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Interaction between *Drosophila* EGF receptor and *vnd* determines three dorsoventral domains of the neuroectoderm

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SUMMARY

Neurogenesis in *Drosophila melanogaster* starts by an ordered appearance of neuroblasts arranged in three columns (medial, intermediate and lateral) in each side of the neuroectoderm. Here we show that, in the intermediate column, the receptor tyrosine kinase DER represses expression of proneural genes, *achaete* and *scute*, and is required for the formation of neuroblasts. Most of the early function of DER is likely to be mediated by the Ras-MAP kinase signaling pathway, which is activated in the intermediate column, since a loss of a component of this pathway leads to a phenotype identical to that in DER mutants. MAP-kinase activation was also observed in the medial column where *esg* and proneural gene expression is unaffected by DER. We found that the homeobox gene *vnd* is required for the expression of *esg* and *scute* in the medial column, and show that *vnd* acts through the negative regulatory region of the *esg* enhancer that mediates the DER signal, suggesting the role of *vnd* is to counteract DER-dependent repression. Thus nested expression of *vnd* and the DER activator *rhomboid* is crucial to subdivide the neuroectoderm into the three dorsoventral domains.

Key words: Neurogenesis, MAP kinase, DER, *vnd*, *Drosophila*, EGF

INTRODUCTION

The massive number and variety of neuronal cells that constitute the nervous systems of higher metazoans originate from a simple monolayer of cells in the neuroectoderm. In the development of the central nervous system of *Drosophila*, the first visible sign of neurogenesis is delamination of neuroblasts from the neuroectoderm, a process that follows strict spatial and temporal patterns. Neuroblasts subsequently undergo stem cell divisions in stereotyped patterns to yield neurons and glia cells. The pattern of the first wave of neuroblast delamination in late stage 8 (~4 hours of development) is arranged in four rows along the anterior-posterior (AP) axis and in three columns in a dorsoventral (DV) direction (Skeath et al., 1992). Many of the neuroblasts at the time of delamination already exhibit unique identities distinguishable by specific combinations of gene expression (Doe, 1992), suggesting that determination of neuroblast identity begins in the neuroectoderm. The dorsoventral subdivision of the neuroectoderm is already apparent in late stage 5 (~3 hour of development) when expression of the genes *muscle-specific homeodomain* (*msh*, D'Alessio and Frasch, 1996) and *ventral nervous system defective* (*vnd*, Jiménez et al., 1995; Mellerick and Nirenberg, 1995) begin in the lateral and medial column, respectively, and *escargot* (*esg*) is expressed in both medial and lateral columns (Yagi and Hayashi, 1997). These patterns of gene expression are followed by the expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*) in the medial and lateral columns in stage 7-8. Primary neurogenesis in vertebrates also occurs in three dorsoventral domains (Chitnis et al., 1995), which are defined by the vertebrate counterparts of *msh* and *vnd* (Su et al., 1991; Saha et al., 1993) expressed in an analogous pattern, suggesting that the mechanism of dorsoventral patterning in the CNS has an ancient origin.

Although a great amount of work has demonstrated the importance of segmentation genes in AP patterning of the neuroectoderm (Patel et al., 1989; Skeath et al., 1992; Chu-LaGraff and Doe, 1993; Bhat and Schedl, 1997), little is known about the mechanism of DV patterning. One good candidate for a regulator of DV patterning in the CNS is the receptor tyrosine kinase DER (*Drosophila* EGF receptor), which was shown to provide DV positional information to the ventral ectoderm in stage 10 (Raz and Shilo, 1993; Kim and Crews, 1993; Golembo et al., 1996; Gabay et al., 1996). Involvement of DER in neuroectoderm patterning in an earlier stage was suggested from the expression of *rhomboid* (*rho*), the rate-limiting component of the processing system that produces the active DER ligand Spitz (Spi; Schweitzer et al., 1995) in the presumptive neuroectoderm of stage 5 embryos (Bier et al., 1990). Indeed, analyses of *esg* (Yagi and Hayashi, 1997) and

msh (D'Alessio et al., 1996) expression demonstrated that DER is required for DV patterning of the neuroectoderm. Upon loss of DER function, the intermediate column of neuroectoderm ectopically expressed *esg* and *msh*. Analysis of the *cis*-regulatory region of *esg* revealed that DER represses transcription of *esg* through a dedicated negative regulatory element that counteracts a ubiquitous activator acting through a separate positive element (Yagi and Hayashi, 1997).

In many developmental contexts, DER affects cell fate decision by activating the Ras-MAPK pathway (Rogge et al., 1991; Diaz-Benjumea and Hafen, 1994). Components of this universal signaling pathway include Sos, the GDP-GTP exchange factor that activates the GTPase Ras, which triggers a sequential activation of protein kinases Raf (MAPKKK), Dsor1 (DMEK/MAPKK) and MAP kinase (MAPK). Activated MAPK enters the nucleus and modulates gene expression by phosphorylating transcription factors (O'Neill et al., 1994; Rebay and Rubin, 1995). One advantage of having several components in a pathway is to provide potential branch points of the pathway for intricate regulation. One example is the terminal patterning system in the early embryo, in which Torso receptor tyrosine kinase acts through Ras-MAPK to activate transcription of *huckebein*. In this case, the loss of Raf had a greater effect on *huckebein* transcription than the loss of Sos or Ras, suggesting that Ras is not the sole activator of Raf in this case (Hou et al., 1995).

In this work, we investigated the role of DER in DV patterning of neuroectoderm and in neurogenesis. We show herein that DER is required to repress transcription of proneural genes and to promote neuroblast formation in the intermediate column of the neuroectoderm. A loss of Ras-MAPK signaling causes a phenotype identical to that of DER mutants, suggesting that MAPK is a sole downstream effector of DER in this case. The spatial and temporal pattern of MAPK activation suggest that MAPK is activated, but failed to affect transcription of its target genes in the medial columns, where *vnd* is expressed. We show that *vnd* counteracts the negative effect of the DER-MAPK signal in the medial column to allow the medial-column-specific gene expression.

