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Report

EGF Signaling and the Origin of Axial Polarity among the Insects

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Summary

The eggs of insects are unusual in that they often have bilateral symmetry when they are laid, indicating that both anterior-posterior (AP) and dorsal-ventral (DV) symmetries are broken during oogenesis [1]. The molecular basis of this process is well understood in *Drosophila melanogaster*, in which symmetry breaking events for both axes depend on the asymmetric position of the oocyte nucleus and on germline-soma signaling mediated by the Tgfα-like epidermal growth factor (EGF) ligand Gurken [2, 3]. Germline-soma signaling interactions centered around the oocyte nucleus have been proposed in other insect species [4, 5], but the molecular nature of these interactions has not been elucidated. We have examined the behavior of the oocyte nucleus and the function of EGF signaling components in the ovaries of the wasp *Nasonia vitripennis*, the beetle *Tribolium castaneum*, and the cricket *Gryllus bimaculatus*. We have found that EGF signaling has broadly conserved roles in mediating the encapsulation of oocytes by the somatic follicle cell layer, in establishing polarity of the egg chambers, and in setting up the DV axis of the embryo. These results provide insights into the evolutionary origins of the unique strategy employed by insects to establish embryonic axial polarity during oogenesis.

Results and Discussion

In *Drosophila melanogaster*, establishment of both the anterior-posterior (AP) and the dorsal-ventral (DV) axes of the embryo depends on signaling provided by the Tgfα-like ligand Gurken (Grk), whose mRNA is localized around the oocyte nucleus and whose protein product activates the epidermal growth factor receptor (EGFR) in the overlying somatic follicle cells. AP symmetry is broken when the oocyte nucleus lies close to the posterior pole of the oocyte, where EGF signaling to the overlying posterior follicle cells, and subsequent back signaling, leads to the repolarization of the oocyte cytoskeleton and to the localization of patterning mRNAs at the poles. Concurrently, the oocyte nucleus migrates to an anterior, cortical location that is asymmetric with regard to the short axis of the oocyte. DV polarity is established when a second EGF signal leads to the differentiation of the follicle cells overlying the oocyte nucleus at this position [2, 3].

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To address whether signaling events between the germline and somatic cells in the ovaries of nondipteran insects contribute to the polarization of the embryonic axes, we have observed the behavior of the oocyte nucleus and have examined the role of EGF signaling components in three insect species that employ the three major modes of insect oogenesis: polytrophic meroistic (*Nasonia vitripennis*), telotrophic meroistic (*Tribolium castaneum*), and panoistic (*Gryllus bimaculatus*) [6] (see Figure S1 available online for details).

Asymmetric Positioning of the Oocyte Nucleus

In all three species, the oocyte nucleus moves to an asymmetric position with respect to the short (DV) axis of the oocyte in the late stages of oogenesis, whereas its final position with respect to the long (AP) axis varies across species (Figures S1B, S1D, and S1F). In addition, a clear morphological asymmetry in the follicle cell layer overlying the cortically localized oocyte nucleus becomes apparent during the late stages of oogenesis in *G. bimaculatus* and *T. castaneum* (Figures S1D and S1F), suggesting that communication between the oocyte (germline) and follicle cells (soma) takes place in these species.

The Expression of EGF Signaling Components during Oogenesis

Because germline-soma communication is mediated by EGF signaling in *D. melanogaster*, we hypothesized that the apparent germline-soma communication seen in the ovaries of the species studied here is also mediated by EGF signaling, and we thus searched for orthologs of EGF components in these organisms.

grk is a rapidly evolving gene, and clear orthologs are difficult to find outside of the Diptera. However, single genes encoding Tgfα-like proteins were found in each of the *N. vitripennis* [7] and *T. castaneum* [8] genomes, as well as by polymerase chain reaction in *G. bimaculatus.* These putative EGF ligands are highly similar to the *D. melanogaster* genes *spitz* and *keren* (Figure S2), which are likely paralogous to *grk* [9, 10].

In *N. vitripennis*, *Nv-tgfα* mRNA is localized within the oocyte near the asymmetrically positioned oocyte nucleus, similar to *grk* in flies. However, this domain of localization extends toward the posterior pole (Figures 1A, 1G, and 1H), a pattern not seen for *D. melanogaster* mRNAs but which has been seen for some honeybee genes [11, 12] and may be related to the structure of the microtubule cytoskeleton of the *N. vitripennis* oocyte at this stage [13]. The *T. castaneum* and *G. bimaculatus tgfα* orthologs (*Tc-tgfα* and *Gb-tgfα*, respectively) are strongly expressed in oocytes starting from early stages, but their mRNAs show no clear localization to the oocyte nucleus (Figures 1B and 1C, respectively).

