Deletion of YAP/TAZ in mouse neocortex impaired the proliferation and differentiation ability of neural progenitor cells

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Abstract

YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are downstream effectors of the Hippo pathway, to activate the expression of transcriptional targets that promote cell growth, cell proliferation, and prevent apoptosis. Aberrant hyper activation of YAP/TAZ causes tissue overgrowth and tumorigenesis, whereas their inactivation impairs tissue development and regeneration. Here we report the deletion of Yap and Taz by Emx1-Cre in mouse neocortex leads to aberrant development and dilated ventricle in adult stage. The phenotype can be detected as early as P0: the cells in ventricular zone is reduced in DKO cortex. Consisted with this, the numbers of Sox2+ cell and Tbr2+ cell are reduced and the ratio of tbr2/Sox2 is also reduced at E17.5 when Yap/Taz are deleted.

Furthermore, the deletion of YAP/TAZ leads to the disrupted the neuroepithelial cell junctions. The result of immunology experiments show that Yap/Taz are highly expressed in the apical surface of neuroepithelials and colocalized with Sox2+ cells in mouse neocortex. Results of BrdU-EdU labeling experiment suggested that the cell cycle exit is increased. Together, our results reveal that Yap/Taz deletion elongated S phase and total cell cycle length, and increased the cell cycle exit ratio, therefore reduced the proliferation ability of progenitor cells. Yap/Taz deletion also reduced the differentiation of Sox2+ cells into Tbr2+ cells through promoting the premature of Sox+ cells. Together, these results suggest that Yap/Taz are essential for epithelial cell proliferation and differentiation, and the deletion can promote hydrocephalus pathogenesis.

Introduction

Hippo pathway is the one of the fundamental pathways that are essential for the early stage development, and the downstream effector of hippo pathway is YAP/TAz, which is isolated in drosophilar as Yokie. Yokie acted as the transcription activator, and its mutants display the reduced tissue proliferation while overexpression leads to tissue overgrowth. Yap is the mammalian homologue of Yokie, while Taz is the Yap paralog in vertebrates. The function of YAP/TAZ in vertebrates is conserved with the function of Yokie in drosophila: overexpression of nonphosphorylated form of YAP or TAZ increases the proliferation of several human or mouse cell lies in vitro; and depletion of YAP/TAZ by RNAi blocks the growth of several human cancer cell lines. YAP/TAZ are primary sensors of the cell's physical nature, as defined by cell structure, shape and polarity. YAP/TAZ activation also reflects the cell "social" behavior, including cell adhesion and the mechanical signals that the cell receives from tissue architecture and surrounding extracellular matrix (ECM) [1].

There are several studies to show that YAP/TAZ are regulated by the cell junction and apical-basal polarity of epithelial cells through phosphorylation of hippo pathway. YAP/TAz are sensors of epithelial polarity, can be inhibited by apical signals and stimulated by basal signals[2].

People have found that the cell junction structure of epithelial cells can inhibit the YAP/TAZ activity. Disrupt the E-cadherin/a-catenin complex decreases YAP phosphorylation and can

promo the nuclear accumulation of YAP. And disruption of tight junction or adherin junctions also turns on the YAP/TAZ nuclear activiation. In addition, YAP2 (one of the YAP isoforms) binds to the TJ component ZO-2 (zonula occludens 2) via a PDZ domain, although this interaction appears to favour nuclear translocation rather than membrane retention [3]. In summary, numerous studies have highlighted the interplay between the Hpo pathway and apico–basal cell polarity/cell–cell contacts. The idea of the Hpo pathway as a sensor of epithelial architecture/integrity rather than a stereotypical ligand–receptor signal transduction pathway is emerging [4]. But there is very limited study to show that YAP/TAZ can regulate the cell junction structure in epithelial cells.

In the nervous system, the neuroepithelial cells are the "stem cells" deriving from actual stem cells in several different stages of neural development. These neural stem cells can differentiate further into multiple types of cells, like neurons, astrocytes and other glial cells. They appear during embryonic development of the neural tube as well as in adult neurogenesis in specific areas of the central nervous system. Neuroepithelial cells are a class of stem cell and have similar characteristics, most notably the ability to self-renew. During the formation of the neural tube, neuroepithelial cells undergo symmetric proliferative divisions that give rise to two new neuroepithelial cells. At a later stage of brain development, neuroepithelial cells begin to self-renew and give rise to non-stem cell progenitors, such as radial glial cells simultaneously by undergoing asymmetric division.

