Autonomic neurocristopathy-associated mutations in PHOX2B dysregulate Sox10 expression

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Introduction

Neural crest cells (NCCs) are transient and multipotent progenitors that give rise to various cell types, including neural, endocrine, pigment, craniofacial, and conotruncal cardiac cells (1, 2). Impaired development of NCCs causes a wide spectrum of disorders, collectively known as neurocristopathies (3, 4). Most neurocristopathies have a strong genetic basis (5), and investigating how a given genetic change influences NCC behavior is crucial for gaining a better understanding of the pathogenesis of neural crest derivatives, which will likely be vital to the development of new strategies for the prevention and treatment of neurocristopathy. Neuroblastoma (NB) and Hirschsprung disease (HSCR), childhood tumors originating from the sympathetic ganglia and adrenal medulla. The risk for these diseases dramatically increases in patients with congenital central hypoventilation syndrome (CCHS) harboring a nonpolyalanine repeat expansion mutation of the Paired-like homeobox 2b (PHOX2B) gene, but the molecular mechanism of pathogenesis remains unknown. We found that introducing nonpolyalanine repeat expansion mutation of the PHOX2B into the mouse Phox2b locus recapitulates the clinical features of the CCHS associated with HSCR and NB. In mutant embryos, enteric and sympathetic ganglion progenitors showed sustained sex-determining region Y (SRY) box10 (Sox10) expression, with impaired proliferation and biased differentiation toward the glial lineage. Nonpolyalanine repeat expansion mutation of PHOX2B reduced transactivation of wild-type PHOX2B on its known target, dopamine β-hydroxylase (DBH), in a dominant-negative fashion. Moreover, the introduced mutation converted the transcriptional effect of PHOX2B on a Sox10 enhancer from repression to transactivation. Collectively, these data reveal that nonpolyalanine repeat expansion mutation of PHOX2B is both a dominant-negative and gain-of-function mutation. Our results also demonstrate that Sox10 regulation by PHOX2B is pivotal for the development and pathogenesis of the autonomic ganglia.

The most common forms of neurocristopathy in the autonomic nervous system are Hirschsprung disease (HSCR), resulting in congenital loss of enteric ganglia, and neuroblastoma (NB), childhood tumors originating from the sympathetic ganglia and adrenal medulla. The risk for these diseases dramatically increases in patients with congenital central hypoventilation syndrome (CCHS) harboring a nonpolyalanine repeat expansion mutation of the Paired-like homeobox 2b (PHOX2B) gene, but the molecular mechanism of pathogenesis remains unknown. We found that introducing nonpolyalanine repeat expansion mutation of the PHOX2B into the mouse Phox2b locus recapitulates the clinical features of the CCHS associated with HSCR and NB. In mutant embryos, enteric and sympathetic ganglion progenitors showed sustained sex-determining region Y (SRY) box10 (Sox10) expression, with impaired proliferation and biased differentiation toward the glial lineage. Nonpolyalanine repeat expansion mutation of PHOX2B reduced transactivation of wild-type PHOX2B on its known target, dopamine β-hydroxylase (DBH), in a dominant-negative fashion. Moreover, the introduced mutation converted the transcriptional effect of PHOX2B on a Sox10 enhancer from repression to transactivation. Collectively, these data reveal that nonpolyalanine repeat expansion mutation of PHOX2B is both a dominant-negative and gain-of-function mutation. Our results also demonstrate that Sox10 regulation by PHOX2B is pivotal for the development and pathogenesis of the autonomic ganglia.
have been identified in isolated and syndromic cases of CCHS, there are strong correlations between the types of PHOX2B mutations and clinical manifestations. Isolated CCHS patients almost exclusively carry mutations that cause an expansion of the second polyalanine repeat (polyalanine repeat expansion mutations [PARMs]) (12, 13). The vast majority of PHOX2B mutations identified in CCHS-HSCR-NB association, however, are non-PARMs (NPARM), which are either missense mutations or nucleotide deletions/insertions causing frameshifts of the open reading frame (ORF) (12). Interestingly, in the CCHS-HSCR-NB association, multifocal NB is common (12), suggesting that such mutations affect the sympathetic nervous system globally and are involved in an early phase of tumor development that establishes susceptibility to NB. The evidence collectively suggests that NPARM PHOX2B in CCHS-HSCR-NB association exerts a common pathogenic effect on autonomic neural crest derivatives. Elucidation of the function of NPARM PHOX2B may thus provide novel insights into autonomic neurocristopathies, HSCR and NB in particular.

In this study, we focused on two versions of NPARM PHOX2B, and investigated the impact on the development of autonomic ganglia by introducing the NPARM PHOX2B into the mouse Phox2b locus via gene targeting. We addressed the following issues: (a) whether NPARM PHOX2B is sufficient for the expressivity of the disease phenotype; (b) what is the earliest developmental process affected by NPARM PHOX2B; and (c) whether there are commonly shared mechanisms underlying the disease phenotype. We show here that NPARM PHOX2B mutations affect autonomic ganglion progenitors and cause in mice a phenotype reminiscent of CCHS-HSCR-NB association. We also show that dysregulation of the expression of sex-determining region Y (SRY) box10 (Sox10) by NPARM PHOX2B is a potentially common factor in the pathogenesis of autonomic neurocristopathy.

Results

Generation of mice harboring human NPARM PHOX2B mutations.

