FIP200 is required for maintenance and differentiation of postnatal neural stem cells

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Despite recent studies showing that inhibition of autophagy depletes the hematopoietic stem cell pool and increases intracellular reactive oxygen species (ROS), it remains unknown whether autophagy is essential in the maintenance of other stem cells. Moreover, it is unclear whether and how the aberrant ROS increase causes depletion of stem cells. Here we report that ablation of FIP200 (also known as Rb1cc1), a gene essential for autophagy induction in mammalian cells, results in a progressive loss of neural stem cells (NSCs) and impairment in neuronal differentiation specifically in the postnatal brain, but not the embryonic brain, in mice. The defect in maintaining the postnatal NSC pool was caused by p53-dependent apoptotic responses and cell cycle arrest. However, the impaired neuronal differentiation was rescued by treatment with the antioxidant N-acetylcysteine but not by p53 inactivation. These data reveal that FIP200-mediated autophagy contributes to the maintenance and functions of NSCs through regulation of oxidative state.

RESULTS
FIP200 deletion leads to SVZ and dentate gyrus defects
To study autophagy in NSCs, we conditionally deleted FIP200, a gene essential for autophagy induction, in these cells by crossing mice with a loxP-flanked FIP200 (ref. 14) with GFAP-Cre transgenic mice, which express Cre recombinase in radial glial cells by means of a human glial fibrillary acidic protein (GFAP) promoter. FIP200fl/fl;GFAP-Cre (designated FIP200GFAP cKO) mice were born at the expected mendelian ratio and without any overt differences from control littermates (FIP200fl/fl and FIP200fl/fl;GFAP-Cre, designated as Ctrl mice) up to 10 weeks of age. Western blotting analysis showed efficient deletion of FIP200 in the SVZ of FIP200GFAP cKO mice (Supplementary Fig. 1a). To analyze potential autophagy defects, we first measured the accumulation of microtubule-associated protein 1A/1B-light chain 3 (LC3-II) and p62 sequestosome-1. LC3-II accumulation was lower in FIP200GFAP cKO mice than in Ctrl mice (Fig. 1a). Furthermore, p62 sequestosome-1 was higher in lysates from FIP200GFAP cKO mice, consistent with autophagy inhibition in these cells. p62 and ubiquitin-positive aggregations were also detected in sections containing the SVZ and dentate gyrus of FIP200GFAP cKO mice (Fig. 1b and data not shown). Together, these results suggest defective autophagy in NSCs of FIP200GFAP cKO mice.

Because autophagy is essential for the clearance of damaged and/or excess mitochondria, which are a principal source of intracellular ROS, we examined possible abnormalities of mitochondria and ROS amounts in FIP200GFAP cKO mice. Analysis of cells in the SVZ by transmission electron microscopy (TEM) showed more mitochondria per nucleus in FIP200GFAP cKO mice than in Ctrl mice at postnatal day (P) 14. Mice had been treated with chloroquine from P7 to P14 to inhibit LC3-II degradation. Furthermore, p62 sequestosome-1 was higher in lysates from FIP200GFAP cKO mice, consistent with autophagy inhibition in these cells. p62 and ubiquitin-positive aggregations were also detected in sections containing the SVZ and dentate gyrus of FIP200GFAP cKO mice. Together, these results suggest defective autophagy in NSCs of FIP200GFAP cKO mice.

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in FIP200KO mice (Fig. 1c, bottom). We verified this aberrant accumulation of larger and more heterogeneous mitochondria in neurospheres derived from NSCs of FIP200KO mice (Fig. 1d). There were approximately 50% more mitochondria per cell in neurospheres from FIP200KO mice (20 ± 2) than in those from Ctrl mice (13 ± 1). We next quantified ROS in vivo using the fluorescent dye dihydroethidium (DHE) as an indicator. ROS were higher in the surrounding granular zone than in the SVZ in Ctrl mice (Fig. 1e, top). ROS amounts were also high in the granular zone of FIP200KO mice (Fig. 1e, bottom), but these were similar to those in Ctrl mice. However, ROS were elevated in the SGZ of FIP200KO mice as compared to Ctrl mice. Similarly, ROS amounts were lower in the SVZ than in the surrounding striatum in Ctrl mice (Fig. 1f, top), but were higher in the SVZ of FIP200KO mice as compared to that in Ctrl mice (Fig. 1f, bottom). Together, these results suggest that, as in other cell types, deficient autophagy upon FIP200 deletion results in increased mitochondrial mass and ROS in NSCs.

**FIP200 ablation impairs NSC maintenance and neurogenesis**

As ROS have been suggested as important regulators for the maintenance of various stem cells, including NSCs, we examined the histology of the dentate gyrus and SVZ, where postnatal NSCs reside. FIP200KO brains at P0 showed apparently normal morphology and cellular organization in the dentate gyrus and SVZ (Supplementary Fig. 1b,c), as well as at all other brain regions (data not shown). At 4 weeks of age, however, the area of dentate gyrus was lower in FIP200KO mice than in Ctrl mice (0.32 ± 0.01 versus 0.17 ± 0.01 mm², n = 5 mice, >4 sections per mouse, P = 0.001) (Fig. 2a). Similarly, FIP200KO mice showed a thinner SVZ with lower cellularity as compared to Ctrl mice (177 ± 7 versus 83 ± 7 cells per section, n = 5 mice, >4 sections per mouse, P = 0.001) (Fig. 2a). Analysis of the SVZ by toluidine blue O staining as well as TEM confirmed the decreased cellularity in FIP200KO mice (Supplementary Fig. 1d,e). By 8 weeks of age, we also observed a significant decrease of the surface area of the olfactory bulb in FIP200KO mice (P = 0.01; Supplementary Fig. 1f,g), suggesting a possible failure of neurogenesis driven by postnatal NSCs in these mice.

