Transcription Factor VAX1 Regulates the Regional Specification of the Subpallium Through Repressing Gsx2

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Received: 14 December 2020 / Accepted: 29 March 2021 / Published online: 5 April 2021
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Abstract
Specification of the progenitors’ regional identity is a pivotal step during development of the cerebral cortex and basal ganglia. The molecular mechanisms underlying progenitor regionalization, however, are poorly understood. Here we showed that the transcription factor Vax1 was highly expressed in the developing subpallium. In its absence, the RNA-Seq analysis, in situ RNA hybridization, and immunofluorescence staining results showed that the cell proliferation was increased in the subpallium, but the neuronal differentiation was blocked. Moreover, the dLGE expands ventrally, and the vLGE, MGE, and septum get smaller. Finally, overexpressed VAX1 in the LGE progenitors strongly inhibits Gsx2 expression. Taken together, our findings show that Vax1 is crucial for subpallium regionalization by repressing Gsx2.

Keywords Vax1 · Gsx2 · Subpallium · Development · Telencephalon

Introduction
The telencephalon originates from the rostral nerve plate. During development, the telencephalon acquires precise subdivisions both along the dorsal-ventral (DV) and the anteroposterior (AP) axis [1–3]. The telencephalon is subdivided into two different territories: pallium and subpallium. The subpallium consists of the lateral ganglionic eminence (LGE), medical ganglionic eminence (MGE), caudal ganglionic eminence (CGE), preoptic area (POA), and septum. The progenitors located in different regions generate different cell types during telencephalic development. For example, the progenitors in the dLGE give rise to the olfactory bulb (OB) interneurons. In contrast, the progenitors in the vLGE mainly contribute to striatal medium-sized spiny neurons (MSNs) [4–6]. Thus, the maintenance of the dLGE, vLGE, MGE, and POA region in the subpallium is crucial for telencephalon development.

Multiple signaling pathways and transcription factors are involved in regionalization of the telencephalon. In the early embryonic stage, Fgf8 and Shh signaling are required for telencephalon patterning. Deletion of Fgf8 leads to structural abnormalities of the dorsal and ventral telencephalon [7, 8]. In Shh−/− mouse embryos, all ventral telencephalic progenitors are missing as detected by loss of ventral markers including Gsx2, Nkx2.1, and Dlx2 [9, 10]. In the subpallium development, several genes have been found responsible for regionalization. Transcription factors Gsx2 and Pax6 play complementary roles in dorsal-ventral patterning of the telencephalon [11]. A ventral to dorsal transformation leads the pallidal primordium into a striatal-like anlage in the Nkx2.1 knockout mice [12, 13]. Otx2 also plays crucial roles in MGE patterning through specifying vMGE fate and repressing POA fate [14]. The mechanisms of activation and mutual repression of the transcription factors in progenitors to establish regional patterning, however, need to be further investigated. For example, the key transcription factor, Gsx2, is not only required for the generation of the striatal MSNs and OB interneurons but is also crucial for the regionalization of the PSB (pallial-subpallial boundary) during forebrain development [4, 11, 15, 16]. The exact mechanism of downregulating its expression in the vLGE/MGE, however, is largely unknown.
The homeobox transcription factor *Vax1* starts to express at approximately embryonic day (E) 8 in mice. *Vax1*-mRNA is detected in the most rostral level of the medial neural plate at E8. As development proceeds, *Vax1* is found in the basal forebrain, optic stalk, optic disk, and medial olfactory placode [17]. Previous studies showed that secreted VAX1 protein regulates retinal axon growth and is required for axon guidance and major tract formation [18]. *Vax1* is also required for the precursor cells proliferating in the SVZ and migrating through the rostral migratory stream (RMS) to the OB [19]. In addition, the generation of cortical interneurons is compromised in the *Vax1*−/− mice [20]. Recently, studies showed that regulatory interactions between *Vax1* and *Pax6* are crucial for stem cell regionalization during olfactory bulb neurogenesis [21]. Interestingly, the other studies showed that *Vax1* and *Six6* were required for SCN circadian pacemaker development and SCN function [22]. Finally, heterozygous deletion of *Vax1* gene causes subfertility in mice and variants in *VAX1* genes are associated with non-syndromic cleft lip in humans [23, 24]. Although *Vax1* plays important roles in subpallium development, the mechanisms of *Vax1* in regulating subpallium regionalization remain largely unknown.

Here, our results show that *Vax1* is crucial for the regionalization of the subpallium. In the *Vax1* mutants, the dLGE expands ventrally, the vLGE and MGE get smaller, and the septum is absent. Furthermore, we found that the expression of Gsx2 in the vLGE and MGE was significantly increased. Lastly, ectopic overexpression of *Vax1* leads to a significant decrease of Gsx2 expression in the LGE. Thus, *Vax1* may regulate subpallium regionalization by repressing Gsx2.

