OTX1 regulates cell cycle progression of neural progenitors in the developing cerebral cortex

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The progenitor cells in the cerebral cortex coordinate proliferation and mitotic exit to generate the correct number of neurons and glial cells during development. However, mechanisms for regulating the mitotic cycle of cortical progenitors are not fully understood. Otx1 is one of the homeobox-containing transcription factors frequently implicated in the development of the central nervous system. Mice bearing a targeted deletion of Otx1 exhibit brain hypoplasia and a decrease in the number of cortical neurons. We hypothesized that Otx1 might be crucial to the proliferation and differentiation of cortical progenitors. Otx1 knockdown by in utero electroporation in the mouse brain reduced the proportion of the G1 phase while increasing the S and M phases of progenitor cells. The knockdown diminished Tbr1+ neurons but increased GFAP+ astrocytes in the early postnatal cortex as revealed by lineage tracing study. Tbr2+ basal progenitors lacking Otx1 were held at the transit-amplifying stage. In contrast, overexpression of wildtype Otx1 but not an astrocytoma-related mutant Y320C inhibited proliferation of the progenitor cells in embryonic cortex. This study demonstrates that Otx1 is one of the key elements regulating cortical neurogenesis, and a loss-of-function in Otx1 may contribute to the overproduction of astrocytes in vivo.

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2The abbreviations used are: VZ, ventricular zone; EdU, 5-ethyl-2′-deoxyuridine; AP, apical progenitor; BP, basal progenitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA; scr-RNA, scramble RNA; KD, knockdown; IUE, in utero electroporation; CPER, cytoplasmic protein extraction reagent.

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Results

Otx1 is expressed in both nucleus and cytoplasm in the developing cerebral cortex

A previous study by immunofluorescence found that OTX1 appeared in the cytoplasm of VZ cells during embryonic development (11). To ascertain the cellular location of this transcription factor, we isolated cytoplasm and nucleus of E13.5 cortical cells, and measured OTX1 by Western blotting. GAPDH and Lamin-B1 were, respectively, determined as markers of the cytoplasmic and nuclear fraction. A band of 31 kDa, the predicted size of OTX1, was found in the GAPDH-enriched cytoplasmic fraction. Noticeably, however, the nuclear fraction that was marked by high Lamin-B1 and low GAPDH contained a significant amount of OTX1 (Fig. 1, A and B), indicating that the transcription factor OTX1 was also present in the nucleus during early development. The specificity of the OTX1 signal measured by Western blot was validated by transfection of pCAGEN-Otx1 into Neuroblastoma N2a cells; the expected band was not detectable until the ectopic expression of Otx1 (Fig. 1, C).

To investigate the role of Otx1 in cortical neurogenesis, we semiquantified its expression in the embryonic cortices of mice. Cortical tissues were collected from brains at E11.5, E13.5, and E15.5 (Fig. 1, D and E), the period when the cortical progenitors undergo proliferation and generate neurons. OTX1 expression gradually increased as the development proceeded (Fig. 1, D and E).

Reducing Otx1 promotes cell cycle progression of cortical progenitors

To explore the function of Otx1, we aimed to inhibit its expression by using shRNA. The efficacy of inhibition was first assessed by Western blotting in vitro. Otx1–shRNA, a construct of Otx1 interference RNA was introduced into the cortical cells prepared from E12.5 dorsal telencephalon. Compared with a scramble RNA (scr-RNA), the Otx1–shRNA reduced OTX1 expression in the primary culture (Fig. 2, A and B). Direct quantification of the inhibition in vivo was not conclusive due to the weak and varied immunofluorescent signal of endogenous OTX1 (data not shown). The RNA interference was thus tested by co-electroporating Otx1–shRNA and V5-tagged Otx1 into the embryonic brain at E13.5. The control group received scr-RNA in place of Otx1–shRNA. We analyzed the expression of V5 in the telencephalon at E15.5 by immunofluorescence. The ratio of V5 positive cells in the transfected population was much less in the cortex receiving Otx1–shRNA compared with that of scr-RNA (Fig. 2, C and D; Otx1–shRNA: 24.5% versus scr-RNA: 48.3%). The results indicate that the shRNA construct was able to inhibit the expression of Otx1.

To explore whether the increase in OTX1 is essential for cortical neurogenesis, we electroporated the Otx1–shRNA along with RFP-pCAGGS into the embryonic brains at E13.5, when APs are generating the deep-layer cortical neurons. Two days after electroporation, the proliferating cells with active DNA synthesis were scored by pulse labeling with EdU that was injected 3 h before harvesting the brains. OTX1 knockdown (KD) increased the proportions of proliferating cells in the
developing cortex. The E15.5 brains transfected with Otx1–shRNA had 15.8% of EdU/H11001 cells in the germinal zone, which rose from 4.8% in the corresponding regions of control brains receiving scr-RNA (Fig. 3, A and B). The generation of upper-layer cortical neurons was similarly examined by electroporating the shRNA into E15.5 brains. Otx1 KD up-regulated the EdU/H11001 population at E17.5 (Fig. 3, C and D). These results indicate that inhibition of OTX1 increased the number of proliferating cells that incorporated EdU during the S-phase of the cell cycle.