The EGFR is highly conserved in sequence among the insects, and its expression in the somatic follicle cells is also conserved among the three species examined here (Figures 1D–1F). In *G. bimaculatus*, the expression is downregulated in the anterior and posterior follicle cells (Figure 1F), possibly as a result of communication between the juxtaposed ovarian follicles along the AP axis of the ovariole. Some heterogeneity in *Nv-EGFR* expression within the follicular epithelium is observed, but its significance is not clear.

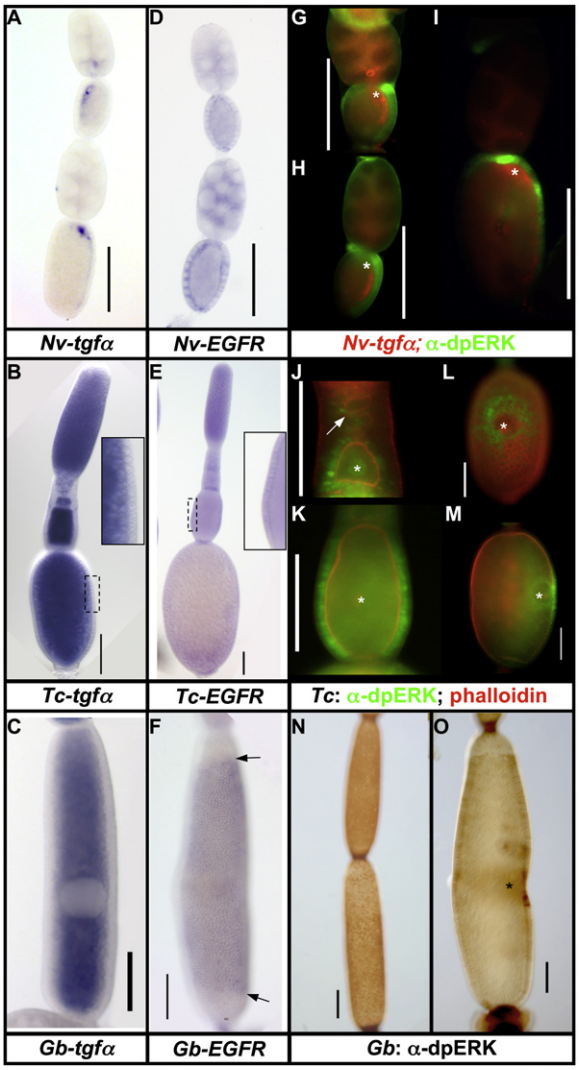

Figure 1. Expression of EGF Components in Insect Ovaries

(A–C) Expression of *tgfα* mRNA in the oocytes of *Nasonia vitripennis* (A), *Tribolium castaneum* (B), and *Gryllus bimaculatus* (C). This mRNA is localized only in *N. vitripennis*, whereas it is ubiquitous in the oocytes of *T. castaneum* and *G. bimaculatus*.

(D–F) Expression of *EGFR* mRNA in the follicular epithelium of *N. vitripennis* (D), *T. castaneum* (E), and *G. bimaculatus* (F) ovarioles. Arrows in (F) mark the borders of *Gb-EGFR* expression along the anterior-posterior (AP) axis of the egg chamber.

(G–I) Expression of *Nv-tgfα* mRNA (red) and dpERK (activated mitogen-activated protein kinase [MAPK]) (green) in progressively older *N. vitripennis* ovarioles.

(J–M) Expression of dpERK in *T. castaneum* ovarioles over the course of oogenesis (dpERK in green, phalloidin in red). dpERK expression is first detected in somatic cells lying just posterior to the very early oocytes (J, white arrow). In slightly older egg chambers, all follicle cells contacting the oocyte express dpERK (J, bottom egg chamber). As the follicle matures (K), dpERK remains strong in all lateral follicle cells but is downregulated in the termini. During vitellogenesis (L, dorsal view; M, lateral view), dpERK is found in the nuclei of the follicle cells surrounding the position of the oocyte nucleus, but not in the cells directly overlying it.