Various types of NPARMS in the PHOX2B gene have been identified in patients with CCHS-HSCR-NB association. One of the features most frequently shared among those mutations is that they cause a frameshift in the ORF, leading to an addition of aberrant amino acid sequences, which are identical among PHOX2B mutant proteins in the last 42 amino acids of the C-terminal region (Figure 1A). Two of these PHOX2B mutants, 931 del5 and 693–700 del8 (hereafter referred to as del5 and del8, respectively), lack nucleotides 931–935 and 693–700 in the ORF (942 nucleotides), respectively. The nucleotide deletion in del5 occurs in box10, the region close to the stop codon of PHOX2B, while, in del8, the nucleotide deletion occurs between the 2 polyalanine repeat regions, resulting in the absence of a 20-polyalanine repeat in the mutant protein (Figure 1A).

To understand how these NPARM PHOX2B mutations affect the development of the nervous system and cause syndromic neurocristopathy, we introduced human genomic fragments of these mutations (extending from the deletion to the new stop codon due to the frameshift) into the mouse Phox2b locus by gene targeting (Figure 1B). Homologously recombined ES clones were isolated (Figure 1C), and injection of these clones into blastocysts yielded several chimeric mice. However, all living offspring from these chimeric mice were WT, and only dead newborn heterozygous mice were occasionally found, suggesting that heterozygous del5 and del8 mutations cause lethality soon after birth.

PHOX2B mutant mouse recapitulate syndromic neurocristopathy. Perinatal lethality by heterozygous PHOX2B mutations was strongly suspected, so we employed intracytoplasmic sperm injection (ICSI) to generate mice heterozygous for del5 and del8 using sperm derived from these chimeric mice (ref. 16; for details see Methods, Supplemental Figure 1, and Supplemental Methods; supplemental material available online with this article; doi:10.1172/JCI63401DS1). This allowed us to examine the phenotype of Phox2bdel5+/− and Phox2bdel8+/− embryos at various developmental time periods, and we confirmed that, in Phox2bdel5+/− and Phox2bdel8+/− embryos, mutant PHOX2B was expressed in all tissues that endogenously express the Phox2b gene (Figure 1D and data not shown).

Caesarian sections revealed that, at E18.5, Phox2bdel5+/− and Phox2bdel8+/− mice were found at the expected Mendelian ratio, indicating that these mutant embryos develop to term. However, Phox2bdel5+/− and Phox2bdel8+/− mice failed to breathe spontaneously (Figure 2A and Supplemental Video 1). Although breathing could be evoked by skin stimulation, all the mutants died within hours after birth. This phenotype was closely similar to that of mice carrying a 7-polyalanine expansion mutation (PARM) in the Phox2b gene (17), the most common genetic hallmark of isolated CCHS.

Histological examination revealed that retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) neurons, which make up respiratory centers in the hindbrain, were missing in Phox2bdel5+/− and Phox2bdel8+/− mutants. In addition, other Phox2b-dependent nuclei, including the facial motor nucleus (nVII) and the dorsal motor nucleus of the vagus (dmnX) (18), were less densely populated or nearly absent in these mutants (Figure 2C and Supplemental Figure 2).

Acetylcholinesterase staining of the gut showed that enteric ganglia were noticeably fewer in the colons of Phox2bdel5+/− mice and absent from the colons of Phox2bdel8+/− mice (Figure 2D). In ganglionized gut segments, we detected a decrease of approximately 20%–50% in the numbers of enteric ganglion cells in the mutant gut (WT vs. del5 vs. del8; 100% vs. 79.3% vs. 49.7%). Moreover, the sympathetic ganglia in both mutant mice were smaller and had thinner nerve fibers (Figure 2E) than those in WT mice. Occasionally, sympathetic ganglia of the mutant mice were found in aberrant locations in the sympathetic chain (Figure 2E) and pararenal areas (Figure 2F). All of the above-mentioned phenotypes were more severe in Phox2bdel5+/− than Phox2bdel8+/− mice.

Overt tumor formation was not detected in the sympathetic ganglia and in the adrenal medulla on gross anatomical analysis or H&E staining of sections (data not shown); it was, however, obvious that the entire structure of the sympathetic ganglia was severely affected in the mutant mice. Collectively, introducing human NPARM PHOX2B into the mouse Phox2b locus resulted in abnormal development of the hindbrain nuclei for respiration and enteric and sympathetic ganglia, demonstrating that Phox2bdel5+/− and Phox2bdel8+/− mice are valuable in vivo tools for studying the molecular mechanisms underlying the CCHS-HSCR-NB association.

NPARM PHOX2B mutations impair neuronal differentiation of the enteric ganglion progenitors. To understand how these PHOX2B mutations affect the development of the enteric and sympathetic ganglia, we performed histological analysis of Phox2bdel5+/− and Phox2bdel8+/− embryos.

The ENS is derived from the vagal and sacral NCCs, with much greater contribution of the former than the latter. In mice, the vagal NCCs invade the foregut mesenchyme at about E9.5 (these cells are hereafter called enteric neural crest–derived cells
ENCCs]) and undergo rostral-to-caudal migration within the gut wall until the most advanced ENCCs reach the anal end at E13.5 to E14.0 (1, 19, 20). Phox2b begins to be expressed as soon as ENCCs enter the foregut region (21). During migration, ENCCs proliferate and differentiate in a partially overlapping manner. The state of differentiation can be monitored by the expression of 2 transcription factors, Phox2b and Sox10. Undifferentiated ENCCs express both Phox2b and Sox10 (Phox2b+, Sox10+), whereas cells committed to neuronal or glial differentiation express only Phox2b or Sox10, respectively (22).

