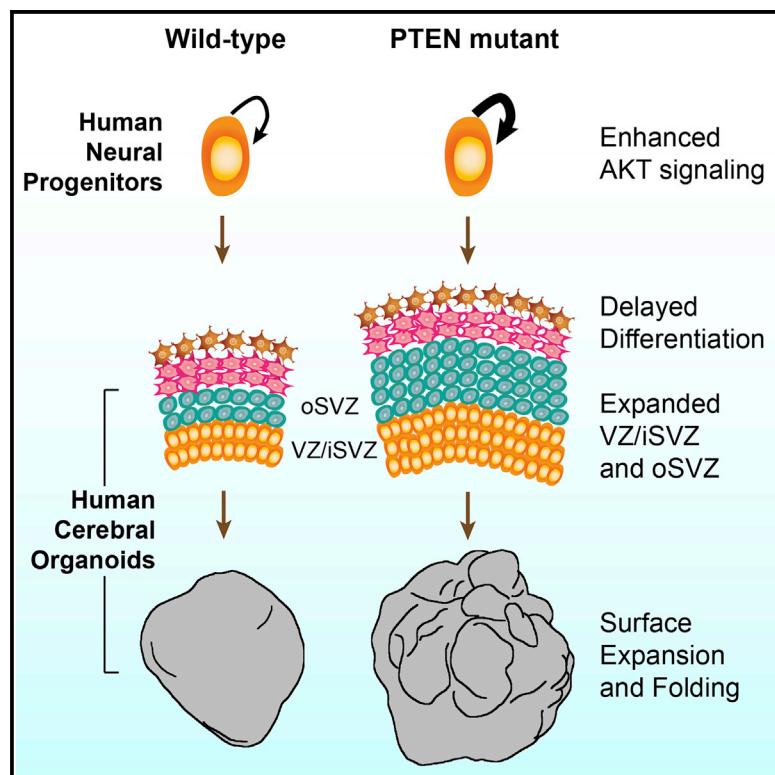


# Induction of Expansion and Folding in Human Cerebral Organoids

## Graphical Abstract



## Authors

Yun Li, Julien Muffat, Attya Omer, ...,  
Lee Gehrke, Juergen A. Knoblich,  
Rudolf Jaenisch

## Correspondence

jaenisch@wi.mit.edu

## In Brief

The growth and structural formation of the human cortex can be modeled *in vitro* using cerebral organoids. In this article, Li, Muffat, and colleagues show that enhancing the PTEN-AKT signaling pathway leads to the generation of human cerebral organoids that are expanded in size and display surface folding.

## Highlights

- PTEN deletion enhances human NP proliferation and sensitivity to growth factors
- PTEN mutant human, but not mouse, cerebral organoids display surface folding
- Folded human organoids contain expanded ventricular and outer progenitors
- Zika virus impairs the formation of expanded and folded human cerebral organoids

# Induction of Expansion and Folding in Human Cerebral Organoids

Yun Li,<sup>1,8</sup> Julien Muffat,<sup>1,8</sup> Attya Omer,<sup>1</sup> Irene Bosch,<sup>2</sup> Madeline A. Lancaster,<sup>3</sup> Mriganka Sur,<sup>4</sup> Lee Gehrke,<sup>2,5,6</sup> Juergen A. Knoblich,<sup>3</sup> and Rudolf Jaenisch<sup>1,7,9,\*</sup>

<sup>1</sup>The Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA

<sup>2</sup>Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

<sup>3</sup>Institute of Molecular Biotechnology of the Austrian Academy of Sciences, 1030 Vienna, Austria

<sup>4</sup>The Picower Institute for Learning and Memory, Cambridge, MA 02139, USA

<sup>5</sup>Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA

<sup>6</sup>Harvard-MIT Program in Health Sciences and Technology, Cambridge, MA 02139, USA

<sup>7</sup>Department of Biology, Massachusetts Institute of Technology, 32 Ames Street, Cambridge, MA 02139, USA

<sup>8</sup>Co-first author

<sup>9</sup>Lead Contact

\*Correspondence: jaenisch@wi.mit.edu

<http://dx.doi.org/10.1016/j.stem.2016.11.017>

## SUMMARY

An expansion of the cerebral neocortex is thought to be the foundation for the unique intellectual abilities of humans. It has been suggested that an increase in the proliferative potential of neural progenitors (NPs) underlies the expansion of the cortex and its convoluted appearance. Here we show that increasing NP proliferation induces expansion and folding in an *in vitro* model of human corticogenesis. Deletion of *PTEN* stimulates proliferation and generates significantly larger and substantially folded cerebral organoids. This genetic modification allows sustained cell cycle re-entry, expansion of the progenitor population, and delayed neuronal differentiation, all key features of the developing human cortex. In contrast, *Pten* deletion in mouse organoids does not lead to folding. Finally, we utilized the expanded cerebral organoids to show that infection with Zika virus impairs cortical growth and folding. Our study provides new insights into the mechanisms regulating the structure and organization of the human cortex.

## INTRODUCTION

A massive increase in the size of the human cerebral neocortex is accompanied by the expansion of the cortical area, and the associated emergence of extensive cortical folding (Bystron et al., 2008; Geschwind and Rakic, 2013; Sun and Hevner, 2014). In the developing human cerebral cortex, radial glial cells, residing in both the ventricular zone (VZ) and the later-formed sub-ventricular zone (SVZ), give rise to intermediate progenitors and, ultimately, post-mitotic neurons (Lui et al., 2011; Rakic, 1988), following an inside-out pattern of migration (Rakic, 1974). Compared with mice, the developing cortex of humans and other gyrencephalic mammals harbor a larger VZ and SVZ,

which are composed of substantially more radial glial cells and intermediate progenitors (Hansen et al., 2010). Whether this increase in neural progenitor (NP) population is directly responsible for the expansion of the human cortex and the evolutionary transformation of a smooth, unfolded cortex to a profoundly folded one remains mostly untested, in no small part because of the inaccessible nature of the developing human cortex. However, recent advances in human pluripotent stem cell technology (Takahashi et al., 2007; Yu et al., 2007) and 3D culture systems (Lancaster et al., 2013; Sasai, 2013) have opened new avenues to investigate human cortical development. Starting with human embryonic stem cells (hESCs) and induced pluripotent stem cells, we have recently reported the generation of cerebral organoids that self-organize into neuralized structures resembling the early developing human cerebral cortex (Lancaster et al., 2013). These *in vitro*-derived neural tissues recapitulate early stages of cortical formation and display human-specific features such as the outer SVZ. Therefore, human cerebral organoids represent a novel method to investigate the molecular, cellular, and anatomical paradigms of early human cortical development.

Cortical folding can be experimentally induced in the developing mouse brain by  $\beta$ -catenin overexpression (Chenn and Walsh, 2002), inhibition of apoptosis (Kuida et al., 1996, 1998), or modification of NP-specific genes such as *Timp1* (Stahl et al., 2013) and *Arhgap11b* (Florio et al., 2015), all likely via direct regulation of the proliferation, survival, and differentiation propensity of NPs. Differential regulation of NP proliferation may also contribute to human-specific cortical pattern formation (Bae et al., 2014). Interestingly, multiple lines of evidence have converged to suggest a physiological involvement of growth factor signaling in regulating human-specific cortical expansion and folding. Introduction of FGF2 into the developing mouse cortex leads to regional formation of gyri (Rash et al., 2013), and human fetal cortical transcriptome revealed progenitor-specific enrichment of platelet-derived growth factor D (*PDGF-D*) (Lui et al., 2014). Existing human genetic evidence strongly suggests that growth factor signaling, in particular through the PTEN-AKT cascade, regulates human cortical formation. Pathological development of a larger brain (megalencephaly) and/or increased gyration (polymicrogyria) have been linked to



mutations that activate the pathway in genes such as *AKT3*, *PTEN*, *PIK3CA*, and *PIK3R2* (DiLiberti, 1998; Lee et al., 2012; Marchese et al., 2014; Mirzaa et al., 2013; Poduri et al., 2012; Rivière et al., 2012; Terrone et al., 2016). Conversely, mutations that impair growth factor signaling have been linked to symptoms of human microcephaly (Boland et al., 2007; Juanes et al., 2015). Genetically modified mice carrying corresponding mutations were shown to mimic, but frequently to a lesser extent, aspects of the phenotypes displayed by human patients. For example, although *AKT3*-activating mutations have been linked to human macrocephaly and polymicrogyria (Lee et al., 2012; Poduri et al., 2012), *Akt3* mutant mice with increased signaling activity displayed increased brain size but otherwise normal cortical patterning with no increase in gyration (Tokuda et al., 2011). Similarly, *Pten* mutant mice also displayed macrocephaly without increased gyration (Groszer et al., 2001; Kwon et al., 2006; Lehtinen et al., 2011). Such phenotypic differences may reflect inherent species differences in signaling regulation, cellular response, and anatomical organization. Therefore, the human genetic evidence strongly suggests an intrinsic and possibly preferential dependence of human NPs on the growth factor signaling cascade.

In the present study, we investigated the direct effect of enhancing human NP proliferation on cortical formation in a 3D cerebral organoid system. We chose the *PTEN* gene because of the human genetics evidence demonstrating its function in cortical development and a substantial body of literature on its role in regulating progenitor cells of various lineages (Hill and Wu, 2009). *PTEN* heterozygous loss-of-function mutations have been associated with human macrocephaly (Butler et al., 2005; Marchese et al., 2014). In the current 3D cerebral organoid system, *PTEN* homozygous mutation was adopted to drive the activation of the phosphoinositide 3-kinase (PI3K)-AKT pathway. Indeed, deletion of the *PTEN* gene increased AKT activity in human NPs, promoted cell cycle re-entry, and transiently delayed neuronal differentiation, resulting in a marked expansion of the radial glia and intermediate progenitor population. Both human and mouse organoids that lacked *PTEN* showed a significant increase in size, but only human organoids displayed substantial surface folding. The degree of expansion and folding was regulated by further tuning the strength of AKT signaling with reduced signaling resulting in smaller and smooth organoids and increased signaling in larger and more folded organoids. These findings lend support to the notion that an increase in the proliferative potential of NPs contributes to the expansion of the human cerebral neocortex and the emergence of surface folding. Last, in light of the current global health emergency regarding Zika virus (ZIKV), we applied the expanded cerebral organoid culture system to investigate a possible link between ZIKV exposure and cortical malformations such as microcephaly and lissencephaly.

