The homeobox gene *mirror* links EGF signalling to embryonic dorso-ventral axis formation through Notch activation

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Recent studies in vertebrates and *Drosophila melanogaster* have revealed that Fringe-mediated activation of the Notch pathway has a role in patterning cell layers during organogenesis[1,2]. In these processes, a homeobox-containing transcription factor is responsible for spatially regulating *fringe* (*fng*) expression and thus directing activation of the Notch pathway along the *fng* expression border. Here we show that this may be a general mechanism for patterning epithelial cell layers. At three stages in *Drosophila* oogenesis, *mirror* (*mirr*) and *fng* have complementary expression patterns in the follicle-cell epithelial layer, and at all three stages loss of *mirr* enlarges, and ectopic expression of *mirr* restricts, *fng* expression, with consequences for follicle-cell patterning. These morphological changes are similar to those caused by *Notch* mutations. Ectopic expression of *mirr* in the posterior follicle cells induces a stripe of *rhomboid* (*rho*) expression and represses *pipe* (*pip*), a gene with a role in the establishment of the dorsal-ventral axis, at a distance. Ectopic Notch activation has a similar long-range effect on *pip*. Our results suggest that Mirror and Notch induce secretion of diffusible morphogens and we have identified TGF-β (encoded by *dpp*) as such a molecule in germarium. We also found that *mirr* expression in dorsal follicle cells is induced by the EGF-receptor (EGFR) pathway and that *mirr* then represses *pip* expression in all but the ventral follicle cells, connecting EGFR activation in the dorsal follicle cells to repression of *pip* in the dorsal and lateral follicle cells. Our results suggest that the differentiation of ventral follicle cells is not a direct consequence of germline signalling, but depends on long-range signals from dorsal follicle cells, and provide a link between early and late events in *Drosophila* embryonic dorsal-ventral axis formation.

