RESEARCH COMMUNICATION

Uncoupling neurogenic gene networks in the *Drosophila* embryo

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The EGF signaling pathway specifies neuronal identities in the *Drosophila* embryo by regulating developmental patterning genes such as *intermediate neurons defective* (*ind*). EGFR is activated in the ventral midline and neurogenic ectoderm by the Spitz ligand, which is processed by the Rhomboid protease. CRISPR/Cas9 was used to delete defined *rhomboid* enhancers mediating expression at each site of Spitz processing. Surprisingly, the neurogenic ectoderm, not the ventral midline, was found to be the dominant source of EGF patterning activity. We suggest that *Drosophila* is undergoing an evolutionary transition in central nervous system (CNS)-organizing activity from the ventral midline to the neurogenic ectoderm.

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Transcriptional control of ligand production is important for the spatiotemporal patterning of cell fate during embryogenesis (Marinić et al. 2013). Limiting components of signaling pathways, such as a ligand or ligand-processing enzyme, are often controlled by multiple enhancers mediating distinct patterns of expression. There are many examples of complex gene control, including the FGF ligands Thisbe and Branchless and the BMP ligands Dpp and Gbb (Housden and Perrimon 2014). However, the specific contributions of individual enhancers to any given signaling process remain unclear. Here we address this question during the patterning of the neurogenic ectoderm in the *Drosophila* embryo, which is controlled by two EGF ligands: Vein and Spitz.

Vein is secreted from its site of synthesis, whereas Spitz requires processing by the membrane-bound Rhomboid protease. Vein and Spitz induce the expression of regulatory genes required for the specification of ventral and lateral neurons (Mayer and Nüsslein-Volhard 1988; Rutledge et al. 1992; Raz and Shilo 1993; Schweitzer et al. 1995; Schnepp et al. 1996; Golembo et al. 1996). High levels of EGF/ERK signaling trigger the expression of the *ventral nervous system defective* (*vnd*) determinant in ventral regions of the neurogenic ectoderm, while low levels activate *intermediate neuroblasts defective* (*ind*) in lateral regions (Ohlen and Doe 2000; Stathopoulos and Levine 2005; Ajuria et al. 2011; Lim et al. 2013, 2015).

Most previous studies on the establishment of this EGF signaling gradient have emphasized the paracrine effects of EGF ligands emanating from the ventral midline (Golembo et al. 1996; Chang et al. 2001; Rousso et al. 2010). However, both *vein* (*vn*) and *rhomboid* (*rho*) are also expressed in ventral regions of the presumptive neurogenic ectoderm ("lateral stripes") prior to the onset of gastrulation (Kosman et al. 1991; Ip et al. 1992; González-Crespo and Levine 1993; Markstein et al. 2004). It has not been possible to assess the relative contributions of the two sites of EGF synthesis, the ventral midline and lateral stripes, in the patterning of the central nervous system (CNS).

Past studies identified separate enhancers for the regulation of *rho* expression in lateral stripes and the ventral midline. Midline expression depends on two separate enhancers, the midline enhancer (MLE) and a putative shadow enhancer (SHA) (Kvon et al. 2014; Pearson and Crews 2014), which is consistent with the view that the midline source of the Vein and Spitz ligands is particularly important for the patterning of the CNS. The role of the lateral stripe pattern, which is controlled by a well-characterized neurogenic ectoderm enhancer (NEE), remains uncertain (Supplemental Table 1).

Here, we used newly developed CRISPR/Cas9 methods to uncouple EGF synthesis in lateral stripes and the ventral midline (Barrangou et al. 2007; Jinek et al. 2012; Gratz et al. 2013). Mutant embryos harboring deletions of defined *rho* enhancers were combined with *vn* mutants, since past studies have shown that Spitz and Vein function in a somewhat redundant fashion to activate *vnd* and *ind* expression in ventral and lateral regions of the developing ventral nerve cord (Skeath 1998; Lim et al. 2015). Spitz activity is fully abolished by the loss of *rho* activity, and, consequently, *rho;vn* double mutants exhibit a complete loss of *ind* expression due to the absence of both Vein and activated Spitz (Hong et al. 2008).

