Mutation in *LBX1/Lbx1* precludes transcription factor cooperativity and causes congenital hypoventilation in humans and mice

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The respiratory rhythm is generated by the preBötzinger complex in the medulla oblongata, and is modulated by neurons in the retrotrapezoid nucleus (RTN), which are essential for selective hypoventilation in response to high CO₂. Here we identify a *LBX1* frameshift (*LBX1*Δ27Ala) mutation in patients with congenital central hypoventilation. The mutation alters the C-terminus but not the DNA-binding domain of *LBX1*. Mice with the analogous mutation recapitulate the breathing deficits found in humans. Furthermore, the mutation only interferes with a small subset of *Lbx1* functions, and in particular with development of RTN neurons that coexpress *Lbx1* and *Phox2b*. Genome-wide analyses in a cell culture model show that *Lbx1*- and wild-type *Lbx1* proteins are mostly bound to similar sites, but that *Lbx1*Δ27Ala is unable to cooperate with *Phox2b*. Thus, our analyses on *Lbx1*- and *Phox2b*-function reveals an unusual pathomechanism; that is, a mutation that selectively interferes with the ability of *Lbx1* to cooperate with *Phox2b*, and thus impairs the development of a small subpopulation of neurons essential for respiratory control.

**Significance**

Maintaining low CO₂ levels in our bodies is critical for life and depends on neurons that generate the respiratory rhythm and monitor tissue gas levels. Inadequate response to increasing levels of CO₂ is common in congenital hypoventilation diseases. Here, we identified a mutation in *LBX1*, a homeodomain transcription factor, that causes congenital hypoventilation in humans. The mutation alters the C-terminus of the protein without disturbing its DNA-binding domain. Mouse models carrying an analogous mutation recapitulate the disease. The mutation spares most *Lbx1* functions, but selectively affects development of a small group of neurons central in respiration. Our work reveals a very unusual pathomechanism, a mutation that hampers a small subset of functions carried out by a transcription factor.

**Author contributions:** L.R.-M., D.M.I., and C.B. designed research; L.R.-M., D.M.I., P.-L.R., M.L., M.W., W.d.W., J.-F.B., and M.C. performed research; J.-F.B. contributed new reagents/analytic tools; L.R.-M., G.F., S.M., and C.B. analyzed data; and L.R.-M. wrote the paper. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1813520115/-/DCSupplemental.

Published online November 28, 2018.

www.pnas.org/cgi/doi/10.1073/pnas.1813520115
protein without affecting its homeodomain. Homozygous mice carrying the analogous mutation (Lbx1<sup>FS/FS</sup>) displayed respiratory deficits that recapitulated the human phenotype. In Lbx1<sup>FS/FS</sup> mice, two Lbx1<sup>-</sup>/Phox2b<sup>+</sup> neuronal subpopulations (in the RTN and in the dorsal hindbrain) were severely affected, but in contrast to Lbx1 null mutants, second-order somatosensory neurons and limb skeletal muscle formed correctly. Genomewide DNA binding analysis of Lbx1<sup>FS</sup> in a cell culture model showed that the mutant variant mostly binds to similar sites as the wild-type protein. However, in contrast to the wild-type protein, Lbx1<sup>FS</sup> is unable to correctly cooperate with Phox2b, and instead overrides its function. Thus, the Lbx1<sup>FS</sup> protein is selectively impaired in a transcriptional cooperativity with Phox2b during neuronal development, but functions correctly in other contexts.