Neurons in the brain originate from a relatively small number of neural stem and progenitor cells (NSPCs)[5]. Before the onset of neurogenesis, neuroepithelial cells (NEs) divide symmetrically to expand their number. When neurogenesis begins, NEs transform into radial glia (RG) cells that can divide to self-renew and generate a neuron (this is termed direct neurogenesis); alternatively, RG cells can divide to self-renew and generate an intermediate progenitor cell (IPC), which can then divide to generate neurons (this is termed indirect neurogenesis). RG cells can also divide to generate outer radial glia (ORG) cells that can themselves divide to self-renew and generate IPCs or neurons.

The VZ has long been thought of as a proliferative niche in the developing cortex. However, in contrast with earlier reports of RGPs losing their proliferative capacity and differentiating when they exit the VZ35, 38. Asymmetric divisions of radial glia progenitors produce self-renewing radial glia and differentiating cells simultaneously in the ventricular zone (VZ) of the developing neocortex. Whereas differentiating cells leave the VZ to constitute the future neocortex, renewing radial glia progenitors stay in the VZ for subsequent divisions. The differential behavior of progenitors and their differentiating progeny is essential for neocortical development [6].

Results

Expression analysis of Yap/TAZ

Yap/Taz are downstream effectors of hippo pathway, to activate the expression of transcriptional targets that promote cell growth, cell proliferation, and prevent apoptosis. Lots of studies have been published about the function of Yap/Taz in the cell proliferation and differentiation. To understand the function of Yap/Taz in the development of nervous system, we detected the expression of Yap/Taz in the neocortex of mouse from E12.5 to E17.5 (Figure1), the result show that Yap/Taz are highly expressed in the progenitor cells(Figure1), which are marked with Sox2. We didn’t detect the expression of Yap/Taz in the intermediate progenitor cells, which are marked with Tbr2. We also found that, at E12.5 Yap/Taz expression are extremely high in the

neural epithelial cells (Figure1). Next we use Emx1-cre deleted Yap/Taz in the neocortex of mouse at E9.5. To determine whether IF result show that the Yap/Taz can be completely deleted in the neocortex (Figure1). Yap/Taz are expressed in neuroprogenitor cells and colocalized with Sox2.

Yap/Taz is essential for normal cortex development

At the adult stage, loss of Yap/Taz in cortex results in the hydrocephalus. In the total four double conditional Yap/Taz knockout mice, all of them displayed the phenotype; while in the single conditional deletion mutant, only 40% of the YapF/F;TazF /+;Emx1-Cre mice display the dilated ventricle phenotype; none of the YapF/+;TazF / Δ;Emx1-Cre mice display the dilated ventricle phenotype (Figure2A,B). This suggests that Yap plays more important role in the development of hydrocephalus.

To determine when the deletion of Yap/Taz leads to the hydrocephalus, we detected the onset time of this phenotype. The result show that the phenotype show up as early as P0 (Figure2C). Conditional deletion of Yap/Taz in neocortex via Emx1-Cre leads to hydrocephalus, and this phenotype can be detected as early as P0.

The size of LV was enlarged at 1 month stage, and statistics show that 83% (5/6) of the mice show the ventricle expansion at P0, this means the most of the hydrocephalus was developed prenatally (Figure2D). The cells linearize the ventricular zone are reduced in the DKO mouse.

Cell polarity are changed in the DKO mouse

To figure out whether the cell polarity in the ventricular zone are changed or not, the polarity marker was used to label the cortex. Result show that the neural epithelial cell polarity are partially lost in the ventricular zone at E17.5 (Figure3). Since the cells line the ventricular zone are reduced in DKO mice, to figure out whether these cells are progenitor cells we stained the Sox2 positive cells in the P0 cortex. The result show that E-cadherin, N-cadherin and ZO-1 localizations were disrupted when YAP/TAZ were deleted. The immunofluorescence experiments indicates that the deletion of YAP/TAZ leads to the disrupted neuroepithelial cell junctions.

Yap/Taz deletion mice have defects in neurogenesis during development

Since the cortex development are defective in P0, we analyzed the progenitor cell in the ventricular zone embryonic stages to figure out when and how the cells are reduced. We used the Sox2 and Tbr2 to label neural progenitor and intermediate progenitor cells from E14.5 to E17, statistic results show that the Sox2+ and Tbr2+ cells are significantly reduced at E17.5(n=3) (Figure4). At the apical surface that the ZO-1 is mislocalized. And the ratio of Tbr2/Sox2 is also reduced at the whole apical surface of ventricular zone. Since Tbr2 is a marker for intermediate progenitor cell, and the Tbr2+ cells are born from SOX2 + cells, this indicates that the when YAP/TAZ are deleted, the differentiate ability of SOX2+ cells to Tbr2+ cells is reduced. The number of Sox2+ cells and Tbr2+ cells are significantly reduced at E17.5 when Yap/Taz are deleted, the ratio of Tbr2/Sox2 is also reduced.

