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Axin Directs the Amplification and Differentiation of Intermediate Progenitors in the Developing Cerebral Cortex

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SUMMARY

The expansion of the mammalian cerebral cortex is safeguarded by a concerted balance between amplification and neuronal differentiation of intermediate progenitors (IPs). Nonetheless, the molecular controls governing these processes remain unclear. We found that the scaffold protein Axin is a critical regulator that determines the IP population size and ultimately the number of neurons during neurogenesis in the developing cerebral cortex. The increase of the IP pool is mediated by the interaction between Axin and GSK-3 in the cytoplasmic compartments of the progenitors. Importantly, as development proceeds, Axin becomes enriched in the nucleus to trigger neuronal differentiation via β-catenin activation. The nuclear localization of Axin and hence the switch of IPs from proliferative to differentiative status are strictly controlled by the Cdk5-dependent phosphorylation of Axin at Thr485. Our results demonstrate an important Axin-dependent regulatory mechanism in neurogenesis, providing potential insights into the evolutionary expansion of the cerebral cortex.

INTRODUCTION

The architectural and functional integrity of the mammalian neocortex requires the tight regulation of neuron production, which is primarily determined by the proliferation and differentiation of neural progenitor cells (NPCs). Perturbation of the proliferation or differentiation process results in either a reduced or excessive number of neurons, which leads to the formation of a smaller or larger brain (i.e., microcephaly or macrocephaly, respectively). In turn, this causes cortical malfunctions such as mental retardation. Cortical neurons in embryonic mouse brains are generated in the proliferative zones by two major types of NPCs: radial glial cells (RGs) (Noctor et al., 2001), and their transit-amplifying neuronal-committed progenies, intermediate progenitors (IPs) (Noctor et al., 2004). RGs located in the ventricular zone (VZ) divide asymmetrically to self-renew and give rise to either a neuron or more commonly an IP. The newly generated IPs migrate to the subventricular zone (SVZ) and divide symmetrically to generate pairs of IPs for one to three cycles before differentiating into neurons (Pontious et al., 2008). Thus, IPs are regarded as the major source of neurons (Pontious et al., 2008), and an increase in IPs relative to RGs may contribute to the expansion of the human cerebral cortex (Martínez-Cerdeño et al., 2006). Importantly, the processes of IP amplification and neuronal differentiation require spatial and temporal coordination to ensure proper neuron generation.

The generation, proliferation, and neuronal differentiation of IPs are determined by both intrinsic regulators and extrinsic signals. The sequential expression of specific transcription factors, i.e., Pax6 → Ngn2 → Tbr2 → NeuroD → Tbr1, is temporally correlated with the RG-to-IP-to-neuron transition and probably contributes to the sequential differentiation of neurons (Englund et al., 2005). Cell-cycle regulation, such as lengthening of the G1 phase and shortening of the S phase, also underlies the sequential RG-to-IP-to-neuron differentiation (Arai et al., 2011; Calegari et al., 2005), implying that cell-cycle regulators control IP amplification and neuronal differentiation. In particular, cyclinD1 and cyclin-dependent kinase 4 (Cdk4) overexpression in RGs increases the generation and expansion of IPs (Lange et al., 2009). Notably, extracellular cues including fibroblast growth factor (FGF), Notch ligands, sonic hedgehog (Shh), Wnt, transforming growth factor β (TGF-β), and retinoic acid (RA) are extensively involved in neurogenesis, probably through the regulation of transcription factors or cell-cycle regulators. While FGF (Kang et al., 2009) and Notch (Mizutani et al., 2007) signaling suppress IP generation, Shh (Komada et al., 2008) signaling induces IP amplification. Furthermore, signaling cascades activated by TGF-β (Vogel et al., 2010) and RA (Siegenthaler et al., 2009) promote neuronal differentiation. Importantly, canonical Wnt signaling has been suggested to play multiple roles in neurogenesis, including IP suppression (Chenn and Walsh, 2002; Gulacsi and Anderson, 2008), IP amplification (Kuwahara et al., 2010; Munji et al., 2011), and neuronal differentiation (Hirabayashi et al., 2004; Munji et al., 2011). Nonetheless, how these pathways are integrated and coordinated to ensure proper IP production and neuronal differentiation remains unclear.

Identifying the molecular switch that governs the transition from the generation/amplification of IPs to neuronal differentiation is critical for understanding mammalian neurogenesis. In this study, we investigated whether the scaffold protein Axin

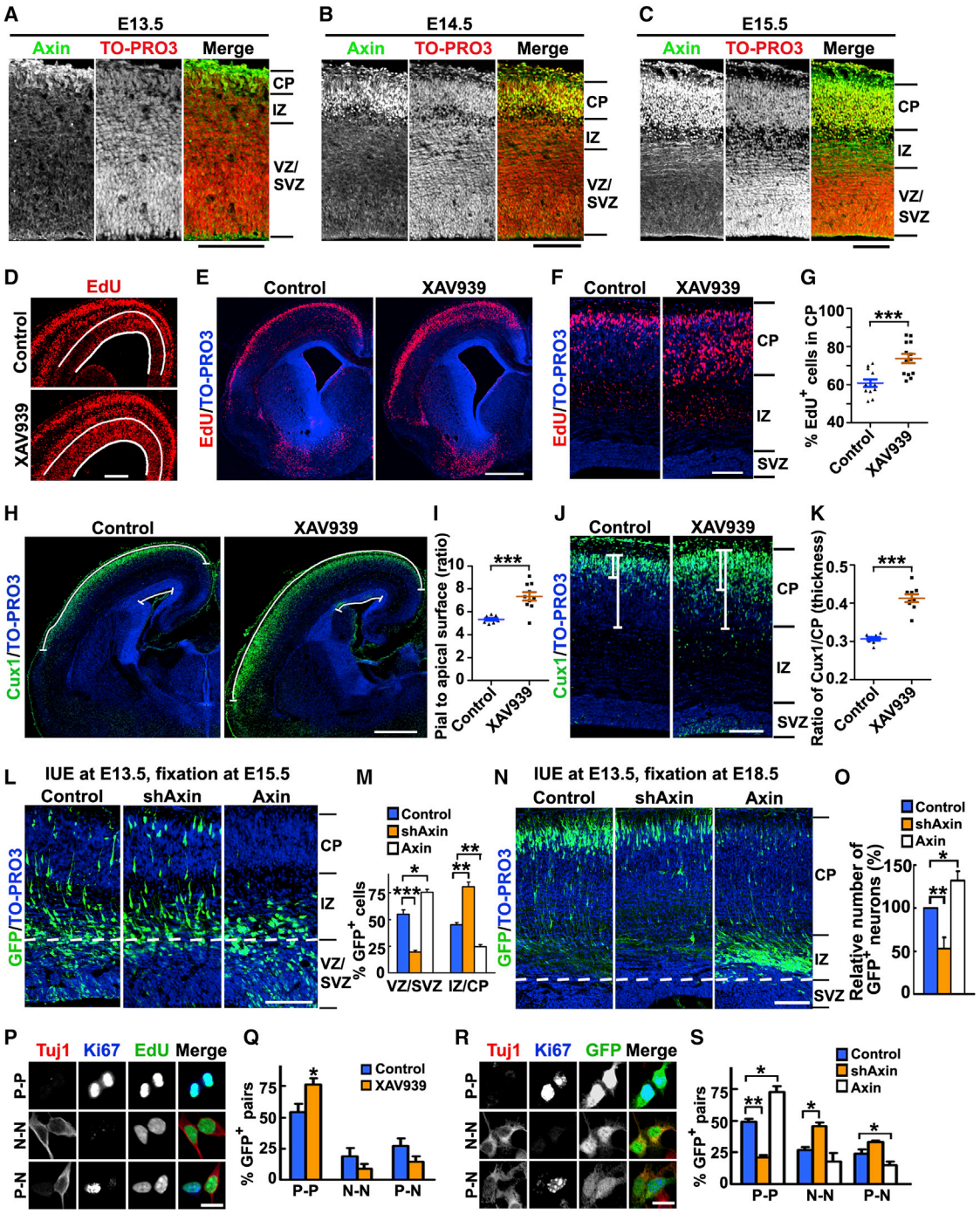

Figure 1. Axin Regulates the Amplification and Neuronal Differentiation of NPCs

(A–C) Axin expression in the mouse cortex during midneurogenesis is presented. Neocortical sections of E13.5 (A), E14.5 (B), and E15.5 (C) mice were collected and stained for Axin. TO-PRO3, a dye that labels DNA.

(D–G) The upregulation of endogenous Axin increased neural progenitor generation, resulting in enhanced neuron production. E13.5 mouse embryos were injected with an Axin stabilizer, XAV939, in utero and received one pulse of EdU labeling 2 hr (D) or 24 hr later (E–G). The embryos were then sacrificed and analyzed at E15.5 (D) or E17.5 (E–G) by EdU and TO-PRO3 staining as indicated. XAV939 administration increased the number of EdU^+ cells in the VZ/SVZ (the region within the white lines) at E15.5 (D) and the CP at E17.5 (E and F).

(H–K) Enhanced levels of endogenous Axin by XAV939 increased cortical surface area and the thickness of the cortical upper layers at E17.5. Representative images of cortical sections (H and J), ratio of pial-to-apical surface area (I), and ratio of the thickness of $Cux1^+$ upper layers to the CP (K) are shown. White lines indicate the length of the pial and ventricular surfaces (H) and the thickness of the $Cux1^+$ upper layer and CP (J). Cux1 is a marker of the upper-layer cortical neurons.

