A role for *extra macrochaetae* downstream of Notch in follicle cell differentiation

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Summary

The *Drosophila* ovary provides a model system for studying the mechanisms that regulate the differentiation of somatic stem cells into specific cell types. Ovarian somatic stem cells produce follicle cells, which undergo a binary choice during early differentiation. They can become either epithelial cells that surround the germline to form an egg chamber ('main body cells') or a specialized cell lineage found at the poles of egg chambers. This lineage goes on to make two cell types: polar cells and stalk cells. To better understand how this choice is made, we carried out a screen for genes that affect follicle cell fate specification or differentiation. We identified *extra macrochaetae* (*emc*), which encodes a helix-loop-helix protein, as a downstream effector of *Notch* signaling in the ovary. EMC is expressed in proliferating cells in the germarium, as well as in the main body follicle cells. EMC expression in the main body cells is Notch dependent, and *emc* mutant cells located on the main body failed to differentiate. EMC expression is reduced in the precursors of the polar and stalk cells, and overexpression of EMC caused dramatic egg chamber fusions, indicating that EMC is a negative regulator of polar and/or stalk cells. EMC and Notch were both required in the main body cells for expression of Eyes Absent (EYA), a negative regulator of polar and stalk cell fate. We propose that EMC functions downstream of Notch and upstream of EYA to regulate main body cell fate specification and differentiation.

Key words: *extra macrochaetae*, Oogenesis, Differentiation, Follicle cells, *Drosophila*

Introduction

Stem cells in adult tissues exhibit the potential to differentiate into a limited variety of cell types. There is considerable interest in understanding the mechanisms of their maintenance and differentiation. A specific example of adult somatic stem cell differentiation is found in the *Drosophila* ovary, which possesses both germline and somatic stem cells. The unit of *Drosophila* oogenesis is the ovariole (Spradling, 1993) (Fig. 1), made up of a number of egg chambers, each of which consists of 16 syncytial germline cells (15 nurse cells and the oocyte) surrounded by a monolayer of follicle cells. The egg chambers in each ovariole grow and are pushed posteriorly by the formation of new egg chambers, so that each ovariole contains multiple egg chambers arrayed from youngest to oldest until the very oldest egg chamber becomes an egg (Fig. 1). Two types of stem cells, germline and somatic, reside in a structure called the germarium at the anterior tip of the ovariole. The germline stem cells produce germline cystoblasts, each of which undergoes four division cycles with incomplete cytokinesis to form a 16-cell cyst. As each cyst ceases division, it encounters somatic stem cells and their progeny and is enveloped, or 'packaged', by a layer of somatic follicle cells. Newly packaged cysts exit the posterior end of the germarium in a process called budding. Intercyst cells, which separate the newly budded egg chamber from the germarium, undergo intercalation movements to form a three- to seven-cell structure called the stalk. At each terminus of each stalk lies a pair of polar cells, which organizes the axial polarity of the egg chamber (Frydman and Spradling, 2001; Grammont and Irvine, 2002; Torres et al., 2003) and induces differentiation of its neighbors during later stages of oogenesis (Grammont and Irvine, 2002; McGregor et al., 2002; Silver and Montell, 2001; Xi et al., 2003).

Although much is known about the maintenance and differentiation of the ovarian germline stem cells (GSC) (Bhat and Schedl, 1997; de Cuevas and Spradling, 1998; Ohlstein and McKearin, 1997; Xie and Spradling, 1998), the somatic stem cells (SSC) are less well understood. Two to three SSC are located in a characteristic position within the germarium. Like germline stem cells, the SSC are thought to reside in a microenvironment, known as a niche, which provides signals that allow their maintenance and prevent their differentiation (Spradling et al., 2001; Xie and Spradling, 2000). These signals probably come from the cap and terminal filament cells, which are found at the anterior tip of the ovary, several cells distant from the SSC. These cells are thought to maintain the SSC by secreting Wingless (Wg) (Song and Xie, 2003). Hedgehog (Hh) is also involved in maintaining SSCs (Zhang and Kalderon, 2001).

Although the progeny of the somatic stem cells ultimately receive a complex series of cues that cause them to differentiate into multiple spatially organized cell types, many of these differentiation events occur relatively late in egg chamber development. The early differentiation of follicle cells within

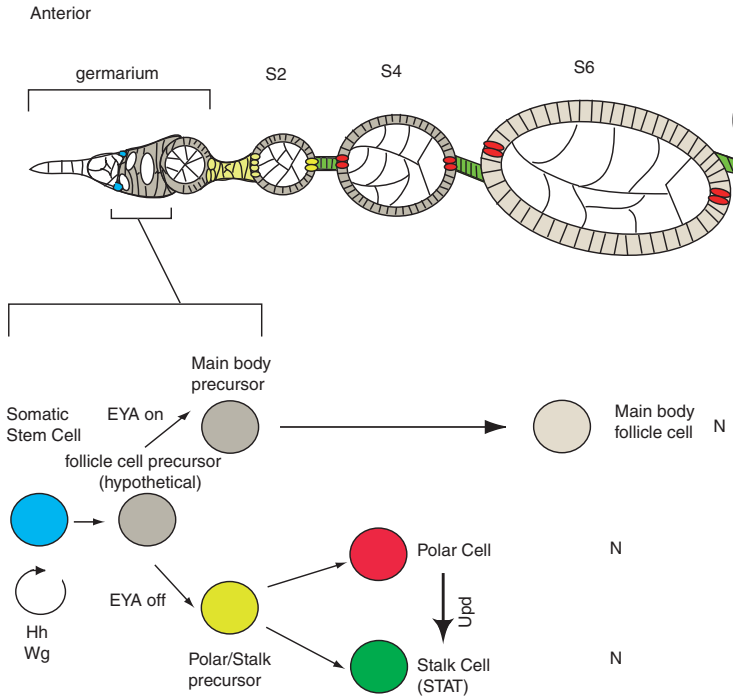

Fig. 1. An ovariole, and early acquisition of cell fates in the follicle cells. The germarium is at the anterior (left, upper bracket), and progressively older egg chamber stages (Spradling, 1993) develop to the posterior (right). Somatic stem cells (blue) produce follicle cell precursors (brown), which form main body precursors that express EYA (brown) and polar/stalk cell precursors (yellow) that ultimately eliminate EYA expression. Main body cells differentiate at stage 6 (light brown). Unpaired is emitted from the polar cells (red) and received by STAT in the stalk cells (green). Polar, stalk and main body cells are all thought to require the activity of Notch (N) for differentiation. NC, nurse cells; FC, follicle cells; O, oocyte.

the germarium is rather simple and involves the choice between two cell lineages: epithelial follicle cells (here termed 'main body cells') that contact the developing cyst; and cells that comprise the precursors of the polar and stalk cells (here termed 'polar/stalk precursors') (Larkin et al., 1996; Tworoger et al., 1999). Mutants that eliminate the formation of the polar and stalk cells, which results in the formation of fused egg chambers, have been identified (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001); one mutant, *eyes absent* (*eya*), autonomously changes the fate of the main body cells into polar cells (Bai and Montell, 2002). However, it is not clear which signals limit EYA expression to main body cells.

