© 2019. Published by The Company of Biologists Ltd | Development (2019) 146, dev170068. doi:10.1242/dev.170068

RESEARCH ARTICLE

Lmx1a drives Cux2 expression in the cortical hem through activation of a conserved intronic enhancer

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ABSTRACT

During neocortical development, neurons are produced by a diverse pool of neural progenitors. A subset of progenitors express the *Cux2* gene and are fate restricted to produce certain neuronal subtypes; however, the upstream pathways that specify these progenitor fates remain unknown. To uncover the transcriptional networks that regulate *Cux2* expression in the forebrain, we characterized a conserved *Cux2* enhancer that recapitulates *Cux2* expression specifically in the cortical hem. Using a bioinformatic approach, we identified putative transcription factor (TF)-binding sites for cortical hem-patterning TFs. We found that the homeobox TF Lmx1a can activate the *Cux2* enhancer *in vitro*. Furthermore, we showed that Lmx1a-binding sites were required for enhancer activity in the cortical hem *in vivo*. Mis-expression of Lmx1a in hippocampal progenitors caused an increase in *Cux2* enhancer activity outside the cortical hem. Finally, we compared several human enhancers with cortical hem-restricted activity and found that recurrent Lmx1a-binding sites are a top shared feature. Uncovering the network of TFs involved in regulating *Cux2* expression will increase our understanding of the mechanisms pivotal in establishing *Cux2* lineage fates in the developing forebrain.

KEY WORDS: Cux2, Lmx1a, Cortical hem, Corticogenesis, Enhancer, Telencephalon

INTRODUCTION

During forebrain development, neural progenitor cells give rise to many different types of neuronal and glial cells that form the various telencephalic structures and circuits. This vast cellular diversity arises through the interplay between early tissue patterning pathways and gene regulatory networks (GRNs). Early in development, multiple tissue organizers and signaling centers provide morphogenic cues that govern regional identity and size. Within these different regions, complex transcriptional programs further diversify multipotent progenitor cells toward specific cell fates. The transcription factors (TFs) that establish the different GRNs to specify cell fates often work by binding gene regulatory elements, such as enhancers, to boost or suppress expression of target genes. Following transcriptional activation of GRNs, neural progenitors divide and eventually differentiate into specified cells. Identifying the signaling and transcriptional networks that establish regional identity and subtype fate specification during embryonic development will greatly enhance our understanding of forebrain development and function.

The TF Cut-like homeobox 2 (Cux2) is dynamically expressed in complex spatiotemporal patterns in the developing mouse forebrain (Zimmer et al., 2004). During early brain development, a subset of neural progenitors weakly express *Cux2* transcripts in a 'salt and pepper' pattern (Franco et al., 2012). We previously fate mapped the lineage output of *Cux2*$^+$ progenitors in the neocortex and found that this subset of neural progenitors are fate restricted to produce late-born corticocortical neurons in upper layers (Franco and Müller, 2013; Franco et al., 2012; Gil-Sanz et al., 2015). Our studies indicated that *Cux2*$^+$ progenitors in the developing forebrain are committed to this fate even before the onset of neurogenesis. This model has been a matter of controversy, as other studies have suggested that neocortical progenitors are homogeneous and multipotent (Eckler et al., 2015; Gao et al., 2014; Guo et al., 2013). However, recent studies using a variety of fate-mapping methods have independently confirmed the existence of neural progenitors that only generate late-born corticocortical neurons (García-Moreno and Molnár, 2015; Llorca et al., 2018 preprint). Nevertheless, a consensus model has yet to emerge and the underlying mechanisms that restrict these progenitors to specific cell fates remain largely unknown.

Cux2 knockout mice do not display any significant phenotype with respect to progenitor cell fate specification (Cubelos et al., 2008), implying that Cux2, although a useful marker for a fate-committed progenitor population, does not necessarily instruct fate in this context. We reasoned that a deeper understanding of Cux2$^+$ cell fate commitment in forebrain progenitors could be achieved by uncovering the upstream GRNs responsible for the complex patterns of *Cux2* expression. Interestingly, neural progenitors in the dorsal telencephalic midline (DTM) strongly express *Cux2* in a more complete pattern than progenitors in adjacent regions, suggesting that this forebrain region might contain critical transcriptional regulators of the *Cux2* locus.

Previous studies have uncovered enhancers active in the developing mouse telencephalon, including an 856 bp element in intron 2 of the *Cux2* genomic locus that could drive strong transgene expression in the DTM (Hasenpusch-Theil et al., 2012; Visel et al., 2008). Here, we have characterized this element as an active enhancer in the developing forebrain and show that it is specifically active in the cortical hem, but not in the adjacent hippocampus or neocortex. We further analyzed this enhancer for possible upstream regulators of *Cux2* expression. Among many bioinformatically identified candidates, we tested several transcription factors known to function in or be expressed within the cortical hem. Using an *in vitro* approach, we demonstrate that Lmx1a is a strong activator of the *Cux2* hem-specific enhancer. We further show by chromatin immunoprecipitation from embryonic forebrain that Lmx1a can bind the endogenous *Cux2* enhancer region. Additionally,

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Received 15 July 2018; Accepted 11 February 2019

Development (2019) 146, dev170068. doi:10.1242/dev.170068

shRNA-mediated knockdown of *Lmx1a in vivo* caused a decrease in *Cux2* enhancer activity in the cortical hem. Conversely, Lmx1a gain of function in the hippocampus, a region normally devoid of Lmx1a expression, increased activity of the *Cux2* enhancer. Finally, we analyzed other enhancers that exhibit specific activity in the cortical hem and identify recurrent Lmx1a-binding sites as a common motif shared between these distinct hem-specific enhancers. Our results suggest Lmx1a functions as an upstream regulator of a conserved *Cux2* enhancer in the cortical hem, and raise the possibility that Lmx1a is a crucial TF in the GRN that specifies cortical hem fate.

RESULTS

Early forebrain expression of *Cux2* begins at the dorsal telencephalic midline

To better understand when and where the earliest transcriptional regulators of *Cux2* are active in the developing telencephalon, we sought to define the temporal and spatial patterns of *Cux2* gene expression. We crossed $Cux2^{Cre/+}$ mice to the *Ai9* Cre-reporter line and used recombination ($tdTomato^{+}$) as a readout of the cumulative transcriptional history of the *Cux2* genomic locus. $Cux2^{Cre/+};Ai9^{fl/+}$ brains were analyzed at E9.5, E10.5, E12.5 and E14.5 (Fig. 1). We found that the earliest consistent pattern of recombined cells in the forebrain first appeared in the dorsal telencephalic midline (DTM) at ~E9.5 (Fig. 1A). At this age, a few recombined cells also began to appear scattered very sparsely throughout the adjacent hippocampal and neocortical neuroepithelium (Fig. 1A). By E10.5, the entire DTM was recombined, and the number of $tdTomato^{+}$ neuroepithelial cells was increased in the neocortex (Fig. 1B). At E12.5 and E14.5, the DTM is reorganized into two distinct structures: the cortical hem and choroid plexus epithelium (Grove et al., 1998). Essentially, all cells in the cortical hem and choroid plexus were recombined at E12.5 (Fig. 1C) and E14.5 (Fig. 1D). In contrast, only a fraction of cells in the adjacent hippocampal primordium and neocortex were recombined (Fig. 1C,D). In fact, we observed a strikingly sharp border of complete-to-sparse recombination at the boundary between the cortical hem and the hippocampal primordium. To trace the long-term lineages of these $Cux2^{+}$ cells in the DTM to later ages, we next used *Cux2-CreERT2* mice (Franco et al., 2012) for temporal fate mapping. We crossed *Cux2-CreERT2* mice to the *Ai9* reporter strain and administered a single dose of tamoxifen at E10.5 (Fig. 1E). At E16.5, many cells in the cortical hem and choroid plexus were recombined (Fig. 1F). By postnatal ages, the cortical hem neuroepithelium transforms in the fimbria. We found many recombined cells in the fimbria at postnatal day 10 after tamoxifen administration at E10.5 (Fig. 1G). These data indicate that forebrain activation of the *Cux2* locus occurs earliest and most uniformly in the DTM, including the cortical hem and choroid plexus.

Cux2 regulatory element contains characteristics of an active enhancer and recapitulates the endogenous *Cux2* expression pattern in the cortical hem

Non-coding gene regulatory elements, such as enhancers, can act as crucial platforms for TFs that drive cell fate decisions (Pattabiraman et al., 2014). To gain insights into some of the transcriptional programs that specify area and subtype fate in the telencephalon, we sought to identify enhancers that could recapitulate *Cux2* expression in the developing forebrain. Previous studies identified an 856 base pair (bp) region within the human *Cux2* gene (hs611) that exhibits extreme human-rodent sequence conservation, suggesting an important functional role for this non-coding element (Visel et al., 2008). Indeed, both the human element (Pattabiraman et al., 2014; Visel et al., 2008, 2013) and the corresponding murine region

