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Original Article

Genetic control of dorsoventral patterning and neuroblast specification in the *Drosophila* Central Nervous System

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ABSTRACT The *Drosophila* embryonic Central Nervous System (CNS) develops from the ventrolateral region of the embryo, the neuroectoderm. Neuroblasts arise from the neuroectoderm and acquire unique fates based on the positions in which they are formed. Previous work has identified six genes that pattern the dorsoventral axis of the neuroectoderm: *Drosophila epidermal growth factor receptor (Egfr), ventral nerve cord defective (vnd), intermediate neuroblast defective (ind), muscle segment homeobox (msh), Dichaete* and *Sox-Neuro (SoxN)*. The activities of these genes partition the early neuroectoderm into three parallel longitudinal columns (medial, intermediate, lateral) from which three distinct columns of neural stem cells arise. Most of our knowledge of the regulatory relationships among these genes derives from classical loss of function analyses. To gain a more in depth understanding of *Egfr*-mediated regulation of *vnd, ind* and *msh* and investigate potential cross-regulatory interactions among these genes, we combined loss of function with ectopic activation of Egfr activity. We observe that ubiquitous activation of Egfr expands the expression of *vnd* and *ind* into the lateral column and reduces that of *msh* in the lateral column. Through this work, we identified the genetic criteria required for the development of the medial and intermediate column cell fates. We also show that *ind* appears to repress *vnd*, adding an additional layer of complexity to the genetic regulatory hierarchy that patterns the dorsoventral axis of the CNS. Finally, we demonstrate that *Egfr* and the genes of the achaete-scute complex act in parallel to regulate the individual fate of neural stem cells.

KEY WORDS: *Egfr, vnd, ind, CNS, dorsoventral patterning*

Introduction

The ability of cells to acquire specific and often unique fates as a function of their position in a developing field is a fundamental process in animal development. The genetic regulatory mechanisms that govern position-dependent cell-fate specification have been studied extensively in the developing vertebrate and *Drosophila* CNS. These studies reveal key parallels between the molecules that pattern the DV axis of the CNS in *Drosophila* and vertebrates. For example, the *vnd* homeodomain-containing gene and its vertebrate homolog *Nkx2.2* regulate cell fate in the medial domains of the *Drosophila* and vertebrate neuroepithelia, respectively (Briscoe *et al.*, 1999, Chu *et al.*, 1998, McDonald *et al.*, 1998). *ind,* another homeodomain gene and its vertebrate homolog *Gsh* appear to control cell fate in the intermediate domain (Valerius *et al.*, 1995, Weiss *et al.*, 1998) while the *msh* and *Msx* homeodomain-containing genes are expressed and control the development of cells in the lateral domain of the *Drosophila* and vertebrate neuroepithelium (Davidson, 1995, Isshiki *et al.*, 1997).

The *Drosophila* CNS is an ideal model system in which to dissect the genetic and molecular mechanisms that pattern and regulate cell-fate specification in the CNS (reviewed in Skeath and Thor, 2003). The *Drosophila* CNS develops from a set of

Abbreviations used in this paper: Ac, achaete; CNS, Central Nervous System; EGF, epidermal growth factor; Egfr, drosophila EGF receptor; Egfr*, embryos that have the Egfr signaling pathway activated throughout the early embryo by using maternal Gal4 driver lines and UAS-s-spi. Eve, even-skipped; Ftz, fushi-tarazu; ind, intermediate neuroblast defective; l'sc, lethal of scute; msh, muscle segment homeobox; NB, neuroblast; Odd, odd-skipped; Pros, prospero; sc, scute; SoxN, Sox-Neuro; vnd, ventral nerve cord defective.

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neural stem cells, called neuroblasts (NBs), that arise from the ventrolateral ectoderm and divide to produce the neurons and glia that populate the CNS. Within each hemisegment of the *Drosophila* CNS every NB acquires a unique fate based on its position and time of formation. A set of six genes collectively referred to here as the "columnar genes" patterns the CNS along the DV axis