(N and O) Expression of dpERK in *G. bimaculatus* ovarioles. *G. bimaculatus* shows a similar pattern to that of *T. castaneum*: in early egg chambers (N), dpERK is expressed in most of the follicle cells. In vitellogenic ovarioles, dpERK is markedly reduced in most of the follicular epithelium except in the follicle cells in the vicinity of the oocyte nucleus (O). Scale bars represent 100 μm; asterisks mark the position of the oocyte nucleus.

We used a cross-reactive antibody against dpERK that recognizes activated mitogen-activated protein kinase (MAPK) in order to detect activation of the EGF signaling cascade in the ovaries of the three species analyzed here.

In *N. vitripennis*, MAPK activation is observed in follicle cells overlying the localized *Nv-tgfα* mRNA, which corresponds to a domain extending from the anterior to the posterior pole of the oocyte (Figures 1G–1I). The activation of MAPK in *N. vitripennis* is dynamic during oogenesis, especially in the follicle cells directly over the oocyte nucleus.

In *T. castaneum*, activated MAPK is initially observed as the oocyte exits the germarium and begins to associate with the somatic cells (Figure 1J, arrow). Later, when the nucleus is centrally located within the oocyte, MAPK is activated in most follicle cells associated with the oocyte (Figure 1J, bottom oocyte). As the egg chamber grows and while the oocyte nucleus remains central, activated MAPK remains highly expressed in all follicle cells along the lateral sides of the oocyte but appears to be downregulated at the termini (Figure 1K). Once the oocyte nucleus moves to the cortex, activated MAPK staining clears from most follicle cells except for a roughly circular, graded domain emanating from, but excluding, the region of the epithelium directly overlying the point of contact of the oocyte nucleus and lateral cortex (Figures 1L and 1M).

A similar pattern is observed in *G. bimaculatus*: before the movement of the oocyte nucleus, dpERK staining is mostly ubiquitous in the follicle cells (Figure 1N). As the follicles mature, dpERK staining is quite dynamic, but staining is consistently very strong in the follicle cells overlying the oocyte nucleus (Figure 1O). This strong dpERK staining is associated with the formation of the kink in the egg chamber that gives rise to the characteristic "banana" shape of late-stage oocytes (Figure 1O; see also Figure S1F).

Function of EGF Signaling in Encapsulation and AP Axis Specification of the Oocyte

The potential role of the EGF pathway in establishing the polarity of the egg chamber and embryo was tested by parental RNA interference (pRNAi) against the *tgfα* genes in the beetle, wasp, and cricket. This treatment caused a severe reduction of fecundity in all three species. This reduced egg production appears to be related to the failure of the somatic follicle cells to properly surround and encapsulate the oocytes (compare wild-type in Figures 2A, 2D, and 2G to *tgfα* pRNAi effects in Figures 2B, 2E, and 2H; see also Figure S3D). A role for the EGF pathway in encapsulation of the oocyte has been detected in *D. melanogaster* [14, 15], and this function of the EGF pathway appears to be ancestral among insects.

In some *tgfα* pRNAi knockdowns, oocytes were encapsulated but showed AP polarity defects. In *T. castaneum*, the oocyte nucleus, rather than moving to a cortical location about midway along the AP axis of the oocyte at the beginning of vitellogenesis (Figure 2A), was instead found at the posterior pole after *Tc-tgfα* RNAi (Figure 2C). That this mislocalization was the result of a defect in axial polarity is supported by the observation that mRNA for the normally anteriorly localized mRNA of *Tc-eagle* [16] was often found, in addition, at the posterior pole after *Tc-tgfα* RNAi (Figures S3A–S3C).

In *N. vitripennis*, *Nv-nos* mRNA is localized at the posterior pole, whereas *Nv-otd1* mRNA is localized at both the anterior and posterior poles of the oocyte (Figure 2D; [13, 17]). After *Nv-tgfα* pRNAi, *Nv-nos* and *otd1* are colocalized in the center