## RESULTS

### ***PTEN* Deletion Enhances Growth Factor-Dependent Proliferation in 2D-Cultured Human Neural Progenitors**

To disrupt the human *PTEN* gene, single guide RNA (sgRNA) was designed to target the first exon (Figure 1A), mimicking a recently reported macrocephalic patient mutation (Figure S1A; Marchese

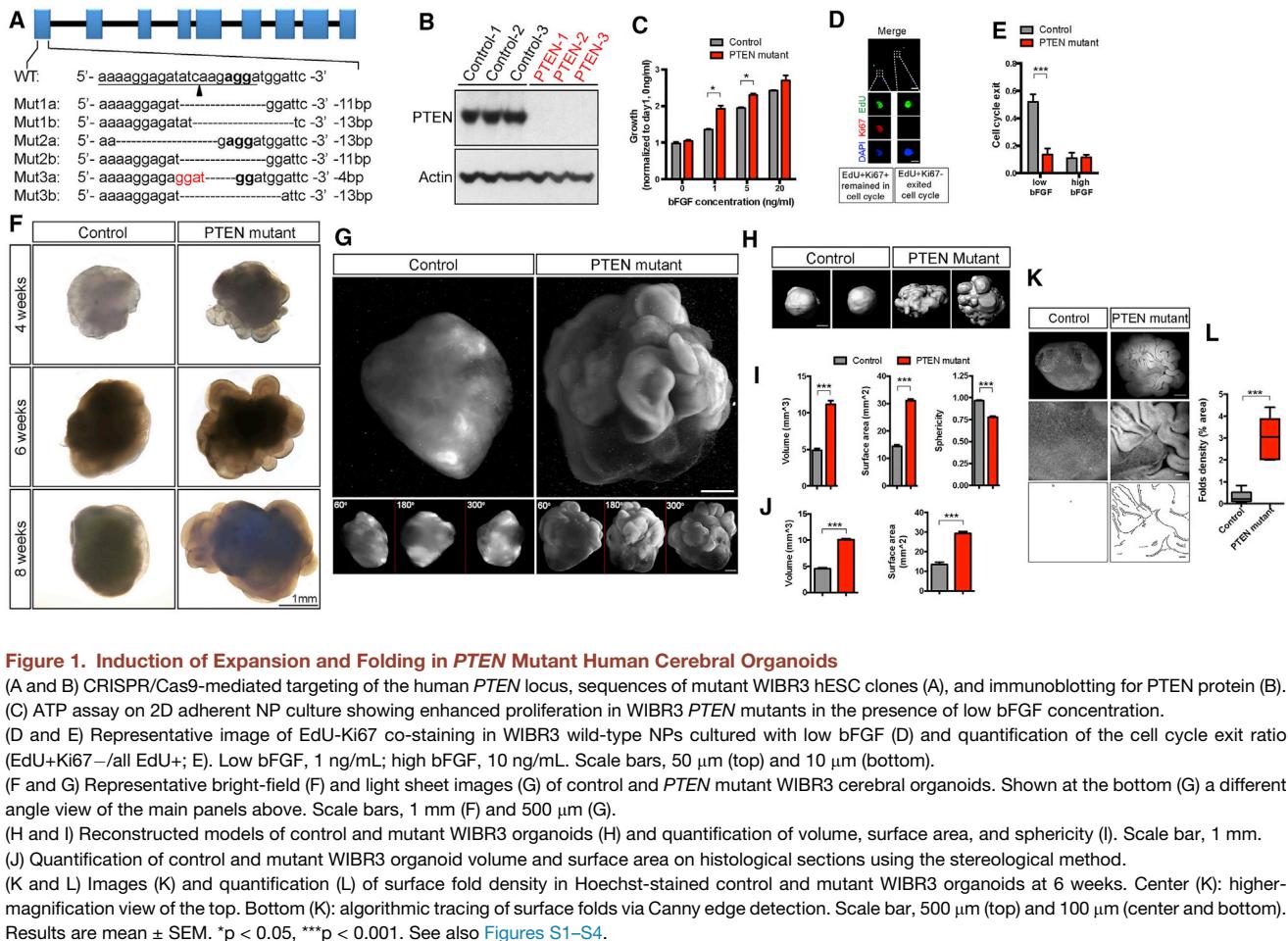
et al., 2014). WIBR3, a previously well characterized hESC line (Lengner et al., 2010), was targeted to generate homozygous frameshift mutations using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (Figure 1A). The absence of *PTEN* protein was confirmed by immunoblotting (Figure 1B). Using the same approach, two additional hESC lines (WIBR1 and WIBR2) were also targeted to generate mutant clones on these independent genetic backgrounds (Figures S1B–S1G).

*PTEN* and its downstream signaling pathway are involved in the regulation of proliferation, differentiation, and apoptosis of various stem/progenitor populations, but little is known of its function in the developing human brain. Analysis of the BrainSpan Atlas of the Developing Human Brain revealed that *PTEN* expression is low in the VZ/SVZ and high in the cortical plate, where differentiated neurons reside, a pattern inversely correlated with the progenitor markers *PAX6* and *TBR2* (Figures S1H–S1K). Similarly, in vitro-derived human NPs express less *PTEN* compared with neurons, suggesting a possible physiological involvement during normal human neural development (Figure S1L). In a 2D adherent culture system, *PTEN* mutant human NPs displayed a significantly higher growth rate in response to low basic fibroblast growth factor (bFGF) (1–5 ng/ml; Figure 1C) and a higher level of cell cycle re-entry, as revealed by 5-ethynyl-2'-deoxyuridine (EdU) pulse-chase assay and Ki67 co-staining (Figures 1D and 1E). Control and mutant NPs had similar proliferation rates in the presence of high bFGF or in the absence of bFGF (Figure 1E). Enhanced proliferation of mutant NPs was also observed in response to low concentrations of PDGF-DD and IGF2, two growth factors implicated in regulating fetal cortical growth (Lehtinen et al., 2011; Lui et al., 2014; Figures S1N and S1O).

### **Expansion and Folding of *PTEN* Mutant Human Cerebral Organoids**

To investigate the effect of *PTEN* deletion on human neural development in a more complex setting, we adopted the cerebral organoid culture system (Lancaster et al., 2013). Consistent with previous reports, hESCs, when differentiated into 3D neural tissues, grew in volume over time and displayed discrete lineage identity (Figures S2A–S2C). At 4 weeks, the organoids were composed mostly of Nestin+ NPs showing a typical radial glial morphology with processes contacting both the apical and basal surfaces of the neuroepithelium. At 8 weeks, these NPs underwent neuronal differentiation and generated a distinct layer of DCX+ neurons located exclusively away from the ventricle, consistent with the apical-basal axis of neural migration (Figure S2C). At 12 weeks and beyond, Nestin+ NPs had greatly diminished, and the organoids were mainly composed of differentiated neurons and glia (Figures S2D and S2E). In wild-type human organoids, *PTEN* gene expression was lower at earlier stages and increased as the organoids differentiated and matured (Figure S1M).

To compare organoid formation of *PTEN* mutant and isogenic control hESCs, equal numbers of dissociated single cells were seeded to form embryoid bodies (EBs). Both control and mutant WIBR3 hESCs generated EBs with comparable overall morphology after 1 week (Figure S3A). At 4 weeks, although control organoids were relatively spherical and smooth, *PTEN* mutant organoids displayed a markedly increased outgrowth



**Figure 1. Induction of Expansion and Folding in PTEN Mutant Human Cerebral Organoids**

(A and B) CRISPR/Cas9-mediated targeting of the human *PTEN* locus, sequences of mutant WIBR3 hESC clones (A), and immunoblotting for PTEN protein (B). (C) ATP assay on 2D adherent NP culture showing enhanced proliferation in WIBR3 *PTEN* mutants in the presence of low bFGF concentration. (D and E) Representative image of EdU-Ki67 co-staining in WIBR3 wild-type NPs cultured with low bFGF (D) and quantification of the cell cycle exit ratio (EdU+Ki67- / EdU+) (E). Low bFGF, 1 ng/mL; high bFGF, 10 ng/mL. Scale bars, 50 μm (top) and 10 μm (bottom). (F and G) Representative bright-field (F) and light sheet images (G) of control and *PTEN* mutant WIBR3 cerebral organoids. Shown at the bottom (G) a different angle view of the main panels above. Scale bars, 1 mm (F) and 500 μm (G). (H and I) Reconstructed models of control and mutant WIBR3 organoids (H) and quantification of volume, surface area, and sphericity (I). Scale bar, 1 mm. (J) Quantification of control and mutant WIBR3 organoid volume and surface area on histological sections using the stereological method. (K and L) Images (K) and quantification (L) of surface fold density in Hoechst-stained control and mutant WIBR3 organoids at 6 weeks. Center (K): higher-magnification view of the top. Bottom (K): algorithmic tracing of surface folds via Canny edge detection. Scale bar, 500 μm (top) and 100 μm (center and bottom). Results are mean ± SEM. \*p < 0.05, \*\*p < 0.001. See also Figures S1–S4.

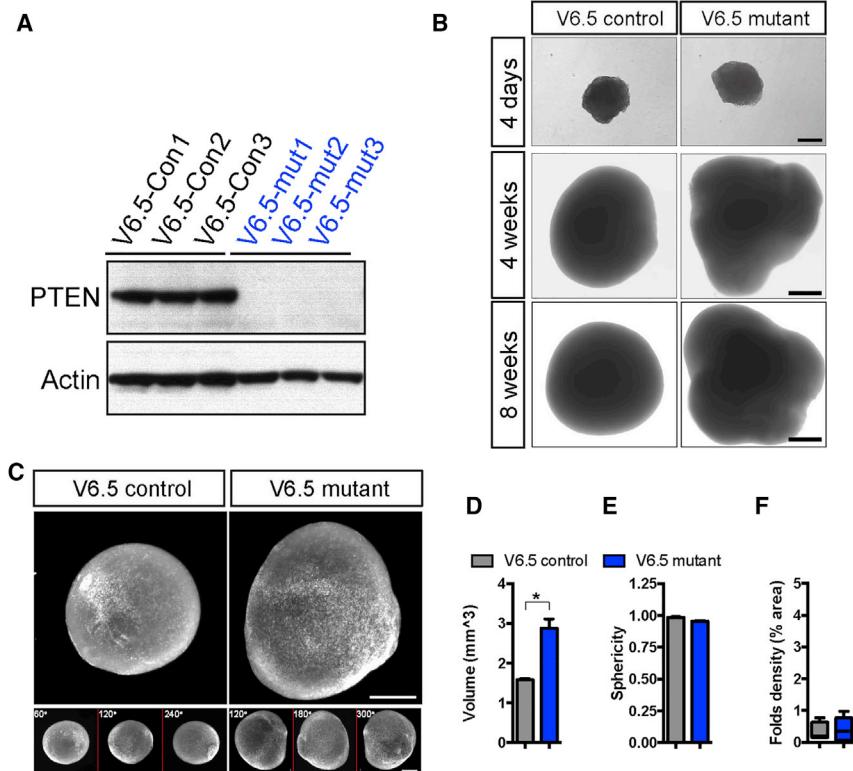
of neuroepithelial tissue surrounding ventricle-like structures (Figure 1F). Neuroepithelial overgrowth was even more evident in mutant organoids at 6 and 8 weeks and led to a drastic increase in size and an overall folded surface (Figure 1F). To better visualize and quantify their 3D morphology, we imaged the organoids on a light sheet fluorescence microscope (Figure 1G). *PTEN* mutants displayed a markedly increased surface area, overall volume, and reduced sphericity compared with controls (Figures 1G–1I). The increases in volume and surface area were separately validated on histological sections (Figure 1J; Figures S3B and S3C). Because the increased surface area was often organized in continuous folds, we further demonstrate a significant increase in fold density in mutant organoids (Figures 1K and 1L). The expansion and folding phenotype was fully penetrant and present in all WIBR3 *PTEN* mutant organoids examined across multiple independent experiments (Figures S3D and S3E) as well as in *PTEN* mutant organoids generated from WIBR1 and WIBR2 hESCs (Figures S4A–S4C).

We explored the potential difference between mouse and human cortical development by generating cerebral organoids from control and *Pten* mutant mouse embryonic stem cells (mESCs). CRISPR/Cas9 gene targeting was used to disrupt the first exon of the *Pten* gene in V6.5 mESCs (Figures S4D and S4E). Three controls and three homozygous frameshift mutants

were selected based on genomic sequence and loss of full-length PTEN protein (Figures 2A; Figure S4D). Equal numbers of dissociated single mESCs were used to generate mouse organoids, following an adapted protocol that utilizes similar media and culturing methods as that for human organoids (Eiraku et al., 2008; Lancaster et al., 2013). Compared with controls, *Pten* mutant mouse organoids became progressively larger but remained smooth throughout development with no surface folds (Figures 2B and 2C; Figure S4F). Imaging using a light sheet fluorescence microscope and subsequent quantification demonstrated that *Pten* mutant mouse organoids had increased overall volume but no change in sphericity compared with controls (Figures 2D and 2E). No detectable change in fold density was present in *Pten* mutant mouse organoids (Figure 2F).

