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Aberrant FGF signaling promotes granule neuron precursor expansion in SHH subgroup infantile medulloblastoma

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Mutations in Sonic Hedgehog (SHH) signaling pathway genes, e.g. *Suppressor of Fused* (SUFU), drive granule neuron precursors (GNP) to form medulloblastomas (MB^{SHH}). However, how different molecular lesions in the Shh pathway drive transformation is frequently unclear, and SUFU mutations in the cerebellum seem distinct. In this study, we show that fibroblast growth factor 5 (FGF5) signaling is integral for many infantile MB^{SHH} cases and that FGF5 expression is uniquely upregulated in infantile MB^{SHH} tumors. Similarly, mice lacking SUFU (Sufu-cKO), ectopically express FGF5 specifically along the secondary fissure where GNPs harbor preneoplastic lesions and show that FGFR signaling is also ectopically activated in this region. Treatment with an FGFR antagonist rescues the severe GNP hyperplasia and restores cerebellar architecture. Thus, direct inhibition of FGF signaling may be a promising and novel therapeutic candidate for infantile MB^{SHH}.

Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in children, with half of cases diagnosed before the age of 5 (Ward et al., 2014; Ostrom et al., 2016). Mutations in *Suppressor of Fused* (*SUFU)* comprise approximately 30% of tumors in infants with Sonic Hedgehog driven MB (MB^{SHH}). Infantile MB^{SHH}, including those with SUFU mutations (MB$^{SHH-SUFU}$) have worse prognosis and higher rates of local recurrence than other MB^{SHH} subtypes (Kool et al., 2014; Schwalbe et al., 2017; Guerrini-Rousseau et al., 2018). Unfortunately, available SHH-targeted treatments for MB^{SHH} act specifically on proteins upstream of SUFU and are therefore ineffective for MB$^{SHH-SUFU}$ patients (Kool et al., 2014). The poor prognosis, early occurrence, and lack of targeted therapy for MB$^{SHH-SUFU}$ patients make detailed understanding of the drivers of oncogenesis in this group of great importance.

Sufu acts as an intracellular modulator of SHH signaling (Matise and Wang, 2011). Briefly, the SHH signaling pathway is initiated after binding of extracellular SHH ligands to the transmembrane receptor Patched 1 (Ptch1). This relieves Ptch1 inhibition of the transmembrane protein, Smoothened (Smo), and enables the initiation of a cascade of intracellular events promoting the activator function of the transcription factors, Gli1, Gli2, or Gli3. Sufu modulates SHH signaling by ensuring the stability of Gli transcription factors or by promoting the formation of the repressor forms of Gli2 (Gli2R) or Gli3 (Gli3R) (Chen et al., 2009; Wang et al., 2010; Lin et al., 2014). Thus, depending on the developmental context, loss of Sufu can lead to activation or repression of SHH signaling. In the developing cerebellum, Sufu dysfunction is associated with abnormal development of granule neuron precursors (GNP), which account for MB^{SHH} (Kim et al., 2011, 2018; Vanner et al., 2014; Kong et al., 2019; Vladoiu et al., 2019; Yin et al., 2019; Jiwani et al., 2020). GNPs populate the external granule layer (EGL) along the cerebellar perimeter where local SHH signals trigger GNP proliferation and differentiation at neonatal stages. However, other mitogenic pathways can also influence GNP behavior (Leto et al., 2016), yet little is known on how Sufu interacts with these pathways. Understanding how SUFU loss-of-function (LOF) affects the activity of concurrent local signaling pathways in granule neuron development may be key towards developing potent targets for anti-tumor therapy.

In this study, we examined regulation of FGF signaling in MB^{SHH} and identified upregulation of FGF5 expression in tumors of infantile MB^{SHH} patients. Similarly, we show ectopic expression of FGF5 in the neonatal cerebellum of mice in lacking SUFU and correlates with the activation of FGF signaling in surrounding EGL-localized cells where GNPs accumulate. Strikingly, acute pharmacological inhibition of FGF signaling results in near-complete rescue of these defects, including a restoration of cerebellar histo-organization. Thus, our findings identify FGF5 as a potential biomarker for a subset of patients with infantile MB^{SHH} who may be responsive to FGFR-targeting therapies.

Results

FGF5 is specifically upregulated in SHH-driven infantile MB.

We previously reported that SUFU LOF in neocortical progenitors result in FGF signaling activation to influence specification and differentiation of neocortical excitatory neurons (Yabut et al., 2020). Thus, we sought to determine if key FGF signaling pathway genes are differentially expressed in MB patient tumors. We performed comparative analysis of the expression dataset from 763 MB patient samples comprised of tumors resected from molecularly distinct MB subgroups: Wingless (Wnt subgroup; MB^{WNT}), MB^{SHH}, Group 3 (MB^{Group3}), and Group 4 (MB^{Group4}) (Cavalli et al., 2017). Strikingly, our analyses show that FGF5 expression is higher in tumors specifically from MB^{SHH} patients compared to other MB subgroups, with approximately 25% of MB^{SHH} tumors exhibiting a two-fold increase (**Fig. 1A-1B**). We also find that FGF5 is

