Development/Plasticity/Repair

Cell Cycle Regulator E2F4 Is Essential for the Development of the Ventral Telencephalon

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Early forebrain development is characterized by extensive proliferation of neural precursors coupled with complex structural transformations; however, little is known regarding the mechanisms by which these processes are integrated. Here, we show that deficiency of the cell cycle regulatory protein, E2F4, results in the loss of ventral telencephalic structures and impaired self-renewal of neural precursor cells. The mechanism underlying aberrant ventral patterning lies in a dramatic loss of Sonic hedgehog (Shh) expression specifically in this region. The E2F4-deficient phenotype can be recapitulated by interbreeding mice heterozygous for E2F4 with those lacking one allele of Shh, suggesting a genetic interaction between these pathways. Treatment of E2F4-deficient cells with a Hh agonist rescues stem cell self-renewal and cells expressing the homeodomain proteins that specify the ventral telencephalic structures. Finally, we show that E2F4 deficiency results in impaired activity of Shh forebrain-specific enhancers. In conclusion, these studies establish a novel requirement for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

Key words: **E2F4; cell cycle; Sonic hedgehog; neural precursors; neural patterning; telencephalon**

Introduction

Forebrain development is characterized by extensive proliferation of neural precursors combined with complex structural transformations regulated by multiple morphogenic signaling pathways (for review, see Fuccillo et al., 2006). This morphogenetic signaling must be tightly coordinated with cell cycle control to ultimately shape the developing brain. Presently, little is known regarding the mechanisms by which the cell cycle machinery is integrated with these key developmental events.

Studies are emerging demonstrating the importance of cell cycle regulatory proteins in nervous system development. In particular, members of the retinoblastoma (Rb) family of cell cycle genes, and genes which in turn regulate Rb activity, have been shown to have important roles both in cell cycle-dependant and developmental processes that go beyond the mechanics of cell division (for review, see McClellan and Slack, 2006). For example, Rb has been shown to have a critical function in regulating terminal mitosis of neuroblasts in the CNS, PNS, and retina (Chen et al., 2002; Ferguson et al., 2002; MacPherson et al., 2003; Marino et al., 2003). Recently, we and others have shown that Rb exhibits non-cell cycle-dependent functions. For example, Rb has been shown to have a cell-autonomous function in ventral telencephalon development, because telencephalon-specific Rb-deficient mice exhibit a migration defect in ventrally derived interneurons (Ferguson et al., 2005). The closely related Rb family member, p107, has also been shown to be an important regulator of precursor self-renewal (Vanderluit et al., 2004). This regulation comes about through modulation of the activity of the fate-determining Notch signaling pathway. Clearly, cell cycle regulators and, in particular, members of the Rb family have functions beyond the regulation of the cell cycle machinery and play a pivotal role in shaping the developing brain.

Rb family proteins execute the function of cell cycle regulation by regulating the activity of E2F transcription factors (for review, see Trimarchi and Lees, 2002). In addition to regulating genes involved in cell cycle progression, E2Fs are ubiquitously expressed and control an array of genes involved in development, differentiation, and apoptosis (Muller et al., 2001). E2F4, a binding partner for Rb and p107, is believed to be a repressor of transcription (Trimarchi and Lees, 2002; Attwooll et al., 2004) and may be involved in the regulation of differentiation. E2F4-deficient mice exhibit growth retardation and developmental defects, including hematopoietic cell maturation and gut epithelial development (Humbert et al., 2000; Rempel et al., 2000). Although E2F4-deficient mice exhibit defects in craniofacial devel-

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opment, the role of E2F4 in nervous system development has never been examined. Because E2F4 is an interacting partner for Rb and p107, and given the key role of Rb proteins in telencephalic development, we questioned whether E2F4 itself may be required for the regulation of brain development.

Here, we show that deficiency of the cell cycle regulatory protein E2F4 results in the loss of ventral telencephalic structures and impaired self-renewal of neural precursor cells. The mechanism underlying aberrant ventral patterning lies in a dramatic loss in Sonic hedgehog (Shh) expression in this region. Treatment with Hh agonist reveals a rescue of stem cell self-renewal and cells expressing homeodomain proteins that specify the ventral telencephalic structures. Finally, we found that E2F4 deficiency resulted in reduced activity of Shh forebrain-specific enhancers demonstrating that E2F4 is required for Shh gene regulation. In conclusion, these studies establish a novel role for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

Materials and Methods

E2F4, Shh, and 447L17βlacZ mice. E2F4$^{+/-}$ mice were generously provided by Dr. Jacqueline Lees (Massachusetts Institute of Technology, Cambridge, MA) (Humbert et al., 2000). 447L17βlacZ transgenic reporter mice were described previously (Jeong et al., 2006). All were maintained on a C57BL/6 background (Charles River Laboratories, Wilmington, MA) except Gli3$^{Xt/+}$ and 447L17βlacZ mice, which were mixed C57BL/6×C3HeB/FeJ and BL6×SJL, respectively. To generate Shh/E2F4, Gli3/E2F4 and 447L17βlacZ/E2F4 embryos, heterozygous Shh$^{+/-}$C57BL/6 (Lewis et al., 2001), Gli3$^{Xt/+}$ (Hui and Joyner, 1993), and transgenic 447L17βlacZ mice were bred with E2F4$^{+/-}$ mice. Animals were genotyped as published previously for E2F4 (Humbert et al., 2000), Shh (Lewis et al., 2001), Gli3 (Hui and Joyner, 1993), and 447L17βlacZ (Jeong et al., 2006). The day of vaginal plug detection was counted as embryonic day 0.5 (E0.5). All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the guidelines of the Canadian Council on Animal Care.

Immunohistochemistry and phenotypic analysis. Pregnant mice were killed with a lethal injection of 65 mg/ml sodium pentobarbital solution (Somnotol) at indicated time points. To analyze neural progenitor proliferation in the embryonic brains, pregnant mouse dams received an intraperitonial injection of 50 mg/kg^{-1} bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO) in 0.9% NaCl 30 min before they were killed. Embryo heads were dissected and fixed in 4% paraformaldehyde (PFA) overnight in phosphate buffer, pH 7.4. Cryoprotection was performed by overnight incubation in 30% sucrose/1× Dulbecco's phosphate buffered saline (DPBS) (Invitrogen, San Diego, CA) solution. Embryos were embedded in a 1:1 mix of 30% sucrose in 1× DPBS/OCT (Sakura Finetek, Tokyo, Japan) and frozen on liquid nitrogen. Coronal sections (14 μm) were cut on a cryostat and mounted on Superfrost slides (Fisher Scientific, Houston, TX). Immunohistochemistry was performed using anti-phosphohistone H3 (1:1000; Upstate Biotechnology, Lake Placid, NY), anti-BrdU (1:50; BD Biosciences, Franklin Lakes, NJ), and anti-βIII-tubulin (mouse monoclonal hybridoma supernatant, 1:50) (Caccamo et al., 1989) primary antibodies. Phosphohistone-H3-positive (PH3+) cell nuclei were counted separately along the dorsal and ventral ventricular zones. BrdU-positive cells were counted in the dorsal and ventral [medial ganglionic eminence (MGE)] 5500 μm^2 areas. The lengths and the areas of the dorsal and ventral regions were measured using Northern Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada). Cells were counted in every 10th coronal section from the most rostral regions of the telencephalon to the preoptic area. PH3+ cells are expressed as a number of cells per 500 μm of the ventricular surface. Cresyl violet staining was performed as per standard protocols. Sections were analyzed using a Zeiss (Oberkochen, Germany) Axioskop 2 upright microscope, and images were captured using Sony Power HAD 3CCD color video camera or QiCam digital video camera (QImaging Corporation, Burnaby, British Columbia, Canada) and Northern Eclipse 6.0 software (Empix Imaging).