**Results**

A Homozygous LBX1 Frameshift Mutation Causes Recessive Congenital Central Hypoventilation Syndrome. We identified two male siblings, offspring from a consanguineous marriage, who displayed hypoventilation during the neonatal period. The parents were unaffected and had a healthy daughter (Fig. 1A). Two sisters from the father/mother side of the patients lost a child to cot death (SI Appendix, Fig. S1A). Both affected siblings studied here required continuous mechanical ventilation after birth because of respiratory insufficiency. They showed recurrent episodes of apnea and signs of central hypventilation during sleep with no response to falling oxygen saturation or hypercapnia. The children were diagnosed with a severe pattern of classic CCHS. Sanger sequencing, microsatellite analysis, and multiplex ligation-dependent probe amplification of DNA from the children did not reveal any mutations in PHOX2B. Lbx1 ablation causes hypoventilation in newborn mice (6, 7). We therefore sequenced LBX1 in the affected individuals and identified a homozygous frameshift mutation in its exon 2 (LBX1<sup>mut</sup> mutation; SI Appendix, Fig. S1B and C). Sanger sequencing of the entire family confirmed that the LBX1<sup>mut</sup> mutation segregated with the phenotype. The mutation was predicted to alter the Lbx1 protein at the C terminus without affecting its homeodomain (SI Appendix, Fig. S1C). Furthermore, the LBX1<sup>FS</sup> variant was absent in control cohorts such as Exome Aggregation Consortium and 1000 Genomes. Ablation of Lbx1 in mice results in a complex phenotype resulting from defects in the development of various hindbrain neuronal subtypes (db1–db4; see scheme in Fig. 1B), as well as deficits in the formation of dorsal spinal cord neurons and limb skeletal muscle (4–10, 23). However, the children carrying the LBX1<sup>FS</sup> mutation did not show any obvious change in limb musculature. We thus reasoned that the LBX1<sup>FS</sup> mutation might selectively impair neurons that participate in the central control of respiration.

**Similarities in the Genome-Wide Binding of Lbx1 and Lbx1<sup>FS</sup>.** To model alterations of Lbx1<sup>FS</sup> function and its binding to DNA in a neuronal context, we looked for a suitable neuronal cell line in the system. P19 murine embryonic teratocarcinoma stem cells differentiate into neurons that express Lbx1 and a HoxA gene code typical of the caudal hindbrain (rhombomeres 4–7) and anterior cervical spinal cord upon retinoic acid treatment (SI Appendix, Fig. S1 D and E) (24). In addition, they express Lmx1b, Pou4f1, and Prx1, the latter at low levels (SI Appendix, Fig. S1F); this combination is indicative of excitatory somatosensory neurons of the spinal cord and hindbrain. We used this model to analyze Lbx1 and Lbx1<sup>FS</sup> binding on a genome-wide scale. The endogenous Lbx1 locus was first mutated in these cells using CRISPR-Cas9, and the resulting Lbx1 mutant cells were transduced with retroviruses encoding flag-tagged Lbx1 or Lbx1<sup>FS</sup> (referred to as Lbx1<sup>-/−</sup> and Lbx1<sup>−/−</sup> cells). Cell clones that expressed comparable levels of Lbx1/Lbx1<sup>FS</sup> were chosen for ChIP-seq analysis. In neurons differentiated from such cells (named Lbx1<sup>-/−</sup> and Lbx1<sup>-/-FS</sup> neurons), we identified 7,537 binding sites for Lbx1, but considerably more (n = 12,343) sites for Lbx1<sup>-FS</sup>. A large fraction (59%) of the Lbx1 sites was also bound by Lbx1<sup>FS</sup> (SI Appendix, Fig. S1G). To analyze how the 1.6-fold increase in Lbx1<sup>FS</sup> binding sites related to binding strength, another important variable for transcription factor function, we performed read enrichment analysis combined with k-means clustering for Lbx1<sup>-/−</sup> and Lbx1<sup>-/-FS</sup>-bound sites. In general, the mean read density for Lbx1<sup>-FS</sup> was lower than for Lbx1 (Fig. 1C). Sites in which Lbx1<sup>-FS</sup> bound more strongly than Lbx1 displayed, on average, low enrichment for both Lbx1<sup>-FS</sup> and Lbx1<sup>-/−</sup> proteins (Fig. 1C). Together, our data show that the Lbx1<sup>-FS</sup> mutant protein can bind to most Lbx1 sites; however, the binding is weaker and less specific than that of the wild-type protein.

