The origin of dorsoventral polarity in *Drosophila*

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In *Drosophila* dorsoventral (DV) polarity arises during oogenesis when the oocyte nucleus moves from a central posterior to an asymmetrical anterior position. Nuclear movement is a symmetry-breaking step and establishes orthogonality between the anteroposterior and the DV axes. The asymmetrically anchored nucleus defines a cortical region within the oocyte which accumulates high levels of *gurken* messenger RNA (mRNA) and protein. Gurken is an ovarian-specific member of the transforming growth factor- α (TGF- α) family of secreted ligands. Secreted Gurken forms a concentration gradient that results in a dorsal-to-ventral gradient of EGF receptor activation in the follicle cells surrounding the oocyte. This leads to concentration-dependent activation or repression of target genes of the EGF pathway in the follicular epithelium. One outcome of this process is the restriction of *pipe* expression to a ventral domain that comprises 40% of the egg circumference. Pipe presumably modifies extracellular matrix components that are secreted by the follicle cells and are present at the ventral side of embryo after egg deposition. Here, they activate a proteolytic cascade that generates a gradient of the diffusible ligand, Spätzle. Spätzle activates the Toll receptor at the surface of the embryo that stimulates the nuclear uptake of the transcription factor Dorsal. This leads to a nuclear concentration gradient of Dorsal that specifies the cell types along the DV axis of the embryo.

Keywords: axis formation; nuclear movement; germline-soma signalling; transforming growth factor- α ; mRNA localization

1. INTRODUCTION

Early insect embryos are characterized by two apparently contradictory features that are rarely found in combination in embryos of other invertebrates and never in embryos of vertebrates. They generate a vast number of undetermined cells despite the fact that the major body axes, the AP and DV axes, are fixed even prior to fertilization and egg deposition. Insects achieve this by producing fairly large yolk-rich eggs that contain precisely positioned spatial cues for axis formation (Schwalm 1988). There are two types of such cues: intracellular cytoplasmic determinants anchored to the oocyte cortex and extracellular signals that emanate from localized regions of the eggshell or ECM (St Johnston & Nüsslein-Volhard 1992). Early embryogenesis is syncytial: the nuclei divide within the yolk-rich cytoplasm and then migrate to the periphery (Schwalm 1988). Here, the transcriptional programme of the nuclei is modulated either directly by the cytoplasmic determinants or indirectly by the extraembryonic signals, which activate cytoplasmic signalling cascades via transmembrane receptors.

These processes have been discovered and extensively analysed in the higher dipteran *Drosophila melanogaster* (St Johnston & Nüsslein-Volhard 1992). However, classical insect embryology (Sander 1976) together with comparative molecular studies (Lall & Patel 2001) suggests that some basic aspects of the picture gained from *Drosophila* apply to

most pterygote insects. I will come back to the question of evolutionary conservation at the end of this article.

The *Drosophila* egg displays a clear axial organization that allows one to predict how the future embryo will develop. The outer eggshell, the chorion, harbours several specialized structures that can serve as markers for the egg axes (figure 1a). The micropyle, containing a channel for sperm entry, is found at the anterior pole where the future head of the embryo will develop, an aeropyle is present at the posterior end, and the respiratory appendages mark the dorsal side (Spradling 1993). Within the egg cytoplasm mRNAs are already localized to the anterior and posterior poles during mid oogenesis where they remain until the beginning of embryogenesis (figure 1b; for a review see Riechmann & Ephrussi 2001). *bicoid* mRNA, the determinant responsible for head and thorax development is localized to the anterior cortex and *oskar* mRNA, the determinant responsible for the formation of the primordial germ cells (pole cells) and the abdomen, is localized to the posterior cortex. We still lack ways to visualize the sources for the extraembryonic signals in the deposited egg. However, the relevant spatial cues can be traced back to the localized expression of certain genes within the follicular epithelium, a monolayer of somatic cells that surrounds the growing oocyte and secretes the eggshells. *pipe* represents the follicle cell-specific gene responsible for establishing the DV axis of the embryo. A *pipe-LacZ* reporter gene marks the cells in late egg chambers that had expressed *pipe* earlier during oogenesis (figure 1c; Sen *et al.* 1998). The broad ventral stripe of *pipe-LacZ* expression corresponds to the region from

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Figure 1. The axial organization of the *Drosophila* egg. (a) Chorion of a deposited egg. The chorion imprints show the shape of the follicle cells that secrete first the inner and then the outer eggshell (Chorion) during late oogenesis. (b) *bcd* and *osk* mRNA distribution during the final stage (14) of egg chamber maturation. (c) *pipe-LacZ* expression in follicle cells during the final stage (14) of egg chamber maturation. The expression is visualized using anti- β -galactosidase antibodies.

which extraembryonic signals emanate and induce ventral fates in the embryo.

An explanation of how these spatial cues arise obviously requires an investigation of how polarity arises during oogenesis. In this article, I will concentrate on the origin of the DV axis. To introduce the topic some background is provided on the morphology of the ovary (Spradling 1993) and on the establishment of AP polarity that occurs prior to, and is a prerequisite for, DV axis formation (Van Eeden & St Johnston 1999; Riechmann & Ephrussi 2001).

The *Drosophila* ovary is composed of independent strings of egg chambers called ovarioles (figure 2). The anterior tip of each ovariole is formed by the germarium, a structure that harbours the germline and somatic stem cells. The egg chambers are assembled at the posterior end of the germarium. A single egg chamber is composed of a cluster of 16 germ cells, 15 nurse cells and the oocyte, surrounded by the monolayer of somatic follicle cells mentioned earlier. Gene expression is largely turned down in the oocyte nucleus. Therefore, oocyte growth depends on products of the polyploid nurse cells that are transported to the oocyte through cytoplasmic bridges (ring canals). During egg chamber formation the oocyte–nurse cell cluster is polarized so that the oocyte is usually positioned posterior to the nurse cells (Gonzalez-Reyes & St Johnston 1998; Godt & Tepass 1998). This polar arrangement of nurse cells and oocyte orients the AP axis of the later embryo and indeed generates a link between the AP axis of the adult female and the future embryo.