Western blotting. Total protein extracts were prepared from proliferating neurosphere cultures according to the protocols described previously (Ferguson et al., 2000). Immunoblotting was performed with antibodies directed against E2F4 (Chemicon, Temecula, CA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were developed using chemiluminescence reagent according to the manufacturer instructions (ECL; GE Healthcare, Arlington Heights, IL).

In situ *hybridization, whole mount β-galactosidase analysis, and reverse transcription-PCR.* Nonradioactive *in situ* hybridization (ISH) was performed on tissue sections and whole-mount embryos and neural tube explants as described previously (Bruhn and Cepko, 1996; Wallace and Raff, 1999). Antisense riboprobes for Shh (Echelard et al., 1993), Gli1 (Hui and Joyner, 1993), Nkx2.1 (Shimamura et al., 1995), Dlx2 (Porteus et al., 1991), and Pax6 were prepared from plasmids [generous gifts from A. P. McMahon (Harvard University, Cambridge, MA), A. L. Joyner (New York School of Medicine, New York, NY), J. L. Rubenstein (University of California at San Francisco, San Francisco, CA), and N. Pringle (University College London, London, UK)]. E2F4 DIG-labeled riboprobe was generated from pBS-IIKS-E2F4 template, containing 0.7 kb cDNA insert, which was amplified by PCR with primers E2F4–1 (ATCGAAGCTTGATTACATCTACAACC) and E2F4–2 (ATATAGGAATTCATTCGTTACTG). β-Galactosidase staining of transgenic 447L17βlacZ reporter embryos was performed as described previously (Zerucha et al., 2000). Embryos were cleared in 1:1 benzyl alcohol:benzyl benzoate, analyzed using Zeiss Stemi 2000-C stereo microscope (Oberkochen, Germany), and images were captured with Sony Power HAD 3CCD color video camera. Reverse transcription (RT)-PCR was performed as described previously (Cregan et al., 2004). The primers for Shh detection (forward 5′-GGAAAGAGGCGGCACCCCAAAAAG-3′, reverse 5′-CTCATCCCAGCCCTCGGTC ACTCG-3′) were designed using DNAStar PrimerSelect software (DNAStar, Madison, WI). cDNA synthesis was performed at 46°C for 45 min, followed by a 2 min initial denaturation step at 94°C. This was followed by 37 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. S12 was used as loading control.

In vitro *neurosphere assay.* Primary and secondary neurosphere cultures were prepared from the telencephalic neuroepithelia of E13.5 mouse embryos as described previously (Vanderluit et al., 2004). Briefly, neuroepithelium of the ganglionic eminences was dissected, mechanically single-celled, counted, and plated at equal cell density (10 cells/μl) for both E2F4−/− and control animals. Multipotentiality was examined by plating proliferating for 4 d neurospheres on poly-L-ornithine-coated dishes in differentiation medium containing 1% fetal bovine serum. After 7 d *in vitro*, cells were fixed with 4% PFA and processed for βIII-tubulin, glial fibrillary acidic protein (GFAP), and O4 immunocytochemistry using anti-βIII-tubulin (mouse monoclonal hybridoma supernatant; 1:50) (Caccamo et al., 1989), anti-GFAP (rabbit polyclonal anti-bovine glial fibrillary acidic protein; 1:400; Dako Cytomation, Carpinteria, CA), and anti-O4 (mouse monoclonal, 1:50; Chemicon) primary antibodies. To label proliferating cells in monolayer progenitor cultures, 10 μM BrdU (Sigma-Aldrich) was added to the culture medium 24 h before fixation. Cells were fixed on day 3 with 1:1 Methanol:Acetone (v/v) for 15 min, washed three times with 1× PBS, and incubated with primary anti-BrdU primary antibodies (1:50; BD Biosciences) overnight. Secondary antibodies goat anti-mouse CY3 (1:1000; Jackson ImmunoResearch, West Grove, PA) were applied for 1 h at room temperature. Cell nuclei were stained with Hoechst (1:250; Sigma). The proportion of neuronal (βIII-tubulin), glial (GFAP), and BrdU-positive cells were calculated as a percentage of the total number of cells (Hoechst-positive nuclei). A minimum of five random fields per genotype were analyzed.

Neurosphere self-renewal rescue experiment was performed using Hh agonist 1.4 (Frank-Kamenetsky et al., 2002) (a kind gift from Curis, Cambridge, MA), which was added to the neurosphere culture media at the time of plating at a final concentration of 20 nM. Data were analyzed for significance by Student's *t* test.

Neural tube explant cultures. Neural tube explant cultures were performed as described previously (Echevarria et al., 2001) with some modifications. E9.5 explants were cultured on 0.4 μm polycarbonate membrane Nunc 10 mm tissue culture inserts (Nalge Nunc International, Naperville, IL) in 24-well Nunclon plates (Nunc, Roskilde, Denmark)

containing explant culture medium (DMEM, 10% FBS, 2 mM glutamine, 100 u/ml penicillin-streptomycin) at 37°C in 5% CO_2, 95% humidity for 48 h. Hh agonist 1.4 was added to the explant culture medium at the time of plating at a final concentration of 20 nM. Explants were fixed in 4% PFA in 1× PBS, pH 7.4, for 2 h at 4°C and processed for whole-mount *in situ* hybridization. Images of the explants were taken with a Zeiss Axioskop 2 upright microscope.

Results

Aberrant development of ventral telencephalon in the absence of E2F4

E2F4 is important for normal embryonic development and is broadly expressed in most tissues (Dagnino et al., 1997; Callaghan et al., 1999). To ask whether E2F4 is differentially expressed in the developing brain, we performed *in situ* analysis on E11.5 embryos. Whereas E2F4 is ubiquitously expressed, the highest levels of E2F4 expression were detected in the ventricular and subventricular zones (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). To determine whether E2F4 was required for forebrain development, we examined E2F4-deficient mice. Recovery of E2F4-deficient embryos at E9.5–E11.5 was at the expected Mendelian ratio. E2F4-deficient embryos (89%; 40 of 45) were smaller and displayed a reduced size of the developing telencephalon (Fig. 1*A*,*C*). Histological characterization revealed that E2F4-deficient embryos exhibited a complete absence or significant reduction of the MGE and lateral ganglionic eminence (LGE) in the ventral telencephalon. In contrast, pallial regions appeared to be unaffected (Fig. 1*B*,*D*). These data suggest that E2F4 plays an essential role in the development of ventral telencephalic structures.

