© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 2539 doi:10.1242/dev.155986

CORRECTION

Correction: A transcription factor network specifying inhibitory versus excitatory neurons in the dorsal spinal cord

Mark D. Borromeo, David M. Meredith, Diogo S. Castro, Joshua C. Chang, Kuang-Chi Tung, Francois Guillemot and Jane E. Johnson

There was an error in the supplementary data of Development **141**, 2803-2812.

In Table S2, on the Ind.replicates tab, some values in column J (Ptf1a-het, sample1_FPKM) were misordered and hence did not correspond correctly with column A (gene); on the master tab, some rows had inadvertently been duplicated. The correct version of Table S2 now appears online.

The authors apologise to readers for this mistake.

© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

RESEARCH ARTICLE

A transcription factor network specifying inhibitory versus excitatory neurons in the dorsal spinal cord

Mark D. Borromeo[1], David M. Meredith[1,*], Diogo S. Castro[2], Joshua C. Chang[1], Kuang-Chi Tung[1], Francois Guillemot[3] and Jane E. Johnson[1,‡]

ABSTRACT

The proper balance of excitatory and inhibitory neurons is crucial for normal processing of somatosensory information in the dorsal spinal cord. Two neural basic helix-loop-helix transcription factors (TFs), Ascl1 and Ptf1a, have contrasting functions in specifying these neurons. To understand how Ascl1 and Ptf1a function in this process, we identified their direct transcriptional targets genome-wide in the embryonic mouse neural tube using ChIP-Seq and RNA-Seq. We show that Ascl1 and Ptf1a directly regulate distinct homeodomain TFs that specify excitatory or inhibitory neuronal fates. In addition, Ascl1 directly regulates genes with roles in several steps of the neurogenic program, including Notch signaling, neuronal differentiation, axon guidance and synapse formation. By contrast, Ptf1a directly regulates genes encoding components of the neurotransmitter machinery in inhibitory neurons, and other later aspects of neural development distinct from those regulated by Ascl1. Moreover, Ptf1a represses the excitatory neuronal fate by directly repressing several targets of Ascl1. Ascl1 and Ptf1a bind sequences primarily enriched for a specific E-Box motif (CAGCTG) and for secondary motifs used by Sox, Rfx, Pou and homeodomain factors. Ptf1a also binds sequences uniquely enriched in the CAGATG E-box and in the binding motif for its co-factor Rbpj, providing two factors that influence the specificity of Ptf1a binding. The direct transcriptional targets identified for Ascl1 and Ptf1a provide a molecular understanding of how these DNA-binding proteins function in neuronal development, particularly as key regulators of homeodomain TFs required for neuronal subtype specification.

KEY WORDS: ChIP-Seq, bHLH transcription factor, Dorsal neural tube, Neuronal subtype specification, Mouse, Chick, Ascl1, Ptf1a

INTRODUCTION

The neurons within the dorsal spinal cord provide the initial integration for somatosensory information originating from the periphery. These neurons relay sensory information to local spinal cord neurons and higher brain centers to modulate and coordinate the appropriate physiological response to environmental stimuli (Liu and Ma, 2011; Ross, 2011). The proper processing of somatosensory information requires the correct balance of excitatory and inhibitory neurons within the dorsal spinal cord. Revealing the genetic programs that give rise to these different classes of neurons will provide insight into neuronal disorders as well as address fundamental concepts in transcriptional control of cell fate determination and neuronal subtype specification.

Specification of excitatory and inhibitory neurons in the developing nervous system relies on combinations of transcription factors (TFs) to activate or repress specific neurogenic programs. The basic helix-loop-helix (bHLH) and homeodomain (HD) families of TFs are particularly important in generating the correct number and subtypes of neurons in the dorsal spinal cord (Cheng et al., 2004, 2005; Glasgow et al., 2005; Gowan et al., 2001; Gross et al., 2002; Helms et al., 2005; Mizuguchi et al., 2006; Muller et al., 2005; Wildner et al., 2006). In the dorsal neural tube, multiple progenitor domains can be identified by neural bHLH factors, such as Ascl1 (previously Mash1) and Ptf1a (Glasgow et al., 2005; Gowan et al., 2001; Helms et al., 2005). Genetic studies have shown that in the absence of one bHLH factor, the neural progenitor cells take on the identity of the neighboring cells, resulting in the transfating of one neuronal type to another. For example, loss of Ptf1a results in a loss of GABAergic neurons and excess glutamatergic neurons in the spinal cord dorsal horn (Glasgow et al., 2005). By contrast, Ascl1 has the opposite effect and is necessary and sufficient for generation of early-born glutamatergic neurons (Chang et al., 2013; Helms et al., 2005; Mizuguchi et al., 2006; Nakada et al., 2004). Robust disruption in HD factor expression is reported as a primary phenotype in Ascl1 and Ptf1a mutants. Many HD factors, such as Pax2 and Tlx3, have been shown to be essential for continued specification of the neuronal subtypes (Batista and Lewis, 2008; Cheng et al., 2004, 2005; Gross et al., 2002; Huang et al., 2008; Pillai et al., 2007). Given the temporal and genetic relationship between bHLH and HD factors, HD factors are prime candidates for being direct transcriptional targets of the bHLH factors in the dorsal spinal cord.

Ptf1a and Ascl1 are Class II bHLH TFs that bind the degenerate DNA motif CANNTG (E-box) as heterodimers with E-proteins, and activate transcription (Beres et al., 2006; Nakada et al., 2004). For decades, researchers have tried to understand how bHLH factors can select and regulate their specific gene targets, given that they bind similar degenerate E-box motifs *in vitro* [reviewed by Bertrand et al. (2002); Lai et al. (2013)]. With advances in chromatin immunoprecipitation (ChIP) and the increasing number of identified cis-regulatory elements that are under the control of bHLH factors, a preference for binding certain E-boxes *in vivo* has been shown, thus explaining some of the functional specificity (Fong et al., 2012; Klisch et al., 2011; Lai et al., 2011; Meredith et al., 2013; Seo et al., 2007). Additional influences on specificity probably involve the epigenetic landscape (Fong et al., 2012; Meredith et al., 2013) and the pool of transcriptional co-factors that are available in a given population of progenitors (Lai et al., 2013). However, Ptf1a and Ascl1 are co-expressed in a subset of neural progenitors, and thus, although they function in a similar cellular environment, they have distinct activities in neuronal specification.

[1]Department of Neuroscience, UT Southwestern Medical Center, Dallas, TX 75390, USA. [2]Instituto Gulbenkian de Ciência, Molecular Neurobiology Laboratory, Oeiras, Portugal. [3]Division of Molecular Neurobiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.
*Present address: Department of Pathology, Brigham & Women's Hospital, Boston, MA 02115, USA.

‡Author for correspondence (jane.johnson@utsouthwestern.edu)

Received 15 November 2013; Accepted 15 May 2014

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

Here, we address a fundamental concept in transcriptional control of cell fate determination by probing how these two related TFs select and regulate distinct neurogenic programs *in vivo*.

Performing ChIP-Seq and RNA-Seq experiments using mouse neural tube tissue, we identify genes that are directly regulated by Ascl1 and Ptf1a. We demonstrate that Ascl1 and Ptf1a activate a glutamatergic or GABAergic specification program by directly regulating distinct subsets of HD factor genes. Additional targets were identified for Ascl1 consistent with its role in regulating several aspects of neurogenesis, whereas targets for Ptf1a illustrate its role in regulating genes encoding components of inhibitory neuron neurotransmitters and synapses. These distinct targets for Ascl1 and Ptf1a reflect differences in the timing of their function, with Ascl1 being expressed earlier during neurogenesis than Ptf1a. The specificity of Ascl1 and Ptf1a function is explained, at least in part, through differential preferences of DNA-binding sequences, as well as by direct and indirect repression of several Ascl1-activated targets by Ptf1a.

RESULTS

Ascl1 and Ptf1a bind largely distinct sites within neural tube chromatin and have distinct E-box sequence preferences

In the dorsal spinal cord, progenitor cells transiently express Ascl1 in the ventricular zone (Fig. 1A,B). As these cells begin to differentiate, a subset of these now postmitotic cells express Ptf1a as they migrate laterally toward the mantle zone (Fig. 1A-B″). Thus, overlap of Ptf1a and Ascl1 expression can be found in a subpopulation of the developing dorsal spinal cord near the ventricular and mantle zone border. The progenitor cells, expressing Ascl1 alone, or with the subsequent expression of Ptf1a, result in the activation of different TFs and neuronal fates in the spinal cord (Fig. 1C) (Glasgow et al., 2005; Nakada et al., 2004). In order to uncover mechanisms by which two neural class II bHLH factors regulate different sets of gene programs that give rise to distinct subtypes of neurons in the dorsal neural tube, we compared and contrasted the genome-wide binding sites of Ascl1 and Ptf1a by ChIP-Seq in E12.5 mouse neural tubes. ChIP-Seq for Ascl1 and Ptf1a have been recently published (Meredith et al., 2013; Sun et al., 2013), but were re-evaluated here and compared using the peak-calling software Homer (Heinz et al., 2010). Using the parameters of a false discovery rate (FDR) cutoff of 0.001, a 4-fold enrichment of sequence tags in the target experiment over control and a cumulative Poisson *P*-value threshold of 0.0001, Ascl1 was found to bind 4082 sites and Ptf1a was found at 7749 sites, with 1588 of those sites bound by both factors (Fig. 2A; supplementary material Table S1). Heat maps show the binding profiles of Ascl1 and Ptf1a (Fig. 2A). The stringent criteria for peak calling discard many low-affinity Ptf1a- and Ascl1-binding events, and visual inspection of the heat maps (Fig. 2A) suggests that the 1588 overlapping sites might be an underestimate. Ascl1 and Ptf1a preferentially bind distal DNA elements (>5-500 kb from transcription start sites) rather than proximal promoters (Fig. 2B). Genes with Ascl1- or Ptf1a-bound sites within 5 kb of their transcription start sites, and those with multiple Ascl1 or Ptf1a sites, were expressed at higher mean levels (*P*-values <0.05) (supplementary material Fig. S1).

