

# Convergence of Dorsal, Dpp, and Egfr Signaling Pathways Subdivides the *Drosophila* Neuroectoderm into Three Dorsal-Ventral Columns

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An important question in neurobiology is how different cell fates are established along the dorsoventral (DV) axis of the central nervous system (CNS). Here we investigate the origins of DV patterning within the *Drosophila* CNS. The earliest sign of neural DV patterning is the expression of three homeobox genes in the neuroectoderm—ventral nervous system defective (*vnd*), intermediate neuroblasts defective (*ind*), and muscle segment homeobox (*msh*)—which are expressed in ventral, intermediate, and dorsal columns of neuroectoderm, respectively. Previous studies have shown that the Dorsal, Decapentaplegic (Dpp), and EGF receptor (Egfr) signaling pathways regulate embryonic DV patterning, as well as aspects of CNS patterning. Here we describe the earliest expression of each DV column gene (*vnd*, *ind*, and *msh*), the regulatory relationships between all three DV column genes, and the role of the Dorsal, Dpp, and Egfr signaling pathways in defining *vnd*, *ind*, and *msh* expression domains. We confirm that the *vnd* domain is established by Dorsal and maintained by Egfr, but unlike a previous report we show that *vnd* is not regulated by Dpp signaling. We show that *ind* expression requires both Dorsal and Egfr signaling for activation and positioning of its dorsal border, and that abnormally high Dpp can repress *ind* expression. Finally, we show that the *msh* domain is defined by repression: it occurs only where Dpp, Vnd, and Ind activity is low. We conclude that the initial diversification of cell fates along the DV axis of the CNS is coordinately established by Dorsal, Dpp, and Egfr signaling pathways. Understanding the mechanisms involved in patterning *vnd*, *ind*, and *msh* expression is important, because DV columnar homeobox gene expression in the neuroectoderm is an early, essential, and evolutionarily conserved step in generating neuronal diversity along the DV axis of the CNS. © 2000 Academic Press

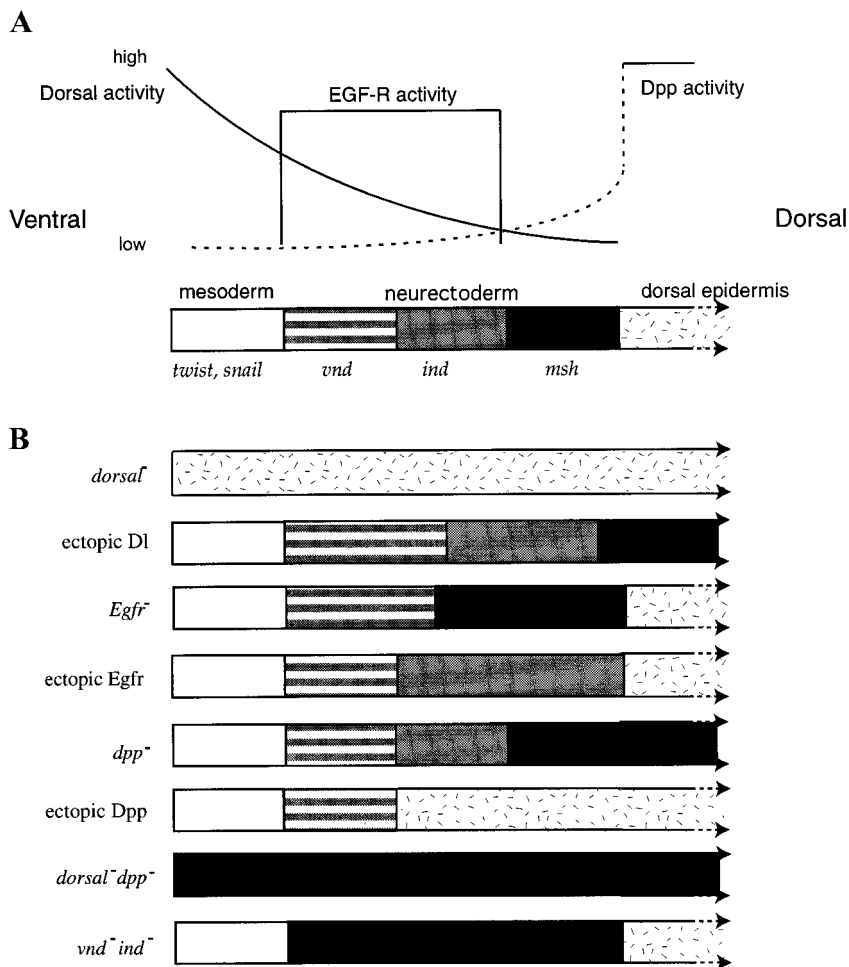
## INTRODUCTION

An early event in *Drosophila* embryogenesis is the subdivision of the embryo into specific domains along the dorsoventral (DV) axis. Three signaling pathways—Dorsal, Decapentaplegic (Dpp), and Epidermal Growth Factor Receptor (Egfr)—work in concert to subdivide the embryo into specific tissue types: mesoderm, neuroectoderm, dorsal epidermis and PNS, and amnioserosa (Fig. 1A). Each tissue is further subdivided into more precise DV domains. For example, the neuroectoderm expresses three homeobox genes in adjacent DV columns: the ventral column expresses *vnd*, the intermediate column expresses *ind*, and the dorsal column expresses *msh* (Mellerick and Nirenberg, 1995; Jiménez *et al.*, 1995; D'Alessio and Frasch, 1996;

Isshiki *et al.*, 1997; Weiss *et al.*, 1998). Here we investigate how the Dorsal, Egfr, and Dpp pathways converge to establish the three domains of homeobox gene expression along the DV axis of the CNS.