### PTEN Deletion Expands the Progenitor Pool

We investigated the cellular organizations that underlie the expansion and folding of the *PTEN* mutant human cerebral organoids. At 4 weeks, both control and mutant human organoids were composed of units of continuous neuroepithelium, as revealed by Nestin staining. *PTEN* mutants contained an array of large and sometimes folded neuroepithelial structures (Figure 3A) that expressed NP markers such as Nestin, Pax6, and Sox2 (Figures 3B and 3C). Because the original organoid culture condition



**Figure 2. Lack of Folding in *Pten* Mutant Mouse Cerebral Organoids**

(A) Immunoblotting shows a complete ablation of wild-type PTEN protein in V6.5 mutant mouse ESCs.

(B and C) Bright-field and light sheet images of control and *Pten* mutant mouse organoids at 4 days, 4 weeks, and 8 weeks. Scale bar, 200  $\mu$ m (top in B) and 500  $\mu$ m (middle, bottom in B, and C). (D–F) Quantification of volume (D), sphericity (E), and fold density (F) on control and *Pten* mutant mouse organoids at 4 weeks.

Results are mean  $\pm$  SEM. \* $p$  < 0.05. See also Figure S4.

provides minimal patterning cues and relies on the intrinsic capacity of hESCs to differentiate into the neural lineage, the majority of the organoids adopt a forebrain identity, as shown by their expression of Pax6 (Figure 3B) and FoxG1 (Figure 7G), but markers of other brain regions can also be observed (Figure S4G). To examine whether organoid expansion and folding can occur from a brain region-specific cellular population, we pre-patterned the EBs specifically toward the dorsal forebrain fate (Li et al., 2013). Treatment with dorsomorphin during the first 7 or 14 days of culture promoted the generation of a dorsal forebrain identity and suppressed markers of other lineages (Figures 3E and 3F; Figures S4H and S4I). At 6 weeks, pre-patterned control forebrain organoids grew to a similar shape and size as un-patterned organoids. Likewise, pre-patterned *PTEN* mutant human forebrain organoids grew larger than controls and developed complex surface folds (Figures 3D and 3G; Figure S4J).

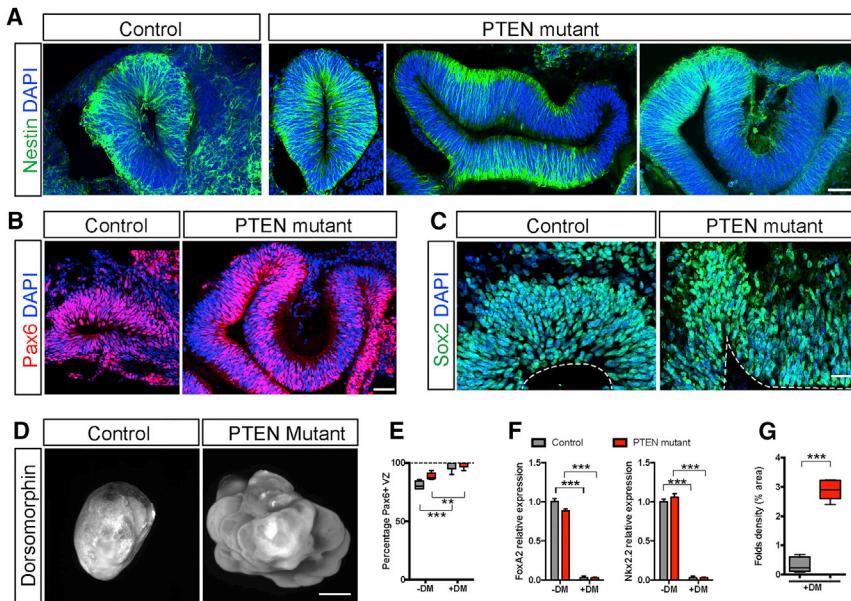
Both control and mutant human organoids contained neuroepithelia organized in a stereotypic manner reminiscent of the early developing cortex, where Ki67+ NPs proliferated at the apical surface of the ventricular zone (Figure 4A). Compared with controls, *PTEN* mutant human organoids harbored significantly more Ki67+ cells at multiple developmental time points (Figures 4A and 4B), further confirmed by qRT-PCR (Figure S5A). Increased proliferation was also observed in *PTEN* mutant organoids derived from WIBR1 and WIBR2 hESCs (Figures S5B–S5D). The enhanced proliferation was prolonged and most prominent at 4–8 weeks and declined over time, with both the control and mutant displaying minimal mitotic activity at 24 weeks (Figure S5A). To investigate the correlation of proliferation and the physical bending of the developing neuroepithe-

lium in *PTEN* mutant human organoids, we measured the distribution of Ki67+ dividing cells relative to their position. We found that areas of the neuroepithelium with an outward curvature contained a significantly higher proportion of proliferating cells compared with un-curved areas (Figures S5E and S5F). This is consistent with the notion that differential proliferation can propel physical buckling and the formation of a curved structure (Kriegstein et al., 2006). Lastly, we found a comparable number and distribution of apoptotic

cells within the VZ of control and *PTEN* mutant human organoids (Figures S5G and S5H).

In *PTEN* mutant human organoids, the increase in proliferation coincided with an expansion of the NP pool, as shown by qRT-PCR for radial glia and the intermediate progenitor markers *PAX6*, *ARHGAP11B* (Florio et al., 2015), and *TBR2* (Figures 4C and 4D; Figure S5I) and immunostaining for *PAX6*, *SOX2*, and *TBR2* (Figures 4F and 4G). Consistent with previous observations, a population of *Sox2*+ NPs detached from the apical VZ in both control and mutants, coinciding with the presence of the outer radial glia marker *HOPX* (Figures 4G and 4J). Mutant human organoids harbored significantly more *HOPX*+ outer radial glial cells; they dispersed into the expanded neuroepithelium in the radial direction (Figures 4G–4I; Figure S5J). Increased abundance of *HOPX* expression in *PTEN* mutant human organoids was further confirmed by qRT-PCR (Figure 4E). A higher proportion of the VZ and outer SVZ progenitors was labeled with Ki67 in mutant organoids (Figures 4J and 4K). Last, *PTEN* mutant human organoids at 12 weeks had increased thickness of the cortical neuroepithelium, further supporting the observation of a radial expansion (Figure 4L). These findings demonstrate that *PTEN* ablation led to expansion of the human NP pool in both the VZ and SVZ.

In contrast to *PTEN* mutant human organoids, *Pten* mutant mouse organoids, which were morphologically smooth, displayed a transient elevation of proliferation over controls, but only within a narrow developmental window (Figures 4M and 4N; Figure S5K). This restricted proliferative capacity of mouse NPs coincides with the smaller steady-state sizes of the mouse organoids compared with human organoids (Figures 1I and 2D). *HOPX* immunopositivity was absent in mouse



**Figure 3. PTEN Deletion Induces Expansion and Folding of Forebrain Neuroepithelia in Human Cerebral Organoids**

(A–C) Immunostaining for NPs using antibodies against Nestin (A), Pax6 (B), and Sox2 (C) in control and mutant WIBR3 organoids at 4 weeks. Scale bars, 50  $\mu$ m (A and B) and 20  $\mu$ m (C).

(D–G) Light sheet images and quantification of control and mutant WIBR3 organoids treated with dorsomorphin (DM) for 14 days, demonstrating expansion and folding (D), increased fold density (G), patterning toward the forebrain fate (E) at the preclusion of non-forebrain lineages (F).

Results are mean  $\pm$  SEM. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001. See also Figure S4.

organoids (Figure 4O). Together, these findings highlight intrinsic disparities between mouse and human NPs in proliferation potential and cellular composition.

### PTEN Deletion Elicits a Transient Delay in Neuronal Differentiation

A hallmark difference between primate and rodent cortical development is the duration of proliferation, with primate NPs undergoing many more rounds of cell division prior to committing to terminal differentiation (Kornack and Rakic, 1998). We investigated whether the increased proliferation in *PTEN* mutant human organoids was coupled with changes in differentiation propensity. At 4 weeks, neuronal differentiation was initiated in control organoids, and DCX+ neurons began to emerge (Figure 5A). In contrast, mutant organoids contained predominantly Nestin+ NPs with few neurons (Figure 5A). qRT-PCR and immunoblotting confirmed that mutant organoids had significantly reduced DCX RNA and protein (Figures S6A–S6C). An EdU pulse-chase experiment on 4-week-old organoids showed that mutants had significantly less cell cycle exit after 24 hr (Figures 5B and 5C). A majority of EdU+ cells in mutant organoids were Sox2+ NPs, in contrast to controls, where a substantial fraction had become DCX+ (Figures 5D–5F). A similar finding of delayed neuronal differentiation was seen in *PTEN* mutant human organoids on the WIBR1 and WIBR2 background (Figure S6D). In contrast, DCX+ or Ctip2+ neurons were already abundantly present in 3-week-old mouse control and *Pten* mutant organoids (Figure 4O; Figure S6E).

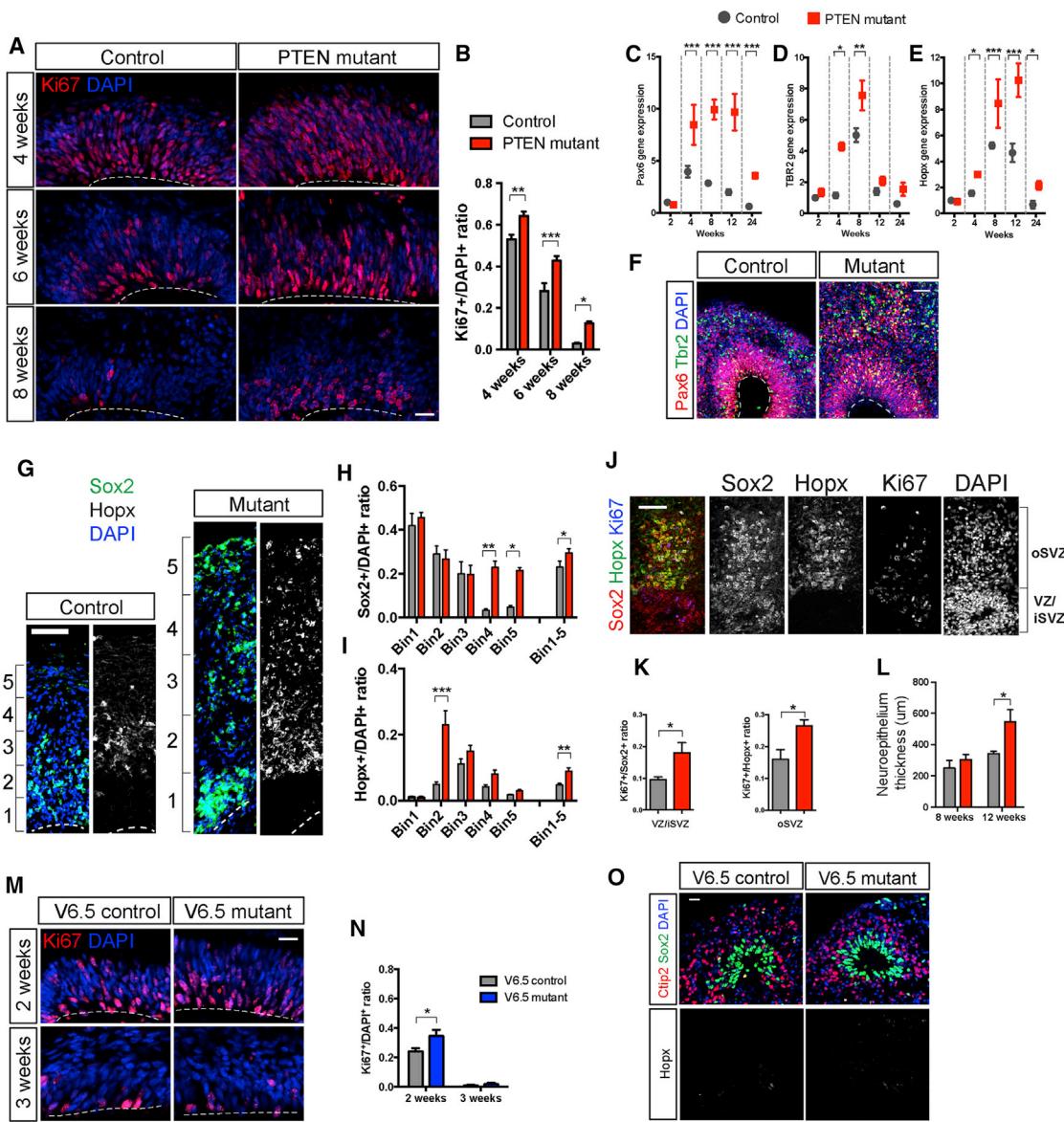
This delay in neuronal differentiation in *PTEN* mutant human organoids was transient and gradually normalized. At 8 weeks, abundant DCX+ neurons were present, and the majority of cells labeled with EdU at 4 weeks had assumed a neuronal phenotype in both controls and mutants (Figures 5G and 5H; Figures S6F and S6H). A small population of EdU+ cells co-expressed HOPX and resided in the outer SVZ, suggesting their identity as outer radial glia (Figures S6G and S6I). Transcriptional analysis of a panel of genes enriched in NPs and neurons demon-

strated reduced expression of neuronal markers in mutants at 4 weeks but normalized expression over time at 10 and 16 weeks (Figures 5I–5K). At 12 weeks, both control and mutant human organoids harbored outer radial glia cells, early- and late-born neurons that were organized in a rudimentary inside-out pattern (Figures 5L–5N). In mature control and mutant human organoids, differentiated neuronal cell types were predominant, and the level of proliferation was diminished (Figure 5K; Figure S5A). At these ages, *PTEN* mutant organoids sustained a folded morphology, underlined by the presence of folded cortical plates composed of differentiated neurons and glia (Figure 5O). We conclude that a transient delay in differentiation allowed amplification of the NP pool and contributed to the expansion and folding of the *PTEN* mutant human organoids.

