Autophagic lipid metabolism sustains mTORC1 activity in TSC-deficient neural stem cells

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Although mammalian target of rapamycin 1 (mTORC1) negatively regulates autophagy in cultured cells, how autophagy impacts mTORC1 signalling, in particular in an in vivo setting, is less clear. Here we show that autophagy supports mTORC1 hyperactivation in neural stem cells (NSCs) lacking tuberous sclerosis complex subunit 1 (Tsc1), thereby promoting defects in NSC maintenance, differentiation and tumorigenesis, and the formation of the neurodevelopmental lesion of tuberous sclerosis complex (TSC). Analysing mice that lack Tsc1 and the essential autophagy gene Rb1-inducible coiled-coil 1 (Rb1cc1, also called Fip200) in NSCs, we find that TSC-deficient cells require autophagy to maintain mTORC1 hyperactivation under energy-stress conditions, likely to provide free fatty acids via lipophagy to serve as an alternative energy source for OXPHOS. In vivo, inhibition of lipophagy or its downstream catabolic pathway reverses defective phenotypes caused by Tsc1-null NSCs and reduces tumourigenesis in mouse models. These results reveal a cooperative function of selective autophagy in coupling energy availability with TSC pathogenesis and suggest a potential new therapeutic strategy to treat people with TSC.
rostral migratory stream (RMS), and had enlarged brains compared with those of Tsc1GFAPKO mice (Fig. 1a,b and Extended Data Fig. 1a,b). Autophagy was functional in Tsc1GFAPKO but reduced in 2cKO SVZ, indicated by p62 aggregates and autophagy flux, consistent with autophagy blockade with Flip200 loss (Fig. 1c,d and Extended Data Fig. 1c–f). Staining of phosphorylated S6 ribosomal protein (pS6RP) and 4EBP1 (p4EBP1), downstream effectors of mTORC1, revealed diminished mTORC1 activity in the SVZ of 2cKO mice (Fig. 1e,f and Extended Data Fig. 1g,h). These results suggested that abnormal Tsc1GFAPKO NSCs were associated with sustained autophagy combined with mTORC1 hyperactivation.

**Autophagy inhibition in Tsc1GFAPKO mice rescues Tsc1-deficient NSCs and suppresses tumourigenesis.** We next examined the cellular basis for the rescue of brain defects in 2cKO mice. At postnatal day (P) 0, Ki67+ cell frequency was increased in the ventricular zone (VZ)/SVZ of Tsc1GFAPKO mice compared to Ctrl (Fig. 1g), contributing to increased cellularity and RMS cell migration at P7 and P21 (see Fig. 1a,b). In Tsc1GFAPKO mice at P21, Ki67+ cell frequency was reduced and apoptosis increased compared with levels in Ctrl mice (Fig. 1i,j). Flip200 deletion in 2cKO mice restored VZ/SVZ proliferation and apoptosis, and NSC proliferation, to Ctrl levels (Fig. 1g–k and Extended Data Fig. 1i–j).

We examined NSC self-renewal by in vitro neurosphere assay using P21 SVZ cells. Primary neurospheres from Tsc1GFAPKO and 2cKO SVZ cells were similar in number and size to those from Ctrl (Fig. 1l and Extended Data Fig. 1k,l). Tsc1GFAPKO secondary neurosphere growth was significantly less than that of Ctrl and 2cKO (Fig. 1m and Extended Data Fig. 1m), suggesting that Flip200 deletion sustained the renewal potential of Tsc1-deficient NSCs. Autophagy in Tsc1GFAPKO and Ctrl neurospheres was similar, and was blocked in 2cKO neurospheres by Flip200 deletion (Extended Data Fig. 1n,o). As previously observed, increased DCX+ neuroblasts were found in Tsc1GFAPKO mice at P21 (Fig. 1n and Extended Data Fig. 2a). Mature neurons (NeuN+ cells) were found in Tsc1GFAPKO SVZ (Fig. 1o). After labelling proliferative SVZ cells with BrdU at P7 and tracing them until P21, we found ∼7% NeuN+ BrdU+ cells in Tsc1GFAPKO SVZ, but almost none in Ctrl SVZ (Fig. 1p,q). This indicates premature differentiation of mutant neuroblasts consistent with either defective migration into RMS or prevention of migration out of the SVZ. Flip200 deletion reversed aberrantly increased doublecortin (DCX)+ neuroblasts, neuronal nuclei (NeuN)+ cells and NeuN+bromodeoxyuridine (BrdU)+ cells, and astrocytes in 2cKO SVZ (Fig. 1n–r and Extended Data Fig. 2b). After culturing neurospheres in vitro to analyse Tsc1GFAPKO SVZ cell differentiation, we found aberrantly increased numbers of β-III tubulin+ cells (Extended Data Fig. 2c) and GFAP+ astrocytes (Extended Data Fig. 2d), and reduced NeuN+ neurons (Extended Data Fig. 2e) and myelin basic protein (MBP)+ cells (Extended Data Fig. 2f); all were rescued in 2cKO neurospheres, consistent with observations in vivo. These results suggest that autophagy is required for the defective maintenance and differentiation of Tsc1-deficient NSCs induced by hyperactivation of mTORC1 in Tsc1GFAPKO mice.

SEN/SEGAs are brain lesions found in people with TSC. SEN-like lesions in the SVZ of Tsc1GFAPKO mice (Extended Data Fig. 2g) were not found until P21. They developed along the lateral wall on the side of striatum, with no regional preference (Fig. 1s). Some lesions appeared as ‘floating’ spheres as tumours protruded and bent in the LV when cross-sectioned (Fig. 1s, arrow). Murine SEN-like cells contained hyperchromatic nuclei surrounded by scanty cytoplasm, resembling NSC, and were pS6RP+ (Extended Data Fig. 2h). As previously reported, proliferation in the SEN-like lesions was lower than in SVZ (∼5% versus ∼20% Ki67+ cells at P21). SEN-like cells were Nestin+ (Extended Data Fig. 2i); most had medium Sox2 expression, compared with strong Sox2 expression in Tsc1GFAPKO mice (Extended Data Fig. 2j); some were NG2+ (Extended Data Fig. 2k);

and most expressed high levels of DCX (Extended Data Fig. 2l). Only a small fraction was GFAP+ (Extended Data Fig. 2j), suggesting they had a mixed lineage with a preference for neuronal differentiation. We found only one SEN-like lesion in ten 2cKO mice at P21, and in none out of six mice at P28 (Fig. 1t and Extended Data Fig. 2g). These results suggest that autophagy supports tumourigenesis with hyperactivated mTORC1 in Tsc1GFAPKO mice.

Many autophagy genes have functions independent of their roles in canonical autophagy. We studied autophagy-specific functions of Flip200 in vivo using our Flip200 4A knock-in mutant mouse, in which the autophagy function of Flip200 is inactivated. Removing the Flip200 autophagy function in Tsc1GFAPKO mice (Extended Data Fig. 2j); some were NG2+ (Extended Data Fig. 2k);
Fig. 1 | Inactivating Fip200 rescued defective postnatal NSCs in Tsc1GFAPcKO mice. a, Haematoxylin and eosin (H&E) staining of P7 and P21SVZ and RMS from Ctrl, Tsc1GFAPcKO and 2cKO mice. b, Mean ± s.e. of the number of SVZ cells at P21 in Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO mice. n = 6 animals. c, Results from immunofluorescence analysis of p62 and DAPI in P21 SVZ from Tsc1GFAPcKO and 2cKO mice. Inset, p62 aggregates. d, Mean ± s.e. of p62 puncta in P21 SVZ of Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO mice. n = 5 animals. e, Immunofluorescence of p56RP and DAPI in P21 SVZ of Tsc1GFAPcKO and 2cKO mice. Bottom panels, boxed areas. f, Mean ± s.e. of p56RP puncta in P21 SVZ of Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO mice. n = 4 animals. g, Mean ± s.e. of the percentage of Ki67+ cells in P0 (g) and P21 (h) SVZ from Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO mice, n = 4 animals. h, Boxed detail, cell #1 and cell #2. Five independent experiments yielded similar results. i, Mean ± s.e. of secondary neurospheres of total P21 SVZ cells of Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO mice. n = 6 animals. j, Mean ± s.e. of DCX+ (m) and NeuN+ (n) cells of P21 SVZ of Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO are shown. n = 4 animals. k, Immunofluorescence of NeuN, BrdU and DAPI in Tsc1GFAPcKO P21 SVZ. BrdU labelled at P7 was retained for 14 d to trace NeuN+ cells. Boxed detail, cell #1 and cell #2. Five independent experiments yielded similar results. l, Mean ± s.e. of the percentage of BrdU+NeuN+ cells of total BrdU+ cells in Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO SVZ, n = 5 animals. m, Mean ± s.e. of GFAP+Nestin+ cells in P21SVZ of Ctrl, Tsc1GFAPcKO, 2cKO and Fip200GFAPcKO mice, n = 5 animals. n, H&E staining of SEN-like lesion (arrow) in Tsc1GFAPcKO P21 brain. Inset, Detail of ‘floating’ lesion. Five independent experiments yielded similar results. o, Frequency of SEN-like lesions in Ctrl, Tsc1GFAPcKO, 2cKO, and Fip200GFAPcKO P21 mice. The numbers of animals in each group are shown in the table. Dotted lines (ce,p) indicate the SVZ boundaries. CC, corpus callosum; E, ependymal; LV, lateral ventricle; RMS, rostral migratory stream; SEN-like, subependymal nodule-like lesion; SVZ, subventricular zone. Scale bars, 100 µm. Data were analysed by one-way analysis of variance (ANOVA) with Tukey’s post-hoc test (b,d,f-k,m-o,q,r) or chi-squared test (t).
Lack of increase in LC3-II with BafA1 indicated decreased autophagy after Fip200 knockdown (Extended Data Fig. 4h–i). Fip200 knockdown decreased hyperactivation of mTORC1 induced by Tsc1 knockdown in cells treated with 2DG (Extended Data Fig. 4j), but not normal culture conditions (see Extended Data Fig. 4e). These results suggested that Fip200-mediated autophagy is necessary to maintain mTORC1 hyperactivation with loss of either Tsc1 or Tsc2 under energy-stress conditions.