To exclude possible off-target effects of vector-driven small interference RNA, we disrupted the fourth exon of mouse Otx1 with CRISPR/Cas9, a powerful tool for genome editing in eukaryotic cells (16, 17). To determine whether expression of the CRISPR/Cas9 system can achieve cleavage of the Otx1 gene, we transfected N2a cells with pX330–gOtx1 and amplified the region spanning the guide RNA sequence from the genomic DNA of N2a cells. By sequencing, we found noisy signals underneath the sequence peaks adjacent to the PAM motif (Fig. 4A). The noise represents the random indel mutations that had been introduced into the genome of N2a cells by pX330–gOtx1. In contrast, N2a cells expressing pX330 without the Otx1-targeting RNA reported a clean sequence of WT Otx1 (Fig. 4A). Furthermore, the primary culture of cortical neurons transfected with pX330–gOtx1 had a reduced level of OTX1 protein compared with that expressing pX330 (Fig. 4, B and C), suggesting that the pX330–gOtx1 was capable of inhibiting Otx1. To knock-out Otx1 in vivo, pX330–gOtx1 was electroporated into E13.5 brains. Otx1 knock-out increased the EdU+ cells in the germinal zone after 2 days of electroporation (control pX330: 6.9 versus pX330-gOtx1: 15.4%) (Fig. 4, D and E). The EdU+ population at E17.5 was also increased by the knock-out introduced at E15.5 (Fig. 4, F and G). The data suggest that the Otx1 mutation enlarged the S phase population, similar to that produced by the KD with the shRNA.

An enhanced S phase may result from either a blockage of G2/M entry or an acceleration of G1-S phase transition. The G1 phase was evaluated by cyclin D1, which was synthesized in the G1 and degraded rapidly as the cell entered the S phase. We studied the expression of cyclin D1 in the embryonic brain electroporated with the Otx1–shRNA, or the scramble, in the presence of RFP–pCAGGS. In E13.5–E15.5 when peak cortical neurogenesis occurs, about half of cells in germinal compartments from the control brain expressed cyclin D1 (50.5%). On the contrary, only 13.3% of the cells with Otx1 KD were cyclin D1 positive (Fig. 5, A and B). The cyclin D1+ population was similarly reduced in the E17.5 brain, 2 days after the transfection with Otx1–shRNA (Fig. 5, C and D). The data suggests that the progenitors had a decreased entry into G1 or an increased exit to S phase when the Otx1 expression was reduced. The mitotic index (the percentage of transfected cells in mitosis) was examined by phosphorylated histone 3 (pH3), which marks the mitotic cells with condensed chromosomes. When E13.5-electroporated brains were examined at E15.5, the proportion of
pH3+ cells were higher in the brain transfected with Otx1–shRNA than that with the control (control scr-RNA: 4.3% versus Otx1–shRNA 8.2%, Fig. 6, A and B). Similarly, Otx1 KD almost doubled the ratio of pH3+ cells in the germinal zone of the E17.5 brain (Fig. 6, C and D). Taken together, our results show that Otx1 KD enhanced the cell cycle progression by increasing cells in S and M phases while reducing the G1 population during cortical development. Our data are consistent with previous studies that the cortical precursors with a shortened G1 phase and a fastened G1/S transition prefer to stay in rather than to exit from the cell cycle (1, 15).

**Otx1 knockdown increases the proliferating APs and BPs at the expense of neurogenesis**

In the cerebral cortex, Pax6-expressing APs and Tbr2-expressing BPs have the capacity of proliferation (18). Tbr2+ cells represent intermediate progenitor cells that have not yet exited the cell cycle. To verify the identity of the proliferative cells that were increased by the Otx1 KD, we first measured the proportions of Pax6-expressing cells at E15.5, 2 days after the electroporation. The percentage of Pax6+ cells in the Otx1–shRNA-electroporated populations was increased from that of control (Otx1–shRNA: 83.7% versus shRNA-Scr: 62%) (Fig. 7B), suggesting that Otx1 KD expands the progenitor pool in the germinal zone.

