Hoxb1 functions in both motoneurons and in tissues of the periphery to establish and maintain the proper neuronal circuitry

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Formation of neuronal circuits in the head requires the coordinated development of neurons within the central nervous system (CNS) and neural crest-derived peripheral target tissues. *Hoxb1*, which is expressed throughout rhombomere 4 (r4), has been shown to be required for the specification of facial branchiomotor neuron progenitors that are programmed to innervate the muscles of facial expression. In this study, we have uncovered additional roles for *Hoxb1*-expressing cells in the formation and maintenance of the VIIth cranial nerve circuitry. By conditionally deleting the *Hoxb1* locus in neural crest, we demonstrate that *Hoxb1* is also required in r4-derived neural crest to facilitate and maintain formation of the VIIth nerve circuitry. Genetic lineage analysis revealed that a significant population of r4-derived neural crest is fated to generate glia that myelinate the VIIth cranial nerve. Neural crest cultures show that the absence of *Hoxb1* function does not appear to affect overall glial progenitor specification, suggesting that a later glial function is critical for maintenance of the VIIth nerve. Taken together, these results suggest that the molecular program governing the development and maintenance of the VIIth cranial nerve is dependent upon *Hoxb1*, both in the neural crest-derived glia and in the facial branchiomotor neurons.

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The vertebrate cranial neural crest cells give rise to many derivatives of the head, face, and neck, including neuronal and glial cells that must act in concert for proper development of the rostral, central, and peripheral nervous systems. The neural crest originates from a migratory population of cells that delaminate from the dorsal neuroepithelium of the neural tube. In the hindbrain, neural crest cells in even-numbered rhombomeres are destined to sequentially populate the corresponding branchial arch tissues (Martin and Jessell 1991; Niederlander and Lumsden 1996; Kulesa and Fraser 1998). The coordinated interactions between the outgrowing motoneurons in the developing brainstem nuclei and the flanking, neural crest-derived branchial arch tissues may provide the means for the establishment of these neuronal circuitries that govern movement and sensory perception of the neck and face (Martin and Jessell 1991).

During formation of rhombomeres in the hindbrain, each segment adopts a unique identity, which can be

characterized by distinct patterns of gene expression (for review, see Wilkenson 1993). Among the differentially expressed genes found in the hindbrain, the family of homeodomain containing transcription factors encoded by the *Hox* genes display overlapping patterns of rhombomere-restricted expression. Thus, each rhombomere expresses a specific combination of *Hox* genes, generating a *Hox* code thought to be in part responsible for determining the identities of individual rhombomeric segments and the tissues originating from them. Several lines of evidence have implicated the dynamic patterns of *Hox* gene activity within the hindbrain as important modulators of cell fates in the CNS, as well as in peripheral tissues derived from the neural crest (Davenne et al. 1999; Rossel and Capecchi 1999; Gauf et al. 2000; Trainor and Krumlauf 2001; Pattyn et al. 2003). However, due to the pleiotropic and interdependent roles of *Hox* gene function within the vertebrate head, it has been difficult to separate these potentially independent roles in establishing and maintaining the neuronal circuitry between the rhombomere-born nuclei and their respective neural crest-derived target tissues.

Through a series of genetic manipulations in the mouse, evidence has accumulated that the *Hox* genes, in

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addition to their role in specifying cells of the CNS, may also be involved in establishing and maintaining the proper circuitry between neurons and their peripheral target tissues (Tiret et al. 1998; del Toro et al. 2001; Watari et al. 2001). Mice harboring both individual and compound mutations of the anteriorly expressed *Hox* genes exhibit overt developmental defects in the anterior CNS and/or craniofacial structures. *Hoxb1* null mutants exhibit distinct defects on the developing facial nerve originating from the r4 domain of the developing hindbrain (Goddard et al. 1996; Studer et al. 1996). Characterized by significant muscular atrophy and facial paralysis, the *Hoxb1* null phenotype has been attributed to the selective loss of the facial branchiomotor neurons (FBMs) that normally innervate the muscles of facial expression. However, compound mutations in *Hoxa1* and *Hoxb1* have also demonstrated a functional role for *Hoxb1* in the normal development of tissues derived from r4 neural crest (Gavalas et al. 1998; Rossel and Capecchi 1999). In these compound mutants, the r4-derived neural crest cells fail to develop and migrate correctly, consequently, all second-arch derivatives are missing. Taken together, these loss-of-function mutations provide genetic evidence that *Hoxb1* is required for the development of both central and peripheral components of the developing nervous system at the level of r4.

To elucidate the roles for *Hoxb1* in these complex processes, we have utilized classical genetic mosaic analysis in addition to Cre/LoxP conditional technology in the mouse. Through genetic mosaic analysis, we show that *Hoxb1* is required for the specification of facial branchiomotor progenitors in a cell-autonomous fashion. By conditionally inactivating *Hoxb1* specifically in the neural crest cell population, we have also uncovered a requirement for *Hoxb1* in the r4-derived peripheral tissues for maintenance and continued development of the VIIth nerve. Moreover, using different temporally restricted neural crest-specific Cre drivers, we have found that this *Hoxb1* function is required early in the neural crest lineage, prior to its migration. Finally, we provide evidence that *Hoxb1* functions in the glial derivatives of r4-born neural crest cells.

Results

Hoxb1 expression in rhombomere 4 is required for facial branchiomotor neuron development

The facial branchiomotor neuron (FBM) populations arise from neuronal progenitors that are born in the ventral neuroepithelium of the vertebrate hindbrain. Specified in a segment-specific manner, these maturing motoneurons organize into specific clusters that give rise to the motor pools that will form the various brainstem motor nuclei. Coincident with the rhombomeric domain in which motoneurons arise, the different brainstem nuclei adopt unique fates that are destined to innervate specific targets originating from the corresponding flanking branchial arch tissues (Martin and Jessell 1991; Niederlander and Lumsden 1996). *Hoxb1*, the most 3' *Hoxb* gene encoded on the murine B cluster, is restricted in

both expression and function to the cells and tissues originating from r4 (Fig. 1A). Utilizing a knock-in loss-of-function allele in which *GFP* has been inserted into the *Hoxb1* locus, *Hoxb1*^{+/GFP} expression can be moni-

Figure 1. *Hoxb1* expression domains and chimeric analysis. (A) Scanning electron micrograph of an E10.5 mouse embryo, highlighting *Hoxb1*-expression domain by green pseudocoloring. (B) Direct imaging of *Hoxb1*^{+/GFP} expression at E9.75 in r4 neural crest cells. (C) *Hoxb1*^{+/GFP} expression at E10.5 in motoneurons of VIIth cranial nerve. *Hoxb1* protein in cross-section through r4 of E11.5 chimera^{Low} embryo (D) and chimera^{High} embryo (E). (F) Differentiation of *Hoxb1*-expressing motoneuron progenitors (green) in r4 of E11.5 chimera^{Low} embryo by colocalization with the general post-mitotic motoneuron marker *Isl1* (red). (G) Cell autonomous loss of post-mitotic motoneurons, as shown by down-regulation of *Isl1* in *Hoxb1*^{-/-} cells in r4 of E11.5 chimera^{High} embryo. (H) Maintenance of *Nkx2.2* (red) in r4 progenitors and *Phox2b* (green) expression in post-mitotic FBMs in chimera^{Low} embryos. (I) Loss of *Nkx2.2* and *Phox2b* expression in visceral motoneuron progenitors in chimera^{High} embryos. Arrows in F and G demarcate nonvisceral motoneurons, which are more numerous in the null mutant. Arrowhead in I points to the expanded population of *Phox2b*-expressing cells that arise in the absence of *Hoxb1*.