MATERIALS AND METHODS

Fly strains

Most of the fly strains used are described in Flybase (1994) and sources were as follows: flb^{IF26} from the Tübingen Stock Center; rho^{del1} from Dr H. Okano; $Draf^{X1}$, $Dsor1^{Gp158}$(Tsuda et al., 1993), ovo^{D1} *FRT101* and FLP^{38} from Dr Y. Nishida; $Dras1^{e2f}$ from Dr Y. Hiromi; Sos^{e46}, $Dras1^{c40b}$ and $Gap1^{BQ}$ from Dr M. Okabe; $msh^{delta\ 68}$ and UAS-*msh* from Dr A. Nose; *Kr-Gal4* and *da-Gal4* from Dr B.-Z. Shilo; vnd^{6} and flb^{1K35} from Dr J. Skeath; ovo^{D1}*FRT40A*, ovo^{D1} *FRT2A* and *FRT82B* ovo^{D1} from Bloomington Stock Center. Mutant chromosomes were balanced over one of the following balancers carrying *lacZ* expression vectors to distinguish mutant embryos: *FM7 ftz-lacZ* (From Dr Yasushi Hiromi), *CyO ftz-lacZ*, *TM3 Ubx-lacZ*, or *TM6B abd-A lacZ*. *esg-lacZ D1* and *D5* were described in Yagi and Hayashi (1997).

In situ hybridization

In situ hybridization was performed as previously described (Tautz and Pfeifle, 1989; Yagi and Hayashi, 1997). The following DNA fragments were used as probes; *ac* and *vnd* from Dr J. Skeath, *sc* from Dr C. Cabrerra, *rho* from Dr H. Okano, *sna* from Dr N. Fuse and *msh* from Dr A. Nose. For identification of mutant embryos, *lacZ* antisense riboprobe was mixed in the hybridization solution to detect *lacZ* expression from the marked balancers.

Antibody staining

Immunostaining was performed as described by Hayashi et al. (1993). Rabbit anti-Sna antibody was obtained from Dr R. Reuter, mouse monoclonal Anti-Eve, from Dr Y. Hiromi, and anti-dpMAPK, from Sigma Chemicals and used as described by Gabay et al. (1997b). Identification of mutant embryos was carried out by staining them simultaneously with anti-β-galactosidase and Cy3-conjugated secondary antibody. Signals were detected with a Vectastain ABC Kit (Vector Lab). TSA indirect system (NEN) was used for further enhancement of the signals.

Double labeling of dpMAPK and *rho*, *sna* and *esg* mRNA

Embryos fixed for anti-dpMAPK staining (Gabay et al., 1997b) were used for double labeling. Antibody staining and in situ hybridization were carried out according to Goto and Hayashi (1997). The dpMAPK signal was enhanced by the use of TSA-indirect (NEN) and Streptavidin-Cy2 (Amersham). Embryos were examined under a confocal microscope (MRC1024, Bio-Rad).

Germline clone

Germline clonal analysis was done by the FLP-DFS technique (Chou and Perrimon, 1992). Mutant females were mated to males carrying mutant chromosomes balanced over one of the blue balancers to distinguish embryos with or without paternal gene products.

RESULTS

DER is required for neurogenesis in intermediate column of the neuroectoderm

To investigate the involvement of DER in neurogenesis, we examined mutant phenotypes of *DER* and its activator *rho* in various stages of neurogenesis. As previously shown (Whiteley et al., 1992, Yagi and Hayashi, 1997), the dorsoventral subdivision of the neuroectoderm in stage-6 embryos is detectable by expression of *esg* (Fig. 1A), which is expressed in the lateral (arrowhead) and medial (arrow) columns but not in the intermediate column. A loss-of-function, temperature-sensitive mutation of DER (flb^{IF26}) and the null mutation of *rho* (rho^{del1}) were used for analysis throughout this work. *flb* and *rho* mutations caused ectopic expression of *esg* in the intermediate column when cultured at 25˚C (Fig. 1B,C). This phenotype was highly penetrant and was also observed in *flb* embryos cultured at 18˚C. No further enhancement of this phenotype was observed in flb^{IF26} embryos cultured at 29˚C (Yagi and Hayashi, 1997) where the mutants show null phenotypes in the epidermis (Clifford and Schüpbach, 1992) and in null flb^{IK35} embryos (data not shown), suggesting that repression of *esg* in the intermediate column requires a relatively high dose of DER signal. To examine the potential role of DER in neurogenesis, we studied expression of the proneural genes *ac* and *sc*, which begin expression in the neuroectoderm of stage-7 embryos in a DV pattern of expression similar to that of *esg* in the previous stage (Fig. 1D,G). In *flb* and *rho* embryos, *ac* and *sc* become ectopically expressed in the intermediate column (Fig. 1E,F,H,I). This phenotype was less penetrant and, occasionally, gaps of *ac* and *sc* expression were observed in