In Lbx1<sup>-/−</sup> and Lbx1<sup>-FS</sup> ChIP-seq tracks revealed occupancy of both factors on intronic and intergenic regions associated with the somatosensory genes Prx1l, Lmx1b, and Pou4f1 (Fig. 1D and SI Appendix, Fig. S2A). The occupancy of Lbx1/Lbx1<sup>FS</sup> on such loci was confirmed by ChIP-qPCR (Fig. 1E and SI Appendix, Fig. S2B). Similar gene expression levels for the three somatosensory genes were observed in neurons differentiated from Lbx1 and Lbx1<sup>-FS</sup> cells, but they were not expressed in Lbx1<sup>-/−</sup> mutant neurons (SI Appendix, Fig. S2C). To test whether these intronic and intergenic regions correspond to enhancer elements, we performed ChIP-qPCR for H3K27ac and H3K27me3, two epigenetic marks associated with active or repressed enhancers, respectively (25). This showed strong enrichment for H3K27ac at the analyzed loci in neurons differentiated from Lbx1 and Lbx1<sup>-FS</sup> cells, whereas H3K27me3 was not enriched (Fig. 1F and SI Appendix, Fig. S2D). Hence, Lbx1/Lbx1<sup>-FS</sup> binding sites on the Lmx1b,
Prox1, and Pou4f1 loci correspond to active enhancers in Lbx1 and Lbx1<sup>FS</sup> neurons.

We next performed de novo motif analysis for Lbx1 and Lbx1<sup>FS</sup> binding sites. In both peak sets, various AT-rich motifs that aligned with previously identified Lbx1-binding sites were overrepresented (26). A closer inspection revealed subtle differences between Lbx1 and Lbx1<sup>FS</sup> sequence preferences (SI Appendix, Fig. S3). The most significantly enriched motif in Lbx1<sup>FS</sup> peaks was a nonpalindromic 8-mer, possibly representing a monomer-binding site, which was identified in Lbx1 sites as the fifth most significant. In contrast, the most overrepresented motif in Lbx1 sites was a 12-mer palindrome, possibly a homodimeric site that was the third-most enriched motif for Lbx1<sup>FS</sup>. Finally, a 16-bp-long nonpalindromic motif was identified in Lbx1, but not Lbx1<sup>FS</sup> sites (SI Appendix, Fig. S3A). This was composed of a partial Lbx1 site at the 3′ end, preceded with a distinct AT-rich 5′ sequence, which could represent the binding site of an Lbx1 cofactor. Interestingly, this AT-rich half-site was reported to be a preferred Phox2b binding site (SI Appendix, Fig. S3B) (27). This raised the possibility that although the general DNA binding of Lbx1<sup>FS</sup> was only mildly compromised, its ability to interact with other factors is more severely impaired.

Hypventilation and Lack of Hypercapnic Reflex in Homozygous Lbx1<sup>FS/FS</sup> Mice. To better understand the deficit in Lbx1<sup>FS</sup> function, we introduced an analogous mutation into the mouse Lbx1 gene (SI Appendix, Fig. S4A). Heterozygous Lbx1<sup>+</sup>/<sup>−</sup> (Lbx1<sup>FS/FS</sup>) mice were viable and fertile, and did not show an obvious phenotype. However, homozygous Lbx1<sup>FS/FS</sup> newborn mice displayed cyanosis and died (n = 18/18) within the first 2 h of life without displaying any apparent deficits in motor behavior. Pletysmographic recordings revealed pronounced respiratory deficits in Lbx1<sup>FS/FS</sup> mice; that is, shallow breathing with frequent and long apneas (Fig. 2 A and B and SI Appendix, Fig. S5 A–C). Importantly, Lbx1<sup>FS/FS</sup> mice lacked the hypercapnic reflex and did not change ventilation (V<sub>E</sub>) and severe hyperventilation (Fig. 2 A and B and SI Appendix, Fig. S5 A–C). In particular, Lbx1<sup>FS/FS</sup> mice displayed longer times between breathing cycles (T<sub>tot</sub>), which led to reduced respiratory minute volumes (V<sub>E</sub>) and severe hyperventilation (Fig. 2 A and B and SI Appendix, Fig. S5 A–C). We concluded that the Lbx1<sup>FS/FS</sup> mutation in mice leads to a respiratory phenotype that resembles the one observed in the studied patients.

