Supplemental Information

Bhlhb5 and Prdm8 form a neural-specific repressor complex

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Supplemental Data

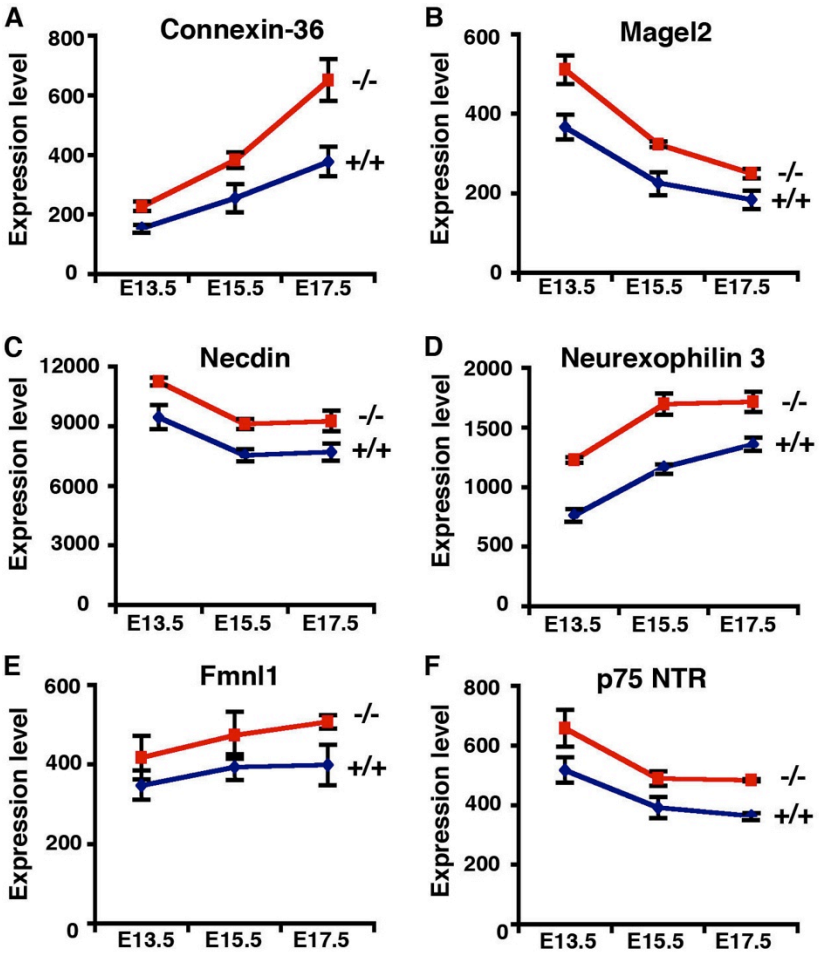

Figure S1 related to Figure 1. Identification of putative Bhlhb5 target genes by transcriptional profiling

A-F) Affymetrix microarray-based gene profiling was performed to identify genes that are misexpressed in the dorsal telencephalon of *Bhlhb5*$^{-/-}$ mice relative to wildtype littermates. Each time point was analyzed using three pairs of mice. Using a false discovery rate of 0.05 or less (see Experimental Procedures), we identified a total of eight genes that were significantly different in *Bhlhb5*$^{-/-}$ mice: *Prdm8* (Figure 1A), *Cdh11* (Figure 7A), *Connexin-36* (A), *Magel2* (B), *Necdin* (C), *Neurexophilin 3* (D), *Fmnl1* (E) and *p75 NTR* (F). Note that all of the misregulated genes are up-regulated upon the loss of *Bhlhb5*, consistent with the idea that they may be direct targets of Bhlhb5-mediated repression. Data are mean +/- SEM, with wild-type (+/+) in blue and *Bhlhb5*$^{-/-}$ (-/-) in red, as indicated. Complete expression profiling data are available online at www.Ross-et-al-2011.hms.harvard.edu.

