


DMRT5, DMRT3, and EMX2 Cooperatively Repress *Gsx2* at the Pallium–Subpallium Boundary to Maintain Cortical Identity in Dorsal Telencephalic Progenitors

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Specification of dorsoventral regional identity in progenitors of the developing telencephalon is a first pivotal step in the development of the cerebral cortex and basal ganglia. Previously, we demonstrated that the two zinc finger *doublesex* and *mab-3 related (Dmrt)* genes, *Dmrt5 (Dmrta2)* and *Dmrt3*, which are coexpressed in high caudomedial to low rostralateral gradients in the cerebral cortical primordium, are separately needed for normal formation of the cortical hem, hippocampus, and caudomedial neocortex. We have now addressed the role of *Dmrt3* and *Dmrt5* in controlling dorsoventral division of the telencephalon in mice of either sex by comparing the phenotypes of single knock-out (KO) with double KO embryos and by misexpressing *Dmrt5* in the ventral telencephalon. We find that DMRT3 and DMRT5 act as critical regulators of progenitor cell dorsoventral identity by repressing ventralizing regulators. Early ventral fate transcriptional regulators expressed in the dorsal lateral ganglionic eminence, such as *Gsx2*, are upregulated in the dorsal telencephalon of *Dmrt3;Dmrt5* double KO embryos and downregulated when ventral telencephalic progenitors express ectopic *Dmrt5*. Conditional overexpression of *Dmrt5* throughout the telencephalon produces gene expression and structural defects that are highly consistent with reduced GSX2 activity. Further, *Emx2;Dmrt5* double KO embryos show a phenotype similar to *Dmrt3;Dmrt5* double KO embryos, and both DMRT3, DMRT5 and the homeobox transcription factor EMX2 bind to a ventral telencephalon-specific enhancer in the *Gsx2* locus. Together, our findings uncover cooperative functions of DMRT3, DMRT5, and EMX2 in dividing dorsal from ventral in the telencephalon.

Key words: Dmrt; dorsoventral patterning; Emx2; Gsx2; pallium–subpallium boundary; telencephalon

Significance Statement

We identified the DMRT3 and DMRT5 zinc finger transcription factors as novel regulators of dorsoventral patterning in the telencephalon. Our data indicate that they have overlapping functions and compensate for one another. The double, but not the single, knock-out produces a dorsal telencephalon that is ventralized, and olfactory bulb tissue takes over most remaining cortex. Conversely, overexpressing *Dmrt5* throughout the telencephalon causes expanded expression of dorsal gene determinants and smaller olfactory bulbs. Furthermore, we show that the homeobox transcription factor EMX2 that is coexpressed with DMRT3 and DMRT5 in cortical progenitors cooperates with them to maintain dorsoventral patterning in the telencephalon. Our study suggests that DMRT3/5 function with EMX2 in positioning the pallial-subpallial boundary by antagonizing the ventral homeobox transcription factor GSX2.

Introduction

The mammalian telencephalon is the largest and most complex region of the mammalian brain, controlling cognitive processes and purposeful actions. It comprises the cerebral cortex dorsally and the amygdala and basal ganglia structures ventrally. Defects in telencephalon development are associated with many human neuropsychiatric and neurological disorders (Gaitanis and Walsh, 2004; Hu et al., 2014). Related to this study, a loss-of-function mutation in the human *DMRT5/DMRTA2* gene has been found to be associated with microcephaly (Urquhart et al., 2016).

Specification of dorsoventral (DV) regional identity in progenitors of the developing telencephalon is a pivotal step in the development of the cerebral cortex and basal ganglia. In the developing telencephalon, as in the spinal cord, bone morphogenetic proteins (BMPs) and Wingless-Int proteins (WNTs) produced dorsally and Sonic Hedgehog (SHH) secreted from ventral sources are implicated in DV specification of the telencephalon (Ericson et al., 1995; Chiang et al., 1996; Backman et al., 2005; Fernandes et al., 2007). Opposition between these morphogens alone does not establish DV telencephalic identity. Rather, interactions among the transcription factor GLI3, FGF signaling from the rostral telencephalic patterning center, and ventral sources of SHH regulate DV patterning (Ohkubo et al., 2002; Shimogori et al., 2004; Hasenpusch-Theil et al., 2017; Grove et al., 1998; Theil et al., 1999; Tole et al., 2000a; Aoto et al., 2002; Kuschel et al., 2003). GLI3 is a transcriptional activator in the presence of high levels of SHH, and a repressor, GLI3R, when levels of SHH are low. SHH promotes ventral identity by maintaining FGF signaling at the rostral telencephalic patterning center in part by suppressing formation of Gli3R, which represses *Fgf8* expression (Ohkubo et al., 2002; Rallu et al., 2002; Rash and Grove, 2007). FGFs at the rostral telencephalic patterning center are in turn required to establish or maintain *Shh* expression in the ventral telencephalon (Storm et al., 2006). FGF signaling further promotes ventral telencephalon development independently of SHH, through regulating expression of the *Foxg1* transcription factor gene (Gutin et al., 2006; Tole and Hébert, 2013). *Pax6* and other homeobox genes, such as *Emx2* (Muzio et al., 2002a,b) expressed throughout the pallium, and the homeobox gene *Gsx2* expressed in the subpallium are also involved in positioning the pallium–subpallium boundary (PSB) (Stoykova et al., 2000; Tor-

esson et al., 2000; Yun et al., 2001; Kroll and O'Leary, 2005; Carney et al., 2009). How these different transcription factors function together to control telencephalon DV patterning and whether there are other players involved remain unknown.

Dmrt3 and *Dmrt5* (*Dmrt2*) encode related transcription factors expressed in a similar high caudomedial to low rostralateral gradient in the cortical primordium. Their loss leads to a similar phenotype, more severe in *Dmrt5* than in *Dmrt3* mutants. In either single null mutant, Wnt and BMP expression at the cortical hem is decreased, and adjacent hippocampus and caudal neocortical areas are reduced in size. In conditional *Dmrt5* mouse models, *Dmrt5* loss or gain of function after hem formation also leads to a reduction of hippocampal size and alters neocortical area map formation, indicating that DMRT5 is not only required for hem formation but also directly controls cortical progenitor proliferation and specification. DMRT3 and DMRT5 are thus crucial regulators of cortical development, acting at different steps of its formation (Konno et al., 2012; Saulnier et al., 2013; Young et al., 2017; De Clercq et al., 2018). DMRT3 and DMRT5 have similar DNA-binding properties (Murphy et al., 2007), suggesting they act redundantly in telencephalic development, implying that analysis of single knock-out (KO) embryos did not reveal their full function. We therefore generated double KO and compared their telencephalic development with that of single KO embryos. Further, we conditionally overexpressed *Dmrt5* in the telencephalon. As *Emx2* is coexpressed with *Dmrt3* and *Dmrt5* in cortical progenitors, we also generated *Dmrt5;Emx2* double KO embryos. Our new findings reveal that DMRT3, DMRT5, and EMX2 cooperate to repress *Gsx2* and maintain DV patterning in the telencephalon.

Materials and Methods

Animals. All mice were maintained on a C57BL/6 or CD1/C57BL6 mixed background and mice of either sex were used. Midday of the day of the vaginal plug discovery was defined as embryonic day (E) 0.5. Animal care was in accordance with Institutional guidelines, and the policies of the National Institutes of Health.

Dmrt3 (Saulnier et al., 2013), *Dmrt5* (De Clercq et al., 2018), *Emx2* (Pellegrini et al., 1996), and *Gsx2^{EGFP}* mice (Wang et al., 2009) were genotyped by PCR as described, respectively, in these articles. *Dmrt3^{+/-}* or *Dmrt3^{-/-}* animals, which are viable, were crossed with *Dmrt5^{+/-}* mice to obtain *Dmrt3^{+/-};Dmrt5^{+/-}* mice. These double heterozygotes were then crossed to obtain *Dmrt3^{-/-};Dmrt5^{-/-}* homozygous double KO embryos. *Dmrt5^{-/-};Emx2^{-/-}* homozygous double mutants were obtained by intercrossing *Dmrt5^{+/-}* heterozygous mutants with *Emx2^{+/-}* heterozygous mutants. *Dmrt5* conditional transgenic (*Dmrt5Tg*) mice were maintained and genotyped as described previously (De Clercq et al., 2018) and crossed to *Foxg1-IRES-Cre* (Kawaguchi et al., 2016) mice to overexpress *Dmrt5* throughout the telencephalon or to *Gsx2-Cre-IRES-EGFP* (*Gsx2-CIE*) (Qin et al., 2016) to overexpress it in ventral telencephalon progenitors. *tetO-Gsx2-IRES-EGFP* mice were crossed to *Foxg1^{TA/+}* mice to obtain *Foxg1^{TA/+};tetO-Gsx2-IRES-EGFP* embryos overexpressing *Gsx2* throughout the telencephalon (Waclaw et al., 2009).

Histology, immunofluorescence (IF), and ISH. Standard H&E staining was performed on 6–8 μ m sections of embryos or brains fixed overnight in 4% PFA/PBS, dehydrated, and paraffin-embedded. For IF, embryos were fixed overnight at 4°C in 4% PFA/PBS, infused in 30% sucrose/PBS overnight, frozen in gelatin (7.5% gelatin, 15% sucrose/PBS) or NEG-50, and cryosectioned (12–20 μ m). Antigen retrieval was performed by boiling the sections in Target Retrieval Solution Citrate, pH 6.0 (Dako). Slides were blocked with 10% normal goat serum, 0.3% Triton X-100 in PBS, and incubated with primary antibodies overnight at 4°C. The incubation with secondary antibodies was performed for 2 h at room temperature. Samples were then mounted in Dako mounting medium. The following primary antibodies were used: rabbit anti-TH (1:500, Immunostar), rabbit anti-DMRT5 (1:2000) (De Clercq et al., 2018), rabbit

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anti-GSX2 (1:500) (Toresson et al., 2000), mouse anti-ASCL1 (1:100, gift from C. Parras laboratory), goat anti-PAX6 (1:200, Santa Cruz Biotechnology), rabbit anti-TBR2 (1:500, Abcam), and chicken anti-GFP (1:1000, Aves Labs). The following secondary antibodies were used: anti-mouse AlexaFluor-488 (1:400, Invitrogen), anti-mouse AlexaFluor-594 (1:400, Invitrogen), anti-rabbit AlexaFluor-488 (1:400, Invitrogen), anti-rabbit AlexaFluor-594 (1:400, Invitrogen), and anti-rabbit AlexaFluor-594 (1:400, Invitrogen). Sections were counterstained with DAPI. Images were acquired with a Carl Zeiss LSM 70 confocal microscope using ZenBlack software or Nikon A1R gallium arsenide phosphide inverted Confocal Microscope and processed using ImageJ and Photoshop software.

ISH on sections and whole-mount ISH of embryos were performed using antisense digoxigenin-labeled riboprobes as described previously (Saulnier et al., 2013; De Clercq et al., 2018). The other antisense probes were generated from the previously described cDNA clones: *Emx1* (Theil et al., 1999), *Ascl1* and *Gad67* (Fode et al., 2000), *Dlx2* (Porteus et al., 1991), *Gsx2* (Toresson et al., 2000), *Sp8* (Sahara et al., 2007), *Isl1* (Huber et al., 2013), *ER81* (Chotteau-Lelièvre et al., 1997), and *sFrp2* and *TGF- α* (Assimacopoulos et al., 2003). Images were acquired with an Olympus SZX16 stereomicroscope and a XC50 camera, using the Imaging software CellSens.

Confocal imaging. Confocal imaging used an LSM-710 confocal microscope (Carl Zeiss) using both $\times 10/0.3$ EC Plan Neofluor and $\times 20/0.8$ PlanApoChromat objectives (Carl Zeiss) and with specific excitation using a 405 nm laser diode, a 488 nm Argon ion laser, and a 594 nm Helium/Neon laser. A specific gallium arsenide phosphide Airyscan detector was used when necessary to increase signal detection and signal/noise ratio. Images were acquired using ZenBlack software (Carl Zeiss). For Tile scan imaging, acquisitions were performed with a 10% overlap of fields, and images were reconstructed using ZenBlack software. For nuclei counting in Figure 5, a homemade automated macro was developed on Fiji software. Briefly, background of images was reduced using a “rolling ball radius” function, and nuclei were segmented through fluorescence intensity using an automated threshold. Nuclei segmented from both “green” and “red” channels were counted automatically through a size selection, and nuclei present in both channels were considered as colocalizing. Brightness and contrast adjustments and image processing were done using Fiji and Photoshop software.