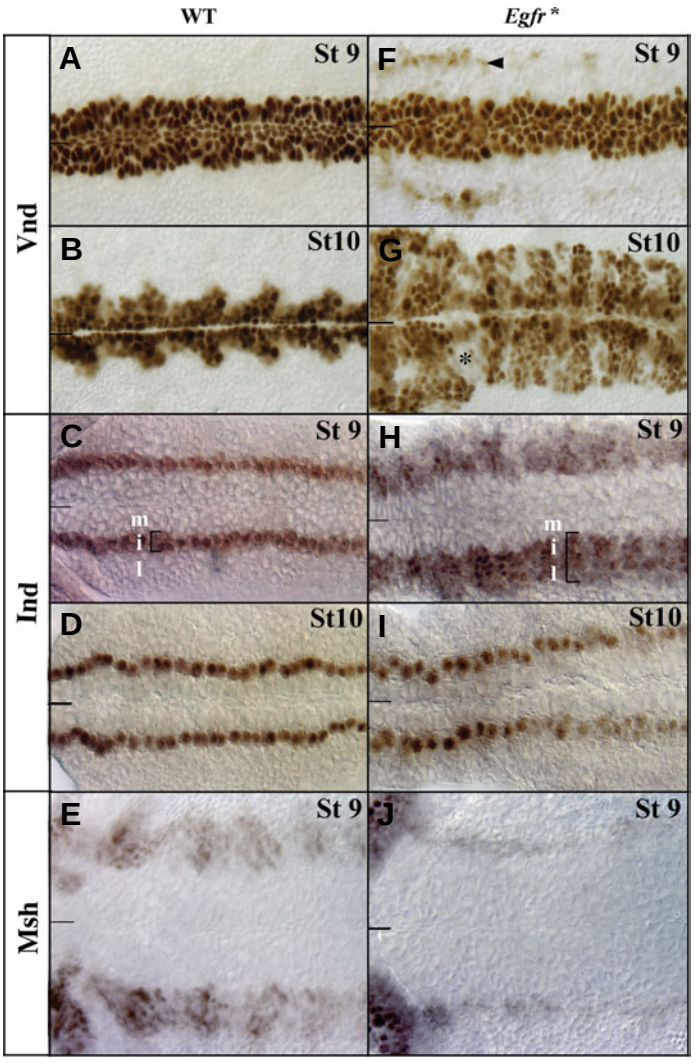

Fig. 1 *Egfr activates the expression of *vnd* and *ind,* but represses *msh* expression.** *High magnification ventral views of the neuroectoderm in wild-type* **(A-E)** *and Egfr** **(F-J)** *embryos labeled for Vnd (A,B,F,G), Ind (C,D,H,I) and Msh (E,J) protein expression. In wild-type embryos,* **(A)** *vnd is expressed in the medial column of the neuroectoderm at stage 9 and* **(B)** *in the neuroectoderm and certain neuroblasts at stage 10. In Egfr* embryos,* **(F)** *ectopic vnd expression first appears in the lateral column during stage 9 (arrowhead) and* **(G)** *is expressed throughout the neuroectoderm by late stage 10, although some intermediate and lateral column neuroectoderm cells do not express vnd (asterisk). In wild-type embryos, ind is expressed in the intermediate column neuroectoderm during stage 9* **(C)** *and neuroblasts during stage 10* **(D).** *In Egfr* embryos, ind expression expands into the lateral column before stage 9* **(H)** *but is restricted to only intermediate column neuroblasts by stage 10* **(I)**. *msh is expressed in the lateral column in wild-type embryos* **(E)** *but is greatly reduced in Egfr* embryos* **(J)**. *Anterior, left; line, ventral midline. m, i, l, indicate positions of medial, intermediate and lateral columns, respectively.*

and functions to enable NBs that develop in different DV positions to acquire position-specific fates (reviewed in Skeath, 1999, Skeath and Thor, 2003). The columnar genes include the *Drosophila EGF receptor* (*Egfr)*, three homeodomain transcription factors - *vnd*, *ind* and *msh* – and *Dichaete* and *Sox Neuro* two high mobility group (HMG) domain transcription factors. These genes interact in a complex genetic hierarchy to partition the DV axis of the CNS into three discrete longitudinal columns: medial, intermediate and lateral.

vnd, *ind* and *msh* were the first genes identified that pattern the CNS along the DV axis. *vnd*, *ind* and *msh* are expressed in the medial, intermediate and lateral columns respectively. *vnd* and *ind* control NB formation and specification within their expression domains (Chu *et al.*, 1998, McDonald *et al.*, 1998, Weiss *et al.*, 1998) while *msh* regulates the differentiation of lateral column NBs. Molecular epistasis tests indicate that *vnd* represses *ind* and that *ind* represses *msh* (McDonald *et al.*, 1998, Weiss *et al.*, 1998). In this genetic hierarchy the more ventrally expressed gene establishes via repression the ventral limit of the gene expressed immediately dorsal to it.

Recently the *Dichaete* and *SoxN* transcription factors have been shown to act in parallel to *vnd* and *ind* to pattern the DV axis of the CNS (Buescher *et al.*, 2002, Cremazy *et al.*, 2000, Overton *et al.*, 2002, Zhao and Skeath, 2002). *Dichaete* is expressed in the medial and intermediate columns while *SoxN* is expressed uniformly throughout the CNS. Genetics studies indicate that *Dichaete* and *SoxN* act in a partially redundant manner to regulate NB formation and fate within each column of the CNS (Buescher *et al.*, 2002, Overton *et al.*, 2002).

Egfr stands atop the genetic regulatory hierarchy that patterns the CNS along the DV axis. *spitz* and *vein* each encode ligands that activate *Egfr* independently of one another (Golembo *et al.*, 1996, Rutledge *et al.*, 1992, Schnepp *et al.*, 1996, Schweitzer *et al.*, 1995). The Spitz precursor is expressed broadly (Rutledge *et al.*, 1992) in its inactive form. *rhomboid* encodes a membrane-spanning protein (Bier *et al.*, 1990) that appears to mediate *Egfr* signaling by helping to process Spitz from its inactive transmembrane form to its active secreted form (Golembo *et al.*, 1996). Localized transcription of *rhomboid* in the ventral domains of the neuroectoderm lead to localized *Egfr* activation (Bier *et al.*, 1990, Gabay *et al.*, 1996, Golembo *et al.*, 1996, Schnepp *et al.*, 1996) in the medial and intermediate columns prior to NB formation (Skeath, 1998) where it positively regulates *vnd*, *ind* and *Dichaete* expression and represses *msh* expression (Gabay *et al.*, 1996, Zhao and Skeath, 2002). *Egfr* promotes the formation of intermediate column NBs by activating *ind* expression (Skeath, 1998, von Ohlen and Doe, 2000) and of late forming medial column NBs likely by maintaining *vnd* expression in the medial column (Zhao and Skeath, 2002). The ability of *Egfr* to activate *ind* also accounts for *Egfr*'s ability to restrict *msh* expression to the lateral column.

In addition to its role in NB formation, *Egfr* also helps specify the fate of early forming medial NBs. In the absence of *Egfr* function, medial NBs arise normally but acquire inappropriate fates about 50% of the time (Skeath, 1998). This activity of *Egfr* appears independent of *vnd* and *achaete/scute* gene expression as expression of these genes in medial NBs is wild-type in *Egfr* mutant embryos. Of note, this mis-specification phenotype of medial NBs is essentially identical to that caused by the

misexpression of the *achaete-scute* complex gene *lethal of scute* (*l'sc*) in the place of *achaete* (*ac*) and *scute* (*sc*) within the medial NB MP2 (Parras *et al.*, 1996, Skeath and Doe, 1996). Although the genetic relationship between *Egfr* and the *ac/sc* genes remains unresolved, the near identity of the phenotypes suggests *Egfr* and the *ac/sc* genes either act in parallel or sequentially in the same pathway to specify medial column NBs.

The aforementioned work provides a solid framework of the genetic hierarchy through which the columnar genes pattern and regulate cell fate along the DV axis of the CNS. Despite this foundation, other studies hint at additional layers of complexity within this hierarchy. For example, the precise genetic relationship between *Egfr* and *vnd* as well as *ind* appears unclear. von Ohlen and Doe (von Ohlen and Doe, 2000) showed that ubiquitous activation of *Egfr* is sufficient to activate *ind* but not *vnd* in the lateral column. In contrast, Gabay *et al.* (Gabay *et al.*, 1996) observed that ubiquitous *Egfr* activity is sufficient to activate *vnd* expression in the lateral column. These results suggest our understanding of the genetic control of DV patterning in the CNS is incomplete.

In this study, we combine ectopic activation of *Egfr* activity with loss of function genetics to dissect the genetic hierarchy of DV patterning in the CNS. Through this work we establish that the genetic requirements for the medial column fate are the presence of *Egfr* activity, *vnd* expression and the absence of *ind* expression while those for the intermediate column fate are the initial presence of *Egfr* activity, *ind* expression and the absence of *vnd* expression. We also find that *ind* can and does repress *vnd* expression in the developing CNS. Finally, we demonstrate that *Egfr* and the *achaete-scute* genes act in parallel to control the fate of individual medial NBs.