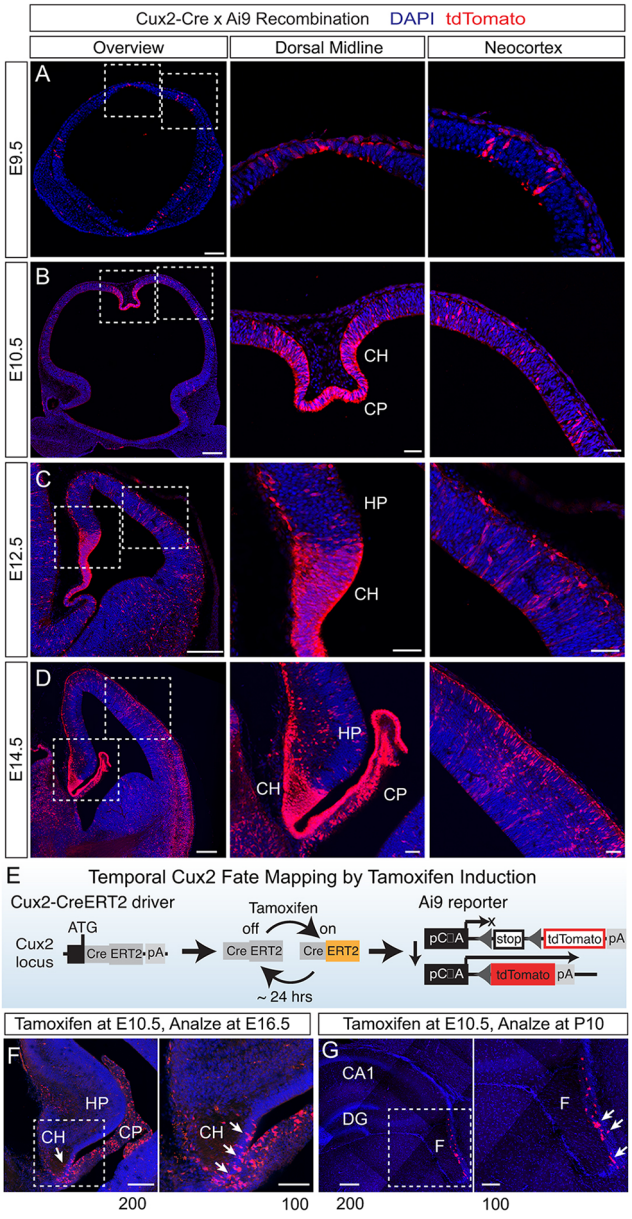

Fig. 1. Spatiotemporal development of *Cux2* expression in murine telencephalic progenitors. (A-D) Coronal sections of forebrains from *Cux2-Cre; Ai9* embryos showing recombination as a cumulative readout of *Cux2* expression. Recombined cells express tdTomato (red). Sections were counterstained for nuclei with DAPI (blue). Boxes outline the areas shown in more detail on the right: the dorsal midline (middle panels) or neocortex (right panels). (A) E9.5: recombination is apparent in the dorsal-most region of the telencephalic neural tube and in scattered cells in the telencephalon. (B) E10.5: recombination becomes robust in the nascent cortical hem and choroid plexus, with salt-and-pepper recombination in the neocortex. (C) E12.5: recombination is nearly ubiquitous in the cortical hem, while still mosaic in the neocortex. (D) E14.5: recombination is complete in the cortical hem and much of the choroid plexus, while the neocortex exhibits a still expanding, mosaic pattern. (E) Temporal fate mapping through tamoxifen induction. *Cux2-CreERT2* mice allow temporary activation of CreERT2 only in $Cux2^{+}$ cells for a period up to 24 h. Subsequently, Cre recombines the *Ai9* allele for permanent labeling of cells with tdTomato. (F) Coronal section of an E16.5 *Cux2-CreERT2;Ai9* forebrain labeled at E10.5 showing Cux2-lineage cells ($tdTomato^{+}$ and arrows) within the cortical hem (dashed box). (G) Coronal section of a P10.5 *Cux2-CreERT2;Ai9* forebrain labeled at E10.5 showing recombined Cux2-lineage ($tdTomato^{+}$) cells (arrows) residing within the cortical hem-derived fimbria (dashed box). Scale bars: 200 µm (A-D,F,G, left); 50 µm (A-D, middle and right); 100 µm (F,G, right). CH, cortical hem; CP, choroid plexus; F, fimbria; HP, hippocampal primordium.

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(Hasenpusch-Theil et al., 2012) can drive restricted expression of a *lacZ* reporter gene in transgenic mouse embryos, indicating their role as functional enhancer elements. This enhancer lies within intron 2 of the *Cux2* gene (Fig. 2A). Using DNaseI hypersensitivity (ENCODE Project Consortium, 2012) and histone modification (Shen et al., 2012) data from the UCSC Genome Browser database (genome.ucsc.edu/) (Kent et al., 2002), we found that this genomic region has characteristics of transcriptionally active chromatin in E14.5 forebrain tissue, including prominent DNaseI hypersensitivity and histone marks H3K4me1 and H3K27ac (Fig. 2B). Together with previously published studies, these data suggest that this region is an active enhancer for *Cux2* in the developing forebrain and led us to characterize it in more detail.

Interestingly, the human and murine elements both exhibited activity patterns in the developing forebrain similar to that of *Cux2*, including strong activity in the DTM (Hasenpusch-Theil et al., 2012; Visel et al., 2008). To better characterize the spatial activity of this candidate cis-regulatory element in the developing mouse forebrain, we first cloned the 856 bp murine enhancer into an expression vector (Wilken et al., 2015) with a minimal promoter (TATA box) driving Cre recombinase. We then introduced the plasmid into the developing forebrain of *Ai9* Cre-reporter mice at E12.5, using *in utero* electroporation (Fig. 3A). We co-electroporated a plasmid expressing GFP from the ubiquitously expressed synthetic CAG promoter (Niwa et al., 1991) as a marker of electroporated cells (Fig. 3A). Electroporations were performed to target different regions of the telencephalon, including the DTM, hippocampal primordium and neocortex. We analyzed patterns of GFP expression and Cre-mediated recombination (tdTomato expression) at E14.5. As controls, we compared recombination patterns in the *Cux2*Enhancer-Cre electroporations with those of the minimal promoter construct alone (MINp-Cre, no enhancer) or with a strong and ubiquitous promoter (CAG-Cre). We found that recombined tdTomato^{+} cells in the MINp-Cre electroporations were very sparse in the DTM, hippocampus (Fig. 3B) and neocortex (Fig. 3E), consistent with weak expression from the TATA box alone. Conversely, the CAG-Cre construct drove recombination ubiquitously throughout the electroporated regions, including in the DTM, hippocampus and neocortex (Fig. 3C,F). Interestingly, recombination in the *Cux2*Enhancer-Cre electroporations was almost completely restricted to the DTM, namely the cortical hem and choroid plexus epithelium (Fig. 3D). Very few tdTomato^{+} cells were present in the hippocampus (Fig. 3D) or the neocortex (Fig. 3G). These data demonstrate that the activity of this *Cux2* enhancer is restricted within the telencephalon to the cortical hem and choroid plexus, recapitulating a specific aspect of the complex endogenous *Cux2* expression pattern.

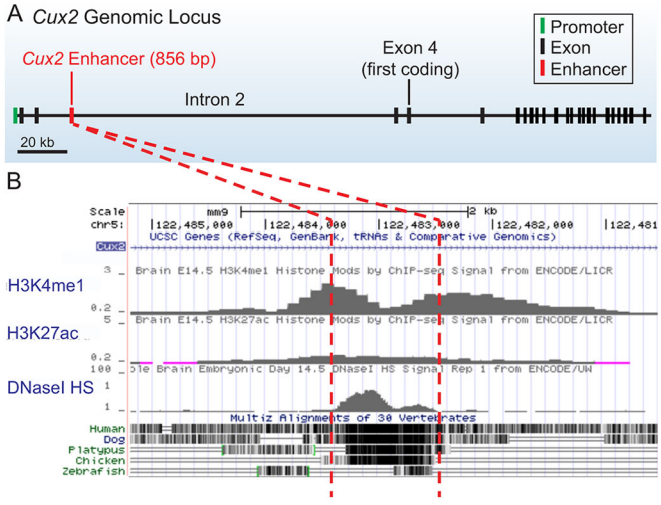

Fig. 2. Genomic location and chromatin characteristics of a *Cux2* enhancer. (A) Schematic of the murine *Cux2* genomic locus, showing the location of an 856 bp cis-regulatory element in the proximal region of intron 2. (B) UCSC Genome Browser data demonstrating key enhancer characteristics for the *Cux2* 856 bp element, including epigenetic marks H3K4me1 and H3K27ac, a prominent DNaseI hypersensitivity peak, and a high degree of evolutionary conservation across taxa.

Developmentally expressed cortical hem transcription factors, Lmx1a and Emx2, activate the *Cux2* enhancer *in vitro*

As pioneering regulators of development, TFs often control gene expression by acting on enhancers. To identify candidate transcriptional regulators of *Cux2* expression in the cortical hem, we analyzed the hem-specific enhancer sequence for predicted TF-binding sites using the JASPAR database (Khan et al., 2018) with a 'predicted' and 'consensus' match threshold of 80% or higher. Our analysis revealed a large list of predicted TF-binding sites, including putative sites for various TFs expressed throughout much of the telencephalon (Table S1). To narrow the list, we focused on a small subset of TFs known to be expressed in the developing cortical hem, including Emx2, Lmx1a and Msx1 (Fig. 4). Consensus binding sequences for these three TFs overlapped with each other in the *Cux2* enhancer in 12 predicted sites with high sequence conservation (Fig. 4A,B). Lmx1a and Msx1 are expressed in the DTM at E8.5 and E9.5, respectively, and their expression continues into adulthood (Failli et al., 2002; Furuta et al., 1997). Emx2 is expressed by neural progenitors in the hippocampus and neocortex beginning at E8.5, with the dorsomedial-most expression domain extending into the cortical hem (Simeone et al., 1992a,b; Tole et al., 2000a; Yoshida et al., 1997). Analysis of the Allen Developing Mouse Brain Atlas (2008) *in situ* hybridization database (developingmouse.brain-map.org) confirmed expression of all three TFs in the cortical hem at E12.5 (Fig. 4C).

As key players in telencephalic patterning, these TFs serve as ideal candidates for regulating *Cux2* expression in the cortical hem during early forebrain development. To begin to test whether these TFs can activate the hem-specific *Cux2* enhancer, the enhancer element was cloned into a minimal promoter vector driving nuclear mCherry expression (Fig. 5A). cDNAs for the candidate TFs were cloned into the bicistronic expression vector pCIG (Hand et al., 2005), which drives expression of both the TF and GFP from the CAG promoter. Each candidate TF plasmid was co-transfected with the *Cux2*Enhancer-mCherry plasmid into murine immortalized neuroectodermal (NE-4C) cells (Schlett and Madarász, 1997). Twenty-four h after transfection, mRNA was collected from the cells and analyzed by RT-qPCR for levels of the *GFP* and *mCherry* reporter transcripts (Fig. 5A). We also used RT-qPCR to verify that each TF was being overexpressed compared with baseline expression in pCIG control transfected cells (fold change for Emx2, 177,711.74±17,580.78; Lmx1a, 220.77±25.97; and Msx1, 53.96±22.38). Compared with the pCIG empty vector negative control, Emx2 and Lmx1a overexpression significantly upregulated activation of the *Cux2* enhancer (Fig. 5B). In contrast, Msx1 did not change *Cux2* enhancer-driven *mCherry* levels compared with control (Fig. 5B).

