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# Binuclear *Drosophila* oocytes: consequences and implications for dorsal-ventral patterning in oogenesis and embryogenesis

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## SUMMARY

**The position of the nucleus along the anterior rim of stage 8 *Drosophila* oocytes presages the dorsal side of the egg and the developing embryo. In this paper, we address the question of whether the oocyte has a previously determined dorsal side to which the nucleus is drawn, or whether nuclear position randomly determines the dorsal side. To do so, we have taken advantage of a genetic system in which *Drosophila* oocytes occasionally become binuclear. We find that (i) the two nuclei migrate independently to their respective positions on the anterior rim, sometimes selecting the same site, sometimes not, (ii) the two nuclei are equivalent in their ability to induce a dorsal-ventral pattern in the overlying follicular epithelium, and (iii) at any position around the anterior circumference of the egg chamber the follicle cell sheet is equally responsive to the Gurken signal associated with the oocyte nuclei. These results argue that the dorsal-ventral axis is determined arbitrarily by the randomly selected position of the nucleus on the anterior rim of the oocyte. Some of the binuclear eggs support embryonic development. However, despite the duplication of dorsal chorion structures, the majority of such embryos show normal dorsal-ventral patterning. Thus, processes exist in the ventral follicular epithelium or in the perivitelline space that compensate for the expansion of dorsal follicle cell fates and consequently allow the formation of a normal embryonic axis.**

Key words: *Broad-Complex*, Axis specification, Nuclear migration, Follicle cell, Patterning, *gurken* signaling

## INTRODUCTION

Patterning in the developing *Drosophila* embryo begins with the establishment of anterior-posterior (AP) and dorsal-ventral (DV) axes. However, the morphogenetic signals responsible for the establishment of the embryonic axes are already asymmetrically placed within the unfertilized egg. Thus it is during oogenesis that the axes are determined (for review see St Johnston and Nüsslein-Volhard, 1992; Ray and Schüpbach, 1996).

The first visible manifestation of DV asymmetry in the developing oocyte is the migration of the nucleus to the future dorsal anterior side of the egg chamber during stage 8 (King, 1970). This migration requires the establishment of proper microtubule polarity, following a major rearrangement of the microtubule cytoskeleton (Theurkauf et al., 1992; Gonzalez-Reyes et al., 1995; Roth et al., 1995). Laser ablation studies have shown that the nucleus plays an important role in DV patterning of the egg chamber (Montell et al., 1991). The nuclear position has functional significance because it determines the localization of *gurken* (*grk*) mRNA. Starting at stage 8 of oogenesis, *grk* mRNA accumulates in the dorsal anterior corner of the oocyte, in tight association with the oocyte nucleus (Neuman-Silberberg and Schüpbach, 1993). The *grk* gene product is a potential ligand for the *Drosophila* EGF receptor (Egfr) and localized activation of the Egfr in the adjacent follicle cells signals them to adopt dorsal fates, which is a prerequisite for patterning of both the egg shell and the embryo.

The positioning of the oocyte nucleus thus appears to determine the site of action of *grk*, which in turn determines the placement of the DV axis. What then determines the location of the oocyte nucleus? It has been suggested that the nucleus arrives randomly at a position on the anterior rim and its location determines the dorsal side (Spradling, 1993; Gonzalez-Reyes et al., 1995; Roth et al., 1995). This hypothesis remains unproved, however, and it is possible that the nucleus, moving on the microtubules, is drawn to a predetermined dorsal side by as yet unknown signals. In the first case, the nuclear migration would be the 'symmetry breaking' event (Turing, 1952; Gonzalez-Reyes et al., 1997) that establishes DV polarity, while in the latter case, it would simply be a necessary, but secondary, component of DV axis determination.

Although Grk signaling determines the DV axis of both egg shell and embryo, it is not known how much spatial information is already contained in the distribution of active Grk protein and how much is gained in subsequent refinement processes. For example, during egg shell formation the dorsal follicle cells differentiate to form two anterior chorionic specializations called dorsal appendages. The formation of these two appendages from a single region of Grk signaling could be a direct read-out of the Grk protein gradient or it