The Lbx1<sup>FS/FS</sup> Mutation Interferes with RTN Formation. We next assessed whether the lack of hypercapnic response in Lbx1<sup>FS/FS</sup> mice was a result of impaired RTN development. RTN neurons locate in the ventral hindbrain and coexpress Lbx1 and Phox2b, but not choline acetyl-transferase (ChAT) (Lbx1<sup>−/−</sup>/Phox2b<sup>+/−</sup>/ChAT<sup>−/−</sup>), and are thus distinguished from the neighboring motor neurons that coexpress Phox2b and ChAT, but not Lbx1 (Lbx1<sup>−/−</sup>/Phox2b<sup>−/−</sup>/ChAT<sup>−/−</sup>). In Lbx1<sup>FS/FS</sup> animals, Phox2b<sup>−/−</sup>/Lbx1<sup>−/−</sup> cells were absent in the RTN region either at embryonic day (E) 14.5 or at birth (Fig. 2 C). However, several other Lbx1<sup>−/−</sup> neuronal types were present and expressed Lbx1 at apparently normal levels. The absence of a functional RTN was confirmed by Ca<sup>2+</sup> imaging (SI Appendix, Fig. S5 D and E). Further analyses demonstrated that RTN precursors (i.e., Lbx1<sup>−/−</sup>/Phox2b<sup>−/−</sup> dB2 neurons) were unchanged in Lbx1<sup>FS/FS</sup> mice at E11.5, but failed to initiate Atoh1 expression during their migration toward the ventral hindbrain at E12.5 (SI Appendix, Fig. S5 F–H). preBötzinger complex neurons have no history of Phox2b or Lbx1 expression (28), and were present and functional in Lbx1<sup>FS/FS</sup> mice (SI Appendix, Fig. S5I). We conclude that in Lbx1<sup>FS/FS</sup> mice, dB2 neuronal precursors are correctly specified, but the subset destined to form the RTN fails to express Atoh1 and does not migrate into the position where the RTN normally resides.

The Lbx1<sup>FS/FS</sup> Mutation Does Not Preclude SpV and Limb Muscle Development. Next we analyzed inhibited and excitatory somatosensory neurons of the spinal trigeminal (SpV) nucleus. These neurons are absent in Lbx1<sup>−/−</sup> null mutant mice, where they instead assumed solitary tract nucleus and inferior olivary nucleus neuronal fates, respectively (7). Interestingly, the SpV was present in Lbx1<sup>FS/FS</sup> mice (Fig. 3 A). Furthermore, the solitary tract and inferior olivary nuclei appeared to have a normal size (Fig. 3 A). Finally, limb muscle development is severely affected in Lbx1<sup>−/−</sup> null mutant mice (8–10), but these muscle groups were present and appeared correctly formed in Lbx1<sup>FS/FS</sup> mice (Fig. 3 B). Together, our analyses demonstrate that the Lbx1<sup>FS/FS</sup> mutation selectively interferes with development of Lbx1<sup>−/−</sup>/Phox2b<sup>−/−</sup> RTN neurons, but in other contexts, the mutant protein functions correctly, as in development of somatosensory SpV neurons and limb muscles.

