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Opposing Regulation of *Sox2* by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells

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SUMMARY

The mechanisms through which cell-cycle control and cell-fate decisions are coordinated in proliferating stem cell populations are largely unknown. Here, we show that E2f3 isoforms, which control cell-cycle progression in cooperation with the retinoblastoma protein (pRb), have critical effects during developmental and adult neurogenesis. Loss of either E2f3 isoform disrupts *Sox2* gene regulation and the balance between precursor maintenance and differentiation in the developing cortex. Both isoforms target the *Sox2* locus to maintain baseline levels of *Sox2* expression but antagonistically regulate Sox2 levels to instruct fate choices. E2f3-mediated regulation of *Sox2* and precursor cell fate extends to the adult brain, where E2f3a loss results in defects in hippocampal neurogenesis and memory formation. Our results demonstrate a mechanism by which E2f3a and E2f3b differentially regulate *Sox2* dosage in neural precursors, a finding that may have broad implications for the regulation of diverse stem cell populations.

INTRODUCTION

Stem cell-fate decisions, such as self-renewal, precursor cell maintenance, and commitment to differentiation have critical outcomes for embryonic development, tissue maintenance, tumor suppression, and regeneration. Cortical development depends on a precisely regulated balance of self-renewal within stem cell-like apical precursors (APs), production of rapidly proliferating basal progenitors (BPs), and differentiation of postmitotic neurons (Englund et al., 2005; Farkas and Huttner, 2008; Hutton and Pevny, 2011) (Figure 1A). Identifying mechanisms that control this balance can inform our understanding of developmental neurogenesis and, more broadly, reveal stem cell biological principles extending to embryonic stem cell differentiation, tumor formation, and tissue regeneration.

The pluripotency factor Sox2 is an established regulator of neural precursor proliferation, self-renewal, and differentiation during development and is also required for maintenance of adult stem cell populations in many different tissues (reviewed in Sarkar and Hochedlinger, 2013). Overexpression of Sox2 in both mouse and chick embryonic neural precursor cells (NPCs) results in maintenance of the $Sox2^+$ population and defective neurogenesis (Bani-Yaghoub et al., 2006; Graham et al., 2003). Conversely, loss of function of Sox2 in neural precursors leads to precursor loss and reduced or aberrant differentiation, depending on the tissue type and degree of Sox2 loss (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004; Graham et al., 2003; Miyagi et al., 2008; Taranova et al., 2006). Taken together, these studies reveal that the function of Sox2 is strongly influenced by dosage; thus, fine-tuning of transcription from the *Sox2* locus is crucial for the generation of the correct proportion of precursors versus differentiated cell types. Interestingly, a recent study finds that the Cyclin-dependent kinase inhibitor 1A (p21) binds a *Sox2* enhancer region to regulate Sox2 expression and adult neurogenesis, linking cell-cycle regulation with Sox2-mediated control of neural stem cell (NSC) expansion (Marqués-Torrejón et al., 2013).

Previous evidence suggests that the cell cycle machinery plays a key role in regulating the proliferative expansion and self-renewal capacity of NPCs (Nishino et al., 2008; Ruzhynsky et al., 2007; Vanderluit et al., 2004). However, how specific cell-cycle regulatory proteins function in this context remains poorly defined. The retinoblastoma pocket protein (pRb) family controls cell-cycle progression by binding and inhibiting the E2f family of transcription factors. E2fs are classified into the "activator" subclass, which drives proliferation and transcription, and the "repressor" subclass, the members of which are thought to repress gene transcription by modifying chromatin structure through association with pocket proteins (Asp et al., 2009). Earlier work has reported that E2f3 is the most highly expressed E2f family member in wild-type and pRb-deficient neural precursors (Callaghan et al., 1999), suggesting that it may be an important regulator of NPC functions. Understanding how the *E2f3* gene functions to regulate the cell cycle is not entirely straightforward, because the two isoforms (*E2f3a* and *E2f3b*) expressed from its locus have identical domains important for DNA binding, transactivation, and pocket-protein binding, and only their N termini are unique. Mice lacking both

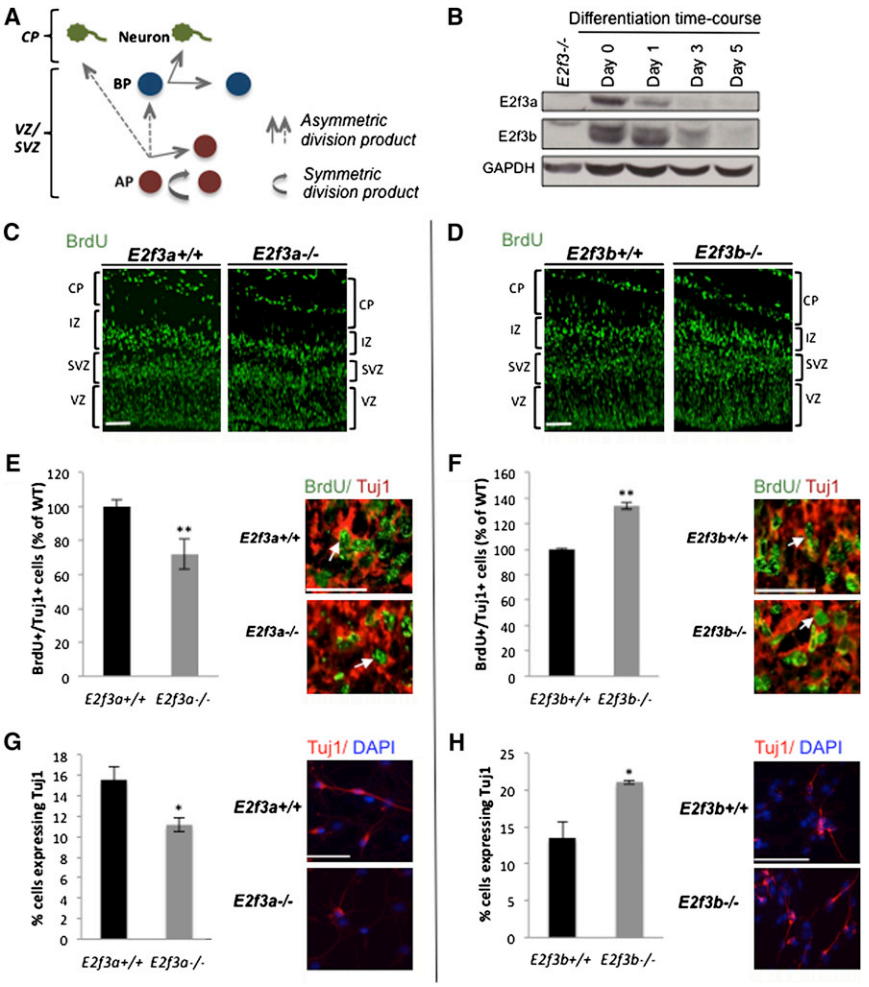

Figure 1. E2f3 Isoforms Are Differentially Required for Neuronal Commitment

(A) Cortical development depends on a finely controlled balance of AP cell proliferation, self-renewal, and differentiation. APs can divide symmetrically to expand their population or asymmetrically to generate one AP and a neuron, glial cell, or BP. BPs generate neurons through asymmetric divisions. CP, cortical plate.

(B) Immunoblot for E2f3 in cultured neurospheres induced to differentiate over 5 days. Both E2f3a and E2f3b are expressed in proliferating neurospheres (day 0), but expression is decreased as differentiation progresses (days 1, 3, and 5). GAPDH was included as a protein loading control.

(C and D) BrdU staining in E14.5 coronal sections following a 24 hr BrdU pulse to identify cells that have exited the cell cycle. Fewer BrdU$^+$ cells are observed in the *E2f3a*$^{-/-}$ SVZ and IZ, whereas more BrdU$^+$ cells are apparent in *E2f3b*$^{-/-}$.

(E and F) Sections described in (C) and (D) were immunostained for BrdU and Tuj1. The number of cells expressing both BrdU and Tuj1 was quantified within a defined area in the SVZ and IZ (arrows identify examples of quantified cells). Results are expressed as a percentage of *E2f3a*$^{+/+}$ average values ± SEM (n = 4).

