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Ankyrin-G Regulates Neurogenesis and Wnt Signaling by Altering the Subcellular Localization of β -catenin

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Abstract

Ankyrin-G is a scaffolding protein required for the formation of the axon initial segment in neurons. Recent genome-wide association studies and whole-exome sequencing have identified *ANKK3*, the gene coding for ankyrin-G, to be a risk gene for multiple neuropsychiatric disorders such as bipolar disorder (BD), schizophrenia, and autism spectrum disorder (ASD). Here, we describe a novel role for ankyrin-G in neural progenitor proliferation in the developing cortex. We found that ankyrin-G regulates canonical Wnt signaling by altering the subcellular localization and availability of β -catenin in proliferating cells. Ankyrin-G loss-of-function increases β -catenin levels in the nucleus, thereby promoting neural progenitor proliferation. Importantly, abnormalities in proliferation can be rescued by reducing Wnt pathway signaling. Together, these results suggest that ankyrin-G is required for proper brain development.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

ANK3; Ankyrin-G; Wnt; Neurogenesis; Neuropsychiatric Disorders

Introduction

Genome-wide association studies (GWAS) have identified many genes that are associated with a risk for mental disorders such as bipolar disorder, schizophrenia and ASD¹⁻⁷. The *ANK3* gene is among the most consistently replicated and statistically significant bipolar risk gene identified in these studies⁶⁻⁸. A number of single nucleotide polymorphisms (SNPs) in *ANK3* have been associated with bipolar disorder, and several of these variants have been associated with altered white matter integrity and changes in cortical-striatal-thalamic circuits, as well as with poorer performance on tasks of sustained attention^{9,10}. A 2012 study described decreased levels of *ANK3* mRNA expression in schizophrenia patients¹¹. Moreover, whole-exome and whole-genome sequencing studies identified putative causative mutations in coding and non-coding regions of *ANK3* in patients with ASD and intellectual disabilities (ID)¹²⁻¹⁴. However, it remains unknown how alterations in *ANK3* may contribute to the pathology of mental illness.

ANK3 encodes a scaffolding protein, ankyrin-G which, in mature neurons, localizes to the axon initial segment (AIS) and the nodes of Ranvier^{15,16}. Ankyrin-G is required for the assembly and maintenance of the AIS, which is established through its interaction with scaffolding and transmembrane proteins, and voltage-dependent sodium and potassium channels^{16,17}. Recently, several studies have suggested that ankyrin-G is required for establishing and maintaining neuronal polarity, suggesting a central role for this protein in establishing intact neural circuitry^{17,18}. Additionally, ankyrin-G has been shown to be a binding partner of E-cadherin in epithelial cells, and is required, along with β -2-spectrin, for the localization of E-cadherin to cell adhesion sites, where it assembles a complex that includes the important Wnt pathway component β -catenin¹⁹.

Canonical Wnt signaling plays an important role in neural progenitor proliferation in the developing central nervous system (CNS), and is also involved in dendrite development, synaptogenesis, and the establishment of axons²⁰⁻²³. Recent studies utilizing comparative genome sequencing of human patients, as well as the examination of neuronal development in rodents, have demonstrated the importance of canonical Wnt signaling in neuropsychiatric disorders such as schizophrenia, bipolar disorder, and autism spectrum disorder (ASD)^{24,25}. For instance, the product of the gene *Disrupted in Schizophrenia 1* (*DISC1*) has been shown to regulate several aspects of cortical development such as neural progenitor proliferation and dendritogenesis²⁶⁻²⁹. Additionally, the autosomal recessive primary microcephaly risk gene *ASPM* was shown to regulate Wnt signaling and progenitor proliferation during cortical development³⁰. These studies highlight the importance of the molecular pathways that underlie early cortical development to the etiology of complex psychiatric and neurodevelopmental disorders.

β -catenin is one of the key components of the Wnt signaling pathway. Upon activation of canonical Wnt signaling, via the interaction of the Wnt peptide with its membrane receptors

LRP and Frizzled, β -catenin is stabilized in the cytoplasm through the inhibition of GSK3 β , which normally promotes the proteolytic degradation of β -catenin. Once stabilized, β -catenin then enters the nucleus, where it binds to TCF/LEF family transcription factors to activate the expression of Wnt target genes²⁰. In addition to its central role in Wnt signaling, β -catenin also binds to type I cadherins at the cell membrane, linking them to the actin cytoskeleton and thereby playing a role in structural organization³¹. Several lines of evidence suggest that altering the levels of β -catenin localized to the catenin-cadherin complex can affect the availability of β -catenin for participation in Wnt signaling^{31–34}. This includes the observation that the sequestration of β -catenin at the cell membrane via overexpression of the cytoplasmic domain of cadherins and the repression of E-cadherin have opposite effects on Wnt signaling. Importantly, it has previously been shown that overexpression of β -catenin can perturb the development of mammalian cortex²⁰.

In the current study, we show that ankyrin-G is highly enriched in the ventricular zone (VZ) of the embryonic brain and is required for proper neural progenitor proliferation. Ankyrin-G loss of function leads to increased neural progenitor proliferation and increased canonical Wnt signaling. This is accompanied by a disruption of the β -catenin/cadherin interaction and increase in the nuclear pool of β -catenin. These results suggest that ankyrin-G can regulate canonical Wnt signaling via the fine-tuning of available levels of β -catenin, thereby ensuring proper brain development. As the importance of canonical Wnt signaling in neuropsychiatric disorders has become increasingly evident, our results shed light on the molecular events that, when influenced by human disease genes, may contribute to the etiology of neuropsychiatric disorders.

Results

Ankyrin-G is Necessary for Proper Embryonic Neural Progenitor Proliferation

To understand whether ankyrin-G has a role in embryonic cerebral cortical development, we first examined the expression pattern of ankyrin-G in the mouse embryonic day 15 (E15) brain, at which period the proliferation and differentiation of cortical neurons are both at high levels³⁵. We found that ankyrin-G is highly expressed in the ventricular surface of developing cortex (Figure 1a). Previous studies have shown that endfeet of radial glial cells at the ventricular surface contact neighboring cells through cell junctions which is composed of two distinct domains: ZO1/mPar3 (apical) and β -catenin/N-cadherin (basal)³⁶. A more careful examination of ankyrin-G expression at the ventricular surface revealed that ankyrin-G is expressed in both of the domains co-localizing with both β -catenin (Figure 1a, top row of images) and ZO1 (Figure 1a, bottom row of images). β -catenin is well known to regulate multiple steps of neurogenesis, including proliferation, differentiation, and radial migration^{21,31}. The similarity in the expression patterns of β -catenin and ankyrin-G encouraged us to ask whether ankyrin-G regulates neural progenitor proliferation at early stages of corticogenesis. To address this question, we selected two small hairpin RNAs (shRNA1 and shRNA2) based on their ability to down-regulate endogenous ankyrin-G in the P19 embryonic carcinoma cell line, as assayed by western blot and quantitative polymerase chain reaction (qPCR; Figure S1a,b). We used these two shRNAs to examine the effect of ankyrin-G knockdown on embryonic neural proliferation. We performed *in*

