Development 139, 1110-1114 (2012) doi:10.1242/dev.076562

Mirror represses *pipe* expression in follicle cells to initiate dorsoventral axis formation in *Drosophila*

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SUMMARY

Dorsoventral (DV) axis formation in *Drosophila* begins with selective activation of EGFR, a receptor tyrosine kinase (RTK), in dorsal-anterior (DA) ovarian follicle cells. A critical event regulated by EGFR signaling is the repression of the sulfotransferase-encoding gene *pipe* in dorsal follicle cells, but how this occurs remains unclear. Here we show that Mirror (Mirr), a homeodomain transcription factor induced by EGFR signaling in DA follicle cells, directly represses *pipe* expression by binding to a conserved element in the *pipe* regulatory region. In addition, we find that the HMG-box protein Capicua (Cic) supports *pipe* expression in ventral follicle cells by repressing Mirr in this region. Interestingly, this role of Cic resembles its function in regulating anteroposterior (AP) body patterning, where Cic supports gap gene expression in central regions of the embryo by repressing Tailless, a repressor induced by RTK signaling at the embryonic poles. Thus, related RTK-Cic repressor circuits regulate the early stages of *Drosophila* DV and AP body axis formation.

KEY WORDS: Dorsoventral patterning, EGFR signaling, *pipe*, Mirror, Capicua, *Drosophila*

INTRODUCTION

Dorsoventral (DV) embryonic patterning in *Drosophila* depends on inductive signals generated during oogenesis that are transmitted to the fertilized embryo (Nilson and Schüpbach, 1999; Moussian and Roth, 2005). During mid-oogenesis, localization of the oocyte nucleus in a dorsal-anterior (DA) position produces a local source of the Gurken (Grk)/TGFα-like secreted factor, which activates EGFR signaling in the overlying DA follicle cells. EGFR then signals via the Ras/Raf/MAPK cassette and represses transcription of the *pipe* gene, thereby restricting its expression to ventral follicle cells (Sen et al., 1998). *pipe* encodes a sulfotransferase that modifies structural components of the eggshell, a process required for subsequent signaling events that activate the Toll receptor on the ventral surface of the embryo (Sen et al., 1998; Moussian and Roth, 2005; Zhang et al., 2009).

The mechanism by which EGFR signaling represses *pipe* expression remains unclear. An important feature is that detectable EGFR signaling activity and *pipe* expression are not precisely complementary: at stage 10A of oogenesis, active phospho-MAPK and known EGFR targets are restricted to the dorsal ~30% of the follicular epithelium, whereas *pipe* expression spans the ventral ~40% region (Sen et al., 1998; Ghiglione et al., 1999; Peri et al., 1999; Reich et al., 1999; Jordan et al., 2000; Zhao et al., 2000). Accordingly, initial analyses suggested that *pipe* repression is not a direct consequence of EGFR signaling, but depends on long-range signals mediated by the homeodomain factor Mirror (Mirr) and the Notch-Fringe pathway (Jordan et al., 2000). However, subsequent studies indicated that Grk/EGFR signaling forms a gradient that reaches ventral positions to directly control *pipe* expression (Pai et al., 2000; Goentoro et al., 2006). In agreement with this, loss of EGFR/Ras/MAPK activity causes cell-autonomous derepression of *pipe* in all lateral and dorsal positions (James et al., 2002; Peri et al., 2002). Furthermore, both Mirr and Notch-Fringe signals were found to be dispensable for *pipe* repression, arguing that this repression might depend on unknown factors (Peri et al., 2002).

Here, we report that Mirror, which is induced by EGFR signaling in DA cells (Jordan et al., 2000; Zhao et al., 2000), directly represses *pipe* expression by binding to a conserved element in its regulatory region. In addition, we find that the HMG-box protein Capicua (Cic) supports *pipe* expression in ventral follicle cells by repressing *mirr* in this region. Together, our results delineate a repressor circuit downstream of EGFR signaling that is essential for asymmetric *pipe* transcription in the follicle cell layer.

