Rho1 regulates signaling events required for proper *Drosophila* embryonic development

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Abstract

The Rho small GTPase has been implicated in many cellular processes, including actin cytoskeletal regulation and transcriptional activation. The molecular mechanisms underlying Rho function in many of these processes are not yet clear. Here we report that in *Drosophila*, reduction of maternal Rho1 compromises signaling pathways consistent with defects in membrane trafficking events. These mutants fail to maintain expression of the segment polarity genes *engrailed* (*en*), *wingless* (*wg*), and *hedgehog* (*hh*), contributing to a segmentation phenotype. Formation of the Wg protein gradient involves the internalization of Wg into vesicles. The number of these Wg-containing vesicles is reduced in maternal *Rho1* mutants, suggesting a defect in endocytosis. Consistent with this, stripes of cytoplasmic β-catenin that accumulate in response to Wg signaling are narrower in these mutants relative to wild type. Additionally, the amount of extracellular Wg protein is reduced in maternal *Rho1* mutants, indicating a defect in secretion. Signaling pathways downregulated by endocytosis, such as the epidermal growth factor receptor (EGFR) and Torso pathways, are hyperactivated in maternal *Rho1* mutants, consistent with a general role for Rho1 in regulating signaling events governing proper patterning during *Drosophila* development.

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Introduction

The specification of pattern during embryonic development requires the proper regulation of the various signaling pathways necessary to determine cell fate. In this context, overactive signaling can be as deleterious as a lack of signaling, necessitating mechanisms to control signaling activity. Downregulation of a number of signaling pathways has been known for some time to involve the endocytosis, and subsequent degradation, of receptor–ligand complexes (reviewed in *Di Fiore and Gill, 1999; Gonzalez-Gaitan, 2003*). Recently, data have accumulated linking endocytosis not only to downregulation of signaling, but also to activation of signaling and dispersal of ligands (e.g., *Parks et al., 2000; Piddini and Vincent, 2003*). In *Drosophila*, endocytosis of receptor–ligand complexes plays a role in the regulation of signaling pathways important in patterning the early embryo, including the activity of signaling pathways downstream of the epidermal growth factor receptor (EGFR; *Sturtevant et al., 1994*) and Torso receptor tyrosine kinases (*Lloyd et al., 2002*). Failure to properly downregulate these pathways in response to signaling results in the ectopic accumulation of their targets (*Lloyd et al., 2002*).

While simple secretion of ligands into the extracellular space is a necessary component of their dispersal, recent data are highlighting the role played by active membrane trafficking processes, particularly in the formation of...
bodies (MVBs; van den Heuvel et al., 1989) spreading out structures identified through EM studies as multivesicular embryo, the Wg protein can also be detected in punctate compartment (Greco et al., 2001). In the early Drosophila embryo, the Wg protein can also be detected in punctate structures termed argosomes, which are then transported from cell to cell through the endocytic compartment (Greco et al., 2001). In the early Drosophila embryo, the Wg protein can also be detected in punctate compartment (Greco et al., 2001). Alternatively, these vesicles have also been suggested to regulate Wg protein distribution through transcytosis of Wg from one cell to another (Pfeiffer et al., 2002).

One class of proteins demonstrated to have a role in the regulation of membrane trafficking, particularly endocytosis, is the Rho family of small GTPases, including Rho and its relatives Rac and Cdc42. Expression of constitutively active (CA) Rho or Rac in mammalian tissue culture cells has been shown to inhibit transferrin-receptor-mediated endocytosis (Lamaze et al., 1996). Microinjection of the Rho-specific inhibitor C3 exoenzyme blocks constitutive endocytosis in Xenopus oocytes, whereas injection of CARh newly stimulates it (Schmalzing et al., 1995). Overexpression of mammalian RhoB, which localizes to endosomes, inhibits the trafficking of EGFR-positive vesicles in tissue culture cells (Adamson et al., 1992), and mammalian RhoD also localizes to early and recycling endosomes and is involved in their trafficking (Murphy et al., 1996, 2001).