In PHOX2B mutant embryos, development of the ENS was affected in the mutant gut soon after the onset of Phox2b expression. At E10.5, ENCCs were present in the mutant stomach, with the density apparently comparable to that of WT. Immunostaining with anti-Sox10 and WT-specific anti-Phox2b (hereafter described as anti-WT Phox2b) revealed that both proteins were expressed in nearly all ENCCs of both WT and mutant embryos. However, by in situ hybridization (ISH), we found that the levels of Sox10 expression in ENCCs were significantly elevated (Figure 3, A–C; signal intensities of WT, del5, and del8 ENCCs = 1 ± 0.04, 1.19 ± 0.08, and 1.38 ± 0.1;

\[ P = 0.02 \text{ and } 0.01 \text{ for WT vs. del5 and for WT vs. del8, respectively, for Sox10 and WT Phox2b double-labeling of the E12.0 gut revealed that ENCCs colonized the entire length of the midgut and a rostral end of the hindgut in WT embryos (Figure 3D). During this period, a delay in gut colonization by mutant ENCCs was readily recognizable in both Phox2b\(^{del5/+}\) and Phox2b\(^{del8/+}\) embryos, and this phenotype was more severe in the latter (Figure 3, D-F). The ENCC density of the mutant gut was lower than that of WT (ENCC numbers in \( \sim 0.04 \text{ mm}^2 \) gut areas; WT vs. del5 vs. del8; 178.9 ± 19.6 vs. 149.0 ± 7.2 vs. 121.5 ± 14.9; \( n = 3, 1, 3; P = 0.026 \).)
for WT and del8). To better understand the cause for the decreased ENCC density in NPARM PHOX2B mutants, we examined cell death and proliferation. No abnormal cell death was detected in the mutant gut, using anti-activated caspase-3 antibody (Supplemental Figure 3). In contrast 5-ethynyl-2′-deoxyuridine (EdU) (or BrdU) and Phox2b double-labeling revealed that proliferation of mutant ENCCs is compromised at E10 (ratio of double-positive cells in Phox2b-positive cells; WT vs. del5 vs. del8; 51.5% ± 4.2% vs. 43.4% ± 4.2% vs. 39.5% ± 0.95%) and restored at E12 (WT vs. del5 vs. del8; 45.2% ± 1.1% vs. 48.1% ± 4.2% vs. 44.0% ± 6.4%). The data suggest that impaired proliferation of immature ENCCs at E10 is the principal cause for the decreased ENCC density in later developmental stages.
At higher magnification of E12 gut, undifferentiated (Phox2b+, Sox10+) neuronal (Phox2b+, Sox10−) and glial (Phox2b−, Sox10+) cells were clearly distinguishable (Figure 3H). This cellular composition was altered in the mutant guts, especially in del8 mutants (Figure 3, I and J); there was a dramatic decrease in neuronal cells (Figure 3, H−J; WT vs. del8: 16.5% ± 1.2% vs. 1.7% ± 0.3%, P = 0.001) and a corresponding increase in undifferentiated cells (WT vs. del8: 59.8% ± 6.9% vs. 77.8% ± 5.3%, P = 0.02). The ratio of
Table 1
Counts of neurons and glia in the SCG

<table>
<thead>
<tr>
<th></th>
<th>Neuron</th>
<th>Glia</th>
<th>Neuron + glia</th>
<th>Ratio of glia (%)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>18236 ± 774.4</td>
<td>7169.0 ± 1012.9</td>
<td>25405.3 ± 1420.6</td>
<td>29.0 ± 2.7</td>
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| Phox2b
(del5/+) | 11480 ± 1425.8A | 6060 ± 205.6B | 17540 ± 1623.8C | 35.7 ± 1.8D |
| Phox2b
(del8/+) | 2909.3 ± 121.0E | 2930.7 ± 258.1F | 5840 ± 246.6G | 51.1 ± 4.3H |

Numbers of neurons and glia were determined by counting the numbers of Phox2b+ and Sox10+ nuclei (see Methods for details). Statistical analysis by Student’s t test. A P = 0.002; B P < 0.1; C P = 0.003; D P = 0.02; E P < 0.001.

A decrease in ganglion size was readily discernible at E10.5 and afterwards (Figure 4B and data not shown), especially in del8 mutants. BrdU and Phox2b labeling at E11.5 (Figure 4D) detected significantly fewer double-positive cells in del8 than WT ganglia (WT vs. del8: 41.9% ± 5.1% vs. 26.6% ± 3%, P = 0.001), whereas cleaved caspase-3–positive cells were detected neither in WT nor in del8 mutant ganglia (data not shown). Thus, reduction in ganglion size is caused at least partly by the impaired proliferation, but not by reduced survival, of ganglion progenitors. Examination of neuronal and glial markers of the sympathetic ganglia revealed that all of these markers are detectable in the sympathetic chain of E10.5 mutant embryos (Figure 4B). However, cells expressing tyrosine hydroxylase (TH) and dopamine β-hydroxylase (Dbh), markers for catecholamine biosynthesis, were noticeably fewer in the mutant than WT ganglia. The difference was less evident with respect to Ascl1 and Ret. This may reflect the physiological expression pattern. Both TH and Dbh are expressed in the vast majority of sympathetic ganglion cells (noradrenergic cells) throughout all developmental time periods. In contrast, Ascl1 is expressed only transiently in early sympathetic ganglion cells (27), and Ret expression is downregulated in the vast majority of ganglion cells after E13.5 (28). That is, markers expressed persistently in differentiated sympathetic neurons were more sensitive in detecting the phenotype of the mutant ganglia. Interestingly, the density of cells expressing Erbb3, a glial marker of the sympathetic ganglia at this developmental age, appeared unchanged or slightly expanded in the mutant ganglia. This likely reflects biased differentiation toward the glial lineage, which is initiated as early as E10 (Figure 4A, and see below).

