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## Bhlhb5 and Prdm8 form a repressor complex involved in neuronal circuit assembly

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### SUMMARY

Although transcription factors that repress gene expression play critical roles in nervous system development, their mechanism of action remains to be understood. Here we report that the Olig-related transcription factor Bhlhb5 (also known as Bhlhe22) forms a repressor complex with the PR/SET domain protein, Prdm8. We find that Bhlhb5 binds to sequence-specific DNA elements and then recruits Prdm8, which mediates the repression of target genes. This interaction is critical for repressor function since mice lacking either *Bhlhb5* or *Prdm8* have strikingly similar cellular and behavioral phenotypes, including axonal mistargeting by neurons of the dorsal telencephalon and abnormal itch-like behavior. We provide evidence that Cadherin-11 functions as a target of the Prdm8/Bhlhb5 repressor complex that must be repressed for proper neuronal development to occur. These findings suggest that Prdm8 is an obligate partner of Bhlhb5, forming a repressor complex that directs neural development in part through the precise regulation of Cadherin-11.

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### SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes eight figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2010.02.025.

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## INTRODUCTION

A key unresolved issue in neurobiology is the nature of the molecular programs that regulate the differentiation of neural precursors into specialized neurons with appropriate connectivity. One family that has emerged as important in this regard is the basic helix-loop-helix (bHLH) containing transcription factors (Bertrand et al., 2002; Ross et al., 2003). For instance, studies investigating neocortical development have revealed that members of the bHLH family, including the Neurogenins and NeuroD family members, orchestrate the formation of glutamatergic neurons (Schuermans and Guillemot, 2002) and many of these are sufficient to activate a pan-neuronal program of gene expression that drives the differentiation of neural precursors into neurons (Farah et al., 2000; Lee et al., 1995; Ma et al., 1996).

Like many other bHLH transcription factors, *Bhlhb5* is broadly expressed in excitatory neurons in the dorsal telencephalon. However, in contrast to the Neurogenins and NeuroD family members, *Bhlhb5* appears not to be a transcriptional activator and does not mediate early steps in neuronal differentiation. Rather, *Bhlhb5* belongs to a subfamily of bHLH factors including *Bhlhb4* (also known as *Bhlhe23*) and the Olig proteins (Olig1–3) that function predominantly as transcriptional repressors. As an example, when Olig2 is fused to the repressor domain of *Engrailed*, this fusion protein, but not an activating Olig2-VP16 fusion protein, recapitulates the function of native Olig2 by specifying neural fate in the chick spinal cord (Zhou and Anderson, 2002). *Bhlhb5* and *Bhlhb4* are likewise thought to mediate repression based on their ability to inhibit the transactivation of NeuroD-responsive target genes in luciferase assays (Bramblett et al., 2002; Peyton et al., 1996; Xu et al., 2002). However, while these findings suggest that the Oligs, *ff*, and *Bhlhb5* form a sub-family of bHLH factors that mediate transcriptional repression, the manner in which these repressors function endogenously to repress transcription and orchestrate neural development remains obscure.

Studies in the spinal cord have provided a framework for understanding the cellular function of Olig proteins, and these studies suggest that a common function of the Oligs is to confer the neuronal identity of neural progenitors. For instance, Olig1 and Olig2 are expressed in select progenitors of the ventral spinal cord and, in the absence of these factors, neural precursors are re-specified to an alternate fate: instead of forming motor neurons and oligodendrocytes, these pMN progenitors inappropriately generate V2 interneurons and astrocytes (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). It is thought that this type of mis-specification occurs because the Oligs function, at least in part, to promote the generation of one subtype of neuron over another by inhibiting the expression of transcription factors that mediate the alternative cell fate choices (Marquardt and Pfaff, 2001).

Though *Bhlhb4* and *Bhlhb5* are closely related to the Oligs, their expression is almost exclusively limited to post-mitotic neurons rather than proliferating neural progenitors, hinting at the possibility that *Bhlhb4* and *Bhlhb5* regulate later aspects of neuronal differentiation (Bramblett et al., 2002; Joshi et al., 2008; Ross et al. 2010). Further evidence in support of this idea comes from studies in the retina where loss of either *Bhlhb4* or *Bhlhb5* results not in the mis-specification of retinal progenitors to alternate fates but rather the loss of subsets of neurons, presumably due to apoptosis. Thus, mice lacking *Bhlhb4* have an absence of rod bipolar cells, whereas *Bhlhb5* mutants are lacking cone bipolar and selective amacrine cells (Bramblett et al., 2004; Feng et al., 2006). Similarly, in the spinal cord, disruption of *Bhlhb5* function results in the selective apoptosis of a specific population of inhibitory interneurons in the superficial dorsal horn that are required for the normal sensation of itch (Ross et al., 2010). Together, these studies raise the possibility that *Bhlhb4*

and *Bhlhb5* are involved in late aspects of neuronal differentiation, such as neural circuit assembly, that may be essential for neuronal survival. However, why certain neurons die in the absence of these transcriptional repressors is unknown, and this gap in knowledge stems in part from a lack of mechanistic understanding of how these transcription factors function in neural development.

A possible clue to this puzzle comes from studies of *Bhlhb5* in the dorsal telencephalon. In mice lacking *Bhlhb5*, neurons of the dorsal telencephalon survive and send out axons, but these projections fail to reach their targets. For instance, corticospinal motor neurons terminate prematurely along the pyramidal tract in the ventral hindbrain and none extend into the spinal cord (Joshi et al., 2008). Moreover, as we report here, *Bhlhb5* mutants also show a complete absence of the three fiber tracts that connect the cerebral hemispheres, suggesting that axonal mistargeting in the absence of *Bhlhb5* is a widespread phenomenon. Since mice lacking *Bhlhb5* show axon targeting defects, we reasoned that one of the roles of *Bhlhb5* in the dorsal telencephalon may be to regulate neuronal connectivity, perhaps by repressing specific genes until they are needed, thereby ensuring that genes are expressed at the right time and place for correct neural circuit assembly. To investigate this possibility, we identified the targets of the *Bhlhb5* transcriptional repressor, hoping to elucidate how this transcription factor functions at a mechanistic level.

Here we show that *Bhlhb5* functions by binding to specific DNA sequence elements and then recruiting the PR/SET domain-containing protein, Pr-domain 8 (*Prdm8*) to mediate the repression of target genes. Our observations suggest that *Bhlhb5* and *Prdm8* are obligate partners for key aspects of neuronal development and, consistent with this idea, we find that mice lacking either *Bhlhb5* or *Prdm8* have strikingly similar cellular and behavioral abnormalities. We use genetic rescue experiments to demonstrate that one important target of the *Prdm8/Bhlhb5* repressor complex is Cadherin-11 (*Cdh11*), a cell-cell adhesion molecule involved in neural circuit assembly. Taken together these experiments have revealed how a bHLH transcription factor associates with a PR/SET-domain repressor protein to regulate genes involved in neural development.

## RESULTS

### ***Prdm8* and *Bhlhb5* regulate common aspects of neural development**

To gain insight into how *Bhlhb5* functions to regulate axon targeting, we sought to determine possible *Bhlhb5* target genes by identifying genes that are misexpressed in *Bhlhb5* mutant mice during the early development of the dorsal telencephalon (E13.5, E15.5 and E17.5). By expression profiling we found a total of eight transcripts that were significantly misregulated (FDR < 0.05) when *Bhlhb5* is disrupted. Notably, all of these genes were upregulated in *Bhlhb5* mutant mice, consistent with the idea that *Bhlhb5* may act directly to inhibit their expression. Of these, *Prdm8* and *Cdh11* were the most significantly misregulated genes, and we selected these for follow-up in the present study (Figures 1A and 7A). Other significantly misregulated genes that we identified are the gap junction protein *Connexin 36*; the MAGE family proteins *Necdin* and *Magel2*, which are inactivated in Prader-Willi syndrome (Nicholls and Knepper, 2001); the neurotrophin receptor *p75 NTR*; the neuropeptide, *Neurexophilin 3*; and the actin-binding protein *Fmnl1* (Figure S1). Of note, several of these genes, including *Cdh11*, *p75 NTR*, *Necdin*, and *Magel2*, are known to mediate axon extension (Lee et al., 2005a; Marthiens et al., 2005; Yamashita et al., 1999), consistent with the idea that *Bhlhb5* may control a program of gene expression that mediates aspects of neural development including axonal outgrowth.