The Notch (N) pathway is involved at multiple steps in the differentiation of follicle cells (Gonzalez-Reyes and St Johnston, 1998), including at mid-oogenesis, when main body follicle cells normally cease proliferation, differentiate and undergo several cycles of genomic endoreplication. *Notch* mutant follicle cells fail to differentiate and, instead of endoreplicating, they overproliferate (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). In addition, Notch appears to be required for the differentiation of the polar cells themselves (Grammont and Irvine, 2001). One important ligand for Notch signaling in the ovary is Delta, which is expressed in the germline at very low levels in the germarium (Deng et al., 2001; Lopez-Schier and St Johnston, 2001) and at high levels at stage 6, when the main body cells differentiate (Bender et al., 1993). Delta signals through the Notch receptor, resulting in a presenilin-dependent cleavage with release of the intracellular domain (N-intra). N-intra binds to Suppressor of Hairless [Su(H)], the *Drosophila* equivalent of CBF1 (Lopez-Schier and St Johnston, 2001), and, together with Mastermind, activates transcription of downstream targets. In neural development, major downstream transcriptional targets of Notch are members of the Enhancer of Split family, which suppress transcription of Achaete and Scute (Van Doren et al., 1991). However, the Enhancer of Split complex does not appear to be required for follicle cell differentiation (Deng et al., 2001; Lopez-Schier and St Johnston, 2001), and it is unclear what the functional downstream targets of Notch are

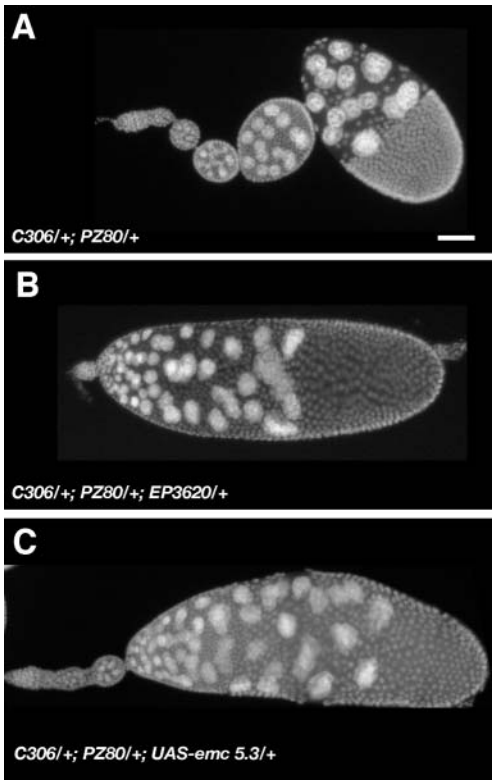

Fig. 2. EMC overexpression phenotype. (A-C) Nuclei of ovarioles, stained with DAPI. The germarium is to the left. (A) A wild-type ovariole. (B,C) Individual ovarioles from females driving gene expression under the control of *C306-Gal4*: (B) *EP3620*; (C) *UAS-emc5.3*. Scale bar: 50 μm.

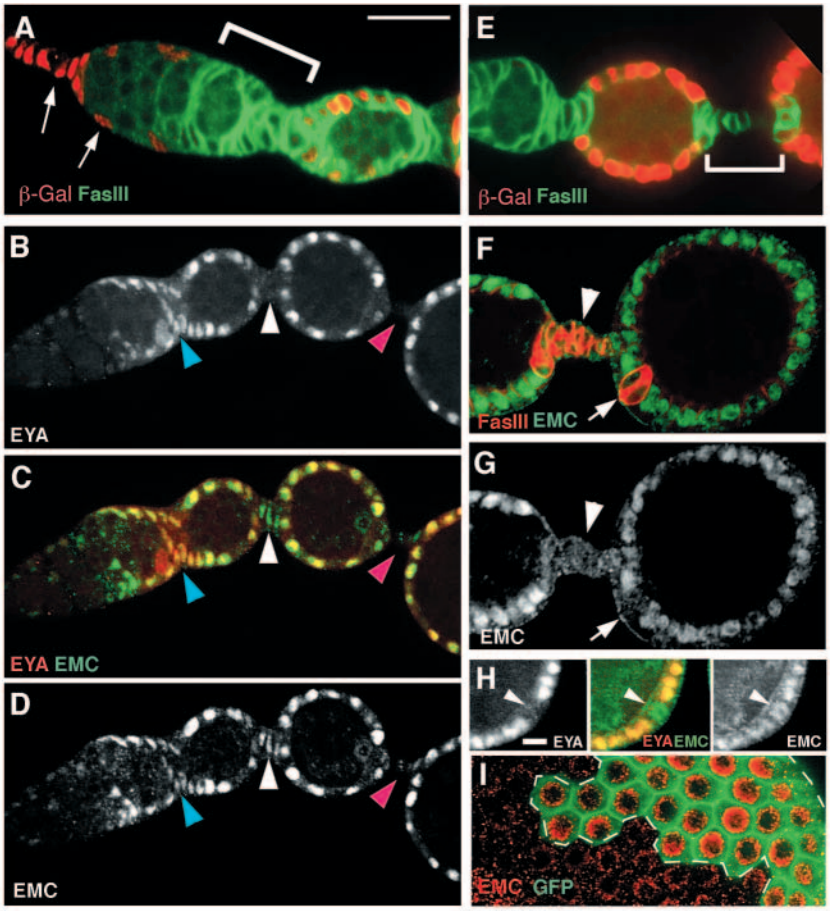

Fig. 3. EMC expression pattern in ovaries. Anterior is to the left. (A,E) Females containing the *emc*^{P5C} enhancer trap, stained with anti-β-galactosidase (red) and with anti-Fas3 (green), showing the germarium and a stage-2 egg chamber (A) or a stage-3 egg chamber and a polar/stalk group (E). Arrows in A indicate terminal filament, cap and inner sheath cells. The bracket in A indicates follicle cells in a germarium. The bracket in E indicates polar and stalk cells. (B-D) The same germarium and early ovariole from a wild-type female stained with anti-EYA (B,C; red in C) and anti-EMC (C,D; green in C). The blue arrowheads indicate intercyst follicle cells in the germarium. The white arrowheads indicate stalk cells between the germarium and a stage-2 egg chamber. The red arrowheads indicate stalk cells between a stage-2 egg chamber and a stage-4 egg chamber. (F,G) The same stage-3 and stage-4 egg chamber and polar/stalk group from a wild-type female stained with anti-Fas3 (red in F) and anti-EMC (F,G; green in F). Arrowheads indicate stalk cells; arrows indicate polar cells. (H) Section of a wild-type stage-6 egg chamber stained with anti-EYA (left panel, and red in middle panel) and anti-EMC (right panel and green in middle panel). Arrowheads indicate a polar cell expressing EMC but not EYA. (I) Posterior of a stage-10 egg chamber possessing a clone of cells (dashed line) that are overexpressing *EP3620* under the control of *ayGal4*. Overexpressing cells are marked with GFP (green). Red indicates EMC. Scale bars: in A, 25 μm in A-G, I; in H, 10 μm in H.

in these cells. Achaete and Scute are members of a tissue-specific class of basic helix-loop-helix (bHLH) genes, which act with Daughterless, a ubiquitously distributed bHLH, to suppress transcription of Delta, and possibly other targets. Daughterless is involved in Notch signaling in the follicle cells (Cummings and Cronmiller, 1994; Smith et al., 2002), but its bHLH partners have not been identified.

