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Development/Plasticity/Repair

LHX2 Interacts with the NuRD Complex and Regulates Cortical Neuron Subtype Determinants Fezf2 and Sox11


In the developing cerebral cortex, sequential transcriptional programs take neuroepithelial cells from proliferating progenitors to differentiated neurons with unique molecular identities. The regulatory changes that occur in the chromatin of the progenitors are not well understood. During deep layer neurogenesis, we show that transcription factor LHX2 binds to distal regulatory elements of Fezf2 and Sox11, critical determinants of neuron subtype identity in the mouse neocortex. We demonstrate that LHX2 binds to the nucleosome remodeling and histone deacetylase histone remodeling complex subunits LSD1, HDAC2, and RBBP4, which are proximal regulators of the epigenetic state of chromatin. When LHX2 is absent, active histone marks at the Fezf2 and Sox11 loci are increased. Loss of LHX2 produces an increase, and overexpression of LHX2 causes a decrease, in layer 5 Fezf2 and CTIP2-expressing neurons. Our results provide mechanistic insight into how LHX2 acts as a necessary and sufficient regulator of genes that control cortical neuronal subtype identity.

Key words: cell fate; chromatin; epigenetics; lamination; progenitor; specification

Significance Statement

The functional complexity of the cerebral cortex arises from an array of distinct neuronal subtypes with unique connectivity patterns that are produced from common progenitors. This study reveals that transcription factor LHX2 regulates the numbers of specific cortical output neuron subtypes by controlling the genes that are required to produce them. Loss or increase in LHX2 during neurogenesis is sufficient to increase or decrease, respectively, a particular subcerebrally projecting population. Mechanistically, LHX2 interacts with chromatin modifying protein complexes to edit the chromatin landscape of its targets Fezf2 and Sox11, which regulates their expression and consequently the identities of the neurons produced. Thus, LHX2 is a key component of the control network for producing neurons that will participate in cortical circuitry.

Introduction

The complex functions of the cerebral cortex arise from circuitry in which distinct neuronal subtypes serve specific functions. The diversity of cortical neurons arises from initially common progenitors in the ventricular zone. Work from several groups has identified a complex network of transcriptional controls for the specification, differentiation, diversity, and plasticity of neuronal subtype identity (Arlotta et al., 2005; Woodworth et al., 2012; Greig et al., 2013). FEZF2 is necessary for the specification of subcerebral projection neurons (SCPN), the dominant population of layer 5 output neurons. It is sufficient for SCPN generation from cortical progenitors during development in vivo (Chen et al., 2005; Molyneaux et al., 2005). Further, SOX4, SOX5, SOX11, and TBR1 are upstream regulators of Fezf2 expression in...
the cerebral cortex, with the SOX factors binding at downstream enhancer element (E4) and TBR1 binding at 3′ noncoding region of Fezf2 (Kwan et al., 2008; Lai et al., 2008; Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011; Shim et al., 2012). It is increasingly understood that dynamic control over timing and levels of expression of key regulators is required to determine the generation of distinct neuronal subtypes in the cortical plate: SCPN in layer 5 versus corticothalamic projection neurons (CThPN) in layer 6 (Lai et al., 2008; Tomassy et al., 2010; Cederquist et al., 2013; Greig et al., 2013). Control of gene expression involves regulation of the chromatin at the relevant loci. However, our understanding of the dynamic chromatin regulation of gene loci involved in neuronal subtype specification remains inadequate. Such understanding is required to address the fundamental open question of how overlapping transcription factors expressed in the ventricular zone are translated into instructions to produce precise sets of neuronal subtypes in a temporal sequence. Here, we report a previously unknown role for the LIM-homeodomain transcription factor LHX2, as a key regulator of Fezf2 and Sox11 that is well positioned to function early in the cascade of mechanisms that specify SCPN identity.