By E11.5, expression of Phox2b and Sox10 was nearly completely segregated in both WT and mutant ganglia. Interestingly, although Sox10+ cells were found almost exclusively at the peripheral margin of WT ganglia (Figure 4C) (26). Sox10+ cells were detected not only at the periphery, but also inside of the mutant ganglia (Figure 4C).

Finally, total numbers of ganglion cells (the superior cervical ganglia [SCG]) were significantly reduced in mutant ganglia at E18.5 (Table 1). Although both neurons (Phox2b+) and glia (Sox10+) were decreased in number, the former were more strongly affected than the latter in mutant ganglia, and the ratio of glial populations in sympathetic ganglia was significantly higher in mutant than in WT ganglia (Figure 4E). No abnormal growth of neuronal and glial cells was observed in NPARM PHOX2B mutant mice at this time period (data not shown).

In summary, commitment, proliferation, and differentiation of neuronal progenitors were impaired in the mutant sympathetic ganglia.

NPARM PHOX2B mutants affect self renewal and proliferation of autonomic neural progenitors. The analysis in vivo revealed that autonomic ganglion development is affected soon after the onset of Phox2b expression, demonstrating that mutant NPARM PHOX2B initially affects the undifferentiated autonomic neural progenitors. To gain a better understanding of how the behavior of autonomic neural progenitors is altered by NPARM PHOX2B, we used neurosphere assays, which allow the examination of self renewal and differentiation capacity of ganglion cell progenitors in vitro (29).
The numbers of primary neurospheres generated from the same numbers of dissociated sympathetic ganglion cells did not differ between WT and mutant ganglia (Figure 5C), although the size of mutant-derived neurospheres was much smaller (Figure 5, A and B). Successive passage of WT ganglion-derived neurosphere cells revealed a transient increase in the rate of neurosphere formation (Figure 5C), suggesting that cells capable of forming neurospheres underwent symmetric division. This self-renewal ability was observed even after passage 3, although there was a gradual decline in the self-renewal rate. In stark contrast, mutant...
ganglion-derived neurosphere cells displayed a dramatic decrease in neurosphere-forming ability after the first passage (Figure 5C), indicating that NPARM PHOX2B impairs the ability to self renew in sympathetic neural progenitors.

On fibronectin-coated dishes, similar to the aforementioned floating culture conditions, neurosphere cells from mutant ganglia grew much more slowly than those from WT ganglia and were almost unable to passage. The impaired expansion of mutant-derived neurospheres was at least partially accounted for by reduced cell division, as pH3 staining revealed significant differences in the numbers of the stained cells (pH3+ cells/total cells; WT vs. del8; 18.2% ± 3.7% vs. 8.8% ± 1.7%; *P < 0.001).

Phox2b and Sox10 double-immunostaining revealed that neurosphere cells derived from WT ganglia (passage 1) were composed mainly of 3 populations; i.e., Phox2b+Sox10+ (60.1% ± 8.32%), Phox2b+Sox10− (34.8% ± 9.0%), and Phox2b−Sox10+ (5.0% ± 2.8%) cells (Figure 5D). In striking contrast, nearly all neurosphere cells derived from mutant ganglia expressed Sox10, and the Phox2b+Sox10+ population (Figure 5D) was dramatically decreased in size (Phox2b+Sox10+ [64.4% ± 11.1%], Phox2b−Sox10+ [8.3% ± 7.1%] and Phox2b−Sox10− [27.2% ± 7.4%]; Figure 5D), suggesting impaired neuronal differentiation. TuJ1 staining revealed young differentiating neurons with octagonal cell bodies and thin neurites in WT ganglion-derived neurosphere cells. These neurons expressed Phox2b, but not Sox10 (Figure 5E). In contrast, TuJ1+ cells observed in mutant ganglion-derived neurosphere cells had flatter cell bodies, and their neurites appeared thicker and shorter. Surprisingly, many of these TuJ1-expressing cells retained Sox10 expression (Figure 5E). Similar results were obtained from the analysis of gut-derived neurosphere cells (Supplemental Figure 4). In summary, NPARM PHOX2B affected self renewal, proliferation, and neuronal differentiation of autonomic neural progenitors, which was associated with abnormally persistent expression of Sox10.

Altered transcriptional properties in NPARM PHOX2B. As with the case of many transcription factors, PHOX2B’s transcriptional function changes in a context-dependent manner. For instance, while PHOX2B typically directly binds and activates DBH promoter (30), overexpression of Phox2b in neural crest stem cells represses Sox10 expression (31). Our analysis revealed that expression of DBH and Sox10 is dramatically altered in embryos heterozygous for NPARM PHOX2B. PHOX2B mutant protein may, therefore, affect expression of DBH and Sox10 by changing the transcriptional activity of the WT PHOX2B allele or by acting directly on the cis-regulatory elements of these genes or both. In order to understand the mode of gene regulation by mutant PHOX2B, we employed a luciferase reporter assay using expression constructs for WT and mutant PHOX2B and promoter/enhancer regions of the DBH or Sox10 in NIH3T3 cells, which do not express either of these genes.

