RESEARCH ARTICLE

Suppressor of Fused regulates the proliferation of postnatal neural stem and precursor cells via a Gli3-dependent mechanism

Hector G. Gomez¹, Hirofumi Noguchi¹, Jesse Garcia Castillo¹, David Aguilar¹, Samuel J. Pleasure¹,²,* and Odessa R. Yabut¹,*

ABSTRACT

The ventricular-subventricular zone (V-SVZ) of the forebrain is the source of neurogenic stem/precursor cells for adaptive and homeostatic needs throughout the life of most mammals. Here, we report that Suppressor of Fused (Sufu) plays a critical role in the establishment of the V-SVZ at early neonatal stages by controlling the proliferation of distinct subpopulations of stem/precursor cells. Conditional deletion of Sufu in radial glial progenitor cells (RGCs) at E13.5 resulted in a dramatic increase in the proliferation of Sox2+ Type B1 cells. In contrast, we found a significant decrease in Gsx2+ and a more dramatic decrease in Tbr2+ transit amplifying cells (TACs) indicating that innate differences between dorsal and ventral forebrain derived Type B1 cells influence Sufu function. However, many precursors accumulated in the dorsal V-SVZ or failed to survive, demonstrating that despite the over-proliferation of Type B1 cells, they are unable to transition into functional differentiated progenies. These defects were accompanied by reduced Gli3 expression and surprisingly, a significant downregulation of Sonic hedgehog (Shh) signaling. Therefore, these findings indicate a potential role of the Sufu-Gli3 regulatory axis in the neonatal dorsal V-SVZ independent of Shh signaling in the establishment and survival of functional stem/precursor cells in the postnatal dorsal V-SVZ.

KEY WORDS: Neurogenesis, Proliferation, Ventricular-subventricular zone, V-SVZ, Sufu, Gli3

INTRODUCTION

Tissue-specific stem cell niches persist at postnatal stages as the source of multiple cell types throughout the life of most animal species. In the mammalian forebrain, particularly in rodents, the ventricular-subventricular zone (V-SVZ) lining the lateral ventricles is a prominent postnatal stem cell niche capable of generating neuronal and glial cell progenies. The V-SVZ are the source of inhibitory neurons (interneurons) that migrate and integrate within the neural network of the olfactory bulb (OB) to influence behaviors including predator avoidance and sex-specific responses (Sakamoto et al., 2011). The adult V-SVZ is composed of Type B1 cells, transit amplifying Type C cells (TACs), Type A cells and a monolayer of ependymal cells along the ventricular wall. Type B1 cells are the primary neural stem cells (NSC) of the V-SVZ, capable of generating TACs that divide into immature cell types that migrate into various forebrain structures where they mature (Lim and Alvarez-Buylla, 2016). Specifically, neurogenic TACs differentiate into immature neurons or Type A cells that migrate through the rostral migratory stream (RMS) and differentiate into molecularly distinct interneuron subtypes of the OB circuitry.

Radial glial cells (RGC) in the lateral ganglionic eminence (LGE) and the neocortex of the embryonic ventral and dorsal forebrain, respectively, produce Type B1 cells (Young et al., 2007). The progeny of RGCs originating from these two regions are identifiable in the V-SVZ. Type B1 cells derived from neocortical RGCs typically produce TACs expressing the transcription factor T-Box Brain Protein 2 (Tbr2) (Brill et al., 2009). On the other hand, Type B1 cells derived from LGE RGCs produce TACs expressing the transcription factor Genetic Screen Homeobox 2 (Gsx2) (Stenman et al., 2003) were significantly increased. However, regulatory mechanisms involved in establishing the production of these Type B1 cell lineages at early neonatal stages are largely unclear. Ensuring the proper timing of differentiation and the number of TACs produced at this early stage are critical to avoid early exhaustion of stem cell sources necessary for later adaptation and homeostatic needs, but also to avoid malformations and tumorigenesis. Therefore, further elucidation of the mechanisms controlling the production of specific cell lineages in the neonatal V-SVZ have far-reaching implications.

Suppressor of Fused (Sufu) is a cytoplasmic protein known for antagonizing Sonic hedgehog (Shh) signaling activity (Ramsbottom and Pownall, 2016). In the developing mammalian forebrain, Sufu modulates Shh signaling to control the proliferation, specification and differentiation of RGCs and their progenies (Yabut et al., 2015, 2016; Yabut and Pleasure, 2018). Sufu exerts this role by controlling the activity of Gli transcription factors, the Shh signaling effectors, by proteolytic processing or protein stabilization to promote the repressor function of Gli and the eventual downregulation of Shh signaling target gene expression. Given the diverse roles of Sufu during corticogenesis in regulating RGCs, which eventually generate or transform to Type B1 cells in the postnatal brain, we wondered whether Sufu function in RGCs influence the generation of Type B1 cells and their progenies, and the establishment of the V-SVZ at neonatal stages. To investigate this, we employed a Cre-loxP approach to selectively delete Sufu in forebrain RGCs that give rise to Type B1 cells in the V-SVZ of the mouse forebrain. We found that loss of Sufu caused a dramatic cellular expansion in the dorsal V-SVZ by postnatal day (P) 7 of the neonatal mouse and persists through early adult stages. This resulted in excess production of Sox2+ Type B1 cells at the expense of TACs. We found that these defects were partially due to a decrease in Gli3 expression, but not due to Shh signaling activation. Taken
together, these studies indicate that Sufu plays a critical role in regulating neural cell precursor generation in the neonatal forebrain.