Activation of the *Cux2* enhancer in the cortical hem requires Lmx1a-binding sites

Our *in silico* data predicted multiple binding sites for Emx2 and Lmx1a that overlapped each other (Fig. 4A,B), which correlated

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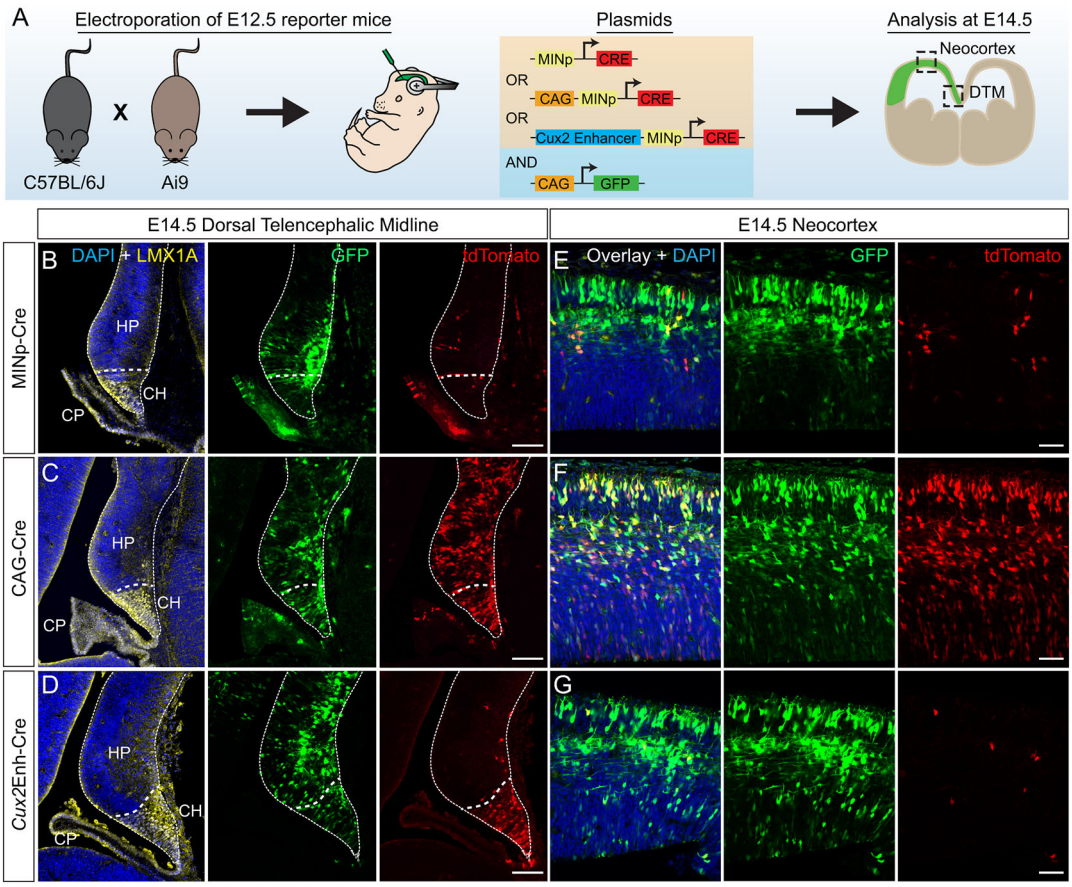

Fig. 3. The *Cux2* enhancer exhibits activity restricted to the cortical hem. (A) Schematic of experimental workflow: E12.5 *Ai9* reporter forebrains were electroporated *in utero* with constructs expressing Cre recombinase driven by either a minimal TATA-box promoter (Minp), a ubiquitous CAG promoter or the *Cux2* enhancer. CAG-GFP was co-electroporated to mark electroporated cells. Forebrains were harvested at E14.5 for analysis. (B-D) Coronal sections of electroporated brains showing the dorsal midline region. All electroporated cells express GFP (green) and recombined cells express tdTomato (red). The boundary between the cortical hem and hippocampal primordium is marked by expression of LMX1A protein (yellow). Sections were counterstained for nuclei with DAPI (blue). Electroporation of Minp-Cre causes minimal recombination in the cortical hem and hippocampal primordium (B), whereas CAG-Cre leads to ubiquitous recombination throughout the electroporated regions (C). *Cux2*Enhancer-Cre drives robust recombination in the cortical hem, but not in the adjacent hippocampal primordium. (E-G) Coronal sections of electroporated brains showing the neocortex. Similar to the dorsal midline, Minp-Cre drives minimal recombination (E) and CAG-Cre drives ubiquitous recombination (F) in the neocortex. The *Cux2* enhancer (G) exhibits no activity in the neocortex. Scale bars: 100 µm in B-D; 50 µm in E-G. CH, cortical hem; CP, choroid plexus; HP, hippocampal primordium.

well with our *in vitro* studies demonstrating that these factors can activate the *Cux2* enhancer (Fig. 5B). To directly test whether these putative TF-binding sequences were required for enhancer activation, we generated a mutant enhancer construct in which the central eight base pairs of 10 out of the 12 predicted binding sites were mutated (see Materials and Methods for details). The mutated *Cux2*Enhancer-mCherry plasmid was then tested (Fig. 5A) for activation by Emx2 and Lmx1a. Compared with the wild-type *Cux2* enhancer, activation of the mutated enhancer by Lmx1a was greatly attenuated (Fig. 5C). Interestingly, Emx2 was still able to upregulate expression from the mutated enhancer (Fig. 5C), raising the possibility of other more crucial Emx2-binding sites within the enhancer.

We next tested the activity of the mutated enhancer *in vivo* by *in utero* electroporation of the mutated enhancer driving Cre recombinase into *Ai9* reporter embryos (Fig. 6A). In contrast to the wild-type *Cux2*Enhancer-Cre that drives recombination specifically and robustly in the cortical hem (Fig. 6B), the TF-binding site mutant *Cux2*Enhancer-Cre construct was unable to drive any recombination at all in the cortical hem (Fig. 6C). These *in vivo* data indicate that at least some of the putative binding sites for homeobox TFs are required for activity of the *Cux2* enhancer in the cortical hem. Together with our *in vitro* data, this suggests that Lmx1a and/or Emx2 may be critical regulators of the *Cux2* enhancer. Although we did not rule out a possible role for Emx2 in regulating the *Cux2* enhancer, we focused on Lmx1a for further functional analysis for two primary reasons: (1) Lmx1a expression is tightly restricted to the cortical hem, in an identical pattern to the Cux2 enhancer; conversely, Emx2 expression extends into the adjacent hippocampal primordium and neocortical neuroepithelium, where the Cux2 enhancer is inactive; and (2) Emx2 over-expression in NE-4C cells could still activate the mutant enhancer, but this mutant enhancer was inactive in the cortical hem.

Lmx1a occupies cortical hem-specific *Cux2* enhancer in the developing telencephalon

Our *in vitro* and *in vivo* interrogation of the *Cux2* enhancer revealed Lmx1a to be a possible regulator of enhancer activity. To determine whether Lmx1a occupies the endogenous *Cux2* enhancer locus in the early forebrain, we harvested whole forebrains from E12.5 mice and performed chromatin immunoprecipitation (ChIP) using three different Lmx1a antibodies (Fig. 7A). Following ChIP, we used

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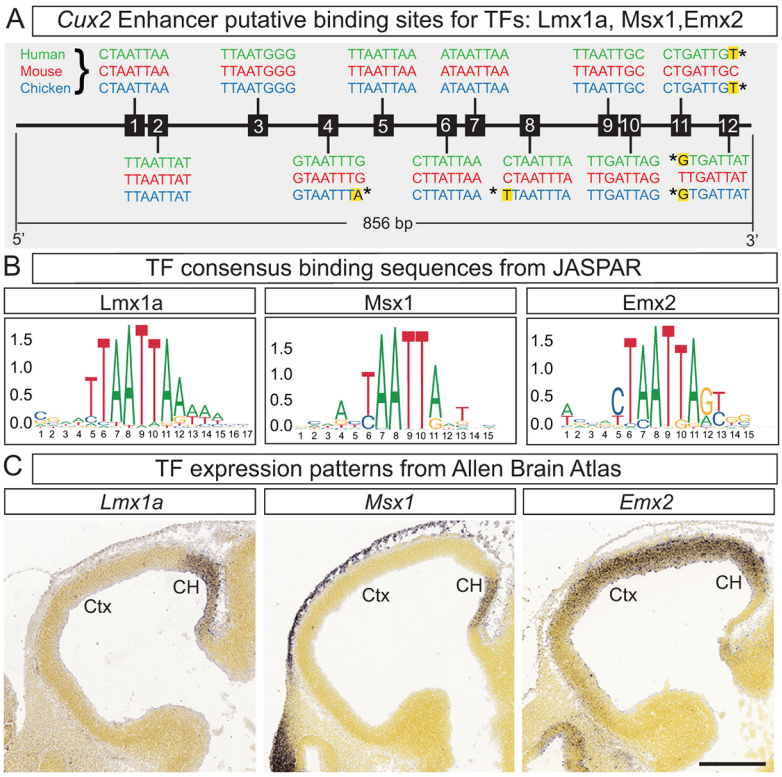

Fig. 4. The *Cux2* enhancer contains multiple predicted binding sites for forebrain-patterning transcription factors expressed in the cortical hem. (A) Schematic of the *Cux2* enhancer with putative binding sites for Emx2, Lmx1a and Msx1, predicted from the JASPAR database at >80% threshold. Predicted sites are numbered 1-12. Color-coded sequences show species conservation for human (*Homo sapiens*), mouse (*Mus musculus*) and chicken (*Gallus gallus*): asterisk indicates a single-base mismatch in one species. (B) JASPAR motifs for consensus binding site sequences of the candidate TFs. (C) Sagittal sections from the Allen Developing Mouse Brain Atlas *in situ* hybridization database showing mRNA expression of candidate TFs in the cortical hem of E11.5 mouse forebrains (Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas: Lmxa1, http://developingmouse.brain-map.org/experiment/show/100058786; Msx1, http://developingmouse.brain-map.org/experiment/show/100092693; Emx2, http://developingmouse.brain-map.org/experiment/show/100047257.) Scale bar: 400 µm. Ctx, neocortex; CH, cortical hem.

qPCR to probe for *Cux2* enhancer enrichment compared with non-specific IgG control ChIP (Fig. 7B) and with a different nearby enhancer region lacking Lmx1a-binding sites (Fig. 7C). Compared with the negative control IgG, the hem-specific *Cux2* enhancer was significantly enriched by ChIP using two out of the three Lmx1a antibodies (Fig. 7B, Aviva and ProSci), and trended toward enrichment in the third (Millipore). None of the three Lmx1a antibodies significantly pulled down a nearby enhancer region that lacked Lmx1a-binding sequences (Fig. 7C), confirming specificity for the *Cux2* cortical hem enhancer region. These data indicate that Lmx1a occupies the *Cux2* enhancer *in vivo*, where it may act to initiate *Cux2* expression specifically in the cortical hem.

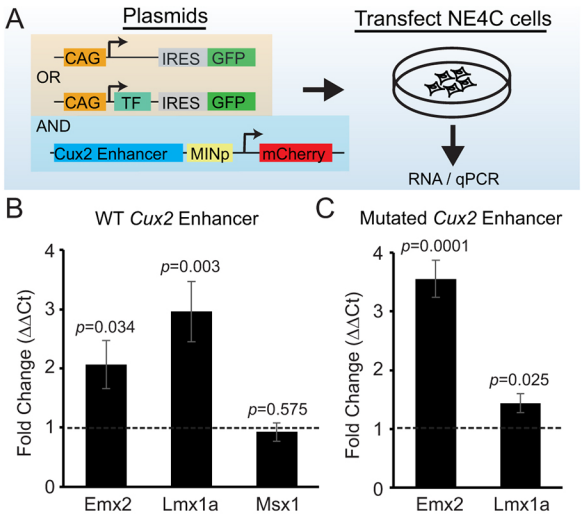

Fig. 5. Lmx1a strongly activates the *Cux2* enhancer *in vitro*. (A) Schematic of experimental workflow. The *Cux2*Enhancer-mCherry plasmid was transfected into NE-4C cells together with either empty pCIG vector (CAG-IRES-GFP) or pCIG that expresses candidate TFs (CAG-TF-IRES-GFP). The effects of candidate TFs on expression of *Cux2*Enhancer-mCherry in NE-4C cells was quantified using RT-qPCR of *mCherry* mRNA. (B,C) RT-qPCR quantification of *mCherry* transcripts. Data are mean fold change (±s.e.m.) over pCIG vector alone (dotted line), calculated using the ΔΔCt method. (B) Expression from the Cux2 enhancer is activated by expression of Emx2 and Lmx1a, but not Msx1. (C) When putative TF-binding sites were mutated in the *Cux2* enhancer, Emx2 still activated the *Cux2* enhancer but Lmx1a-mediated activation of the enhancer was greatly attenuated.

