WNT signaling increases proliferation and impairs differentiation of stem cells in the developing cerebellum

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SUMMARY

The WNT pathway plays multiple roles in neural development and is crucial for establishment of the embryonic cerebellum. In addition, WNT pathway mutations are associated with medulloblastoma, the most common malignant brain tumor in children. However, the cell types within the cerebellum that are responsive to WNT signaling remain unknown. Here we investigate the effects of canonical WNT signaling on two important classes of progenitors in the developing cerebellum: multipotent neural stem cells (NSCs) and granule neuron precursors (GNPs). We show that WNT pathway activation in vitro promotes proliferation of NSCs but not GNPs. Moreover, mice that express activated β-catenin in the cerebellar ventricular zone exhibit increased proliferation of NSCs in that region, whereas expression of the same protein in GNPs impairs proliferation. Although β-catenin-expressing NSCs proliferate they do not undergo prolonged expansion or neoplastic growth; rather, WNT signaling markedly interferes with their capacity for self-renewal and differentiation. At a molecular level, mutant NSCs exhibit increased expression of c-Myc, which might account for their transient proliferation, but also express high levels of bone morphogenetic proteins and the cyclin-dependent kinase inhibitor p21, which might contribute to their altered self-renewal and differentiation. These studies suggest that the WNT pathway is a potent regulator of cerebellar stem cell growth and differentiation.

KEY WORDS: WNT, β-catenin, Stem cell, Cerebellum, Self-renewal, Mouse

INTRODUCTION

WNT proteins play crucial roles in nervous system development (Ille and Sommer, 2005; Salinas and Zou, 2008). Mutations in *Wnt1* cause severe defects in the midbrain, hindbrain and developing spinal cord (McMahon and Bradley, 1990; McMahon et al., 1992; Ikeya et al., 1997) and ablation of *Wnt3a* results in loss of the hippocampus (Lee et al., 2000). Conversely, ectopic expression of β-catenin (a key activator of the canonical WNT signaling pathway) in neural precursors leads to expansion of the progenitor pool and enlargement of the forebrain and spinal cord (Chenn and Walsh, 2002; Chenn and Walsh, 2003; Zechner et al., 2003). In addition to its mitogenic effects in some parts of the CNS (Megason and McMahon, 2002; Ille et al., 2007), WNT signaling can also regulate cell fate determination (Lee et al., 2004), differentiation (Hirabayashi et al., 2004), axon growth (Ouchi et al., 2005), synapse formation (Hall et al., 2000; Zaghetto et al., 2007) and myelination (Fancy et al., 2009). Thus, WNTs may have distinct effects on different cell types in the developing nervous system.

Among the most widely studied functions of WNT signaling is in the establishment of the midbrain-hindbrain boundary that gives rise to the cerebellum (McMahon and Bradley, 1990). However, the role of the pathway at later stages of cerebellar development is less

well understood. At postnatal stages, Wnt7a is required for axonal branching by granule neurons and facilitates their formation of synapses with mossy fibers (Lucas and Salinas, 1997; Hall et al., 2000). In addition, recent studies have shown that deletion of β-catenin in nestin-expressing progenitors results in premature neuronal differentiation and hypoplasia of the cerebellar vermis, suggesting that WNT signaling might regulate growth and differentiation in the embryonic and early postnatal cerebellum (Schuller and Rowitch, 2007). Perhaps the most striking evidence for the importance of WNT signaling in the cerebellum is the association between WNT pathway mutations and the cerebellar tumor medulloblastoma. Germline mutations in the adenomatous polyposis coli (*APC*) gene, a negative regulator of canonical WNT signaling (Hamilton et al., 1995), result in Turcot's syndrome, a disorder characterized by increased incidence of colon cancer and brain tumors (often medulloblastomas). In addition, 15-20% of sporadic medulloblastomas harbor activating mutations in β-catenin or inactivating mutations in *APC* (Zurawel et al., 1998; Huang et al., 2000; Clifford et al., 2006; Thompson et al., 2006). Recent studies suggest that WNT-associated medulloblastomas can arise from cells outside the cerebellum, in the dorsal hindbrain (Gibson et al., 2010); however, it remains possible that some of these tumors originate from progenitors within the cerebellum. The ability of progenitors in the cerebellum to proliferate in response to WNT signaling remains poorly studied.

The cerebellum contains two distinct germinal zones: the ventricular zone (VZ), which contains multipotent neural stem cells (NSCs) that give rise to the majority of cerebellar neurons and glia, and the external granule layer (EGL), which contains granule neuron precursors (GNPs) that give rise to a single cell type, the granule neuron (Goldowitz and Hamre, 1998; Wang and Zoghbi, 2001). To determine which of these cells is susceptible to the mitogenic effects of WNT signaling, we isolated NSCs and GNPs

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and tested their ability to proliferate following infection with β-catenin-encoding retroviruses. In addition, we used transgenic mice carrying a Cre-inducible allele of β-catenin to examine the effects of activating the WNT pathway in stem cells and progenitors in vivo. Our studies reveal that WNT signaling is not mitogenic for GNPs. By contrast, activation of the WNT pathway does promote proliferation of NSCs in the VZ, and these cells undergo expansion during embryonic development. However, this expansion is accompanied by loss of the ability to undergo self-renewal or differentiation and by failure to form most differentiated cell types in the cerebellum. These studies suggest that WNT signaling plays an important role in regulating the growth and differentiation of stem cells in the developing cerebellum.

MATERIALS AND METHODS
Mice
Catnb$^{lox(ex3)/+}$ mice (Harada et al., 1999), Apc$^{lox/lox}$ mice (Colnot et al., 2004) and Catnb$^{lox(ex2-6)}$ (Brault et al., 2001) mice have been described previously. hGFAP-Cre (Zhuo et al., 2001) mice and hGFAP-green fluorescent protein (hGFAP-GFP) (Zhuo et al., 1997) mice were from Jackson Laboratories, and Math1-Cre mice (Schuller et al., 2008; Yang et al., 2008) were provided by David Rowitch at UCSF. All mice were maintained in the Cancer Center Isolation Facility at Duke University. All experiments were performed in accordance with national guidelines and regulations, and with the approval of the Duke University Animal Care and Use Committee.

Immunostaining
Tissue was harvested from embryos or neonates, fixed in 4% paraformaldehyde (PFA), equilibrated in 25% sucrose at 4°C and frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA). For bromodeoxyuridine (BrdU) staining, pregnant females were injected with 100 mg/kg BrdU prior to embryo harvest. Cryosections (12 μm) were blocked with 10% goat serum in 0.1% Triton X-100 at room temperature and primary antibodies were applied overnight at 4°C. Antibodies included rabbit anti-Ki67 (Abcam, Cambridge, MA, USA; 1:100) or mouse anti-Ki67 (BD Biosciences, San Jose, CA, USA; 1:100), rabbit anti-Sox1 (gift of Larysa Pevny, University of North Carolina, Chapel Hill; 1:1500), rabbit anti-β-catenin (Abcam; 1:2000), rabbit anti-calbindin (EpitMics, Burlingame, CA, USA; 1:250), rabbit anti-Pax2 (Covance, Princeton, NJ, USA; 1:250), rabbit anti-Pax6 (Covance; 1:300), mouse anti-S100β (Sigma; 1:500), rabbit anti-Map2 (Chemicon/Millipore, Temecula, CA, USA; 1:1000), rabbit anti-BLBP (Chemicon; 1:500), mouse anti-GFAP [cocktail of monoclonal antibodies (clones 4A11, 1B4, 2E1), BD Pharmingen; 1:100] and rat anti-BrdU (Abcam; 1:100). Secondary antibodies included goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) used at 1:200. Noggin, Bmp2 and Bmp7 proteins were purchased from Peprotech (Rocky Hill, NJ, USA) and used at 200 ng/ml. p21shRNA retroviral plasmid (Fasano et al., 2007) was a gift from Sally Temple at the Neural Stem Cell Institute, Rensselaer, NY, USA. Cre retrovirus was a gift from Tannishtha Reya (University of California, San Diego).