The manner by which the polar arrangement of the oocyte–nurse cell cluster determines the AP axis of the embryo is complex and comprises the reciprocal exchange of signals between the oocyte and the surrounding follicle cells (Gonzalez-Reyes & St Johnston 1994; Gonzalez-Reyes *et al.* 1995; Roth *et al.* 1995). First, the posteriorly localized oocyte signals to the adjacent terminal follicle cells and induces them to adopt a posterior fate. The oocyte signal is the TGF- α -like ligand Grk that is secreted

Figure 2. Structure of ovariole and intermediate stage egg chambers. In stages 6–8, the MT polarity is indicated by + and – signs within the oocyte (yellow). Anterior (green) and posterior (pink) terminal follicle cells are distinguished from mainbody follicle cells (white).

Figure 3. Nuclear movement determines orthogonality of the body axes. (a) Wild-type. A posterior-to-anterior movement forces the nucleus to acquire an asymmetrical position. AP and DV axes are oriented orthogonally to each other. (b) If nuclear movement does not occur, AP and DV axes are parallel to each other.

Figure 4. Egg phenotypes resulting from aberrant oocyte nucleus behaviour. (a) Wild-type. (b) An egg derived from a binuclear egg chamber. Dorsal chorion structures are duplicated. (c) An egg derived from an egg chamber in which the oocyte nucleus remained in a posterior position. Dorsal chorion structures are present as a symmetric ring at the posterior pole. (d) An egg derived from an egg chamber in which the nucleus did not remain anchored at the dorsal-anterior pole. Dorsal chorion structures are absent.

Figure 5. Two models for the movement of the oocyte nucleus. The nucleus is depicted during its migration to the anterior pole (left). Three types of MTs are shown (red): cortical MTs extending the plus ends preferentially to the posterior pole of the oocyte, cortical MTs extending the plus ends preferentially to the centre of the oocyte and MTs forming a cage around the nucleus whose polarity has not been determined. (a) The dynein-dynactin complexes that move the nucleus are anchored to the oocyte cortex. This anchorage could occur through an interaction of ARP1, a subunit of the dynactin complex, with spectrin, a typical component of the cell cortex (e.g. Muresan *et al.* 2001). The motor domains of the cortical dynein contact the MT cage of the nucleus. To ensure polar movement, the MTs of the cage require to be polarized with regard to the AP axis of the oocyte. (b) The dynein-dynactin complexes responsible for nuclear movement are anchored to the nucleus. They might be either identical to the dynein-dynactin complexes that organize the nuclear MT cage or they might represent a distinct population connected to the cage as depicted here. The motor domains contact cortical MTs that are polarized along the AP axis with their minus ends pointing to the anterior pole. This generates a nuclear movement towards the anterior pole.

and activates the *Drosophila* Egfr in abutting follicle cells. If this signalling process is disrupted in *grk* or *egfr* mutants, egg chambers develop with two anterior poles indicating that anterior development is the groundstate for the terminal follicle cells. Later, the posterior follicle cells signal back to the oocyte. The ligands and receptors employed in this 'back signalling' process have not been identified. However, we know that 'back signalling' has a profound effect on MT polarity of the oocyte. In early stages of oogenesis the MTs are nucleated from a MTOC present at the posterior of the oocyte (Theurkauf *et al.* 1992). From there they extend through the ring canals into the nurse cells and fulfil important functions in nurse cell-to-oocyte transport. 'Back signalling' destabilizes the posterior MTOC. As at the same time a diffuse anterior MTOC emerges, the overall polarity of the MT network is reversed. An anterior-posterior gradient of MTs forms with most minus ends at the anterior and plus ends extending to the posterior of the oocyte. This polarized

MT network is required for the correct polar transport of *bicoid* mRNA to the anterior pole and of *oskar* mRNA to the posterior pole of the oocyte. The polar localization of these mRNAs together with subsequent anchoring processes to the oocyte cortex directly determines the AP axis of the later embryo (for a review see Palacios & Johnston 2001). The polarized MT network also leads to the posterior-to-anterior movement of the oocyte nucleus that establishes the DV polarity of the egg chamber and, through a rather indirect multi-step process, the DV axis of the embryo.

2. ASYMMETRICAL NUCLEAR POSITIONING AND THE ORIGIN OF DORSOVENTRAL POLARITY OF THE EGG CHAMBER

(a) *Three general features of dorsoventral axis induction by nuclear positioning*

During mid oogenesis the *Drosophila* oocyte acquires a half-ellipsoidal shape, being pointed at the posterior pole and with a circular cross-section at the anterior pole facing the nurse cells (figure 3). We assume that the nucleus stays anchored cortically during its posterior-to-anterior movement. Therefore, the geometry of the oocyte forces the nucleus to follow an asymmetrical trajectory with regard to the AP axis, so that when it arrives at the anterior pole it is forced to occupy a particular point on the circular circumference. This point corresponds to the dorsal side of the egg chamber as can be seen from the course of later development in which dorsal chorion structures differentiate close to the nucleus (figure 3a). In the mature egg, when the nucleus completes meiosis II, it is still present in a dorsal-anterior position.

Several observations have demonstrated that nuclear movement is not just correlated with, but indeed establishes, DV polarity. Laser ablations of the nucleus cause a loss of dorsal chorion structures (Montell *et al.* 1991). If nuclear movement is prevented as in mutants of *mago* or when the Grk signal is delayed, eggs develop that have a normal AP axis, but lack DV polarity orthogonal to the AP axis (figure 3b; Micklem *et al.* 1997; Peri & Roth 2000). They are rotationally symmetrical expressing the same cell fates along their DV circumference. Such eggs may indeed possess dorsal chorion structures in a ring at the posterior pole (figure 4c). Thus, the posteriorly localized oocyte nucleus leads to a dorsalizing signal that is identical along the DV circumference. As these eggs exhibit a sequence of different DV chorion fates along the AP axis. One may say that they have a DV axis that is parallel to the AP axis (figure 3b). This phenotype reveals that nuclear movement establishes the perpendicular orientation that the DV axis normally possesses with respect to the AP axis.

