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Conditional deletion of *Hand2* reveals critical functions in neurogenesis and cell type-specific gene expression for development of neural crest-derived noradrenergic sympathetic ganglion neurons

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ABSTRACT

Neural crest-derived structures that depend critically upon expression of the basic helix–loop–helix DNA binding protein *Hand2* for normal development include craniofacial cartilage and bone, the outflow tract of the heart, cardiac cushion, and noradrenergic sympathetic ganglion neurons. Loss of *Hand2* is embryonic lethal by E9.5, obviating a genetic analysis of its in-vivo function. We have overcome this difficulty by specific deletion of *Hand2* in neural crest-derived cells by crossing our line of floxed *Hand2* mice with Wnt1-Cre transgenic mice. Our analysis of *Hand2* knock-out in neural crest-derived cells reveals effects on development in all neural crest-derived structures where *Hand2* is expressed. In the autonomic nervous system, conditional disruption of *Hand2* results in a significant and progressive loss of neurons as well as a significant loss of TH expression. *Hand2* affects generation of the neural precursor pool of cells by affecting both the proliferative capacity of the progenitors as well as affecting expression of *Phox2a* and *Gata3*, DNA binding proteins important for the cell autonomous development of noradrenergic neurons. Our data suggest that *Hand2* is a multifunctional DNA binding protein affecting differentiation and cell type-specific gene expression in neural crest-derived noradrenergic sympathetic ganglion neurons. *Hand2* has a pivotal function in a non-linear cross-regulatory network of DNA binding proteins that affect cell autonomous control of differentiation and cell type-specific gene expression.

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Introduction

The basic helix–loop–helix (bHLH) DNA binding protein *Hand2* (dHand, Thing-2, Hed) is expressed in a subset of neural crest-derived cells where it has been shown to function in various aspects of cell specification, lineage segregation, and cell type-specific gene expression (Firulli et al., 2003; Ruest et al., 2004; Howard, 2005; Olson, 2006; Hendershot et al., 2007). The cell types expressing *Hand2* are quite diverse and include noradrenergic sympathetic ganglion neurons, enteric neurons, craniofacial cartilage and bone, as well as multiple components in the heart. Generation of a diverse array of derivatives is one hallmark of the neural crest but mechanisms underlying development of this cellular diversity remain obscure. It is generally accepted that patterns of differential gene expression account for and can be used to demonstrate fate maps of early lineage segregation for many neural crest-derived cell types. For neural crest-derived cell types where expression of *Hand2* is necessary for proper development, both extrinsic factors and other *cis*-regulatory elements have been identified. Development of noradrenergic sympathetic ganglion neurons is perhaps the best described.

A network of DNA binding proteins, whose expression is regulated downstream of bone morphogenetic proteins and which is required for differentiation of autonomic neurons has been described (Groves et al., 1995; Lo et al., 1999, Pattyn et al., 1999; Howard et al., 2000; Coppola et al., 2005; Ernsberger and Rohrer, 2006; reviewed in Bertrand et al., 2002; Goridis and Rohrer, 2002; Howard, 2005). Based on gain-of-function, loss-of-function and gene knock-in studies, the homeodomain DNA protein *Phox2b* has been identified as a likely “master” gene required for specification and differentiation of all classes of autonomic neurons (Pattyn et al., 2000; Coppola et al., 2005). In addition, this core network includes the homeodomain DNA protein *Phox2a* and the bHLH DNA binding protein *Ascl1* (*Mash1*) (Guillemont et al., 1993; Sommer et al., 1995; Hirsch et al., 1998; Morin et al., 1997; Pattyn et al.,

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1997, 1999; Hirsch et al., 1998; Lo et al., 1998; Bertrand et al., 2002; Howard, 2005). Loss-of-function of *Phox2b*, *Phox2a* and *Ascl1* result in either the complete loss of peripheral autonomic neurons (*Phox2b*) or loss of subsets of neurons (*Ascl1*) or some aspects of cell type-specific gene expression (*Ascl1* and *Phox2a*). This pattern of affect suggested that neuronal diversity might be dependent upon additional transcriptional regulators that were recruited in a specific manner in segregated populations of neural crest-derived precursor cells. The restricted neuronal expression pattern of *Hand2* to enteric and sympathetic ganglion neurons suggested that *Hand2* might serve this function.

The contribution of *Hand2* to the specification and cell type-specific gene expression in noradrenergic neurons has thus far been demonstrated *in-vitro* and in-vivo using gain-of-function and loss-of-function approaches depending upon retrovirus-meditated gene transfer and knock-down using antisense oligonucleotides (Howard et al., 1999, 2000; Hendershot et al., 2007). Loss-of-function of *Hand2 in-vitro* results in a significant decrease in the number of neural crest-derived cells differentiating as neurons expressing noradrenergic marker proteins; these data suggested a function for *Hand2* in both neurogenesis and cell type-specific gene expression. Since both overexpression and ectopic expression of *Hand2* support neurogenesis and expression of noradrenergic and VIPergic marker genes we posited that *Hand2* might be both sufficient and necessary for the development of subsets of enteric and noradrenergic sympathetic ganglion neurons. Considering that *Hand2* null mice die at E9.5–10, prior to development of the majority of peripheral autonomic neurons, we generated a conditional allele of *Hand2* to be able to dissect genetically the function of *Hand2* in neurogenesis and cell type-specific gene expression in neural crest-derived autonomic neurons. Knock-out of *Hand2* in neural crest-derived precursors of sympathetic noradrenergic neurons results in a substantial reduction in the generation of noradrenergic neurons, decreased expression of the noradrenergic marker genes tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) as well as altered expression of the noradrenergic determinants *Phox2a* and *Gata3*. These results support our hypothesis that *Hand2* affects neurogenesis and cell type-specific gene expression as well as maintenance of sympathetic ganglion neurons. Our results indicate an obligate role for *Hand2* in maintaining the proliferative capacity of the neural precursor pool as well as an essential role in expression of noradrenergic characteristics.

Materials and methods

Targeting strategy and mouse strains

Prior to initiation of this work, all breeding procedures, animal care and experimental protocols were approved by the Medical University of Ohio (renamed University of Toledo Health Sciences Campus) Animal Care and Use Committee. The complete strategy for targeting *Hand2* is published elsewhere (Hendershot et al., 2007). $Hand2^{fl/fl}$ mice are phenotypically normal, fertile and viable suggesting that the introduction of loxP sites in the 5′ UTR does not negatively impact transcription at the targeted locus. Normal expression of *Hand2* has been confirmed by qRT-PCR. Targeted deletion of *Hand2* is embryonic lethal at around E12 although we have had several embryos survive until birth at which time they died. Based on the cardiac phenotype we observed in $Hand2^{flneo/flneo}$ embryos and $Hand2^{fl/fl/Wnt1\text{-}Cre}$ embryos we conclude that loss of *Hand2* in the neural crest is lethal due to substantial loss of norepinephrine (Lim et al., 2000; Moriguchi et al., 2006). To rescue $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos (Lim et al., 2000; Kaufman et al., 2003), pregnant dams are fed water containing a cocktail of catecholamines (100 μg/ml L-phenylephrine, 100 μg/ml isoproterenol, and 2 mg/ml ascorbic acid) beginning at embryonic day (E) 8. This pharmacological approach allowed us to rescue $Hand2^{flneo/flneo}$ and $Hand2^{fl/fl/;Wnt1\text{-}Cre}$ embryos from pre-term death, a phenotype observed in other mouse models in which norepinephrine is absent or curtailed (Lim et al., 2000; Thomas et al., 1995; Zhou and Palmiter, 1995). To generate reporter mice, homozygous R26RYFP females were mated to hemizygous Wnt1-Cre males and $Hand2^{fl/+;Wnt1\text{-}Cre}$ males were mated to $Hand2^{fl/fl;R262YFP}$ females. The description of targeting *Phox2a* in the *Phox2b* locus and obtaining $Phox2b^{KI2a}$ knock-in mice has been described elsewhere (Coppola et al., 2005).

Expression of transcript encoding Hand2

Expression of transcript encoding *Hand2* was determined based on *in-situ* hybridization or RT-PCR according to our published procedures (Wu and Howard, 2002; Ruest et al., 2004, Liu et al., 2005; Hendershot et al., 2007). Briefly, E11 $Hand2^{fl/+}$ and $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos were emersion fixed in 4% paraformaldehyde, washed in PBS, cryoprotected in 30% sucrose, embedded in Cryo-gel™ (Instrumedics, Inc., St. Louis, MI) and sectioned (20 μm) using a Leica 1800 cryostat. A digoxigenin-labeled *Hand2* riboprobe was hybridized at 65 °C and detected following color development using NBT/BCIP or BM purple AP (Roche Diagnostics Corp., Indianapolis, IN). To determine quantitatively the levels of *Hand2* transcript expression, total RNA was purified from the jaw of E11 $Hand2^{wt/wt}$, $Hand2^{flneo/+}$ and $Hand2^{flneo/flneo}$ embryos using the RNeasy Mini kit (Qiagen Inc. Valencia, CA) according to the manufacturer's directions. We employed real time RT-based PCR, using β-actin as internal control. The relative levels of transcript encoding *Hand2* were calculated from the difference in CT values for *Hand2* and compared to those for β-actin; a detailed description of the protocol as well as the equations can be found in Liu et al. (2005).

Immunocytochemistry

The antibodies used for the studies presented in this manuscript are detailed in Table 1. Embryos were emersion fixed in 4% paraformaldehyde overnight at 4 °C, extensively washed in PBS and stored in 30% sucrose unless otherwise stated. Immunostaining was done according to our established procedures (Howard et al., 1999, Wu and Howard, 2002, Liu et al., 2005, Hendershot et al., 2007). Tissue sections were blocked in a solution containing 0.1 M Tris, pH. 7.5, 1.5% NaCl, 0.3% Triton X-100 and 10% horse serum for 3×10 min. Primary antibody is applied in this same solution but containing 4% horse serum and incubated overnight at 4 °C. Following washing in 0.1 M Tris pH 7.5, 15% NaCl, secondary antibody was applied in this same solution with 4% horse serum for 3 h at room temperature. Sections are then washed 3×5 min in 0.1 M Tris, pH 7.5, 15% NaCl and mounted in Vectashield (Vector Laboratories, Burlingame, CA) or Fluoromount-G (Southern Biotech, Birmingham, Al). To view sites of antibody binding in whole embryos (Young et al., 1999; Hendershot et al., 2007) samples were incubated overnight in PBS containing 0.3% Triton X-100 with gentle shaking at 4 °C. We used our standard blocking step and primary antibody was applied as described above and incubated for two days at 4 °C with gentle shaking. Following extensive washing secondary antibody was applied and embryos incubated overnight at 4 °C with shaking. Whole embryos were mounted on depression slides in Fluoromount-G (Southern Biotech, Birmingham, AL). For zebrafish, embryos were processed for

Table 1
Antibodies

Antigen/ antibody	Dilution	Secondary antibody	Source
Gata3 (rabbit)	1:1000	Biotin conjugated goat anti-rabbit	Gift of James Douglas Engel, University of Michigan
GFP (chicken)	1:500	Donkey anti-chicken FITC Goat anti-chicken FITC	Aves Labs Inc., Tigard, OR
GFP (rabbit)	1:500	Donkey anti-rabbit FITC Goat anti-rabbit FITC	Molecular Probes, Eugene, OR
Hu (human)	1:25,000	Goat anti-human Cy2 Donkey anti-human FITC Donkey anti-human TRITC	Miles Epstein University of Wisconsin, Madison Wisconsin
Ki67 (rabbit)	1:300	Donkey anti-rabbit FITC Goat anti-rabbit FITC	Abcam, Cambridge, MA
Mash1 (mouse)	1:50	Biotin conjugated goat anti-mouse	BD Pharmingen, San Jose, CA
Phox2a (rabbit)	1:1000	Biotin conjugated goat anti-rabbit	J-F. Brunet, École Normale Supérieure Paris, France
Phox2b (rabbit)	1:1000	Biotin conjugated goat anti-rabbit	J-F. Brunet, École Normale Supérieure Paris, France
Sox10 (goat)	1:50	Donkey anti-goat TRITC Donkey anti-goat FITC	Santa Cruz, Santa Cruz, CA
TH (rabbit)	1:200	Donkey anti-rabbit TRITC Goat anti-rabbit TRITC	Pel Freez, Rogers, AK
Neuron-specific β-tubulin/TuJ1 (rabbit)	1:500	Donkey anti-rabbit TRITC	Covance, Berkeley, Ca

immunocytochemistry as previously described (Raible and Kruse, 2000). Differentiated neurons were revealed with the anti-Hu mAb 16A11 (Molecular Probes) (Marusich et al., 1994). Tyrosine Hydroxylase (TH) expressing neurons were identified with an anti-TH mouse monoclonal antibody (Chemicon) (An et al., 2002). Both mAbs were visualized using an Alexa Fluor 568 anti-mouse IgG antibody (Molecular Probes).