The ventral side of the embryo is patterned by maternally contributed Dorsal protein, a member of the Rel/NF- $\kappa$ B family, which is selectively transported into ventral nuclei in a graded fashion such that the highest levels of Dorsal protein are found in the most ventral nuclei (reviewed in Anderson, 1998). Nuclear localization of Dorsal is regulated by Cactus protein, which binds to Dorsal and prevents its nuclear localization (Whalen and Steward, 1993). In embryos lacking Cactus, high levels of Dorsal protein accumulate in both ventral and dorsal nuclei, leading to dorsal cells acquiring ventral fates. Peak levels of Dorsal in ventral regions activate the *twist* and *snail* genes resulting in mesodermal cell fates (reviewed in Rusch and Levine, 1996). Intermediate levels of Dorsal can directly or indi-

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**FIG. 1.** Summary of dorsal-ventral patterning in the *Drosophila* embryo. (A) Schematic depiction of the limits of Dpp, Egfr, and Dorsal signaling. Egfr signaling is active in the intermediate and ventral columns. Dorsal is active ventrally in tissues that give rise to mesoderm and ventral ectoderm. Dpp signaling is active dorsally, outside the neuroectoderm; dashed line indicates that the activity of Dpp is lower but uncharacterized within the neuroectoderm. (B) Summary of the results from this paper and others. See Materials and Methods for genotypes; see text for details.

rectly activate neuroectoderm-specific genes including *vnd* and *rhomboid* (*rho*); these genes are not expressed in the mesoderm because they are repressed by Snail (Mellerick and Nirenberg, 1995; Ip *et al.*, 1992; Thisse *et al.*, 1991; Jiang *et al.*, 1991). Thus, the Dorsal gradient promotes the formation of mesoderm and ventral neuroectoderm, positions the boundary between them, and activates *vnd* expression; however, its role in promoting or repressing *ind* and *msh* expression is unknown.

The dorsal surface of the embryos is patterned by zygotically expressed Dpp, a secreted protein of the TGF $\beta$  family. *dpp* transcription is repressed by Dorsal, thus limiting *dpp* expression to the dorsal surface of the embryo. The Dpp activity gradient is hard to predict, but it is clearly high dorsally and much lower within the neuroectoderm due to expression of the Dpp pathway antagonists *brinker* (*brk*)

and *short gastrulation* (*sog*) within the neuroectoderm (Biehs *et al.*, 1996; Jazwinska *et al.*, 1999). Embryos with reduced Dpp activity show an expansion of neuroectoderm at the expense of dorsal structures (amnioserosa and dorsal epidermis; D'Alessio and Frasch, 1996; Jazwinska *et al.*, 1999; Irish and Gelbart, 1987; Ferguson and Anderson, 1992a,b; Wharton *et al.*, 1993) and have been reported to show an expansion of the *vnd* within the neuroectoderm (Mellerick and Nirenberg, 1995). In contrast, ectopic Dpp activity leads to expansion of dorsal tissues at the expense of neuroectoderm (D'Alessio and Frasch, 1996; Jazwinska *et al.*, 1999; Ferguson and Anderson, 1992a,b). Thus, the Dpp gradient promotes the formation of dorsal epidermal tissues and establishes the dorsal boundary of the neuroectoderm, but its role in specifying different DV domains within the neuroectoderm has not been fully explored.

The Egfr signaling pathway has also been implicated in DV patterning within the CNS (Skeath, 1998; Yagi *et al.*, 1998). Egfr is ubiquitous, but its ligand Spitz is restricted to the ventral midline of the neuroectoderm. An additional activating ligand, Vein, is also expressed in the ventral neuroectoderm and is important for robust activation of the Egfr pathway (Schnepp *et al.*, 1996; Golembo *et al.*, 1999). Consistent with these data, a reporter for active Egfr signaling (diphosphorylated MAP kinase) is detected in the ventral and intermediate columns of the neuroectoderm (Skeath, 1998; Yagi *et al.*, 1998). Embryos lacking Egfr function show early defects in neuroblast formation in the intermediate column of the neuroectoderm (Skeath, 1998; Yagi *et al.*, 1998), and late defects in gene expression within the ventral neuroectoderm (Yagi *et al.*, 1998). However, it is not known whether Egfr is involved in establishing *vnd*, *ind*, or *msh* expression domains, nor whether it acts by modulating Dorsal or Dpp activity.

## MATERIALS AND METHODS

### Fly Lines

*y w* flies were used as the wild-type stock. Embryos lacking Dorsal function (*dorsal* embryos) were derived from homozygous *dorsal*<sup>1</sup> mothers. Embryos with ectopic Dorsal function were derived from homozygous *cactus*<sup>1</sup> mothers.

Embryos lacking Dpp function (*dpp* embryos) were homozygous *dpp*<sup>H94</sup> embryos derived from *dpp*<sup>H94</sup>/*CyO23* flies. Embryos with ectopic Dpp function (ectopic Dpp) embryos were either homozygous *sog*<sup>U2</sup> embryos derived from *sog*<sup>U2</sup>/*FM7 ftzlacZ* flies (Fig. 7E) or homozygous *sog*<sup>YS06</sup> *4xdpp* embryos derived from *sog*<sup>YS06</sup>/*FM7 ftzlacZ*; *Dp(2;2)DTD48/CyO23*, *Pdpp+* flies (Figs. 3E and 4G), or homozygous *brk*<sup>M68</sup> *sog*<sup>YS06</sup> embryos derived from *brk*<sup>M68</sup> *sog*<sup>YS06</sup>/*FM7 ftzlacZ* flies (Figs. 3F, 4H, and 7F). The latter two stocks were provided by Jazwinska and Roth (Jazwinska *et al.*, 1999).

Embryos lacking Egfr function (*Egfr* embryos) were either homozygous *Egfr*<sup>flb-1K35</sup> embryos or homozygous *rho*<sup>7M</sup> *vn*<sup>dddRy</sup> embryos (Figs. 5D and 5H) derived from *Egfr*<sup>flb-1K35</sup>/*CyO ftzlacZ* or *rho*<sup>7M</sup> *vn*<sup>dddRy</sup>/*TM3 ftzlacZ* flies (obtained from Jim Skeath, Washington University, St Louis, MO). *rho*<sup>7M</sup> *vn*<sup>dddRy</sup> and *Egfr*<sup>flb-1K35</sup> embryos have an indistinguishable phenotypes (Skeath, 1998). Ectopic activation of the Egfr pathway was accomplished by overexpression of *rho* using a heat shock-inducible promoter (Sturtevant *et al.*, 1993). *Hs-rho* embryos, 2–3 h old, were heat shocked at 37°C for 25 min and then allowed to recover for 1 h at 25°C before fixing.