The demonstration that an asymmetrical anterior position of the oocyte nucleus is necessary for the establishment of the DV axis does not, however, prove that the nuclear movement defines the dorsal side of the egg chamber. It is conceivable that the nucleus moves towards a position of the egg chamber that has been selected through other processes. A DV polarization of individual egg chambers by the overall architecture of the ovary had, for example, been proposed for another higher dipteran, the house fly, *Musca domestica* (Kleine-Schonnefeld &

Engels 1981). However, this is unlikely to be true for *Drosophila*. First, the DV axes of different egg chambers within an ovariole are randomly oriented with regard to each other and with regard to the DV axis of the female (S. Roth, unpublished data). Second, certain genetic conditions allow the formation of egg chambers in which the nurse cells and the oocyte contain two nuclei (Roth *et al.* 1999). In such binuclear oocytes both nuclei move to the anterior cortex and induce dorsal chorion fates (figure 4b). A statistical evaluation shows that they choose their position randomly with regard to each other. Without making very specific assumptions this behaviour is most compatible with the complete lack of DV asymmetry prior to the nuclear movement. Thus, nuclear movement is likely to be a true symmetry-breaking step that randomly determines the dorsal side of the egg chamber.

Anterior nuclear positioning has a third general property besides establishing orthogonality of the body axes and defining the dorsal side by symmetry breaking. After the nucleus has reached its anterior-dorsal position it has to be maintained there for a considerable period of time to fulfil its function in axis formation. If the nucleus migrates correctly but later slides back to the posterior pole, as in *cnc* mutants, the resulting egg chambers completely lack DV polarity (figure 4d; Guichet *et al.* 2001). Thus, the nucleus does not just initiate a process that perpetuates itself. Rather, nuclear migration has to be followed by stable nuclear anchoring to allow sufficient time for axis induction.

(b) *Molecular mechanisms of nuclear positioning*

The posterior-to-anterior migration of the oocyte nucleus takes place when the MT network of the oocyte becomes repolarized. In *grk* mutants this repolarization does not occur and MTOCs form at both ends of the oocyte. Such a 'bipolar' MT distribution leads to defects in nuclear positioning (Gonzalez-Reyes *et al.* 1995; Roth *et al.* 1995). In most cases the nucleus remains at the posterior pole, but it can also be found at the anterior pole and in rare cases in intermediate positions. Thus, the nucleus predominantly localizes to regions of high density of MT minus ends. The requirement of an intact MT network for nuclear movement also follows from experiments in which flies are treated with colchicine (Koch & Spitzer 1983; Saunders & Cohen 1999). Mild treatments already prevent normal nuclear movement. Together these observations suggest a simple picture in which the nucleus moves with the help of minus-end directed MT-dependent motor proteins along MT tracks that nucleate at the anterior of the oocyte.

However, several observations indicate that this scheme is too simple. First, two different populations of MTs appear to be involved in nuclear movement and anchorage. MTs not only assemble from the cortex of the oocyte, but also from the periphery of the nucleus itself so that the nucleus becomes surrounded by a MT cage (Theurkauf *et al.* 1992; Januschke *et al.* 2002; figure 5). Treatments that affect this MT cage not only prevent anterior-dorsal positioning of the nucleus, but also lead to its detachment from the lateral cortex. This suggests an essential role for the cage in anchoring of the nucleus to the oocyte cortex. Second, there are two pools of dynein and components of the dynactin complex within the oocyte (Li *et al.* 1994;

Swan *et al.* 1999). Dynein and dynactin are present at the cortex and enriched at the posterior pole of the oocyte. In addition, a layer of dynein and dynactin is found surrounding the nucleus. The perinuclear dynactin accumulation appears to be independent of MTs, but itself is required for the formation of the nuclear MT cage (Januschke *et al.* 2002). Third, both minus- and, surprisingly, also plus-end directed motors are required for anterior-dorsal positioning of the nucleus (Brendza *et al.* 2002; Duncan & Warrior 2002; Januschke *et al.* 2002).

The precise role of dynein in nuclear positioning is difficult to assess since interference with dynein leads to the disassembly of the nuclear cage and the subsequent lateral detachment of the nucleus. In kinesin I mutants the nucleus moves to the anterior pole, but cannot maintain its anterior position. During oocyte growth the nucleus slides back; however, it always remains attached cortically.

There are two possibilities to explain the requirement for kinesin I. Either kinesin I acts directly by moving the nucleus towards the plus ends of certain MT populations or it is only indirectly involved by improving the efficiency of dynein function. As shown by Cha *et al.* (2001, 2002) a considerable number of oocyte MTs emanate from the lateral cortex and extend their plus ends into the centre of the oocyte. During growth of the oocyte and anterior-to-posterior migration of the follicle cells, lateral domains of the oocyte cortex might be displaced towards the posterior pole. If the nucleus were tightly attached to the lateral cortex, this process could potentially cause its slide backward during oocyte growth. Therefore, kinesin I might be required to cause a weak detachment from the lateral cortex by moving the nucleus along inwards-pointing MTs. This might allow the nucleus to remain anchored to the interface between oocyte and nurse cells in a dorsal-anterior position. The other alternative to explain the role of kinesin I is linked to the observation that opposite polarity motors often collaborate in the same transport process (Gross *et al.* 2002). For example, the efficient dynein-dependent transport of *bcd* mRNA to the anterior pole requires kinesin I (Januschke *et al.* 2002; Duncan & Warrior 2002). This is likely to be owing to the fact that the dynein distribution itself changes in kinesin I mutants. Instead of a posterior accumulation, dynein is enriched at the anterior pole (Brendza *et al.* 2002; Duncan & Warrior 2002; Januschke *et al.* 2002). The posterior accumulation of dynein might replenish the pool of motor proteins required for sustained posterior-to-anterior transport. If the anterior maintenance of the nucleus (prior to stable anchoring) requires continuous motor activity it is conceivable that this process also depends on the replenishment of posterior dynein pools (Cohen 2002).

