TET3 prevents terminal differentiation of adult NSCs by a non-catalytic action at Snrpn

Ten-eleven-translocation (TET) proteins catalyze DNA hydroxylation, playing an important role in demethylation of DNA in mammals. Remarkably, although hydroxymethylation levels are high in the mouse brain, the potential role of TET proteins in adult neurogenesis is unknown. We show here that a non-catalytic action of TET3 is essentially required for the maintenance of the neural stem cell (NSC) pool in the adult subventricular zone (SVZ) niche by preventing premature differentiation of NSCs into non-neurogenic astrocytes. This occurs through direct binding of TET3 to the paternal transcribed allele of the imprinted gene Small nuclear ribonucleoprotein-associated polypeptide N (Snrpn), contributing to transcriptional repression of the gene. The study also identifies BMP2 as an effector of the astrocytic terminal differentiation mediated by SNRPN. Our work describes a novel mechanism of control of an imprinted gene in the regulation of adult neurogenesis through an unconventional role of TET3.
In the mammalian brain two regions generate new neurons throughout adulthood: the subventricular zone (SVZ) in the walls of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. The process of neurogenesis in these adult neurogenic niches is continually sustained by the activity of neural stem cells (NSCs) which are characterized by their ability to balance self-renewal with multipotential differentiation into astrocytes, oligodendrocytes, and neurons. NSCs are lineage-related to radial glial cells, therefore, they exhibit astrocytic characteristics, such as glial fibrillary acidic protein (GFAP) + filaments or astrocyte-specific glutamate aspartate transporter (GLAST). NSCs also express the transcription factor Sox2 (Sry-related HMG box). In the SVZ in particular, NSCs also known as type B cells have a long basal process contacting blood vessels and extend an apical process ending in a primary cilium that protrudes into the ventricle. The walls of the lateral ventricles thus show a typical organization where the apical process of type B cells are surrounded by a rosette of epithelial ependymal cells forming structures known as rosettes. Activated and quiescent NSCs appear to coexist in the SVZ. Once activated, NSCs give rise to transit-amplifying progenitors or type C-cells, which in turn, generate neuroblasts, or type A-cells. These cells migrate anteriorly forming the rostral migratory stream (RMS) to the olfactory bulb (OB), where they mature into functional neurons involved in olfactory discrimination. Subventricular NSCs are also capable of producing some oligodendroblasts that migrate to the corpus callosum.

Signaling between NSCs and other cells of their micro-environment or niche is critical for adult neurogenesis, through its interaction with NSCs intrinsic programs of gene expression. Spatial and temporal gene regulation of gene expression in NSCs is established and maintained by the coordinated interaction between transcription factors and epigenetic regulators. Most mammalian genes are expressed from both maternally and paternally inherited chromosomal homologs. In contrast, imprinted genes are expressed from one parental copy only and the gene copy inherited from the other progenitor remains repressed. Their monallelic expression makes these loci very vulnerable as mutation or deregulation of the sole expressed allele can compromise expression and lead to severe developmental defects. Interestingly, we have previously shown that selective absence of imprinting can occur in particular lineages to modulate the dosage of imprinted genes for cell-specific functions in physiological contexts. These context-dependent changes may be important for cell plasticity during normal development and tissue regeneration. Indeed, we have previously described that in the SVZ the paternally expressed gene Delta-like homolog 1 (Dlk1), an atypical Notch ligand, plays a relevant function in postnatal neurogenesis. Dlk1 is canonically expressed elsewhere in the brain, however, it shows a selective absence of imprinting in subventricular NSCs with biallelic expression being required for stem cell maintenance and, ultimately, neurogenesis to the OB. Another implanted gene, the Insulin-like growth factor 2 (Igf2), which is canonically expressed from the paternally inherited allele, is biallelically expressed in the choroid plexus resulting in a higher dose of Ig2 secreted into the cerebrospinal fluid to regulate NSC proliferation. Dlk1 is also implicated in neuronal memory-associated genes, further supporting non-conventional functions of TET proteins.

There are many intriguing questions concerning the role of genomic imprinting and gene dosage during the neurogenesis process. Here we demonstrate a function for the non-catalytic form of TET3 in the maintenance of adult NSC state by preventing the differentiation of type B cells into non-neurogenic astrocytes. The mechanism involves direct binding and repression of TET3 to the imprinted gene Snrpn. We also show that astrocytic differentiation of adult NSCs in response to Snrpn deregulation involves increased production of BMP2. Our study provides important new insights into the role of TET3 in neurogenesis and emphasizes the importance of correct regulation of imprinted genes in the neurogenic niche.

**Results**

**Tet3 deficiency causes depletion of the NSC pool in vivo.** Tet3 dioxygenases convert 5mC to 5hmC resulting in the removal of the methylated cytosine in somatic tissues, including the brain. Quantitative RT-PCR (qPCR) assays showed that Tet2 and Tet3 were the most abundant members of the Tet dioxygenases in the adult SVZ, being also highly expressed in NSCs isolated from the early postnatal SVZ (Fig. 1a). However, only Tet3 expression was maintained postnatally in the stem cell pool and in differentiated cells (Fig. 1a). Immunofluorescent analyses with antibodies to TET3 and to cell-identity antigens in wild-type adult brains revealed nuclear staining for TET3 protein in the GFAP/SOX2+ population located close to the lateral ventricles (Fig. 1b), whereas no expression was observed in
doublecortin+ (DCX+) neuroblasts (Supplementary Figure 1a). TET3 staining was also observed in mature neurons of the striatal parenchyma (Supplementary Figure 1b). Furthermore, TET3 was present in all proliferating SOX2+ cells in neurosphere cultures from the adult SVZ (Fig. 1c). Consistent with the analyses at the mRNA level the TET3 protein was detected in Nestin+ progenitors and GFAP+ astrocytes at 3 and 7 days of in vitro differentiation, respectively (Fig. 1d). Taken together, these data suggested a role for TET3 in adult NSCs.