utero electroporation at E13, using a combination of either a non-targeting scrambled shRNA (Control) or ankyrin-G shRNA expression constructs, in addition to a GFP expression construct to label electroporated cells. This was followed by pulse labeling either at E15 or E16 with 5-bromo-2-deoxyuridine (BrdU), which incorporates into the newly synthesized DNA of replicating cells during the S phase of the cell cycle. The brains were harvested either 2 or 24 hours following the BrdU injection and immunolabeled with antibodies against BrdU, the proliferative marker Ki67 (which is only absent from cells in G₀ phase³⁰), and GFP. We found that ankyrin-G knockdown resulted in a significant increase in BrdU incorporation in the GFP-positive (GFP⁺) cell population compared to controls after both 2 hours (Figure 1b,c) and 24 hours (Figure S2a,b) of BrdU labeling, indicating an overall increase in cell proliferation during the BrdU labeling period. Additionally, the number of Ki67-negative (Ki67⁻) cells within the GFP⁺ BrdU⁺ population was significantly decreased following ankyrin-G knockdown (Figure 1b,d and Figure S2a,c), indicating that ankyrin-G knockdown reduces the number of cells exiting the cell cycle. To further demonstrate that ankyrin-G is important for neural progenitor proliferation, a complementary experiment was conducted using embryonic *Ank3* +/- mice in which the brain-specific isoform of the ankyrin-G is deleted. A previously published study demonstrated that *Ank3* +/- mice exhibit bipolar disorder-like behavioral alterations such as reduced anxiety and increased motivation for reward³⁷. E15 *Ank3* +/- brains were pulse-labeled with BrdU, harvested, and immunolabeled with antibodies against BrdU and Ki67. Images covering the same area were taken from 5–7 consecutive brain slices per animal. Consistent with our *in utero* electroporation assay, we observed a significant increase in the number of BrdU-positive cells in *Ank3* +/- mice compared to wild-type littermates (Figure 1e, f). The increase in BrdU labeled cells was accompanied by a significant decrease in cell-cycle exit (Figure 1e,g). These results further confirm that ankyrin-G is a positive regulator of neural progenitor proliferation.

Consistent with the observation of increased proliferation, we also observed a significant increase in mitotic activity in the *in utero* transfected GFP⁺ population in ankyrin-G knockdown animals based on immunoreactivity for phosphohistone H3 (PHH3)³⁰, which labels mitotic cells (Figure 1h,i). In addition, we observed a reduced percentage of GFP⁺ cells in the cortical plate (CP) following ankyrin-G knockdown compared to controls (Figure 1j). Reduction in GFP⁺ cells in the cortical plate could be due to either a delay in neuronal differentiation, or an early neuronal migration defect. To rule out the latter possibility, we analyzed the distribution of GFP⁺ cells exclusively in the intermediate zone/cortical plate population and found no significant difference between control and ankyrin-G knockdown conditions (data not shown). The fact that the increase in GFP⁺ cells in the ventricular zone (VZ) correlates with the reduction in GFP⁺ cells in the CP is suggestive of a neuronal differentiation phenotype. To test this possibility, we examined immunoreactivity for Tuj1, a marker of differentiated neurons³⁰. Our results showed that ankyrin-G knockdown reduced the percentage of GFP⁺ cells overlapping with the Tuj1⁺ cells in the cortex, which are mostly localized to the cortical plate and intermediate zone (IZ; Figure 1k,l). Overall, these results suggest that ankyrin-G knockdown increases progenitor proliferation through reducing cell cycle exit and neuronal differentiation in the developing brain.

Ankyrin-G Regulates Wnt Reporter Activity

The observation of abundant ankyrin-G expression in the ventricular zone in a pattern similar to β -catenin expression and the up-regulation of neural progenitor proliferation following ankyrin-G knockdown, combined with the well-known role of Wnt signaling in proliferation, led us to ask whether ankyrin-G plays a role in canonical Wnt signaling, of which β -catenin is a core component. Additionally, the Wnt pathway has recently been implicated in several psychiatric disorders via its regulatory function in cortical neurogenesis^{26,30}. To measure Wnt-mediated transcriptional activity, we used a luciferase reporter construct containing seven copies of the TCF/LEF-binding site (8XSuperTOPFLASH), which can be activated by β -catenin downstream of canonical Wnt signaling^{38,39}. Murine P19 cells were transfected with either control or ankyrin-G-targeting shRNA constructs together with the luciferase reporter construct. We found that, following knockdown of ankyrin-G, TCF/LEF-reporter activity was increased in the absence of Wnt3a stimulation compared to controls (Figure 2a, left). Wnt3a treatment increased the TCF/LEF reporter activity by ~2 fold, and AnkG knockdown further upregulated the luciferase activity by ~1.5 fold (Figure 2a, right). These results suggest that ankyrin-G negatively regulates the canonical Wnt signaling pathway. To further decipher this mechanism, we examined the protein expression levels of several canonical Wnt pathway components following ankyrin-G knockdown in P19 cells. We observed that neither the protein (Figure 2b,c) nor the mRNA (Figure S1c) levels of β -catenin were altered following ankyrin-G knockdown compared to control conditions in P19 cells. Moreover, we did not detect differences in GSK3 β or phospho-GSK3 β (pY216 GSK3 β) abundance at the whole-cell level following ankyrin-G knockdown (Figure 2b,c), suggesting that ankyrin-G does not directly regulate the abundance of these canonical Wnt pathway components.

Ankyrin-G Knockdown Alters the Subcellular Localization of β -catenin

In addition to its central role in Wnt-mediated TCF/LEF transcription, β -catenin is a core member of a cell-cell adhesion complex comprised of cadherins and catenins (the cadherin-catenin complex)³¹. Although it was previously thought that the cadherin-bound pool of β -catenin cannot be made available for Wnt signaling, recent studies have suggested instead the existence of an interplay between Wnt signaling and this cell adhesion complex^{31–34}. Specifically, studies show that sequestration of β -catenin at cell-cell adhesion sites via E-/N-cadherin overexpression down-regulates Wnt signaling and that the absence of E-cadherin in embryonic stem cells results in the accumulation of free β -catenin in the nucleus⁴⁰. Importantly, ankyrin-G has been identified to be a molecular partner of E-cadherin in epithelial cells and is required for the proper accumulation of E-cadherin to cell-cell contact sites¹⁹. Therefore, we set out to evaluate how ankyrin-G knockdown affects the integrity of the adherens junctions, as well as to determine changes in the subcellular localization and levels of β -catenin following ankyrin-G knockdown. First, following ankyrin-G knockdown in P19 cells, we examined the association of β -catenin and E-cadherin by immunoprecipitation followed by western blot analysis. We found that the amount of E-cadherin co-immunoprecipitated with β -catenin was markedly decreased in ankyrin-G knockdown conditions compared to controls (Figure 2d,e). We then repeated this experiment using brain lysates from E14 *Ank3* +/- mice. Consistent with the shRNA knock-down assay,

the interaction between β -catenin and E-cadherin is significantly reduced in *Ank3* +/- mice compared to wild-type littermates (Figure 2f, g).

