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The Sox-domain containing gene *Dichaete/fish-hook* acts in concert with *vnd* and *ind* to regulate cell fate in the *Drosophila* neuroectoderm

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SUMMARY

In the *Drosophila* embryonic central nervous system, neural stem cells, called neuroblasts, acquire fates in a position-specific manner. Recent work has identified a set of genes that functions along the dorsoventral axis to enable neuroblasts that develop in different dorsoventral domains to acquire distinct fates. These genes include the evolutionarily conserved transcription factors *ventral nerve cord defective* and *intermediate neuroblasts defective*, as well as the *Drosophila EGF receptor*. We show that the Sox-domain-containing gene *Dichaete/fish-hook* also plays a crucial role to pattern the neuroectoderm along the DV axis. *Dichaete* is expressed in the medial and intermediate columns of the neuroectoderm, and mutant analysis indicates that *Dichaete* regulates cell fate and neuroblast formation in these domains. Molecular epistasis tests, double mutant analysis and dosage-sensitive interactions demonstrate that during these processes, *Dichaete* functions in parallel with *ventral nerve cord defective* and *intermediate neuroblasts defective*, and downstream of EGF receptor signaling to mediate its effect on development. These results identify *Dichaete* as an important regulator of dorsoventral pattern in the neuroectoderm, and indicate that *Dichaete* acts in concert with *ventral nerve cord defective* and *intermediate neuroblasts defective* to regulate pattern and cell fate in the neuroectoderm.

Key words: *Drosophila*, Sox-domain proteins, *Dichaete*, Neuroectoderm

INTRODUCTION

How cells acquire specific and often unique fates as a function of their position in a developing cellular field is a central question in developmental biology. One of the best model systems in which to explore the link between pattern formation and cell-type specification is the *Drosophila* embryonic central nervous system (CNS). The *Drosophila* CNS develops from the reiterative divisions of neural stem cells (reviewed by Goodman and Doe, 1993). Neural stem cells, called neuroblasts (NBs), segregate from the neuroectoderm into the interior of the embryo in five temporally distinct waves (SI-SV) and form an invariant and roughly orthogonal pattern of 30 NBs per hemisegment. Based on its position, each NB acquires a unique fate and divides in a stem cell manner to produce a unique and nearly invariant family of neurons and/or glia. Cell transplantation and genetic experiments support the model that the fate of a NB is predetermined by the fate of the cells within the neural equivalence group from which it segregates (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Udolph et al., 1995). Thus, to link pattern formation to cell type specification, it is crucial to identify the genes and elucidate the genetic pathways that regulate pattern and cell fate in the neuroectoderm.

Work from many laboratories has begun to define the genetic mechanisms that establish pattern along the anteroposterior (AP) and dorsoventral (DV) axes of the *Drosophila* embryo. These studies show that segment polarity gene activity divides each segment along the AP axis into four parallel transverse rows (reviewed by Bhat, 1999). Along the DV axis, the activity of the *Drosophila EGF receptor* (*Egfr*), *ventral nerve cord defective* (*vnd*), *intermediate neuroblasts defective* (*ind*) and *muscle segment homeobox* (*msh*; *Dr* – FlyBase) genes initially divides the neuroectoderm into three parallel longitudinal columns (reviewed by Skeath, 1999). Superimposition of the expression patterns of these genes subdivides the neuroectoderm into a checkerboard pattern of cell clusters that corresponds to the pattern of neural equivalence groups from which individual NBs arise. Functional studies indicate that these genes regulate NB fate by controlling the precise combination of genes expressed within each cell cluster (reviewed by Bhat, 1999; Skeath, 1999). These data have led to the model that the coordinated action of the segment polarity genes, as well as of *Egfr*, *vnd*, *ind* and *msh* specify the fate of the cells in an equivalence group and with it the fate of the NB that segregates from the group.

We focus on the genetic regulatory mechanisms that control DV pattern and cell fate in the neuroectoderm. Previous research has shown that *Egfr*, *vnd*, *ind* and *msh* subdivide the early neuroectoderm into three longitudinal columns – medial, intermediate and lateral – from which early forming NBs arise. Active *Egfr* signaling occurs in the medial and intermediate

columns prior to the first wave of NB formation and persists in the medial column throughout neurogenesis. *Egfr* activates *ind* expression in the intermediate column and promotes intermediate column NB fates. In addition, *Egfr* acts in the medial column to help specify the individual fates of medial NBs (Skeath, 1998; Udolph et al., 1998; von Ohlen and Doe, 2000; Yagi et al., 1998). *vnd* expression marks the medial neuroectodermal column throughout neurogenesis. *vnd* promotes medial column fates, at least in part by repressing *ind* expression. *vnd* also promotes SI and SII medial NB formation but appears to play only a limited role in SIII-SV medial NB formation (Chu et al., 1998; Jimenez and Campos-Ortega, 1990; McDonald et al., 1998; Skeath et al., 1994; Jimenez et al., 1995). *ind* expression marks the intermediate column during the first two, but not subsequent, waves of NB formation. *ind* promotes the formation and fate of SI and SII intermediate NBs, and represses *msh* expression in the intermediate column (Weiss et al., 1998). *msh* expression marks the lateral column during the first two waves of NB formation. However, *msh* does not affect lateral column gene expression and its function in the lateral column is not well defined (Buescher and Chia, 1997; Isshiki et al., 1997; Skeath, 1999). Despite the well-defined roles *Egfr*, *vnd* and *ind* play in regulating DV pattern and cell fate in the neuroectoderm, existing evidence suggests additional genes regulate these processes. For example, neither *Egfr* nor *ind* is active in the intermediate column after SII NB formation (Skeath, 1998; Weiss et al., 1998), and most late-forming medial column NBs develop normally in *vnd* mutant embryos (Chu et al., 1998; McDonald et al., 1998).

One candidate regulator of DV pattern in the neuroectoderm is the Sox-domain-containing gene *Dichaete* or *fish-hook*. (We refer to the gene as *Dichaete*.) *Dichaete* belongs to the conserved Sox family of high-mobility group domain DNA-binding proteins (Nambu and Nambu, 1996; Russell et al., 1996). Sox proteins regulate the transcription of target genes through their ability to bind DNA and to partner with a wide variety of different transcription factors (Kamachi et al., 2000). Many vertebrate Sox-domain-containing genes are expressed in the neural plate/tube (Cremazy et al., 2000; Wegner, 1999); however, their function in neural development remains unclear. In *Drosophila*, *Dichaete* is initially expressed in seven transverse pair-rule stripes in early embryos, where it regulates segmentation (Nambu and Nambu, 1996). Later, *Dichaete* is activated in the ventral half of the neuroectoderm during gastrulation. Late-stage *Dichaete* mutants exhibit severe defects in CNS development, consistent with *Dichaete* playing a role in DV neural patterning (Nambu and Nambu, 1996). However, the possibility that these defects arise because of a direct role for *Dichaete* in the DV patterning of the neuroectoderm or indirectly as a consequence of the role of *Dichaete* in segmentation was not investigated.