MATERIALS AND METHODS

Drosophila strains and genetic analyses

The following stocks were used: $mirr^{1825}$ (Collins and Cohen, 2005), $fs(1)K10^{1}$ (Wieschaus et al., 1978), *pipe-lacZ* (Sen et al., 1998), *UAS-mirr* (McNeill et al., 1997), cic^{fetE11}, cic^{fetU6} (Goff et al., 2001), *FRT82B* cic^{Q474X} (Tseng et al., 2007) and *FRT82B* $Ras^{\Delta C40b}$ (James et al., 2002). iro^{EGP} deficiencies were generated using FRT-bearing transposon insertions from Exelixis (Thibault et al., 2004; Parks et al., 2004). Mosaic analyses were performed by FLP-FRT-mediated mitotic recombination (Xu and Rubin, 1993). Ectopic *mirr*-expressing clones were generated using the GAL4/UAS flip-out technique and an *hs-flp*; *UAS-CD8-GFP*; *tub>*y^{+}*Gal80>Gal4* line, resulting in GFP-labeled clones. Transgenic lines were established by standard P-element transformation.

Immunohistochemistry and in situ hybridization

The following primary antibodies were used: mouse anti-β-galactosidase (40-1a, 1:200 dilution) and mouse anti-Grk (1D12, 1:50) (Developmental Studies Hybridoma Bank). Mirr expression was visualized using a rat polyclonal antibody that recognizes all three Iro proteins (our unpublished data). Signals were detected using fluorochrome-conjugated secondary antibodies and DAPI. Whole-mount in situ hybridization was performed using digoxygenin-labeled RNA probes.

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Accepted 6 January 2012

DNA constructs

pipe enhancer fragments were amplified by PCR using the following primers: *m2-f* (5′-AGCTACTCACTTTCTTGGTAC-3′) and *m2-r* (5′-AGCCAAACTTCGGGATCTAAG-3′) to amplify module *M2*; and *m3-f* (5′-CTACTAAAAGTATTCACTCTAG-3′) and *m3-r* (5′-ATTTCAGC-ATTTGGTACCAAG-3′) for *M3*. *M1* was generated by ligating *M2* and *M3* fragments through their common *Kpn*I site. Reporter constructs were assembled in *pC4PLZ* (Wharton and Crews, 1993). $M1^{r1mut}$ and $M2^{r1mut}$ were made by replacing the 39 bp *r1* sequence with a *pC4PLZ* polylinker fragment (*Bam*HI-*Not*I) of the same size.

In vitro DNA binding assays

Recombinant Mirr protein was synthesized by coupled transcription-translation (TNT, Promega). EMSA experiments were performed by incubating 0.5-2 μl TNT reactions with ~50 ng ^{32}P-end-labeled probes, 1 μg BSA and 1 μg poly(dI-dC) in a final volume of 20 μl supplemented with binding buffer (60 mM Hepes pH 7.9, 20 mM Tris-HCl pH 7.9, 300 mM KCl, 5 mM EDTA, 5 mM DTT, 12% glycerol). Complexes were resolved on 5% non-denaturing polyacrylamide gels run in 0.5× TBE at 4°C, and visualized by autoradiography. The control (c) probe in Fig. 2I is 5′-GAGTTTGGAGAAAAACACGTGTTAAGCT-3′ (Mirr binding site is underlined) (Bilioni et al., 2005).

RESULTS AND DISCUSSION

Mirr mediates cell-autonomous repression of *pipe* in dorsal follicle cells

Although previous clonal analyses indicated that Mirr is not involved in *pipe* regulation (Peri et al., 2002), we reasoned that Mirr function in this context could be masked by redundant activities of the two other components of the *iroquois* complex (Iro-C), *araucan* (*ara*) and *caupolican* (*caup*), which encode homeodomain proteins highly related to Mirr (Gómez-Skarmeta et al., 1996; McNeill et al., 1997). Therefore, we generated follicle cell clones mutant for a deficiency, iro^{EGP7}, that removes the whole of Iro-C (Fig. 1A). Strikingly, iro^{EGP7} clones showed full, cell-autonomous derepression of *pipe-lacZ* expression at all positions where *pipe* is normally off (Fig. 1B). We then used two additional deficiencies, iro^{EGP5} and iro^{EGP6}, that specifically abolish *mirr* and *ara/caup* function, respectively (Fig. 1A). These analyses revealed a requirement for Mirr, but not Ara or Caup, in *pipe* repression: only iro^{EGP5} caused ectopic *pipe* expression equivalent to that seen with iro^{EGP7} (Fig. 1C,D and supplementary material Fig. S1). In addition, we found that *mirr* is the only member of Iro-C that is detectably expressed in follicle cells (Fig. 1E-G).