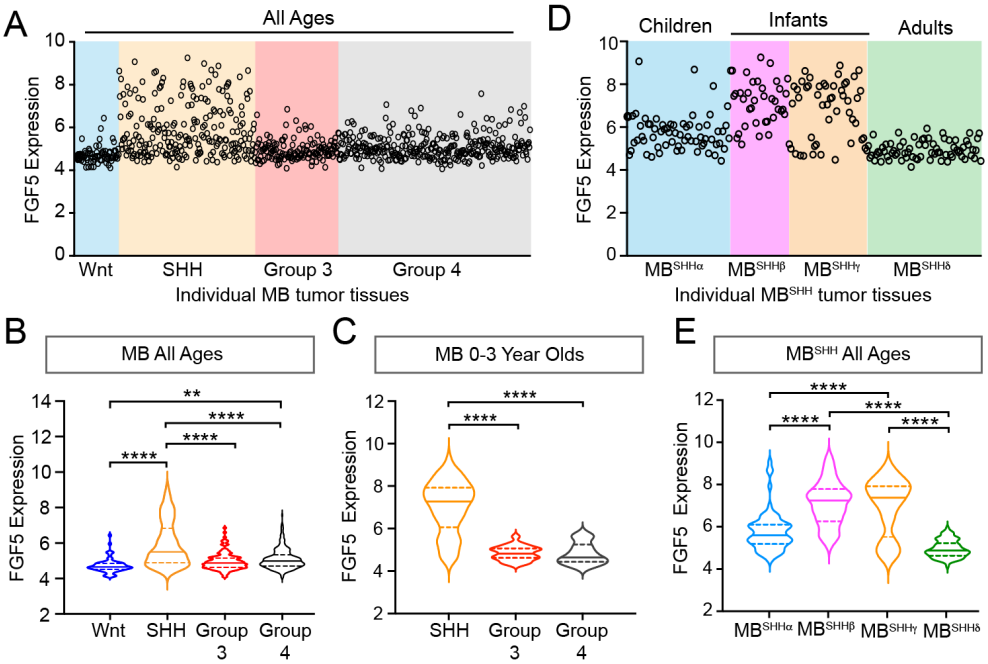

Figure 1: Upregulated FGF5 expression in MB^{SHH} tumors from infant patients. (A) Levels of FGF5 expression in human MB tumors of all ages from GEO expression dataset #GSE85217(Cavalli et al., 2017). **(B, C)** Statistical analysis of FGF5 expression levels associated with MB tumor subtypes from patients across all ages (B) and 0-3 year old MB patients (C). **p<0.01, ****p<0.0001. **(D, E)** Graph represents FGF5 expression levels in human MB^{SHH} tumors of all ages from GEO expression dataset #GSE85217 (D) and corresponding plots (E) showing statistically higher FGF5 expression in tumors from infants with MB^{SHH} compared to tumors from children or adults with MB^{SHH}. ****p<0.0001.

uniquely upregulated in MB^{SHH} tumors from patients within the 0-3 years old age group, but not patients within the same age group in other MB subtypes (**Fig. 1C**). Further examination across all MB^{SHH} tumors stratified which subgroups express the highest levels of FGF5 expression. Infantile tumors, largely belonging within the SHHb and SHHg subgroups (Cavalli et al., 2017), exhibit higher FGF5 levels compared to tumors from children (SHHa) or adults (SHHd) (**Fig. 1D**). By all measures, the proportion of SHHb and SHHg tumors with relatively high levels of FGF5 is significantly increased (~30%) compared to other MB^{SHH} subgroups (**Fig. 1E**). Taken together, these findings strongly suggest that FGF signaling is specifically disrupted in infantile-onset MB^{SHH}.

Region-specific expansion of GNPs in the P0 Sufu-cKO cerebellum coincides with increased FGF5 expression

FGF signaling has been implicated in cerebellar development, particularly in granule neuron development (Yaguchi et al., 2009; Yu et al., 2011) leading us to wonder if and how aberrant FGF signaling may be contributing to oncogenicity of GNPs. Since mutations in SUFU drive infantile MB^{SHH}, we generated the mutant mice (*hGFAP-Cre;Sufu$^{fl/fl}$*, hereto referred as Sufu-cKO), in which Sufu is conditionally deleted in GNPs (Zhuo et al., 2001) to examine FGF expression and activity. Sufu-cKO mice exhibit profound defects in cerebellar development. At P0, a timepoint at which GNP proliferation and differentiation is ongoing, there is a visible increase in measured cerebellar size and expansion of Pax6-positive (Pax6+) GNPs in the EGL of Sufu-cKO cerebellum compared to controls (**Fig. 2A-C**). Notably, the expansion of Pax6+ GNPs specifically localize along the secondary fissure (EGL Region B, arrow in **Fig. 2A**) compared to other EGL areas (EGL Regions A and C) in the P0 Sufu-cKO cerebellum (**Fig. 2D**). We then proceeded to examine whether these defects correlated with abnormal FGF5 expression. *In situ* hybridization (ISH) using FGF5-specific riboprobes show high expression of FGF5 (FGF5^{high}) immediately adjacent to the secondary fissure in the P0 control cerebellum (**Fig. 2E**). Strikingly, in the P0 Sufu-cKO cerebellum, there is anterior expansion of FGF5^{high} expression regions (outlines, **Fig. 2F**), coinciding with areas near the secondary fissure where GNP expansion is most severe (**Fig. 2A, 2D**). Further, while FGF5-expressing (FGF5+) cells are largely excluded from the EGL of the control cerebellum, a substantial number of FGF5+ cells are ectopically localized in the EGL Region B of the Sufu-cKO cerebellum (**Fig. 2E**). FGF5 mRNA molecules are visibly higher in FGF5-expressing cells in the Sufu-cKO EGL compared to controls (arrows, **Fig. 2F**). These findings implicate FGF5 as a potential instigator of region-specific defects in GNP differentiation present in the P0 Sufu-cKO cerebellum.

FGF signaling drives GNP proliferation in the P0 Sufu-cKO cerebellum

FGF5 is a ligand for fibroblast growth factor receptors 1 (FGFR1) and 2 (FGFR2), both of which are expressed in the developing cerebellum, particularly in IGL regions where FGF5-expressing cells localize (Clements et al., 1993; Ornitz et al., 1996). Binding of FGF5 to these receptors triggers the activation of multiple intracellular signaling pathways including the mitogen activated protein kinase (MAPK) pathway to control cellular activities driving NSC progression (**Fig. 3A**) (Ornitz and Itoh, 2015). Immunostaining with antibodies to detect MAPK pathway activation reveals increased MAPK activity in the EGL of the P0 Sufu-cKO cerebellum. Regional distribution of cells labeled with phospho-Erk1/2 (pErk1/2+), a marker for activated MAPK signaling, shows that the abnormally expanded EGL of the secondary fissure in the P0 Sufu-cKO cerebellum increase in these cells (**Fig. 3B**). Quantification of the number of pERK1/2+ cells in this area reveals that this is significant compared to controls (**Fig. 3C**). Many pERK1/2+ cells are also proliferative as indicated by Ki67 labeling (boxed areas, **Fig. 3B**) and the numbers of these dual labeled cells are significantly higher in the Sufu-cKO cerebellum compared to controls (**Fig. 3D**). These findings indicate that the increase in FGF5 correlates with the activation of FGF signaling in GNPs and demonstrates a likely role in regulating the abnormal proliferation and pre-neoplastic lesion in mutant mice.

Given the role of SUFU in regulating Gli transcription factors to modulate SHH signaling activity (Kim et al., 2018; Yin et al., 2019), we examined whether the activation of FGF signaling occurred concurrently with SHH signaling activation. Surprisingly, Gli protein levels in the P0 control and Sufu-cKO cerebellum show marked reduction of Gli1, Gli2, Gli3, and Ptch1 levels (**Supplementary Fig. 1A**); this is inconsistent with

elevated SHH signaling we anticipated in the absence of SUFU. To directly examine this in specific EGL regions, we compared the cerebellum of P0 control and Sufu-cKO mice carrying the SHH reporter transgene Gli1-LacZ (Ahn and Joyner, 2005). In these mice, SHH signaling activity is absent or very low throughout the entire P0 Sufu-cKO cerebellum but is highly active in a region-specific manner in controls (**Supplementary Fig. 1B**). Furthermore, while some LacZ+ cells are detectable in EGL regions A and C and adjacent ML and IGL, LacZ+ cells are completely absent in EGL region B and adjacent areas of the P0 Sufu-cKO cerebellum (**Supplementary Fig. 1B-1C**). These findings indicate that the accumulation of GNPs does not rely on active SHH signaling, particularly in Region B where there is a severe expansion of GNPs in the absence of SUFU.