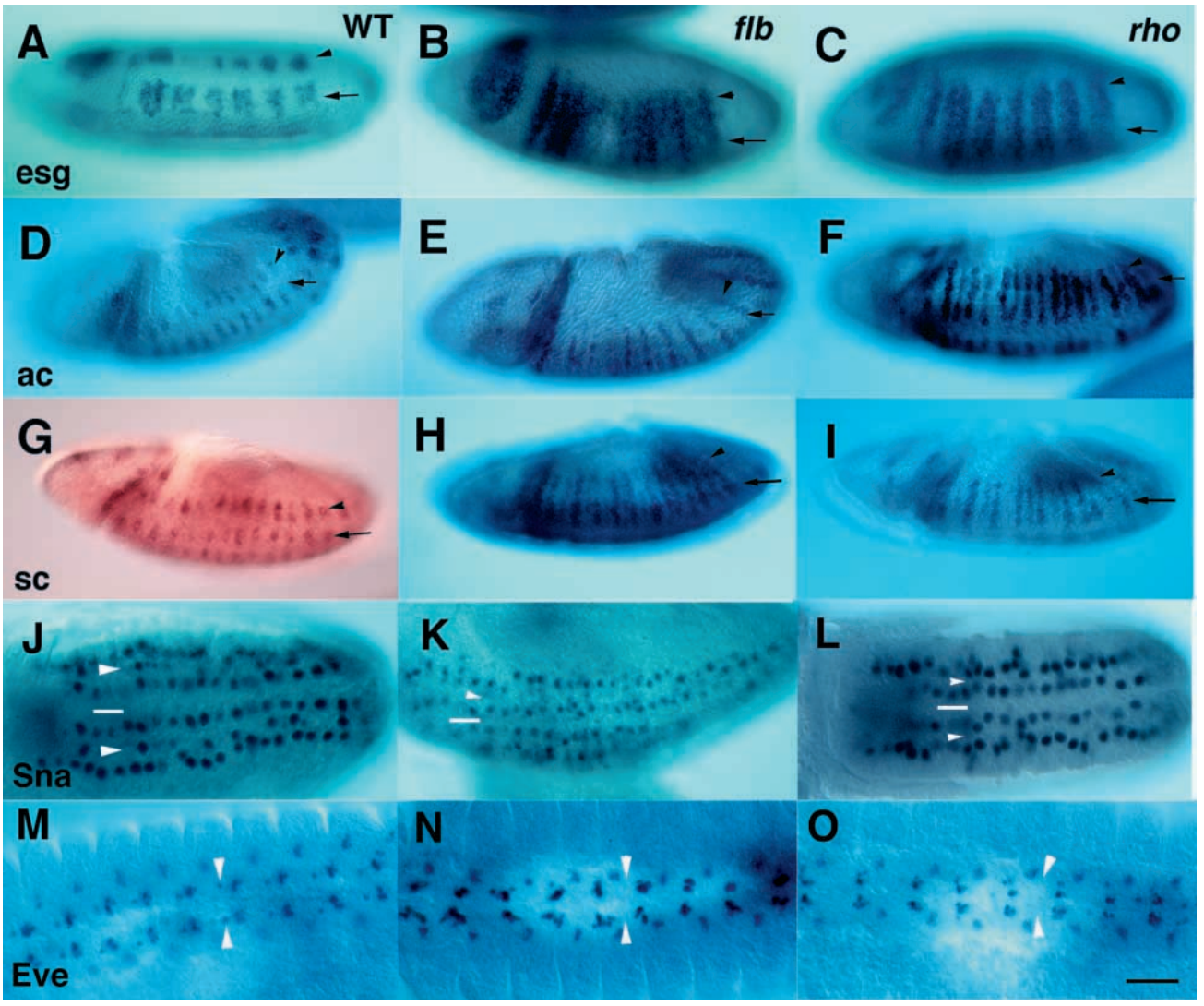

Fig. 1. Effects of a loss of DER signal on expression patterns of neuroectoderm and neuronal markers. (A-C) In situ hybridization with *esg* probe, stage 5-6 embryo, lateral view. (D-F) In situ hybridization with *ac* probe, stage-7 embryo, lateral view. (G-I) In situ hybridization with *sc* probe, stage-7 embryo, lateral view. (J-L) Antibody staining with anti-Sna, ventrolateral view of stage-9 embryos. (M-O) Antibody staining with anti-Eve, stage-13 embryo, ventral view. (A,D,G,J,M) Wild-type embryos; (B,E,H,K,N) flb^{If26} mutant embryos; (C,F,I,L,O) rho^{del1} mutant embryos. In the mutant embryos, *esg*, *ac* and *sc* expression were expanded into the intermediate column of the neuroectoderm (A-I). Neuroblasts of the intermediate column (J, white arrowhead) were frequently not observed in *flb* and *rho* mutants (K,L, white arrowhead). L is an embryo with the most severe case of the phenotype, in which an almost complete loss of neuroblasts occurs in the intermediate column. Frequently, Eve-positive RP2 motor neuron did not appear in *flb* and *rho* mutants (M-O, white arrowhead). White bars indicate the ventral midline. Arrow, medial column; arrowhead, lateral column (A-I), neuroblast of intermediate column (J-L), RP2 motor neuron (M-O). Bar in O, 100 μm (A-I), 50μm (J-O).

the intermediate column. Since *sc* expression was similarly derepressed in flb^{IK35} embryos, these phenotypes are likely to represent the near null phenotype of DER in the neuroectoderm. These data indicate that, in the intermediate column, the DER signal represses not only *esg* but also proneural genes, which are known to play key roles in neurogenesis. Effect of DER on neuroblast formation was monitored by the neuroblast marker Snail (Sna, Fig. 1J-L). Anti-Sna staining revealed three columns of SI neuroblasts in the control embryo, in which the intermediate column is distinguishable by the delayed onset of formation and number of Sna-positive cells (Fig. 1J, white arrowhead). In *flb* and *rho* mutants, Sna-positive neuroblasts in the intermediate position were frequently missing (Fig. 1K,L, white arrowhead), with higher frequency in *flb* embryos. In *rho* mutant embryo, frequency of the loss of intermediate column neuroblasts was variable between embryos.

To further examine the effect of the loss of DER signaling on the late events of neurogenesis, we traced the progeny of one of the intermediate neuroblasts, NB4-2. NB4-2 gives rise to the RP2 motor neurons, which can be identified by the expression of Even-skipped (Eve) and its unique position (Fig. 1M). Loss of RP2 neurons in stage 13 was observed (over half the case examined) with the frequency of loss slightly higher

in *flb* than in *rho* (Fig. 1N,O, white arrowhead), reflecting the earlier defect in neuroblast formation in stage 9. The variability in the *flb* phenotype observed in stage 9 and 13 might be due to the hypomorphic nature of the flb^{IF26} allele used, or to a limited requirement for DER in the late stage of neurogenesis.

Requirement for Ras-MAPK signaling in the neuroectoderm

It is known that Ras-MAPK signaling cascade is the major target of DER in many tissues (Rogge et al., 1991; Tsuda et al., 1993; Diaz-Benjumea and Hafen, 1994; Hsu and Perrimon, 1994). To understand whether Ras-MAPK signaling also mediates the DER signal in the neuroectoderm and to determine the relative contribution of each component of the pathway, we examined the expression of *esg* and *sc* in embryos that lacked one of the Ras-MAPK signaling components. We studied the phenotype of mutants lacking either *Sos*, *Ras1*, *Draf* or *Dsor1*. To remove the maternal store of those gene products, we used the FLP-DFS technique developed by Chou and Perrimon (1992). As in wild-type embryos, embryos mutant for any of the four genes examined expressed *esg* in three separate domains, procephalic neurogenic region, amnioserosa and neuroectoderm. In all cases, the anterior limit of the procephalic expression and the posterior limit of neuroectodermal expression were expanded to the terminus, consistent with the previous report that Ras-MAPK is required for the terminal fate specification controlled by Torso receptor tyrosine kinase (Hou et al., 1995; Tsuda et al., 1993; Fig. 2). The terminal-loss phenotype was stronger in *Draf* and *Dsor1* mutants than in *Sos* and *Ras1* mutants, confirming the results of previous analysis of *huckebein* expression (Hou et al., 1995; Tsuda et al., 1993). In the trunk, initial subdivision into amnioserosa, dorsal ectoderm, neuroectoderm and mesoderm appeared normal, based on the expression pattern of *esg* in amnioserosa and in neuroectoderm. This observation suggests that global DV patterning by the gradient of Dorsal protein (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989) does not require Ras-MAPK. All mutants exhibited specific defects within the neuroectoderm where *esg* expression was derepressed in the intermediate column. Essentially the same phenotype was also observed with *sc* expression, suggesting the loss of Ras-MAPK signaling has the same consequence as the loss of DER. In contrast to the case of the terminal-loss phenotype, all four mutants showed qualitatively the same phenotype in the neuroectoderm. The neuroectoderm phenotype in *Ras1* mutants was not rescued by a paternal copy of the wild-type gene, suggesting that a relatively high dose of the Ras signal is required for repression of *esg* and *sc* in the neuroectoderm. We also examined embryos mutant for $Gap1^{BQ}$, the negative regulator of Ras, but found no significant change in *esg* and *sc* expression (not shown). In later stages, the mutant embryos showed defects in germ band extension and many embryos showed defects in gastrulation, precluding further analysis of neurogenesis.

