Molecular Pathogenesis of Genetic and Inherited Diseases

Pbx3 Deficiency Results in Central Hypoventilation

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**Pbx proteins comprise a family of TALE (three amino acid loop extension) class homeodomain transcription factors that are implicated in developmental gene expression through their abilities to form hetero-oligomeric DNA-binding complexes and function as transcriptional regulators in numerous cell types. We demonstrate here that one member of this family, Pbx3, is expressed at high levels predominantly in the developing central nervous system, including a region of the medulla oblongata that is implicated in the control of respiration. Pbx3-deficient mice develop to term but die within a few hours of birth from central respiratory failure due to abnormal activity of inspiratory neurons in the medulla. This partially phenocopies the defect in mice deficient for Rnx, a metaHox homeodomain transcription factor, that we demonstrate here is capable of forming a DNA-binding complex with Pbx3. Rnx expression is unper-
turbed in Pbx3-deficient mice, but its ability to enhance transcription *in vitro* as a complex with TALE proteins is compromised in the absence of Pbx3. Thus, Pbx3 is essential for respiration and, like its DNA-binding partner Rnx, is critical for proper development of medullary respiratory control mechanisms. Pbx3-deficient mice provide a model for congenital central hypoventilation syndrome and suggest that Pbx3 mutations may promote the pathogenesis of this disorder. (Am J Pathol 2004, 165:1343–1350)**

Pbx proteins comprise a set of four TALE (three amino acid loop extension) class homeodomain transcription factors^{1–4} that are implicated in developmental gene expression. They form hetero-oligomeric DNA-binding complexes and function as transcriptional regulators in cells of different developmental lineages.⁵ Pbx proteins are

orthologs of *Drosophila extradenticle*,⁶ which contributes to axial and limb patterning,⁷ eye development,⁸ and patterning of the embryonic peripheral nervous system.⁹ Similarly, loss-of-function studies in knockout mice demonstrate a role for Pbx1 in patterning and morphogenesis.^{10–13} Most organs are abnormally developed in *Pbx1*^{−/−} embryos and some of the observed deficiencies in organogenesis and cellular differentiation appear to result from compromise of orphan Hox proteins.¹² To further define the developmental roles of Pbx proteins and their potential isotype-specific contributions, we have determined the expression pattern of Pbx3 and created *Pbx3*-deficient mice. We report here that lack of Pbx3 results in neonatal death due to central respiratory failure, a phenotype similar to that of mice deficient for Rnx,¹⁴ a metaHox protein that is also expressed in the medulla and capable of associating with Pbx3 in a higher-order DNA-binding transcriptional complex. Our studies implicate Pbx3 as being critical for development of central control of respiration in vertebrates and suggest the broader hypothesis that mutations of Pbx3, its cofactors, or its regulators may promote the pathogenesis of congenital central hypoventilation syndrome.

Materials and Methods

Construction of Pbx3 Null Mice

The *Pbx3* gene was mutated by deletion of a *HindIII*-*BamHI* fragment spanning exon 3 and replacement with a PGK neo cassette (from the pNT vector) in opposite transcriptional orientation. The *Pbx3* deletion creates an early frame-shift mutation that causes termination of the *Pbx3* reading frame upstream of its dimerization and

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DNA-binding domains. A 12-kb *Xba*I-*Eco*R1 fragment of genomic DNA spanning the disrupted *Pbx3* exon 3 was then cloned into the targeting vector. Homologous recombinant clones were identified by Southern blot analyses. Out of 132 informative clones, 8 were identified as homologous recombinants. Euploid clones were microinjected into C57BL/6 host blastocysts, which were then implanted in pseudo-pregnant females. Chimeric male mice were backcrossed with wt C57BL/6 females to obtain germline transmission of the targeted allele. Phenotypes were analyzed in neonates derived from third backcross generations on a C57BL/6 background.

Antibodies and Immunohistochemistry

Monoclonal antibodies were raised against maltose-binding protein (MBP) fusion proteins containing the 80 or 30 carboxy-terminal amino acids of human *Pbx3a* or *Pbx3b*, respectively. Specificity for *Pbx3* proteins was established by Western blot analysis of *in vitro* translated *Pbx* family proteins (*Pbx1*, 2, and 3). Anti-Rnx rabbit antisera were raised against an affinity-purified MBP-Rnx fusion protein containing the 75 C-terminal amino acids of mouse Rnx. Immune sera were purified by Protein A Sepharose affinity chromatography (Amersham-Pharmacia Biotech, Piscataway, NJ) and their specificity determined by Western blot analysis of *in vitro* translated Hox11 family proteins.