Pax6+ APs asymmetrically divide to give birth to either a neuron or a basal progenitor (BP) (2). Newborn BPs are characterized by Tbr2+ and Pax6+, the latter of which inherits from APs and remains to be down-regulated (18, 19). We observed an increase in the proportion of Pax6 and Tbr2 double positive cells in the electroporated populations compared with the control (Otx1–shRNA: 30.4% versus shRNA-Scr: 13.5%) (Fig. 7, A and C). Notably, expansion of the Tbr2+/Pax6+ population (16.9%) accounted for most of the increase in the Pax6+ cells (20.5%) at E15.5, indicating a shift toward the production of Tbr2+ precursors in the Pax6 progeny. Furthermore, the BPs with a reduced Otx1 were more likely proliferative because the percentage of EdU+/cycling cells in the Tbr2+ transfected population was higher in the KD group than in control (Fig. 7, D and E, control: 5.1% versus KD: 15.8%). The results indicate that knocking down Otx1 favors the generation of Tbr2+ precursors rather than exiting from the mitotic cell cycle. The BPs may be held at the transit-amplifying stage by the Otx1 KD.
An extension of the proliferative cell division during the period of neurogenesis will invariably reduce the number of differentiating neurons. To assess whether \( Otx1 \) regulates the cortical neurogenesis, we introduced the interference RNA into the E13.5 brains and examined the expression of Tbr1, a marker of early postmitotic neuron in the cortex (18). Tbr1\(^+\)/H11001 neurons cannot be labeled and quantified until 3 days of electroporation (data not shown). E16.5 brains transfected with \( Otx1\)-shRNA had a reduced Tbr1\(^+\) population in the emerging cortical plate (\( Otx1\)-shRNA: 2.8 \% versus shRNA-Scr: 17.2\%) (Fig. 8, A and B). Our results establish that \( Otx1 \) KD increases the proliferative divisions at the expense of neurogenic cell divisions in the cerebral cortex.

\( Otx1 \) knockdown raises the production of astrocytes

The cortical progenitors differentiate into neurons during the embryonic development, yet around the birth, they start to transform into astrocytes, the largest glial population in the...
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Figure 5. Otx1 knockdown reduces G1 cyclin in the cortical progenitors. A and C, confocal microphotographs showing cyclin D1-expressing cells (green) in the E15.5 (A) and E17.5 (C) brains, after electroporation with control scr-RNA or Otx1 shRNA in the presence of RFP (red) at E13.5 (A) and E15.5 (C). Arrowheads indicate RFP⁺, cyclin D1⁺ cells. Scale bars = 20 μm. B and D, statistics of cyclin D1⁺ cells in the transfected population from A and C, respectively. SVZ, subventricular zone.

mammalian brain (20). To determine the fate of the proliferative progenitors that were increased by the Otx1 KD, we traced the lineage of the cortical progenitors that had received Otx1 shRNA with Tol2 transposon. With the Tol2 transposase, a transgene flanked by Tol2 cis-sequences is integrated into the genomic DNA of the mitotic progenitor and inherited by all progeny including neurons and glial cells (21). This method allowed the labeling of glial cells after birth by the expression of GFP or RFP (22). Otx1 shRNA or scr-RNA was introduced into the cortex by in utero electroporation in the presence of pT2K–CAGGS–EGFP and pCAGGS–T2TP. The shRNA without Tol2 cis-sequences was diluted and lost following multiple cell divisions before the progenitors produced the differentiated glial cells. Glia generation was determined at the early neonatal stage P3 when the local proliferation of glia had not fully begun (23). Most GFAP-positive astrocytes appeared in the vicinity of white matter (23). The percentage of progeny (GFP positive) that expressed GFAP were almost doubled in the cortex (Fig. 8, C and D), suggesting that the early inhibition of Otx1 increased the gliogenesis. Taken together, we conclude that the Otx1 KD reduced the neuron but increased glia production in the brain.

Figure 5. Otx1 knockdown reduces G1 cyclin in the cortical progenitors. A and C, confocal microphotographs showing cyclin D1-expressing cells (green) in the E15.5 (A) and E17.5 (C) brains, after electroporation with control scr-RNA or Otx1 shRNA in the presence of RFP (red) at E13.5 (A) and E15.5 (C). Arrowheads indicate RFP⁺, cyclin D1⁺ cells. Scale bars = 20 μm. B and D, statistics of cyclin D1⁺ cells in the transfected population from A and C, respectively. SVZ, subventricular zone.

Overexpression of WT Otx1 diminishes the proliferating precursors

The KD experiments suggest that Otx1 is essential for the embryonic progenitors to exit from the cell cycle. We sought to determine whether Otx1 overexpression is sufficient to increase the mitotic exit leading to neuron generation. Otx1-pCAGEN was transfected into mouse brains at E12.5. Brains with the ectopic expression of OTX1 were examined at E14.5 with immunofluorescence. Overexpression of Otx1 led to dramatic decreases in the proportions of G1 and S phase cells as measured by the expression of cyclin D1 and the incorporation of EdU, respectively (cyclin D1: control 19.6 % versus overexpression 0.5%; EdU: control 16.7 % versus overexpression 0.4%) (Fig. 9, A–D). The mitotic index as measured by pH3 staining was concurrently diminished (control 6.9 % versus overexpression 0.98%) (Fig. 9, E and F). The lack of cells in G1, S, and M phases upon Otx1 overexpression imply that the progenitors may have exited from the cell cycle. Indeed, the postmitotic neurons as labeled by TuJ1 staining were increased in the Otx1 overexpression brain (control 8.8 % versus overexpression 21.3%) (Fig. 9, G and H).
To explore whether a transcriptional regulation is involved in Otx1 control of the cell cycle, we studied the structure of the OTX1 protein. The C terminus of the Drosophila Otd protein harbors a conservative transcriptional activation domain (24). Mutations in the C terminus of human OTX1 (D318H, Y319C, K320T) have been identified in astrocytoma samples isolated from different patients (http://cancer.sanger.ac.uk/cosmic).3 To test whether this region is crucial to the function of OTX1, we introduced mutant Y320C, a mouse homolog of human Y319C, into the E12.5 brain by in utero electroporation (IUE). Otx1–Y320C was unable to reduce the proportion of EdU+/H11001 cells in the transfected cells the same as WT Otx1. In fact, Y320C increased EdU+/H11001 cells slightly (Fig. 9, I and J). The result indicates that the C terminus of Otx1 may participate in controlling the mitotic cycle of the cortical progenitors in vivo.

