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Focal Reduction of αE-catenin Causes Premature Differentiation and Reduction of β-catenin Signaling During Cortical Development

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Abstract

Cerebral cortical precursor cells reside in a neuroepithelial cell layer that regulates their proliferation and differentiation. Global disruptions in epithelial architecture induced by loss of the adherens junction component αE-catenin lead to hyperproliferation. Here we show that cell autonomous reduction of αE-catenin in the background of normal precursors in vivo causes cells to prematurely exit the cell cycle, differentiate into neurons, and migrate to the cortical plate, while normal neighboring precursors are unaffected. Mechanistically, αE-catenin likely regulates cortical precursor differentiation by maintaining β-catenin signaling, as reduction of αE-catenin leads to reduction of β-catenin signaling in vivo. These results demonstrate that, at the cellular level, αE-catenin serves to maintain precursors in the proliferative ventricular zone, and suggest an unexpected function for αE-catenin in preserving β-catenin signaling during cortical development.

Keywords

Cerebral cortex; Ventricular zone; Precursors; Neuroepithelium; Cell fate; Alpha Catenin; Beta Catenin; Wnt

Introduction

During embryonic development, polarized multipotent neural precursor cells reside in a proliferative epithelium called the ventricular zone (VZ). The precursors in the VZ are polarized neuroepithelial cells, with distinct apical and basolateral domains (Hinds and Ruffett, 1971; Shoukimas and Hinds, 1978). Apical processes extend to contact the ventricular surface where they expand slightly to form apical endfeet joined to each other by adherens junctions (Hinds and Ruffett, 1971; Shoukimas and Hinds, 1978). These apical processes are enriched for a number of molecules involved in adhesion including β-catenin, N-cadherin, pp120, paxillin (Chenn et al., 1998) and αE-catenin (Stocker and Chenn, 2006).

Although recent studies support the idea that adherens junction components play vital roles in regulating tissue organization, interpretation of tissue-wide loss of cell adhesion in the developing cortex is complicated by the concurrent loss of epithelial architecture which is

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sufficient to significantly alter cell proliferation (Bilder et al., 2000). Conditional loss of N-cadherin from the developing cortex caused architectural disruptions and massive cortical disorganization (Kadowaki et al., 2007). Similarly, tissue-wide conditional deletion of β-catenin also caused loss of epithelial integrity, delamination, and apoptosis of neuroepithelial precursors (Junghans et al., 2005). Tissue-wide elimination of αE-catenin also led to a massive disorganization of the cortical neuroepithelium and hyperproliferation of cortical progenitors (Lien et al., 2006).

In the developing cortex, overexpression of β-catenin caused decreased cell cycle exit and overproduction of precursors (Chenn and Walsh, 2002). Experiments reducing β-catenin signaling without overt disruption of epithelial architecture supported the idea that the cell-autonomous signaling function of β-catenin regulated neural precursor proliferation (Woodhead et al., 2006). Although these experiments suggested the importance of the signaling function of β-catenin in neural development, it remains unclear if β-catenin at cell junctions impacted neural development.

Whether cell adhesion might regulate β-catenin signaling *in vivo* remains poorly understood. Observations that β-catenin is both an adherens junction molecule that binds cadherin and α-catenin as well as the key mediator of the canonical Wnt signaling pathway led to suggestions that β-catenin activity may be responsive to cell adhesion (Nelson and Nusse, 2004). Overexpression of cadherins (Gottardi et al., 2001; Zhu and Watt, 1996) or α-catenin (Giannini et al., 2000) has been demonstrated to reduce β-catenin-mediated transcriptional activation, while knockdown experiments in culture have suggested that α-catenin may regulate β-catenin signaling by preventing the formation of a β-catenin-TCF-DNA complex (Giannini et al., 2000). However, the regulation of β-catenin activity in tissue is likely more complex. While increases in expression of several Hedgehog-pathway genes was observed following tissue-wide loss of αE-catenin in developing cortex (Lien et al., 2006), no apparent changes were noted in β-catenin signaling (Lien et al., 2008). Because of potential secondary effects caused by tissue disruption and subsequent disregulation of multiple signaling pathways, we sought to examine cell-autonomous function of αE-catenin in cortical development in vivo in the background of normal tissue architecture.

Materials and Methods

Animals

All mice (*Mus musculus*) were treated according to protocols reviewed and approved by the IACUC of Northwestern University. Timed pregnant mice were obtained from Charles River Laboratories (Wilmington, MA). Homozygous floxed αE-catenin mice (containing loxP sites flanking exon 2 of αE-catenin; *Ctnna1*$^{\text{lox(Ex2)}}$) were acquired from E. Fuchs (Rockefeller University). Transgenic mice expressing Cre-recombinase driven by a nestin enhancer element were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were genotyped according to The Jackson Laboratory protocols available at http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=697.

DNA and shRNA constructs

pCAG-IRES2-GFP, pCAG-eGFP, pCAG-mCherry, pTOP-dGFP, and pCAG-Cre-IRES2 are as described in (Woodhead et al., 2006). Additionally, a pCAG-Cre construct lacking GFP was utilized in conjunction with the Wnt signaling reporter construct pTOP-dGFP. A stabilized β-catenin construct that has been NH_2-terminally truncated to eliminate several GSK3β phosphorylation sites, one containing a fused GFP tag: pCAG-Δ90-β-catenin-GFP and another without: pCAG-Δ90-β-catenin, were used to rescue β-catenin signaling. Short-hairpin RNAi constructs targeting αE-catenin as well as control shRNA constructs were purchased from Open

Biosystems (Huntsville, AL) (pSHAGM2-eGFP, accession number: pEGFP U76561; pSHAGM2-NonSilencing; pSHAGM2-αEcatenin-2010-E-4 clone ID: V2MM_67879, accession number: NM_009818, anti-sense sequence: TTTATCAAAGTTGTTGAGAGCG; pSHAGM2-aEcatenin-2697-H-3 clone ID: V2MM_67396, accession number: NM_009818, anti-sense sequence: ATTGTTGGAGATTGAACAGGCC; pSHAGM2-aEcatenin-2226-B-3 clone ID: V2HS_165579, accession number: NM_009818, anti-sense sequence: TTACTATTGGTGTTTACCAGGG).

In utero electroporation

For in utero injection, timed-pregnant mice at embryonic day 13.5 (E13.5) were electroporated as described in (Woodhead et al., 2006). For the studies, 0.75 μg/μl DNA was used for the pCAG-GFP, pCAG-mCherry, pCAG-Cre-IRES2-GFP, pTOP-dGFP, pCAG-Cre, pSHAGM2-eGFP, pSHAGM2-NonSilencing, pCAG-Δ90-β-catenin-GFP, or pSHAGM2-αEcatenin-2010-E-4 plasmids. Rescue experiments contained 0.75 μg/μl DNA of both pCAG-Δ90-β-catenin-GFP and pSHAGM2-αEcatenin-2010-E-4, or of both pCAG-Cre-IRES2-GFP and pCAG-Δ90-β-catenin. All shRNA constructs were also co-electroporated with 0.25 μg/μl DNA of pCAG-GFP or pCAG-mCherry.

Primary cortical culture

Primary cortical cultures were generated from E13.5 embryos using minor modifications to the method described by (Murphy et al., 1989). Dorsolateral corticies were isolated from E13.5 embryos by dissection. After dissection, cells were disassociated with 0.25% trypsin and washed initially in complete media then in DMEM (Invitrogen) with 2 mM L-glutamine, 1 mM *N*-acetyl-cysteine, 1 mM sodium pyruvate, and B27 and N2 supplement (final 1x), with 25 ng/ml FGF2. Isolated cells were nucleofected immediately with constructs of interest, then plated on to poly-D-lysine-treated tissue culture plates. Cells were nucleofected using the AMAXA nucleofection kit following the manufacturer's protocol at an efficiency of approximately 50% (AMAXA Biosystems, Gaithersburg, MD). Treated cells were incubated at 37°C, 5% CO_2 in the described media.

Cortical disassociation studies

Ctnna1$^{lox(Ex2)}$ mice were crossed to create timed pregnant females with all embryos carrying homozygous floxed αE-catenin alleles. Embryos were co-electroporated with an internal control expression plasmid (mCherry) along with our GFP-expressing Cre-recombinase construct (pCAG-Cre-IRES2-GFP). Forty-eight hours after treatment dorsolateral corticies were isolated by dissection and briefly disassociated with 0.25% trypsin. Following disassociation the cells were plated onto poly-D-lysine treated coverslips at a density of 5×10^5 cells per well in complete DMEM. Cortical cells were incubated at 37°C for 2 hours, then fixed with 4% paraformaldehyde for 10 minutes at room temperature, and were subsequently stained for GFP and αE-catenin. Immunofluorescent intensity for αE-catenin was quantified for disassociated electroporated cells using ImageJ software (http://rsb.info.nih.gov/ij/) to calculate the values from images taken using a Zeiss UV LSM510 confocal microscope (Oberkochen, Germany).