Consistent with previous reports (30), transfection of WT PHOX2B significantly transactivated the DBH reporter. Such transactivation capacity, however, was abrogated by the mutations (Figure 6A). In order to test whether mutant PHOX2B influences the transcriptional capacity of WT PHOX2B, we transfected NIH3T3 cells with a constant amount of WT PHOX2B along with varying concentrations of mutant PHOX2B, and assayed for transactivation of the DBH-luciferase reporter gene. Expression and amounts of WT and NPARM PHOX2B proteins in this experimental setting were also examined by Western blot analysis (Supplemental Figure 5). Although both WT and NPARM PHOX2B proteins were clearly detectable, the amount of NPARM PHOX2B was 22%–26% of that of WT PHOX2B (lanes 1, 2 and 7, 8), suggesting that NPARM PHOX2B is less stable than WT PHOX2B. Nonetheless, we found that reporter activity was significantly reduced in a dose-dependent fashion (Figure 6B). The inhibitory effect by NPARM PHOX2B was evident even when protein expression levels of NPARM PHOX2B did not reach that of WT PHOX2B (Figure 6B and Supplemental Figure 5, lanes 3, 4 and 9, 10). Mutant PHOX2B,
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Mice heterozygous for NPARM
these mutations on development of the murine nervous system.


The Journal of Clinical Investigation

Collectively, we conclude that mutant PHOX2B acts as both
dominant negative and gain-of-function, leading to impaired
neuronal differentiation and dysregulation of Sox10 expression.

Discussion
Neurocristopathies in the autonomic nervous system, including
HSCR and NB, are one of the most common forms of developmen-
tal disorder in pediatric practice, but the exact pathogenic mecha-
nisms underlying these conditions remain elusive. By introduc-
ing nonpolyalanine expansion mutations of the PHOX2B gene, which
were identified specifically in CCHS-HSCR-NB association, into
the mouse Phox2b locus, we examined the biological influences of
these mutations on development of the murine nervous system.
Mice heterozygous for NPARM PHOX2B died at birth due to lack
of spontaneous breathing, a phenotype reminiscent of CCHS.
Histological examination revealed that the RTN, a respiratory
center in the brainstem, was absent. These mutant mice also dis-
played HSCR-like distal colon aganglionosis and size reduction
and ectopic formation of the sympathetic ganglia. Thus, introd-
uction of NPARM PHOX2B affects development of an identical set
of neuronal populations in human and mouse, revealing that the
pathogenic effects of NPARM PHOX2B are significantly conserved
in mammals. Investigation of the earliest developmental event
affected by these mutations led to the identification of Sox10 dys-
regulation as a potentially common cause for HSCR-NB associa-
tion. This study reports for the first time, to our knowledge, the
biological impact of NPARM PHOX2B in vivo, and the strikingly
conserved pathogenic effects in NPARM PHOX2B mutant mice
underscores the power of mouse genetic approaches for exploring
mechanisms underlying human diseases.

Functional impairment of PHOX2B mutant proteins. PMRs of the
PHOX2B gene are found in the vast majority of isolated CCHS
cases, whereas NPARMs (including del5 and del8) are much
more common in CCHS with HSCR and/or NB (12, 13). Our
study delineates the functional differences between PARM and
NPARM PHOX2B in the physiological context. Mice carrying
PARm in the Phox2b gene display respiratory distress with selective
loss of RTN/pFRG neurons (17). Mice with NPARM PHOX2B,
in contrast, exhibit deficits in a much broader range of neuronal
populations (autonomic ganglia, nVII, and dmnnX). This can be
accounted for, at least in part, by differences in the transcriptional
properties of these Phox2b mutants. For instance, although
PARm and NPARM PHOX2B fail to transactivate the DBH pro-
moter, the dominant-negative effect of NPARM PHOX2B on WT
PHOX2B is much stronger than that of PARM PHOX2B (ref. 34
and this study). Furthermore, the NPARM PHOX2B we examined
(del5 and del8) acquired an ability to transactivate Sox10 via its
enhancers. Collectively, the data suggests that NPARM PHOX2B
exerts more detrimental effects on the nervous system development
than PARM by a combination of dominant-negative and
gain-of-function effects.

Notably, the del5 mutant protein retains 98% of the amino acid
sequences of WT PHOX2B. This suggests that the alterations in
transcriptional activity of del5 are largely attributed to the C-ter-
nial 42 amino acids aberrantly generated by ORF frameshift.
It therefore seems reasonable to speculate that these aberrant
sequences can recruit, via the C-terminal region, coactivators or
corepressors, which do not interact with WT PHOX2B.

Finally, although sustained Sox10 expression by del5 and del8
is a potential cause of impaired autonomic ganglion develop-
ment, this mechanism does not apply to the deficits in the brain
nuclei, as we were not able to detect aberrant Sox10 expression
in Phox2b-expressing cells in the hindbrain. Although it remains
unknown whether del5 and del8 can transactivate genes other
than Sox10 in these cells, our preliminary data suggest that del8
PHOX2B physically interacts with a set of proteins that do not
bind to WT PHOX2B (M. Nagashimada, unpublished observa-
tions). Further characterization of the acquired function via
aberrant C-terminal sequences will be required to attain a more
comprehensive understanding of the molecular basis for CCHS-
HSCR-NB association.

