Supporting Information

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SI Materials and Methods

Immunocytochemistry, in Situ Hybridization, and X-Gal Histochemistry. Embryos were harvested at different developmental stages, drop-fixed for 1–2 h in 4% (wt/vol) paraformaldehyde at room temperature (RT), and equilibrated in 30% (wt/vol) sucrose at 4 °C overnight. Samples were then embedded in optimum cutting temperature (OCT) compound and sectioned at 20 μm on a cryostat. Immunocytochemistry, nonradioactive in situ hybridization, and whole-mount and section X-Gal histochemistry were performed as described (1). For immunocytochemistry, the following antibodies and reagents were used: chicken anti-lacZ (1:1,000; Abcam), chicken anti-GFP (1:500; Abcam), rabbit anti-Ki67 (1:300; Abcam), rabbit anti-pHistone3 (1:500; Santa Cruz), rabbit anti-Tuj1 (1:300; Covance), goat anti-LHX2 (1:500; Santa Cruz), goat anti-LHX9 (1:500; Santa Cruz), mouse anti-ISL1/2 (1:200; DSHB), rabbit anti-PAX2 (1:200; Santa Cruz), mouse anti-LIM1/2 (1:200; DSHB), mouse anti-ROBO3 (1:20; R&D Systems), and rabbit anti-activated caspase 3 (1:200; R&D Systems). The following secondary antibodies were used: donkey anti-rat Cy3 (1:1,000; Jackson Immunoresearch) and Alexa-conjugated antibodies (1:1,000; Molecular Probes). For nonradioactive in situ hybridization, the following probes were used: *Barhl1* (1918–2541 of AB043980), *Barhl2* (1), *Lhx2* (1149–1806 of AF124734), *Lhx9* (466–788 of AF134761), *Atoh1* (178–1233 of NM_007500), *Gdf7* (gift of D. Kingsley, Stanford University, Stanford, CA), and *Ntn1* and *Slit2* (gift of M. M. Tessier-Lavigne, Rockefeller University, New York, NY). 5-Ethynyl-2′-deoxyuridine dU (Invitrogen) was administered i.p. at 50 mg/kg of body weight. The pregnant dams were killed 30 min after injection. Fluorescent images were acquired and analyzed by a Zeiss LSM 510 confocal microscope. Other pictures were taken with a Nikon Eclipse TE2000-U inverted microscope with a Nikon DXM1200F digital camera. GraphPad Prism was used for all statistical analysis.

Retrograde Labeling with FD. Retrograde labeling of spinal cord neurons was performed as previously described (2). Briefly, E13.5 embryos were harvested and dissected ventrally, and the thoracic vertebrae were removed to expose the spinal cord. One side of the spinal cord was cut, and crystallized FD (Molecular Probes) was applied to the lateral aspect of the VF, slightly away from the midline. Embryos were then incubated in oxygenated artificial cerebrospinal fluid (aCSF) at 37 °C for 6–8 h, fixed in 4% (wt/vol) paraformaldehyde/PBS, vibratome-sectioned at 30 μm, and processed for immunolabeling with anti-GFP antibody.

***Lhx2* Sequence Analysis and Electrophoretic EMSA.** We analyzed *Lhx2* genomic sequences using a 30-way Multiz alignment and conservation tool from the University of California, Santa Cruz Genome Browser. Sequence alignment was performed by ClustalX algorithm. For EMSA, *Barhl2* expression plasmid was constructed by inserting the full-length *Barhl2* cDNA with an HA tag at C-terminal into pcDNA 3.1 expression vector (Invitrogen). EMSAs were performed using in vitro-translated BARHL2-HA proteins. The protein product was generated by a T7/T3-coupled reticulocyte lysate system (Promega) using *Barhl2* expression plasmid. DNA oligonucleotides were annealed and radiolabeled with γ-^{32}P ATP. Binding reactions were carried out at RT for 20 min with labeled probes in amounts corresponding to 5×10^5 cpm and 4 μL of the BARHL2-HA protein lysates. Competition was performed by adding excess amounts of cold oligonucleotides to the reaction mixtures. Supershift was performed by adding 0.5 μg of anti-HA antibody (Santa Cruz) with the protein lysates for 20 min on ice before the radiolabeled probe. Free and bound probes were resolved on a 5% (wt/vol) nondenaturing polyacrylamide gel.