We next used immunohistochemistry to determine whether the reduced interaction between E-cadherin and β -catenin might lead to alterations in the subcellular localization of β -catenin. P19 cells were transfected with either nuclear GFP-expressing scrambled shRNA (Control) or ankyrin-G shRNA constructs for 48 hours, then fixed and immunolabeled with antibodies against ankyrin-G and either β -catenin or E-cadherin. In control cells, as previously observed¹⁹, E-cadherin is localized to the cell membrane, as suggested by the immunoreactivity surrounding the cell. However, ankyrin-G knockdown disrupted the localization of E-cadherin to the cell periphery, resulting instead in a distribution that is more diffuse throughout the cell (Figure 3a, b). This disrupted E-cadherin phenotype was observed in over 85% of ankyrin-G knockdown cells, compared to 20% of control cells (Figure 3a). Consistent with previous observations, β -catenin is mostly localized to the plasma membrane in control shRNA-transfected cells (Figure 3c). In ankyrin-G knockdown cells, however, β -catenin localization to the cell periphery was less conspicuous (Figure 3c, d).

The β -catenin on the cell membrane is presumably in a complex with E-cadherin³¹. Therefore, since the overall levels of β -catenin do not change in the absence of ankyrin-G (Figures 2b, c and S1c), we hypothesized that reduced levels of E-cadherin/ β -catenin at the plasma membrane following ankyrin-G knockdown might increase the levels of free, non-membrane-bound β -catenin that would then be available for Wnt signaling. We measured nuclear β -catenin levels in transfected cells expressing control shRNA or ankyrin-G shRNAs, as well as nuclear GFP, and observed a marked increase in the amount of β -catenin immunoreactivity localized to the nucleus (Figures 3c, d, e). Finally, to confirm that this phenomenon is observed in the brain *in vivo*, we examined the localization of intracellular β -catenin in cortical progenitor cells in sections from *in utero* transfected E16 embryos as well as from E15 *Ank3* +/- brains. These sections were immunolabeled with antibodies against β -catenin, Ki67 (to label cycling cells) and DAPI. In both *in utero* ankyrin-G knockdown and *Ank3* +/- animals, a greater level of β -catenin immunoreactivity was observed throughout the cytoplasm and the nucleus of Ki67⁺ cells compared to those from control animals (Figure 3f and h, respectively). Additionally, consistent with the *in vitro* assay, increased levels of nuclear β -catenin are observed in proliferating cortical neural progenitor cells (Figure 3f–i). These data suggest that ankyrin-G knockdown reduces the levels of E-cadherin localized to cadherin-catenin complexes at the plasma membrane, which in turn results in an increased abundance of β -catenin in the nucleus, which is then available for the activation of Wnt signaling.

Increased GSK3 β Levels Ameliorated Heightened Wnt Signaling Caused by Ankyrin-G Loss of Function

Our findings suggest that ankyrin-G is a negative regulator of the Wnt signaling pathway, since reduced levels of ankyrin-G lead to upregulated TCF/LEF reporter activity and nuclear β -catenin accumulation. To directly test this hypothesis, we examined the effect of the overexpression of GSK3 β , which phosphorylates β -catenin and targets it for ubiquitin-

dependent proteasomal degradation^{41,42}, upon on the ankyrin-G loss of function phenotype. In P19 cells, increased TCF/LEF luciferase reporter activity, observed as the result of ankyrin-G shRNA knockdown, was completely reversed by the overexpression of GSK3 β (Figure 4a). These results indicate that ankyrin-G functionally interacts with other Wnt signaling proteins such as GSK3 β and that the effects of ankyrin-G knockdown on Wnt signaling activity can be normalized by reducing β -catenin abundance. Together these results further suggest that ankyrin-G is a negative regulator of Wnt signaling

In Vivo Defects in Neurogenesis Caused by Ankyrin-G Knockdown Can Be Rescued via Depletion of β -catenin Levels in the Developing Cortex

Given that the ankyrin-G-mediated increase in TCF/LEF activity can be normalized via the expression of a core member (GSK3 β) of the Wnt signaling pathway, we hypothesized that the neurogenesis defects caused by *in vivo* knockdown of ankyrin-G could be rescued via similar manipulations. To examine whether the overexpression of GSK3 β can normalize the effects of ankyrin-G knockdown, we performed *in utero* electroporation at E13 with control or ankyrin-G shRNA and co-expressed either control empty vector or GSK3 β overexpression construct. Pulse labeling with BrdU was performed at E16 and brains were harvested 2 hours later. As shown earlier, knockdown of ankyrin-G resulted in increased BrdU incorporation in the GFP⁺ cell population, indicating increased proliferation (Figure 4b, c, e). Consistent with the TCF/LEF luciferase assay, the co-expression of GSK3 β with ankyrin-G shRNA led to levels of BrdU incorporation that were indistinguishable from the control condition (Figure 4b, d, e). Additionally, overexpression of GSK3 β was able to rescue the reduced cell cycle exit phenotype associated with ankyrin-G down-regulation (Figure 4b, c, d, f). Finally, we have observed normal GFP⁺ cell distribution upon co-expression of GSK3 β with ankyrin-G shRNA (Figure 4g). These findings demonstrate that the neurogenesis defects resulting from the *in vivo* knockdown of ankyrin-G can be rescued via dampening Wnt signaling through modulation of other core members of this signaling pathway. These *in vivo* neurogenesis data, in addition to our *in vitro* TCF/LEF reporter data, suggest that ankyrin-G exerts its effect on neural progenitor proliferation via controlling the pool of β -catenin participating in Wnt signaling (Figure 4h).

Discussion

In the current work, we describe a novel role for ankyrin-G in brain development. Extensive work has addressed the role of ankyrin-G as a scaffolding protein required for neuronal polarity and the formation of the AIS^{15,16}. Our findings indicate that ankyrin-G is also an important regulator of neural progenitor cells in the developing cortex. In the current literature, one of the hypotheses posits that early neurodevelopmental changes predispose the individual to the development of schizophrenia in adulthood^{43,44}. Furthermore, autism spectrum disorders are often associated with increased brain size and malformation of multiple brain regions including the cortex, suggesting alteration in aspects of neurogenesis, such as the proliferation and differentiation of cortical neurons. Our study further supports these findings and also suggests that neurodevelopmental changes likely also underlie bipolar disorder etiology.

Recently, it was shown that ankyrin-G regulates the production of olfactory bulb interneurons in the adult brain via its up-regulation in radial glia destined to become SVZ ependymal cells⁴⁵. In this environment, ankyrin-G is required for the SVZ niche assembly through the lateral adhesion of neural progenitors⁴⁵. Consistent with this study, we show that ankyrin-G regulates proliferation and new neuron production in the developing brain via a mechanism that involves E-cadherin localization to the cell membrane and alterations in subcellular β -catenin levels. Our results suggest, for the first time, that ankyrin-G modulates subcellular compartmentalization of β -catenin, and thereby controls the pool of cellular β -catenin available to participate in Wnt signaling in cortical progenitor cells (Figure 4h).

