Development 137, 1035-1044 (2010) doi:10.1242/dev.046417

Wnt signaling and its downstream target N-myc regulate basal progenitors in the developing neocortex

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SUMMARY

Basal progenitors (also called non-surface dividing or intermediate progenitors) have been proposed to regulate the number of neurons during neocortical development through expanding cells committed to a neuronal fate, although the signals that govern this population have remained largely unknown. Here, we show that N-myc mediates the functions of Wnt signaling in promoting neuronal fate commitment and proliferation of neural precursor cells in vitro. Wnt signaling and N-myc also contribute to the production of basal progenitors in vivo. Expression of a stabilized form of β-catenin, a component of the Wnt signaling pathway, or of N-myc increased the numbers of neocortical basal progenitors, whereas conditional deletion of the *N-myc* gene reduced these and, as a likely consequence, the number of neocortical neurons. These results reveal that Wnt signaling via N-myc is crucial for the control of neuron number in the developing neocortex.

KEY WORDS: Neocortical development, Basal progenitors, Neural precursor cells, The Wnt–β-catenin pathway, N-myc, Mouse

INTRODUCTION

The mammalian neocortex is a complex and highly organized six-layer structure responsible for cognitive function, sensory perception and consciousness (Molyneaux et al., 2007). This structure has undergone pronounced development during evolution, with an accompanying increase in neuronal number (Dehay and Kennedy, 2007). Neocortical neurons are derived from apical progenitors, which divide on the apical surface of the embryonic brain (surface division) (Temple, 2001). Although some apical progenitors directly undergo terminal differentiation into neurons (‘direct neurogenesis’), recent studies have revealed that other apical progenitors differentiate into basal progenitors (also called non-surface dividing or intermediate progenitors) that then divide once or twice in the non-surface area before terminal differentiation (‘indirect neurogenesis’) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Wu et al., 2005). Basal progenitors are largely restricted to the neuronal fate (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Pinto and Gotz, 2007; Wu et al., 2005) and have been proposed to contribute to an increase in the number of neocortical neurons that are generated from apical progenitors by further round(s) of cell division. Basal progenitors differ from apical progenitors in their molecular features: basal progenitors express high levels of Svet1, Tbr2 (Eomes – Mouse Genome Informatics), Math2/Nex (Neurod6 – Mouse Genome Informatics) and Cux2, whereas apical progenitors highly express Pax6, Emx2, Hes1 and Hes5 (Englund et al., 2005; Kawaguchi et al., 2008a; Nieto et al., 2004; Tarabykin et al., 2001; Wu et al., 2005; Zimmer et al., 2004). Importantly, the number of basal progenitors has increased during animal evolution concomitant with the increase of neocortical neurons (Cheung et al., 2007; Martinez-Cerdeno et al., 2006; Smart et al., 2002). The production of basal progenitors by apical progenitors may therefore have played a major role in the evolution of the cortex. Despite the significance and postulated roles of basal progenitors in regulating the number of neocortical neurons, the signaling pathways that govern the development and function of basal progenitors are largely unclear.

The Wnt–β-catenin pathway (or the canonical Wnt pathway) regulates the transcription of its target genes via the stabilization and nuclear translocation of cytoplasmic β-catenin (Ctnnb1 – Mouse Genome Informatics) (Clevers, 2006; Logan and Nusse, 2004). Several Wnt ligands and their receptors are expressed in the developing neocortex (for a review, see Hirabayashi and Gotoh, 2005). Indeed, transgenic mice harboring a reporter gene responsive to β-catenin/Tcf transcription complex show high reporter activity in the developing neocortex (Backman et al., 2005; Maretto et al., 2003), indicating that the Wnt–β-catenin pathway is activated within this region. The abrogation of the Wnt–β-catenin pathway in Lrp6- or Ryk-deficient mice resulted in the impairment of neocortical neuron production (Lyu et al., 2008; Zhou et al., 2006). It is therefore of great interest to elucidate which progenitor populations and which steps in neuron production are being affected by this pathway. Accumulating evidence suggests that activation of the Wnt–β-catenin pathway promotes proliferation of neural precursor cells (NPCs) that include apical progenitors and basal progenitors (Chenn and Walsh, 2002; Woodhead et al., 2006; Wrobel et al., 2007; Zechner et al., 2003). For instance, persistent expression of a stabilized form of β-catenin in NPCs results in an enlarged brain accompanied by excess proliferation of NPCs, presumably due to increased cell cycle re-entry (Chenn and Walsh, 2002; Wrobel et al., 2007). In addition to the proliferation-

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Accepted 19 January 2010

stimulating roles of Wnt signaling, it has been shown that the Wnt–β-catenin pathway instructs neuronal differentiation of NPCs during neocortical development (Guillemot, 2007; Hirabayashi et al., 2004; Israsena et al., 2004; Lyu et al., 2008; Muroyama et al., 2004; Zhou et al., 2006). Expression of stabilized β-catenin promoted neuronal differentiation in the neocortex, whereas expression of Axin, an inhibitor of the Wnt–β-catenin pathway, suppressed neuronal differentiation, indicating the role of this pathway in neurogenesis (Hirabayashi et al., 2004). It is thus well established that the Wnt–β-catenin pathway regulates proliferation and neuronal differentiation of NPCs. However, it remains to be shown whether Wnt signaling also regulates basal progenitors.

Considering that Wnt signaling appears to play many roles in the neocortex and exerts its specific functions in a context-dependent manner, it is possible that the target genes for this signaling might be context dependent (Clevers, 2006). The proneural basic helix-loop-helix (bHLH) genes neurogenin (Ngn; Neurog – Mouse Genome Informatics) 1 and Ngn2 are known to be direct targets for the β-catenin/Tcf transcription complex in inducing neuronal differentiation (Hirabayashi et al., 2004; Israsena et al., 2004), but it is unknown which Wnt-induced genes are responsible for stimulating proliferation. In this study, we show that N-myc (Mycn – Mouse Genome Informatics) is a pivotal target for Wnt signaling in promoting the proliferation of neocortical NPCs. Surprisingly, we found that N-myc is also responsible for mediating the ability of Wnt to promote neocortical neuronal differentiation. Furthermore, we have obtained data demonstrating the roles of Wnt signaling and N-myc in the differentiation of apical progenitors into basal progenitors and increase in neuronal number during neocortical development.

MATERIALS AND METHODS

Mice

The generation of mutant mice has been reported previously: *Ctnnb1*$^{lox(ex3)/+}$ (β-catenin$^{lox(ex3)/+}$) mice (Harada et al., 1999), *Mycn*$^{lox/lox}$ (*N-myc*$^{lox/lox}$) mice (Knoepfler et al., 2002) and *Tg*(*Nes-Cre*)*1Kag* (*Nes-Cre*) mice (Isaka et al., 1999). Mice were maintained in accordance with the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Primary neocortical NPC culture and in vitro assays

Neocortical NPCs were isolated from the dorsal cerebral cortex of mouse embryos at embryonic day (E) 10.5 or 11.5. Isolation of NPCs, NPC culture, immunocytochemical analysis, neurosphere assay, RNA extraction, qPCR analysis and chromatin immunoprecipitation (ChIP) assay were performed as described previously (Hirabayashi et al., 2004; Hirabayashi et al., 2009). For clonal analysis, NPCs were separated into two portions and one portion of NPCs were plated on poly-D-lysine (PDL)-coated dishes (500 cells/mm^2) and cultured with fibroblast growth factor (FGF) 2 for use as feeder cells. The other portion of NPCs were infected with control or N-myc retroviruses, and incubated in suspension for 1 day with FGF2. The infected cells were completely dissociated, plated at a low density (1.6-5.3 cells/mm^2) on feeder NPCs and incubated for 2 days with FGF2. Then the cells were fixed and immunostained. Each clone was distinguished by green fluorescent protein (GFP) expression, and the number of clones obtained under these conditions was about 0.4 clone/mm^2. Following this, the numbers of cells and differentiated cells (βIII-tubulin$^+$ cells or GFAP$^+$ cells) in each clone were determined. Data represent the percentage of non-neuron clones (containing no βIII-tubulin$^+$ cells), neuron-containing clones (containing both βIII-tubulin$^+$ cells and βIII-tubulin$^-$ cells) and neuron-only clones (containing only βIII-tubulin$^+$ cells), and of glial clones. Data represent the mean±s.d. values of three independent samples.

Purification of recombinant Wnt3a

Mouse Wnt3a protein was purified to near homogeneity from Wnt3a-conditioned medium as described previously (Kishida et al., 2004; Willert et al., 2003), and used for experiments at a final concentration of 0.50-1.0 μg/ml. The vehicle was used as a control (2.5-5.0 mM NaCl, 0.005-0.01% CHAPS). The vehicle treatment did not significantly affect neuronal differentiation of NPCs compared with untreated NPCs (data not shown).

Retroviral infection in utero, BrdU labeling, immunohistochemistry and in situ hybridization

These in vivo experiments were performed as described previously (Hirabayashi et al., 2009; Ishii et al., 1998; Kawaguchi et al., 2008b). Digoxigenin-labeled cRNA probes were transcribed from *N-myc* cDNA (FANTOM2, MGI:1901364).