In order to evaluate the function of TET3 in the adult SVZ, a murine genetic model was generated by crossing male mice carrying loxP sites flanking part of the T3 gene (Tet3loxP/loxP)42,43 with female mice expressing the Cre-recombinase under the control of the Ifap promoter (Gfap-cre+/-)44. Importantly, X-gal histochemistry and immunostaining for β-galactosidase in the adult brain of ROSA26R;Gfap-cre+/0 mice (Gfap-cre/LACZ) and control mice (Gfap-control/LACZ) showed positive staining in the SVZ, RMS and olfactory bulbs of the adult Gfap-cre/LACZ brains.
Fig. 2 Removal of Tet3 in the SVZ GFAP+ population causes a depletion of the neural stem cell pool in vivo. a qPCR for Tet3 in adult brain, SVZ, astrocytes and NSCs from Tet3-Gfapcontrol and Tet3-Gfapcre mice. Data are expressed relative to Gapdh. Mean is indicated in the boxplots as a “+” symbol. b Schematic drawing of the BrdU injection protocol (upper panel). Number of BrdU-label-retaining cells (BrdU-LRCs) that are GFAP+ and GFAP/Ki67+ in the SVZ of Tet3-Gfapcontrol and Tet3-Gfapcre mice (lower panel). c Immunohistochemistry panoramic images of Tet3-Gfapcontrol and Tet3-Gfapcre SVZ stained for BrdU-labeled retaining cells (LRCs; red). d Immunohistochemistry images for BrdU-LRCs (red) and GFAP (green) in the SVZ of mice from both genotypes. e Immunohistochemistry images for GFAP (green), γ-tubulin (red) and β-catenin (blue) in SVZ whole-mount preparations identifying pinwheel structures in both genotypes (left panels). Total number of pinwheels and differentiated astrocytes, found in the SVZ of Tet3-Gfapcontrol and Tet3-Gfapcre mice (right panels). g Confocal reconstructions of whole-mount staining for the neuroblast marker DCX in the rostral migratory stream (RMS) of mice from both genotypes. Inserts show high-magnification images of DCX in chains (left panels). h Quantification of the number of newborn neurons incorporating in the granular (GL) and periglomerular (PGL) layers in the olfactory bulbs (OB) of Tet3-Gfapcontrol and Tet3-Gfapcre mice (left panel). Immunohistochemistry images for GFAP-LRCs (red) in the granular layer of the OBs from both genotypes. V lateral ventricle lumen. DAPI was used to counterstain DNA. All error bars show s.e.m. Unpaired two-tailed Student’s t-test was used. P-values and number of mice used are indicated. Scale bars in (c), 100 μm; in (d and g), 30 μm (inserts in (d), 7 μm); in (f), 15 μm; in (h), 40 μm. Source data are provided as a Source Data file (Supplementary Figure 2a) and colocalized with GFAP+ cells (Supplementary Figures 2bc), corroborating the specific deletion of Tet3 in the adult GFAP+ stem cell population. Significant expression of Cre-recombinase in Tet3loxP/loxP_Gfap-cre+/- (Tet3-Gfapcre) compared to Tet3loxP/loxP_Gfap-cre0/0 (Tet3-Gfapcontrol) control mice was confirmed in SVZ-derived NSCs (Supplementary Figure 2d). Cre expression in NSCs isolated from Tet3-Gfapcre deficient SVZ correlated with reductions in Tet3 mRNA and protein without compensatory changes in Tet1 and Tet2 (Fig. 2a and Supplementary Figures 2e–g). As expected, body and brain weights were not affected in Tet3-Gfapcre mice compared to controls (Supplementary Figures 2h).
Two-month-old Tet3-Gfapcre and Tet3-Gfapcontrol mice were injected with the nucleoside analog BrdU three weeks before sacrifice (Fig. 2b). BrdU is specifically retained in some slowly proliferating NSCs (label-retaining cells, LRCs) and in OB newborn neurons that ceased to divide and underwent terminal differentiation soon after the injection.45 The number of GFAP+/LRCs was significantly reduced in TET3-deficient mice (Fig. 2b-d and Supplementary Figure 3a) and fewer of them were positive for the proliferation antigen Ki67 (Fig. 2b and Supplementary Figure 3b), suggesting a role for TET3 in regulating the number of activated NSCs within the SVZ. Conversely, related to the decrease in activated NSCs in Tet3-deficient mice, we found a higher proportion of GFAP+/cells with detectable levels of the calcium-binding protein S100β, a marker of terminally differentiated astrocytes (Fig. 2e). Type B cells are elongated and bear an apical process that extends through the cells of the ependymal monolayer, to end in a primary cilium immersed in the cerebrospinal fluid. Monociliated B cells form structures known as pinwheels, and can, therefore, be readily identified in whole-mount preparations of the SVZ, following immunofluorescence for GFAP and the basal body marker γ-tubulin. In line with the previous findings, we observed a decrease in the number of GFAP/γ-tubulin+/Type B cells contacting the ventricle in whole-mounts of the Tet3-Gfapcre SVZ (Fig. 2f). Interestingly, we also found increased proportions of monociliated GFAP+/cells with cell bodies completely intercalated within the ependymal layer, a feature that has been reported to increase with aging.46 (Fig. 2f). Less densely populated DCX+/neuroblast chains in the RMS (Fig. 2g) and fewer BrdU+/newly-generated neurons in the granular and periglomerular layers of the OB were found in Tet3-Gfapcre mice (Fig. 2h) likely as a result of the reduced number of NSCs in the TET3 mutant SVZ. However, no changes were observed in the density of newly-formed oligodendrocytes, scored as BrdU-LRC/OLIG2+/cells in the corpus callosum (CC) of Tet3-deficient mice (Supplementary Figure 3c). These data together indicate that TET3 is required for the maintenance of the NSC pool in the SVZ of adult mouse brains by preventing their premature differentiation into terminally differentiated astrocytes thus limiting neurogenesis in vivo.