To further explore potential NSC defects in FIP200KO mice, we determined the number of NSCs and their progeny by using various lineage markers. During embryonic development, radial glial cells in the ventricular zone are NSCs and express radial glial cell marker-2 (RC2) (ref. 23). Postnatally, radial glia are transformed into NSCs in the SVZ or dentate gyrus as GFAP-positive, nestin-positive cells and are the source of continuous neurogenesis throughout life. In newborn mice (P0), we found comparable numbers of radial glia or NSCs (RC2+GFAP+nestin+), neuroblasts (PSA-NCAM+) and Olig2+ cells (oligodendrocytes and neural progenitor cells) in FIP200KO mice and Ctrl mice (Supplementary Fig. 2a,b). However, the pool of NSCs was significantly reduced in the dentate gyrus of FIP200KO mice at P28 in comparison to that in Ctrl mice, as measured by their radial glial morphology with expression of both GFAP and nestin (27 ± 3 versus 8 ± 2 cells per section, n = 5 mice, >4 sections per mouse, P < 0.001) (Fig. 2b) or both GFAP and SOX2 (23 ± 2 versus 12 ± 1 cells per section, n = 5 mice, >4 sections per mouse, P < 0.01) (Fig. 2c). GFAP+nestin+ (48 ± 3 versus 24 ± 5 cells per section, n = 5 mice, >4 sections per mouse, P < 0.01) (Fig. 2d) and GFAP+SOX2+ (47 ± 2 versus 25 ± 2 cells per section, n = 5 mice, >4 sections per mouse, P < 0.01) (Fig. 2e) were similarly lower in the SVZ of FIP200KO mice than in that of Ctrl mice. Staining with PSA-NCAM showed a significant decrease of neuroblasts in the SVZ (65 ± 2 versus 26 ± 7 cells per section, n = 5 mice, >4 sections per mouse, P < 0.01), rostral migratory stream (RMS) and dentate gyrus (688 ± 33 versus 190 ± 30 cells per mm², n = 5 mice, >4 sections per mouse, P = 0.001) of FIP200KO mice than Ctrl mice (Fig. 2f), suggesting a defect in NSC-mediated neurogenesis. Consistent with this observation of impaired neurogenesis, we found a less densely populated RMS (Supplementary Fig. 2c) as well as significantly reduced number of cells positive for the neuronal marker NeuN in the internal granular layer of the olfactory bulb (4,954 ± 236 versus 3,406 ± 589 cells per mm², n = 5 mice, >4 sections per mouse, P < 0.05) and the granular zone of the dentate gyrus (601 ± 25 versus 221 ± 19 cells per section, n = 5 mice, >4 sections per mouse, P < 0.001)
FIP200 regulates NSC apoptosis and proliferation

To complement the above analysis using various markers and further study potential mechanisms of FIP200 in the regulation NSC, we first examined self-renewal of NSC from FIP200GFAP cKO mice by assessing the abilities of dissociated neural cells from the SVZ of the mutant and control mice to form neurospheres in vitro. The numbers and sizes of the primary neurospheres formed by the SVZ cells isolated at P0 were comparable between FIP200GFAP cKO and Ctrl mice, but the secondary neurospheres formed by the dissociated cells from the primary neurospheres were significantly fewer and smaller in size for FIP200GFAP cKO than for Ctrl mice (Fig. 3a–c). For SVZ cells isolated at P28, we found significantly less formation of both primary and secondary neurospheres by FIP200GFAP cKO neural cells (Fig. 3d,e). Similarly, we found an approximately 70% decrease in neurosphere formation for neural cells isolated from the hippocampus (containing the dentate gyrus) of P28 FIP200GFAP cKO mice as compared to those from Ctrl mice (data not shown). We also found that, consistent with observations in vivo (Fig. 2b,d,f,g), culturing of dissociated cells from neurospheres in differentiation medium showed significantly less differentiation to Tuj1+ neurons (7.3 ± 0.3 versus 3.4 ± 0.3%, n = 3 mice, >1,000 cells per genotype, P < 0.05) but slightly more differentiation to astrocytes by neurospheres derived from FIP200GFAP cKO NSCs than by those derived from Ctrl NSCs (75 ± 3 versus 82 ± 5%, n = 3 mice, >1,000 cells per genotype, P > 0.05). These results suggest a potential function of FIP200 in the regulation of self-renewal and neurogenesis of NSCs.
Recent studies have shown that FIP200 and several other Atg genes affect the survival of neurons and other cells.\textsuperscript{24–26} To determine whether elevated apoptosis is responsible for the progressive loss of NSCs in postnatal FIP200\textsuperscript{GFAP} cKO brains, we analyzed apoptosis in the SVZ, dentate gyrus and other brain regions. At P0, several regions of FIP200\textsuperscript{GFAP} cKO brain, including the cortex, cerebellum, medulla and midbrain, showed significantly more apoptosis than those of Ctrl mice (P < 0.01; Supplementary Fig. 3a–f). Double staining with neural markers showed that most apoptotic cells in both FIP200\textsuperscript{GFAP} cKO and Ctrl mice were postmitotic neurons, as identified by NeuN\textsuperscript{+} staining, but not NSCs or progenitors (nestin\textsuperscript{+}) or oligodendrocytic cells (Olig2\textsuperscript{+}) (Supplementary Fig. 3g). In the SVZ and SGZ of the dentate gyrus, where NSCs reside and which have no or few neurons, comparable degrees of apoptosis were observed for FIP200\textsuperscript{GFAP} cKO and Ctrl mice (Supplementary Fig. 3h,i). In contrast to P0, at P28 we found a higher fraction of apoptotic cells in FIP200\textsuperscript{GFAP} cKO mice than in Ctrl mice in both the SVZ (3.3 \pm 0.3 vs. 4.6 \pm 0.4 apoptotic cells per 100 cells, n = 5 mice) and dentate gyrus (15 \pm 2 vs. 27 \pm 3 apoptotic cells per mm\textsuperscript{2}, n = 5 mice, >4 sections per mouse, P < 0.05) (Fig. 3f), suggesting that elevated apoptosis upon FIP200 deletion is responsible for the progressive loss of NSCs at postnatal stages.