**Materials and Methods**

**Mice**

All experiments conducted in this study were in accordance with guidelines from Fudan University. *Vax1tm1b(KOMP)MBP* (*Vax1*+/−) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). *Vax1*−/− mice were obtained from crossing *Vax1*+/− with *Vax1*+/− mice. All mice were maintained in a mixed genetic background of C57BL/6J and CD1. Noon of the day of vaginal plug detection was considered E0.5 and the day of birth was defined as P0. Genotyping of *Vax1*−/− mice by PCR uses the following primers:

- **Vax1-WT-F:** CCAAGGGGGCTAACTTCATA
  
- **Vax1-WT-R:** TTCCTCTTCTGCTTGTTGG
  
- **Vax1-Mut-F:** CGGTGCCTACATTACCATG
  
- **Vax1-Mut-R:** AAGCCCTGTTTCTGAC

**Secondary antibodies**

| secondary antibodies | cat number |
|----------------------|------------|
| Alexa®488-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) | 711-545-152 |
| Cy5-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) | 711-165-152 |
| Alexa®488-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) | 715-545-151 |
| Cy5-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) | 715-165-151 |
| Alexa®488-conjugated AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L) | 703-545-155 |
| Cy5-conjugated AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L) | 703-165-155 |
| Alexa®488-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) | 705-545-147 |
| Cy5-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) | 705-165-147 |
| Alexa®488-conjugated AffiniPure Donkey Anti-Guinea Pig IgG (H+L) | 712-165-150 |
| Cy5-conjugated AffiniPure Donkey Anti-Guinea Pig IgG (H+L) | 706-165-148 |

In *In situ* RNA Hybridization

In situ hybridization was performed on 20-μm-thick cryosections using digoxigenin riboprobes, as previously...
described [27]. Probes were made from P0 wild-type mouse brain cDNA amplified by PCR using the following primers:

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| Vax1 | TCCTGTCTAGAACAGAGCTGATGGG | CTGTGGTTTTGGTTAAATTGCGAG |
| Gsx2 | CCTTTGTTCGAGTCCCAGACACC | AAAGGGACTTCCAGAGACCTGGAT |
| Etv1 | TTCATGGCCTCCCCTACCTAAAATC | CCTTCGTTGTAGGGGTGAGGGTT |
| Tshz1 | GAGAAGGTCACGGGCAAGGTCAGC | GAGGCGGAGACACAGCAGTCTGCCA |
| Prokr2 | ATGGGACCCCAGAACAGA | ATAGATACGCTACCTAAGATGAG |
| Isl1 | ATGGGAGACATGGGCGATCC | CATGCCTCAATAGGACTGGCTACC |
| Drd1 | ATGGCTCCTAACACTTCTACCATGG | TCAGGTTGAATGCTGTCCGCTGTG |
| Drd2 | CGGGAGCTGGAAGCCTCGA | TGCAGGGTCAAGAGAAGGCCG |
| Lhx6 | CAGAGAGGGCGCGCATGGTCACCT | AATTGGGGGGGGGGTCTTTGGCAC |
| Lhx8 | GCCTTAGTGTGGCTGAGAGA | AGGATGGTAGGCTTTGTAAACT |
| Nkx2.1 | TCCTAGTCAAAGACGGCAAACCCT | TAAAGAGAAAGGAAGGGGGAAAGA |
| Gbx1 | GATGAAGAGAAGCTAGAGCCCCCA | GGTTCAGATCCTGTGACTTCCGGT |
| Gbx2 | GTTCGCTATTCGAAGTACAGCCAGT | ATGTTCGGGAAACCAGACAAAATGG |
| Cdeca7 | AGACCTCTCGAGACGGTTTGGCTA | ATGCTGAGCTTCAATCCTACCT |
| Cnnd2 | GGGCTAGCAGATGGCAACAGC | GCCCATCGGCAAAGAGAGAGA |
| Ascl1 | CTAACAGGCAGGGCTGGA | TAAAGAGAAAGGAAGGGGGAAAGA |
| Dlx1 | ATGGCATTGGGAGGCGGCGGCGCAG | ATGGCATTGGGAGGCGGCGGCGCAG |
| Dlx2 | TTCCTGCTGCCGGGCTGAGG | ATGTTCGGGAAACCAGACAAAATGG |
| Dlx5 | CAGGCTTCACGGCTGACCGCAG | ATGTTCGGGAAACCAGACAAAATGG |
| LacZ | CTCAATGCGGCTGGTGAAGACTA | GGGTGGTGTTTGTTTTCATCATATT |

**RNA-Seq**

RNA sequencing (RNA-Seq) analysis was performed as previously described [27]. The ganglionic eminence from the E13.5 Vax1−/− and littermate WT and lateral ganglionic eminence from the E16.5 Vax1−/− and littermate WT were dissected (n = 3). Gene expression levels were reported in FPKM (fragments per kilobase of exon per million fragments mapped).