Yap/Taz deletion neuronal progenitors show premature cell cycle exit

To figure out whether the reduced cell number are caused by proliferation, we used the BrdU labeling, and then counted the cell number of BrdU+Sox2+ to analyzed the proliferation ability of neural progenitor cells. Results show that the Yap/Taz deletion elongated S phase and total Cell cycle length, and increased the cell cycle exit ratio, therefore reduced the proliferation ability of progenitor cells. Disrupted differentiation Results of BrdU-EdU labeling experiment suggested that the cell cycle exit is increased, indicates therefore reduced the proliferation ability of progenitor cells.

Neuron born ability was reduced when Yap/Taz are deleted

Yap/Taz deletion also reduced the ability of Sox2+ cells differentiate into Tbr2+ cells. Yap/Taz are expressed in Sox2+ cell, deletion of Yap/Taz lead to reduced proliferation and differentiation of Sox2+ cell.

Conditional deletion of Yap/Taz in neocortex via Emx1-Cre leads to hydrocephalus, and this phenotype can be detected as early as P0. IF results show that Yap/Taz are expressed in neuroprogenitor cells and colocalized with Sox2. Both the Sox2+ cells and Tbr2+ cells are significantly reduced at E17.5 when Yap/Taz are deleted, the ratio of Tbr2/Sox2 is also reduced.
and increased the cell cycle exit ratio, therefore reduced the proliferation ability of progenitor cells.
Yap/Taz are expressed in Sox2+ cell, deletion of Yap/Taz lead to reduced proliferation and differentiation of Sox2+ cell. Yap/Taz deletion also reduced the ability of Sox2+ cells differentiate into Tbr2+ cells. Through cell cycle analysis we found that Yap/Taz deletion increased the cell cycle exit, and therefore reduced the proliferation ability of Sox2+ cells. The improperly increased expression of Tuj1+ cells in the DKO cortex indicates the premature of Sox2+ cells. Therefore, our data indicates the deletion of Yap/Taz in neocortex both reduced the proliferation and differentiation ability of neural progenitor cells.

Yap/Taz deletion reduced the differentiation of Sox2+ into Tbr2+ cells because of the premature since Sox2+ cells expressed Tuj1 improperly.

Yap/Taz are expressed in neuroprogenitor cells and conditional deletion of Yap/Taz in neocortex via Emx1-Cre leads to hydrocephalus.

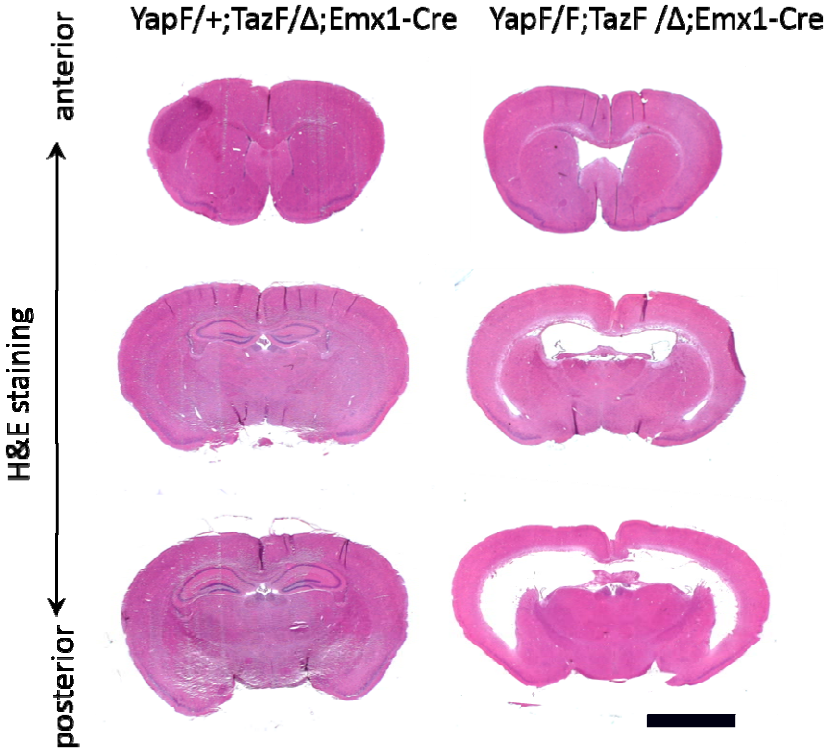
The number of Sox2+ cells and Tbr2+ cells are significantly reduced at E17.5 when Yap/Taz are deleted, the ratio of Tbr2/Sox2 is also reduced, indicates the reduced differentiated ability of Sox2+ cells to Tbr2+ cells.

ZO-1 immunofluorescence experiments show that the deletion of YAP/TAZ leads to the disrupted neuroepithelial cell junctions.