Embryos lacking both Dorsal and Dpp function (*dorsal dpp* embryos) were of the genotype *dorsal*<sup>1</sup> *dpp*<sup>H48</sup>/*dpp*<sup>H94</sup>, derived from homozygous *dorsal*<sup>1</sup> mothers obtained from *DP(2;1)G146dpp+*; *dorsal*<sup>1</sup> *dpp*<sup>H48</sup> *wg*<sup>SP-1</sup>/*SM6b* flies (Panzer *et al.*, 1992) crossed to *dpp*<sup>H94</sup>/*CyO23*. Of 93 embryos scored for *ind* expression, 9 had a ring of *ind* around the head region (due to expansion of a dorso-lateral head domain of *ind* expression); this phenotype is not seen in either single mutant and was observed at a ratio of 1:10, consistent with the ratio of 1:12 *dorsal dpp* embryos observed in a previous study (Panzer *et al.*, 1992). The “*ind* head ring” was then used to identify *dorsal dpp* embryos in subsequent experiments.

*vnd ind* embryos were obtained from *vnd*<sup>6</sup>/*FM6*; *ind*<sup>RR108</sup>/*TM3 ftzlacZ* flies.

### mRNA and Protein Detection in Embryos

Embryos were collected and fixed according to standard procedures (Mc Donald *et al.*, 1998). Primary antibodies used were rabbit anti-Vnd (1:20; Mc Donald *et al.*, 1998), rabbit anti-Msh (1:500; T. Isshiki and A. Nose), rat anti-Ind (1:250; Weiss *et al.*, 1998), mouse anti-diphosphorylated MAP kinase (1:2000; Gabay *et al.*, 1997; Sigma), mouse anti-β-galactosidase (1:500; Promega), and rabbit anti-β-galactosidase (1:5000, Cappel). Fluorescent images were collected using a Bio-Rad confocal microscope. Histochemical images were collected using a Zeiss Axioplan and a Sony DKC-5000 digital camera. Standard methods were used for RNA *in situ* hybridizations (Tautz and Pfeiffle, 1989). *ind* and *msh* cDNA clones have been described (Isshiki *et al.*, 1997; Weiss *et al.*, 1998); *vnd* cDNA was a gift from Dervela Mellerick (Michigan).

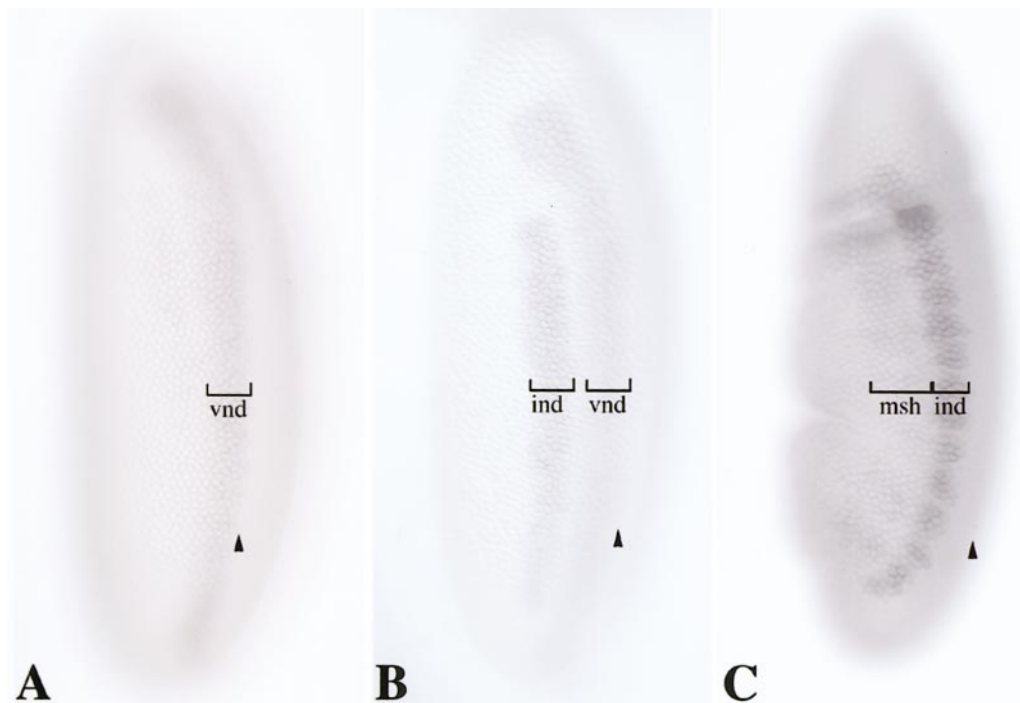
## RESULTS

### Initiation of *vnd*, *ind*, and *msh* Expression

Previous studies have shown that *vnd*, *ind*, and *msh* are expressed in adjacent domains of the neuroectoderm, from ventral to dorsal, respectively (Mellerick and Nirenberg, 1995; Jiménez *et al.*, 1995; D’Alessio and Frasch, 1996; Isshiki *et al.*, 1997; Weiss *et al.*, 1998); however, the timing and spacing of their initial expression patterns have not been investigated. Using double-label *in situ* hybridization, we find that early stage 5 embryos express *vnd* in a narrow domain similar to its final width; *ind* and *msh* are not detected (Fig. 2A; staging according to Campos-Ortega and Hartenstein, 1985). By the end of stage 5, both *vnd* and *ind* are expressed with a one to two cell wide gap; again in domains similar to their final widths (Fig. 2B). The gap fills in during development resulting in the precise juxtaposition of the *vnd* and *ind* domains (Weiss *et al.*, 1998). Expression of *msh* in the trunk is not detected until stage 7 (Fig. 2C). Thus, the timing of gene expression progresses from ventral to dorsal: *vnd* is detected first, *ind* appears soon after, and *msh* is observed last.

### Activation and Patterning of the *vnd* Expression Domain

To investigate the mechanisms establishing the domain of *vnd* expression we examined embryos lacking, or ectopically expressing, each of the three known signaling pathways active along the DV axis (Dorsal, Dpp, and Egfr). We confirm that embryos lacking Dorsal function (called *dorsal* embryos; derived from homozygous *dorsal* mothers, see Materials and Methods) fail to express *vnd* (Mellerick and Nirenberg, 1995; Fig. 3B). Conversely, embryos where Dorsal is ectopically activated (called ectopic Dorsal embryos; derived from homozygous *cactus* mothers, see Materials and Methods) show a dorsal expansion of *vnd* expression, to a width of 7 to 9 cell diameters instead of the normal width of 5 cell diameters (Fig. 3C). Our results do not reveal whether Dorsal regulates *vnd* directly, or indirectly via repression of Dpp within the neuroectoderm. There is good precedent for considering the latter mechanism: *dorsal*