RNA sequencing (RNAseq). RNA was extracted using the RNeasy mini kit from QIAGEN and prepared for sequencing using the TruSeq RNA Sample Prep Kit version 2. Four WT samples and five samples of each genotype were analyzed. Sequence reads have been obtained using an Illumina HiSeq 2000 in single-end mode (51 bp). Sequencing quality was checked and approved using the FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and one Dmrt5 KO sample that had too low number of reads was removed. Reads were aligned to the mouse genome reference (genome assembly mm10 downloaded from www.ensembl.org) using the STAR alignment software (<https://www.ncbi.nlm.nih.gov/pubmed/23104886>, version 2.3) allowing for 2 mismatches within 51 bp. Gene expression quantification was performed using the featureCounts tool (<https://www.ncbi.nlm.nih.gov/pubmed/24227677>). Count normalization and differential gene expression analyses were conducted using R/Bioconductor (version 3.2) and the DESeq2 package (<https://www.ncbi.nlm.nih.gov/pubmed/25516281>, version 1.8.2). PCA and hierarchical clustering were applied to normalized count data. Two outlier samples were removed (one from control group after inspection of sample cluster plots and the one from the Dmrt5 KO group with low number of reads). Genes were annotated using the biomaRt package (<https://www.ncbi.nlm.nih.gov/pubmed/19617889>, version 2.24). Differentially expressed genes were selected based on a log₂-fold-change and false discovery rate cutoff of 1 and 0.05, respectively. Gene Ontology analysis was performed using DAVID 6.7 (<http://david.abcc.ncifcrf.gov>) in the annotation category BP-FAT. Strongly enriched categories had a score of ≤ 0.05 after Benjamini multiple test correction. RNAseq data have been deposited at NCBI GEO (GSE108611).

Plasmid construct and transgenesis. The 1.8 kb of the *Gsx2* locus (chr5: 75481187–75479361, mm9 assembly) was amplified by PCR with the oligonucleotides forward 5'-CGCACCGTTGGGGATTCTAA-3' and re-

verse 5' TCTCTCAATTCCCAGGGGTCA-3' and using DNA from BAC clone RP23–382i10 as template. The resulting fragment was subcloned 5' of the globin basal promoter in the SpeI and NotI sites of a reporter vector (BGZA) containing the chick β -globin promoter, the lacZ reporter gene, and an SV40 polyadenylation cassette (Yee and Rigby, 1993). The 1.3 kb fragment of the *Gsx2* locus (chr5: 75480316–75481596, mm9 assembly) was amplified by PCR with the oligonucleotides forward 5'-GGC GCGCCACCCTTTTGTGGTTGTTAAGACTTAG-3' and reverse 5'-ACAAAAAGCAGGCTGTTGTCGTTTCAGGTGGCAAGG-3' using DNA from BAC clone RP24–223O21 as template, and was inserted into a vector containing *Cre-IRES-EGFP*. Transgenic embryos were generated by pronuclear injection at the Transgenesis platform of the De Duve Institute (UCL, Leuven) using fertilized eggs from B6CBAF1/Cl crosses (for fragment A) or at the Transgenic Animal and Genome Editing Core at Cincinnati Children's Hospital Medical Center by microinjection of fertilized eggs from FVN mice (for fragment B). Transgenic embryos were identified by PCR using tail DNA. Expression of the transgene was analyzed in E12.5 embryos. LacZ activity in transgenic embryos was detected by whole-mount X-gal staining. Expression of the GFP transgene was examined by DAB staining of E12.5 brain sections with chicken anti-GFP antibodies (1:1000, Aves Labs), followed by biotinylated donkey anti-chicken IgY (1:200, Jackson ImmunoResearch Laboratories) and ABC solution (Vector Laboratories).

Electrophoretic mobility shift assay (EMSA). EMSAs were performed using extracts from HEK293 cells transfected with *Dmrt3*, *Dmrt5*, and/or *Emx2* pEFX expression vectors. The double-stranded oligonucleotide used the Dmrt and/or Emx2 binding sites (BSs) (italicized) as follows: Dmrt BS1, 5'-GCTGGGTACTACTGGAAGGA-3'; Dmrt BS2, 5'-TAGTCACTGTTTCATTAGGC-3'; Dmrt BS3, 5'-GACTTTTCGATACATTCCTA-3'; Emx2 BS1, ACCTCCCCTCTTCCTTCCTAATTAA TGACCAT-3'; and DMRT BS3/EMX2 BS2, 5'-GACTTTTCGATACATTCCTAATTGACTGAGGG-3'. Briefly, double-stranded biotin-labeled probes and nonlabeled competitors were prepared by mixing equal molar ratio of two complementary oligos in annealing buffer (10 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, and 50 mM NaCl). The paired oligos were heated to 95°C for 5 min; then the temperature was reduced slowly to room temperature on hot blot. DNA binding reaction was performed in binding buffer (10 mM Tris-acetate, pH 7.9, 5 mM magnesium acetate, 25 mM potassium acetate, 0.1 μ M ZnSO₄, and 50 μ g/ml BSA), including 0.5 μ g sonicated herring sperm DNA and 1 pmol double-stranded DNA probe. A total of 23 pmol of competitor (23-fold molar excess to probe) was used in each reaction as indicated. Protein-DNA complexes were separated by electrophoresis at 50 V for 3 h on 5% polyacrylamide minigels in 0.5 \times TBE at 4°C. DNA was transferred to Hybond⁺ membrane using Bio-Rad Trans-blot SD SemiDry Electrophoretic Transfer Cell and crosslinked by UV light for 5 min. Biotin-labeled DNA was detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific Pierce).

Experimental design and statistical analysis. Quantification of the dorsal surface area of the cortical hemisphere of E12.5 and E18.5 animals was obtained by taking measurements from images of whole brains. Photographs were taken with an Olympus SZX16 stereomicroscope and a XC50 camera. Measurements were done using ImageJ software. All quantified data are expressed as mean \pm SD values with the value obtained for WT set to 1. Significance tests were performed using a two-tailed Student's *t* test; *p* values < 0.05 were regarded as statistically significant. For each genotype, at least 5 embryos were examined.

In *Dmrt* single and double KO and transgenic *Dmrt5*-overexpressing embryos, for each marker and age, 2–4 embryos were analyzed. In *Dmrt5*;*Emx2* double mutants, markers were only examined in 2 embryos due to the difficulties of obtaining them. For each of the markers used and in embryos of the different genotypes analyzed, photographs of sections from the rostral to caudal or from the lateral to medial part of the telencephalic vesicles were taken and assembled into series. Comparable coronal sections, taken at rostral to mid telencephalon levels (based on landmarks provided by the ganglionic eminences), and sagittal sections, taken at the level of the olfactory bulb (OB), are shown.

Mice with conditional misexpression of *Dmrt5* throughout the telencephalon (using the *Foxg1-IRES-Cre* driver line) show a ventral expan-

sion of the normally dorsally expressed gene, *Emx1*. The amount of expansion was measured in images of coronal brain sections through E12.5 conditional transgenic and control embryos. Images were obtained with an Axioscope microscope fitted with Axiovision software (Carl Zeiss). Five 12.5 transgenic embryos and 5 controls, from 3 different litters were used. To quantify the ectopic spread of *Emx1* expression, a consistent landmark between control and mutant mice was essential. The demarcation between the LGE and MGE was such a landmark, given that the MGE appeared essentially unaltered in *Dmrt5*^{Tg/+}; *Foxg1*^{TRES-Cre/+} embryos. We therefore measured how close the expression of *Emx1* came to this landmark, focusing on expression in the medial and central parts of the LGE where *Emx1* is not expressed in WT mice. Measurements were made using ImageJ, and the two groups were statistically compared with the paired-sample Student's *t* test.

Results

Dmrt3; *Dmrt5* double KO shows greater defects in the cerebral cortex than either single KO embryos

We generated *Dmrt3*; *Dmrt5* double mutants by intercrossing the *Dmrt3* and *Dmrt5* single mutants (Konno et al., 2012; Saulnier et al., 2013; De Clercq et al., 2018). *Dmrt3*; *Dmrt5* double KO die at birth, as do *Dmrt5* single KO mice (Saulnier et al., 2013); therefore, embryos were analyzed just before birth (E18.5). We found that the dorsal surface area approximately correlated with the number of null *Dmrt3* and *Dmrt5* alleles in an individual genotype, such that cortical hemispheres in *Dmrt3*; *Dmrt5* double KO were strikingly smaller than either single KO embryos ($-63 \pm 3.4\%$ compared with $-43 \pm 7.6\%$ in *Dmrt5* KO and $-13 \pm 10.4\%$ in *Dmrt3* KO embryos) (Fig. 1*A,B*). This more severe reduction of the cerebral hemispheres in double KO embryos was already apparent at E12.5 ($-52.5 \pm 0.04\%$ compared with $-43.7 \pm 0.06\%$ in *Dmrt5* KO and with $-19.9 \pm 0.32\%$ in *Dmrt3* KO embryos).

In *Dmrt3*; *Dmrt5* double KO, the reduction of caudal neocortical areas, hippocampus, and other dorsomedial telencephalic structures, such as the cortical hem, is more dramatic than in single KO embryos (data not shown). An obvious external difference between single KO and *Dmrt3*; *Dmrt5* double KO embryos was that the telencephalic vesicles of the double KO embryos show no discernible OB (Fig. 1*A,C*). However, when analyzed with gene and protein expression markers of the OB, including *Tbx21* whose expression is limited to OB mitral cells (Faedo et al., 2002; Kahoud et al., 2014), the residual pallium of the double KO embryos appears dominated by an OB-like structure (OBLS) (Fig. 1*D*). Thus, the *Dmrt3*; *Dmrt5* double KO have more substantial defects than either single KO embryos, suggesting that *Dmrt3* and *Dmrt5* compensate for the loss of one another.

Loss of both *Dmrt3* and *Dmrt5* causes ventralization of the pallial neuroepithelium

To determine how DMRT3 and DMRT5 interact to regulate gene expression in the developing telencephalon, we dissected the dorsal telencephalon from E12.5 *Dmrt3* KO, *Dmrt5* KO, *Dmrt3*; *Dmrt5* double KO and WT embryos and performed a transcriptome analysis of the dissected tissue by RNAseq (Fig. 2*A*). Applying a minimal cutoff of twofold change and a significance level of $p < 0.05$, 68 differentially expressed genes were identified in the *Dmrt3* single KO, 146 in the *Dmrt5* single KO embryos, and 553 in the double KO embryos. The majority of differentially expressed genes were downregulated (67 in *Dmrt3* KO, 126 in *Dmrt5* KO, and 422 in *Dmrt3*; *Dmrt5* double KO), and the overlap between the single and double KO embryos was very high (Fig. 2*A*). Gene Ontology analysis of the deregulated genes identified in the double KO embryos revealed an enrichment in genes in-