In addition to its roles in endocytosis, Rho has been linked to a wide variety of cellular functions, including cytoskeletal regulation, transcriptional activation, cell cycle progression, and others, though it is not clear in all cases how directly Rho is involved (reviewed in Hall, 1998; Mackay and Hall, 1998). Our previous work identified a segmentation defect due to a failure to maintain segment polarity gene expression as one of the phenotypes associated with loss of maternal Rho1 (Magie et al., 1999). In this paper, we describe a general role for Rho1 in signaling pathways involving membrane trafficking during early development in Drosophila. Reduction of maternal Rho1 activity results in the misregulation of a number of signaling pathways, including those mediated by the EGFR and Torso receptor tyrosine kinases, as well as aberrant Wg protein localization that results in a failure to properly activate the Wg signaling pathway and maintain expression of the segment polarity genes En, Wg, and Hh. The segmentation phenotype of maternal Rho1 mutants suggests that the effects of Rho1 on segment polarity gene signaling are limiting in this context.

Results and discussion

Maternal Rho1 mutants fail to maintain segment polarity gene expression and exhibit aberrant Wingless protein localization

To examine the phenotypes associated with loss of maternally deposited Rho1, we utilized a change-of-function mutation in an RNA polymerase II subunit, wimp, to reduce expression of maternal Rho1 (Magie et al., 1999, 2002; Parkhurst and Ish-Horowicz, 1991). It is not possible to completely eliminate maternal Rho1 function, as germline clones of Rho1 cannot be generated due to cell inviability (Magie et al., 1999). The reduction in Rho1 expression is achieved by generating females doubly heterozygous for wimp and Rho11B, a strong Rho1 allele, then mating them to wild-type males (see Materials and methods and Supplemental Fig. 1). The resulting embryos will hereafter be referred to as maternal Rho1 mutants.

Endocytic processes have been shown to be involved in the dispersal of some ligands (Entchev et al., 2000; Greco et al., 2001; Strigini and Cohen, 2000). This is particularly important in the context of morphogen gradient formation, both for morphogens that act long range such as Dpp, as well as short-range morphogens such as Hedgehog (Hh) and Wg. In the Drosophila embryo, proper regulation of segment polarity gene products, and in particular the distribution of Wg protein, is crucial to the proper patterning of the embryonic epidermis (Hays et al., 1997; O’Keefe et al., 1997).

Embryos with reduced maternal Rho1 exhibit a segmentation phenotype due in part to improper maintenance of segment polarity gene expression (74% penetrance; n = 126; Magie et al., 1999). In particular, expression of the segment polarity genes en, wg, and hh, while initiated normally, is not maintained properly, leading to the fusion or absence of stripes (Figs. 1A–F). Because we cannot make clones due to the requirement of Rho1 for cell viability, we are not able to directly assess if the Wg- or En/Hh-expressing cell or both require Rho1 function for proper maintenance of segment polarity gene expression.

The segmentation phenotype associated with reduced maternal Rho1 could result from different biochemical mechanisms. One possibility is that, similar to the involvement of Ras in MAPK signaling, Rho1 acts directly in a signal transduction pathway leading to transcriptional activation. Alternatively, Rho1 could affect the Wg signal transduction pathway through its effects on a general cellular process such as regulation of the cytoskeleton or membrane trafficking. To distinguish between these possibilities, we examined Wg protein distribution in maternal Rho1 mutants relative to wild type (Figs. 1H–H’) and wimp/+ controls (Figs. 1I–I’). In wild-type stage 9 embryos, Wg is expressed in a stripe two cells wide and can be detected in multivesicular bodies (MVBs) up to a distance of several cells away (Figs. 1H–H’; van den Heuvel et al., 1989).
formation of these vesicular structures has been shown to require endocytosis, as they are absent in embryos mutant for shibire (shi), which encodes the Drosophila homolog of Dynamin and cannot undergo endocytosis (Figs. 1J–J’; Bejsovec and Wieschaus, 1995). Similar to shi mutants, maternal Rho1 mutants also exhibit fewer Wg-positive
vesicles than wild-type embryos (Figs. 1K–K'), though the total amount of Wg protein present in these mutants is the same as wild type (Fig. 1G). This effect is rescued by the presence of a Rho1 transgene expressed under the control of its own promoter (Figs. 1L–L'; see Materials and methods). The presence of Wg protein in vesicular structures spreading out from cells that actively express it indicates the importance of membrane trafficking in the formation of the Wg protein gradient.