**FIG. 2.** Ventral to dorsal progression in the initiation of *vnd*, *ind*, and *msh* expression. Anterior is up; arrowhead indicates position of the ventral midline of the CNS. (A) Early stage 5 embryo showing *vnd* mRNA expression (light purple). *ind* mRNA expression is just being initiated. (B) Stage 5 embryo showing expression of *vnd* mRNA (light purple) and *ind* mRNA (dark purple); initially there is a gap of 1–2 cells between *ind* and *vnd* domains. (C) Stage 7 embryo showing expression of *ind* mRNA (dark purple) and *msh* mRNA (light purple); no gap is observed between *ind* and *msh* domains.

embryos show elevated Dpp activity in the neuroectoderm (Biehs *et al.*, 1996; Jazwinska *et al.*, 1999), and Dpp has been proposed to repress *vnd* expression (Mellerick and Nirenberg, 1995). Surprisingly, we find that embryos lacking both Dorsal and Dpp function (*dorsal dpp* embryos; see Materials and Methods) have a lack of detectable levels of *vnd* protein in the trunk region of the embryo (Fig. 4D), showing that loss of *vnd* expression in *dorsal* embryos is not due to de-repression of Dpp activity in the neuroectoderm. We conclude that Dorsal is necessary to activate *vnd* expression, probably directly, and that increased Dorsal levels can expand the *vnd* domain.

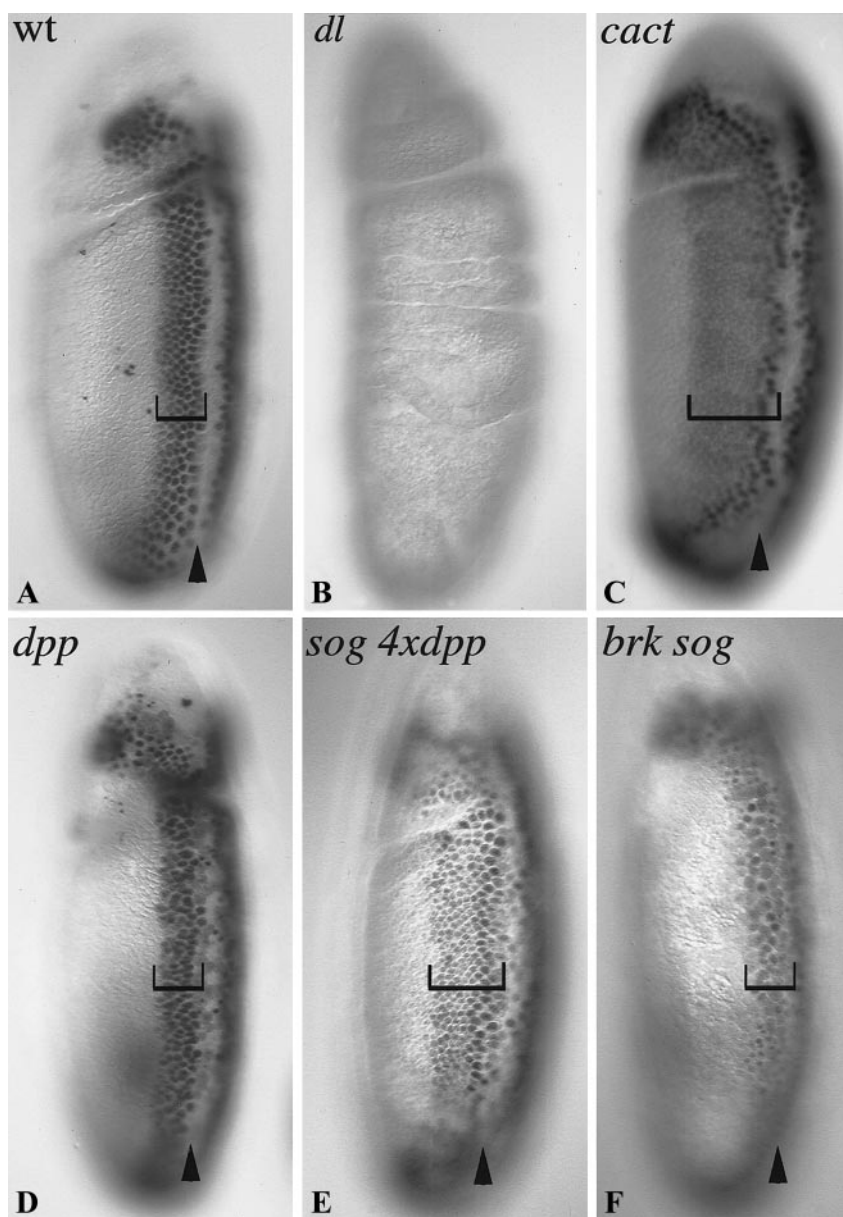
It has been proposed that Dpp signaling represses *vnd* expression and thus establishes the dorsal border of the *vnd* domain (Mellerick and Nirenberg, 1995). In contrast, we find that embryos with severely reduced Dpp activity (*dpp* embryos, see Materials and Methods) show no change in the pattern of *vnd* expression (Fig. 3D). Moreover, two different genetic backgrounds leading to ectopic Dpp activity (ectopic Dpp embryos, see Materials and Methods) show no repression of *vnd* expression (Figs. 3E and 3F). These results show that Dpp is not required to activate *vnd* expression, nor to establish the dorsal border of *vnd* expression (see Discussion).

Embryos lacking Egfr signaling (*Egfr* embryos, see Mate-

rials and Methods) have normal early *vnd* expression followed by premature loss of expression, indicating that Egfr signaling is not required for initiating *vnd* expression (Gabay *et al.*, 1997; data not shown). To determine if Egfr signaling is sufficient to induce *vnd* expression, we examined *vnd* expression in embryos where Egfr signaling is ectopically activated (*Hs-rho* embryos, see Materials and Methods). Embryos with ectopic Egfr activity have a normal pattern of *vnd* expression, despite the expanded expression of diphosphorylated MAP kinase (data not shown), a marker for Egfr activity (Gabay *et al.*, 1997). Thus, the timing and pattern of Egfr signaling play no role in establishing the initial domain of *vnd* expression, although it does have a late function in maintaining *vnd* levels.