Antibodies, qPCR primers, plasmids and retroviruses

pMXs vector was used for the production of recombinant retroviruses, as described previously (Hirabayashi et al., 2009; Morita et al., 2000). Detailed information about qPCR primers and plasmids can be supplied upon request. Antibodies used in immunocytochemistry, immunohistochemistry and ChIP analysis were: mouse antibodies to βIII-tubulin (TuJ1, Covance) 1:1000, GFAP (Chemicon) 1:500, NeuN (Chemicon) 1:500 and BrdU (BD Biosciences) 1:80, chick antibody to GFP (Abcam) 1:2000, rat antibody to Ctip2 (Abcam) 1:500, goat antibody to Tcf3 (M-20, Santa Cruz) and rabbit antibodies to GFP (MBL) 1:1000, Pax6 (Chemicon) 1:1000, Tbr2 (Chemicon) 1:1000, Tbr1 (Abcam) 1:1000, Cux1 (anti-CDP M-222, Santa Cruz Biotechnology) 1:200, GFAP (DAKO) 1:2000, and phosphorylated histone H3 (pH3) (Cell Signaling Technology) 1:200. Alexa-labeled secondary antibodies, TO-PRO-3 and Hoechst 33342 (for nuclear staining) were from Molecular Probes.

Statistical analysis

A statistical analysis was performed using the unpaired two-tailed Student's *t*-test between control and experimental conditions and one-way ANOVA. A *P*-value of <0.05 was considered statistically significant. In culture experiments, similar results were obtained in at least three independent experiments. In mutant mouse experiments, similar results were obtained in at least five brains.

RESULTS

N-myc is a direct downstream target of the Wnt–β-catenin pathway in the developing neocortex

The Wnt–β-catenin pathway promotes both proliferation and neuronal differentiation of neocortical NPCs. Wnt signaling induces the expression of Ngn1/2, and thus promotes neocortical neuronal differentiation (Hirabayashi et al., 2004; Israsena et al., 2004). However, it remains unclear which target genes are responsible for the promotion of NPC proliferation by Wnt signaling. In other systems, members of the Cyclin D and the Myc families, such as Cyclin D1, c-Myc and N-myc, have been implicated in mediating the proliferative effects of Wnt signaling (He et al., 1998; Megason and McMahon, 2002; Shtutman et al., 1999; Shu et al., 2005; ten Berge et al., 2008; Tetsu and McCormick, 1999; van de Wetering et al., 2002). We used DNA microarray analysis to identify genes upregulated or downregulated by treatment with recombinant Wnt3a in an NPC culture prepared from E11.5 mouse neocortex. The data have been deposited with ArrayExpress (Accession number E-TABM-923). We found that *N-myc* is upregulated by Wnt3a treatment at 3, 6 and 12 hours (see Table S1 in the supplementary material). Quantitative RT-PCR analysis confirmed that the level of *N-myc* mRNA was significantly increased by Wnt3a treatment for 3 hours (Fig. 1A, Cyclin D1; see Fig. S1 and its legend in the supplementary material). We next investigated whether β-catenin, the downstream mediator of Wnt signaling, promotes the expression of *N-myc*. NPC culture prepared from E11.5 mouse neocortex was infected with retroviruses encoding either GFP alone or stabilized β-catenin (S33Y mutant) and GFP. We observed that stabilized β-catenin overexpression increased the level of *N-myc* mRNA (Fig. 1B). Moreover, retroviral infection

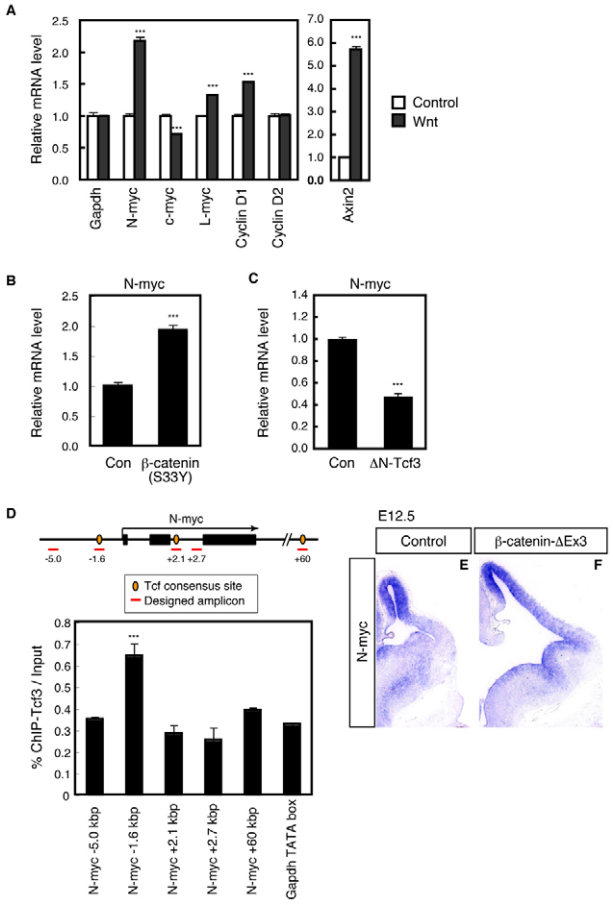

Fig. 1. N-myc is a direct downstream target of the Wnt–β-catenin pathway in the developing mouse neocortex. (**A**) Neural precursor cells (NPCs) isolated from murine developing neocortex at E10.5 were plated on PDL-coated dishes and incubated in the presence of Fgf2 with recombinant Wnt3a or vehicle control for 3 hours, and total RNA extracted. The mRNA levels of Myc and Cyclin D families were determined by qPCR analysis. Note that expression of *Axin2*, a typical Wnt target, was induced in this condition. (**B**,**C**) E11.5 NPCs were infected with a retrovirus encoding GFP (Con), or with both GFP and β-catenin-S33Y (a dominant stable mutant of β-catenin; B), or with both GFP and ΔN-Tcf3 (an N-terminal truncated form of Tcf3 that suppresses the β-catenin pathway; C). The cells were then incubated in the presence of FGF2 for 3 days. The abundance of *N-myc* mRNA was determined by qPCR analysis. (A-C) Data represent mRNA levels normalized to that of *Gapdh*. (**D**) Chromatin complex was immunoprecipitated from E11.5 neocortical lysates with anti-Tcf3. The immunoprecipitates were subjected to qPCR analysis. The designed PCR amplicons are indicated by red lines. (**E**,**F**) In situ hybridization for *N-myc* mRNA of coronal sections of E12.5 β-catenin$^{+/+}$;*Nes-Cre* (control; E) or β-catenin$^{lox(ex3)/+}$;*Nes-Cre* (β-catenin-ΔEx3, a dominant stable mutant; F) cortex. Data represent mean±s.e.m. ***, $P<0.001$.

with ΔN-Tcf3, an N-terminal truncated form of Tcf3 (Tcf711 – Mouse Genome Informatics) that suppresses the β-catenin pathway (Merrill et al., 2001), significantly reduced the level of *N-myc* mRNA, indicating that the Wnt–β-catenin pathway regulates *N-myc* expression in NPCs (Fig. 1C).

We next investigated whether Tcf3, a transcription regulator of the Wnt–β-catenin pathway, directly associates with the promoter/enhancer of the *N-myc* gene in NPCs by ChIP assay. Several Tcf-consensus sites are present in *N-myc* promoter/enhancer regions (Shu et al., 2005; Tam et al., 2008). Among them, we examined the association of Tcf3 protein with evolutionarily conserved sites at –1.6, +2.1, and +60 kb relative to the transcription start site of the *N-myc* gene, and found that Tcf3 was enriched around the –1.6 kb site in E11.5 neocortical NPCs (Fig. 1D). This suggests that *N-myc* gene expression is directly regulated by the β-catenin/Tcf transcription complex.

We then examined the expression patterns of *N-myc* in the developing telencephalon. In situ hybridization analyses of E11.5 and 12.5 mouse embryos revealed that *N-myc* transcripts were highly expressed in the dorsal neocortex (Fig. 1E; see Fig. S1A in the supplementary material), where a β-catenin/Tcf reporter gene (BAT-Gal) was activated with a similar pattern (Backman et al., 2005; Machon et al., 2007; Maretto et al., 2003).

We then asked whether expression of stabilized β-catenin also induces N-myc expression in vivo. A mouse containing the Cre recombinase transgene under the control of the nestin (*Nes*) promoter and enhancer (*Nes-Cre*) was crossed with a knock-in mouse in which exon 3 of the β-catenin gene is flanked by loxP sites [β-catenin$^{lox(ex3)}$] (Harada et al., 1999). Deletion of exon 3 in these β-catenin$^{lox(ex3)/+}$;*Nes-Cre* mice (referred to as β-catenin-ΔEx3 mice) results in the stabilization of β-catenin protein, as this domain contains phosphorylation sites crucial for degradation. We found that *N-myc* mRNA was increased in β-catenin-ΔEx3 mice in the germinal zone of telencephalon, in particular in the ventral side of the neocortex and the lateral ganglionic eminence, where endogenous *N-myc* level is normally low (Fig. 1E,F). These results indicate that stabilized β-catenin can induce N-myc production in the developing neocortex.