Blockade of FGF signaling dramatically rescues the Sufu-cKO phenotype

To determine if activated FGF signaling drives GNP defects in the Sufu-cKO cerebellum, we pharmacologically inhibited FGF signaling using the competitive FGFR1-3 antagonist AZD4547 (Gudernova et al., 2016). For this experiment, 1 μl of AZD4547 (5mg/ml) was - delivered via intraventricular (IV) injection for 5 consecutive days beginning at P0 and the cerebellum analyzed 3 days after treatment at P7 (**Fig. 3E**). Strikingly, AZD4547 treatment results in near complete rescue of the GNP phenotype by P7 in the Sufu-cKO cerebellum, displaying a cerebellar morphology indistinguishable from controls with normal foliation and cellular organization (**Fig. 3F**). Indeed, in the cerebellum of AZD4547 -treated P7 Sufu-cKO mice, proliferating Ki67+ cells largely exclusively localize in the EGL while NeuN+ cells densely pack the IGL and not the EGL (**Fig. 3G**). Notably, NeuN expression appears in cells localized at the border of the EGL and ML, where Ki67+ cells are absent, indicating that post-mitotic cells successfully began differentiation as observed in controls (boxed regions, **Fig. 3G**). Thus, our findings confirm that inhibition of FGF signaling in proliferating GNPs of the Sufu-cKO cerebellum ensure normal progression of GNP differentiation.

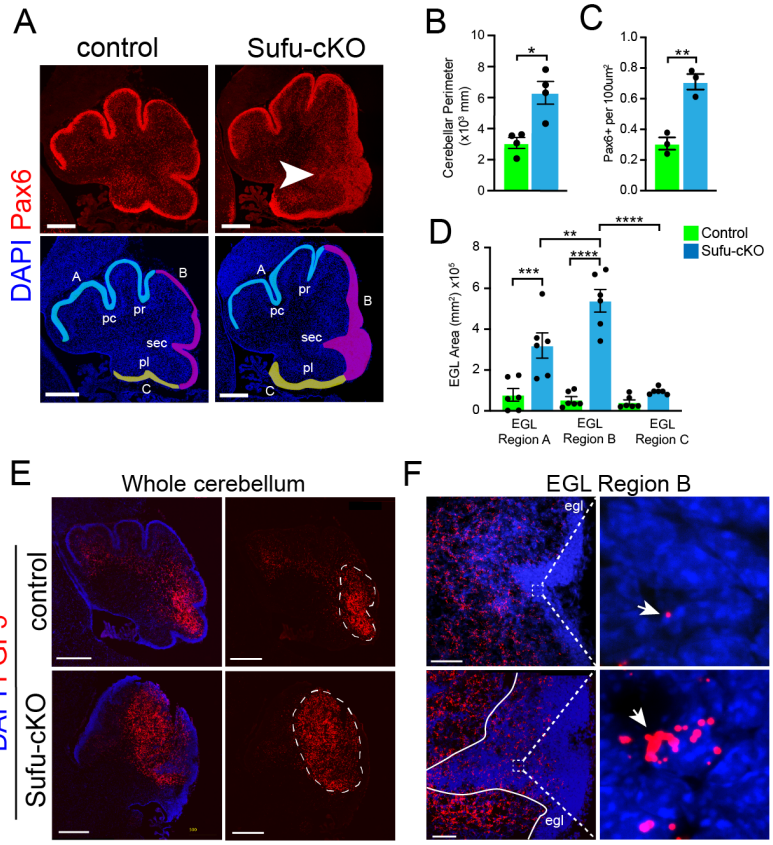

Figure 2: Increased FGF5 expression coincides with region-specific expansion of GNPs in the P0 Sufu-cKO cerebellum.

(A) Pax6 (red) and DAPI (blue) immunofluorescence staining of the P0 Sufu-cKO and control cerebelli. Arrow points to severely expanded EGL region B in the P0 Sufu-cKO cerebellum. EGL regions are designated in DAPI-labeled sections as A (light blue), B (magenta), and C (yellow). Each region encompasses specific fissures: the preculminate (pc) and primary (pr) fissures for Region A, the secondary (sec) fissure for Region B, and the posterolateral (pl) fissure for Region C. Scale bars: Scale bars = 250 μm. **(B-D)** Quantification and comparison of the cerebellar perimeter (B), total area occupied by densely packed Pax6+ cells (C), and size of specific EGL regions (D) between P0 Sufu-cKO and control cerebelli. **(E)** Fluorescent *in situ* hybridization using RNAScope probes against FGF5 mRNA (red) and DAPI labeling in the P0 Sufu-cKO and control whole cerebellum. Areas with high levels of FGF5 expression are outlined. Scale bars = 500 μm. **(F)** FGF5, as detected by fluorescent *in situ* hybridization, is ectopically expressed by cells in the EGL and adjacent regions of Region B. Boxed areas within the EGL show DAPI-labeled cells expressing visibly high levels of FGF5 (arrowheads) in the EGL of P0 Sufu-cKO cerebellum compared to controls. Scale bars = 50 μm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

FGF5 expression is increased in the developing cerebellum of Sufu;p53-dKO mice

Our findings indicate a critical role for FGF signaling in driving GNP hyperplasia, making GNPs vulnerable to neoplastic lesions resulting in tumorigenesis, when SUFU is absent in the developing cerebellum. Indeed, we find that Pax6+ GNPs within the neonatal Sufu-cKO EGL display an increase in double-strand breaks, especially in Region B (DSBs; **Fig. 4A-4B**), as detected by immunostaining for phosphorylated H2AX (gH2AX), an early marker for DSBs (Mah et al., 2010). Nevertheless, as previously reported, tumors do not readily form in the Sufu-cKO cerebellum (Yin et al., 2019), indicating either timely repair of DSBs or the induction of apoptosis in GNPs with significant genomic instability. Indeed, double-labeling with cleaved Caspase 3 (CC3) and gH2AX show a significantly higher number of double-labeled cells in EGL Regions A and B (**Supplementary Fig. 2A-2B**). Among the downstream targets of DSB repair pathways is p53, which when activated mediates cell death to suppress tumor formation (Gao et al., 2000). In the P0 Sufu-cKO cerebellum, p53 protein is present, albeit significantly reduced (**Fig. 4C**). The reduction in p53 may be driving an increase in DSBs yet still sufficient to induce apoptotic pathways. Supporting this, conditional ablation of both p53 and SUFU in GNPs (*hGFAP-Cre;Sufu$^{fl/fl}$;p53$^{fl/fl}$* or Sufu;p53-dKO) results in the formation of massive tumors in the cerebellum within 2 months after birth (**Fig. 4F**), indicating the failure to activate critical apoptotic pathways. However, tumors do not form in mice lacking p53 (Marino et al., 2000) or SUFU alone (**Fig. 4F**). These findings suggest that in the absence of SUFU, the failure of GNPs to transition into fully differentiated granule neurons compromises genomic stability and renders GNPs extremely vulnerable to tumor formation with a second molecular hit.