(G and H) Neurospheres were expanded in vitro and, upon first passage, were cultured in differentiation media on poly-L-ornithine-coated dishes for 3 days, PFA fixed, and immunostained for Tuj1 and DAPI. *E2f3a*$^{-/-}$ possesses fewer Tuj1$^+$ cells; *E2f3b*$^{-/-}$ has more Tuj1$^+$ cells. Results are presented as the percentage of DAPI$^+$ cells expressing Tuj1 ± SEM (n = 4).

For (E)–(H): *p < 0.05, **p < 0.01. Scale bars represent 50 μm. See also Figure S1.

isoforms die perinatally due to cardiac defects (King et al., 2008), whereas those deficient in either isoform are fully viable (Danielian et al., 2008; Tsai et al., 2008), suggesting functional overlap. Tissue- and cell-type-specific analysis of pRb and E2f knockout mice suggests that E2f3a is generally a potent activator of transcription and proliferation, whereas E2f3b induces proliferation weakly and promotes differentiation (Asp et al., 2009; Chong et al., 2009; Danielian et al., 2008), but whether individual E2f3 isoforms make a distinct contribution to developmental and adult neurogenesis is currently unknown.

Here, we use mouse models deficient for either E2f3 isoform to reveal that E2f3a and E2f3b antagonistically regulate Sox2 expression in NSCs. In E2f3b-null animals, where E2f3a is the dominant isoform, we find that E2f3a represses *Sox2* in cooperation with the pRb family member p107, reduces precursor self-renewal, and promotes differentiation. Conversely, in E2f3a-null animals, where E2f3b is the dominant isoform, we find that E2f3b activates *Sox2* expression by recruiting RNA Polymerase II to its promoter, which leads to increased self-renewal and precursor expansion at the expense of differentiation. Knockdown of *Sox2* in E2f3a-deficient NPCs restored basal levels of self-renewal. Importantly, we find that adult E2f3a-null mice have impaired neurogenesis and a reduced capacity for hippocampal-dependent contextual learning, underscoring how the antagonism between E2f3 isoforms is conserved to regulate adult neurogenesis and affect memory formation.

RESULTS

E2f3 Isoforms Are Expressed in NPCs

E2f3 is a potent cell-cycle regulator and a highly expressed E2f family member in NPCs (Callaghan et al., 1999; McClellan et al., 2007), suggesting a potential role for E2f3 in this cell type. Interestingly, we observed that expression of both E2f3 isoforms is enriched in NPCs but reaches negligible levels by day 5 of differentiation in vitro (Figure 1B; Figures S1A and S1B available online), pointing to a regulatory role for both isoforms within the proliferating precursor pool. We asked whether E2f3 isoforms play an important role in regulating neural stem and progenitor cell-fate decisions by examining mouse lines deficient for *E2f3a* and *E2f3b* (Chen et al., 2007; Tsai et al., 2008).

E2f3a and E2f3b Deficiency Impacts NPC-Fate Decisions in an Opposing Manner

We first asked whether loss of E2f3 isoforms impacts NPC-fate decisions by performing a neuronal commitment assay. Mice

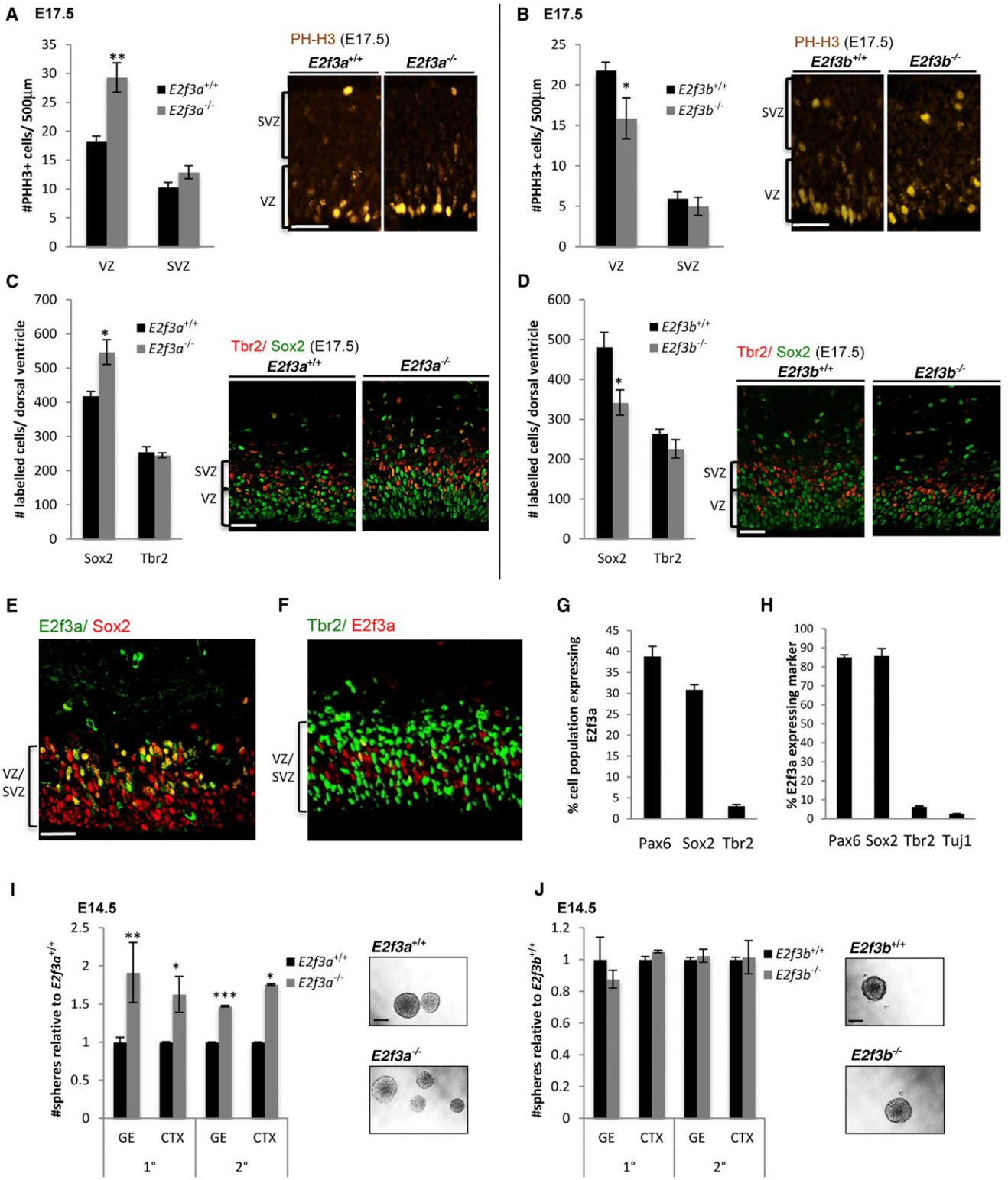

Figure 2. E2f3 Isoforms are Differentially Required for Regulation of NPC Numbers and Self-Renewal

(A and B) PH-H3 staining in E17.5 coronal sections to label mitotic cells. PH-H3$^+$ cells were quantified along the dorsal surface of the lateral ventricle in either the VZ or SVZ, and numbers were normalized to a defined ventricular length (500 µm). Quantification demonstrates an expansion in *E2f3a*$^{-/-}$ and a decrease in *E2f3b*$^{-/-}$, specifically in the VZ (n = 4).

(C and D) Quantification of Sox2$^+$ and Tbr2$^+$ cells within the dorsal cortex at E17.5 demonstrates an increased number of Sox2$^+$ cells in *E2f3a*$^{-/-}$ and fewer Sox2$^+$ cells in *E2f3b*$^{-/-}$ (n = 4).

