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The Doublesex Homolog *Dmrt5* is Required for the Development of the Caudomedial Cerebral Cortex in Mammals

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Regional patterning of the cerebral cortex is initiated by morphogens secreted by patterning centers that establish graded expression of transcription factors within cortical progenitors. Here, we show that *Dmrt5* is expressed in cortical progenitors in a high-caudomedial to low-rostrolateral gradient. In its absence, the cortex is strongly reduced and exhibits severe abnormalities, including agenesis of the hippocampus and choroid plexus and defects in commissural and thalamocortical tracts. Loss of *Dmrt5* results in decreased *Wnt* and *Bmp* in one of the major telencephalic patterning centers, the dorsomedial telencephalon, and in a reduction of Cajal–Retzius cells. Expression of the dorsal midline signaling center-dependent transcription factors is downregulated, including *Emx2*, which promotes caudomedial fates, while the rostral determinant *Pax6*, which is inhibited by midline signals, is upregulated. Consistently, *Dmrt5*$^{-/-}$ brains exhibit patterning defects with a dramatic reduction of the caudomedial cortex. *Dmrt5* is increased upon the activation of Wnt signaling and downregulated in *Gli3*$^{xt/xt}$ mutants. We conclude that *Dmrt5* is a novel Wnt-dependent transcription factor required for early cortical development and that it may regulate initial cortical patterning by promoting dorsal midline signaling center formation and thereby helping to establish the graded expression of the other transcription regulators of cortical identity.

Keywords: choroid plexus, cortical hem, *Emx2*, telencephalon, *Wnt/Bmp*

Introduction

The cerebral cortex derives from the embryonic dorsal telencephalon. It can be subdivided into distinct regions. The most lateral region becomes the 3-layered paleocortex (olfactory piriform cortex). The most posterior and medial region develops into the archicortex (including the hippocampus). The neocortex, which is the larger region, is positioned between the two other regions. It has a very sophisticated architecture, being organized radially into 6 layers and tangentially into distinct areas that serve specialized functions.

Regionalization of the dorsal cortical primordium and arealization are thought to be initiated by signaling molecules secreted from localized inductive centers. Two major patterning centers are most directly implicated in telencephalon patterning. The first is the anterior neural ridge, which later becomes the commissural plate (CoP), which is located at the rostromedial pole of the telencephalon and secretes Fgfs. The second is the roof plate and cortical hem (CH) at the dorsal/caudal midline and immediate adjacent territories, which produce a variety of Wnt and Bmp ligands critical for hippocampus development. These signals are thought to establish in cortical progenitors graded expression of transcription factors that are crucial for the regionalization and subsequent arealization of the cortex (Hébert and Fishell 2008; O'Leary and Sahara 2008; Rakic 2009; Borello and Pierani 2010; Mallamaci 2011). These include *Emx2*, *Lhx2*, *Pax6*, *Foxg1*, *Sp8*, and *COUP-TF1*. How these secreted ligands and transcription factors function together is still not well understood. Whether additional transcription factors are implicated in the regionalization of the cortical primordium is unknown.

The *Dmrt* (*d*oublesex and *m*ab-3-*r*elated *t*ranscription factor) genes encode a large family of evolutionarily conserved transcription factors sharing the DM domain DNA-binding motif, whose function in sex determination and sexual dimorphism has been well studied in invertebrates and vertebrates (Ross et al. 2005; Kimura et al. 2008; Matson et al. 2010). In vertebrates, most *Dmrt* genes are expressed in the developing gonads. Like doublesex and mab-3, *Dmrt1* has been shown to play a critical role in mammalian male sex differentiation (Raymond et al. 1998; Matson et al. 2010). However, some of them are also expressed and function in non-gonadal tissues. For example, *Dmrt3–5* are strongly expressed in the embryonic forebrain (Hong et al. 2007). In the simple chordate *Ciona*, *Dmrt1*, the ortholog of vertebrate *Dmrt4–5*, is essential for the development of anterior neural plate derivatives (Tresser et al. 2010). In zebrafish, a recent analysis of a *Dmrt5* (*Dmrta2*) mutant has revealed that it regulates neurogenesis in the telencephalon (Yoshizawa et al. 2011). The role of *Dmrt* genes in mammalian forebrain development is unknown.

In this study, we show that *Dmrt5* is expressed within cortical progenitors in a high-caudomedial to low-rostrolateral gradient and that its inactivation disrupts the development of the caudomedial cerebral cortex. Together, our data suggest that *Dmrt5* is essential for the specification of regional fate in dorsal telencephalic progenitors and that it may act through the promotion of *Wnt* and *Bmp* expression in the embryonic dorsal midline signaling center and through the modulation

of the expression of the transcription factors that impart distinctive regional identities on cortical progenitors.

Materials and Methods

Generation and Genotyping Strategy of Dmrt5 Knockout Mice

To generate *Dmrt5* null mice, a targeting vector containing a neomycin phosphotransferase resistance cassette (PGK-neo) was inserted by homologous recombination into the wild-type (WT) *Dmrt5* allele, replacing the entire *Dmrt5* ORF. The PGK-neo cassette was inserted into a pBKSII vector as a SacI–BamHI fragment in between 2 kb 5′ and 5 kb 3′ genomic *Dmrt5* BamHI–SacI fragments isolated from PAC clone 403-P18. The *DTA* gene was also inserted at the 3′ extremity of the construct. The linearized targeting construct was electroporated in embryonic stem (ES) cells. After G418 selection, DNA from 650 resistant clones was analyzed by PCR using the external primer: 5′-AGGCTCAACGCCCCTTCTTA-3′ and the internal primer: 5′-AGCGCCTCCCCTACCCGGTA-3′. Three positive clones were identified. The *Dmrt5* null mouse strain was generated by injection of one positive ES cell clone into 129/Sv blastocysts. The chimeric mice were mated to WT C57Bl/6 mice to generate heterozygous animals that were then intercrossed to generate F2 offspring for analysis.

To distinguish between the WT and *Dmrt5* null allele, mice were genotyped by PCR using genomic DNA from the tail of postnatal and late embryonic stages or yolk sac from earlier embryos and primers: Fwd 5′-CGAATCTTTCGGACACTGTAGA-3′; Rev WT 5′-CCAGAC CCTCAAGCACTCAA-3′; Rev knockout (KO) 5′-AGCGCCTCCCCTA CCCGGTA-3′. Morning of the vaginal plug is E0.5 and the first 24 h after birth is P0. Animal care was in accordance with institutional guidelines.

Reverse transcription (RT)-PCR and Southern Analysis

RT–PCR analysis was performed on total RNA isolated from E15 WT and *Dmrt5* null brain and from adult testis. RNAs were reverse transcribed with the "iScript cDNA Synthesis Kit" (Bio-Rad). The sequences of the primers used to detect *Dmrt5* are the following: Fwd 5′-CTTACGAAGTCTTTGGCTCG-3′ and Rev 5′-TTCTTTGTCAGCCT CCGAAC-3′. Primers used to detect the expression of the *Dmrt5* adjacent genes *Faf1* and *Elav4* are for *Faf1*: Fwd 5′-GGCGTCCAA CATGGACCGGG-3′ and Rev 5′-CTGCAGGGCACCAGCATGGC-3′; and for *Elav4*: Fwd 5′-CGAAGCGCTGCGAGACCCAA-3′ and Rev 5′-GG CGGGCGTAGGACACCTT-3′. Amplification of *HPRT* (primers: Fwd 5′-CCTGCTGGATTACATTAAAGCACTG-3′ and Rev 5′-GTCAAGGGC ATATCCAACAACAAAC-3′) was used as a control.

Southern blot analysis was performed on genomic DNA digested with HindIII (for the 5′ Southern) or EcoRV (for the 3′ Southern) using external probes (500 bp for the 5′ Southern and 600 bp for the 3′ Southern). In each case, the predicted fragments were observed (6.8 kb for the WT allele, 8.2 kb for the targeted allele in the 5′ Southern, 14.6 kb for the WT allele, and 11.7 kb for the targeted allele in the 3′ Southern). An internal Neo probe was also used with HindIII digests to detect a single 8.2 kb *Dmrt5* targeted allele (see Supplementary Fig. 2; data not shown).

Histology, Immunohistochemistry, and Immunofluorescence Staining

Standard hematoxylin and eosin staining was performed on 6–8 µm sections of embryos fixed overnight in Bouin, dehydrated, and paraffin-embedded.

For immunohistochemistry (IHC), embryos or brains were fixed overnight in 4% paraformaldehyde, dehydrated in ethanol, paraffin-embedded, and sectioned (6–8 µm). Sections were washed in xylene and rehydrated in ethanol. Antigen retrieval was performed by boiling the sections in Target Retrieval Solution Citrate, pH 6.0 (DAKO). Sections were then treated with 0.3% H_2O_2, blocked in a 5% lamb serum solution, and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: Anti-2H3a (mouse, 1:500, DSHB), anti-L1 (rabbit, 1:500, Fritz G. Rathjen), anti-Lim1/2 (mouse, 1:10, DSHB), anti-Pax6 (rabbit, 1:200, PRB-278P, Covance), anti-Tbr2 (rabbit, 1:500, ab23345, Abcam), anti-Reelin (mouse, 1:750, A. Goffinet), anti-Calretinin (rabbit, 1:1000, AB5054, Millipore), anti-Ctip2 (rat, clone 25B6, 1:500, ab18465, Abcam), anti-phospho-histone H3 (PH3, rabbit, 1:200, 06-570, Millipore), and anti-cleaved Caspase 3 (rabbit, 1:1000, 9661, Cell Signaling). Sections were incubated with anti-rabbit (1:200, 2AB02B, AbD Serotec), anti-mouse (1:200, B0529, Sigma), or anti-rat (1:200, BA-9400, Vector) secondary antibodies. Detection was performed with the ABC Kit (Vectastain) according to the manufacturer's instructions and visualized by DAB (Sigma). Images were acquired with an Olympus SZX2-ILLB or an Olympus XC50 camera, using the Imaging software Cell*.

For immunofluorescence staining (IF), brains were fixed overnight in 4% paraformaldehyde, infused in 30% sucrose/phosphate-buffered saline (PBS) overnight, frozen in gelatin (7.5% gelatin, 15% sucrose/PBS), and sectioned in 20 µm cryostat sections. Sections were rinse in PBS. Antigen retrieval was performed by boiling the sections in Target Retrieval Solution Citrate, pH 6.0 (DAKO). Sections were then permeabilized in 0.3% Triton X-100, blocked in 5% goat serum, and incubated with primary antibodies overnight at 4°C. The following antibodies were used: Ctip2 (rat, clone 25B6, 1:250, ab18465, Abcam), Tbr1 (rabbit, 1:1000, ab31940, Abcam), Satb2 (mouse, 1:100, ab51502, Abcam), and Cux1 (rabbit, 1:100, sc-13024 Santa-Cruz). Sections were incubated with Alexa Fluor 488 goat anti-rabbit (1:400, A-11008, Invitrogen), Alexa Fluor 594 goat anti-rabbit (1:400, A-11012, Invitrogen), Alexa Fluor 488 goat anti-mouse (1:400, A-11017, Invitrogen), or Alexa Fluor 594 goat anti-rat (1:400, A-11007, Invitrogen) secondary antibodies. Wide-field images were acquired with a Leica DM 4000B equipped with an EBQ10 lamp and leica filters (i3, Tx2, and A).