In utero electroporation internal control and analysis

Internal control analysis was performed as in (Woodhead et al., 2006). In the homozygous floxed animals (*Ctnna1*$^{lox(Ex2)}$), we co-electroporated an internal control expression plasmid (mCherry) along with our GFP-expressing experimental construct (pCAG-Cre-IRES2-GFP) and compare the distribution and fate of cells expressing the GFP-tagged construct (any GFP^+ cell) with control cells expressing only mCherry (Fig. S1). In this way, experimental conditions (target tissue, surgical variability, electroporation location and effectiveness,

timing, etc.) could be compared between cells expressing the experimental construct versus only the control mCherry.

Immunohistochemistry

Immunohistochemistry was performed as described in (Woodhead et al., 2006). Primary antibodies used were anti-GFP rabbit polyclonal antibody (1:1000; Invitrogen, Carlsbad, CA), anti-GFP chicken polyclonal antibody (1:1000; Abcam, Cambridge, MA), anti-dsRed rabbit polyclonal antibody (1:1000; Clontech, Mountain View, CA), anti-αE-catenin rabbit polyclonal antibody (1:200, Abcam), anti-Pax6 mouse monoclonal antibody (1:400; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-Tbr2 rabbit polyclonal antibody (1:500; Abcam), anti-Tbr1 rabbit polyclonal antibody (1:1000; Chemicon, Temecula, CA), anti-TuJ1 rabbit polyclonal antibody (1:500; Covance, Princeton, NJ), anti-actin mouse monoclonal antibody (1:500; Chemicon), anti-activated caspase-3 rabbit polyclonal antibody (1:200; Cell Signaling, Beverly, MA), anti-5 bromo-2′-deoxyuridine (BrdU) rat monoclonal antibody (1:250; Serotec, Oxford, UK), anti-N-cadherin monoclonal antibody (1:250; BD Transduction Laboratories, San Jose, CA), anti-β-catenin monoclonal antibody (1:250, BD Transduction Laboratories), anti-Phalloidin polyclonal antibody directly conjugated to alexa 546 (1:500; Invitrogen), and anti-Ki67 rabbit polyclonal antibody (1:500; Novocastra Laboratories, Newcastle, UK). Secondary antibodies used were Alexa 488-goat anti-rabbit IgG antibody (1:500; Invitrogen), Alexa 488- goat anti-chicken IgG antibody (1:500; Invitrogen), Alexa 555- goat anti-rabbit IgG antibody (1:500; Invitrogen), Alexa 555- goat anti-mouse IgG antibody (1:500; Invitrogen), Alexa 555- goat anti-rat IgG antibody (1:500; Invitrogen), Alexa 647-goat anti-mouse IgG antibody (1:500; Invitrogen), Alexa 647- goat anti-rabbit IgG antibody (1:500; Invitrogen), HRP-conjugated goat anti-rabbit IgG antibody (BioRad, Hercules, CA), and HRP-conjugated goat anti-mouse IgG antibody (BioRad). Immunofluorescent images were captured using a Zeiss UV LSM510 confocal microscope.

Western analysis

Cell lysates were collected from cortical cultures 48 hours after plating/nucleofection and boiled in sample buffer containing 2-mercaptoethanol. Proteins were separated on 6.5% acrylamide gels by electrophoresis then transferred onto Immobilon membranes (Millipore, Billerica, MA). Blots were blocked with 5% non-fat milk in Tris-buffered saline containing Tween-20 (TBS-T) then probed with antibodies of interest in block solution overnight at 4°C. Following primary treatment, blots were washed with TBS-T then incubated with secondary anti-bodies in TBS-T for an hour. Signals were detected with the ECL Western blotting detection system (Amersham Pharmacia Biotech, Chalfont St Giles, UK).

Cell-cycle exit analysis

Swiss-Webster E13.5 embryos were electroporated with pCAG-GFP alone or pCAG-GFP (0.25 μg/μl) and pSHAGM2-αEcatenin-2010-E-4 (0.75 μg/μl) plasmids, and homozygous floxed αE-catenin (*Ctnna1*$^{lox(Ex2)}$) E13.5 embryos were electroporated with pCAG-Cre-IRES2-GFP or pCAG-GFP only. Dams were injected intraperitoneally with BrdU (50 mg/kg body weight) at E14.5, and 24 h after BrdU injection, embryo brains were fixed and processed for BrdU, Ki67, and GFP expression. To determine the fraction of cells that had exited the cell cycle within comparable electroporated sections, GFP and BrdU double-labeled cells were counted first, and then Ki67 expression was determined as either positive or negative. Cell-cycle exit index was calculated as the fraction of BrdU pulse labeled, electroporated cells that exited the cell cycle (GFP^+, $BrdU^+$, and $Ki67^-$), over the total number of electroporated cells that had been pulse labeled with BrdU (GFP^+ and $BrdU^+$) (Chenn and Walsh, 2002).

Statistical Analysis

All statistical analysis was performed using the SPSS 16.0 program (SPSS Inc, Chicago, IL).

Results

Focal elimination of αE-catenin causes cells to prematurely exit the ventricular zone

Conditional loss of αE-catenin in neural precursors during embryonic cortical development resulted in severely disrupted cortical organization and brain enlargement (Fig. 1), in agreement with earlier findings (Lien et al., 2006). While observations that proliferation appeared misregulated following tissue-wide αE-catenin loss (Lien et al., 2006) lent further support to the importance of epithelial architecture in regulating cell proliferation (Bilder et al., 2000), the cell-autonomous function of αE-catenin during neural differentiation remained less clear.

To examine the cell-autonomous function of αE-catenin in cortical precursor development, we used two independent approaches to generate focal loss of function regions within the context of an otherwise normal neocortex using *in utero* electroporation. First, we introduced shRNA against αE-catenin into embryonic day 13.5 (E13.5) cortical precursors. Transfection of primary cortical precursors confirmed that the shRNA construct utilized was able to effectively reduce αE-catenin protein levels (Fig. S2). In a complementary approach, we introduced a Cre-recombinase expression plasmid into homozygous floxed αEcatenin (*Ctnna1*$^{lox(Ex2)}$) embryos by in utero electroporation. Analysis 48 hours after manipulation of neocortical cells isolated from embryos electroporated with Cre-recombinase (pCAG-Cre-IRES2-GFP) and a mCherry internal control (pCAG-mCherry), revealed that protein levels of αE-catenin were significantly reduced in Cre-expressing cells (Fig. 2).

In vivo, focal reduction of αE-catenin with both approaches decreased the proportion of electroporated cells in the VZ and increased the proportion in the CP versus control cells (Fig. 3). Furthermore, analysis of internal control cells showed that neighboring cells targeted by electroporation but did not receive Cre-recombinase (i.e. expressing only the mCherry construct) have a distribution of cells within the cortex similar to other control electroporation approaches. Together, the observations that internal control cells are indistinguishable from all other control cells suggest that the observed effects are cell-autonomous (Fig. S1). Control animals for shRNA comparison were electroporated with fluorescent protein constructs alone or with shRNA specific controls (shRNA targeting GFP or non-silencing shRNA) into Swiss-Webster embryos. No differences in the cortical distribution of electroporated cells were observed between the control treatments in Swiss-Webster mice (Fig. S3). Homozygous floxed αE-catenin mice (*Ctnna1*$^{lox(Ex2)}$) or wild-type mice of the same strain were electroporated with fluorescent protein constructs only or Cre-recombinase, respectively, for control animals to compare with Cre experimental manipulations. There were no differences in the cortical distribution of electroporated cells between the control treatments for the floxed αE-catenin mice (Fig. S3). These observations suggest that reduction of αE-catenin in a cell-autonomous manner causes cells to prematurely exit the VZ.

Loss of αE-catenin increases neuronal differentiation

In the developing cortex, migration from the VZ is closely coupled with cell cycle exit and neuronal differentiation (Takahashi et al., 1996). To determine whether precursors differentiated into neurons following reduction of αE-catenin, we examined the proportion of electroporated cells that expressed a transcription factor found in postmitotic neurons (Tbr1) (Hevner et al., 2001). Both αE-catenin loss-of-function treatments showed a significant increase in the proportion of electroporated cells expressing Tbr1 (4.7%, Cre compared to 1.3% for respective controls; and 16.6% shRNA compared to 3.4% for controls) (Fig. 4). Additionally in the Cre-mediated loss of function treatment, we also investigated the proportion

of electroporated cells that expressed the early pan-neuronal marker class III β-tubulin (TuJ1) (Joshi and Cleveland, 1989) We observed a significant increase in the proportion of targeted cells expressing TuJ1 in the loss of function treatment (80.7% Cre, vs. 54.2% control) (Fig. 4). Together, these studies suggest that reduction of αE-catenin in a cell-autonomous manner increases the proportion of cells that exit the VZ and differentiate into neurons.

Reduction in αE-catenin causes increased cell cycle exit

To determine whether the increased proportion of neurons generated following loss of αE-catenin function resulted from a shift in the number of precursors that differentiate instead of remaining as proliferative neural progenitors, we examined cell-cycle exit and re-entry by quantifying the fraction of electroporated cells that continued to divide 24 hours after BrdU pulse labeling (Fig. 5). Both αE-catenin loss of function approaches revealed that reduction of αE-catenin led to increased cell-cycle exit compared to their respective controls (62.3% shRNA vs. 39.6% control; and 60.9% Cre vs. 34.6% control) (Fig. 5). Thus, the increased neuronal differentiation that follows the reduction in αE-catenin appears to be directly related to increased cell-cycle exit and not due to an additional effect on neuronal differentiation.

