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Mechanisms of Gurken-dependent *pipe* regulation and the robustness of dorsoventral patterning in *Drosophila*

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SUMMARY

The restriction of Pipe, a potential glycosaminoglycan-modifying enzyme, to ventral follicle cells of the egg chamber is essential for dorsoventral axis formation in the *Drosophila* embryo. *pipe* repression depends on the TGFα-like ligand Gurken, which activates the *Drosophila* EGF receptor in dorsal follicle cells. An analysis of *Raf* mutant clones shows that EGF signalling is required cell-autonomously in all dorsal follicle cells along the anteroposterior axis of the egg chamber to repress *pipe*. However, the autoactivation of EGF signalling important for dorsal follicle cell patterning has no influence on *pipe* expression. Clonal analysis shows that also the *mirror-fringe* cassette suggested to establish a secondary signalling centre in the follicular epithelium is not involved in *pipe* regulation. These findings support the view that the *pipe* domain is directly delimited by a long-range Gurken gradient. Pipe induces ventral cell fates in the embryo via activation of the Spätzle/Toll pathway. However, large dorsal patches of ectopic *pipe* expression induced by *Raf* clones rarely affect embryonic patterning if they are separated from the endogenous *pipe* domain. This indicates that potent inhibitory processes prevent *pipe* dependent Toll activation at the dorsal side of the egg.

Key words: EGF signalling, *mirror*, *fringe*, *rhomboid*, *dec*-marked clones, Long-range inhibition, *pipe*, *spätzle*

INTRODUCTION

During *Drosophila* oogenesis, the anterior cortical localization of Gurken (Grk) within the oocyte marks the future dorsal side of the egg and embryo. Grk is a TGFα-like signalling molecule, which activates the *Drosophila* homologue of the EGF receptor (Egfr) expressed in the overlying follicular epithelium (Neuman-Silberberg and Schüpbach, 1993; Ghiglione et al., 2002; Shmueli et al., 2002). Egfr activation specifies dorsal fates in follicle cells close to the site of Grk production. Concomitantly, a ventral region of the follicular epithelium is delimited that, after egg deposition, provides spatial cues for the dorsoventral (DV) axis of the embryo (for a review, see Nilson and Schüpbach, 1999).

The events that follow Grk signalling might be quite complex as exemplified by the patterning of the dorsal follicular epithelium (Wasserman and Freeman, 1998). In dorsally located follicle cells close to the oocyte nucleus, vesicles of Grk protein can be detected, suggesting high levels of signalling activity (Peri et al., 1999; Queenan et al., 1999). In these cells, Grk initiates the transcription of *rhomboid* (*rho*) and several inhibitors of the EGF signalling pathway. *rho*, in turn, activates another TGFα-like ligand, Spitz (Spi), which is present as an inactive precursor throughout the entire follicular epithelium. Cleaved Spi is diffusible and activates Egfr together with Grk in a broad dorsal-anterior zone, leading to a positive feedback on Egfr pathway activation (Wasserman and Freeman, 1998). The ensuing interplay between activators and inhibitors of EGF signalling locates a group of follicle cells on each side of the dorsal midline, which gives rise to respiratory chorion specializations, the so called dorsal appendages (DAs). DA specification and *rho* induction require, in addition to Grk signalling, the presence of Decapentaplegic (Dpp), a TGFβ ligand that emanates from a ring of anterior follicle cells (Twombly et al., 1996; Deng and Bownes, 1997; Dobens et al., 2000; Peri and Roth, 2000). This explains why the DAs form only at the anterior pole of the egg while Grk signalling is setting up the entire DV axis of the eggshell and the embryo.

Grk-dependent gene activation at the dorsal side is accompanied by repression of *pipe*, which encodes a potential glycosaminoglycan-modifying enzyme with crucial function for embryonic axis formation. *pipe* expression is delimited to a ventral domain spanning about 40% of the egg circumference (Sen et al., 1998). Thus, the border of *pipe* expression is far from the region of strong Egfr signalling, as defined by Grk uptake into larger vesicles. The following molecular observations have supported the idea of an indirect action of Grk signalling on *pipe* transcription. The homeodomain transcription factor Mirror (Mirr) is activated by Grk at the dorsal side and this in turn restricts *fringe* (*fng*) expression to the ventral side of the egg chamber (Jordan et al., 2000; Zhao et al., 2000b). As for DV patterning of the eye and wing imaginal discs (Irvine, 1999), it has been proposed that the Notch receptor is activated in cells straddling the border of the

fng domain and that this leads to the production of diffusible molecules able to repress *pipe* at a distance (Jordan et al., 2000). Ectopic *mirr* expression at the posterior of the egg chamber appears to support this model as it leads to long-range repression of *pipe* (Jordan et al., 2000).

However, recent experiments have provided evidence for a direct influence of Grk on *pipe* repression (Pai et al., 2000). The EGF receptor seems to be activated also in the ventral region of the follicular epithelium where *pipe* is expressed. *Cbl* has been found to be required in ventral follicle cells to lower EGF pathway activation presumably by targeting the activated EGF receptor complex into the ubiquitination-dependent degradation pathway (Pai et al., 2000). Ectopic activation of the EGF pathway in ventral *Cbl* mutant cell clones leads to cell-autonomous repression of *pipe*. As this depends on Grk signalling, Grk may work as a long-range signalling molecule able to stimulate ventral follicle cells and directly repress *pipe* transcription. However, a gradient mechanism for delimiting the *pipe* domain does not exclude the presence of secondary refinement processes involving either the Egfr feedback or *mirr*- and *fng*-dependent mechanisms. A Grk gradient might crudely define the *pipe* expression domain while the observed sharp on-off border of *pipe* transcription might result from subsequent signalling events.

pipe expression at the ventral side of the egg chamber, in an unknown way, initiates a proteolytic cascade that leads to the production of an extracellular ligand (Spätzle, Spz) (Morisato and Anderson, 1994). This ligand is believed to activate the transmembrane receptor Toll which relays the extracellular signal into the embryo (Morisato and Anderson, 1994). The activated signalling pathway stimulates the nuclear import of Dorsal protein which forms a gradient with peak levels along the ventral midline of the syncytial blastoderm embryo (for a review, see Morisato and Anderson, 1995). Dorsal, a member of the NF-κB family of transcription factors, regulates the expression of zygotic DV genes in a concentration-dependent manner and thereby specifies the different cell fates along the embryonic DV axis (for a review, see Rusch and Levine, 1996).

It is currently not known how much precision in *pipe* expression is needed in order to allow normal DV patterning of the embryo. The clonal analysis of Nilson and Schüpbach (Nilson and Schüpbach, 1998) has shown that *pipe* is required in a ventral region that is approximately 8-12 cells wide to induce the ventralmost embryonic cell fate, the mesoderm (as visualized by *twist* expression). The normal expression domain of *pipe*, however, is at least twice as wide. Studies with egg chambers containing two oocyte nuclei indicate that a narrowing of the *pipe* domain is compatible with normal development (Roth et al., 1999). The opposite change, an enlargement of the *pipe* domain, demonstrates that the processes initiated by *pipe* have self-regulatory properties. If the *pipe* domain is expanded beyond a certain limit, two partial DV axes form within one embryo (Roth and Schüpbach, 1994; Morisato, 2001). All these observations indicate that complex regulatory processes intervene between *pipe* expression and embryonic axis formation.