involved in neuron differentiation, forebrain development, neuron fate, and cell fate commitment (data not shown). Among the downregulated genes in the double KO embryos were, as expected (Saulnier et al., 2013; De Clercq et al., 2018), genes of the WNT and BMP signaling pathways associated with the cortical hem and downstream targets genes linked to hippocampus and choroid plexus development (*Wnt3a*, *Bmp6*, *Lmx1*, *Emx1*, *Lhx2*, *Nfix*, *Rspo1–3*, *Foxj1*, *Ttr*, *Mcidas*, *Gmnc*, *Ccno*, *Dnah6*). Partially overlapping this list were transcription regulators of dorsal telencephalic fates, including *Emx1*, *Emx2*, *Lhx2*, *Tbr2/Eomes*, the neuronal factors *Ngn2/Neurog2*, *NeuroD1*, *Math2/NeuroD6*, *Nhlh1*, and the glutamatergic neuronal gene *Vglut1*. Among upregulated genes were unexpectedly, many transcriptional determinants of ventral telencephalic cell fates, including *Gsh2/Gsx2*, *Dlx2*, *Dlx5*, *Sp8*, *Sp9*, *Ascl1*, ventral neuronal telencephalic genes (*Gad1/Gad67*, *Gad2*, *Scl30a3*, *Slc32a1*), and OB interneuron genes (*Foxp2*, *TH*, *calbindin*) (Fig. 2*B,C*). The dorsal determinant *Pax6* was also slightly increased, but its change of expression level was below the applied twofold change cutoff. Genes encoding general regulators of neurogenesis, such as *MyT1*, *Myt1l*, and general neuronal markers (*Dcx*, *Mapt*, *Nefh*) were, in contrast, not differentially expressed. The most highly upregulated and downregulated genes together with the change of their expression in the different KO genotypes are shown in Figure 2*B,C*. For the vast majority of the deregulated genes identified, the changes observed in the *Dmrt3*; *Dmrt5* double KO embryos relative to WT controls were in the same direction as those observed in the two single KO embryos, suggesting that *Dmrt3* and *Dmrt5* are redundant in several aspects of cortical development. The changes were generally greater than additive and the deregulation observed in *Dmrt5* KO was always much stronger than in *Dmrt3* KO embryos. For a given target, the difference between the WT and *Dmrt3* KO expression levels ($E_{WT} - E_{Dmrt3\ KO}$) and between the *Dmrt5* KO and *Dmrt3*; *Dmrt5* double KO ($E_{Dmrt5\ KO} - E_{Dmrt3/5\ DKO}$) represent the contribution of *Dmrt3* to gene expression in the presence or absence of *Dmrt5*. For many of the transcripts, this $E_{WT} - E_{Dmrt3\ KO}$ value is smaller than the $E_{Dmrt5\ KO} - E_{Dmrt3/5\ DKO}$ value. The greater effect of the loss of *Dmrt3* in the double KO embryos than in the WT context suggests that *Dmrt5* indeed partially compensates for the loss of *Dmrt3*.

Distinct domains are distinguished in the early telencephalic neuroepithelium (Fig. 3). The dorsal telencephalon, or pallium, is subdivided into the medial, dorsal, lateral, and ventral pallium (MP, DP, LP, and VP). MP generates the hippocampus, and the DP gives rise to the neocortex. The LP and the VP contribute neurons to olfactory cortex and the amygdala. The ventral telencephalon, or subpallium, has three proliferative subdomains, the lateral, medial, and caudal ganglionic eminences (LGE, MGE, and CGE), which develop into the striatum, globus pallidus, and parts of the amygdala. The MGE and CGE are also the source of GABAergic interneurons, which migrate tangentially into the cortex (Anderson et al., 1997; Puelles et al., 2000; Flames et al., 2007). To confirm the differential gene expression revealed by the RNA-seq analysis, we performed ISH of selected deregulated genes on brain sections of E12.5 embryos (Fig. 3). We first examined patterning genes, such as *Emx1*, *Emx2*, *Pax6*, and *Ngn2*, expressed in the cortex of WT mice, and *Dbx1* expressed selectively in the VP, which lies adjacent to the PSB (Fig. 3*A*). For genes expressed in the subpallium, we analyzed *Gsx2*, *Dlx2*, and *Ascl1* (Toresson et al., 2000; Yun et al., 2001; Tole et al., 2005).

The dorsal regulators *Emx1*, *Emx2*, and *Ngn2* were strongly decreased in cortical progenitors of the double KO, more severely than in either single KO embryos. Expression of these genes re-

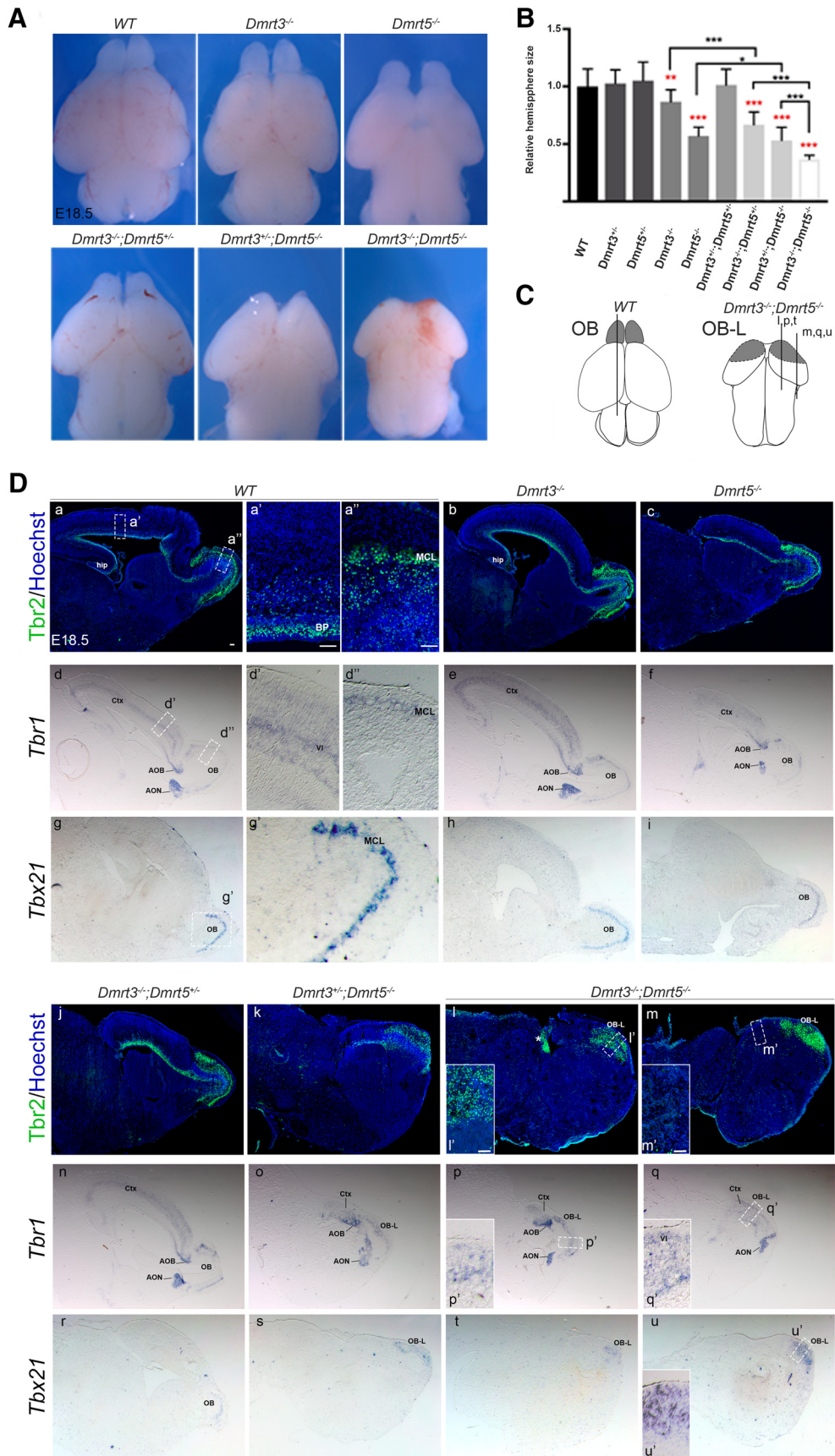


Figure 1. Cortices of *Dmrt3*;*Dmrt5* double KO embryos are more severely reduced in size than either single KO embryos and contain a prominent OBLs. **A**, Dorsal views of E18.5 brains of the indicated genotypes. **B**, Quantification of dorsal cortical surface area compared with WT controls (red) or as indicated. *******p* < 0.01, ********p* < 0.001. *n* ≥ 5. **C**, Diagram showing the telencephalon of E18.5 WT embryos and the size reduction and absence of the OBs observed in *Dmrt3*^{-/-};*Dmrt5*^{-/-}. **D**, Sagittal sections through E18.5 brains processed by IF or ISH for *Tbr1*, *Tbr2*, and *Tbx21* OB mitral projection neuron markers. In the cortex, *Tbr2* is also detected in SVZ progenitors and *Tbr1* in layer VI neurons. For WT and double KO embryos, high-magnification (Figure legend continues.)

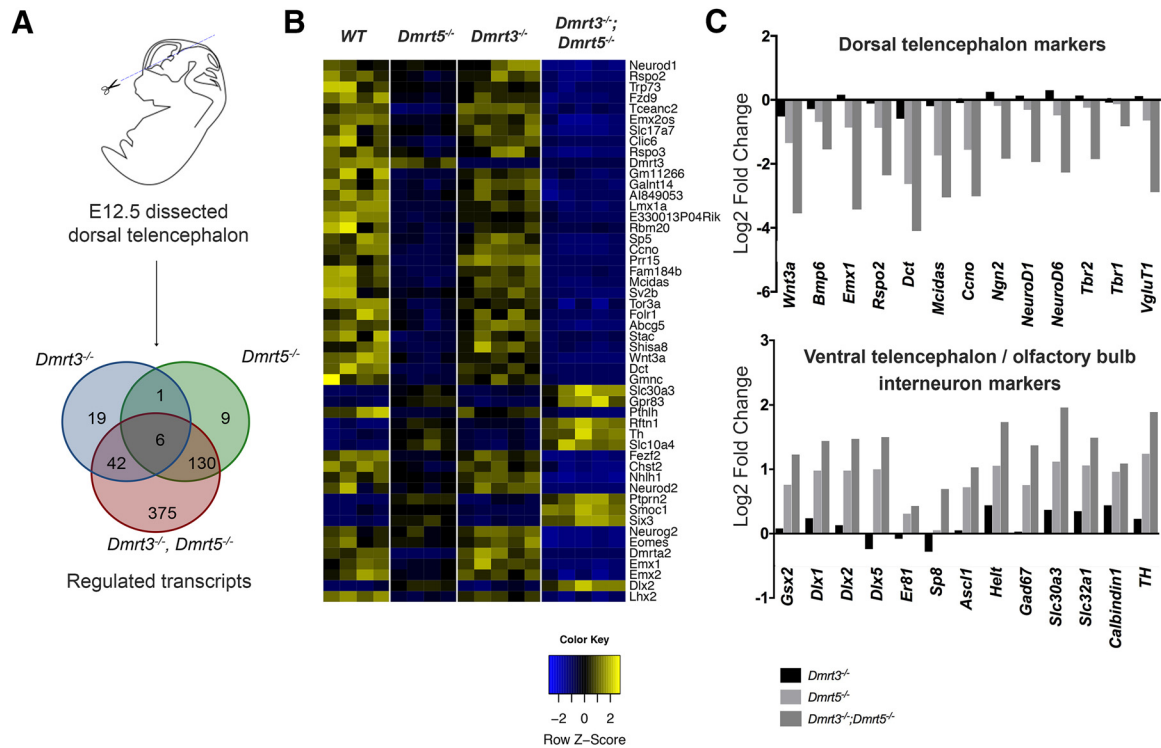


Figure 2. Genome-wide transcriptome analysis reveals that DMRT3 and DMRT5 cooperate to regulate cortical gene expression and play a role in early telencephalon DV patterning. **A**, Dissected dorsal telencephalic tissues analyzed by RNA-seq and Venn diagram showing the overlap of differentially expressed genes (both upregulated and downregulated) identified in *Dmrt3* KO, *Dmrt5* KO, and double KO E12.5 embryos. **B**, Heatmap for the 50 most significantly regulated genes (according to *p* value) in a comparison between WT and double KOs. Yellow represents upregulated genes. Blue represents downregulated genes. **C**, Examples of identified differentially expressed dorsal and ventral genes with log₂-fold changes observed in each genotype.

remained detectable only in the dorsomedial telencephalon (Fig. 3A, open arrows). *Dbx1* expression, which remains detectable in the VP of both single KO embryos, was lost in the double KO embryos (Fig. 3A, open arrow). In contrast, *Pax6*, which is upregulated in the cortex of *Dmrt* single KO (Saulnier et al., 2013; De Clercq et al., 2018), remained expressed in the cortex of the double KO embryos but was reduced at the PSB. *Gsx2*, *Dlx2*, and *Ascl1* whose expression is restricted to the subpallium in WT embryos, extended dorsally into the lateral part of the telencephalon in the double KO (Fig. 3A, arrows), a phenotype that was not observed in either single *Dmrt* KO embryos.