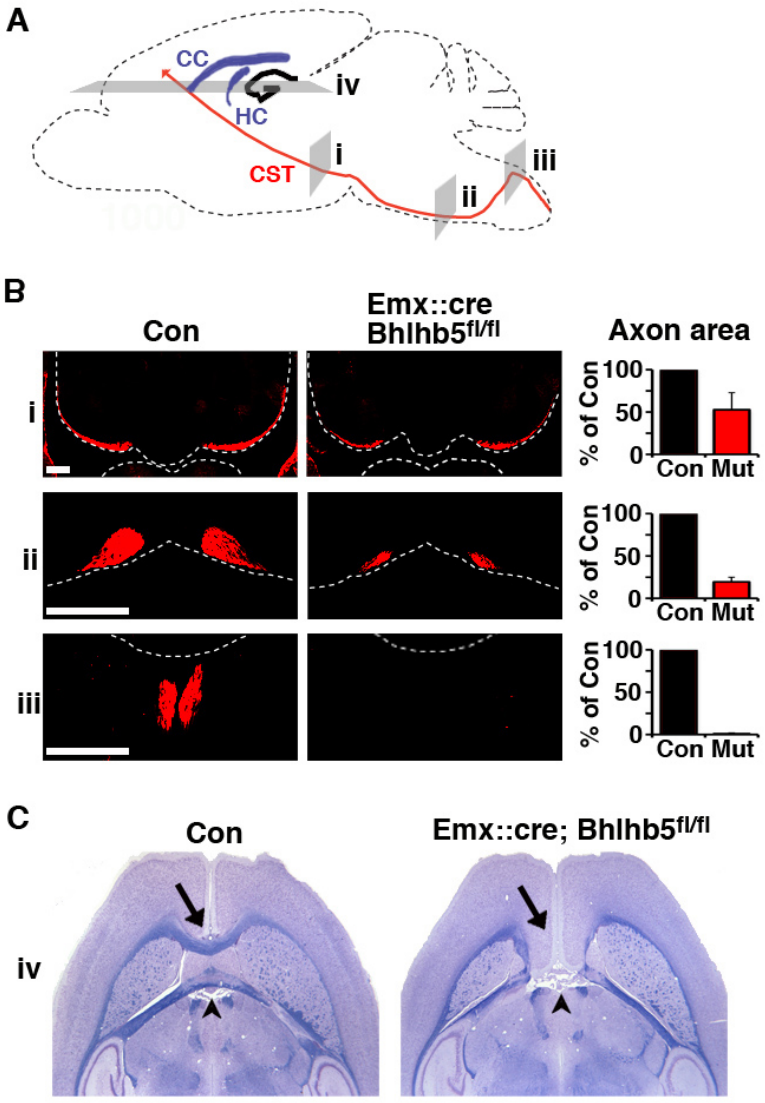

Figure S2 related to Figure 2. Bhlhb5 is required in the dorsal telencephalon for major axon tracts **A)** Sagittal schematic of the brain illustrating path of the corticospinal tract (CST), the corpus callosum (CC) and the hippocampal commissure (HC). Roman numbers illustrate locations and planes of sections shown in (B) and (C). **B)** Representative coronal sections from P7 mice immunostained with PKCγ, a protein that marks the corticospinal tract. Sections are from mice that are selectively lacking *Bhlhb5* in the dorsal tel-encephalon (Emx::cre;Bhlhb5$^{fl/fl}$) or control littermates (Con) that are heterozygous for Bhlhb5 in the dorsal telencephalon (Emx::cre; Bhlhb5$^{fl/wt}$). The area of PKCγ staining was quantified using matched sections from three pairs of mice, and are expressed as percent of control. Similar results were obtained when the corticospinal tract was labeled genetically using the Emx::cre line and a cre-responsive eYFP reporter (data not shown). These data indicate that, upon loss of *Bhlhb5* in the dorsal telencephalon, only a third of corticospinal motor neurons extend axons beyond the basilar pons and none extend axons beyond the pyramidal decussation. **C)** Representative horizontal sections from adult mice co-stained with luxol fast blue and cresyl violet. Arrow indicates corpus callosum and arrowhead indicates hippocampal commissure; both are significantly reduced upon loss of *Bhlhb5* in the dorsal telencephalon. Almost complete agenesis of the corpus callosum and hippocampal commissure was observed in 20 of 20 *Bhlhb5* mutant mice, which were on a mixed C57BL/6J:129S6 background, and never observed in control littermates. In *Bhlhb5* mutant mice, these colossal projections appeared to be stalled in Probst bundles adjacent to the midline. Conditional knockout of Bhlhb5 (Bhlhb5$^{fl/fl}$) was generated in the Greenberg lab and is described in (Ross et al., 2010); Emx::cre mice were obtained from Kevin Jones (Gorski et al., 2002) and are currently available from Jaxlabs.

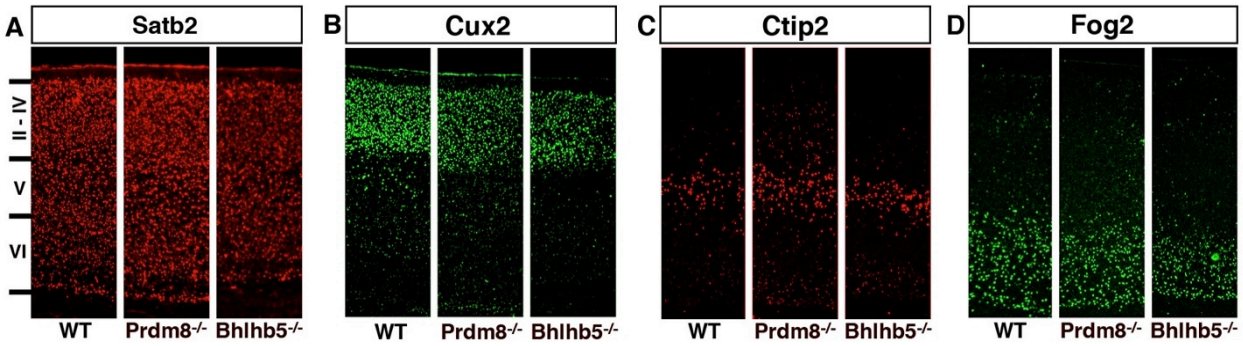

Figure S3 related to Figure 2. Cortical lamination is normal in mice lacking either *Prdm8* or *Bhlhb5* Coronal sections from P6 mice of the indicated genotype were stained using antibodies against layer-specific markers **A)** Satb2, **B)** Cux2, **C)** Ctip2 and **D)** Fog2. No differences in layering were observed in either *Bhlhb5* or *Prdm8* mutant mice. These results are consistent with previous studies that reported normal survival, migration and layering in the cortex of *Bhlhb5* null mice (Joshi et al., 2008). Note that mice lacking either *Bhlhb5* or *Prdm8* show a slightly thinner cortex by P6. This phenotype may potentially be a secondary consequence to the fact that both mutants are somewhat runted compared to wild-type littermates, since this diminution in cortical thickness is not observed upon Emx::cre-mediated loss of *Bhlhb5* (data not shown).