Embryos were fixed in formalin and embedded in paraffin using standard procedures. Sectioned tissues were stained with anti-*Pbx3a* (0.008 mg/ml), anti-*Pbx3b* (0.04 mg/ml), or anti-Meis¹⁵ (0.043 mg/ml) monoclonal antibodies followed by biotinylated rabbit anti-mouse IgG secondary antibody (1:150). Immune complexes were visualized using horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) and DAB chromagen (Sigma, St. Louis, MO). For Rnx/*Pbx3* co-localization, paraffin sections were stained with anti-Rnx (1:25) and *Pbx3a* (0.16 mg/ml) antibodies. Secondary antibodies (1:150) consisted of Texas red-conjugated goat anti-rabbit IgG (Accurate Antibodies, Westbury, NY) and FITC-conjugated goat anti-mouse IgG (Accurate Antibodies). Stained slides were mounted in DAPI-containing slide mount medium with anti-quench (Vector Labs, Burlingame, CA) and examined by fluorescence microscopy.

Respiratory Analyses

Whole body plethysmography was performed on P0 mice less than 1 day of age.¹⁶ Respiratory frequency, tidal volume, and minute volume were calculated from 50 to 70 respiratory cycles during quiet breathing. Medulla and spinal cords for *in vitro* preparations were isolated from P0 mice under deep ether anesthesia as described previously.^{14,17,18} Respiratory-like activity corresponding to the inspiration rhythm was monitored at the C4/C5 ventral root through a glass capillary suction electrode and a high-pass filter with a 0.3 seconds time constant. Values are presented as mean $\pm$ SD. Statistical significance was determined by analysis of variance (analysis of variance)

for all three genotypes. Significant differences between groups were identified using post-hoc Bonferroni tests. Membrane potentials of inspiratory neurons in the ventrolateral medulla¹⁹ were recorded using conventional whole-cell patch-clamp methods.²⁰ The membrane potentials were recorded with a single-electrode voltage-clamp amplifier (CEZ-3100, Nihon Kohden, Tokyo, Japan) after compensation of the series resistance (25 to 50 mol/L Ω) and capacitance.

DNA-Binding and Transcriptional Assays

Proteins used in DNA-binding assays were produced *in vitro* from SP6 expression plasmids using a coupled rabbit reticulocyte lysate system (Promega, Madison, WI). The DNA probe consisted of a gel-purified, end-labeled, double-stranded oligonucleotide encoding a modified *HoxB2* r4 enhancer,¹⁵ containing consensus *Pbx* and *Meis* sites and a *Hox* site that was altered to favor binding by Rnx.²¹ Transient transfection assays were performed as described²² using a pGL3 luciferase reporter (driven by SV40 early promoter containing one copy of the modified *HoxB2* r4 enhancer) and internal control (pCMV-1 β -gal) plasmids. Luciferase activity was measured in light units using a Monolight 2010 luminometer; β -gal activity was used to normalize luciferase activity to account for differences in transfection efficiency.

Results

Pbx3 Is Expressed in the Central and Peripheral Nervous Systems

Pbx3 expression was studied using immunohistochemical staining methods using highly specific monoclonal antibodies directed against two different isoforms (*Pbx3a* and *Pbx3b*) that arise from differential splicing of the *Pbx3* RNA.³ *Pbx3a* was present throughout the mesenchyme at early gestational days, but was progressively more restricted to tissues of the developing central nervous system (CNS) by E11.5 (data not shown). At E15, nuclear *Pbx3a* (and to a much lesser extent *Pbx3b*) staining was observed in the dorsal thalamus, cerebellum, pons, medulla, dorsal root ganglia, and neurons in both the ventral and dorsal columns of the spinal cord (Figure 1A to D). Staining was most intense throughout the medulla (Figure 1, A and D) and was present in regions that overlap with those involved in central control of respiration.²³ *Pbx3a* was also found at high levels in myenteric neurons and at lower levels in smooth muscle myocytes of the intestine (Figure 1, E and F). Even at late gestational days, *Pbx3a* was not exclusively confined to the nervous system, as lower levels were also observed in chondrocytes throughout the axial and appendicular skeleton (Figure 1C).