### Activation and Patterning of the *ind* Expression Domain

We have previously shown that Vnd represses *ind* expression and thus establishes the ventral border of the *ind* domain (Weiss *et al.*, 1998; Mc Donald *et al.*, 1998), however, the inputs that activate *ind* expression and set its dorsal border are unknown. In *dorsal* embryos, stripes of *ind* expression are not detected at any stage of development (Fig. 5B); in contrast, ectopic Dorsal embryos show an

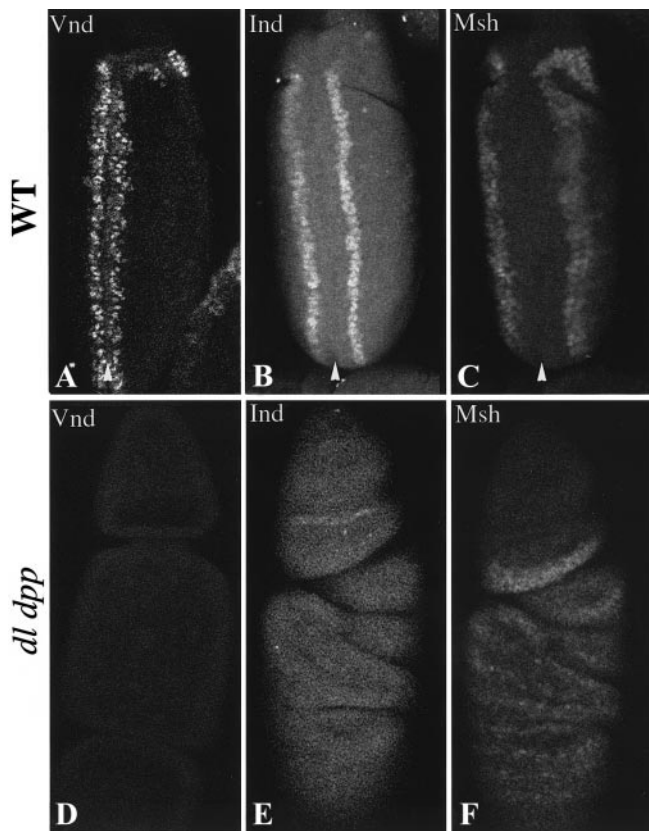


**FIG. 3.** Establishing the *vnd* expression domain. Vnd protein staining in stage 8 embryos, anterior is up; arrowhead indicates ventral midline of CNS. Genotypes are as labeled and described under Materials and Methods. Width of Vnd stripe is indicated by brackets. (A) Wild-type embryo: Vnd is detected in the ventral column of neuroectoderm. (B) *dorsal* embryo: Vnd is not detected. (C) Embryo with ectopic Dorsal activity: Vnd is expanded dorsally. (D) *dpp* embryo: Vnd expression is similar to wild type. (E) Embryo with ectopic Dpp activity: Vnd expression may show a slight dorsal expansion. (F) Embryo with ectopic Dpp activity: Vnd expression is similar to wild type.

expansion of the *ind* expression domain and a shift in the ventral border of *ind* expression toward a more dorsal position (Fig. 5C). Thus, elevated Dorsal activity will expand the *ind* expression domain, consistent with Dorsal acting as a concentration-dependent activator of *ind* expression. The shift of the *ind* ventral border in ectopic Dorsal embryos is likely due to the expansion of *vnd* (Fig. 3C),

because *vnd* is a known repressor of *ind* expression (Weiss *et al.*, 1998; Mc Donald *et al.*, 1998).

In *Egfr* embryos, we do not detect *ind* expression (Fig. 5D); we confirm the loss of *Egfr* signaling in these embryos by the absence of activated MAP kinase (data not shown). Conversely, ectopic *Egfr* embryos show a dorsal expansion of *ind* expression (Fig. 5E), as well as the



**FIG. 4.** Regulation of *vnd*, *ind*, and *msh* expression: epistasis between *dorsal* and *dpp*. All embryos are stage 10, anterior is up; ventral midline of CNS, arrowhead. (A–C) Wild-type embryos showing (A) Vnd protein, (B) Ind protein, (C) Msh protein. (D–F) *dorsal dpp* double mutant embryos showing (D) no Vnd expression, (E) no Ind expression except the “head ring,” and (F) ubiquitous Msh expression around the DV axis.

expected ubiquitous expression of activated MAP kinase (data not shown).

In order to discern the relationship between Dorsal and Egfr pathways, we performed epistasis experiments with loss- and gain-of-function mutations. In embryos with ectopic activation of both Dorsal and Egfr (see Materials and Methods), the domain of *ind* expression expands beyond that observed in either genetic background alone (compare Fig. 6B with Figs. 5C and 5E); we also observe the expected ubiquitous activation of MAP kinase (Fig. 6F). In embryos with ectopic Egfr but lacking Dorsal (see Materials and Methods), we observe ubiquitous activated MAP kinase (Fig. 6G), yet there is absolutely no *ind* expression (Fig. 6C). In the converse experiment, embryos with ectopic Dorsal but no Egfr function (see Materials and Methods) show a brief initiation of *ind* expression with a slight dorsal expansion (similar to embryos with ectopic Dorsal only), but the expression decays prematurely (Fig. 6D); we confirm that Egfr signaling is abolished in the neuroectoderm by lack of