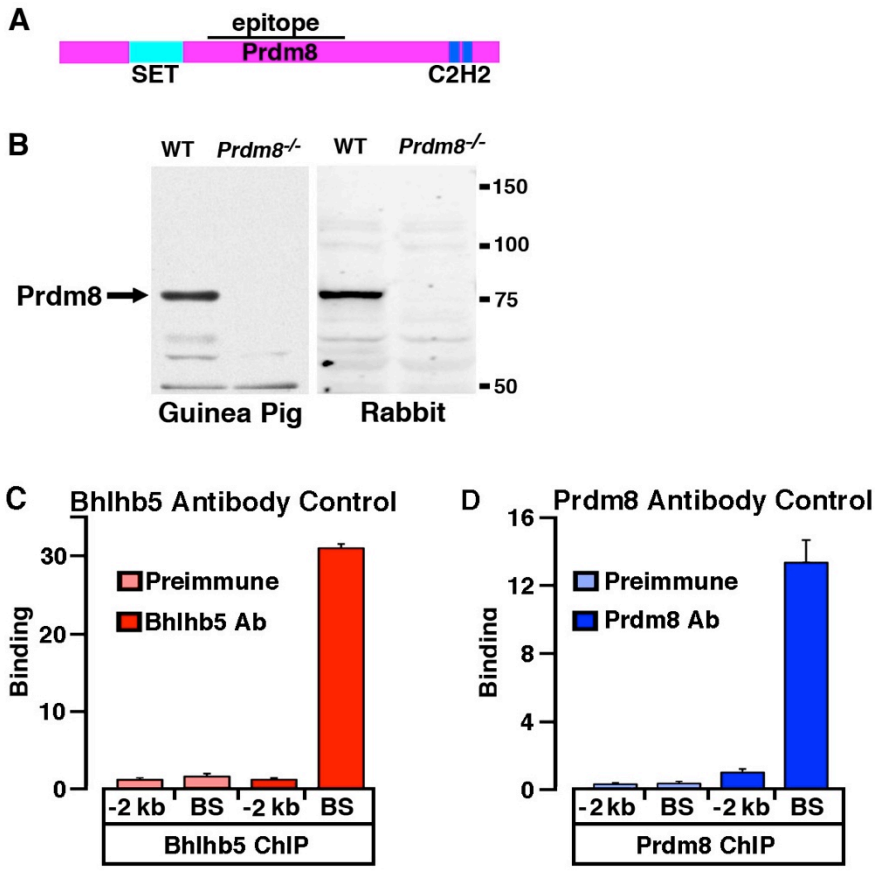

Figure S4 related to Figures 3 and 5. Generation and specificity of antibodies **A)** Schematic of Prdm8 protein illustrating the SET domain (Suppressor of variegation 3-9 (Su(var)3-9)/Enhancer of zeste/Trithorax) and the pair of C_2H_2 zinc fingers. Black bar indicates region of the Prdm8 protein (amino acids 228 – 457) used to make a Prdm8-GST fusion protein for the generation of Prdm8 antibodies. **B)** Western blot showing the specificity of Prdm8 antibodies. Cortical lysates from P0 wild type (WT) or *Prdm8*$^{-/-}$ mice were subjected to immunoblotting using sera from animals (guinea pig or rabbit, as indicated) injected with the Prdm8-GST fusion protein. Prdm8 runs as a single band at ~ 75 kDA on a 10% acrylamide gel, which is lacking in *Prdm8*$^{-/-}$ mice. **C-D)** ChIP-qPCR at the *Bhlhb5* promoter using either Bhlhb5 antisera, Prdm8 antisera, or preimmune sera from the appropriate animal (as indicated) reveals that the Bhlhb5 and Prdm8 antibodies are specific in ChIP experiments. Significantly enriched binding of Bhlhb5 (C) and Prdm8 (D) at the binding site (BS) relative to a negative control region 2 kb upstream (-2kb) is observed with antisera but not with preimmune sera. Binding represents enrichment of gDNA over input (x10^{-3}). For all ChIP experiments, one of two negative controls was used to control for specificity. We either performed ChIP using preimmune sera as a negative control for the antibody (as illustrated here) or we used tissue from appropriate knockout mice (Figures 6B, S8A and S8B).