DiI Labeling

Brains were fixed overnight in 4% paraformaldehyde. To retrogradely label neurons in the dorsal thalamus, brains were sagitally hemidissected. Crystals of 1.1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, D282, Invitrogen) were placed with a fine tungsten wire in the thalamus. Samples were kept in PBS-azide 0.01% for 2 weeks at 37°C in the dark. Brains were then washed in PBS, embedded in gelatine, and cut at 100 µm on a vibroslice (M752 vibroslice, Campden Instruments). Sections were mounted onto slides and analyzed by fluorescence with an Olympus XC50 camera, using the Imaging software Cell*. For each genotype and for each cortical labeling point, at least 3 hemibrains were processed.

In Situ Hybridization

An antisense *Dmrt5* probe was synthetized by linearizing EST AI592924 (GenBank) with EcoRI and transcribing it with T3. The *Dmrt3* probe was synthesized by linearizing EST BU054807 with MluI and transcribing it with T3. The *p75* probe was synthesized by linearizing EST BC038365 with SalI and transcribing it with T7. The other antisense probes were generated from the following previously described cDNA clones: *Lef1*, *Fzd10*, *Id3*, *Emx2*, *Hes5* (Muzio et al. 2005), *Axin2* (Bluske et al. 2009), *Cux2*, *Nfib*, *Nurr1/Nr4a2*, *Rspo1*, *Rspo2*, *Rspo3* (Hasenpusch-Theil et al. 2012), *Lhx2*, *Wn2b*, *Ttr*, *Wnt3a* (Monuki et al. 2001), *Bmp4* and *Bmp7* (Furuta et al. 1997), *Msx1*, *Wnt5a*, *Gli3* (Grove et al. 1998), *Math2*, *Ngn2* (Schuurmans et al. 2004), *Mash1* (Chou et al. 2009), *Dbx1* (Causeret et al. 2011), *Auts2*, *Tbr1* (Bedogni et al. 2010), *COUP-TF1* (Zhou et al. 2001), *EphrinA5*, *EphA7*, *RZRβ*, *Cad8* (Bishop et al. 2002), *Sp8* (Zembrzycki et al. 2007), *Fgf8*, *Fgf17*, *RORβ* (Fukuchi-Shimogori and Grove 2003), *ER81*, *Dlx2*, *Nrp2*, *Satb2* (Chou et al. 2009), *Foxg1*, *Lmx1a* (Imayoshi et al. 2008), *Pax6* (Hamasaki et al. 2004), *KA1*, *Scip*1, *EphB1* (Bulchand et al. 2001), *Reelin* (Yoshida et al. 2005), *Prox1* (Xie et al. 2010), and *Wnt8b* (Lee et al. 2000).

Whole-mount embryo and section in situ hybridization (ISH) were performed as previously described (Wilkinson and Nieto 1993), using digoxigenin-labeled riboprobes, an alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragment (1:2000, Roche), and NBT/BCIP substrates. For section ISH, 20 µm cryostat sections of 4%

paraformaldehyde fixed, 30% sucrose/PBS-infused tissue frozen in gelatin (7.5% gelatin, 15% sucrose/PBS) were used. Double ISH was performed as previously described (Hauptmann and Gerster 1994), using digoxigenin-labeled *Wnt3a* and fluorescein-labeled *Dmrt5* riboprobes, an AP-conjugated anti-fluorescein Fab fragment (1:2000, Roche), and a Magenta phosphate substrate (Sigma).

RNA Isolation and RT–qPCR

Total RNA was extracted from telencephalon using the "RNAspin mini" (GE Healthcare) according to the manufacturer's protocol. DNase treatment was performed using the "Rnase free—Dnase set" (Qiagen), followed by cDNA synthesis ("iScript cDNA Synthesis Kit," Bio-rad) starting from equal amounts of RNA.

RT–qPCRs were performed on a StepOnePlus Real Time PCR system (Applied Biosystems) using SYBER Green MESA GREEN qPCR MasterMix Plus (Eurogentec) with a program optimized by the manufacturer. Gene expression was normalized to the expression of 2 reference genes: Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Reference genes were validated using the Genorm program. The primers were designed using the PrimerBank (Spandidos et al. 2010) and Primer3 (Rozen and Skaletsky 2000). The primers were verified for specificity with Primer-Blast from NCBI. The PCR efficiency was estimated with the Applied Biosystems software using a calibration dilution curve for each primer set. Primers used were as follows: *Dmrt3* forward primer GCGCAGCTTGCTAAACCAG, *Dmrt3* reverse primer CCTCTGATCGGTGTCTTTGTCA; *Dmrt4* forward primer CCAACTTTCGAGGTTTTCCA, *Dmrt4* reverse primer GATGATCCCAGAGAATGGTGA; *Dmrt5* forward primer GCGCCAACAGAGGAGGAG, *Dmrt5* reverse primer ACGCTCACTGGTAGCGATG; *Emx2* forward primer GTCCACCTTCTACCCCTGG, *Emx2* reverse primer CCACCACGTAATGGTTCTTCTC; *Lhx2* forward primer TGGCAGCATCTACTGCAAAG, *Lhx2* reverse primer TGTGCATGTGAAGCAGTTGA; *Pax6* forward primer AGGGCAATCGGAGGGAGTAA, *Pax6* reverse primer CAGGTTGCGAAGAACTCTGTTT; *Gli3* forward primer CACAGCTCTACGGCGACTG, *Gli3* reverse primer CTGCATAGTGATTGCGTTTCT; *Sp8* forward primer CACTCGACGATTTCGAAGGG, *Sp8* reverse primer GGAAGCCTTTACCGAAGGAAG; *COUP-TF1* forward primer AAAGTG GGCATGAGGCGGGA, *COUP-TF1* reverse primer CCCGTTTGTGA GTGCATACTG.

Explant Culture

Organotypic slice cultures of E13.5 embryonic mouse telencephalon in the presence of either DMSO or 5, 25, or 50 µM Chir (Cambridge BioScience) were performed as described previously (Hasenpusch-Theil et al. 2012).

Statistical Analysis

To quantify the dorsal surface area of the cortex of *Dmrt5* mutants and their littermate, brains from E18.5 embryos were dissected and fixed overnight in 4% paraformaldehyde. The brains were then photographed under a Nikon dissection microscope, slide images were digitized, and the dorsal surface measured using Imaging Software NIS-Elements. Measurements were made blind to genotype.

Quantification of the thickness of the cortical wall of *Dmrt5* null mice and WT embryos has been done on coronal sections at the level of the caudomedial telencephalon based on hematoxylin and eosin histological images using the Imaging software Cell*.

Quantifications of cells expressing PH3 and Tbr2 have been done on 3 sections/embryo at caudal levels of the telencephalic vesicles (n = 2–4). Images were acquired with a Zeiss Axioskop microscope.

For quantification of cells expressing Tbr2, a rectangle of 100 µm width was fixed in medial regions and at caudal levels of the telencephalic vesicles. Quantification of apical PH3-positive cells at E11.5 and E14.5 was done similarly. Apical PH3-positive cells at E9.5 and basal PH3-positive cells at E11.5 and E14.5 were counted throughout the entire medial part of the telencephalon. At E18.5, PH3-positive cells were counted through the entire dorsal telencephalon (n = 3 for each stage analyzed). All counts performed without the use of a rectangle marquee were normalized with respect to the size of the vesicles. Positive cells were counted in Adobe CS5 Photoshop. All statistical analyses were done with Student's t-test; values of $P < 0.05$ were regarded as statistically significant.

Results

Dmrt5 is Expressed in Mouse Cortical Progenitors in a High-Caudomedial to Low-Rostrolateral Gradient

Dmrt5 was identified as a downregulated gene in a microarray screen comparing the transcriptome of WT and *Mdm4* KO brains, which display a high level of apoptosis and a severe deficit in neurogenesis (Martoriati et al. 2005). Embryonic expression of *Dmrt5* was studied from E9.5 by ISH on whole embryos and on sections. We found that at E9.5, *Dmrt5* is strongly expressed in the developing dorsal telencephalon, except in the dorsal midline. Outside the telencephalon, *Dmrt5* was also found in the optic stalk, the lateral head ectoderm, and in the maxillary and mandibular processes (Fig. 1*A*,*B*). At E10.5, *Dmrt5* expression was also detected in the ventral midbrain, eyes, nasal placodes and hypophysis. From that stage, *Dmrt5* was detected in a high-caudomedial

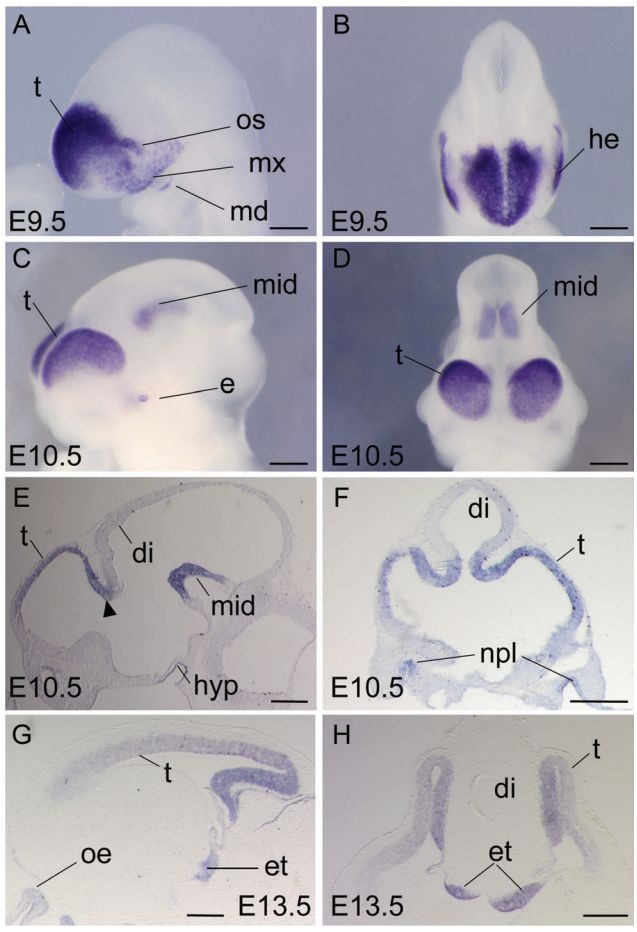

Figure 1. *Dmrt5* expression in the mouse developing brain. (*A*–*D*) Whole-mount ISH of *Dmrt5* at E9.5 (*A* and *B*) and E10.5 (*C* and *D*). (*E*–*H*) Sagittal (*E* and *G*) and coronal (*F* and *H*) brain sections. Arrowhead in (*E*) indicates *Dmrt5* expression at the telen-diencephalic boundary. di, diencephalon; e, eye; et, eminentia thalami; he, head ectoderm; hyp, hypophysis; md, mandibular process; mid, midbrain; mx, maxillary process; npl, nasal placode; oe, olfactory epithelium; os, optic stalk; t, telencephalon. Scale bars: 200 µm in *A* and *B*; 500 µm in *C*–*H*.

to low-rostrolateral gradient in the telencephalic evagination and was restricted to progenitors. *Dmrt5* staining in the dorsomedial part of the telencephalic evagination includes the primordia of the CH and choroid plexus. No expression was detected in the roof plate. From E11.5, *Dmrt5* was also detected in the developing diencephalon. At E13.5, *Dmrt5* expression in the choroid plexus has strongly decreased. From that stage, additional expression was detected in scattered cells located in the most marginal portion of the cerebral walls, most abundant in the medial area (Fig. 1*C–H*; see Supplementary Fig. 1*A–H*). The marginal zone of the cortex contains Cajal–Retzius (CR) cells, one of the earliest neuronal cell types in the mammalian telencephalon that express *Reelin*, a key molecule in the orchestration of the radial migration of neurons (Ogawa et al. 1995). We therefore asked whether these *Dmrt5*$^{+}$ cells also express *Reelin*. Using ISH combined with immunohistochemistry (IHC), we detected colocalization of *Dmrt5* transcripts and Reelin protein, indicating that *Dmrt5* is expressed in CR cells (see Supplementary Fig. 1*I,J*). Together, these data identified *Dmrt5* as a likely candidate for a cortical regulatory factor.