One of the most interesting questions that still remains unanswered is where the dynein motors are localized that produce the force for nuclear movement. Do they reside in the oocyte cortex and move the nuclear MT cage relative to the cortex (figure 5a) or do they travel with the nucleus and move along MTs of the cortex (figure 5b)? Only the latter model provides an easy explanation for how the motor proteins sense the overall polarity of the oocyte MT network (Januschke *et al.* 2002).

3. THE OOCYTE NUCLEUS AS A SIGNALLING CENTRE

(a) *gurken* RNA localization

The asymmetrically localized nucleus becomes a signalling centre since the mRNA of the secreted TGF- α -like ligand Grk accumulates in an anterior-dorsal cap around it (Neuman-Silberberg & Schupbach 1993). *grk* mRNA is probably transported into the oocyte from the nurse cells and also produced by the oocyte nucleus itself (Saunders & Cohen 1999). However, the expression of *grk* mRNA by the nucleus is insufficient to explain the localization of the transcripts. Polar transport processes involving dynein and kinesin I are crucial (Brendza *et al.* 2002; Januschke *et al.* 2002). Again, dynein is likely to be the primary motor while kinesin I might be involved in replenishing the dynein pools. In an elegant series of experiments, MacDougall *et al.* (2003) have shown that *grk* transcripts are transported in a two-step process, both requiring dynein, first from the posterior of the oocyte to the anterior pole and subsequently from there to the nucleus. They suggest that each step depends on a different population of MTs, the first on MTs with minus ends at the anterior cortex and the second on MTs with minus ends close to the nucleus. Thus, the nuclear cage mentioned in §2b appears to have MTOC activity for long-range (re)organization of the MT network of the oocyte. This conclusion had also been drawn from the analysis of *cnc* mutant oocytes in which the nucleus does not maintain its anterior position (Guichet *et al.* 2001). The incorrect positioning of the nucleus is accompanied by global changes of the MT network of the oocyte and by the mislocalization of *bcd* and *osk* mRNAs while *grk* mRNA preserves its cap-like attachment to the nucleus.

The two-step process of *grk* mRNA localization poses an interesting problem. How do mRNA-motor complexes distinguish between different MT populations and how are the different MT populations marked in the first place? Dynein-dynactin complexes loaded with *bcd* mRNA should be transported to and remain at the anterior cortex, while those loaded with *grk* mRNA should be subject to a second transport step toward the nucleus. Deletions within the *grk* 3'UTR allow anterior localization of *grk* mRNA but prevent its transport to the nucleus (Thio *et al.* 2000). This suggests that specific factors distinguish between anterior and anterior-dorsal transport of *grk*. The heterogeneous nuclear RNA-binding protein Squid plays a central role in this process. It regulates both *grk* localization and translation and binds directly to the *grk* 3'UTR (Norvell *et al.* 1999).

(b) *Gurken* secretion

As is the case for other localized mRNAs there is some indication for translational control mechanisms that ensure that the bulk of *grk* mRNA translation takes place only when the RNA is correctly localized (for reviews see Norvell *et al.* 1999; Johnstone & Lasko 2001). The local translation of *grk* mRNA close to the nucleus leads to the anterior-dorsal accumulation of Grk protein that enters the secretory pathway. Secreted Grk is released and activates the *Drosophila* Egfr present in the overlying follicle cells. This initiates the second round of Grk signalling that establishes DV polarity in the follicular epithelium. The direct binding between Grk and Egfr has been demon-

Figure 6. The oocyte nucleus as signalling centre. (a) Cross-section through the anterior pole of a stage 10A oocyte. Gurken protein (blue) is concentrated between the oocyte nucleus and plasmamembrane of the oocyte. (b) Enlarged view of the dorsal side of a section similar to that shown in (a). Blue dots within the follicle cells reveal the uptake of Gurken protein. Abbreviations: fc, follicle cells; n, oocyte nucleus.

Figure 7. Dorsoventral patterning of the follicular epithelium. (a) Lateral view of stage 10 egg chamber showing *kekkon* and *pipe* mRNA distribution in the follicular epithelium. (b) Cross-section through a stage 10 egg chamber showing *kekkon* mRNA. (c) Cross-section through a stage 10 egg chamber showing *pipe* mRNA. Abbreviations: fc, follicle cells; n, oocyte nucleus; nc, nurse cells.

strated (Shmueli *et al.* 2002) and the uptake of Grk protein by follicle cells close to the Grk source can be visualized (figure 6; Peri *et al.* 1999; Queenan *et al.* 1999).

As a TGF- α -like ligand Grk has a single transmembrane domain and has to be proteolytically processed to be active (Ghiglione *et al.* 2002). Grk function appears to be entirely restricted to the female germline. However, *Drosophila* has two other TGF- α -like ligands, Spitz and Keren, which act in somatic tissues. Spitz is involved in a large number of signalling processes (Schweitzer & Shilo 1997). The function of Keren is currently not known (Reich & Shilo 2002). The biochemistry of ligand activation has

Figure 8. The specification of the respiratory appendages. (a) The distribution of activated (dephosphorylated) MAPkinase (mitogen-activated protein kinase) reveals the pattern of Egfr signalling in the dorsal follicle cells of a stage 10 egg chamber. (b) Expression of *Broad-Complex* in groups of cells on either side of the dorsal midline. These cells will form the respiratory appendages. Their expression domains correspond to regions of intermediate MAPkinase activation.