tored throughout embryogenesis (Gaufo et al. 2000). The earliest wave of *Hoxb1* expression observed in r4 corresponds to a migratory population of neural crest cells that are entering the 2nd branchial arch (Fig. 1B). By embryonic day 10.5 (E10.5), *Hoxb1* is down-regulated in the neural crest derivatives, while being maintained in the motoneuron progenitors that are differentiating in the ventral neuroepithelium. Specifically, strong *Hoxb1*^{+/GFP} expression can be seen in the outgrowing axons of the FBMs that will make up the VIIth motor nucleus. At this time, extensive axonal outgrowth has already facilitated the innervation of the 2nd arch tissues (Fig. 1C).

Adult mice bearing null mutations of *Hoxb1* are completely lacking the motoneurons of the facial nucleus and, consequently, exhibit facial paralysis (Goddard et al. 1996; Studer et al. 1996). Embryological analysis revealed misspecification of r4 progenitors that normally give rise to FBMs (Gaufo et al. 2000). Complete absence of *Hoxb1* thus precludes the analysis of later stages of FBM differentiation and development. As a first attempt to discern the differential roles for *Hoxb1* in the motoneurons versus the neural crest-derived tissues in forming a functional facial branchiomotor circuit, we conducted a genetic chimera analysis. By forming morula aggregates comprised of cells from both wild-type and *Hoxb1*^{-/-} embryos, we were able to generate midgestation embryos and adults that exhibited variable levels of mutant mosaicism within the hindbrain (Fig. 1D,E; data not shown), which was also reflective of the overall levels of chimerism throughout the embryo. Offspring with high numbers of *Hoxb1*^{-/-} cells (Chimera^{High}) exhibited variable levels of facial paralysis and muscular atrophy, as seen in the null mutants. Offspring containing low numbers of *Hoxb1*^{-/-} cells (Chimera^{Low}) largely resembled wild-type mice (data not shown). The colocalization of *Hoxb1* and *Isl1* proteins in the motoneuron precursors allowed us to follow the clonal differentiation of *Hoxb1*^{+/+} or *Hoxb1*^{-/-} cell types in a mosaic background (Fig. 1F,G). *Isl1*, as a general motoneuron marker, identifies all motoneurons present in the developing hindbrain. Rhombomere 4 gives rise to predominantly visceral motoneurons, which have been shown to require *Hoxb1* activity during neuronal progenitor specification and FBM differentiation. In Chimera^{Low} embryos, *Hoxb1*-dependent FBM progenitors can be seen differentiating in a ventriculopial manner by the concomitant expression of *Isl1* and the homeobox-containing transcription factor, *Phox2b* (Pattyn et al. 2000; Fig. 1F; data not shown). Whereas the *Hoxb1*^{-/-} mutant cells lose all of the FBM properties, such as caudal migration and axonal extension to VIIth nerve targets (data not shown). In Chimera^{High} embryos, very few cells express the *Hoxb1* protein. As a result, neuronal progenitors fail to maintain *Nkx2.2*, *Phox2b*, and *Isl1* in the domain that normally gives rise to FBMs (Fig. 1G,I; data not shown). However, the few remaining wild-type cells that express *Hoxb1* maintain a normal developmental program, resulting in proper differentiation and a mosaic behavioral output of VIIth nerve targets. Interestingly, in the absence of developing FBM pools, r4 neuronal specification

is generally compromised, resulting in the ectopic expansion of an undefined *Isl1* population of cells (Fig. 1G), and presumably a transformation into 5HT-expressing serotonergic cell fates (Pattyn et al. 2003; data not shown). Consistent with previously published work (Gaufo et al. 2000; Cooper et al. 2003), we show that *Hoxb1* is required cell autonomously for early neuronal specification.

However, due to the disparity of timing between *Hoxb1* expression in neural crest cells (E8.0–E9.5) and r4 motoneurons (E10.5–E12.5), in addition to the complexity of axon–2nd arch interactions, germ-line loss-of-function analysis is unable to resolve peripheral tissue-specific roles for *Hoxb1* in forming the VIIth nerve, facial branchiomotor circuit. To address this issue, we turned to Cre/LoxP-mediated conditional mutagenesis.

The conditional allele of *Hoxb1* (*b1*^C) is illustrated in Figure 2A. Two mutant LoxP511–Cre recognition sites were targeted into the *Hoxb1* locus so as to flank the entire *Hoxb1* coding sequence. Mice heterozygous for the conditional allele were identified by Southern transfer analysis (Fig. 2B). The *NeomycinR* selection cassette, flanked by FRT sites and used to obtain the ES cell line containing the *b1*^C allele, was removed from the germ line of these mice by breeding to a *Flp deleter* strain (Rodriguez et al. 2000) and verified by Southern transfer analysis (Fig. 2C). In the absence of Cre, mice homozygous for this conditional allele are fertile and indistinguishable from the control wild-type mice.

For somatic removal of the *Hoxb1*^C allele in a tissue-specific manner, we took advantage of both new and previously described Cre drivers. For removal of *Hoxb1*^C in premigratory neural crest, and all of its derivatives, we utilized the *Wnt1*–Cre transgenic driver (Chai et al. 2000). Lineage analysis of the *Wnt1*–Cre driver in a *ROSA26* background (Soriano 1999) confirmed expression in the dorsal neural tube, in addition to all neural crest derivatives (Fig. 3C,D). To target neural crest after delamination from the neural tube for the expression of Cre, and concomitant removal of *Hoxb1* function, we generated an *AP2*–Cre driver that functions specifically in the post-migratory neural crest cells (Macatee et al. 2003). *ROSA26* lineage analysis revealed the lack of X-Gal staining in the dorsalmost neural tube, with strong staining observed in all neural crest derivatives (Fig. 3A,B). The differential activity of the *Wnt1*–Cre versus the *AP2*–Cre driver afforded us the ability to define the temporal requirement for *Hoxb1* in the neural crest and its derivatives. To demonstrate the temporal difference in expression patterns between *AP2* and *Wnt1*, we analyzed *AP2* expression in the presence of a *ROSA26* allele activated in the *Wnt1* lineage domain at stage E10.5 (Fig. 3E–G). Although most of the cells marked by the *Wnt1* lineage colocalize with *AP2*, a subset of tissues exhibit unique patterns of differential expression, mainly *AP2* in the ectoderm, *Wnt1* in the dorsalmost neural tube, and a few cells deep in the arch that have down-regulated *AP2* expression. As a control to test the efficacy of the conditional system, we utilized the *Deleter*–Cre (Schwenk et al. 1995), which is expressed from the two-cell stage in