Results of BrdU-EdU labeling experiment suggested that the cell cycle exit is increased, indicates the reduced proliferation ability of progenitor cells.

The increased expression of Tuj1+ cells in the DKO cortex indicates the premature of Sox2+ cells.

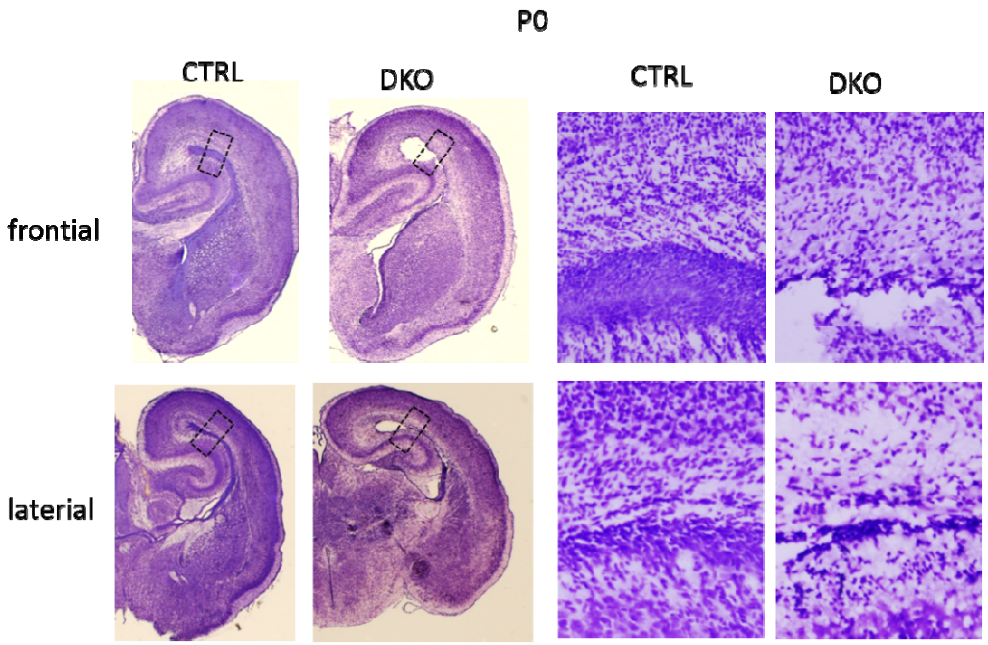
Figure legend



genotype	No phenotype	Small hole	Large hole
YapF/+;TazF/+;Emx1-Cre	**8/8 (100%)**		
YapF/F;TazF/+;Emx1-Cre	**3/5 (60%)**	**1/5 (20%)**	**1/5 (20%)**
YapF/+;TazF/Δ;Emx1-Cre	**4/4 (100%)**		
YapF/F;TazF/Δ;Emx1-Cre			**4/4 (100%)**

Figure1, Enlarged ventricles in YapF/F;TazF /Δ;Emx1-Cre mouse.

A–F) H&E staining of paraffin-embedded coronal sections of P30 YapF/+;TazF/Δ;Emx1-Cre and YapF/F;TazF /Δ;Emx1-Cre mouse. Representative adult mouse brain coronal sections are shown. Extensive dilation of the lateral ventricles is evident in the mutants at P30 (B), and tissue loss in the cortex is observed in more caudal sections (G) statistical analysis of the phenotype of YapF/F;TazF /Δ;Emx1-Cre mouse and the control mouse.



genotype	No phenotype	Small hole	Large hole
YapF/+;TazF/+;Emx1-Cre	**4/4 (100%)**		
YapF/F;TazF/+;Emx1-Cre	**1/1 (100%)**		
YapF/+;TazF/Δ;Emx1-Cre		**1/1 (100%)**	
YapF/F;TazF/Δ;Emx1-Cre	**1/6 (17%)**		**5/6 (83%)**

Figure2, H&E staining of paraffin-embedded coronal sections of P0 YapF/+;TazF/Δ;Emx1-Cre and YapF/F;TazF/Δ;Emx1-Cre mouse. Cells in the ventricular zone is dramatically reduced both in frontial and laterial position