De novo motif analysis (Heinz et al., 2010) of the Ascl1- and Ptf1a-called peaks returned the canonical E-box (CANNTG) (Fig. 2C), the known class II bHLH consensus-binding site (Murre et al., 1989). We found that 98% of Ascl1- and 85% of Ptf1a-bound sites contained a generic CANNTG E-box within 75 bp of the peak center. The specific primary E-box motifs show that, whereas the CAGCTG E-box is enriched in both Ascl1- and Ptf1a-bound sites, Ptf1a peaks are also enriched within the CATCTG/CAGATG E-box (Fig. 2C). The primary E-box motifs are commonly found near the peak centers (Fig. 2C,D), and on average there are two or more E-boxes in each peak (supplementary material Fig. S1). An example of a Ptf1a-bound site that is not shared with Ascl1 is shown for the inhibitory neuronal specification gene *Pax2* (Fig. 2E). The DNA sequence under the summit of the Ptf1a peak within *Pax2* shows a highly conserved sequence that contains a TC/GA core E-box. Additionally, the average Ascl1 binding is lowest at Ptf1a peaks that contain only a TC/GA core E-box compared with Ptf1a peaks that have a GC core E-box (Fig. 2F). A total of 1332 Ptf1a peaks were classified as peaks with a TC/GA E-box only; among these sites, only 145 overlap with Ascl1 peaks. Thus, *in vivo* Ptf1a can bind DNA with GC or TC/GA E-boxes; however, Ascl1 preferentially binds to regions with the GC E-box. These results suggest that these two bHLH factors regulate distinct gene expression programs through recognition of distinct DNA binding motifs.

Enrichment of non-E-box transcription factor motifs within Ascl1- and Ptf1a-bound genomic regions

The specific E-box sequence influences binding site selection, but cooperation from additional TFs may also modulate Ascl1 and/or Ptf1a binding. Therefore, we searched for additional sequence motifs enriched within the regions bound by both Ptf1a and Ascl1 (Fig. 2G) and in peaks specific to Ascl1 or Ptf1a (Fig. 2H). We found that sites shared by Ptf1a and Ascl1 are enriched with the GC E-box, Sox, HD, Rfx and Pou motifs (Fig. 2G). In fact, regardless of how the data are binned, shared sites, Ascl1 only or Ptf1a only are all enriched for these motifs. Moreover, Ptf1a is a component of a trimeric complex that includes Rbpj in addition to the heterodimeric E-protein partner; this complex is required for Ptf1a function (Beres et al., 2006; Hori et al., 2008; Masui et al., 2008). With over 1500 sites shared by Ptf1a and Ascl1, we anticipated an enrichment of the Rbpj binding site. Indeed, embedded within the Rfx motif is the canonical Rbpj binding site known as the TC-box (Fig. 2G,

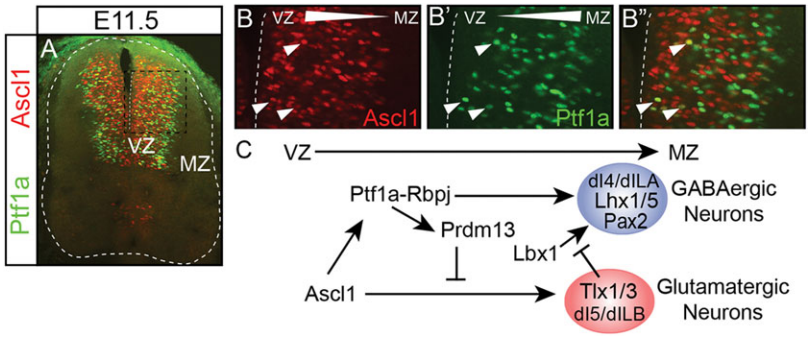

Fig. 1. Ascl1 and Ptf1a overlap in the dorsal neural tube cells. (A-B″) Immunofluorescence for Ascl1 and Ptf1a in mouse E11.5 neural tube. Black dashed box in A marks the magnified region in B-B″. Arrowheads indicate examples of Ascl1 and Ptf1a colocalization. (C) A summary of the known TF network involved in generating excitatory and inhibitory populations in the dorsal spinal cord.

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

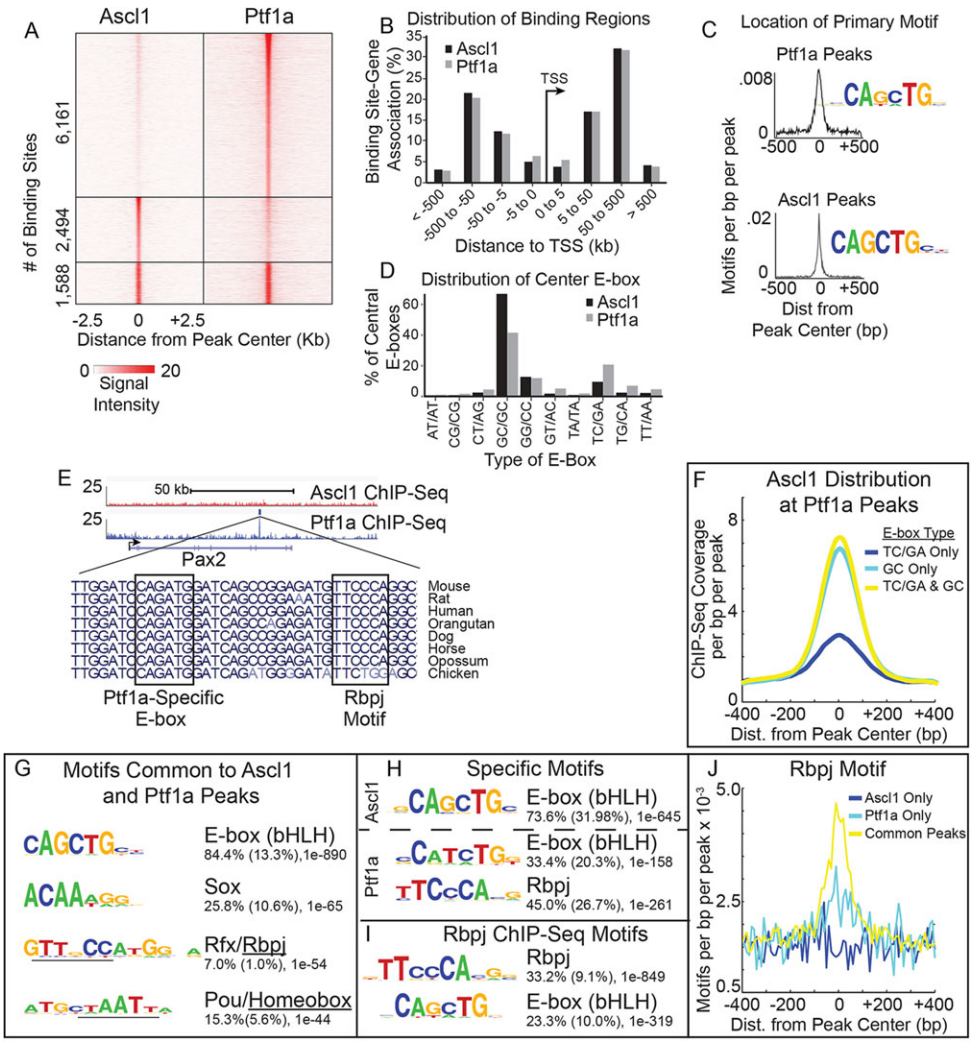

Fig. 2. Ptf1a- and Ascl1-binding sites in the neural tube reveal distinct and overlapping preferences. (A) Heat map of ChIP-Seq signal intensity ±2.5 kb around peak centers bound by Ascl1, Ptf1a or both in E12.5 mouse neural tube. (B) Distribution of bound sites with respect to transcriptional start sites (TSS). (C) *De novo* motif analysis. Density plots show the primary motif enriched near the peak center. (D) Distribution of E-box core type found nearest to peak centers. (E) Example of a Ptf1a-bound site, not shared by Ascl1, located within the *Pax2* gene. (F) Coverage of Ascl1 ChIP-Seq fragments at Ptf1a peaks that contain a GA/TC E-box only, a GC E-box only or both GA/TC and GC E-boxes. (G) *De novo* motif analysis shows that the GC core E-box, Sox, Rfx/Rbpj and Pou/homeobox motifs are enriched in sites shared by Ptf1a and Ascl1. (H) Motifs enriched in Ascl1 sites over Ptf1a, or in Ptf1a sites over Ascl1. (I) Motif analysis of Rbpj ChIP-Seq sites (from E12.5 neural tube) identified the known Rbpj motif and a Ptf1a-like E-box motif. (J) The frequency of finding the Rbpj motif in Ascl1 only, Ptf1a only or Ascl1/Ptf1a shared sites.

underlined sequence). Thus, Rfx motifs could be bound by Rfx factors, by Rbpj or by both.

