Sorbs3 Modulates ErbB3 Signaling Required for Dorsal Root Ganglia Development

Sarah Jeanine Malmquist

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Reading Committee:

David Raible, Chair

Cecilia Moens

Ajay Dhaka

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Abstract

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Sarah Jeanine Malmquist

Chair of the Supervisory Committee:

Professor David W. Raible

Department of Biological Structure

The process of development, through which a single fertilized egg becomes a multicellular adult organism, is one of the most fascinating aspects of life. With intricate molecular guidance, thousands of specialized cell types arise from multipotent progenitors in spatiotemporal precision. In the first chapter of this dissertation, I review the processes that shape the development of one group of cells, the sensory neurons and glia that comprise the dorsal root ganglia. These cells types are derived from the multipotent and migratory neural crest. As such, the study of their development provides a beautiful model for understanding the coordination of the processes of migration and differentiation, common to many aspects of both development and disease progression. In the second chapter, I review how the ErbB receptor tyrosine kinases control several migratory processes in development. Sorbs3 is a scaffold protein that has been implicated in many aspects of cell migration and cell signaling; I

review the processes shaped by this protein in the third chapter. In the fourth chapter, I present a study of the identification of a mutant lacking dorsal root ganglia neurons, and the identification of a role for Sorbs3 in mediating ErbB-dependent development of these neurons. Finally, I present implications of my work to development and disease, along with a discussion of open questions remaining in the field in the fifth chapter.

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Chapter I

The Development of the Sensory Dorsal Root Ganglia

INTRODUCTION

In the beginning, we were all single cells. Then, through the patient work of billions of years of change, we, too, were changed into the finest examples of the endless forms most beautiful that populate our world. Our original cell became two, then four; our tissues and even minds were shaped through the intricate process of development. Understanding this process is understanding who we are.

Of course, differentiation is a crucial step in the process of developing a multicellular organism. In differentiation, cells respond to environmental cues that direct them toward the assumption of a single cell fate. Pluripotent cells lose the ability to assume all fates as they become increasingly lineage restricted, progressing down the path toward a single differentiated state. Ectodermal progenitors first chose to become neuroectoderm, then chose glial fates and finally begin to express proteins found in myelinating Schwann cells, for example.

Integral also to development, and many times linked to differentiation as a driving force for development, is the migration of cells from their original location in the embryo to sometimes distant environs. In even the simplest organisms, migration and differentiation are often linked. In the echinoderm embryo, for example, primary mesenchyme cells delaminate from an apical layer into the blastocoel and differentiate into the primitive skeletal cells, a process which allows larvae to develop a basic skeletal system (first described by Dan and Okazaki, 1956; reviewed in Kominami and Takata, 2004). Strikingly, coordination of these same two mechanisms is what allows the human brain to be perhaps the most complex biological structure in the universe: neural progenitor cells differentiate and migrate sometimes long distances to their final locations, forming precise connections that allow for higher order cognition.

In addition to the spatial and temporal coupling of migration and differentiation, these processes are also physically coupled in many cell types. Exquisite signal amplification, interaction and coordination allows the same signals to be translated into both cellular motion and gene expression; for example, the same transcriptional regulators control both the neuronal identity and laminar location of cortical neurons (reviewed in Kwan et al., 2012). Understanding how the coordination of these processes can direct developmental events will not only allow us to grasp the beauty of the developmental process, but provides hope to the amelioration of diseases, such as cancers, in which the intricate mechanisms of the development are recapitulated in dysregulation.

The first chapter of this dissertation contains a review of processes involved in the migration and differentiation of one class of cells, the neurons of the dorsal root ganglia (DRG) in the zebrafish, from the early steps of specification through migration and differentiation, as well as some of the unanswered questions remaining in the field. In Chapter 2, I review the processes through which one group of proteins, the ErbB receptor tyrosine kinases, function in the migration of several cell types during development. In Chapter 3, I review how one gene, *sorbs3*, controls several of the molecular aspects of cell migration. Finally, I detail a series of experiments I performed to examine how *sorbs3* works to pattern the developing DRG neurons through the ErbB activity in Chapter 4 and discuss the future of this research briefly in Chapter 5.

THE NEURAL CREST

The neural crest (NC) is a useful model for studying the basic processes of migration and differentiation that are crucial to the development of all vertebrate tissues. It is a multipotent progenitor population found in all vertebrates that migrates after its specification and differentiates into diverse tissues that have been classically attributed to all three primary germ layers. While all vertebrates have NC, recent evidence suggests that lower chordates, even those that lack migratory NC-like cells, also express markers of this tissue (Martinez-Morales et al., 2007, reviewed in Hall and Gillis, 2012). Early vertebrate ancestors probably co-opted these genes originally used in other developmental processes to function in the development of the NC. The

appearance of markers of the NC in the evolution of chordates appears to be coincident with the expansion of the anterior part of the nervous system into a complicated brain, and with the switch from filter feeding to using the jaws for eating (Gans and Northcutt, 1983). Most of the facial bones and musculature, as well as the placodes that develop into the paired sensory organs and nerves of the face have a significant NC component, and their development seems crucial for supporting a central anterior nervous center.

In addition to the facial bones and musculature, NC gives rise to all of the peripheral sensory nerves and all of the glia of the peripheral nervous system. Pigment cells that populate the skin are also derived from the NC, as is the enteric nervous system and the autonomic neurons of the sympathetic ganglia. In addition, the NC gives rise to numerous ectomesenchymal derivatives: dentin-producing cells in teeth, parts of the heart and endocrine cells of the adrenal medulla, thyroid, intestine and carotid artery. While it has long been known to contribute to the mesenchyme of unpaired fins in fish, it has recently been suggested to give rise to the plastron, or ventral shell, of turtles and the ventral bones of alligators (Gilbert et al., 2007). The NC precursors to these cell types all migrate from their initial site of specification, the junction of the forming neural tube and the overlying ectoderm, to more ventral locations, and in some instances transverse nearly the entire axial length of the developing embryo before reaching their final destinations.

NEURAL CREST SPECIFICATION

NC development is marked by the activity of three groups of genes, timed together and coordinated with each other, to control the induction, migration and specification of derivatives. Another hallmark of NC development is the reiterated use of genes, first in induction, then migration, then differentiation. In this chapter, I will focus on the development of the zebrafish NC, drawing on data obtained other models to provide insights as well.

FGF, Wnt, BMP and Delta/Notch signaling have all been implicated in the induction of the NC in tetrapods. The NC is induced at the margins of the neuroectoderm, and this process is controlled by notochord-modulated BMP signaling. The notochord secretes BMP antagonists, such as chordin and noggin, which reduce

the levels of BMP activity in the areas directly dorsal to it. High levels of BMP activity at the regions most distal to the notochord induce the formation of non-neural tissues, low levels induce the formation of neurectoderm, and the NC arises at intermediate concentrations of BMP signaling received at the neural plate border. In the anterior, a tissue called the preplacodal ectoderm is induced between the non-neural ectoderm and the NC; this will, with NC, contribute to the paired sense organs of the head. This gradient of BMP signaling allows for the formation of different tissues, including neural tube, NC, preplacodal tissue in the anterior and Rohon-beard sensory neurons in the trunk, and at the highest concentrations non-neural ectoderm. When BMP4 or BMP2 are depleted in zebrafish embryos, NC fails to develop (Neave et al., 1997; Nguyen et al., 1998; Barth et al., 1999), consistent with data from other vertebrates. Similarly, early inhibition of BMP signaling by overexpressing the inhibitor *chordin* leads to dorsalization and loss of the NC **(**Tucker et al., 2008). Fine perturbations of the BMP gradient can lead to expansion of one of the tissue types controlled by BMP at the expense of others. For example, when high concentrations of the BMP inhibitor dorsomorphin are added to embryos, it completely blocks the formation of NC, but low concentrations allow the expansion of the NC domain into surrounding tissues including the preplacodal ectoderm, consistent with the gradient model (Kwon et al., 2010).

When all of the secreted BMP agonists *chordin*, *noggin*, and *follistatin* are removed, crest still is able to develop (Ragland and Raible, 2004), suggesting that other signals or mechanisms are also required for the induction of zebrafish NC. It has been proposed that overlapping gradients of BMP and FGF signaling work together to pattern the zebrafish embryo, both dorsoventrally and anteriorposteriorly and to induce NC (Kudoh et al., 2004). Experiments in *Xenopus* have demonstrated that the interactions between Wnt and FGF signaling are also involved in induction of the NC (Monsoro-Burq et al., 2005; Hong et al., 2008a). In the zebrafish, Wnt is signaling required for NC induction, but not for most later events in NC development; blocking signaling through Wnt8 at early timepoints blocks induction of NC in embryos, but later blocks do not prevent subsequent developmental steps (Lewis et al., 2004). While the exact mechanism of NC induction through these signals may differ slightly in the zebrafish, it undoubtedly relies on the actions of Wnt, FGF and BMPs, and is probably close to the

model proposed in the avian gene regulatory network of Betancur et al. (Betancur et al., 2010).

After the earliest steps of NC induction occur, two more sets of genes are required for the maturation of the NC; while the basic principles of their actions are the same, there are some differences between species in their action (reviewed in Stuhlmiller and García-Castro, 2012). Coordinated action of Wnt/BMP and FGF signaling results in the upregulation of a group of genes termed neural plate border specifiers, including Msx1/2, Pax3/7, Zic1, Dlx3/5, Hairy2, Id3, and Ap2 in birds and mammals**.** Many of these genes are controlled in the same way in zebrafish. For example, several enhancer sequences that promote the expression of NC genes *pax3a* and *zic3* are controlled by partially overlapping action of Wnt, BMP and FGF signaling in zebrafish (Garnett et al., 2012). In the zebrafish, the transcription factor *prdm1a* and the viral gene *crestin* are also required for neural plate border specification (Artinger et al., 1999; Hernandez-Lagunas et al., 2005; John and Garrett-Sinha, 2009; Rubinstein et al., 2000; Luo et al., 2001). Upregulation of the neural plate border specifiers is followed shortly by that of other genes called NC specifiers, which include Snail2, FoxD3, Sox9/10, Twist, cMyc, and Ap2 in mammals and birds; a similar set of genes are controlled by the neural plate border specifier genes in zebrafish. For example, morpholino oligonucleotide knockdown of zebrafish Msx homologs results in downregulation of *foxD3*, *snail2*, *sox10* and *pax7* expression and loss of NC, along with loss of the anterior preplacodal ectoderm (Phillips et al., 2006). NC specifier genes control the behavior of NCCs in such aspects as migration, survival and differentiation. Loss of the NC specifier genes causes loss of some or all NC in the zebrafish (Thisse et al., 1995; Lister et al., 2006; Stewart et al., 2006; Carney et al., 2006; Yan et al., 2005; Hong et al., 2008b; Li et al., 2003; Li and Cornell, 2007; O'Brien et al., 2004; Knight et al., 2005; Hoffman et al., 2007).

Several different subpopulations of NC arise at different levels and give rise to different derivatives, which will be discussed later. The signals involved in specification of the NC differ somewhat at different axial levels of the developing embryo, and these differences reflect differences in neural tube formation and other developmental processes along the rostral-to-caudal axis of the developing organisms (reviewed in

Duband, 2010). The complicated derivatives of the NC make it crucial for understanding development and disease. It is also a useful system in which to study the migration and differentiation of cells in living animals responding to extracellular signals.

The cranial NC arises in the midbrain and hindbrain regions and gives rise to diverse structures of the head region of the zebrafish (reviewed in Klymkowsky et al., 2010). Three streams, from rhombomeres 1/2, 4 and 6/7 migrate ventrally, populate the pharyngeal arches and differentiate into the bones, muscles and connective tissue of the zebrafish face and endocranium, as well as the fifth, seventh, ninth and tenth cranial nerves. Another anterior stream that migrates near the developing eye also contributes to the developing endocranium. Cranial NC-derived pigment cells populate the face and neck, and cranial NC also contributes to the development of the ear, teeth, and other structures. Cells from the cranial NC mix with placodal cells to produce paired sense organs of the head.

The trunk NC is derived along the length of the body from about the 2nd somite through the end of the tail. It gives rise to both the pigment cells that populate the skin and much of the peripheral nervous system, including the neurons and glia of the dorsal root and sympathetic ganglia, along with the peripheral glia associated with the CNS-derived motor roots. Trunk NCCs can take either a lateral path of migration, traversing from dorsal to ventral in a path distal to the developing somite, or a more medial path, travelling between the somite and the neural tube. Those that take the lateral path for the most part differentiate into pigment cells. In the medial path, precursors migrate from dorsal to ventral in streams, one per somite, following approximately the same track as the previously deposited motor axons. Some NC precursors pause at a more dorsal level in discrete clusters; these differentiate into the neurons and glia of the dorsal root ganglia. Trunk NCCs that travel more ventrally, to the level of the dorsal aorta, become the sympathetic ganglia. Upon reaching their axial level, they do not initially form discrete clusters, but are dispersed widely along the entire width of the somite. Some degree of mixing of precursors descended from different streams occurs before the ganglia later coalesce.

The enteric nervous system is derived from NC specified at the most posterior segments (the sacral NC), as well as from the vagal crest that arises from the neck region of the zebrafish embryo. These populations of cells migrate throughout the length of the developing gut and populate it with the neurons and glia that regulate the peristalsis of the digestive system (reviewed in Shepherd and Eisen, 2011). Finally, a group of precursors specified in rhombomeres 1-6 have been mapped to fates including the cardiac outflow tract and cardiomyocytes, which is a greater contribution to the heart than that provided by avian or mammalian cardiac NC (Sato and Yost, 2003; Li et al., 2003). Induction of this group of cells is at least partially dependent on Wnt3a (Sun et al., 2008).

NEURAL CREST CELL MIGRATION AND FATE DECISIONS

Much of our understanding of the general mechanisms cells use to migrate and differentiate comes from the study of the NC. Analysis of migrating and differentiating NCCs *in vivo* offers a unique opportunity to examine the mechanisms cells use to move and differentiate in the context of the whole developing animal. Much remains to be understood of the developmental processes that occur within the NC. In addition, understanding the mechanisms of NC migration and differentiation holds promise for the development of NCC-based therapies for injuries such as stroke and neurocristopathic diseases.

General Mechanisms of Epithelial-to-Mesenchymal Transitions

Before migration, the NC undergoes an epithelial-to-mesenchymal transition (EMT), a process through which stationary cells are transformed into motile ones through changes in their behavior, morphology and gene expression. In this process, NCCs become competent to delaminate, or leave the neuroepithelium in which they have been specified. This same process is integral to developmental events including gastrulation and neurulation, as well as in the invasive motility of metastatic cancer cells (reviewed in Lim and Thiery, 2012; Kawauchi, 2012). Many of the NC specifier genes are involved in a complex regulatory network that, in part, regulates EMT (reviewed in Duband, 2010; Betancur et al., 2010); while there are some differences between species, many of the genes have the same action in zebrafish as in other species.

In EMT, certain adhesion molecules are downregulated, allowing junctions with neighboring cells to be broken. Members of the Snail/Slug, Twist and Zeb families of transcriptional repressors are responsible for the downregulation of molecules such as E-cadherin, tight junctional complex members and polarity factors (reviewed in Peinado et al., 2007; Barrallo-Gimeno and Nieto, 2005; Vandewalle et al., 2009; Wheelock et al., 2008). These genes are all part of the suite of NC specifier genes: their inclusion in this group highlights the requirement of EMT in the early development of the NC. Other adhesion molecules, such as N-cadherins, are upregulated during EMT, and this promotes the formation of adhesion complexes characteristic of motile cells. The change in the ratio of E-cadherins to N-cadherins that occurs during EMT has been termed the "cadherin switch"; besides transcriptional regulation of the genes for the cadherins, the switch often also involves changes in their stabilization at the cell surface by proteins such as p120catenin (reviewed in Wheelock et al., 2008). In zebrafish, NC fails to migrate when N-cadherin is overstabilized by the protein Ovo, for example (Piloto and Schilling, 2010).

In addition to the N-cadherin that is ubiquitous to all neural tube cells, mice, birds and zebrafish NCCs express an extra N-cadherin, termed cadherin-6 or -6B, and its regulation may play a role in the delamination process (Inoue et al., 1997; Nakagawa and Takeichi, 1995; Liu et al., 2008). After delamination, the NC again begins to express E-cadherins. The "reverse" cadherin switch from the N-cadherins, Cadherin-6 and -6B to the E-cadherin Cadherin-7 after delamination of the NC has been relatively well studied in chick (reviewed in Duband, 2010), but little is known about this process in zebrafish. Cadherin-7 is not expressed in zebrafish NC, and the E-cadherin involved in the switch remains to be found (Espeseth et al., 1998; Liu et al., 2008). In *Xenopus,* loss of cadherin-11 prevents NCCs from migrating, and expression of a version lacking the extracellular domain induces precocious migration (Hadeball et al., 1998; Vallin et al., 1998); it is likely that cadherin-11 could have the same function in *Xenopus* as Cadherin-7 in mouse. Although cadherin-11 has been implicated in the development of tissues derived from preplacodal ectoderm in zebrafish, its role in the migration of the NC has not been examined (Clendenon et al., 2009; Clendenon et al., 2012).

Besides the cadherin switch, other processes are also important for EMT, and genes required for these processes affect the delamination of the NC. Many of these processes are tied to the cadherin switch. Cadherins and junctional complexes regulate the small GTPases that are crucial for cell motility. Changes in cell-matrix adhesion also are important for NCC delamination. This is accomplished through action of matrix metalloproteases to degrade the basal lamina, changes in expression of basal lamina components, and regulation of integrins that alter adhesions between the cell and the matrix (reviewed in Duband, 2010). Understanding the complex regulation of EMT in NCCs is critical for understanding how cells later transition between a migratory population and a stationery differentiated population, and a model showing the molecular changes known to accompany it is shown in Figure 1.

Cell Migration and Focal Adhesions

The process of NC migration is dependent on actin and myosin-based cell contractility (reviewed in Clay and Halloran, 2010). The actin cytoskeleton is connected to the extracellular matrix through which cells move via focal adhesions, integrin-based structures that both coordinate movement via actin and coordinate signaling via the localized activity of receptor–ligand complexes. During migration, cells form actin-based protrusions at their leading edges and then form transient integrin based focal contacts that link the cytoskeleton and the matrix; these complexes can be solidified into focal adhesions when signals direct cells to slow their migration (reviewed in Ridley et al., 2003). Since NC migration and differentiation are tied, and since different structures form at different axial levels, proper migration and cessation of it must be coordinated in order for the correct development of NC-derived tissues. The activity of focal adhesions can coordinate the movement of cells, their directionality and their ability to stop moving when required.

While recent work has begun to characterize migration of NCCs in vivo through timelapse imaging (reviewed in Clay and Halloran, 2010), relatively little is known about the molecules that actually perform the work of migration in NCCs, although some data has implicated focal adhesion components in NC migration. In the zebrafish, integrins are required for the migration of enteric NC and for the development of the crest-derived

facial cartilage (Nagy et al., 2009; Crump et al., 2004). Integrin is also upregulated in migrating NCCs (Duband and Thiery, 1990) and is required for NCC migration in other systems (Alfandari et al., 2003; Strachan and Condic, 2003). Neuroblastomas, highly metastatic cancers derived from the NC, have elevated levels of integrins and their migration depends on the activity of Focal Adhesion Kinase (FAK; Wu et al., 2008). Vinculin, a core component of focal adhesions, is expressed in migrating NCCs and is upregulated as the cells progress through migration (Duband and Thiery, 1990). Additionally, focal adhesions containing the protein vinculin have been observed in cultured cardiac NCCs, and disruption of them inhibits migration (Xu et al., 2006).

The interplay between the activities of the small GTPases Rac and Rho coordinates the directional movement of cells (reviewed Hall, 2005; Parri and Chiarugi, 2010). Rac is active at the leading edge of cells to promote lamellipodia formation, and Rho is active at the retracting tail. In general, the downstream effectors of Rac on the leading edge mediate actin polymerization, pushing cells forward, while the downstream effectors of Rho mediate actin depolymerization, retracting the tails of cells. Rho is also associated with the formation of adhesions and stress fibers mediating more solid interaction between cells and the matrix beginning at the tail. Rac and Rho interact both with cadherin-based cell-cell junctions and with integrin based cell-matrix junctions. Inhibition of either myosin–II or Rho can block the migration of zebrafish hindbrain level NCCs by interrupting the polymerization and localization of actin (Berndt et al., 2008), suggesting that coordination of migration is dependent on the integration of these signals; the exact roles of Rho in NC migration do appear to be context dependent, however, with different populations of crest responding to manipulation of Rho signaling in different ways (Clay and Halloran, 2010). A third GTPase, Cdc42, is thought to mediate cell polarity as well.

In order for the NC to migrate in the ventral direction, an axis of polarity must be established before the small GTPases can do the work of pushing the cell in the forward direction. The small GTPases are activated at the correct parts of the cell when the axis is set up by Wnts and syndecans. In zebrafish and *Xenopus*, Wnt11r is required for NC migration, but not for the induction of NC (Matthews et al., 2008a; De Calisto et al., 2005). FRET analysis of the small GTPases in isolated NCCs has shown that Wnt11r

promotes Rho activity, Syndecan4 activity inhibits Rac activity, and that Rac inhibits Rho (Matthews et al., 2008b). These signals together promote the actin polymerization at the leading edge of the NCCs as they begin to migrate. The link between the activities of these molecules and focal adhesions remains to be elucidated.

Once NCC migration is initiated, it progresses based on attractive cues from targets, repulsive cues from neighboring tissues and interactions with other NCCs that differ between organisms and axial levels (reviewed in Raible, 2006; Clay and Halloran, 2010). In zebrafish, the chemoattractant Sdf1 is required for cranial NC migration into the pharyngeal arches and for proper migration of lateral trunk NC that gives rise to pigment cells, but not the ventromedial crest that gives rise to other tissues (Olesnicky Killian et al., 2009; Svetic et al., 2007). Cardiac NCC migration appears to be enhanced by FGF8 expression in the zebrafish, independent of effects on NC induction (Sato et al., 2011). Chemoattractants guiding the trunk NC are not yet known.

Mutations that disrupt the formation or patterning of the somites result in aberrant NC migration, suggesting that signals from somites are important for patterning the trunk NC (Raible et al., 1992). Live imaging studies have shown that zebrafish trunk NC is repelled by contacts with the lateral somites so that each stream of crest migrates in the center of the somite (Jesuthasan, 1996); this differs from the axial locations of migration within the somites of other organisms, but the mechanisms guiding this repulsion are not known. Cranial NC migration is restricted to certain zones by Semaphorin signaling (Yu and Moens, 2005), but no such mechanism is known to restrict trunk NCCs to the correct positions for migration in the zebrafish.

Is NC Migration Collective Cell Migration?

Thevaneau and Mayor (2011) have proposed two criteria for deciding whether cells are moving as part of a collective: first, cells are migrating in a coordinated directional fashion in such a way that the movement of cells within the group correlates in some way with the average movement of the group as a whole, and second, cell-cell interactions affect the migratory behavior of the cells within the group. Similarly, Rørth defines collective migration as “collections of cells moving together and affecting one another while doing so” (Rørth, 2012). Under these definitions, cells migrating as part

of an epithelium are considered collectively migrating cells, while mesenchymal cells may or may not be collective. The migrating NC takes on different characteristics depending on both the axial level and organism, and can therefore fall into different levels on the gradient of collectiveness, with some populations of neural crest having more influence on each other during migration than others.

In some cases, it is clear that NC does migrate collectively. In the frog, the cephalic NC exhibits many hallmarks of an epithelium at the beginning of migration; both *in vitro* and *in vivo* relatively stable cell junctions and polarization of Rac1 to the leading edge allow the cells to pull each other along, even those that are individually incapable of migration due to lack of protrusions or cell cycle state or are not responding to guidance cues (Theveneau et al., 2010). Non-canonical Wnt/PCP signaling is required for the polarization and directional migration of this cell type *in* vivo and *in* vivo*,* and results in upregulation of RhoA at sites of cell-cell contact (De Calisto et al., 2005; Carmona-Fontaine et al., 2008b). Wnt11r is required for the migration, but not the induction, of *Xenopus* NC (Matthews et al., 2008a). Cell-cell interactions in this population promote what is known as “contact inhibition of locomotion”, a phenomenon described over 50 years ago in fibroblast cells, where encounters with other migrating cells cause cells to pause and change their direction of migration (Abercrombie and Heaysman, 1953). The "collectiveness” of the *Xenopus* cranial NC is actually required for its response to the chemotactic factor Sdf1, as individual cells cannot migrate as well towards Sdf1 as groups of cells, and since depletion of N-cadherin activity disrupts directional migration (Theveneau et al., 2010). As migration progresses, the cells take on more mesenchymal characteristics, but still maintain at least some degree of contact that allows them to continue to move as a group.

Based on the criteria in (Theveneau and Mayor, 2011), the vagal crest that populates the intestine and the dorsolateral trunk NC that gives rise to pigment cells are not true examples of collective cell migration. In both of these cell populations, cell spreading to populate the tissue seems to be dependent on repulsion of cells from one another; when the number of cells is depleted, the distal regions are not populated by NC. For example, when embryos are injected with a morpholino oligonucleotide against *phoxb2* that blocks the development of enteric neurons, the distal regions of the hindgut

are more affected than the proximal regions (Elworthy et al., 2005). In *sox10* mutants, the distal region of the large intestine is not colonized by the NC, leading to disorders such as aganglionic megacolon (reviewed in Mollaaghababa and Pavan, 2003). While a limited number of transient contacts have been observed and modeled in the migrating enteric crest (Young et al., 2004; Simpson et al., 2007; Landman et al., 2007), contacts do not appear to influence the migrating behavior of other cells.

