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PUBLISHER'S NOTE

Publisher's Note: Modulation of dorsal root ganglion development by ErbB signaling and the scaffold protein Sorbs3 by Malmquist et al. Development doi:10.1242/dev.084640

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This Publisher's Note relates to the article 'Modulation of dorsal root ganglion development by ErbB signaling and the scaffold protein Sorbs3' by Malmquist et al. (2013).

A Correspondence article in Development (Bostaille et al., 2017) has identified the causative mutation for the *ouchless* phenotype and demonstrates that this mutation is associated with the *adgra2* gene, rather than the *sorbs3* gene as reported by Malmquist et al. (2013). We refer the reader to the Correspondence article for further details.

The senior author of Malmquist et al. (2013), David Raible, is an author on the Correspondence and investigations are currently ongoing in the laboratory to reassess the results and conclusions presented in this paper. Development is publishing this note to make readers aware of this issue, and we will provide further information once Dr Raible and colleagues have completed their analysis.

This course of action follows the advice set out by COPE (Committee on Publication Ethics), of which Development is a member.

References

Malmquist, S. J., Abramsson, A., McGraw, H. F., Linbo, T. H. and Raible, D. W. (2013). Modulation of dorsal root ganglion development by ErbB signaling and the scaffold protein Sorbs3. *Development* **140**, 3986-3996.

Bostaille, N., Gauquier, A., Stainier, D. Y. R., Raible, D. W. and Vanhollebeke, B. (2017). Defective *adgra2* (*gpr124*) splicing and function in zebrafish *ouchless* mutants. *Development* **144**, 8-11.

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Modulation of dorsal root ganglion development by ErbB signaling and the scaffold protein Sorbs3

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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*, that specifically fails to develop DRG neurons, although 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 that are 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 helps to integrate ErbB signals to promote DRG neurogenesis through the activation of MAPK and upregulation of *neurogenin1*.

KEY WORDS: Dorsal root ganglia, Neural crest, ErbB, Neurogenin 1, Sorbs3, Zebrafish

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). 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 that migrate medially, between the neural tube and somite, give rise to sensory neurons and glia of the segmentally arranged bilateral dorsal root ganglia (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 by 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. Although a common neuro-glial 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, 1986; Serbedzija et al., 1989; 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 assume glial fates instead 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; Riethmacher et al., 1997; Honjo et al., 2008; Morris et al., 1999). ErbB3 is required for several early processes crucial to DRG neuron development, including NC migration (Budi et al., 2008; Honjo et al., 2008) and glial development (Riethmacher et al., 1997; Erickson et al., 1997; Garratt et al., 2000; Lyons et al., 2005). The requirements for ErbB receptors in multiple stages of DRG development complicate our understanding of their distinct roles. ErbB receptors are also implicated in both the initiation and progression of several cancers (reviewed by Feigin and

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Muthuswamy, 2009), and thus unraveling methods of integration during differentiation and migration during development could have clinical implications.

We have isolated a zebrafish mutant, *ouchless*, in which most DRG fail to develop whereas other NC-derived tissues appear unaffected. NC migration and condensation at sites of DRG appear unchanged, 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; Mitsushima et al., 2006; Mitsushima et al., 2007; Mizutani et al., 2007). 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 we 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*.

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. They were raised under standard conditions in EM (Westerfield, 1994) and staged according to Kimmel et al. (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 previously (Lister et al., 1999; Owens et al., 2008). 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 narrowing it to 0.34 Mb with *ca37* and *ca48* as flanking markers. These simple sequence length polymorphisms (SSLP) are amplified with the following primers (5′-3′): ca-48 fwd, TGGCACCTTAAACTGATACTC; ca48 rev, GCCTTCAAATTCACCATAAA; ca37fwd, TGAGTGAAGT-GAAGTAAGCCT; ca37 rev, GTTGTGGCAAGTTAGTTGG.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature or overnight at 4°C, then immunostained as described previously (Ungos et al., 2003). Antibodies were used with 20% goat serum 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; a gift from B. Appel), 1:1000; rabbit αMBP (Lyons et al., 2005; a gift from W. Talbot), 1:50. Embryos were imaged as described previously (McGraw et al., 2008).

In situ hybridization

RNA *in situ* hybridization was performed as described previously (Andermann et al., 2002). Digoxigenin-labeled 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, CCCGATCCTC-AGGCTATG; *pdlim2*F, GCGATGAGAACCAGAACTAC; *pdlim2*R, GTTTGTGTGCGTGTATATCTT.

RT-PCR and cell sorting

Four hundred to 450 dechorionated embryos were dissociated for 20-30 minutes in 5.5 ml of digestion solution [0.25× trypsin; HyClone), 5 mM EDTA, 10 μl DNaseI (Invitrogen) in 1× PBS] with trituration through a micropipette tip. Kill solution (1.4 ml) [50% FBS (Gibco), 5 mM $CaCl_2$ in 1× PBS] was added to stop digestion. Cells were centrifuged for 3 minutes at 1300 ***g***, rinsed with 1× PBS, centrifuged for 3 minutes at 1300 ***g*** and resuspended in 1 ml of suspension solution [900 μl of 1× Leibovitz's L15 media (Gibco), 10% FBS and 0.8 mM $CaCl_2$]. Eos+ cells were sorted with a FACSAria flow cytometer. RNA was isolated using RNeasy (Qiagen); cDNA was amplified from DNase-treated RNA using Superscript III (Invitrogen); RT-PCR was performed using Ssofast supermix (Bio-Rad) on a CFX connect cycler and analyzed using CFX manager 3.0 software (Bio-Rad). Primers used for amplification from cDNA were *bactinF* (CCCAAGGCCAACAGGGAAAA), *bactinR* (GGTGCCCATCT-CCTGCTCAA), *sox10F* (ATCCCGAGTACAAGTACCAGCCAC), *sox10R* (TGCAGGCTCTGTAATGCGATTGG), *sorbs3F* (TCTTCCA-CATCCCAGTCTCC), *sorbs3R* (CACATGCTTGGTGAAGAACG).