Results

Ubiquitous Egfr activity changes the dorsoventral subdivision of the neuroectoderm

To gain a more in depth understanding of *Egfr*-mediated regulation of *vnd, ind* and *msh*, we assayed the effect of ubiquitous *Egfr* activity on *vnd*, *ind* and *msh* expression in the neuroectoderm and on NB fate. We activated the *Egfr* signaling pathway throughout the early embryo by using maternal Gal4 driver lines and UAS-s-*spi*. We refer to these embryos as *Egfr** embryos. Ubiquitous expression of the active diphosphorylated-form of MAP kinase, a marker of *Egfr* activity (Gabay *et al.*, 1996), confirmed ubiquitous activation of the *Egfr* pathway in these embryos (data not shown).

We observe that ubiquitous activation of *Egfr* expands the expression of *vnd* and *ind* into the lateral column and reduces that of *msh* in the lateral column (Fig. 1). The *vnd* and *ind* expression profiles in the lateral column exhibit significant temporal and spatial differences. In *Egfr** embryos, *ind* expression expands into the lateral column prior to stage 9 (Fig. 1H), but is restricted to intermediate column NBs in a pattern that closely resembles wild-type by stage 10 (compare Figs. 1D, I). In contrast, *vnd* expression comes on in the lateral column towards the end of stage 9 (Fig. 1F). At this stage the intermediate column is devoid of *vnd* expression. However, by stage 10 we detect *vnd* expression throughout the neuroectoderm, although a few intermediate column cells still lack *vnd* expression

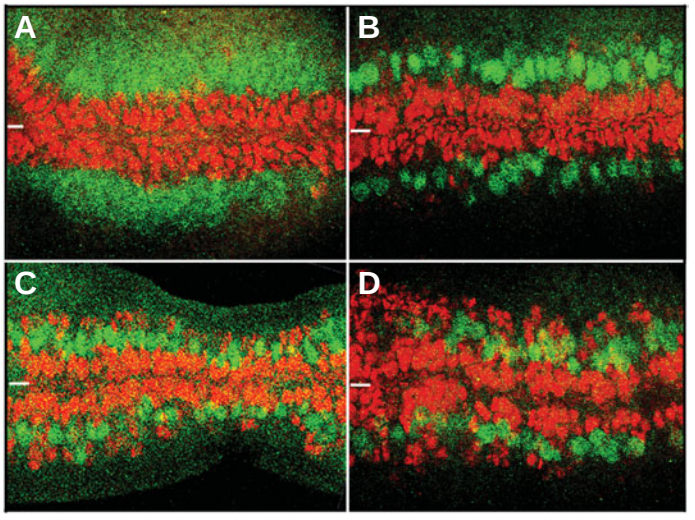

Fig. 2. Mutually-exclusive expression of *vnd* (red) *and ind* (green) in *Egfr embryos.** *Ventral views of Egfr* embryos labeled for vnd and ind.* **(A)** *At early stage 9, vnd is expressed in the medial column as per wild-type embryos, while ind expression expands into the lateral column.* **(B)** *At late stage 9, ind expression is restricted to intermediate column neuroblasts, while vnd expression is still restricted to the medial column.* **(C)** *At early stage 10, vnd expression expands into the lateral column and ind is expressed only in intermediate column neuroblasts.* **(D)** *During late stage 10, vnd is expressed throughout the neuroectoderm, while Ind remains restricted to intermediate column neuroblasts. There is very limited co-expression of vnd and ind throughout the CNS at all developmental stages. Anterior, left; line, ventral midline.*

(Fig. 1G, asterisk). Thus, *Egfr* activity is sufficient to activate both *vnd* and *ind* in the lateral column although to different extents and at different times. The inability of ubiquitous *Egfr* activity to activate *vnd* or *ind* outside the CNS suggests the presence of factors, such as *dpp*, that actively suppress, or the absence of factors that are necessary for *Egfr*-mediated activation of, *vnd* and *ind* expression in this domain.

*Egfr**-mediated activation of *vnd* and *ind* in the lateral column was unexpected in light of previous experiments that demonstrated that *vnd* represses *ind* (McDonald *et al.*, 1998) and that ubiquitous *Egfr* activity leads to the expansion of *ind*, but not *vnd*, in the lateral column (von Ohlen and Doe, 2000). Our double label immunofluorescence studies show that *vnd* and *ind* exhibit mutually exclusive expression patterns in the CNS of *Egfr** embryos at all developmental stages (Fig. 2). Thus, although *vnd* and *ind* are expressed in the same DV domains in these embryos, on a cell-by-cell basis their expression patterns remain mutually exclusive consistent with the known ability of *vnd* to repress *ind*.

Different columnar gene expression in the neuroectoderm leads to different neuroblast fates

The sequential expression of *ind* and *vnd* in the lateral column of *Egfr** embryos prior to and during the first wave (SI) of NB formation led us to investigate the formation and fate of these NBs. We first assayed NB formation and observed a significant decrease in the formation of each lateral column SI NB, as NB 2-5, NB 3-5, NB 5-6 and NB 7-4 are present in 20.7%, 36.7%, 20.2% and 3.7% of hemisegments, respectively (n =