From this list of putative *Bhlhb5* target genes, we focused initially on *Prdm8*, a protein belonging to the PRDI-BF1 and RIZ homology domain containing family that have recently

emerged as key mediators of development (Baudat et al., 2010; Berg et al., 2010; Ohinata et al., 2005; Parvanov et al., 2010; Seale et al., 2008). Members of this family are transcriptional regulators that are characterized by the presence of a SET domain, a signature motif found in members of the histone methyltransferase superfamily. Consistent with this, several Prdm proteins, including Prdm8, have been reported to have intrinsic histone methyltransferase activity (Eom et al., 2009; Hayashi et al., 2005; Kim et al., 2003; Wu et al., 2010), while others are known to function as repressors by recruiting histone modifying enzymes (Ancelin et al., 2006; Davis et al., 2006; Duan et al., 2005; Gyory et al., 2004). Since Prdm8 is significantly over-expressed upon the loss of *Bhlhb5* (Figures 1A, 1B and 1C), we reasoned that Prdm8 might function as part of a linear repressor cascade in which *Bhlhb5* represses Prdm8 and Prdm8 represses other targets. The other possibility that we considered was that *Bhlhb5* and Prdm8 function together, and that Prdm8 is up-regulated in the absence of *Bhlhb5* due to a mis-regulated negative feedback loop.

To begin to investigate these possibilities, we investigated whether mice lacking *Bhlhb5* or *Prdm8* share any common phenotypes. As reported previously, we observe that the axons from corticospinal motor neurons of *Bhlhb5* mutant mice terminate prematurely and fail to enter the spinal cord (Figures 2A, S2A, S2B and (Joshi et al., 2008)). In addition, we noted that loss of *Bhlhb5* in the dorsal telencephalon resulted in the almost complete absence of the three fiber tracts that connect the cerebral hemispheres: the corpus callosum, hippocampal commissure and the anterior commissure (Figures 2B and S2C). Strikingly, we found that these major axon tracts are also mistargeted in *Prdm8* mutant mice, which showed a similar absence of the corticospinal tract (Figure 2A) as well as agenesis of the corpus callosum and hippocampal commissure (Figure 2B). In contrast, cortical layering is unaffected in both *Bhlhb5* and *Prdm8* knockout mice (Figure S3). Thus, both *Bhlhb5* and *Prdm8* are required for the correct targeting of projection neurons of the dorsal telencephalon.

In addition to showing common axonal targeting defects, we found that *Bhlhb5* and *Prdm8* mutant mice have similar behavioral abnormalities. For example, both mutants show abnormal itching behavior that results in the formation of skin lesions, which is observed in 100% of *Bhlhb5* mutant mice and ~75% of *Prdm8* mutant mice (Figure 2C). Furthermore, ~5% of mice lacking either *Bhlhb5* or *Prdm8* occasionally display an unusual movement in which they walk on their forepaws (Figure 2D). The remarkable similarity of the cellular and behavioral phenotypes observed in mice lacking either *Bhlhb5* or *Prdm8* strongly suggests that these factors function together, possibly as obligate partners of the same transcriptional repressor complex.

### **Bhlhb5 and Prdm8 form a transcriptional repressor complex**

If *Bhlhb5* and *Prdm8* form a protein complex that represses transcription, we would expect *Bhlhb5* and *Prdm8* to i) be expressed in the same subsets of neurons, ii) inhibit the same target genes, and iii) bind together to the same regulatory elements in DNA. Thus, we set out to test each of these possibilities.

We began by generating antibodies to Prdm8 (Figure S3) to characterize its expression pattern. At the subcellular level, both *Bhlhb5* and *Prdm8* show a similar distribution in the nucleus (Figures 3A and 3B). Moreover, analysis of sections from wild type mice at a variety of embryonic and early post-natal ages revealed that *Bhlhb5* and *Prdm8* show a high degree of co-localization in select subpopulations of differentiating neurons. For instance, *Bhlhb5* and *Prdm8* are both expressed in the intermediate zone and the cortical plate of dorsal telencephalon from E13.5 – E17.5 (Figure S5A). By P0, both factors are highly expressed in superficial layers of the cortex (Figure 3B). In other regions of the nervous system (where *Bhlhb5* and *Prdm8* are expressed more sparsely) the co-expression of these

two proteins is even more apparent; *Bhlhb5* and *Prdm8* clearly mark a shared subset of neurons in the diencephalon (Fig. 3B, inset ii), the brainstem (Figure S5B) and the spinal cord (Ross et al., 2010). This co-expression in specific populations of neurons suggests that *Bhlhb5* and *Prdm8* might work together to regulate common aspects of neuronal differentiation.

Next we investigated whether the same genes are misexpressed in *Bhlhb5*<sup>-/-</sup> and *Prdm8*<sup>-/-</sup> mice. To obtain an unbiased view, we independently determined the gene expression profiles of each mutant, analyzing mRNA expression in the dorsal telencephalon of mice. This approach allowed us to investigate whether the genes that are misregulated in *Bhlhb5* mutant mice are also misregulated in *Prdm8* mutant mice, and vice versa, rather than simply testing previously discovered *Bhlhb5* targets in the *Prdm8* mutant mice. For these new gene profiling experiments, we chose P0 as the time point, both for practical reasons and with the hope of discovering new target genes.

Interestingly, we found that just as *Prdm8* mRNA is upregulated in *Bhlhb5* mutant mice, so *Bhlhb5* mRNA is upregulated in *Prdm8* mutant mice (Figures 4A and 4B). But what about other *Bhlhb5* target genes—are they likewise upregulated in *Prdm8* mutant mice? We found that loss of either *Bhlhb5* or *Prdm8* resulted in changes in gene expression in a small number of genes and, remarkably, all of the genes that were significantly upregulated in one mutant were also upregulated in the other. These genes include *Antxr2*, *Connexin36*, *NMDA3A* and *Paqr3* (Figures 4C, 4D, 4E and 4F) as well as *Fgf5* and *Netrin1* (data not shown). We therefore conclude that *Bhlhb5* and *Prdm8* inhibit the expression of a common set of genes, consistent with the possibility that they function together as part of the same repressor complex.

We next investigated more directly the possibility that *Bhlhb5* and *Prdm8* form a repressor complex by characterizing *Bhlhb5* occupancy throughout the genome and testing whether *Prdm8* is bound to the same genomic loci as *Bhlhb5*. The genomic binding sites of *Bhlhb5* in the dorsal telencephalon were mapped by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). As a negative control, we also performed ChIP-seq using tissue from *Bhlhb5*<sup>-/-</sup> mice, thereby confirming the specificity of the *Bhlhb5* antibody. In these ChIP-seq experiments, we identified ~ 2300 specific *Bhlhb5* binding sites, representing approximately one binding site per million bases (see Ross-et-al-2011.hms.harvard.edu to visualize genomic data online). In addition to describing *Bhlhb5* binding sites in the brain across the genome, the identification of these sequences allowed us to uncover an eight nucleotide consensus binding motif for *Bhlhb5*: CATATGNTNT (Figure 5A). Thus, *Bhlhb5* binds to a sequence element consisting of a canonical E-box (underlined), a motif common to many members of the basic helix-loop-helix family, together with several other key nucleotides that likely confer additional sequence specificity.

Having identified genomic *Bhlhb5* binding sites in the brain, we were in a position to ask whether *Prdm8* binds to the same DNA sequence elements. To address this question, we chose several genes from the ChIP-seq data to test, including two genes that showed *Bhlhb5* binding in the proximal promoter, *Bhlhb5* itself and *Repressor Protein 58 (RP58)* (Figures 5B and 5C). In addition, we selected one of the putative *Bhlhb5* target genes identified by expression profiling, *Cdh11*, which showed *Bhlhb5* binding within its first intron (Figure 5D). First, we confirmed that *Bhlhb5* bound to these genomic locations by ChIP-qPCR, comparing the binding of *Bhlhb5* at its putative binding site to that observed at a negative control region 2 – 3 kilobases away (Figures 5E, 5F and 5G). Next, we performed ChIP-qPCR using specific antibodies to *Prdm8* and examined whether the *Bhlhb5* binding sites are likewise occupied by *Prdm8*. Notably, these experiments revealed that each of the loci tested that are bound by *Bhlhb5* are also bound by *Prdm8* (Figures 5H, 5I, and 5J). To



ensure that the binding of *Bhlhb5* and *Prdm8* at these loci is specific we performed a number of negative controls. We observed no binding at these sites when preimmune antisera is used instead of immune antisera (e.g., Figures S4C and S4D) and none of these sites is bound by the transcription factors *Npas4*, *CREB* or *SRF* (Kim et al., 2010), thereby confirming specificity. To address whether the precise correspondence in binding sites for *Bhlhb5* and *Prdm8* is a widespread phenomenon, we tested twelve other genomic loci, including all of the putative *Bhlhb5* binding sites that are found within 200 kb of genes that are misregulated in the *Bhlhb5* knockout mouse. In general, a very good correspondence in binding between *Bhlhb5* and *Prdm8* was observed, suggesting that the vast majority of *Bhlhb5* binding sites are also occupied by *Prdm8* (Figure S7). Consistent with this idea, we found that *Bhlhb5* and *Prdm8* are associated with one another under the conditions used for ChIP, as revealed by co-immunoprecipitation and western blotting (Figure 5K). Taken together, these data strongly indicate that *Bhlhb5* and *Prdm8* are bound concurrently to common DNA elements throughout the genome where they repress transcription.