Confocal microscopy

Confocal images were captured using an Olympus BX51WI upright microscope equipped with a laser scanning Bio-Rad Radiance 2000 Imaging System. To acquire images, fluorescent FITC-coupled and TRITC-coupled secondary antibodies were excited using argon and HeNE laser lines, respectively. Confocal *Z*-stacks were obtained at 10× magnification (n.a. = 0.3). Images were acquired, and *Z*-stack projections were made using Bio-Rad LaserSharp imaging software. Images were montaged in Adobe Photoshop.

Statistics

Data are presented as the mean ± S.E.M unless otherwise stated. Statistical significance was determined using Student's unpaired *t* test or ANOVA and Bonferroni post hoc test, unless otherwise stated.

Results

Our *in-vitro* studies demonstrated that loss-of-function of *Hand2* manifests as both a significant reduction in the number of differentiated neural crest-derived neurons and as loss in expression of the cell type-specific markers, TH and norepinephrine (NE; Howard et al., 1999). In order to substantiate these conclusions and define the molecular basis for these effects we generated a conditional targeted mutation of *Hand2* (Hendershot et al., 2007). We bred homozygous floxed-*Hand2* (*Hand2*$^{fl/fl}$) mice for selective deletion of *Hand2* in neural crest-derived cells by crossing them with mice expressing Cre recombinase under control of the Wnt-1 promoter. It is well established that the Wnt1-Cre transgene allows floxed genes to be targeted in the neural crest (Danielian et al., 1998; Jiang et al., 2000; Brewer et al., 2004). Cre-mediated recombination in neural crest-derived cells was confirmed by crossing hemizygous Wnt1-Cre mice to R26R;YFP indicator mice (generous gift of Drs. F. Constantini, Columbia University and Miles Epstien, University of Wisconsin, Madison). Expression of YFP in neural crest-derived structures demonstrated the expected sites of Cre-mediated recombination (Figs. 1B and C). Deletion of *Hand2* was confirmed based on *in-situ* hybridization (Fig. 1D) and semi-quantitative RT-PCR (Fig. 1E). Loss of *Hand2* expression is embryonic lethal at around E12 likely due to lack of norepinephrine and cardiac defects (Srivastava et al., 1997; Lim et al., 2000). In order to determine the consequences of loss of *Hand2* beyond E12, we fed pregnant dams a cocktail of catecholamine intermediates (see Materials and methods for details) to produce pharmacological rescue of embryos (Lim et al 2000; Kaufman et al., 2003). These rescued embryos die at birth. The ability to generate embryos older than E12 allowed us to do a detailed analysis of the effects of *Hand2* knock-out that could not be completed in E12 or younger mutant embryos (Morikawa et al., 2007). Comparison of E14 *Hand2*$^{fl/fl}$ and *Hand2*$^{fl/fl;Wnt1\text{-}Cre}$ embryos (Fig. 1A) shows that loss of *Hand2* results in embryos that are smaller in size with readily apparent defects in craniofacial development. *Hand2*$^{fl/fl}$ mice are fertile and phenotypically normal suggesting that insertion of loxP sites does not affect expression of *Hand2*; we generate embryos in the expected Mendellian ratio (141 conditional knock-out embryos of 564 embryos generated from 69 litters). Wnt1-mediated knock-out of *Hand2* in neural crest-derived cells revealed effects of loss of *Hand2* in

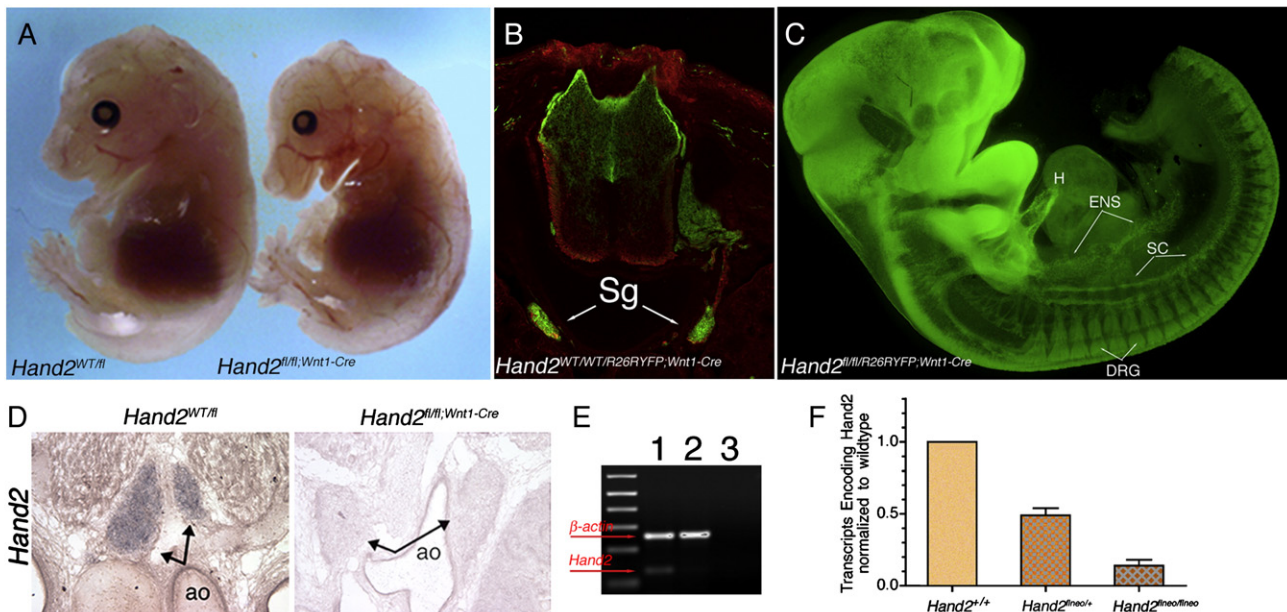

Fig. 1. Targeted deletion of *Hand2*. (A) *Hand2*$^{fl/fl;Wnt1\text{-}Cre}$ embryos are small compared to *Hand2*$^{wt/fl}$ E14 littermates. Visual inspection reveals craniofacial abnormalities including shortened mandible. We also observed malformations of craniofacial bone structure and defects in heart development (data not shown). (B) *R26R;Wnt1-Cre* reporter mice were generated to visualize the expression pattern of Wnt1 to confirm sites of *Hand2* deletion. Immunostaining for GFP (green) and TuJ1 (red) demonstrates sites of Wnt1-Cre-mediated excision in the dorsal spinal cord and the sympathetic ganglion (Sg) in a 20 μm frozen section from an E14 *Hand2*$^{wt/wt;R26R/Wnt1\text{-}Cre}$ mouse. (C) To visualize sites where Wnt1-Cre-mediated excision could be expected in our *Hand2* mutant embryos, we imaged an E10 *Hand2*$^{fl/fl;ROSAYFP/Wnt1\text{-}Cre}$ reporter embryo; YFP is expressed in all sites where Wnt1 is expressed. This three dimensional view allows visualization of 1) vagal neural crest-derived cells contributing to heart (H) development; 2) neural crest contributions to craniofacial structures; 3) dorsal root ganglia (DRG); 4) the enteric nervous system (ENS) migratory stream and the sympathetic chain (SC). Since YFP remains after Wnt1 expression is extinguished, this pattern provides a visual history of neural crest cell migration and localization. (D) We confirmed targeted deletion of *Hand2* using *in-situ* hybridization with digoxygenin-labeled RNA probe specific for *Hand2*. Transcript encoding *Hand2* was not detected in sympathetic ganglia (black arrows) visualized in sections derived from *Hand2*$^{fl/fl;Wnt1\text{-}Cre}$ E16 embryos compared to robust expression in sympathetic ganglia in *Hand2*$^{wt/fl}$ embryos. (E) The absence of transcript encoding *Hand2* in *Hand2*$^{fl/fl;Wnt1\text{-}Cre}$-derived tissue was confirmed based on RT-PCR. Total cellular RNA was purified from the jaw of an E11 *Hand2*$^{fl/fl}$ (control) embryo (lane 1) and a *Hand2*$^{fl/fl;Wnt1\text{-}Cre}$ embryo (lane2) RT-PCR run as described in experimental procedures. Expression of transcript encoding β-actin was used to control for possible loading artifacts and demonstrates equivalent input. Transcript encoding *Hand2* is absent in tissue taken from mutant embryos (lane 3: no RT control). (F) RNA was purified from E11 *Hand2*$^{+/+}$, *Hand2*$^{+/-}$ and *Hand2*$^{flneo/flneo}$ jaw. qRT-PCR was used to assess expression of transcript encoding *Hand2*. The level of transcript encoding *Hand2* (normalized to *Hand2*$^{+/+}$) is reduced 50% in *Hand*$^{+/-}$ heterozygous embryos and 76% in *Hand2*$^{flneo/flneo}$ embryos.

all neural crest-derived structures dependent upon *Hand2* for early aspects of their development.

Our initial analysis revealed that in embryos where the neomycin cassette remained in the genome ($Hand2^{flneo/flneo}$), expression of *Hand2* was abnormal (Fig. 1F). We had anticipated the possibility of neomycin neighbor effects generating a *Hand2* hypomorphic allele. Indeed, homozygous $Hand2^{flneo/flneo}$ pups die perinatally, likely as a consequence of their inability to suckle. Gross analysis of E18 embryos revealed that all $Hand2^{flneo/flneo}$ embryos develop cleft secondary palate (data not shown). To confirm that *Hand2* expression was affected, the level of transcript encoding *Hand2* was examined in the jaw of E11 $Hand2^{flneo/flneo}$ embryos. Based on qRT-PCR, $Hand2^{flneo/flneo}$ embryos expressed only around 14% of transcript encoding *Hand2* compared to $Hand2^{wt/wt}$ littermates (Fig. 1F). As expected, $Hand2^{flneo/+}$ embryos expressed about 50% of *Hand2* transcript compared to wild type littermates. Previous studies have highlighted the importance of gene dosage for both Hand1 and *Hand2* in cardiac development (McFadden et al., 2005) and this study extends this finding. The phenotypic characteristics we describe in both $Hand2^{fl/fl;Wnt1\text{-}Cre}$ and $Hand2^{flneo/flneo}$ embryos supports previous data suggesting that *Hand2* protein function is dosage dependent (McFadden et al., 2005). The more severe cardiac and craniofacial phenotypes (data not shown) we observed in the *Hand2* hypomorph embryos compared to embryos where *Hand2* was deleted in neural crest-derived cells underscores the complex functional interactions that the Twist family of bHLH proteins exhibits in-vivo (Firulli et al, 2003, 2005); our data indicate a complex pattern of interaction between different cell types that express *Hand2*. Interestingly, examination of the sympathetic chain ganglia in *Hand2* hypomorphs did not show a significant reduction in the number of differentiated neurons. However, there was a trend toward a reduction in the number of cells expressing TH (data not shown). This suggested that both penetrance and gene dosage might contribute to the degree of phenotypic anomaly developing as a consequence of *Hand2* misexpression.

Introduction of loxP sites into the genome flanking a portion of the *Hand2* coding sequence does not affect transcription providing an essentially wild type control for knock-out littermates. This is in contrast to another line of floxed *Hand2* mice where insertion of loxP sites does affect *Hand2* transcription (Morikawa et al., 2007). Because of the acknowledged importance of gene dosage in Hand gene function, the *Hand2* hypomorph allele will provide a valuable tool for future examination of phenotypic response to loss of *Hand2* as well as interaction of *Hand2* with other transcriptional regulators.