Dmrt5 Deletion Leads to a Strong Reduction of Posterior Midline Cortical Structures

To elucidate *Dmrt5* function, *Dmrt5* null mice were generated (see Supplementary Fig. 2*A–C*). While heterozygous mice were healthy and fertile, about 95% of homozygous *Dmrt5*$^{-/-}$ mice died within a few hours after birth. Surviving animals were growth retarded and presented neurological abnormalities (see Supplementary Fig. 2*D–F*). While E18.5 *Dmrt5*$^{+/-}$ brains were indistinguishable from WT brains (data not shown), E18.5 *Dmrt5*$^{-/-}$ brains exhibited a strong reduction in the surface area of the cerebral hemispheres ($-38.6 \pm 5.9\%$, $n = 16$, $P < 0.001$) and olfactory bulbs (Fig. 2A). Hematoxylin and eosin staining of E18.5 sagittal brain sections revealed that the cortical wall of the mutants has a decreased thickness ($-35.8 \pm 10.1\%$, $n = 3$, $P < 0.05$) and a poorly differentiated structure. On average, the proportional size of the ventricular zone (VZ), subventricular zone (SVZ), and cortical plate was similar in mutant and control embryos. The subplate appeared much less well formed (Fig. 2B). Defects in the subplate were confirmed by ISH or IHC using *EphA7*, *p75*, and Calretinin (see below). Remarkably, the caudomedial portion of the cortex of *Dmrt5* mutants including the hippocampus, dentate gyrus, and choroid plexus was missing. A strong impairment of commissural axonal tracts crossing the midline was also evident in mutant mice (Fig. 2C). Anteriorly, the limit of the neocortex with the olfactory bulbs was abnormal. The VZ often appeared disorganized, showing in some cases bulges protruding to the pial surface (Fig. 2D). Development of the efferent fibers from the hippocampus was also poor (Fig. 2E). L1 and 2H3 neurofilament IHC confirm these observations. These data revealed that mutant forebrains have also severe defects in the thalamocortical axon (TCA) and cortifugal axon (see Supplementary Fig. 3*A* and *D*). DiI injections into the dorsal thalamus confirm the TCA defects (see Supplementary Fig. 3*E*). The anlagen of the cerebral hemispheres was already smaller than normal at E10.5. Unexpectedly, the cortical wall, which is smaller in *Dmrt5*$^{-/-}$ embryos at E18.5, was thicker in *Dmrt5*$^{-/-}$ embryos at E11.5 and of about the same size as controls at E14.5 (see Supplementary Fig. 4 and

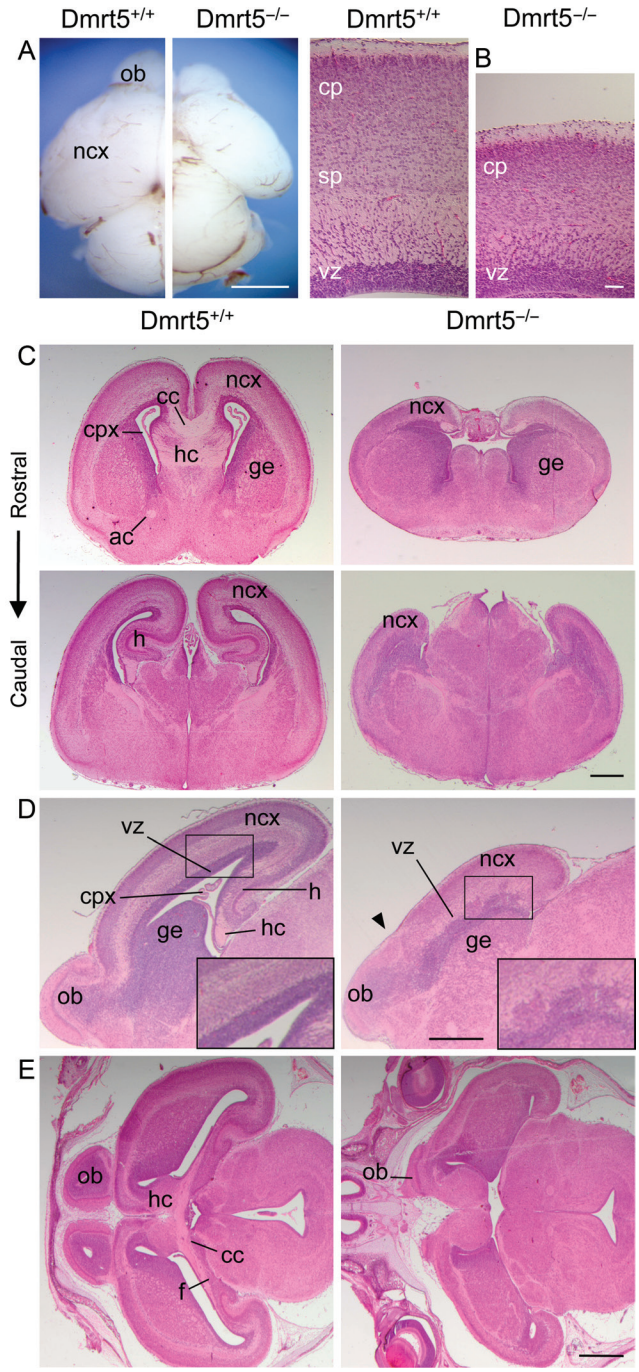

Figure 2. Reduced cerebral cortex with midline defects in *Dmrt5*$^{-/-}$ mice. (*A*) Dorsal views of P0 control and *Dmrt5*$^{-/-}$ brains. (*B*) High magnification view of sagittal sections of E18.5 control and mutant brains. (*C–E*) Hematoxylin and eosin staining of coronal (*C*, at the rostral level, upper panels, and the caudal level, lower panels), sagittal (*D*) and horizontal (*E*) sections of E18.5 control and mutant brains. Note the abnormal limit of the neocortex with the olfactory bulbs (arrowhead in *D*). ac, anterior commissure; cc, corpus callosum; cp, cortical plate; cpx, choroid plexus; f, fimbria; ge, ganglionic eminences; h, hippocampus; hc, hippocampal commisure; ncx, neocortex; ob, olfactory bulbs; sp, subplate; vz, ventricular zone. Scale bars: 200 μm in *A* and *B*; 500 μm in *C* and *D*; 1 mm in *E*.

see below). Thus, the loss of *Dmrt5* causes early alterations in the development of the telencephalic vesicles leading to a strong reduction at E18.5 of the caudomedial cortex.

To understand the cause of the observed phenotype, we first examined the extent of apoptosis in the *Dmrt5*$^{-/-}$

neocortex by anti-cleaved Caspase-3 immunostaining at E9.5, E11.5, and E14.5. We found no increase in apoptosis in the entire cortex of *Dmrt5* mutants compared with WT embryos (see Supplementary Fig. 5). Next, we quantified the mitotic progenitors labeled with an antibody against PH3 based on their location (apical in the VZ for the radial glial cell [RGCs] and basal in the SVZ for the intermediate progenitor cell [IPCs]) at the same stages and at E18.5. We counted the number of mitotic progenitors in medial regions and at caudal levels of the telencephalic vesicles in *Dmrt5* mutants and wild types. We found that there is no significant difference in the total number of proliferating cells in the neuroepithelium of *Dmrt5*$^{-/-}$ embryos compared with controls at E9.5 (Fig. 3A). However, at E11.5, we observed a slight increase in the number of apical (1.86-fold) and a larger increase in basal (6-fold) mitotic progenitors in *Dmrt5*$^{-/-}$ embryos compared with controls (Fig. 3B). We thus also tested in E11.5 and later embryos the expression of Tbr2 which labels specifically basal progenitors. In accordance with our PH3 results, we found at E11.5 an increase in Tbr2-positive cells (3.15-fold) in the mutants with respect to the controls (Fig. 3*E*,*E*′). At E14.5, we observed that mitotic rates return to normal for both RGCs and IPCs in the *Dmrt5* mutants. This normalization of the mitotic rate also comes with a smaller increase in Tbr2-positive cells (1.25-fold) in *Dmrt5* mutants (Fig. 3*C*,*F*,*F*′). At E18.5, we observed a reduction in both apical (1.8-fold) and basal (1.28-fold) progenitors in *Dmrt5*$^{-/-}$ cortices. No differences in the number of Tbr2-positive cells could be detected anymore between mutants and controls (Fig. 3*D*,*G*,*G*′). Thus, in *Dmrt5* mutant cortices, a transient burst of proliferation with an expansion of the population of basal progenitors occurs at E11.5, followed by a reduction later of apical progenitors. Those defects in cell proliferation are likely to contribute to the observed phenotype.

Reduction of Wnt and Bmp Signals in the Caudomedial Cortex and Decrease in CR Cells in Dmrt5$^{-/-}$ *Embryos*

The CH region is a Wnt and Bmp-rich tissue that plays an essential role in the specification of the hippocampus (Lee et al. 2000; Muzio and Mallamaci 2005; Fernandes et al. 2007). The structural perturbations observed in the forebrain of *Dmrt5*$^{-/-}$ mice prompted us to investigate expression of the signaling molecules produced by the CH region. We first looked by in situ at the *Wnt* family members *Wnt2b*, *Wnt3a*, and *Wnt5a*, whose expression in the telencephalon is restricted to the CH and *Wnt8b* expressed in the eminentia thalami, in the CH, and in a high-medial to low-lateral gradient in the dorsomedial telencephalon (Grove et al. 1998). At E12.5, *Wnt2b* and *Wnt5a* were undetectable in *Dmrt5*$^{-/-}$ embryos (Fig. 4*A*,*B*). *Wnt3a*, which is the earliest *Wnt* selectively expressed in the CH (Lee et al. 2000), was still expressed but at a reduced level (Fig. 4C). *Wnt8b* expression in the dorsomedial telencephalon was strongly reduced in the *Dmrt5* mutants but was still detected in the eminentia thalami visualized by immunostaining using the Lim1/2 marker (Fig. 4D). We also looked at the expression of *Wnt* receptors, nuclear effectors, and cytoplasmic modulators. The *Wnt* receptor gene *Fzd10* expressed in the CH, as well as the *Wnt* nuclear effector *Lef1*, normally restricted to the dorsomedial cortex (Muzio et al. 2005), were downregulated in mutants (Fig. 4*E*,*F*). Several modulators including *Axin2* which is upregulated in the area of strong canonical Wnt signaling (Jho et al. 2002) and *Rspo1-3* strongly expressed in the CH (Hasenpusch-Theil et al. 2012) were also reduced in *Dmrt5*$^{-/-}$ embryos. The remaining Rspo1-3 signal detected in the caudal telencephalon of the mutants likely corresponds to the signal detected in the eminentia thalami in the controls (Fig. 4*G*–*J*). Thus, canonical Wnt signaling is strongly reduced in *Dmrt5*$^{-/-}$ caudomedial cortical progenitors.