In this study, we have re-examined *pipe* regulation in the follicular epithelium and its significance for embryonic patterning. In order to test the two models for *pipe* repression we have generated marked clones for components of the relevant signalling pathways and directly observed their influence on *pipe* transcription. Our analysis allows us to discriminate between the proposed models and it reveals that Grk signalling directly represses *pipe* from all dorsal follicle cells along the AP axis. No secondary signalling events appear to contribute to the refinement of the *pipe* domain, suggesting that Grk is the only ligand taking part in the process. We also analysed how ectopic *pipe* expression influences embryonic development. Surprisingly, even large dorsal patches of *pipe* fail to affect dorsoventral patterning of the embryo. Different models accounting for these findings are discussed.

MATERIALS AND METHODS

Fly stocks and genetic mosaic analysis in the follicular epithelium

The following *Drosophila melanogaster* strains were used: Oregon R; rho^{7M43} *FRT80B* (Wasserman and Freeman, 1998); Raf^{LE78} *FTR101* (T. Schüpbach, unpublished); dec^{VA28} *FRT101* (Nilson and Schüpbach, 1998); $mirr^{e48}$ *FRT80B* (McNeill et al., 1997); dof^{1} *FRT82B* (Vincent et al., 1998); fng^{13} *FRT80B* (Papayannopoulos et al., 1998); *FRT82B ubi-nls GFP* and *FRT 80B* (gifts from S. Luschnig); *FRT101 GFP* (Brennan et al., 1999); *pipe-lacZ* (Sen et al., 1998). Follicle cell clones were generated with the FLP/FRT technique (Xu and Rubin, 1993) using the follicle cell-specific recombination line *e22c-GAL4, UAS-FLP* (Duffy et al., 1998) or in the case of *dec*-marked clones *hs FLP TM3, Sb* (gift from T. Schüpbach). Clones of genetically marked cells expressing *λtop* (*UAS-λtop*; Queenan et al., 1997) and *rho* (*UAS-rho*) (Sapir et al., 1998) were generated using the combined *Gal4, flip-out* system (*hs-flip*; *actin>CD2>Gal4*; *UAS-GFP*) (Pignoni and Zipursky, 1997; Dobens et al., 2000). Flip-out clones were induced in larvae applying a 30 minutes heat shock at 37°C.

Induction and detection of *dec*-marked follicle cell clones

A dec^{VA28} Raf^{LE78} *FRT101* was used to generate *dec*-marked *Raf* clones visible in the chorion of deposited eggs (Nilson and Schüpbach, 1998). To induce expression of the FLP recombinase flies were heat shocked twice, 6-8 hours apart, at 37°C for 1 hour. The females were mated to Oregon R males and the deposited eggs were aged to allow completion of embryonic development and then mounted in Hoyer's medium.

In situ hybridization and immunohistochemistry

pipe transcripts (Sen et al., 1998) were detected by in situ hybridization with a digoxigenin-labelled antisense probe (Tautz and Pfeifle, 1989). For immunofluorescence antibody staining, the ovaries were incubated overnight with anti-β-galactosidase antibodies (1:1000, pre-adsorbed to fixed tissue; Cappel). The secondary antibodies were from Jackson Laboratories.

RESULTS

Dynamics of *pipe* mRNA and *pipe* reporter gene expression

In order to understand the mechanisms of *pipe* repression, we have first re-examined the dynamics of *pipe* mRNA expression in wild-type egg chambers (Sen et al., 1998). We were specifically interested in comparing the expression pattern of the endogenous *pipe* mRNA with that of a *pipe* reporter line (*pipe-lacZ*) (Sen et al., 1998), which, in the absence of anti-Pipe antibodies, was used in the course of the mosaic analysis.

In early stage 9 egg chambers, *pipe* is expressed in two distinct domains of the follicular epithelium, a posterior-ventral domain and a broad anterior domain overlying the nurse cells (Fig. 1A,F). During stage 9, the follicle cells migrate towards the posterior of the egg chamber. This generates a population of stretched follicle cells overlying the nurse cell cluster at the anterior of the egg chamber and a population of columnar-shaped cells, or main-body follicle cells, abutting the growing oocyte at the posterior (Spradling, 1993). As the anterior to posterior migration of the follicular epithelium takes place, the two *pipe* expression domains approach each other (Fig. 1B,C,G,H) and finally they fuse forming a stripe at stage 10A of oogenesis (Fig. 1D,I) (Sen et al., 1998). Up to this stage we could not detect any difference between the expression of the endogenous *pipe* mRNA and that of the *pipe* reporter construct. Thus, as our following study concentrates on stage 9 and 10A egg chambers, the *pipe lacZ* construct can be used with sufficient accuracy to monitor *pipe* expression.

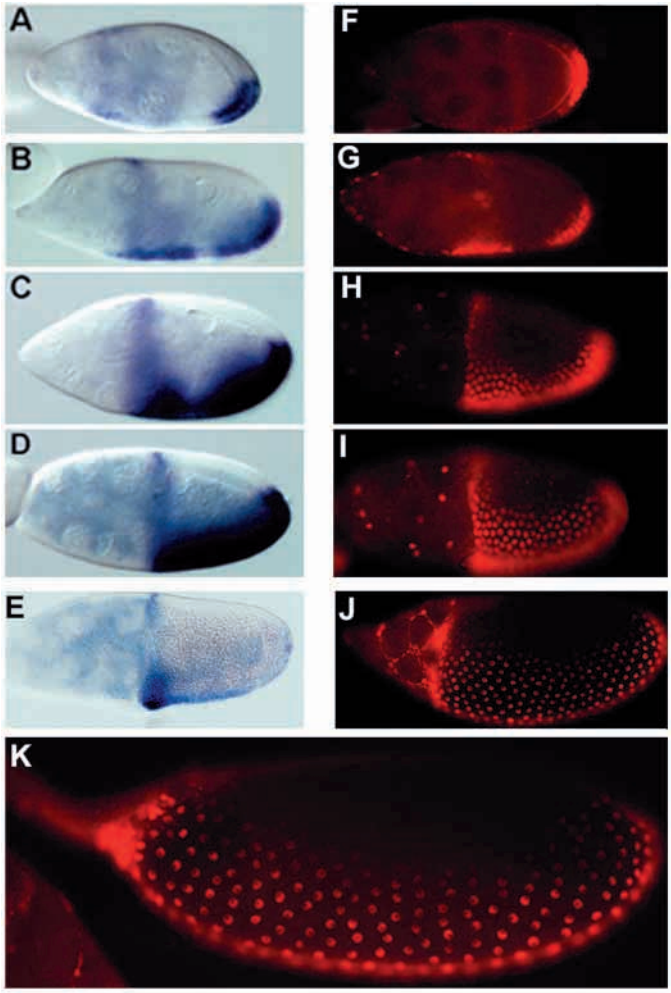

Fig. 1. Expression of *pipe* mRNA and a *pipe* reporter construct in wild-type ovaries. All egg chambers are oriented with the anterior towards the left and dorsal towards the top. (A-E) *pipe* mRNA distribution. (F-K) *pipe-lacZ* expression visualized using anti-β-galactosidase antibodies. (A,F) During early stage 9, *pipe* is expressed in two domains, a weak anterior domain and a strong posterior-ventral domain. (B,G,C,H) The two domains of expression approach each other during stage 9 and (D,I) fuse, forming a solid ventral stripe at early stage 10. (E) *pipe* mRNA disappears during stage 10 starting from the posterior pole. (J) Stage 12. (K) Stage 14.