Deletion of Hand2 in neural crest affects development of sympathetic chain ganglia

A large body of data suggest that *Hand2* might mediate some aspects of the intracellular responses to BMP-mediated signaling required for neural crest-derived precursors of noradrenergic neurons to differentiate where they ganglionate around the dorsal aorta (Schneider et al., 1999; Howard et al., 2000; Liu et al., 2005; Sarkar and Howard, 2006). Two aspects of development of noradrenergic sympathetic ganglion neurons attributed to *Hand2* are neurogenesis and cell type-specific gene expression (Howard et al., 1999, 2000; Xu et al., 2003; Rychlik et al., 2003). To determine whether *Hand2* is functional in both of these aspects of noradrenergic neuron development in-vivo, we counted the number of cells expressing the pan-neuronal marker Hu and the catecholaminergic marker TH present in the sympathetic chain ganglia of $Hand2^{fl/fl}$ and of $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos (Fig. 2). Using serial 20 μm cross-sections, the number of Hu^+ and TH^+ cells was counted in every section over a distance covering 3 to 5 ganglia in E10 through E18 embryos. The sympathetic ganglia begin to form shortly after neural crest cells initiate migration around E8.5 and by E9.5 ganglionic neurons begin to express pan-neuronal (Hu) and cell type-specific (TH) markers. To insure that comparable axial levels were being assessed, samples from $Hand2^{fl/fl}$ and $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos were matched based on landmarks including the forelimb, liver and vertebrae.

At E12 when both neuronal and cell type-specific makers are expressed along the rostral–caudal extent of the neural axis, there is a significant reduction in the number of cells expressing either Hu or TH as well as a comprehensive decrease in the proportion of cells expressing both markers (Figs. 2A–D). With increasing developmental age (E18), there was a continual decrease in both neurons and expression of TH (Figs. 2E–H). At P0 a few scattered cells remain that express TH and TuJ1 but the sympathetic chain is absent. Expression of TH and TuJ1 in the locus coeruleus and midbrain noradrenergic centers (Supplementary Fig. 1) appear comparable in the P0 $Hand2^{fl/fl;Wnt1\text{-}Cre}$ mice (inset) compared to $Hand2^{fl/fl}$ mice suggesting that the effect in the periphery is specific to loss of *Hand2*. To determine if these *Hand2* functions are evolutionarily conserved we assessed the expression of Hu and TH in the zebrafish *hands off* mutant fish that have a deletion of the *Hand2* gene (Yelon et al., 2000). At 96 hpf we observed a clear reduction in the number of cells expressing both Hu and TH in the *hands off* mutant embryos as compared to wild type siblings (Supplementary Fig. 2). These results are intriguing as it has been reported that loss of *Hand2* in the zebrafish results in decreased expression of transcripts encoding TH but has no effect on expression of transcripts encoding Hu (Lucas et al., 2006). A potential explanation for these conflicting results may be the different experimental endpoints and method of analysis used in our study as compared to the previous one. Together our results suggest that *Hand2* has a maintenance function in addition to a role in neurogenesis and cell type-specific gene expression. Ascribing a function to *Hand2* in neurogenesis presented a challenge since there was not a comprehensive loss of neurons initially as the sympathetic chain ganglia develop either in murine or fish embryos (Lucas et al., 2006; Morikawa et al., 2007; D'Autrèaux et al., 2007).

To address this issue we first examined patterns of neural crest cell migration (Figs. 3A, B). We did not anticipate an effect on neural crest cell migration, as there is no evidence that *Hand2* is expressed in migrating neural crest-derived cells. Using *Sox10* as a marker of neural crest cells we compared the expression pattern in $Hand2^{fl/fl}$ (Fig. 3A) and $Hand2^{fl/fl;Wnt1\text{-}Cre}$ (Fig. 3B) embryos at E10. We reconstructed midline views from 1 μm optical sections and compared the localization of $Sox10^+$ cells in dorsal root ganglia (DRG), trigeminal ganglia (TG), and sympathetic ganglia. Neural crest-derived cells migrated to each of these structures and there was no apparent difference in the development of the dorsal root or trigeminal ganglia. We confirmed that deletion of *Hand2* does not affect neural crest cell migration by analyzing YFP expression in $Hand2^{fl/fl;Wnt1\text{-}Cre}$;R26RYFP reporter E10 embryos (Figs. 1C and 3C). The embryo shown (Fig. 1C) was photographed in whole-mount making the three dimensional pattern of neural crest cell migration readily visible. In addition to extensive migration in the head, migration into the dorsal root ganglia (DRG), sympathetic chain (SC), branchial arches, heart (H) and the migratory stream into the gastrointestinal tract (ENS) appear normal. Cell counts showed that comparable numbers of YFP^+ cells migrated and localized around the dorsal aorta in R26R/Wnt1-Cre reporter embryos compared to $Hand2^{fl/fl;R26R/Wnt1\text{-}Cre}$ embryos (Figs. 3C and 8). On average there were 18.7±0.64 YFP^+ cells per R26R/Wnt1-Cre reporter ganglion section compared to 16.9±0.9 YFP^+ cells in the $Hand2^{fl/fl;R262RYFP;Wnt1\text{-}Cre}$ E10 embryos (Fig. 3D). Similarly, the number of cells expressing *Sox10* was equivalent (10.6±0.85 vs. 9.4±0.71) in the reporter compared to the *Hand2* knock-out reporter embryos. To confirm that ganglia development was affected in the $Hand2^{fl/fl;R26R/Wnt1\text{-}Cre}$ reporter embryos, we also counted the number of cells expressing Hu (Figs. 3C, D). As expected, there was a significant reduction in the number of Hu^+ cells in the $Hand2^{fl/fl;R26R/Wnt1\text{-}Cre}$ embryos (3.1±0.59 Hu^+ cells) compared to the R26R/Wnt1-Cre reporter embryos (11.7±1.1 Hu^+ cells). In total, there is no evidence that loss of *Hand2* expression impacts neural crest

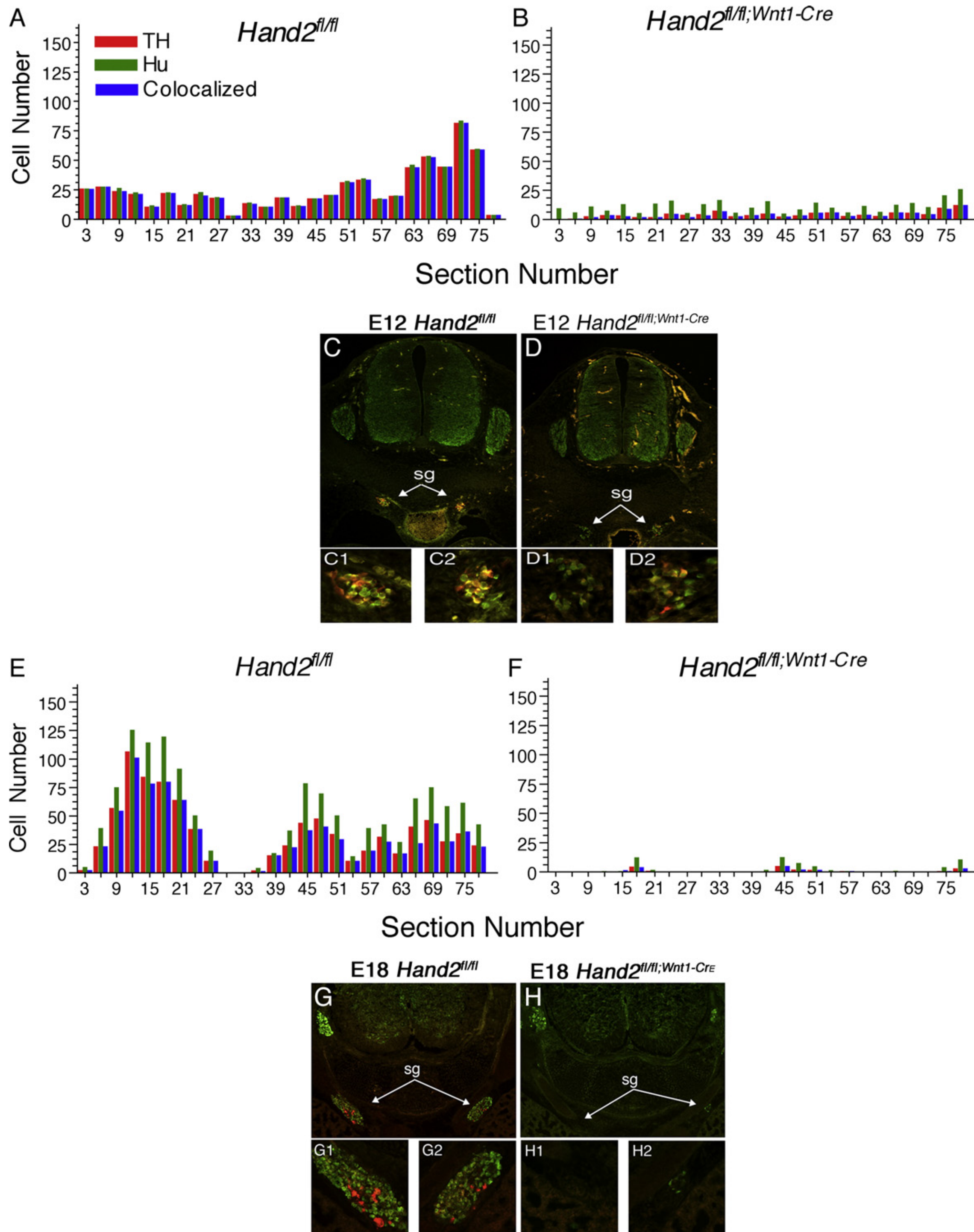

Fig. 2. Conditional deletion of *Hand2* affects development of sympathetic chain ganglia. (A) The number of cells expressing TH and Hu were counted in 84 consecutive tissue sections from E12 *Hand2*$^{fl/fl}$ (control) embryos and compared to [panel B] similar sections obtained from *Hand2*$^{fl/fl;Wnt1\text{-}Cre}$ mutant embryos. There was a significant decrease in cells expressing the pan-neuronal marker Hu (green) and cells expressing the catecolaminergic marker TH (red). Co-localization of TH in Hu expressing cells (blue) was also significantly decreased in mutant embryos suggesting that both neurogenesis and cell type-specific gene expression are compromised by deletion of *Hand2*. Each bar represents the mean value of counts from three consecutive sections. Visual inspection of immunolabeled sympathetic chain ganglia (SG) in control (C) and mutant (D) thoracic level sections shows a significant decrease in size of ganglia as well as significant loss of Hu and TH immunoreactivity (IR). C1–D2 show higher power views of SG shown in panels C and D. A similar, albeit more severe, loss is demonstrated in E18 embryos (E–H). Cell counts and data presentation are as described above for E12 embryos. Panels C, D, G, and H were photographed at 10× magnification. Panels C1–H2 were photographed at 40– magnification. In addition to the impact of *Hand2* on differentiation of SG, these data confirm previous studies (Kasemeier-Kulesa et al., 2005) showing that neural crest-derived cells initially form a continuous line of cells along the dorsal aorta (A, B) and only later in development do discrete ganglia form (E, F). Although few cells are evident at E18, the few that remain distribute normally.

cell migration. Since the neural crest-derived progenitor cells clearly reach their final destination along the dorsal aorta where they will form the sympathetic chain ganglia but where neuronal differentiation is already affected by loss of *Hand2*, we next asked whether deletion of *Hand2* affected the number of progenitor cells present in the anlagen of the sympathetic chain ganglia.