Lmx1a gain of function extends spatial competence for *Cux2* enhancer activation

In the developing telencephalon, Lmx1a is expressed strongly throughout the DTM, where it functions to promote cortical hem fate and suppress hippocampal and neocortical fate (Caronia-Brown et al., 2014; Chizhikov et al., 2010; Failli et al., 2002). The previously described sharp border of *Lmx1a* expression between the cortical hem and hippocampus is very similar to that of *Cux2*, which is expressed strongly throughout the cortical hem (Franco et al., 2012; Saulnier et al., 2013; Zimmer et al., 2004) but only weakly in a limited number of progenitors and neurons in the hippocampus and neocortex (Franco et al., 2012; Zimmer et al., 2004) (Fig. 1C,D). As Lmx1a is not expressed at all in the developing hippocampus where *Cux2* expression is initially weak, this provided us with an opportunity to assess whether mis-expression of Lmx1a is sufficient to ectopically activate the *Cux2* enhancer. To test this possibility, we co-electroporated our CAG-Lmx1a-IRES-GFP and *Cux2*Enhancer-Cre constructs into the medial cortex of $Ai9^{fl/+}$ Cre-reporter embryos *in utero* at E12.5. We allowed the embryos to continue developing until E14.5 and analyzed the percentage of electroporated cells (GFP^{+}) that activated the *Cux2* cortical hem enhancer ($tdTomato^{+}$) (Fig. 8A). Lmx1a overexpression in the hippocampus was verified by IHC (Fig. 8C, arrowheads). Compared with the empty vector control (Fig. 8B,B′), Lmx1a mis-expression (Fig. 8C,C′) resulted in a significant increase in

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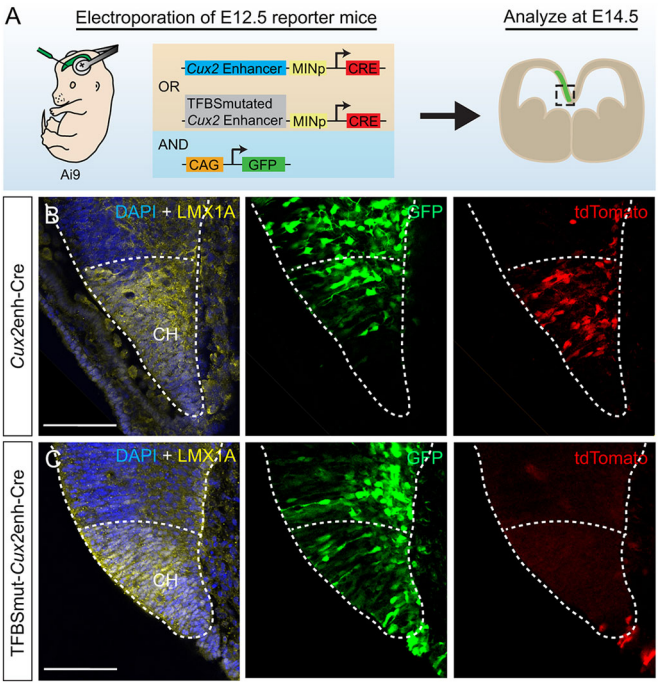

Fig. 6. TFBS-mutated *Cux2* enhancer activity abolished *in vivo*. (A) Schematic of experimental workflow. *Ai9* reporter embryos were electroporated *in utero* at E12.5 with either the wild-type *Cux2*enhancer-Cre plasmid or the mutated version that is no longer activated by Lmx1a. CAG-GFP was co-electroporated as a marker of the electroporated cells. Forebrains were harvested at E14.5 for analysis of recombination. (B,C) Coronal sections of electroporated brains showing the dorsal midline region, including cortical hem. Boundary between the cortical hem and hippocampal primordium is marked by expression of LMX1A protein (yellow). Sections were counterstained for nuclei with DAPI (blue). Activation of the wild-type *Cux2* enhancer in electroporated GFP$^+$ cells led to robust recombination in only the cortical hem (B), whereas the TFBS-mutated enhancer lost all activity in the cortical hem (C). Scale bars: 100 µm. CH, cortical hem.

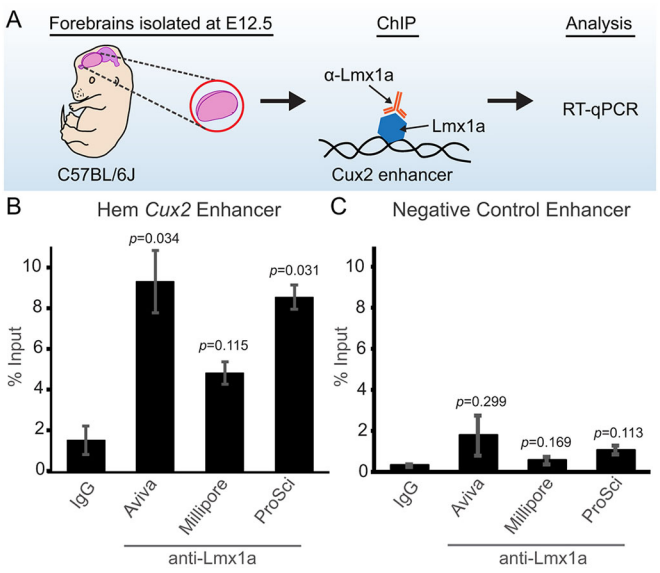

Fig. 7. ChIP-qPCR of E12.5 forebrains confirms Lmx1a occupancy of the endogenous *Cux2* enhancer locus. (A) Schematic of experimental workflow. E12.5 *C57BL/6J* forebrains were harvested for chromatin immunoprecipitation (ChIP)-qPCR validation of Lmx1a enrichment at the endogenous *Cux2* enhancer genomic locus. (B) qPCR of *Cux2* enhancer enrichment using three different anti-Lmx1a antibodies, compared with the anti-rabbit IgG negative control. Data are mean percentage input (±s.e.m.). (C) Lmx1a ChIP-qPCR quantification shows no significant enrichment of a nearby enhancer region that lacks Lmx1a binding sites. Data are mean percentage input (±s.e.m.)

ectopic enhancer activation in the hippocampal primordium adjacent to the cortical hem (Fig. 8D). These data indicate that Lmx1a is sufficient to activate the *Cux2* enhancer *in vivo*.

Lmx1a knockdown abolishes activation of the *Cux2* enhancer in the cortical hem

The results of our Lmx1a gain-of-function experiments as well as our *in vitro* results with the mutated *Cux2* enhancer point to a sufficiency and necessity, respectively, for Lmx1a to activate this enhancer in a spatially restricted manner. To further determine whether Lmx1a is necessary to activate the *Cux2* enhancer, we designed a loss-of-function strategy to knockdown Lmx1a expression in the cortical hem *in vivo* by *in utero* electroporation (Fig. 9A). We co-electroporated constructs expressing either a non-targeting control shRNA or Lmx1a-targeting shRNA along with *Cux2*Enhancer-mCherry and CAG-IRES-GFP into the DTM of E12.5 embryos. We allowed the embryos to continue developing until E14.5 and quantified the number of mCherry$^+$ cells among the electroporated (GFP$^+$) population (Fig. 9A). Lmx1a knockdown resulted in a ∼50% decrease in Lmx1a protein signal in electroporated cells (Fig. 9B,C). Compared with non-targeting controls, Lmx1a knockdown resulted in a drastic decrease in the number of electroporated cells that activated the *Cux2* enhancer (mCherry$^+$GFP$^+$/GFP$^+$) (Fig. 9D-F). Furthermore, in the remaining knockdown cells that did activate the Cux2 enhancer, there was a significant decrease in the levels of mCherry reporter expression compared with non-targeted control cells (Fig. 9D,E,G). Together, these data indicate that Lmx1a is necessary for *Cux2* enhancer activation in the cortical hem, thereby providing further support for the role of Lmx1a as a critical regulator of *Cux2* expression.

Multiple Lmx1a-binding sites are a shared feature among cis-regulatory elements active in the cortical hem

Previous work has revealed a number of forebrain enhancers, including some that appear to have restricted cortical hem activity similar to the murine *Cux2* enhancer (Pattabiraman et al., 2014; Visel et al., 2008). Images of transgenic animals from the VISTA Enhancer Browser (Visel et al., 2007) show that human enhancer elements hs411, hs611 and hs643 can drive cortical hem-specific *lacZ* expression in transgenic mouse embryos (Fig. 10A-C). We reasoned that all three enhancers might share features that regulate their activity through a common mechanism, given their very similar spatial and temporal transcriptional activity. To uncover common features between hs611, hs411 and hs643, the sequences of all three genomic regions, together with the murine *Cux2* hem enhancer, were analyzed using Analysis of Motif Enrichment (McLeay and Bailey, 2010) through the MEME Suite web portal (meme-suite.org/tools/ame). Compared with 1004 shuffled control sequences, the most enriched motif shared by all four elements was a TTAATTAA motif (P=1.48e-6 by Fisher's exact test). We then ran this top-enriched motif through the Tomtom tool (Gupta et al., 2007) in MEME Suite to compare it with databases of known motifs. The sequence was identified as an Lmx1a consensus binding motif by JASPAR, Jolma and Uniprobe databases (Fig. 10D), indicating that the Lmx1a consensus binding motif is the top enriched motif found in common between all four enhancer sequences.

