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Prdm13 mediates the balance of inhibitory and excitatory neurons in somatosensory circuits

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SUMMARY

Generating a balanced network of inhibitory and excitatory neurons during development requires precise transcriptional control. In the dorsal spinal cord, Ptf1a, a basic helix-loop-helix (bHLH) transcription activator, maintains this delicate balance by inducing homeodomain (HD) transcription factors such as Pax2 to specify the inhibitory lineage, while suppressing HD factors such as Tlx1/3 that specify the excitatory lineage. We uncover the mechanism by which Ptf1a represses excitatory cell fate in the inhibitory lineage. We identify *Prdm13* as a direct target of Ptf1a and reveal that Prdm13 actively represses excitatory cell fate by binding to regulatory sequences near the *Tlx1* and *Tlx3* genes to silence their expression. Prdm13 acts through multiple mechanisms including interactions with the bHLH factor Ascl1 to repress Ascl1 activation of *Tlx3*. Thus, Prdm13 is a key component of a highly coordinated transcriptional network that determines the balance of inhibitory versus excitatory neurons in the dorsal spinal cord.

INTRODUCTION

The central nervous system (CNS) is composed of a balanced number of excitatory and inhibitory neurons that are interconnected to form neuronal networks essential for information processing. In the dorsal spinal cord, which provides the first level of central somatosensory processing, this excitatory/inhibitory balance is required to integrate sensory information including pain, touch and proprioception coming into the spinal cord from dorsal root ganglia, before it is relayed to supraspinal brain regions or locally for reflex responses (Liu and Ma, 2011; Ross, 2011). Disruption in this balance can lead to sensory disorders such as hyperalgesia and allodynia (Fitzgerald, 2005; Tavares and Lima, 2007). Given the importance of the dorsal horn neurons in processing all somatosensory modalities, understanding how these neurons are specified during embryogenesis to generate the correct composition of neurons is critical. Here we identify a key component of the transcriptional machinery that controls how neurons in the somatosensory circuit are generated, and how regulated specification of these neurons leads to a correct excitatory/inhibitory balance during development.

Excitatory and inhibitory neurons in the spinal cord arise from progenitor populations within the ventricular zone of the dorsal neural tube (Gross et al., 2002; Muller et al., 2002). These

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progenitors are competent to give rise to neurons in either class depending on the basic helix-loop-helix (bHLH) transcription factors that are expressed (Helms and Johnson, 2003; Zhuang and Sockanathan, 2006). For example, ectopic expression of the bHLH factors *Ascl1* (previously *Mash1*) or *Atoh1* (previously *Math1*) result in an increase of excitatory neurons at the expense of neighboring neuronal subtypes (Gowan et al., 2001; Helms et al., 2005). On the other hand, the bHLH factor *Ptf1a* is required for the generation of local circuit inhibitory neurons, and in its absence excess local circuit excitatory neurons form (Glasgow et al., 2005).

These functions for the bHLH factors in neuronal subtype specification highlight a fundamental question in generating neuronal diversity. A transcription factor with the ability to re-direct the fate of a cell requires two activities; it must activate lineage specific gene expression, and it must repress expression of genes in the alternate lineage. The current understanding of the transcriptional network that controls the excitatory/inhibitory balance in the dorsal horn of the spinal cord has *Ptf1a* and *Ascl1* acting upstream of homeodomain (HD) factors such as *Pax2* and *Lbx1* to specify GABAergic (inhibitory) lineages, whereas *Ascl1* alone acts upstream of *Tlx1* and *Tlx3* to specify glutamatergic (excitatory) lineages (Batista and Lewis, 2008; Brohl et al., 2008; Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005; Helms et al., 2005; Mizuguchi et al., 2006; Wildner et al., 2006). Since *Ascl1* and *Ptf1a* are transcriptional activators (Beres et al., 2006; Nakada et al., 2004), the simple model has *Ascl1* directly activating glutamatergic lineage genes, and *Ptf1a* directly activating GABAergic lineage genes. However, because both *Ascl1* and *Ptf1a* are expressed in progenitors to the GABAergic lineages, how the glutamatergic genetic program in this lineage is suppressed remains unclear (Beres et al., 2006; Hori et al., 2008; Krapp et al., 1996; Krapp et al., 1998; Masui et al., 2008). Here we identify a PRDM class transcription repressor, *Prdm13*, as the missing component through which *Ptf1a* can suppress genes in the glutamatergic lineage in the dorsal spinal cord.

The PRDM family of transcription factors is defined by a PR domain and a variable number of zinc finger domains (for review see Fog et al., 2012; Fumasoni et al., 2007; Hohenauer and Moore, 2012). The PR domain has 20–30% identity to the SET domain that is found in a class of histone methyltransferases (HMTs), which function to silence transcription. Some PRDM members exhibit intrinsic HMT activity, such as *Prdm2*, 8, and 9, whereas most PRDM members lack this enzymatic activity. However, PRDM factors can recruit other histone-modifying enzymes to mediate their transcriptional activity. Some PRDM factors have been shown to be essential for regulating cell fate decisions. For example, *Prdm16* specifies brown fat fate while suppressing white fat or muscle (Kajimura et al., 2010; Seale et al., 2008). In the nervous system, *Prdm1* specifies photoreceptor over bipolar neuron fate (Brzezinski et al., 2010; Katoh et al., 2010). Recently, it was reported that *Prdm8* regulates *Cadherin-11* to ensure correct targeting of neurons in the cortex (Ross et al., 2012), and *Prdm14* regulates *Isl1* to ensure correct axon growth of motoneurons in zebrafish (Liu et al., 2012).

Despite the emergence of *Prdm* family members as important regulators of neural development, to date very little is known about their roles in cell fate specification and differentiation in the CNS. In this study, we identify *Prdm13* as a critical component of the transcription network that regulates neuronal diversity and the excitatory/inhibitory balance in the dorsal spinal cord. In particular, we show that *Prdm13* phenocopies that of *Ptf1a* in the CNS, where *Prdm13* is both necessary and sufficient to promote *Pax2*⁺/GABAergic over *Tlx1/3*⁺/glutamatergic neuronal fate. Moreover, we present evidence that *Prdm13* directly represses the glutamatergic neuron specifier genes *Tlx1* and *Tlx3*, thereby indirectly resulting in a derepression of the *Pax2*⁺/GABAergic lineage (Cheng et al., 2004; Cheng et al., 2005). Finally, we reveal a mechanism suggesting *Prdm13* physically interacts with *Ascl1* to

silence *Ascl1*'s transcriptional activity on the *Tlx3* gene. Together, our findings put *Prdm13* at the center of the bHLH and HD transcription factor cascade that governs cell fate decisions, and its function is central to the generation of a balanced circuitry of excitatory and inhibitory neurons required for somatosensory information processing.

RESULTS

Ptf1a activity is dominant over *Ascl1* in specification of dorsal spinal cord neurons

During development of the dorsal spinal cord, progenitor populations are defined by expression of bHLH transcription factors including *Ascl1* and *Ptf1a* (see diagram Fig. 1A) (Glasgow et al., 2005; Helms et al., 2005). At embryonic day 10.5 (E10.5), *Ascl1* is present in progenitors to interneurons dI3, dI4, and dI5, whereas *Ptf1a* is co-expressed with *Ascl1* in progenitors to dI4. Functionally, overexpression of *Ascl1* in ovo in chick neural tube induces expression of the homeodomain (HD) factors *Tlx1/3*, *Isl1*, and *Lmx1b* (dI3 and dI5) at the expense of *Pax2* (dI4) (Fig. 1C',C'',E,F) (Helms et al., 2005; Mizuguchi et al., 2006; Wildner et al., 2006). In contrast, overexpression of *Ptf1a* represses these factors, and instead induces *Pax2* and *Lhx1/5* (Fig. 1B',B'',E,F) (Glasgow et al., 2005). In addition to the regulation of these HD factors by *Ascl1* and *Ptf1a*, cross-talk between the HD factors also occurs since *Tlx1/3* repress the ability of *Lbx1* to induce *Pax2* (Cheng et al., 2005) (see diagram Fig. 1A). Indeed, ectopic expression of *Lbx1* in the chick neural tube induces *Pax2*, but only in regions outside of *Tlx1/3* expression (Fig. S1B''). The output from this regulatory network is the specification of either an excitatory (vGlut2+) or inhibitory (*Gad1+*) neuronal phenotype (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005; Gross et al., 2002; Muller et al., 2002).

Since progenitors to dI4 express both *Ascl1* and *Ptf1a*, some mechanism must exist to repress *Ascl1* specification activities when *Ptf1a* is present. *Ptf1a* appears sufficient to repress *Ascl1* activity since ectopic expression of *Ptf1a* represses *Tlx1* and *Tlx3* (Fig. 1C'). To test this further, we drove ectopic expression of *Ascl1* and *Ptf1a* simultaneously in the chick neural tube. In this paradigm, *Ptf1a* was dominant over *Ascl1*, inhibiting both ectopic and endogenous *Tlx1/3* expression while continuing to activate *Pax2* (Fig. D',D'',E,F). Given that *Ptf1a* acts as a transcriptional activator (Beres et al., 2006), we hypothesized that *Ptf1a* induces a transcriptional repressor as one of its downstream targets, and this repressor functions to mediate the suppression of glutamatergic lineage genes.