**Cloning of the pCAG-Vax1-ires-GFP Expression Plasmid and In Vivo Electroporation**

The Vax1 CDS was cloned from WT P0 cDNA amplified by PCR using the following primers: F-ATGTTCGGGAAACCAGACAAAATGG; R-AAATCAGTCCAGACCTGGAT. It was then inserted into the pCAG-ires-GFP vector, using XbaI and EcoRI restriction sites. DNA-sequencing was performed to make sure no mutation was generated during the cloning. In vivo electroporation was performed at E16.5 or P0. Plasmids pCAG-GFP (Addgene #11150) or pCAG-Vax1-ires-GFP (final concentration of 1-2 mg/ml, 1 μl each embryo) were mixed with 0.05% Fast Green (Sigma), and injected into the lateral ventricle using a beveled pulled glass micropipette. Five electrical pulses (duration: 50 ms) were applied at 65V or 130V across the uterine wall with a 950-ms interval between pulses. Electroporation was performed using a pair of 7-mm platinum electrodes (BTX, Tweezer electrode 45-0488, Harvard Apparatus) connected to an electroporator (BTX, ECM830). Pups were analyzed at E18.5 or P3.

**Quantification**

The numbers of BrdU and FOXP1 positive cells in the LGE or MGE VZ/SVZ were quantified in 9 randomly chosen 20-μm sections from each mouse. Three control and Vax1 mutant mice from each group were analyzed at E13.5 or E16.5. The integrated density of Cdca7, Cnnd2, Ascl1, Dlx1, Dlx2, Dlx5, and Isl1 measured by Adobe Photoshop CC in the LGE SVZ were quantified in 9 randomly chosen 20-μm sections from each mouse [28]. Three control and Vax1 mutant mice from each group were analyzed at E16.5. For quantification of GFP+ and GFP+/GSX2+ cells in the mouse lateral ventricular zone at E18.5 and P3, images were collected with an Olympus VS 120 microscope using a ×20 objective. Six 20-μm-thick coronal sections from rostral, intermediate, and caudal levels of the striatum were selected (n = 5 mice per group). We selected the dorsal-most lateral ventricular VZ/SVZ region (1500 pix × 1500 pix) to quantify the number of GFP+ and GFP+/GSX2+ cells in each group.

**Microscopy**

Figures were imaged with an Olympus VS 120 microscope. Images were merged, cropped, and optimized in Adobe Photoshop CC without distortioning the original information.
Results

Increase of Cell Proliferation in the Vax1−/− Subpallium VZ-SVZ

The ventral homeodomain protein 1 (VAX1) is highly expressed in the subpallium progenitors from embryonic day 12.5 (E12.5) to adult, which would suggest Vax1 plays an important role in forebrain development (see Supplementary Fig. S1a-f, Additional File 1). To investigate whether Vax1 regulates cell proliferation, we first performed 30-min BrdU pulse labeling at E16.5 and BrdU immunofluorescence staining. The number of BrdU+ cells in the Vax1−/− LGE was gradually increased from rostral to caudal compared to controls (Fig. 1a-c, j). This means the number of cells in S phase of the cell cycle was increased after Vax1 deletion. But, the number of BrdU+ cells in the LGE and MGE is comparable between Vax1−/− and control mice at E13.5 (see Supplementary Fig. S2 a-d, Additional File 1). Since Cdc47 is a cell division cycle-associated gene and Ccnd2 promotes cell cycle progression from G1 to S phase, we then checked these genes at E16.5. In situ RNA hybridization of Ccnd2-mRNA showed that Ccnd2+ cells in the dLGE and postnatal SVZ travel through the RMS into the OB where they mature into OB interneurons. At E16.5, we found that these dLGE markers (Gsx2, Etv1, Sp8, Tshz1, and Prokr2) were significantly increased in the Vax1−/− mice (Fig. 3a-o). Gsx2 was heavily expressed in progenitors of the dLGE and its expression showed a ventral-low to dorsal-high gradient in the subpallium of the controls, whereas more Gsx2+ cells were seen in the VZ/SVZ of the Vax1−/− mice. The obvious ventral to dorsal gradient in Gsx2+ cell numbers in the Vax1−/− mice disappeared (Fig. 3a-c). Even more remarkably, Prokr2, Tshz1, Etv1, and Sp8 showed an increased gradient expression in the dLGE (Fig. 3d-o). Gsx2 is expressed in proliferating progenitors, Sp8 is found in some dividing cells, and Prokr2 is observed in post-mitotic immature interneurons, which suggests the blockage of neuronal differentiation in the dLGE after Vax1 gene deletion. The expression of these markers (Gsx2, Sp8, and Prokr2) was also significantly increased in the Vax1−/− mice at P0 (data not shown). Taken together, the progenitors and immature neurons in the dLGE are significantly increased in the Vax1−/− mice.