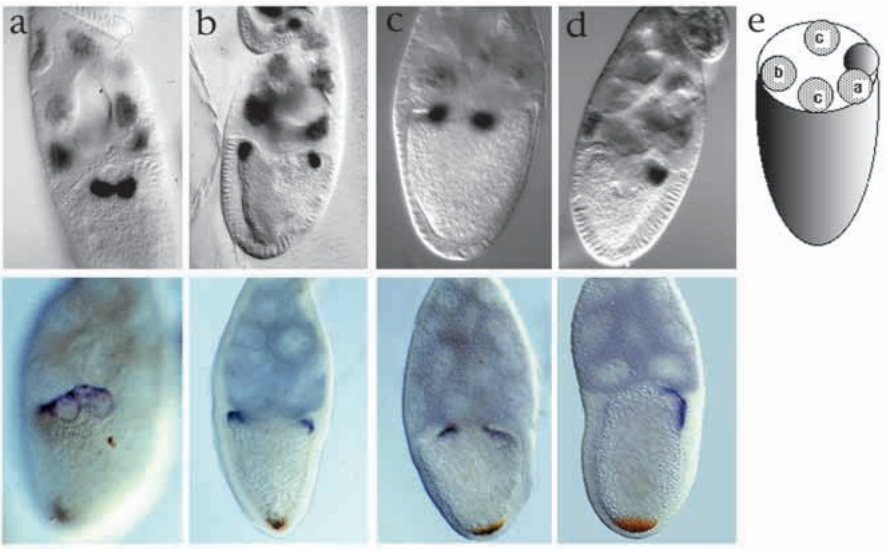


**Fig. 1**. Examples of binuclear egg chambers in *sqh*-A21 germline clones, showing three 'classes' of nuclear spacing. (a) Adjacent, (b) opposite, and (c) intermediate. (d) A mononuclear *sqh*-A21 egg chamber. Oocyte nuclei in top panel of each set are stained for β-galactosidase expression. The bottom panel of each set shows *grk* and *osk* mRNA distribution of similar oocytes. *grk* message is associated with each nucleus, regardless of number or location. *osk* localization at the posterior pole is normal, confirming the integrity of the AP axis. (e) A schematic cross section of a binuclear egg chamber showing the three classes. The unlabeled nucleus is the 'fixed point', relative to which the second nucleus is classed as a, b or c, by its location.

might require additional signaling processes, mediated for example by lateral inhibition, which would define the region giving rise to each dorsal appendage. The situation is even more complicated for the embryonic axis since the flow of spatial information is more indirect. Here Grk signaling to the dorsal side delimits by inhibition a domain of ventral follicle cells. These follicle cells give rise to a prepattern of unknown nature, which in the mature egg is present either in the inner egg shell (the vitelline membrane) or in the fluid-filled space surrounding the embryo (perivitelline space) (Nilson and Schüpbach, 1998). This prepattern then directs the production in the perivitelline space of an extraembryonic ligand, which in turn induces the embryonic DV axis (Stein et al., 1991).

In this paper, we first test the randomness of nuclear migration by taking advantage of a system in which the developing oocyte is occasionally binuclear owing to a defect in cystoblast cytokinesis. We find that, when two nuclei are present, they migrate independently to the anterior edge of the oocyte. Each nucleus is associated with localized *grk* RNA, and each is capable of inducing a local dorsalizing signal in the adjacent follicle cells, leading to both molecular and morphological differentiation, and resulting in eggs with 3 or 4 dorsal appendages. We conclude then that nuclear migration is truly random and it is therefore the symmetry breaking event that determines the DV axis within the oocyte.

Secondly, the binuclear egg chambers with randomly positioned nuclei provided us with a tool to investigate the transmission of spatial information from the oocyte to the follicular epithelium and back to the embryo. We observe that the signals from both oocyte nuclei initially induce dorsal follicle cell fates in an independent fashion so that the resulting patterns can be explained by a superposition of those induced by each nucleus alone. However, the later refinement giving rise to the regions from which the dorsal appendages emerge seems to result from a more indirect interpretation of the Grk signal. Interestingly, despite the greater fraction of the follicle cell layer that has adopted dorsal fates, embryos of eggs sporting 3 or 4 dorsal appendages often have a normal DV axis. Therefore, it appears that the formation of the signals in the ventral egg shell that induce the embryonic axis is buffered in some way, either at the level of ventral follicle cell patterning or during formation of the extraembryonic ligand in the perivitelline space, so as to assure proper embryonic axis formation even from eggs with an abnormal expansion of the dorsal egg shell.

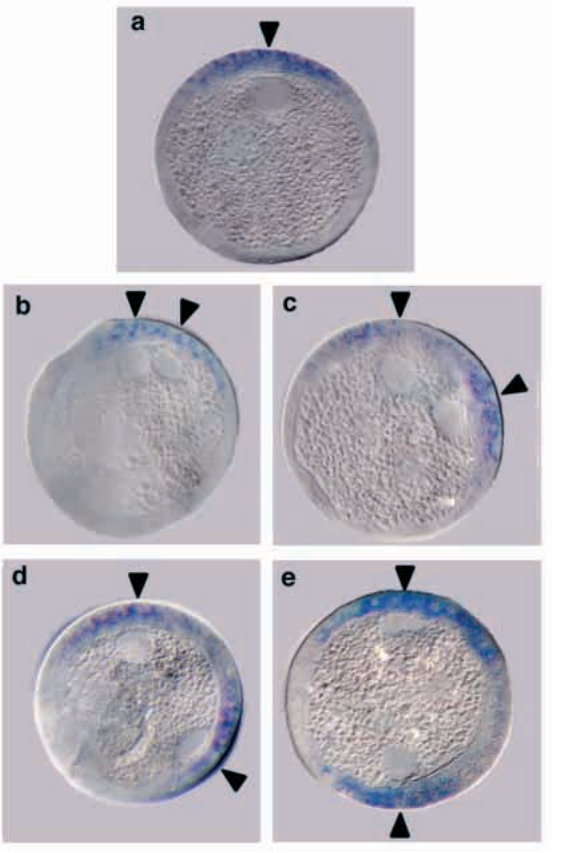


**Fig. 2**. Cross sections through stage 10 egg chambers showing oocyte nuclei and *kekkon*-1 expression in the follicular epithelium. (a) Mononuclear wild-type egg chamber. (b-e) Binuclear egg chambers derived from *sqh*-A21 germline clones. (b) Adjacent nuclei. (c,d) Nuclei with intermediate spacing. (e) Opposite nuclei.