During stage 10, *pipe* transcripts disappear first from the posterior follicle cells (Fig. 1E) and by the end of stage 10, *pipe* mRNA is no longer detected in the follicular epithelium (data not shown). *pipe-lacZ*, however, remains visible until completion of egg development probably due to the high stability of the β-galactosidase (Fig. 1J,K). The stage 10 *pipe* domain is transformed into a ventral stripe that spans the entire AP axis of the mature egg and covers 40% of its circumference (Fig. 1K). This stripe indeed corresponds to the region which harbours the nuclear Dorsal gradient of the later embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989).

Cell-autonomous requirement of EGF signalling for *pipe* repression

EGF signalling in somatic follicle cells is transduced by the canonical MAPK cascade in which GTP-activated Ras interacts with the effector protein Raf (MAPKKK), which in turn phosphorylates the downstream component Dsor1 (MAPKK). The ensuing phosphorylation of Rolled (MAPK) links Egfr activity to changes in gene regulation (Wassarman et al., 1995). We analysed *Raf* mutant clones to investigate how blocking the cascade affects *pipe* expression.

Homozygous *Raf* mutant clones were detected by the absence of GFP. Clones induced at the dorsal side show a strong cell-autonomous de-repression of *pipe* (Fig. 2A-F). No difference could be observed among dorsal clones located at distinct AP positions of the egg chamber (compare Fig. 2A-C with D-F). Moreover, clones at the dorsal side did not affect endogenous *pipe* expression at the ventral side (Fig. 2A-C and data not shown). This demonstrates that MAPK signalling is required for *pipe* repression along the entire AP axis and that cells in which MAPK signalling is blocked do not receive other inputs that lead to *pipe* repression. Even in very narrow *Raf* clones (Fig. 2E) *pipe* transcription is not repressed, which clearly excludes the possibility of even short-range repressing signals being produced by neighbouring cells. Furthermore, as the lateral *pipe* expression border could be established even if some dorsal cells did not receive the Grk signal, we conclude that *pipe* repression does not result from a relay mechanism, which is initiated at the dorsal side. Similar results have been obtained in experiments inducing *Ras* mutant clones (James et al., 2002)

The MAPK cascade acts downstream of several receptor tyrosine kinases (RTKs) (Wassarman et al., 1995). Thus, *Raf* mutant cell clones could impair signalling initiated by other RTKs as well as Egfr. For some RTKs, such as Torso and Sevenless, an early involvement in DV axis specification can be excluded (Schweitzer and Shilo, 1997). However, loss of *Raf* function could also impair signalling initiated by the FGF receptors. Their role in establishing the DV polarity has not been investigated so far. Dof was identified as a specific component of the FGF signalling pathway in *Drosophila* and it has been shown to be essential in the signal transduction cascade downstream of the two identified receptors (Michelson et al., 1998; Vincent et al., 1998; Imam et al., 1999). We have made use of the *dof* mutation in order to investigate if FGF signalling is involved in shaping the *pipe* domain. Homozygous *dof* mutant clones, marked by the absence of GFP expression, do not affect *pipe* expression, suggesting that

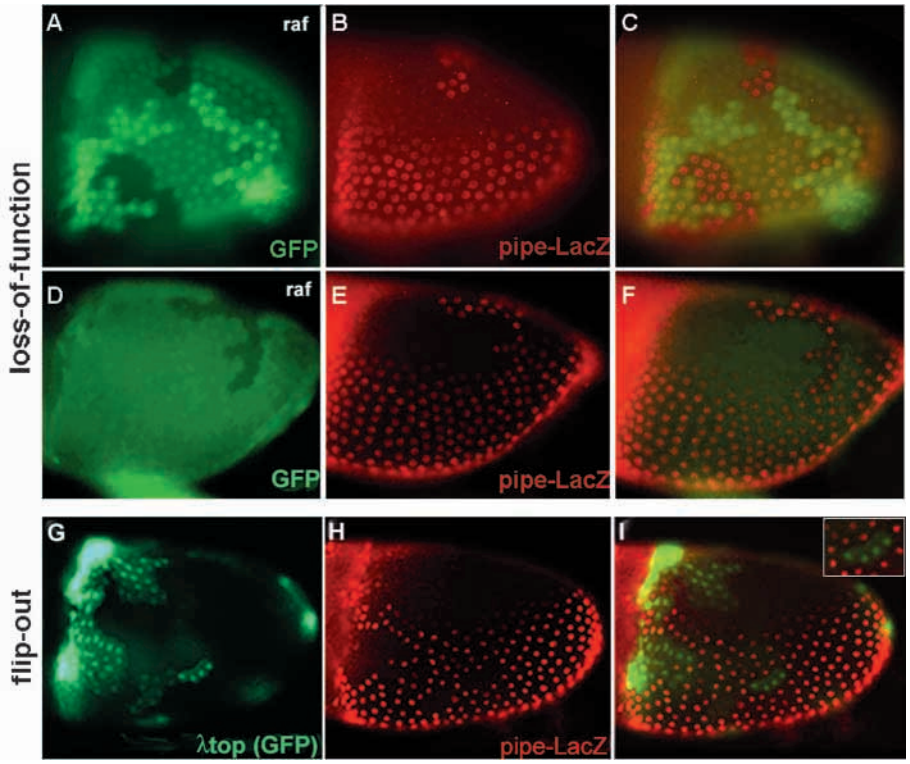

Fig. 2. *Raf* loss-of-function and *λtop* flip-out clones affect *pipe* expression in a cell-autonomous manner. (A-I) Stage 10 egg chambers (anterior towards the left and dorsal towards the top). Anterior (A) and posterior (D) clones of mutant cells lacking *Raf* are marked by the absence of GFP (green) expression. (B,E) *pipe* expression is visualized using a *pipe-lacZ* construct (red). (C,F) The merged pictures show that *pipe* is de-repressed in *Raf* mutant clones (lack of green) located at the dorsal side. De-repression occurs in a cell-autonomous manner and it is not influenced by the position of the clone along the AP axis. (G-I) *λtop* flip-out clones. (G) *λtop*-expressing cells are marked by GFP expression (green). (H) *pipe* expression is visualized using a *pipe-lacZ* construct (red). (C) The merged pictures show that *λtop* expression leads to cell-autonomous repression of *pipe*.

signalling initiated by FGF receptor activation does not play a role on *pipe* regulation (Fig. 3A-C). This observation confirms that the effects of *Raf* clones on *pipe* expression are due to a lack of EGF signalling.

