bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Ttc21b is required in Bergmann glia during postnatal cerebellar development

Driver A.M.[1], Shrumrick C.[1], Stottmann R.W.[1,2]#

[1]Division of Human Genetics
[2]Division of Developmental Biology
Department of Pediatrics
Cincinnati Children's Hospital Medical Center
Cincinnati, OH 45229

author for correspondence
rolf.stottmann@cchmc.org
phone: 513-636-7136
fax:513-636-4373

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Abstract

Proper cerebellar development is dependent on tightly regulated proliferation, migration, and differentiation events. Disruptions in any of these leads to a range of cerebellar phenotypes from ataxia to childhood tumors. Animal models have shown proper regulation of *sonic hedgehog* (*Shh*) signaling is crucial for normal cerebellar architecture and increased signaling leads to cerebellar tumor formation. Primary cilia are known to be required for the proper regulation of multiple developmental signaling pathways, including *Shh*. One of the genes important for primary cilia form and function, is *Tetratricopeptide Repeat Domain 21B* (*Ttc21b)*. As *Shh* is a known mitogen in the cerebellum, we hypothesized loss of *Ttc21b* may contribute medulloblastoma formation. Surprisingly, *Ttc21b* ablation in Bergmann glia did not result in tumor formation but did cause ectopic granule cells to accumulate in the lower/ posterior lobes of the cerebellum. Additionally, we found that Shh signaling was reduced in the cerebella of ablated animals rather than increased. When we ablated *Ttc21b* in the purkinje cells, the phenotype was significantly less severe. These results suggest that *Ttc21b* expression is required for Bergmann glia structure and signaling in the developing cerebellum, and in some contexts, augments, rather than attenuates, Shh signaling.

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Introduction

Development of the cerebellum includes significant postnatal growth and morphological change. In humans, disrupted cerebellar development can result in a wide variety of structural malformations including cerebellar hypoplasia, failure of proper foliation, and tumor formation (Ten Donkelaar and Lammens, 2009). *Sonic hedgehog* (*Shh*) signaling has become a well-recognized pathway involved in early cerebellar development (Dahmane and Ruiz i Altaba, 1999; Wallace and Raff, 1999). The Shh ligand, secreted from Purkinje neurons, serves as a mitogenic factor promoting granule cell proliferation and differentiation of Bergmann glia (Dahmane and Ruiz i Altaba, 1999; Wallace and Raff, 1999; Wechsler-Reya and Scott, 1999). The downstream GLI transcription factors also have dynamic expression patterns during early cerebellar development (Corrales et al., 2004).

Altered levels of *Shh* signaling has severe cerebellar developmental consequences and can also cause tumor formation. Loss of *Shh* signaling results in a reduction of granule cell proliferation and cerebellar hypoplasia (Corrales et al., 2006; Lewis et al., 2004; Spassky et al., 2008). In addition to hypoplasia, conditional ablation of *Gli2* with an *En1-Cre* led to abnormal Purkinje cell and Bergmann glia morphology (Corrales et al., 2006). Deletion of *Gli1 and Gli2* leads to further disruptions in foliation and patterning (Corrales et al., 2006). In human, impaired SHH signaling has been implicated in the cerebellar anomalies seen in both Joubert and Meckel Syndrome (Aguilar et al., 2012). Conversely, activation of SHH signaling via loss of *Patched*-1 or constitutively activated Smoothened mutations results in medulloblastoma formation (Goodrich and Scott, 1998; Hallahan et al., 2004; Han et al., 2009; Oliver et al., 2005;

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Yang et al., 2008). Interestingly, constitutive activation of Gli2 does not result in medulloblastomas, but does produce ectopic granule cells and non-medulloblastoma type 2 tumors (Han et al., 2009). While these tumors were actively proliferating, they lacked traditional medulloblastoma markers and had downregulated Gli3 (Han et al., 2009).

The primary cilium is a non-motile sensory organelle that has now been well established to be critical for proper modulation of the SHH pathway (Bangs and Anderson, 2017). Many proteins are trafficking through the primary cilium via intraflagellar transport (IFT). GLI proteins in particular require IFT through the primary cilium for proper processing into transcriptional repressor/activator forms (Liu et al., 2005; Tran et al., 2008). Loss of cilia and/or IFT leads to multiple defects affecting SHH signal transduction, including perturbation of GLI processing patterns. Consistent with this role in SHH signal transduction, loss of primary cilia causes cerebellar hypoplasia (Spassky et al., 2008) and modulates the effects of other mutations on tumor formation (Han et al., 2009).

Among the IFT proteins, *Tetratricopeptide Repeat Domain 21B* (*Ttc21b; Ift139*) is required for normal ciliary retrograde transport (Tran et al., 2008) and early forebrain patterning and development (Snedeker et al., 2017; Stottmann et al., 2009). Loss of *Ttc21b* has been shown to result in abnormal GLI3 processing, and thus increased Shh signaling (Stottmann et al., 2009; Tran et al., 2008). In humans, *TTC21B* is one of the most commonly mutated genes in ciliopathy patients, and variants are present in

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

disorders of the cerebellum such as Joubert Syndrome (Davis et al., 2011). Given the expression of *Ttc21b* in the early postnatal cerebellum, we hypothesized loss of *Ttc21b* during postnatal development may result in increased SHH signaling and cerebellar tumor formation, especially medulloblastoma. To investigate this and circumvent the perinatal lethality of *Ttc21b* mutants (Tran et al., 2008), we used the Cre-loxP system to separately ablate *Ttc21b* in Bergmann glia and Purkinje cells. Surprisingly, loss of *Ttc21b* resulted in ectopic granule cells in the posterior lobes of the cerebellum but not in tumor formation. Analysis of the affected lobes showed abnormal morphology of both Bergmann glia and Purkinje cells, as well as reduced Shh signaling. These results demonstrate a unique requirement for *Ttc21b* in cerebellar astrocytes and the first demonstration of reduced Shh signaling upon loss of *Ttc21b*.

Materials and Methods

Mouse husbandry

All animals were maintained through a protocol approved by the Cincinnati Children's Hospital Medical Center IACUC committee. Mice were housed in a vivarium with a 12-hour light cycle with food and water *ad libitum*. All mouse alleles used in this study were previously published: *Ttc21b*^{alien} is a null allele of *Ttc21b* (Tran et al., 2008), *Ttc21b*$^{tm1a(KOMP)Wtsi\text{-}lacZ}$ (*Ttc21b*^{lacZ}) for analysis of *Ttc21b* expression via the LacZ expression cassette (Tran et al., 2014), *Ttc21b*^{flox} (Snedeker et al., 2017; Tran et al., 2014); a Cre recombinase reporter allele B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$*/J* (*ROSA*$^{dTom/EGFP}$) (Muzumdar et al., 2007); FVB-Tg(GFAP-cre)$^{25Mes/J}$ (GFAP-Cre) (Zhuo et al., 2001) and B6.129-Tg(Pcp2-cre)$^{2Mpin/J}$ (Pcp2-Cre)(Barski et al., 2000). *Gli1*^{tm2AlJ}*/J* (*Gli1*^{lacZ}) mice were used to measure *Gli1*

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

expression (Bai et al., 2002). For adult analysis, 4-month-old animals were sedated and transcardial perfusion with paraformaldehyde was performed using standard procedures. Brains were subsequently harvested and fixed for 48 hours in 4% paraformaldehyde at room temperature (RT) and embedded in paraffin. For postnatal brain collections, pups were sedated with isoflurane to minimize discomfort followed by decapitation. Brains were collected and fixed for 24 hours at 4°C and embedded in O.C.T. medium (Tissue Tek) for cryosectioning or in paraffin.