Thus, Mirr is essential for repression of *pipe* in dorsal follicle cells. Based on further analyses (supplementary material Fig. S1), it is possible that previous negative results were caused by residual Mirr activity of the $mirr^{e48}$ allele (Peri et al., 2002). In fact, *pipe* is highly sensitive to Mirr repression, as this repression occurs even in cells abutting the normal *pipe* domain where Mirr levels are very low or undetectable (Jordan et al., 2000; Zhao et al., 2000) (Fig. 1B and supplementary material Fig. S1). Furthermore, this is consistent with evidence that low EGFR/Ras/MAPK levels mediate *pipe* repression in lateral and dorsal-posterior cells close to its border of expression (Pai et al., 2000; Peri et al., 2002; James et al., 2002) (Fig. 3B).

Mirr binds to a conserved element in the *pipe* regulatory region

To further study *pipe* regulation, we identified two highly conserved sequence motifs in its 5′ flanking region (designated *r1* and *r2*; Fig. 2A). Using different *lacZ* reporters, we defined a minimal *pipe* enhancer module, *M2*, which includes the *r1* element and recapitulates endogenous *pipe* expression (Fig. 2A-D). As expected, *M2-lacZ* responded to EGFR regulation, as indicated by the loss of ventral-anterior expression in *fs(1)K10* mutant ovaries, which display ectopic EGFR activity (Wieschaus et al., 1978; Neuman-Silberberg and Schüpbach, 1993) (Fig. 2E). In addition, we found that clonal ectopic expression of Mirr repressed *M2-lacZ* in a cell-autonomous manner (Fig. 2H), consistent with EGFR signaling mediating *pipe* repression via Mirr.

We then found that *pipe* regulation crucially depends on the *r1* sequence, as mutation of this element resulted in dorsal derepression of reporter constructs ($M1^{r1mut}$-*lacZ* and $M2^{r1mut}$-*lacZ*, Fig. 2F,G). These mutant constructs, particularly $M2^{r1mut}$-*lacZ*, exhibit weaker and more variable expression than *M2-lacZ* throughout the epithelium, raising the possibility that the *r1* element includes sequences required for both repression in DA cells and full activation in ventral cells. Based on these results, we postulated that Mirr might repress *pipe* expression by binding to the *r1* element. EMSA experiments (Fig. 2I) showed that recombinant Mirr protein binds strongly to the *r1* sequence, but not to the mutated *r1mut* sequence that caused dorsal derepression in vivo. Using a set of mutations along the *r1* sequence (*mut A-D*), we then found that Mirr preferentially binds to a site containing the sequence ACACGA (Fig. 2I), which resembles the ACAnnTGT motif defined as a minimal Mirr binding site in vitro (Bilioni et al., 2005).