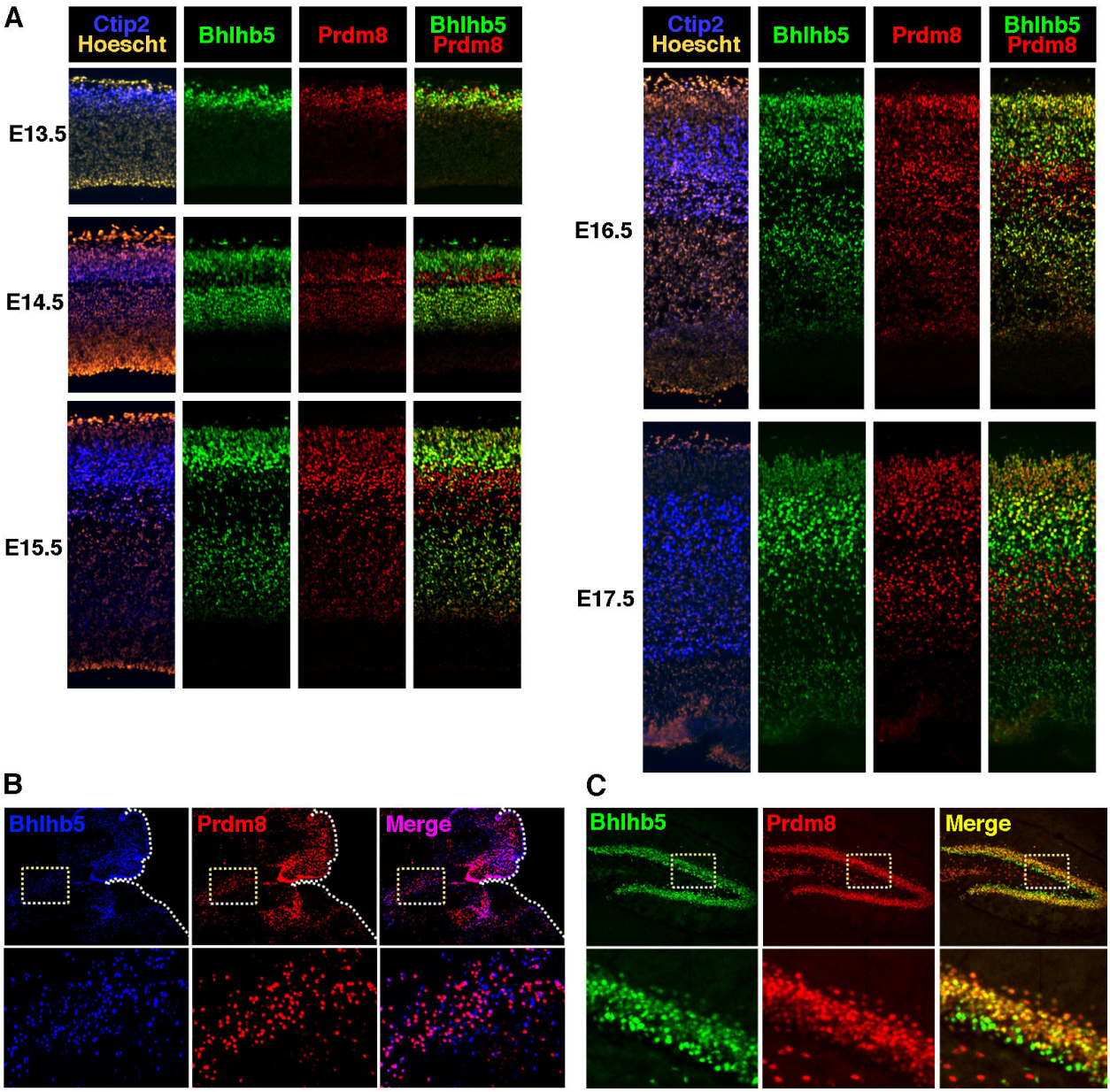

Figure S5 related to Figure 3. Bhlhb5 and Prdm8 are colocalized in subsets of neurons **A)** Time course of cortical development reveals that Bhlhb5 and Prdm8 show dynamic expression in differentiating neurons. Coronal sections of the indicated ages were stained with the nuclear marker Hoescht (yellow), the layer 5/6 marker Ctip2 (blue), Bhlhb5 (green) or Prdm8 (red), as indicated. Note that all glutamatergic neurons appear to co-express Bhlhb5 and Prdm8 at some point during development but that, at any given time, many neurons appear to express reciprocal levels of Bhlhb5 and Prdm8, suggesting that their expression is coordinately regulated as part of a precisely controlled network. **B)** Bhlhb5 and Prdm8 show a high degree of co-localization throughout the nervous system, as illustrated in this sagittal section of the hindbrain and cerebellum at P2, where ~ 75% of neurons that express either Bhlhb5 or Prdm8 also express the other factor. Boxed insets in upper panels are enlarged in lower panels. **C)** Colocalization of Bhlhb5 and Prdm8 persists throughout development, as illustrated in this in this coronal section of dentate gyrus at P30 where > 90% of neurons that express one factor also express the other. Boxed insets in upper panels are enlarged in lower panels. Previous studies have also shown that Bhlhb5 and Prdm8 are likewise co-localized in neurons of the developing spinal cord (Ross et a., 2010).

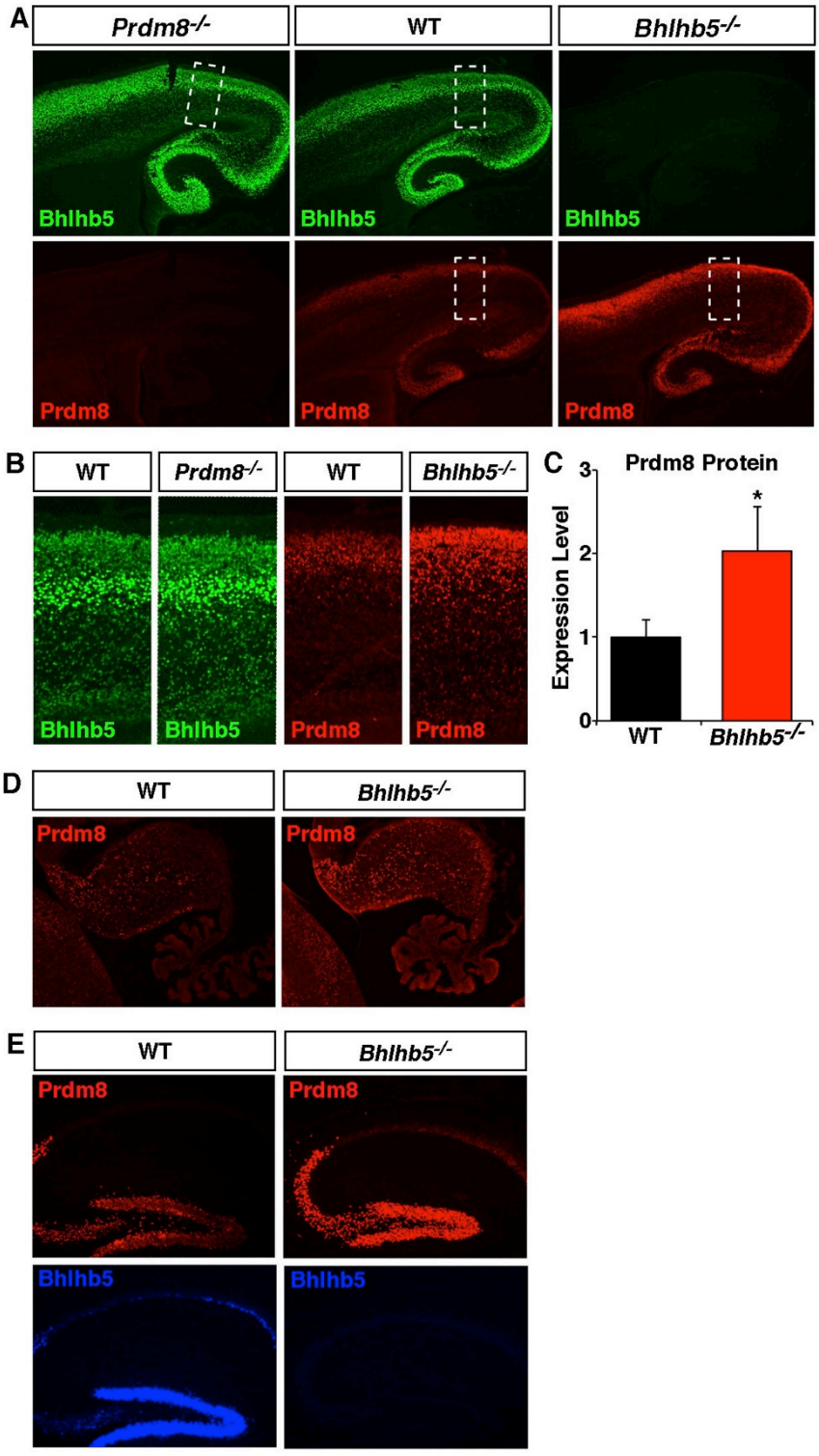

Figure S6 related to Figure 4. Analysis of possible cross-regulation between Bhlhb5 and Prdm8 **A - B)** Prdm8 protein is over-expressed in *Bhlhb5* mutant mice. Sagittal sections from mice of the indicated genotype at P0 were co-stained with antibodies to Bhlhb5 (green) and Prdm8 (red). Boxed insets are enlarged in (B). Note that in Prdm8 mutant mice, there is a small but significant up-regulation of Bhlhb5 message (Figure 4A), which was not detected at the protein level. **C)** Quantitative western blotting of lysates from the dorsal telecephalon of mice at P0 reveals a 2-fold increase in Prdm8 protein in *Bhlhb5* mutant mice. n = 4, $p < 0.05$, t-test. **D)** Loss of Bhlhb5 results in the upregulation of Prdm8 in many regions of the nervous system, as illustrated here in sagittal sections of the differentiating cerebellum in wild type (WT) and *Bhlhb5*$^{-/-}$ mice at E15.5. **E)** Loss of Bhlhb5 results in the up-regulation of Prdm8 in the adult brain, as illustrated here in the hippocampus. These results suggest that loss of the Bhlhb5/Prdm8 repressor complex results in the up-regulation of Prdm8 mRNA and protein, possible through indirect mechanisms or via binding at genomic loci that remain unidentified. Loss of this repressor complex also has a modest effect on the expression of Bhlhb5 mRNA, and this effect is likely to be direct via binding of the Bhlhb5/Prdm8 repressor complex to the proximal promoter of the Bhlhb5 gene. However, this observed increase in Bhlhb5 mRNA is not reflected in an increase in Bhlhb5 protein in *Prdm8* knockout mice.