We sought to confirm that upregulated FGF signaling also occur in the tumor-prone Sufu;p53-dKO. As expected, the

neonatal cerebellum of Sufu-cKO, display EGL expansion due to excess proliferative (Ki67+) and Pax6+ cells in the P0 Sufu;p53-dKO cerebellum but not in p53-cKO and control cerebelli (**Fig. 4G**). Further expansion of Pax6+ GNPs in the P0 Sufu;p53-dKO is also most severe along the secondary fissure (EGL Region B) compared to other EGL areas (EGL Regions A and C) of the P0 Sufu;p53-dkO cerebellum (**Fig. 4G-4H**). As with our observations in the P0 Sufu-cKO cerebellum, ISH for FGF5 in the P0 Sufu;p53-dKO cerebellum show ectopic FGF5 expression. Particularly, FGF5+ cells are expanded anteriorly and detected specifically around the secondary fissure of the P0 Sufu;p53-dKO cerebellum (**Fig. 4I**). There is also ectopic MAPK signaling activity in the P0 Sufu;p53-dKO cerebellum, with significantly higher numbers of pErk1/2+ cells within the EGL, many of which are proliferative as marked by co-labeling with Ki-67, within the expanded EGL of the secondary fissure (**Fig. 4J**). These findings indicate that as in the P0 Sufu-cKO cerebellum, ectopic FGF5 expression triggers FGF signaling in GNPs in the P0 Sufu;p53-dKO cerebellum and may facilitate oncogenic transformation and tumor growth of GNPs.

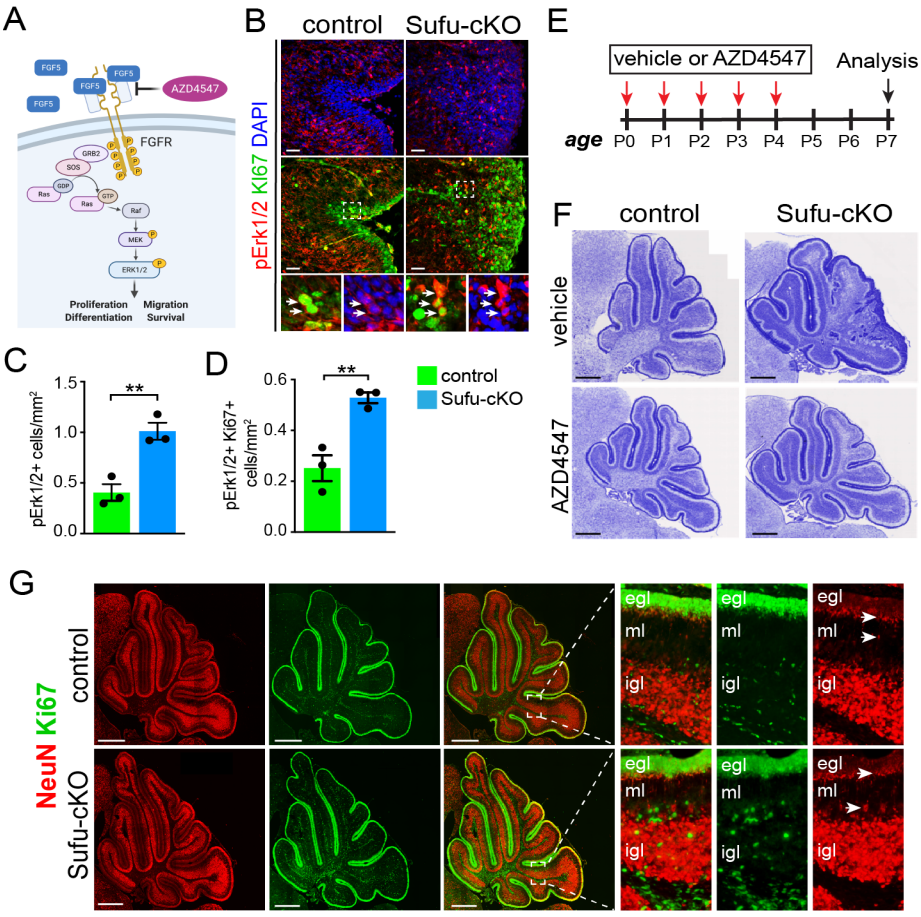

Figure 3: Ectopic activation of FGF signaling in the EGL of P0 Sufu-cKO cerebellum. (A) Schematic diagram showing the activation of FGF signaling activity, upon binding of FGF5 to extracellular domains of FGFR, via the MAPK signal transduction pathway. **(B)** Double-immunofluorescence staining with Ki67 (green) and phospho-Erk1/2 (pErk1/2; red), a marker of activated MAPK signaling in the P0 Sufu-cKO and control cerebelli. Boxed regions show pErk1/2+ and Ki67+ cells (arrowheads) in the control and Sufu-cKO EGL. Scale bars = 50 μm. **(C, D)** Quantification of pErk1/2+ cells (C) and double-labeled pErk1/2+ and Ki67+ cells (D) in the P0 Sufu-cKO and control EGL Region B. **p<0.01. **(E)** Experimental design of rescue studies performed by intraventricular administration of FGFR1-3 pharmacological inhibitor, AZD4547 or vehicle controls. **(F)** Nissl staining of the P7 control and Sufu-cKO treated with either AZD4547 or vehicle, 2 days after treatment. Scale bars = 500 μm. **(G)** NeuN and Ki67 double immunofluorescence staining of the P7 control and Sufu-cKO treated with either AZD4547 or vehicle. Boxed regions show localization and organization of NeuN+ and Ki67+ cells in distinct cerebellar layers. Arrows point to areas of the EGL and IGL where NeuN+ cells are beginning to be expressed.