Prdm13* is a direct downstream target of *Ptf1a

In order to better understand the transcriptional network that controls the specification of inhibitory and excitatory neuronal subtypes in the dorsal spinal cord, we identified direct downstream targets of *Ptf1a*. Targets were defined by intersecting gene lists identified via *Ptf1a* ChIP-Seq with genes identified as being *Ptf1a*-dependent using expression profiling of *Ptf1a* mutant neural tubes compared to heterozygote or wildtype tissues. For ChIP-Seq experiments localizing *Ptf1a* to chromatin in vivo, chromatin from mouse E12.5 neural tube was immunoprecipitated with antibodies to *Ptf1a*. Tissue from the telencephalon of the same embryos was used as a negative neural control as no *Ptf1a* is present in this tissue. High-throughput sequencing of these samples, followed by peak detection revealed *Ptf1a* localized to thousands of sites within the genome (DMM, MDB, and JEJ, unpublished). For identifying genes whose expression is *Ptf1a*-dependent, we performed RNA-Seq from *Ptf1a* lineage cells sorted from neural tubes of *Ptf1a* mutants and heterozygotes at E11.5. These analyses revealed *Prdm13*, a member of the PRDM class of proteins, whose expression decreased 4.5-fold in the *Ptf1a* mutant, as a particularly interesting target (Fig. 2A). Given the importance of the PRDM family of factors to cell fate decisions in other tissues (for review see Fog et al., 2012; Hohenauer and Moore, 2012) and the lack of functional data for

Prdm13, we chose to study this factor to determine its function in neuronal subtype specification in the dorsal neural tube.

ChIP-Seq for Ptf1a identified 7 binding regions surrounding the *Prdm13* gene (Fig. 2A). Ptf1a is a component of a trimeric transcription complex (PTF1) that also includes an E-protein and Rbpj and binds a DNA motif containing an E-box and a TC-box with constrained spacing requirements (Beres et al., 2006; Henke et al., 2009; Hori et al., 2008; Masui et al., 2007). Of the 7 regions surrounding the *Prdm13* gene bound by Ptf1a, 5 contain consensus PTF1 binding motifs (Fig. 2B, underlined sequence). To determine if these Ptf1a-bound regions are occupied by the PTF1 complex, we performed ChIP-Seq on neural tube tissue with antibodies to Rbpj (DMM, MDB, and JEJ, unpublished). Examination of the *Prdm13* locus confirmed that Rbpj co-localizes with Ptf1a in those regions that contain a consensus PTF1 motif (Fig. 2A). All five of the PTF1 sites are conserved across mammalian species, and 4 of them are conserved to zebrafish (Fig. 2A,B asterisks).

To verify that *Prdm13* is a direct target of Ptf1a, we examined the *Prdm13* expression pattern relative to Ptf1a, and in response to increased or decreased levels of Ptf1a. In the chick, *Prdm13* is expressed in the ventricular zone in the dorsal neural tube (Fig. 2D, control side), and ectopic expression of Ptf1a via in ovo electroporation resulted in a dramatic increase in endogenous *cPrdm13* mRNA levels (Fig. 2C,D). In mouse embryos, the pattern of *Prdm13* expression is strikingly neural specific and is almost exclusively restricted to Ptf1a domains (Fig. 2E–J'). At E10.5–E12.5, Ptf1a is present in the dorsal neural tube from spinal regions (Fig. 2I,I', Fig. S2) to the developing cerebellum (Fig. 2F,F'), and in a subset of cells in the diencephalon (Fig. 2G–H'). *Prdm13* is present in all these domains, including the retina at E16.5 (Fig. 2J,J'), but is not detected in the pancreas, the other major site of Ptf1a expression. Notably in the caudal neural tube, although *Prdm13* is restricted to the dorsal region, it appears to be present more broadly in the ventricular zone than Ptf1a. *Prdm13* overlaps progenitor regions from dl2–dl6 (Fig. 2I', Fig. S2) and is expressed from E9 to E14.5 (Fig. S2). However, even in the neural tube, *Prdm13* appears enriched in the Ptf1a domains. In *Ptf1a* null embryos, *Prdm13* is abolished in rostral domains (Fig. 2F''–H''), indicating that expression of *Prdm13* in these regions is dependent on *Ptf1a*. Within the caudal neural tube, *Prdm13* mRNA is reduced only at the lateral border of the ventricular zone where Ptf1a is expressed (Fig. 2I–I'', arrows; Fig. S2), suggesting that additional, Ptf1a-independent mechanisms also regulate *Prdm13* in this region.

In summary, the neural restricted patterns of *Prdm13* and Ptf1a are strikingly similar with a major component of *Prdm13* expression being Ptf1a-dependent. Together with the ChIP-Seq results, we conclude that *Prdm13* is a neural specific, direct downstream target of Ptf1a.

Prdm13 phenocopies Ptf1a function in the dorsal neural tube

To begin to uncover the function of Prdm13 during neural development, we misexpressed mouse Ptf1a and Prdm13 in chick neural tubes at stage HH12–13 via in ovo electroporation. The exogenous expression was confirmed by specific immunofluorescence for Ptf1a and mRNA in situ hybridization for *Prdm13* (Fig. 3B–D), while GFP indicated electroporation efficiency across the dorsoventral axis (Fig. 3A). Transverse sections of thoracic neural tube were collected at HH24–25 and examined by immunofluorescence for alterations in neuronal sub-type specification relative to the non-electroporated side. Ectopic expression of either Ptf1a or Prdm13 dramatically decreased the number of Tlx1/3⁺ cells (2.5 and 2.0-fold, respectively) while increasing the number of dl4 Pax2⁺ cells (1.5-fold in each case) (Fig. 3B'–C'', E–F). Electroporation of empty vector, in contrast, did not alter the number of the neurons generated in either population (Fig. 3A'–A'', E–F). Co-electroporation of Prdm13 and Ptf1a together more strongly induced Pax2 expression than either alone (Fig. 3D'–F).

Similar trends were seen with additional markers for dorsal interneuron populations including *Isl1/2*, *Lmx1b*, and *Lhx1/5* (Fig. S3).

Because other members of the PRDM family are primarily repressors, we hypothesized that *Prdm13* might induce *Pax2* by indirect means, such as by relieving repression by another factor. For example, derepression of *Lbx1*, which induces *Pax2* in the absence of *Tlx1/3* (Cheng et al., 2005), might account for the increase in *Pax2* levels seen with *Prdm13* misexpression. Indeed, *Lbx1* was also induced by ectopic expression of *Ptf1a* and *Prdm13*, particularly in regions dorsal to its native domain (Fig. 3O–S, arrows). Since *Ptf1a* and *Prdm13* also inhibit *Tlx3* in the *dl3* domain, the induction of *Pax2* may be indirect through the unopposed activity of ectopic *Lbx1*.

In the dorsal neural tube, *Tlx1/3* cells give rise to *vGlut2*⁺ excitatory neurons, and *Pax2* cells give rise to *Gad1*⁺ inhibitory neurons. To determine whether *Ptf1a* and *Prdm13* are sufficient to modulate the neurotransmitter phenotypes as well, we performed mRNA in situ hybridization for *Gad1* and *vGlut2* on the electroporated neural tubes. An increase in *Gad1* and decrease in *vGlut2* confirms that the ectopic *Pax2* expressing neurons from *Ptf1a* and *Prdm13* overexpression adopt a GABAergic identity (Fig. 3T–W', arrows indicating *dl3* and *dl5* regions with ectopic *Gad1* or reduced *vGlut2*). Thus, *Prdm13* serves a similar function as *Ptf1a* by promoting the *Pax2*⁺ GABAergic lineage and suppressing the *Tlx1/3*⁺ glutamatergic fate.

A closer look at the pattern of *Pax2*⁺ neurons in the *Ptf1a* and *Prdm13* electroporated neural tubes revealed that the normal *dl5* gap between *dl4* and *dl6* was filled with *Pax2*⁺ cells (Fig. 3 compare A'' with B''–C'', arrows). This suggested that ectopic expression of *Ptf1a* and *Prdm13* alter the fate of progenitor cells, an interpretation consistent with previous conclusions from analysis of the *Ptf1a* knock out mouse (Glasgow et al., 2005). Indeed, closer examination of the *dl3*–*dl5* domain revealed that *Prdm13* electroporated cells are strongly biased to a *Pax2*⁺:*Tlx1/3*[−] fate (Fig. 3G–N). In the empty vector control, 34% of the dorsal GFP⁺ cells were *Tlx1/3*⁺. When *Ptf1a*, *Prdm13*, or both were ectopically expressed, GFP⁺:*Tlx1/3*⁺ cells were rarely seen and the percentage dropped to 2–4% (Fig. 3H–J). In contrast, the number of dorsal GFP⁺:*Pax2*⁺ cells increased from 12% in the control to 39–40% with ectopic *Ptf1a* or *Prdm13* (Fig. 3L–M). The percentage increased to 53% GFP⁺:*Pax2*⁺ cells when *Ptf1a* and *Prdm13* were introduced simultaneously, consistent with the collaborative effect noted above (Fig. 3N). We confirmed that the loss of *Tlx1/3*⁺ cells and enrichment of *Pax2*⁺ cells is not an artificial result of selective cell death, as assayed by immunostaining for Caspase 3 (data not shown). These findings indicate both *Ptf1a* and its target *Prdm13* can alter the fate of progenitor cells and bias them to the *Pax2*⁺/inhibitory neuronal lineage.