We next examined proliferation of NSCs in the SVZ and dentate gyrus of FIP200\textsuperscript{GFAP} cKO and Ctrl mice, as recent studies also suggested that deletion of FIP200 could result in decreased proliferation in breast cancer cells.\textsuperscript{19} At P0, rates of proliferation in the SVZ and dentate gyrus of FIP200\textsuperscript{GFAP} cKO and Ctrl mice were comparable between the two genotypes, as measured by either bromodeoxyuridine (BrdU) incorporation assay or Ki67 staining, a marker for cell proliferation (Supplementary Fig. 4a,d). At P28, we found fewer proliferative cells in the SVZ and dentate gyrus in FIP200\textsuperscript{GFAP} cKO mice than in Ctrl mice. However, the fractions of proliferative cells were similar in the SVZ (27 \pm 2 and 25 \pm 1 BrdU\textsuperscript{+} cells per 100 cells, n = 5 mice, >500 cells per genotype, P > 0.05) and only slightly lower in the dentate gyrus of FIP200\textsuperscript{GFAP} cKO mice compared to those of Ctrl mice (62 \pm 2 vs. 52 \pm 2 BrdU\textsuperscript{+} cells per mm\textsuperscript{2}, n = 5 mice, >4 sections per mouse, P < 0.05) (Fig. 3g, and see Ki67 labeling in Supplementary Fig. 4e–g), as the overall cellularity and area of these regions were also significantly lower (P < 0.001) in the mutant mice. Because of the aberrantly elevated genesis of astrocytes in the SVZ and dentate gyrus of FIP200\textsuperscript{GFAP} cKO mice (Fig. 2b,d), cells which could also proliferate, we stained with further neural markers to identify the nature of proliferative cells. The proliferative NSCs (that is, GFAP\textsuperscript{+}nestin\textsuperscript{+}BrdU\textsuperscript{+} cells) as either a fraction of total BrdU\textsuperscript{+} cells (3.8 \pm 0.5 versus 1.3 \pm 0.4% (SVZ) and 4.2 \pm 0.6 versus 1.7 \pm 0.4% (SGZ), n = 5 mice, >200 BrdU\textsuperscript{+} cells per genotype and >4 sections per mouse, respectively, P < 0.001) or total GFAP\textsuperscript{+}nestin\textsuperscript{+} cells (3.6 \pm 0.5 versus 1.2 \pm 0.4% (SVZ) and 3.8 \pm 0.6 versus 0.9 \pm 0.3% (SGZ), n = 5 mice, >200 GFAP\textsuperscript{+}nestin\textsuperscript{+} cells per genotype and >4 sections per mouse, respectively, P < 0.001 and P < 0.01, respectively) were indeed lower in both the SVZ and dentate gyrus of FIP200\textsuperscript{GFAP} cKO mice than in Ctrl mice. We also observed similar reduction of dividing NSCs in FIP200\textsuperscript{GFAP} cKO mice by labeling proliferating cells using Ki67 (Supplementary Fig. 4h–k). These data suggest that reduced proliferation could also contribute to the gradual loss of postnatal NSCs in FIP200\textsuperscript{GFAP} cKO mice.

Lastly, to validate the decreased numbers of NSCs in FIP200\textsuperscript{GFAP} cKO mice \textit{in vivo}, we performed BrdU retention experiments by labeling with three consecutive injections at P7, followed by detection of BrdU\textsuperscript{+} cells in the SVZ and SGZ 3 weeks after the pulse labeling. We found similar numbers of BrdU\textsuperscript{+} cells in the SVZ and dentate gyrus between FIP200\textsuperscript{GFAP} cKO and Ctrl mice at P7 (data not shown). Notably, the number of BrdU\textsuperscript{+} cells remaining in the lateral walls of the SVZ and SGZ of dentate gyrus was significantly lower in FIP200\textsuperscript{GFAP} cKO mice than in Ctrl mice (7.6 \pm 0.5 versus 3.3 \pm 0.3 (SVZ) and 11 \pm 1 versus 5 \pm 1 (SGZ) cells per section, n = 5 mice, >4 sections per mouse, P < 0.001) (Fig. 3h), suggesting a loss of self-renewing NSCs \textit{in vivo} in the mutant mice. Together, these results suggest that FIP200 deletion reduces NSC self-renewal by affecting both apoptosis and proliferation, leading to progressive depletion of postnatal NSCs in FIP200\textsuperscript{GFAP} cKO mice.

\textbf{P53 ablation rescues self-renewal of FIP200-null NSCs}

Although multiple signaling pathways could be affected by ROS, one important downstream target of ROS is the p53 tumor suppressor, which is a transcription factor that activates a plethora of genes whose products induce cell cycle arrest, differentiation, senescence and...
apoptosis in response to DNA damage or other stress conditions. Therefore, it is possible that changes in expression and activity of Trp53, encoding p53, could contribute to NSC defects upon FIP200 deletion and aberrant increase in ROS in FIP200GFAP cKO mice. To test such a possibility, we first quantified Trp53 expression in neurospheres derived from FIP200GFAP cKO and Ctrl mice. Expression of Trp53 was greater in neurospheres from FIP200GFAP cKO mice than from Ctrl mice (Fig. 4a). Moreover, reverse transcription and quantitative PCR showed substantial elevation, relative to their expression in neurospheres from Ctrl mice, in the expression of several p53 target genes, including Cdkn1a (p21), Bbc3 (PUMA) and Fas in neurospheres from FIP200GFAP cKO mice (Fig. 4b), although other targets, such as Bax, Tiger (963003F20Rik) and Sfn (14-3-3sigma), did not show appreciable differences (data not shown). These results suggest that NSC defects in FIP200GFAP cKO mice may be dependent at least in part on elevation of p53 and its downstream targets.

To explore the above possibilities directly, we generated double conditional knockout mice with deletion of FIP200 and Trp53 (FIP200GFAP-Trp53GFAP cKO, designated as 2cKO), as well as Trp53GFAP cKO mice (designated as Trp53GFAP cKO), as a control, by crossing FIP200GFAP cKO and Trp53GFAP cKO mice (from the US National Cancer Institute’s Mouse Models of Human Cancers Consortium). Both 2cKO and Trp53GFAP cKO mice were obtained at the mendelian ratio, and analysis of neurospheres derived from these mice verified efficient deletion of Trp53 in NSCs (Fig. 4a). Consistent with this, the elevated expression of Cdkn1a, Bbc3 and Fas was also abrogated completely or partially in neurospheres from 2cKO mice (Fig. 4b). Analysis of the numbers and sizes of the neurospheres formed from 2cKO mice showed significant elevation in both of these parameters compared to those from FIP200GFAP cKO mice (Fig. 4c,d). We then sectioned the neurospheres and stained them with various markers. Ki67 staining (Fig. 4e) as well as the BrdU incorporation assay (Fig. 4f) revealed significantly fewer proliferative cells in neurospheres from FIP200GFAP cKO mice than in those from Ctrl mice, which is consistent with observations in vivo (Fig. 3g). Notably, the reduced proliferation of FIP200-null NSCs or progenitors was completely rescued by Trp53 inactivation (Fig. 4e,f). Also similarly to data from the dentate gyrus and SVZ in vivo (Fig. 3f), we observed a higher percentage of apoptotic cells in the neurospheres from FIP200GFAP cKO mice, which was markedly reduced in the neurospheres from 2cKO mice (Fig. 4g,h). As expected, most cells in neurospheres of all samples were nestin+ (Fig. 4i) and RC2+ (data not shown), confirming their NSC or progenitor nature. Together, these results indicate that the increased Trp53 expression after FIP200 deletion and aberrant ROS increase contribute to the defective self-renewal by inducing apoptosis and inhibiting proliferation of NSCs.