**RESULTS**

**Expansion of the V-SVZ in the P7 hGFAP-Cre;Sufu<sup>fl/fl</sup> mice**

The postnatal V-SVZ structure is composed of distinct dorsal and ventral domains (Fig. 1A). The rudimentary dorsal and ventral domains can be distinguished anatomically and molecularly at birth. The wild-type dorsal V-SVZ domain expresses dorsal V-SVZ marker, Pax6, while the lateral wall along the ventral V-SVZ domain expresses the marker, Dlx2 (Fig. S1; Brill et al., 2008). These areas are densely populated and, in the case of the ventral V-SVZ, are composed of several cell layers (Fig. 1C). Over time, a progressive reduction in V-SVZ cell density occurs (Fig. 1E,G). The ventral V-SVZ forms a one-cell-layer-thick structure, while the area occupied by the dorsal V-SVZ dramatically decreases (Fig. 1I). These observations indicate that critical regulatory events are actively shaping the V-SVZ cellular structure at early neonatal stages.

Type B1 cells are produced in late embryonic stages from RGCs of the embryonic forebrain and generate the majority of cells populating the V-SVZ. We previously showed that embryonic regulation of RGC proliferation and specification are tightly...
controlled by the Shh signaling pathway at embryonic stages. To determine how Shh signaling affects Type B1 cells in the developing V-SVZ, we generated a conditional mouse knockout for Sufu, a known antagonist of Shh signaling using the hGFAP-Cre mouse line in which Cre recombinase is specifically active in RG cells of the late stage embryonic forebrain, at E13.5 (Zhuo et al., 2001). Generating the hGFAP-Cre;Sufufl/fl mice (Fig. 1B) allowed us to target Sufu deletion in RGCS from all progenitor domains of the dorsal and ventral forebrains. At P0, we examined coronal sections from V-SVZ regions of hGFAP-Cre;Sufufl/fl and control littersmates and found no obvious anatomical differences (Fig. 1C,D) and that the dorsal and ventral V-SVZ domains correctly formed in the mutant V-SVZ, as determined by the clear demarcation of Pax6+ dorsal V-SVZ and Dlx2+ ventral V-SVZ domains (Fig. S1). By P7, we found a dramatic enlargement of the dorsal V-SVZ in mutant mice compared to control littersmates, while the ventral V-SVZ was comparable between the two genotypes (Fig. 1E,F; data not shown). Quantification of the overall dorsal V-SVZ area confirmed that no significant difference in the overall size of the dorsal V-SVZ was observed between controls and mutants at P0 (Fig. 1I; 278,512±39,546 µm² for n=3 controls and 338,946±48,133 µm² for n=3 mutants; P-value=0.369). However, the mutant dorsal V-SVZ was expanded approximately three-fold compared to control littersmates by P7 (Fig. 1I; 126,984±99,151 µm² for n=4 controls and 514,863±86,674 µm² for n=4 mutants; P-value =0.0043). By P28, despite a considerable reduction in the overall area of the dorsal V-SVZ in both genotypes, the mutant dorsal V-SVZ remained significantly enlarged compared to controls (Fig. 1G-I; 23,323±2067 µm² for n=7 controls and 97,439±12,890 µm² for n=5 mutants; P-value =0.0001). These observations indicate that Sufu does not play a role in the initial patterning of the dorsal and ventral V-SVZ domains. However, Sufu appears to control the expansion of dorsal V-SVZ cell types at neonatal stages likely influencing the final number of cells residing in this domain at early adult stages.

**Accumulation of proliferating cells in the dorsal V-SVZ of the P7 hGFAP-Cre;Sufufl/fl mice**

The dorsal V-SVZ is populated by actively proliferating precursors, including immature Type A cells that divide and migrate into the OB. To examine whether the increase in cell number in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ is due to the failed migration of Type A cells, we labeled proliferating precursors in the V-SVZ of either P0 or P1 littersmates by intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU) and examined the location of BrdU+ cells in the P7 hGFAP-Cre;Sufufl/fl dorsal V-SVZ (2G, 0.003417±0.0004118 cells per 100 µm² for n=3 controls and 0.007374±0.001042 cells per 100 µm² for n=3 mutants; P-value=0.0242). However, the proportion of proliferating BrdU+ cells did not significantly differ between controls and mutants (Fig. 2H; 3.008±0.2010% of BrdU+ cells in n=3 controls and 3.771±0.4467% BrdU+ cells for n=3 mutants; P-value=0.1975). Our findings indicated that loss of Sufu resulted in the continuous proliferation of cells within the dorsal V-SVZ of the P7 hGFAP-Cre;Sufufl/fl mice.

**Persistent cell proliferation in the dorsal V-SVZ of the P7 hGFAP-Cre;Sufufl/fl mice**

NSCs in the V-SVZ include slowly-dividing quiescent populations able to retain S-phase labels such as BrdU – and are referred to as label-retaining cells – for extended periods (Cotsarelis et al., 1990; Codega et al., 2014). To exclude the possibility that BrdU+ cells in the dorsal V-SVZ are label-retaining quiescent NSCs (qNSC), we examined the proportion of cells that remained proliferative by immunostaining with the mitotic marker Phospho-Histone H3 (Ph-H3) following BrdU labeling (Fig. 2A). We found that, unlike controls (Fig. 2D), many of the accumulated BrdU+ cells in the P7 hGFAP-Cre;Sufufl/fl dorsal V-SVZ expressed Ph-H3, and were therefore still proliferative (Fig. 2E). Quantification of double-labeled cells verified the presence of a significantly higher proportion of Ph-H3+ and BrdU+ double-labeled cells in the P7 hGFAP-Cre;Sufufl/fl dorsal V-SVZ (Fig. 2G, 0.003417±0.0004118 cells per 100 µm² for n=3 controls and 0.007374±0.001042 cells per 100 µm² for n=3 mutants; P-value=0.0242). However, the proportion of proliferating BrdU+ cells did not significantly differ between controls and mutants (Fig. 2H; 3.008±0.2010% of BrdU+ cells in n=3 controls and 3.771±0.4467% BrdU+ cells for n=3 mutants; P-value=0.1975). Our findings indicated that loss of Sufu resulted in the continuous proliferation of cells within the dorsal V-SVZ of the P7 hGFAP-Cre;Sufufl/fl mice.