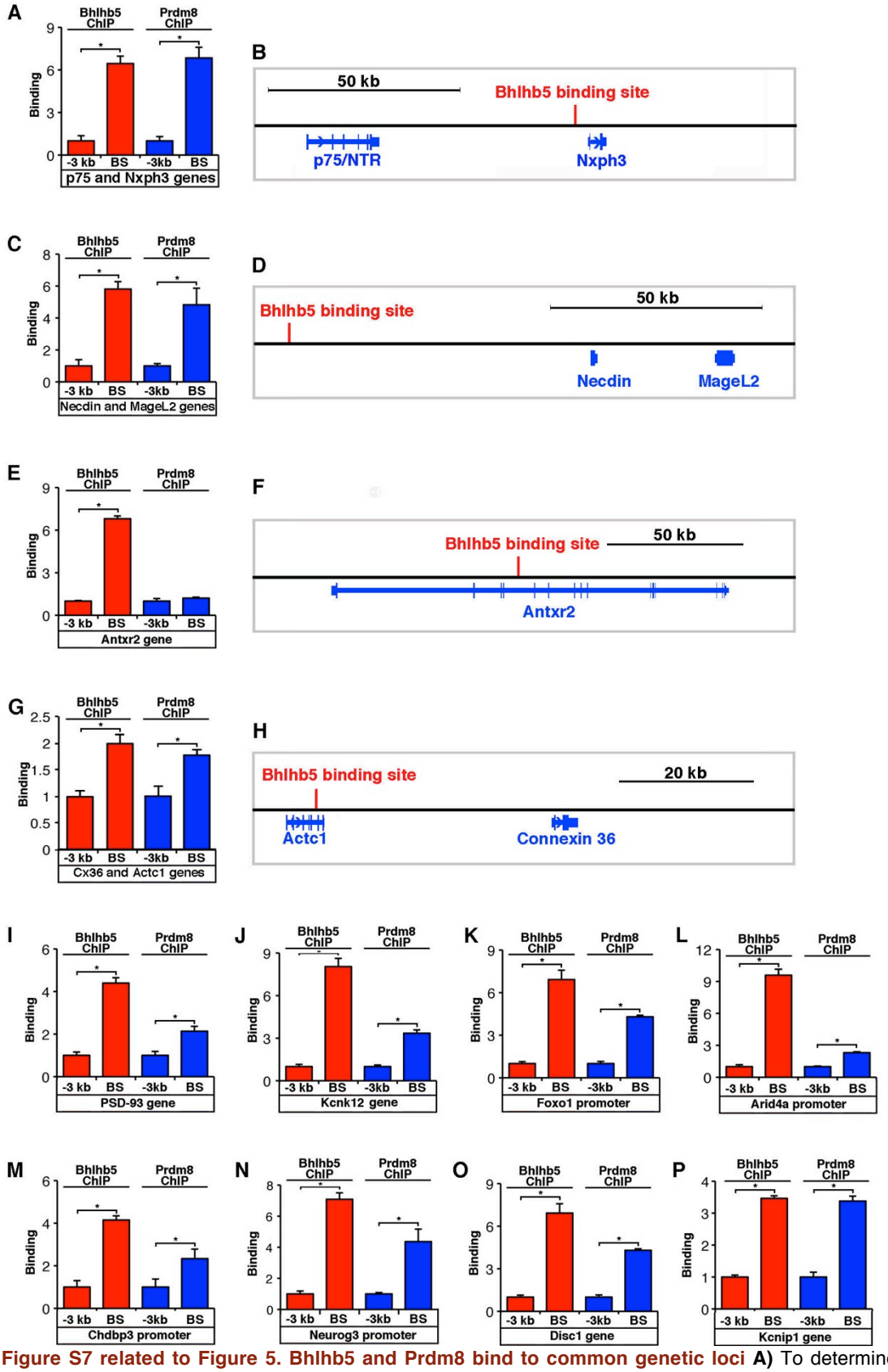

Figure S7 related to Figure 5. Bhlhb5 and Prdm8 bind to common genetic loci A) To determine the extent to which Bhlhb5 and Prdm8 target the same genetic loci, 12 additional Bhlhb5 binding sites were selected from the

Bhlhb5 ChIP-seq data for further testing. These loci included included all putative Bhlhb5 target genes (as identified by gene profiling experiments) with nearby Bhlhb5 binding sites. Thus, of the 13 genes that are significantly misregulated in *Bhlhb5* and mutant mice from E13.5 –E17.5 (Figures 1A, 7A and S1) or P0 (Figure 4), seven have Bhlhb5 binding sites within 200 kb of the transcriptional start, as identified by ChIPseq. These genes are *Bhlhb5* itself (Figure 5B), *Cdh11* (Figure 5D), *p75/NTR* and *neurexophilin 3 (Nxph3)* (B), *Necdin and MageL2* (D) and the *Anthrax toxin receptor 2 (Antxr2)* (F) and *Connexin 36 (Cx36)* (H). We tested these, along with 8 other putative Bhlhb5 binding sites (G – N), by performing ChIP-qPCR using antibodies to Bhlhb5 (red) or Prdm8 (blue). For each loci tested, we found significant enrichment of Bhlhb5 at the binding site (BS) relative to a negative control regions ~ 3kb upstream (red bars). At the vast majority of these loci (11 of 12), we likewise observed a significant enrichment of Prdm8 binding (blue bars). Thus, a strong degree of correspondence in binding between Bhlhb5 and Prdm8 was observed. The y-axis (Binding) represents the enrichment at the binding site (BS) relative to a negative control regions ~ 3kb upstream. For B, D, F and H, genomic loci are illustrated showing the location of the Bhlhb5 binding site (red bar) relative to the target gene. Somewhat surprisingly, our ChIP-seq experiments did not identify Bhlhb5 binding sites at all of the genes that were found to be misregulated in *Bhlhb5* mutant mice. For instance, none were observed near the *Prdm8* gene. Since we only identified ~2300 Bhlhb5 binding sites by ChIP-seq (rather than the >10,000 binding sites observed for many other transcription factors), it is possible that some loci were missed in these experiments because they were below the detection limit. Alternatively, genes lacking Bhlhb5 binding sites may represent indirect targets.