Histology and Immunohistochemistry

For histological analysis, brain sections were collected on a Leica microtome at a thickness of 5μM. Sections were de-paraffinized, hematoxylin and eosin stained, and sealed using Cytoseal (Thermo Scientific). Images were obtained with a Zeiss Stereoscope (all paired images are shown at the same magnification). For DAB immunohistochemistry, sections were de-paraffinized and bleached with hydrogen peroxide. Sections were incubated overnight in primary antibody (NeuN, 1:500; Ki67 1:500) at RT. Sections were then washed in PBS and incubated for two hours in biotinylated swine-anti rabbit secondary antibody (1:500; Vector Laboratories) for 2 hours at RT. Sections were then washed in PBS and incubated in ABC solution (Vector Laboratories) for 1 hour at RT. Sections were then washed and visualized using DAB for 3 minutes followed by a PBS wash, ethanol dehydration, xylene incubation and mounted Cryoseal (Thermo). For fluorescent immunohistochemistry, sections were collected on a Leica cryostat at 12μM thickness and washed in PBS prior to antigen retrieval with sodium citrate buffer (as appropriate for each antibody). Sections were

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

then washed with PBS and incubated in blocking buffer (PBS+ 4% normal goat serum and 0.1% Triton-X) for 1 hour at RT. Sections were incubated overnight at 4°C in primary antibody diluted in blocking buffer: Calbindin 1:1000 (Abcam), GFAP 1:500 (Abcam), pHH3 1:500 (SIGMA), ARL13B 1:500 (Abcam), TTC21B 1:200(Novus Biologicals). Sections were then washed and incubated in Alexa Fluor-conjugated secondary antibodies diluted in blocking buffer (1:500, Life Technologies) followed by PBS washes and DAPI counterstain (1:1000) for 10 minutes. To visualize the GFP expressed by the *ROSA*$^{dTom/EGFP}$ Cre reporter allele, sections were only incubated for 1 hour at RT with anti-GFP antibody (1:100, Life Technologies) followed by washes and DAPI counterstain. Sections were sealed with ProLong Gold (Life Technologies) and images were obtained on a Nikon C2 confocal microscope. All paired images were taken at the same magnification.

Section in situ hybridization and lacZ staining

For RNA section *in situ* hybridization (SISH) and LacZ staining, sections were collected on a Leica cryostat at a thickness of 20µM. The in situ hybridization was done according to standard protocols. For LacZ staining sections were washed in PBS followed by fixation in cold 0.5% PFA on ice followed by PBS wash and incubation in LacZ buffer on ice. Sections were then incubated in X-Gal solution at 4°C for 1-3 days after which they were washed in PBS, counterstained in Eosin, and dehydrated. Sections were sealed with Cytoseal (Thermo) and images were taken on a Zeiss Stereoscope with all paired images taken at the same magnification.

Results

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Ttc21b is enriched in the Purkinje cell layer of the developing cerebellum

We used a *Ttc21b*^{lacZ} allele to determine the pattern of *Ttc21b* expression in the developing cerebellum. LacZ staining from P1-P13 showed expression in the Purkinje cell layer (PCL,) and the the deep cerebellar nuclei (DCN: Fig 1. A-H). In the more mature cerebellum, expression was also noted in the internal granule layer (Fig 1. G,H). The Purkinje cell layer at these stages is comprised of both Purkinje cells and the cell bodies of Bergmann glia (Yamada and Watanabe, 2002). We performed immunohistochemistry to both verify the fidelity of the lacZ reporter expression, and to further define the cell type(s) expressing *Ttc21b.* We observed TTC21B in the cytoplasm of the Purkinje cells and in puncta consistent with primary cilia, a previously demonstrated site of *Ttc21b* expression (Fig 1. I,K,M). We also saw TTC21B in Bergmann glia (BG) cells highlighted by a GFAP antibody. In Bergman glia, the only TTC21B immunoreactivity was in puncta consistent with primary cilia, and not in the cytoplasm as seen in the Purkinje cells. We confirmed this ciliary localization of TTC21B with three dimensional optical reconstructions also are consistent with a, consistent with previous results (Fig 1. O). Thus, we conclude *Ttc21b* is expressed in both cells types in the early postnatal Purkinje cell layer, with cytoplasmic and ciliary localization in Purkinje cells, but only ciliary localization in Bergmann glial cells.

GFAP-Cre ablation of Ttc21b results in ectopic granule cells in the cerebellum

Given the known roles of SHH signaling in early postnatal cerebellar development and the role of *Ttc21b* in regulating Shh signaling, we sought to determine if *Ttc21b* was required in the developing cerebellum. To circumvent the perinatal

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

lethality of *Ttc21b* null mutations (Tran et al., 2008), we used a conditional genetic approach and performed a genetic ablation using GFAP-Cre (Zhuo et al., 2001) (Fig S1. A-C). Consistent with previous reports, GFAP-Cre stimulated recombination was observed in the telencephalic hemispheres and in the cerebellum. To confirm the cell types exhibiting Cre activity, we used a Cre recombinase reporter allele, *ROSA*$^{dTom/EGFP}$, in combination with immunohistochemistry. We see significant co-localization of GFP immunoreactivity indicating Cre activity with GLAST marking immature astrocytes (Fig S1. G-I), but no such co-localization in the Calbindin-positive Purkinje cells (Fig S1. D-F).

We genetically ablated *Ttc21b* from Bergmann glia by crossing *GFAP-Cre;Ttc21b*$^{aln/wt}$ mice with *Ttc21b*$^{flox/flox}$ animals to generate *GFAP-Cre;Ttc21b*$^{flox/aln}$ mice lacking functional *Ttc21b* in the entire GFAP-expressing lineage. Histological analysis of *GFAP-Cre;Ttc21b*$^{flox/aln}$ animals at P7 showed dense areas of granule cells in the nodular region of the cerebellum which were not seen in control animals (Fig 2. A,B,arrowhead ; (White and Sillitoe, 2013). This phenotype was also observed in 4-month-old adult cerebella ($n \geq 4$). Interestingly, the ectopic cells were found in the posterior cerebellum (lobes IX and X; Fig 2. C-F). We demonstrated these cells are differentiated granule cells with immunohistochemistry for NeuN as a marker for differentiated neurons. Ki67 immunostaining for proliferating cells indicated these are not mitotically active (Fig 2G-J). Although we initially hypothesized loss of *Ttc21b* may lead to medulloblastoma formation, we conclude that there is no morphological or molecular evidence of medulloblastoma formation, or any aberrant postnatal cell

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

proliferation of any kind in any *GFAP-Cre;Ttc21b*$^{flox/aln}$ we have analyzed to date ($n \geq 8$). This suggests that the ectopic cells, while displaced, are not forming tumors.