Generation of *Pbx3*-Deficient Mice

To assess the functional role of *Pbx3*, gene targeting was used to generate *Pbx3*-deficient (*Pbx3*^{-/-}) mice (Figure

Figure 1. Pbx3 expression in the CNS. Immunohistochemistry was performed using a monoclonal antibody directed against Pbx3a on paraffin-embedded tissue sections. **A:** A sagittal section near the midline of a wild-type C57BL/6 embryo at E15.5 shows presence of Pbx3a protein, as evidenced by intense nuclear staining (brown), in the dorsal thalamus (T), the roof of the midbrain, and the cortex and internal cerebellar primordia (CB). Pbx3 is most widely expressed in the hindbrain, particularly in the medulla. **B:** A coronal section from E15.5 shows Pbx3a protein in the medulla and cerebellar primordia (CB). **C:** Transverse section through the spinal cord shows Pbx3 in both the ventral and dorsal portions of the spinal cord (SC). Less intense staining can be seen in the dorsal root ganglia (DRG). Robust staining of chondrocytes in the vertebra (V) is present. **D:** A coronal section of the caudal medulla shows abundant Pbx3 throughout the medulla, including cells in the ventral respiratory column (indicated by red box). **E** and **F:** Transverse sections of the bowel (B) at E15.5 show expression of Pbx3a in nuclei of myenteric ganglion cells (arrows) and smooth muscle myocytes of the muscularis. Magnifications: **A, B, and D,** $\times 25$; **C,** $\times 60$; **E,** $\times 40$; **F,** $\times 400$.

2A). Chimeric male mice carrying the *Pbx3* null allele were bred with wild-type C57BL/6 mice until the allele was transmitted through the germline as determined by Southern blot analysis (Figure 2B). Mice heterozygous for the targeted *Pbx3* gene were viable and fertile. They developed normally and showed no histopathological abnormalities. Intercross matings of *Pbx3*^{+/-} mice, however, did not yield viable *Pbx3*^{-/-} mice at 3 weeks of age (0 of 155 pups, $P < 0.001$). A high number of dead pups were observed at P0, and most were found to be null for Pbx3. Analysis of all neonates showed a Mendelian distribution of genotypes (wild-type,

23 of 93, 25%; *Pbx3*^{+/-}, 49 of 93, 53%; *Pbx3*^{-/-}, 21 of 93, 23%) indicating that the absence of Pbx3 did not result in embryonic lethality. Western blot analysis confirmed the lack of Pbx3 protein in the brains of late gestation *Pbx3*^{-/-} embryos (Figure 2C).

Close observation showed that *Pbx3*^{-/-} pups were viable at birth with intact heart rate but died within a few hours. They appeared apneic and developed marked cyanosis shortly after birth (Figure 2D). Gross anatomical examination of *Pbx3*^{-/-} mice showed that all internal organs were present, properly developed, and of appro-

Figure 2. Construction of *Pbx3* null mice. **A:** A schematic representation of the mouse *Pbx3* cDNA, genomic locus, targeting vector, and mutated allele following homologous recombination. In the *Pbx3* cDNA, sequences derived from exon 3 and the homeodomain (HD) are shown as **shaded boxes**. Approximately 20 kb of the *Pbx3* locus flanking exon 3 (**white box**) are depicted along with mapped restriction sites. The targeting construct carries a *PGK-neo* cassette, which replaces *Pbx3* exon 3, and the *HSV-tk* gene, shown as **solid white boxes**. The transcriptional orientation of the *Pbx3* arms of homology is opposite to that of the *PGK-neo* cassette. The 5' and 3' external probes used for Southern blot analyses are shown as **solid black boxes** below the mutated allele. Restriction enzymes sites: B, BamHI; E, *Eco*RI; H, *Hind*III; N, *Nhe*I; X, *Xba*I. **B:** Southern blot analysis of *Pbx3* alleles. DNA from ES cells (CG8.2) and mouse tissues was analyzed with probes and enzymes indicated beneath the panel. Wt (11.5 kb) and mutant (8 kb) *Pbx3* alleles are indicated to the right. **C:** Western blot analysis of *Pbx3* expression. Protein extracts prepared from brain tissue of E16 embryos were subjected to Western blot analysis using monoclonal antibodies specific for the Pbx3a and Pbx3b isoforms. Genotypes determined by Southern blotting are listed at the **top** of the panel. **Left lane** contains *in vitro* translated Pbx3a and Pbx3b proteins. Migrations of Pbx3 protein isoforms and β -actin loading controls are indicated on the **left**. **D:** Gross appearance of pups within a few hours of birth shows marked cyanosis of the *Pbx3* newborn (**right**) compared to a wt littermate (**left**).