Discussion

Homeobox-containing transcription factors are indispensable for the development of the central nervous system. Transcription factor OTX1 is a murine homolog of the Drosophila orthodenticle (otd) gene containing a bicoid-like DNA-binding homeodomain. In the developing human brain, OTX1 expression was located in the proliferative zones of the neocortex (25). During murine embryonic development, increasing expression of Otx1 was observed in the ventricular cells (10). We confirmed the expression of Otx1 from E11.5 to E15.5 when the neural progenitors undergo the shifting from a self-expanding to a neuron-generating model of cell division (Fig. 1). By preventing the increase of the Otx1 expression, we found that Otx1 KD expanded the proliferative pool while shrinking the number of newborn neurons in the embryonic cortex. The proliferative progenitors increased during pregnancy cannot give rise to neurons but produces glial cells after birth. The results indicate that the altered balance of proliferation and differentiation of cortical progenitors may account for the impaired neuron generation and brain hypoplasia in Otx1 knock-out mice (12, 14).

Although Otx1 is primarily expressed in the deep-layer neurons of postnatal brains, Otx1 deletion reduces neurons in the upper layers as well (14). We show that Otx1 KD increases the self-renewal of Tbr2+ BPs (Fig. 7), suggesting that BP cells with

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Figure 6. Otx1 interference RNA enhances the mitotic index. A and C, the control scr-RNA or Otx1 shRNA (red) was electroporated into E13.5 (A) and E15.5 (C) brains. Two days later pH3 (green) was detected by immunofluorescence. Arrowheads indicate RFP + pH3 + cells. Scale bar = 20 μm. B and D, comparison of the pH3+ proportions between the control and shRNA-transfected cells in the E15.5 (B) and E17.5 (D) brains. SVZ, subventricular zone.
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Otx1 KD were likely kept in the transit-amplifying stage without proceeding to the mitotic exit. Therefore, by interfering with the generation, OTX1 regulates the number of neurons in the supragranular layer where the gene may not express. Although we did not perform IUE at the early time points to study the proliferation and mitotic exit of APs, an increased proliferation with a concurrent inhibition of mitotic exit may explain the reduction of neurons in the infragranular layers of the knock-out animal as well (14).

If Otx1 plays an essential role for the regulation of cortical neurogenesis, we wondered whether a deficit in the expression and function of Otx1 might cause neurological or psychiatric diseases. Patients with a 2p15-p16.1 microdeletion that includes OTX1 exhibited autism spectrum disorders with severe mental retardation. Furthermore, single nucleotide polymorphisms rs2018650 and rs13000344 in the OTX1 locus were associated with autism (26), suggesting that the expression may be important for proper development of the cerebral cortex. From COSMIC, an expert-curated database of somatic mutations, five point mutations of OTX1 (c.952G→C, c.955T→G, c.956A→G, c.958A→G, and c.959A→C) have been identified from astrocytoma. To our surprise, the mutations give rise to substitutions in three consecutive amino acids located in the C terminus of the OTX1 protein (Asp318, Tyr319, Lys320). Although the exact functional significance of these mutations to cancer development is as yet unknown, the consecutive pattern implies that they are unlikely random mutations. Our data demonstrated that in the mouse, the homolog mutant Y320C failed to inhibit the cell cycle re-entry as the WT Otx1 did. Thus, the human Y319C mutation may destroy the capability of OTX1 to regulate the progenitor cell cycle. Because suppression of Otx1 by the siRNA elevated the number of GFAP+ cells, a loss-of-function mutation, if occurring early in the embryonic development, may lead to an excessive production of astrocytes after birth. Our study may shed new light on the contribution of OTX1 mutations to the formation of astrocytoma.