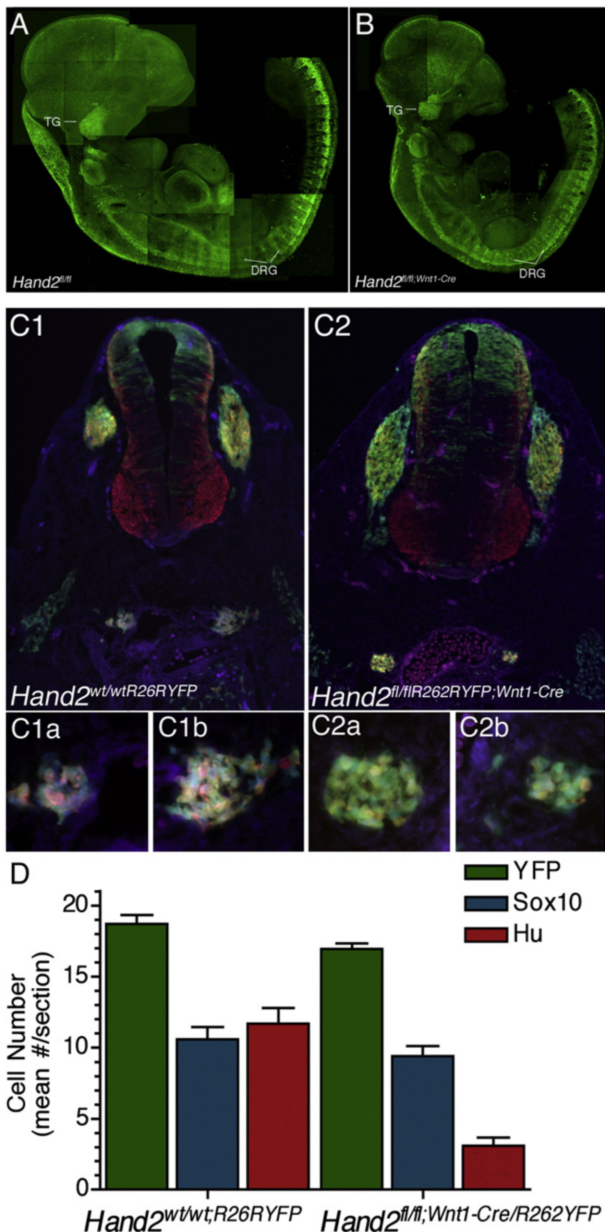

Fig. 3. Neural crest-derived cell migrate normally in the absence of *Hand2*. To ask whether neural crest-derived cells migrated normally and reached their final sites of localization in the trigeminal ganglion (TG), dorsal root ganglia (DRG) and sympathetic chain we used whole-mount immunostaining with a *Sox10* antibody. To visualize sites of IR, 1 μm serial confocal images were reconstructed from (A) $Hand2^{fl/fl}$ and (B) $Hand2^{fl/fl;Wnt1\text{-}Cre}$ E10 embryos. Neural crest-derived cells migrate normally in the absence of *Hand2*. (C) To demonstrate that $Hand2^{fl/fl;R26R/Wnt1\text{-}Cre}$ reporter embryos have analogous phenotypic characteristics to $Hand2^{fl/lf;Wnt1\text{-}Cre}$ embryos, E10 reporter and reporter *Hand2* mutant embryos were fixed and expression of *Sox10* (blue), GFP (green) and Hu (red) was examined in tissue sections. Migration and neuronal differentiation was determined by counting the number of cells expressing each of these markers in serial 20 μm sections. The average number of cells expressing each marker over a distance of 3–5 somites was compared (D); data from three embryos is presented as mean ± S.E.M. The number of cells expressing YFP (detected with anti-GFP antibody) or *Sox10* was equivalent in control and mutant embryos suggesting neither a loss of neural crest-derived cells nor an effect on migration. The significant reduction in the number of cells expressing Hu indicates that *Hand2* is excised in the $Hand2^{fl/fl;R26RYFP/Wnt1\text{-}Cre}$ embryos. Tissue sections [C1 and C2] were photographed at 10× magnification and panels C1a–C2b were photographed at 40× magnification.

Hand2 maintains the neural precursor pool of cells

As a means to understand the mechanism of *Hand2* function in neurogenesis, we asked whether the significant decrease in noradrenergic sympathetic ganglion neurons we observe in *Hand2* mutant embryos is due to early onset cell death or alterations in the proliferation of the neural precursor pool of cells. We did not find increased cell death either by TUNEL or activated caspase 3 immunoreactivity (IR); at E10 there was little if any cell death in the anlagen of the sympathetic chain ganglia (data not shown). However, there was a significant effect on cell proliferation in response to loss of *Hand2* (Fig. 4). Initially we compared the number of cells expressing *Sox10*, as a marker of neural crest-derived progenitor cells, located around the dorsal aorta in E10 embryos. The number of cells in the forming sympathetic ganglia expressing *Sox10* IR was counted in 20 μM sections over the entire length of the neural axis. The mean number of $Sox10^{+}$ cells per ganglion section in $Hand2^{fl/fl}$ embryos was 14.3 ± 0.3 compared to 13 ± 1.4 cells per ganglion section in $Hand2^{fl/fl;\mathrm{Wn1\text{-}Cre}}$ embryos. As expected, there was no difference in the numbers of $Sox10^{+}$ cells in the normal compared to the mutant embryos supporting our previous conclusion that neural crest cell migration is not effected by the loss of *Hand2* expression. Interestingly, there was a significant ($P<0.001$) difference in the number of $Sox10^{+}$ cells that also expressed the proliferation marker, Ki67. On average, 87 ± 6% of $Sox10^{+}$ cells were proliferating per ganglion section in control embryos compared to 8 ± 2% of $Sox10^{+}$ cells in the mutant ganglion sections (Figs. 4C, D). Since sympathetic neuroblasts undergo several rounds of cell division before they are born (Rothman et al., 1978; Rohrer and Thoenen, 1987), we counted the number of cells expressing the neuronal maker Hu and the proliferation marker Ki67 in E10 embryos (Figs. 4A, B); for these cell counts, alternate sections from the same embryos were immunostained for Hu and Ki67 or *Sox10* and Ki67. Of the cells expressing Hu (13.4 ± 1 wt vs. 6.6 ± 1 mutant) an average of 51 ± 10% of control but only 13 ± 0.6% of mutant cells also express Ki67 (range control = 29% to 70% and mutant = 3% to 12%). This significant ($P<0.01$) reduction in the proportion of proliferating cells in the mutant embryos suggests that the decrease in neuron numbers is due to lack of generation of neuronal precursor cells as well as a reduction in the proliferation capacity of neuroblasts. Because we observed a progressive decrease in the number of cells expressing Hu and TH in the mutant, we assessed the expression of *Sox10*, Ki67 and Hu at E12 to determine whether there was also a progressive loss of proliferating cells in either the $Sox10^{+}$ pool or in the neurons (Figs. 4E–H). We observe a significant ($P<0.05$) reduction in the number of cells expressing *Sox10* per ganglion section between E10 and E12 in both control and mutant embryos (Figs. 4G, H). From E10 to E12 there is also a shift in the population of cells expressing the proliferation marker Ki67 from the pool of $Sox10^{+}$ cells. At E12, less than 2% of cells co-express Ki67 and *Sox10*, in either the control or mutant (Figs. 4G, H). Interestingly, there is a significant increase in the percentage of cells that co-express Hu and Ki67 (Figs. 4E, F) in control embryos, with 79.8 ± 7.1% of Hu^{+} cells also expressing Ki67 compared to 5.4 ± 0.23% of Hu^{+} cells in mutant sympathetic ganglia. This increase in cell proliferation does not represent a substantial increase in the number of Hu^{+} cells in control E12 ganglia compared to the number of cells present at E10. Already by E12, the number of proliferating neuroblasts has increased in the control and significantly decreased in the mutant. These studies indicate that loss of *Hand2* is reflected in a substantial change in the proliferative capacity of both neuroblasts (young neurons) and neuronal precursor cells in the developing sympathetic chain ganglia. Based on these results, we examined the expression of the transcription factors shown to be necessary for some aspect of the specification and/or differentiation of sympathetic noradrenergic neurons.

Feed-forward and feed-back transcriptional regulation

Hand2 is one member of a network of transcriptional regulators identified as sufficient and/or necessary for some aspect of neurogenesis or neurotransmitter specification/expression, both *in-vitro* and in-vivo, of autonomic noradrenergic neurons (Howard et al., 1999, 2000;

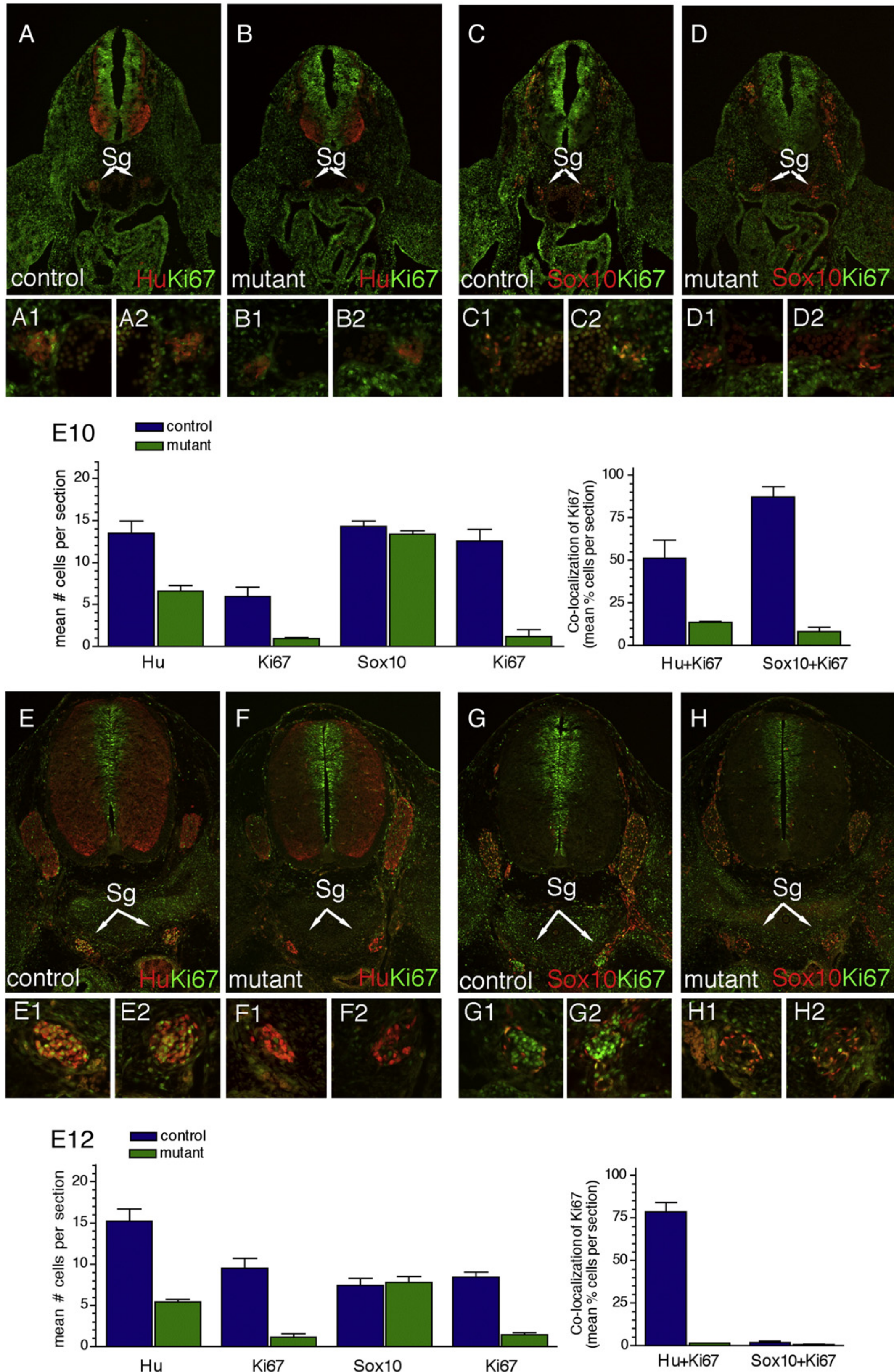

Fig. 4. Generation of neural precursor cells is affected by loss of *Hand2* expression. To determine the effect of *Hand2* on cell division (Ki67), neurogenesis (Hu) and precursor cells (*Sox10*), cells expressing Hu and Ki67 or *Sox10* and Ki67 were counted on consecutive sections over a 3 to 5 somite length at E10 (A–D) or E12 (E–H). Cell counts from 3 embryos in each condition were combined and presented as mean ± S.E.M. per tissue section. In addition to a significant decrease in the number of cells expressing Hu in the mutant, interestingly, the number of *Sox10*+ cells is not affected at E10 by loss of *Hand2*. However, the ability to generate *Sox10*+ cells is significantly decreased in the mutant. By E12, few if any *Sox10*+ cells are proliferating in mutant embryos. The proliferative capacity of young neurons is also significantly lower in the mutant cells compared to control. With increasing developmental age, the *Sox10*+ cells move to the periphery of the ganglion (compare C, D to G, H). Abbreviations: SG, sympathetic ganglion.