Plasmid Constructs, Cell Culture, and Luciferase Assay. BARHL2 expression plasmid was constructed by inserting the full-length *Barhl2* cDNA into the pcDNA 3.1 expression vector (Invitrogen). To generate *Lhx2*-luciferase reporter constructs, a 3.9-kb *Lhx2* 5′-promoter fragment (−3423 to +466 bp relative to the transcription start site) was PCR-amplified and then ligated into pGL3-Basic vector (Promega). The mutated constructs were derived from a 3.9-kb sequence using PCR-based site-directed mutagenesis. All constructs were verified by DNA sequencing.

HEK293 cells were plated and cultured in 24-well plates in DMEM with 5% (vol/vol) FBS. Transfections were carried out with TransIT-2020 transfection reagent (Mirus) when cells reached 80% confluence. The cells were transfected with 200 ng of each reporter and expression construct, along with 10 ng of the pRL-TK Renilla luciferase reporter plasmid for the internal control of cell-transfected efficiency. Transfected cells were lysed and assayed 48 h later using a Dual-Luciferase Reporter Assay Kit (Promega). The value of firefly luciferase activity was normalized to that of Renilla luciferase activity.

1. Joshi PS, et al. (2008) Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. *Neuron* 60:258–272.

2. Moran-Rivard L, et al. (2001) Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. *Neuron* 29:385–399.

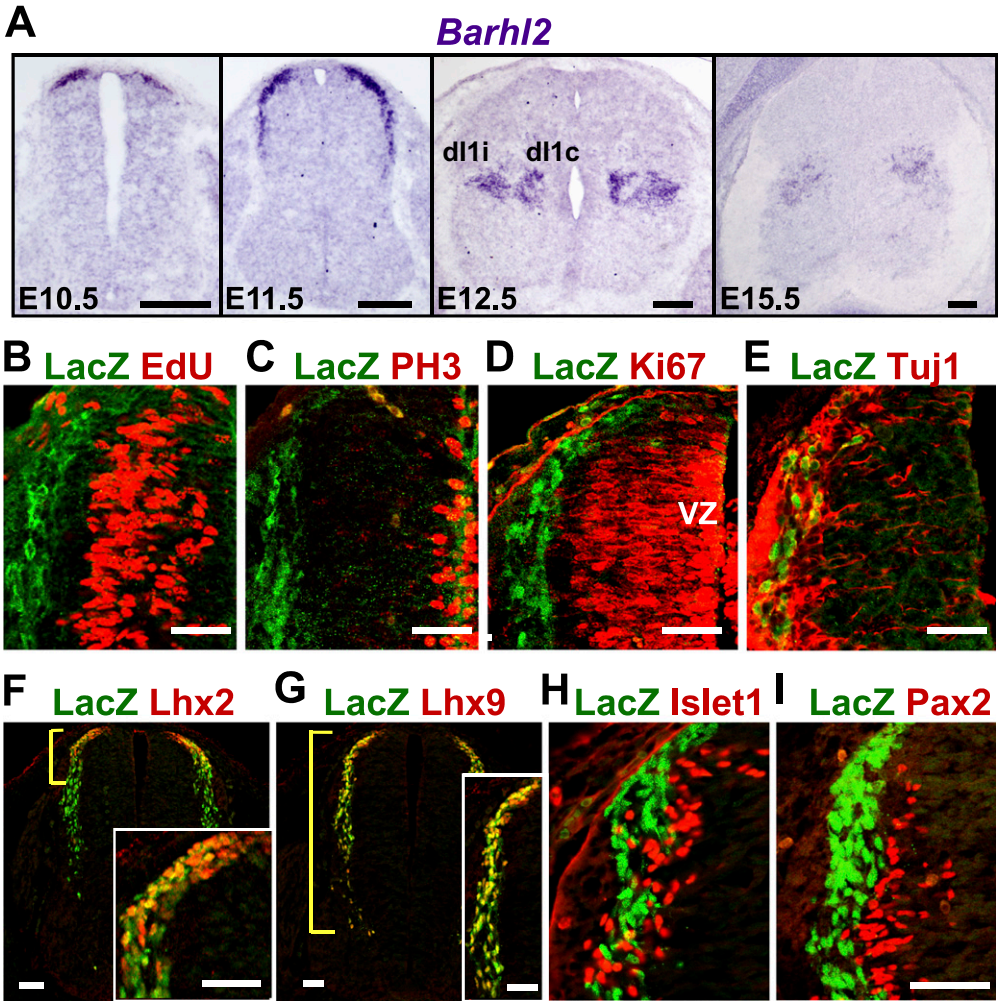


Fig. S1. *Barhl2* is expressed in postmitotic dI1 interneurons. (*A*) In situ hybridization on E10.5–E15.5 cervical spinal cord sections. *Barhl2* is expressed in newly postmitotic, migrating, and postmigrational dI1 neurons. *Barhl2* is expressed by both dI1i and dI1c subtypes (E12.5). *Barhl2* expression becomes weaker from E15.5 onward. (*B–I*) Double immunolabeling for *Barhl2*-lacZ (green) and cell type-specific markers (red) on E11.5 *Barhl2*$^{lacZ/+}$ cervical spinal cord