EPIDERMAL GROWTH FACTOR DEPENDENT REGULATION OF DROSOPHILA NERVOUS SYSTEM DEVELOPMENT ALONG THE DORSO-VENTRAL AXIS

by

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Abstract

The *Drosophila* embryonic nervous system develops from an array of neural precursor cells called, neuroblasts. These neuroblasts give rise to all the cell types that populate the mature central nervous system (CNS). The CNS originates from a bilateraly symmetric neurectoderm that is subdivided into three domains along the dorso-ventral (DV) axis. One of these domains is defined by the expression of the Homeodomain protein ventral nervous system defective (*vnd*). Regulation of neuroblast designation is very precise and controlled. Extensive research has been done on neuroblast formation along the anteroposterior axis, most of which indicates that neuroblast selection within a cluster of neurectodermal cells is controlled by segmentation genes. However, much more research is required to elucidate the function of genes along the DV axis. Early studies indicate that *vnd* is required for neuroblast formation in the ventral column. Here, we show that *vnd* function, but not expression, is dependent on MAPK activity downstream of *Drosophila EGF-R (DER)*. Specifically, we show that *vnd* activity is eliminated in EGF-R mutant embryos in a stage specific manner by evaluating *vnd*'s ability to inhibit intermediate neuroblast defective (ind), muscle segment homeobox (msh), and the newly identified neural tube development player, neu3. Finally, we show that *DER* functionality in the ventral column is entirely dependent on the processing protein *rhomboid (rho)* in later stage embryos.

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Chapter 1

Introduction/Literature Review

Early embryonic patterning

During early *Drosophila* embryogenesis, several cell determination signals specify different tissue types. One of these tissues is the neuroectoderm which gives rise to neural progenitor cells, called neuroblasts. These cells must have proper directionality, timing, and cellular properties to develop into a working spinal cord. In essence, the embryo must develop a bilateral neurectoderm domain with precise boundaries within each subdomain. Of the three subdomains, each encodes a different homeodomain containing transcription factor: *ventral nervous system defective (vnd), intermediate nueroblast defective (ind)*, and *muscle segment homeobox* (*msh*) (Chu et al., 1998; Isshiki et al., 1997; Mc Donald et al., 1998; Weiss et al., 1998). These three subdomains run the length of the anterior-posterior (AP) axis and are bilaterally symmetric along the dorsoventral (DV) axis. The neurectoderm forms along the ventral midline and expand dorsally as well as anteriorly/posteriorly. This process is highly regulated using a variety of cellular and transcriptional signals. In vertebrates, complications in this process can give rise to a variety of congenital neurological disorders due to the highly conserved nature of the system.

Ventral nerve cord development begins with the designation of proneural clusters. These clusters are determined by the expression patterns of the *Acheate-scute* complex (A-SC) genes. Certain levels of A-SC expression, along with other pro-neural genes, among them the pair-rule genes, create a cluster of cells that all can become neuroblasts although only one will do so (Skeath, et. al. 1994). The cell that expresses the highest level of A-SC will form the new neuroblast. Once the neuroblast has formed, cell nonautonomous genes are expressed that laterally inhibit proneural genes in neighboring cells,

preventing the formation of other neuroblasts (Skeath, et al. 1994) through the Notch pathway (Skeath. 1998).

Researchers have done extensive work in *Drosophila* nervous system development along the AP axis. The A-SC is an essential complex in designating proneural clusters along the AP axis (Skeath. 1998). A-SC genes are uniquely expressed in S1 and S2 neural clusters early in development (McDonald, et al. 1998) and appear to designate cell clusters as proneural clusters. However, complete *a-sc* knockouts show that only 25% of the neuroblast clusters are lost (Skeath and Doe, 1996). This indicates that other proneural genes must also have a role in designating neural clusters. One such gene is the DV gene *vnd*. This gene affects A-SC expression at alternating neuroblast clusters along the AP axis (Skeath et al., 1994)(McDonald, et al. 1998). Without *vnd,* AS-C is not expressed within the ventral column which clearly indicates that *vnd* is required for AS-C activation in the ventral column. Tandem *vnd*; A-SC deletions result in the loss of up to 50% of all neuroblast clusters most of which are in the ventral column (Skeath et al., 1994)(McDonald, et al. 1998). This indicates that *vnd* and A-SC share a similar or parallel function in neuroblast formation.

Subdivision of the neurectoderm

vnd is the most ventral of three bilateral DV-axis genes (from ventral to dorsal*): vnd, intermediate neuroblast defective (ind),* and *muscle segment homeobox (msh)* (Chu et al., 1998; Isshiki et al., 1997; Mc Donald et al., 1998; Weiss et al., 1998). Each of these genes has repressive, cell nonautonomous features that help determine the precise patterning of each gene within a column (Figure 1A). Establishing each of these domains requires two steps. First, *vnd* is activated by *Dorsal,* an NF-κB like transcription factor, and *ind*/*msh* are activated by some unknown mechanism. Mitogen Activated Protein Kinase (MAPK), downstream of the EGF-R, clearly plays a role in *ind* activation but once a signal

has been transduced, the precise activator of *ind* remains a mystery (Von Ohlen and Doe, 2000). The dorsal boundary of the neurectoderm and thus *msh* expression is established through *Dpp* repression (Von Ohlen and Doe, 2000). *Dorsal* then represses *Dpp* on the ventral border allowing *msh* to express properly (Von Ohlen and Doe, 2000). Once the genes are activated, the repression hierarchy is established. Repression typically acts ventral to dorsal. For instance, *vnd* represses *ind* which in turn represses *msh* (Mc Donald et al., 1998; Von Ohlen and Doe, 2000)(Chu,H. 1998). However, *ind* can repress *vnd* expression in late stage embryos (data not shown). In wild-type embryos, *Dorsal* shows a decreasing gradient from the mesoderm to the dorsal side of the neurectoderm (reviewed in: (Anderson, 1998). EGF-R is evenly expressed in the ventral and intermediate columns of the neurectoderm, and *Dpp* is only expressed in the epidermis because *Dorsal* represses *Dpp* expression (Skeath, 1998; Yagi et al., 1998)(Von Ohlen,and Doe 2000). When *Dorsal* is knocked out, *Dpp* expands into the neurectoderm and mesoderm and the tissue transforms into epidermis (D'Alessio and Frasch, 1996). When the EGF-R is knocked out, *ind* expression is lost and *vnd* and *msh* fill the intermediate column (Von Ohlen,and Doe. 2000). It is unknown whether EGF-R expression affects *vnd.* When *vnd* is knocked out, the ventral domain establishes an intermediate-like column, *ind* expression is expanded into the ventral column, *achaete* is no longer expressed in the ventral column, and several ventral column neuroblasts are lost (Mellerick,et al. 2002a; 2002b). Conversely, when *vnd* is overexpressed into the intermediate column, that column develops a ventral column fate (Mellerick, et al. 2002a; 2002b). In this case, *ind* expression is lost and *Ac* is over-expressed in the intermediate column ((Mellerick, et al. 2002a; 2002b), Figure 2).