**Maternal Rho1 mutants exhibit defective secretion of Wg**

Actin cytoskeletal regulation has been linked to regulation of both the endocytic and secretory pathways (reviewed in Stamnes, 2002). To determine if defective secretion/exocytosis in maternal Rho1 mutants also contributes to the lack of Wg-containing vesicles, we examined the amount of extracellular Wg in wild type, wimp/+, shibire, and maternal Rho1 mutants by incubating embryos in primary antibody prior to permeabilization (Fig. 2; see Materials and methods). In wild type and wimp/+ control embryos, extracellular Wg can be seen surrounding the cells that produce it (Figs. 2A–B'; Pfeiffer et al., 2002). In shibire mutants, the amount of extracellular Wg is increased relative to wild type, as expected for a mutant that can exocytose, but not properly internalize, Wg (Figs. 2C–C'). In maternal Rho1 mutants, the amount of extracellular Wg is reduced relative to wild type, indicating an additional role for Rho1 in secretion of Wg (Figs. 2D–D') and suggesting an underlying role for Rho1 in multiple actin-based processes. As with conventional Wg staining (Figs. 1L–L'), this phenotype is rescued by the presence of a Rho1 transgene (Figs. 2E–E').

S2R+ cells treated with Rho1 dsRNA cannot respond properly to Wg signaling

In many systems, Wg signaling results in the cytoplasmic accumulation of β-catenin, which then enters the nucleus, binds transcription factors of the TCF/LEF family, and activates transcription from target genes (reviewed in Barker et al., 2000; Novak and Dedhar, 1999). S2R+ cells express the Wg receptor D-Frizzled2 (Dfz2; Yanagawa et al., 1998). Treatment of these cells with conditioned medium containing Wg results in the cytoplasmic accumulation of Armadillo (Arm), the Drosophila homolog of β-catenin (compare Figs. 3A and B; Yanagawa et al., 1998).

Treatment of S2 cells with Rho1 dsRNA has been shown to result in a multinucleate phenotype due to defects in cytokinesis, mimicking defects in cellularization exhibited by maternal Rho1 mutants (Supplemental Fig. 2; Somma et al., 2002). Rho1 dsRNA treatment in S2R+ cells treated with Wg attenuates the Wg-induced accumulation of Arm and DE-Cad (Figs. 3C, F, and J). Cells not exposed to Wg-conditioned medium show little Wg staining (Fig. 3G). Upon treatment with Wg, punctate staining could be seen in treated cells (Fig. 3H) due to the internalization of Wg. Treatment of cells with Rho1 dsRNA abolishes this punctate staining pattern (Fig. 3I), and Wg staining is more restricted
to the cell surface than in wild-type cells, suggesting that similar to what we observe in embryos, Wg internalization is compromised.

