

Published in final edited form as:

*J Neurosci.* 2010 August 4; 30(31): 10551–10562. doi:10.1523/JNEUROSCI.5563-09.2010.

## LIM-homeobox gene *Lhx5* is required for normal development of Cajal-Retzius cells

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### Abstract

Cajal-Retzius (C-R) cells play important roles in the lamination of the mammalian cortex via reelin secretion. The genetic mechanisms underlying the development of these neurons have just begun to be unraveled. Here we show that two closely related LIM homeobox genes *Lhx1* and *Lhx5* are expressed in *reelin*<sup>+</sup> cells in various regions in the mouse telencephalon at or adjacent to sites where the C-R cells are generated, including the cortical hem, the mantle region of the septal/retrobulbar area and the ventral pallidum. Whereas *Lhx5* is expressed in all of these reelin-expressing domains, *Lhx1* is preferentially expressed in the septal area and in a continuous domain spanning from lateral olfactory region to caudomedial territories. Genetic ablation of *Lhx5* results in decreased *reelin*<sup>+</sup> and *p73*<sup>+</sup> cells in the neocortical anlage, in the cortical hem and in the septal, olfactory, and caudomedial telencephalic regions. The overall reduction in number of C-R cells in *Lhx5* mutants is accompanied by formation of ectopic *reelin*<sup>+</sup> cell clusters at the caudal telencephalon. Based on differential expression of molecular markers and by fluorescent cell tracing in cultured embryos, we located the origin of reelin<sup>+</sup> ectopic cell clusters at the caudomedial telencephalic region. We also confirmed the existence of a normal migration stream of reelin<sup>+</sup> cells from the caudomedial area to telencephalic olfactory territories in wild-type embryos. These results reveal a complex role for *Lhx5* in regulating the development and normal distribution of C-R cells in the developing forebrain.

### Keywords

cortex; homeodomain; *Lhx1*; migration; mouse; telencephalon

### Introduction

Cajal-Retzius (C-R) cells, which constitute one of the earliest neuronal cell types in the mammalian telencephalon, are involved in the establishment of the cortical laminar

organization by providing the secreted glycoprotein reelin. Loss of reelin function prevents preplate splitting and alters radial migration of cortical plate neurons (reviewed in Soriano and Del Rio, 2005).

Several findings have revealed the cortical hem as the main source of C-R cells and additional sites for their generation have been shown at the septal/retrobulbar area (sep), and near the pallial-subpallial boundary (psb) at the ventral pallium (VP) (Meyer et al., 1998; Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006; García-Moreno et al., 2007; Hanashima et al., 2007; Imayoshi et al., 2008; Inoue et al., 2008). More recently, origin sites at the thalamic eminence have also been proposed (Abellan et al., 2009; Tissir et al., 2009). C-R cells migrate tangentially to populate the dorsal telencephalon, but issues regarding their precise site of origin, migratory routes, and relative contribution of each of the different subpopulations, are still controversial.

A number of transcription factors and nuclear proteins have been implicated in the control of C-R cell differentiation, migration, or survival. Early observations pointed to a role of genes such as *Tbr1*, *Pax6*, *Emx1* and *Emx2* (Mallamaci et al., 2000; Hevner et al., 2001; Muzio and Mallamaci, 2003; Stoykova et al., 2003). The transcription factor *Foxg1* represses the neocortical reelin<sup>+</sup> cell fate *in vivo* (Hanashima et al., 2004; Muzio and Mallamaci, 2005) and *in vitro* (Shen et al., 2006) (Hanashima et al., 2007). Factors related to particular reelin-expressing cell subpopulations have recently been identified: *p73* (Meyer et al., 2002), *p21* (Siegenthaler and Miller, 2008) and *Zic1-3* (Inoue et al., 2008) for septal- and hem-derived cells, *Er81* for septal-derived cells (Zimmer et al., 2010) and *Ebf2* for cells of VP origin (Hanashima et al., 2007). A microarray analysis designed to uncover the molecular determinants of C-R cell differentiation revealed a number of genes enriched in the transcriptome of mouse C-R cells (Yamazaki et al., 2004). Among them, known markers of C-R cells such as reelin, calretinin and *p73*, and the LIM homeobox genes *Lhx1* and *Lhx5* were found to be highly upregulated. *Lhx1* and *Lhx5* were recently confirmed to be expressed in C-R cells in chick, mouse, and macaque (Abellan et al., 2009).

In this study, we analyzed the role of *Lhx1* and *Lhx5* in C-R cell development. We found that both *Lhx1* and *Lhx5* are expressed in the septum and in a continuous domain that spans the olfactory cortex territory and the caudo-medial region adjacent to the diencephalon. Moreover, early *Lhx5* expression was found in midline structures including the cortical hem. Deletion of *Lhx5* caused an overall reduction of C-R cell density throughout the developing cortex and the appearance of reelin<sup>+</sup> ectopic cell clusters at the caudal end of the telencephalic vesicle. These results thus reveal a complex role for *Lhx5* and additionally suggest an early role for *Lhx1* in C-R cell development.

## Materials and methods

### Animals

Mouse lines maintained in a CD-1 background and carrying the following transgenic alleles were used: *Lhx5*-null (*Lhx5*<sup>-/-</sup>) (Zhao et al., 1999), *Lhx1*<sup>tau-lacZ</sup> (*Lhx1*<sup>tlz</sup>) (Kania et al., 2000) and *Ebf2-GFP* (GENSAT, <http://www.gensat.org/>) (Gong et al., 2003). Mice were crossed to generate mutant embryos lacking *Lhx5* function (*Lhx5*<sup>-/-</sup> or *Lhx5*<sup>-/-</sup>; *Lhx1*<sup>tlz</sup>). Controls were either wild-type or heterozygous littermates. Genotyping was performed by PCR using primers and conditions referenced for each mouse line. The day of detection of the vaginal plug was considered as embryonic day (E) 0.5. Staging of the embryos was confirmed using limb development as a reference (Kaufman, 1992). For whole-embryo cultures, wild-type and *Lhx5*<sup>-/-</sup> mutant embryos in CD1 or C57/B6 background were used. Pregnant females were euthanized by cervical dislocation or using CO<sub>2</sub> with minimum distress for the animal. Animals were housed and handled in compliance with National Institutes of Health

regulations, Mexican governmental guidelines regarding the use of laboratory animals for research purposes NOM-062-ZOO-1999, the University of California Chancellor's Animal Research Committee, the European Commission guidelines (86/609/CEE) and the committee for animal care and use of the Cajal Institute.

### Tissue preparation

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and washed in PBS. Brains were extracted and the telencephalic vesicles were carefully isolated. To prepare frozen sections, tissue was submerged in 30% sucrose/PBS overnight and embedded in Tissue Tek OCT compound (Miles, Elkhart, IN). Coronal sections (10 µm) were cut and mounted on Superfrost-plus slides (Thermo-Fisher Scientific, Waltham, MA), dried for 30 min and stored at -70°C. For histological analysis, brains were dehydrated, embedded in paraffin, and sectioned (20 µm). The sections were rehydrated and Nissl-stained following standard protocols.

### In situ hybridization (ISH)

Single chromogenic and double fluorescent ISH (FISH) was performed as described elsewhere (Varela-Echavarría et al., 1996; Ramírez-Amaya et al., 2005). Digoxigenin (DIG) or fluorescein (FITC)-labeled antisense riboprobes were synthesized by *in vitro* transcription using the following cDNA templates: *Cux2* and *Er81* (Zimmer et al., 2004), *Lhx1* (Fujii et al., 1994), *Lhx5* (Sheng et al., 1997), *reelin* (D'Arcangelo et al., 1997), *Wnt3a* (Parr et al., 1993), *Wnt5a* (Muzio and Mallamaci, 2005) and the IMAGE clones (Invitrogen, Carlsbad, CA): *Ebf2* (No. 6306804), *p21* (No. 5326128), *p73* (No. 6826464) and *Tbr1* (No. 6817237). For double FISH, DIG-labeled *Lhx5* or *Ebf2* probe was mixed with FITC-labeled *reelin* probe during the hybridization step and detected sequentially. Detection was carried out essentially as described in Ramírez-Amaya et al. (2005), with some modifications. After RNase A treatment, quenching of endogenous peroxidase was performed by using 1% H<sub>2</sub>O<sub>2</sub>/1X SSC. The slides were then incubated for 30 min in Blocking Reagent (TSA with 5% goat serum, PerkinElmer Life Science, Waltham, MA). Overnight incubation with an anti-DIG-HRP antibody (Roche, Basel, Switzerland, 1:800 in TSA) was performed at 4°C. Slides were washed in Tris-buffered saline (supplemented with 0.05% Tween-20, TBS-T) and signal was revealed with a Cyanine 3 substrate kit (PerkinElmer Life Science), 1:100 for 45 min. Sections were then treated with 1% H<sub>2</sub>O<sub>2</sub>/TBS to quench residual HRP activity. Incubation with anti-FITC-HRP antibody (Jackson ImmunoResearch, West Grove, PA, 1:400 in TSA) was carried out overnight at 4°C. After washing in TBS-T, the FITC-HRP conjugated antibody was detected using a FITC substrate kit (PerkinElmer Life Science), 1:100 for 15 min. Nuclear counterstaining was performed with DAPI (1:500 in TBS) prior to slide mounting (Vectashield Mounting Medium, VECTOR, Burlingame, CA).

### X-gal staining and immunohistochemistry (IHC)

Sections were washed in PBS, blocked in 5% heat-inactivated goat serum/0.1% Tween-20/PBS for 1 h and incubated with primary antibodies overnight at 4°C in the same solution. Sections were then washed with PBS and incubated for 1 h with fluorophore-conjugated secondary antibodies. For detection of bromodeoxyuridine (BrdU), sections were treated additionally with 2N HCl for 20 min at 37°C, neutralized with 0.1 M borate buffer (pH 8.5) for 10 min, washed with PBS, and then processed for IHC. To analyze the expression of  $\beta$ -galactosidase ( $\beta$ -gal), brains were fixed for 30 min and processed for X-gal staining prior sectioning. The following antibodies were used for IHC: mouse monoclonal anti-reelin [164-496, Calbiochem, Darmstadt, Germany and MAB5364 (Clone G10), Chemicon, Billerica, MA]; rabbit anti-Lhx1/Lhx5 (Tsuchida et al., 1994); rabbit anti-Lhx1 (Poche et al., 2007); goat anti- $\beta$ -gal (4600-1409, Biogenesis, Kinston, NH), rabbit anti-calbindin D-28K (CB38, Swant, Bellinzona, Switzerland), rabbit anti-calretinin (7699/4, Swant), rabbit-anti-

Tbr1 (AB9616, Chemicon) and mouse monoclonal anti-BrdU (347580, BD Biosciences, San Jose, CA). Secondary antibodies coupled to Alexa 488, 546, 568 and 594 and Alexa 488 and 568-streptavidin were from Invitrogen (Carlsbad, CA). Biotin-SP-AffiniPure goat anti-rabbit and goat anti-mouse IgG (H+L) were from Jackson ImmunoResearch.