dB2 Neurons Are Responsible for the Breathing Deficits Observed in Lbx1<sup>FS/FS</sup> Mice. To assess whether the breathing deficits observed in Lbx1<sup>FS/FS</sup> mice exclusively depend on dysfunction of dB2 derivatives, we conditionally restricted the Lbx1<sup>FS</sup> mutation to the dB2 lineage by using Phox2b<sup>cre</sup> (Phox2b<sup>cre</sup>;<Lbx1<sup>FS/lox</sup>) mice (see SI Appendix, Fig. S6A for a scheme of the strategy). In such animals, neurons with a history of Phox2b expression carried an Lbx1<sup>FS</sup> genotype, but other cells (Lbx1<sup>FS/lox</sup>) retained one copy of a fully functional Lbx1<sup>FS</sup> allele (SI Appendix, Fig. S4B). In dB2-Lbx1<sup>FS</sup> animals, RTN neurons were absent (Fig. 4 A). Pletysmographic recordings of dB2-Lbx1<sup>FS</sup> animals showed a full recapitulation of the physiological phenotype observed in Lbx1<sup>FS/FS</sup> animals (i.e., severe hypoventilation, lack of the hypercapnic reflex, frequent apneas; SI Appendix, Fig. S6 C–G;
summarized in Fig. 4B), as well as lethality (n = 12/12) within the first 2 h of life. Thus, all respiratory deficits associated with the Lbx1F5 mutation are the result of a selective developmental deficit in the dB2 neuronal lineage.

RTN neurons arise from rhombomere 5 (29). We next restricted the Lbx1F5 mutation to rhombomeres 3 and 5, using Egr2F2 to only recombines cells in these rhombomeres (Egr2F2/−; Lbx1F5lox/lox; named Egr2-Lbx1F5F5 mice; see SI Appendix, Fig. S6B for a scheme of the strategy). As expected, RTN neurons were absent in Egr2-Lbx1F5F5 animals (Fig. 4A). Plethysmographic recordings of Egr2-Lbx1F5F5 mice showed that they were unable to respond to high CO2 levels in the air (SI Appendix, Fig. S6C–G). Nevertheless, Egr2-Lbx1F5F5 mice did not display apneas and survived the postnatal period (n = 11/11), with a mild hyperventilation that was observed in their early postnatal life (SI Appendix, Fig. S6D and H). The response of Egr2-Lbx1F5 mice to high levels of CO2 improved with maturation, but even adult mutants presented a blunted hypercapnic reflex (SI Appendix, Fig. S6H). This phenotype, largely similar to the one observed in dB2-Lbx1F5 mice (Fig. 4C), implies that several neuronal groups originating from dB2 precursors participate in the control of breathing.

We next used intersectional lineage tracing to specifically label dB2 derivatives with Tomato fluorescent protein, using Lbx1F5-cre/−; Phox2bCre/−; Ai65+− animals (see SI Appendix, Fig. S7A for a scheme of the strategy). TomatoF2/Lbx1F5/Phox2bF5 cells were found, in addition to the RTN, around the trigeminal motor nucleus in rhombomeres 1 and 2 (a population known as perIV neurons, as well as in the dorsal part of rhombomeres 3–6 (SI Appendix, Fig. S7B–D). We compared development of these two dB2 derivatives (perIV neurons and neurons in the dorsal part of the hindbrain) in strains displaying the most severe breathing phenotype (i.e., Lbx1F5F5, dB2-Lbx1F5F5) and the milder breathing deficit (Egr2-Lbx1F5F5). Lbx1F5/Phox2bF5 perIV neurons were present in normal numbers in all analyzed strains (quantified in Fig. 4B). However, the number of dorsally located Lbx1F5/Phox2bF5 neurons was severely reduced in Lbx1F5F5 and dB2-Lbx1F5F5 animals, but not obviously affected in Egr2-Lbx1F5F5 mice (Fig. 4B and SI Appendix, Fig. S7E). Thus, the absence of the RTN combined with the reduction of the dorsal Lbx1F5/Phox2bF5 population correlates with the severe breathing phenotype observed in Lbx1F5F5 and dB2-Lbx1F5F5 mutants.