P53 does not mediate FIP200 regulation of neurogenesis

To validate the rescue of NSC defects of FIP200GFAP cKO mice by Trp53 inactivation in vivo, we further examined the SVZ and dentate gyrus in 2cKO mice. Histological analysis showed that the reduced cellularity of the SVZ and dentate gyrus area in FIP200GFAP cKO mice was rescued in 2cKO mice, up to 80% of the values in Ctrl mice (Fig. 5a–c). More importantly, the pool of NSCs in the dentate gyrus of 2cKO mice, as assessed by radial glial morphology with double-positive staining for nestin and GFAP or for or SOX2 and GFAP, was significantly larger than that in FIP200GFAP cKO mice (Fig. 5d–h). Similarly, numbers of NSCs in the SVZ of 2cKO mice were also greater than those in FIP200GFAP cKO mice and were comparable to those in Ctrl mice, suggesting a more complete rescue in this region (Fig. 5i–m). Consistent with in vitro data from the neurosphere analysis, the elevated apoptosis in SVZ and dentate gyrus of FIP200GFAP cKO mice was rescued in 2cKO mice (Fig. 5n,o). Moreover, analysis of the number of NSCs by BrdU retention and...
Figure 5 Trp53 deletion rescues NSCs defects in FIP200GFAP cKO mice. (a–c) Hematoxylin and eosin staining of the SVZ and dentate gyrus (DG) of P28 mice (a). Arrows mark cells in the SVZ. Mean ± s.e.m. of the SVZ cellularity (b) and DG area (c) per section are shown. (d–m) Immunofluorescence of the DG (d–h) and SVZ (i–m) of P28 mice (d,i,j). Boxed areas are shown in more detail in insets (e,j) and/or panels below (i,j). Arrows mark GFAP+nestin+ and GFAP+SOX2+ NSCs with radial glial morphology (d,e), and arrowheads mark GFAP+nestin– and GFAP+SOX2– astrocytes. Mean ± s.e.m. of the number of GFAP+nestin+ and GFAP+SOX2+ radial glia (f,h), and NSCs (k,m), and GFAP+nestin– astrocytes (g,i) per section are shown. (n,o) Mean ± s.e.m. of the number of TUNEL+ cells per 100 SVZ cells (n) or per 1 mm² DG area (o) of P28 mice. (p–r) BrdU retention in the SVZ and DG of P31 mice. BrdU+ cells are marked by arrows (p). Mean ± s.e.m. of the number of BrdU+ cells per section are shown (q,t). Mean ± s.e.m. of percentage of GFAP+nestin+BrdU+ cells of total BrdU retained cells in the SVZ (s) and SGZ (t) of P31 mice. n = 5 mice, ≥2 sections per mouse, >500 cells counted per mouse in n, >20 BrdU+ cells counted per mouse. Dotted lines indicate the boundaries of the SVZ and DG (a,i,j,p) or granular zone (GZ; d,e,p). E, ependymal cells; LV, lateral ventricle; ML, molecular layer; ST, striatum. NS, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

lineage tracing experiments confirmed that Trp53 inactivation was sufficient to restore NSC numbers in FIP200GFAP cKO mice (Fig. 5p–t). Together, these results suggest that the aberrantly increased p53 expression and apoptosis was mainly responsible for the shrinkage of the dentate gyrus and SVZ and the reduction in number of NSCs in FIP200GFAP cKO mice.

Despite the rescued numbers of NSCs observed in 2cKO mice, PSA-NCAM staining showed numbers of neuroblasts in the dentate gyrus and SVZ of 2cKO mice similar to the reduced numbers in FIP200GFAP cKO mice (Fig. 6a–c). Consistent with this, the reduced numbers of NeuN+ neurons were also not rescued in 2cKO mice (Fig. 6d–f). These results suggest that Trp53 inactivation does not rescue defects in neuronal differentiation in FIP200-deficient NSCs. We therefore examined the neurogenesis steps from neuroblasts to neurons in these mice by determining the fractions of neurons with BrdU label retention (that is, those cells labeled as proliferating neuroblasts just before the last mitosis to postmitotic neurons) in the dentate gyrus and olfactory bulb. There was a significantly smaller NeuN+BrdU+ fraction in the dentate gyrus of FIP200GFAP cKO mice than in that of Ctrl mice (P < 0.01; Fig. 6g,h). Moreover, 2cKO mice showed similarly reduced fractions of NeuN+BrdU+ cells in the dentate gyrus as those of FIP200GFAP cKO mice, supporting the notion that defects in neuronal differentiation of FIP200-null NSCs are independent of Trp53 activation. Results were similar in olfactory bulb (Fig. 6i and Supplementary Fig. 5a).

Besides a lack of rescue for neurogenesis defects, we also noted that numbers of SVZ GFAP+nestin+ astrocytes in 2cKO mice remained higher than those in Ctrl mice, although they were not as high as those in FIP200GFAP cKO mice (Fig. 5i). Similarly, although the numbers of radially shaped cells in the granular zone of 2cKO mice were significantly rescued (P < 0.01), the elevated GFAP staining of cells with astrocyte morphology was still present in the SGZ of
2cKO mice, in amounts similar to those in FIP200GFAP cKO mice (Fig. 5d,g). Notably, examination of remaining BrdU+ cells at P28 (Fig. 5p) also showed a greater fraction of GFAP+nestin−BrdU+ cells in FIP200GFAP cKO mice than in Ctrl mice, which was also only partially reduced in the SVZ (Fig. 6j) and was comparably high in the dentate gyrus (Fig. 6k) of 2cKO mice. These results suggest that the excess of GFAP+ differentiated astrocytes caused by FIP200 deletion (and not rescued by Trp53 inactivation) was generated from NSCs (rather than proliferating or upregulating GFAP through a more indirect mechanism). Taken together, these results suggested that FIP200 regulates self-renewal of NSC and differentiation of NSCs and their progeny neuroblasts through p53-dependent and p53-independent mechanisms, respectively. They also imply that other targets of increased ROS could be responsible for the p53-independent, differentiation defects in FIP200GFAP cKO mice.

**ROS depletion rescues NSC defects in FIP200GFAP cKO mice**

To investigate the hypothesis that the neuronal differentiation defects in 2cKO mice were caused by aberrantly high ROS but independent of p53, we first examined whether ROS were reduced by Trp53 inactivation in these mice, as previous studies have reported that p53 can regulate ROS and autophagy. We found that 2cKO mice showed comparably elevated ROS levels as those in FIP200GFAP cKO mice in both the SGZ (Fig. 6l) and SVZ (Fig. 6m). Moreover, the elevation in p62 aggregates caused by FIP200 deletion and defective autophagy also remained in the SVZ and dentate gyrus of 2cKO mice (Supplementary Fig. 5b). Therefore, inactivation of Trp53 did not rescue the autophagy defects and consequent ROS increase in FIP200GFAP cKO mice. These results are consistent with the idea that aberrant ROS levels were genetically upstream of increased Trp53 expression and that other downstream targets of ROS could be responsible for the p53-independent NSC differentiation and neurogenesis defects in FIP200GFAP cKO mice.