Liu et al., 2005; Müller and Rohrer, 2002; Moriguchi et al., 2006; Hendershot et al., 2007; Morikawa et al., 2007). Because some neurons differentiate in the absence of *Hand2* in the developing sympathetic chain, it was important to determine the expression pattern of the other identified DNA binding proteins in this network. We reasoned that we would identify which, if any, of these DNA binding proteins had altered expression in response to loss of *Hand2*. We first counted the number of cells expressing the autonomic determination factor, *Phox2b* on serial 20 μm cross-sections of E10 mutant and control embryos. There was no significant difference at E10 in the number of cells expressing *Phox2b* in $Hand2^{fl/fl}$ compared to $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos (Figs. 7C, D) suggesting that loss of *Hand2* does not impact the initial number of neural progenitor cells that might differentiate into neurons. To confirm that *Hand2* is expressed downstream of *Phox2b*, we examined *Hand2* expression in $Phox2b^{-/-}$ embryos (Fig. 5C). In the absence of *Phox2b*, transcripts encoding *Hand2* are not expressed in the anlagen of the sympathetic chain ganglia supporting a model where *Hand2* is genetically downstream of *Phox2b* (data for Fig. 5C was graciously provided by Christo Goridis).

Gain-of-function studies in avian embryos suggested that *Hand2* could induce expression of *Phox2a* (Howard et al., 2000) and time of appearance of transcript encoding *Phox2a* in cells localized around the dorsal aorta placed *Phox2a* downstream of *Hand2* (Howard et al., 2000). We confirmed this expression pattern by asking whether expression of *Phox2a* in the absence of *Phox2b* could support expression of *Hand2* by using the knock-in $Phox2b^{KIa}$ mouse line (Coppola et al., 2005). We thus posited that expression of *Hand2* would be reduced or absent in the mutant embryos (Figs. 5A, B). The absence of transcript encoding *Hand2* in the $Phox2b^{KIa}$ embryos (Fig. 5A) suggests that *Hand2* is genetically upstream of *Phox2a*. Our previous functional studies also placed *Hand2* upstream of *Phox2a* (Liu et al., 2005). Re-examination of the epistatic relationship between *Phox2b*, *Hand2* and *Phox2a* was important because of the potential impact of *Hand2* expression on *Phox2a* and subsequently *Gata3*, Hu and TH.

We found that in $Hand2^{fl/fl;Wnt1\text{-}Cre}$ E10 embryos there is a significant (57%, $P<0.01$) reduction in the number of cells expressing *Phox2a* in the developing sympathetic ganglia compared to $Hand2^{fl/fl}$ embryos (Figs. 6A, B). As development proceeds, there is a substantial reduction of cells expressing *Phox2a* in $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos (Figs. 6D, F, H) compared to normal littermates (Figs. 6C, E, G). The fact that some cells do express *Phox2a* raised the possibility that initial expression of *Phox2a* is not induced by *Hand2* in all cells that express *Phox2a* or that some neurons dependent upon Phox2 proteins develop independently of *Hand2*. We tested this possibility in two ways. Firstly, we demonstrated that expression of *Ascl1* (Mash1) is not affected by loss of *Hand2* (Figs. 7A, B) indicating that initial expression of *Phox2a* may require only *Phox2b* and *Ascl1*. However, it is important to note that the number of cells expressing *Phox2b* is significantly decreased by E12 in *Hand2* mutant embryos and by E16 the number of cells expressing *Phox2b* is substantially reduced in $Hand2^{fl/fl/Wnt1\text{-}Cre}$ embryos (Supplementary Fig. 3); we find that *Phox2b* expression is not extinguished in $Hand2^{fl/fl}$ embryos throughout development. One testable hypothesis that emerged from these data was that there are neurons in the sympathetic chain ganglia which do not depend upon *Hand2* but which are dependent upon other members of the "noradrenergic" DNA binding protein network. Examination of the expression of *Gata3* in *Hand2* mutant embryos supports this idea.

Expression of *Gata3* has been shown to affect both neurogenesis and expression of TH in sympathetic neural precursor cells (Lim et al., 2000; Tsarovina et al., 2004; Moriguchi et al., 2006). We reasoned that if *Gata3* is required for neurogenesis and dependent upon expression of *Hand2*, that we should observe an effect on expression of *Gata3*, Hu and TH in response to loss of *Hand2*. We posited that we could distinguish between a dual function of *Hand2* in both neurogenesis and neurotransmitter expression from a single role in determining neurotransmitter identity by demonstrating an early reduction in cells

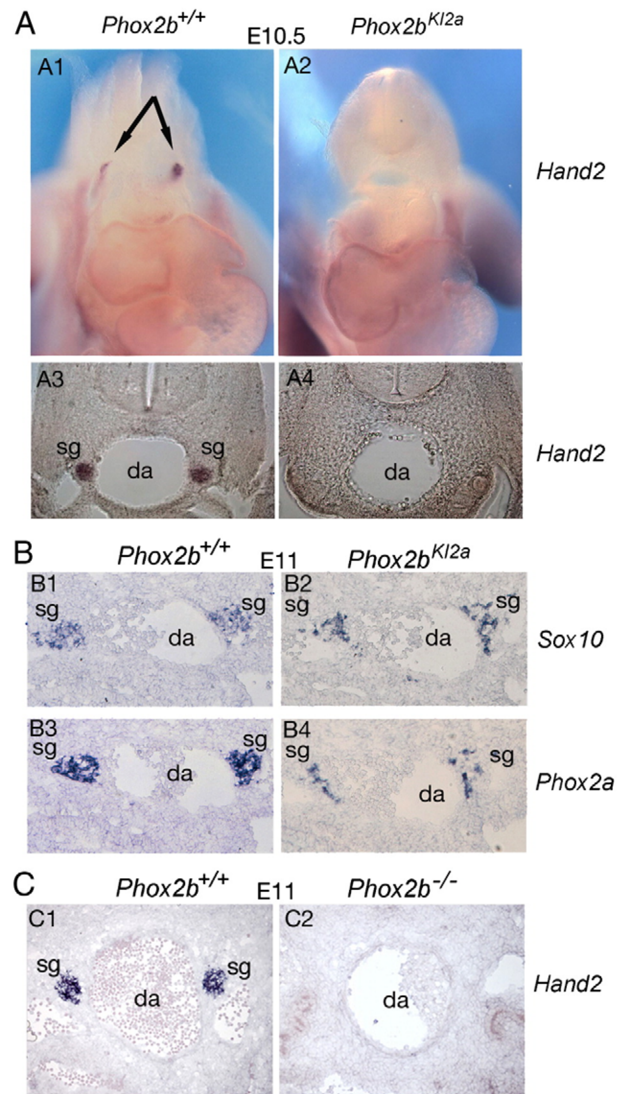

Fig. 5. *Hand2* is not expressed in $Phox2b^{Ki2a}$ or $Phox2b^{LacZ/LacZ}$ embryos. (A) *In-situ* hybridization, using a specific *Hand2*-digoxygenin-labeled ribroprobe was used to ask if transcript encoding *Hand2* was expressed in *Phox2a* knock-in mice (Coppola et al., 2005). In these mice, *Phox2a* is expressed from the *Phox2b* promoter. Transcript encoding *Hand2* is expressed in control embryos (A1, A3) but is absent in $Phox2b^{KI2a}$ embryos (A2, A4). (B) Comparison of the expression patterns of *Sox10* in *Phox2b* wild type E11 (B1) sympathetic ganglia (sg) to $Phox2b^{Ki2a}$ (B2) demonstrates that neural crest-derived cells localize around the dorsal aorta in the absence of *Phox2b*. *Phox2a* is expressed in the *Phox2b* domain (B4 compared to B3) around the dorsal aorta (da) but in the absence of *Phox2b*, transcripts encoding *Hand2* are not expressed (C2 compared to C1). This places *Hand2* upstream of *Phox2a* and downstream of *Phox2b*.

which differentiated as neurons. Using 10 μm serial cross-sections, we counted cells expressing *Gata3*, Hu, TH and *Sox10* at E10 (Fig. 8 top) and E12 (Figs. 8A–E) in $Hand2^{fl/fl}$ embryos and compared this to samples from $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos. At E10 there is no difference in the number of cells expressing *Sox10* in control (11±0.4) compared to mutant (11±0.4) embryos (Fig. 8 top). This suggests that any effect on the number of cells expressing Hu, TH or *Gata3*, could be ascribed to a function of *Hand2*. We observed a significant reduction in the number of cells expressing Hu ($P<0.05$), TH ($P<0.05$) and *Gata3* ($P<0.001$) when cell counts from mutant embryos were compared to cell counts from control embryos (Fig. 8). On average there are eleven neurons per section in $Hand2^{fl/fl}$ embryos and five neurons in $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos. Of these neurons, on average seven in $Hand2^{fl/fl}$ and two in $Hand2^{fl/fl;Wnt\text{-}Cre}$ embryos expresses TH. The percentage (70% vs. 30%) of the Hu^+ that are also TH^+ is significantly reduced ($P<0.01$) by loss of

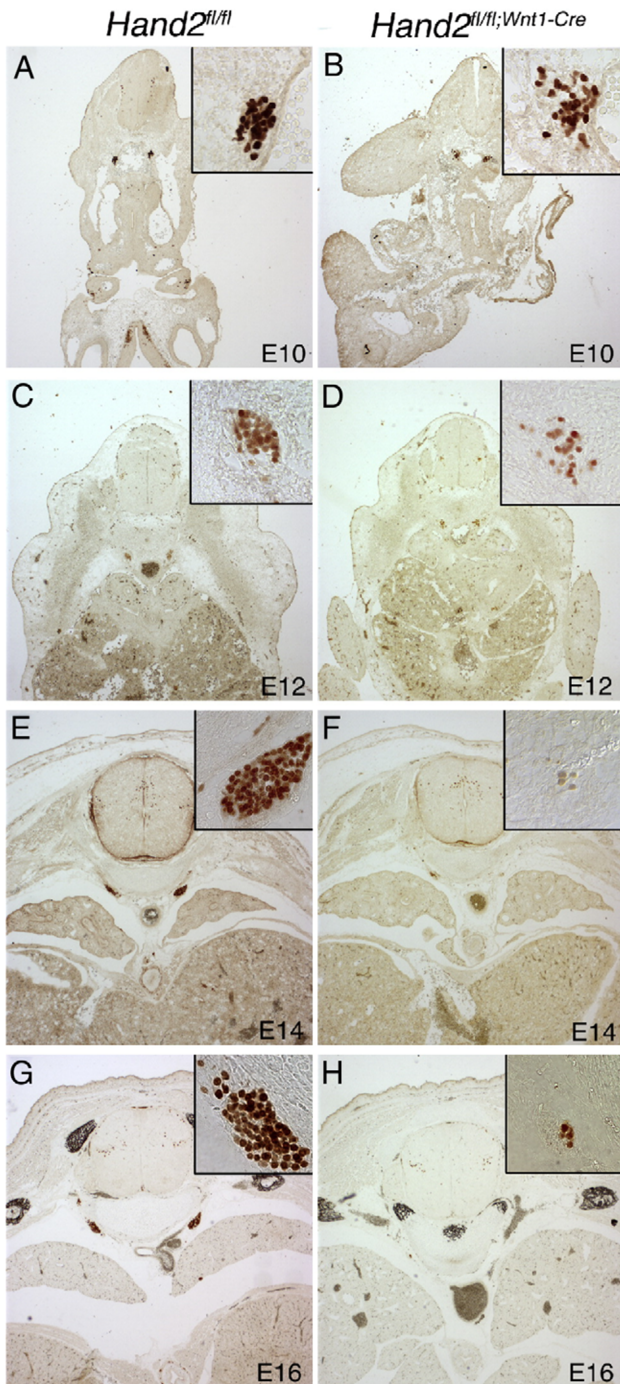

Fig. 6. Expression of *Phox2a* is affected by deletion of *Hand2* in neural crest-derived cells. Matched tissue sections from control (A, C, E, G) and *Hand2* mutant (B, D, F, H) embryos were immunolabeled using an anti-*Phox2a* antibody at E10 (A, B), E12 (C, D), E14 (E, F) and E16 (G, H). Comparison of mutant and control sympathetic ganglia demonstrates a progressive decrease in the number of cells expressing *Phox2a* in the mutant embryos. Bright field images were taken at 4× magnification and the inset images of sympathetic ganglia were taken at 40× magnification.