sections. (*B–E*) *Barhl2*-lacZ is expressed in postmitotic neurons. *Barhl2*-lacZ does not colocalize with S-phase marker EdU (*B*), M-phase marker PH3 (*C*), or Ki67 (*D*), expressed by cycling progenitors in all phases of the active cell cycle. (*E*) *Barhl2*-lacZ colocalizes with the postmitotic neuronal marker Tuj1. (*F–I*) *Barhl2*-lacZ is specifically expressed by dI1 neurons. *Barhl2*-lacZ colabels with dI1 markers LHX2 (*F*) and LHX9 (*G*). (*Insets*) Magnified views from bracketed areas in *F* and *G*, respectively. *Barhl2*-lacZ is not expressed in other spinal interneuron classes, such as ISL1^{+} dI3 neurons (*H*) and PAX2^{+} dI4, dI6, and ventral interneurons (*I*). VZ, ventricular zone. (Scale bars: *A*, 100 μm; *B–I*, 50 μm.)

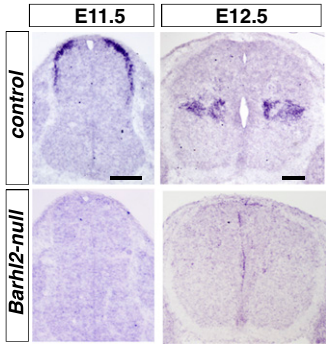


Fig. S2. Absence of *Barhl2* transcripts in *Barhl2*-null mice. In situ hybridization on control and *Barhl2*-null cervical spinal cord sections at E11.5 and E12.5 reveals complete absence of *Barhl2* transcripts in *Barhl2*-nulls. (Scale bars: 100 μm.)

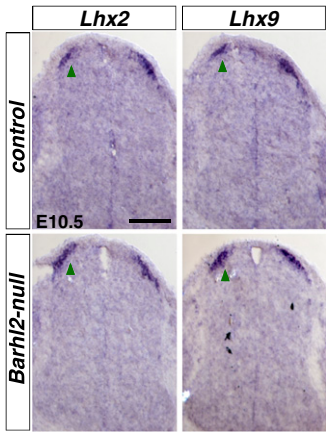


Fig. S3. Early expression of *Lhx2* and *Lhx9* is unaltered in *Barhl2*-null spinal cord. In situ hybridization on control and *Barhl2*-null cervical spinal cord sections. The expression of *Lhx2* and *Lhx9* at the dorsal margin of the E10.5 spinal cord (green arrowheads) is unperturbed in *Barhl2*-nulls. (Scale bars: 50 μm.)

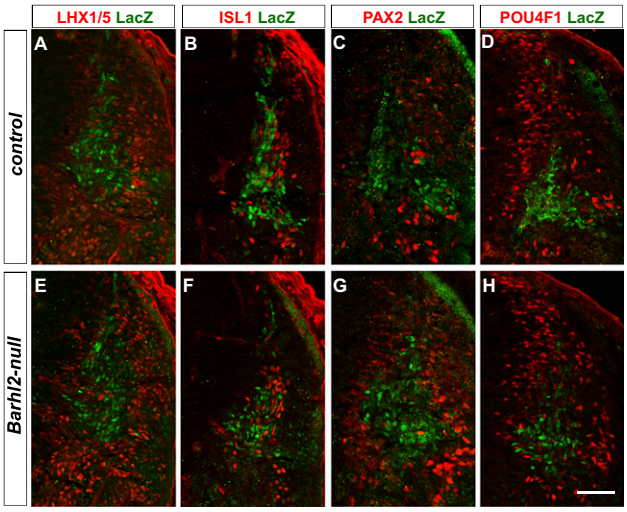


Fig. S4. dI2–dI6 markers are not expressed in *Barhl2*-null dI1 interneurons. Double immunolabeling on E12 control (*A*–*D*) and *Barhl2*-null (*E*–*H*) cervical spinal cord sections demonstrates that *Barhl2*-lacZ^{+} dI1 neurons do not ectopically express LHX1/5 (dI2 and dI4 markers) (*A* and *E*), ISL1 (dI3 marker) (*B* and *F*), PAX2 (dI4 and dI6 markers) (*C* and *G*), or POU4F1 (dI5 marker) (*D* and *H*) in *Barhl2*-nulls. (Scale bars: 100 μm.)