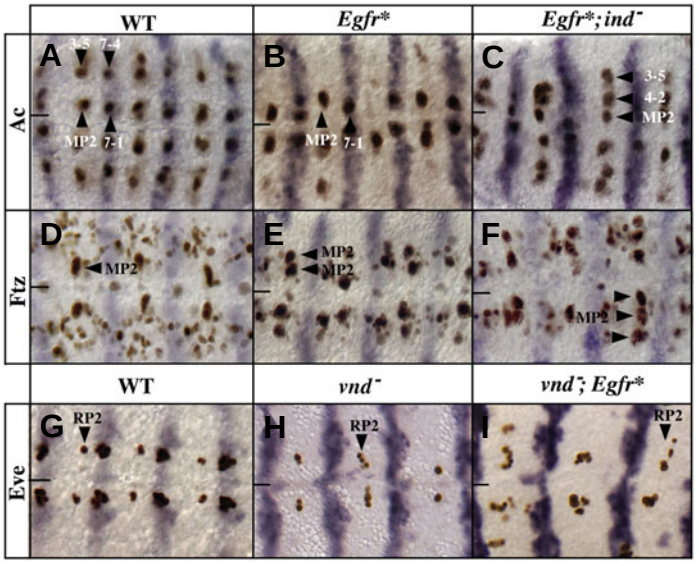

Fig. 3 (Left). Neuroblast specification in wild-type, *Egfr*, Egfr*; ind-, vnd-* and *vnd-; Egfr embryos.** *High magnification views of the ventral neuroectoderm of wild-type* **(A, D, G)**, *Egfr** **(B, E)**, *Egfr*; ind-* **(C, F)**, *vnd-* **(H)** *and vnd-; Egfr** **(I)** *embryos. At stage 9,* **(A)** *in wild-type embryos, lateral NB 3-5 and 7-4 normally express achaete (Ac) protein.* **(B)** *With ectopic Egfr activity, most lateral column neuroblasts lose Ac expression.* **(C)** *In Egfr*; ind- embryos, not only lateral neuroblast 3-5 recovers Ac expression, but also intermediate column neuroblast 4-2 expresses Ac ectopically. At stage 11,* **(D)** *only neural precursor MP2 expresses Ftz in wild-type embryos.* **(E)** *In Egfr* embryos, MP2 is duplicated.* **(F)** *In Egfr*; ind- embryos, MP2 is triplicated.* **(G)** *In wild-type embryos, RP2, the progeny of intermediate column neuroblast 4-2 expresses Eve.* **(H)** *In vnd- embryos, RP2 is duplicated.* **(I)** *In vnd-; Egfr* embryos, RP2 is triplicated. Anterior, left; line, ventral midline. Blue stripe: engrailed protein expression which labels the posterior boundary of each segment.*

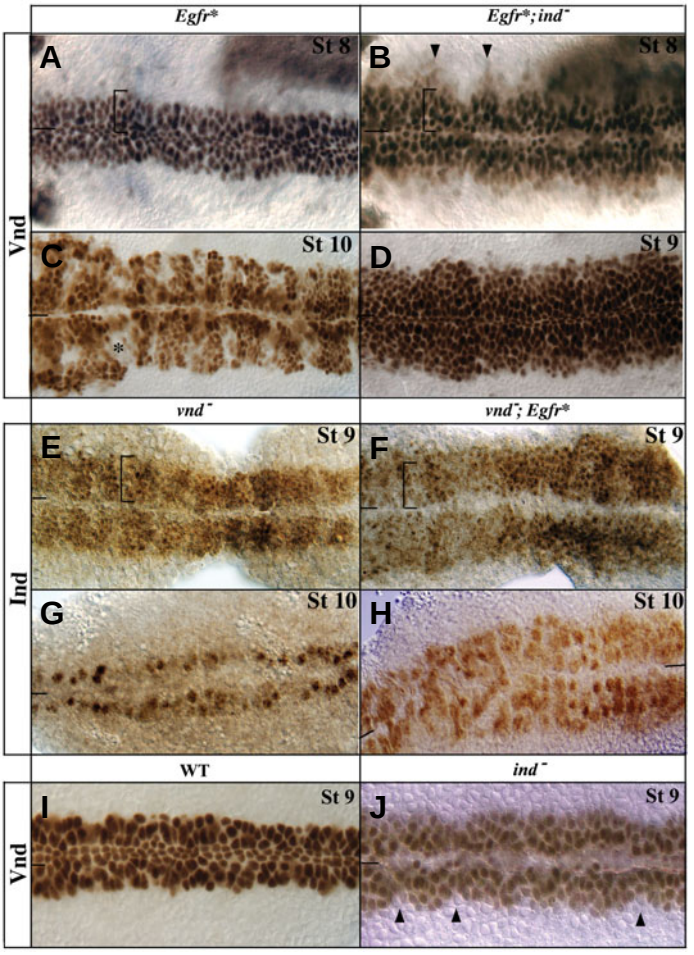

Fig. 4 (Right). Vnd expression in *Egfr*, Egfr*; ind-* embryos (A-D), *ind* expression in *vnd-, vnd-; Egfr embryos (E-H) and *ind* represses *vnd* expression (I - J).** *Ventral views of stage 8* **(A)** *Egfr*,* **(B)** *Egfr*; ind- embryos and high magnification ventral views of the CNS in* **(C)** *Egfr* and* **(D)** *Egfr*; ind- embryos labeled for Vnd protein.* **(A)** *At stage 8, vnd is expressed in the medial column in Egfr* embryos as per wild-type embryos.* **(B)** *In Egfr*; ind- embryos, ectopic vnd appears in the intermediate column from stage 8.* **(C)** *In Egfr* embryos, vnd is expressed in most but not all cells in the neuroectoderm at stage 10.* **(D)** *In contrast, vnd is expressed uniformly throughout Egfr*; ind- embryos even at an earlier stage (stage 9).* **(E-H)** *High magnification views of the ventral neuroectoderm of vnd* **(E, G)** *and vnd-; Egfr** **(F, H)** *mutant embryos labeled for Ind protein.* **(E)** *In vnd mutant embryos, ind is expressed in the medial and intermediate columns at stage 9 and* **(G)** *ind is restricted into one row of neuroblasts at stage 10.* **(F)** *In vnd-; Egfr* embryos, ind is expressed throughout the CNS at stage 9 and* **(H)** *is expressed in neuroblasts throughout the CNS at stage 10. Anterior, left; line, ventral midline.* **(I-J)** *High magnification views of the ventral neuroectoderm of wild-type* **(I)** *and ind- embryos* **(J)** *at stage 9 labeled with Vnd protein. Notice the irregular dorsal border of Vnd expression in ind- embryos compared with the sharp border of Vnd expression in wild-type embryos.*

430 hemisegments scored for each NB). Next, we assayed the fate of these NBs at two different times – as they formed and either immediately prior to or just after their first division. At early stages many lateral column NBs display traits consistent with an intermediate column fate, while at later stages these NBs display medial column fates. For example, in wild-type embryos the medial and lateral column SI NBs of row 3 and 7 (MP2, 3-5, 7-1, 7-4) express Achaete (Ac, Fig. 3A) while those of row 1 (1-1, 2-5) express Odd-skipped (Odd, Table 1). However, in *Egfr** embryos, lateral NBs in rows 3 and 7 either do not express Achaete or express it at very low levels (Fig. 3B) while the lateral NB of row 1 does not express Odd (Table 1). Thus, in *Egfr* embryos a significant fraction of lateral NBs that form fail to display traits indicative of lateral NBs but rather initially appear most similar to intermediate column NBs, consistent with the early expression of *ind* in the lateral column of *Egfr** embryos. The NBs that do exhibit Ac and Odd expression likely acquire their normal lateral column NB identity because at that time *vnd* has not initiated expression in the lateral column.

