue. Rather, the individual retinal determination genes function independently of the network in the ovary.

DISCUSSION

Eyes absent is a repressor of polar cell fate

Our results reveal a novel role for the retinal determination gene, *eya*, in polar cell specification during *Drosophila* oogenesis. The data demonstrate that *eya* is required to suppress polar cell fate in the epithelial follicle cells. The evidence for this is that EYA protein is absent from polar cells in wild-type egg chambers as soon as the polar cells express markers such as A101. Furthermore, loss of EYA can transform

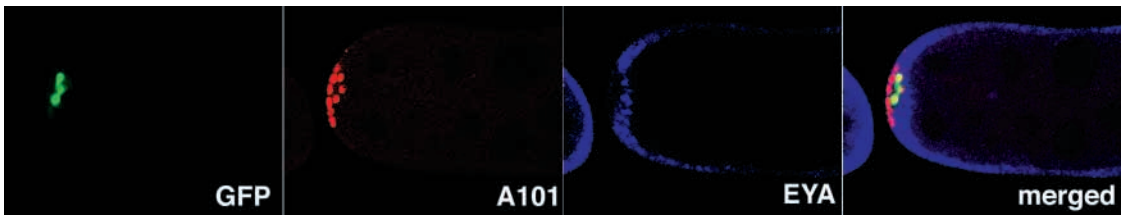

Fig. 7. The effect of activated N on EYA expression and polar cell fate. GFP (green) is expressed in cells in which activated N is expressed. A101 staining (red) labels polar cells, among which three are GFP-positive cells. EYA expression is shown in blue. Overlap of green and red appears yellow. Overlap of blue and yellow would appear white.

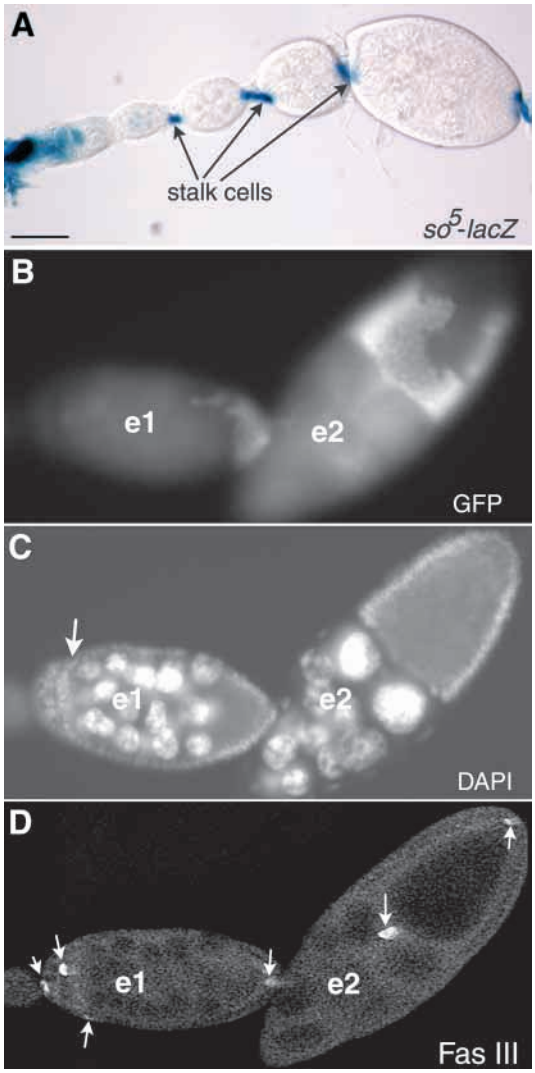

Fig. 8. The *so* mutant phenotype in the ovary. (A) Nomarski optics images of a wild-type ovariole stained for β-galactosidase activity to reveal the expression pattern of *so*^{5}*-lacZ* in stalk cells (arrows) after stage 3. (B-D) Fluorescence images of two *so* mutant egg chambers (e1 and e2). (B) In the e1 egg chamber most follicle cells are mutant for *so* (lack GFP). In the e2 egg chamber, about half of the follicle cells are mutant for *so* and lack GFP (e2). (C) DAPI staining of the same two egg chambers. (D) Polar cells (arrows), revealed by anti FAS3 staining, develop normally. Scale bar: 50 μm.

other epithelial follicle cells into polar cells in a cell autonomous fashion. Finally, ectopic expression of *eya* is capable of suppressing normal polar cell fate and compromising the normal functions of polar cells, such as promotion of border cell migration.