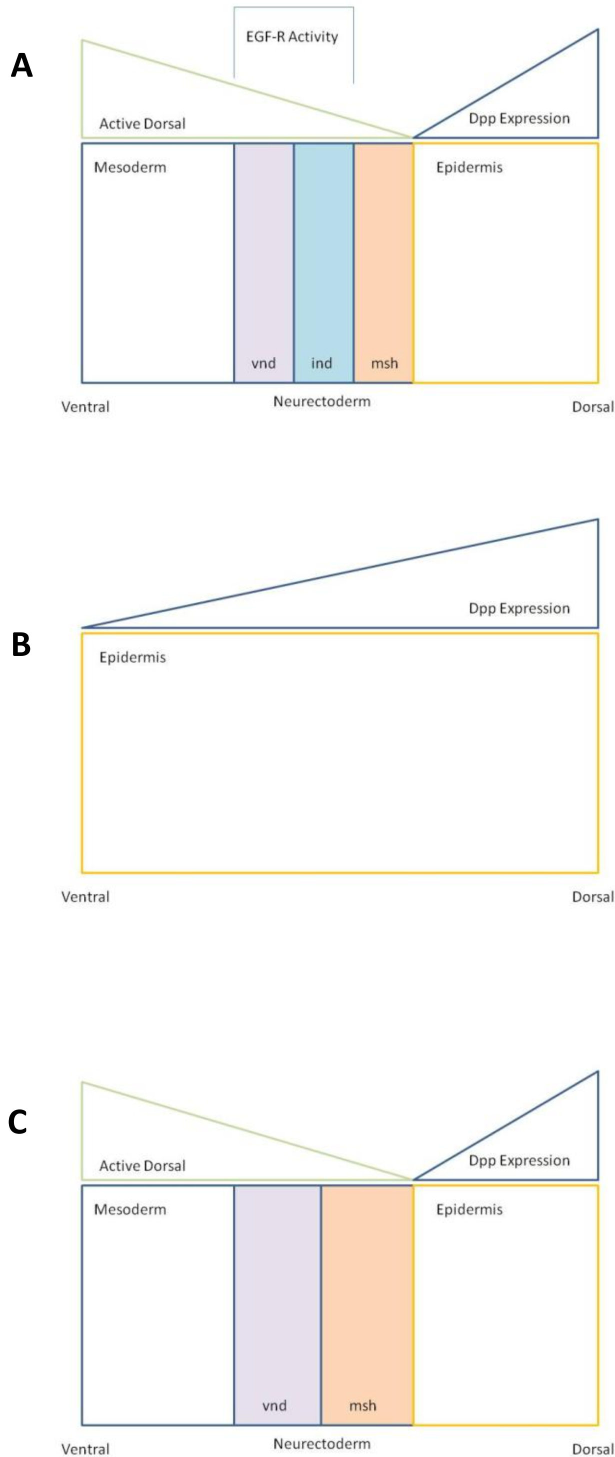

Fig. 1. Schematic of DV axis expression patterns. (A) Wild type expression patterns. *Dorsal* expression shows a decreasing gradient from ventral to dorsal. *Dpp* shows a decreasing gradient from dorsal to ventral. EGF-R activity is uniform in the ventral and intermediate domain of the neurectoderm. *vnd*, *ind*, and *msh* all show normal expression patterns. (B) *Dorsal* knockout. Mesoderm, *vnd*, *ind*, EGF-R, and *msh* expression is lost. The epidermis overtakes the DV axis. (C) EGF-R knockout. *ind* expression is lost. *vnd* and *msh* expand towards the intermediate domain to fill the void left by *ind* (Figure developed from Von Ohlen and Doe 2000).

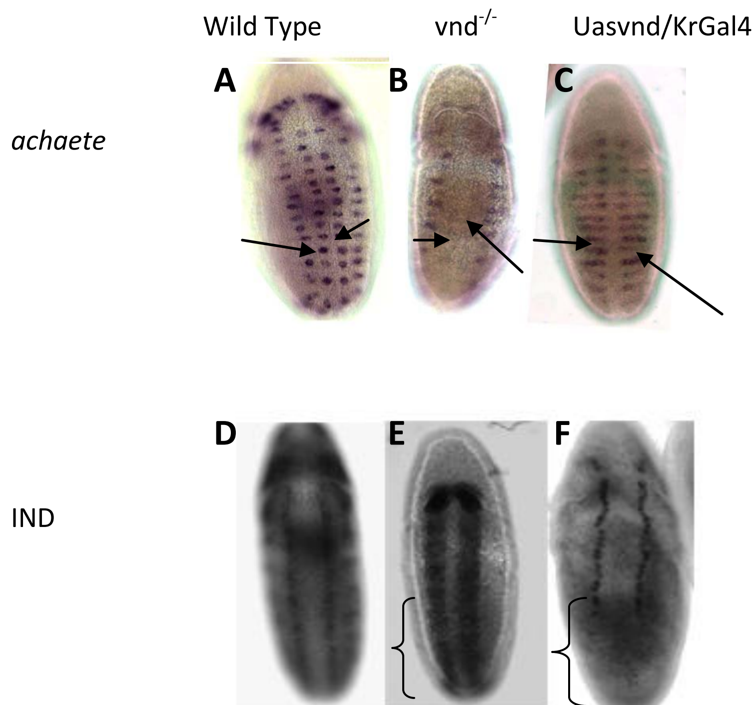

Fig. 2. *In Situ* hybridization of *Drosophila* embryos reveals stimulatory activity of *vnd* on achaete but repression activity on *ind*. Embryos from wild type (A), vnd$^{-/-}$ (B, vnd^{438}), and Uasvnd/KrGal4 (C) backgrounds are stained for *achaete* expression. Embryos from wild type (D), vnd$^{-/-}$ (E, vnd^{438}), and Uasvnd/KrGal4 (F) backgrounds are stained for *ind* expression. Arrows point *achaete* expression in wildtype (A), lack of *achaete* expression in *vnd* mutants (B), and *achaete* over expression in ectopic *vnd* embryos(C). Brackets show areas of expansion or inhibition.

The expression of *vnd*, *ind*, and *msh* is initially regulated by the precise activity patterns of three signaling pathways: Dorsal, Dpp, and the EGF-R (Figure 1B and 1C). Dorsal is primarily active in the ventral column with moderate activity in the intermediate column and low activity in the dorsal column (Figure 1A). Dpp is just the opposite with high expression in the dorsal epidermis and has a minimal effect in the neurectoderm (Figure 1A). The EGF-R is evenly expressed in the ventral and intermediate column but is not active in the dorsal column. Dorsal knockouts result in the total loss of the entire neurectoderm (Figure 1B). Partly because Dorsal is required for the expression of certain genes like *vnd* and *ind* (Von Ohlen, Doe. 2000) and also Dorsal prevents Dpp from being expressed in the neurectoderm, changing it into epidermis (Von Ohlen, Doe. 2000). Interestingly, even though EGF-R is

active in the *vnd* domain, EGF-R knockouts do not result in the loss of *vnd* expression initially (during late stages, *vnd* does depend on EGF-R signaling). It is unknown whether the loss of EGF-R activity changes the activity of *vnd* itself (Von Ohlen, Doe. 2000).

Vnd structure

The structure and function of the 80 Kd VND and its motifs have been well characterized (Figure 3). Residues 154-193 encode one transcriptional repression motif and one activation motif from residues 293-528. The activation motif consists of a glutamine-rich domain and two acidic domains. VND contains an EH-1 domain, or tinman motif. This motif is also found in the vertebrate NKx-2.2 homologue (Stepchenko. 2004). This small region is conserved in vertebrates and is known to interact with the co-repressor, Groucho (Stepchenko. 2004, Zongxin et. al. 2005, and Nirenberg 2004). Interactions between Groucho and VND are critical to repressional functions *in vivo* and remain unclear. Phosphorylation of Groucho down regulates its repressional functions *in vitro* yet phosphorylation of VND appears to be vital to VND's function (Cinnamon,E. 2008, Ze'ev Paroush personal communication). Finally, VND contains the DNA binding domain, homeodomain (HD). The HD recognizes the DNA binding sequence 5'-TCAAGTGG-3'. Note that the HD in VND recognizes a completely different sequence than the HD's in antenapedia transcription factors, making this particular domain unique (Stepchenko. 2004).

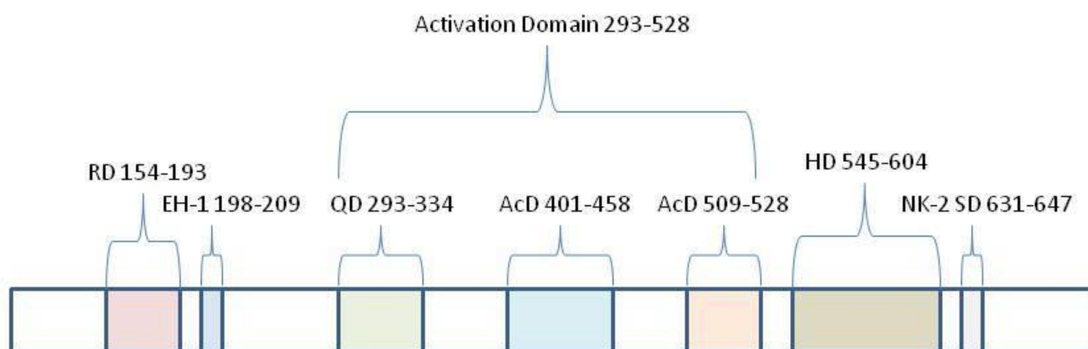

Fig. 3. VND protein sequence contains several important structural domains. The Repression Domain (RD) includes residues 154-193. The EH-1 or tinman region includes residues 198-209. The Activation Domain runs from amino acid 293-528 and includes the glutamine rich domain (QD 293-334) and two acidic domains (AcD 401-458 and AcD 509-528). The homeodomain (HD) runs from 545-604 and the NK-2 specific domain (NK-2 SD) includes 631-647.