Figure 2. *Hoxb1* conditional allele. (A) Schematic representation of the *Hoxb1* conditional allele. In the targeting vector, the entire *Hoxb1*-coding region (solid boxes) was flanked with two lox511 sites and the *GFPneo* fusion selection marker was inserted in the 3' UTR (hatched box). *Thymidine kinase* (*TK1*) negative selection marker was placed 3' of the gene. The *GFPneo* marker was engineered for removal using the flanking FRT sites and a *Flp*-expressing mouse deleter strain. (A) *AccI*; (B) *BsrGI*; (E) *EcoRI*; (N) *NdeI*; (S) *Sau3AI* converted to *SalI*. Roman numerals indicate the position of hybridization probes. (B) Southern transfer analysis of the targeted ES clones. Genomic DNA was codigested with *NdeI* and *BsrGI* and probed with external hybridization probe I (see A). In this section, lanes 2 and 4 are correctly targeted. The presence of both lox511 sites was examined by digesting with *AccI*, and hybridizing to probe II (data not shown). (C) Removal of the positive selection marker with the *Flp* transgene. Mouse-tail genomic DNA was digested with *EcoRI* and hybridized with probes II and III simultaneously. Animals harboring the conditional *Hoxb1* allele, but lacking the *GFPneo* selection marker (in this example, lanes 3,4), were subsequently bred to homozygosity, omitting the *Flp* transgene.

all somatic tissues, resulting in complete recombination throughout the embryo (data not shown).

Conditional inactivation of *Hoxb1* in premigratory neural crest phenocopies null mutants

Crossing the various *Cre* drivers into the conditional *Hoxb1*^C background generated conditional mutants; crosses were set up as (male *Hoxb1*^{1-/-}, *Cre*^{+/-}) X (female *Hoxb1*^{C/C}). We established that the *Hoxb1*^C allele efficiently recombines in vivo by detecting conditional mutations using Southern transfer analysis from genomic DNA isolated from all driver lines analyzed (data not shown). Using immunohistochemistry directed against the *Hoxb1* protein, efficiency of *Hoxb1*^C recombination was verified in situ at stage E9 using the different *Cre*-driver lines. Serving as a control, *Hoxb1*^{C/-} embryos, devoid of *Cre*, exhibit the normal domains of *Hoxb1* expression in both the neuroepithelium and r4-derived neural crest (Fig. 3H). *Hoxb1*^{C/-} embryos inheriting the *AP2-Cre* driver lack *Hoxb1* expression specifically in the migratory neural crest (Fig. 3I), whereas embryos harboring the *Wnt1-Cre* driver exhibit a broader domain of *Hoxb1*^{C/-} recombination, encompassing cells of the dorsalmost neuroepithelium and all of the neural crest derivatives. (Fig. 3J). When crossed to the *Deleter-Cre* line, the *Hoxb1*^C allele is recombined in all cells, resulting in the complete absence of *Hoxb1* protein throughout the embryo (data not shown). Taken together, these data verify that the *Hoxb1*^C allele is differentially recombined in a tissue/cell-specific manner in the presence of the different *Cre* drivers.

To better understand the roles for *Hoxb1* in the presumptive neural crest derivatives in establishing the proper FBM circuitry, we analyzed the adult phenotypes of the conditional mutants. Interestingly, by removing *Hoxb1* in the premigratory neural crest, we were able to phenocopy the null mutant behavior. *Hoxb1* null mu-

tants are unable to blink their eyes, retract their ears, or move their whiskers, a phenotype consistent with defects in FBM development. However, the expressivity of the conditional mutant phenotype was variable with incomplete penetrance. Of the mice that harbored the appropriate alleles (*Hoxb1*^{C/-}, *Wnt1-Cre*^{+/-}) 35% (14/40) were completely unable to respond to forced air directed at their face, whereas 10% (4/40) exhibited partial facial paralysis. Control littermates (*Hoxb1*^{C/-}) reacted adversely to the forced air by squinting their eyes and retracting their ears and whiskers (Fig. 4A,B). Upon dissecting the skin from the face of the *Hoxb1*^{C/-}; *Wnt1-Cre*^{+/-} mutants, we observed the loss of VIIth cranial nerve branches that normally innervate the muscles of facial expression (Fig. 4C,D). Hematoxylin and eosin staining (H&E) of paraffin sections taken through the brainstem of adult controls and *Hoxb1*^{C/-}; *Wnt1-Cre*^{+/-} mice revealed the specific loss of basophilic-staining motoneuron cell bodies that normally contribute to the facial nucleus (Fig. 4E,F).

Hoxb1 is only expressed transiently in r4 neural crest cells, prior to, and during the earliest stages of migration. To address potential roles for *Hoxb1* in later stages of neural crest cell development, we inactivated its function following delamination from the neural tube using a migratory neural crest driver, *AP2-Cre*. Surprisingly, we did not observe any behavioral phenotypes in adult *Hoxb1*^{C/-}; *AP2-Cre*^{+/-} conditional mutants. The marked differences in mutant phenotypes resulting from the use of the two *Cre*-drivers, demonstrates that whatever role *Hoxb1* confers on the r4 neural crest to maintain VIIth nerve function is conferred early, prior to the delamination and migration from the neural tube.

Early motoneuron specification and development is intact in the conditional mutants

Hoxb1 protein is required for maintaining *Hoxb1* expression in r4 (Popperl et al. 1995). Taking advantage of the

Figure 3. Cre drivers used for tissue-specific ablation of *Hoxb1*. (A) Lateral view of E10.5 embryo harboring the *AP2-Cre* and *ROSA26* alleles reacted with X-Gal. (B) Dorsal view of A. (C) Lateral view of E10.5 embryo harboring the *Wnt1-Cre* and *ROSA26* alleles reacted with X-Gal. (D) Dorsal view of C. (E–G) Colocalization of the AP2 protein (demarcating presumptive neural crest cells) with the Wnt1 lineage in r4 of E9.5 embryos. (H–J) Immunohistochemistry monitoring loss of Hoxb1 protein in the differential domains of E9 embryos targeted for conditional inactivation of the *Hoxb1*^C allele. (H) Normal domains of Hoxb1 protein in r4 of *Hoxb1*^{C/-} controls. (I) Loss of Hoxb1 protein specifically from migratory neural crest cells in embryos harboring the *AP2-Cre* driver. (J) Loss of Hoxb1 protein in premigratory neural crest cells and the corresponding 2nd branchial arch derivatives. (ba2) 2nd branchial arch, (nc) neural crest cells.

autoregulated nature of the endogenous *Hoxb1* locus, we used a *GFP* reporter to assist in determining the efficacy and domains of *Hoxb1*^C inactivation. By generating conditional mutant offspring that are *Hoxb1*^{C/GFP}; *Cre*^{+/-}, in which (^{GFP}) represents the *GFP*-knock-in null allele, we can detect the conditional inactivation of *Hoxb1* through the concomitant loss of *GFP* transcription (Fig. 5D).