The avian trunk NC does not begin its migration as an epithelium, but as a true mesenchymal population (reviewed in Kuo and Erickson, 2010). It does, however, exhibit cell-cell interactions, a defining feature of collective migration. Timelapse imaging of avian trunk NC has shown that cells often migrate in chains, and extend protrusions to contact the cells in front of and behind them (Kasemeier-Kulesa et al., 2006; Kulesa and Fraser, 1998). Early experiments in cultured NC show that cell-cell interactions are required for directional migration of avian trunk NC *in vivo* (Rovasio et al., 1983). In concordance with these data, computational models of this system have predicted that, while an ECM "path of least resistance" may promote the directional migration of NCCs toward cues, it is not sufficient to drive it; cell-cell contact is crucial to this process for both leading and following cells to travel on the correct path in the correct direction (Wynn et al., 2012). The chain-like migration of trunk NCCs in *Xenopus* and zebrafish appears, like the frog cephalic crest, to be dependent on non-canonical Wnt/PCP signaling and the control of small GTPases Rac1 and Rho (Matthews et al., 2008b; Carmona-Fontaine et al., 2008a), providing further evidence that these cells may be indeed migrating collectively. Timelapse imaging of zebrafish ventromedial trunk NC has also shown what appears to be chain-like migration (Prendergast et al., 2012), but further analysis of the behavior of migrating cells will help determine whether or not cell-cell contacts are present throughout this migration.

What Comes First: Migration or Specification?

Many early experiments using chick-quail chimeras led to the hypothesis that the NC was multipotent and that cells transplanted from one axial location to another would adopt new fates characteristic of their new surroundings (reviewed in Le Douarin, 1999); this supports the idea that NCCs respond to cues in their environment with respect to

their fates. When researchers labeled individual premigratory cells in chick (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989), mouse (Serbedzija et al., 1994) or *Xenopus (*Collazo et al., 1993), they again found that cells often gave rise to clones of cells that sometimes included multiple cell types, suggesting that specification could and did happen during or after migration had commenced.

In birds, several experiments have suggested that the NCCs that become pigment cells are fate restricted, as well as restricted to the dorsolateral migratory path, before they leave the neural tube (reviewed in Kuo and Erickson, 2010). The cells that enter the dorsolateral migratory path begin to do so later than the cells that enter the ventromedial path in avians, mice and zebrafish; this could mean that the time of exit regulates fate, or that prespecification controls the time of exit. Indeed, a lineage-restricted neural population appears earlier than a lineage-restricted pigment precursor population, although unspecified progenitors capable of generating both cell types exist after the onset of migration as well (Henion and Weston, 1997). Once cells begin to express markers of either neuronal or pigment precursors, however, their fates appear to be restricted to that lineage (Luo et al., 2003). In contrast, markers of glial fates appear to be expressed early in most NCCs, but expression becomes progressively restricted to those that will give rise to glia (Henion et al., 2000).

Experiments in zebrafish suggest that some degree of specification may happen earlier in this system. Individually labeled zebrafish cranial NC precursors usually contributed to a single pharyngeal arch and a single cell type, and potency was somewhat dictated by their position in the NC zone, with neurogenic cells found more laterally and chondrogenic cells found nearer to the developing neural tube (Schilling and Kimmel, 1994). Individually labeled zebrafish trunk NCCs most often contributed to a single cell type as well, and sometimes expressed markers of that cell fate during migration; around 20 percent of cells did form multiple types of progeny, but usually first gave rise to type-specific daughters that then proliferated (Raible and Eisen, 1994). These experiments highlight possible differences in the timing of specification and migration in zebrafish, but also demonstrate that cell fate is not completely set prior to the onset of migration.

General Mechanisms of NC Fate Specification

In addition to specification prior to migration and to migratory location controlling fates, there are some additional general mechanisms known to govern the differentiation of NC cells into their final fates. Lateral inhibition, particularly through the Notch-Delta receptor/ligand system, influences the fates of cells once they reach their postmigratory locations. Through this two-way signaling system, neighboring cells prevent each other from assuming the same fate. Depleting Notch signaling results in a gain of Rohon-Beard neurons at the expense of NC, for example (Cornell and Eisen, 2002). In the zebrafish DRG, the balance of neurons to glia is controlled by Notch; when notch signaling is perturbed, more neurons differentiate than normally would (McGraw et al., 2012). Additionally, lateral inhibition signaling is also important in other NC populations and perturbation of it results in gains in some derivatives at the expense of others (Mead and Yutzey, 2012; Tsarovina et al., 2008; Singh et al., 2011; reviewed in Cornell and Eisen, 2005).

The reiterated activation of transcription factors is also characteristic of the development of many NC tissues. Prime examples of this phenomenon are the SoxE transcription factors Sox8, Sox9 and Sox10, which control multiple aspects of NC development, and are required for the development of NC and non-NC derivatives in all vertebrates (reviewed in Kelsh, 2006; Stolt and Wegner, 2010; Haldin and LaBonne, 2010). In the zebrafish, several lethal alleles of *sox10* have been isolated, and homozygous mutants show defects in specification of pigment cells, both neurons and glia of the dorsal root and sympathetic ganglia, enteric neurons, and oligodendrocytes as well as defects in NC migration and survival (Kelsh and Eisen, 2000; Dutton et al., 2001; Carney et al., 2006). Sox10 is expressed in NCCs in the trunk prior to and during migration, but expression is lost as differentiation proceeds in most derivatives. The one exception to this is glial cells, where expression is maintained until adulthood; action of Sox10 is required yet again for differentiation of glial cells. Similarly, Sox9 orthologs are required both for the induction of NC and then again later for the differentiation of craniofacial skeleton, otic placode and pectoral fin mesenchyme (Yan et al., 2005). In addition, while *prdm1a* is required for NC specification and regulates *sox10* expression (Olesnicky et al., 2010), 3 homologs of prdm1 are required again, in

combination, for the specification of several types of cranial NC derivatives (Ding et al., 2012).

Finally, the activity of ErbB receptors controls multiple aspects of NC differentiation, as well as other processes in NC development (reviewed in Birchmeier, 2009). In the mouse, Sox10 is required for the expression of ErbB3, a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinase receptors (Sonnenberg-Riethmacher et al., 2001). Similarly, Sox10 controls expression of zebrafish ErbB3 through a conserved enhancer element (Prasad et al., 2011). When ErbB3 or ErbB2, its preferred dimerization partner in the NC, are depleted in mouse, NCCs show defects in migration, and both mouse and zebrafish Schwann cells show aberrant migration (Britsch et al., 1998; Lyons et al., 2005). Similar defects are observed in the *ErbB2* mutant mouse, when the expression of ErbB2 in the heart is rescued allowing the embryos to develop to stages when a NC phenotype can be observed (Woldeyesus et al., 1999). The roles of ErbB receptors in the migration of the NC will be discussed further in Chapter 2 of this dissertation. Deletion of *ErbB3* causes defects in the differentiation of all peripheral glia, including those of the DRG, as well as apoptosis of the cell bodies of the DRG neurons (Britsch et al., 1998; Reithemacher et al., 1997). A partial loss of DRG neurons and sympathetic ganglia neurons is also seen in fish mutant for *erbb3b* or for *erbB2*, as well as a pigment cell defect that is not observed in the mouse (Honjo et al., 2008; Budi et al., 2008). DRG development requires at least two ErbB2/ErbB3 ligands, Neuregulin 1 (Nrg1) and one of two orthologs of mammalian Neuregulin 2, Nrg2a, though signaling through Nrg2b is not required (Honjo et al., 2008). Another isoform of Nrg1, called glial growth factor 2, is also involved in ErbB3-mediated development of the enteric nervous system and promotes gliogenesis, although it has not yet been implicated in enteric neurogenesis (Chalazonitis et al., 2011). Ligand binding and receptor dimerization of ErbB receptors can activate signaling through many different pathways (reviewed in Feigin and Muthuswamy, 2008). It is still unknown which signaling mechanisms downstream of ErbB control NCC migration, differentiation and survival.

ErbB receptors, which will be reviewed in the next chapter, are often localized to focal adhesions and can influence their behavior and cell motility (Kim et al., 2005; Duan et al., 2011; Rafiq et al., 2012). Activity through focal adhesion signaling has the potential to be another common mechanism involved in NC development and differentiation in addition to their more known roles in NC migration, although this research area remains to be explored. Interestingly, cardiac NCCs that give rise to the cardiac outflow tract require focal adhesion kinase (FAK) for differentiation but not for migration (Vallejo-Illarramendi et al., 2009). In addition, integrin signaling through focal adhesions and ErbB activity are known to coordinate other NC developmental processes, such as myelination of Schwann cells (reviewed in Newbern et al., 2011). Taken together with the known reiterated role of ErbB signaling in NC, these ideas support the notion that focal adhesions can be centers of signaling as well as migration coordinators for the NC, and the DRG in particular.

DORSAL ROOT GANGLIA PRECURSOR SPECIFICATION

Cell Types of the DRG

In invertebrates, the peripheral nervous system (PNS) is derived from the ectoderm in the same manner as the central nervous system; vertebrate PNS is, for the most part, derived from the NC. The neurons and glia of the sensory DRG are derived from the NC, as are the neurons and glia of the sympathetic ganglia.

The DRG are composed of the cell bodies of several different types of sensory neurons – thermoreceptors, nociceptors, mechanoreceptors and proprioceptors - as well as at least 2 distinct types of glial cells. In mammals, several different classes of thermoreceptive neurons respond to different temperatures, as well as to the compounds we commonly associate with “hot” or “cold” feelings, such as capsaicin and menthol. Some of these neurons respond to warm or cool, non-noxious, temperatures, while others respond to painful hot or extreme cold temperatures; nociceptive neurons can also respond to other stimuli, such as pH or other painful compounds (reviewed in Dhaka et al., 2006). Mechanoceptive neurons respond to painful, as well as non-painful touch. Proprioceptors detect the relative position of body limbs and are crucial for balance and coordinating movement. Expression of selective ion channels in all of

these types of neurons control which stimuli cause an action potential and subsequent neuron firing (reviewed in Waxman et al., 1999; Scott, 1992). All of these neurons send projections to the periphery, as well as to the spinal cord; different subtypes of neurons project to specific laminae of the spinal cord and integrate sensory information into specific circuits in the central nervous system. The collection of sensory neuron subtypes in zebrafish is just beginning to be understood. Recent work suggests that the same channels may not be responsible for the sensation of the same modalities as in mammals (Low et al., 2011; Prober et al., 2008). Understanding of sensory circuitry in the zebrafish is also in its infancy, but the transparent relatively simple nervous systems of these embryos make them attractive models for its study.

DRG contain both myelinating and non-myelinating glia (reviewed in Le Douarin et al., 1991; Jessen and Mirsky, 2005). Myelinating glia derived from the DRG also wrap the CNS-derived motor root. In many cases interaction with nerve fibers determines whether an individual glia will wrap around the nerve, as a Schwann cell, or will form a Remak bundle, though not wrap the nerve. Other glia act as satellite cells, and serve to provide tropic support to the neuronal cell bodies within the DRG, as well as a potential source of stem cells. Tropic signaling is bidirectional; glia require neuronal signals for their survival as well.

DRG Precursor Migration and Specification

In the first step of cell migratory path restriction that eventually produces the DRG, a population of NC embarks on a migratory path that is closer to the neural tube than the dorsolateral path taken by pigment cell precursors. Depending on the organism, this ventromedial path can be through the developing somites, as is the case for some mouse NCCs, or between the somites and the neural tube, as is the case in the zebrafish. In the zebrafish, trunk cells traverse a path near the center of each somite toward their ventral targets, but in mouse and chick they migrate through the anterior part of the sclerotome and in *Xenopus* they migrate at the posterior part of the somite. Additionally, about half of cells that will become melanocytes, black pigment cells, migrate through the ventromedial path in zebrafish, while precursors to the other pigment cell types in zebrafish and almost all pigment cells in mouse and avians

migrate solely in the dorsolateral path (Kelsh et al., 2009). Almost all *Xenopus* pigment precursors migrate through the ventromedial path and later reach the epidermis by crossing the somite (Collazo et al., 1993). Cues that coordinate migration and fate restriction, therefore, may differ slightly between organisms.

What directs NCCs to the proper path? In most cases, NCCs are not permitted to enter the dorsolateral path, but it appears that cells fated to become pigment cells in avians acquire the special ability to do so as they become fated to the pigment lineage. Cells fated to become pigment from late stage embryos that are transferred to the neural tube in earlier embryos migrate through the dorsolateral pathway earlier than host pigment precursors, suggesting that it is the pigment precursors themselves, not the environment, that undergoes a change to allow this dorsolateral migration (Erickson and Goins, 1995). Expression of Robo 1/2 by neuroglial precursors prevents them from entering the dorsolateral path, but the mechanism rendering pigment precursors refractory to this signal is unknown (Jia et al., 2005). Several molecules that are upregulated as pigment precursors become fate restricted have been implicated in the ability of these cells to traverse the dorsolateral path. Use of alternative forms of the EphB receptors in chicken pigment precursors and neuroglial precursors allows the former to migrate in the dorsolateral path while preventing the latter from doing so. (Harris et al., 2008; Santiago and Erickson, 2002). In the chicken embryo, the ectoderm and dermamyotome express the chemoattractant endothelin-3, and pigment progenitors express the receptor EDNRB2. Misexpression of the EDNRB2 in neural or glial progenitors, which normally express EDNRB3, along with knockdown of Eph/ephrin signaling, neural and glial progenitors to migrate into the dorsolateral path (Harris et al., 2008; Dupin and Le Douarin, 2003).

Once path choice is established, NCCs then must be conduced to migrate segmentally and to pause at the correct axial level. Signals from both the somites and neural tube are important for these aspects of the development of the DRG. When neural tube is transplanted to a different location in the body, or when it is flipped dorsoventrally, the ganglion still always develops near its dorsal aspect in avian embryos (Weston, 1963; Gvirtzman et al., 1992; Spence et al., 1996; Bronner-Fraser and Stern, 1991). It is relatively unclear what molecules expressed by the tissues at the

level of the presumptive DRG are required for these cells to pause and differentiate there, and the pathway may not be conserved across vertebrates. In culture, mouse DRG precursors express CXCR4 and can chemotax to sources of SDF-1, which is expressed along their migratory routes (Belmadani et al., 2005). In chick, however, SDF-1 is not expressed by tissues along the ventromedial path until after migration onset, suggesting that other mechanisms may be at play (Rehimi et al., 2008). The mechanism utilized in zebrafish for guiding NCCs to the dorsal neural tube is an open question. In addition, NCCs that give rise to the DRG migrate further ventrally in relation to the neural tube than their tetrapod counterparts, so signals emanating from another more ventral source such as the notochord, may be important.

Both attractive and repulsive signaling are known to participate in patterning the neuroglial derivatives of the trunk NC. While the evidence for a chemoattractant guiding the migration of the DRG precursors is sparse, it is relatively clear that expression of Sdf-1 by the dorsal aorta allows the CXCR4-expressing precursors of the SG to migrate to this location in mouse and chick (Kasemeier-Kulesa et al., 2010; Saito et al., 2012). Expression of N-cadherin, along with Eph-ephrin signaling allows the initially longitudinally dispersed precursors of the SG to coalesce into discrete ganglia (Kasemeier-Kulesa et al., 2005; Kasemeier-Kulesa et al., 2006). Robo expression by trunk NCCs appears to prevent them from entering the Slit-expressing intestine, while allowing vagal and sacral crest to do so (Jia et al., 2005; De Bellard et al., 2003).

In birds, the DRG always forms in the anterior half of the somite, but the posterior half is required as well; when NC is forced to migrate through a somite that contains two anterior halves or two posterior halves, the ganglion does not properly form (Kalcheim and Teillet, 1989; Goldstein et al., 1990). The coordinated action of F-spondin, versican, and semaphorins expressed in the caudal scleratome appears to restrict the migration of the NC anteriorly (reviewed in Kuo and Erickson, 2010; Theveneau and Mayor, 2012). The anatomy of the zebrafish, however, is not equivalent to that of the avian embryo. Dorsal root ganglion precursors migrate at the anteriorposterior level corresponding to the center of each somite rather than its anterior, and they migrate farther, to the level of the notochord, in the fish, so the signaling mechanisms involved in positioning the presumptive DRG may differ from those utilized by the avian embryo.

Recent work in zebrafish has, however, identified a novel conserved role for muscle-specific kinase (MUSK) in the maintenance of the NC through the correct ventromedial path; without MUSK expression, its ligand Wnt11r or PCP signaling, cell migration is not confined to the center of the somite in zebrafish or to the anterior somite in mice (Banerjee et al., 2011).

The extracellular matrix undoubtedly plays crucial roles in the migration and pausing of the NC. Several molecules are expressed in the path of the migrating DRG precursors, including fibronectin, laminin, collagen and vitronectin, but it is unknown with which the major cell-matrix interactions occur (Erickson and Perris, 1993). Some evidence supports the role of focal adhesions in specific interactions with the ECM. Blocking α4 integrin does prevent a substantial portion of the migration of chick NC, suggesting that either fibronectin or thrombospondin-1 are important ECM components for NC migration (Kil et al., 1998). In FRAP experiments, the fibronectin receptor was found to be a component of focal adhesions in both stable and motile cultured avian NCCs (Duband et al., 1988). Modification of ECM components may also be crucial to DRG development. In the zebrafish RECK mutant, which lacks a matrix metalloprotease, DRG neuron differentiation fails and embryos exhibit a defect in the final positioning of NCCs in the DRG anlagen (Prendergast et al., 2012). RECK is known to interact with ErbB signaling (Hsu et al., 2006; Kitajima et al., 2011), raising the possibility that the two work together to regulate the migration and differentiation of the DRG. This idea is consistent with the observation that DRG precursors in ErbB mutant zebrafish also fail to migrate to the site of the presumptive DRG and instead continue ventrally (Honjo et al., 2008). In contrast ErbB mutant mice show extra precursors in the DRG at the expense of SG cells, although these precursors fail to differentiate as neurons or glia (Britsch et al., 1998). The anatomical differences between organisms undoubtedly contribute to this difference. Regardless, the relationship between the ECM, ErbBs and DRG differentiation warrants further study.

DRG NEURON AND GLIA DEVELOPMENT

DRG neurogenesis

The first known marker in the specification of the DRG neurons in all species is the expression of members of a family of basic-helix-loop-helix transcriptions factors, the *neurogenin* genes. These genes mark cells that are committed to the neural lineage, but have not yet begin to express true neuronal markers, and are therefore often termed proneural genes. In the mouse and chick, an initial wave of migrating NCCs expresses *neurogenin-2* and gives rise to the large-diameter proprioceptive neurons of the DRG. A later wave of *neurogenin-1* (*neurog-1*) expressing neurons gives rise to smaller diameter nociceptive neurons. While loss of both genes leads to loss of all neurons in the DRG, *neurog-1* expression allows later development of the proprioceptive population initially lost in *neurog-2* mutants (Ma et al., 1999; Parras et al., 2002). Both of these genes are known to be direct transcriptional regulators of markers of mature neurons, such as *NeuroD* (Kageyama and Nakanishi, 1997), therefore understanding how they are expressed is crucial to understanding how NCCs adopt neural fates.

The zebrafish genome contains only one neurogenin gene, *neurog-1 (*Korzh and Strähle, 2002), and its expression is necessary for the specification of all sensory neurons in the DRG (Andermann et al., 2002; Cornell and Eisen, 2002). The development of the zebrafish DRG is simple compared to that of the mouse in several ways. Only about a dozen NCCs migrate into each segment, without the waves of migration observed in mice. While many neurons differentiate simultaneously in the embryonic mouse DRG, only one or a few cells initially differentiate as neurons in each zebrafish DRG, with more neurons added throughout the lifetime of the animal (An et al., 2002; McGraw et al., 2012). In addition, relatively little apoptosis is seen in the developing zebrafish DRG, while about half of the neurons that differentiate in the mouse DRG die before birth (reviewed in Marmigère and Ernfors, 2007). Perhaps the addition of a new *neurogenin* gene was crucial for the ability of complex vertebrates to develop complex nervous systems.

neurogenin overexpression is sufficient to cause the development of ectopic neurons in fish, frogs, birds and mice, but it is not sufficient to drive all cells to the sensory neurogenic fate (Blader et al., 1997; Ma et al., 1996; Perez et al., 1997; Lo et al., 2002; Parras et al., 2002; Zirlinger et al., 2002). Other transcription factors, including Brn3a and islet1, have been implicated in DRG neurogenesis, by promoting the expression of markers of mature neurons and preventing cell death in specified neuronal progenitors as well (reviewed in Pavan and Raible, 2012).

It is not clear how neuronal expression of *neurog-1* is initiated, however. Expression of *neurog-1* is regulated by *sox10*, such that in *sox10* mutant zebrafish, most neurons fail to develop, but the few neurons that remain suggest that *sox10* is not completely required. (Carney et al., 2006). In the *sox10* mutant mouse, *neurog-1+* neurons do develop, but then die due to lack of support from glia that are missing in the mutant (Sonnenberg-Riethmacher et al., 2001; Britsch et al., 2001), suggesting that other factors may be involved in the control of *neurog-1* expression. Wnt signaling appears to be upstream of *sox10* in the promotion of neurogenesis. When Wnts are depleted in mouse, *sox10+* precursors fail to differentiate as neurons and expression of a constitutively active form of β-catenin causes cells to stop migrating early and form ectopic ganglia that express *neurog-2 (*Hari et al., 2002; Kléber et al., 2005; Lee et al., 2004). In zebrafish, however, constitutively active Wnt signaling promotes generation of pigment cells at the expense of neurons (Dorsky et al., 1998). Recent work suggests that a model involving repeated action of Wnts at several discrete timepoints prior to, during, and after the onset of migration could resolve these discrepant observations (Hari et al., 2012).

Many unanswered questions remain surrounding the neurogenesis of DRG neurons. It seems that coordinated action of several pathways is intricately regulated to promote neurogenesis. It is unclear how these pathways combine to allow only one neuron to initially differentiate in the DRG of the zebrafish, while many neurons differentiate in waves in other organisms. Understanding how the precursor pool in the zebrafish DRG is regulated to produce neurons at discrete points in development rather than all at once is not only crucial for understanding how this system develops, but may be critical for clinical applications dealing with regeneration of human peripheral nerves.

A simplified model for neurogenesis, as well as initiation of fate choices in other cell types, is shown in Figure 2.

DRG gliogenesis

A major mechanism of gliogenesis in the DRG appears to be repression of *neurog-1* expression, and this is regulated by Notch/Delta signaling. The Notch/Delta pathway of lateral inhibition has been implicated in the development of many types of cells, including CNS neurons and glia (Lathia et al., 2008; Chenn, 2009). In the rodent DRG, Notch is expressed in differentiating neurons, while surrounding cells express the Delta ligand; inhibition of Notch signaling leads to increased differentiation of neurons at the expense of glia, while increased Notch signaling allows fewer neurons to differentiate (Wakamatsu et al., 2000; Tsarovina et al., 2008; Morrison et al., 2000; Kubu et al., 2002; Taylor et al., 2007). In addition, when Notch signaling is perturbed, markers normally restricted to either the neuronal or glial lineages are coexpressed, suggesting that cells that begin the initial program of gliogenesis are redirected toward the neural fate without Notch (Hu et al., 2011). Consistent with findings in other organisms, zebrafish *notch1a* mutants have more neurons than normal, suggesting a similar role for notch in the differentiation of glia in this system as well. Activation of the *hairy/enhancer of split (hes/hey)* transcriptional repressors by Notch signaling is a probable mechanism in limiting *neurog-1* expression in the NCCs of the DRG (reviewed in Korzh and Strähle, 2002; Kageyama et al., 2008). Although this idea has not been explored in the trunk NC, development of structures derived from the cardiac NC does involve these transcription factors under the control of Notch signaling (de la Pompa and Epstein, 2012). In addition, they have also recently been shown to be important for the balance of neuronal subtypes in the mouse DRG (Mukhopadhyay et al., 2009)

Besides its roles in the migration and survival of NCCs, ErbB signaling appears to be crucial for the differentiation of glia. In the *ErbB2* mutant mouse, Schwann cell precursors fail to be specified and differentiation is aberrant (Riethmacher et al., 1997). Terminal differentiation of Schwann cells in zebrafish also requires ErbB2 and ErbB3, independently from their requirements in NCC or Schwann cell migration (Lyons et al., 2005). *sox10* expression is maintained in cells directed toward the glial lineage long

after it is downregulated in the NC, and, since *sox10* regulates the expression of ErbB3, it may allow a threshold of expression necessary for gliogenesis to occur in these cells that is not reached in other NC derivatives. It is worth noting that, while *sox10* mutant mice lack both Schwann cells and satellite glia, only Schwann cell differentiation is hampered by the loss of ErbB3, at least initially (Britsch et al., 2001). There are at present no markers for satellite glia in the zebrafish, however, and in fact almost nothing is known about their development in any systems.