Nkx2-2 function in vertebrates

In vertebrates, reverse genetic analysis shows the VND-like protein, Nkx2-2, is vital to forming oligodendrocytes in the spinal cord (Cornell,and Von Ohlen. 2000). Oligodendrogenesis is largely controlled by the Sonic Hedgehog (Shh)-Notch signaling pathway. In this pathway, Shh expression levels control transcription factor expression along the spinal cord (Qi,Y. 2001). These transcription factors then dictate the genesis of, among others, neurons, oligodendrocytes, and glial cells. Nkx2-2 is one such transcription factor (Cornell, Von Ohlen. 2000). In Nkx2-2 mutant mice, oligodendrocytes fail to form properly and intermediate domain proteins such as Olig1 and Olig2 develop ventral-like expression patterns (Qi, et al. 2001). These two proteins, related to the olig family from *Drosophila,* help define the pre-oligodendroglial domain along with the receptor for platelet-derived growth factor α. Although oligodendrogenisis is initiated by Shh signaling, it's these proteins that create the precursor cell

domains. Interaction between these proteins and Nkx2.2 create a neurectoderm structure similar to that in *Drosophila* (Cornell and Von Ohlen, 2000).

The two domains in VND that share sequence homology to Nkx2.2 are the homeodomain and the EH-1/tinman domain. The tinman domain binds to the universal co-repressor, Groucho (Mellerick and Modica, 2002).This co-repressor enhances the weak repression capabilities of VND suggesting that Groucho is required for VND-mediated repression *in vivo* (Zhang H., et. al. 2008). Groucho-mediated repression is regulated by EGFR signaling *in vivo* (Cinnamon, et al. 2008). Phosphorylation of Groucho derepresses the activity of the Groucho-protein complex, allowing transcription of the repressed gene to continue (Cinnamon, et al. 2008). However, the VND-Groucho complex still has strong repressor activity so there must be other mechanisms by which VND repressor function is maintained. This may include direct interaction with other co-factors or modifications such as phosphorylation (Zhang et al., 2008).

Vnd interacts with the HMG domain proteins Dichaete and SoxNeuro

Acting as an antagonist to Groucho is the Sox-family protein Dichaete (Zhao,G. 2002). Dichaete, or fish-hook, contains the high mobility group DNA interacting domain and acts in parallel to both VND and IND functions. In the medial column, Dichaete and VND coordinate to initiate neuroblast formation (Zhao,G. 2002). Genetic knockouts of *dichaete* result in severe defects in neuroblast patterning similar to *vnd* knockouts. Double knockouts of *vnd*; *dichaete* result in a phenotype (loss of neuroblast cluster formation) that is more pronounced than the single mutants (Zhao, et al. 2007) (Overton et al., 2002). However, the double knockout shows that the phenotype is three times more profound than what is predicted if the two genes operated independently of each other (Zhao, et al. 2007). This genetic interaction suggests that *dichaete* and *vnd* synergize in establishing the ventral column of neuroblasts (Zhao, et al. 2007). A similar interaction is seen between d*ichaete* and *ind*. Dichaete also has an ortholog,

SoxNeuro (SoxN). SoxN is also a member of the Sox-family and contains the DNA interacting domain, high mobility group. Loss-of-function SoxN mutants show that neuroblast formation is severely affected in the intermediate and lateral columns but not the medial column (Buescher, et al. 2002) (Overton et al., 2002). However, Dichaete acts in a parallel pathway to SoxN in the medial column; double mutants are indicative of this. SoxN also interacts genetically with *vnd* and *ind* (Buescher, et al. 2002) (Overton et al., 2002).

Neu3 function in neurogenisis

Clearly, *vnd* functions in neuroblast formation within the ventral column, but exactly how remains unclear. Many VND targets, including *ind*, *Ac*, *Odd*, *Prospero*, *Huckebein*, and *msh*, have been identified (Mc Donald et al., 1998)(Zhang H., et. al., 2008). Recently, a new *vnd* target has been characterized: *neu3*. *neu3* encodes the matrix metalloprotease, Meltrin (Meyer,H. 2010). In mice and insects, Meltrin is commonly expressed in the peripheral nervous system, skeletal muscles, and bones (Meyer, et al. 2010). Its exact function in neurogenesis is unknown, however. Its expression pattern in *Drosophila* embryos mimics that of *ind* but *neu3* reacts differently than *ind* when EGF-R signaling is lost (Meyer, et al. 2010). In fact, *neu3* expression does not change at all in EGF-R mutant embryos (Fig. 4C). Also, recent evidence shows that VND represses *neu3* expression in the KrGal4 region when *vnd* is under the control of the UAS promoter (Figure 4). This unique feature may make *neu3* a good indicator of VND activity in EGF-R mutants.

Fig. 4. *neu3* is regulated by *vnd* but not EGF. *neu3* expression patterns visualized via *in situ* stain in wildtype (A), Uasvnd/KrGal4 mutant embryos (B), and DER mutant embryos (C). Expression of *neu3* is not seen in the Gal4 region of the Uasvnd mutant embryo. Loss of EGFR signaling does not affect *neu3* expression patterns. Bracket indicates the area of *neu3* inhibition

EGF-R signaling in neurogensis

EGF-R signaling in *Drosophila* controls many developmental and metabolic processes. This signaling operates through the receptor tyrosine kinase, Drosophila EGF-R (DER, Skeath. 1998). There are two ligands for the DER: *spitz* and *vein*. Vein is a secreted signaling protein that directly activates DER signaling (Golembo et al., 1999; Schnepp et al., 1996)(Skeath,J.B. 1998). Spitz encodes an inactive transmembrane protein that, when cleaved and processed from the membrane, becomes a secreted signaling molecule (Lanoue and Jacobs, 1999). Cleavage of Spitz requires the function of an additional membrane bound protein, *rhomboid* (*rho*) (Lanoue and Jacobs, 1999). *rho, vein* double mutant embryos are phenotypically identical to DER mutant embryos with regards to neurectoderm patterning (Skeath, et al. 1994), suggesting that both *vein* and *rho* are required for maximal activation of the DER pathway in the early embryo. Recent studies show that in early embryonic development, *vein* and *spitz* operate in

parallel pathways (Cinnamon,E. 2008). However, later in development, signaling through the EGF-R occurs entirely through the rho-spitz pathway (Skeath, et al. 1994). Because *ind* requires the activity of DER to be expressed and maintain that expression, in late stage embryos, *ind* expression must depend on both *vein* and *spitz/rho* expression.

As mentioned earlier, whether VND transcription factor function is affected by EGF-R activity remains unknown. In DER mutant embryos neuroblast formation fails to occur in the intermediate column (Skeath. 1998). *ind* expression, a gene that plays a crucial role in intermediate column neuroblast formation, is lost in DER mutants (Von Ohlen and Doe, 2000). Some neuroblast clusters in the medial column form incorrectly in DER mutants, indicating that the EGF-R influences neuroblast formation in the medial column (Skeath,J.B. 1998). Unlike *ind*, however, *vnd* expression is not initially lost in DER mutants (Yagi,Y. 1998). It is unclear whether the function of *vnd* depends on phosphorylation by MAPK downstream of the EGF-R. However, Groucho phosphorylation down-regulates its repressive capabilities, suggesting that phosphorylation of the Groucho-Vnd complex may reduce *vnd's* ability to repress target proteins like *ind* and *neu3* (Cai. 2003). This is unlikely, however, considering that the EGFR is active in both the ventral and intermediate domain and *vnd* still represses *ind* and *neu3*. It may be that Groucho phosphorylation, when bound to *vnd*, is thwarted by some unknown mechanism controlled by a phosphatase. Another possibility is that Groucho cannot be phosphorylated when bound to vnd because of conformational restraints. It is also possible that additional modifications or co-factor interactions are able to over-ride the reduced Groucho function in the presence of MAPK activity. Regardless, it is highly probable that a Groucho-vnd complex is fully functional under an active MAPK pathway despite the fact that other Groucho complexes lose functionality when phosphorylated.