Tet3 is required to maintain self-renewal of adult NSCs. Individual cells dissected from the postnatal SVZ can proliferate in medium containing basic fibroblast growth factor (FGF2) and/or epidermal growth factor (EGF) to produce multipotent clonal aggregates, called neurospheres.45,47 To investigate cell-intrinsic properties of TET3, we derived neurospheres from adult Tet3-Snrpn+ mice (Fig. 3a and Supplementary Figure 4f). This led to the reactivation of a small proportion of cells with the capacity to form neurospheres in non-adherent conditions after 12 days in vitro (12 DIV) (Supplementary Figure 4f). The number of neurosphere-forming cells in Tet3-Gfapcre NSCs was significantly reduced, indicating that the bias toward a more differentiated astrocytic phenotype in the absence of TET3 correlated with a reduction in stemness (Supplementary Figure 4f). This suggests that TET3 can directly promote the neurogenic potential of the multipotent stem cell-like astrocytes by preventing their premature differentiation.

TET3 prevents terminal astrocytic differentiation of NSCs. Adult NSCs are multipotent cells that can form neurons, astrocytes, and oligodendrocytes ex vivo. Indeed, when neurosphere cultures are induced to differentiate in vitro they undergo an orderly series of intermediate steps of proliferation and cell fate restriction resembling those occurring in vivo.48,49 and resulting in the differentiation of derived neural progenitors into different neural cell types (Supplementary Figure 4a). Because the in vivo data suggested that TET3 deficiency resulted in increased astrocytic differentiation, we monitored the in vitro behavior of neural progenitors derived from Tet3-Gfapcre and control neurospheres plated under adherent conditions in the absence of mitogens. During the first 2 days (2-DIV) of differentiation, a decrease in the neural progenitor marker Nestin together with an increase in the terminally differentiated astrocytic marker S100β was observed in Tet3-Gfapcre compared to control cultures (Fig. 3g), whereas no change in the levels of expression of the neuronal βIII-tubulin gene (Tubb3) was found (Supplementary Figure 4c). At the protein level, the deficiency in TET3 resulted in reduced proportions of Nestin+cells after 2-DIV of differentiation (Fig. 3h, i). Higher proportions of cells that were strongly positive for S100β, a protein largely absent from neurogenic GFAP+/cells correlating with loss of neurosphere-forming potential,49 was also observed after 7-DIV of differentiation (Fig. 3h, i). Conversely, neuronal and oligodendrogial differentiation was normal as no significant differences were found in the percentage βIII-tubulin+ or O4+ cells, respectively (Supplementary Figures 4d,e). In order to test whether enhanced astrocytic differentiation in the absence of Tet3 was consistently accompanied by a reduction in the capacity of NSCs to form neurons, 7-DIV differentiated NSCs cultures were detached and replated under proliferating conditions with mitogens (Supplementary Figure 4a). This led to the reactivation of a small proportion of cells with the capacity to form neurospheres in non-adherent conditions after 12 days in vitro (12 DIV) (Supplementary Figure 4f). The number of neurosphere-forming cells in Tet3-Gfapcre NSCs was significantly reduced, indicating that the bias toward a more differentiated astrocytic phenotype in the absence of TET3 correlated with a reduction in stemness (Supplementary Figure 4f). This suggests that TET3 can directly promote the neurogenic potential of the multipotent stem cell-like astrocytes by preventing their premature differentiation.