Knockdown of *Ptf1a* or *Prdm13* leads to a reduction of *Pax2*⁺ cells

To gain further insight into the role of *Prdm13* in neuronal specification, we generated specific short hairpin RNAs (shRNA) to knockdown endogenous *Ptf1a* or *Prdm13* in the chick neural tube. Constructs containing shRNAs to *Ptf1a*, *Prdm13*, or *DsRed* (control) were electroporated as above (Fig. 4A–C), and mRNA in situ hybridization was performed to verify knockdown (Fig. 4H–K, black arrows). Reduction of *Ptf1a* and *Prdm13* levels led to a marked decrease in the number of *Pax2*⁺ cells (1.7 and 2.0-fold for *Ptf1a* and *Prdm13*, respectively) (Fig. 4B''–C'', G). There was a modest increase in the number of *Tlx1/3*⁺ cells with *Prdm13* knockdown; however, in the *Ptf1a* knockdown there was no significant difference (Fig. 4B'–C', F). Electroporation of control shRNA did not alter the expression of *Tlx1/3* nor *Pax2* (Fig. 4A'–A'', F, G). There was no increase in cell death in electroporated hemicords as detected by Caspase3 expression (data not shown). Misexpression of mouse *Ptf1a* or *Prdm13* with their respective shRNA constructs rescued the knockdown

phenotypes, demonstrating specificity of the shRNA (Fig. S4). These results demonstrate that Prdm13, like Ptf1a, is required to generate the correct number of Pax2⁺ neurons in the dorsal spinal cord.

Ptf1a requires Prdm13 to increase Pax2⁺ and decrease Tlx1/3⁺ cells

Results detailed above strongly suggest that Prdm13 functions as a downstream effector of Ptf1a during neuronal sub-type specification. If Prdm13 is required for Ptf1a activity, then knocking down Prdm13 when Ptf1a is ectopically expressed should abrogate Ptf1a activity. Indeed, the increase in Pax2⁺ cells and the decrease in Tlx1/3⁺ cells normally seen with ectopic Ptf1a expression was inhibited when Prdm13 was simultaneously knocked down (Fig. 4E'-E'', F, G, compare with gray bar). Conversely, knockdown of Ptf1a did not affect the activity of ectopically expressed Prdm13, consistent with Ptf1a acting upstream of Prdm13 (Fig. 4D'-D'', F-G). These experiments support the requirement for Prdm13 as a downstream effector of Ptf1a.

Prdm13 requires its zinc finger motifs for activity and acts as a repressor

The PRDM family of transcription factors is characterized by a PR domain and a variable number of zinc fingers, but the functional contribution of each of these domains remains unclear for any given member of the family (for review see Fog et al., 2012; Hohenauer and Moore, 2012). To better understand the mechanism behind Prdm13 function, we generated mutants of Prdm13 and tested their activity in chick neural tube (Fig. 5A-C). Although the PR domain has been shown to be critical for the function of other PRDM family members, this domain was dispensable for Prdm13 activity, as the truncated form of Prdm13 containing the four zinc fingers (Z1234) retained full activity (Fig. 5B-C, representative images are shown in Fig. S5I', I''). Furthermore, the zinc fingers themselves are required for Prdm13 activity since mutations designed to disrupt these domains (Prdm13 mZ1234) also disrupted Prdm13 function (Fig. 5B-C, Fig. S5J', J'').

Members of the PRDM family of factors have been reported to function either as transcriptional repressors or activators depending on the cellular context. To determine whether the neuronal specification activity observed with Prdm13 reflects its function as a transcriptional repressor or an activator, we fused it with engrailed repressor (EnR) (Smith and Jaynes, 1996) or with the VP16 activator (Triezenberg et al., 1988) and tested these chimeric proteins for activity in the chick neural tube. The activity of Prdm13-EnR was indistinguishable from that of wild-type Prdm13 in that the number of Tlx1/3⁺ neurons was dramatically reduced while Pax2⁺ neurons increased (Fig. 5B-C, Fig. S5). In contrast, expression of Prdm13-VP16 resulted in a decrease in Pax2⁺ cells. Prdm13-VP16 also slightly repressed Tlx1/3, although the effect was much less pronounced than that of Prdm13-EnR or Prdm13 (Fig. 5B-C, Fig. S5). Taken together, Prdm13 appears to exert its effects by acting as a transcriptional repressor, an activity that requires its zinc finger domains.

Prdm13 suppresses generation of Tlx1/3⁺ neurons by antagonizing Ascl1 activity

Given that the bHLH factor Ascl1 specifies opposing programs (dI3 and dI5) to Ptf1a and Prdm13 at this stage (Fig. 1) (Brohl et al., 2008; Helms et al., 2005; Mizuguchi et al., 2006; Nakada et al., 2004), we hypothesized that Prdm13 might function through interfering with Ascl1 activity. We tested this by co-electroporating Ascl1 and Prdm13 into the chick neural tube. Ectopic Ascl1 results in a dramatic increase of Tlx1/3⁺ neurons and a loss of Pax2⁺ neurons (Fig. 5D'-D'', F-G). Strikingly, when Ascl1 and Prdm13 are expressed simultaneously, Ascl1 is no longer able to induce Tlx1/3. Instead, we observed a 3.3-fold decrease in Tlx1/3⁺ neurons and a 1.6-fold increase in Pax2⁺ cells relative to the control side, similar to effects of Prdm13 misexpression alone (Fig. 5E'-E'', F-G, compare with

Fig. 3C'-C'',E-F). Prdm13 does not alter endogenous *Ascl1* levels (Fig. S5A',B',C); therefore, Prdm13 antagonizes *Ascl1* activity without inhibiting expression of *Ascl1* itself. Thus, while *Ascl1* is expressed in both progenitors to dI4 and dI5 neurons, it is the presence or absence of enriched Prdm13 levels that dictate the decision between these cell fates.

Prdm13 complexes with *Ascl1* to directly repress *Tlx3* expression

We used ChIP-Seq to localize Prdm13 to chromatin *in vivo* as a means to uncover how Prdm13 suppresses expression of *Tlx1* and *Tlx3*. We performed ChIP-Seq with antibodies to Prdm13 using chromatin from E11.5 neural tubes. We found Prdm13 enriched at regions upstream of both the *Tlx1* and *Tlx3* genes (Fig. 6A). No enrichment was detected with these antibodies in chromatin isolated from E11.5 mouse telencephalon tissue where Prdm13 is absent (Fig. 6A). Furthermore, Prdm13 was not found within a 300 kb region surrounding the *Pax2* gene consistent with Prdm13 induction of this gene being indirect. Enrichment of the *Tlx1* and *Tlx3* regions was confirmed in ChIP-qPCR experiments. Again, there was enrichment of the *Tlx1* and *Tlx3* sites in neural tube that was not seen with telencephalon tissue or with a control genomic region (Fig. 6B). These data suggest that Prdm13 inhibits glutamatergic identity by binding near the glutamatergic specifier genes *Tlx1* and *Tlx3*, and directly repressing their transcription. This in turn may facilitate Lbx1-mediated induction of *Pax2* and GABAergic neuronal specification (see Fig. 7 for model).

To explore how Prdm13 antagonizes *Ascl1*, we asked whether *Ascl1* was also found at the Prdm13 bound *Tlx1* and *Tlx3* sites. ChIP with *Ascl1* antibodies from E11.5 neural tubes showed significant enrichment at the *Tlx3* site but not the *Tlx1* (Fig. 6C). This enrichment was similar to that seen with a known target of *Ascl1*, *Dll1* (Castro et al., 2006). Enrichment at the *Tlx3* site was not detected from limb tissue where *Ascl1* is not expressed or from a control genomic region. This finding suggests a model whereby *Ascl1* binds and activates expression of *Tlx3*, but when Prdm13 levels increase downstream of *Ptf1a*, a novel transcription repressor complex that includes *Ascl1* and Prdm13 is formed. Supporting this model, specific binding between *Ascl1* and Prdm13 is detected in co-IP experiments when epitope-tagged versions of the proteins are expressed in HEK 293 cells (Fig. 6D).

Two distinct mechanisms, one *Ascl1*-dependent and one *Ascl1*-independent, are suggested for Prdm13 suppression of the glutamatergic gene expression program in cells fated to become GABAergic neurons (see Fig. 7). To verify that Prdm13 and *Ascl1*-bound regions function as enhancers, we tested reporter constructs derived from the *Tlx1* and *Tlx3* genomic loci. *eTlx1::GFP* and *eTlx3::GFP* reporters were electroporated into the chick neural tube at HH12-13 and assayed 48 hours later. Strikingly, *eTlx3::GFP* was largely restricted to the dI3 and dI5 domains and *eTlx1::GFP* to the dI5 domain reflecting the expression of their respective gene loci (Fig. 6E,I). Furthermore, co-electroporation of the reporter constructs with *Ascl1* resulted in a dramatic induction of *eTlx3::GFP* and only a slight induction of *eTlx1::GFP*, consistent with *Ascl1* localizing to *Tlx3* but not *Tlx1* (Fig. 6F,J,M,N). This dramatic activation of *eTlx3::GFP* by *Ascl1* was abrogated when Prdm13 was also expressed (Fig. 6H,L). Misexpression of Prdm13 alone with the reporter constructs similarly inhibited their activity in dI3 and dI5 domains (Fig. 6G,K). Thus, in addition to identifying enhancers for *Tlx1* and *Tlx3* that are directly repressed by Prdm13, we suggest a mechanism whereby Prdm13 may be recruited to the *Tlx3* enhancer by *Ascl1* to directly repress *Tlx3*.