The LGE is subdivided into a dorsal (dLGE) and a ventral domain (vLGE) (Yun et al., 2001). dLGE progenitors express *Sp8* and generate OB interneurons, whereas vLGE progenitors express *Isl1* and give rise to striatal GABAergic projection neurons (Stenman et al., 2003; Waclaw et al., 2006, 2009; Ehrman et al., 2013). We examined their expression in single and double KO embryos. *Sp8*, normally restricted to the dLGE SVZ, showed a similar expression in single KO but expanded dorsally the double

KO embryos (Fig. 3B, arrow). *Isl1* expression in single KO embryos was similar to WT controls. In double KO, *Isl1* appears only moderately affected, with only very weak staining detectable in the cortex (Fig. 3B).

We also examined expression of dorsal and ventral regulator genes in sagittal sections (Fig. 4). Expression of *Ngn2* and *Emx1* retracted caudodorsally. *Pax6* expression, upregulated in the single KO, also appeared to retract in double KO embryos. This was accompanied by a dorsal expansion of *Gsx2* and *Dlx2* expression in the anterior telencephalon. In the brain of E12.5 *Dmrt3*;*Dmrt5* double KO embryos, subpallial gene expression expand dorsally both in the lateral and anterior telencephalon (Fig. 4, arrows). We found that *Er81* expression, restricted at E12.5 in WT embryos to the OB primordium (OBP) (Stenman et al., 2003; Allen et al., 2007; Besse et al., 2011), was also extensively expanded caudally (Fig. 4, arrow). Together, these results indicated that in E12.5 *Dmrt3*;*Dmrt5* double KO, there is an expansion of dLGE and OBP domains, as characterized by gene expression, into the dorsal telencephalon, and a concomitant shrinkage of the dorsal pallium (Figs. 1C, 3C).

The expansion of dLGE and OBP domains in E12.5 *Dmrt3*;*Dmrt5* double KO could be the consequence of the collapse of the dorsal telencephalon and invasion of ventral progenitors or of a respecification, namely, ventralization of dorsal telencephalic progenitors. To test the second hypothesis, we performed double immunostaining with antibodies for both the dorsal PAX6 and ventral GSX2 markers on coronal brain sections of E12.5 single and double KO embryos and WT controls. In the lateral cortex of the double KO embryos, but not in the single KO and WT embryos, many cells were found to be positive for both markers (Fig. 5), suggesting that indeed dorsal telencephalic progenitors were

(Figure legend continued.) views of the cortex (*a'*, *d'*, *m'*, *q'*) and OBs (*a'*, *d'*, *p'*, *u'*) are shown. For the double KO brains, two sections are shown (*i*, *j*, *m*, *n*), taken at the levels indicated in the diagram. In the *Dmrt3*^{+/-};*Dmrt5*^{-/-} and double KO embryos, the presence of a prominent OBLS occupying most of the reduced cortex (shown schematically in the diagram compared with controls) in which *Tbr1*, *Tbr2*, and *Tbx21* are expressed and form a cluster or appear as a disorganized layer (*l'*, *p'*, *u'*). Note also in the cortex the disappearance of cortical *Tbr2*⁺ basal progenitors (compare *a'* and *m'*) and more diffuse *Tbr1* expression (compare *d'* and *q'*). *Region of strong ectopic expression of *Tbr2* outside the cortex in the double KO embryos. AOB, Accessory OB; AON, anterior olfactory nucleus; BP, basal progenitor; Ctx, cortex, OB-L, OB-like structure; Hip, hippocampus; MCL, mitral cell layer; OB, olfactory bulb. Scale bars, 50 μm.

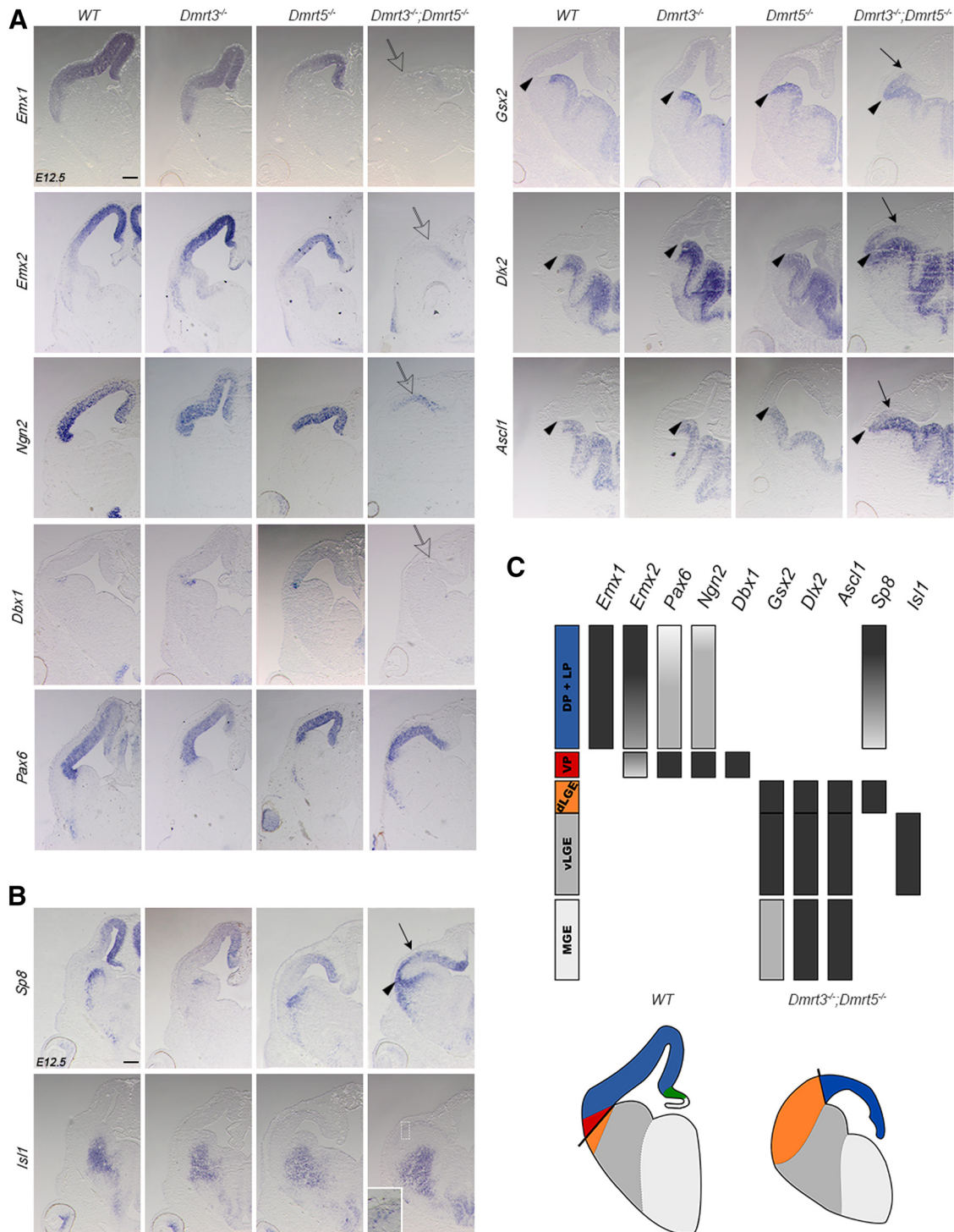


Figure 3. Downregulation of dorsal determinants and expansion of dLGE markers in the lateral telencephalic neuroepithelium of *Dmrt3;Dmrt5* double KO embryos. **A, B**, Coronal brain sections of E12.5 embryos hybridized with the indicated markers. Arrowheads indicate the region of the PSB. Arrows point to the shifted dorsal limit of ventral markers expressed ectopically in the pallium of the double KO embryos. Open arrows indicate the strongly downregulated expression of *Emx1*, *Emx2*, and *Ngn2* that remains detectable only in the dorsomedial telencephalon and the absence of *Dbx1*. Inset in the *Isl1* panel of the *Dmrt* double KO represents a high-magnification view of the boxed region where some *Isl1* ectopic staining in the pallium is observed. **C**, Diagram showing the expression domain of the different markers used in the telencephalon of E12.5 WT embryos and the reduction of the ventral pallium and an expansion of the dLGE domain as observed in *Dmrt3*^{-/-};*Dmrt5*^{-/-}. Scale bar, 200 μ m.

acquiring a ventral identity, perhaps corresponding to that in WT of the few cells present at the PSB that coexpress *Pax6* and *Gsx2*, or a hybrid fate.

To assess the consequences of the ventralization of dorsal progenitors in *Dmrt3;Dmrt5* double KO embryos, we exam-

ined TH, found in a subpopulation of OB interneurons, *Gad67*, a GABAergic interneuron marker, and *Math2*, a marker of glutamatergic cortical neurons in sagittal sections of E18.5 brains. In accordance with our observation of an expansion of the dLGE and OBP at E12.5, compared with single KO and controls,

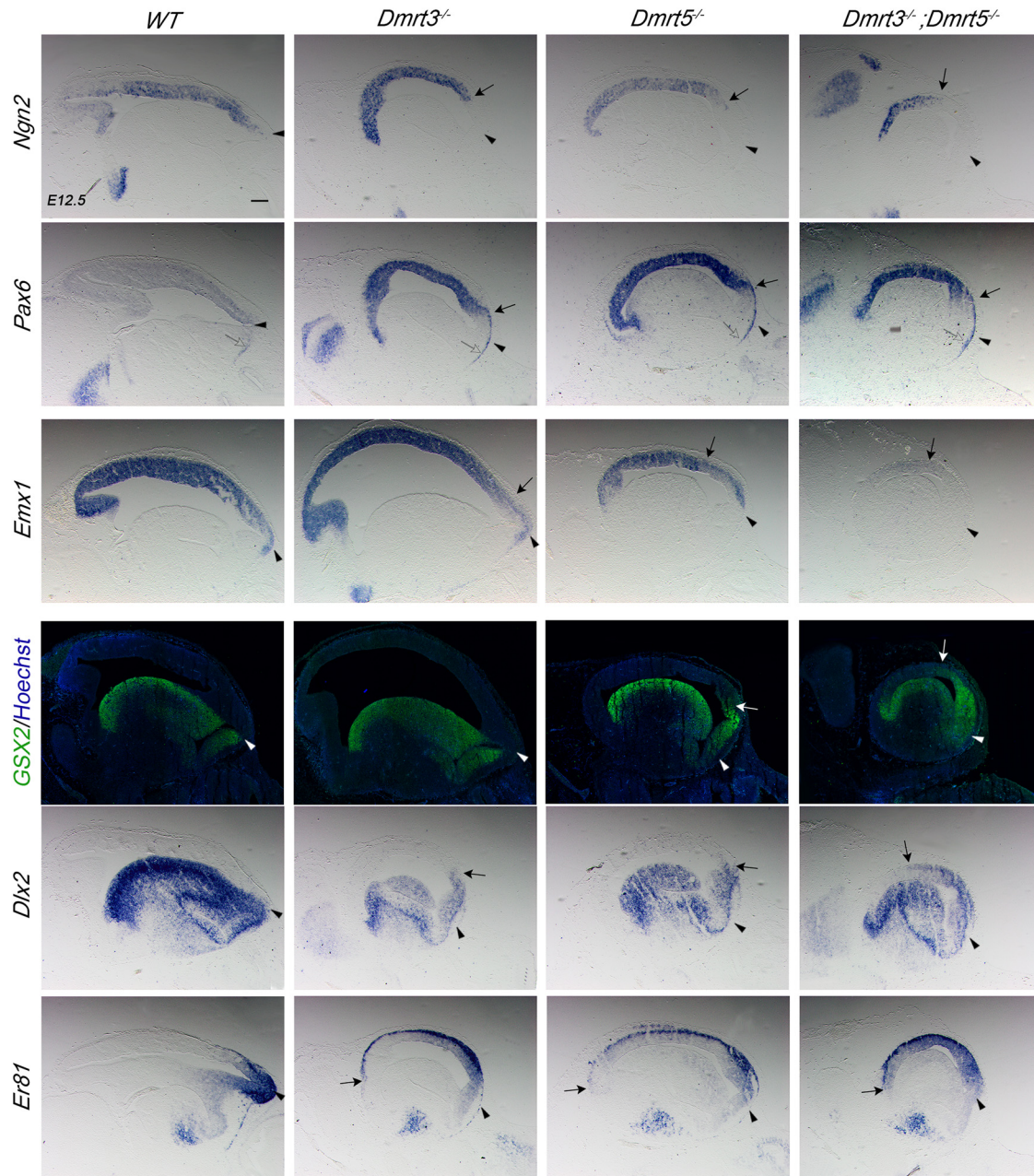


Figure 4. Subpallial gene expression expands in the rostral telencephalon of *Dmrt3*;*Dmrt5* double KO embryos. Sagittal sections of E12.5 brains processed by ISH or IF with the indicated markers, with the rostral part to the right. Arrowheads indicate PSB. Arrows indicate the rostral or caudal boundaries of the gene expression domains. These boundaries are shifted dorsally in the *Dmrt3*;*Dmrt5* double KO embryos. Open arrows indicate stronger *Pax6* staining in a stream of postmitotic cells that derive from dLGE progenitors in the mantle zone of the rostral part of cortical neuroepithelium of the single and double KO embryos. Scale bar, 200 μ m.