(legend continued on next page)

(Axin1) is the key molecular control. Originally identified as a tumor suppressor, the multidomain protein Axin is well characterized as a "master" scaffold for various signaling proteins including Wnt, Notch, RA, TGF-β, p53, and c-Jun N-terminal kinase (JNK)—all of which are known to control neurogenesis (Guo et al., 2008; Lyu et al., 2003; Muñoz-Descalzo et al., 2011; Rui et al., 2004). Importantly, the disruption of Axin or Axin-like proteins in *Xenopus* and zebrafish embryos leads to the development of small brains (Heisenberg et al., 2001; Yamamoto et al., 1998), resembling human microcephaly. Furthermore, the human *Axin* gene is located on the short arm of chromosome 16 at position 13.3 (16p13.3), where an unidentified recessive gene that causes microcephaly is located (Brooks et al., 2006; Kavaslar et al., 2000). These findings prompted us to determine whether and how Axin regulates embryonic neurogenesis during brain development.

Here, we show that the level and subcellular localization of Axin in NPCs determine whether they undergo amplification or neuronal differentiation. The interaction between cytoplasmic Axin and GSK-3β is critical for the amplification of the IP pool, whereas the interaction between Axin and β-catenin in the nucleus promotes neuronal differentiation. Intriguingly, the phosphorylation of Axin at Thr485 by Cdk5 shifts the subcellular localization of Axin from the cytoplasm to nucleus upon NPC differentiation, thus acting as a molecular switch that causes IPs to switch from amplification to differentiation.

RESULTS

Increased Axin Enhances Cortical Neuron Production

Axin was strongly expressed in the developing mouse neocortex from embryonic day 13.5 (E13.5) to E15.5 (Figure S1A available online). Although Axin expression was prominent in neuron-residing intermediate zone/cortical plate (IZ/CP), the protein was also detected in the VZ/SVZ, where NPCs are predominantly located (Figures 1A–1C), and was expressed in cultured NPCs (Figure S1B). As a first step to investigate whether Axin plays an important role in embryonic neurogenesis, we examined the functional consequence of increasing the endogenous level of Axin in mouse cortices at E13.5 by in utero intraventricular microinjections of a tankyrase inhibitor, XAV939 (Huang et al., 2009), which allows the transient stabilization of Axin protein (Figures S1C and S1D). After injection, Axin levels increased by 57.3% ± 5.3% at E14.5 and 29.6% ± 3.4% at E15.5 in mouse cortices (Figure S1D). Intriguingly, XAV939 injection enhanced the production of newly generated cells at E15.5 (labeled with 5-ethynyl-2′-deoxyuridine [EdU]) incorporation at E13.5), with a greater percentage of EdU^+ NPCs in the VZ/SVZ (Figure 1D; Control, 38.6% ± 3.7%; XAV939, 61.2% ± 4.3%). The enlarged NPC pool ultimately led to the generation of more upper-layer cortical neurons ($Cux1^+$; labeled with EdU at E14.5) in the CP by E17.5 (Figures 1E–1G, S1E, and S1F), possibly at the expense of deeper-layer neurons ($Ctip2^+$; Figures S1G and S1H). Robust cortical neuron production contributes to the expansion of cortical surface, which is critical for the evolutionary enlargement of the mammalian cerebral cortex (Rakic, 2009). Consistent with this notion, XAV939-injected brains exhibited greater cortical surface area (Figures 1H and 1I) and thicker upper cortical layers ($Cux1^+$; Figures 1J, 1K, and S1I–S1K) than the controls. Collectively, enhanced Axin protein levels expand the neural progenitor pool, which augments cortical neuron production, leading to tangential and radial expansion of the cerebral cortex during development.

To further investigate the functional roles of Axin, we examined the effects of Axin overexpression or knockdown in the VZ/SVZ of the mouse cortex using in utero electroporation (Fang et al., 2011) (Figures S1B and S1L). Axin overexpression increased the proportion of GFP^+ NPCs in the VZ/SVZ at E15.5 (Figures 1L and 1M), further supporting a role of Axin in maintaining/amplifying NPCs. In contrast, Axin silencing at E13.5 resulted in a remarkable reduction of GFP^+ cells in the VZ/SVZ at E15.5 with a concomitant increase in the proportion of GFP^+ cells in the IZ/CP; this suggests premature depletion of NPCs and precocious neuronal differentiation (Figures 1L and 1M). Concordantly, Axin knockdown increased the percentage of cells with enhanced neuron-specific promoter activity (i.e., cells marked by GFP expression driven by the NeuroD promoter; Figures S1M and S1N). Premature neuronal differentiation is closely associated with early cell-cycle exit. Indeed, Axin-depleted cells underwent premature cell-cycle exit as shown by a significantly higher proportion of GFP^+ EdU^+ cells in the VZ/SVZ negative for the cell proliferation marker, Ki67 (arrows in Figures S1O and S1P), with no obvious cell death (Figure S1Q). The precocious or suppressed neuronal differentiation upon Axin knockdown and overexpression ultimately resulted in reduced and increased numbers of upper-layer cortical neurons at E18.5, respectively (Figures 1N, 1O, and S1R). Notably, a significant number of Axin-overexpressing neurons were stacked in the IZ, suggesting that Axin has an alternative function in neuronal migration probably through the regulation of neuronal polarization (Fang et al., 2011). To further demonstrate that the protein level of Axin is critical for regulating NPC neurogenesis, we used in vitro pair-cell analysis to follow the division of NPCs (Figures 1P–1S). Stabilization (Figures 1P and 1Q) and overexpression of Axin (Figures 1R and 1S) both resulted in the amplification of NPCs (Figures 1Q and 1S), whereas Axin knockdown increased the tendency of

(L and M) Precise levels of Axin are critical for proper neurogenesis. In utero electroporation of E13.5 mouse embryos with pSUPER (Control), pSUPER-Axin shRNA (shAxin), and pCAGI2G-Axin (Axin) was performed (L), and the percentages of GFP^+ cells in the VZ/SVZ and IZ/CP at E15.5 were quantified (M).
(N and O) Axin knockdown and overexpression at E13.5 reduced and increased neuron production by E18.5, respectively. The number of GFP^+ neurons in the IZ/CP of the Control group was set as 100%, and the relative number of GFP^+ neurons in the shAxin or Axin groups was quantified (O).
(P–S) Axin regulated NPC amplification and neuronal differentiation in vitro. (P and Q) XAV939 treatment increased neural progenitor amplification. Cultured NPCs were prelabeled with EdU for 1 hr and treated with XAV939 for 24 hr. Tuj1, a neuronal marker; Ki67, a mitotic cell marker. (R and S) Loss of Axin depleted NPCs, whereas overexpression expanded the NPC pool. NPCs were dissociated from the mouse cortex at E14.5 after Axin knockdown or overexpression at E13.5. DCX, immature neuronal marker. (Q and S) Quantification of the three types of cell division: P-P, N-N, and P-N. P, neural progenitor cell; N, neuron.
Scale bars, 500 μm (D, E, and H), 100 μm (A–C, F, J, L, and N), and 10 μm (P and R). Error bars indicate SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Control; Student's t test. See also Figure S1.

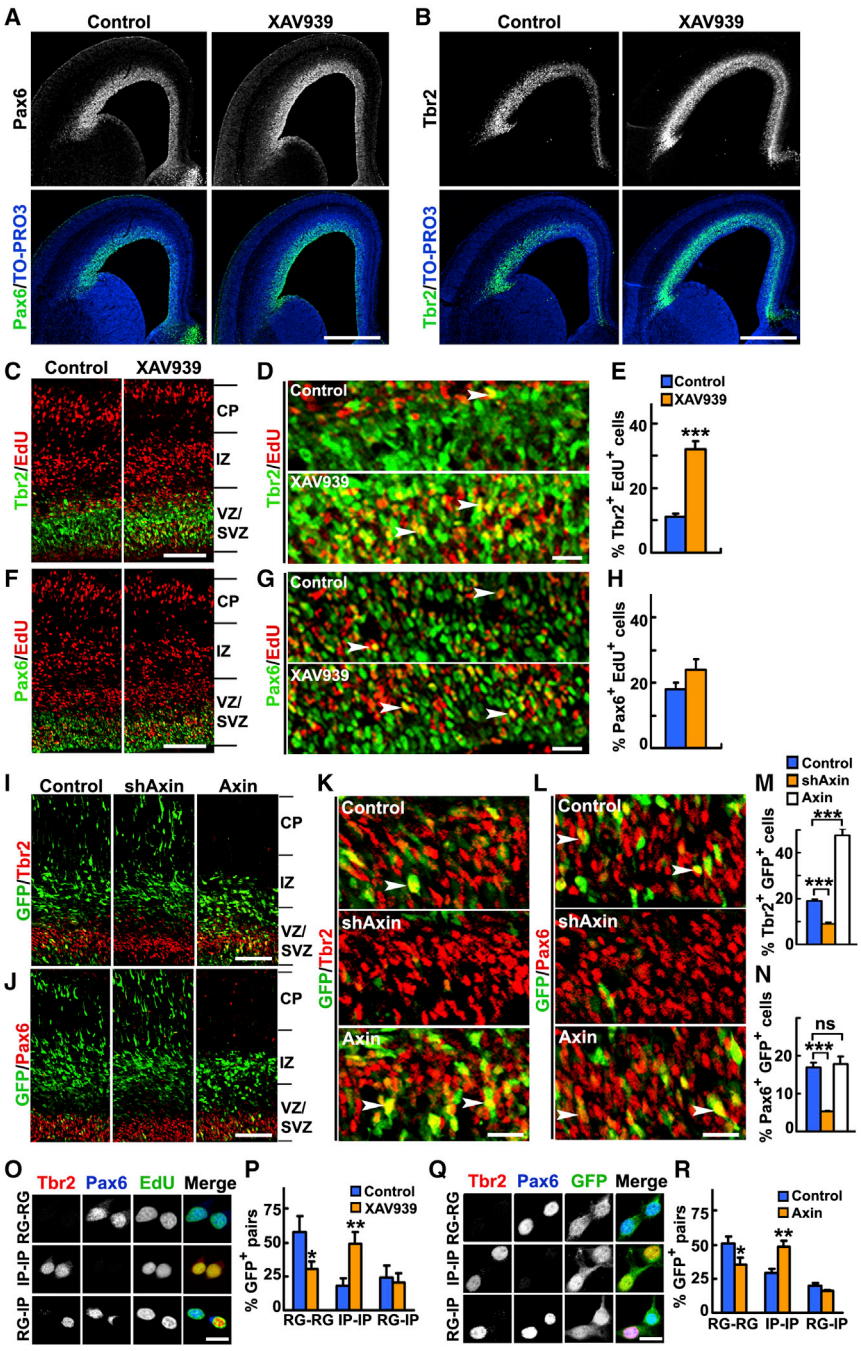

Figure 2. Increased Axin Levels Enhance IP Generation and Amplification

(A–H) Increasing endogenous Axin levels expanded the IP pool without affecting the number of RGs. (A and B) XAV939 injection at E13.5 increased the number of IPs ($Tbr2^+$) without affecting the pool of RGs ($Pax6^+$) at E15.5.

