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Wnt/β-catenin Signaling Regulates Sequential Fate Decisions of Murine Cortical Precursor Cells

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ABSTRACT

The fate of neural progenitor cells (NPC) is determined by a complex interplay of intrinsic programs and extrinsic signals, very few of which are known. β-catenin transduces extracellular Wnt signals, but also maintains adherens junctions integrity. Here, we identify for the first time the contribution of β-catenin transcriptional activity as opposed to its adhesion role in the development of the cerebral cortex by combining a novel β-catenin mutant allele with conditional inactivation approaches. Wnt/β-catenin signaling ablation leads to premature NPC differentiation, but, in addition, to a change in progenitor cell cycle kinetics and an increase in basally dividing progenitors. Interestingly, Wnt/β-catenin signaling affects the sequential fate switch of progenitors, leading to a shortened neurogenic period with decreased number of both deep and upper-layer neurons and later, to precocious astrogenesis. Indeed, a genome-wide analysis highlighted the premature activation of a corticogenesis differentiation program in the Wnt/β-catenin signaling-ablated cortex. Thus, β-catenin signaling controls the expression of a set of genes that appear to act downstream of canonical Wnt signaling to regulate the stage-specific production of appropriate progenitor numbers, neuronal subpopulations, and astroglia in the forebrain. STEM CELLS *2014; 00:000–000*

INTRODUCTION

Proper development of the forebrain requires precise interpretation of temporally- and spatially-restricted cues by the NPC, which initially expand by symmetric division in the ventricular zone (VZ) [1] [2]. As neurogenesis progresses, the apical precursors produce sequentially all the neurons of the six-layered cerebral cortex. At the end of embryogenesis VZ precursors switch to production of glia, which integrate into the established neuronal network [3].

Several distinct progenitors differing in gene expression, proliferation kinetics and mitotic morphology reside in the murine VZ [4]. The most numerous radial glia (RG) maintain both an apical and a basal process while undergoing multiple asymmetric divisions to generate either a neuron or a more restricted intermediate progenitor cell (IPCs) for the subventricular zone (SVZ) [5, 6]. Short neural precursors (SNPs), in contrast, extend only an apical process during mitosis and mostly directly

produce neuronal progeny [7, 8]. Both of these apical progenitors (APs) can generate bipolar subapically dividing progenitors (SAPs), whose numbers are very limited in the murine dorsal VZ [9]. However, SAPs significantly contribute to the neuronal lineage amplification in gyrencephalic species alongside another unique mammalian precursor termed basal radial glia (bRG) [10, 11]. The bRG residing in the outer SVZ are predominantly expressing Pax6, undergoing more than one division and mostly displaying a basal or apical-only anchor [12, 13].

The mechanisms regulating the progressive restriction of this diversity of progenitors and their fate are just starting to emerge. Isolated cortical progenitors and embryonic stem cells in culture can recapitulate the sequential generation of neurons from different layers, followed by gliogenesis, pointing to the existence of an inherent timing program [14-17]. However, these studies do not exclude the presence of an environmental signal secreted by the clones themselves, which drives the orderly progression of fate.

Several Wnt ligands are secreted already at early stages from the hem, the caudo-medial margin of the expanding neocortex, from the choroid plexus of the hindbrain [18] and later from broader neocortical domains [19]. In the Wnt/β-catenin pathway (or canonical Wnt pathway) binding of Wnt ligands to the LRP5/6-Frizzled receptors releases β-catenin from the GSK3β-Axin-APC-CKIα destruction complex. It can thus translocate to the nucleus and activate transcription together with members of the lymphoid enhancer-binding factor (Lef)/T-cell factor (Tcf) transcription factor family [20]. At early neurogenesis persistent Wnt signaling stimulates the proliferation of VZ precursors and promotes deep neuron laminar fate at the expense of upper neuron identity [21-23]. Similarly, β-catenin knockdown results in enhanced cell cycle exit and premature differentiation or leads to an increase in IPCs [23, 24]. Abrogation of Wnt signaling *in vitro* and via LRP6 co-receptor knockout at mid-neurogenesis, however, leads to decreased neuronal output both via APs and IPCs, suggesting a later role of β-catenin in promoting differentiation [25-28]. Independently of its transcription function, β-catenin also associates with adhesion junction proteins such as α-catenin and N-cadherin to maintain the apicobasal polarity of APs [29]. Indeed, loss of adhesion coupling can either promote or inhibit proliferation of APs, depending on the downstream effectors [30-32]. Hence, the precise role of β-catenin signaling in forebrain development needs to be defined while preserving cell-cell adhesion.

In order to examine solely the transcription function of β-catenin from the onset of neurogenesis, we combined a recently generated transcription-defective allele of β-catenin, whose adhesion function is intact, with conditional inactivation approaches [33]. We show that ablation of Wnt signaling results in depletion of the AP pool concomitant with an increase in basal precursor cells. Most strikingly, we reveal unexpected roles of Wnt signaling in regulating the proper temporal progression of cortical neural progenitors from early to late neuron production, and later to gliogenesis through an elaborate transcription factor network.

Materials and Methods

Animals and genotyping

Mouse experiments were performed in accordance with Swiss guidelines and approved by the Veterinarian Office of the Kanton of Zürich, Switzerland. Floxed *Ctnnb1* mice [34] were crossed to *Ctnnb1*^{dm} mice and *Emx1-Cre* [35] driver line to generate *E1C-Ctnnb1*$^{dm/flox}$ and *E1C-Ctnnb1*$^{flox/flox}$ mutants. In order to monitor Wnt signaling activity, the mice were additionally crossed to BAT-gal reporter line [36]. All the animals were bred on a C57/BL6 background.

Immunohistochemistry, X-gal staining, cell cycle analysis and luciferase assay

Detailed in Supporting Information. **In situ hybridization, quantitative real-time PCR and microarray analysis**

Detailed in Supporting Information.

Imaging and quantification analysis

Epifluorescence and confocal micrographs were taken with a Leica DMI6000 B or a CLSM Leica SP8 upright microscope, processed with Adobe Photoshop and quantified manually using ImageJ. For each quantification, 3 mutants and 3 controls from at least 2 different litters, ≥3 sections per animal at different rostro-caudal levels were analyzed, except for calculating the saturation point in Fig. 3K where 2 animals of each genotype were used. A radial unit was defined as a 100μm-wide stripe measured at the ventricular surface with perpendicular borders given by the direction of radial glial cell fibers. PHH3 nuclei were considered basal if they were localized more than 2 DAPI nuclei away from the ventricle. Measurements of the thickness of the VZ, SVZ, IZ and CP were done in ImageJ on micrographs stained for Tbr2-Ctip2 and mutant cortex values were normalized against the thickness of the respective zone of their littermate control. Statistical analysis was performed on Microsoft Excel using unpaired, two-tailed Student's t-test.

Results

A novel mutant β-catenin allele disrupts Wnt signaling but not cell-cell adhesion in the neocortex

In order to investigate the role of canonical Wnt signaling in the development of the neocortex independently of β-catenin-mediated cell adhesion, we employed a novel β-catenin allele - *Ctnnb1*^{dm} (Fig. 1A). In this allele, a substitution of amino acid 164 in the first Armadillo

repeat from aspartate to alanine (D164A) prevents binding of Bcl9/Bcl9L, the most important N-terminal transcriptional co-activator of β-catenin. Further, a prematurely introduced STOP codon at position 673 in exon 13 induces a protein truncation and abrogates interactions with C-terminal binding partners. To avoid the early lethality of such homozygous Wnt/β-catenin signaling mutants at embryonic day 7.5 (E7.5) [33], we ablated Wnt signaling specifically in cortical progenitors by combining a forebrain-specific *Emx1-Cre* (*E1C*) strain with the previously described conditional β-catenin null allele *Ctnnb1*^{flox} [34] and with the novel *Ctnnb1*^{dm} [35] (Supporting information Fig. S1). In parallel, we used the *E1C-Ctnnb1*$^{flox/flox}$ signaling- and adhesion-mutant for comparison.