A preferential change in the frequency of apoptosis, which occurs during normal cortical development (Kuan et al., 2000), could be partially responsible for the alterations caused by elimination of αE-catenin. To investigate this potential change, we examined apoptotic cell death by comparing the expression of an activated form of caspase-3. Cleaved caspase-3 plays a crucial role in the most distal effector pathway in apoptosis (Nicholson et al., 1995). Activated caspase-3 positive cells were confirmed by visualization of condensed nuclei labeled with the DNA binding dye Hoechst 33342. Analysis revealed that elimination of αE-catenin via either approach did not significantly change cell death, both within electroporated regions as well as in the surrounding tissue compared to controls (compiled from all respective controls) (Fig. 6). Together, these findings suggest that cell-autonomous loss of αE-catenin causes cortical precursors to exit the cell cycle and differentiate into neurons.

Reduction of αE-catenin causes a reduction in β-catenin signaling

How does reduction of αE-catenin lead to premature cell cycle exit and neuronal differentiation? Prior studies have suggested that β-catenin signaling also functions in a cell-autonomous manner in cortical precursors to maintain precursors in the cell cycle (Woodhead et al., 2006). Because αE-catenin binds directly to β-catenin during cadherin mediated adhesion (Drees et al., 2005; Yamada et al., 2005), it has been hypothesized that α-catenin may be able to influence canonical Wnt signaling by sequestering β-catenin to the adherens junction (Nelson and Nusse, 2004). Although tissue-wide loss of αE-catenin apparently resulted in no apparent change in β-catenin-mediated signaling (Lien et al., 2008), our observations that focal reduction of αE-catenin mimics the phenotype of focal reduction in β-catenin signaling (Woodhead et al., 2006) led us to examine whether focal reduction of αE-catenin without global tissue disruption might influence β-catenin signaling.

To examine the role of αE-catenin on the transcriptional activity of β-catenin in the developing VZ, we utilized a reporter construct that expresses a destabilized GFP variant under the control of a β-catenin responsive promoter (pTOP-dGFP) (Dorsky et al., 2002). Constructs mediating loss of αE-catenin function were co-electroporated with the TOPdGFP reporter (the Cre-recombinase construct used did not contain a GFP signal). Beta-catenin transcriptional activity during neocortical development is most prominent in the VZ (Woodhead et al., 2006), as such analysis was undertaken 24 hours after electroporation at E13.5. Once again, the respective controls did not differ from one another and are presented as a single control group. We found that reduction of αE-catenin reduced the number of cells exhibiting β-catenin mediated Wnt

signaling as visualized by destabilized GFP expression from a TOP responsive promoter (Fig. 7, S4).

Rescue of β-catenin signaling following reduction of αE-catenin restores proliferation

The changes observed following the focal reduction of αE-catenin closely mirror findings from previous research where cell-autonomous reduction of β-catenin mediated signaling caused increased cell-cycle exit and premature neuronal differentiation (Woodhead et al., 2006). To examine whether the premature cell-cycle exit we observed following αE-catenin reduction was due to reduction of β-catenin signaling, we examined the consequences of restoring β-catenin signaling by introducing a stabilized form of the protein (Δ90-β-catenin) along side the reduction of αE-catenin. This construct has previously been shown to activate β-catenin mediated signaling and increase neural precursor cell-cycle re-entry *in vivo* (Chenn and Walsh, 2002). Following electroporation of only a control fluorescent construct or only stabilized β-catenin the proportion of targeted cells that expressed the proliferative marker Ki67 was similar (for WT Swiss-webster strain: 52.9% for controls and 49.9% for Δ90-β-catenin; for floxed αE-catenin mice: 63.5% for controls and 60.4% for Δ90-β-catenin). Consistent with our cell cycle exit analysis, reduction of αE-catenin by either approach decreased the proportion of electroporated cells that were proliferative (31.0% for shRNA; 32.2% for Cre). When both shRNA targeting αE-catenin and stabilized β-catenin were introduced into embryonic cortical tissue, the proportion of Ki67 positive cells was returned to control levels (47.7% for shRNA rescue) (Fig. 8, S5). Similarly, when both Cre-recombinase and stabilized β-catenin were introduced into *Ctnna1*$^{lox(Ex2)}$ embryo brains, we observed a partial rescue of the proliferative phenotype (46.0% for Cre rescue) (Fig. 8, S5). However, while the rescue approaches restored the number of cells in the VZ, neither rescue approach was able to return the cortical distribution of targeted cells back to control levels (Fig. S6). These findings suggest that TCF-mediated β-catenin signaling underlies the proliferative phenotype but is unlikely solely responsible for all of the changes observed following cell-autonomous loss of αE-catenin. Together, the presented data indicate that cell-autonomous loss of αE-catenin causes cortical progenitors to reduce β-catenin signaling, prematurely migrate from the VZ, exit the cell cycle, differentiate into neurons, and migrate to the developing cortical plate.

Discussion

Here we find that reduction in αE-catenin in neural precursors leads to premature cell-cycle exit, neuronal differentiation, and migration to the cortical plate. We also find that decreased αE-catenin leads to lowered endogenous levels of β-catenin signaling in VZ precursors. Moreover, the decline in proliferation seen following the reduction of αE-catenin can be rescued by restoring β-catenin signaling activity. Our findings differ dramatically from those observed when αE-catenin is deleted on a tissue-wide level from the entire neuroepithelium (Lien et al., 2006) (Fig. 1). Tissue-wide knockout of αE-catenin in the developing cerebral cortex leads to hyperproliferation (Lien et al., 2006). Although loss of epithelial organization following αE-catenin deletion apparently caused misregulation of Hedgehog signaling, a mechanistic link between αE-catenin and the hedgehog signaling pathway remains unclear. In contrast, here we find that in the context of a non-disrupted VZ, cell-autonomous reduction of αE-catenin caused premature exit from the ventricular neuroepithelium coupled with cell-cycle withdrawal and a reduction in β-catenin signaling. Our findings suggest that in the background of a normal tissue epithelium the cell-autonomous function of αE-catenin in cortical development is to retain these cells in the proliferative zone, likely through the maintenance of β-catenin signaling in neural precursors.

Although the differences between the previously described tissue-wide knockout of αE-catenin and the current findings described here are striking, there are several important differences

between the two approaches: 1) only a subset of cells is targeted by the cre/shRNA approach in otherwise normal tissue, 2) the cre/shRNA approach is unlikely completely effective in eliminating αE-catenin, and 3) the timing and spatial distribution of knockdown by the cre/shRNA approach is determined by the electroporation. The tissue-scale loss of function embryos began expression of Cre recombinase at embryonic day (E)11.5, while electroporated embryos began recombination/silencing at E13.5. The different developmental time frames could account for some of the variation between the two approaches if the role αE-catenin plays in neocortical development changes over the course of neurogenesis. Alternatively, the differences could be attributed to changes in β-catenin function that appear over the same developmental time frame (Hirabayashi et al., 2004). Finally, it is also possible that the phase of the cell cycle that αE-catenin is lost could yield disparate results, since in the conditional knockout, recombination likely occurs during all phases, while the electroporation targets cells in M phase (Navarro-Quiroga et al., 2007).

Furthermore, differences in the two approaches may avoid responses to tissue damage that might be activated in the genetic deletion of αE-catenin. Many signaling pathways and growth factor receptors are polarized to specific membrane domains (Chenn et al., 1998; Woods et al., 1997), and their mislocalization in disrupted tissue may affect signaling pathways that regulate controlled growth (Bilder et al., 2000). Tissue-wide knockout of αE-catenin appears to result in non-autonomous misregulation of unrelated signaling pathways, such as the increases in hedgehog target genes observed in the conditional knockout (Lien et al., 2006). Other compensatory responses have been described following global knockout in the developing cortex that can mask gene function; for example, while RNAi approaches revealed a key function of DCX in neuronal migration no apparent phenotype was observed in the developing cortex following global DCX knockout, suggestive of compensation following knockout (Bai et al., 2003). Future studies may lend insight into whether compensatory mechanisms normalize β-catenin signaling in the tissue-wide knockout.

The observation that αE-catenin binds directly to β-catenin at adherens junctions (Drees et al., 2005; Yamada et al., 2005) has raised the hypothesis that it might regulate canonical Wnt signaling by regulating β-catenin availability (Nelson and Nusse, 2004). While this proposed model provides an attractive link between adhesion and proliferation, the evidence connecting αE-catenin to Wnt signaling has only been demonstrated in vitro (Giannini et al., 2000; Hwang et al., 2005; Simcha et al., 1998). Furthermore, loss of αE-catenin on a tissue-wide scale in vivo resulted in changes in a variety of signaling pathways, but there was no apparent modification in Wnt signaling (Lien et al., 2006; Lien et al., 2008; Vasioukhin et al., 2001). Our data indicates that, in contrast to previously observed results, canonical Wnt signaling is actually reduced following the loss of αE-catenin (Fig. 7).