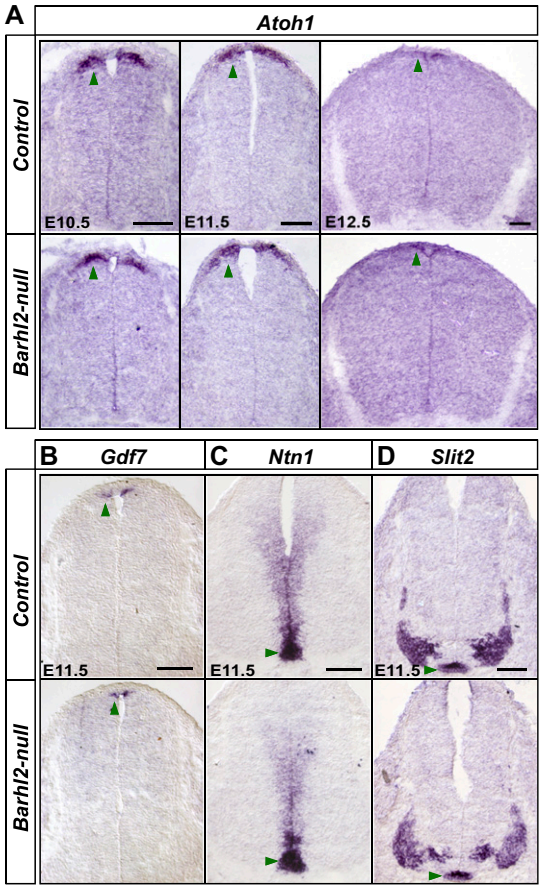


Fig. S5. Molecular identity of the roof plate, floor plate, and dI1 progenitors is largely preserved in *Barhl2*-nulls. The dI1 progenitor marker *Atoh1* (*A*), roof plate marker *Gdf7* (*B*), floor plate markers *Ntn1* (*C*) and *Slit2* (*D*) are unaltered in *Barhl2*-nulls. Green arrowheads indicate the roof plate in *A*, floor plate in *B* and *C*, and dI1 progenitors in *D*. (Scale bars: 50 μm.)