### AKT Activation Mediates the Expansion and Folding of *PTEN* Mutant Human Cerebral Organoids

To confirm that *PTEN* deficiency was responsible for the phenotypes, we transduced mutant hESCs with a lentivirus vector encoding GFP or a *PTEN*-GFP fusion protein (Figures 6A and 6B) and generated organoids. At 6 weeks, although GFP-transduced mutant human organoids were expanded and folded, *PTEN*-GFP-transduced mutants formed smooth organoids (Figure 6C; Figure S7A) and showed normalized levels of proliferation and neuronal differentiation (Figures S7B–S7D).

AKT activation is a well-documented downstream effect of *PTEN* deletion (Worby and Dixon, 2014). *PTEN*, *AKT1*, *AKT2*, and *AKT3* are expressed in the developing human cortex (Figures S1H and S7E). In control human organoids, phospho-AKT (pAKT) could be detected, albeit at a relatively low level (Figures S7F and S7H). *PTEN* deletion led to a significant upregulation of pAKT (Figure 6D; Figures S7F–S7I). In both control and mutants, pAKT-positive staining was enriched in the VZ and co-localized with progenitor markers such as Nestin, Sox2, and phospho-Vimentin (Figure 6D; Figures S7F and S7G). In mature human organoids comprised of mostly differentiated neurons, the pAKT level was reduced but could still be detected with tyramide signal-amplified immunostaining as well as immunoblotting (Figures S7J and S7L). In 12-week-old organoids, acute exposure to exogenous BDNF elicited a rapid

**Figure 4. PTEN Deletion Enhances Proliferation and Expands the NP Pool**

(A and B) Representative images (A) and quantification (B) of Ki67 immunostaining in control and *PTEN* mutant WIBR3 cerebral organoids. Scale bar, 20 μm.

(C–E) Quantitative RT-PCR analysis of the radial glial marker Pax6 (C), the intermediate progenitor marker Tbr2 (D), and the outer radial glia marker Hopx (E) demonstrates their temporally specific overexpression in *PTEN* mutant WIBR3 organoids compared with controls.

(F) Immunostaining for Pax6 and Tbr2 shows increased numbers of positive cells in *PTEN* mutant WIBR3 organoids.

(G–I) Representative images of the expanded neuroepithelium in 12-week-old control and *PTEN* mutant WIBR3 organoids stained with Sox2 and the outer radial glia marker Hopx. Quantification was conducted by measuring and dividing the entire span of the neuroepithelium into five equal portions (bins), showing significantly more Sox2+ (H) and Hopx+ (I) cells in *PTEN* mutant WIBR3 organoids in the expanded neuroepithelium. Scale bar, 100 μm.

(J and K) Representative images of Sox2, Hopx, and Ki67 co-staining in a *PTEN* mutant WIBR3 organoid (J) and quantification showing increased proliferation of VZ/ISVZ (inner sub-ventricular zone) as well as oSVZ (outer sub-ventricular zone) NPs in *PTEN* mutants compared with controls (K).

(L) Total neuroepithelial thickness at 8 and 12 weeks in control and *PTEN* mutant WIBR3 organoids.

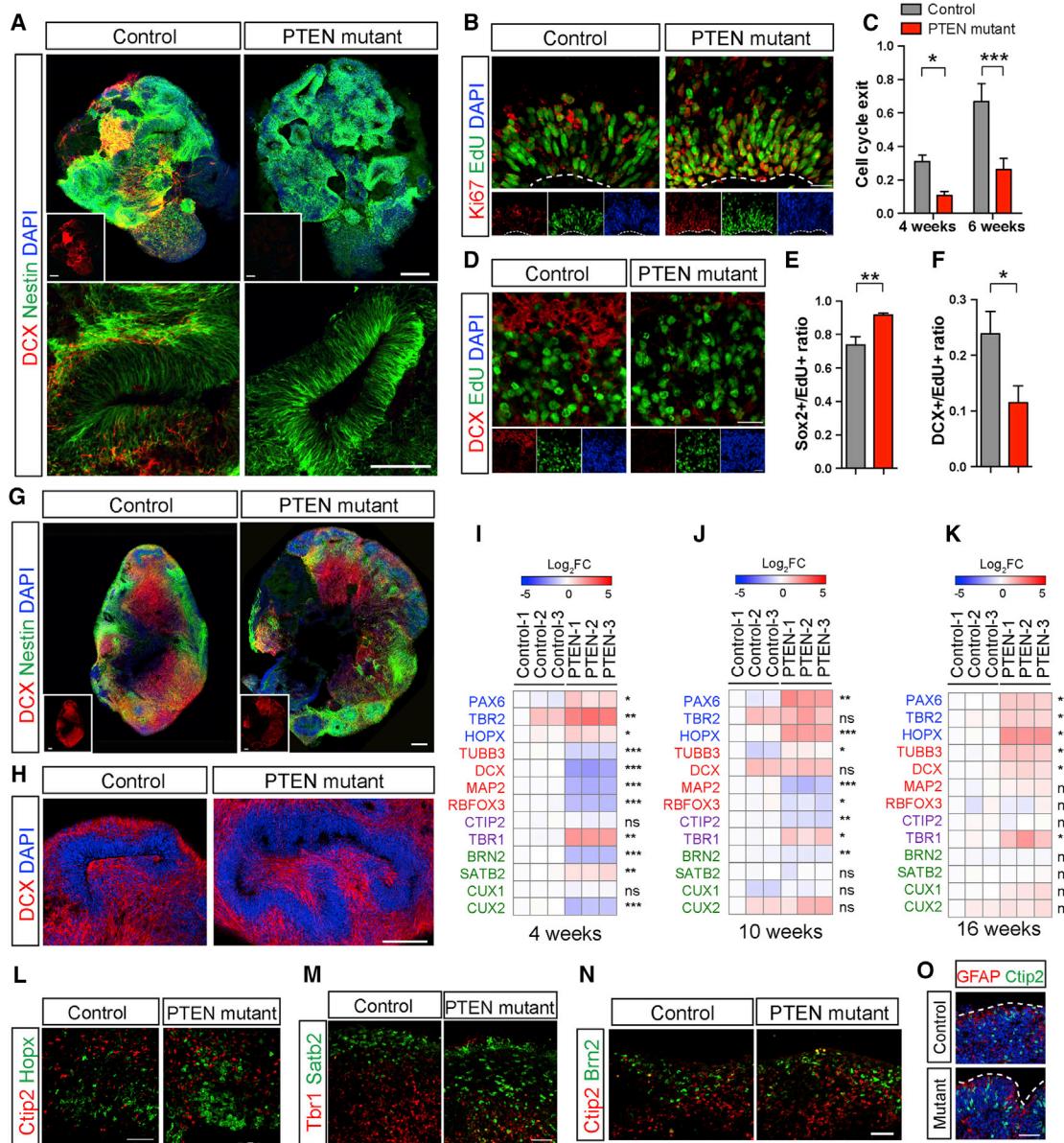
(M and N) Representative images (M) and quantification (N) of Ki67 immunostaining in control and *Pten* mutant mouse cerebral organoids. Scale bar, 20 μm.

(O) Immunostaining for the NP marker Sox2, the outer radial glia marker Hopx, and the neuronal marker Ctip2 in 3-week-old control and *Pten* mutant mouse organoids, showing lack of Hopx immunopositivity. Scale bar, 20 μm.

Results are mean ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S5.

elevation in AKT signaling, suggesting that scarcity of relevant neurotrophic factors in the culture medium may contribute to the reduced baseline pAKT activity (Figures S7K and S7L). This

transient and cell type-specific activation of AKT signaling in NPs echoed the pattern of enhanced proliferation in *PTEN* mutant human organoids and strongly indicated that increased

**Figure 5. Transient Delay in Neuronal Differentiation Expands the NP Pool**

(A) Immunostaining for DCX and Nestin at 4 weeks in control and PTEN mutant WIBR3 cerebral organoids. Scale bar, 200 μm.

(B and C) Representative images of EdU-Ki67 co-staining in 4-week-old control and mutant WIBR3 organoids (B) and quantification of the cell cycle exit ratio (EdU+/Ki67-/all EdU+) (C) in 4- and 6-week-old organoids. Scale bars, 20 μm.

(D) Immunostaining for DCX and EdU in 4-week-old control and mutant WIBR3 organoids. Scale bars, 20 μm.

(E and F) Quantitative cell fate analysis of EdU+ cells in 4-week-old organoids, showing that PTEN mutant EdU+ cells have increased retention as Sox2+ NPs (E) and a decreased propensity for differentiation into DCX+ immature neurons (F).