First, we examined the efficiency of recombination in the dorsal telencephalon at E12.5 using antibodies against the N- and C-termini of β-catenin (Fig. 1B-C). In controls, β-catenin protein recognized by both antibodies is enriched at the apical surface of the neuroepithelium and at adherens junctions. As expected, in the *E1C-Ctnnb1*$^{dm/flox}$ signaling mutant immunoreactivity against the truncated C-terminus was absent, while the N-terminal part of the protein was concentrated apically and at cell-cell junctions. The preserved integrity of the neuroepithelium in controls and *E1C-Ctnnb1*$^{dm/flox}$ mutants was also visualized by immunostaining for the adhesion proteins N-cadherin, ZO-1 and α-E-catenin (Fig. 1E-J). In contrast, the null mutant *E1C-Ctnnb1*$^{flox/flox}$ was devoid of β-catenin immunoreactivity (Fig. 1D), leading to loss of adherens junctions in recombined regions (Fig. 1G, J). The disrupted tissue integrity observed in the β-catenin null mutant reinforces the importance of separating the adhesion from the Wnt-signaling function of β-catenin using the β-catenin signaling mutant.

To confirm that Wnt/β-catenin signaling is functionally inactive, we employed the BAT-gal reporter transgene, in which β-catenin activity drives the expression of β-galactosidase in Wnt signaling-active cells [36]. In controls, highest β-galactosidase activity was detected medially and caudally; however, no X-gal signal was visible in *E1C-Ctnnb1*$^{dm/flox}$ cortices (Fig. 1K-L). To confirm our *in vivo* observations, we isolated neural precursor cells from E12.5 embryos and electroporated them with SuperTOPFlash, a specific Wnt/β-catenin reporter construct, or the scrambled SuperFOPFlash reporter as control [37]. Formation of active β-catenin-Tcf/Lef complexes after Wnt3a stimulation of control cells induced a 20-fold increase in luciferase signal (Fig. 1M). In contrast, *E1C-Ctnnb1*$^{dm/flox}$ mutants were defective in stimulating luciferase activity, demonstrating the failure of the *Ctnnb1*^{dm} allele to mediate Wnt/β-catenin signaling while supporting normal adhesion.

Absent Wnt/β-catenin transcriptional activity abolishes hippocampal development

A canonical Wnt3a signal from the hem is required to maintain the proliferation of the caudo-medial cortex, while β-catenin/LEF/TCF-mediated transcription is essential for hippocampal specification [19, 38]. Since Wnt/β-catenin signaling was lost in the *E1C-Ctnnb1*$^{dm/flox}$ pallium already by E12.5, we evaluated forebrain coronal sections at E18.5, just before mutant embryos died. In both *E1C-Ctnnb1*$^{dm/flox}$ and *E1C-Ctnnb1*$^{flox/flox}$ embryos a shortened cortical wall, smaller lateral ventricles and absence of hippocampal structures were detected (Supporting information Fig. 2A-C). Unlike controls in which *Wnt3a* mRNA was present in the hem ventral to the hippocampal anlage, *Wnt3a* expression in β-catenin mutants was shifted dorsally (Supporting information Fig. 2D-F), similarly to *BMP6* expression and to BMP signaling activity as assessed by immunostaining for pSmad1/5/8 (Supporting information Fig. 2J-O). In situ hybridization for *Wnt8b*, normally expressed both in the hem and in the presumptive hippocampal primordium, further implied the absence of the hippocampus (Supporting information Fig. 2G-I). In agreement, at E14.5 the dentate gyrus marker Prox1 could not be detected in *E1C-Ctnnb1*$^{dm/flox}$ embryos (Supporting information Fig. 2P-Q) [39]. Thus, we could confirm the essential role of canonical Wnt signaling for hippocampal development.

Further, we assessed dorso-ventral patterning since Wnt/β-catenin signaling has been implicated in maintaining dorsal fate and preventing ventralization prior to the onset of neurogenesis (E9.0-E11.5) [40, 41]. However, *in situ* hybridization for Neurog2, a transcription factor (TF) specifying early neurons in the dorsal cortex [42], for ventral proneural TF Mash1 [43] and for its direct target Dlx2 [44] demonstrated normal domains of expression both in controls and in β-catenin-ablated embryos (Supporting information Fig. S3). In addition, normal enrichment of Pax6 rostrally and CoupTFI caudally was confirmed in *E1C-Ctnnb1*$^{dm/flox}$ mutants (Supporting information Fig. S4) [45]. These results indicate that loss of Wnt/β-catenin signaling in dorsal forebrain progenitors after E9.5 affects neither dorso-ventral nor rostro-caudal forebrain patterning.

β-catenin signaling regulates progenitor differentiation

The cortical wall defects of β-catenin mutants at E18.5 could result from a disturbed balance between NPC proliferation and differentiation or from apoptosis. Notably, the number of NPC positive for the TF Sox2, implicated in neural precursor maintenance [46], was similar in *E1C-Ctnnb1*$^{dm/flox}$ and control animals at E12.5 (Fig. 2A-B, G). In contrast, expression of the early neuronal marker Doublecortin (Dcx) was increased in the mutant forebrain already at E12.5 and even more pronounced at E14.5 (Fig. 2A-G). Similarly, premature differentiation was observed in *E1C-Ctnnb1*$^{flox/flox}$ mutants at E12.5 but accompanied by a loss of neuroepithelial integrity and intermingling of Dcx^{+} neurons and Sox2^{+} progenitors (Fig. 2C, F). Since loss of adhesion coupling has been associated with secondary defects [47], we further examined *E1C-Ctnnb1*$^{dm/flox}$ mutants only, the phenotype

of which is attributed solely to canonical Wnt signaling ablation.

To confirm the precocious neurogenesis in β-catenin signaling mutants, the fraction of cells that have exited the cell cycle was assessed by a 20h pulse-labeling of $Ki67^+$ proliferating cells using the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Supporting information Fig. S5A-B). Indeed, the proportion of $BrdU^+Ki67^-$ cortical progenitors, which have exited the cell cycle, was significantly higher in mutant than in control cells (Fig. 2H). In addition, we measured the thickness of the VZ, SVZ, the intermediate zone (IZ) and cortical plate (CP) from E12.5 to E18.5 (Fig. 2I). We detected a consistent decline in the VZ thickness after E12.5, accompanied by a transient thickening of the SVZ, IZ and CP. At E18.5, the mutant VZ, SVZ, IZ and CP were significantly thinner than in controls. Finally, we noted no differences in the apoptotic rate of *E1C-Ctnnb1*$^{dm/flox}$ mutants and controls at E14.5 (Supporting information Fig. S6A-C). In summary, the absence of Wnt/β-catenin transduction resulted in enhanced neurogenesis from E12.5, which eventually led to precocious depletion of the VZ precursor pool in the absence of apoptosis.