Neurogenesis pathway

Using genetic and molecular data, a pathway can be drawn (Figure 5). In early embryogenesis, Dorsal signals to *vnd* first and then *ind* to initiate expression of each of these genes (Von Ohlen, Doe. 2000). During this time, DER also begins signaling in the ventral and intermediate columns. DER signaling directly activates *ind* expression but is not required for initiation of *vnd* expression. It remains unclear whether MAPK downstream of EGF-R activity contributes to VND function. DER signaling is directly stimulated by the neuregulin like protein, Vein (Skeath, et al. 1994). Vein is only expressed in the ventral and intermediate column and is downregulated beginning in stage 10 embryos (Skeath. 1998). In the ventral column, the DER is stimulated by Spitz. Spitz functionality is controlled by the membrane-bound protein, Rho (Lanoue and Jacobs, 1999)(Skeath. 1998). In *rho* mutant embryos, Spitz cannot be properly processed for signaling to the DER. Once *vnd* and *ind* expression patterns are properly established, *msh* expression begins. The lateral edge of the *msh* domain is regulated by Dpp expression. Dpp repressed up to the far lateral side of the embryo by Dorsal (von Ohlen, Doe. 2000). It is unclear what stimulates the expression of *msh*. Vnd, using groucho as a cofactor, inhibits *ind* expression to establish the ventral domain. Ind represses and positions the ventral boundary of *msh* although it remains unclear if Groucho is a cofactor for *ind* as well (Von Ohlen. 2007). Also expressed in the intermediate domain is *neu3*. VND has shown the ability to inhibit the expression of this gene when over-expressed into the intermediate column (Meyer,H. 2010). The function of *neu3* during embryonic development is currently unknown as embryos mutant for *neu3* have no obvious defects in nervous system development (Meyer,H. 2010). However, *neu3* is clearly regulated by VND and therefore can be used as a readout of the VND repressor function (Meyer, et al. 2010). Once the three domains are established, VND and MSH stimulate and maintain A-SC expression. VND does this through the co-activator Dichaete (Zhao,G. 2002). MSH may have a similar co-activator but that remains unclear.

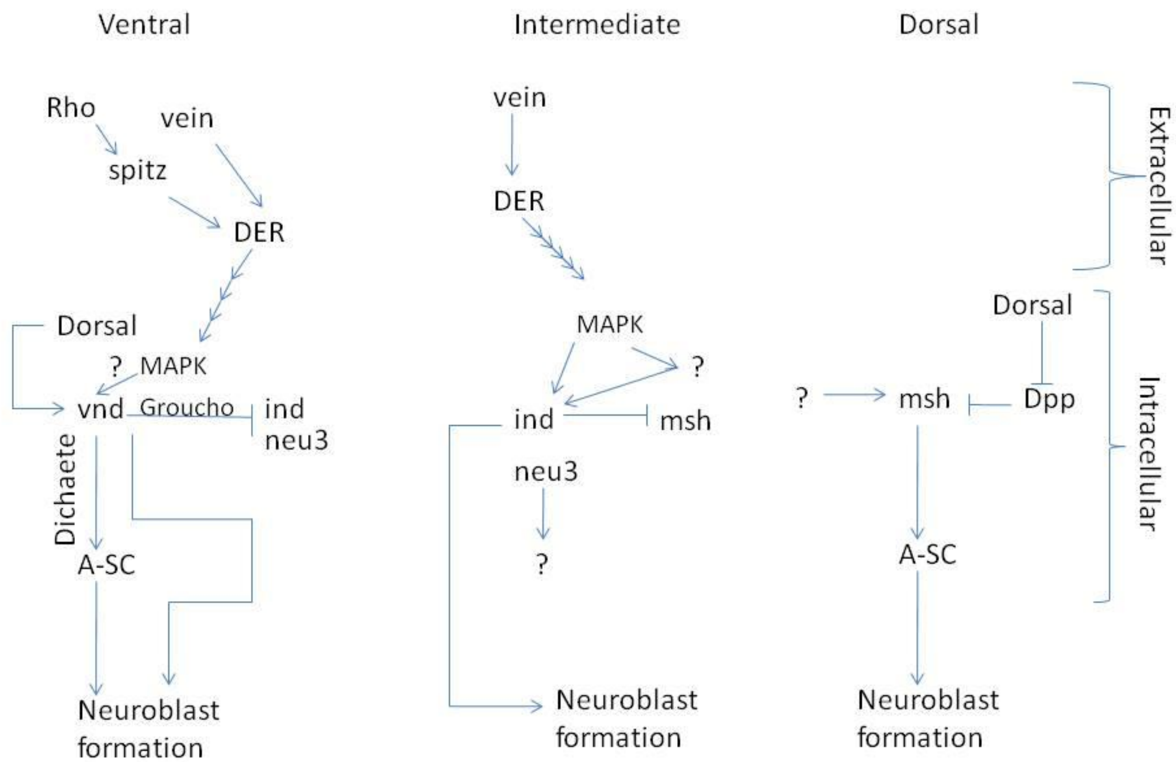

Fig. 5. Pathway of neuroblast formation in embryogenesis. In the ventral column, Rho cleaves spitz which signals to the DER or the DER is directly stimulated by Vein. This activates MAPK for Ind expression and possibly Vnd function. Vnd requires cofactors like the co-repressor Groucho or the co-activator Dichate. Vnd functions to activate and regulate the A-SC, ind, neu3, and neuroblast formation.

Chapter 2

Abstract

The *Drosophila* embryonic nervous system develops from an array of neural precursor cells called, neuroblasts. These neuroblasts give rise to all the cell types that populate the mature central nervous system (CNS). The CNS originates from a bilateraly symmetric neurectoderm that is subdivided into three domains along the dorso-ventral (DV) axis. One of these domains is defined by the expression of the Homeodomain protein ventral nervous system defective (*vnd*). Regulation of neuroblast designation is very precise and controlled. Extensive research has been done on neuroblast formation along the anteroposterior axis, most of which indicates that neuroblast selection within a cluster of neurectodermal cells is controlled by segmentation genes. However, much more research is required to elucidate the function of genes along the DV axis. Early studies indicate that *vnd* is required for neuroblast formation in the ventral column. Here, we show that *vnd* function, but not expression, is dependent on MAPK activity downstream of *Drosophila EGF-R (DER)*. Specifically, we show that *vnd* activity is eliminated in EGF-R mutant embryos in a stage specific manner by evaluating *vnd*'s ability to inhibit intermediate neuroblast defective (ind), muscle segment homeobox (msh), and the newly identified neural tube development player, neu3. Finally, we show that *DER* functionality in the ventral column is entirely dependent on the processing protein *rhomboid (rho)* in later stage embryos.

Introduction

During early *Drosophila* embryogenesis, cell determination signals spawn precise neural progenitor cells which then form the neural tube structure. These cells must have proper directionality, timing, and cellular properties to develop into a working neural tube. For instance, the embryo must develop a

bilateral neurectoderm domain with precise boundaries within each subdomain. In the three subdomains, each encodes a different developmental transcription factor: *ventral nervous system defective (vnd), intermediate nueroblast defective (ind),* and *muscle segment homeobox (msh)*. These three subdomains run anterior-posterior (AP) and are bilateral along the dorsoventral (DV) axis (Chu,H. 1998). The neurectoderm must run along the ventral midline and expand dorsally as well as anteriorly/posteriorly. In vertebrates, complications in this process can give rise to a variety of congenital neurological disorders. This process is highly regulated by a variety of cellular and transcriptional signals.

vnd, ind, and *msh* expression are initially regulated by the precise activity patterns of three signaling pathways: Dorsal, Dpp, and the EGF-R. Dorsal is primarily active in the ventral column with moderate activity in the intermediate column and low activity in the dorsal column (Von Ohlen, and Doe. 2000). Dpp is just the opposite, showing high expression in the dorsal column, moderate expression in the intermediate column, and low expression in the ventral column. The EGF-R is evenly expressed in the ventral and intermediate column but is repressed in the dorsal column (Von Ohlen,and Doe. 2000). Dorsal knockouts result in the total loss of the entire neurectoderm, at least in part because Dorsal is required for the expression of certain genes like *vnd* and *ind* (Von Ohlen, and Doe. 2000). Dorsal also prevents Dpp from overexpressing itself into the neurectoderm, which would change it into epidermis (Von Ohlen, Doe. 2000). Interestingly, even though EGF-R is active in the *vnd* domain, EGF-R knockouts do not result in the loss of *vnd* expression. It is unknown whether the loss of MAPK activity changes the activity of *vnd* itself (Von Ohlen, Doe. 2000).

Clearly, *vnd* is very important in neuroblast formation but exactly how this is accomplished remains unclear. Many *vnd* targets, including *ind*, *Ac, Odd, Prospero, Huckebein,* and *msh*, have been identified. Recently, a new *vnd* target has been characterized: *neu3*. *neu3* encodes meltrin, a matrix

metalloprotease. In mice and insects, meltrin is commonly expressed in the peripheral nervous system, skeletal muscles, and bones (Meyer, et al. 2010). Its exact function in neurogenisis is unknown, however. Its expression pattern in *Drosophila* embryos mimics that of *ind* but *neu3* does not react like *ind* when EGF-R signaling is lost. In fact, *neu3* expression does not change at all in EGF-R mutants. Also, recent evidence shows that, like *ind*, *vnd* represses *neu3* expression in the KrGal4 region when the UAS promoter controls *vnd* (Meyer,H. 2010). This unique feature may make *neu3* a good indicator of *vnd* activity in EGF-R mutants.