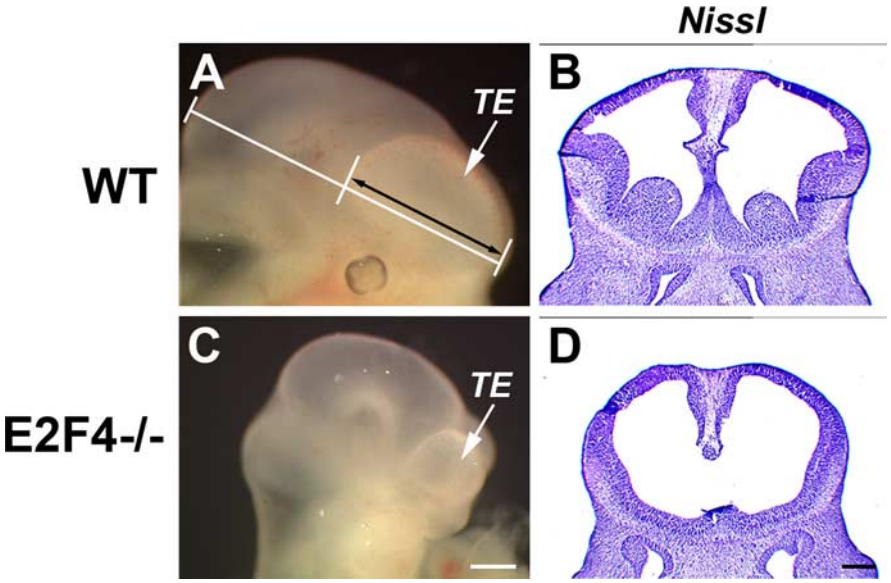

Figure 1. Reduced size of the developing ventral telencephalon in E2F4-deficient mouse embryos. ***A***, ***C***, Anterolateral view of the wild-type (WT) (***A***) and E2F4-deficient (E2F4−/−) (***C***) E11.5 mouse embryos. Note the size difference between WT ($n = 13$) and E2F4−/− ($n = 17$ of 20) developing telencephalic hemispheres (TE; black double-ended arrow) relative to the total anteroposterior length of the developing head (white line). Scale bar: ***A***, ***C***, 0.5 mm. ***B***, ***D***, Coronal sections through the brains of WT and E2F4−/− E11.5 mouse embryos stained with cresyl violet. Scale bar, 250 μm.

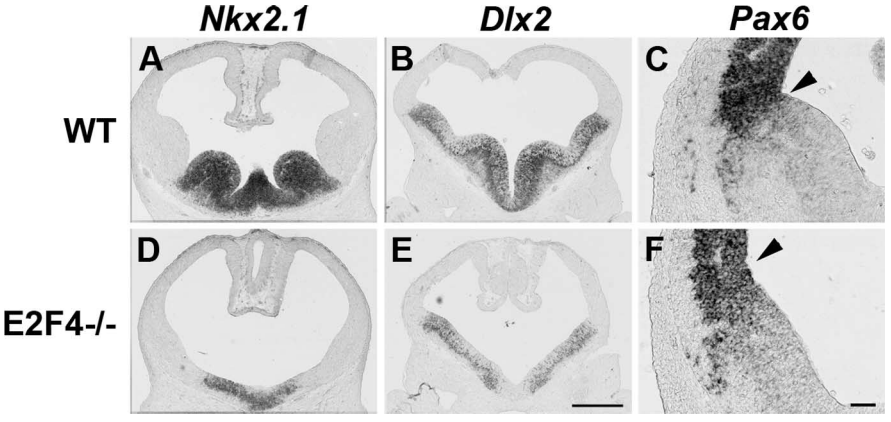

Figure 2. Aberrant early ventral telencephalic patterning in E2F4-deficient mouse embryos. ***A–F***, Region-specific mRNA expression of homeobox patterning genes Nkx2.1 (***A***, ***D***), Dlx2 (***B***, ***E***), and Pax6 (***C***, ***F***) in the ventral telencephalon of E11.5 mouse embryos analyzed by *in situ* hybridization. Note the ventral shift of Pax6 expression at the dorsoventral boundary (***F***, black arrowhead). At least five animals of each genotype were examined. Scale bars: ***A***, ***B***, ***D***, ***E***, 500 μm; ***C***, ***F***, 125 μm.

To further define the requirement of E2F4 in telencephalic development, we asked whether there was any perturbation in the expression of homeodomain-containing transcription factors Nkx2.1, Dlx2, and Pax6. Nkx2.1 expression, which is normally restricted to the ventral midline and MGE (Corbin et al., 2003), was markedly reduced in the ventral telencephalon of the E2F4-deficient mice (Fig. 2*A*,*D*). Consistent with a reduction in ventral structures, we found that Dlx2, which is expressed in ventricular and subventricular zones of the ventral telencephalon, was also decreased in the E2F4 null telencephalon (Fig. 2*B*,*E*). Pax6 expression is normally localized to the cortical proliferative zones of the developing brain (Walther and Gruss, 1991). In the absence of E2F4, the expression pattern of Pax6 was similar to the wild-type embryos in the rostral telencephalon, whereas in the caudal regions, the ventral boundary of Pax6 was extended into the ventral telencephalon (Fig. 2*F*) (data not shown), suggesting a moderate dorsalization of the caudal region of the developing telencephalon. The reduced expression of homeodomain proteins that specify ventral telencephalic structures (Nkx2.1, Dlx2) is consistent with the loss of the MGE and LGE. At midneurogenesis (E15.5), the ventral defect continues to persist, although some growth of ventral telencephalic structures has occurred. E2F4-deficient embryos exhibit enlarged lateral ventricles because of the impaired development of the MGE and LGE (supplemental Fig. 2*C*, available at www.jneurosci.org as supplemental material). Furthermore, we observed reduced expression of LIM (the three gene products Lin-11, Isl-1, and Mec-3) homeodomain transcription factor Lhx6, which labels postmitotic neurons in the ventral telencephalon, as well as MGE-derived migrating interneurons in the dorsal telencephalon (supplemental Fig. 2*D*, available at www.jneurosci.org as supplemental material) (Grigoriou et al., 1998; Lavdas et al., 1999). These results demonstrate that E2F4 deficiency results in a significant delay in the development of the ventral telencephalon.

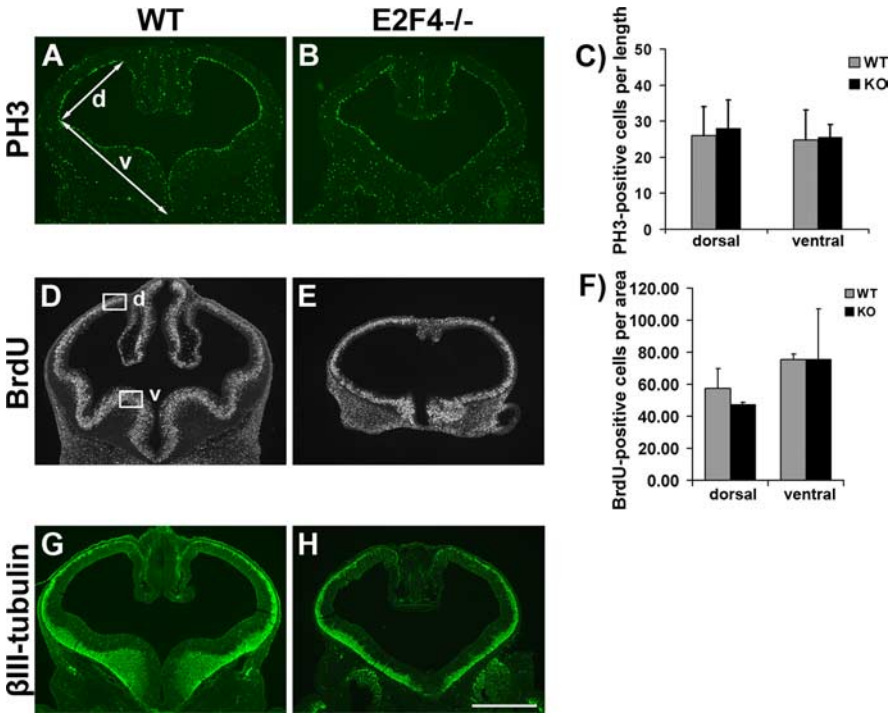

Figure 3. Proliferation and early neuronal differentiation appears normal in the developing telencephalon. ***A***, ***B***, ***D***, ***E***, Coronal sections (14 μm) through the telencephalon of E11.5 embryos were processed for PH3 and BrdU immunohistochemistry. ***C***, The mean count of PH3-positive cells per 500 μm of the dorsal (d) and ventral (v) ventricular surface of wild-type (WT) ($n = 5$) and E2F4−/− ($n = 3$) embryos. Error bars indicate SD. ***F***, Relative number of BrdU-positive cells per 5500 μm^2 area in the dorsal (d; white box) and ventral (v; white box) telencephalic regions of WT ($n = 3$) and E2F4−/− ($n = 3$) embryos after 30 min BrdU pulse. Error bars indicate SD. ***G***, ***H***, Early differentiating neurons are present in the mantle region of the embryonic telencephalon of E2F4-deficient mice (***E***) as shown by the βIII-tubulin immunoreactivity (green). Scale bar, 500 μm. KO, Knock-outs.