(C–H) After XAV939 injection at E13.5 for 2 hr, the embryos received one pulse of EdU labeling. The brain sections were then analyzed at E15.5 by EdU and neural progenitor marker (Pax6 or Tbr2) staining as indicated. (D and G) High-magnification images of the VZ/SVZ. Arrowheads indicate $Tbr2^+$ EdU^+ IPs (D) and $Pax6^+$ EdU^+ RGs (G). (E and H) Analyses of the percentages of $Tbr2^+$ EdU^+ IPs or $Pax6^+$ EdU^+ RGs over total EdU^+ cells.

(I–N) Axin is required for IP production and amplification. Mouse embryos were electroporated with pSUPER (Control), pSUPER-Axin shRNA (shAxin), or pCAGI2G-Axin (Axin) in utero at E13.5 (I and J), and the percentages of $Tbr2^+$ GFP^+ and $Pax6^+$ GFP^+ cells at E15.5 were quantified (M and N). (K and L) High-magnification images of the VZ/SVZ. Arrowheads indicate $Tbr2^+$ GFP^+ IPs (K) and $Pax6^+$ GFP^+ RGs (L).

(O–R) Increased Axin level in RGs promoted IP production in vitro. In vitro pair-cell analyses after XAV939 treatment (O) and Axin overexpression in RGs (Q) are shown. (P and R) Quantification of the three types of cell differentiation (i.e., RG-RG, IP-IP, RG-IP).

Scale bars, 500 μm (A and B), 100 μm (C, F, I, and J), 20 μm (D, G, K, and L), and 10 μm (O and Q). Error bars indicate SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Control; Student's t test. ns, not significant. See also Figure S2.

NPCs to divide and differentiate into two neurons (Figure 1S). Thus, the regulation of Axin protein levels in NPCs at midneurogenesis is critical for generating the proper number of neurons during brain development, probably through the control of NPC amplification and neuronal differentiation.

Cytoplasmic Axin Promotes the Amplification of IPs

There are two major cell types of NPCs: RGs and IPs. Although RGs are predominantly found in the mouse cortex for maintaining the NPC pools, IPs are transient, amplifying neurogenic progenitors that contribute to cortical neuron production. Intriguingly, although the number of $Pax6^+$ RGs in the cortex of XAV939-injected embryos was not substantially different from that of the controls at E15.5 (Figure 2A), there were more $Tbr2^+$ IPs in the XAV939-injected brains than the controls (Figure 2B). Given that XAV939 administration did not inhibit neuronal differentiation (Figures 1E–1G) or affect cell survival (data not shown), the increased number of IPs may be due to enhanced IP generation. Therefore, we labeled mitotic RGs with EdU 2 hr after XAV939 injection and traced the fates of their progenies at E15.5. XAV939-injected brains exhibited a markedly increased proportion of $Tbr2^+$ EdU^+ IPs (Figures 2C–2E), whereas the $Pax6^+$ EdU^+ RG pool remained relatively unchanged (Figures 2F–2H); this suggests that Axin upregulation enhances IP generation. Consistent with this finding, Axin overexpression at E13.5 resulted in a significant increase in the IP population (Figures 2I, 2K, and 2M) without affecting the RG pool at E15.5 (Figures 2J, 2L, and 2N). The expansion of IPs may be attributable to either increased proliferation of IPs or enhanced

differentiation from RGs. The proportion of mitotic IPs ($pH3^+$ $Tbr2^+$) remained relatively unchanged when Axin was stabilized or overexpressed (Figures S2A–S2H), suggesting that Axin does not markedly affect IP proliferation. Furthermore, Axin overexpression at E12.5 led to an enlarged IP pool and concomitantly a reduced number of deeper-layer neurons (Figures S2I–S2O); this indicates that Axin expression causes a shift of neuronal differentiation from RGs toward IP generation. Collectively, Axin upregulation in midneurogenesis enhances IP amplification, which contributes to increased upper-layer neuron production ($Cux1^+$; Figures 1E–1K). In addition, consistent with the observation that Axin knockdown resulted in premature neuronal differentiation (Figures 1L and 1M), shAxin-electroporated brains exhibited significant reductions in the populations of both RGs and IPs (Figures 2I–2N), suggesting that Axin is required for the maintenance/amplification of RGs and IPs. Furthermore, in vitro pair-cell analysis revealed that both stabilization (Figures 2O and 2P) and overexpression of Axin (Figures 2Q and 2R) in RGs increased the number of IP-IP progeny pairs, supporting a role of Axin in facilitating IP generation and amplification.

Nuclear Accumulation of Axin Switches IP Amplification to Differentiation

Next, we investigated how increased Axin levels enhance IP generation. Axin was mainly localized to the cytoplasm of NPCs in the VZ/SVZ at E13.5 (Figure 3A), whereas the protein was gradually enriched in the nuclei of a subset of NPCs (E13.5–E15.5, Figures 3A and S3A–S3C). Therefore, we hypothesized that the subcellular localization of Axin is regulated differently in different types of NPCs. To further characterize the subcellular localization of Axin in NPCs, cultured NPCs were prepared from embryonic mouse cortices and stained for Axin. Although Axin was predominantly expressed in the cytoplasm (83.2% ± 6.8% of total Axin) and was weakly detectable in the nuclei (16.8% ± 3.3% of total Axin) of RGs ($nestin^+$), the protein became more enriched in the nuclei of $Tbr2^+$ IPs (53.3% ± 3.1% of total Axin; Figure 3B). Furthermore, when NPCs were cultured in neuronal differentiation medium for 3 hr, Axin level increased significantly in the nucleus (3.8 ± 0.7-fold) and decreased significantly in the cytoplasm (by 60.4% ± 6.2%) in differentiating NPCs (Figure 3C). Therefore, these findings indicate that Axin accumulates in the nuclei of NPCs in response to differentiation signals.

The nucleocytoplasmic shuttling of Axin is tightly controlled by the nuclear localization signal (NLS) and nuclear export signal (NES) of the protein (Cong and Varmus, 2004). To elucidate the specific roles of cytoplasmic and nuclear Axin, we generated two point mutants of Axin, allowing the protein to be expressed specifically in the cytoplasm (Axin-NLSm) or nucleus (Axin-NESm) (Figure 3D). Like wild-type Axin, the overexpression of cytoplasmic Axin (Axin-NLSm) at E13.5 increased the proportion of GFP^+ cells in the VZ/SVZ at E15.5 (Figures 3E and 3F), suggesting that cytoplasmic Axin enhances NPC expansion. Furthermore, the re-expression of Axin-NLSm in Axin-knockdown NPCs also led to NPC pool expansion (Figures 3G–3L) specifically through the enlargement of the IP population (Figures 3H, 3J, and 3L). In contrast, the expression of nuclear Axin (Axin-NESm) (Figures 3E and 3F) or re-expression of the protein in Axin-knockdown NPCs depleted the GFP^+ NPCs in the VZ/SVZ and promoted the differentiation of NPCs into neurons (Figures 3G–3L). Together with the nuclear accumulation of Axin in cultured NPCs upon differentiation (Figures 3A–3C), these findings strongly suggest that Axin in different subcellular compartments of NPCs specifically regulates the amplification and differentiation of NPCs; cytoplasmic Axin in RGs enhances IP amplification, whereas Axin in the nucleus of IPs promotes neuronal differentiation of IPs.

Phosphorylation-Dependent Subcellular Localization of Axin Controls Neurogenesis

Next, we investigated the molecular mechanism that controls the trafficking of Axin between the cytoplasm and nucleus. Treating RGs with leptomycin B led to the nuclear accumulation of Axin (Cong and Varmus, 2004) (Figure S4A), suggesting that the nuclear enrichment of Axin is regulated by nuclear export. It was noted that the Cdk5-dependent phosphorylation site (Thr485) is located close to the NES of Axin (amino acids 413–423) (Fang et al., 2011) (Figure 4A). Although Axin phosphorylation at Thr485 (p-Axin) could be detected in wild-type mouse neocortices at E13.5, this specific phosphorylation was markedly reduced in $cdk5^{-/-}$ littermates (by 45.5% ± 4.3%; Figures 4B, S4B, and S4C), indicating that Cdk5 is a major kinase that phosphorylates Axin during neurogenesis in vivo. Importantly, the nuclear level of Axin was reduced in $cdk5^{-/-}$ neocortices (by 68.2% ± 5.1%) accompanied by an increased level of cytoplasmic Axin (2.0 ± 0.3-fold; Figure 4B). These results suggest that Cdk5-dependent Axin phosphorylation is critical for controlling the nuclear localization of Axin in the embryonic cerebral cortex.