appropriate size; however the lungs were not inflated indicating an absence of appropriate ventilation. Neurofilament staining of E10.5 embryos showed no gross abnormalities of cranial nerve ganglia, indicating correct specification of hindbrain rhombomeres and associated cranial nerve nuclei (data not shown). The brain and spinal cord appeared grossly normal in *Pbx3*^{-/-} mice at P0 and no histological abnormalities of the cranial nerves, ganglia, or brainstem nuclei were observed. No apparent neuronal loss or gliosis was observed in the brainstem, including areas of the ventral medulla implicated in respiratory control and associated with high-level *Pbx3* expression.

Pbx3-Deficient Mice Exhibit Central Hypoventilation

The physiological abnormality in *Pbx3*^{-/-} mice was examined by monitoring spontaneous inspiration, using whole body plethysmography.¹⁶ The respiratory pattern in *Pbx3*^{-/-} mice exhibited a marked irregularity of the tidal volume in each respiratory cycle (Figure 3, A and B). In addition, the average respiratory frequency of 81 $\pm$ 35 per minute in *Pbx3*^{-/-} mice was significantly lower than that observed in *Pbx3*^{+/-} and wild-type (wt) mice (Table 1). As a consequence, the mean respiratory minute volume of 0.72 $\pm$ 0.29 ml/min in *Pbx3*^{-/-} mice was significantly lower than that of wt (1.57 $\pm$ 0.62 ml/min; *P* < 0.01) and *Pbx3*^{+/-} mice, suggesting that hypoventilation was a likely cause of death within several hours after birth.

To localize the level of the defect in *Pbx3*^{-/-} mice, C4 ventral nerve root activity was recorded in medulla-spinal cord preparations *in vitro* as a measure of respiratory output. Ventral C4/C5 activity has been shown to be synchronous with phrenic nerve discharge and contraction of inspiratory intercostal muscles.¹⁷ The C4 ventral root recordings from *Pbx3*^{-/-} mice showed a characteristic pattern of motoneuronal outputs, consisting of small

bursts at a frequency of 6 to 13 per minute, interspersed with large bursts appearing at a frequency of 0.2 to 0.5 per minute (Figure 3C). The latter bursts were usually followed by apnea (up to 30 seconds), or by bursts with minimum activity whose amplitude gradually recovered with time. Mean values of parameters of C4 inspiratory activity revealed a significantly higher frequency but lower amplitude of the bursts in the medulla-spinal cord preparations of *Pbx3*^{-/-} mice (Table 1). This reduced amplitude likely produces insufficient pressure changes by ventilatory movement in some respiratory cycles lead-

Figure 3. Respiratory analysis of *Pbx3*-deficient mice. **A:** Spontaneous inspiratory activity of newborn mice. Plethysmographic recordings correspond to spontaneous inspiration in *Pbx3*^{-/-}, *Pbx3*^{+/-} and wt littermates, respectively. **B:** Amplitude histograms of tidal volume (VT) in different genotypes calculated from recording data shown in **A**. The number of respiratory cycles measured were 144 (*Pbx3*^{-/-}), 196 (*Pbx3*^{+/-}) and 192 (wt). **C:** C4 ventral root inspiratory activity in medulla-spinal cord preparations of newborn mice. Faster sweep representations are shown on the **right** for parts denoted by **bars** on the **left**. **D:** Membrane potential recordings of an inspiratory neuron in the ventrolateral medulla of a *Pbx3*^{-/-} mouse. **Top tracing**, membrane potential trajectory from an inspiratory neuron recorded using conventional whole-cell patch-clamp method; **bottom tracing**, simultaneous recording of C4 ventral root inspiratory activity. Coordinate patterns are seen in which inspiratory bursts with short duration are followed by a subsequent large burst of long duration followed by apnea.