An intriguing hypothesis is that Notch and ErbB signaling both function in gliogenesis, and possibly neurogenesis, through interactions with adhesion complexes. It is well known that integration of signals through Notch and adhesions are important in the development of other tissues, such as the zebrafish lateral line (reviewed in Aman and Piotrowski, 2011). In addition, both Notch and the ErbB receptor EGFR are crucial for maintaining integrin localization and stable adhesions in cultured cells (Arora et al., 2012). It will be interesting to see whether more links between these two signaling pathways and adhesions will be uncovered in the NC, but given the migratory mutipotent nature of the population, it does not seem unlikely.

LATER DORSAL ROOT GANGLIA DEVELOPMENT

After the initial steps of neuronal specification, DRG neurons become further specified to functional subtypes of neurons including varieties of thermoreceptors, mechanoreceptors and proprioceptors. Their development in the mouse has recently been extensively reviewed (Pavan and Raible, 2012; Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012; Ernsberger, 2009). Survival of these specialized neuronal precursors depends on trophic signals including the neurotrophins. These molecules are secreted by glia and bind to the Trk class of receptors, which are expressed in varying combinations on subtypes of nociceptive neurons. Ret expression helps distinguish peptidergic from non-peptidergic precursors, and the survival of these precursors is dependent on GDNF ligands. Runx1 and Runx3 are also required for the development of neuronal subtypes in the DRG. Loss of one or more of these factors can cause complex phenotypes in the DRG. The development and function of these factors in the zebrafish DRG is just beginning to be examined (Phila Gau, unpublished), and

many unanswered questions remain. Discovering whether these receptors confer the same specificity to zebrafish neurons as the do in mammals is a fascinating question.

Neurons are continually added to the zebrafish DRG throughout larval and juvenile periods (An et al., 2002; McGraw et al., 2008; McGraw et al., 2012; Honjo et al., 2008; Honjo et al., 2011). The cellular origin of these new neurons remains a bit of a mystery, but it is known that their development depends on both ErbB and Notch/Delta signaling. When ErbB activity is lost, additional neurons are not added to the DRG (Budi et al., 2008; Honjo et al., 2008). In contrast, when Notch signaling is inhibited, additional neurons are added to the DRG in later development (McGraw et al., 2012). Notch appears to regulate the rate at which neurons are added. It is unclear whether Notch signaling is required to establish the progenitor pool that gives rise to the new DRG neurons, but this seem likely given the role of Notch in the establishment of other NC progenitor pools during development (Tsarovina et al., 2008; Okamura and Saga, 2008). An experiment in which the development of later neurons was blocked by early treatment of fish with an inhibitor of Notch signaling, which may obliterate the progenitor pool at the expense of extra neurons, could help to demonstrate this.

An intriguing possibility is that the same progenitor pool used to supply DRG neurons is the one from which the late embryonic and larval pigment precursors descend. In zebrafish lacking ErbB2 or ErbB3, late neuronal addition and late pigment cells are both absent (Honjo et al., 2008; Budi et al., 2008), and ErbBs are required not at the time of differentiation of these precursors, but during the first 48 hours when a progenitor pool is established (Budi et al., 2008; Hultman et al., 2009; Hultman and Johnson, 2010). In the mouse, a requirement for ErbB2 in establishing the progenitor pool that gives rise to new Schwann cells after injury has also been shown, supporting the role of ErbBs in NC progenitor establishment (Garratt et al., 2000). Studies of early NC development suggest that most systems have precursors that can give rise to both pigment cells and glia during early development, but recent research has shown that Schwann cell precursors located near the DRG nerve can give rise to melanocytes in adult mice (Adameyko and Lallemend, 2010; Adameyko et al., 2009). Additionally, precursors located near the DRG can give rise to the later-developing pigment cells in zebrafish (Budi et al., 2011). Adult enteric neuron precursors also persist close to

mature neurons and glia in the gut (Liu et al., 2009) suggesting that the milieu of ganglia is a common location for precursor pools. Conversely, differentiated human melanocytes can give rise to Schwann cells (Chi et al., 2011), and murine melanoblasts can produce not only melanocytes, but also mature neurons and glia (Motohashi et al., 2009), suggesting that these lineages are further connected. Investigating the mechanisms through which these common progenitor pools are established is an exciting area for further study and could have many potential therapeutic benefits.

CONCLUDING REMARKS

The neural crest is remarkable and complex. The intricacies of its development are fascinating, and many unanswered questions remain. How do the molecules that control EMT affect the differentiation of NC derivatives? The localization of the small GTPases undoubtedly controls migration, but could they also be involved in differentiation? The interaction between the matrix and focal adhesions could be a major signaling center that controls this juncture of functions, so research is needed to see what role they play in receiving and transmitting differentiation signals from the environment to the nucleus to elicit expression of fate-specific genes. How is the precursor pool that gives rise to new neurons in the DRG, and also pigment cells, established? If Notch/Delta signaling is indeed important for the addition of new neurons, manipulation of this pathway could be an option for the differentiation of either neurons or glia in clinical settings. Do satellite cells in the DRG divide to produce new neurons as they do in other systems? Developing or uncovering markers for satellite glia in the zebrafish, with its rapidly-developing simple and transparent peripheral nervous system, could be an important task in understanding the science of satellite cells in disease processes. And, most importantly, how can we isolate and manipulate cells such as these as a source for potential cell-based therapies? Understanding the molecules that regulate the differentiation of neuron precursors is crucial to answer this question.

NC diseases, or neurocristopathies, are relatively common forms of birth defects, which makes the study of the development of the NC particularly important (reviewed in Etchevers et al., 2006; Keyte and Hutson, 2012). In addition, neuroblastomas are some

of the most common forms of cancer in infants and children, while melanomas and other cancers of the neural crest affect thousands of adults each year. Finally, neural crest stem cells hold the hope for regeneration of new tissues including nerves damaged in injury or stroke. Understanding the functions of ErbB receptors has led to life-changing cancer treatments, such as the development of trastuzimab (trade name Herceptin), a humanized monoclonal antibody that recognizes the human ErbB receptor that is upregulated in about 20% of human breast cancers (Witton et al., 2003). Similarly, understanding the process of melanocyte differentiation has contributed to several new strategies for melanoma treatment (reviewed in Tsao et al., 2012). I hope that my work, presented in this dissertation, will not only add to our understanding of the fascinating process of development, but also someday help in a small way to ameliorate some of the effects of disease.

Figure 1: Epithelial-to-Mesenchymal Transition in Neural Crest Cells.
Premigratory neural crest cells exhibit epithelial morphology, and are connected via adherens junctions through the N-cadherins Cadherin-6 and Cadherins-6B as well as integrins. In mesenchymal neural crest cells, Cadherin-7 and Cadherin-11 are expressed. The small GTPases Rac and Rho are also activated, at the leading edge and retracting tail, respectively. N-cadherins and Rho both act to repress the activity of Rac.

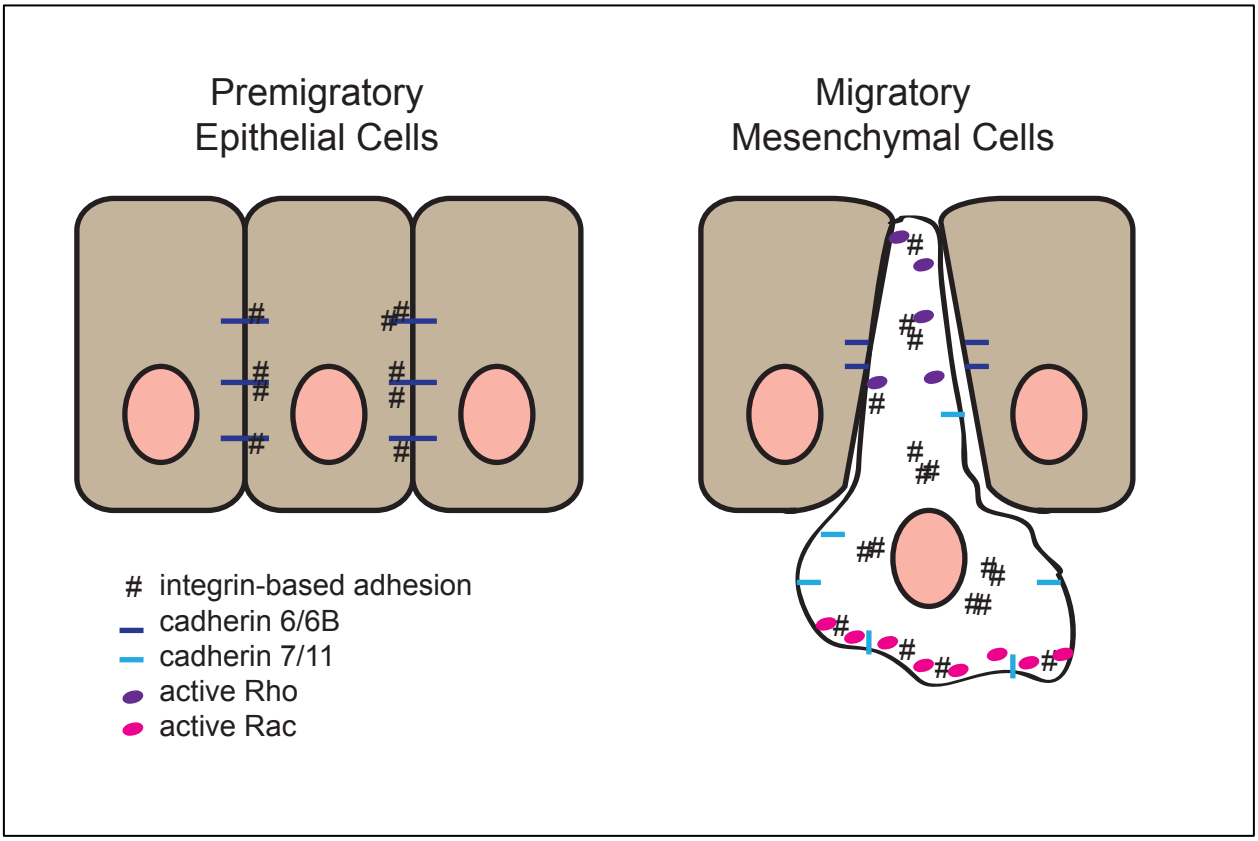
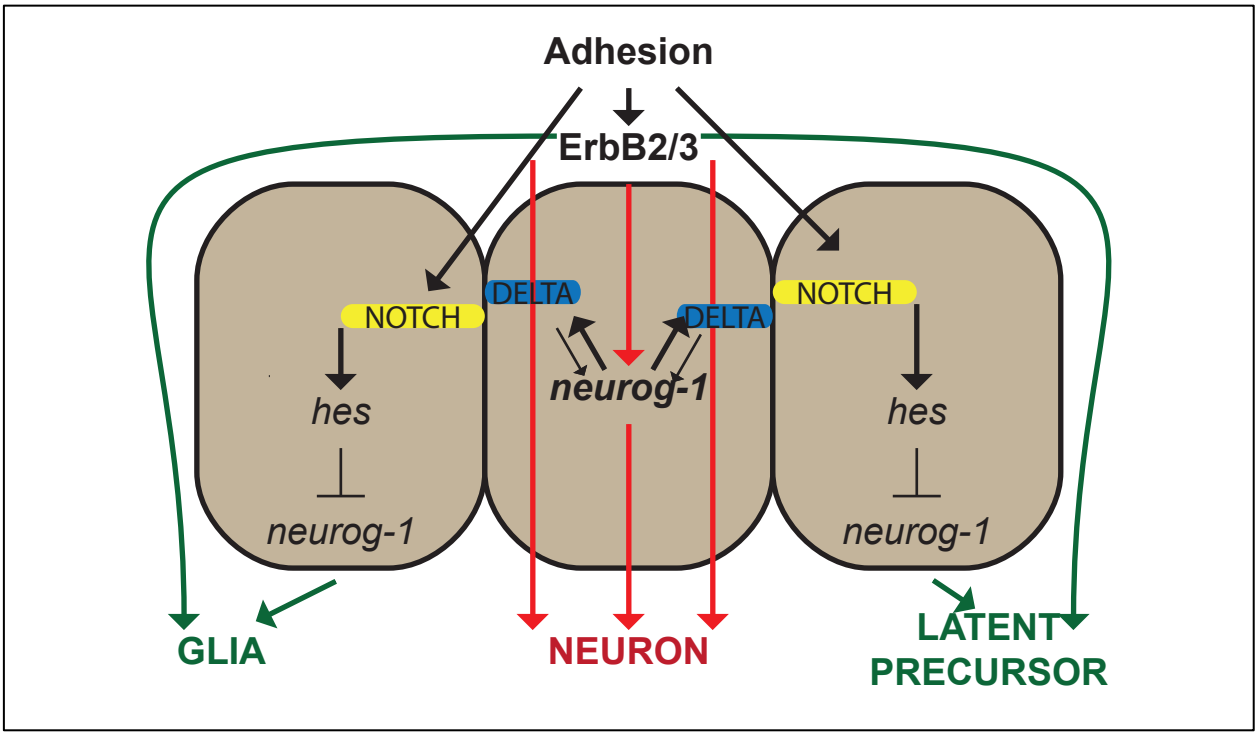


Figure 2: ErbB and Notch/Delta signaling regulate NC fate decisions. In the NC precursors that give rise to the DRG, adhesion may regulate the localization of both ErbB2/3 and Notch/Delta. ErbB2/3 activity may directly promote neurogenesis in some cells through the activation of *neurog-1*, perhaps through MAPK. Active *neurog-1* activates Delta, which inhibits the activity of Notch. Delta exerts positive feedback on *neurog-1.* In non-neurogenic cells Notch activity may activate *hes* genes, which repress *neurog-1*. This allows non-neural fates, which are also directly promoted by the action of ErbB2/3 signaling. How these other fates are specified is unknown.



Chapter II

The Roles of ErbB Signaling in Developmental Cell Migration: A Review

Glossary box

ECM – extracellular matrix

EGFR – Epidermal growth factor receptor

EMT – epithelial –to-mesenchymal transition

HER – human epidermal growth factor receptor

MAPK – mitogen activated protein kinase

MMP – matrix metalloprotease

PI3K – phosphoinositide-3 kinase

PLCgamma – phospholipase C gamma

RTK - receptor tyrosine kinase

SUMMARY

Coordinate cell migration is crucial for many developmental processes, and recent work has implicated the ErbB family of receptors in several developmental migrations. ErbBs are receptor tyrosine kinases, and members of the Epidermal Growth Factor receptor (EGFR) family; upon ligand binding, they dimerize and are then capable of activating several signaling cascades, including MAP kinase, PI3 kinase and phospholipase-c gamma signaling. Activation of these cascades can lead to changes in cell biology and gene expression. This review will highlight recent work in two model systems, the fruitfly *Drosophila melanogaster* and the zebrafish *Danio rerio*, that have implicated ErbBs as crucial players in the migratory development of many organs. Activation of MAPK and PI3K by ErbB signaling appears to be a common mechanism used by cells to control migration during border cell migration, gastrulation, heart development and NC migration, as well as in regeneration. Intriguingly, analysis of mutant phenotypes suggests that ErbBs often function to suppress migration in incorrect directions, rather than to promote migratory behaviors.

INTRODUCTION

The complexities of multicellular animals are in large part due to cell migration - convergence and extension movements allow the formation of the three germ layers during gastrulation, changes in cell shape and position allow complex organs like the heart to form from relatively simple tubular precursors, and the synapses and connections formed through migration of neurons allow the precise wiring of our brains. The sophisticated process of cell migration involves response to several signals and coordinated rearrangement of the actin cytoskeleton (reviewed by Montell, 2008; Rørth, 2009; Ilina and Friedl, 2009). Migrating cells must extend processes, make transient contacts with their neighbors and with the extracellular matrix, then respond to various cues by breaking or solidifying those contacts in a polarized way.

The ErbB family of membrane-associated receptors has recently been implicated in several developmental processes that require migration. ErbBs, named after the avian erythroblastomatosis virus protein B, are receptor tyrosine kinases that dimerize upon ligand binding, allowing autophosphorylation of tyrosine residues in their cytoplasmic domains, which then serve as docking sites for adapter proteins that activate several signaling pathways (see figure 1). Homo- and heterodimerization of these receptors allows for specificity in ligand binding and in downstream signaling, which, coupled with differences in temporal and spatial expression patterns of receptors, ligands and effectors, gives ErbBs powerful developmental potential (reviewed in Citri and Yarden, 2006); a whole genome duplication event in the teleost lineage has created even more combinations available for signaling in the zebrafish. ErbB2 and ErbB3 have unique structures that make them especially intriguing molecules for signaling: ErbB2 lacks a ligand binding domain, but is the most potent in downstream signaling, while the kinase domain of ErbB3 has been inactivated by point mutations, requiring it to heterodimerize with another receptor to transmit signals.

Despite the importance of the ErbB receptors in many developmental pathways, they are notorious for their roles in cancer progression, a context in which migration also plays an important role (Hynes and MacDonald, 2009). ErbBs are capable of signaling through several pathways that can potentially influence changes in cytoskeletal

structure, polarity and gene expression required for migration and metastases (reviewed in Feigin and Muthuswamy, 2008). The ErbBs can activate proteins with their intrinsic kinase activity, or through the recruitment of SH2 domain containing adaptor proteins, such as Shc and Grb2, to their phosphorylated tyrosines, eliciting cascades of enzyme activation leading to transcription factor activity, second messenger signaling and changes in the cytoskeleton and cell structure.

It appears that many of the pathways ErbBs use during development are reiterated in cancer progression, though most recent reviews have focused on cancer. This chapter will focus on the developmental roles for ErbBs in migration in two systems, the fruitfly *Drosophila melanogaster* and the zebrafish *Danio rerio*. Many discoveries in these two systems, which are both amenable to screening and to imaging studies, have recently brought to light the diverse migratory processes in which ErbBs are required. Since the zebrafish genome contains 7 ErbB genes, while the fruitfly genome encodes only a single receptor, EGFR, comparison of their roles in migration brings to light functional conservations and redundancies, and also highlights pathways that are conserved between vertebrates and invertebrates. This review will focus on the recent phenotypic analysis of ErbB mutants and the insights that these studies have brought to understanding how ErbBs function during migration. It will also describe recent work done to characterize downstream signaling pathways important for migration. Future studies could help to further elucidate the functions of these receptors in development and migration, and may give insight into the treatment of diseases like cancer; these will be addressed at the end of the review.

EGFR IN *DROSOPHILA* BORDER CELL MIGRATION

The single ErbB in the fruitfly, EGFR, has multiple roles in development; border cell migration is a well studied example of developmental migration in which EGFR plays a role. During oogenesis in *Drosophila*, a cluster of somatic cells in each egg chamber, the border cells, moves dorsally, then posteriorly through a field of germ cell-derived nurse cells toward the developing oocyte (reviewed in Montell, 2003). The tightly associated cluster of border cells consists of about eight cells, two of which, the polar cells, are not inherently motile and are carried along by the rest, though they are

required for migration. In this type of migration, position within the cluster of the cells is not set, and outer cells often change positions during migration, though the polar cells remain in the center of the cluster during migration (Prasad et al., 2007). The relatively simple genome of the fruitfly, which is amenable to manipulation to produce gain- or loss-of-function mutations in desired genes in whole organisms or clusters of cells, as well as the easy observation of the border cells in dissected egg chambers makes this an excellent system in which to study migration.

Despite the relative simplicity of the fruitfly, the signaling required for border cell migration appears to be complex. EGFR and the PDGF-and VEGF- receptor PVR act during border cell migration, and coexpression of dominant negative forms of both receptors causes a more dramatic migration phenotype than either one alone (Duchek et al., 2001). Two EGFR ligands, Keren and Spitz, guide border cell migration (McDonald et al., 2006). EGFR activates different signaling cascades in the two phases of border cell migration. In the early posterior phase, EGFR appears to act redundantly with PVR through the small GTPase Rac and the atypical Rac exchange factor Mbc to cause actin accumulation; in later dorsal migration, EGFR signaling acts through PLC-gamma and MAPK (Bianco et al., 2007). The migration needed for dorsal closure, another developmental process, in fruitfly requires activation of Jun kinase through MAPK, along with changes in cell shape (Harden et al., 2002). Rac is also needed for thorax closure in the fruitfly (Ishimaru et al., 2004). These results suggest that the Rac and MAPK signaling pathways may be common to many migration processes (see table 1).

Mixed clone experiments show that cells within the border cell cluster that have higher expression of EGFR are more often found at the leading edge of the cluster and form protrusions in the direction of migration. Cells lacking EGFR are still able to form protrusions, but they more frequently made in other directions. Expression of constitutively active EGFR causes cells to stop forming protrusions altogether, suggesting that EGFR actually functions by suppressing formation of protrusions in the wrong direction (Prasad et al., 2007). The observation that EGFR is actually prohibitive of migration in the fruitfly is intriguing, given the positive roles EGFRs play in vertebrate

migration, and this suggests that the functions of the multiple EGFRs, ligands and effectors may have diverged since their duplication in the ancestors of vertebrates.

COMPLEX ERBB SIGNALING IN GASTRULATION

While much basic cell biological knowledge has come from the study of invertebrates like the fruitfly, the translational potential, yet ease of use of basal vertebrates such as the zebrafish has been a driving force for the development of these models for the study of developmental migration. Genome duplications have produced 4 ErbBs (EGFR, ErbB2, ErbB3 and ErbB4) in tetrapods and 7 in zebrafish (see table 1); complex interactions between these receptors allow for intricate developmental pathways that give rise to complex organ systems. The rapid external development of and ease of transgenesis and genetic manipulation in the zebrafish have made it particularly useful for the study of developmental processes.

During the process of gastrulation, several types of cell migration in coordination result in an embryo with specified germ layers and axes. Studies of the function of ErbBs in gastrulation have been complicated by earlier requirements for the downstream effectors MAPK and PI3K in mesoderm induction (Carballada et al., 2001; Sivak et al., 2005), but recent work has identified a role for ErbBs in controlling both convergent extension movements in the trunk mesoderm and in migration of the head mesoderm of the gastrulating *Xenopus laevis* embryo (Nie and Chang, 2007b). When ErbB4, one of 4 ErbBs in *Xenopus* is depleted in embryos with morpholino oligonucleotides, gastrulating cells fail to form adhesions with the extracellular matrix and fail to extend filopodia in the direction of migration. Both MAPK and PI3K overexpression can rescue the effects of morpholino oligonucleotide knockdown of ErbB4 on gastrulation (Nie and Chang, 2007a), but MAPK can more efficiently rescue cell adhesion, while PI3K can more efficiently rescue filopodia formation, indicating crosstalk, but specificity, of the signaling pathways in these 2 migratory processes.

Similar experiments in the zebrafish confirm the importance of PI3K signaling in gastrulation. In the zebrafish, loss of P13K, and its signaling through PKB, causes decreases in motility during gastrulation (Montero et al., 2003). Overexpression of PTEN, a negative regulator of PI3K, or inhibition of PI3K with the chemicals wortmannin

or LY294002 causes a similar phenotype in zebrafish (Finkielsztein and Kelly, 2009). These experiments indicate a conserved role for PI3K signaling in migration during gastrulation. There are 2 duplicates of ErbB4 in the zebrafish genome, but it is not known whether they are each required for gastrulation.

ERBBS AND CELL POLARITY IN THE ZEBRAFISH

Polarization of cells is important so that they can migrate in the correct direction. Cells need to send processes, such as filopodia or lamellipodia, at only their leading edge, and then form adhesions with the matrix within those protrusions, while retracting their trailing edges. In the fruitfly, a complex that includes the protein *lethal giant larvae (lgl)* is localized to the basolateral side of polarized cells. Intriguing research suggests that lgl2, one of the two zebrafish orthologs of this protein, functions as a tumor suppressor and is required to prevent polarization of epidermal cells through ErbB2 (Reischauer et al., 2009). When *lgl* is inactivated, ErbB2 is activated. Cells in the epidermis then become polarized and exhibit increased growth, lamellipodia formation and migration as a sheet. In this case, ErbB2 works to promote cell migration through polarization.

In other cases, it appears that ErbBs can act to suppress polarization in the developing zebrafish. Using selective plane illumination microscopy of live zebrafish hearts, Scherz et al. (2008) found that the atrioventricular valves in fish treated with pan- ErbB inhibitors have defects caused by lack of invagination of these cells and a change in their shape, possibly due to disruptions in the polarity of the cells or defects in actin polymerization that accompany an epithelial-to-mesenchymal transition (EMT) they undergo during development. Similar defects in mice mutant for the ErbB ligand HB-EGF and for TACE, an enzyme required for ligand processing (Jackson et al., 2003), suggest that ErbBs can hold in check the polarization of cells that is required for EMT. ErbB are thus capable of both preventing and promoting migration through changing cell polarity. Because the inhibitors in these experiments target all 7 zebrafish ErbBs, and because several ErbBs are expressed in the developing heart, the identity of the receptors that repress polarity is unknown. Potentially, the functions of these various receptors in polarity determination could be unique.

ERBBS, THE NEURAL CREST, AND REGENERATION

Another context in which ErbBs may serve to prevent migration is in the development of the neural crest (NC). The multipotent NC arises at the intersection of the ectoderm and mesoderm during gastrulation and neurulation in vertebrates, then migrates in a stereotyped manner to different locations and differentiates into multiple cell types. Thus, the NC is a useful model for studying the coupling of migration to differentiation. In the zebrafish, transgenic lines have been used to screen for defects in NC development; several mutants in ErbBs have been isolated in such screens, and study of them has revealed more roles for ErbB in development.