Morpholino oligonucleotide (MO) injections

A MO predicted to block splicing of exons 5 and 6 of Sorbs3 with the sequence TTCCGACAGGGAAAGCACATACC was obtained from Gene Tools. Two to 6 ng MO in water + 0.2% Phenol Red was injected into a one-cell embryos carrying the TgBAC(*neurog1:dsred)* transgene (Drerup and Nechiporuk, 2013). DsRed-positive DRG were counted at 2-4 days. Fish were anesthetized in MS-222, then fixed for 2 hours at room temperature or overnight at 4°C 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). One to 2 μl DNA, 0.1 M KCl and 0.2% Phenol Red was injected into *ouchless; Tg(neurog1:GFP)* embryos at the one-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 and 0.2% Phenol Red and injected into *ouchless; Tg(neurog1:EGFP)* embryos. GFP^+ DRG were counted on one side of the embryos at 48 hpf, 72 hpf and 96 hpf using a Nikon SMZ1500 fluorescent dissecting microscope.

Transplants

Mosaic embryos were generated by cell transplantation at early gastrula stages as described previously (Carmany-Rampey and Moens, 2006). Donor embryos were injected at the one-cell stage with 1 nl 0.2% rhodamine-dextran (Sigma) and cells from sphere stage donors were transplanted into shield stage host embryos. Melanophore-deficient *nacre* hosts (Lister et al., 1999) 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. Embryos were genotyped as described previously for *neurog1* (Golling et al., 2002) or for *reck* (Prendergast et al., 2012).

Statistics

Contingency tables were analyzed using Statistics To Use (http://www.physics.csbsju.edu/stats/). All other statistics were generated using GraphPad Prism for MacOSX version 5.0 (GraphPad, 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

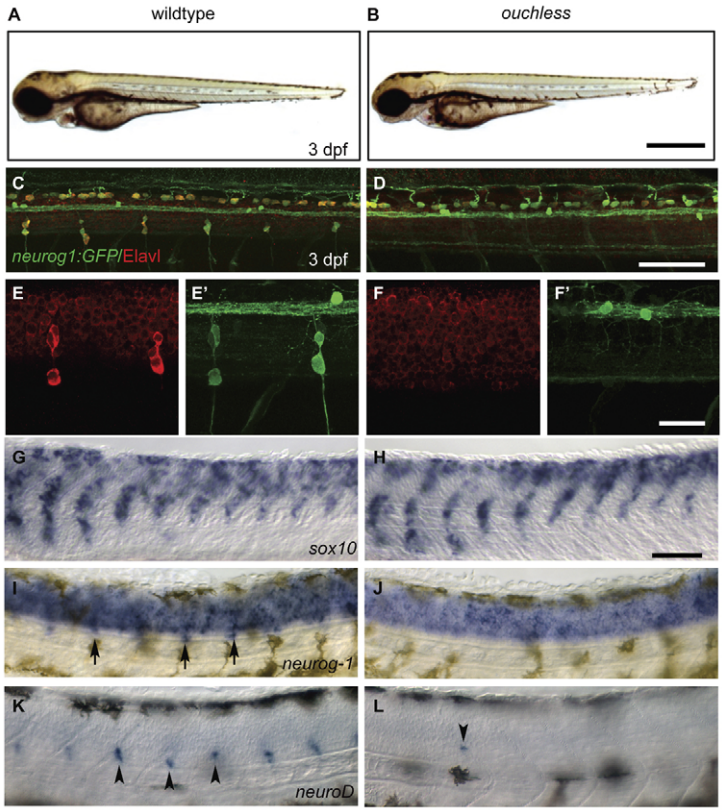

Fig. 1. *ouchless* mutants lack DRG sensory neurons. (A,C,E,G,I,K) Wild-type or (B,D,F,H,J,L) *ouchless* mutant embryos. (**A**,**B**) Bright-field images at 3 dpf. (**C**,**D**) Lateral view of 3 dpf *Tg(neurog1:EGFP)* embryo, immunostained for EGFP (green) and Elav1 (red). DRG are located ventral to 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: 500 μm in A,B; 100 μm in C,D,G-L; 25 μm in E-F′.

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 siblings (Fig. 1A,B), lacks most DRG at 3 dpf. 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 (Fig. 1C,D). The few DRG that develop are most frequently found in the first eight segments. *ouchless* DRG contain fewer neurons than those in wild type (supplementary material Fig. S1C,D). Spatial arrangement of each remaining ganglion, as well as orientation of axonal projections, appears normal in mutants (supplementary material Fig. S1A,B). More neurons differentiate in wild-type DRG as animals age (McGraw et al., 2012); by contrast, *ouchless* fish show reduced neuron addition in the few DRG that form (supplementary material Fig. S1C-D). *ouchless* mutants do not add DRG after 3 dpf, suggesting the defect does not result in a simple developmental delay. Although *ouchless* mutants eventually grow to adult stages, they never gain normal numbers of DRG (not shown). We were not able to find any changes in Elavl expression in or morphology of sympathetic ganglia (SG), enteric neurons or cranial ganglia in mutants (supplementary material Fig. S2). These results indicate a specific requirement for *ouchless* in formation of DRG, but not other NC-derived 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 differentiation of neurons and glia. As other NC-derived cells 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 wild type and *ouchless* mutants (Fig. 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. Segmented clusters of cells ventral to the spinal cord express *neurog1* in wild-type embryos, but only occasional *neurog1*$^+$ cells could be detected in *ouchless* embryos (Fig. 1I,J). *neuroD*, a marker of maturing neurons (Blader et al., 1997), was expressed in a similar sporadic pattern in *ouchless* at 36 hpf (Fig. 1K,L), suggesting that the few cells that initiate the neuronal program continue their differentiation. These results suggest that 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 (supplementary material Fig. 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 (Fig. 2A). This interval contained two annotated genes, *pdlim2* and *sorbs3*. We sequenced the entire coding sequence of both genes from wild type and *ouchless* but were unable to identify a lesion, suggesting that mutation in a regulatory region may be responsible.