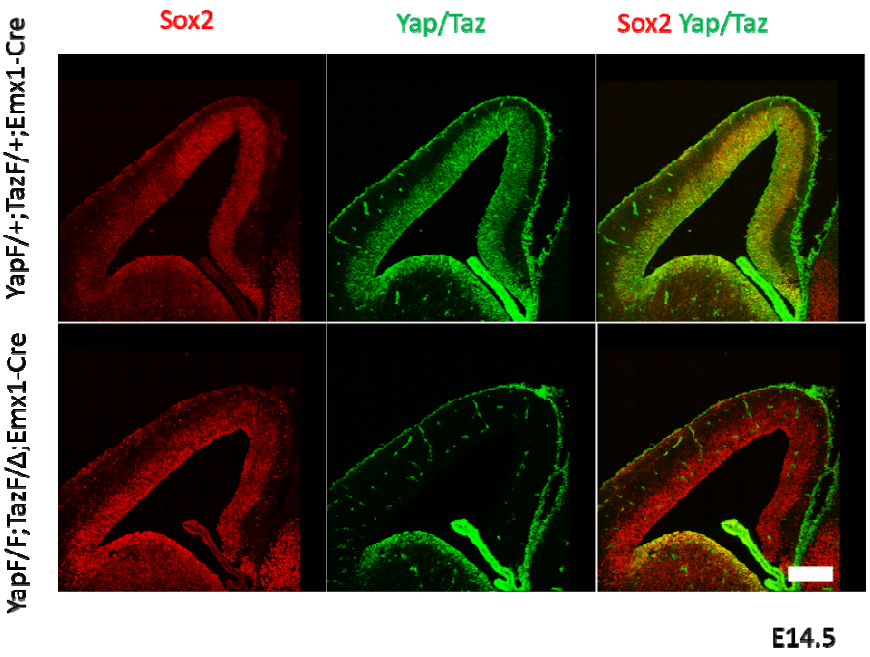


Figure3, IF staining to detect the expression of YAP/TAZ in YapF/+;TazF/Δ;Emx1-Cre and YapF/F;TazF/Δ;Emx1-Cre mouse cortex at E14.5. DAPI staining shows the overall structure of YapF/+;TazF/Δ;Emx1-Cre and YapF/F;TazF/Δ;Emx1-Cre mouse, the SOX2 staining the progenitor cells, the loss of YAP/TAZ signal in the neocortex indicates the complete deletion of YAP/TAZ with Emx1-cre.

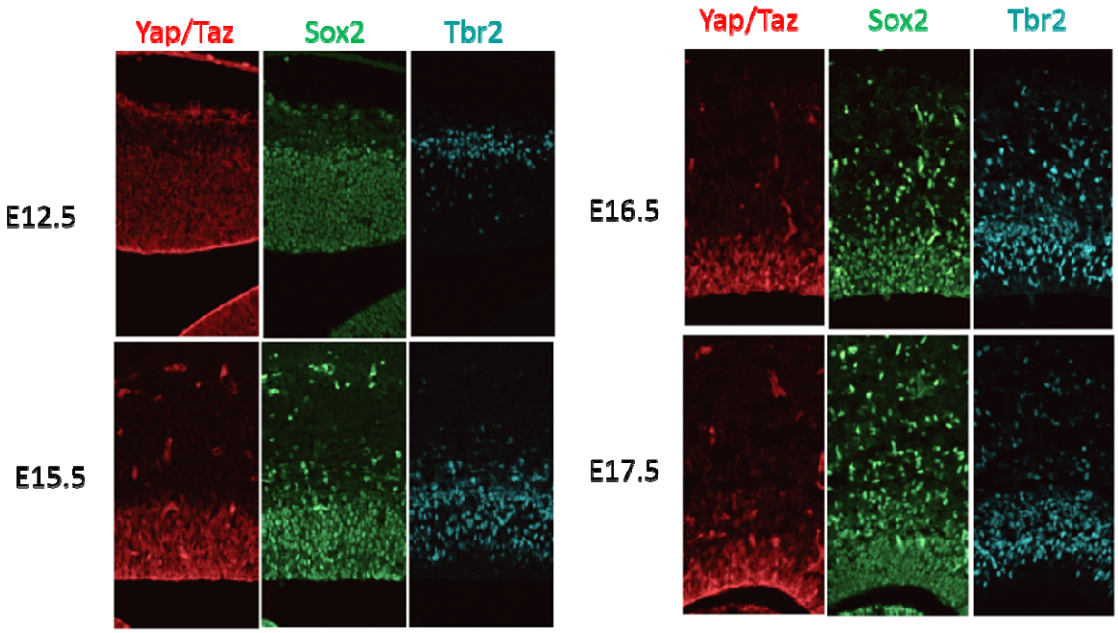


Figure4, IF staining to detect the expression of YAP/TAZ in neocortex at E12.5 and E17.5. SOX2 staining shows the progenitor cells and Tbr2 staining shows the intermediate progenitor cells.