Extra macrochaetae (EMC) is a helix-loop-helix protein that is unable to activate transcription because it lacks a DNA-binding basic domain (Ellis et al., 1990; Garrell and Modolell, 1990). Instead, it inhibits the transcriptional activity of bHLH proteins by binding to them and sequestering them (Martinez et al., 1993; Van Doren et al., 1991; Van Doren et al., 1992). EMC is a transcriptional target of Notch in the wing (Baonza et al., 2000) and in the eye (Baonza and Freeman, 2001). In addition to its functions in Notch signaling, EMC is required for cell proliferation (Baonza and Garcia-Bellido, 1999). The mammalian counterparts of EMC, the Id (Inhibitor of DNA binding, and Inhibitor of Differentiation) family, are involved in myriad differentiation events, primarily as inhibitors of differentiation. Id proteins interact with the cell cycle machinery (Lasorella et al., 2001; Ruzinova and Benezra, 2003; Zebedee and Hara, 2001) and are upregulated in a number of metastatic cancers (Duncan et al., 1992). Here we identify a role for EMC in follicle cell differentiation, and identify it as an effector of Notch signaling in the ovary.

Materials and methods

Drosophila genetics

Fly culture and crosses were performed according to standard procedures. Egg chamber stages are according to Spradling (Spradling, 1993). Wet yeast was added 1 day before all dissections. For the EP screen, flies from the Rørth EP collection (Rørth et al., 1998) were obtained from Exelixis (CA) or from the Szeged Drosophila Stock Centre (http://gen.bio.u-szeged.hu/gen), crossed either to *C306-Gal4; PZ80/CyO* or to *PZ80/CyO; HS-Gal4* and grown at room temperature (or, if inviable at room temperature, at 18°C). *PZ80* (Karpen and Spradling, 1992) is an insertion into the non-essential *Fas3* gene (Patel et al., 1987). *C306-Gal4* (*C306*) drives expression in the germarium and in egg chambers (Manseau et al., 1997). Non-balancer F1 males and females were placed on wet yeast, and either heat shocked in a 37°C water bath for 1 hour (*HS-Gal4*), then placed at 25°C for 2 days, or placed at 29°C (*C306*) for at least 5 days with dry yeast. Three females were dissected from each line, fixed and stained for β-galactosidase activity (see below), examined under a dissecting microscope and scored (score = number of examples observed of fused egg chambers or of egg chambers with extra polar cells). EP candidates scoring ⩾3 were crossed again to whichever Gal4 driver was not used in the first cross, and five to seven females were dissected and stained from each cross. Candidates scoring ⩾1 on the second cross were crossed again to both drivers and to *C306; A101/TM3* [*A101* (Bier et al., 1989) is an enhancer trap in the gene *neuralized*] and re-examined, and those that scored ⩾3 on at least three out of five total tests were retained.

UAS-emc 5.3 and *emc*^{P5C} (an enhancer trap in *emc*) were obtained from A. Garcia-Bellido (Baonza et al., 2000; Ellis et al., 1990) and were either crossed to *C306* in parallel with *EP3620* (*UAS-emc 5.3*) or dissected as 2-day-old homozygotes (*emc*^{P5C}).

To examine EMC or EYA expression in FLP-OUT clones, *EP3620* or *UAS-NΔ34A* [(Doherty et al., 1996) obtained from Ken Irvine] were crossed to *hsFLP; ayGal4*, P[*w*$^{+}$*; UAS-mCD8GFP]/TM3* [*ayGal4* (Ito et al., 1997) is a transgene in which Gal4 is expressed in clones of