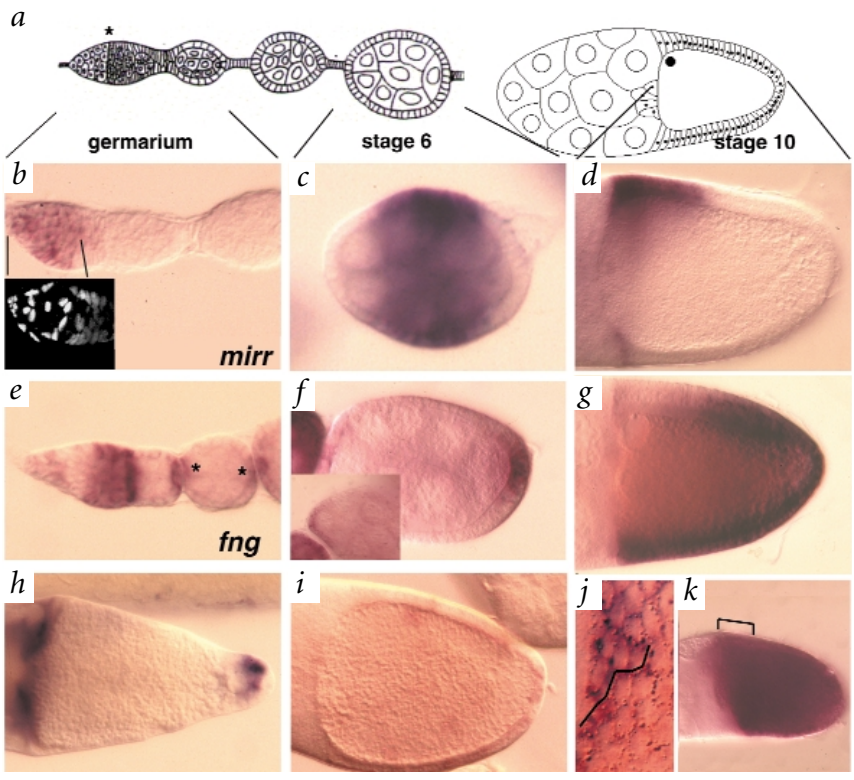

Fig. 1 The expression patterns of *mirr* and *fng* are complementary during *Drosophila* oogenesis. ***a***, The stages of oogenesis in *Drosophila*. In the germarium, the most anterior structure of the ovariole, the follicle cells, which are derived from stem cells (asterisk), encapsulate the 16-cell germline cysts. The completion of encapsulation requires differentiation of two groups of follicle cells, stalk cells and polar cells, which cease dividing as they separate the newly formed egg chamber from the germarium[16]. The remaining follicle cells continue to divide until stage 6 of oogenesis. In subsequent stages, the oocyte matures in the encapsulated cyst. The anterior-posterior polarity of the oocyte is established by the beginning of stage 7, and dorsal-ventral polarity is established by late stage 10. In the germarium, *mirr* (***b***) is expressed in the anterior-most region and *fng* (**e**) in the posterior region. The *mirr-lacZ* line shows expression in the somatic inner sheath cells as well as in the most anterior follicle cells (***b***, inset). A similar pattern was detected using anti-MIRR antibody. *fng* is expressed in the follicle cells at the posterior part of the germarium. *fng* expression in the polar cells at stages 1–5 (**e**, asterisks) defines the non-dividing cells and marks a boundary with those cells that continue to divide. Neither *mirr* nor *fng* are expressed in the interfollicular stalk cells. At stage 6, *mirr* (***c***) is expressed in the lateral and *fng* (***f***) in the terminal follicle cells of the egg chamber (inset in *f* shows *fng* expression in anterior follicle cells). At stage 10, *mirr* (***d***) is expressed in the dorsal anterior and *fng* (***g***) in the remaining follicle cells. *mirr* expression is detected in ~42 cells, whereas *fng* expression is excluded from ~44 cells. In double *in situ* hybridization (***j***), no white cells are detected between *mirr* (blue, above the line) and *fng* (brown, below the line) expressing cells. *mirr* expression is also detected in centripetally migrating cells. *mirr* expression in dorsal anterior columnar follicle cells is not observed in grk^{DC29}/grk^{2B6} mutant egg chambers (***h***); the centripetal follicle cells still express *mirr*. Overexpression of *mirr* (*UAS-mirr/+; Gal4 109(3)9/+*) reduces (***i***) and loss of function (clones of $mirr^{l(3)6D1}$) expands (***k***) the *fng* expression domain.

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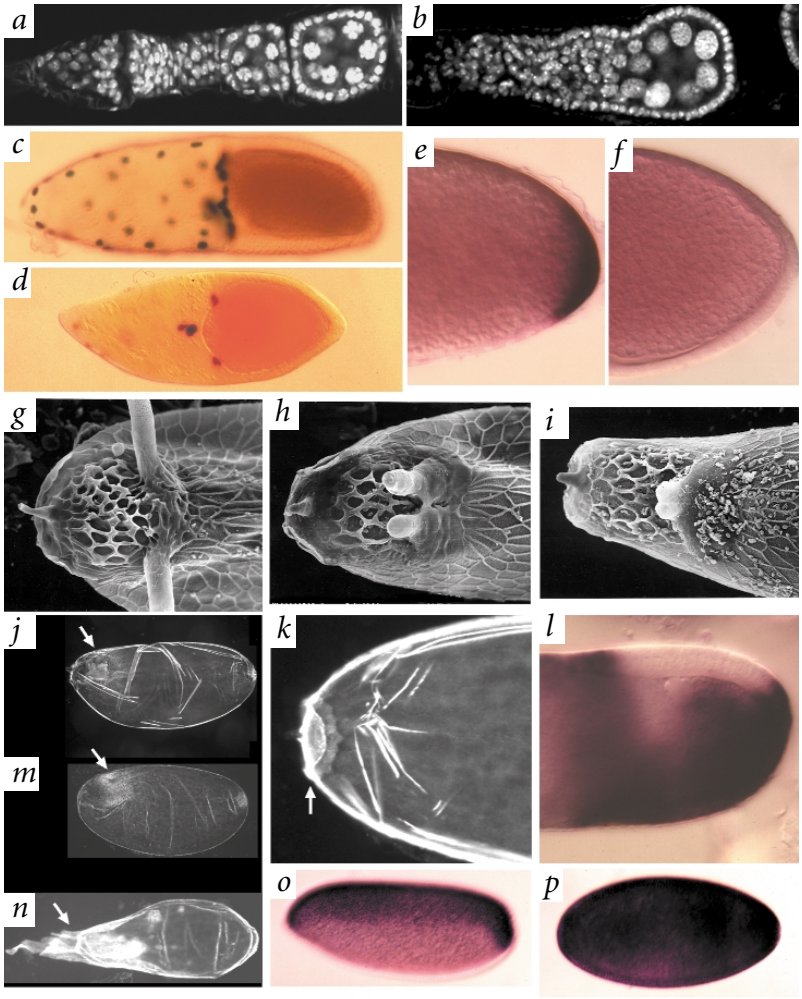