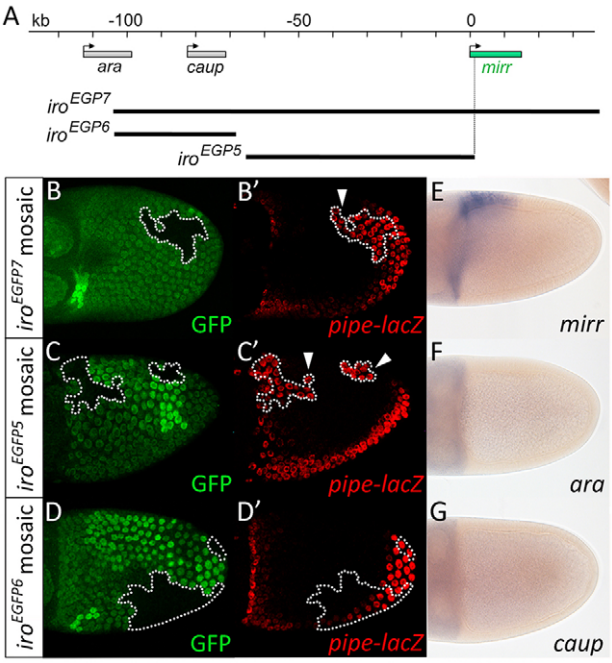

Fig. 1. Mirr represses *pipe* expression in dorsal follicle cells. (**A**) The Iro complex. Horizontal lines indicate the regions deleted in three iro^{EGP} deficiencies. iro^{EGP5} removes the first *mirr* exon and adjacent upstream sequences. (**B-D′**) Stage 10 mosaic egg chambers carrying iro^{EGP7} (B), iro^{EGP5} (C) and iro^{EGP6} (D) clones marked by absence of GFP (green, outlined); B′-D′ show *pipe-lacZ* expression (red). Dorsolateral (B,D) or lateral (C) views are shown. Arrowheads indicate ectopic *pipe-lacZ* expression. (**E-G**) Stage 10 egg chambers stained for *mirr* (E), *ara* (F) and *caup* (G) mRNA expression.

Cic supports *pipe* expression by repressing Mirr

Previous studies showed that Cic is required for *pipe* expression in ventral follicle cells; accordingly, embryos derived from *cic* mutant females are severely dorsalized (Goff et al., 2001). However, how