Cell isolation and flow cytometry
Cells were isolated from embryonic (E14.5) or neonatal (P0) cerebellum as described (Oliver et al., 2005). Briefly, tissue was digested in a solution containing 10 units/ml papain (Worthington) and 250 U/ml DNase, and triturated to obtain a single-cell suspension.

To isolate prominin 1$^+$ lineage-negative (Prom1$^+$ Lin$^-$) cells from wild-type (WT) or mutant mice, cells were suspended in FACS buffer (Dulbecco's PBS with 5% FCS). Cells were stained for 1 hour with phycoerythrin (PE)-conjugated rat anti-prominin 1 antibody (clone 13A4, eBioscience, San Diego, CA, USA; 1:200) and with antibodies specific for lineage markers [polysialylated neuronal cell adhesion molecule (PSA-NCAM), Chemicon/Millipore, 1:200; O4, Chemicon/Millipore, 1:100; TAPA-1 (CD81), eBioscience, 1:250]. After staining with FITC-conjugated

secondary antibodies, cells were washed and analyzed or sorted using a FACS-Vantage SE flow cytometer (BD Biosciences). PE-conjugated rat IgG was used as a negative control.

In vitro analysis of proliferation, differentiation and neurosphere formation
To analyze the effects of β-catenin on proliferation, cells were cultured in Neurobasal medium with B27 supplement (NB-B27, Invitrogen) on poly-D-lysine (PDL)-coated chamber slides (5×10^4 cells/well). β-catenin-IRES-YFP or control YFP viruses were added and cells incubated for 48 hours before staining with anti-Ki67. To assay incorporation of tritiated thymidine, cells were cultured in PDL-coated 96-well plates at 2×10^5 cells/well. β-catenin-IRES-GFP or control GFP viruses were added and cells incubated for 48 hours before being pulsed with methyl-[^{3}H]thymidine (GE Healthcare, Piscataway, NJ, USA). After 16 hours, cells were harvested using a Mach IIIM manual harvester 96 (Tomtec, Hamden, CT, USA), and incorporated radioactivity was quantitated using a Wallac MicroB microplate scintillation counter (Perkin Elmer, Waltham, MA, USA).

To analyze the differentiation potential of WT or mutant NSCs, Prom1$^+$ Lin$^-$ cells were plated on PDL-coated coverslips in NB-B27 plus 1% FBS, and cultured for 3 days before fixation with 4% PFA, permeabilization with 0.1% Triton X-100, and blocking with 10% goat serum. Cells were stained with anti-Map2 and anti-S100β.

To measure neurosphere formation, Prom1$^+$ Lin$^-$ cells were cultured at 2000 cells/ml in uncoated wells containing Neurocult with proliferation supplement (Stem Cell Technologies, Vancouver, BC, Canada) plus 25 ng/ml bFGF (Invitrogen) and 25 ng/ml EGF (Peprotech). Cells were cultured for 7 days and photographed under bright-field microscopy. To examine self-renewal, spheres were dissociated in 0.05% trypsin/EDTA (Invitrogen) and replated at 2000 cells/ml.

Real-time RT-PCR
mRNA was isolated using the RNAqueous kit (Ambion, Austin, TX, USA) and treated with DNA-free DNase (Ambion). cDNA was synthesized using oligo(dT) and Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR reactions were performed in triplicate using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. Gene expression was normalized to actin (*Actb*), and expression of each gene was compared between WT and G-Cat mice from the same litter. Primer sequences are listed in supplementary material Table S1.

Statistical analysis
Statistical significance was determined by Student's *t*-tests and, in the case of gene expression, by performing a two-factor ANOVA to identify genotype-by-gene interaction and post-hoc two-tailed *t*-tests (for *P*-value).

RESULTS
Identification of cells in the developing cerebellum that proliferate in response to WNT signaling
Although previous studies have shown that WNT signaling can promote the proliferation of progenitors in the cortex and spinal cord (Chenn and Walsh, 2002; Zechner et al., 2003), mitogenic effects of WNT signaling have not been described in the cerebellum. To determine whether cerebellar progenitors are responsive to canonical WNT signaling, we isolated cells from the embryonic cerebellum and tested their ability to proliferate following infection with retroviruses encoding activated β-catenin. Cells from embryonic day (E) 13.5 or E17.5 were infected with control (GFP) or β-catenin-IRES-GFP viruses for 48 hours, pulsed with tritiated thymidine and assayed for thymidine incorporation. Whereas cells from E13.5 cerebellum exhibited a 2.5-fold increase in proliferation following infection with β-catenin-encoding viruses, cells from E17.5 showed no response (Fig. 1A). E13.5
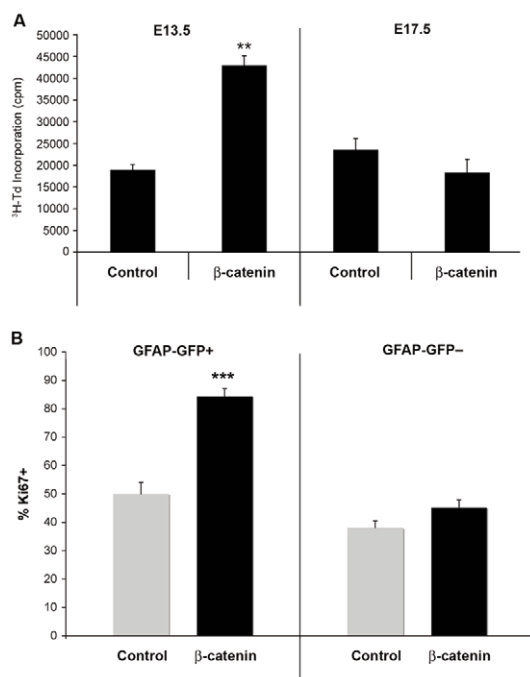
Fig. 1. β-catenin increases the proliferation of a subpopulation of cells of the developing cerebellum. (**A**) Cells from the cerebellum of E13.5 or E17.5 wild-type (WT) mice were infected with control (GFP only) or β-catenin-IRES-GFP retroviruses for 48 hours, pulsed with tritiated thymidine (^{3}H-Td) and cultured overnight before being assayed for ^{3}H-Td incorporation. Data represent the mean (±s.e.m.) of triplicate samples. (**B**) GFP$^+$ or GFP$^-$ cells were FACS sorted from cerebella of E14.5 hGFAP-GFP transgenic mice and infected with control (YFP only) or β-catenin-IRES-YFP retroviruses for 48 hours. There was no significant difference in infection rates of GFAP$^+$ and GFAP$^-$ cells (45% and 40%, respectively). Cells were stained with Ki67 antibodies and the percentage of Ki67$^+$ cells was counted. Data represent the mean (±s.e.m.) percentage of Ki67$^+$ cells in six fields **, $P<0.01$; ***, $P<0.0001$; Student's t-test.

cells also proliferated in response to soluble Wnt1 protein (supplementary material Fig. S1). These studies suggested that WNT signaling is mitogenic for cells in the early embryonic cerebellum.

