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 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 AP body patterning.

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)/TGFβ-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: mirr¹¹²¹ (Collins and Cohen, 2005), fs(1)K1¹⁰⁷ (Wieschaus et al., 1978), pipe-lacZ (Sen et al., 1998), UAS-mirr (McNeill et al., 1997), cic¹⁶⁶¹¹ (Goff et al., 2001), FRT82B cicΔ⁷⁴⁷ (Tseng et al., 2007) and FRT82B RaxΔ⁴⁹⁸ (James et al., 2002). iroEGP 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>y¹⁵⁹ 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 digoxigenin-labeled RNA probes.
DNA constructs
pipe enhancer fragments were amplified by PCR using the following primers: m2-f (5’-AGCTACTACCTTTCCGTGAC-3’) and m2-r (5’-AGCGAATTCCTCATGAGAATTG-3’) to amplify module M2; and m3-f (5’-CTCTAAAGTATTCTACTTATG-3’) and m3-r (5’-ATTTTACGTATTGTACCAAG-3’) for M3. M1 was generated by ligating M2 and M3 fragments through their common KpnI site. Reporter constructs were assembled in pC4PLZ (Wharton and Crews, 1993). M1r1mut and M2r1mut were made by replacing the 39 bp r1 sequence with a pC4PLZ polylinker fragment (BamHI-NolI) 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 32P-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’-GAGTTTGGAGAAAAACACGTGT-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, iroEGP7, that removes the whole of Iro-C (Fig. 1A). Strikingly, iroEGP7 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, iroEGP5 and iroEGP6, 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 iroEGP5 caused ectopic pipe expression equivalent to that seen with iroEGP7 (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 mirr45 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).

Mirk 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 (M1r1mut-lacZ and M2r1mut-lacZ, Fig. 2F,G). These mutant constructs, particularly M2r1mut-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 ACAnnTGT motif defined as a minimal Mirr binding site in vitro (Bilioni et al., 2005).

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
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. 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)K101 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.

Fig. 3. Cic regulates pipe indirectly through the r1 element. (A-C) Stage 10 mosaic egg chambers carrying cicfetU6 (A), RasG12D (B) and RasG12D cicfetU6 (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 cicfetE11/cicfetU6 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 cicfetE11/cicfetU6 egg chamber carrying iroG5S mutant clones marked by absence of GFP (F, green); F' shows pipe-lacZ expression (red). The asterisk indicates residual pipe-lacZ expression in the cicfetE11/cicfetU6 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 Bowman, 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, M1r1mut-lacZ and M2r1mut-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 iryCic clones in cig/cig/cig clones 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).

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 (K) 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 K 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; Lühr 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 (Technum et al., 2011; Fuchs et al., 2012).

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Competing interests statement
The authors declare no competing financial interests.

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