To directly test the above hypothesis more rigorously, we employed a widely used antioxidant, NAC, to scavenge ROS and examined the effects on NSCs. After daily treatment with NAC for 21 d, starting from P7, the elevated ROS in the dentate gyrus and SVZ of...
**Figure 7** Rescue of NSCs maintenance in FIP200<sup>GFAP</sup> cKO mice by scavenging abnormally elevated ROS. (a–c) Hematoxylin and eosin staining (a) of the SVZ and dentate gyrus (DG) of P28 mice treated by NAC or vehicle (Veh) control. Dotted lines indicate boundaries for the SVZ and DG. Arrows mark cells in the SVZ. Mean ± s.e.m. of the SVZ cellularity (b) and DG area (c) per section are shown. (d–m) Immunofluorescence of the DG (d–h) and SVZ (i–m) of P28 mice treated by NAC or vehicle control. Representative images are shown in d,g (DG) and k,m (SVZ). Dotted lines indicate boundaries of the granular zone (GZ) (d,g) and SVZ (k,m). Boxed areas in g,k,m are shown in more detail in insets (g,m) and/or panels below (k,m). Lines indicate boundaries of the GZ (d,g,k) and SVZ (k,m). Arrows mark GFAP<sup>nestin</sup> and GFAP<sup>SOX2</sup> NSCs with radial glial morphology (d,g), and arrowheads mark GFAP<sup>nestin</sup> and GFAP<sup>SOX2</sup>– radial glia (e,h), and NSCs (i,l) and GFAP<sup>nestin</sup>– astrocytes (f,j) per section are shown. (n,o) Mean ± s.e.m. of the number of GFAP<sup>nestin</sup> and GFAP<sup>SOX2</sup> radial glia (e,h); and NSCs (i,l), and GFAP<sup>nestin</sup>– astrocytes (f,j) per section are shown. Mean ± s.e.m. of the SVZ cellularity (**p < 0.01). Consistent with these data from neurosphere culture studies in vitro, histological analysis showed restoration of the SVZ cellularity and the dentate gyrus area in NAC-treated responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice to levels comparable to those in Ctrl mice (Fig. 7a–c). Moreover, the decrease in the pool of NSCs in the dentate gyrus, as measured by GFAP<sup>nestin</sup> and GFAP<sup>SOX2</sup>– cells with a radial glial morphology (Fig. 7), was also rescued by NAC in responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice, to values similar to those in Ctrl mice (Fig. 7d,e,g,h). Results were similar for NSCs in the SVZ of responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice. NAC restored both of these parameters in FIP200-null NSCs from responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice to values similar to those from Ctrl mice (Supplementary Fig. 6c,f). Consistent with these data from neurosphere culture studies in vitro, histological analysis showed restoration of the SVZ cellularity and the dentate gyrus area in NAC-treated responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice to levels comparable to those in Ctrl mice (Fig. 7a–c). Moreover, the decrease in the pool of NSCs in the dentate gyrus, as measured by GFAP<sup>nestin</sup> and GFAP<sup>SOX2</sup>– cells with a radial glial morphology (Fig. 7), was also rescued by NAC in responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice, to values similar to those in Ctrl mice (Fig. 7d,e,g,h). Results were similar for NSCs in the SVZ of responder (but not non-responder) FIP200<sup>GFAP</sup> cKO mice.
(Fig. 7i–k–m). Lastly, the elevated apoptosis in SVZ and dentate gyrus of FIP200GFAP cKO mice was also reversed by NAC in responder (but not non-responder) FIP200GFAP cKO mice (Fig. 7n,o). Together, these results indicate that suppression of the aberrant ROS levels and consequent \( p53 \) increase by NAC treatment rescued defective self-renewal caused by FIP200 deletion.

In contrast to Trp53 inactivation, reversal of elevated ROS by NAC also alleviated the defects in neurogenesis and differentiation of NSCs in FIP200GFAP cKO mice. The reduced numbers of neuroblasts in the dentate gyrus, SVZ and RMS of responder (but not non-responder) FIP200GFAP cKO mice were significantly increased after NAC treatment (Fig. 8a–c). Similarly, the number of neurons in the dentate gyrus and olfactory bulb was also rescued in responder (but not non-responder) FIP200GFAP cKO mice by NAC treatment (Fig. 8d–f). Consistent with these results, the fraction of NeuN+ neurons retaining BrdU labeling was also rescued in responder (but not non-responder) FIP200GFAP cKO mice, supporting restoration of neuronal production in these mice by NAC treatment (Supplementary Fig. 6g–i). Besides rescued neurogenesis, the aberrant increase of astrocytes in the mutant mice was abrogated, as the elevated numbers of astrocytes (GFAP+nestin− cells) were reduced to a level similar to that in Ctrl mice after NAC treatment (Fig. 7d,f,j,k).

Several transcription factors and signaling pathways, such as the Notch and bone morphogenetic protein (BMP) pathways, are important in the regulation of NSC differentiation and neurogenesis. Therefore, we next examined the expression of several genes of these pathways in neurospheres derived from Ctrl, FIP200GFAP cKO and 2cKO mice, as well as those from FIP200GFAP cKO mice treated with NAC (both responder and non-responder) to explore the potential p53-independent mechanisms regulating NSC differentiation downstream of elevated ROS after FIP200 deletion. We found that the expression of Notch and several target genes was not changed by FIP200 deletion (Supplementary Fig. 7), suggesting that Notch pathway is not involved. Notably, the expression of PAX6 (a neurogenic transcription factor) was lower in neurospheres from FIP200GFAP cKO mice in comparison to that from Ctrl mice, as measured at both mRNA and protein levels (Fig. 4b,h). Moreover, the expression and activity of STAT3, which both cooperates with the BMP pathway and can activate the BMP pathway to promote astrogenesis, were increased in FIP200GFAP cKO neurospheres, as assessed by mRNA expression and phosphorylation of STAT3 (Fig. 4b,h). The changes for PAX6 and STAT3 were not rescued by p53 inactivation in 2cKO mice (Fig. 4b,h), suggesting that they were regulated in a p53-independent manner. Consistent with the rescue of both NSC maintenance and differentiation phenotypes, NAC treatment of FIP200GFAP cKO mice restored expression of PAX6 and STAT3 in responder (but not non-responder) mice to levels comparable to those in Ctrl mice (Supplementary Fig. 8a,b).
be expected to respond to the treatment uniformly. As expected, NAC reversed the elevated expression of Trp53 and its targets Cdkn1a, Bbc3 and Fas in neuropheres from FIP200-null NSCs (Supplementary Fig. 8c). NAC also rescued defects in secondary neurosphere propagation of NSCs from P0 FIP200GFAP cKO mice (Supplementary Fig. 8d–f). Similarly to results in responder mice, NAC reversed the abnormal expression of PAX6 and STAT3 in these cells (Supplementary Fig. 8g,h). These results suggest that FIP200-mediated autophagy may support neurogenesis and astrogenesis by maintaining the normal amounts and activities of PAX6 and/or STAT3 in a p53-independent manner. Taken together, our studies demonstrate that suppression of abnormal increase in ROS rescued both p53-dependent and p-53-independent NSC defects in FIP200GFAP cKO mice, implying that the increased ROS after FIP200 deletion is most likely an underlying molecular mechanism for the deficient self-renewal and differentiation of NSCs in these mice.