To determine which cells in the embryonic cerebellum proliferate in response to WNT signaling, we fractionated cells prior to infection with β-catenin-encoding viruses. The human *GFAP* (hGFAP) promoter is expressed in the VZ of the embryonic cerebellum, and FACS-sorted hGFAP-GFP$^+$ cells can generate neurospheres and differentiate into neurons, astrocytes and oligodendrocytes (Schuller et al., 2008; Spassky et al., 2008; Yang et al., 2008). Thus, the hGFAP promoter marks stem cells in the embryonic cerebellum. To determine whether these cells can proliferate in response to WNT signaling, we FACS sorted GFP$^+$ and GFP$^-$ cells from the cerebellum of hGFAP-GFP mice (Zhuo et al., 1997) and infected them with retroviruses encoding β-catenin. After 48 hours, we stained cells with antibodies against the proliferation marker Ki67 and counted Ki67$^+$ cells. As shown in Fig. 1B,C, β-catenin significantly increased the proliferation of GFAP$^+$ cells but not of GFAP$^-$ cells. These results suggest that WNT responsiveness is associated with a GFAP$^+$ population in the embryonic cerebellum.

We have previously demonstrated that the postnatal cerebellum also contains NSCs (Lee et al., 2005). These represent less than 1% of the cells in the cerebellum during the first postnatal week, but can be identified based on their expression of prominin 1 (CD133) and their lack of neuronal and glial lineage markers (Prom1$^+$ Lin$^-$). To determine whether postnatal cerebellar NSCs are also responsive to WNT signaling, we sorted Prom1$^+$ Lin$^-$ cells from P7 cerebellum and treated them with Wnt1 protein or infected them with β-catenin-encoding retroviruses. Both Wnt1 protein and β-catenin increased the proliferation of these cells in vitro (supplementary material Fig. S2A,B). By contrast, GNPs, which represent the majority of cells in the postnatal cerebellum [and express the transcription factor Math1 (Atoh1 – Mouse Genome Informatics)] did not proliferate in response to WNT1 protein or β-catenin-encoding retroviruses (supplementary material Fig. S2C; data not shown). Together, these studies suggest that WNT signaling is mitogenic for NSCs in the embryonic and postnatal cerebellum.

Activation of β-catenin expands NSCs in the cerebellar ventricular zone

Having observed that cerebellar NSCs proliferate in response to WNT signaling in vitro, we tested their responsiveness to WNT signaling in vivo. We used Catnb$^{lox(Ex3)}$ mice, in which exon 3 of the β-catenin gene is flanked by loxP sites (Harada et al., 1999; Zechner et al., 2003). When this exon is deleted by Cre recombinase, the resulting protein cannot be phosphorylated and degraded and therefore accumulates in the nucleus, where it constitutively turns on expression of WNT target genes. To activate WNT target gene expression in cerebellar NSCs, we crossed Catnb$^{lox(Ex3)}$ mice with hGFAP-Cre mice (Zhuo et al., 2001), which express Cre in the cerebellar VZ (Schuller et al., 2008; Spassky et al., 2008; Yang et al., 2008). To verify that β-catenin was activated in the VZ of hGFAP-Cre; Catnb$^{lox(Ex3)}$ (G-Cat) mice, we stained sections from E14.5 wild-type (WT) or G-Cat animals with anti-β-catenin antibodies. As shown in Fig. 2A-D, β-catenin was predominantly cytoplasmic in the VZ of WT mice, but was strongly expressed in nuclei of cells within the VZ of mutant mice. These data suggest that G-Cat mice have constitutive activation of the WNT pathway in the cerebellar VZ.
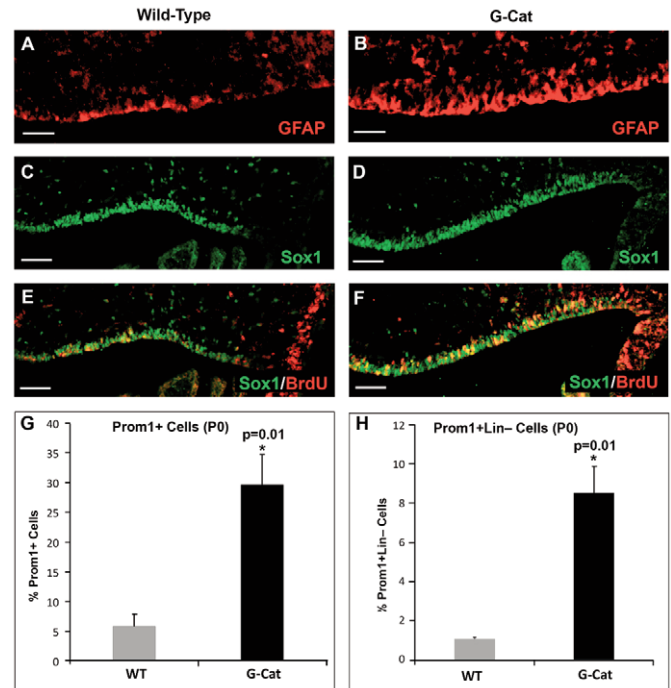
To assess the effect of WNT signaling on proliferation in the embryonic cerebellum, we examined BrdU incorporation. E14.5 WT and mutant mice were exposed to BrdU 30 minutes before being sacrificed, and cerebellar sections were stained with anti-BrdU antibodies. As shown in Fig. 2E,F, the VZ of mutant mice contained significantly more BrdU$^+$ cells than that of WT mice (92±8 versus 38±5 cells/section). We also used anti-Ki67 to assess proliferation in WT and mutant cerebella (Fig. 2G-I). At E14.5, we found a 3-fold increase in the proportion of Ki67$^+$ cells in the VZ of mutant mice compared with WT mice, confirming that WNT signaling increases proliferation in the embryonic VZ (Fig. 2I). Constitutively active β-catenin also caused increased proliferation at E13.5, E16.5 and P0, although the effects were most pronounced at earlier stages (Fig. 2J). These data suggest that activated β-catenin promotes proliferation in the cerebellar VZ during embryonic development.