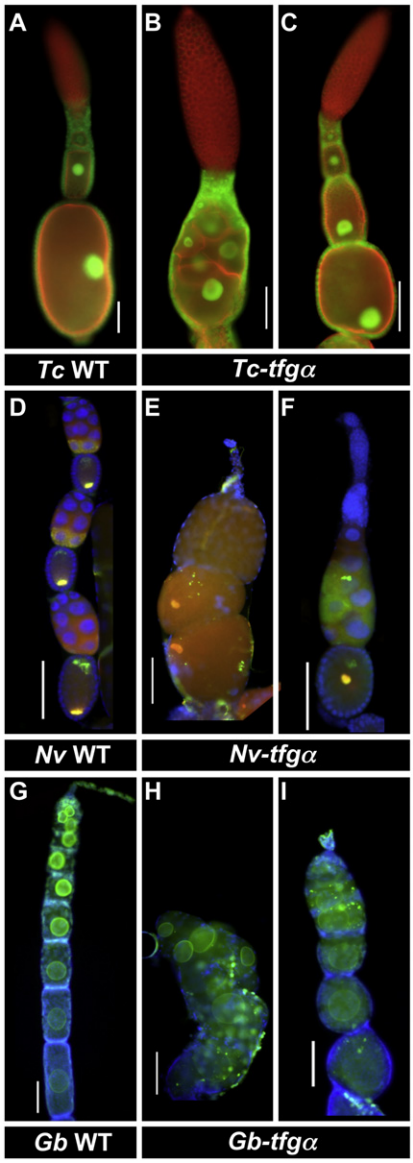

Figure 2. Effects of *tgfα* pRNAi during Oogenesis in *T. castaneum*, *N. vitripennis*, and *G. bimaculatus*

(A–C) *T. castaneum* ovarioles stained with phalloidin (red) and α-nuclear pore antibody (green). In wild-type (WT), each oocyte becomes encapsulated by a single layer of follicle cells, and the oocyte nucleus moves to a cortical location about midway along the long axis of the oocyte in late oogenesis (A). In strongly affected *Tc-tgfα* parental RNA interference (pRNAi) ovarioles, oocytes are not encapsulated and form a mass of germline cells that are not separated by follicle cells (B). In presumably weaker knockdowns, oocytes are encapsulated, but the oocyte nucleus is often found at an ectopic posterior location (C). See also Figure S3.

(D–F) Expression of DAPI (blue), *Nv-otd1* (green), and *Nv-nos* (red) in wild-type (D) and *Nv-tgfα* pRNAi (E and F) ovarioles from *N. vitripennis*. *Nv-tgfα* pRNAi ovarioles show defects in encapsulation of the oocytes by follicle cells (E), as well as improper localization of normally anteriorly and posteriorly localized mRNAs (E and F). In general, there is also a major reduction in the production of germline cells.

(G–I) Ovarioles of *G. bimaculatus* stained for α-nuclear pore (green) and DAPI (blue). Egg chambers in wild-type ovarioles show a linear arrangement and an increasingly elongated cylindrical shape during early oogenesis (G). In strongly affected *Gb-tgfα* pRNAi ovarioles (H), oocytes are arranged chaotically and are not encapsulated by a complete layer of follicle cells. In moderately affected ovarioles, oocytes are encapsulated but take on a more rounded shape (I). Scale bars represent 100 μm.

of the oocyte rather than at the posterior (Figure 2F; Figure S3D), which is highly reminiscent of what is seen for *oskar* mRNA in *grk*, *EGFR*, and *cornichon* mutants of *D. melanogaster* [2, 3]. In other cases, encapsulation defects are combined with polarity defects in compound egg chambers (Figure S3E).

AP polarity defects in *G. bimaculatus* ovaries are more difficult to assess because no localized mRNAs are known and because the oocyte nucleus localization does not appear to be sensitive to reduction of EGF signaling. However, we observed misshapen early egg chambers that were more spherical than cylindrical in shape (Figure 2I), indicating a defect in the polarized growth of the egg chambers along the AP axis after reduction of EGF signaling.

The Function of EGF Signaling in DV Axis Specification

Despite the disruption of AP polarity in oocytes observed in both *T. castaneum* and *N. vitripennis* after *tgfα* pRNAi, we did not observe major AP patterning defects in blastoderm-stage embryos of either species (Figures S4A–S4J). However, in both *T. castaneum* and *N. vitripennis*, pRNAi against *tgfα* transcripts led to variable defects in DV patterning.