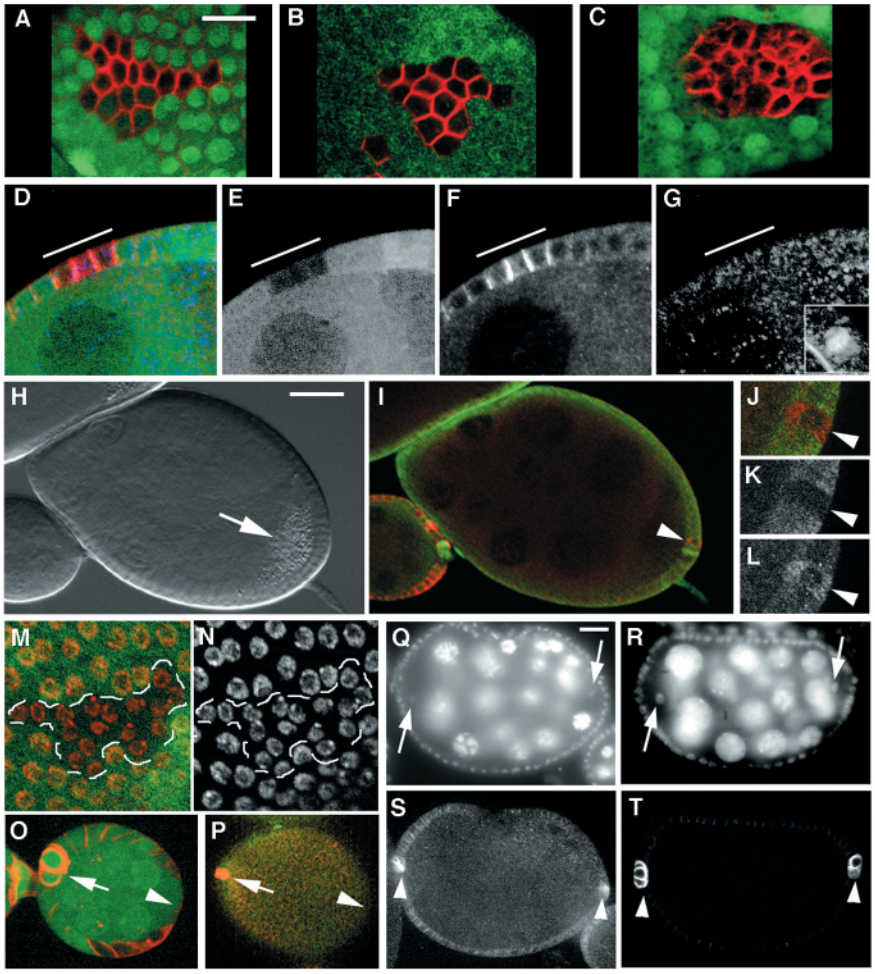

Fig. 4. Loss-of-function phenotypes of *emc* (A,B,D-Q,S) compared with those of *Notch* (C,R,T). Mutant cells are marked by the absence of GFP (A-E,I-K,M,O,P; green in A-D,I,J,M,O,P). (A-C) clones of cells mutant for emc^{1} (A), emc^{AP6} (B) or N^{55E11} (C), stained with anti-Fas3 (red). (D-G) A portion of the same egg chamber showing a clone of emc^{1} mutant cells marked by the loss of GFP (D,E; green in D) expressing elevated Fas3 (D,F; red in D) but not extra PZ80 (D,G; blue in D). Inset in G: PZ80 expression in polar cells from the same experiment. The lines indicate *emc* mutant cells. (H,I) A brightfield (H) and a fluorescent section (I) of the same stage-8 egg chamber possessing a small clone of emc^{AP6} mutant cells (arrowhead) marked by the absence of GFP (I, green) and stained with anti-Cyclin B antibody (I, red). The arrow indicates yolk. The arrowhead indicates mutant cells magnified in J-L. (J-L) Magnified region of I, marked with GFP (J,K; green in J), and stained with anti-Cyclin B antibody (J,L; red in J). Arrowheads indicate an *emc* mutant cell expressing Cyclin B. (M,N) Posterior of the same stage-10 egg chamber, showing a clone of emc^{AP6} mutant cells (inside dashed line) and their heterozygous neighbors (green, outside dashed line) stained with DAPI (M,N; red in M). (O,P) Single sections (O) and a Z-stack (P) of different egg chambers from animals possessing *emc* mutant clones marked with GFP (green) and stained with anti-Fas3 antibody (red). Arrowheads indicate expected position of polar cells. Arrows indicate polar cells. (Q-T) Fused egg chambers from different females possessing clones of cells mutant for emc^{1} (Q,S) or N^{55E11} (R,T) stained with DAPI (Q,R) and with anti-Fas3 antibody (S,T). Arrows indicate oocyte nuclei; arrowheads indicate polar cells. Scale bars: in A, 10 μm in A-G,J-P; in H, 25 μm in H,I; in Q, 25 μm in Q-T.

cells when FLP is induced by heat shock; expression of *UAS-mCD8GFP* (Lee and Luo, 1999) marks the clones; both were obtained from the Bloomington Stock Center (http://fly.bio.indiana.edu)]. Non-balancer females were heat-shocked in a water bath for 1 hour at 37°C, then placed at 25°C or 29°C with wet yeast for 2 or 3 days before dissecting.

For analysis of loss-of-function phenotypes, mutant clones were generated using the FLP/FRT system (Xu and Rubin, 1993) and *hs-FLP*; adult females were heat shocked in a 37°C water bath twice in 1 day (4 to 6 hours between heat-shocks), and then placed on wet yeast at 25°C for 1 to 7 days. Clones were marked using *Ubi-nlsGFP* (*emc* and *N*) (Davis et al., 1995) or using anti-EYA antibody (eya^{54C2}) (Bonini et al., 1993). N^{55E11}, *FRT18A/FM7* (Grammont and Irvine, 2001) and emc^{1}, *FRT80B/TM6b* [a strong hypomorphic allele of *emc* (Ellis et al., 1990)] were obtained from Ken Irvine and Bloomington, respectively. eya^{54C2}, *FRT40A* was described previously (Bai and Montell, 2002). emc^{AP6}, *FRT2A/TM6b* [emc^{AP6} is a null allele obtained from Pascale Heitzler (Ellis, 1994; Heitzler et al., 1996)] and emc^{EP3620}, *FRT2A/TM6b* were generated by standard methods. Clones of emc^{1}, *FRT80B* were generated in parallel with clones of N^{55E11}, *FRT18A*, and with clones of a wild-type *FRT80B* chromosome. They were analyzed in a *PZ80*-containing background 3 days after heat shock, since they exhibited no phenotype 2 days after heat shock. We were unable to observe clones of emc^{1} mutant cells more than 3 days after heat shock. Clones of emc^{AP6}, *FRT2A*, and of emc^{EP3620}, *FRT2A* were analyzed in parallel with clones of a wild-type *FRT2A* chromosome and were found as many as 7 days after heat shock.

In order to examine EMC overexpression using *Upd-Gal4*, a driver expressed only in polar cells and their precursors (Bai and Montell, 2002) (obtained from Doug Harrison), *Upd-Gal4; UAS-mCD8GFP/CyO* was crossed to *w; PZ80/CyO; EP3620/TM6b* or *UAS-LacZ* or *w; PZ80/CyO; UAS-mCD8GFP*. Non-balancer flies were placed at 29°C for 3 to 5 days before dissection. This was modified for *Upd-Gal4* temperature shifts: females and several males of the appropriate genotypes in a vial were fed with wet yeast overnight at 18°C, incubated in a 33°C water bath for 6 hours, then returned to 18°C with fresh wet yeast for 24 hours before dissecting, fixing and staining with anti-βGal antibody and 4,6-diamidino-2-phenylindole (DAPI). Each egg chamber from stages 4 through 9 was scored for the number of polar cells in the expected anterior and posterior polar cell groups.

Immunofluorescence and immunohistochemistry

Ovary dissections were performed in Grace's medium containing 10% fetal calf serum. For the EP screen, whole ovaries were fixed in a 96-well plate for 10 minutes in 4% formaldehyde (Polysciences, PA) in 0.1 mol/l potassium phosphate buffer. Fifty percent of the lines were then washed with NP-40 wash (50 mmol/l Tris pH 7.4, 150 mmol/l NaCl, 0.5% NP-40, 1 mg/ml bovine serum albumin) and stained overnight at 4°C with 1:1 7G10 anti-Fas3 antibody [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA] and DAPI as described below. The remaining 50% of the lines were washed once in PBT (0.1 mol/l PBS + 0.5% Triton-X-100), then incubated overnight at 37°C with 0.2% X-GAL in staining solution (10 mmol/l phosphate buffer pH 7.2, 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 3 mmol/l $K_4[FeII(CN)_6]$, 3 mmol/l $K_3[FeIII(CN)_6]$, 0.3% Triton-X-100). They were then incubated with

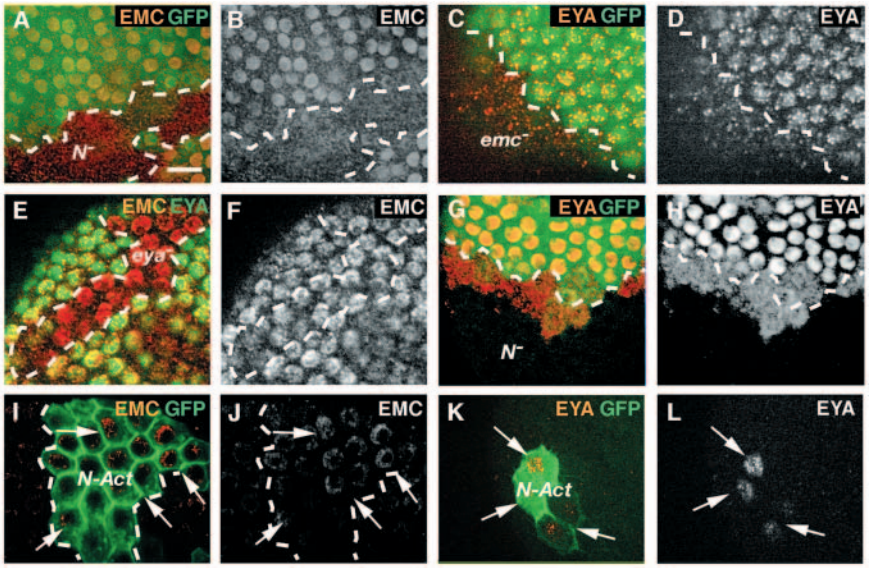

Fig. 5. Molecular epistasis of Notch, EMC and EYA. (A-H) Loss-of-function clones of N^{55E11} (A,B,G,H,), emc^{1} (C,D) or eya^{54C2} (E,F) mutant cells marked with GFP (A,C,G), or anti-EYA (E, green) and stained with either anti-EMC (A,B,E,F; red in A,E) or anti-EYA (C,D,G,H; red in C,G). The dashed lines indicate clones of mutant cells. (I-L) FLP-OUT clones of cells expressing activated Notch (*UAS-NΔ34A:* designated N-Act) in two separate stage-10 egg chambers, marked with GFP to show NΔ34A-expressing cells in green (I,K) and stained with anti-EMC (I,J; red in I) or with anti-EYA (K,L; red in K). The arrows indicate cells overexpressing EMC (I,J) or EYA (K,L). The dashed lines (I,J) and arrows (K,L) indicate cells overexpressing UAS-N*Δ34A*. Scale bar: 10 μm.