In this study, the endogenous OTX1 was detected both in the cytoplasmic and nuclear fractions of embryonic brains (Fig. 1, A and B). Otx1 has a nuclear localization signal in the N terminus of the protein (11) but does not appear to have a nuclear exporting signal as predicted by LocNES (27). It is unclear what prevents the endogenous OTX1 from completely getting into the nucleus in the embryonic cortex. The embryonic cortex might express a binding partner that blocks the endogenous OTX1 from moving into the nucleus. Alternatively, the nuclear localization signal of OTX1 may be disabled by unknown modifications. For example, sequential phosphorylation events of transcription factor FoxO trigger its nuclear exclusion (28). We speculate that the endogenous OTX1 may have already satu-

Figure 7. Knocking down Otx1 increases Pax6+ and Tbr2+ cycling BPs. The control scr-RNA or Otx1 shRNA were electroporated into E13.5 mouse brains. A, the transfected cells (green) were examined 2 days later for the expression of Pax6 (red) and Tbr2 (purple) in the VZ. Arrowheads indicate triple positive cells (GFP+ Pax6+ Tbr2+). Scale bar = 20 μm. B and C, Otx1 KD increased the ratios of Pax6+ (B) and Pax6+ Tbr2+ cells (C) in the transfected population. D, expression of EdU (green) and Tbr2 (purple) in the transfected cells (red) in the E13.5 brains. Arrowheads indicate RFP+ EdU+ Tbr2+ cells. Scale bar = 20 μm. E, the proportion of proliferative Tbr2+ cells in the transfected populations. SVZ, subventricular zone.
rated the binding factor or the modifying enzyme in the cytoplasm, thus partly moved into the nucleus (Fig. 1A). As a result, the cytoplasmic factors are no longer available to stop any exogenous OTX1 from concentrating into the nucleus (Fig. 2C). The ectopically expressed OTX1 promoted the mitotic exit leading to cortico-neurogenesis, likely by its transcriptional regulation inside the nucleus. We do not know whether the cytoplasmic OTX1 has unconventional functions or merely provide a reservoir for the nuclear pool. It would be interesting to know if the nuclear entry of OTX1 consists of a critical step regulating the generation of cortical neurons.

In conclusion, by manipulating the gene expression in vivo, this study revealed an essential role of Otx1 in cell cycle regulation of cortical progenitors. We found that Otx1 is required for the mitotic exit leading to neuron generation. The results may improve our understanding of the regulation on neurogenesis and gliogenesis in the cerebral cortex.

Experimental procedures

Ethics statement

All procedures were approved by Wenzhou Medical University and performed in accordance with the guidelines and regulations established by Institutional Animal Care and Use Committee (IACUC).

Plasmids

For Otx1 expression, the full-length cDNA of Otx1 (NM_011023) was cloned into pCAGEN (Addgene). To create V5-Otx1-pCAGEN, a V5 tag (GKPIPNPLLGLDST) was inserted into Otx1-pCAGEN following the Otx1 start codon by PCR cloning. For inhibition of Otx1 expression, we used Otx1 shRNA with targeting sequence AGTCACTAACTGGCTATATC in the 3′-untranslated region. The scrambled control sequence is 5′-GTCAAAGCACTTCGTAGTCTA, which does not match with any sequences of mouse genes by blast search. Both the targeting and the scramble sequences were cloned into pSUPER. Mouse Otx1 was analyzed for potential CRISPR/Cas9 targets in silico by CRISPR gDNA design tool DNA2.0 (www.dna20.com/eCommerce/cas9/input). The target sequence with the highest predicted on-target score (100) against Otx1 (CACCAGCTCCCGTGCTGCTCCGC) plus the NGG was synthesized and subcloned into pX330 vector (Addgene plasmid number 42230) (29). The vector pX330 was taken as the control that expressed Cas9 nuclease without a Otx1 targeting
sequence. The point mutation Y320C was introduced into Otx1 by PCR-based site-directed mutagenesis using the following primers: 5'-CGGGCTCCTTGCAATCCAAGCAATCGGC-AGA-3' and 5'-TCTGCCGATTGCTTGGATTGCAAGGAG-CCCG-3'. All plasmids were verified by sequencing and purified using EndoFree plasmid maxi kit (Qiagen).

In utero electroporation

ICR mice were housed on a 12-h light/dark cycle with food and water ad libitum. Mating was monitored, and pregnancy was dated from the morning when a vaginal plug was detected (E0.5). In utero electroporation was performed as described previously (30, 31). Briefly, timed-pregnant female mice were anesthetized with an injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) diluted in sterile 0.9% saline. The uterine horns were carefully taken out following cutting the abdomen. Approximately 3 μg of plasmid DNA mixed with 1 μg of RFP-pCAGGS or GFP-pCAGGS and 0.025% of Fast Green was pressure injected into the lateral ventricles of embryos via pulled glass capillaries. Five pulses of current (40 mV for 50 ms for each) were delivered to the brains using an electroporator (BTX, T830). The uterus was relocated into the peritoneal cavity, and the abdomen wall and skin were sutured.

Figure 9. Overexpression of Otx1 enhances mitotic exit and neuron generation. Mouse brains receiving pCAGEN (control), pCAGEN-Otx1 (Otx1), and pCAGEN-Otx1-Y320C (Otx1-Y320C) at E12.5 were examined by E14.5. A, C, E, G, and I, confocal microphotographs showing the expression (green) of EdU (A and I), cyclin D1 (C), pH3 (E), or Tuj1 (G) in the electroporated cells (red). The colocalized cells are indicated by arrowheads. Scale bar = 20 μm. B, D, F, H, and J, percentages of EdU (B and J), cyclin D1 (D), pH3 (F), or Tuj1 (H) positive cells in the transfected populations.
For determining the efficacy of Otx1-shRNA, we injected the lateral ventricles of E13.5 embryos with 0.2 μg of V5-Otx1-pCAGEN, 0.8 μg of RFP-pCAGGS, together with 3 μg of Otx1-shRNA or scr-RNA. RFP + V5+ -expressing cells were examined by confocal microscopy at E15.5. For lineage tracing study, 1.4 μg of Otx1-shRNA or scr-RNA was electroporated into dorsal telencephalon in the presence of 1 μg of pT2K-CAGGS-EGFP and 1 μg of pCAGGS-T2TP.