At later stages most lateral NBs express markers consistent with a medial fate. For example, by stage 11 MP2, the row 3 medial column NB can be uniquely identified by the expression of Odd, Fushi-tarazu (Ftz) and nuclear Prospero (Pros) protein and the first-born progeny of NB 4-2, the row 3 intermediate column NB, can be identified by Eve expression. Using these markers, we observe that more than 20% of hemisegments contain an ectopic MP2 located more laterally in row 3 (Fig. 3E, Table 1) relative to less than 2% for NB 4-2 (Table 1). As we only observe a lateral column NB in 36.7% of hemisgements, this suggests that ~60% of all lateral column NBs in this position

acquire a medial fate. Thus, most lateral column NBs in this position acquire a medial NB fate. Molecular markers for other medial column NBs support the model that most lateral column NBs acquire a medial NB identity (Table 1). In addition, these results also indicate plasticity in the competence of lateral column NBs to acquire individual fates in *Egfr** embryos as they initially display traits consistent with an intermediate fate (majority) and lateral fate (minority) but in the end most such NBs appear to acquire medial *vnd*-dependent fates. This suggests the activation of *vnd* expression in the lateral domain predominates over the initial expression of *ind* in this domain with respect to NB specification and also highlights at least a limited competency of NBs to respond to changing cues by altering their identities.

ind inhibits vnd expression

Although *ind* has not been shown to repress *vnd* the spatiotemporal dynamics of *vnd* and *ind* expression in *Egfr** embryos raised the possibility that *ind* represses *vnd* expression in the CNS of *Egfr** embryos. If this occurs, we would expect removal of *ind* function in an otherwise *Egfr** background to allow earlier and more uniform expression of *vnd* in the CNS. In support of this, we detect *vnd* expression first in the intermediate column instead of lateral column in the *Egfr* ind*⁻ embryos by stage 8 (Fig. 4B) and uniform *vnd* expression in all neuroectodermal cells by mid stage 9 (Fig. 4D). The earlier ectopic activation as well as uniform expression of *vnd* in the *Egfr* ind*⁻ embryos indicate that *ind* represses *vnd* expression in the intermediate and lateral columns in *Egfr** embryos.

Although *ind* has not been reported to regulate *vnd* expression, our demonstration that *ind* represses *vnd* under conditions of uniform *Egfr* activity led us to ask if *ind* normally helps establish the sharp lateral limit of *vnd* expression in wild-type embryos. In stage 9 embryos *vnd* is expressed in two-bilaterally symmetric columns of neuroectodermal cells that flank the ventral midline. These rows are two-three cells wide and exhibit a sharp lateral boundary (Fig. 4I). In *ind*⁻ embryos the two columns of *vnd*⁺ positive cells are present however they display a jagged lateral boundary and their width varies from two-to-five cells (Fig. 4J). The erratic and expanded lateral boundary of *vnd* expression reveals that *ind* helps establish the precise lateral boundary of *vnd* expression. Although this is the first demonstration of cross-repressive interactions between the genes that pattern the DV axis of the *Drosophila* CNS such interactions are a common theme in the DV patterning of the vertebrate CNS.

Egfr ind*⁻ embryos exhibit a significant reduction of NB formation in intermediate and lateral columns (Table 1); this phenotype is similar to that observed for *ind* mutant embryos for the intermediate column. Consistent with the transformation of the entire CNS to the medial column, NBs in all three columns acquire the medial column fate. In fact, relative to *Egfr** embryos, *Egfr* ind*⁻ embryos exhibit a two-to-seventeen fold increase of lateral column NBs acquiring a medial fate (Table 1), indicating a much stronger medial transformation of the neruoectoderm. For example, NBs in all three columns express Ac in row 3 and 7 (Fig. 3C) and various molecular markers reveal a triplication of the medial column NBs 1-1, MP2 and 7-1 (Fig. 3F, Table 1). NBs of intermediate or lateral fates are rarely if ever observed (data not shown). The significant reduction in NB formation in the intermediate and lateral columns likely accounts for the low frequency at which we observe triplication of medial NBs within one AP row (Table 1). Together these results establish the genetic requirements sufficient for medial column fate in the CNS as ubiquitous *Egfr* activity in the absence of *ind* function.

Egfr can promote ind expression throughout the neuroectoderm in the absence of vnd function

As *vnd* is known to repress *ind*, the transient expression of *ind* in the lateral column of *Egfr** embryos may arise as a result of *vnd*-mediated repression. To test this we assayed *ind* expression in *vnd*⁻; Egfr** embryos and found these embryos express *ind* throughout the entire neuroectoderm between stages 7 and 9 (Fig. 4F). Thus, loss of *vnd* function together with ubiquitous activation of *Egfr* appears sufficient to re-specify the entire neuroectoderm towards the intermediate column fate. Subsequent to stage 9 in *vnd*⁺; Egfr** embryos, *ind* expression

TABLE 1

NEUROBLAST SPECIFICATION IN *Egfr AND *Egfr* ind*⁻ EMBRYOS**

Genotype	NB 3-5		NB 7-4		NB 2-5	
	% Ac	n	%Ac	n	%Odd	n
WT	100	144	100	144	100	152
*Egfr**	22.1	446	0.9	348	1.8	448

Genotype	GMC 1-1 duplication		GMC 7-1 duplication		MP2 duplication						RP2 duplication	
	% Eve	n	%Eve	n	% Odd	n	% Ftz	n	% Pros	n	%Eve	n
*Egfr**	5.0	426	0.4	426	21.1	342	20.7	488	24.0	384	1.8	446
Egfr ind*⁻	19.0 (d) 3.9 (t) 22.1 (total)	558	7.3 (d) 1.9 (t) 9.2 (total)	558	ND		48.5 (d) 7.6 (t) 56.1 (total)	542	56.0 (d) 9.1 (t) 65.1 (total)	496	ND	

Upper panel shows the percentage of lateral column NBs that exhibit their normal traits. Lower panel shows the percentage of lateral column NBs that acquire medial or intermediate column fates. Percentage indicates the percent formation of an Ac-positive, Odd-positive, Eve-positive, Ftz-positive or Pros-positive neuron or neuroblast. Eve is expressed in the first-born progeny (GMC 1-1 and GMC 7-1) of NB 1-1 and 7-1, the row 1 and row 7 medial column NB. n, number of hemisegments scored; ND, not determined; d, duplication; t, triplication

remains in all NBs throughout the CNS but disappears from the neuroectoderm (Fig. 4H). Therefore, additional timing mechanisms must be involved in down-regulation of *ind* expression as despite the presence of uniform *Egfr* activity *ind* expression is still extinguished in the neuroectoderm by stage 10.