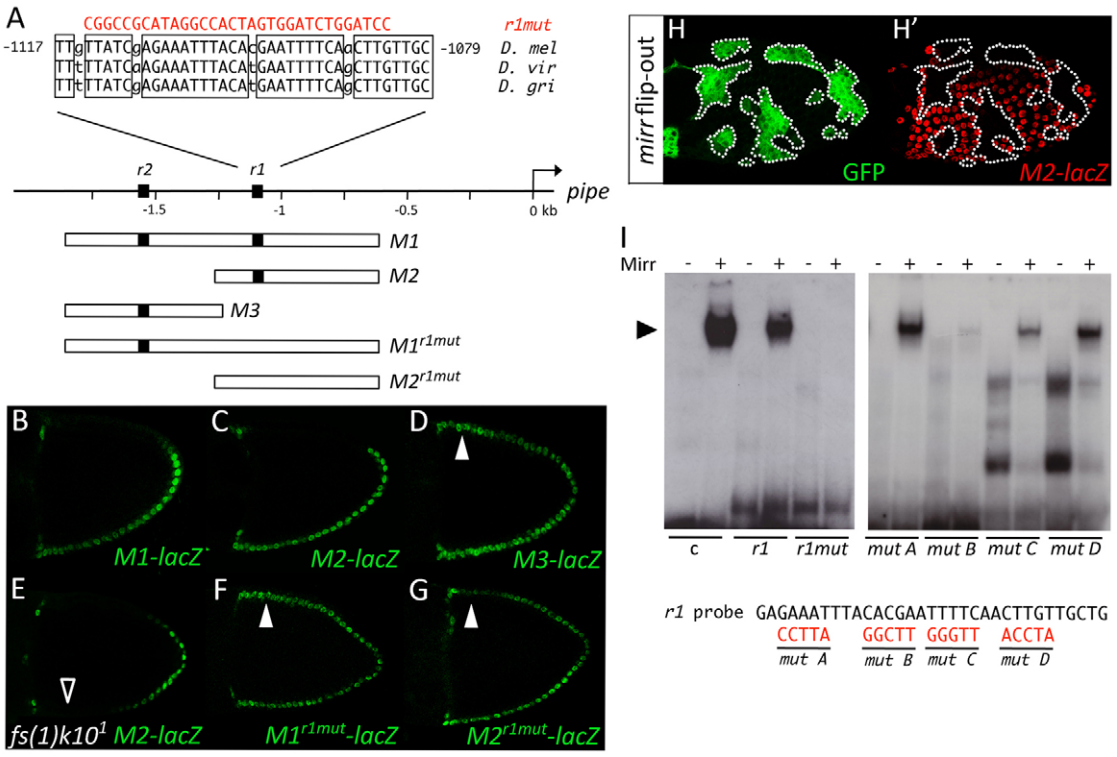

Fig. 2. Mirr binds to a conserved regulatory element in *pipe*. (**A**) The *pipe* upstream region showing *r1* sequences from *D. melanogaster*, *D. virilis* and *D. grimshawi* above; the *r1mut* sequence is also indicated. DNA fragments analyzed for enhancer activity are depicted beneath. (**B-G**) Lateral views of transgenic stage 10 egg chambers stained for *lacZ* reporter expression. Solid arrowheads indicate ectopic expression of constructs lacking the *r1* element. The open arrowhead in E indicates loss of ventral expression in the *fs(1)K10*^{1} background. In all cases, dorsal is up as confirmed by the dorsal position of the oocyte nucleus under Nomarski optics (not shown). (**H**,**H'**) Ventrolateral views of a stage 10 mosaic egg chamber carrying *mirr* overexpression clones marked by expression of GFP (H, green, outlined); H' shows *M2-lacZ* expression (red). (**I**) EMSA analysis of in vitro synthesized Mirr protein binding to different oligonucleotide probes. Lanes labeled (–) contain TNT extract without *mirr* plasmid. The control probe (c) contains the ACAcgTGT sequence (Bilioni et al., 2005). The *r1mut* sequence is indicated in Fig. 2A; *mut A-D* probes contain the changes indicated in red.

Cic affects *pipe* expression is not understood (Goff et al., 2001; Atkey et al., 2006). Since Cic often represses genes induced by RTK signaling pathways (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Atkey et al., 2006; Löhr et al., 2009; Ajuria et al., 2011), we hypothesized that Cic might support *pipe* transcription indirectly by repressing Mirr in ventral cells. Consistent with this idea, loss of *cic* function causes ectopic *mirr* expression, although only in anterior regions of the egg chamber (Goff et al., 2001; Atkey et al., 2006) (supplementary material Fig. S2).

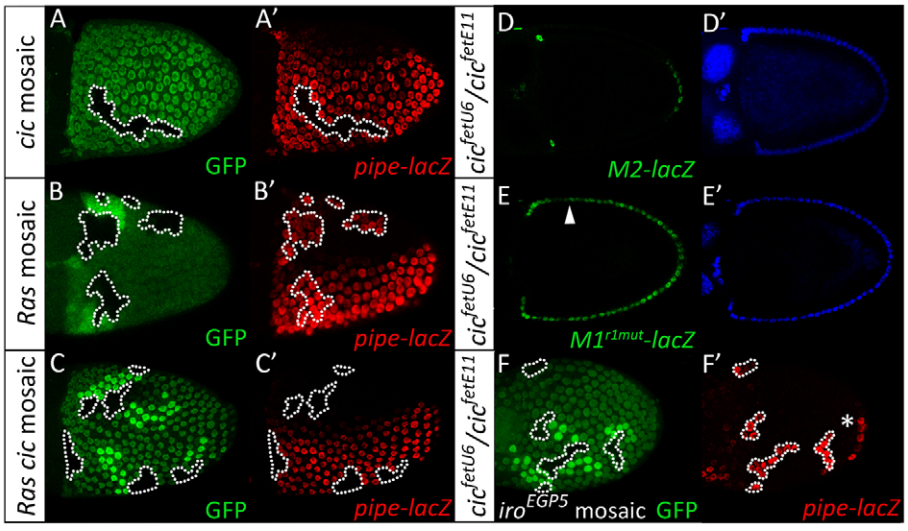

Fig. 3. Cic regulates *pipe* indirectly through the *r1* element. (**A-C'**) Stage 10 mosaic egg chambers carrying *cic*^{Q474X} (A), *Ras*$^{\Delta C40b}$ (B) and *Ras*$^{\Delta C40b}$ *cic*^{Q474X} (C) mutant clones marked by absence of GFP (green, outlined); A'-C' show *pipe-lacZ* expression (red). Ventrolateral (A) or lateral (B,C) views are shown. (**D-E'**) Lateral views of stage 10 *cic*^{fetU6}/*cic*^{fetE11} egg chambers stained for *lacZ* reporter expression; D' and E' show DAPI staining (blue). Dorsal is up (see Fig. 2 legend). (**F**,**F'**) Lateral view of a stage 10 *cic*^{fetU6}/*cic*^{fetE11} egg chamber carrying *iro*^{EGP5} mutant clones marked by absence of GFP (F, green); F' shows *pipe-lacZ* expression (red). The asterisk indicates residual *pipe-lacZ* expression in the *cic*^{fetU6}/*cic*^{fetE11} background.