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were given a single bromodeoxyuridine (BrdU) injection and were sacrificed 24 hr later for the purpose of visualizing $BrdU^+$ cells that had exited the cell cycle and initiated differentiation. There were visibly fewer $BrdU^+$ cells migrating into the subventricular zone (SVZ) and intermediate zone (IZ) of *E2f3a*$^{-/-}$ mice, but more $BrdU^+$ cells in these regions in E2f3b knockouts (Figures 1C and 1D), suggesting a differential commitment to neurogenesis. Newly committed cells that have undergone terminal mitosis can be identified by double labeling with BrdU and differentiation markers, including βIII-tubulin (TuJ1) and Doublecortin (DCX). These BrdU-positive cells are also negative for the proliferation marker Ki67. *E2f3a*$^{-/-}$ mice exhibited a significant reduction in newly committed cells that colabeled for BrdU and Tuj1 (Figure 1E) or BrdU and DCX (Figure S1C) and cells that were negative for Ki67 ($BrdU^+/Ki67^-$) (Figure S1D). In contrast, these same experiments revealed that E2f3b-deficient brains contain significantly more committed cells (Figure 1F; Figures S1E and S1F). These results were further supported in vitro by quantification of newly committed cells in neurosphere cultures induced to differentiate. Here again, E2f3a-deficient NPCs exhibited a reduction in differentiation, whereas E2f3b-deficient precursors had an increase in differentiating cells (Figures 1G and 1H). Deficiency of either E2f3 isoform does not lead to compensatory expression changes of other pRb and E2f family members (Figures S1G–S1J), demonstrating the specificity of E2f3-isoform-dependent phenotypes. Thus, deficiency of E2f3 isoforms impacts NPC-fate decisions in distinct ways: E2f3a loss reduces, whereas E2f3b deficiency increases, commitment to a neuronal fate.

Second, to determine whether E2f3 isoforms are similarly required to regulate the size of the neural precursor pool in an opposing manner, we quantified the number of proliferating NPCs during forebrain development by performing a 2 hr BrdU incorporation (S phase) and phosphohistone H3 (PH-H3) immunostaining (M phase). E2f3a loss resulted in an expanded neural precursor pool (Figures S2A and S2C), specifically affecting the $Sox2^+$ stem-like APs in the ventricular zone (VZ) (Figures 2A and 2C; Figure S2E) and culminating in a 38% increase in the size of this population by embryonic day 17.5 (E17.5) (Figure 2A). Loss of E2f3b resulted in an average 25% decrease in precursor numbers throughout development (Figures S2B and S2D), again specifically affecting Sox2-expressing stem-like APs (Figures 2B and 2D; Figure S2F). Concomitant with the expanded precursor population in *E2f3a*$^{-/-}$ brains, the neuronal output at birth was significantly reduced (e.g., a 24% decrease in later-born neurons, layers I–III) (Figure S3A). In contrast, neuronal output was increased in E2f3b knockouts (Figure S3B). Thus, E2f3a and E2f3b are differentially required for the regulation of both the expansion of the AP population and the commitment of AP cells to a neuronal fate.

E2f3 Is Expressed in Stem-like $Sox2^+$ APs

The impact of E2f3 isoforms on cell-fate decisions within the AP population suggests that E2f3 is expressed in $Sox2^+$ precursors. Using an N-terminal E2f3a-specific antibody, we detected E2f3a protein within NPCs in the ganglionic eminence (GE), a ventrally located tissue that gives rise to inhibitory interneurons (Wonders and Anderson, 2006), as well as the VZ and SVZ surrounding the lateral ventricle (Figures S4A and S4B). Importantly, E2f3a colocalizes with a subset of Sox2-expressing cells in the GE (Figure S4C) and the dorsal cortex (Figures 2E, 2G, and 2H) (also marked by Pax6 [Figure S4D]). Conversely, little E2f3a colocalization was found in committed BPs, which express Tbr2 (Figures 2F–2H), or in $Tuj1^+$ neurons (Figure 2H). Quantification of cells colabeled with E2f3a and cell-cycle phase markers revealed that E2f3a is highly enriched in S phase, during which 83% of $E2f3a^+$ cells coexpressed BrdU following a 2 hr pulse (Figures S4E–S4G). E2f3a expression in S phase precursors supports a role in NPC-fate decisions, given that a recent study suggests that fate decisions in the developing brain are controlled by gene expression patterns during S phase (Arai et al., 2011). Thus, E2f3a is expressed predominantly in $Sox2^+$ self-renewing precursors.

E2f3a Is Required for Regulation of NSC Self-Renewal

We asked next whether E2f3 isoforms modulate the self-renewal capacity of the stem cell-like AP population. Loss of E2f3a increased the number of primary and secondary neurospheres generated by both cortical and GE-derived NPC populations by 1.4- to 2-fold at E14.5 (Figure 2I) and E17.5 (Figure S4H). Loss of E2f3b, however, showed no effect (Figure 2J; Figure S4I). To ask whether E2f3a deficiency affects the mode of AP cell division, we measured the orientation of mitotic spindle poles in control and E2f3a-deficient brains. APs undergo mitosis at the apical surface of the lateral ventricle, and the orientation of the mitotic spindle pole and cleavage furrow during cytokinesis has been linked with the resulting fate of daughter cells (Das and Storey, 2012; Farkas and Huttner, 2008; Godin et al., 2010) (see Supplemental Information for detailed methods). In E2f3a knockouts, we observed 1.5-fold more APs with a cleavage angle within the vertical 75°–90° range, associated with symmetric (self-renewing) cell divisions (Figures S4J and S4K). In contrast, there was a corresponding 2.7-fold decrease in the number of divisions within the 0°–15° range, suggesting a reduction in asymmetric, differentiative cell divisions. These results suggest that E2f3a-deficient brains exhibit an increased proportion of AP cells undergoing symmetric cell divisions, consistent with our in vitro studies showing enhanced neural precursor self-renewal.

(E) Colocalization of E2f3a (green) with Sox2 (red) in the dorsal cortex (E14.5).
(F) Lack of colocalization between E2f3a (red) and the BP marker Tbr2 (green) in the dorsal cortex (E14.5).
(G) Quantification of the percentage of all $E2f3a^+$ cells per section in the dorsal cortex (E14.5) coexpressing Sox2, Pax6, Tbr2, or Tuj1 (n = 3).
(H) Quantification of the percentage of Pax6-, Sox2-, or Tbr2-expressing cells per section in the dorsal cortex (E14.5) that also express E2f3a (n = 3).
(I and J) Increased number of primary and secondary neurospheres in *E2f3a*$^{-/-}$ precursors derived from both GE and dorsal cortex (CTX) (I); E2f3b knockouts generate the same number of neurospheres as wild-types (J). Included in the right side are phase-contrast images of neurospheres from the indicated genotypes (n = 5–7).
For (A)–(J), results are presented as the mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001). The scale bar represents 100 μm. See also Figures S2–S24.

Opposing Regulation of the *Sox2* Gene by E2f3 Isoforms

To identify target genes through which E2f3 isoforms regulate NPC properties, we performed a genomic chromatin immunoprecipitation (ChIP)-on-chip screen to identify E2f3 binding sites and associated target genes in NPCs (L.M.J., Y. Liu, D.S.P., R.S.S., and A.B., unpublished data). From three independent samples of wild-type, *E2f3a*$^{-/-}$, and *E2f3b*$^{-/-}$ E14.5 GE neurospheres, we identified the gene encoding the pluripotency factor *Sox2* as a potential target of E2f3 (Figure 3A). Enrichment levels for E2f3 at the *Sox2* promoter were comparable in wild-type and E2f3a- and E2f3b-deficient cells, indicating that both isoforms bind this locus. Previous studies have shown that changes in Sox2 expression can have dramatic effects on the maintenance and differentiation capacity of neural precursor populations, wherein elevated Sox2 leads to expansion of the precursor pool and impaired neurogenesis, and decreased Sox2 results in loss of NPCs and dose-dependent defects on neurogenesis (Bani-Yaghoub et al., 2006; Graham et al., 2003; Pevny and Nicolis, 2010; Taranova et al., 2006). As precursor numbers and neurogenesis are disrupted in E2f3a- and E2f3b-deficient brains, *Sox2* was a strong candidate to account for these biological effects. We first validated by conventional ChIP that E2f3 binds to the *Sox2* promoter, at an enrichment level comparable to that of previously established E2f3 target genes (Figure 3B). An E2f consensus motif (CTTCCCGC) was identified within the center of the E2f3 binding peak, 371 bp upstream of the transcriptional start site (TSS) (Figure 3A), and is conserved in the murine and human genomes. This E2f3-bound region is transcriptionally responsive to E2f3 activity, as indicated by a 2-fold increase in luciferase activity from a *Sox2* promoter fragment (800 bp upstream to 285 bp downstream of the *Sox2* TSS) following cotransfection of a full-length E2f3 construct (Figures 3C and 3D). Furthermore, point mutations within the E2f consensus motif reduced luciferase activity by 50% (Figures 3C and 3D), demonstrating a functional E2f consensus site at 371 bp upstream of the *Sox2* TSS.