1:400 DAPI from 1 mg/ml stock (Sigma, MO) in PBT for 40 minutes at room temperature, and rinsed twice with PBT. Samples were mounted in 50% glycerol/PBT or examined in a depression slide.

For immunofluorescence, the following primary antibodies were used: mouse anti-Fas3 7G10 1:10 (DSHB); rabbit anti-β-galactosidase 1:400 (Cappel; NC); rabbit anti-EMC 1:1000 [a gift from Y. Jan (Baonza et al., 2000)]; mouse anti-EYA 10H6 (Bonini et al., 1993) 1:50 (DSHB); rabbit anti-Phosphohistone H3 1:1000 (Upstate Biotechnology, NY); mouse anti-Cyclin B 1:10 (DSHB). Ovarioles were fixed for 10 minutes at room temperature in 4% formaldehyde in 0.1 mmol/l phosphate buffer, rinsed twice in NP-40 wash, stained in the appropriate antibody diluted in NP-40 wash overnight at 4°C, washed for 1 hour at room temperature in NP-40 wash, and stained with appropriate secondary antibodies diluted in NP-40 wash for 1 hour at room temperature. Secondary antibodies used (Molecular Probes, OR) were Alexa 488 anti-rabbit, Alexa 488 anti-mouse, Alexa 568 anti-rabbit, Alexa 568 anti-mouse, Alexa 647 anti-rabbit and Alexa 647 anti-mouse, each at 1:400. DAPI was added to the secondary incubation at 1:400. After incubation with secondary antibody, samples were rinsed twice in NP-40 wash and mounted in Vectashield (Vector Laboratories, CA). Fluorescent images were captured on an Ultraview spinning disk confocal microscope, a Zeiss Axioplan, or a Zeiss Apotome.

Results

An overexpression screen for genes affecting follicle cell fate and differentiation

In order to identify genes involved in cell fate decisions in the ovary, we performed a genetic screen using the EP system (Rørth, 1996). Briefly, this screen takes advantage of the ability of Gal4 protein to activate expression of genes

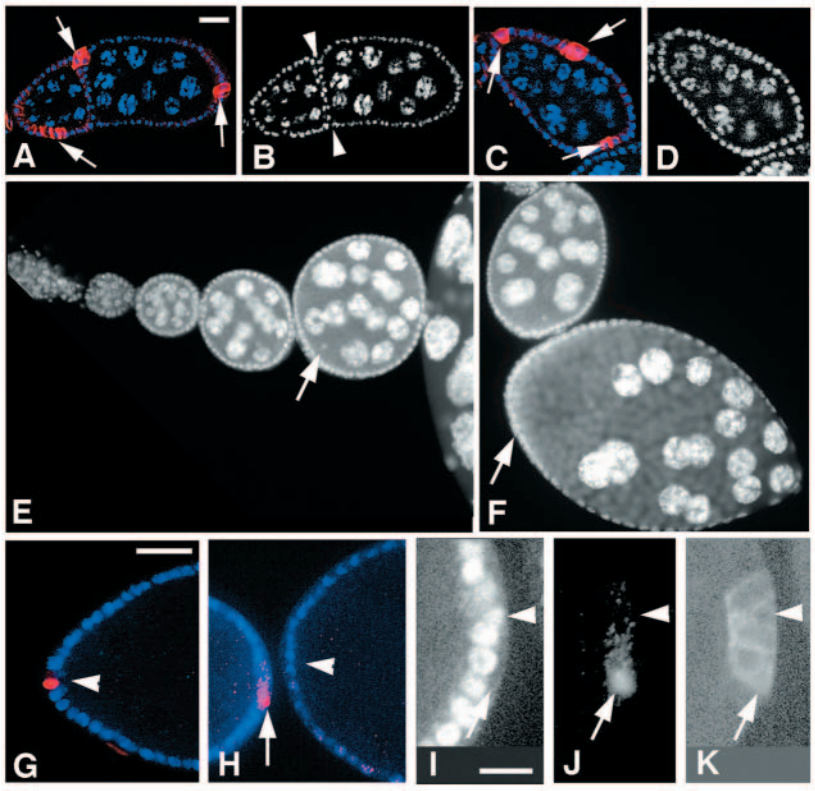

Fig. 6. The effect of EMC overexpression on polar cell differentiation and on expression of EYA. (A-D) Two examples of fused egg chambers in which *EP3620* is overexpressed using *FLP-OUT Gal4*, stained with anti-Fas3 antibody (A,C; red) and DAPI (A-D; blue in A,C), possessing three polar cell groups (arrows) rather than four, and having either a layer of cells between the fused egg chambers (B, arrowheads) or no such layer (D). (E-K) *EP3620* (E,F,H-K) or *UAS-LacZ* (G) overexpressed using *Upd-Gal4*. (E,F) An ovariole (E) or two egg chambers in a different ovariole (F) stained with DAPI, showing an egg chamber with the oocyte positioned toward the anterior rather than the posterior of the ovariole (arrows). (G,H) Portions of the anterior end of an egg chamber (G) and the posterior end of one egg chamber (H, left) and anterior end of its neighbor (H, right) expressing *UAS-LacZ* (G) or *EP3620* (H), and showing expression of EYA (blue), and the polar cell marker *PZ80* (red). The arrowheads indicate the shape of the anterior end of the egg chamber and the position of the expected polar cell. The arrow indicates the region magnified in I-K. (I-K) EYA expression (I), polar cells (J) and Gal4-expressing cells (marked with GFP, K). The arrows indicate a polar cell expressing Gal4 (and presumably EMC) but not EYA. The arrowheads indicate a cell expressing Gal4 and EYA but not PZ80. Scale bars: in A, 25 μm in A-F; in G, 25 μm in G,H; in I, 10 μm in I-K.