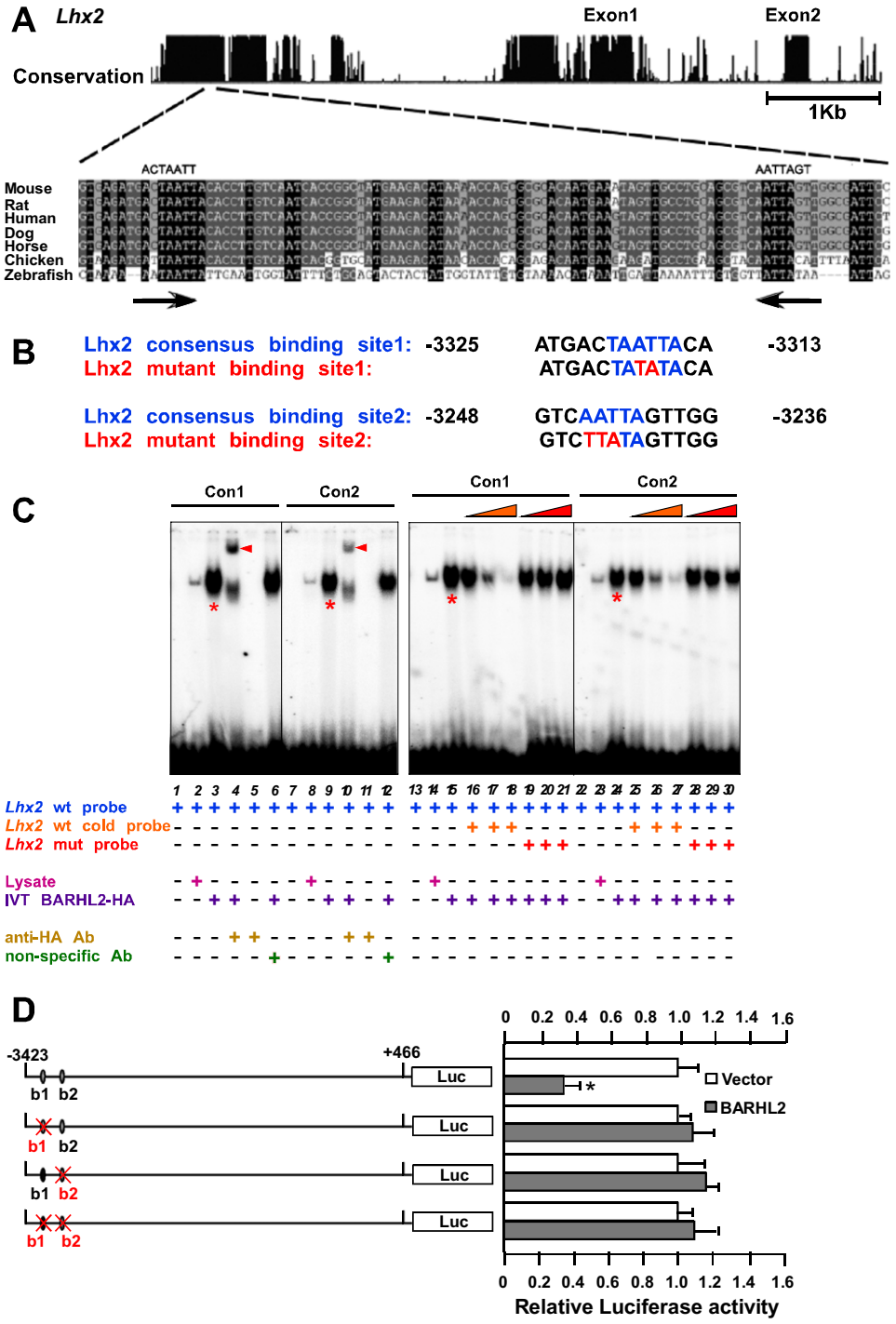
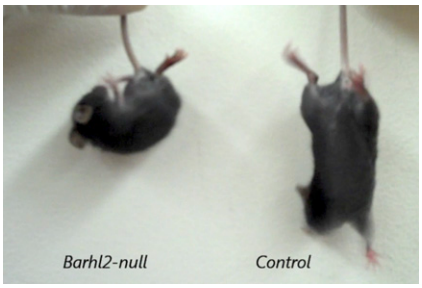


Fig. S6. BARHL2 directly regulates the *Lhx2* gene. (*A*) Schematic showing conserved sequence domains in the *Lhx2* gene and sequence alignment of the region with putative BARHL2 binding sites depicting evolutionary conservation across multiple species. (*B*) Two *Lhx2* oligonucleotides with putative BARHL2 binding sequences in forward (Con1) and reverse (Con2) orientations; mutant oligonucleotides with nonconserved nucleotides (red) replace the core TAAT motif. (*C*) EMSA. Radiolabeled *Lhx2* oligonucleotides incubated with in vitro-translated BARHL2-HA protein produce a discrete band shift (lanes 3, 9, 15, and 24; red asterisks), which is substantially diminished by an excess of unlabeled oligonucleotides (lanes 16–18 and 25–27). Incubation of *Lhx2* oligonucleotides with BARHL2-HA and an HA antibody results in a supershift (lanes 4 and 10; red arrowheads). The supershift is not observed in the absence of BARHL2-HA (lanes 5 and 11) or in the presence of control antibody (lanes 6 and 12). Mutant *Lhx2* oligonucleotides do not block the binding activity (lanes 19–21 and 28–30). (*D*) (*Left*) Schematics of luciferase (Luc) reporter constructs containing WT *Lhx2* 5′-promoter sequences and single- or double-mutant constructs. Ovals represent the two BARHL2 binding sites (b1 and b2) in the promoter region. The mutated binding sites are indicated in red. (*Right*) Regulation of luciferase activity from WT and mutant constructs by BARHL2. Each histogram represents the mean ± SD of results from three separate triplicate experiments (*$P < 0.01$).



Movie S1. *Barhl2*-null mice display an unsteady and staggering gait as well as a motor impairment phenotype revealed by the tail suspension test.

Movie S1