To directly assess endocytic activity in these cells, we added fluorescently labeled dextran (70 kDa) to the Wg-conditioned medium prior to treating the cells. Wild-type cells show an internalization of both Wg and dextran, with Wg-containing endosomes comprising a subset of those present within the cell (Figs. 3K–K*; yellow endosomes marked with arrows in Fig. 4K). These Wg-containing conditioned medium prior to treating the cells. Wild-type cells show an internalization of both Wg and dextran, with Wg-containing endosomes comprising a subset of those present within the cell (Figs. 3K–K*; yellow endosomes marked with arrows in Fig. 4K). These Wg-containing
endosomes are reduced in number in cells treated with 
Rho1 dsRNA, as shown by the lack of yellow staining in 
Figs. 3L–L (Fig. 3M; \( n = 50, P < 0.00001 \)). While some 
endosomes form in Rho1 dsRNA-treated cells (Figs. 3
L–L), they are smaller and reduced in number relative to 
wild-type cells (\( n = 50, P = 0.026 \)).

Cytoplasmic Arm accumulation is aberrant in maternal 
Rho1 mutants

As in S2R+ cells, activation of Wg signaling in the 
embryo also leads to accumulation of cytoplasmic Arm 
(Peifer et al., 1994). In stage 9–10 embryos, stripes of 
cytoplasmic Arm accumulation can be seen in cells 
responding to Wg signaling (Fig. 4). Consistent with 
previously published results, these stripes are narrower in 
shibire mutants relative to wild type or wimp controls due to 
the failure to form a proper Wg protein gradient (compare 
Figs. 4A and B with Fig. 4C; Bejsovec and Wieschaus, 
1995). Similarly, maternal Rho1 mutants also exhibit 
narrower overall stripe width (Figs. 4D–E; \( n = 10, P < 
0.001 \) for shibire; \( n = 10, P < 0.001 \) for maternal Rho1). The 
similarity between the shibire and maternal Rho1 mutant 
phenotypes suggests that the aberrant Wg protein distribu-
tion in these embryos results in their failure to properly 
activate Wg signaling (Fig. 4F).

Maternal Rho1 mutants exhibit general endocytosis defects

Membrane trafficking is an important aspect of the 
regulation of many developmental signaling pathways 
(Gonzalez-Gaitan, 2003). Initial links between endocytosis 
and signal transduction were shown to involve the 
attenuation of signal by internalizing receptor–ligand 
complexes and targeting them for lysosomal degradation, 
as in the case of epidermal growth factor receptor (EGFR) 
signaling (Dickson et al., 1983). To determine whether 
maternal Rho1 mutants exhibit a general defect in mem-
brane trafficking, we examined the activity of signaling 
pathways known to require endocytosis for their proper 
function (Fig. 5). EGFR signaling is under feedback 
control whereby activation of signaling leads to endocy-
tosis of active receptor and attenuation of the signal 
(Sturtevant et al., 1994). The accumulation of dual-
Fig. 5. Maternal Rho1 mutants are generally defective in endocytosis. (A–D) Wild type (A), wimp/+ (B) maternal Rho1 mutant (C), and prd (D) mutant embryos labeled with antibodies to dpERK and visualized with immunofluorescence. Note the ectopic accumulation of dpERK in Rho1 mutants relative to wild type, wimp/+, and prd controls. (E) Drosophila EGFR (DER) was immunoprecipitated from wild type, wimp/+, and maternal Rho1 mutant embryo lysates. Western analysis was used to identify the overall amount of DER protein immunoprecipitated and its phosphorylation state. The graph indicates the amount of phosphotyrosine detected relative to the total amount of DER protein. (F–I) Wild type (F), wimp/+ (G), and maternal Rho1 mutant (H and I) embryos labeled with antibodies against Tll. Brackets indicate the extent of the anterior and posterior expression domains. Note the posterior cellularization defect indicated by the arrow in I. (J) Quantitation of the extent of Tll expression domains, expressed as percentage embryo length. (K) Western analysis of Torso protein levels in 0–4 and 4–8 h wild type and maternal Rho1 embryo lysates. Fifty micrograms of total protein loaded per lane, tubulin levels shown as loading control. Scale bars: 100 μm.
phosphorylated extracellular signal-related kinase (dpERK) in response to phosphorylation by EGFR has been used as a marker for EGFR signaling, and Drosophila mutants such as hepatocyte growth factor-related tyrosine kinase substrate (hrs) that are unable to properly target activated EGFR for degradation show higher levels of dpERK accumulation compared to wild type (Lloyd et al., 2002). Similar to hrs mutants, maternal Rho1 mutants exhibit ectopic accumulation of dpERK relative to wild type and wimp/+ controls (Figs. 5A–C). This effect is not simply due to patterning defects, as the segmentation mutant prd, while missing segments, exhibits dpERK staining levels similar to wild type within each segment (Fig. 5D). To verify that this effect is due to the failure to attenuate EGFR signaling, we immunoprecipitated the Drosophila EGFR from wild type, wimp/+ , and maternal Rho1 mutant embryo lysates. If active receptor is not endocytosed and degraded properly, the phosphorylated form of EGFR accumulates to higher levels (Boni-Schnetzler and Pilch, 1987). In maternal Rho1 mutants, we observe higher levels of phosphorylated EGFR relative to wild type, indicating that dpERK accumulation is likely due to overactive EGFR signaling (Fig. 5E).