EGF-R signaling in *Drosophila* controls many developmental and metabolic processes. This signaling operates through the receptor tyrosine kinase, DER (Skeath. 1998). Like most receptor tyrosine kinases, DER requires a ligand for signaling to be transduced. In this case, two ligands are present: *spitz* and *vein*. Vein is a secreted signaling protein that directly activates DER signaling. Spitz encodes an inactive transmembrane protein that, when cleaved and processed from the membrane, becomes a secreted signaling molecule. Another membranous protein, rhomboid (Rho), cleaves *spitz* from the membrane for signaling to the DER. *Vein/Rho* mutant embryos are phenotypically identical to DER mutant embryos but not as single mutants (Skeath, et al. 1994), suggesting that both *vein* and *rho* act in parallel pathways to DER. Recent studies show that in early embryonic development, *vein* and *spitz* operate in parallel pathways (Skeath, et al. 1994). However, later in development, signaling through the EGF-R is done entirely through the *rho*-*spitz* pathway (Skeath, et al. 1994). Because *ind* requires the DER in order to begin expression and maintain that expression, it can be concluded that in late stage embryos *ind* patterning is dependent on *spitz* and *rho* expression.

As mentioned earlier, whether *vnd* function is affected by EGF-R signaling remains unknown. In DER mutant embryos neuroblast formation fails to occur in the intermediate column (Skeath, et al. 1994). Expression of *ind*, a gene that plays a crucial role in intermediate column neuroblast formation, is lost in

DER mutants (Von Ohlen and Doe, 2000). Some neuroblast clusters in the medial column form incorrectly in DER mutants, indicating that the EGF-R has some influence in neuroblast formation in the medial column. Unlike *ind*, however, *vnd* expression is not lost in DER mutants. Until recently, whether the function of *vnd* depends on phosphorylation from the EGF-R remained unclear. Groucho, a universal co-repressor that binds VND, when phosphorylated, down-regulates its repressive capabilities, thus suggesting that phosphorylation of the Groucho-Vnd complex may reduce VND's ability to repress target proteins like IND and meltrin (Cai, et al. 2003). This is, however, unlikely considering that the EGFR is active in both the ventral and intermediate domain, and *vnd* can still repress *ind* and *neu3*. It may be that Groucho phosphorylation, when Groucho is bound to Vnd, is thwarted by some unknown mechanism controlled by a phosphatase. It may also be that Groucho cannot be phosphorylated when bound to *vnd* due to conformational restraints. Regardless, it is highly possible that a Groucho-VND complex remains fully functional under an active MAPK pathway despite the fact that other Groucho complexes lose functionality when phosphorylated.

Our objective in this study has three parts: (1) examine the *in vitro* phosphorylation of MAPK on VND; (2) Characterize *vnd, ind,* and *neu3* phenotypes in *rho*$^{-/-}$ and DER$^{-/-}$ mutant embryos; and (3) elucidate the importance of phosphorylation of VND in its function. Here we hypothesize that (1) VND is phosphorylated; (2) IND but not VND or Meltrin portray phenotypes in *rho* and DER mutants; and (3) VND depends on phosphorylation from EGF-R regulated MAPK to repress target genes.

Materials and Methods

In vitro analysis of vnd phosphorylation

Full length *vnd* cDNA was constructed along with genetic mutations at S46A, T49A, T256A, and S412A. Potential phosphorylation sites were identified using prediction programs motif scan (http://scansite.mit.edu) and Kinasephos (http://kinasephos.mbc.nctu.edu). Mutagenesis of Vnd cDNA was done in pTOPO4 using the Stratagene Quick Change Mutagenesis multi Kit following manufacturer's directions. Primers were as follows *vnd* T49A TCCTCCCCAGCC*G*CGCCGAAGGCCCCG; *vnd* T46A GTGAGTCCGTCC*G*CCCCAGCCACGCCG; *vnd* S412A CAGCAGCCCGCCGCTCCGGATAGCACC; and *vnd* T265A GCCGTGTCGCCCGCGCCAGCGGGCGTT. Then 6x His6 genetic constructs were added to the c-terminal end using ATGCACCACCACCACCACCACACCACGTCGGCGTCCTTG forward primer and CCCAGTCCCGTGAATGCCCAGTTC reverse primer. Fragments were amplified using an annealing temperature of 60°C and a melting temperature of 72°C. His6-tagged *vnd* variants were expressed using the Promega TnT rabbit reticulocyte lysate (RRL) expression system. Expressed proteins were pulled down using the Promega MagZ^{tm} protein purification system. Half of the isolated proteins were applied to an *in vitro* MapKinase phosphorylation system. This system includes 100 units of active MAPK and 200 µM of ATP. Phosphorylation shifts were visualized on 7.5% SDS-PAGE polyacrylamide gels and silver stained. If a shift occurred was observed then RRL's were split, phosphorylated for 30 minutes or 90 minutes, and loaded onto an SDS-PAGE with 5 µL sample loading diluted to 20 µL. Shifts were revisualized using western blot procedure. Rabbit anti-Vnd antibody was diluted 1:2000 (Sofia Georgieva, Russian Academy of Sciences, Moscow, Russia). Antibody-antigen complexes were visualized using horseradish peroxidase.

Genetics

Wildtype genetic backgrounds were examined in Yellow-white embryos. Mutant embryonic lines were rho^{7m}/tm3ftz, DER^{f2}/cyoftz, $Krgal4rho^{7m}$/$Uasvndrho^{7m}$, and UasRhoind/+; Krgal4ind/ind. Rho^{7m}/tm3ftz and DER^{f2}/cyoftz fly lines were self crossed (from Bloomington Stock Center). $Krgal4rho^{7m}$/Tm3ftz males were crossed to $Uasvndrho^{7m}$/Tm3ftz females (McDonald,J.A. 1998). UasRho/UasRho; ind/tm3 males were crossed with +/+; Krgal4ind/tm3ftz females (Greenspan, R.J. 1997). All other rho and egfr pathway mutant fly lines were gifts from Jocelyn McDonald (Cleveland Clinic) or Amanda Simcox (Ohio State).

Immuno staining and in situ hybridization

Rho^{7m}/tm3ftz mutant flies were used in the expression *vnd* profiling. mRNA of *vnd* and *lacz* was hybridized to *vnd* antisense RNA probe using the standard *in situ* protocol (Tautz, D. 1989). *vnd* and *lacz* mRNA probe was diluted 1:100. The same experiment was performed on DER^{f2}/cyoftz fly lines. Double label immunohistochemistry was performed on rho^{7m}/tm3ftz and DER^{f2}/cyoftz for *ind* and *lacz*. The anti-IND antibody was diluted 1:2000 and the anti-lacz antibody was diluted 1:500 (Von Ohlen, Moses. 2009). Double label *in situ* hybridization was performed with neu3 and lacz mRNA (Meyer, et al. 2010) on rho^{7m}/tm3ftz and DER^{f2}/cyoftz fly lines. All mRNA probes were diluted 1:100. *In situ* stains were performed on Wildtype, vnd-, Krgal4xUASvnd, and rho- Krgal4xUASvnd fly lines. The embryos were stained for neu3 and examined under a microscope. All probes were diluted 1:100. Wildtype, UASrhoxKrGal4, and UASrho/+; Krgal4 ind- were immunostained for vnd to observe vnd expansion into the intermediate column. VND antibody was diluted 1:2000. The same embryos were examined using *in situ* staining for *neu3*.

Results

vnd is phosphorylated in vitro by recombinant MAPK

Full length VND, when detected on a western blot, showed a fair p42 ERK-induced phosphorylation shift (Figure 1A). MAPK phosphorylation shifts for specific point mutations were also observed using a western blot procedure (Figure 1B). The bands were detected at the 80 kD range calculated for VND. S46A and T49A mutants showed a minor shift whereas T256A and S412A mutants showed a slightly larger shift. Shifts were lost at the 90 minute interval due to active phosphatases present in the RRL.