To determine whether E2f3 isoforms regulate Sox2 levels in vivo and in vitro, we measured Sox2 protein levels in GE-derived tissue and cultured neurospheres, because NPCs from this region are predominantly Sox2^{+} (Figures S5A and S5B). We show that E2f3a and E2f3b regulate Sox2 expression in a reciprocal manner. Specifically, *E2f3a*$^{-/-}$ neurospheres and GE tissue exhibited a 2.3- and 6-fold increase, respectively, in Sox2 levels (Figures 3E and 3F). In contrast, *E2f3b*$^{-/-}$ neurospheres and GE tissue express Sox2 at 40% and 30% of wild-type levels (Figures 3G and 3H). These results suggest an opposing role for E2f3 isoforms in the regulation of the *Sox2* gene.

E2f3a Represses NSC Self-Renewal through *Sox2* Regulation

To directly determine whether E2f3a represses self-renewal by regulating *Sox2*, we asked whether *Sox2* knockdown could rescue the enhanced self-renewal phenotype observed in E2f3a-deficient cultures. *E2f3a*$^{+/+}$ or *E2f3a*$^{-/-}$ neurospheres were infected with a bicistronic lentivirus expressing GFP and one of two short hairpin Sox2 (shSox2) or scrambled control sequences. Importantly, each shSox2 construct reduced Sox2 expression in *E2f3a*$^{-/-}$ cells to a level comparable to that of GFP-infected wild-type cells (Figures 4A and 4B). shSox2-mediated knockdown of *Sox2* in *E2f3a*$^{-/-}$ cultures restored neurosphere numbers (Figures 4C and 4D) and self-renewal capacity (Figure 4E) back to basal levels. To determine whether elevated Sox2 can account for the increased self-renewal in *E2f3a*$^{-/-}$ precursors, we overexpressed Sox2 in wild-type cultures (Figure 4F). Sox2 overexpression in *E2f3a*$^{+/+}$ precursors increased self-renewal (Figure 4G) and correspondingly decreased neurogenesis (Figure 4H) to levels observed in E2f3a-deficient cells. Furthermore, overexpression of Sox2 in *E2f3a*$^{-/-}$ precursors, which already express elevated Sox2, did not increase neurosphere numbers further. Thus, E2f3a functions to maintain Sox2 levels below a specific threshold, beyond which precursor self-renewal and cell-fate decisions are markedly disrupted.

E2f3 Isoforms Recruit Distinct Transcriptional Cofactors to the *Sox2* Promoter

To determine the mechanism by which E2f3a and E2f3b antagonistically regulate Sox2 expression, we identified the regulatory factors recruited to the *Sox2* locus by each isoform. We confirmed that both isoforms bind the *Sox2* promoter within a 200 bp region surrounding the conserved E2f motif (upstream binding site [US]) and at the TSS, given that E2f3 enrichment is similar in wild-type, *E2f3a*$^{-/-}$, and *E2f3b*$^{-/-}$ neural precursors (Figure 5A). Consistent with E2f3b as an activator of *Sox2* expression, in *E2f3a*$^{-/-}$ cells, in which only the E2f3b isoform is present, we observed enrichment of RNA polymerase II (Pol II) at and beyond the TSS (Figure 5B) and the trimethyl-H3K4 (H3K4me3) chromatin modification (Figure 5C), as well as a decrease in H3K27me3 (Figure 5C). Each of these changes are associated with transcriptional activation, demonstrating that in the absence of E2f3a, Sox2 expression is elevated due to an increased ratio of bound E2f3b-Pol II complexes. Conversely, binding of the repressive pocket protein p107 was significantly enriched in the absence of E2f3b, where only E2f3a is present, and was decreased in *E2f3a*$^{-/-}$ cells (Figure 5D). These findings show that E2f3a functions as a repressor at the *Sox2* promoter by recruiting p107. Supporting this conclusion, GE tissue from p107-deficient animals exhibited a 2.2-fold increase in Sox2 levels compared to wild-type controls (Figure 5E).

The percentage of precursor cells in each cell-cycle phase was not altered by E2f3a or E2f3b deficiency (Figures S6A and S6B); thus, the changes in binding enrichments we observed in E2f3 isoform-deficient cells could not be explained by disrupted cell-cycle dynamics, but rather by altered enrichment of these factors. We show that E2f3 isoforms regulate *Sox2* expression in an opposing manner, whereby E2f3a recruits the transcriptional repressor p107 and E2f3b recruits activator complexes to the promoter. This reveals a novel mechanism for regulation of the pluripotency factor *Sox2*, through the cell cycle effectors E2f3a and E2F3b.

A Common Mechanism of E2f3a-Mediated *Sox2* Regulation in Embryonic and Adult NSCs

The requirement for controlled Sox2 expression in NSCs extends from development to adulthood (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004; Pevny and Nicolis,

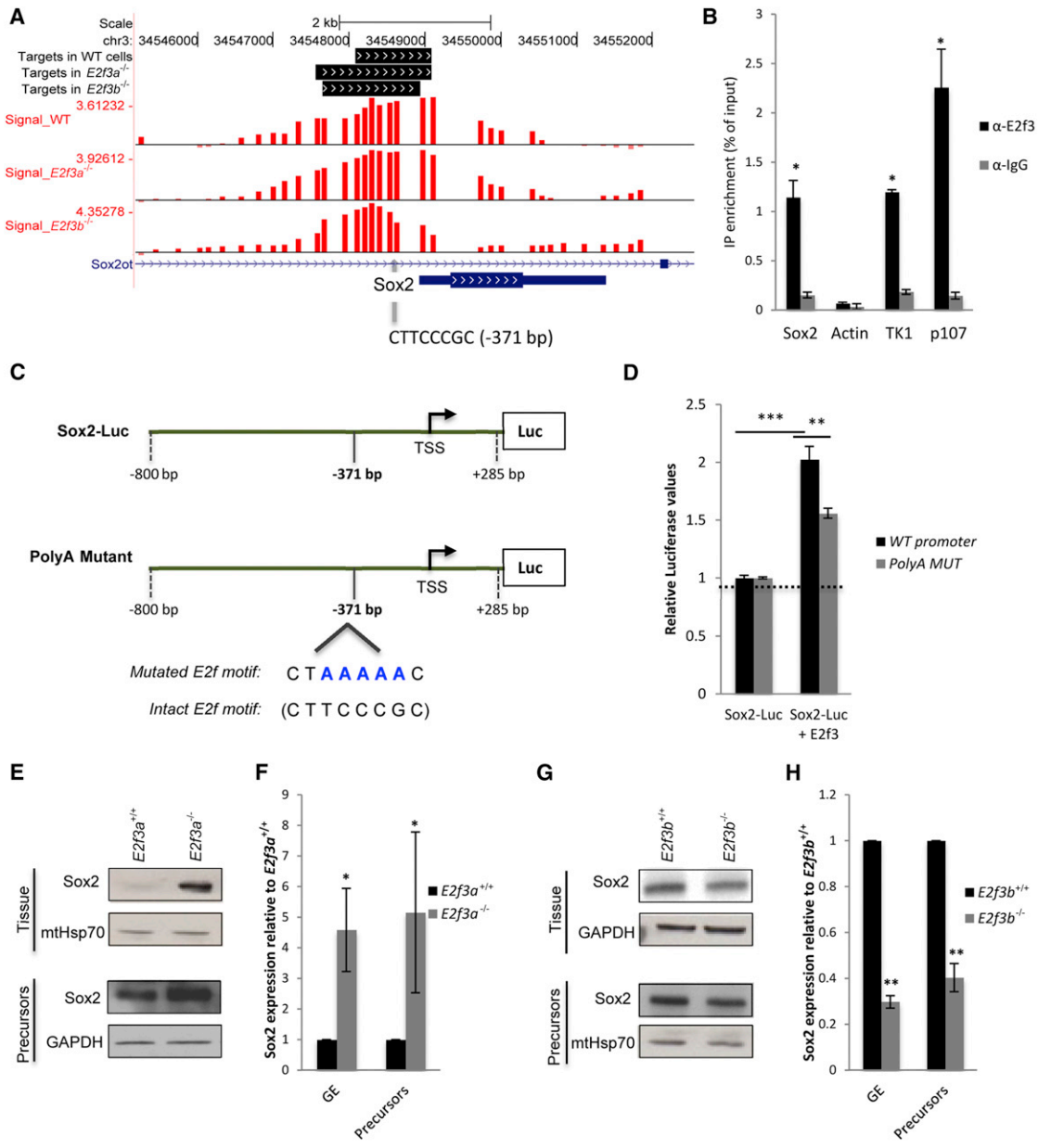

Figure 3. E2f3 Isoforms Regulate *Sox2* Expression in an Opposing Manner

(A) Binding-peak profiles for E2f3 from *E2f3a*$^{+/+}$, *E2f3a*$^{-/-}$, and *E2f3b*$^{-/-}$ ChIP-on-chip experiments, generated with the UCSC Genome Browser (http://genome.ucsc.edu/). E2f3 binding peaks extend throughout the proximal promoter region and the TSS. An E2f consensus motif was identified at 371 bp upstream of the TSS. WT, wild-type.