N-myc mediates Wnt-induced proliferation and neuronal differentiation of NPCs

N-myc is a member of the Myc family of transcription factors (Eilers and Eisenman, 2008), and conditional deletion of the *N-myc* gene has revealed that it plays an essential role in the proliferation of neocortical NPCs and brain mass (Knoepfler et al., 2002). Given that the Wnt–β-catenin pathway directly induces N-myc production in neocortical NPCs in vitro and in vivo (this study) and that expression patterns of *N-myc* in telencephalon are similar to those previously observed using the BAT-Gal reporter (this study) (Backman et al., 2005), we hypothesized that N-myc may mediate the proliferative effects of Wnt signaling. To test this, we deleted the *N-myc* gene in a neocortical NPC culture prepared from E11.5 *N-myc*$^{lox/lox}$ mice by infecting cells with a retrovirus encoding the Cre recombinase and GFP (Cre) (Knoepfler et al., 2002). Cell proliferation was monitored by BrdU incorporation after BrdU treatment for 2.5 hours. Pretreatment of the control culture with Wnt3a for 12 hours increased the percentage of BrdU-labeled cells among GFP-positive NPCs [Fig. 2A, see Fig. S2A,B in the supplementary material; consistent with previous reports (Hirsch et al., 2007; Muroyama et al., 2004)]. By contrast, in cells deleted for N-myc, Wnt3a had little effect on the percentage of BrdU-labeled cells among GFP-positive NPCs (Fig. 2A), indicating that N-myc is necessary for Wnt-stimulated proliferation of neocortical NPCs. Deletion of the *N-myc* gene did not reduce BrdU incorporation of NPCs in the absence of Wnt3a treatment (Fig. 2A), suggesting that N-myc is required for Wnt3a-induced NPC proliferation and not for the basal machinery of proliferation per se. We next examined the effect of N-myc overexpression on NPC proliferation by infecting a neocortical NPC culture with retroviruses encoding either GFP alone or N-myc and GFP. We observed that N-myc overexpression significantly increased the percentage of BrdU-positive cells among

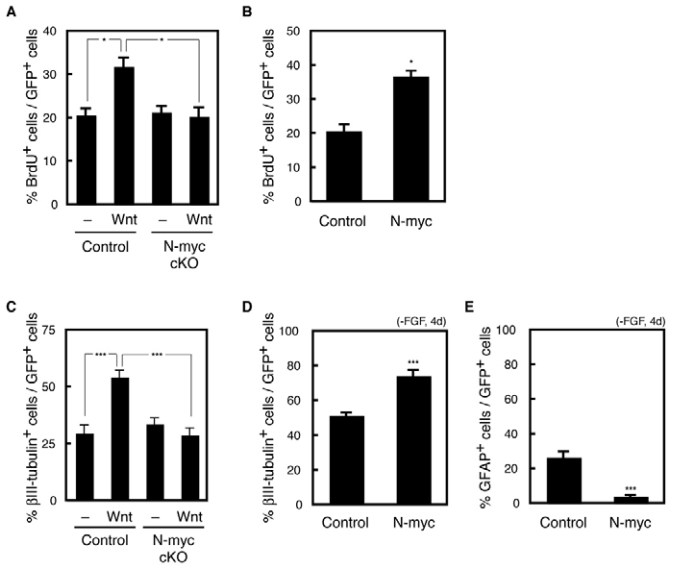

Fig. 2. N-myc mediates Wnt-induced proliferation and neuronal differentiation of NPCs. (**A**) E11.5 NPCs isolated from *N-myc*$^{lox/lox}$ mice were infected with a retrovirus encoding GFP (control) or with both GFP and Cre recombinase (N-myc cKO) and incubated for 2 days with FGF2. Then, cells were incubated with either Wnt3a or control for 12 hours, followed by labeling with BrdU (10 μg/ml) for 2.5 hours. The percentage of BrdU$^+$ cells among GFP$^+$ cells was determined by immunostaining. (**B**) E10.5 neocortical NPCs were infected with a retrovirus encoding GFP (control) or with both GFP and N-myc. Then, the cells were incubated for 2 days with FGF2, followed by labeling with BrdU for 2.5 hours. The mitotic index was determined as in A. (**C**) E11.5 N-Myc-cKO NPCs and control NPCs prepared as in A were incubated with either Wnt3a or control in the presence of FGF2 for 24 hours, and then incubated without Wnt3a or FGF2 for 50 hours. The percentage of βIII-tubulin$^+$ cells among GFP$^+$ cells was determined by immunostaining. (**D**,**E**) E11.5 NPCs were incubated in suspension for 6 days with FGF2 and EGF, followed by infection with a retrovirus encoding GFP (control) or with both GFP and N-myc. Infected NPCs were incubated for 4 days without FGF2 or EGF (differentiation condition), and the percentage of βIII-tubulin$^+$ cells (D) or GFAP$^+$ cells (E) among GFP$^+$ cells determined by immunostaining. (A-E) Data represent mean±s.e.m. *, $P<0.05$; ***, $P<0.001$.

GFP-positive cells (Fig. 2B), indicating that N-myc expression is sufficient to promote NPC proliferation in vitro. These results indicate that N-myc mediates Wnt-induced proliferation of neocortical NPCs.

We then examined whether N-myc is positively or negatively involved in Wnt promotion of neuronal differentiation, as Myc proteins have been shown to promote or inhibit differentiation processes in a context-dependent manner (see Discussion). Neocortical NPCs prepared from E11.5 *N-myc*$^{lox/lox}$ mice were labeled with GFP by infection with a retrovirus encoding GFP. Treatment of these cells with Wnt3a for 24 hours increased the proportion of cells expressing the neuronal marker βIII-tubulin among GFP-positive cells (detected after 2 days in the absence of FGF2) (Fig. 2C). By contrast, when the *N-myc* gene was deleted by infection with a retrovirus encoding both GFP and Cre recombinase, Wnt3a treatment did not increase the proportion of βIII-tubulin-positive cells among GFP-positive cells (Fig. 2C). This result suggests that N-myc is necessary for Wnt stimulation of neuronal differentiation.

We then examined whether expression of N-myc promotes neuronal differentiation. NPCs, prepared from E11.5 neocortex and cultured for 6 days in the presence of FGF2 (for maintenance of the undifferentiated state), were infected with a retrovirus encoding GFP alone or both GFP and N-myc and cultured for 4 days in the absence of FGF2 (for induction of differentiation). N-myc production significantly increased the percentage of cells expressing βIII-tubulin among GFP-positive cells and reduced that of cells expressing the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 2D,E). These results together indicate that N-myc mediates Wnt-induced neuronal differentiation of NPCs.

N-myc promotes neuronal fate commitment of multipotent NPCs

This increase in βIII-tubulin-positive cells could be due either to N-myc promotion of neuronal fate commitment among multipotent NPCs or to the selective proliferation of progenitors already committed to neuronal fate (neuronal progenitors, NPs), or both. We thus examined the fate of NPCs using a clonal assay to avoid any effects due simply to selective expansion. NPCs infected with a retrovirus encoding GFP alone or both GFP and N-myc were plated at a clonal density (i.e. low density: 1.6-5.3 cells/mm^2) within a high-density culture of uninfected NPCs (500 cells/mm^2). We observed that N-myc expression markedly increased the percentage of neuron-containing clones at the expense of non-neuron clones (Fig. 3A), including astrocyte-containing clones (Fig. 3B). This clearly indicates that N-myc can promote the neuronal lineage choice of multipotent NPCs.

As N-myc induces neuronal fate commitment, we asked whether N-myc affects the multipotent NPC population. We thus performed a neurosphere assay, which is commonly used to monitor the abundance of multipotent and self-renewable NPCs [see Yoshimatsu et al. (Yoshimatsu et al., 2006) and references cited therein]. In this assay, neocortical NPCs were cultured in suspension at a low density (<15,000 cells/ml) so that most of the resulting cell aggregates (neurospheres) were derived from single cells. Under the conditions used in this study, the majority (>95%) of neurosphere-forming cells were multipotent, as these neurospheres contained neurons, astrocytes and oligodendrocytes upon differentiation (data not shown). Consistent with previous reports (Hirabayashi et al., 2004; Muroyama et al., 2004), we observed here that a pulse treatment of NPC culture with recombinant Wnt3a for 24 hours reduced the numbers of secondary neurospheres (Fig. 3C). Importantly, expression of N-myc also significantly reduced the numbers of primary and secondary neurospheres formed from the E10.5 neocortical NPC culture (Fig. 3D, and data not shown). These results suggest that N-myc does not increase the numbers of 'sphere-forming' multipotent NPCs but rather reduces this population.