Previous studies have suggested that GFAP-expressing cells in the embryonic cerebellum include multipotent NSCs as well as mature astrocytic cells (Schuller et al., 2008; Spassky et al., 2008; Yang et al., 2008). To determine whether the proliferating cells in the VZ of G-Cat mice were NSCs, we stained sections from E14.5 WT and mutant mice with antibodies specific for GFAP and the NSC marker Sox1. Mutant animals exhibited increased numbers of
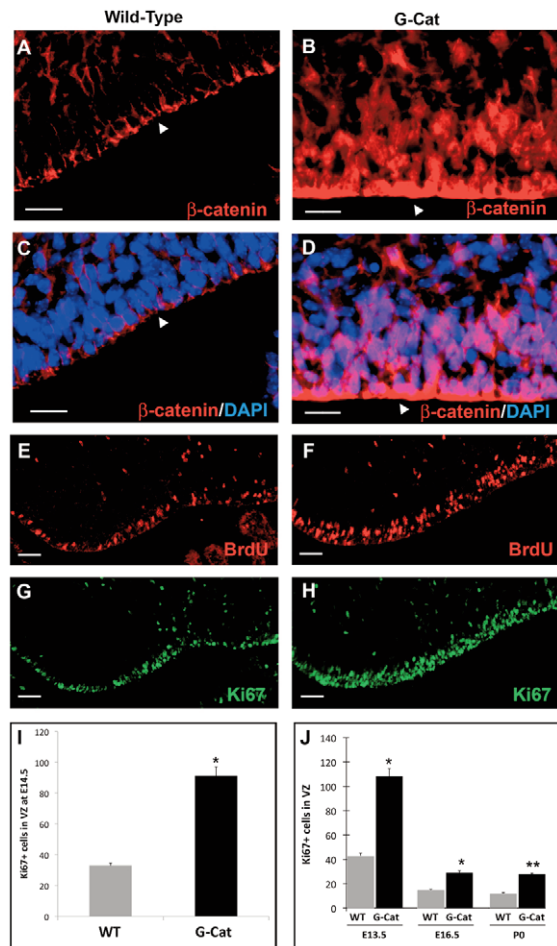
Fig. 2. Activated β-catenin promotes proliferation in the cerebellar ventricular zone. (**A,B**) β-catenin immunostaining (red) of E14.5 cerebellum reveals increased expression in the ventricular zone (VZ, arrowheads) of hGFAP-Cre; Catnb$^{lox(Ex3)}$ (G-Cat) mice (B) compared with WT mice (A). (**C,D**) Colocalization with DAPI (blue) highlights the cytoplasmic localization of β-catenin in WT mice (C) and nuclear localization in G-Cat mice (D). Arrowheads indicate the VZ. (**E-H**) Staining with anti-BrdU (red, E,F) or anti-Ki67 (green, G,H) shows increased proliferation in the VZ of E14.5 G-Cat mice (F,H) compared with WT mice (E,G). (**I,J**) Number of Ki67$^+$ cells per section in the VZ of WT and G-Cat mice at E14.5 (I) and at E13.5, E16.5 and P0 (J). Data represent the mean (±s.e.m.) number of Ki67$^+$ cells per section of six sections. *, $P<0.01$; **, $P<0.001$. Scale bars: 50 μm.

Fig. 3. Activated β-catenin increases the number of stem cells in the embryonic and neonatal cerebellum. (**A-F**) Cerebellar sections from E14.5 WT (A,C,E) or G-Cat (B,D,F) mice were stained with anti-GFAP antibodies (red in A,B) or anti-Sox1 (green) and anti-BrdU (red) antibodies (C-F). Note the increased expression of GFAP and Sox1 and the colocalization of Sox1 and BrdU in the mutant VZ. (**G,H**) Increased numbers of progenitors (G) and stem cells (H) in the cerebellum white matter in G-Cat mice compared with WT mice at P0. Cells isolated from the cerebellum of P0 WT and G-Cat mice were stained with antibodies specific for prominin 1 (Prom1) and for lineage (Lin) markers (PSA-NCAM, O4 and TAPA-1). The percentage of Prom1$^+$ progenitors (G) and Prom1$^+$ Lin$^-$ stem cells (H) was analyzed by flow cytometry. Data represent mean (± s.e.m.) percentages from three separate experiments. *, $P<0.01$. Scale bars: 50 μm.

GFAP$^+$ and Sox1$^+$ cells in the embryonic cerebellar VZ (Fig. 3A-D). We also co-stained sections from these animals with Sox1 and BrdU antibodies, and observed that almost all of the BrdU$^+$ cells in the VZ expressed Sox1 (Fig. 3E,F). Similar results were obtained using antibodies specific for Sox2 (data not shown), supporting the notion that the proliferating cells in the VZ were NSCs. To determine whether there was a similar expansion of NSCs in the postnatal cerebellum, we isolated cells from G-Cat mice at P0 and analyzed the percentage of Prom1$^+$ cells (stem cells and progenitors) and Prom1$^+$ Lin$^-$ cells (stem cells). Both populations were increased ~6-fold in mutant compared with WT mice (Fig. 3G,H). Together, these studies suggest that activation of WNT signaling in GFAP$^+$ cells expands the stem cell pool in the embryonic and postnatal cerebellum.

Continued expression of β-catenin disrupts cerebellar development

The fact that β-catenin promoted the proliferation of cerebellar NSCs suggested that it might lead to increased production of neurons and glia and expansion of the cerebellum. Unfortunately, almost all G-Cat mice died shortly after birth, making it impossible to study the long-term consequences of β-catenin activation in these animals. However, even at late embryonic and early postnatal stages the phenotype of mutant mice was not consistent with a purely mitogenic effect of WNT signaling. At E16.5, mutant mice had smaller cerebella than WT mice and, despite expansion of the VZ, exhibited a marked reduction in the thickness of the rhombic lip and EGL (supplementary material Fig. S3A-D). At P0, mutant mice had an even more severe phenotype: their cerebella were significantly smaller than those of WT mice and lacked the foliation characteristic of this stage of development (Fig. 4A,B). Moreover, staining of the cerebellum with lineage markers revealed marked defects in neuronal and glial differentiation. The region normally occupied by the EGL contained few cells and none of these expressed Ki67 or Pax6, which are markers of proliferating GNPs (Fig. 4A-D). Some calbindin$^+$ Purkinje cells were present, but these were very disorganized compared with those in the WT cerebellum (Fig. 4E,F). Pax2$^+$
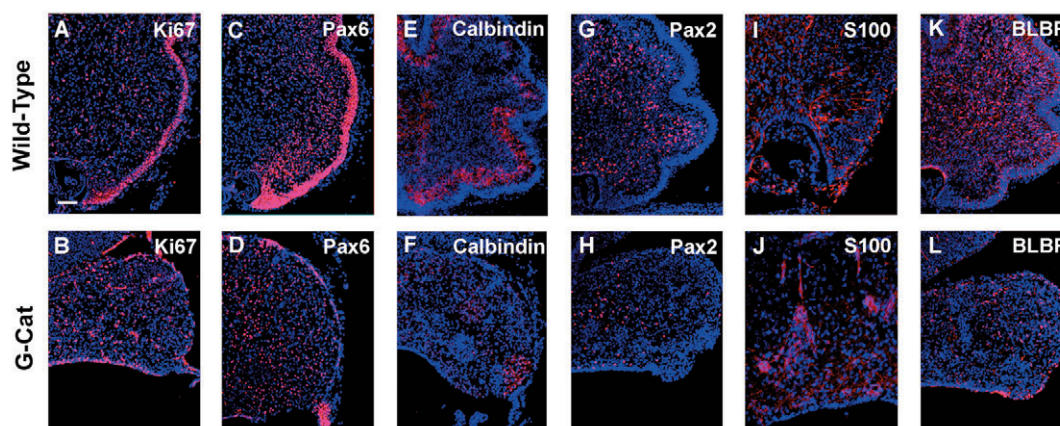
Fig. 4. Activation of β-catenin in stem cells impairs neuronal and glial differentiation. (A-L) Sections from P0 WT (A,C,E,G,I,K) and G-Cat (B,D,F,H,J,L) cerebella were stained with antibodies specific for Ki67 (A,B) to label proliferating cells, Pax6 (C,D) to label granule neuron precursors (GNPs), calbindin (E,F) to mark Purkinje neurons, Pax2 (G,H) to detect stellate and basket interneuron precursors, and S100β (I,J) or BLBP (K,L) to detect glial cells. All sections were counterstained with DAPI. Note the marked reduction in progenitors and differentiated cells in G-Cat mice. Scale bar: 50 µm.