In *T. castaneum*, the most common (34 of 71) phenotype can be interpreted as a lateralization of the embryo, because *Tc-cact* (representing the most ventral embryonic fates; Figure 3A) is missing from the main body of the embryo, whereas *Tc-sog* expression, which normally marks more lateral fates, expands to completely encircle the blastoderm (Figure 3B). Strikingly, *Tc-cact* and *Tc-sog* expression patterns that are perpendicular to their normal domains are also observed (8 of 71) (Figure 3C). Occasionally (5 of 71), duplications of the DV axis occur, in which multiple domains of *Tc-cact* and *Tc-sog* expression are observed (Figure 3D). We have found that these defects in marker gene expression are correlated with changes in the pattern of Tc-Dorsal nuclear uptake in the blastoderm-stage embryos (Figures S4K–S4O), indicating that, as in *D. melanogaster* [18, 19], maternal EGF signaling acts upstream of Dorsal gradient formation in *T. castaneum*.

In *N. vitripennis*, a major expansion of ventral fates (marked by *Nv-twist*) with a simultaneous shift of lateral fates (marked by *Nv-vnd*) is the most common (34 of 54) phenotype observed (compare Figures 3E and 3F). Partial axis duplications (7 of 54) are also seen in these experiments (Figure 3G), as are major disruptions of ventral patterning, in which the borders of *Nv-twi* and *Nv-vnd* are neither parallel to nor continuous along the long axis of the embryo (6 of 54) (Figure 3H). Finally, embryos in which *Nv-twi* is only activated in small, chaotically distributed patches are observed at low frequency (4 of 54) (data not shown).

The observation of partial axis duplications and chaotic induction of ventral fates after *tgfα* pRNAi indicates that the DV patterning systems of *N. vitripennis* and *T. castaneum* have some self-regulatory properties that are normally constrained by maternal EGF signaling to give a single source of ventralizing activity along the AP axis of the embryo. Such a role for EGF signaling is well described in *D. melanogaster*, where strong *grk* mutations lead to duplicated peaks of ventralizing activity rather than a simple expansion of ventral fates [20]. Self-regulatory properties, albeit of a different nature, have been described for the Dorsal gradient in *T. castaneum* [21], and a similar system could exist in *N. vitripennis*.

We were not able to obtain embryos from *G. bimaculatus* after *Gb-tgfα* pRNAi, but we were able to detect a role for

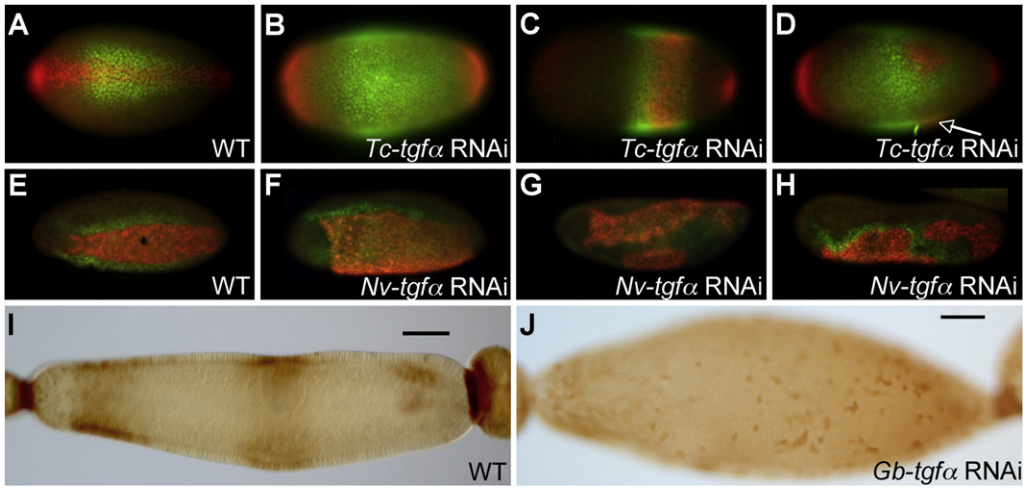

Figure 3. EGF Signaling Is Required for Proper DV Patterning in Insects

(A–D) Disruption of dorsal-ventral (DV) polarity in *T. castaneum* after *Tc-tgfα* pRNAi, as revealed by the expression of *Tc-sog* (green) and *Tc-cact* (red).

(A) Wild-type expression of *Tc-sog* and *Tc-cact* (ventral view).