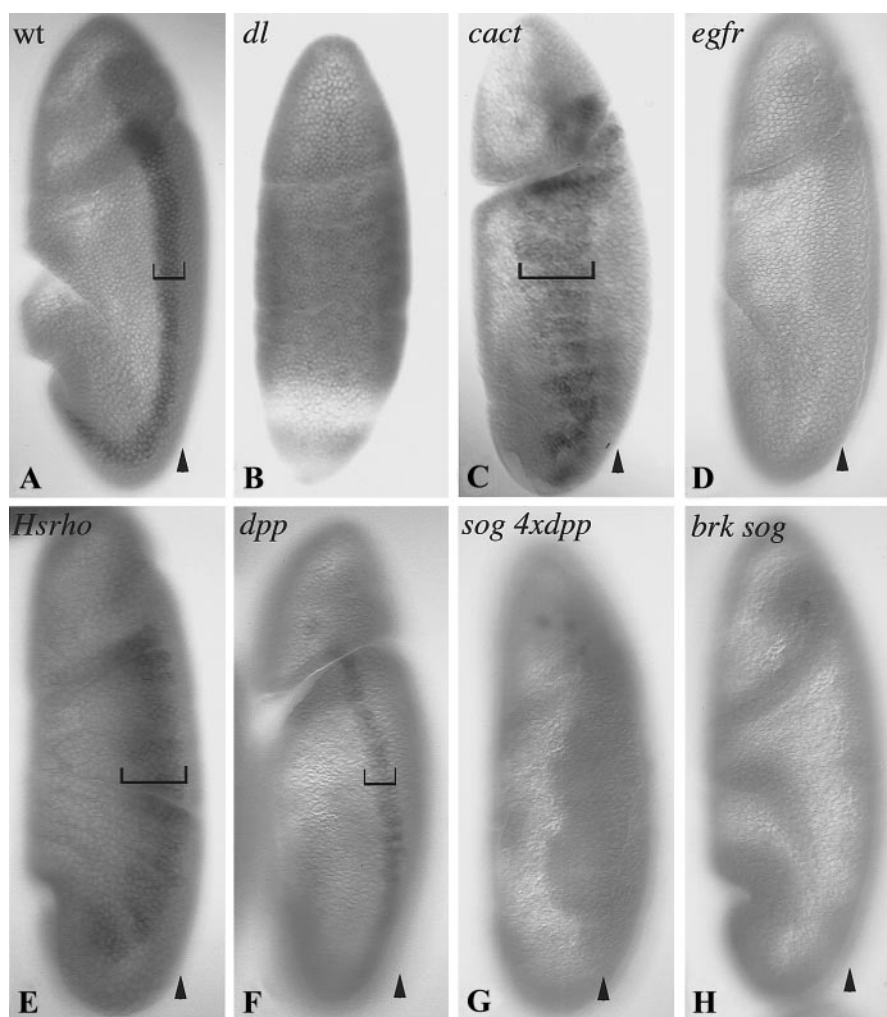
activated MAP kinase (Fig. 6H). Taken together, our results show that (1) both Dorsal and Egfr are required to activate *ind* expression; (2) ectopic expression of either Dorsal or Egfr in an otherwise wild-type embryo can expand the *ind* expression domain; (3) ectopic expression of both Dorsal and Egfr expands *ind* more than either alone; and (4) overexpression of Dorsal in the absence of Egfr can transiently activate *ind* expression, but not vice versa, suggesting that Dorsal is a more potent activator of *ind* expression. We conclude that Dorsal and Egfr normally act together to activate *ind* expression, and that the dorsal border of the *ind* domain is set by the dorsal border of Egfr signaling (see Discussion).

We assayed *ind* expression in *dpp* embryos and found it to be normal (Fig. 5F). However, ectopic Dpp embryos of two different genotypes showed significant repression of *ind* expression (Figs. 5G and 5H). Thus, *ind* expression requires that Dpp activity be kept low. Because Dpp can repress *ind* expression, and because *dorsal* embryos have high Dpp in the neuroectoderm, we assayed *dl dpp* embryos for rescue of *ind* expression. We found no *ind* expression in *dl dpp* embryos except a ring of *ind* expression in the head (Fig. 4E), showing that the loss of *ind* expression in *dorsal* embryos is not due to ectopic Dpp activity.

#### Activation and Patterning of the *msh* Expression Domain

*msh* is expressed in the most dorsal column of neuroectoderm beginning at stage 7. At this time of development, the *msh* domain may be exposed to low levels of Dpp, but both Egfr activity and Dorsal protein are not detectable. We find that *dorsal* embryos lack *msh* expression (Fig. 7B), whereas ectopic Dorsal embryos show an expansion of *msh* expression around the dorsal circumference of the embryo (Fig. 7C). Dorsal is unlikely to be a direct activator of *msh* expression, however, because *dorsal dpp* double mutants show widespread expression of *msh* throughout the embryo (Fig. 4F). We conclude that Dorsal keeps Dpp activity low within the neuroectoderm, thus allowing *msh* expression (see Discussion). Consistent with this conclusion, a reduction in Dpp activity expands *msh* expression dorsally (Fig. 7D; D'Alessio and Frasch, 1996), while a slight increase in Dpp activity in the neuroectoderm (*sog* embryos, see Materials and Methods) leads to a partial reduction in Msh expression (Fig. 7E; D'Alessio and Frasch, 1996), and high level ectopic Dpp in the neuroectoderm (*brk sog* embryos, see Materials and Methods) represses *msh* expression (Fig. 7F). Thus, *msh* is expressed only where Dpp activity is low.

The entire neuroectoderm has low Dpp activity, due to expression of the Dpp inhibitors *brk* and *sog*. What keeps *msh* expression off in the ventral and intermediate columns of the neuroectoderm? Previously, we reported that in *ind* mutant embryos, *msh* expression shows a slight ventral expansion (Weiss *et al.*, 1998; Fig. 7H). Here we show that in *vnd ind* double mutant embryos (see Materials and Methods), *msh* expression is detected throughout the neu-



**FIG. 5.** Establishing the *ind* expression domain. *ind* mRNA expression in stage 7 embryos, anterior is up, ventral to left; ventral midline of CNS, arrowhead. Genotypes are as labeled and described under Materials and Methods. Width of *ind* stripe indicated by brackets. (A) Wild-type embryo: *ind* is expressed in the intermediate column of neuroectoderm. (B) *dorsal* embryo: *ind* expression is not detected. (C) Embryo with ectopic Dorsal activity: *ind* expression is expanded slightly dorsally. (D) *Egfr* embryo: *ind* is not expressed. (E) Embryo with ectopic *Egfr* activity: *ind* expression is expanded slightly dorsally. (F) *dpp* embryo: *ind* expression is the same as in wild type. (G) Embryo with ectopic Dpp activity (*sog 4xdpp*): *ind* expression is repressed in the trunk. (H) Embryo with ectopic Dpp activity (*brk sog*): *ind* is not expressed in the trunk.

roectoderm (Fig. 7I). Thus, *msh* has the potential to be expressed in the entire neuroectoderm, but is normally restricted to the dorsal column due to repression by Vnd and Ind.

*Egfr* signaling also modulates *msh* expression. In embryos lacking *Egfr* signaling, *msh* expands slightly into the intermediate column (D'Alessio and Frasch, 1996); this is likely an indirect effect caused by the loss of *ind* expression (Fig. 5D), because *ind* mutant embryos show an identical phenotype (Weiss *et al.*, 1998). Ectopic *Egfr* leads to a loss of *msh* expression (data not shown) and an expansion of *ind* expression (Fig. 5E); again, the *msh* phenotype is likely

caused by the expansion of *ind*, since misexpression of *ind* can repress *msh* (data not shown).