## MATERIALS AND METHODS

### Fly stocks and crosses

Flies were raised on standard corn meal *Drosophila* medium at 25°C. The markers and chromosomes are described in Lindsley and Zimm (1992), except as noted. The stocks of $FRT^{101}$ and $ovo^{D1}$ $FRT^{101}$/ Y; $hs\text{-}FLP^{38}$ used to generate germline clones (Chou and Perrimon, 1992) were obtained from Dr N. Perrimon.

Homozygous germline clones of *y w* $sqh^{AX3}$ $sn^{3}$ $FRT^{101}$ containing the transgene P[$w^{+}$, *sqh*-A21] on chromosome-2 were induced by the DFS-FRT technique (Chou and Perrimon, 1992), as described (Jordan and Karess, 1997). Induction of germline clones in such flies is recognized by their ability to generate and lay eggs (loss of $ovo^{D}$ phenotype). To detect oocyte nuclei, the enhancer trap insertion P[$w^{+}$, *lacZ*] $orb^{PZ107}$ on chromosome 3 (Spradling et al., 1995) was introduced by the cross *y w* $sqh^{AX3}$ $sn^{3}$ $FRT^{101}$/FM7c; P[w+*sqh*-A21] X $ovo^{D1}$ $FRT^{101}$/Y;$hs\text{-}FLP^{38}$ *orb-lacZ*/TM3. From this cross, females nominally *y w* $sqh^{AX3}$ $sn^{3}$ $FRT^{101}$/$ovo^{D1}$ $FRT^{101}$ ; hs-FLP/P[$w^{+}$, *sqh*-A21]; P[*orb-lacZ*]/+ were selected for egg chamber dissection.

### Observations of egg chambers and embryos

For examination of egg and embryo morphology, the clone-bearing flies were mated to wild-type males and allowed to lay eggs for 24 hours, and then aged for 24 hours. Dark-field photographs were taken to document the number and arrangement of extra dorsal appendages. Later the chorion and the vitelline membrane were removed by hand and dark-field photographs of the flattened cuticular preparations were taken. This allowed a direct comparison between chorion phenotype and embryonic phenotype.

In situ hybridization with antisense *grk*, *kekkon-1*, *Broad-Complex*, *rhomboid*, *zerknüllt* and *short gastrulation* riboprobes was performed as described by Tautz and Pfeifle (1989) with modification. For detection of *oskar* RNA in double in situ hybridizations, a biotin-labeled antisense riboprobe was generated and after hybridization directly detected using the Vectastain ABC (peroxidase) kit (Vector Laboratories, PK-6100). Detection of β-galactosidase activity to mark the oocyte nuclei is described in Cooley et al. (1992). Antibody stainings were done as described in Roth et al. (1989).

## RESULTS

### Independent migration of the oocyte nuclei

In the course of a study on the importance of phosphorylation sites for the activity of nonmuscle myosin light chain (encoded by the *spaghetti-squash* gene (*sqh*)) during oogenesis, we observed that germline clones expressing the mutant transgene *sqh*-A21 (lacking an important site of phosphorylation) in an otherwise *sqh*-null mutant background reduced the efficiency of cystoblast cytokinesis (Jordan and Karess, 1997). Approximately 10-20% of the egg chambers in such clones had apparently sustained a failure of cytokinesis during the fourth round of cystocyte division since they comprised exactly seven binuclear nurse cells and one oocyte, which was itself binuclear (Fig. 1a-c). Both nuclei present in one oocyte become associated with *grk* mRNA (Fig. 1, bottom panels in each set), each in amounts similar to that produced by one wild-type oocyte nucleus. As a control for the integrity of the AP axis, the accumulation of *oskar* message at the posterior pole was also examined (Ephrussi et al., 1991), and was found to be entirely normal in the binuclear oocytes (Fig. 1, bottom panels).

The relative positions of the two nuclei were determined in 157 oocytes from stages 8-10. To aid in the identification of the oocyte nucleus, we analyzed egg chambers after *grk* in situ hybridization or we generated *sqh*-A21 clones in the presence of an enhancer trap line which leads to the accumulation of *lacZ* activity in germline nuclei (see Materials and Methods).

Three classes of distribution of the two nuclei were defined by visually dividing the oocyte into quadrants: adjacent (Fig.