Discussion

Our studies identify a mechanism by which the combinatorial effects of oncogenic *SUFU* mutations and other concurrent developmental signaling pathways, make GNPs vulnerable to oncogenic transformation leading to infantile MB^{SHH}. Using mice lacking SUFU in GNPs, we find that ectopic FGF5 expression correlates with an increase in FGF signaling, particularly in areas where proliferating GNPs reside, resulting in GNP hyperplasia, preneoplastic lesions, and patterning defects. Inhibition of FGF signaling through pharmacological blockade of FGFR1-3 prevents hyperplasia and associated cerebellar architectural abnormalities. Strongly supporting a role for FGF5, we also find elevated levels of FGF5 gene expression specifically in infantile MB^{SHH} patients, but not in other MB subgroups. These findings indicate that FGF-targeting compounds may be a promising therapeutic option for infantile MB^{SHH} patients, with elevated levels of FGF5 in tumor tissues acting as a potential biomarker.

Expansion of Pax6+ GNPs in the newborn Sufu-cKO cerebellum (**Fig. 2**) occur in similar anatomic subregions where infantile MB tumors typically arise (Tan et al., 2018). Interestingly, these subregions have low levels of Gli1 reporter activity, Ptch1 expression, and SHH ligand expression (Corrales et al., 2004). Spatially distinct regulation of granule neuron development by Sufu may therefore rely on non-canonical SHH signaling beyond Smo, or through yet undefined downstream interactions resulting in control of FGF signaling activity. Supporting this, we find ectopic expression of FGF5 and deregulation of FGF signaling as marked by the excessive intracellular activation of MAPK signaling in the P0 Sufu-cKO cerebellum. Similarly in previous studies, ectopic expression of FGF ligands, FGF8 and FGF15, are associated with degradation of Gli3R because of SUFU deletion, resulting in regional patterning and precursor specification and differentiation defects (Kim et al., 2011, 2018; Jiwani et al., 2020; Yabut et al., 2020). Taken together, these findings indicate that SUFU acts at the intersection of SHH and FGF signaling to modulate GNP behavior (as summarized in our working model in **Fig. 8**). We postulate that SUFU exerts this role via stabilization of Gli transcription factors, since we observed a decrease in Gli1, Gli2, and Gli3 protein level in the newborn Sufu-cKO cerebellum. Supporting this, Yin et al., (2019) found that reducing Gli2 levels rescued GNP hyperplasia and patterning defects in the Sufu-cKO neonatal cerebellum. Further studies are required to elucidate the involvement of Gli-dependent mechanisms in controlling FGF5 gene expression in the neonatal cerebellum.

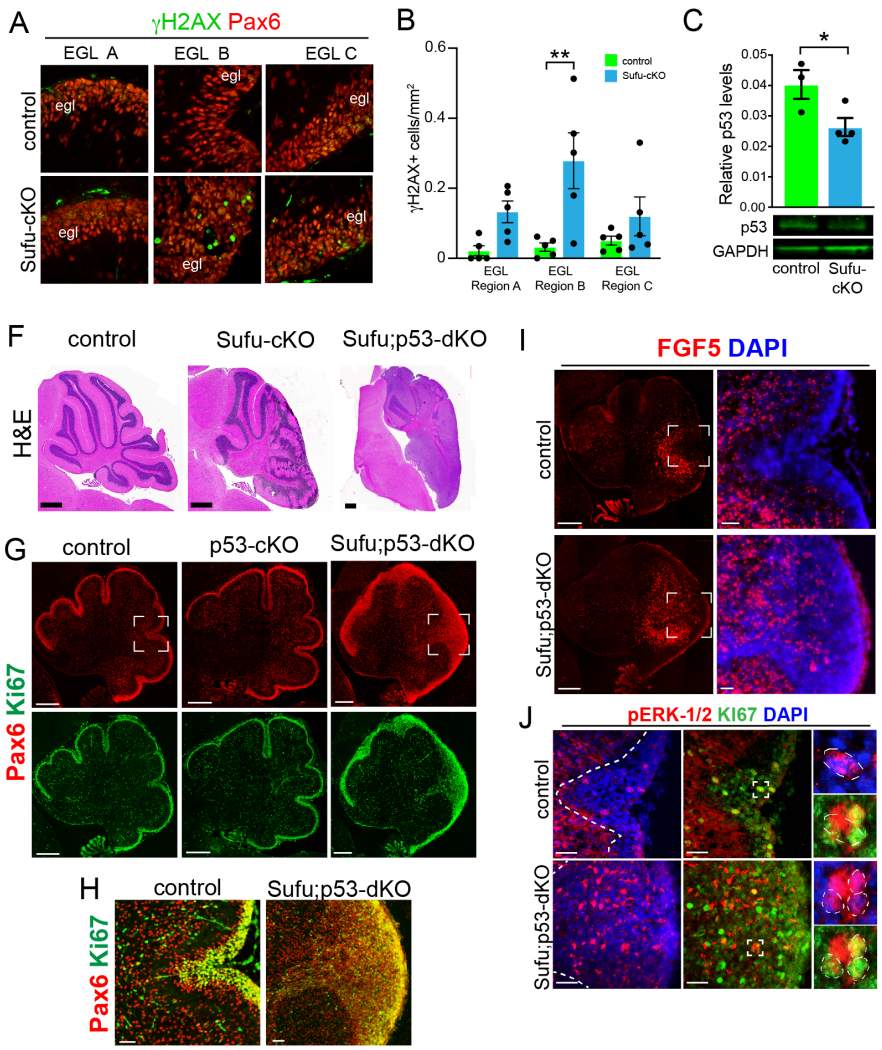

Figure 4: Evidence of pre-neoplastic lesions and high rates of cell death in Sufu-cKO granule neuron precursors. (A) Double-immunofluorescence staining with Pax6 (red) and (γH2AX (green), a marker for double-strand DNA breaks in specific EGL regions of the P0 Sufu-cKO and control cerebelli. **(B)** Quantification of (γH2AX+ cells in each cerebellar region of P0 control and Sufu-cKO mice. **p<0.01. **(C)** Western blot analysis of p53 protein levels in P0 control and Sufu-cKO cerebellar protein lysates. *p<0.05**. (F)** Hematoxylin and Eosin (H&E) staining of P60 control, Sufu-cKO, Sufu;p53-dKO cerebelli. Scale bars = 500 μm. **(G, H)** Double-immunofluorescence staining against Pax6 (red) and Ki67 (green) in the P0 control, p53-cKO, and Sufu;p53-dKO cerebellum (G). Boxed regions in G are magnified in H demonstrating the expansion of the EGL in the P0 Sufu;p53-dKO cerebellum compared to littermate controls. Scale bars = 200 μm (A) and 50 μm (B). **(I)** Fluorescent *in situ* hybridization using RNAScope probes against FGF5 mRNA (red) and DAPI labeling in the P0 Sufu;p53-dKO and control cerebellum. Boxed areas are enlarged to show ectopic localization of FGF5+ cells in the EGL of Sufu-p53-dKO cerebellum unlike in controls. Scale bars = 200 mm and 50 mm (boxed area). **(J)** Double-immunofluorescence staining with Ki67 (green) and phospho-Erk1/2 (pErk1/2; red) in the P0 Sufu;p53-dKO and control cerebelli. Boxed regions show cells double-labeled with pErk1/2+ and Ki67+ cells in the control and Sufu;p53-dKO EGL Region B. Scale bars = 25 μm.