We demonstrate that *Dichaete* plays a crucial role to pattern the neuroectoderm along the DV axis. Our expression studies show that *Dichaete* is expressed in the medial and intermediate neuroectodermal columns throughout all waves of NB formation. Loss-of-function studies indicate that *Dichaete* regulates cell fate and neuroblast formation in the medial and intermediate column. Genetic interactions, as well as molecular epistasis tests, demonstrate that *Dichaete* functions in parallel to *vnd* and *ind*, and downstream of *Egfr* to regulate pattern and cell fate in the neuroectoderm. Work from vertebrates indicates that Sox-domain-containing proteins regulate transcription by partnering with different transcription factors (Kamachi et al., 2000). Together with results presented here, these data support a model whereby Dichaete physically associates with Vnd and Ind to regulate gene expression and NB formation within the medial and intermediate columns of the neuroectoderm.

MATERIALS AND METHODS

Genetics

Wild-type patterns of gene expression were examined in Oregon R embryos. Mutant lines used were: *Egfr,* allele flb^{IK35} (Clifford and Schupbach, 1994); ind^{RR108} and $ind^{16.2}$ (Weiss et al., 1998); $vnd^{\Delta 38}$ (Chu et al., 1998); $Dichaete^{87}$, $Dichaete^{96}$, provided by John R. Nambu (Mukherjee et al., 2000); and H162, an enhancer trap line inserted into the *seven-up* gene and referred to *as svp-lacZ* (Mlodzik et al., 1990). We used standard genetic means to create fly lines or embryos multiply mutant for the following genes: (1) $Dichaete^{87}$ *svp-lacZ*; (2) $Dichaete^{87}$ ind^{RR108}; (3) $vnd^{\Delta 38}$; $Dichaete^{87}$; (4), $vnd^{\Delta 38}$; flb^{IK35}; (5) $vnd^{\Delta 38}$; ind^{RR108}; and (6) $vnd^{\Delta 38}$; $Dichaete^{87}$ and ind^{RR108}.

Immunohistochemistry of whole mount embryos

Single- and double-label immunohistochemistry, and RNA in situ analysis were performed as described elsewhere (Skeath, 1998). For the active MAP kinase antibody, we used biotinyl tyramide (NEN Life Science Products) to amplify the signal following the manufacturer's protocol. We used the following antibodies at the indicated dilutions: mouse anti-Achaete (1:3) (Skeath and Carroll, 1991); rabbit anti-*Vnd* (1:10) (McDonald et al., 1998); rabbit anti-Dichaete (1:1000) (Mukherjee et al., 2000); rat anti-Ind (1:250) (Weiss et al., 1998); rabbit anti-Msh (1:600) (Isshiki et al., 1997); rabbit anti-Eve (1:2000) (Frasch et al., 1986); mouse anti-Engrailed 4D9 (1:5) (Patel et al., 1989a); mouse anti-βgal (1:2000; Promega); mouse anti-Pros MR1A (1:3) (Spana and Doe, 1995); and mouse anti-Active MAP kinase (1:2000; Sigma) (Gabay et al., 1996).

RESULTS

Genetic identification of additional genes that pattern the neuroectoderm along the DV axis

vnd, *ind* and *Egfr* are key factors that regulate pattern and cell fate along the DV axis of the neuroectoderm. To ask if *Egfr* pathway activity depends on *vnd* or *ind*, we assayed MAPK activity in homozygous *vnd* or *ind* single mutant embryos. In both backgrounds, the initial activation of *Egfr* signaling in the medial and intermediate columns is normal. Thus, in the early neuroectoderm, *Egfr* acts either upstream or in parallel to *vnd* and *ind*. To investigate whether *Egfr* acts upstream of *vnd* or *ind*, we assayed *vnd* and *ind* expression in embryos homozygous mutant for the *Egfr* null allele flb^{IK35} (referred to as *Egfr* mutant embryos). *ind* expression is absent in *Egfr* mutant embryos, indicating that *Egfr* activates *ind* expression in the intermediate column (data not shown) (von Ohlen and Doe, 2000). By contrast, *vnd* expression in *Egfr* mutant embryos appears normal through the onset of stage 8 (Fig. 1). However, during stage 8, *vnd* expression begins to dissipate in medial column cells, and by early stage 10 these cells no longer express *vnd* (Fig. 1) (Gabay et al., 1996). Conversely, medial column NBs that form in *Egfr* mutant embryos express *vnd*

normally and retain *vnd* expression throughout embryogenesis (Fig. 1). Thus, *Egfr* functions to maintain *vnd* expression in the neuroectoderm but is dispensable for *vnd* expression in NBs. These data indicate that *Egfr* resides atop the genetic hierarchy known to subdivide the neuroectoderm along the DV axis.

Our results suggest that *Egfr* patterns the neuroectoderm, at least in part, through its regulation of *vnd* and *ind*. To determine if additional genes act downstream of *Egfr* in this process, we compared the phenotypes of embryos singly mutant for *Egfr* and *ind*. We reasoned that if *Egfr* patterns the intermediate column solely through regulation of *ind*, then *Egfr* and *ind* mutant embryos should exhibit identical intermediate column phenotypes. To compare the early CNS phenotypes of *Egfr* and *ind*, we carried out a precise analysis of *msh* expression and the NB pattern. In both cases, *Egfr* exhibits a more severe phenotype than *ind* (Fig. 2). *msh* expression expands more medially in *Egfr* mutant embryos than in *ind* mutant embryos (Fig. 2). In addition, lateral NBs are most often separated from medial NBs by a gap in *ind* mutant embryos, while lateral NBs develop immediately adjacent to medial NBs in *Egfr* mutant embryos (Fig. 2). These data indicate a greater disruption to the intermediate column in *Egfr* mutant embryos than in *ind* mutant embryos. These phenotypic differences are consistent with the presence of additional genes acting downstream of *Egfr* and in parallel to *ind* to control cell fate in the intermediate column. However, *Egfr* maintains *vnd* expression in the neuroectoderm; thus, these data do not exclude the possibility that the differences in phenotype between *Egfr* and *ind* arise due to the late regulation of *vnd* expression by *Egfr*.

To test whether the phenotypic differences between *ind* and *Egfr* mutant embryos are an indirect result of the regulation of *vnd* expression by *Egfr*, we asked whether these differences were equalized in double mutants where *vnd* function is also removed. In *vnd*; *ind* mutant embryos, *msh* is expressed throughout the neuroectoderm, although its expression is higher in the lateral column relative to the medial column (Fig. 2). By contrast, *msh* is expressed at uniformly strong levels throughout the neuroectoderm in *vnd; Egfr* mutant embryos (Fig. 2). Thus, removal of *vnd* and *Egfr* causes a stronger derepression of *msh* in the neuroectoderm than loss of *vnd* and *ind*. These results suggest that additional gene(s) act downstream of *Egfr* and in parallel to *vnd* and *ind* to regulate DV pattern in the neuroectoderm. They also suggest that in the absence of *vnd* and *Egfr* function, the entire neuroectoderm acquires a lateral column fate.