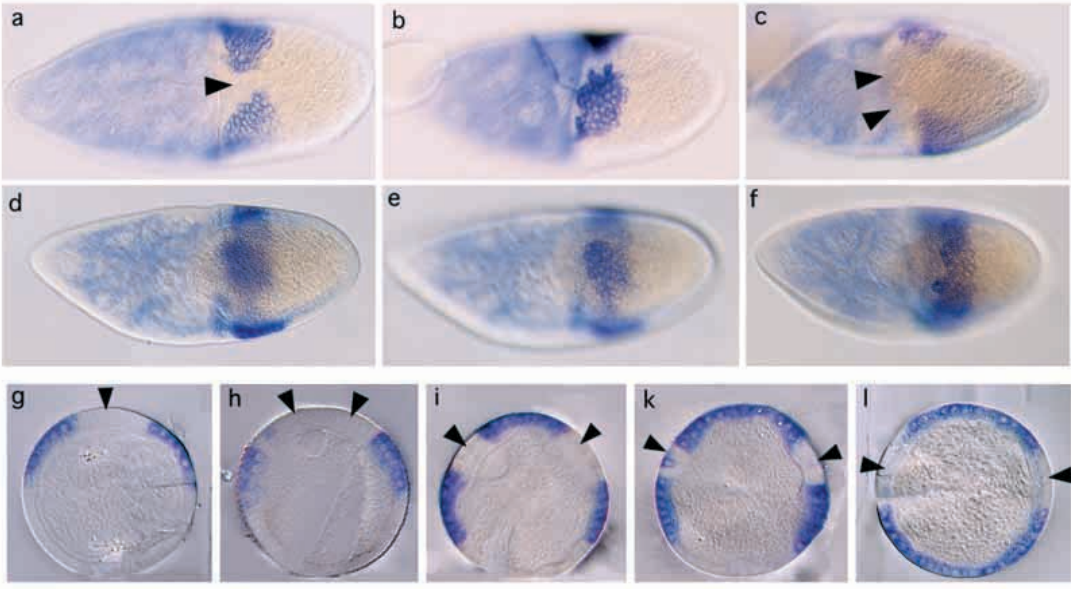


**Fig. 3**. The expression of the *Broad-Complex* (*BR-C*) in *sqh*-A21 egg chambers. (a-f) Stage 10 egg chambers showing *BR-C* expression. The position of the oocyte nucleus is indicated with an arrowhead. (a) Dorsal surface view of a mononuclear egg chamber. The two patches are separated by a 4- to 6-cell-wide gap of expression, corresponding to the position of the oocyte nucleus. (b) Lateral surface view of a mononuclear egg chamber showing a patch of 45 *BR-C*-expressing cells. (c) Binuclear *sqh-A21* egg chamber in which the two nuclei occupy adjacent positions. The gap between the two patches is 12 cells wide. (d-f) Three different views of the same binuclear egg chamber with nuclei occupying opposite positions in the oocyte. Four partially separated patches can be found. (d) Optical midsection showing two opposite patches in focus while the two other patches are above and below the focal plane. (e) Surface view of the egg chamber shown in d. The *BR-C* patch comprises 42 cells. (f) Large *BR-C* domain comprising approximately 80 cells. (g-l) Cross sections through stage 10 egg chambers. The positions of the nuclei (indicated with an arrowhead) were usually determined from adjacent sections. The patches of *BR-C* expression are normally located just posterior to, and on either side of, the oocyte nucleus. (g) Mononuclear egg chamber. (h) Binuclear egg chamber with adjacent nuclei. (i) Binuclear egg chamber with nuclei occupying intermediate positions. The third *BR-C* patch located between the nuclei has normal size. (k) Binuclear egg chamber with nuclei occupying intermediate positions. The third *BR-C* patch located between the nuclei is larger than in wild type. (l) Binuclear egg chamber with nuclei occupying opposite positions.

1a), opposite (Fig. 1b) or intermediate (Fig. 1c). A tendency for the nuclei to migrate to a predetermined dorsal region should result in both nuclei being found predominantly within the same sector. If migration of the nuclei is random, they should be found equally frequently together as opposed and more frequently in the intermediate class. The frequencies of the three classes should ideally follow the binomial distribution (25% adjacent, 25% opposite and 50% intermediate).

For 157 binuclear oocytes examined (Table 1), the nuclei were 44 times adjacent, 42 opposite and 71 intermediate. These numbers are not significantly different from that expected (by $\chi^2$ test, $P>0.4$) We conclude that there is no tendency for the nuclei to comigrate and therefore that there is no tropism of nuclei for a preexisting dorsal signal.

## Follicle cell patterning in binuclear oocytes

In wild-type oocytes, the dorsalizing signal encoded by the *grk* RNA induces in the overlying follicle epithelium the expression of the genes *kekkon 1 (kek 1*) and *Broad-Complex* (*BR-C*), which appear to be necessary for the adoption of dorsal fates in these cells (Ruohola-Baker et al., 1993, Musacchio and Perrimon, 1996, Queenan et al., 1997, Deng and Bownes, 1997). In normal stage 10B egg chambers, *kek 1* is expressed in a patch of follicle cells constituting about one-fifth of the egg chamber circumference, centered over the oocyte nucleus (Fig. 2a). In binuclear oocytes, each of the two foci of *grk* RNA accumulation sends a dorsalizing signal to the adjacent follicle cells, inducing them to express *kek 1* and to adopt the dorsal fate (Fig. 2b-e). *kek 1* expression is seen above each oocyte nucleus. Depending on the distance between the two nuclei, the *kek 1* expression domains may be fused or distinct. When distinct, the width of each domain (10-12 cells) is similar to that seen in mononuclear egg chambers. This suggests that, at the level of *kek 1* expression, no negative interaction is occurring between the two signaling sites.

