Development/Plasticity/Repair

Transcription Factor Short Stature Homeobox 2 Is Required for Proper Development of Tropomyosin-Related Kinase B-Expressing Mechanosensory Neurons

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Dorsal root ganglia (DRG) contain somatosensory neurons of diverse sensory modalities. Among these different types of sensory neurons, the molecular mechanisms that regulate the development and specification of touch neurons are the least well understood. We took a candidate approach and searched for transcription factors that are expressed in subsets of DRG neurons, and found that the transcription factor Shox2 (short stature homeobox 2) is expressed in subpopulations of TrkB (tropomyosin-related kinase B)- and Ret-expressing neurons at neonatal stages. Since TrkB is a known marker that is selectively expressed in touch sensory neurons, we decided to examine the function of Shox2 in specifying TrkB-positive DRG neurons. Conditional deletion of *Shox2* in neural crest cells (which give rise to all DRG neurons) caused a 60 ~ 65% reduction in the number of TrkB-expressing neurons. It also resulted in an increase in coexpression of TrkC in Ret-positive sensory neurons. Deletion of *Shox2* in differentiating DRG neurons at later time points caused only a moderate reduction in TrkB expression. Overexpression of Shox2 in all neural crest cells resulted in a small increase in the number of TrkB-expressing neurons. Finally, *Shox2* deletion also caused reduced touch sensory axonal innervation to layers III/IV of the spinal cord. Together, our findings identify Shox2 as an essential but not sufficient component of the transcription programs required in neural progenitor cells for the proper specification of subsets of TrkB-expressing touch/mechanosensory neurons.

Introduction

Somatosensory neurons located in dorsal root ganglia (DRG) consist of many different types that detect diverse modalities of sensory stimuli. All DRG neurons are originated from neural crest cells (NCCs) (Ma et al., 1999; Chai et al., 2000; Szeder et al., 2003). We are interested in identifying molecular mechanisms that enable NCCs to differentiate into touch/mechanosensory neurons. Different DRG neurons have unique molecular compositions. Receptors for neurotrophic factors are among the best characterized markers for sensory neurons. It has been shown that TrkA (tropomyosin-related kinase A) and Ret receptors are mainly expressed in nociceptive and thermal sensory neurons (Chen et al., 2006; Kramer et al., 2006; Luo et al., 2007). TrkC is expressed primarily in proprioceptive neurons innervating the skeletal muscles (Hippenmeyer et al., 2005; Sedý et al., 2006; Inoue et al., 2007; Hasegawa and Wang, 2008). TrkB is expressed by a subpopulation of cutaneous low-threshold touch neurons (González-Martínez et al., 2004; Shimizu et al., 2007; Perez-Pinera et al., 2008). An early-born population of Ret-expressing neurons develops into rapid-adapting mechanosensory neurons (Bourane et al., 2009; Luo et al., 2009). Signaling through neurotrophic receptors is important for neuron survival, axon growth, innervation of central and peripheral targets, and proper differentiation into specialized and modality-specific sensors (Marmigère and Ernfors, 2007; da Silva and Wang, 2011).

Significant progresses have been made in elucidating the transcriptional programs specifying nociceptive and proprioceptive neurons. For example, the transcription factor *Runx1* is essential for differentiation and diversification of nociceptive neurons into peptidergic and nonpeptidergic lineages (Chen et al., 2006; Kramer et al., 2006; Marmigère and Ernfors, 2007; Inoue et al., 2008), whereas *Runx3* and *Er81* are important for the specification of TrkC-expressing proprioceptive sensory neurons (Levanon et al., 2001; Hippenmeyer et al., 2005; Kramer et al., 2006; Inoue et al., 2007). Recently, MafA was shown to be involved in the development of Ret-positive rapid-adapting mechanoreceptors (Bourane et al., 2009). However, the transcription factors that enable progen-

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itor cells to differentiate into TrkB-expressing mechanosensory neurons remain unclear.

We searched for transcription factors expressed in a subset of DRG neurons and found the gene encoding Shox2 (short stature homeobox 2), a homeobox transcription factor, is dynamically expressed during DRG development. The mouse *Shox2* gene displays 99 and 73% similarity to human *Shox2* and *Shox*, respectively. Mutations in human *Shox* cause short stature and Leri–Weill dyschondrosteosis (Marchini et al., 2007; Binder, 2011). Mice only have the *Shox2* gene. Thus, it appears that mouse Shox2 assumes the functions of both human Shox and Shox2. *Shox2* mutant mice show defects in bone, heart, and palate development (Yu et al., 2005; Cobb et al., 2006; Blaschke et al., 2007). However, the role of Shox2 in neuronal development has not been examined. Here we performed loss- and gain-of-function analyses to determine the role of Shox2 in DRG development. We discovered that Shox2 is important for the development of TrkB-expressing mechanosensory neurons.

Materials and Methods

Mice. $Shox2^{flox/flox}$ (Cobb et al., 2006), *Wnt1-Cre* (Danielian et al., 1998), $Advillin^{Cre/+}$ (Zhou et al., 2010), $Isl1^{Cre/+}$ (Srinivas et al., 2001; Yang et al., 2006), and $Advillin^{PLAP/+}$ (Hasegawa et al., 2007) mice have all been previously described. $Rosa^{CAG\text{-}STOP\text{-}Shox2/+}$ mouse was generated by inserting a cassette of "chicken βActin promoter-LoxP-neo-polyA-LoxP-Shox2-polyA" into the *Rosa26* locus via homologous recombination. Genotyping of the $Rosa^{CAG\text{-}STOP\text{-}Shox2/+}$ mice was performed by PCR. PCR primers were designed as follows: *Rosa/01*, 5-CACTTGCTCTCCCAAAGTCG-3; *Rosa/02*, 5-TAGTCTAACTCGCGACACTG-3; and *CAG/02*, 5-GTTATGTAACGCGGAACTCC-3. The wild-type allele produces a 560 bp fragment with *Rosa/01* and *Rosa/02* primers, whereas the knock-in allele results in a 300 bp fragment with *Rosa/01* and *CAG/02* primers. Furthermore, primers were designed to specifically detect the Shox2 cDNA in the $Rosa^{CAG\text{-}STOP\text{-}Shox2}$ allele: *RShox2/01*, 5-GTGTCCCCTGAACTGAAGGA-3; and *RShox2/02*, 5-GCCTGAACCTGAAAGGACAA-3. The knock-in allele produces a 400 bp fragment using the *RShox2/01 and RShox2/02* primers. All experiments were conducted according to protocols approved by The Duke University Institutional Animal Care and Use Committee.

In situ *hybridization.* The mouse cDNA fragments of the neurotrophic receptors and Shox2 were amplified by PCR with the antisense primers containing the T7 promoter sequence. *In vitro* transcription was then performed from the PCR-amplified template using T7 RNA polymerase (Roche) with Digoxigenin-UTP (Roche) for the synthesis of the antisense probes. *In situ* hybridization was performed according to standard methods (Hodge et al., 2007). Fluorescent two-color *in situ* hybridization was performed according to standard methods (Hasegawa and Wang, 2008).

Immunostaining. Immunostaining was performed according to standard methods (Hodge et al., 2007). The following antibodies were used: Alexa Fluor 488-conjugated IB4 (Invitrogen), anti-Caspase 3 (active) (1:1000; R&D Systems), anti-CGRP (calcitonin gene-related peptide) antibody (1:2000; Millipore Bioscience Research Reagents/Invitrogen), anti-PGP9.5 antibody (UltraClone), anti-vGluT1 antibody (1:1000; Millipore), Alexa 488-labeled anti-rabbit IgG (1:400; Invitrogen), Alexa 488-labeled anti-guinea pig IgG (1:400; Invitrogen), and Cy3-labeled anti-rabbit IgG (1:400, Jackson Laboratories).

Alkaline phosphatase staining. Alkaline phosphatase staining was performed according to standard methods (Hasegawa et al., 2007).