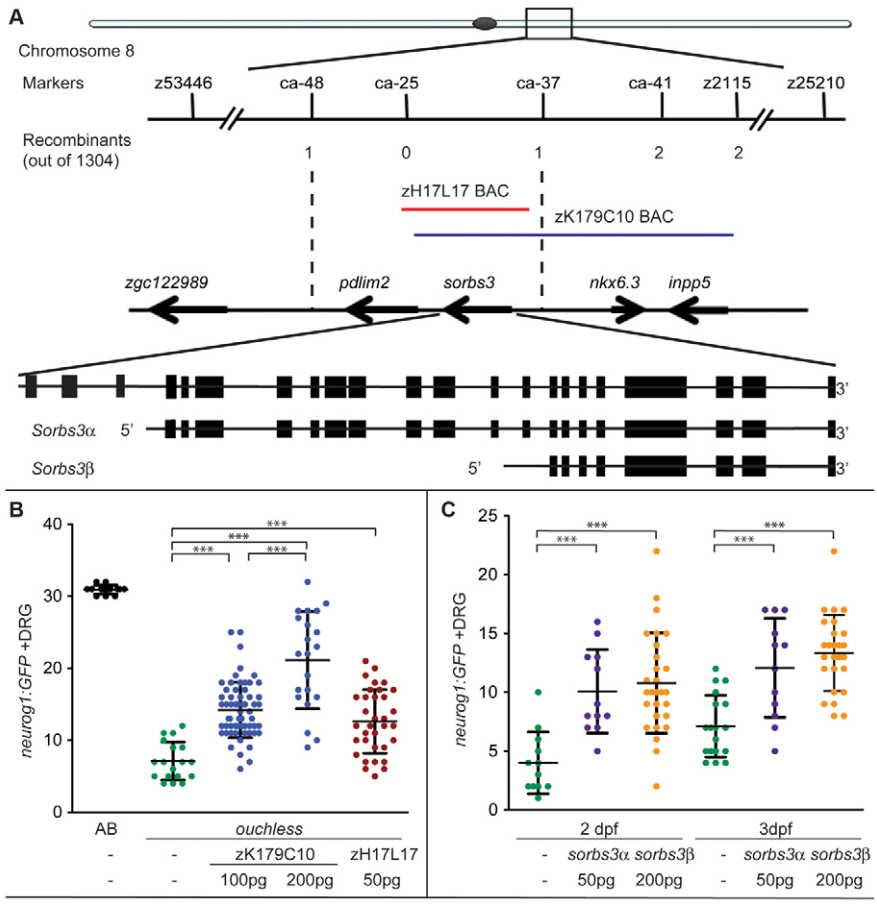

Fig. 2. *ouchless* affects the *sorbs3* gene.
(**A**) Lesion in *ouchless* was mapped to 0.34 Mb region on chromosome 8, between markers z53446 and z2115. Number of recombinants is shown below the solid line; dotted vertical lines indicate the recombination interval that contains the *ouchless* lesion. BACs zH17L17 (red line) and zK179c10 (blue line), used for rescue 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 4-21 and 14-21, used for rescue in C, are shown. (**B**) *Tg(neurog1:EGFP)* or *ouchless; Tg(neurog1:EGFP)* fish injected with the 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 s.d. (**C**) *ouchless; Tg(neurog1:EGFP)* fish injected at the one-cell stage with the 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 indicate s.d., ***P<0.001 (t-test).

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 gene *sorbs3* and two other genes outside of the recombination interval but not *pdlim2*, into *ouchless* embryos (Fig. 2A,B). This resulted in a dose-dependent rescue of the number of DRG at 3 dpf. 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 (not shown). Taken together with recombination mapping data, this suggests that the *ouchless* lesion 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 isoform, Sorbs3α, contains 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 5′ sequences, including the sorbin domain (Kioka et al., 1999). The zebrafish *sorbs3* locus contains 21 exons and is predicted to encode a protein with the same domain arrangement as the mammalian form (Fig. 2A). We have isolated transcripts containing message complementary to exons 14-21, which probably encodes Sorbs3β, and longer transcripts that encode isoforms corresponding to mammalian Sorbsα. Sorbs3 mRNAs 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 which the *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 to 3 dpf, and at 3 dpf animals were fixed and stained with EGFP and Elavl antibodies to label differentiated neurons. mRNA (50 pg) encoding a long isoform of *sorbs3* was able to partially rescue the DRG defect (Fig. 2C). Higher concentrations of mRNA were deleterious to embryos. We observed no changes in DRG development with overexpression of *sorbs3* in wild-type embryos. mRNA for the shorter *sorbs3b* isoform was also able to rescue DRG defects (Fig. 2C). This suggests that the N-terminal region of Sorbs3, including the sorbin domain, is dispensable for DRG development and that the crucial region is contained in the C-terminal SH3 domains.

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

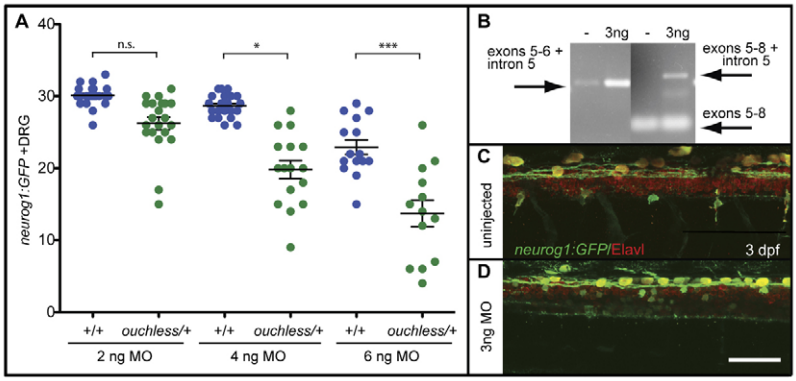

Fig. 3. *sorbs3* is required for DRG neurogenesis. (**A**) *Tg(neurog1:EGFP)* or *ouchless/+; Tg(neurog1:EGFP)* embryos were injected with amounts of MO shown, fixed at 3 dpf and immunostained for EGFP/Elavl. Each dot indicates number of DRG on one side of one fish; error bars indicate s.d. ***P<0.001 (*t*-test); *P<0.05; n.s., not significant. Data were analyzed using two-way ANOVA, showing that genotype accounts for 30.4% of variance (P<0.0001), MO dose accounts for 36% of variance (P<0.001) and interaction for 3.81% (P=0.0039). Bonferroni multiple comparisons post-hoc test shows significant differences between genotypes at 2 ng, 4 ng and 6 ng MO (P<0.01, P<0.0001, P<0.0001). (**B**) *Tg(neurog1:EGFP)* were injected with 3 ng MO, and cDNA prepared at 24 hpf. PCR indicated mis-splicing of the *sorbs3* transcript. (**C**,**D**) Images of 3 dpf *Tg(neurog1:EGFP)* embryos, immunostained for EGFP (green) and Elav1 (red). Scale bar: 50 μm.