To further examine follicle cell patterning in binuclear egg chambers, we monitored the distribution of *BR-C* expression, which allows simultaneous visualization of the dorsalmost and dorsolateral follicle cell regions. In mononuclear egg chambers *BR-C* expression refines to two dorsolateral patches comprising 40- 50 follicle cells at stage 10B (Fig. 3a,b,g), which will later form the dorsal appendages. The follicle cell region between the two patches is about 6 cells wide and directly overlies the oocyte nucleus. This region will later give rise to the dorsalmost part of the chorion between the two dorsal appendages (the wild-type chorion pattern is shown in Fig. 4a).

In binuclear egg chambers in which the two nuclei are adjacent to each other, the two *BR-C* patches are shifted to more lateral positions and the gap between the patches is enlarged to a width of 12-18 cells (Fig. 3c,h). If the nuclei are an intermediate distance apart, three patches are found: two of normal size and a third patch of variable size between them at the midpoint defined by the two nuclei (Fig. 3i,k). This patch may comprise between 35 and 90 cells depending on the distance between the two nuclei. No patches of strongly expressing *BR-C* cells comprising less than 35 cells were observed, indicating that there may be a threshold below which no *BR-C* expression occurs, but above which patches form of approximately the size seen in wild type. If the nuclei occupy opposite positions in the oocyte, up to four *BR-C* patches can be seen. However, adjacent patches are often fused laterally to form one contiguous expression domain comprising 80 to 100 cells (Fig. 3d-f,l). Thus, although there might be mechanisms that define the lower size limit of a *BR-C* domain, no upper size limit regulation could be detected.

Taken together, these observations confirm that proximity to the oocyte nucleus is sufficient to determine dorsal fates in the overlying follicle cells, that each nucleus is independently capable of inducing dorsal fates and that all parts of the follicular epithelium along the circumference of the egg chamber are equally responsive to Grk signaling. The follicle cell patterns found in binuclear egg chambers are determined by the distance between the nuclei and can be largely explained by a superposition of signals emanating from each nucleus. The only observed deviation from this rule appears to be a threshold for the smallest size of *BR-C* expression domains.

**Table 1. Distribution of nuclei in binuclear oocytes**

| Total | Opposite | Adjacent | Intermediate |
|---|---|---|---|
| 157 (100%) | 42 (27%) | 44 (28%) | 71 (45%) |

By $\chi^2$ test, this distribution is not significantly different from the expected ratio ($P>0.4$).

## Chorion and embryonic phenotypes derived from binuclear oocytes

A fraction of the eggs derived from *sqh*-A21 clones were deposited and were found to have three or four dorsal appendages (Fig. 4a-d). This duplication of dorsal chorion structures is in agreement with the duplication or expansion of the expression domains of *kek 1* and *BR-C* in the follicular epithelium. The four appendages were found in two pairs separated by a variable fraction of egg circumference. When three appendages were present, the central one was often visibly the result of a fusion of two (Fig. 4b). Apparently eggs with two pairs of dorsal appendages diametrically opposed (Fig. 4d) are only rarely oviposited, since their frequency is far below that of egg chambers with opposed nuclei (Table 1). Interestingly, no eggs with three appendages were found in which the central appendage was incomplete or which had only residual dorsal appendage material in a central position. This observation is consistent with our finding that the size of the smallest *BR-C* expression domains between two nuclei was close to that of wild-type domains.

Binuclear eggs can be fertilized and begin development. We analyzed the DV pattern formation of embryos derived from *sqh*-A21 germline clones, both those with two nuclei (as revealed by duplication of dorsal appendages) and those apparently mononuclear (Figs 4, 5). Eggs showing signs of embryonic development were individually mounted and photographed to document the number and arrangement of the extra dorsal appendages. Each embryo was then dissected to remove the chorion and the vitelline membrane so that cuticular phenotypes could be observed. This allowed a direct comparison between chorion and embryonic phenotypes. Surprisingly, only 36% of the embryos developing in egg shells with three or four dorsal appendages showed signs of partial dorsalization as revealed by a reduced head skeleton and a