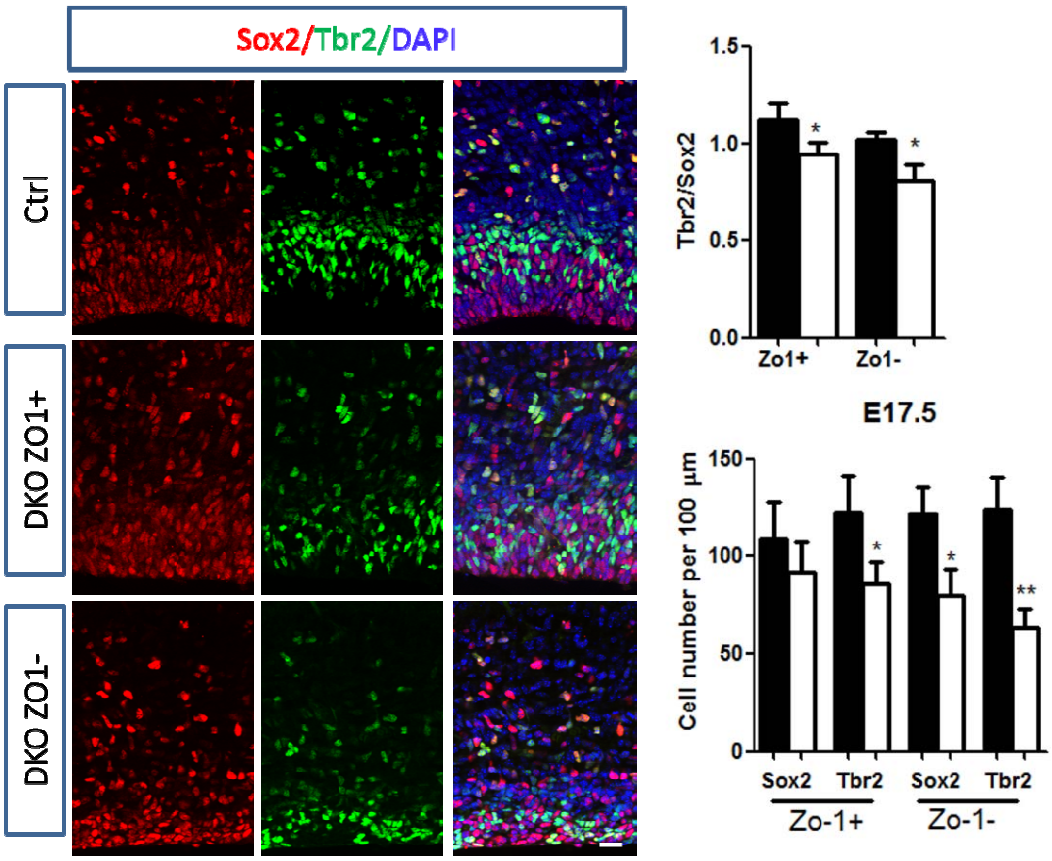


Figure5, Loss of Yap/Taz reduced the apical progenitor pool by late neurogenesis in the cortex. (A) SOX2 and TBR2 immunostaining in E17.5 control and YapF/F;TazF/Δ;Emx1-Cre cortex. Scale bar: 100 µm. (B) Quantification of SOX2⁺ and TBR2⁺ cells in E17.5 control and YapF/F;TazF/Δ;Emx1-Cre cortex (*n*□=□3). (C) Quantification of SOX2⁺ (both within the VZ and beyond the VZ) as a percentage of total TBR2⁺ cells in E17.5 control and YapF/F;TazF/Δ;Emx1-Cre cortex (*n*□=□3). Data expressed as mean ± S.E.M. **P*<0.05. ***P*<0.01