Purkinje cell and Bergmann glial cell development are disrupted upon loss of Ttc21b

We next sought to determine why the ectopic cells may be present in the *GFAP-Cre;Ttc21b*$^{flox/aln}$ animals. We initially reasoned they may be granule cells that did not properly migrate from the site of formation in the external granule layer. This migration occurs along the radially oriented Bergmann glial fibers as part of normal granule cell development (Xu et al., 2013). To assess this hypothesis, we performed an immunohistochemical analysis of Bergmann glial cell development.

Immature Bergmann glia were specified in approximately the appropriate numbers and appear to have established radial fibers by P1 as shown by immunoreactivity for both Nestin and GLAST (Fig. 3A-D). Purkinje cells were specified and migrated to the appropriate position in the *GFAP-Cre;Ttc21b*$^{flox/aln}$ cerebella at P1 (Fig 3. E,F). However, by P11 the *GFAP-Cre;Ttc21b*$^{flox/aln}$ animals had disrupted Bergmann glia fibers in the lower lobes of the cerebellum (Fig 3. G,H) failing to form appropriate scaffolds for migration. Interestingly, anterior lobes where we did not note ectopic cells appeared to have normal Bergmann glia in the *GFAP-Cre;Ttc21b*$^{flox/aln}$ animals (Fig 3. K,L). At P11, the Purkinje cell bodies were irregularly aligned and the dendritic arbors were more extensive in the *GFAP-Cre;Ttc21b*$^{flox/aln}$ animals as compared to control (Fig 3. K,L).

SHH signaling is reduced in GFAP-Cre;Ttc21b$^{flox/aln}$ *cerebella*

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

As the primary cilium is a major hub for Shh signaling and loss of *Ttc21b* has been shown to affect SHH signaling, we then investigated SHH pathway activity in the *GFAP-Cre;Ttc21b*$^{flox/aln}$ animals with the Gli1^{lacZ} allele (Lee et al., 1997). LacZ expression was reduced in the Purkinje cell layer of *Gli1*^{lacZ}*;GFAP-Cre;Ttc21b*$^{flox/aln}$ animals (Fig 4. A-D, arrow). Since *Gli1* is not expressed by Purkinje cells, these results suggest a loss of SHH signaling activity in Bergmann glia (Corrales et al., 2004). RNA section *in situ* hybridization for another *Shh* transcriptional target, *Patched-1* (*Ptch1*) also showed reduced expression in the Purkinje cell layer of the *GFAP-Cre;Ttc21b*$^{flox/alien}$ animals as compared to controls at P11 (Fig 5. E,F,arrowhead). Expression of the *Shh* itself, however, was at similar levels between *GFAP-Cre;Ttc21b*$^{flox/aln}$ and control animals, suggesting that Purkinje cells secretion of SHH is not affected (Fig 5. G,H). Similar to the results from the Calbindin immunohistochemistry, the pattern of *Shh* expressing cells was disrupted specifically in the lower lobe of the *GFAP-Cre;Ttc21b*$^{flox/aln}$ cerebellum. Taken together, these data show that *Shh* signaling is reduced in the same tissues where we observed ectopic cells in the adult *GFAP-Cre;Ttc21b*$^{flox/aln}$ cerebellum.

Purkinje cell ablation of Ttc21b results in milder ectopic granule cell phenotype

Since we observed *Ttc21b* expression in both Purkinje cells and Bergmann glia, we performed a similar genetic ablation of *Ttc21b* in the Purkinje cells with the *Pcp2-Cre* (Barski et al., 2000) (Fig. S2). At 1 month of age, histological analysis again revealed small areas of ectopic cells in the outer layer of the *Pcp2-Cre;Ttc21b*$^{flox/aln}$ cerebella (Fig 5. A-D, arrowhead). This was also periodically seen in 4-month-old *Pcp2-*

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Cre;Ttc21b$^{flox/aln}$ as well (Fig 5. E-H, n=4). Immunohistochemistry for NeuN again suggesting these cells were differentiated granule cells which failed to migrate to the IGL (Fig 5. I,J).

Discussion

Here we have demonstrated a role for *Ttc21b* in the both the Bergmann glia astrocytes and Purkinje cells of the developing mouse cerebellum. Using a GFAP-Cre genetic ablation of *Ttc21b* we showed a few granule cells become trapped in the outer layer of cerebellum, specifically in lobes IX and X. This entrapment appears to be due to a failure of granule cell migration as the Bergmann glia, substrates for tracts for granule cell migratory, have reduced radial projections in the lower lobes of the ablated animals. In contrast, a Pcp2-Cre Purkinje cell specific ablation has a similar, but much less severe, phenotype, with only sparse areas of ectopic granule cells. Additionally, while GFAP-Cre recombination occurs in Bergmann glia, we noted affects on Purkinje cell development, supporting previous suggesting a critical interaction between these two cells types is required for proper Purkinje cell dendritic development. We also observed ablation of *Ttc21b* resulted in a reduction in SHH signaling in the Purkinje cell layer, which is the first time we have a reduction on the Shh pathway upon loss of *Ttc21b*.

The radial fibers of the Bergmann glia serve as primary migratory tracts for granule cells in the developing cerebellum (Xu et al., 2013) and disruptions of these radial fibers have been previously shown to create ectopic granule cells (Frick et al., 2012; Inouye et al., 1992). We only observed disruption of mature Bergmann glia in the affected lobes of the mutant suggesting this is a primary cause of the failed granule cell migration. Additionally, we noted abnormalities in Purkinje cell morphology in GFAP-

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Cre;*Ttc21b*$^{flox/aln}$ animals consistent with numerous interactions between Bergmann glia and Purkinje neurons (Bellamy, 2006; Lordkipanidze and Dunaevsky, 2005; Wang et al., 2012). The ectopic granule cell phenotype was much less severe in the *Pcp2-Cre; Ttc21b*$^{flox/aln}$ animals. This could suggest differing cell-specific requirements for *Ttc21b*. Indeed, patients with *TTC21B* variants have shown cytoskeletal abnormalities in human podocytes in addition to abnormal cilia suggesting multiple roles for *TTC21B* (Huynh Cong et al., 2014). Additionally, *Pcp2-Cre* recombination does occur later in development than GFAP-Cre (P5). Thus the ultimate *Ttc21b* recombination may occur after many cells have already completed migration.