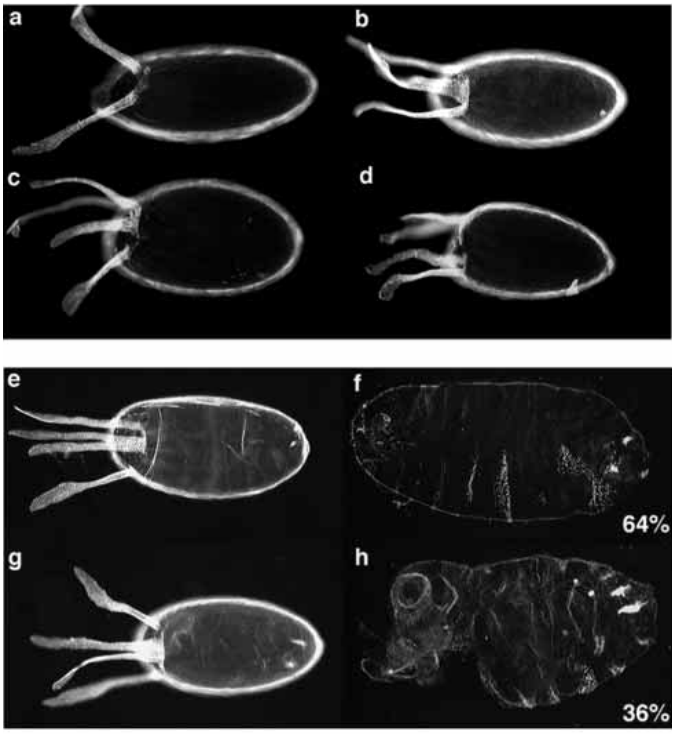


**Fig. 4.** Chorion and embryonic phenotypes. Dark-field photographs of eggs (a-e,g) and cuticle preparations (f,h). Anterior is to the left. (a) Wild-type egg showing two dorsal appendages anteriorly. (b) *sqh-A21* egg with three dorsal appendages. (c) *sqh-A21* egg with four equally spaced dorsal appendages. (d) *sqh-A21* egg with pairs of dorsal appendages on opposite sides. (e,f) *sqh-A21* egg with four dorsal appendages and cuticle of larva derived from the same egg showing normal DV patterning. (g) *sqh-A21* egg with four dorsal appendages and cuticle of larva derived from the same egg showing weak dorsalization. From 41 eggs with extra dorsal appendages, 26 (64%) contained larvae with normal DV pattern. The remainder showed weak dorsalization as evidenced by a reduction of the head skeleton and a loss ventral denticles. No correlation was seen between strength of the chorion phenotype and cuticular defects.

narrowing of the ventral denticle belts. This phenotype could be scored clearly despite the fact that all embryos have posterior segmentation defects. [The latter are an independent phenotype of *sqh*-A21-derived eggs, probably caused by a failure of axial nuclear migration that results in a delayed arrival of syncytial blastoderm nuclei at the posterior pole (Jordan and Karess, 1997)]. Interestingly, there was no apparent correlation between the strength of the chorion phenotype and the embryonic phenotype. That is, dorsalized embryos were not more likely to be found in those eggs that had four clearly separated dorsal appendages than in those with three appendages. An example of an egg with four dorsal appendages and normal DV cuticle pattern is shown in Fig. 4e. The rarely oviposited eggs with two pairs of dorsal appendages on opposite sides did not support embryonic development. No abnormalities in the DV axis features were seen among the eggs with only two dorsal appendages.

A more detailed representation of the DV pattern of embryos developing in eggs with extra dorsal appendages was obtained using molecular markers. Ventral, lateral and dorsal regions of the early embryonic DV pattern were visualized by detecting *twi* and *rho* expression, respectively (Fig. 5a,b). 65% of the embryos showed no dorsal-ventral alteration of *twi* and *rho* expression, as was expected from the analysis of cuticle phenotypes. Most of the remaining embryos revealed a weak dorsalization, causing a partial deletion of ventral and lateral regions of the pattern accompanied by an expansion of dorsal regions (Fig. 5e,f). This dorsalization was usually confined to a region between 50% and 90% egg length (where the posterior pole is 0%) along the AP axis. Only a small fraction of embryos showed severe deletions of ventral and lateral fates along most of the AP axis (Fig. 5g,h). Similar results have been obtained using *zerknüllt* as dorsal and *short gastrulation* as ventrolateral markers (data not shown). Since all the embryos examined developed in eggs with at least one extra dorsal appendage, they have been derived from binuclear egg chambers with at least three *BR-C* expression domains. In contrast to mononuclear egg chambers in which dorsal follicle cell fates are derived from approximately 40% of the egg circumference, in egg chambers with three *BR-C* domains at least 70% of egg circumference gives rise to dorsal follicle cell fates. Thus, a severe dorsalization of the follicular epithelium is compatible with normal or almost normal embryonic DV pattern formation.

## DISCUSSION

By observing the consequences of having two nuclei within a single oocyte, we have attempted to distinguish between two competing hypotheses for the establishment of DV polarity on the oocyte: (1) nuclear position around the anterior of the circumference of the oocyte is random, and therefore it is the primary ‘symmetry-breaking event’ establishing the DV axis, and (2) the nucleus, although required for inducing the DV axis, is drawn to a predetermined presumptive dorsal side of the oocyte by as yet unidentified signals. The results presented here indicate that the distribution of the two nuclei along the anterior edge of the oocytes conforms to that predicted for random, independent migration, and that each nucleus is equally capable of inducing the dorsal fate in the adjacent follicle cells. These data are inconsistent with any model invoking a dorsally directed attraction of the nuclei, since such models predict that both nuclei would be found more often than not at the same dorsal anterior position.