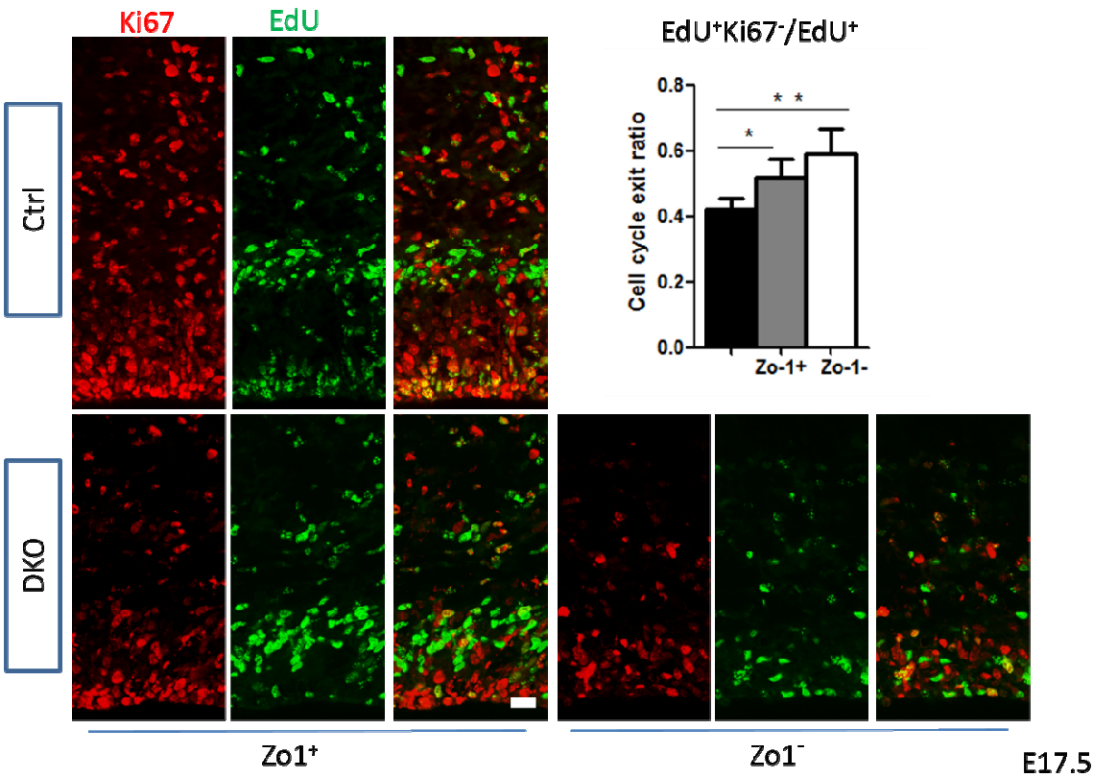


Figure6. Increased cell cycle exit in the YapF/F;TazF/Δ;Emx1-Cre cortex during early neurogenesis. (A) Pregnant female mice were subjected to a 24-hour BrdU pulse prior to sacrifice. Ki67 and BrdU immunostaining in E17.5 control and YapF/F;TazF/Δ;Emx1-Cre cortex. Scale bar: 50 µm. Arrowheads indicate BrdU+Ki67− cells that have exited the cell cycle. Cell cycle exit indices were calculated by measuring the ratio of BrdU+Ki67− cells to total BrdU+ cells in E17.5 control and YapF/F;TazF/Δ;Emx1-Cre cortex (n□=□3). Data expressed as mean ± S.E.M. *P<0.05.

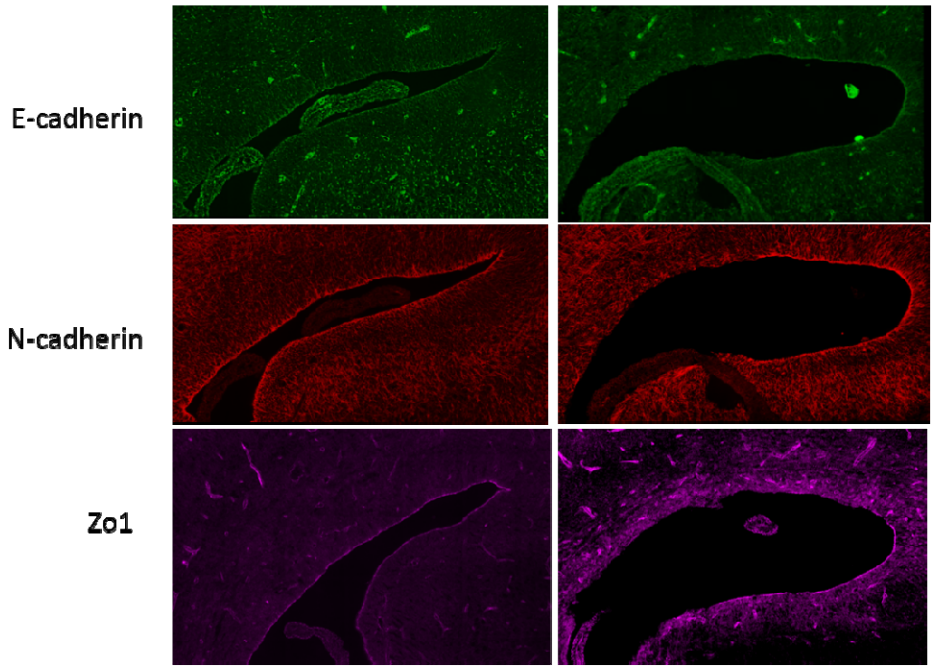


Figure7. Loss of epithelial feature of YapF/F;TazF/Δ;Emx1-Cre neural epithelial cells. (A) Apical junction complex marker N-Cadherin (green) E-Cadherin (red) and ZO-1immunostained cortical section of embryos at E17.5, showing the loss of adhesive junctions in YapF/F;TazF/Δ;Emx1-Cre neural epithelial cells.