(B) RT-PCR analysis of E2f3 ChIP experiments shows E2f3 binding at the *Sox2* promoter with an enrichment value similar to that for other known E2f targets. Plotted is the mean from at least three independent experiments ± SEM (n = 4).

(C) Model for luciferase experiments. E2f3-dependent activity was tested from a Sox2 promoter fragment covering −800 bp to +285 bp relative to the TSS. For E2f consensus site mutation, five core nucleotides were replaced with adenine.

(D) E2f3a drives Sox2-luciferase activity. Mutation of the E2f consensus motif reduced E2f3-mediated transcription by 50% (n = 4–6).

(E and G) Immunoblot for Sox2 from cultured E2f3a (E) or E2f3b (G) neural precursors or GE tissue. GAPDH and mtHsp70 were included as protein-loading controls.

(F and H) Quantification by densitometry of immunoblots shows that E2f3a knockouts express significantly more Sox2 (F), whereas E2f3b knockouts have lower Sox2 levels (H).

For all quantifications, data are plotted as the mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001). See also Figure S5.

2010); thus, we hypothesized that E2f3-dependent *Sox2* regulation is also important in adult NSCs. To evaluate the role of E2f3a in the adult we generated animals containing two modified E2f3 alleles: one *floxed* allele and a second E2f3a-deficient allele (*E2f3-flox/3a*$^{-}$). To acutely remove E2f3a, we infected cultured SVZ precursors from adult *E2f3-flox/3a*$^{-}$ animals with

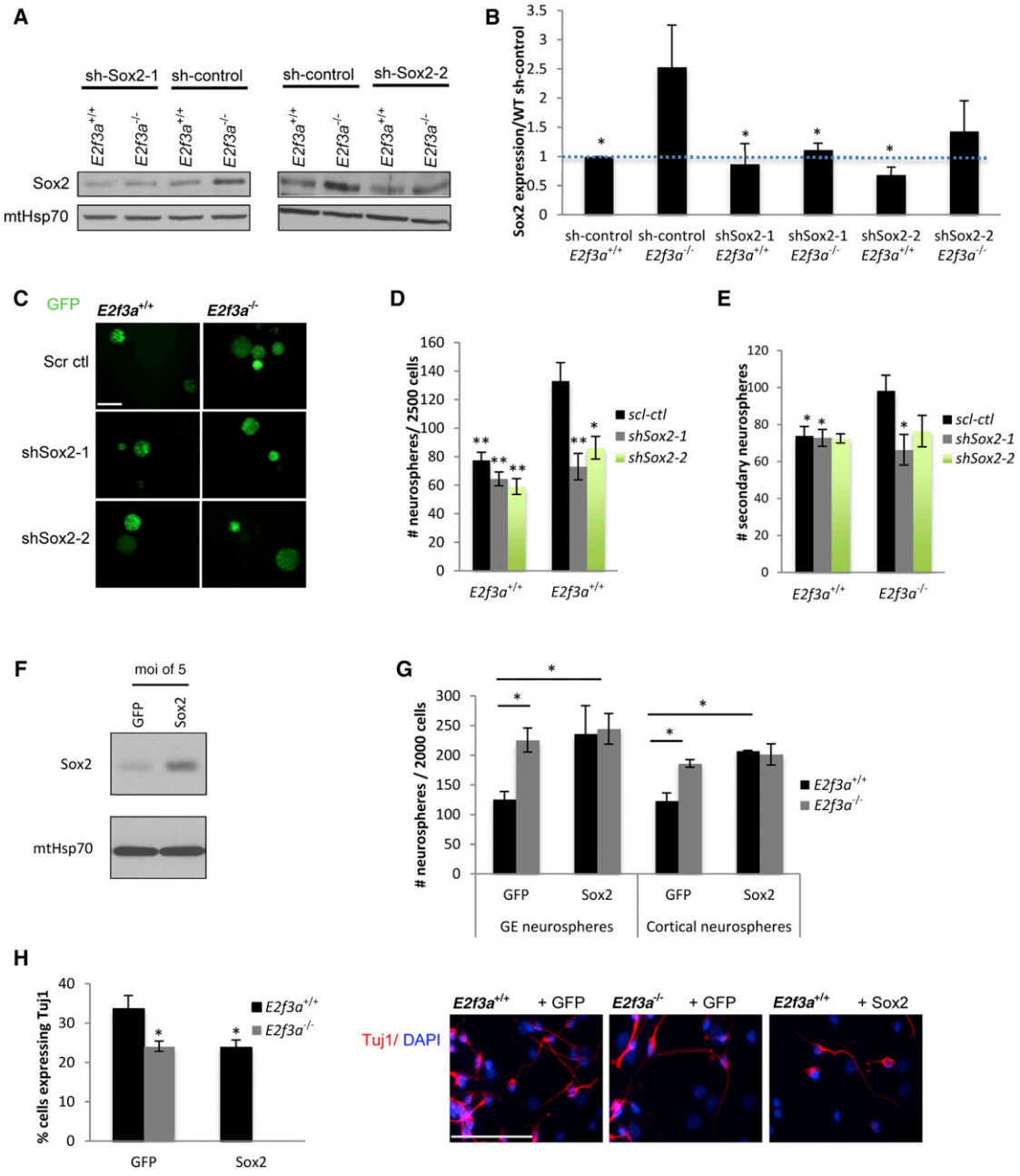

Figure 4. Regulation of NSC Self-Renewal by E2f3a Is Sox2-Dependent

(A and B) Immunoblot analysis (A) and densitometry quantification (B) of Sox2 expression in cultured GE neurospheres (E14.5) 5 days post infection (p.i.) with scrambled (Scr) control or shSox2 lentiviruses (n = 3). mtHsp70 was included as a measure of protein loading.

(C) Representative images of GFP-positive neurospheres 7 days p.i. Scale bar represents 200 μm.

(D and E) Infected cultures were plated immediately for primary neurosphere assays (D) and one week later were used in secondary neurosphere assays (E). Neurosphere numbers are restored in E2f3a knockouts following *Sox2* knockdown (n = 4).

(F) Immunoblot demonstrating increased Sox2 expression in neurospheres infected with a Sox2-expressing lentivirus compared to GFP-infected cells, 4 days p.i.

(G) Cells were plated 7 days p.i. for secondary neurosphere assays. Sox2 overexpression in wild-type cells increases self-renewal (n = 3).

(H) *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ neurospheres were cultured in differentiation media on poly-L-ornithine plates 7 days p.i. and were fixed and stained for Tuj1 and DAPI after 6 days. The percentage of DAPI^{+} cells that express Tuj1 was quantified. Sox2 overexpression inhibits neuronal differentiation in vitro. Scale bar represents 50 μm (n = 3).

Significance was determined for all samples compared to *E2f3a*$^{-/-}$ (B, D, and E) or *E2f3a*$^{+/+}$ (G and H) cells infected with control virus. All data are presented as the mean ± SEM (*$p < 0.05$, **$p < 0.01$).