### **A *Bhlhb5* homodimer binds specifically to DNA and then recruits *Prdm8* to mediate repression**

Our experiments provided several lines of evidence in support of the idea that *Bhlhb5* and *Prdm8* form a neural repressor complex: these factors are co-localized in neurons where they bind to the same genomic loci, and loss of either factor results in highly similar cellular and behavioral phenotypes, as well as the upregulation of a common set of genes. However, the discovery of this neural repressor complex left open a key remaining question—how does each component of the *Bhlhb5/Prdm8* repressor complex function at a molecular level to repress gene expression?

As a first step to gain molecular insight into the nature of the *Bhlhb5* repressor complex, we investigated whether *Bhlhb5* forms a homo or heterodimer. Many members of the basic helix-loop-helix family of transcription factors bind DNA as a heterodimer with E-proteins (*E2A*, *E2-2* and/or *HEB*) and of these, only *E2-2* (also known as *Tcf4*) is expressed in post-mitotic neurons of the dorsal telencephalon (see [www.stjudebgem.org](http://www.stjudebgem.org)). We therefore considered the possibility that *Bhlhb5* might dimerize with *E2-2*. Alternatively, given that the *Olig2*, which is closely related to *Bhlhb5*, forms avid homodimers, we also tested whether *Bhlhb5* might likewise partner with itself (Lee et al., 2005b; Li et al., 2011). To distinguish between these possibilities, we performed co-immunoprecipitation of tagged constructs expressed in heterologous cells. These experiments revealed that myc-tagged *Bhlhb5* is able to pull down HA-tagged *Bhlhb5* but not HA-tagged *E2-2*, suggesting that *Bhlhb5* more readily forms a homodimer than a heterodimer with E-proteins (Figure 6A).

Next we addressed the molecular role of *Prdm8* within this repressor complex. Some members of the *Prdm* family have been shown to function as sequence specific transcription factors, while others are known to function as co-factors to mediate transcriptional repression (Davis et al., 2006; Duan et al., 2005; Gyory et al., 2004; Hayashi et al., 2005; Kim et al., 2003). Given the phenotypic similarity between *Bhlhb5* and *Prdm8* mutant mice, we first considered the hypothesis that a *Bhlhb5* dimer is recruited to specific DNA elements through its consensus binding motif and then recruits *Prdm8* to mediate transcriptional repression. If so, we reasoned that *Bhlhb5* would bind normally to its DNA targets in the absence of *Prdm8*, but that *Prdm8* would not associate with these sites in the absence of *Bhlhb5*.

To address this hypothesis, we performed ChIP-qPCR from the dorsal telencephalon of wild type or mutant mice and analyzed the binding of *Bhlhb5* and *Prdm8* at the *RP58* promoter. As we had shown above (Figures 5F and 5I), we again found that both *Bhlhb5* and *Prdm8* display robust binding to the proximal promoter of *RP58* in wild type mice (Figure 6Bi).

Furthermore, *Bhlhb5* shows similar binding to the *RP58* promoter when ChIP-qPCR was performed in *Prdm8* mutant mice, indicating that the binding of *Bhlhb5* at this promoter is not dependent on *Prdm8* (Figure 6Bii). In sharp contrast, however, we found that *Prdm8* was not bound to the *RP58* promoter in *Bhlhb5* mutant mice (Figure 6Biii). Note that the observed absence of *Prdm8* binding at this site is not due to a general absence of *Prdm8* protein in *Bhlhb5*<sup>-/-</sup> mice (e.g., see Figure 1C). Thus, the inability of *Prdm8* to bind to the *RP58* promoter in the absence of *Bhlhb5* suggests that *Prdm8* requires *Bhlhb5* for targeting to this genetic locus. Furthermore, the dependence of *Prdm8* on *Bhlhb5* for sequence-specific targeting to DNA appears to be a general phenomenon since we observed similar results when we tested several other genomic loci including the *Bhlhb5* promoter (Figure S8A) and the *Bhlhb5* binding site in the first *Cdh11* intron (Figure S8B).

Based on these findings, we suggest a model in which *Bhlhb5* functions by binding to specific DNA elements possibly as a homodimer and then recruiting *Prdm8* to mediate the repression of target genes (Figure 8A). When the *Bhlhb5* alone is present, it can bind to target genes, but it cannot repress them. Likewise, when *Prdm8* alone is present, target genes are also not repressed, in this case because *Prdm8* does not bind to DNA in the absence of *Bhlhb5*. Thus, both factors are required to mediate transcriptional repression of a specific set of target genes so that, when either *Bhlhb5* or *Prdm8* is knocked out, common genes are upregulated and highly similar phenotypes result.

### **Cdh11 is a critical target of the Bhlhb5/Prdm8 repressor complex**

With this new insight into *Bhlhb5* function, we next turned our attention to identifying a mechanistic link between the misexpressed target genes and the neural phenotypes observed upon the loss of the *Bhlhb5/Prdm8* repressor complex. We focused on the genes that are misregulated in *Bhlhb5* mutant mice at the time of the axon targeting defects (i.e. from E13.5 – E17.5). One of these genes is *Cdh11*, a classic type II cadherin that mediates homophilic cell-cell adhesion (Kimura et al., 1995). *Cdh11* mRNA is expressed in differentiating neurons of the cortical plate, including layer V projection neurons that form the corticospinal tract (Kimura et al., 1996). Furthermore, we found that *Cdh11* protein is highly expressed in the axons of corticofugal neurons at E16.5, when these projection neurons are extending their projections through the internal capsule (Figure 7C, white arrows), consistent with the idea that *Cdh11* may play a role in their guidance. In addition, the subcortical expression pattern of *Cdh11* is suggestive of a possible role in regulating the connectivity of corticospinal motor neurons. In particular, *Cdh11* is specifically expressed in a number of intermediate subcortical targets where corticospinal motor neurons form collaterals, namely the red nucleus, the basilar pons, and the inferior olive (Kimura et al., 1996). Importantly, *Bhlhb5* and *Prdm8* bind to an intron within the *Cdh11* gene (Figures 5D, 5G and 5J) and *Cdh11* mRNA is up-regulated in both *Bhlhb5* and *Prdm8* mutant mice during embryonic development (Figures 7A and 7B). Upon loss of *Bhlhb5*, the overall level of *Cdh11* protein appears elevated, whereas the pattern of *Cdh11* expression is unaffected (Figures 7C and D), suggesting that a *Bhlhb5/Prdm8* repressor complex may function to restrain the level of *Cdh11* rather than its distribution.