To explore the role of Cdk5-mediated Axin phosphorylation, we examined how Axin phosphorylation is regulated in NPCs. Although basal levels of p-Axin were detected in the nuclear compartments of cultured NPCs, protein phosphorylation was markedly upregulated upon neuronal differentiation (by 2.1 ± 0.3-fold; Figure 4C). Furthermore, in the developing cortex, p-Axin was prominently detected in the nuclei of a subset of NPCs, primarily the IPs (arrowheads in Figures 4D, S4E, and S4F). The proportion of p-$Axin^+$ NPCs increased progressively from E13.5 to E15.5 (Figure 4E), and p-Axin was found in the VZ/SVZ of the mouse neocortex at a rostrolateral-high to caudomedial-low gradient (Figures S4G–S4N)—a spatial profile similar to the gradient of neurogenesis (Caviness et al., 2009). These observations suggest that increased phosphorylation of Axin at Thr485 is associated with neurogenesis. Notably, the mitotic RGs lining the ventricular surface of the VZ were prominently labeled with p-Axin (Figure S4D); this phosphorylation was not substantially reduced in $cdk5^{-/-}$ cortices (Figure S4C), indicating that Axin phosphorylation in these mitotic cells is likely mediated by other kinase(s). Importantly, *cdk5* knockout (Figure 4F) or blockade of Cdk5 activity by the overexpression of the dominant-negative Cdk5 mutant (Cdk5-DN) resulted in an expansion of the IP pool at E15.5 (Figures 4G–4J); this further supports a role of Cdk5 in the regulation of neuronal differentiation of IPs, probably through phosphorylation and hence the nuclear localization of Axin.

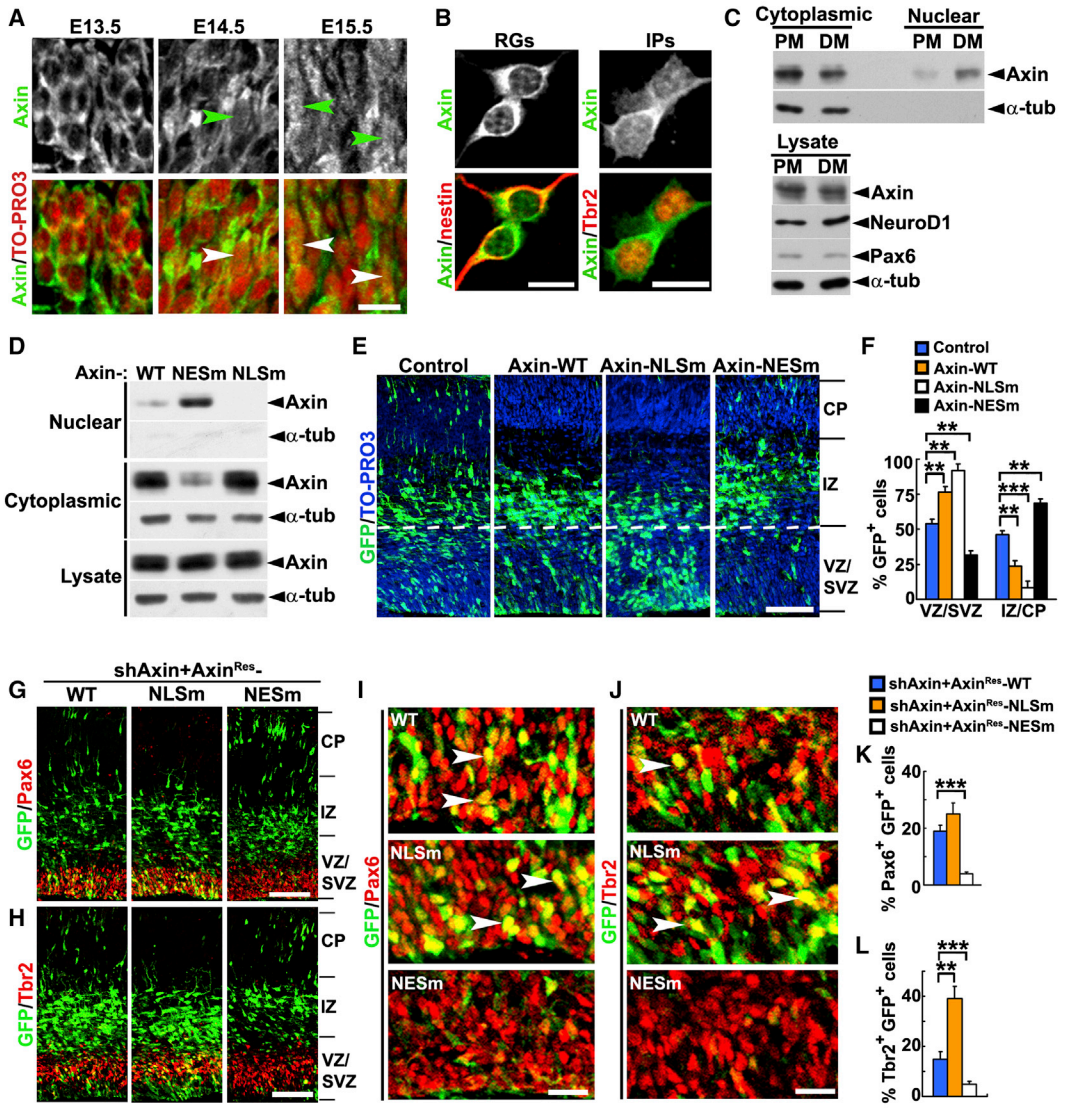

Figure 3. The Translocation of Axin from the Cytoplasm to the Nucleus in NPCs Regulates the Shift from IP Amplification to Neuronal Differentiation

(A) Axin accumulated in the nuclei of NPCs upon development. The proportion of NPCs with nuclear expression of Axin (arrowheads) increased progressively from E13.5 to E15.5.

(B) Axin staining in cultured NPCs (RGs, nestin$^+$; IPs, Tbr2$^+$) is shown.

(C) Axin was upregulated in the nuclei of cultured NPCs upon neuronal differentiation. PM, proliferation medium; DM, neuronal differentiation medium. Note that the level of NeuroD1, a proneural protein, was upregulated in NPCs upon DM stimulation. α-Tubulin (tub) served as a cytoplasmic marker and loading control.

(D–F) Cytoplasmic Axin amplified the NPC pool, whereas nuclear Axin promoted neuronal differentiation. (D) Subcellular distribution of Axin and its point mutants (Axin-NESm and Axin-NLSm) in HEK293T cells is shown. (E and F) E13.5 mouse embryos were electroporated with Axin mutants (Axin-NLSm, cytoplasmic Axin; or Axin-NESm, nuclear Axin) in utero and examined at E15.5. Distribution of GFP$^+$ cells (F).

(G–L) Cytoplasmic Axin amplified the IP pool. In utero electroporation of shAxin together with the shRNA-resistant Axin-WT and its mutants (NLSm and NESm) is shown. (I and J) High-magnification images of the VZ/SVZ. Arrowheads indicate GFP$^+$ Pax6$^+$ RGs (I) and GFP$^+$ Tbr2$^+$ IPs (J). Analyses of the percentages of Pax6$^+$ GFP$^+$ RGs (K) and Tbr2$^+$ GFP$^+$ IPs over total GFP$^+$ cells (L) are presented.

Scale bars, 100 μm (E, G, and H), 20 μm (I and J), and 10 μm (A and B). Error bars indicate SEM. ***$p < 0.001$, **$p < 0.01$ versus Control (F) and shAxin+Axin^{Res}-WT (K and L); Student's t test. See also Figure S3.

Next, we investigated the role of the Cdk5-dependent Axin phosphorylation in neuronal differentiation by examining the effects of overexpressing phospho-mimetic (Axin-TE) and phospho-deficient Axin (Axin-TA) mutants in NPCs. Axin-TE mutant was concentrated in the nucleus, whereas Axin-TA mutant was exclusively detected in the cytoplasm (Figure 5A). Re-expression of the Axin-TE mutant in Axin-knockdown cortices promoted the neuronal differentiation of NPCs.

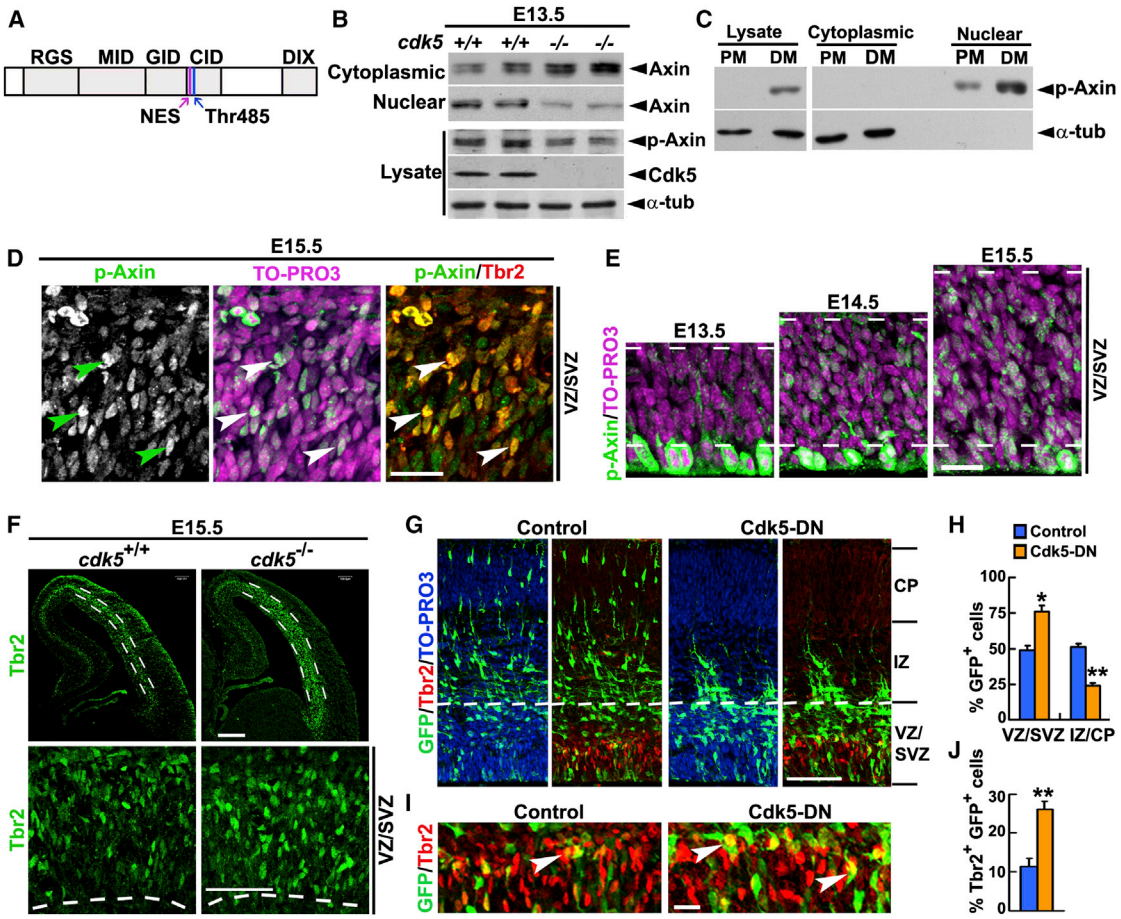

Figure 4. Cdk5-Dependent Axin Phosphorylation Regulates Neurogenesis

(A) Schematic representation of Axin protein.