EYA protein is first lost in the cells along the border between regions 2b and 3 of the germarium. Those cells are likely to be the cells that separate cysts and may be the early progeny of the polar/stalk cell precursors. Therefore, loss of EYA expression appears to be the earliest available marker for this lineage.

Although EYA is normally missing from both polar cells and stalk cells, the expression of the mature stalk cell marker 93F was never observed in *eya* mosaic clones. Hence, loss of *eya* transformed the epithelial follicle cells only into polar cells, not into stalk cells. However, mis-expression of *eya* early in oogenesis leads to the absence of stalk cells and generates compound egg chambers, sometimes containing normal pairs of polar cells. It seems that the formation of compound egg chambers depends more directly on the loss of stalk cells than on the loss of polar cells. This was also the case when EYA expression was forced exclusively in the polar cells. Although the function of polar cells was compromised, the stalk cells still formed compound egg chambers were not observed. Further evidence for the crucial role of stalk cells in separating egg chambers is the compound egg chamber phenotype resulting from loss of *so*, a gene that is only expressed in the stalk cells. Therefore, repression of EYA appears to be required for stalk cell formation, which is in turn essential to separate egg (Dobens and Raftery, 2000).

Why does loss of *eya* lead only to ectopic polar cells, not to stalk cells in the epithelial follicle layer? One possible reason is that the stalk cells, as opposed to polar cells and epithelial follicle cells, normally form in the absence of direct contact with germline cell. Thus, signals from the germline might prevent stalk cell fate in cells that directly contact the germline.

One germline signal that is known to play a role in polar cell specification is Delta, which signals from the germline to Notch in the soma to control the differentiation of polar cells (Lopez-Schier and St Johnston, 2001). Epithelial follicle cells do not respond to Delta in the same way, presumably because, unlike polar cells, they do not express *fringe* (Grammont and Irvine, 2001; Zhao et al., 2000). *fringe* encodes a glucosyltransferase that potentiates the ability of the Notch receptor to be activated by its ligand, Delta. Mutation of either *Notch* or *fringe* leads to the disappearance of polar cells (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). As a result, EYA-negative cells are not found in the follicles. Mis-expression of either FNG or activated Notch produces ectopic polar cells only at the poles of the egg chamber, whereas loss of EYA can cause polar cells to form throughout the follicle epithelium. Thus Notch signaling appears to be necessary, but not sufficient to repress EYA expression and lead to polar cell formation. Surprisingly, activated Notch also can produce ectopic polar cells cell-nonautonomously at the poles of the egg chamber. The reason for this could be that activated Notch signaling might activate the expression of Delta, which, in turn, can activate Notch signaling in the adjacent cells.

Another signaling pathway that impinges on polar and stalk cell fates is the JAK/STAT pathway (McGregor et al., 2002). The ligand for this pathway is unpaired (UPD), which is expressed specifically in polar cells. The ligand interacts with a receptor, which in turn activates the tyrosine kinase known as Hopscotch (HOP). HOP activity results in phosphorylation and nuclear translocation of the transcription factor STAT92E. In the ovary, UPD secreted from polar cells functions to suppress polar cell fate in stalk cells. It has been proposed that N signaling specifies a pool of cells competent to become polar and stalk cells and the UPD/JAK/STAT pathway distinguishes polar versus stalk fates (McGregor et al., 2002). Thus, whatever signal normally represses EYA in the polar/stalk lineage presumably acts prior to UPD/JAK/STAT as EYA repression occurs in both polar and stalk cells, possibly in the

common precursor cell. The observation that forcing EYA expression in polar cells under the control of *upd-GAL4* can repress polar cell fate suggests that this fate remains malleable for some time after its normal specification. The relatively low penetrance of this phenotype (~30%) might be due to the late expression of *upd-GAL4* relative to the normal timing of EYA repression.

Ectopic HH signaling and polar cell formation

Loss of EYA results in the production of ectopic polar cells virtually anywhere in the egg chamber. At first glance, this phenotype looks very similar to that of ectopic activation of the HH pathway, either by overexpression of HH or by loss of the negative regulators PTC, PKA or COS2. Indeed the ectopic polar cells that form in *ptc*, *Pka* or *cos2* mutant clones lack EYA. However, ectopic HH signaling has additional effects besides ectopic polar cell formation, whereas loss of EYA does not. We observed several different cell types in the *ptc*, *Pka* or *cos2* mutant clones. There were EYA-positive but FAS3-negative cells, which may correspond to differentiated epithelial follicle cells. There were also cells expressing both EYA and FAS3, which could be immature, undifferentiated precursor cells. Finally, there were the EYA-negative but FAS3-postive polar cells. In this study, we show that the production of ectopic polar cells caused by ectopic activation of the HH pathway occurs by repression of EYA. But what is the normal relationship between HH signaling and polar cell formation, and why does excessive HH signaling generate ectopic polar cells as well as other cell types?