Our data suggest a model in which ankyrin-G regulates β -catenin anchoring at the progenitor cell membrane via its interaction with E-cadherin. Thus, when ankyrin-G is down regulated, the localization of E-cadherin to the cell membrane is reduced, and β -catenin becomes available to act at the nucleus, where it modulates gene transcription (Figure 4h). In support of this model, it has previously been shown that ankyrin-G down-regulation decreases N-cadherin attachment to the cell membrane in postnatal radial glia progenitors during the SVZ niche formation⁴⁵. Therefore, by regulating the levels of nuclear β -catenin available for Wnt signaling, ankyrin-G acts to negatively regulate progenitor cell proliferation. Accordingly, we were able to counteract the effects of ankyrin-G knockdown upon cortical progenitor proliferation by dampening Wnt signaling via the overexpression of GSK3 β , major component of this pathway.

These findings support a hitherto unknown role for ankyrin-G in canonical Wnt signaling. One of the most commonly-used medications for the treatment of bipolar disorder, lithium, has been shown to inhibit the function of GSK3 β , and this has been suggested to underlie some of lithium's therapeutic effects⁴⁶. This mechanism of lithium action further implicates the canonical Wnt pathway in psychiatric disorders. Although the current study does not have a direct line of evidence suggesting the loss of function of ankyrin-G manifests itself in behavioral changes, a recent study using the same *Ank3*^{+/-} mice line demonstrated that loss of ankyrin-G results in bipolar disorder-like behavioral alterations such as reduced anxiety which can be considered as higher risk taking, and increased motivation for reward both of which are features of bipolar disorder³⁷. Additionally, they demonstrated that behavioral changes caused by ankyrin-G knockdown can be reversed by lithium administration³⁷. However, little is known about the contribution of Wnt signaling to the etiology of mental illnesses.

We showed previously that deficits in neuronal proliferation via modulation of GSK3 β / β -catenin signaling underlie neurodevelopmental phenotypes reminiscent of psychiatric disorders, such as schizophrenia²⁶. Furthermore, a recent analysis of GWAS data examining pathways implicated in bipolar disorder found that the cadherin- and Wnt-signaling pathways are enriched in this disease and confirmed *ANK3* as a bipolar risk gene⁸. Our study makes the novel observation that ankyrin-G may serve as a common regulator of both cadherin- and Wnt-signaling pathways by altering the availability of β -catenin. We demonstrate the impact of ankyrin-G loss of function on neurogenesis in the embryonic brain, supporting the importance of Wnt signaling in the etiology of neurodevelopmental disorders. These results give new insights into the study of psychiatric disease etiology, and

support the idea that alterations in neuronal proliferation are an important element underlying the pathology of mental illness.

Finally, it is worth discussing that ankyrin-G is also highly expressed at the apical domain of radial glial progenitor endfeet at the ventricular surface. Mammalian Par3 (mPar3) is exclusively expressed at apical domain during interphase where β -catenin expression is minimal³⁶. Additionally, it was shown that mPar3 regulates asymmetric cell division of radial glial progenitors via Notch signaling in the developing neocortex. Therefore, we cannot rule out the possibility that ankyrin-G may also interact with mPar3 in radial glial progenitors, and through which it regulates neural progenitor proliferation. This regulation would be expected to be a β -catenin-independent pathway. Future studies will be needed to address whether ankyrin-G also interacts with mPar3 in neural progenitors in the developing neocortex.

Materials and Methods

DNA constructs

shRNA constructs used in this study were cloned into pLKO.2 PU6-shRNA/PUBiC-eGFP vector available from Broad Institute. Control non-targeting shRNA (Sigma, SHC002) and shRNA2 targeting Ank3 were obtained from the Petryshen lab at MGH. shRNA1 targeting Ank3 is obtained from Broad Institute. The sequences can be found on Table S1. Full length HA-GSK3 β (kind gift from Dr. Yingwei Mao at Penn State University, PA) were previously used in Mao et al 2009²⁶. Super8XTOPFLASH, a gift from Dr. R. Moon (University of Washington, WA) and a Renilla-Luc-TK reporter (pRL-TK, Promega) were used for testing TCF transcriptional activity. pCAGIG-Venus was provided by Dr. Zhigang Xie (Boston University, MA).

Animals

Swiss Webster pregnant female mice were purchased from Taconic (Hudson, NY, USA) for *in utero* electroporation. *Ank3* +/- mice were kind gifts from Bennett Lab (Duke University) and have been described elsewhere^{37,47}.

Cell Culture

P19 embryonic carcinoma cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (10566-016, Invitrogen) containing 10% FBS and penicillin/streptomycin.

Antibodies and Reagents

The following primary antibodies were used in this study: rabbit anti-ankyrin G antibody (H-215, Santa Cruz Biotechnology), rabbit anti- β -catenin antibody (H-102, Santa Cruz Biotechnology), mouse anti- β -catenin antibody (610153, BD Transduction Laboratories), mouse anti-ZO-1 antibody (33-9100, Invitrogen), chicken anti-GFP antibody (GFP-1020, Aves Labs), mouse anti-BrdU antibody (M0744, Clone Bu20a, DakoCytomation), rabbit anti-Ki67 antibody (Clone SP6, Lab Vision/Thermo Scientific), rabbit anti-phospho-Histone H3 (Ser10) antibody (06-570, Millipore), mouse anti-GSK3 β antibody (610202, BD

Transduction), mouse anti-pY216 GSK3 β antibody (ab75745, Abcam), anti-actin (Sigma A5316, clone AC-74) and mouse anti-E-cadherin antibody (610181, BD Transduction). Alexa-conjugated secondary antibodies (Jackson ImmunoResearch) were used for IHC and ICC. Recombinant human Wnt-3a was purchased from R&D Systems (Catalog number: 5036-WN). BrdU (5-Bromo-2'-deoxyuridine) was purchased from Sigma-Aldrich (B5002-5G).

Immunohistochemistry

Brain Sections—Embryonic cortical brains were drop-fixed overnight in 4% formaldehyde (FA) and then transferred to 30% sucrose/PBS solution at 4°C. Brains were embedded in O.C.T. compound (Electron Microscopy Sciences) and sliced into 14 – 20 μ m sections using cryostat. Cryosections were rehydrated with 1 \times PBS and blocked for 1 – 2 hours with blocking solution (1 \times PBS + 10% Donkey Normal Serum + 0.3% Triton-X). Following blocking, the cryosections were incubated with primary antibodies overnight at 4°C. Incubation with secondary antibodies were performed for 1 hour at room temperature. Finally, cryosections were mounted using ProLong Gold Antifade Reagent (Invitrogen).

Cell Cultures—Cell cultures were plated onto cover slips in 24-well plates. Following transfection with Lipofectamine 2000, cells were fixed with 4% FA at room temperature for 10 min and then washed 3 times with 1 \times PBS. Following 30 min blocking, they were incubated with primary antibodies for 45 – 60 min, washed again with 1 \times PBS, incubated with secondary antibodies for 30 min and finally washed and mounted for imaging.