MAPK activation pattern in early neuroectoderm

rho is initially expressed in the medial half of neuroectoderm (Bier et al., 1990), but repression of *esg*, *ac* and *sc* transcription by DER and Ras-MAPK occurred only in the intermediate column, posing a question as to whether the site of MAPK activation and the transcriptional repression exactly correspond or not. The spatial and temporal pattern of MAPK activation has been described by the use of an antibody that specifically reacts with the phosphorylated and activated form of MAPK (diphospho-MAPK=dpMAPK), which showed that dpMAPK is distributed in a broad domain in the neuroectoderm in stage 5-7 embryos (Gabay et al., 1997a,b). The result was confirmed here, which showed that dpMAPK is distributed in an 8- to 10-cell-wide area in the neuroectoderm in stage-5 embryos and becomes restricted to the ventral region at the end of gastrulation (Fig. 3A-C,M). This rapidly evolving pattern of dpMAPK expression made it difficult to determine the exact correlation between distribution of dpMAPK and the DV

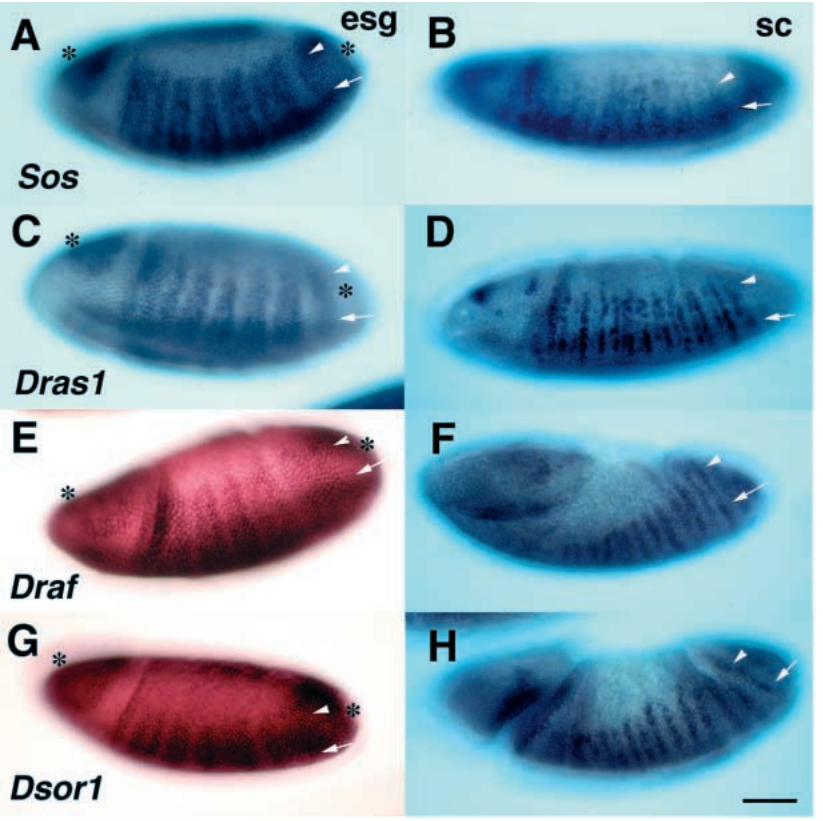

Fig. 2. Germline mosaic analysis of the components of MAPK pathway in DV patterning of the neuroectoderm. (A,C,E,G) In situ hybridization with *esg* probe, stage-6 embryo, lateral view. (B,D,F,H) In situ hybridization with *sc* probe, stage-7 embryo, lateral view. (A,B) Sos^{e46} mutant embryos. (C,D) $Dras1^{e2f}/Dras1^{c40b}$ mutant embryos derived from $Dras1^{e2f}$ female germline. (E,F) $Draf^{X1}$ mutant embryos. (G,H) $Dsor1^{Gp158}$ mutant embryos. In all mutant embryo, *esg* and *sc* are expressed in the intermediate column. Mutant embryos also show the loss of the terminal region (anterior and posterior extent of *esg* and *sc* expression is expanded, *). Arrow, medial column; arrowhead, lateral column. Bar, 100 μm.