Reciprocal regulation between Phox2b and Sox10 is central to develop-
ment and pathology of the autonomic ganglia. Our in vivo analysis iden-
tifies Sox10 dysregulation as a molecular event shared by develop-
ing enteric and sympathetic ganglia of NPARM PHOX2B mutant
mice and provides insight into the development and pathology
of these ganglia (Figure 7). NCCs contributing to these ganglia
initially express Sox10, but not Phox2b. Soon after they reach the
site of ganglion formation (gut mesenchyme and para/preverte-
bral areas for the enteric and sympathetic ganglia, respectively),
they begin to express Phox2b. These Phox2b/Sox10 double-positive
cells are bipotential progenitors whose future identity (either
neurons or glia) is as yet undetermined. As differentiation pro-
ceeds, Phox2b- and Sox10-expressing cell populations are segre-
The most common surgical intervention for HSCR patients is the removal of an aganglionic gut segment followed by end-to-end anastomosis between the remaining gut and the anus. Some HSCR patients, however, exhibit intestinal problems even after surgery, and defects in the ganglionic gut regions are suspected (37). NPARM PHOX2B mutant mice exhibited paucity in neuronal differentiation and low ENCC density in the ganglionic gut. This implies that structural and functional deficits in the ENS would persist even postnatally in these mice (even if the respiratory problems were rescued) and presumably in patients exhibiting CCHS-HSCR-NB association as well. More careful management of postoperative gastrointestinal function is required for such patients.

*Studies in NPARM PHOX2B knockin mice provide novel insight into NB susceptibility.* To date, various types of PHOX2B mutation have been reported in isolated and syndromic NB cases (12, 38–41). This study provides vital information about how such mutations influence the sympathetic ganglion development in a physiological context. Although our PHOX2B mutant mice displayed severe deficits in development of the sympathetic ganglia, overt tumor formation was not observed, at least at birth. Moreover, we failed to detect increased growth of the PHOX2B mutant sympathetic ganglion cells in any developmental time periods. Rather, sympathetic ganglion progenitors were less proliferative in these mutants. These results appear contrary to the previous observation that overexpression of NPARM PHOX2B mutants in chick sympathetic ganglion cells leads to increased proliferation of those cells (42). This may be because different types of NPARM PHOX2B mutants were used in that study. Although the precise reason for this discrepancy remains unknown, our mouse genetic system clearly demonstrates that single-copy expression of the NPARM PHOX2B allele does not confer growth advantages in sympathetic ganglion progenitors in vivo.

Our neurosphere assay also supports this observation, as the number of secondary neurospheres and the neurosphere diameter, which respectively reflect the self-renewal ability and capacity of Sox10 may also exert adverse effects on the proliferation of ENCC, and reciprocal regulation between Sox10 and Phox2b is required for maintaining appropriate levels of Sox10 expression.

### Figure 7
Schematic showing mechanisms underlying autonomic neurocristopathy by NPARM PHOX2B. Regulation Phox2b and Sox10 expression by reciprocal suppression between these 2 transcription factors plays an important role in generating appropriate numbers of neurons and glia in the enteric and sympathetic ganglia (left). NPARM PHOX2B impairs Phox2b’s inhibitory activity on Sox10 expression and simultaneously transactivates the Sox10 gene, leading to biased differentiation of bipotential progenitors toward glial lineage.
progenitor proliferation, were significantly smaller in mutant than in WT ganglion-derived neurospheres. Given these findings, we conclude that the NPARM PHOX2B mutations that we examined are insufficient to support NB formation, at least by birth, and that additional gene mutations must therefore be required for tumors to develop. Should the NPARM PHOX2B per se be insufficient for NB tumorigenesis, we nevertheless speculate that NPARM PHOX2B does confer NB susceptibility via aberrant expression of Sox10. Several lines of evidence support this hypothesis. First, Sox10 takes part in a program that maintains multipotency in NCCs (31), suggesting that persistent expression of Sox10 can keep cells at least partially in an undifferentiated state. Second, cells in the glial lineage in the peripheral nervous system, or Schwann cell precursors, express Sox10 and are capable of producing multiple cell types (43, 44). They can also be reprogrammed and transdifferentiate (45–47) in response to growth factors (44, 48) or upon injury (49). Given their multipotency and plasticity, glial cells overproduced as a consequence of NPARM PHOX2B mutations could be a source of tumors, such as NB. One recent study on a nodular NB discovered that Schwann cells in the tumor were clonal but did not carry mutations found in neuroblastoma regions (50). The authors speculate that an initial genetic hit occurs in bipotential progenitors and additional genetic changes in neuronal progenitors, which take place later in development, cause neuroblastic tumors. An alternative possibility based on those data, however, is that initial genetic hit occurs in Schwann cells or their precursors and additional genetic changes inducing dedifferentiation/reprogramming of those cells may lead to the establishment of neuroblastic tumors. In either case, Sox10-expressing cell population appears to play an important role in tumor development.

Although Sox10 expression is detectable in many original tumors of NB patients (51), our preliminary analysis failed to detect Sox10 in the majority of NB cell lines. The exact identity of the Sox10-expressing cell population in the original tumors is currently unknown. More detailed studies on Sox10/PHOX2B expression in NB tumor samples and tumor-initiating cells (52) will be required for future study. We also have to acknowledge, however, the limits of our experimental system, as we are unable to obtain direct evidence indicating the physiological relevance of aberrant Sox10 expression with respect to NB susceptibility. New genetic experiments using a variety of NB mouse models are needed to achieve a better understanding of the contribution of Sox10-expressing cells in the pathogenesis of NB.