ErbB knockout mice have defects in NC development in several tissues, including the dorsal root ganglia and pigment cells (Britsch et al., 1998; Riethmacher et al., 1997). Since live imaging is not feasible in the internally developing mouse embryo, the molecular and cellular nature of these defects has remained unclear. Recent work in zebrafish has illuminated these developmental defects. In zebrafish mutant for ErbB3b, NC precursors of the DRG migrate to the sites of DRG formation, but fail to pause and differentiate into neurons and glia and continue to migrate, suggesting that ErbB3 is required for them to stop migrating once they recognize their targets or in the target recognition process itself (Honjo et al., 2008). Treatment of fish with inhibitors of ErbBs during a defined window of DRG development results in recapitulation of the ErbB3-/- phenotype (Budi et al., 2008), suggesting a role for this pathway in the target recognition process in migration. Schwann cells of the DRG, which are also derived from the NC, require ErbB2 and ErbB3 for the initiation and maintenance of migration in the mouse, however (Lyons et al., 2005). ErbBs also have other roles in Schwann cell development, including roles in promoting gliogenesis and glial survival, so the defect seen in mouse Schwann cells could be due to complex interactions between signaling pathways downstream of ErbBs; future work is needed to understand these roles.

Although ErbB2 and ErbB3 appear to both promote and prevent migration in the NC, their role in the migration required for the regeneration of the zebrafish tail is positive. Fish lacking ErbB2 or ErbB3 cannot regenerate clipped fins as wildtype fish can; treating fish with inhibitors of MAPK or PI3K also blocks regeneration, which

suggests that ErbBs work through these pathways to allow migratory cells to recolonize fins (Rojas-Muñoz et al., 2009).

CONCLUSIONS

While ErbB signaling consistently plays a role in many migratory developmental processes (reviewed in table 1), the exact nature of these roles remains unclear. Signaling through ErbB receptors can elicit numerous signaling cascades to direct changes in many cellular processes, these processes can have differing effects on cell migration. In migration, it appears that MAPK and PI3K signaling are utilized in many contexts, but other pathways surely play important roles as well. How these pathways are coordinated to regulate changes in cell motility in both development and cancer remains an unanswered question. The multiplicity of receptors, ligands and effectors in the zebrafish amplifies the signaling potential found in the fruitfly EGFR signaling system providing more opportunities for fine-tuning, but also more opportunities for indiscriminate crosstalk between downstream pathways.

The development of new genetic tools will allow more careful dissection of the complexities of ErbB signaling. With complex knockouts, and new genetic tools the cumulative effects of the loss-of-function of ErbB receptors, ligands or downstream effectors, could allow elucidation of their effects on migration. The mechanisms cells use to select pathways downstream of ErbB signaling are not well characterized, but scaffold proteins may be important for both promoting downstream signaling and preventing crosstalk, as has been shown for MAPK signaling in other systems (reviewed in Pullikuth and Catling, 2007). Scaffold proteins may also serve to couple ErbB signaling to other signaling pathways crucial to migration as well. The dynamics of focal adhesion formation and disassembly, a process that is crucial for migration, may be coordinated to ErbB2/3 signaling, since these receptors localize to focal adhesions (Kim et al., 2005). The protein PINCH can interact with both ErbB2/3 and focal adhesion components, suggesting that this or other scaffold proteins may coordinate their outputs (reviewed in Legate et al., 2006). Since ErbBs are important for non-migratory developmental processes, including cell survival, differentiation and

proliferation, investigating the coordination of these functions could also yield insights into the mechanisms of development and of disease.

Figure 1: ErbB receptors activate multiple signaling pathways. In this example, and ErbB2/3 heterodimer has formed upon binding to a neuregulin (Nrg) ligand. ErbB2 is unable to bind ligands, but can form dimers with other bound receptors. Adapter molecules, including Grb2, Shc, and Sos, are recruited to the phosphorylated tyrosines on the intracellular domain of activated ErbB2. ErbB3 has no activated kinase activity, but ErbB2 can activate MAPK, PLC gamma and PI3K pathways, both directly and through these adapter molecules.

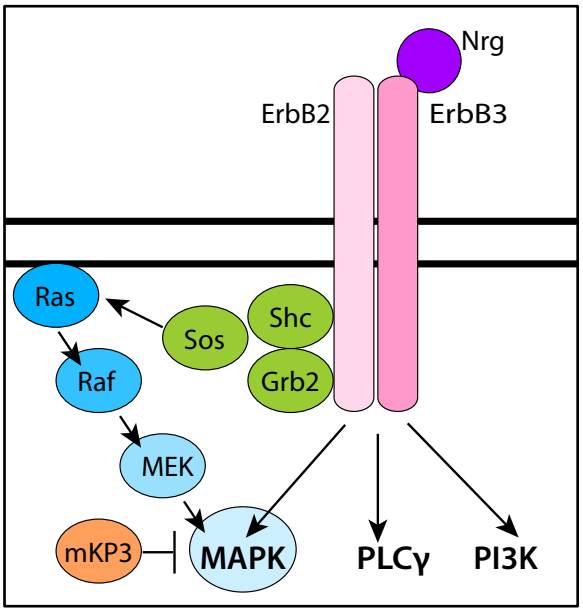


Table 1: ErbB receptors, ligands and developmental processes

Receptor	Ligands	Developmental processes
D. melanogaster EGFR	Keren, Spitz	Border cell migration
D. rerio ErbB1a/b	EGF, amphiregulin, TGFα, EPR, HB-EGF, BTC	Branching morphogenesis, skin innervation
D. rerio ErbB2	(no ligand binding)	Neural crest development, gliogenesis, regeneration
D. rerio ErbB3a/b	Neuregulin 1-4	Neural crest development, gliogenesis, regeneration
D. rerio ErbB4a/b	Neuregulin 1-4, HB-EGF, BTC, EPR	Gastrulation

Chapter III

Sorbs3: A Scaffold Protein Regulating Adhesion, Motility, and Receptor Signaling: A Review

INTRODUCTION

During migration, cells must coordinate signals from multiple sources, which together influence their structures and fates as well as their motility. This process is integral to proper development of embryos and homeostasis of adult tissues, but is often misregulated in disease, particularly cancers. Recent work has highlighted the importance of the scaffold protein Sorbs3, or vinexin, in the formation and maintenance of focal adhesions formed during cell migration, and identified it as an important focus of future research regarding their function in development and disease. Sorbs3 is a member of the SoHo family of scaffold proteins, know to bind specifically to interacting partners and regulate such diverse cellular processes as migration, lipid metabolism and Mitogen Activated Protein Kinase (MAPK) signaling**.** In this review, I will first discuss the structure and overall function of Sorbs3 and its paralogs, and then highlight recent findings implicating the Sorbs3 in cellular processes, particularly focal adhesion dynamics and signaling cascades. Finally I will discuss evidence for Sorbs3's role in development and disease, and bring up some unanswered questions about its diverse functions.

SORBS3 IS A SCAFFOLD PROTEIN

The SoHo (sorbin homology) family of proteins is composed of three proteins, ArgBP2 (Arg-binding protein2)/sorbs1, CAP (c-Cbl associated protein)/ponsin/sorbs2 and vinexin/sorbs3. These proteins share characteristic domain architecture, having an N-terminal sorbin homology domain followed by 3 c-terminal Src-homology 3 (SH3) domains, and several splice isoforms are produced from each gene. All of these domains are known to mediate interactions with binding partner proteins, and their amino acid sequences are highly similar; interdomain sequences in the three proteins share little similarity, however they all contain numerous PxxP motifs, potential sites of

interaction with other SH3-domain containing proteins. The genomes of invertebrates, including *c. elegans* and *Drosophila*, contain a single gene with this domain organization, suggesting the paralogs arose through gene duplication.

Numerous proteins that contain modular binding domains use them to spatially and temporally sequester members of signaling cascades to coordinate their activities; these proteins have been termed scaffold proteins, and are important regulators of several cellular processes (reviewed in Scott and Pawson, 2009; Pawson and Scott, 2010). While some of the first characterized scaffold proteins were shown to direct simple, unidirectional signaling cascades, such as the Ste 11MAPK scaffolded by Ste5 in yeast (Choi et al., 1994), more recently, scaffold proteins including the SoHo family have been implicated in the coordination of complex pathways that appear to have multiple possible outputs. All three vertebrate SoHo family members were identified based on their interactions with other proteins, and their initial interacting partners highlight the diversity of their binding functions. CAP was isolated in a screen for SH3 ligand binding proteins, and was initially found to associate with the E3 ubiquitin ligase c-Cbl and the Ras GTPase afadin (Mandai et al., 1999; Ribon et al., 1998a; Ribon et al., 1998b). ArgBP2 was first identified for binding to the non-receptor tyrosine kinases c-Abl and c-Arg (Wang et al., 1997). Vinexin was isolated in a screen for proteins that bind to vinculin, a component of cell-matrix adhesions (Kioka et al., 1999). Since their identification, the proteins have been shown to interact with a number of different classes of proteins and have been implicated in diverse, but somewhat overlapping, cellular processes. These are highlighted in Figure 1. Of note, all three SoHo paralogs have been implicated in cell adhesion and migration, as well as in ERK signaling, and interactions between each of them and the focal adhesion proteins vinculin and paxillin have been demonstrated in cell culture systems.

Several different isoforms the Sorbs3 protein are found in mammals. A short isoform, Sorbs3β, is expressed almost ubiquitously in both embryonic and adult mice (Kawauchi et al., 2001; Kioka et al., 1999). This isoform appears to be translated from a unique intronic promoter and lacks the N-terminal portion of the protein, including the sorbin domain and the inter-domain region. In contrast, the longer isoform Sorbs3α has more restricted expression – in the developing mouse embryo, its expression is

restricted to the retinal pigment epithelium, cardiac outflow tract and AC canal of the heart, hindbrain, pons and gonad, while in the adult mouse it is most highly expressed in muscle. (Kawauchi et al., 2001; Kioka et al., 1999). There is also evidence for several other differentially spliced long isoforms (Martens et al., 2004; Matsuyama et al., 2005; Paz et al., 2007); at least two of these are expressed in both the developing and neonatal somatic gonad, as well as in germ cells. It is possible that the sorbin domain contained in these long isoforms helps to target it to the membrane through its interaction with flotillin (Kimura et al., 2001) and that differential splicing allows different isoforms to form unique interactions with other proteins and to participate in specialized cellular functions, some of which are highlighted in Figure 2.

SORBS3 IS A COMPONENT OF FOCAL ADHESIONS

Sorbs3 was first identified in focal adhesions as an interactor with vinculin. In order to migrate, cells must cycle through the establishment and dissolution of adhesions with the surrounding extracellular matrix (ECM) and neighboring cells, and when cells reach their final destinations, they need to solidify these transient adhesions into permanent junctions. The first step in the formation of a cell-ECM junction is the interaction of the intracellular actin network with integrins, via adaptor proteins such as talin and paxillin, in small structures called focal complexes located near the leading edges of cells. As other proteins including vinculin are recruited to the complexes, they enlarge and mature into focal adhesions. Focal adhesions can either be disassembled after the cell has moved forward, using the adhesions as anchors or can be solidified into fibrillar adhesions and actin stress fibers once cells have stopped moving. The protein vinculin plays an important role in this process, being found at nascent as well as mature adhesions and cycling in and out of adhesion complexes in migrating cells (reviewed in Carisey and Ballestrem, 2011).

Sorbs3 is localized to several cellular compartments including the cell membrane, particularly at sites of adhesion and leading edges of migrating cells (Kioka et al., 1999; Wakabayashi et al., 2003; Townson et al., 2003; Martens et al., 2004), (Mitsushima et al., 2006a; Mitsushima et al., 2006c), but also cytosol and nucleus (Tujague et al., 2004; Bour et al., 2005; Chen et al., 2005). The localization of Sorbs3

appears to be dependent on its binding to other proteins. Membrane localization appears to be dependent on the interaction with vinculin, which only occurs when vinculin is in its "open," or active conformation (Chen et al., 2005). In its closed conformation, the proline rich region of vinculin, through which it binds to the first and second SH3 domains of Sorbs3, as well as CAP/ponsin and ArgBP2 (Kioka et al., 1999; Mandai et al., 1999; Zhang et al., 2006; Kawabe et al., 1999) is masked through intramolecular interactions between the vinculin head and tail regions (Ziegler et al., 2006). Vinculin peptides that bind to the third SH3 domain of Sorbs3 are atypical SH3 domain ligands and have a relatively weak affinity for Sorbs3 (Zhang et al., 2007), suggesting that binding of Sorbs3 alone is not sufficient to activate vinculin. In fact, several studies show that activation of vinculin by breaking the tight intermolecular bonds between its head and tail domains requires the binding to several other proteins (reviewed in (Carisey and Ballestrem, 2011). Sorbs3 can co-precipitate with vinculin in adherent cells that contain focal adhesions, but not in suspended cells (Takahashi et al., 2005), suggesting that Sorbs3 is recruited to focal adhesions to bind vinculin after it has been activated. Although Sorbs3 probably does not activate vinculin, it may be able to stabilize vinculin at focal adhesions. Expression of Sorbs3α, a full-length isoform, increases the amount of vinculin found at focal adhesions, as well as the size of focal adhesions and actin stress fibers (Kioka et al., 1999). Additionally, the interaction of Sorbs3's SH3 domains with vinculin promotes the accumulation of f-actin at stress fibers (Takahashi et al., 2005).

In addition to vinculin, Sorbs3 also interacts with other proteins found at focal adhesions, including paxillin (Gehmlich et al., 2007), SOCS7 (Martens et al., 2004), SHIP2 (Paternotte et al., 2005), WASP (Mitsushima et al., 2006c), actin (Mitsushima et al., 2006b) and β-dystroglycan (Thompson et al., 2010). Paxillin, itself a scaffold protein, is one of the first components to localize to nascent adhesions (Laukaitis et al., 2001), and presumably interacts with the last SH3 domain of Sorbs3, as it does with CAP (Gehmlich et al., 2007). β-dystroglycan interacts with the last SH3 domain of Sorbs3, allowing Sorbs to link vinculin bound to its first two SH3 domains with β-dystroglycan in spreading myoblasts (Thompson et al., 2010). Both paxillin and SHIP2 serve as scaffolds or docking proteins for several other components of adhesions; Sorbs3 may

thus allow combinatorial linking of several structural and signaling components of focal adhesions.

While vinculin is found at focal adhesions, leading edges, cell-cell junctions, and in the cytosol, several other proteins that interact with Sorbs3 have more specific localization patterns. For example, Sorbs3 binds to and prevents the dephosphorylation of WAVE2, which is found at leading edges but not focal adhesions (Mitsushima et al., 2006c); WAVE2 promotes actin polymerization by activating the Arp2/3 complex. In contrast, Sorbs3 also binds to the guanylate cyclase kinase family member lp-dlg, also known as Discs Large homolog 5(DLG5), which is only localized to cell-cell contacts, not cell-ECM contacts such as focal adhesions (Wakabayashi et al., 2003). Experiments in both mouse neural progenitor cells and embryonic fibroblasts have demonstrated that DLG5 is necessary for the stabilization of the cadherin-catenin complex found at apical junctions (Nechiporuk et al., 2007). Sorbs3 could therefore be a general stabilizer, like vinculin, of most cell contacts/adhesions, with the more specific roles of complexes defined by other components.

Sorbs3 and the Ableson kinases c-Abl and v-Abl interact via the third SH3 domain of Sorbs3, and Sorbs3 is a substrate of their kinase activity (Mitsushima et al., 2006b). Interestingly, c-Abl also phosphorylates several other Sorbs3 binding partners, and this activity may promote the stabilization of adhesions. For example, WAVE2 is phosphorylated and activated by c-Abl and its coactivators Abi-1/2 (Stuart et al., 2006), (Leng et al., 2005). WASP is also phosphorylated by c–Abl, but this impairs is recruitment to focal adhesions, particularly its association with the crucial focal adhesion component zyxin (Maruoka et al., 2012). Paxillin (Lewis and Schwartz, 1998) and zyxin, another crucial component of focal adhesions are also substrates of c-Abl and its coactivators (Maruoka et al., 2012). However, zyxin is not able to bind to the SH3 domains of Sorbs3 (Li et al., 2004) The relationship between binding of sorbs3 to c-Abl and c-Abl phosphorylation of Sorbs3, its binding partners, and other focal adhesion components is complex and require further study to understand completely.

SORBS3, RECEPTORS AND SIGNALING CASCADES

Sorbs3 has been shown to interact with several types of growth factor and hormone receptors. In the nucleus, Sorbs3β has been shown to interact directly with the AF1 substrate of gamma-retinoic acid receptor (RARγ), but not with RARα or RXRα, where it inhibits RARγ-dependent gene expression (Bour et al., 2005). Sorbs3 associates with only unphosphorylated, inactive RARγ, and only in the absence of retinoic acid. In the presence of retinoic acid, RARγ becomes phosphorylated and dissociates from Sorbs3β, and can occupy promoters of retinoic acid-responsive genes (Lalevée et al., 2010). Sorbs3 is thus a RAR corepressor. Sorbs3 also interacts with glucocorticoid receptor, androgen receptor and estrogen receptors (ER), both alpha and beta subtypes. Sorbs3 can enhance the activation of all of these receptors by activating kinases, with the exception of ERβ (Tujague et al., 2004). Intriguingly, Sorbs3 expression results in an increase in ER expression, but in a decrease in ER phosphorylation. Sorbs3β also binds to SAFB2, an ER co-repressor (Townson et al., 2003), but the effect of this on ER-mediated gene expression is unknown. It appears that Sorbs3's scaffolding activity can both promote and inhibit signaling by preventing and allowing spatial proximity of interacting proteins.

Much more work has been done to describe the interactions of Sorbs3 with the Epidermal Growth Factor Receptor (EGFR) pathway than with any of the above receptors. EGFRs are ErbB type receptor tyrosine kinases that, upon binding of their ligands, dimerize and phosphorylate themselves, and then activate multiple signaling cascades either through their intrinsic kinase activities or through recruitment of adapter molecules to their phosphorylated tyrosine residues. As of yet, there has been no evidence of a direct physical interaction between the receptor and Sorbs3, but several downstream components do have direct binding; indeed, signaling through EGFR is highly influenced by Sorbs3, and vice versa. Other receptor tyrosine kinases (RTKs) are also influenced by Sorbs3. Sorbs3 is required for the anchorage independent activation of ERK by fibroblast growth factor (FGF; Mitsushima et al., 2007), and platelet-derived growth factor (PDGF)-induced phosphorylation of Sos can block a Sos-Sorbs3 interaction (Akamatsu et al., 1999). It is likely that Sorbs3 can interact with

several RTK pathways that are initiated at sites of Sorbs3 localization, such as focal adhesions, along with those in the nucleus.

Sorbs3 appears to modulate several steps in RTK pathways: receptor activation, adapter protein binding, and downstream effector stabilization. First, Sorbs3 may be important for maintaining the activated states of RTKs themselves. Mitsushima et al showed that expression of Sorbs3 can increase the phosphorylation of several residues on EGFR, including those required for MAPK signaling downstream of EGF. Sorbs3 appears to allow EGFR, through its intrinsic kinase activity, to maintain its own phosphorylation, but is not required for the initial phosphorylation of EGFR. They also showed that Sorbs3 decreases endocytosis of EGFR and its interaction with the ubiquitin ligase c-Cbl. Interestingly, a direct physical interaction between EGFR and Sorbs3 does not appear to be required for these effects, but the presence of the first two SH3 domains, which interact with several other components of the signaling pathway, is required (Mitsushima et al., 2006c).

Sorbs3 also appears to be important in the recruitment or binding of adapter molecules to activated RTKs. The third SH3 domain of Sorbs3 interacts with the adapter protein Sos in cultured cells, and expression of Sorbs3 enhances the activation of the MAPK c-Jun kinase (JNK) signaling through EGF, presumably through Sos binding, as SH3 mutations that abrogate Sos binding also block JNK response to EGF (Akamatsu et al., 1999). There is evidence that MAGUK family members, such as DLG1, can act as scaffold proteins by specifically binding phosphorylated residues on binding partners (Zhu et al., 2011) and point mutations in the third SH3 domain of Sorbs3 can block the ability of the MAGUK protein DLG5, as well as Sos, to bind to Sorbs3 (Wakabayashi et al., 2003). Since so many of Sorbs3's binding partners also have multiple protein-protein interaction motifs, it is quite probable that some of them also serve as adapter proteins to recruit other signaling cascade molecules to activated RTKs as well.

Much of the work on Sorbs3s interaction with downstream effectors of RTK pathways has centered on the MAPK pathway. The region of Sorbs3 between the Sorbin and SH3 domains physically interacts with the MAPKKK c-Raf (Matsuyama et

al., 2005), while the MAP kinases ERK1 and 2 bind to both this region and the region of Sorbs3 between the second and third SH3 domains, which contains a DEF motif (Mitsushima et al., 2004; Mitsushima et al., 2007). ERK1/2 can phosphorylate murine Sorbs3 at serine 139, which is upstream of the SH3 domains, and this causes a conformational change in the Sorbs3 protein (Mitsushima et al., 2004). Phosphorylation of Sorbs3 by ERK seems to occur after it is recruited to sites of existing ERK activation, such as cell membranes (Mitsushima et al., 2004) leading edges (Mizutani et al., 2007a) and synapses (Ito et al., 2007). In turn, Sorbs3 can promote the activity of ERK. Expression of Sorbs3 allows EGF-mediated ERK activation downstream of adhesion signaling (Suwa et al., 2002), as well as anchorage-independent activation of ERK by FGF (Mitsushima et al., 2007). While Sorbs3 has no intrinsic kinase activity itself, it works to prevent the dephosphorylation of ERK by deactivating phosphatases including MKP3 (Mitsushima et al., 2007). Interestingly, ERK and MTOR are responsible for the decrease in Sorbs3 expression seen in v-src transformed cells (Umemoto et al., 2009b), suggesting that there may also be a negative regulatory loop linking Sorbs3 and ERK activity.

INTEGRATING FOCAL ADHESIONS AND SIGNALING

Sorbs3 seems to promote the maturation of focal adhesions into more stable permanent structures, and thus may promotes the cessation of migration. It has been shown to increase adhesion in several studies (Kioka et al., 1999; Paternotte et al., 2005; Umemoto et al., 2009b); increases in adhesion and cell spreading are indicative of tighter associations of cells and their substrates and usually lead to decreased cell migration. Sorbs3 may allow cells to stop migrating, and integration of signals from EGFR is probably crucial to this activity. In cultured cells, activation of ERK by EGF requires adhesion, but Sorbs3 expression allows EGF-mediated activation of ERK without adhesions (Suwa et al., 2002), suggesting that it can promote the formation of mature adhesions by bypassing the early signaling steps needed for them. Adhesion/spreading activates ERK and allows vinexin to translocate to the periphery of adherent cells (Mitsushima et al., 2004) suggesting there is possibly a positive feedback loop between adhesions, ERK signaling and Sorbs3. Indeed, while ERK phosphorylation is not required for the localization of Sorbs3 to focal adhesions, ERK

phosphorylation of vinexin is partially responsible for the decreased migration seen in Sorbs3 expressing cells (Misutani et al., 2007a).

Sorbs3 increases adhesion when coexpressed with Src homology 2 (SH2) domain-containing inositol polyphosphate 5-phosphatase 2 (SHIP2.), which is localized to focal adhesions and catalyzes the dephosphorylation of PIP3 at membranes (Paternotte et al., 2005). This might promote adhesion through several mechanisms, including its phosphatase activity and docking protein function (reviewed in (Erneux et al., 2011). SHIP2 may also promote adhesion through EGFR. ErbB receptors undergo receptor-mediated endocytosis to turn down signaling; treatment of cells with shRNA for either SHIP2 or its mediator PR130/B”α1 causes both an increase in endocytosis of the receptor and a decrease in its association with the Ubiquitin ligase c-Cbl (Prasad and Decker, 2005; Zwaenepoel et al., 2010). Sorbs3 itself binds to c-Cbl (Mitsushima et al., 2006b); this could prevent associations between c-Cbl and its substrates and their ubiquitination and subsequent degradation. Indeed, several key components of adhesion complexes and EGF signaling are regulated by c-Cbl, including not only EGFR and SHIP2, but also several other components of EGFR signaling pathways (reviewed in Sorkin and Goh, 2009) and adhesions (reviewed in Huang, 2010). Sorbs3 therefore seems to promote adhesions through multiple mechanism, integrating protein stability, activation of cascades and structurally supporting adhesions. Together, these activities could powerfully regulate cellular processes.

SORBS3 IN DEVELOPMENT AND DISEASE

Emerging research is beginning to uncover the roles that Sorbs3 plays in developmental processes. Kioka et al. have recently showed that Sorbs3 is required for wound healing *in vivo* and for EGFR dependent migration of keratinocytes *in vitro* (Kioka et al., 2010). Surprisingly, the mouse strain generated by this group appears to have no major developmental phenotypes, despite lack of all isoforms of Sorbs3. Compensation by the other SoHo paralogs may explain the relatively normal development of these mutant mice; to date, no multiple knockout mice have been generated or examined. There are, however, some observations that implicate Sorbs3 in at least two developmental processes, testes development and neurogenesis.