Autophagy maintains OXPHOS to sustain ATP production for hyperactivated mTORC1. Autophagy is proposed to provide substrates for oxidative metabolism under energy stress\(^1,4\). We studied mitochondrial oxygen consumption rate (OCR) and found that Tsc2 KO MEFs had increased ATP-related and maximum OCR (Fig. 2h and Extended Data Fig. 4k,l), indicative of high mitochondrial OXPHOS. After glucose deprivation (Extended Data Fig. 4k) or 2DG treatment (Extended Data Fig. 4l), both ATP-related and maximum OCRs were significantly reduced in WT but were maintained in Tsc2 KO MEFs. Autophagy inhibition by Spautin1, CQ or Atg7 KO reduced OCRs in Tsc2 KO MEFs (Fig. 2i,j and Extended Data Fig. 4m,n), consistent with reduced ATP (see Extended Data Fig. 4c,d). Similarly to Tsc2 KO MEFs, Tsc1 knockdown (KD) HEK293 cells (Fig. 2k,m) and WT MEFs expressing constitutively active Rheb (Rheb-CA MEF) (Fig. 2l,n) maintained OCR in glucose-free conditions in an autophagy-dependent manner. Lastly, increased ATP-related and maximum OCR in Tsc1GFAP cKO neurospheres were reversed in 2cKO (Fig. 2o). Together, these results suggest a role for autophagy in maintaining elevated OXPHOS to sustain ATP production and mTORC1 hyperactivation under energy stress.

Lipophagy produces FFAs as an energy source for hyperactivation of mTORC1. We explored potential autophagy-dependent energy sources that could fuel mTORC1 activity in energy-stressed Tsc-deficient cells. Glutamine did not restore mTORC1 hyperactivation in Tsc2 KO MEFs after autophagy inhibition (Extended Data Fig. 5a), in contrast with previous results in pancreatic cancers\(^1,4\). Amino-acid carbon skeletons were unlikely energy sources, as similar ammonia concentrations were found in WT and Tsc2 KO MEFs under normal and glucose-free conditions (Extended Data Fig. 5b). Glycogen content was comparable in WT and Tsc2 KO MEFs after normal and glucose-free conditions (Extended Data Fig. 5b). β-oxidation of FFAs supports bioenergetics in Tsc-deficient cells during energy stress. FFAs can be metabolized to produce ATP through catabolic β-oxidation. We analysed β-oxidation under glucose-free conditions and found higher activity in Tsc2 KO MEFs, reversed by Spautin1 treatment (Fig. 3f). We then cultured Tsc2 KO MEFs with medium and long-chain FFA. After autophagy inhibition, we found that both FFAs abrogated ATP loss by Spautin1 and CQ under glucose-free (Fig. 3m), but not normal, culture conditions (Extended Data Fig. 5g). Consistent with ATP production, under energy stress, long-chain FFAs increased the OCR (Fig. 3n) and restored S6RP phosphorylation (Fig. 3o) in Spautin1-treated Tsc2 KO but not WT MEFs. These results demonstrate a role for autophagy-mediated lipid catabolism in Tsc-deficient cells under energy-stress conditions.

![Image](https://example.com/image.png)

**Fig. 2 | Autophagy supplies ATP to sustain hyperactivation of mTORC1 in Tsc-deficient cells under energy stress.** a. Lysates from Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO neurospheres in glucose-free medium for 2 h probed for phosphorylated and total S6RP. Three independent experiments yielded similar results. b. Mean ± s.e. of mean fluorescence intensity (MFI) of pS6RP from dissociated Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. c. Mean ± s.e. of percentage of pAMPK-α2 cells in P21 SVZ of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. d. Mean ± s.e. of percentage of pAMPK-α2 cells in P21 SVZ of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. e. Mean ± s.e. of percentage of pACC and DAPI in P21 SVZ of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. Scale bars in (c) and (e), 100 μm. f. Mean ± s.e. of percentage of pACC-α2 cells in P21 SVZ of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. n = 5 animals. g. Mean ± s.e. of percentage of pAMPK-α2 cells in P21 SVZ of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. n = 5 animals. h. Mean ± s.e. of ATP content of isolated primary neurospheres from Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. n = 5 animals. i. Mean ± s.e. of ATP-related and maximum OCR in Tsc1GFAP cKO neurospheres in glucose-free medium for 2 h probed for phosphorylated and total S6RP. Three independent experiments yielded similar results.
We then examined FFA β-oxidation directly and found that treatment with the β-oxidation inhibitors ETO, trimetazidine (TMZ)\(^{52}\) and ranolazine (Rano)\(^{53}\) triggered bioenergetic stress characterized by phosphorylation of AMPK, Raptor and ACC (Fig. 4a and Extended Data Fig. 6a,c), and reduced phosphorylation of S6K and S6RP (Fig. 4b and Extended Data Fig. 6b,d) in Tsc2 KO...
MEFs under glucose-free conditions and with 2DG treatment. ETO inhibited mTORC1 activation under energy stress, but not normal culture in Tsc1GFAP cKO neurospheres (Fig. 4c). ETO and Rano dissociated mTOR from LAMP2 structures in Tsc2 KO MEFs under glucose deprivation (Extended Data Fig. 6e, bottom panels, and Extended data Fig 6f; compare with Extended Data Fig. 3d, second row, right three panels, and Extended Data Fig. 3E), but not normal culture conditions (Extended Data Fig. 6e, top panels, and Extended Data Fig. 6f). Lastly, inhibition of AMPK by compound C prevented mTORC1 suppression by ETO (Extended Data Fig. 6g) in Tsc2 KO MEFs, providing further support that FAO inhibitors blocked mTORC1 via AMPK in TSC-deficient cells under energy stress.

All β-oxidation inhibitors reduced ATP levels in Tsc2 KO (Fig. 4d) but not WT (Extended Data Fig. 6h) MEFs under energy stress. Similar results were obtained for TSC1 KD cells treated with ETO (Extended Data Fig. 6i). ETO and Rano also decreased ATP levels under energy stress in Tsc1GFAP cKO neurospheres (Fig. 4e). We also observed a corresponding decrease of maximum OCR in Tsc2 KO MEFs by all inhibitors under energy stress (Fig. 4f,g). These results suggest that Tsc-deficient cells have an increased dependence on FFA β-oxidation fuelled by FFAs to maintain hyperactivated mTORC1 under energy stress.

All three β-oxidation inhibitors also increased the number of LDs co-localized with lysosomes in Tsc2 KO but not in WT MEFs under glucose deprivation (Fig. 4h,i). Co-localization of LDs with LC3+ autophagosomes increased together with FFAs (Fig. 4j,k), suggesting there is feedback to increase lipophagy when β-oxidation is inhibited. These results further support the notion that FFAs generated by lipophagy of LDs are used to sustain mTORC1 hyperactivation in Tsc-deficient cells under energy stress.

Lysosomal acid lipase digests LDs to release FFAs in Tsc-deficient cells. We next explored potential lipase(s) for lipophagy of LDs. We used lipase inhibitors, including Orlistat, for lysosomal acidic lipase (LAL)54, atglistat for adipose TG lipase (ATGL)55 and JZL184 for monoacylglycerol lipase56. Similar to a previous report suggesting a role for LAL in autophagy, we found that Orlistat, but not atglistat or JZL184, significantly reduced glycerol release (Fig. 5a) and increased the number of LDs (Fig. 5b) in Tsc2 KO MEFs with glucose starvation. No inhibitor affected glycerol release in normal and increased the number of LDs (Fig. 5c) in Tsc2 KO MEFs with glucose-free conditions and with 2DG treatment. ETO inhibited mTORC1 activation under energy stress, but not normal culture in Tsc1GFAP cKO neurospheres (Fig. 4c). ETO and Rano dissociated mTOR from LAMP2 structures in Tsc2 KO MEFs under glucose deprivation (Extended Data Fig. 6e, bottom panels, and Extended data Fig 6f; compare with Extended Data Fig. 3d, second row, right three panels, and Extended Data Fig. 3E), but not normal culture conditions (Extended Data Fig. 6e, top panels, and Extended Data Fig. 6f). Lastly, inhibition of AMPK by compound C prevented mTORC1 suppression by ETO (Extended Data Fig. 6g) in Tsc2 KO MEFs, providing further support that FAO inhibitors blocked mTORC1 via AMPK in TSC-deficient cells under energy stress.