LHX2 has a fundamental role in early cortical specification, in which it acts as a cortical selector gene (Mangale et al., 2008). However, Lhx2 continues to be expressed in the ventricular zone throughout the period of cortical neurogenesis and displays intriguing spatiotemporal dynamics (Bulchand et al., 2003). Whereas neurons of deep layers 5 and 6 rapidly repress Lhx2 expression, superficial layer neurons continue to express LHX2 from their birth dates through maturity (Bulchand et al., 2003). We hypothesized that the dynamic regulation of Lhx2 might be important for the production of particular neuronal subtypes that predominates in deep versus superficial layers.

In this paper, we report the first evidence that LHX2 regulates cortical neuronal subtype identity. Loss of LHX2 function causes a striking increase in the layer 5 neurons expressing high levels of Fezf2 and CTIP2, indicating SCPN fate (Arloleta et al., 2005; Molyneaux et al., 2005). It also causes a reduction in layer 6 neurons expressing TBR1 (predominantly CThPN fate) (Hevner et al., 2001; McKenna et al., 2011). We performed chromatin immunoprecipitation followed by sequencing (ChIP-Seq), and identified LHX2 occupancy on distal regulatory elements associated with Fezf2 and its regulator, Sox11. To elucidate the mechanisms by which LHX2 might regulate Fezf2 and Sox11, we performed protein IP followed by mass spectrometry and identified members of the nucleosome remodeling and histone deacetylase (NuRD) complex of chromatin regulators to be binding partners of LHX2. The transcription start sites (TSSs) and the LHX2 binding sites of both Fezf2 and Sox11 are epigenetically modified in an LHX2-dependent manner and display histone marks corresponding to the activity of NuRD complex. Finally, we show that LHX2 overexpression causes a decrease in the high Fezf2/CTIP2+ expressing (SCP) population. Together, these results demonstrate that LHX2 is both necessary and sufficient to regulate the numbers of deep layer 5 corticofugal projection neurons that express the Fezf2/CTIP2 signature and that it functions by modulating the epigenetic marks on key factors that control cortical neuronal subtype identity.

Materials and Methods

Mice. All animal protocols were approved by the Institutional Animal Ethics Committee (Tata Institute of Fundamental Research, Mumbai, India) according to regulations formulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. The floxed LIM homeobox2 (Lhx2) line (Lhx2lox/lox) and Emx1CreERT2 lines used in this study have been described previously by Mangale et al. (2008). The Emx1CreERT2 (Jin et al., 2000) was obtained as a gift from Prof. Yuqing Li at University of Florida College of Medicine. The floxed Lhx2 line was a gift from Prof. Edwin Momoki at the University of California, Irvine.

Timed pregnant female mice were obtained from the Tata Institute animal breeding facility, and embryos of both sexes were used for the experiments. Noon of the day the vaginal plug was observed was considered embryonic day (E) 0.5. Early-age embryos were staged by somite number and genotyped using PCR. Animals were genotyped and assigned to groups accordingly. Controls used for each experiment were age-matched littermates.

ChIP sequencing. For each ChIP sequencing experiment, 50 μg chromatin and 4 μg antibody were used per IP. To obtain chromatin, brains from E12.5 embryos were harvested and the neocortical tissue was isolated in cold 0.5% glucose in PBS with 1× Protease inhibitor mixture (Sigma). The tissue was cross-linked immediately after harvesting with 1% formaldehyde (Thermo Scientific). Chromatin was sonicated using a Covaris S220 sonicator for 18 cycles of 60 s ON and 30 s OFF (5% Duty cycle, 2 Intensity and 200 cycles per burst) to get chromatin within the size range of 100–500 bp. The following antibodies were used for ChIP: goat α-LHX2 (Santa Cruz Biotechnology SC19344), goat IgG (Bangalore Genei). The protein-DNA complex was pulled down using Protein A–G magnetic beads (Dynabeads, Invitrogen). The immunoprecipitated DNA was purified using phenol-chloroform-isomyl alcohol (Ambion).