Fig. 2 *mirr* function is required for encapsulation in the germarium, differentiation of terminal follicle cells at stage 6 and establishment of dorsal-ventral polarity at stages 8–10. *a*,*b*, Sixteen-cell germline cysts fail to separate from germaria defective for *mirr* function. Wild-type (***a***) and $mirr^{l(3)6D1}/mirr^{e48}$ (***b***) germaria (20% of the germaria) are shown. *c*–*f*, Overexpression of MIRR results in loss of termini. Wild-type expression patterns of an anterior marker, L53B, in the anterior follicle cells (***c***) and a posterior marker, pnt^{P1}, in the posterior follicle cells (***e***). In *UAS-mirr/+; 109(3)9 Gal4/+*, both anterior (***d***) and posterior (***f***) markers are lost or reduced in all of the egg chambers. *g*–*m*, Loss of MIRR or Notch function ventralizes the eggshell. Operculum and dorsal appendages of wild type (***g***) and $mirr^{l(3)6D1}/mirr^{e48}$ (***h***, 68%; ***i***, 9%; ***j***, 23%) are shown. In wild-type, stage-14 egg chambers the operculum has 43±5.2 cell imprints, whereas in $mirr^{l(3)6D1}/mirr^{e48}$ there are only 30±2.6 imprints. Mosaics for $mirr^{l(3)6D1}$ result in ventralization (arrow indicates the collar around the anterior circumference) of the egg chamber (***k***). The normal ventral *pip* expression is expanded toward the dorsal-lateral follicle cells in large, directed MIRR ($mirr^{l(3)6D1}$) mosaics (***l***). Smaller (18–20 cells) marked mosaics also showed abnormalities in *pip* expression. Of the eggs laid by N^{ts} females after 14 h at the restrictive temperature, 80% are defective in the dorsal anterior region. The strongest phenotype is a complete loss of dorsal appendages (***m***, arrow) comparable to the phenotype detected with *mirr*-mutant females (*j*, arrow). *n*–*p*, Overexpression of *mirr* in follicle cells dorsalizes the eggshell (***n***) and the embryo (***p***). Embryonic expression of *dpp* in wild-type (***o***) is shown. Overexpression of MIRR during oogenesis using a *hs-mirr* construct results in an expansion of the embryonic *dpp* pattern (*p*, 75%). Morphological evidence indicates that overexpression of *mirr* in follicle cells using the GAL4/UAS system can also result in dorsalization of the embryo (data not shown). Ectopic expression of *UAS–mirr* using 8.2 Gal4 results in expansion of dorsal appendages (*n*, arrow; 100%). A similar phenotype occurs 18–23 h after heat shocking *hs-mirr/+* adult fllies. Thus, these egg chambers were a stage 9 at the time of the heat shock.

The somatic follicle cells of the *Drosophila* egg chamber provide a simple system to study patterning of an epithelial cell layer. These cells derive from stem cells located in the most anterior structures of the ovary[3] (Fig. 1*a*). Most of the follicle cells are not lineage restricted, but differentiate by inductive cell-cell communication events. Signals from subgroups of follicle cells have roles in such processes as control of germline division and establishment of the anterior-posterior and dorsal-ventral axes of the egg, and ultimately the embryo[4,5].

We found that in oogenesis the expression patterns of *mirr* and *fng* are complementary. The expression patterns define borders between cells with specific developmental roles: the encapsulation of 16-cell germline cysts which culminates in their separation from the germarium; the boundary between terminal and lateral follicle cells at stage 6; and the boundary between dorsal anterior and all other follicle cells at stages 8–10 (Fig. 1). In the germarium, *mirr* is expressed in the inner sheath cells and the anterior-most follicle cells, whereas *fng* is expressed in the follicle cells in the posterior part of the germarium (Fig. 1*b*,*e*). The follicle cells at the expression boundary encapsulate the 16-cell germline cysts and subsequently separate the newly formed egg chamber from the germarium. At stage 6, when the follicle cells in the termini of the egg chamber differentiate from the lateral follicle cells[7,8] and establish the oocyte anterior-posterior (A-P) polarity[9–11], *mirr* expression was detected in the lateral region, complementary to *fng* expression in the termini (Fig. 1*c*,*f*). As the follicle cells migrate posteriorly at stages 8 and 9, we detected *mirr* expression in the most dorsal anterior follicle cells (Fig. 1*d*,*j*), whereas *fng* was expressed in all other follicle cells (Fig. 1*g*,*j*). At this point, signalling between the oocyte and the follicle cells establishes the dorsal-ventral axis of the follicle-cell layer and the future embryo[4]. The complementary expression patterns of *mirr* and *fng* throughout oogenesis are likely to be a consequence of MIRR repression of *fng* expression. Follicle-cell clones made with a loss-of-function allele of *mirr* result in an expansion of the *fng* expression pattern into dorsal anterior follicle cells (Fig. 1*k*), and, conversely, overexpression of *mirr* results in the loss of *fng* expression (Figs 1*i* and 3*e*,*f*; and data not shown).