In the wildtype cerebellum, FGF5-expressing cells localize within the IGL surrounding the secondary fissure at P0 (**Fig. 4B**) (Ozawa et al., 1996; Hattori et al., 1997; Yaguchi et al., 2009) where Gli1 reporter activity (**Fig. 4A**), and SHH and Ptch1 expression are lowest (Corrales et al., 2004). By P4, FGF5 expression is detected in cells within the IGL throughout the cerebellum (Yaguchi et al., 2009). However, by P14, a timepoint at which most granule neurons have differentiated, FGF5 is no longer detected in the IGL or other cerebellar regions per Allen Developing Mouse Brain Atlas (www.brain-map.org). The strict spatiotemporal expression of FGF5 strongly supports a stage-specific role for regulating GNP development, particularly along the secondary fissure, to ensure timely differentiation and maturation of granule neurons. Indeed, deregulation of FGF5 expression, as we observed in the P0 Sufu-cKO cerebellum, results in the inability of adjacent GNPs to normally differentiate and instead continued massive proliferation.

Our findings are contrary to previous reports of the proliferation-suppressive roles of FGFs, particularly FGF2, in GNPs specifically carrying *Ptch1* mutations (Fogarty et al., 2007; Emmenegger et al., 2013). However, in contrast to the Ptch1-cKO cerebellum, the newborn Sufu-cKO cerebellum still express Ptch1 and exhibit reduced Gli1, Gli2, and Gli3 protein levels (**Supplementary Fig. 1A**). These key molecular differences may activate unique signaling networks in Sufu-cKO GNPs. Additionally, there are 18 FGF ligands which differ significantly in molecular features and binding specificities to distinct combinations of FGFR splice variants (Ornitz and Itoh, 2015). For example, unlike FGF2 which act in an autocrine manner, FGF5 can exert both autocrine and paracrine functions, bind different combinations of FGFRs, and is dynamically expressed in distinct regions of the developing cerebellum. Thus, in-depth studies are needed to elucidate the exact mechanisms triggered by abnormally high levels of FGF5 in the developing cerebellum, particularly since FGF5 overexpression is known to drive cancer development and progression, including brain tumors (Allerstorfer et al., 2008).

The high occurrence of *SUFU* mutations in infantile MB indicate the selective vulnerability of the developing cerebellum to neoplastic effects of SUFU dysfunction. Notably, the timing of SUFU LOF is critical; conditional deletion of SUFU in neural stem cells prior to granule neuron specification (using the hGFAP-Cre line) results in GNP hyperplasia, whereas conditional deletion of SUFU after granule neuron specification (using the Math1-Cre line) does not lead to these defects (Jiwani et al., 2020). Thus, SUFU-associated infantile MB^{SHH} is a likely consequence of defects stemming from early stages of granule neuron lineage specification at embryonic stages.

Unfortunately, tumors initiated at embryonic stages are typically undetectable until several months after birth when tumorigenesis has significantly progressed. Thus, therapeutics for infantile MB must successfully curtail tumorigenic mechanisms at postnatal stages and minimally affect normal GNPs elsewhere in the cerebellum. Towards this goal, inhibiting localized FGF5 and FGF signaling activity may provide new paths toward the design of targeted treatments. Since we confirmed the occurrence of high FGF5 levels in a subset of infantile MB^{SHH} patients, FGF5 levels may be useful as a

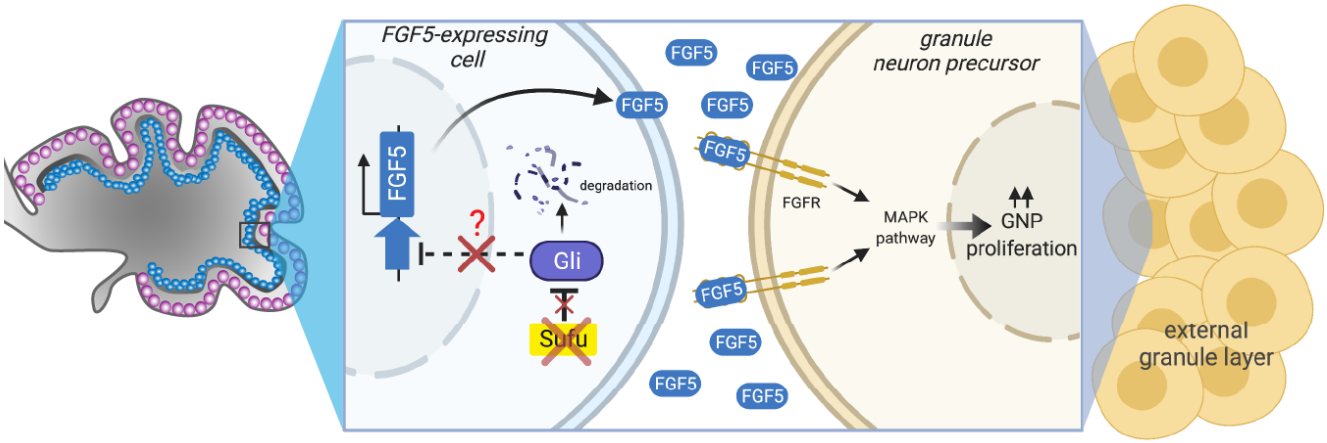

Figure 5: Loss of Sufu function drives excess proliferation of granule neuron precursors via FGF signaling activation. The schematic diagram summarizes how Sufu LOF facilitate expansion of GNPs at early stages of cerebellar development by driving FGF signaling activation via ectopic expression of FGF5.

diagnostic biomarker for this patient population. This may predict the lack of efficacy of SHH-targeting compounds in curtailing tumor growth but could instead significantly impede cerebellar development. Importantly, detection of elevated FGF5 levels may identify patients who will be responsive to FGF-targeting treatments. Ultimately, we hope these studies facilitate the design of much needed precision medicines to address the distinct oncogenic mechanisms specifically and effectively in infantile MB^{SHH} patients while enabling normal progression of cerebellar development.

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Competing interest statement

The authors declare no competing financial interest.

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Materials and Methods

Animals

Mice carrying the floxed Sufu allele (Sufu^{fl}) were kindly provided by Dr. Chi-Chung Hui (University of Toronto) and were genotyped as described elsewhere(Pospisilik et al., 2010). *hGFAP-cre* (Stock #004600) and Gli1-LacZ (Stock #008211) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice designated as controls did not carry the *Cre* transgene and may have either one of the following genotypes: *Sufu*$^{fl/+}$ or *Sufu*$^{fl/fl}$. 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).