TH-positive cells were detected more caudally and *Gad67* was increased in the dorsal telencephalon of the double KO embryos. Compared with single KO and controls, *Math2* expression was in contrast dramatically reduced overall (Fig. 6), suggesting some respecification of cortical neuroblasts.

Misexpression of *Dmrt5* in the subpallium represses ventral telencephalic markers

To perform *Dmrt5* gain-of-function experiments, we used a *Dmrt5* Cre/loxP conditional transgenic mouse model we recently generated. In our previous work, the *Dmrt5* conditional transgenics (designated here *Dmrt5*Tg mice) were crossed with *Emx1*-Cre mice to study the role of DMRT5 in neocortical area map formation (De

Clercq et al., 2018). *Emx1*-Cre drives excess *Dmrt5* only in the dorsal, not ventral, telencephalon, and Cre lox recombination begins in the most medial telencephalon at approximately E10, spreading laterally so that full recombination in lateral cortex is as late as E11.5. In the present study, *Dmrt5*Tg mice were crossed with different Cre driver lines, *Foxg1*-IRES-Cre or *Gsx2*-Cre-IRES-EGFP (*Gsx2*-CIE). In *Dmrt5*^{Tg/+};*Foxg1*^{IRES-Cre/+} embryos, *Dmrt5* is overexpressed in both the dorsal and ventral telencephalon from E8.5 (Kawaguchi et al., 2016), thus much earlier and more broadly than with *Emx1*-Cre. In *Dmrt5*^{Tg/+};*Gsx2*-CIE embryos, *Dmrt5* is overexpressed selectively in the ventral telencephalon from E10.5 (Qin et al., 2016).

As expected, in *Dmrt5*^{Tg/+};*Foxg1*^{IRES-Cre/+} embryos, *Dmrt5* transgene expression detected by GFP ISH filled the entire telen-

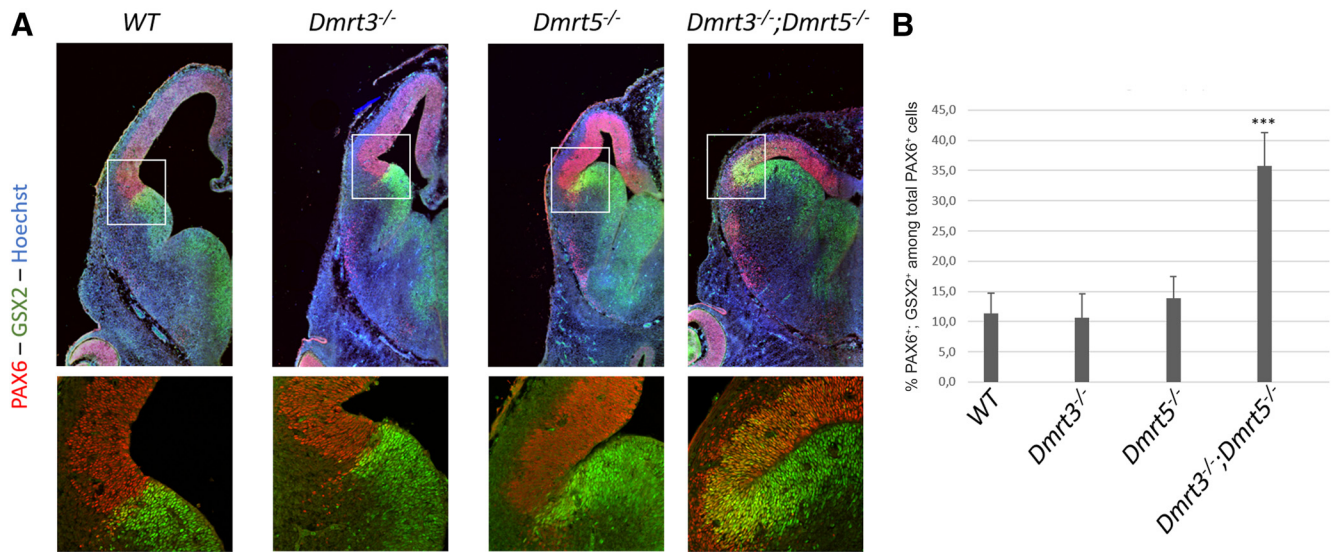


Figure 5. Dorsal telencephalon cells express ventral markers in *Dmrt3*;*Dmrt5* double KO embryos. **A**, Coronal section through E12.5 brains of the indicated genotypes processed by IF for Pax6 and Gsx2. Bottom, high-magnification views of the PSB. **B**, Histograms showing the number of double-positive cells among Pax6-positive cells in the boxed area. *** $p < 0.001$.

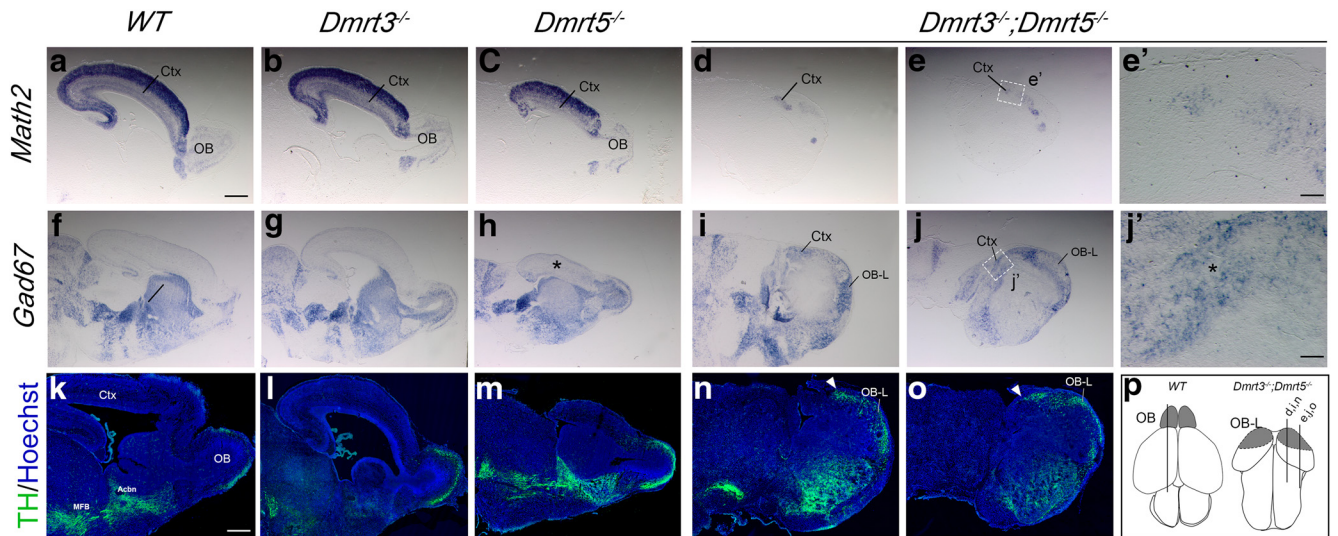


Figure 6. Expression of GABAergic, glutamatergic, and OB interneuron markers in the cortex of E18.5 *Dmrt3* and *Dmrt5* single KO and double KO embryos. **a, o**, Sagittal sections through brains of the indicated genotypes processed by ISH or IF for the indicated markers. **p**, The line in the schematic of the brain of WT and double KO embryos indicates the positions of the sections. For the double KO brains, two sections are shown for each marker. **e', j'**, A high magnification of the *Math2* and *Gad67* staining in the residual cortex of the double KO. There is slight upregulation of *Gad67* in both single and double mutants (*) and a dramatic loss of *Math2* in the cortex of the double KO embryos. TH-positive cells that are not correctly laminated and form a large cluster in the OB-L structures of the double KO that extend more caudally in the dorsal telencephalon than in WT embryos (arrowheads). Ctx, Cortex; MFB, medial forebrain bundle; Acbn, accumbens nucleus. Scale bars: 500 μ m; **h, p**, 50 μ m. OB, olfactory bulb; OB-L, olfactory bulb like.

cephalon (data not shown). In E12.5 *Dmrt5*^{Tg/+};*Foxg1*^{IRES-Cre/+} embryos, we examined the expression of ventral (*Gsx2*, *Dlx2*, and *Ascl1*) and dorsal (*Emx1* and *Ngn2*) telencephalic markers. We also analyzed *sFrp2*, *Tgf- α* , *Dbx1* expressed in the VP (Assimacopoulos et al., 2003) and *Sp8* marking the dLGE (Fig. 7).

In E12.5 control embryo sections, *Emx1* expression did not enter the LGE and only extended along the far lateral edge of the LGE in a territory that may be presumptive olfactory cortex. By contrast, in *Dmrt5*^{Tg/+};*Foxg1*^{IRES-Cre/+} E12.5 embryos, the *Emx1* expression domain expanded ventrally into the central and medial LGE (Fig. 7A, arrow). This expansion of *Emx1* expression in *Dmrt5*^{Tg/+};*Foxg1*^{IRES-Cre/+} E12.5 embryos, compared with controls, was quantified by measuring how close the central sector of *Emx1* expression came to the boundary between the LGE and the

MGE. The latter boundary served as a consistent landmark because the MGE appeared morphologically unaffected by overexpression of *Dmrt5*. For *Dmrt5*^{Tg/+};*Foxg1*^{IRES-Cre/+} E12.5 embryos the mean distance from the *Emx1* expression front and the LGE/MGE boundary was 133.3 μ m (SEM, 25.3 μ m; SD 75.9 μ m), and for control embryos, 273.8 μ m (SEM, 18.2 μ m; SD, 54.6 μ m). The two groups were significantly different ($p = 0.0004$). Notable by eye, but not quantified, the extension of *Emx1* expression increased from rostral to caudal, reaching the MGE/LGE boundary in more caudal sections. Similarly, *Ngn2* expression, normally confined to the cerebral cortical primordium, expanded into almost all the LGE territory (Fig. 7A, arrow).