To further investigate Cic function, we performed mosaic analyses and found that Cic is required cell-autonomously for the expression of *pipe* (Fig. 3A). Since mutations in negative regulators of the EGFR pathway, such as Cbl, cause ventral *pipe* repression by upregulating EGFR signaling (Pai et al., 2000), we tested whether Cic might act similarly. To this end, we performed epistasis analyses using *cic* and *Ras* mutations. Whereas *Ras* clones cause ectopic *pipe* expression in dorsal cells and do not affect *pipe* expression in ventral cells (Fig. 3B) (James et al., 2002), *Ras cic* clones lacked *pipe* expression in all positions (Fig. 3C). This indicates that loss of *pipe* expression in *cic* mutant cells does not result from increased EGFR signaling in those cells.

We also analyzed the effect of *cic Ras* clones on Mirr expression and found that, like *cic* clones, they cause ectopic Mirr expression only in anterior regions (supplementary material Fig. S2). This is consistent with the observation that both *grk cic* and *cic* mutant ovaries exhibit expanded *mirr* expression (Atkey et al., 2006) and supports a model in which *mirr* is normally activated by EGFR signaling and by an independent anterior signal that may depend on Decapentaplegic (Twombly et al., 1996; Deng and Bownes, 1997; Peri and Roth, 2000), with Cic being required to counteract this latter input and repress *mirr* in ventral-anterior follicle cells (Atkey et al., 2006) (see below).

Although these observations are partly consistent with our hypothesis that Cic supports *pipe* expression by repressing Mirr, they raise a paradox: although Cic is required for *pipe* expression in both anterior and posterior follicle cells, loss of *cic* function derepresses *mirr* only in anterior cells. Since Mirr normally represses *pipe* in lateral and dorsal-posterior follicle cells where Mirr is undetectable, we reasoned that loss of Cic function in ventral-posterior cells might induce similar low-level expression of Mirr that is nevertheless sufficient to repress *pipe*. If this is correct, Cic should be dispensable for the expression of mutant *pipe* enhancers that lack the *r1* motif and are insensitive to Mirr. Indeed, whereas *M1-lacZ* and *M2-lacZ* require Cic activity for expression in ventral follicle cells, $M1^{r1mut}$*-lacZ* and $M2^{r1mut}$*-lacZ* do not (Fig. 3D,E; data not shown). This indicates that Cic regulates *pipe* expression in ventral cells by acting indirectly through the Mirr-responsive *r1* element. Furthermore, we have confirmed that Mirr represses *pipe* in the absence of Cic by generating iro^{EGP5} clones in cic^{fetU6}/cic^{fetE11} mutant ovaries that normally lack *pipe* expression (Goff et al., 2001) (Fig. 3D). We find that *pipe* expression is derepressed in all such mutant clones, irrespective of their position (Fig. 3F; data not shown), indicating that ectopic, low-level activity of Mirr accounts for the loss of *pipe* expression in *cic* ovaries (see also Fig. 4B).

Conclusions

Mirr had previously been implicated in the specification of dorsal cell fates during eggshell patterning (Atkey et al., 2006), but it was assumed to be dispensable for establishing embryonic polarity (Peri et al., 2002; Moussian and Roth, 2005; Atkey et al., 2006). Accordingly, it is well accepted that EGFR signaling in the follicle cell layer bifurcates into one branch that regulates DV axis formation and another that controls eggshell patterning, with *pipe* being required only in the first branch and Mirr only in the second. However, our results clearly identify Mirr as a common mediator of EGFR signaling responses in both developmental processes (Fig. 4A).