TET3 regulates NSCs maintenance through repression of Snrpn. To understand the molecular mechanism underlying the differentiation defects observed in Tet3-deficient NSCs, a RNA-seq was performed in control and Tet3-Gfapcre neurosphere cultures. RNA-seq analysis confirmed the downregulation of Tet3 mRNA levels in Tet3-Gfapcre compared to control cultures (LogFC in Tet3-Gfapcre neurosphere cultures: −1.0074 with a FDR = 3.97 × 10^{-17}). Tet3 depletion resulted in 97 significantly downregulated and 96 upregulated genes (Fig. 4a; Supplementary Figure 5a; Supplementary Data set 1 and 2). Interestingly, Gene Set Enrichment Analysis (GSEA) revealed specific changes in pathways promoting cell growth such as PI3K-Akt, MAPK, and Wnt signaling pathways and genes involved in synaptic plasticity in Tet3-Gfapcre compared to Tet3-Gfapcontrol NSCs (Supplementary Figure 5b).
Non-canonical regulation of imprinted genes has been shown to regulate adult neurogenesis\textsuperscript{[16,17,31]}. Moreover, the brain expresses high levels of 5hmC, suggesting a potential contribution of TET3 to the epigenetic state at imprinted regions in adult NSCs. Based on the RNA-seq datasets, we next focused on changes in expression of all known imprinted genes (Supplementary Dataset 3). From around 150 imprinted genes analyzed, three showed a significant change in mRNA expression. Ctnm3 (Contactin 3) was downregulated in Tet3-Gfap\textsuperscript{cre} NSCs, whereas Cobl (Cordon-bleu WH2 repeat) and Snrpn (Small nuclear
Depletion of Tet3 in NSCs increases the expression of the imprinted gene Snrpn. a Volcano plot for genes differentially expressed by RNAseq in Tet3-Gfapcre compared to control NSCs. Downregulated genes are in red and upregulated genes in yellow. Imprinting genes are showed in purple. b qPCR for Cntn3, Cobl, and Snrpn in NSCs from both genotypes. c qPCR for Snrpn in NSCs from both genotypes in proliferating conditions after 2 or 3 days of differentiation. The pick of expression of the gene occurs after 2 DIV of differentiation. d qPCR for Snrpn in Tet3-Gfapcontrol and Tet3-Gfapcre NSCs that have been lentivirus transduced with a shRNA for SNRPN. A shRNA SCRAMBLE was used as a control. e Immunocytochemistry images for SNPRN (red) in Tet3-Gfapcontrol and Tet3-Gfapcre NSCs 7 days after the shRNA experiment. f Number of secondary spheres formed after Tet3 downregulation in Tet3-Gfapcre cultures. g Growth curves showing the total number of cells formed after 4 passages in Tet3-Gfapcontrol and Tet3-Gfapcre neurosphere cultures that have been interfered with the shRNA for Snrpn. h qPCR for the astrocytic differentiation marker S100β after 2 DIV of differentiation in NSCs that have been lentivirus transduced with a shRNA for SNRPN. i Percentage of S100β+ cells (relative to total number of DAPI) in shRNA interfered Tet3-Gfapcontrol and Tet3-Gfapcre cultures. j Immunocytochemistry images for S100β (red) and GFAP (green) in interfered NSCs of both genotypes after 7-DIV of differentiation. Data are expressed relative to Gapih. DAPI was used to counterstain DNA. All error bars show s.e.m. One-way ANOVA and Tukey post-test were applied. P-values and number of samples are indicated. Mean is indicated in the box and whiskers plots as “±”. Scale bars in (e and j), 40 µm (high-magnification images 15 µm).
NSCs was increased to normal in Tet3-Gfapcre neurosphere cultures infected with the shSNRPN (Fig. 4g). To assess whether this rescue in the self-renewal and expansion capacities in Tet3-deficient cultures might be due to an independent effect of Snrpn on proliferation, neurospheres size was determined, however no changes were found in mean diameter in any condition (Supplementary Figures 6b, c) suggesting a functional role of Snrpn in NSCs self-renewal. Consistently, lentiviral delivery of the shSNRPN in Tet3-deficient cells restored S100β levels to normal after 2 and 7-DIV of differentiation (Fig. 4h and Supplementary Figure 6d). Moreover, the percentage of S100β+cells in Tet3-Gfapcre differentiated cultures was restored to wild-type levels (Fig. 4i, j). No changes in the levels of Tubb3 mRNA or in the proportion of βIII-tubulin+cells were observed after knockdown of Snrpn levels in Tet3-Gfapcre neurospheres (Supplementary Figures 6d,e). Notably, downregulation of Snrpn in Tet3-Gfapcontrol cells, caused an increase in neurosphere formation and cell growth and decreased the proportion of differentiated S100β+cells (Fig. 4d–j). These data suggested that Tet3 directly promotes the neurogenic potential of multipotent stem cell-like astrocytes via regulation of Snrpn, antagonizing their premature terminal differentiation into mature astrocytes.

Snrpn controls BMP to mediate differentiation of NSCs. Because our data indicated that Tet3 deficiency results in increased astrogiogenesis, we focused on Bone Morphogenetic Proteins (BMPs), cytokines that are strong promoters of gliogenesis in vivo and in vitro. Real-time PCR analysis of the expression of these molecules revealed an enrichment of Bmp2 mRNA in the absence of Tet3 that was completely restored to normal levels by interference of the Snrpn gene (Fig. 5a). BMP ligands signal through a tetrameric complex that is formed by BMP type II receptor (BMPR-II) and different classes of BMP type I receptors. Activation of BMPR-IA or -IB results in the addition of BMPR-II, as well as by the interference of shRNA for SNRPN (left). NSCs were treated with 100 ng/ml of the BMP natural antagonist Noggin. Immunocytochemistry images for pSMAD1/5+astrocytes (Supplementary Figures 6f–h), indicating that differentiating NSCs are responsive to BMPs. Notably, increased levels of nuclear pSMAD1/5 were detected in Tet3-Gfapcre compared to Tet3-Gfapcontrol NSCs (Fig. 5b). Furthermore, this was antagonized by the addition of Noggin, a natural antagonist of BMP and by the downregulation of the Snrpn gene (Fig. 5b).