Sequencing libraries were prepared using SOLiD ChIP-Seq library preparation kit, and sequencing was performed on the SOLID 4 System (Applied Biosystems). Five biological replicates were performed, and reads were aligned to the reference genome mm9 using bowtie 1. Peaks were called using the MACS 1.4 program with default settings. The UCSC browser was used for data visualization.

ChIP-qPCR. In each ChIP-qPCR experiment for validation of binding of LHX2 and/or NuRD complex protein members on LHX2 binding regions and TSS, 10 μg chromatin and 2 μg antibody was used per IP. For each Histone mark ChIP-qPCR, 5 μg chromatin and 1 μg antibody was used per IP. The following antibodies were used for ChIP: goat α-LHX2 (Santa Cruz- SC19344), goat IgG (Bangalore Genei), rabbit anti-Kdm1a/ LSD1 (Abcam, #ab17721), rabbit anti-HDAC2 (Abcam, #ab7029), rabbit anti-RBBP4 (Abcam, #ab38135), rabbit anti IgG (as control IgG for NuRD complex protein ChIPs) (Sigma, #18140), anti-Histone H3 antibody (ab1791), rabbit anti-H3K4me3 (Diagenode, C15410030), rabbit anti-H3K9ac (Diagenode, C15410004).

ChIP was performed as described above. For LHX2 and NuRD complex protein members, individual enrichment over the control genomic region was assessed by performing ChIP-qPCR with primers specific for these regions using the SYBR Green master mix (Roche). For histone and 1× ChIP signals in histone mutants using a Dounce homogenizer.

Information of the primers used for ChIP-qPCR is given (5′ to 3′) as follows: LHX2 BR on Fezf2: forward, TAGGCACTGGGAGGGACTGTA; reverse, TGGGACAGGAAGAAAAAGCG; TSS Fezf2: forward, CCCTGGTGTCGCCGCTTAAC; reverse, CGGCCACATCTCAATGAAT; LHX2 BR on Sox11: forward, GCCGAACAGGCGGTCCAT; reverse, GGAACAATACACGGGTCTCC; TSS Sox11: forward, CACTACTCCCACCGCAAC; reverse, GCACCTCGCGGATTTCTTT; and control genomic region: forward, GGTTGACACGAGGAAATCT; reverse, GCCATATCCTGAGGATTC.