Quantification methods. For every developmental stage, at least three embryos/pups from two to three different litters were analyzed. *In situ* (or immuno) signal-positive DRG neurons were counted from randomly selected sections. For counting TrkA- or Ret-positive neurons, $N = 20$ randomly selected sections from each animal are counted; for counting Shox2-, TrkC-, TrkB-, or activated caspase-3-positive neurons, $N = 40 \sim 50$ randomly selected sections from each animal are counted; and for counting MafA-, *Runx3*-, or parvalbumin-positive neurons, $N = 30 \sim 35$ randomly selected sections from each animal are counted. On each section, the area of the DRG was measured using MetaMorph software. The number of cells per unit area is then calculated and averaged over all embryos/animals. *p* values were calculated using Student's *t* test. Placenta alkaline phosphatase (PLAP) staining intensity (from 50 randomly selected sections of animals of two different litters), and vGluT1 staining intensities and areas (from 60 randomly selected sections of animals of two different litters) were measured using MetaMorph software and were set to artificial units. *p* values were calculated with Student's *t* test.

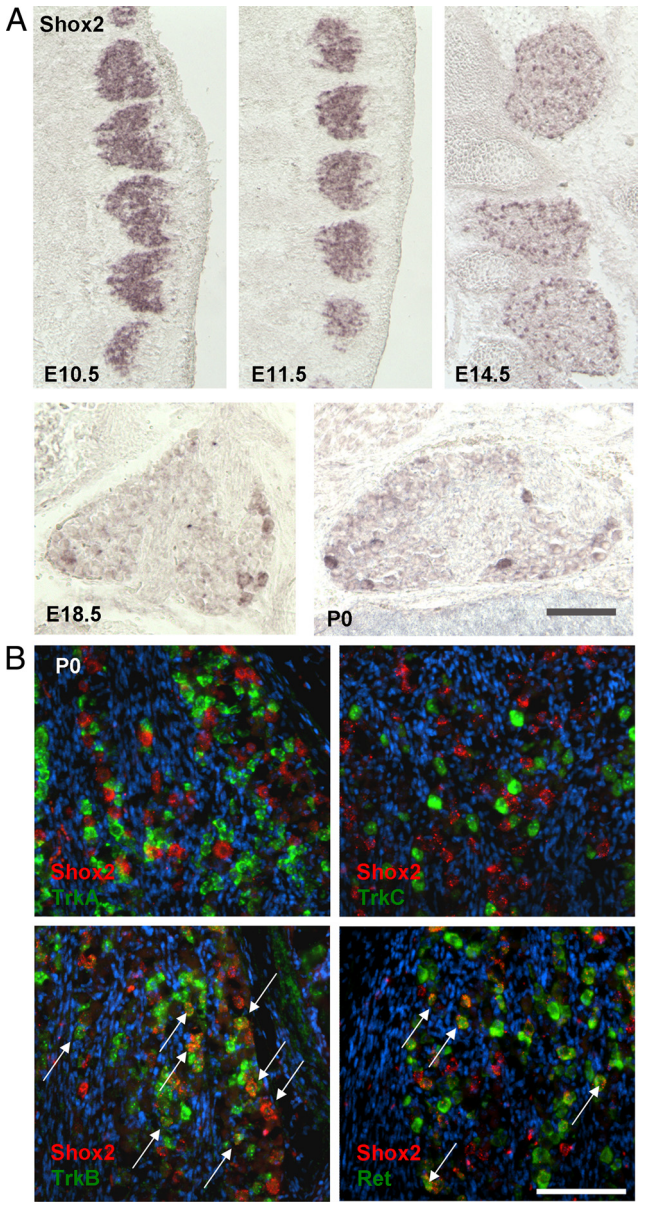

Figure 1. Shox2 expression in the developing DRG. ***A***, *In situ* hybridization experiments show that at E10.5 and E11.5 *Shox2* is expressed throughout the entire DRG; at E14.5, *Shox2* expression begins to downregulate in subsets of DRG neurons; and by E18.5 and P0, *Shox2* is only present in a small number of DRG neurons. ***B***, Two-color *in situ* hybridization with probes for *Shox2* (red) and different receptors (*TrkA*, *TrkB*, *TrkC*, and *Ret*) (green). Arrows indicate cell bodies in the ganglia showing colocalization of *Shox2* with either *TrkB* or *Ret*. Note that not all *TrkB*- or *Ret*-positive cell bodies have *Shox2* expression at P0. Scale bar, 100 μm.

Results

Shox2 expression pattern in the developing mouse DRG

In situ hybridization was performed to examine the expression of *Shox2* in the developing mouse DRG. At early stages beginning at embryonic day 10.5 (E10.5), *Shox2* is expressed throughout the

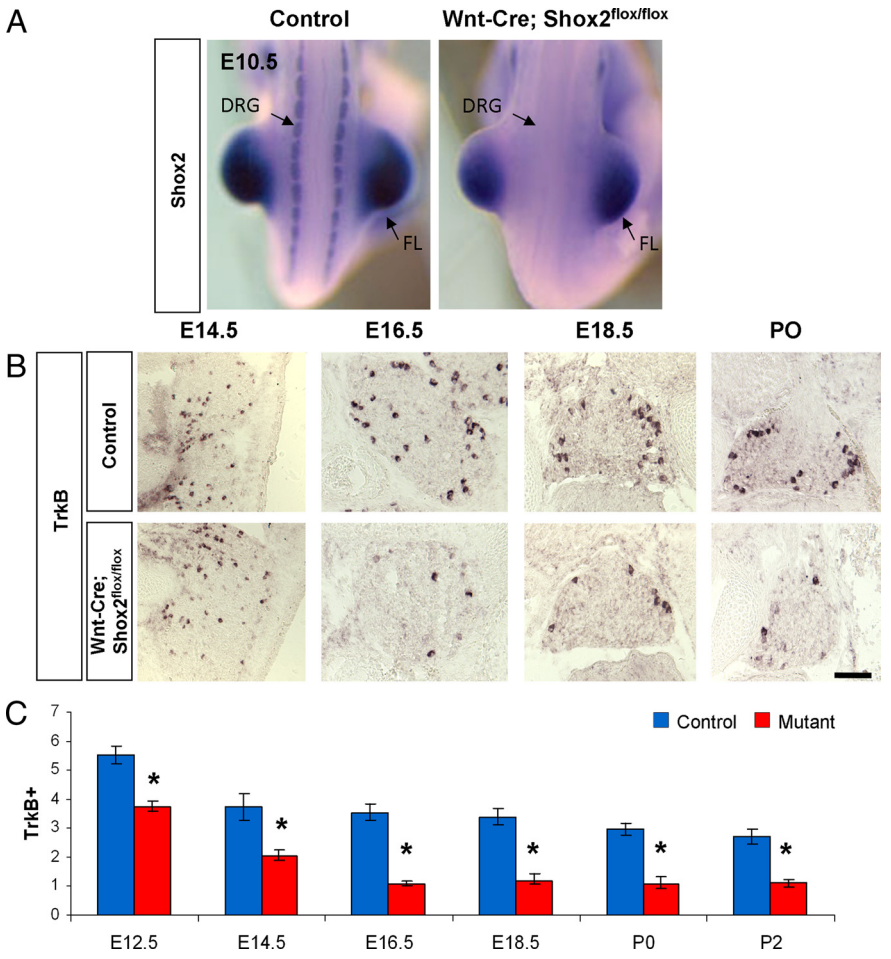

Figure 2. Reductions in the number of *TrkB*-expressing DRG neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ embryos. ***A***, *In situ* hybridization reveals the absence of *Shox2* expression in DRGs of *Wnt1-Cre; Shox2*$^{flox/flox}$ mice at E10.5. ***B***, Representative images of *TrkB* expression in control and *Shox2*-deleted DRGs at E14.5, E16.5, E18.5, and P0. ***C***, Quantifications of the numbers of *TrkB*-expressing DRG neurons per unit area in control and *Wnt1-Cre; Shox2*$^{flox/flox}$ embryos. *$p < 0.001$. Error bars represent ±SEM. Scale bar, 100 μm. FL, Forelimb.

ganglion (Fig. 1*A*). By E14.5, its expression begins to downregulate in the majority of sensory neurons (Fig. 1*A*). This downregulation continues after E14.5, and by E18.5 only a small population of DRG neurons retains stable expression of *Shox2*, which persist into adulthood (data not shown) (Fig. 1*A*). The dynamic expression pattern of *Shox2* led us to hypothesize that Shox2 is involved in the specification and development of subtypes of DRG neurons. To test this, we performed fluorescent two-color *in situ* hybridization experiments to examine the potential coexpression of *Shox2* with *Trks* or *Ret* receptors (which are markers for different somatosensory lineages). We observed that at postnatal day 0 (P0), *Shox2* colocalizes with subsets of *TrkB*- or *Ret*-expressing neurons and is absent from the *TrkA*- and *TrkC*-expressing cells (Fig. 1*B*). Upon quantification, we found that 65% of *Shox2*-positive cells colocalize with 65% of *TrkB* neurons, and the remaining 35% of *Shox2*-expressing cells colocalizes with a small number of *Ret*-positive DRG neurons. Since *TrkB* has been shown to be a marker for subsets of mechanosensory neurons, and *Ret* is expressed in some of the rapid-adapting mechanosensory neurons in addition to nociceptive neurons, the coexpression results suggest that Shox2 may be involved in the differentiation of NCCs into mechanosensory neurons.