MO injection at these concentrations causes few other phenotypes, suggesting that the decrease in DRG formation is specific (Fig. 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 (Fig. 4A). This pattern continues through 24 hpf, but expression is downregulated by 30 hpf (Fig. 4E). Strong *sorbs3* expression is found in the pronephros and more moderate expression in forebrain and skin. Although *ouchless* embryos show similar *sorbs3* expression patterns to wild-type embryos in head and pronephros, they exhibit lower levels in the neural tube (Fig. 4B,F). In transverse sections at the level of the yolk extension of wild-type embryos, *sorbs3* expression is concentrated to the ventral neural tube, but there is also weaker expression in cells resembling NC ventrolateral to the spinal cord (Fig. 4C, arrows). These could not be found in *ouchless* mutants (Fig. 4D). By RT-PCR analysis, expression of *sorbs3* mRNA in *ouchless* embryos is approximately twofold less than wild-type levels at 28-30 hpf (Table 1). Using fluorescence-activated cell sorting, we confirmed that both NC and non-NC cells express *sorbs3* mRNA in wild-type embryos at 28-30 hpf (Table 1).

sorbs3 is required cell-autonomously for DRG neurogenesis

As *sorbs3* is expressed both in NC and in surrounding cells, we constructed mosaic embryos to elucidate its tissue-specific requirement for DRG development. We transplanted donor cells from 3 hpf embryos into the prospective NC domain at 6 hpf. We used *nacre* hosts, which lack NC-derived melanophores 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 wild-type cells transplanted into *ouchless* hosts should be able to generate sensory neurons. If, on the other hand, *sorbs3* acts non-cell autonomously, wild-type 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. Of 44 transplants, 31 (70%) contained pigmented cells, demonstrating transfer of NC precursors, and of those 48% had DRG derived from donors (Fig. 5A,B, Table 2). In complementary experiments, transplanting *ouchless* cells (*neurog1:gfp*+, rhodamine

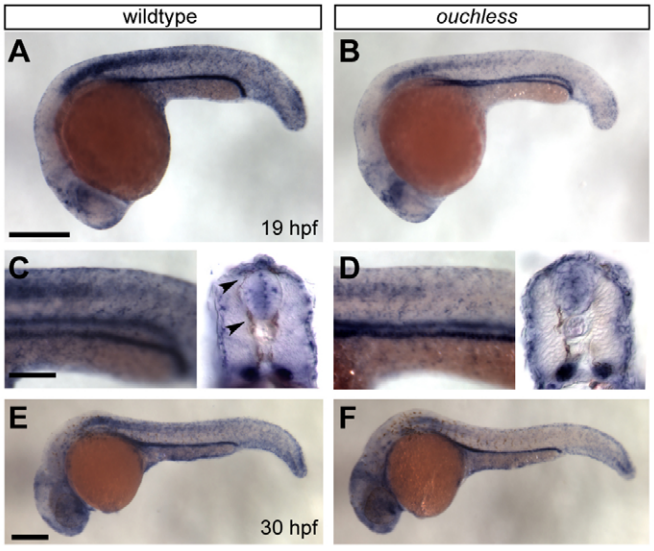

Fig. 4. *sorbs3* expression is altered in *ouchless*. (**A**) *In situ* hybridization for *sorbs3* mRNA (19 hpf) in wild-type fish reveals expression in the anterior neural tube, eye, brain, pronephros and surface cells. (**B**) *sorbs3* expression in the neural tube and surface cells is decreased in *ouchless*, but expression is unchanged in other tissues. (**C**,**D**) Region dorsal to the yolk extension of 19 hpf wild-type and *ouchless* embryos, and transverse sections at the level of the yolk extension at 30 hpf. Cells outside the neural tube in the position of the migrating NC are indicated by arrowheads. (**E**,**F**) Expression of *sorbs3* at 30 hpf in wild type (E) and *ouchless* (F). Scale bars: 250 μm in A,B,E,F; 100 μm in C,D (left); 25 μm in C,D (right).

Table 1. *sorbs3* is expressed in NC and non-NC cells

A

	ΔCt *sox10*	ΔCt *sorbs3*
AB (*n*=9)	8.08±0.21	9.87±0.23
ouchless (*n*=3)	8.27±0.17	10.85±0.11
	P=0.1785 (n.s.)	***P*<0.001

B

	ΔCt *sox10*	ΔCt *crestin*	ΔCt *foxd3*	ΔCt *sorbs3*
Eos^{+} (*n*=3)	6.51±1.90	5.52±1.29	7.68±1.07	12.13±0.17
Eos^{-} (*n*=3)	8.80±0.93	9.04±0.74	9.82±0.25	12.11±0.95
	P=0.0519 (n.s.)	**P*=0.0149	**P*=0.0281	*P*=0.9730 (n.s.)