Wnt signaling and N-myc increase NPs in vitro

Given that Wnt signaling and its downstream target N-myc reduce multipotent NPCs (Fig. 3A-D) while promoting BrdU incorporation in Fig. 2A,B, it is plausible that they increase NPs. We therefore measured the effects of Wnt3a treatment on the percentage of cells positive for Tbr2, an NP marker (Englund et al., 2005; Sessa et al., 2008), in vitro. NPC culture prepared from E11.5 mouse neocortex was infected with a retrovirus encoding GFP to label mitotic NPCs, treated with either vehicle control of recombinant Wnt3a for 24 hours, and then cultured in the absence of FGF2 to promote differentiation. Wnt3a treatment increased the proportion of cells expressing Tbr2 among GFP-positive cells (Fig. 3E). Consistently, Wnt3a treatment decreased the percentage of cells positive for the undifferentiated NPC marker Pax6 (Fig. 3F). We further examined the effect of N-myc overexpression on the Tbr2-positive cell population by infecting neocortical NPC culture with retroviruses encoding either GFP alone or N-myc and GFP. N-myc overexpression significantly increased the

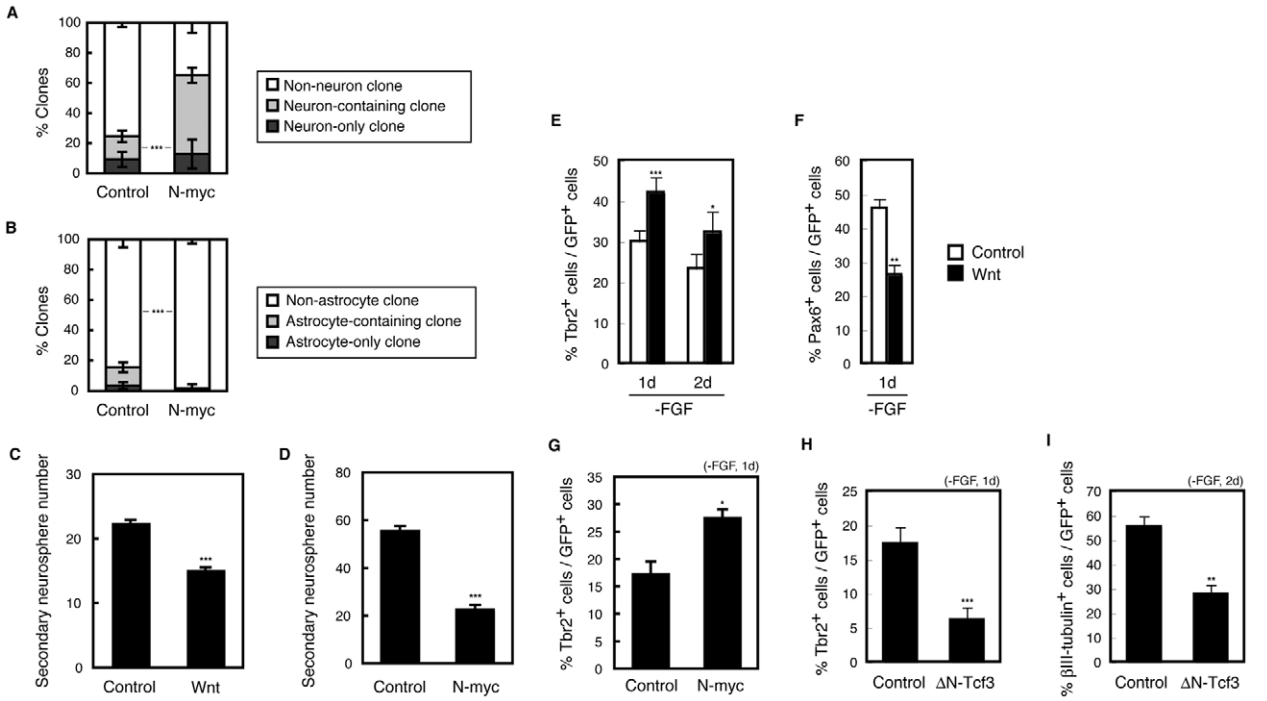

Fig. 3. Wnt signaling and N-myc promote neuronal fate commitment of multipotent NPCs and increase NPs in vitro. (**A**,**B**) E11.5 NPCs were incubated in suspension for 6 days with FGF2 and EGF, followed by infection with a retrovirus encoding GFP (control) or with both GFP and N-myc. Infected NPCs were plated at a clonal density and incubated for 2 days with FGF2 (undifferentiated condition). Then, the cells were stained with anti-GFP and either anti-βIII-tubulin (A) or anti-GFAP (B), and subjected to a clonal assay. The numbers of differentiated cells (βIII-tubulin$^+$ cells or GFAP$^+$ cells) and total cells in each clone were examined, and clones were classified and sorted based on whether the clone included differentiated cells. (**C**) E10.5 NPCs were incubated in a suspension culture with either Wnt3a or control in the presence of FGF2 for 24 hours (primary sphere). Cells were plated in suspension at a low density (1000 cells/well in a 96-well dish) and incubated for 9 days in the presence of FGF2 without Wnt3a (secondary spheres). Data represent the number of cell aggregates formed (neurospheres). (**D**) E10.5 NPCs were infected with a retrovirus encoding GFP (control) or with both GFP and N-myc, and incubated in a suspension culture for 4 days in the presence of FGF2 and EGF. The cells were plated as in C (3000 cells/well) and incubated for 7 days with FGF2 and EGF. Then, the numbers of GFP$^+$ and GFP$^-$ neurospheres were counted (secondary spheres). Data represent the numbers of GFP$^+$ neurospheres. Under these conditions, the number of GFP$^-$ neurospheres (i.e. neurospheres formed from uninfected cells) in N-myc-infected cultures was not significantly different from that in control culture (control, 28.2±1.8; N-myc, 29.4±1.3). (**E**,**F**) E11.5 NPCs were plated on PDL-coated dishes and infected with a retrovirus encoding GFP to label mitotic cells, followed by incubation with FGF2 for 6 hours. The cells were incubated with Wnt3a protein or vehicle control for 24 hours in the presence of FGF2, followed by incubation without FGF2 for the indicated days to promote differentiation. The percentage of Tbr2$^+$ cells (E) and Pax6$^+$ cells (F) among GFP$^+$ cells was determined by immunostaining. (**G-I**) E11.5 NPCs were infected with a retrovirus encoding GFP (control), or with both GFP and N-myc (G), or with both GFP and ΔN-Tcf3 (H,I). The cells were incubated with FGF2 for 2 days, followed by incubation without FGF2 for 1 day (G,H) or 2 days (I) to promote differentiation. The percentage of Tbr2$^+$ cells (G,H) and βIII-tubulin$^+$ cells (I) among GFP$^+$ cells was determined by immunostaining. (A,B,E-I) Data represent mean±s.e.m. (C,D) Data represent mean±s.e.m of three independent samples. *, P<0.05; **, P<0.01; ***, P<0.001.

percentage of Tbr2-positive cells among GFP-positive cells (Fig. 3G). These results indicate that Wnt signaling and N-myc may increase NPs in vitro.

We next investigated whether the Wnt–β-catenin pathway is required for the generation of Tbr2-positive cells in vitro. NPC culture prepared from E11.5 mouse neocortex was infected with retroviruses encoding either GFP alone or ΔN-Tcf3 and GFP and cultured in the absence of FGF2 to promote differentiation. ΔN-Tcf3 overexpression decreased the percentage of Tbr2-positive cells and that of βIII-tubulin-positive cells among GFP-positive cells (Fig. 3H,I). These results indicate that repression of Wnt signaling may reduce the production of NPs.

Wnt signaling and N-myc increase basal progenitors in the developing neocortex

To investigate whether Wnt signaling promotes production of basal progenitors in vivo, we infected a small proportion of neocortical NPCs with a retrovirus encoding both stabilized β-catenin (S33Y mutant) and a nuclear-localized GFP (H2B-GFP) or a control retrovirus encoding H2B-GFP alone. Expression of stabilized β-catenin in the neocortical NPCs of E12.5 mice significantly increased the percentage of Tbr2-positive cells among GFP-positive cells at E14.0 (Fig. 4A). Concomitantly, expression of stabilized β-catenin reduced the percentage of Pax6-positive cells (Fig. 4B). As Tbr2 or Pax6 is a basal progenitor marker or an apical progenitor marker, respectively, in vivo (Englund et al., 2005; Sessa et al., 2008), these results indicate that the Wnt–β-catenin pathway increases the number of basal progenitors in vivo. In this condition, expression of stabilized β-catenin reduced the percentage of GFP-positive cells in the ventricular zone (VZ) and increased that in the cortical plate (CP) compared with control, consistent with our previous results demonstrating Wnt-induced neurogenesis (Fig. 4C and see its legend) (Hirabayashi et al., 2004).

We then examined the effect of N-myc overexpression on the number of basal progenitors. Expression of N-myc by retroviral infection reduced the proportion of GFP-positive cells in the VZ and increased the proportion in the intermediate zone (IMZ) compared with the control (Fig. 4D,D′,G). Furthermore, retroviral introduction