**Cell culture**

Mouse neuroblastoma N2a cells were cultivated in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified atmosphere with 5% CO₂. Transfection of N2a cells was performed with Lipofectamine 2000 (Invitrogen). For preparing the primary culture of cortical neurons, E12.5 dorsal telencephalon was dissected and placed into ice-cold Hanks’ balanced salt solution containing 25 mM HEPES buffer. Tissues were transferred to a pre-warmed trypsin solution and dissociated by trituration with fire-polished glass pipettes. Cells were resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected using a NEPA21 Super Electroporator (NEPA GENE). The gene-transfected cells were plated at a density of 5 × 10⁵ cells/ml in dishes coated with polyornithine (Sigma, 0.001%) and laminin (Invitrogen, 5 mg/ml), and cultured in Neurobasal containing 2% B27 supplement, 1% penicillin/streptomycin.

**EdU labeling, immunofluorescence, and antibodies**

For in vivo labeling of S phase cells, timed-pregnant mice were injected with EdU (Ribobio) at 50 mg/kg of mouse body weight intraperitoneally 3 h before sacrifice. EdU-positive cells were detected by immunostaining analysis using Cell-Light™ EdU Apollo®488 In Vivo Imaging Kit (Ribobio).

The embryonic brains of mice were dehydrated in 30% sucrose after fixature at 4 °C with 4% paraformaldehyde. The samples were then embedded in OCT compound and cut into 16-μm thick sections with a Cryostat (HM505E, Microm, Germany). Immunostaining was performed with a standard procedure: brain sections were incubated with primary antibodies at 4 °C overnight and incubated with appropriate fluorescence-conjugated secondary antibodies at room temperature for 2 h. The following primary antibodies were used: goat anti-GFP (1:1000, Novus Biologicals, NB100–1770), rabbit anti-RFP (1:500, Abcam, ab62341), rabbit anti-V5 (1:1000, Sigma, V8137), rabbit anti-Tbr2 (1:300, Abcam, ab23345), rabbit anti-Tbr1 (1:200, Millipore, 1951083), rabbit anti-GFAP (1:400, Abcam, ab7260), mouse anti-Tuj1 (1:100, Promega, G7121), and rabbit anti-cyclin D1 (1:200, Abcam, ab16663). For mouse anti-pH3 (1:500, Abcam, ab14955) and mouse anti-Pax6 (1:1000, Developmental Studies Hybridoma Bank) staining, heat-mediated antigen retrieval was performed for 5 min at 95 °C in a Dako antigen retrieval reagent before incubation in the normal goat serum. Immunofluorescence images were obtained using a Zeiss LSM 710 confocal microscope.

**DNA sequencing**

pX330 or pX330-gOtx1 was transfected into N2a cells 48 h before the genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). Genomic DNA corresponding to the single guide RNA target site was PCR-amplified using Platinum® TaqDNA Polymerase High Fidelity (Invitrogen) with the following primers: 5'-CAGACACGCTAGCCAGATTTTCCAGA-3' (forward) and 5'-CCCTAAGTTCTAGACTGAGGCCCTTTAGTA-3' (reverse). The PCR amplicon was verified by sequencing.

**Nuclear and cytoplasmic protein extraction**

Nuclear and cytoplasmic proteins of cortical tissues were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Briefly, cortical tissues were dissected from E13.5 fetal brains and mixed with 200 μl of cytoplasmic protein extraction reagent (CPER) A and 10 μl of CPER B supplemented with 1 mM phenylmethylsulfonyl fluoride. The tissues were mechanically homogenized and incubated on ice for 15 min, followed by centrifugation at 1,500 × g for 5 min to obtain clear supernatants as the cytoplasmic 1. The pellets were resuspended in 200 μl of the CPER A by vortexing and incubated for 15 min on ice. The samples were mixed with 10 μl of cold CPER B and centrifuged at 16,000 × g for 5 min at 4 °C to collect the supernatants (cytoplasmic 2). The insoluble pellet was resuspended in 50 μl of nuclear protein extraction reagent supplemented with 1 mM phenylmethylsulfonyl fluoride and vortex repeatedly on ice. The nuclear extracts were obtained by centrifugation at 16,000 × g for 10 min. The nuclear and cytoplasmic fractions were then subjected to Western blotting for measuring the expression of OTX1, with GAPDH and Lamin-B1 as the cytoplasmic and nuclear markers, respectively.