Significant reduction in the number of *TrkB*-expressing DRG neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ mouse

To examine the function of Shox2 in DRG neuron development, we used *Wnt1-Cre* (Danielian et al., 1998) to conditionally delete the *Shox2* gene in all neural crest-derived cells including DRG neurons. We crossed *Wnt1-Cre* mice with *Shox2*$^{flox/flox}$ mice (Danielian et al., 1998; Cobb et al., 2006) to obtain conditional mutant *Wnt1-Cre; Shox2*$^{flox/flox}$ (as well as control *Wnt1-Cre; Shox2*$^{flox/+}$) embryos and neonates. Figure 2*A* shows the absence of *Shox2* expression in DRGs from *Shox2* mutant embryo at E10.5. In our hands, all *Shox2* mutant mice die within 2 d after birth due to an anterior cleft palate defect (data not shown), which limited our characterization of the role of Shox2 to embryonic and neonatal stages.

Using *in situ* hybridization, we examined the expression of *Trks* and *Ret* receptors in developing DRG at different stages in the mutant and control embryos. There is no observable difference in the number of *TrkA*- or *Ret*-positive cells at all time points examined, suggesting that Shox2 does not play a major role in the development of nociceptive lineage (Fig. 3*C*,*D*). In contrast, we observed a significant decrease in the number of *TrkB*-expressing cells in the *Shox2*-deleted DRG compared with controls at all time points examined (Fig. 2*B*,*C*). On average, there is a 60 ~ 65% loss of *TrkB*-expressing DRG neurons at stages after E16.5 (Fig. 2*C*), which is consistent with the fact that 65% of *TrkB* neurons express *Shox2*. Finally, the proprioceptive lineage marker *TrkC* showed a mild increase in the *Shox2*-deleted DRG at perinatal stages starting after E18.5 (Fig. 3*A*,*B*). The detailed method used for quantifying the numbers of different types of DRG neurons in these and subsequent results is described in Materials and Methods.

Previous studies have shown that the transcription factors MafA and Runx3 are involved in the development of the Ret-positive mechanosensory neurons and TrkC-positive proprioceptive neurons, respectively (Kramer et al., 2006; Inoue et al., 2007; Bourane et al., 2009). We used *in situ* hybridization to examine the expression of both of these transcription factors in mutant and control mice. We found that there was no difference in the number of DRG neurons expressing either *MafA* or *Runx3* in the *Shox2*-deleted versus control DRG at P0 or P2 (Figure 3*E–G*). The result suggests that Shox2 does not regulate *MafA* or *Runx3* expression and, by extension, probably does not play a major role in the development/specification of Ret-positive rapid-adapting mechanosensory neurons or TrkC-expressing proprioceptive neurons.

Loss of *TrkB*-expressing DRG neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ mouse is not caused by elevated apoptosis

We next investigated whether apoptosis could account for the observed loss of *TrkB*-expressing neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ DRG. We used anti-activated caspase-3 antibody to detect cell

death and found no statistically significant difference in the number of apoptotic cells in *Shox2*-deleted versus control DRG at all time points examined (Fig. 4*A*,*B*). Note that both *Shox2*-deficient and control DRGs show increased numbers of caspase-3-positive cells at E14.5 (Fig. 4*B*), a time point of naturally occurring cell death of developing sensory neurons, as shown previously (Raff et al., 1993; White et al., 1998). Thus, the loss of TrkB expression in *Shox2* mutant mice is not likely due to apoptosis of sensory neurons.

TrkC coexpression in subsets of *TrkB*- or *Ret*-positive sensory neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ mouse

It is known that during early DRG neurogenesis, the transient population of *TrkC*/*TrkB*-double-positive progenitor neurons later differentiate into *TrkB*-single-positive or Ret-single-positive mechanosensory neurons, or *TrkC*-single-positive proprioceptive neurons (Kramer et al., 2006; Marmigère and Ernfors, 2007). However, there appears to be a small percentage of sensory neurons that maintains coexpression of *TrkB*/*TrkC* or *Ret*/*TrkC*. We thus examined the coexpression of *TrkC* in the remaining *TrkB*-expressing, as well as in *Ret*-expressing, DRG neurons in *Shox2* mutant mice.

We first performed fluorescent two-color *in situ* hybridization to detect *TrkB* and *TrkC* mRNA simultaneously. In control DRGs at E14.5, ~33% of *TrkB*-positive neurons also express *TrkC*. By E16.5, <10% of *TrkB* cells still express *TrkC* in wild-type DRGs. This number is further reduced during postnatal development (Fig. 5*A*). In *Wnt1-Cre; Shox2*$^{flox/flox}$ DRGs, there is an apparent increase in the relative percentage of *TrkB*/*TrkC*-double-positive cells. However, when we quantified the actual average numbers of *TrkB*/*TrkC*-double-positive DRG neurons per unit area, there is no statistically significant difference between control and *Shox2* mutant DRGs (Fig. 5*B*). This result suggests that in the wild-type mouse *TrkB*/*TrkC*-double-positive mechanosensory neurons normally belong to the 35% TrkB-positive but Shox2-negative populations, and thus their number is unaffected by *Shox2* deletion.