Several *Bmp* family members that are required for the formation of the cortical plate and choroid plexus are also expressed within cortical dorsal midline cells (Furuta et al. 1997; Currle et al. 2005; Cheng et al. 2006; Fernandes et al. 2007). Two of these, *Bmp4* and *Bmp7*, which mark the choroid plexus and the ventral portion of the CH, were also strongly reduced in mutants (Fig. 4*K*,*L*). Reduced *Wnt3a* and *Bmp7* expression was already detectable in the abnormal telencephalic dorsal midline of mutants at E10.5 (see Supplementary Fig. 6*A*,*B*). We also investigated the expression of *Fgfs* produced by the CoP. By ISH on sections and in whole mounts, *Fgf8* and *Fgf17* expression in the bridge region between the two hemispheres in the mutants was similar in intensity to that of control embryos (see Supplementary Fig. 6*C*–*F*). Thus, while *Fgfs* are not dramatically affected in the CoP, *Wnt* and *Bmp* are deficient in the dorsal midline in the telencephalon of *Dmrt5* mutants.

As the CH is a major source of CR cells (Yoshida et al. 2006), we examined the CR cell markers *Reelin* and Calretinin in *Dmrt5*$^{-/-}$ embryos. As expected, based on the observed CH defects, *Reelin* was severely reduced at E18.5. However, a few positive cells remained in the most medial portion of the cortex. Some *Reelin*$^{+}$ cells also accumulated in the form of aggregates at the border between the neocortex and the piriform cortex. Similar results were obtained using Calretinin at E15.5 (see Supplementary Fig. 7). Thus, the differentiation of CR cells is severely impaired in *Dmrt5* mutants. Together, these observations indicate that the loss of the caudomedial cortex is likely caused by the reduction of Wnt and Bmp signals at the dorsal midline signaling centers.

Altered Expression of Transcriptional Regulators of Cortical Development in Dmrt5$^{-/-}$ *Embryos*

The expression of several cortical transcription factors is known to be dependent on signaling molecules from the CH and surrounding regions. Among them, *Emx2*, which promotes caudomedial fates and is expressed similarly to *Dmrt5*, is upregulated by Wnt and Bmp ligands (Theil et al. 2002). *Lhx2* is a Bmp-regulated selector gene that is expressed similarly to *Dmrt5* except that, unlike *Dmrt5*, *Lhx2* is not expressed in the CH and choroid plexus. In mice in which *Lhx2* is disrupted between E8.5 and E10.5, the neocortex and the hippocampal primordium are lost at the expense of an expanded CH and choroid plexus (Monuki et al. 2001; Mangale et al. 2008). When *Lhx2* is deleted later, between E10.5 and E11.5, lateral cortical progenitors generate olfactory cortex rather than lateral neocortex (Chou et al. 2009). Genes encoding the homeodomain transcription factor *Cux2*, the orphan nuclear receptor *Nurr1/Nr4A2*, *Nfib*, and *Dmrt3* are recently identified Wnt targets that show restricted expression in the dorsomedial telencephalon and may thus also function in early cortical regionalization (Hasenpusch-Theil et al. 2012). Given the deficiency of *Wnt* and *Bmp* in the caudomedial part of the telencephalon of *Dmrt5* mutants, we suspected that expression of those genes would be altered and examined

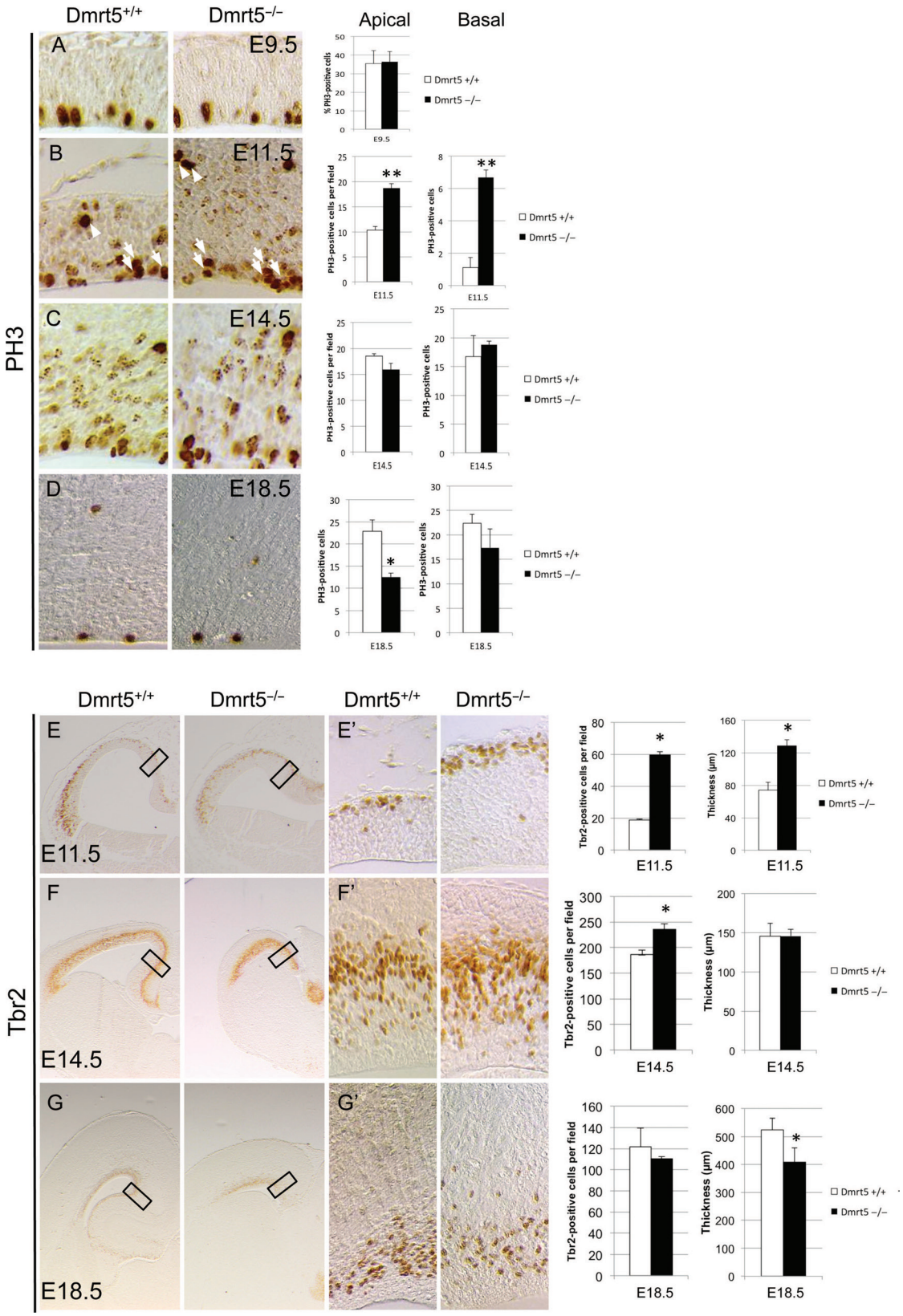

Figure 3. Altered cell proliferation in the telencephalon of *Dmrt5*$^{-/-}$ mice. (*A–D*) PH3 IHC on coronal sections of *Dmrt5*$^{-/-}$ and control embryos at the indicated stages. Quantification of the results is shown on the right. Note the transient slight increased in apical and larger increase in basal PH3 cells at E11.5 and later slight decrease in PH3-positive cells in *Dmrt5*$^{-/-}$ embryos. (*E–G*) Tbr2 IHC on coronal sections of *Dmrt5*$^{-/-}$ and control embryos at the indicated stages. High magnifications of the rectangles in (*E–G*) are shown in (*E′–G′*). Quantification of the results and thickness of the cortical wall is shown on the right. Note the transient increase of the number of Tbr2-positive cells and of the thickness of the cortical wall at E11.5, and the decrease of the thickness at E18.5 in *Dmrt5*$^{-/-}$ embryos (*$P < 0.05$ and **$P < 0.01$). Error bars indicate standard deviation (SD).

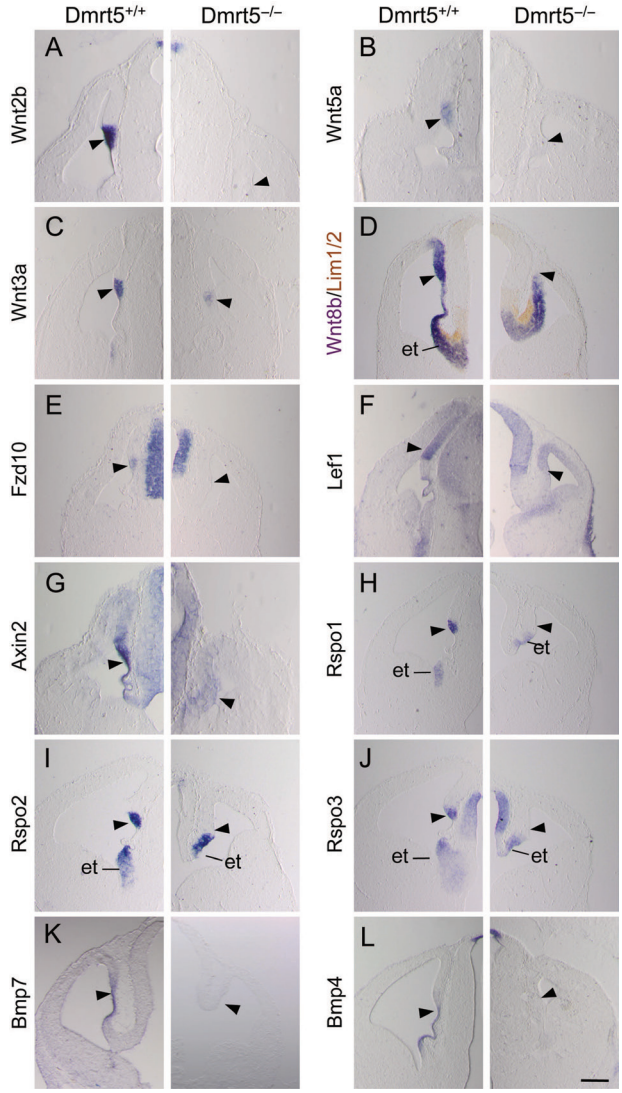

Figure 4. Reduction of *Wnt* and *Bmp* genes and genes encoding *Wnt* signaling components in the caudomedial cerebral cortical wall of *Dmrt5* mutants. (*A–C* and *E–L*) Coronal forebrain sections of mutant and control mice at E12.5 analyzed by ISH for the indicated genes. (*D*) ISH for *Wnt8b* combined to immunoperoxidase staining for Lim1/2, a marker of diencephalic cells. Note that all tested genes were undetectectable or strongly reduced in the CH region (arrowheads) of E12.5 mutant mice. et, eminentia thalami. Scale bars: 500 μm.

their expression by ISH at E12.5. We found that the *Emx2* gradient is maintained in *Dmrt5*$^{-/-}$ cortices but at a lower level than in controls. *Lhx2* was also reduced in the mutants to a low level similar to that detected in the ventral telencephalon and its normal graded pattern of expression was no longer apparent. RT–qPCR on RNA extracted from isolated E12.5 mutant or WT telencephalons confirmed the downregulation of *Emx2* and *Lhx2* in the mutants (Fig. 5*A*,*B*). As Lhx2 selector activity occurs only during early development, we analyzed its expression in earlier E9.5–E10.5 embryos. At E9.5, *Lhx2* appeared similar in mutant and control embryos (data not shown). At E10.5, consistent with the decrease of *Bmps* observed at that stage in the mutants and its bimodal regulation and suppression by high levels of *Bmps* (Monuki et al. 2001), *Lhx2* appeared slightly decreased and the negative domain of expression in the dorsal midline was reduced (see Supplementary Fig. 8*A*,*B*). *Cux2*, which is strongly expressed in the CH, was lost in the mutant. The low expression of *Nurr1* in the CH and its higher expression in the choroid plexus and eminentia thalami were reduced in *Dmrt5*$^{-/-}$ embryos, the remaining signal likely corresponding to the eminentia thalami. *Nfib*, expressed in a high-medial to low-lateral gradient in dorsal telencephalic progenitors, was also strongly reduced in *Dmrt5* mutants. In contrast, the level of *Dmrt3* appeared similar in mutants compared with controls (see Supplementary Fig. 9*A–D*).