**Ectopic Expression of Somatosensory Genes in Lbx1F5/F5 Phox2b Expressing Neurons.** To assess whether the absent dB2 neurons in Lbx1F5/F5 mice assumed an aberrant neuronal fate, we extended our intersectional genetic lineage tracing to Lbx1F5 (Lbx1F5/−; Phox2bF5/F5; Ai65F5/−, see SI Appendix, Fig. S7A) mutant mice. This demonstrated that ectopic TomatoF5 cells appeared in the somatosensory SpV nucleus of Lbx1F5 mice, which were not observable in control animals (Fig. 5A and SI Appendix, Fig. S8A). These ectopic TomatoF5 cells coexpressed markers of excitatory somatosensory neurons such as Prrx1 or Lmx1b (Fig. 5A and B and SI Appendix, Fig. S8B). Thus, the Lbx1F5 mutation selectively affects the development of an Lbx1F5/Phox2bF5 dB2 subpopulation that adopts an aberrant somatosensory fate.

We next modeled the (dys)function of Lbx1F5 in Phox2bF5 neurons, using our cell culture model. For this, Lbx1 mutant P19 mice were transduced with retroviruses encoding a HA-tagged version of Phox2b (hereafter Phox2b cells) alone or in combination with flag-tagged Lbx1 or Lbx1F5 (Lbx1/F5/Phox2b/Phox2b cells). We then sequenced the transmitters of neurons differentiated from these cells. Hierarchical expression clustering showed that Phox2b, Lbx1/Phox2b and Lbx1F5/Phox2b neurons were clearly distinct from Lbx1 neurons and clustered separately (Fig. 5C). Nevertheless, Lbx1 and Lbx1F5/Phox2b neurons were more closely related to each other than to Lbx1/Phox2b or Phox2b neurons (Fig. 5C). Interestingly, the Prrx1, Lmx1b, and Pou4F1 somatosensory genes were among the

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.1813520115)

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**Fig. 3.** Development of somatosensory neurons and limb muscle in Lbx1F5/FS mice. (A) Histological analysis of somatosensory neurons of the Sp5, viscerosensory neurons of the nucleus of the solitary tract (NTS), and neurons of the inferior olive (IO) in control (Left), Lbx1 null (Lbx1F2/−; Middle), and Lbx1F5/FS (Right) mutant newborn mice. Pax2 (green) and Lmx1b (red) antibodies distinguish inhibitory and excitatory somatosensory neurons of the spinal trigeminal nucleus, respectively. NTS neurons express Lmx1b, and inferior olivary neurons express FosP2 (blue). (B) Histological analysis of limb muscles in control, Lbx1F2/−, and Lbx1F5/FS newborn mice, using antibodies against laminin (Lam, red) and desmin (green). Confocal tile scan modus was used to acquire photomicrographs, and assembled using ZEN2012 software (10% overlap between tiles). Photomicrographs were mounted on a black frame to maintain figure panel proportions.

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**Fig. 4.** Conditional mutagenesis restricts the Lbx1F5 mutation to specific neuronal subpopulations. (A) Analysis of Lbx1F5 (red) Phox2bF5 (green) RTN neurons (arrowheads) in control, dB2-Lbx1F5F5 and Egr2-Lbx1F5F5 newborn mice. DAPI (blue) was used as a counterstain, and the facial (nVII) motor nucleus is indicated. Confocal tile scan module was used to acquire photomicrographs, and assembled using ZEN2012 software (10% overlap between tiles). (B) Top) Comparison of dB2 neuron numbers: RTN neurons [one-way ANOVA, F (3, 15) = 883.4; dorsal Lbx1F5/Phox2bF5 neurons [one-way ANOVA, F(3, 15) = 370] and perIV neurons [one-way ANOVA, F(3, 15) = 0.1562] in control (n = 6) Lbx1F5 (n = 4); Egr2-Lbx1F5 (n = 4), and dB2-Lbx1F5 (n = 4) mice. (B, Bottom) Comparison of ventilatory minute volumes in normal air and in hypercapnia in control (n = 20) Lbx1F5F5 (n = 11), Egr2-Lbx1F5F5 (n = 11), and dB2-Lbx1F5F5 (n = 12) mice (unpaired nonparametric Mann–Whitney U test).
most significant and differentially expressed genes in Lbx1<sup>FS</sup>/Phox2b<sup>+</sup> neurons compared with Phox2b or Lbx1/Phox2b<sup>+</sup> neurons (Fig. 5D). Thus, Phox2b represses these somatosensory genes alone or even when Lbx1 is present, but this does not occur when Lbx1<sup>F</sup>S and Phox2b are coexpressed, a change reminiscent of the one observed in vivo where Lbx1<sup>FS</sup>/Phox2b<sup>+</sup> (dB2) neurons assumed an aberrant somatosensory fate.