Indeed, we observed increased LAL in Tsc2 KO MEFs (Extended Data Fig. 7d). In glucose-starved Tsc2 KO MEFs, expression of LAL shRNA (Extended Data Fig. 7e) reduced glycerol release (Extended Data Fig. 7f) and FFA content (Extended Data Fig. 7g), accompanied by corresponding increases in LDs (Extended Data Fig. 7h) and TG (Extended Data Fig. 7i). LAL small hairpin RNA (shRNA) also decreased mitochondrial OCR, ATP levels and mTORC1 activation under energy stress (Extended Data Fig. 7j–l). Supplementation with BSA–palmitate restored ATP and mTORC1 hyperactivation in Tsc2 KO MEFs lacking LAL in glucose limiting conditions (Fig. 5) and Extended Data Fig. 7k). Together, these results demonstrate LAL to be the major lipase for the production of FFAs from lipophagy of LDs to sustain hyperactivated mTORC1 in Tsc-deficient cells under energy stress.

**Lipophagy inhibition restores function of Tsc1-deficient NSCs and inhibits tumourigenesis.** To evaluate targeting lipophagy to counteract mTORC1 hyperactivation as new therapies, we examined pharmacological inhibition of autophagy in Tsc1GFAP cKO mice. We injected autophagy inhibitor CQ (50 mg per kg (body weight)) and glycosylation inhibitor 2DG (500 mg per kg (body weight)), individually or in combination, intraperitoneally into P7 Ctrl and Tsc1GFAP cKO mice every other day for 14 d. Combined CQ+2DG inhibited murine SEN-like lesions, but each alone did not (Fig. 6a). Lesions were also eliminated by positive control rapamycin (2.5 mg per kg (body weight), daily i.P.). Combined CQ+2DG, but neither...
alone decreased S6RP phosphorylation (Fig. 6b and Extended Data Fig. 7m) and increased AMPK phosphorylation and ACC (Fig. 6c,d and Extended Data Fig. 7n,o). mTORC2 was unaffected (Extended Data Fig. 7p,q). Treatment with 2DG, CQ and the combination all reduced ERK signalling comparably (Extended Data Fig. 7r,s). The combination treatment restored the pool of GFAP⁺Nestin⁺...
Fig. 4 | β-oxidation of FFAs to sustain hyperactivation of mTORC1 in energy-stressed Tsc-deficient cells. a, b, Lysates from WT and Tsc2 KO MEFs in FBS-free normal, glucose-free and/or 2DG media for 2 h. ETO was preincubated for 12 h, followed by 2-h energy stress. a, Phosphorylated and total Raptor, phosphorylated and total ACC and vinculin. b, Phosphorylated and total S6K, phosphorylated and S6RP and vinculin. c, Lysates from Ctrl and Tsc1 cKO neurosphere in FBS-free normal, glucose-free and 2DG media for 2 h. ETO was preincubated for 12 h, followed by 2-h energy stress. Phosphorylated and total S6K, and phosphorylated and total S6RP are shown. Three independent experiments gave similar results in a–c. d, Mean ± s.e. of ATP content of Tsc2 KO MEFs treated with ETO (200 μM), TMZ (1 mM) and Rano (200 μM) in FBS-free normal, glucose-free and 2DG media for 2 h. Inhibitors were preincubated for 12 h. n = 3 independent experiments. e, Mean ± s.e. of ATP of Tsc1 cKO primary neurospheres treated with ETO (200 μM) or Rano (200 μM) in normal or glucose-free media. Inhibitors were preincubated for 12 h. n = 5 independent experiments. f, g, Mean ± s.e. of maximum OCR of ETO-, TMZ- and Rano-treated WT and Tsc2 KO MEFs under glucose-free (f) or 2DG treatment (g). n = 4 independent experiments. h, Immunofluorescence of LDs, LAMP2 and DAPI in ETO-treated WT and Tsc2 KO MEFs under FBS-free normal and glucose-free media for 2 h. Regions in yellow boxes are shown in detail in the right panels. i, j, Mean ± s.e. of percentage of LDs found on LAMP2+ lysosomes (i) and on LC3+ puncta (j) (out of total LDs) of ETO-, TMZ- and Rano-treated Tsc2 KO MEFs in FBS-free normal and glucose-free media for 2 h. n = 5–10 (i) and 10 (j) independent experiments. k, Mean ± s.e. of FFA content of ETO-, TMZ- and Rano-treated Tsc2 KO MEF in FBS-free normal, glucose-free and 2DG media for 2 h. n = 5 independent experiments. Scale bar, 10 μm. Data were analysed using a two-tailed Student’s t test (d–g, i, k).
and GFAP\(^+\)Sox2\(^+\) NSCs (Fig. 6c and Extended Data Fig. 8a). Combination CO\(^+\)2DG decreased proliferation and apoptosis (Fig. 6f,g and Extended Data Fig. 8b), decreased aberrant neogenesis and premature neuronal differentiation and increased astrogenesis (Fig. 6h–j) of Tsc1\(^\text{GrA}^+\)cKO, but single treatment had no effect on mTORC1 activity or the maintenance and differentiation of SVZ NSC in Ctrl mice (Fig. 6b–j).

We next tested \(\beta\)-oxidation inhibitors ETO, Rano and TMZ with or without 2DG in mice. The combination blocked tumourigenesis, but \(\beta\)-oxidation inhibitors alone did not (see Fig. 6a).
Fig. 6 | Targeting lipophagy to rescue defects in postnatal Tsc1-deficient NSCs. a, SEN-like lesions in Tsc1cKO mice treated with vehicle (number of mice with lesions/total), 2DG, CQ, etomoxir, ranolazine, trimetazidine, CQ + 2DG, etomoxir + 2DG, ranolazine + 2DG or trimetazidine + 2DG, or positive control, rapamycin; na, not available; ns, not significant. b–d, Mean ± s.e. of the percentages of pS6RP+ (b), and pAMPK+ (c), and pACC+ cells (d) in P21 SVZ of CQ + 2DG-treated Ctrl, and vehicle–, 2DG–, CQ–, and CQ + 2DG–treated Tsc1cKO mice. n = 4 (b) and 5 (c,d) independent experiments. e, Mean ± s.e. of GFAP+ Nestin+ NSCs in SVZ of CQ + 2DG-treated Ctrl, and vehicle–, 2DG–, CQ–, and CQ + 2DG–treated Tsc1cKO mice. n = 6 independent experiments. f, Mean ± s.e. of GFAP+ Nestin+BrdU+ cells of total GFAP+ Nestin+ cells in SVZ of CQ + 2DG-treated Ctrl, and vehicle–, 2DG–, CQ–, and CQ + 2DG–treated Tsc1cKO mice. n = 6 independent experiments. g, Mean ± s.e. of TUNEL+ cells in SVZ of CQ + 2DG-treated Ctrl, and vehicle–, 2DG–, CQ–, and CQ + 2DG–treated Tsc1cKO mice. n = 3 independent experiments. h, Mean ± s.e. of the percentage of DCX+ cells (i), NeuN+ cells (j) and GFAP+Nestin+ cells (j) from SVZ of CQ + 2DG-treated Ctrl, and vehicle–, 2DG–, CQ–, and CQ + 2DG–treated Tsc1cKO mice. n = 3 independent experiments. k–m, Mean ± s.e. of percentage of pAMPK+ (k), pACC+ (l) and pS6RP+ cells (m) in SVZ of vehicle–, 2DG–, ETO–, Rano–, TMZ–, ETO + 2DG–, Rano + 2DG–, and TMZ + 2DG–treated Ctrl and Tsc1cKO mice. n = 5 independent experiments. n, Mean ± s.e. of GFAP+Nestin+ NSCs (number of cells) in SVZ of vehicle–, 2DG–, ETO–, Rano–, TMZ–, ETO + 2DG–, Rano + 2DG–, and TMZ + 2DG–treated Ctrl and Tsc1cKO mice. n = 5 independent experiments. o, Mean ± s.e. of GFAP+Nestin+BrdU+ cells of total GFAP+Nestin+ cells in SVZ of Ctrl and Tsc1cKO mice treated with vehicle, 2DG, ETO, Rano, TMZ, ETO + 2DG, Rano + 2DG and TMZ + 2DG. n = 5 independent experiments. p, Mean ± s.e. of TUNEL+ cells in SVZ of Ctrl and Tsc1cKO mice treated with vehicle, 2DG, ETO, Rano, TMZ, ETO + 2DG, Rano + 2DG and TMZ + 2DG. n = 5 independent experiments. q, Working model of sustained hyperactivated mTORC1 in Tsc-deficient NSCs. Data were analysed by chi-square test (a) or one-way ANOVA with Tukey’s post-hoc test (b–p).
Phosphorylation of AMPK and ACC increased (Fig. 6k,l), and mTORC1 signalling was reduced (Fig. 6m) in Tsc1GFAP-CreKO, but not Ctrl, mice. The combination rescued defective NSC maintenance, proliferation and apoptosis (Fig. 6n,p and Extended Data Fig. 8c,d), and neurogenesis and neuronal differentiation defects (Extended Data Fig. 8e,f) in Tsc1GFAP-CreKO but not Ctrl mice. β-oxidation inhibitors alone did not alter AMPK and ACC phosphorylation, mTORC1 activation or proliferation and apoptosis in Tsc1GFAP-CreKO mice (Fig. 6k–p). Each suppressed aberrant neurogenesis and neuronal differentiation (Extended Data Fig. 8e,f) without affecting SEN-like lesion formation (see Fig. 6a) in Tsc1GFAP-CreKO mice. Together, these results indicate that combined inhibition of glycolysis and lipophagy, or their downstream catabolic processes, prevents tumourigenesis, exhaustion and abnormal NSC differentiation, establishing the therapeutic potential of autophagy inhibition for Tsc-deficient conditions.