Another signal transduction pathway whose activity is regulated by endocytosis is that of the receptor tyrosine kinase Torso, which is involved in the specification of terminal pattern in Drosophila (Sprenger and Nusslein-Volhard, 1993). Accumulation of the transcription factor tailess (ill), a downstream target of Torso signaling, reflects the level of Torso activity (Ghiglione et al., 1999). In wild-type embryos, Tll is expressed at the posterior pole of the embryo and in a stripe near the anterior pole (Fig. 5F). In the membrane trafficking mutant hrs, the anterior Tll stripe is shifted posteriorly 50% and the posterior domain is expanded by 25% (Lloyd et al., 2002). The effect on Tll expression we observe in maternal Rho1 mutants is of similar magnitude as that reported for hrs, with a posterior shift of the anterior stripe by 27% and an expansion of the posterior domain by 25% relative to wimp/+ controls (Figs. 5F–I; bottom bars in Fig. 5I; n = 10, P < 0.002 for the anterior stripe, n = 10, P < 0.0001 for the posterior domain). Additionally, maternal Rho1 mutants exhibit terminal cellularization defects similar to those resulting from endocytosis defects in hrs mutants (Lloyd et al., 2002) and mutants affecting Torso signaling such as pole hole (arrow in Fig. 5I; Perrimon et al., 1986). To verify that the expansion of Tll expression is due to a failure to properly downregulate Torso protein levels, we performed Western analysis of 0–4 h wild type and maternal Rho1 embryo lysates (Fig. 5K). As expected, Torso protein is present in 0–4 h wild-type lysates, but absent from 4 to 8 h wild-type lysates. In maternal Rho1 mutant lysates, however, the Torso protein persists in 4–8 h lysates, indicating that the expansion of Tll expression is due to the failure to downregulate Torso signaling. Taken together, the misregulation of EGFR and Torso signaling pathways suggests that maternal Rho1 mutants are generally compromised in endocytosis.