In order to find motifs enriched specifically in the Ptf1a-bound sites, we performed *de novo* motif analysis using all Ptf1a peaks, but used Ascl1-bound sites as the background. This strategy revealed the TC/GA core E-box and a stronger consensus sequence for the Rbpj binding site than that found within peaks shared by Ascl1 and Ptf1a (Fig. 2G,H). The highest frequency of the Rbpj motif is found near the center of Ptf1a-bound sites (Ptf1a only and shared), whereas Ascl1-only sites display a low frequency of the motif (Fig. 2J). In the reciprocal *de novo* motif analysis of Rbpj-binding sites from Rbpj ChIP-Seq of E12.5 neural tube (Meredith et al., 2013), the Rbpj motif is enriched along with an E-box that is similar to the Ptf1a primary motif (Fig. 2I). This is consistent with a role for Rbpj in influencing the selection of Ptf1a binding.

Conversely, we searched for Ascl1-specific co-factors, using Ascl1 peaks called and using Ptf1a sites as background. We did not find any other transcription factor motifs enriched beyond the preferential binding of Ascl1 to the GC core E-box (Fig. 2H). This suggests that Ascl1-specific co-factor binding sites, if present, occur at a low frequency and are not detected by this approach, or that the co-factor shares a redundant motif with Ptf1a sites. Thus, the primary distinction between Ascl1- and Ptf1a-bound regions is the presence of the CAGATG/CATCTG E-box and a strong Rbpj consensus motif in Ptf1a-bound regions. Furthermore, Sox, HD, Pou and Rfx factors found with the E-box in these regions begin to define potential motif combinations that suggest the presence of a neural-specific enhancer.

We used a chick electroporation reporter assay to test the requirement for E-box and Sox motifs for activity in two identified enhancers (supplementary material Fig. S2). In both *ePrdm13::GFP* (a Ptf1a target) and *eTlx3::GFP* (an Ascl1 target), enhancers drive GFP expression in the dorsal neural tube, but requirement for the E-box and Sox motifs was different for each motif in each enhancer. For example, *ePrdm13::GFP* activity depends on the Sox motif, but not on two CA E-box sites (supplementary material Fig. S2D,E,I), whereas *eTlx3::GFP* activity depends on a GC E-box, but not the Sox motif (supplementary material Fig. S2G,H,J). Thus, enhancers identified through Ascl1 and/or Ptf1a binding can activate transcription in the correct tissue, but transcription factor motifs identified as enriched in the genomic data sets are not consistently required for enhancer activity in these assays.

Ascl1 and Ptf1a have opposite actions in neuronal subtype specification

It is well-established that Ascl1 and Ptf1a influence the expression of several HD factors that mark specific neuronal populations and function in the maturation of those neurons in the dorsal neural tube [reviewed by Lai et al. (2013)]. To summarize prior studies

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

that revealed the genetic network regulating neuronal subtype specification in the E11.5 dorsal neural tube, and to introduce the populations used in the current study for the ChIP-Seq and RNA-Seq experiments, we show here the expression patterns of Ascl1 and Ptf1a and some of the HD factors that specify the excitatory (Tlx1/3) and inhibitory (Pax2, Lhx1/5) neurons in the wild type (WT) and mutants of *Ascl1* and *Ptf1a* (Fig. 3).

The opposing functions of Ascl1 and Ptf1a in specifying neuronal subtype in the dorsal neural tube are illustrated by the changes in HD factor expression in *Ascl1* and *Ptf1a* mutant mouse embryos. The excitatory neuronal populations in the dorsal neural tube (dI3, dI5 and dIL^{B}), marked by the HD factors Tlx1 and Tlx3 (Fig. 3G), are drastically reduced at E11.5 in the *Ascl1* null (Fig. 3H), whereas in the *Ptf1a* null they are markedly increased (Fig. 3I). By contrast, inhibitory neuronal populations (dI4 and dIL^{A}), marked by Pax2, Lhx1 and Lhx5 (Fig. 3J,M), are lost in the *Ptf1a* null (Fig. 3L,O). These inhibitory markers are also diminished in the *Ascl1* null (Fig. 3K,N), a phenotype probably secondary to the dependence of *Ptf1a* expression on Ascl1 at this stage (Fig. 3E) (Mizuguchi et al., 2006). There are additional complexities and feedback loops in the transcriptional network, including repressive interactions of Tlx1/3 on Pax2 levels (Cheng et al., 2005), and involvement of other TFs, such as Prdm13 (Chang et al., 2013) (see diagram Fig. 1C). In summary, Ascl1 and Ptf1a are at the head of a transcription factor network that is crucial in generating the GABAergic (inhibitory) and glutamatergic (excitatory) neuronal populations in the dorsal spinal cord. As the HD factors are genetically downstream of the bHLH factors, we hypothesized that they are direct transcriptional targets of Ascl1 and Ptf1a in the dorsal spinal cord. In the following sections, we provide evidence supporting this hypothesis; we also identify a cohort of genes directly regulated by Ascl1 and Ptf1a that allow these factors to direct neuronal differentiation and neuronal subtype specification.

In order to identify the transcriptomes downstream of Ascl1 and Ptf1a, we used fluorescence-activated cell sorting (FACS) to isolate dorsal neural tube populations plus and minus Ascl1 and Ptf1a, and performed RNA-Seq. For the Ascl1-lineage cells, we used $Ascl1^{GFP/+}$ knock-in embryos (Leung et al., 2007) compared with $Ascl1^{GFP/null}$ embryos that completely lack Ascl1 protein. Because GFP is more stable than Ascl1, Ascl1-expressing progenitor cells and their immediate progeny were isolated in this paradigm (supplementary material Fig. S3A). For the Ptf1a lineage, we isolated cells from transgenic embryos in which mCherry is driven by a 12.4 kb genomic region that directs expression to the dorsal neural tube overlapping, but not restricted to, the *Ptf1a*-expression domain (supplementary material Fig. S3B) (Meredith et al., 2009). The *12.4kbPtf1a::mCherry* line was crossed to the $Ptf1a^{Cre}$ knock-in mouse (Kawaguchi et al., 2002), and mCherry+ cells were isolated from *Ptf1a−/+* and *Ptf1a−/−* neural tubes. The identity and purity of the samples was confirmed (supplementary material Fig. S3C,D, see brackets).

RNA-Seq from the *Ascl1* heterozygotes compared with the *Ascl1* mutants identified 1173 genes with a significant difference in gene expression (*P*-value <0.05), including 449 Ascl1-activated (higher in heterozygotes than in nulls) and 724 Ascl1-repressed (lower in heterozygotes than in nulls) (Fig. 3P, red dots in left panel). Comparing *Ptf1a*-heterozygous cells with *Ptf1a* null cells revealed 361 genes showing a significant change in expression, including 132 Ptf1a-activated and 229 Ptf1a-repressed (Fig. 3P, red dots in right

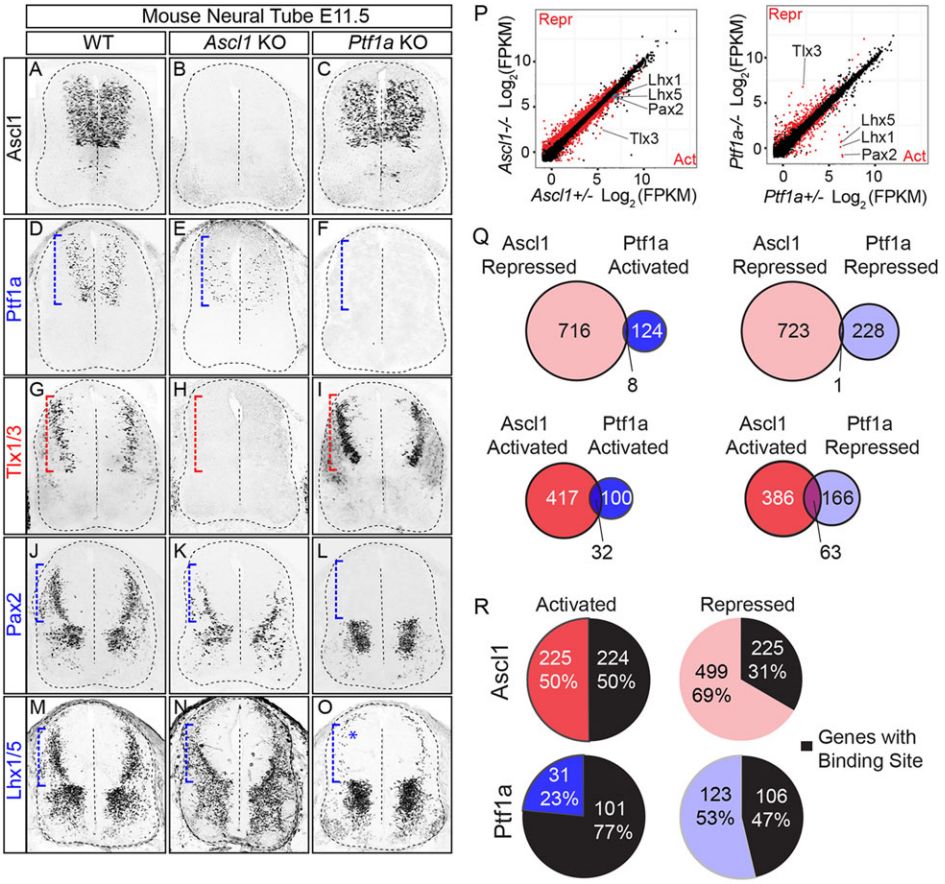

Fig. 3. bHLH factors Ascl1 and Ptf1a have opposite actions in neuronal subtype specification in the developing spinal cord. (A-O) Immunofluorescence for Ascl1, Ptf1a, Tlx1/3, Pax2 and Lhx1/5 on mouse E11.5 neural tube from wild-type and *Ascl1* or *Ptf1a* null embryos. Brackets indicate where Ascl1 and Ptf1a are expressed and where phenotypes are detected in the mutants. Red indicates a marker of excitatory neurons, and blue indicates markers of inhibitory neurons or their precursors. Asterisk in O indicates a Ptf1a-independent Lhx1/5+ population (dI2) that is unaffected in the *Ptf1a* null. (P) RNA-Seq data show transcript levels (FPKM) in Ascl1 lineage cells from *Ascl1* control or null embryos (left plot), and Ptf1a lineage cells from *Ptf1a* control or null embryos (right plot). Genes with a significant change in expression in the mutants versus controls are marked in red (*P*-value <0.05). Genes above the diagonal are repressed (Repr) and genes below the diagonal are activated (Act). (Q) The number of genes activated or repressed by Ptf1a or Ascl1. (R) The total number of genes activated or repressed by Ascl1 or Ptf1a, as determined by RNA-Seq, and the number of genes that contain an Ascl1- or Ptf1a-binding site assigned by GREAT (McLean et al., 2010) (black fill, direct targets).