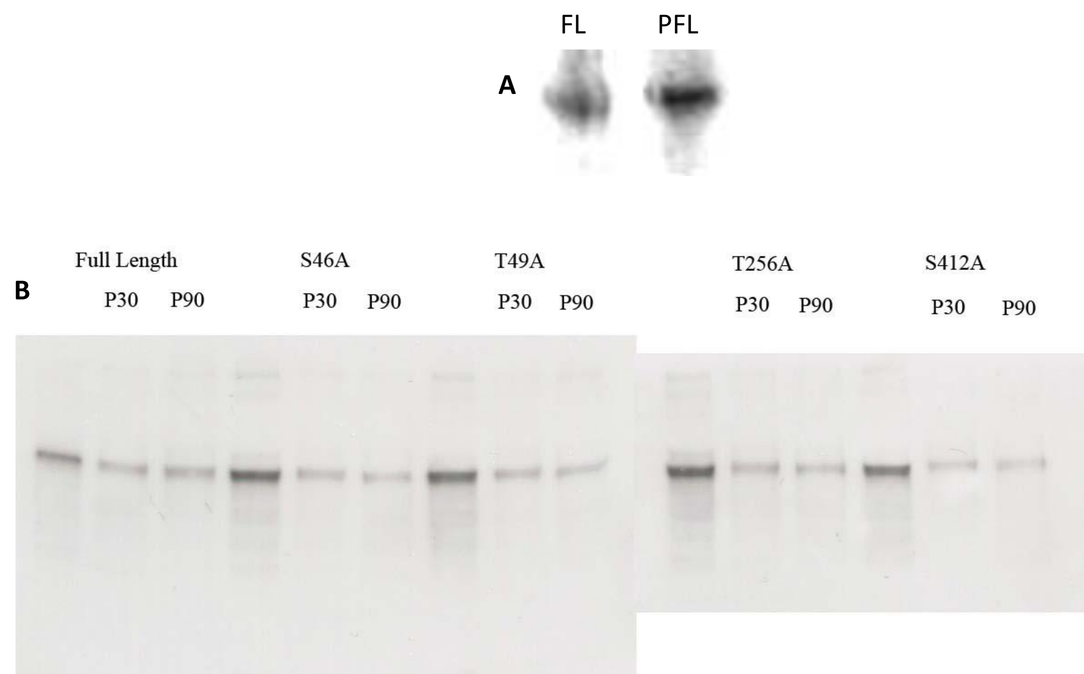

Fig. 1. VND shows phosphorylation shifts for various point mutations. Full length VND (A) and VND with four point mutations (B) show a significant phosphorylation shift. Full length and mutated VND were incubated in MAPK for 30 minutes (P30) and 90 min (P90). PFL indicates phosphorylated full length VND.

MAPK does not affect vnd expression

In situ hybridizations reveal that *vnd* expression is unaffected by *Rho*$^{-/-}$ mutations (Figure 2A,B). However, it is possible that the vein-mediated phosphorylation of *vnd* pathway is still functional. Therefore, DER^{f3}/cyoftz mutant flies were generated and self-crossed to each other to produce DER homozygous mutants. After *in situ* hybridization of *vnd*, no obvious phenotype was observed (Figure 2C). Whether the lack of phosphorylation affects *vnd* activity remains unclear.

MapK regulates ind gene expression

Ind immunostaining using the Rho^{7m}/tm3ftz fly line revealed that Ind expression was relatively unaffected in Stage 9 embryos (Figure 3B). In stage 11 embryos, however, Ind expression begins to expand into the ventral column where VND would normally inhibit expression (Figure 3E). In the DER^{f3}/cyoftz fly line, IND expression was lost in stage 9 embryos (Figure 3C). However, in stage 11 embryos, IND expression begins to turn back on. Small pockets of protein patterning can be seen (Figure 3F). Note that the pockets are mainly in the ventral column, not the intermediate column where IND is normally expressed.

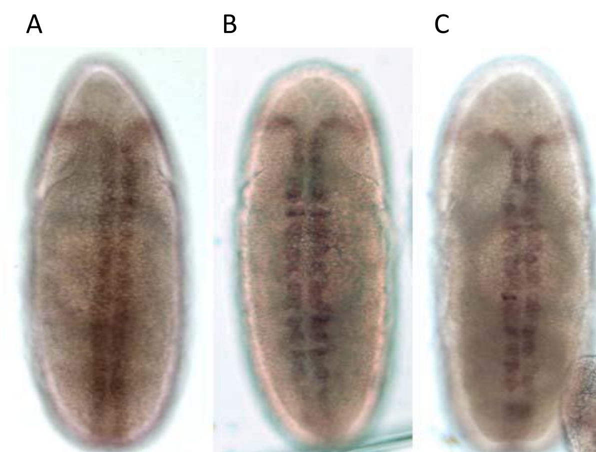

Fig. 2. Stage 9 mutant and wildtype embryos stained for *vnd* mRNA do not show a distinct phenotype. Shown is a wild type embryo (A), Rho$^{-/-}$ mutant embryo (B), and a DER$^{-/-}$ mutant embryo (C).

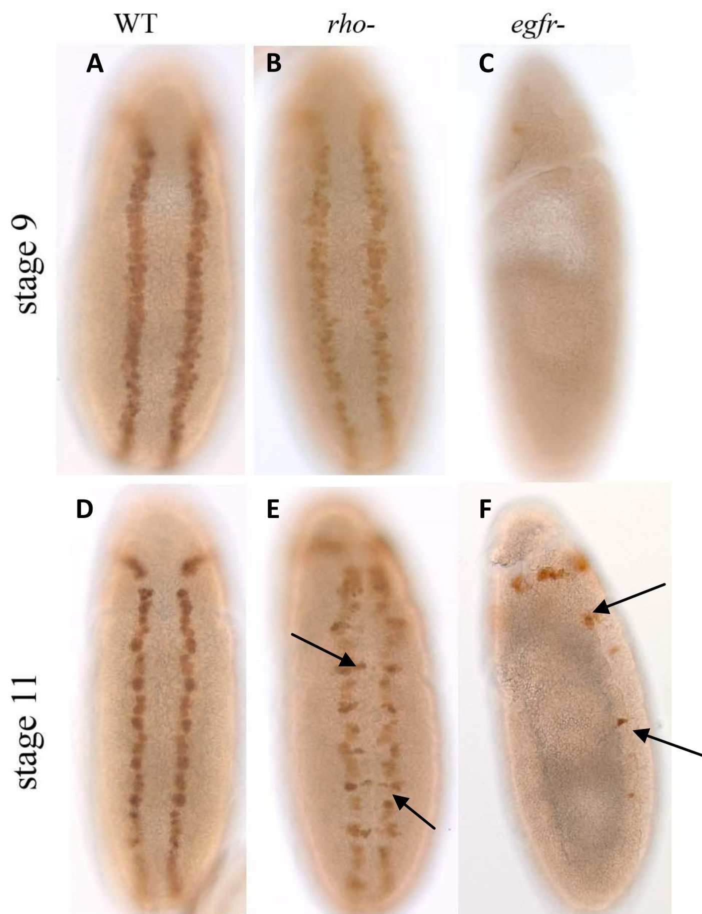

Fig. 3. Ind is misregulated in EGF-R mutants. IND expression is not affected in stage 9 Rho$^{-/-}$ mutants (B) but IND expansion into the ventral column can be seen in stage 11 Rho$^{-/-}$ embryos (E). IND expression is completely lost in stage 9 DER$^{-/-}$ mutants but small pockets of IND expression can be seen in the ventral column of stage 11 DER$^{-/-}$ embryos. Arrows point to IND expansion into the ventral column.

Neu3 expression is independent of phosphorylation

In situ stains for *neu3* in EGF-R mutants reveal that *neu3* expression is independent of the EGF pathway (Figure 4). Rho$^{-/-}$ and DER$^{-/-}$ mutant embryos show no specific change from the phenotype of wildtype embryos. This indicates that neu3 would be an excellent test gene for *vnd* overexpression under *rho* or DER mutant backgrounds. Since neu3 has an identical expression pattern to *ind* and it is immune to changes in the EGF pathway, *neu3* can be utilized to evaluate *vnd* function.

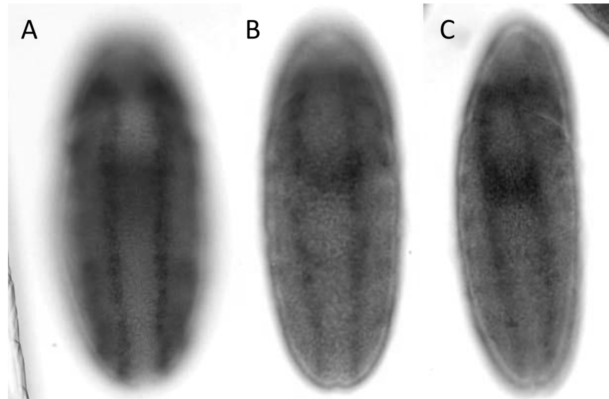

Fig. 4. *In situ* stains show that *neu3* does not respond to EGF-R mutants. Shown here is wildtype (A), rho^{7m}/tm3ftz (B), and DER^{f2}/cyoftz (C).

vnd function is dependent on phosphorylation

KrGal4Rho/UasvndRho embryonic lines were developed to elucidate the functionality of *vnd* in a background that lacks EGF-R signaling in the ventral column. Rho is a major corner stone for EGF-R signaling in the ventral column and does not affect *vnd* expression. But it remains unknown whether or not Rho mutants affect *vnd* function. To answer this question, KrGal4Rho/UasvndRho mutant embryos were first immunovisualized for *ind*. Wild-type embryos can be seen with the *lacz* staining (data not shown). In a typical KrGal4/Uasvnd embryo, *ind* is inhibited at stages 9 and 11 in the KrGal4 region (Fig. 5B). In KrGal4Rho/UasvndRho mutants, however, *ind* is inhibited in stage 9 embryos but not in stage 11. In stage 11 embryos, *ind* expression can be seen in small pockets in the intermediate column where its expression would normally be inhibited. This clearly indicates that *vnd's* ability to inhibit *ind* is dependent upon phosphorylation from the *vein* pathway in early stage embryos. But once *vein* activity

drops off, that pathway is no longer active leaving *vnd* unphosphorylated and unable to inhibit *ind* across the KrGal4 domain.