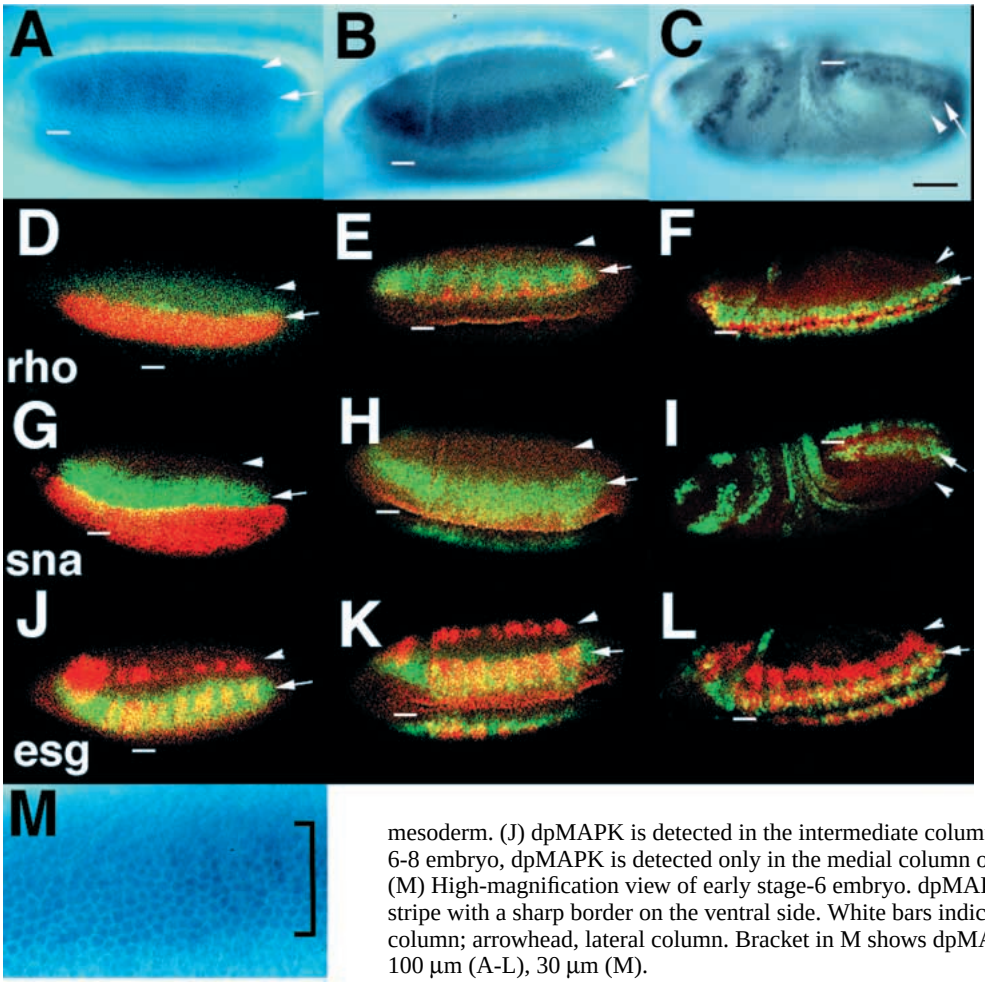

Fig. 3. Localization of dpMAPK in stage 5-8 embryos. Immunochemical staining with anti-dpMAPK (A-C,M) and fluorescent double labeling with anti-dpMAPK and hybridization (D-L). Hybridization probes were *rho* (D-F), *sna* (G-I) and *esg* (J-L). (A,D,G,J,M) Stage-5-6 embryos; (B,E,H,K) stage-6-7 embryos; (C,F,I,L) stage-8 embryos. (A) At first dpMAPK is detected as a broad band in the neuroectoderm. (B,C) In later stages, the dpMAPK signal becomes a narrow band. (D) In stage 5-6, dpMAPK is detected in the same region that expresses *rho*. (E) dpMAPK is still detectable after *rho* expression narrowed down to the midline stripes. (F) In stage 8, dpMAPK is detected in a stripe of about 2 cell wide along both sides of the ventral midline. (G-I) dpMAPK expression does not overlap with *sna* expression in the prospective mesoderm. (J) dpMAPK is detected in the intermediate column where *esg* is repressed. (K,L) In stage 6-8 embryo, dpMAPK is detected only in the medial column of neuroectoderm where *esg* is expressed. (M) High-magnification view of early stage-6 embryo. dpMAPK is detected in an 8- to 10-cell-wide stripe with a sharp border on the ventral side. White bars indicate ventral midline. Arrow, medial column; arrowhead, lateral column. Bracket in M shows dpMAPK-positive domain. Scale bar in C: 100 μm (A-L), 30 μm (M).

subdomains in the neuroectoderm. We therefore used the protocol of Goto and Hayashi (1997) to double label embryos with dpMAPK and antisense RNA probes to study the spatiotemporal relationship between expression of dpMAPK, its activator *rho* and its downstream target, *esg*. Initial expression of dpMAPK overlapped with that of *rho* in stage-5 embryos (Fig. 3D); dpMAPK expression remained in this broad domain when *rho* expression became restricted to the medial column at gastrulation in stage 6 (Fig. 3E), and finally narrowed down to a 2- to 3-cell-wide stripe that abutted the stripe of *rho* at stage 7 (Fig. 3F). Comparison with the mesodermal marker *sna* showed that the ventral border of dpMAPK expression abutted the neuroectoderm-mesoderm border (Fig. 3G-I). Examination of histochemically stained material revealed a sharp ventral border of dpMAPK expression, which gradually declined in the dorsal direction, resembling the pattern of *rho* expression (Fig. 3M). In flb^{IF26} mutant embryos cultured at 25˚C, dpMAPK staining was not detectable (data not shown). These results demonstrate that MAPK activation in the neuroectoderm is dependent on DER and follows the spatial expression pattern of *rho*, but persists for some time after termination of *rho* transcription. The latter observation may reflect perdurance of Rho or its target protein, Spitz (Spi). Alternatively, a ligand other than Spi, such as Vein (Schnepp et al., 1996) might be activating DER.

We next determined the position of the dorsal limit of dpMAPK expression relative to the three separate columns of neuroectoderm revealed by *esg* expression. In stage 5, the dorsal limit of dpMAPK reached halfway within the intermediate column (Fig. 3J) and subsequently retracted to the medial column in stage 6 and 7 (Fig. 3K,L). These data indicate MAPK is activated at least in the ventral half of the intermediate column of the neuroectoderm when it is required to repress transcription of *esg*. We conclude that transcription of *esg* is repressed by a marginal level of MAPK activation.

vnd is required for the medial column determination

Why did the high level of dpMAPK in the medial column fail to repress transcription of *esg*, *ac* and *sc*. One possibility is that a factor is present in the medial column that antagonizes or overcomes the events downstream of dpMAPK. A candidate for such a gene is *vnd*, which is expressed in the medial column in late stage 5 and was shown to be required for expression of *ac* (Skeath et al., 1994). We examined expression of *esg* and *sc* in *vnd* null mutant embryos and found that their expression in the medial column was lost (Fig. 4A,B). To understand how *vnd* controls gene expression in the medial column, we searched for a target for *vnd* in the *cis*-regulatory regions of *esg* enhancer. Expression of *esg* is regulated by the neurogenic enhancer, which was divided into two regions, the activator