**DISCUSSION**

Here we demonstrate that autophagy is critical in controlling ROS amounts in postnatal NSCs for their maintenance and differentiation. Deletion of the essential autophagy gene FIP200 by GFAP-Cre resulted in aberrant increase in ROS and gradual depletion of NSCs. Moreover, inhibition of autophagy by Spautin-1 (ref. 36) significantly reduced neurosphere formation from the SVZ cells in culture (P < 0.001; Supplementary Fig. 9a–c), further supporting an autophagy function of FIP200 in NSC regulation. Unlike the increased proliferation upon FIP200 deletion in HSCs13, FIP200-null NSCs with elevated ROS showed decreased proliferation and increased apoptosis, which were rescued by NAC treatment, suggesting that deficiency in autophagy and increased ROS may affect NSCs and HSCs through distinct cellular mechanisms. Despite the previous studies in HSCs11,12, the present study is the first, to our knowledge, to provide the mechanistic basis underlying the progressive loss of NSCs or any other stem cells caused by aberrant increase of ROS.

It is noteworthy that FIP200 deletion caused gradual depletion of postnatal NSCs but did not affect embryonic NSCs. Because deletion of FIP200 in NSCs would also lead to its inactivation in their progeny, including astrocytes, which mostly differentiate and proliferate in the postnatal brain, one possibility is that FIP200-null astrocytes affected postnatal NSCs in the adult brain. However, coculture of embryonic NSCs with neonatal astrocytes from FIP200GFAP cKO mice did not significantly affect their neurosphere formation when compared to those cocultured with astrocytes from Ctrl mice or alone (P > 0.05; Supplementary Fig. 9d), suggesting that such potential effects from FIP200-null astrocytes are less likely and that the different response to FIP200 deletion between embryonic and postnatal NSCs is mostly intrinsic to NSCs. It is also possible that both embryonic and postnatal NSCs are susceptible to autophagy inhibition and aberrant ROS elevation, but the accumulation of ROS is a gradual process in the autophagy-deficient NSCs. Therefore, damage could be observed in only postnatal NSCs but not embryonic NSCs owing to the longer time for ROS accumulation to reach a threshold in these cells. Lastly, an alternative but not mutually exclusive possibility is that embryonic and postnatal NSCs may have intrinsic differences in their autophagy activity and consequently differential responses to loss of FIP200 in FIP200GFAP cKO mice. Indeed, analysis of brain samples from embryonic and postnatal FIP200GFAP cKO mice showed that autophagy activity in regions containing NSCs was minimal at embryonic day 17.5 but could be readily detected at P14, with further increase at P28, as measured by LC3-II levels (Supplementary Fig. 9c). Notably, inactivation of autophagy by FIP200 deletion in FIP200GFAP cKO mice caused accumulation of p62 and elevated ROS in P14 and P28 SVZ, but not in embryonic day 17.5 ventricular zone, where postnatal and embryonic NSCs respectively reside (Supplementary Figs. 9f and 10a). Therefore, the very low autophagy activity in embryonic NSCs and thus the absence of elevated ROS upon FIP200 deletion in these cells could account for their resistance to loss of FIP200 and autophagy.

Our findings here that treatment of FIP200GFAP cKO mice with NAC rescued all NSC defects in the majority of mice and that the rescue of NSC defects correlated with the ROS amounts in the treated mice (that is, rescued in responders but not in non-responders) highlight the importance of ROS in the regulation of NSCs. Consistent with our findings, several previous studies have suggested a role of increased ROS in NSC defects upon deletion of other genes, including FoxO family members37–39. Notably, recent studies have suggested that FoxO1 can control autophagy in cancer cells40 and that FoxO3 regulates autophagy in muscles41. Thus, it will be worthwhile to examine possible autophagy defects in these mice, which might also contribute to the ROS elevation and NSC phenotypes caused by FoxO deficiency.

Our studies also imply that autophagy is important for NSC maintenance and differentiation by regulating oxidative stress under physiological conditions. Adult NSCs are located in hypoxic niches; for example, the SGZ of the dentate gyrus42. As hypoxia could cause elevation of ROS and oxidative damage, it would be important for NSCs to keep a ROS low, especially in quiescence. Indeed, we observed lower ROS in the SVZ and SGZ than in cells in surrounding regions of Ctrl mice, and ROS were elevated in FIP200GFAP cKO mice, suggesting that autophagy may limit ROS to protect NSCs in the hypoxic niche. In contrast to our observations and other studies43, a recent report showed higher ROS in NSCs in the SVZ, which was suggested to be associated with the proliferative or ‘activated’ state of NSCs13. Notably, we also observed some heterogeneity in ROS amounts in the SVZ (Fig. 1f), although ROS were uniformly low in the SGZ (Fig. 1e), which was not examined in ref. 13. It is therefore possible that NSCs with the higher ROS amounts in the SVZ are proliferative, whereas those with lower amounts are quiescent. Further analysis by triple labeling showed very low ROS in GFAP+SOX2+ NSCs of the Ctrl SVZ (Supplementary Fig. 10b), which showed some heterogeneity in DHE staining. In contrast, ROS was elevated in GFAP+SOX2+ NSCs in the SVZ of FIP200GFAP cKO mice (Supplementary Fig. 10b), supporting the hypothesis that aberrantly elevated ROS are responsible for the gradual depletion of postnatal NSCs. Future studies will be necessary to resolve the potentially complex regulation of NSCs by ROS in both positive and negative manners.

Previous studies have suggested Trp53 and its target genes Cdkn1a and Bbc3 as negative regulators of self-renewal in various stem cells, including NSCs44–46. Ectopic expression of constitutively active p53 leads to depletion of various adult stem cells, including NSCs45, whereas germline Trp53 deletion increases proliferation, survival and self-renewal of NSCs46,47. However, our study found that conditional inactivation of Trp53 in somatic cells caused little or no phenotype in the SVZ and dentate gyrus, consistent with a previous study that used the same GFAP-Cre-mediated inactivation of Trp53 (ref. 48). These results suggest that p53 does not normally regulate NSC functions unless these stem cells are under stress, such as in culture (as in previous studies) or high ROS (this study).