IP and Western blotting: mass spectrometry. For mass spectrometric analysis of the LHX2 interacting proteins, IP was performed using 10 μg of protein sample and 20 μg of antibody. Brains from E15 embryos were harvested in cold 0.5% glucose in PBS with 1× Protease inhibitor mixture. For IP, samples were lyzed in TNN buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.9% NP-40, 1 mM PMSE, 1× protease inhibitor mixture, and 1× PIP signal in histone mutants) using a Dounce homogenizer. The lysate was centrifuged at 13,000 rpm for 45 min at 4°C to remove membranes and other debris. Supernatant was collected. Goat anti-
LHX2 (Santa Cruz Biotechnology, SC 19344) was used for LHX2 pull-down, and goat IgG (Bangalore Genei) was used as the negative control. The magnetic beads used for IP along with the precipitated proteins were resuspended in Laemmli buffer (63 mM Tris-HCL, 2% SDS, 0.0025% bromophenol blue, 10% glycerol) without β-mercaptoethanol and boiled at 65°C for 5 min. Proteins obtained by IP were resolved on 8% SDS-containing gel and then transferred on a PVDF membrane (Roche) in transfer buffer containing 20% methanol and 0.01% SDS at 196 V for 100 min. LHX2 affinity purified mouse monoclonal antibody, which was used as a control for all the reverse IPs. IPs were performed as previously described for mass spectrometric analysis. The magnetic beads used for IP along with the precipitated proteins were resuspended in Laemmli buffer (63 mM Tris–HCL, 2% SDS, 0.0025% bromophenol blue, 10% glycerol) without β-mercaptoethanol and boiled at 65°C for 5 min for all the IPs other than the LHX2 IP probed with LSD1. LSD1 has a high molecular weight, which makes the visualization of the band difficult in the IP lanes as IgG produces a dark band at higher molecular weights. For this particular IP, the beads were resuspended in Laemmli buffer with β-mercaptoethanol and boiled at 95°C for 3 min to degrade the IgG, which resulted in a band at ~55 kDa, thus making the band corresponding to LSD1 clearly visible. The resuspended samples were run on SDS-containing gel and then transferred on a PVDF membrane (Roche) in transfer buffer containing 20% methanol and 0.01% SDS at 90 V for 100 min. LHX2 affinity purified mouse monoclonal antibody, custom made from Bioclon, was used at a dilution of 1:1000 for probing the blots for the LHX2 IPs and reverse IPs. The monoclonal antibody was validated before use. The aforementioned antibodies (anti-KDM1A, anti-HDAC2, and anti-RBBP4) were used for probing the Western blots for the LHX2 IPs and reverse IPs. The monoclonal antibody was used at a concentration of 1:400, Molecular Probes, catalog #A11008) for TBR1. Goat anti-rabbit antibody conjugated to Alexa-488 (1:400, Molecular Probes, catalog #A11077) for CTIP2. Goat anti-mouse antibody conjugated to Alexa-647 (1:400, Molecular Probes, catalog #A21235) for SATB2. Tissue processing for immunohistochemistry was performed as described by Subramanian et al. (2011). For each control and experimental condition, ≥100 cells were counted from each of five biological replicates for Figure 1 and from each of three biological replicates for Figure 5.

In utero electroporation. All procedures conducted followed the guidelines prescribed by the Institutional Animal Ethics Committee. Swiss mice obtained from the Tata Institute of Fundamental Research animal breeding facility were used for electroporation. E12.5 timed pregnant mice were anesthetized using isoflurane (Forane, Abbott India). The surgical procedure performed has previously been described by Subramanian et al. (2011). The 3–4 μl plasmid DNA of concentration ~2 μg/μl dissolved in nuclease free water and mixed with Fast Green dye was injected into the lateral ventricle of the embryos using a fine-glass microcapillary. For electroporation, a BTX CUY21 electroporator [32 V (E12.5), 4 pulses, 50 μs pulse length, ~1.0 s pulse interval was used. Electric pulses were delivered using 3 mm paddle electrodes. The cortex was targeted by placing the positive electrode on the side of the dorsal wall of the lateral ventricle. The uterine horns were replaced, and the incision was sewn with surgical sutures. Animals were kept on a 27°C warm plate for half an hour for post-surgical recovery. An oral suspension of Meloxican (Melonex, United Pharmaceuticals) was mixed with the water in the feeding bottles of the dams (0.6 μl/ml) as an analgesic and given to the animals until 2 d after surgery. DNA constructs Lhx2-GFP and pCAGires-2-EGFP were used as described by Subramanian et al. (2011).

Imaging. Bright-field images were taken using a Zeiss Axioplan 2 + microscope, Nikon Digital Sight DS-F12 camera, and Nikon NIS 4.0 imaging software. Images of immunohistochemistry were obtained using a Zeiss LSM 5 Exciter-Axioimager M1 imaging system and Zeiss LSM510 imaging system. Image stacks were generated by scanning at intervals of 4 μm for lower magnification and at intervals of 0.8 μm for higher magnification using filters of the appropriate wavelengths. The stacks were analyzed, merged, and projected using ImageJ software from the National Institutes of Health. Figure panels were prepared using Adobe Photoshop CS6.

Statistical analysis. Statistical analysis was performed using the unpaired two-tailed Student’s t test. Error bars indicate SEM. All primary data from immunohistochemistry and ISH experiments were analyzed by one investigator and then confirmed by a second, independent investigator.