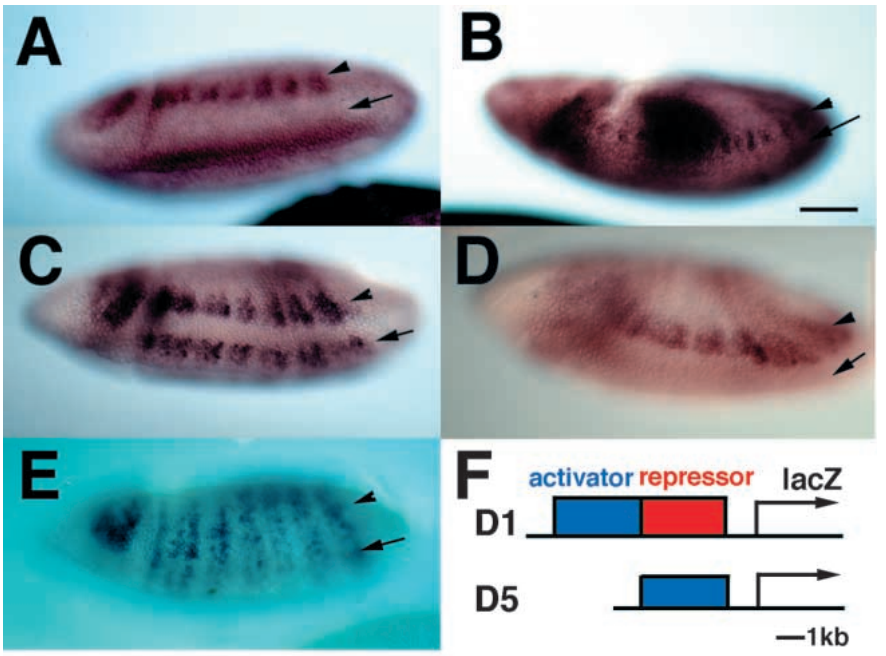

Fig. 4. *vnd* is required for the medial cell fate. (A,B) In situ hybridization of vnd^{6} mutant embryo. (A) *esg* probe; (B) *sc* probe. Expression of *esg* and *sc* in the ventral column was lost in mutant embryos. (C-E) Expression of *esg-lacZ* reporter genes in control (C,E) and vnd^{6} (D) mutant embryos. (C) Construct D1 shows identical expression pattern as *esg* in the neuroectoderm. (D) In *vnd* mutant embryo, *lacZ* expression was lost in the ventral column, indicating *vnd* is acting through this enhancer element. (E) Expression of construct D5 in control embryos was similar to the pattern of *esg* expression in DER mutants. This pattern did not change in *vnd* mutants (data not shown). (F) Structures of the neurogenic enhancer and construct D1 and D5. The neurogenic enhancer was divided into two regions, one of which activates expression in the entire neuroectoderm (activator region: blue) and the other which mediates DER signal (repressor region: red; Yagi and Hayashi, 1997). Reporter construct D1 contains both regions but D5 has the activator region only. Arrow, medial column. Arrowhead, lateral column. Bar, 100 μm.

region, which mediates activation in the entire neuroectoderm, and the repressor region, which mediates DER-dependent repression (Yagi and Hayashi, 1997; Fig. 4F). We thus examined expression of the *esg-lacZ* fusion genes in the *vnd* mutant background. The construct *esg-lacZ D1* containing the complete neurogenic enhancer reproduced neuroectodermal expression of *esg* and was regulated by *vnd* in the same manner as *esg* (Fig. 4C,D; Yagi and Hayashi, 1997). In contrast, the construct *esg-lacZ D5* lacks the repressor region for the DER-mediated regulation and was expressed in all three columns (Fig. 4E; Yagi and Hayashi, 1997). We collected embryos from a cross between vnd^{6}/*FM7ftz-lacZ* females and *y w; esg-lacZ D5* males, and detected *lacZ* expression. Although half of *ftz-lacZ*$^{-}$ embryos were expected to be vnd^{-}, no alteration of *esg-lacZ D5* expression pattern was observed in 23 embryos scored. We thus conclude that *vnd* does not regulate *esg-lacZ D5* and that the target site for *vnd* regulation is included in the repressor region.

To ask whether *vnd* affects the pattern of DER activation, we examined expression of *rho* in *vnd* mutants but found no change in its expression pattern in stage 5-7 embryo (data not shown). Similarly, *vnd* expression pattern was not altered in *flb* and *rho* mutants (flb^{IF26}, flb^{IK35}, rho^{del1}) in stage 6 embryo (data not shown; Gabay et al., 1996), suggesting DER and *vnd* do not cross-regulate each other in this stage. These results indicate that *vnd* is not involved in activation of *esg* or DER, but rather in counteracting the negative effect of DER.

msh is not involved in regulation of *escargot*

Expression of *msh* starts at stage 6 in the lateral column of the neuroectoderm and is required for proper cell fate determination of neuroblasts formed in the lateral column in later stages (Isshiki et al., 1997). Upon loss of DER function, expression of *msh* expands to the intermediate column (D'Alessio and Frasch, 1996), suggesting that *msh* is negatively regulated by DER. To determine whether *msh* is involved in lateral fate determination in the neuroectoderm in the pregastrulation stage, we examined the effect of a loss and a gain of *msh* function on expression of *esg*. We did not observe any alteration of *esg* expression in *msh* null mutants or in embryos expressing *msh* under the control of the *Krüppel* or *daughterless* enhancer up to stage 8 (data not shown). We therefore conclude that, while *msh* is regulated by DER, *msh* by itself does not contribute to the subdivision of the neuroectoderm in the pregastrulation stage.

DISCUSSION

DER signaling initially occurs in a broad domain in the neuroectoderm in stage 5-6 embryos (Gabay et al., 1997a,b; this work) and later becomes restricted to the midline, where it continues to affect the patterning in the ventral ectoderm (Raz and Shilo, 1993; Kim and Crews, 1993; Golembo et al., 1996, Gabay et al., 1996) and CNS (Skeath, 1998). The relationship between genes involved in DER signaling also changes with time (Gabay et al., 1996). Here we will limit our discussion to the early (stage 5-6) function of DER in neurogenesis.