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

panel). As expected, the HD TFs Tlx1, Tlx3 (Fig. 3P) and Lmx1b – which mark excitatory neurons in the dorsal spinal cord – require Ascl1 for expression, as detected by a significant decrease in their transcript levels in the *Ascl1* mutant versus *Ascl1* heterozygous populations. Conversely, *Tlx1*, *Tlx3* and *Lmx1b* significantly increase in the absence of Ptf1a (Fig. 3P and Fig. 4A; supplementary material Table S2). Among the genes that require Ptf1a for expression are those encoding the inhibitory neuronal markers Pax2, Lhx1 and Lhx5 (Fig. 3P and Fig. 4A; supplementary material Table S2). These inhibitory neuronal markers also require Ascl1, as seen in the more subtle decrease in *Pax2*, *Lhx1* and *Lhx5* in *Ascl1* mutants (Fig. 3P and Fig. 4A; supplementary material Table S2). These results are consistent with the immunohistochemistry (Fig. 3A-O) and confirm the robustness of these data.

The majority of genes that change when Ascl1 or Ptf1a are mutated are non-overlapping, suggesting distinct functions for these bHLH TFs (Fig. 3Q). However, the subset of Ptf1a-activated genes that are also activated by Ascl1 include Pax2 and Lhx1/5 and distinctly define dI4/dIL^{A} Inhibitory neurons (32 genes, supplementary material Table S4). Coregulation of these genes by Ptf1a and Ascl1 may reflect the dependence of some *Ptf1a* expression on Ascl1 (Fig. 3D,E). More strikingly, 63 of Ptf1a-repressed genes are activated by Ascl1 (Fig. 3Q). This subset of genes specifically marks the dI3/5, dIL^{B} neurons and includes those encoding the HD factors Tlx1 and Tlx3 (Fig. 3G-I and Fig. 4A). This group also contains genes that code for factors, such as Cbln1 and Cbln2, which are involved in forming connections that promote synapse formation in glutamatergic neurons (Cagle and Honig, 2014; Ito-Ishida et al., 2012). Thus, genes activated by Ascl1, but repressed by Ptf1a, define a subset of excitatory neurons in the dorsal spinal cord (Fig. 3).

Homeodomain neuronal specification factors are direct downstream targets of Ascl1 and Ptf1a

Direct downstream targets of Ascl1 and Ptf1a are enriched in genes that influence neuronal subtype specification. We define direct downstream targets here as genes that (1) show a significant change of expression between controls and mutants, and (2) have an Ascl1- or Ptf1a-binding site within the regulatory region of the gene, as identified by the GREAT algorithm (McLean et al., 2010). We identified 449 putative targets regulated directly by Ascl1 (224 activated and 225 repressed). For Ptf1a, 207 putative targets were identified (101 activated and 106 repressed) (Fig. 3R). The high number of repressed genes predicted in this analysis was unexpected, as Ascl1 and Ptf1a are primarily known as transcriptional activators.

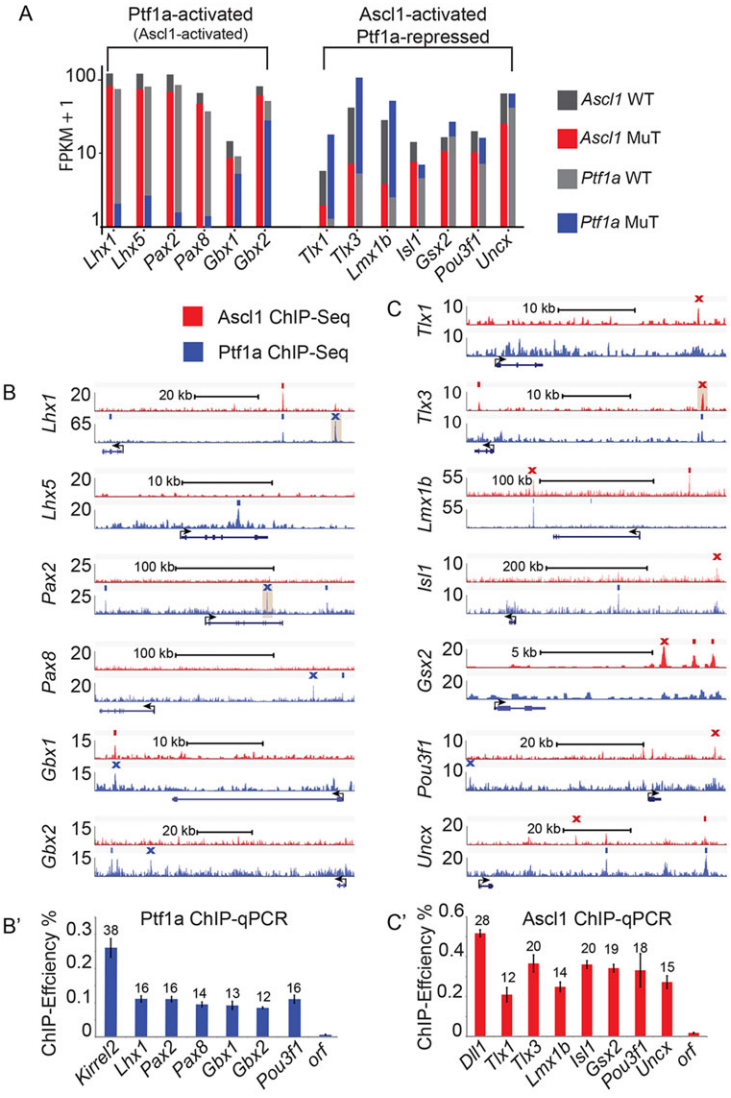

Fig. 4. Ascl1 and Ptf1a directly regulate the homeodomain factors involved in neuronal specification. (A) Expression levels in dorsal neural tubes of *Ascl1* or *Ptf1a* control and mutant embryos of the HD factor genes that are directly regulated by Ptf1a (left panel) or by Ascl1 (right panel). *y*-axis is in $\log_{10}$ scale. Directly activated targets of Ptf1a partially depend on Ascl1 expression, whereas direct targets of Ascl1 are repressed by Ptf1a. All changes in gene expression are significant (*P*-value <0.5), except *Pax8* and *Gbx2* in the *Ascl1* null, and *Isl1* and *Gsx2* in the *Ptf1a* null. (B,C) Genomic regions around Ptf1a targets (B) or Ascl1 targets (C) displaying Ascl1 (red track) and Ptf1a (blue track) ChIP-Seq data. Binding sites called by Homer are indicated by tick marks or an x (validated by qPCR) above each track. Beige box marks sequences previously shown to drive dorsal neural tube expression in transgenic animals (Chang et al., 2013; Meredith et al., 2013). (B′,C′) ChIP-qPCR validating several Ptf1a- and Ascl1-binding sites found in the ChIP-Seq assay. *Kirrel2* and *Dll1* are known targets of Ptf1a and Ascl1 (Castro et al., 2006; Henke et al., 2009; Meredith et al., 2013). The number above each bar indicates the fold of enrichment found at that site over the negative control (*Kirrel2* open reading frame, orf).

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

We looked specifically for HD factor genes as direct targets of the bHLH factors. We found that Ptf1a directly activates a set of HD factor-encoding genes: *Pax2*, *Lhx1*, *Pax8*, *Lhx5*, *Gbx1* and *Gbx2* (Fig. 4A-B′; supplementary material Table S3). Most of these are involved in development of the dorsal horn spinal cord GABAergic neurons (John et al., 2005; Luu et al., 2011; Pillai et al., 2007). By contrast, direct targets of Ascl1 comprise a different set of HD factor genes: *Tlx3*, *Tlx1*, *Lmx1b*, *Isl1*, *Gsx2*, Pou3f1 and Uncx (Fig. 4A,C,C′; supplementary material Table S3); some of which contribute to the proper development of dorsal horn spinal cord glutamatergic neurons (Avraham et al., 2010; Ding et al., 2004; Mizuguchi et al., 2006; Zou et al., 2012). Most of these Ascl1- and Ptf1a-binding sites identified by ChIP-Seq near the HD factor genes were validated by ChIP-qPCR (Fig. 4B′,C′; see supplementary material Fig. S4). Genomic regions containing the Ptf1a sites near *Lhx1* and *Pax2*, and the Ascl1 binding site near *Tlx3* (Fig. 4B,C, peaks highlighted in beige), drive reporter activity in the dorsal neural tube in transgenic animals (Chang et al., 2013; Meredith et al., 2013), thereby validating their function.