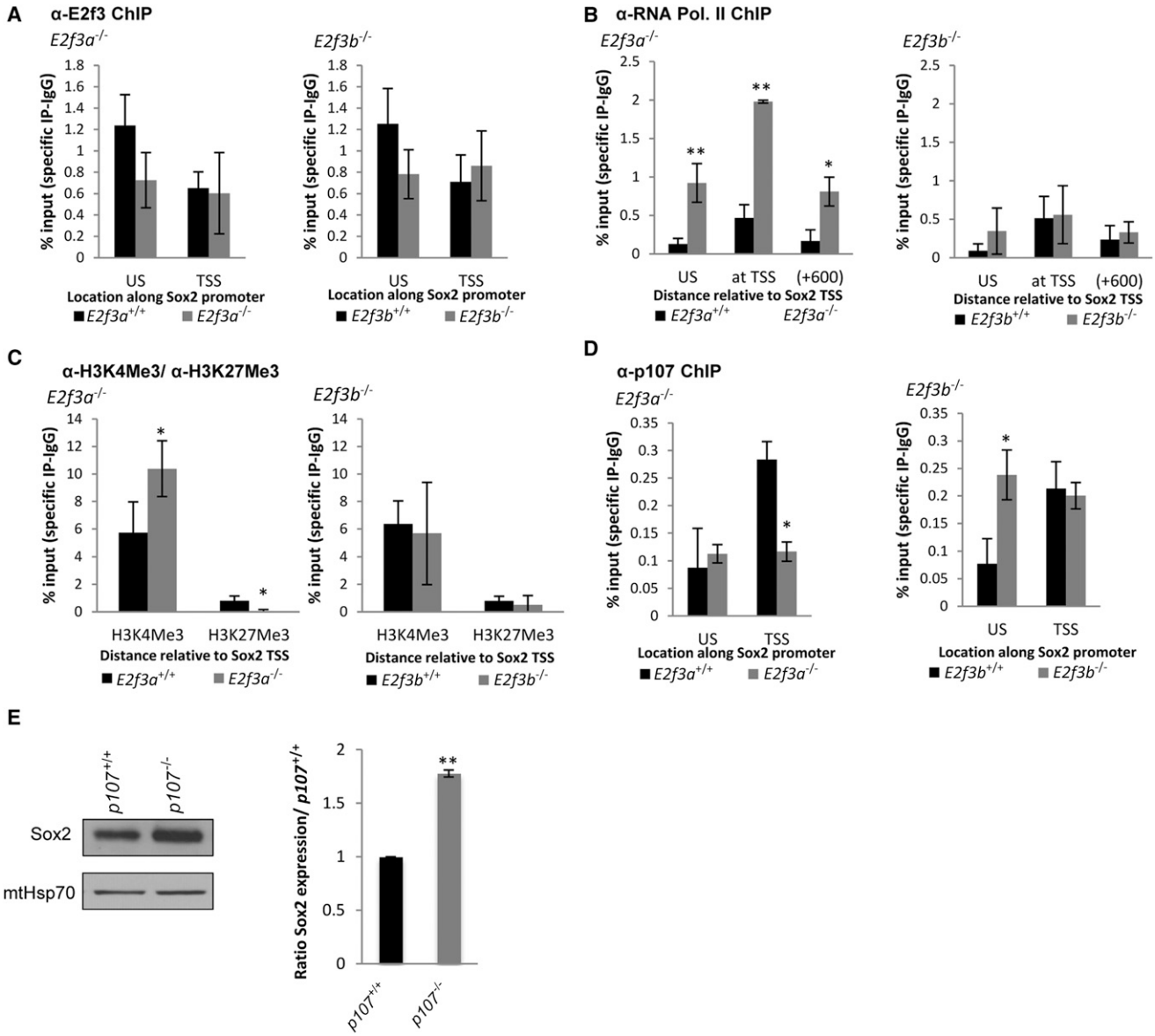

Figure 5. E2f3 Isoforms Recruit Distinct Transcriptional Cofactors to the *Sox2* Promoter

(A–D) ChIP was performed in *E2f3a*$^{-/-}$, *E2f3b*$^{-/-}$, and wild-type (both *E2f3a*$^{+/+}$ and *E2f3b*$^{+/+}$) GE-derived neurospheres using antibodies for E2f3 (A), Pol II (B), H3K4me3 and H3K27me3 (C), and p107 (D). Chromatin enrichment was quantified by RT-PCR using primers designed to amplify 200 bp regions centered on either the upstream (US) E2f binding motif or the TSS of the *Sox2* promoter. For all panels, we have plotted values for the specific antibody IP with immunoglobulin G (IgG) values subtracted (n = 3–5).

(E) Immunoblot analysis and densitometry quantification of *p107*$^{+/+}$ and *p107*$^{-/-}$ GE tissue shows a significant increase in Sox2 levels in the absence of p107 (n = 3). See also Figure S6.

Data for (A)–(E) are plotted as the mean ± SEM (*p < 0.05, **p < 0.01).

a Cre-expressing lentivirus, which removes *E2f3a* but leaves *E2f3b* intact (Figure 6A). As with embryonic *E2f3a*$^{-/-}$ precursors, Cre-infected cells exhibited increased neurosphere self-renewal (Figure 6B) and were impaired in their ability to generate neurons (Figure 6C). Importantly, these self-renewal and neurogenic changes were accompanied by increased Sox2 expression (Figure 6D). Furthermore, the absence of E2f3a reduced enrichment of E2f3 (Figure 6E) and p107 (Figure 6F) and significantly increased recruitment of Pol II (Figure 6G) at the *Sox2* promoter. Thus, NSCs maintain a common mechanism of E2f3a-dependent *Sox2* regulation from development to adulthood.

Loss of E2f3a Disrupts Neurogenesis and Cognitive Function in the Adult Brain

Given that E2f3a regulates *Sox2* in both embryonic and adult NSCs, we asked whether absence of E2f3a had a functional consequence in the adult brain. We evaluated the levels of