Uniform *ind* expression throughout the neuroectoderm in the *vnd*$^{+}$; *Egfr** embryos suggests that the entire CNS acquires intermediate fate. Consistent with this, we observe triplication of GMC 4-2, the first progeny of intermediate column NB 4-2 in row 3 (Fig. 3I), suggesting that all row 3 NBs acquire intermediate column NB 4-2 fate. In addition, we have not observed expression of markers specific for NBs with medial or lateral fates (data not shown). These data suggest a complete transformation of the entire CNS to the intermediate fate and establish that the presence of ubiquitous Egfr activity and the absence of *vnd* are sufficient to promote the intermediate fate throughout the entire CNS.

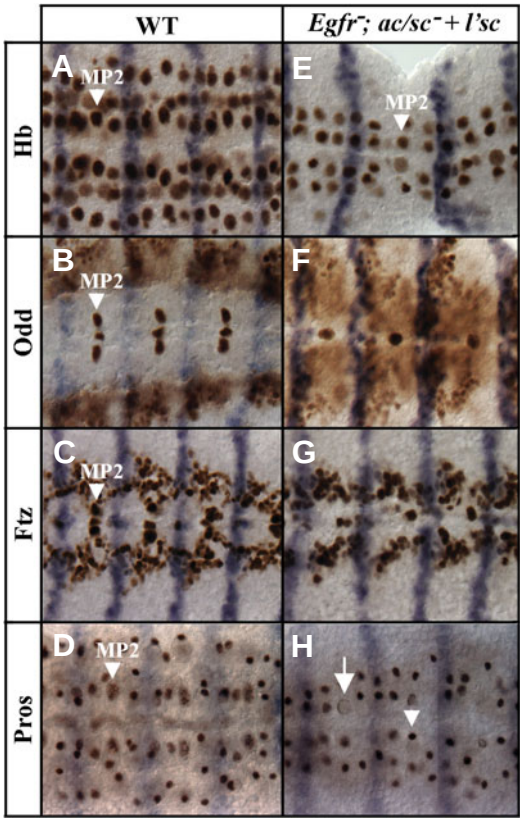

Fig. 5 MP2 formation and specification in wild-type embryos and in embryos that are mutant for *Egfr* and have *l'sc* expression replaces *ac/sc* expression in MP2 *(Egfr*$^{-}$*, ac/sc*$^{-}$*+l'sc).* *High magnification ventral views of the developing CNS of* **(A-D)** *wild-type and* **(E-H)** *Egfr*$^{-}$*, ac/sc*$^{-}$*+l'sc embryos labeled for Hunchback* **(A, E)**, *Odd* **(B, F),** *Ftz* **(C, G)** *and Pros* **(D, H)** *proteins. Hunchback protein labels all the neuroblasts.* **(A)** *In wild type embryos, three neuroblast columns form. MP2 is right in the middle between two Engrailed stripes in the most medial column (arrowhead). MP2 always expresses Odd* **(B)**, *Ftz* **(C)** *and localizes Pros protein to the nucleus* **(D)**. **(E)** *In Egfr*$^{-}$*, ac/sc*$^{-}$*+l'sc embryos, two neuroblast columns develop because of Egfr mutation. MP2 (arrowhead) forms 92.5% of the time. However, MP2 seldom expresses Odd* **(F)**, *Ftz* **(G)** *or localizes Pros in the nucleus. Instead, it localizes Pros protein either to the nucleus of GMC (arrowhead)* **(H)** *or to the cell cortex of GMC (arrow)* **(H)**, *or neuroblast. Anterior, left; line, ventral midline. Blue stripe: engrailed protein expression which labels the posterior boundary of each segment.*

Egfr activity and the achaete/scute *genes converge to specify medial column neuroblast fate*

Previous studies indicate that *Egfr* activity and the activity of *achaete/scute* genes help specify the fate of medial column NBs (Parras *et al.*, 1996, Skeath, 1998, Skeath and Doe, 1996, Udolph *et al.*, 1998). The roles of the *Egfr* and *ac/sc* genes in medial column NB specification have been best studied for MP2. MP2 arises from a cluster of *ac* and *sc* expressing cells and is unique in that it is the only NB that localizes Pros protein to the nucleus (all other NBs localize Pros to the cell cortex) and also expresses Odd and Ftz. Loss of *Egfr* activity has little to no effect on NB formation in the MP2 position but only half of these NBs display the defining characteristics of MP2. All other NBs localize Pros to the cell cortex and fail to express Odd and Ftz – traits consistent with a NB fate distinct from MP2 (Parras *et al.*, 1996, Skeath, 1998, Skeath and Doe, 1996, Udolph *et al.*, 1998). Replacing *ac* and *sc* gene expression with that of *l'sc* leads to an essentially identical phenotype with respect to NB formation and specification in the MP2 position (Skeath and Doe, 1996; Parras *et al.,* 1996).

The similarity of the phenotypes suggests the *Egfr* and *ac/sc* genes may act in the same pathway, or alternatively in parallel to control MP2 fate and more general medial column NB fate. To distinguish these models we created *Egfr* mutant embryos that express *l'sc* in the place of *ac* and *sc* in MP2 (see Materials and Methods). Consistent with the idea that *Egfr* and the *ac/sc* genes act in parallel we observed that a NB almost always forms in the MP2 position (92.5%; n=482) in this background but that this NB almost never displays traits indicative of MP2 (Fig. 5). For example, NBs in this position rarely express Odd (3%, n=506), Ftz (1.6%, n=494) or nuclear Pros (0.8%, n=370) (Fig. 5). Rather, these NBs localize Pros to the cell cortex (arrow, Fig. 5H) or to the nucleus of a GMC (arrowhead, Fig. 5H) consistent with acquiring traits characteristic of other neuroblasts. As a control, we verified that misexpression of *l'sc* in the place of *ac* and *sc* in the MP2 position essentially always promotes the formation of a NB in the MP2 position but that this NB acquires the MP2 fate only approximately half of the time (Skeath and Doe, 1996; data not shown). Taken together these results indicate that the *Egfr* signaling pathway and the proneural *ac/sc* genes act in parallel to control MP2 fate.

Discussion

The genetic control of dorsoventral patterning and cell fate in the Drosophila *CNS*

The Dorsal gradient initiates patterning of the CNS via the transcriptional regulation of the expression *vnd*, *rhomboid* and *zen* (Stathopoulos and Levine, 2002). Dorsal-mediated activation of *rhomboid*, the rate-limiting factor in Egfr-signaling and *vnd* establishes the initial expression domains of two of the earliest positive activators of CNS patterning along the DV axis. Similarly, Dorsal-mediated repression in the ventral and ventrolateral ectoderm limits the expression of *zen* and *decapentaplegic (dpp)* to the dorsal ectoderm (Rusch and Levine,

1996). Dpp functions as a morphogen and defines via a repressive mechanism the lateral limit of the developing CNS (Skeath *et al.*, 1992).