**Loss of Sufu drives the proliferation of Type B1 cells in the dorsal V-SVZ**

Given the abnormal cell expansion and the continuous proliferation of cells in the dorsal V-SVZ of the P7 hGFAP-Cre; Sufufl/fl mice, we analyzed the behavior of specific cell types through labeling with V-SVZ cell specific markers (Fig. 3A). First, we conducted immunostaining with Sox2, a transcription factor highly expressed in the dorsal V-SVZ Type B1 cells (Ellis et al., 2004). At P7, Sox2-expressing (Sox2+) cells were present and accumulated along the lining of the ventricular wall and the periphery of the dorsal V-SVZ of both control and mutant mice (Fig. 3B,C). High magnification analysis of the dorsal V-SVZ showed two distinct populations of Sox2+ cells (Fig. 3F,G): cells expressing high levels of Sox2 (Sox2+high, white arrowheads) and cells expressing low levels of Sox2 (Sox2+low, yellow arrowheads). Sox2+high cells co-expressed Nestin (Nestin+), indicating that Sox2+high cells are proliferating Type B1 Cells (Codega et al., 2014). Thus we focused our subsequent analysis on Sox2+high cells. These cells were increased in the P7 hGFAP-Cre;Sufufl/fl dorsal V-SVZ, particularly in regions where Sox2+high cells were typically scant in the controls (boxed areas, Fig. 3B,C,F,G). When quantified, we did not find any significant difference per unit area in the total number of Sox2+high cells (Fig. 3D, 0.6895±0.04573 cells per 100 µm² for n=3 controls and 0.5037±0.0773 cells per 100 µm² for n=3 mutants; P-value=0.1074). However, we found that the total number of Sox2+high cells dramatically increased per dorsal V-SVZ of mutant mice (Fig. 3E, 351.8±59.82 cells, n=3 controls and 0.1308±0.01477 cells per 100 µm² for n=3 controls and 0.1789±0.03221 cells per 100 µm² for n=3 mutants; P-value=0.2463) and OB (0.1225±0.002195 cells per 100 µm² for n=3 controls and 0.1457±0.01775 cells per 100 µm² for n=3 mutants; P-value=0.2650). Overall, these observations indicate that cells within the dorsal V-SVZ of mutant mice were able to migrate despite of the accumulation of BrdU+ cells in the mutant dorsal V-SVZ.
1521±391.5 cells, \( n = 3 \) mutants; \( P \)-value=0.0418). Further quantification of Sox2\(^{+}\)\(^{\text{high}}\) Nestin\(^{+}\) cells showed that Type B1 cells specifically increased in the dorsal V-SVZ of mutant mice (Fig. 3H; 0.0602±0.009856 cells per 100 \( \mu \)m\(^2\) in controls and 0.2426±0.04155 cells per 100 \( \mu \)m\(^2\) in mutants, \( n = 3 \) control/mutant mice, \( P \)-value=0.0130). These findings showed that Sox2\(^{+}\)\(^{\text{high}}\) Type B1 cells proliferated proportionately as the dorsal V-SVZ expanded.

Reduced transit amplifying cells in the dorsal V-SVZ of P7 hGFAP-Cre;Sufu\(^{fl/fl}\) mice

Type B1 cells in the dorsal V-SVZ generate neurogenic TACs (Fig. 3A). Thus, we investigated how the increase in proliferating Sox2\(^{+}\)\(^{\text{high}}\) cells affected TACs in the dorsal V-SVZ by immunostaining with TAC-specific markers, Gsx2 or Tbr2 (Brill et al., 2009; López-Juárez et al., 2013). At P7, we observed weak Gsx2 expression in GFAP\(^{+}\) cells in the ventricular lining (Fig. S2A–F, blue arrows) whereas strong Gsx2 expression was detected in GFAP\(^{-}\)cells in the ventricular lining and outside this region (Fig. S2A–F, white arrows) in both P7 control and hGFAP-Cre;Sufu\(^{fl/fl}\) dorsal V-SVZ. These indicate that Gsx2\(^{+}\)/GFAP\(^{-}\) cells in the ventricular lining represented Type B1 cells that have acquired a neuronal precursor fate. The increase in Gsx2 expression, coupled with the loss in GFAP expression, likely marks further differentiation of Type B1 cells into TACs as they migrate away from the ventricular lining.
lining. Interestingly, we observed fewer Gsx2+ cells in the ventricular lining of the hGFAP-Cre;Sufu<sup>fl/fl</sup> dorsal V-SVZ, pointing to a reduction in the formation of Gsx2+ TACs. Indeed, we found obvious differences in the overall distribution of Gsx2+ cells between the dorsal V-SVZ of control and hGFAP-Cre;Sufu<sup>fl/fl</sup> mice. Visibly, Gsx2+ cells were sparsely distributed in the mutant dorsal V-SVZ unlike controls (Fig. 4A,B). Quantification confirmed a significant reduction in the density of Gsx2+ cells in mutants compared to controls (Fig. 4C, 0.1533±0.02214 cells per 100 µm<sup>2</sup> for n=5 controls, 0.07684±0.008522 cells per 100 µm<sup>2</sup> for n=5 mutants; P-value=0.0122), although the overall number of Gsx2+ cells in the dorsal V-SVZ did not significantly differ (Fig. S2M, 98.6±14.38 cells per dorsal V-SVZ in n=5 controls, 146.7±21.38 cells per dorsal V-SVZ in n=5 mutants; P-value=0.098). Also, the proliferative capacity of Gsx2+ TACs, as determined by co-labeling with Ki67, did not significantly differ between mutant and control dorsal V-SVZ (Fig. 4D,F; 23.24±6.655% of cells in controls, 28.62±4.27% of...
Delayed maturation of ependymal cells in the dorsal V-SVZ of P7 hGFAP-Cre;Sufufl/fl mice