To further substantiate these data, we used a luciferase reporter construct that contains two copies of two distinct highly conserved BMP-responsive elements (BREs) and the associated regulatory motifs of the natural human and mouse Id1 promoter as a pSMAD activity sensor (BRE-tk-luciferase)34. Consistent with the data described above, we found an increase in the basal activity of the BRE reporter in Tet3-Gfapcre when compared with wild-type cells, and this increase was efficiently abrogated by the addition of Noggin, as well as by the interference of Snrpn (Fig. 5c). Taken together, these results demonstrate that BMP signaling is augmented in the absence of Tet3 and that control of BMP2 by SNRPN is functionally responsible for the enhanced astrocytic differentiation of Tet3-deficient NSCs.

**Fig. 5** BMP2 mediates terminal differentiation of Tet3-deficient NSCs. a qPCR for Bmp2 in Tet3-Gfapcontrol and Tet3-GfapshRNA interfered NSCs after 2 DIV of differentiation. Data are expressed relative to Gapdh. b Percentage of pSMAD1/5+cells relative to total cells in Tet3-Gfapcontrol and Tet3-GfapshRNA NSCs that have been interfered with a shRNA for SNRPN (left). NSCs were treated with 100 ng/ml of the BMP natural antagonist Noggin. Immunocytochemistry images for pSMAD1/5+cells (right). DAPI was used to counterstain DNA. c BRE-tk-luciferase reporter activity in Tet3-Gfapcontrol and Tet3-GfapshRNA shRNA interfered neurospheres and treated with Noggin. All error bars show s.e.m. One-way ANOVA and Tukey post-test were applied. P-values and number of samples are indicated. Mean is indicated in the box and whiskers plots as “+”. Scale bars in (b), 40 µm (high-magnification images 15 µm). Source data are provided as a Source Data file.
suggested that formation of 5hmC might also participate in the function and/or maintenance of the undifferentiated state in the adult neurogenic niches. Notably, upon Tet3 knockdown no changes in global 5mC or 5hmC levels, determined by ELISA, were observed in adult Tet3-Gfapcontrol and Tet3-Gfapcre neurosphere cultures (Supplementary Figure 7d). Consistently, immunohistochemistry for 5mC within the Tet3-Gfapcre SVZ revealed no significant changes in global 5hmC in Tet3-deficient NSCs in vivo (Supplementary Figure 7c).

Snrpn belongs to the cluster of imprinted genes associated with Prader–Willi Syndrome and is canonically expressed from the paternally inherited chromosome55. The mouse Snrpn gene has a germline derived differentially methylated region (DMR) that is associated with imprinted Snrpn expression, and is required for imprinting control of the domain (Supplementary Figure 8a)56,57. We have previously shown the expected paternal expression of Snrpn in normal tissue and adult NSCs16, but loss of imprinting of the gene could explain the upregulation of Snrpn in Tet3-Gfapcre NSCs. To test this, bisulfite sequencing of the Snrpn DMR was performed in Tet3-Gfapcontrol and Tet3-Gfapcre neurosphere cultures (Supplementary Figure 8a, b). Both control and mutant neurospheres showed 50% methylation indicating retention of imprinting. Bisulfite sequencing cannot distinguish between 5mC and 5hmC; therefore, to specifically assess 5hmC levels at the Snrpn DMR, a hydroxymethylation immunoprecipitation using antibodies against 5hmC (hydroxyMeDIP) was developed in Tet3-Gfapcontrol and Tet3-Gfapcre NSC cultures. Consistent with the bisulfite sequencing analysis, no differences in 5hmC levels were observed at the Snrpn promoter (Supplementary Figure 8c), suggesting that there was no loss of imprinting of the Snrpn gene.

The remarkable increase of Snrpn expression independently of a change to its DMR methylation and hydroxymethylation in Tet3-deficient NSCs, prompted us to explore the possibility that TET3 might have non-catalytic functions in NSCs. We first used chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) to assess direct binding of TET3 to the chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) to assess direct binding of TET3 to the chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) to assess direct binding of TET3 to the chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) to assess direct binding of TET3 to the chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) to assess direct binding of TET3 to the chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) to assess direct binding of TET3 to the chromatin immuno

Discussion

The formation of new neurons in the adult brain throughout life involves the activation of pools of NSCs while preserving the stem cell number. Emerging evidence indicates that imprinted genes regulate the cell-type function of adult NSCs16,17,51. This study demonstrates a role for the DNAse1 gene TET3 in the self-renewal and hence maintenance of NSCs within the adult SVZ. We show that loss of Tet3 results in the differentiation of neurogenic progenitors both in vivo and in vitro and compromises the maintenance of the NSC pool in this niche leading to reduced OB neurogenesis. More interestingly, our work indicates that TET3 regulates self-renewal by repressing transcription of the imprinted gene Snrpn through mechanisms that are independent of its catalytic function in modulating methylation.