Our data indicate that a number of signaling pathways important during early development in Drosophila are compromised in maternal Rho1 mutants. Our observation that secretion of Wg protein is aberrant in these mutants together with the endocytosis defects we observe in S2R + cells treated with Rho1 dsRNA and in maternal Rho1 embryos indicates that Rho1 plays a general role in membrane trafficking processes in the early embryo. The biochemical mechanisms through which Rho proteins affect membrane trafficking are currently unclear. One possibility is that the function of Rho1 in this process is a byproduct of its regulation of the actin cytoskeleton. In yeast, there is evidence that the actin cytoskeleton is important in endocytosis, as mutations in actin and some actin-binding proteins inhibit endocytosis (Lanzetti et al., 2001; Munn, 2001). In addition, yeast Rho1 has been shown to be involved in endocytosis of the α-receptor (deHart et al., 2003). In mammalian cells, treatment with pharmacological agents that perturb actin structure can affect endocytosis in a cell-type-specific way. In polarized epithelial cells, for example, treatment with the actin-depolymerizing drug cytochalasin D inhibits endocytosis specifically at the apical, but not the basolateral surface (Gottlieb et al., 1993). RhoA has also been implicated in endocytosis in polarized epithelial cells (Leung et al., 1999). In Drosophila, Rho1 has clear roles in actin cytoskeletal regulation during oogenesis and embryogenesis, consistent with the notion that Rho1 may be acting primarily through its effects on the actin cytoskeleton (Johndrow et al., in press; Magie et al., 1999, 2002).

Our observation that the segmentation phenotype in maternal Rho1 mutants is the result of general defects in membrane trafficking processes (both secretion and endocytosis) and not a primary effect on transcriptional activation has important implications for the interpretation of data linking Rho to disparate cellular processes. While current data cannot exclude the possibility that Rho directly acts in transcriptional activation or through many disparate mechanistic pathways, data are accumulating that suggest Rho may act primarily as a regulator of the actin cytoskeleton and other functions it has been linked to are indirect effects. For instance, the ability of Rho to influence transcriptional activation through the serum response factor (SRF; Geneste et al., 2002), as well as affect cell cycle progression (Roovers and Assoian, 2003), is due to its direct effects on actin cytoskeletal regulation. Identifying the molecular mechanisms underlying each of Rho’s activities will be crucial to determining whether Rho1 has direct effects on a number of pathways or has a small number of primary functions that indirectly affect other functions. Investigations of Rho GTPase function in genetically amenable model organisms are providing a diversity of developmental contexts in which to examine all aspects of Rho biology, and
the ability to examine specific, loss-of-function phenotypes will continue to aid identification of the mechanisms underlying Rho function.

**Materials and methods**

**Fly stocks**

Flies were cultured and crossed on yeast–cornmeal–molasses–malt extract medium at 25°C. The Rho1 allele used in this study is Rho1^{1B}/CyO, an imprecise P-element excision line that removes the Rho1 coding region C-terminal to amino acid 52 and produces no protein detectable by immunofluorescence (Supplemental Fig. 1). Other alleles used were shi^{1} (Poodry, 1990) and wimp/TM3 (Parkhurst and Ish-Horowicz, 1991).

**Construction of the Rho1 rescue construct and transformant lines**

The Rho1 rescue construct was made by subcloning a 7-kb HindIII–MluI fragment of genomic DNA containing the Rho1 locus into the pCasper4 transformation vector lacking Rho1. The 3.7-kb EcoRI–BamHI fragment within the coding region was excised and replaced with the corresponding 1.2-kb fragment from Rho1 coding region C-terminal to amino acid 52 and produces no protein detectable by immunofluorescence (Supplemental Fig. 1). Other alleles used were shi^{1} (Poodry, 1990) and wimp/TM3 (Parkhurst and Ish-Horowicz, 1991).

**Cell culture**

*Drosophila* cell lines used in this study were S2, S2R+ (S. Yanagawa), and S2hs-Wg (S. Cumberledge). Cells were grown at 25°C in Schneider’s medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 25 mM glutamine, penicillin, and streptomycin.

**RNAi in cells**

To generate Rho1 dsRNA, the Rho1 ORF was cloned into pBluescript in both orientations. Templates were produced by linearizing the constructs with *KpnI* or *BamHI*. ssRNA was transcribed from the T7 promoter of each template using the Megascript RNA production kit (Ambion), annealed, and dsRNA purified as per the kit protocol. Cells were washed 1× in Schneider’s medium without FBS and resuspended in medium without FBS containing 45 μg Rho dsRNA/mL (experimental) or no dsRNA (control). Cells were placed in 6-well culture dishes coated with gelatin and incubated at 25°C for 1 h. Two milliliters of Schneider’s medium with FBS was added to each well and the cells were incubated at 25°C for 3 days.