We also examined the expression of the other transcription factors playing crucial roles in cortical development: *Pax6*, *Foxg1*, *COUP-TF1*, *Sp8*, and *Gli3*. *Pax6* is downregulated by Emx2 and inhibited by Wnt signaling (Machon et al. 2007; Ivaniutsin et al. 2009). It is expressed in an opposite pattern to *Emx2* and promotes rostrolateral fates (Bishop et al. 2000; Hamasaki et al. 2004). *Foxg1* is a forkhead gene downregulated by Wnt that suppresses hem fate. It is expressed in the entire telencephalon with the exception of the caudomedial regions (Muzio and Mallamaci 2005). *COUP-TF1* represses frontal/motor cortical areas. It is expressed in a high-caudolateral to low-rostromedial gradient in the cortex, and hence, its expression is similar to *Dmrt5* along the rostrocaudal axis but opposite to *Dmrt5* along the mediolateral axis (Zhou et al. 2001; Armentano et al. 2007). *Sp8* promotes frontal/motor areas. It is induced by Fgf8 and is expressed at highest levels in the medial and anterior part of the dorsal telencephalon, thus similarly to *Dmrt5* along the L-M axis and in an opposite manner along the A-P axis (Sahara et al. 2007; Zembrzycki et al. 2007). *Gli3* is detected from early stages of neurulation throughout the telencephalic anlagen and is then progressively downregulated in the ventral portion (Aoto et al. 2002; Fotaki et al. 2006). In *Dmrt5* mutants, *Pax6* was upregulated in the medial and posterior cortical anlage of *Dmrt5* mutants where it is normally less intensively expressed. Similar changes were observed by RT–qPCR and at the protein level (Fig. 5C; see Supplementary Fig. 8*C–E*). The *Foxg1* negative domain of expression was absent in the mutants (see Supplementary Fig. 9*E*). In the telencephalon of *Dmrt5*$^{-/-}$ mutants, *COUP-TF1* was expressed in a gradient along both axes as in wild types with apparently no dramatic change in the level of its expression (see Supplementary Fig. 9*F*). In accordance with our observation that *Fgf8* in the CoP appears not dramatically affected in the mutants, no significant changes in *Sp8* expression were detected in the cortex between controls and mutants (see Supplementary Fig. 9*G*). *Gli3* expression appears also unaffected in the mutants (see Supplementary Fig. 9*H*). *COUP-TF1*, *Sp8*, and *Gli3* expression was also not significantly affected in E12.5 mutant embryos by RT–qPCR (see Supplementary Fig. 9*I*). Thus, among the known transcription factors controlling cortical identity, *Emx2*, *Lhx2*, and *Pax6* are the most severely affected in *Dmrt5* mutants. While *Emx2* and *Lhx2* are decreased, the rostral determinant *Pax6* is upregulated in the cortex of *Dmrt5* mutants. The alteration of their expression is likely to contribute to the observed cortical defects.

Mediolateral Patterning Defects and Laminar Expression of Cortical Markers in the Cortex of Dmrt5 Mutants

To investigate the role of *Dmrt5* in mediolateral cortical patterning, we first examined the expression of *Transthyretin*

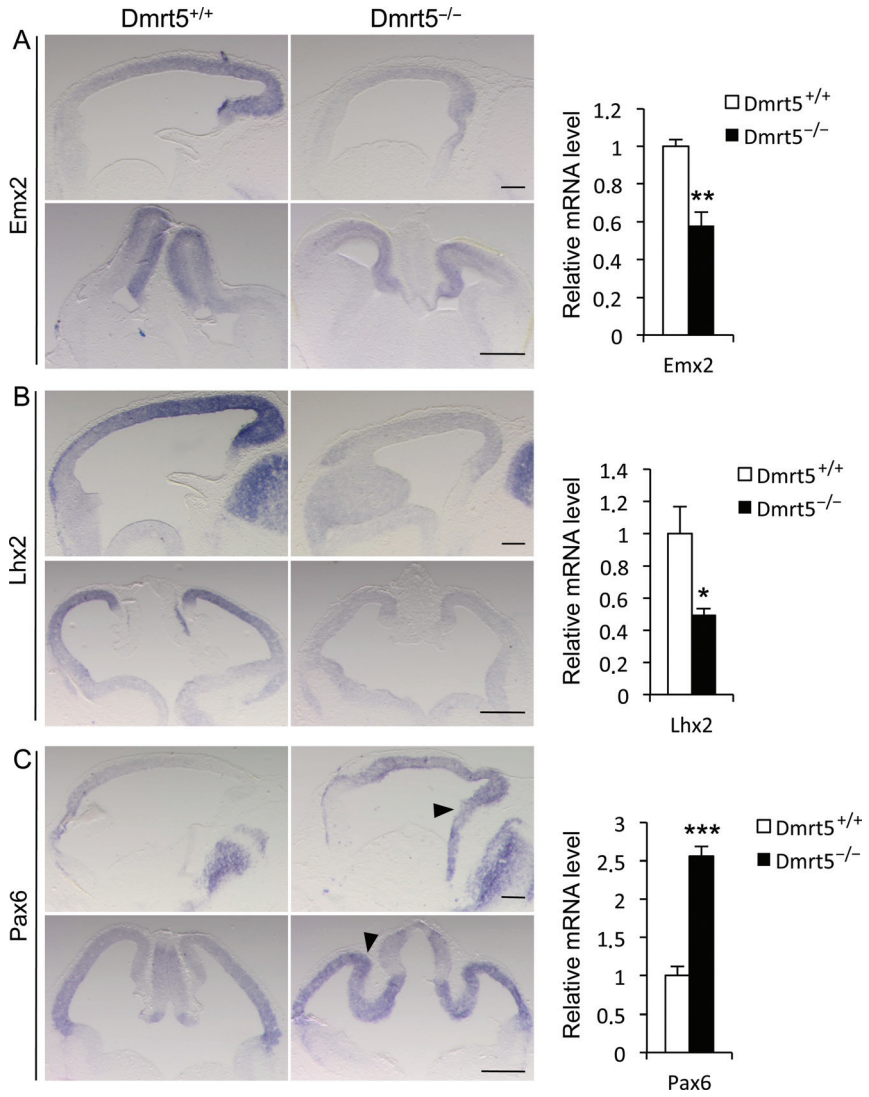

Figure 5. Downregulation of *Emx2* and *Lhx2* and upregulation of *Pax6* in the cortex of E12.5 *Dmrt5* mutants. (*A–C*) ISH for the indicated markers on sagittal (upper panels) and coronal (lower panels) sections. Sagittal sections are shown with anterior to the left. The arrowheads in (*C*) indicate *Pax6* upregulation in the medial and posterior part of the cortex of the mutants. Quantitative RT–qPCR analysis of *Emx2*, *Lhx2*, and *Pax6* in the cortex of WT and mutant E12.5 embryos is shown on the right. Results are normalized to the level of expression in the WT forebrain. Error bars show SD of 3 independent experiments. Scale bars: 200 μm in upper panels; 500 μm in lower panels (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

(*Ttr*) and of the homeobox transcription factor *Msx1*, which are expressed in the choroid plexus. Neither gene was transcribed in *Dmrt5* mutants. The homeobox gene *Lmx1a*, which is expressed in both the choroid plexus and CH, was also absent (Fig. 6*A–C*). *EphB1*, an early marker of the hippocampal primordium, *KA1*, a marker of the hippocampal CA3 field, and *Prox1*, a marker of the dentate gyrus, were also undetectable in mutants (Fig. 6*D–F*). Strikingly, a site of *EphB1* expression in the lateral part of the cortex in WT animals was found more dorsally in the cortex of mutants, suggesting possible additional defects in the lateral telencephalon. We therefore investigated the expression of additional markers expressed regionally along the mediolateral axis of the dorsal telencephalon in *Dmrt5*$^{-/-}$ mice. *Scip*, which is expressed in the lateral neocortex, was detected, although reduced, throughout the cortex in the mutants (Fig. 6G). The neurogenic gene *Ngn2*, whose expression is activated initially in the lateral cortical field, was upregulated within the medial cortical wall (Fig. 6*H*). The antineural *Id3* gene, which is confined to the medial pallium, and the dorsomedial subdomain of expression of the Notch mediator *Hes5* (Muzio et al. 2005) were in contrast downregulated (Fig. 6*I,J*). *ER81*, expressed in a high-ventral to low-dorsal gradient in the cortical plate, *Dbx1*, expressed in the anti-hem, and *Nrp2*, expressed in the paleocortex, were all detected more medially in the lateral wall of the telencephalon (Fig. 6*K–M*). Ctip2 expression in layer II of the piriform cortex and olfactory tubercles appeared shifted medially, whereas expression in layer V of the neocortex extended less ventrally (Fig. 6N).