Given the reduction in Gsx2+ and Tbr2+ TACs in the dorsal V-SVZ, we wondered if loss of Sufu also affected the formation of non-proliferative ependymal cells. Ependymal cells control the structural integrity of the V-SVZ. These cells are arranged in a single-cell layer along the ventricular lining to form a barrier between the ventricle lumen and the brain parenchyma (Lim and Alvarez-Buylla, 2016). Although the majority of ependymal cells are specified and generated from RGs at embryonic stages, their maturation into functional ependymal cells begins at birth and concludes by P20 (Spassky et al., 2005). Indeed at P7, we observed sparsely distributed ependymal cells, labeled with the ependymal cell marker S100β (S100β+), along the ventricular wall of the control dorsal V-SVZ (Fig. 4O). In contrast, in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ we observed fewer S100β+ cells along the ventricular wall (Fig. 4M,N) and was significantly reduced compared to controls (Fig. 4O; 0.17±0.0044 cells per 100 µm² for n=3 controls and 0.09±0.0259 cells per 100 µm² for n=3 mutants; P-value=0.0363). Nevertheless, by P28, we observed neatly lined S100β+ ependymal cells along the ventricular wall of the control and hGFAP-Cre;Sufufl/fl dorsal V-SVZ (Fig. S3). These findings indicate that despite the delay in maturation, Sufu deletion does not impede the formation and maturation of ependymal cells along the ventricular lining of the dorsal V-SVZ.

Loss of Sufu downregulated Gli3 expression and Shh signaling activity

Similar to the P7 hGFAP-Cre;Sufufl/fl V-SVZ, deletion of Gli3 in NSCs of the developing brain causes expansion of the dorsal V-SVZ in neonatal mice (Petrova et al., 2013; Wang et al., 2014). We examined if changes in Gli3 levels occurred in the P7 hGFAP-Cre;Sufufl/fl V-SVZ. Quantitative PCR analysis showed that Gli3 mRNA was reduced in dissected V-SVZ of P7 hGFAP-Cre;Sufufl/fl mice (Fig. 5A; 1±0.09995 relative expression level in controls, 0.4697±0.02742 levels in mutants, n=3 mice/genotype, P-value=0.0069). These findings suggest that loss of Sufu results in diminished expression of Gli3 causing the defects observed in the hGFAP-Cre;Sufufl/fl mice. As with mice lacking Gli3 in the V-SVZ (Wang et al., 2014), we did not observe ectopic activation of Shh signaling in the dorsal V-SVZ of the P7 hGFAP-Cre;Sufufl/fl V-SVZ by visualizing cells that express LacZ under the control of the Gli1 promoter (LacZ+) (Ahn and Joyner, 2005). Surprisingly, far fewer LacZ+ cells were observed in the dorsal V-SVZ of P0 and P7 mutant mice (Fig. 5C,D and Fig. S4). This observation correlated with the reduced Gli1 expression in the dorsal V-SVZ of mutant mice (Fig. 5B; 1±0.1909 relative expression level in controls, 0.4898±0.03958 levels in mutants, n=3 mice/genotype, P-value=0.0387). Further supporting these observations, expression of the Shh target gene, Ptc1, was also comparable between control and mutant mice (Fig. 5B; 1±1628 relative expression level in controls, 0.8080±0.2008 levels in mutants, n=3 mice/genotype, P-value=0.4839). These results showed that Sufu deletion in the neonatal dorsal V-SVZ does not ectopically activate Shh signaling. Furthermore, these findings indicate that Shh signaling does not drive the uncontrolled expansion of Sox2+ Type B1 cells in the neonatal hGFAP-Cre;Sufufl/fl dorsal V-SVZ.

Normal migration and maturation of OB interneurons in the P28 hGFAP-Cre;Sufufl/fl mice