Within the CNS, *vnd* and *rhomboid* exhibit differential sensitivity to the dorsal gradient with *vnd* being activated solely within the medial column and *rhomboid* in both the intermediate and medial columns (Bier *et al.*, 1990, Mellerick and Nirenberg, 1995). As *rhomboid* is the limiting factor in Egfr signaling, its presence activates *Egfr*-signaling activity in the medial and intermediate columns (Golembo *et al.*, 1996, Urban *et al.*, 2001). In wild-type embryos, *Egfr* activity maintains *vnd* expression in the medial column and is necessary to promote *ind* expression in the intermediate column (Gabay *et al.*, 1996, von Ohlen and Doe, 2000). The ability of *vnd* to repress *ind* expression explains the restriction of *ind* expression to the intermediate column. *vnd* expression persists throughout most of the medial column until the end of embryogenesis; in contrast, *ind* expression is extinguished in the intermediate column neuroectoderm by stage 10 after the first two (of five) waves of NB segregation.

Our work adds a new regulatory relationship into the genetic regulation of CNS patterning as we find that *ind* helps establish the lateral limit of *vnd* expression. *ind* could perform this function via the direct repression of *vnd*, a possibility supported by our gain-of-function and loss-of-function experiments. If this model is correct, the mutual repression of *vnd* and *ind* would bear striking similarity to the reciprocal repressive interactions observed for the class I and class II homeodomain proteins that pattern the DV axis of the vertebrate CNS (Briscoe *et al.*, 2000). In this context, it is important to note that the vertebrate ortholog of *vnd*, Nkx2.2, is a class II protein that plays a key role in patterning some of the ventral-most regions of the vertebrate CNS. Alternatively or additionally, *vnd* and *ind* could establish their mutual sharp boundary indirectly via the regulation of other factors. For example, differential regulation of homophilic cell-adhesion molecules could account for the observed phenotype. Differential expression of cell-adhesion molecules on medial versus intermediate column cells would cause these cells to associate preferentially with cells from the same column and result in a sharp boundary between the two cell populations that minimized interaction. Loss of such differences would reduce the requirement to minimize interactions and likely result in a jagged boundary. Additional work is necessary to identify the precise mechanism through which *ind* helps establish the lateral limit of *vnd* expression. Previous work has shown that misexpression of *ind* along the anterior–posterior axis using the Krüppel enhancer failed to repress *vnd* expression in the medial column (Cowden and Levine, 2003). However, this is not contradictory to our finding. Our work suggests that *ind* can repress *vnd* in the intermediate and lateral columns but not in the medial columns. It is likely that some factors that are present in the intermediate and lateral columns but are absent in the medial column help *ind* to repress *vnd*.

In addition, our work, for the first time, demonstrates that *Egfr* and *vnd* are sufficient to confer medial fate and that *Egfr* and *ind* are sufficient to confer intermediate fate. Although loss-of-function studies have shown that both *Egfr* and *vnd* are necessary for NBs to acquire medial fate, it is not clear whether *Egfr* functions solely through *vnd*. McDonald *et al.* (McDonald *et al.*, 1998) have shown that ectopic *vnd* expression results in partial transformation of lateral column into medial column. Our work shows that ectopic *Egfr* activity can induce the expression of *vnd* and together *Egfr* and *vnd* fully transform the lateral column into the medial column. Therefore, *Egfr* likely plays additional roles in determining medial cell fate other than maintaining *vnd* expression in the neuroectoderm. However, it remains unclear whether *Egfr* contributes to the intermediate column NB fate determination other than through its regulation of *ind* and whether *ind* by itself is sufficient to confer intermediate fate. Further studies are necessary to dissect the regulatory mechanisms that control intermediate column NB fate specification. In addition, while our work did not address the roles of Dichaete and Sox-Neuro, we have reported that ubiquitous EGFR signaling activates Dichaete expression throughout the neuroectoderm (Zhao and Skeath, 2002). Because Dichaete and SoxNeuro cooperates with *vnd* in the mediate column and *ind* in the intermediate column in NB fate specification (Buescher *et al.*, 2002, Overton *et al.*, 2002, Zhao and Skeath, 2002), they are likely to act as co-factors with Vnd and Ind in *Egfr** embryos to specify NB fate in the lateral column.

Temporal regulation of gene expression during CNS patterning

Our experiments also underline the importance of temporal regulation of gene expression during CNS patterning. This is most notable with respect to the dynamic regulation of *ind* and *vnd* expression by *Egfr* signaling. Previous work suggested that the spatial dynamics of *Egfr* activity in the CNS account for the transient nature of *ind* expression in the intermediate column. Prior to NB formation *Egfr* activity is present in the intermediate column and activates *ind* expression in this domain. Once NBs begin to form *Egfr* activity disappears from the intermediate column and *ind* expression is also lost from intermediate column neuroectodermal cells. These data supported a simple regulatory relationship in which the presence of *Egfr* activity is necessary for *ind* expression in the intermediate column. However, while *Egfr* is necessary to activate *ind* in the intermediate column and sufficient to activate *ind* in the entire CNS (Figure 3), we find that *ind* expression turns over at its normal time even in the presence of ubiquitous and prolonged *Egfr* activity in the CNS (Fig. 3). Thus, even though *Egfr* activity is necessary and sufficient for the activation of *ind*, once activated *ind* expression in the CNS appears to become independent of *Egfr* activity and other factors must regulate its temporally precise downregulation in the CNS.

Similarly, *vnd* also exhibits differential sensitivity to *Egfr* activity as a function of time. In contrast to *ind*, *Egfr* activity is not necessary to activate *vnd* expression in the medial column, however, *Egfr* activity is required later to maintain *vnd* expression in this domain. Thus, *vnd* and *ind* exhibit opposite responses to the *Egfr* signaling – *ind* is activated but not maintained by *Egfr* activity while *vnd* is maintained but not activated by this pathway. It is interesting to note that *vnd* becomes competent to respond to *Egfr* signaling about the time *ind* loses its ability to respond to this signal. While the differential competency of the *vnd* and *ind* promoters to *Egfr* signaling is essential for proper DV patterning of the CNS, the molecular bases of these differences remain unknown. Some of the specificity

likely resides within the promoters or regulatory regions of the genes themselves. However, since both promoters are *Egfr*-responsive albeit at different times additional levels of regulation appear necessary to explain the complexity in regulation. Alteration to higher order chromatin structure is known to play a key role in controlling the competency of different promoters to respond to specific signals (McKinsey *et al.*, 2002)and is a clear candidate to help mediate the differential responses of *ind* and *vnd* to *Egfr*-activity. However, how chromatin structure affects the ability of *ind* and/or *vnd* to respond to *Egfr*-activity remains unexplored. Future work that addresses the influence of modulation of chromatin structure on the ability of these and other genes to respond differentially to the same inputs should shed light on basic principles of gene regulation during development.