Discussion

In mice with Fip200 and Tsc1 deletion, we found that autophagy deficiency rescued defective NSCs caused by mTORC1 hyperactivation. The working model (Fig. 6q) outlines a new paradigm of mTORC1 regulation by selective lipophagy and lipid catabolism required to fuel mTORC1 hyperactivation in Tsc-deficient NSCs. mTORC1 hyperactivation in Tsc-deficient cells increases aerobic glycolysis, FA synthesis and lipid storage6–8. In SVZ tissue in vivo and various cells under energy stress in vitro, autophagy was sustained, despite Tsc deficiency, to meet increased energy demands. mTORC1 hyperactivation increases protein synthesis9,10 and must be coordinated with cellular energy status11,12. Under nutrient-rich conditions, increased glycolytic activity and OXPHOS13 provide energy. In the absence of glycolysis, lipophagy mechanisms described here could support elevated protein synthesis and energy expenditure. Nevertheless, we recognize that the absence of tracing experiments for metabolites to provide direct evidence for each intermediate of the working model (see Fig. 6a) is a limitation of the study. Despite this, both genetic and pharmacological approaches in animal and cell models clearly established a role for lipophagy to meet energy demands in Tsc1-null NSCs. These results add FAs to a growing list of metabolic outputs from autophagy upstream of mTORC1.

Fip200 single-knockout mice are depleted of NSCs9,9, but Fip200 knockout in the Tsc1-deficient background rescued NSC maintenance in this study, further highlighting the metabolic rewiring by mTORC1 hyperactivation in NSCs. Previously, we proposed that Fip200 regulates normal NSCs via its functions that are distinct from canonical autophagy, dependent on autophagy-related 5 (Atg5), Atg7 or Atg16L1 (ref. 3). In Tsc-deficient conditions with energy limitation, however, the autophagy function of Fip200 becomes required for neoplastic phenotypes driven by mTORC1 hyperactivation. Importantly, blocking only Fip200 autophagy function, like ablation of the Fip200 gene, rescued NSC phenotypes in 2eK1 mice. Autophagy inhibition by gene ablation has been shown to decrease tumourigenesis and progression in several mouse models of cancer, including breast, lung and pancreatic cancers22–24. Our studies advance autophagy inhibition for cancer therapy, and establish new links between autophagy, lipid catabolism and bioenergetics. In vivo inhibition of tumourigenesis by targeting either lipophagy or lipid catabolism could be a new therapeutic strategy for TSC and other diseases with mTORC1 hyperactivation.

Methods

Animals. Fip200f/f, Fip200fl/fl, Tsc1f/f and hGFAP-Cre transgenic mice have been described previously25,26,27, Fip200f/f;Tsc1f/f;hGFAP-Cre, Fip200f/f;Tsc1f/f;hGFAP-Cre and control mice were maintained on a mixed C57B6 (87.5%) with FVB (12.5%) background. Age- and littermate-matched control and mutant mice were used for analysis to minimize the impact of modifier genes. Mice were housed and handled according to local, state and federal regulations. All experimental procedures were carried out according to the guidelines of Institutional Animal Care and Use Committee at the University of Cincinnati. Genotyping for Fip200, Tsc1 and Cre alleles was performed by polymerase chain reaction (PCR) analysis of tail DNA, essentially as described previously28.

Cells and cell culture. HEK293 cells from the American Type Culture Collection (ATCC) were cultured in DMEM with 10% FBS under growth conditions. HEK293 cells infected with human TSC1 and Fip200 shRNAs, or scrambled shRNA, were selected using 1 μg ml−1 hygromycin B for selection. Atg5 knockout MEFs and paired WT MEFs were gifted by N. Mizushima from the University of Tokyo. Atg5 KO MEFs and WT MEFs were transfected with rat Rhbds16–17 or empty plasmid and were cultured in DMEM with 10% FBS and 1 μg ml−1 puromycin for 3 d. MEFs were purchased from Gibco (cat. no. A11960025). We used 100 nM bafloycin A1 for 2 h to inhibit autophagosome degradation.

CRISPR knockout of Atg7 and AMPKα1 in MEflo cells. Two pairs of sgRNA targeting Atg7 (#1, 5′-TGGGCAAGCACGAGTTGAC-3′) and #2, 5′-GAACAGTACGGGCTGAG-3′) and two pairs of sgRNA targeting AMPKα1 (#1, 5′-TTATTGTGGCAGCATATTGG-3′ and #2, 5′-GTGGCGAAACGAGGTGAC-3′) were subcloned into lentiviral vectors essentially as described previously29,30.

ViruProduction and infection. Human scrambled shRNA, Fip200 shRNA and Tsc1 shRNA encoded in pGPZ lentiviral vector were purchased from Open Biosystems. Mouse scrambled shRNA, LAL shRNA #1 and #2 were purchased from Sigma. Package vectors of pSPAX2 and pMD2.G were mixed with CRISPR lentivectors and selected with puromycin (2–4 μg ml−1) for 1 week. Single clones were screened by limited dilution in 96-well plate. The selected clones were cultured in DMEM with 10% FBS.

Neurosphere-formation assay and in vitro differentiation. Neurons were cultured in neurobasal medium supplemented with B27, 10 ng ml−1 basic fibroblast growth factor (bFGF) and 20 ng ml−1 epidermal growth factor (EGF) in ultra-low attachment dishes, essentially as described in our previous reports3–5. Neurons with diameter larger than 50 μm were counted 7–9 d after culturing. For their passage, primary neurons were cultured by centrifugation, then incubated with the calcium chelator BAPTA-AM (50 μM) for 30 min and then washed twice in cold PBS for 10 min. These cells were stained with βIII-tubulin, NeuN, GFAP, MBP and DAPI for lineage analysis.

Antibodies and reagents. The primary antibodies used in our studies were as follows. Mouse anti-Nestin (cat. no. Rat-401, DSHB), immunohistochemistry (IHC), 1:50; anti-GFAP (cat. no. 5G8795, Sigma), IHC, 1:10,000; anti-total S6 ribosomal protein (Ser240/244) (cat. no. 22188, Cell Signaling), IHC, 1:100; anti-phosphorylated S6 ribosomal protein (Ser240/244) (cat. no. 22181, Cell Signaling), IHC, 1:100; anti-β-actin (cat. no. A2228, Sigma), western blot (WB), 1:4,000; anti-GAPDH (cat. no. G8795, Sigma), WB, 1:10,000; anti-β-III tubulin (cat. no. 20924, Sigma), WB, 1:5,000; anti-NeuN (cat. no. MAB377, Millipore), IHC, 1:500; rabbit anti-Ki67 (cat. no. M3606, Spring BioScience), IHC, 1:200; anti-phospho-p62 (cat. no. BML-PW9860, Enzo), WB, 1:1,000; IHC, 1:200; anti-GAPDH (cat. no. 50334, DAKO), IHC, 1:200; anti-phosphorylated (Ser65) eEF2 (cat. no. 9451, Cell Signaling), WB, 1:1,000; anti-phosphorylated (Thr70) eEF2 (cat. no. 2855, Cell Signaling), IHC, 1:100; WB, 1:1,000; anti-total eEF2 (cat. no. 9644, Cell Signaling), WB, 1:1,000; anti-Fip200 (cat. no. 12436, Cell Signaling), WB, 1:1,000; anti-phosphorylated Fip200 (Thr202/Tyr204) ERK1/2 (cat. no. 4307, Cell Signaling), IHC, 1:100; anti-phospho-LC3B (cat. no. 218350, Cell Signaling), WB, 1:1,000; anti-total LC3B (cat. no. 2075, Cell Signaling), WB, 1:1,000; anti-phosphorylated S6 ribosomal protein (Ser235/236) (cat. no. 3440, Cell Signaling), WB, 1:1,000; IHC, 1:200; anti-phospho-LC3B (cat. no. 218350, Cell Signaling), WB, 1:1,000; anti-total LC3B (cat. no. 2075, Cell Signaling), WB, 1:1,000; anti-phosphorylated-Raptor (Ser792) (cat. no. 2083, Cell
in immunofluorescent staining. Nuclei were stained with DAPI and mounted with Vectashield mounting medium (Vector Laboratories). For IF and detection of BrdU in the same tissue, IF was carried out first and samples were post-fixed with 4% PFA in 0.1 M PBS at room temperature before nuclear decondensation with 0.02% SDS for 20 min. After permeabilization, samples were then incubated with lipase for 20 min, followed by a 30-min incubation with Triglyceride Reaction Mix. The optical density at 570 nm was measured, and TG amount was calculated using a standard curve.