We were therefore able to obtain mutant embryos containing a midline-only source of EGF signaling by the targeted deletion of the *rho* NEE in vn^{-}/vn^{-} mutant embryos. These embryos exhibit incomplete patterns of *ind* expression, suggesting that the midline is not sufficient to pattern the CNS. Surprisingly, the reciprocal experiment, restricted processing of Spitz in lateral stripes, produces an essentially normal *ind* expression pattern. These findings underscore the importance of *rho* and *vn* lateral stripes in the formation of the EGF signaling gradient and patterning of the CNS. Genome-editing methods provide a powerful means for deleting defined enhancers within endogenous loci to uncouple the complex

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regulatory control of critical developmental patterning genes such as *rho*.

Results and Discussion

Differential activation of *vnd* and *ind* in ventral and lateral regions of the neurogenic ectoderm is controlled by Spitz and Vein EGF ligands (Ohlen and Doe 2000; Stathopoulos and Levine 2005; Ajuria et al. 2011; Lim et al. 2013, 2015) emanating from ventral regions of the neurogenic ectoderm (summarized in Fig. 1A). We used CRISPR/Cas9 genome-editing methods to determine the relative contributions of EGF signaling in lateral stripes (ventral neurogenic ectoderm) and the ventral midline by deleting defined *rho* enhancers: MLE, NEE, and SHA (summarized in Fig. 1B; Barrangou et al. 2007; Jinek et al. 2012; Gratz et al. 2013). The SHA and MLE mediate expression in the midline in response to the Sim activator, whereas the NEE activates *rho* in the presumptive neurogenic ectoderm (lateral stripes) in response to the maternal dorsal gradient.

Midline-specific EGF signaling was obtained by deleting the *rho* NEE. Mutant embryos exhibit a slight delay in the onset of *ind* expression but nonetheless grow to adulthood (Supplemental Fig. S1A,B″). Similar patterns of expression were also observed in rho^{-}/rho^{-} mutant embryos (Skeath 1998; Hong et al. 2008; Lim et al. 2013) as well as mutants harboring different combinations of *rho* enhancer deletions (Supplemental Fig. S1). These observations are consistent with previous findings that Vein alone is sufficient for the normal induction of *ind* expression (Supplemental Fig. S1). In order to assess the individual contributions of rho^{+} gene activity in the midline and neurogenic ectoderm, it was necessary to introduce *rho* NEE deficiency homozygotes into a vn^{-}/vn^{-} mutant background.

The resulting double mutants, *rho* ΔNEE;vn^{-}/rho ΔNEE;vn^{-}, completely lack both *rho* and *vn* lateral stripes (e.g., Fig. 1F). The only source of EGF signaling arises from the midline-specific expression of *rho* and localized processing of Spitz (Fig. 1E,H). Double-labeling methods using *rho* and *ind* RNA probes indicate a significant delay in the activation of *ind* (Fig. 1, cf. D and G). Moreover, the late *ind* expression pattern is incomplete and displays considerable variation between embryos (Fig. 1H). These observations suggest that the midline processing of the Spitz ligand is not sufficient for proper induction of *ind* expression and patterning of the nerve cord.

Most previous studies of EGF-mediated patterning of the CNS emphasized the role of the ventral midline as the source of ligand production (Golembo et al. 1996; Chang et al. 2001; Rousso et al. 2010). However, embryos with individual deletions of the MLE and SHA exhibit normal *rho* and *ind* expression patterns (Supplemental Fig. S1A–A″,C–D″). To more accurately assess the role of the midline, we produced embryos that selectively produce only lateral stripes of Spitz activity. This was achieved by creating *sim;vn* double-mutant embryos, which have transient lateral stripes of *rho* expression in the absence of midline activity (Fig. 2). Sim encodes a basic helix–loop–helix (bHLH)-PAS activator that functions as a "master regulator" of ventral midline differentiation. It is expressed in the presumptive midline prior to the onset of gastrulation, where it coordinates the expression of >50 different target genes, including *rho* and *vein*. sim^{-}/sim^{-} mutant embryos display a severe loss of *rho* and *vn* expression in the ventral midline (Crews et al. 1988; Thomas et al. 1988; Nambu et al. 1990, 1991; Chang et al. 2001; Kearney et al. 2004; Hong et al. 2013).

sim;vn double mutants produce active Spitz ligands in lateral stripes due solely to the transient activity of the *rho* NEE. The initial induction of *ind* expression is virtually indistinguishable from that seen in normal embryos (e.g., Fig. 2A,D) despite the loss of midline targets of EGF signaling such as *orthodenticle* (Supplemental Fig. S2). We expected premature loss of *ind* expression due to the absence of sustained *rho* expression in the midline. However, *ind* expression was found to persist in advanced-stage embryos long after the loss of the transient *rho* lateral stripes (Fig. 2E,F). This pattern is significantly less variable than that seen when Spitz is processed solely in the midline (Fig. 1H). We therefore conclude that transient lateral stripes of EGF signaling, rather than stable midline processing, provide the dominant source of EGF activity in the patterning of the *Drosophila* CNS (see below).