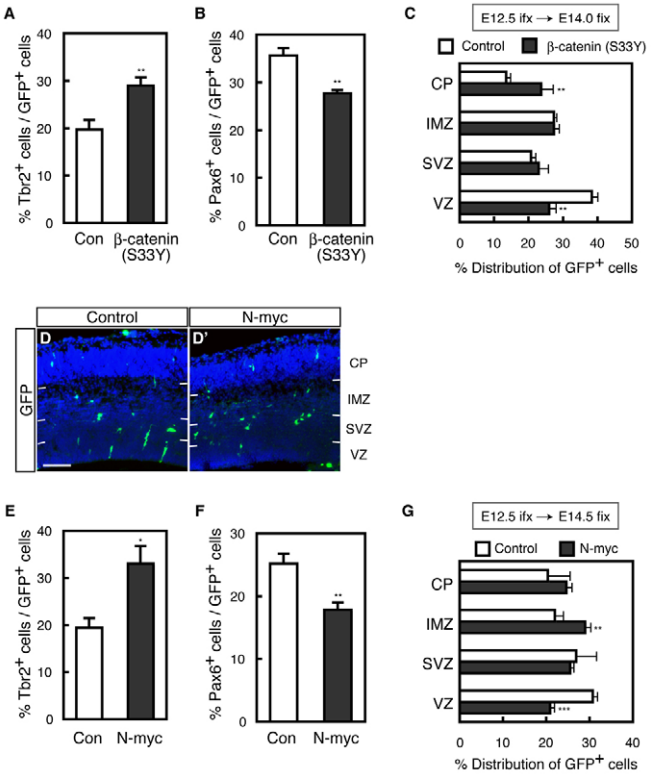

Fig. 4. Retroviral expression of stabilized β-catenin and N-myc reduces the proportion of apical progenitors and increases that of basal progenitors in the developing neocortex. (**A-G**) Retroviruses were injected into the lateral ventricle of E12.5 mice. These viruses encode H2B-GFP (control or Con; A-C,E-G), both H2B-GFP and β-catenin-S33Y (A-C), both H2B-GFP and N-myc (E-G), GFP alone (control; D), or both GFP and N-myc (D′). After 1.5 days (A-C) or 2.0 days (D-G), brains were fixed and examined. (D,D′) Typical sections stained with anti-GFP and Hoechst. (A,B,E,F) Brain sections were immunostained with anti-GFP and anti-Tbr2 for basal progenitors (A,E) or anti-Pax6 for apical progenitors (B,F). The percentage of Tbr2$^+$ cells (A,E) and of Pax6$^+$ cells (B,F) among GFP$^+$ cells was determined. (C,G) The distribution of the GFP$^+$ cells in the developing neocortex. Notably, expression of stabilized β-catenin increased the percentage of Tbr2$^+$ cells in the VZ (control, 23±4%; β-catenin-S33Y, 43±3%) as well as in the SVZ and IMZ (control, 24±2%; β-catenin-S33Y, 38±2%). (A-C,E-G) Data represent mean±s.e.m. of typical brain. *, P<0.05; **, P<0.01; ***, P<0.001. CP, cortical plate; IMZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar: 50 μm.

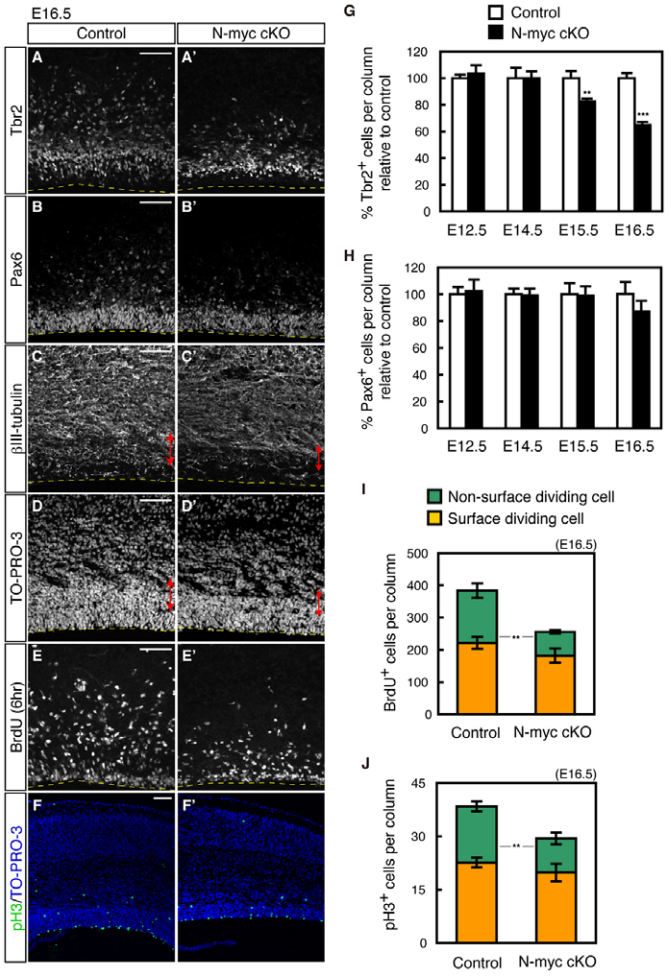

Fig. 5. Conditional deletion of *N-myc* reduces the number of basal progenitors in the developing neocortex. (**A-F′**) Coronal sections of control (A-F) and *N-myc*$^{lox/lox}$*;Nes-Cre* (N-Myc-cKO, A′-F′) neocortex at E16.5 immunostained as indicated. (E,E′) Immunostaining for BrdU in E16.5 cortex 6 hours after BrdU injection in order to label mitotic cells around M phase. Yellow dashed lines represent the ventricular surface. Red arrows indicate the SVZ. (**G**,**H**) Quantification of Tbr2$^+$ cells (G) and Pax6$^+$ cells (H) in the control and N-Myc-cKO neocortex at E12.5, E14.5, E15.5 and E16.5. (**I**,**J**) Quantification of BrdU$^+$ cells (I) and pH3$^+$ cells (J) at the ventricular surface (surface division) and above the ventricular surface (non-surface division). (G-J) Cell number was counted in a unit section of 460 μm width (G-I) and 800 μm width (J). Data represent the mean±s.e.m. **, P<0.01; ***, P<0.001. Scale bars: 100 μm.

of N-myc at E12.5 significantly increased the percentage of Tbr2-positive cells among GFP-positive cells and reduced that of Pax6-positive cells in the neocortex at E14.5 (Fig. 4E,F). These results suggest that activation of Wnt signaling, as well as its target N-myc, is sufficient to increase the basal progenitor population in the developing neocortex.

N-myc is necessary for the expansion of basal progenitors

We further investigated whether N-myc regulates the production of basal progenitors during neocortical development by conditional deletion of the *N-myc* gene. In the *N-myc*$^{lox/lox}$*;Nes-Cre* mouse (N-Myc-cKO mouse), the levels of *N-myc* mRNA in the neocortex were reduced to ~30% at E12.5 and to ~3% at E14.5 compared with wild-type mice, indicating successful gene deletion (data not shown). The radial thickness of the subventricular zone (SVZ) detected by nuclear and βIII-tubulin staining appears to be reduced in N-Myc-cKO mice at E16.5 (Fig. 5C-D′). Indeed, the number of Tbr2-positive cells per unit width (per column) was markedly reduced in N-Myc-cKO mice at E15.5 and 16.5, compared with control mice (Fig. 5A,A′,G), whereas that of Pax6-positive cells was less affected by the *N-myc* gene deletion (Fig. 5B,B′,H). These results suggest that N-myc is in part necessary for the production of basal progenitors in vivo.

We further analyzed the numbers of basal progenitors and apical progenitors by comparing dividing cells located above the ventricular surface (non-surface division) versus cells dividing at the ventricular

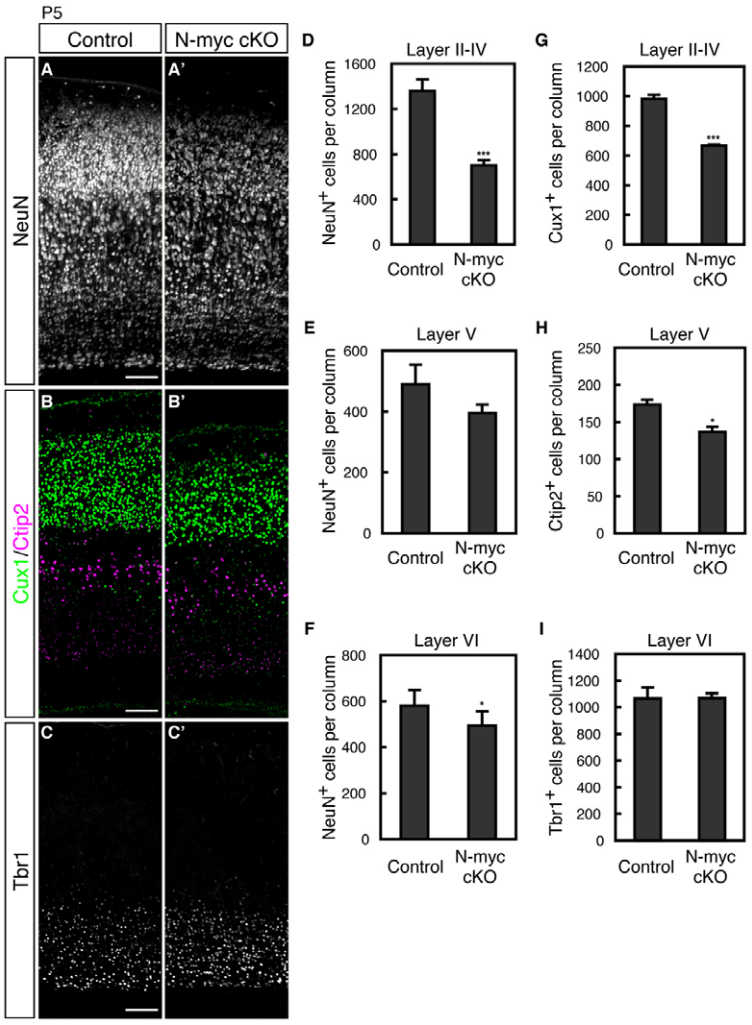

Fig. 6. Conditional deletion of *N-myc* reduces the number of neocortical neurons, particularly in the upper layers. (**A-C′**) Coronal sections of control (A-C) and *N-myc*$^{lox/lox}$*;Nes-Cre* (N-Myc-cKO, A′-C′) neocortex taken from equivalent coronal levels (at the level of the anterior hippocampus) at postnatal day 5 (P5) and immunostained as indicated. (**D-I**) Quantification of marker-positive cells. Cell number was counted in a unit section of 510 μm width. (D-F) The numbers of NeuN$^+$ cells in layers II-IV (D), layer V (E) and layer IV (F) were determined. Data represent the mean±s.e.m. *, $P<0.05$; ***, $P<0.001$. Scale bars: 100 μm.

surface (surface division), which represent basal and apical progenitors, respectively. Staining for pH3 allowed detection of M-phase dividing cells and revealed that the numbers of non-surface dividing cells was markedly reduced by deletion of the *N-myc* gene, whereas those of surface dividing cells were reduced to a lesser extent (Fig. 5F,F′,J). Similar results were obtained when cells around M phase were detected 6 hours after a pulse BrdU labeling (Fig. 5E,E′,I). These results together indicate that conditional deletion of the *N-myc* gene reduces the number of basal progenitors in the developing neocortex.