The dramatic increase in Type B1 cells and the decrease in TACs in the dorsal V-SVZ at P7, led us to investigate if the number of...
interneurons in the OB have been compromised. Immunostaining for Doublecortin (Dcx), a marker for immature neurons, showed Dcx-expressing (Dcx+) cells in the dorsal V-SVZ and RMS indicating that TACs were able to differentiate into immature neurons (Fig. 6A–D). To confirm that proliferating TACs in the P7 dorsal V-SVZ differentiated into interneurons in the OB, we treated neonatal pups with three pulses of BrdU every 12 h beginning at P6 to efficiently label and fate map cells that were actively proliferating in the P7 V-SVZ cells (Fig. 6E). Results from these experiments showed that BrdU-labeled V-SVZ cells successfully migrated and integrated into various OB layers by P28 (Fig. 6F,G). We found that the total number of BrdU+ cells in the OB of control and mutant mice did not significantly differ (Fig. 6H, 0.08992±0.01195 cells per 100 µm² for n=3 controls and 0.08334±0.004048 cells per 100 µm² for n=3 mutants; P-value=0.6297), indicating that proper numbers of immature neurons originating from the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ migrated into the OB. Quantification of BrdU+ cells in each OB layer revealed a significant increase in the density of BrdU+ cells in the mutant mice compared to controls (n=3 controls/mutants). (Fig. 4. Sufu deletion causes a reduction in transit amplifying cells (TACs) and ependymal cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ. (A,B) Immunofluorescence staining against Gsx2, a marker for TACs, shows that Gsx2+ cells are present in both control (A) and mutant (B) V-SVZ. Scale bars: 250 µm. (C) Quantification of Gsx2+ cells in the P7 dorsal V-SVZ shows a significant increase in the density of Gsx2+ cells in the mutant mice compared to controls (n=3 controls/mutants). (D,E) Double immunofluorescence staining against Gsx2, co-labeled with the proliferation marker Ki67, shows that proliferating Gsx2+ cells are present in both control and mutant dorsal V-SVZ (n=3 controls/mutants). (G,H) Immunofluorescence staining against dorsal forebrain neurogenic progenitor cell marker, Tbr2, shows a visible decrease in the number of Tbr2+ cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ (H) compared to controls (G). Scale bars: 250 µm. (I) Quantification of Tbr2+ cells in the dorsal V-SVZ shows a significant increase in the number of Ki67+ cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ compared to controls. Scale bars: 250 µm. Arrowheads mark double-labeled cells. (L) Quantification of proliferating Tbr2+ and Ki67+ cells, verify no significant differences between control and mutant dorsal V-SVZ (n=3 controls/mutants). (M,N) Immunofluorescence staining against ependymal cell marker, S100β, shows a decrease in the density of S100β+ cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ compared to controls. Scale bars: 200 µm. (O) Quantification of S100β+ cells in the dorsal V-SVZ shows a significant increase in the density of ependymal cells in the mutant P7 hGFAP-Cre; Sufufl/fl mice compared to controls (n=3 controls/mutants). *P-value ≤0.05; **P-value ≤0.03. (Fig 6. Sufu deletion causes a reduction in transit amplifying cells (TACs) and ependymal cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ. (A,B) Immunofluorescence staining against Gsx2, a marker for TACs, shows that Gsx2+ cells are present in both control (A) and mutant (B) V-SVZ. Scale bars: 250 µm. (O) Quantification of Tbr2+ cells in the dorsal V-SVZ shows a significant increase in the density of Tbr2+ cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ (H) compared to controls (G). Scale bars: 250 µm. (I) Quantification of Tbr2+ cells in the dorsal V-SVZ shows a significant increase in the density of Tbr2+ cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ compared to controls (n=3 controls/mutants). (J,K) Double immunofluorescence staining against Ki67 and Tbr2, shows proliferating Tbr2+ cells in both control (J) and mutant (K) P7 dorsal V-SVZ. Scale bars: 50 µm. Arrowheads mark double-labeled cells. (L) Quantification of proliferating Tbr2+ and Ki67+ cells, verify no significant differences between control and mutant dorsal V-SVZ (n=3 controls/mutants). (M,N) Immunofluorescence staining against ependymal cell marker, S100β, shows a decrease in the density of S100β+ cells in the P7 hGFAP-Cre; Sufufl/fl dorsal V-SVZ compared to controls. Scale bars: 200 µm. (O) Quantification of S100β+ cells in the dorsal V-SVZ shows a significant decrease in the density of ependymal cells in the mutant P7 hGFAP-Cre; Sufufl/fl mice compared to controls (n=3 controls/mutants). *P-value ≤0.05; **P-value ≤0.03.
the number of BrdU+ cells of the granule cell layer (GCL) (0.1309±0.01715 cells per 100 µm² for n=3 controls and 0.1098±0.01342 cells per 100 µm² for n=3 mutants; P-value=0.3871), internal plexiform layer (IPL) (0.06176±0.01272 cells per 100 µm² for n=3 controls and 0.07677±0.00666 cells per 100 µm² for n=3 mutants; P-value=0.3550), or mitral cell layer (MCL) (0.1176±0.02794 cells per 100 µm² for n=3 controls and 0.1147±0.01621 cells per 100 µm² for n=3 mutants; P-value=0.9313), between controls and mutants. These findings indicate that despite the reduction in TACs in the P7 dorsal V-SVZ, Sufu deletion did not dramatically alter the number of interneurons, which are typically generated by neuronal precursors labeled cells (Fig. 7J 69.66±7.761% of n=3 controls/mutants). Scale bars: 200 µm. *P-value ≤0.05; **P-value ≤0.03.

**A greater number of proliferating Type B1 cells at P7 remained in the P28 dorsal V-SVZ of hGFAP-Cre;Sufuβ/β' mice**

The lack of any significant changes in OB interneurons prompted us to further examine the fate of NSCs and TACs in the dorsal V-SVZ of hGFAP-Cre;Sufuβ/β' mice. We found that cells labeled with BrdU at P7 (Fig. 7A) remained in the dorsal V-SVZ of mutant mice at P28 unlike controls (Fig. 7B–E). Quantification showed a dramatic increase in BrdU+ cells in the P28 mutant dorsal V-SVZ compared to controls (Fig. 7H; 15.7±1.099 cells per dorsal V-SVZ for n=5 controls and 98.45±24.39 cells per dorsal V-SVZ for n=5 mutants; P-value=0.0095). We also found a significantly higher number of BrdU+ cells expressing Sox2 (Fig. 7F,G and 110.7±0.8456 cells per dorsal V-SVZ for n=5 controls and 49.2±11.6 cells per dorsal V-SVZ for n=5 mutants; P-value=0.0107). Although we found a significantly higher number of BrdU+ cells co-expressing Sox2 in mutants, we did not find a disproportionate increase of double-labeled cells (Fig. 7J 69.66±7.761% of n=5 controls, 51.91±5.226% of n=5 mutants, P-value=0.0944). These findings indicate that many proliferating Type B1 cells in the P7 dorsal V-SVZ remained in this region at P28, likely contributing to the enlarged size of the P28 hGFAP-Cre;Sufuβ/β' dorsal V-SVZ.

**Increased cell death in the P7 hGFAP-Cre;Sufuβ/β' mice dorsal V-SVZ**

We previously observed expansion of specific neocortical progenitors in the embryonic neocortex of conditional Sufu
knockouts and found that many were unable to survive (Yabut et al., 2016). Therefore, we investigated if cells in the dorsal V-SVZ of the neonatal hGFAP-Cre;Sufufl/fl mice were similarly unstable and became apoptotic. We conducted immunostaining against the cell death marker, cleaved Caspase-3 (Cl-Casp3), and observed many dying cells (Cl-Casp3+) along the dorsal V-SVZ of mutant mice whereas Cl-Casp3+ cells in the dorsal V-SVZ of control mice were less frequent (Fig. 8A–D). Indeed, quantification of Cl-Casp3+ cells reflected these observations and showed a significant increase in apoptotic cells in the dorsal V-SVZ of P7 hGFAP-Cre;Sufufl/fl mice (Fig. 8E; 0.184±0.08815 cells per 100 µm² for n=3 controls and 0.5282±0.0672 cells per 100 µm² for n=3 mutants; P-value=0.0360). These findings suggest that despite the massive expansion of precursor cells in the dorsal