Several isoforms of Sorbs3 are expressed in the developing and adult brains of mice, rats and humans (Kawauchi et al., 2001; Ito et al., 2007; Ito et al., 2008; Tujague et al., 2004). Sorbs3 is enriched in nerve terminals and the filopodia of nerve growth cones, where in its phosphorylated form it colocalizes with activated ERK (Ito et al., 2007), consistent with its localization in cultured cells. Interaction of Sorbs3 and the proteins synaptophysin and p140Cap may modulate its activity at synapses. When p140Cap binds to Sorbs3, it can both suppress the interaction of Sorbs3 with WASP and cause Sorbs3 to localize to the perinuclear area instead of to focal adhesions (Ito et al., 2008). Thus, p140Cap may alter focal adhesion dynamics through the sequestration of Sorbs3. Like most migrating cells, growing neurons are polarized, partially through the activity of small GTPases such as Rho, Rac and Cdc42. Sorbs3 interacts with the Rho effector rhotekin (Nagata et al., 2009) in neurons as well as other cells, and activated Cdc42 is able to disrupt this interaction, while Rho itself is not. It is possible that Sorbs3 may work to coordinate signals from polarity proteins to direct neurite outgrowth, but this remains to be investigated.

Prior to the full knockout of Sorbs3, an isoform specific knockout of Sorb3α/γ was generated, also without major phenotypes (Matsuyama et al., 2005). Expression of the short isoform Sorbs3β was unaffected in this mouse. Decreased activated ERK was observed in E12.5 XY gonads of this line, however, as was associated decreased expression of *sox9,* a gene required for proper testes development, although its levels recovered during later fetal development. Interestingly, treatment of male mice with 17-beta estradiol (E(2) from 2 weeks prefertilization to P30, was shown to decrease the amount of Sorbs3α/δ normally seen in developing male germ cells at P30, while having no effect on fetal or postnatal expression of Sorbs3γ in Sertoli cells, or Sorbs3β (Paz et al., 2007), suggesting that Sorbs3 may be required specifically in the adult testes for spermatogenesis.

Sorbs3 is also highly expressed in the adult human testis, and binding of the androgen receptor to the region between the sorbin and SH3 domains of Sorbs3 can enhance the activity of the androgen receptor through its ligand (Tujague et al., 2004), further suggesting roles in spermatogenesis. Development of sperm required closely regulated interactions between the spermatogonia and their neighboring Sertoli cells, so

cell-cell adhesions mediated by Sorbs3 have the potential to be involved in this regulation. The relationship between the varying Sorbs3 isoforms and testes development, remains to be ascertained.

Sorbs3 has been implicated in several cancer studies, but it seems that its promotion or prevention of oncogenic processes may depend on the context in which it acts. An increase in Sorbs3 expression has been noted in two androgen-independent prostate cancer lines, but was not found in an androgen-dependent prostate cancer line (Mizutani et al., 2007b). Interestingly, the efficacy of paxlitaxel in preventing growth in one line was blocked by expression of Sorbs3, but was restored when treated with Sorbs3 shRNA; Sorbs3 has an oncogenic role in this case. Decreased Sorbs3 expression is associated with increased survival in chronic myeloid leukemia patients treated with imatinib (Villuendas et al., 2006). Imatinib targets the oncogenic form of c-Abl, the BCR-ABL fusion protein. Although a direct interaction between BCR-ABL and Sorbs3 has not been shown, based on its interactions with c-Abl, it is possible that Sorbs3 association with BCR-ABL may promote its kinase activity and oncogenic function. In this case, Sorbs3 also has an oncogenic role.

In v-src transformed cells, however, Sorbs3 appears to act as a tumor suppressor. Restoration of Sorbs3 in 3T3 cells that have been treated with v-src can decreased their elevated migration levels; interestingly the increase in migration of these cells is dependent on a loss of Sorbs3 expression mediated by MTOR and ERK, as well as an increase in the degradation of Sorbs3 protein (Umemoto et al., 2009b). Sorbs3 is itself phosphorylated by v-src, and this attenuates its binding to vinculin (Umemoto et al., 2009a), which could lead to loss of focal adhesion stability and increased migration. A similar decrease in adhesion is seen in cells infected with the hepatitis B virus; the hepatitis B protein X interacts with Sorbs3 and blocks its interaction with focal adhesion proteins, delaying the formation of focal adhesions (Tan et al., 2006). A recent study has also implicated the deletion of a region containing the Sorbs3 gene with poor prognosis in hepatocellular carcinoma (HCC) patients, and has shown that Sorbs3 can act as a tumor suppressor in HCC lines and in rats (Roessler et al., 2011).

CONCLUSIONS

The SoHo proteins have emerged as a multifunctional group that is capable of integrating many signals and regulating many cellular processes. Through integration of signals from adhesions and from receptors for surface growth factor receptors, along with hormone receptors in the nucleus, they are capable of influencing gene expression, cell structure and motility. It appears that multiple isoforms of the three paralogs may have overlapping, but possibly distinct functions, and that they may be able to compensate for each other during development, as many crucial duplicated genes do. What are the unique contributions of each isoform of Sorbs3? While Sorbs3β is found in the nucleus and cytosol, longer isoforms are most often localized to the plasma membrane. Interactions with the upstream portion of the protein may allow it more flexibility in function than the shorter isoform, but could also provide more opportunities for it to sequester binding partners away from one another. Since the expression of these long isoforms is somewhat more restricted to specific cell types, especially in adults, compared to the more ubiquitous Sorbs3β, analysis of its functions in certain cell types could shed light on its specific roles, as will analysis of the regulation of Sorbs3 isoform expression.

How does Sorbs3 contribute to disease progression? As a scaffold protein, Sorbs3 could possible work to both promote signaling pathways and to sequester pathway members apart from one another; evidence for both of these functions has been shown in developing cells, but how does this promote or prevent cancer progression? Observing the effect of Sorbs3 expression on activation of oncogenic pathways in cancer cell lines may give insight into the mechanisms cells use to integrate multiple signaling pathways to promote cell division or metastasis, for example. Computational modeling may prove to be a useful tool for analyzing the complex regulation of Sorbs3 dependent processes in the context of different cell types and protein milieus.

Figure 1: The SoHo family of proteins has conserved domain structure and overlapping functions. ArgBP2, Cap/ponsin, and Sorbs3/vinexin each contain an N-terminal sorbin domain and 3 C-terminal SH3 domains. These domains all mediate specific protein-protein interactions. The sorbin domain of all three proteins interacts with Flotillin, which is targeted to lipid rafts. The first two SH3 domains of all three proteins interact with components of focal adhesions, including vinculin and paxillin. After Roignot et al., 2009.

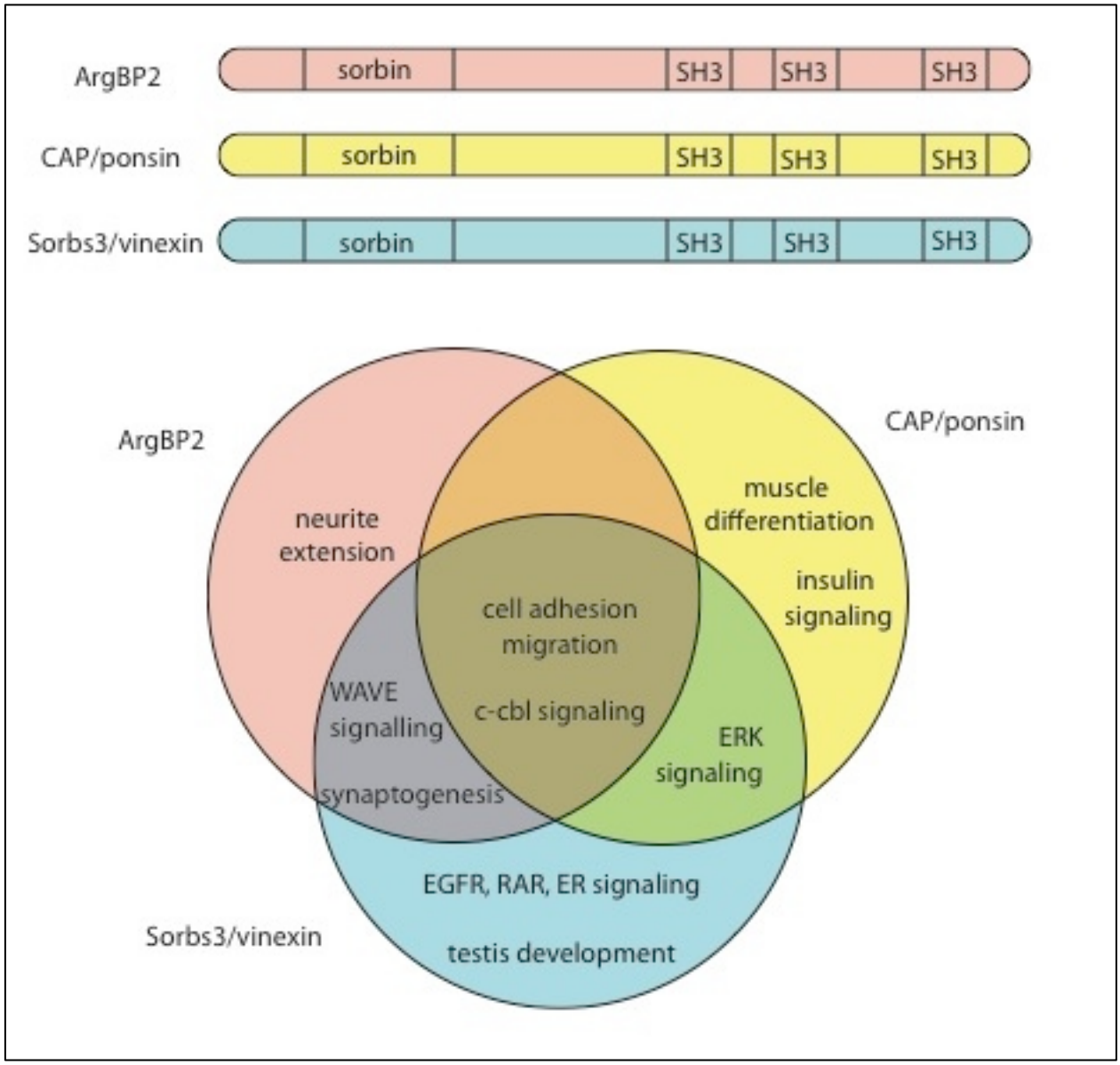
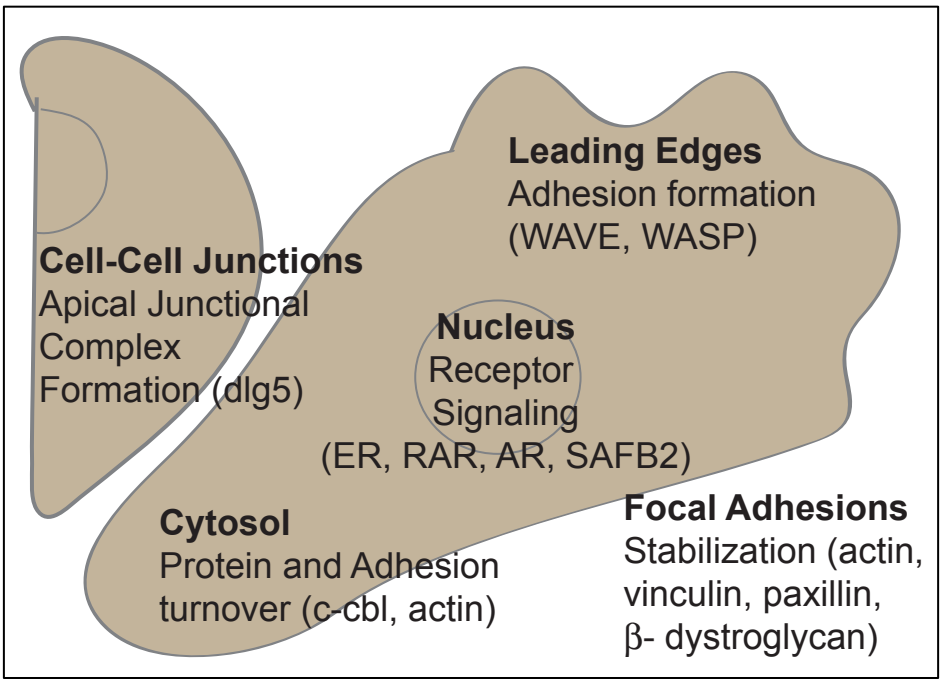


Figure 2: Sorbs3 localization and interactions. Sorbs3 localizes to several sites in cells (shown in bold text) where it interacts with many proteins (in parentheses) to carry out its functions. AR, androgen receptor; ER, estrogen receptor; RAR, retinoic acid receptor.



Chapter IV

Dorsal Root Ganglia Development Requires Modulation of ErbB Signaling by the Scaffold Protein Sorbs3

SUMMARY

The multipotent cells of the vertebrate neural crest (NC) arise at the dorsal aspect of the neural tube, then migrate throughout the developing embryo and differentiate into diverse cell types, including the sensory neurons and glia of the dorsal root ganglia (DRG). As multiple cell types are derived from this lineage, it is ideal for examining mechanisms of fate restriction during development. We have isolated a mutant, *ouchless*, which specifically fails to develop DRG neurons, though other NC derivatives develop normally. This mutation affects the expression of Sorbs3, a scaffold protein known to interact with proteins involved in focal adhesions and several signaling pathways. *ouchless* mutants share some phenotypic similarities with mutants in ErbB receptors, EGFR homologs implicated in diverse developmental processes and associated with several cancers**,** and *ouchless* interacts genetically with an allele of *erbB3* in DRG neurogenesis. However, the defect in *ouchless* DRG neurogenesis is distinct from ErbB loss of function in that it is not associated with a loss of glia. Both *ouchless* and *neurogenin1* heterozygous fish are sensitized to the effects of ErbB chemical inhibitors, which block the development of DRG in a dose-dependent manner. Inhibitors of MEK show similar effects on DRG neurogenesis. We propose a model in which Sorbs3 integrates ErbB signals to promote DRG neurogenesis through the activation of MAPK and upregulation of *neurogenin1.*

INTRODUCTION

Organisms must coordinate the processes of differentiation and migration, both to effect normal embryonic development and to prevent diseases such as metastatic cancer. A particularly interesting system in which to study cell fate specification in the context of migration is the neural crest (NC) cell lineage. The NC arises at the conjunction of neural and non-neural ectoderm; cells then migrate and differentiate into

a variety of tissue types (Le Douarin and Dupin, 2003). Signals for specification/induction, epithelial-to-mesenchymal transition, migration and differentiation all converge in this lineage. Consequently, it can be thought of as a microcosm of developmental signaling in the embryo as a whole and as a useful model for studying the coordination of these processes.

In the trunk, NC cells arise at the dorsal aspect of the neural tube, then migrate ventrally and differentiate. NC cells that migrate medially, between the neural tube and the somite, give rise to the sensory neurons and glia of the dorsal root ganglia (DRG). NC cells coalesce to form segmentally arranged bilateral DRG near the ventralmost aspect of the neural tube. In mammals, DRG precursor migration proceeds in waves of tens to hundreds of cells, followed by significant apoptosis to produce ganglia that contain the full complement of neurons in neonates. In zebrafish, however, the initial migratory population of NC is composed of only 10-12 cells per somite, of which only one or two cells assume a sensory neuronal fate (Raible et al., 1992; Raible and Eisen, 1994; McGraw et al., 2008; McGraw et al., 2012). This relatively simple developmental pattern makes the zebrafish particularly amenable to the study of the earliest processes of DRG sensory neurogenesis.

Some degree of cell fate specification occurs prior to NC migration, but environmental cues also influence the fates of cells in different migratory paths (reviewed in Marmigère and Ernfors, 2007; Pavan and Raible, 2012). The exact mechanisms required for the integration of migration and differentiation signals in the developing NC are not known. While a common neuroglial progenitor population appears to give rise to neurons and glia, the timing of migration may influence whether these cells are associated with DRG (Le Douarin et al., 1986; Serbedizija et al., 1994; Raible and Eisen, 1996; Wright et al., 2010). Upregulation of neuron-specific transcription factors such as *neurogenin1 (neurog1)* and *neurog2* are some of the first events in DRG neurogenesis (Greenwood et al., 1999; Ma et al., 1999; Perez et al., 1999; McGraw et al., 2008). Mechanisms that drive only some cells to activate these transcription programs while others instead assume glial fates are not well understood, although lateral inhibition via Notch/Delta signal (Hu et al., 2011; Mead and Yutzey,

2012), Hedgehog signaling (Ungos et al., 2003), and Wnt signaling (Lee et al., 2004) have been implicated.

The ErbB family of receptor tyrosine kinases plays diverse roles during development, and the paralogs *erbB2* and *erbB3* influence DRG formation in both mammals and zebrafish (Britsch et al., 1998; Reithmacher 1997; Honjo et al., 2008; Morris et al., 1999). ErbB3 is required for several early processes crucial to DRG neuron development, including NC migration (Honjo et al., 2008; Budi et al., 2008) and glial development (Erickson et al., 1997; Riethmacher et al., 1997; Lyons et al., 2005; Garratt et al., 2000). The requirements for ErbB receptors in multiple stages of DRG development complicate our understanding of their distinct roles. ErbBs are also implicated in both the initiation and progression of several cancers (reviewed in Feigin and Muthuswamy, 2009), and unraveling their methods of integrating the processes of differentiation and migration during development could have clinical implications.

We have isolated a zebrafish mutant, *ouchless*, in which most DRG fail to develop, while other NC-derived tissues appear unaffected. NC migration and condensation at the sites of DRG appear unaffected, yet most DRG progenitors fail to undergo neurogenesis. The mutant affects the expression of the scaffold protein gene *sorbs3*, also known as *vinexin* (Kioka et al., 1999), which is known to interact with the ErbB pathway in cell culture (Akamatsu et al., 1999; Suwa et al., 2002; Mitsushima et al., 2004; 2006a; 2006c; 2007; Mizutani et al., 2007a). Here, we show that *sorbs3* is both necessary for DRG neurogenesis and sufficient to rescue the *ouchless* DRG phenotype. We further show that *ouchless* is required cell autonomously with respect to the NC for DRG neuron differentiation and present evidence suggesting that *sorbs3* acts in the same pathway as *erbB3*. We propose that *sorbs3* acts to modulate a subset of *erbB* signaling required specifically for DRG neurogenesis, and integrates this signal with others to promote expression of the proneural transcription factor *neurog1*. This study is the first to implicate Sorbs3 in a developmental process, and addresses the functions of ErbB receptors in NC differentiation, making it significant in the fields of both developmental and cancer biology.

MATERIALS AND METHODS

Fish lines and care

AB, *ouchless* (*sorbs3*^{w35}), *picasso* (*erbB3b*^{wpr2e2}; (Budi et al., 2008), *neurogenin1* (*neuroD3*^{hi1089}*; (*Golling et al., 2002) *Tg*(*sox10:nlsEos*) and *reck (reck*^{w12}*,* Prendergast et al., 2012) embryos were obtained from natural spawning or *in vitro* fertilization, were raised under standard conditions in EM (Westerfield, 1994), and were staged according to (Kimmel et al., 1995). Zebrafish care followed standard procedures approved by the University of Washington Institutional Care and Use Committee.

Isolation of *ouchless*

ENU mutagenesis and early pressure screens were carried out as described (Owens et al., 2008; Lister et al., 1999). Using bulk segregant analysis, the *ouchless* lesion was linked to Chromosome 8, then fine resolution mapping of a total of 1304 individuals narrowed the region to between markers *z53446* and *z25210*. We designed primers to amplify polymorphic CA-repeats scattered throughout the region, and further narrowed the region to 0.34 Mb with *ca37* and *ca48* as flanking markers. Both are simple-sequence-length-polymorphisms (SSLP) that are amplified with the following primers: ca-48 fwd 5'- *tggcaccttaaactgatactc*-3' and ca48 rev 5'- *gccttcaaattcaccataaa*-3'; ca37fwd 5'-*tgagtgaagtgaagtaagcct* -3' and ca37 rev 5'-*gttgtggcaagttagttgg*-3'.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde for 2h at room temperature or overnight at 4°C, then immunostained as described (McGraw et al., 2008). Antibodies were used at the following concentrations: mouse αElavl (mAB 16A11, Invitrogen) 1:500, rabbit αGFP (Invitrogen) 1:1000, mouse αGFP (Invitrogen) 1:100, rabbit αSox10 (Park et al., 2005) 1:1000, rabbit αMBP (Lyons et al., 2005) 1:50, with 20% goat serum. Embryos were imaged as described (McGraw et al., 2008).

In situ hybridization

RNA *in situ* hybridization was performed as in (Andermann et al., 2002). Digoxigenin-labelled probes for *sox10* (Dutton et al., 2001), *neurog1* (Korzh et al., 1998), *crestin*

(Rubinstein et al., 2000) and *neuroD* (Blader et al., 1997) were generated as described. *sorbs3* riboprobes were generated by amplifying fragments from cDNA clones, inserting them into the pCRII-TOPO vector (Clontech), linearizing the vector and transcribing with RNA polymerase using the following oligos: *sorbs3*F AACCCCTGATAAAGCATGTAC; *sorbs3*R CCCGATCCTCAGGCTATG; *pdlim2*F GCGATGAGAACCAGAACTAC; *pdlim2R* GTTTGTGTGCGTGTATATCTT.

Morpholino oligonucleotide (MO) injections

A MO predicted to block the splicing of exons 3 and 4 of Sorbs3 with the sequence TTCCGACAGGGAAAGCACATACC was obtained from Gene Tools**.** 4ng MO in water + 0.2% phenol red was injected into one-cell embryos carrying the TgBAC(*neurog1:dsred)* transgene (Drerup et al., *in prep*) . DsRed positive DRG were counted at 2 – 4 days. Fish were anesthetized in MS-222, then fixed for 2 hours rt or overnight at 4° in 4% paraformaldehyde.

BAC/mRNA rescue experiments

BAC DNA was prepared from clones zK179C10 and zH17L17 (ImaGenes GmbH) using the Nucleobond Extra-Midi Plus kit (Machery Nagel). 1-2 μL DNA 0.1M KCL + 0.2% phenol red was injected into *ouchless; Tg(neurog1:GFP)* embryos at the 1 cell stage. Embryos were fixed at 3 days postfertilization (dpf) and stained with mouse α-Elavl and rabbit α-GFP antibodies. *sorbs3* coding regions were inserted into pCS2MT, and mRNA was synthesized from linearized DNA using the mMessage mMachine *in vitro* transcription kit (Ambion.) mRNA was diluted in water + 0.2% phenol red and injected into *ouchless; Tg(neurog1:EGFP)* embryos. GFP+ DRG were counted on one side of the embryos at 48hpf, 72hpf and 96hpf using a Nikon SMZ1500 fluorescent dissecting microscope.

Transplants

Mosaic embryos were generated by cell transplantation at early gastrula stages as described (Carmany-Rampey and Moens, 2006). Donor embryos were injected at the 1-cell stage with 1nL 0.2% rhodamine-dextran (Sigma) and cells from sphere stage donors were transplanted into shield stage host embryos. Melanocyte-deficient *nacre*

hosts were used to identify mosaic embryos in which transplanted cells had contributed to NC.

Inhibitor Treatments

ErbB inhibitors PD158780 (Tocris Bioscience) and AG1478 (Tocris Bioscience) and MEK inhibitor PD0325901 (Stemgent) were dissolved in DMSO. Embryos were treated with drug and a final concentration of 1%DMSO at 18 hpf, and replenished with fresh drug and media at 42 hpf. At 70-72 hpf, embryos were fixed and stained with mouse α-Elavl and rabbit α-GFP antibodies. Where appropriate, embryos were genotyped as in Golling et al., 2002 for *neurog1* or Prendergast et al., 2012 for *reck*.

Statistics

Contingency tables were analyzed using Statistics To Use (Kirkman 1996). All other statistics were generated using GraphPad Prism for MacOSX version5.0 (GraphPad software, La Jolla, CA.)

RESULTS

ouchless mutants lack DRG sensory neurons

To discover genes with essential functions in sensory neuron development, we conducted a screen for mutants with abnormal DRG. Fish were examined at 3 dpf using antibody against Elavl, which marks differentiated neurons. The *ouchless* mutant, though viable and morphologically indistinguishable from wild type (wt) siblings (Figure 1a,b), lacks most DRG at 3dpf. DRG development was examined using the *Tg(neurog1:EGFP)* line (McGraw et al., 2008), where GFP is expressed in newly-specified neurons and subsequently downregulated in mature Elavl+ cells. We found severe reductions in DRG in *ouchless* mutants (Figure 1c,d). The few DRG that develop are most frequently found in the first eight segments. *ouchless* DRG contain fewer neurons than those in wt animals (Supplemental Figure 1c,d). The spatial arrangement of each remaining ganglion, as well as the orientation of axonal projections, appears normal in mutants (Supplemental Figure 1a,b). More neurons

differentiate in each wt DRG as larvae age; by contrast, *ouchless* fish show reduced neuron addition in the few DRG that form (Supplemental Figure 1c-d). Thus, *ouchless* embryos do not appear to exhibit a delay in initial formation of ganglia, since they do not add DRG after 3dpf. Even though existing DRG in *ouchless* eventually grow to contain neuronal numbers comparable to adult wt animals, mutants never gain a normal number of DRG (data not shown). In addition to DRG, NC in zebrafish gives rise to sympathetic ganglia (SG), enteric neurons and part of the cranial ganglia. We were not able to find any changes in Elavl expression in or morphology of these neurons in mutants (Supplemental Figure 2). These results indicate a specific requirement for *ouchless* in the formation of DRG, but not other NC neurons.