Vax1 Promotes Neuronal Differentiation in the Subpallium VZ-SVZ

The bHLH transcription factor, Ascl1, is heavily expressed in ventral forebrain progenitors, and Ascl1 is crucial for neurogenesis [29]. At E16.5, the number of Ascl1+ cells in the Vax1−/− subpallium VZ (ventricular zone) was significantly increased, compared to controls (Fig. 2a-c, m). Dlx1 and Dlx2 are mainly expressed in the VZ and SVZ of the subpallium. Dlx5 appears later than Dlx1/2, and it is expressed in more differentiated neurons in the SVZ (subventricular zone) and MZ (mantle zone). Our results showed that Dlx1+, Dlx2+, and Dlx5+ cells in the Vax1−/− subpallium were increased markedly (Fig. 2d-i, m). Accumulation of Ascl1+, Dlx1+, Dlx2+, and Dlx5+ immature neurons in the Vax1−/− VZ and SVZ suggests that neural progenitors in the subpallium could not normally exit the cycle and differentiate. Therefore, Vax1 promotes neuronal differentiation in the subpallium VZ-SVZ.

The Expansion of the dLGE Domain in the Vax1−/− Mice

The progenitors in the dLGE mainly give rise to olfactory bulb interneurons. Strong expression of Gsx2 in the dLGE promotes the activation and lineage progression of OB interneuron progenitors. Sp8 is expressed in migrating neuroblasts in the embryonic dLGE, postnatal SVZ and most OB interneurons, and the OB interneuron defects in conditional inactivation of Sp8 mice [6]. Sp8 regulates the expression of Tshz1 and Prokr2 [30]. Prokr2 and Tshz1 are expressed in the SVZ-RMS-OB and the neuroblasts tangential and radial migration were severely defected in Prokr2-KO mice [25, 31]. Etv1+ cells in the dLGE and postnatal SVZ travel through the RMS into the OB where they mature into OB interneurons. At E16.5, we found that these dLGE markers (Gsx2, Etv1, Sp8, Tshz1, and Prokr2) were significantly increased in the Vax1−/− mice (Fig. 3a-o). Gsx2 was heavily expressed in progenitors of the dLGE and its expression showed a ventral-low to dorsal-high gradient in the subpallium of the controls, whereas more Gsx2+ cells were seen in the VZ/SVZ of the Vax1−/− mice. The obvious ventral to dorsal gradient in Gsx2+ cell numbers in the Vax1−/− mice disappeared (Fig. 3a-c). Even more remarkably, Prokr2, Tshz1, Etv1, and Sp8 showed an increased gradient expression in the dLGE (Fig. 3d-o). Gsx2 is expressed in proliferating progenitors, Sp8 is found in some dividing cells, and Prokr2 is observed in post-mitotic immature interneurons, which suggests the blockage of neuronal differentiation in the dLGE after Vax1 gene deletion. The expression of these markers (Gsx2, Sp8, and Prokr2) was also significantly increased in the Vax1−/− mice at P0 (data not shown). Taken together, the progenitors and immature neurons in the dLGE are significantly increased in the Vax1−/− mice.