It is striking that the HD factor genes directly activated by Ascl1 are defined as repressed by Ptf1a (Fig. 4A). The repression by Ptf1a probably involves both direct and indirect mechanisms: there is no binding of Ptf1a near genes such as *Tlx1* and *Gsx2* (suggesting indirect mechanisms), but Ptf1a is found near *Tlx3*, *Isl1*, *Lmx1b*, *Uncx* and *Pou3f1* (suggesting direct mechanisms). Thus, Ascl1 directly activates a distinct set of HD factor genes for specifying the glutamatergic lineage, and Ptf1a appears to directly and indirectly repress this set of genes. Taken together, these findings indicate that the bHLH factors Ascl1 and Ptf1a are at the head of a transcription factor network controlling distinct subsets of HD factor genes necessary for the generation of excitatory and inhibitory neurons in the dorsal spinal cord.

Ascl1 directly regulates genes involved in multiple processes of neurogenesis

Like other proneural bHLH factors, Ascl1 coordinates the transition from a neural progenitor cell to a differentiated neuron, which is reflected in the Gene Ontology (GO) analysis of Ascl1 targets (Bertrand et al., 2002; Wang et al., 2013) (Fig. 5; supplementary material Table S5). This cellular transition requires several processes to occur within a short window of time, such as cell-cycle exit, cell migration and cell type-specific gene expression. Indeed, Ascl1 directly regulates differentiation and specification not only through the HD factors, but through a larger complement of TFs (31 out of 224 activated target genes are classified as encoding proteins with transcription factor activity; Fig. 5C). Ascl1 also targets components of the Notch signaling pathway, such as *Dll1*, *Dll3*, *Mfng*, *Numbl* and *Hes5*. Additional aspects of neurogenesis regulated by Ascl1 include genes involved in neuronal projection and axon guidance, such as *Nfasc*, *Epha2*, *Ephb3*, *Sema7a*, *Sema6b*, *Dcc*, *Plxna2*, *Pak3*, *Rgs3* and *Slit*. Moreover, Ascl1 regulates several genes found in synaptic terminals that regulate neurotransmitter release, such as *Snap25* and *Syt6*. Notably, Ascl1 does not directly regulate genes involved in generating or transporting the neurotransmitter glutamate, and unlike the reported function for Ascl1 in the developing telencephalon (Castro et al., 2011), in the caudal neural tube Ascl1 does not activate many genes associated with proliferation.

Although Ascl1 is primarily known as an activator of transcription, it appears to promote differentiation by repressing neural stem maintenance genes, such as *Sox2* and *Pax3* (Nakazaki et al., 2008; Pevny and Nicolis, 2010). In addition, Ascl1 represses

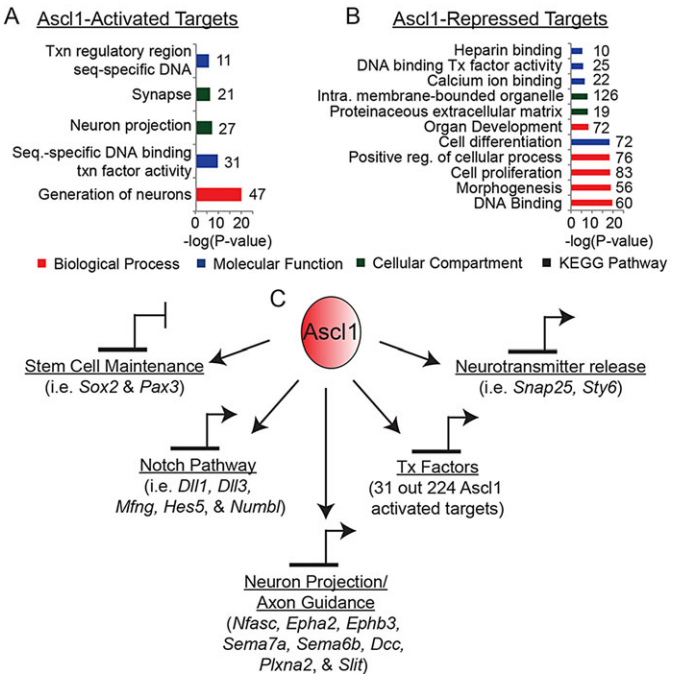

Fig. 5. Ascl1 directly regulates several components of the neurogenic program in the developing spinal cord. (A,B) GO/KEGG of direct Ascl1-activated (A) or Ascl1-repressed (B) targets. The colors denote the GO category or KEGG pathway the terms were derived from, and the adjacent numbers indicate the number of target genes in that category. (C) Summary of Ascl1 gene targets involved in different steps of the neurogenic program. For a complete list of Ascl1 target genes see supplementary material Table S3.

genes that function in early dorsal-ventral patterning of the spinal cord, such as *Wnt1* (Augustine et al., 1995; Lee and Jessell, 1999) and the sonic hedgehog pathway gene *Gli3* (supplementary material Table S5) (Persson et al., 2002). Ascl1 also represses several genes involved broadly in differentiation of non-neuronal lineages. The mechanisms by which Ascl1 represses transcription are currently not yet understood.

Ptf1a represses the glutamatergic fate and directly upregulates components of the GABAergic machinery

Similar to Ascl1, Ptf1a turns on a cascade of TFs that function in neuronal differentiation and specification (Fig. 6A,E). Approximately a quarter (24 out of 101) of Ptf1a-activated targets have transcriptional activity. Ptf1a directly activates neural genes that are specific to the GABAergic program, including genes encoding the TFs Prdm13, Lhx1, Lhx5 and Pax2, which are important for maturation of these neurons (Chang et al., 2013; Pillai et al., 2007). Nine of the target genes encode synaptic proteins, such as Sv2c (Fig. 6D), Sez6 and Iqsec3, which have all been shown to localize specifically at inhibitory synapses (Fukaya et al., 2011; Gronborg et al., 2010; Gunnersen et al., 2009). By contrast to Ascl1, Ptf1a activates genes involved in GABA biosynthesis and transport pathways, such as *Gad1* (GAD67), *Abat* (GABA transaminase), *Slc32a1* (Viaat) and *Slc6a5* (Glyt2) (Fig. 6C-E). Additional Ptf1a-regulated genes contribute to the extracellular matrix and to cell adhesion, such as *Adamts4*/*20* and *Adamtsl1*, *Nrxn1*, *Vcan*, *Gpc3/4*, *Ccbe1*, *Nphs* and *Kirrel2*, or encode subunits of voltage-gated calcium channels, such as *Cacna2d2*, *Cacna2d3* and *Cacna1g*.

Ptf1a not only activates genes necessary for the GABAergic lineage, but also represses genes involved in the glutamatergic fate. Approximately one-third (33 out of 106) of the genes repressed by

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

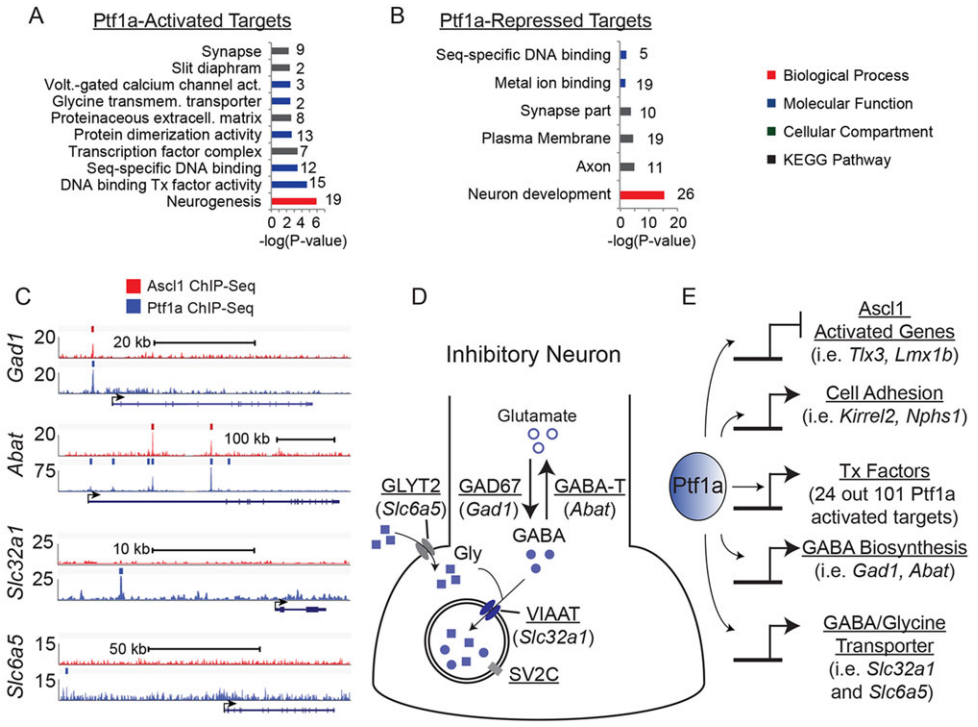

Fig. 6. Ptf1a directly opposes Ascl1 target genes and activates several components of the GABAergic machinery. (A,B) GO/KEGG of Ptf1a-activated (A) or Ptf1a-repressed (B) targets The colors denote the GO category or KEGG pathway the terms were derived from, and the adjacent numbers indicate the number of target genes in that category. (C) ChIP-Seq around Ptf1a target genes involved in GABA biosynthesis and GABA/glycine transport. Binding sites called by Homer are indicated by tick marks above each track. (D) Ptf1a target genes that are specifically found in inhibitory presynaptic terminals (in parentheses). (E) Summary of Ptf1a gene targets involved in different steps of inhibitory neuron specification. For a complete list of Ptf1a target genes see supplementary material Table S3.

Ptf1a are directly activated by Ascl1, notably HD factors Tlx3 and Lmx1b (Fig. 4; supplementary material Table S3). Ptf1a has recently been shown to indirectly repress the glutamatergic fate through the activation of the target gene *Prdm13* that interacts with Ascl1 to block its transcriptional activity (Chang et al., 2013). Thus, inhibition of Ascl1 targets by Ptf1a could be explained through this indirect mechanism. However, the ChIP-Seq revealed that Ascl1 and Ptf1a commonly occupy the same genomic regions around 22 of 33 Ascl1-activated, Ptf1a-repressed genes, suggesting Ptf1a repression of the glutamatergic fate might also involve direct binding by Ptf1a.