Finally, many genetic changes have been reported in HSCR and NB patients, and among these, those with the NAPRM PHOX2B mutation represent only a small fraction (7, 53). Nonetheless, detailed developmental studies of such rare conditions are important, as lower incidence often correlates with higher physiological impact (e.g., mortality) and because multiple types of the causative gene mutations are insufficient to support NB formation, at least by birth, and that additional gene mutations must therefore be required for tumors to develop. Should the NPARM PHOX2B per se be insufficient for NB tumorigenesis, we nevertheless speculate that NPARM PHOX2B does confer NB susceptibility via aberrant expression of Sox10. Several lines of evidence support this hypothesis. First, Sox10 takes part in a program that maintains multipotency in NCCs (31), suggesting that persistent expression of Sox10 can keep cells at least partially in an undifferentiated state. Second, cells in the glial lineage in the peripheral nervous system, or Schwann cell precursors, express Sox10 and are capable of producing multiple cell types (43, 44). They can also be reprogrammed and transdifferentiate (45–47) in response to growth factors (44, 48) or upon injury (49). Given their multipotency and plasticity, glial cells overproduced as a consequence of NPARM PHOX2B mutations could be a source of tumors, such as NB. One recent study on a nodular NB discovered that Schwann cells in the tumor were clonal but did not carry mutations found in neuroblastoma regions (50). The authors speculate that an initial genetic hit occurs in bipotential progenitors and additional genetic changes in neuronal progenitors, which take place later in development, cause neuroblastic tumors. An alternative possibility based on those data, however, is that initial genetic hit occurs in Schwann cells or their precursors and additional genetic changes inducing dedifferentiation/reprogramming of those cells may lead to the establishment of neuroblastic tumors. In either case, Sox10-expressing cell population appears to play an important role in tumor development.

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Methods

**Generation of Phox2b<sup>del5</sup> and Phox2b<sup>del8</sup> knockin mice.** A WT full-length PHOX2B cDNA containing 3′-untranslated region was obtained from human NB cell lines (TGW and NBLS) by PCR. Deletion mutations (931 del5 and 693–700 del8; adenine for the first methionine of PHOX2B cDNA is assigned as 1) were introduced by PCR-based mutagenesis. The targeting vectors were designed such that they introduce the PHOX2B deletion/frameshift mutations, spanning from the deletion to the new stop codon due to the frame shift into the corresponding region of the exon 3 of the Phox2b gene. The vectors also contained a neomycin resistance cassette (neo) flanked by loxP sites. Linearized targeting constructs were electroporated into the 129/Ola ES cell line EB3/5 (a gift from Hitoshi Niwa, RIKEN Center for Developmental Biology) (54). G418-resistant clones were screened by Southern blot analysis using an external probe located immediately downstream of the short arm. Properly targeted ES clones were injected into the blastocysts (see Supplemental Methods for more details). All studies were carried out on F2 mice with a hybrid 129/Ola:C57BL/6 or 129/Ola:BDF1 background. Primer sequences for PCR screening of Phox2b<sup>del5</sup> and Phox2b<sup>del8</sup> mice were forward (5′-CTGTCTTGCGGCTCCTCTGTTAGGA) and reverse (5′-ATCTCTCACCGCAGCGCCAGGCTGCG), where 249-bp and 358-bp PCR products were amplified for the WT and mutant alleles, respectively.

**Histological analysis.** ISH, immunohistochemistry, and acetylatedin- terase histochemistry were performed as described elsewhere (55–57). All riboprobes for ISH were synthesized using the DIG RNA Labeling Kit (Roche) as specified by the manufacturer. The primary antibodies used for immunohistochemistry were rabbit anti-Phox2b (1:1000, a gift from J-F. Brunet, CNRS UMR, Paris, France), guinea pig anti-Phox2b (1:500, homemade, raised against the C-terminal region of WT Phox2b [ref. 15]; specificity confirmed by complete overlap of the signals in double staining of mouse embryo sections with rabbit and guinea pig anti-Phox2b antibodies; note that both anti-Phox2b antibodies used in this study recognize only WT Phox2b, as their epitopes are absent in NPARM PHOX2B), goat anti-Sox10 (1:300, Santa Cruz Biotechnology Inc.), sheep anti-TH (1:500, Millipore), and rat anti-Brdu (1:300, Abcam). To detect signals, appropriate secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 (Invitrogen), or Cy3 (Jackson ImmunoResearch Laboratories) were used (1:500).

To examine RTN/pFRG neurons, consecutive parasagittal sections of E18.5 mouse brain were subjected to Phox2b immunohistochemistry and thionin staining. To estimate the numbers of ENCCs at E18.5, guts were subjected to AChE histochemistry. ENCCs were counted in 3 randomly selected areas (0.365 mm<sup>2</sup> each) for each genotype. To quantify signal intensities of Sox10 transcripts in ENCCs (E10), ISH was performed on consecutive frozen sections of mouse embryos, and color developing was stopped within 6 hours (before saturation of the reaction). Gray-scaled images of ISH photos were inverted to white-on-black, and the signal deposits were quantified by ImageJ (NIH). This method can reveal an increase or decrease in gene expression, although the values may not directly reflect the actual transcript numbers. For the detection of proliferating cells, Brdu (Sigma-Aldrich) or Edu (Invitrogen) was injected intraperitoneally into pregnant mice (6 mg/100 g body weight) 2 hours before dissection. ENCC density in E12 gut was examined by counting the numbers of Sox10<sup>+</sup> and/or Phox2b<sup>+</sup> nuclei in 3 randomly chosen areas (0.04 mm<sup>2</sup> each). To determine the ratio of Phox2b<sup>+</sup> and/or Sox10<sup>+</sup> cells in ENCCs of WT and del8 mutant gut, Sox10<sup>+</sup> and/or Phox2b<sup>+</sup> nuclei were counted in randomly selected gut areas (700–1700 nuclei were counted for each embryo).