There are several reasons that may explain the disparity between our findings and previously published work that did not detect changes in β-catenin/TCF signaling. The sensitivity of the electroporated destabilized-GFP construct used here is likely more able to detect reductions of signaling on a cellular level than alternative approaches using the more stable β-galactosidase reporter previously described (Lien et al., 2008). Spatial and temporal gradients in β-catenin/Wnt signaling in the developing cortex (Machon et al., 2007) make evaluation of relative changes in signaling in the conditional knockout challenging because it is difficult to control the timing and location of knockout with the tissue analyzed. In contrast, the electroporation approach allows for 1) more precise targeting of the analysis of signaling following manipulation of gene expression and 2) examining cell-autonomous function by preserving tissue integrity with relatively low numbers of cells affected. Finally, in the conditional knockout model, widespread tissue disruption and concomitant alterations in other signaling pathways (Lien et al., 2006) might have obscured changes in β-catenin/TCF signaling.

Although the in utero electroporation approaches allowed us to examine cell-autonomous gene function in the developing cortex, it is clear from the tissue-wide knockout studies, that disrupting αE-catenin can lead to non-autonomous effects on the developing cortex (Lien et al., 2006) (Fig. 1). We did not observe any evidence of epithelial disruption in our focal knockdown/knockout studies, suggesting that the fraction of cells electroporated in each cortex was insufficient to lead to non-cell-autonomous effects on neighboring cells. Furthermore, we did not detect differences in cell behavior at the periphery of electroporated regions compared to the central focus. Analysis of internal controls ($mCherry^+$/Cre-electroporated cells in the same brains) showed that neighboring electroporated cells that did not receive the experimental construct behaved the same as cells in other control approaches, providing evidence that the observed effects of αE-catenin knockdown are cell-autonomous (Fig. S1).

Consistent with previously reported data suggesting delayed neuronal differentiation following β-catenin overexpression, increased β-catenin by electroporation at E13.5 led to had a slight increase in the proportion retained in the ventricular zone compared to controls (Fig. S6). However, unlike in transgenic mice overexpressing stabilized β-catenin from ~E10.5, we did not observe increased cell cycle re-entry in our acute E13.5 electroporations. This may represent either a ceiling to the effect on proliferation by β-catenin at this age or related to amount or duration of β-catenin activity. Moreover, rescuing β-catenin signaling following focal reduction of αE-catenin also did not seem to rescue the distribution of targeted cells (Fig. S6). This suggests that the physical retention of cells within the ventricular zone is complex and likely dependent upon both adhesion and β-catenin signaling.

The rescue experiments also suggest that while β-catenin can restore the proliferative reduction caused by αE-catenin reduction, the control of progenitor retention in the VZ by αE-catenin adhesion is more complex. While the early appearance of neurons in the cortical plate is consistent with accelerated migration, if the phenotype is due purely to accelerated migration, we would not expect the changes in cell cycle exit we observed. However, without doing live imaging, we cannot rule out increased rates of cortical neuronal migration that may also contribute to the changes in distribution of electroporated cells. Our findings suggest that reducing E-catenin reduces β-catenin signaling; αE-catenin also likely functions in physically retaining cells in the ventricular zone. β-catenin would not be expected to restore changes in adhesion. Part of the observed increased exit from the VZ noted following αE-catenin reduction may be secondary to reduction of physical adhesion to other progenitors. Addition of Δ90β-catenin does not decrease the number of cells migrating to the cortical plate; furthermore, we also note that the Δ90β-catenin combined with αE-catenin loss may increase the number of cells found in the cortical plate. This abnormal cellular distribution may be a result of decreased cell adhesion caused by Δ90β-catenin.

In some rescue brains (shRNA+Δ90-β-catenin), we observed epithelial disruption (Fig. S8), suggesting that the additive effect of α-catenin reduction and over-expression of Δ90-β-catenin caused perturbation in adhesion. This finding is consistent with previous observations that the Δ90-β-catenin construct alters cell adhesion in cell culture (Barth et al., 1997). Despite the alterations in architecture, expression of Δ90-β-catenin rescued the proliferative defect caused by αE-catenin knockdown, regardless of cell-polarity and tissue disruptions. Together, these findings suggest that reduction of β-catenin signaling function, rather than changes in adhesion or polarity, is the primary cause of the reduction in proliferation observed following focal αE-catenin knockdown/knockout.

Knockdown using shRNA approaches can have a variety of non-specific off-target effects that confound interpretation of data. While rescue experiments can help in demonstrating shRNA specificity, such experiments with αE-catenin are likely to be technically challenging. Overexpression of αE-catenin can inhibit canonical Wnt/β-catenin signaling (Giannini et al.,

2000; Simcha et al., 1998), and inhibition of Wnt/β-catenin signaling in the developing cortex leads to premature cell-cycle exit and neuronal differentiation (Woodhead et al., 2006). These findings suggest that a rescue experiment would have to precisely match the degree of rescue with knockdown. To complement the silencing approach, we chose to eliminate αE-catenin through the electroporation of a Cre-expressing plasmid into cortical precursors of floxed αE-catenin mice. A possible shortcoming of our two different approaches is that the mechanisms facilitating the elimination have different time courses and efficiencies. Furthermore, it is also possible that elimination of αE-catenin in either manipulation is incomplete and that some active residual protein may remain. While quantifying the extent of protein reduction in vivo is not technically possible using current methods, the similarity of the findings using the two distinct approaches reduces the potential concern of confounding off-target effects from methodology. Our observations that both approaches lead to reduction in β-catenin mediated Wnt signaling, premature departure from the ventricular zone, increased cell-cycle exit, cortical neuronal differentiation, and that proliferation in the ventricular zone can be restored by increasing β-catenin signaling provide support for the possibility that retention in the ventricular zone influences precursor self-renewal by maintaining β-catenin signaling.

Coordinate regulation of β-catenin signaling and cell adhesion

Neural precursors may require adherens junctions for physical attachment to the apical surface of the neuroepithelium. Severing of the apical process of cortical precursors causes them to translocate from the ventricular zone to the cortical plate (Miyata and Ogawa, 2007), raising the suggestion that physical tension underlies a mechanism to propel precursors from the ventricular zone. Loss of adherens junctions may thereby initiate migration from the ventricular zone during the process of neuronal differentiation. The molecular trigger for migration remains unknown, but our data suggest that reduction of β-catenin signaling is sufficient to lead to migration. Our experiments suggest an interplay between adhesion and maintenance of β-catenin signaling, as the increased cell-cycle exit and premature neuronal differentiation mirror experiments where β-catenin mediated signaling was suppressed cell-autonomously (Woodhead et al., 2006). Our findings that loss of αE-catenin reduces β-catenin signaling raise the possibility that cell-cell adhesion and β-catenin signaling might be coordinately regulated by αE-catenin.

Unfortunately the presented studies cannot delineate whether the observed effects are specifically through adhesion or through a novel function of αE-catenin, independent of cadherin-mediated adhesion. Thus, the molecular mechanism by which αE-catenin leads to reduction in β-catenin signaling in VZ precursors remains unclear. While αE-catenin might conceivably stabilize β-catenin from degradation, most existing evidence suggests that α-catenin bound β-catenin does not function in signaling (Gottardi and Gumbiner, 2004); indeed over-expression of α-catenin reduces β-catenin signaling in culture (Giannini et al., 2000; Hwang et al., 2005; Simcha et al., 1998). In the developing VZ, however, α-catenin adhesion might be necessary to preserve precise physical interactions between neural precursors necessary to transduce signaling pathways such as Wnt/β-catenin. Wnts are highly hydrophobic (Mikels and Nusse, 2006), and close cell apposition between signaling and responding cells may be necessary to maintain signaling in tissues. Further understanding of how cell adhesion can maintain Wnt/β-catenin signaling in tissues will lend insight into the mechanisms that regulate organ growth in development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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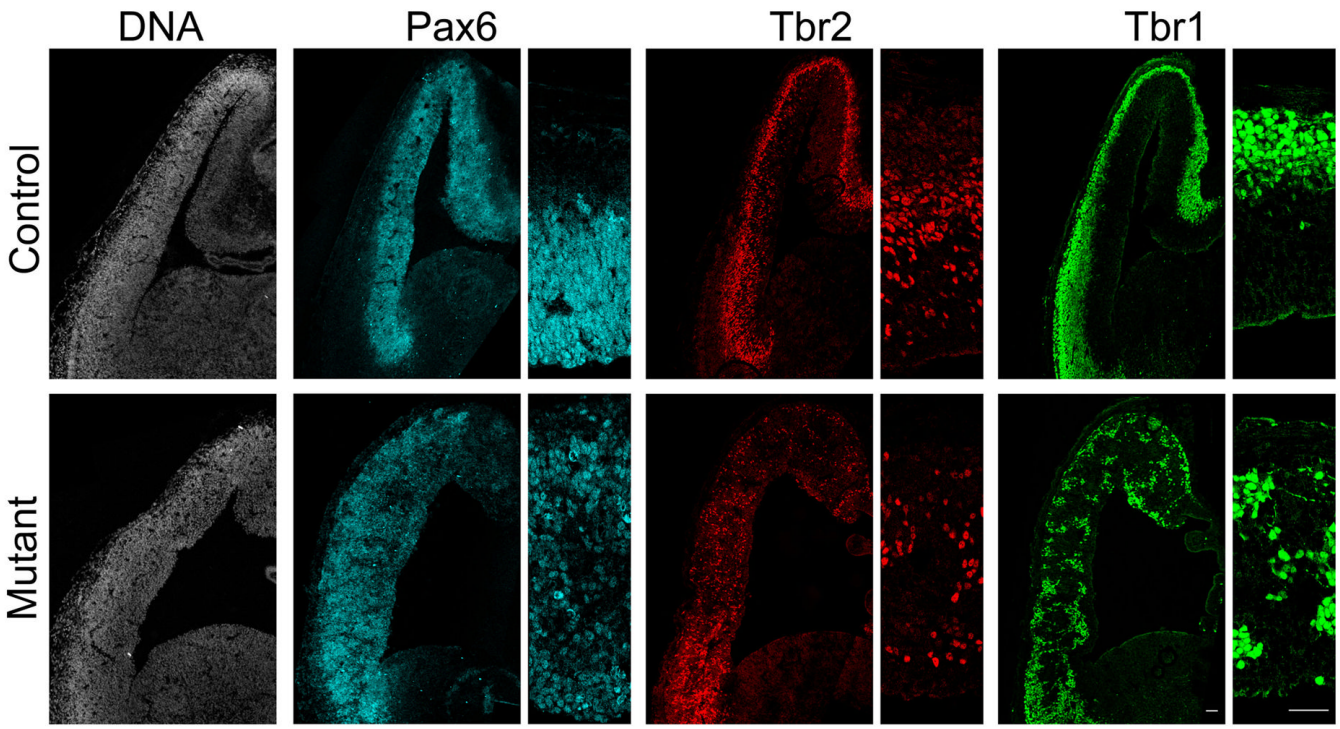