### BrdU- labeling

A BrdU solution (10mg/ml) was injected intraperitoneally (100 mg/kg) into pregnant mice at E11.5. After 30 min, pregnant mice were euthanized by cervical dislocation and embryos were rapidly extracted and fixed in 4% PFA/PBS followed by chromogenic ISH and IHC, as described above.

### Whole-embryo cultures

The procedure for embryo culture has been described in detail elsewhere (de Carlos et al., 1996). A 10 mM solution of carboxy-fluorescein diacetate succinimidyl ester in DMSO (CFDA SE, 557 M.W., V12883, Invitrogen) was injected with the aid of a pressure device under a dissecting microscope. The injected embryo was transferred to a glass bottle containing 4 ml of culture medium, which was placed in a rotor housed in an incubator for one day at 36°C with continuous gassing (95% O<sub>2</sub>, 5% CO<sub>2</sub>). The embryos were cultured in heat-inactivated rat serum obtained by centrifugation of blood immediately after extraction from adult donor animals. Serum was filtered through a 0.45 µm filter and supplemented with 2 mg/ml glucose and 200 IU/ml of a penicillin-streptomycin mix (Gibco, BRL, Grand Island, NY) just before use.

### Image acquisition

Bright-field and fluorescence images were obtained under Nomarski/bright-field and fluorescence/confocal microscopy, respectively, and processed with Adobe Photoshop software (Adobe Systems, San Jose, CA) equally for all genotypes analyzed. For double ISH/IHC (Suppl. Fig. 1), fluorescence and bright-field hybridization images were sequentially acquired from the same field. The bright-field image was digitally converted into a false-color image and displayed alone or merged with the fluorescence image for comparison. For confocal images, a 3–6 µm confocal slice was scanned sequentially for the different emissions and averaged (n=8) using an inverted Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany).

### Signal quantification and statistical analysis

ISH signal with the DIG-labeled *reelin* riboprobe was analyzed semi-quantitatively by determining pixel intensity of the reaction product using the Image J software (NIH, Bethesda, MD). Telencephalic vesicles of E12.5 mutant (n=6) and control (n=6) embryos were subjected to ISH in equal conditions. Bright-field pictures of laterally-oriented whole brains were acquired with identical lighting and camera settings and a 0.16 mm<sup>2</sup> square box at the lateral cortex was selected for analysis. For each individual telencephalic hemisphere, the positioning of the square was achieved by drawing a line at its base and making a triangle by extending two lines at 45° with respect to the base line. The square was then centered at the crossing of the lines as shown in Fig. 3E, F. Individual images obtained from each selected square were equally processed with Adobe Photoshop to improve signal to noise ratio. Images were further analyzed by a blind experimenter using the Multithreshold Isodata plugin of the Image J software that allowed the detection of above-threshold pixels as positive signal (red label in Fig. 3E', F'). The relative proportion of pixels above and below threshold was expressed as a percent of the area covered by signal. Statistical analysis using the SPSS software (SPSS Inc, Chicago, IL) was performed by applying an unpaired *t*-test to the data, giving a significant difference between groups with a *p* value < 0.005.



## Results

### ***Lhx5* is expressed in *reelin*-expressing territories in the developing mouse telencephalon**

To determine the expression patterns of *Lhx5* in relation to the proposed sites for C-R cell generation, we performed whole-mount ISH on the telencephalon from E10.5 to E12.5, the time window of C-R cell production in the mouse (Hevner et al., 2003). The expression of *reelin*, a specific marker for C-R cells, was analyzed in parallel.

As described previously (Zhao et al., 1999), *Lhx5* was strongly expressed in the dorsal telencephalic midline at E10.5 throughout the whole thickness of the medial neuroepithelial wall, including the cortical hem and the ventrally located choroid plexus territory. In addition, *Lhx5* was expressed in the septal area and in scattered cells in the dorsal telencephalon (Fig. 1A, C). The septal expression consisted of a continuous domain from the septum to the lateral side of the rostroventral telencephalon around the retrobulbar region. At this early stage, *Reelin*<sup>+</sup> cells were concentrated at the rostral pole, although faint expression could already be detected at the caudo-medial telencephalic wall (CMTW) (Fig. 1B, D).

At E11.5, the expression of *Lhx5* persisted in the septal area and at the rostral and caudal ends of the cortical hem (Fig. 1G). A clearly discernible stripe of *Lhx5*<sup>+</sup> cells was detected superficially in a lateral region corresponding to the prospective olfactory cortex (pOC). This domain of expression seemingly followed the lateral olfactory tract (LOT), curving caudally and ventrally reaching a site in the ventral region of the CMTW adjacent to its boundary with the diencephalon (vCMTW; arrows in Fig. 1E, G). Interestingly, the region occupied by the lateral component of this domain appeared to coincide with the location of the *reelin*<sup>+</sup> domain derived in part from the *Dbx1*<sup>+</sup>-lineage at the VP/PSB (Fig. 1F) (Bielle et al., 2005). Moreover, *reelin* was found to be expressed in the septal area and, to a lesser extent in the cortical hem, resembling the expression of *Lhx5*. In contrast to *Lhx5*, many *reelin*<sup>+</sup> cells were also detected in neocortical regions, confirming that migration of C-R cells is already taking place at this stage (Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006; García-Moreno et al., 2007).

ISH analysis on E12.5 brains in whole mount and in sections showed that *Lhx5* and *reelin* were expressed in the domain that spans the pOC and extends into the vCMTW as well as in the septal region at the borders of the telencephalon. In the cortical hem, while *reelin* was expressed at all rostro-caudal levels, *Lhx5* was only expressed at its caudal-most end (Fig. 1I–L, M–P). At this stage, numerous *reelin*- and few *Lhx5*-positive cells were found scattered in the dorsal telencephalon (Fig. 1I–L, M–P). Comparing the medial telencephalic expression of *Lhx5* between E11.5 and E12.5, it can be noted that the expression in the cortical hem becomes restricted to its caudo-ventral part by E12.5 and that the domain in the vCMTW appears to recede ventrally (Fig. 1G, K). This contrasts with the expression of *Lhx5* spanning the whole extent of the cortical hem neuroepithelium at earlier stages (Fig. 1C, G). Expression of *Lhx5* by this stage appeared generally in the outermost layer of the developing telencephalon, suggesting that it is mainly confined to post-mitotic cells except in restricted rostral septal and caudo-medial regions (Fig. 1M, O). This was addressed directly by labeling mitotically-active cells with a 30-min BrdU pulse followed by *Lhx5* ISH and BrdU immunodetection in E11.5 embryos. This analysis revealed that the *Lhx5*<sup>+</sup> cells occupying the mantle zone at olfactory and caudal septal regions were not labeled by BrdU and, hence, were not proliferating (Suppl. Fig. 1C–D).

Given the similarities between the expression patterns of *Lhx5* and *reelin* we asked whether *Lhx5*<sup>+</sup> cells in the developing telencephalon also expressed *reelin*. Using double FISH we were able to detect extensive cellular colocalization of *Lhx5* and *reelin* transcripts in the

marginal zone of the septal and pOC domains at E12.5 (Fig. 2A–C and data not shown). By this stage, the expression of *Lhx5* in the cortical hem is dramatically reduced compared to the high expression level observed at earlier stages, before the onset of *reelin* expression.

In order to examine cellular colocalization with reelin proteins, we performed double immunostaining on sections of E12.5 embryos with an antibody that recognizes both *Lhx5* and *Lhx1*, and an antibody against reelin. As shown in Fig. 2D, E, *Lhx5*<sup>+</sup> nuclei were found in reelin<sup>+</sup> cells in the marginal zone of both the septal and pOC regions (see below for description of *Lhx1* expression).

### **Deletion of *Lhx5* causes reduction and abnormal distribution of Cajal-Retzius cells**

Based on the observation of *Lhx5* expression at the dorsal telencephalic midline including the cortical hem and later in the reelin<sup>+</sup> cells in the marginal zone in the septum, olfactory cortex and ventro-medial telencephalon, we hypothesized that proper development of subsets of reelin<sup>+</sup> cells is dependent on *Lhx5* function. To determine such role, we analyzed null-mutant embryos lacking *Lhx5* function (Zhao et al., 1999). The telencephalon of mutant embryos was often slightly smaller than that of control littermates. Using whole-mount ISH we found a marked reduction in expression of *reelin* in the cortical hem as well as in the septal and pOC regions of *Lhx5* mutants (Fig. 3). As early as E11.5, numerous C-R (*reelin*<sup>+</sup>) cells were normally found throughout the cortical neuroepithelium (Fig. 3A). In contrast, only few such cells could be detected in the *Lhx5* mutant, and these were mainly located at the caudal telencephalic pole (Fig. 3B). This difference was more pronounced at E12.5 (Fig. 3E–H). Quantification of *reelin* ISH signal revealed a significant reduction in mutants compared to control littermates (control: 100%, mutant: 41.215.6%,  $p < 0.005$ ) (Fig. 3E, F). Notably, at the caudal telencephalon, ectopic *reelin*<sup>+</sup> cell clusters were consistently detected in the mutant brains from E11.5 onwards ( $n = 24/24$ ) (Fig. 3B, D, F, H). At E12.5, two *reelin*<sup>+</sup> ectopic clusters were generally found in the mutant telencephalon: a large one in the caudal pole extending from the medial to the lateral side of the telencephalon, and a small lateral cluster sometimes seen continuous to the large cluster. Despite some variability in their dorso-ventral location, both clusters seemed to be associated with the *reelin*<sup>+</sup> domain in the pOC (Fig. 3F, H). The reduction of the different *reelin*<sup>+</sup> domains and the presence of *reelin*<sup>+</sup> cell ectopias at the marginal layer of the caudal telencephalon in *Lhx5* mutants were further confirmed by examination of coronal sections of the brain from embryos at E12.5 (Fig. 3I–P).