Based on these observations, we hypothesized that *Cdh11* might be a target of the *Bhlhb5/Prdm8* repressor complex whose upregulation in the absence of *Bhlhb5* or *Prdm8* leads to a disruption of the formation of the corticospinal tract. Specifically, over-expression of *Cdh11* in axons of corticospinal motor neurons might impede their progress due to enhanced adhesion to *Cdh11*-expressing intermediate targets. This might then prevent these *Cdh11*-overexpressing axons from extending past *Cdh11*-expressing intermediate targets and into the spinal cord. If so, we reasoned that reducing the level of *Cdh11* in *Bhlhb5* mutant mice might at least partially rescue the axon extension defects in corticospinal motor neurons. To test this idea we obtained *Cdh11* mutant mice (*Cdh11*<sup>-/-</sup>), which lack functional *Cdh11* due

to a targeted disruption of the extracellular domain and most of the transmembrane domain (Horikawa et al., 1999). Importantly, mice lacking *Cdh11* show normal targeting of corticospinal axons (Figure 7G and data not shown). We compared the size of the corticospinal tract in mice lacking both *Bhlhb5* and *Cdh11* (*Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup>) to littermates in which *Bhlhb5* alone is disrupted (*Bhlhb5*<sup>-/-</sup>). For these experiments, we used anatomical landmarks to carefully identify matched coronal sections for each genotype, and then quantified the area marked by PKC $\gamma$ , a protein that labels axons of the corticospinal tract. This analysis revealed that, relative to wild type mice, both *Bhlhb5*<sup>-/-</sup> single mutants and *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> double mutants show a significant reduction in the size of corticospinal tract as it passes through the caudal brain. However, the severity of this defect was significantly reduced in the *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> double mutants, which showed a significantly larger area of PKC $\gamma$  staining than that observed in mice lacking *Bhlhb5* alone, both in the ventral medulla and in the dorsal funiculus of the cervical spinal cord (Figures 7E, 7F and 7G). These findings indicate that reducing the level of expression of *Cdh11* in *Bhlhb5* mutant mice results in a partial rescue of the area of the corticospinal tract, suggesting that *Cdh11* is one of the *Bhlhb5*/*Prdm8* target genes that must be repressed for proper axon targeting of corticospinal motor neurons to occur.

We next considered the possibility that upregulation of *Cdh11* expression in the absence of the *Bhlhb5*/*Prdm8* complex might contribute to other phenotypes observed in *Bhlhb5* and *Prdm8* mutant mice. Consistent with this idea, we observed that, whereas virtually all *Bhlhb5*<sup>-/-</sup> mice developed skin lesions by eight weeks of age, almost none of the *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> double mutants had skin lesions at this time. To confirm this observation, we carefully documented the age of onset of skin lesions in littermates that were either single (*Bhlhb5*<sup>-/-</sup>) or double (*Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup>) mutants. Although mice of both genotypes eventually developed skin lesions, the appearance of lesions occurred significantly later in *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> mice compared to *Bhlhb5*<sup>-/-</sup> mice (13 vs. 6 weeks, respectively, n = 12 littermate pairs; Figure 7H), whereas mice lacking *Cdh11* alone never developed skin lesions. Thus, loss of *Cdh11* significantly delays the onset of skin lesions in *Bhlhb5* mutant mice. The partial genetic rescue of two phenotypes (skin lesions and corticospinal tract axon mistargeting) observed in *Bhlhb5*<sup>-/-</sup> mice supports the idea that *Cdh11* is a target of the *Bhlhb5*/*Prdm8* repressor complex, and suggests that the precise regulation of this adhesion molecule through repression is critical for proper neural development.

## DISCUSSION

We examined how *Bhlhb5* functions at a molecular level and discovered that it couples specifically with *Prdm8* to form a neuronal repressor complex. This conclusion is supported by our observation that a common set of genes is aberrantly over-expressed upon loss of either factor, likely accounting for the striking phenotypic similarities between *Bhlhb5* and *Prdm8* knockout mice. In addition, our studies reveal that *Bhlhb5* and *Prdm8* bind to common genetic loci and, importantly, each requires the other for its activity: *Prdm8* can no longer target these loci in the absence of *Bhlhb5*, whereas *Bhlhb5* can bind target genes but cannot repress them without *Prdm8* (Figure 8A). Finally, genetic rescue experiments reveal that *Cdh11* is one of the targets of the *Bhlhb5*/*Prdm8* repressor complex whose upregulation in the absence of the *Bhlhb5*/*Prdm8* repressor complex contributes to the abnormal phenotype observed in *Bhlhb5* and *Prdm8* mutant mice. Taken together, these findings suggest that *Bhlhb5* and *Prdm8* are obligate components of a transcriptional repressor complex. *Bhlhb5* binds to specific DNA within the regulatory regions of its target genes and recruits *Prdm8* to mediate repression of the transcription of these targets. In the absence of *Bhlhb5* or *Prdm8* the gene targets are upregulated resulting in abnormal development.



## The coupling of bHLH factors and Prdm-related proteins as a common theme in neural development

Since Bhlhb5 and Prdm8 belong to conserved gene families, our finding that these two factors specifically interact raises the question as to whether the functional association between bHLH factors and Prdm-related proteins is a more general occurrence. Phylogenetic analysis of SET-domain containing proteins reveals that Prdm8 is most closely related to Prdm13, and recent studies show that Prdm8 and Prdm13 are expressed in non-overlapping patterns in the developing nervous system (Fumasoni et al., 2007; Kinameri et al., 2008). However, we unexpectedly discovered that the gene with which Prdm8 shares the highest degree of similarity (as revealed using a blastn algorithm comparing murine genes) is not a member of the Prdm family, but rather the zinc-finger protein, Zfp488. This gene shares 82% similarity with Prdm8 over 80 amino acids in the C-terminus (Figure 8C), suggesting that Prdm8 and Zfp488 may share a common ancestor. The idea that Prdm8 and Zfp488 are ancestrally related proteins is noteworthy because Zfp488 was recently shown to interact physically and functionally with one of Bhlhb5's closest relatives, Olig2 (Wang et al., 2006). This intriguing connection suggests the possibility that the interaction between bHLH transcription factors and Prdm-related proteins is a general mechanism for the regulation of gene expression during neural development (Figure 8B). In this regard, it is notable that both Prdm13 and Olig3 are expressed in Class A progenitors in the dorsal spinal cord, consistent with the idea that these two factors may also couple selectively to mediate transcriptional repression in these cells (Kinameri et al., 2008; Muller et al., 2005).

We provide phenotypic and mechanistic evidence that Bhlhb5 and Prdm8 are obligate partners for certain aspects of development. However, it is likely that Prdm8 functions without Bhlhb5 in some contexts. This prediction is based on the observation that, while Prdm8 shows significant overlap with the Bhlhb5 expression domain, there are regions of the nervous system that express Prdm8 but not Bhlhb5. For instance, Prdm8 (but not Bhlhb5) is widely expressed in the developing diencephalon, throughout neurons of the dorsal root ganglia (DRG), and in rod bipolar cells of the retina (S.E. Ross, unpublished observation). This somewhat broader pattern of expression of Prdm8 relative to Bhlhb5 suggests that Prdm8 may have additional partners to which it can couple, and one attractive candidate in this regard is Bhlhb4: loss of function studies have revealed that Bhlhb4 is required for the survival of rod bipolar cells and, furthermore, that this factor is expressed, like Prdm8, in the embryonic diencephalon and DRG (Bramblett et al., 2002; Bramblett et al., 2004).

### Molecular function of the Bhlhb5/Prdm8 repressor complex

Because Prdm8 contains a SET domain that is characteristic of histone methyltransferases, it is possible that it may directly mediate repression of target genes by methylating target gene-associated histones. Consistent with this idea, Prdm8 has been shown to methylate histone H3K9 *in vitro* (Eom et al., 2009), a modification associated with transcriptional repression. Likewise, the tumor suppressor Prdm2 and the meiotic recombination determinant Prdm9 also show intrinsic histone methyltransferase activity (Hayashi et al., 2005; Kim et al., 2003). However, several other Prdm family members, including Prdm1, Prdm5 and Prdm6, appear to mediate repression indirectly by recruiting the histone methyltransferase, G9A (Davis et al., 2006; Duan et al., 2005; Gyory et al., 2004). Thus, it is not yet clear whether Prdm8 functions directly or indirectly to mediate transcriptional repression. In either case, however, Prdm8 appears to be required for the repression of Bhlhb5 target genes.

A curious aspect of the Bhlhb5/Prdm8 repressor complex is that, while each requires the other to repress target gene expression, we do not observe a perfect coincidence the

expression of *Bhlhb5* and *Prdm8*. Indeed, in many cases, the expression of these two factors appears to be somewhat reciprocal—neurons with highest levels of *Bhlhb5* tend to have low levels of *Prdm8*, and vice versa. This disparity in expression level implies that *Bhlhb5* and *Prdm8* do not always exist as part of a functional repressor complex, and furthermore suggests that the expression of these factors is very tightly controlled, possibly to limit the degree and/or the duration of gene repression mediated by *Bhlhb5/Prdm8*. In keeping with this idea, we find that the *Bhlhb5/Prdm8* repressor appears to curb its own activity by restricting the expression of *Prdm8*, which is upregulated in *Bhlhb5* knockout mice. These observations suggest that *Bhlhb5* and *Prdm8* are part of a complex regulatory network that needs to be precisely coordinated for proper development.

