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 accelerating respiration in response to high CO\(_2\). Here we identify a *LBX1* frameshift (*lbx1*\(^{-}\)) 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*\(^{+}\) is unable to cooperate with *Phox2b*. Thus, our analyses on *Lbx1*\(^{+}\) (dys)function reveal 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.

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eurons represent the most diverse cell population in animals. How this diversity is specified and maintained is incompletely understood. Available evidence shows that multiple transcription factors cooperate to control common as well as neuron-specific gene expression programs (1, 2). The combinatorial binding of such factors to regulatory elements in chromatin is key for gene expression (3). Homeodomain transcription factors, among them *Lbx1* and *Phox2b*, impose specific neuronal fates during development. In mice, *Lbx1* specifies distinct neuronal subtypes in the spinal cord and hindbrain (4–7), and it is also essential for limb muscle development (8–10). *Phox2b* controls development of central and peripheral visceral neurons (11, 12). In the hindbrain, a single neuronal population (*db2* neurons) coexpresses *Lbx1* and *Phox2b* and depends on both factors for proper development (6, 7, 13). A subpopulation of *db2* neurons forms the retrotrapezoid nucleus (RTN), a small group of cells in the ventral hindbrain that is central for the hypercapnic reflex; that is, the acceleration of breathing in response to increased partial pressure of CO\(_2\) levels (13–16).

Breathing is regulated unconsciously by the nervous system. The respiratory rhythm is generated by the preBötzinger complex located in the ventral hindbrain (17), and is modulated by RTN neurons and by other neuronal populations. Congenital central hypoventilation syndrome (CCHS, also known as Ondine’s curse; OMIM 209880) is a rare, life-threatening disorder characterized by slow and shallow breathing resulting from a deficiency in autonomic control of respiration.

Patients with CCHS are hypercapnic; that is, they have abnormally high levels of CO\(_2\) in the blood and lack the hypercapnic reflex (18). Atypical heterozygous expansions of alanine repeats in *PHOX2B* are the most common genetic causes of CCHS (12, 19), but similar phenotypes can also be caused by genetic abnormalities in *RET*, *EDN3*, and *MYO1H* (20, 21). The introduction of a frequent *PHOX2B* (*PHOX2B*\(^{+12\text{Ala}}\)) mutation into the murine genome precludes development of RTN neurons, causes loss of the hypercapnic reflex, produces severe hypoventilation, and results in neonatal lethality (13). However, the selective elimination of RTN neurons accounts only for the loss of the hypercapnic reflex, but not for the severe hypoventilation and the neonatal lethality observed in *Phox2b*\(^{+12\text{Ala}}\) mutant mice (22).

In this study, we report on a consanguineous family with two CCHS-diagnosed children that tested negative for *PHOX2B* mutations. The children carried a homozygous frameshift mutation in *LBX1* (*LBX1*\(^{+}\)) that alters the C terminus of the
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<sup>−/−</sup> 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 hypoventilation 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<sup>−/−</sup> ablation causes hypoventilation in newborn mice (6, 7). We therefore sequenced Lbx1<sup>−/−</sup> in the affected individuals and identified a homozygous frameshift mutation in its exon 2 (LBX1<sup>FS</sup> mutation; SI Appendix, Fig. S1B and C). Sanger sequencing of the entire family confirmed that the LBX1<sup>FS</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<sup>−/−</sup> 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/FS</sup> mutation did not show any obvious change in limb musculature. We thus reasoned that the LBX1<sup>FS/FS</sup> mutation might selectively impair neurons that participate in the central control of respiration.