Table 1. *In Vivo* and *in Vitro* Respiratory Activity of *Pbx3*-Deficient Mice

Genotype	<i>In vivo</i> respiratory activity measured by plethysmography		
	Frequency (per min)	Tidal volume (μ l)	Minute volume (ml/min)
+/+ (<i>n</i> = 7)	124 $\pm$ 24	12.6 $\pm$ 4.5	1.57 $\pm$ 0.62
+/- (<i>n</i> = 9)	124 $\pm$ 38	11.8 $\pm$ 4.9	1.47 $\pm$ 0.91
-/- (<i>n</i> = 8)	81 $\pm$ 35*	9.0 $\pm$ 2.5	0.72 $\pm$ 0.29†

Genotype	C4 inspiratory activity recorded <i>in vitro</i>		
	Frequency (per min)	Duration (ms)	Amplitude (μ V)
+/+ (<i>n</i> = 8)	6.0 $\pm$ 0.7	934 $\pm$ 371	246 $\pm$ 73 _s
+/- (<i>n</i> = 15)	8.1 $\pm$ 1.4	958 $\pm$ 275†	159 $\pm$ 52 [‡]
-/- (<i>n</i> = 11)	9.0 $\pm$ 2.1*	713 $\pm$ 239†	65 $\pm$ 35*

*, *p* < 0.01 compared to +/+ and +/-.†, *p* < 0.05 compared to +/+ and +/-.‡, *p* < 0.05 compared to +/+.s, *p* < 0.01 compared to +/+.

ing to a significant decrease of minute respiratory volume in *Pbx3*^{-/-} mice.

To investigate whether the altered inspiratory pattern of *Pbx3*^{-/-} mice may result from abnormal function of respiratory centers in the medulla, we assessed the membrane potentials of inspiratory neurons in this region.^{19,24,25} The membrane potential trajectory of inspiratory neurons (*n* = 6) in *Pbx3*^{-/-} mice correlated with the pattern of C4 motoneuronal outputs (Figure 3D). The coordinate patterns indicate that an underlying basis for respiratory failure in *Pbx3*^{-/-} mice is, at least in part, dysfunction of central respiratory networks in the medulla.

Pbx3 and *Rnx* Are Co-Expressed in Neurons of the Medulla

Neonatal death due to a central hypoventilation syndrome has been reported in *Rnx*-deficient mice.¹⁴ *Rnx* is a member of the *Hox11* orphan homeobox gene family,^{26,27} whose representatives contain DNA-binding homeodomains that differ in their recognition helix from those encoded by clustered *Hox* genes,²⁸ but contain conserved motifs (hexapeptide) implicated in Pbx dimerization.²⁹ Immunofluorescence analysis demonstrated that both *Rnx* and *Pbx3a* proteins were present in the nuclei of neurons of the caudal portion of the medulla (Figure 4A). Since the nuclear localization of Pbx proteins is dependent on dimerization with TALE class homeoproteins of the *Meis/Pknox1* family,³⁰ we also examined the distribution of *Meis* protein expression in the medulla. *Meis* proteins were present in the nuclei of neurons whose distribution extensively overlapped with those expressing *Pbx3a* (Figure 4B), consistent with a *Meis*-mediated mechanism for *Pbx3* nuclear import.³¹ Furthermore, the nuclear co-localization of *Rnx* and *Meis* proteins was unaltered in *Pbx3*^{-/-} mice (Figure 4C), demonstrating that neither the expression nor nuclear localization of either protein was dependent on *Pbx3*.

Pbx3 Forms a DNA-Binding Transcriptional Complex with *Rnx*

Expression of *Rnx*, *Pbx3*, and *Meis* in the medulla raised the possibility that they function together as a hetero-

oligomeric complex whose transcriptional activity may be altered by the lack of *Pbx3*. Therefore, their ability to form a DNA-binding complex was examined on a modified endogenous enhancer that contained consensus sites for all three proteins. This genetically defined response element (*Hoxb2 r4*) has been shown to rely on combinatorial interactions between *Hox* and TALE proteins to mediate appropriate *HoxB2* developmental expression *in vivo*.^{15,32} For the current studies, we modified the *Hox* consensus site to optimize for recognition by the divergent homeodomain of *Rnx*.²¹ As expected, *in vitro*-produced *Pbx3a* and *Meis1a* formed a dimeric complex on this enhancer element (Figure 5A, lane 5). Addition of *Rnx* resulted in the formation of an additional slower migrating band consistent with formation of a trimeric complex (Figure 5A, lane 6), similar to those observed in previous studies of *Hoxb1* and *Hoxa9*.^{15,33} This indicated that *Rnx* has the capacity to assemble into a higher order, trimeric DNA-binding complex with TALE homeodomain proteins *Pbx3* and *Meis1a*.