DISCUSSION

Over two decades of research have established the genetic requirement for neural bHLH TFs in generating the correct number and composition of neurons in the central nervous system. However, a mechanistic understanding of bHLH factor function is only just emerging, as transcriptional targets are beginning to be identified through genome-wide strategies utilizing ChIP-Seq. In this study, we identify numerous direct transcriptional targets of two bHLH factors in the mouse dorsal neural tube. Ascl1 and Ptf1a provide an informative model pair of factors for uncovering how two related TFs function to specify distinct cell fates from a common progenitor domain. We show that Ascl1 and Ptf1a directly activate sets of genes encoding HD factors that are known specifiers of the glutamatergic or GABAergic neuronal fates, respectively. In addition, Ptf1a represses many of the Ascl1-activated HD genes, ensuring that the glutamatergic phenotype is repressed in GABAergic neurons. Furthermore, Ascl1 targets genes early in the differentiation process, whereas Ptf1a targets genes required for the activity of GABAergic neurons, thus reflecting the temporal difference in their expression and the fundamental differences in their function. The list of target genes for Ptf1a and Ascl1 in the developing neural tube generated by this study will serve as a rich dataset for further probing the functions of these essential TFs (supplementary material Table S3).

Multiple mechanisms for crossrepression of Ascl1 and Ptf1a in neuronal subtype specification

Through identifying HD factor genes as direct targets of Ascl1 and Ptf1a, our current studies have provided mechanistic insights into fundamental processes of cell fate determination, specifically those generating the balance of excitatory and inhibitory neurons in the dorsal spinal cord. Experiments testing the function of Ascl1 and Ptf1a have shown that each will induce one cell fate while suppressing the other (Chang et al., 2013; Glasgow et al., 2005; Helms et al., 2005; Hori et al., 2008). Because Ascl1 and Ptf1a are known as transcriptional activators, identifying the HD genes that function in specifying the relevant neurons as direct targets of these bHLH factors provides a simple model for generation of the excitatory and inhibitory neurons, respectively. However, it is much less clear how the alternative cell fates are repressed. Our results provide multiple insights into possible mechanisms for this crossrepression.

Understanding how Ascl1 can repress the Pax2-defined GABAergic lineages stems from our data showing that Ascl1 directly activates *Tlx1* and *Tlx3*, and from previous studies that demonstrated the crossrepression between the Tlx factors and *Pax2* (Cheng et al., 2005). Tlx1 or Tlx3 suppress the GABAergic fate by antagonizing the ability of the HD factor Lbx1 to induce *Pax2* expression (see diagram Fig. 1C). Thus, the higher the levels of Tlx1 and Tlx3 driven by Ascl1, the lower the levels of Pax2. Consistent with this indirect mechanism for Ascl1 suppression of the Pax2 GABAergic lineage, Ascl1 did not directly suppress Ptf1a-activated target genes, as determined by ChIP-Seq. Instead, we found that several of the Ptf1a-activated genes were also activated by Ascl1. Taken together, it appears that Ascl1 activates the glutamatergic lineage through direct regulation of HD factors such as the *Tlx1/3* genes, but only indirectly represses the opposing lineage.

By contrast, Ptf1a appears to use both direct and indirect mechanisms for suppressing the glutamatergic neuronal fate. We recently identified *Prdm13* as a direct downstream target of Ptf1a that provides an indirect mechanism for Ptf1a suppression of the glutamatergic fate (Chang et al., 2013). In this case Prdm13,

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

induced by Ptf1a, directly suppresses Ascl1 activity in inducing its targets, such as *Tlx1* and *Tlx3* (see diagram Fig. 1C). In addition, we show here that Ptf1a may suppress several Ascl1 targets directly, although the specific mechanism used is unclear. In several cases, Ptf1a binds near Ascl1-activated genes through sites that do not overlap with Ascl1 sites (i.e. *Tlx3*, *Uncx* and *Pou3f1*). This would suggest a mechanism by which Ptf1a recruits a transcriptional repressor and blocks Ascl1 activity from a distant site; a capability that has never been shown for Ptf1a. In other cases, such as binding around *Lmx1b*, Ascl1 and Ptf1a locate to the same genomic site. This suggests that competition might occur between Ptf1a and Ascl1. Consistent with this possibility, Ptf1a has been found to suppress the ability of Ascl1 to activate an E-box reporter in a transcription assay (Obata et al., 2001). Given that Ptf1a and Ascl1 can bind a common CAGCTG E-box *in vivo*, Ptf1a could compete with Ascl1 for these E-boxes and passively block Ascl1 activity, as the Ptf1a/E-protein heterodimer is a poor activator (Beres et al., 2006). Taken together, Ptf1a may deploy several mechanisms to ensure the proper repression of a subset of Ascl1 target genes, thus resulting in repression of glutamatergic lineage genes in the GABAergic neurons.

Ascl1 and Ptf1a bind neuronal enhancers enriched with specific transcription factor motifs

Ptf1a- and Ascl1-bound genomic regions *in vivo* are enriched with multiple transcription factor family motifs, such as Sox, Pou, HD and Rfx. Members of these families have been shown to play important roles during neuronal development (Ashique et al., 2009; Bergsland et al., 2011; Castro et al., 2006; Lodato et al., 2013). In addition, *de novo* motif analysis of Sox2 and Brn2 (a Pou factor) ChIP-Seq regions from cultured neural progenitor cells found enrichment of the same set of motifs (Sox, Pou, HD, Rfx and E-box) plus NFI (Lodato et al., 2013). Thus, combinations of these motifs are emerging as indicators of a possible neural specific enhancer. Different motif combinations have been reported in other cell lineages, such as for macrophage-specific enhancers (Pu.1, C/EBP and AP1) (Heinz et al., 2010, 2013) or for muscle lineage enhancers (E-box, AP1, Meis, Runx and SP1) (Cao et al., 2010). We found no significant space constraints between the DNA recognition motifs across 150 bp, consistent with a presumptive collaborative binding to select enhancers that would not necessarily require direct protein-protein interactions between the different TFs. We tested the requirement for the E-box and Sox motifs in active enhancers bound by Ascl1 and Ptf1a, and found that their presence was necessary in one case but in not the other. This illustrates our inability to predict a motif required for enhancer activity in reporter assays, and highlights the need for additional approaches to test the importance of any given transcription factor motif. For example, the motif may have been used to open or close chromatin at different stages within the lineage progression than the stage being tested in a reporter assay (Heinz et al., 2010; Stergachis et al., 2013).

Ptf1a binds a distinct E-box to regulate its specification program

Ascl1 and Ptf1a ChIP-Seq data have provided valuable insight into how two bHLH factors can regulate select target genes. Motif analysis from these data shows that Ptf1a binds a common E-box with Ascl1 (CAGCTG), but also has a preference for a distinct E-Box (CATCTG/CAGATG). Other genome-wide studies have shown that bHLH factors, such as Atoh1 and Neurod2, have an E-box preference similar to Ptf1a (GC and TC/GA cores) (Fong et al., 2012; Klisch et al., 2011; Lai et al., 2011). Interestingly, E-box preferences for a specific bHLH factor can change depending on the cell context; for example, Ptf1a in the developing pancreas preferentially binds E-boxes with GC and GG/CC cores, but not the TC/GA core preference found in the neural tube (Meredith et al., 2013). Several studies have suggested that E-box binding by bHLH factors is heavily dictated by the availability of the site; the chromatin landscape of each tissue type would thus strongly influence the target selection of a transcription factor (Fong et al., 2012; Meredith et al., 2013). In this study, both Ascl1 and Ptf1a are functioning in a similar chromatin landscape, yet sequence preferences were still detected. Examination of the genes near Ptf1a binding sites that contain just the specific GA/TC core E-box alone revealed 41 out of 101 Ptf1a-activated genes, and include known specification factors, such as *Pax2* (Fig. 2E), *Pax8*, *Lhx5*, *Gbx2* and *Prdm13*. Thus, Ptf1a preference for a specific E-box, distinct from Ascl1, is probably an important mechanism in activating genes for the GABAergic neuronal identity and function.

MATERIALS AND METHODS

Mouse strains

Ptf1a^{Cre} (*p48*^{Cre}) was used as the *Ptf1a* null (Kawaguchi et al., 2002); *12.4Ptf1a::mCherry* transgenic mice were used for isolation of *Ptf1a* lineage cells from E11.5 WT or *Ptf1a* null neural tubes (Meredith et al., 2009). *Ascl1*^{GFP} (*Ascl1*^{tm1Reed}/J) (Leung et al., 2007) was used for isolation of *Ascl1* lineage cells from E11.5 control or *Ascl1* null (Guillemot et al., 1993) neural tubes. PCR genotyping was performed as described (Glasgow et al., 2005; Kim et al., 2007; Meredith et al., 2009). All procedures on animals follow NIH Guidelines and were approved by the UT Southwestern Institutional Animal Care and Use Committee.

In ovo chick electroporation and GFP measurement

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

Immunofluorescence and tissue processing

Mouse E11.5 embryos and chick HH24-25 were processed as previously described (Chang et al., 2013). Immunofluorescence was performed using the following primary antibodies: rabbit anti-Ascl1 [1:10,000; J.E.J. group, generated from bacterially expressed rat Ascl1 (aa 1-232)], guinea pig anti-Ptf1a (1:5000; J.E.J. group-generated) (Hori et al., 2008), rabbit anti-Pax2 (1:1000; Life Technologies, 71-6000), rabbit anti-Tlx1/3 (1:20,000; gift from T. Müller and C. Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), mouse anti-Lhx1/5 (1:100; Developmental Studies Hybridoma Bank, 4F2) and mouse anti-myc (1:100, ATCC, CRL-1729). Fluorescence imaging of upper limb level sections was carried out on a Zeiss LSM 510 confocal microscope.