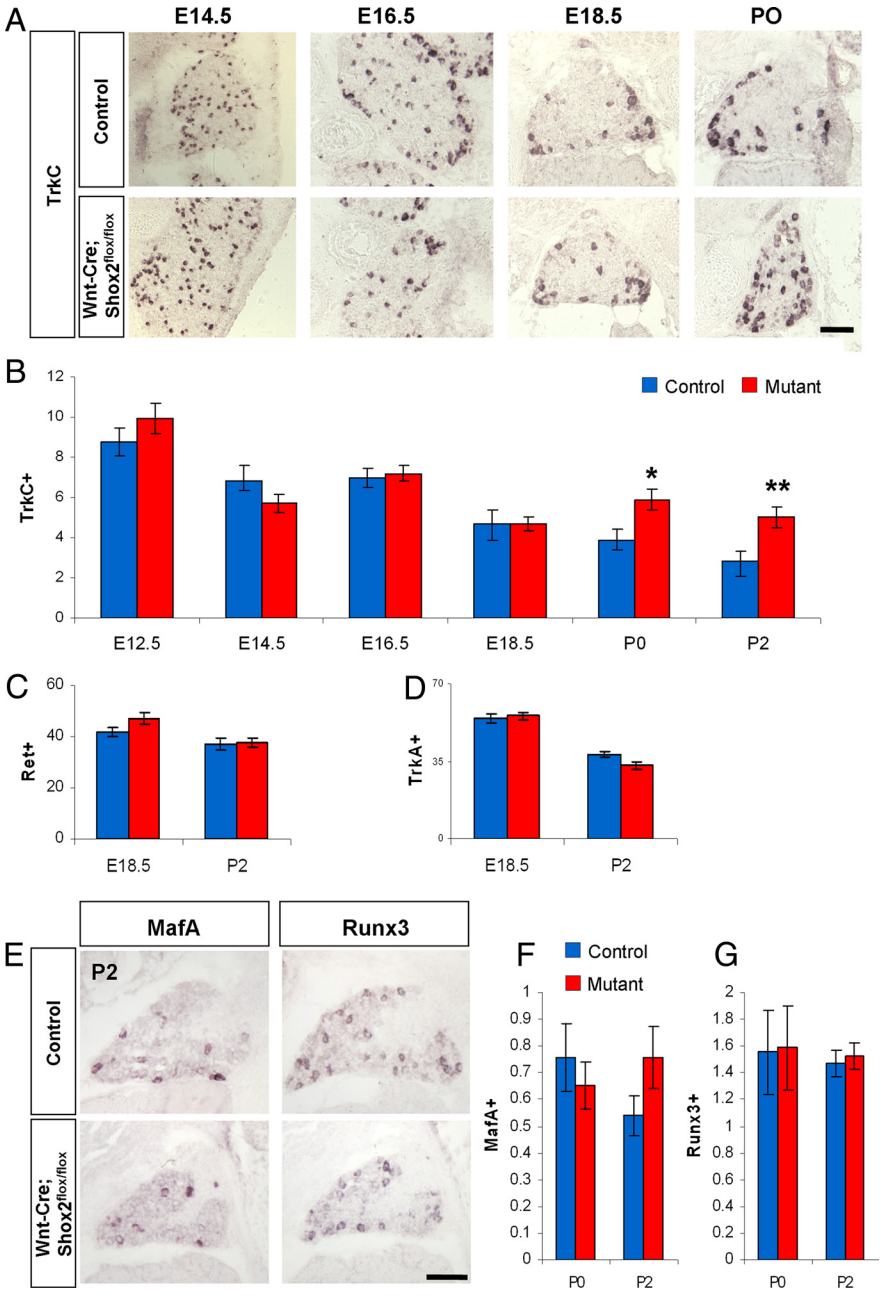

Figure 3. Increases in the number of *TrkC*-expressing DRG neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ mice at perinatal stages. ***A***, Representative images of *TrkC* expression in control and *Shox2*-deleted DRG at E14.5, E16.5, E18.5, and P0. ***B***, Quantifications of the numbers of *TrkC*-expressing DRG neurons per unit area in control and *Wnt1-Cre; Shox2*$^{flox/flox}$ embryos at six different stages. Note that there is an increase in the number of *TrkC*-expressing neurons in the mutant at P0 (*$p < 0.005$) and P2 (**$p < 0.001$). ***C***, ***D***, Quantification of the number of Ret- and TrkA-expressing neurons in control and *Wnt1-Cre; Shox2*$^{flox/flox}$ mice at E18.5 and P2. Scale bar, 100 μm. ***E***, Representative images of *MafA* and *Runx3* expression in control and *Shox2*-deleted DRGs at P2. ***F***, ***G***, Quantifications show no significant differences in the numbers of cells expressing *MafA* or *Runx3* per unit area between the control and *Shox2*-deleted DRGs at P0 and P2. Error bars represent ±SEM.

Since *Shox2* is also expressed in a subset of *Ret*-positive neurons (Fig. 1*B*), we also examined coexpression of *TrkC* in *Ret*-expressing sensory neurons. Again, using two-color *in situ* hybridization, we found that there is a small percentage of *Ret*-positive cells also expressing *TrkC* at perinatal stages in both control and *Shox2* mutant DRGs. Interestingly, the average number of *Ret*/*TrkC*-double-positive neurons per unit area is increased in the mutant (Fig. 5*C*,*D*). This result is consistent with the observed increase in the total number of *TrkC*-expressing DRG neurons and suggests that normally the function of Shox2 in Ret-positive neurons may be to suppress TrkC expression.

To examine whether any of the *Ret*/*TrkC*-double-positive cells in *Shox2*-deleted DRGs differentiate toward a proprioceptive neuron fate, we performed two-color *in situ* hybridization to detect *Ret* and *Parvalbumin* (*PV*) simultaneously. Parvalbumin is a known marker for TrkC expressing proprioceptive sensory neurons (Arber et al., 2000). *Ret*/*PV*-double-positive cells are rarely seen in either control or *Shox2*-mutant DRG, and there is no statistically significant difference in the total number of *PV*-

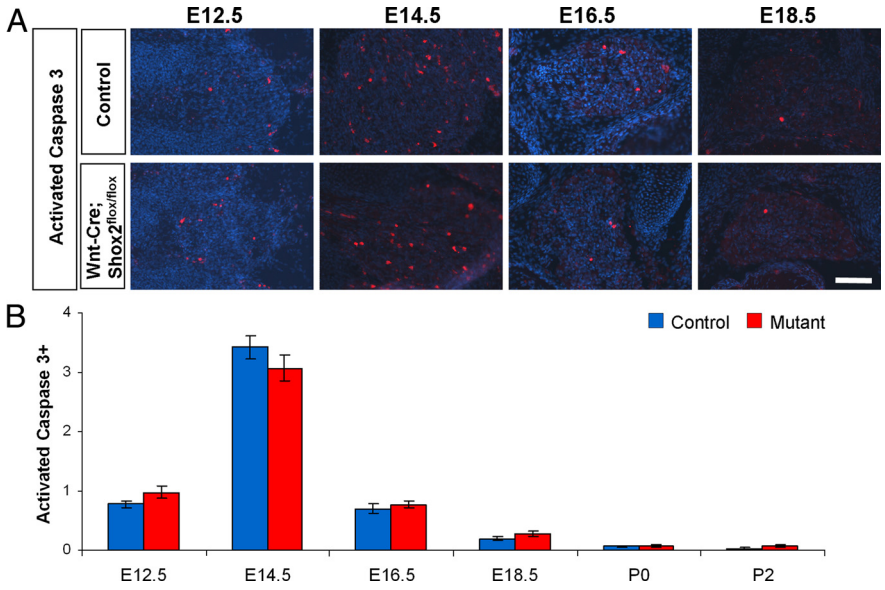

Figure 4. Apoptosis is normal in *Wnt1-Cre; Shox2*$^{flox/flox}$ DRGs. ***A***, Representative images of immunostaining with anti-activated-caspase-3 in control and *Shox2*-deleted DRGs at E12.5, E14.5, E16.5, and E18.5. ***B***, Quantifications show no significant differences in the amount of apoptotic cells between control and *Shox2*-deleted DRGs. Note that at E14.5, a time point of naturally occurring cell death, there is an increase in activated-caspase-3-positive cells in both control and mutant DRGs. Error bars represent ±SEM. Scale bar, 100 μm.

positive neurons between control and mutant mice (data not shown) (Fig. 5*E*). This result suggests that the increased number of *Ret/TrkC*-double-positive neurons in Shox2-mutant DRG do not appear to be proprioceptive neurons. To determine whether any of the *Ret/TrkC*-double-positive neurons belong to the late-born Ret-positive nociceptive neurons, we performed two-color *in situ* using *MrgD* and *TrkC* probes. MrgD is expressed exclusively in Ret/*Runx1*-expressing nonpeptidergic nociceptive neurons (Liu et al., 2008). We did not find any *MrgD/TrkC*-double-positive neurons in either control or *Shox2*-mutant mice (Fig. 5*F*). Thus, by exclusion, the increased *Ret/TrkC*-double-positive neurons in the Shox2 mutant are mechanosensory neurons.

Together, *Shox2* deletion resulted in a significant loss of *TrkB* expression and a mild increase of *TrkC* coexpression in small number of *Ret*-positive mechanosensory neurons. These data suggest that Shox2 is essential for the proper expression of neurotrophic receptors during the differentiation of mechanosensory neurons.

Developmental time-dependent requirement of Shox2 for proper TrkB expression in DRG neurons

Previous studies have found time-dependent roles of certain transcription factors [*ETS* genes, Islet1 (Isl1)] in DRG neuron development (Hippenmeyer et al., 2005; Sun et al., 2008). We therefore wanted to determine the time window when Shox2 is required for the development of a subset of *TrkB*-expressing sensory neurons. We used *Avil*$^{Cre/+}$ (Zhou et al., 2010) to delete the *Shox2* gene at later stages by generating *Avil*$^{Cre/+}$*; Shox2*$^{flox/flox}$ mice. *Advillin* is a gene whose expression is largely restricted to peripheral sensory neurons. Advillin is weakly expressed in a few DRG neurons at E12.5 and reaches peak expression at E16.5 (Hasegawa et al., 2007; Zhou et al., 2010; da Silva et al., 2011). *Avil*$^{Cre/+}$-mediated deletion of *Shox2* is completed at E18.5 (Fig. 6*A*).

The *Avil*$^{Cre/+}$*; Shox2*$^{flox/flox}$ mice are viable and fertile, and appear normal. Using *in situ* hybridization, we found that the number of *TrkB*-positive cells is only moderately decreased in these mutants compared with the controls (Fig. 6*B*,*C*). Furthermore, there is no difference in the numbers of *TrkC*-, *TrkA*-, or *Ret*-positive neurons between *Avil*$^{Cre/+}$*; Shox2*$^{flox/flox}$ and the control DRGs (Fig. 6*D–F*). Thus, deleting *Shox2* at stages after E12.5 resulted in a milder loss of *TrkB* expression and no effect on *TrkC* expression, suggesting that Shox2 is required primarily at the very early stages of mechanosensory neuron differentiation to ensure normal Trk receptor expression.