### A possible role for *Cdh11* in neural circuit formation

One of the consequences of disrupting the function of the *Bhlhb5/Prdm8* repressor complex is that *Cdh11* is aberrantly over-expressed, and our findings suggest that this misexpression has detrimental consequences for neuronal development. Previous studies have revealed that cadherins form a large family of cell-cell adhesion molecules that have been proposed to serve as an adhesive code underlying neural assembly (Takeichi, 2007). Classic type II cadherins, in particular, are expressed in restricted groups of synaptically interconnected neurons in a manner that is highly suggestive of a role in neural circuit formation (Inoue et al., 1998; Suzuki et al., 1997). We speculate that the importance of *Cdh11* for neural connectivity is masked in loss-of-function studies, possibly as a consequence of redundancy in the mechanisms that mediate neural circuit assembly. Here we have revealed a possible role for *Cdh11* in circuit development by observing the effects of a *Cdh11* gain-of-function—that is, overexpression of *Cdh11* due to loss of the *Bhlhb5/Prdm8* repressor complex. We suggest that *Cdh11* may be involved in the interaction between axons from corticospinal motor neurons and intermediate subcortical targets, such as the red nucleus, the basilar pons, and the inferior olive, which also express *Cdh11*. If so, overexpression of *Cdh11* in axons of corticospinal motor neurons may impede their progress beyond these intermediate targets and into the spinal cord. In addition, our genetic rescue experiments implicate *Cdh11* as one target of the *Bhlhb5/Prdm8* repressor complex in the spinal cord, where *Bhlhb5/Prdm8*-mediated repression is required for the proper function of neural circuits that mediate itch sensation. Thus, *Bhlhb5/Prdm8*-mediated repression may be required to ensure that *Cdh11* is expressed at the right time and place for proper connectivity and function of motor and sensory circuits. Formal proof that *Cdh11* is a direct target of the *Bhlhb5/Prdm8* repressor complex will require analysis of the axonal projection and itch phenotypes in a knock-in mouse in which the *Bhlhb5* binding site within *Cdh11* gene is disrupted.

Since the loss of *Cdh11* in *Bhlhb5* mutant mice results only in a partial rescue of axon extension in corticospinal neurons, it is likely that other misexpressed genes also contribute to this phenotype. In this regard, it is noteworthy that several additional putative *Bhlhb5/Prdm8* target genes, including *p75 NTR*, *Necdin*, *MageL2*, and *Netrin* have been shown to play roles in axon extension and/or axon guidance (Lee et al., 2005a; Marthiens et al., 2005; Serafini et al., 1994; Yamashita et al., 1999). Furthermore, netrin signaling is required for the guidance of corticospinal tract axons (Finger et al., 2002). Thus, our work identifies several interesting candidates that warrant further study since they may contribute to the axon targeting defects observed when either *Bhlhb5* or *Prdm8* function is disrupted.

## EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for details on animal husbandry and colony management, immunohistochemistry, gene expression analysis by qPCR, ChIP-Seq library construction, ChIP, read alignment, the identification of *Bhlhb5* binding sites, the

identification of Bhlhb5 consensus binding motif, co-immunoprecipitation, the generation of phylogenetic trees, and quantitative western blotting.

### Quantification of corticospinal tract axon area

Five pairs of littermate adult mice (~ 8 wks) that were lacking either *Bhlhb5* alone (*Bhlhb5*<sup>-/-</sup>) or both *Bhlhb5* and *Cdh11* (*Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup>) were perfused with 4% PFA and the brain was dissected out with the cervical spinal cord attached. For each sample, serial sections (20 μm) were collected from the cortex through to the cervical spinal cord. Every 5<sup>th</sup> section was co-stained with PKCγ (which marks the corticospinal tract), as well as NeuN and Hoescht to assist with matching levels between samples. Matched images corresponding to two regions were selected for analysis: I) caudal to the basilar pons and II) caudal to the pyramidal decussation. Images were analyzed in Metamorph. A constant threshold was applied to all images and the dorsal funiculus was masked. We then computed the area above threshold, which was normalized to the area observed in wild type mice. All measurements were conducted blind to genotype.

### Generation of Phylogenetic trees

Phylogenetic trees of murine Bhlhb5- and Prdm8-related proteins were created using the amino acid sequences of each murine protein and the ClustalW algorithm, with MyoD and G9A as the outgroups, respectively. Apart from Zfp488, which we added based on our discovery of high similarity in protein sequences between Prdm8 and Zfp488 (Evalue 3e-28), the decision of which family members to include in the phylogenetic analysis was based on previous analyses for bHLH (Ledent et al., 2002; Ledent and Vervoort, 2001; Stevens et al., 2008) and Prdm families (Fumasoni et al., 2007).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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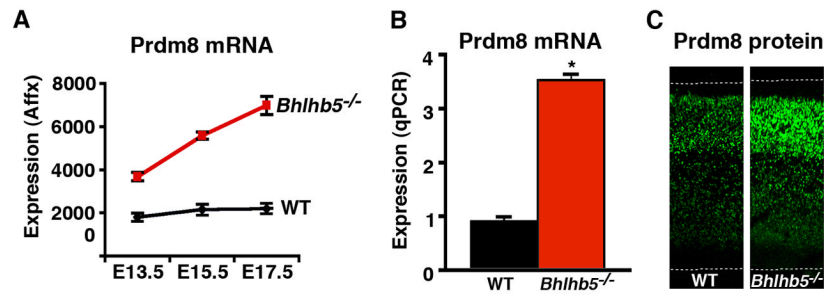
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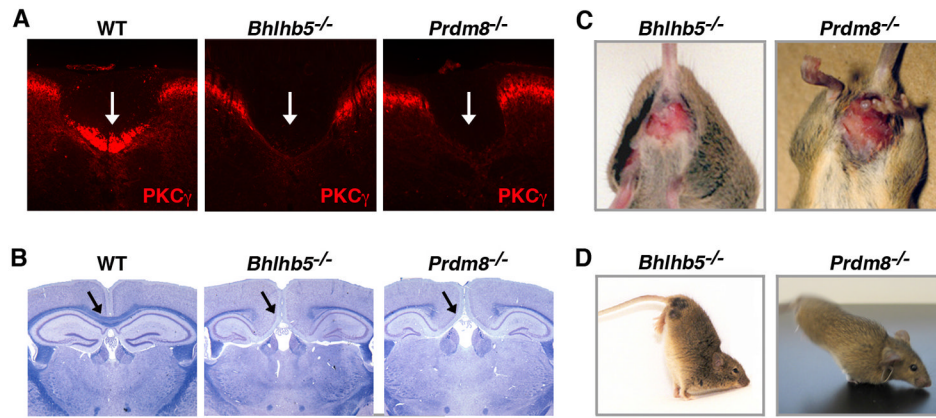
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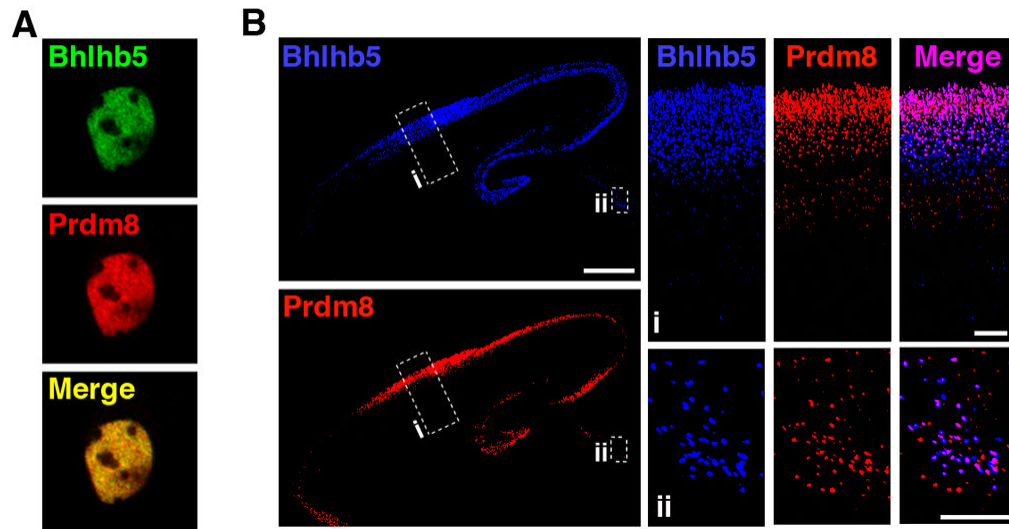