Yap/Taz are expressed in Sox2+ neural progenitor cells, loss of Yap/Taz in mouse cortex cause the hydrocephalus after birth, this phenotype can be detected as early as P0. Yap/Taz deletion elongated S phase and total Cell cycle length, and increased the cell cycle exit ratio, therefore reduced the proliferation ability of progenitor cells. Yap/Taz deletion also reduced the differentiation into Tbr2+ cells.

Yap/Taz are expressed in Sox2+ cell, deletion of Yap/Taz lead to reduced proliferation and differentiation of Sox2+ cell. Through cell cycle analysis we found that Yap/Taz deletion elongated both S phase length and total Cell cycle length, also increased the cell cycle exit, and therefore reduced the proliferation ability of Sox2+ cells. Yap/Taz deletion reduced the differentiation of Sox2+ into Tbr2+ cells because of the premature since Sox2+ cells expressed Tuj1 improperly.

Mice

Deletion of *Yap/Taz* in the mouse forebrain was achieved by mating *Yap/Taz loxP* mice with heterozygous *Emx1Cre* knock-in mice, as previously described. All mice were maintained according to the protocol approved by the Animal Care and Use Committee of St Jude Children's Research Hospital. Emx1-Cre (stock no. 005628) was obtained from the Jackson Laboratory.

EdU labeling

For in vivo labeling of EdU, pregnant mice were injected intraperitoneally with EdU at 1 ml per 100 g body weight (GE Healthcare Life Sciences) and embryos were collected and processed for immunofluorescence analysis 24 hours later. To detect EdU positive cells, sections were first treated with 1N HCl at 45°C for 30 minutes prior to immunofluorescence analysis. Then washed with TBST, and processed as above using an anti-EdU antibody. For costaining using an anti-EdU antibody and other primary antibodies, sections were first stained with the other primary then stained with the anti-EdU antibody.

Immunostaining

Mouse brains were dissected in PBS and fixed overnight in 4% paraformaldahyde in PBS at 4°C. Brains were then cryoprotected using 30% sucrose in PBS overnight at 4°C and embedded in OCT for cryosectioning. Frozen sections were washed with 0.2% Triton X-100 in TBS (TBST) and incubated in the blocking solution (1% goat serum in TBST) for 1 hour at room temperature. Sections were incubated with primary antibodies diluted in the blocking solution overnight at 4°C, washed with TBST, and incubated with secondary antibodies (Jackson ImmunoResearch) diluted at 1:1000 in the blocking solution for 2 hours at room temperature. Sections were counterstained with DAPI, washed in TBST, and mounted in ProLong Gold antifade reagent (Invitrogen).

Antibodies for immunostaining

Primary antibodies: Mouse anti-ZO-1 (Alexa 488conjugated) (Invitrogen 339188); rabbit anti-Phospho-histone H3 (Chemicon 06-570); rabbit anti-Ki67 (Vector Labs VP-RM04); rat anti-BrdU (Abcam ab6326); goat anti-Lhx2 (Santa Cruz sc-19342); goat anti-Sox2 (Santa Cruz sc-17320); rabbit anti-Sox2(Cell Signaling 3728); rabbit anti-Tbr2 (Abcam ab23345); rat anti-Tbr2 (Alexa 647 conjugated, eBiosciences 51-4875); rabbit anti-Yap/Taz (Cell Signaling 8418 1:500); rabbit anti-YAP (pS127) (Cell Signaling 4911); mouse anti-TUJ1 (1:200; Covance),

Secondary antibodies: donkey anti-rabbit Alexa Fluor 488 (1:500; Invitrogen), donkey anti-goat Dylight 549 (1:1000; Jackson ImmunoResearch), donkey anti-chicken Dylight 649 (1:1000; Jackson ImmunoResearch) and donkey anti-mouse Dylight 549 (1:1000; Jackson ImmunoResearch

Microscopy and quantification

Images were captured using a Leica DMI-6000B inverted microscope (Leica Microsystems) equipped with a digital camera (ORCA-ER, Hammamatsu). Image capture was achieved using Openlab v5.0 (Perkin Elmer) and processed using Volocity v5.4 (Perkin Elmer) and Photoshop CS5 (Adobe). To analyze P7 cortical layer thickness, three different measurements separated by 100 µm were obtained perpendicular to the lateral cortical axis in equivalent regions from three sequential cryosections per brain ($n = 5$). For P7 and embryonic cortical cell quantifications, 200 µm-wide columns perpendicular to the lateral cortical axis were quantified for immunopositive cells in equivalent areas from three adjacent cryosections per brain ($n = 3$–5).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., Version 4.02), and all results are expressed as the mean ± standard error of the mean. *P* values were generated using Student's *t* test (unpaired, two-tailed) to compare between two independent data sets and a value of $^*P<0.05$ was considered statistically significant.

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