Results
Lhx2 is strongly expressed in the ventricular zone at embryonic day (E) 12.5 and E15.5 (Fig. 1A, white asterisks) but is not detectable in postmitotic deep layer neurons accumulated in the cortical plate from E15.5 onwards. By postnatal stages, deep layer neurons do not detectably express Lhx2 (Fig. 1A, black asterisks). We generated cortex-specific Lhx2 conditional mutant brains using Emx1CreEC, which we have previously characterized as a reagent that achieves near-complete recombination in the dorsal telencephalon by E11.5, a day later than the commonly used Emx1CreF1 line (Shetty et al., 2013). The Emx1CreF2 line is crucial to the present study because it spares the neocortex (Shetty et al., 2013); removing LHX2 from the dorsal telencephalon before E11.5 using Emx1CreF3 causes a transformation of neocortex to paleocortex (Chou et al., 2009). For the rest of this study,
EnxICreYL will be referred to as simply “EnxICre,” and the resulting brains will be referred to as “cortex-specific conditional mutant brains.”

In cortex-specific Lhx2 conditional mutant mice (EnxICreYL) (Shetty et al., 2013), as well as in the pan-CNS Lhx2 conditional mutant mice (Nestin Cre) (Chou and O’Leary, 2013), the cortex is thinner than in control brains. Although all layers are specified, the later born superficial layers are considerably thinner in mutant brains compared with controls. In these studies, the deep layers 5 and 6 did not appear to be as drastically affected (Chou and O’Leary, 2013; Shetty et al., 2013). In the present study, we discovered a previously unreported phenotype in the deep layers 5 and 6 of cortex-specific Lhx2 conditional mutant brains.

On close examination of postnatal day (P) 7 brains, we found that Tbr1-expressing layer 6 neurons are reduced in Lhx2 conditional mutant brains compared with controls. In contrast, layer 5 is substantially expanded as revealed by the expression of ER81, Id2, high-level Fezf2, and CTIP2, a downstream effector of FEZF2 (Fig. 1 B, C, E) (Arlotta et al., 2005; Molyneaux et al., 2005; Chen et al., 2008; Rouaux and Arlotta, 2013). Cell counts of CTIP2-expressing and TBR1-expressing cells as a percentage of the total DAPI-stained population (layers 5 + 6) confirms the increase (CTIP2) and decrease (TBR1) in the respective populations (Fig. 1 D, E). This suggested that LHX2 may regulate neuronal subtype fate specification.

To investigate this possibility, we harvested cortical primordia from embryonic telencephalic hemispheres at E12.5, and performed ChIP using anti-LHX2 antibody, followed by sequencing (ChIP-Seq; Fig. 2A). We screened the loci associated with LHX2 binding regions for known regulators of cortical neuronal subtype identity. Two candidates, Fezf2 and its known transcriptional regulator Sox11 (Shim et al., 2012), displayed LHX2 occupancy on putative distal regulatory regions.

During deep layer neurogenesis, both these factors promote layer 5 SCPN fate, and FEZF2 represses layer 6 TBR1 + CThPN fate (Molyneaux et al., 2005; Han et al., 2011; McKenna et al., 2011). The LHX2 occupancy peaks for the Fezf2 and Sox11 loci were at positions distant from the TSS, 7.8 and 266 kb, respec-
LHX2 occupies enhancer elements of cortical neuron subtype regulators Fezf2 and Sox11. A, Diagram illustrating dissection of cortical primordia. B, The LHX2 binding site sequence reported in the literature was also found in the LHX2 binding regions of Fezf2 and Sox11. C–F, ChIP-Seq data showing UCSC genome browser tracks of the LHX2 occupancy profile at the Fezf2 (C) and Sox11 (E) loci. Each LHX2-binding region was validated by ChIP followed by qPCR analysis (D, F). G, H, Examination of Fezf2 and Sox11 at E12.5 (G), 1 d after cortex-specific loss of LHX2, reveals an increased expression of Fezf2 and Sox11 in the ventricular zone (white asterisks) and an increased accumulation of Fezf2- and Sox11-expressing postmitotic cells (arrowhead). High-magnification images are displayed alongside. H, By E13.5, the increase in the ventricular zone has attenuated (white asterisks), but the expression in the cortical plate has expanded (arrowhead). Error bars indicate SEM. Scale bar, 100 μm. *p < 0.05. **p < 0.001.
latory elements of Fezf2 and Sox11, and loss of LHX2 results in upregulation of these genes in the cortical ventricular zone during the time of peak production of SCPNs.