We next used the JASPAR database to search all three human enhancer elements for putative Lmx1a-binding sites (>85%

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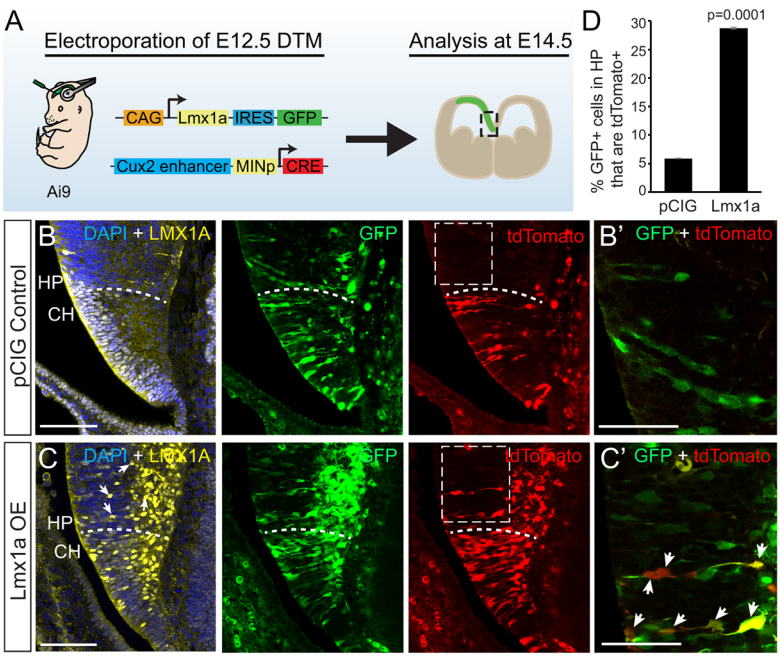

Fig. 8. Ectopic activation of the *Cux2* cortical hem enhancer by Lmx1a overexpression. (A) Schematic of experimental workflow. *Ai9* embryos were electroporated *in utero* at E12.5 with a constitutive Lmx1a expression plasmid along with *Cux2*Enhancer-Cre, targeting the DTM. Forebrains were harvested at E14.5 for quantification of the percentage of electroporated cells that were recombined. (B-C′) Coronal sections of electroporated brains showing the dorsal midline region. (B,C) Boundary between the cortical hem (CH) and hippocampal primordium (HP) is marked by expression of LMX1A protein (yellow) and a dotted line. Lmx1a overexpression was confirmed by immunohistochemistry (arrows in C). Sections were counterstained for nuclei using DAPI (blue). (B′,C′) Higher magnification views of the boxed areas in B,C, respectively, showing tdTomato expression in electroporated GFP$^+$ cells; arrows indicate double-positive cells. Compared with controls (B,B′), Lmx1a gain of function in the hippocampal primordium resulted in a significant increase in the number of recombined cells outside the cortical hem. (D) Quantification showing the mean percentage (±s.e.m.) of electroporated cells (GFP$^+$) that are recombined (tdTomato$^+$) outside the Lmx1a$^+$ cortical hem in control versus Lmx1a electroporations. Scale bars: 100 µm in B,C; 50 µm in B′,C′.

threshold). Similar to the murine *Cux2* cortical hem enhancer, hs611, hs411 and hs643 were all predicated to contain multiple high-threshold Lmx1a-binding sites (Fig. 10E). To determine whether Lmx1a was required for activation of any of these hem enhancers, we generated an hs643-mCherry reporter construct and electroporated it into the DTM at E12.5. Analysis at E14.5 confirmed that hs643 activity was restricted to the cortical hem (Fig. 10F,F′). Next, we knocked down Lmx1a expression, as in

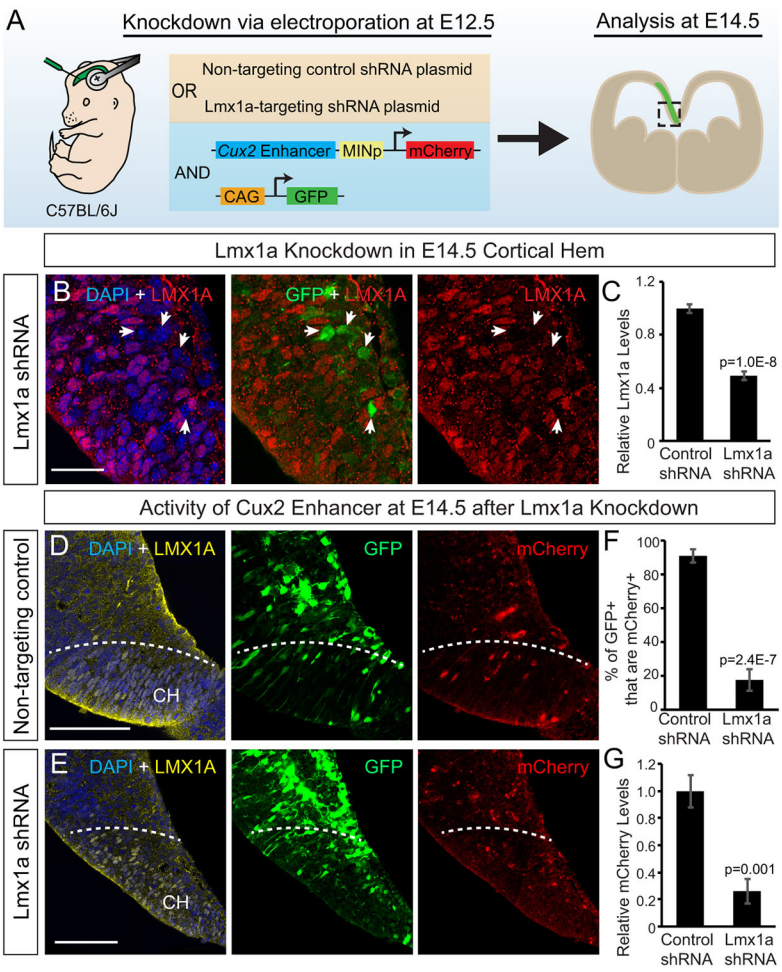

Fig. 9. Endogenous knockdown of Lmx1a greatly attenuates *Cux2* enhancer activation. (A) Schematic of experimental workflow. Knockdown of endogenous *Lmx1a* mRNA was induced by *in utero* electroporation of E12.5 *C57BL/6J* embryos with constructs expressing either targeting or non-targeting shRNAs for Lmx1a together with *Cux2*Enhancer-mCherry and CAG-GFP. Forebrains were harvested at E14.5 to quantify the percentage of electroporated (GFP$^+$) that expressed mCherry. (B,C) Lmx1a knockdown resulted in a decrease in relative Lmx1a average signal intensity in electroporated GFP$^+$ cells. Arrows in B indicate electroporated Lmx1a knockdown cells. (D,E) Coronal sections of electroporated brains showing the cortical hem (CH). The boundary between the cortical hem and hippocampal primordium is marked by expression of Lmx1A protein (yellow) and a dotted line. Sections were counterstained for nuclei with DAPI (blue). (F) Quantification showing mean percentage (±s.e.m.) of electroporated cells that expressed mCherry in control versus Lmx1a knockdown cells in the cortical hem. (G) Relative mCherry expression quantified only in electroporated cells that expressed mCherry above background levels. Compared with control, Lmx1a knockdown resulted in a significant decrease in the number of mCherry$^+$/GFP$^+$ cells in the cortical hem, as well as a decrease in mCherry intensity in mCherry$^+$/GFP$^+$ cells. Scale bars: 25 µm in B; 100 µm in D,E.

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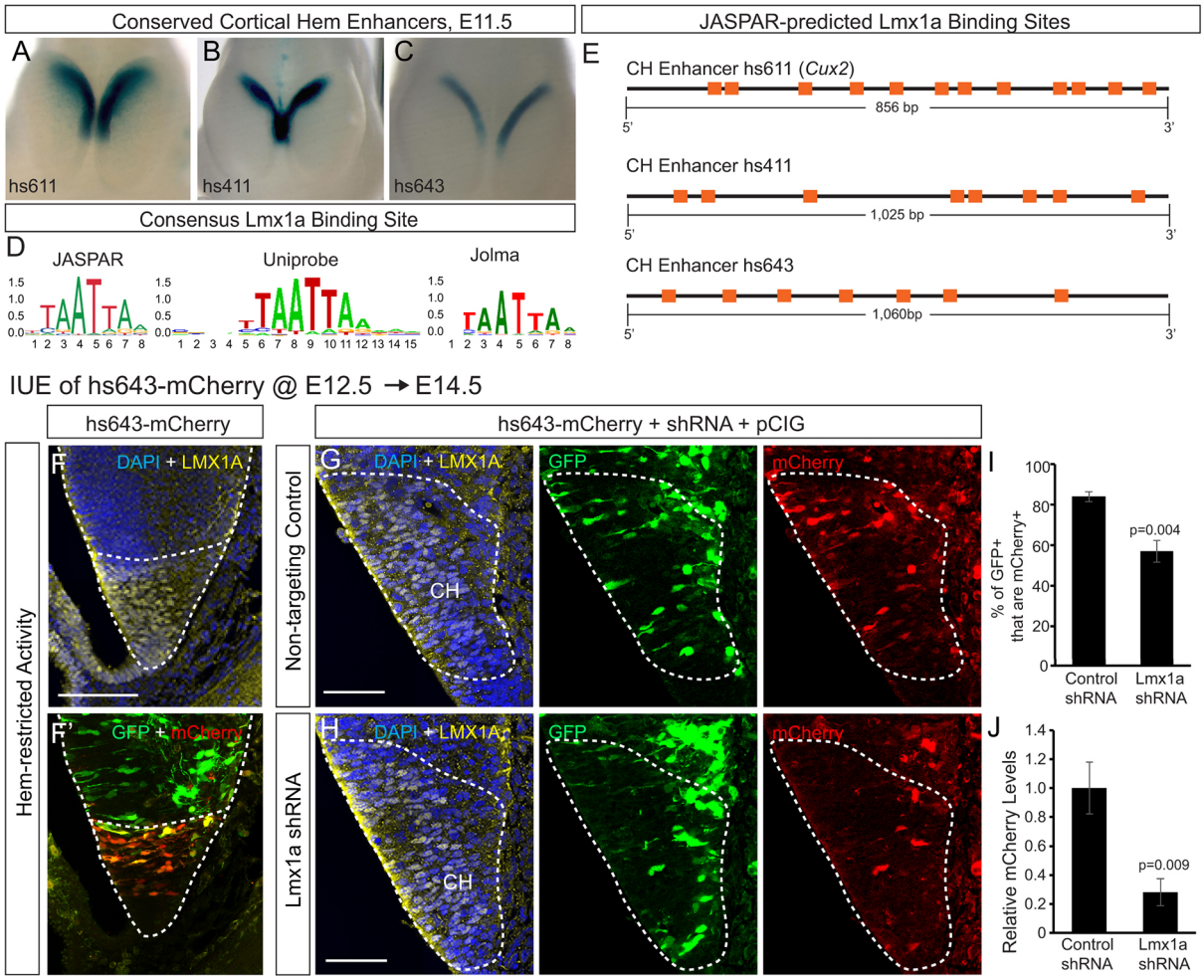

Fig. 10. Lmx1a-binding sites are a common feature of enhancers active in the developing cortical hem. (A-C) Examples of human enhancer elements driving *lacZ* expression in transgenic mouse embryos. Whole-mount staining images from the Vista Enhancer Browser show activity of hs611 (A), hs411 (B) and hs643 (C) in the cortical hem region. (D) Analysis of Motif Enrichment followed by Tomtom comparison to known motifs identified Lmx1a consensus binding sites as the most significantly enriched motif in the four hem-expressed enhancers. (E) Using the JASPAR database, all cortical hem enhancers were predicted to contain seven or more high-threshold Lmx1a-binding sites. (F,F′) Coronal sections of brains electroporated with hs643-mCherry and CAG-GFP, showing the spatially restricted activity of enhancer Hs643 within the Lmx1a^{+}(yellow) cortical hem. (G,H) Cortical hems electroporated with either control (G) or Lmx1a-targeting shRNA (H) and hs643-mCherry and CAG-GFP. (I,J) Control and Lmx1a-knockdown cortical hems were quantified for the percentage of electroporated GFP^{+} cells that expressed mCherry (I) and for relative mCherry levels of mCherry^{+}/GFP^{+} cells (J). Compared with control, Lmx1a knockdown resulted in a significant decrease in the number of mCherry^{+} cells in the cortical hem, as well as a decrease in the relative expression of mCherry. Boundary between the cortical hem and hippocampal primordium is marked by expression of Lmx1A protein (yellow) and a dotted line. Sections were counterstained for nuclei with DAPI (blue). Scale bars: 100 µm in F,F′; 50 µm in G,H.