As a positive control, demonstrating the efficacy of the conditional allele through the lack of *GFP* activity, we generated *Hoxb1*^{C/GFP}; *Cre-deleter*^{+/-} embryos. In these animals, no *GFP* signal is present in r4 or its derivatives (Fig. 5A). In a similar approach, we analyzed *Hoxb1*^{C/GFP}; *Wnt1-Cre*^{+/-} embryos and found that *GFP* expression was comparable in r4 to that of *Hoxb1*^{C/GFP} controls, confirming proper expression of *Hoxb1* in the CNS component of conditional mutants (Fig. 5B,C). Maintenance of *Hoxb1* expression in r4 of *Hoxb1*^{C/GFP}; *Wnt1-Cre*^{+/-} embryos correlated with proper development and specification of motoneuron progenitors through E12.5, as shown by the presence of Hoxb1 (*GFP*), *Isl1*, and *Phox2b* (Fig. 5E,F, insets; data not shown), differentiation markers of post-mitotic FBMs (Pfaff et al. 1996). These data demonstrate that the loss of *Hoxb1* in the periphery does not affect the early developmental programs governing FBM differentiation. Also, *Hoxb1* is not required in the periphery for maintaining its own transcription in r4.

To determine the developmental time point at which the loss of *Hoxb1* in the neural crest derivatives affects the development of the FBMs, we compared the numbers of *Isl1*-positive cells in the facial nuclei of *Hoxb1*^{C/-}; *Wnt1-Cre*^{+/-} mutants to that of control littermates at different stages of embryogenesis (Fig. 6A). At E12.5, there were no differences in motoneuron numbers between conditional mutants and control littermates. We first observe a loss of motoneurons in *Hoxb1*^{C/-}; *Wnt1-Cre*^{+/-} mutants at E14.5, in which the average neuron number was 152 ± 29 (sd), compared with 182 ± 21 (sd) in control littermates ($p = 0.02$). Curiously, this is the time point in which normal neuronal pruning, through apoptosis, is initiated during development. By E16.5, the majority of cell loss is complete (Fig. 6B,C). At this time, *Hoxb1*^{C/-}; *Wnt1-Cre*^{+/-} mutants are reduced to an average number of 59 ± 40 (sd), whereas control littermates retained a constant number of 174 ± 7 (sd; $p = 0.002$). Of particular interest was the observation that at E16.5 *Hoxb1*^{C/-}; *AP2-Cre*^{+/-} conditional mutants exhibited a loss of motoneurons significantly different ($p = 0.01$) from that of controls, 147 ± 8 (sd) versus 174 ± 7 (sd), respectively (Fig. 5C,D), revealing a cellular phenotype undetectable through adult behavioral phenotyping.

In all tissues analyzed, the loss of *Isl1* expression coincided with the positive reaction to TUNEL (data not shown), indicating that the primary mechanism of mo-

Figure 4. Conditional loss of *Hoxb1* in premigratory neural crest (*Wnt1-Cre* domain) phenocopies adult null mutant. (A) Wild-type behavior in response to forced air blown in the face. (B) *Wnt1-Cre; Hoxb1* conditional mutant behavioral phenotype in response to forced air blown in the face. (C) Nerve branches that make up the VIIth cranial nerve in control animals (arrow). (D) Conditional ablation of *Hoxb1* in the *Wnt1-Cre* expression domain results in the loss of VIIth cranial nerve. (E) H&E staining of VIIth nucleus motoneurons in control animals. (F) Loss of motoneurons in the adult CNS of *Wnt1-Cre; Hoxb1* conditional mutants.

toneuron loss is through programmed cell death. Taken together, these data suggest a role for *Hoxb1* in the premigratory neural crest population required later during the development of CNS-born FBMs.

Conditional loss of Hoxb1 in the periphery results in axon outgrowth defects

Having determined the time window of FBM apoptosis due to conditional loss of *Hoxb1* in the neural crest derived tissues, we were able to focus our studies on the causative defects originating in the periphery. Because we did not observe any difference in motoneuron loss between mutants and control littermates until E14.5, we directed our peripheral analysis to earlier developmental stages. Taking advantage of the autoregulated *GFP* reporter system, which transiently expresses *Hoxb1* (*GFP*) in the r4 neural crest and maintains high levels of expression in the FBMs, we evaluated the extent of recombination in the 2nd arch tissues and imaged the outgrowing VIIth nerve axons as they extended into the periph-

ery. Through this analysis, it became evident that (1) excision of the *Hoxb1* conditional allele by *Wnt1-Cre* was largely complete (>90% of the cells that would normally express *Hoxb1-GFP* lacked *GFP* expression) in the 2nd arch derivatives (data not shown); (2) the early steps in FBM specification and neural tube exit are not affected; and (3) the first noticeable phenotype in the periphery was at E12.5. At this time, *Hoxb1*^{C/-}; *Wnt1-Cre*^{+/-} mutants reproducibly exhibited defects in axonal branching and/or fasciculation, with an average number of branch points being significantly lower in mutants compared with controls (Fig. 7A–D; data not shown). These results allowed us to identify the first time point that *Hoxb1* programmed cells may act in forming the peripheral FBM circuitry. However, it was still unclear as to what types of cells or tissues *Hoxb1*-expressing neural crest cells formed and how they affected this biological process.

Hoxb1-expressing neural crest cells give rise to glial cells of the PNS

To address the fates of *Hoxb1*-expressing neural crest cells, we turned to genetic lineage analysis using the *ROSA26* reporter system (Soriano 1999). We mapped the *Hoxb1*-expressing neural crest derivatives originating from r4 using a *Hoxb1-Cre* mouse line (Arenkiel et al. 2003).

By crossing the *Hoxb1-Cre* driver to *ROSA-GFP* mice (Mao et al. 2001), we were able to identify the *Hoxb1* lineage that gives rise to the peripheral components of the developing VIIth cranial nerve at E12.5, the stage in which we first observe the axonal defects in the periphery. At this point in development, the VIIth nerve has bifurcated into two main branches; one that dives deep into the muscle precursors of the neck, as the other navigates rostrally to form terminal synapses with the muscles of facial expression. We were interested in determining the tissue types derived from the *Hoxb1* expression domain that may influence VIIth nerve development in the periphery at the time of axonal defects. Transverse sections taken through the anterior region of the embryonic face revealed that none of the target or support tissues associated with the VIIth nerve branches, other than glial progenitors, were descendants from the *Hoxb1* expression domain (Fig. 8A–D). Colocalization of *GFP*-positive cells with multiple molecular markers demonstrated that peripheral *Hoxb1* derivatives involved in the FBM circuit give rise primarily to glia. An example of this colocalization is the overlapping expression of *GFP* with the HMG containing transcription factor *Sox10* (Britsch et al. 2001; Paratore et al. 2001; Fig. 8D). We followed the *Hoxb1* lineage to adulthood, and found that these cells localize to the main fascicle of the VIIth cranial nerve that is innervating the muscles of facial expression (Fig. 8E,F). In addition to the FBMs that make up the neuronal component of the VIIth nerve, as shown by colocalization with the cytoskeletal neuronal marker β -III-Tubulin (Tuj1; Fig. 8G,H), *Hoxb1*-expressing neural crest also give rise to a significant percentage