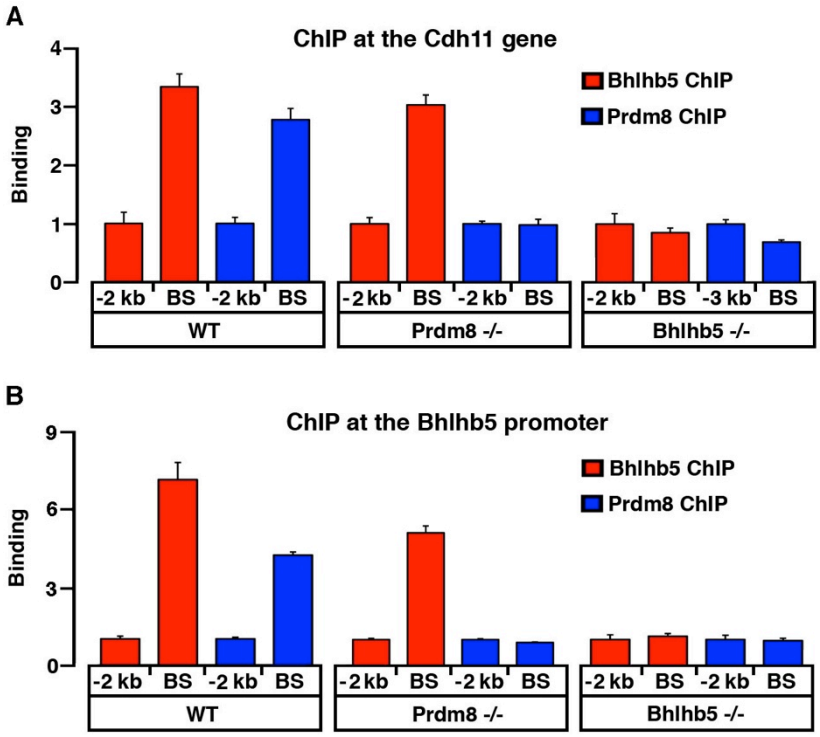

Figure S8 related to Figure 6. Bhlhb5 is required for the targeting of Prdm8 ChIP-qPCR was performed using antibodies to Bhlhb5 (red) or Prdm8 (blue) and analyzed for binding at a region in the first intron of *Cdh11* (A) and the *Bhlhb5* promoter (B). In WT mice, there is enriched Bhlhb5 binding at the identified Bhlhb5 binding site (BS) relative to the negative control region, 2 kb upstream (-2 kb). In *Prdm8*$^{-/-}$ mice, Bhlhb5 still binds to the *Cdh11* intron and *Bhlhb5* promoter. In *Bhlhb5*$^{-/-}$ mice, Prdm8 no longer binds to the *Cdh11* intron or the *Bhlhb5* promoter, indicating that Bhlhb5 is required for Prdm8 targeting to this loci. Note that the *Bhlhb5* mutant mice used in these experiments contain an intact *Bhlhb5* promoter. Data are normalized to binding at the negative control region, 2 kb upstream of the binding site in question. Data are representative of at three independent experiments.

Supplemental Experimental Procedures

Animal husbandry and colony management

The *Bhlhb5* null allele was generated previously and described in (Ross et al., 2010). Prdm8$^{-/-}$ mice were generated in the McInnes lab, details to be provided elsewhere. In brief, IRES-eGFP was targeted to the second and third coding exons of the Prdm8 gene. The absence of Prdm8 mRNA and protein in *Prdm8*$^{-/-}$ mice was confirmed by RT-PCR (Figure 4B) and western blotting (Figure S4B), respectively. Furthermore, using antibodies directed to the N-terminus we found no evidence for the expression an N-terminal peptide fragment of Prdm8, indicating that the Prdm8 mutant is a complete null (data not shown). *Cdh11*$^{-/-}$ mice were from the Takeichi lab (Horikawa et al., 1999). To investigate the role of Cdh11 as a putative Bhlhb5 target gene, *Bhlhb5*$^{-/+}$;*Cdh11*$^{-/+}$ compound heterozygotes were intercrossed and littermates were analyzed. All mice were maintained on a mixed C57BL/6J:129S6 background. Note that we were careful to ensure that the agenesis of the corpus callosum and hippocampal commissures was not due to the background strain, a possible concern because 129 strains are known to have colossal projection defects. The use of animals was approved by the Animal Care and Use Committee of Harvard Medical School.

Immunohistochemistry

Mice (P0 and above) were fixed with 4% paraformaldehyde in PBS by intracardial perfusion. Tissue from embryonic mice was removed by dissection and drop-fixed. Tissues were post-fixed for 1 h at 4 °C, washed extensively with PBS, cryopreserved in 20% sucrose in PBS overnight, embedded in OCT, and frozen. Sections were cut at 20 μm on a cryostat and placed on slides. For immunostaining, sections were blocked in 10% goat serum and 0.25% Triton-X in PBS for 1 h at room temperature. Sections were incubated with primary antibodies in block overnight at 4 °C. Slides were washed 4 times for 5 min in PBS containing 0.1% Triton-X. Detection was carried out using Alexa Fluor secondary antibodies diluted 1:500 in block. Sections were counterstained with the nuclear dye Hoechst 33342 for one min at room temperature. Sections were washed, as above, and coverslips were applied. Primary antibodies were as follows: Bhlhb5 amino-terminal antibody (rat, 1:1000 or rabbit, (1:10,000), generated in the Greenberg lab; see (Ross et al., 2010) for details); Prdm8 (rabbit or guinea pig, 1:1000, generated in the Greenberg lab to a GST-Prdm8 fusion protein; see Figure S4 for details); PKCγ (rabbit, 1:500, Santa Cruz) and Cdh11 (mouse, 1:1000, Zymed), Satb2 (mouse, 1:500, Abcam), Cux1 (rabbit, 1:1000, Santa Cruz), Ctip2 (rat, 1:1000, Abcam) and Fog2 (rabbit, 1:1000, rabbit).

Identification of Bhlhb5 target genes through gene expression profiling

RNA was made from the dorsal telencephalon of wildtype and Bhlhb5-/- mice at three developmental times, E13.5, E15.5 and E17.5. Three biological replicates were used for each time point, and pairs of wildtype and Bhlhb5-/- mice were from the same litter. Expression levels were obtained for ~ 12,000 probe sets from Affymetrix 3' Expression mouse gene chips. In order to assess fold changes in expression irrespective of trends in time, for each gene the 9 paired differences (3 replicates, 3 times) of the logarithms of measured expression levels between each WT/Bhlhb5-/- pair were analyzed for significance using a paired t-test (8 degrees of freedom). This was repeated after randomly permuting the 9 WT/Bhlhb5-/- pairings of levels for each gene to obtain a null set for comparison. The resulting sets of p-values for unpermuted vs. permuted data yielded 9 genes (Bhlhb5 and 8 others) with a false discovery rate (FDR) of 0.05 or less, which are shown in Figures 1A, 7A, and S1). Complete expression profiling data are available online at www.Ross-et-al-2011.hms.harvard.edu.