We investigated the role of MIRR by examining egg chambers from females with altered MIRR function. In *mirr*-mutant females, defects in encapsulation of the 16-cell cyst and separation from the germarium were observed (Figs 2*b* and 4*g*). At stage 6 *mirr* expression was excluded from the follicle cells at the termini of the egg chamber (Fig. 1*c*), and ectopic expression of *mirr* throughout the follicle cells beginning at this stage reduces the size of the expression domain of anterior (Fig. 2*c*,*d*, *dpp* and L53B, and data not shown) and posterior terminal markers (Fig. 2*e*,*f*, pnt^{P1}). Thus, *mirr* expression must be excluded from the termini for proper differentiation of those regions. Ectopic expression of MIRR also perturbs the oocyte anterior-posterior axis (data not shown), consistent with the function of posterior terminal follicle cells in establishment of this axis[7–11]. Later in oogenesis, when dorsal and ventral follicle cells are differentiating, *mirr* is expressed in dorsal anterior follicle cells (Fig. 1*d*) and reduction or loss of dorsal structures is evident in *mirr* loss-of-function egg chambers (Fig. 2*g*–*k*). Loss of *mirr* function results

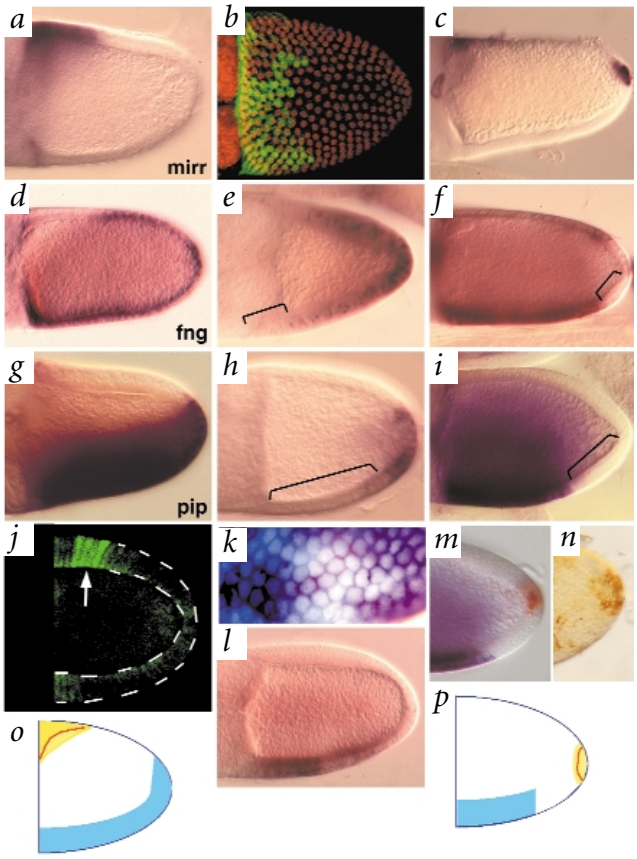

Fig. 3 MIRR and constitutively active Notch affect *pip* expression at a distance. In wild type at stage 10, *mirr* is normally expressed in the dorsal anterior follicle cells (***a***,***o***, yellow), *fng* is expressed in the rest of the follicle cells (***d***), *pip* is expressed in the the ventral and posterior follicle cells (***g***,***o***, blue), and *rho* is expressed as two stripes, one on each side of the dorsal midline[18] (*o*, red). Localized Notch activation, assayed in ovaries from fllies of the genotype*hs-N*$^{+}$*GV3/+*; *UAS-GFP/+*, is also seen in the dorsal anterior follicle cells (***j***, green is GFP). We used 55B Gal4 to express *UAS–mirr* in the anterior follicle cells (***b***, green is GFP, red is PI). Whereas *fng* is repressed in only the anterior-most follicle cells (***e***), *pip* is repressed in a larger area in 73% of the egg chambers (***h***, bracket). Double *in situ* hybridization revealed that ectopic expression of *mirr* (blue) causes repression of *pip* (purple) at a distance approximately fiiv cell diameters from the source of *mirr* expression (***k***). We used A62 Gal4 to overexpress *UAS–mirr* (***c***,***f***,***i***,***m***,***n***,***p***) or *UAS*–constitutively active Notch (***l***) in the posterior follicle cells. Ectopic expression of MIRR in the posterior follicle cells (*c*, purple; *m*, orange; *p*, yellow) represses *fng* (*f*, diameter 5 cells, bracket), represses *pip* at a distance (*i*, diameter 10 cells, bracket; *m*, blue; *p*, blue), induces a ring of *rho* expression (*n*,*p*, red) and causes defects in the patterning of posterior embryonic structures. Posterior expression of constitutively active Notch also causes repression of *pip* at a distance (*l*).