The vLGE-Derived Striatal MSNs Were Reduced in Vax1 Mutant Mice

We showed above that the dLGE was expanded; next, we wanted to know whether the vLGE and MGE-derived neurons changed or not. Isl1 was highly expressed in D1-type MSNs [32, 33]. At E16.5, Isl1+ cells were significantly reduced in the LGE SVZ and MZ of the Vax1−/− mice (Fig. 4a-g). More than 90% striatal MSNs are expressed Foxp1. The number of FOXP1+ cells in the Vax1−/− mouse SVZ was greatly reduced at E16.5 (Fig. 4h-n and h’-m’). Striatal projection neurons were mainly composed of dopamine D1 receptor (Drd1) expressing MSNs and dopamine D2 receptor (Drd2) expressing MSNs. In situ hybridization of Drd1-mRNA and Drd2-mRNA showed that Drd1+ and Drd2+ MSNs were also reduced in Vax1 mutant mice (data not shown). These results suggest striall MSNs are decreased in Vax1−/− mice at E16.5. At P0, we can see a thicker SVZ in Vax1−/− mice from the nuclear staining of DAPI (see Supplementary Fig. S3 a, b, Additional File 1). The partial sequences of exon 2 and exon 3 of the Vax1 gene were replaced by a LacZ reporter gene in the Vax1−/− mice. In situ hybridization of LacZ-mRNA showed a marked increase of LacZ+ cells, suggesting that
most Vax1 mutant cells were still there (see Supplementary Fig. S3 c, d, Additional File 1). Ki67 is expressed in dividing cells and the KI67+ region was the thicker LacZ+ SVZ region in Vax1−/− mice (see Supplementary Fig. S3 e, f, Additional File 1), whereas FOXP1+ cells were rarely detected (see Supplementary Fig. S3 g-h, Additional File 1). Altogether, these results indicated the blockage of striatal MSN differentiation in the Vax1−/− mice.

Only about 6% Vax1−/− mice can survive to P20, and we were fortunate to have collected two P20 Vax1−/− mouse brains. We then performed DAPI, and in situ hybridization staining of Drd1 and Drd2. We observed the absence of the septum and preoptic area in the Vax1 mutant mice, the change of the striatum shape, and that Vax1 mutants had enlarged lateral ventricles (see Supplementary Fig. S4 a, a’, Additional File 1). The Vax1 mutants had a low density of Drd1-mRNA+ and Drd2-mRNA+ cells in the medial striatum (see Supplementary Fig. S4 b-c’, Additional File 1). Taken together, our results showed the number of vLGE-derived striatal MSNs was reduced in the Vax1−/− mice.

The MGE Domain Had Defects in the Vax1−/− Mice

Since the dLGE domain is enlarged and the vLGE domain is atrophic in the Vax1−/− mice, we wanted to further investigate the change of the MGE domain in the Vax1 mutants. We then performed in situ hybridization staining of Nkx2.1, Lhx6, and Lhx8 at E13.5. The induction and regional pattern formation of MGE are dependent on the transcription factor Nkx2.1 [13]. Nkx2.1 plays an important role in maintaining the specificity and differentiation of MGE progenitors [12, 34]. The Nkx2.1+ MGE domain in the Vax1−/− mice was significantly smaller
than that in the controls (Fig. 5a–c'). Note that the septum got smaller in the Vax1−/− mice (Fig. 5a–c'). LIM-homeodomain transcription factors Lhx6 and Lhx8, are expressed in the MGE SVZ, and are crucial for the migration and differentiation of MGE-derived neurons. In Vax1−/− mice, the expression of the Ascl1, Dlx1, Dlx2, and Dlx5 was increased in the LGE SVZ of Vax1−/− mice (Student's t-test, *P < .05, **P < .01, ***P < .001, n = 3 mice per group, mean ± SEM). Scale bars: 500 μm in l' for a–l'.

transcription factors have been found to be expressed in the globus pallidus, such as Nkx2.1, Gbx1, Gbx2, Arx, Dlx1, Evl, Lhx6, and Lhx8 [35]. In order to know clearly the changes in the globus pallidus, we performed in situ hybridization staining of Nkx2.1, Gbx1, and Gbx2 at E12.5 in the Vax1−/− and control mice. We also detected Evl, Nkx2.1, Lhx6, and Lhx8 at E18.5. There was a reduction in globus pallidus neurons after Vax1 deletion (see Supplementary Fig. S5, Fig.S6, Additional File 1). In summary, the MGE and septum domain get smaller and MGE-derived neurons are decreased in the Vax1−/− mice.
RNA-Seq Analysis Provided Further Molecular Evidence for Defects in Vax1 Mutant Mice

To characterize the molecular changes in Vax1−/− mice, we performed RNA-Seq analysis. Gene expression profiles from the embryonic day (E) 13.5 and E16.5 ganglionic eminence (GE) were analyzed. Changed expression of genes revealed by the RNA-Seq was consistent with our staining results, above. At E13.5, there was a remarkable increased expression of Gsx2 and Sp8 in the GE, with significant decrease of Isl1, Ebf1, Foxp1, Tac1, Otx2, Lhx8, Gbx2, and Gbx1 (Table 1). At E16.5, the dLGE markers (Sp8, Etv1, Tshz1) were upregulated and the vLGE-derived neuronal markers (Fopx1, Foxo1, Iklf1, Ebf1, Gpr88, Tac1, Sox8, Zfp503, Drd1, Ppp1r1b, Gpr6, Is1, Zc512, and Adora2a) were downregulated, with an upregulation of genes that promote cell cycle and maintenance of the progenitor state (Table 2). The RNA-Seq analysis and staining results showed that the dLGE cells invaded ventrally into the vLGE, causing the dLGE region gets larger while the vLGE and MGE regions get smaller in the Vax1−/− mice compared to controls.
Increased Expression of Gsx2 in the vLGE and MGE in Vax1 Mutant Mice