Transcription factors usually act in multimeric complexes to achieve their functions (Vernimmen and Bickmore, 2015). To identify binding partners of LHX2, we performed IP from cortical tissue using anti-LHX2 antibody, and ran the resulting samples on an SDS-PAGE gel. Silver staining revealed bands that were differentially enriched in comparison with a control IgG IP (Fig. 3A). These were excised and the proteins identified using mass spectrometry. Mass spectrometry was performed for 2 additional biological replicates of LHX2-IP material, for which all bands were excised and sequenced. The resulting dataset was manually curated for known chromatin modifiers. Three subunits associated with the NuRD complex, LSD1, RBBP4, and HDAC2 (Wang et al., 2009), were identified as potential binding partners of LHX2. These were individually validated in three biological replicates, by performing LHX2 IP followed by Western blotting using antibodies against each candidate partner. Reverse validation of interactions was performed by performing IP for each binding partner and probing the Western blot using anti-LHX2. D, Diagram illustrating the NuRD complex and members that bind LHX2. E, ChIP-qPCR-based occupancy analysis demonstrating the binding of LSD1, HDAC2, and RBBP4 to either the TSS and/or the LHX2 binding region (LHX2 BR) on Fezf2 and/or Sox11. y-axis indicates fold enrichment over IgG at the respective loci. CHD4, Chromodomain helicase DNA binding protein 4; HDAC2, histone deacetylase 2; LSD1, lysine-specific histone demethylase 1; MBD, methyl CpG binding domain protein; MTA1/2, metastasis-associated protein 1/2; RBBP4, retinoblastoma binding protein 4. A, The image of the silver-stained gel has been cropped to remove the lanes corresponding "unbound" fractions of control IgG and LHX2 IP that were on the left of the marker lane. B, C, The Western blots are cropped from full-length Western blots. The original uncropped images are available upon request. Error bars indicate SEM. *p < 0.05. **p < 0.001. ***p < 0.0001.
minished, and the active marks are enriched (Fig. 4B), thereby leading to an aberrant expression of Fezf2 and Sox11.

Loss of LHX2 results in increased activation/expression of target genes, and correlated with this, an increase in the number of layer 5 Fezf2/CTIP2 + neurons. We tested whether overexpression of Lhx2 would have the opposite effect of reducing the numbers of these neurons. We used an LHX2-GFP construct for in utero electroporation at E12.5 (Fig. 5A, B). The quantifications demonstrate that LHX2 overexpression causes a 50% decrease in CTIP2-expressing cells and a 59% increase in SATB2-positive cells in layer 5, consistent with a role for LHX2 in modulating relative numbers of neurons with distinct molecular subtype signatures (Fig. 5D).

In summary, our results show that loss or overexpression of Lhx2 regulates key genes that specify neuronal subtype identity. LHX2 appears to achieve this regulation by recruiting chromatin-modifying proteins, and loss of LHX2 causes changes in the epigenetic marks associated with its target genes. These findings position LHX2 as a critical component of the network of factors that control the production of an appropriate proportion of neuronal subtypes in the developing cerebral cortex.