DISCUSSION

A balance of excitatory and inhibitory neuronal input is crucial for normal nervous system function. Since dorsal horn neurons in the spinal cord form the first level of central processing of somatosensory input from the periphery, this balance is required for normal

perception of pain, touch, and sense of body position. Generation of neuronal diversity in the spinal cord involves the specification of GABAergic and glutamatergic neurons from neighboring and interspersed progenitors in the dorsal neural tube ventricular zone just as they are transitioning to post-mitotic neurons. The network of transcription factors required for this process includes members of the bHLH and HD families. In particular, *Ascl1*⁺ proliferating progenitors in the dorsal neural tube are competent to become either glutamatergic during early neurogenesis (around E10.5) or both GABAergic and glutamatergic after E12.5. In the absence of *Ptf1a*, *Ascl1* induces expression of *Tlx1/3* and *Lmx1b*. These HD factors in turn induce the glutamatergic program while suppressing the ability of *Lbx1* to induce *Pax2* and the GABAergic program (Cheng et al., 2005; Helms et al., 2005) (Fig. 7 for model). In contrast, a subset of these *Ascl1*⁺ progenitors begins to express *Ptf1a*, which activates expression of the HD factors *Pax2* and *Lhx1/5* and the resulting GABAergic program (Glasgow et al., 2005; Wildner et al., 2006) (Fig. 7). However, for correct specification of these neurons, the glutamatergic program must be repressed. Here we identify the zinc finger transcription factor *Prdm13* that provides this critical function through repression of *Ascl1* activity and direct repression of *Tlx1* and *Tlx3* expression (Fig. 7).

Mechanisms of *Prdm13* action in neuronal specification

The PRDM family of factors is interesting in the variety of mechanisms they reportedly use to regulate transcription (for review Fog et al., 2012; Hohenauer and Moore, 2012). They contain two major recognizable motifs, the PR domain and zinc fingers. The PR domain is similar to the SET domain found in histone lysine methyltransferases (HMT), and thus, some members of the PRDM family have been shown to have this enzymatic activity. However, the sequence homology between PR domains and the SET domain reveals that the PR domain of *Prdm13* is substantially divergent. We show that the PR domain is dispensable for *Prdm13* activity in our assays since a truncated *Prdm13* (amino acids 185-755) that lacks the PR domain but contains all four zinc fingers is sufficient to induce *Pax2*⁺ cells and repress *Tlx1/3* at levels similar to that seen with the full-length protein. Furthermore, the zinc fingers are required for *Prdm13* function since mutations designed to disrupt each zinc finger abolished *Prdm13* activity (Fig. 5).

Zinc finger domains in other PRDM factors have been shown to be involved in a variety of activities including DNA binding, protein-protein interaction, intrinsic HMT activity, and recruitment of other histone-modifying proteins. *Prdm1*, 3, 5, 9, and 14 can directly bind DNA (Fog et al., 2012). In contrast, *Prdm8*, which shows the highest similarity to *Prdm13*, does not directly bind DNA but rather binds chromatin in a complex with *Bhlhb5* (Ross et al., 2012). Results from *Prdm13* ChIP indicate it localizes with chromatin, but like *Prdm8*, it may do so indirectly. This is supported by three findings in our data. First, for efficient ChIP, a long-arm cross-linker compound treatment, ethylene glycol-bis (succinimidyl succinate) (Zeng et al., 2006) was required. Second, no zinc finger type binding motif was identified from the ChIP-Seq results in regions bound by *Prdm13* (data not shown). And third, we provide evidence for *Prdm13* interacting with *Ascl1* to repress *Ascl1* target genes (Fig. 6). However, these results do not exclude the possibility that *Prdm13* also binds DNA directly. Indeed, ChIP for *Prdm13* in *Ascl1* mutant neural tubes still showed *Prdm13* bound to the *Tlx3* enhancer (data not shown). Thus, *Prdm13* is functioning through mechanisms distinct from those seen with *Prdm8*, and may repress its targets by combinations of direct and indirect binding to DNA.

We demonstrate here that *Prdm13* is a key factor in ensuring glutamatergic lineage genes are silenced during generation of GABAergic neurons. *Prdm13* suppresses the glutamatergic lineage and induces the GABAergic lineage through transcriptional repression of the glutamatergic specifier factors *Tlx1* and *Tlx3* (Fig. 7). This is supported by 1) ectopically

expressed Prdm13 reduces the number of Tlx1/3⁺ cells in the dorsal neural tube, 2) Prdm13 functions as a transcriptional repressor, 3) Prdm13 occupies genomic regions near both the *Tlx1* and *Tlx3* genes in sequence conserved across mammalian species, and 4) Prdm13 bound *Tlx1* and *Tlx3* regions have enhancer activity that was blocked by ectopic Prdm13. Since Prdm13 was not localized to the *Pax2*, *Lhx1/5*, or *Lbx1* genes (Fig. 6 and data not shown), and Prdm13 is acting as a transcriptional repressor, the increase in expression of these GABAergic lineage genes upon overexpression of Prdm13 is likely indirect. Indeed, induction of Pax2 can be explained by Prdm13's direct suppression of Tlx1 and Tlx3, known to block Lbx1 induction of Pax2 as mentioned above (Cheng et al., 2005) (see Model Fig. 7).

Additional functions for Prdm13

Ptf1a not only functions in specifying the excitatory/inhibitory balance in the dorsal spinal cord, but it also controls the balance of GABAergic interneurons with that of the glutamatergic granule cells in the cerebellum (Hoshino et al., 2005; Pascual et al., 2007). Similarly, Ptf1a is required in the retina for generation of GABAergic and glycinergic amacrine and horizontal neurons and suppression of glutamatergic retinal ganglia neurons (Dullin et al., 2007; Fujitani et al., 2006; Jusuf and Harris, 2009; Nakhai et al., 2007). By mRNA *in situ* hybridization, *Prdm13* reflects Ptf1a in cerebellum and retina, and in *Ptf1a* mutants, *Prdm13* is dramatically attenuated in these domains. This suggests Prdm13 may suppress non-Ptf1a lineage gene expression in these regions as it does in the dorsal neural tube.

Non-Ptf1a dependent activities are also suggested by *Prdm13* expression detected as early as E9 in the dorsal neural tube ventricular zone (Fig. S1). This ventricular zone expression spans the region where dl2 to dl6 arise, and it is not affected in the *Ptf1a* mutant. A hint that Prdm13 may also function in neuronal proliferation and differentiation was seen in the Prdm13 overexpression experiments in chick. Normally, Ascl1 drives neuronal differentiation when overexpressed, however, when Prdm13 was co-expressed with Ascl1, it disrupted this phenotype (Fig. 5, compare the distribution of Ascl1⁺ cells in D and E). Thus, Prdm13 may function to modulate Ascl1 activity (and other bHLH factors) in their role in neuronal differentiation as well as their role in controlling cell fate decisions.

Switching Ascl1 from transcriptional activator to repressor

Ascl1 has been shown to be essential in the development of multiple neural and neuroendocrine lineages, particularly during early stages of differentiation from progenitors (Bertrand et al., 2002). Most recently it was identified as an essential component of a transcription factor cocktail that can directly reprogram or transdifferentiate fibroblasts to neurons (Yang et al., 2011). At a mechanistic level, Ascl1 heterodimerizes with E-proteins (such as Heb and E2a), binds DNA, and activates transcription (Johnson et al., 1992). Interactions with the global co-activator p300, a histone acetyltransferase, have been reported (Yamamoto et al., 2001). Our current findings suggest Ascl1 may make a repressor complex with Prdm13, switching Ascl1 from an activator to a repressor with respect to the glutamatergic specifier gene *Tlx3*. Supporting this model (Fig. 7) we show 1) Ascl1 and Prdm13 localize to the same genomic location upstream of the *Tlx3* gene, 2) Ascl1 and Prdm13 co-immunoprecipitate, and 3) Ascl1 induces expression of the Tlx1 and Tlx3 enhancer reporters, activities repressed by Prdm13. An alternative model would have Prdm13 blocking Ascl1 from binding the *Tlx3* enhancer. Further experiments are required to definitively distinguish between these models. Nevertheless, Prdm13 is instrumental to silencing the gene program for the glutamatergic fate via Ascl1 as cells progress towards the GABAergic neuronal lineage.

In summary, PRDM factors utilize diverse mechanisms to modulate transcription, and broadly function in progenitor populations to control lineage decisions. This study places Prdm13, a zinc finger transcription factor, in a critical role connecting the neuronal specification functions of bHLH and HD factors, and highlights the importance of silencing transcription of gene programs in the opposing lineage.