**Western blotting**

The proteins extracted from the cortical cells were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, transferred to PVDF membranes, blocked in TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1% Tween 20) containing 5% (w/v) dry milk. The proteins were determined with rabbit anti-OTX1 (1:500, Santa Cruz, sc-292314), mouse anti-α-tubulin (1:5000, Sigma, t6199), rabbit anti-Lamin B1 (1:1000, Abcam, ab16048), or rabbit anti-GAPDH (1:2000, Cell Signaling Technology, 3683) antibodies. The specific bands in the blots were analyzed by a Western blot infrared imaging system (LI-COR Biosciences) after incubation with the corresponding secondary antibodies. The density of each band was measured by ImageJ.

**Cell counting and statistics**

The boundaries between different compartments in the cerebral cortex were identified based on histological features as observed by DAPI staining (32). Three sections were taken as replicate samples for each experiment. Brains from three independent experiments were processed for each experimental condition unless specified otherwise. All data are presented as mean ± S.D. Student’s t test was designed for the comparison of

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**OTX1 regulates mitotic exit of cortical progenitors**

**DNA sequencing**

pX330 or pX330-gOtx1 was transfected into N2a cells 48 h before the genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). Genomic DNA corresponding to the single guide RNA target site was PCR-amplified using Platinum® TaqDNA Polymerase High Fidelity (Invitrogen) with the following primers: 5’-CAGACACGCTAGCCAGATTTTCCAGA-3’ (forward) and 5’-CCCTAAGTTCTAGACTGAGGCCCTTTAGTA-3’ (reverse). The PCR amplicon was verified by sequencing.

**Nuclear and cytoplasmic protein extraction**

Nuclear and cytoplasmic proteins of cortical tissues were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Briefly, cortical tissues were dissected from E13.5 fetal brains and mixed with 200 μl of cytoplasmic protein extraction reagent (CPER) A and 10 μl of CPER B supplemented with 1 mM phenylmethylsulfonyl fluoride. The tissues were mechanically homogenized and incubated on ice for 15 min, followed by centrifugation at 1,500 × g for 5 min to obtain clear supernatants as the cytoplasmic 1. The pellets were resuspended in 200 μl of the CPER A by vortexing and incubated for 15 min on ice. The samples were mixed with 10 μl of cold CPER B and centrifuged at 16,000 × g for 5 min at 4 °C to collect the supernatants (cytoplasmic 2). The insoluble pellet was resuspended in 50 μl of nuclear protein extraction reagent supplemented with 1 mM phenylmethylsulfonyl fluoride and vortex repeatedly on ice. The nuclear extracts were obtained by centrifugation at 16,000 × g for 10 min. The nuclear and cytoplasmic fractions were then subjected to Western blotting for measuring the expression of OTX1, with GAPDH and Lamin-B1 as the cytoplasmic and nuclear markers, respectively.

**Western blotting**

The proteins extracted from the cortical cells were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, transferred to PVDF membranes, blocked in TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1% Tween 20) containing 5% (w/v) dry milk. The proteins were determined with rabbit anti-OTX1 (1:500, Santa Cruz, sc-292314), mouse anti-α-tubulin (1:5000, Sigma, t6199), rabbit anti-Lamin B1 (1:1000, Abcam, ab16048), or rabbit anti-GAPDH (1:2000, Cell Signaling Technology, 3683) antibodies. The specific bands in the blots were analyzed by a Western blot infrared imaging system (LI-COR Biosciences) after incubation with the corresponding secondary antibodies. The density of each band was measured by ImageJ.

**Cell counting and statistics**

The boundaries between different compartments in the cerebral cortex were identified based on histological features as observed by DAPI staining (32). Three sections were taken as replicate samples for each experiment. Brains from three independent experiments were processed for each experimental condition unless specified otherwise. All data are presented as mean ± S.D. Student’s t test was designed for the comparison of
two groups. Differences were considered statistically significant as: *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \).

**Author contributions**—J.-G. C. conceived the study. J.-G. C. and B. H. designed the experiments. B. H. and X. L. analyzed data. B. H., X. L., X. T., W. Z., D. Z., Y. F., and X. S. performed the experiments. B. H. and J.-G. C. wrote the manuscript.

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## References

1. Gotz, M., and Huttner, W. B. (2005) The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788

2. Haubensak, W., Attardo, A., Denk, W., and Huttner, W. B. (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7, 136–144

3. Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131, 3313–3345

4. Kwan, K. Y., Sestan, N., and Anton, E. S. (2012) Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex. *Development* 139, 1535–1546

5. Kriegstein, A., Noctor, S., and Martinez-Cerdeno, V. (2006) Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. Nature reviews. *Neuroscience* 7, 883–890

6. Kolwi, M., and Doe, C. Q. (2013) Temporal fate specification and neural progenitor competence during development. *Nat. Rev. Neurosci.* 14, 823–838