in expansion of the ventral expression of *pip* (refs 12,13), a gene required for embryonic dorsal-ventral axis formation, to the lateral and dorsal follicle cells in stages 9 and 10 (Fig. 2*l*). In contrast, ectopic expression of *mirr* in the ventral follicle cells at this stage causes dorsalization of the eggshell and embryo (Fig. 2*n–p*). Thus MIRR is required for proper dorsal-ventral axis formation and *mirr* expression must be restricted to the dorsal region for correct ventral patterning to occur. In summary, either loss of MIRR function or ectopic expression of MIRR disrupts follicle-cell differentiation at the three stages of oogenesis in which a MIRR-FNG expression border is observed.

In a number of developmental systems, regulation of *fng* by a homeobox gene has a role in establishing a domain in which Notch is activated[1,2]. We thus compared the phenotypes observed in *mirr* and *Notch* (*N*) mutants during oogenesis. In oogenesis, Notch activity is required in the germarium[8,9,14–16] and for the formation of the termini at stage 6 (refs 7,8) as described above for MIRR. We tested whether Notch function is also required for dorsal-ventral patterning of follicle cells by analysing the eggs laid by N^{ts} females at the restrictive temperature. The strongest phenotype observed in eggs laid by N^{ts} females is similar to that observed in eggs laid by *mirr* loss-of-function females: a complete loss of the dorsal appendages (Fig. 2*j*,*m*). In addition, the ventral *pip* expression domain was defective in N^{ts} females and restricted due to expression of constitutively active Notch (Fig. 3*l*, and data not shown). Thus Notch, like MIRR, functions to restrict *pip* expression to the ventral region and to organize dorsal structures, and loss of either MIRR or Notch function affects follicle cells on both sides of the MIRR-FNG expression border.

Activation of Notch at a *fng* expression border has been seen in wing and eye development. In the wing this border acts as an organizing centre by producing a morphogen, Wingless, which acts on cells on both sides of the border[17]. At stage 9 in oogenesis