**Fig. 1.** Genome-wide characterization of chromatin binding of a frameshift mutant LBX1 associated with CCHS in humans. (A) Pedigree of a family with two children diagnosed with CCHS (black). (B, Left) Transverse section of the developing hindbrain stained with Olig3 (red) and Lbx1 (green) antibodies at E11.5. Olig3 is expressed in the dorsal hindbrain, whereas Lbx1 is expressed in dB1–dB4 neurons. (B, Right) Scheme showing genes expressed in progenitor cells and neurons of the dorsal hindbrain. Lbx1 and Phox2b are coexpressed in dB2 neurons. (C, Left) Heat maps showing read tracks at sites occupied by Lbx1 and Lbx1<sup>FS</sup> ± 5 kb around the binding sites. (C, Right) Lbx1 higher (blue) and Lbx1<sup>FS</sup> (pink) mean read densities (MRDs) for distinct classes of binding sites. (D) ChiP-seq tracks illustrating Lbx1 and Lbx1<sup>FS</sup> occupancy on intergenic and intronic regions of Proxl1. (E) ChiP-qPCR analysis using antibodies against flag-tag to validate Lbx1 and Lbx1<sup>FS</sup> occupancy on the highlighted regions displayed in D (n = 4 independent replicates). (F) H3K27ac (Left) and H3K27me3 (Right) ChiP-qPCR analysis performed on chromatin prepared from Lbx1<sup>−/−</sup> and Lbx1<sup>FS/FS</sup> differentiated neurons (n = 4 independent replicates).
**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>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. Plethysmographic 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 hypcapnic reflex and did not change ventilation (V<sub>E</sub> and T<sub>tot</sub>) when exposed to high levels of CO<sub>2</sub> in air (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 hypcapnic 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>+/Phox2b<sup>+</sup>/ChAT</sup>), and are thus distinguished from the neighboring motor neurons that coexpress Phox2b and ChAT, but not Lbx1 (Lbx1<sup>−/Phox2b<sup>−</sup>/ChAT</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 C<sup>34</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.

**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>命名为dB2-Lbx1<sup>FS/FS</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/FS</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. 4A). Plethysmographic 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 hypventilation, lack of the hypcapnic 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 Lbx1FS 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 Lbx1FS mutation to rhombomeres 3 and 5, using Egr2FScre/cre;Phox2bFlox/Flox animals (Egr2FScre/cre;Lbx1FSlox/lox named Egr2-Lbx1FS mice; see SI Appendix, Fig. S6B for a scheme of the strategy). As expected, RTN neurons were absent in Egr2-Lbx1FS animals (Fig. 4A). Phlethysmographic recordings of Egr2-Lbx1FS mice showed that they were unable to respond to high CO2 levels in the air (SI Appendix, Fig. S6 C–G). Nevertheless, Egr2-Lbx1FS 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. S6 D and H). The response of Egr2-Lbx1FS 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 after conditional mutation of Phox2b2/2ala in rhombomeres 3 and 5 (22), 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 Lbx1+/−;Pax2Ftact/lox;Egr2Ftact/lox;Ai65 animals (see SI Appendix, Fig. S7A for a scheme of the strategy). Tomato+/Lbx1+/Phox2b+ cells were found, in addition to the RTN, around the trigeminal motor nucleus in rhombomere 1 and 2 (a population known as perIV neurons, as well as in the dorsal part of rhombomeres 3–6 (SI Appendix, Fig. S7 B–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., Lbx1FS/FS, dB2-Lbx1FS) and the milder breathing deficit (Egr2-Lbx1FS). Lbx1+/Phox2b+ perIV neurons were present in normal numbers in all analyzed strains (quantified in Fig. 4B). However, the number of dorsally located Lbx1+/Phox2b+ neurons was severely reduced in Lbx1FS/FS and dB2-Lbx1FS animals, but not obviously affected in Egr2-Lbx1FS mice (Fig. 4B and SI Appendix, Fig. S7E). Thus, the absence of the RTN combined with the reduction of the dorsal Lbx1+/Phox2b+ population correlates with the severe breathing phenotype observed in Lbx1FS/FS and dB2-Lbx1FS mutants.