interneuron progenitors (Fig. 4G,H) and glial cells [S100β and BLBP (Fabp7 – Mouse Genome Informatics) staining, Fig. 4I-L] were detectable but in reduced numbers, and, in the case of glia, with aberrant localization and morphology. Thus, the increased NSC proliferation induced by β-catenin is accompanied by marked defects in neuronal and glial differentiation.

The dramatic loss of neuronal progenitors in G-Cat mice could reflect a failure of NSCs to differentiate along the neuronal lineage or the reduced proliferation or survival of neuronal progenitors once they have been generated. To test whether expression of β-catenin in GNPs impairs their growth or survival, we crossed Catnb$^{lox(Ex3)}$ mice with Math1-Cre transgenic mice, which express Cre in cells of the granule lineage (Schuller et al., 2008; Yang et al., 2008). Analysis of cerebella from Math1-Cre; Catnb$^{lox(Ex3)}$ (M-Cat) mice at E14.5 revealed an intact EGL with relatively normal numbers of proliferating (Ki67$^+$) cells (supplementary material Fig. S4A,B). At P0, proliferation could be detected in the anterior EGL, but few proliferating cells were seen on the posterior surface of the cerebellum (supplementary material Fig. S4C,D). Although these animals exhibited normal viability and no overt signs of neurological dysfunction, their cerebella were smaller and contained fewer lobes than those of WT animals (supplementary material Fig. S4E,F). These results suggest that expression of activated β-catenin does not prevent formation of the EGL, but might impair proliferation or long-term survival of GNPs.

Deletion of *Apc* impairs the function of cerebellar NSCs

A possible explanation for the defects in G-Cat mice is that the non-degradable (exon 3-lacking) β-catenin protein in these animals leads to high levels of WNT pathway activation, which might be toxic for stem cells and progenitors. To examine this possibility, we used an alternative approach to activating WNT signaling in these cells: inactivation of the Apc protein. Apc is part of the 'destruction complex' that targets β-catenin for degradation in the absence of WNT stimulation; inactivating mutations in Apc allow for accumulation and nuclear translocation of β-catenin, thereby promoting WNT target gene transcription (Willert and Jones, 2006).

To study the consequences of Apc inactivation for cerebellar NSCs we crossed hGFAP-Cre mice with Apc$^{lox/lox}$ mice, which carry loxP sites surrounding exon 14 of the *Apc* gene. This exon is crucial for interaction of Apc with β-catenin and axin (another component of the destruction complex), so following Cre-mediated deletion Apc is unable to target β-catenin for degradation. To verify that this approach resulted in WNT pathway activation, we stained cerebella from E14.5 hGFAP-Cre; Apc$^{lox/lox}$ (G-APC) mice with anti-β-catenin. Deletion of *Apc* exon 14 resulted in nuclear accumulation of β-catenin in the VZ (supplementary material Fig. S5A,B). Similar to G-Cat mice, G-APC mice showed increased proliferation in the VZ at E14.5 (Ki67 staining, supplementary
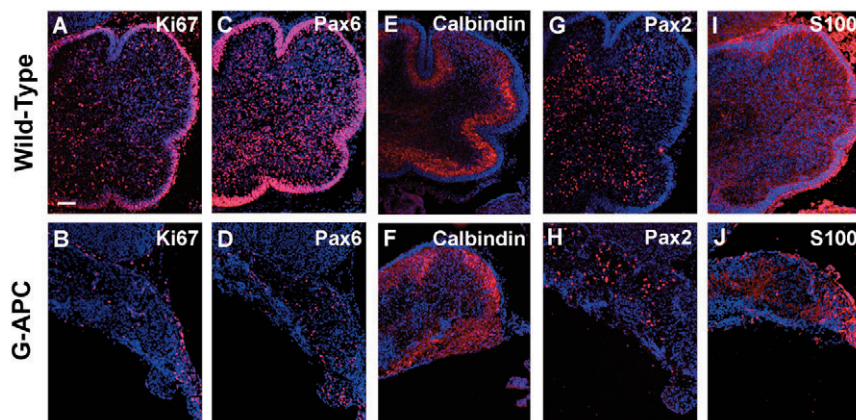
Fig. 5. Deletion of *Apc* in stem cells also impairs differentiation. (A-J) Sections from P0 WT (A,C,E,G,I) and hGFAP-Cre; Apc$^{lox/lox}$ (G-APC; B,D,F,H,J) cerebella were stained with antibodies specific for Ki67 (A,B) to label proliferating cells, Pax6 (C,D) to label GNPs, calbindin (E,F) to mark Purkinje neurons, Pax2 (G,H) to detect stellate and basket interneuron precursors, and S100β (I,J) to detect glial cells. Sections were counterstained with DAPI. Note the marked reduction in progenitors and differentiated cells in G-APC mice. Scale bar: 50 µm.
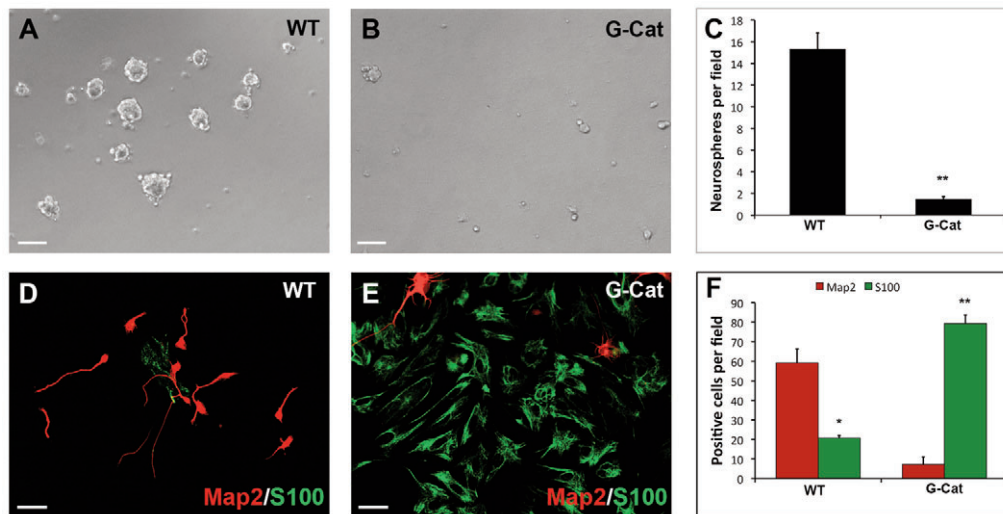
Fig. 6. β-catenin-expressing stem cells exhibit aberrant self-renewal and differentiation in vitro. (**A**,**B**) Prom1$^+$ Lin$^-$ cells were sorted from P0 WT (A) or G-Cat (B) mouse cerebellum and cultured at clonal density in serum-free medium containing 25 ng/ml EGF and bFGF for 7 days before being photographed under bright-field. Passageable neurospheres were consistently obtained from WT but not from mutant cerebellum. (**C**) Average neurosphere numbers (±s.e.m.) per field from eight fields in P0 WT or G-Cat cerebellum. (**D**,**E**) Prom1$^+$ Lin$^-$ cells were sorted from P0 WT (D) or G-Cat (E) cerebellum and cultured for 3 days in medium containing 1% FBS. Cells were stained with antibodies specific for the neuronal marker Map2 (red) and the glial cell marker S100β (green). (**F**) Quantitation (mean ± s.e.m.) of Map2/S100β staining from five fields. *, $P<0.01$; **, $P<0.001$. Whereas WT stem cells differentiated predominantly into neurons, G-Cat showed a marked skewing toward glial lineages. Scale bars: 25 μm in A,B; 50 μm in D,E.