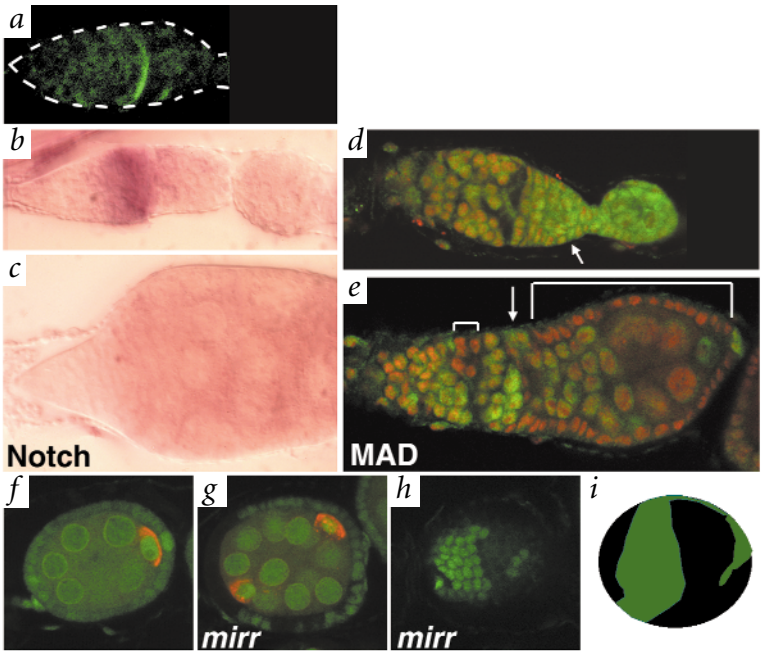

Fig. 4 *dpp* is expressed in the germarium near a region of Notch activity in a Notch-dependent manner. The TGF-β pathway and MIRR are required in the follicle cells for the encapsulation process. Wild type (*b*,*d*,*f*), N^{ts} (*c*), *mad12* clones (e) and *mirrl(3)8A5* clones (*g*,*h*,*i*) are shown. ***d***,***e***, Red is nuclear PI, green is the cytoplasmic arm of β-gal; arrows show the overlay of these two colours in wild-type follicle cells. *f*,*g*,*h*,*i*, Red is ORB, green is GFP. We found that *dpp* is expressed in the follicle cells in the mid-germarium (***b***) near a stripe of Notch activity (***a***, green is GFP, ovaries from fllies of genotype*hs-N*$^{+}$*GV3/+*; *UAS-GFP/+*) in a Notch-dependent manner (***c***, no *dpp* expression in N^{ts} ovaries at restrictive temperature). Furthermore, in follicle-cell clones of MAD (***e***, *HSFlp; mad*^{12} *FRT40A/armadillo-lacZ FRT40A*, clones lack the cytoplasmic arm of β-gal, large bracket shows a large follicle-cell clone) or MEDEA, encapsulation defects of 16-cell cysts were observed (31/31 and 6/6, respectively). Follicle-cell clones of MIRR (***g***,***h***, *HSFlp; ubiqGFP FRT79/ mirr*$^{(3)8A5}$ *FRT79*, clones lack GFP) also show encapsulation defects resulting in compound egg chambers with 32 germline cells and two oocytes (red, ORB). ***i***, Depiction of the egg chamber in (***f***) and (*g*) shows a large clone in follicle cells (black, bottom of the circle) that failed to migrate and separate the two egg chambers from each other.

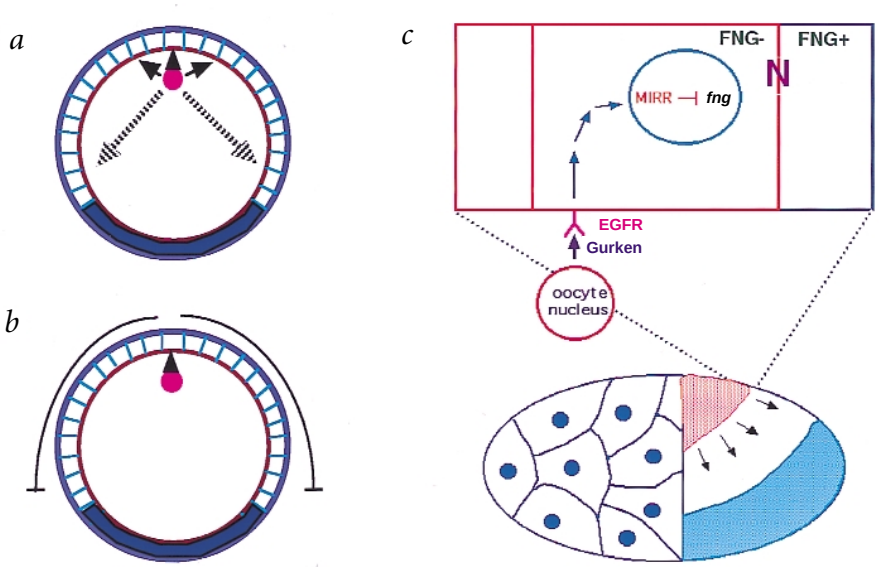

Fig. 5 Model for MIRR function at stage 9 in oogenesis. Our data show that expression of *pip* in the ventral follicle cells is not a direct consequence of a graded germline signal by Gurken (***a***), but depends on MIRR-dependent long-range signals from dorsal follicle cells (***b***). ***c***, *mirr* expression is induced by the EGFR pathway. MIRR then represses expression of FNG, a repressor of Notch activity, thereby activating Notch and inducing expression of secreted molecules that repress *pip* expression.