Western Blot Analysis

Transfected cells were lysed and run on 8% SDS-polyacrylamide gels at 60 – 120 constant voltage to separate, and transferred onto Immobilon-P PVDF membranes (Millipore) at constant current. Membranes were blocked using 5% BSA prepared in TBS-T (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 30 min at room temperature. Membranes were incubated with the primary antibodies overnight at 4°C. Next, they were washed 3 times with TBS-T, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE) for 1 hour at room temperature. Following washing with TBS-T, immunoreactivity signals were detected by enhanced chemiluminescence (Perkin Elmer).

qPCR

Total RNA was collected using the Rneasy Plus Kit (Qiagen) 48 hours after transfection. Reverse transcription of the mRNA transcripts to produce cDNA for QPCR was achieved using the SuperScript III Reverse Transcriptase (Invitrogen). qPCR was performed using SsoFast Evagreen Supermix (Bio-Rad) on CFX96 Real-Time PCR Detection System (Bio-Rad). The reactions were run in triplicates and average of these triplicates were used for statistical analysis. β -actin was used as internal control. Primer sequences used for qPCR can be found in Supplemental Table 1.

Immunoprecipitation

48 hour post-transfection, transfected cells were lysed in 1× lysis buffer (150 mM NaCl, 0.1% NP40, 50 mM Tris, pH7.5, 5 mM EDTA) with protease inhibitors. Whole-tissue lysates of *Ank3* +/- mice brains were prepared by homogenization in 500 µl solution including 50 mM Tris, 120 mM NaCl, 0.5% NP-40 with protease inhibitors. 0.5 mg of protein from each condition was added to protein A sepharose beads (GE Healthcare) conjugated with β-catenin antibody, and incubated overnight at 4°C. The beads were then washed with RIPA and lysis buffers before boiling in Laemmli sample buffer. Following SDS-Page to separate the proteins, blots were probed with E-cadherin antibody.

In utero Electroporation

The Institutional Animal Care and Use Committee of Massachusetts Institute of Technology approved all experiments. *In utero* electroporation was performed as described elsewhere⁴⁸. Briefly, pregnant Swiss Webster mice were anesthetized by intraperitoneal injections of Ketamine 1% / Xylazine 2 mg/ml, the uterine horns were exposed, and the plasmids mixed with Fast Green (Sigma) were microinjected into the lateral ventricles of embryos. Five pulses of current (50 ms on / 950 ms off) were delivered across the head of the embryos. The following voltages were used for different ages: 28–30 V for E13 and 32–35 for E15. In the DNA mixture, the shRNA plasmid concentration was 2 to 3-fold higher than that of pCAGIG-Venus. For rescue experiments with GSK3β, the ratio between the ankyrin-G shRNA and GSK3β was 3:1 in co-expression DNA mixture.

Luciferase Assay

Luciferase assays were performed as described elsewhere²⁶. P19 cells at 1×10⁵ cell/well density were plated into 24-well plates without antibiotics. Cells were transfected with 0.8 µg of shRNA plasmid along with 50 ng of Super8xTOPFLASH and 10 ng of pRL-TK. The media was replaced with one containing antibiotics 2 hours after transfection. Either 24 or 36 hours after transfection, cells were stimulated with recombinant human Wnt3a for either 16 or 12 hours, respectively, in Wnt-stimulated condition. TCF/LEF reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega). For the rescue experiments, 0.2 µg of HA-GSK3β was co-transfected with 0.6 µg of ankyrin-G shRNA. Firefly luciferase activity was normalized to Renilla luciferase activity in all conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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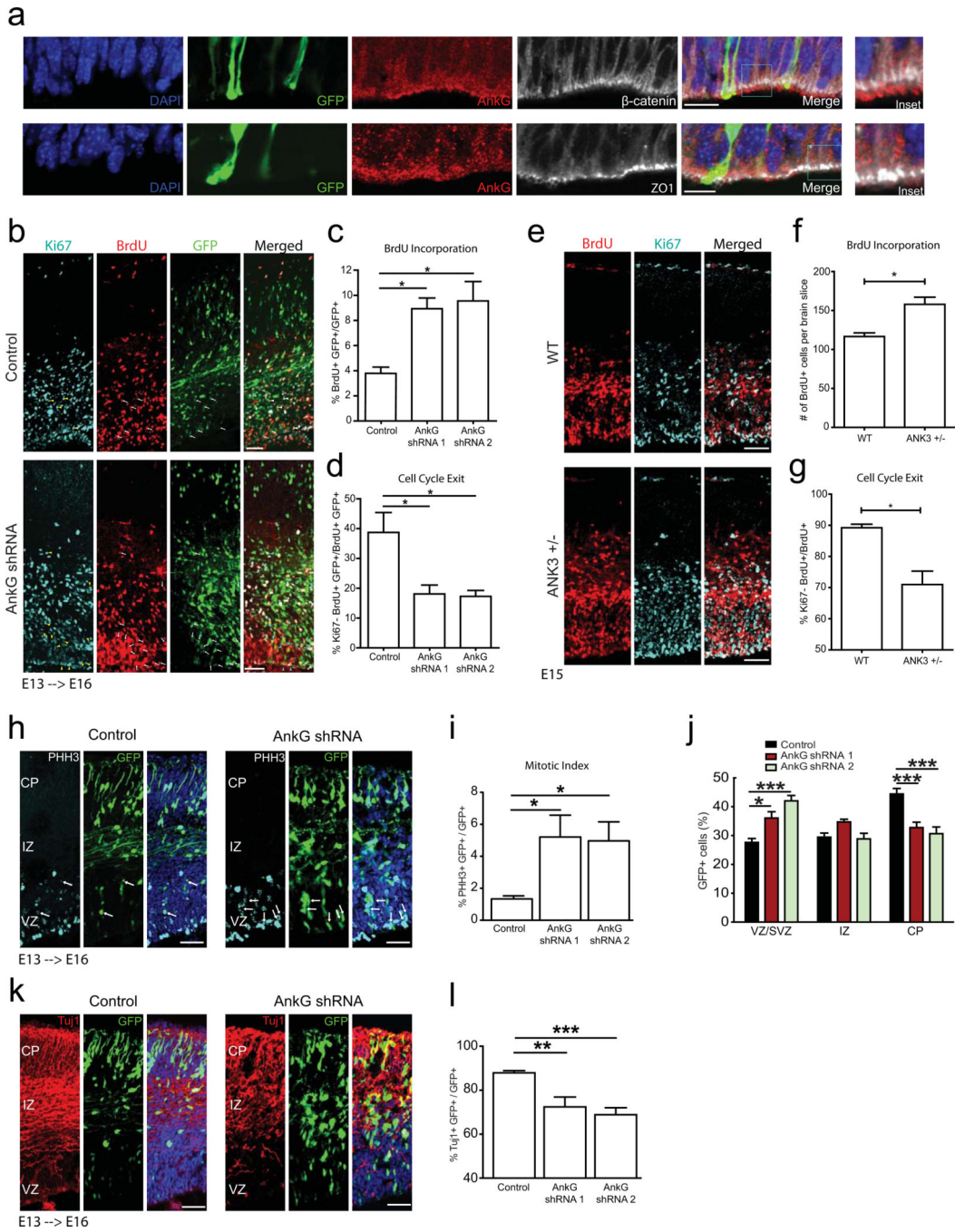