Figure 9. *pipe* expression and the nuclear Dorsal gradient. (a) Cross-section through a stage 10 egg chamber. *pipe* is expressed in the ventral 40% of the circumference. (b) Cross-section through a syncytial blastoderm embryo. Nuclear uptake of Dorsal protein is restricted to the ventral 40% of the circumference. Abbreviations: fc, follicle cells; n, oocyte nucleus.

been best studied in the case of Spitz (Lee *et al.* 2001; Urban *et al.* 2001; Tsruya *et al.* 2002), and what has been found there seems to apply to Grk (Ghiglione *et al.* 2002; Urban *et al.* 2002). Both Spitz and Grk need to be cleaved so that the active secreted ligand is generated. In the case of Spitz cleavage does not occur as for vertebrate TGF- α predominantly at the plasmamembrane by TACE, an ADAM family metalloprotease, but rather within the Golgi apparatus by Rhomboids 1 and 3, which are members of a novel class of seven transmembrane proteases (Lee *et al.* 2001; Urban *et al.* 2001). Grk is presumably cleaved by another member of the Rhomboid family, Rhomboid 2 (or Brother of Rhomboid) although Grk cleavage might already occur within the ER (Guichard *et al.* 2000; Urban *et al.* 2001; Ghiglione *et al.* 2002). Passage of Spitz through the secretory pathway requires one additional component, the single-pass transmembrane protein Star that mediates the ER-to-Golgi transport of Spitz (Lee *et al.* 2001; Tsruya *et al.* 2002). An involvement of Star in Grk secretion has also been suggested so that

Figure 10. The Dorsal gradient and its zygotic target genes. Cross-sections through cellular blastoderm embryos. (a) Dorsal protein distribution, (b) *short gastrulation* and *snail* expression and (c) *short gastrulation* and *decapentaplegic* expression. Abbreviations: sog, *short gastrulation*; sna, *snail*; dpp, *decapentaplegic*. High nuclear concentrations of Dorsal protein activate the expression of zygotic genes like *snail* that are required for mesoderm formation. High and low nuclear concentrations of Dorsal protein activate zygotic genes that are required for the formation of the neuroectoderm. Such genes are in turn repressed by *snail* and therefore confined to broad lateral domains as exemplified by *short gastrulation*. High and low nuclear concentrations of Dorsal protein also repress several zygotic genes that, in the absence of Dorsal, are activated uniformly in all cells of the early embryo. In the presence of Dorsal they are confined to the dorsal half of the embryo. Among those, *decapentaplegic* has a central role in specifying the non-neurogenic ectoderm. (For a review, see Stathopoulos & Levine 2002.)

the basic mechanisms of Spitz and Grk processing and transport appear to be similar (Ghiglione *et al.* 2002). However, one factor seems to be specific for Grk. The small hydrophobic transmembrane protein cornichon is essential for Grk secretion (Roth *et al.* 1995; Queenan *et al.* 1999; Ch. Bökel, S. Dass and S. Roth, unpublished results). Cornichon is the founding member of a family of proteins that have so far been identified in all eukaryotic cells. Like its homologue Erv14 in yeast (Powers & Barlowe 1998, 2002), cornichon appears to be involved in ER-to-Golgi transport and we currently believe that it presents a specific cargo receptor that recruits Grk into COPII vesicles mediating the anterograde transport from ER to Golgi (Ch. Bökel, S. Dass and S. Roth, unpublished results). As Star has a similar function it is currently not clear why Grk but not Spitz requires an additional factor for the same transport step.

Judging by the appearance of vesicles in the abutting follicle cell, the bulk of Grk secretion occurs in a very restricted domain immediately above the oocyte nucleus (figure 6). However, clonal analysis with a mutation in a negative regulator of the Egfr pathway has shown that Grk actually activates the receptor even at the ventral side of the egg chamber (Pai *et al.* 2000). This implies that the active Grk ligand spreads around the entire oocyte circumference and that it probably forms a steep concentration gradient extending from the dorsal to the ventral side. It is not clear how this gradient forms. Either the secreted form of Grk diffuses in the space between the follicle cells and the oocyte membrane or there is some spreading of secretory vesicles containing Grk within the oocyte cytoplasm. We also cannot exclude the possibility that the small amounts of *grk* mRNA that are found in an anterior ring around the entire circumference (MacDougall *et al.* 2003) are translated to some extent.

4. PATTERN FORMATION IN THE FOLLICULAR EPITHELIUM INDUCED BY GURKEN SIGNALLING

Along the DV axis of the egg chamber there are at least three types of follicle cell that can be distinguished by their

gene expression pattern, by the eggshell structures they secrete and by the function they fulfil for embryonic axis induction (figure 7). These are the dorsal cells that express *kekkon* (Ghiglione *et al.* 1999) and give rise to dorsal chorion structures including the respiratory appendages, the ventral cells that express *pipe* and provide the cues for the embryonic DV axis (Nilson & Schupbach 1998; Sen *et al.* 1998) and the lateral cells that neither express *kekkon* nor *pipe*. Grk signalling to the dorsal side might induce secondary signalling cascades that define these expression domains. Alternatively, being present in a concentration gradient Grk might act as a morphogen, i.e. *kekkon* and *pipe* might be direct target genes whose activation or repression depends on particular concentration thresholds of Grk. Genetic mosaic studies support the latter view (Pai *et al.* 2000; James *et al.* 2002; Peri *et al.* 2002). Mutant follicle cell clones, which cannot transmit the Grk signal (e.g. clones of mutations in downstream components of Egfr signalling, like *Ras* or *Raf*) show ectopic expression of *pipe* in a cell-autonomous manner. Conversely, if the Egfr pathway is ectopically activated in cell clones, autonomous activation of *kekkon* as well as autonomous repression of *pipe* is observed. Thus, Grk appears to have a direct and long-range function in regulating target gene expression in the follicular epithelium.