N-myc is in part necessary for neuronal production

Finally, we investigated the role of N-myc in the generation of neocortical neurons in vivo by examining the postnatal phenotypes of N-Myc-cKO mice. We examined the numbers of neurons at each layer by immunostaining for NeuN, a mature neuronal marker. *N-myc* gene deletion markedly reduced the numbers of layer II-IV neurons per column in the somatosensory field of the neocortex (Fig. 6A,A′,D). Consistent with this, the numbers of neurons positive for Cux1, a marker for layer II-IV neurons, were also significantly reduced in N-Myc-cKO mice (Fig. 6B,B′,G). *N-myc* gene deletion had less effect on the deep-layer neurons, as detected by immunostaining with NeuN, Ctip2 or Tbr1 (Fig. 6A-C′,E, F, H, I). It is not clear why deletion of N-myc had a weaker effect on the deep-layer neurons compared with the upper-layer neurons, but this could be due to incomplete elimination of N-myc protein at the time point when deep-layer neurons are generated, or to the selective function of N-myc on neuronal production of different layers considering a proposed role of basal progenitors in the expansion of upper-layer neurons (Dehay and Kennedy, 2007; Smart and McSherry, 1982; Wu et al., 2005).

DISCUSSION

Although it has been suggested that basal progenitors play a pivotal role in determining the number of neocortical neurons, the molecular mechanisms regulating the generation of basal progenitors have just begun to be explored. Here we show that Wnt signaling and its downstream target N-Myc play a key role in the production of basal progenitors. Expression of N-myc or stabilized β-catenin increases, while conditional gene deletion of *N-myc* decreases the numbers of basal progenitors found in the developing neocortex, as determined by the numbers of Tbr2-positive cells and non-surface dividing cells. The increase in basal progenitors by the Wnt–N-myc axis can be ascribed to either: (1) differentiation of apical progenitors into basal progenitors; or (2) proliferation (and survival) of basal progenitors, or both. The observation that retroviral expression of stabilized β-catenin or N-myc in the neocortex reduced the number of apical progenitors while increasing that of the basal progenitors supports a role for the former mechanism (Fig. 7A).

Members of the Myc family have been reported to be involved in differentiation processes in other cell types, including epithelial, neural crest and hematopoietic stem cells (for a review, see Eilers

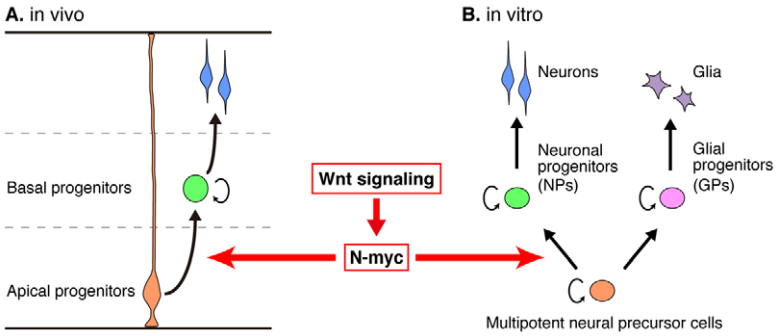

Fig. 7. A model for fate regulation of neural progenitor populations by Wnt signaling and N-myc. (**A**) Wnt signaling and its downstream target N-myc promote differentiation of apical progenitors into basal progenitors in vivo (Figs 4, 5). (**B**) Wnt signaling and N-myc promote neuronal fate commitment of multipotent 'sphere-forming' NPCs and production of NPs in an in vitro culture (Figs 2, 3).

and Eisenman, 2008; Watt et al., 2008), although, to our knowledge, previous reports have not directly demonstrated that Myc is involved in fate commitment by a lineage-tracing analysis. In this study, the clonal analysis suggests that N-myc instructs commitment of NPC fate into the neuronal lineage at the expense of the glial lineage and reduces multipotent neurosphere-forming NPCs (Fig. 7B). This function of N-myc is similar to the reported function of Wnt signaling (Hirabayashi et al., 2004; Kleber and Sommer, 2004; Muroyama et al., 2004).

We do not know what transcriptional targets of N-myc are involved in instructing neurogenesis. Possible candidates include the proneural gene Ngn1, as deletion of N-myc was observed to cause a decrease in the level of *Ngn1* mRNA in the developing neocortex (data not shown). As Ngn1 is also a direct target of the β-catenin/Tcf transcription complex (Hirabayashi et al., 2004; Israsena et al., 2004), it would be interesting to examine the interaction between N-myc and these transcription factors on the *Ngn1* promoter. The Myc family has also been shown to function in the regulation of the global chromatin state (Knoepfler et al., 2006), in addition to its function as a classical transcription factor; thus it is possible that mechanisms other than direct target gene activation are also involved in N-myc regulation of neurogenesis and proliferation of NPCs.

In this study, we also provide evidence that N-myc is directly regulated by the β-catenin/Tcf transcription complex and mediates the functions of Wnt signaling to stimulate neocortical NPC proliferation and differentiation: (1) Wnt3a treatment and stabilized β-catenin expression induced N-myc expression, whereas expression of a dominant-negative form of Tcf3 reduced N-myc expression in NPC cultures; (2) misexpression of stabilized β-catenin in the ventral telencephalon induced ectopic N-myc expression in vivo; (3) N-myc is expressed in the developing neocortex in a pattern similar to that of a Tcf reporter transgene; (4) Tcf3 directly binds to a Tcf-consensus site 1.6 kb upstream of the N-myc gene; (5) Wnt stimulation of proliferation and differentiation in NPC cultures was abrogated by deletion of the *N-myc* gene. These results provide evidence that N-myc is a key downstream mediator of Wnt–β-catenin signaling in the developing neocortex. It is of note that N-myc is not the only downstream target responsible for the functions of Wnt signaling in the neocortex (see Fig. S3 and its legend in the supplementary material).

The Wnt–β-catenin pathway exerts multiple functions in a context-dependent manner. For instance, persistent expression of stabilized β-catenin in NPCs (i.e. β-catenin-ΔEx3 mouse) results in overproliferation of apical progenitors and horizontal/tangential expansion of the cortex in addition to the reduction of Tbr2-positive basal progenitors (see Fig. S3A,B in the supplementary material; data not shown), as reported previously (Chenn and Walsh, 2002; Wrobel et al., 2007). However, when the same stabilized β-catenin was expressed by retroviral infection in a small proportion of NPCs located at the VZ, it had the opposite effect: increasing the numbers of basal progenitors and decreasing the number of apical progenitors. This difference does not appear to be due to the differential requirement of N-myc, as N-myc gene deletion rescued both proliferative and differentiating effects of activation of β-catenin (Fig. 2A,C; regarding in vivo effects, see also Fig. S3 in the supplementary material). This difference might be rather due to the aberrant brain architecture generated in the β-catenin–ΔEx3 mice, to other non-cell autonomous effects of β-catenin or to differences in the levels or timing of active β-catenin expression. Indeed, different levels of active β-catenin expression result in different outcomes in hair follicle stem cells (Lowry et al., 2005).

Although it has previously been postulated that β-catenin exerts its different functions via distinct targets (Clevers, 2006), we observe that both the proliferating and neurogenic functions of Wnt–β-catenin signaling in the developing neocortex are mediated in common by N-myc (Fig. 2A,C). It is noteworthy that c-Myc can also exert distinct functions depending on its expression levels, such as in epithelial stem cells (Watt et al., 2008), raising the possibility that the levels of N-myc might determine the cellular output. Importantly, heterozygous mutation of *N-MYC* (*MYCN*) in humans causes Feingold syndrome (OMIM 164280), comprising several defects including microcephaly, supporting the notion that the levels of N-myc in the nervous system are crucial for determining the neuronal number and brain size. It is also possible that N-myc alters its function in a developmental-stage-dependent manner. This possibility is consistent with our previous finding that canonical Wnt signaling promotes proliferation of neocortical neural precursor cells at a relatively early stage (E10.5) but promotes their differentiation at a relatively late stage (E13.5) (Hirabayashi and Gotoh, 2005; Hirabayashi et al., 2004).