DRG NC precursors are specified and migrate, but fail to differentiate as neurons in *ouchless*

DRG development is the result of several coordinated events, including NC specification, precursor migration, and the specification and differentiation of neurons and glia. Since other NC-derived cells, including autonomic neurons and larval pigment, develop normally in *ouchless* mutants, we reasoned that NC specification and migration were unlikely to be perturbed. Indeed, *sox10* and *crestin* expression by *in situ* hybridization showed no differences in the pattern of ventrally migrating cells at 24 hours post- fertilization (hpf) between wt and *ouchless* mutants (Figure 1g,h; data not shown).

The earliest marker for newly formed DRG sensory neurons is *neurog1*, which can be detected as early as 24 hpf by *in situ* hybridization. Segmented clusters of cells ventral to the spinal cord express *neurog1* in wt embryos, but only occasional *neurog1+* cells could be detected in *ouchless* embryos (Figure 1i, j). *neuroD*, a marker of maturing neurons (Blader et al., 1997), was expressed in a similar sporadic pattern in *ouchless* at 36 hpf (Figure 1k,l), suggesting that the few cells that initiate the neuronal program continue their differentiation. These results suggest that the lack of DRG neurons in mutants originates from impaired specification of the correct number of *neurog1+* precursors. Other peripheral neurons that require *neurog1* function, such as Rohon-Beard cells and cranial ganglia (Andermann et al., 2002), develop normally

(Supplemental Figure S2), suggesting that defects in the initiation of *neurog1* expression are limited to migrating trunk NC.

ouchless affects the zebrafish vinexin gene, sorbs3

We next sought to determine the gene affected in *ouchless* embryos. Bulk segregation analysis localized the mutation to chromosome 8, confirmed by analysis of 1304 individual embryos to a region between marker *z53446* and *z25210*. Analysis of de novo generated SSLP markers narrow the region to the 342 kb flanked by markers ca37 and ca48 (Figure 2a). This interval contained two annotated genes, *pdlim2* and *sorbs3*. We sequenced the entire coding sequence of both genes from wt and *ouchless* cDNA. We were unable to identify a lesion, suggesting that *ouchless* is a mutation in a regulatory region for one of these two genes.

We used a rescue approach to further limit the region containing *the ouchless* lesion. We injected the bacterial artificial chromosome (BAC) clone zK179C10, which includes the genomic region of *sorbs3* and two other genes outside of the recombination interval but not *pdlim2* into one-cell stage *ouchless* embryos (Figure 2a,b). This resulted in a dose dependent rescue of the number of DRG at 3 dpf; embryos injected with 200 ng of BAC developed 21.1 ± 6.7 *neurog1:EGFP/*Elavl+ DRG compared to 7.1 ± 2.6 DRG in controls. We confirmed this result using a second BAC, zH17L17, which contains only the *sorbs3* gene. The other two genes contained in zK179C10 were sequenced and no lesions found. Furthermore, analysis of their mRNA expression patterns by *in situ* hybridization did not reveal expression consistent with roles in DRG development (data not shown). Taken together with the recombination mapping data, this suggests that the lesion in *ouchless* affects the *sorbs3* gene.

The mouse *sorbs3* gene, which encodes several alternatively spliced isoforms of the Sorbs3/Vinexin protein, consists of 19 exons. A long isoforms, *sorbs3*α, contain a sorbin homology domain in the N-terminus and 3 C-terminal src homology 3 (SH3) domains. An intronic promoter controls transcription of a shorter *sorbs3β* isoform that lacks the upstream portion of the protein, including the sorbin domain (Kioka et al, 1999). The zebrafish *sorbs3* locus contains twenty-one exons and is predicted to encode a protein with the same domain arrangement as the mammalian form (Figure

2a). We have isolated transcripts containing message complementary to exons 14-21, which likely encodes *sorbs3*β, and longer transcripts that encode isoforms corresponding to mammalian *sorbs*α. *sorbs3* messages are appropriately spliced in *ouchless*; we confirmed that splice donor and acceptor sites for each exon are unaffected in mutants with genomic DNA sequencing.

sorbs3 is necessary and sufficient for DRG neuron development

To test whether re-expression of *sorbs3* was sufficient to rescue mutants, we generated mRNA expression constructs in the pCS2-MT vector in which *sorbs3* coding sequences were fused to an N-terminal myc tag. mRNA was injected into one-cell *ouchless; Tg(neurog1:EGFP)* embryos. DRG were counted each day from 2-3 dpf, and at 3 dpf animals were fixed and stained with EGFP and Elavl antibodies to label differentiated neurons. 50 pg mRNA encoding a long isoform of *sorbs3* was able to partially rescue the DRG defect (Figure 2c). Higher concentrations of mRNA were deleterious to embryos. We observed no changes in DRG development with overexpression of *sorbs3* in wildtype embryos. mRNA for the shorter *sorbs3b* isoform was also able to rescue the DRG defect in *ouchless* embryos (Figure 2c). This suggests that the N-terminal portion of Sorbs3, including the Sorbin domain, is dispensable for DRG development and that the critical region may be contained in the C-terminal SH3 domains.

To validate that *ouchless* affects *sorbs3* function*,* we designed a morpholino oligonucleotide (MO) to block the splicing of the fifth and sixth exons of *sorbs3,* injected it into one-cell stage *Tg(neurog1:EGFP)* embryos, fixed the embryos at 3 dpf and stained with GFP and Elavl antibodies to assess DRG development. We observed a dose-dependent loss of DRG in injected embryos; WT embryos injected with 6 ng MO developed significantly fewer DRG than uninjected embryos, suggesting that *sorbs3* is required for DRG development (Figure 3a). Furthermore, fish heterozygous for *ouchless* developed significantly fewer DRG than their WT counterparts when injected with as little as 2 ng MO. This sensitization to the effects of MO provides additional support to the hypothesis that the *ouchless* lesion affects *sorbs3*. Mis-splicing of cDNA after MO injection was confirmed using RT-PCR (Figure 3b). MO depletion at these

concentrations causes few other phenotypes suggesting that the decrease in DRG formation is specific (Figure 3c.)

sorbs3 neural tube expression is affected in *ouchless*

We designed an *in situ* hybridization probe that recognizes mRNA corresponding to exons 4-21 of *sorbs3*. At 19 hpf, *sorbs3* is expressed in the anterior neural tube, with expression decreasing posteriorly (Figure 4a). This pattern continues through 24 hpf, but expression is downregulated by 30 hpf (Figure 4e). *In situ* hybridization also revealed strong *sorbs3* expression in the pronephros and more moderate expression in the forebrain and cells populating the epidermis. While *ouchless* embryos show similar *sorbs3* expression patterns in head and pronephros, they exhibit lower levels in the neural tube than wt embryos (Figure 4b,f). In transverse sections at the level of the yolk extension of wt embryos, *sorbs3* expression is concentrated to the ventral neural tube, but there is also weaker expression in cells ventrolateral to the spinal cord resembling NC (Figure 4c, arrow). These could not be found in *ouchless* mutants (Figure 4d, arrow). *sorbs3* expression is spatially and temporally consistent with a role in NC/DRG development, and the decrease in the neural tube in *ouchless* mutants suggests that the DRG defect could be correlated with a specific loss of expression in this tissue.

sorbs3 is required cell-autonomously for DRG neurogenesis

Since *sorbs3* is expressed both in cells resembling NC and in surrounding cells of the neural tube, we constructed genetic mosaic embryos to elucidate the tissue-specific requirement of *sorbs3* expression for DRG development. We transplanted donor cells from dome-stage (3 hpf) embryos into the prospective NC domain at shield stage (6 hpf). We used *nacre* hosts, which lack NC-derived pigment due to a mutation in the *mitfa* gene (Lister et al., 1999) to confirm that NC was successfully targeted by transplantation.

We reasoned that if *sorbs3* is required cell-autonomously in the NC, then wt cells transplanted into *ouchless* hosts should be able to generate sensory neurons. If, on the other hand, *sorbs3* acts non-cell autonomously, wt cells should not be able to give rise to DRG more frequently than *ouchless* mutant cells. Chimeras of rhodamine labeled

Tg(neurog1:gfp) donor cells transplanted into *nacre* hosts were analyzed at 3 dpf for pigment, *Tg(neurog1:gfp)* and Elavl expression, and rhodamine distribution. Out of 44 transplants 31 (70%) contained pigmented cells, demonstrating the transfer of NC precursors, and of those 48% had DRG with cells from donors (Figure 5a,b,i). In complementary experiments, transplanting *ouchless* cells (*neurog1*:*gfp*+, rhodamine labeled) into *nacre* embryos, resulted in a comparable percentage of pigmented larvae (66%, 37/56) but *ouchless* cells never gave rise to DRG (Figure 5d,e,i). In both sets of experiments, the *nacre* host cells gave rise to normal Elavl+ DRG neurons (Figure 5c,f). These results show that *sorbs3* is required cell-autonomously for DRG development, but do not exclude a second, non-cell autonomous function for *sorbs3.* We addressed this question by transplanting wt cells into *ouchless* mutants and found that wt cells were able to generate sensory neurons, indicating that DRG precursors do not require extrinsic *sorbs3* for their development. (Figure 5g,h). Taken together, these data suggest a cell-autonomous role for *ouchless* within DRG precursors.

ouchless is required for metamorphic pigment formation

Despite their severe DRG defects, a proportion of the *ouchless* homozygous mutants survive until adulthood and are able to reproduce. Though grossly indistinguishable from their wt and heterozygous siblings as larvae (Figure 1a,b), adult mutants can be identified by their slow growth and a distinct adult pigment pattern defect. Mutants largely fail to produce the metamorphic pigment cells that begin to appear at 3-4 weeks and do not fully form the adult pigment pattern of wt zebrafish (Figure 6a,b). This eventually results in broken melanophore stripes that consist of few and dispersed melanophores surrounded by iridophores and xanthophores in adults (Figure 6c,d). The mutant pattern phenotype is strikingly similar, though less severe than that of the *picasso* mutant, shown to be a mutation in the *erbb3b* gene (Budi et al., 2008), Figure 6e,f). *erbb3b* mutants also have a DRG phenotype resembling that of *ouchless* mutants (Honjo et al., 2008).

DRG precursor condensation is normal in *ouchless*

In cultured cells, the third SH3 domain of Sorbs3 binds to Son-of-sevenless (Sos), and this binding activity is known to modulate ErbB receptor tyrosine kinase

signaling (Akamatsu et al., 1999). This, in combination with the phenotypic similarities of *ouchless* and *erbB* mutants (Honjo et al., 2008; Budi et al., 2008; Figure 2), led us to hypothesize that Sorbs3 might be a downstream modulator of ErbB signaling during the development of DRG. We therefore sought to examine which ErbB3-dependent developmental processes were perturbed in *ouchless.*

In the absence of *erbb3*, neither DRG sensory neurons nor glia develop in zebrafish embryos because NC cells, instead of pausing to condense and form ganglia, continue to migrate ventrally (Honjo et al., 2008; Britsch et al., 1998; Lyons et al., 2005). This is in contrast to *ouchless*, where migration of NC is grossly normal (Figure 1). However, a subtle defect in the ErbB signaling cascade could interfere with NC condensation and lead to the defects in neurogenesis observed in *ouchless* mutants. To examine this possibility, we visualized DRG progenitor condensation at 24 and 36 hpf in wt, *ouchless*, and *erbB3b* embryos with high-resolution confocal microscopy, using an antibody specific for Sox10. Sox10-positive NC cells, migrating ventrally in a comb-like pattern over the trunk at 24 hpf, became arranged into distinct clusters adjacent to the ventral neural tube in both wt and *ouchless* embryos by 36 hpf (Figure 6g,h,j,k). Consistent with previous results (Honjo et al., 2008), *erbB3b* mutants did not form any Sox10-expressing aggregates at 36 hpf even though initial NC migration was normal at 24 hpf (Figure 6i,l). Thus, while *erbB3* mutants have dramatic defects in DRG condensation, this process appears to be independent of the changes in *sorbs3* found in *ouchless* mutants.

Peripheral gliogenesis is normal in *ouchless.*

ErbB signaling, besides being required for DRG progenitor condensation, has a separate function required for the development of glial cells in the DRG and other tissues (Britsch et al., 1998; Riethmacher et al., 1997; Pogoda et al., 2006; Lyons et al., 2005; reviewed in Britsch, 2007). It is possible that the lack of neurons seen in *ouchless* DRG is the result of a deficit in glia and the trophic support they provide, but if this were the case we would expect to see initial development, then subsequent death, of DRG sensory neurons in *ouchless,* as is seen in *erbB* mutants (Sharghi-Namini et al., 2006; Chen et al., 2003; Riethmacher et al., 1997). We sought to examine the development of

peripheral glia in *ouchless* mutants to determine whether this ErbB-dependent process is also dependent on Sorbs3.

The dearth of early markers of DRG satellite glia prevented us from directly examining the development of these cell types, so we examined the development of other peripheral glia cell types known to be affected in *erbB* mutants. We stained fish at 5 dpf for myelin basic protein (MBP), a marker for myelinating glia (Brösamle and Halpern, 2002) and found MBP+ cells surrounding both the posterior lateral line nerve (PLL) in both wt and *ouchless* mutants (Figure 6m,n). The PLL of *erbb3b* mutants lacked MBP staining (Figure 6o), as described previously (Lyons et al., 2005). Patterning of the lateral line system, which is dependent on peripheral glial cells (Grant et al., 2005), is also normal in *ouchless* mutants (data not shown). To exclude the possibility that the lack of a glial phenotype in *ouchless* may be masked by the hypomorphic nature of the allele, we injected *ouchless* embryos carrying *Tg*(*sox10:nlsEos*) with 6ng *sorbs3* MO and examined the peripheral glia of the DRG at 30hpf and of the lateral line at 5dpf, and found no differences compared to WT fish (Figure 6p-s). Thus, the development of peripheral glia, while requiring ErbB function, does not seem to require *sorbs3.*

sorbs3 and *erbB3* interact genetically for DRG neurogenesis

To further assess the idea that Sorbs3 and ErbB functions intersect, we tested for genetic interactions. Fish heterozygous for either *ouchless* or *erbB3b* were indistinguishable from wt (Table 1). Those heterozygous for both *ouchless* and *erbB3b* showed significantly fewer DRG ($p>0.05$) than either wt or *ouchless/+* fish, but did not differ significantly from *erbB3b/+* larvae. To further characterize this subtle difference, we scored whether DRG were missing in the trunk or in the tail. Fish of all genotypes occasionally lacked DRG in late-developing posterior segments, but we found that the number of fish lacking DRG in the trunk region of double heterozygous fish was significantly larger than all other genotypes (Table 1). While this genetic interaction is subtle, it suggests that *sorbs3* and *erbBb3* may function in the same biochemical pathway.

We reasoned that if Sorbs3 acts in the same pathway as ErbB3, heterozygous *ouchless* larvae might be sensitized to further disruptions in that pathway. Lyons et al. (2005) have previously shown that treatment of *erbB3* heterozygotes with pharmacological ErbB inhibitors shows a dose-dependent effect on myelin gene expression. We tested whether similar interactions might occur to regulate DRG development using two inhibitors: AG1478 (Levitzki and Mishani, 2006; Lyons et al., 2005) and PD158780 (Frohnert et al., 2003; Rewcastle et al., 1998; Fry et al., 1997). Treating fish from 18-72 hpf with either inhibitor resulted in a specific and dose-dependent loss of DRG (Figure 7a, data not shown). PD158780 showed less toxicity than AG1478, so we continued using this inhibitor.

Activation of ErbB signaling is known to initiate several signaling cascades, including MAP kinase activation. We treated fish with the MEK (MAP kinase kinase) inhibitor PD0325901over a range of concentrations from 18-72 hpf, then counted the number of *neurog1:gfp* positive DRG. PD0325901 showed dose dependent effects on DRG development (Figure 7b), similar to that for ErbB inhibitors. These data suggest that the MAP kinase pathway is required for the development of DRG.

We next sought to determine whether heterozygous mutant embryos were sensitized to ErbB inhibition. When treated at a suboptimal concentration of 0.5μM PD158780, both *ouchless/+* and *erbB3b/+* fish showed sensitization to inhibition as compared to wt fish (Figure 7c). *neurog1* appears to be downstream of both ErbB3 and Sorbs3, as its expression is rarely initiated in *picasso* or *ouchless* DRG. We therefore tested whether *neurog1* heterozygotes were also sensitized to ErbB inhibition. When *neurog1/+* embryos (Golling et al., 2002) were treated with ErbB3 inhibitors, they developed significantly fewer DRG than their wt siblings (Figure 7c). Embryos heterozygous for *reck* loss of function*,* another gene necessary for DRG development (Prendergast et al., 2012), are not sensitized to ErbB inhibition, (Figure 7c), suggesting that ErbB inhibitor treatment is specifically affecting a developmental pathway that includes a novel link to *sorbs3* and *neurog1.*

DISCUSSION

In this study, we have identified and characterized a zebrafish mutant, *ouchless,* which develop only a small subset of the normal complement of DRG, fail to add neurons to the DRG that do develop at a normal rate, and fail to develop normal numbers of adult melanocytes. All of these phenotypes are reminiscent of a subset of those seen in mutants for the EGFR receptor tyrosine kinases ErbB2 and ErbB3 (Budi et al., 2008; Honjo et al., 2008; Honjo et al., 2011). The formation of zebrafish adult pigment cells has been shown to be from progenitor cells that are sometimes localized to peripheral nerves and DRG, and that specification of these cells is dependent on *erbB3b* (Budi et al., 2011). Together these observations support the idea that ErbB and Sorbs3 are required in the same population of progenitor for the differentiation of all of these cell types.

ouchless affects the expression of *sorbs3*, a scaffold protein known to interact with components of focal adhesions as well as effectors of ErbB signaling, including Sos, c-raf, and ERK (Kioka et al., 1999; Akamatsu et al., 1999; Mitsushima et al., 2004; Gehmlich et al., 2007; Mitsushima et al., 2007; Matsuyama et al., 2005). Our experiments support the hypothesis that Sorbs3 and ErbB2/3 are part of the same genetic pathway. ErbB2/3 and Sorbs3 are required for *neurog1* expression in the DRG, *ouchless* and the *erbB3b* allele *picasso* interact genetically in DRG neurogenesis, and *neurog1/+* embryos are also sensitized to the DRG-depleting effects of ErbB signaling. Taken together, these results suggest a crucial role for Sorbs3 in DRG development and shed light on the processes of neuron differentiation through ErbB signaling.

Sorbs3 has been previously implicated in neuronal function but not initial specification or differentiation. Sorbs3 is expressed in the developing rat and mouse brains (Kawauchi et al., 2001), and is localized to developing growth cones and filopodia, as well as to synapses (Ito et al., 2008; Ito et al., 2007). However both isoform-specific and complete knockouts of Sorbs3 in mice do not have any apparent DRG or pigment phenotypes (Matsuyama et al., 2005; Kioka et al., 2010). There are several possible explanations for the differences between phenotypes of *ouchless* mutants in zebrafish and mouse Sorbs3 knockouts. One possibility is that mammalian

paralogs of Sorbs3, CAP/Ponsin/Sorbs1 and ArgBP2/Sorbs2 (Kioka et al., 2002), may compensate for the loss of Sorbs3 in most developmental processes, including the development of DRG. Transcriptome analysis of mouse embryos suggests that at least one of these paralogs is expressed in developing DRG (Diez-Roux et al., 2011). Alternatively these phenotypic differences, like those observed between the mouse and fish after ErbB loss of function, may be a result of distinct regulatory pathways for DRG development within these organisms.

Our experiments support a novel connection between Sorbs3 and ErbB signaling. We propose a model in which Sorbs3 acts as a scaffold to bind components of ErbB signaling complexes to promote the expression of *neurog1* and subsequent differentiation of DRG neurons. This function of Sorbs3 would likely occur within the precursors of DRG neurons, since Sorbs3 is required cell-autonomously. While Sorbs3 has not been directly implicated to modulate ErbB2/3 signaling, there is extensive evidence for its interaction with EGFR signaling. Sorbs3 binds to the adapter protein Sos (Akamatsu et al., 1999), and this binding is required for the activation of the MAPKs ERK and JNK by the ErbB ligand EGF (Akamatsu et al., 1999). Sorbs3 binds to ERK1/2, as well as c-Raf (Mitsushima et al., 2004); (Matsuyama et al., 2005), and prevents the dephosphorylation, and thus promotes the kinase activities of, both EGFR and ERK1/2 (Mitsushima et al., 2006a; Mitsushima et al., 2007). At the same time, ERK itself phosphorylates Sorbs3, which inhibits cell migration (Mitsushima et al., 2004; Mizutani et al., 2007a). Expression of Sorbs3 in v-src transformed cells also suppresses their enhanced migration phenotype (Umemoto et al., 2009a). EGFR and ErbB2/3 signaling share many of these signaling components including Sos, Raf and ERK (Yarden and Sliwkowski, 2001), suggesting that similar interactions may underlie Sorbs3 and ErbB2/3 functions during DRG sensory neuron development.

While MAPK signaling has been implicated in regulating several aspects of mammalian neuron development, the roles of specific signaling components in DRG specification remain unclear. ERK5, which is activated downstream of ErbB2/3 (Esparis-Oganda et al., 2002), regulates *neurog1* during cortical neurogenesis (Cundiff et al., 2009), suggesting it might play a similar role during DRG development. Activated ERK2

is found in developing NC and peripheral nervous system (Corson et al., 2003), its activity is required for some aspects of NC development (Newbern et al., 2008), and its phosphorylation can activate neurogenesis programs (Kim et al., 2004). However, while genetic inactivation of ERK1/2 results in significant effects on sensory neuron axon outgrowth and survival, loss of ERK1/2 or ERK5 results in little gross changes in initial DRG formation (Newbern et al., 2011). The components of the MAPK signaling pathway that are involved in zebrafish sensory neuron specification remain to be identified.

Disruption of ErbB2/3 signaling has a greater phenotypic effect than that resulting from interference with Sorbs3. In particular, glial development appears normal in *ouchless* mutants in contrast to disruptions caused by loss of ErbB signaling. These observations suggest that while ErbB2/3 plays multiple roles in NC development, the outcomes of signaling are refined by Sorbs3 interaction through integration of other signals. A candidate for an additional signaling pathway modulated by Sorbs3 is focal adhesion signaling. Sorbs3 is often localized to, promotes the assembly of, and binds to several components of focal adhesions (Kioka et al., 1999; Gehmlich et al., 2007; Thompson et al., 2010). Several integrins, extracellular components of focal adhesions, have been implicated in NC migration, and loss of these proteins cause defects in NC development (reviewed in Perris and Perissinotto, 2000). Focal adhesions are signaling centers, and are often characterized by high concentrations of ErbB2/3 dimers (reviewed in Pinon and Wehrle-Haller, 2011), poising ErbBs and other focal adhesion components to physically interact. The coordination of ErbB and focal adhesion signaling in the DRG is plausible, given their synergism in other systems. ErbB2 promotes the formation and stabilization of focal adhesions in breast carcinoma (Zaoui et al., 2010; Marone et al., 2004; Zaoui et al., 2008). ErbB signaling is also known to promote focal adhesions in cultured adult neurons, which facilitates neurogenesis (Grimm et al., 2010; Grimm et al., 2009). Scaffolding of adhesion signals has already been demonstrated in the NC. Nedd9, a member of the p130Cas family of scaffold proteins is required for the integration of integrin-based adhesions and NC migration (Aquino et al., 2009). Interestingly, p130Cas family proteins are known to coordinate signals from adhesions and ErbB2 to promote aggressiveness and metastases in several cancer models (reviewed in Cabodi et al., 2010).

A critical step in DRG development regulated by Sorbs3 and by ErbB2/3 may be NC cell migration. Sorbs3 has been implicated in keratinocyte cell migration and wound healing, events also regulated by EGFR signaling (Kioka et al., 2010). In addition, Sorbs3 overexpression inhibits cell migration in vitro, and phosphorylated forms localize to the leading edge of migrating cells (Mitsushima et al., 2004; Mizutani et al, 2007a; Uemoto et al., 2009b). There is a large body of evidence that ErbB receptors regulate cell migration (reviewed in Feigin and Muthuswamy, 2009), and zebrafish NC cell migration is altered after loss of *erbB2/3* (Honjo et al., 2008). While we have found no gross deficits in *ouchless* mutants or after *sorbs3* MO injection, whether there are subtle effects on migration of specific DRG precursors within the migrating NC population will require more detailed analysis.