Figure 5. Conditional loss of *Hoxb1* in premigratory neural crest does not affect initial motoneuron specification. *Hoxb1* autoregulation in the hindbrain, as shown by *GFP* expression, can be used to monitor *Hoxb1* ablation. (A) Dorsal view of an E11.5 embryo harboring the *Cre-Deleter*, *Hoxb1* conditional, and *Hoxb1 GFP* null alleles. (B) Dorsal view of E11.5 control embryo harboring the *Hoxb1* conditional allele and null-*GFP* allele in the absence of *Cre*. (C) Dorsal view of an E11.5 embryo in which *Hoxb1* has been ablated in the *Wnt1* domain. (B,C, inset) *GFP* expression in cross-section through the VIIth nucleus. (D) Model of *Hoxb1* autoregulation, in which *Hoxb1* transcription requires the presence of its gene product. (E,F) *Isl1* immunoreactivity in motoneurons of the VIIth nucleus, control and *Wnt1-Cre* conditional mutant, respectively.

of glial cells associated with the VIIth nerve. More specifically, we were able to colocalize *GFP* expression with the Schwann cell marker Myelin Basic Protein (MBP; Fig. 8I,J). Interestingly, it appears as if the r4-born FBMs are closely associated with neural crest-derived glia that also expressed *Hoxb1*. In fact, <5% of the FBM axons are ensheathed by glia that are not expressing the *GFP* lineage marker. This correlation suggests a paradigm in which proper FBM circuitry is established early in embryogenesis through the coordinated programs of r4-born neurons and their r4-derived glial support cells.

Loss of Hoxb1 function does not affect glial progenitor specification

Several studies have described genetic mechanisms by which *Hox* genes function to control cell-fate decisions along the axis of the developing embryo, primarily through influencing progenitor pools for proper specification. We have previously shown that the initial allocation and overall number of neural crest cells derived from r4 is normal in *Hoxb1*^{-/-} mutants (Goddard et al. 1996). This was verified by immunohistochemical analysis directed against the general neural crest markers, transcription factor AP2 (Schorle et al. 1996), and the low-affinity neurotrophin receptor P75 (Morrison et al.

1999; data not shown). However, we wished to determine whether neural crest cell fate specification is compromised in *Hoxb1*^{-/-} mutant mice. To determine whether *Hoxb1* functions in the r4-derived neural crest lineage to specify glial progenitor cell fates, we turned to an in vitro analysis using primary neural crest cell cultures.

E8.5 embryos (6–9 somites) were removed from pregnant females, and segments from presumptive r3–r5 were dissected from the hindbrain. Mesenchymal and endodermal tissues were removed from explants, and the remaining neural tubes were transferred to laminin-coated culture dishes containing a defined growth medium. Over a period of 72 h, neural crest cells were allowed to delaminate from the neural tube explant, migrate onto the laminin substrate, and express early differentiation markers.

Neural crest populations were identified first by morphological criteria, such as location relative to explant, cell shape, and clonal expansion (Fig. 9A). Identity was then verified by immunohistochemistry using the neural crest-specific marker AP2 (Leask et al. 1991; Schorle et al. 1996; Fig. 9B). To determine whether *Hoxb1* influences the early steps in allocating peripheral glial progenitors, we assayed for the glial progenitor marker Sox10 in *Hoxb1*^{-/-} versus wild-type neural crest cell cul-

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Figure 6. Loss of motoneurons occurs during later stages of embryogenesis in the *Hoxb1*, neural crest-specific conditional mutants. (A) Graph illustrating the developmental loss of VIIth nucleus motoneurons in the CNS of conditional mutants. (B) Cross section through VIIth nucleus of *Hoxb1*^{c/-} control embryo immunoreacted with Isl1 antibody. (C) Cross-section through VIIth nucleus of *Wnt1-Cre; Hoxb1*^{c/-} embryo immunoreacted with Isl1 antibody. (D) Cross-section through VIIth nucleus of *AP2-Cre; Hoxb1*^{c/-} embryo immunoreacted with Isl1 antibody. (B–D) Sections of E16.5 embryos.

tures. We did not observe a significant difference in the number of *Sox10*-expressing neural crest cells between mutant and wild-type cell cultures (Fig. 9C–E). Consistent with these results, we also did not find a significant difference in expression of *Sox10*, *GFAP*, and *PLP* through E14.5 between mutant and wild-type embryos (Fig. 9F,G; data not shown). However, fewer overall numbers of glial cells are observed with the loss or absence of FBMs at later stages in the mutant mice. Taken together, these data suggest that the initial glial specification program is intact in the *Hoxb1* mutants.

Discussion

It has been postulated that the principal role for the *Hox* genes is to provide positional information to cells along the body axes of the developing embryo, so as to, in part, coordinate the formation of tissue at a given axial level. Such a need is particularly apparent for the formation of neural circuits along the rostro caudal axis of the body. For this reason, the observation that *Hox* genes, such as *Hoxb1*, were found to be expressed at a specific axial level both in the central nervous system as well as in the neural crest emanating from that level, was particularly satisfying (Wilkenson et al. 1989; Hunt et al. 1991). This observation leaves open the question of how *Hox* genes

function at the cellular and molecular level to contribute to the coordinated formation of neural circuits involving, for example, motoneurons generated within the CNS and the peripheral target tissues. Herein, we have explored this question with respect to the role of *Hoxb1* in the formation and/or maintenance of the facial motoneuron circuit (i.e., that involving the FBMs).

In addition to expression of *Hoxb1* in the FBMs and r4-neural crest, loss-of-function mutations in *Hoxb1*, and in its paralogous family member, *Hoxa1*, also support a pleiotropic role for these *Hox* genes in the formation of the facial motoneuron circuit. *Hoxb1* null mutants exhibit severe facial paralysis and muscular atrophy, a phenotype that has been shown to result from the loss of FBMs (Goddard et al. 1996; Studer et al. 1996). In addition, studies of compound mutant mice harboring both *Hoxb1* and *Hoxa1* loss-of-function alleles have revealed a specific role for *Hoxb1* in tissues derived from the neural crest originating from r4 (Gavalas et al. 1998; Rossel and Capecchi 1999). Through expression analysis using a *Hoxb1-GFP* reporter allele, we elucidated the dynamic, differential expression pattern of *Hoxb1* in cells and tissues derived from r4. Interestingly, following the strong expression at E9.5 in r4-neural crest, within 24 h *Hoxb1* is rapidly down-regulated in the crest-derived tissues and strongly up-regulated in the FBMs that

Figure 7. VIIth nerve axonal outgrowth is compromised by E12.5 in conditional mutants. Autoregulation of the *GFP* (null) allele can be used to monitor the integrity of VIIth nerve outgrowth. (A) *Hoxb1-GFP* expression in *Hoxb1 c/-* control animals (5 $\times$). (B) A 25 $\times$ magnification of inset shown in A. (C) *Hoxb1-GFP* expression in *Wnt1-Cre; Hoxb c/-* conditional animals (5 $\times$). (D) A 25 $\times$ magnification of inset shown in C, highlighting the onset of axonal defects that arise in the periphery of conditional *Hoxb1* mutants.

are programmed to innervate these same tissues. This is surprisingly similar to the scenario that has been described for the ETS-containing transcription factors, in which it has been proposed that the sensory-motor system is formed through the coordinate expression of a transcription factor in both the central and peripheral components of the nervous system in order to establish and maintain proper neuronal circuitry (Lin et al. 1998; Arber et al. 2000; Livet et al. 2002).