## DISCUSSION

Expression of *vnd*, *ind*, and *msh* follows a ventral to dorsal progression: *vnd* is expressed first, followed by *ind* and lastly *msh*. There is a gap between the initial *vnd* and *ind* domains, suggesting that each gene is independently activated at a precise DV position. Subsequently, *ind* can be expressed in the ventral domain, but this is normally

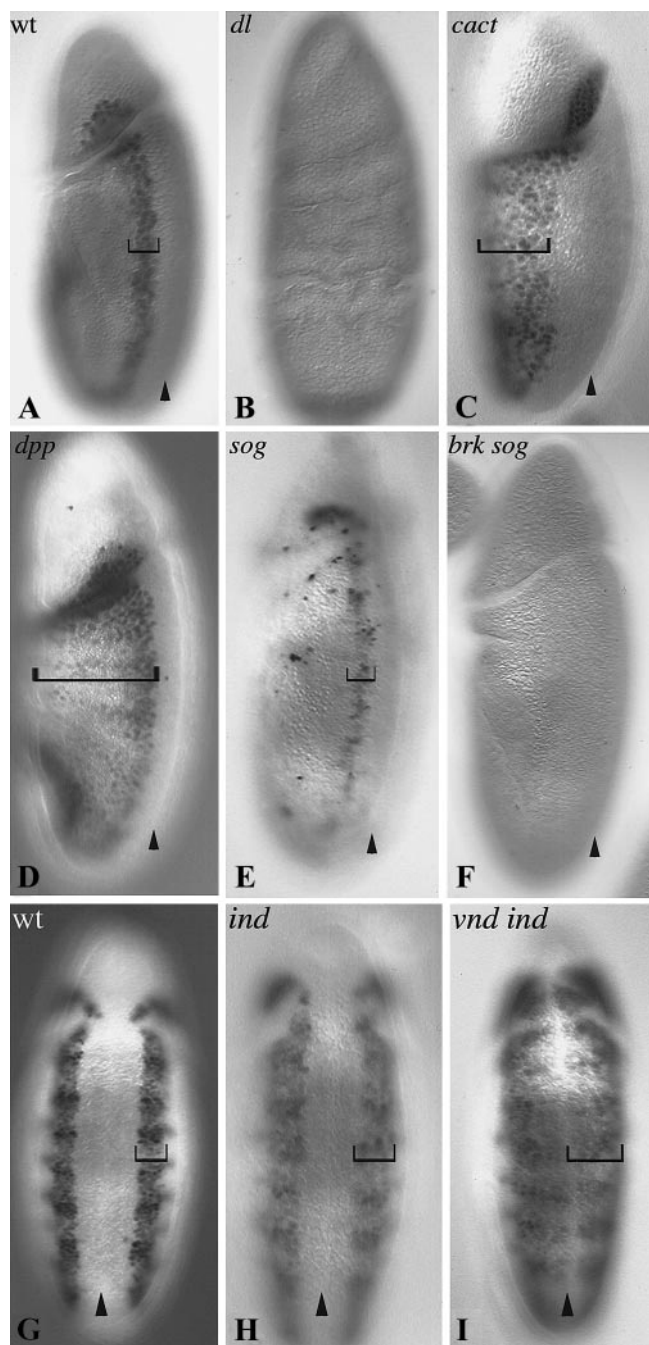


**FIG. 6.** Regulation of *ind* expression: epistasis between *dorsal* and *egfr*. (A–D) *ind* mRNA expression and (E–H) activated MAP kinase expression in stage 7 embryos, anterior is up, ventral to left; ventral midline of CNS, arrowhead. Genotypes are as labeled and described under Materials and Methods. Width of *ind* or activated MAP kinase domains indicated by brackets. (A, E) Wild-type embryos: (A) *ind* is expressed in the intermediate column of neuroectoderm; (E) activated MAP kinase is expressed in the ventral and intermediate columns of neuroectoderm. (B, F) Embryos with ectopic Dorsal and Egfr: (B) *ind* expression expands dorsally to cover the dorsal surface of the embryo; (F) activated MAP kinase is detected throughout the DV axis of the embryo. (C, G) Embryos with ectopic Egfr activity in the absence of Dorsal function: (C) *ind* expression is not detected; (G) activated MAPK is detected throughout the DV axis of the embryo. (D, H) Ectopic Dorsal activity in the absence of Egfr function: (D) *ind* expression is expanded slightly dorsally but is not maintained past stage 7. (H) activated MAPK is not detected in neuroectoderm, although it is still detected in the mesoderm (out of focus).

prevented by Vnd-mediated repression (Weiss *et al.*, 1998; Mc Donald *et al.*, 1998). Because *ind* is capable of repressing *vnd* expression (Weiss *et al.*, 1998), if *ind* were to be expressed first in both the ventral and the intermediate columns, it might fully inhibit the expression of *vnd*. Thus, the temporal pattern of *vnd* and *ind* expression is likely to be important for establishing their final spatial pattern of gene expression.

The activation and borders of *vnd* expression appear to be wholly dependent on the Dorsal morphogen gradient. High levels of Dorsal in the mesoderm/mesectoderm anlagen can activate *twist*, *snail*, and *vnd* (Thisse *et al.*, 1991; Ip *et al.*, 1992), but Snail activity represses *vnd* expression (Meller-

ick and Nirenberg, 1995). Intermediate levels of Dorsal are sufficient to activate *vnd*, but not *snail*, thus establishing the ventral column of neuroectoderm. It is unclear how the dorsal border of Vnd is positioned, but it may be dependent on the concentration of nuclear Dorsal, because if Dorsal levels are increased in dorsal cells, there is a corresponding expansion of the *vnd* domain. In contrast to a previous report (Mellerick and Nirenberg, 1995), we find no evidence that Dpp signaling establishes the dorsal border of the *vnd* domain. We observe no change in the width of the *vnd* domain in *dpp* embryos, and we fail to observe repression of *vnd* in ectopic Dpp embryos. In fact, elevated Dpp activity in the neuroectoderm (in *sog 4xdpp* embryos) gives a slight