However, it might be argued that, in the binuclear oocytes, one nucleus has bound a unique dorsally located ‘docking site.’ The second nucleus, having found the docking site occupied, migrated to another position, which would still be random with respect to the first, resulting in a distribution of nuclei identical to what we observe in Table 1. We cannot formally exclude this possibility, but for several reasons it appears unlikely. In the absence of a dorsally directed tropism, both nuclei would have to migrate anteriorly at first, and then randomly but circumferentially until one found the dorsally positioned binding site. The second nucleus would then continue to wander around the anterior rim. There is no evidence for such circumferential nuclear migration and its existence would pose a second problem since, during this migration, *grk* RNA presumably remains associated with the migrating nuclei and would be transmitting its dorsalizing signal to the follicular epithelium passing overhead. Secondly, we have shown that the

two oocyte nuclei are indistinguishable in their ability to localize *grk* RNA and induce dorsal fates in the follicle cells, and the follicle cell layer itself is uniformly responsive to the Grk signal (discussed further below). Such equivalence in mRNA localization and signaling from either of the two nuclei to the overlying follicular epithelium would be surprising if one nucleus were correctly bound to a docking site, altering the cytoskeletal organization of the subcortical region between itself and the oocyte membrane, while the other roamed free around the anterior circumference.

It appears therefore that prior to nuclear migration from its posterior position (before stage 8), the oocyte has no intrinsic DV polarity. The axis is defined by the position at which the nucleus comes to rest on the anterior rim of the oocyte. A simple model to explain nuclear migration, first proposed by Theurkauf et al. (1992), is that the initial random association of nucleus with subcortical microtubules determines its final anterior resting place.

In binuclear oocytes, Grk signaling to the follicular epithelium occurs simultaneously in two different sectors along the circumference of the egg chamber. This situation in principle should have allowed the detection of any DV asymmetries present in the follicle cells before signaling had occurred, such as localized sensitivity or resistance to the Grk signal. The uniformity with which Grk signaling induces *kek 1* and *BR-C* expression at all positions of the follicular circumference confirms that such asymmetries do not exist and that the follicular epithelium is indeed completely devoid of dorsal-ventral information before the arrival of germline signals. Furthermore, the induction of *kek 1* and *BR-C* expression simultaneously at different sites along the follicular epithelium shows no interference, that is, there is no tendency for adjacent regions of *kek 1* or *BR-C* expression to influence each other. The observed patterns can be explained largely by the superposition of signals emanating from the two nuclei. However, one exception to this rule was noted. The two nuclei must be a certain minimum distance apart to allow the expression of *BR-C* in the patch of follicle cells between them. Beyond this distance, the smallest *BR-C* domains that we observed had a size similar to that of a normal *BR-C* patch giving rise to one dorsal appendage. Accordingly, the extra dorsal appendages seen on *sqh*-A21 eggs were never abnormally small. They were either of normal size or appeared to be fusions of two appendages. The mechanisms that could explain such a minimal size threshold are not known. It could be that a certain amount of Grk signaling is required to initiate a secondary signaling cascade which spreads a limited distance from the region of initiation. The distribution pattern of activated MAP kinase in the follicular epithelium suggests the existence of such a mechanism (Wasserman and Freeman, 1998; Peri et al., 1999). Activated MAP kinase, presumably reflecting the activation of the EGF receptor in the follicular epithelium by Grk, is first seen in a narrow domain overlying the highest concentrations of Grk protein. Subsequently the activation domain dramatically expands and resolves into a complex pattern that presages the position of the dorsal appendages, although during that time no change in Grk protein distribution has occurred, indicating the existence of a secondary mechanism of signal amplification.

The *BR-C* expression pattern in binuclear egg chambers allows one to correlate the degree of dorsalization of the follicular epithelium with the chorion phenotype of deposited eggs. The formation of three dorsal appendages requires the presence of *BR-C* expression in 70% of the egg chamber circumference instead of 40% as in wild type. Thus, in such egg chambers the size of the ventral follicle cell region from which the prepattern of the embryonic axis emerges is reduced by half as compared to wild type. Despite such dramatic changes in follicle cell patterning, eggs with three or even four dorsal appendages support the development of embryos with mostly normal DV axes. This is surprising since it is well established that the DV axis of the embryo emerges from spatial cues of the vitelline membrane (Stein et al., 1991; Roth, 1993; Nilson and Schüpbach, 1998), cues that themselves