Dichaete is expressed in the medial and intermediate neuroectodermal columns

Based on its restricted expression pattern in the ventral region of the neuroectoderm (Fig. 3) (Nambu and Nambu, 1996), we identified the Sox-domain-containing gene *Dichaete* as a likely candidate to act downstream of *Egfr* to regulate DV pattern in the neuroectoderm. To investigate whether *Dichaete* contributes to neuroectodermal patterning, we first determined the precise limits of *Dichaete* expression in the neuroectoderm using the expression of *msh* and *achaete* (*ac*) to mark different longitudinal columns (Fig. 3 and data not shown). *msh* is expressed in the lateral column; *ac* is expressed in neural equivalence groups (proneural clusters) in the medial and lateral columns of rows 3 and 7. Within the neuroectoderm, *Dichaete* expression begins during stage 7. *Dichaete* is expressed uniformly in the ventral region of the neuroectoderm with a lateral expression boundary that precisely abuts the medial limit of *msh* and *ac* expression in the lateral column (Fig. 3; data not shown). Within the neuroectoderm, *Dichaete* expression is restricted to the medial and intermediate columns through late stage 12, at which point *Dichaete* expression expands to include the entire neuroectoderm (data not shown). Thus, in contrast to the transient presence of *Egfr* and *ind* activity in the intermediate column, *Dichaete* is expressed in the intermediate and medial columns throughout all waves of NB formation.

During our analysis of *Dichaete* expression in the neuroectoderm, we noticed that most medial and intermediate NBs do not express *Dichaete* at detectable levels. To determine the pattern of *Dichaete* expression in neuroblasts we co-labeled wild-type embryos for *Dichaete* and *hunchback*, a marker of all neuroblasts (Kambadur et al., 1998). We observe, in general, that newly formed medial and intermediate column NBs express weak levels of *Dichaete* but that most older NBs in these domains do not express *Dichaete.* Exceptions to this exist, as two conspicuous NBs express *Dichaete* – one in the medial column of row 4 and one in the intermediate column of row 3 (data not shown). These data suggest that newly formed medial and intermediate NBs retain residual *Dichaete* expression from the neuroectoderm but that *Dichaete* expression is downregulated in most medial and intermediate NBs once they form.

In contrast to medial and intermediate NBs, many lateral NBs activate *Dichaete* at specific points in their lineages. NB 7-4 is the first lateral NB to activate *Dichaete* expression during late stage 10 (Fig. 3). *Dichaete* expression in lateral NBs is dynamic. NBs 5-6, NB 2-5 and eventually NB 3-5 express *Dichaete* (Fig. 3; data not shown for NB 3-5). Thus, all medial and intermediate column neuroectodermal cells express *Dichaete* but most medial and intermediate NBs do not express *Dichaete*. Conversely, lateral NBs but not neuroectodermal cells express *Dichaete*. These data are consistent with *Dichaete* regulating cell fate in the medial and intermediate neuroectodermal columns, and at specific points in the lineage of lateral NBs. We focus on the role *Dichaete* plays to regulate DV pattern and cell fate in the medial and intermediate neuroectodermal columns.

Dichaete regulates cell fate and NB formation in the medial and intermediate columns

The restricted expression of *Dichaete* in the medial and intermediate columns suggests that *Dichaete* regulates cell fate and NB formation in this region. However, *Dichaete* mutant embryos exhibit AP patterning defects, owing to an early requirement in segmentation (Nambu and Nambu, 1996). The segmental defects are largely restricted to the abdominal segments; thoracic segments appear largely normal (Soriano and Russell, 1998). These segmentation defects could obscure a role for *Dichaete* during neuroectodermal patterning. Thus, we restricted our analysis of *Dichaete* function in the neuroectoderm to thoracic segments.

To investigate whether *Dichaete* patterns the neuroectoderm, we followed early neural development in embryos mutant for the $Dichaete^{87}$ and $Dichaete^{96}$ null alleles (Nambu and Nambu, 1996). We first tested whether *Dichaete* regulates gene expression in the neuroectoderm by following *ac* and *msh*

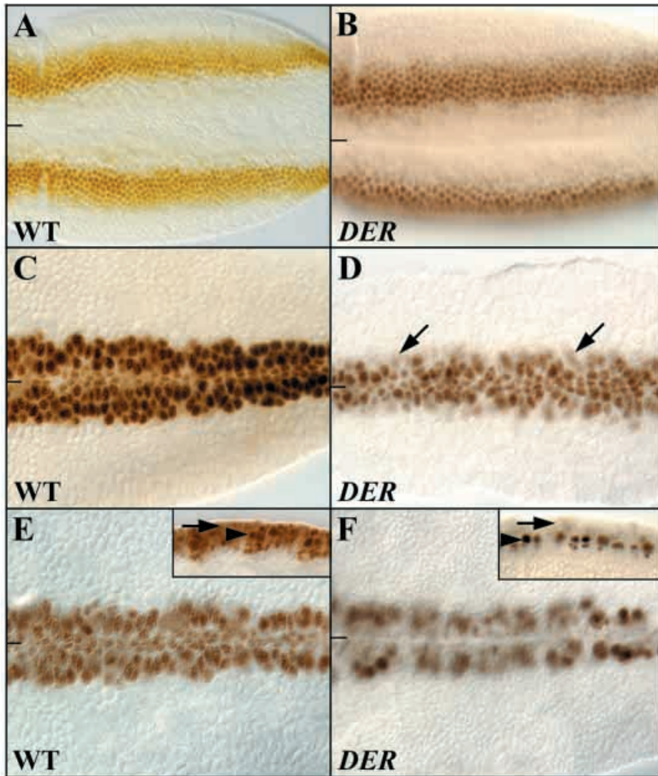

Fig. 1. *Egfr* maintains *vnd* expression in the medial column. High magnification ventrolateral and ventral views of wild-type (A,C,E) and *Egfr* (*DER*; B,D,F) embryos labeled for *vnd* expression. Insets in E,F show high-magnification lateral views of *vnd* expression in stage 10 wild-type and *Egfr* embryos. (A,B) During stage 6, *vnd* expression becomes detectable in an approx. six-cell-wide column on either side of the ventral midline in wild-type and *Egfr* embryos. (C) By late stage 8 in wild-type embryos, *vnd* expression narrows to a three-cell-wide column on either side of the ventral midline. These cells identify the medial column and stage 10 embryos (E) maintain this pattern of *vnd* expression. Inset in E shows that neuroectodermal cells (arrow) and NBs (arrowhead) express *vnd.* (D) In *Egfr* mutant embryos, *vnd* expression begins to dissipate in the ventral neuroectoderm by stage 8 (arrows) and by stage 10 (F) *vnd* expression is completely absent from the neuroectoderm. Inset in F shows that NBs (arrowhead) but not neuroectodermal cells (arrow) express *vnd* in *Egfr* mutant embryos. Anterior is towards the left and the line indicates the ventral midline.

expression. Normally, *ac* is expressed in the medial and lateral, but not intermediate, proneural clusters of rows 3 and 7 during the first wave of NB formation (Fig. 4) (Skeath and Carroll, 1992). In *Dichaete* mutant embryos, we observe a partial derepression of *ac* expression in the intermediate column (Fig. 4). We find that roughly 50% of the cells within the intermediate column of rows 3 and 7 express *ac*. *ac* expression in the medial column appears normal, as do the AP limits of *ac* expression in the thoracic segments. In contrast to *ac*, we detect no obvious alterations to *msh* expression in the neuroectoderm (data not shown). As *ac* is a key determinant of neural fate, we interpret its derepression in the intermediate column to indicate that *Dichaete* regulates cell fate in this column. However, our *msh* results indicate that lateral fates are specified normally in *Dichaete* mutant embryos.