Discussion

We present an extensive mechanistic analysis of how LHX2, a transcription factor expressed in the cortical ventricular zone, can exert regulatory control on mechanisms that confer subtype identity to cortical neurons. Emx1Cre acts in the ventricular zone, and postmitotic neurons arising from these progenitors would also be expected to carry any alleles that undergo recombination as a result of the Emx1Cre action. Therefore, an obvious question is whether the effects of LHX2 removal are relevant in the ventricular zone progenitors themselves, or in the newly postmitotic neurons they produce. A recent study that used NexCre to delete LHX2 specifically in postmitotic neurons starting from E11 offers clarity on this issue (Zembrzycki et al., 2015). This study elegantly demonstrated that NexCre action is not seen in proliferating progenitors in the ventricular zone but is seen in newly postmitotic cells (Zembrzycki et al., 2015, their Figure S2) and that molecular specification of the different cortical layers is not affected (Zembrzycki et al., 2015, their Fig. 3). Therefore, the function of LHX2 we uncovered in the present study appears to be a novel role for LHX2 that operates in ventricular zone progenitors, the effects of which are seen by way of molecular dysregulation of its target genes in the ventricular zone itself, and also evident in altered proportions of cortical neuronal subtypes in maturity.

Loss of LHX2 in the cortical ventricular zone has been reported to cause premature neurogenesis, resulting in a thicker cortical plate during the early stages of corticogenesis and as a consequence, depletion of the progenitor pool resulting in thinner superficial layers (Chou et al., 2013). However, by postnatal stages the combined effect of these two opposing phenomena result in a cortex with diminished thickness compared with that of wild-type brains (Chou et al., 2013; Shetty et al., 2013). A defect involving premature neurogenesis would be expected to leave the earliest born layer 6 relatively unaffected and have a progressively greater effect on the later born layers due to progenitor depletion. Our present study uncovers a paradoxical phenotype, such that layer 6 is thinner than normal, and layer 5 is expanded compared with control brains (Fig. 1). This is not explained by a progressive depletion of the progenitor pool (Chou et al., 2013) but, rather, suggests an entirely different defect: that of neuronal subtype fate specification. Removal of LHX2 leads to upregulation of Fezf2 in the ventricular zone, which is known to suppress Tbr1 (Mckenna et al., 2011). The decrease in layer6 TBR1 neurons may not be a consequence of direct regulation by LHX2, but an indirect result of Fezf2 upregulation in the absence of LHX2. Therefore, in addition to its previously described role in regulating progenitor proliferation, this multifunctional transcription factor also regulates neuronal subtype identity. This is the first report of such a function for LHX2.

To provide a comprehensive understanding of the mechanisms at play, studies of gene regulation should include both an examination of the protein complexes in which the regulatory molecule acts, as well as its occupancy on target gene loci. We report a novel molecular role for LHX2, in which it partners with proteins HDAC2, LSD1, and RBBP4, that are found together in the NuRD-HDAC complex. However, these proteins display differential occupancy on Fezf2 and Sox11 that RBBP4 does not appear to bind the Fezf2 locus on either the TSS or the LHX2 BR, whereas LSD1 binds both these regions on both Fezf2 and Sox11, and HDAC2 does so only at the LHX2 BR on both loci. This raises the possibility that may not act together within the holo-NuRD complex but act independently or in other combinations in a site-specific context.
LHX2 is necessary and sufficient to regulate molecular subtype identity. A, B, Electroporation of control GFP (A) or LHX2-GFP (B) at E12.5 and examination at P5 reveal GFP-expressing cells in the region of electroporation, overlapping with CTIP2-expressing layer 5 cells. Individual high-magnification confocal images of GFP, CTIP2, and SATB2, and the corresponding merged images of GFP/CTIP2 and GFP/SATB2 are shown alongside the low-magnification GFP/CTIP2 image. C, Diagram illustrating in utero electroporation at E12.5, and examination of brain sections at P5. D, The percentage of electroporated (GFP-expressing) cells that also express CTIP2 or SATB2 reveals a striking decrease in CTIP2-expressing cells and an increase in SATB2-expressing cells upon electroporation of LHX2-GFP. E, A gradient of Lhx2 expression is seen in a series of sagittal sections at E12.5. Scale bars: A, B, 500 μm (low-magnification images), 50 μm (high-magnification images); E, 500 μm. Error bars indicate SEM. **p < 0.001.