As a first attempt to address this issue, we utilized a genetic mosaic approach in which we generated chimeric animals comprised of both wild-type and *Hoxb1*^{-/-} cells. Through this analysis, we were able to define a specific role for *Hoxb1* in the initial specification of FBM progenitors, independent of influence from neural crest-derived peripheral tissues, confirming previous work conducted in the background of the germ-line null mutant and ruling out an early non-cell autonomous role for *Hoxb1* in motoneuron specification. Unlike the observation that peripheral cues are required for the induction and maintenance of the ETS gene *Pea3* in motor neurons of the CNS, resulting in proper cell body localization and axonal arborization (Livet et al. 2002), *Hoxb1* initiates its autoregulatory expression in the CNS independent of peripheral cues. Moreover, in contrast to *Pea3* not being required for general MN specification, *Hoxb1*, acting cell-autonomously in the CNS, is absolutely necessary to initiate the appropriate FBM differentiation program.

We next turned our attention toward understanding the roles for *Hoxb1* in the neural crest lineage during the formation of the FBM circuit.

Using a conditional mutagenesis approach, uncoupling the functions of *Hoxb1* in the neural crest from that of the developing motoneurons, we have uncovered a novel function for *Hoxb1* in maintaining the FBM circuitry at later developmental stages in the periphery. Further, we show that conditional loss of *Hoxb1* in the periphery does not influence the normal developmental program governing the proper specification of FBMs that are born in the CNS. By somatic inactivation of *Hoxb1* specifically in the neural crest, we generated conditional mutants that phenocopied the *Hoxb1* germ-line null mutant phenotype, although with incomplete penetrance and variable expressivity.

There are a few potential explanations for the incomplete penetrance and variable expressivity. First, coordinated development of multiple tissues involving complex circuitry is biologically plastic (Trainor and Krumlauf 2000a,b). Mutant cells, or tissues, may be replaced, displaced, or reprogrammed by ones that can act in their place for proper function. For example, *Hoxa1* may compensate for the loss of *Hoxb1* in r4 neural crest tissues. This hypothesis is testable by generating double conditional mutants. Secondly, a specific temporal requirement for *Hoxb1* may exist in the developing neural crest population originating from r4. Interestingly, in this study, we are only able to phenocopy the null mutants if we remove *Hoxb1* from the premigratory neural crest population using the *Wnt1-Cre* driver. Although we observe a phenotypic difference at the cellular level, we do not observe a behavioral phenotype if we remove *Hoxb1* function in the postmigratory crest population using *AP2-Cre*. Although we cannot rule out the possibility that *Hoxb1* has an additional role in this circuitry through an unknown activity in the interneuron population arising from the dorsal neural tube, another domain subject to inactivation by *Wnt1-Cre*, we favor a temporal requirement for *Hoxb1* early in crest development. Ultimately, this would have to be tested by inactivating *Hoxb1* specifically in the dorsal region of the neural tube, while sparing the premigratory neural crest pool, or rescuing *Hoxb1* function in these same cells in a *Hoxb1*^{-/-}; *Wnt1-Cre* background. Finally, incomplete penetrance and variable expressivity of the conditional phenotype could arise from the inherent technical imprecisions of the Cre/loxP conditional mutagenesis system as applied to the mouse. This may result in *Wnt1-Cre* not triggering excision of *Hoxb1* sufficiently early, or at variable extents, in the premigratory neural crest to consistently abolish all *Hoxb1* activity in these cells. Nevertheless, our results demonstrate a nonautonomous role for *Hoxb1* in maintaining proper FBM circuitry, beyond that of specification, in the ventral neural tube.

Having evaluated the loss of CNS-born motoneurons as a bioassay to determine when conditional ablation of *Hoxb1* in the periphery manifested its phenotype, we were able to delineate that peripheral defects begin prior to E14.5. Utilizing a *GFP* reporter assay, driven by auto-

Figure 8. Genetic lineage analysis reveals that *Hoxb1* neural crest gives rise to myelinating cells of the VIIth cranial nerve. (A–D) Lineage analysis using *ROSA-GFP* and *Hoxb1-Cre* in transverse sections through anterior facial structures to identify *Hoxb1* derivatives and potential VIIth nerve interactions. (A) Colocalization of β -III-Tubulin with *Hoxb1*-derived VIIth nerve bundles traversing through first arch derivatives. (B) PECAM expression in blood vessels associated with, but nonoverlapping with the *Hoxb1* lineage. (C) Nonoverlapping expression of Myogenin-expressing facial muscle precursors and the VIIth nerve. (D) Sox10 expression in the first arch derivatives shows colocalization with VIIth nerve glia, and nonoverlapping expression in cartilage. This particular antibody cross-reacts with both Sox9 and Sox10 proteins, facilitating detection of both glial and chondrocyte progenitors (M. Wegner, pers. comm.). (E) GFP and DAPI expression in cross-section through adult peripheral nerve highlighting *Hoxb1* lineage. (F) A 100 $\times$ magnification of E. (G) Cross-section through the VIIth peripheral nerve immunoreacted with an antibody against the neuronal marker Tuj1, colocalizing GFP activity with axons extending from the motoneurons of the facial nucleus. (H) A 100 $\times$ magnification of G. (I) Cross-section through the VIIth peripheral nerve immunoreacted with an antibody against myelin basic protein (MBP), colocalizing MBP-expressing glia reactivity to the *Hoxb1* lineage. (VIIIn) Seventh cranial nerve, (Vg) fifth cranial ganglia, (ba1) first branchial arch, (S) sensory tract, arrow-*Hoxb1*-derived Schwanns cell, arrowhead-FBM axon associated with a non-*Hoxb1*-derived Schwanns cell.

regulation of the *Hoxb1* locus, we showed that the first discernable phenotype of the VIIth nerve in the periphery occurred at E12.5, which was defective in axonal branching and outgrowth into 2nd arch derived tissues. A similar phenotype has also been described for the IXth nerve in *Hoxa3* mutants (Manley and Capecchi 1997; Watari et al. 2001). This observation supports the hypothesis that loss of *Hox* gene function in the neural crest lineage may generally affect mechanisms governing motoneuron axonal path finding and/or growth.