Table 1. Phenotypes observed when EMC is overexpressed using *Upd-gal4*

Defect observed	EP3620 (*n*=401*)		UAS-GFP (*n*=253*)	
	n†	%‡	*n*†	%‡
Anterior polar cells missing	41	10.2	1	0.4
[(Rounded at anterior)	8	2	1	0.4]
Posterior polar cells missing	42	10.5	1	0.4
Extra (4 or more) polar cells in group	17	4.2	4	1.6
Oocyte mispositioned	19	4.7	0	0
Fused or mispackaged egg chambers	2	0.5	0	0

Types and frequencies of egg chamber defects in females expressing *EP3620* or *UAS-GFP*, under the control of *Upd-Gal4*, at 29°C for 5 days.
*Total egg chambers analyzed.
†Number of egg chambers possessing the defect shown.
‡Percent of total egg chambers possessing the defect shown.
'Rounded at anterior' is a subset of 'anterior polar cells missing'.

located adjacent to upstream activating sequences (UAS sites). The EP is a P-element containing 14 UAS sites upstream of a basal promoter (Rørth, 1996). The Rørth EP collection contained about 2300 stocks, each with a random EP insertion into the genome, and therefore should separately drive expression of a wide variety of genes when crossed to a stock expressing Gal4. We crossed lines from the EP collection to Gal4 lines that can drive target gene expression in follicle cells in the germarium, and examined the ovaries for effects on polar cell formation or egg chamber packaging. *EP*s *3620* and *0415* produced egg chambers containing multiple germline cysts with high penetrance, indicating that they affected packaging (Fig. 2, compare A with B, and data not shown). These EPs are inserted upstream of the *extra macrochaetae* (*emc*) open reading frame and oriented in the correct direction to overexpress *emc* (Berkeley Drosophila Genome Project). Overexpression of EMC using a *UAS-emc* transgene (Baonza and Garcia-Bellido, 1999) produced an indistinguishable phenotype (Fig. 2C).

Expression of EMC in the ovary

In order to further understand the role of EMC, we examined its expression pattern (Fig. 3). An enhancer trap insertion into *emc*, emc^{P5C}, has previously been used to examine EMC expression in the wing (Baonza et al., 2000; Baonza and Garcia-Bellido, 1999). We compared its expression with that of Fas3, a marker of follicle cell differentiation. Fas3 is not expressed in somatic cells in germarium region 1, but it is expressed in undifferentiated follicle cells (in germarium regions 2B and 3, and in early egg chambers), and in early polar and stalk cells (Ruohola et al., 1991). It is reduced in differentiated main body follicle cells and stalk cells but is expressed in polar cells throughout oogenesis. By contrast, EMC had a complex pattern of expression. emc^{P5C}*-lacZ* was expressed in the terminal filament and inner sheath cells of the germarium (Fig. 3A, arrows), and in the main body follicle cells of egg chambers (Fig. 3E), but not in the early follicle cells (Fig. 3A, bracket) or in polar or stalk cells (Fig. 3E, bracket). An anti-EMC antibody revealed additional features of EMC expression (Fig. 3B-D,F-H): EMC was expressed in the undifferentiated follicle cells of the germarium (Fig. 3D). Its expression was reduced in follicle cells in the intercyst regions between region 2 and region 3 cysts (Fig. 3C,D, blue arrowheads). It was maintained in main body cells but further reduced in stalk and polar cells of stage-1-4 egg chambers (Fig. 3C,D white and red arrowheads; Fig. 3F,G, arrows). Its expression in stalk cells remained low (Fig. 3F,G, arrowheads), but, in polar cells after stage 4, returned to a level indistinguishable from that exhibited by their neighbors (Fig. 3H, arrowheads). The overall level of expression was somewhat reduced from stage 6 to stage 8, and at stage 9 expression in the oocyte-associated follicle cells was further reduced, although still detectable (Fig. 3I, outside dashed lines, and data not shown). In order to confirm that *EP3620* overexpressed EMC, we also examined expression of EMC when expression of *EP3620* was induced using a FLP-OUT cassette that only expresses Gal4 in GFP-marked clones of cells. As expected, induction of *EP3620* resulted in elevated expression of EMC (Fig. 3I, inside dashed lines) in a mosaic fashion in the Gal4-expressing cells.

Loss-of-function analysis of emc

The wild-type EMC expression pattern suggested that EMC might be involved in differentiation in the germarium and in main body cells. In order to examine this, we generated mutant clones with loss-of-function alleles of *emc* (Fig. 4). Small clones of *emc* (Fig. 4A,B) displayed elevated Fas3 expression, but control, wild-type clones did not (data not shown). This is similar to the phenotype of *Notch* mutant clones in main body cells (Fig. 4C). emc^{1} clones in main body cells failed to express the polar-cell-specific enhancer trap *PZ80* (Fig. 4D-G, lines; compare with inset in G), indicating they did not become polar cells but were undifferentiated cells. Undifferentiated *Notch* mutant cells proliferate beyond the programmed cessation of proliferation at stage 6. In order to determine whether proliferation was still occurring in *emc* mutant cells, we examined expression of Cyclin B, a marker for cell proliferation, in follicle cells of egg chambers during stages 7 through 9. In wild-type follicle cells at these stages Cyclin B is not present because the cells have ceased cell division. Cyclin B was expressed in *emc* mutant clones (Fig. 4H-L, arrowheads in I-L: 5 out of 66 mutant clones).

Undifferentiated *Notch* mutant follicle cells are more densely distributed than their heterozygous neighbors (Lopez-Schier and St. Johnston, 2001; Deng et al., 2001), because the extra cell divisions are not accompanied by additional cell growth; thus, the cells become smaller as they divide. Undifferentiated cells mutant for a null allele of *emc* (emc^{AP6}) are denser than their neighbors (area of mutant cell/area of wild-type cell = 1.8; *n*=10) (Fig. 4M,N, compare nuclei inside and outside dashed lines), indicating that these cells are unable to change from proliferation to endoreplication at the proper time.

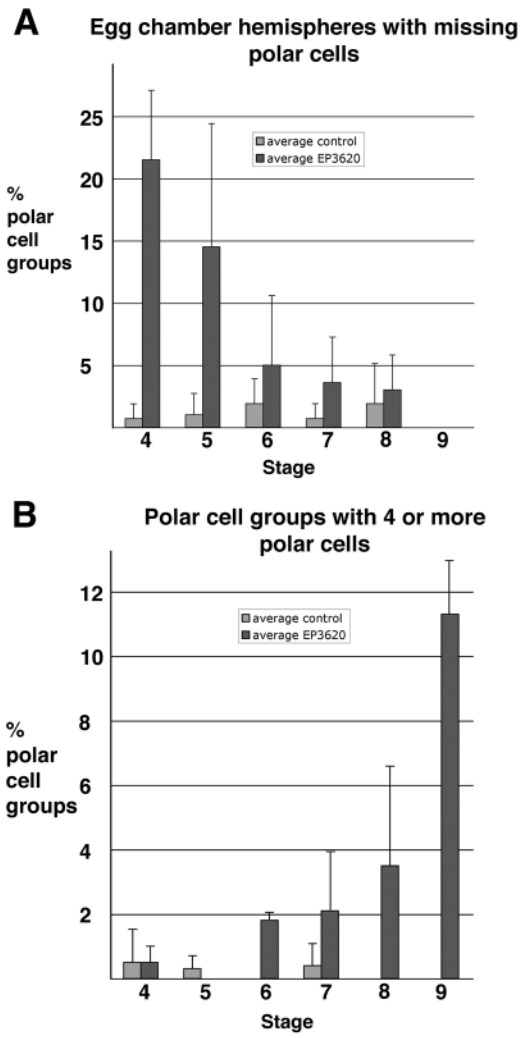

Fig. 7. Graphs showing the relationship between loss of polar cells, gain of polar cells and egg chamber stage in temperature-shift experiments. *Upd-Gal4/+; P280/+; EP3620/+*: dark gray; *Upd-Gal4/+; P280/+; UAS-mCD8GFP/+*: light gray. Egg chamber stage is plotted on the *x*-axis and percentage of expected polar cell groups in egg chambers of that stage that show loss of all polar cells (A) or that show four polar cells (B) is plotted on the *y*-axis. In each case, the value plotted is the average of three experiments. Error bars: standard deviation.