7. Yang, Y. J., Balts, A. E., Mathew, R. S., Murphy, E. A., Evrny, G. D., Gonzalez, D. M., Wang, E. P., Marshall-Walker, C. A., Barry, B. J., Murn, J., Tatarakis, A., Mahajan, M. A., Samuels, H. H. S., Shi, Y., Golden, J. A., Mahajahn, M., Shenvah, R., and Walsh, C. A. (2012) Microcephaly gene links trithorax and REST/NRSF to control neural stem cell proliferation and differentiation. *Cell* 151, 1097–1112

8. Beatty, J., and Laughlin, R. E. (2006) Genomic regulation of natural variation in cortical and noncortical brain volume. *BMC Neurosci.* 7, 16

9. Frantz, G. D., Weimann, J. M., Levin, M. E., and McConnell, S. K. (1994) Otx1 and Otx2 define layers and regions in developing cerebral cortex and cerebellum. *J. Neurosci.* 14, 5725–5740

10. Zhang, Y. A., Okada, A., Lew, C. H., and McConnell, S. K. (2002) Regulated expression of the early mammalian telencephalon: A major site of neurogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3196–3201

11. Gao, P., Postiglione, M. P., Krieger, T. G., Hernandez, L., Wang, C., Han, Z., Streicher, C., Papusheva, E., Insolera, R., Chugh, K., Kodish, O., Huang, K., Simons, B. D., Luo, L., Hippenmeyer, S., and Shi, S. H. (2014) Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell* 159, 775–788

12. Dehay, C., and Kennedy, H. (2007) Cell-cycle control and cortical development. *Nat. Rev. Neurosci.* 8, 438–450

13. Beatty, J., and Laughlin, R. E. (2006) Genomic regulation of natural variation in cortical and noncortical brain volume. *BMC Neurosci.* 7, 16

14. Gao, P., Postiglione, M. P., Krieger, T. G., Hernandez, L., Wang, C., Han, Z., Streicher, C., Papusheva, E., Insolera, R., Chugh, K., Kodish, O., Huang, K., Simons, B. D., Luo, L., Hippenmeyer, S., and Shi, S. H. (2014) Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell* 159, 775–788

15. Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., and Church, G. M. (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826

16. Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bullone, A., Kowalczyk, T., and Hevner, R. F. (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.* 25, 247–251

17. Dehay, C., and Kennedy, H. (2007) Cell-cycle control and cortical development. *Nat. Rev. Neurosci.* 8, 438–450

18. Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bullone, A., Kowalczyk, T., and Hevner, R. F. (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.* 25, 247–251

19. Arai, Y., Pulvers, J. N., Haffner, C., Schilling, B., Nüsslein, I., Calegari, F., and Huttner, W. B. (2011) Neural stem and progenitor cells shorten S-phase on commitment to neuron production. *Nat. Commun.* 2, 154

20. Kriegstein, A., and Alvarez-Buylla, A. (2009) The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* 32, 149–184

21. Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131, 3313–3345

22. Kwan, K. Y., Sestan, N., and Anton, E. S. (2012) Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex. *Development* 139, 1535–1546

23. Kriegstein, A., Noctor, S., and Martinez-Cerdeno, V. (2006) Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. Nature reviews. *Neuroscience* 7, 883–890

24. Kowli, M., and Doe, C. Q. (2013) Temporal fate specification and neural progenitor competence during development. *Nat. Rev. Neurosci.* 14, 823–838

25. Yang, Y. J., Balts, A. E., Mathew, R. S., Murphy, E. A., Evrny, G. D., Gonzalez, D. M., Wang, E. P., Marshall-Walker, C. A., Barry, B. J., Murn, J., Tatarakis, A., Mahajan, M. A., Samuels, H. H. S., Shi, Y., Golden, J. A., Mahajahn, M., Shenvah, R., and Walsh, C. A. (2012) Microcephaly gene links trithorax and REST/NRSF to control neural stem cell proliferation and differentiation. *Cell* 151, 1097–1112

26. Beatty, J., and Laughlin, R. E. (2006) Genomic regulation of natural variation in cortical and noncortical brain volume. *BMC Neurosci.* 7, 16

27. Xie, D., Marquis, K., Pei, J., Fu, S. C., Cagátay, T., Grishin, N. V., and Chook, Y. M. (2015) LocNES: a computational tool for locating classical NESs in XPO1 genes with autism spectrum disorders. *Eur. J. Hum. Genet.* 23, 1264–1270

28. Xu, D., Marquis, K., Pei, J., Fu, S. C., Cagátay, T., Grishin, N. Y., and Chook, Y. M. (2015) LocNES: a computational tool for locating classical NESs in XPO1 genes with autism spectrum disorders. *Eur. J. Hum. Genet.* 23, 1264–1270

29. Van der Heide, L. P., Hoekman, M. F., and Smidt, M. P. (2004) The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem. J.* 380, 297–309

30. Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308

31. Chen, J. G., Sin, R. M., Kwan, K. Y., and Sestan, N. (2005) Zfp312 is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17792–17797

32. Saijo, T., and Nakatsui, N. (2001) Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev. Biol.* 240, 237–246

33. Nguyen, L., Besson, A., Heng, J. I., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J. M., and Guillermot, F. (2006) p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. *Genes Dev.* 20, 1511–1524