EXPERIMENTAL PROCEDURES

Mouse strains and tissue preparation

Mutant mouse strains have been previously described. *Ptf1a^{Cre}* (*p48^{Cre}*), where the *Ptf1a* coding region is replaced by that coding for Cre recombinase was used as the *Ptf1a* null (Kawaguchi et al., 2002). *12.4Ptf1a::mCherry* transgenic mice, where a 12.4 kb regulatory sequence from 3' of the *Ptf1a* gene drives expression of mCherry (Meredith et al., 2009), was used for fluorescence activated cell sorting (FACS) of *Ptf1a* lineage cells from E11.5 wildtype or *Ptf1a* null neural tubes. PCR genotyping was performed as previously described (Glasgow et al., 2005; Meredith et al., 2009). All procedures on animals follow NIH Guidelines and were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Chromatin Immunoprecipitation (ChIP-Seq)

Tissue source for ChIP was mouse E11.5 or 12.5 neural tubes. *Ptf1a*, *Ascl1* and *Prdm13* negative tissue for control included telencephalon and/or limbs from the same embryos. ChIP was performed as previously described (Holmstrom et al., 2011; Masui et al., 2007), with details in the Supplemental Experimental Procedures (SEP). Notably, the *Prdm13* ChIP was treated with formaldehyde along with the long-arm cross-linker, ethylene glycol-bis (succinimidyl succinate), to increase ChIP efficiency (Zeng et al., 2006). Antibodies used for *Ptf1a* and *Ascl1* ChIP are as published (Castro et al., 2006)(Beres et al., 2006). Lab generated antisera were used for *Rbpj* (TX857, rabbit anti-*Rbpj*) and *Prdm13* (TX970, rabbit anti-*Prdm13*), details provided in the SEP. NEBNext ChIP-Seq Sample Prep Master Mix Set 1 was used for library generation. Detailed methods and primers used for ChIP qPCR are listed in SEP. ChIP efficiency (CE) was calculated relative to input as $CE = (2^{Ct_{input} - Ct_{ChIP}}) \times DF \times 100\%$, where DF is the dilution factor between input and ChIP sample.

Plasmid description

FLAG-tagged *Prdm13* and variations used for expression in the chick neural tube were inserted into the pCIG vector, which drives expression through a combined CMV early enhancer/chicken β -actin promoter and contains an IRES-NLS-GFP (Megason and McMahon, 2002). *Prdm13* mZ1234 contains point mutations in each zinc finger (Z1;C185A, H207A, Z2;C622A, H638A, Z3;C650A, H666A, Z4;C679A, H695A). *Ptf1a* and *Ascl1* expression constructs used pMiWIII as previously described (Hori et al., 2008; Nakada et al., 2004). The *Lbx1* expression vector was as described (Muller et al., 2002). The engrailed repressor (EnR) (Smith and Jaynes, 1996) or the VP16 activator (Triezenberg et al., 1988) were fused at the C-terminal end of *Prdm13* in pMiWIII. The *eTlx1::GFP* and *eTlx3::GFP* reporter constructs include mm9 coordinates (*eTlx1*, chr19:45217747-45218583) and (*eTlx3*, chr11:33134077-33134502), respectively, cloned into a GFP reporter cassette with a β -globin basal promoter (Lai et al., 2011). All constructs used were sequence verified.

The pSilencer 1.0-U6 vector (Ambion) was used in knock down experiments where several sets of 21-mer oligonucleotides were selected from chick *Ptf1a* or *Prdm13* mRNA sequences (listed in the SEP). Additionally, shRNA targeting *DSred* was used as control (Rao et al.,

2004). The shRNA constructs were co-electroporated with pCIG so the GFP could be used to assess electroporation efficiency.

In ovo chick electroporation

Fertilized white Leghorn eggs were obtained from the Texas A&M Poultry Department (College Station, TX) and incubated at 37°C for 48 hours until stage HH12-13 (Hamburger and Hamilton, 1992). Supercoiled plasmid DNA (1–2.5 µg/µl each) were injected into the lumen of the closed neural tube, and embryos were electroporated as previously described (Timmer et al., 2001). After 48 hours incubation at 37°C, stage HH24-25 embryos were harvested and processed for immunofluorescence or in situ hybridization.

Immunofluorescence and In Situ hybridization

Immunofluorescence was performed as previously described (Glasgow et al., 2005; Hori et al., 2008). A list of primary antibodies used is listed in SEP. Fluorescence imaging was carried out on a Zeiss LSM 510 confocal microscope. For each experiment, multiple sections from at least six different embryos were analyzed and used for quantification. Cell counting was blind to the condition and was performed manually using ImageJ. The quantitative results are presented as a ratio of the number of marker positive cells on the electroporated side divided by the number on the non-electroporated side. Only sections with confirmed high efficiency expression across the dorsal ventral axis were used in the analysis. Mean GFP pixel intensity for *eTlx1/3::GFP* expression was measured by ImageJ. Significant differences between control and experimental samples were calculated using a two-tailed two-sample equal variance (homoscedastic) Student's t test (* indicates $P < 0.001$) in Microsoft Excel. SEM is shown.

In situ hybridization was performed as previously described (Lai et al., 2011). Chick *Gad1* and *vGlut2* (Cheng et al., 2004), and chick *Ptf1a* (gift T. Reh) probes were used. Plasmids for generating chick and mouse *Prdm13* probes were cloned by PCR into pBluescript with the primers listed in SEP.

Co-immunoprecipitation

HEK293T cells were transfected with Flag-tagged Prdm13, myc-tagged Ascl1 or Hook3 (control) expression constructs using FuGene 6 (Roche Life Sciences), and collected 48 hours later. Anti-Flag antibodies conjugated to protein A/G beads (Santa Cruz) were added to cleared lysate. Co-immunoprecipitation was assessed by western blot using anti-myc (A-14, Santa Cruz, 1:2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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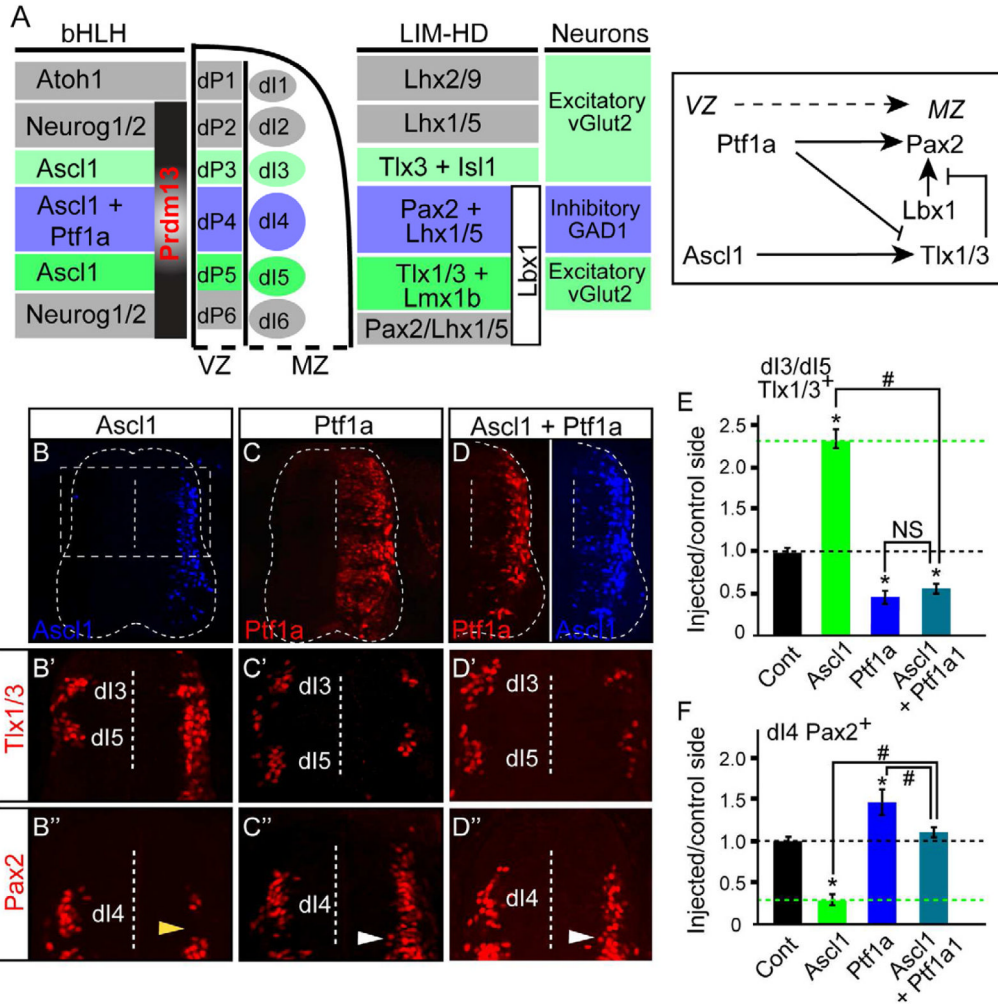


Figure 1. Opposing activities of Ascl1 and Ptf1a on neuronal subtype specification
 (A) Diagram showing the pattern and interacting network of bHLH and LIM-HD transcription factors in the dorsal spinal neural tube. (B–F) Stage HH12–13 chick embryos were electroporated with expression vectors for Ptf1a and/or Ascl1 and harvested at HH24–25. Transverse sections are shown with the electroporated side on the right. Ascl1 (B,D blue) and Ptf1a (C,D red) detects the ectopically expressed proteins. Dashed box in (B) is the region of the neural tube shown in (B'–D''). Dashed vertical line indicates the ventricle. (B'–F) Dorsal neural tube sections show the dI3 and dI5 neuronal populations marked by Tlx1/3 antibody (B'–D'), or dI4 marked by Pax2 (B''–D''). (E,F) the ratio of Tlx1/3⁺ or Pax2⁺ neurons on the electroporated side versus the control side showing that overexpression of Ascl1 causes a dramatic increase in Tlx1/3⁺ and decrease in Pax2⁺ neurons, while Ptf1a, or Ascl1 plus Ptf1a, causes the opposite. Note that ectopic Pax2⁺ cells (white arrowheads) are found in the dI5 domain not normally expressing Pax2 (yellow arrowhead). Error bars are reported as SEM. p-values < 0.001 between sample and control (*) or between samples (#). NS, not significant. Supplementary Fig. S1 shows Lbx1 also induces Pax2 (associated with panel A).