DER-MAPK signal determines cell fate in the intermediate column

Insect neuroblasts are selected from a group of equipotential cells in the neuroectoderm and acquire a specific fate according to their position (Doe, 1992). In order to obtain a fixed number of neuroblasts with a specific repertoire of cell identity, it is essential that the neuroectoderm be subdivided into domains in a reproducible manner, so that a single neuroblast is selected from each of the domains. Failure in this subdivision process is expected to cause misspecification or a loss of neuroblasts. In this work, we investigated the mechanism of DV patterning in the neuroectoderm by studying the function of DER. Upon a

loss of DER function, the intermediate column neuroectoderm was incorrectly specified as evidenced by ectopic expression of *esg* and proneural genes *ac* and *sc* (Fig. 1B,C,E,F,H,I), leading to a failure in the formation of intermediate column neuroblasts (Fig. 1K,L,N,O). The activated form of MAPK was detectable in the intermediate column neuroectoderm in stage 5-6 embryos (Fig. 3), but was not detectable at this position or in intermediate column neuroblasts later in stage 7. From these observations, we propose that DER acts in the neuroectoderm to provide the positional information necessary to specify neuroblasts that are formed at a later stage.

The ectopic expression of *esg, ac* and *sc* in the neuroectoderm of DER mutant suggests that the intermediate column of neuroectoderm acquired characteristics of either the lateral or medial column. D'Alessio and Frasch (1996) reported that *msh* expression in the lateral column expands into the intermediate column. Gabay et al. (1996) and this work reported that expression of *vnd* in the medial column in the blastoderm stage did not change in *flb* mutants. This suggests that intermediate column in DER signal mutants is lost and is replaced by expanded lateral column. On the contrary, we previously observed that, in *Star* (s) and *spi* mutants, where DER activity is mildly reduced, both medial and lateral expression of *esg* expanded into the position of the intermediate column (Yagi and Hayashi, 1997). These results suggest that a loss of DER function leads to a loss of the cell fate of the intermediate column, which is replaced by that of the lateral and medial columns. Such expanded lateral (and medial) columns give rise to a single column of neuroblasts in a timing and pattern characteristic of lateral (and medial) SI neuroblasts. We currently do not understand why the expanded lateral column neuroectoderm produced only one set, instead of two, of neuroblasts despite expression of proneural genes *ac* and *sc*.

A variety of evidence suggests that a high dose of MAPK activation is required to correctly specify the intermediate column neuroectoderm in stage 5-6. First, embryos of the temperature-sensitive allele of DER (flb^{If26}) cultured at 18˚C showed a fully penetrant phenotype of ectopic expression of *esg* in the intermediate column. Second, paternal copies of the genes of the Ras-MAPK pathway (*Sos*, *Ras1*, *Draf*, *Dsor1*) failed to rescue the phenotype. Third, removal of *rho* was sufficient to derepress *esg*, *ac* and *sc* expression in the intermediate column, although *vein*, encoding a putative ligand of DER, is also expressed in the neuroectoderm in stage 6 and can potentially activate DER (Schnepp et al., 1996). In addition, loss of *Sos*, *Ras1*, *Draf* or *Dsor1* showed the same severity of phenotype in the neuroectoderm (Fig. 2), in contrast to their effect on terminal patterning where loss of *Draf* or *Dsor1* had a greater effect compared to the loss of *Sos* or *Dras1*. This would indicate that the levels of *Sos* and *Ras1* that are insufficient to fully promote the terminal patterning are sufficient to shut down the DER signal and repress *esg* and *sc*. We have shown that expression of dpMAPK forms a gradient, the highest level being detectable at the ventral side, with a reduction in the dorsal direction, to cover the ventral half of the intermediate column of the neuroectoderm, reflecting the graded expression pattern of its activator *rho* (Bier et al., 1990). This indicates that transcription of *esg, ac* and *sc* is repressed by a marginal level of activated MAPK. Insufficient activation of DER or Ras-MAPK would be expected to restrict the domain of MAPK activation, which leads to a failure to repress *esg, ac* and *sc* in the intermediate column. We suggest that a high dose of DER signaling is required to expand the domain of MAPK activation to cover the intermediate column of the neuroectoderm.

In contrast to the near absolute requirement for DER for repression of *esg, ac* and *sc*, neuroblast formation in the intermediate column was not completely eliminated by flb^{IF26} or rho^{del1} mutations. One possibility is that an additional DER activator such as the putative DER ligand Vein (Schnepp et al., 1996), appears slightly later than *rho* to stimulates DER to partially rescue the phenotype of flb^{IF26} or rho^{del1}. This idea is consistent with our previous observation in *spi* and *S* mutants (Yagi and Hayashi, 1997). In these mutants, initial expression of *esg* in the intermediate column neuroectoderm was partially derepressed till stage 6, but returned to the normal pattern in stage 7, suggesting delayed onset of an additional DER activation partially complemented the defect.

pointed (*pnt)* and *yan* are known to encode transcription factors that are targets of the MAPK pathway in the eye and ventral ectoderm (O'Neill et al., 1994; Gabay et al., 1996), and the expression pattern of *pnt* and *yan* in stage 5-6 showed a gradient in the neuroectoderm (Klämbt, 1993; Gabay et al., 1996). However, zygotic loss of either of the genes did not alter the pattern of *esg* expression (Yagi and Hayashi, 1997). Therefore either maternal products of *pnt* and *yan*, or some unknown factor act as a downstream transcription factor of MAPK in the early neuroectoderm.

How does the DER signal determine the intermediate column?

Here we consider possible regulatory mechanisms of DV patterning of the neuroectoderm based on the current results as well as on our previous results from an analysis of *cis*-acting elements in the *esg* promoter (Yagi and Hayashi, 1997). Our previous study showed that all aspects of *esg* expression in the neuroectoderm are reproducible by the action of its neurogenic enhancer, which is composed of activator and repressor regions. The activator region potentially promotes transcription of *esg* throughout the circumference of stage 5-6 embryos, but is normally repressed in the dorsal ectoderm and amnioserosa by *decapentaplegic* (*dpp*), and in the mesoderm by *sna* and *twist* (*twi*). The repressor region is the target of DER-dependent repression in the intermediate column. Identification of separate *cis*-acting elements for activation and repression excludes a possibility of DER acting directly upon transcription factors activating transcription. It is also unlikely that *dpp, sna* or *twi* acts at a distance to affect *esg* expression within the neuroectoderm, since the dorsal and ventral borders of the intermediate column defined by the expression of *esg* were not altered in any of these mutants (Yagi and Hayashi, 1997). To explain the subdivision of the neuroectoderm by DER, we previously proposed that a combination of opposing gradients of DER ligand and receptor creates a peak of DER activity in the intermediate position (Yagi and Hayashi, 1997). This model, however, proved not to be correct because this and other works showed that the peak of DER activity detected with anti-dpMAPK antibody is located in the medial column (Gabay et al., 1997a,b, this study).