An additional marker for C-R neurons is *p73*, a nuclear protein of the p53 family of tumor-suppressors. *p73* has been shown to be expressed during early telencephalic development in *reelin*<sup>+</sup> cells, particularly in the septum and cortical hem (Meyer et al., 2004; Yoshida et al., 2006; Inoue et al., 2008; Siegenthaler and Miller, 2008). By whole-mount ISH, we detected *p73*<sup>+</sup> cells in control embryos from E10–11.5. The *p73*<sup>+</sup> cells were present along the dorsal midline from septal to cortical hem regions and throughout the telencephalic vesicle albeit not as numerous as *reelin*<sup>+</sup> cells in the same regions (Fig. 4A, C, E). At E11.5, *p73* expression strongly labeled the vCMTW region whereas few scattered cells were found along the pOC (Fig. 4C, E). By E12.5, increased *p73* expression was observed in the developing neocortex, cortical hem, septum and in a continuous domain spanning pOC and vCMTW regions (Fig. 4G, I). In contrast, the vCMTW region of *Lhx5* mutants appeared abnormal from E10.5 to E12.5 by *p73* expression; clear malformations were detected as the typical curved shape at the caudal end of the hem territory was missing (Fig. 4B, F, J). Similar to *reelin*, in E12.5 *Lhx5* mutants, decrease in number of *p73*<sup>+</sup> cells was observed in the dorsal telencephalon, and in the septal, pOC, and vCMTW regions defined by *Lhx5* expression (Fig. 4H, J). Notably, only the rostral-most region of the pOC in the mutant contained *p73*<sup>+</sup> cells (Fig. 4H). Like *reelin*, *p73* expression labeled a large medio-lateral ectopic cell cluster at the caudal telencephalic pole (compare Fig 3F, H with Fig. 4H, J) and

a small lateral domain (Fig. 4H). Since *reelin* and *p73* expression was missing in the caudal cortical hem and in the vCMTW region adjacent to the diencephalon, and a nearby stream of ectopic cells was labeled instead, we deemed likely that the cells of these domains were abnormally located in the mutants.

Variable cellular ectopias were also found later, at E18.5, in the cortex of the *Lhx5* mutants. On Nissl-stained sections, we noticed that these ectopias were composed of cell clusters surrounded by cell-free areas, and mostly localized in occipital and perirhinal cortices (n=2/2) (Suppl. Fig. 2A–C). Despite the early alterations in C-R cell numbers, preplate splitting and cortical lamination did not seem to be affected in *Lhx5* mutants, as assessed by Nissl staining and by the expression of the laminar markers *Tbr1*, *Er81* and *Cux2* (Suppl. Fig. 2D–G).

In summary, lack of *Lhx5* function results in a strong reduction in C-R cells in the dorsal telencephalon and a marked decrease in *reelin* and *p73* expression in the cortical hem, septum, pOC, and vCMTW territories at E12.5. Moreover, ectopic cell clusters expressing *reelin* and *p73* were found in the caudal region of the mutant telencephalon from early developmental stages. These results indicate that *Lhx5* is required for the development and normal distribution of C-R cells.

### **Lack of *Lhx5* results in cortical hem shortening and reduction of *p21*<sup>+</sup> cells**

Since the number and distribution of C-R cells was found to be severely affected in *Lhx5* mutants, and these mutants display abnormalities in midline structures such as the hippocampus (Zhao et al., 1999), we further analyzed entire telencephalic vesicles for the expression of *Wnt5a* and *Wnt3a*, two markers normally expressed in the cortical hem (Yoshida et al., 2006) (Fig. 5). At E11.5, the expression of *Wnt5a* was absent from most of the hem territory in *Lhx5* mutants, in keeping with our previous findings (Fig. 5A, B) (Zhao et al., 1999). Likewise, *Wnt3a* expression was dramatically reduced from the cortical hem, although residual expression could be detected rostrally (Fig. 5C, D). Moreover, the cortical selector gene *Lhx2* (Mangale et al., 2008) was ectopically expressed in the medial telencephalic regions (Zhao et al., 1999), suggesting that most of the cortical hem territory adopts a neocortical fate in the mutants.

We also analyzed the expression of *p21*, a cyclin-dependent kinase inhibitor expressed in *reelin*<sup>+</sup> and *p73*<sup>+</sup> cells in the septum, cortical hem and in C-R cells at early stages of their development in the adjacent cortical neuroepithelium (Siegenthaler and Miller, 2008). Consistent with the cortical hem shortening and the decreased number of *reelin*<sup>+</sup> and *p73*<sup>+</sup> cells along the telencephalic midline, fewer *p21*<sup>+</sup> cells were found in the septum, cortical hem and medial telencephalon at E11.5 and E12.5, with the caudal domain of the hem being the most affected (Fig. 5E–H). Although no ectopic clusters were labeled by *p21*, aberrant expression was detected in the caudomedial region that appeared to give rise to the large ectopic cell clusters (arrows in Fig. 5F, H, see below).

### ***Lhx1* and *Ebf2* expression identifies subsets of *reelin*<sup>+</sup> cells affected in *Lhx5* mutants**

To better dissect the *reelin*<sup>+</sup> cell phenotype in *Lhx5* mutants, we analyzed the expression of *Lhx1*, a LIM-HD factor closely related to *Lhx5* that is also expressed in C-R cells (Abellan et al., 2009). At E10, scattered *Lhx1*<sup>+</sup> cells appeared in the rostral-most region of the wild-type telencephalon (not shown). By E11.5 strong *Lhx1* expression was found in the septal and vCMTW regions but it was excluded from the cortical hem (Fig. 6A, C). BrdU labeling at this stage confirmed that *Lhx1* is expressed in post-mitotic cells in the septal and pOC regions (Suppl. Fig. 1A, B). At E12.5, we detected strong *Lhx1* expression in the pOC region and in scattered cells in the entire developing cortex (Fig. 6E, G and Suppl. Fig. 1E–G). By

double labeling of *Lhx1* and *reelin* on sections from wild-type embryos or embryos carrying the *Lhx1<sup>tlz</sup>* reporter allele (Kania et al., 2000) we detected *Lhx1/reelin*-double positive cells in pOC, vCMTW, septal, and neocortical regions (Suppl. Fig. 3A–H). We also found that cells labeled with the *Lhx1* reporter in all those regions expressed calretinin, a calcium-binding protein found in a large proportion of C-R cells but also present in other cell types (Suppl. Fig. 3I–L). Thus, a subset of C-R cells can be readily identified in the developing neocortex by double *reelin/Lhx1* expression.

ISH of E11.5 *Lhx5* mutants revealed a near-total absence of *Lhx1* expression in the pOC and a thinner *Lhx1<sup>+</sup>* domain in the vCMTW region (Fig. 6B, D). Interestingly, this last domain extended farther dorsally in the mutant than in controls, which appears complementary to the shortening defect of the cortical hem (Fig. 6C, D and Fig. 5A–D). By E12.5, we observed a dramatic decrease in *Lhx1* expression in the regions harboring *reelin<sup>+</sup>* cells in the septal, pOC, and vCMTW territories and in the neocortical primordium (Fig. 6E–H). Moreover, ectopic cell clusters located at the caudal *Lhx5<sup>-/-</sup>* telencephalon at E11.5 and E12.5 expressed *Lhx1* (asterisks in Fig. 6). Immunostaining at E12.5 revealed that those clusters coincide with those detected by *reelin* expression (Fig. 3E–H and 6W, X).

Similar results were obtained by analyzing the expression of the *Lhx1<sup>tlz</sup>* reporter in relation to the endogenous *Lhx1* gene expression. We confirmed the dramatic reduction of *Lhx1<sup>+</sup>* cells in the *Lhx5* mutant telencephalon and its presence in caudal ectopias (Fig. 6I–V). Our results thus reveal *Lhx1* expression in *reelin<sup>+</sup>* cells in pOC, vCMTW, and septal regions and in a subset of *reelin<sup>+</sup>* cells in the neocortex whose development is largely dependent on *Lhx5* function. Moreover, *Lhx5* seems to be required for the proper migration of *reelin/Lhx1*-expressing cells at the caudal telencephalon.

To further evaluate the effect of the lack of *Lhx5* in the development of *reelin<sup>+</sup>* subpopulations, we analyzed the expression of *Ebf2*, proposed to label VP-derived C-R cells (Yamazaki et al., 2004; Hanashima et al., 2007). We first evaluated the presence of *Ebf2* in C-R cells and in pOC regions by double FISH in control embryos and by *reelin* immunostaining of embryos carrying the GFP reporter gene expressed from the *Ebf2* promoter. Expression of *Ebf2* was detected in *reelin<sup>+</sup>* cells in the pOC and in most *reelin<sup>+</sup>* cells in the surface of the developing neocortex at E12.5 (Fig. 7A–E). We also compared expression of *Ebf2* in the telencephalon of *Lhx5* mutant and control littermates by whole-mount ISH (Fig. 7F–M). In E11.5 control embryos, strong *Ebf2* signal was detected throughout the telencephalic vesicle. At E12.5, the signal was particularly strong in the pOC and vCMTW regions (Fig. 7F, H, J). Analysis of *Lhx5* mutants revealed decreased expression in the lateral and caudal neocortical territories at E11.5, although in pOC regions it seemed unchanged at E12.5. Notably, marked *Ebf2* expression was detected in ectopic cell clusters at this stage (Fig. 7H–K). Large medio-lateral and small lateral clusters were detected by *Ebf2* expression as with the *reelin* probe.

### **Ectopic *reelin<sup>+</sup>* cell clusters in the caudal *Lhx5<sup>-/-</sup>* telencephalon originate from the caudo-medial telencephalic wall**

Seeking evidence to determine the origin of ectopically migrating cells, we performed lineage tracing experiments in cultured whole mouse embryos. The fluorescent permeable tracer CFDA was first injected in the germinative ventricular zone in the caudal part of the cortical hem (Fig. 8A–B, Suppl. Fig. 4A–I). We assessed the migratory behavior of cells derived from this region at E11.5 after 24 hours of *in toto* culture. In keeping with our previous results (García-Moreno et al., 2007), we found that injections in the cortical hem of control embryos label groups of cells that migrate tangentially towards the neocortex in a rostro-lateral direction. In contrast, equivalent injections in *Lhx5* mutant embryos labeled cells that failed to migrate tangentially and stayed close to the injection site (Fig. 8A–B,

Suppl. Fig. 4B-I). Notably, labeled cells were not found at ectopic positions in the mutant telencephalon. These data suggests that, at least 24 hours post-injection, the extent of cell migration from the caudal cortical hem is hampered in *Lhx5* mutant embryos, a finding that is consistent with the hem abnormalities detected by gene expression analysis.