(B) The phosphorylation of Axin by Cdk5 is critical for the nuclear localization of Axin. Cytoplasmic and nuclear fractions of forebrains from E13.5 *cdk5*$^{-/-}$ mice and wild-type littermates were subjected to western blot analysis for Axin and Axin phosphorylated at Thr485 (p-Axin).

(C) The p-Axin protein accumulated in the nuclear fraction of cultured NPCs upon neuronal differentiation. α-Tubulin served as a cytoplasmic marker and loading control.

(D) The p-Axin protein was mainly concentrated in the nuclei of IPs. The majority of p-Axin^{+} NPCs were Tbr2^{+} IPs (nuclei were labeled with TO-PRO3).

(E) The expression of p-Axin increased progressively in the nuclei of NPCs in the VZ/SVZ from E13.5 to E15.5.

(F–J) Blockade of Cdk5 activity resulted in IP expansion at E15.5. (F) *cdk5*$^{-/-}$ mice exhibited a wider and much more densely packed band of IPs at E15.5. (G–J) Overexpression of a Cdk5 dominant-negative mutant (Cdk5-DN) inhibited neuronal differentiation (G and H) and increased the number of IPs (I and J). High-magnification images show the costaining of GFP and Tbr2 (arrowheads in I).

Scale bars, 200 μm (F, upper panels), 100 μm (F, lower panels; G), and 20 μm (D, E, and I). Error bars indicate SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ versus Control; Student's t test. See also Figure S4.

Meanwhile, Axin-TA mutant inhibited neuronal differentiation and led to the amplification of NPCs (Figures 5B and 5C), mainly IPs (Figures 5D–5I). Furthermore, the impaired neurogenesis due to Cdk5-DN expression was partially rescued by the coelectroporation of either the Axin-TE or Axin-NESm mutant (Figures 4G–4J and S5A–S5D). These findings provide further evidence that Cdk5-dependent Axin phosphorylation and hence the phosphorylation-dependent nuclear localization of the protein are critical for promoting neuronal differentiation. To further investigate how p-Axin regulates the neuronal differentiation of IPs, we examined the regulation of p-Axin in IPs with respect to cell-cycle progression. The S phase cells in developing mouse brains were pulse labeled with EdU. The EdU-labeled cells in the upper VZ and lower SVZ were in the S and G2 phases within the first 0.5 and 2 hr after EdU injection, respectively, and required 14 hr to reach the late G1 phase (Britz et al., 2006). p-Axin was concentrated in the nuclei of most progenies of the EdU-labeled cells at the G1 phase (80.1% ± 8.9%; Figure 5J) but was barely detected in the EdU^{+} cells in the S or G2 phases (13.4% ± 4.1%; Figure 5K). The observation that most p-Axin^{+} cells in the upper VZ and lower SVZ were IPs (Figure 4D) suggests that p-Axin^{+} IPs exit cell cycling at the G1 phase (Dehay and Kennedy, 2007). Therefore, we conclude that the Cdk5-dependent phosphorylation of Axin at Thr485 maintains the nuclear accumulation of Axin in IPs and promotes neuronal differentiation.

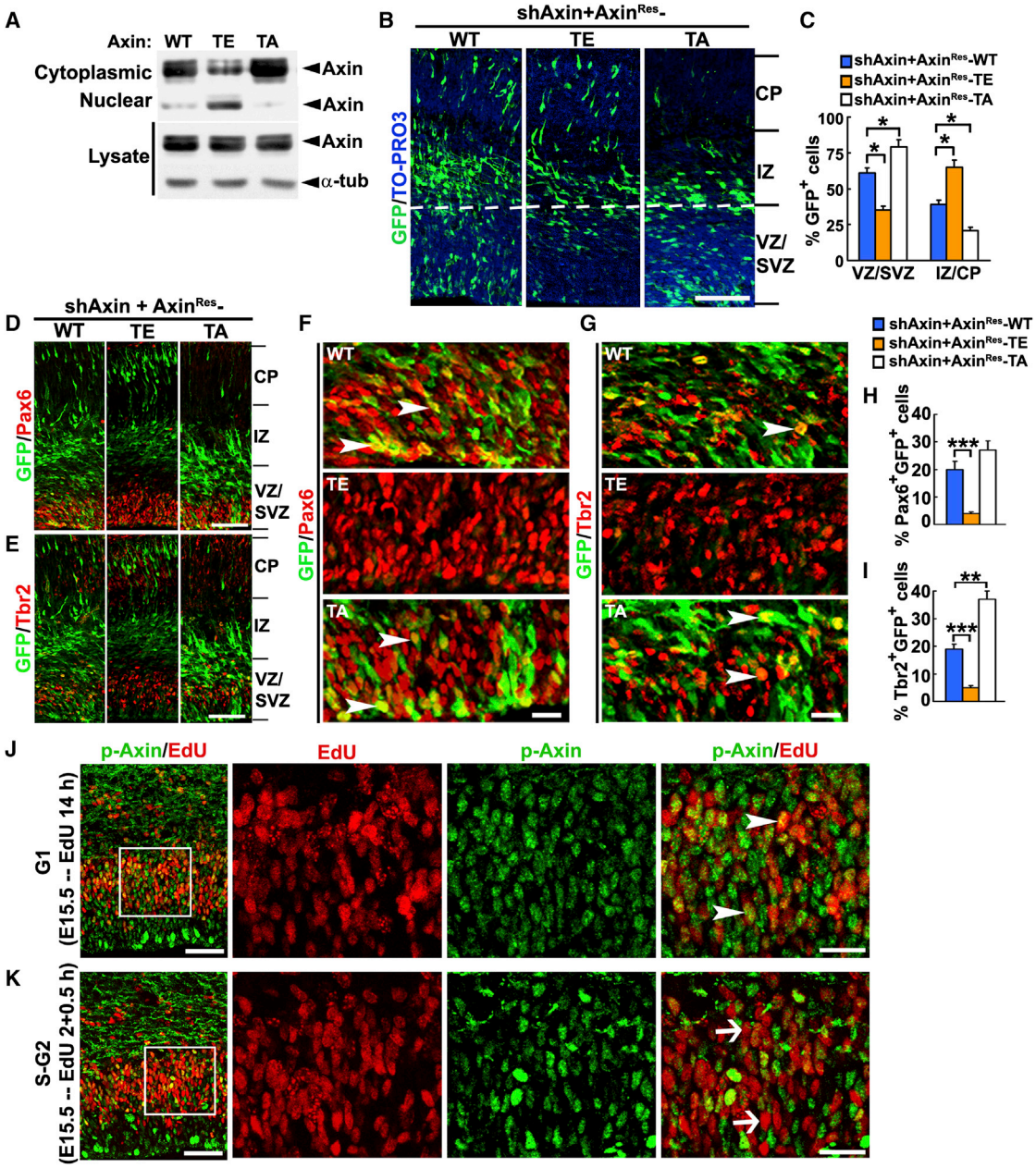

Figure 5. The Phosphorylation of Axin at Thr485 Is Required for the Neuronal Differentiation of IPs

(A) Subcellular distribution of Axin-WT and its mutants (TE, a phospho-mimetic mutant; TA, a phospho-deficient mutant) in HEK293T cells is shown.

(B–I) The phosphorylation of Axin at Thr485 promotes neuronal differentiation. Mouse embryos at E13.5 were electroporated with shAxin together with shRNA-resistant Axin-WT, Axin-TE, or Axin-TA. (B and C) Re-expression of Axin-TE in Axin-knockdown cortices increased the percentage of GFP^+ cells in the IZ/CP, whereas Axin-TA mutant enhanced the percentage of GFP^+ cells in the VZ/SVZ. (D–G) Images of fluorescent staining for Pax6 and Tbr2 are shown. (F and G) High-magnification images of the VZ/SVZ are presented. Arrowheads indicate $Pax6^+$ GFP^+ RGs (F) and $Tbr2^+$ GFP^+ IPs (G). Analyses of the percentages of $Pax6^+$ GFP^+ RGs (H) and $Tbr2^+$ GFP^+ IPs (I) over total GFP^+ cells are shown.

(J and K) The p-Axin protein accumulated in the nuclei of IPs at the G1 phase, but not at the S or G2 phase. Pregnant mice at day 15.5 of gestation were intraperitoneally injected with EdU, and newborn cells in the embryonic brains at different cell-cycle phases were identified by their positive labeling with EdU after discrete time periods. In the upper VZ and lower SVZ (boxed area), p-Axin protein was detected in the nuclei of the majority (~80%) of EdU^+ cells in the G1 phase (J) but barely found in the nuclei of cells in the S and G2 phases (K). Arrowheads (J) and arrows (K) indicate EdU^+ cells with or without nuclear p-Axin, respectively. Scale bars, 100 μm (B, D, and E), 50 μm (J and K, right panels), and 20 μm (F and G; J and K, left panels). Error bars indicate SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus shAxin+$Axin^{Res}$-WT; Student's t test. See also Figure S5.