β-catenin signaling regulates the generation of distinct basal progenitor populations

The enhanced neurogenesis detected in *E1C-Ctnnb1*$^{dm/flox}$ animals could indicate a changed mode of AP division, shorter AP cell cycle allowing generation of more progeny in a given time period, or enhanced production of basal progenitors. To distinguish between these possibilities, we first examined markers of APs, Pax6, and of IPCs, Tbr2 [48] (Fig. 3A-B, E-J). At E12.5, the number of Pax6-expressing cells per radial unit was similar in control and β-catenin signaling-deficient cortex. However, it declined by E14.5, as also evidenced by quantifying the precursor subtypes in 5 bins across the cortical wall (Supporting information Fig. S5G-L). Instead, the number of proliferating $Tbr2^+Ki67^+$ IPCs was 3-fold increased in the mutant cortex at E12.5. In addition, a significantly higher proportion of Pax6/Tbr2-double positive APs transitioning to IPCs was apparent in the *E1C-Ctnnb1*$^{dm/flox}$ embryos at both stages (Fig. 3C). These data point to a disturbed balance between AP self-renewing (generating more $Pax6^+$ cells) versus differentiative divisions (generating $Tbr2^+$ cells) in β-catenin signaling mutants. To corroborate our observations, we analyzed NPC proliferation by staining for the mitotic marker phospho-histone 3 (pHH3). While the overall mitotic rate was unchanged, a significant shift from apical to basal divisions was observed in the signaling mutant (Supporting information Fig. S5C-F). Taken together with the assessment of stunt growth (Fig. 2I), these data suggest that loss of β-catenin signaling gradually depletes the AP pool at the expense of a transient increase of IPCs.

Apart from IPCs, the number of another basal progenitor, the bRG, might also be affected by the absence of Wnt/β-catenin signaling. While bRG are predominantly $Pax6^+$ and located at the outer SVZ, the presence of a basal-only process is controversial in the light of recent findings in primates, where bRG with an apical-only or with both apical and basal processes were identified [49]. Therefore, we focused on presumptive bRG as $Pax6^+$ cells basal of the $Tbr2^+$ SVZ (Fig. 3D-J). Strikingly, similarly to IPCs, we detected an increase in this basal $Pax6^+$ population in *E1C-Ctnnb1*$^{dm/flox}$ mutants, even when scored against the population of IPCs (0.12±0.04 in controls vs. 0.26±0.08 in mutants, p=0.046, data not shown).

The appearance of more IPCs in the course of neurogenesis and of bRG is associated with changes in cell cycle kinetics and with oblique/horizontal spindle orientation of APs divisions, respectively [12, 13, 50-52]. Estimations of the cell cycle length by cumulative EdU labeling at E12.5 [53] showed no significant difference in overall cycling time between mutant and control APs (Fig. 3K). However, commitment to a neural fate of cortical precursors is linked to both lengthening of G1 and to shortening of S-phase due to a diminished requirement for stringent DNA quality control [54]. Interestingly, we observed a significant shortening of S-phase (co: 5.4h, mt: 3.8h, $p<0.01$) concomitant with a prolongation of G1-phase (co: 8.9h, mt: 9.9h, $p<0.01$) of mutant APs, suggesting that Wnt signaling prevents fate-associated changes in the cell cycle. We next calculated the angle of division of APs after immunostaining for the centrosome marker γ-tubulin (Fig. 3L-O). In agreement with the slight increase in the number of bRG, we detected a few oblique divisions in the mutant cortices. Collectively, these data suggest that the absence of Wnt/β-catenin signaling promotes indirect neurogenesis by affecting not only the AP division mode but also their cell cycle kinetics and spindle orientation.

β-catenin – mediated transcription regulates laminar fate and onset of gliogenesis

Since we observed defects in the proliferation of APs and expansion of the basal precursor pool at E12.5 (Fig. 3A-K), we assessed whether the ablation of Wnt/β-catenin transduction influences neuronal specification and cortical lamination. We examined E18.5 cortices for layer-specific markers – Reelin for the marginal zone (MZ)/layer 1, Tbr1 for layer 6 (L6), Ctip2 for layer 5, and Cux1 for layers 2-4 and SVZ IPCs progenitors [16]. The number of reelin$^+$ Cajal-Retzius neurons was significantly increased in signaling mutants compared to wild-type (Fig. 4A-B, G). Likewise, Tbr1 protein expression was not restricted to the mutant layer 6, but was found in upper-layer and nascent immature neurons (Fig. 4A-B). Contrary to Tbr1, the thickness of the deep Ctip2 neuronal layer was significantly reduced, bringing it in close proximity to the Cajal-Retzius cells of layer 1 (Fig. 4C-D, G). Similarly, in *E1C-Ctnnb1*$^{dm/flox}$ mutants fewer deep Foxp2 cells were detected (data not shown), suggesting that early neurons were not overproduced, but that *Tbr1* expression was impaired instead. In addition, the number of late $Cux1^+$ neurons of layers 2-4 was de-

creased to 72% of control, and hardly any Cux1-expressing IPCs were detected in the SVZ at this stage (Fig. 4E-F, G). Except for the Tbr1-expressing neurons, however, the relative position of the distinct neuronal subtypes was largely preserved in the Wnt/β-catenin signaling-depleted pallium. Indeed, immunostaining for the NPC-specific neurofilament protein nestin and the fatty acid-binding protein Blbp demonstrated that the neuroepithelial scaffold in *E1C-Ctnnb1*$^{dm/flox}$ embryos was intact even at late corticogenesis (Supporting information Fig. S7A, C). In support of this, confocal reconstruction of mitotic E14.5 RG after pHH3 and phospho-vimentin immunodetection confirmed the presence of basally-oriented processes in mutant RG (Supporting information Fig. S7B, D). In conclusion, by restricting neurogenic divisions to the appropriate time frame, canonical Wnt signaling controls the progenitor output for different neuronal subtypes, but not their lamination.

After sequential generation of neurons of all layers APs give rise to astrocytes detected at E18.5 with the astrocytic markers glial acidic fibrillary protein (GFAP) and Aldh1l1. GFAP-expressing glial progenitors are restricted to the VZ of control cortex, with few GFAP$^+$ astrocytic fibers detected at the basal CP. Surprisingly, in *E1C-Ctnnb1*$^{dm/flox}$ embryos, stronger GFAP immunoreactivity was observed in the VZ and individual GFAP$^+$ mature astrocytes had already migrated basally (Fig. 4H-I). Similarly, a significant increase in the number of Aldh1l1$^+$ astrocytes in the IZ/CP was detected in the mutant cortices (Supporting information Fig. S7E-H) [55]. Finally, the astrocytic nature of these basally located cells was also confirmed by quantifying Blbp$^+$nestin$^-$ cells outside the VZ/SVZ (Supporting information Fig. S7A, C) [56]. Therefore, in addition to regulating neuronal layer production, Wnt/β-catenin signaling prevents the premature initiation of astrogenesis.