If the role of EGF signalling was to promote other downstream signalling events able to repress *pipe* at a distance, ectopic activation of EGF signalling at the ventral side should lead to cell non-autonomous repression of *pipe* in surrounding cells. We tested this by analysing clones that expressed a ligand-independent activated form of the EGF receptor (λtop) (Queenan et al., 1997). The clones were generated using the Gal4 flip-out system (Pignoni and Zipursky, 1997). λtop-expressing cells that are ventrally located and marked by GFP expression act autonomously to repress *pipe* (Fig. 2G-H). Lateral clones just outside the endogenous *pipe* domain do not affect endogenous *pipe* expression (Fig. 2G-I). These observations suggest that the levels of EGF signalling we obtained by ectopic expression of λtop do not initiate secondary signalling cascades involved in *pipe* repression.

The positive feedback on EGF pathway activation via *rhomboid* is not required for *pipe* repression

As our data so far were obtained using a component downstream of the EGF receptor we cannot distinguish whether Grk is the only EGF ligand responsible for *pipe* repression or whether there is also a contribution from Spi (Wasserman and Freeman, 1998). To test whether Spi in principle is able to repress *pipe*, we induced marked clones ectopically expressing *rho*, which encodes a proteolytic activator of Spi (Urban et al., 2001; Lee et al., 2001). Ventral clones of cells expressing *rho* marked by GFP show repression of *pipe* transcription (Fig. 3G-I). This occurs in a cell non-autonomous manner. *pipe* is repressed not only within the clone, but also in surrounding cells. Thus, either Grk or Spi can achieve Egfr activation leading to *pipe* repression. The non-autonomy can be explained by the molecular nature of Rho and Spi. Rho activates Spi, which diffuses leading to the expansion in width of the EGF activation domain (Schweitzer et al., 1995; Golembo et al., 1996; Urban et al., 2001; Lee et al., 2001).

However, these findings do not prove an involvement of Spi in normal *pipe* regulation. Nor does the reported hatching of larvae from eggs carrying *spi* or *rho* clones disprove such an involvement (Wasserman and Freeman, 1998) (see Discussion). Therefore, we have re-examined the potential role of *spi* in *pipe* repression by inducing large marked *rho* mutant clones in the follicular epithelium. Large *rho* mutant clones detected by the absence of GFP expression and covering the dorsal anterior half of the egg chamber do not lead to the de-repression of *pipe*. Furthermore, the sharpness of the on-off transition of *pipe* expression is not altered (Fig. 3D-F). These observations indicate that Grk is the only EGF ligand responsible for repression of *pipe* at the dorsal side of the egg chamber and that *rho* and thus *spi* are not involved in the process.

The *mirror-fringe* cassette is not required for *pipe* repression

mirr encodes a homeodomain transcription factor which is involved in establishing the equator, a boundary where dorsal and ventral cells meet in the eye imaginal disc (McNeill et al., 1997). *mirr* represses *fng* at the dorsal side of the eye imaginal disc and thereby localizes a stripe of Notch activity to the border between *fng* expressing and non-expressing cells (Papayannopoulos et al., 1998; Yang et al., 1999). During mid-oogenesis, *mirr* is expressed in a Grk-dependent manner at the dorsal-anterior side of the egg chamber and delimits *fng* expression to the ventral side (Jordan et al., 2000; Zhao et al., 2000a; Zhao et al., 2000b). The lateral confrontation between *fng*-expressing and non-expressing cells was proposed to establish, via Notch activation, a signalling centre that leads to

the repression of *pipe* at a distance (Jordan et al., 2000; Zhao et al., 2000b).

In order to test this model, we have generated *mirr* and *fng* mutant clones in the follicular epithelium. These clones were marked by the absence of GFP and had variable sizes and random locations. For *mirr* it was not possible to detect *pipe* expression using *pipe-lacZ*, as the only available strong loss-of-function allele of *mirr* is derived from an enhancer trap screen and possesses residual *lacZ* activity (Helen McNeill, personal communication). Thus, potential mosaic ovaries were first examined for GFP expression to verify the presence of clones. Subsequently, *pipe* mRNA expression was monitored by in situ hybridization using ovaries from the same females. No change in the wild-type pattern of *pipe* expression was observed in stage 10A egg chambers (0/223). In a parallel experiment in which we generated *Raf* clones, we could easily detect ectopic *pipe* expression at the dorsal side of stage 10A egg chambers (19/116). Both the absence of *pipe* derepression at the dorsal side and the lack of changes in the endogenous *pipe* domain in egg chambers carrying *mirr* clones contrasts with the proposed model in which the boundary of *mirr* expression defines an organizing centre important for *pipe* repression. This suggests that dorsal *fng* repression by *mirr* might not be required to restrict *pipe* expression. This notion is confirmed by the analysis of *fng* mutant clones. Large anterior or posterior *fng* clones marked by the absence of GFP did not affect *pipe* expression (Fig. 4A-F). *fng* clones at the ventral side lead to a confrontation between *fng* expressing and non-expressing cells within the *pipe* domain (Fig. 4A-F). According to the model, this should establish a signalling centre able to repress *pipe*. However, at the ventral side of mosaic egg chambers, the *pipe* domain extends continuously from wild-type to *fng* mutant cells.

Together these data suggest that the MAPK pathway activated by Grk does not act via the *mirr-fng* cassette to repress *pipe*. This is in agreement with the cell-autonomous effects on *pipe* expression exerted by *Raf* (Fig. 2) and *Ras* mutant clones (James et al., 2002).