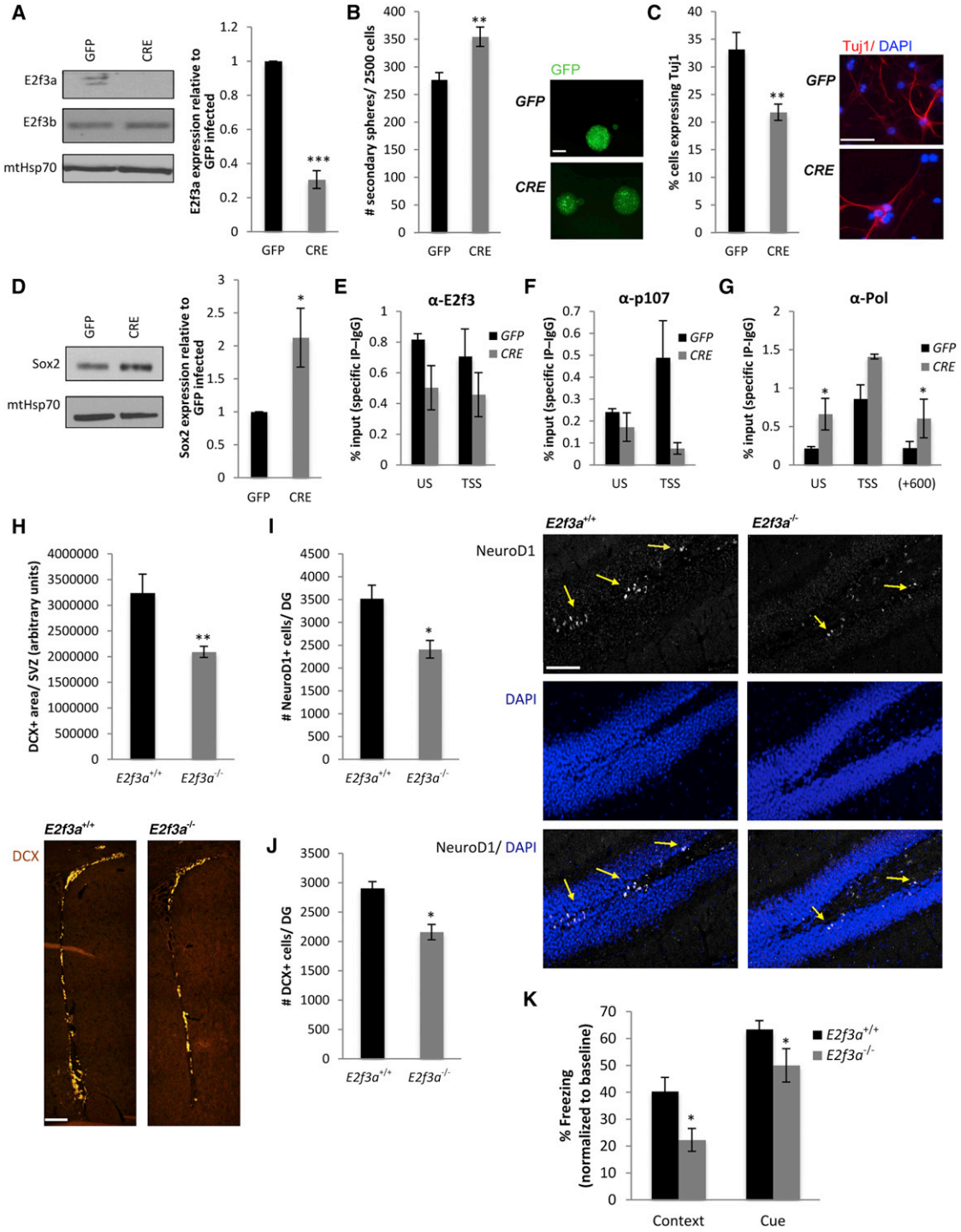

Figure 6. E2f3a Regulates *Sox2*, Neurogenesis, and Cognitive Function in the Adult Brain

(A) Immunoblot showing loss of E2f3a, but not E2f3b, in *E2f3-flox/E2f3a*$^{-}$ adult SVZ precursors 4 days after Cre infection. Results were quantified by densitometry, using mtHsp70 as a loading control (n = 4).

(B) Infected cells were plated for neurosphere assays 7 days p.i., and regenerated neurospheres were counted 6 days later. Cre-infected cells generated more neurospheres than did controls. The image to the right shows GFP-expressing neurospheres infected with either control (GFP) or GFP-CRE virus 7 days p.i. (n = 7).

(C) Neurospheres were plated in differentiation media on poly-L-ornithine-coated dishes 7 days p.i. and were fixed and stained for Tuj1 and DAPI after 6 days. The percentage of DAPI^{+} cells expressing Tuj1 was quantified. Cre-infected cells have a reduced capacity to generate neurons (n = 5).

(D) Elevated Sox2 in Cre- versus GFP-infected *E2f3-flox/E2f3a*$^{-}$ adult precursors (5 days p.i.), quantified by densitometry using mtHsp70 as a loading control (n = 3).

(E–G) RT-PCR analysis of ChIP assays for E2f3 (E), p107 (F), and Pol II (G) in GFP- or Cre-infected *E2f3-flox/E2f3a*$^{-/-}$ precursors (n = 4).

(legend continued on next page)

neurogenesis in *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ adult brains in two distinct neurogenic regions, the SVZ and the dentate gyrus (DG) of the hippocampus, wherein neurogenesis is required for olfactory function and hippocampal memory formation, respectively. As determined by NeuroD1 and DCX staining, the number of committed neurons was significantly decreased, by 35% in the SVZ (Figure 6H) and 31% in the DG (Figures 6I and 6J), revealing an impairment in adult neurogenesis that had progressed since the late stages of development. We also found that *E2f3a*$^{-/-}$ mice are significantly impaired in their ability to learn and remember the association between an aversive experience and environment in the classical fear-conditioning paradigm (Wehner and Radcliffe, 2004). In this test, animals are trained to acquire a learned response to an aversive stimulus (foot shock) that is associated with a specific environment (context) and a tone (cue). Following training, animals are tested for their ability to have learned that the context or cue is associated with the aversive stimulus through measuring their freezing behavior during exposure to each condition. *E2f3a*$^{-/-}$ mice exhibited a significant 45% decrease in freezing behavior associated with amygdala- and hippocampus-dependent contextual learning (Marín-Burgin and Schinder, 2012) and a more subtle 21% decrease in freezing associated with amygdala-dependent cue learning (Figure 6K) (Wehner and Radcliffe, 2004). The reduced freezing in *E2f3a*$^{-/-}$ versus control mice was not due to differences in the unconditioned freezing behavior, as assessed during training and before tone presentation in the cue trial (Figures S7A and S7B), nor to differences in the foot-shock threshold (Figure S7C). These results suggest that E2f3a influences the formation of associative memories and that its loss reveals defects in at least two telencephalic structures, with the most severely affected function (contextual learning) being that which is influenced by adult neurogenesis (Marín-Burgin and Schinder, 2012). Thus, E2f3a is required for regulation of neural precursor maintenance and neurogenesis in both the embryonic and adult brain, and this role significantly impacts cognitive function.

DISCUSSION

This study presents two key discoveries. First, we show that E2f3 isoforms play opposing roles in regulating the balance between neural precursor self-renewal and differentiation during developmental neurogenesis. Loss of E2f3a leads to neurogenic defects in adulthood, underscoring the importance of E2f3 in mediating fate choices in both embryonic and adult NSCs. Second, we report a transcriptional mechanism by which E2f3 isoforms antagonistically regulate levels of *Sox2* expression. Alteration of *Sox2* expression by loss of either E2f3 isoform shifts the equilibrium between precursor expansion and differentiation, thereby affecting downstream generation of cortical neurons and ultimately cognitive function.

Based on our findings and previous reports of E2f3 isoform expression patterns (Adams et al., 2000), we predict that E2f3a is predominant during S phase, whereas E2f3b is expressed throughout the cell cycle. Thus, at different phases of the cell cycle, E2f3a and E2f3b isoforms dynamically fine-tune Sox2 expression levels. We present a model of E2f3-dependent *Sox2* regulation in which both E2f3 isoforms, in a see-saw-like fashion, regulate levels of Sox2 in proliferating NSCs to ensure the proper balance of precursor expansion and differentiation (Figure 7A). When E2f3b is lost, E2f3a-p107-mediated repression is not balanced by E2f3b-mediated activation, leading to lower Sox2 levels and increased neurogenesis at the expense of precursor expansion (Figure 7B). Conversely, E2f3a deficiency leads to activation by E2f3b that is not balanced by E2f3a-mediated repression, resulting in elevated Sox2 levels and, consequently, increased precursor self-renewal at the expense of neurogenesis (Figure 7C). This functional model illustrates the requirement for a balance between E2f3a and E2f3b transcriptional activities to maintain the correct dosage of Sox2 in stem and progenitor cells.

Although E2f3a, E2f3b, and p107 are highly expressed in NPCs, these proteins become rapidly downregulated as cells undergo differentiation. Uncovering other mechanisms by which long-term repression of *Sox2* is maintained as cells differentiate will therefore be important. Notably, a recent study has shown that the Cyclin-dependent kinase inhibitor (CKI) p27 is required for repression of *Sox2* during the differentiation of pluripotent stem cells (Li et al., 2012). p27 is recruited to the *Sox2* SRR2 enhancer and functions in a complex together with p130 and E2f4 to silence *Sox2* expression during differentiation. As the pocket protein p130 is highly expressed in differentiated cells and plays a key role in long-term silencing of cell-cycle genes, it may well play a crucial role in silencing *Sox2* in postmitotic neurons. Another recent study demonstrated that the CKI p21 represses *Sox2* expression in adult NSCs (Marqués-Torrejón et al., 2013). In adult NSCs it has been shown that p21 represses *Sox2* through the SRR2 enhancer and that its loss results in excessive *Sox2* expression and precursor exhaustion. This exhaustion, however, is preceded by an initial expansion of the precursor pool (Kippin et al., 2005; Marqués-Torrejón et al., 2013). In E2f3a-deficient embryonic and adult NSCs, we found that elevated levels of Sox2 overexpression lead to increased self-renewal; however, it is also possible that E2f3a-deficient NSCs may exhaust with time, following extended passaging in vitro or with advanced animal age. It is also conceivable that E2f3 may participate in the recruitment of p21 to its SRR2-enhancer binding site. However, given that the E2f3 and p21 binding sites have been identified in distinct regulatory domains of the *Sox2* gene and that E2f-independent mechanisms of p21 recruitment to *Sox2* have been suggested (Marqués-Torrejón et al., 2013), it is probable that they regulate *Sox2* expression by distinct mechanisms. Examining these questions will be an

(H–J) Neurogenesis was measured in the adult brain by quantifying the total area of DCX staining along the SVZ (n = 4) (H) and the number of NeuroD1$^+$ or DCX$^+$ cells in the DG of the hippocampus (n = 3) (I and J).
(K) *E2f3a*$^{-/-}$ mice spent 45% and 21% less time freezing in the context ($p = 0.015$) and after the auditory cue ($p = 0.047$), respectively, following fear-conditioning training (n = 18 for *E2f3a*$^{+/+}$; n = 13 for *E2f3a*$^{-/-}$).
For (A)–(K), data are presented as the mean ± SEM. Scale bar represents 100 μm for (B), (H), and (J) and 25 μm for (C) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). See also Figure S7.