Prior reports of *Ttc21b*^{alien} mutants have shown increased SHH signaling in addition to abnormal GLI processing. Embryonically, loss of *Ttc21b* led to increased GLI2 and abnormal proteolytic processing of GLI3 (Tran et al., 2008). Inappropriate SHH activity has also been demonstrated in embryonic forebrain of *Ttc21b*^{alien} mutants with abnormal GLI3 processing (Stottmann et al., 2009; Tran et al., 2008). In a postnatal deletion of *Ttc21b*, increased GLI2 activity led to polycystic kidneys (Tran et al., 2014). In contrast to these findings we report a reduction in downstream *Patched* and *Gli1*, consistent with reduced Shh signaling.

While loss of *Ttc21b* occurred throughout the cerebellum, we only observed the ectopic granule cell phenotype in the posterior lobes. While all 3 GLI transcription factors are expressed in the developing cerebellum, *Gli1* becomes restricted to the Bergmann glia in the Purkinje cell layer (Corrales et al., 2004). However, it is unlikely that the phenotype reported here is due to an effect on *Gli1* as *Gli1* homozygous null mutants have a phenotypically normal cerebellum (Park et al., 2000). Both *Gli2* and *Gli3*

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

null mice have abnormal cerebellar development, with primary defects in foliation (Blaess et al., 2008). GLI2 is enriched in the Purkinje cell layer in the posterior lobes of the developing cerebellum and has been shown to be the major effector of positive SHH signaling (Corrales et al., 2004). Additionally, loss of *Gli2* has been shown to result in abnormal Bergmann glia morphology and Purkinje cell alignment (Corrales et al., 2006). Therefore, the reduced SHH noted here may be primarily due to an effect on *Gli2* rather than Gli3.

The role of both ciliary genes and SHH signaling is a rapidly expanding field of study in human cerebellar development. SHH signaling is dysregulated in cells from Joubert syndrome and Meckel syndrome patients (Aguilar et al., 2012). Interestingly, *TTC21B* variants are associated with `Joubert syndrome patients (Romani et al., 2013). Our study shows *Ttc21b* has a role in the glial cerebellar cells and loss of *Ttc21b* in different tissues can lead to increased and decreased SHH signaling.

Figure Legends:

Figure 1: *Ttc21b* is expressed in the postnatal mouse cerebellum. (A-H) LacZ expression from the *Ttc21b*^{lacZ} reporter is evident in the Purkinje cell layer of the cerebellum from P1-P13. Significant *Ttc21b* expression is also noted in the IGL at P13 (I-O). Immunohistochemistry for Calbindin in Purkinje cells (I) shows significant overlap with TTC21B (K,M). TTC21B is seen in both the cytoplasm (asterisk) and in cilia (e.g.,

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

circled) of Purkinje cells. TTC21B co-localization is also seen with the astrocyte marker GFAP (J) where TTC21B is found in primary cilia, but not the cytoplasm (L,N, arrowheads). Three-dimensional reconstructions are consistent with a ciliary co-localization pattern (O, arrowhead). Boxes in A,C,E,G indicate areas of focus in B,D,F,H, respectively.

Figure 2: Ablation of *Ttc21b* from astrocytes results in ectopic granule cells in the cerebellum. H&E staining at P7 (A,B) and 4 months of age (C-F) show granule cells present in the outer layers of the cerebellum of *GFAP-Cre;Ttc21b*$^{fx/aln}$ animals which are not found in controls. NeuN immunoreactivity indicates these ectopic cells are differentiated granule cells (G,H, arrowheads). Ki67 immunostaining for proliferative cells is negative (I,J) suggesting these are not proliferating tumors.

Figure 3: Purkinje cells and Bergmann glia development is disrupted in the *GFAP-Cre;Ttc21b*$^{fx/aln}$ cerebella. (A-D) Immunostaining for immature astrocytes at P1 with Nestin (A,B) and GLAST (C,D) appears similar between control and *GFAP-Cre;Ttc21b*$^{fx/aln}$ animals (C-D). (E,F) Calbindin immunostaining highlights the presence of Purkinje cells at P1 in both control and *GFAP-Cre;Ttc21b*$^{fx/aln}$ animals. (G-J) P11 GFAP-positive mature astrocytes (G,H) and Calbindin-positive Purkinje cells (I-L) appear abnormal in the lower lobes of the *GFAP-Cre;Ttc21b*$^{fx/aln}$ cerebella. (K,L) Anterior lobes still show normal Bergmann glia morphology in all animals. Scale bars 50µm.

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 4: Shh signaling is reduced in *GFAP-Cre;Ttc21b$^{fx/aln}$* cerebella. (A-D) P11 *Gli1*-LacZ activity is reduced in in the Purkinje cell layer (arrows) of the *GFAP-Cre;Ttc21b$^{fx/aln}$* animals (B,D) as compared to control (A,C). (E,F) *Patched1* expression is also reduced in the *GFAP-Cre;Ttc21b$^{fx/aln}$* cerebella at P11 (F) compared to control (E), especially in the Purkinje cell layer (open arrowhead). (G,H) *Shh* expression appears similar between control (G) and *GFAP-Cre;Ttc21b$^{fx/aln}$* (H) but the organization of Shh cells again appears disorganized in the lower lobe of the cerebellum in ablated animals.

Figure 5: Ablation of *Ttc21b* with *Pcp2-Cre* results in similar, but milder, ectopic granule cell phenotype. (A,B) Small areas of displaced granule cells (arrowhead) are present in *Pcp2-Cre;Ttc21b$^{fx/aln}$* brains at 1 month of age. (B,D: boxes in A,B show areas highlighted in C, D). These cells are still visible at 4 months of age in the *Pcp2-Cre;Ttc21b$^{fx/aln}$* animals (H, arrowhead) and are immuno-positive for NeuN indicating these cells are differentiated granule cells (J, arrowhead).

Figure S1: GFAP-Cre recombination occurs in the Bergmann glia of the cerebellum. (A,B) GFAP-Cre: *ROSA$^{dTom/EGFP}$* GFP expression indicating Cre recombination appears in the cerebral cortex, hippocampus and cerebellum at P7. (D-H) Immunohistochemistry at P3 shows that the Purkinje cell marker Calbindin does not co-localize with GFP staining (marking Cre activity, D,G) while the astrocyte marker GLAST does strongly co-localize with GFP (E,H).

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure S2: Pcp2-Cre recombination occurs in the Purkinje cells of the cerebellum. (A,B) Staining in a *Pcp2-Cre*;ROSA^{lacZ} cerebellum shows strong recombination in the Purkinje cell layer of the cerebellum at P10. (C,D) *Pcp2*-Cre: *ROSA*$^{dTom/EGFP}$ GFP fluorescence shows this signal is present in the cerebellum at 1 month of age.