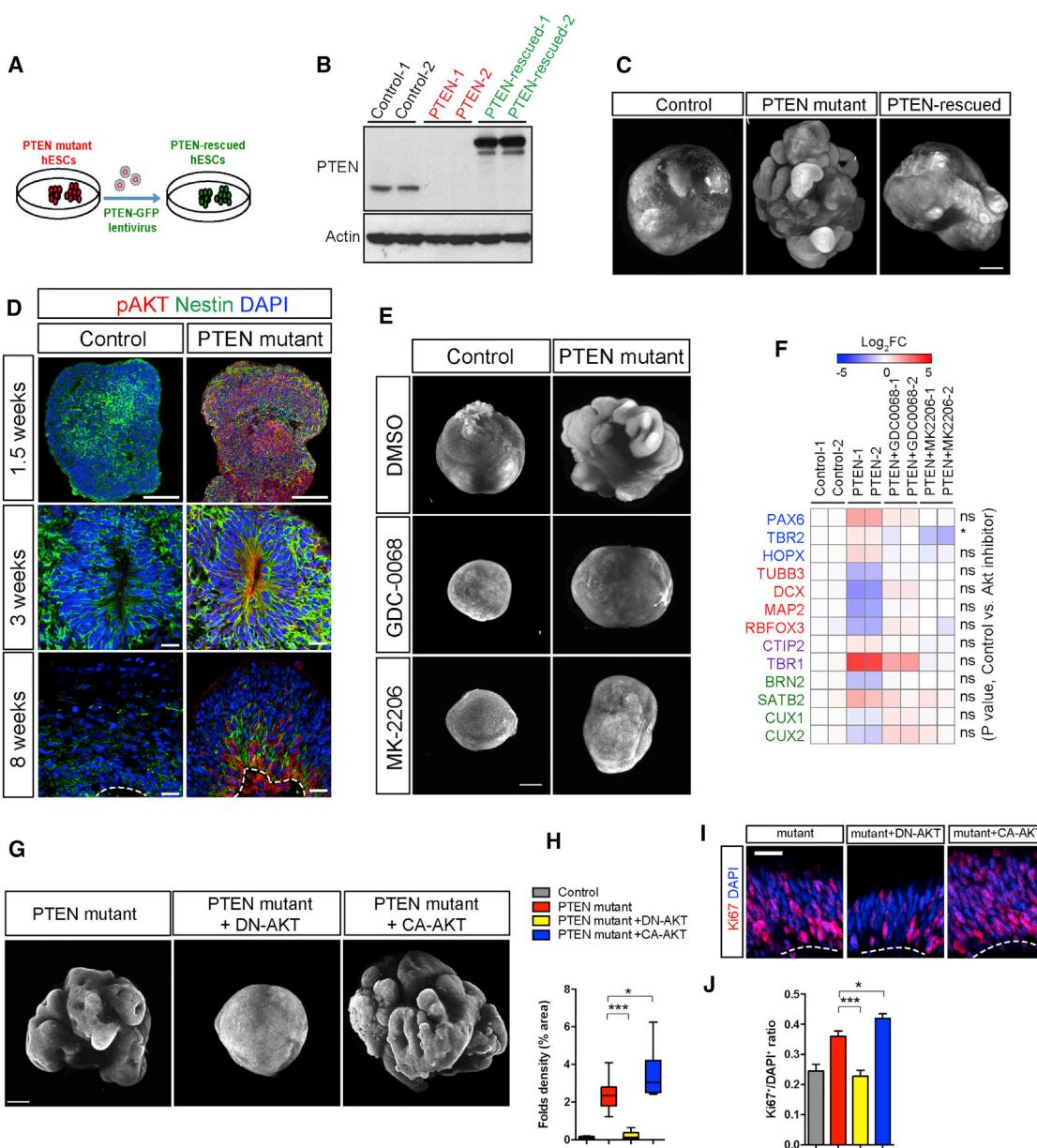
(G and H) Immunostaining for DCX (G and H) and Nestin (G) at 8 weeks. Scale bar, 200 μm.

(I–K) Differential gene expression analyses by qRT-PCR on control and mutant WIBR3 organoids at 4 (I), 10 (J), and 16 (K) weeks. Genes analyzed are representative of NPs (PAX6, TBR2, and HOPX), pan-neuronal markers (TUBB3, DCX, MAP2, and RBFOX3), early-born neurons (CTIP2 and TBR1), and late-born neurons (BRN2, SATB2, CUX1, and CUX2). The majority of neuronal markers were transiently downregulated in PTEN mutants at 4 weeks but greatly normalized at 16 weeks. Gene expressions are normalized to control-1 at each time point. The p value reflects controls versus mutants.

(L–N) Immunostaining for markers of outer radial glia (Hopx, L), early-born neurons (Tbr1 [M] and Ctip2 [N]), and late-born neurons (Satb2 [M] and Brn2 [N]) in control and mutant WIBR3 organoids at 12 weeks. Scale bars, 50 μm.

(O) PTEN mutant WIBR3 organoids had a folded cortical plate at 16 weeks. The images show immunostaining for markers of neurons (Ctip2) and astrocytes (GFAP). White dashed lines outline the cortical surface. Scale bars, 50 μm.

Results are mean ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S6.



**Figure 6. PTEN-AKT Signaling Controls Expansion and Folding in Human Cerebral Organoids**

- (A) Schematic depicting the strategy to rescue PTEN mutant WIBR3 hESCs via lentivirus re-expression of PTEN.
- (B) Immunoblotting for PTEN shows the presence of PTEN-GFP fusion protein in rescued PTEN mutant WIBR3 hESCs.
- (C) Light sheet images of Hoechst-stained WIBR3 organoids at 6 weeks, generated from control, PTEN mutant, and PTEN-rescued hESCs. Scale bar, 500  $\mu$ m.
- (D) Immunostaining images of pAKT and Nestin in control and mutant WIBR3 organoids. Increased pAKT immunostaining is present in mutants at 1.5 weeks, prior to the onset of expansion and folding (top). Increased pAKT signal concentrates in Nestin+ NPs and is highest at the apical surface. Scale bars, 100  $\mu$ m (top) and 20  $\mu$ m (center and bottom).
- (E) Light sheet images of control and mutant WIBR3 organoids at 6 weeks, treated with AKT inhibitors GDC-0068 (1uM) or MK-2206 (100nM). Scale bar, 500um.
- (F) Differential gene expression analyses by quantitative RT-PCR on control and mutant WIBR3 organoids, treated with AKT inhibitors GDC-0068 (1uM) or MK-2206 (100nM). Gene expressions are normalized to control-1. P value reflects controls versus AKT inhibitor treated mutants.
- (G) Light sheet images of 6-week-old human organoids generated from PTEN mutant WIBR3 alone or transduced with a lentivirus encoding dominant negative (DN)-AKT or constitutive active (CA)-AKT. Scale bars, 500um.
- (H) Quantitative analysis of the density of surface folds in 6-week-old organoids from PTEN mutant alone, or PTEN mutant transduced with DN-AKT or CA-AKT.
- (I and J) Representative images (I) and quantification (J) of Ki67 immunostaining in 6-week-old organoids from PTEN mutant alone or PTEN mutant transduced with DN-AKT or CA-AKT. Scale bar, 20  $\mu$ m.
- Results are mean  $\pm$  SEM. \*p < 0.05, \*\*\*p < 0.001. See also Figure S7.

AKT signaling drove the expansion of the NP pool and, consequently, the expansion and folding of cortical tissue.

To directly investigate whether AKT activation was responsible for the expansion and folding phenotypes, we treated developing *PTEN* mutant human organoids with GDC-0068 or MK-2206, known inhibitors of AKT activation. Although both inhibitors at higher concentrations were deleterious for cellular growth and prevented the formation of viable EBs, lower concentrations allowed the generation of mutant human organoids with comparable size and morphology as non-treated controls (Figure 6E) but blocked the formation of expanded and folded organoids, accompanied by normalized proliferation and neural marker gene expression (Figures 6E and 6F; Figure S7M). Furthermore, a lentivirus-transduced dominant negative form of *AKT* normalized proliferation and blocked the formation of folded neuroepithelium in mutant organoids (Figures 6G–6J). In contrast, a constitutive active form of *AKT* further enhanced proliferation as well as the expansion of neuroepithelium to form organoids with even greater size and surface complexity than the *PTEN* mutant alone (Figures 6G–6J). These findings demonstrate that AKT signaling exerts an essential, tunable control on the expansion and folding of cortical neuroepithelium.

### ZIKV Infection Impairs Expansion and Folding in *PTEN* Mutant Cerebral Organoids

The generation of cerebral organoids with more complex, human-like structures enhances our ability to model brain malformation because of genetic or environmental causes. To that end, we applied our 3D culture system to study the consequence of ZIKV infection. Although the underlying mechanism remains to be elucidated, the recent outbreak of ZIKV in Central and South America is associated with severe teratogenic consequences. Accumulating evidence indicates that affected fetuses and newborns display a plethora of neurological abnormalities, including microcephaly, lissencephaly, pachygyria, and cortical atrophy (Hazin et al., 2016). Although recent studies in 2D and 3D human neural cultures have demonstrated the tropism of ZIKV toward NPs and its effect on organoid formation (Cugola et al., 2016; Garcez et al., 2016; Onorati et al., 2016; Qian et al., 2016; Tang et al., 2016), none utilized a 3D culture system that mimics key developmental features of the human cortex, such as expansion and surface folding.

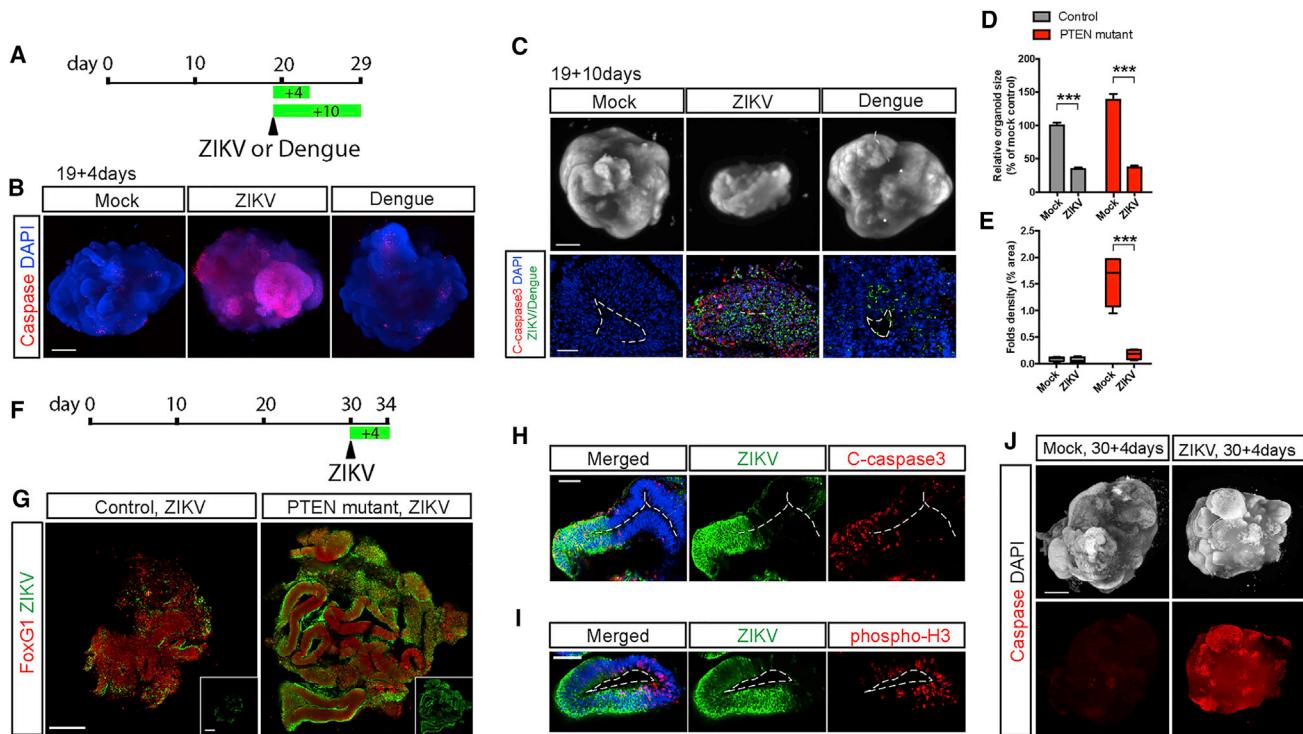
ZIKV exposure during early pregnancy is correlated with risks of major brain malformation, such as microcephaly and lissencephaly. In organoids, ZIKV infection at the onset of surface folding (day 19) leads to widespread initiation of apoptosis (Figures 7A and 7B). 10 days post infection, ZIKV severely hampered organoid growth both in size and surface folding (Figures 7C–7E). Cellular analysis revealed broad detection of viral envelope protein in infected organoids, coinciding with marker of apoptosis (Figure 7C). In contrast, infection with an equal amount of Dengue virus did not trigger increased apoptosis or broad viral amplification (Figures 7B and 7C). Therefore, ZIKV exposure at this early stage severely disrupted organoid expansion and fold formation. We next examined the effect of ZIKV on 4-week-old organoids, when neuronal differentiation is initiated in control organoids and delayed in mutants (Figure 5A). Although a low amount of ZIKV led to partial infection of control organoids at this stage, *PTEN* mutant organoids displayed a

markedly increased susceptibility to infection (Figures 7F and 7G). Regions of organoid expansion and folding were amply infected with ZIKV (Figure 7G) and correlated with increased apoptosis (Figure 7H) and decreased proliferation (Figure 7I). Staining for activated caspase on whole mutant organoids further demonstrated a global increase in apoptosis 4 days after ZIKV exposure (Figure 7J). Together, these findings demonstrate the profound consequences of ZIKV exposure during early neural development on the expansion and folding of the developing cortex *in vitro* and illustrate the strength of the genetically modified cerebral organoid system in modeling complex structural malformations of the developing human brain.