To determine the cellular identity of the r4-derived neural crest tissue that directly influences the development of the VIIth cranial nerve, we used a genetic lineage approach that utilized a previously described *Hoxb1-Cre* driver (Arenkiel et al. 2003) and *ROSA-GFP* reporter. This proved to be very informative, because it identified the cells and tissues that initially associate with the outgrowing VIIth nerve axons in addition to the terminally differentiated cells and tissues that make up the mature

VIIth nerve. At mid-embryogenesis, when axons first encounter the periphery, they associate with the cranial sensory ganglia, coalescing with axons from the sensory neurons and glia to form a fascicle as the nerve grows into the 2nd arch. Cells of the ganglia are derived from both neural crest and placodal origin (D'Amico-Martel and Noden 1983; Stark et al. 1997), giving rise to a complex structure that relays environmental information from the periphery back to the motoneurons in the CNS to complete a functional neuronal circuit. When we conducted the lineage analysis of r4 neural crest cells, we found that the *Hoxb1* expressing neural crest preferentially give rise to glial progenitors (Arenkiel et al. 2003). At E10.5, most of the *Hoxb1*-derived neural crest cells associated with VIIth/VIIIth ganglia express the glial progenitor marker *Sox10*. In the adult, this lineage persisted within the VIIth nerve branch as nonneuronal *Hoxb1*-derived cells and colocalized with the Schwann cell marker *MBP*. By E12, the anterior branch of the VIIth

Figure 9. Glial progenitor specification is not affected in *Hoxb1*^{-/-} neural crest cells. (A) Brightfield DIC image of 72-h neural crest culture. (B) Immunohistochemical identification of neural crest cells by expression of the migratory crest marker AP2 (green). (C) A 63× magnification of wild-type neural crest cells expressing the glial progenitor marker *Sox10* (yellow). (D) A 63× magnification of *Hoxb1*^{-/-} neural crest cells expressing *Sox10* (yellow). In all panels, nuclei are marked with DAPI (blue). (E) Graph representing relative fraction of *Sox10*-expressing cells in 72-h neural crest cultures. Data reflects results from 12 independent samples for each genotype. (F) Immunohistochemistry directed toward *Sox10* (glia) and *Tuj1* (neurons) expression in an E11.5 wild-type embryo. (G) *Sox10* and *Tuj1* expression in an E11.5 *Hoxb1*^{-/-} embryo.

nerve that is destined to innervate the muscles of facial expression turns rostrally, exiting the tissues derived from the second branchial arch and entering first arch derivatives. Unable to colocalize *GFP* expression in the *Hoxb1* lineage with any other tissues types thought to be important for peripheral circuitry, we concentrated on a role for *Hoxb1* in the glial lineage. Consistent with the proposed functional register between neurons and neural crest-derived, flanking-arch tissues that express the same *Hox* code (Niederlander and Lumsden 1996), we observed a strong correlation between neurons born in r4 and the subsequent ensheathment by glia derived from that same segment. Albeit r4 neural crest give rise to many other cell and tissue types derived from the 2nd branchial arch, such as cartilage, muscle, and connective tissue, lineage analysis in the adult (Arenkiel et al. 2003) has yet to identify these other tissues in association with

the FBM circuitry as originating from the *Hoxb1* domain. It is possible that transient interactions between the *Hoxb1*-expressing neural crest cells and other 2nd-arch derivatives, such as muscle precursors, at an earlier developmental time point somehow program the outgrowing FBMs for proper pathfinding and synapse maintenance. However, we have been unable to detect any defects in these tissues in either the germ-line or conditional *Hoxb1* mutants. Moreover, our data support glial progenitor cells as the significant derivative of the *Hoxb1*-expressing neural crest associated with this circuit, and suggests mechanisms whereby these cells participate in the maintenance and outgrowth of the VIIth nerve.

Evaluating the potential for neural crest cultures to give rise to *Sox10*-expressing cells in vitro, no significant difference in glial specification between wild-type and mutant neural crest cultures was discerned. Although the overall specification program appears to be intact, by the time FBMs begin to die in our conditional model, glial cells are compromised in a concordant fashion. This may be an indication of other problems that arise later in the glial specification program, migration, or even within a subset of misspecified glial progenitors that would go undetected through our in vitro analysis. However, these observations are consistent with the idea that initial glial fate decisions are not compromised in *Hoxb1* mutants, and that *Hoxb1* neural crest derivatives are acting later in the overall maintenance of the r4 circuitry. In this scenario, *Hoxb1* in the neural crest-derived glial cells is initiating a molecular program, independent of specification, required later for direct communication with the maturing FBMs. Compromising the regulation of these downstream targets results in loss of FBMs through programmed cell death. This is a compelling idea, as in our system, glial cells are initially intact, but are inevitably lost upon prolonged interaction and failure to support the proper branching and fasciculation of the cognate FBMs. In fact, it may be worth speculating that *Hoxb1* expression in the VIIth nerve glial cells may play a much more active role in FBM branching and/or target recognition rather than, or in addition to, a general support role. For example, in *Sox10*^{-/-} and *ErbB3*^{-/-} mutant mice, glial cells are invariably missing, resulting in general axonal growth and branching defects prior to early neuronal cell death (Riethmacher et al. 1997; Britsch et al. 2001; Paratore et al. 2001). This is in contrast to our phenotype in which FBMs are correctly specified, send out initial fascicles that undergo a main bifurcation, but are ultimately lost near the time at which they normally make connections with their terminal targets. This may be an indication of molecular defects in the glial cells, resulting in failure to sustain guidance or provide general support. Potential candidates for regulation by *Hoxb1* include axon guidance molecules, such as the *Ephs* and *Ephrins* (Wang and Anderson 1997), or growth factors belonging to the neurotrophin family (Oorschot and McLennan 1998; Oppenheim et al. 2000).

Taken together, the data presented in this study are consistent with a functional role for *Hoxb1* in both the

developing FBMs and neural crest-derived peripheral glia toward the establishment and maintenance of a functional neuronal circuit. Specification of neural crest-derived structures and associated nervous tissue has been postulated to be a developmentally coordinated program, in which the proper formation of one tissue is directly dependent upon the other. This study provides insight at the cellular level into how the vertebrate embryo properly coordinates such complex developmental programs through the dynamic regulation of *Hox* genes in multiple cell and tissue types at a given axial level.

By undertaking a detailed genetic and molecular analysis of *Hoxb1*, we have uncovered novel functions for *Hoxb1* in FBM development and identified the cells and tissues in which it acts. Although we have yet to determine the specific molecular mechanisms by which *Hoxb1* governs the formation and maintenance of FBM circuitry, we are now poised to begin to identify specific downstream targets involved in this process. Through the use of this system, in conjunction with microarray analysis, attempts at identifying downstream targets for *Hoxb1* are underway.

Materials and methods

Generation of genetic chimeras

Chimeric embryos and adult mice were generated from morula derived from intercrosses between wild-type BL6 to wild-type BL6, and *Hoxb1*^{-/-} to *Hoxb1*^{-/-} mice. Wild-type and *Hoxb1* mutant morulas were aggregated and transferred into pseudo-pregnant BL6 females. Chimeras were harvested at E11 or allowed to develop to adulthood.