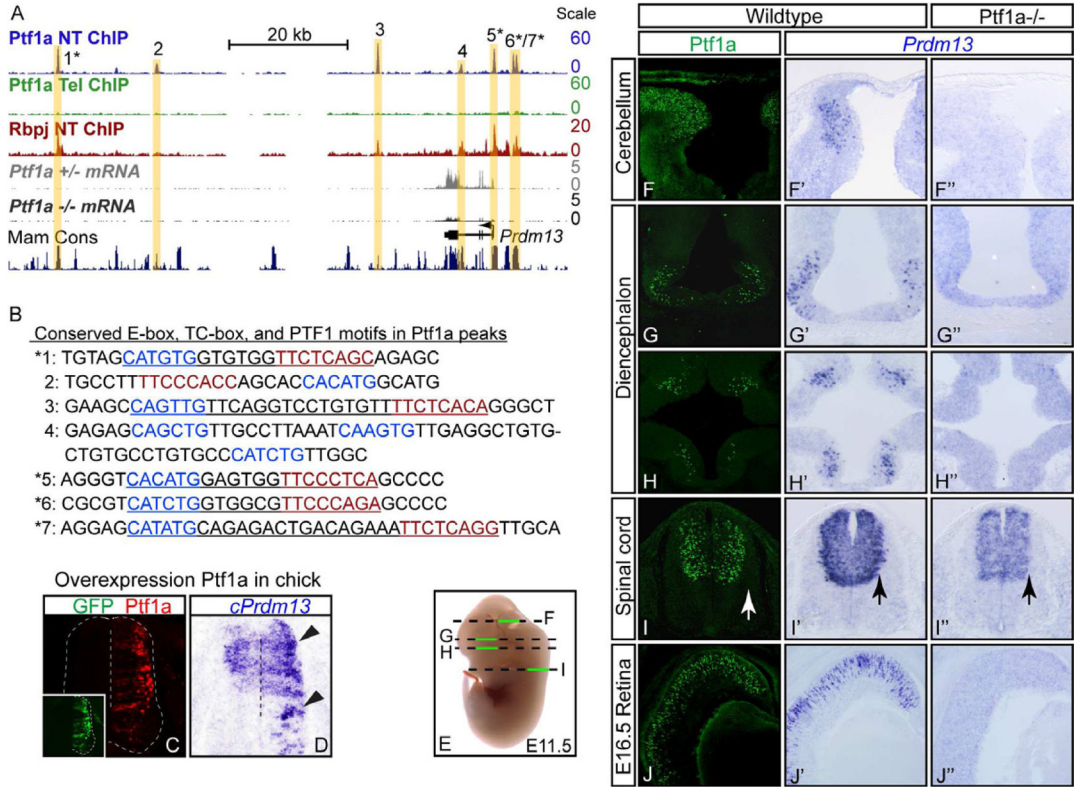


Figure 2. Prdm13 is a direct downstream target of Ptf1a

(A) The mouse genomic region surrounding *Prdm13* showing ChIP-Seq for Ptf1a from E12.5 neural tube (blue) and telencephalon (green) or Rbpj from neural tube (red), and RNA-Seq from FACS isolated Ptf1a lineage cells from E11.5 *Ptf1a*^{+/-} (gray) or *Ptf1a*^{-/-} embryos (black). Seven Ptf1a peaks are highlighted with yellow bars. The location of the *Prdm13* transcript is shown, as is a histogram of mammalian conserved regions (UCSC Genome Browser). (B) Sequence under the apex of each Ptf1a ChIP-Seq peak. DNA regions 1–7 showing the E-box (blue), TC-box (red), and the PTF1 motifs (underlined). * peaks that are conserved from mouse to zebrafish and contain the consensus PTF1 motif. (C–D) Overexpression of mouse Ptf1a (C, red) in chick neural tube induces *cPrdm13* mRNA (D, arrowheads). Inset: GFP indicates the electroporation efficiency. (E) Diagram of section planes for images shown in (F–I). (F–J'') Cross sections of mouse E11.5 or E16.5 show Ptf1a protein (F–J), and *Prdm13* mRNA in wildtype (F'–J') and *Ptf1a*^{-/-} (F''–J''). Note in cerebellum, diencephalon, and retina *Prdm13* is lost in the *Ptf1a* mutant, but in the spinal cord, only the lateral domain that reflects the Ptf1a pattern is lost (arrows). Supplementary Fig. S2 includes expression data for Ptf1a and *Prdm13* in the neural tube at additional embryonic stages and genomic coordinates for Ptf1a peaks 1–7 from panel B.

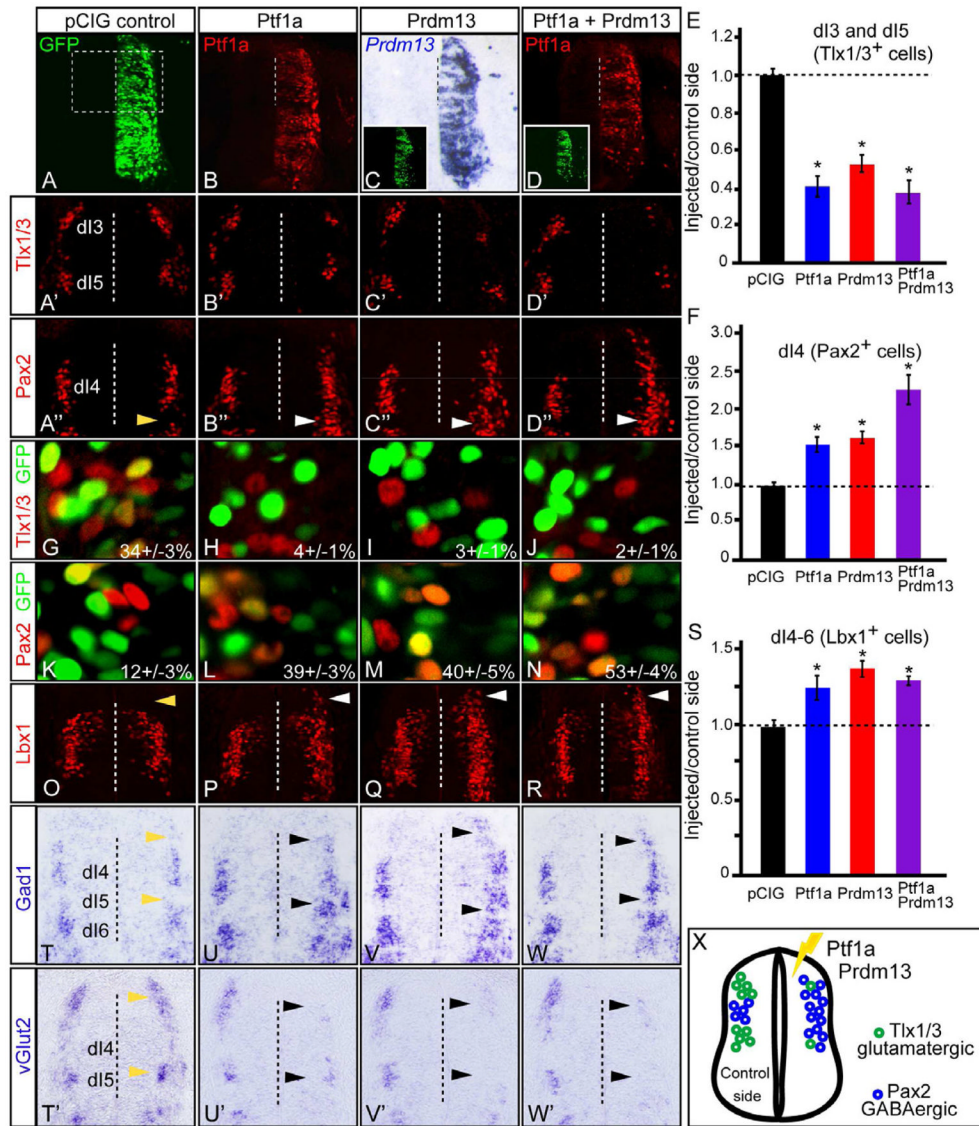


Figure 3. Ptf1a and Prdm13 induce inhibitory and suppress excitatory neuronal markers
 Stage HH12-13 chick embryos were electroporated with empty vector (control), Ptf1a, Prdm13, or both. Transverse sections through stage HH24-25 are shown with the electroporated side on the right. (A–D) GFP (green) shows electroporation efficiency along the dorsoventral axis. Ptf1a protein (red) and *Prdm13* mRNA show ectopic expression. Dashed box in (A) is the region of the neural tube shown in (A'–D'', O–R). (A'–F) Dorsal neural tube sections from (A–D) indicate the dl3 and dl5 neuronal populations marked by Tlx1/3, or the dl4 neurons marked by Pax2. (E,F) the ratio of Tlx1/3⁺ or Pax2⁺ neurons on the electroporated side versus the control side showing that overexpression of Ptf1a and Prdm13 cause a dramatic decrease in Tlx1/3⁺ and increase in Pax2⁺ neurons relative to control. Note that ectopic Pax2⁺ cells (white arrowheads) are found in the dl5 domain not normally expressing Pax2 (yellow arrowhead). (G–N) Electroporated cells, visualized by GFP, co-localized with Tlx1/3 (G–J) or Pax2 (K–N). The percentage of GFP cells that co-express Tlx1/3 or Pax2 in the dorsal mantle zone is shown. Ectopic expression of Ptf1a and/or Prdm13 reduces the percentage of Tlx1/3;GFP double positive cells from 34% to 2–4%, and increases Pax2;GFP double positive cells from 12% to 39–53%. (O–S) Dorsal neural