Progenitor proliferation and early neuronal differentiation are not affected by the loss of E2F4

Because the Rb/E2F pathway is primarily known for its role in cell cycle regulation and E2F4 deficiency results in a dramatic reduction in ventral telencephalic structures, we asked whether cell proliferation was impaired in the ventricular zones of E2F4 null mice. Cell proliferation was measured using an M phase marker, PH3, and by BrdU incorporation to count cells that have entered the S phase. Cells expressing PH3 were counted along the entire dorsal or ventral regions of the ventricular zones, whereas BrdU expressing cells were counted in the dorsal neuroepithelium and MGE (Fig. 3*D*, boxed areas). E2F4 mutant embryos did not exhibit any difference in the number of proliferating cells in the dorsal or ventral ventricular zone regions of the telencephalon (Fig. 3*A–F*). Similarly, the differentiation of early born neurons *in vivo* was not affected by the loss of E2F4 as determined by the expression of βIII-tubulin in the mantle region of the developing telencephalon (Fig. 3*H*). The number of βIII-tubulin-positive cells, however, is clearly reduced in the region of the medial ganglionic eminence, consistent with the impaired development of ventrally derived structures. These findings suggest that E2F4 deficiency does not affect the rate of cell division in neural progenitor cells or the timing of the onset of neuronal differentiation in the telencephalon.

Reduced number of neurosphere-forming cells in the telencephalon of E2F4-deficient mouse embryos

Because no defect was found in the rate of progenitor proliferation in E2F4-deficient embryos, we next asked whether the loss of ventral telencephalic structures results from a decrease in the number of neural stem cells. Because there is no unambiguous marker for neural stem cells *in vivo*, we used an *in vitro* neurosphere assay (Reynolds and Weiss, 1996) to quantify stem cells from the brains of wild-type and E2F4-deficient mice. When plated at equal densities, neuroepithelial cells from the telencephalon of E2F4-deficient embryos at E13.5 gave rise to 77% fewer primary neurospheres compared with wild type, indicative of a reduction in the number of sphere-forming cells in the mutant brains (Fig. 4*A*). To confirm that neurospheres exhibited the properties of stem cells, we assessed their self-renewal capacity and multipotentiality. The self-renewal capacity of primary neurospheres from E2F4 null embryos was markedly reduced relative to controls as demonstrated by the number of secondary spheres generated (Fig. 4*B*). Neurospheres from E2F4-deficient embryos were still multipotent, as demonstrated by their ability to differentiate into βIII-tubulin-positive (early neuronal), GFAP-positive, and O4-positive (glial) cells (Fig. 4*C–F*). To ask whether E2F4 affects cell cycle regulation in neural precursors, we measured BrdU incorporation in monolayer cultures of neural progenitors *in vitro*. No differences in the proliferation index were detected in cultures derived from wild-type and E2F4-deficient mouse embryos, indicating that E2F4 deficiency does not affect the rate of cell proliferation (Fig. 4*G*). Consistent with *in vivo* findings, loss of E2F4 does not affect the rate of progenitor proliferation, but instead our results show a reduction in the size and self-renewal capacity of the neural precursor pool in the telencephalon.

Sonic hedgehog signaling is impaired in the forebrains of E2F4-deficient mice

Because E2F4 deficiency specifically affects ventral telencephalic patterning and stem cell self-renewal, we examined the regulation of genes that direct the development of this region. Although several candidates were examined, we focused our studies on the components of the Sonic hedgehog pathway. Shh was assessed because of the following: (1) previous studies revealed a requirement for Shh for specification of the ventral telencephalon (Chiang et al., 1996; Rallu et al., 2002b); (2) Shh promotes stem cell self-renewal (Machold et al., 2003; Palma et al., 2005), and (3) the E2F4-deficient phenotype was strikingly similar to the phenotype of the Shh pathway mutants (Fuccillo et al., 2004). To ask whether there was a deregulation in Shh signaling, we examined Shh regulation in the telencephalon of E2F4-deficient and wild-type littermates. We first asked whether loss of E2F4 disrupted Shh expression at earlier developmental time points. At E7–E8.5, the time of appearance of Shh in the forebrain anlage (Shimamura and Rubenstein, 1997; Rallu et al., 2002b), there was no difference in the levels of Shh mRNA in the rostral neural plate (Fig. 5*A*,*E*) (data not shown). By E10.5, however, there was a decrease in Shh mRNA rostral to the zona limitans interthalamica (ZLI) and an absence of the signal in the ventral telenceph-

alon (Fig. 5*F*). In other areas of the developing embryos such as the ventral spinal cord and notochord, there was no detectable difference in the expression of Shh at E11.5 (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Thus, findings from whole-mount *in situ* hybridization demonstrate that Shh disruption occurs after E8 and specifically in the ventral telencephalon and rostral diencephalon of E2F4 null embryos. The timing of Shh disruption occurs after the division of the eyefields and telencephalic hemispheres consistent with the absence of a cyclopia and holoprosencephaly (characteristic features of Shh null phenotype) (Chiang et al., 1996) in E2F4-deficient embryos. We therefore examined Shh regulation specifically at the time when ventral telencephalic structures are developing.

At E11.5, Shh is normally expressed in the ventral telencephalic midline, as well as in the base of MGE (Fig. 5*C*). In E2F4-deficient embryos, Shh was absent from the ventral midline and greatly reduced in the mantle region of the MGE (Fig. 5*G*). To address whether the reduced expression of Shh in E2F4 mutants correlated with reduced Shh pathway activity, we examined the expression of Gli1, a primary target gene of the pathway (Lee et al., 1997). In wild-type embryos, Gli1 was strongly expressed in the ventricular and subventricular zones of ventral telencephalon between the MGE and LGE (Fig. 5*D*). In contrast, Gli1 expression was greatly diminished in E2F4-deficient littermates, consistent with decreased Shh activity in this region of the brain (Fig. 5*H*).

To determine whether the disruption of Shh signaling results from a cell autonomous defect in E2F4-deficient neural precursor cells, we asked whether Shh was also reduced in neurospheres cultured from E2F4-deficient brains. RT-PCR revealed a reduction in the levels of Shh mRNA in E2F4-deficient neurospheres (Fig. 5*I*). Decreased Shh expression in neurosphere cultures is consistent with an impairment of self-renewal capacity found in E2F4-deficient precursors. Together, these data indicate that E2F4 deficiency leads to a cell autonomous impairment of Shh signaling in the developing ventral telencephalon.