(A) cDNA isolated from AB or *ouchless* embryos at 28 hpf was analyzed for expression of *sox10* or *sorbs3*.
(B) *Eos*-expressing NCC were isolated from dissociated wild-type *Tg(sox10:nlsEos)* embryos at 28 hpf. cDNA was analyzed for the expression of *sox10*, *crestin*, *foxd3* or *sorbs3*. ΔCt values relative to β-actin are shown with standard deviations
P values are given for *t*-test. **P*<0.05, ***P*≤0.001.

labeled) into *nacre* embryos, resulted in a comparable percentage of pigmented larvae (66%, 37/56) but *ouchless* cells never gave rise to DRG (Fig. 5D-F). In both sets of experiments, the *nacre* host cells gave rise to normal Elavl^{+} DRG neurons (Fig. 5C,F). These results showed that *sorbs3* is required cell-autonomously for DRG development, but did not exclude a second, non-cell autonomous function for *sorbs3*. We addressed this question by transplanting wild-type cells into *ouchless* mutants and found that wild-type cells were able to generate sensory neurons, indicating that DRG precursors do not require extrinsic *sorbs3* for their development (Fig. 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 homozygous *ouchless* mutants survive until adulthood and are able to reproduce. Though grossly indistinguishable from wild-type and heterozygous siblings as larvae (Fig. 1A,B), adult mutants can be identified by their slow growth. 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 wild-type zebrafish (Fig. 6A,B). This eventually results in broken melanophore stripes that consist of few and dispersed melanophores surrounded by iridophores and xanthophores in adults (Fig. 6C,D). The mutant pattern phenotype is strikingly similar, though less severe than that of *picasso* mutants, which have been shown to possess a mutation in the *erbb3b* gene (Budi et al., 2008) (Fig. 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*

The phenotypic similarities of *ouchless* and *erbb* mutants led us to hypothesize that Sorbs3 might be a downstream modulator of ErbB signaling during the development of DRG. 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). 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; Lyons et al., 2005; Britsch et al., 1998). To examine this possibility, we visualized DRG progenitor condensation at 24 and 36 hpf in wild-type, *ouchless* and *erbb3b* embryos. Sox10-positive NC cells, migrating ventrally in a comb-like pattern over the trunk at 24 hpf, were arranged in distinct clusters adjacent to the ventral neural tube in both wild-type and *ouchless* embryos by 36 hpf (Fig. 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 (Fig. 6I,L). Thus, although *erbb3* mutants have dramatic defects in DRG condensation, this process appears to be independent of the changes in *sorbs3* found in *ouchless* mutants.

Table 2. Quantification of transplant experiments

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)

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 (Riethmacher et al., 1997; Britsch et al., 1998; Lyons et al., 2005; Chen et al., 2003; Sharghi-Namini et al., 2006; Pogoda et al., 2006) (reviewed by Britsch, 2007). 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 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 wild type and *ouchless* mutants (Fig. 6M,N). The PLL of *erbb3b* mutants lacked MBP staining (Fig. 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 but defective in *erbb3* mutants (data not shown). To exclude the possibility that the lack of a glial phenotype may be due to potential residual *sorbs3* function in *ouchless* mutants, we injected *ouchless* embryos carrying *Tg(sox10:nlsEos)* with 6 ng *sorbs3* MO. We examined the peripheral glia of the DRG at 30 hpf and of the lateral line at 5 dpf, and found no differences compared with wild-type fish (Fig. 6P-S). Thus, the development of peripheral glia, although requiring ErbB function, does not seem to require *sorbs3*.

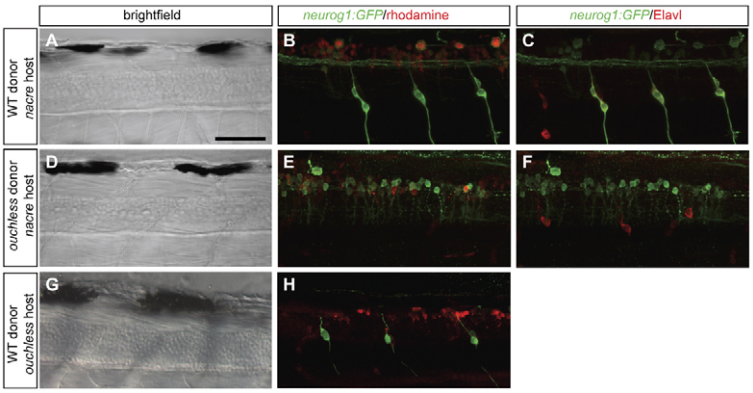

Fig. 5. *sorbs3* is required in the NC for DRG neurogenesis. All embryos are shown at 3 dpf. (A,D,G) Bright-field image showing donor-derived pigment cells. (B,E,H) EGFP immunofluorescence (green) and rhodamine (red). (C,F) EGFP (green) and Elavl (red) immunofluorescence. (**A-C**) Wild-type *Tg(neurog1:EGFP)* donors to *nacre* host show robust contribution to DRG. (**D-F**) *ouchless*; *Tg(neurog1:EGFP)* to *nacre* host. No donor cells are found in DRG. (**G**,**H**) Wild-type *Tg(neurog1:EGFP)* donor to *ouchless* host. Wild-type cells form DRG in mutants.

sorbs3 and *erbb3* interact in the same pathway 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 wild type (Table 3). Those heterozygous for both *ouchless* and *erbb3b* showed significantly fewer DRG ($P>0.05$) than either wild-type 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 tail 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 3). Although this genetic interaction is subtle, it suggests that *sorbs3* and *erbb3* 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. (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 (Lyons et al., 2005; Levitzki and Mishani, 2006) and PD158780 (Fry et al., 1997; Rewcastle et al., 1998; Frohnert et al., 2003). Treating fish from 18-72 hpf with either inhibitor resulted in a specific and dose-dependent loss of DRG (Fig. 7A, data not shown). PD158780 showed less toxicity than AG1478, so we continued using this inhibitor.