We then applied the fluorescent tracer focally in E11.5 mutant embryos at the apparent site of origin of ectopically-migrating cells that form the large cluster to determine their origin. The injection site in the ventricular zone was directed to the dorsal end of the *Lhx1*, *p73*, and *reelin* expression domain in the vCMTW region (Fig. 8C, D and Suppl. Fig. 4B-2). When making this injection we took into account that, as described above, this domain extended farther dorsally in the mutants than in wild-type embryos. After one day of *in toto* culture, labeled cells in *Lhx5* mutants were found to migrate caudally in a pattern similar to that suggested by the ectopic expression of *reelin*, *p73*, *Lhx1* and *Ebf2* (n=3) (compare Fig. 8C, D with Figs. 3H, 4J, 6H and 7K). Moreover, most aberrantly-migrating cells expressed *reelin* and were localized at the caudo-medial cortex, at a location where *reelin*<sup>+</sup> cell ectopias were found by ISH (Fig. 8E-H). These experiments indicate that in *Lhx5* mutants, *reelin*<sup>+</sup> cells originating at E11.5 from the vCMTW region migrate aberrantly and form ectopic clusters in the caudal telencephalon.

### Ventral migratory route of *reelin*<sup>+</sup> cells originated at the caudomedial telencephalon

The demonstration that ectopic clusters of *reelin*<sup>+</sup> cells derive from the vCMTW region in *Lhx5* mutants suggested that this region could normally contribute to migratory *reelin*<sup>+</sup> cells in the nearby territory. Moreover, a previous study revealed the migration of *reelin*<sup>+</sup> cells from a broad region in the CMTW to the lateral telencephalon via a ventral route (Takiguchi-Hayashi et al., 2004). To map the site of origin of these migratory cells, we performed fluorescent tracer injections in E11.5 wild-type embryos and then cultured the embryos for 24 hours (n=6). Injections in the ventricular zone were directed to the vCMTW region (Suppl. Fig. 4A-2) and showed labeled cells that migrated radially to the external layer of the telencephalon and then tangentially in a ventral and lateral route around its caudal pole to converge rostrally in the pOC (Fig. 9A-I). The region populated by these caudo-medial-derived cells likely corresponds to the primordium of entorhinal and piriform cortices and matched with mantle regions labeled by *reelin*, *p73*, *Lhx5*, *Lhx1*, and *Ebf2* expression (Figs. 1, 4, 6, and 7). To further characterize the migratory cells along this path we performed immunohistochemical analysis of the CFDA-injected brains using several markers (Fig. 9J-O). Migratory cells appeared to belong to a pallial lineage, since most of them expressed the marker *Tbr1* (Fig. 9L) (Hevner et al., 2001; Hevner et al., 2003). Moreover, about 50% of the labeled cells expressed *reelin* and *calretenin* (Fig. 9K,M-O) and did not express *calbindin* (Fig. 9J), an expression profile suggestive of C-R cells (Hevner et al., 2003; Stoykova et al., 2003; García-Moreno et al., 2007). These data show that the vCMTW region adjacent to the diencephalon-telencephalon border gives rise to a number of migratory cells, including *reelin*-positive cells, that are born at E11.5 and travel long distances along the pOC, where they remain at least up to E12.5.

## Discussion

In this study we investigated the role of LIM-HD transcription factors *Lhx5* and *Lhx1* in the development of Cajal-Retzius cells in mice. We were able to identify three main regions rich in *reelin* expression at or adjacent to proposed sites of origin of C-R cells: rostrally, in the septal/retrobulbar area; medially, in the cortical hem; and laterally, near the VP/PSB region in a continuous domain also extending medially into the ventral CMTW region adjacent to the diencephalon. *Lhx1* and *Lhx5* were expressed in *reelin*<sup>+</sup> cells in septal, pOC, and vCMTW regions as well as in the neocortical anlage. The cortical hem, main source of C-R



cells, expressed high levels of *Lhx5* but lacked *Lhx1* expression at early developmental stages (E10.5–11.5).

The deletion of *Lhx5* resulted in a drastic reduction of C-R cells throughout the neocortex from early stages as assessed by the expression of *reelin*, *p73*, *Lhx1* and *Ebf2*. Moreover, whereas the septal, pOC, and vCMTW regions showed decreased expression of these markers, it was the telencephalic midline that appeared more severely affected. As early as E10.5, *p73*<sup>+</sup> cells were found in aberrant positions at the caudal region of the telencephalon. This correlates with the impairment in dorsal midline specification in *Lhx5* mutants revealed by the medial expansion of neocortical regions (*Lhx2*<sup>+</sup>) at the expense of the hem (*Wnt5a*<sup>+</sup>, *Wnt3a*<sup>+</sup>) and choroid plexus (*Bmp7*<sup>+</sup>) (Zhao et al., 1999 and this study). The severe shortening of the cortical hem in *Lhx5* mutants is likely to account for the decreased *p21* expression domain at the caudo-medial telencephalon and for most of the reduction in C-R cell numbers. Indeed, fluorescent labeling of mutant embryos in a region that in wild-type embryos would correspond to the caudal cortical hem, yielded few migratory cells and those few stayed close of the injection point.

In wild-type mice we confirmed that *Ebf2*, shown previously to be expressed in mature C-R cells (Chowdhury et al., 2010) of putative VP origin (Yamazaki et al., 2004; Hanashima et al., 2007), is expressed in *reelin*<sup>+</sup> cells in the neocortex and in the pOC and vCMTW regions at E12.5. Moreover, *Lhx1*, a gene predominantly expressed in *reelin*<sup>+</sup> and *calretinin*<sup>+</sup> cells in septal, pOC, and vCMTW regions but absent from the cortical hem at the peak of C-R cell generation, was also found in a fraction of neocortical *reelin*<sup>+</sup> cells. Hence, the observation in *Lhx5* mutants of decreased *Ebf2* and *Lhx1* expression in the neocortical primordium and in the septal, pOC, and vCMTW regions suggests that *Lhx5* controls the differentiation of C-R cells from other potential sources in addition to the cortical hem. As in previous studies (Bielle et al., 2005; Yoshida et al., 2006; Tissir et al., 2009), the drastic reduction of C-R cells in the dorsal telencephalon of *Lhx5* mutants did not result in *reeler*-like phenotypes such as cortical layer inversion, suggesting that compensatory mechanisms ensure a minimum of C-R cells to accomplish their main functions.

In the mutant embryos we found that the domain in the vCMTW region defined by expression of *Lhx1* which was extended dorsally in comparison to control embryos, gave rise to cells expressing *reelin*, *p73*, *Lhx1* and *Ebf2* that migrate and cluster ectopically in the caudal telencephalon (see diagram in Suppl. Fig. 4D). Their site of origin, however, appears to be a domain with properties that differ from those of the *reelin*-expressing domains found in this region in wild type embryos; it expresses *p21*, a marker of early differentiated hem-derived C-R cells as well as *Lhx1*, a gene expressed predominantly in the vCMTW region. Thus, ectopic cell migration in *Lhx5* mutants could be related to altered intrinsic properties of specific *reelin*<sup>+</sup> cell subsets or, alternatively, to non-cell autonomous modifications in the caudal telencephalon related to the loss of cortical hem and choroid plexus territories. Small clusters (*reelin*<sup>+</sup>, *Ebf2*<sup>+</sup>, and *p73*<sup>+</sup>) associated to the pOC were also found laterally with some variability in their location but their origin was not determined. At birth, aberrant cell clustering persisted caudally in olfactory cortical regions and, interestingly, cellular ectopias have also been reported in embryos carrying mutant alleles of *p73*, *Zic1-3* and *Tbr1*, also involved in C-R cell development (Hevner et al., 2001; Meyer et al., 2004; Inoue et al., 2008). Another medial telencephalic region in which migration may be altered in mutant embryos is the septal territory. At E11.5 there is a clear signal reduction only with the *reelin* probe. At E12.5, however, there appears to be a signal reduction with *reelin*, *p73*, *p21*, and, more dramatically, with the *Lhx1* probe. Aberrant migration of *reelin*<sup>+</sup> cells from or to this region might cause the differences observed with the various markers at both stages.

Recent studies have uncovered the prospective olfactory cortex as a complex territory where heterogeneous cell populations born both at local and distant telencephalic locations converge (Tomioka et al., 2000; García-Moreno et al., 2008). In keeping with this, the reelin-expressing population found in this region appears to be heterogeneous as well containing C-R and other olfactory cells. Reelin-expressing cells in this region have been shown to contain VP and dorsal midline derivatives (Bielle et al., 2005; Imayoshi et al., 2008) and our studies in wild-type mice revealed that it also contains cells originating at a site in the vCMTW region that reach the pOC by tangential migration. Hence, the reelin/*Lhx5*/*Lhx1*-expressing population in this region may also be a heterogeneous cell group with cell types from various sources. We found that in the migrating population from the vCMTW region, most cells possess a phenotype similar to that of C-R cells, as they express reelin, calretinin, and *Tbr1*, lack calbindin expression, and are located in the outermost layer of the telencephalon. At least a fraction of reelin-expressing cells, however, remains in this region at E13.5 (not shown) and thus appears to correspond to olfactory cell types rather than to C-R cells. These cells could have a guidance role, as has been described for LOT cells (Tomioka et al., 2000), but further investigation is required to determine their final location, identity and function.

Previous studies have proposed that reelin<sup>+</sup> migratory cells are generated at various sites of the medial telencephalic wall in addition to C-R cells from the cortical hem. These include the prospective choroid plexus (Imayoshi et al., 2008) and a broad region in the CMTW (Takiguchi-Hayashi et al., 2004). Another study proposed the thalamic eminence, a region rich in *p73*, *Lhx5*, and *reelin* expression as a source of C-R cells (Abellan et al., 2009; Tissir et al., 2009), but this remains to be confirmed. In our experiments in wild-type embryos, labeling was aimed at the ventricular surface of the caudomedial *Lhx1*/*Lhx5* expression domain adjacent to the telencephalon-diencephalon border and hence, it is not likely to label migratory cells at the mantle zone of this region in transit from other regions. Other migratory populations potentially originating from adjacent caudal subdomains such as the caudal cortical hem, the prospective choroid plexus, or even the diencephalon, however, may follow this route to the olfactory region. A precise characterization of all reelin<sup>+</sup> populations that originate at or migrate through this region will lead to a better understanding of this developmentally complex region.