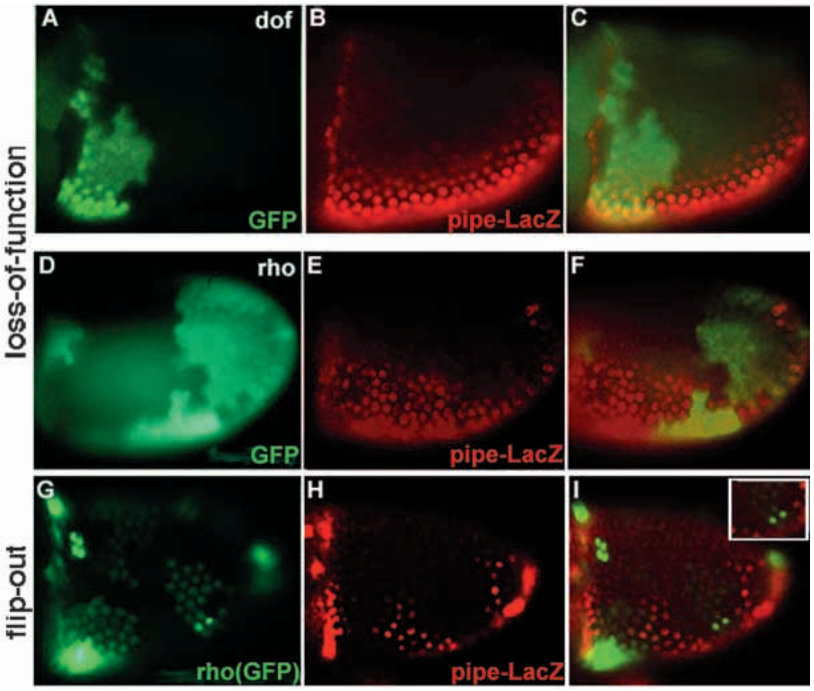

Fig. 3. *pipe* expression is not affected by *dof* and *rho* loss-of-function clones, but by ectopic expression of *rho*. (A-I) Stage 10 egg chambers (anterior towards the left and dorsal towards the top). (A) A large clone of mutant cells missing *dof* is marked by the absence of GFP expression (green). (B) *pipe* expression is visualized using a *pipe-lacZ* construct (red). (C) The merged pictures show that *pipe* expression is not affected by loss of *dof* activity. (D) A large clone lacking *rho* function is marked by the absence of GFP. (E) *pipe* expression is visualized using the *pipe-lacZ* construct. (F) The merged pictures show that *pipe* expression is not affected by loss of *rho* activity. (G) *rho* flip-out clones marked by GFP expression. (H) *pipe* expression is visualized using the *pipe-lacZ* construct. (I) The merged pictures show that ectopic *rho* expression (green) leads to cell-non-autonomous repression of *pipe* (red).

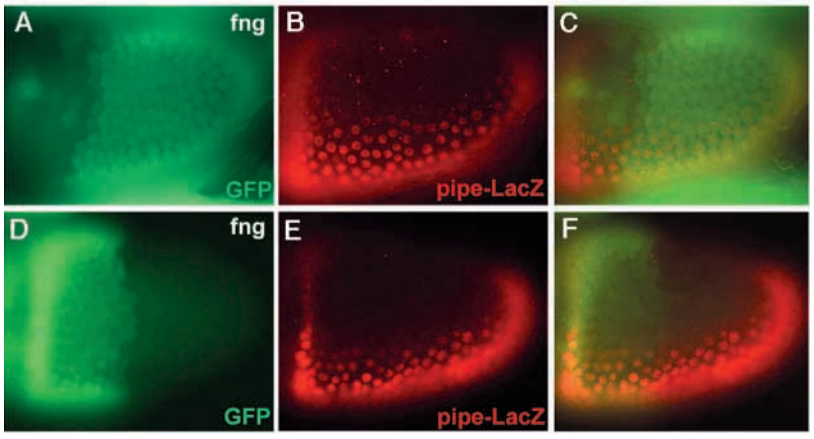

Fig. 4. *fringe* mutant clones do not affect *pipe* expression. Large anterior (A) and posterior (D) clones that lack *fng* are marked by the absence of GFP expression (green). (B,E) *pipe* expression is visualized using the *pipe-lacZ* construct (red). (C,F) The merged pictures show that *fng* clones do not affect *pipe* expression.

Ectopic *pipe* expression has frequently no effects on DV patterning of the embryo

The observation that *Raf* mutant clones in the dorsal half of the egg chamber lead to ectopic *pipe* expression without affecting the endogenous *pipe* domain provides us with an interesting tool for studying regulatory properties of embryonic DV patterning. Ectopic patches of *pipe* should initiate the proteolytic cascade leading to Spz activation at ectopic positions within the egg and thus affect the embryonic DV axis. We used a *Raf FRT* chromosome which also carried a mutation of *defective chorion 1* (*dec*) to mark the mutant clones in the chorion of deposited eggs (Nilson and Schüpbach, 1998). Eggs were collected at different time intervals after heat shock-induced mitotic recombination to sample specimens carrying clones of different sizes. The

eggs were allowed to complete embryonic development, mounted and inspected with dark field optics to visualize both the clones within the chorion and the cuticle secreted by the embryo.

As expected, *Raf* clones frequently disrupted the development of the dorsal appendages (DAs) if the clones extended into dorsal-anterior regions [see James et al. (James et al., 2002) for the function of Ras in DA formation]. However, as *dec* is not a reliable marker for terminal regions of the eggshell and as anterior *Raf* clones frequently prevent embryonic development, these clones are under-represented in our collection.

The cuticle pattern of embryos from mosaic eggs was carefully analysed to detect defects in DV patterning (Figs 5, 6). Several structures of the mature larval cuticle (Fig. 5A$_4$), including most parts of the head skeleton (hs), the dorsal epidermis carrying dorsal hairs (de) and the tracheal system with its posterior openings (Filzkörper, fk), are derived from dorsal or dorsolateral positions of the blastoderm embryo and thus require absence or very low levels of Toll signalling for their formation (Roth et al., 1991). The ventral denticle belts (vd) are derived from ventrolateral positions of the blastoderm corresponding to intermediate levels of Toll signalling. The regions of high Toll signalling that give rise to the mesoderm invaginate and thus do not contribute to the cuticle (for a review, see Morisato and Anderson, 1995).

Ventral *Raf* clones did not affect embryonic development. Fig. 5A shows the example of an egg with a large ventral clone spanning most of the AP axis, which contains a larva with normal cuticle pattern. Comparison with Fig. 1K indicates that this clone largely lies within a region corresponding to the normal *pipe* domain and therefore did not significantly alter *pipe* expression. Conversely, large *Raf* clones extending from the ventral to the dorsal side of the egg cause local distortions of the embryonic DV pattern (15/15). The egg harbouring a huge dorsal anterior clone shown in Fig. 5B contains a larva whose head skeleton and thoracic cuticle are largely deleted, while the ventral denticle rows of the first abdominal segment (arrow) are expanded. Judging from an independently performed analysis of *twist* protein expression in eggs carrying *Raf* clones, we assume that these cuticular deletions result from the local expansion of mesoderm at the expense of cuticle secreting ectoderm.