material Fig. S5C,D). G-APC mice also died shortly after birth and had much smaller cerebella than WT littermates at P0 (Fig. 5). Moreover, whereas WT cerebella contain proliferating (Ki67$^+$) Pax6$^+$ GNPs on their surface (Fig. 5A,C), mutant cerebella had few such cells (Fig. 5B,D). As with G-Cat mice, staining of G-APC cerebella with anti-calbindin antibodies revealed disorganization of Purkinje neurons (Fig. 5E,F). Staining for Pax2 (Fig. 5G,H) and S100β (Fig. 5I,J) also showed marked reductions in the numbers of interneurons and glia in mutant cerebella. These results suggest that WNT pathway activation – either through stabilization of β-catenin or loss of Apc – causes increased proliferation of NSCs and defective differentiation of these cells into neuronal and glial progenitors.

Overexpression of β-catenin impairs NSC self-renewal and differentiation in vitro

The above studies suggest that WNT pathway activation causes cerebellar NSCs to proliferate but does not result in increased numbers of differentiated cells. To understand the basis for this defect, we examined the effects of β-catenin on self-renewal. Cells were isolated from WT and G-Cat mice at P0 and cultured in serum-free medium containing bFGF (FGF2) and EGF. As we have shown previously (Lee et al., 2005), Prom1$^+$ Lin$^-$ cells from WT mice form neurospheres under these conditions (Fig. 6A,C). By contrast, NSCs from mutant cerebellum formed neurospheres at a significantly lower frequency ($P<0.01$; Fig. 6B,C). Moreover, mutant neurospheres were smaller and could not give rise to secondary neurospheres, indicating a defect in self-renewal. Similar results were observed when NSCs were isolated from the cerebellum at E14.5 (data not shown). Thus, constitutive activation of β-catenin in GFAP$^+$ cells causes transient proliferation of NSCs but impairs their ability to undergo self-renewal. This defect in self-renewal could explain the reduction in cerebellar mass in these mice.

We next asked whether WNT signaling affects the differentiation of cerebellar NSCs in vitro. Although NSC differentiation is often assayed by generating neurospheres and then withdrawing growth factors, the impaired neurosphere-forming capacity of mutant NSCs precluded such analysis. Instead, we isolated Prom1$^+$ Lin$^-$ cells from P0 WT and mutant mice and cultured them in the presence of 1% FBS for 3 days. Cells were stained with antibodies specific for Map2 (Mtap2 – Mouse Genome Informatics; to detect neuronal differentiation) and S100β (to detect glial differentiation). Whereas NSCs from WT animals predominantly generated neurons (60% Map2$^+$, 22% S100β$^+$), NSCs from mutant mice almost exclusively generated glia (7% Map2$^+$, 80% S100β$^+$) (Fig. 6D-F). These data, which are consistent with the marked depletion of neurons in G-Cat mice in vivo, suggest that activation of β-catenin in GFAP$^+$ cells markedly alters the differentiation potential of postnatal cerebellar NSCs.

Loss of β-catenin causes self-renewal defects and increased neuronal differentiation in vitro

Given the effects of β-catenin overexpression on stem cell self-renewal and differentiation, we examined the effects of loss of β-catenin on these processes. We used Catnb$^{lox(ex2-6)}$ mice, in which exons 2-6 of β-catenin are flanked by loxP sites. Introduction of Cre into these cells results in excision of exons 2-6 and loss of β-catenin expression. Prom1$^+$ Lin$^-$ cells were isolated from Catnb$^{lox(ex2-6)}$ mice at P0, infected with control (GFP) viruses (supplementary material Fig. S6A) or Cre-IRES-GFP viruses (supplementary material Fig. S6B) and cultured in stem cell media in the presence of bFGF and EGF. After 10 days, total and GFP-expressing neurospheres were counted. The percentage of GFP$^+$ neurospheres was significantly lower in Cre-infected cultures than in control cultures (supplementary material Fig. S6A,C). Additionally, the neurospheres from Cre-infected cells were smaller and more irregularly shaped than those from control cells (supplementary material Fig. S6B,C).
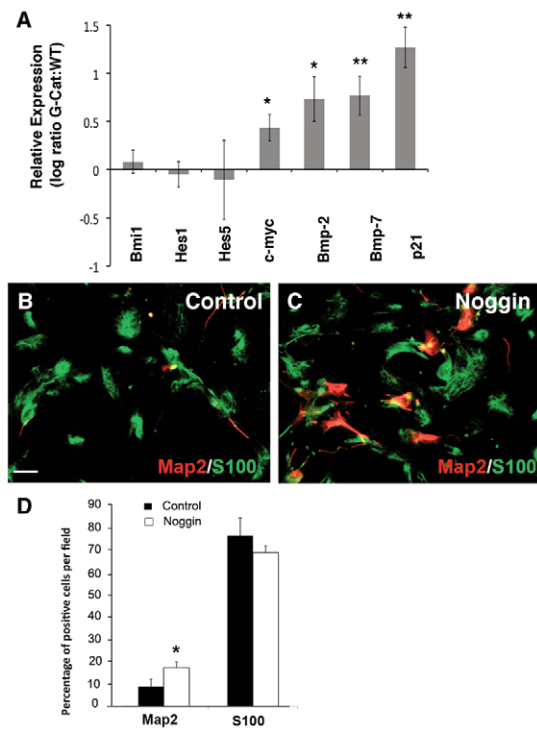
Fig. 7. Stem cells from G-Cat mice express elevated levels of BMPs and p21. (**A**) Quantitative RT-PCR was performed on Prom1^{+} Lin^{−} cells isolated from P0 WT and G-Cat mice using primers for the indicated genes. Mice from four litters were analyzed, and after normalization to actin the expression of each gene in G-Cat cells was compared with that in WT littermates to calculate a ratio of gene expression for each litter. Data are plotted as the mean (±s.e.m.) log ratio (G-Cat:WT). *, $P<0.02$; **, $P<0.05$. (**B-D**) Noggin promotes neuronal differentiation. Prom1^{+} Lin^{−} cells were isolated from P0 G-Cat mice, treated with noggin protein (200 ng/ml) and cultured for 3 days in serum-containing media to promote differentiation. Cells were stained for Map2 (red) and S100β (green). Quantitation (mean ± s.e.m.) of Map2/S100β staining is from six fields. *, $P<0.01$. Scale bar: 50 μm.