Convergent control of neuroblast specification

Our genetic studies indicate that the activities of *Egfr* and the *ac/sc* genes converge to specify the fate of MP2 and possibly other NBs. Additional work on genes that regulate NB fate suggests that distinct convergent signals may play a general role in NB specification. For example, the transcription factor *huckebein* is expressed in NB 4-2 and its associated proneural cluster and helps promote the fate of some of the neurons that develop in the 4-2 lineage (Chu-LaGraff *et al.*, 1995). However, in the absence of *huckebein* function, the 4-2 lineage retains many of its wild-type characteristics (Chu-LaGraff *et al.*, 1995). Thus additional intrinsic and extrinsic cues likely converge with *huckebein* to control the fate of NB4-2 and enable it to elaborate its proper cell lineage. Similar, albeit less detailed observations, have been made for *runt* and *msh* (Buescher and Chia, 1997, Dormand and Brand, 1998, Isshiki *et al.*, 1997). These genes are expressed in specific NBs and the cell clusters from which they delaminate. Each gene appears to regulate only a subset of the distinguishing characteristics of the neuronal lineages that arise from their respective NBs yet none of them appears deterministic for a specific NB fate. Thus, we speculate that convergent regulation of NB fate by multiple intrinsic and extrinsic factors is a general theme in CNS development and that classical double and triple mutant analyses will be essential to reveal convergent pathways involved in NB as well as neuronal specification.

Materials and Methods

Fly strains and genetics

Wild-type patterns of gene expression were examined in Oregon R embryos. Ectopic activation of the *Egfr* signaling pathway was accomplished by using UAS/Gal4 system (Brand and Dormand, 1995). The following fly lines were used: *In(1)y^{3PL} sc^{8R}*; *scabrous*-Gal4 (*sca*-Gal4) and UAS-*l'sc*. *tub*-Gal4 fly is kindly provided by D. St Johnston (Jazwinska *et al.*, 1999). This fly line carries a transgene in which the DNA binding domain of GAL4 fused to the VP16 transcriptional activation domain expressed from the α4-tubulin promoter. UAS-*s-spi* on the second chromosome is kindly provided by A. Michelson (Schweitzer *et al.*, 1995). We mobilized UAS-*s-spi* to the third chromosome by providing with transposes. When we ubiquitously express it with *tub*-Gal4 driver, we got identical phenotype in both CNS patterning and cell fate specification as UAS-*s-spi* in the second chromosome. All our analyses were performed using UAS-*s-spi* on the third chromosome because it is healthier. Mutant lines used were: *Egfr: flb^{1k35}* and *flb^{1F26}* (Clifford and Schupbach, 1994); *ind^{RR108}* (Weiss *et al.,* 1998); *vnd^{Δ38}* (Chu *et al.*, 1998);

To ectopically express *s-spi* in *ind* mutant background, virgin females from *tub*-GAL4/Cyo-*ftz* lacZ; *ind^{RR108}*/+ were crossed to *ind^{RR108}*-UAS-*s-spi*/TM3-*ftz* lacZ at 25ºC. Embryos resulting from the cross were fixed and stained for one of the markers and β–galactosidase. We identified embryos of the appropriate genotype by the lack of β–galactosidase expression as well as by expected phenotypic ratios and the specific observed phenotypes.

To express ectopically *s-spi* in *vnd* mutant background, virgin females from *vnd^{Δ38}*/FM7-*ftz* lacZ; *tub*-GAL4 were crossed to UAS-*s-spi* male at 25ºC. We fixed, stained and scored embryos resulting from the cross for each markers and β–galactosidase and identified embryos of the appropriate genotype as noted in the prior paragraph.

For MP2 specification experiments, two fly lines were used: *In(1)y^{3PL}sc^{8R}; flb^{lF26} sca*-Gal4 / CyO-*ftz*-lacZ carries a deletion of *ac/sc* genes and *Egfr* temperature sensitive mutation (restriction temperature 28ºC). The *sca*–Gal4 insertion activates genes placed after the Gal4 upstream activating sequence (UAS) throughout the neuroectoderm at stage 8-9. *In(1)y^{3PL}sc^{8R}; flb^{1K35}* UAS - *l'sc* / CyO-*ftz*-lacZ carries a deletion of *ac/sc* genes and *Egfr* null mutation. *In(1)y^{3PL}sc^{8R}* deletes the *ac* gene and the enhancer sequences of *sc* (the coding sequence of *sc* is still present) which results in lack of *ac* and *sc* expression in MP2. Virgin females from *In(1)y^{3PL}sc^{8R}; flb^{lF26} sca*-Gal4 / CyO-*ftz*-lacZ were crossed to *In(1)y^{3PL}sc^{8R}; flb^{1K35}* UAS-*l'sc* / CyO-*ftz*-lacZ males or vise versa at 29ºC. Embryos resulting from the cross were fixed and stained for one of the markers and β–galactosidase. Embryos that lacked β–galactosidase expression in the *ftz* pattern were scored for the various markers in the MP2 position. These embryos have a genotype of *In(1)y^{3PL}sc^{8R}; flb^{1K35}* UAS - *l'sc* / *flb^{lF26} sca* - Gal4. Control were carried out on embryos of the following genotypes: *In(1)y^{3PL}sc^{8R}; flb^{lF26} sca*-Gal4 / *flb^{lF26} sca*-Gal4, *In(1)y^{3PL}sc^{8R}; flb^{1K35}* UAS - *l'sc* / *flb^{1K35}* UAS - *l'sc* and *In(1)y^{3PL}sc^{8R}; sca*-Gal4 / *UAS-l'sc.* All embryos were raised at 29ºC.

Immunohistochemistry of whole mount embryos

Single- and double-label immunohistochemistry analyses were performed as described elsewhere (Skeath *et al.*, 1992). 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, 1992); rabbit anti-Vnd (1:10) (McDonald *et al.*, 1998); 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.*, 1989); mouse anti-βgal (1:2000; Promega); mouse anti-Pros MR1A (1:3) (Spana and Doe, 1995); mouse anti-Ftz (Kellerman *et al.*, 1990); rabbit anti-Odd (1:2000; kindly provided by Ellen Ward); GP anti-Hb (1:400; kindly provided by David Kosman) and mouse anti-Active MAP kinase (1:2000; Sigma) (Gabay *et al.*, 1996).

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