Interestingly, the initial DV subdivision of the follicular epithelium into broad zones is followed by an intricate patterning mechanism at the dorsal-anterior side required for the formation of the two respiratory appendages. This patterning mechanism is initiated by a positive feedback on Egfr signalling: Grk induces the expression of *rho* in dorsal follicle cells and *rho* in turn proteolytically activates Spitz (Wasserman & Freeman 1998). Production of activated Spitz leads to increased Egfr signalling and thus to an autocrine self-amplification of the signal. Spitz is diffusible and thus this signal amplification has the potential to spread throughout the follicular epithelium. However, this spreading is prevented in two ways. First, *rho* can be induced only in anterior follicle cells since its activation requires both TGF- α and TGF- β signalling (Peri & Roth

2000). The TGF- β ligand, Dpp, is produced from an anterior ring of follicle cells (Twombly *et al.* 1996) and spreads only for a certain distance towards the posterior. This delimits the region where *rho* can be activated and thereby prevents Spitz autoactivation from spreading to the posterior pole. Second, the threshold to initiate autoactivation is high and thus remains critically dependent on a certain level of Grk signalling. This assumption follows from the phenotypic series of *grk* alleles and in particular from the observation that reducing the dose of Grk by half leads to a fusion of the respiratory appendages (Neuman-Silberberg & Schupbach 1994; Peri *et al.* 1999). The dependency on Grk signalling restricts Spitz autoactivation to the dorsal side of the egg chamber.

Autoactivation alone would not constitute a pattern formation mechanism. Indeed, it is the combination of self-amplification with inhibitory processes that leads to patterning (Meinhardt 1982). In our case three different inhibitors are induced in dorsal follicle cells upon Egr activation (Peri *et al.* 1999): Kekk, a single-pass transmembrane protein (Ghiglione *et al.* 1999), Sprouty, an intracellular component (Reich *et al.* 1999; Kramer *et al.* 1999; Casci *et al.* 1999), and Argos a secreted, diffusible, inhibitory ligand of the Egr (Schweitzer *et al.* 1995; Golembo *et al.* 1996). Among these three inhibitors Argos might have the most important patterning function. It is expressed at the dorsal midline in regions of highest levels of Egr signalling (Queenan *et al.* 1997; Wasserman & Freeman 1998; Peri *et al.* 1999). According to Wasserman & Freeman (1998), Argos locally represses Egr signalling at the dorsal midline and this leads to the formation of two peaks of Egr activity that define the regions on either side of the dorsal midline where the respiratory appendages form. However, experiments with a constitutive active Egr receptor indicate that cells with submaximal levels of Egr activity give rise to the respiratory appendages while highest levels correspond to cells at the dorsal midline between the two appendages (Queenan *et al.* 1997; Peri *et al.* 1999). Therefore, we suggest that the spreading of Argos from its expression domain at the dorsal midline leads to a suppression of Egr signalling in more lateral cells and thereby defines the regions giving rise to the respiratory appendages. This model is in agreement with the observed activation pattern of the Egr pathway during the critical stages of pattern formation (figure 8). It is also supported by an excellent theoretical study in which computer simulations have been used to model the patterning of the dorsal appendages (Shvartsman *et al.* 2002; Steven Wiley *et al.* 2003).

5. THE INDUCTION OF THE EMBRYONIC AXIS

The expression of *pipe* in the ventral follicle cells is required for the establishment of embryonic DV polarity (Nilson & Schupbach 1998; Sen *et al.* 1998). Females mutant for *pipe* produce eggs with normal eggshell polarity. However, the embryos that develop in such eggs are completely dorsalized, i.e. they lack DV polarity in gastrulation and produce dorsalmost cell fates around the entire embryonic circumference. *pipe* encodes a heparan sulphate 2-O-sulphotransferase and thus is probably involved in the modification of glycosaminoglycan containing ECM components (Sen *et al.* 1998, 2000). The

particular ECM components modified by Pipe have not been identified so far, but it is likely that the *pipe* expressing cells deposit these components into the space between oocyte and follicle cells before the latter start to produce the inner and the outer eggshell, the vitelline membrane and chorion, respectively. Consequently, after egg deposition the modified ECM components should be present either on the vitelline membrane facing the embryo or directly on the surface of the embryo. Here, they are believed to initiate a proteolytic cascade that comprises a chain of four serine proteases and culminates in the activation of the extracellular ligand Spätzle (for a review see Morisato & Anderson 1995). Spätzle has structural similarities to nerve growth factor (Morisato & Anderson 1994; Mizuguchi *et al.* 1998; DeLotto & DeLotto 1998). It activates the transmembrane receptor Toll that is evenly expressed on the surface of the embryo (Hashimoto *et al.* 1991). Activated Toll relays the extracellular signal to the embryonic nuclei by regulating the nuclear import of the NF- κ B transcription factor Dorsal.

The way *pipe* induces the embryonic axis poses two interesting problems. First, the width of the ventral domain specified by *pipe* has to be constant along the entire AP axis of the future embryo (compare figures 1c and 7a). This is a prerequisite for long-germ development (see the evolutionary considerations that follow) in which not only all segments along the AP axis, but also their DV subdivision are specified simultaneously. Thus, the ventral region of the follicular epithelium defined by *pipe* expression during mid oogenesis has to expand in a very regular way when the egg elongates and the initial ventral stripe of *pipe* must be set up with high accuracy (Peri *et al.* 2002). This seems not to be a trivial task since Grk essentially emanates from a point-like source in the vicinity of the asymmetrically localized oocyte nucleus. Thus, the patterns of Grk-dependent gene expression in the follicular epithelium should be essentially of radial symmetry. A deviation from radial symmetry might result from morphogenetic movements of the follicle cells or from an AP modulation of transcription. During mid oogenesis the follicle cells in contact with the nurse cells become thin and flat while the follicle cells in contact with the oocyte form a high epithelium. These morphogenetic changes are accompanied by a migration of anterior follicle cells toward the posterior pole. As during the migration phase the nucleus is already asymmetrically localized, dorsal follicle cells pass the source of Grk signalling. This might help to convert the point-like Grk source into a stripe-like pattern of *pipe* repression. It is also possible that Grk-independent activators of *pipe* transcription exist that are not uniformly distributed along the AP axis of the egg chamber. They might counteract Grk repression in a differential way along the AP axis.