The fact that both gain and loss of β-catenin result in decreased neurosphere-forming capacity suggests that the level of β-catenin is crucial for maintenance of self-renewal capacity.

We then asked whether loss of β-catenin affects the differentiation of stem cells. Prom1^{+} Lin^{−} cells were isolated from Catnb[lox(ex2-6)] mice, infected with viruses, and maintained in 3% FBS-containing media for 3 days to allow for differentiation. Cells were then stained for Map2 (supplementary material Fig. S6D,E) and S100β (supplementary material Fig. S6F,G) to mark differentiated neuronal and glial cells. Interestingly, we found an increase of Map2^{+} cells among Cre-infected cells compared with control cells (supplementary material Fig. S6D,E,H). Loss of β-catenin had no effect on the percentage of S100β^{+} cells as compared with control cells (supplementary material Fig. S6F-H). These data suggest that loss of β-catenin skews stem cell differentiation toward neuronal lineages.

Activation of β-catenin causes increased expression of BMPs and p21

Our studies suggested that WNT signaling promotes the proliferation of cerebellar NSCs but interferes with their capacity for self-renewal and differentiation. To gain insight into the molecular basis for these findings, we isolated RNA from WT and mutant Prom1^{+} Lin^{−} cells and analyzed expression of WNT target genes and regulators of proliferation and self-renewal. RT-PCR analysis revealed no consistent differences in expression of the polycomb transcription factor *Bmi1* or the Notch target genes *Hes1* and *Hes5* (Fig. 7A), each of which has been implicated in NSC self-renewal (Nakamura et al., 2000; Ohtsuka et al., 2001; Molofsky et al., 2003). By contrast, expression of *c-Myc*, a common target of the WNT pathway (He et al., 1998; Rohrs et al., 2009), was elevated in NSCs isolated from G-Cat mice (Fig. 7A).

Although increased expression of *c-Myc* provided a potential explanation for the increase in proliferation induced by β-catenin, the basis for the defects in self-renewal and differentiation in G-Cat mice remained unclear. Previous reports have suggested that in neuroepithelial progenitors from the dorsal CNS, WNT signaling not only induces proliferation but also increases the expression of bone morphogenetic proteins (BMPs) (Ille et al., 2007). BMPs, in turn, inhibit WNT-induced proliferation and self-renewal (Ille et al., 2007) and promote glial differentiation (Bonaguidi et al., 2005; See et al., 2007). This led us to examine whether the self-renewal and differentiation defects in G-Cat mice are mediated by BMPs. As shown in Fig. 7A, mutant cells exhibited a significant increase in the expression of *Bmp2* and *Bmp7*. In addition, expression of the cyclin-dependent kinase inhibitor *p21* (*Cdkn1a* – Mouse Genome Informatics), which is a common target of BMPs, was also markedly elevated in NSCs from G-Cat mice. These findings suggest that overexpression of β-catenin in NSCs results in increased BMP/p21 signaling.

To determine whether the BMP pathway regulates self-renewal of cerebellar stem cells, we performed both gain- and loss-of-function experiments. Prom1^{+} Lin^{−} cells from WT mice were cultured at low density in the presence of growth factors along with recombinant Bmp2 or Bmp7 (200 ng/ml). After 7 days, stem cells treated with either Bmp2 or Bmp7 showed a marked decrease in neurosphere formation compared with control cells (supplementary material Fig. S7A). Conversely, Prom1^{+} Lin^{−} stem cells treated with the BMP inhibitor noggin produced more neurospheres than control cells (supplementary material Fig. S7B). Cells infected with viruses encoding short hairpin RNA specific for *p21* (p21shRNA) also showed increased neurosphere formation (supplementary material Fig. S7B). These studies suggested that BMP signaling interferes with the self-renewal of cerebellar stem cells.

To determine whether increased BMP signaling could explain the impaired self-renewal and differentiation of stem cells from G-Cat mice, we isolated Prom1^{+} Lin^{−} cells from P0 G-Cat mice and treated them with noggin or p21shRNA. Neither noggin treatment nor p21 knockdown was able to rescue the neurosphere defect in these cells (data not shown). However, noggin significantly increased neuronal differentiation and slightly decreased glial differentiation of G-Cat cells (Fig. 7B-D). Knockdown of p21 promoted the neuronal differentiation of WT stem cells (supplementary material Fig. S8A-E), although it was not able to rescue the differentiation defects in G-Cat stem cells (supplementary material Fig. S8F-J). Together, these data suggest that the BMP pathway regulates the self-renewal and differentiation of WT stem cells, but is not sufficient to account for the defects in self-renewal and differentiation seen in β-catenin-overexpressing mice.

DISCUSSION
WNT signaling plays a crucial role in the development of many parts of the CNS and, in particular, has been implicated in the establishment of the midbrain-hindbrain boundary that gives rise

to the cerebellum. The fact that a subset of human medulloblastomas contains activating mutations in β-catenin or loss-of-function mutations in APC suggests that WNT signaling might also be mitogenic for a population of cells in the developing cerebellum. To identify such a population, we screened cells from various stages of cerebellar development for WNT responsiveness. Our studies suggest that NSCs from the embryonic and neonatal cerebellum are capable of proliferating in response to WNT pathway activation. However, the increased proliferation of these cells in vivo is accompanied by marked defects in self-renewal and differentiation. Based on these studies, we conclude that WNT signaling induces proliferation but impairs differentiation of cerebellar stem cells.

Although WNT signaling is known to be mitogenic for NSCs in other parts of the CNS (Chenn and Walsh, 2002; Chenn and Walsh, 2003; Zechner et al., 2003), it has not been shown to promote proliferation in the cerebellum. We observed WNT pathway-induced proliferation both in vitro, upon treatment of NSCs with WNT protein or infection with β-catenin-encoding retroviruses, and in vivo, following activation of β-catenin or deletion of *Apc* using the hGFAP-Cre transgene. It is interesting that in previous studies examining the effects of WNT pathway activation in the CNS, there was no mention of mitogenic effects in the cerebellum (Chenn and Walsh, 2002; Chenn and Walsh, 2003; Zechner et al., 2003); it is unclear whether these studies did not examine this region, or whether the promoters used [nestin, *Brn4* (*Pou3f4*)] did not target the same population of NSCs at the same stage of development. However, recent studies have demonstrated that deletion of β-catenin results in loss of the cerebellar vermis (Schuller and Rowitch, 2007), and these investigators speculated that this might result from inadequate proliferation. Our studies support this notion and suggest that, at least early in development, the mitogenic effects of WNT signaling might contribute to expansion of the cerebellar primordium.