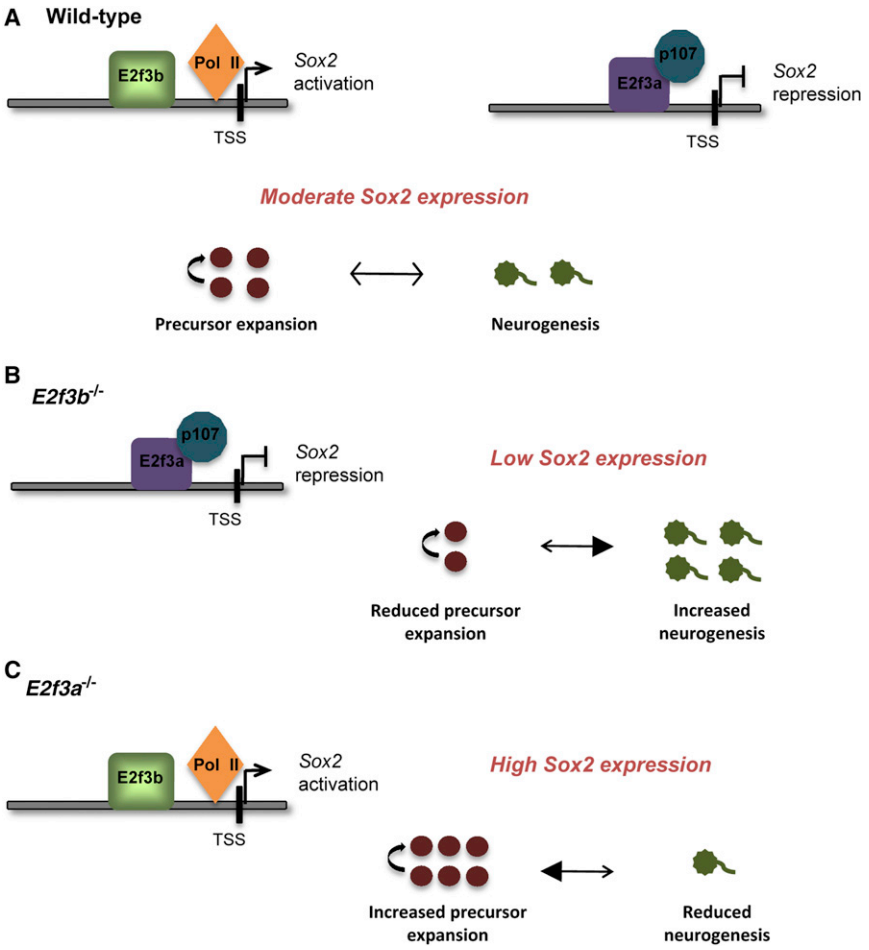

Figure 7. Model of E2f3 Function: E2f3 Isoforms Regulate *Sox2* Transcription in an Opposing Manner to Direct NSC Fate Choice

(A) In wild-type conditions, E2f3b activates and E2f3a represses *Sox2* transcription, allowing for dynamic fine-tuning of Sox2 levels. This fine-tuning maintains the proper balance between neurogenesis and expansion of the NPC pool.

(B) In the absence of E2f3b, E2f3a-p107-mediated repression dominates and reduces Sox2 levels, thereby increasing neurogenesis at the expense of precursor expansion.

(C) In the absence of E2f3a, E2f3a repression unbalances E2f3b-mediated activation, thus increasing Sox2 levels and promoting NPC expansion at the expense of neurogenesis.

important focus for future studies and will contribute to our understanding of molecular events underlying *Sox2* gene silencing in differentiating cells.

Through our identification of *Sox2* as a functional target gene of the pRb/E2f pathway in neural precursors, we have linked the cell-cycle machinery with pluripotent gene regulation in a biologically relevant context. E2f-dependent regulation of *Sox2* has clear functional consequences in the developing brain; however, we suggest that this may be a common feature of stem cell regulation and the pluripotent state, given that both Sox2, pRb, and E2f proteins are expressed in diverse tissue-specific, pluripotent, and cancer stem cell populations (Arnold et al., 2011; Galderisi et al., 2006; Pevny and Nicolis, 2010). In addition, the pRb binding proteins RBBP4 and RBBP9 have recently been implicated in regulation of the pluripotent state in human stem cells, and E2f motifs have been identified in the promoters of key pluripotency factors, including *NANOG*, *POU5F1*, *FOXD3*, and *SOX2* (O'Connor et al., 2011). ChIP-based experiments have further demonstrated that E2fs are found at the promoters of a large number of pluripotency-related genes (Chen et al., 2008; O'Connor et al., 2011), although direct functional consequences for these interactions have not previously been described. In conclusion, these studies point to the possibility that E2fs may regulate other pluripotency factors in addition to *Sox2* and support the idea that E2f3-dependent *Sox2* regulation is a fundamental mechanism in tissue-specific, tumorigenic, and pluripotent stem cell populations.

EXPERIMENTAL PROCEDURES

Mouse Models

Germline *E2f3a*- and *E2f3b*-null mice were originally generated by G. Leone and were maintained on an FVB/N background (Chen et al., 2007; Tsai et al., 2008). Animal experiments were approved by the University of Ottawa's Animal Care Committee, which abides by the guidelines of the Canadian Council on Animal Care. *E2f3-flox/E2f3a*$^{-}$ mice were generated by crossing *E2f3a*$^{-/-}$ mice with *E2f3-flox/flox* animals maintained on an FVB/N background. p107-deficient mice were generated as previously described (LeCouter et al., 1998). All adult mice analyzed were 2 months of age or older.

Neural Precursor Cultures

Neural precursors were obtained by dissection of the ventral (GE) or dorsal (cortex) telencephalic tissue of developing embryos; neurosphere and in vitro differentiation assays were performed as previously described (Vanderluit et al., 2004), with the exception of the lentivirus experiments, in which neural precursors were plated at a density of 5 cells/μl 7 days post infection and the number of regenerated neurospheres were counted after 6 days in culture. All neurosphere assays were performed with brain samples from four to seven independent animals, from at least two separate experiments.

Western Blotting, Immunohistochemistry, Cell Counts, Primers, and Antibodies

Details are described in Supplemental Experimental Procedures.

Statistical Analysis

All statistical comparisons in this study were performed using an unpaired two-tailed t test. Differences were considered significant with a p value of <0.05 (*), **p < 0.01, ***p < 0.001.

Lentiviral Infections

shRNA lentiviral expression constructs were obtained from Open Biosystems and included scrambled control (catalog no. RHS4346), shSox2-1 (clone ID 153337), and shSox2-2 (clone ID 153339) plasmids. Neurosphere cultures were infected with lentiviral particles at a multiplicity of infection (moi) of 30. For Sox2 overexpression, the *Sox2* coding sequence was subcloned into the multiple cloning site (MCS) of an internal ribosome entry site-GFP

backbone, viruses were produced, and neurospheres were infected at an moi of 5. For Cre-expression experiments, GFP- or Cre-coding sequences were subcloned into the MCS of a pWPXLD plasmid, and cells were infected at an moi of 10.

Luciferase Reporter Assays

Reporter assays were performed in HEK293T cells as previously described (Andrusiak et al., 2011). E2f consensus site mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit and primer-design software from Stratagene.

ChIP

ChIP analysis was performed as previously described (Andrusiak et al., 2011) in proliferating neurospheres, except that immunocomplexes were captured using Dynabeads Protein A. RT-PCR was used to quantify ChIP enrichment values. Each experiment was performed on at least three independent samples. ChIP-on-chip experiments were performed as previously described (Liu et al., 2010).

Fear-Conditioning Analysis

Details are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.02.001.

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