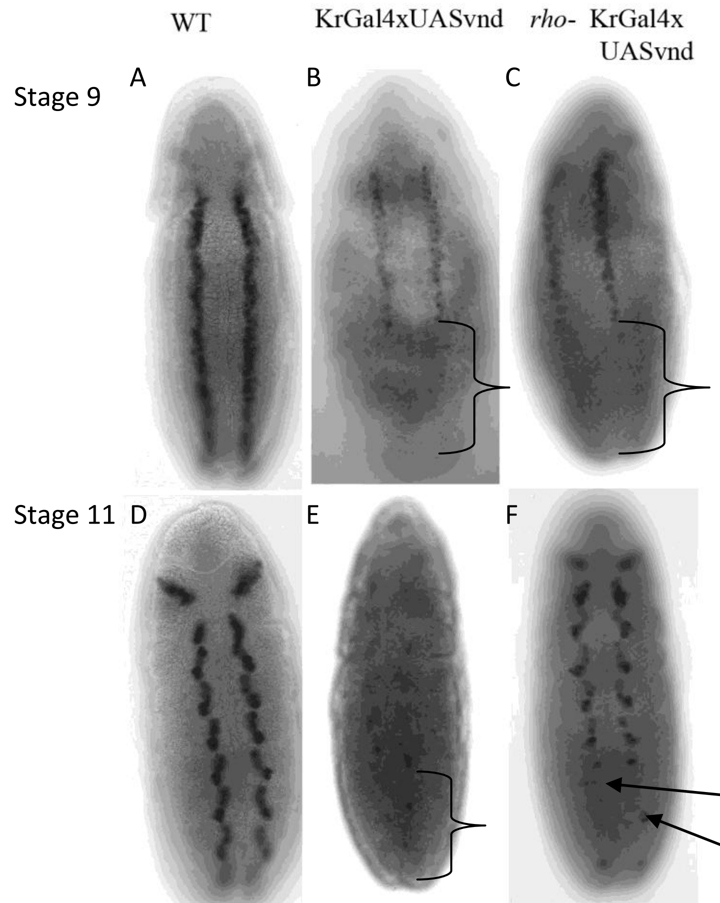

Fig. 5. IND immune-staining shows a loss of vnd inhibition in late stage embryos. Shown is Wildtype (A), KrGal4/Uasvnd (B), stage 9 Krgal4rho/Uasvndrho (C), and stage 11 Krgal4rho/Uasvndrho (D). Areas of inhibition are shown in brackets. Arrows show pockets of derepression.

A similar phenotypic profile was seen in KrGal4Rho/UasvndRho embryos *in situ* stained for the meltrin gene, *neu3*. Meltrin is expressed in the intermediate column in the same general pattern as *ind*. When VND is under KrGal4-controlled promotion, VND can also inhibit *neu3* (Figure 6B). Again, under mutant

rho backgrounds, VND lacks the ability to properly inhibit *neu3* in late stage embryos although in early stages, it can (Fig. 6C, D). This again indicates that *vnd* requires the Rho pathway in later stages because *vein* is no longer active.

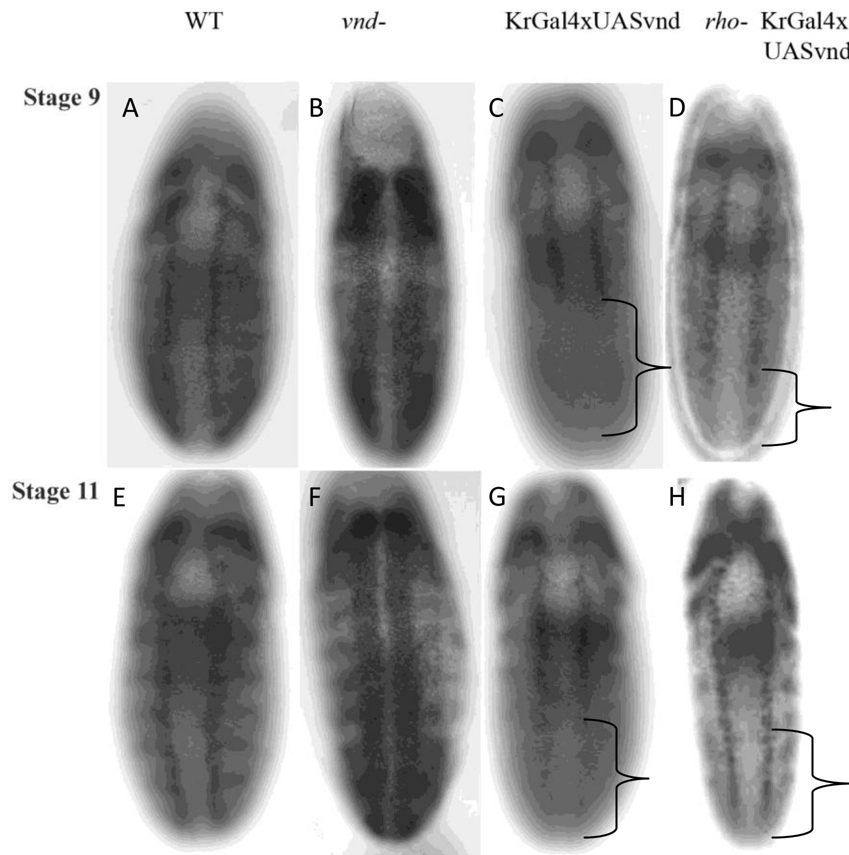

Fig. 6. *In situ* staining for *neu3* reveals a loss in vnd function in late stage *rho* mutant embryos. Shown are the expression profiles of *neu3* in stage 9 or 11 wildtype, vnd$^{-/-}$, Krgal4/Uasvnd, and Krgal4rho/Uasvndrho genetic backgrounds. Brackets show areas of repression and derepression (H).

vnd is hyperactive in an ectopic rho background

To test whether *vnd* has repressive activities in the intermediate column when *rho* is ectopically expressed, we constructed UasRho/+; Krgal4ind/ind embryonic lines. This expresses *rho* ectopically across the KrGal4 domain, allowing Vnd to be phosphorylated in the intermediate domain in late stage embryos. An *ind* mutant allows *vnd* to expand into the intermediate domain (Figure 7E,F). To test Vnd activity in the intermediate column, we stained for neu3 mRNA. As expected, *neu3* is inhibited in the UasRho/+; Krgal4ind/ind genetic background (Figure 6C) but not in the UasRho/Krgal4 background (6B).