tube sections from (A–D) showing the dI4–6 neuronal populations marked by Lbx1. Quantification (S) showing that Ptf1a and Prdm13 overexpression cause an increase in Lbx1⁺ neurons relative to control. The white arrowheads indicate ectopic Lbx1 expression detected in the dI3 region not normally expressing Lbx1 (compare to control (O) yellow arrowhead). (T–W') *In situ* hybridization with *Gad1* or *vGlut2* probes showing that Ptf1a and Prdm13 overexpression increase *Gad1* and decrease *vGlut2* (black arrowheads) when compared to either the empty vector control (yellow arrowheads) or the control side in each neural tube. (X) Diagram summarizing the overexpression phenotypes. Error bars are reported as SEM. * p-values<0.001. Supplementary Fig. S3 includes analysis of the Prdm13 phenotype with additional markers of the dorsal interneuron populations including *Isl1/2*, *Lmx1b* and *Lhx1/5*.

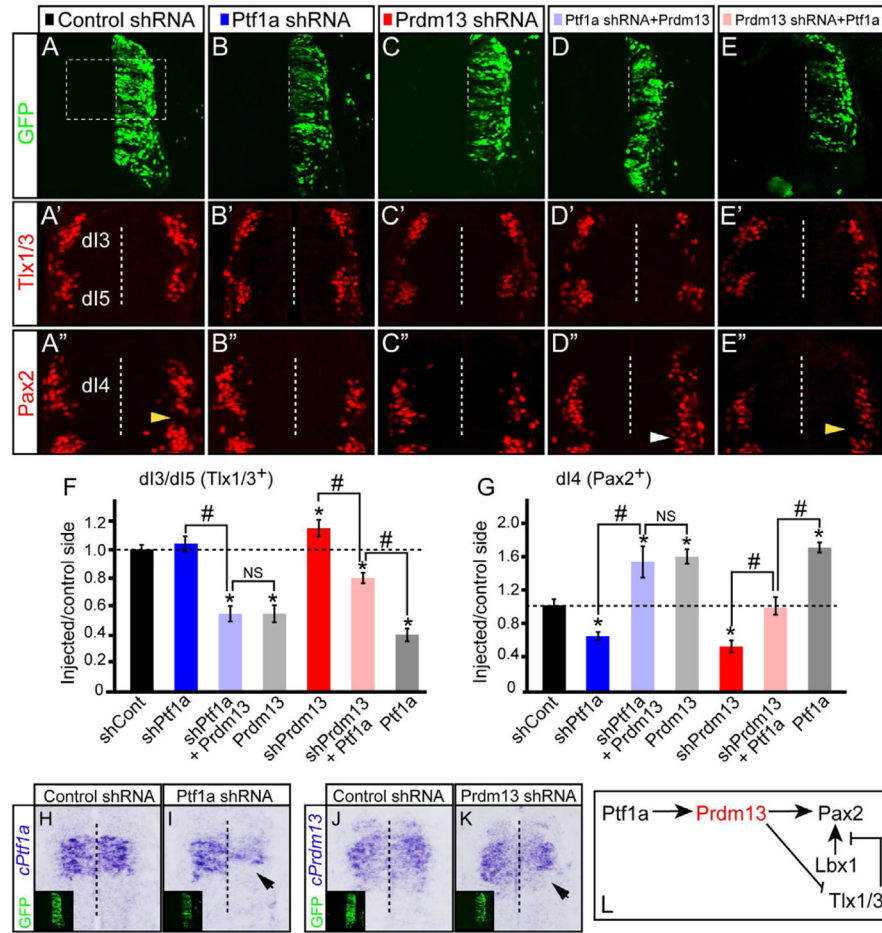


Figure 4. Ptf1a requires Prdm13 to specify inhibitory neurons and suppress excitatory neurons Stage HH12-13 chick embryos were electroporated with expression or shRNA vectors to modulate levels of Ptf1a and Prdm13. Transverse sections through stage HH24-25 are shown with the electroporated side on the right. (A–E) GFP indicates the electroporation efficiency along the dorsoventral axis. The dashed box is the region shown in (A'–E''). (A'–E', F) Tlx1/3 shows Prdm13 knockdown results in an increase in dI3/dI5 neurons (C'). Epistasis experiments show Prdm13 does not require Ptf1a for this activity in repressing Tlx1/3 (D') but Ptf1a activity is abrogated with shPrdm13 (E'). (A''–E'', G) Pax2 shows a significant decrease in the number of dI4 neurons when Ptf1a or Prdm13 are knocked down compared to control shRNA (B'', C'', G). Epistasis experiments show Prdm13 does not require Ptf1a for this activity in inducing Tlx1/3 (D'') but Ptf1a activity is abrogated with shPrdm13 (E''). Note that ectopic Pax2⁺ cells (white arrowhead) are found in the dI5 domain not normally expressing Pax2 (yellow arrowheads). Error bars are reported as standard error of the mean. p-values < 0.001 between sample and control (*), and between samples (#). NS, not significant. (H–K) *cPtf1a* or *cPrdm13* probes indicate the knockdown efficiency (arrows). Inset: GFP indicates the electroporation efficiency. (L) Diagram summarizing the conclusion that Prdm13 is a functional downstream target of Ptf1a that induces Pax2 and suppresses Tlx1/3. Supplementary Fig. S4 includes rescue control experiments for the shRNA.

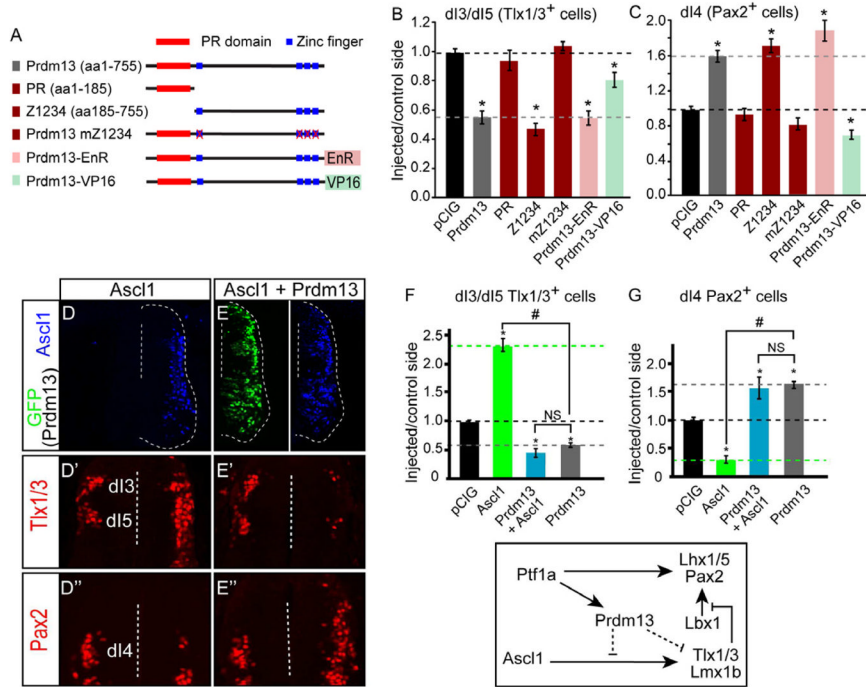


Figure 5. Prdm13 Blocks Ascl1 Activity through Repression Requiring Its Zn Fingers
 (A) Diagram of the Prdm13 mutations tested. PR domain (red boxes) and Zn finger domains (blue boxes) are indicated. Engrailed repressor (EnR) or activator VP16 were fused in frame at the C-terminal end of Prdm13.

(B-C) Quantification of experiments testing the Prdm13 mutants indicated. The black dashed line indicates the pCIG control ratio of injected/control side. The gray dashed line indicates the phenotype of the full-length Prdm13. The PR domain is dispensable for Prdm13 activity, but the four Zn fingers are required. The Prdm13-EnR fusion mimics the Prdm13 phenotype, whereas the Prdm13-VP16 does not.

(D-E'') Stage HH12-13 chick embryos were electroporated with Ascl1 (D) or Ascl1 plus Prdm13 (E). Transverse sections through stage HH24-25 are shown with the electroporated side on the right. Ascl1 detects the ectopically expressed protein (blue), and Prdm13 is indicated by GFP. Dashed lines indicate location of the ventricle. Dorsal neural tube sections from (D and E) show Tlx1/3 defining the dl3 and dl5 (D'-E') and Pax2 defining dl4 neuronal populations (D''-E''). Ectopic Ascl1 causes a dramatic increase in the number of Tlx1/3⁺ neurons with a decrease in Pax2⁺ cells, whereas addition of Prdm13 reverses this phenotype.