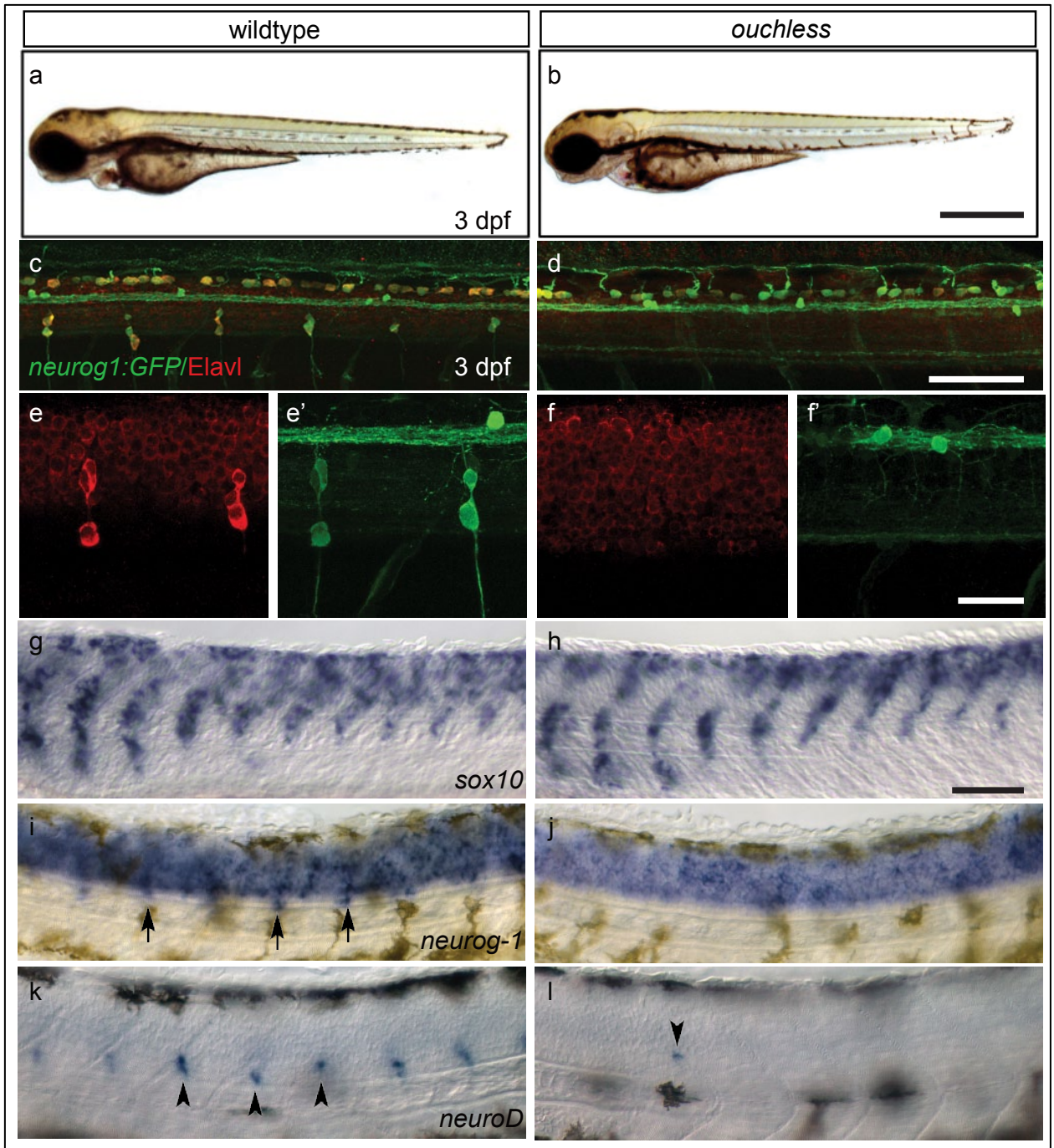
ErbB signaling is already a well-established player in several cancers, including those of the NC. The Sorbs3 paralog ArgBP2 has been implicated in anti-oncogenic processes (Roignot et al., 2009), raising the possibility that Sorbs3 could be an important modulator of ErbB signaling in cancers as well. As many developmental signaling processes are recapitulated in error to cause cancer, further study of the role of the Sorbs3 protein in developmental processes may provide new insight into cancer biology.

Table 1: *ouchless* and *picasso* interact genetically in DRG neurogenesis.

genotype	n	Mean DRG per fish side ± SD (***)	fraction fish missing at least 1 DRG		
			missing in any segment (n.s.)	*missing in trunk (***)*	*missing in tail (n.s.)*
WT	67	31.71±0.68	0.313	0.045	0.284
ouchless/+	72	31.66±0.86	0.306	0.028	0.375
erbB3b/+	38	31.54±0.83	0.343	0.086	0.286
ouchless/+; *erbB3b*/+	69	30.79±2.39	0.464	0.246	0.348

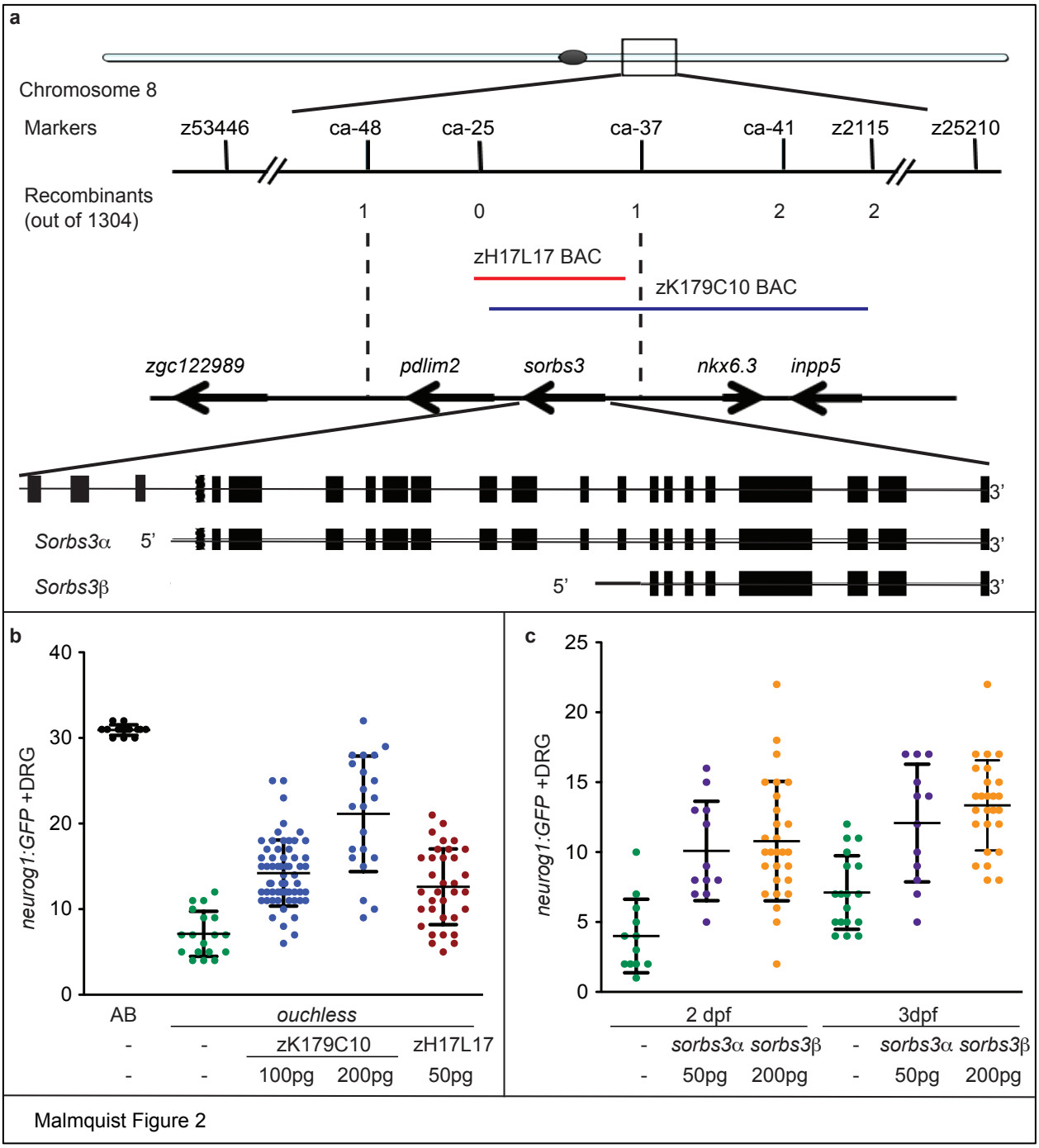
3 dpf *Tg(neurog-1;EGFP)* embryos were scored for DRG in each segment. Kruzkal-Wallis one-way test on mean DRG per fish was significant ($p = 0.0002$). Dunn's multiple comparison post-hoc test showed significant differences between *ouchless/+;erbB3b/+* and wt ($p < 0.001$) and *ouchless/+* ($p < 0.01$). Comparisons between other genotypes were not significant. X^2 test of fraction of fish missing DRG shows a significant difference between groups lacking DRG in trunk (segments 1-16; $X^2 = 22.4$, $p < 0.0005$), and no significant difference between groups with respect to total ($X^2 = 4.83$, $p = 0.184$) or tail (segments 17-34; $X^2 = 0.846$, $p = 0.834$).

Figure 1: *ouchless* mutants lack DRG sensory neurons. (a,c,e,g,i,k) wt or (**b,d,f,h,j,l**) *ouchless* mutant embryos. (**a,b**) brightfield images at 3 dpf. (**c,d**) lateral view of 3 dpf *Tg(neurog1:EGFP)* embryo*,* immunostained for EGFP (green) and Elav1(red). DRG are located ventral to the spinal cord. (**e, f**) high magnification of two segments of a *Tg(neurog1:EGFP)* showing colocalization of Elavl (**e,f; red**) or EGFP (**e',f'; green**). (**g,h**) *in situ* hybridization for *sox10* at 24 hpf*.* (**i,j**) *in situ* for *neurog1* at 30 hpf, showing DRG neurons ventral to the spinal cord (arrows). (**k,l**) *in situ* for *neuroD* at 36 hpf, showing DRG neurons (arrowheads). Scale bars: a, b = 500μm ; c, d, g-l = 100μm; e-f = 25μm.



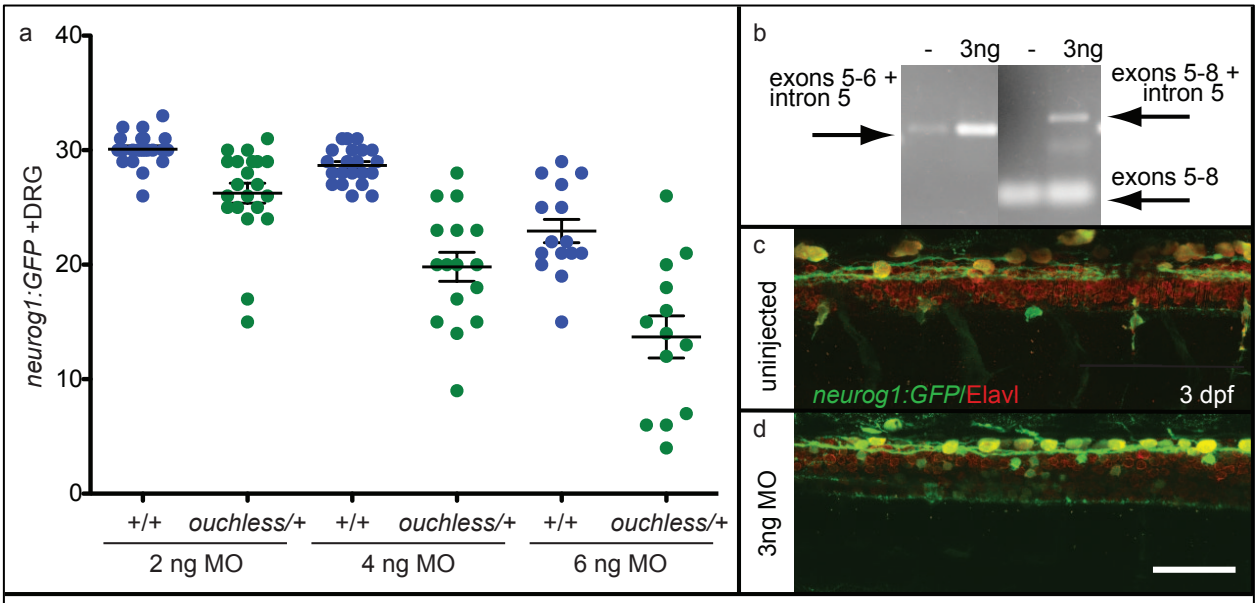
Malmquist Figure 1

Figure 2: *ouchless* affects the *sorbs3* gene. (a) The lesion in *ouchless* was mapped to a 0.34 Mb region on chromosome 8, between markers between markers z53446 and z2115. Number of recombinants is shown below the solid line; dotted vertical lines indicate the recombination interval that contains the *ouchless* lesion. BACS zH17L17 (red line) and zK179c10 (blue line), used for rescue experiments, span parts of this interval. The two genes within the recombination interval, *pdlim2* and *sorbs3*, as well as three others outside the interval, are indicated by arrows in the direction of transcription. The *sorbs3* locus contains at least 21 exons indicated by black boxes and 21 introns (not to scale). Two transcripts containing exons 2-21 and 15-21, used for rescue in **(c),** are shown. **(b)** *Tg(neurog1:EGFP)* or *ouchless; Tg(neurog1:EGFP)* fish injected with amounts of BAC DNA shown were fixed at 3 dpf and immunostained for EGFP and Elavl. Each dot indicates the number of EGFP/Elavl+ DRG on one side of one fish, error bars indicate SD. **(c)** *ouchless; Tg(neurog1:EGFP)* fish injected at the one-cell stage with amounts of *sorbs3* mRNA shown were assessed for EGFP+ DRG at 2 and 3 dpf. Each dot indicates the number of DRG on one side of one fish. error bars = SD.



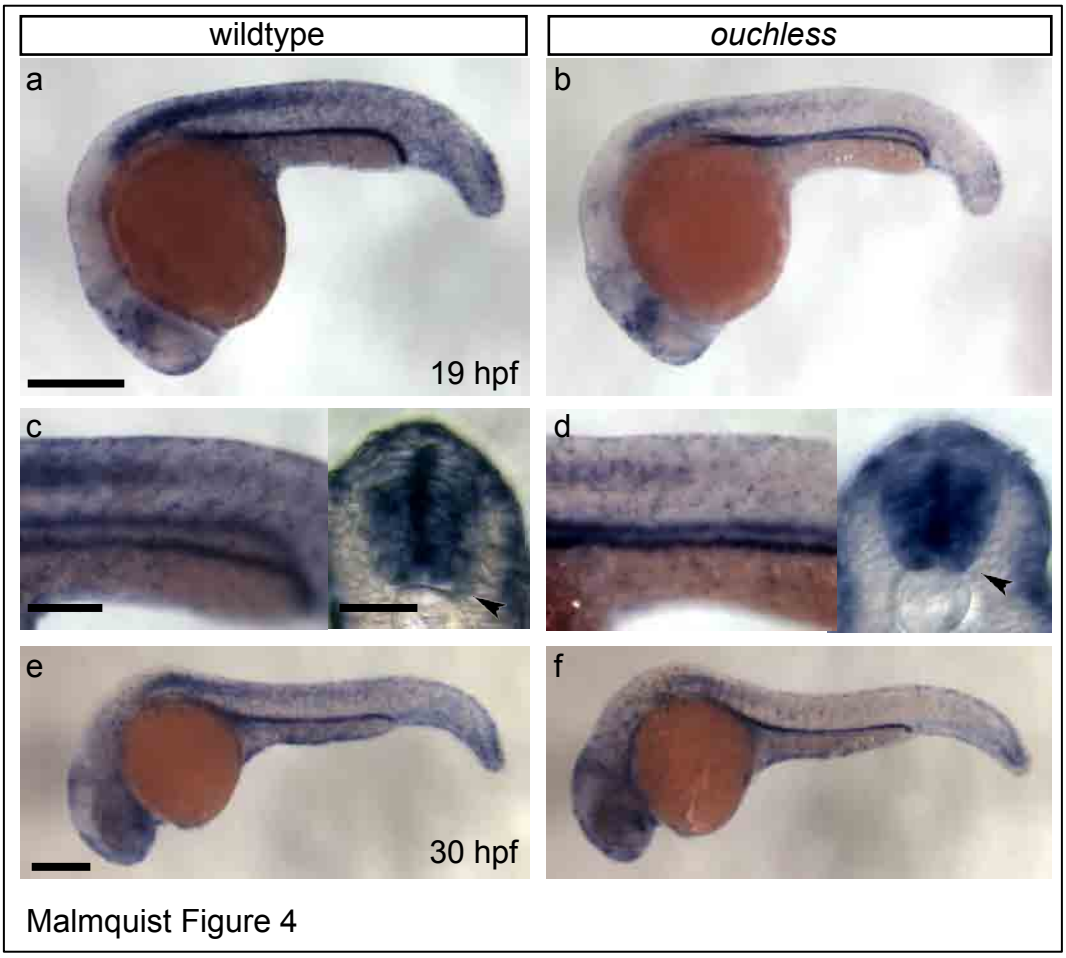
Malmquist Figure 2

Figure 3: *sorbs3* is required for DRG neurogenesis. (a) *Tg(neurog1:EGFP)* or *ouchless/+; Tg(neurog1:EGFP)* embryos were injected with the amounts of MO shown, fixed at 3 dpf and immunostained for EGFP/Elavl. Each dot indicates the number of DRG on one side of one fish, error bars indicate SD. **(b)** *Tg(neurog1:EGFP)* were injected with 3 ng MO, and cDNA prepared at 24 hpf. PCR indicated missplicing of the *sorbs3* transcript. **(c,d)** images 3 dpf *Tg(neurog1:EGFP)* embryos, immunostained for EGFP (green) and Elav1(red). Scale bar = 50μm.



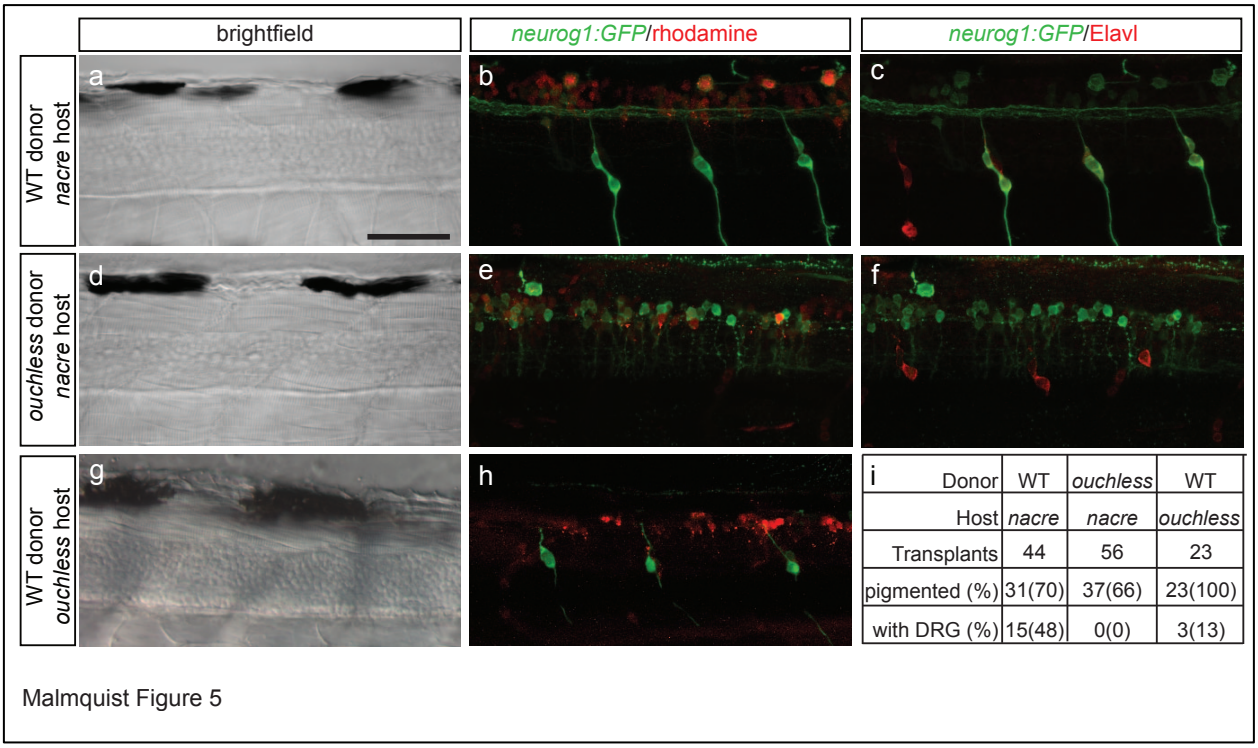
Malmquist Figure 3

Figure 4: *sorbs3* expression is altered in *ouchless.* (a) *in situ* hybridization for *sorbs3* mRNA in 19 hpf wt fish reveals expression in the anterior neural tube, eye, brain, pronephros and surface cells. **(b)** *sorbs3* expression in the neural tube and surface cells is decreased in *ouchless* at 19 hpf, but expression is unchanged in other tissues. **(c,d)** region dorsal to the yolk extension of 19 hpf wt and *ouchless* embryos, and transverse sections at the level of the yolk extension. Cell outside the neural tube in the position of the developing DRG is indicated by arrowhead. **(e,f)** expression of *sorbs3* at 30 hpf in WT and *ouchless*. Scale bars: a,b,e,f = 250μm; c,d = 100μm; c,d insets = 25μm.



Malmquist Figure 4

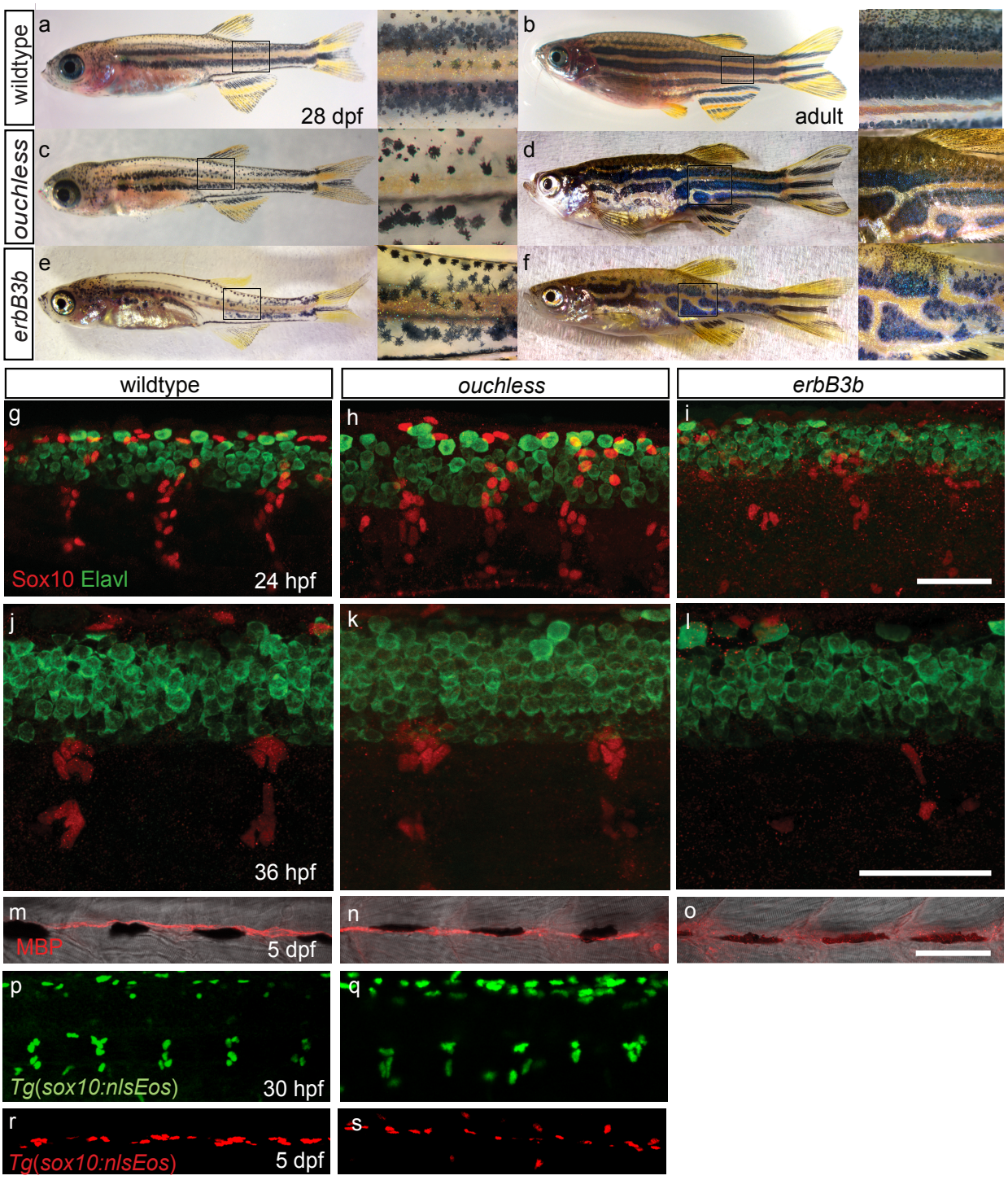
Figure 5: *sorbs3* is required in the NC for DRG neurogenesis All embryos are shown at 3 dpf. **(a,d,g)** brightfield image showing donor-derived pigment cells. **(b,e,h)** EGFP immunofluorescence (green) and rhodamine (red). **(c,f)** EGFP (green) and Elavl (red) immunofluorescence. **(a-c)** wt *Tg(neurog1:EGFP)* donors to *nacre* host chimera. Donors show robust contribution to DRG. **(d-f)** *ouchless*;*Tg(neurog1:EGFP)* to *nacre* host chimera. No donor cells are found in DRG. (**g-h)** wt *Tg(neurog1:EGFP)* donor to *ouchless* host chimera. Wt cells form DRG in mutant hosts. (**i)** quantification of transplant experiments.