**FIG. 7.** Establishing the *msh* expression domain. Msh protein staining in early stage 8 (A–F) or late stage 9 (G–I) embryos, anterior is up; ventral midline of CNS, arrowhead. Genotypes are as labeled and described under Materials and Methods. Width of *msh* stripe is indicated by brackets. (A) Wild-type embryo: Msh is detected in the dorsal column of neuroectoderm. (B) *dorsal* embryo: Msh is not detected. (C) Embryo with ectopic Dorsal activity: Msh expression is both shifted dorsally and expanded to span the dorsal surface of the embryo. (D) *dpp* embryo: Msh expression expands dorsally to cover the dorsal surface of the embryo. (E) Embryo with low level ectopic Dpp activity (*sog*): Msh expression is weakly repressed. (F) Embryo

expansion of the *vnd* domain (Fig. 3E), and even higher levels of Dpp (in *brk sog* embryos) still fail to repress *vnd* expression (Fig. 3F), despite eliminating much of the remaining CNS (Jazwinska *et al.*, 1999). The reason the *vnd* domain is expanded in *sog 4xdpp* embryos remains unclear; however, we feel that our combined results clearly demonstrate that *dpp* signaling does not repress *vnd* and therefore cannot position the dorsal border of *vnd*. All existing data are consistent with Dorsal acting as a direct, concentration-dependent activator of *vnd* expression. In contrast, the Egfr and Dpp signaling pathways have no role in establishing the correct *vnd* expression pattern, although Egfr is required to maintain *vnd* expression later in embryogenesis (Gabay *et al.*, 1996).

Initiation and maintenance of *ind* expression require both Dorsal and Egfr signaling pathways, but not Dpp activity. The ventral border of *ind* expression is established by dorsal limit of *vnd* expression (Weiss *et al.*, 1998). The dorsal border of *ind* expression has more complex regulation. Dpp repression does not establish the dorsal border of *ind*, since the *ind* domain is normal in *dpp* embryos. In contrast, both Dorsal and Egfr are required to activate *ind* and set its dorsal border. In wild-type embryos, the domains of *ind* and activated Egfr have identical dorsal borders. When Egfr activity is increased throughout the embryo, *ind* expression shows a partial dorsal expansion, showing that the dorsal border of Egfr activity sets the precise dorsal border of *ind* expression. Ectopic Dorsal activity can also expand the *ind* domain (without affecting the Egfr activation domain), showing that sufficiently high levels of nuclear Dorsal protein can independently activate *ind* expression. As expected, when Egfr activity and nuclear Dorsal levels are simultaneously increased there is a complete dorsal expansion of the *ind* domain. The data presented here suggest that *ind* expression is activated by both Dorsal and Egfr pathways, limited ventrally by Vnd, and limited dorsally by lack of Dorsal and Egfr activity. Our data do not distinguish between a linear pathway in which Egfr signaling activates or potentiates Dorsal to allow *ind* transcription and a parallel pathway in which Dorsal and Egfr signaling act independently to activate *ind* expression.

Although Dpp is not required for any aspect of *ind* expression in wild type embryos, ectopic Dpp signaling in the neuroectoderm can repress *ind* expression. This shows that Dpp signaling must be kept low in the intermediate column to allow *ind* transcription and raises the possibility

with high level ectopic Dpp activity (*brk sog*): Msh expression is completely repressed. (G) Wild-type embryo: Msh expression is detected in the dorsal column of neuroectoderm. (H) *ind* mutant embryo: Msh expression expands ventrally into the *ind* domain. (I) *vnd ind* double mutant embryo: Msh expression expands to the ventral midline.

that the loss of *ind* expression seen in *dorsal* embryos is an indirect effect, due to the de-repression of Dpp activity within the neuroectoderm. *dorsal dpp* double mutants fail to express *ind*, however, proving that loss of *ind* expression in *dorsal* mutants is not due to de-repression of Dpp within the neuroectoderm. We propose that Dorsal must both activate *ind* expression and repress Dpp signaling to allow *ind* expression.

*msh* is expressed in a DV domain that has low Vnd, Ind, and Dpp activity. Overexpression of any of these genes will repress *msh* expression, and *dorsal dpp* embryos that lack all *vnd*, *ind*, and *dpp* expression show ectopic *msh* expression around the DV axis. Thus, the borders of the *msh* domain are defined by repression: Vnd and Ind ventrally, and Dpp dorsally. What activates *msh* expression? *msh* expression could be activated by "basal" transcription factors present uniformly in the early embryo. Alternatively, *msh* expression may be induced by a low level of ubiquitous TGF $\beta$  activity, similar to the observed activation of zebrafish *msh* homologs (reviewed in Mayor *et al.*, 1999). The *screw* gene encodes a TGF $\beta$ -like protein expressed at low levels throughout the embryo, and although it has no striking CNS phenotype (Arora *et al.*, 1994), it would be interesting to see if *screw dpp* embryos lose dorsal *msh* expression, or whether *screw dorsal dpp* embryos lose global *msh* expression.

The patterned expression of *vnd*, *ind*, and *msh* within the neuroectoderm appears to have been evolutionarily conserved between insects and vertebrates. Murine, zebrafish, and chick embryos express homologous genes in DV columns of the developing CNS: several *Nkx* genes (similar to *vnd*) are expressed ventrally, *Gsh1/2* genes (similar to *ind*) are expressed in intermediate columns, and *Msx* genes (similar to *msh*) are expressed in the dorsal CNS (reviewed in Arendt and Nübler-Jung, 1999; Cornell and Von Ohlen, 2000). Although the *vnd/Nkx*, *ind/Gsh*, *msh/Msx* patterns appear to be conserved between insects and vertebrates, the regulatory inputs that establish these patterns appear different. In vertebrates, Sonic hedgehog signaling patterns the ventral CNS and induces expression of *Nkx* family members (Ericson *et al.*, 1995; Qiu *et al.*, 1998; Barth and Wilson, 1995; Ericson *et al.*, 1997). In *Drosophila*, the ventral CNS is patterned by Dorsal and Egfr, which induce expression of *vnd* and *ind* genes, whereas *hedgehog* mutants show normal initiation of *vnd* and *ind* gene expression (S. Cheesman, T. Von Ohlen, and C. Q. Doe, unpublished observations). In vertebrates, the Dpp-related BMP2/4 proteins activate *msx* expression (Suzuki *et al.*, 1997), whereas Dpp represses *msx* expression in *Drosophila*. Clearly further analysis, including examination of DV pattern formation in different phyla, will be necessary to understand how the conserved *vnd/Nkx*, *ind/Gsh*, *msh/Msx* expression patterns are generated by such diverse regulatory inputs and how they direct diverse cell fate decisions.

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