(F-G) The ability of Prdm13 to block this activity of Ascl1 is quantitatively shown. All error bars are reported as SEM. p values < 0.001 between sample and control (*), and between samples (#). NS, not significant. Diagram summarizing the conclusion that Prdm13 antagonizes Ascl1 neuronal subtype specification activity. Figure S5 shows Ascl1 expression levels are not altered by overexpression or knockdown of Prdm13. Additionally, representative images for Tlx1/3 and Pax2 for chick electroporation experiments with the Prdm13 truncations are shown.

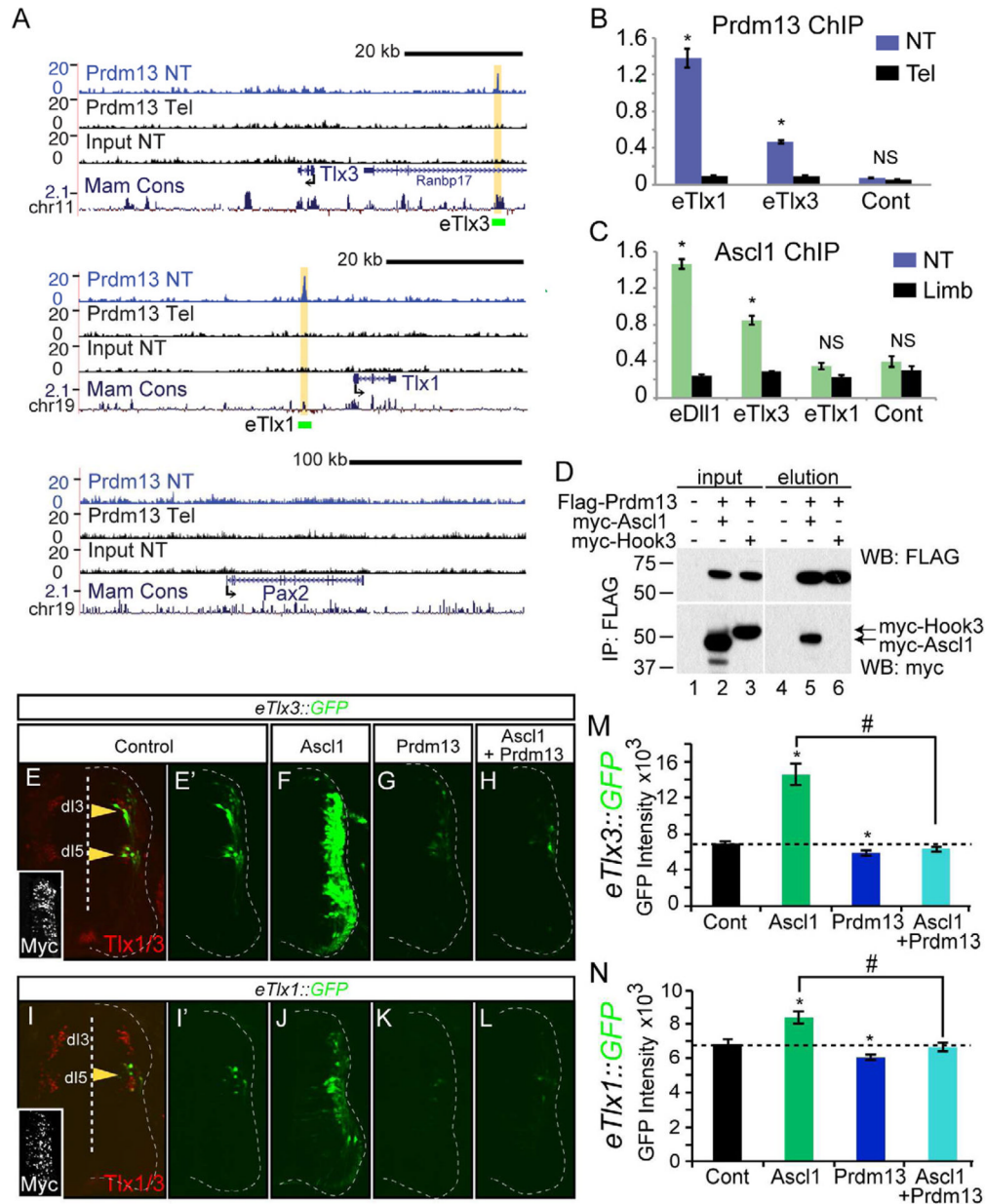


Figure 6. Prdm13 directly suppresses Tlx1 and Tlx3 through multiple mechanisms
 (A) UCSC Genome Browser showing mouse genomic regions surrounding *Tlx3*, *Tlx1*, and *Pax2* including Prdm13 ChIP-Seq from neural tube (NT, blue), and two negative controls including Prdm13 ChIP-Seq from telencephalon (Tel) and input from NT, all from E11.5 mouse embryos. Prdm13 occupies chromatin near the *Tlx1* and *Tlx3* genes at sequence conserved across mammals (yellow highlight). Green bars labeled *eTlx1* and *eTlx3* are the regions used in the GFP reporter constructs in (E–N). (B) ChIP-qPCR validating Prdm13 ChIP-Seq at the *eTlx1* and *eTlx3* loci in (A). Tel tissue is negative for Prdm13 and serves as a control for specificity of the antibody. (C) ChIP-qPCR for Ascl1 at the *eTlx1* and *eTlx3* loci in (A). Limb tissue is negative for Ascl1 and serves as a control for specificity of the antibody. *eDIII* is a known target of Ascl1. (D) Co-IP experiments of epitope tagged versions of the proteins transfected into HEK 293 cells indicate specific interaction between Ascl1 and Prdm13 (lane 5), but not the negative control, myc-Hook3 (lane 6). (E–N) Chick

neural tube electroporated with either *eTlx3::GFP* or *eTlx1::GFP* reporter and expression vectors for *Ascl1*, *Prdm13*, or both as indicated. Only the electroporated side is shown and the neural tube is outlined with a dashed line. (E) The *eTlx3::GFP* signals are restricted and co-localize with *dI3/dI5* markers, *Tlx1/3* (yellow arrowheads). (E'–H) *Ascl1* dramatically induces *eTlx3::GFP* and this is blocked by *Prdm13*. (I) The *eTlx1::GFP* signals are restricted to *dI5* neurons (yellow arrowhead). (I'–L) Induction by *Ascl1* is more subtle than that seen with *eTlx3::GFP* but *Prdm13* is still repressive. Inset: *myc* immunofluorescence indicates the electroporation efficiency. (M and N) shows the quantification of these data by measuring pixel intensity. Error bars are reported as SEM. * and # p-value<0.001 relative to control or between samples indicated.

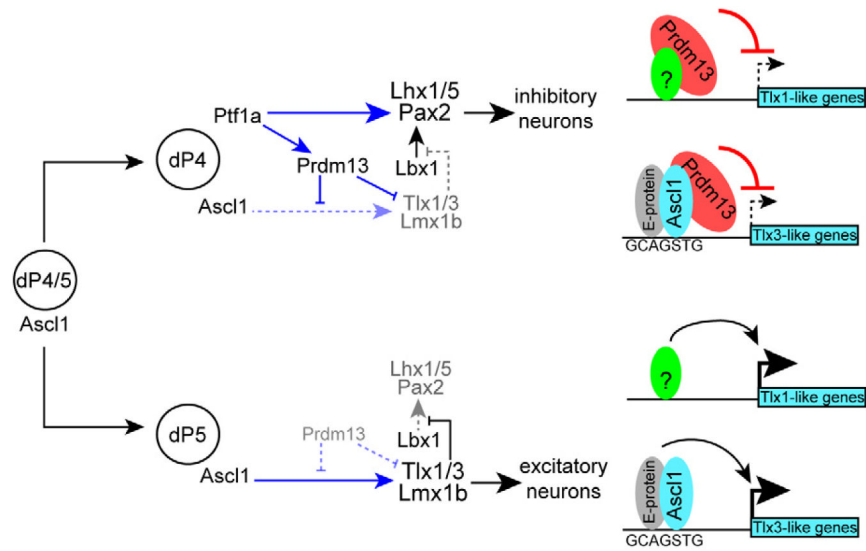


Figure 7. Transcriptional network controlling the balance of inhibitory and excitatory neurons in the dorsal spinal cord

Progenitors in the ventricular zone express *Ascl1*. In the absence of *Ptf1a*, these cells become excitatory neurons (dP5 lineage) through *Ascl1* induction of the HD factors *Tlx1/3* and *Lmx1b*. *Tlx1/3* repress the *Lbx1*-dependent induction of *Pax2* and the inhibitory neuronal program. *Ascl1* regulates *Tlx3* directly whereas *Tlx1* may be indirect. *Ptf1a* is upregulated in a subset of the *Ascl1* progenitor cells directing their fate to inhibitory neurons (dP4 lineage) through induction the HD factors *Pax2* and *Lhx1/5*. Additionally, *Ptf1a* directly increases levels of *Prdm13* that in turn suppresses *Tlx1* and *Tlx3*, ensuring a suppression of the glutamatergic program in the inhibitory neurons. *Prdm13* works through at least two mechanisms; one being *Ascl1*-dependent possibly involving a novel transcriptional repressor complex for *Tlx3*-like genes, and the other *Ascl1*-independent requiring some other activator (green circle) for *Tlx1*-like genes.