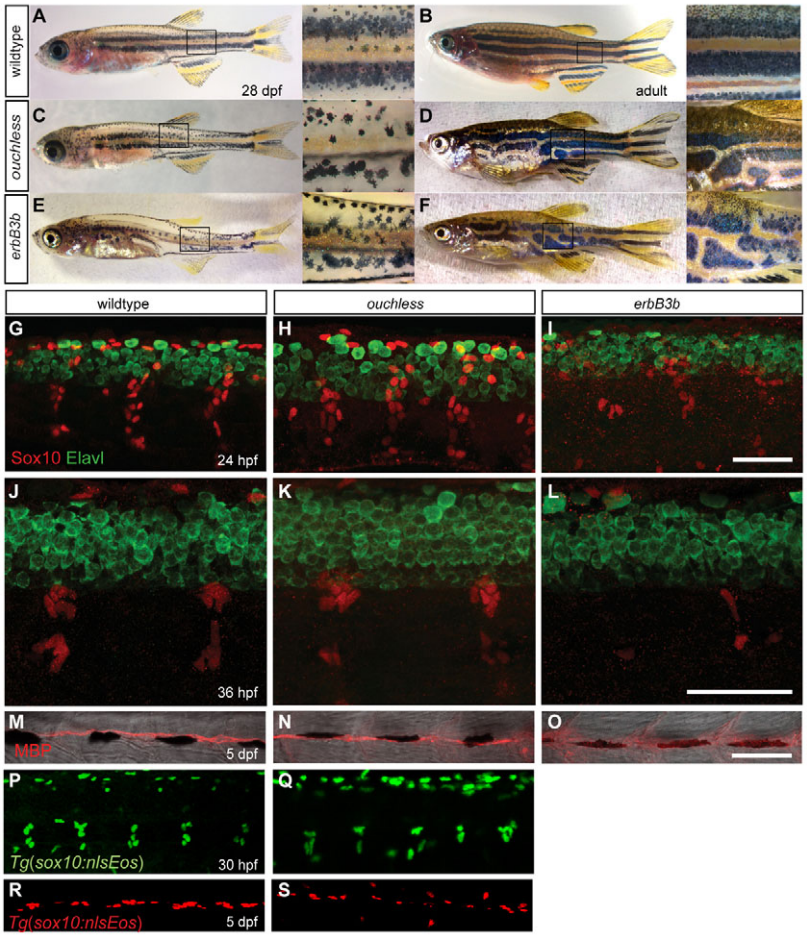

Fig. 6. *ouchless* and *erbb3b* share a subset of phenotypes. (**A-F**) Bright-field images of wild-type (A,B), *ouchless* (C,D) and *erbb3b* (E,F) fish at 28 dpf (A,C,E) and as adults (B,D,F). Boxes indicate positions of insets. Both *ouchless* and *erbb3b* fish have fewer melanophores than wild type at 28 dpf and disrupted stripe patterns as adults. (**G-I**) Immunofluorescence of Sox10$^+$ NC cells (red) and Elavl spinal neurons (green) in wild type, *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 site of 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. Disorganization of glia seen in *erbb3b* is absent in *ouchless*. (**P-S**) Imaging of glial cells in *Tg(sox10:nlsEos)* transgenic line in trunk segments at 30 hpf (P,Q) and posterior lateral line nerve at 5 dpf (R,S). Scale bars: 50 μm.

Table 3. *ouchless* and *erbb3* interact genetically in DRG neurogenesis

Genotype	*n*	Mean DRG per fish side±s.d.***	Fraction of fish missing at least one DRG		
			Missing in any segment (n.s.)	Missing in trunk‡‡‡	Missing in tail (n.s.)
Wild type	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

Tg(neurog-1;EGFP) embryos at 3 dpf were scored for DRG in each segment. Kruskal-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 wild type (P<0.001) and *ouchless/+* (P<0.01). Comparisons between other genotypes were not significant. χ^2 test of fraction of fish missing DRG shows a significant difference between groups lacking DRG in trunk (segments 1-16; χ^2=22.4, ‡‡‡P<0.0005), and no significant difference between groups with respect to total (χ^2=4.83, P=0.184) or tail (segments 17-34; χ^2=0.846, P=0.834).

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 PD0325901 over 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 (Fig. 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 0.5 μM PD158780, both *ouchless/+* and *erbb3b/+* fish showed sensitization to inhibition when compared with wild-type fish (Fig. 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 wild-type siblings (Fig. 7C). Embryos heterozygous for *reck* loss of function, another gene necessary for DRG development (Prendergast et al., 2012), are not sensitized to ErbB inhibition, (Fig. 7C), suggesting that ErbB inhibitor treatment is specifically affecting a developmental pathway that includes a novel link to *sorbs3* and *neurog1*.

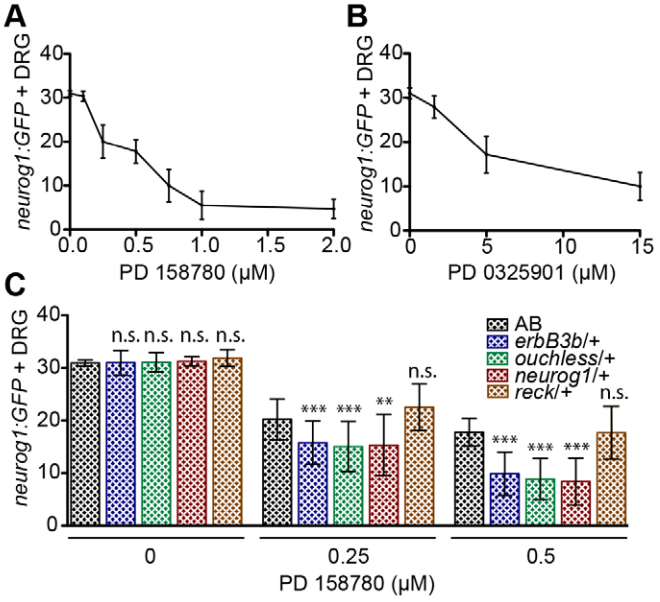

Fig. 7. ErbB3 and Sorbs3 are part of the same biochemical pathway. (**A**,**B**) *Tg(neurog1:EGFP)* embryos treated from 18-72 hpf in EM + 1% DMSO + PD158780 (A) or PD0325901 (B), stained for GFP and Elavl at 72 hpf. Data are mean±s.d. of DRG on one side. (**C**) AB, *ouchless/+*, *erbb3b/+*, *neurog1/+* and *reck/+* embryos, all carrying *Tg(neurog1:EGFP)*, were treated from 18-72 hpf in EM + 1% DMSO + PD158780, then stained for GFP and Elavl at 72 hpf. Data are mean±s.d. of DRG on one side. ***P<0.001 in *t*-test, **P<0.01, n.s., not significant.