Canonical Wnt signaling regulates timely sequential neurogenesis in the neocortex

Since we didn't observe augmented neuronal apoptosis (Supporting information Fig. S6A-C), the thinner CP in *E1C-Ctnnb1*$^{dm/flox}$ embryos might result from a premature switch of progenitors to production of subsequent-layer neurons. Thus, we investigated whether each neuronal layer is generated in the correct temporal order by neuronal birthdating. Embryonic neuronal progenitors undergoing S-phase were labeled with BrdU by intraperitonial injections of pregnant mothers at E12.5 or E15.5 when early and late neurons, respectively, are at the peak of their production [57]. Upon immunostaining, the percentage of marker-specific subtypes labeled with BrdU per total marker-positive cells at each stage was calculated. Birthdating of E12.5 neurons (Fig. 5A-E) showed that more early Ctip2$^+$ neurons were generated in mutants (33±5% in controls vs 45±6% in mutants) and β-catenin-signaling-ablated progenitors had already switched to producing late Cux1$^+$ neurons destined for layers 2-4 (9±2% in controls vs 20±4% in mutants). Indeed, we detected more Cux1+ upper-layer neurons present on E14.5 coronal sections from *E1C-Ctnnb1*$^{dm/flox}$ embryos (Supporting information Fig. S6D, E). By E15.5 the generation of lower layer neurons had already declined in both controls and mutants. However, production of upper layer Cux1$^+$ neurons at E15.5 was severely diminished to 13±0% in *E1C-Ctnnb1*$^{dm/flox}$ cortices compared to 22±2% in controls (Fig. 5F-J). Altogether, we conclude that the defective neurogenesis in *E1C-Ctnnb1*$^{dm/flox}$ pallium is due to precocious production of neurons of subsequent layers and later, to an earlier switch to astrogenesis.

Absence of β-catenin – mediated signaling triggers premature activation of a corticogenesis transcriptional program

To identify the targets of Wnt/β-catenin signaling independent of β-catenin-sustained adhesion, we performed microarray-based expression analysis on directly isolated dorso-medial pallium from control and *E1C-Ctnnb1*$^{dm/flox}$ embryos at E11.5. Among the most downregulated genes were some known Wnt/β-catenin signaling targets such as *Sp5*, *Tnfrsf19* (*Troy*), *N-myc*, *Lef1* and *Axin2* (Fig. 6A, red), validating the microarray data. β-catenin preferentially acts as a transcriptional activator or can serve as a repressor depending on the cofactor context [58]. Thereby, we identified novel DNA-binding proteins which have not been previously linked to Wnt/β-catenin signaling and whose expression was decreased or strongly (more than 2-fold) upregulated in mutant tissues (Fig. 6A, blue). We set out to determine if their temporal NPC-specific expression fits with a role in regulating sequential fate shifts. For this purpose, we performed bioinformatic analysis on microarray data from APs collected either from E11 cortex tissue (that largely consists of APs and very few neurons) and APs isolated from E14.5 and E18.5 cerebral cortex by flow cytometry based on the expression of the apical membrane protein Prominin1 and human GFAP promoter (Fig. 6B) [59]. At E11 APs mostly self-renew, followed by a peak of differentiative divisions producing either neurons or IPCs at E14.5 and finally convert to gliogenesis by E18.5. Notably, previously confirmed regulators of sequential fate decisions such as *Hmga2* [60], *Foxg1* and its targets *Rgmb*, *Magel2* and *Nr4a2* [61] demonstrated dynamic, highly significant changes in their temporal expression (Fig. 6B, black). When we assessed the levels of the DNA-binding proteins affected upon β-catenin signaling loss (Fig. 6B, red and blue), two patterns emerged. First, the NPC-specific expression of transcripts downregulated in the absence of Wnt transduction peaked at early stages and then significantly declined (7 out of 10 TFs). Second, mRNA levels of astrogenesis-associated genes, for example *Nfix* and *Etv5*, increased after mid-neurogenesis, consistent with their suggested role in glia. Thus, the dynamic changes in the expression of these genes at key

corticogenesis stages suggest that they might be controlling the orderly fate switches.

Given that cortical Wnt/β-catenin signaling ablation affected mostly progenitor proliferation and sequential differentiation, we used these two functional categories to classify the microarray-identified TF. Indeed, when we validated our gene chip results by quantitative real-time PCR (qPCR), the mRNA levels of *Tcfap4*, *Sp5* and *N-myc* which repress cell cycle inhibitors p21 and/or p27 were decreased (Fig. 6B) [62-65]. Similarly, the expression of TF *Eya2* and *Dach1,* which form an activating complex positively affecting precursor proliferation and survival [66], was also reduced in the absence of Wnt/β-catenin signaling. Concomitantly, the levels of the neurogenesis-implicated proteins *Rora* and *Prdm12* were disregulated [67, 68]. Finally, the expression of *Prtg*, required to maintain an early progenitor state in the retina [69], and of *Dmrt3*, which belongs to a family acting downstream of Pax6 in the cortex [70], was downregulated.

Interestingly, the upregulated TF *Etv5* was recently identified as part of an early transcriptional network coordinating temporal neurogenesis downstream of the repressor FoxG1 [61]. Apart from the known FoxG1 target *Wnt8b*, some of the most rapidly downregulated genes upon FoxG1 re-expression were *Dmrt3*, *Eya2* and *Dach1*, whose levels were also significantly decreased upon β-catenin signaling loss. Intriguingly, these TFs were suggested to regulate the temporal competence of NPC to early neurogenesis. In addition, *Etv5*, which is increased in both *FoxG1* gain-of function and β-catenin loss-of-function cortices, was shown to promote astrogenesis in late NPC [71]. Also, in the Wnt signaling-ablated brain, the glia-inhibiting TF *Emx2* and the two members of the Nuclear Factor I family *Nfix* and *Nfib* also showed altered expression [72]. Thus, our data collectively suggest that Wnt/β-catenin signaling regulates stage-specific TF targets to maintain an orderly progression from NPC proliferation to neurogenesis and eventually, gliogenesis.

We then tested if the TF Dach1, Eya2, Etv5 and Nfix, which are implicated in orderly progression of corticogenesis, are direct Wnt/β-catenin signaling targets. First, we examined the DNA upstream of the transcription start site (TSS) for regions conserved between human and mouse (Fig. 6D, conservation regions denoted as pink peaks) and designed different primer pairs against selected regions. We carried out chromatin immunoprecipitation (ChIP) using an anti-β-catenin antibody on chromatin derived from E13.5 cortical tissue and analyzed the immunoprecipitate by qPCR. *Axin2* intron1 and the *Sp5* TSS served as positive and the promoter regions of GATA and β-globin as negative controls [65]. Interestingly, β-catenin interacted with the promoter regions of Dach1, Eya2, Etv5 and also Nfix, suggesting that β-catenin signaling regulates a TF cascade to exert its effects on the sequential fate of NPC.

DISCUSSION

Despite the fundamental importance of the canonical Wnt pathway for the development of the neocortex, its specific role has been difficult to address due to the dual function of its downstream effector β-catenin as a transcriptional activator and adhesion protein. Therefore, we employed a newly generated β-catenin mutant strain, in which β-catenin cannot interact with its N- or C-terminal transcriptional coactivators, but can still fully sustain cell-cell adhesion. Apart from an *in vitro* assay, we demonstrated the functional ablation of the Wnt/β-catenin pathway using the highly sensitive BAT-gal Wnt/β-catenin signaling reporter line by E12.5. Through this model, we found that canonical Wnt signaling within progenitor cells regulates the duration of the S- and G1-phases, both of which are key factors for NPC fate decisions, and apical progenitor maintenance. Unexpectedly, we also uncovered a Wnt/β-catenin pathway role in the timely regulation of corticogenesis by controlling a set of TF with stage-specific function.