### DISCUSSION

Efforts in the investigation of human brain development have been hindered by a lack of access to the fetal human brain. Given the substantial species difference between humans and the currently available model organisms pertinent to cortical morphology and function, it is essential to develop an alternative *in vitro* system that recapitulates key features of human cortical formation. Here, using a genetically modified cerebral organoid system, we address the direct consequences of increased proliferation in human cortical development. Compared with control organoids, *PTEN* mutant human organoids develop a markedly expanded VZ/SVZ, increased overall size, and a larger surface area organized into continuous cortical folds. This *in vitro* expansion is triggered by increased proliferation, transiently delayed neuronal differentiation, and, thereby, an amplified overall NP population. Such amplification abides by the cellular and anatomical identity of the human developing cortex and results in a greater number of NPs first in the VZ and, subsequently, in the outer SVZ. In *PTEN* mutant human organoids, the genetically modified neural tissue expands tangentially to generate longer sheets of neuroepithelium and radially to form thicker neuroepithelium. Such modes of *in vitro* structural expansion are consistent with the radial unit hypothesis of neocortical expansion (Rakic, 1988), suggesting that increased proliferation contributes to folding. We envision future work to incorporate spatially and temporally specific control of the *PTEN*-AKT signaling pathway to drive NP proliferation in an inducible and reversible manner, allowing the precise dissection of the contribution of tangential and radial expansion to cortical expansion and folding.

The comparison of mouse and human organoids revealed a marked species difference in the capacity of developing expanded and folded neural tissues. In contrast to the drastic effects of *PTEN* deletion in human organoids, *Pten* mutant mouse organoids grew larger but did not form surface folds, coinciding with a short window of enhanced proliferation and a lack of an outer SVZ. The smooth appearance of mouse *Pten* mutant organoids echoes the lack of cortical folding in *Pten* mutant mouse brains, further indicating that features inherent to human NPs, such as the capacity of a prolonged period of proliferation and the generation of an outer radial glia population, may underlie human-specific cortical tissue expansion and folding. Recent studies in mice demonstrated that overexpression of *ARHGAP11B*, a human-specific gene enriched in the NPs of the developing cortex, can endow mouse NPs with enhanced proliferation, leading to the emergence of cortical folding (Florio



**Figure 7. ZIKV Infection Impairs Expansion and Folding in Human Cerebral Organoids**

(A and B) Schematic (A) and light sheet images (B) of PTEN mutant WIBR3 cerebral organoids on day 23 (19 + 4 days) showing widespread caspase activity induced by ZIKV but not Dengue virus. Scale bar, 500  $\mu$ m.

(C) Light sheet images and immunostaining of mutant WIBR3 organoids on day 29 (19 + 10 days) show reduced organoid size and increased apoptosis caused by ZIKV but not Dengue virus. C-caspase 3, cleaved caspase 3 as detected by immunostaining. Scale bars, 500  $\mu$ m (top) and 50  $\mu$ m (bottom).

(D and E) Quantitative analysis of organoids on day 29 (19 + 10 days) shows reduced size (D) and loss of surface fold density (E) upon ZIKV exposure.

(F and G) Schematic (F) and representative images (G) of immunostaining for ZIKV-infected control and mutant WIBR3 organoids on day 34 (30 + 4 days). Scale bar, 200  $\mu$ m.

(H and I) Representative images of immunostaining in mutant WIBR3 organoids show that ZIKV infection coincides with elevated apoptosis (cleaved caspase 3, H) and reduced proliferation (phosphorylated H3, I). Scale bars, 50  $\mu$ m.

(J) Light sheet images show that mutant WIBR3 organoids treated with ZIKV on day 30 displayed widespread apoptosis, as revealed by whole-mount caspase activity staining. Scale bar, 500  $\mu$ m.

Results are mean  $\pm$  SEM. \*\*\*p < 0.001.

et al., 2015). These findings raise the possibility that further genetic engineering may shed light on the mechanism and plasticity of mouse and human cortical development.

Our study highlights the essential role for the PTEN-AKT signaling pathway in regulating human neural development. Gain- and loss-of-function mutations of genes in this pathway have been reported in patients with major brain malformation (Boland et al., 2007; DiLiberti, 1998; Juanes et al., 2015; Lee et al., 2012; Marchese et al., 2014; Mirzaa et al., 2013; Poduri et al., 2012; Rivière et al., 2012; Terrone et al., 2016), suggesting that a precise level of AKT activity is pivotal to brain formation. Our study in human organoids demonstrates that AKT activity is enriched in the NP population in both control and mutants. Inhibition of AKT activity via pharmacological or genetic means results in smaller organoids, reminiscent of microcephaly in patients with AKT heterozygous loss-of-function mutation (Boland et al., 2007). We note that, unlike NPs, PTEN mutant mature neurons within the organoids display less AKT activity. Acute stimulation with BDNF was sufficient to increase AKT activity, suggesting the reduced basal AKT activity may stem from the

lack of neurotrophic growth factors in the current culture system. Future investigation of the effects of AKT activation in response to various trophic factors in mature human neurons could provide novel insights into normal and pathological neural functions. Another limitation of the current culture system is that it inadequately reflects human fetal brain development because it does not support the formation of folded non-mutant human cerebral organoids. This is not surprising, considering its historical origin from the minimal culture media aimed at supporting the survival and differentiation of mouse NPs and EBs (Eiraku et al., 2008). We found that activating the PTEN-AKT pathway, a central signaling cascade downstream of various growth factors, is sufficient to induce folding. Interestingly, embedding in an extracellular matrix (Matrigel) rich in growth factors spurs a rapid yet transient phase of growth in both wild-type and mutant human organoids. Thus, it is likely that a combination of physiologically relevant growth factors could also induce folding in wild-type human organoids. Further microfluidic and microfabrication designs can be implemented to improve oxygen and nutrient exchange, maintain tissue health and homeostasis,

and provide more faithful recapitulation of the *in vivo* developmental process.

In the current study, we utilized an isogenic genetic system to demonstrate that the cerebral organoid system allows experimental manipulation of distinct, isolated driving forces of cortical development such as proliferation. This experimental framework can be broadly utilized to model other genetic and environmental factors that determine the size and shape of a developing human cortex and explore pathological cortical formation such as microcephaly, macrocephaly, polymicrogyria, lissencephaly, and double cortex syndrome. We used this system to model microcephaly and lissencephaly resulting from ZIKV exposure. Our findings revealed major organoid growth and folding defects because of ZIKV but not Dengue virus exposure, indicating a strong pathological difference between these two closely related flaviviruses.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - ESC culture
- METHOD DETAILS
  - Neural differentiation
  - CRISPR/Cas9 gRNA design
  - CRISPR/Cas9-mediated genome editing of ESCs
  - Cerebral organoid culture
  - Lentivirus production and transduction
  - ZIKV and Dengue virus production and infection
  - ATP assay
  - EdU click-it assay
  - Histology and imaging
  - RNA extraction, reverse transcription and quantitative PCR
  - Protein purification and immuno-blotting
  - Analysis of BrainSpan data
- QUANTIFICATION AND STATISTICAL ANALYSIS

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2016.11.017>.

## AUTHOR CONTRIBUTIONS

Y.L., J.M., and R.J. conceived the project, designed the experiments, interpreted the results, and wrote the paper with input from all authors. A.O. participated in cell culture, sample preparation, and data analysis. I.B. and L.G. provided Zika and Dengue viruses for the study and participated in the design of the virus infection experiments. M.A.L., J.A.K., and M.S. advised on the study design and helped establish the organoid culture system.

## ACKNOWLEDGMENTS

The authors thank Dongdong Fu, Raaji Alagappan, Tenzin Lungjangwa, Sean Corcoran, Laure Freland, and Grisilda Bakiasi for technical support and Danielle Feldman and members of the Jaenisch lab for helpful discussions. We thank Magdalena Renner for advice on cerebral organoid cultures; Wendy

Salmon, Dr. Christian Cortes Campos, and Dr. Ryann Farn for help with light sheet imaging; Dr. Bryan Luikart for the *PTEN-GFP* plasmid; and Dr. Michael Robinson for the *AKT* plasmids. Y.L. received funding from a Simons Foundation postdoctoral fellowship, an International Rett Syndrome Foundation postdoctoral fellowship, and a NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation. J.M. received support from the European Leukodystrophy Association and a NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation. A.O. was supported by a Jerome and Florence Brill Graduate Student Fellowship. Work in the R.J. laboratory was supported by grants from the Simons Foundation (SFARI 204106 and SFLIFE 286977); NIH Grants HD 045022, R01-MH104610, and R01-NS088538; the ELA Foundation; the Emerald Foundation; Biogen; and the Simons Center for the Social Brain at MIT. Work in the L.G. laboratory was supported by NIH AI100190. R.J. is an advisor to Stemgent and a cofounder of Fate Therapeutics and Fulcrum Therapeutics.

Received: September 6, 2016

Revised: October 24, 2016

Accepted: November 29, 2016

Published: December 29, 2016

## REFERENCES

- Bae, B.I., Tietjen, I., Atabay, K.D., Evrony, G.D., Johnson, M.B., Asare, E., Wang, P.P., Murayama, A.Y., Im, K., Lisgo, S.N., et al. (2014). Evolutionarily dynamic alternative splicing of GPR56 regulates regional cerebral cortical patterning. *Science* 343, 764–768.
- Boland, E., Clayton-Smith, J., Woo, V.G., McKee, S., Manson, F.D., Medne, L., Zackai, E., Swanson, E.A., Fitzpatrick, D., Millen, K.J., et al. (2007). Mapping of deletion and translocation breakpoints in 1q44 implicates the serine/threonine kinase AKT3 in postnatal microcephaly and agenesis of the corpus callosum. *Am. J. Hum. Genet.* 81, 292–303.
- Butler, M.G., Dasouki, M.J., Zhou, X.P., Talebizadeh, Z., Brown, M., Takahashi, T.N., Miles, J.H., Wang, C.H., Stratton, R., Pilarski, R., and Eng, C. (2005). Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. *J. Med. Genet.* 42, 318–321.
- Bystron, I., Blakemore, C., and Rakic, P. (2008). Development of the human cerebral cortex: Boulder Committee revisited. *Nat. Rev. Neurosci.* 9, 110–122.
- Chenn, A., and Walsh, C.A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* 297, 365–369.
- Cugola, F.R., Fernandes, I.R., Russo, F.B., Freitas, B.C., Dias, J.L., Guimarães, K.P., Benazzato, C., Almeida, N., Pignatari, G.C., Romero, S., et al. (2016). The Brazilian Zika virus strain causes birth defects in experimental models. *Nature* 534, 267–271.
- DiLiberti, J.H. (1998). Inherited macrocephaly-hamartoma syndromes. *Am. J. Med. Genet.* 79, 284–290.
- Eiraku, M., Watanabe, K., Matsuo-Takahashi, M., Kawada, M., Yonemura, S., Matsumura, M., Wataya, T., Nishiyama, A., Muguruma, K., and Sasai, Y. (2008). Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* 3, 519–532.
- Florio, M., Albert, M., Taverna, E., Namba, T., Brandl, H., Lewitus, E., Haffner, C., Sykes, A., Wong, F.K., Peters, J., et al. (2015). Human-specific gene ARHGAP11B promotes basal progenitor amplification and neocortex expansion. *Science* 347, 1465–1470.
- Fricano, C.J., Despensa, T., Jr., Frazel, P.W., Li, M., O'Malley, A.J., Westbrook, G.L., and Luikart, B.W. (2014). Fatty acids increase neuronal hypertrophy of Pten knockdown neurons. *Front. Mol. Neurosci.* 7, 30.
- Garcez, P.P., Loiola, E.C., Madeiro da Costa, R., Higa, L.M., Trindade, P., Delvecchio, R., Nascimento, J.M., Brindeiro, R., Tanuri, A., and Rehen, S.K. (2016). Zika virus impairs growth in human neurospheres and brain organoids. *Science* 352, 816–818.
- Geschwind, D.H., and Rakic, P. (2013). Cortical evolution: judge the brain by its cover. *Neuron* 80, 633–647.

- Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X., and Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene *in vivo*. *Science* 294, 2186–2189.
- Hansen, D.V., Lui, J.H., Parker, P.R., and Kriegstein, A.R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* 464, 554–561.
- Hazin, A.N., Poretti, A., Turchi Martelli, C.M., Huisman, T.A., Di Cavalcanti Souza Cruz, D., Tenorio, M., van der Linden, A., Pena, L.J., Brito, C., Gil, L.H., et al.; Microcephaly Epidemic Research Group (2016). Computed Tomographic Findings in Microcephaly Associated with Zika Virus. *N. Engl. J. Med.* 374, 2193–2195.
- Hill, R., and Wu, H. (2009). PTEN, stem cells, and cancer stem cells. *J. Biol. Chem.* 284, 11755–11759.
- Juanes, M., Guercio, G., Marino, R., Berensztein, E., Warman, D.M., Ciaccio, M., Gil, S., Bailez, M., Rivarola, M.A., and Belgorosky, A. (2015). Three novel IGF1R mutations in microcephalic patients with prenatal and postnatal growth impairment. *Clin. Endocrinol. (Oxf.)* 82, 704–711.
- Kornack, D.R., and Rakic, P. (1998). Changes in cell-cycle kinetics during the development and evolution of primate neocortex. *Proc. Natl. Acad. Sci. USA* 95, 1242–1246.
- Kriegstein, A., Noctor, S., and Martínez-Cerdeño, V. (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat. Rev. Neurosci.* 7, 883–890.
- Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R.A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384, 368–372.
- Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., and Flavell, R.A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 94, 325–337.
- Kwon, C.H., Luikart, B.W., Powell, C.M., Zhou, J., Matheny, S.A., Zhang, W., Li, Y., Baker, S.J., and Parada, L.F. (2006). Pten regulates neuronal arborization and social interaction in mice. *Neuron* 50, 377–388.
- Lambeth, C.R., White, L.J., Johnston, R.E., and de Silva, A.M. (2005). Flow cytometry-based assay for titrating dengue virus. *J. Clin. Microbiol.* 43, 3267–3272.
- Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. *Nature* 501, 373–379.
- Lee, J.H., Huynh, M., Silhavy, J.L., Kim, S., Dixon-Salazar, T., Heiberg, A., Scott, E., Bafna, V., Hill, K.J., Collazo, A., et al. (2012). De novo somatic mutations in components of the PI3K-AKT3-mTOR pathway cause hemimegalencephaly. *Nat. Genet.* 44, 941–945.
- Lehtinen, M.K., Zappaterra, M.W., Chen, X., Yang, Y.J., Hill, A.D., Lun, M., Maynard, T., Gonzalez, D., Kim, S., Ye, P., et al. (2011). The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron* 69, 893–905.
- Lengner, C.J., Gimelbrant, A.A., Erwin, J.A., Cheng, A.W., Guenther, M.G., Welstead, G.G., Alagappan, R., Frampton, G.M., Xu, P., Muffat, J., et al. (2010). Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. *Cell* 141, 872–883.
- Li, Y., Wang, H., Muffat, J., Cheng, A.W., Orlando, D.A., Lovén, J., Kwok, S.M., Feldman, D.A., Bateup, H.S., Gao, Q., et al. (2013). Global transcriptional and translational repression in human-embryonic-stem-cell-derived Rett syndrome neurons. *Cell Stem Cell* 13, 446–458.
- Lui, J.H., Hansen, D.V., and Kriegstein, A.R. (2011). Development and evolution of the human neocortex. *Cell* 146, 18–36.
- Lui, J.H., Nowakowski, T.J., Pollen, A.A., Javaherian, A., Kriegstein, A.R., and Oldham, M.C. (2014). Radial glia require PDGF $\beta$ -PDGFR $\beta$  signalling in human but not mouse neocortex. *Nature* 515, 264–268.
- Marchese, M., Conti, V., Valvo, G., Moro, F., Muratori, F., Tancredi, R., Santorelli, F.M., Guerrini, R., and Sicca, F. (2014). Autism-epilepsy phenotype with macrocephaly suggests PTEN, but not GLIALCAM, genetic screening. *BMC Med. Genet.* 15, 26.
- Mirzaa, G.M., Rivière, J.B., and Dobyns, W.B. (2013). Megalencephaly syndromes and activating mutations in the PI3K-AKT pathway: MPPH and MCAP. *Am. J. Med. Genet. C. Semin. Med. Genet.* 163C, 122–130.
- Onorati, M., Li, Z., Liu, F., Sousa, A.M., Nakagawa, N., Li, M., Dell'Anno, M.T., Gulden, F.O., Pochareddy, S., Tebbenkamp, A.T., et al. (2016). Zika Virus Disrupts Phospho-TBK1 Localization and Mitosis in Human Neuroepithelial Stem Cells and Radial Glia. *Cell Rep.* 16, 2576–2592.
- Poduri, A., Evrony, G.D., Cai, X., Elhosary, P.C., Beroukhim, R., Lehtinen, M.K., Hills, L.B., Heinzen, E.L., Hill, A., Hill, R.S., et al. (2012). Somatic activation of AKT3 causes hemispheric developmental brain malformations. *Neuron* 74, 41–48.
- Qian, X., Nguyen, H.N., Song, M.M., Hadiono, C., Ogden, S.C., Hammack, C., Yao, B., Hamersky, G.R., Jacob, F., Zhong, C., et al. (2016). Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. *Cell* 165, 1238–1254.
- Rakic, P. (1974). Neurons in rhesus monkey visual cortex: systematic relation between time of origin and eventual disposition. *Science* 183, 425–427.
- Rakic, P. (1988). Specification of cerebral cortical areas. *Science* 241, 170–176.
- Rash, B.G., Tomasi, S., Lim, H.D., Suh, C.Y., and Vaccarino, F.M. (2013). Cortical gyration induced by fibroblast growth factor 2 in the mouse brain. *J. Neurosci.* 33, 10802–10814.
- Rivière, J.B., Mirzaa, G.M., O'Roak, B.J., Beddaoui, M., Alcantara, D., Conway, R.L., St-Onge, J., Schwartzentruber, J.A., Gripp, K.W., Nikkel, S.M., et al.; Finding of Rare Disease Genes (FORGE) Canada Consortium (2012). De novo germline and postzygotic mutations in AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes. *Nat. Genet.* 44, 934–940.
- Sasai, Y. (2013). Next-generation regenerative medicine: organogenesis from stem cells in 3D culture. *Cell Stem Cell* 12, 520–530.
- Stahl, R., Walcher, T., De Juan Romero, C., Pilz, G.A., Cappello, S., Irmler, M., Sanz-Aquela, J.M., Beckers, J., Blum, R., Borrell, V., and Götz, M. (2013). Trnp1 regulates expansion and folding of the mammalian cerebral cortex by control of radial glial fate. *Cell* 153, 535–549.
- Stelzer, Y., Shivalila, C.S., Soldner, F., Markoulaki, S., and Jaenisch, R. (2015). Tracing dynamic changes of DNA methylation at a single-cell resolution. *Cell* 163, 218–219.
- Sun, T., and Hevner, R.F. (2014). Growth and folding of the mammalian cerebral cortex: from molecules to malformations. *Nat. Rev. Neurosci.* 15, 217–232.
- Takahashi, K., Okita, K., Nakagawa, M., and Yamanaka, S. (2007). Induction of pluripotent stem cells from fibroblast cultures. *Nat. Protoc.* 2, 3081–3089.
- Tang, H., Hammack, C., Ogden, S.C., Wen, Z., Qian, X., Li, Y., Yao, B., Shin, J., Zhang, F., Lee, E.M., et al. (2016). Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. *Cell Stem Cell* 18, 587–590.
- Terrene, G., Voisin, N., Abdullah Alfaiz, A., Cappuccio, G., Vitello, G., Guex, N., D'Amico, A., James Barkovich, A., Brunetti-Pierri, N., Del Giudice, E., and Reymond, A. (2016). De novo PIK3R2 variant causes polymicrogyria, corpus callosum hyperplasia and focal cortical dysplasia. *Eur. J. Hum. Genet.* 24, 1359–1362.
- Tokuda, S., Mahaffey, C.L., Monks, B., Faulkner, C.R., Birnbaum, M.J., Danzer, S.C., and Frankel, W.N. (2011). A novel Akt3 mutation associated with enhanced kinase activity and seizure susceptibility in mice. *Hum. Mol. Genet.* 20, 988–999.
- Worby, C.A., and Dixon, J.E. (2014). Pten. *Annu. Rev. Biochem.* 83, 641–669.
- Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1920.

**STAR★METHODS****KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
See <a href="#">Table S1</a>	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
Dorsomorphin	Stemgent	04-0024
GDC-0068	Selleckchem	S2808
MK-2206	Selleckchem	S1078
Critical Commercial Assays		
CellTiter-Glo Luminescent Cell Viability Assay	Promega	G7571
CellEvent Caspase-3/7 Green Detection Reagent	Invitrogen	C10423
Click-iT EdU Imaging Kit	Invitrogen	C10337
TSA Plus Cyanine 3 System	Perkin Elmer	NEL744001KT
Experimental Models: Cell Lines		
Human: WIBR1/2/3 hESCs	<a href="#">Lengner et al., 2010</a>	N/A
Human: WIBR1/2/3 PTEN mutant hESCs	This study	N/A
Mouse: V6.5 mESCs	<a href="#">Stelzer et al., 2015</a>	N/A
Mouse: V6.5 Pten mutant mESCs	This study	N/A
Sequence-Based Reagents		
Primers: see <a href="#">Table S2</a>	This study	N/A

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for reagents may be directed to, and will be fulfilled by the Lead Contact, Dr. Rudolf Jaenisch ([jaenisch@wi.mit.edu](mailto:jaenisch@wi.mit.edu)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS****ESC culture**

HESC lines WIBR1, WIBR2 and WIBR3 were previously described ([Lengner et al., 2010](#); [Li et al., 2013](#)) and cultured in 5% O<sub>2</sub> on mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) in hESC medium, containing DMEM/F12 (Invitrogen), 15% fetal bovine serum (Hyclone), 5% knockout serum replacement (Invitrogen), 1% non-essential amino acids (Invitrogen), 1mM glutamine (Invitrogen), 0.1mM b-mercaptoethanol (Sigma) and 4ng/ml bFGF (R&D Systems). Cultures were passaged manually or with 1mg/ml collagenase type IV (Invitrogen) every 5-7 days. Mouse ESC line V6.5 was expanded on MEFs using mESC medium containing fetal bovine serum and leukemia inhibitory factor as previously reported ([Stelzer et al., 2015](#)).

All experiments involving cells from human subjects were performed in compliance with MIT COHUES protocol 0612002068.

**METHOD DETAILS****Neural differentiation**

Differentiation of hESCs to NPs in 2D adherent culture was performed as previously described ([Li et al., 2013](#)). Confluent hESCs were passaged onto matrigel-coated dishes using collagenase type IV, and cultured in mTeSR medium (Stem Cell Technologies) containing dorsomorphin (2.5uM, Stemgent) for 1-3 days until near confluent. To induce neural differentiation, increasing amounts of N2 media (25, 50, 75, 100%) were added to the mTeSR medium every other day while maintaining 2.5uM dorsomorphin. N2 medium contains 1X N2 supplement (Invitrogen), 50% DMEM/F12, 50% Neurobasal, 0.075% Bovine serum albumin, and 2mM GlutaMAX (Life Technologies). Cells were subsequently passaged with trypsin and cultured on matrigel-coated dishes in N2 media with the addition of 20ng/ml bFGF. Rho-associated protein kinase (ROCK) inhibitor Y27632 (10uM, Stemgent) was added to the medium for the first 24 hr. NPs were expanded and maintained in N2 medium with 20ng/ml bFGF. All 2D culture experiments were performed on WIBR3-derived NPs unless otherwise specified.