By scoring eggs with different clone sizes, we tried to define the minimal clone size that causes a disturbance of normal development. Thirty-four eggs each harbouring several clones in dorsal or lateral positions which ranged from two to 16 cells in size contained normal larvae (a representative example is shown in Fig. 6A). For bigger clones (>30 cells), their position relative to the normal *pipe* domain was essential with regard to their effects on DV patterning. Dorsal clones that did not extend to the ventrolateral side of the egg could comprise up to 70 cells without affecting embryonic development (5/5; Fig. 6B). However, clones of similar size extending from the ventral to the dorsal side of the egg invariably caused cuticle defects (7/7; Fig. 6C). The larvae exhibit deletions of cuticular elements in a region along the anteroposterior axis, which corresponds to the position of the clone in the eggshell (Fig. 6C). These observations suggest that ectopic patches of *pipe* are effective in inducing ventral structures if they exceed a crucial size limit (~30 cells) and enlarge the endogenous *pipe* domain. However, their effects are suppressed if they are located dorsally at a distance from the endogenous *pipe* domain. It is particularly striking that such dorsal clones can span up to 14 cells in DV dimension and occupy more than 30% of the AP axis (Fig. 6B and data not shown) without affecting the formation of the dorsal epidermis. *pipe* domains of this size have been shown to be sufficient to induce mesoderm formation at the ventral side of the egg (Nilson and Schüpbach, 1998). These results suggest that either potent inhibitory processes prevent *pipe* function at the dorsal side of wild-type eggs or that the consequences of ectopic expression of early zygotic DV genes are corrected during later development.

To investigate the latter possibility, we analysed *twist* (*twi*) expression in cellular blastoderm and early gastrulating embryos derived from females in which *dec Raf* follicle cell clones had been induced. Parallel egg lays, which were not stained, were used for chorion preparations to determine the clone size of a particular batch. *twi* is normally expressed in a 20 cell wide ventral stripe extending along the entire AP axis (Thisse et al., 1988). The cells of the *twi* domain invaginate by

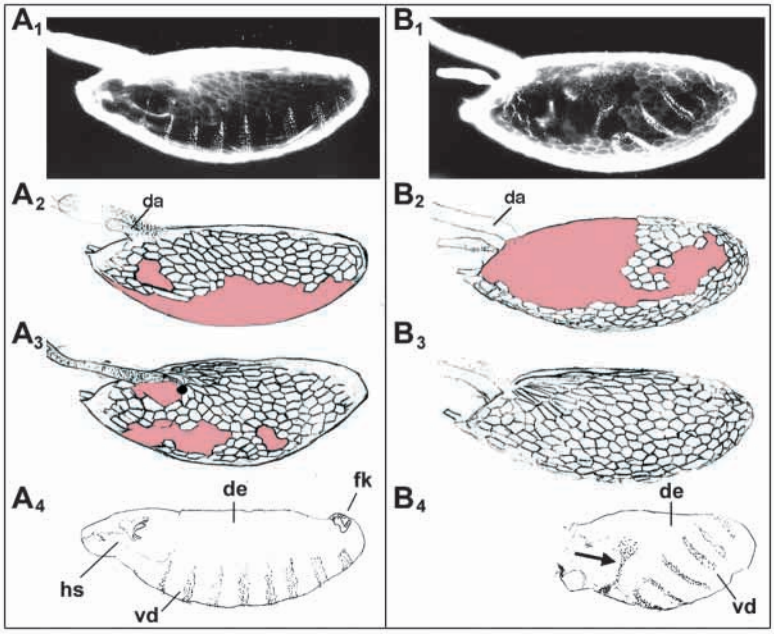

Fig. 5. The effects of large *dec*-marked *Raf* clones on the larval cuticle. (A$_1$,B$_1$) Darkfield micrographs of the lateral surface of eggs focusing on the outer eggshell (chorion). The chorion derived from *dec* follicle cell clones appears to be more transparent. Anterior is towards the left and dorsal to the top. (A$_2$,B$_2$,A$_3$,B$_3$) Camera lucida drawings of the left and right sides of the same eggs. The clones are marked in pink. (A$_4$,B$_4$) Camera lucida drawings of the larval cuticle present inside the eggs. da, dorsal appendage; de, dorsal epidermis; fk, filzkörper; hs, head skeleton; vd, ventral denticles. (A) Egg carrying a large ventral clone that contains a larva with normal cuticle. (B) Egg carrying a large dorsolateral clone. Deletions of head and thorax structures and the expansion of ventral denticle rows of the first abdominal segment (arrow) might be caused by local ventralization.

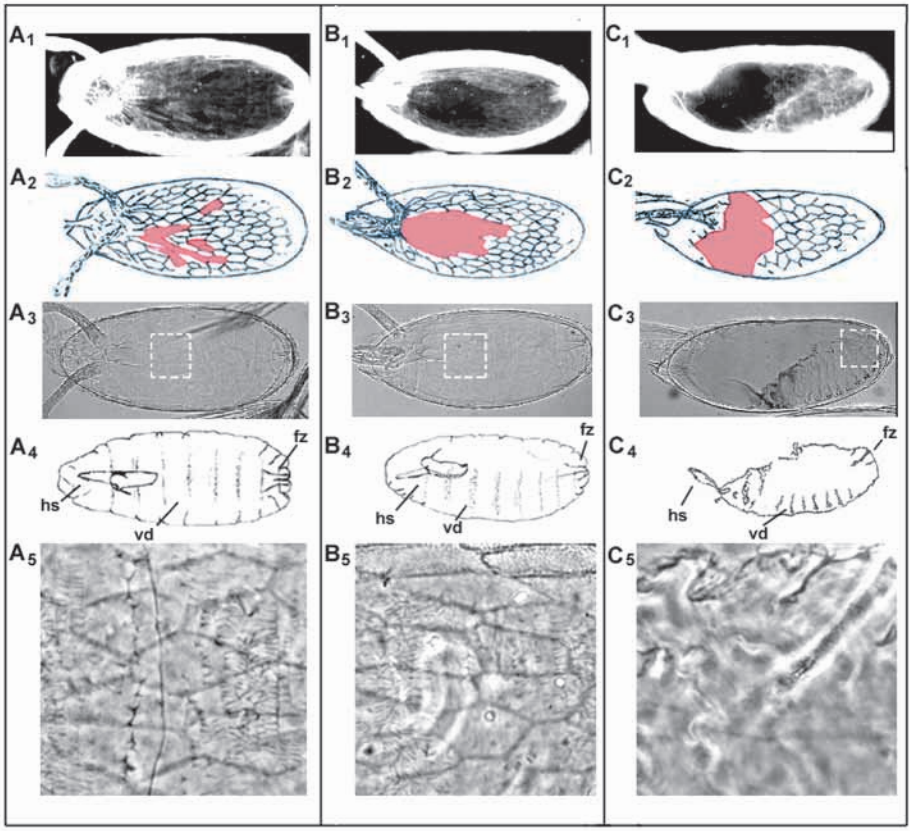

Fig. 6. The effects of *dec*-marked *Raf* clones of variable sizes on the larval cuticle. Darkfield micrographs of the (A_1,B_1) dorsal or (C_1) lateral surface of eggs focusing on the outer egg shell (chorion). The chorion derived from *dec* follicle cell clones appears to be more transparent. Anterior is towards the left. (A_2,B_2,C_2) Camera lucida drawings of the pictures shown in A_1,B_1,C_1. The *dec* clones are marked in pink. (A_3,B_3,C_3) Nomarski micrographs showing the larval cuticle inside the eggs. (A_4,B_4,C_4) Camera lucida drawings of the larval cuticles. (A_5,B_5,C_5) Phase-contrast micrographs showing magnified views of the regions of the cuticle boxed in A_3,B_3,C_3. (A) Egg carrying multiple dorsal *dec Raf* clones that contains a larva with normal cuticle. At the dorsal side facing the clones the cuticle harbours dorsal hairs (A_5). (B) Egg carrying a large dorsal *dec Raf* clone, which comprises about 40 follicle cells and spans up to 10 follicle cells in the mediolateral dimension. The larva that developed inside this egg has a normal cuticle. At the dorsal side facing the clone, the cuticle harbours dorsal hairs (B_5). (C) Egg carrying a large lateral *dec Raf* clone that comprises about 40 follicle cells. The larva that developed inside this egg shows cuticular defects in the head and thorax region which might be caused by a local ventralization. At the posterior pole, the presence of normally sized ventral denticle belts (vd) and extended filzkörper (fk, B_5) demonstrates normal DV patterning.