MIF (2 x 10^6) cells were extracted with homogenization buffer and washed with PBS. Cell lysates were then incubated with primary antibodies at 4°C overnight, and the samples were then incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). Western blotting was performed with an ECL Western Blotting Detection System. For TUNEL assay, the samples were treated with 2 M HCl at room temperature for 10 min. Slices were then incubated with 0.1% Triton X-100 and 0.2% sodium deoxycholate in 0.2% SDS for 20 min, followed by a 30-min incubation with Triglyceride Reaction Mix. The optical density at 570 nm was measured, and TG amount was calculated using a standard curve.

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Seahorse extracellular assay for oxygen consumption rate. Measurement of OCR by XF24 microplate-based respirometry from intact cells was performed using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). We seeded 20,000 cells per well in Seahorse XF Cell Culture Microplates and incubated overnight at 37 °C with 5% CO₂. Mitochondria stress tests were initiated according to the manufacturer’s instructions by replacing the growth medium in each well with 500 µl of Seahorse XF Base Medium (supplemented with 10 mM glucose, 2 mM t-glutamine and 1 mM sodium pyruvate for normal conditions, or without glucose only for glucose-deprivation conditions) prewarmed at 37 °C. Glycolysis inhibitor 2DG (10 mM) was included in Seahorse XF Base medium for some experiments. Cells were then incubated at 37 °C in a non-CO₂ incubator for 60 min to allow temperature and pH equilibration. Following the equilibration period, cells were loaded into the XF24 extracellular flux analyser and further equilibrated for 20 min prior to the first measurement. After an OCR baseline measurement, the minimum oxygen consumption was determined by injection of 4 µM oligomycin (Olig) and sequentially the maximal respiration rate was assessed upon injection of 0.5 µM FCCP (f luorourocethoxy carbonylcyanide phenylhydrazone). Finally, the non-mitochondrial oxygen consumption was evaluated after injection of both 0.5 µM rotenone and antimycin (R/A). The measurements were normalized to cell number, which was determined using CyQuant DNA dye (Invitrogen). Analysis of data was carried out using Wave software and the XF Mito/Glycolysis stress test report generator (Seahorse Bioscience).

Spatulini (10 µM) and chloroquine (20 µM) were incubated with cells overnight before experiments. ETO, Rano or TMZ prepared in assay medium (75 µM) was injected after FCCP and OCR was measured 3 times before injection of rotenone and antimycin.