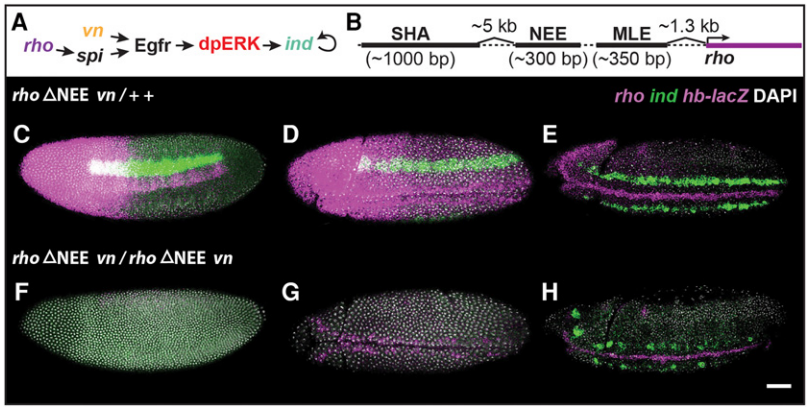

Figure 1. Late *rho* enhancers insufficient for normal *ind* expression. (*A*) The EGFR pathway with arrows showing activation. (*B*) The locations of known *rho* enhancers in relation to the genes. Black bars are the enhancers for the *rho* gene (purple bar). The dotted line indicates intergenic region. (*C–H*) The *rho* ΔNEE,*vn* double mutant is orientated with anterior to the left and posterior to the right. Images from early NC14 (*C,F*), gastrulation (*D,G*), and germ band elongation (*E,H*). (*C–E*) Heterozygous mutants. (*F–H*) Homozygous mutants. (Magenta lines) *rho* expression; (anterior magenta) *hb*-lacZ was used to visualize heterozygous mutants; (green lines) *ind* expression; (gray dots) DAPI. Bar, 50 µm.

The maintenance of the *ind* expression pattern in *sim;vn* double mutants raises the possibility that autoregulatory mechanisms might be responsible for sustained expression at later stages of embryogenesis (Von Ohlen et al. 2007). For example, positive autofeedback is responsible for maintaining *sim* expression in the ventral midline of advanced-stage embryos (Kasai et al. 1992). To explore whether a similar mechanism might be used to regulate *ind*, we induced ectopic EGF signaling by placing the *rho* coding sequence under the control of *ind* regulatory sequences (Fig. 3).

Normally, *rho* lateral stripes straddle the ventral border of the *ind* expression pattern (e.g., Fig. 1C). The use of *ind* regulatory sequences causes a significant expansion of the *rho* expression pattern into more dorsal

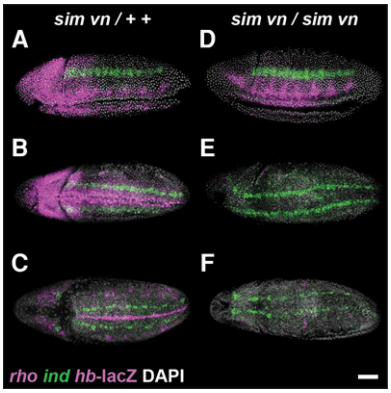

Figure 2. Early *rho* expression leads to stable late *ind* expression. The *sim,vn* heterozygous mutants (*A–C*) and homozygous mutants (*D–F*) are orientated with anterior to the left and posterior to the right. Images are from early NC14 (*A,D*), gastrulation (*B,E*), and germ band elongation (*C,F*). (Magenta lines) *rho* expression. (*A–C*) (Anterior magenta) *hb*-lacZ was used to visualize heterozygous mutants; (green lines) *ind* expression; (gray dots) DAPI. Bar, 50 µm.

regions of the neurogenic ectoderm. This expanded pattern leads to ectopic EGF signaling and dpERK activity in lateral regions of the developing nerve cord (Fig. 3, cf. A and E). The initial *ind* expression pattern is considerably broader than the wild-type pattern (Fig. 3, cf. C and G) but nonetheless refines to a nearly normal pattern following gastrulation (Fig. 3, cf. D and H). This refinement occurs in spite of expanded EGF signaling, suggesting the occurrence of compensatory mechanisms of activation and repression of *ind* expression during development.