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

References

Aguilar, A., Meunier, A., Strehl, L., Martinovic, J., Bonniere, M., Attie-Bitach, T., Encha-Razavi, F., Spassky, N., 2012. Analysis of human samples reveals impaired SHH-dependent cerebellar development in Joubert syndrome/Meckel syndrome. Proc Natl Acad Sci U S A 109, 16951-16956.
Bai, C.B., Auerbach, W., Lee, J.S., Stephen, D., Joyner, A.L., 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 129, 4753-4761.
Bangs, F., Anderson, K.V., 2017. Primary Cilia and Mammalian Hedgehog Signaling. Cold Spring Harb Perspect Biol 9.
Barski, J.J., Dethleffsen, K., Meyer, M., 2000. Cre recombinase expression in cerebellar Purkinje cells. Genesis 28, 93-98.
Bellamy, T.C., 2006. Interactions between Purkinje neurones and Bergmann glia. Cerebellum 5, 116-126.
Blaess, S., Stephen, D., Joyner, A.L., 2008. Gli3 coordinates three-dimensional patterning and growth of the tectum and cerebellum by integrating Shh and Fgf8 signaling. Development 135, 2093-2103.
Corrales, J.D., Blaess, S., Mahoney, E.M., Joyner, A.L., 2006. The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. Development 133, 1811-1821.
Corrales, J.D., Rocco, G.L., Blaess, S., Guo, Q., Joyner, A.L., 2004. Spatial pattern of sonic hedgehog signaling through Gli genes during cerebellum development. Development 131, 5581-5590.
Dahmane, N., Ruiz i Altaba, A., 1999. Sonic hedgehog regulates the growth and patterning of the cerebellum. Development 126, 3089-3100.
Davis, E.E., Zhang, Q., Liu, Q., Diplas, B.H., Davey, L.M., Hartley, J., Stoetzel, C., Szymanska, K., Ramaswami, G., Logan, C.V., Muzny, D.M., Young, A.C., Wheeler, D.A., Cruz, P., Morgan, M., Lewis, L.R., Cherukuri, P., Maskeri, B., Hansen, N.F., Mullikin, J.C., Blakesley, R.W., Bouffard, G.G., Program, N.C.S., Gyapay, G., Rieger, S., Tonshoff, B., Kern, I., Soliman, N.A., Neuhaus, T.J., Swoboda, K.J., Kayserili, H., Gallagher, T.E., Lewis, R.A., Bergmann, C., Otto, E.A., Saunier, S., Scambler, P.J., Beales, P.L., Gleeson, J.G., Maher, E.R., Attie-Bitach, T., Dollfus, H., Johnson, C.A., Green, E.D., Gibbs, R.A., Hildebrandt, F., Pierce, E.A., Katsanis, N., 2011. TTC21B contributes both causal and modifying alleles across the ciliopathy spectrum. Nat Genet 43, 189-196.
Frick, A., Grammel, D., Schmidt, F., Poschl, J., Priller, M., Pagella, P., von Bueren, A.O., Peraud, A., Tonn, J.C., Herms, J., Rutkowski, S., Kretzschmar, H.A., Schuller, U., 2012. Proper cerebellar development requires expression of beta1-integrin in Bergmann glia, but not in granule neurons. Glia 60, 820-832.
Goodrich, L.V., Scott, M.P., 1998. Hedgehog and patched in neural development and disease. Neuron 21, 1243-1257.
Hallahan, A.R., Pritchard, J.I., Hansen, S., Benson, M., Stoeck, J., Hatton, B.A., Russell, T.L., Ellenbogen, R.G., Bernstein, I.D., Beachy, P.A., Olson, J.M., 2004. The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. Cancer Res 64, 7794-7800.

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Han, Y.G., Kim, H.J., Dlugosz, A.A., Ellison, D.W., Gilbertson, R.J., Alvarez-Buylla, A., 2009. Dual and opposing roles of primary cilia in medulloblastoma development. Nat Med 15, 1062-1065.
Huynh Cong, E., Bizet, A.A., Boyer, O., Woerner, S., Gribouval, O., Filhol, E., Arrondel, C., Thomas, S., Silbermann, F., Canaud, G., Hachicha, J., Ben Dhia, N., Peraldi, M.N., Harzallah, K., Iftene, D., Daniel, L., Willems, M., Noel, L.H., Bole-Feysot, C., Nitschke, P., Gubler, M.C., Mollet, G., Saunier, S., Antignac, C., 2014. A homozygous missense mutation in the ciliary gene TTC21B causes familial FSGS. J Am Soc Nephrol 25, 2435-2443.
Inouye, M., Hayasaka, S., Funahashi, A., Yamamura, H., 1992. Gamma-radiation produces abnormal Bergmann fibers and ectopic granule cells in mouse cerebellar cortex. J Radiat Res 33, 275-281.
Lee, J., Platt, K.A., Censullo, P., Ruiz i Altaba, A., 1997. Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development 124, 2537-2552.
Lewis, P.M., Gritli-Linde, A., Smeyne, R., Kottmann, A., McMahon, A.P., 2004. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. Dev Biol 270, 393-410.
Liu, A., Wang, B., Niswander, L.A., 2005. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. Development 132, 3103-3111.
Lordkipanidze, T., Dunaevsky, A., 2005. Purkinje cell dendrites grow in alignment with Bergmann glia. Glia 51, 229-234.
Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., Luo, L., 2007. A global double-fluorescent Cre reporter mouse. Genesis 45, 593-605.
Oliver, T.G., Read, T.A., Kessler, J.D., Mehmeti, A., Wells, J.F., Huynh, T.T., Lin, S.M., Wechsler-Reya, R.J., 2005. Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. Development 132, 2425-2439.
Park, C., Longo, C.M., Ackerman, S.L., 2000. Genetic and physical mapping of the cerebellar deficient folia (cdf) locus on mouse chromosome 6. Genomics 69, 135-138.
Romani, M., Micalizzi, A., Valente, E.M., 2013. Joubert syndrome: congenital cerebellar ataxia with the molar tooth. Lancet Neurol 12, 894-905.
Snedeker, J., Schock, E.N., Struve, J.N., Chang, C.F., Cionni, M., Tran, P.V., Brugmann, S.A., Stottmann, R.W., 2017. Unique spatiotemporal requirements for intraflagellar transport genes during forebrain development. PLoS One 12, e0173258.
Spassky, N., Han, Y.G., Aguilar, A., Strehl, L., Besse, L., Laclef, C., Ros, M.R., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 2008. Primary cilia are required for cerebellar development and Shh-dependent expansion of progenitor pool. Dev Biol 317, 246-259.
Stottmann, R.W., Tran, P.V., Turbe-Doan, A., Beier, D.R., 2009. Ttc21b is required to restrict sonic hedgehog activity in the developing mouse forebrain. Dev Biol 335, 166-178.
Ten Donkelaar, H.J., Lammens, M., 2009. Development of the human cerebellum and its disorders. Clin Perinatol 36, 513-530.
Tran, P.V., Haycraft, C.J., Besschetnova, T.Y., Turbe-Doan, A., Stottmann, R.W., Herron, B.J., Chesebro, A.L., Qiu, H., Scherz, P.J., Shah, J.V., Yoder, B.K., Beier, D.R., 2008. THM1 negatively modulates mouse sonic hedgehog signal transduction and affects retrograde intraflagellar transport in cilia. Nat Genet 40, 403-410.

bioRxiv preprint doi: https://doi.org/10.1101/181230; this version posted August 27, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Tran, P.V., Talbott, G.C., Turbe-Doan, A., Jacobs, D.T., Schonfeld, M.P., Silva, L.M., Chatterjee, A., Prysak, M., Allard, B.A., Beier, D.R., 2014. Downregulating hedgehog signaling reduces renal cystogenic potential of mouse models. J Am Soc Nephrol 25, 2201-2212.