Figure 1. Ankyrin-G regulates progenitor cell proliferation in developing cortex
a) Ankyrin-G (red) is highly expressed in the ventricular zone of developing cortex. **b)** Images of E16 mouse cortices electroporated at E13 with non-targeting (left panel, Control) and ankyrin-G-directed small hairpin (right panel, AnkG shRNA) and GFP expression plasmid. Single-pulse BrdU was injected 2 hour prior to brain dissection. Images were stained for GFP (green), BrdU (red) and Ki67 (white). Arrows indicate BrdU, GFP double-positive cells, and arrowheads indicate Ki67, BrdU, GFP triple positive cells. **c)** Ankyrin-G knockdown resulted in increased BrdU incorporation (Control, n=3; shRNA1 and shRNA2,

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n=4). **d**) Ankyrin-G knockdown decreased cell cycle exit (Control, n=3; shRNA1 and shRNA2, n=4). **e**) Images of E15 *Ank3* +/- brains stained for BrdU (green) and Ki67 (white). Images were taken by keeping the width of the images constant, and covering all the cortical layers (VZ, SVZ, IZ and CP). **f**) Number of BrdU-positive cells per brain slice is significantly increased in *Ank3* +/- animals compared to wild type littermates (WT, n=2; *Ank3* +/-; n=3). **g**) Cell cycle exit in the BrdU-positive population is decreased in *Ank3* +/- animals compared to wild type littermates (WT, n=2; *Ank3* +/-; n=3). **h**) Images of E16 mouse cortices electroporated at E13. Images were stained for GFP (middle images in each set), PHH3 (left images in each set) and DAPI. Arrows indicate GFP, PHH3 double-positive cells. **i**) Ankyrin-G knockdown increased mitotic index as measured by PHH3 staining (Control, n=4; shRNA1 and shRNA2, n=3). **j**) Distribution of GFP⁺ cells in different cortical zones 72 hours after transfection at E16. Consistent with proliferative effect of ankyrin-G, percentage of GFP⁺ cells increased in the VZ/SVZ after ankyrin-G knockdown (Control, n=7; shRNA1, n=5 and shRNA2, n=6). **k**) Images of E16 mouse cortices electroporated at E13. Images were stained for GFP (middle images in each set), Tuj1 (left images in each set) and DAPI. **l**) Fraction of GFP⁺ cells overlapping with Tuj1 staining is reduced after ankyrin-G knockdown (Control, n=7; shRNA1, n=5 and shRNA2, n=8). All analyses, one-way analysis of variance (one-way ANOVA) followed by Dunnett's Multiple Comparison Test, except panel (f) and (g) where Unpaired t Test is used; *, P<0.05; **, P<0.01; ***, P<0.001. Scale bar: 10µm (a), 50µm (b, e) and 100µm (h, k).

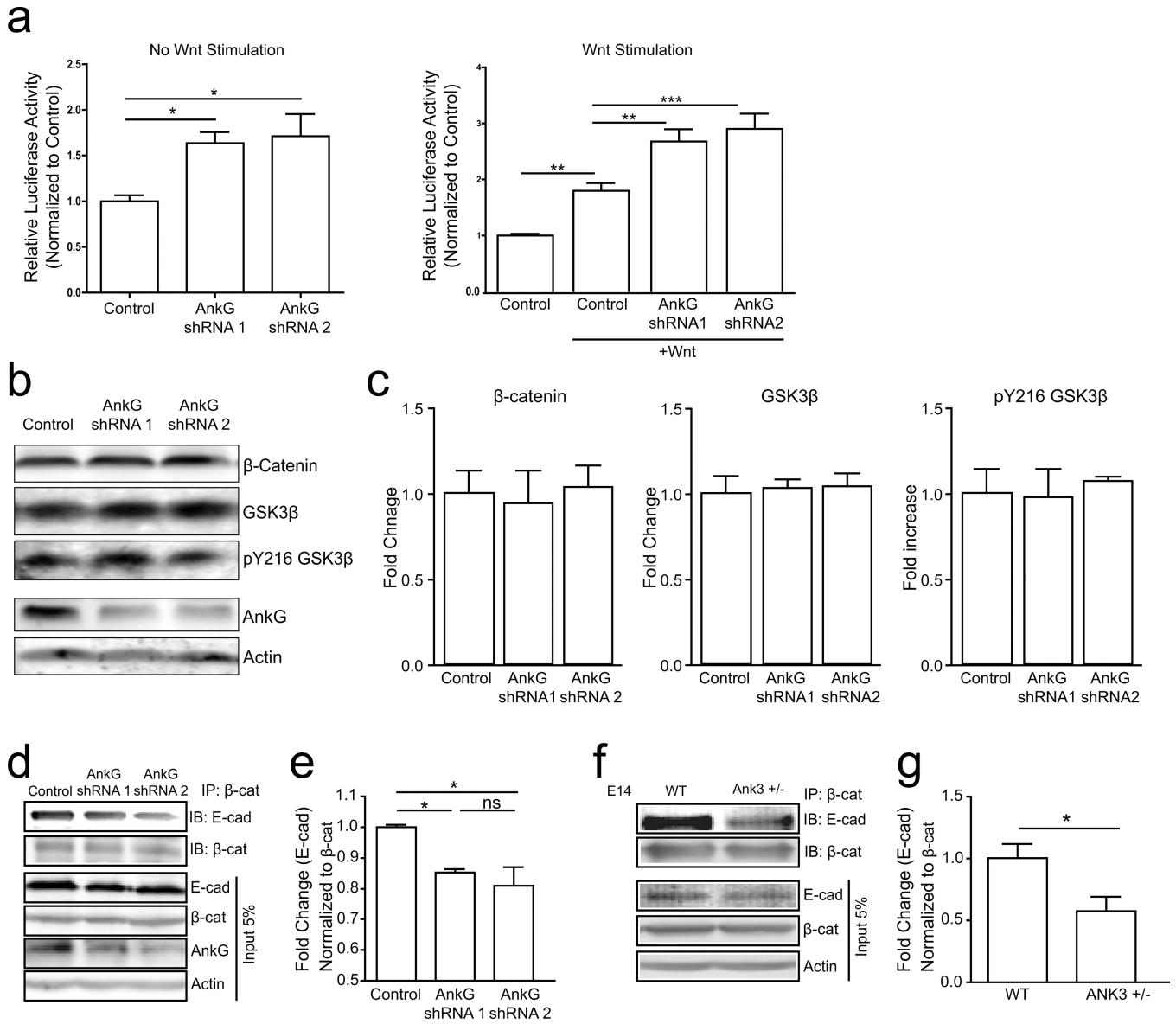


Figure 2. Ankyrin-G regulates Wnt signaling

a) Ankyrin-G negatively regulates canonical-Wnt signaling. Ankyrin-G knockdown resulted in significant increase in luciferase activity with (right) or without (left) Wnt3A stimulation (No Wnt stimulation; n=4, Wnt stimulation; n=8). **b)** Ankyrin-G knockdown does not alter expression of canonical-Wnt signaling proteins. Sample western blots shown for several components of canonical-Wnt signaling. **c)** Quantification of protein expression levels does not show significant difference after ankyrin-G knockdown compared to control, except ankyrin-G levels (please see Supplementary Figure 1 for ankyrin-G quantification) (n = 3, in all cases). **d)** Ankyrin-G knockdown reduces interaction between E-cadherin and β-catenin in P19 cells. Immunoprecipitation (IP) with β-catenin antibody followed by immunoblotting (IB) E-cadherin antibody. Input, 5% of the total protein used for immunoprecipitation **e)** Fold change of E-cadherin levels normalized to loading immunoprecipitated β-catenin (Control and shRNA2; n=5; shRNA1, n=3). **f)** Ankyrin-G knockdown reduces interaction