Fig. 9A, and tested the effects of knockdown on hs643 enhancer activity compared with non-targeting control shRNA (Fig. 10G,H). Similar to our results with the Cux2 enhancer, we found that Lmx1a knockdown decreased both the percentage of electroporated cells that expressed the mCherry reporter (Fig. 10I) and the levels with which the positive cells expressed the reporter (Fig. 10J). These data support a role for Lmx1a as a genuine regulator of hs643 in the cortical hem. As a common feature among cortical hem enhancers, the presence of multiple Lmx1a-binding sites may indicate that Lmx1a sits near the top of the GRN that is active in the developing cortical hem, perhaps as a pioneering TF.

DISCUSSION

Using a combination of *in silico*, *in vitro* and *in vivo* approaches, we characterized a *Cux2* gene regulatory element with the goal of uncovering key TFs involved in the transcriptional regulation of *Cux2* in neural progenitors. We showed that this *Cux2* enhancer recapitulates a specific aspect of the complex *Cux2* expression pattern in the developing forebrain, namely strong and precise activity in the cortical hem. Further analysis uncovered the LIM homeobox transcription factor Lmx1a as a positive regulator of the *Cux2* cortical hem enhancer. Comparison of three cortical hem-specific human enhancer elements revealed recurring Lmx1a-binding sites were the most shared motif, raising the possibility that Lmx1a is master transcriptional regulator of the GRN that controls cortical hem identity.

Cux2 expression as a tool to uncover regulators of cell-fate decisions

We previously identified a subset of neural progenitors in the developing forebrain that are fate restricted to produce only corticocortical projection neurons in the neocortex (Franco and Müller, 2013; Franco et al., 2012; Gil-Sanz et al., 2015). These progenitors can be identified by expression of the transcription

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factor *Cux2*, and lineage-traced using *Cux2-Cre* and *Cux2-CreERT2* knock-in mice. Although Cux2 itself does not appear to control cell fate decisions in the forebrain (Cubelos et al., 2008, 2010), its restricted expression in defined subsets of neural progenitors may be a useful tool for uncovering transcriptional regulators of cell fate during forebrain patterning. The *Cux2* expression pattern in the developing forebrain is complex and dynamic (Franco et al., 2012; Gil-Sanz et al., 2015; Zimmer et al., 2004), suggesting that control of the *Cux2* locus may involve multiple transcriptional regulatory mechanisms. Using our *Cux2-Cre* mice crossed to a Cre-reporter line, we identified the DTM as one of the earliest sites of *Cux2* expression in the developing forebrain. In contrast to the salt-and-pepper pattern of *Cux2* expression in the adjacent hippocampus and neocortex, we find that essentially all neural progenitors in the cortical hem belong to the *Cux2* lineage. This raised the interesting possibility that *Cux2* expression in different parts of the developing forebrain are controlled by distinct mechanisms. This may also be the case for other transcription factors in the forebrain. For example, an identified enhancer region for Emx2 recapitulates most aspects of Emx2 expression in the forebrain, except for expression in the cortical hem, which is likely controlled by a different unidentified enhancer (Suda et al., 2010). Identification of the various cis-regulatory elements that drive differential *Cux2* expression, and the transcription factors that regulate these elements, may therefore lead to a better understanding of the GRNs that control tissue patterning and subtype fate specification in the developing forebrain.

Identification and characterization of a cortical hem-specific *Cux2* enhancer

Previous studies have identified genomic regions within intron 2 of the human and murine *Cux2* genes that can recapitulate *Cux2* expression in the DTM of transgenic mice (Hasenpusch-Theil et al., 2012; Visel et al., 2008). We found that the murine *Cux2* element exhibits features of an active enhancer in the E14.5 forebrain, including DNaseI hypersensitivity and histone modifications associated with transcriptionally active chromatin. This region also displays high levels of conservation from human to chicken, pointing towards an important functional role. Using *in utero* electroporation to test the *in vivo* activity of this region, we show that it drives expression specifically and robustly in the cortical hem neuroepithelium, but not in progenitors in the adjacent hippocampus or neocortex. These data indicate that this element is a developmentally active enhancer specific for the cortical hem. It will be interesting in future studies to determine which exact features of this enhancer are required for *Cux2* expression in the cortical hem, and whether this regulatory element is active in other *Cux2* expression domains that share features with the cortical hem, such as the rhombic lip in the hindbrain (Capaldo and Iulianella, 2016).

Lmx1a is a crucial regulator of the *Cux2* hem-specific enhancer

Using a bioinformatics approach, we identified several putative TF-binding sites in the *Cux2* enhancer. As known regulators of cortical hem development (Chizhikov et al., 2010; Tole et al., 2000b), Emx2, Lmx1a and Msx1 are a promising group of candidate TFs with the potential to regulate the *Cux2* enhancer in the cortical hem. Although we chose to focus here on these three candidates, this short list likely does not represent the full complement of TFs that can regulate Cux2 expression via this enhancer. Indeed, our analysis revealed a much longer list of putative TF-binding sites that we did not test further. For example, our analysis confirmed two putative Tcf-Lef1-binding sites that were identified in a previous study on the *Cux2* enhancer (Hasenpusch-Theil et al., 2012). Interestingly, that study showed that, although *Cux2 enhancer-lacZ* transgenic animals normally expressed the *lacZ* reporter specifically in the cortical hem, when the Tcf-Lef1-binding sites were mutated the reporter expression was much stronger and expanded beyond the cortical hem. These data raise the intriguing possibility that Wnt signaling through Tcf-Lef1 may actually repress activity of the *Cux2* enhancer outside the cortical hem. It will be interesting in future to test whether this pathway or potentially other repressors may function in combination with Lmx1a to restrict the Cux2 enhancer activity to the cortical hem, which would have important implications for understanding regional patterning in the developing forebrain.

With respect to the TFs we did test, we did not identify any effect of Msx1 on activity of the *Cux2* enhancer *in vitro*, indicating it may not be a direct regulator of *Cux2* cortical hem expression. On the other hand, both Emx2 and Lmx1a activated transcription from the *Cux2* enhancer. The majority of the predicted Emx2- and Lmx1a-binding sites overlapped each other, reflective of the similarity of their consensus binding motifs. Interestingly, mutation of these putative binding sites drastically reduced responsiveness of the enhancer to Lmx1a, but not to Emx2. This may suggest substantial redundancy in the number of Emx2-binding sites in the *Cux2* enhancer, or that there are Emx2-binding sites remaining that were not mutated and are more critical. Importantly, the mutated enhancer showed no activity in the cortical hem or adjacent hippocampus, suggesting that activation by Lmx1a is more relevant *in vivo* than activation by Emx2. This would be in line with our further experiments showing that the *Cux2* enhancer is not active in the hippocampus or neocortex, where Emx2 is strongly expressed but Lmx1a is absent. However, we do not rule out the possibility that Emx2 may still play an important role in activating the Cux2 hem enhancer, either directly or indirectly. Future studies using *Emx2* loss and gain of function *in vivo* should shed light on the role that Emx2 may play in regulating *Cux2* expression in the cortical hem, or possibly in the hippocampus and neocortex.

In further support of Lmx1a as an activator of *Cux2* expression, we showed that Lmx1a protein was significantly enriched at the *Cux2* enhancer genomic locus in isolated E12.5 forebrains compared with a second putative *Cux2* enhancer found within the same intron but that is devoid of Lmx1a-binding sites. As expression of both *Lmx1a* and *Cux2* commences much earlier than E12.5 in the DTM (Failli et al., 2002; Mangale et al., 2008), we would predict that Lmx1a regulates *Cux2* expression in the cortical hem during the earliest stages of forebrain development. We also found that modulating Lmx1a levels in the DTM affects *Cux2* enhancer activity. Whereas Lmx1a knockdown *in vivo* resulted in a substantial reduction in the ability of cortical hem cells to activate the *Cux2* enhancer plasmid, overexpression in the hippocampus resulted in ectoptic activation of the *Cux2* enhancer outside the cortical hem. Taken together with our mutated Lmx1a-binding site experiments, these results demonstrate that Lmx1a is sufficient and necessary for *Cux2* enhancer activation in the DTM. Interestingly, however, Lmx1a mis-expression in the neocortex did not result in activation of the *Cux2* enhancer construct (data not shown). We also noticed that the further away the hippocampal electroporated cells were from the cortical hem, the less likely they were to express the reporter plasmid (data not shown). This result may suggest the presence of additional factors that regulate *Cux2* expression. For example, Lmx1a may require a transcriptional co-activator for maximum activity that is missing from the neocortex. Alternatively

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or additionally, there may be unidentified transcriptional repressors of the *Cux2* enhancer that are expressed specifically in the hippocampus and neocortex. Further studies will be required to fully elucidate the mechanisms that control the complex expression pattern of *Cux2* in the different forebrain regions.

Lmx1a as a common activator of cortical hem GRNs

When we compared the murine and human Cux2 enhancers with two other conserved human elements that show activation in the cortical hem, we found that the most enriched motif in all four enhancers corresponds to the Lmx1a consensus binding site. Together with the fact that Lmx1a is one of the earliest markers of the DTM (Failli et al., 2002; Mangale et al., 2008), these data suggest that Lmx1a may sit near the top of the GRN involved in regulating cortical hem cell fate. In support of this idea, we demonstrated that endogenous Lmx1a knockdown greatly attenuated activation of one of these hem-specific enhancers, hs643. Additionally, previous work has shown that cortical hem identity is lost in *dreher* mutant mice in which *Lmx1a* is inactivated by a missense mutation (Chizhikov et al., 2010). The sharp border of Lmx1a expression between the cortical hem and adjacent hippocampal primordium further make it an ideal candidate for establishing precise patterns of gene expression during early patterning of the developing forebrain.

An important unanswered question is what lies upstream of Lmx1a during these early patterning stages? Previous work has reported that *Lmx1a* expression can be activated by BMP4 in the developing forebrain (Srinivasan et al., 2014; Watanabe et al., 2016). As a morphogenetic pathway that is specifically expressed within the cortical hem and choroid plexus, BMP signaling could potentially initiate the Lmx1a-dependent GRN that leads to specific DTM fates. Interestingly, previous studies have reported that upregulation of BMP signaling both in the developing chick olfactory epithelium (Wittmann et al., 2014) and murine mandibular neural crest cells (Bonilla-Claudio et al., 2012) results in significant upregulation of *Cux2* expression. Additionally, *Cux2* expression appears coincident with BMP4 within the mesenchyme of the developing mouse limb bud (Iulianella et al., 2003). How BMPs activate *Cux2* expression in these contexts has not been determined, but it would be interesting to test whether BMP signaling can drive *Cux2* expression in multiple tissues through Lmx1a-mediated activation of the conserved enhancer.