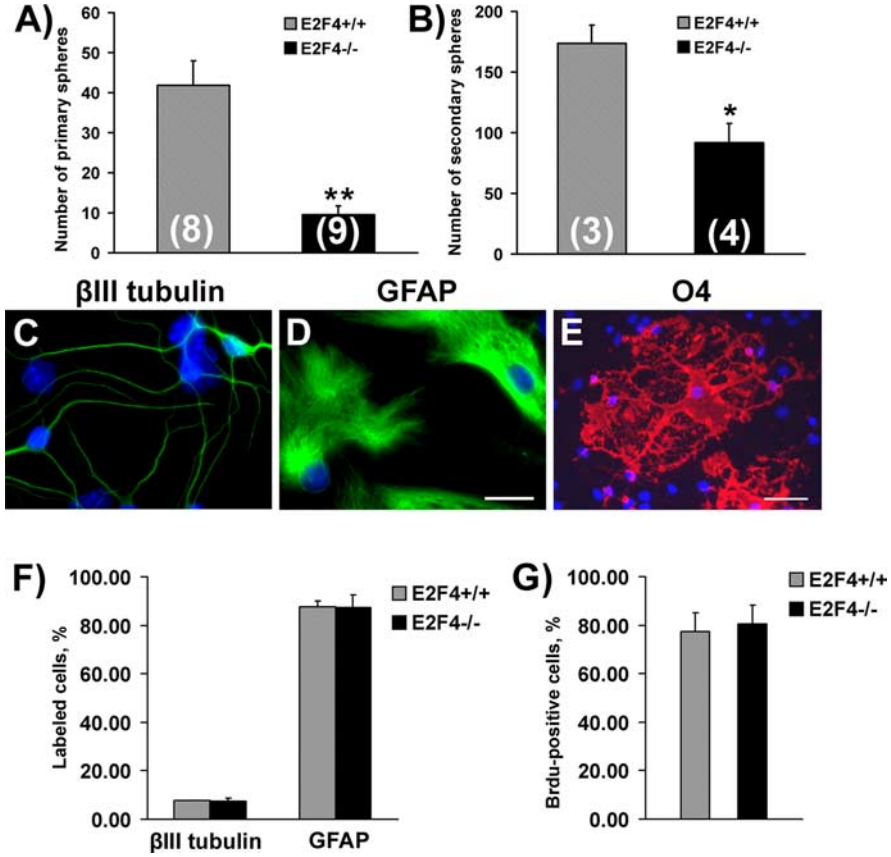

Figure 4. Reduced number and self-renewal capacity of neural precursors in E2F4-deficient mouse embryos. Cultures from E2F4−/− embryos generated significantly lower numbers of primary (***A***) and secondary (***B***) neurospheres versus their wild-type (WT) control littermates. ***A***, Dissociated telencephalic neuroepithelial cells from E13.5 mouse embryos were cultured in 500 μl of serum-free medium containing 25 ng/ml basic fibroblast growth factor (bFGF) at a density of 10 cells/μl. ***B***, Single primary neurospheres were dissociated and cultured in serum-free medium with bFGF. Primary and secondary spheres were counted after 7 d *in vitro*. The numbers on the bar graph indicate the number of embryos per genotype analyzed. Error bars indicate SEM (*$p < 0.02$; **$p < 0.0001$; *t* test, two-tail). ***C–E***, Neurons, astrocytes, and oligodendrocytes in differentiating neurospheres derived from telencephalic neuroepithelia of E13.5 E2F4−/− mouse embryos. After 7 d *in vitro* in differentiating medium with FBS, cells were fixed with 4% PFA in 1× PBS and processed for βIII-tubulin (***C***; neurons, green), GFAP (***D***; astrocytes, green), and O4 (***E***; oligodendrocytes, red) immunocytochemistry. Cell nuclei were stained with Hoechst (blue). Scale bars: ***C***, ***D***, 25 μm; ***E***, 50 μm. ***F***, Percentage of βIII-tubulin- and GFAP-positive cells out of the total number of cells (Hoechst-labeled nuclei) in differentiated neurospheres. Neurospheres from two embryos per genotype were analyzed. Data are presented as means ± range. ***G***, Proliferation analysis in neural progenitor cultures from wild-type and E2F4-deficient embryos. Cells from wild-type ($n = 4$) and E2F4−/− ($n = 5$) mouse embryos were fixed on day 3 after induction of differentiation. BrdU (10 μM) was added to the culture medium 24 h before the end of the culture period. The proportion of proliferating cells is estimated as a percentage of the total number of cells (Hoechst-positive nuclei). Data are presented as means ± SEM.

Genetic interaction between Shh and E2F4 in ventral telencephalic patterning

Because Shh is disrupted in E2F4-deficient brains, we asked whether there was a genetic interaction between E2F4 and the Shh pathway. To address this question, compound Shh and E2F4 heterozygous mice were generated. Compound heterozygous mice for E2F4 and Shh exhibited a reduction in Shh expression in the ventral telencephalon. Because mice heterozygous for either E2F4 or Shh are similar to wild type, we asked whether compound heterozygous mice exhibit an exacerbated phenotype, thus revealing a genetic interaction. In contrast to the single E2F4 (Fig. 6*A–C*) or Shh (data not shown) heterozygous mice, double heterozygous E2F4/Shh mice (Fig. 6*G–I*) displayed a telencephalic phenotype similar to that found in E2F4 deficiency (Fig. 6*D–F*). Shh/E2F4 double-deficient embryos could not be recovered, suggesting that the combined null mutation is lethal before E11 and that the loss of E2F4 exacerbates Shh deficiency. These studies demonstrate a novel genetic interaction between E2F4 and Shh.

Another member of the Shh signaling pathway with a known role in dorsoventral telencephalic patterning is the Gli3 transcription factor, which functions as an antagonist of Shh signaling (Rallu et al., 2002a,b). Gli3 functions as a transcriptional repressor in the absence of Shh activity, and its repressive effects are relieved after activation of the Shh pathway (Cayuso and Marti, 2005). Spontaneous mutant extra-toes (Xt) heterozygous Gli3 mice ($Gli3^{Xt/+}$) display normal telencephalic structures,

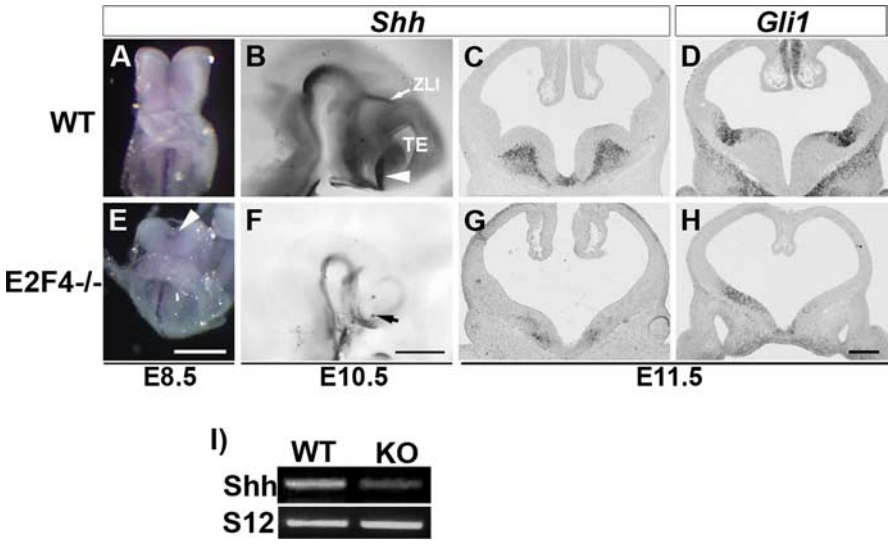

Figure 5. Deregulation of Shh and Gli1 expression in the ventral telencephalon and in neurosphere cultures from E2F4-deficient mice. ***A***, ***B***, ***E***, ***F***, Whole-mount *in situ* hybridization for *Shh* mRNA of E8.5 (***A***, ***E***) and E10.5 (***B***, ***F***) wild-type (WT) (***A***, ***B***) and E2F4−/− (***E***, ***F***) mouse embryos. Note that Shh mRNA expression is present in the rostral neural plate ventral midline of E2F4-deficient embryo at E8.5 (***E***, white arrowhead) similar to the control littermate. At E10.5, Shh is detected in the ventral telencephalon of WT embryos (***B***, white arrowhead), whereas it is absent in E2F4-deficient mice (***F***, black arrow). TE, Telencephalon. ZLI is indicated by a white arrow. Scale bars, 500 μm. ***C***, ***D***, ***G***, ***H***, Coronal sections through the telencephalon of WT (***C***, ***D***) and E2F4−/− (***G***, ***H***) E11.5 embryos were analyzed for Shh (***C***, ***G***) and Gli1 (***D***, ***H***) mRNA expression (*in situ* hybridization). Scale bar, 250 μm. ***I***, RT-PCR analysis of mRNA extracted from WT and E2F4−/− secondary neurospheres. Shh mRNA level is reduced in neurospheres from E2F4-deficient mouse embryos ($n = 3$). S12 was used as loading control.