between E-cadherin and β -catenin in E14 *Ank3* +/- mice brain lysates. Immunoprecipitation (IP) with β -catenin antibody followed by immunoblotting (IB) E-cadherin antibody. Input, 5% of the total protein used for immunoprecipitation. **g**) Fold change of E-cadherin levels normalized to immunoprecipitated β -catenin (WT, n=7; *Ank3* +/-, n=6). All analyses, one-way analysis of variance (one-way ANOVA) followed by Dunnett's Multiple Comparison Test, except for panel (a) where Tukey's Multiple Comparison Test is used, and panel (g) where Unpaired t Test is used; *, P<0.05; **, P<0.01; ***, P<0.001.

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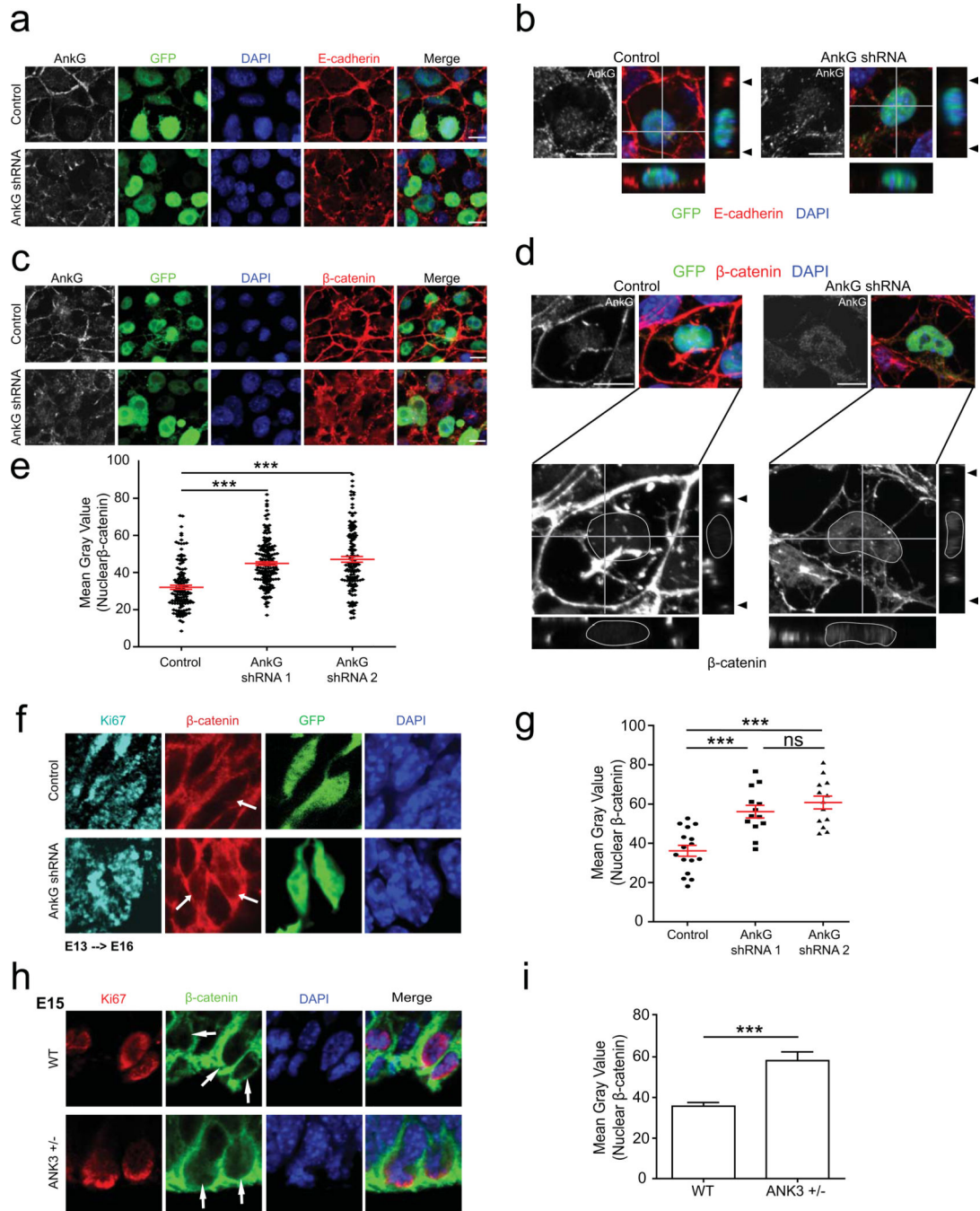


Figure 3. Ankyrin-G knockdown increased nuclear beta-catenin levels

a) Top panels are control cells transfected with non-targeting shRNA (Control) and lower panels are cells transfected with ankyrin-G targeting shRNA (AnkG shRNA). Ankyrin-G knockdown disrupts E-cadherin localization to cell membrane. P19 cells are stained for ankyrin-G (white), E-cadherin (red), GFP (green) and DAPI (blue). **b)** Orthogonal images of single cells showing that ankyrin-G knockdown disrupt E-cadherin localization to cell membrane. Arrows point to E-cadherin expression on the cell membrane. **c)** Ankyrin-G knockdown disrupts beta-catenin localization to cell membrane, and increases nuclear beta-catenin

levels. P19 cells are stained for ankyrin-G (white), β -catenin (red), GFP (green) and DAPI (blue). **d**) Orthogonal images of single cells showing that nuclear β -catenin levels are increased after ankyrin-G knockdown in P19 cells. Cell nuclei are circled in each image. Arrows point to β -catenin expression on the cell membrane. **e**) Quantification of nuclear β -catenin levels showing increased levels of β -catenin after ankyrin-G knockdown (Control, n=127; shRNA1, n=165; shRNA2, n=130; three different cultures in all cases). **f**) Ankyrin-G knockdown increases nuclear β -catenin levels in proliferating neural progenitors *in vivo*. Images of E16 mouse cortices electroporated at E13 with non-targeting (top panel, Control) and ankyrin-G-directed small hairpin (bottom panel, AnkG shRNA) and GFP expression plasmid. Images were stained for GFP (green), Ki67, cell cycle marker (white), β -catenin (red) and DAPI (blue). Arrows indicate Ki67, GFP double-positive cells. **g**) Quantification of nuclear β -catenin levels showing increased levels upon ankyrin-G knockdown compared to control (Control, n= 16; shRNA1 and shRNA2, n=13; two different animals per condition). **h**) Loss of ankyrin-G also results in increased nuclear β -catenin levels in proliferating neuroprogenitors *in vivo*. E15 brain slices from wild-type (top panels) and *Ank3* +/- animals are stained for Ki67 (red), β -catenin (green) and DAPI (blue). **i**) Quantification of nuclear β -catenin levels showing increased levels in *Ank3* +/- animals compared to wild-type littermates (WT, n= 16; *Ank3* +/-, n=22; two different animals per condition). All analyses, one-way analysis of variance (one-way ANOVA) followed by Dunnett's Multiple Comparison Test, except for panel (i) where Unpaired t Test is used; *, P<0.05; **, P<0.01; ***, P<0.001. Scale bar: 10 μ m