Fig. 6. Type A cells are produced and able to differentiate into interneuron subtypes in the P28 hGFAP-Cre;Sufufl/fl OB. (A–D) Immunofluorescence staining against DCX, which labels Type A cells, shows that Type-A cells are generated in the dorsal V-SVZ of the P7 control and mutant mice and are able to migrate through the RMS. Scale bars: (A,B) 250 µm, (C,D) 500 µm. (E) Schematic of the experimental design to identify the progeny of proliferating cells in the P7 dorsal V-SVZ and determine their localization and identity in the OB at P28. (F,G) Immunofluorescence staining against BrdU shows visible confirmation of proliferating Type A cells migrating into the OB layers of P28 hGFAP-Cre;Sufufl/fl mice and controls. Scale bars: 100 µm. (H) Quantification of BrdU+ cells in each OB layer shows a significant increase in BrdU+ cells in external plexiform layer (EPL) of mutant mice compared to controls. However, no other significant increase was observed in the remaining layers of the OB (n=3 controls/mutants). (I,J) Immunofluorescence staining against the dorsal V-SVZ derived interneuron cell marker, Calretinin in the P28 OB. Scale bars: 100 µm. (K) Quantification of Calretinin+ cells in the OB confirms no significant difference in individual layers of the OB or as a whole (n=3 controls/mutants). (L,M) Immunofluorescence staining against ventral V-SVZ derived interneuron cell marker, Calbindin. Scale bars: 100 µm. (N) Quantification of Calbindin+ cells in the OB confirms there is no significant difference in individual layers of the OB or as a whole (n=3 controls/mutants). RMS, rostral migratory stream; OB, olfactory bulb; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer; **P-value ≤ 0.03.
V-SVZ, many of these cells failed to survive and differentiate into mature cell types.

**DISCUSSION**

The postnatal V-SVZ is composed of multiple neuronal precursor populations that sustain lifelong neurogenesis in rodents. Our study provides insights into the molecular mechanisms involved in the formation of a molecularly distinct neurogenic domain, the dorsal V-SVZ. We showed that the cytoplasmic adaptor protein, Sufu, plays important roles in controlling precursor number and viability. Genetic ablation of Sufu in RG cells at late embryonic stages caused a dramatic expansion of the dorsal V-SVZ, but not the ventral V-SVZ. This expansion is due to the uncontrolled proliferation and organization of Sox2+ Type B1 cells, resulting in deregulated production of TACs via Gli3-dependent mechanisms, and independent of Shh signaling activity. Our novel findings establish a crucial role for Sufu in maintaining precursor populations in the neonatal dorsal V-SVZ.

Fig. 7. Cells generated in the dorsal V-SVZ at P7 accumulate in the dorsal V-SVZ of P28 hGFAP-Cre;Sufufl/fl mice. (A) Schematic of the experimental design to identify the progenies of proliferating cells in the P7 dorsal V-SVZ. Three pulsed intraperitoneal injections at 12-h intervals of S-phase label BrdU was administered starting at P6 to label proliferating cells in the V-SVZ and determine if any BrdU-labeled cells remained in the dorsal V-SVZ at P28. (B–E) Immunofluorescence staining against BrdU shows an obvious increase in the number of BrdU+ cells in the dorsal V-SVZ of the P28 hGFAP-Cre;Sufufl/fl mouse. Scale bars: 200 µm. (F,G) Double-immunofluorescence staining shows that many BrdU+ cells co-labeled Sox2 in the P28 dorsal V-SVZ of control and mutant mice. Scale bars: 100 µm. (H) Quantification of BrdU+ cells in the V-SVZ demonstrated a significant decrease in hGFAP-Cre;Sufufl/fl mice compared to controls (n=5 controls/mutants). (I, J) Quantification of BrdU+/Sox2+ cells in the V-SVZ indicates a significant increase in hGFAP-Cre;Sufufl/fl mice compared to controls (I) although no difference is observed in the percentage of BrdU+/Sox2+ cells (J; n=5 controls/mutants). **P-value ≤ 0.03; ***P-value ≤ 0.01.
We found that loss of Sufu at late embryonic stages did not disrupt the formation of the V-SVZ. Mice lacking Sufu formed anatomically distinct dorsal and ventral V-SVZ domains capable of generating predicted subpopulations of interneuron subtypes in the OB. This indicates that progenitor specification, as determined by their localization along the dorsoventral V-SVZ axis, was not severely disrupted. However, the dorsal V-SVZ was expanded in mice lacking Sufu, as a result of the persistent proliferation of Sox2+ Type B1 cells. These findings implied that loss of Sufu at late embryonic stages maintained NSCs in a highly proliferative state at neonatal stages, preventing their differentiation into Tbr2+ and Gsx2+ TACs. Thus, Sufu must play a role in modulating the cell cycle progression of NSCs in the dorsal V-SVZ to ensure a timely production of specific NSC lineages.