forming the ventral furrow and later give rise to the mesoderm. In embryos that developed in eggs with small randomly distributed clones (up to the size of 30 cells) no defects in *twi* expression were observed (0/150; Fig. 7A). Eggs carrying larger clones (30 to 120 cells) contained a small number of embryos with abnormal *twi* domains (17/228). In some embryos the *twi* stripe does not follow the ventral midline, but shifts to lateral positions (8/17; Fig. 7B,C). Although these embryos have a normal DV pattern at a given AP position, they have lost bilateral symmetry (see Discussion). In other embryos the *twi* domain expands at a certain region of the AP axis and here the ventral furrow often bifurcates (9/17; Fig. 7D,E). We have not observed isolated patches of *twi*-expressing cells in lateral or dorsal positions. Ectopic regions of *twi* expression were always connected to the endogenous *twi* domain. Similar results have been obtained with dorsally localized *Ras* clones (James et al., 2002). These findings suggest that ectopic *pipe* at restricted dorsal positions is not effective in inducing the high levels of Toll signalling required for *twi* expression.

Fig. 7. Effects of *Raf* clones on *twist* expression. *twi* and *ftz* protein distribution in gastrulating embryos. All embryos are shown from the ventral side. Anterior is towards the left. (A) Embryo showing normal *twi* expression. The *twi*-expressing cells invaginate along the ventral midline (ventral furrow). The embryo is derived from an egg collection in which the eggs carried several *Raf* clones, ranging from 2-16 cells in size. (B-E) Embryos derived from eggs carrying clones with an average size of 30-60 cells. (B,C) The ventral furrow is displaced to lateral positions as it runs along the AP axis. (D,E) Local expansions of the *twi* domain are accompanied by a bifurcation of the ventral furrow. In some embryos, similar effects were also seen at more posterior positions.

DISCUSSION

In normal development, the DV morphogen gradient of the embryo is set up within blastoderm cells that abut a ventral stripe of the eggshell. Using a *pipe-lacZ* transgene, one can show that this region of the eggshell is secreted by follicle cells that have expressed *pipe* during stage 10 of oogenesis (Sen et al., 1998) (Fig. 1I-K). *pipe* activity is required within this region for the induction of ventral cell fates in the embryo, as has been shown earlier by clonal analysis (Nilson and

Schüpbach, 1998). This demonstrates the importance of *pipe* regulation for embryonic axis formation and raises several interesting questions. First, how does Grk precisely delimit the *pipe* domain, whose borders presumably correspond to low levels of Grk signalling activity? Are there parallel signalling cascades and secondary refinement processes involved as commonly found in pattern formation of the early embryo or imaginal discs? Second, how precisely must the expression of *pipe* be regulated in order to form a normal embryonic axis? What is the regulatory capacity of the system in case of misregulations occurring during oogenesis? These latter questions are directly related to the problem of how the ventral *pipe* domain directs the formation of the embryonic DV morphogen gradient.

Cell-autonomous *pipe* regulation

Many important patterning events are regulated in a redundant or partially redundant fashion (Tautz, 1992). In addition, gradient mechanisms are often followed by secondary refinement processes (Gurdon and Bourillot, 2001). Therefore, the two contrasting models of *pipe* regulation, invoking either a direct repression by Grk (Pai et al., 2000) or a Grk-dependent secondary signalling cascade (Jordan et al., 2000), might not exclude each other. However, our data find no support for the involvement of secondary signalling cascades. *Raf* clones lead to completely cell-autonomous de-repression of *pipe*. Similar results have been obtained with *Ras* follicle cell clones (James et al., 2002). The *Raf* and *Ras* loss of function is likely to reflect only the loss of EGF signalling, as FGF signalling does not play a role in the *pipe* regulation (Fig. 3A-C). The observation that loss of *rho* has no effects on *pipe* in addition suggests that Spi is not involved and that indeed Grk is the only TGFα-like ligand defining the border of the *pipe* expression domain.

It has been reported that embryos from eggs carrying *rho* or *spi* clones show normal development (Wasserman and Freeman, 1998). However, in these experiments the clones were not marked, so that it was not certain whether the clones had been large enough to affect the *pipe* domain. Furthermore, even if they had affected the *pipe* domain, downstream regulatory processes might correct the changes. The observation that *pipe* expression was completely normal in egg chambers carrying huge *rho* clones suggests that the *pipe* domain has its final shape before Egfr autoregulation takes place. Indeed, the enhancement and refinement of *rho* expression occurs during stage 10, and thus at a time were *pipe* expression disappears (Fig. 1E) (Peri et al., 1999).

Several observations had suggested that *fng*, and thus Notch signalling, played a role in *pipe* repression. The best evidence was derived from experiments in which *mirr* activity was manipulated, assuming that *mirr* acts via *fng* (Jordan et al., 2000; Zhao et al., 2000b). This indeed seems to be the case in early oogenesis, where *mirr* and *fng* mutant follicle cell clones cause virtually identical phenotypes (Grammont et al., 2001; Jordan et al., 2000). During midoogenesis *mirr* also represses *fng* and this appears to be important for chorion patterning (Jordan et al., 2000; Zhao et al., 2000a). Furthermore, ectopic expression of *mirr* leads to non-autonomous *pipe* repression (Jordan et al., 2000). This appears to be in conflict with our finding that neither *mirror* nor *fringe* loss-of-function clones show effects on *pipe* expression (Fig. 4A-F). However, both observations can be reconciled. Ectopic *mirr* expression induces *rho*, which activates the diffusible Egfr ligand Spi (Schweitzer et al., 1995). This leads to non-autonomous repression of *pipe* as *rho* flip-out clones demonstrate (Fig. 3G-I).