Statistical analysis. Lengths, areas and the number of cells from comparable sections were quantified using the Image software package. Statistical significance was evaluated by Student’s t-test, one-way ANOVA, two-way ANOVA and Pearson’s correlation coefficient in GraphPad Prism v.5.0, and differences between groups for frequency of SEN-like structures were assessed using Extreme Limiting Dilution Analysis (ELDA), as described previously, with P < 0.05 indicating statistical significance. The number of animals used for quantification is indicated in the figure legends.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding author upon request. Gel source data are shown in Source Data for Figs. 1–5.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Analyses of autophagy, mTORC1 signalling and NSCs from SVZ tissues and neurospheres. a, Brain size (upper panel) and H&E staining of sagittal sectioned brain (lower panel) of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice at P21. Five independent experiments gave similar results. b, H&E staining of sagittal sectioned brain at two positions of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice at P0 shown on the right. n = 5 independent experiments. c, d, The levels of p62, Fip200 and Tsc1 (c) and LC3 (d) in isolated SVZ tissue of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice treated with or without CQ for 14 d. Three independent experiments gave similar results. e, Means ± s.e. of the autophagy flux (calculated as LC3-II with CQ divided by LC3-II without CQ as shown in b). n = 3 independent experiments. f, g, Immunofluorescence of p62 (f), p4EBP1 (phosphorylated at T37/46) (g) and DAPI in SVZ of Tsc1GFAP cKO and 2cKO mice at P21. Bottom panels in g show details of p4EBP1+ cells in boxed area. h, Mean ± SE of the percentage of p4EBP1+ cells in SVZ of Ctrl, Tsc1GFAP cKO, 2cKO, and Fip200GFAP cKO mice at P21. n = 4 independent experiments. i, j, Mean ± SE of the number of GFAP+Sox2+NSC (i) and the number of GFAP+Nestin+BrdU+ cells (j) in SVZ of Ctrl, Tsc1GFAP cKO, 2cKO, and FIP200GFAP cKO mice at P21. n = 6 independent experiments. k–m, Mean ± SE of the number (k) and size (l, m) of primary (k, l) and secondary (m) neurospheres from SVZ cells of Ctrl, Tsc1GFAP cKO, 2cKO, and FIP200GFAP cKO mice at P21. n = 4 (k), 3 (l, m) independent experiments. n, Lysates from primary neurospheres of Ctrl, Tsc1GFAP cKO, 2cKO, and FIP200GFAP cKO mice examined by immunoblotting with indicated antibodies. Three independent experiments gave similar results. o, The level of LC3 in neurospheres of Ctrl, Tsc1GFAP cKO, 2cKO, and FIP200GFAP cKO mice treated with or without BafA1. Three independent experiments gave similar results. The dotted lines indicated the boundaries of SVZ with LV (f, g). E: ependymal layer; LV: lateral ventricle; ST: striatum; SVZ: subventricular zone. Bar = 100 mm. Data were analyzed by one-way ANOVA with Tukey’s post-hoc test (b, e–m).
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Fip200 KO and Fip200-4A knock-in mutant rescued defective phenotypes in Tsc1GFAP cKO SVZ. a,b, Immunofluorescence of DCX (a), GFAP (b) and DAPI in SVZ of Tsc1GFAP cKO and 2cKO mice at P21. Four independent experiments gave similar results. c–f, Mean ± s.e. of the percentage of β-III tubulin + cells (c), NeuN + cells (d), GFAP + cells (e) and MBP + cells (f) from differentiated neurospheres of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice. n = 4 (c,d), 5 (e,f) independent experiments. g, Frequency of SEN-like structure in Ctrl, Tsc1GFAP cKO, 2cKO, 2cKI, and Fip200GFAP cKO mice at P7, P14 and P28. The animal numbers used at different postnatal stages are indicated. (h–l) Immunofluorescence of pS6RP (h), nestin and Ki67 (i), GFAP and Sox2 (j), NG2 (k), DCX and GFAP (l) in SEN-like lesion in Tsc1GFAP cKO brain at P21. Three independent experiments gave similar results. m, Immunofluorescence of pS6RP and DAPI in SVZ of Tsc1GFAP cKO and 2cKI mice at P21. Five independent experiments gave similar results. n, Mean ± s.e. of the number of pS6RP + cells in SVZ of Ctrl, Tsc1GFAP cKO, 2cKI and Fip200GFAP cKI mice at P21. n = 5 independent experiments. o, H&E staining of SVZ from Ctrl, Tsc1GFAP cKO, 2cKI and Fip200GFAP cKI mice at P28. Five independent experiments gave similar results. p, Mean ± s.e. of SVZ cell number of Ctrl, Tsc1GFAP cKO, 2cKI and Fip200GFAP cKI mice at P21. n = 5 independent experiments. q, r, Mean ± s.e. of the number of GFAP + nestin + NSCs (q) and GFAP + Sox2 + NSC (r) in SVZ of Ctrl, Tsc1GFAP cKO, 2cKI and Fip200GFAP cKI mice at P21. n = 5 independent experiments. The dotted lines indicated the boundaries of SVZ or SEN-like lesion with LV. E, ependymal layer; LV, lateral ventricle; SEN, subependymal nodule-like lesion; ST, striatum; SVZ, subventricular zone. Scale bar, 100 μm (a,b,m,o), 40 μm (h–l). Data were analysed by one-way ANOVA with Tukey’s post-hoc test (c–f,n,p–r) or Chi-square test (g).
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Autophagy sustained mTORC1 hyperactivation in Tsc-deficient MEFs under energy stresses. **a**, Lysates from WT and Tsc2 KO MEFs treated with DMSO or BafA1 examined by immunoblots with antibodies for LC3 and actin. Three independent experiments gave similar results. **b**, Mean ± s.e. of the number of LC3 puncta of DMSO or Spautin1 treated WT and Tsc2 KO MEFs in normal medium and glucose-free medium (without FBS) with or without BafA1 for 2 hours. Spautin1 was preincubated for 12 h. n = 6–12 independent experiments for at least 200 cells. **c**, Lysates from DMSO- or Spautin1-treated WT MEFs and Tsc2 KO MEFs under normal, glucose-free and amino-acid-free conditions (without FBS) for 2 h were examined with indicated antibodies. Spautin1 was preincubated for 12 h. Three independent experiments gave similar results. **d**, Immunofluorescence of mTOR, LAMP2 and DAPI in WT MEF, Tsc2 KO MEF and Tsc2/Atg7 2KO MEF under normal medium (without FBS), glucose-free medium (without FBS) or glucose-free medium with 10 μM Spautin1 (without FBS) for 2 h. Spautin1 was preincubated for 12 h. Four independent experiments gave similar results. **e**, Mean ± s.e. of the Pearson correlation coefficient (PCC) of mTOR co-localization on LAMP2+ structure in WT MEF, Atg7 KO MEF, Tsc2 KO MEF and 2KO MEF under normal medium, glucose-free medium and glucose-free medium (without FBS) with Spautin1 for 2 h. n = 5 independent experiments for at least 200 cells. **f**, Lysates from WT MEFs and Tsc2 KO MEFs treated with DMSO or Spautin1 were examined by immunoblots with antibody for LC3 and vinculin. BafA1 was used to block autophagosome degradation, and Spautin1 was preincubated for 12 h before experiments. Three independent experiments gave similar results. Mean ± s.e. of the relative level of LC3II of DMSO- or Spautin1-treated WT MEFs and Tsc2 KO MEFs with or without BafA1 for 2 h on the right. n = 3 independent experiments. **g**, Lysates from WT MEFs, Atg7 KO MEFs (2 independent clones), Tsc2 KO MEFs and 2KO MEFs (2 independent clones) were examined by immunoblots with antibodies as indicated. Three independent experiments gave similar results. **h**, Lysates were extracted from WT MEF, Atg7 KO MEF (clone 1), Tsc2 KO MEF and 2KO MEF (clone 1) in normal medium, glucose-free medium and 2DG-supplemented medium (without FBS) for 2 h. The levels of phosphorylated S6K, total S6K, phosphorylated S6RP, total S6RP and vinculin were examined. Three independent experiments gave similar results. Scale bar, 10 μm. Data were analysed by two-tailed Student’s t test (**b, f**) or Pearson’s correlation coefficient (**e**).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Analysis of the role of autophagy, TORC1 hyper-activation, ATP content and AMPK targeting phosphorylation in Tsc-deficient neurospheres, MEF, 293 cells and SVZ. a, Lysates extracted from WT and Tsc2 KO MEF in normal, glucose-free and 2DG-supplemented media (without FBS) for 2 h were examined by immunoblot as indicated. Cells were preincubated with DMSO or 10 μM Spautin1 for 12 h. Three independent experiments gave similar results. b, Lysates were extracted from WT, Atg7 KO, Tsc2 KO and 2KO MEF in normal, glucose-free and 2DG-supplemented media (without FBS) for 2 h. The levels of phosphorylated Raptor, total Raptor, phosphorylated ACC and total ACC were examined. Three independent experiments gave similar results. c, Mean ± s.e. of the ATP content of Tsc2 KO MEFs in normal, glucose-free and 2DG-supplemented media (without FBS) supplemented with 10 μM Spautin1 or 20 μM CQ for 2 h was shown. The autophagy inhibitors were preincubated for 12 h. n = 3–9 independent experiments. d, Mean ± s.e. of the ATP content of Tsc2 KO MEFs and 2KO MEFs in normal, glucose-free and 2DG-supplemented media (without FBS) for 2 h. n = 7–8 independent experiments. e, Lysates from Ctrl, Fip200 KD, Tsc1 KD, and 2KD 293 cells were examined by immunoblots with indicated antibodies. Four independent experiments gave similar results. f,g, Means ± s.e. of the protein levels of Tsc1 (f) and Fip200 (g) in Ctrl, Fip200 KD, Tsc1 KD, and 2KD 293 cells were shown. n = 4 independent experiments. h, The level of LC3-II was examined in Ctrl, Fip200 KD, Tsc1 KD, and 2KD 293 cells treated with or without BafA1. Three independent experiments gave similar results. i, Mean ± s.e. of the level of LC3-II in Ctrl, Fip200 KD, Tsc1 KD and 2KD 293 cells treated with or without BafA1 was shown. n = 3 independent experiments. j, Lysates were prepared from Ctrl, Fip200 KD, Tsc1 KD and 2KD 293 cells treated with 25 mM 2DG (without FBS) for 2 h and analysed by immunoblot with antibodies as indicated. Three independent experiments gave similar results. k,l, Means ± s.e. of ATP-related and maximum OCR in WT MEFs, Atg7 KO MEFs, Tsc2 KO MEFs and 2KO MEFs under glucose-free conditions (k) and normal or 2DG treatment conditions (l), n = 9–11 (k) and 4 (l) independent experiments. m,n, Mean ± s.e. of ATP-related and maximum OCR in WT MEFs, Tsc2 KO MEFs and 2KO MEFs under glucose-free conditions (m) and 2DG-treatment conditions (n). n = 8 independent experiments. Data were analysed using two-tailed Student’s t test (c,d,i,k–n), one-way ANOVA with Tukey’s post hoc test (f,g).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Energy stressed Tsc-deficient cells activated lipolysis through autophagy. a, Lysates were extracted from DMSO- or Spautin1-treated WT and Tsc2 KO MEFs supplemented with 20 mM glutamine under normal, glucose-free (upper panels), or 2DG-treatment conditions (lower panels) for 2 h. The levels of phosphorylated S6K and total S6K were examined by immunoblot with antibodies as indicated. Three independent experiments gave similar results. b,c, Mean ± s.e. of the ammonia content (b) and the glycogen content (c) in WT and Tsc2 KO MEFs under normal and glucose-free media (for c, with or without BafA1 treatment) for 2 h. n = 6 (b) and 2 (c) independent experiments. d, Mean ± s.e. of the content of FFAs in Ctrl, Tsc1 KD, 2KD and Fip200 KD 293 cells under normal and glucose-free media. n = 6 independent experiments. e, Immunofluorescence for lipid droplets, LC3 and DAPI in DMSO-, BafA1- and Spautin1-treated Tsc2 KO MEFs under normal or glucose-free conditions (without FBS) for 2 h. Four to nine independent experiments for at least 200 cells gave similar results. f, Immunofluorescence for LDs, LAMP2 and DAPI in DMSO or Spautin1-treated WT and Tsc2 KO MEFs under normal or glucose-free conditions (without FBS) for 2 h. Six to ten independent experiments for at least 200 cells gave similar results. g, Mean ± s.e. of ATP content in CQ or Spautin1-treated Tsc2 KO MEFs under normal conditions and supplemented with BSA or BSA–palmitate for 2 h. n = 3–6 independent experiments. h–l, Representative images of LDs (indicated by Oil Red O staining) in SVZ of Ctrl (H and K), Tsc1GFAPcKO (i, l), and 2cKO (j) mice treated with (k, l) or without (h–j) ETO at P21. Five independent experiments gave similar results. m, Mean ± s.e. of the percentage of cells with lipid droplets in SVZ of Ctrl, Tsc1GFAPcKO and 2cKO mice treated with or without ETO at P21. n = 5 independent experiments. Scale bars, 10 μm. Data were analysed by two-tailed Student’s t test (b,g) or one-way ANOVA with Tukey’s post-hoc test (c,m).