TET3 deficiency causes an active apoptosis of neural progenitors derived from ESCs, which results in a reduction of neuronal production30. This context differs from ours, as in adult NSCs Tet3 deficiency does not modify apoptosis or survival rates. Rather, when Tet3 is absent in the GFAP+ population in vivo, a depletion of the adult NSC pool is observed, and this is due to their terminal differentiation into non-neurogenic astrocytes. As a consequence of the continuous depletion of the NSC pool in Tet3-deficient mice, the process of neurogenesis to the OB is impaired, a finding recapitulated in neurosphere cultures in vitro. Interestingly, no changes in the proportion of the cell cycle phases are observed in mutant cultures indicating that TET3 promotes self-renewal capacity without affecting their overall proliferation rate. Differentiation of both mutant and wild-type NSCs into the three cell lineages of the central nervous system supports premature differentiation of NSCs into non-neurogenic astrocytes in Tet3-deficient NSCs.

Gene expression analyses have revealed the molecular bases of these phenotypic observations, with a significant upregulation of the Snrpn gene in Tet3-deficient NSCs. Snrpn belongs to the Prader–Willi imprinted gene cluster and is expressed from the paternally inherited chromosome55. Patients with Prader–Willi syndrome lack expression of Snrpn and exhibit neurological problems including learning difficulties and hypoplasia59,60. Snrpn encodes the RNA-associated Smn protein implicated in pre-mRNA editing, which contributes to tissuespecific alternative splicing61. It is well-established that Snrpn is highly expressed in brain and its expression increases markedly during postnatal brain development61. Our data indicate that TET3 regulates Snrpn dosage in an imprint-independent manner and that this is responsible for the premature differentiation of NSCs that we observe. Our findings suggest that Snrpn overexpression influences the NSC pool in adults, a function that has not previously been associated with Prader–Willi Syndrome and which has implications for our understanding of the etiology of this disorder.
The effects of BMPs in astroglial differentiation have been reported extensively for both late fetal and postnatal progenitors\(^5\), and most frequently, BMP-induced astroglial differentiation is related to deficiencies in the production of other cell lineages\(^5\). Moreover, BMP4 overexpression appears to accelerate the differentiation of some radial glia, suggesting that multipotential progenitors terminally differentiate when exposed to BMPs\(^5\). Our findings show that Snrpn overexpression correlates with elevated levels of this morphogen, suggesting that premature differentiation of Tet3-deficient NSCs into astrocytes may be partly the consequence of the augmented activity of the BMP signaling. Moreover, the presence of the BMP antagonist Noggin that is produced by ependymal cells\(^6\) could be balancing the Snrpn function in the SVZ, preventing the differentiation of the NSC pool. Indeed, we showed that addition of Noggin to Tet3-deficient cultures, restored the terminal astrocytic differentiation phenotype observed. Further work will be necessary to elucidate whether Snrpn has a role on the pre-mRNA editing of BMP genes.

**TET enzymes regulate the balance of DNA methylation and demethylation by dynamic conversion of 5mC into 5hmC, 5fC, mRNA relative expression**

|       | EV | Tet3 | Tet3 CDmut |
|-------|----|------|------------|
| Tet3-Glap\(^{+/+}\)NSCs | 2.00 ± 0.20 | 1.50 ± 0.15 | 1.00 ± 0.05 |
| Tet3-Glap\(^{-/-}\)NSCs | 1.50 ± 0.15 | 1.00 ± 0.05 | 0.50 ± 0.02 |

**Relative arbitrary units**

|       | EV | Tet3 | Tet3 CDmut |
|-------|----|------|------------|
| pSMA1 | 801 ± 9 a.u. | 614 ± 6 a.u. | 644 ± 11 a.u. |
and ScaC\textsuperscript{10,41}. Several other reports have shown non-catalytic functionalities of the TET proteins\textsuperscript{23,36}. Concretely, TET3 has been implicated in the stabilization of thyroid hormones nuclear receptors promoting transcriptional activation where TET3 catalytic domain is not implicated and a TET3 catalytic dead mutant can rescue the developmental defects caused by TET3 knockdown\textsuperscript{64}. Most notably, TET3 interacts with O-linked N-acetylglucosamine transfersase (OGT) to influence transcription\textsuperscript{4,35}. Although our data show significant amounts of 5mC in GFAP-expressing cells within the SVZ and in proliferating cells in vitro, global levels of 5mC/5hmC are not altered in Tet3-deficient NSCs suggesting non-catalytic effects of TET3 in this neurogenic niche. Indeed, we demonstrate that TET3 binds to the Snrpn promoter at the paternal transcribed and unmethylated allele acting as a transcriptional repressor of the gene by a catalytic-independent activity mechanism, supporting a multifunctional role for TET3 in adult NSCs.

Adult niches preserve the undifferentiated property of their resident stem cells, but contribution of epigenetic mechanisms to this process has remained largely unexplored. Our data have elucidated the action of TET3, preventing terminal differentiation and exhaustion of NSCs by transcriptional repression of the Snrpn gene independently of its methylation. We also described how this regulation acts in concert with niche signals such as BMP and Noggin to modulate the proper differentiation of the NSC pool. Further work will be relevant to elucidate the structural, genetic and epigenetic requirements of this regulatory mechanism and its contribution to neurogenic phenotypes.