To assess RNAi efficiency, cell lysates were made by resuspending 1 well of cells in 200 μL L-buffer + protease inhibitors, sonicating 3× 10 s, and spinning out cellular debris. Lysates were separated by SDS-PAGE and analyzed by Western blot.

**Wg treatment of S2R+ cells**

Wg-conditioned medium was produced by culturing S2hs-Wg cells in Schneider’s medium + FBS, heat shocking them at 37°C for 30 min, and allowing them to recover at least 3 h. Following this recovery period, the S2hs-Wg cells were spun down and the medium added to S2R+ cells that were then incubated at least 3 h at 25°C. The biochemical responses of these cells to Wg exposure were assessed by making lysates and analyzing them by SDS-PAGE and Western blots, or by fixing and staining cells (see below).

**Immunofluorescence**

**Cells**

Cells were grown in 6-well culture dishes coated with gelatin and containing a coverslip. Following the experimental treatment (RNAi + Wg exposure), the coverslips were removed and the cells fixed in 4% formaldehyde in PBS for 15 min. They were washed 3× in PBS + 0.1% Tween, then incubated in 1× antibody for 1 h. They were then washed 3× in PBS + 0.1% Tween, then incubated in 2× antibody + propidium iodide as described (Magie et al., 2002) for 1 h. They were then washed 3× in PBS + 0.1% Tween, visualized on a Leica TCS confocal microscope, and processed with Adobe Photoshop.

Experiments examining dextran uptake were conducted by adding 0.25 μg/mL Texas-Red-conjugated Dextran 70 kDa (Molecular Probes) to the Wg-conditioned medium used for Wg treatment. Following Wg treatment, cells were washed 3× in PBS to remove noninternalized dextran, then fixed as described.

**Embryos**

Embryos were prepared and detection of proteins was performed as described (Parkhurst et al., 1990). Propidium iodide staining was done as described (Magie et al., 2002).

1× antisera used: anti-DE-cadherin (H. Oda; 1:100); anti-Dah (T. Hsieh; 1:50); anti-DER (N. Baker; 1:500); anti-Wg and anti-Arm (Developmental Studies Hybridoma Bank; 1:100 and 1:50); anti-phosphotyrosine (4G10; Upstate Biotechnology; 1:1000); anti-Rho1 P1D9 (Magie et al., 2002); anti-Hh (I. Guerrero; 1:600); and anti-dpERK (Cell Signaling Technologies; 1:500).

2× antisera used: anti-mouse or anti-rat Alexa 488, or anti-rabbit Alexa 568 (Molecular Probes; 1:2000).
Quantification of Arm stripe widths was performed by independently identifying the limits of stripe and interstripe regions with dotted lines, splitting the difference when these did not overlap exactly. The percentage of the total distance between stripes 2 and 7 that was covered by Arm expression was determined for each embryo scored, and these results averaged for each genotype.

Extracellular Wg staining

Embryos were dechorionated by hand, lined up on double stick tape, and covered with 4% formaldehyde in PBS without detergent. The vitelline envelope was punctured with a glass needle and the embryos fixed for 10'. They were then devitel linized by hand, incubated in 1° anti-Wg (1:50) in PBS for 1 h, washed 3× in PBS + 0.1% Tween, incubated in 2° anti-mouse Alexa 488 (Molecular Probes; 1:2000) in PBS + 0.1% Tween 30 min, washed 3× in PBS + 0.1% Tween, and mounted for visualization by confocal microscopy. Control experiments utilizing a primary antibody that recognizes α-Spectrin, a cytoplasmic protein, indicate that this technique specifically labels extracellular proteins (data not shown).

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Appendix A. Supplementary data


References