Second, as the target of *pipe* is likely to be an ECM component, we assume that it is not diffusible and its local concentration probably reflects the levels of *pipe* expression. The *pipe* domain comprises the ventral 40% of the egg chamber circumference and within this domain *pipe* expression levels are uniform. The Dorsal protein gradient of the later embryo forms within the ventral 40% of the embryonic circumference and thus within the segment of the egg circumference specified by *pipe* (Sen *et al.* 1998; Morisato 2001; figure 9). However, this correspon-

dence does not offer an explanation of how the graded distribution of nuclear Dorsal arises that has a sharp ventral peak (15% of the circumference) centred within the region specified by *pipe* (figure 9). This becomes even more obvious from experiments in which the *pipe* domain is expanded or in which the amount of Spätzle precursor is increased. In these cases a partial duplication of the DV pattern is observed (Roth & Schüpbach 1994; Morisato 2001). Dorsal gradients with peaks on either side of the ventral midline are formed and consequently the ventralmost cell fates are duplicated. Such pattern alterations suggest that, as in the case of respiratory appendage formation, the activation process is linked to inhibitory processes. A closer look at the biochemistry of the protease cascade activating Spätzle reveals formal analogies to the patterning mechanism discussed for the respiratory appendages. The proteolytic cascade involves both positive and negative feedback mechanisms: downstream proteases have been proposed to either proteolytically activate or inactivate upstream proteases (Dissing *et al.* 2001; LeMosy *et al.* 2001). The former would constitute a positive, the latter a negative feedback loop. Furthermore, the cleavage of the precursor form of Spätzle not only generates the active ligand for Toll, but also a diffusible peptide that might function as an inhibitor of the protease cascade (Morisato 2001). Taken together these results suggest that *pipe* only defines the limits of a region in which a self-regulative pattern forming system generates the gradient of active Spätzle. This extraembryonic gradient in turn initiates the formation of the intracellular Dorsal nuclear gradient that determines the cell fates along the DV axis of the embryo (figure 10; see figure caption and, for a review, see Stathopoulos & Levine 2002).

6. EVOLUTIONARY CONSIDERATIONS

Like the *Drosophila* egg, typical insect eggs show a clear axial organization with bilateral symmetry (Hinton 1981; Gutzeit & Sander 1985). They are banana shaped, although the pronouncement of the curvature and the length might vary greatly ranging from ones that are long and thin to those that are short, thick and almost spherical. The curvature allows a prediction of the dorsal and the ventral side of the egg and differences between the two ends permit the identification of the anterior and posterior poles. Thus, just from the mere shape of insect eggs we have to conclude that, as in *Drosophila*, the orientations of the body axes of the future embryo are determined during oogenesis. However, does this also apply to the particular molecular mechanisms of axis formation that operate in *Drosophila*?

At least one aspect appears to be conserved to some degree. A correlation between the position of oocyte nucleus and the orientation of the DV axis is found in many insect orders indicating that the establishment of DV polarity in insects relies on an ancient mechanism. In most cases the asymmetrical position of the oocyte nucleus marks the future dorsal side. An impressive study on a hemimetabolous insect, the house cricket *Acheta domesticus* (Orthoptera), was conducted by Netzel (1965, 1968). In *Acheta* the nucleus first moves into the posterior half of the oocyte and then in a perpendicular direction towards one side of the follicular epithelium that will

become the dorsal side of the egg. The follicle cells close to the nucleus as well as those opposite to it undergo characteristic shape changes that even in the late 1960s led Netzel to conclude that the nucleus acts as a signalling centre and induces a pattern of different cell fates within the follicular epithelium. The situation observed in crickets appears to be prototypic for many insects. However, exceptions are found even within the orthopterans. In the grasshopper *Schistocerca americana* the nucleus just moves to the posterior pole where it remains in a symmetrical position with regard to the DV axis of the egg (Patel *et al.* 2001). Despite this, the DV axis of the egg and embryo appears to be determined during oogenesis since the shape of *Schistocerca* eggs has an overt DV asymmetry. In both *Acheta* and *Schistocerca* the position of the nucleus corresponds to the region of the egg that later forms the germ rudiment (Schwalm 1988). The germ rudiment harbours the anlagen of the embryo proper while the remainder of the egg gives rise to extraembryonic tissue. In short-germ insects the germ rudiment may occupy only a small fraction of the egg and comprise only the anlagen of the head or the head and thorax, while the posterior parts of the embryo develop from a growth zone. As the short-germ mode of development is ancestral compared to the long-germ mode found in holometabolous insects, it is possible that in addition to specifying DV polarity the oocyte nucleus has an evolutionarily ancestral role in determining the region of the egg that gives rise to the embryo proper.

Despite the phenotypic similarities and dissimilarities it is difficult to say how many of the actual signalling events are conserved between different insect species. Attempts to clone *grk* from insects outside the *Drosophilids* have failed so far (S. Roth, unpublished data). The *gurken* homologue from *Drosophila virilis* shares only 34% overall sequence identity with *gurken* from *Drosophila melanogaster*, although *grk* mRNA localization during oogenesis is identical in both species (Peri *et al.* 1999). The genome sequence of the lower dipteran *Anopheles gambiae* has been found to contain only one TGF- α homologue that is equally related to *grk* and *spitz* (Holt *et al.* 2002; P. Kalscheuer and S. Roth, unpublished data). Thus, despite its fundamental role in the establishment of both body axes in *Drosophila*, *grk* appears to be a fast-evolving gene. It shares this property with other maternally localized mRNAs like *bicoid* (Stauber *et al.* 2002) and *oskar* (Webster *et al.* 1994).

The molecular processes involved in axis induction during oogenesis might be the subject of fast evolutionary change because there is considerable variability in ovary types among insects (Büning 1994). Further, there might be several molecular alternatives for inducing an axis of polarity. The process appears to be mechanistically less demanding than the pattern formation in the later embryo that with high precision generates the complex arrangement of embryonic cell types. This poses the question as to whether later processes involved in DV patterning in the embryo would be more conserved.