Comparison of molecular profiles in Bhlhb5-/- and Prdm8-/- mice.

RNA was made from the dorsal telencephalon of Bhlhb5-/-and Prdm8-/- mice, and their respective wildtype littermates at P0. Four pairs of mice were analyzed for a total of 16 samples. Expression levels were obtained for ~ 12,000 probe sets from Affymetrix 3' Expression mouse gene chips. As a preliminary screen, genes that showed a change in ex-pression ($p < 0.01$, t-test) upon loss of either Bhlhb5 or Prdm8 were re-tested by qPCR using independent biological samples (see Gene expression analysis by qPRC in Supplemental Experimental Proceedures). By this method, all genes that showed a significant change in expression in Bhlhb5-/- mice also showed a significant change in expression in Prdm8-/- mice, and vice versa (t-test, $p < 0.05$). Complete expression profiling data are available online at www.Ross-et-al-2011.hms.harvard.edu.

Gene expression analysis by qPCR

The dorsal telencephalon from at least three independent pairs of wild type/mutant mice was dissected out and homogenized into Trizol reagent (Invitrogen). The RNA was isolated using the Trizol protocol and then further purified using the Qiagen RNeasy MinElute Kit. In order to make cDNA from this RNA, 2 μg of RNA was digested with DNase I and reverse-transcribed using the SuperScriptIII kit (Invitrogen). 1/20th of the resulting cDNA sample was used in each qPCR reaction. QPCR was performed using SYBR green detection. Each primer set included in the analysis had melt curves that were consistent with the amplification of a single product in the expected size range. A standard

curve was used for each primer pair, in triplicate. Levels of mRNA were normalized to βTubulinIII or ActinB. Data are average +/- SEM of the biological replicates.

ChIP-Seq library construction

Two ChIP-seq libraries were made from either wild type mice or *Bhlhb5*$^{-/-}$ mice (as a negative control). The dorsal telencephalon from three embryonic mice (E17.5) were pooled for each ChIP-Seq library. Littermate pairs of WT & *Bhlhb5*$^{-/-}$ mice were used to reduce variability. ChIP for Bhlhb5 was performed as described below. The immunoprecipitated DNA fragments were repaired by the End-It DNA End Repair Kit (Epicentre Biotechnology) according to the manufacturer's instructions. The end-repaired ChIP DNA fragments were purified by MinElute Reaction Cleanup Kit (Qiagen) and eluted in 20 µl EB buffer. The resulting DNA fragments were ligated with P1 and P2 adaptors for the SOLiD genome for 20 min at room temperature using the Quick Ligase Kit (NEB), followed by purification using the MinElute Reaction Cleanup Kit (Qiagen). The purified, adaptor-ligated ChIP DNA fragments were subject to 6% native-PAGE for an in-gel PCR reaction. A gel slice containing 175–200 bp adaptor-ligated ChIP DNA fragments (corresponding to 125–150 bp genomic fragment sizes) was cut and shredded. PCR Platinum Supermix (100–200 µl, Invitrogen), 50 pmol of PCR primers (available upon request), 0.5 *µl Taq* DNA polymerase (NEB), and 0.15 *µl Pfu* DNA polymerase (Invitrgen). Turbo DNA polymerase (Stratagene) were added into the shredded gel slice. The adaptor-ligated ChIP DNA fragments were amplified by 15 cycles of in-gel PCR. After the PCR reaction, gel pieces were filtered out by 0.45 µm filter spin column, and the amplified ChIP-Seq library was purified by the MinElute PCR purification kit (Qiagen). The library was purified by one more round of 6% PAGE. A gel slice containing 200–250 bp PCR products (110–150 bp fragment size) was cut and shredded, and the amplified library was extract out of the gel by passive elution in elution buffer (1.5 M ammonium acetate in TE). Gel pieces were filtered out by filter spin column, and the resulting ChIP-Seq library was purified using the Qiaquick PCR purification kit (Qiagen).

ChIP

The dorsal telencephalon of at least 3 P0 animals was used for each ChIP experiment. P0 mice were selected for ChIP experiments rather than the E17.5 mice used for ChIP-seq experiments since the use of newborn mice did not require sacrificing pregnant mice. The tissue was crosslinked in 1% formaldehyde for 10 min at room temperature and briefly dounced. The crosslinking reaction was quenched by incubation with 125 mM glycine for 5 min at room temperature. Tissue was washed three times with cold PBS and further homogenized with 4 - 8 dounce strokes in Buffer I [50 mM HEPES KOH pH 7.5, 140 mM NaCl, 1mM EDTA pH 8.0, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, and a protease inhibitor cocktail tablet (Roche MiniTab)]. Crosslinked nuclei were spun down (2 min, 16,000 rpm) and washed with Buffer II [200 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 10 mM Tris pH 8.0, and a protease inhibitor cocktail tablet (Roche MiniTab)]. The nuclei were then resuspended in 1.5 mL Buffer [1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 10 mM Tris pH 8.0, and a protease inhibitor (Roche MiniTab)] and sonicated using a Misonix 3000. The sonication was performed for 12 cycles of 15 s with a 2 min interval each at a power setting of 6-7.5. Sonicated lysates were centrifuged at 14,000 rpm for 20 min to remove insoluble cellular debris. Lysates were adjusted to IP buffer conditions [1mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 10 mM Tris-Cl pH 8.0, 0.3 M NaCl, 0.1% DOC, and a protease inhibitor (Roche MiniTab)] before preclearing. Lysates were pre-cleared for 2 h at 4 º C with protein A beads (Invitrogen) equilabrated in IP buffer. Five percent of the pre-cleared lysate was taken as the unenriched input sample. Pre-cleared lysates were incubated overnight at 4 ºC with 5 µL serum antibody to Bhlhb5 or Prdm8, or the appropriate preimmune serum. The immunoprecipitation was performed with 40 µL per IP of Protein A/G PLUS agarose beads (Santa Cruz). Beads were washed twice in low salt buffer [0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-Cl pH 8.0, and 150 mM NaCl], twice in high salt buffer [0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-Cl pH 8.0, and 500 mM NaCl], twice in LiCl buffer [0.25 M LiCl, 1% NP40, 1% DOC, 1 mM EDTA, 10 mM Tris ph 8.1], and once in room temperature TE. All washes are performed for 5 min at 4 ºC unless otherwise noted. The immunoprecipitates were eluted from the beads twice by incubation in 100 µL of elution buffer [10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS] at 65 ºC. The crosslinking reaction in the eluates and the input samples was reversed by overnight incubation at 65 ºC. All samples were digested with Proteinase K (140 µg per sample, 1 h at 37 ºC), extracted twice with phenol, and once with chloroform. Ethanol precipitation was used to form a DNA pellet, which was treated with RNase A (10 µg per sample, 1 h at 37 ºC) and purified over a QiaQuick PCR purification column.