**Methods**

**Animals and in vivo manipulations.** GFAP-cre (6.Cg-Tg(Fgf-cre)73.12Mvs/J) mice were obtained from Jackson Laboratory and genotyped as described\textsuperscript{44}. GFAP-cre mice were generated using a 15 kb mouse Gfap promoter cassette containing all introns, promoter regulatory elements, exons, and 2 kb of 3′ and 2.5 kb of 5′ flanking regions of the mouse Gfap gene\textsuperscript{44}. Gfap expression is prevented by the removal of a small region in exon 1. Tet3\textsuperscript{loxp/loxp} mice contain LoxP sites flanking exon 5 of Tet3 gene\textsuperscript{45}. Expression of Cre-recombinase results in a deletion of this region and a frame-shift from exon 6 that affects all downstream exons until a premature stop codon in exon 7. To generate specific deletion of Tet3 in GFAP positive cells, heterozygous GFAP-cre transgenic animals were bred to Tet3\textsuperscript{loxp/loxp}. Additionally, The Jackson Laboratory reports that the GFAP-cre line have cre expression in the male germ line. To avoid this problem, GFAP-cre females and Tet3\textsuperscript{loxp/loxp} males were used to generate the experimental animals. Animals were genotyped by PCR analysis of DNA, extracted from mouse ear-punch tissue with DNA sequences after ChIP-qPCR after chromatin immunoprecipitation (ChIP) of wild-type NSCs. Two regions (R1 and R2) of the Snrpn promoter (pSnrpn) were analyzed. Cln6 promoter was used as a control for TET3 binding. A non-relevant distal region (R3) was analyzed as a control of specificity. Values are shown as the fold change over the IgG. b DNA sequences after ChIP-qPCR for TET3 in F1 hybrid NSCs derived from Mus musculus domesticus (abbreviated, BL6) and Mus musculus castaneus (abbreviated, Cast) mice (BxC), showing the two diagnostic strain-specific polymorphisms at the Snrpn promoter in the input sample. A more abundant presence of the “A” nucleotide, corresponding to the paternal allele (Cast), is observed. c qPCR for Tet3, Snrpn and Bmp2 genes in Tet3-Gfap\textsuperscript{loxp/loxp} NSCs that had been nucleofected with Tet3 or with Tet3 with a mutated catalytic domain (Tet3 CDMut). An empty vector was used as a control for nucleofecion. d Number of secondary spheres formed in Tet3-Gfap\textsuperscript{loxp/loxp} cultures after nucleofection with Tet3 variants. A rescue in the self-renewal capacity was observed. e Immunohistochemistry images for TET3 (red) in Tet3-Gfap\textsuperscript{loxp/loxp} NSCs that had been nucleofected with Tet3, Tet3 CDMut or EV (upper panels). Immunocytochemistry images for GFAP (green) and S100\textsuperscript{b} (red) are also shown (lower panels). f Percentage of S100\textsuperscript{b}-positive cells in Tet3-Gfap\textsuperscript{loxp/loxp} NSCs that had been nucleofected with Tet3, Tet3 CDMut, or EV. g Immunohistochemistry images for pSMAD5/1-5 (red) in nucleofected Tet3-Gfap\textsuperscript{loxp/loxp} NSCs. h BRE-tk-luciferase reporter activity in Tet3-Gfap\textsuperscript{loxp/loxp} NSCs that had been nucleofected with Tet3, Tet3 CDMut, or EV. All error bars show s.e.m. One-way ANOVA and Tukey post-test were applied. P-values and number of samples are indicated. Mean is indicated in the box and whiskers plots as “±”.

"s" Scale bars, 30 μm. Source data are provided as a Source Data file.
performed immunocytochemistry as described 18. Primary and secondary antibodies and dilutions used are listed in Supplementary Table 2 and Supplementary Table 3. For cell cycle analysis 1 × 10^6 dissociated cells were stained using the BD CyclotestTM Plus DNA kit in accordance with the manufacturer’s protocol. Cell cycle phases were assessed in a FACS Verse flow cytometer (BD) and analyzed with FlowJo® software.

Luciferase reporter assays. For luciferase assays, we electroporated 2–2.5 × 10^6 freshly dissociated Tet3-Gfp (GFP) and 1.5 × 10^6 NSC-NSE cells using the Amaxa Nucleofector Kit V, following the instructions provided by the manufacturer. All samples were resuspended in 2 µg of the B-tek reporter construct driving the expression of the firefly luciferase and Renilla luciferase plasmid in a 1:20 ratio. We lysed cells after 48 h and obtained cell extracts using the Dual Luciferase Reporter kit (Promega), measured luciferase activity using a VICTOR3 reader and calculated the ratio of firefly to Renilla luciferase.