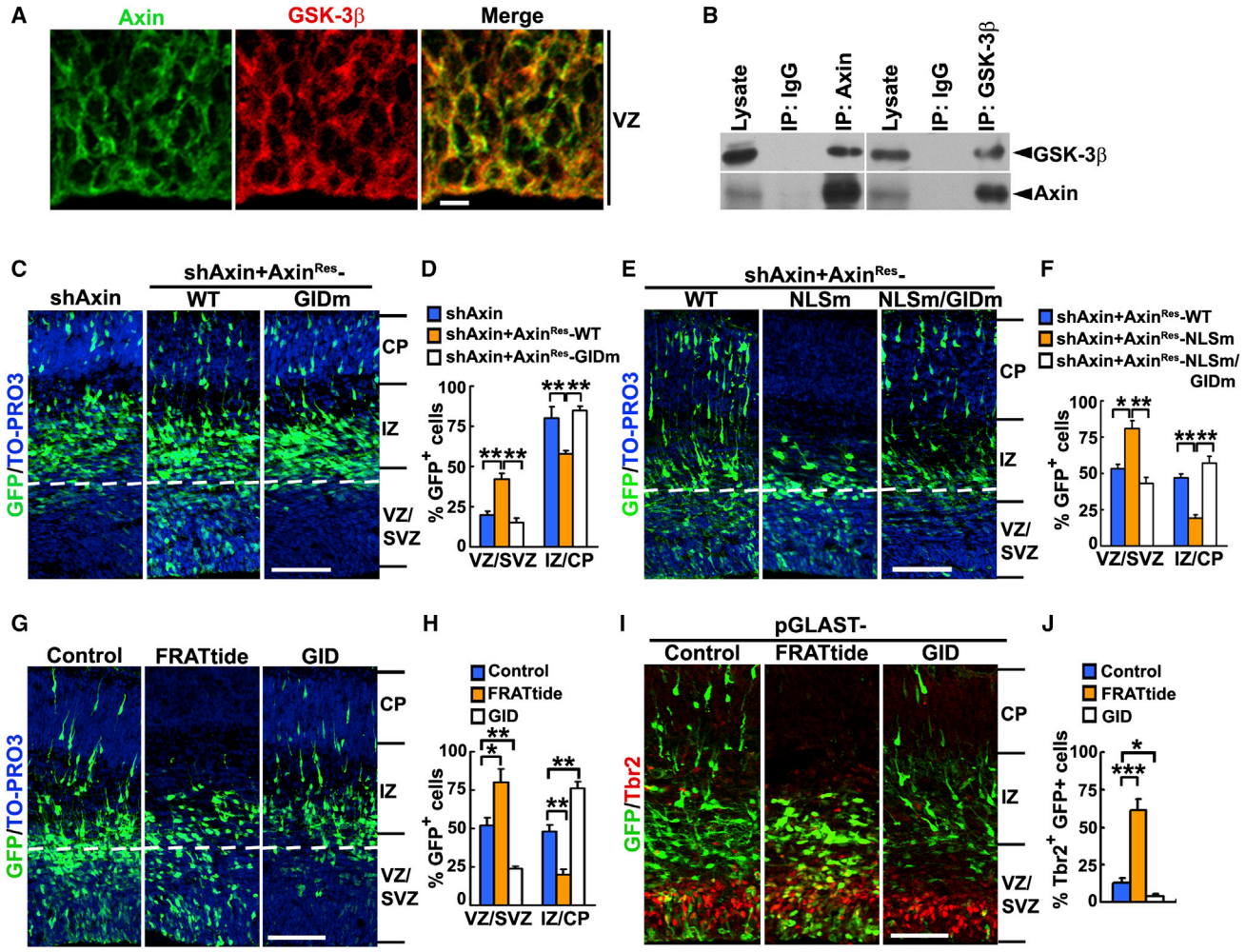

Figure 6. Cytoplasmic Axin Promotes IP Amplification via a Mechanism Dependent on Its Interaction with GSK-3β

(A) Axin and GSK-3β were highly expressed and colocalized in the cytoplasm of NPCs in the VZ.

(B) Axin interacted with GSK-3β in the cytoplasmic fraction of cultured NPCs from E13.5 brains.

(C–F) The binding of cytoplasmic Axin to GSK-3β is required to maintain and expand the NPC pool. E13.5 mouse embryos were electroporated in utero with constructs as indicated and analyzed at E15.5. GIDm, the Axin point mutant that cannot interact with GSK-3β; NLSm, the Axin mutant expressed exclusively in the cytoplasm. (D and F) Quantification of the distribution of GFP^+ cells is shown.

(G–J) Modulating the interaction between Axin and GSK-3β by small peptides regulated the amplification of the NPC pool (G and H) and the generation of IPs from RGs (I and J). FRATtide, a peptide that enhanced Axin-GSK-3β interaction; GID, a peptide that abolished Axin-GSK-3β interaction. The expressions of small peptides were driven by a constitutive promoter (pCAGI2G; G) or the radial glia-specific promoter (pGLAST; I).

Scale bars, 100 μm (C, E, G, and I) and 10 μm (A). Error bars indicate SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus shAxin+$Axin^{Res}$-WT (D and F) or Control (H and J); Student's t test. See also Figure S6.

Axin-GSK-3β Interaction Mediates IP Amplification

How does cytoplasmic Axin amplify IPs? The size of the IP pool is negatively regulated by multiple pathways including Wnt (Munji et al., 2011), Notch (Mizutani et al., 2007), and FGF (Kang et al., 2009) signaling; each of these pathways can be modulated by a key regulator, GSK-3 (Kim et al., 2009b). Axin colocalized and interacted with GSK-3β in the cytoplasmic compartment of NPCs at E13.5 (Figures 6A, 6B, and S6A). Notably, the re-expression of an Axin point mutant that failed to bind GSK-3β (GIDm) (Fang et al., 2011) in Axin-knockdown cortices abolished the ability of cytoplasmic Axin to enhance NPC amplification, resulting in early neuronal differentiation (Figures 6C–6F, S6B, and S6C). Therefore, our findings suggest that cytoplasmic Axin expands the NPC (i.e., IP) population in a GSK-3β-dependent manner. To confirm this finding, we utilized small peptides, FRATtide (Bax et al., 2001) and GID peptide (Hedgepeth et al., 1997), which can enhance and block Axin-GSK-3β interaction, respectively (Figures S6D and S6E), and examined their effects on the regulation of the fate of NPCs. FRATtide expression led to the enlargement of the NPC pool (Figures 6G and 6H) and promoted the generation of IPs from RGs (Figures 6I, 6J, S6F, and S6G); meanwhile, GID peptide depleted the NPC pool (Figures 6G–6H) and promoted the direct neuronal differentiation of RGs (Figures 6G–6J, S6F, and S6G). Thus, Axin in the cytoplasm

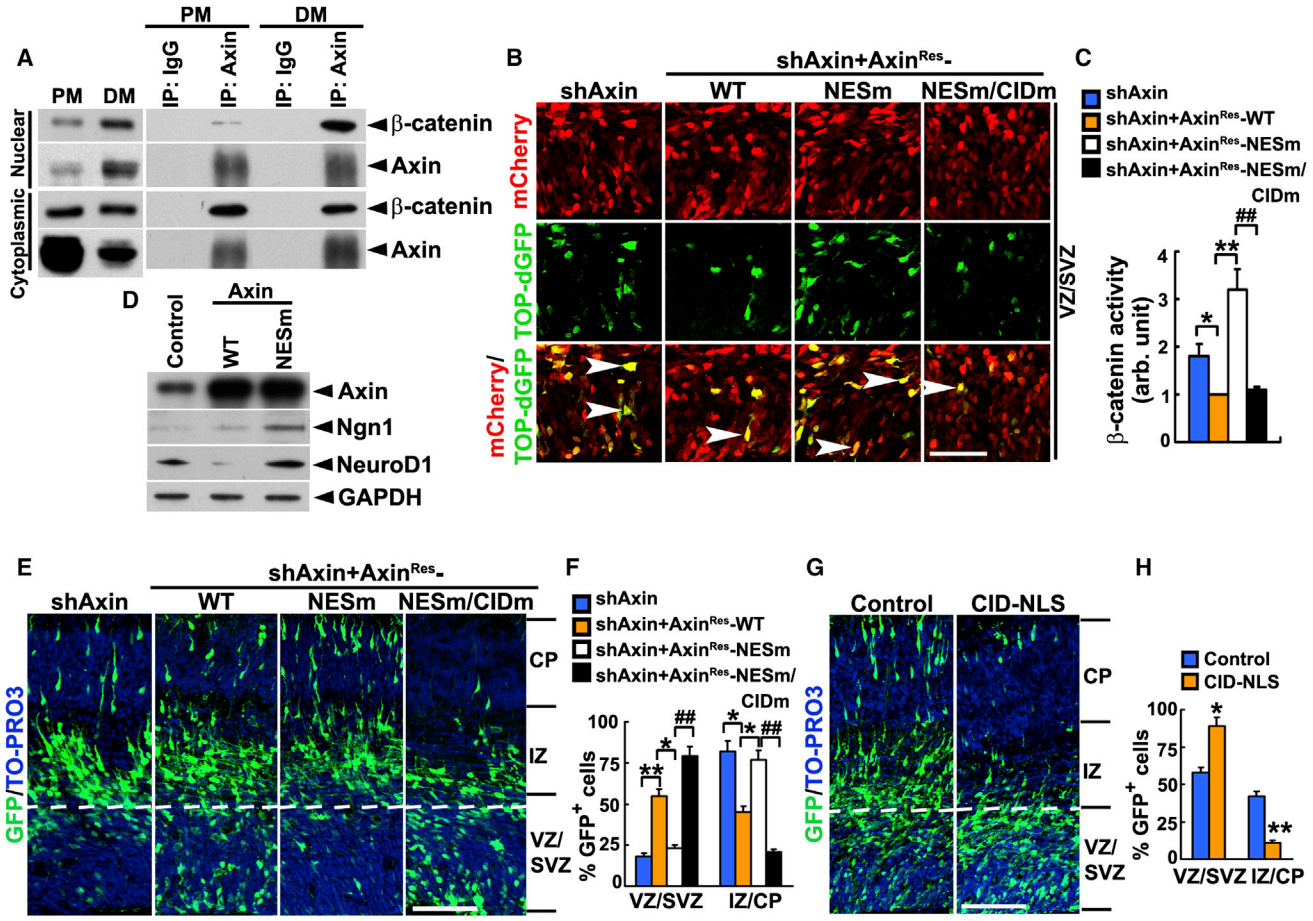

Figure 7. Nuclear Axin Enhances Neuronal Differentiation via Binding to β-Catenin

(A) Protein levels of Axin and β-catenin and their interaction were markedly increased in the nuclei of cultured NPCs upon neuronal differentiation.