Conclusions

In this study, we identify a conserved enhancer and its transcriptional activator, Lmx1a, as an important mechanism for driving restricted expression of *Cux2* in the developing forebrain. We further show that recurrent Lmx1a-binding sites are a common motif shared in multiple enhancers with similarly restricted activities. These studies provide a template for future studies aimed at identifying other *Cux2* cis-regulatory elements that control its complex expression during forebrain development, and ultimately the upstream GRNs that specify different cell fates among the forebrain progenitor pool.

MATERIALS AND METHODS

Animals

Mice used for experiments were housed and handled in accordance with protocols approved by the UC Anschutz Medical Campus IACUC committee. The following mouse lines were used in this study: *Cux2-Cre* and *Cux2-CreERT2* (Franco et al., 2012, 2011; Gil-Sanz et al., 2015), *Ai9* (Madisen et al., 2010) and *C57BL/6J* (JAX Stock #000664). Mice of both sexes were analyzed in all experiments. Embryos were produced from timed-pregnant females, with noon on the day of the plug being designated E0.5. Tamoxifen induction of the *Cux2-CreERT2* line was performed by intraperitoneal injection of 2 mg tamoxifen (Sigma) dissolved in sunflower oil (Sigma) into pregnant mothers at the indicated age. Progesterone (Sigma) was co-administered at half the concentration of tamoxifen to prevent late abortions caused by the mixed-estrogen effects of tamoxifen. For postnatal analysis of tamoxifen-induced animals, pups were delivered by cesarean section at E19.5 and provided with a foster mother until analysis.

Plasmids and *in utero* electroporation

The murine *Cux2* enhancer was cloned from the endogenous genomic locus (NCBI37/mm9 chr5:122,482,512-122,483,367) using a Gblock (IDT) with 5′ and 3′ arms homologous to the multiple cloning site in the backbone vector. The Gblock was cloned by Gibson assembly into the pMinp vector (Wilken et al., 2015), immediately upstream of the TATA box. mCherry or Cre recombinase with a nuclear localization signal (Lewandoski and Martin, 1997) were cloned immediately downstream of the TATA box. Hs643-mCherry was cloned in a similar manner using a Gblock corresponding to human genomic locus GRCh38/hg38 chr9:23,004,731-23,005,790. To generate the TF-binding site mutant version of the *Cux2* enhancer, we synthesized a Gblock in which the central 8 bp of putative binding sites identified (excluding sites #8 and 10, Fig. 4A) were mutated to 5′ AAGCGCAA3′. Transcription factor cDNAs were either obtained from Addgene (Lmx1a, 45070; Msx1, 34998) or from IDT as Gene blocks (Emx2) and cloned into the *Sac*I and *Xma*I sites of the pCIG vector (Hand et al., 2005), between the CAG promoter and the IRES-GFP cassette. *In utero* electroporation of plasmids (0.5-1 mg/ml) was carried out as previously described (Franco et al., 2012; Gil-Sanz et al., 2013) on E12.5 embryos of timed-pregnant mice. Embryos were harvested for analysis at E14.5.

Immunohistochemistry

Brains from E9.5-14.5 embryos were dissected and fixed for 2 h at room temperature in 4% paraformaldehyde. Forebrains were sectioned on a vibrating microtome (Leica VT1200S) at 100 µm increments, or on a cryostat (Leica CM1520) at 15-30 µm increments. Immunohistochemistry was performed on tissue sections as described previously (Winkler et al., 2018) using the following antibodies: rabbit anti-Lmx1a (1:1000, Millipore, RRID:AB_10805970) and rabbit anti-RFP (1:500, LifeSpan Biosciences, RRID:AB_945213). Donkey secondary antibodies conjugated to Alexa Fluor 488, Rhodamine Red-X or Alexa Fluor 647 were purchased from Jackson ImmunoResearch and used at 1:500. Sections were imaged using a Zeiss LSM 780 confocal microscope.

Cell culture and qRT-PCR

Experiments were performed using the immortalized mouse neuroectodermal NE-4C cells (ATCC, CRL-2925) grown in Dulbecco's minimal essential media (MEM; Corning 10-010-CV) with 4 mM L-glutamine (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen) and penicillin (0.0637 g/l)-streptomycin (0.1 g/l). We did not re-authenticate or test for contamination after receiving the cells from ATCC. Cells were plated on 12-well plates and grown to ∼70% confluency prior to transfection. Cells were transfected with either CAG-Emx2, Lmx1a or Msx1-IRES-GFP, together with *Cux2*Enhancer-mCherry or the TF-binding site mutated *Cux2*Enhancer-mCherry for 4-6 h with Lipofectamine 3000 (Invitrogen), with subsequent media change. At 24 h after transfection, RNA was isolated from cells with an RNeasy Plus Kit (Qiagen) and reverse transcribed into cDNA using an iScript RT Kit (Bio-Rad). Expression of *mCherry*, *GFP* and the housekeeping gene cyclophilin A was assessed by qRT-PCR (Bio-Rad CFX Connect R-T System). Fold change was calculated using the delta-CT method for both *GFP* and *mCherry*, relative to cyclophilin A. Fold changes of *mCherry* mRNA were normalized to those of *GFP*, to account for variations in transfection efficiency. The following primers were used: cyclophilin A forward, GAGCTGTTTGCAGACAAAGTTC; cyclophilin A reverse, CCCTGGCACATGAATCCTGG; *eGFP* forward, ACGTAAA-CGGCCACAAGTTC; *eGFP* reverse. AAGTCGTGCTGCTTCATGTG; *mCherry* forward, GATAACATGGCCATCATCAAGGA; *mCherry* reverse, CGTGGCCGTTCACGGAG.

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Chromatin immunoprecipitation and qPCR

Chromatin-immunoprecipitation (ChIP) experiments for Lmx1a were performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling). Eight to ten E12.5 *C57BL/6J* forebrains per experiment (n=3 biological replicates) were harvested and then fixed with 1% EM-grade paraformaldehyde for 10 min (Electron Microscopy Sciences). The chromatin was sheared to ∼500 bp fragments by sonication (Diagenode Bioruptor ultrasonicator) at 10×30 s cycles ON/OFF at 100% power. ChIP was performed at 4°C overnight on 10 µg of DNA with 2 µg of three different rabbit antibodies against Lmx1a (Aviva, RRID:AB_387461; Millipore, RRID:AB_10805970; ProSci, RRID:AB_2316106), anti-Histone H3 as a positive control for enhancer pulldown and amplification (Cell Signaling), and anti-rabbit IgG as a negative control (Cell Signaling). After DNA purification, RT-qPCR was performed for the cortical hem *Cux2* enhancer (Chr5:122,482,512-122,483,367) (forward, CCACACTGTCTG-GGGACAGAAAGA; reverse, GCAGCCCTGGAAGCATGTAATTTG) as well as for a negative control region using SsoAdvanced Sybr Green (Bio-Rad). The negative control locus used was a putative enhancer located distally to the cortical hem-specific enhancer in *Cux2* intron 2 (Chr5:122,375,401-122,375,550) (forward, CCCCTCTTGAAGCTTCCT; reverse, GGTGATAGATGCAGGAGG).

Quantitative analysis of Lmx1a gain of function

CAG-Lmx1a-GFP or empty vector control along with the *Cux2*Enhancer-Cre constructs were electroporated into forebrains of E12.5 *Ai9*$^{fl/+}$ reporter embryos followed by quantification of tdTomato expression at E14.5. The electroporated and quantified region included the area spanning ∼150 µm directly above the cortical hem (demarcated by Lmx1a immunohistochemistry), corresponding to the hippocampal primordium with normally no *Cux2* enhancer activity. Harvested brains were sectioned and stained for Lmx1a, and imaged using a confocal microscope. The percentage of GFP$^+$ cells outside the hem that were also expressing tdTomato was quantified. Single-plane confocal images were used for quantification.

Quantitative analysis of Lmx1a loss-of-function

To knock down endogenous Lmx1a expression and assess *Cux2* enhancer activity, an Lmx1a-targeting shRNA construct (Sigma, SHCLNG-NM_033652, TRCN0000433282) was co-electroporated with the *Cux2*Enhancer-H2B-mCherry (or Enhancer hs643-H2B-mCherry) and pCIG constructs into the DTM of E12.5 *C57BL/6J* embryos. For control experiments, a pLKO.1 non-mammalian shRNA construct (Sigma, SHC002) was electroporated. Knockdown analysis was performed at E14.5. Specifically, harvested forebrains were sectioned, stained for Lmx1a (Millipore, RRID:AB_10805970) and imaged on a confocal microscope at the exact same acquisition settings for every image. Knockdown of Lmx1a was confirmed by measuring relative Lmx1a signal intensity in GFP$^+$ cells between knockdown and control cells (Zeiss Zen Blue 2.3, average pixel intensity). To quantify the effects of Lmx1a knockdown on enhancer activity, the percentage of GFP$^+$ cells in the cortical hem that were also expressing mCherry above background levels was quantified, as well as the relative intensity of mCherry expression only in the mCherry$^+$ cells, compared with control. Single-plane confocal images were used for quantification.

Statistics

All comparisons made were between two groups using a two-tailed, two-sample equal or unequal variance Student's t-tests to analyze all data. Equality of variance was determined using a Bartlett's test. The standard error of the mean (s.e.m.) is reported on all graphs.

Acknowledgements

We thank Joseph Brzezinski (University of Colorado – Anschutz Medical Campus, USA) for providing the minimal promoter plasmids.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.P.F., S.J.F.; Methodology: S.P.F., S.J.F.; Formal analysis: S.P.F., S.J.F.; Investigation: S.P.F., B.E.D., S.J.F.; Resources: B.E.D.; Writing - original draft: S.P.F., S.J.F.; Writing - review & editing: S.P.F., S.J.F.; Supervision: S.J.F.; Project administration: S.J.F.; Funding acquisition: S.J.F.

Funding

This work was supported by the Children's Hospital Colorado Program in Pediatric Stem Cell Biology and The Boettcher Foundation (S.J.F.).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.170068.supplemental

References

Bonilla-Claudio, M., Wang, J., Bai, Y., Klysik, E., Selever, J. and Martin, J. F. (2012). Bmp signaling regulates a dose-dependent transcriptional program to control facial skeletal development. *Development* **139**, 709-719.

Capaldo, E. and Iulianella, A. (2016). Cux2 serves as a novel lineage marker of granule cell layer neurons from the rhombic lip in mouse and chick embryos. *Dev. Dyn.* **245**, 881-896.

Caronia-Brown, G., Yoshida, M., Gulden, F., Assimacopoulos, S. and Grove, E. A. (2014). The cortical hem regulates the size and patterning of neocortex. *Development* **141**, 2855-2865.