Loss of Sufu drastically reduced the production of Tbr2+ TACs. Tbr2+ TACs typically originate from RGCs in the embryonic neocortical progenitors whereas Gsx2+ neural progenitors that originate from the embryonic ganglionic eminence (Stenman et al., 2003; Brill et al., 2009) were significantly increased. We previously reported that loss of Sufu results in the increase in proliferation of neocortical progenitors (resulting in an increase in superficial layer projection neurons in the neocortex) and oligodendrogenesis in the E16.5 neocortex of hGFAP-Cre;Sufufl/fl mice (Yabut et al., 2016; Winkler et al., 2018). Thus, two possibilities could explain the reduction in Tbr2+ TACs: 1) neocortical progenitors that generate V-SVZ NSCs were re-specified towards the gliogenic lineage, and/or 2) exhaustion of the neocortical RGC pool has occurred. This would prevent neocortical RGCs to generate Type B1 cells in the dorsal V-SVZ. Lineage tracing of embryonic neocortical progenitors will determine if proportions of RGCs in the E16.5 neocortex, a time at which Type B1 cells are thought to be specified (Fuentelba et al., 2015; Furutachi et al., 2015), failed to generate Type B1 cells in the neonatal V-SVZ. Nevertheless, these findings indicate that in addition to previously identified roles of Sufu in corticogenesis, Sufu also functions to ensure the proper production of dorsal V-SVZ NSCs generating specific TAC subtypes. Since Tbr2+ TACs uniquely generate glutamatergic OB neurons (Brill et al., 2009), further investigation is required to determine if glutamatergic OB neurons are significantly reduced or if physiological disturbances in the OB circuitry has occurred as a consequence of abnormal production TACs.

Sufu antagonizes Shh signaling by mediating the proteolytic processing of Gli transcription factors to either inhibit activator function or promote the formation of transcriptional repressor forms. In the developing forebrain, we have previously shown that Sufu acts to regulate the stability and processing of Gli2 and Gli3 proteins at early stages of corticogenesis that resulting in an increase in Gli2R and Gli3R levels, while it functions to promote the generation of Gli3R alone at later stages (Yabut et al., 2015). Here, we found that loss of Sufu affected Gli transcription in dorsal V-SVZ cells. We found that Gli1 and Gli3 mRNA levels were significantly reduced in the hGFAP-Cre;Sufufl/fl V-SVZ. Gli3 is typically highly expressed in the dorsal V-SVZ to exert its repressor function during the establishment of the V-SVZ (Petrova et al., 2013; Wang et al., 2014). These findings showed that in the absence of Sufu, Gli3 transcription is not efficiently maintained in the neonatal V-SVZ. Loss of Sufu may have resulted in the repression of Gli3 expression by deregulation of yet unidentified transcription factors that typically promote Gli3 transcription, or indirectly caused by defects in Gli3 protein processing triggering other transcriptional repressors to inhibit Gli3 expression. Elucidating the molecular steps by which Sufu alters Gli3 expression and activity in dorsal V-SVZ NSCs could provide novel insights on the diverse mechanisms utilized by Sufu to control Gli3 function.

Deletion of Sufu and the subsequent reduction in Gli3 transcription in the neonatal hGFAP-Cre;Sufufl/fl mice resulted in defects that phenocopy Gli3R-cKO mice (Wang et al., 2014). Similar to our findings, ectopic activation of Shh signaling did not occur in Gli3R-cKO V-SVZ at neonatal stages. This could be because responsiveness to Shh signals by neonatal dorsal V-SVZ cells, such as in NSCs and TACs, do not occur until after P7 (Ahn and Joyner, 2005; Palma et al., 2005; Wang et al., 2014). Supporting this, conditional deletion of Smo in V-SVZ NSCs at P0, using the mGFAP-Cre driver, does not cause any obvious proliferation defects in the V-SVZ until after P15 (Petrova et al., 2013). This would indicate that the Sufu-Gli3 regulatory axis alone is critical in the control of progenitor populations in the dorsal
V-SVZ niche, independent of its canonical roles as regulators of Shh signaling activity. Indeed, previous studies have shown that Gli3R functions to regulate gp130/STAT3 signaling in NSCs at early neonatal stages for proper establishment of the V-SVZ niche (Wang et al., 2014).

In summary, our studies identified multiple roles for Sufu in establishing appropriate cell number and identity in the neonatal dorsal V-SVZ. We found that Sufu maintains neurogenic precursor populations in the dorsal V-SVZ via regulation of Gli3. These findings underscore the importance of Sufu as a key regulator of stem/progenitor populations not only in the developing embryonic forebrain but also in establishing postnatal neurogenic niches such as the V-SVZ. These results have potential implications in how neural stem/progenitor populations are established and sustained in the postnatal neurogenic niche, how defects in proliferation could predispose these cells to a number of neurological diseases and malignancies, and provide insights on potential molecular strategies that can be utilized for regenerative therapies.

**MATERIALS AND METHODS**

**Animals**
Mice carrying the floxed Sufu allele (Sufu<sup>fl</sup>) were kindly provided by Dr Chi-Chung Hui (University of Toronto) and were genotyped as described elsewhere (Pospisilik et al., 2010). The IGFAP-Cre (Stock #004600) was obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice designated as controls did not carry the IGFAP-Cre transgene and may have either one of the following genotypes: Sufu<sup>fl/fl</sup> or Sufu<sup>fl/+</sup> (Fig. 1A). All mouse lines were maintained in mixed strains, and analysis included male and female pups from each age group, although sex differences were not included in data reporting. All animal protocols were in accordance to the National Institute of Health regulations and approved by the UCSF Institutional Animal Care and Use Committee (IACUC).

**Quantitative PCR**
Total RNA was isolated from dissected V-SVZ of P7 mice using TRIzol<sup>™</sup> Reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions, and each sample was reverse-transcribed using a SuperScript IV cDNA Synthesis Kit (Invitrogen). Quantitative PCR reactions were performed using a KAPA SYBR Fast qPCR Kit (KAPA Biosystems) with ROX as reference dye, and transcript expression was measured via Applied Biosystems 7500 Real-Time PCR System (Life Technologies). Expression levels of each gene were normalized to RNA polymerase II subunit A (polr2a) and calculated relative to the control. The following primers were used: Gli3 Fw: CCGACGGAGTGTCCTTGTGTC; Gli3 Rv: AACATG-GCGTCTCAAAGGAAAG; Gli3 Fw: AAGCCTCTCAAGATCAGGAC; Gli3 Rv: TTGGTCCTCCGCGGTTC; Gli3 Fw: TGACAAAGGCGGACTACAGTG; Gli3 Rv: AGGTAGCTCTGAGGGCTCT; Polr2a Fw: CATCAAGAGGATGCGTTC; Polr2a Rv: CCATTAGTCCCCCTCACTGATTC; Polr2a Fw: CCGACGGAGTGTCCTTGTGTC; Gli3 Rv: AACATG-GCGTCTCAAAGGAAAG; Gli3 Fw: AAGCCTCTCAAGATCAGGAC; Gli3 Rv: TTGGTCCTCCGCGGTTC; Gli3 Fw: TGACAAAGGCGGACTACAGTG; Gli3 Rv: AGGTAGCTCTGAGGGCTCT; Polr2a Fw: CATCAAGAGGATGCGTTC; Polr2a Rv: CCATTAGTCCCCCTCACTGATTC.