Our results indicate that the main effect of the deletion of *Lhx5* in C-R cell development is due to impaired dorsal midline development. Since we did not detect an increase in apoptotic cell death at E11.5 and E12.5 in mutant embryos in other regions of the telencephalon where *Lhx5* is normally expressed [(Zhao et al., 1999) and data not shown], this transcription factor is likely to be involved in the control of early cell differentiation in those regions rather than in proliferation. Consistent with this, *Lhx5/1* have been implicated in the control of post-mitotic differentiation of specific cellular subsets in the hippocampus, cerebellum, retina and spinal cord (Zhao et al., 1999; Poche et al., 2007; Zhao et al., 2007). Moreover, our data reveals that *Lhx5* is also required for proper migration of a subset of reelin<sup>+</sup> cells. Overall, our findings show that *Lhx5* plays important roles in the development of C-R cells and of reelin<sup>+</sup> olfactory cells and that *Lhx1* is expressed in particular subsets of these cells from early developmental stages. As transcription factors, *Lhx5/1* must exert their function through the regulation of downstream target genes. Given the potential of LIM protein-protein interactions, intricate regulatory mechanisms between members of LIM-HD factors in the telencephalon are thus expected during the development of early telencephalic populations including C-R cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Part of this research was performed at the laboratory of Heiner Westphal at the National Institute of Child Health and Human Development, NIH, Bethesda, MD while A. V.-E. held a National Research Council Associateship Award. The work was supported by funds from the intramural research program of NICHD/NIH, The Wellcome Trust (GR071174AIA), Agencia Española de Cooperación Internacional (A/010585/07), BFU2007-60351 / BFI from Spanish MICINN and Mexican CONACYT (40286M). A. M. was supported by a fellowship from DGAPA-UNAM, D. F.-P. by a fellowship from CONACYT, F.G.-M. by a postdoctoral contract from the Comunidad Autónoma de Madrid - OLFACTOSENSE Consortium P-SEM-0255-2006 and M.L.C. by a Spanish predoctoral I3P fellowship. Technical support was provided by Anaïd Antaramián, Adriana González, Pilar Galarza, Martín García, Cristina González, Nydia Hernández, Alberto Lara, and Omar González. *Lhx1<sup>tlz/+</sup>* founder mice were kindly donated by Tom Jessell. We thank Tom Curran, Antonello Mallamaci and Magdalena Götz for providing probes and Richard Behringer for the rabbit Lhx1 antibody.

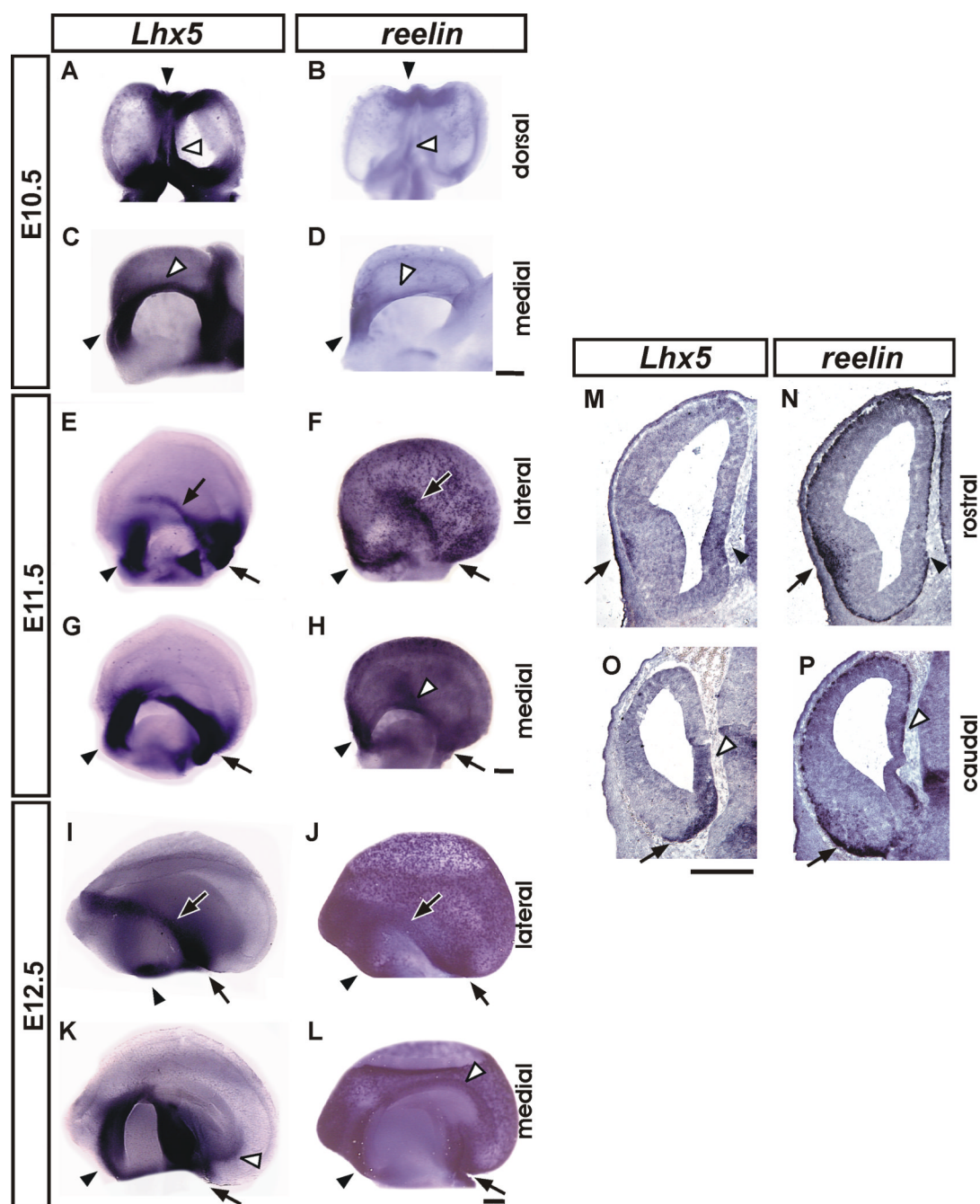
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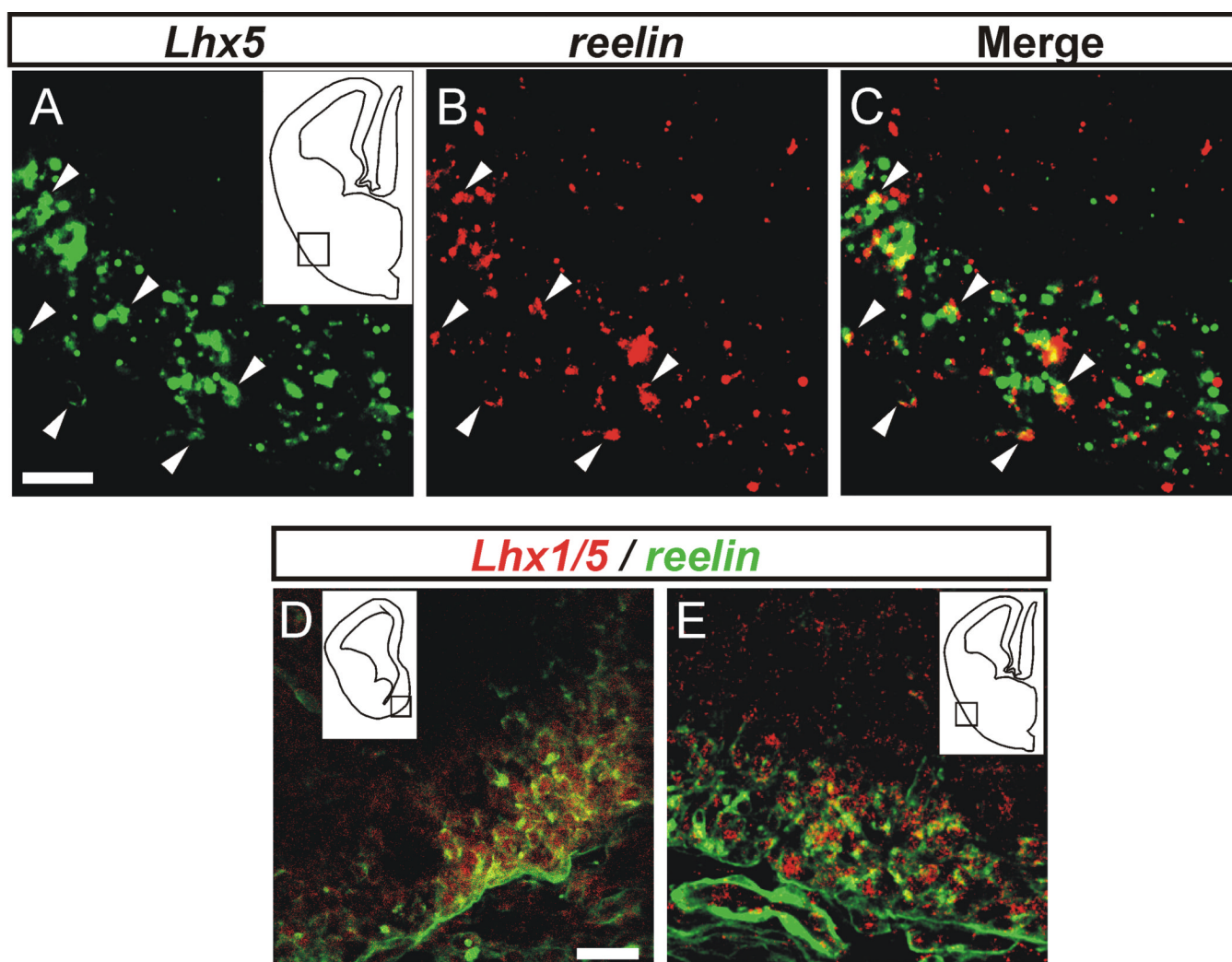
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**Figure 1.**