Effects of induced constitutive expression of *Shox2* in all neural crest-derived cells

To gain further insight into the functions of Shox2, we asked whether overexpressing Shox2 in all DRG neurons would have a dominant effect on *Trk* receptor expression. To achieve this, we created a knock-in mouse that allows Cre-dependent overexpression of Shox2. Briefly, a CAG promoter followed by loxP-STOP-loxP cassette, followed by Shox2 cDNA and polyA was inserted into Rosa26 locus (*Rosa*$^{CAG\text{-}STOP\text{-}Shox2}$). The knock-in mice were crossed with *Wnt1-Cre* to obtain *Wnt1-Cre; Rosa*$^{CAG\text{-}STOP\text{-}Shox2/+}$ mice. *In situ* hybridization confirmed the overexpression of *Shox2* mRNA in all DRG cells (Fig. 7*A*). *Wnt1-Cre; Rosa*$^{CAG\text{-}STOP\text{-}Shox2/+}$ mice die immediately at birth, due to a completely cleft palate (data not shown). We examined the expression of *Trk*s and *Ret* in these mice at E18.5 and P0 stages. Upon quantification, we found a 20% increase in the number of *TrkB*-positive neurons in DRGs from *Shox2* overexpression embryos (Fig. 7*B*,*C*). However, no apparent changes in *TrkA*, *TrkC*, and *Ret* expression were detected in these mice compared with the controls (Fig. 7*D–F*). Thus, although Shox2 is necessary for inducing and/or maintaining *TrkB* expression in subsets of mechanosensory neurons, it is not sufficient to induce *TrkB* or suppress *TrkC* expression in all DRG neurons.

Defects in mechanosensory neuron central innervations in *Shox2*-deficient mice

Finally, we examined the consequences of loss of Shox2 on the peripheral and central axonal projections of mechanosensory DRG neurons. To visualize the axonal projections, we used the *Avil*$^{PLAP/+}$ mice in which human PLAP is inserted into the Advillin locus (Hasegawa et al., 2007). We crossed *Wnt1-Cre; Shox2*$^{flox/+}$ males with *Avil*$^{PLAP/+}$*; Shox2*$^{flox/flox}$ females to generate *Avil*$^{PLAP/+}$*; Wnt1-Cre; Shox2*$^{flox/flox}$ mutant mice and their littermate controls. Since no mutant mice survive past P2, we examined axonal projection at E18.5, P0, and P2. At these stages, the peripheral axons have reached their targets, but have not yet fully differentiated into specialized sensory endings (Albuerne et al., 2000; Hasegawa et al., 2007), thereby preventing us from definitively determining the exact morphological subtypes of neurons that are affected by *Shox2* deletion. Using PLAP staining and anti-PGP9.5 staining, we did not detect any apparent differences in the general peripheral sensory projections into the hairy or the glabrous skin (data not shown).

However, PLAP staining on spinal cord sections from control or *Avil*$^{PLAP/+}$*; Wnt1-Cre; Shox2*$^{flox/flox}$ mutant mice revealed that

the *Shox2*-deleted DRG neurons showed reduced central axonal innervation to layers III/IV of the spinal cord compared with the control (Fig. 8). Note that layer III/IV stained strongly for PLAP in the control (Fig. 8*A*, arrow), but the corresponding region in the *Wnt1-Cre; Shox2*$^{flox/flox}$ mutant spinal cord stained much weaker (Fig. 8*A*). On average, there is a 10% reduction in the PLAP staining intensity in *Shox2* mutant ($p < 0.001$). Since layers III/IV receive inputs from mechanosensory neurons, including *TrkB*- and *Ret*-expressing touch neurons, this observation suggests that Shox2 deficiency causes central innervation defects in subsets of mechanosensory neurons. Note that the reduced PLAP staining in layer III/IV was not due to changes in the expression of *Advillin* locus as PLAP staining in the DRG cell bodies and peripheral axons was equally intense, and *in situ* hybridization showed a similar level of *Advillin* expression in both control and Shox2-deleted DRGs (data not shown).

To confirm the central axon innervation defects with an independent method, we also used *Isl1*$^{cre/+}$ (Srinivas et al., 2001) to conditionally delete *Shox2*. Isl1 is expressed in all sensory neurons beginning at E10; thus, in these mice Shox2 is deleted at early stages of DRG neuron differentiation. In *Isl1*$^{cre/+}$*; Shox2*$^{flox/flox}$ mice, we observed a 53% reduction in the number of *TrkB*-expressing cells, only slightly less than what we observed in *Wnt1-Cre; Shox2*$^{flox/flox}$ mice ($p < 0.001$; data not shown). We used anti-vGlut1 staining to specifically visualize mechanosensory afferent termini (whereas *Avil*$^{PLAP/+}$ labels all axons including nociceptive afferents). The average vGluT1 staining intensity in layer III/IV showed an 11% reduction in *Isl1*$^{cre/+}$*; Shox2*$^{flox/flox}$ dorsal spinal cord compared with that in controls (Fig. 8*B*) ($p < 0.002$). As a control, the vGLUT staining signals in DRG neuronal cell bodies were indistinguishable between control and Shox2 mutant mouse (Fig. 8*B*, insets). In addition, the average area covered by vGluT1-positive mechanosensory axons in dorsal horn spinal cord is also reduced by ~20% in *Isl1*$^{cre/+}$*; Shox2*$^{flox/flox}$ mice (Fig. 8*B*) ($p < 0.001$). Not surprisingly, nociceptive innervation to the dorsal horn as revealed by anti-CGRP and IB4 staining (to visualize peptidergic and nonpeptidergic nociceptive central axonal projections, respectively) was unchanged in *Shox2* mutant mice (Fig. 8*C*). Together, our study uncovered an important requirement for the transcription factor Shox2 for the proper development, differentiation, and central innervation of a subset of TrkB-expressing mouse mechanosensory neurons.