Given the results of the present work showing that *vnd* counteracts the negative regulatory effect of DER, we propose a model shown in Fig. 5A. Gradient of nuclear localized Dorsal protein induces expression of dorsoventrally regulated genes

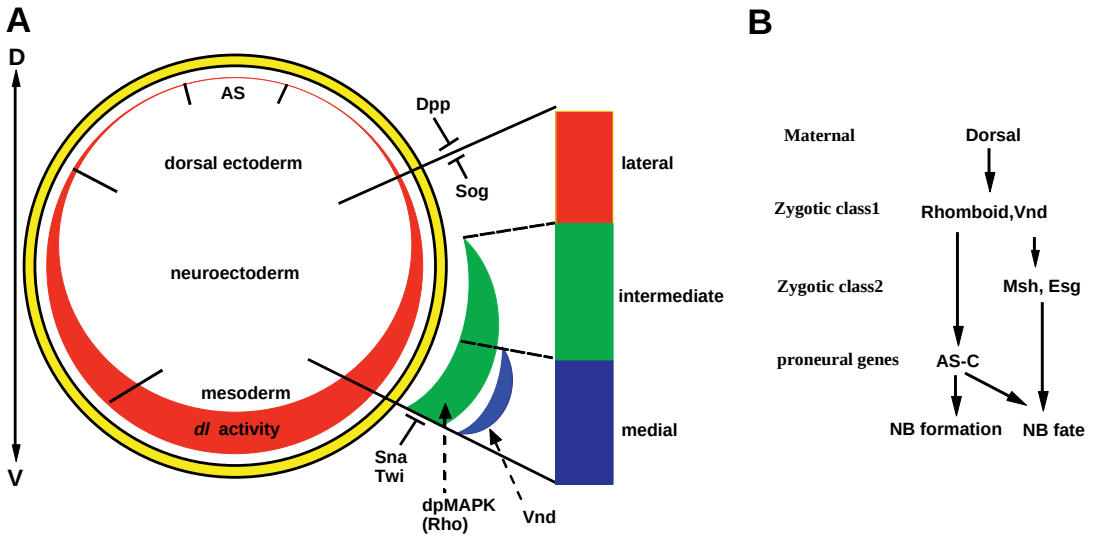

Fig. 5. Model of the neuroectoderm subdivision along the DV axis. (A) In the prospective neuroectoderm of the stage-5 embryo, the gradient of *dorsal* (*dl*) transcription factor activates genes (*rho*, *vnd*, *dpp*, *short gastrulation* (*sog*), *sna*, *twi* and others), which promote dorsoventral differentiation. *rho* determines the activation domain of DER and MAPK. Activated MAPK has two roles. It suppresses lateral cell fate by repressing expression of genes such as *msh*. Activated MAPK also promotes intermediate cell fate by repressing expression of lateral/medial genes *esg*, *ac* and *sc*. In the medial column, *vnd* antagonizes/overcomes the negative effect of activated MAPK to promote expression of *esg*, *ac* and *sc*, and medial cell fate. Lateral fate may be the default state. AS, amnioserosa; D, dorsal; V, ventral. (B) Hierarchy between dorsoventral patterning genes in the neuroectoderm. The gradient of nuclear Dorsal protein promotes expression of zygotic class 1 and class 2 genes. Class 1 genes regulate expression of class 2 genes and proneural genes, and are essential for cell fate determination within the neuroectoderm. We distinguish class 2 genes from class 1 genes because they are not directly involved in subdivision of the neuroectoderm, but rather in later events of neurogenesis. Expression of class 1 and class 2 genes are maintained in the midline in later stages and are likely to be regulated by a complex cross regulation in later stage (Gabey et al., 1996).

such as *dpp*, *sna*, *twi*, which determines the extent of the neuroectoderm, and the nested expression domains of *rho* and *vnd*. *rho* determines the domain of MAPK activation, which covers the medial and intermediate columns. *vnd* is expressed in the medial column where it counteracts DER signal to allow expression of *esg*. Thus the three columns in the stage 5-6 neuroectoderm are distinguished by unique combinations of activated MAPK and *vnd* expression. In the lateral column, neither of them are activated or expressed, and *esg* transcription is activated by default. In the intermediate column, MAPK is activated and repress *esg* transcription. In the medial column, *vnd* counteracts activated MAPK to allow the default pathway to activate *esg* transcription. It is possible that proneural genes are also regulated by the same mechanism. Loss of the DER signal leaves two domains, one with and the other without expression of *vnd*, the pattern likely to be reflected in the appearance of only two neuroblast columns in the later stage. Thus we propose that the primary role of DER signal in this stage is to define the intermediate domain to the neuroectoderm which is otherwise separated into two domains. It is possible that DER signal and *vnd* have later roles in promoting neuroblast formation in the intermediate and in the medial column, respectively.

Hierarchy between DV patterning genes

This study revealed a new aspect of the regulatory hierarchy between DV pattering genes. Based on expression timing and interaction, we grouped the four genes expressed in the neuroectoderm into 2 classes (Fig. 5B). Maternal DV genes instruct the formation of the nuclear Dorsal protein gradient (Chasan and Anderson, 1993), which controls expression domains of zygotic class 1 genes in stage 5-6 embryos (*rho*, Ip et al., 1992; *vnd*, Mellerick and Nirenberg, 1995). Expression of class 2 genes are regulated by class 1 genes (*esg*, this study; *msh*, D'Alessio and Frasch 1996). Class 1 gene *rho* and *vnd* do not regulate each other's expression, and class 2 gene *msh* does not regulate *esg* expression (this study). We infer that *msh* does not regulate class 1 genes, because class 1 gene-dependent expression of *esg* was unaltered in *msh* mutants. Later in stage 8, proneural genes starts to be expressed in a class 1 gene-dependent manner (Skeath et al., 1994; this study). The role of class 2 genes on proneural gene expression is likely to be in later stage of neural cell specification. *msh* was shown to affect neuroblast formation and identity (Isshiki et al., 1997). Role of *esg* in neurogenesis is less clear at this moment but it was shown that Esg modulates Scute-dependent transcriptional activation in tissue culture cells (Fuse et al., 1994) and inhibits neurogenesis when expressed in proneural cells (N. Fuse and S. H., unpublished result). Use of early neuroectodermal markers enabled us to recognize the earliest role of DER in development and the regulatory hierarchy between zygotic DV genes. Further analyses should reveal a link between the flow of DV positional information and determination of position specific characters of the neuroblast.

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