In contrast, *Gsx2* and *Dlx2* expression was downregulated overall and did not reach along the entire DV length of the LGE

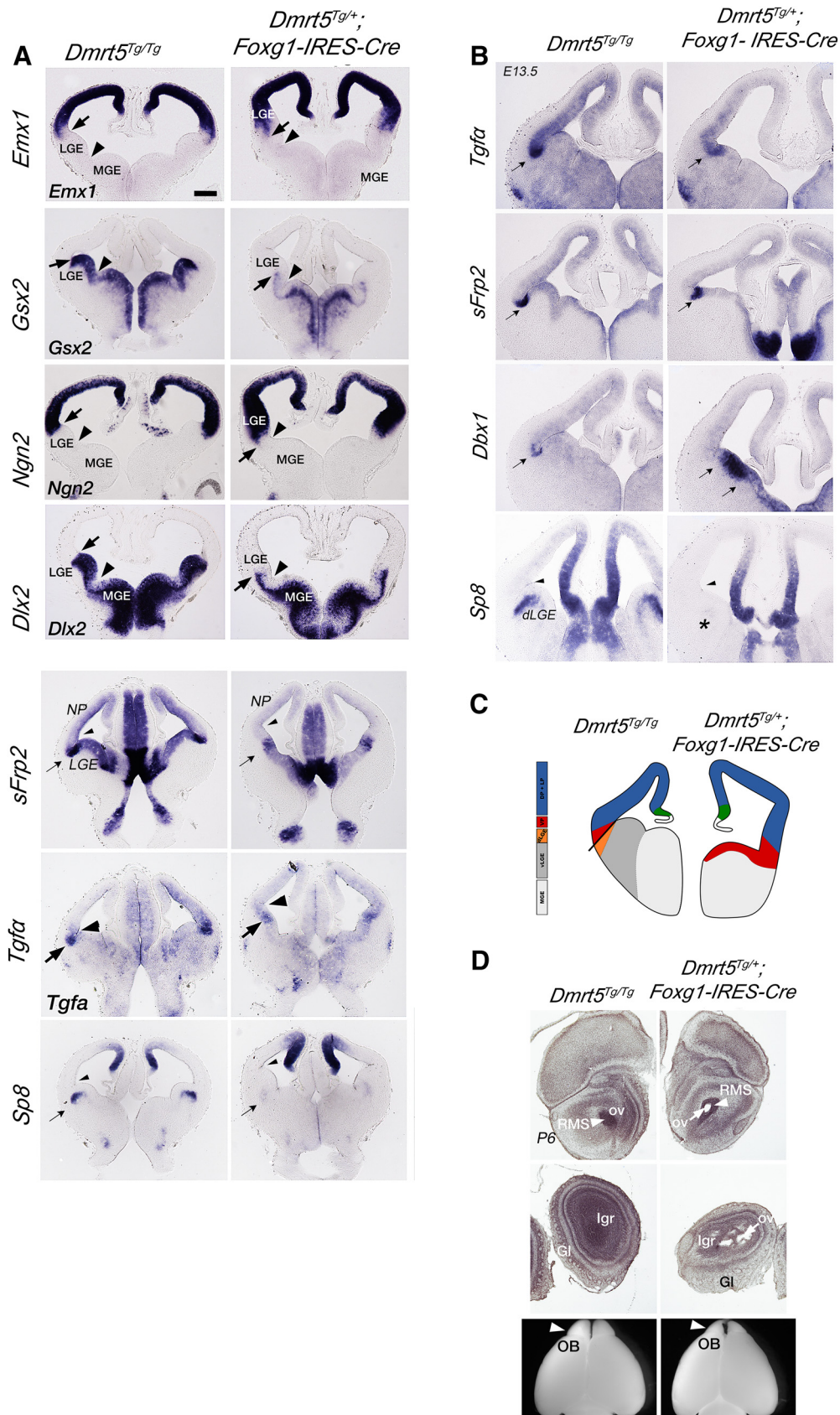


Figure 7. Repression of ventral and expansion of dorsal telencephalic and ventral pallium markers in the subpallium of *Dmrt5^{Tg/+};Foxg1-IRES-Cre* embryos. **A, B**, Coronal sections through the telencephalon of transgenic and control embryos at E12.5 (**A**) and E13.5 (**B**) processed by ISH for the indicated markers. **A**, Top 8 panels, Arrowheads indicate the boundary of the MGE and LGE, a constant landmark between control and mutant mice, used for quantification of *Emx1* expansion. Arrows point to the ventral limit of *Emx1* and *Ngn2* expression, or the dorsal limit of *Gsx2* and *Dlx2* expression. Notably, in *Foxg1-IRES-Cre* embryos, the arrows and arrowheads are closer together than in controls, indicating the dorsalization of the telencephalon when *Dmrt5* is overexpressed. **A**, Bottom 6 panels, arrowheads indicate the PSB, arrows indicate the mis-located expression of *sFrp2* and *Tgfa* when *Dmrt5* is overexpressed, and the near loss of (*Figure legend continues*.)

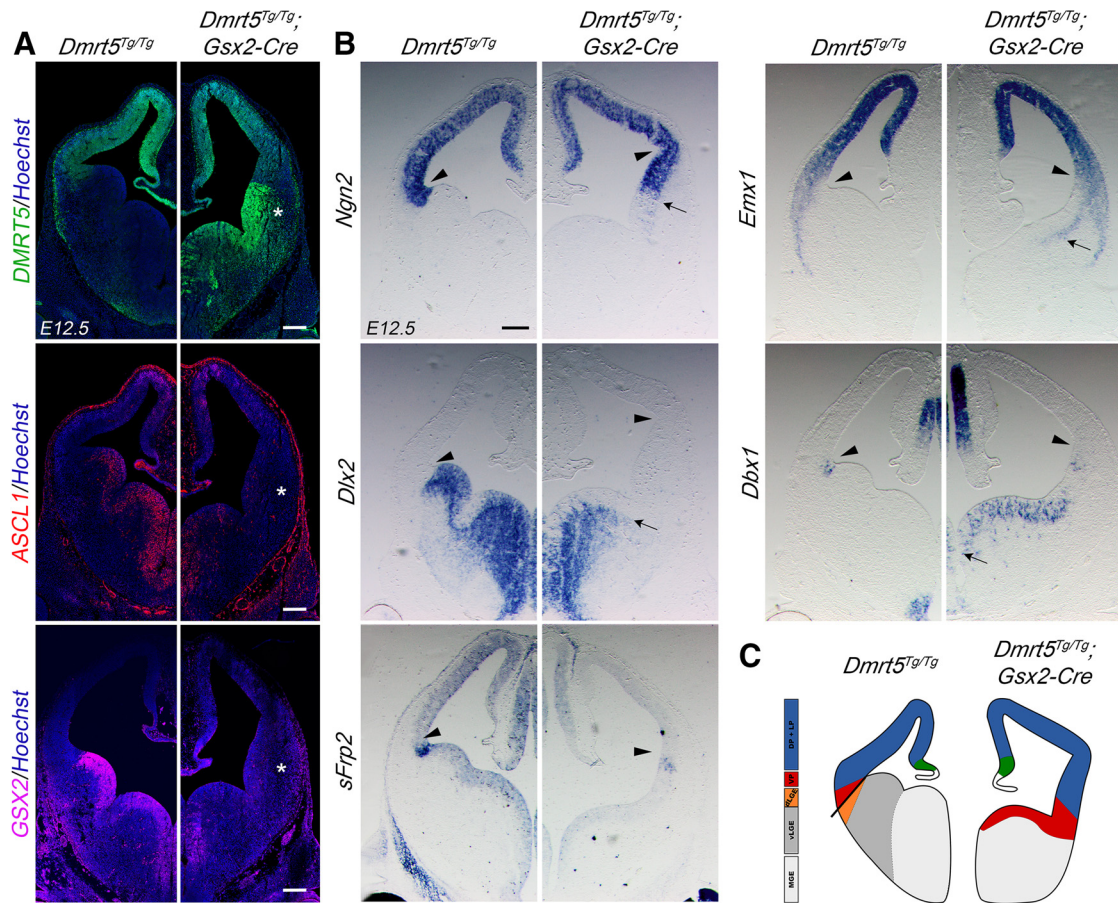


Figure 8. Repression of ventral and expansion of dorsal and ventral pallium markers in the subpallium of *Dmrt5^{Tg/Tg};Gsx2-Cre* embryos. **A, B**, Coronal sections through the telencephalon of E12.5 embryos processed by IF (**A**) or ISH (**B**) with the indicated markers. **A**, *The ectopic expression of *Dmrt5* and downregulation of *Ascl1* and *Gsx2* in the ventral telencephalon. **B**, Arrowheads indicate the region of the PSB. Arrows point to the expansion and shifted ventral limit of *Ngn2*, *Emx1*, and *Dbx1* and downregulation and shifted dorsal limit of *Dlx2* expression in the subpallium of the transgenics. **C**, Diagram showing the expansion of the ventral pallium as observed in *Dmrt5^{Tg/Tg};Gsx2-Cre* embryos. Scale bar, 200 μ m.

(Fig. 7A, arrow). The strongest expression of both *sFrp2* and *Tgf- α* was shifted ventrally, and *Sp8* in the dLGE was virtually absent (Fig. 7A, arrow). At E13.5, *Tgf- α* and *sFrp2* appeared to have recovered a normal expression level at the PSB, but expression of *Dbx1*, a selective marker of the VP, was greatly expanded and shifted ventrally. *Sp8* remained almost absent in the dLGE (Fig. 7B). Thus, at E13.5, the PSB boundary expressed some appropriate genes, yet the VP area as marked by *Dbx1* had expanded (Fig. 7C). An enlarged VP was previously observed in the *Gsx2* null mutant (Yun et al., 2001; Carney et al., 2009; Waclaw et al., 2010).

Sp8 in the dLGE is required for OB interneurons to begin migration through the embryonic rostral migratory stream (Waclaw et al., 2006). In *Dmrt5^{Tg/+};Foxg1^{IRE5-Cre/+}* brains at P6, consistent with the virtual loss of *Sp8* expression in the dLGE, the ventricle of the OB was slightly open rather than filled with mi-

grating interneurons as in WT mice, suggesting that interneurons were not reaching the OB correctly (Fig. 7D).

In *Dmrt5^{Tg/Tg};Gsx2-Cre* embryos, *Dmrt5* overexpression was only detected in the ventral telencephalon, as expected. As observed in *Dmrt5^{Tg/+};Foxg1^{IRE5-Cre/+}* embryos, *Dmrt5* ectopic expression induces loss of the ventral (*Gsx2*, *Dlx2*, *Ascl1*) and gain of the dorsal (*Emx2*, *Ngn2*, *Dbx1*) markers tested (Fig. 8), suggesting that the ventral expansion of dorsal telencephalic and ventral pallial markers is largely caused by the downregulation of ventral determinants.

EMX2 interacts with DMRT5 to control telencephalic DV patterning, and both transcription factors can bind to a ventral telencephalic-specific enhancer of the *Gsx2* locus

Emx2 is coexpressed with *Dmrt3* and *Dmrt5* in cortical progenitors in a similar graded manner. As in *Dmrt5* or *Dmrt3* single mutants, the hippocampus and caudomedial neocortex are reduced in *Emx2*-deficient mice (Yoshida et al., 1997; Tole et al., 2000b). This reduction too is more dramatic in *Dmrt5/Emx2* double KO than in single KO embryos (Fig. 9A), suggesting that *Dmrt5* and *Emx2* genes interact in controlling telencephalic growth and patterning. Supporting such an interaction, ISH showed that, in E12.5 *Dmrt5/Emx2* double KO brains, the pallial transcription factor gene *Ngn2* was switched off, whereas gene expression domains of *Gsx2*, *Dlx2*, and *Ascl1*, normally confined

(Figure legend continued.) *Sp8* expression. **B**, Arrowheads indicate the PSB. Arrows indicate restored expression of *sFrp2* and *Tgf- α* to the PSB, and upregulated expression of *Dbx1*. Asterisk marks continued absence of *Sp8*. **C**, Diagram showing the ventral expansion of DP and VP markers as observed in the telencephalon of *Dmrt5^{Tg/Tg};Foxg1^{IRE5-Cre}* embryos. **D**, Dorsal view of P6 brains and eosin staining of OBs. Arrowheads point to the RMS. Arrow indicates the OV. RMS, Rostral migrating stream; OV, olfactory ventricle; GL, glomerular layer; lgr, internal granule layer; LGE, lateral ganglionic eminence; dLGE, dorsal lateral ganglionic eminence; MGE, medial ganglionic eminence; OB, olfactory bulb; NP, neocortical primordium.

to the subpallium, extended dorsally, a phenotype not observed in either *Dmrt5* or *Emx2* single KO embryos (Fig. 9B).

GSX2 is crucial for ventral identity in the telencephalon. In *Gsx2* mutants, the VP expands into the dLGE (Toresson et al., 2000; Yun et al., 2001; Carney et al., 2009; Waclaw et al., 2010). Conversely, *Gsx2* misexpression in the pallium results in increased expression of ventral telencephalic markers and repression of dorsal ones (Toresson et al., 2000; Yun et al., 2001; Carney et al., 2009; Waclaw et al., 2009), including *Dmrt5* (Fig. 10).