(B–D) Representatives of different classes of *Tc-tgfα* pRNAi phenotypes (see Results and Discussion). Arrow in (D) indicates the out-of-focus additional ectopic spot of *Tc-cact* expression.

(E–H) Disruption of DV polarity in *N. vitripennis* after *Nv-tgfα* pRNAi, as revealed by the expression of *Nv-twi* (red) and *Nv-vnd* (green).

(E) Wild-type expression pattern of *Nv-twi* and *Nv-vnd* (ventral-lateral view).

(F–H) Expression of *Nv-twi* and *Nv-vnd* in *Nv-tgfα* pRNAi embryos representing different phenotypic classes (see Results and Discussion for details).

(I and J) After *Gb-tgfα* RNAi, the bilaterally symmetric banana shape and strong activation of MAPK signaling (brown) over the oocyte nucleus of late egg chambers (I) are lost, giving rise to egg chambers with radial symmetry (J).

this gene in generating asymmetry along the short (DV) axis of the egg chamber: *Gb-tgfα* pRNAi produces radially symmetric late egg chambers (Figure 3J) rather than the normal banana shape (Figure 3I). The degree to which asymmetry is lost appears to correlate with the degree of EGF signaling knockdown (Figures S4O–S4Q).

We propose that the DV patterning role of EGF signaling is related to the activation of this pathway in the follicle cells overlying the asymmetric oocyte nucleus (Figures 1I, 1L, 1M, and 1O) and that this signal acts as a dorsalizing influence to the embryo. This idea is supported by earlier observations in crickets that the position of the oocyte nucleus marks the future dorsal side of the egg [22], as well as our observation that *Nv-tgfα* mRNA persists on the dorsal side of freshly laid *N. vitripennis* eggs (data not shown). In *T. castaneum*, we used the position of the polar body, marked with EGFP, as a proxy for the final position of the oocyte nucleus and found that the position of the polar body is strongly associated with the future dorsal side of the embryo (Figure S5).

The Evolution of DV Patterning in the Insects

In sum, the most parsimonious explanation of our observations is that the EGF pathway had functions in mediating germline-soma communication and in establishing axial polarity in the most recent common ancestor of crickets, wasps, and beetles. In addition, because asymmetric activation of the EGF pathway is correlated with the position of the oocyte nucleus in all species examined and the final position of this organelle is strongly associated with the future dorsal side of the embryo, the migration of the oocyte nucleus to the cortex likely represents a symmetry-breaking event for DV patterning. How could such a system have originated?

The role of EGF signaling in mediating the encapsulation of the oocyte by the follicle cells probably predated the role of this pathway in DV patterning, because the latter process depends on the former. In addition, the eggshell structures produced by follicle cells were likely a crucial adaptation of insects to a terrestrial lifestyle [23]. Similarly, the migration of the oocyte nucleus to an asymmetric cortical location likely also predated any role in breaking DV symmetry, because the interaction of the egg cortex with the products of meiosis is critical for the formation and regulation of the polar bodies [24]. We propose that mechanisms later evolved to concentrate EGF signaling activity around an already asymmetrically localized oocyte nucleus and to allow transmission of this asymmetric signal to the overlying follicle cells to be used later during embryogenesis. This hypothesis is summarized in Figure 4.

The recruitment of EGF signaling and the oocyte nucleus for roles in DV patterning likely took place in a common ancestor of the Orthoptera and Holometabola, and analysis of this system in more basally branching insect or hexapod lineages will be required to precisely map its evolutionary origin.

Experimental Procedures

Additional experimental procedures can be found in the Supplemental Experimental Procedures.

In Situ Hybridization and Immunohistochemistry

Single-color in situ hybridization was performed as described in [25]. Two-color in situ hybridizations involved the use of α-biotin or α-fluorescein::AP antibodies, with Fast Red and HNPP (Roche) as substrates to give a red fluorescent signal, and α-digoxigen::POD antibodies, with AlexaFluor 488 tyramide (Invitrogen) as a substrate, following the method of [26].

Immunohistochemistry was performed as described in [27].

Antibodies used are as follows: α-nuclear pore (Sigma N8786) 1:2000, α-dpERK clone MAPK-YT (Sigma M-8159) 1:1000, α-Nv-Hb [28] 1:1000, α-Tc-Dl (J. Cande, M. Levine) 1:50, α-mouse or rabbit::AP 1:2500, and α-mouse or rabbit::POD 1:100.