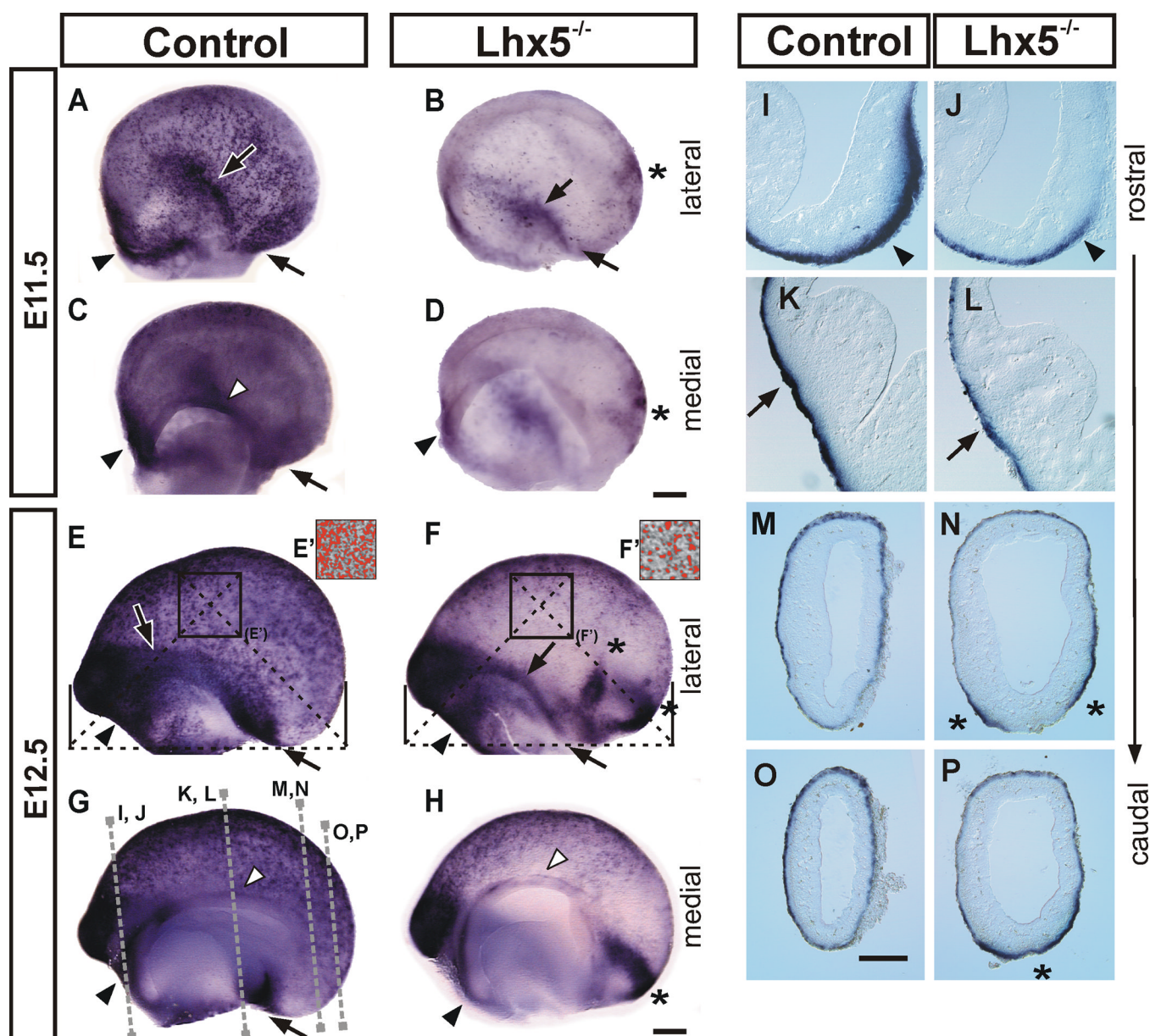
*Lhx5* and *reelin* expression in the developing mouse telencephalon. Expression was analyzed by whole-mount *in situ* hybridization on telencephalic vesicles from E10.5 (A–D), E11.5 (E–H) and E12.5 (I–L), shown in dorsal, medial and lateral views as indicated (rostral is to the top in A and B and to the left in C–L). (M–P) E12.5 coronal telencephalic sections of comparable rostral (M, N) and caudal (O, P) levels were analyzed by ISH for *Lhx5* (M, O) and *reelin* (N, P) (medial to the right). Black arrowheads point to the septal domain; black arrows indicate the location of the prospective olfactory cortex (pOC) domain and white arrowheads mark the location of the cortical hem. Scale bars: 400µm (A–D), 200µm (E–P).



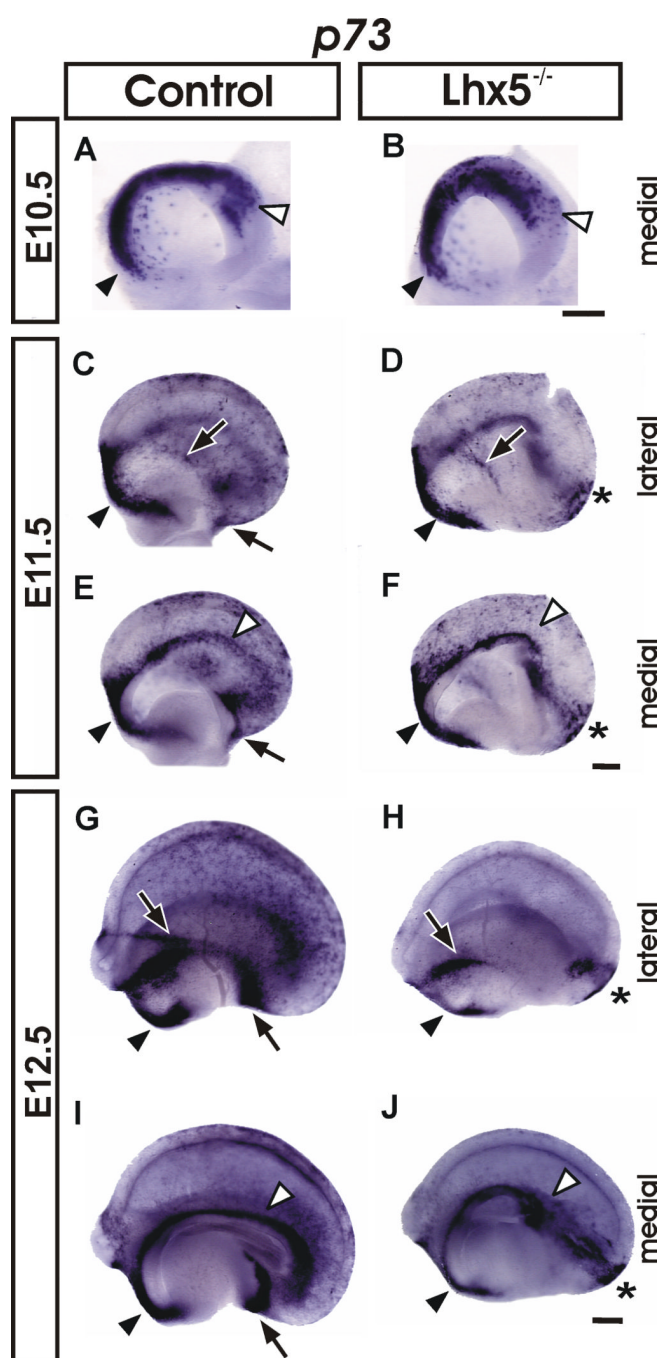
**Figure 2.**

Expression of *Lhx5/Lhx1* in *reelin*<sup>+</sup> cells. (A–C) Double fluorescent *in situ* hybridization on E12.5 coronal sections showing cellular colocalization of *Lhx5* (A) and *reelin* (B) transcripts on a 3μm-optical section acquired from the pOC domain, as indicated in the diagram in A. (C) Merged images (A and B). Arrowheads point to examples of colocalization of the *Lhx5/reelin* transcripts. (D and E) Double immunostaining of Lhx5/1 (red) and reelin (green) of coronal brain sections from E12.5 embryos. Location of images from septal (D) and pOC (E) domains are indicated in insets. Scale bar: 20μm.

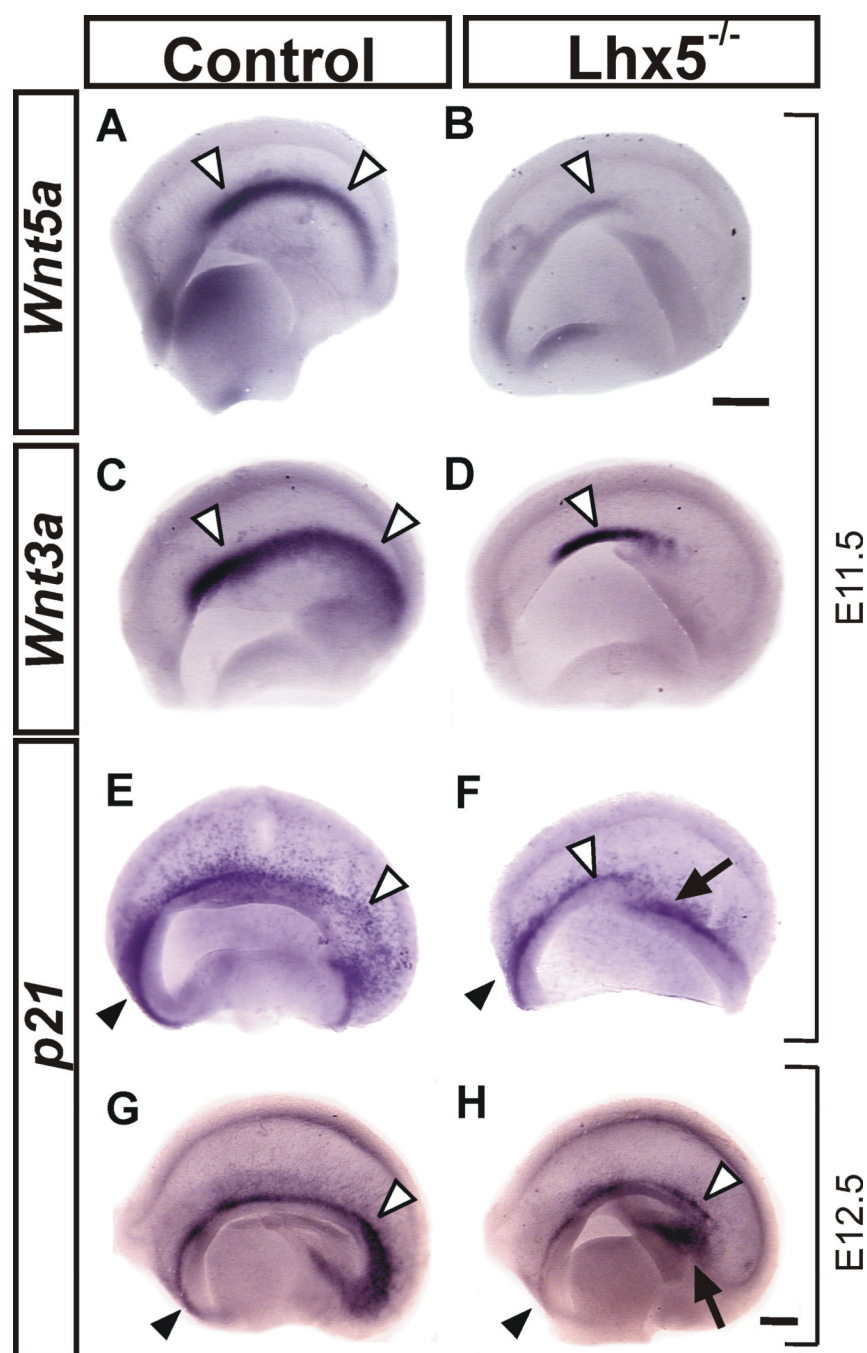




**Figure 3.** Abnormal development of Cajal-Retzius cells in *Lhx5* null mutant embryos. Whole-mount *reelin* *in situ* hybridization on E11.5 (A–D) and E12.5 (E–H) telencephalic vesicles from control and mutant (*Lhx5*<sup>−/−</sup>) littermates. As indicated, lateral and medial views of the telencephalon are shown (rostral is to the left). (E' and F') Over-threshold *reelin*<sup>+</sup> signal (red overlay) in control and mutant embryos, respectively, obtained from equivalent regions in the lateral cortex (squared boxes in E and F, see Materials and Methods for details). (I–P) Coronal sections showing expression of *reelin* in control and *Lhx5*<sup>−/−</sup> telencephalic hemispheres at the approximate rostro-caudal levels indicated by dotted lines in G (medial is to the right). For all panels: Black arrowheads, septal domain; black arrows, prospective olfactory cortex (pOC) domain; white arrowheads, cortical hem; asterisks, ectopic *reelin*<sup>+</sup> cell clusters. Scale bar: 200µm.