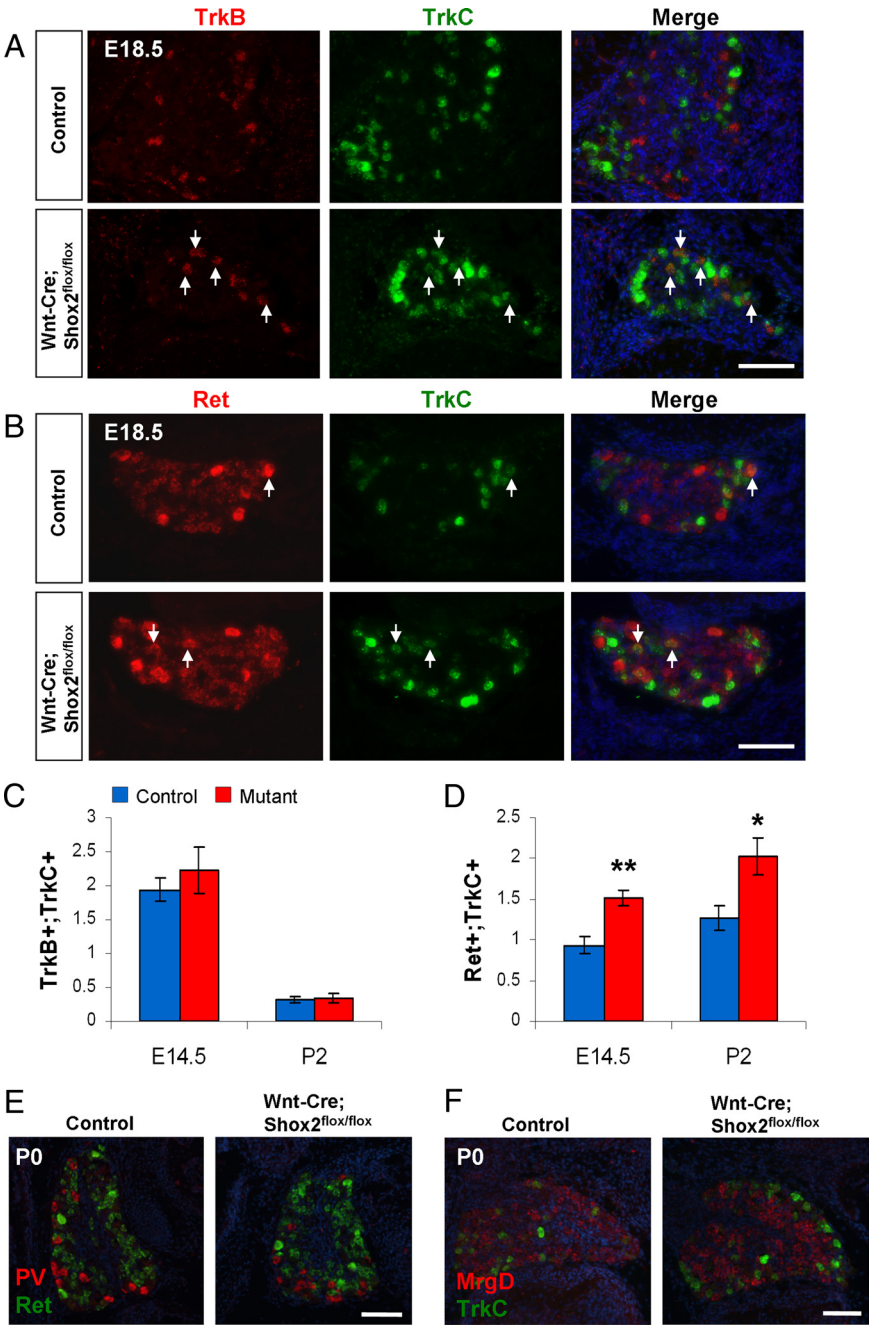

Figure 5. Increased coexpression of *Ret* and *TrkC* in DRG neurons in *Wnt1-Cre; Shox2*$^{flox/flox}$ mice. ***A***, Representative results of two-color *in situ* hybridization using *TrkB* (red) and *TrkC* (green) probes at E18.5. Arrows indicate neurons in the DRGs that are positive for both receptors. ***B***, Representative results of two-color *in situ* hybridization using *Ret* (red) and *TrkC* (green) probes at E18.5. Arrows indicate cell bodies in the DRGs that are positive for both receptors. ***C***, Quantification of the average number (per unit area) of *TrkB/TrkC*-double-positive neurons revealed no significant difference between the control and *Shox2*-deleted mice. ***D***, Quantification of the average number (per unit area) of *Ret/TrkC*-double-positive neurons showed a significant increase in the *Shox2*-deleted versus control DRGs. ***E***, Representative images of two-color *in situ* hybridization results with *Parvalbumin* (red) and *Ret* (green) probes at P0. ***F***, Representative images of two-color *in situ* hybridization results with *MrgD* (red) and *TrkC* (green) probes at P0. *$p < 0.01$, **$p < 0.001$. Error bars represent ± SEM. Scale bars, 100 μm.

Discussion

In the mammalian peripheral somatosensory system, mechanosensory and proprioceptive lineages arise from the same progenitor populations, through the first wave of neurogenesis from the precursor NCCs that migrate into the site of the future DRGs at E9 (Fode et al., 1998; Ma et al., 1999). The initially TrkC/TrkB-double-positive progenitor neurons eventually differentiate into

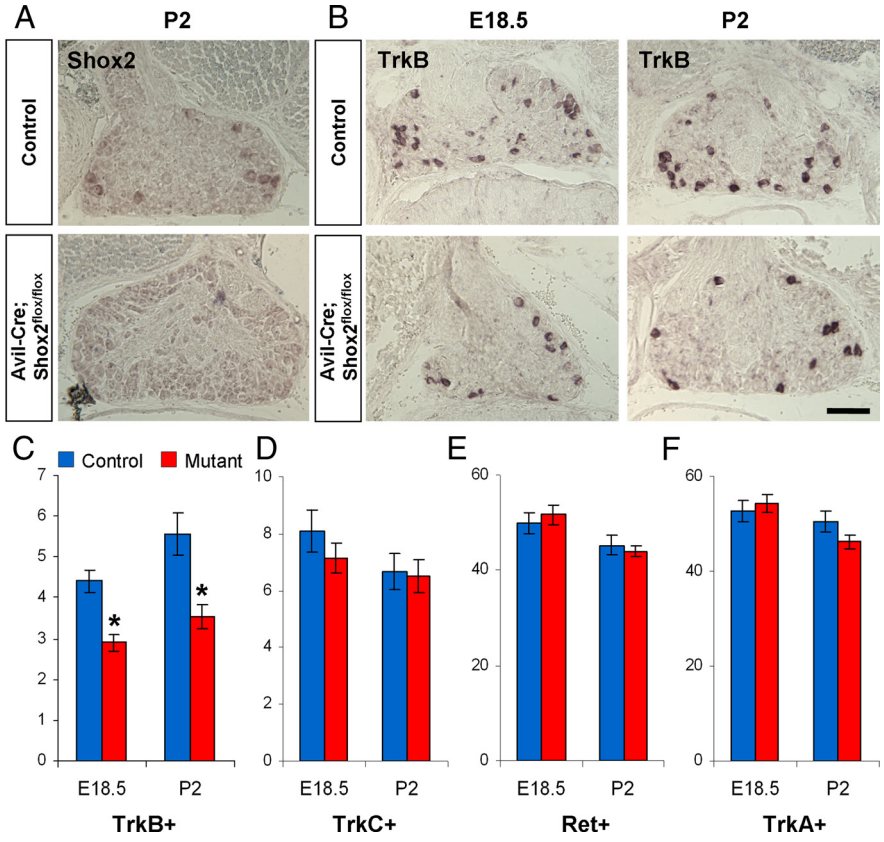

Figure 6. Mild reduction in the number of *TrkB*-expressing cells in $Avil^{Cre/+}$; $Shox2^{flox/flox}$ mice. ***A***, *In situ* hybridization confirms the loss of *Shox2* expression in the DRGs from $Avil^{Cre/+}$; $Shox2^{flox/flox}$ mice. ***B***, Representative images of *TrkB* expression in control and $Avil^{Cre/+}$; $Shox2^{flox/flox}$ mice at E18.5 and P2. ***C–F***, Quantification of the numbers of *TrkB*- (***C***), *TrkC*- (***D***), *Ret*- (***E***), and *TrkA*-expressing (***F***) DRG neurons per unit area in control and $Avil^{Cre/+}$; $Shox2^{flox/flox}$ mice at E18.5 and P2. *$p < 0.001$. Error bars represent ±SEM. Scale bar, 100 μm.

TrkC-single-positive proprioceptive neurons, as well as TrkB- or Ret-single-positive touch sensory neurons, although a very small number of mechanosensory neurons are TrkB/TrkC or Ret/TrkC double positive. Previously, the molecular mechanisms regulating the specification of TrkB-expressing mechanosensory neuron lineage are unknown. In this study, we discovered that Shox2 is an essential, although not sufficient, component required for the proper development of a subpopulation of TrkB-positive DRG neurons.

Wnt1-Cre-mediated deletion of *Shox2* in NCCs results in a 60–65% reduction in the number of *TrkB*-expressing DRG neurons at stages after E16.5. Shox2 deletion also caused a small increased in *TrkC*/*Ret*-double-positive neurons at later stages. These findings suggest that Shox2 is important for ensuring TrkB expression and may contribute to TrkC repression in Ret-expressing touch sensory neurons, although at present we do not yet know whether such effects of Shox2 are direct or indirect.

Later deletion of *Shox2* using $Avil^{Cre/+}$ resulted in only a moderate decrease in TrkB-positive neurons than that observed in *Wnt1-Cre*-mediated deletion mice, suggesting that Shox2 is required in progenitor and early-born neurons to promote their differentiation into TrkB-expressing touch neurons. The onset of Advillin expression occurs at E12.5 and reaches a maximum at E16.5. It is likely that only those neurons that express Cre at early stages (E12.5) are affected and lose TrkB expression, whereas those that express Cre after E12.5 are not affected by *Shox2* deletion. Perhaps once the stable high-level expression of TrkB is established, Shox2 is no longer needed for the maintenance of TrkB expression. In our gain-of-function studies, overexpression of Shox2 in all NCC progenitor cells only mildly increased the number of *TrkB*-positive cells, but had no effects on other *Trks* or *Ret* receptor expressions, including *TrkC*. Together, Shox2 is necessary for inducing or maintaining TrkB expression in a subpopulation of mechanosensory neurons, but alone is not sufficient to induce TrkB expression in nonmechanosensory neurons. A model summarizing previous and current finding related to proprioceptive and mechanosensory neuron specification is shown in Figure 9.