Wallace, V.A., Raff, M.C., 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901-2909.

Wang, F., Xu, Q., Wang, W., Takano, T., Nedergaard, M., 2012. Bergmann glia modulate cerebellar Purkinje cell bistability via Ca2+-dependent K+ uptake. Proc Natl Acad Sci U S A 109, 7911-7916.

Wechsler-Reya, R.J., Scott, M.P., 1999. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron 22, 103-114.

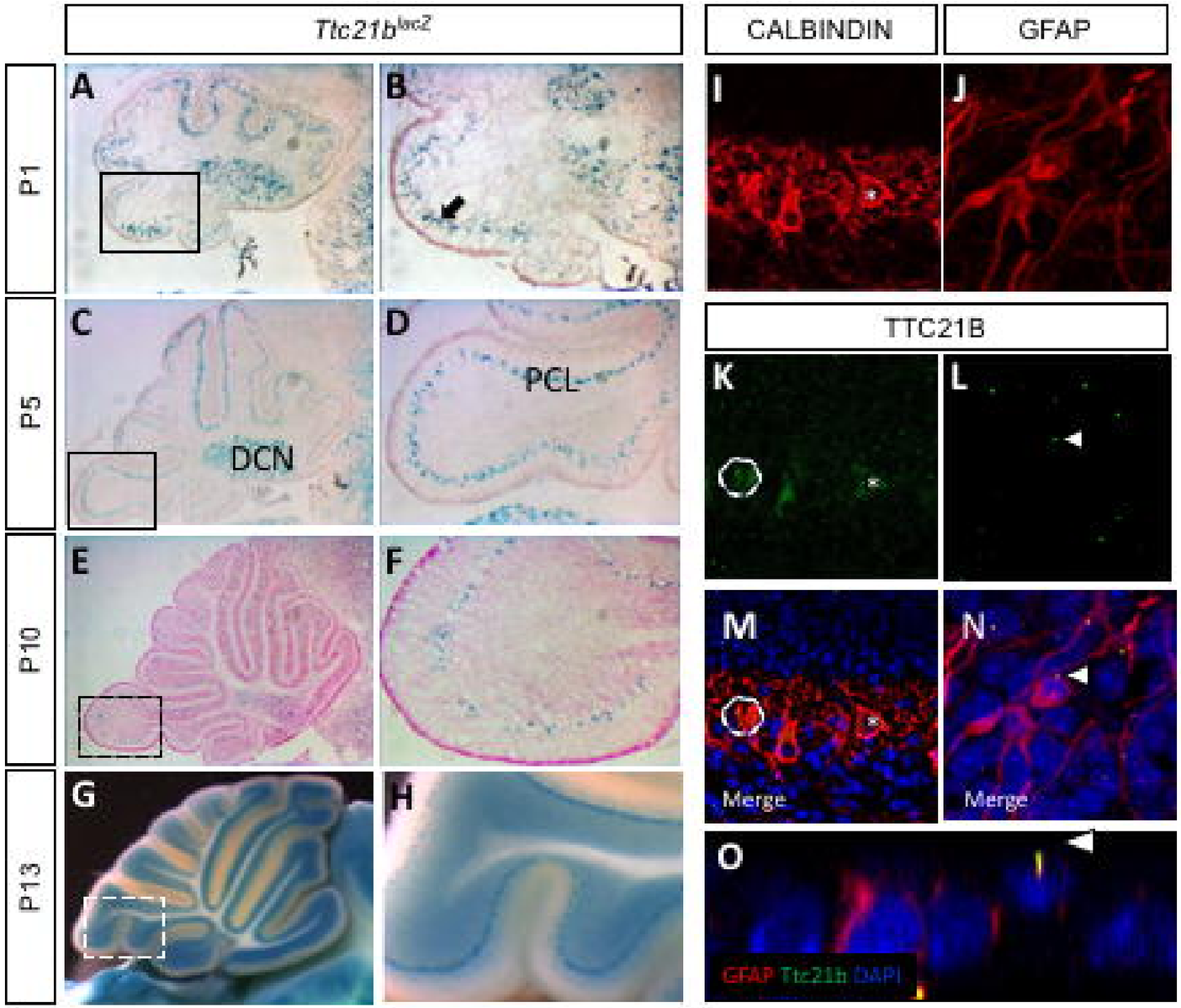
White, J.J., Sillitoe, R.V., 2013. Development of the cerebellum: from gene expression patterns to circuit maps. Wiley Interdiscip Rev Dev Biol 2, 149-164.