Our data also show that Cic affects *pipe* expression by repressing *mirr* throughout the *pipe* domain. It should be noted that, although Cic is normally downregulated by EGFR signaling in DA cells (Astigarraga et al., 2007), this downregulation does not play a major role in modulating the effect of Cic on Mirr, nor in inducing Mirr expression in response to EGFR signaling. Indeed, Cic derivatives that are insensitive to EGFR-mediated downregulation do not repress Mirr in DA cells, implying that EGFR signaling induces Mirr by mechanisms that do not require downregulation of Cic (Atkey et al., 2006; Astigarraga et al., 2007) (see above).

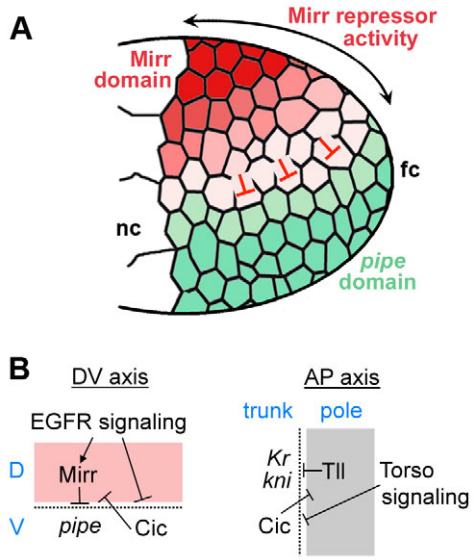

Fig. 4. Roles of Mirr and Cic in *pipe* regulation. (**A**) Mirr-dependent regulation of *pipe* in follicle cells. The Mirr gradient peaks at the dorsal-anterior region (red). Lateral and dorsal-posterior follicle cells (pale red) contain very low levels of Mirr protein that are nevertheless sufficient to repress *pipe*, thereby restricting its expression to ventral cells (green). nc, nurse cells; fc, follicle cells. (**B**) Model of regulatory networks involved in dorsoventral (DV) and anteroposterior (AP) embryonic patterning. Both processes require RTK signals that, together with other inputs, induce expression of repressor factors (Mirr and Tll). Note that Torso signaling induces *tll* expression simply by relieving Cic repression (Astigarraga et al., 2007; Cinnamon et al., 2008). In regions where RTK signaling is low or absent, Cic represses *mirr* and *tll*, thereby supporting the expression of key patterning genes such as *pipe*, *Kr* and *kni*.

Finally, the role of Cic in DV patterning is analogous to its function in the anteroposterior (AP) axis, where it supports the expression of gap genes such as *Kruppel* (*Kr*) and *knirps* (*kni*) in the segmented trunk of the embryo. In this case, Cic supports gap gene expression by repressing Tailless (Tll), a repressor of *Kr* and *kni* induced by Torso RTK signaling at the posterior embryonic pole (Fig. 4B) (Paroush et al., 1997; Jiménez et al., 2000; Goff et al., 2001; Morán and Jiménez, 2006; Löhr et al., 2009; Ajuria et al., 2011). Therefore, similar interactions between Cic and RTK-induced repressors are essential for the early subdivision of both DV and AP embryonic axes.

Note added in proof

Two related papers on *pipe* regulation have appeared while this article was in revision (Technau et al., 2011; Fuchs et al., 2012).

Acknowledgements

We thank A. Olza for *Drosophila* injections; L. Bardia for support with confocal analyses; Z. Paroush, J. Bernués, G. Pyrowolakis, J. Relat and S. Shvartsman for discussions; and A. Casali, J. Botas, G. Darras, I. Hariharan, Y. Kim, E. Martín-Blanco, J. Martínez, H. McNeill, L. Nilson, G. Pyrowolakis, S. Shvartsman, D. Stein and the Bloomington *Drosophila* Research Center for reagents and strains.

Funding
This work was funded by grants from the Spanish Ministry of Science and Innovation (MICINN) [BFU2008-01875/BMC to G.J. and BFU2008-03762/BMC and CONSOLIDER CDS2007-00008 to S.C.]; an institutional grant from Fundación Ramón Areces to the CBMSO; and the Generalitat de Catalunya (2009SGR-1075). G.J. is an ICREA investigator.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076562/-/DC1

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