NPC undergo several rounds of symmetric divisions before they switch to asymmetric divisions, which generate IPCs, bRG, or neurons. Previous reports suggested that cell-autonomous Wnt signaling promotes APs expansion by increased cell cycle re-entry and by delaying the transition from APs to IPCs [21, 23, 73]. In contrast, other studies demonstrated a functional relevance of β-catenin signaling in inducing IPCs differentiation through N-myc and Neurog1/2 after E13 [25, 27, 28, 74]. Yet another report revealed a crucial role of β-catenin in maintaining the structural integrity of the neuroepithelium and in cell survival in the early telencephalon [75]. Thus, although some studies implied a role of Wnt/β-catenin signal transduction in neural differentiation, the orderly progression of corticogenesis was not possible to examine because of tissue disruption, compensatory mechanisms, or technical limitations of *in utero* electroporation. Indeed, disrupted neuroepithelial integrity often results in an increase in basally dividing cells and defective lamination [32, 47, 76-78], emphasizing the importance of studying Wnt signaling function in the context of intact progenitor adhesion. Using our signaling-defective, adhesion-active β-catenin strain, we showed that Wnt/β-catenin signaling maintains the APs pool at a size appropriate for the stage-specific generation of stem cell derivatives (Fig. 2-3). In β-catenin signaling mutant embryos, the basal mitoses and the number of proliferating $Tbr2^+$ IPCs were enhanced at E12.5, followed by a decreased number of $Pax6^+$ APs at E14.5 and shrinkage of the VZ. This VZ precursor depletion in mutant embryos was accompanied by a shortening of S-phase and lengthening of G1, which is characteristic for proliferating progenitors committing to neuronal fate [50, 51, 54]. Strikingly, although the main function of β-catenin signaling is not to regulate the spindle orientation of APs in general, the increased number of APs divisions with oblique spindle orientations reflected the enhanced number of bRG-like

cells in β-catenin signaling mutants [52]. Hence, we propose that canonical Wnt signaling controls the timely transition of apical progenitors to a more committed state, reflected by increased generation of different types of basal progenitors upon inactivation of β-catenin signaling.

As corticogenesis proceeds, the neural progenitors become progressively restricted in their fate potential, first generating neurons of deep layers, then more superficial neurons to finally transition to gliogenesis, while resetting to an earlier fate has proven difficult [79]. Compelling evidence suggests that this sequential timing of neurogenesis is a complex interplay between an intrinsic timing property of individual progenitors and extrinsic signals. Indeed, neurons for example express Notch ligands, thus activating Notch signaling in precursors and inducing demethylation of astrocytic promoters via nuclear factor I [80]. Furthermore, embryonic neurons secrete the cytokine cardiotrophin-1, which activates the gp130-JAK-STAT pathway in APs, thus promoting timely astrogenesis [81]. The neuron-specific Sip1 restricts the production of Ntf and Fgf in postmitotic neurons to ensure sufficient number of each neuronal subtype is generated before the onset of gliogenesis [57]. However, intrinsic mechanisms themselves can drive the orderly progression of fate. Isolated cortical progenitors *in vitro* exhibit the orderly generation of different types of neurons and subsequently glia, as they would *in vivo* [14, 15]. Further supporting the existence of an internal timing property, both mouse and human embryonic stem cells can be sequentially differentiated into the distinct types of dorsal pyramidal neurons, which efficiently integrate into the mouse brain [16, 82]. Here we demonstrate that in an environment of intact precursor interactions, Wnt/β-catenin signaling is required to prevent premature progression to a later fate. When we ablated β-catenin transcriptional activity, the progenitors precociously proceeded to generating early neurons and then, late neurons, diminishing the final neuronal output (Fig. 5). While both populations were affected, Ctip2$^+$ deep neurons of layer 5 exhibited a stronger decrease, supporting a role of canonical Wnt pathway in specifying this neuronal type [22]. The reduction in upper neurons in spite of abundant IPCs and bRG-like cells presumably resulted from premature depletion of the APs pool and switch to astrogenesis (Fig. 2-3). Indeed, contrary to control cortices, we observed no Cux1$^+$ IPCs in the SVZ of mutant forebrains at E18.5, but extensive Blbp$^+$, Aldh1l1$^+$ and GFAP$^+$ mature astrocytes in the cortical wall (Fig. 4 and Fig. S7). Therefore, canonical Wnt signaling disruption led to precocious termination of neurogenesis at the expense of astrogenesis.

Interestingly, we identified a temporal TF cascade downstream of β-catenin signaling which is crucial at different corticogenesis phases. At early stages, we identified changes in the cell cycle and division mode of NPC, which coincided with downregulation of the pro-proliferative TF *Sp5*, *N-myc*, *AP4*, *Dach1* and *Eya2*. Strikingly, Dach1 and Eya2, together with Dmrt3, are suggested to regulate neurogenic competence in the brain [61]. The precocious decrease in their expression along with their promoters being bound by β-catenin suggests that in β-catenin signaling-ablated embryos a neurogenesis program is prematurely activated. In addition, β-catenin associated upstream of the *Etv5* TSS, possibly exerting a negative regulation through TCF3 [58]. Increased levels of *Etv5* accompanied by a decrease in glia-repressing *Emx2* TF levels can probably account for the premature glial generation in *E1C-Ctnnb1*$^{dm/flox}$ mutants. Thus, Wnt/β-catenin signaling acts upstream of a complex and dynamic temporal TF network to control progenitor fate shifts.

Conclusion

In summary, we describe an unrecognized role of canonical Wnt signaling as a mechanism within the precursor niche, which regulates the timely fate switch from early to late neurogenesis and subsequently to astrogenesis. Such a role could only be uncovered in the context of a transcriptionally-impaired, but adhesion-functional β-catenin, where no neuroepithelial integrity defects were apparent.

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Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interests.

Author Contributions

K.D.: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing; M.Z.: Collection and/or assembly of data, Data analysis and interpretation; L.Z.: Collection and/or assembly of data, Data analysis and interpretation; T.V.: Conception and design, Provision of study material, Final approval of manuscript; C.C.: Conception and design, Collection and/or assembly of data, Data analysis and interpretation; M.O.: Data analysis and interpretation; M.S.: Provision of study material; R.H.: Conception and design; M.G.: Provision of study material, Final approval of manuscript, Financial support; K.B.: Conception and design, Provision of study material, Final approval of manuscript, Financial support; L.S.: Conception and design, Data analysis and interpretation, Manuscript writing, Financial support.

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Figure 1. A novel β-catenin allele abrogates Wnt signaling in the absence of adhesion defects. (A): Representation of the β-catenin alleles used in this study, where black boxes denote coding exons and numbers on top – Armadillo repeats. *Ctnnb1*^{dm} allele bears a mutation D164A in Armadillo repeat 1 and a premature STOP codon, while *Ctnnb1*^{flox} has two loxP sites (red arrowheads): before its transcription start (ATG) and after exon 6. β-catenin was inactivated in the forebrain using a dorsal cortex-specific *Emx1-Cre* (E1C) line. At E12.5 similarly to controls **(B)**, β-catenin^{dm} protein preserved its enrichment at the ventricular surface **(C)** whereas no β-catenin protein was detectable in the *E1C-Ctnnb1*$^{flox/flox}$ forebrain **(D)**. Also, β-catenin^{dm} still co-localized with its interaction partner N-cadherin at adherens junctions **(F)** while the neuroepithelial integrity was lost in the *E1C-Ctnnb1*$^{flox/flox}$ cortex **(G)**. Similarly, staining for α-E-catenin and ZO-1 detected intact junctions in *E1C-Ctnnb1*$^{dm/flox}$ **(I)** forebrains compared to *E1C-Ctnnb1*$^{flox/flox}$ **(J)**. **(K-L):** Using the BAT-gal Wnt/β-catenin signaling reporter onto *E1C-Ctnnb1*$^{dm/flox}$ embryos, we demonstrated the loss of transcriptional activity in *E1C-Ctnnb1*$^{dm/flox}$ dorsal cortices, but not in the non-recombined thalamus. **(M)**: In addition, contrary to controls, mutant neural progenitors electroporated in vitro with SuperTOPFlash β-catenin signaling reporter construct failed to induce luciferase activity in response to Wnt stimulation, ** p = 0.01. Error bars indicate s.d. Abbreviations: VE, ventricle; TH, thalamus. Bar: A-L, 100 μm.