Of the many roles reported for LHX2 in the literature, this is perhaps the most surprising, given that Lhx2 appears to be expressed in all progenitors at E10.5-E11.5 (Rincón-Limás et al., 1999; Lu et al., 2000) and displays continued expression in a gradient throughout the period of cortical neurogenesis (Bulchand et al., 2003). At E12.5, Lhx2 expression displays a gradient in the cortical primordium, from caudomedial high to rostrolateral low (Nakagawa et al., 1999) (Fig. 5E). The fact that Lhx2 overexpression at E12.5 is able to dramatically reduce the number of postnatal CTIP2 expressing neurons in layer 5, and loss of Lhx2 is able to increase them, indicates that levels of Lhx2 in the E12.5 neuroepithelium may play an important role in governing the neuronal number corresponding to this population, together with other regulators across the rostrocaudal axis. Work from several groups has reported that regulators of neuronal subtype identity, such as Fezf2, COUP-TFI, CB1R, and Cux2, display widespread to limited expression in the ventricular zone (Hirata et al., 2004; Nieto et al., 2004; Zimmer et al., 2004; Chen et al., 2005; Molyneaux et al., 2005; Faedo et al., 2008; Cubelos et al., 2010; Tomassy et al., 2010; Díaz-Alonso et al., 2012, 2015; Rodríguez-Tornos et al., 2016). Of these, Fezf2 is expressed widely in the ventricular zone at E12.5 but is seen only in postmitotic neurons by E15.5 (Hirata et al., 2004), and neurons in all cortical layers appear to originate from FEZF2+ lineage (Guo et al., 2013). Although this immediately suggests a strong parallel with the temporal fate specification model that has been elegantly described in Drosophila neuroblasts (Kohwi and Doe, 2013), the function of ventricular Fezf2, if any, in determining postmitotic identity of subcerebral projection neurons remains to be eluci-
dated, and our finding that loss of LHX2 results in an upregulation of Fezf2 in the ventricular zone itself adds further motivation to exploring this question.

Our study motivates a mechanistic analysis of the transcription factor network that controls cortical neuronal subtype identity, from an epigenetic angle. It is important to unravel what terms, such as induction or suppression, actually entail at the level of the chromatin. Such an analysis may offer some clarity on why some genes appear more responsive to regulatory controls (e.g., whether they are in an epigenetically “poised” state), with both active and repressive marks, in which case they can be induced or suppressed with short time delays (Bernstein et al., 2006; Mikkelsen et al., 2007; Hirabayashi and Gotoh, 2010). In some cases, transcription factors may compete for or sequester key components of chromatin remodeling complexes and cause some cases, transcription factors may compete for or sequester in these cells (cortical progenitors and possibly drives the expression of Fezf2) (Cederquist GY, Azim E, Shnider SJ, Padmanabhan H, Macklis JD (2013)). This enhancer is most strongly expressed in cortical progenitors and possibly drives the expression of Fezf2 in these cells (Eckler MJ, 2014). How the activation or repression is achieved via this distal regulatory element will almost certainly be a fascinating story of epigenetic regulation of Fezf2. Moreover, in a role strikingly similar to that of LHX2, a recent study demonstrated that transcription factor CTIP1, expressed by newly postmitotic neurons, functions to specify SCPN versus CThPN identity in the cortex (Woodworth et al., 2016). It is in such a context that our results are of immediate interest because we position LHX2 among the known regulators of neuronal subtype identity that are expressed in the ventricular zone, the interactions of which are likely to determine area specific complements of deep layer neuronal subtypes along the rostrocaudal and mediolateral axes of the developing cortex.

References

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