Although activation of WNT signaling promotes proliferation of NSCs, this proliferation appears to be transient: G-Cat mice exhibit increased numbers of dividing Sox1^{+} cells in the VZ at embryonic stages, but these cells do not expand indefinitely in vivo and cannot form neurospheres in vitro. One explanation for the failure of mutant NSCs to form neurospheres is that activation of the WNT pathway allows cells to proliferate at high density but does not favor growth under neurosphere-forming conditions (at low density in EGF and bFGF). Alternatively, it is possible that aberrant expression of β-catenin affects the cell surface expression of cadherins and thereby interferes with cell-cell interactions that are necessary for cells to grow as neurospheres (Yagita et al., 2009). Finally, WNT pathway activation, while promoting proliferation, might change the mode by which NSCs divide; for example, by shifting the balance from symmetric renewal (whereby a stem cell gives rise to two stem cells) or asymmetric division (whereby stem cells give rise to one stem cell and one differentiated cell) to symmetric differentiation (wherein both progeny undergo differentiation). This might explain both the inability to form neurospheres in vitro and the lack of prolonged expansion in vivo.

In addition to its effects on proliferation and self-renewal, WNT pathway activation also severely disrupts NSC differentiation. In vitro, this is manifest as a marked skewing toward glial cells at the expense of neuronal lineages. In vivo the effects are even more striking, with a reduction in most classes of neurons and a decrease in the overall size of the cerebellum. In principle, these defects could reflect either a failure of NSCs to commit to neuronal lineages or the impaired growth and survival of neuronal

progenitors or neurons once they are formed. The fact that few Purkinje and granule neurons can be detected in the cerebellum of G-Cat mice, and that purified NSCs from these animals differentiate predominantly into S100β[+] glia, support the former possibility. However, our observations that M-Cat mice have a normal EGL but produce fewer GNPs suggest that impaired proliferation might also contribute to the reduction in the number of neurons, at least in the granule lineage. Consistent with this, a recent study has demonstrated that loss of Apc in GNPs results in inhibition of cell proliferation (Lorenz et al., 2011). Although WNT signaling promotes neurogenesis in many parts of the nervous system (Chenn and Walsh, 2003; Hirabayashi et al., 2004; Hirsch et al., 2007), its ability to interfere with neuronal differentiation in the cerebellum is not without precedent. For example, β-catenin has been reported to inhibit neuronal differentiation in embryonic stem cells and in progenitors within the spinal cord (Haegele et al., 2003; Ille et al., 2007). Likewise, in the hematopoietic system, activation of β-catenin increases proliferation of stem cells (Reya et al., 2003; Willert et al., 2003) but can also cause a profound block in differentiation along myeloid, lymphoid and erythroid lineages (Kirstetter et al., 2006; Scheller et al., 2006). Finally, stabilization of β-catenin in the airway epithelium blocks Clara cell differentiation (Reynolds et al., 2008). Thus, impaired differentiation is not an uncommon effect of WNT pathway activation.

To understand the mechanisms by which β-catenin alters NSC renewal and differentiation, we examined expression of genes that have been identified as targets of the WNT pathway in other cells. Our results showed that *c-Myc*, a common target of WNT signaling (He et al., 1998), was elevated in NSCs from G-Cat mice compared with control NSCs. Increased expression of *c-Myc* could account for the NSC proliferation we observed in embryonic and postnatal G-Cat mice, but is difficult to reconcile with the defects in self-renewal and differentiation observed in these animals. In this regard, our finding that NSCs from G-Cat mice express increased levels of Bmp2 and Bmp7 might be important. BMPs have been reported to be induced by β-catenin and to counteract the mitogenic effects of canonical WNT signaling in the dorsal neural tube (Ille et al., 2007). In addition, BMPs frequently induce expression of the cyclin-dependent kinase inhibitor p21 (Franzen and Heldin, 2001; Yamato et al., 2001; Pouliot and Labrie, 2002), which has also been shown to inhibit self-renewal of NSCs (Kippin et al., 2005; Fasano et al., 2007). The fact that we observe significant upregulation of both BMPs and p21 raises the possibility that this pathway contributes to the self-renewal defects in G-Cat mice. However, our inability to rescue these defects with noggin or p21shRNA implies that other pathways might be involved as well. Notably, in addition to their inhibitory effects on proliferation, BMPs are also potent inducers of glial differentiation (Bonaguidi et al., 2005; Imura et al., 2008). Thus, increased expression of BMPs might also explain the skewing of NSC differentiation toward the glial lineage that we observed in vitro and in vivo. Our observation that noggin can restore neuronal differentiation of G-Cat stem cells supports a role for BMP signaling in this process.

Although G-Cat mice do not live long enough to develop brain tumors, our studies have implications for understanding WNT-associated medulloblastoma. First, our observation that β-catenin does not promote the proliferation of GNPs in vitro or in vivo suggests that unlike Shh-associated tumors (Yang et al., 2008), WNT-associated tumors are unlikely to arise from GNPs. By contrast, the fact that WNT is mitogenic for NSCs in the cerebellar VZ and postnatal white matter raises the possibility that these

represent cells of origin for the disease. The idea that SHH- and WNT-driven tumors come from different cells of origin is supported by genomic analysis (Thompson et al., 2006; Cho et al., 2010; Northcott et al., 2010), which indicates that these tumors have very distinct gene expression profiles. Although recent studies have shown that WNT-associated tumors can arise from progenitors in the dorsal brainstem (Gibson et al., 2010), our results raise the possibility that some of these tumors could also originate from embryonic or postnatal NSCs in the cerebellar VZ.

Whatever the cell of origin for WNT pathway tumors, it seems likely that activation of WNT signaling alone is insufficient to drive tumorigenesis. This is supported by our own studies, in which activation of β-catenin or deletion of *Apc* drives proliferation but not long-term self-renewal of NSCs. Similarly, activation of β-catenin in BLBP-expressing cells does not cause tumors on its own, but can synergize with loss of p53 (Trp53) to promote medulloblastoma formation (Gibson et al., 2010). Interestingly, analysis of human WNT pathway tumors indicates that they frequently lose one copy of chromosome 6 (Clifford et al., 2006; Thompson et al., 2006), suggesting that loss of a gene on this chromosome might be a key event in tumorigenesis. Identification of WNT-responsive cells in the cerebellum will facilitate analysis of specific genes that may cooperate with the WNT pathway to promote uncontrolled growth or tumor formation.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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DEVELOPMENT

Table S1. Primers for qPCR

Gene	Primer	Sequence
Actin	F	TATTGGCAACGAGCGGTTCC
	R	GGCATAGAGGTCTTTACGGATGTC
Bmi1	F	CAACTTCTCCTCGGTCTTCA
	R	AGCTGATGCTGCCAATGGCTCCA
Hes1	F	GCCAATTTGCCTTTCTCATC
	R	ACATGGAGTCCGAAGTGAGC
Hes5	F	CCTCACCTCAAGGTCCACAT
	R	TGTTCTCCCACATGACCAAG
c-Myc	F	CTCTCCTTCCTCGGACTCG
	R	GGTTTGCCTCTTCTCCACAG
Bmp2	F	TGGACGTGCCCCCTAGTGCT
	R	GGATGCCGCGGCGAACTTCT
Bmp7	F	GCGCAGCCAGAATCGCTCCA
	R	ATGGCGTGGTTGGTGGCGTT
p21	F	CTGTCTTGCACTCTGGTGTCTGAG
	R	TTTTCTCTTGCAGAAGACCAATCTG

F, forward; R, reverse.