Hand2. These results demonstrate that in the absence of *Hand2* both neurogenesis and expression of catecholaminergic marker genes are affected in mice.

These data indicate that loss of *Hand2* impacts both neurogenesis (Hu) and cell type-specific gene expression (TH). Further, this analysis indicates that *Gata3* expression impacts neurogenesis to a greater degree than it does neurotransmitter marker gene expression. Interestingly, our data indicate that there is a population of neurons in the sympathetic chain that develop independent of *Hand2*, potentially accounting for the presence of a small cohort of neurons which differentiate in the sympathetic anlagen in $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos. This conclusion is supported by analysis of E12 embryos (Figs. 8A–E). At E12, there remains a significant decrease in the number of cells expressing *Gata3* (19.03 ± 0.6 vs. 8.4 ± 0.4; $P < 0.001$), Hu (19.86 ± 1.0 vs. 8.6 ± 0.5; $P < 0.001$) and TH (19.67 ± 1.1 vs. 2.4 ± 0.2; $P < 0.001$) in the mutant embryos compared to control embryos. Considering that all neurons appear to express *Gata3*, but greater than 50% of those cells do not express TH (at either E10 or E12), our data indicate that it is those neurons that develop independent of a requirement for *Hand2*. Our data suggest *Gata3* is expressed genetically downstream of *Hand2* in *Hand2*-dependent neural precursor cells (Tsarovina et al., 2004; Moriguchi et al., 2006).

Discussion

The goal of the current study was to establish a genetic model that would allow us to determine the function of *Hand2* in generation of sympathetic noradrenergic neurons. Studies *in-vitro* (Howard et al., 1999, 2000; Liu et al., 2005) and in-vivo (Howard et al., 2000; Lucas et al., 2006; Hendershot et al., 2007) implicated *Hand2* as a pivotal transcriptional effector mediating some aspects of neural development and expression of the catecholaminergic marker gene TH and the noradrenergic marker gene DBH in the generation of noradrenergic sympathetic ganglion neurons. Using pharmacological intervention (Lim et al., 2000; Kaufman et al., 2003) to rescue embryos where *Hand2* was excised in neural crest-derived cells we were able to analyze in some detail the consequences of loss of *Hand2* in sympathetic

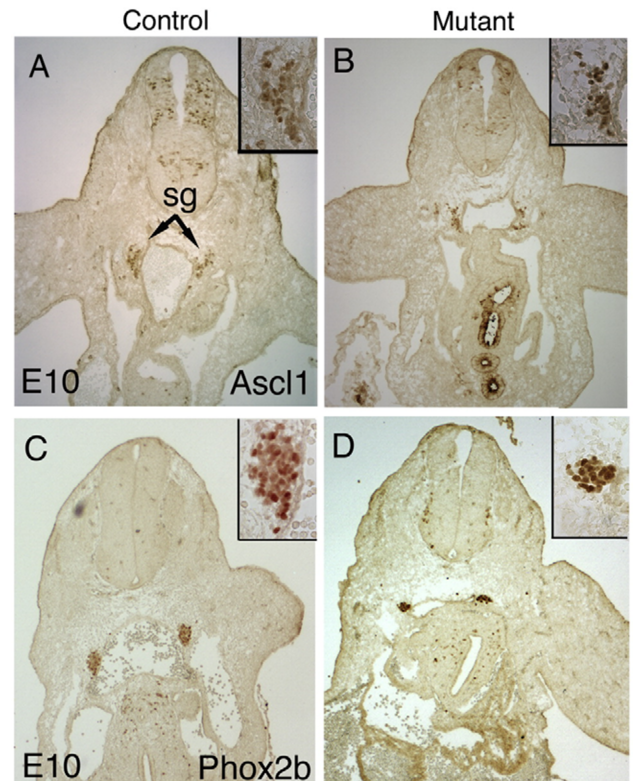

Fig. 7. Expression patterns of *Ascl1* and *Phox2b* in control and *Hand2* mutant embryos. Expression of *Ascl1* (A, B) and *Phox2b* (C, D) was examined in matched cross-sections from E10 control (A, C) or *Hand2* mutant (B, D) embryos. No difference in initial expression of these DNA binding proteins was evident. Expression of *Ascl1* was downregulated in both control and mutant embryos by E12. Expression of *Phox2b* is maintained through P0 in control animals (data not shown). At E18, the few neurons that remain in the mutant embryos maintain expression of *Phox2b* (data not shown). Tissue sections were imaged at 10× magnification and inset images were taken at 40× magnification.

ganglia anlagen. Based on the data presented here we suggest that *Hand2* is a multifunctional transcriptional regulator affecting neurogenesis and cell type-specific gene expression in autonomic noradrenergic neurons. This conclusion is at odds with a recent report published while this manuscript was in preparation (Morikawa et al., 2007).

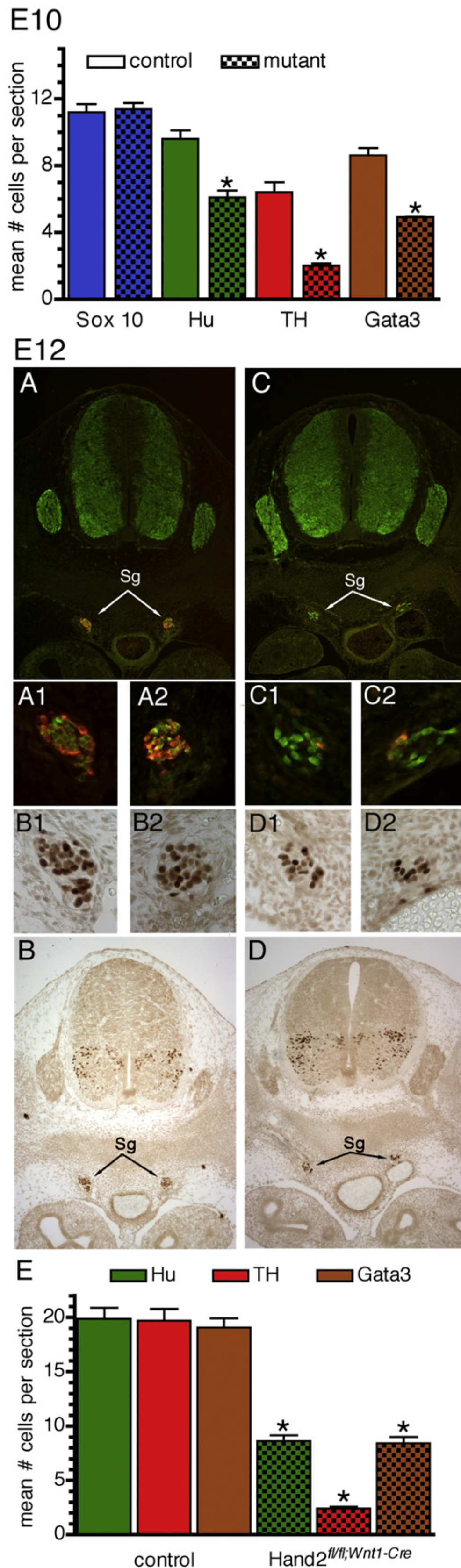

Fig. 8. The number of cells expressing *Gata3* is reduced in *Hand2* mutant embryos. The number of cells expressing *Sox10* (blue), Hu (green), TH (red) and *Gata3* (brown) was counted on serial 10 μM frozen tissue sections from control (open bars) and mutant (hatched bars) embryos at E10 (top graph) and E12 (A–E). At E10, Triple IR for *Sox10*, Hu and TH was used for cell counts; *Gata3* IR was done on the sister sections. For E12 embryos, sections were doubled labeled for Hu and TH and *Gata3* IR was done on the sister sections. Cells aggregated next to the dorsa aorta were counted. The mean number of cells per tissue section expressing each marker from compiled counts shows a significant reduction in neurons as well as a significant reduction in cells that express TH in the absence of *Hand2*. The number of cells expressing *Gata3* is significantly reduced in *Hand2* mutant embryos at E10 and E12. Interestingly, the number of cells expressing *Gata3* more reflects the number of neurons (Hu, green) than it does the number of neurons that also express TH (red). The pattern of *Gata3* IR in the neural tube and sympathetic chain ganglia coincides with the expression pattern reported for mRNA encoding *Gata3* or the LacZ expression pattern in a *Gata3*/LacZ knock-in mouse line (Lakshmann et al., 1999; Smith et al., 2002). Data are presented as the mean ± S.E.M. from three control and three mutant embryos counted over a distance of three to five sympathetic ganglia for E10 and E12. The anti-*Gata3* antibody was kindly provided by J.D. Engel, University of Michigan.

Hand2 functions in neurogenesis is to maintain the precursor pool of cells

Neurogenesis is a multistep process, some steps of which can be interrogated and others for which there are currently no definitive markers. We have taken advantage of available markers of neural differentiation (Hu and neuron specific β-tubulin (TuJ1)) and neurotransmitter expression (TH) to assess the effect of loss of *Hand2* function in neural crest-derived cells that will localize around the dorsal aorta and give rise to sympathetic chain ganglia. The migration and localization of progenitor cells does not appear to be affected by loss of *Hand2* (and see Taylor et al., 2007; Morikawa et al., 2007; D'Autrèaux et al., 2007). At E10, the earliest time-point examined, neural crest-derived cells have migrated to the dorsal aorta and form a chain of *Sox10* expressing cells dispersed along the rostral to caudal extent of the developing neural axis. Thus, the pool of precursor cells potentially able to differentiate into noradrenergic neurons is generated in both $Hand2^{fl/fl}$ and $Hand2^{fl/fl;Wnt1\text{-}Cre}$ embryos. However, the size of this pool is affected by loss of *Hand2*. Normally, neural crest-derived cells that express *Sox10* and are proliferating maintain the pluripotency of the precursor pool as well as assuring that an appropriate number of neurons will be generated (Kim et al., 2003). In the absence of *Hand2*, the number of *Sox10*-expressing cells that proliferate is significantly reduced, suggesting that *Hand2* has a role in cell cycle maintenance (Howard et al., 1999). Importantly, these data indicate that in the absence of *Hand2* *Sox10* no longer maintains neurogenic potential (Kim et al., 2003) in the precursor cells. Since the number of cells in the anlagen of sympathetic chain ganglia expressing *Sox10* is equivalent in control and mutant embryos, we posit that neurogenesis does not proceed efficiently in the absence of *Hand2* because the necessary pool of precursor cells with neurogenic potential is not generated and/or maintained. An alternative hypothesis is that there is a cohort of neurons that develop independent of *Hand2* (discussed below). One interesting aspect of neurogenesis in sympathetic chain ganglia is that young neurons continue to divide (Rothman et al., 1978, 1980; Rohrer and Thoenen, 1987; DiCicco-Bloom et al., 1990). We find that at E10, about 13% of the Hu^+ cells in *Hand2* mutants are dividing and that this number has substantially decreased by E12. Between E10 and E12 the proportion of $Sox10^+$ cells that also proliferate decrease and proliferating cells become concentrated in the middle of the condensing ganglia with a ring of non-proliferating cells located to the periphery; the majority of proliferating cells do not express Hu at this time suggesting a lag between down-regulation of *Sox10* and expression of Hu. The fact that some $Sox10^+$ cells also express Hu (15–25%) suggests that sympathetic ganglion neurons are generated from both a pool of proliferating progenitor/precursor cells and young proliferating

neurons (Tsarovina et al., 2008). These pools of proliferating cells are not maintained in the absence of *Hand2*.