Figure 1. Tissue-wide loss of αE-catenin function in nervous tissue causes cortical disorganization

Homozygous floxed αE-catenin (*Ctnna1*$^{lox(Ex2)}$) embryos (embryonic day 13.5) expressing nestin driven Cre-recombinase (Mutant) and controls (heterozygotes expressing Cre and homozygotes lacking Cre expression) were immunostained for an apical progenitor marker (Pax6), a basal progenitor marker (Tbr2), and an early layer neuronal marker (Tbr1). Nuclei were labeled with Hoechst 33342. Bar: 50 μm.

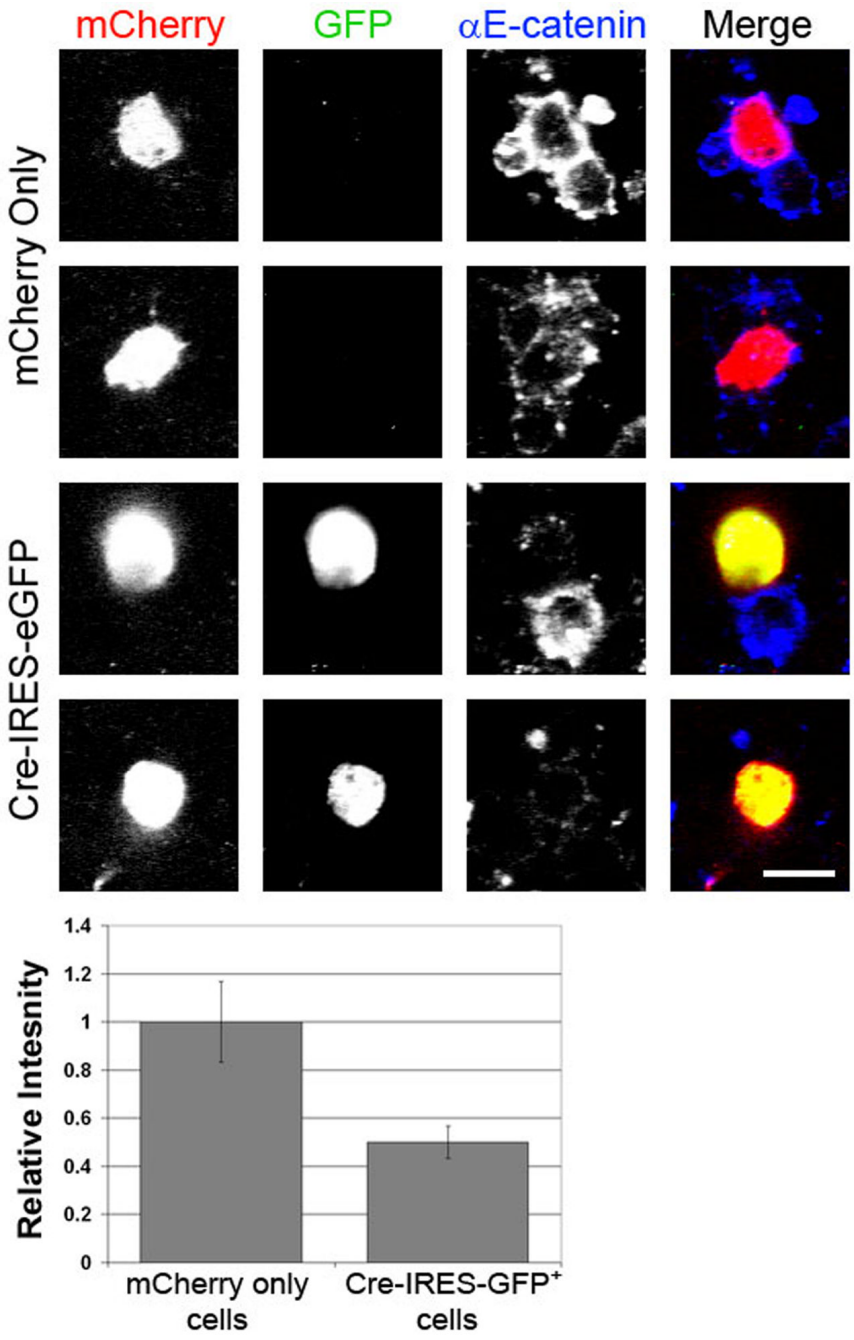

Figure 2. αE-catenin protein is reduced after Cre electroporation in floxed αE-catenin neocortical tissue

Floxed homozygous *Ctnna1*$^{lox(Ex2)}$ embryos were electroporated with Cre-recombinase (pCAG-Cre-IRES2-GFP) and a mCherry internal control (pCAG-mCherry) at E13.5 and analyzed 48 hours post surgery (E15.5). Disassociated cells plated onto poly-D-lysine treated coverslips were fixed, and subsequently stained for GFP and αE-catenin. The measured immunofluorescent intensity was compared between cells expressing Cre-recombinase (GFP$^+$ cells) and internal controls exclusively expressing mCherry (GFP$^-$, mCherry$^+$ cells) and demonstrated that protein levels of αE-catenin were significantly reduced in Cre expressing cells ($F_{[1,46]} = 7.46$, $p = 0.008$). Data presented as mean ± s.e.m.; Bar: 10 μm.