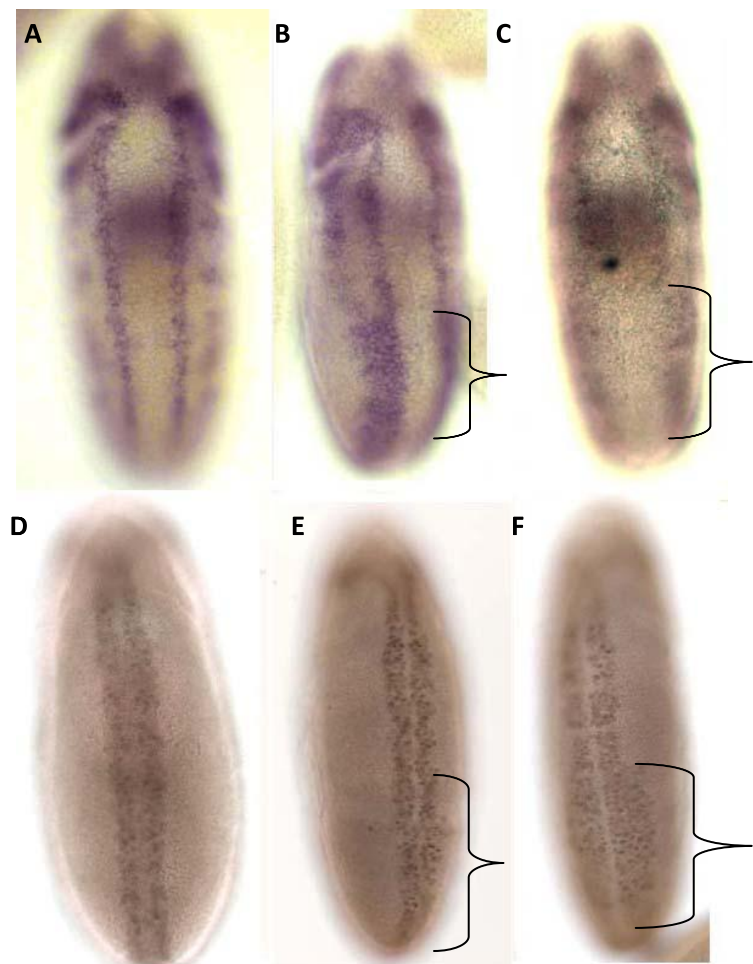

Fig. 7. *In situ* and antibody stains in UasRho/+; Krgal4ind/ind embryos reveal inhibitory function of vnd on neu3 in the intermediate column. *In situ* stains for neu3 in wildtype (A), UasRho/Krgal4 (B), and UasRho/+; krgal4ind/ind (C). Zones of inhibition are shown in backets. Also shown are antibody stains for vnd in wildtype (D), UasRho/Krgal4 (E), and UasRho/+; krgal4ind/ind (E). Brackets show areas of inhibition (C) of *neu3* and expansion of VND (F).

Discussion

The EGF pathway plays a central role in *Drosophila* development. Early studies indicate that an active EGF pathway, run through the DER, must be present to induce *ind* transcription in the intermediate column but not *vnd* in the ventral column. Here we provide evidence that *vnd* requires phosphorylation from a MAPK pathway to maintain it's repressive activities. Futhermore, we also demonstrate that the EGF signals through two distinct pathways with precise timing mechanisms. The first pathway is a two step signal involving the Rho-dependent activation of Spitz. Active Spitz is one of the ligands for DER, the receptor for EGF. MAPK signals from the DER can then phosphorylate effector proteins for transcriptional control. The second pathway involves a ubiquitously expressed ligand, Vein. This ligand is expressed evenly in the intermediate and ventral columns until stage 10 when *vein* signaling shuts down leaving the Rho-Spitz pathway as the sole regulator of MAPK activity (Figure 8).

We provide significant genetic and physical evidence that *vnd* phosphorylation upregulates its repressive activity in the intermediate column. We have identified a MAPK target via *in vitro* MAPK assays. Phosphorylation shifts show that Vnd can be phosphorylated by p42 MAPK *in vitro*. Mutational analysis reveals that S46 and T49 are possible MAPK targets. Further mutational analysis and standardization is required to properly indentify the MAPK target but this is the first evidence that VND is phosphorylated by p42 MAPK. S46A and T49A double mutational analysis would provide an accurate insight into this residue targeting by MAPK. Regardless, VND is clearly capable of undergoing phosphorylation by the MAPK downstream from DER.

Fly lines with DER and Rho knockouts reveal that *vnd* expression does not depend on phosphorylation from MAPK. However, *vnd* function may require MAPK phosphorylation. Ubitquitously expressed *vnd* requires an active MAPK pathway to inhibit both *neu3* and *ind* in late stage embryos. Ectopically expressed *vnd* with an active MAPK pathway do not share the same phenotype indicating that *vnd*-

mediated inhibition of intermediate column proteins by *vnd* requires phosphorylation. This is further confirmed in UasRho/+ ; Krgal4ind/ind mutant embryos where *vnd* expansion into the intermediate column occurred. In this case, *neu3* expression is inhibited by *vnd*.

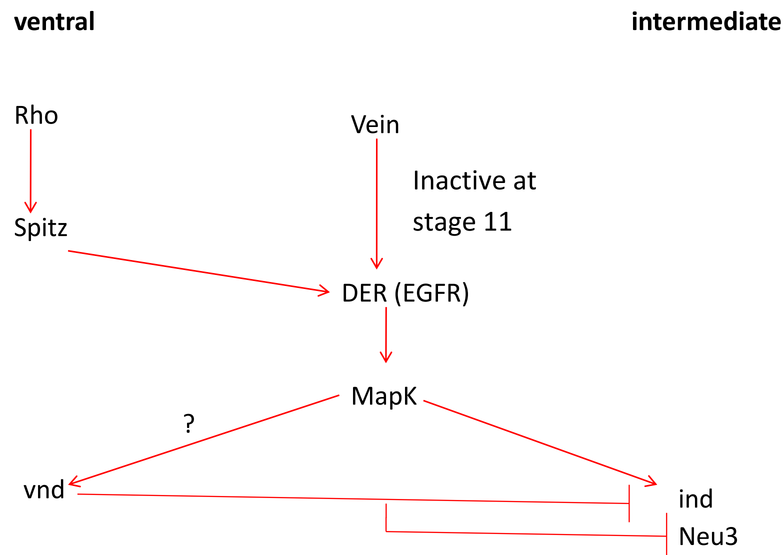

Fig. 8. MapK signaling in the ventral and intermediate columns of *Drosophila* embryos. Vein signaling is deactivated at stage 11.

Late stage embryos seem to show a decrease in *vein* signaling. *Rho* mutant embryos, stained for *ind*, reveal an expansion of *ind* expression into the ventral column. This is evidence that *vnd* requires phosphorylation to prevent *ind* expansion into this column. This also demonstrates that *vein* signaling shuts down sometime between stage 9 and 11 leaving Rho as the only active EGF pathway. *DER*

mutants, stained for *ind,* show a loss of function phenotype in stage 9 embryos as seen in figure 3. However, in stage 11 embryos, *ind* expression begins to turn back on. Interestingly, the small pockets of *ind* expression are only seen in the ventral column, not the intermediate column. This provides evidence that *ind* expression is activated by an unknown activator in the intermediate column. This activator requires phosphorylation in the intermediate column to initiate *ind* expression in wildtype embryos. It is also very possible that this activator is repressed by phosphorylated *vnd a*nd expands into the ventral column when *vnd* is not functional. This would explain why *ind* expression is seen in the ventral column in *DER* mutant embryos only in this case, phosphorylation of the activator, is not required to induce *ind* expression.

Recent studies have indicated that phosphorylation of the *vnd* co-repressor, Groucho, down regulates Groucho and thus *vnd* activity (Cornell, Von Ohlen. 2000; Paroush. Personal Communications). The results of this study partly contradict this. It has been found that phosphorylation of Groucho by EGFR is, at the very least, partly required for *ind* promotion (Paroush. Personal Communications). In fact, r*as* mutants, which show a similar *ind* phenotype to *DER* mutants, are partly restored when Groucho is knocked-out in tandem (Paroush. Personal Communications). However, because the phenotype is not fully restored, a Groucho-independent mechanism must induce *ind* expression, and that mechanism requires signals from the EGF-R. Futhermore, Groucho appears to maintain its phosphorylated state even when EGF-R signaling has ceased. This indicates that Groucho remains inactive for several hours after phosphorylation from MAPK. If *vnd* inhibition of *neu3* and *ind* depends upon Groucho function, then phosphorylation of Groucho from the *vein* pathway should continue to have an effect on Groucho function well into stage 11 embryos. We did not see that effect, which indicates that either *vnd* inhibition of *ind* and *neu3* is a Groucho-independent process or Groucho phosphorylation is inhibited by an unknown mechanism in the ventral column. It is possible that Groucho cannot be phosphorylated due to the heterodimeric conformation it forms when complexed to *vnd*. It is also possible that Groucho

is stoichiometrically inhibited and does not play a significant role in the *vnd*-mediated inhibition of *ind* and *neu3*. Clearly, more research is required to elucidate this contradiction.

In conclusion, *vnd* inhibition of *ind* and *neu3* is a mechanism under the control of MAPK signaling. There is a strong possibility that *vnd* function is partially or entirely controled by the EGF pathway despite the fact that *vnd* expression is not. Signaling to the DER is done through two pathways: 1. Spitz is cleaved from the membrane by Rho which signals to the DER; and 2. Vein signals directly to the DER. The first pathway is active in the ventral column, and the second pathway is active in both the ventral and intermediate column but becomes inactive sometime during stage 10. This pathway is likely important in *vnd*-controled regulation of neuroblast formation in the ventral column.

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