Chizhikov, V. V., Lindgren, A. G., Mishima, Y., Roberts, R. W., Aldinger, K. A., Miesegaes, G. R., Currle, D. S., Monuki, E. S. and Millen, K. J. (2010). Lmx1a regulates fates and location of cells originating from the cerebellar rhombic lip and telencephalic cortical hem. *Proc. Natl. Acad. Sci. USA* **107**, 10725-10730.

Cubelos, B., Sebastián-Serrano, A., Kim, S., Moreno-Ortiz, C., Redondo, J. M., Walsh, C. A. and Nieto, M. (2008). Cux-2 controls the proliferation of neuronal intermediate precursors of the cortical subventricular zone. *Cereb. Cortex* **18**, 1758-1770.

Cubelos, B., Sebastián-Serrano, A., Beccari, L., Calcagnotto, M. E., Cisneros, E., Kim, S., Dopazo, A., Alvarez-Dolado, M., Redondo, J. M., Bovolenta, P. et al. (2010). Cux1 and Cux2 regulate dendritic branching, spine morphology, and synapses of the upper layer neurons of the cortex. *Neuron* **66**, 523-535.

Eckler, M. J., Nguyen, T. D., McKenna, W. L., Fastow, B. L., Guo, C., Rubenstein, J. L. R. and Chen, B. (2015). Cux2-positive radial glial cells generate diverse subtypes of neocortical projection neurons and macroglia. *Neuron* **86**, 1100-1108.

ENCODE Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57-74.

Failli, V., Bachy, I. and Rétaux, S. (2002). Expression of the LIM-homeodomain gene Lmx1a (dreher) during development of the mouse nervous system. *Mech. Dev.* **118**, 225-228.

Franco, S. J. and Müller, U. (2013). Shaping our minds: stem and progenitor cell diversity in the mammalian neocortex. *Neuron* **77**, 19-34.

Franco, S. J., Martinez-Garay, I., Gil-Sanz, C., Harkins-Perry, S. R. and Müller, U. (2011). Reelin regulates cadherin function via Dab1/Rap1 to control neuronal migration and lamination in the neocortex. *Neuron* **69**, 482-497.

Franco, S. J., Gil-Sanz, C., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S. R., Ramos, C. and Müller, U. (2012). Fate-restricted neural progenitors in the mammalian cerebral cortex. *Science* **337**, 746-749.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Gao, P., Postiglione, M. P., Krieger, T. G., Hernandez, L., Wang, C., Han, Z., Streicher, C., Papusheva, E., Insolera, R., Chugh, K. et al. (2014). Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell* **159**, 775-788.

García-Moreno, F. and Molnár, Z. (2015). Subset of early radial glial progenitors that contribute to the development of callosal neurons is absent from avian brain. *Proc. Natl. Acad. Sci. USA* **112**, E5058-E5067.

Gil-Sanz, C., Franco, S. J., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S. and Müller, U. (2013). Cajal-Retzius cells instruct neuronal migration by coincidence signaling between secreted and contact-dependent guidance cues. *Neuron* **79**, 461-477.

Gil-Sanz, C., Espinosa, A., Fregoso, S. P., Bluske, K. K., Cunningham, C. L., Martinez-Garay, I., Zeng, H., Franco, S. J. and Müller, U. (2015). Lineage tracing using Cux2-Cre and Cux2-CreERT2 mice. *Neuron* **86**, 1091-1099.

Grove, E. A., Tole, S., Limon, J., Yip, L. and Ragsdale, C. W. (1998). The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. *Development* **125**, 2315-2325.

Guo, C., Eckler, M. J., McKenna, W. L., McKinsey, G. L., Rubenstein, J. L. R. and Chen, B. (2013). Fezf2 expression identifies a multipotent progenitor for neocortical projection neurons, astrocytes, and oligodendrocytes. *Neuron* **80**, 1167-1174.

Gupta, S., Stamatoyannopoulos, J. A., Bailey, T. L. and Noble, W. S. (2007). Quantifying similarity between motifs. *Genome Biol.* **8**, R24.

Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, J. I.-T., Guerrier, S., Boutt, E., Peters, E., Barnes, A. P., Parras, C. et al. (2005). Phosphorylation of

Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. *Neuron* **48**, 45-62.

Hasenpusch-Theil, K., Magnani, D., Amaniti, E.-M., Han, L., Armstrong, D. and Theil, T. (2012). Transcriptional analysis of Gli3 mutants identifies Wnt target genes in the developing hippocampus. *Cereb. Cortex* **22**, 2878-2893.

Iulianella, A., Vanden Heuvel, G. and Trainor, P. (2003). Dynamic expression of murine Cux2 in craniofacial, limb, urogenital and neuronal primordia. *Gene Expr. Patterns* **3**, 571-577.

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M. and Haussler, D. (2002). The human genome browser at UCSC. *Genome Res.* **12**, 996-1006.

Khan, A., Fornes, O., Stigliani, A., Gheorghe, M., Castro-Mondragon, J. A., van der Lee, R., Bessy, A., Chèneby, J., Kulkarni, S. R., Tan, G. et al. (2018). JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. *Nucleic Acids Res.* **46**, D260-D266.

Lewandoski, M. and Martin, G. R. (1997). Cre-mediated chromosome loss in mice. *Nat. Genet.* **17**, 223-225.

Llorca, A., Ciceri, G., Beattie, R., Wong, F. K., Diana, G., Serafeimidou, E., Fernandez-Otero, M., Streicher, C., Arnold, S. J., Meyer, M. et al. (2018). Heterogeneous progenitor cell behaviors underlie the assembly of neocortical cytoarchitecture. *bioRxiv* 494088.

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R. et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* **13**, 133-140.

Mangale, V. S., Hirokawa, K. E., Satyaki, P. R. V., Gokulchandran, N., Chikbire, S., Subramanian, L., Shetty, A. S., Martynoga, B., Paul, J., Mai, M. V. et al. (2008). Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. *Science* **319**, 304-309.

McLeay, R. C. and Bailey, T. L. (2010). Motif Enrichment Analysis: a unified framework and an evaluation on ChIP data. *BMC Bioinformatics* **11**, 165.

Niwa, H., Yamamura, K.-I. and Miyazaki, J.-I. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.

Pattabiraman, K., Golonzhka, O., Lindtner, S., Nord, A. S., Taher, L., Hoch, R., Silberberg, S. N., Zhang, D., Chen, B., Zeng, H. K. et al. (2014). Transcriptional regulation of enhancers active in protodomains of the developing cerebral cortex. *Neuron* **82**, 989-1003.

Saulnier, A., Keruzore, M., De Clercq, S., Bar, I., Moers, V., Magnani, D., Walcher, T., Filippis, C., Kricha, S., Parlier, D. et al. (2013). The doublesex homolog Dmrt5 is required for the development of the caudomedial cerebral cortex in mammals. *Cereb. Cortex* **23**, 2552-2567.

Schlett, K. and Madarász, E. (1997). Retinoic acid induced neural differentiation in a neuroectodermal cell line immortalized by p53 deficiency. *J. Neurosci. Res.* **47**, 405-415.

Shen, Y., Yue, F., McCleary, D. F., Ye, Z., Edsall, L., Kuan, S., Wagner, U., Dixon, J., Lee, L., Lobanenkov, V. V. et al. (2012). A map of the cis-regulatory sequences in the mouse genome. *Nature* **488**, 116-120.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992a). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-690.

Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M. and Boncinelli, E. (1992b). Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J.* **11**, 2541-2550.

Srinivasan, S., Hu, J. S., Currle, D. S., Fung, E. S., Hayes, W. B., Lander, A. D. and Monuki, E. S. (2014). A BMP-FGF morphogen toggle switch drives the ultrasensitive expression of multiple genes in the developing forebrain. *PLoS Comput. Biol.* **10**, e1003463.

Suda, Y., Kokura, K., Kimura, J., Kajikawa, E., Inoue, F. and Aizawa, S. (2010). The same enhancer regulates the earliest Emx2 expression in caudal forebrain primordium, subsequent expression in dorsal telencephalon and later expression in the cortical ventricular zone. *Development* **137**, 2939-2949.

Tole, S., Goudreau, G., Assimacopoulos, S. and Grove, E. A. (2000a). Emx2 is required for growth of the hippocampus but not for hippocampal field specification. *J. Neurosci.* **20**, 2618-2625.

Tole, S., Ragsdale, C. W. and Grove, E. A. (2000b). Dorsoventral Patterning of the Telencephalon Is Disrupted in the Mouse Mutant extra-toesJ. *Dev. Biol.* **217**, 254-265.

Visel, A., Minovitsky, S., Dubchak, I. and Pennacchio, L. A. (2007). VISTA Enhancer Browser–a database of tissue-specific human enhancers. *Nucleic Acids Res.* **35**, D88-D92.

Visel, A., Prabhakar, S., Akiyama, J. A., Shoukry, M., Lewis, K. D., Holt, A., Plajzer-Frick, I., Afzal, V., Rubin, E. M. and Pennacchio, L. A. (2008). Ultraconservation identifies a small subset of extremely constrained developmental enhancers. *Nat. Genet.* **40**, 158-160.

Visel, A., Taher, L., Girgis, H., May, D., Golonzhka, O., Hoch, R. V., McKinsey, G. L., Pattabiraman, K., Silberberg, S. N., Blow, M. J. et al. (2013). A high-resolution enhancer atlas of the developing telencephalon. *Cell* **152**, 895-908.

Watanabe, M., Fung, E. S., Chan, F. B., Wong, J. S., Coutts, M. and Monuki, E. S. (2016). BMP4 acts as a dorsal telencephalic morphogen in a mouse embryonic stem cell culture system. *Biology Open* **5**, 1834-1843.

Wilken, M. S., Brzezinski, J. A., La Torre, A., Siebenthall, K., Thurman, R., Sabo, P., Sandstrom, R. S., Vierstra, J., Canfield, T. K., Hansen, R. S. et al. (2015). DNase I hypersensitivity analysis of the mouse brain and retina identifies region-specific regulatory elements. *Epigenet. Chromatin* **8**, 8.

Winkler, C. C., Yabut, O. R., Fregoso, S. P., Gomez, H. G., Dwyer, B. E., Pleasure, S. J. and Franco, S. J. (2018). The dorsal wave of neocortical oligodendrogenesis begins embryonically and requires multiple sources of sonic hedgehog. *J. Neurosci.* **38**, 5237-5250.

Wittmann, W., Iulianella, A. and Gunhaga, L. (2014). Cux2 acts as a critical regulator for neurogenesis in the olfactory epithelium of vertebrates. *Dev. Biol.* **388**, 35-47.

Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S. and Aizawa, S. (1997). Emx1 and Emx2 functions in development of dorsal telencephalon. *Development* **124**, 101-111.

Zimmer, C., Tiveron, M.-C., Bodmer, R. and Cremer, H. (2004). Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. *Cereb. Cortex* **14**, 1408-1420.

Table S1

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