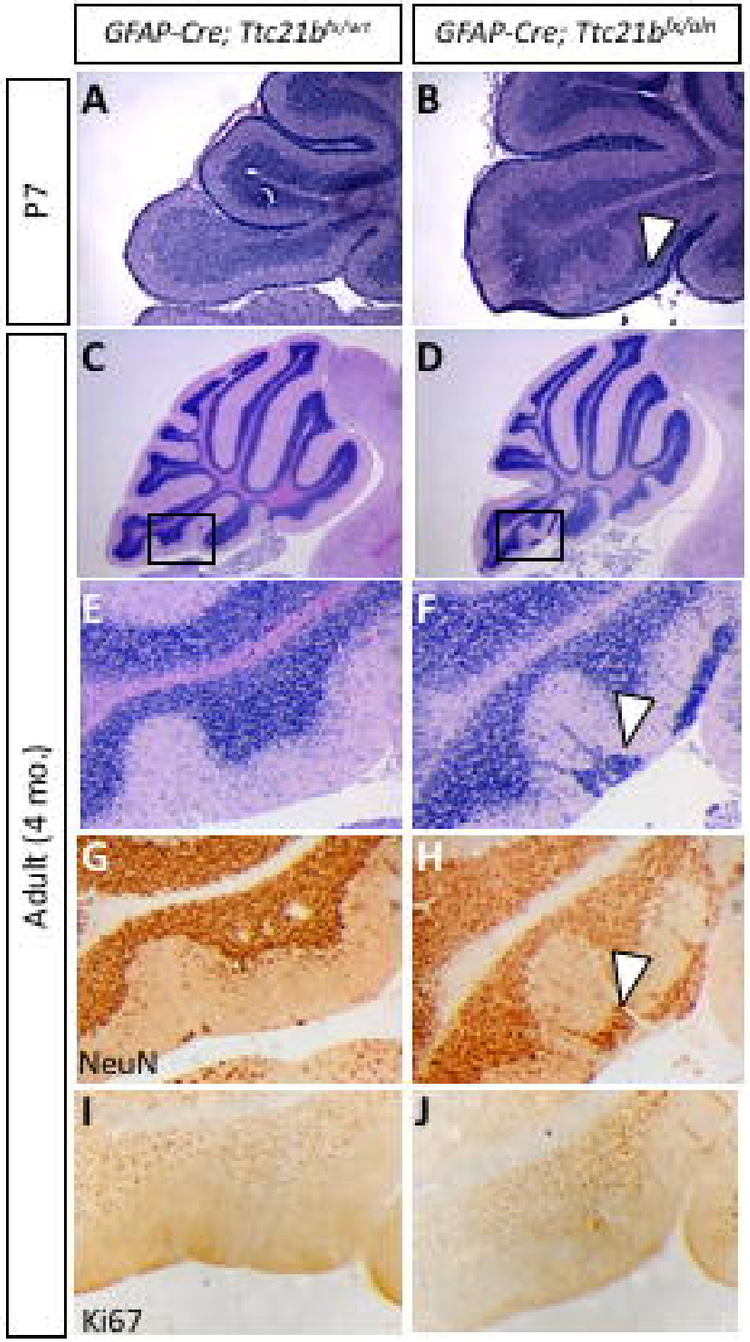
Xu, H., Yang, Y., Tang, X., Zhao, M., Liang, F., Xu, P., Hou, B., Xing, Y., Bao, X., Fan, X., 2013. Bergmann glia function in granule cell migration during cerebellum development. Mol Neurobiol 47, 833-844.

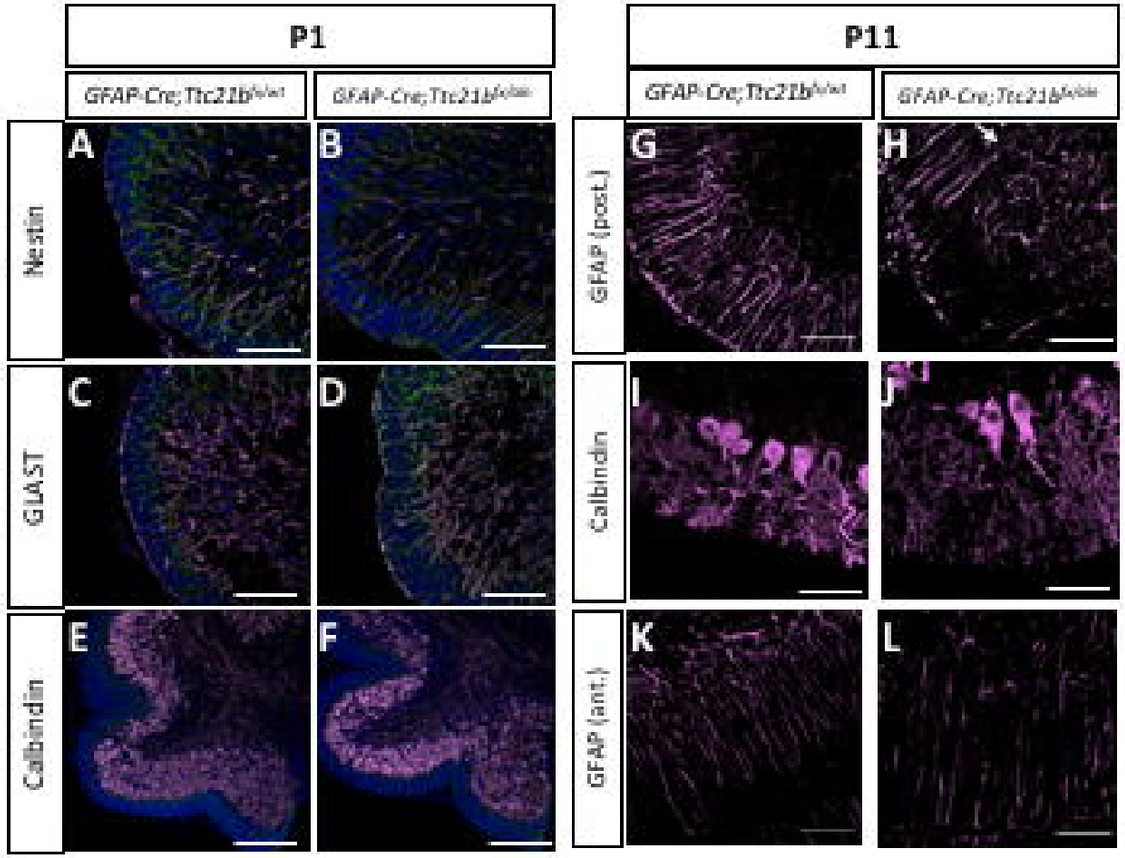
Yamada, K., Watanabe, M., 2002. Cytodifferentiation of Bergmann glia and its relationship with Purkinje cells. Anat Sci Int 77, 94-108.