Previous studies showed that Vax1 is a fundamental regulator of ventral identity for retinal ganglion cells and Gsx2 is a key regulator of dLGE identity for OB interneurons [4, 36–39]. In an attempt to understand the relationship between Vax1 and Gsx2, we performed immunofluorescence or in situ RNA hybridization staining of Gsx2 or Vax1 from immediate adjacent 20-μm sections in WT mice at E11.5 and E16.5. We observed Gsx2 expression as a ventral-low to dorsal-high gradient in the subpallium (Fig. 6a, c). In contrast, Vax1 was expressed in a ventral-high to dorsal-low gradient along the subpallium (Fig. 6b, d). The expression pattern of Vax1 is largely complementary to that of Gsx2, which supports our hypothesis that Vax1 may be crucial for subpallium regionalization. To test this, we performed immunofluorescence or in situ RNA hybridization of Gsx2 at early stages, and our results showed that the expression pattern of Gsx2 (ventral-low to dorsal-high) was disrupted in the Vax1−/− mice at E11.5 and E13.5 (Fig. 7a-d). Gsx2 was highly expressed in the whole subpallium VZ and SVZ in the Vax1−/− mice, which suggests that Vax1 may be an inhibitor to suppress the expression of Gsx2 in the subpallium VZ and SVZ. Altogether, these results suggest that Vax1 may regulate subpallium regionalization by repressing Gsx2.

Vax1 Inhibits the Expression of Gsx2 in the Progenitor Cells

To further investigate whether Vax1 inhibits the expression of Gsx2 during forebrain development, we overexpressed Vax1...
in the lateral neural progenitors by electroporation and analyzed the impact on *Gsx2* expression. A *Vax1* expression plasmid (pCAG-Vax1-GFP) or a control vector (pCAG-GFP) was electroporated into the lateral ventricular wall at E16.5 or P0 in the WT mice (Fig. 8a, e). Two or three days later, animals were analyzed and the number of GFP/GSX2 double positive cells was measured in the dorsal-lateral region (Fig. 8a, e). The number of GFP/GSX2 double positive cells showed a significant decrease (about 5-fold) in the pCAG-Vax1-GFP mice compared to control mice (Fig. 8b-d and f-h). Thus, we conclude that in progenitor cells, *Vax1* has the capacity to act as a negative regulator of *Gsx2* expression.

**Table 1** The significantly changed gene at E13.5

| Gene name | *Vax1*−/− (FPKM) | WT (FPKM) | P-value  | Gene name | *Vax1*−/− (FPKM) | WT (FPKM) | P-value |
|-----------|------------------|-----------|----------|-----------|------------------|-----------|---------|
| Gsx2      | 23.14            | 13.38     | 1.14E-05 | Sp8       | 14.54            | 9.97      | 0.000109|
| Bmp2      | 0.33             | 0.15      | 0.019487 | Gsx1      | 1.35             | 3.34      | 1.60E-07|
| Wnt8b     | 0.26             | 0.01      | 0.000507 | Olig2     | 24.76            | 33.79     | 0.009027|
| Col2a1    | 9.47             | 3.43      | 1.03E-08 | Lhx8      | 7.22             | 18.93     | 7.45E-08|
| Rpl21     | 9.39             | 3.54      | 9.74E-06 | Gbx2      | 1.54             | 3.56      | 2.27E-05|
| Pcdhga9   | 9.16             | 1.67      | 2.00E-06 | Gbx1      | 4.07             | 8.90      | 5.27E-08|
| Zfp125    | 6.61             | 2.51      | 0.018824 | Isl1      | 13.07            | 19.52     | 0.000176|
| Pcdhgb5   | 5.09             | 1.22      | 0.003204 | Ebf1      | 9.35             | 13.38     | 0.000134|
| Znb9      | 5.05             | 0.75      | 0.000337 | Pcdhgb8   | 0.60             | 14.38     | 3.11E-07|
Discussion