(B and C) Nuclear interaction between Axin and β-catenin enhanced the transcriptional activity of β-catenin. E13.5 mouse embryos were electroporated with the TOP-dGFP/mCherry construct together with various plasmids (as indicated) in utero and examined at E15. The transcriptional activity of β-catenin was monitored by the expression of a destabilized GFP driven by the β-catenin-responsive element, TOP-dGFP (B), and the ratio of the number of GFP^+ $mCherry^+$ cells (arrowheads) to the number of $mCherry^+$ cells was quantified (the ratio of the shAxin+$Axin^{Res}$-WT group was set as 1; C). arb. unit, arbitrary unit.

(D) Overexpression of nuclear Axin in cultured NPCs increased the expression of β-catenin proneural target proteins (Ngn1 and NeuroD1).

(E and F) Nuclear interaction between Axin and β-catenin was required for neuronal differentiation. (E) CIDm, the Axin point mutant unable to bind to β-catenin; NESm, the Axin mutant whose expression was confined to the nucleus. (F) Quantification of the distribution of GFP^+ cells is shown.

(G and H) Blockade of the nuclear interaction between Axin and β-catenin by a nuclear-targeting CID peptide, CID-NLS, inhibited neuronal differentiation.

Scale bars, 100 μm (E and G) and 50 μm (B). Error bars indicate SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ versus shAxin+$Axin^{Res}$-WT (C and F) or Control (H); ##$p < 0.01$ versus shAxin+$Axin^{Res}$-NESm (C and F); Student's t test. See also Figure S7.

of RGs enhances IP amplification via a mechanism dependent on its interaction with GSK-3β.

Axin-β-Catenin Interaction Enhances Neuronal Differentiation

Next, we investigated how nuclear Axin promotes neuronal differentiation. Axin was progressively enriched in the nuclei of NPCs upon neuronal differentiation (Figures 3C and 7A). The neuronal differentiation of progenitors was marked by the prominent upregulation of proneural target genes of β-catenin (including Ngn1 and NeuroD1) (Hirabayashi et al., 2004; Kuwabara et al., 2009) together with reduced levels of antineural β-catenin targets (e.g., Cyclin D1 and N-myc) (Clevers, 2006; Kuwahara et al., 2010) (Figure S7A). Axin interacted with β-catenin in the nuclear compartments of differentiating NPCs (Figure 7A). Although the nuclear accumulation of β-catenin is important for its transcriptional activity, the nuclear accumulation of Axin and hence its interaction with β-catenin were not prerequisites for the nuclear localization of β-catenin; this is because Axin level was significantly reduced (Figure 4B), whereas β-catenin level remained unchanged (Figures S7B and S7C), in the nuclei of *cdk5*$^{-/-}$ cells. Nonetheless, the interaction between Axin and β-catenin was critical for nuclear Axin to enhance β-catenin transcriptional activity in the NPCs of the mouse neocortex (Figures 7B, 7C, S7D, and S7E), as indicated by the TOP-dGFP reporter assay (Zhang et al., 2010b). Axin overexpression in NPC nuclei increased the levels of proneural targets of β-catenin, Ngn1 and NeuroD1, by 4.3 ± 0.3-fold and

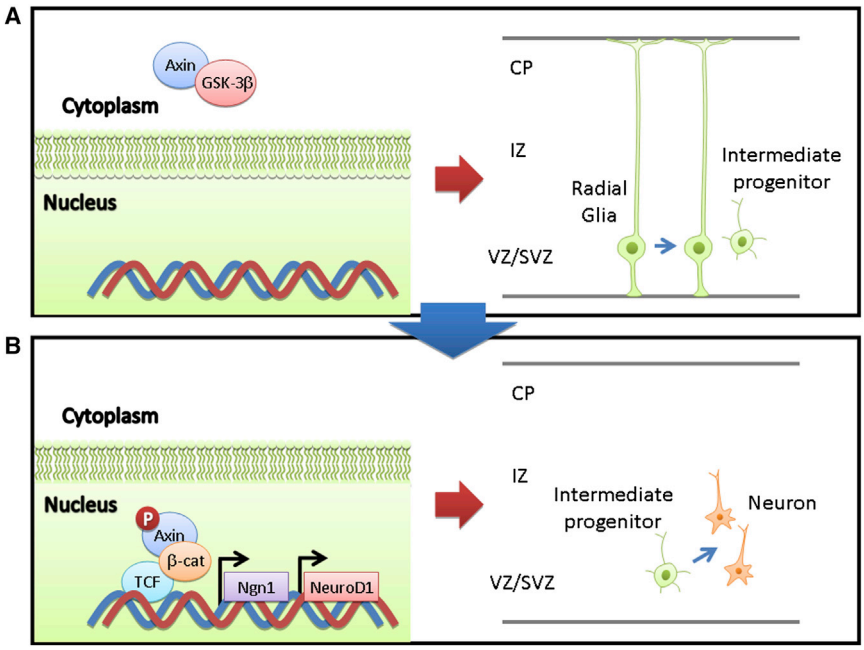

Figure 8. Subcellular Axin Localization Determines the Amplification and Neuronal Differentiation of NPCs during Embryonic Neurogenesis via the Activation of Specific Signaling Networks in NPCs

(A) Axin in the cytoplasm of RGs maintains RG self-renewal and promotes IP generation through its interaction with GSK-3β.

(B) Upon development, Axin is phosphorylated at Thr485 and becomes enriched in the nuclei of NPCs, particularly IPs, triggering neuronal differentiation through binding to β-catenin.

0.7 ± 0.2-fold, respectively (Figure 7D). Intriguingly, blocking the interaction between nuclear Axin and β-catenin by expressing the Axin point mutant (CIDm) that was unable to bind β-catenin in the nucleus (Xing et al., 2003) inhibited neuronal differentiation and maintained the NPC pool (Figures 7E and 7F), particularly IPs (Figures S7F and S7G). To further confirm the importance of the interaction between Axin and β-catenin in the nucleus, we designed a small peptide CID based on the protein sequence of the β-catenin-interacting domain of Axin (Xing et al., 2003) and tagged the peptide with an SV40 T-antigen NLS to enable specific targeting of the CID peptide into the nucleus. CID-NLS effectively blocked the interaction between Axin and β-catenin (Figure S7H) and significantly inhibited neuronal differentiation in the mouse neocortex (Figures 7G and 7H). These observations collectively indicate that nuclear Axin promotes neuronal differentiation in a β-catenin-dependent manner.

DISCUSSION

The fate decision of NPCs between amplification and differentiation controls the number of neurons produced during brain development and ultimately determines brain size. However, it is unclear how the NPCs make this fundamental choice. Here, we show that the subcellular localization of a signaling scaffold protein, Axin, defines the activation of specific signaling networks in NPCs, thereby determining the amplification or neuronal differentiation of NPCs during embryonic development (Figure 8). Cytoplasmic Axin in NPCs enhances IP generation, which ultimately leads to increased neuron production, whereas nuclear Axin in IPs promotes neuronal differentiation. Intriguingly, the Cdk5-dependent phosphorylation of Axin facilitates the nuclear accumulation of the protein, thereby functioning as a "brake" to prevent the overproduction of IPs and induce neuronal differentiation.

Axin Governs the Expansion of the Mammalian Cerebral Cortex through IP Pool Amplification

The drastic increase in the size of the cerebral cortex in the human brain, which is thought to underpin our unique higher-cognitive functions, is associated with a disproportionate expansion of cortical neurons, especially the upper-layer neurons. The expansion of cortical surface may result from increased numbers of neuroepithelial (NE) cells and RGs (Rakic, 2009) or from an amplified IP pool (Pontious et al., 2008). NE/RG augmentation evidently controls the global enlargement of cortical surface (Chenn and Walsh, 2002; Vaccarino et al., 1999). The amplification of a subset of RGs expressing the transcription factor Cux2 was recently suggested to facilitate upper-layer neuron expansion (Franco et al., 2012). However, there is a lack of experimental evidence indicating whether IP amplification also substantially contributes to the expansion of upper-layer cortical neurons and the cerebral cortex. Nonetheless, upper-layer neurons are generated during mid- and late neurogenesis (Molyneaux et al., 2007), at which time IPs play the primary role in neuron production. Moreover, the enlargement of IP-residing SVZ is temporally correlated with the increased number of upper-layer neurons and expanded cortical surface (Zecevic et al., 2005). Therefore, it is tempting to speculate that the amplification of IPs during mid- and late corticogenesis has facilitated the evolutionary expansion of the cerebral cortex. Our present findings demonstrate that increasing Axin levels during midcorticogenesis, which leads to the transient amplification of IPs without affecting the RG pool, is sufficient to expand the surface of the neocortex (Figures 1 and 2). Previous studies show that Axin expression is tightly regulated by different posttranslational modifications including deubiquitination (Lui et al., 2011), SUMOylation (Kim et al., 2008), methylation (Cha et al., 2011), and phosphorylation (Yamamoto et al., 1999), which increase the stability of Axin; meanwhile, polyubiquitination (Kim and Jho, 2010) and poly-ADP-ribosylation (Huang et al., 2009) lead to its degradation. Thus, the adaptive evolution of the *Axin* gene that regulates its posttranslational modifications and hence its expression level might be involved in the evolutionary expansion of the cerebral cortex.