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Regulation of activation of mTORC1, AMPK and ATP content by inhibitors for β-oxidation and AMPK in energy-stressed Tsc-deficient cells. a–d, Lysates were extracted from WT MEFs and Tsc2 KO MEFs in normal medium, glucose-free medium and 2DG-supplemented medium (without FBS) for 2 h. Cells were preincubated with β-oxidation inhibitors of Rano (a, b) and TMZ (c, d) for 12 hours and followed by 2 hours treatment. In a and c, the levels of phosphorylated Raptor, total Raptor, phosphorylated ACC, total ACC and vinculin were examined. In b and d, the levels of phosphorylated S6K, total S6K, phosphorylated S6RP, total S6RP and vinculin were examined. Three independent experiments gave similar results. e, Immunofluorescence of mTOR, LAMP2 and DAPI in ETO and Rano treated Tsc2 KO MEFs under normal and glucose-free medium for 2 h. Five independent experiments for at least 200 cells gave similar results. f, Mean ± s.e. of the Pearson correlation coefficient of mTOR localization on LAMP2+ structure in Tsc2 KO MEF treated with ETO or Rano under glucose-free conditions. n = 5 independent experiments. g, Lysates from vehicle-, ETO- or Compound C (CC)-treated Tsc2 KO MEFs in normal, glucose-free and 2DG supplemented media (without FBS) for 2 h were examined by immunoblot using antibodies as indicated. Cells were preincubated with ETO with or without CC for 24 h. Three independent experiments gave similar results. h, Mean ± s.e. of the ATP content of β-oxidation inhibitors of ETO-, TMZ- and Rano-treated WT MEFs in normal, glucose-free and 2DG media (without FBS) for 2 h. The β-oxidation inhibitors were preincubated for 12 h. n = 3–6 independent experiments. i, Mean ± s.e. of ATP content in ETO-treated Ctrl and Tsc1 KD 293 cells under glucose deprivation conditions for 2 h. n = 5 independent experiments. Scale bar, 10 μm. Data were analysed by two-tailed Student’s t test (h, i) or Pearson’s correlation coefficient (f).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Lipophagy released FFAs in energy-stressed mTORC1 hyperactivated Tsc-deficient cells. a, b, Mean ± s.e. of glycerol release from (a) and the number of LDs in (b) WT MEFs under normal and glucose-free media (without FBS) for 2 h supplemented with DMSO, orlistat, atglistat or JZL were shown. n = 3 (a) and 5 (b) independent experiments. c, Immunofluorescence of LAMP2, mTOR and DAPI in Orlistat-treated WT and Tsc2 KO MEFs under glucose-free medium (without FBS) for 2 h. Insets in details on right panels. Three independent experiments gave similar results. d, Lysates from WT and Tsc2 KO MEFs were examined by immunoblot of LAL and actin. Three independent experiments gave similar results. e, Lysates from scrambled shRNA and two individual LAL shRNAs infected WT MEF were examined by immunoblot of LAL and actin. Three independent experiments gave similar results. f, g, Mean ± s.e. of the glycerol release (f) and FFA content (g) from scrambled-shRNA- and LAL-shRNA-#1-infected WT and Tsc2 KO MEF in normal and glucose-free media for 2 h were shown. n = 3 (f) and 6 (g) independent experiments. h, i, Mean ± s.e. of the number of LDs (h) and the content of TG (i) in scrambled-shRNA- and LAL-shRNA-#1-infected Tsc2 KO MEFs under normal medium and glucose-deprivation conditions. n = 5 independent experiments. j, Mean ± s.e. of ATP-related OCR of scrambled-shRNA- and LAL-shRNA-#1-infected WT and Tsc2 KO MEFs under glucose deprivation conditions. n = 4 independent experiments. k, Mean ± s.e. of the ATP content of scrambled-shRNA- and LAL-shRNA-#1-infected Tsc2 KO MEFs in normal, glucose-free and 2DG media with or without supplement of BSA–palmitate for 2 h were shown. n = 3 independent experiments. l, Lysates from scrambled-shRNA- and LAL-shRNA-#1-infected WT and Tsc2 KO MEFs in normal, glucose-free and 2DG-supplemented media for 2 hours. The levels of phosphorylated S6K, total S6K, phosphorylated S6RP, total S6RP and vinculin were examined by Western blot as indicated. Three independent experiments gave similar results. m–o, Immunofluorescence of pS6RP (m), pACC (n), pAMPK (o) and DAPI in SVZ of Tsc1GFAPcKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. Five independent experiments gave similar results. p, Immunofluorescence of pAKT and DAPI in SVZ of Tsc1GFAPcKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. Five independent experiments gave similar results. q, Mean ± s.e. of the percentage of pAKT+ cells in SVZ of Tsc1GFAPcKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. n = 5 independent experiments. r, Immunofluorescence of pERK and DAPI in SVZ of Tsc1GFAPcKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. Five independent experiments gave similar results. s, Mean ± s.e. of the percentage of pERK+ cells in SVZ of Tsc1GFAPcKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. n = 5 independent experiments. Dotted lines indicated the boundaries between SVZ and LV. E, ependymal layer; LV, lateral ventricle; ST, striatum; SVZ, subventricular zone. Scale bar, 10 μm (c); 100 μm (m–p,r). Data were analysed by two-tailed Student’s t test (a,b,f–k), one-way ANOVA with Tukey’s post-hoc test (q,s).
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | AMPK activation stimulated autophagy and lipophagy was required in Tsc-deficient cells. Postnatal developmental defects of cerebral cortex in Tsc2−/− cKO mice were rescued in 2cKO mice. a, Mean ± s.e. of the number of GFAP+Sox2+ NSCs in SVZ of Ctrl mice treated with CQ+2DG and Tsc2−/− cKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. n = 6 independent experiments. b, Mean ± s.e. of the number of GFAP+Nestin+BrdU+ cells in SVZ of Ctrl mice treated with CQ+2DG and Tsc2−/− cKO mice treated with vehicle, 2DG, CQ and CQ + 2DG. n = 6 independent experiments. c, Mean ± s.e. of the number of GFAP+Sox2+ NSCs in SVZ of Tsc2−/− cKO mice treated with vehicle, 2DG, ETO, Rano, TMZ, ETO + 2DG, Rano + 2DG and TMZ + 2DG. n = 5 independent experiments. d, Mean ± s.e. of the number of GFAP+Nestin+BrdU+ cells in SVZ of Ctrl and Tsc2−/− cKO mice treated with vehicle, 2DG, ETO, Rano, TMZ, ETO + 2DG, Rano + 2DG and TMZ + 2DG. n = 5 independent experiments. e, f, Mean ± s.e. of the number of DCX+ cells (e) and NeuN+ cells (f) in SVZ of Ctrl and Tsc2−/− cKO mice treated with vehicle, 2DG, ETO, Rano, TMZ, ETO+2DG, Rano+2DG and TMZ+2DG. n = 5 independent experiments. g, Lysates from WT and Tsc2 KO MEFs in normal or glucose-free media were examined by immunoblots with antibodies as indicated. Three independent experiments gave similar results. h, Lysates from WT MEFs, AMPKα1 KO MEFs (2 independent clones), Tsc2 KO MEFs and 2KO MEFs (2 independent clones) were examined by immunoblots with antibodies, as indicated. Three independent experiments gave similar results. i, Lysates were extracted from WT MEFs, AMPKα1 KO MEFs, Tsc2 KO MEFs and 2KO MEFs in glucose-free media with or without BafA1 for 2 h. The levels of LC3-II and vinculin were examined. Three independent experiments gave similar results. j, H&E staining of sagittal sectioned brain indicating hydrocephaly of Tsc2−/− cKO lateral ventricle, but not in Ctrl, 2cKO, 2cKI and Fip200−/− cKO mice at P21. Arrows indicated SEN-like structures in Tsc2−/− cKO brain. Three independent experiments gave similar results. k, Mean ± s.e. of brain weight of Ctrl, Tsc2−/− cKO, 2cKO and Fip200−/− cKO mice at P7, P14 and P21. n = 3 independent experiments. l, H&E staining of sagittal sectioned brain indicating thicker cortex of Tsc2−/− cKO brain, but not in Ctrl, 2cKO, 2cKI and Fip200−/− cKO mice at P21. The sagittal sections for middle of the brain (middle position) and 1 mm lateral to the middle position were stained. The dashed lines indicated the boundaries of cortex and CC. Four independent experiments gave similar results. m–o, Mean ± s.e. of cortex thickness of Ctrl, Tsc2−/− cKO, 2cKO and Fip200−/− cKO mice at two different section positions (m,n) at P7, P14, and P21. 2cKO mice at different section position (o) at P7, P14, and P21. 2cKI mice at different section position (o) at P7, P14, and P21. 2cKI mice at different section position (o) at P7, P14, and P21. n = 4 (m,n) and 5 (o) independent experiments. p–r, Mean ± s.e. of cortex cell size (p) and cortex cell number (q) of Ctrl, Tsc2−/− cKO, 2cKO and Fip200−/− cKO mice by H&E staining at P21, and cortex TUNEL-positive cell number (r) at P7 and P21. n = 4 (p,q) and 3 (r) independent experiments. CC, corpus callosum; hippo, hippocampus; I-VI indicates layers of cerebral cortex. Scale bar, 50 μm. Data were analysed by one-way ANOVA with Tukey’s post-hoc test (a–f,o–r) or one-way ANOVA (k,m,n,r).
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Postnatal developmental defects of cerebral cortex in Tsc1GFAP cKO mice were rescued in 2cKO mice and by rapamycin treatment. a, Immunofluorescence of pS6RP, NeuN and DAPI in cortex of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice at P21. Inset shows details of pS6RP and NeuN in boxed area. Four independent experiments gave similar results. b, c. Mean ± s.e. of the percentage of pS6RP NeuN+ of NeuN+ cells (b) and the cell size of NeuN+ cells (c) in the cortex of Ctrl, Tsc1GFAP cKO, 2cKO, and Fip200GFAP cKO mice at P21. n = 4 independent experiments, at least 300 cells counted. d, Immunofluorescence of GFAP and DAPI in SVZ of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice at P21. Four independent experiments gave similar results. e, Mean ± s.e. of the relative percentage of GFAP+ cells in the cortex of Ctrl, Tsc1GFAP cKO and 2cKO mice at P21. n = 4 independent experiments, at least 300 cells counted. f, Immunofluorescence of pS6RP, NeuN and DAPI in the cortex of rapamycin treated Ctrl and Tsc1GFAP cKO mice at P21. Inset shows details of pS6RP and NeuN in boxed area. The dashed lines indicated the boundaries of cortex and CC. Four independent experiments gave similar results. g, Mean ± s.e. of the percentage of pS6RP NeuN+ of NeuN+ cells in the cortex of rapamycin treated Ctrl and Tsc1GFAP cKO mice at P21. n = 4 independent experiments, at least 300 cells counted. h, Mean ± s.e. of the brain weight of rapamycin treated Ctrl and Tsc1GFAP cKO mice at P21. n = 3 independent experiments. i, H&E staining of sagittal sectioned brain indicating cortex of Ctrl, Tsc1GFAP cKO, 2cKO, 2cKI and Fip200GFAP cKO mice at P21. The sagittal sections for middle of the brain (middle position) and 1 mm lateral to the middle position were stained. The dashed lines indicated the boundaries of cortex and CC. Four independent experiments gave similar results. j, Mean ± s.e. of cortex thickness of Ctrl, Tsc1GFAP cKO, 2cKO and Fip200GFAP cKO mice at two different section positions at P21. n = 3 (middle) or 4 (~1 mm) independent experiments. CC, corpus callosum; hippo, hippocampus; I-VI indicates layers of cerebral cortex. Scale bar, 50 μm. Data were analysed by one-way ANOVA with Tukey’s post-hoc test (b,c,e,h,j).
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