We examined effects of *emc* mutations on development of polar and stalk cells. Notch signaling is required for differentiation of all the follicle cells, including main body, polar and stalk cells (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). As with *Notch*, egg chambers with *emc* mutant clones lacked polar cells at one end or the other (Fig. 4O,P, arrowheads), demonstrating a requirement for EMC in polar cell specification or differentiation. However, in contrast to *Notch*, the *emc* polar cell phenotype was partially penetrant (31.3%: 21 out of 67 clones).

Notch mutant egg chambers can be fused to their neighbors (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). Likewise, fused *emc* mutant egg chambers could be found, possessing 32 germline nuclei (*n*=12). The presence of two oocyte nuclei (Fig. 4, compare Q and R, arrows) suggested

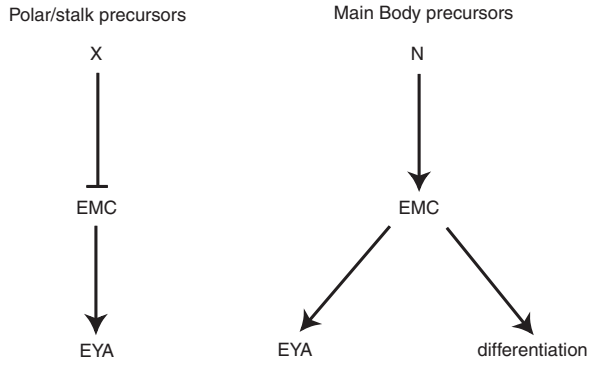

Fig. 8. Model for the relationship between Notch, EMC and EYA during oogenesis. Left: in polar/stalk precursors, an unknown pathway (X) inhibits expression of EMC. In the absence of EMC, EYA is reduced. Right: in main body precursors, Notch promotes EMC expression, and EMC promotes EYA expression, leading to either the establishment or maintenance of main body cell identity. In addition, EMC activity promotes differentiation.

that these egg chambers probably resulted from fusion of two distinct cysts rather than from an extra round of germline nuclear division. Fused egg chambers in ovarioles containing *emc* mutant clones, like those observed in ovarioles containing *Notch* mutant clones, possessed polar cells only at the termini of the fused cysts (Fig. 4 compare S and T, arrowheads), suggesting that fusion resulted from defects in polar cell differentiation or polar/stalk precursor specification, rather than from defects in stalk cell differentiation or morphogenesis.

If EMC affected polar cell precursor specification in addition to polar cell differentiation, then we would expect that clones generated in the germarium (i.e. those observed ⩾4 days after heat shock) would produce fused egg chambers more frequently than those generated in existing egg chambers (i.e. those observed 1-2 days after heat shock). However, fused egg chambers were observed at the same low frequency in both cases. Thus, current evidence does not support a role for EMC in generating polar/stalk cell precursors.

Relationship between EMC, Notch and EYA

EMC functions downstream of Notch in some tissues, and *emc* and *Notch* mutant clones share some phenotypic characteristics, so we examined whether expression of EMC in the main body cells depended upon Notch. EMC expression was greatly reduced in large *Notch* clones on the main body at stage 6 (*n*=36) (Fig. 5A,B). By contrast, EMC expression was normal in mutant clones of *STAT*, another gene required for follicle cell differentiation (data not shown).

We next examined the relationship between EMC and EYA. EYA is a negative regulator of both polar and stalk cell fate. Forced expression of EYA leads to elimination of both cell fates and thus to fused egg chambers, and loss of *eya* transforms main body cells into polar cells (Bai and Montell, 2002). EMC and EYA expression are similar, and EMC overexpression in the stalk leads to fused egg chambers, so we tested whether EMC was required for EYA expression. Consistent with this hypothesis, EYA was reduced in clones of *emc* on the main body (*n*=23; Fig. 5C,D). As expected, EMC was not reduced in *eya* clones (*n*=14; Fig. 5E,F). Since EMC regulates EYA and Notch regulates EMC, we examined EYA

expression in *Notch* mutant cells. As expected, EYA was reduced in *Notch* mutant cells on the main body (n=23; Fig. 5G,H). We observed that in cells at the border of the clone, EYA was expressed but was cytoplasmic. This suggests local non-autonomous effects and thus that there is communication between follicle cells on the main body. In order to confirm that Notch promotes expression of EMC and EYA in main body follicle cells, we examined their expression in FLP-OUT Gal4 clones in which expression of activated Notch (*UAS-NΔ34A*) was forced (Fig. 5I-L). As EYA and EMC are expressed at high levels in main body cells until stage 8, we analyzed their expression only in clones of cells covering the oocyte at stage 10. These cells normally express EMC only at low levels and do not express EYA. We found a partially penetrant elevation in the expression of both EMC (11 out of 37 clones) (Fig. 5I,J, arrows and inside dashed lines) and EYA (29 out of 60 clones) (Fig. 5K,L, arrows).

The similarity between the expression patterns of EMC and EYA, the dependence of EYA on EMC in main body cells, and the formation of fused egg chambers when each is overexpressed, suggest that EMC is a regulator of EYA expression in all the follicle cells. Therefore we tested whether forcing EMC expression in the polar cells could induce EYA expression and affect polar cell formation. We generated FLP-OUT Gal4 clones of cells that expressed EMC. The polar cells were rarely targeted and out of several thousand clones observed, we obtained just six that resulted in fused egg chambers or the elimination of a single polar cell pair from one end of the egg chamber (Fig. 6A-D, arrows in Fig. 6A,C, and data not shown).

The low frequency of these effects precluded us from closely examining the effect of EMC overexpression on polar cell development using these drivers. Therefore, we used a Gal4 driver specific to the polar cells, *Upd-Gal4* (Bai and Montell, 2002; Silver and Montell, 2001). When we drove expression of *UAS-LacZ* (data not shown) or *UAS-GFP* (Table 1), egg chambers appeared normal and polar cells developed normally. By contrast, when we drove *UAS-emc*, polar cells were absent from one end or other of about 20% of egg chambers (Table 1; Fig. 6G,H, arrowheads). Thus, expression of EMC can prevent polar cells from forming. In addition, the oocyte was sometimes mispositioned (Table 1; Fig. 6E,F, arrows) and egg chambers were sometimes round in shape at the anterior end (Table 1; compare Fig. 6G,H, arrowheads), phenotypes that are observed when polar cells are not formed (Grammont and Irvine, 2002).

We expected that these phenotypes would occur if EMC affected EYA expression in polar cells, and, indeed, egg chambers in which polar cells were missing from one end exhibited EYA expression in all the cells at that end (Fig. 6H, arrowhead). In some cases, cells expressed both EYA and GFP, indicating that EMC was driving expression of EYA in those cells (Fig. 6I,K, arrowheads). Thus, EMC can force expression of EYA in the polar cells, and this affects their fate and function.