the *mirr-fng* expression border (Fig. 3*a*,*d*,*o*) and a region of localized Notch activation (Fig. 3*j*) are approximately 10 cell diameters from the ventral *pip* expression border (Fig. 3*g*). Nevertheless, reduction of *mirr* expression expands the *pip* domain laterally (Fig. 2*l*). If a MIRR-FNG border activates Notch locally to produce a morphogen that represses *pip*, a reduction of *pip* expression should be seen upon expansion of the *mirr* expression domain or ectopic activation of Notch. To examine this, we expressed *mirr* ectopically in anterior follicle cells (Fig. 3*b*,*e*,*h*,*k*). *pip* repression occurred 5–7 cell diameters beyond the *mirr* expression domain (Fig. 3*k*), showing that the effect of MIRR on *pip* is non-cell autonomous and supporting the idea that a MIRR-FNG border generates a *pip*-repressing agent. To further test the effect of ectopic MIRR expression, we expressed MIRR in posterior follicle and tested the effect on *pip* and *rho*, which is normally expressed as two stripes on the dorsal region at stage 10 (Fig. 3*o*; ref. 18). Such ectopic *mirr* expression induces a ring of *rho* expression and represses *pip* at a distance (Fig. 3*i*,*m*,*n*,*p*). Expression of constitutively active Notch in posterior follicle cells also repressed *pip* expression at a distance (Fig. 3*l*). These results suggest that MIRR and Notch induce secretion of a diffusible molecule which represses *pip*. Although we do not know what the Notch-dependent diffusible molecule is at stage 9, we found *dpp* (encoding TGF-β; ref. 19) to be expressed in follicle cells in the mid-germarium (Fig. 4*b*) near a stripe of cells showing localized Notch activity (Fig. 4*a*) in a Notch-dependent manner (Fig. 4*c*). Furthermore, in follicle cell clones of MAD or MEDEA (downstream effectors of the TGF-β pathway[19]), we saw encapsulation defects of 16-cell cysts (Fig. 4*e*, and data not shown). This phenotype is similar to *Notch*- and *mirr*-mutant phenotypes in germarium (Fig. 4*c*,*g*–*i* and 2*b*), suggesting that TGF-β may be a morphogen induced by Notch activity in the germarium.

Local activation of Notch in a number of developmental systems is achieved by spatially restricted expression of a homeodomain protein that either represses or induces *fng* expression, generating a border of *fng* expressing and non-expressing cells. It is less clear how the initial asymmetric expression of the homeobox protein is generated. Because the dorsal anterior expression of *mirr* is characteristic of a number of genes regulated by the EGFR pathway[4], we analysed *mirr* expression in mutants that lack Gurken, one of the ligands for EGFR. In these egg chambers, the dorsal anterior pattern of *mirr* expression was reduced or lost, showing that activation of the EGFR pathway is necessary for *mirr* expression (Fig. 1*h*). The patterns of oogenesis in the germaria at stage 6 and in the centripetally migrating cells were unaltered, however, indicating that either another EGFR ligand[20] or another pathway regulates *mirr* expression at these stages (Fig. 1*h*, and data not shown).

Results from several developmental systems have led to the idea that the trio of a homeobox gene, FNG and Notch are fundamental to organogenesis[1,2]. We suggest, based on our results, that MIRR, FNG and Notch are part of a conserved mechanism for dividing epithelial cell layers into domains that is not restricted to organogenesis. Furthermore, our data suggest that MIRR integrates the EGFR and Notch pathways in oogenesis: *mirr* transcription is induced by the EGFR pathway, and MIRR in turn spatially regulates *fng* expression leading to a Notch activation border (Fig. 5). Finally, we propose that the link between EGFR pathway signalling in the dorsal follicle cells and the differentiation of the ventral follicle cells suggested by genetic studies[4] is mediated by MIRR. The EGFR pathway induces *mirr* expression, which leads to creation of a Notch-FNG border in lateral follicle cells from which molecules are secreted that repress *pip* expression. PIP regulates the activity of a protease cascade that activates Toll and ultimately determines the dorsal-ventral pattern of the *Drosophila* embryo[13]. Our data show that expression of *pip* in the ventral follicle cells is not a direct consequence of a graded germline signal by Gurken (Fig. 5*a*), but depends on MIRR-dependent long-range signals from dorsal follicle cells (Fig. 5*b*,*c*). MIRR therefore connects the well-studied events in early and late *Drosophila* dorsal-ventral axis formation.