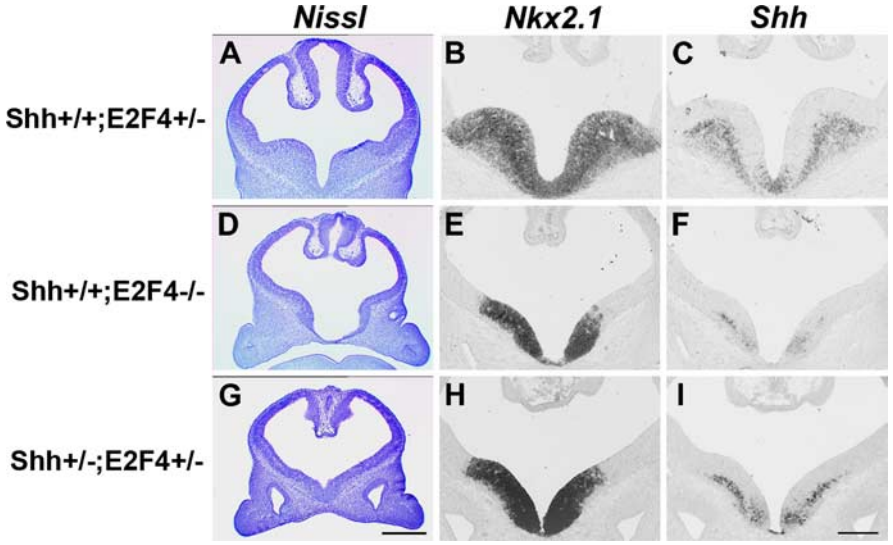

Figure 6. Altered telencephalic patterning in combined double heterozygous (Shh+/−;E2F4+/−) mouse embryos. ***A***, ***D***, ***G***, Cresyl violet staining of coronal sections through the telencephalon of E2F4 heterozygous (Shh+/+;E2F4+/−), E2F4-deficient (Shh+/+;E2F4−/−), and Shh;E2F4 double heterozygous (Shh+/−;E2F4+/−) E11.5 embryonic brains. A minimum of four embryos per genotype were examined. Scale bar, 500 μm. ***B***, ***C***, ***E***, ***F***, ***H***, ***I***, *In situ* hybridization analysis of Nkx2.1 and Shh mRNA expression in the MGE and ventral telencephalic midline of E11.5 embryos. Scale bar, 200 μm.

whereas Gli3$^{Xt/Xt}$ homozygous embryos have severe craniofacial abnormalities often combined with the loss of the dorsal midline, exencephaly, and perinatal lethality (Buscher et al., 1997). Because removal of Gli3 rescues the Shh-deficient phenotype in the forebrain (Rallu et al., 2002b), we asked whether the phenotype we observed in the telencephalon of E2F4-deficient mice could also be rescued by disruption of Gli3 activity. Analysis of E11.5 mouse embryos demonstrates that heterozygosity for Gli3 restores the size of ventral telencephalic structures and the expression of Nkx2.1 and Shh in E2F4-deficient embryos (Fig. 7*G–I*). These results highlight the importance of E2F4 in normal dorsoventral patterning during early telencephalic development.

Shh agonist rescues defective ventral telencephalic patterning and self-renewal capacity of neural precursors in E2F4-deficient embryos

Because Shh signaling activity is decreased in the absence of E2F4, we next asked whether replacing Shh could rescue the development of ventral structures. To address this question, we treated E9.5 embryonic neural tube explants (Echevarria et al., 2001) from E2F4-deficient mice with a Smo receptor agonist (Frank-Kamenetsky et al., 2002) and evaluated whether Nkx2.1, which is known to be induced by Shh (Gaiano et al., 1999; Wilson and Rubenstein, 2000), could be restored. Activation of Shh signaling by the exogenous agonist rescued the expression of Nkx2.1 in explants from E2F4-deficient mice (Fig. 8*E*). These findings indicate that Shh signaling downstream of the Smo receptor is preserved in the absence of E2F4, and activation of the Shh pathway is sufficient to partially restore cells that express homeodomain genes that specify ventral telencephalic structures in E2F4-deficient mice. We also asked whether replacing Shh could restore stem cell self-renewal in E2F4-deficient neural precursors. Addition of the Hh pathway agonist rescues the self-renewal capacity of E2F4-deficient neural precursor cells (Fig. 8*F*). Although the Hh agonist has no significant effect on the self-renewal capacity of wild-type neurospheres, Shh dependency was demonstrated by the inhibition of neurosphere formation after the addition of the Hh pathway antagonist cyclopamine (data not shown). In conclusion, these results establish that the Shh pathway is functional in E2F4-deficient embryos and that replacement of Hh agonists can restore precursor self-renewal and cells expressing ventrally derived markers in tissue explants. Because replacing Hh agonist partially rescues defects in E2F4-deficient tissue, we next asked whether E2F4 may be required for the regulation of Shh gene expression in the ventral telencephalon.

E2F4 deficiency leads to aberrant activation of Shh brain enhancers

Given the widespread E2F4 expression (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Dagnino et al., 1997; Callaghan et al., 1999), we questioned how Shh regulation could be disrupted specifically in a spatial and temporal manner in E2F4-deficient embryos. One of the mechanisms to achieve differential regulation of *Shh* expression along the rostrocaudal axis of the neural tube is through the regulation

of region-specific Shh enhancers (Epstein et al., 1999; Jeong et al., 2006). Because the expression of *Shh* in the rostral diencephalon and ventral telencephalon is controlled by *Shh* brain enhancer 2 (SBE2), SBE3, and SBE4 (Jeong et al., 2006), we asked whether the activity of these enhancers is deregulated in the absence of E2F4. To answer this question, we interbred E2F4 mutant mice with transgenic mice carrying a 447L17βlacZ reporter construct, which contains the SBE2, SBE3, and SBE4 regulatory sequences. The activity of these enhancers specifically targets reporter gene expression to the sites of *Shh* transcription in the developing forebrain (Jeong et al., 2006). First, consistent with the unaffected regulation of Shh in other areas, we observed similar reporter activity in the notochord of wild-type and E2F4-deficient transgenic embryos (Fig. 9*C*,*F*, arrowheads). At E9.5, strongly expressing *lacZ* regions were present in the wild-type ventral forebrain from the middle of diencephalon and converging at the midline rostrally at the level of the optic vesicle (Fig. 9*A*,*B*). In the absence of E2F4, the reporter activity is barely detectable in the region of the optic vesicle (Fig. 9*D*,*E*). By E10.5, the intensity of X-gal staining in the ventral forebrain of E2F4-deficient embryos is increased but still failed to extend into the ventral telencephalon (Fig. 9*C*,*F*). These results demonstrate that there is a prominent developmental delay in the activation of the Shh enhancer activity in the absence of E2F4. Thus, the impairment in the activation of the brain-specific enhancers can account for the region-specific disruption of Shh activity seen in E2F4-deficient embryos. In conclusion, the results of our studies examining Shh gene expression combined with Shh enhancer activity in E2F4 null embryos demonstrate that E2F4 is required for the development of the ventral telencephalon by affecting the region-specific regulation of Shh.