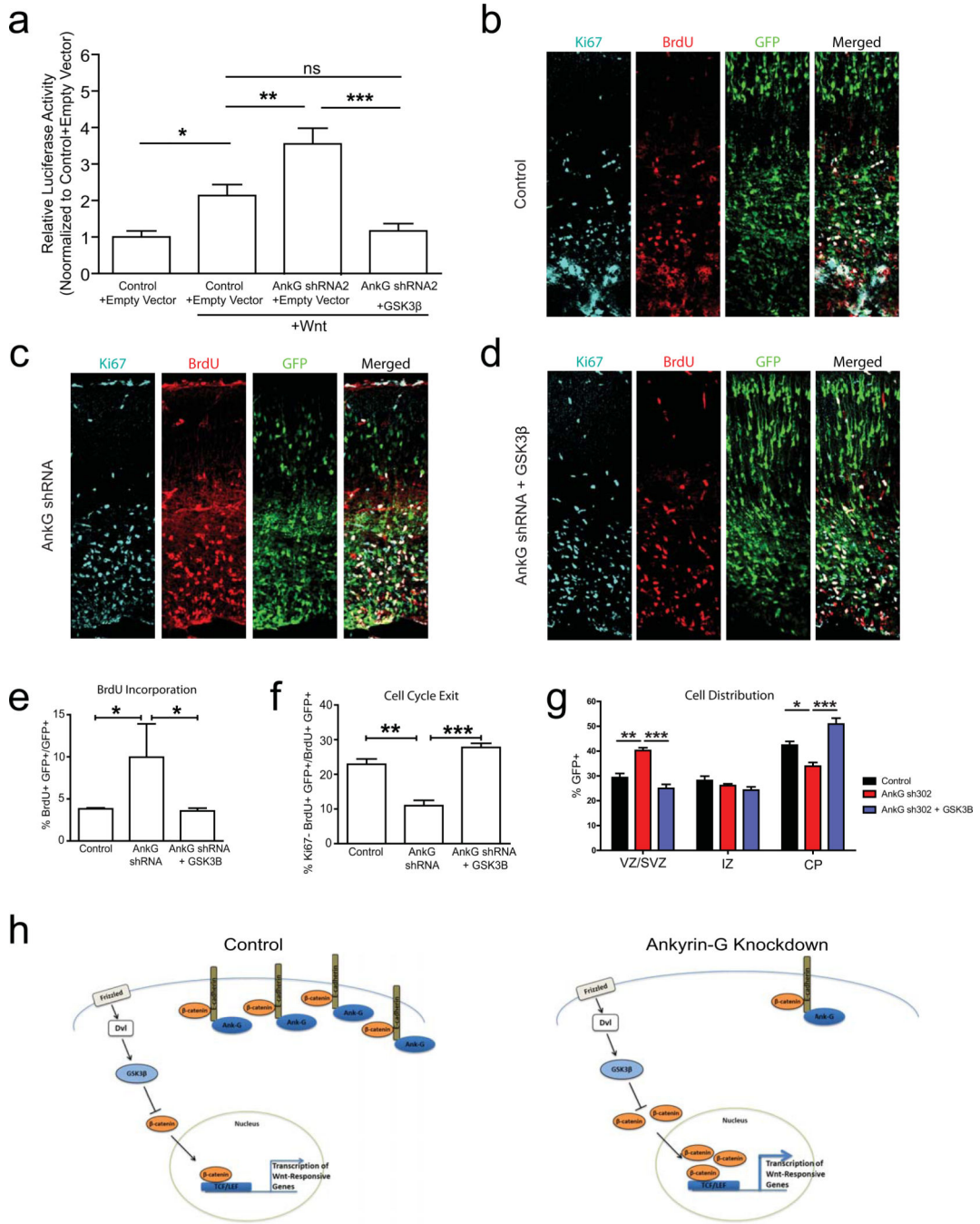


Figure 4. Dampening canonical Wnt-signaling rescues phenotypes associated with ankyrin-G knockdown

a GSK3β overexpression rescues increased Wnt-mediated luciferase activity (in all cases, n=4; Tukey’s Multiple Comparison test). **b–d** Images of E16 mouse cortices electroporated at E13 with non-targeting (**b**, Control) or ankyrin-G-directed small hairpin along with either empty vector (**c**, AnkG shRNA) or GSK3β overexpression construct (**d**, AnkG shRNA + GSK3β), and GFP expression plasmid. Images were stained for GFP (green), BrdU (red) and Ki67 (blue). **e** Increased BrdU incorporation associated with ankyrin-G knockdown is

reduced to control levels when ankyrin-G shRNA is co-expressed with GSK3 β (Control + Empty Vector, n=4; AnkG shRNA + Empty Vector, n=2; AnkG shRNA+ GSK3 β , n=4; Tukey's Multiple Comparison Test). **f**) Co-expression of GSK3 β with ankyrin-G shRNA rescues the cell cycle exit phenotype assayed using Ki67 proliferative marker (Control + Empty Vector, n=4; AnkG shRNA + Empty Vector, n=2; AnkG shRNA+ GSK3 β , n=4; Tukey's Multiple Comparison Test). **g**) Distribution of GFP⁺ cells in different cortical zones 72 hours after transfection at E16. Co-expression of GSK3 β with ankyrin-G shRNA reduced the percentage of GFP⁺ cells in the VZ compared to ankyrin-G knockdown condition (Control + Empty Vector, n=4; AnkG shRNA + Empty Vector, n=2; AnkG shRNA+ GSK3 β , n=4; Tukey's Multiple Comparison Test). **h**) Working model for ankyrin-G function during cortical development. Left, in control condition ankyrin-G localizes E-cadherin to plasma membrane where it participates in cadherin-catenin complex. Right, in ankyrin-G knockdown condition E-cadherin localized to plasma membrane is reduced, which in turn results in increased free β -catenin available for Wnt signaling. Upregulation of Wnt signaling results in increased neural progenitor proliferation in developing cortex. *, P<0.05; **, P<0.01; ***, P<0.001. Scale bar: 100 μ m