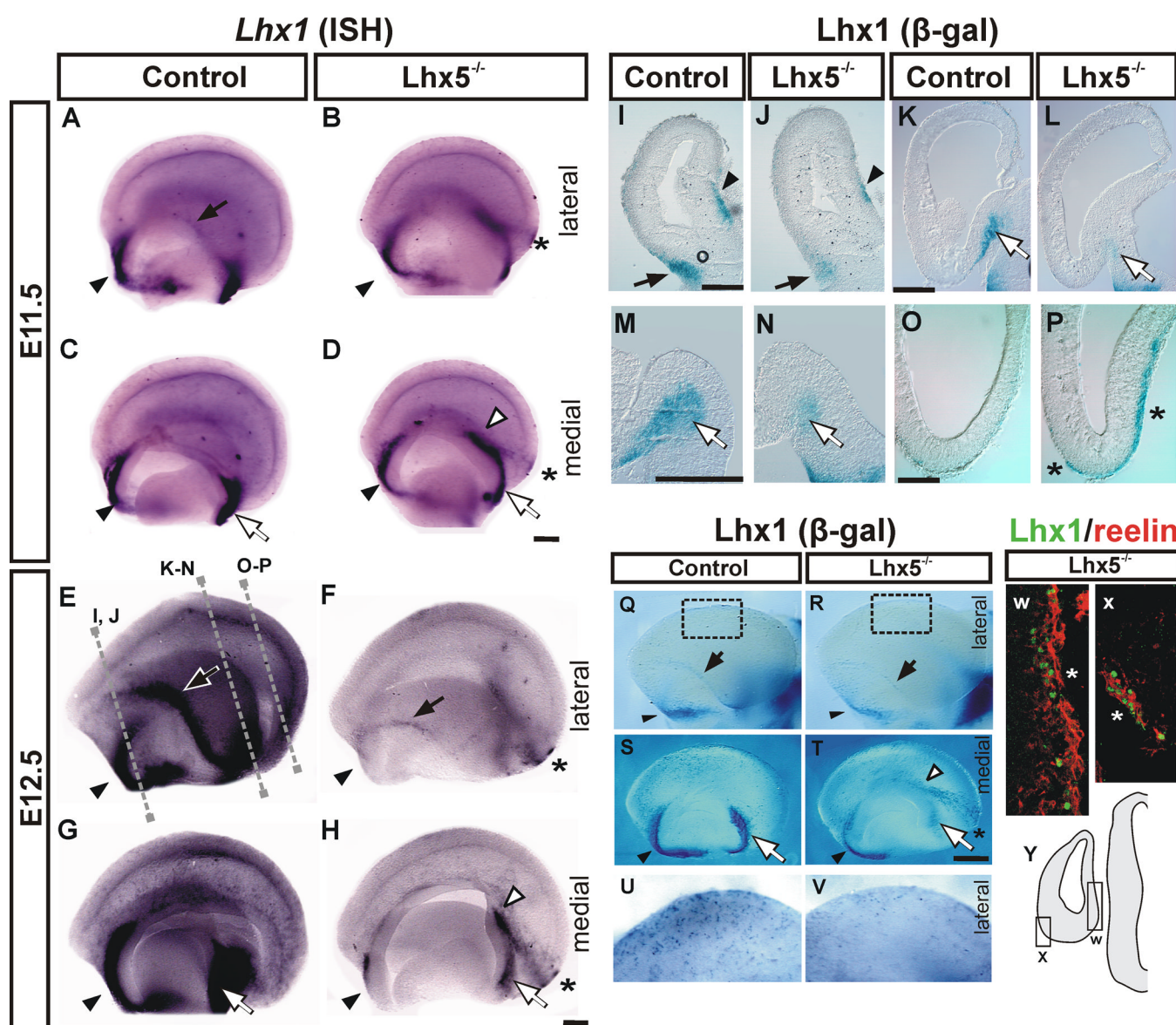


**Figure 4.** *p73* expression is altered in *Lhx5* mutants. Whole-mount *in situ* hybridization on telencephalic vesicles from control and mutant embryos at E10.5 (A–B), E11.5 (C–F) and E12.5 (G–J) (rostral is to the left). Black arrowheads point to the septal domain; black arrows indicate the location of the prospective olfactory cortex (pOC) domain; white arrowheads mark the location of the cortical hem and asterisks indicate ectopic cell clusters. Scale bar: 200μm.

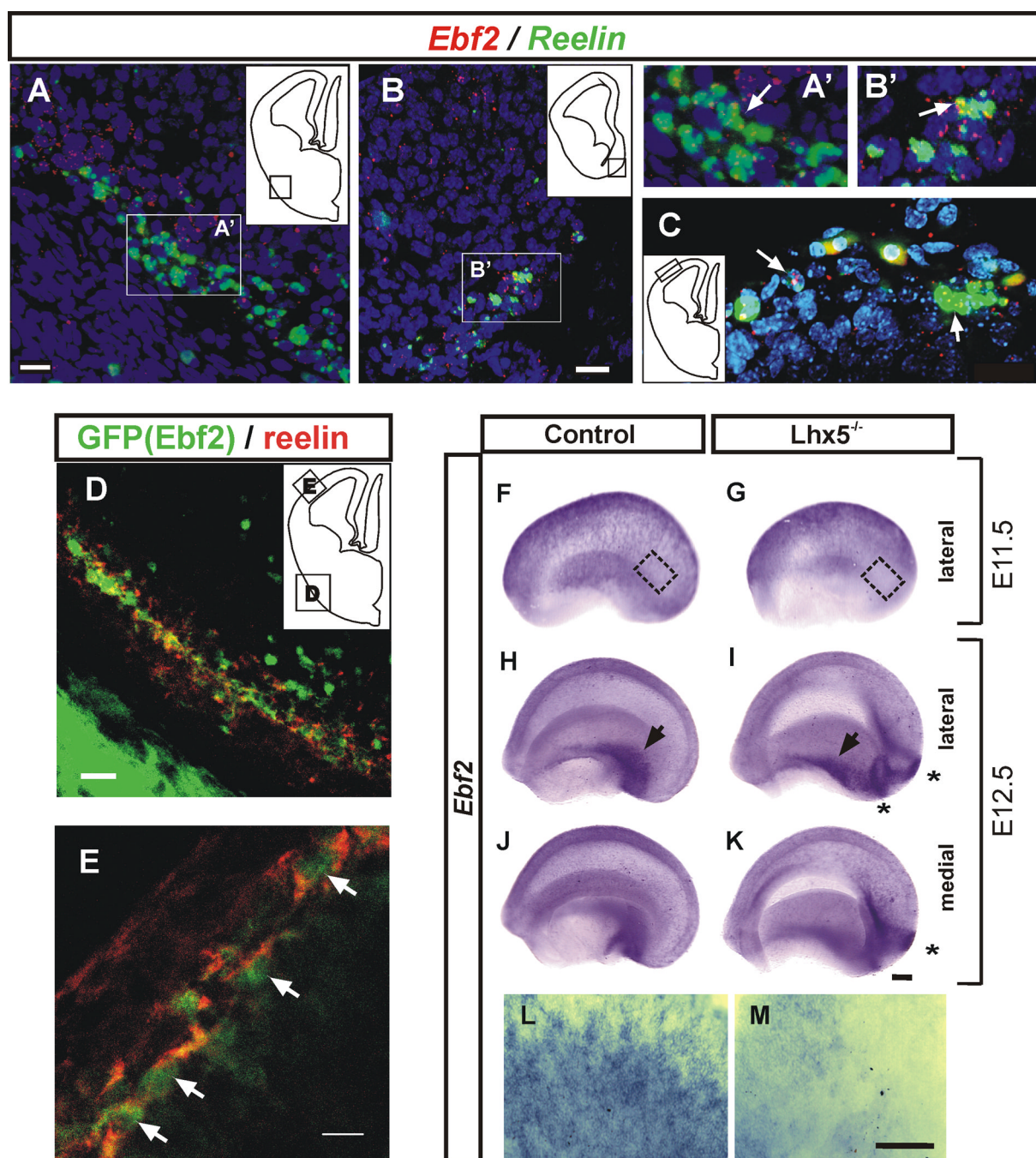


**Figure 5.** Cortical hem alterations in *Lhx5* mutants. Medial views of telencephalic vesicles from control and mutant embryos showing the expression of the hem-specific markers *Wnt5a* (A, B) and *Wnt3a* (C, D) at E11.5. (E–H) Hem-derived C-R cells labeled by *p21* expression at E11.5 and E12.5. Note the shortening of the cortical hem in *Lhx5* mutants by *Wnt3a* expression, the dramatic reduction of *Wnt5a* expression levels and the decreased numbers of *p21*<sup>+</sup> cells in the medial telencephalon of *Lhx5* mutants. In all frames, rostral is to the left. White arrowheads point to the cortical hem, black arrowheads to the septal region, and the arrows indicate the approximate site of origin of the ectopic cell clusters. Scale bar: 200μm.





**Figure 6.** *Lhx1* labels specific C-R cell subpopulations affected by *Lhx5* mutation. (A–H) Whole-mount *Lhx1* *in situ* hybridization of control and *Lhx5* mutants at E11.5 (A–D) and E12.5 (E–H). Medial and lateral views of the telencephalon are shown (rostral is to the left). (I–V) Detection of *LacZ* activity from the *Lhx1*<sup>fl</sup> allele by X-gal staining on coronal sections (I–P, approximate locations of the sections are indicated by dotted lines in E) and whole telencephalic preparations (Q–V) from E12.5 control and *Lhx5* mutant embryos. U and V show higher magnification views of the dorsal cortex from boxed areas in Q and R, respectively. (W, X) Double immunostaining of reelin (red) and *Lhx1* (green) in a caudal telencephalic coronal section. Diagram in Y indicates location of ectopic clusters shown in W (lateral) and X (medial). For all panels: Black arrowheads, septal region; white arrowheads, ectopic *Lhx1*<sup>+</sup> cells; black arrows, prospective olfactory cortex (pOC) domain; white arrows, medial expression domain at the vCMTW; asterisks, ectopic cell clusters. Scale bar: 200μm.