In summary, loss-of-function analysis shows that none of the signalling cascades that, upon ectopic activation, affects *pipe* is indeed involved in Grk-dependent *pipe* repression. This supports the idea that Grk directly defines the *pipe* border (Pai et al., 2000) and places high demands on the accuracy of Grk signalling and its co-ordination with the morphogenetic movements of the follicular epithelium. Grk emanates essentially from a point-like source in the vicinity of the asymmetrically localized oocyte nucleus (Neuman-Silberberg and Schüpbach, 1993). However, *pipe* forms a ventral stripe in the follicular epithelium, which has the same width along the entire AP axis of the egg. Slight mislocalization of Grk prevents the formation of this stripe (Roth and Schüpbach, 1994). During migration of the follicle cells towards the oocyte, dorsal follicle cells pass the oocyte nucleus. This might help to convert the point-like Grk source into a stripe-like pattern of *pipe* repression (Sapir et al., 1998). Furthermore, our data do not exclude the existence of Grk-independent activating signals for *pipe* that might contribute to the shape of the *pipe* domain. Recently, the HMG-box transcription factor Capicua has been shown to be required for *pipe* activation (Goff et al., 2001). However, *capicua* is evenly expressed in stage 9/10A egg chambers and thus seems not to contribute to the spatial regulation of *pipe* (Goff et al., 2001). Finally, it is conceivable that even morphogenetic processes during late oogenesis, which lead to egg chamber elongation and define the shape of the mature egg, contribute to making the stripe even along the AP axis.

pipe expression and embryonic patterning

Pipe is located in the Golgi complex and presumably modifies a non-diffusible extracellular matrix (ECM) component (Sen et al., 2000). Furthermore, *pipe* expression levels are uniform in the ventral 40% of the follicular epithelium (Sen et al., 1998). Thus, although the protein distribution of Pipe is not known, Pipe activity itself is unlikely to shape the nuclear Dorsal gradient of the embryo, which reaches its highest levels in a ventral domain encompassing 15% of the egg circumference (Costa et al., 1994; Rusch and Levine, 1996).

The expansion of the *pipe* domain in *grk* or *Egfr* mutants leads to a partial duplication of the DV pattern, which results from a Dorsal gradient with two peaks (Roth and Schüpbach, 1994; Morisato, 2001). Interestingly, this phenotype can also be generated by the overexpression of Spz (Morisato, 2001). The proteolytic processing of Spz generates N-terminal and C-terminal fragments. The C-terminal fragment presumably corresponds to the Toll activating ligand, while the N-terminal fragment appears to play an inhibitory role. On the basis of these observations, the following model has been proposed (Morisato, 2001). The proteolytic activation of Spz initially occurs in a broad region corresponding to the *pipe* domain. The C-terminal fragment is immediately bound by Toll and therefore does not diffuse far. The N-terminal fragment, however, diffuses and inhibits Spz production. In wild type, this process shapes the Dorsal gradient. In mutants with expanded *pipe* domain, the inhibitor accumulates ventrally, while it can leave the activation domain laterally. This leads to an activation profile with two peaks and consequently to a

partial DV pattern duplication. Experimental evidence for alternative inhibitory mechanisms exists (Misra et al., 1998; Dissing et al., 2001; LeMosy et al., 2001) that could account for the pattern duplications. In all these mechanisms, the inhibition is linked to the active proteolytic cascade.

The analysis of ectopic *pipe* expression in a wild-type background, which results from *Raf* clones, supports these models and allows the assessment of some of their parameters. First, partial pattern duplications are observed in the regions, which have expanded *twi* domains (Fig. 7D,E). At the point where the expansion starts, the ventral furrow bifurcates, suggesting the formation of a nuclear Dorsal distribution with two peaks. This shows that the pattern forming system can act locally in a restricted segment of the AP axis (comprising less than 30% egg length). Second, large *Raf* clones located at the dorsal side did not affect embryonic development (Fig. 6B). We expect that these clones lead to patches of ectopic *pipe* not connected to the endogenous *pipe* domain. In their study of *pipe* loss-of-function clones, Nilson and Schüpbach (Nilson and Schüpbach, 1998) concluded that 8- to 12-cell wide *pipe* domains at the ventral side were sufficient to initiate the high levels of Toll signalling required for *twi* expression. Our observations suggest that even wider *pipe* domains (occupying more than 30% of the AP axis) are unable to induce *twi* expression if they are at the dorsal side of a wild-type embryo. Similar conclusions have been drawn from the analysis of *Ras* mutant clones (James et al., 2002).

Judging from the dorsal ectoderm, which forms underneath large dorsally restricted *Raf* clones, we propose that such clones completely lack the ability to initiate Toll signalling. Although at the molecular level we have tested only *twi* expression, it is unlikely that these clones induce zygotic genes such as *short gastrulation*, the activation of which can be achieved by low levels of Toll signalling (Rusch and Levine, 1996). The phenotypic series of *dorsal* group genes shows that even low levels of Toll signalling suppress the dorsalmost cell fate, the amnioserosa (Roth et al., 1991). This, in turn, results in characteristic abnormalities in germband extension and patterning of the dorsal epidermis that we have not observed in eggs carrying dorsally located *Raf* mutant clones.

The apparent inability of isolated dorsal patches of *pipe* to induce Toll signalling can be explained in two ways. The normal ventral *pipe* domain might produce so much inhibitor diffusing to the dorsal side that the activation processes initiated by dorsal *pipe* expression are completely suppressed. Alternatively, independent inhibitory signals might be present on the dorsal side that have already originated during oogenesis (Araujo and Bier, 2000). We favour the first hypothesis, as it allows a common explanation for partial axis duplication and dorsal inhibition (Morisato, 2001). Theoretical models that can be used to simulate such behaviour require that long-range inhibition (lateral inhibition) is combined with autocatalytic processes that have a short range (local activation) (Meinhardt, 1989). A positive feedback loop involving the Easter protease might be part of the proteolytic cascade that leads to the cleavage of Spz (Dissing et al., 2001; LeMosy et al., 2001).

A pattern that forms by a system of local activation and lateral inhibition is to some degree independent from the spatial inputs that initiate the process (Meinhardt, 1989). This provides the system with considerable robustness exemplified in our experiments by the suppression of the effects of ectopic *pipe* at the dorsal side. It also may explain the twisting of the DV axis that we have observed in some embryos (Fig. 7B,C). Large lateral *pipe* clones may shift the centre of activation away from its normal position along the ventral midline. The system locally corrects for changes in *pipe* expression, generating a normal DV pattern at a given AP position. However, the overall bilateral symmetry of the pattern is lost. This, in turn, suggests that the bilateral symmetry of the embryo can be traced back to the evenness with which Grk signalling is received in both lateral halves of the follicular epithelium. Random cell clones unable to receive Grk can disrupt this situation. In conclusion, our findings, together with earlier work (Nilson and Schüpbach, 1998; Sen et al., 1998; Morisato, 2001), suggest the following: the *pipe* domain defines the side of the egg where the Dorsal gradient reaches peak levels, its lateral expansion determines the number of peaks (normally one) and its even AP extension is responsible for the bilateral symmetry of the embryo. However, the fine-grained spatial information reflected in the slope of the Dorsal gradient results from a self-organizing pattern-forming process.

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