**Figure 1. Screen for *Bhlhb5* target genes identifies the PR/SET-domain protein, Prdm8**  
**A)** Affymetrix microarray-based gene profiling was performed to identify genes that are misexpressed in the dorsal telencephalon of *Bhlhb5*<sup>-/-</sup> mice. Three embryonic stages (E13.5, E15.5 and E17.5) were investigated, and each time point was analyzed using three independent pairs of littermate mice. Using a false discovery rate (FDR) of 0.05 or less, we identified a total of eight genes, and all of these genes were up-regulated in *Bhlhb5*<sup>-/-</sup> mice (also see Figures 7A and S1). Of these, Prdm8 (shown) was the most significantly misregulated. Data are mean  $\pm$  SEM, with wild type (WT) in black and *Bhlhb5*<sup>-/-</sup> in red, as indicated. **B)** Quantitative PCR confirms that Prdm8 mRNA transcripts are upregulated in the dorsal telencephalon of *Bhlhb5*<sup>-/-</sup> mice relative to WT littermates at E17.5. Data are normalized to WT and are presented as mean  $\pm$  SEM of biological replicates. \* indicates significant difference relative to controls ( $p < 0.05$ , t-test). **C)** Prdm8 protein is upregulated in the cortex of *Bhlhb5*<sup>-/-</sup> mice at E16.5. Matched coronal sections were stained with a Prdm8 antibody raised against a GST-Prdm8 fusion protein. Also see Figure S4 for details on the generation of Prdm8 antibodies and Figure S6 for further analysis of the upregulation of Prdm8 in other regions of the nervous system and at other times during development.



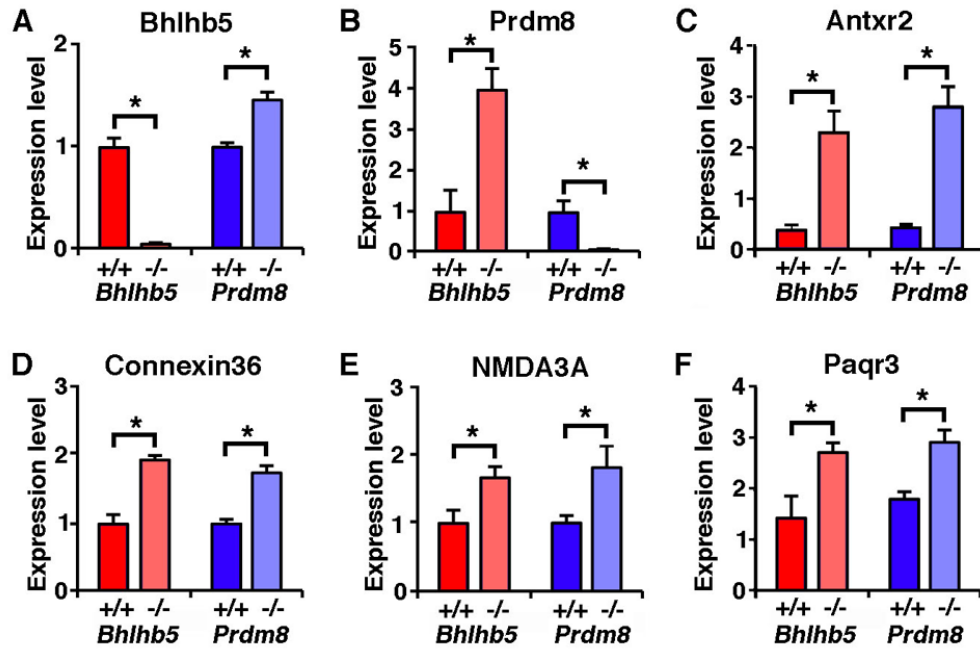
**Figure 2. Mice lacking either *Bhlhb5* or *Prdm8* have similar phenotypes**

**A)** Absence of the corticospinal tract in the spinal cord of mice lacking either *Bhlhb5* or *Prdm8*. In wild type mice (WT), PKC $\gamma$  immunostaining labels the axons from corticospinal motor neurons as they descend in the dorsal funiculus of the spinal cord (white arrow). In both *Bhlhb5*<sup>-/-</sup> and *Prdm8*<sup>-/-</sup> mice, this axon tract is absent at all levels of the spinal cord. Similar results are observed when this fiber tract is labeled genetically (data not shown). Note that PKC $\gamma$  also labels a subset of lamina II neurons in the dorsal horn, which are unaffected by the loss of *Bhlhb5* or *Prdm8*. Images are of representative cervical spinal cords from adult mice (8 wks). **B)** Agenesis of the corpus callosum and hippocampal commissure in mice lacking either *Bhlhb5* or *Prdm8*. In wild type mice (WT), dense bundles of axons connect the two hemispheres of the dorsal telencephalon (black arrow). In *Bhlhb5*<sup>-/-</sup> and *Prdm8*<sup>-/-</sup> mice, there is severe agenesis of these colossal fiber tracts. This axon targeting phenotype was observed in 20/20 *Bhlhb5*<sup>-/-</sup> mice, 5/7 *Prdm8*<sup>-/-</sup> mice, and none of the corresponding wild type littermates. Images are of representative coronal brain sections from adult mice stained with luxol fast blue and cresyl violet. **C)** Mice lacking either *Bhlhb5*<sup>-/-</sup> or *Prdm8*<sup>-/-</sup> show elevated scratching behavior that results in the development of skin lesions. Photos illustrating characteristic lesions are shown. All *Bhlhb5*<sup>-/-</sup> mice develop skin lesions at approximately 6 wks of age; ~75% of *Prdm8*<sup>-/-</sup> mice eventually develop skin lesions. **D)** Both *Bhlhb5*<sup>-/-</sup> and *Prdm8*<sup>-/-</sup> mice show an unusual movement in which they walk on their forepaws. Note that this ‘handstand’ phenotype, which appears to be secondary to abnormal contraction of the hindpaws, is only observed in a small fraction (~1–5%) of *Bhlhb5* or *Prdm8* mutants. Also see Figure S2 for a more detailed analysis of axon targeting defects and Figure S3 for analysis of cortical lamination, which is unaffected by the loss of either *Bhlhb5* or *Prdm8*.



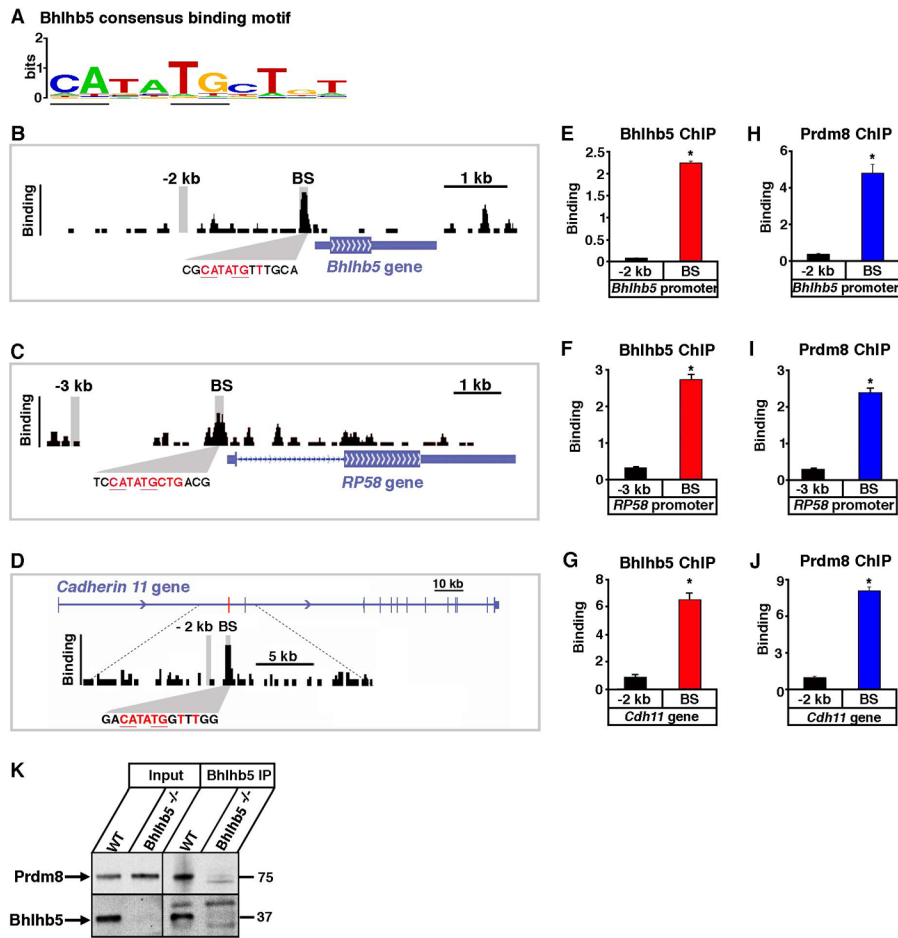
**Figure 3. Bhlhb5 and Prdm8 are colocalized in subsets of neurons**