ChIP-Seq

Detailed descriptions of Ptf1a, Rbpj (Meredith et al., 2013) and Ascl1 (Castro et al., 2011) E12.5 NT ChIP protocols have previously been published. Ptf1a antibody (5 µg; Santa Cruz, sc-69320X) was used for ChIP-qPCR validation. All ChIP-Seq samples are available on the GEO database. For the Ptf1a ChIP-Seq sample (GSM1150324), we used the telencephalon Ptf1a ChIP-Seq sample (GSM1347011) as control, as the telencephalon is a neural tissue of similar developmental stage that does not express Ptf1a. Both Rbpj (GSM1150327) and Ascl1 (GSM1347006) ChIP-Seq samples were compared with their respective E12.5 neural tube input (GSM1150340 and GSM1347007).

Sequence reads were mapped to the mm9 genome assembly with Bowtie (Trapnell et al., 2009). Only unique reads were included and were

Development (2014) 141, 2803-2812 doi:10.1242/dev.105866

normalized to 10 million reads. Peak calling was performed by HOMER (annotatePeaks.pl - size 5000 -hist 10 -ghist) (Heinz et al., 2010) using an FDR cutoff of 0.001. An additional cutoff of a cumulative Poisson *P*-value of <0.0001 and a 4-fold enrichment of normalized sequenced reads in the treatment sample over the control/input sample were used. A common binding site between two samples was called when the peak summits of each sample were found within 150 bp of each other.

mRNA isolation and sequencing (RNA-Seq)

Neural tubes from the *12.4kbPtf1a::mCherry;Ptf1a*$^{Cre/+}$, *12.4kbPtf1a::mCherry;Ptf1a*$^{Cre/Cre}$, *Ascl1*$^{GFP/+}$ or *Ascl1*$^{GFP/-}$ lines were dissociated in DMEM/F12 with 0.25% trypsin, and GFP or mCherry positive cells were purified by FACS. Total RNA was purified with a Mini RNA Isolation Kit (Zymo). An mRNA-Seq kit (Illumina) was used for mRNA (polyA) isolation and sequencing library preparation.

RNA-Seq data are available on the GEO database (GSE55831). Sequence reads were aligned to the mm9 genome assembly using TopHat v2.0.9 (Trapnell et al., 2009). All default settings were used except '-G option' and '–no-novel-juncs'. If a biological replicate was available, it was specified and used to build an expression level model determined by the FPKM method of Cuffdiff v2.1.1 (Trapnell et al., 2013, 2010). The options used were multiple read correction (-u) and the bias correction (-b). A gene was considered to be expressed if it had an FPKM >1. For a gene to be called as differentially expressed, it required a *P*-value <0.05. Scatter plots and expression bar plots were created by Cummerbund (Trapnell et al., 2012).

GO classification and ChIP-Seq peak gene annotation

Distance to gene and gene annotations for ChIP-Seq peaks were obtained using GREAT v1.82 (McLean et al., 2010). GREAT assigns a gene to a binding region if the region falls within 5 kb 5′ or 1 kb 3′ of the transcription start site (basal region), with a maximum extension of 1000 kb in either direction. If the binding region falls within the basal region of multiple genes, then more than one assignment is made. All parameters were left at their default settings. Webgestalt (Wang et al., 2013) at default settings was used for GO and KEGG pathway analysis.

Motif discovery and density plots

All tests for motif discovery were conducted with the HOMER package v4.2, using the following settings: -size 150 – S 10 – bits using 150 bp around each peak summit (Heinz et al., 2010). All Ascl1- or Ptf1a-binding sites limited to 150 bp around each peak summit were used for *de novo* analysis. HOMER uses a hypergeometric statistical analysis to determine motif enrichment, using a random background sequence with similar GC content to the test sample. To find the Ascl1 or Ptf1a factor-specific motifs, all Ascl1 or Ptf1a peaks were used as the treatment, whereas the peak regions not being tested were specified as the background. When searching for transcription factor motifs, no mismatches to the motif matrix were allowed. The E-box and Rbpj motif density plots were generated in HOMER (annotatePeaks.pl –size 1000 –hist 10).

Acknowledgements

We thank Dr Hung-Chung Huang for assistance with the bioinformatics analysis and the University of Texas Southwestern Microarray Core facility, particularly Chaoying Liang, for her technical assistance with the next-generation sequencing.

Competing interests

The authors declare no competing financial interests.

Author contributions

M.D.B., D.M.M. and J.E.J. initiated the project. M.D.B., D.M.M. and J.C.C. performed experiments. D.S.C. and F.G. provided Ascl1 ChIP-Seq data and experimental insight. K.C.T. provided bioinformatics analysis; M.D.B. prepared the manuscript together with J.E.J. All authors provided scientific insight and edited the manuscript.

Funding

This work was supported by grants from the National Institutes of Health [F31 NS705592 to M.D.B., R01 HD037932 and R01 NS032817 to J.E.J. and F31 NS061440 to D.M.M.]. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105866/-/DC1

References

Ashique, A. M., Choe, Y., Karlen, M., May, S. R., Phamluong, K., Solloway, M. J., Ericson, J. and Peterson, A. S. (2009). The Rfx4 transcription factor modulates Shh signaling by regional control of ciliogenesis. *Sci. Signal.* **2**, ra70.

Augustine, K. A., Liu, E. T. and Sadler, T. W. (1995). Interactions of Wnt-1 and Wnt-3a are essential for neural tube patterning. *Teratology* **51**, 107-119.

Avraham, O., Hadas, Y., Vald, L., Hong, S., Song, M.-R. and Klar, A. (2010). Motor and dorsal root ganglion axons serve as choice points for the ipsilateral turning of dI3 axons. *J. Neurosci.* **30**, 15546-15557.

Batista, M. F. and Lewis, K. E. (2008). Pax2/8 act redundantly to specify glycinergic and GABAergic fates of multiple spinal interneurons. *Dev. Biol.* **323**, 88-97.

Beres, T. M., Masui, T., Swift, G. H., Shi, L., Henke, R. M. and MacDonald, R. J. (2006). PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. *Mol. Cell. Biol.* **26**, 117-130.

Bergsland, M., Ramskold, D., Zaouter, C., Klum, S., Sandberg, R. and Muhr, J. (2011). Sequentially acting Sox transcription factors in neural lineage development. *Genes Dev.* **25**, 2453-2464.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Cagle, M. C. and Honig, M. G. (2014). Parcellation of cerebellins 1, 2, and 4 among different subpopulations of dorsal horn neurons in mouse spinal cord. *J. Comp. Neurol.* **522**, 479-497.

Cao, Y., Yao, Z., Sarkar, D., Lawrence, M., Sanchez, G. J., Parker, M. H., MacQuarrie, K. L., Davison, J., Morgan, M. T., Ruzzo, W. L. et al. (2010). Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming. *Dev. Cell* **18**, 662-674.

Castro, D. S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I. J., Parras, C., Hunt, C., Critchley, J. A., Nguyen, L., Gossler, A., Göttgens, B. et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. *Dev. Cell* **11**, 831-844.

Castro, D. S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., Drechsel, D., Lebel-Potter, M., Garcia, L. G., Hunt, C. et al. (2011). A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes Dev.* **25**, 930-945.

Chang, J. C., Meredith, D. M., Mayer, P. R., Borromeo, M. D., Lai, H. C., Ou, Y.-H. and Johnson, J. E. (2013). Prdm13 mediates the balance of inhibitory and excitatory neurons in somatosensory circuits. *Dev. Cell* **25**, 182-195.

Cheng, L., Arata, A., Mizuguchi, R., Qian, Y., Karunaratne, A., Gray, P. A., Arata, S., Shirasawa, S., Bouchard, M., Luo, P. et al. (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. *Nat. Neurosci.* **7**, 510-517.

Cheng, L., Samad, O. A., Xu, Y., Mizuguchi, R., Luo, P., Shirasawa, S., Goulding, M. and Ma, Q. (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. *Nat. Neurosci.* **8**, 1510-1515.

Ding, Y.-Q., Yin, J., Kania, A., Zhao, Z.-Q., Johnson, R. L. and Chen, Z.-F. (2004). Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. *Development* **131**, 3693-3703.

Fong, A. P., Yao, Z., Zhong, J. W., Cao, Y., Ruzzo, W. L., Gentleman, R. C. and Tapscott, S. J. (2012). Genetic and epigenetic determinants of neurogenesis and myogenesis. *Dev. Cell* **22**, 721-735.

Fukaya, M., Kamata, A., Hara, Y., Tamaki, H., Katsumata, O., Ito, N., Takeda, S., Hata, Y., Suzuki, T., Watanabe, M. et al. (2011). SynArfGEF is a guanine nucleotide exchange factor for Arf6 and localizes preferentially at post-synaptic specializations of inhibitory synapses. *J. Neurochem.* **116**, 1122-1137.

Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V. E. and Johnson, J. E. (2005). Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. *Development* **132**, 5461-5469.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-232.

Gronborg, M., Pavlos, N. J., Brunk, I., Chua, J. J. E., Munster-Wandowski, A., Riedel, D., Ahnert-Hilger, G., Urlaub, H. and Jahn, R. (2010). Quantitative comparison of glutamatergic and GABAergic synaptic vesicles unveils selectivity for few proteins including MAL2, a novel synaptic vesicle protein. *J. Neurosci.* **30**, 2-12.