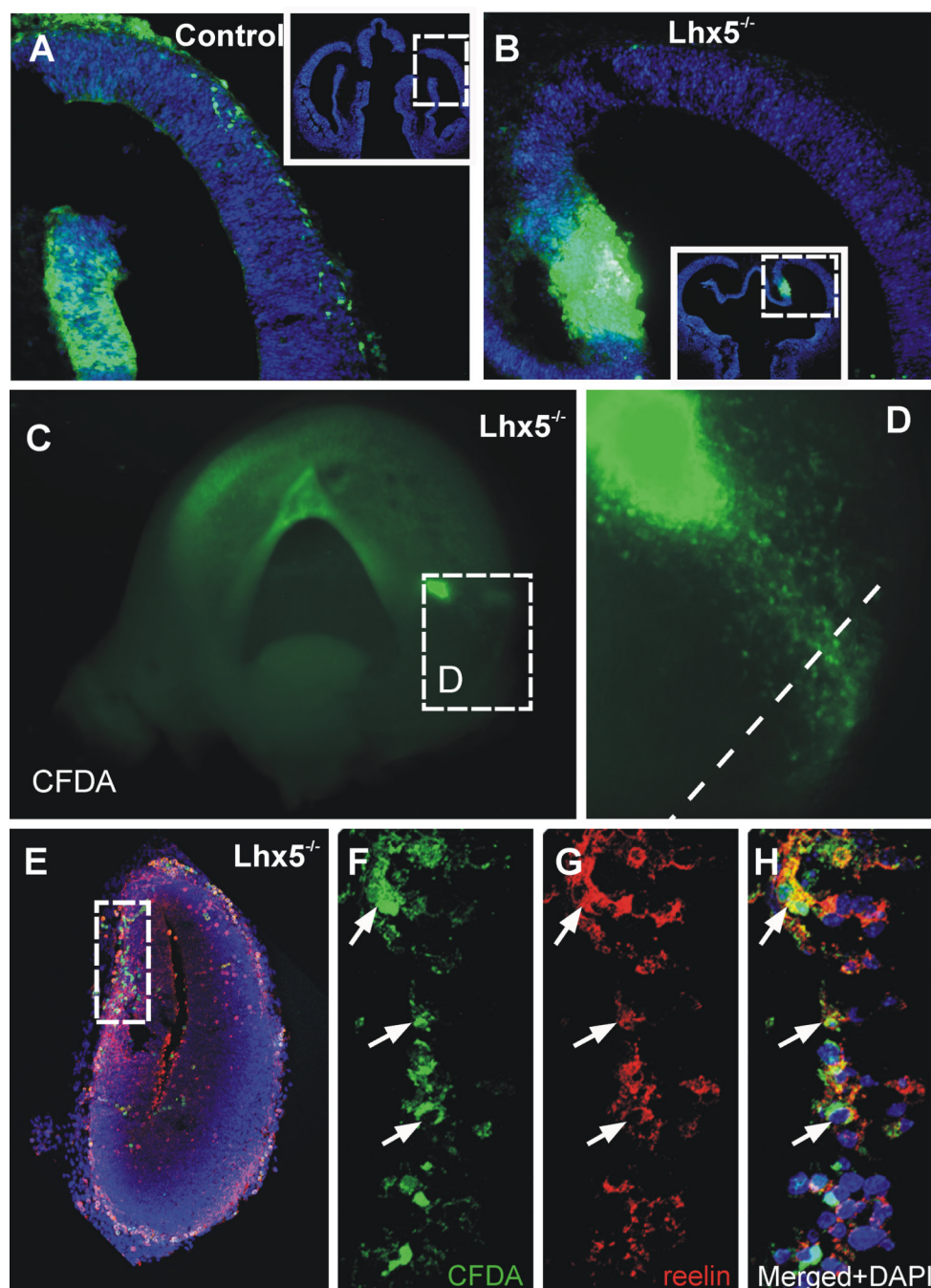


**Figure 7.**

Expression of *Ebf2* in C-R cells is affected in *Lhx5* mutants. (A–C) Double FISH of *Ebf2* (red) and *reelin* (green) showing cellular colocalization at E12.5. DAPI (blue). (A) prospective olfactory cortex (pOC). (B) vCMTW. (A' and B') are magnified views of the boxed areas in A and B, respectively). (C) Colocalization in mantle regions of the lateral pallium. Examples of cellular colocalization are indicated by arrows. (D–E) Confocal images of the pOC domain and marginal zone of the lateral pallium from E12.5 embryos, respectively, showing that *Ebf2*-GFP<sup>+</sup> cells (green) coexpress *reelin* (red). Insets in A–D indicate the location of the fields shown in each image. (F–M) E11.5 and E12.5 telencephalic vesicles from control (F, H and J) and *Lhx5* mutant (G, I and K) littermates

analyzed by whole-mount *in situ* hybridization (rostral to the left; medial and lateral views as indicated). (L, M) High magnification of lateral cortex at E11.5 as indicated by insets in F and G, respectively. Black arrows: prospective olfactory cortex (pOC) domain; asterisks, ectopic *reelin*<sup>+</sup> cell clusters. Scale bar: 200μm.



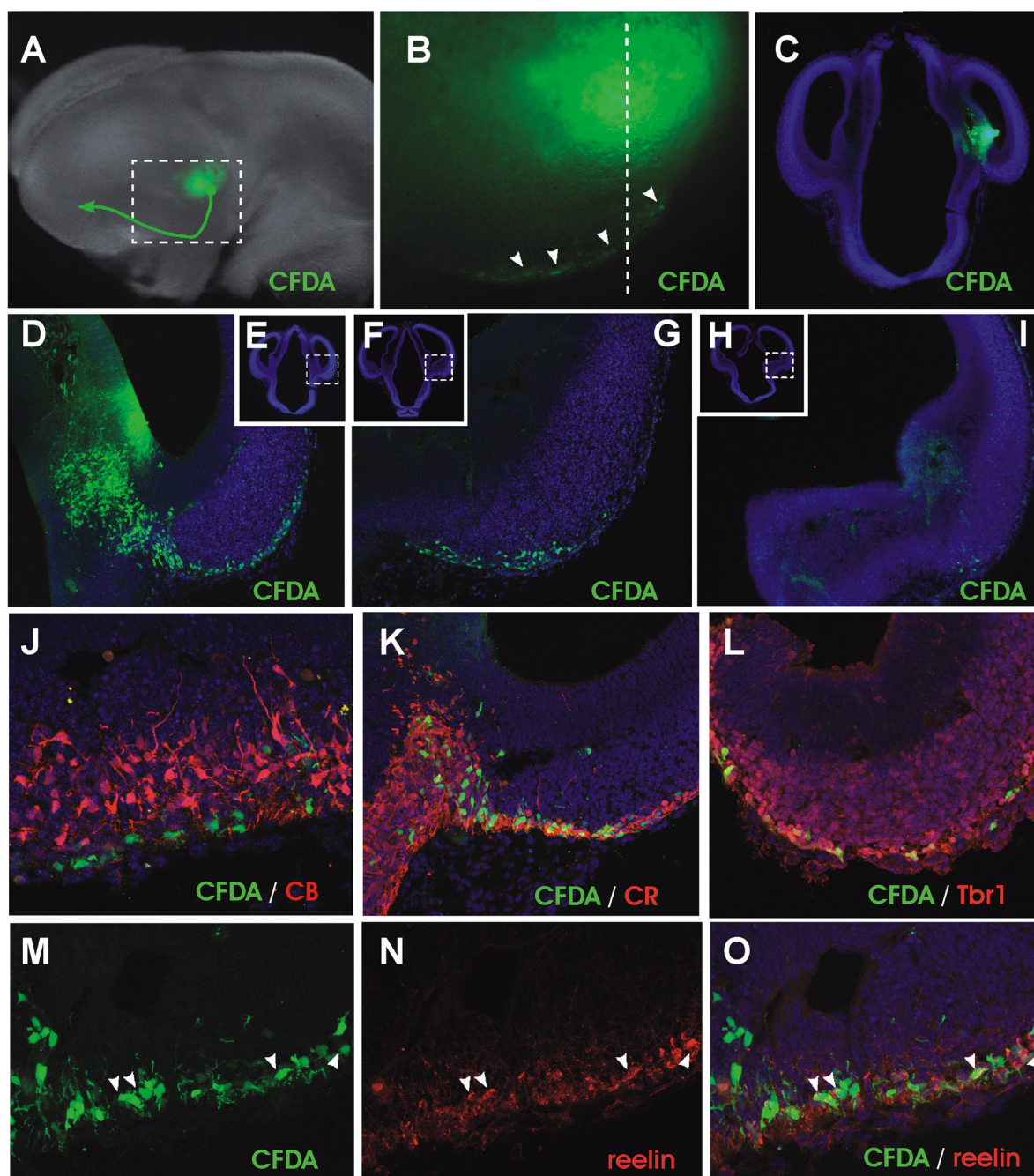


**Figure 8.**

Reelin-expressing cells migrate aberrantly in *Lhx5* mutants. (A and B) Coronal sections of E11.5 control and mutant embryos injected with CFDA (green) in the cortical hem and cultured for 24 hours. Migrating cells in the mutant migrate scarcely towards the dorsal telencephalon (insets show location of images in A and B). (C–H) *Lhx5* mutant embryo injected at the origin of the cellular ectopia and cultured *in toto* for 24 h. (C) Medial view of a telencephalic vesicle showing the injection site at the vCMTW region (rostral is to the left). (D) Magnified view of the boxed area in C showing cells migrating towards the caudal pole of the telencephalon. (E–H) Immunostaining of Reelin on coronal sections of the same injected embryo (dotted lines in D indicate the approximate plane of section). (F–H)

Confocal optical section of the boxed area in E, showing CFDA-labeled cells (green) expressing reelin (red). DAPI (blue). Arrows point to examples of cellular colocalization.





**Figure 9.**

Cell migration from the ventral region of the CMTW. (A) Example of injection of the fluorescent tracer CFDA (green) into an E11.5 wild-type embryo followed by 24 hours of culture. Arrow indicates the migratory route of labeled cells. (B) Close-up of the boxed area shown in A. Arrowheads point to migratory cells in the ventral telencephalon. (C) Coronal section at point indicated by dotted line in B showing the injection site. (D–I) Examples of sections (caudal to rostral) showing the migratory path of labeled cells towards and along the prospective olfactory cortex (pOC). D, G, and I are close-up views of the boxed areas shown in E, F, and H, respectively. (J–O) Immunohistochemical analysis of migrating cells.

Note that no expression of calbindin (CB) was detected among labeled cells (J) while calretinin (CR), Tbr1 and reelin were expressed by a fraction of them (K–O).