**A)** Double-labeling of neurons with antibodies to Bhlhb5 (green) and Prdm8 (red) reveals that Bhlhb5 and Prdm8 are localized (merge, yellow) to the nucleus where they show a diffuse pattern of expression, suggesting that they may modulate the expression level of transcribed genes found within euchromatic DNA. **B)** Immunohistochemistry of sagittal sections from wild type mice at P0 using antibodies to Bhlhb5 (blue) and Prdm8 (red) reveals that both proteins are co-expressed to a large extent in superficial layers of the cortical plate and in the developing hippocampus. Scale bar = 500  $\mu$ m. Insets are enlarged on the right, top (i), showing co-localization (purple) of Bhlhb5 and Prdm8 in the superficial cortex, and bottom (ii), showing co-localization in sparse subsets of neurons in the diencephalon. Scale bar = 100  $\mu$ m. Also see Figure S5 for a more extensive analysis of Bhlhb5 and Prdm8 co-localization at other times during development and in other regions of the nervous system.



**Figure 4. Mice lacking either *Bhlhb5* or *Prdm8* have a common molecular profile**  
 Affymetrix microarray-based gene profiling was performed to identify genes that are misexpressed in the dorsal telencephalon of either *Bhlhb5*<sup>-/-</sup> or *Prdm8*<sup>-/-</sup> mice at P0. Each of the mutant strains was compared to its respective littermate control, using four independent biological replicates per genotype, and observations were validated by quantitative qPCR. **A)** *Bhlhb5* mRNA is significantly upregulated in *Prdm8*<sup>-/-</sup> mice. **B)** *Prdm8* mRNA is significantly upregulated in *Bhlhb5*<sup>-/-</sup> mice. **C – F)** Common genes are similarly misregulated in both *Bhlhb5*<sup>-/-</sup> and *Prdm8*<sup>-/-</sup> mice, including *Antxr2* (C), *Connexin36* (D), *NMDA3A* (E) and *Paqr3* (F). Data are presented as mean  $\pm$  SEM of biological replicates and \* indicates significant difference relative to controls ( $p < 0.05$ , t-test). Of note, most of the genes that are misregulated in *Bhlhb5* mutant mice at P0 (i.e., as illustrated here) are different that those that are misregulated from E13.5 – E17.5 (Figure S1), suggesting that *Bhlhb5* may regulate different genes at different times during development. Also see Figure S6 for analysis of possible cross-regulation of *Bhlhb5* and *Prdm8* at the protein level.

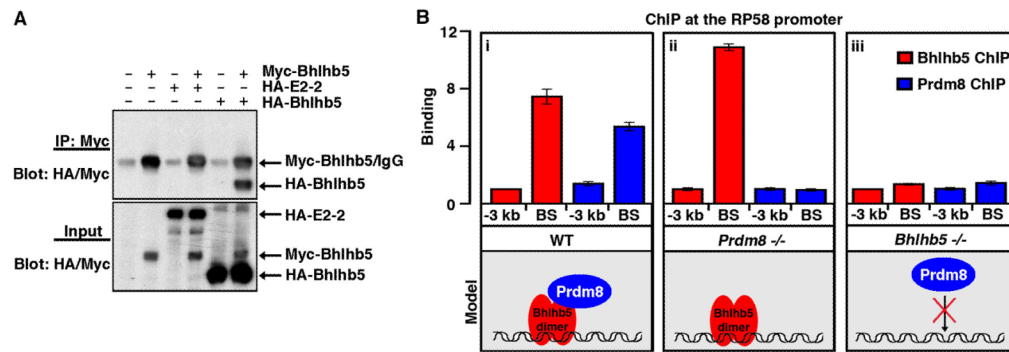




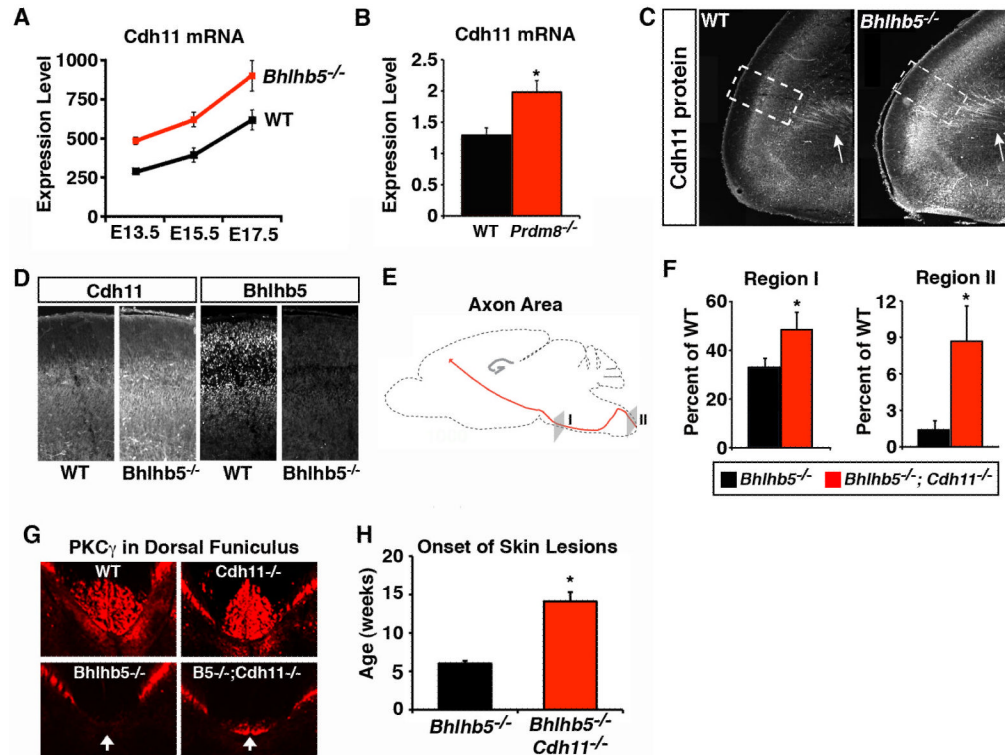
**Figure 5. Bhlhb5 and Prdm8 bind to common genomic loci**

DNA sequences to which Bhlhb5 is bound in the dorsal telencephalon at E17.5 were identified by chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq). **A**) Bhlhb5 consensus binding motif identified by the analysis of genomic Bhlhb5 binding sites. E-box is underlined. **B – D**) ChIP-seq data shows Bhlhb5 binding at its own proximal promoter (**B**), the proximal promoter of *RP58* (**C**) and the first intron of *Cdh11* (**D**). The height of the black bars indicates the number of input-normalized ChIP-Seq reads, representing the amount of Bhlhb5 binding. Consensus binding motifs for Bhlhb5 are found within the Bhlhb5 binding site at each gene, shown in red. Vertical gray bars indicate the genomic regions amplified in subsequent ChIP-qPCR experiments, either at the Bhlhb5 binding site (BS) or at a negative control region located –2 or –3 kb away, as indicated. The binding site for Bhlhb5 within the first intron of *Cdh11* is indicated by the red bar. **E – G**) ChIP-qPCR using antibodies to Bhlhb5 confirms that Bhlhb5 is bound to its own proximal promoter (**E**), the proximal promoter of *RP58* (**F**) and the first intron of *Cdh11* (**G**). Experiments show significantly enriched Bhlhb5 binding at the identified Bhlhb5 binding site (BS) relative to the negative control region. **H – J**) ChIP-qPCR using antibodies to Prdm8 reveals that Prdm8 is also bound to the Bhlhb5 binding site at the proximal promoter of *Bhlhb5* (**H**), the proximal promoter of *RP58* (**I**) and the first intron of *Cdh11* (**J**). Experiments show significantly enriched Prdm8 binding at the identified Bhlhb5 binding site (BS) relative to the negative control region. Also see Figure S7, in which 12 additional genomic loci are tested for the co-occupancy of Bhlhb5 and Prdm8. For **E – J**, chromatin immunoprecipitates were prepared from the dorsal telencephalon of P0 mice and y-axis