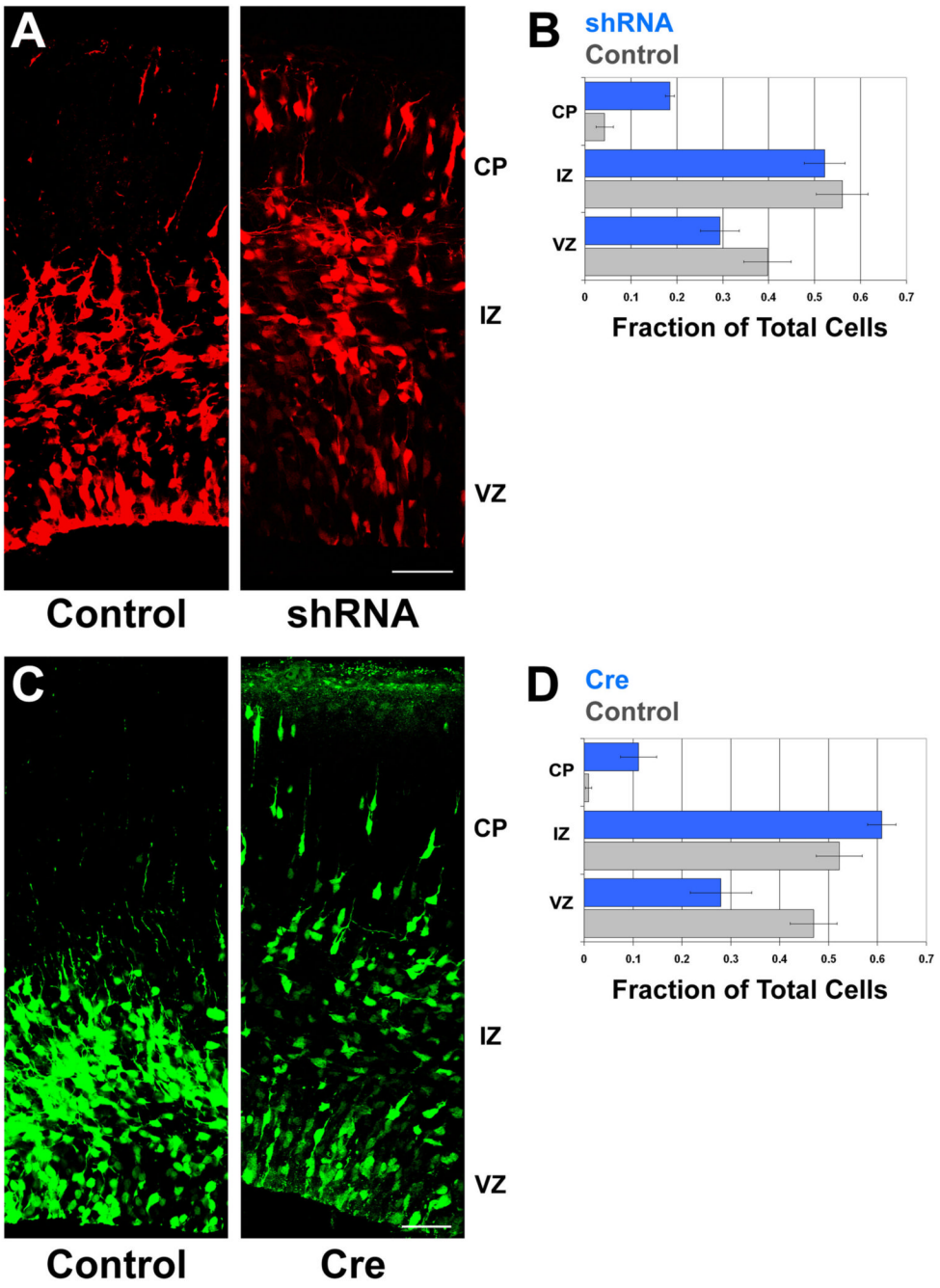

Figure 3. Focal elimination of αE-catenin causes cells to prematurely exit the ventricular zone

Both loss of function experimental treatments and their respective controls were electroporated at E13.5 and analyzed at E15.5. **A**) Left: Control treatment electroporated with pCAG-mCherry. Right: Experimental treatment co-electroporated with pCAG-mCherry and pSHAGM2-αEcatenin-2010-E-4. **B**) Distribution indexes for shRNA/control with data presented as the mean ± s.e.m.; shRNA v. control $\chi^2_{[2]} = 852$, $p < 0.001$ (control n = 6 brains, 2213 cells; shRNA n = 3 brains, 1757 cells). **C**) Left: Control treatment where homozygous floxed αE-catenin (*Ctnna1*$^{lox(Ex2)}$) brains were electroporated with pCAG-eGFP. Right: Experimental treatment where homozygous floxed brains were electroporated with pCAG-Cre-IRES-GFP. **D**) Distribution indexes for Cre/control with data presented as the mean ±

s.e.m.; Cre v. control $\chi^2_{[2]}$= 1620, $p < 0.001$ (control n = 5 brains, 2341 cells; Cre n = 4 brains, 1847 cells). Both loss of function treatments caused cells to exit the ventricular zone layer earlier than controls. CP denotes cortical plate, IZ designates intermediate zone, and VZ represents the ventricular zone. Bar: 50 μm.

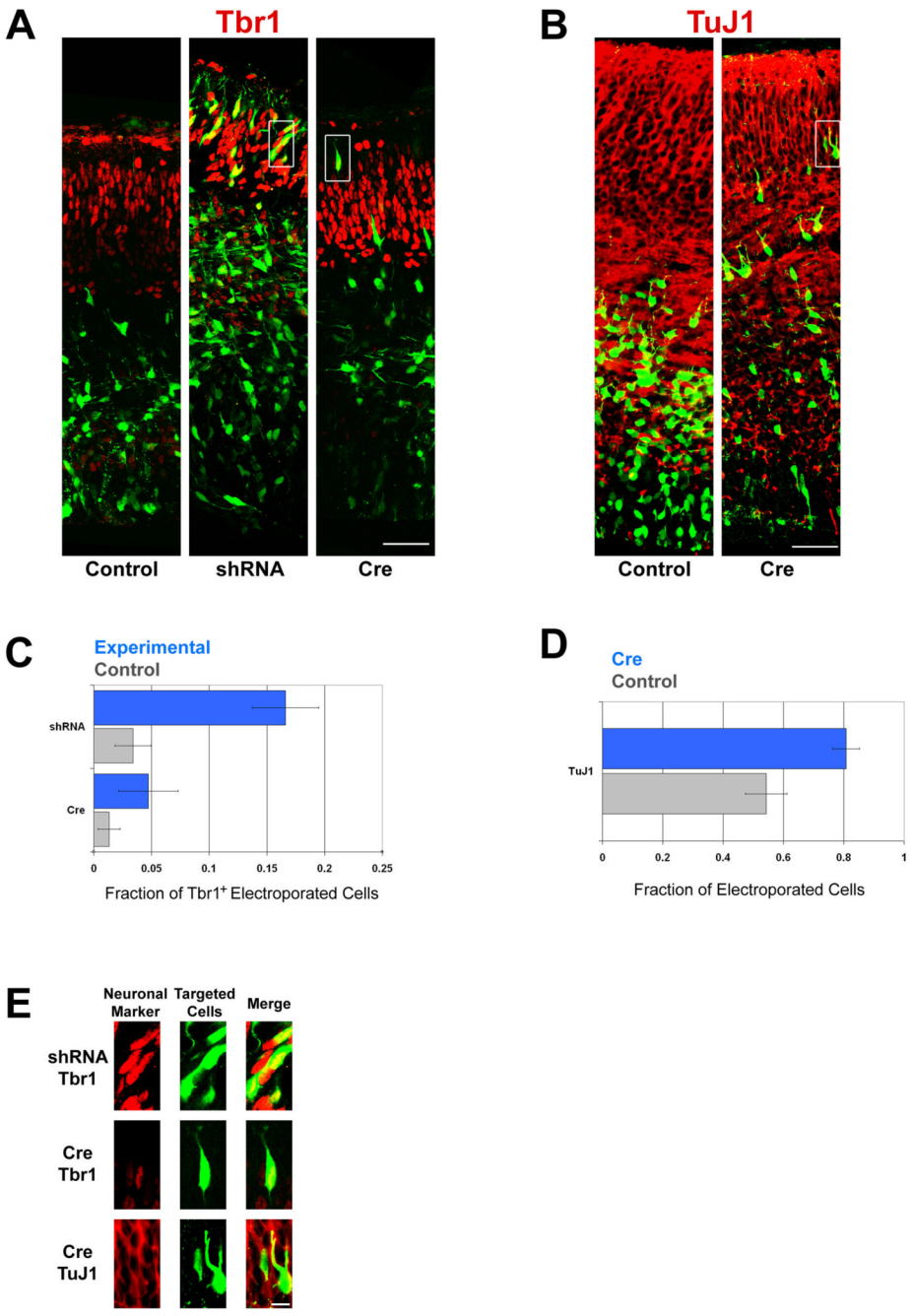

Figure 4. Focal elimination of αE-catenin increases neuronal differentiation

Electroporation of constructs into embryos was performed at E13.5 and subsequently analyzed at E15.5. Control: control treatment electroporated with pCAG-eGFP displayed, various shRNA and Cre controls were also performed in addition to fluorescent only electroporations into both strains. shRNA: experimental embryos electroporated with pCAG-eGFP and pSHAGM2-αEcatenin-2010-E-4. Cre: homozygous floxed αE-catenin embryos electroporated with pCAG-Cre-IRES-GFP. Experimental and control sections were stained for the early layer neuronal marker Tbr1 (**A**), or the pan-neuronal marker class III β-tubulin (TuJ1) (**B**), both shown in red (bar: 50 μm). Histograms displaying the proportion of precursors expressing the respective markers are presented as the mean ± s.e.m. for shRNA/control and Cre/control with

Tbr1 (**C**) and TuJ1 (**D**). There is a significant increase in the proportion of electroporated cells expressing Tbr1 in both loss of function treatments (Cre v. control $\chi^2_{[1]}= 9.26$, $p = 0.002$; shRNA v. control $\chi^2_{[1]}= 272$, $p < 0.001$). There is also a significant increase in the fraction of TuJ1 expressing electroporated cells in Cre treated animals (Cre v. control $\chi^2_{[1]}= 225$, $p < 0.001$). **E**) Higher magnification of cells targeted for loss of αE-catenin function that reside in the cortical plate, confirming that highlighted cells in (**A**) and (**B**) express the appropriate neuronal markers (bar: 10 μm). Both loss of function treatments increased neuronal differentiation relative to their respective controls. Control n = 6 brains, 2213 cells; shRNA n = 3 brains, 1757 cells; floxed control n = 4 brains, 2107 cells; Cre n = 4 brains, 1847 cells.