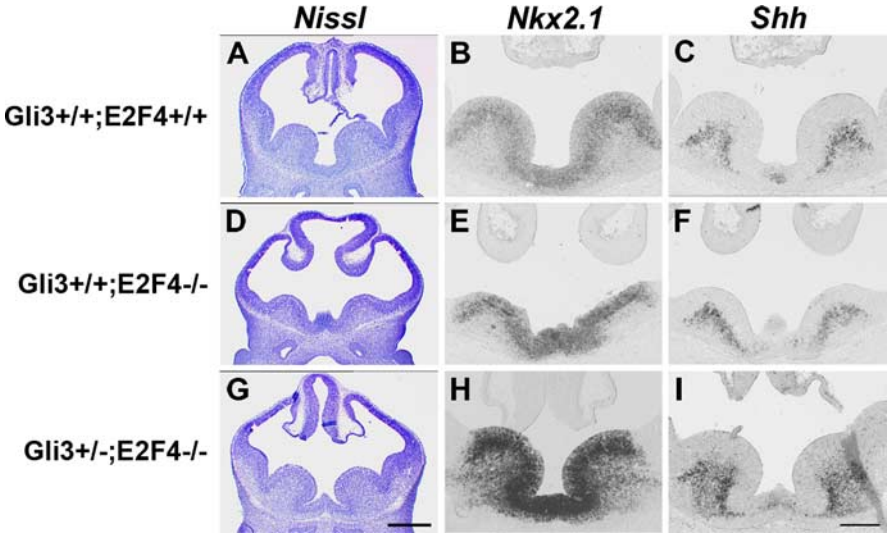

Figure 7. Partial removal of Gli3 restores ventral telencephalic development in Gli3+/−;E2F4−/− compound mutant mice. Cresyl violet staining (***A***, ***D***, ***G***) and *in situ* hybridization for Nkx2.1 (***B***, ***E***, ***H***) and Shh (***C***, ***F***, ***I***) mRNA of WT (Gli3+/+;E2F4+/+), E2F4-deficient (Gli3+/+;E2F4−/−), and combined Gli3 heterozygous;E2F4-deficient (Gli3+/−;E2F4−/−) ($n = 4$ of 5) of E11.5 embryonic brain coronal sections. Scale bars: ***A***, ***D***, ***G***, 500 μm; ***B***, ***C***, ***E***, ***F***, ***H***, ***I***, 200 μm.

Discussion

The results of these studies show for the first time that the cell cycle regulatory protein E2F4 is required for normal development of the ventral telencephalon. Animals lacking E2F4 exhibit a dramatic loss of ventral telencephalic structures characterized by the absence or reduction of the lateral and medial ganglionic eminences. Homeodomain proteins, including Nkx2.1 and Dlx2, which specify these regions, are absent or expressed at very low levels. Whereas a cell cycle defect was not detected in existing progenitor cells, there was an impairment in the self-renewal capacity of neural stem cells. Examination of the mechanism underlying the telencephalic defect found in E2F4 deficiency revealed a striking reduction in Shh, a morphogen essential for the development of ventral structures. These studies define a novel requirement for E2F4 in regulating the specification of the ventral telencephalon.

E2F4 is a regulatory target for Rb family members and has been found to interact with all three proteins, namely p107, p130, and Rb (Trimarchi and Lees, 2002). Whereas the Rb family of cell cycle proteins are well known for their role in the regulation of proliferation, new evidence is emerging that reveals novel functions for these proteins beyond cell cycle control (McClellan and Slack, 2006). For example, p107 has been shown to regulate the neural precursor pool, specifically by controlling the self-renewal capacity of neural stem cells (Vanderluit et al., 2004). The mechanism by which p107 mediates this effect was through the repression of the Notch signaling pathway, because p107-deficient brains exhibit enhanced levels of the Notch1 intercellular domain and elevated Hes1. Loss of only one Hes1 allele restores the neural precursor pool back to wild-type levels. As with p107, studies examining the role of Rb have also identified cell cycle-independent functions. It is well established that Rb is required for the regulation of terminal mitosis in multiple cell types (Callaghan et al., 1999; Lipinski and Jacks, 1999); however, more recently, Rb has been shown to regulate neuronal migration (Ferguson et al., 2005). Conditional mutants, in which Rb has been deleted in the telencephalon, have revealed defects associated with migration that could not be reconciled with ectopic proliferation. In the Rb-deficient ventral telencephalon, several neuronal populations arising from this region fail to migrate to their final destinations within the cortical plate. A search for E2F targets, which may be deregulated in Rb null mice, has revealed genes known to play important roles in controlling neuronal migration (McClellan et al., 2007). Given that Rb deficiency exhibits a striking defect in ventral telencephalic development, we asked whether the loss of Rb-interacting partners, such as the E2F transcription factors, affected this same region. We therefore examined development of the brain in E2F4 null mice.

E2F4 in early brain development

Whereas previous studies have revealed that E2F4 is important during embryogenesis, the mechanisms by which it impacts development remain poorly understood. E2F4 is required for proliferation and maturation of erythroid lineage and for the normal development of the intestinal epithelium and craniofacial structures (Humbert et al., 2000; Rempel et al., 2000; Deschenes et al., 2004; Kinross et al., 2006). Closer examination of the intestinal epithelium of E2F4-deficient mice suggests that E2F4 has a role in the regulation of differentiation (Deschenes et al., 2004). Here, we report a novel previously unidentified requirement for E2F4 in early patterning of the ventral telencephalon. Specifically, E2F4-deficient mouse embryos display a severe phenotype char-