To determine whether the different cortical cell types are specified in the *Dmrt5* mutant, we first analyzed by ISH the expression of a panel of 6 layer-specific neocortical markers (*Lhx2*, *Cux2*, *Satb2*, *RORβ*, *ER81*, and *Tbr1*). In the mutants, expression of these markers appeared in a pial to ventricular order similar to that in wild types. The ventral border of their expression as well as that of the pan-neuronal marker *Math2*

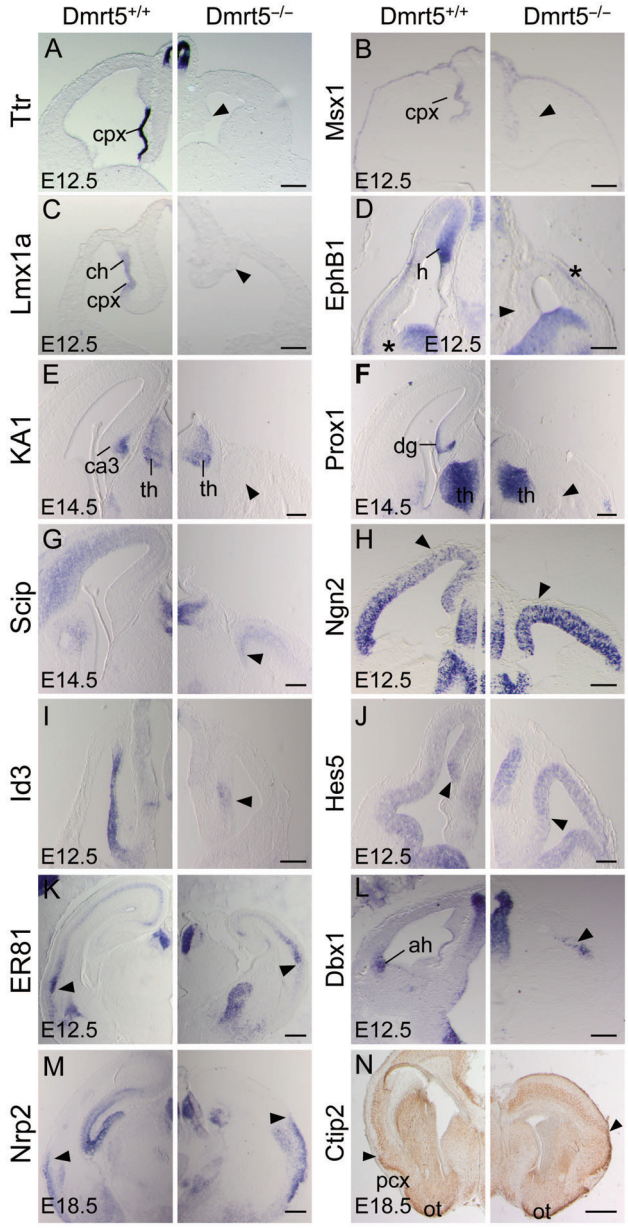

Figure 6. The Absence of choroid plexus and hippocampal markers and altered pattern of mediolateral regional markers in the dorsal telencephalon of *Dmrt5*$^{-/-}$ embryos. (*A–M*) Coronal forebrain sections processed by ISH for the indicated markers at the indicated stages. (*A–J*) While *Ttr*, *Msx1*, *Lmx1a*, *EphB1*, *KA1*, *Prox1*, *Scip*, *Id3*, and *Hes5* medial markers were strongly reduced in the mutants (arrowheads), *Ngn2* expression spreads from the lateral part of the cortex toward the dorsomedial cortex (arrowheads in *H*). (*K–M*) *ER81*, *Dbx1* and *Nrp2* as well as a site of *EphB1* expression in the lateral part of the cortex (asterisk in *D*) appeared all shifted medially in the lateral cortical wall of mutant mice (arrowheads). (*N*) Olfactory cortex specific Ctip2 expression appears shifted medially while neocortical-specific expression extends less ventrally in the mutant. The limit between the neocortex and piriform cortex is indicated (arrowheads). ah, anti-hem; ch, cortical hem; cpx, choroid plexus; dg, dentate gyrus; h, hippocampal primordium; ot, olfactory tubercles; pcx, piriform cortex; th, thalamus. Scale bars: 500 μm in *A–M*; 1 mm in *L*.

was shifted medially, providing further evidence for mediolateral patterning defects. Their expression was in some cases more diffuse than in controls (see Supplementary Fig. 10*A* and *B*). Double immunofluorescence for Cux1 (layers II–IV) together with Ctip2 (layers V and VI) or for Satb2 (layers II–IV) and Tbr1 (layer VI and subplate) confirm the expression of markers of all layers and their more diffused distribution in the cortex of *Dmrt5* mutants (see Supplementary Fig. 10*C*).

To determine the effects of *Dmrt5* ablation on dorsoventral patterning of the telencephalon, we examined the expression of *Mash1* and *Dlx2*, 2 markers of the ventral telencephalon. Those markers were unchanged, indicating that the telencephalon does not become ventralized in the mutants (see Supplementary Fig. 10*D*). Thus, genetic evidence confirms our histological observations, indicating that *Dmrt5* ablation leads to dramatic cortical patterning defects, including agenesis of the hippocampus and choroid plexus, and a disorganization and reduction of the cortical plate.

Reduction of Caudal Neocortical Regions in Dmrt5 Mutants

We next investigated on sagittal sections of E18.5 control and mutant embryos the expression of genes with restricted or graded expression patterns across the embryonic neocortex that relate to its future organization into areas (*EphrinA5*, *EphA7*, *Cad8*, *RZRβ*, *Auts2*, and *p75*). *EphrinA5* is expressed predominantly in multiple layers of the prospective somatosensory area located in between the frontal motor and caudal visual areas. In mutants, we found that *EphrinA5* is expressed at a lower level and is restricted to the most caudal part of the cortex (Fig. 7*A*). *EphA7* and *Cad8* high expression domain mark the region of the motor cortex. In mutants, their domain of high expression appeared to occupy most of the reduced cortex. The domain of low *EphA7* expression centered on the future somatosensory area was reduced and shifted caudally (Fig. 7*B*,*C*). *RZRβ*, *Auts2*, and *p75* normally exhibit strongly graded patterns of expression across the neocortex. *RZRβ* and *Auts2* are broadly expressed in the cortical plate in a high-rostral to low-caudal gradient. The low-affinity nerve growth factor receptor *p75* is expressed in the caudal half of the cortical plate. In *Dmrt5* mutants, while *RZRβ* and *Auts2* were still detectable and appeared to expand to almost the caudal end of the cortex, *p75* was severely reduced and confined to the caudal-most cortex (Fig. 7*D–F*). Therefore, a dramatic reduction of caudal neocortical areas occurs in *Dmrt5*$^{-/-}$ mice.

Activation of Wnt/β-Catenin Signaling Induces Dmrt5 Expression

The high-caudomedial to low-rostrolateral *Dmrt5* gradient in the dorsal telencephalon suggests that *Dmrt5* might be regulated by midline signals. To determine whether it is regulated by Wnt signaling, we analyzed the consequences of ectopic activation of this pathway on *Dmrt5* expression using organotypic slice cultures of E13.5 mouse telencephalon. Slices were cultured for 24 h in the presence of DMSO or various concentration of Chir, which selectively inhibits GSK3β and therefore activates Wnt signaling, and processed for ISH for *Dmrt5* and *Axin2* as a positive control. As previously reported, *Axin2* was induced by Chir and at the highest dose was expressed through the whole telencephalic ventricular zone (Hasenpusch-Theil et al. 2012) (Fig. 8*A–D*). *Dmrt5* expression was also induced at the lowest Chir concentration, and at the highest concentration, ectopic expression was detected in the ventricular zone of the lateral ganglionic eminence (Fig. 8*E–H*). These results indicate that *Dmrt5* expression is controlled by Wnt signaling, suggesting the existence of a positive feedback loop between Wnt and Dmrt5.

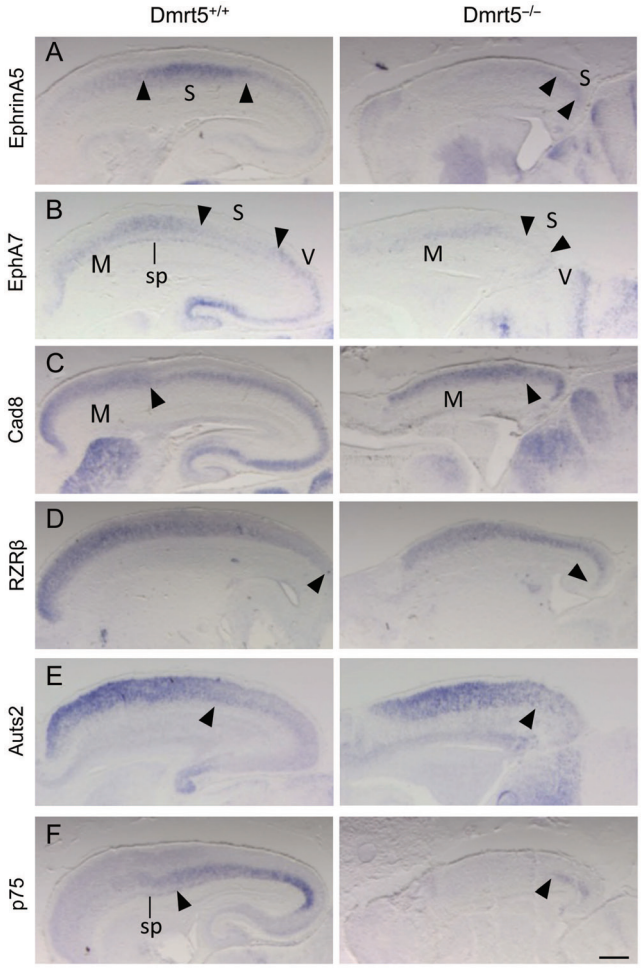

Figure 7. Reduction of caudomedial neocortical areas in *Dmrt5* mutants. Sagittal brain sections were analyzed by ISH for the indicated genes. (*A–C*) *EphrinA5* domain of high expression and *EphA7* domain of low expression that demarcate the somatosensory areas (delimitated by arrowheads) is contracted and detected in the most caudal part of the cortex in *Dmrt5*$^{-/-}$ embryos. *EphA7* and *Cad8* high expression that mark the rostrally located motor area (with caudal limits indicated by arrowheads) occupies most of the reduced cortex in mutants. (*D–F*) *RZRβ* and *Auts2* are expanded and *p75* constricted caudally in mutants. Arrowheads mark the approximate caudal and rostral limit of their expression, respectively. Note the reduced *EphA7* and *p75* in the subplate. M, motor; S, somatosensory; sp, subplate; V, visual areas. Scale bars: 500 μm.

Dmrt3–5 Expression in Gli3$^{xt/xt}$*, Emx2*$^{-/-}$*, and Pax6*$^{sey/sey}$ *Mutants*

Little is known about the genetic hierarchy underlying Wnt-regulated hippocampal development. Wnt gene expression is severely affected in *Gli3*$^{xt/xt}$ mutants (Grove et al. 1998; Theil et al. 1999) and is known to regulate the expression of the homeobox *Emx2* gene (Theil et al. 2002; Suda et al. 2010). To determine whether Gli3 and Emx2 influence *Dmrt5* expression, we investigated its expression in *Gli3*$^{xt/xt}$ and *Emx2*$^{-/-}$ mutants. In addition, we examined *Dmrt5* expression in *Pax6*$^{sey/sey}$ mutants as Emx2 and Pax6 interact with each other to control regionalization of the cortical primordium (Muzio et al. 2002). In all 3 mutants, we used ISH on sections of E11.5–E12.5 embryos and RT–qPCR analysis of RNA extracted from dissected E10.5–E12.5 cortices to analyze *Dmrt5* expression. In our RT–qPCR analysis, we also examined the expression of the closely related *Dmrt3* and *Dmrt4* genes that are coexpressed with *Dmrt5* in the developing forebrain (Smith et al. 2002; Balciuniene et al. 2006). In *Gli3*$^{xt/xt}$ mutants, *Dmrt3* and *Dmrt4* but not *Dmrt5* were downregulated at E10.5. Later at E12.5, all 3 genes were downregulated in *Gli3*$^{xt/xt}$ mutants. *Emx2*, used as control, was reduced as reported previously (Theil et al. 1999) (Fig. 8*I*,*J*). In *Emx2*$^{-/-}$ mutants, *Dmrt3* was reduced while *Dmrt5* and *Dmrt4* and *Pax6* used as controls (Muzio et al. 2002) were unchanged (Fig. 8*K*). In *Pax6*$^{sey/sey}$ mutants, *Dmrt3* and *Dmrt4* were reduced but *Dmrt5* and *Emx2* used as controls (Muzio et al. 2002) were not significantly changed (Fig. 8*L*). ISH revealed a graded *Dmrt5* expression in *Gli3*$^{xt/xt}$ and *Emx2*$^{-/-}$ mutants similar to that in normal embryos but a downregulation in the rostral telencephalon of *Gli3*$^{xt/xt}$ mutants (Fig. 8*M–O*). In *Pax6*$^{sey/sey}$ mutants, the *Dmrt5* graded expression appeared to spread slightly more anteriorly and laterally, thus flattening its gradient (Fig. 8*P*). These data indicate that *Gli3* but not *Emx2* is required for normal *Dmrt5* expression in the cortex and that Pax6 may antagonize its expression. They also reveal that *Dmrt3–5*, although coexpressed in the developing forebrain, are differentially regulated by Gli3, Emx2, and Pax6.