CRISPR/Cas9 genome-editing methods were used for the selective removal of defined developmental enhancers controlling the complex *rho* expression pattern during *Drosophila* embryogenesis (summarized in Supplemental Table 2). This approach has the potential to reveal new mechanisms of gene control. For example, the larval cuticles of *rho* ΔNEE,*vn* and *sim;vn* show no morphological defects (Supplemental Fig. S3), yet the removal of the 5′ *rho* SHA and NEE appears to cause a delay in the onset of *rho* MLE activity in the ventral midline (Supplemental Fig. S4). The specific deletion of the *rho* NEE led to an incomplete and variable *ind* expression pattern, suggesting that the ventral midline may not be the central organizer of CNS patterning suggested by earlier studies (Golembo et al. 1996; Chang et al. 2001; Rousso et al. 2010). Instead, we showed that transient lateral stripes of EGF signaling are sufficient to induce *ind* expression, while subsequent compensatory mechanisms maintain this expression during embryogenesis.

The ventral midline is thought to be the ancestral mode of EGF signaling in the developing CNS of insects and other arthropods. For example, the ventral midline of the crustacean *Parhyale* plays a dominant role in the patterning of the CNS (Vargas-Vila et al. 2010). Disruption of sim^+ gene activity or ablation of midline cells causes severe patterning defects, whereas *sim* mutants in *Drosophila* exhibit essentially normal patterning of the nerve cord (except for the differentiation of mesectoderm cells arising from the midline) (Vargas-Vila et al. 2010; Lynch and Roth 2011). We propose that *Drosophila* represents a transitional patterning system in which the ancestral midline mode of EGF signaling is replaced by lateral stripes (Fig. 4). Rhomboid does not exhibit obvious lateral stripes of expression in other insects such as flour beetles and mosquitoes. This novel pattern of expression appears to coincide with the duplication of *rhomboid* genes in the Drosophilids (Crocker et al. 2010; Rousso et al. 2010).

Drosophila appears to use two distinct gene regulatory networks for deploying EGF signaling during embryogenesis. The expression of EGF signaling components in the ventral midline depends on the Sim activator, which in turn is induced by Notch signaling (Zinzen et al. 2006). In contrast, lateral stripes of *rho* and *vn* expression depend on dorsal and bHLH activators, including proneural determinants such as Daughterless and Achaete/Scute (González-Crespo and Levine 1993). The latter mode of EGF signaling is dominant in *Drosophila*, but the retention of midline patterning activity might render the system robust to genetic and environmental variation. This view of network evolution, the co-option of novelty while retaining ancestral mechanisms, might be a common property of animal development.

Materials and methods

CRISPR–Cas9 (Supplemental Tables 3–5)

Guide RNA (gRNA) The protocol was followed based on the publication by the O'Connor-Giles laboratory, and the unique PAM recognition sites were designed with the CRISPR optimal target finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder; Gratz et al. 2014). The two unique PAM recognition sites were inserted into individual pU6b-gRNA vectors by BbsI. PAM sites were verified in the injection line. The pU6b-gRNA was obtained from the Perrimon laboratory (Supplemental Material; Ren et al. 2013).

Donor vectors The p2xattP-dsRed donor vector was modified from the O'Connor-Giles laboratory pHD-DsRed-attP vector by the addition of an inverted attP site 3′ of the dsRed cassette (Supplemental Material). An ~1-kb 5′ homology arm was inserted with NheI and SacII. An ~1-kb 3′ homology arm was inserted with SbfI and AscI (modified MCS). The GFP donor vector (gift from Thomas Gregor's laboratory) used XhoI/AscI and AgeI/NotI for the 5′ and 3′ homology arms, respectively. CRISPR vectors (donor vector and two unique guide vectors) were injected into nos-Cas9 (y sc v;{nos-Cas9}attP40/CyO [Bestgene: TH00788.N]). The double enhancer deletions were produced by injecting previous dsRed CRISPR fly lines with the GFP donor vector (Supplemental Material), two unique