ind normally represses *ac* expression in the intermediate column, because in *ind* mutant embryos, *ac* expression is completely derepressed within rows 3 and 7 of the intermediate column (Weiss et al., 1998). The *Dichaete* and *ind* phenotypes demonstrate that both genes are necessary for intermediate column fates. To determine if *Dichaete* and *ind* function in a linear pathway to regulate intermediate cell fates, we followed *ind* expression in *Dichaete* mutant embryos and *Dichaete* expression in *ind* mutant embryos. *ind* expression is normal in

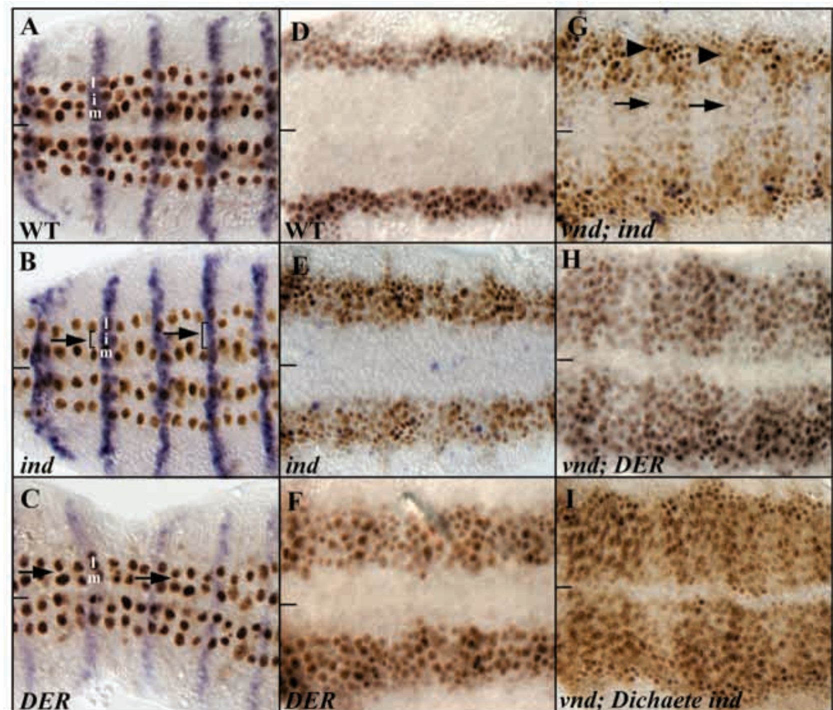

Fig. 2. *Egfr* regulates DV pattern in the neuroectoderm through genes other than *vnd* and *ind*. High-magnification ventral views of the neuroectoderm of stage 9 (A-C) and late stage 8 (D-I) wild-type (A,D), *ind* (B,E), *Egfr* (*DER*; C,F), *vnd; ind* (G), *vnd*; *Egfr* (*vnd;DER*; H) and *vnd; Dichaete ind* (I) mutant embryos labeled for NBs (A-C) or *msh* expression (D-I). (A) In wild-type embryos, NBs occupy three columns: medial (m), intermediate (i) and lateral (l). (B) In *ind* embryos, intermediate NBs do not form and medial and lateral NBs are separated by a gap (bracket and arrows). (C) In *Egfr* embryos, intermediate NBs do not form and medial and lateral NBs reside adjacent to each other (arrows). (D) In wild-type embryos, *msh* expression is restricted to the lateral column. (E) In *ind* embryos *msh* expression expands into the intermediate column. (F) In *Egfr* embryos, *msh* expression expands into the intermediate column and partially into the medial column. (G) In *vnd; ind* embryos, *msh* is expressed throughout the neuroectoderm with higher expression laterally (arrowheads) than ventrally (arrows). (H) In *vnd; Egfr* embryos and in (I) *vnd; Dichaete ind* embryos, *msh* is expressed uniformly throughout the neuroectoderm. Anterior is towards the left and the line indicates the ventral midline.

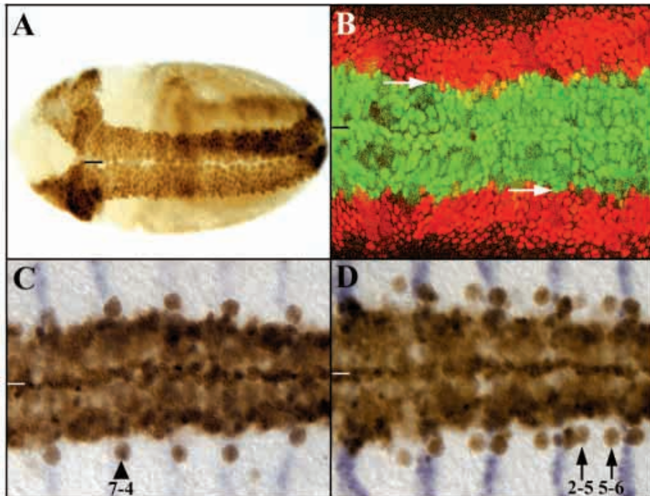

Fig. 3. *Dichaete* is expressed in the medial and intermediate neuroectodermal columns and in lateral NBs. Low- (A) and high- (B-D) magnification ventral views of wild-type stage 8 (A,B) or stage 11 (C,D) embryos labeled for *Dichaete* (A), *Dichaete* (green) and *msh* (red) (B), or *Dichaete* (brown) and *engrailed* (blue) (C,D). (A) In wild-type embryos, *Dichaete* is expressed in the ventral region of the neuroectoderm. (B) The lateral limit of *Dichaete* expression abuts precisely the medial limit of *msh* expression in the lateral column (arrows). (C) In early stage 11 embryos, *Dichaete* expression persists in the medial and intermediate columns; however, lateral column NB 7-4 now expresses *Dichaete* (arrowhead). (D) In late stage 11 embryos two additional lateral column NBs (NB 2-5 and NB5-6) express *Dichaete* (arrows). Anterior is towards the left and the line indicates the ventral midline.