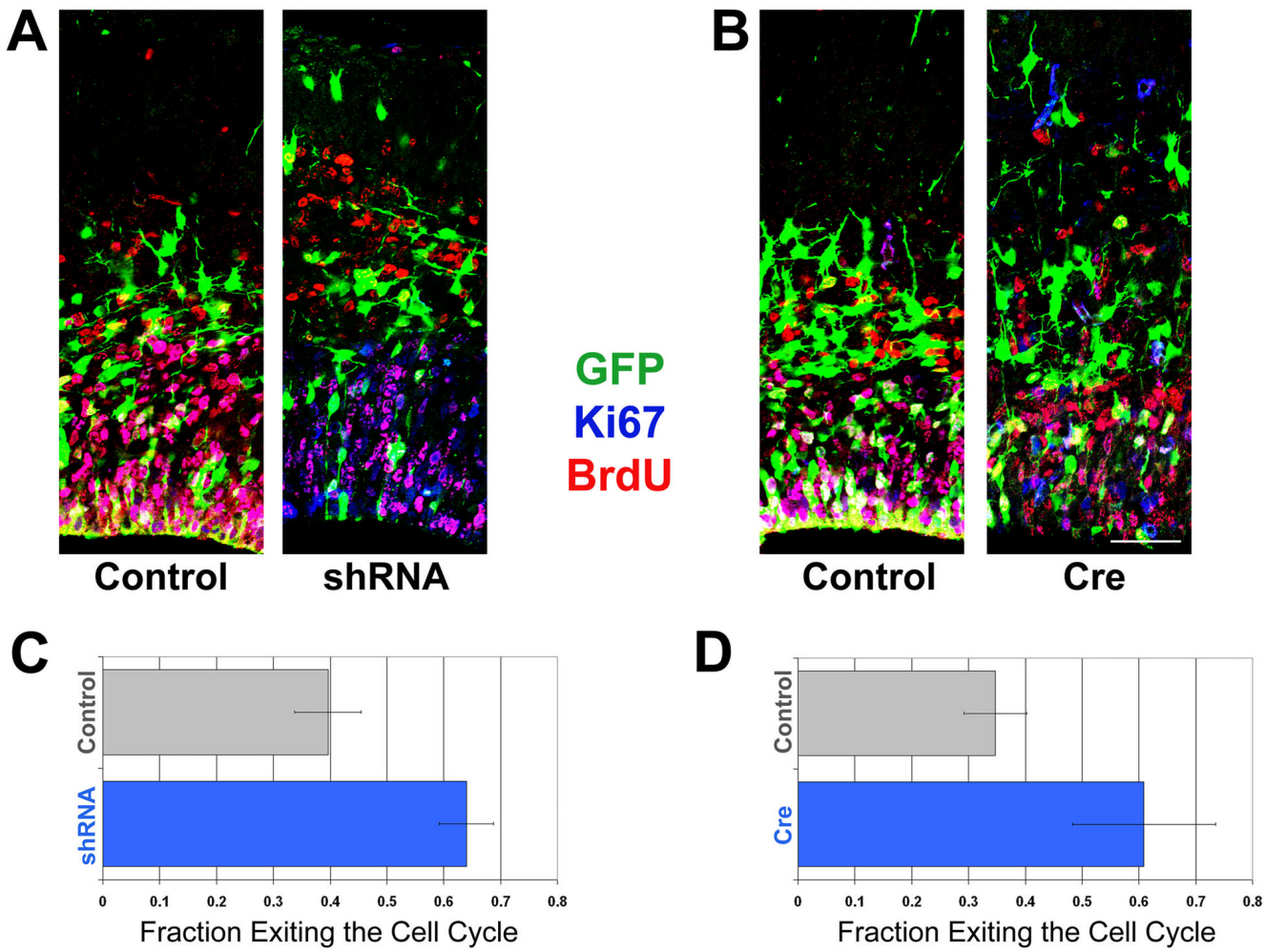

Figure 5. Focal elimination of αE-catenin increases the rate of cell-cycle exit

A single pulse of BrdU was administered at E14.5. Cortical tissue was analyzed at E15.5. Brain slices were stained with antibodies against BrdU (red) and Ki67 (blue). GFP-positive cells (green) labeled with BrdU and Ki67 (triple positive) have re-entered the cell cycle. GFP-positive cells labeled with BrdU but not Ki67 have exited the cell cycle (no longer dividing). The comparison of double labeled to triple labeled cells provides the cell cycle exit index (or quit fraction). **A**) pCAG-GFP (Control) or pCAG-GFP and pSHAGM2-αEcatenin-2010-E-4 (shRNA) were electroporated into embryonic brains at E13.5. **B**) pCAG-GFP (Control) or pCAG-Cre-IRES-GFP (Cre) were electroporated into *Ctnna1*$^{\text{lox(Ex2)}}$ embryonic brains at E13.5. Loss of αE-catenin increased the proportion of cells exiting the cell cycle compared to controls in both experimental treatments. **C**) Cell cycle exit indexes for shRNA/control with data presented as the mean ± s.e.m.; shRNA v. control $\chi^2_{[1]}= 96.7$, $p < 0.001$ (control n = 3 brains, 243 cells; shRNA n = 3 brains, 222 cells). **D**) Cell cycle exit indexes for Cre/control with data presented as the mean ± s.e.m.; Cre v. control $\chi^2_{[1]}= 74$, $p < 0.001$ (floxed control n = 3 brains, 281 cells; Cre n = 3 brains, 253 cells). Bar: 50 μm.

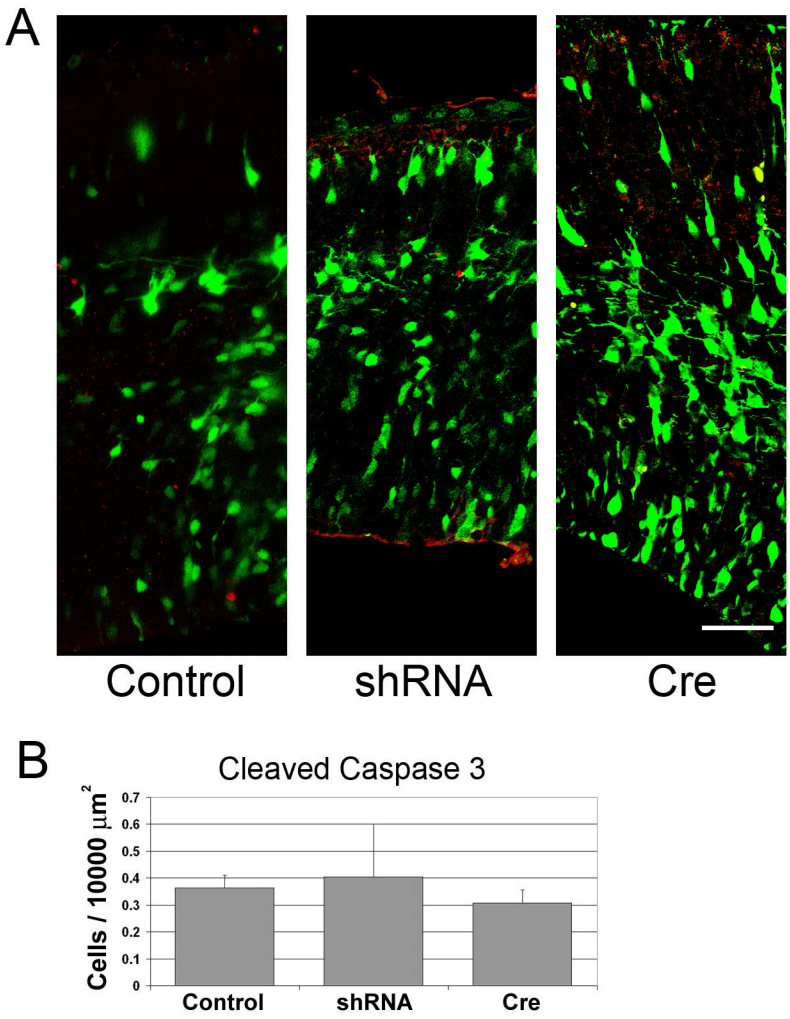

Figure 6. No change in cell death is observed after αE-catenin reduction. A
) Embryonic brains were electroporated at E13.5 with pCAG-GFP alone (Control), pCAG-GFP and pSHAGM2-αEcatenin-2010-E-4 (shRNA), or pCAG-Cre-IRES-GFP (Cre; into *Ctnna1*$^{lox(Ex2)}$ animals). Brains were analyzed at E15.5 for cleaved caspase-3 expression (red). Cell death was not altered by elimination of αE-catenin both within electroporated regions as well as in the surrounding tissue. **B**) Data presented as the mean ± s.e.m. (control n = 6 brains; shRNA n = 4 brains; Cre n = 3 brains). ANOVA: $F_{[2,10]} = 0.229$, $p = 0.800$. Bar: 50 μm.