i Donor	WT	*ouchless*	WT
Host	*nacre*	*nacre*	*ouchless*
Transplants	44	56	23
pigmented (%)	31(70)	37(66)	23(100)
with DRG (%)	15(48)	0(0)	3(13)

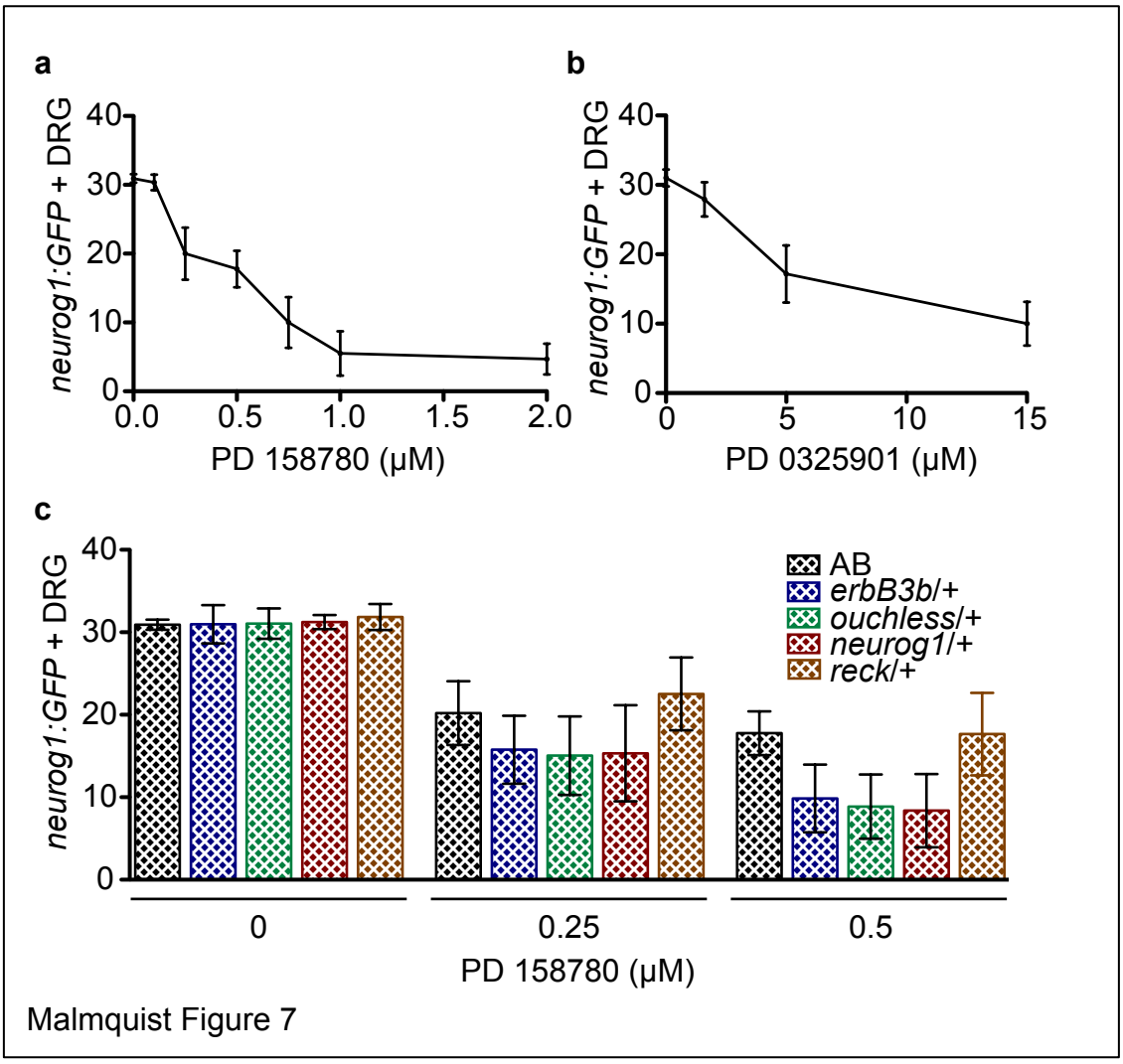
Malmquist Figure 5

Figure 6: *ouchless* and *erbB3b* share a subset of phenotypes Brightfield images of WT **(a, b)**, *ouchless* **(c, d)** and *erbB3b* **(e, f)** fish at 28 dpf **(a,c,e)** and as adults **(b,d,f)**. Boxes indicate positions of high-magnification insets. Both *ouchless* and *erbB3b* fish have fewer melanophores than wt at 28 dpf and disrupted stripe patterns as adults. **(g-i)** immunofluorescence of Sox10+ NC cells (red) and Elavl spinal neurons (green) in AB, *ouchless*, and *erbB3b* mutants at 24 hpf. NC pattern is disrupted in *erbB3b,* but not in *ouchless*. **(j-l)** immunofluorescence of Sox10+ DRG precursor cells (red) and Elavl spinal neurons (green) in AB, *ouchless*, and *erbB3b* mutants at 24 hpf. NC condensation at the site of the presumptive DRG is disrupted in *erbB3b,* but not in *ouchless*. **(m-o)** immunofluorescence of MBP in the lateral line glia of AB, *ouchless* and *erbB3b* at 5 dpf. The disorganization of glia seen in *erbB3b* is absent in *ouchless.* Scale bars = 50μm. **(p-s)** images of *Tg(sox10:nlsEos)* in *ouchless* embryos injected with 6ng *sorbs3* MO **(q, s)** show no additional glial defects when compared to wt embryos **(p, r). (p-q)** DRG precursors at 30 hpf, **(r-s)** lateral line glia at 5dpf.

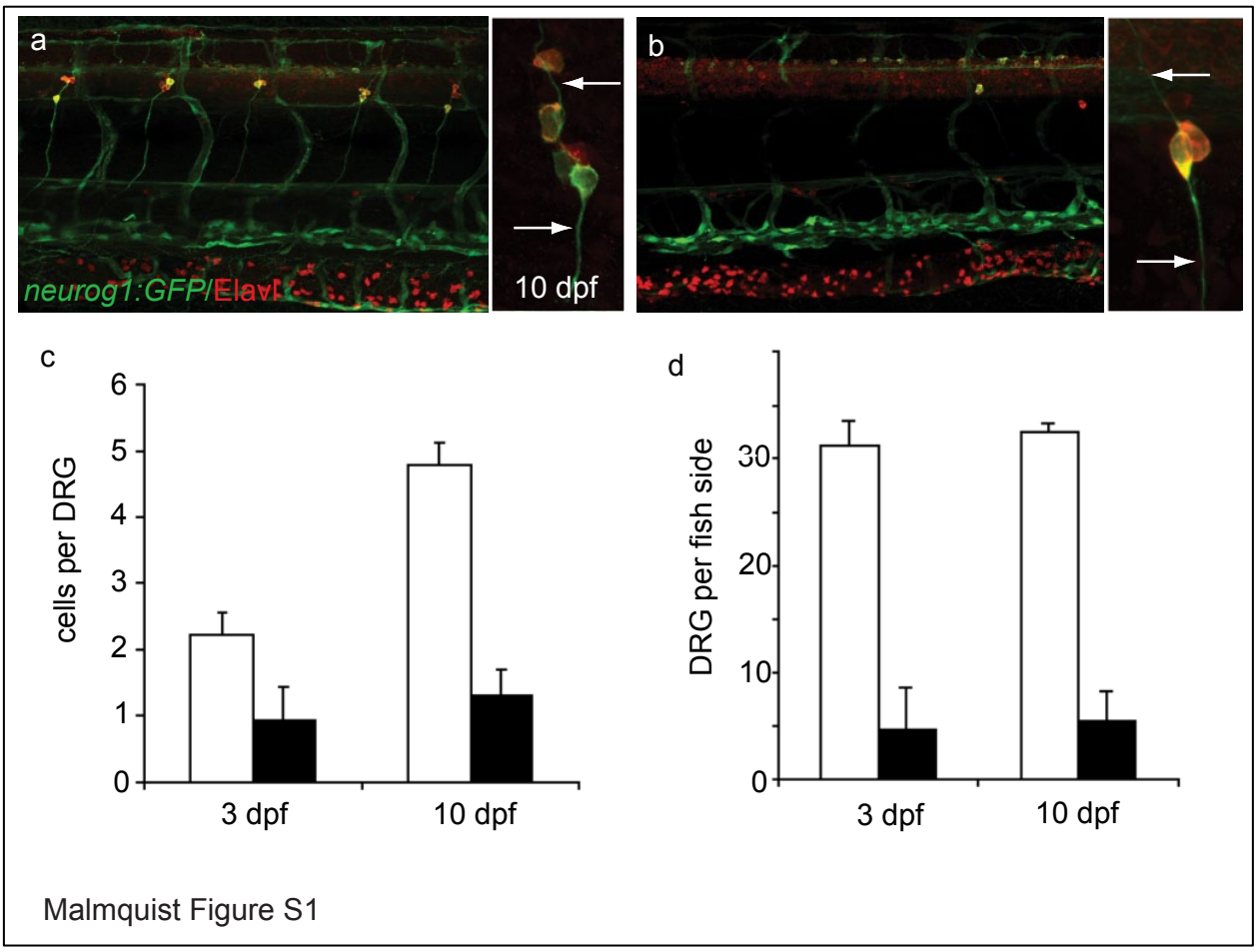


Malmquist Figure 6

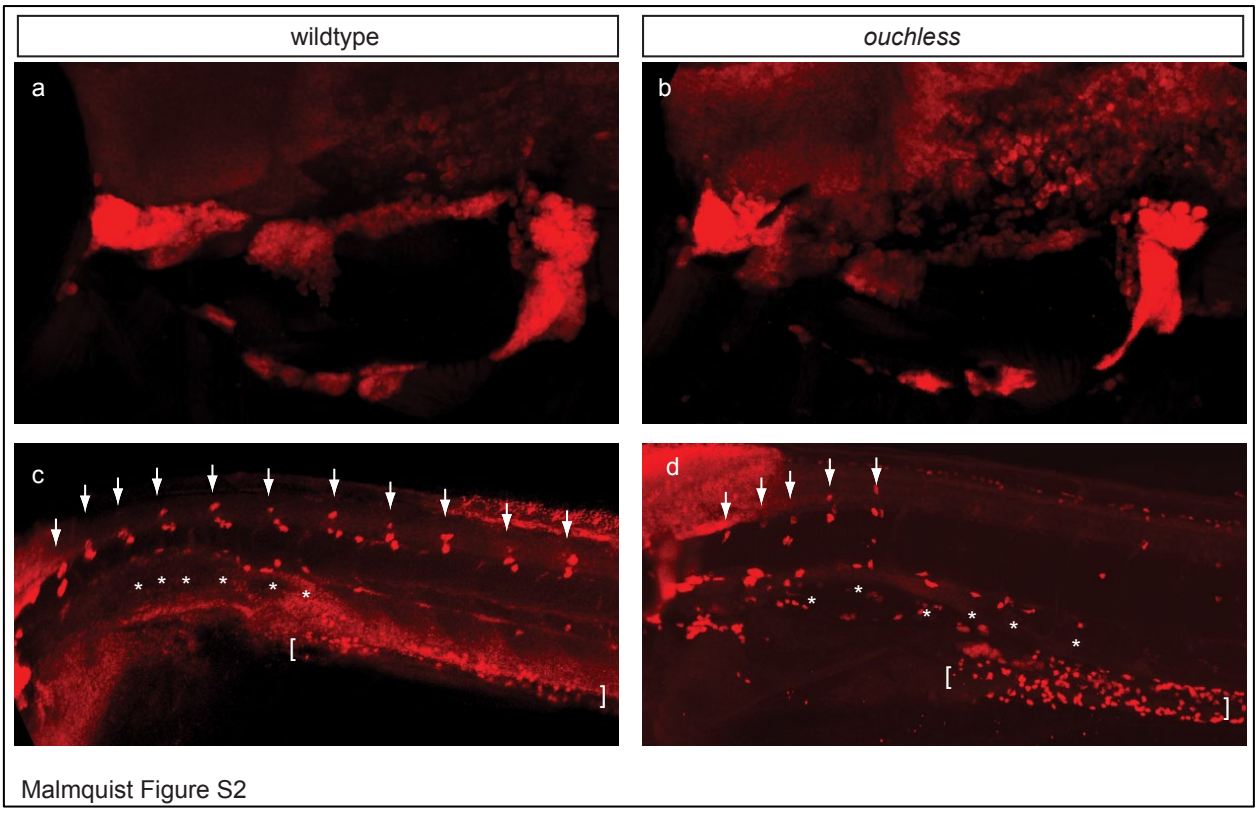
Figure 7: ErbB3 and Sorbs3 are part of the same biochemical pathway.
Tg(neurog1:EGFP) embryos treated from 18-72 hpf in EM + 1% DMSO + PD158780 **(a),** or PD0325901 **(b),** fixed and stained for GFP and Elavl at 72 hpf. Data shown are mean + SD of DRG on one side. **(c)** AB, *ouchless/+, erbB3b/+*, *neurog1/+,* and *reck/+* embryos, all carrying *Tg(neurog1:EGFP)*, were treated from 18-72 hpf in EM + 1% DMSO + PD158780, then fixed and stained for GFP and Elavl at 72 hpf. Data shown are mean + SD of DRG on one side.



Supplementary Figure 1: DRG neuron addition is altered in *ouchless.* (a) lateral view of 10 dpf transgenic zebrafish expressing *Tg(neurog1:EGFP),* immunostained for EGFP (green) and Elavl (red). High magnification view of a single DRG composed of six EGFP/Elavl + neurons. **(b)** lateral view of 10 dpf *ouchless; Tg(neurog1:EGFP)* larva, immunostained for EGFP (green) and Elav1(red), and high magnification view of a single DRG composed of two EGFP/ElavL1 + neurons. Arrows in **(a)** and **(b)** indicate dorsal and ventral axonal projections. **(c)** quantification of the number of EGFP/ElavL1+ cells per DRG in WT (white bars) and *ouchless* (black bars) at 3 dpf and 10 dpf. **(d)** quantification of the number of EGFP/ElavL1+ DRG per fish side in WT (white bars) and *ouchless* (black bars) at 3 dpf and 10 dpf. For panels c and d, n= 6 fish and error bars are SEM.



Supplementary Figure 2: Other neurons are unaffected in *ouchless.* (a)confocal image of a 4 dpf WT larvae immunostained with Elavl, showing cranial ganglia. **(b)** image of a 4 dpf *ouchless* larvae, showing normal cranial ganglia morphology. **(c)** confocal image of a 7 dpf WT larvae immunostained with ElavL1, showing DRG (arrows), SG (asterisks) and enteric neurons (brackets). **(d)** image of a 7dpf *ouchless* larvae. SG and enteric neurons are present in segments lacking DRG.



Chapter 5

Summary and Future Directions

I began this dissertation with a brief review of the development of the neurons of the DRG, from the initial step in neural crest induction, through the migration and differentiation into neurons, and into the later development of neurons through precursor populations that are specified early in embryogenesis. In the second chapter, I reviewed how the ErbB receptor tyrosine kinases are known to be involved in several migratory developmental processes, including the migration of the NC. In the third chapter, I reviewed how one protein, Sorbs3, has been shown to interact with several signaling pathways including ErbB signaling, and provided some speculation about how its action could tie together interactions through focal adhesions and some of these signaling networks. In the fourth chapter, I describe how Sorbs3 is required for DRG neurogenesis and propose a mechanism by which it modulates ErbB signaling to promote cell fate choice. I have proposed a model in Figure 1 of this chapter for how the interaction of ErbB2/3 and Sorbs3 promote DRG neurogenesis.

While much work has detailed the interactions of Sorbs3 with many of its binding partners, little is known about how these interactions can influence signaling, especially through ErbB. In order to understand whether the interaction of Sorbs3 and the Sos ErbB adapter seen in cell culture is observed in zebrafish NC, I would like to generate transgenic lines expressing both Sorbs3 and ErbB2 under the control of the *sox10* promoter, so as to observe their localization in the NC in live fish. This system has the potential to uncover many aspects of the function of Sorbs3, not least of which is that it will be the first examination of the interaction of these two proteins *in vivo* in an intact organism. The zebrafish is an attractive system in which to study the migration of cells *in vivo*, since large numbers of rapidly and externally developing embryos can be obtained and manipulated easily. Constructs are easily injected into one-celled embryos and can be rapidly screened for transgenesis, while timelapse imaging studies such as those employed by Prendergast et al., (2012) can be used to image living, developing fish continuously over the entire length of time from initial migration of the DRG to differentiation of the first neurons.

Given their pattern of localization in other tissues and its interaction with Sorbs3 show in Chapter 4, I predict that ErbB2 and ErbB3 localize to focal adhesions in the NC. It will interesting to see whether ErbB2/3 actually play a role in maintaining the structure of these adhesions and whether this is part of the mechanism through which mutants develop mispatterned DRG. To investigate this, the localization of focal adhesions components, or possibly Sorbs3, could be observed in developing transgenic fish treated with the ErbB inhibitor PD158780 or when ErbB signaling is constitutively activated. If the ErbB receptors do indeed play a role in stabilizing NC adhesions, the adhesion components imaged may show different patterns of localization in perturbed embryos.

The aforementioned experiment would test the interaction of two components in the development of the NC. A broader question that could be addressed through a series of experiments such as this one is how ErbB2/3, Sorbs3, Notch, Delta and focal adhesion components, such as vinculin, affect the localization of one another and the development of subpopulations of NC. Each of these components could be depleted using a combination of mutant lines, MO and pharmacological inhibitors, and the localization of the other components could be investigated using imaging of transgenic animals. This type of comprehensive *in vivo* study would be almost impossible to perform in many other animal models.

While the data presented in Chapter 4 presents several lines of evidence that Sorbs3 and ErbB interact genetically, it is not clear that this interaction results in activation of the MAPK pathway, although inhibition of this pathway does affect neurogenesis in the DRG. In other systems, antibodies against activated forms of MAPK can be used to detect changes in this pathway, but these are difficult to work with in zebrafish tissue. A more feasible and exciting way to detect the activity of MAPK in the developing zebrafish DRG would be to use a transgenic reporter of MAPK activity, such as the one described by Harvey et al., (2009). With this FRET- based reporter, the dynamics of ERK activity could be observed at a single cell level.

In the zebrafish, only one or two neurons initially differentiate out of the dozen or so cells that initially populate the ganglia. There are several open questions left in this

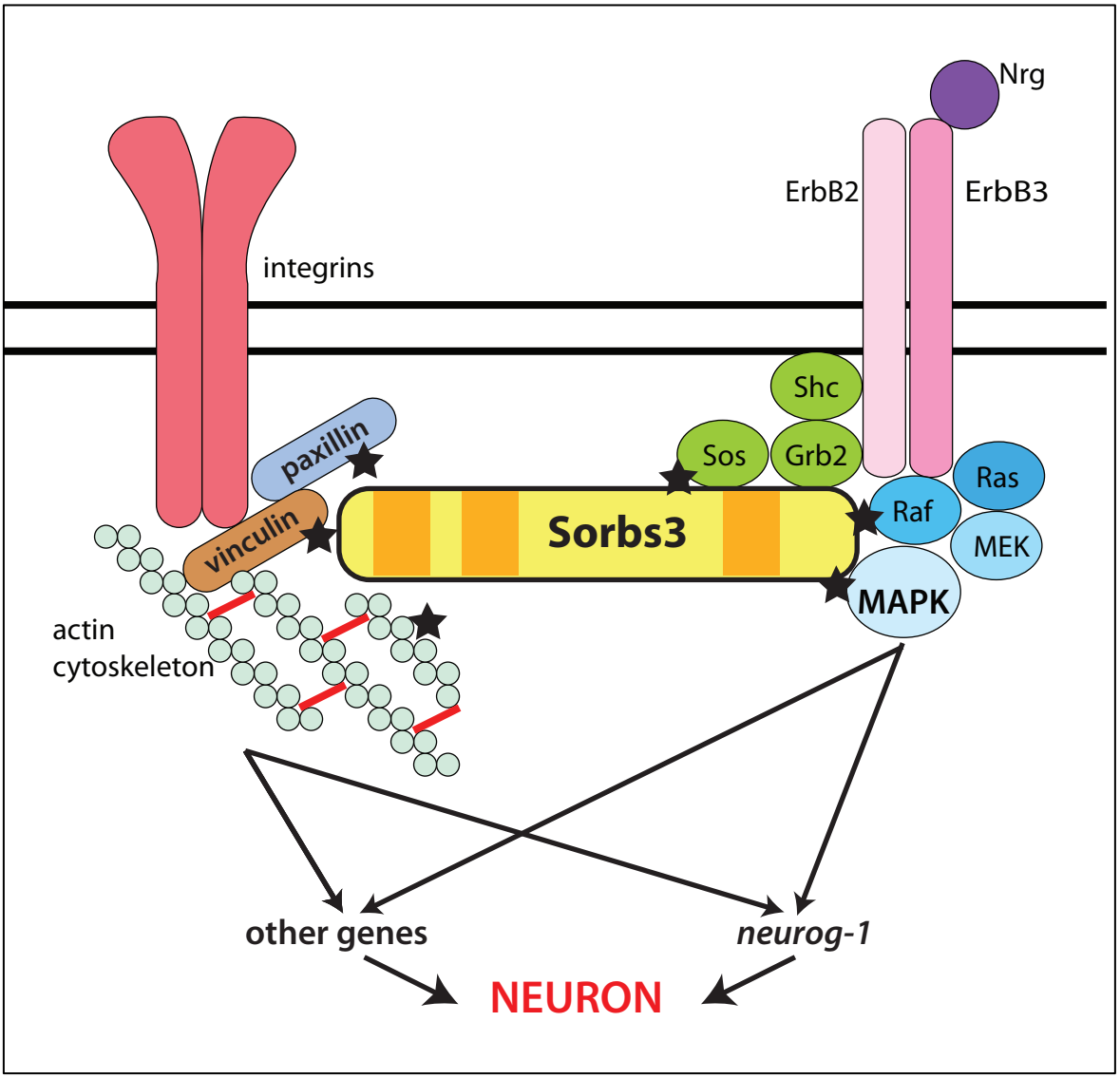
observation. First, how are the cells that initially become neurons different from the others? Using the transgenics described above, a unique signaling event or protein localization pattern required for the differentiation of the first neurons could possibly be observed in one or two of the cells of the presumptive ganglia. While it may be difficult to observe the differences in the activities of the individual cells of the same ganglia, this marker of neural fate may be more easy to observed in more of the cells if the Notch pathway was blocked, promoting neurogenesis of more than the normal number of neurons in the ganglia.

A second question that remains is the nature of the fates of the non-neuronal DRG precursors. Although it is known that some of the cells differentiate into myelinating glia, it is not clear whether the entire population of non-Schwann glial cells are capable of dividing or differentiating to produce later born neurons or pigment cells. There are no known markers that allow differentiation of these cell types in the zebrafish, with the exception of later-differentiating myelin structural proteins in Schwann cells. One possible way to uncover new markers of the precursors that probably persist in the DRG is to compare the expression of genes in the WT DRG with those in the DRG of *ouchless* or *picasso* mutants. Since the glia and precursor sNCCs of the DRG can be isolated, by flow cytometry, from the differentiated neurons of the DRG and non-NC cells based on Eos expression in the *Tg*(*sox10:nlsEos*) line (Andy Prendergast, unpublished), a sample of cells large enough for microarray analysis could be collected to analyze for differential expression of genes, particularly for genes associated with “stemness.” Potentially, these genes could serve as markers of the precursor population residing in the DRG. Comparison of their expression with that of Schwann cell markers and could help to characterize the initial makeup of the DRG, and describe the glial populations therein. Investigating the satellite glia and precursor population in the DRG is of particular clinical relevance. These populations could be conduced to give rise to new neurons, as well as other cell types, with use in regenerative therapies for diseases and injury. In addition, the satellite glia of the DRG have been found to have causative roles in the persistence of chronic neuropathic pain (reviewed in Ohara et al, 2009).

Finally, the role of Sorbs3 in the development of other organ systems remains

relatively unexplored, but my work has demonstrated that it is expressed in several tissues and is required for the development of one, the DRG. Using the *ouchless* mutant and other tools I described in Chapter 4, it will be interesting to examine the role of Sorbs3 in the development of other tissues, including those in which the *sorbs3* mRNA is expressed. ErbB signaling has been implicated in the development and pathology of several of these tissues, including the brain and kidney (reviewed in Birchmeier, 2009, Zeng et al., 2009), and an interaction with Sorbs3 could also be important in these processes.

Figure 1: Sorbs3 integrates signals from focal adhesions and ErbB receptors to promote DRG neuron fate. Sorbs3 protein is shown interacting with components of focal adhesions and downstream effectors of ErbB signaling. Known physical interactors of Sorbs3 are marked with a star. Activation of MAPK, along with activation other downstream effectors of ErbB signaling, promote the activation of *neurog-1*. They may also influence other unknown genes that promote neurogenesis. The activity of focal adhesion components may also activate both genes. The combined effects of these genes promote DRG neurogenesis.



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