Discussion

Regionalization of the cerebral cortex is initiated by morphogens secreted from patterning centers located at the perimeter of the dorsal telencephalon that establish within cortical progenitors differential expression of transcription factors that determine their area identity. In this study, we show that the transcription factor *Dmrt5* is expressed in cortical apical progenitors in a high-caudomedial to low-rostrolateral gradient. We generated a *Dmrt5* KO mouse and found that it exhibits significant brain abnormalities, including a severe reduction of the caudomedial cortex, a loss of CR cells, a decrease in *Wnt* and *Bmp* signaling in the embryonic dorsomedial telencephalon, and an alteration of the downstream transcription factors, including *Emx2*, *Lhx2*, and *Pax6*. Finally, we observed that *Dmrt5* expression is activated by Wnt/β-catenin signaling and downregulated in *Gli3* mutants. These findings establish Dmrt5 as a novel Wnt-dependent key transcriptional regulator of early cortical development.

Roles for Dmrt5 in the Gene Network Regulating Early Caudomedial Cortical Development

The earliest and most severe defect observed in *Dmrt5* mutants is the reduction of the cortical surface, detectable as early as E10.5, associated with the loss of the caudomedial cortex, including the CH expressing a number of signals essential for cortical midline structure formation. Such a phenotype is reminiscent of that observed in roof plate ablated embryos (Currle et al. 2005; Cheng et al. 2006). As in *Dmrt5* mutants, midline genes such as *Wnt2b*, *Wnt3a*, *Bmp4*, and *Lmx1a* and the graded cortical transcription factors *Lhx2* and *Emx2* are also reduced in those roof plate ablated mutants. *Pax6*, *Foxg1*, and *Ngn2* are however relatively unaffected in those mutants, which contrast with their upregulation seen in *Dmrt5* mutants. A similar shrinkage and disorganization of the caudomedial cortex has also been reported in hem-ablated embryos (Yoshida et al. 2006). These similarities highlight the importance of *Wnt/Bmp* expression at the dorsal midline for telencephalon development and strengthen arguments for the

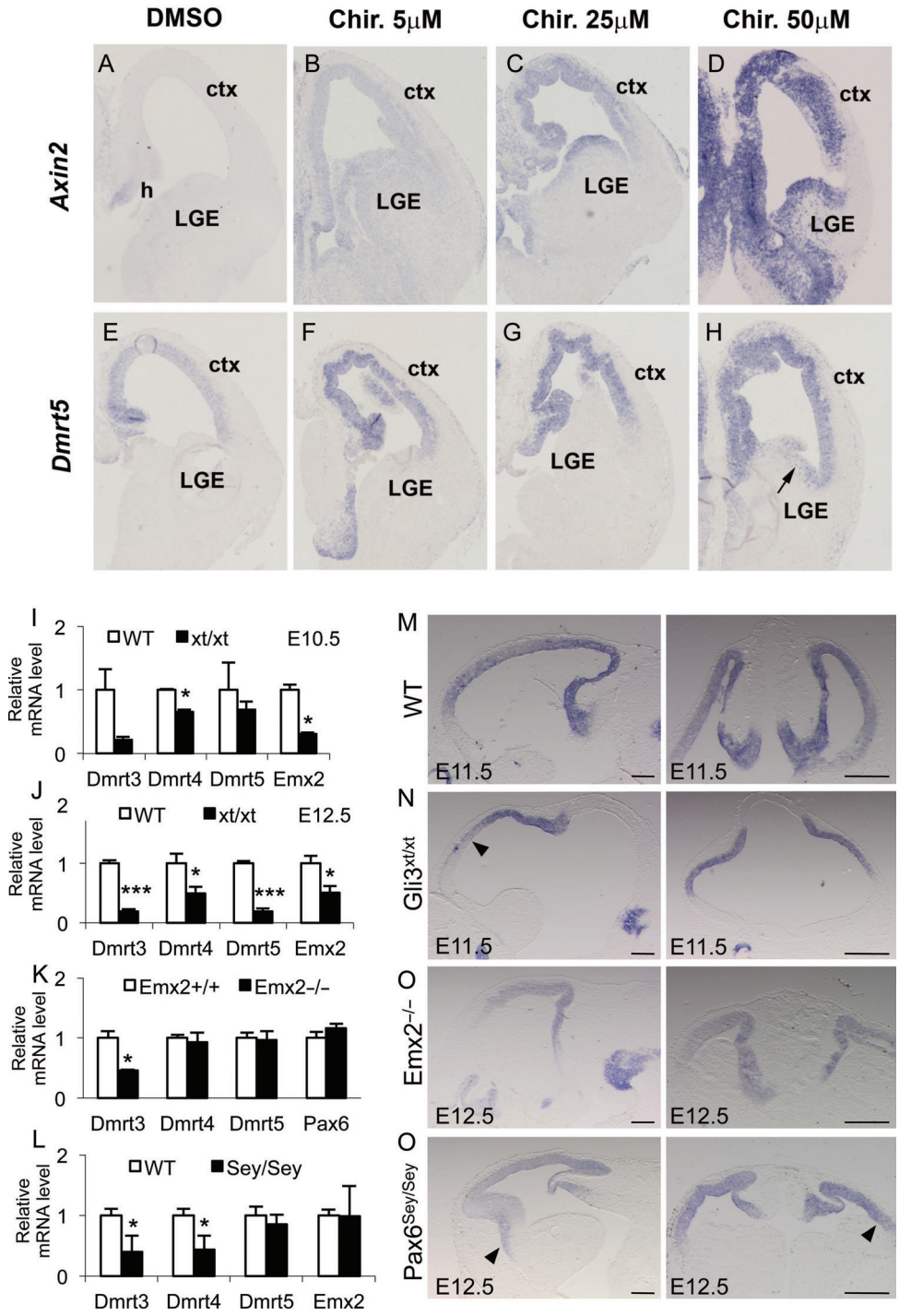

Figure 8. *Dmrt5* expression in the cortex is upregulated by activation of Wnt/β-catenin, downregulated in *Gli3*$^{xt/xt}$ embryos, and unchanged in *Emx2*$^{-/-}$ a *Pax6*$^{sey/sey}$ embryo. (*A–H*) *Dmrt5* expression in Chir-treated and control ex vivo cortical explants. Note the activation of *Dmrt5* throughout the telencephalic ventricular zone similar to that observed for *Axin2* used as a positive control after addition of Chir in a concentration-dependent manner. Note also the *Dmrt5* ectopic expression detected at high dose in the ventricular zone of the lateral ganglionic eminence (arrow). (*I–P*) *Dmrt5* expression in *Gli3*$^{xt/xt}$, *Emx2*$^{-/-}$, and *Pax6*$^{sey/sey}$ embryos. (*I–L*) Quantitative RT–qPCR analysis of *Dmrt3*, *4*, and *5* in the cortex of WT and *Gli3*$^{xt/xt}$ at E10.5 (*I*) and E12.5 (*J*) and in *Emx2*$^{-/-}$ (*K*) and *Pax6*$^{sey/sey}$ (*L*) at E12.5. Results are normalized to the level of expression in the WT forebrain. Error bars show SD of 3 independent experiments (*$P < 0.05$ and ***$P < 0.001$). (*M–P*) Sagittal (left panels) and coronal (right panels) brain sections of WT (*M*), Gli3$^{xt/xt}$ (*N*), *Emx2*$^{-/-}$ (*O*), and *Pax6*$^{sey/sey}$ (*P*) embryos, processed by ISH for *Dmrt5* at the indicated stage. Note in (*N*) the reduction anteriorly and in (*P*) the slight extension on *Dmrt5* anteriorly and laterally (arrowheads). Ctx, cortex; h, hem; LGE, lateral ganglionic eminences. Scale bars in (*M–P*): 200 μm, left panels; 500 μm, rights panels.

primacy of their defective expression in the *Dmrt5* null phenotype.

In the dorsal telencephalon, both *Emx2* and *Emx1* have graded expression similar to that of *Dmrt5*. In the double *Emx1;Emx2* mutants, but not in the single *Emx1* or *Emx2* mutants, the dorsomedial telencephalon fails to develop, indicating that the two genes cooperate in its formation (Shinozaki et al. 2002, 2004; Bishop et al. 2003). The similarity of the *Dmrt5* and *Emx1;Emx2* phenotypes and the reduction of *Emx1* and *Emx2* expression in the *Dmrt5* mutant

telencephalon (Fig. 5; data not shown) suggest that some of the telencephalic defects observed in the *Dmrt5* mutants may be attributable to the deficiency of *Emx* gene expression. The *Dmrt5* phenotype is also very similar to that observed in *Gli3* mutants. Indeed, in *Gli3* mutants, the dorsal telencephalon is abnormally small and fails to develop dorsomedial structures, including the hippocampus and CH. As in $Dmrt5^{-/-}$ embryos, loss of *Gli3* results in a reduction and/or loss of *Wnt/Bmp* expression in the dorsomedial telencephalon (Grove et al. 1998; Theil et al. 1999; Tole et al. 2000; Fotaki et al. 2011). *Gli3* mutants however also show dorsoventral patterning defects and an expansion of *Fgf8* (Theil et al. 1999; Tole et al. 2000; Aoto et al. 2002; Kuschel et al. 2003), defects not detected in *Dmrt5* mutants.

While *Dmrt5* inactivation does not affect the global dorsoventral patterning of the telencephalon nor apoptosis, the size and decreased thickness of the cortical hemispheres, the disorganization of the cortical ventricular zone, and the frequency of PH3 positive cells are altered in *Dmrt5* mutants. These results suggest that the *Dmrt5* phenotype cannot be purely due to misspecification and that it might have a role in corticogenesis. As apical progenitors did not decrease in number at E11.5, it is unclear whether the transient increase in Tbr2-positive cells observed at that stage in $Dmrt5^{-/-}$ mice is due to apical progenitors exiting prematurely from the cell cycle and differentiating into IPCs. The decrease of Wnt signals known to control initiation of neurogenesis in the cortex (Machon et al. 2007), the reduction of *Emx2* that is required for the proliferation of cortical progenitors (Heins et al. 2001; Muzio et al. 2005), the repression of the antineurogenic *Id3* and *Hes5* genes, and the upregulation of *Pax6* and of its downstream target, the proneural gene *Ngn2* (Scardigli et al. 2003), observed in the mutants support however this premature differentiation hypothesis. Additional studies will be required to provide further evidence for those changes in proliferation and determine rigorously the cell cycle kinetics in these mutants.