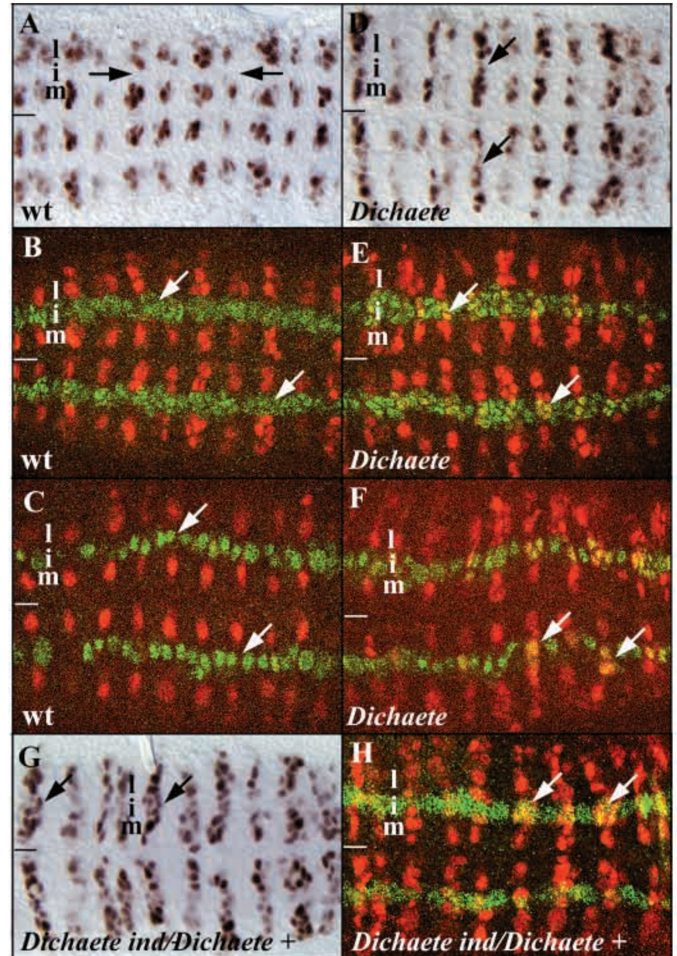

Fig. 4. *Dichaete* regulates cell fate in the intermediate column. High-magnification ventral views of stage 9 wild-type (A-C), *Dichaete* (D-F) and *Dichaete ind/ Dichaete* + (G,H) mutant embryos labeled for *ac* (A,D,G) or *ac* (red) and *ind* (green; B,C,E,F,H). (A) In wild-type embryos *ac* is expressed in proneural cell clusters in the medial and lateral but not intermediate (arrows) columns of rows 3 and 7. (B,C) Normally, the expression of *ind* and *ac* is mutually exclusive in the neuroectoderm (arrows, B) and NBs (arrows, C). (D-F) In *Dichaete* embryos, *ac* expression is partially derepressed in the intermediate column (arrows, D) and intermediate column neuroectodermal cells (arrows, E) and NBs (arrows, F) inappropriately co-express *ac* and *ind*. (G,H) In *Dichaete* embryos heterozygous for *ind, ac* expression is strongly derepressed in the intermediate column (arrows, G) and *ac* and *ind* expression again colocalize in intermediate column neuroectodermal cells (arrows, H). Anterior is towards the left and the line indicates the ventral midline.

Dichaete mutant embryos (Fig. 4) and *Dichaete* expression is normal in *ind* mutant embryos (data not shown). Thus, *ind* and *Dichaete* are regulated independently of each other.

Double labeling *Dichaete* mutant embryos for *ac* and *ind*, and double labeling *ind* mutant embryos for *ac* and *Dichaete* revealed an interdependent relationship between *Dichaete* and *ind*. In *Dichaete* mutant embryos, a significant number of row 3 and 7 intermediate column cells and NBs co-expressed *ac* and *ind* – an occurrence never observed in wild-type embryos (Fig. 4). Thus, the ability of *ind* to repress *ac* in the intermediate column requires *Dichaete* activity. Reciprocally, in *ind* mutant embryos, all row 3 and 7 intermediate column cells co-express *ac* and *Dichaete* (data not shown). Thus, the ability of *Dichaete* to repress *ac* in the intermediate column requires *ind* activity.

Next, we assayed whether *Dichaete* regulates NB formation in the medial and intermediate columns. To do this, we followed the development of individual NBs using a panel of molecular markers that identify specific NBs or their progeny (Doe, 1992; Kambadur et al., 1998; Patel et al., 1989b). We used *Svp-lacZ* to label the medial column SI NBs 5-2 and 7-1, as well as SIII NB 4-1; *castor* expression to label the medial column SIII NB 6-1; and *eve* expression to label the first-born progeny of SI medial column NBs 1-1 and 7-1, and of SII intermediate column NB 4-2. In *Dichaete* mutant embryos, NBs 1-1 (99% formation; *n*=200), 5-2 (99.6%; *n*=226) and 7-1 (97.8%; *n*=226) develop normally. Thus, SI medial column NBs form normally in the absence of *Dichaete* function. By contrast, we observe defects in the formation of SII and SIII NBs in *Dichaete* mutants (Fig. 5; Table 1). For example, we fail to detect a Svp-*lacZ*-positive NB 4-1 in 30.9% of thoracic hemisegments (*n*=256) or a Castor-positive NB 6-1 in 7.9% of hemisegments (*n*=240). In addition, we fail to detect an Eve-positive RP2 neuron in 12.9% of hemisegments (*n*=210), suggesting the absence of NB 4-2 in these hemisegments. Together with our expression analyses, these phenotypic studies demonstrate that *Dichaete* acts in the neuroectoderm to promote the formation of late-forming NBs in the medial and intermediate columns.

Table 1. *Dichaete* interacts genetically with *vnd* and *ind*

	Loss of NB 6-1		Loss of NB 4-1			Loss of RP2	
Genotype	% Castor	*n*	% Svp-*lacZ*	*n*	Genotype	% Eve	*n*
Wild type	0	250	3.6	248	Wild type	0	172
$vnd^{\Delta 38}$	64.5	276	60.7	168	ind^{RR108}	100	144
$Dichaete^{87}$	7.9	240	30.9	256	$Dichaete^{87}$	12.9	210
$vnd^{\Delta 38}$; $Dichaete^{87}$	90.9	132	89.2	102	$Dichaete^{87}$ ind^{RR108}/$Dichaete^{87}$ +	36.4	250
					$Dichaete^{87}$ $ind^{16.2}$/$Dichaete^{87}$ +	42.4	250

Dichaete acts downstream of *Egfr* and in parallel to *vnd* and *ind* in the neuroectoderm

Our loss of function analyses identify *Dichaete* as a regulator of DV pattern and cell fate in the neuroectoderm. To place *Dichaete* within the known genetic regulatory hierarchy that governs DV pattern in the neuroectoderm, we performed systematic molecular epistasis tests for *Dichaete*, *ind*, *vnd* and *Egfr*. Initially, we assayed *vnd* and *ind* expression, as well as *Egfr* activity in *Dichaete* mutant embryos. *Dichaete* mutant embryos exhibit no obvious defects to the expression of *vnd* or *ind*, or the activity of *Egfr* (Fig. 4; data not shown). Thus, *Egfr*, *vnd* and *ind* function upstream or in parallel to *Dichaete*.

To investigate whether *Egfr*, *vnd* or *ind* regulate *Dichaete*, we assayed *Dichaete* expression in embryos mutant for each gene. We observe no alterations to the initial pattern of *Dichaete* expression in *vnd* or *ind* mutants, or in embryos doubly mutant for *vnd* and *ind* (Fig. 6). *Dichaete* expression remains normal in *ind* mutant embryos throughout embryogenesis. However, by stage 11 in *vnd* and *vnd*; *ind* mutant embryos *Dichaete* expression narrows inappropriately to an irregularly patterned stripe two-to-four cells wide immediately adjacent to the ventral midline (Fig. 6). These results show that *Dichaete* is regulated independently of *ind* and is activated independently of *vnd*, but that *vnd* helps maintain *Dichaete* expression in the neuroectoderm.

In contrast to *vnd* and *ind*, the initial pattern of *Dichaete* in *Egfr* mutant embryos is greatly reduced in the intermediate column and moderately reduced in the medial column during early neurogenesis (stage 8; Fig. 6). By stage 11, *Dichaete* expression narrows inappropriately to a thin and irregular stripe zero-to-three cells wide immediately adjacent to the ventral midline; *Dichaete* expression in the ventral midline is normal (Fig. 6). These data identify *Egfr* as a key positive regulator of *Dichaete* in the neuroectoderm, and indicate that at least one other gene acts with *Egfr* to activate *Dichaete* expression in the medial column.