DISCUSSION

In this study, we have identified and characterized a zebrafish mutant, *ouchless*, which develops only a small subset of the normal complement of DRG, fails to add neurons to the DRG that do develop at a normal rate and fails to develop normal numbers of adult melanophores. All of these phenotypes are reminiscent of a subset of those seen in mutants for the EGFR receptor tyrosine kinases ErbB2 and ErbB3 (Honjo et al., 2008; Budi et al., 2008; Honjo et al., 2011). Zebrafish adult pigment cells form from progenitor cells that are sometimes localized to peripheral nerves and DRG, and 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.

The *ouchless* mutation affects the expression of *sorbs3*, a scaffold protein known to interact with components of focal adhesions, as well as with effectors of ErbB signaling, including Sos, Raf and ERK (Kioka et al., 1999; Akamatsu et al., 1999; Mitsushima et al., 2004; Matsuyama et al., 2005; Mitsushima et al., 2007; Gehmlich et al., 2007). Our experiments support the hypothesis that Sorbs3 and ErbB2/3 are part of the same genetic pathway. ErbB2/3 and Sorbs3 are both required for *neurog1* expression in DRG precursors, *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. Although we favor a model where Sorbs3 directly modulates ErbB signaling, we cannot rule out that interactions may be indirect through parallel pathways.

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., 2007; Ito et al., 2008). 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.

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, as Sorbs3 is required cell-autonomously. Although 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; Suwa et al., 2002). Sorbs3 binds to ERK1/2, as well as to c-Raf (Mitsushima et al., 2004; Matsuyama et al., 2005), and prevents the dephosphorylation, thus promoting the kinase activities of both EGFR and ERK1/2 (Mitsushima et al., 2006; Mitsushima et al., 2007). At the same time, ERK itself phosphorylates Sorbs3, which inhibits cell migration (Mitsushima et al., 2004; Mizutani et al., 2007). Expression of Sorbs3 in v-src transformed cells also suppresses their enhanced migration phenotype (Umemoto et al., 2009). 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.

Although 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 (Esparís-Ogando 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, although 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 specific components of the MAPK signaling pathway that are involved in zebrafish sensory neuron development 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, which are extracellular components of focal adhesions, have been implicated in NC migration, and loss of these proteins cause defects in NC development (reviewed by Perris and Perissinotto, 2000). Focal adhesions are signaling centers, and are often characterized by high concentrations of ErbB2/3 dimers (reviewed by 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) and cultured adult neurons (Grimm et al., 2009; Grimm et al., 2010). 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 by Cabodi et al., 2010).

A crucial 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., 2007; Umemoto et al., 2009). There is a large body of evidence that ErbB receptors regulate cell migration (reviewed by Feigin and Muthuswamy, 2009), and zebrafish NC cell migration is altered after loss of *erbb2/3* (Honjo et al., 2008). Although 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 and Soubeyran, 2009), raising the possibility that Sorbs3 could also be an important modulator of ErbB signaling in cancers. 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.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
S.J.M., A.A. and D.W.R. conceived experiments; S.J.M., A.A., H.F.M. and T.H.L. performed experiments; S.J.M., A.A. and D.W.R. analyzed data and wrote the manuscript.

Supplementary material
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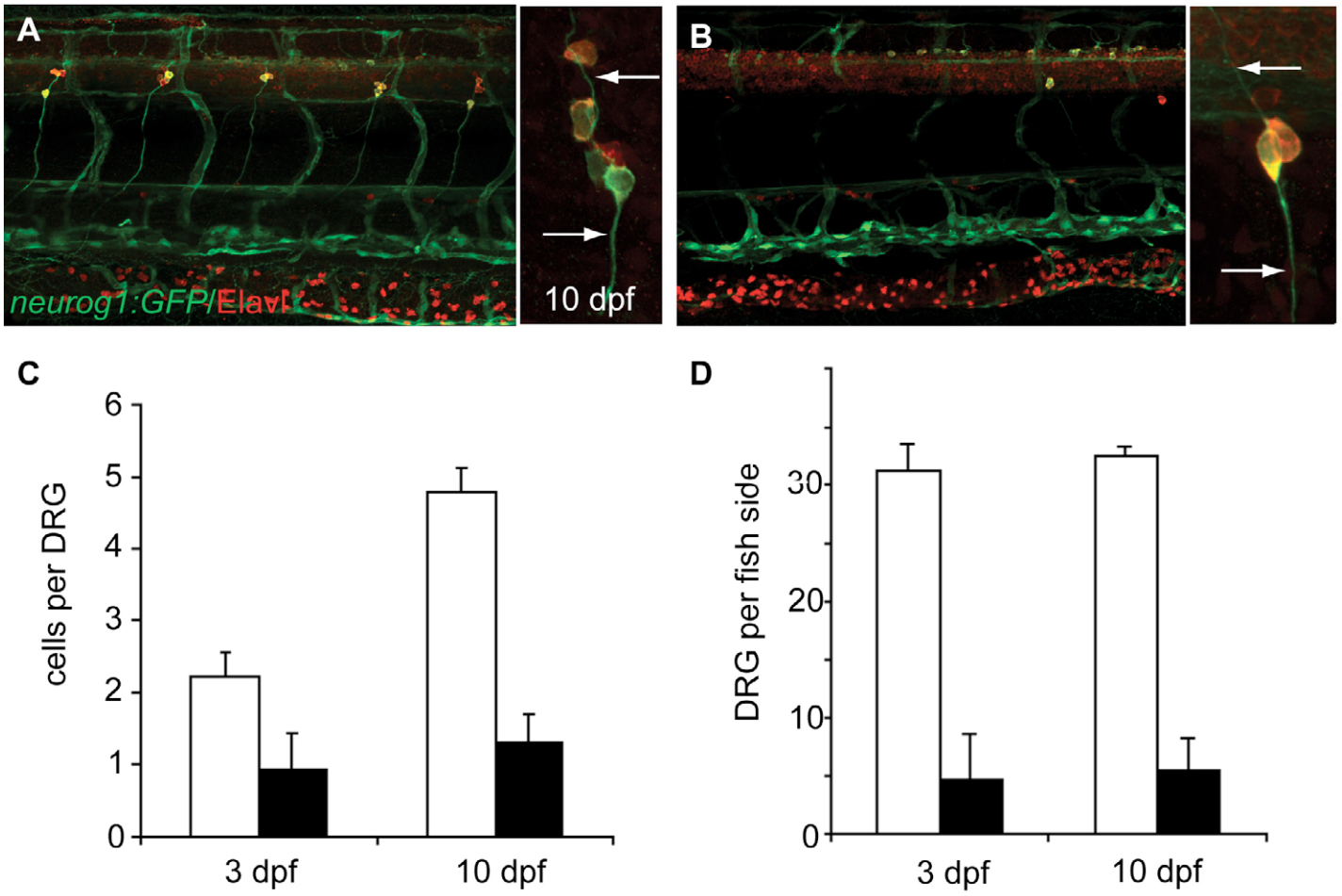