To ensure the development of a cerebral cortex of the proper size, the amplification and neuronal differentiation of IPs need to

be precisely controlled. A reduced number of IPs due to precocious depletion of NEs/RGs (Buchman et al., 2010) or inhibition of IP generation/proliferation (Sessa et al., 2008) ultimately lead to the generation of fewer cortical neurons, resulting in a smaller cortex—a characteristic feature of human microcephalic syndromes. In contrast, the overexpansion of IPs (Lange et al., 2009) generates an excessive number of neurons, which is associated with macrocephaly and autism (McCaffery and Deutsch, 2005). Our findings demonstrate that Axin strictly controls the process of indirect neurogenesis to ensure the production of a proper number of neurons. Although cytoplasmic Axin simultaneously maintains the RG pool and promotes IP amplification to sustain rapid and long-lasting neuron production, subsequent enrichment of Axin in the nuclei of IP daughter cells triggers neuronal differentiation and prevents the overexpansion of IPs. In addition, the results demonstrate that Cdk5-mediated phosphorylation regulates the nucleocytoplasmic shuttling of Axin, thereby controlling the switching of NPCs from proliferative to differentiation status.

Axin Phosphorylation Triggers the Differentiation of IPs into Neurons

Our findings show that Axin phosphorylation in IPs triggers neuronal differentiation in a rostrolateral-high to caudomedial-low gradient correlated with the spatial gradient of neurogenesis (Figures 4 and 5) (Caviness et al., 2009). Thus, the gradient of Axin phosphorylation may provide a quantitative tool for evaluating the temporal and spatial gradient of IP differentiation into neurons. Importantly, nuclear Axin phosphorylation is rapidly induced in IP daughter cells in the G1 phase, which is the stage when progenitor cells actively respond to neurogenic signals (Dehay and Kennedy, 2007); this suggests that the timing of Axin phosphorylation-dependent IP differentiation is regulated by diffusible extracellular signals (Tiberi et al., 2012). Therefore, understanding how Axin phosphorylation is regulated in IPs by extracellular cues and niches should shed new light on the molecular basis underlying the gradient-specific differentiation of IPs.

Our findings also highlight the importance of Cdk5 in embryonic neurogenesis. Although Cdk5 plays critical roles in neuronal development (Jessberger et al., 2009) and is implicated in the neurogenesis of cultured neural stem cells (Zheng et al., 2010), it remains unclear whether Cdk5 regulates embryonic neurogenesis. Our findings provide in vivo evidence that Cdk5 is required for the neuronal differentiation of IPs, at least in part through phosphorylating Axin. Intriguingly, although *cdk5*$^{-/-}$ cortices exhibited an accumulation of IPs and reduced neuron production during early-mid neurogenesis (Figure 4), the brain size of these mutant mice remained unchanged by the end of neurogenesis (Dhavan and Tsai, 2001). This may be due to the compensatory increase of neuron production from the expanded pool of IPs during the mid-to-late neurogenesis stages. Therefore, elucidating how Cdk5 is involved in different stages of neurogenesis may provide insights into the molecular control of neuronal number and subtypes.

Subcellular Axin Signaling Coordinates the Generation, Amplification, and Differentiation of IPs

Several factors that regulate the generation and amplification of IPs have been identified (Pontious et al., 2008). Nonetheless, key questions remain open: how RGs determine to differentiate into IPs instead of neurons, how RG-to-IP transition and IP differentiation are coordinated, and how IP amplification and differentiation are balanced. The present results show that the interaction between cytoplasmic Axin and GSK-3β maintains the RG pool and promotes IP production (Figure 6). The signaling mechanisms underlying the action of Axin-GSK-3β interaction require further investigation. We hypothesize that Axin regulates IP differentiation from RGs via various molecular mechanisms. First, the Axin-GSK-3β complex may reduce the level of Notch receptor or β-catenin (Muñoz-Descalzo et al., 2011; Nakamura et al., 1998), leading to the suppression of Notch- and Wnt-mediated signaling, respectively (Gulacsi and Anderson, 2008; Mizutani et al., 2007; Woodhead et al., 2006). Given that Axin and GSK-3β can associate with the centrosome (Fumoto et al., 2009; Wakefield et al., 2003) and mitotic spindle (Izumi et al., 2008; Kim et al., 2009a), Axin-GSK-3β interaction may also modulate cleavage plane orientation (Postiglione et al., 2011). Furthermore, Axin-GSK-3β can interact with and affect the microtubule-binding activity of adenomatous polyposis coli (APC) (Nakamura et al., 1998), which is required for establishing the apical-basal polarity and asymmetric division of RGs (Yokota et al., 2009). Finally, interaction with Axin can cause GSK-3β inhibition (Fang et al., 2011), which may enhance IP amplification (Kim et al., 2009b) through the activation of Shh signaling (Komada et al., 2008).

The timing of IPs to undergo cell-cycle exit balances the proliferative and neurogenic divisions of IPs and switches the RG-to-IP transition to the neuronal differentiation of IPs. We show that the interaction between Axin and β-catenin in the nucleus switches the division of IPs from proliferative to neurogenic by enhancing the neurogenic transcriptional activity of β-catenin (Figure 7). Indeed, Axin and β-catenin are required for the signal transduction of Wnt (Hirabayashi et al., 2004; Munji et al., 2011), RA (Otero et al., 2004), and TGF-β (Zhang et al., 2010a), which triggers and promotes neuronal differentiation. Thus, Axin in the nucleus may serve to transduce and converge multiple neurogenic signaling pathways to β-catenin during neurogenesis. However, the mechanism by which nuclear Axin enhances the transcriptional activity of β-catenin requires further investigation. Given that β-catenin exerts its transcriptional regulation of target genes through association with T cell factor/lymphoid enhancer factor (Tcf/Lef), we hypothesize that nuclear Axin facilitates β-catenin/Tcf/Lef complex formation to enhance transcription (Shitashige et al., 2008).

Although Axin was previously recognized as a negative regulator of canonical Wnt signaling, suppressing cell division by recruiting GSK-3β and β-catenin into the β-catenin destruction complex for β-catenin degradation (Ikeda et al., 1998), the present results show that cytoplasmic Axin and nuclear Axin act distinctly from canonical Wnt signaling through specific binding to GSK-3β and β-catenin, respectively. Therefore, our findings corroborate the notion that Wnt signaling components play multifaceted roles in NPCs during neurogenesis independent of canonical Wnt signaling as demonstrated in previous studies (Kim et al., 2009b; Yokota et al., 2009).

In conclusion, the present study identified distinct roles of Axin in IP amplification and neuron production. Our results

demonstrate that the modulation of Axin levels, subcellular localization, phosphorylation, and its interaction with key signaling regulators (e.g., GSK-3β and β-catenin) in NPCs ultimately control neuron production and expansion of the cerebral cortex. Given that Axin is a key regulator of the switch from IP amplification to differentiation, the characterization of the signals that control this switch will not only advance our current understanding of how the cerebral cortex expands during evolution but also provide important insights into neurodevelopmental disorders such as microcephaly.

EXPERIMENTAL PROCEDURES

Procedures regarding shRNA, expression constructs, chemicals, reagents, antibodies, mice, NPC culture, pair-cell analysis, in vivo β-catenin transcriptional activity assay, image acquisition, and quantitative analysis can be found in the Supplemental Experimental Procedures. All animal procedures were conducted in accordance with the Guidelines of the Animal Care Facility of the Hong Kong University of Science and Technology (HKUST) and were approved by the Animal Ethics Committee in HKUST.

Intraventricular Microinjection

The embryos of timed-pregnant ICR mice at E13.5 were anesthetized with pentobarbital (5 mg × ml^{-1}) and exposed and transilluminated to visualize the cerebral ventricles (Fang et al., 2011). XAV939 (1 mM) was microinjected into the lateral ventricles. After 2 hr or at E14.5, the pregnant mice were intraperitoneally injected with one pulse of the nucleoside analog, EdU (30 mg × kg^{-1}). The injected fetuses were harvested at E15.5 or E17.5, intracardially perfused with 4% paraformaldehyde (PFA), and subjected to EdU staining. At least six brains were analyzed for each condition.

In Utero Electroporation and Transfection

In utero electroporation of embryos at E12.5 or E13.5 was performed as described previously (Fang et al., 2011). At least three independent experiments were performed, and at least six brains were analyzed for each condition. The final concentration of plasmids used for each condition can be found in the Supplemental Experimental Procedures. Mouse embryonic NPCs were transfected using the Amaxa Nucleofector Kit (Lonza) following the Amaxa optimized protocol (program: A033) for mouse neural stem cells.

In Utero EdU Labeling

To examine cell-cycle exit, EdU was injected into pregnant mice 24 hr after electroporation. Twenty-four hours after injection, the brains were processed, and EdU was detected using the Click-iT EdU Alexa Fluor Imaging Kit (Invitrogen). To correlate the regulation of phospho-Axin with cell phase distribution, EdU (30 mg × kg^{-1}) was intraperitoneally injected into pregnant ICR mice at E15.5. The cell cycle in E15.5 mice is ~18 hr long, comprising an ~12 hr G1 phase, ~4 hr S phase, and ~2 hr G2/M phase. To label the S and G2 phases of NPCs, E15.5 embryos were subjected to two pulses of EdU, 2 and 0.5 hr prior to harvesting, respectively. To label the late G1 phase progenitors, the embryos were collected 14 hr after EdU injection (Britz et al., 2006).

Western Blot, Nuclear Coimmunoprecipitation, and Immunohistochemical Analyses

Western blotting, immunoprecipitation, and immunohistochemistry were performed as described previously (Fang et al., 2011). Cytosolic and nuclear fractionation was performed using the Nuclear/Cytosol Extraction Kit (BioVision). Nuclear coimmunoprecipitation was carried out using the Nuclear Complex Co-IP Kit (Active Motif).

Statistical Analysis

Statistical analyses were performed with Student's t test using GraphPad Prism (GraphPad Software). All bar graphs represent mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.06.017.

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