ChIP binding was measured by quantitative real-time PCR on the final samples (gDNA fragments) using SYBR Green detection (Applied Biosystems, Foster City, CA). A standard curve based on the input gDNA fragments was performed for each primer in triplicate, and each sample was measured in triplicate. All ChIP-qPCR experiments are representative of at least three independent experiments. In addition, each ChIP experiment was performed with a negative control, either ChIP using preimmune serum (e.g., Figures S4C and S4D) or ChIP from tissue from knockout mice (Figures 6B, S8A and S8B).

Read alignment (mapping sequencing reads to the genome)

ChIP-Seq sequencing reads were aligned using the large genomes matching pipeline from Life Technologies with parameters –e 3 –t 35 –z 10. These parameters dictate that 0–3 color-space mismatches are allowed, a 35-bp read is aligned, and after 10 hits on a given chromosome, the aligner no longer looks for further matches. ChIP-Seq reads were aligned to the mouse NCBI genome version 37. Only reads aligning to a single genomic position with a tolerance of 0–3 color-space mismatches were used for findings reported in this manuscript. After ChIP-Seq reads were aligned, they were extended to 120 bp to match the length of the DNA fragments that were sequenced. To compensate for differences in total sequencing read depth among samples, all ChIP-Seq read counts were first normalized to their equivalent numbers assuming 10 million total reads per sample for display in the UCSC genome browser.

Identification of Bhlhb5 binding sites

To determine Bhlhb5-specific binding sites, we developed an algorithm to identify the sequences specifically enriched in the ChIP samples from wildtype neurons relative to the negative control ChIP samples from *Bhlhb5*$^{-/-}$ neurons. First we determined the false detection ratio by using a sliding window with a width of 240 bp for every 10 bp in the mouse genome. Repetitive regions are excluded from our analysis since 35 bp reads cannot be assigned uniquely to such regions. For each window, we calculated the statistic D = R-N where R is the number of reads in the sample from wild type mice, and N is the number of reads from the negative control *Bhlhb5*$^{-/-}$ sample. By considering the marginal distributions of R and N, we note that they both can be well approximated by a Poisson distribution with parameters l_R and l_N, respectively. It follows that D is a Skellam distribution (Skellam, 1946).

$$\Pr(D = d) \approx \mathrm{Ske}(d; \lambda_R, \lambda_N) = e^{-(\lambda_R+\lambda_N)} \left(\frac{\lambda_R}{\lambda_N}\right)^{d/2} I_{|d|}\left(2\sqrt{\lambda_R \lambda_N}\right)$$

where $I_{|d|}$ (z) is the modified Bessel function of the first kind of order Idl. When comparing the number of reads in a given window from two different samples, care must be taken to correct for differences in the total amount of sequenced reads. The construction of the null distribution takes unequal numbers of reads in the two samples into account by shifting the mode of the distribution. To determine the number of reads required for a 240 bp window to be significant, we use the local false detection rate (locFDR) framework (Efron, 2008). Using this methodology, we assume that the density of D, f(D), can be written as the mixture $f(D)= p_0f_0(D)+p_1f_1(D)$, where f_0 is the null density, f_1 is the density of windows corresponding to true peaks and $p_0+p_1 = 1$ with $p_0>0.9$. The locFDR, fdr(D) is related to the more familiar FDR [24] through FDR = E[locFDR(D)ID>=d], where E is the expectation with respect to the mixture density f and d is the threshold for the statistic D.

We used a locFDR cut-off of .001, which corresponds to D = 4 for a 240 bp window, as described in (Kim et al.). Overlapping windows were merged resulting in 2337 peaks, representing Bhlhb5 binding sites. When the same algorithm was applied to the ChIP sequencing from *Bhlhb5*$^{-/-}$ samples (i.e. negative control), only 159 peaks were detected, suggesting that that the majority of peaks identified in WT samples are specific.

The ChIP-Seq data are available online at Ross-et-al-2011.hms.harvard.edu.

Identification of Bhlhb5 consensus binding motif

To investigate the common motifs of the Bhlhb5 binding sites, we first extracted the DNA sequences found at each peak. We searched for overrepresented motifs using the software Weeder using the default settings (Pavesi et al., 2001). The best 10 bp motif is shown in Figure 5A, which corresponds very well to the known E-box (Ross et al., 2010).

Co-immunoprecipitation in heterologous cells

Mouse Bhlhb5 was subcloned into a pCS2 expression vector containing either 6 N-terminal myc tags or 3 N-terminal HA tags. HA-tagged E2-2B was a kind gift form C. Ampero (Sobrado et al., 2009). HEK293T cells were grown to ~60% confluence, transfected with the tagged plasmids for 48 hours, and lysed in ice-cold lysis buffer (0.2% Triton X-100, 300 mM NaCl, 50 mM Tris pH 8.0, 5 mM NaF, supplemented with complete protease inhibitor tablet from Roche). Lysates were rotated at 4 ºC for 30 min, and subsequently cleared by centrifugation (14,000 rpm for 5 min at 4 °C). For immunoprecipitations, supernatants were incubated with 2 µg of anti-myc (9E10, Abcam) for 8 hours followed by addition of protein A/G PLUS beads (Santa Cruz) for 1 h with rotation at 4 ºC. Samples were subsequently washed three times (10 min rotation between washes) with ice-cold lysis buffer and separated by SDS PAGE on a 4 – 20% gradient gel (Invitrogen) and subjected to Western Blotting using antibodies to HA (9F10, Roche) and myc (Abcam).

Quantitative western blotting Lysates of the dorsal telencephalon were subjected to western blotting using antibodies to Prdm8 and actin. Proteins levels were visualized by chemiluminescence and quantified by infrared fluorescence detection (Odyssey). To control for protein loading differences, all conditions were normalized to actin.

Supplemental References

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