FIP200 deletion also affected NSC differentiation, with reductions in neurogenesis, as consistent with previous studies showing altered differentiation in HSCs with loss of FIP200 or Atg7 (refs. 11,12) or in preadipocytes lacking Atg5 or Atg7 (refs. 49,50). In FIP200GFAP cKO mice, the diminished production of new neurons could be the consequence of reduced pool of NSCs, decreased proliferation or survival...
of NSCs or their progeny, or reduced neuronal lineage commitment. Notably, although it rescued the deficiency in the pool of NSCs, Trp53 ablation did not restore neurogenesis, suggesting that the reduced NSC pool is not solely responsible and that more ROS targets are important for the defective neurogenesis. We found that the neurogenic transcription factor PAX6 was decreased, whereas the expression and activity of STAT3, which both cooperates with the BMP pathway and can activate the BMP pathway to promote astrogenesis, were increased in neurospheres derived from FIP200ΔF K(O) mice. Moreover, the altered expression of PAX6 and STAT3 was rescued by NAC treatment but not by p53 ablation, suggesting that elevated ROS may decrease neurogenesis and/or increase astrogenesis through PAX6 and/or BMP signaling in a p53-independent manner.

In conclusion, our study identified a role for FIP200-dependent autophagy in the maintenance of postnatal, but not embryonic NSCs, and in neurogenesis of NSCs. Our results also demonstrate that autophagy is a critical mechanism in controlling ROS amounts to ensure the self-renewal of postnatal NSCs.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.W. conducted the study, analyzed the data and wrote the manuscript; C.-L.L. generated some mouse lines; Z.C.B. genotyped the mice; Y.Z. analyzed the data; J.-L.G. conceived and supervised the study, analyzed the data and co-wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. FIP200GFAP, Trp53GFAP and GFAP-Cre transgenic mice were described previously. Male and female mice with 50% FVB and 50% C57/B6 backgrounds were used in experiments. Age- and littermate-matched control and mutant mice were randomly collected on the basis of their genotypes. Mice were housed and handled according to local, state and federal regulations. All experimental procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee at University of Michigan. Genotyping for FIP200, Trp53 and Cre alleles was performed by PCR analysis of tail DNA, essentially as described previously. About 250 mice were used.

Antibodies and reagents. Primary antibodies used were mouse anti-nestin (1:50, Rat-401, anti-PSA-NCAM (1:50, S5A), anti-Brdu (1:200, G34G4), anti-RC2 (1:100, C-19) (all from DSHB, IA), rhodamine-conjugated anti-Brdu (1:200, Millipore, MA), anti-β-actin (1:5,000), anti-vinculin (1:5,000; both from Sigma, MO) and anti-NeuN (1:500, Millipore, MA); rabbit anti-p53 (1:1,000, Leica, Germany), anti-Ki67 (1:100, Spring Bioscience, CA), anti-p62 (1:200 for immunofluorescence and 1:1,000 for western blot, Enzo, PA), anti-GFAP (1:400, Leica, Germany), anti-Ki67 (1:100, Cell Signaling, MA), anti-PAX6 (1:100, Abcam, Cambridge, UK), anti-Tuj1 (1:100, Sigma, MO), anti-SOX2 (1:500, Millipore, MA) and anti-phosphor-STAT3 (1:500, Cell Signaling; MA); and rat anti-Olig2 (1:1,000, BioLegend, CA). Rabbit anti-FIP200 (1:1,000) and rabbit anti-Olig2 (1:500) were as described previously. Secondary antibodies were goat anti-rabbit IgG-FITC (1:400), goat anti-rabbit IgG Texas Red (1:400), goat anti-mouse IgG-FITC (1:200), goat anti-mouse IgG–Texas Red (1:400), goat anti-mouse IgM-rhodamine (1:200), goat anti-mouse IgG–HRP (1:5,000), goat anti-rabbit IgG–HRP (1:5,000) (all from Jackson Immunology, PA). Chloroquine, N-acetylcysteine (NAC) and dihydroethidium (DHE) were purchased from Sigma (St Louis, MO).

Histology, immunofluorescence and transmission electron microscopy. Mice were killed using CO2 and a complete tissue set was collected during necropsy. Fixation was carried out for 16 h at 4°C using 4% (w/v) freshly made, prechilled PBS-buffered paraformaldehyde (PFA). The brains were separated into hemispheres and then embedded in paraffin and sectioned at 5 μm. Slides from histologically comparable positions (triangular lateral ventricle with intact RMS) were stained with hematoxylin and eosin (H&E) for routine histological examination or left unstained for immunofluorescence (IF). H&E-stained sections were examined under immunofluorescence staining. Nuclei were stained with DAPI and slides mounted with Vectashield mounting medium (Vector Laboratories, CA). Digital photography was carried out as described previously.

The TEM studies of the SVZ samples were performed as described previously. In brief, wild-type and FIP200GFAP cKO mice were perfused through the heart with a mixture of 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed for 2 h with the same fixative. Brains were sectioned as 200-μm slices with a vibratome (Leica, Germany). Before postfixation for 1 h with 1% osmium tetroxide, slices were washed in 0.1 M cacodylate buffer pH 7.2. The slices were dehydrated through a series of ethanol solutions, infiltrated with epoxy resins and flat-embedded. Slices with the lateral ventricle and the SVZ were trimmed and mounted on blocks and sectioned with an ultramicrotome. Ultrathin sections were counterstained with uranyl acetate and lead citrate and analyzed with a Philips CM-10 transmission electron microscope. Neurospheres were settled by gravity and fixed with 1.25% glutaraldehyde in 0.1 M phosphate buffer. The subsequent postfixation and staining procedures for neurospheres were same as those for the SVZ tissues.