To investigate how *GSX2* and *DMRT5* interact, we first compared by IF their expression in *Gsx2^{GFP/+}* knock-in embryos and found that the two proteins abut at the PSB (Fig. 10). Their exclusive expression at the PSB, the upregulation of *Gsx2* observed in *Dmrt3;Dmrt5* and *Dmrt5;Emx2* double KO embryos, along with its downregulation in response to overexpression of *Dmrt5*, suggest that *DMRT5* may regulate cortical identity in telencephalic progenitors via repression of *Gsx2* transcription. We therefore searched the *Gsx2* locus for noncoding, evolutionarily conserved potential enhancer sequences (Bejerano et al., 2004; Pennacchio et al., 2006) with potential *DMRT3/5* BSs (Murphy et al., 2007). We focused on one such conserved site of ~0.5 kb that has been described as an enhancer bound by *PAX6* that is active in the forebrain but not the lens (Sun et al., 2015), and is located 8.8 kb downstream of *Gsx2* transcribed sequences. Two overlapping fragments, encompassing this conserved region, one of 1.8 kb including upstream sequences and the other of 1.3 kb containing downstream sequences and including another conserved region (fragments A and B, respectively, Fig. 11A) were cloned and fused to a reporter gene (Constructs A and B). We found that both fragments tested exhibit specific enhancer activity in the ventral forebrain in E12.5 transgenic embryos, suggesting that it is the conserved region common to the two fragments that is responsible for the activity (Fig. 11B). This conserved region contains two potential *DMRT3/5* BSs as well as one 5'-ATTA-3', which represent the core of potential homeobox transcription factor-binding motifs (Berger et al., 2008). This 5'-ATTA-3' motif is adjacent to the second *Dmrt3/5* BS. Upstream of this conserved region, within the cloned genomic fragments, we found one additional *Dmrt3/5* BS and one additional 5'-ATTA-3' motif (Fig. 11A). To test for direct binding of *DMRT3/5* and *EMX2* to

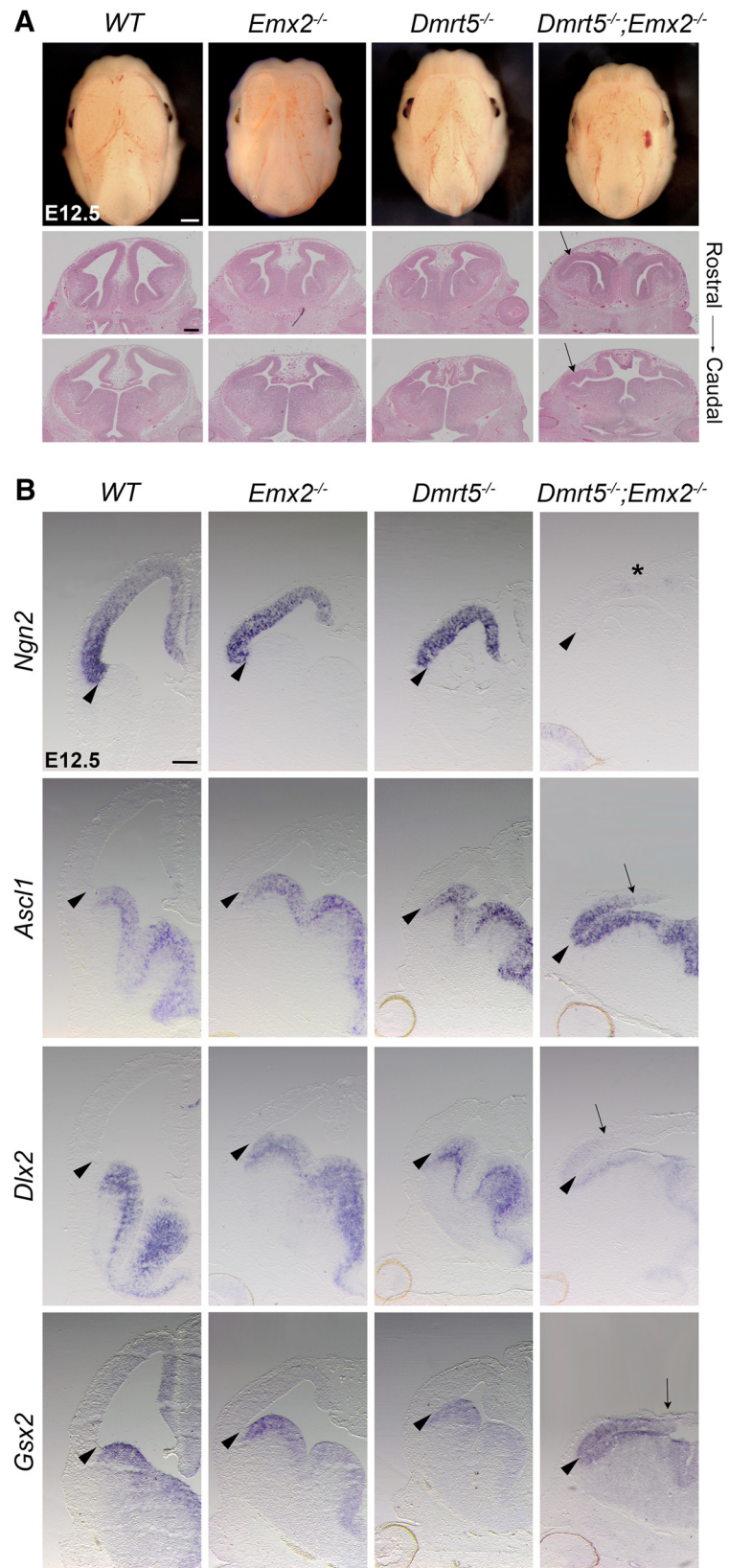


Figure 9. *DMRT5* and *EMX2* cooperate in telencephalon DV patterning. **A**, Dorsal views of the head of E12.5 embryos. H&E staining of E12.5 brain coronal sections of the telencephalon, at rostral and caudal levels. Arrows point to the caudomedial cortex of the *Dmrt5;Emx2* double mutants, more severely reduced than in single mutants. **B**, Coronal brain sections of E12.5 embryos processed by ISH with the indicated markers. Arrowheads indicate the region of the PSB. Arrows point to the dorsal limit of *Gsx2*, *Dlx2*, and *Ascl1* expression, shifted dorsally in the *Dmrt5*^{-/-};*Emx2*^{-/-} double KO embryos. *Dramatic reduction of *Ngn2* in the cortex of the double KO embryos. Scale bar, 200 μm.

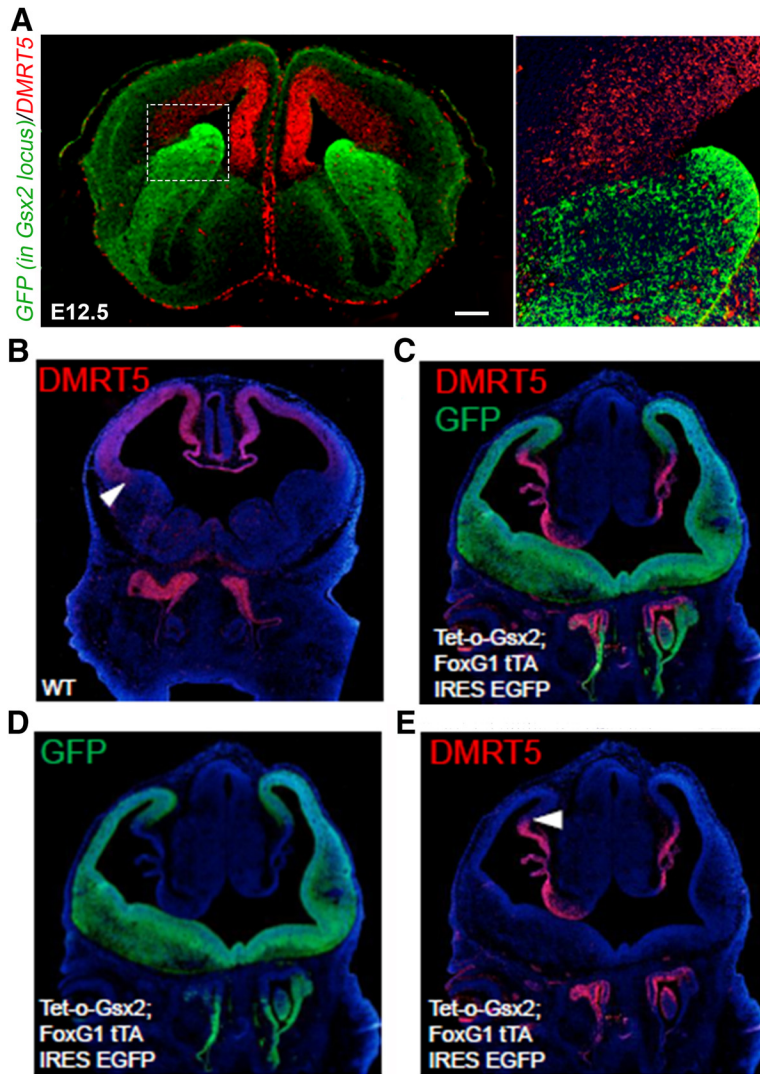


Figure 10. DMRT5 forms a boundary with GSX2 at the PSB, and the overexpression of *Gsx2* represses *Dmrt5* expression. **A**, Coronal sections of the head of E14.5 *Gsx2^{GFP/+}* knock-in embryos processed by IF with DMRT5 and GFP antibodies showing that, at the PSB, cells expressing DMRT5 do not express GFP, hence, GSX2. Boxed area is shown at a high magnification on the left. Oe, Olfactory epithelium. Scale bar, 200 μ m. **B–E**, Coronal sections through the telencephalon of E12.5 WT or *Foxg1^{ITA/+}; tet-O-Gsx2-IRES-EGFP* double-transgenic embryos immunostained with the indicated antibodies. In these transgenic embryos, *Gsx2* is misexpressed throughout the embryonic telencephalon and *Dmrt5* is reduced. **B**, **E**, Arrows point to the ventral limit of high *Dmrt5* expression.

these putative BSs, we performed EMSAs using extracts prepared from HEK293 cells transfected with *Dmrt3*, *Dmrt5*, or *Emx2* expression plasmids. In first EMSA assays, using oligonucleotides containing the different Dmrt and Emx2 potential BSs, we found that DMRT5 and DMRT3 both bind strongly to BS3 but not to the other sites. EMX2 can bind to both identified BSs (data not shown). We then examined the ability of DMRT3, DMRT5, and EMX2 to bind to an extended oligonucleotide encompassing both Dmrt BS3 and Emx2 BS2, in the presence or absence of an identical competitor oligonucleotide containing mutated Dmrt and Emx2 BSs. Figure 11C shows that, as expected, the binding of DMRT3, DMRT5, and EMX2 to the Dmrt BS3/Emx2 BS2 oligonucleotide is abolished in the presence of WT but not mutated competitors. Interestingly, in the presence of EMX2, the complex of DMRT3 appears more intense and to run faster than in its absence. Although no change in mobility shift was observed, DMRT5 binding in the presence of EMX2 appears also more

intense than in its absence, suggesting cooperative interactions between DMRT3/5 and EMX2.

Discussion

We have previously identified DMRT5 and DMRT3 as important regulators of the development of caudomedial cortical structures acting downstream of dorsal WNT midline signals and controlling *Wnt* expression in a feedback loop (Hasenpusch-Theil et al., 2012; Saulnier et al., 2013; De Clercq et al., 2018). In the present study, we show that the size of the cortical hemispheres is drastically smaller in the *Dmrt3*; *Dmrt5* double KO than in the single KO embryos, further highlighting their importance in the control of the proliferative state of progenitor cells. This dramatic reduction in size of the cortex of the double KO embryos may due to premature differentiation and exhaustion of the pool of progenitors as observed in *Dmrt5^{-/-}* embryos (Young et al., 2017), which remains to be explored. We also show that the development of caudomedial telencephalic structures, such as the cortical hem and hippocampus, is more severely reduced in the *Dmrt3*; *Dmrt5* double KO than in the single KO embryos. Together, these observations indicate that DMRT3 and DMRT5 have similar overlapping function in several aspects of cortical development and partially compensate for one another. *Dmrt5* is upregulated in the absence of *Dmrt3*, and *Dmrt3* is downregulated in *Dmrt5* KO embryos. The two factors appear thus to function in the same cascade, but it remains unclear which one is upstream of the other.