At present, we do not know the downstream targets of Shox2, nor do we know whether Shox2 only regulates Trk receptor expression or whether it also regulates other genes involved in other aspects of the touch/mechanosensory neuronal development. In other sensory lineages, the transcription factors Runx3 and Runx1 control all of the gene expression programs relevant to proprioceptive or nociceptive sensory neurons development and differentiation, respectively (Chen et al., 2006; Kramer et al., 2006; Inoue et al., 2008). On the other hand, the transcription factor Klf7 is required only for *TrkA* gene expression by binding to an enhancer element in the TrkA promoter. The loss of Klf7 leads to increased apoptosis of nociceptive sensory neurons. However, Klf7 did not appear to regulate other aspects of the differentiation program of TrkA-positive neurons (Lei et al., 2005). Future work is needed to determine the transcription targets of Shox2.

In *Shox2*-deficient mice, the central afferent innervations from mechanosensory neurons to layers III/IV in the spinal cord are reduced. Again, this could be a direct consequence of failed differentiation of a subset of TrkB-expressing touch neurons, or a secondary effect due to the loss of TrkB expression. Unlike the wealth of marker genes known for proprioceptive or nociceptive neurons, a very limited number of molecular markers are known to specifically label TrkB-expressing mechanosensory neurons. We, therefore, could not examine other molecular aspects of mechanosensory neuron development and differentiation in *Shox2* mutant mice. Nonetheless, Shox2 is the first transcription factor discovered that affects TrkB expression in mechanosensory DRG neurons.

References

Albuerne M, De Lavallina J, Esteban I, Naves FJ, Silos-Santiago I, Vega JA (2000) Development of Meissner-like and Pacinian sensory corpuscles in the mouse demonstrated with specific markers for corpuscular constituents. Anat Rec 258:235–242.

Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2000) ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell 101:485–498.

Binder G (2011) Short stature due to SHOX deficiency: genotype, phenotype, and therapy. Horm Res Paediatr 75:81–89.

Blaschke RJ, Hahurij ND, Kuijper S, Just S, Wisse LJ, Deissler K, Maxelon T, Anastassiadis K, Spitzer J, Hardt SE, Schöler H, Feitsma H, Rottbauer W, Blum M, Meijlink F, Rappold G, Gittenberger-de Groot AC (2007) Targeted mutation reveals essential functions of the homeodomain transcription

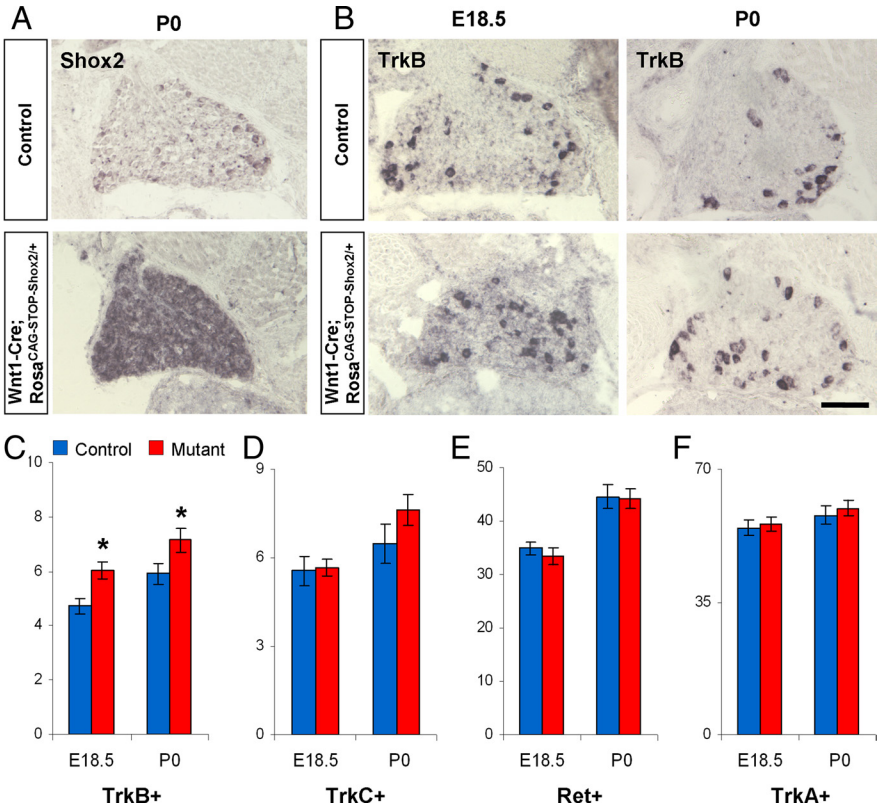

Figure 7. Overexpression of *Shox2* in all sensory neurons results in a mild increase in the number of *TrkB*-positive cell. ***A***, Representative *in situ* hybridization images confirming the overexpression of *Shox2* in all DRG neurons in *Wnt1-Cre; Rosa*$^{CAG\text{-}STOP\text{-}Shox2}$ mice. ***B***, Representative images of *TrkB in situ* hybridization results in control and *Shox2* overexpression mice. ***C–F***, Quantifications of the numbers of *TrkB*- (***C***), *TrkC*- (***D***), *Ret*- (***E***), and *TrkA*-expressing (***F***) DRG neurons per unit area in control and *Shox2* overexpression mice at E18.5 and P0. $^*p < 0.05$. Error bars represent ±SEM. Scale bar, 100 μm.

Bourane S, Garces A, Venteo S, Pattyn A, Hubert T, Fichard A, Puech S, Boukhaddaoui H, Baudet C, Takahashi S, Valmier J, Carroll P (2009) Low-threshold mechanoreceptor subtypes selectively express MafA and are specified by Ret signaling. Neuron 64:857–870.

Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127:1671–1679.

Chen CL, Broom DC, Liu Y, de Nooij JC, Li Z, Cen C, Samad OA, Jessell TM, Woolf CJ, Ma Q (2006) Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron 49:365–377.

Cobb J, Dierich A, Huss-Garcia Y, Duboule D (2006) A mouse model for human short-stature syndromes identifies Shox2 as an upstream regulator of Runx2 during long-bone development. Proc Natl Acad Sci U S A 103:4511–4515.

da Silva S, Wang F (2011) Retrograde neural circuit specification by target-derived neurotrophins and growth factors. Curr Opin Neurobiol 21:61–67.

da Silva S, Hasegawa H, Scott A, Zhou X, Wagner AK, Han BX, Wang F (2011) Proper formation of whisker barrelettes requires periphery-derived Smad4-dependent TGF-{beta} signaling. Proc Natl Acad Sci U S A 108:3395–3400.

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol 8:1323–1326.

Fode C, Gradwohl G, Morin X, Dierich A, LeMeur M, Goridis C, Guillemot F (1998) The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron 20:483–494.

González-Martínez T, Germanà GP, Monjil DF, Silos-Santiago I, de Carlos F, Germanà G, Cobo J, Vega JA (2004) Absence of Meissner corpuscles in the digital pads of mice lacking functional TrkB. Brain Res 1002:120–128.

Hasegawa H, Wang F (2008) Visualizing mechanosensory endings of TrkC-expressing neurons in HS3ST-2-hPLAP mice. J Comp Neurol 511:543–556.

Hasegawa H, Abbott S, Han BX, Qi Y, Wang F (2007) Analyzing somatosensory axon projections with the sensory neuron-specific *Advillin* gene. J Neurosci 27:14404–14414.

Hippenmeyer S, Vrieseling E, Sigrist M, Portmann T, Laengle C, Ladle DR, Arber S (2005) A developmental switch in the response of DRG neurons to ETS transcription factor signaling. PLoS Biol 3:e159.

Hodge LK, Klassen MP, Han BX, Yiu G, Hurrell J, Howell A, Rousseau G, Lemaigre F, Tessier-Lavigne M, Wang F (2007) Retrograde BMP signaling regulates trigeminal sensory neuron identities and the formation of precise face maps. Neuron 55:572–586.