To address these questions, we have to consider the normal role of HH signaling in the ovary. Expression of HH protein has only been observed in the terminal filament and cap cells at the extreme anterior tip of the germarium (Forbes et al., 1996). The normal function of HH appears to be to regulate somatic stem cell fate and proliferation (Zhang et al., 2001). Loss of HH signaling in somatic stem cells results in the loss of stem cell fate. Conversely, overexpression of HH leads to overproduction of stem-cells. Despite the fact that ectopic expression of HH leads to ectopic polar cells, HH signaling does not appear to specify polar cell fate normally. The best direct evidence for that is that *smo* mutant cells, which cannot transduce HH signals, are still capable of generating normal polar cells at normal positions (Zhang and Kalderon, 2000). In addition, normal polar cells can develop in the absence of *ci* (this study).

Why, then, does ectopic HH signaling produce ectopic polar cells? It has been previously argued that excessive HH signaling might maintain follicle cells, and the polar/stalk cell lineage in particular, in a precursor state for an abnormally long period of time (Tworoger et al., 1999; Zhang and Kalderon, 2000). Thus, delayed specification of polar cells would permit more proliferation than usual in this lineage. This model might explain the presence of extra polar cells at the two poles of the egg chamber, where the polar cells normally reside. However, it does not explain the presence of ectopic polar cells elsewhere in the egg chamber, or why there are three different cell types present in the *ptc*, *Pka* and *cos2* mutant clones. Based on the normal role of HH in regulating proliferation and maintenance of stem cells and their immediate progeny, the prefollicle cells, we propose that ectopic HH signaling might cause ectopic prefollicle cell fates within the epithelial follicle layer of early egg chambers. As these cells undergo further proliferation, and then differentiation, they produce the various follicle cell types observed in the *ptc*, *Pka* and *cos2* clones. Additional, as yet unknown, signals might determine which specific fates the differentiating cells adopt. However, the normal mechanisms that function to coordinate follicle cell fates spatially are obviously lacking in the mutant clones, as the three types of cells appear in random locations relative to each other. This provides an explanation for how ectopic HH signaling might produce polar cells all over the egg chamber, rather than only at the two poles of the egg chamber, where the polar/stalk precursors normally reside.

The relationship between CI and EYA

Ectopic HH signaling produces numerous effects in the *Drosophila* ovary, which include regulating proliferation of somatic cells as well as specification of polar cells (Forbes et al., 1996; Zhang and Kalderon, 2000). Both of these effects appear to be achieved through the cell autonomous action of CI. This raises the question of how different effects are elicited by the same signal. The data presented here indicate that ectopic HH activates polar cell fate by repressing *eya* expression, the function of which is required to repress polar cell fate. As loss of *eya* does not mimic ectopic hedgehog in causing extra proliferation, it is not yet clear what factors act downstream of ectopic HH to affect proliferation.

The relationship between EYA and CI is not a simple linear one. Although EYA expression is repressed by CI^{AC}, mutations in *eya* also alter the balance between CI^{AC} and CI^{R}, without affecting overall *ci* expression. CI^{AC} is upregulated in *eya* mutant follicle cells. In addition, some of the ectopic polar cells in *eya* mosaic egg chambers express *ptc-lacZ*, which is an indicator for activation of CI (Aza-Blanc and Kornberg, 1999). Thus, there appears to be mutual repression between CI^{AC} and EYA. One place in the mammalian embryo where a similar relationship between CI and EYA might exist is in patterning the eye field. HH is normally expressed at the midline where it represses eye development. In the absence of HH, a single cyclopic eye forms at the midline (Macdonald et al., 1995). The three mammalian homologs of EYA are all expressed in the eye primordium (Xu et al., 1997) and therefore it may be that the antagonism between HH and EYA revealed in this study is also employed in the mammalian embryo to repress midline eye development.

Concluding remarks

It is clear that the effect of the ectopic HH signaling on the specification of the polar cell fate is through the repression of EYA. What still remains unknown is the spatially localized signal that normally represses EYA expression in polar and stalk cells. As Notch signaling is necessary, but not sufficient, to define polar cells, it is likely that there is an additional, spatially localized signal required for specifying polar cell fate. Clearly, EYA is a key regulator that represses polar and stalk cell fates. Whatever the regulatory signal that normally specifies polar cell fate, it must regulate EYA expression to determine a polar versus non-polar cell fate in the follicular epithelium.

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