Gross, M. K., Dottori, M. and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* **34**, 535-549.

Guillemot, F., Lo, L.-C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463-476.

Gunnersen, J. M., Kuek, A., Phipps, J. A., Hammond, V. E., Puthussery, T., Fletcher, E. L. and Tan, S.-S. (2009). Seizure-related gene 6 (Sez-6) in amacrine cells of the rodent retina and the consequence of gene deletion. *PLoS ONE* **4**, e6546.

Hamburger, V. and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. *Dev. Dyn.* **195**, 231-272.
Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C., Singh, H. and Glass, C. K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* **38**, 576-589.
Heinz, S., Romanoski, C. E., Benner, C., Allison, K. A., Kaikkonen, M. U., Orozco, L. D. and Glass, C. K. (2013). Effect of natural genetic variation on enhancer selection and function. *Nature* **503**, 487-492.
Helms, A. W., Battiste, J., Henke, R. M., Nakada, Y., Simplicio, N., Guillemot, F. and Johnson, J. E. (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. *Development* **132**, 2709-2719.
Henke, R. M., Meredith, D. M., Borromeo, M. D., Savage, T. K. and Johnson, J. E. (2009). Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. *Dev. Biol.* **328**, 529-540.
Hori, K., Cholewa-Waclaw, J., Nakada, Y., Glasgow, S. M., Masui, T., Henke, R. M., Wildner, H., Martarelli, B., Beres, T. M., Epstein, J. A. et al. (2008). A nonclassical bHLH Rbpj transcription factor complex is required for specification of GABAergic neurons independent of Notch signaling. *Genes Dev.* **22**, 166-178.
Huang, M., Huang, T., Xiang, Y., Xie, Z., Chen, Y., Yan, R., Xu, J. and Cheng, L. (2008). Ptf1a, Lbx1 and Pax2 coordinate glycinergic and peptidergic transmitter phenotypes in dorsal spinal inhibitory neurons. *Dev. Biol.* **322**, 394-405.
Ito-Ishida, A., Miyazaki, T., Miura, E., Matsuda, K., Watanabe, M., Yuzaki, M. and Okabe, S. (2012). Presynaptically released Cbln1 induces dynamic axonal structural changes by interacting with GluD2 during cerebellar synapse formation. *Neuron* **76**, 549-564.
John, A., Wildner, H. and Britsch, S. (2005). The homeodomain transcription factor Gbx1 identifies a subpopulation of late-born GABAergic interneurons in the developing dorsal spinal cord. *Dev. Dyn.* **234**, 767-771.
Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J. and Wright, C. V. E. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat. Genet.* **32**, 128-134.
Kim, E. J., Leung, C. T., Reed, R. R. and Johnson, J. E. (2007). In vivo analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis. *J. Neurosci.* **27**, 12764-12774.
Klisch, T. J., Xi, Y., Flora, A., Wang, L., Li, W. and Zoghbi, H. Y. (2011). In vivo Atoh1 targetome reveals how a proneural transcription factor regulates cerebellar development. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 3288-3293.
Lai, H. C., Klisch, T. J., Roberts, R., Zoghbi, H. Y. and Johnson, J. E. (2011). In vivo neuronal subtype-specific targets of Atoh1 (Math1) in dorsal spinal cord. *J. Neurosci.* **31**, 10859-10871.
Lai, H. C., Meredith, D. M. and Johnson, J. E. (2013). bHLH Factors in neurogenesis and neuronal subtype specification. In *Comprehensive Developmental Neuroscience: Patterning and Cell Type Specification in the Developing CNS and PNS* (ed J. Rubenstein and P. Rakic), pp. 333-354. Amsterdam: Elsevier.
Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.
Leung, C. T., Coulombe, P. A. and Reed, R. R. (2007). Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nat. Neurosci.* **10**, 720-726.
Liu, Y. and Ma, Q. (2011). Generation of somatic sensory neuron diversity and implications on sensory coding. *Curr. Opin. Neurobiol.* **21**, 52-60.
Lodato, M. A., Ng, C. W., Wamstad, J. A., Cheng, A. W., Thai, K. K., Fraenkel, E., Jaenisch, R. and Boyer, L. A. (2013). SOX2 co-occupies distal enhancer elements with distinct POU factors in ESCs and NPCs to specify cell state. *PLoS Genet.* **9**, e1003288.
Luu, B., Ellisor, D. and Zervas, M. (2011). The lineage contribution and role of Gbx2 in spinal cord development. *PLoS ONE* **6**, e20940.
Masui, T., Swift, G. H., Hale, M. A., Meredith, D. M., Johnson, J. E. and Macdonald, R. J. (2008). Transcriptional autoregulation controls pancreatic Ptf1a expression during development and adulthood. *Mol. Cell. Biol.* **28**, 5458-5468.
McLean, C. Y., Bristor, D., Hiller, M., Clarke, S. L., Schaar, B. T., Lowe, C. B., Wenger, A. M. and Bejerano, G. (2010). GREAT improves functional interpretation of cis-regulatory regions. *Nat. Biotechnol.* **28**, 495-501.
Meredith, D. M., Masui, T., Swift, G. H., MacDonald, R. J. and Johnson, J. E. (2009). Multiple transcriptional mechanisms control Ptf1a levels during neural development including autoregulation by the PTF1-J complex. *J. Neurosci.* **29**, 11139-11148.
Meredith, D. M., Borromeo, M. D., Deering, T. G., Casey, B. H., Savage, T. K., Mayer, P. R., Hoang, C., Tung, K.-C., Kumar, M., Shen, C. et al. (2013). Program specificity for Ptf1a in Pancreas versus Neural Tube Development correlates with distinct collaborating cofactors and chromatin accessibility. *Mol. Cell. Biol.* **33**, 3166-3179.
Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q. and Goulding, M. (2006). Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. *Nat. Neurosci.* **9**, 770-778.
Müller, T., Anlag, K., Wildner, H., Britsch, S., Treier, M. and Birchmeier, C. (2005). The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. *Genes Dev.* **19**, 733-743.
Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B. et al. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.
Nakada, Y., Hunsaker, T. L., Henke, R. M. and Johnson, J. E. (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification. *Development* **131**, 1319-1330.
Nakazaki, H., Reddy, A. C., Mania-Farnell, B. L., Shen, Y.-W., Ichi, S., McCabe, C., George, D., McLone, D. G., Tomita, T. and Mayanil, C. S. K. (2008). Key basic helix-loop-helix transcription factor genes Hes1 and Ngn2 are regulated by Pax3 during mouse embryonic development. *Dev. Biol.* **316**, 510-523.
Obata, J., Yano, M., Mimura, H., Goto, T., Nakayama, R., Mibu, Y., Oka, C. and Kawaichi, M. (2001). p48 subunit of mouse PTF1 binds to RBP-Jkappa/CBF-1, the intracellular mediator of Notch signalling, and is expressed in the neural tube of early stage embryos. *Genes Cells* **6**, 345-360.
Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Böse, J., Rüther, U., Ericson, J. and Briscoe, J. (2002). Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. *Genes Dev.* **16**, 2865-2878.
Pevny, L. H. and Nicolis, S. K. (2010). Sox2 roles in neural stem cells. *Int. J. Biochem. Cell Biol.* **42**, 421-424.
Pillai, A., Mansouri, A., Behringer, R., Westphal, H. and Goulding, M. (2007). Lhx1 and Lhx5 maintain the inhibitory-neurotransmitter status of interneurons in the dorsal spinal cord. *Development* **134**, 357-366.
Ross, S. E. (2011). Pain and itch: insights into the neural circuits of aversive somatosensation in health and disease. *Curr. Opin. Neurobiol.* **21**, 880-887.
Seo, S., Lim, J.-W., Yellajoshyula, D., Chang, L.-W. and Kroll, K. L. (2007). Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. *EMBO J.* **26**, 5093-5108.
Stergachis, A. B., Neph, S., Reynolds, A., Humbert, R., Miller, B., Paige, S. L., Vernot, B., Cheng, J. B., Thurman, R. E., Sandstrom, R. et al. (2013). Developmental fate and cellular maturity encoded in human regulatory DNA landscapes. *Cell* **154**, 888-903.
Sun, W., Hu, X., Lim, M. H. K., Ng, C. K. L., Choo, S. H., Castro, D. S., Drechsel, D., Guillemot, F., Kolatkar, P. R., Jauch, R. et al. (2013). TherMos: estimating protein-DNA binding energies from in vivo binding profiles. *Nucleic Acids Res.* **41**, 5555-5568.
Timmer, J., Johnson, J. and Niswander, L. (2001). The use of in ovo electroporation for the rapid analysis of neural-specific murine enhancers. *Genesis* **29**, 123-132.
Trapnell, C., Pachter, L. and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105-1111.
Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J. and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511-515.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L. and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562-578.
Trapnell, C., Hendrickson, D. G., Sauvageau, M., Goff, L., Rinn, J. L. and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* **31**, 46-53.
Wang, J., Duncan, D., Shi, Z. and Zhang, B. (2013). WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res.* **41**, W77-W83.
Wildner, H., Müller, T., Cho, S.-H., Bröhl, D., Cepko, C. L., Guillemot, F. and Birchmeier, C. (2006). dILA neurons in the dorsal spinal cord are the product of terminal and non-terminal asymmetric progenitor cell divisions, and require Mash1 for their development. *Development* **133**, 2105-2113.
Zou, M., Li, S., Klein, W. H. and Xiang, M. (2012). Brn3a/Pou4f1 regulates dorsal root ganglion sensory neuron specification and axonal projection into the spinal cord. *Dev. Biol.* **364**, 114-127.