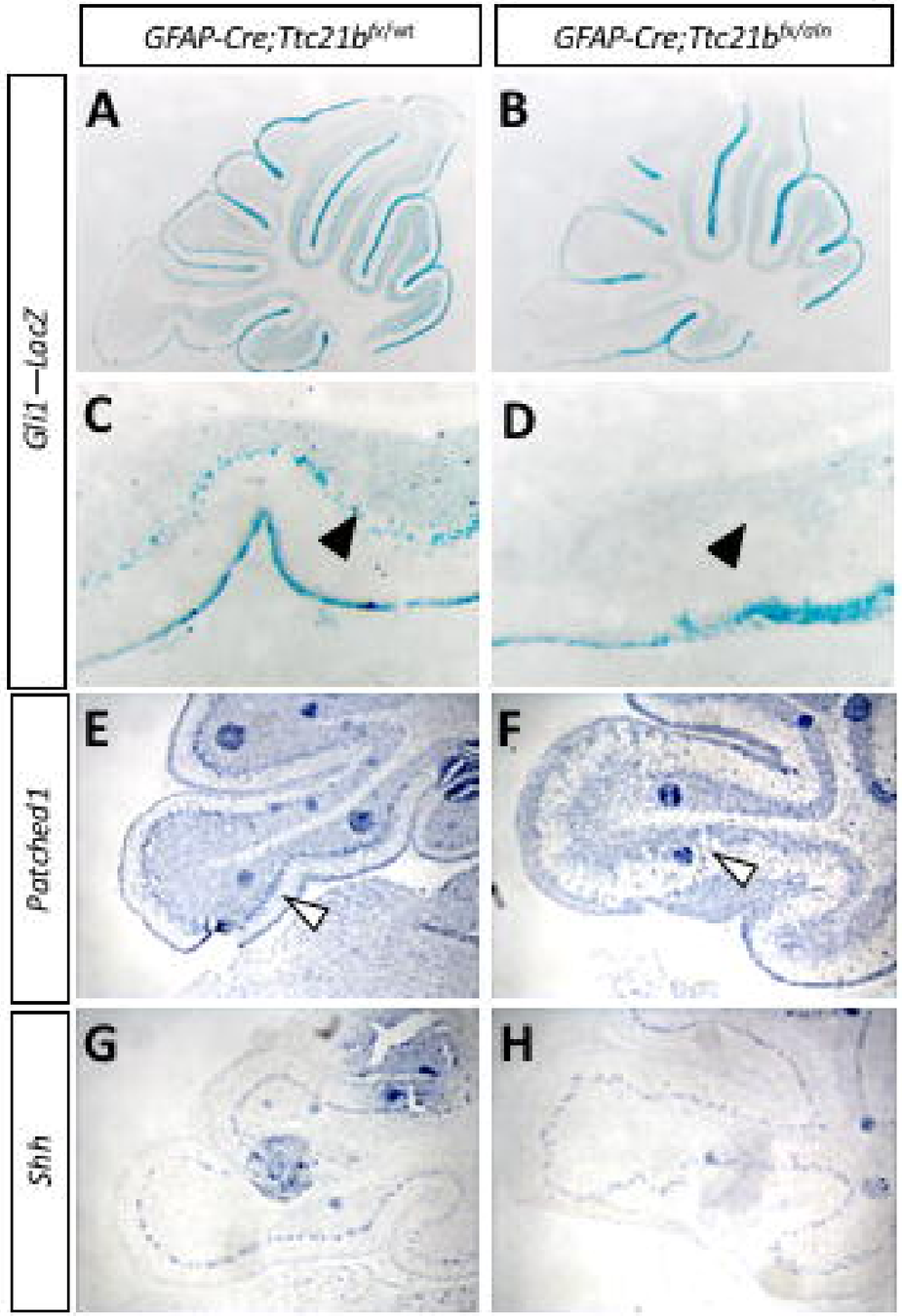
Yang, Z.J., Ellis, T., Markant, S.L., Read, T.A., Kessler, J.D., Bourboulas, M., Schuller, U., Machold, R., Fishell, G., Rowitch, D.H., Wainwright, B.J., Wechsler-Reya, R.J., 2008. Medulloblastoma can be initiated by deletion of Patched in lineage-restricted progenitors or stem cells. Cancer Cell 14, 135-145.

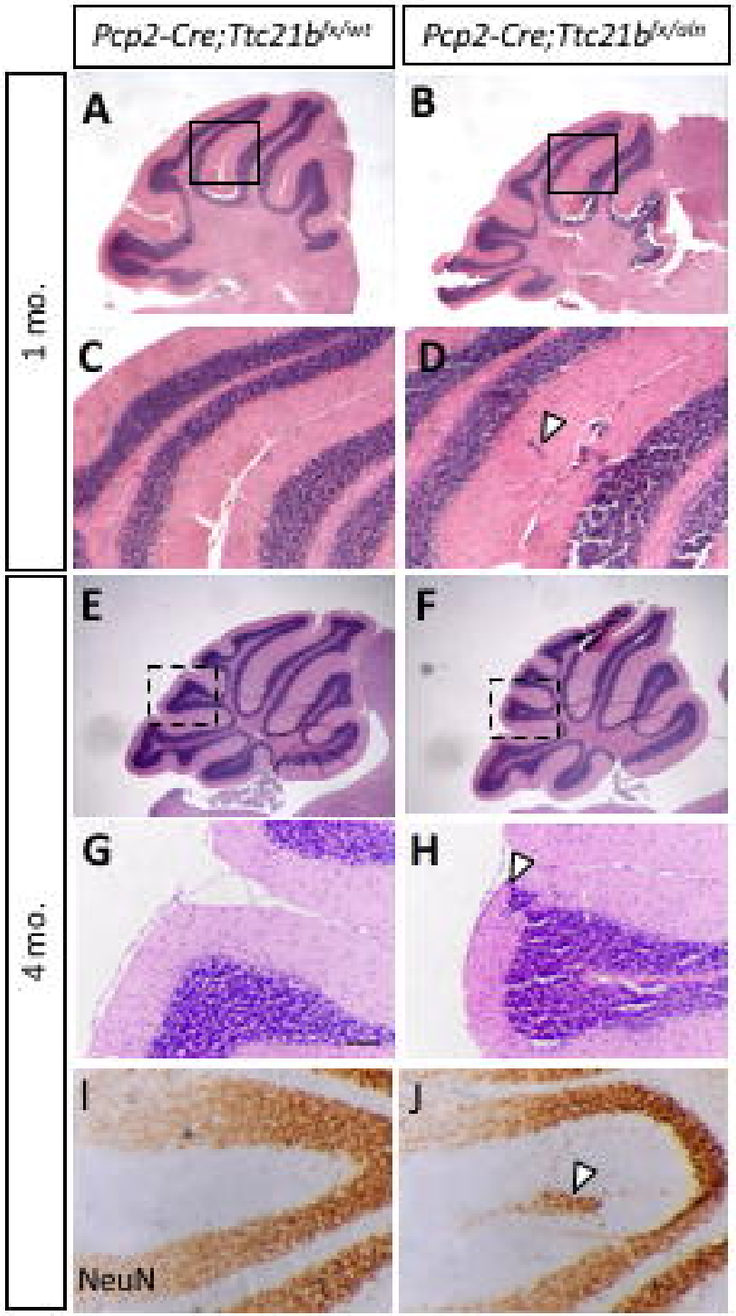
Zhuo, L., Theis, M., Alvarez-Maya, I., Brenner, M., Willecke, K., Messing, A., 2001. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. Genesis 31, 85-94.


Ttc21b^lacZ
CALBINDIN
GFAP
TTC21B
P1
P5
P10
P13
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
DCN
PCL
Merge
Merge
GFAP Ttc21b DAPI


GFAP-Cre; Ttc21b$^{lx/wt}$
GFAP-Cre; Ttc21b$^{lx/ln}$
P7
Adult (4 mo.)
A
B
C
D
E
F
G
H
I
J
NeuN
Ki67


P1
P11
GFAP-Cre;Ttc21b
GFAP-Cre;Ttc21b
Nestin
GLAST
Calbindin
GFAP (post.)
Calbindin
GFAP (ant.)
A
B
C
D
E
F
G
H
I
J
K
L


GFAP-Cre;Ttc21b fx/wt
GFAP-Cre;Ttc21b fx/gtn
Gli1 –lacZ
Patched1
Shh
A
B
C
D
E
F
G
H


Pcp2-Cre;Ttc21b/x/wt
Pcp2-Cre;Ttc21b/x/oin
1 mo.
4 mo.
A
B
C
D
E
F
G
H
I
J
NeuN