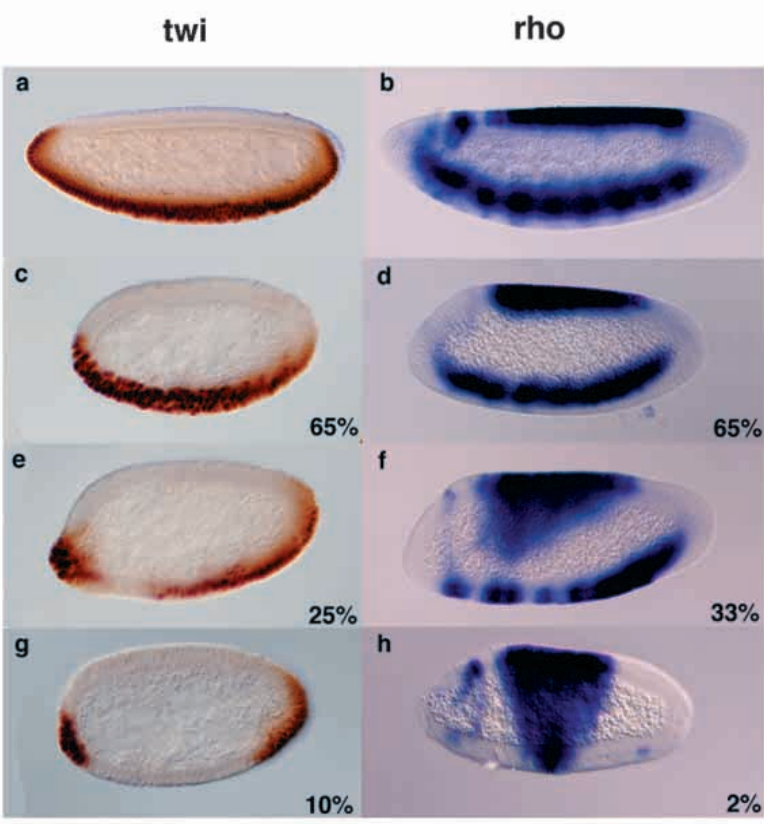


**Fig. 5.** Dorsal-ventral pattern formation in embryos from *sqh-A21* eggs with extra dorsal appendages. Optical midsections of whole-mount preparations of embryos at cellular blastoderm stage. Anterior the left and dorsal up. (a,c,e,g) *twi* protein expression detected with anti-*twi* antiserum. (b,d,f,h) *rho* RNA distribution detected with labeled *rho* antisense RNA. (a,b) Wild type. *twi* is expressed in the ventralmost region of the DV pattern giving rise to mesoderm. *rho* is expressed in a dorsal and in a ventrolateral domain. (c-h) Embryos from eggs with three or four dorsal appendages. (c,d) Normal DV pattern. (e,f) Weak dorsalization. *twi* expression shows a gap anteriorly, the ventrolateral *rho* domain is partially deleted and the dorsal domain expanded. (g,h) Strong dorsalization. Most of the twi domain is deleted. The dorsal *rho* domain is expanded at the expense of the ventrolateral *rho* domain. 36 embryos were analyzed for *twi* and 114 for *rho* expression. The numbers indicate the frequencies with which the different phenotypes occurred.

depend on Grk signaling (Schüpbach, 1987; Roth and Schüpbach, 1994; Queenan et al., 1997). The formation of these spatial cues depends on the somatic dorsal group genes *nudel* (*ndl*), *windbeutel* (*wind*) and *pipe* (*pip*) (Stein et al., 1991). By generating clones of mutant follicle cells, it has been shown that *wind* and *pip* are required in a ventral domain of approximately 8-12 follicle cells to generate these cues (Nilson and Schüpbach, 1998). The cues are believed to initiate a protease cascade, which leads to the formation of an active ligand for the transmembrane receptor *Toll*. Ventral *Toll* activation relays the extraembryonic signal to the cytoplasm and controls the nuclear import of Dorsal protein (the product of the *dorsal* gene) so that a ventral-to-dorsal nuclear gradient of Dorsal protein is established (for review, see Morisato and Anderson, 1995). Dorsal protein acts as a concentration-dependent transcriptional activator and repressor to specify the different domains of zygotic gene expression along the embryonic DV axis (for review see Rushlow and Roth, 1996).

How is it possible, considering these experimental results, that a large proportion of *sqh-A21* eggs with expanded dorsal egg shell regions support normal embryonic DV patterning? Firstly, the establishment of the ventral prepattern in the follicular epithelium might have size-regulatory properties; i.e., its width might be maintained despite the expansion of dorsal follicle cell regions. Such a mechanism has been suggested to explain the partial duplication of the embryonic DV axis caused by loss-of-function mutations of *grk* and Egfr (Roth and Schüpbach, 1994). Secondly, ventral cues might indeed be affected by the expansion of dorsal fates, becoming narrower in the case of two oocyte nuclei as compared to wild type, but as long as their width does not fall below a certain limit, normal formation of the extraembryonic signals in the perivitelline cleft might still be possible. Feedback mechanisms might occur at the level of the protease cascade leading to the activation of the Toll ligand. Some observations regarding the processing and modification of Easter, the last serine protease in the cascade leading to Toll-ligand activation, do suggest the existence of regulatory feedback mechanisms that could compensate for changes during the initiation of the cascade (Misra et al., 1998).

Currently it is not possible to distinguish between these two alternatives for compensation since the components whose expression is restricted to the ventral follicular epithelium have still to be described. However, it is instructive to compare the binuclear egg chambers to those derived from *fs(1)K10* and *squid* mutations. *fs(1)K10* and *squid* are required for the tight localization of *grk* mRNA to the nucleus (Neumann-Silberberg and Schüpbach, 1993, 1994). In mutant *fs(1)K10* and *squid* egg chambers, some or all of the *grk* mRNA forms an anterior ring around the entire circumference of the oocyte. Although, in weak mutations of these genes, only small amounts of *grk* RNA are delocalized and the majority of the RNA still accumulates at the site of the nucleus, embryonic patterning is dramatically affected (Roth and Schüpbach, 1994). Thus, the decisive aspect of *grk* signaling with respect to embryonic patterning might be its complete absence from some ventral portion of the follicle cell circumference rather than the size of the dorsal domain that receives the *grk* signal. This may explain the need for the tight association of *grk* RNA with the nucleus. In addition, the flexibility in the size of the region that can adopt dorsal fates without affecting embryonic patterning may provide the basis for the enormous evolutionary diversity in the number and size of dorsal appendages found among different *Drosophila* species (Hinton, 1981).

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