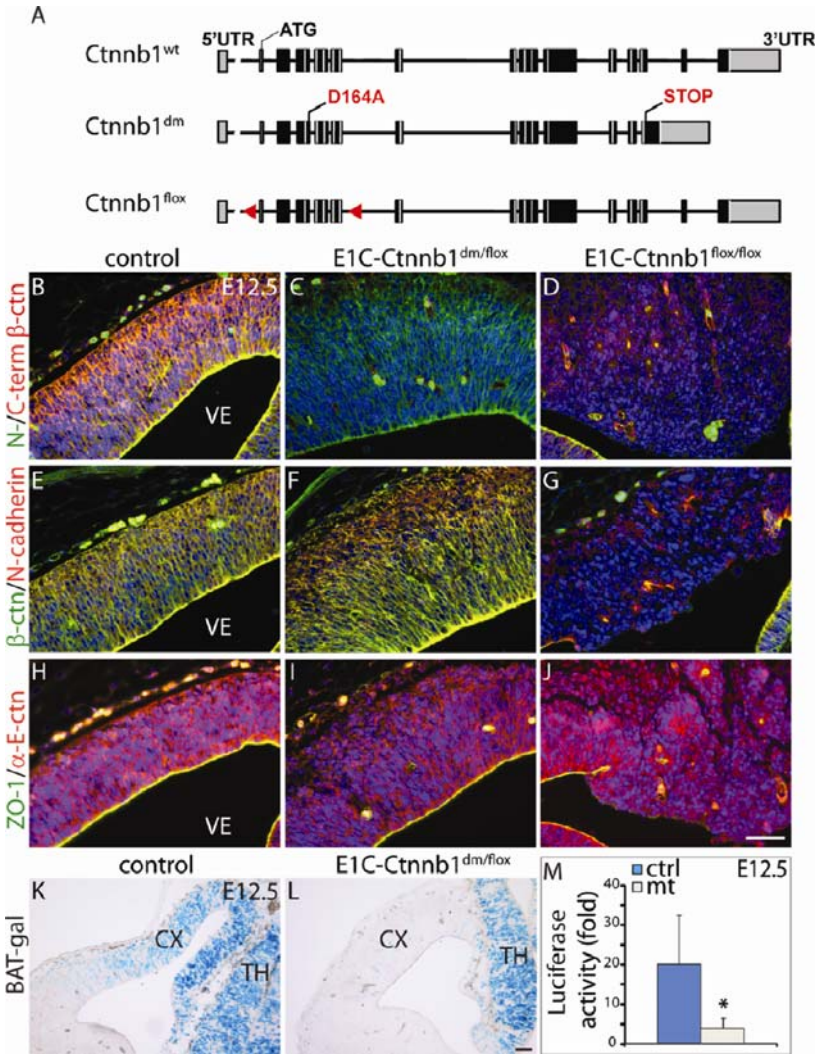

Figure 2. Specific Wnt/β-catenin signaling ablation affects neurogenesis without tissue disintegration. (A-F): Immunohistochemistry for the NPC-specific marker Sox2 and the neuron-specific protein doublecortin (Dcx) at E12.5 and E14.5 revealed precocious differentiation in both *E1C-Ctnnb1*$^{dm/flox}$ **(B, E)** and *E1C-Ctnnb1*$^{flox/flox}$ **(C, F)** cortices. However, only in the latter intermingling of these populations due to adhesion defects was observed **(C, F)**. Quantification of the number of NPC and neurons at E12.5 confirmed an increase in neurogenesis in signaling mutants vs. controls **(G)**, * p = 0.005. As expected, increased cell cycle exit of progenitors also contributed to the premature neurogenesis **(H)**, * p = 0.003. **(I):** Measurement of the thickness of the VZ, SVZ, IZ and CP demonstrated depletion of VZ progenitors after E12.5 concomitant with a transient increase in basally dividing cells resulting in a significantly thinner CP by the end of neurogenesis, p<0.05. Abbreviations: VE, ventricle. Error bars indicate s.d. Bar: 100 μm.