Methods

Fly stocks and genetic crosses. We used *mirr* alleles $mirr^{l(3)6D1}$ or $mirr^{(3)8A5}$ (ref. 18) and $mirr^{e48}$ (ref. 6). Defects similar to those shown in Fig. 2*h*,*i* were also seen with $mirr^{p69Df7}$ and $mirr^{B1\text{-}12}$ (refs 6,21,22) and with homozygous $mirr^{P2}$ (ref. 6) alleles. We used the following fly stocks: grk^{DC29} and grk^{2B6} (ref. 23), *109(3)9 Gal4* (ref. 8), *55B Gal4* (ref. 24), *A62 Gal4* (ref. 25), *8.2 Gal4*, *UAS-mirr* (ref. 6), *UAS-caN* (ref. 14), mad^{12}, Med^{26}, *HSFlp;FRT40A armadillo-lacZ, HSFlp;FRT82B armadillo-lacZ* (ref. 5), *UAS-GFP* (from Bloomington Stock Center), *HSFlp; ubiqGFP FRT79*, $mirr^{l(3)8A5}$ *FRT79*, $hs\text{-}N^{+}GV3/Cyo$ (ref. 26). *109(3) Gal4* and *8.2 Gal4* are expressed throughout the follicle cells beginning at stage 6. *E22c Gal4* is expressed in region 2 of the germarium[27]. L53b was used to mark anterior follicle cells. We used the Gal4/UAS system[24] or *hs-mirror/Cyo* (ref. 6) to overexpress *mirr*. Well-yeasted *hs-mirr*/+ 3–5-day-old adult flies were heat-shocked at 39 °C for 15 min and then allowed to lay eggs on apple-juice agar plates. We collected

eggs at 5–6 h stages after heat shock and immediately fixed the developing embryos for *in situ* hybridization. N^{ts} adult females were placed at the restrictive temperature 29 °C and the eggs were collected on apple-juice agar plates or ovaries dissected for *in situ* hybridization. We analysed phenotypes from flies that had been at the restrictive temperature for less than 14 h. Unless noted, >100 egg chambers were analysed in all experiments.

We generated clones of mutant cells by HSFlp-mediated mitotic recombination as described[28]. The clones were induced by 2-h heat shocks on 2 consecutive days of third instar larvae. Ovaries were dissected 5 d after eclosion and fixed for antibody staining. Directed mosaics were performed using $FRT^{3L\text{-}2A}$ (from Bloomington Stock Center) and *e22c Gal4, UAS-FLP/Cyo* (ref. 27). Adult flies of the genotype *e22c Gal4, UAS-FLP/+; mirr* $^{ll(3)6D1}$ $FRT^{3L\text{-}2A}/FRT^{3L\text{-}2A}$ were allowed to lay eggs on apple-juice agar plates for eggshell analysis. Ovaries from these flies were also dissected in PBS and fixed for *in situ* hybridization. The strongest ventralized phenotype appeared after 6 d (mosaic frequency 30%). To visualize the localization of Notch activity, we heat-shocked adult flies of the genotype, *hs-*N^{+}*GV3/+; UAS-GFP/+* (ref. 26) 3 times at 37 °C for 1 h over a 24-h period. After the last heat shock the flies recovered for 2 h before being dissected in PBS, fixed for 20 min and mounted as described below for confocal microscopy.

***In situ* hybridization.** We carried out X-gal staining and whole-mount *in situ* hybridization with digoxigenin-labelled DNA or RNA probes as described[8,25]. RNA probes were made from *mirr* cDNA (ref. 6), *fng* cDNA and *pip* cDNA (ref. 13). DNA probes were made from pnt^{P1} cDNA (ref. 28) and *dpp* cDNA (ref. 8). The *dpp* mRNA pattern in germarium was only detectable with a RNA probe. We performed double labelling for whole-mount *in situ* hybridization as described[29]. Ovaries were mounted in 70% glycerol.

Confocal and electron microscopy. We carried out antibody staining as described[14]. The following antisera were used: polyclonal anti-β-galactosidase (1:1,500, Cappel), anti-MIRR (1:1,000), anti-ORB (1:2, Developmental Studies Hybridoma Bank, Orb 6H4) and Alexa 488 conjugated anti-rabbit secondary (1:500, Molecular Probes). For staining nuclei, ovaries were fixed, rinsed 3× each in PBS and PBT (PBS+0.1% Triton X-100), then incubated in PBT with RNase A (20 μg/ml; 37 °C, 1 h). Samples were incubated in OliGreen (1:200, Molecular Probes) for 45 min or propidium iodine (PI) for 10 min (ref. 16), washed 2×15 min in PBT, then mounted in 70% glycerol/1×PBS/2% n-propyl gallate. We collected optical sections on an MRC 600 laser scanning confocal microscope (Biorad Microsciences Division).

We prepared eggshells for scanning electron microscopy as described[30], except that before immersion in Tetramethylsilane, eggs were immersed in absolute ethanol and acetone for 10 min each.

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