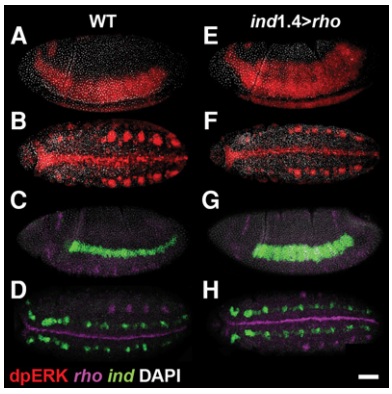

Figure 3. Early ectopic *ind* expression compensates for normal late expression. (*A–D*) Wild type (WT). (*E–H*) Homozygous *rho* overexpression. Images were taken at gastrulation (*A,E,C,G*) and germ band extension (*B,F,D,H*). All images are anterior to posterior. (Red lines) dpERK expression; (purple lines) *rho* expression; (green lines) *ind* expression; (gray dots) DAPI. Bar, 50 µm.

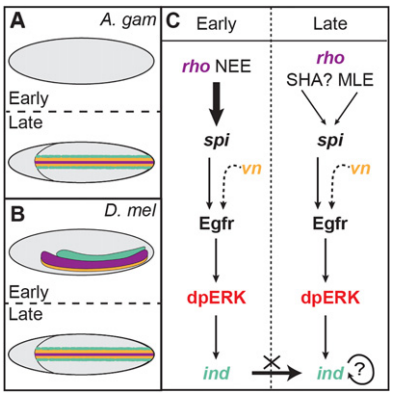

Figure 4. The ancestral *rho* MLE shifts its role to NEE for midline *ind* expression. (*A*) Mosquitos (*Anopheles gambiae*) rely primarily on the midline expression of *rho* for late *ind* expression. (*B*) Fruit fly (*Drosophila melanogaster*) *ind* regulation has a unique lateral expression followed by the ancestral late stage mediated mostly by *rho*. (*C*) *A. gambiae* has no lateral regulation of *ind* (X) and roles of *rho* SHA, and *ind* autoregulation are unknown (?). *D. melanogaster* seems to have acquired a major role for the *rho* NEE (large arrow) for regulating *ind* (arrow) while minimizing the role of the midline *rho* MLE and SHA (small arrows). (Purple) *rho*; (orange) *vn*; (green) *ind*.

guide vectors, and the nos-Cas9 plasmid. All lines were PCR-validated and imaged. All injections were performed by Bestgene. Primers were made by IDT.

Immunostaining and fluorescent in situ hybridization (FISH)

Immunostaining and FISH protocols were performed as described elsewhere (Lim et al. 2015). Sheep anti-digoxigenin (DIG; 1:125; Roche), mouse anti-biotin (1:125; Jackson ImmunoResearch), and rabbit anti-dpERK (1:100; CST4370) were used as primary antibodies. DAPI (1:10,000; Molecular Probes) was used to stain for nuclei, and Alexa fluor conjugates (1:500; Invitrogen) were used as secondary antibodies.

Fly recombination

;;vn,sim The vn^{L6}/TM3,*hb*-lacZ was crossed to sp/+;sim^{2}/TM3,*hb*-lacZ. The virgin female *vn/sim* flies were crossed to dsRed/TM3,*hb*-lacZ. Recombination was assayed by crossing individual recombinant males to virgins of each mutant line. Validated lines exhibited TM3 from the assay (n = 137).

;;vn,rho ΔNEE The vn^{L6}/TM3,*hb*-lacZ line was crossed to *rho* ΔNEE/TM3,*hb*-lacZ. The female virgin *vn/rho* ΔNEE flies were crossed to TM3,*hb*-lacZ. Individual males were tested for recombination by crossing to virgin *vn* mutants. Validated lines exhibited both TM3 and dsRed from the assays (n = 63).

Microscopy and image processing

Fluorescent imaging was performed on Nikon A1-RS scanning confocal (Fig. 1, 2) and Zeiss 880 (Fig. 3) microscopes with a 20× objective. For pairwise comparisons of wild-type and mutant backgrounds, embryos were collected, stained, and imaged together under the same experimental conditions. Broken embryos, embryos with intact vitelline membrane, or embryos undergoing mitosis were excluded from the analysis. The contrast and brightness for images were adjusted in ImageJ relative to the wild-type images for the final figure panels, thus allowing quantitative comparisons between two sets of images.

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