In this study, we investigated the function of the Vax1 gene in subpallium regionalization using Vax1−/− mice. Our results showed an increase in cell proliferation and the blockage of neuronal differentiation in the subpallium of the Vax1−/− mice. More importantly, Vax1 and Gsx2 display largely complementary patterns of expression in the subpallium (Fig. 9a). We found that the Gsx2 was highly expressed in the whole subpallium VZ and SVZ caused by Vax1 gene deletion (Fig. 9b). Overexpression of Vax1 in the LGE progenitors strongly inhibits the expression of Gsx2. Thus, we proposed that Vax1 may regulate the subpallium regionalization by suppressing Gsx2 expression.
The dLGE, vLGE, and MGE Domain in the Vax1 Mutant Mice

The full name of the Vax1 gene is ventral anterior homeobox 1. The Vax1 gene is also a member of the Emx and Not gene families [17]. The genes of the Emx and the Not gene families play an important role in the patterning development of the regions where they are expressed. In the Vax1 mice, the progenitors in the dLGE/dSVZ domain are significantly increased, vLGE-derived striatal projection neurons are significantly decreased, MGE progenitors are decreased, and the septal area is absent. This suggests that the Vax1 gene regulates the regionalization of the subpallium. It seems that the dLGE region significantly expanded ventrally and the vLGE, MGE, and septum got smaller in Vax1 mice.

The dLGE progenitors mainly give rise to OB interneurons. Prokr2, Tshz1, Etv1, and Sp8 showed increased gradient expression in the dLGE/dSVZ at E16.5 and P0. Since Gsx2 is expressed in proliferating progenitors, Sp8 is found in some dividing cells, and Prokr2 is observed in post-mitotic immature interneurons, this would suggest the blockage of neuronal differentiation in the dLGE after Vax1 gene deletion. Despite the significant increase of dLGE/dSVZ progenitors, the blockage of neuronal differentiation and defects of migration result in Vax1 mice with a severely hypoplastic OB [40].

There is a reduction of Ebf1+, Isl1+, Foxp1+, Drd1+, and Drd2+ striatal projection neurons in the striatum of Vax1 mutant mice. Despite the increase of cell proliferation in the Vax1 subpallium VZ-SVZ, the smaller vLGE and blockage of striatal MSN differentiation in the Vax1 mice, thereby, led to the developmental defects of the striatum in the Vax1 mice.

MGE contributes approximately 70% of cortical interneurons and most globus pallidal projection neurons...
The MGE domain got smaller and MGE progenitors were decreased in the \textit{Vax1}−/− mice, which leads to a reduction of cortical interneurons [20] and globus pallidal neurons.

The septum is an important structure that connects with many important brain structures. It begins to develop in the early embryonic stage, from E10.5 to E14.5 when septal neurons are mainly produced [43]. Progenitors in the septum contribute to not only septal neurons but also OB interneurons [39, 44]. The septum is absent in the \textit{Vax1}−/− mice, suggesting that \textit{Vax1} plays an important role in its development.

The Relationship Between \textit{Vax1} and \textit{Gsx2}

There was an increased expression of \textit{Gsx2} in the vLGE and MGE in \textit{Vax1} mutant mice. The transcription factor \textit{Gsx2} plays a powerful role in the development of the telencephalon [37] and is crucial for the dorsal-ventral patterning [16]. \textit{Gsx2} and \textit{Pax6} play important roles in the maintenance of the pallium and subpallium boundary [11, 45]. In \textit{Gsx2} mutant mice, dLGE progenitor cells express markers of the ventral pallium (\textit{Pax6}, \textit{Ngn2}, \textit{Tbr2}, and \textit{Dbx1}) during early telencephalic development. The dLGE is respecified into a ventral pallium-like structure [11, 37, 46, 47]. \textit{Vax1} (ventral-high,
dorsal-low) and Gsx2 (ventral-low, dorsal-high) display largely complementary patterns of expression in the developing subpallium, suggesting that Vax1 may regulate ventral telencephalic development by repressing Gsx2. We performed in vivo electroporation to overexpress Vax1 in the LGE progenitors, and our results showed that Vax1 can strongly inhibit the expression of Gsx2. These results suggested that Gsx2 was downregulated in the progenitors due to Vax1 expression. In addition, to further investigate the mechanism of the upregulation of the Gsx2 in the Vax1 mutants, the Vax1;Gsx2 double mutants may provide more evidence.