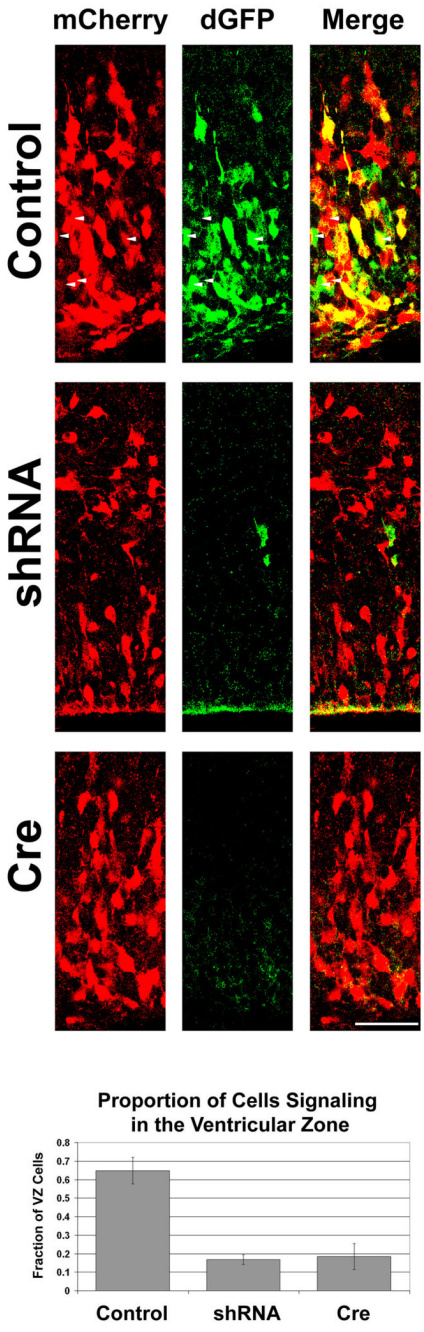

Figure 7. Focal elimination of αE-catenin reduces β-catenin signaling transcriptional activity

β-catenin mediated Wnt signaling in the ventricular zone was examined through expression of destabilized GFP controlled by the TOP promoter. **A**) E13.5 embryos were electroporated with pTOP-dGFP and pCAG-mCherry in the Control treatment and analyzed at E14.5. Only electroporated cells residing in the ventricular zone were included in the analysis, and the proportion of cells expressing dGFP was compared to the total number of electroporated cells in the VZ. Arrows indicate cells of interest. Additional control treatments were also performed using various shRNA constructs and homozygous floxed animals (images not shown). Experimental loss of function treatments were electroporated with the TOP-dGFP reporter, mCherry, and pSHAGM2-αEcatenin-2010-E-4 (shRNA) (**B**), or pCAG-Cre (Cre; into

Ctnna1$^{lox(Ex2)}$ animals) (**C**). Loss of αE-catenin function reduced the proportion of cells in the ventricular zone that display β-catenin mediated Wnt signaling. **D**) Data presented as the mean ± s.e.m. (control n = 9 brains, 997 cells; shRNA n = 4 brains, 442 cells; Cre n = 3 brains, 609 cells); shRNA v. control: $\chi^2_{[1]} = 146$, $p < 0.001$; Cre v. control: $\chi^2_{[1]} = 156$, $p < 0.001$. Bar: 50 μm.

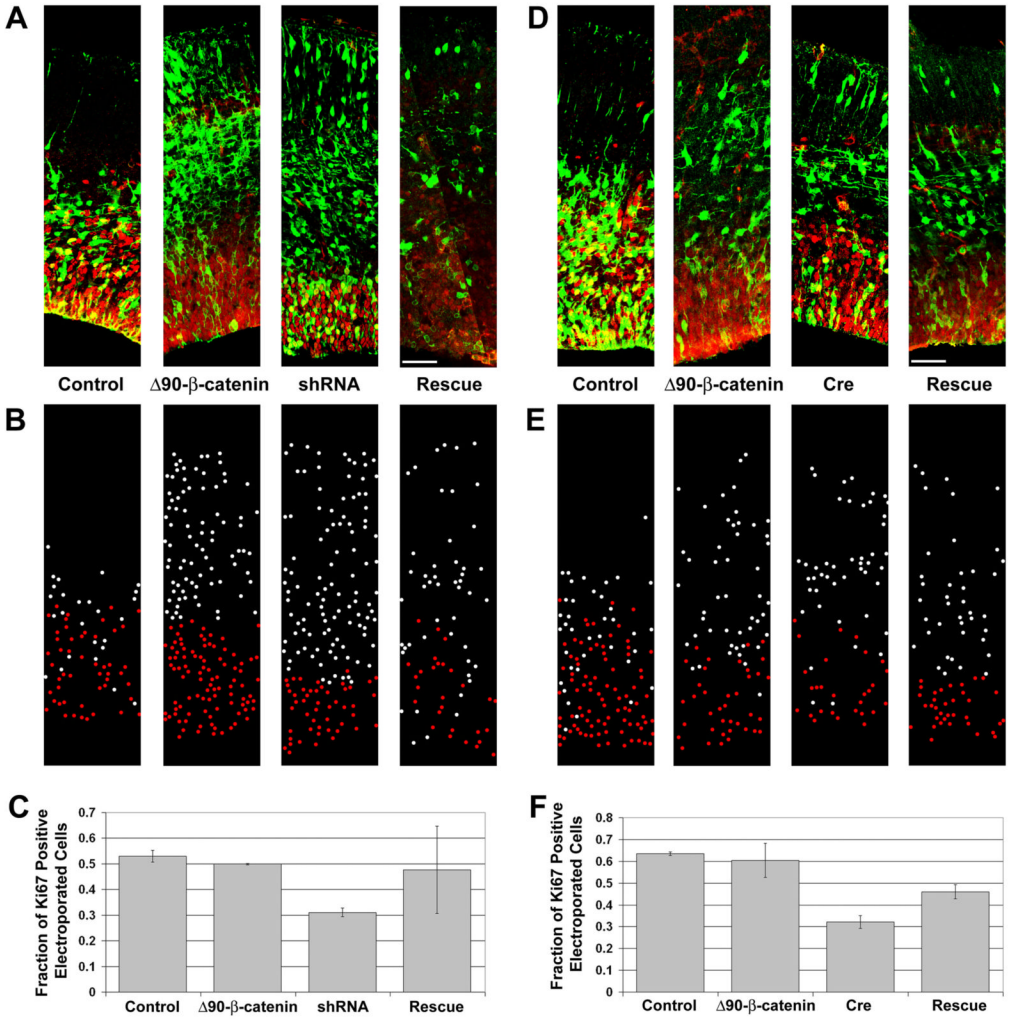

Figure 8. Rescue of β-catenin signaling following elimination of αE-catenin function restores proliferation

Beta-catenin signaling was rescued by introducing a stabilized variant of the protein (Δ90-β-catenin) which lacks GSK3β phosphorylation sites that normally target the protein for degradation. **A**) E13.5 embryos were electroporated with pCAG-GFP alone (control), pCAG-Δ90-β-catenin-GFP (Δ90-β-catenin), pSHAGM2-αEcatenin-2010-E-4 and pCAG-GFP (shRNA), or pSHAGM2-αEcatenin-2010-E-4 and pCAG-Δ90-β-catenin-GFP (rescue). All treatments were immunostained for the proliferative marker Ki67 (shown in red). **B**) Visual representation of the individual cells where all those cells targeted by electroporation that express Ki67 are labeled with red marks, while electroporated cells that do not express Ki67 are labeled with white marks. **C**) A histogram displaying the proportion of electroporated cells expressing Ki67 are presented as the mean ± s.e.m. (control n = 9 brains, 1448 cells; Δ90-β-catenin n = 3 brains, 388 cells; shRNA n = 6 brains, 856 cells; rescue n = 4 brains, 381 cells); Δ90-β-catenin v. control: $\chi^2_{[1]} = 1.75$, $p = 0.186$; shRNA v. control: $\chi^2_{[1]} = 148$, $p < 0.001$; rescue v. control: $\chi^2_{[1]} = 0.72$, $p = 0.397$; rescue v. shRNA: $\chi^2_{[1]} = 85.1$, $p < 0.001$. The co-expression of a stabilized β-catenin with shRNA targeting αE-catenin returned the proportion of proliferative cells to control levels. **D**) E13.5 homozygous floxed αE-catenin (*Ctnna1*$^{lox(Ex2)}$) embryos were electroporated with pCAG-GFP alone (control), pCAG-Δ90-

β-catenin-GFP (Δ90-β-catenin), pCAG-Cre-IRES-GFP (Cre), or pCAG-Cre-IRES-GFP and pCAG-Δ90-β-catenin (rescue), then immunostained for Ki67 (red). **E**) Visual representation of the individual cells where all those cells targeted by electroporation that express Ki67 are labeled with red marks, while electroporated cells that do not express Ki67 are labeled with white marks. **F**) A histogram displaying the proportion of electroporated cells in floxed αE-catenin embryos expressing Ki67 are presented as the mean ± s.e.m. (control n = 3 brains, 614 cells; Δ90-β-catenin n = 3 brains, 314 cells; Cre n = 5 brains, 645 cells; rescue n = 5 brains, 908 cells); Δ90-β-catenin v. control: $\chi^2_{[1]} = 0.44$, $p = 0.506$; Cre v. control: $\chi^2_{[1]} = 262$, $p < 0.001$; rescue v. control: $\chi^2_{[1]} = 121$, $p < 0.001$; rescue v. Cre: $\chi^2_{[1]} = 78.5$, $p < 0.001$. The co-expression of a stabilized β-catenin with Cre-recombinase significantly increased the proportion of proliferative cells compared to Cre treated tissue, but did not completely restore levels to that of controls. All scale bars: 50 μm.