More unexpectedly, we observed that double KOs have a dorsal telencephalon that is ventralized. In addition, the OBs, the most rostral cortical structure, take over the cortex, thus suggesting “rostroventralization” of the dorsal telencephalon, in accordance with the high caudal to low rostral gradient of expression of *Dmrt3/5* genes. Conversely, in *Dmrt5*-overexpressing embryos, DP genes expand ventrally, although some PSB markers remain in place, and OBs are smaller than in WT mice. In addition, *Dbx1* expression expands, supporting an expansion of the VP. These findings revealed a new early role for DMRT5 and DMRT3 in DV telencephalic patterning, which could not have been predicted from examining the phenotype of either single KO line alone. This role fits with the timing of activation in the developing telencephalon. Both *Dmrt3* and *Dmrt5* expression is detected from the open neural plate stage in the prospective dorsal telencephalon when patterning is initiated (data not shown).

As noted, WNT and BMP signaling molecules and downstream dorsal telencephalic transcription factors are dramatically reduced in the cortex of the double KO embryos. The reduction of these pathways is likely to be involved in the ventralization. In

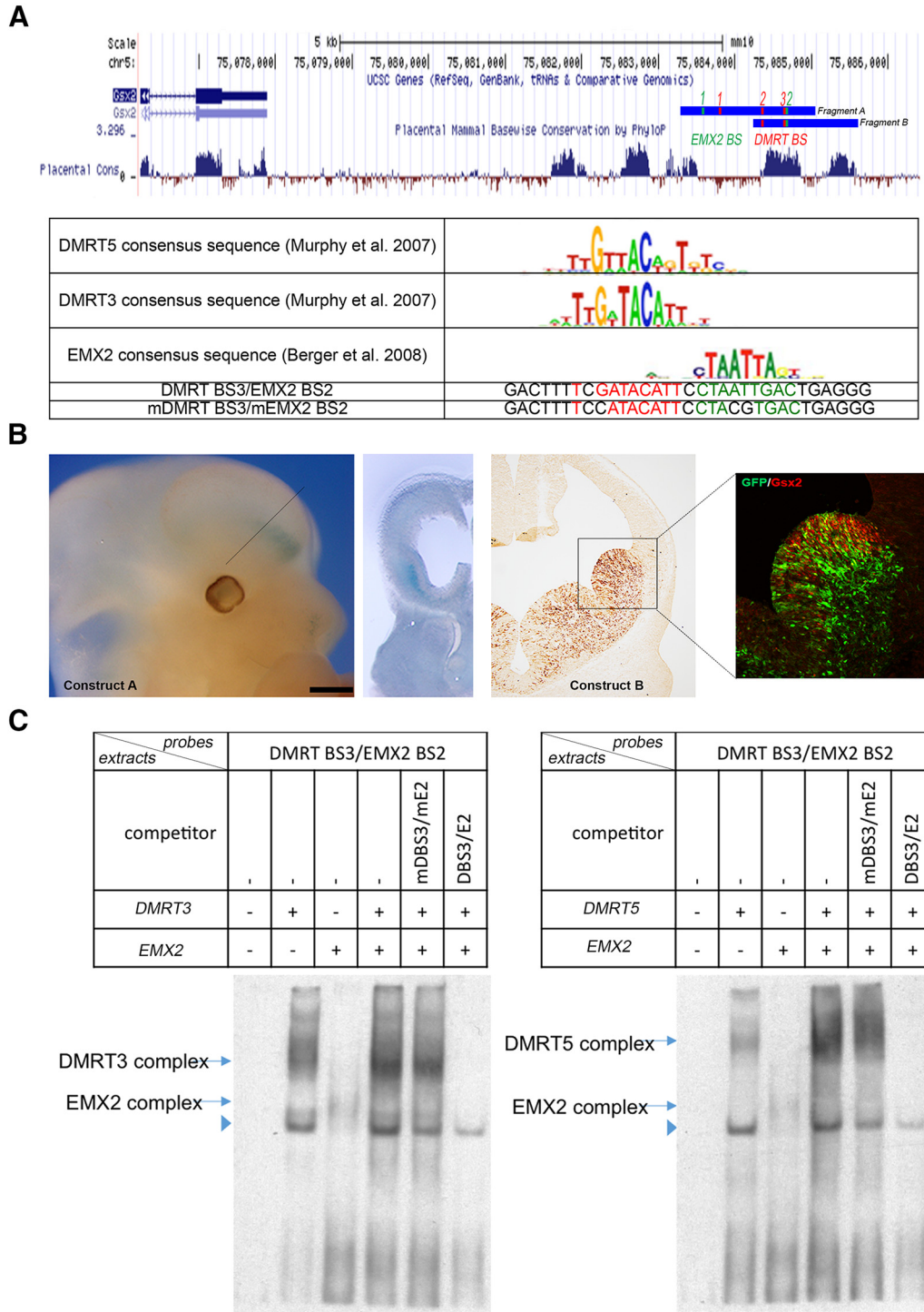


Figure 11. DMRT5 and EMX2 bind a *Gsx2* ventral-specific telencephalon enhancer. **A**, UCSC genome browser view of the *Gsx2* locus with the location of the two cloned fragments tested in transgenic embryos. The identified putative DMRT3/5 and EMX2 BSs are shown. **B**, Left, A lateral view and a coronal section of the head of a E12.5 *Gsx2* 1.8 kb enhancer-LacZ reporter transgenic embryo (Construct A). Scale bars: lateral view, 500 μ m; coronal section, 200 μ m. Dashed line indicates the level of the section. Right, A coronal section of the brain of a *Gsx2* 1.3 kb enhancer-GFP reporter transgenic embryo (Construct B) processed by DAB immunostaining for GFP and a high-magnification view of the LGE region processed by IF for both GSX2 (red) and GFP (green). **C**, EMSA showing *in vitro* binding of cellular extracts containing DMRT3, DMRT5, and EMX2 or control extracts to BS3 of the *Gsx2* enhancer. DMRT3/5 and EMX2 complex formation is competed by WT enhancer oligonucleotides but not by oligonucleotides containing mutations in the DMRT and EMX2 BSs. Arrowhead indicates a nonspecific band.

contrast, FGF signals appear unaffected, and SHH and downstream targets, such as *Nkx2.1*, appear normally restricted to the MGE (data not shown). *Gli3R* is another major player in telencephalic DV patterning (Theil et al., 1999; Tole et al., 2000a; Rallu et al., 2002; Kuschel et al., 2003). RNA-seq analysis of the cortex

of E12.5 single and double *Dmrt* KO embryos did not detect a significant difference in *Gli3* mRNA levels compared with WT controls. This unaltered expression of *Gli3* in double *Dmrt* KO embryos has been confirmed by ISH and qRT-PCR experiments (data not shown). Given the similarity of the telencephalic pat-

terning defects observed in *Dmrt3;Dmrt5* and *Gli3^{Pdn/Pdn}* mutants, whether a reduction of Gli3R activity occurs in the *Dmrt* double KO embryos is a hypothesis that remains to be tested. A more significant role in the ventralization of double *Dmrt* KO embryos is likely to be played by the transcription factor GSX2. In the ventral telencephalon, GSX2 is known to be required for the specification of LGE progenitors. GSX2, further, represses the expression of *Dbx1* and other VP markers (Yun et al., 2001; Carney et al., 2009). Our data indicate that it also represses *Dmrt5* expression. GSX2 acts in the specification of LGE progenitors upstream of the homeobox *Dlx* and proneural *Ascl1* genes (Wang et al., 2009, 2013). Thus, the increase of *Dlx2* and *Ascl1* and the loss of dorsal and ventral pallium markers in *Dmrt3;Dmrt5* double KO embryos may be a consequence of the dorsal expansion of *Gsx2*. The large expansion of *Gsx2* and other subpallial markers in the cortical neuroepithelium when *Dmrt3* and *Dmrt5* are lost is intriguing because it occurs despite continued strong *Pax6* expression in the cortex, which is reduced only close to the PSB. PAX6 has been shown to repress *Gsx2* expression to position the PSB (Toresson et al., 2000; Yun et al., 2001; Carney et al., 2009), but PAX6 may be not sufficient for this role in the absence of DMRT3 and DMRT5.

Gsx2 is expressed at high level in dLGE progenitors that generate *ER81*- and *Sp8*-expressing OB interneurons, and at lower levels in vLGE progenitors giving rise to *Isl1*-expressing striatal projection neurons (Toresson and Campbell, 2001; Yun et al., 2001; Stenman et al., 2003; Waclaw et al., 2006; Ehrman et al., 2013). *Gsx2* mutants have reduced vLGE and dLGE and exhibit a significant reduction of their derivatives, striatal projection, and OB interneurons (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001, 2003). Conversely, in *Gsx2* conditional transgenics, GSX2 sequentially favors striatal and OB fates (Waclaw et al., 2009). In *Dmrt* double KO embryos, we observed at E12.5 strong ectopic expression of *Sp8* but no or low *Isl1* indicating a predominant expansion of dLGE. Accordingly, at E18.5, TH-positive interneurons appear abundant in the OBLS structure and *Gad67*, expressed in OB interneurons, is increased. At the same stage in the double mutant cortex, we also observed a strong reduction of *Math2*, suggesting that some respecification of cortical neuroblasts is occurring as a consequence of the observed early patterning defects.

Upon ectopic expression of *Dmrt5* throughout the telencephalon, we observed at E12.5–E13.5 a loss of *Gsx2* expression and downstream dLGE gene expression markers, and an expansion of DP and some VP gene expression markers into the subpallium. Other PSB markers appeared less affected. *SFrp2* expression shifted ventrally at E12.5 but recovered at E13.5. In *Gsx2* mutants, a strong LGE phenotype at early stages goes away later, due to increased *Gsx1* expression (Toresson and Campbell, 2001; Yun et al., 2003; Carney et al., 2009). Whether there is a compensatory increase in *Gsx1* in the *Dmrt5^{Tg/Tg};Foxg1-IRES-Cre* that rescues the location of *sFrp2* expression remains to be investigated. An alternative possibility is that the PSB repositions itself. The VP, however, does not, and instead expands in subpallial territory, thus dissociating the VP from the PSB. A similar dorsalizing phenotype was observed when *Dmrt5* is selectively misexpressed in the ventral telencephalon. This indicates that the ventral expansion of DP and VP gene expression markers is indirect and caused by downregulation of ventral determinants by DMRT5. *Gsx2* is again a good DMRT3/5 target candidate, as *SHH* and *Nkx2.1* were found to be unaffected in *Dmrt5^{Tg/Tg};Gsx2-CIE* embryos (data not shown). A further suggestion is that dorsal identity in

the telencephalon is a “ground state,” which must be suppressed by ventral determinants.

Finally, our findings indicate that *Emx2*, a target of *Dmrt5* (Saulnier et al., 2013), cooperates with *Dmrt3* and *Dmrt5* in repressing *Gsx2*, and that these transcription factors bind to an evolutionarily conserved, PAX6-bound element, located downstream of *Gsx2*. This *Emx2* involvement in *Dmrt*-dependent *Gsx2* repression echoes a previous report that coablation of *Emx2* and *Pax6* results into generalized spreading of subpallial programs into dorsal telencephalon and suppression of pallial programs (Muzio et al., 2002a). Intriguingly, despite the prominent ventralization of dorsal telencephalon occurring in mid-neuronogenic *Emx2^{-/-};Pax6^{-/-}* and *Dmrt3^{-/-};Dmrt5^{-/-}* embryos, vestigial and abortive pallial specification can still be detected at earlier developmental stages in both double KO embryos.

In conclusion, our findings suggest that DMRT3/5 transcription factors and GSX2 mutually antagonize one another and that DMRT3/5 cooperate with EMX2 and PAX6 to establish and maintain DV patterning in the telencephalon. How DMRT3 and DMRT5 interact with EMX2, and possibly PAX6 and other dorsal regulators such as LHX2, to control the positioning of the PSB and, more globally, telencephalon patterning remains to be investigated.

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