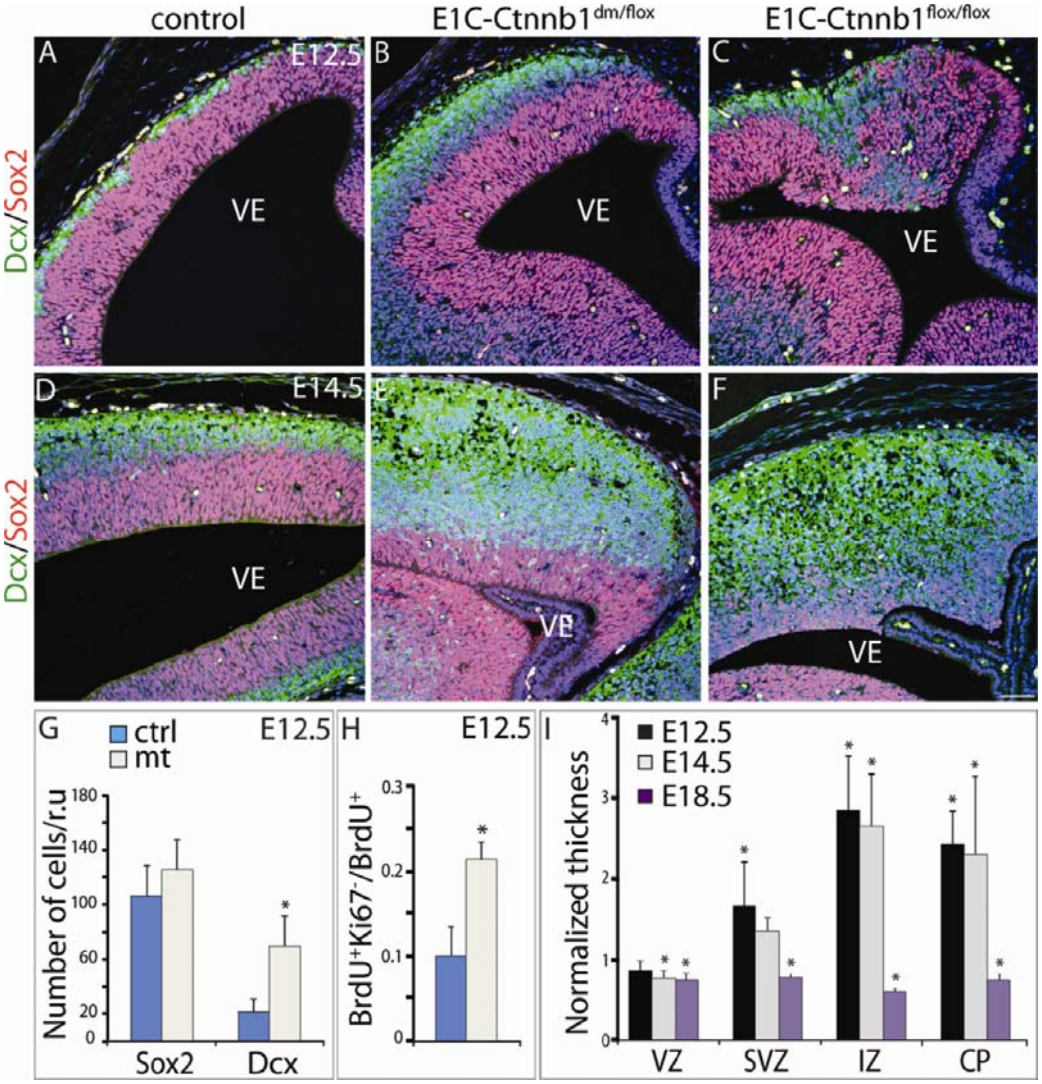

Figure 3. Wnt/β-catenin signaling ablation increases the number of basally located progenitors. (A-B): APs were distinguished from proliferating IPCs by immunostaining for Pax6 and Tbr2/Ki67, respectively. **(C):** Quantification of the numbers of APs, APs transitioning to IPCs (i.e. $Pax6^+/Tbr2^+$ cells, named AP→ IPC) and IPCs at E12.5 and E14.5. While $Pax6^+$ APs were spared in *E1C-Ctnnb1*$^{dm/flox}$ forebrains at E12.5, their numbers declined by E14.5 ($p = 0.006$) due to enhanced AP→IPC transition (E12.5: $p = 0.001$, E14.5: $p = 0.029$) concomitant with a transient increase in IPCs at E12.5 ($p = 0.023$). Micrographs **(E-J)** and quantification **(D)** of $Pax6^+$ residing basal to the SVZ at E14.5 cortices demonstrate more presumptive bRG in the *E1C-Ctnnb1*$^{dm/flox}$ mutants. **(K):** Strikingly, although the total cell cycle length was similar, *E1C-Ctnnb1*$^{dm/flox}$ APs shortened their S-phase and prolonged their G1 compared to control APs. Saturation was reached in both genotypes at 15h. **(L-M):** Cleavage angle analysis on confocal micrographs using DAPI to identify mitotic cells in ana- and telophase and the centrosome marker γ-tubulin. **(N-O)** A few nonvertical divisions were detected in the absence of β-catenin signaling. Error bars indicate s.d. Abbreviations: AP, apical progenitor; rRG, basal radial glia; IPC, intermediate progenitor cell; AP→ IPC, AP transitioning to IPC; r.u. = radial unit. Bar: A-B, E, H, 50 μm, M, 5 μm.

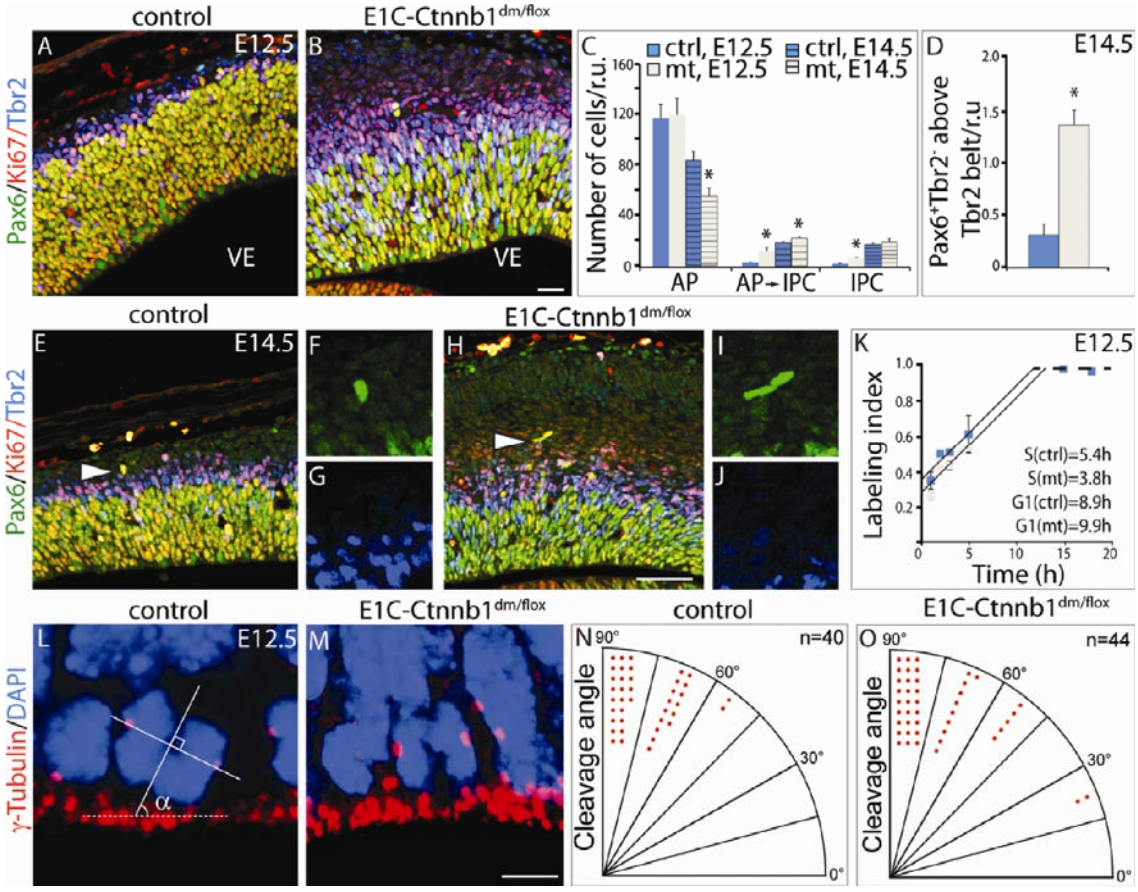

Figure 4. Wnt/β-catenin signaling regulates laminar fate and onset of gliogenesis. (A-B): At E18.5, in Wnt signaling mutants the population of early reelin^{+} (Rln) Cajal-Retzius neurons was expanded, similarly to the deep-layer Tbr1^{+} neurons (double-pointed arrows indicate layer thickness). **(C-G):** To the contrary, lower Ctip2^{+} and upper-layer Cux1^{+} cells are depleted, * p = 0.005, ** p = 0.029, *** p = 0.027. Furthermore, a precocious switch of mutant progenitors from neurogenesis to astrogenesis was evidenced by extensive GFAP labeling in the proliferative zone and in the cortical plate (arrows), where mature astrocytes migrate **(H-I)**. For additional astrocyte markers, refer to Figure S7. Abbreviations: MZ, marginal zone; SVZ, subventricular zone. Bar: 50 μm.