**Fig. 8** The expression of Gsx2 was suppressed by Vax1 in progenitor cells. (a) Experimental design for the electroporation of progenitor cells in the lateral wall with pCAG-GFP or pCAG-Vax1-GFP at E16.5 and analysis at E18.5. (b) There are some GSX2/GFP double positive cells (arrows) observed in control mice. (c) Very few GSX2/GFP double positive cells were observed in Vax1 overexpressing (Vax1-OE) mice. (d) Quantification GFP/GSX2 double positive cells in control or Vax1 overexpressing mice. (e) Experimental design for the electroporation of progenitor cells in the lateral wall with pCAG-GFP or pCAG-Vax1-GFP at P0 and analysis at P3. (f) Representative images showing the GFP/GSX2 double positive cells in the control mice. White arrows: GFP/GSX2 double positive cells. (g) Representative images showing the downregulation of GSX2 in GFP positive cells after electroporation of lateral progenitor cells by Vax1. (h) Quantification images showing the downregulation of GSX2 in GFP positive cells after electroporation of lateral progenitor cells by Vax1. Student’s t-test, ***P < .001, n = 5 mice per group, mean ± SEM. Scale bars: 20 μm for b, c, f, and g.

**Gsx2 Cooperated with Pax6 and Vax1 Which Are Essential for Telencephalic Regionalization**

The telencephalon is subdivided into molecularly and functionally distinct progenitor regions along the dorsal-ventral (DV) axis that generate different subclasses of neurons. A number of developmental control genes are specifically expressed by progenitors in either the pallium or subpallium. For instance, progenitor cells in the pallium express Emx1, Pax6, and Ngn2 [11, 48]. In the pallium, Pax6 is critical for the progenitors’ identity. The dLGE expands to the pallium in the Pax6−/− mice as detected by dLGE markers such as Dlx1/2, Sp8, and En1 [49]. Progenitors in the dLGE express Gsx2, Sp8, En1, Tshz1, and Prokr2. In this region, Gsx2 is a core regulator for dLGE identity. Gsx2 function was found to be essential to maintain the molecular identity of early striatal progenitors and in its absence the pallium regulatory genes Pax6 and Ngn2 are ectopically expressed in the LGE [16]. Progenitors in the MGE or POA express Nkx2.1, Lhx6, Lhx8, and Otx2. In the Nkx2.1 mutant, the MGE gets smaller and the LGE progenitors invade into the MGE primordium [13, 35]. Otx2 was also reported to be essential for promoting vMGE identity and repressing POA identity [14]. In this study, we found that Vax1 was highly expressed in the subpallium (dorsal-low,
Vax1 functions as a repressor to inhibit Gsx2 expression for subpallium regionalization. Overall, we speculated that Vax1, Gsx2, and Pax6 are the key regulators of the progenitors’ regionalization during telencephalic development.

The Increase of Cell Proliferation and the Blockage of Neuronal Differentiation in the Vax1<sup>−/−</sup> Mice

In the Vax1<sup>−/−</sup> mouse subpallium, the expression of Gsx1 is downregulated (Table 1 and Table 2) [20]. Gsx2 maintains LGE progenitors in an undifferentiated state, whereas Gsx1 promotes progenitor maturation and the acquisition of neuronal phenotypes [50]. Therefore, that is the reason for the increase of cell proliferation and the blockage of neuronal differentiation in the Vax1<sup>−/−</sup> subpallium.

Supplementary Information  The online version contains supplementary material available at https://doi.org/10.1007/s12035-021-02378-x.

Author Contribution  All authors had full access to the data and take responsibility for the integrity of the results and the accuracy of the data analysis. Y.W., Z.Y., and Z.Z. designed the research and Y.W., Z.S., and Z.W. performed experiments and analysis. L.Y., G.L., Z.S., Y.D., H.D., Z.L., Y.Y., and X.L. helped conduct experiments. Y.W., Z.Y., and Z.Z. drafted the manuscript.

Funding  Research grants to Z. Yang from the National Key Research and Development Program of China (2018YFA0108000), National Natural Science Foundation of China (NSFC 31820103006, 31630032 and 31425011), and research grant to Y. You (NSFC 31700889).

Data Availability  The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate  All experiments conducted in this research study were in accordance with guidelines from Fudan University.

Consent for Publication  Not applicable.

Conflict of Interest  The authors declare no competing interests.

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Fig. 9  A model of Vax1 regulating subpallium regionalization. (a) The expression patterns of Vax1 and Gsx2 are largely complementary. Gsx2 was expressed in a ventral-low to dorsal-high gradient while the Vax1 was expressed in a ventral-high to dorsal-low gradient along the subpallium. (b) Increased expression of Gsx2 in the vLGE and MGE leads to the abnormal cell proliferation and differentiation of the subpallium neurons ventral-high). Vax1 functions as a repressor to inhibit Gsx2 expression for subpallium regionalization. Overall, we speculated that Vax1, Gsx2, and Pax6 are the key regulators of the progenitors’ regionalization during telencephalic development.

Natural Science Foundation of China (NSFC 31820103006, 31630032 and 31425011), and research grant to Y. You (NSFC 31700889).
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