(Binding) represents enrichment of gDNA over input ( $\times 10^{-3}$ ). For each ChIP experiment, antibody and/or knockout controls were performed in parallel, and in each case, these showed extremely low apparent binding, indicating that the ChIP experiments were specific (e.g., Figures S4C, S4D, 6A, 6B and S8). Data are representative of at least three independent experiments. \* indicates significant ( $p < 0.05$ , t-test). **K**) *Bhlhb5* and *Prdm8* co-associate in neurons. Co-immunoprecipitation experiments (using ChIP conditions to preserve protein-DNA complexes) were performed to address whether *Bhlhb5* and *Prdm8* exist in a common complex. *Bhlhb5* was immunoprecipitated from the dorsal telencephalon of WT or *Bhlhb5*<sup>-/-</sup> mice and subjected to western blotting using antibodies to *Prdm8* or *Bhlhb5*, as indicated. Data are representative of four independent experiments. This interaction appears to be specific since the transcriptional activator CREB was not observed in *Bhlhb5*-associated complexes (data not shown). Note that we were unable to co-immunoprecipitate *Bhlhb5* and *Prdm8* under native conditions, possibly because the interaction between *Bhlhb5* and *Prdm8* is indirect, or because they interact selectively in the presence of DNA.



**Figure 6. A Bhlhb5 dimer recruits Prdm8 to form a repressor complex at specific DNA targets**  
**A)** Bhlhb5 forms a homodimer. HEK293T cells were transfected with the indicated constructs, immunoprecipitated with antibodies to myc, and blotted using antibodies to both myc and HA, revealing that myc-tagged Bhlhb5 can co-immunoprecipitate HA-tagged Bhlhb5 but not HA-tagged E2-2. Top blot shows the immunoprecipitated protein (IP); bottom blot shows 2.5% of input. Note that myc-Bhlhb5 (with 6 myc tags) and the IgG heavy chain have the same apparent molecular weight (~ 50 kDa), whereas HA-Bhlhb5 (with 3 HA tags) is ~ 40 kDa. The isoform of E2-2 used in these experiments was the longer form, E2-2B. **B)** ChIP experiments performed using tissue from knockout mice reveal that Prdm8 cannot target to Bhlhb5 binding sites in the absence of Bhlhb5. In WT mice, Bhlhb5 (red) and Prdm8 (blue) bind to the proximal promoter of *RP58*, as revealed by ChIP-qPCR for Bhlhb5 and Prdm8 (i). In *Prdm8*<sup>-/-</sup> mice, Bhlhb5 still binds to the proximal promoter *RP58* (ii). In *Bhlhb5*<sup>-/-</sup> mice, Prdm8 can no longer bind to Bhlhb5 binding sites, indicating that Bhlhb5 is required for Prdm8 targeting to this loci (iii). Similar results were seen for the Bhlhb5 binding sites at the *Cdh11* and *Bhlhb5* genes (see Figure S4). Data are representative of three independent experiments. The y-axis (Binding) represents enrichment of gDNA over input ( $\times 10^{-3}$ ).

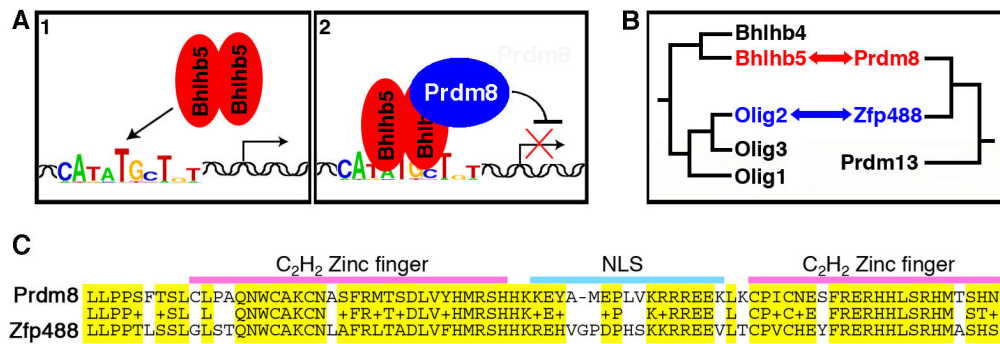


**Figure 7. *Cdh11* is a key target of the *Bhlhb5/Prdm8* repressor complex**

**A)** Affymetrix microarray-based gene profiling identifies *Cdh11* as a gene that is significantly upregulated in the dorsal telencephalon of *Bhlhb5*<sup>-/-</sup> mice (FDR < 0.05) from E13.5 – E17.5. Data are mean  $\pm$  SEM of biological replicates, with WT in black and *Bhlhb5*<sup>-/-</sup> in red, as indicated. **B)** *Cdh11* mRNA is also upregulated in the dorsal telencephalon of *Prdm8*<sup>-/-</sup> mice relative to WT littermates at E14.5, as shown by qPCR. Data are normalized to WT and are presented as mean  $\pm$  SEM of biological replicates. \* indicates significant difference relative to controls ( $p < 0.05$ , t-test). **C–D)** *Cdh11* protein is upregulated in the dorsal telencephalon of *Bhlhb5*<sup>-/-</sup> mice. Sagittal sections from mice of the indicated genotype at E16.5 were stained with antibodies to *Cdh11*. Note the high *Cdh11* expression in the axons of corticofugal fibers in the internal capsule (white arrows). Boxed insets are enlarged in D, which also shows the corresponding immunostaining with antibodies to *Bhlhb5*. **E)** Sagittal schematic of the brain illustrating the path of the corticospinal tract. Regions I and II (gray) indicate the location of sections in the coronal plane that were used to quantify the area of the corticospinal tract in *Bhlhb5*<sup>-/-</sup> mice and *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> double mutant mice (F). **F)** Quantification of the axon area in regions I and II. Relative to WT mice, there is a dramatic loss of axon area in the *Bhlhb5*<sup>-/-</sup> mice (black bars) and this loss is partially rescued in *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> double mutant mice (red bars). Five pairs of adult littermate mice were analyzed. \* indicates significant ( $p < 0.05$ , t-test). **G)** Representative images showing the corticospinal tract stained with PKC $\gamma$  in WT, *Cdh11*<sup>-/-</sup>, *Bhlhb5*<sup>-/-</sup>, and *Bhlhb5*<sup>-/-</sup>; *Cdh11*<sup>-/-</sup> mice. Matched cervical sections (corresponding to region II) from adult littermate mice are shown. The corticospinal tract is almost absent in the cervical spinal cord of *Bhlhb5*<sup>-/-</sup> mice but a partial rescue is seen in *Bhlhb5*; *Cdh11*<sup>-/-</sup> double mutant mice. The loss of *Cdh11* alone has no effect on the formation of the corticospinal tract. **H)** *Bhlhb5* mutant mice develop skin lesions by ~ 6 weeks of age. The onset of skin lesions is significantly delayed to ~13 weeks in *Bhlhb5*<sup>-/-</sup>;

*Cdh11*<sup>-/-</sup> double mutant mice. n = 12 littermate pairs; \* indicates significant difference (p < 0.05, t-test.)





**Figure 8. Model: Mechanism of repression**

**A)** A Bhlhb5 homodimer binds to its consensus binding element (1) and then recruits Prdm8 to mediate repression (2). Bhlhb5 requires Prdm8 to mediate repression, whereas Prdm8 requires Bhlhb5 for proper targeting to Bhlhb5 target genes. **B)** A schematic illustrating phylogenetic relationships among murine Bhlhb5-related proteins and Prdm8-related proteins, including Zfp488, a zinc finger protein that shows a very high degree of similarity with Prdm8 over the C-terminus (including the C<sub>2</sub>H<sub>2</sub> zinc fingers) but lacks the SET domain characteristic of other Prdm family members. Our study reveals that Bhlhb5 and Prdm8 are obligate partners and others have shown that Olig2 interacts physically and functionally with Zfp488 (Wang et al., 2006), suggesting that the interaction between bHLH transcription factors and Prdm-related proteins may be a general mechanism of repression during neuronal development. Note that branch lengths are not scaled to distances. **C)** Prdm8 and Zfp488 show a very high degree of similarity across their C-termini. Amino acids 609 – 687 (for murine Prdm8) and 258 – 337 (for murine Zfp488) are shown. Identity = 53/80 (66%); Positives = 66/80 (82%); Gaps = 1/80 (1%). E-value, 3-e28. NLS, nuclear localization sequence.