Acquisition of noradrenergic characteristics depends upon Hand2

The significant reduction in cells expressing the neuronal marker Hu as well as the cell type-specific marker TH indicates that loss of *Hand2* affects both the generation of neurons as well as the expression of catecholaminergic marker genes. These two aspects of neuron differentiation appear to occur in parallel. In *Hand2$^{fl/fl}$* embryos the vast majority of cells that express Hu also express TH, this is not the case in *Hand2$^{fl/fl;Wnt1\text{-}Cre}$* embryos where the majority of neurons that develop do not express TH. Previous studies indicated that *Hand2* functions in neurogenesis downstream of MapK and BMP4-mediated signaling in parallel with direct regulation of expression and function of DBH (Liu et al., 2005; Rychlik et al., 2003; Xu et al., 2003). The fact that only a small proportion of the neurons that develop in the *Hand2* mutant express TH raises the possibility that *Hand2* also directly regulates transcription of TH and suggests that *Hand2* is not dispensable for the acquisition of noradrenergic characteristics (and see Morikawa et al., 2007).

Based on our previous studies showing that ectopic expression (Howard et al., 2000, Hendershot et al., 2007) or overexpression (Howard et al., 1999) of *Hand2* supports neurogenesis as well as expression of noradrenergic marker genes, we predicted that knock-out of *Hand2* would result in complete absence of neurons in the developing sympathetic chain ganglia in-vivo, which in fact, it did not. One explanation for this apparent paradox is that *Hand2* is not completely excised in our mutant embryos. Variable excision has been reported when using Wnt1-Cre driver mice (Taylor et al., 2007) but our inability to detect mRNA encoding *Hand2* in *Hand2$^{fl/fl;Wnt1\text{-}Cre}$* embryos does not support this possibility. A more likely scenario is that some neural precursor cells not restricted to the noradrenergic lineage remain in the anlagen of the sympathetic chain. This is difficult to assess experimentally since in the sympathetic ganglia non-noradrenergic neurons also express noradrenergic characteristics early in development. Conversely, the majority of neurons also express cholinergic marker genes at early time points (Huber and Ernsberger, 2006). To ask whether there is a change in neurotransmitter specification in the absence of *Hand2*, we examined expression of the vesicular acetylcholine transporter (VChAT) as a marker of cholinergic neurons; we were not able to detect any significant effect of loss of *Hand2* on early expression of VChAT, suggesting either a poor choice of marker or that neurotransmitter specification/expression would not manifest at this stage. It might also be argued that our results reflect generation of mosaic embryos; we do no think this is the case. We did not observe augmented or different effects on expression of Hu or TH in embryos from crosses of *Hand2$^{fl/del}$* embryos, suggesting that mosiacism does not account for our results. Our results suggest that a cohort of neural precursor cells are generated in the absence of *Hand2* but that these cells are not maintained, resulting in a progressive decrease of neurons with increased developmental time. This conclusion is supported by the decrease but not complete loss of neurons in the *Hand2* mutant zebrafish, *hands off*.

In the zebrafish there is only one *Hand* gene (Angelo et al., 2000). Although structurally this gene is similar to *Hand2*, its early expression pattern and function seem more akin to Hand1 (Shepherd, unpublished results). In light of previous studies where no apparent role for *Hand2* in neurogenesis was identified in the zebrafish (Lucas et al., 2006), we chose to re-examine the phenotype of the *Han^{s6}* mutant. Interestingly, in the mouse and fish, based on expression of both pan-neuronal (Hu) and cell-type-specific (TH) markers, we conclude that *Hand2* does function in neurogenesis and/or neuronal differentiation in the zebrafish. Differences in the endpoints (expression of mRNA vs. protein) or method of quantification reported by others (area vs. cell counts) are likely reasons for the differences in experimental outcomes or interpretation of data. Our data suggest that *Hand2* is required to maintain the neural precursor pool of cells and that it affects neuronal differentiation, as well as affecting expression of cell type-specific genes. Examination of the patterns of expression of the transcriptional regulators comprising the sympathetic noradrenergic locus supports these conclusions.

Hand2 regulates expression of Phox2 and Gata3

We undertook an examination of expression patterns of *Phox2b*, *Phox2a*, *Ascl1* and *Gata3* in order to determine whether expression of *Hand2* influences expression/function of these other DNA binding proteins as well as to establish the epistatic relationship between *Hand2* and these other transcriptional regulators. We suggest that *Hand2* controls feed-forward and feed-back cross-regulation of *Phox2b*, *Phox2a* and *Gata3* required to maintain the precursor pool and support differentiation of noradrenergic neurons. We define the *Phox2b*-expressing cells as autonomic progenitors; the survival of these cells is dependent upon *Phox2b* and this function may be mediated by *Hand2* (Pattyn et al., 1999, 2000; Stanke et al., 1999; Coppola et al., 2005; Tsarovina et al., 2008). The number of *Phox2b*-expressing cells present in the anlagen of the sympathetic chain ganglia is equivalent in control and *Hand2* mutant embryos at E10, suggesting that the neural progenitor pool is not initially affected by loss of *Hand2*. Since *Hand2* is expressed downstream of *Phox2b*, any effect of *Hand2* on *Phox2b* expression should manifest later in development. This suggests a model (Fig. 9) whereby both feed-forward and feed-back transcriptional regulation occurs in the noradrenergic pathway (Goridis and Rohrer, 2002). If correct, this model would predict a decrease in the number of *Phox2b*-expressing cells with advanced developmental age, as we have reported. Lack of effect of *Hand2* deletion on either timing or extent of *Ascl1* expression supports the idea that *Ascl1* functions in parallel with other transcription factors in the network and suggests that initial expression of *Phox2a* may be *Ascl1*-dependent.

Our earlier studies in avian embryos demonstrated that ectopic expression of *Hand2* (Howard et al., 2000) could induce expression of *Phox2b* (feed-back) as well as *Phox2a*, NF160, TH, and DBH (feed-forward). In addition to demonstrating a complex pattern of cross-regulation in this transcription factor network, we and others have shown that *Hand2* can induce expression and effect function of *Phox2a* (Howard et al., 2000, Xu et al., 2003; Rychlik et al., 2003; Liu et al., 2005). The lack of *Hand2* expression in embryos derived from a

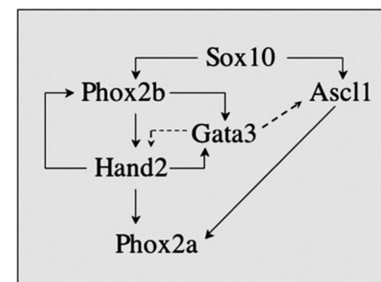

Fig. 9. Working model of the transcription factor network necessary for specification and differentiation of noradrenergic sympathetic ganglion neurons. *Phox2b* and *Ascl1* are independently induced and require *Sox10*. *Hand2* is induced downstream of *Phox2b* and does not require *Ascl1* (Howard, unpublished data). Following initial induction, *Hand2* can feed-back and regulate subsequent/maintained expression of *Phox2b*. *Hand2* induces expression of *Phox2a* and *Phox2a* does not feed-back and regulate *Hand2*. *Ascl1* can induce *Phox2a* but sustained expression of *Phox2a* requires *Hand2*. *Gata3* is expressed downstream of *Phox2b* and is cross-regulated by *Hand2*. *Gata3* negatively regulates expression of *Ascl1* and may feed-back and regulate sustained expression of *Phox2b* and *Hand2* (Moriguchi et al., 2006). In this network, *Phox2b*, *Gata3* and *Hand2* are essential for specification, neurogenesis and neurotransmitter expression of noradrenergic sympathetic ganglion neurons. *Phox2a* may not be dispensable but is likely not essential (Coppola et al., 2005).

Phox2a knock-in (*Phox2a* expression was driven by the *Phox2b* promoter; Coppola et al., 2005) line of mice, as well as the diminished number of cells expressing *Phox2a* in our *Hand2* mutant embryos, places *Phox2a* downstream of *Hand2* and implicates its necessity for expression of cell type-specific marker genes. In addition to *Phox2a*, *Gata3* has been implicated as a *Hand2* downstream target gene (Tsarovina et al., 2004; Lucas et al., 2006), functioning in aspects of neuronal differentiation and expression of TH (Tsarovina et al., 2004; Moriguchi et al., 2006).

An essential function of *Gata3* in neurogenesis and cell type-specific gene expression, although not completely defined, is clearly demonstrated in *Gata3*-null embryos and in gain-of-function studies in avian embryos (Lim et al., 2000; Tsarovina et al., 2004; Moriguchi et al., 2006). *Gata3*-null embryos die in the absence of pharmacological intervention due to low levels of norepinephrine biosynthesis (Lim et al., 2000; Moriguchi et al., 2006). These embryos exhibit diminished expression of both TH and DBH, compromised adrenal chromaffin cell survival and effects on sympathetic ganglion neuronal differentiation. In avian embryos, expression of Gata2 appears after *Phox2b*, *Hand2* and *Phox2a*, suggesting that we should have found decreased expression of *Gata3* in *Hand2* mutant embryos. At E10–12, a time at which we observe influences of *Hand2* on the number of cells expressing *Phox2a*, Hu, and TH, we did observe a significant decrease in the number of cells expressing *Gata3* as well as a significant reduction in the number of neurons. The disparity in the number of differentiated neurons expressing TH and the number of cells expressing *Gata3* suggests differential regulatory pathways for neuronal differentiation and expression of neurotransmitter. This raises the possibility that *Hand2* is not the only factor regulating expression of *Gata3*. Indeed, while loss-of-function of *Gata3* results in a decrease in both neuron numbers and expression of TH, ectopic expression of Gata2 in avian embryos supports neurogenesis but the neurons do not express noradrenergic characteristics (Tsarovina et al., 2004); this is similar to our observation that expression of *Gata3* is a better indicator of neural development than it is of neurotransmitter specification/expression. Gain-of-function and temporal appearance place Gata2 downstream of *Hand2* in chick embryos (Tsarovina et al., 2004) and zebrafish (Lucas et al., 2006). However, regulation of *Hand2* in murine embryos downstream of Gata4 in the heart (McFadden et al., 2000), *Gata3* in the branchial arches (Ruest et al., 2004) and in *Gata3*-null embryos (Tsarovina et al., 2004; Moriguchi et al., 2006) supports the conclusion that expression of *Gata3* does not occur in a linear network but rather fits into our model (Fig. 9) of a cross-regulatory transcription factor network regulating specification and differentiation of sympathetic noradrenergic neurons (Howard et al., 2000; Goridis and Rohrer, 2002; Liu et al., 2005; Moriguchi et al., 2006; Sarkar and Howard, 2006). We add support for the proposition that cell autonomous transcriptional regulation occurs in a non-linear network comprising both feed-forward and feed-backward interactions (Goridis and Rohrer, 2002; Moriguchi et al., 2006).

Conditional deletion of *Hand2* in neural crest-derived cells has shown *Hand2* to be essential for development of sympathetic noradrenergic neurons. A more clearly defined set of gene regulatory interactions in this lineage will have to await identification of lineage specific *cis*-regulatory elements.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.036.

References

An, M., Luo, R., Henion, P.D., 2002. Differentiation and maturation of zebrafish dorsalroot and sympathetic ganglion neurons. J. Comp. Neurol. 446, 267–275.

Angelo, S., Lohr, J., Lee, K.H., Ticho, B.S., Breitbart, R.E., Hill, S., Yost, H.J., Srivastava, D., 2000. Conservation of sequence and expression of *Xenopus* and zebrafish dHAND during cardiac. branchial arch and lateral mesoderm development 95, 231–237.

Bertrand, N., Castro, D.S., Guillemot, F., 2002. Proneural genes and the specification of neural cell fates. Nat. Rev., Neurosci. 3, 517–530.

Brewer, S., Feng, W., Huang, J., Sullivan, S., Williams, T., 2004. Wnt1-Cre-mediated deletion of AP-2 alpha causes multiple neural crest-related defects. Dev. Biol. 267 (1), 135–152.

Coppola, E., Pattyn, A., Guthrie, S.C., Goridis, C., Studer, M., 2005. Reciprocal gene replacements reveal unique functions for *Phox2* genes during neural differentiation. EMBO J. (24), 4392–4403.

Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K., McMahon, A.P., 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr. Biol. 8 (24), 1323–1326.

D'Autrèaux, F.D., Morikawa, Y., Cserjesi, P., Gershon, M.D., 2007. *Hand2* is necessary for terminal differentiation of enteric neurons from crest-derived precursors but not for their migration into the gut or for formation of glia. Development 134, 2237–2249.

DiCicco-Bloom, E., Townes-Anderson, E., Black, I.B., 1990. Neuroblast mitosis in dissociated culture: regulation and relationship to differentiation. J. Cell Biol. 110, 2073–2086.

Ernsberger, U., Rohrer, H., 2006. *Phox2a* and *Phox2b*: essential transcription factors for neuron specification and differentiation. In: Thiel, G. (Ed.), Transcription Factors in the Nervous System — Development, Brain Function and Disease. Wiley-VCh, Weinheim, pp. 53–73.

Firulli, B.A., Howard, M.J., McDaid, J.R., McIlreavy, L., Dionne, K.M., Centonze, V.E., Cserjesi, P., Virship, D.M., Firulli, A.B., 2003. PKA, PKC, and the protein phosphatase 2A influence HAND factor function: a mechanism for tissue-specific transcriptional regulation. Mol. Cell 12, 1225–1237.

Firulli, B.A., Krawchuk, D., Centonze, V.E., Vargesson, N., Virshup, D.M., Conway, S.J., Cserjesi, P., Laufer, E., Firulli, A.B., 2005. Altered Twist1 and *Hand2* dimerization is associated with Saethre–Chotzen syndrome and limb abnormalities. Nat. Genet. 4, 373–381.

Goridis, C., Rohrer, H., 2002. Specification of catecholaminergic and serotonergic neurons. Nat. Rev. Neurosci. 3, 531–541.

Groves, A.K., George, K.M., Tissier-Seta, J.-P., Engel, J.D., Brunet, J.-F., Anderson, D.J., 1995. Differential regulation of transcription factor gene expression and phenotypic markers in developing sympathetic neurons. Development 121, 887–901.

Guillemont, F., Lo, L.-C., Johnson, J.E., Auerbach, A., Anderson, D.J., Joyner, A., 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75, 463–476.

Hendershot, T.J., Liu, H., Sarkar, A.A., Giovannucci, D.R., Clouthier, D.E., Abe, M., Howard, M.J., 2007. Expression of *Hand2* is sufficient for neurogenesis and cell type-specific gene expression in the enteric nervous system. Dev. Dyn. 1, 93–105.

Hirsch, M.-R., Tiveron, M.-C., Guillemot, F., Brunet, J.-F., Goridis, C., 1998. Control of noradrenergic differentiation and *Phox2a* expression by MASH1 in the central and peripheral nervous system. Development 125, 599–608.

Howard, M.J., Foster, D.N., Cserjesi, P., 1999. Expression of HAND gene products may be sufficient for the differentiation of neural crest-derived cells into catecholaminergic neurons in avians. Dev. Biol. 215, 62–77.

Howard, M.J., Stanke, M., Schneider, C., Wu, X., Rohrer, H., 2000. The transcription factor dHAND is a downstream effector of BMPs in sympathetic neuron specification. Development 127, 4073–4081.

Howard, M.J., 2005. Mechanisms and perspectives on differentiation of autonomic neurons. Dev. Biol. 277, 271–286.

Huber, K., Ernsberger, U., 2006. Cholinergic differentiation occurs early in mouse sympathetic neurons and requires *Phox2b*. Gene Exp. 13, 133–139.

Jiang, X., Rowitch, D.H., Soraino, P., McMahon, A.P., Sucov, H.M., 2000. Fate of mammalian cardiac neural crest. Developmnet 127, 1607–1616.

Kaufman, C.K., Zhou, P., Pasolli, H.A., Rendl, M., Bolotin, D., Lim, K.C., Dai, X., Alegre, M.L., Fuchs, E., 2003. GATA-3: an unexpected regulator of cell lineage determination in skin. Genes Dev. 17, 2108–2122.

Kim, J., Lo, L., Dormand, E., Anderson, D.J., 2003. *Sox10* maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron 38, 17–31.

Kasemeier-Kulesa, J.C., Kulesa, P.M., Lefcort, F., 2005. Imaging neural rest dynamics during formation of dorsal root ganglia and sympathetic chan. Development 132, 235–245.

Lakshmann, G., Lieuw, K.H., Lim, K.-C., Gu, Y., Grosveld, F., Engel, J.D., Karis, A., 1999. Localization of distinct urogenital system-, central nervous system-, and endocardium-specific transcriptional regulatory elements in the Gata-3 locus. Molec. Cell Biol. 19, 1558–1568.
Lim, K.-C., Lakshmanan, G., Crawford, S.E., Gu, Y., Grosveld, F., Engel, J.D., 2000. *Gata3* loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. Nat. Genet. 25, 209–212.
Liu, H., Margiotta, J.F., Howard, M.J., 2005. BMP4 supports noradrenergic differentiation by a PKA-dependent mechanism. Dev. Biol. 2, 521–536.
Lo, L.-C., Tiveron, M.-C., Anderson, D.J., 1998. MASH1 activates expression of the paired homeodomain transcription factor *Phox2a*, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development 125, 609–620.
Lo, L.-C., Morin, X., Brunet, J.F., Anderson, D.J., 1999. Specification of neurotransmitter identity by Phox2 proteins in neural crest stem cells. Neuron 22, 693–705.
Lucas, M.E., Müller, F., Rüdiger, R., Henion, P.D., Rohrer, H., 2006. The bHLH transcription factor *Hand2* is essential for noradrenergic differentiation of sympathetic neurons. Development 20, 4015–4024.
Marusich, M.F., Furneaux, H.M., Henion, P.D., Weston, J.A., 1994. Hu neuronal proteins are expressed in proliferating neurogenic cells. J. Neurobiol. 25, 143–155.
Moriguchi, T., Takako, N., Hamada, M., Maeda, A., Fujioka, Y., Kuroha, T., Huber, R.E., Hasegawa, S.L., Rao, A., Yamamoto, M., Takahashi, S., Lim, K.C., Engel, J.D., 2006. *Gata3* participates in a complex transcriptional feedback network to regulate sympathoadrenal differentiation. Development 19, 3871–3881.
McFadden, D.G., Charite, J., Richardson, J.A., Srivastava, D., Firulli, A.B., Olson, E.N., 2000. A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. Development 127, 5331–5341.
McFadden, D.G., Barbosa, A.C., Richardson, J.A., Schneider, M.D., Srivastava, D., Olson, E.N., 2005. The Hand1 and *Hand2* transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development 1, 189–201.
Morikawa, Y., D'Autréaux, F., Gershon, M.D., Cserjesi, P., 2007. *Hand2* determines the noradrenergic phenotype in the mouse sympathetic nervous system. Dev. Biol. 1, 114–126.
Morin, Z., Cremer, H., Hirsch, M.-R., Kapur, R.P., Goridis, Brunet, J.-F., 1997. Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene *Phox2a*. Neuron 18, 411–423.
Müller, F., Rohrer, H., 2002. Molecular control of ciliary neuron development: BMPs and downstream transcriptional control in the parasympathetic lineage. Development 129, 5707–5717.
Olson, E.N., 2006. Gene regulatory networks in the evolution and development of the heart. Science 313, 1922–1927.
Pattyn, A., Morin, X., Cremer, H., Goridis, C., Brunet, J.-F., 1997. Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis. Development 124, 4065–4075.
Pattyn, A., Morin, X., Cremer, H., Goridis, C., Brunet, J.-F., 1999. The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. Nature 399, 366–370.
Pattyn, A., Goridis, C., Brunet, J.-F., 2000. Specification of the central noradrenergic phenotype by the homeobox gene *Phox2b*. Mol. Cell. Neurosci. 3, 235–243.
Raible, D.W., Kruse, G.J., 2000. Organization of the lateral line system in embryonic zebrafish. J. Comp. Neurol. 421, 189–198.
Rohrer, H., Thoenen, H., 1987. Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. J. Neurosci. 7, 3739–3748.
Rothman, T.P., Gershon, M.D., Holtzer, H., 1978. The relationship of cell division to the acquisition of adrenergic characteristics by developing sympathetic ganglion cell precursors. Dev. Biol. 65, 321–341.
Rothman, T.P., Specht, L.A., Gershon, M.D., Joh, T.H., Teitelman, G., Pickel, V.M., Reis, D.J., 1980. Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system. Proc. Natl. Acad. Sci. U. S. A. 77, 6221–6225.
Ruest, L.B., Xiang, X., Lim, K.C., Levi, G., Clouthier, D.E., 2004. Endothelin-A receptor-dependent and -independent signaling pathways in establishing mandibular identity. Development 18, 4413–4423.
Rychlik, J., Gerbasi, V., Lewis, E.J., 2003. The interaction between dHAND and Arix at the dopamine-β-hydroxylase promoter region is independent of direct dHAND binding to DNA. J. Biol. Chem. 278, 49652–49660.
Sarkar, A.A., Howard, M.J., 2006. Perspectives on integration of cell extrinsic and cell intrinsic pathways of signaling required for differentiation of noradrenergic sympathetic ganglion neurons. Auton. Neurosci. 126–127, 225–231.
Schneider, C., Wicht, H., Wegner, E.J., Rohrer, H., 1999. Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. Neuron 24, 861–870.
Smith, E., Hargrave, M., Yamada, T., Begley, C.G., Little, M.H., 2002. Coexpression of SCL and *Gata3* in the V2 interneurons of the developing mouse spinal cord. Dev. Dyn. 224, 231–237.
Srivastava, D., Thomas, T., Lin, Q., Kirby, M.L., Brown, D., Olson, E.N., 1997. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat. Genet. (2), 154–160.
Sommer, L., Shah, N., Rao, M., Anderson, D.J., 1995. The cellular function of MASH1 in autonomic neurogenesis. Neuron 15 (6), 1245–1258.
Stanke, M., Junghans, D., Geissen, M., Goridis, C., Ernsberger, U., Rohrer, H., 1999. The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. Development 126, 4087–4094.
Taylor, M.K., Yeager, K., Morrison, S.J., 2007. Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems. Development 13, 2435–2447.
Thomas, S.A., Matsumoto, A.M., Palmiter, R.D., 1995. Noradrenaline is essential for mouse fetal development. Nature 374, 643–646.
Tsarovina, K., Pattyn, A., Stubbusch, J., Muller, F., Wees, J., Schneider, C., Brunet, J.-F., Rohrer, H., 2004. Essential role of GATA transcription factors in sympathetic neuron development. Development 131, 4775–4786.
Tsarovina, K., Schellenberger, J., Schneider, C., Rohrer, H., 2008. Progenitor cell maintenance and neurogenesis in sympathetic ganglia involves Notch signaling. Molec. Cell. Neurosci. 37, 20–31.
Wu, X., Howard, M.J., 2002. Transcripts encoding HAND genes are differentially expressed and regulated by BMP4 and GDNF in developing avian gut. Gene Expr. 10, 291–305.
Xu, H., Firulli, A.B., Zhao, X., Howard, M.J., 2003. *Hand2* synergistically enhances transcription of dopamine-β-hydroxylase in the presence of *Phox2a*. Dev. Biol. 262, 183–193.
Yelon, D., Ticho, B., Halpern, M.E., Ruvinsky, I., Ho, R.K., Silver, L.M., Stainier, D.Y., 2000. The bHLH transcription factor *Hand2* plays parallel roles in zebrafish heart and pectoral fin development. Development 12, 2573–2582.
Young, H.M., Ciampoli, D., Hsuan, J., Canty, A.J., 1999. Expression of Ret-, p75(NTR)-, *Phox2a*-, *Phox2b*-, and tyrosine hydroxylase-immunoreactivity by undifferentiated neural crest-derived cells and different classes of enteric neurons in the embryonic mouse gut. Dev. Dyn. 2, 137–152.
Zhou, Q.Y., Palmiter, R.D., 1995. Dopamine-deficient mice are severally hypoactive, adipsic, and aphagic. Cell 83, 1197–1209.