To assess the functional consequences of trimeric *Rnx/Pbx3/Meis* interactions, transient transfection assays were performed. For these studies, a reporter gene that contained the modified enhancer situated upstream of the SV40 promoter was used in human embryonic kidney cells, which are permissive for *Hox* transcriptional activity. Co-transfection of expression constructs *Pbx3a* and *Meis1a* displayed minimal activation above background (Figure 5B), as previously noted for comparable TALE heterodimers.^{15,33} Co-transfection of *Rnx*, *Pbx3a*, and *Meis1a*, however, resulted in a 7.5-fold activation above background levels of transcription obtained with *Rnx* alone. In contrast, *Rnx* and *Meis1a* in the absence of *Pbx3a* displayed a reduced level of activation that approximated only a third of that observed in the presence of all three homeodomain proteins (Figure 5B). These data reveal trimeric interactions between *Rnx*, *Pbx3a*, and *Meis1a* function to promote heightened transcriptional activation, and highlight the critical role of *Pbx3a* in trimeric transcriptional activity.

Discussion

The respiratory pattern in *Pbx3*^{-/-} mice is characterized by irregular amplitude of inspiration and reduced respi-

Figure 4. Co-localization of Pbx3 and Rnx in hindbrain neurons. **A:** Immunohistochemical analysis of Pbx3a in a coronal section of the medulla oblongata in wt E15 embryo (magnification, $\times 25$). **Red box** corresponds to the fields examined by immunofluorescence at higher magnification. Pbx3a (green) and Rnx (red) are co-localized in neurons as evidenced by the merged image (yellow) obtained by fluorescence microscopy (magnification, $\times 600$). DAPI stain (blue) highlights nuclei. **B:** Immunohistochemical analysis of wt embryos at E15 shows similar patterns of expression for Pbx3a and Meis proteins in coronal sections of the medulla (magnification, $\times 40$). **C and D:** Immunohistochemical analysis shows nuclear expression of Meis and Rnx in $Pbx3^{-/-}$ mice (magnification, $\times 125$).

ratory frequency. Although a higher than normal frequency of C4 inspiratory neuronal activity is observed in the *in vitro* brainstem preparations of $Pbx3^{-/-}$ mice, the burst amplitude of the phrenic motoneurons is significantly smaller than wt. This reduced amplitude likely produces insufficient pressure changes by ventilatory movement in some respiratory cycles leading to a significant decrease of minute respiratory volume in $Pbx3^{-/-}$ mice, and appears insufficient to fully inflate the lungs. Importantly, the coordinate patterns of membrane potentials for inspiratory neurons in the ventrolateral medulla and C4 motoneuronal outputs indicate that an underlying basis for respiratory failure in $Pbx3^{-/-}$ mice is dysfunction of central respiratory networks in the medulla. We cannot exclude a possibility that abnormality of afferent inputs contributes to respiratory dysfunction *in vivo*.

Dysfunction of central respiratory networks has been reported in *Rnx*-deficient mice.¹⁴ *Rnx* is one of three *Hox11*-related orphan homeobox genes that encode homeodomain-containing transcription factors, which also possess conserved motifs implicated in Pbx dimerization. Our studies herein demonstrate that Pbx3 forms a DNA-binding complex with Rnx and enhances its transcriptional properties. Although the three family members (*Hox11*, *Rnx*, and *Enx*) display extensively overlapping patterns of embryonic expression, mice deficient for each