Inoue K, Ito K, Osato M, Lee B, Bae SC, Ito Y (2007) The transcription factor Runx3 represses the neurotrophin receptor TrkB during lineage commitment of dorsal root ganglion neurons. J Biol Chem 282:24175–24184.

Inoue K, Shiga T, Ito Y (2008) Runx transcription factors in neuronal development. Neural Dev 3:20.

Kramer I, Sigrist M, de Nooij JC, Taniuchi I, Jessell TM, Arber S (2006) A role for Runx transcription factor signaling in dorsal root ganglion sensory neuron diversification. Neuron 49:379–393.

Lei L, Laub F, Lush M, Romero M, Zhou J, Luikart B, Klesse L, Ramirez F, Parada LF (2005) The zinc finger transcription factor Klf7 is required for TrkA gene expression and development of nociceptive sensory neurons. Genes Dev 19:1354–1364.

Levanon D, Brenner O, Negreanu V, Bettoun D, Woolf E, Eilam R, Lotem J, Gat U, Otto F, Speck N, Groner Y (2001) Spatial and temporal expression pattern of Runx3 (Aml2) and Runx1 (Aml1) indicates non-redundant functions during mouse embryogenesis. Mech Dev 109:413–417.

Liu Y, Yang FC, Okuda T, Dong X, Zylka MJ, Chen CL, Anderson DJ, Kuner R, Ma Q (2008) Mechanisms of compartmentalized expression of Mrg class G-protein-coupled sensory receptors. J Neurosci 28:125–132.

Luo W, Wickramasinghe SR, Savitt JM, Griffin JW, Dawson TM, Ginty DD (2007) A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. Neuron 54:739–754.

Luo W, Enomoto H, Rice FL, Milbrandt J, Ginty DD (2009) Molecular identification of rapidly adapting mechanoreceptors and their developmental dependence on ret signaling. Neuron 64:841–856.

Ma Q, Fode C, Guillemot F, Anderson DJ (1999) Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev 13:1717–1728.

Marchini A, Rappold G, Schneider KU (2007) SHOX at a glance: from gene to protein. Arch Physiol Biochem 113:116–123.

Marmigère F, Ernfors P (2007) Specification and connectivity of neuronal subtypes in the sensory lineage. Nat Rev Neurosci 8:114–127.

Perez-Pinera P, García-Suarez O, Germanà A, Díaz-Esnal B, de Carlos F, Silos-Santiago I, del Valle ME, Cobo J, Vega JA (2008) Characterization of sensory deficits in TrkB knockout mice. Neurosci Lett 433:43–47.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. Science 262:695–700.

Sedý J, Tseng S, Walro JM, Grim M, Kucera J (2006) ETS transcription factor ER81 is required for the Pacinian corpuscle development. Dev Dyn 235:1081–1089.

Shimizu S, Ichikawa H, Nakagawa H, Kiyomiya K, Matsuo S (2007) Effect of BDNF depletion on the formation of Ruffini endings in vibrissa follicles and the survival of their mechanoreceptive neurons in trigeminal ganglion. Brain Res 1154:95–104.

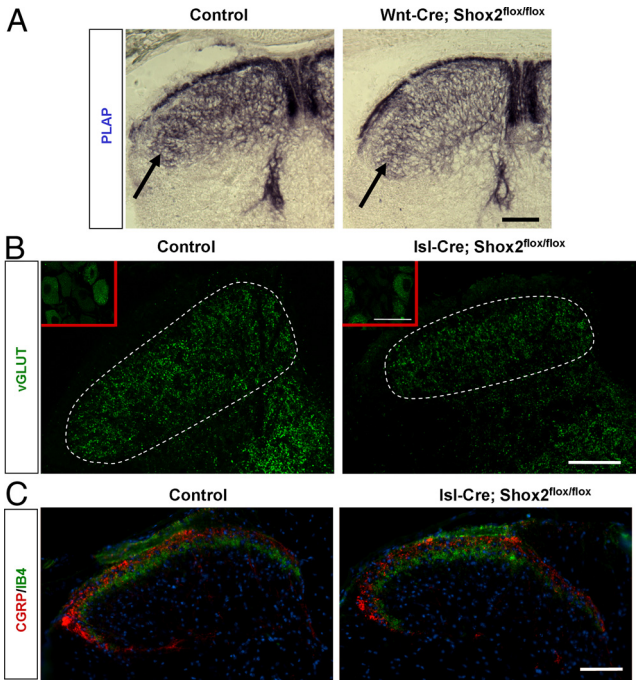

Figure 8. Reduced mechanosensory central innervation in the spinal cord in *Shox2*-deleted mice. ***A***, Representative images of sensory afferent projections in the spinal cord from control (*Wnt1-Cre; Shox2*$^{flox/+}$;*Avil*$^{PLAP/+}$) and *Shox2*-deleted mice (*Wnt1-Cre; Shox2*$^{flox/flox}$;*Avil*$^{PLAP/+}$) as revealed by PLAP staining at P0. Arrows point to a densely stained band in layer III/IV in the control that is only moderately stained in the mutant. ***B***, Representative images of immunofluorescence staining with anti-vGluT1 in the spinal cord of control (*Isl1-Cre; Shox2*$^{flox/+}$) and *Shox2*-deleted (*Isl1-Cre; Shox2*$^{flox/flox}$) mice. Inset shows the anti v-GluT1 immunofluorescence signal in the DRG of control and *Shox2*-deleted mice. ***C***, Representative images of immunofluorescence staining with anti-CGRP (red) and anti-IB4 (green) in the spinal cord of control and *Shox2*-deleted mice. Blue is DAPI. Scale bars: ***A***, ***C***, 100 μm; ***B***, 50 μm.

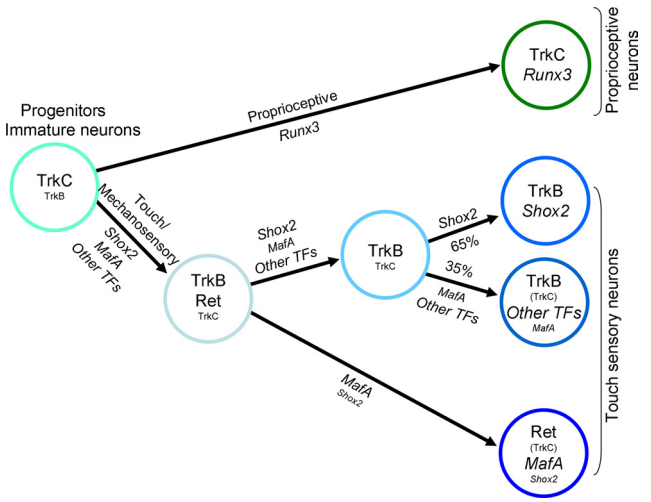

Figure 9. A model for the development and diversification of proprioceptive and touch sensory neurons. Schematic model shows the transcription factors involved in the progressive specification of TrkB/TrkC-double-positive progenitor/immature neurons into proprioceptive and different types of touch sensory neurons.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1:4.

Sun Y, Dykes IM, Liang X, Eng SR, Evans SM, Turner EE (2008) A central role for Islet1 in sensory neuron development linking sensory and spinal gene regulatory programs. Nat Neurosci 11:1283–1293.

Szeder V, Grim M, Halata Z, Sieber-Blum M (2003) Neural crest origin of mammalian Merkel cells. Dev Biol 253:258–263.

White FA, Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD (1998) Widespread elimination of naturally occurring neuronal death in *Bax*-deficient mice. J Neurosci 18:1428–1439.

Yang L, Cai CL, Lin L, Qyang Y, Chung C, Monteiro RM, Mummery CL, Fishman GI, Cogen A, Evans S (2006) Isl1Cre reveals a common Bmp pathway in heart and limb development. Development 133:1575–1585.

Yu L, Gu S, Alappat S, Song Y, Yan M, Zhang X, Zhang G, Jiang Y, Zhang Z, Zhang Y, Chen Y (2005) Shox2-deficient mice exhibit a rare type of incomplete clefting of the secondary palate. Development 132:4397–4406.

Zhou X, Wang L, Hasegawa H, Amin P, Han BX, Kaneko S, He Y, Wang F (2010) Deletion of PIK3C3/Vps34 in sensory neurons causes rapid neurodegeneration by disrupting the endosomal but not the autophagic pathway. Proc Natl Acad Sci U S A 107:9424–9429.