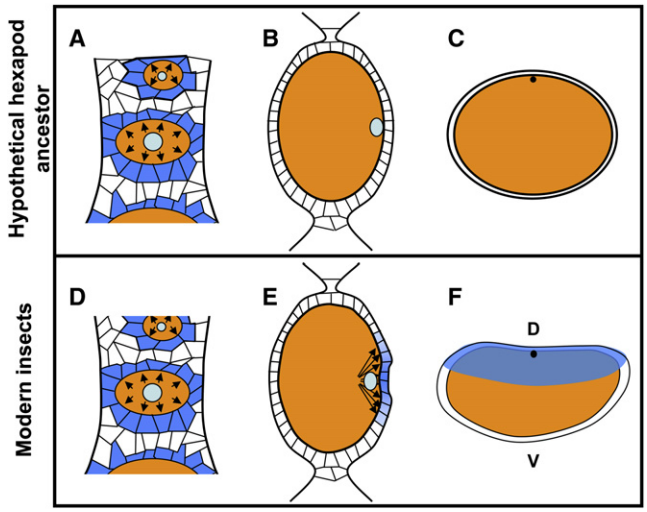

Figure 4. Model for the Evolutionary Origin of the Insect DV Patterning Strategy

(A–C) Schematic representation of features present in a hypothetical insect ancestor that predated the use of germline-soma signaling for DV axis establishment. Because the ovariole structure of ovaries is synapomorphic for insects, a mechanism for germline-soma communication in mediating the encapsulation of oocytes (orange ovals) by somatic cells was present in a common insect ancestor. Those somatic cells (marked in blue in A), receiving the epidermal growth factor (EGF) signal emanating from the oocyte (represented by outward-pointing arrows), will give rise to the follicle cells, which later give rise to eggshell structures such as the vitelline membrane and chorion but which at this stage have no role in receiving or transmitting patterning information. In this hypothetical ancestor, the oocyte nucleus (light blue) moved to a cortical location (B) to allow the formation of the polar body (black spot) in the egg after laying, but it originally had no role in embryonic patterning (C).

(D–F) Features present in modern insects, based on results presented in this manuscript. The role in EGF signaling in recruiting follicle cells to the oocyte (A) is maintained (D). The oocyte nucleus still moves to the cortex (E), because this event is still critical for regulation of meiosis. In this ancestor, however, EGF signaling becomes strongly associated with the position of the oocyte nucleus, allowing asymmetric activation of this pathway (blue gradient in E) in the overlying follicle cells. This patterning information is stored in the follicle cells, where it is incorporated into the eggshell (transparent blue in F) and is then later transmitted to the embryo to provide a basis for patterning the now maternally established DV axis.

Antibodies were detected either with AP-conjugated secondary antibodies, with either NBT/BCIP or Fast Red TR/HNPP (Roche) as substrates, or with POD conjugates, with either DAB or Alexa Fluor 488 tyramide (Invitrogen) as substrates.

Alexa Fluor 555-conjugated phalloidin (Invitrogen) was used at a dilution of 1:100 in PBT (1× phosphate-buffered saline [PBS], 0.1% Tween-20) for 1 hr to detect F-actin.

RNAi

Young *N. vitripennis* pupae were injected as in [29]. Adult *T. castaneum* females were injected as in [30]. *G. bimaculatus* adult females were injected with 5 μl of double-stranded RNA (dsRNA) solution via the method in [31].

Concentrations of dsRNA used were: *Nv-tgfα*: 0.8 μg/μL, *Tc-tgfα*: 0.6 μg/μL, and *Gb-tgfα*: 0.5 μg/μL.

Embryo and Ovary Dissection and Fixation

All *N. vitripennis* embryos were collected and hand devitellinized by the method in [29]. Because of the large reduction in fecundity, *Tc-tgfα* pRNAi embryos were also prepared by this method to minimize the loss of embryos inherent in the methanol devitellinization protocol used for wild-type eggs.

Cuticle preparations were performed as described in [29].

Ovaries from all species were dissected from adult females quickly in PBS with fine forceps. The peritoneal sheath was then removed with 27 gauge hypodermic needles. The ovaries were then fixed in 5% electron microscopy-grade formaldehyde in PBT (1× PBS, 0.1% Tween-20) for 30 min, either used immediately or dehydrated in methanol, and stored at −20° until needed.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.cub.2010.04.023.

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