display major phenotypes at sites where the mutated gene is singularly expressed,^{14,34–36} which is the medulla for *Rnx*. The respiratory pattern in $Rnx^{-/-}$ mice is characterized by more frequent and prolonged apneas than $Pbx3^{-/-}$ mice, possibly reflecting the contributions of non-dependent functions of the respective proteins. The respiratory defect in *Rnx*-deficient mice has recently been proposed to result from compromised development of the nucleus of the solitary tract, a major relay station for visceral sensory afferents in the medulla,³⁷ as opposed to primary defects of rhythmic respiratory neurons. The *Rnx*-related *Hox11*, as well as *Rnx*, is also required for proper development of most relay sensory neurons.³⁸ More refined analyses are required to determine whether Pbx3 may contribute to development of relay somatic sensory neurons and whether the Pbx3 respiratory defect results from compromise of *Rnx* and/or *Hox11* function. Nevertheless, deficiencies of either Pbx3 or *Rnx*, which are capable of forming active transcriptional complexes *in vitro*, result in abnormal respiration attributable in part to dysfunction of hindbrain neurons.

Mice deficient for clustered *Hox* genes display reorganization of the hindbrain (*Hoxa1*, *a2*),^{39–41} defects in cranial nerve nuclei (*Hoxb1*, *b2*, and *a2*),^{41–44} or branchial arch homeotic transformation (*Hoxa2*).⁴¹ These do not appear to be features of $Pbx3^{-/-}$ mice, despite

Figure 5. Pbx3 forms a DNA-binding transcriptional complex with Rnx and Meis. **A:** Electrophoretic mobility shift assay (EMSA) was performed with *in vitro* produced Pbx3a, Meis1a, and Rnx proteins. The DNA probe consisted of a modified HoxB2 r4 enhancer, which contains sites for Pbx, Meis, and Hox binding. The Hox site was altered to provide optimal binding for Rnx. A trimeric complex with slower mobility than the dimeric Pbx3a-Meis1a complex (lane 5) was observed in the presence of Pbx3a, Meis1a, and Rnx (lane 6). Lane contents are indicated at top. **B:** A transient transfection assay was performed using a reporter construct containing the modified HoxB2 r4 enhancer positioned upstream of a minimal promoter/luciferase gene. Expression constructs for Pbx3a, Meis1a, and Rnx were co-transfected alone or in combination. Increased transcriptional activation was observed in the presence of Pbx3a, Meis 1a, and Rnx compared to that observed with various dimeric combinations. Lane contents are indicated below.

previous studies implicating Pbx TALE homeodomain proteins in the regulation of *Hoxb1* and *Hoxb2* expression in the embryonic hindbrain.^{15,32,45,46} Hindbrain patterning phenotypes, however, have been noted in *Pbx1*^{-/-} embryos,¹⁰ suggesting independent roles for Pbx family proteins in hindbrain development. *Pbx3*^{-/-} mice also contrast with *Krox-20*^{-/-} mice, in which rhombomeres 3 and 5 are eliminated and deficits in rhythmic activity were localized to the pontine reticular formation, not the ventral medullary respiratory center.⁴⁷

Defects in the amplitude and frequency of breathing are characteristic of congenital central hypoventilation syndrome (CCHS), which results in potentially lethal hypoxia. Thus, *Pbx3*^{-/-} mice may be a model for CCHS, similar to previous suggestions for *Rnx*-deficient mice,¹⁴ implicating downstream pathways regulated by heterooligomeric Pbx3 transcriptional complexes as critical for development of hindbrain neurons involved in control of respiration. The molecular pathogenesis of CCHS is known for only the small subset of patients harboring mutations of the RET/GDNF and EDNRB/EDN3 receptor/ligand pairs.⁴⁸ Up to 50% of patients with CCHS also

manifest Hirschsprung's disease,⁴⁹ a clinical constellation referred to as Haddad syndrome, characterized by congenital failure of autonomic control of ventilation and gastrointestinal motility.⁵⁰ Mice that are deficient for *Enx*, another *Hox11*-related orphan homeobox gene, are characterized by hyperganglionic megacolon.³⁶ Interestingly, Pbx3 is also expressed at high levels in myenteric ganglionic neurons of the ileum and colon raising the possibility that loss of Pbx3 may also compromise *Enx* function, which, as a close homolog of Rnx, would be expected to bind DNA as a complex with Pbx3. Due to the demise of *Pbx3*^{-/-} mice within hours of birth, we were unable to evaluate for development of megacolon. Nevertheless, our studies suggest the broader hypothesis that mutations of Pbx3, its cofactors, targets, or regulators may promote the pathogenesis of a subset of CCHS, which warrants further investigation.

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