Next we analyzed chromatin modifications of the previously characterized enhancers of Prx11, Lmx1b, and Pou4f1 somatosensory genes. In Lbx1/Phox2b neurons, ChiP-qPCR showed a modest enrichment of Lbx1 and Phox2b at the analyzed loci (Fig. 5E and SI Appendix, Fig. S9). Moreover, the chromatin mark H3K27me3 was enriched in those sites, demonstrating that the enhancers are repressed. However, when the chromatin of Lbx1<sup>FS</sup>/Phox2b neurons was used for ChiP-qPCR experiments, Lbx1, Phox2b, and H3K27ac were significantly enriched at the Prx11, Lmx1b, and Pou4f1 enhancers (Fig. 5E and SI Appendix, Fig. S9). Thus, enhancer sequences of the Prx11, Lmx1b, and Pou4f1 genes are activated when Lbx1<sup>FS</sup> and Phox2b are recruited to these sites, but repressed when Lbx1 and Phox2b are recruited.

**Discussion**

Respiratory disorders in humans range from irregular and unstable respiration to the complete loss of breathing control. The most common causes of congenital hypoventilation are dominant mutations in *PHOX2B* that affect the formation of the RTN. Here we show that a homozygous frameshift mutation in *LBX1* causes severe congenital hypoventilation that resembles classical CCHS. We used cell culture and mouse models to investigate the (dys)function caused by the frameshift mutation, which alters the C-terminal sequence of the protein but spares its homeodomain. In most developmental contexts, the mutant protein exerts its role correctly; that is, the mutation only interferes with small subsets of Lbx1 functions. Our analysis has thus revealed a very unusual pathomechanism of a transcription factor mutation that results in a severe respiratory disorder.

**Lbx1<sup>FS</sup> Protein Correctly Functions in Most Developmental Contexts**

Our cell culture modeling of Lbx1<sup>FS</sup> binding showed that Lbx1<sup>FS</sup> and Lbx1 largely bind to similar sites genome-wide, which is in agreement with conserved functionality of the Lbx1<sup>FS</sup> protein in most developmental contexts. Motif analyses revealed subtle differences between the binding preferences of Lbx1 and Lbx1<sup>FS</sup>. In particular, a specific motif was present in Lbx1, but not in Lbx1<sup>FS</sup> binding sites, which consists of a 16-bp-long nonpalindromic sequence that is composed of an Lbx1-monodimer site combined with a half-site of another factor. Interestingly, the sequence that represents the second half-site corresponds to the preferred binding motif previously identified for Phox2b (27). This observation suggested a failure of Lbx1<sup>FS</sup> to cooperate productively with Phox2b.

Lbx1 and Phox2b are known to functionally repress each other: When Lbx1 is mutated, supernumerary Phox2b viscerosensory neurons arise (7). Vice versa, mutation of Phox2b results in the appearance of supernumerary somatosensory Lbx1 neurons (30). Remarkably, development of the dB2 lineage depends on both Lbx1 and Phox2b and relies on the repression of
the somatosensory genes (7, 13). Lbx1-dependent differentiation of somatosensory neurons can be modeled in vitro and occurs in the presence of Lbx1 and Lbx1FS. Interestingly, coexpression of Phox2b represses somatosensory genes in Lbx1+ but not Lbx1FS+ neurons. In the presence of Phox2b, the altered C-terminal sequence of Lbx1 might impede the correct recruitment of coregulatory factors, thus accounting for the fact that Lbx1 is unable to correctly cooperate with Phox2b.