Which Wnt ligands are responsible for the activation of N-myc and consequent regulation of basal progenitors in the developing brain? Wnt7a is expressed in NPCs at the VZ and might be important for increase in cells localized in the SVZ (Viti et al., 2003). Wnt7b, which is expressed in the deep-layer neurons (neurons at the layer VI), might elicit a feed-forward signal to increase the number of basal progenitors that in turn contribute to the generation of the upper-layer neurons (Rubenstein et al., 1999). It is plausible that extracellular signals other than Wnt ligands are also involved in the activation of N-myc and regulation of basal progenitors. N-myc is induced by Shh signaling in cerebellar granule cells (Hatton et al., 2006; Kenney et al., 2003; Oliver et al., 2003), and a recent report shows that Shh protein is localized in the IMZ of the neocortex and contributes to the production of basal progenitors (Komada et al., 2008). Growth factors expressed in NPCs such as Fgf2 and

epidermal growth factor (Egf) might also participate in the activation of N-myc. Growth factor receptors activate the PI3K (Pik3r1 – Mouse Genome Informatics) pathway, which induces phosphorylation and stabilization of N-myc protein (Kenney et al., 2004). In addition, Egfr as well as Frs2, an adaptor of Fgfr/Egfr, have been shown to regulate the production of basal progenitors (Yamamoto et al., 2005). The RNA-binding protein HuC/D is another candidate that could regulate N-myc function in basal progenitors, as it binds to and stabilizes *N-myc* mRNA and is localized in the SVZ (Lazarova et al., 1999; Miyata et al., 2004).

As a mechanism of neocortical expansion during animal evolution, the increase of basal progenitors is considered to be a key event, given that basal progenitors increase the number of neurons from a given number of apical progenitors through extra cell division and that the number of basal progenitors dramatically increases during animal evolution (Cheung et al., 2007; Martinez-Cerdeno et al., 2006; Smart et al., 2002). The observation in this study that N-myc deletion decreases Tbr2-positive cells and non-surface dividing cells without marked reduction of Pax6-positive cells supports the notion that Wnt signaling, via N-myc, promotes differentiation from apical progenitors to basal progenitors and promotes indirect neurogenesis. It would be interesting to investigate possible roles of this signaling pathway in the neocortical expansion during animal evolution in future studies.

Acknowledgements

We thank Drs Roel Nusse and Akira Kikuchi for instruction on Wnt3a purification; Dr Noriaki Kojima and Ms Akashi Izumi for microarray analysis; Drs Robert N. Eisenman and Marc Lamphier for critical reading of the manuscript; Dr Ryoichiro Kageyama for Nes-Cre mice; Drs Toshio Kitamura, Theodore Fotsis, Shigeaki Kato and Randall T. Moon for plasmids; and members of the Gotoh laboratory for help and discussion. A.K. is a research fellow of the Japan Society for the Promotion of Science (JSPS). This work was supported in part by Grants-in-Aid from Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, by Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency, by Grants-in-Aid for JSPS fellows from JSPS, and by the Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms), MEXT, Japan.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.046417/-/DC1

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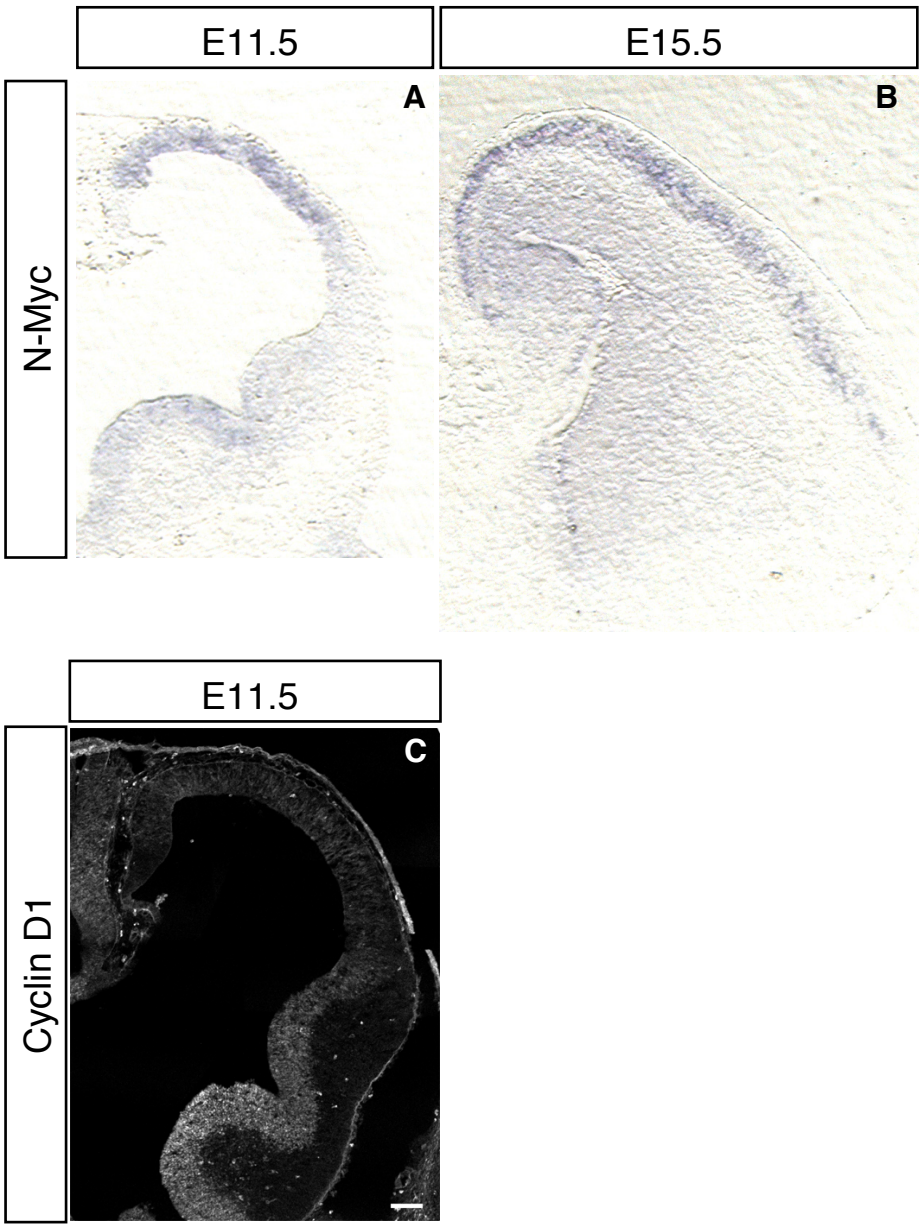
Table S1

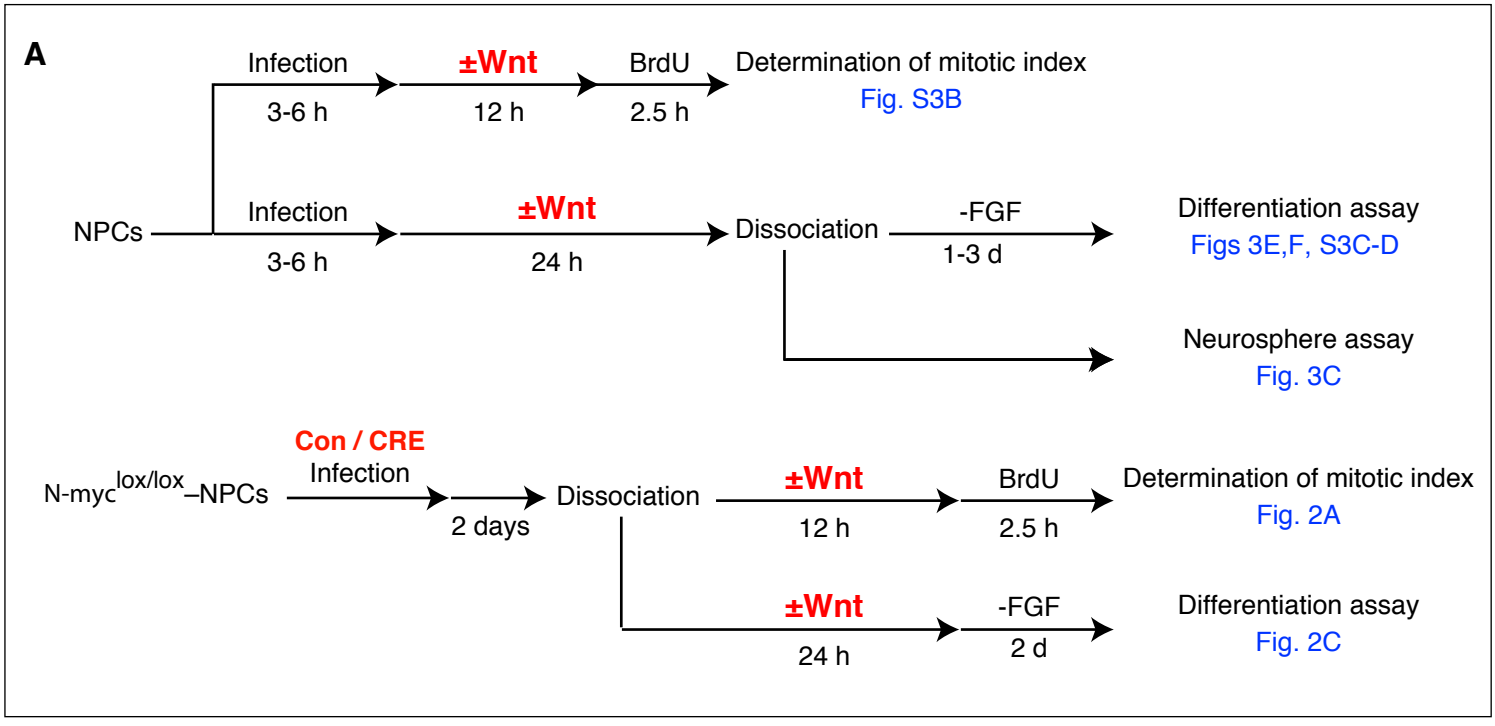
Cell cycle related genes (upregulated)