Expression studies. RNAs were extracted with RNAsesy mini kit (Qiagen) including DNase treatment, following the manufacturer’s guidelines. For quantitative PCR, 1 µg of total RNA was reverse transcribed using random primers and RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific), following the manufacturer’s instructions. PCR amplification was performed in a final volume of 20 µl containing 1 µl of cDNA sample and the reverse transcribed RNA was amplified by PCR with appropriate Taqman probes (Supplementary Table 4). Quantitative PCR was used to measure gene expression levels normalized to 18S, the expression of which did not differ between the groups. qPCR reactions were performed in a Step One or a Step One Plus Real-Time PCR System (Applied Biosystems) with SYBR green thermocycling was also performed in a final volume of 12 µl containing 1 µl of cDNA sample, 0.2 µM of each primer (Supplementary Table 1), and 5 µl of cDNA sample, 0.2 µM of each primer (Supplementary Table 1), and SYBR® Premix ExTaq™ (Takara) according to the manufacturer instructions. A standard curve made up of doubling dilutions of pooled cDNA from the samples being assessed was run on each plate, and quantification was performed relative to the standard curve.

RNAseq. Library preparation and high-throughput sequencing were performed by the Central Service for Experimental Research (SCSIE) at the University of Valencia. RNA-seq libraries were generated from triplicated samples per condition using the Illumina TruSeq stranded mRNA Sample Preparation Kit v2 following the manufacturer’s protocol. The RNA-seq libraries were sequenced using Illumina NextSeq 500. Analysis of the RNAseq data was performed by EpiDisease S.L. The percentage of 5hmC and 5mC was calculated by comparing a standard curve of hydroxymethylated and methylated DNA standards (5-hmc, 5-mc, & cytosine DNA standard pack; Diagenode), respectively. The amount of total DNA examined was previously measured by the Quant-iT PicoGreen dsDNA Reagent and Kits (Invitrogen) according to the manufacturer’s protocol. The percentage of 5hmC and 5mC was calculated by dividing the amount of hydroxymethylated and methylated DNA by that of total DNA, respectively.

ChiP and ShmDIP. For ChiP five 100 cm dishes with wild-type NSCs isolated from the adult SVZ were cross-linked and chromatin isolated. Chromatin was sheared to an average size of 200–500 bp using a Bioruptor sonicator (Diagenode, USA). After shearing, chromatin was precipitated with 10 µg of rabbit IgG (Santa Cruz, cat. no. sc-207) and 20 µl of protein G magnetic beads (Dynabeads®, cat. no. 10030D) for 3 h at 4 °C with rotation. A volume of 10 µg of TET3 antibody (Supplementary Table 2) or rabbit IgG were added and incubated overnight at 4 °C on a rotation wheel. Chromatin was precipitated with 10 µl protein G beads for 3 h at 4 °C. An aliquot of chromatin before the immunoprecipitation was used as input. Beads were then washed followed by crosslink reversal and protein digestion. Finally, DNA was purified using MiniElute PCR purification kit (Qiagen) following manufacturer’s instructions. To analyze the TET3 interaction with the Snrpn promoter, ChiP enriched DNA was analyzed by qPCR using SYBR green primers (Supplementary Table 1). Pull-downs using non-immune rabbit IgG were used as control for non-specific enrichments. The comparative Ct method was used to calculate fold enrichment levels normalizing to input DNA and non-specific IgG. Uncropped scans of the PCR electrophorograms are shown in Supplementary Figure 1b.

To study TET3 binding to the paternal or maternal alleles a ChiP for TET3 was performed in NSCs derived from adult F1 mice hybrids offspring from Mus musculus domesticus (C57BL/6j) females and Mus musculus castaneus (CAST/EiJ) males (Bc hybrids NSCs), in which we identified two SNPs between the two subspecies at the Snrpn promoter (Supplementary Figure 8d). SNP1 was a “C” nucleotide in BL6 and an “A” nucleotide in Cast mice. SNP2 was a “G” nucleotide in BL6 and an “A” nucleotide in Cast mice. Genomic DNA sequences were obtained after PCR with primers within Region 2 at the Snrpn DMR (Supplementary Table 1). To enrich DNA samples in 5hmC modification, an immunoprecipitation for 5hmC was performed using 5 µg of sheared DNA with anti-5hmC rabbit IgG antibodies (Roche) at 55 °C for 30 min. Finally, samples were purified with 0.5 µl of 5hmC capture kit (Qiagen) following the manufacturer’s instructions. To calculate 5hmC enrichment at the Snrpn-DMR, qPCR was done with specific primers (Supplementary Table 1). A standard curve made up of doubling dilutions of PCR products from unbound and bound fractions was run on each plate. The enrichment between bound and unbound fractions was calculated estimating relative concentration with the standard curve. To ensure the specificity of 5mC and 5hmC antibodies, genomic DNAs were spiked with 0.3 ng of synthetic Arabidopsis sp. (Diagenode) sequence containing either 5-C or 5hmC and subjected to immunoprecipitation. No cross-binding was detected (Supplementary Figure 8c).

Cloning of Tet3 variants and NSCs line construction. Tet3 was cloned from cDNA from embryoid bodies and recombined into pDONR221 (Invitrogen). Mutations abolishing catalytic activity (HKD catalytic triad to YKA) 24,25 were introduced using the QuickChange II mutagenesis system (Agilent) using primers
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Author contribution
R.M.-L., A.L.-U. and S.R.F. performed most of the experiments. M.I. helped with methylation studies. C.K. contributed to Tet3 variants overexpression experiments. S.R.F. initiated, designed, and led the study and wrote the manuscript. R.M.-L., A.L.-U., A.C.F.-S. and W.R. contributed to experimental design, data analysis, discussion, and writing of the paper.

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