**Lbx1/Lbx1 in CCHS.** Here we demonstrate that the hypomorphic Lbx1FS mutation selectively interferes with the development of specific dB2 neuronal populations. Physiologically, Lbx1FS/FS mice display a plethora of respiratory deficits: slow and irregular breathing, lack of hypocapnic reflex, and frequent and prolonged apneas. Together, these deficits appear to result in neonatal lethality. We observed that the conditional restriction of the Lbx1FS mutation to the dB2 lineage (dB2-Lbx1FS mice) fully recapitulates the physiological phenotypes observed in Lbx1FS/FS mice. In contrast, the conditional restriction of the Lbx1FS mutation to rhombomeres 3 and 5 (Egr2-Lbx1FS mice) impaired RTN neuron development, abolished the hypocapnic reflex, and caused mild hypventilation, but not abnormal apneas or neonatal lethality. Interestingly, similar or even identical phenotypes are observed when the Phox2b+2ala mutation is restricted to rhombomeres 3 and 5 (22). Thus, the Lbx1FS mutation causes respiratory deficits that are in part, but not completely, a result of the loss of RTN neurons.

Last, we report in this study that dB2 precursors produce, in addition to the RTN and periV cells, an additional not previously described group of Lbx1+/Phox2b+ neurons that locate dorsally in rhombomeres 3–6. Because of the complexity of the developmental deficits displayed by Lbx1 null mutant mice, the contribution of individual cell populations to respiratory deficits had previously not been assessable. We used here intersecional genetic strategies to show that the combined deficits in development of RTN and the dorsal Lbx1+/Phox2b+ population correlated with severe hypventilation and neonatal lethality.

Further studies will be needed to define the connectivity and the exact function of this dorsal neuronal population.

**Materials and Methods**

**Research Involving Humans and Mice.** Venous blood and genomic DNA samples from humans were obtained by standard procedures. Written informed consent was obtained from all individuals. Experimental procedures and animal handling were conducted according to institutional protocols and guidance approved by the Max Delbrueck Center (Berlin), CNRS (Gif sur Yvette), Max Planck Institute for Genetics (Berlin), and the Ethics Committee of the Charité Universitätsmedizin (Berlin). Details on mouse strains are provided in **SI Appendix, SI Materials and Methods**.

**Histology.** Development of dB2 neuronal derivatives was assessed on 20-μm transverse hindbrain sections from control and mutant mice. Details on antibodies and in situ probes used in this study are provided in **SI Appendix, SI Materials and Methods**.

**Cell Cultures.** P19 embryonic teratocarcinoma cells were obtained from ATCC (CRL-1825) and differentiated into neurons using 1 μm retinoic acid (Sigma), as described (31). Details on CRISPR-CAS9 mutation of Lbx1 in P19 cells, retroviral infection, CHIP, and deep sequencing experiments are provided in **SI Appendix, SI Materials and Methods**.

**Physiology.** Unrestrained plethysmographic recordings of individual mouse pups were carried out as described (32). Further details on plethysmographic recordings and Ca2+ imaging studies can be found in **SI Appendix, SI Materials and Methods**.

**ACKNOWLEDGMENTS.** We thank Christo Goridis (Institut de Biologie, École Normale Supérieure) and Elijah Lowenstein (Max Delbrueck Center) for a critical reading of the manuscript, and Sven Buchert, Petra Stallerow, Claudia Päseler, and Sandra Autran for technical support. Funding for this work was provided by the European Commission (Marie Curie Fellowship 302477 to L.R.H.-M.), Deutsche Forschungsgemeinschaft (SFB 665), Excellence cluster NeuroCure and Helmholtz Association (C.B.), Agence Nationale pour la Recherche (ANR-15-CE16-0013-02 to J.-F.B. and G.F.), European Molecular Biology Organization (long-term fellowship 408-2016 to P.-L.R.), and Fondation pour la Recherche Médicale (DEQ20120323709 to G.F.).

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