Interactions between secreted ligands and transcription factors controlling cortical regionalization are very complex. Emx2 promotes Wnt signaling, which in turn promotes its expression (Theil et al. 2002; Muzio et al. 2005). In this way, a robust positive feedback loop is established in the caudomedial cortex contributing to reinforcing *Emx2* expression (Mallamaci 2011). *Gli3* controls *Wnt* gene expression which in turn activates its expression and induces several *Wnt* targets, including direct targets such as *Emx2* and *Dmrt3* (Theil et al. 1999, 2002; Fotaki et al. 2011; Hasenpusch-Theil et al. 2012). Our data show that *Dmrt5* which is required for *Wnt* gene expression in the midline is regulated by Wnt and that Gli3 is required at least for the maintenance of *Dmrt5* in the forebrain. In contrast, the absence of *Emx2* does not affect its expression. These data, together with the observation that *Gli3* is unaltered in *Dmrt5* mutants, suggest that *Dmrt5* may act downstream of *Gli3* and upstream of *Emx2* genes in the *Wnt/Bmp*-dependent genetic cascade controlling development of the dorsomedial telencephalon (Fig. 9). Whether as in the case of *Emx2* (Theil et al. 2002), Bmp signaling plays a role in the regulation of *Dmrt5* is unknown. Whether *Gli3* regulates directly *Dmrt5* expression or whether its loss in *Gli3* mutants is indirect, due to a blocking of Wnt signaling, as recently shown for *Dmrt3* (Hasenpusch-Theil et al. 2012), remains also to be investigated. Whether Dmrt5 regulates directly or indirectly *Wnt* and *Emx* genes requires also further investigations.

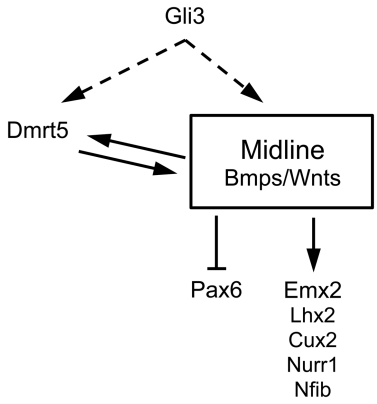

Figure 9. Summary of the interactions identified in this work between *Dmrt5* and the secreted ligands and Wnt/Bmp regulated graded transcription factors expressed during early patterning of the cerebral cortex. Wnt and Gli3 control *Dmrt5* expression. Direct or indirect action of Gli3 on *Dmrt5*, through regulation of the Wnt signaling pathway, is indicated by dashed lines. Dmrt5 in turn is required for *Wnt* and *Bmp* expression in the dorsal midline signaling center. In the absence of Dmrt5, this feedback loop is not maintained, which is likely responsible for the downregulation of Wnt target genes. *Pax6* is upregulated in *Dmrt5* mutants, presumably through its negative regulation by Wnts and Emx2.

We found that *Dmrt3*, like *Dmrt5*, is reduced in *Gli3* mutants. In contrast to *Dmrt5*, *Dmrt3* is also decreased in *Pax6* and *Emx2* mutants, suggesting that it may function downstream of the 3 factors. *Dmrt4*, like *Dmrt5*, is reduced in *Gli3* mutants and unaltered in *Emx2* mutants. In contrast to *Dmrt5*, *Dmrt4* is downregulated in *Pax6* mutants, suggesting a possible function downstream of this factor.

Reduction of CR and Subplate Cells in Dmrt5 Mutants

CR cell formation is dramatically impaired in *Dmrt5* mutants, as previously reported in *Emx2* mutants (Mallamaci et al. 2000; Shinozaki et al. 2002). This deficit of CR cells is probably a consequence of the reduction of the hem, which is a major source of CR cells (Yoshida et al. 2006). Hem-derived CR cells have been shown to predominantly populate the caudomedial and dorsolateral pallium, while CR cells found in the rostromedial and lateral pallium are derived from the septum and anti-hem, respectively (Bielle et al. 2005). It is thus likely that the remaining CR cells found mainly in the medial part of the cortex of *Dmrt5* mutants derive from the rostromedial source. The few remaining cells found in the lateral neocortex may be derived from the anti-hem. Their abnormal clustering may be due to altered intrinsic properties, or alternatively, to non-cell autonomous modifications in the caudal telencephalon related to the loss of CH and choroid plexus territories. Aberrant clustering of CR cells has been previously reported in the *Gli3* compound mutant $Gli3^{Xt/Pdn}$ (Friedrichs et al. 2008) and in embryos carrying mutant alleles of other genes involved in CR cells development (Miquelajauregui et al. 2010). As migrating CR neurons have been shown to express a repertoire of signaling factors and to signal to VZ progenitors (Borello and Pierani 2010), their absence likely contribute to the early regionalization defects observed in *Dmrt5* mutants. We showed that from E13.5, *Dmrt5* is expressed in CR cells. Whether it plays a role in the maintenance CR cells of the marginal zone requires further investigation.

Our analysis indicates that the subplate is reduced in the cortex of *Dmrt5* mutants at E15.5 and E18.5 similar to Emx2 and Gli3 mutants (Mallamaci et al. 2000; Shinozaki et al. 2002; Friedrichs et al. 2008). The fact that this early neuronal population is affected in the mutant, despite the absence of *Dmrt5* expression in subplate cells and main restriction of *Dmrt5* expression in the VZ, further suggests a role for Dmrt5 in the control of the timing of the exit of apical progenitors from the cell cycle or the differentiation-survival program of their descendants.

Dmrt5 is Required for Cortical Regionalization

In addition to these early midline defects, our histological and molecular analyses at E18.5 have revealed that the caudal neocortex is dramatically reduced in *Dmrt5* mutants. Although rostral regions are expanded in proportional sizes, their absolute sizes appeared rather similar in mutants and wild types. The contraction of the caudal neocortex of *Dmrt5* mutants may be caused in part by its decreased growth. It is also possible to be due to alteration of positional identity of cortical progenitors. Those changes may be the consequence of the reduction of *Emx2* which promotes caudomedial fates, as small changes in its expression have substantial effects on arealization (Hamasaki et al. 2004). Emx2 has been show to pattern the neocortex by regulating Fgf positional signaling (Fukuchi-Shimogori and Grove 2003; Cholfin and Rubenstein 2008). However, a direct cell-intrinsic mechanism has also been implicated (Hamasaki et al. 2004). As *Fgf8* and *Fgf17* appear not dramatically affected in the mutants, alteration of these intrinsic Emx2-dependent patterning mechanisms is likely to be responsible for the mutant phenotype. COUP-TF1 is another transcription factor that plays an essential direct role in cortical arealization by regulating *Emx2* and *Pax6* expression without apparently altering *Fgf8* expression (Armentano et al. 2007). The contribution of the upregulation of the rostral determinant *Pax6* to the *Dmrt5* phenotype is also unclear, as its overexpression has no substantial effect on area patterning (Manuel et al. 2007). We also found that the loss of *Dmrt5* has substantial effects on cortical mediolateral patterning. In its absence, choroid plexus, CH, and hippocampal markers are switched off, which is consistent with the observation that *Foxg1*, which restricts the CH to the dorsomedialmost telencephalon (Muzio and Mallamaci 2005b), is expressed in the entire cortex. Neocortical markers extend less laterally and paleocortical markers are detected slightly more medially in the reduced cortex of the $Dmrt5^{-/-}$ embryos. This phenotype fits with our observation that *Lhx2* becomes downregulated in already specified cortical progenitors (at E10.5) in $Dmrt5^{-/-}$ embryos, during the critical period of time where it controls regional fate within telencephalic progenitors (Chou et al. 2009).

Mild Cortical Lamination Defects and Severe Forebrain Axonal Connection Abnormalities in Dmrt5 Mutants

The absence of *Reelin* expression in the neocortical marginal zone of $Dmrt5^{-/-}$ mice suggested that radial neuronal migration could be perturbed in these mice. We therefore investigated laminar ordering in E18.5 *Dmrt5* mutants. Despite the massive loss of CR cells, we found that neocortical layers are ordered correctly, as previously observed in hem-ablated embryos (Yoshida et al. 2006). As in hem-ablated embryos, expression of layer-specific neuronal markers showed however a variable degree of diffuseness in *Dmrt5* mutants relative to wild types. As in *Emx2* and in $Gli3^{Xt/Pdn}$ mutants, βIII-tub positive neurons were also present in the cortical VZ/SVZ of the *Dmrt5* mutants (data not shown) which may correspond to neuronal cells coming out of the cell cycle and migrating, slower than normal, from the VZ toward their final radial location (Mallamaci et al. 2000). In addition to potential migration abnormalities, post-migratory mechanisms involved in laminar consolidation could also explain these mild defects.

Dmrt5 mutants have major defects in the pathfinding of most cortical axons. Similar defects have been observed in *Emx1;Emx2* double mutant mice and in the *Gli3* hypomorphic mutant Polydactyly Nagoya (Pdn) (Shinozaki et al. 2002; Bishop et al. 2003; Magnani et al. 2010). The cortex appears to fail to send axons to the striatum. TCAs fail to enter the cortex and a large proportion of them are stopped at the border region between the diencephalon and telencephalon. The reason why cortical efferent axons are affected is not clear. Their inability to pathfind may be a cell autonomous defect or it may be secondary to a loss of instructive cues within the cortex. Because *Dmrt5* is not expressed in the region of the thalamus that gives rise to the TCA projection neurons, it is likely that the TCA defects are non-cell autonomous to them and that these defects are secondary to the cortical alterations. Pioneer neurons from the subplate are well known to play an important guidance role in thalamocortical projections (Allendoerfer and Shatz 1994; Zhou et al. 1999; Kanold and Luhmann 2010). Their absence in *Dmrt5* mutants is thus likely to play a role in the observed thalamocortical tract defects. As *Dmrt5* is also expressed in the prethalamus and hypothalamus, in the eminentia thalami, and in a narrow strip of the medial ventral telencephalon (see Supplementary Fig. 1; data not shown), additional studies are also required to determine whether *Dmrt5* in these regions influence the trajectory of TCA axons.

All major telencephalic commissures (corpus callosum, hippocampal commissure, and anterior commissure) are hypoplastic in $Dmrt5^{-/-}$ embryos. The origin of the commissure defects observed in $Dmrt5^{-/-}$ embryos, also found in *Emx2* and *Gli3* mutants (Naruse et al. 1990; Yoshida et al. 1997), is unknown. Disturbance of commissure formation is likely to result from intrinsic defects in cortical neurons or from failure of the environment to produce an extracellular environment that is suitable for navigation (Lindwall et al. 2007). Midline structures that are required for corpus callosum formation, glial wedge, induseum griseum neurons, and glia are most likely severely perturbed in the $Dmrt5^{-/-}$ embryos given the fact that the whole rostral midline structures are mostly absent. As their formation is dependent on appropriate Bmp and Wnt signaling levels (Wang et al. 2006; Sánchez-Camacho et al. 2011), we speculate that the absence of the corpus callosum in the mutant is a secondary consequence of the reduction of Bmp and Wnt from midline structures and the downregulation of transcription targets of midline signals such as *Nfib* known to be required for the development of midline glial populations (Piper et al. 2009). Proper patterning of the cortical septal boundary and hence accurate distribution of guidepost neurons at later stages has been demonstrated recently to be crucial for callosal development in *Rfx3* mutants (Benadiba et al. 2012).

Recent studies using embryonic stem cells have shown that *Dmrt5* is an important regulator of ventral mesencephalic neural fate specification (Gennet et al. 2011). Our analysis of the defects of *Dmrt5*$^{-/-}$ mice indicate that it also plays a crucial role in caudomedial cerebral cortex development, likely via the control of the production of midline signaling molecules during early forebrain development. In the future, it will be interesting to determine whether *Dmrt5* also has a role in later forebrain development and whether *Dmrt3* and *Dmrt4* have redundant function with *Dmrt5* during hippocampus formation.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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