To investigate whether *vnd* acts with *Egfr* to promote *Dichaete* expression in the medial column, we followed *Dichaete* expression in *vnd*; *Egfr* mutant embryos. The initial pattern of *Dichaete* in these embryos is the same as that observed in *Egfr* mutant embryos (Fig. 6). However, by stage 11, *Dichaete* expression is completely absent from the neuroectoderm, although *Dichaete* expression is normal in the ventral midline. These results indicate that *vnd* and *Egfr* collaborate to maintain *Dichaete* expression in the neuroectoderm.

To determine if *Egfr* activity is sufficient to activate *Dichaete* expression, we used the GAL4/UAS system (Brand and Perrimon, 1993) to activate *Egfr* signaling throughout the early *Drosophila* embryo. Ubiquitous *Egfr* signaling activates *Dichaete* expression throughout the neuroectoderm but not in the dorsal ectoderm (data not shown). Thus, *Egfr* is necessary and sufficient to activate *Dichaete* in the neuroectoderm. However, in the dorsal ectoderm, either factors exist that inhibit the ability of *Egfr* to activate *Dichaete* or this domain lacks co-factors required for *Egfr* to activate *Dichaete*. Our molecular epistasis tests place *Egfr* upstream of *Dichaete* and indicate that *vnd*, *ind* and *Dichaete* function largely in parallel to regulate pattern and cell fate in the neuroectoderm.

Dichaete interacts genetically with *vnd* and *ind* to regulate cell fate in the neuroectoderm

The parallel genetic activities of *Dichaete*, *vnd* and *ind*, the co-expression of *Dichaete* with *vnd* and *ind*, and the similarity of the early *Dichaete* CNS phenotype to those of *vnd* and *ind* (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998) led us to test whether *Dichaete* interacted genetically with *vnd* and *ind*. To ascertain whether *Dichaete* interacted with *vnd* we made *vnd*; *Dichaete* double mutants and assayed the formation of medial column SIII NBs 4-1 and 6-1. In *Dichaete* mutant embryos, NBs 4-1 and 6-1 formed in 69.1% (*n*=256) and in 92.1% (*n*=240) of hemisegments, respectively (Fig. 5; Table 1). Although a previous report indicated little to no effect of *vnd* function on SIII-SV NB formation (Chu et al., 1998), in *vnd* mutant embryos we found that NBs 4-1 and 6-1 formed in 39.3% (*n*=168) and 35.5% (*n*=276) of hemisegments, respectively (Fig. 5; Table 1). In *vnd*; *Dichaete* mutant embryos NBs 4-1 and 6-1 formed in 10.8% (*n*=102) and 9.1% (*n*=132) of hemisegments, respectively (Fig. 5; Table 1). The increased defects in NB formation in *vnd*; *Dichaete* mutant embryos relative to either single mutant confirms that *Dichaete* and *vnd* do not act in a linear pathway to regulate NB formation, rather they demonstrate that *Dichaete* and *vnd* function in parallel to control NB formation in the medial column.

We should note that defects in NB formation in *vnd; Dichaete* mutant embryos are more severe than would be expected if these genes function independently. For example, if two genes act independently to promote NB formation then the frequency of NB formation in the double mutant would be the product of the individual probabilities that the indicated NB will form in each single mutant. Thus, if *vnd* and *Dichaete* function independently, we would expect NB 4-1 to form 27.2% of the time ($0.393\times0.691=0.272$) and NB 6-1 to form 32.7% of the time ($0.355\times0.921=0.327$) in *vnd; Dichaete* mutant embryos. However, NBs 4-1 and 6-1 form ~10% of the time in *vnd*; *Dichaete* mutant embryos – roughly threefold more severe than predicted for independently acting genes. These results reveal a genetic interaction between *Dichaete* and *vnd*. Furthermore, we interpret these results to suggest that the activities of *vnd* and *Dichaete* are more convergent than parallel with respect to NB formation.

Next, we tested for genetic interactions between *Dichaete*

and *ind*. The partial derepression of *ac* expression and the incomplete loss of an Eve-positive RP2 neuron are the most sensitive assays for *Dichaete* function in the intermediate column. However, strong alleles of *ind* cause a complete derepression of *ac* expression, and a complete loss of RP2 neurons in this domain (Weiss et al., 1998). Thus, an analysis of *Dichaete ind* double mutant embryos using these markers would be uninformative. To circumvent this problem, we tested whether *ind* dominantly enhances the *Dichaete* intermediate column *ac* and RP2 phenotypes. Embryos heterozygous for *ind* exhibit wild-type *ac* expression and RP2 formation. However, *Dichaete ind/Dichaete* + mutant embryos exhibit enhanced derepression of *ac* expression and an approximately threefold enhancement of the RP2 loss phenotype relative to *Dichaete* mutant embryos (Fig. 4; Table 1). The dominant enhancement of the *Dichaete* phenotype by *ind* reveals a genetic interaction between *Dichaete* and *ind*.

Our initial interest in *Dichaete* arose from our observation that *vnd*; *Egfr* mutant embryos exhibit a more severe neuroectodermal phenotype than *vnd*; *ind* mutant embryos. This suggested that at least one other gene acts downstream of *Egfr*, and in parallel to *vnd* and *ind* to pattern the early neuroectoderm and led to our analysis of *Dichaete*. To determine if the continued function of *Dichaete* in *vnd*; *ind* mutant embryos can explain the phenotypic differences between *vnd*; *ind* and *vnd*; *Egfr* mutant embryos, we followed *msh* expression in *vnd*; *Dichaete ind* triple mutant embryos. In this background, we observe a complete and uniform derepression of *msh* expression throughout the neuroectoderm (Fig. 2). The *msh* phenotype of *vnd*; *Dichaete ind* embryos is essentially identical to that of *vnd*; *Egfr* embryos, and more severe than that of *vnd*; *ind* embryos (Fig. 2). Thus, with respect to *msh* expression the difference between the *vnd; ind* and *vnd; Egfr* mutant phenotypes appears to result from the persistent function of *Dichaete* in *vnd; ind* mutant embryos.

DISCUSSION

Prior work has underlined the pivotal role *Egfr*, *vnd* and *ind* play to regulate DV pattern and cell-fate in the neuroectoderm (reviewed by Skeath, 1999). The results in this paper indicate that additional genes act with this genetic trio to pattern the neuroectoderm. We identified *Dichaete* as a key regulator of DV pattern in the neuroectoderm. *Dichaete* is expressed in the medial and intermediate columns and regulates cell fate and NB formation in these domains. Within the neuroectoderm, *Dichaete* acts downstream of *Egfr* and in parallel to *vnd* and *ind* (Fig. 7). Together with biochemical research on Sox-domain-containing genes in vertebrates (reviewed by Kamachi et al., 2000) our work supports a model (Fig. 7) in which Dichaete protein physically associates with Vnd and Ind to regulate target gene expression and NB formation in distinct neuroectodermal columns.