DP controller (version 1.2.1.10B, OLYMPUS); DP Manager (Version 1.2.1.107, OLYMPUS), Zen 2010B SP1 [Version 6.0.0.485, Zeiss].

Data analysis

Image J (NIH) with the Coloc 2 plugin (3.0.0), and Seahorse Wave Controller [version, 2.6, Agilent], Prism [version 5.0, GraphPad Software].

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- **Sample size**: No sample-size calculations were performed. Sample size was determined according to previous experimental observations.
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|-----|-----------------------|
| ☒   | Antibodies           |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology        |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data        |

**Methods**

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |

**Antibodies**

- **Antibodies used**
  - anti-Nestin (Cat: Rat-401, PMID: 23403289, DSHE, IA), IHC, 1:50;
  - anti-GFAP (Cat# 3607, PMID: 28794441, Cell Signaling, MA), IHC, 1:100;
  - anti-mTOR (Cat#: 14-2190-82, PMID: 18803507, ThermoFisher Scientific, CA), IF, 1:100;
  - Alexa 647 conjugated anti-β-III (Cat: B35133, PMID: 28754836, ThermoFisher Scientific, CA), IHC, 1:50;
  - anti-beta-actin (Cat: A2228, PMID: 21498634, Sigma, MO), WB, 1:4,000;
  - anti-GAPDH (Cat: G8795, PMID: 23222489, Sigma, MO), WB, 1:10,000;
  - anti-vinculin (Cat: V9264, PMID: 23685627, Sigma, MO), WB, 1:5,000;
  - anti-NeuN (Cat: MAB377, PMID: 26373451, Millipore, MA), IHC, 1:500;
  - anti-iKi67 (Cat: M30060, PMID: 26402841, Spring Bioscience, CA), IHC, 1:200;
  - anti-p62 (Cat: BML-PW9860, PMID: 22562096, Inzio, PA), WB, 1:1,000, IHC, 1:200;
  - anti-GFAP (Cat: 20334, PMID: 8229641, Dako, CA), IHC, 1:200;
  - anti-phosphorylated (Ser65) 4EBP1 (Cat: 9451, PMID: 28778841, Cell Signaling, MA), WB, 1:1,000;
  - anti-phosphorylated (Thr37/46) 4EBP1 (Cat: 2855, PMID: 26913956, Cell Signaling, MA), IHC, 1:100, WB, 1:1,000;
  - anti total 4EBP1 (Cat: 9644, PMID: 29720666, Cell Signaling, MA), WB, 1:1,000;
  - anti-FIP200 (Cat: 12436, PMID: 27305347, Cell Signaling, MA), WB, 1:1,000;
  - anti-beta-III tubulin (Cat: 3568, PMID: 25726526, Cell Signaling, MA), IF, 1:100;
  - anti-NG2 (Cat: AB5320, PMID: 25902404, Millipore, MA), IHC, 1:100;
  - anti-phosphorylated (Thr202/Tyr204) ERK1/2 (Cat: 4370, PMID: 22027685, Cell Signaling, MA), IHC, 1:100;
  - anti-phosphorylated 56 Kinase (Thr389) (Cat: 9234, PMID: 28928465, Cell Signaling, MA), WB, 1:1,000;
  - anti total 56 Kinase (Cat: 2708, PMID: 28835610, Cell Signaling, MA), WB, 1:1,000;
  - anti-phosphorylated 56 ribosomal protein (Ser240/244) (Cat: 5364, PMID: 27026523, Cell Signaling, MA), IHC, 1:500, WB, 1:3,000;
  - anti total 56 ribosomal protein (Cat: 2317, PMID: 29162813, Cell Signaling, MA), WB, 1:3,000;
  - anti-Lc3 (Cat: 2775, PMID: 28574081, Cell Signaling, MA), IF, 1:100, WB, 1:1,000;
  - anti-phosphorylated (Ser473) pAKT (Cat: 4060, PMID: 24670654, Cell Signaling, MA), IHC, 1:100;
  - anti-phosphorylated AMPK (Thr172) (Cat: 2535, PMID: 27409632, Cell Signaling, MA), IHC, 1:100, WB, 1:1,000;
  - anti AMPK (Cat: 5831, PMID: 27172265, Cell Signaling, MA), WB, 1:1,000;
  - anti-phosphorylated-Raptor (Ser792) (Cat: 2083, PMID: 23352126, Cell Signaling, MA), WB, 1:1,000;
  - anti total Raptor (Cat: 2280, PMID: 28613987, Cell Signaling, MA), WB, 1:1,000;
  - anti-phosphorylated ACC (Ser79) (Cat: 3661, PMID: 28552492, Cell Signaling, MA), IHC, 1:100, WB, 1:1,000;
  - anti total ACC (Cat: 3662, PMID: 28552492, Cell Signaling, MA), WB, 1:1,000;
  - anti-phosphorylated-Ulk1 (Ser757) (Cat: 6888, PMID: 30784596, Cell Signaling, MA), WB, 1:1,000;
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

HEK293 cell line is from ATCC, TSC2 knock-out mouse embryonic fibroblast (MEF) and paired wild type MEF were gifted from Diane Finger in the Department of Cell and Developmental Biology, University of Michigan. Atg5 KO MEF and paired wild type MEF were gifted from Noboru Mizushima in University of Tokyo. Primary neurospheres were isolated from wildtype and mutant mice.

Authentication

HEK293 cell line was obtained from cell bank of ATCC and authenticated by Short tandem repeat profiling service. No further authentication has been performed since HEK 293 cells arrived in lab. TSC2 KO MEF and Atg5 KO MEF were tested to have deletion of TSC2 as described in PMID: 14561707 and PMID: 15525940, respectively.

Mycoplasma contamination

All the cell lines used in this study are tested for mycoplasma contamination monthly. The results are negative.

Commonly misidentified lines

(See ITAC register) No cell line in the list.

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Laboratory animals

FIP200f/f, FIP200f/k, Tsc1f/f, FIP200f/f, Tsc1f/f, hGFAP-Cre transgenetic and control mice. This study were maintained in the mixed C57Bl6 (87.5%) with FVB (12.5%) background. Age and littermate-matched control and mutant mice were used for analysis. Mice were used at postnatal day 0 and 21. Both sex of mice were used in our experiments.

Wild animals

We do not use wild animals.

Field-collected samples

We do not use field-collected samples.

Ethics oversight

IACUC of the University of Cincinnati

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
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- A numerical value for number of cells or percentage (with statistics) is provided.
Methodology

| Sample preparation          | Neural cells from cultured neurospheres of isolated SV2 cell. |
|----------------------------|---------------------------------------------------------------|
| Instrument                 | FACS Vantage SE-dual laser, three-line flow cytometer or an FACSCanto (BD Biosciences) |
| Software                   | FlowJo                                                        |
| Cell population abundance  | The neural cells from neurospheres are almost 100% pure neural progenitor/stem cell population. The purity had been established by staining of neurospheres with NSC markers of nestin, RC2 and Sox2. |
| Gating strategy            | Neural cells without antibody (pS6RP) staining were used as negative control to set gating threshold. |

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.