In addition to loss of polar cells, extra polar cells were sometimes formed when EMC was expressed either with *Upd-Gal4* (Table 1) or other drivers such as *HS-gal4* (data not shown). In wild-type ovarioles, EMC is reduced in immature polar cells from stage 1 through stage 4 but is expressed in mature polar cells at stage 5 and above (see Fig. 3). This coincides with stages in which a reduction of polar cell number occurs in wild-type ovarioles: up to four or five polar cell precursors are observed in polar cell groups belonging to stage-2 and stage-3 egg chambers, but two to three of these precursor cells die upon maturation into polar cells at stage 4 or 5 (Besse and Pret, 2003). We hypothesized that the ability of EMC to induce loss or gain of polar cells was a consequence of differing roles of EMC at different times during polar cell development. Specifically, we thought that EMC might be a negative regulator of polar cells in stage 1 through stage 4, and a positive regulator subsequent to this. In order to test this hypothesis, we took advantage of the temperature-dependence of Gal4 activity. We shifted flies expressing *UAS-emc* under the control of *Upd-Gal4* to high (Gal4-active) temperature for 6 hours in order to express EMC in immature polar cells, and then down to low (Gal4-inactive) temperature for 24 hours to allow these cells to mature. We then determined in which egg chambers polar cells were lost, and in which egg chambers extra polar cells were formed. Based on the known durations of various stages (Spradling, 1993), we can estimate at what stage the egg chambers expressed EMC, and thus determine at what stages polar cell development was inhibited or enhanced by EMC. Egg chambers that were in stage 2 or 3 during EMC expression, and were in stage 4-8 when they were analyzed, showed more frequent loss of polar cells (Fig. 7A). By contrast, extra polar cells were observed most frequently in egg chambers that were at stage 9 at analysis (Fig. 7B), which were at stage 5 during EMC expression. Thus, *UAS-emc* inhibits polar cell development at stages 2 and 3, but promotes it at stage 5.

Discussion

In this work, we demonstrated a number of similarities in the phenotypic effects of Notch and EMC in egg chamber development, and we conclude that EMC is a downstream effector of Notch in the differentiation of follicle cells. In other tissues, genes of the Enhancer of split complex are major downstream effectors of Notch signaling, but they do not appear to be required for differentiation of the follicle cells (Lopez-Schier and St Johnston, 2001). By contrast, EMC is expressed in most somatic cells of the ovariole and has a loss-of-function phenotype similar to that of Notch. Like Notch, EMC is required for the formation of polar cells, and we observe egg chambers lacking polar cells at one end when *emc* clones are generated. *emc* mutant cells also fail to downregulate Fas3 expression at stage 6, and the cells fail to enter endoreplication at the proper time, indicating that *emc* is required for follicle cell differentiation. In addition, EMC expression in the main body follicle cells is dependent on Notch and can be induced by forced expression of activated Notch. Taken together, this leads us to propose that EMC is an effector of Notch activity in the follicle cells.

Notch and EMC are both involved in separation of adjacent egg chambers. Follicles mutant for *Notch* or *emc* include egg chambers containing multiple germline cysts and lacking intervening polar and stalk cells. Recent work has shown that Notch signaling is not required for early packaging (Torres et al., 2003), but its role in specifying polar/stalk precursors, which might occur subsequent to packaging, has not been addressed specifically. However, Notch is required autonomously for polar cell differentiation (Grammont and

Irvine, 2001; Lopez-Schier and St Johnston, 2001), and at least one pair of differentiated polar cells is required to induce a stalk. Therefore, the packaging defects observed in *Notch* mutants may be due solely to either the failure of polar cells to differentiate or to a particular role in specifying polar/stalk precursors. The fused egg chambers we observed in *emc* mutants could also be due to a role in one or both processes. If *emc* were involved separately in both processes, then we would expect to see a dramatic increase in the frequency of fused egg chambers when clones were generated in the germarium, where precursors are formed. Since we did not see such an increase, we do not posit a role for *emc* in polar/stalk precursor formation; however, without a better understanding of polar/stalk precursors, this cannot be confirmed.

emc is probably not the only effector downstream of *Notch* in the ovariole, since *em*c mutant clones exhibit some differences from *Notch* mutant clones. In general the effects of *emc* were less penetrant than those of *Notch*. For example, *emc* mutant cells, like *Notch* mutant cells, exhibited persistent Phosphohistone H3 (data not shown) and Cyclin B labeling but at low frequency and at low levels. In addition, although *emc* is involved in polar cell specification or differentiation, it is only partially required for this, since normal polar cells could be observed even in clones of a null *emc* allele. Thus, there are likely to be additional downstream effectors of Notch in the ovary.

Molecular epistasis indicates that Notch signals through EMC to induce or maintain EYA expression in the main body follicle cells. EYA expression is lost in large *Notch* mutant follicle cell clones and can be induced by forced expression of activated Notch in the follicle cells. EYA is involved in inhibiting polar and stalk cell fate, so one might expect that *Notch* or *emc* loss-of-function mutants would make extra polar cells. This is not the case, however; it seems likely that the reason *Notch* and *emc* mutant cells do not become polar cells is that they fail to differentiate. EYA does not appear to be required for differentiation, but rather for main body cell fate, since *eya* mutant cells differentiate into polar cells (Bai and Montell, 2002). Thus, the Notch pathway must branch downstream of EMC, with one pathway leading to EYA expression and repression of polar cell fate, and a separate pathway leading to differentiation (Fig. 8).

EMC can have both positive and negative effects on the number of polar cells. Although this might initially seem mysterious, it can be explained by the dynamic expression of EMC in ovaries. EMC is expressed in the germarium, but it is reduced in polar/stalk precursors. Its expression remains low in stalk cells but, in polar cells, returns to the same level as that of their neighbors around the time of differentiation. Our temperature-shift experiments show that forced expression of EMC in immature polar cells can lead to expression of EYA, which is the presumed cause of loss of polar cells. By contrast, forced expression of EMC in maturing polar cells appears to lead to potentiation of polar cell number, i.e. polar cell number per group is not reduced from four to two, as in wild-type, but remains at four polar cells per group. This is probably due to a role for EMC in polar cell differentiation, because loss-of-function *emc* clones can result in loss of polar cells.

Three lines of evidence suggest that EMC may be a key regulator of EYA expression. First, EMC and EYA expression are similar in multiple respects. Both are upregulated in follicle cells in region 2B of the germarium, and in main body follicle cells from stages 2 through 6. Both are downregulated in the polar/stalk lineage from the germarium through stage 3, and in the oocyte-associated follicle cells at stages 7 through 9. Second, EMC is required for EYA expression in the main body. Third, EMC and EYA produce similar overexpression phenotypes, including fused egg chambers and the loss of polar cells, and, when EMC is overexpressed in the polar cells, EYA expression is induced. Taken together, these data suggest that the expression of EYA in the follicle cells is largely regulated by EMC. EMC is a helix-loop-helix protein that lacks the basic DNA-binding domain of the bHLH transcription factors. It normally opposes the activity of bHLH transcription factors by sequestering them in non-productive complexes. Thus, the dependence of EYA on EMC is likely to be indirect. Presumably, EMC inhibits a bHLH protein that inhibits expression of EYA. The identity of this protein remains an interesting subject for further study.

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