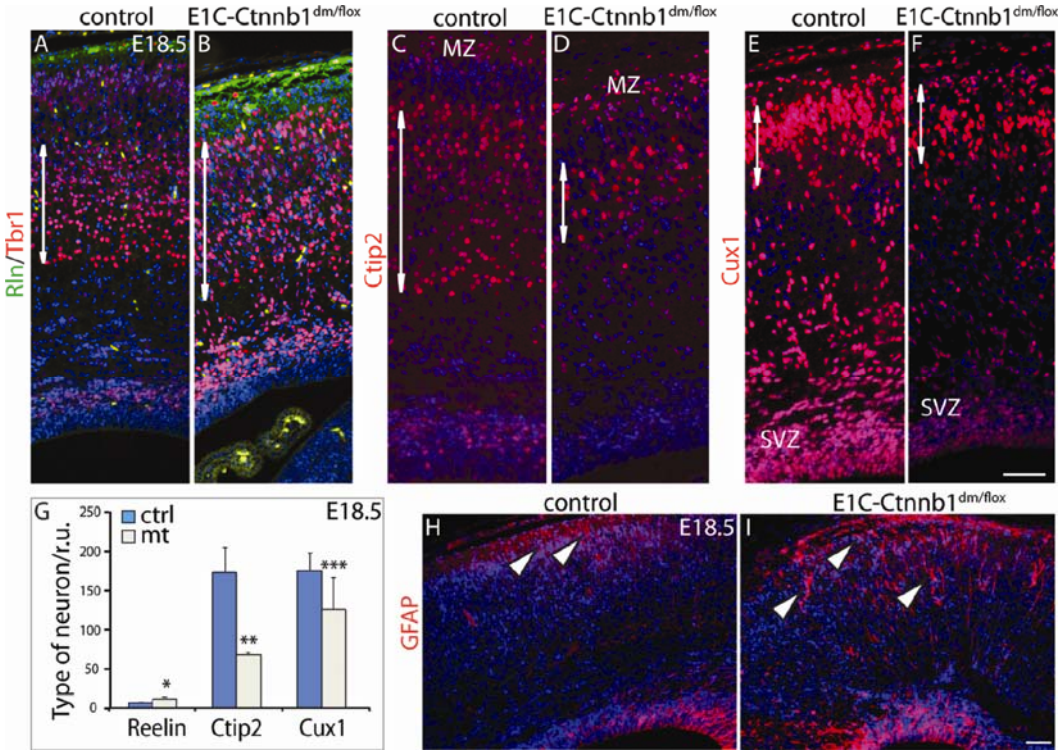

Figure 5. Canonical Wnt signaling regulates the temporal order of neurogenesis. Neuronal birthdating was performed by injection of BrdU at E12.5 and E15.5 to label early- and late-born neurons, respectively, and analysis of neuronal populations at E18.5. Birthdating at E12.5 showed enhanced production of deep-layer Ctip2^{+} (p = 0.004) and upper-layer Cux1^{+} cells (p = 0.011) in *E1C-Ctnnb1*$^{dm/flox}$ embryos **(A-E)**. By E15.5, the generation of deep-layer neurons has almost ceased, but the number of BrdU-labeled upper-layer Cux1^{+} neurons was severely diminished in mutant cortices **(F-J)**, p = 0.002. Error bars indicate s.d. Bar: 50 μm.

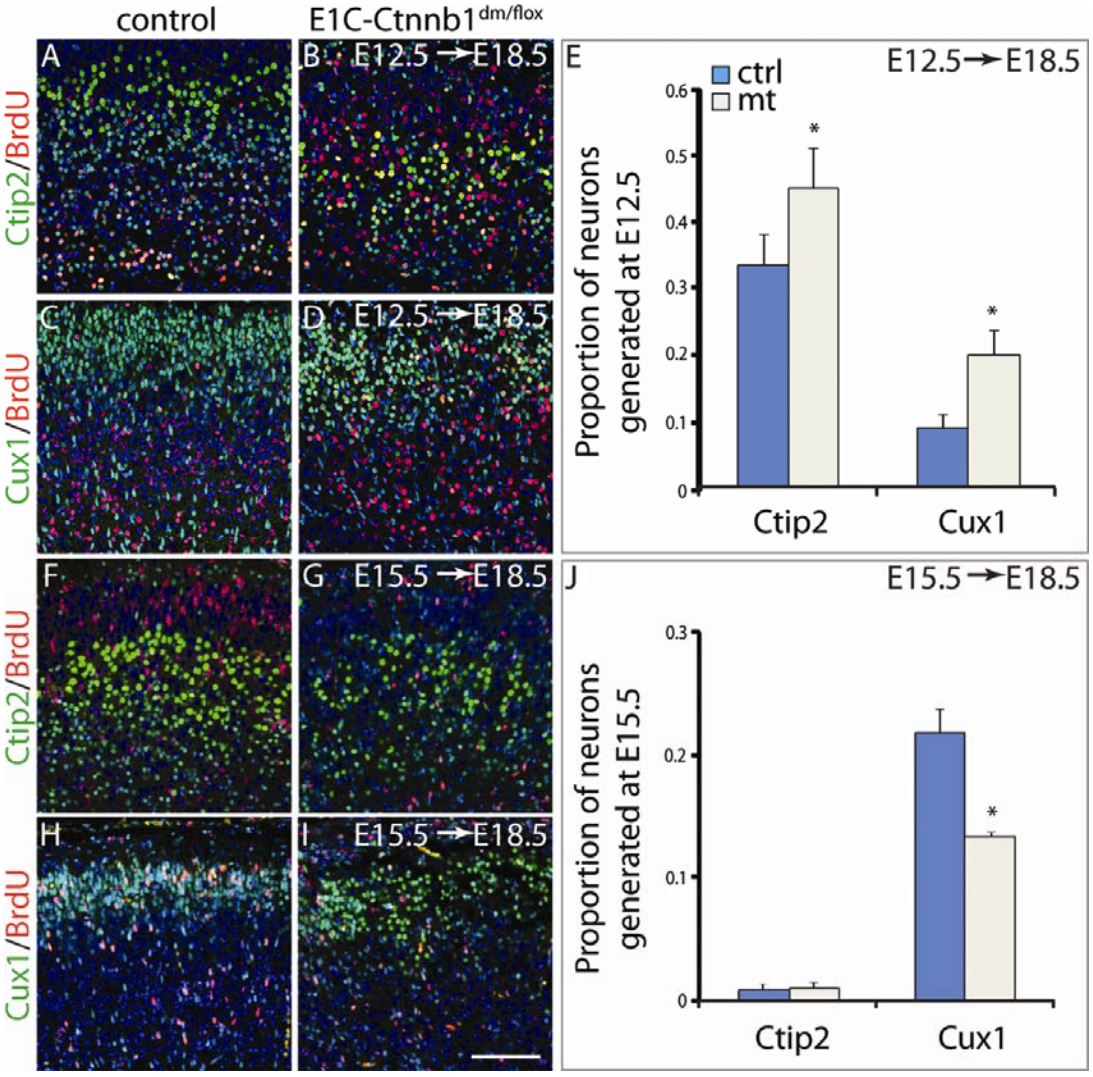

Figure 6. Wnt/β-catenin signaling controls a TF cascade in cortical progenitors. (A): Heat map of significantly affected transcripts after inactivation of β-catenin – mediated signaling, where known Wnt signaling targets are colored red and TF verified by qPCR – in blue. **(B):** Heat map of precursor-specific TF whose expression significantly changes from E11.0, E14.5 to E18.5 in dorsal forebrain, where reported regulators of sequential fate are colored black and TF selected for analysis in red and blue, as in **(A)**, $p < 0.05$, one-way ANOVA. **(C):** qPCR was performed to confirm microarray expression changes in DNA-binding proteins reported to regulate proliferation or differentiation, * $p < 0.05$, *t*-test. **(D):** Primers for ChIP (red arrows) for selected TF were designed in DNA regions close to the TSS that were conserved between human and mouse. **(E):** Real-time PCR analysis of DNA fragments precipitated in a representative ChIP assay using an anti-β-catenin antibody shows enrichment of β-catenin occupancy at the promoter regions of Dach1, Nfix, Eya2 and Etv5 when compared to positive and negative controls.