In vivo treatment with FGFR inhibitor

The FGFR1-3 inhibitor, AZD4547 (Selleck Chemicals, #S2801) was dissolved sequentially in 4% Dimethyl Sulfoxide (DMSO), 30% Polyethylene glycol (PEG), 5% Tween-80, and water to make a 1mM solution. 1 ul of 1mM AZD4547, or vehicle only as control, was injected into the lateral ventricle (~1 mm from the cerebellar midline) of pups for 5 days from P0/P1 using a 2.5 ml syringe (Model 62 RN; Hamilton Scientific). Pups remained with the mother until perfusion at P7 for analysis.

Immunohistochemistry and LacZ Staining

Perfusion, dissection, and immunofluorescence and LacZ staining were conducted according to standard protocols as previously described(Yabut et al., 2015). Briefly, P0/P1 brain tissues were fixed after dissection by direct immersion in 4% paraformaldehyde (PFA) and P7 and older postnatal brains fixed by intracardial perfusion followed by 2 h post-

fixation. All fixed brains were cryoprotected a 15-30% sucrose gradient prior to cryosectioning. 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. A heat-induced antigen retrieval protocol was performed on selective immunohistochemistry using 10 M Citric Acid at pH 6.0. Primary antibodies were diluted in 10% serum diluted in PBS with 0.2% Triton containing 4'6-diamidino-2-phenylindole (DAPI); sections were incubated in primary antibody overnight at room temperature. The following antibodies were used: rabbit anti-Pax6 (1:250 dilution; Cat. #: 901301, Biolegend); rabbit anti-NeuN (1:250 dilution; Cat. #: PA5-784-99, Invitrogen); mouse anti-Calretinin (1:250, Cat. #: AB5054, Millipore); rabbit anti-phospho-Erk1/2 (1:250 dilution; Cat. #: 4370, Cell Signaling); γH2AX (1:100 dilution; Cat. #: 05-636, Millipore); mouse anti-Ki67 (1:100 dilution; Cat. #: 550809 BD Biosciences) and cleaved-Caspase 3 (1:250 dilution; Cat. #: 9661S, Cell Signaling). 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 (SouthernBiotech).

In Situ Hybridization RNAScope ISH was conducted for FGF15 and Ptch1. RNAscope probes for Mm-FGF5 were designed commercially by the manufacturer (Advanced Cell Diagnostics, Inc.). RNAScope Assay was performed using the RNAscope Multiplex Fluorescent Reagent Kit V2 according to manufacturer's instructions. Detection of the probe was done with Opal 570 or Opal 520 reagent (Akoya Biosciences).

Western Blot Analysis

Western blot analyses were conducted according to standard protocols. Soluble extracts were loaded onto Criterion, 4-15% Tris-HCI 4 SDS-PAGE gels (Bio-Rad), separated at 120V, and transferred to PVDF membrane at 30V for 2 hours or overnight at 4°C. Membranes were blocked with 3% milk/1X TBS-T (Tris-buffered saline with 0.1% Tween 20) or 5% BSA/1X TBS-T for 1hr at room temperature, and incubated with primary antibodies diluted in blocking buffer overnight at 4°C, and secondary antibodies (1:5000 dilution; IR-Dye antibodies, LI-COR) for 1 hour at RT. Membranes were washed in 1X TBS-T and scanned using the Odyssey Infrared Imaging System (LI-COR). Primary antibodies were used as follows: rabbit anti-Gli1 (1:1000; Abcam); goat anti-Gli2 (1:1000; R&D Systems), rabbit anti-Gli3 (1:100; Santa Cruz), rabbit anti-Pax6 (1:1000 dilution; Cat. #: 901301, Biolegend); rabbit anti-NeuN (1:1000 dilution; Cat. #: PA5-784-99, Invitrogen); rabbit anti-GABA A Receptor α6 (1:1000 dilution; Cat. #. PA5-77403, GABRA6; Invitrogen), and a-Tubulin (1:5000 dilution; Cat. #: ab4074, Abcam). Quantification and analysis were conducted using the Odyssey Image Studio Software (LI-COR). Protein levels were normalized to GAPDH protein levels. Levels of NeuN and GABRA6 were quantified in correlation with Pax6 levels (NeuN/Pax6 or GABRA6/Pax6) to determine the proportion of Pax6+ cells expressing mature granule neuron markers.

Image Analysis and Acquisition

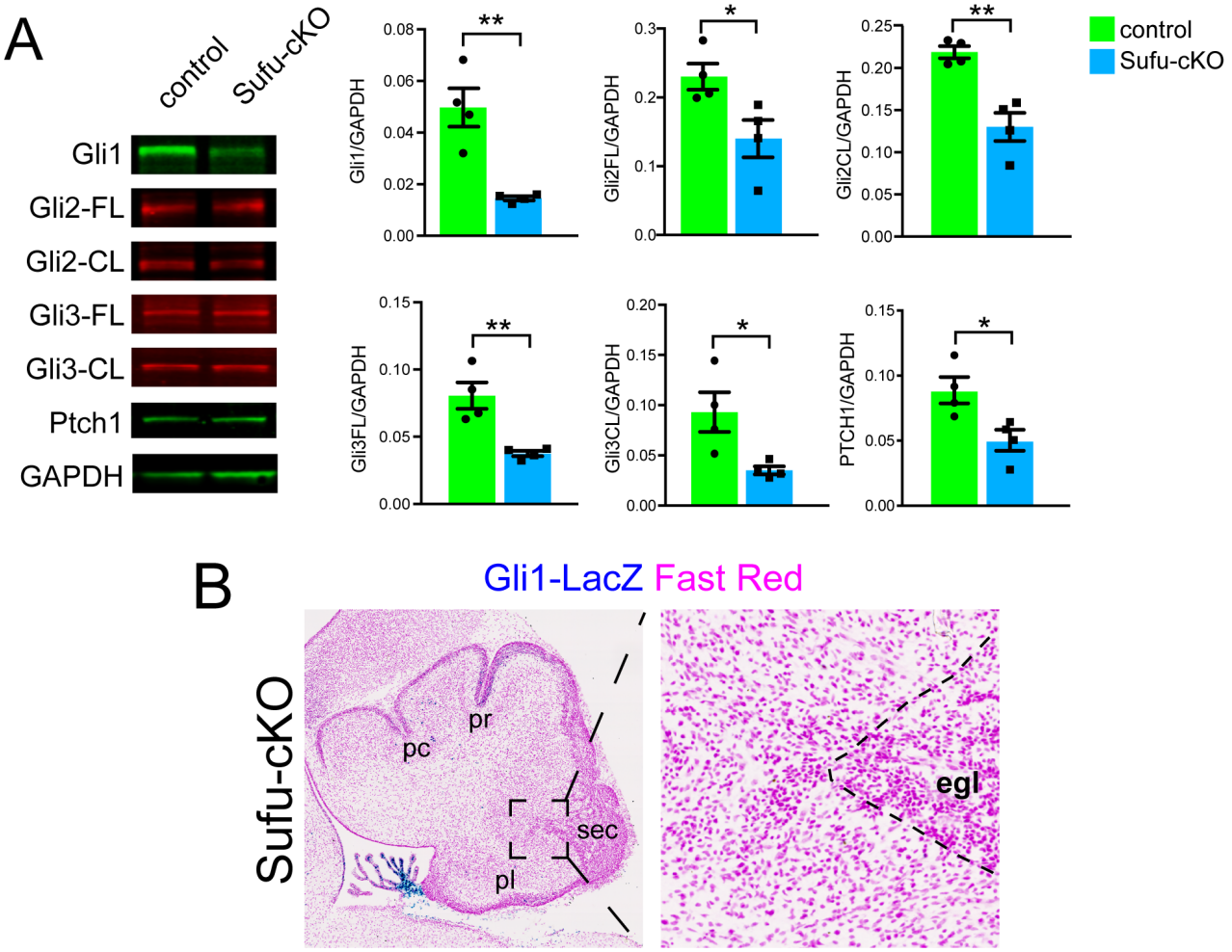
Images were acquired using a Nikon E600 microscope equipped with a QCapture Pro camera (QImaging), Zeiss Axioscan Z.1 (Zeiss, Thornwood, NY, USA) using the Zen 2 blue edition software (Zeiss, Thornwood, NY, USA), or the Nikon Ti inverted microscope with CSU-W1 large field of view confocal and Andor Zyla 4.2 sCMOS camera. All images were imported in tiff or jpeg format. Brightness, contrast, and background were adjusted equally for the entire image between controls and mutant using the "Brightness/Contrast" and "Levels" function from "Image/Adjustment" options in Adobe Photoshop or NIH ImageJ without any further modification. NIH Image J was used to threshold background levels between controls and mutant tissues to quantify fluorescence labeling. To quantify cell density, positively labeled cells within defined EGL regions, as defined in **Fig. 2A**, were counted. Measurements were obtained and averaged from 2-3 20 m thick and histologically matched sections encompassing the cerebellar midline (vermis).