### CRISPR/Cas9 gRNA design

To generate gRNA expression vector, annealed oligonucleotides for the human or mouse PTEN exon 1 locus were ligated into the BbsI restriction site of a modified pX330 vector containing a CMV-EGFP cassette. The gRNA sequence for human PTEN was 5'-aaa caaaaggagatatacaag-3', which contains an EcoRV restriction site. The gRNA sequence for mouse Pten was 5'-agatcggttagcagaaaa caaaagg-3'. To evaluate the targeting efficiency of human PTEN gene, the CRISPR/Cas9 construct was transiently transfected into HEK293 cells, and PCR fragment was generated from genomic DNA followed by EcoRV restriction digest to evaluate targeting efficiency, using primers described in [Table S2](#). Cel-1 assay was used to evaluate the efficiency of gRNA toward the mouse Pten gene as previously described ([Li et al., 2013](#)).

### CRISPR/Cas9-mediated genome editing of ESCs

CRISPR/Cas9-mediated genome editing in hESCs was performed as previously described with modifications ([Li et al., 2013](#)). Briefly, WIBR3 hESCs were cultured in ROCK inhibitor Y27632 (10uM) for 24 hr prior to electroporation. Cells were harvested using trypsin/EDTA solution and re-suspension in phosphate buffered saline (PBS) as single cells. Electroporation was performed using 25ug of gRNA expression vector and 25ug of PGK-puro plasmid. Cells were plated on DR4 MEFs, and cultured in hESC medium containing ROCK inhibitor Y27632 for the first 24 hr. Puromycin (2ug/ml) was added to hESC medium 48 hr after electroporation for 2 days, and culture was maintained in hESC medium until colonies emerged. Individual hESC clones were picked and expanded 14 days after electroporation. PCR on genomic DNA and EcoRV restriction digest were performed to identify candidate clones, followed by DNA sequencing. CRISPR/Cas9-mediated genome editing in mESCs was performed as previously described ([Stelzer et al., 2015](#)).

### Cerebral organoid culture

Cerebral organoids were generated from WIBR3 hESCs using a previously reported protocol ([Lancaster et al., 2013](#)), with some modifications. Briefly, hESCs were dissociated from MEFs using collagenase type IV, and further separated from residual MEFs by gravity separation, before trypsinization of hESC colonies to generate single cells. A total of 9000 cells were then plated into each well of an ultra-low-attachment 96-well plate (Corning) to form single EBs, in medium containing DMEM/F12, 20% KSR, 3% fetal bovine serum, 2mM GlutaMAX, 1% non-essential amino acids, 50nM b-mercaptoethanol and 4ng/ml bFGF. ROCK inhibitor Y27632 (50uM) was included in the first 24 hr. EBs were maintained in 96-well plates for 6 days, then transferred to ultra-low-attachment 24-well plates (Corning), in neural induction medium containing DMEM/F12, 1X N2 supplement, 1% non-essential amino acids, 2mM GlutaMAX and 1ug/ml heparin (Stem Cell Technologies). On days 10-12, EBs were embedded in droplets of matrigel, and were allowed to gel at 37C. Embedded EBs were subsequently cultured in neural maturation medium containing 50% DMEM/F12, 50% Neurobasal, 0.5X N2 supplement, 0.5X B27 supplement, 2mM GlutaMAX, 2.5ng/ml human insulin, 0.5% non-essential amino acids, and 25nM b-mercaptoethanol. Droplets were cultured in stationary condition in 6cm suspension dishes for 4 days, followed by transfer to an orbital shaker (Unimax-1010, Heidolph Brinkmann) rotating continuously at 80rpm. Mouse cerebral organoids were generated as previously described ([Lancaster et al., 2013](#)). Multiple independent experiments were performed to generate cerebral organoids from WIBR1 (n = 3), WIBR2 (n = 3), WIBR3 (n > 15) and V6.5 (n = 3). Each experiment generates 24-96 individual organoids of each condition (genotype and treatment). Various concentrations of AKT inhibitors GDC-0068 (Selleckchem) or MK-2206 (Selleckchem) were added to growth medium for EBs on day 1. To pre-pattern organoids toward the dorsal forebrain fate, 2.5uM dorsomorphin was added to the culture on day 0 for 7 or 14 days. To stimulate phospho-AKT in 12-week organoids, BDNF (100ng/ml, Peprotech) was added for 30 min. All human cerebral organoid experiments were performed on WIBR3-derived control and mutant lines unless otherwise specified.

### Lentivirus production and transduction

FUW-GFP, FUW-GFP-PTEN lentivirus constructs were previously reported ([Fricano et al., 2014](#)). DN-AKT and CA-AKT cDNA were subcloned into FUW-GFP vector to generate the FUW-DN-AKT and FUW-CA-AKT lentivirus constructs. VSVG-coated lentiviruses were generated in HEK293 cells. Briefly, HEK293 cells were transfected using X-tremeGENE 9 (Sigma), with a mixture of lentiviral construct and second-generation packaging plasmids. Culture medium was changed 12 hr after and collected 96 hr after transfection. Virus-containing medium was filtered through 0.45um filter and concentrated via ultracentrifugation. To transduce hESCs with lentivirus, hESCs cultured on MEFs were first dissociated from MEFs using collagenase type IV, and further dissociated using Trypsin/EDTA to generate single cells. hESCs were plated onto matrigel-coated dishes and fed mTeSR medium containing 10uM ROCK inhibitor Y27632, and lentivirus particles. hESCs were subsequently passaged as single cells at clonal density to MEFs, and single clones were manually picked and expanded. Expression of lentiviral-transgenes was evaluated using GFP fluorescent, quantitative RT-PCR and immuno-blotting.

### ZIKV and Dengue virus production and infection

ZIKV strain MR766 was obtained from ATCC and expanded in Vero cells. Dengue virus serotype 2 strain 16681 was expanded in C3/36 mosquito cells. To establish titered viral stocks, virus-containing cell supernatants were harvested, and viral titer was determined by infecting Vero cells, followed by quantitative flow cytometry analysis of Flavivirus envelope immuno-staining ([Lambeth et al., 2005](#)) to calculate Vero cell infectious units. To infect cerebral organoids, the number of cells on the surface was estimated from the diameter of the 3D organoid and was used to calculate the amount of virus applied. An MOI of 1 based on Vero cell infectious units was used for all experiments for ZIKV and Dengue virus (actual MOI for the entire organoid was greatly less than 0.01). ZIKV or Dengue virus was added to culture medium and replaced with fresh medium 24 hr later. Organoids were analyzed 4 or 10 days post-infection.

### ATP assay

Cell growth rate was measured using an ATP assay with the CellTiter-Glo Luminescent kit according to the manufacturer's instructions (Promega). NPs were plated in matrigel-coated 96-well plates (black wall, clear bottom, Falcon) at 20,000 cells per well, in the presence or absence of bFGF (0–20ng/ml), PDGF-DD (0–10ng/ml) or IGF2 (0–10ng/ml). ATP assay was performed on day 1 and day 3. Luminescence was recorded on a Tecan microplate reader.

### EdU click-it assay

EdU (10μM, Life Technologies) was added to N2 medium for NPs, or neural maturation medium for cerebral organoids for 2 hr, after which cells or tissues were collected immediately, or washed with PBS and fed fresh medium until collection. N2 medium contained 1 or 20ng/ml of bFGF. EdU click-it assay was performed on fixed cells or tissue sections per manufacturer's instruction (Invitrogen), followed by immuno-staining.

### Histology and imaging

Cells and tissues were fixed with 4% (w/v) paraformaldehyde in PBS. Frozen or paraffin sections were prepared from tissues (EBs and organoids). Following membrane permeabilization with PBS containing 0.3% triton, cells and tissue sections were blocked with 3% normal donkey serum. Primary antibodies were against Brn2, Cleaved-caspase 3, Ctip2, Doublecortin, Flavivirus, FoxA2, FoxG1, GFAP, phospho-H3, HOPX, Ki67, Nkx2.2, NeuN, Nestin, PAX6, phospho-AKT, Satb2, Sox2, Tau, Tbr1, Tbr2, TH, phospho-Vimentin, and visualized by secondary antibodies conjugated with Alexa 488, 568, 594, 647 (Life Technologies), followed by counter-staining with DAPI. Detailed antibody information is described in [Table S1](#). Tyramide Signal Amplification was performed using TSA Plus Cyanine 3 System per manufacturer's instruction (Perkin Elmer). Detection of activated caspase 3/7 in live organoids was performed using a CellEvent Caspase-3/7 Green Detection Reagent (ThermoFisher). Fluorescent images of immuno-staining were captured on a Zeiss LSM-700 confocal microscope. Light-sheet fluorescent microscopy was performed on Hoechst-stained organoids using a Zeiss Z.1 Lightsheet microscope. Reconstruction of multi-view light sheet images was performed using ZEN (Zeiss) and Imaris (Bitplane) softwares. Quantification of volume, surface area and sphericity were performed using Imaris. Alternatively, stereological quantification was performed on every 5 section of organoids, stained with DAPI. The area of the organoid and the length of the outline were measured to calculate volume and surface area. A Canny Edge Detection plugin of ImageJ was used to measure fold density in images of Hoechst-stained organoids, some were manually stitched from multiple images.

### RNA extraction, reverse transcription and quantitative PCR

Cells and tissues were homogenized and total RNA extracted using the RNeasy kit (QIAGEN) following manufacturer's instructions. Total RNA concentrations were measured using NanoDrop ND-1000 spectrophotometer. RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen) with random hexamer primers according. Transcript abundance was determined by quantitative PCR using SYBR Green PCR mix (Applied Biosystems), with primer pairs against ARHGAP11B, BRN2, CTIP2, CUX1, CUX2, DCX, FOXA2, HOPX, MAP2, MKI67, NKX2.2, PAX6, RBFOX3, SATB2, TBR1, TBR2, TUBB3 and GAPDH. Primer sequences are described in [Table S2](#). Raw Ct values were normalized to GAPDH. Heatmap visualization of quantitative PCR results was generated using gene-E software.

### Protein purification and immuno-blotting

Total protein was extracted from cells and tissues using RIPA lysis buffer (Millipore) containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 0.25% deoxycholic acid 1% NP-40, 1mM EDTA, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail 2 and 3 (Sigma). Total protein from the supernatant was measured using BCA protein assay (Pierce). Primary antibodies were against Actin, PTEN, phospho-AKT, AKT, and visualized with HRP-conjugated secondary antibodies, followed by LumiGlo Chemiluminescent Kit (KPL) in accordance with the manufacturer's instructions. Detailed antibody information is described in [Table S1](#). Membranes blotted for phospho-proteins were stripped and re-probed with antibodies against total proteins. Quantification of band intensity was performed using ImageJ. Values for phospho-proteins were normalized to total proteins, and all others were normalized to Actin.

### Analysis of BrainSpan data

Gene expression data was obtained from the BrainSpan Prenatal LMD Microarray database, which contains transcriptional profiling of ~300 distinct brain regions from human prenatal brain. Expression levels of genes of interest at week 15–16 were aggregated according to top-level structure designation, and plotted based on z-scores.

### QUANTIFICATION AND STATISTICAL ANALYSIS

All data values were presented as mean ± SEM. Student's t tests were applied to data with two groups. ANOVA analyses were used for comparisons of data with greater than two groups. Post hoc group comparisons were performed with Bonferroni test. A value of  $p < 0.05$  was considered significant.