**Immunohistochemistry and BrdU-Labeling**
Perfusion, dissection, immunofluorescence and Nissl staining were conducted according to standard protocols as previously described (Siegenthaler et al., 2009). Cryostat sections were air dried and rinsed 3× in PBS plus 0.2% Triton before blocking for 1 h in 10% normal lamb serum diluted in PBS with 0.2% Triton to prevent nonspecific binding. Primary antibodies were diluted in 10% serum diluted in PBS with 0.2% Triton containing 40,6-diamidino-2-phenylindole (DAPI); sections were incubated in primary antibody overnight at room temperature. The following antibodies were used: mouse anti-BrdU (1:50 dilution; BD Pharmingen, #347580; Franklin Lakes, NJ, USA), rabbit anti-Phospho-Histone H3 (1:250 dilution; Millipore, #06-570; Billerica, MA, USA), rabbit anti-Sox2 (1:1000 dilution; Abcam, #ab29494; Cambridge, UK), rabbit anti-Thrb (1:500 dilution; Abcam, #ab32345), rabbit anti-cleaved Caspase-3 (1:300 dilution; Cell Signaling Technology, #9661S; Madison, WI, USA), mouse anti-Calretinin (1:250 dilution; Millipore, MAB168), mouse anti-Ki67 (1:250 dilution; BD Biosciences, #550089; USA), rabbit anti-GS2X2 (1:250 dilution; gift from Kenneth Campbell; Toresson et al., 2000; mouse anti-Olig2: 1:250 dilution; Millipore, #MABN50), rabbit anti-Calbindin (1:1000 dilution; Swant, #CB-38; Switzerland), rabbit anti-Pdgfra (1:1000; gift from William Stallcup; Nishiyama et al., 1996; rabbit anti-Doublecortin; 1:250 dilution; Abcam, #ab18723), mouse anti-S100B (1:100; Sigma-Aldrich, #S2532; St. Louis, MO, USA). For 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, #1028079001) labeling, early postnatal mice were treated with 50 µg/g BrdU by intraperitoneal injection at P0-P1 prior to dissection at P7-P8. For BrdU-labeling at P28, mice were treated with 50 µg/g BrdU pulse by intraperitoneal injection from P6 to P7 every 12 h for 36 h for a total of 3 BrdU treatments. To detect primary antibodies, we used species-specific Alexa Fluor-conjugated secondary antibodies (1:500; Invitrogen) in 1X PBS-T for 1 h at room temperature, washed with 1X PBS, and coverslipped with Fluoromount-G (Southern Biotech).

**Image analysis and acquisition**
Images were acquired using a Nikon E600 microscope equipped with a QCapture Pro camera (QImaging) or Zeiss Axioscan Z.1 (Zeiss, Thornwood, NY, USA) using the Zen 2 blue edition software (Zeiss). NIH ImageJ was used to quantify raw, unedited images. All analyses were conducted in at least two to three 20-µm-thick sections that were histologically matched at the rostral-caudal level between genotypes.

V-SVZ analysis: for measurement of VZ/SVZ thickness, the length of densely populated cell or DAPI+ regions adjacent to the lateral ventricles was measured and designated as the dorsal V-SVZ or ventral V-SVZ as defined in Fig. 1B. DAPI-dense regions were also used to define and measure the SVZ, RMS, and the OB to quantify BrdU-localization along sagittal sections. S100b+ cells were counted by measuring a 25-µm-thick region from the ventricular lining of the dorsal V-SVZ. Cells labeled with cell-specific markers were quantified within the dorsal V-SVZ to measure the number of cells per 100 µm<sup>2</sup> or per V-SVZ.

OB analysis: a slice of the OB containing all layers, as designated according to their anatomical features (as defined in Fig. 6B), was used for cell quantification. Cells that express cell-specific markers (Calr+, Calb+, or BrdU+) were counted in each layer.

**Statistics**
All experiments were conducted in triplicate with a sample size of n=3–6 embryos/animals per genotype. Unpaired Student’s t-test was conducted using Prism 7 (GraphPad) for pairwise analysis of control and mutant genotypes. Values of P<0.05 were considered statistically significant. Graphs display the mean±standard error of the mean (s.e.m.).

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
Conceptualization: S.J.P., O.R.Y.; Methodology: H.G.G., H.N., D.A., S.J.P., O.R.Y.; Validation: H.G.G., H.N., J.G.C., O.R.Y.; Formal analysis: H.G.G., H.N., J.G.C., D.A., O.R.Y.; Investigation: H.G.G., H.N., J.G.C., D.A., S.J.P., O.R.Y.; Data curation: H.G.G., O.R.Y.; Writing - original draft: H.G.G., H.N., J.G.C., S.J.P., O.R.Y.; Investigation: H.G.G., H.N., J.G.C., D.A., S.J.P., O.R.Y.; Data curation: H.G.G., H.N., J.G.C., S.J.P., O.R.Y.; Writing - review & editing: H.G.G., H.N., S.J.P., O.R.Y.; Supervision: S.J.P., O.R.Y.; Project administration: S.J.P., O.R.Y.; Funding acquisition: S.J.P., O.R.Y.

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**Supplementary information**
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