Neurosphere formation assay, in vitro differentiation and cryosectioning. The neurosphere formation assay was performed as previously described. In brief, the SVZ tissues were isolated under a dissection microscope and cut into ~1 mm3 cubes. They were digested in 0.2% trypsin to obtain single-cell suspensions. The cells were cultured in neurobasal medium supplemented with B27 (Invitrogen, CA), 10 ng/ml bFGF and 20 ng/ml EGF (Invitrogen, CA) in Ultra-Low Attachment dishes (Corning, MA). Neurospheres with diameters larger than 50 μm were counted 7–9 days after culturing. For their passage, primary neurospheres were collected by centrifugation and then incubated in 0.2% trypsin at 37 °C for 10 min with pipetting ~50 times. After addition of trypsin inhibitor supplemented with DNase I ( Worthington, NJ) to stop digestion, dispersed cells were collected by centrifugation at 400g for 10 min, counted and then cultured for the secondary neurospheres formation and quantified as described above. In some experiments, the dispersed cells from neurospheres were cultured for differentiation in neurobasal medium supplemented with B27 (Invitrogen, CA), 10 ng/ml bFGF and 5% (v/v) FBS in 48-well plates coated with 150 μg/ml poly-D-lysine (Biomedical Technologies, MA) and 20 μg/ml laminin (Invitrogen, CA) for another 7 d. Differentiated cells were stained for TuJ1 and GFAP for lineage analysis. In other experiments, neurospheres were fixed in 4% PFA for 30 min and then washed twice in cold PBS for 10 min. Fixed neurospheres were consecutively transferred to 20% and 30% sucrose to settle overnight. They were embedded in O.C.T. (Sakura, CA), cryosectioned at 10 μm (3050 Cryostat, Leica, Germany) and the slices kept at −80 °C before used for IF.

In some experiments, primary astrocytes were isolated from P0 Ctrl and FIP200GFAP cKO mice and cultured in DMEM with 10% FBS for 10 d. They were then replated in 0.4-mm pore size Transwell membrane inserts (Costar) for 7 d. The cultured astrocytes were then rinsed and fed with neurosphere growth medium, and the Transwells containing the cells were placed above freshly plated embryonic NSCs isolated from E17.5 embryos. The cultures were fed with fresh neurosphere growth media every 4 d. The number of neurosphere was determined as described above 10 d after co-culture.

BrdU incorporation assay and TUNEL assay. For in vitro experiments, BrdU was added to the neurosphere culture medium 2 h before cell collection at a final concentration of 10 μM. For in vivo studies, BrdU was administered intraperitoneally (i.p.) at 100 μg/gram body weight three times at 2-h intervals. Mice were either killed 2 h after the last injection (short-term incorporation) or 3 weeks later (long-term retention), and tissues were processed as described above. For BrdU detection, the samples were treated with 2 M HCl at 22–25 °C for 20 min to denature the nucleotides and then neutralized with 0.1 M sodium borate at 22–25 °C for another 20 min. After three exchanges in PBS (5 min each), slides were incubated with mouse anti-BrdU and secondary antibodies as described under immunofluorescence staining. Nuclei were stained with DAPI and slides mounted with Vectashield mounting medium (Vector Laboratories, CA). For IF and detection of BrdU in the same tissue, IF was carried out first and samples were postfixed with 4% PFA at 22–25 °C for 20 min before nucleotide denaturation with HCl. Histological examination and digital photography were carried out as described above.

Apoptotic cells were detected by TUNEL method according to the protocol provided by the manufacturer within the In situ Cell Death Detection Kit-TMR Red (Roche, Germany).

DHE administration. Mice were injected i.p. with DHE prepared as follows. DHE powder was dissolved in dimethylsulfoxide (DMSO) to create a DHE stock solution (10 mg/ml). The DHE injectate (27 mg/kg) was produced by adding DHE stock solution to PBS and maintained at 40 °C. Mice were anesthetized 18 h after DHE injection and were perfused with 4% PFA in PBS. Samples were postfixed in 4% PFA at 4 °C for 1 week and transferred to 20%, 30% sucrose overnight at 4 °C. Brains were then embedded in O.C.T. compound and frozen on dry ice. Cryosectioning at 10 μm thickness was performed in 3050 Cryostat machine (Leica, Germany) and sectioned samples were kept at −80 °C before IF. For IF in DHE stained samples, IF was carried out as described above.

Chloroquine and NAC administration. To show autophagy defects in cko mice, chloroquine was administrated i.p. into Ctrl and FIP200GFAP cKO mice from P7 for 7 consecutive days at a concentration of 50 mg/kg in sterile PBS to block LC3-II degradation. For in vitro experiments, NAC was added daily to the
culture medium of neurospheres at a final concentration of 50 µM. For *in vivo* administration of NAC, freshly prepared NAC (10 mg/ml in PBS at pH 7.4) was subcutaneously injected into Ctrl and FIP200GFAP cKO mice at a dose of 67 mg/kg once daily from P7 to P28. After 21 d of NAC treatment, mice were injected DHE as described above. In some experiments, brains from NAC treated mice were split into halves at the end of treatment. One hemisphere was used for *in vitro* neurosphere assay and the other for IF and DHE staining. In other experiments, NAC-treated mice were labeled first with BrdU for long-term BrdU retention at P7 before the injection of NAC.

**RNA extraction, reverse transcription and quantitative real-time PCR.** Total RNA was extracted from neurospheres using TRIZol reagent (Ambion, CA) and an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturers’ instructions. Reverse transcription and quantitative real-time PCR using the SYBR Green PCR Core reagents system (Qiagen, Germany) were carried out as described previously. The primers for mouse *Pax6*, *Stat3*, *Trp53*, *Notch1*, *Hes1*, *Hes5*, *Hey1* (sequences will be sent upon request), *p21*, *Bbc3*, *Fas* (sequences as described previously) and internal control (sequence as described previously) were obtained from Fischer Scientific (Fischer Scientific, PA).

**Protein extraction, SDS-PAGE and western blotting.** The cortices were collected from embryos of Ctrl and FIP200GFAP cKO mice at E17.5. The SVZ tissues from Ctrl and FIP200GFAP cKO mice were microdissected from 300-µm-thick coronal sections of P14 and P28 brains and used for protein extraction by homogenization in modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 1 mM sodium EDTA) supplemented with protease inhibitors (5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). In other experiments, cells from neurospheres were obtained by centrifugation and lysed with the same lysis buffer. After removing tissue and cell debris by centrifugation at 13,000 r.p.m. in a Biofuge 13 (Heraeus, Germany) for 10 min at 4 °C, protein concentration was determined using Bio-Rad protein assay reagent. The lysates were boiled for 5 min in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 12.5% glycerol, 1% SDS, 0.01% bromphenol blue) containing 5% β-mercaptoethanol. They were then analyzed by SDS-PAGE followed by western blotting using various antibodies, as described previously.

**Statistical analysis.** Lengths, areas and the number of cells from comparable sections were quantified using the ImageJ software package. Data collection and analysis were not performed blind to the conditions of the experiments. No data points were excluded in the analysis. Data distribution was assumed to be normal, but this was not formally tested. Statistical significance was evaluated by two-tailed Student's *t*-test using GraphPad Prism (version 4.0), with *P* < 0.05 as indicative of statistical significance. Sample sizes were not determined a priori but are similar to sample sizes used in previous similar studies (for example, refs. 13,48). The number of animals used for quantification is indicated in the figure legends. Most of the experiments were successfully repeated at least three times and all at least two times.

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