**Ectopic Expression of Somatosensory Genes in Lbx1FS/Phox2bExpressing Neurons.** To assess whether the absent dB2 neurons in Lbx1FS/FS mice assumed an aberrant neuronal fate, we extended our intersectional genetic lineage tracing to Lbx1FS (Lbx1cre;Phox2bFlpFrt;Ai65SorFl/+), see SI Appendix, Fig. S7A) mutant mice. This demonstrated that ectopic Tomato+ cells appeared in the somatosensory SpV nucleus of Lbx1FS mice, which were not observable in control animals (Fig. 5A and SI Appendix, Fig. S8A). These ectopic Tomato+ cells coexpressed markers of excitatory somatosensory neurons such as Prrx1 or Lmx1b (Fig. 5 A and B and SI Appendix, Fig. S8B). Thus, the Lbx1FS mutation selectively affects the development of an Lbx1+/Phox2b+ dB2 subpopulation that adopts an aberrant somatosensory fate.

We next modeled the (dys)function of Lbx1FS in Phox2b+ 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 Lbx1FS (Lbx1/Phox2b and Lbx1FS/Phox2b cells). We then sequenced the transcripomes of neurons differentiated from these cells. Hierarchical expression clustering showed that Phox2b, Lbx1/Phox2b, and Lbx1FS/Phox2b neurons were clearly distinct from Lbx1 neurons and clustered separately (Fig. 5C). Nevertheless, Lbx1 and Lbx1FS/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
most significant and differentially expressed genes in Lbx1^{FS}/Phox2b^{+} neurons compared with Phox2b or Lbx1/Phox2b^{+} neurons (Fig. 5D). Thus, Phox2b represses these somatosensory genes alone or even when Lbx1 is present, but this does not occur when Lbx1^{FS} and Phox2b are coexpressed, a change reminiscent of the one observed in vivo where Lbx1^{FS}/Phox2b^{+} (dB2) neurons assumed an aberrant somatosensory fate.

Next we analyzed chromatin modifications of the previously characterized enhancers of Prrxl1, 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^{FS}/Phox2b neurons was used for ChiP-qPCR experiments, Lbx1, Phox2b, and H3K27ac were significantly enriched at the Prrxl1, Lmx1b, and Pou4f1 enhancers (Fig. 5E and SI Appendix, Fig. S9). Thus, enhancer sequences of the Prrxl1, Lmx1b, and Pou4f1 genes are activated when Lbx1^{FS} 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 hypventilation 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 hypventilation 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^{FS} Protein Correctly Functions in Most Developmental Contexts.** Our cell culture modeling of Lbx1^{FS} binding showed that Lbx1^{FS} and Lbx1 largely bind to similar sites genome-wide, which is in agreement with conserved functionality of the Lbx1^{FS} protein in most developmental contexts. Motif analyses revealed subtle differences between the binding preferences of Lbx1 and Lbx1^{FS}. In particular, a specific motif was present in Lbx1, but not in Lbx1^{FS} 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^{FS} 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 Lbx1β. Interestingly, coexpression of Phox2b represses somatosensory genes in Lbx1β but not Lbx1β mice. In the presence of Phox2b, the altered C-terminal sequence of Lbx1β might impede the correct recruitment of coregulatory factors, thus accounting for the fact that Lbx1β is unable to correctly cooperate with Phox2b.

**Lbx1/Lbx1β in CCHS.** Here we demonstrate that the hypomorphic Lbx1β mutation selectively interferes with the development of specific dB2 neuronal populations. Physiologically, Lbx1β mice display a plethora of respiratory deficits: slow and irregular breathing, lack of hypercapnic reflex, and frequent and prolonged apneas. Together, these deficits appear to result in neonatal lethality. We observed that the conditional restriction of the Lbx1β mutation to the dB2 lineage (dB2-Lbx1β mice) fully recapitulates the physiological phenotypes observed in Lbx1β mice. In contrast, the conditional restriction of the Lbx1β mutation to rhombomeres 3 and 5 (Egr2-Lbx1β mice) impaired RTN neuron development, abolished the hypercapnic reflex, and caused mild hypopituitarism, but not abnormal apneas or neonatal lethality. Interestingly, similar or even identical phenotypes are observed when the Phox2bΔC-fl allele is restricted to rhombomeres 3 and 5 (22). Thus, the Lbx1β 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 Lbx1β/Phox2bα 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 intersectional genetic strategies to show that the combined deficits in development of RTN and the dorsal Lbx1β/Phox2bα population correlated with severe hypopituitarism 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 Ethic 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.

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