It is already clear that several aspects of the zygotic level of DV patterning are highly conserved even between insects and vertebrates (Ferguson 1996; Lall & Patel 2001). Therefore, we will consider here only those processes that are maternally controlled in *Drosophila*. A role for the Toll–Dorsal cascade in DV patterning has been

Figure 11. Oocyte nuclear positioning and Dorsal gradient in *Tribolium*. (a) Optical midsection through a *Tribolium* egg chamber. (b) Cross-section through an egg chamber of similar stage as shown in (a). The nucleus is asymmetrically positioned; however, it has not been possible so far to prove that the nucleus marks the dorsal side. (c) Ventral surface view of a *Tribolium* blastoderm embryo. Accumulation of Dorsal protein is highest in nuclei straddling the ventral midline. Abbreviations: fc, follicle cells; n, oocyte nucleus.

suggested for Coleopterans. In the red flour beetle, *Tribolium castaneum*, a dorsal homologue exists that is maternally expressed (Chen *et al.* 2000). As in *Drosophila*, the *Tribolium* Dorsal protein forms a nuclear concentration gradient with the peak levels in regions where the ventralmost cell fates of the mesoderm arise (figure 11c). However, there are interesting differences in the way the signalling cascade is regulated. *Toll*, which is maternally expressed in *Drosophila* (Hashimoto *et al.* 1988), appears to be a zygotic gene in *Tribolium* (Maxton-Küchenmeister *et al.* 1999). Its transcription is positively regulated by the *Tribolium* nuclear Dorsal gradient. As *Toll* promotes the nuclear import of Dorsal this establishes a positive feedback mechanism. Such feedback mechanisms might explain the greater regulatory potential with regard to DV patterning, which has been found in many short germ insects (Sander 1976). For example, longitudinal fragmentations of preblastoderm embryos of the leaf hopper *Euscelis* lead to the formation of segmented germ bands in each egg fragment (Sander 1971). In such cases, DV patterning seems to be initiated from different positions along the embryonic circumference including the dorsal side of the egg. In normal development the initiation of the process at the ventral side inhibits the same process to be initiated in other parts of the egg. This inhibition has to be long range and, presumably, is blocked or reduced by the respective experimental manipulations. It will be interesting to find out how the residual potential to generate pattern duplications in *Drosophila* (Roth & Schüpbach 1994; Morisato 2001)

relates to this much more dramatic twinning phenomena found in other insects.

As the Dorsal gradient in *Tribolium* forms with remarkable precision along the entire AP axis of the blastoderm embryo, spatial cues must be present in the egg that, like in *Drosophila*, presage the region where the active *Toll* ligand is produced. Currently, we have no information on the molecular nature of these cues or on the way they are linked to asymmetries in the egg chamber. In *Tribolium*, the oocyte nucleus moves to one side of the egg chamber late in oogenesis (figure 11a,b). However, we have been unable to detect specific changes in the follicle cells upon asymmetrical nuclear positioning that would allow us to determine the DV polarity of the egg chamber (S. Roth, unpublished data).

The existence of a *Toll*–Dorsal signalling pathway required for DV patterning in a beetle indicates that this mechanism is likely to be common to all holometabolous insects. So far the pathway has not been studied in more primitive hemimetabolous insects. It is, however, interesting to note that the *Toll* pathway fulfils an essential role in innate immunity in both insects and vertebrates (Hoffmann *et al.* 1999). This suggests that the immune function of the pathway is evolutionarily very old and might represent its ancestral function. In this scenario the role of the pathway in axis formation has to be derived from its earlier role in pathogen defence. The transition from immune function to axis formation might have occurred during early insect evolution (Chen *et al.* 2000).

If so, it will be fascinating to explore how the respective evolutionary transitions have occurred and how the link was established between egg polarization during oogenesis and embryonic axis induction mediated by Toll–Dorsal signalling.

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Discussion

R. G. Edwards (*Reproductive BioMedicine Online*, Dry Drayton, Cambridge, UK). I was very impressed by the cortical location of the proteins and that they showed the same distribution. Could you comment on the importance of the cortex in patterning? Could you tell us if this cortical location persists in later stages and if you find it in other species, especially mammals?

S. Roth. The importance of cortical domains in *Drosophila* can be understood from the way early embryonic development proceeds. Early embryogenesis is syncytial. The nuclei first divide in the centre of a large yolk-rich egg and subsequently migrate to the periphery. There they are influenced by signals emanating from cortically localized determinants. Some of these signals remain very local, such as those responsible for primordial germ cell formation at the posterior pole of the embryo. Here, the pole plasma, which is restricted to a narrow domain, determines the first nuclei reaching the cortex to become pole cells (primordial germ cells). The anteriorly localized determinant bicoid acts differently. *bicoid* mRNA is localized to the cortex. It is the source of a long range Bicoid protein gradient that acts as a morphogen to determine the different regions of the head and thorax of the embryo. The precise localization of proteins and mRNAs within the cortex of a large yolk-rich egg allows insect embryos to combine high cell numbers with early cell specification. As a result in some species, like in *Drosophila*, embryogenesis can be very fast (about one day from egg deposition to hatching). Cortical domains are important in many invertebrate embryos, such as in nematodes (*Caenorhabditis elegans*), in tunicates or in embryos with spiral cleavage. However, to my knowledge in all of these cases the localization of the determinants occurs after fertilization. *Drosophila* seems to be very special in that all this information is put into the egg long before egg deposition and fertilization. I am not aware of the importance of cortical domains in early mammalian development.

GLOSSARY

AP: anteroposterior
cnc: *cap'n'collar*
 Dpp: decapentaplegic
 DV: dorsoventral
 ECM: extracellular matrix
 Egfr: EGF receptor
 ER: endoplasmic reticulum
 Grk: Gurken
 mRNA: messenger RNA
 MT: microtubule
 MTOC: microtubule organizing centre
rho: *rhomboid*
 TGF- α : transforming growth factor- α
 TGF- β : transforming growth factor- β