Human MB Gene Expression:

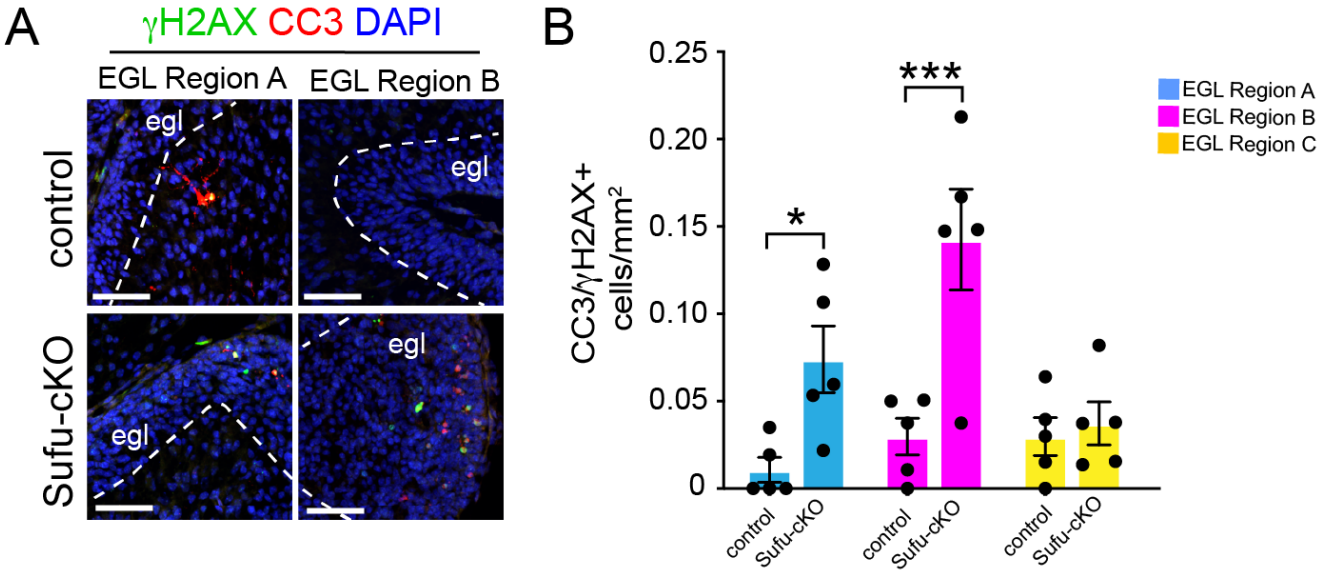
Expression values of FGF5 (ENSG00000138675) were obtained using Geo2R(Barrett et al., 2013) from published human MB subtype expression dataset GEO Accession no. GSE85217 (Cavalli et al., 2017). Analyses were conducted according to reported clinical data, subgroup and subtype classifications accompanying the dataset.

Statistics:

Prism 8.1 (GraphPad) was used for statistical analysis. Two sample experiments were analyzed by Student's *t* test and experiments with more than two parameters were analyzed by ANOVA. In 1- or 2- way ANOVA, when interactions were found, follow-up analyses were conducted for the relevant variables using Holm-Sidak's multiple comparisons test. All experiments were conducted at least in triplicate with sample sizes of n = 3−6 embryos/animals/slices per genotype. P value ≤0.05 was considered statistically significant. Graphs display the mean ± standard error of the mean (SEM).



Supplementary Figure 1: Reduced SHH signaling activity in the P0 Sufu-cKO cerebellum. **(A)** Western blot analysis of cerebellar protein lysates from P0 control and Sufu-cKO mice showing significantly lower levels of total and cleaved versions of Gli transcription factors in the P0 Sufu-cKO cerebellum. *p<0.05, **p<0.01 **(B)** b-galactosidase activity (blue), representing the Gli1-LacZ transgene, is largely absent in areas adjacent to the EGL along the secondary (sec) fissure of the P0 Sufu-cKO cerebellum.



Supplementary Figure 2: Evidence of pre-neoplastic lesions and high rates of cell death in Sufu-cKO granule neuron precursors. (A) Double-immunofluorescence staining with (γH2AX (green) and cleaved-caspase 3 (CC3; red), a marker for apoptotic cells, and DAPI labeling in regions A and B. Scale bars = 50 μm **(B)** Quantification of the density of cells labeled with CC3 and (γH2AX within each EGL regions. *$p<0.05$, ***$p<0.001$.