Fig. S1. DRG neuron addition is altered in *ouchless.* (**A**) Lateral view of 10 dpf transgenic zebrafish expressing *Tg(neurog1:EGFP),* immunostained for EGFP (green) and Elavl (red). High magnification view of a single DRG composed of six EGFP/Elavl^{+} neurons. (**B**) Lateral view of 10 dpf *ouchless; 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,B indicate dorsal and ventral axonal projections. (**C**) Quantification of the number of EGFP/ElavL1^{+} cells per DRG in wild type (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 wild type (white bars) and *ouchless* (black bars) at 3 dpf and 10 dpf. For C,D, *n*=6 fish and error bars are s.e.m.

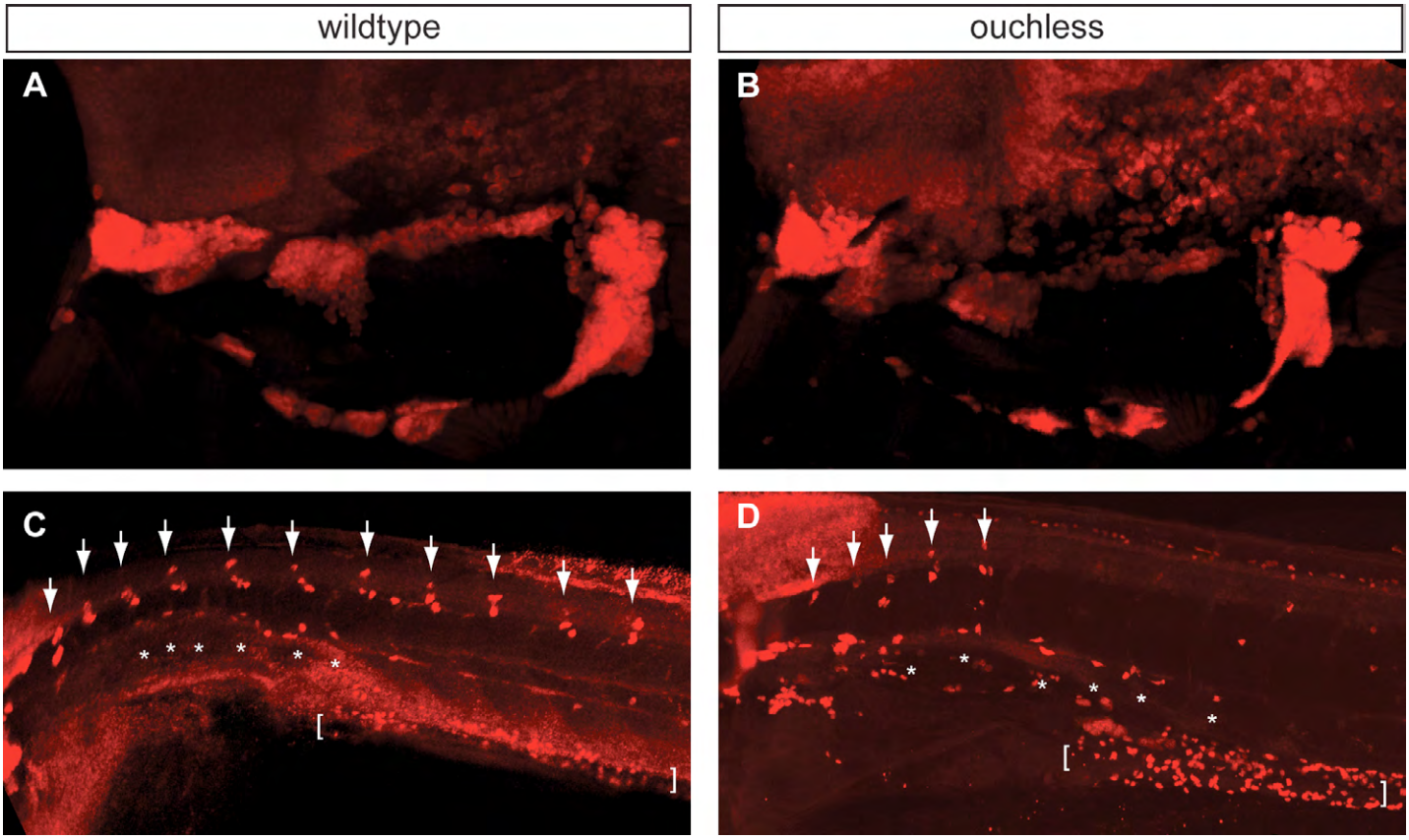

Fig. S2. Other neurons are unaffected in *ouchless.* (**A**) Confocal image of a 4 dpf wild-type larvae immunostained with Elavl, showing cranial ganglia. (**B**) Image of a 4 dpf *ouchless* larvae, showing normal cranial ganglia morphology. (C) Confocal image of a 7 dpf wild-type larvae immunostained with ElavL1, showing DRG (arrows), SG (asterisks) and enteric neurons (brackets). (**B**) Image of a 7 dpf *ouchless* larva. SG and enteric neurons are present in segments lacking DRG.