Our interest in *Dichaete* arose owing to our observation that removal of *vnd* and *Egfr* function caused a stronger derepression of *msh* expression in the neuroectoderm than removal of *vnd* and *ind* function. These results contrast slightly with previous research that did not identify a phenotypic difference between *vnd*; *ind* and *vnd*; *Egfr* mutant embryos (von Ohlen and Doe, 2000). This work analyzed *msh* expression in the neuroectoderm at a later stage (late stage 9) than ours. At late stage 9, we also observe identical alterations to *msh* expression in *vnd; ind* mutant embryos relative to *vnd; Egfr* mutant embryos. However, the *msh* expression pattern is dynamic – rapidly changing from uniform expression in the lateral column during stage 8 to a segmentally modulated pattern of cell clusters located within the lateral half of the neuroectoderm by stage 10. We attribute the differences in our observations to the different stages used to assay the effects of *vnd*, *ind* and *Egfr* on neuroectodermal development in the two studies.

Dichaete exhibits region specific functions in the neuroectoderm

Dichaete is expressed and regulates cell fate in the medial and intermediate neuroectodermal columns. However, *Dichaete* carries out distinct functions in each domain: *Dichaete* represses *ac* expression in the intermediate column but has no effect on *ac* expression in the medial column where *Dichaete* and *ac* are co-expressed.

How might *Dichaete* exhibit region specific effects on putative target genes? Work from vertebrate systems suggests that individual Sox-domain-containing proteins exhibit a widespread ability to partner with different transcription factors (reviewed by Kamachi et al., 2000). Thus, Dichaete protein could exhibit column-specific functions via its association with different transcription factors in different domains. The formation of distinct protein complexes containing Fish could alter the output of Fish activity in at least two ways. Different protein complexes that contain Fish could exhibit different effects on transcription: repression versus activation. Alternatively, different Fish-containing protein complexes could exhibit distinct DNA-binding properties and therefore bind distinct recognition sites. These two possibilities are not mutually exclusive, and different Fish-containing protein complexes may both bind different recognition sites and exert different transcriptional effects on target genes.

Examples of both forms of regulation are known. In the early *Drosophila* embryo, the transcription factor Dorsal activates one set of target genes ventrally and represses a distinct set dorsally (Jiang et al., 1993). On its own, Dorsal functions as a transcriptional activator. However, in the dorsal region of the embryo, the interaction of Dorsal with a co-factor that binds to adjacent sites on target promoters converts Dorsal to a repressor. Although less well-defined mechanistically, the vertebrate Sox2 protein appears capable of activating or repressing target gene expression depending on cell-type and the target promoter (Botquin et al., 1998). In addition, work on vertebrate Sox domain proteins indicates that the composition of Sox-protein containing complexes modulates the DNA-binding specificity of these complexes. For example, in lens cells, Sox2 interacts with the DNA-binding factor δEF3 and binds to a bipartite recognition site on the δ-crystallin enhancer (Kamachi et al., 1998; Kamachi et al., 1999). In embryonic stem cells, Sox2 interacts with Oct3/4 and binds to a different recognition site in the Fgf4 minimal enhancer (Ambrosetti et al., 1997). In both enhancers, Sox2 binds to the same individual sequence. However, the specificity for the entire recognition site in one enhancer over the other arises as a consequence of the interaction of Sox2 with different transcription factors in different cell types and the distinct DNA-binding preferences of the entire complex.

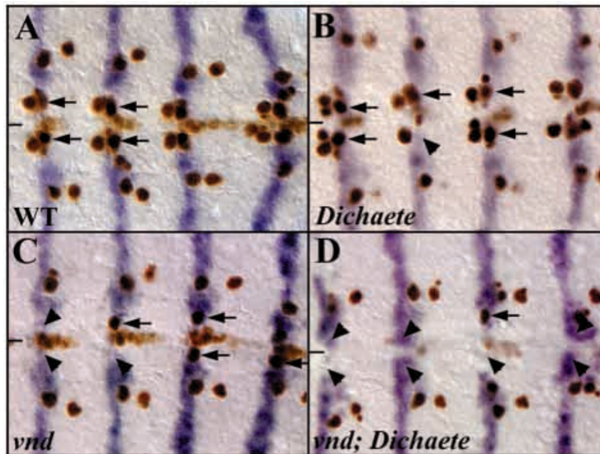

Fig. 5. *Dichaete* regulates NB formation. High-magnification ventral views of stage 11 wild-type (A), *Dichaete* (B), *vnd* (C) and *vnd*; *Dichaete* (D) embryos labeled for Castor protein. (A) At stage 11 in wild-type embryos, a Castor-positive NB 6-1 forms in every hemisegment (arrows). (B) In *Dichaete* mutant embryos NB 6-1 forms in most hemisegments (arrows indicate Castor-positive NB6-1; arrowheads indicate the absence of NB6-1). (C) In *vnd* mutant embryos NB 6-1 forms in roughly one-third of all hemisegments (arrows); arrowhead indicates the absence of NB6-1. (D) In *vnd; Dichaete* double mutant embryos, NB 6-1 rarely forms (arrow); arrowheads indicate the absence of NB 6-1. Anterior is towards the left and the line indicates the ventral midline.

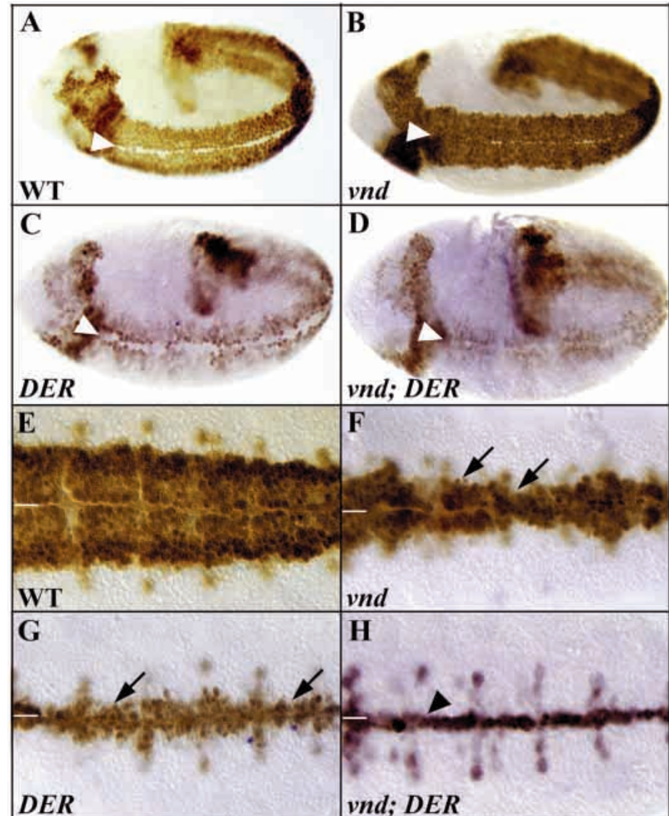

Fig. 6. *Egfr* regulates *Dichaete* expression in the neuroectoderm. (A-D) Ventrolateral views of whole-mount stage 8 (A-D) and high-magnification ventral views of stage 11 (E-H) wild-type (A,E), *vnd* (B,F), *Egfr* (*DER*; C,G) and *vnd; Egfr* (*vnd; DER*; D,H) mutant embryos labeled for *Dichaete*. (A) In stage 8 wild-type embryos *Dichaete* is expressed in the medial and intermediate columns. (B) In *vnd* embryos, *Dichaete* expression is normal during stage 8. (C,D) In stage 8 in *Egfr* (C) or *vnd; Egfr* (D) embryos, *Dichaete* expression is strongly reduced in the intermediate column and moderately reduced in the medial column. (E) In stage 11 wild-type embryos, *Dichaete* is expressed in the medial and intermediate columns, and in one or more lateral column NBs. (F) In stage 11 *vnd* embryos, *Dichaete* is expressed in an irregular stripe two-to-four cells wide immediately adjacent to and on either side of the ventral midline (arrows). (G) In stage 11 *Egfr* embryos, *Dichaete* is expressed in an irregular stripe of cells zero to three cells wide immediately adjacent to and on either side of the midline (arrows). (H) In stage 11 *vnd; Egfr* embryos, *Dichaete* is not expressed in the neuroectoderm but is expressed in the ventral midline (arrowhead) and lateral NBs. Anterior is towards the left; white arrowheads (A-D) and lines (E-H) indicate ventral midline.