Construction of the *Hoxb1* conditional allele

Hoxb1 genomic DNA was isolated from a 129Sv mouse genomic library (Stratagene). To introduce the upstream lox511 sequence, an EcoRV site was engineered in the 5'UTR of the *Hoxb1* gene, followed by insertion of an oligonucleotide to produce the following sequence context: GGGataactctgatagata cattatacgaagtattcTGCTCTCCCCAAACGGCCCGACCCTCC TTCGGCCTCTACATG (the newly introduced sequence is in lowercase, the *Hoxb1* start codon is in bold). Similarly, the downstream lox511 site along with the *EGFPneo* fusion gene (flanked with FRT sites) was inserted in an artificial *AscI* site placed 37 bp from the *Hoxb1* stop codon. A total of 10.8 kb of genomic sequence was included in the targeting vector (Fig. 2A), which was linearized with *Sall*, electroporated in ES cells, and selected on G418 and FIAU. Positive ES clones were identified by Southern blotting, using a 0.7-kb 5' external probe on *NdeI* + *BsrGI*-digested genomic DNA. Mice derived from one positive ES clone were mated with *Flpe*-expressing mice (Rodriguez et al. 2000), and the progeny were screened for the absence of the *EGFPneo* selection cassette (Fig. 1C).

X-gal staining

Embryos harboring both the *ROSA26* reporter and either the *Wnt1-Cre*, *AP2-IRES-Cre*, or *Deleter-Cre* driver alleles were removed from pregnant females in PBS with 2 mM MgCl₂. Embryos were fixed [2% paraformaldehyde, 1.25 mM EGTA, 2 mM MgCl₂, 0.1 M PIPES at pH 6.9], washed in PBS, 2 mM MgCl₂,

and immersed into X-gal staining solution [15 mM K₃Fe(CN)₆, 15 mM K₄Fe(CN)₆·3H₂O, 2 mM MgCl₂, 0.01% Na Deoxycholate, 0.02% NP40 in PBS at pH 7.2]. Staining was carried out overnight at room temperature with rocking.

Adult phenotyping analysis

Hoxb1 conditional models were phenotypically characterized by two criteria. First, the ability or inability to respond to forced air blown in the face. Behavior responses included closure of eyelids, pinning back of ears, and retraction of whiskers. Secondly, the mice were examined for compromised integrity of the VIIth cranial nerve. Analysis included the visualization of branch loss in addition to motoneuron loss within the facial nucleus. H&E staining was done by standard protocols on paraffin-embedded brainstems cut at 10 μm.

Immunohistochemistry

Embryonic and adult tissues were dissected in PBS and fixed for 3 h at 4°C with 4% paraformaldehyde in PBS (pH 7.2). Samples were washed in PBS, equilibrated to 30% sucrose, and embedded in O.C.T. Serial sections were cut at 10 μm, washed in PBS, and preincubated in blocking solution (2% BSA, 5% NGS, 0.1% triton in PBS, at pH 7.2). Primary antibodies were diluted in blocking solution and applied overnight at 4°C. The primary antibodies used in this study were as follows: α-GFP polyclonal (Molecular probes; 1:1000), α-Nkx2.2 monoclonal (Developmental Hybridoma; 1:50), α-Islet1 monoclonal (Developmental Hybridoma; 1:50), α-AP2 monoclonal (1:50), α-Phox2b polyclonal (C. Goridis, CNRS, Paris, France; 1:1000), α-PECAM monoclonal (Pharmacia; 1:100), α-Myogenin (Developmental Hybridoma; 1:50), α-Tuj1 monoclonal (Covance; 1:1000), α-Sox10 monoclonal (Dr. Michael Wegner, Institute für Biochemie, Universität Erlangen, Erlangen, Germany; 1:50), α-Hoxb1 polyclonal (Covance; 1:300), α-MBP monoclonal (Chemicon; 1:75). Alexafluor-conjugated secondary antibodies (α-Rb or α-Ms; Molecular probes) were used at a dilution of 1:500 in blocking solution and incubated at 4°C for 4 h.

GFP expression in *Hoxb1*^{stf/+} reporter embryos was analyzed through in situ enhancement of the GFP signal. Embryos were fixed as above, washed, and preincubated in blocking solution (2% BSA, 2% milk, 0.5% triton in PBS at pH 7.2). Whole-mount immunohistochemistry was carried out using the α-GFP polyclonal (Molecular probes; 1:1500) diluted in blocking solution, followed by secondary enhancement using the Alexafluor FITC-conjugated α-Rb antibody (Molecular probes; 1:500). Finally, tissues were washed 5 × 1 h each in PBS/.1 Triton X-100, cleared by equilibration into 95% glycerol, and imaged by confocal microscopy.

For motoneuron cell counts, entire brainstems were cryo-sectioned at 10 μm and the number of IsII-positive cells present in the facial nuclei were counted. The five centralmost sections, containing the highest number of motoneuron cell bodies, were used to determine an average number of neurons per section in each nucleus. Facial nuclei were treated independently in all animals analyzed.

Immunodetection and fluorescence confocal microscopy was carried out using Bio-Rad MRC1024 instrumentation and software.

Neural crest cultures

E8.5 embryos (6–9 somites) were dissected into PBS (Mg²⁺, Ca²⁺, at pH 7.2) from timed pregnant females. Neural tube explants spanning r3–r5 were isolated, and flanking mesodermal and ven-

tral endodermal tissues were removed with a tungsten needle. Clean neural-tube explants were transferred to laminin-coated culture dishes containing a defined neural crest growth medium. Culture dishes and medium was prepared as previously described (Strachan and Condic 2003). Laminin was bound to culture dishes at a concentration of 20 μ g/mL. Cultures were grown for 72 h at 37°C at 7.5% CO₂, changing medium every 24 h. Immunohistochemistry was carried out by rinsing cells in PBS, fixing in 4% paraformaldehyde in PBS for 15 min, washing 3 $\times$ 5 min each in PBS, preincubation in blocking solution (10% NGS, 0.1% BSA, 0.3% Triton X-100 in PBS) for 15 min at room temperature, and incubation of primary antibodies for 3 h at room temperature in blocking solution. Primary antibodies were used at the following concentrations: α -Sox10 monoclonal (Dr. Michael Wegner; 1:25), α -AP2 (3B5) monoclonal (Developmental Hybridoma; 1:25). Secondary detection was carried out using the Alexafluor FITC-conjugated α -Ms antibody (Molecular probes; 1:300) after incubation for 2 h in blocking solution at room temperature, and washing 4 $\times$ 10 min each in PBS. Visualization and cell counts were done on a Zeiss inverted epifluorescent microscope using 3-Eye imaging software. Statistical analysis was carried out on a sample size of 12 independent cultures for each genotype.

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Hoxb1 functions in both motoneurons and in tissues of the periphery to establish and maintain the proper neuronal circuitry

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