The size of the sympathetic ganglia at E10 was examined by staining consecutive sections (transverse) of embryos with anti-Phox2b and anti-Sox10 antibodies. Areas containing Phox2b<sup>+</sup> and/or Sox10<sup>+</sup> cells were measured on every third section using ImageJ.

**Cell culture.** Enteric and sympathetic ganglion progenitors were isolated and cultured as reported previously (58). In brief, cells were isolated from the sympathetic ganglia and gut of E13.5 embryos and cultured in a neurosphere medium (DMEM-low [Gibco; Invitrogen], 20 ng/ml recombi-
nant human bFGF [R&D Systems], 20 ng/ml IGF1 [R&D Systems], 1% N2 supplement [Gibco; Invitrogen], 2% B27 supplement [Gibco; Invitrogen], 50 mM 2-mercaptoethanol, 15% chick embryo extract [ref. 8], 35 μg/ml [110 nM] retinoic acid [Sigma-Aldrich], and penicillin and streptomycin [Meiji]] in nonadhesive culture plates treated with F127 (Sigma-Aldrich). All cultures were maintained at 37°C in 5% CO2 balanced air.

For immunocytochemical characterization of neurosphere cells, neurospheres were plated on dishes coated with poly-D-lysine (Sigma-Aldrich) and fibronectin (Biomedicinal Technologies).

For the neurosphere formation assay (nonadherent cultures), 5,000 neurosphere cells were seeded in a 35-mm well, treated with F-127, and cultured for 9–11 days. Frequency of neurosphere formation (%) was calculated by dividing the numbers of neurospheres by 5,000. Proliferation of neurosphere cultures was assessed by double immunocytochemistry with rabbit anti-phospho histone H3 (1:1000; Upstate) and anti-Sox10 and/or anti-Phox2b antibodies.

Quantification of Sox10 transcripts in developing gut. Total RNAs were isolated from E12 gut using Trizol (Invitrogen). cDNAs were generated using Superscript III (Invitrogen) and Random Primers (Promega). Sox10 mRNA levels were analyzed using intron-spanning primers (forward and reverse, 5′-CAGGCTCACTAAGAGTGC-3′ and 5′-CTTGCACCAGT-GCCAGCTTG-3′, respectively) with Power SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 real-time PCR system (Applied Biosystems). Levels of β-actin transcript were used for normalization.

Cell counts of the sympathetic ganglion cells. Mice were perfused with 4% paraformaldehyde, and the SCG were dissected. Consecutive frozen sections (16-μm thickness) of the SCG were stained with anti-Phox2b and anti-Sox10 antibodies, and nuclear profiles were counted in every fourth section. The numbers of neurons and glia were determined by multiplying the total numbers of nuclear profiles positive for Phox2b and Sox10, respectively, by 4. Three mice (E18.5) for each genotype were examined.

Luciferase assay. WT human PHOX2B assay and mutant (del5 and del8) cDNA were cloned into the pCAGGS expression vector (59). The luciferase reporter constructs containing a 978-bp DBH promoter fragment (30) or Sox10 enhancers (298-bp U1, 547-bp U2 or 396-bp U3; alternatively known as MCS7, MCS5 or MCS4, respectively) (32, 33) were prepared using pGL3 or pGL4.25 vectors (Promega).

NIH 3T3 cells were seeded at a density of 5.5 × 104 cells in each well of 12-well plates 24 hours before transfection. A DNA mixture consisting of effector (500 ng), luciferase reporter vector (500 ng), and pCS2-β-gal (200 ng) was transfected for 24 hours with Lipofectamine LTX and PLUS (Invitrogen). Lysate preparation, luciferase, and β-gal assays were performed as described (60). Luciferase activities were normalized to β-gal activities. For each experiment, values from 3 samples were averaged and presented with SD. To confirm expression of WT and NPARM PHOX2B proteins, FLAG-tagged (N-terminal) WT and NPARM PHOX2B expression vectors were constructed and transfected to NIH3T3 cells employing a condition identical to that used for experiments with non-FLAG-tagged constructs. Cell lysates were subjected to Western blot analysis using anti-FLAG (Sigma-Aldrich) and anti-α-tubulin antibody (Sigma-Aldrich).

Study approval. All animal studies were approved by the RIKEN Center for Developmental Biology.

Statistics. Data are presented as mean ± SD. For all experiments, we calculated the difference between groups with 2-tailed Student’s t test.

Acknowledgments. The authors thank Kazuto Kobayashi for providing genomic fragments of the DBH gene. They also thank Hiroshi Sasaki, Ken-ichi Wada, Yohei Yonekura, Toko Kondo, Kaori Hamada, and Chihiro Nishiyama for their excellent technical assistance. This work was supported by RIKEN and MEXT (Grant-in-Aid for Scientific Research on Innovative Areas, Cellular and Molecular Basis for Neuro-Vascular Wiring).

Received for publication February 15, 2012, and accepted in revised form June 21, 2012.

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13. Renier D, Chachat V, Chedotal A, Babinet C, Burglen L, Nishiyama for their excellent technical assistance. This work was supported by RIKEN and MEXT (Grant-in-Aid for Scientific Research on Innovative Areas, Cellular and Molecular Basis for Neuro-Vascular Wiring).

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