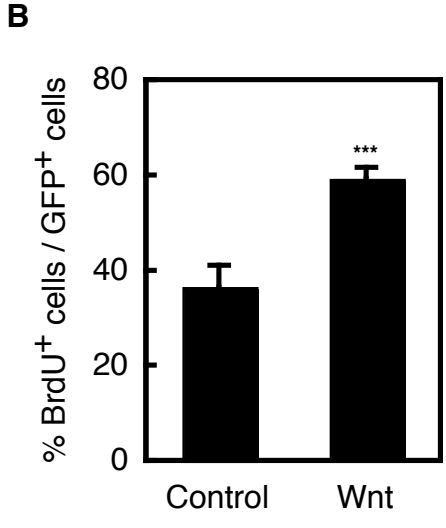
Gene	Probe ID	Fold change Wnt / Control		
		3 hr	6 hr	12 hr
Axin2	1436845_at	**4.5**	**3.3**	**3.2**
Id3	1416630_at	**2.4**	**2.5**	**2.5**
Jag1	1434070_at,1421106_at	**2.0**	**2.0**	**2.0**
Nedd9	1450767_at	**2.0**	**1.5**	**1.7**
Bmp7	1418910_at	**1.7**	**1.6**	**1.9**
Tm4sf9	1431530_a_at	**1.4**	**1.7**	**2.0**
N-myc	1417155_at	**1.7**	**1.8**	**1.4**
Hira	1436241_s_at	**1.4**	**1.8**	**1.7**
Rasl11b	1423854_a_at	**1.6**	**1.5**	**1.7**
Smc2l1	1429658_a_at	**1.5**	**1.6**	**1.6**
Tgfb2	1450923_at,1423250_a_at	**1.4**	**1.6**	**1.7**
Hes1	1418102_at	**1.5**	**1.5**	**1.7**
Tgif	1422286_a_at	**1.6**	**1.5**	**1.5**
Fgf15	1418376_at	**1.6**	**1.4**	**1.5**

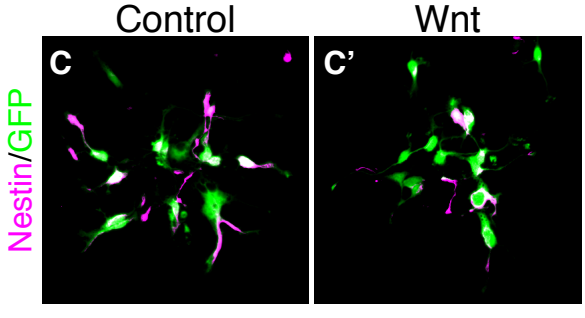
Cell cycle related genes (downregulated)

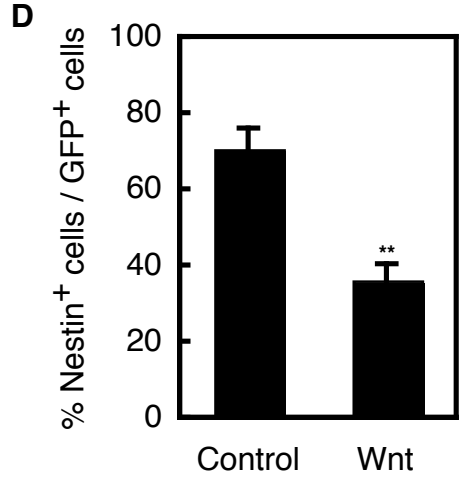
Gene	Probe ID	Fold change Wnt / Control		
		3 hr	6 hr	12 hr
1190002H23Rik	1438511_a_at	**0.53**	**0.22**	**0.44**
Morf4l1	1438597_x_at	**0.17**	**0.57**	**0.33**
Rad21	1455938_x_at	**0.22**	**0.43**	**0.35**
Cav2	1417327_at	**0.15**	**0.47**	**0.52**
Cav1	1449145_a_at	**0.61**	**0.32**	**0.37**
Sesn1	1438931_s_at	**0.38**	**0.60**	**0.48**
Btg1	1437455_a_at	**0.42**	**0.63**	**0.45**
Jun	1417409_at,1448694_at	**0.53**	**0.45**	**0.54**
Ptch1	1428853_at	**0.61**	**0.50**	**0.61**

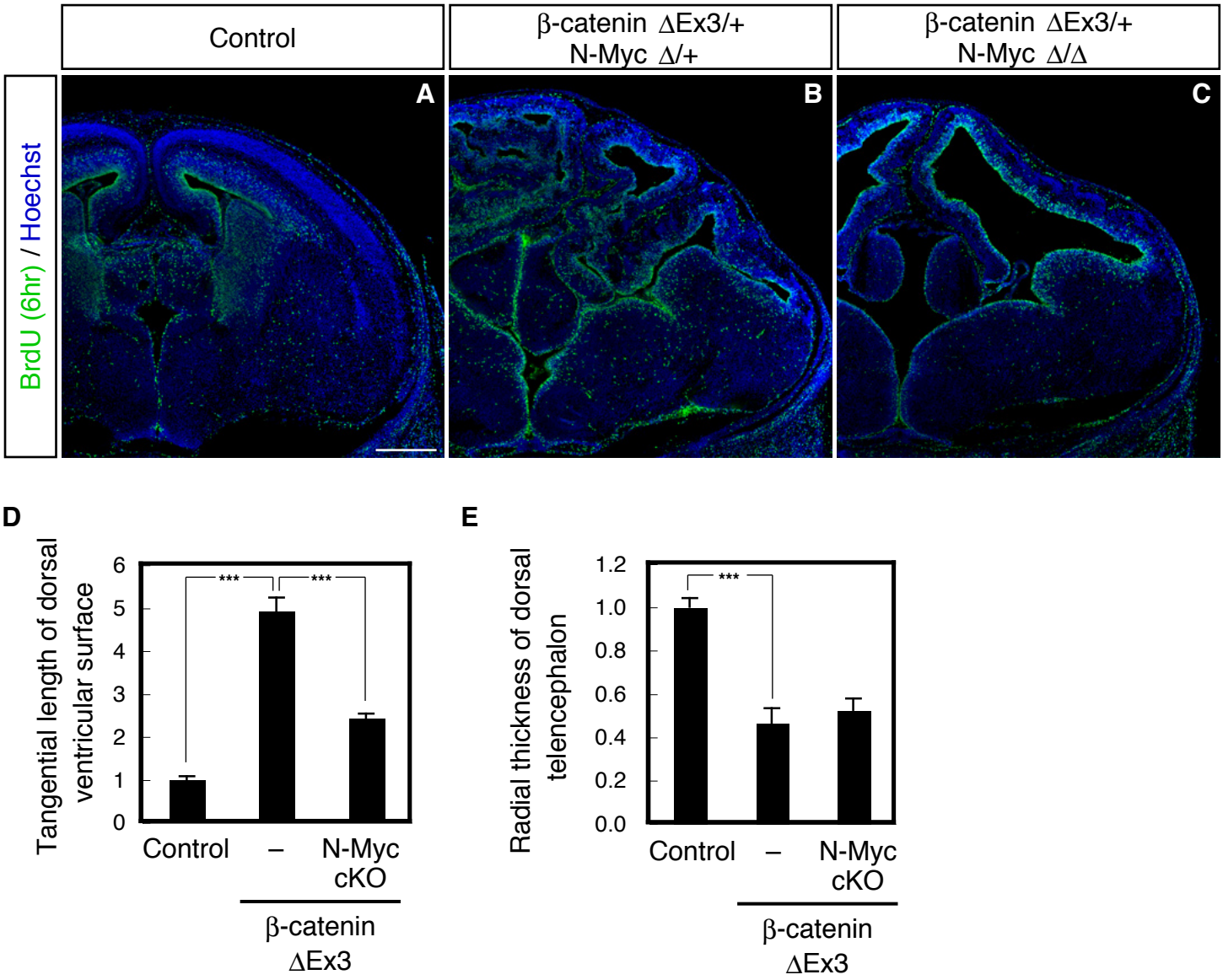

E11.5
E15.5
N-Myc
A
B
E11.5
Cyclin D1
C


A
Infection
3-6 h
±Wnt
12 h
BrdU
2.5 h
Determination of mitotic index
Fig. S3B
NPCs
Infection
3-6 h
±Wnt
24 h
Dissociation
-FGF
1-3 d
Differentiation assay
Figs 3E,F, S3C-D
Neurosphere assay
Fig. 3C
N-myc lox/lox –NPCs
Con / CRE
Infection
2 days
Dissociation
±Wnt
12 h
BrdU
2.5 h
Determination of mitotic index
Fig. 2A
±Wnt
24 h
-FGF
2 d
Differentiation assay
Fig. 2C


B
% BrdU+ cells / GFP+ cells
80
60
40
20
0
***
Control
Wnt


Control
Wnt
Nestin/GFP
C
C'


D
% Nestin+ cells / GFP+ cells
100
80
60
40
20
0
**
Control
Wnt


Control
β-catenin ΔEx3/+
N-Myc Δ/+
β-catenin ΔEx3/+
N-Myc Δ/Δ
BrdU (6hr) / Hoechst
A
B
C
D
Tangential length of dorsal ventricular surface
0
1
2
3
4
5
6
***
***
Control
–
N-Myc cKO
β-catenin ΔEx3
E
Radial thickness of dorsal telencephalon
0.0
0.2
0.4
0.6
0.8
1.0
1.2
***
Control
–
N-Myc cKO
β-catenin ΔEx3