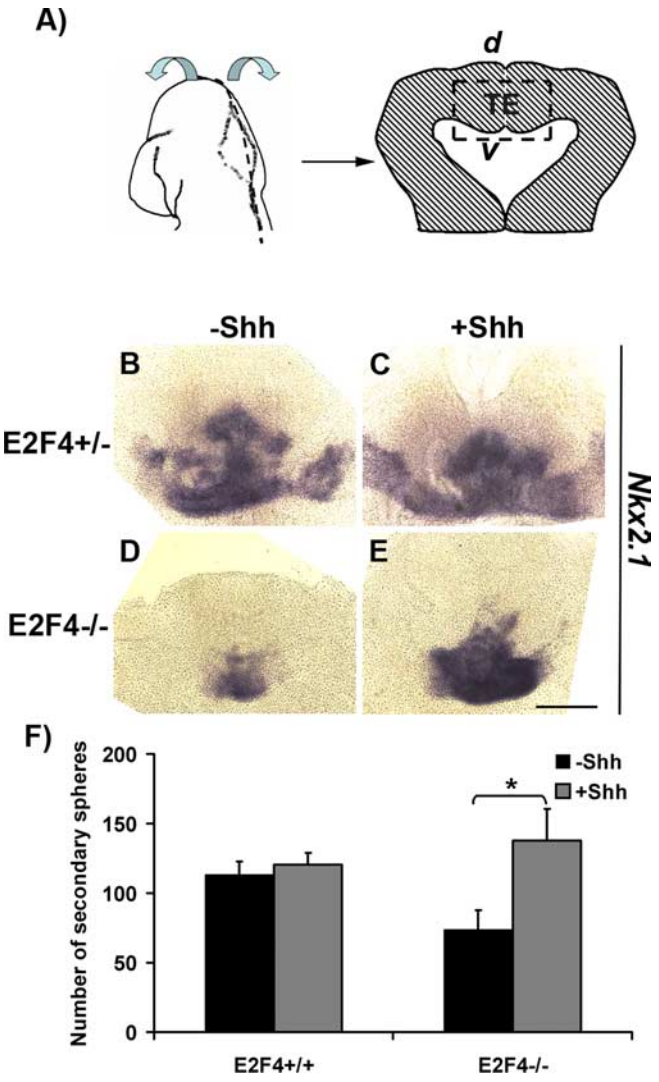

Figure 8. Shh agonist partially restores neurosphere self-renewal capacity and cells expressing Nkx2.1 in the E2F4−/− ventral telencephalon. ***A***, Diagram of the explant culture experiment. The posterolateral view of the head of the developing E9.5 embryo is shown (left). The dashed black line indicates the cut along the dorsal midline. The blue arrows show the directions the neural tube is opened. A diagram of the opened neural tube explant is shown (right); the filled area represents luminal (ventricular) surface of neural tube. The dashed black box indicates the area represented in ***B–E***. d, Dorsal; v, ventral; TE, telencephalon. ***B–E***, *In situ* hybridization for Nkx2.1 mRNA of E9.5 explants cultured with or without 20 nM Shh agonist for 48 h ($n = 3$). Scale bar, 250 μm. ***F***, Self-renewal capacity of E2F4−/− neurospheres is restored with the addition of Shh agonist ($n = 3$). Error bars indicate SEM (*$p < 0.05$; *t* test, two-tail).

acterized by the loss of the ventral telencephalic structures. Analysis of region-specific patterning markers revealed a dramatic reduction in their expression in the ganglionic eminences and telencephalic ventral midline of E2F4-deficient embryos. Whereas Rb is known to regulate progenitor proliferation and migration (Ferguson et al., 2002, 2005), and the mechanism by which this occurs is through the E2F pathway (McClellan et al., 2007), our present findings are the first to reveal a requirement for E2F4 in early patterning of the ventral forebrain.

E2F4 mutant embryos exhibit aberrant Shh signaling

The results of the secondary neurosphere assay indicate that in the absence of E2F4, the self-renewal capacity of neural stem cells is significantly reduced. Consistent with the known role of the Shh pathway in regulation of embryonic and adult stem cell compartments (Lai et al., 2003; Palma et al., 2005), we observed reduced levels of Shh transcripts in neural precursor cells of E2F4-deficient mice. The dependence of the self-renewal capacity of E2F4-deficient neural stem cells on Shh is further supported by the ability of the exogenous Shh agonist to rescue the number of secondary spheres in E2F4-deficient cultures. These data demonstrate that E2F4 is required to regulate the self-renewal capacity of neural precursor cells during early telencephalic development by modulating Shh signaling.

Our *in vivo* studies reveal that defective telencephalic development in E2F4-deficient embryos results from a temporal and region-specific reduction of Shh signaling. The importance of Shh in patterning of the ventral telencephalon is demonstrated by holoprosencephaly and cyclopia, a phenotype exhibited by whole embryo Shh knock-outs (Chiang et al., 1996; Wallis and Muenke, 1999). Unlike animals lacking Shh, E2F4-deficient mice do not develop cyclopia and holoprosencephaly, suggesting that patterning defects occur after the division of the eye field and separation of telencephalic hemispheres. The ventral phenotype exhibited by E2F4 deficiency is strikingly similar to that found in conditional mutants of Smo, the obligate transducer of Shh signaling. Complete deletion of Smo in the mouse telencephalon at E9 results in the loss of ganglionic eminences and a reduction of Nkx2.1 and Mash1 expression, with the ventral expansion of dorsal markers Pax6, Ngn2, and Emx2 (Fuccillo et al., 2004). E2F4 deficiency results in impaired Shh expression in the ventral telencephalon after E8.5 when the division of the telencephalic hemispheres is complete.

Because E2F4 is ubiquitously expressed, one might ask why Shh expression is impaired only in the ventral forebrain in E2F4-deficient mice. A number of reasons could account for such a temporal and region-specific activation of Shh. One explanation is that the disruption of Shh signaling is not complete in the absence of E2F4. Thus, the reduced activity of Shh may be marginally sufficient for the development of the dorsal pallial structures but not for the induction of the ventral-most patterning genes. Partial restoration of cells expressing Nkx2.1 in response to exogenous Shh agonist supports this interpretation. Alternatively, and most likely, is the more recent finding demonstrating that the temporal and region-specific expression of Shh throughout the embryo is regulated by region-specific regulatory elements (Epstein et al., 1999; Jeong et al., 2006). Because each enhancer is unique, these results would suggest that E2F4 is required for normal activation of the brain-specific enhancers that direct Shh expression in the ventral telencephalon (Jeong et al., 2006). Our results with LacZ reporter mice demonstrating impaired activation of Shh brain enhancers are consistent with this interpretation.

In conclusion, these results demonstrate that the cell cycle regulatory protein E2F4 is essential for ventral telencephalic patterning. The deficit in ventral telencephalic structures is accompanied by a defect in neural stem cell self-renewal. The mechanism underlying aberrant ventral patterning lies in a dramatic loss in Shh expression in this region. Treatment with Hh agonist reveals a rescue of stem cell self-renewal and cells expressing homeodomain proteins that specify the ventral telencephalic structures. Finally, we found that E2F4 deficiency resulted in reduced activity of Shh forebrain-specific enhancers. In conclusion, these studies establish a novel genetic interaction between E2F4 and the Shh pathway and identify a novel function for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

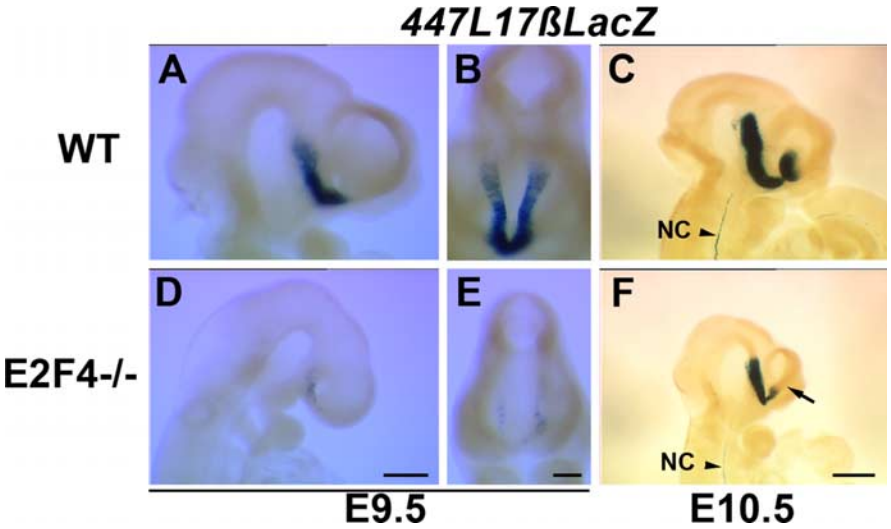

Figure 9. Aberrant activity of *Shh* regulatory elements in the ventral forebrain of E2F4-deficient mouse embryos. Whole-mount *lacZ* expression (X-gal) of E9.5 (***A***, ***B***, ***D***, ***E***) and E10.5 (***C***, ***F***) WT (***A–C***) and E2F4−/− (***D–F***) embryos carrying 447L17βlacZ reporter construct. Note the reduced reporter activity in E2F4-deficient embryos at E9.5 (***D***, ***E***) and failure to expand into the ventral telencephalon at E10.5 (***F***, black arrow), whereas reporter activity is present in the notochord (NC) (***C***, ***F***, black arrowheads). Scale bars: ***A***, ***D***, 250 μm; ***B***, ***E***, 125 μm; ***C***, ***F***, 500 μm.

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