Based on these data, we expect *Dichaete* to associate with different transcription factors in the medial and intermediate columns to carry out its column-specific effects on target genes. The results in this paper identify Vnd and Ind as excellent candidates to be column-specific factors that associate with Dichaete and enable Dichaete to regulate transcription in a region specific manner. First, Dichaete is co-expressed with Vnd in the medial column and Ind in the intermediate column. Second, the neuroectodermal *Dichaete* mutant phenotype is similar to those of *vnd* and *ind*. Third, *Dichaete* functions in parallel to *vnd* and *ind* in the neuroectoderm. Fourth, *Dichaete* exhibits dose-sensitive interactions with *ind* and genetic interactions with *vnd* – consistent with these proteins interacting physically. Based on these data, we speculate that physical interactions between Dichaete and Vnd in the medial column and Dichaete and Ind in the intermediate column mediate the ability of distinct Dichaete protein complexes to bind to and to activate or to repress distinct target genes (Fig. 7). Validation of this model awaits the determination of whether Dichaete associates with Vnd or Ind, and how these proteins regulate target gene activity. However, recent results provide precedence for the model as genetic interactions between *Dichaete*, *single-minded* and *drifter* during midline development in the *Drosophila* CNS led to experiments that showed Dichaete physically associates with the Single-minded and Drifter proteins (Ma et al., 2000).

Do additional genes pattern the DV extent of the neuroectoderm?

Our results place *Dichaete* within the known genetic regulatory hierarchy that controls pattern and cell fate along the DV extent of the neuroectoderm (Fig. 7). In the future, we expect many additional genes to join this pathway. For example, the Sox-domain-containing gene *sox-neuro* is expressed throughout the entire neuroectoderm (Cremazy et al., 2000) and it may exhibit region-specific effects in the neuroectoderm in a manner similar to that proposed by us for *Dichaete*. In addition, the Ras-pathway antagonist *yan* is expressed in the lateral half of the neuroectoderm during early neurogenesis and may help regulate pattern and cell fate in this domain (G. Z. and J. B. S., unpublished). A complete understanding of the genetic and molecular mechanisms that pattern the neuroectoderm requires the identification of all such genes and the elucidation of how

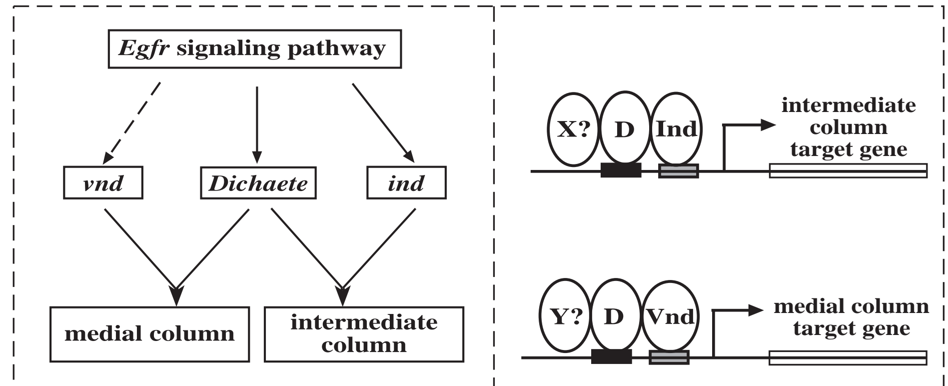

Fig. 7. The genetic regulatory hierarchy that regulates DV pattern in the neuroectoderm (left) and the molecular pathway through which *Dichaete, vnd* and *ind* might regulate target gene expression (right). Left: *Egfr* stands atop the genetic pathway that regulates DV pattern in the neuroectoderm. *Egfr* activates *ind* in the intermediate column and *Dichaete* in the medial and intermediate columns, while maintaining *vnd* expression in the medial column. *vnd* is activated independently of *Egfr* and plays a supporting role in regulating *Dichaete* expression. *Dichaete* activity appears to converge with that of *vnd* in the medial column and that of *ind* in the intermediate column to regulate DV pattern and cell fate. Right: a model that proposes that physical interactions between Dichaete and Vnd, as well as Dichaete and Ind, mediate the ability of Dichaete to carry out distinct function in different columns. Other proteins (X/Y) are likely required in these processes.

these genes interact to regulate cell fate along the DV axis of the neuroectoderm.

Phylogenetic conservation of DV patterning in the CNS

As first noted by D'Alessio and Frasch (D'Alessio and Frasch, 1996), there is a remarkable conservation of gene expression patterns along the DV axis of the *Drosophila* neuroectoderm and the vertebrate neural tube. Members of the vertebrate *vnd*/Nkx2.2 gene family are expressed and control cell fate within the ventral/medial domain of the neural tube (Pabst et al., 1998; Price et al., 1992). *Gsh1* and *Gsh2*, the vertebrate homologs of *ind*, are expressed in an intermediate position in the neural tube (Hsieh-Li et al., 1995; Valerius et al., 1995), while murine orthologs of *msh* are expressed in the most lateral region of the neural tube (Davidson, 1995).

The expression patterns of Sox-domain-containing genes in the *Drosophila* neuroectoderm and the vertebrate neural tube are also similar. For example, most vertebrate Sox genes are expressed throughout the entire neural plate (Wegner, 1999) similar to the expression of *sox-neuro* throughout the *Drosophila* neuroectoderm (Cremazy et al., 2000). In addition, chick Sox21 expression is expressed in the ventral half of the early vertebrate neural tube (Rex et al., 1997) – reminiscent of the *Dichaete* expression pattern. In the vertebrate neural tube, Sox domain proteins are likely to exhibit region specific effects via their interaction with transcription factors expressed in spatially restricted patterns. The proteins encoded by the *Nkx2.2*, *Gsh1*/*2* and Msx genes are excellent candidates to interact with Sox proteins in this context. Future research in both flies and vertebrates will identify the partners of Sox proteins and the precise molecular mechanisms through which Sox-protein-containing complexes regulate pattern and cell fate in developing nervous systems. As this research progresses, it will be exciting to see the extent of conservation as well as divergence between the molecular logic employed by Sox proteins and their cohorts in *Drosophila* and vertebrates to regulate neural development.

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