Smoothened and ARL13B are critical in mouse for superior cerebellar peduncle targeting

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Summary statement
Joubert syndrome is diagnosed by the hindbrain “molar tooth sign” malformation. Using mouse models, we show loss of the ciliary GTPase ARL13B, mutations in which lead to Joubert syndrome, result in two features of the molar tooth sign: hypoplasia of the cerebellar vermis and inappropriate targeting of the superior cerebellar peduncles. Furthermore, we demonstrate that loss of vertebrate Hedgehog signaling may be the underlying disrupted mechanism as we extend its role in axon guidance to the superior cerebellar peduncles.
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Abstract

Patients with the ciliopathy Joubert syndrome present with physical anomalies, intellectual disability, and a hindbrain malformation described as the “molar tooth sign” due to its appearance on an MRI. This radiological abnormality results from a combination of hypoplasia of the cerebellar vermis and inappropriate targeting of the white matter tracts of the superior cerebellar peduncles. ARL13B is a cilia-enriched regulatory GTPase established to regulate cell fate, cell proliferation and axon guidance through vertebrate Hedgehog signaling. In patients, mutations in ARL13B cause Joubert syndrome. In order to understand the etiology of the molar tooth sign, we used mouse models to investigate the role of ARL13B during cerebellar development. We found ARL13B regulates superior cerebellar peduncle targeting and these fiber tracts require Hedgehog signaling for proper guidance. However, in mouse the Joubert-causing R79Q mutation in ARL13B does not disrupt Hedgehog signaling nor does it impact tract targeting. We found a small cerebellar vermis in mice lacking ARL13B function but no cerebellar vermis hypoplasia in mice expressing the Joubert-causing R79Q mutation. Additionally, mice expressing a cilia-excluded variant of ARL13B that transduces Hedgehog normally, showed normal tract targeting and vermis width. Taken together, our data indicate that ARL13B is critical for control of cerebellar vermis width as well as superior cerebellar peduncle axon guidance, likely via Hedgehog signaling. Thus, our work highlights the complexity of ARL13B in molar tooth sign etiology.
Joubert Syndrome and Related Disorders (JSRD) are recessive congenital disorders with a variety of symptoms including developmental delay, intellectual disability, abnormal respiratory rhythms, ataxia, oculomotor apraxia, polydactyly, craniofacial defects, retinal dystrophy, nephronophthisis, and hepatic fibrosis (Parisi et al. 2007, Bachmann-Gagescu et al. 2020). While the exact prevalence of JSRD is not known, published statistics range from 1:80,000 to 1:100,000, but these may be underestimates as suggested by a recent study (Brancati et al. 2010, Nuovo et al. 2020). The characteristic neuroanatomical feature of JSRD is the molar tooth sign (MTS), which is caused by hypoplasia of the cerebellar vermis and thickened, elongated superior cerebellar peduncles (SCPs) that fail to decussate (Maria et al. 1997; Yachnis and Rorke 1999; Poretti et al. 2007). However, little is known about the mechanistic etiology of this hindbrain malformation. This is especially significant as several symptoms of JSRD arise from defects in the hindbrain: cerebellar dysfunction commonly causes ataxia and hypotonia, while some patients manifest life-threatening breathing problems (Parisi 2019).

To date, mutations in over 35 genes cause JSRD, and their associated proteins almost always localize to the primary cilium or the centrosome (Parisi 2019). Thus, JSRD is classified as a ciliopathy, a category of human disease stemming from ciliary dysfunction. One of the genes implicated in JSRD is ARL13B, which encodes a regulatory GTPase highly enriched in cilia (Cantagrel et al. 2008; Bachmann-Gagescu et al. 2015; Thomas et al. 2015; Shaheen et al. 2016; Rafiullah et al. 2017). As a GTPase, ARL13B is expected to have multiple effector proteins which interact with...
specific ARL13B residues. ARL13B can function as a guanine exchange factor (GEF) for ARL3, mutations in which also lead to JSRD (Gotthardt et al. 2015; Ivanova et al. 2017). JSRD-causing mutations in either ARL3 or ARL13B can disrupt their interaction or ARL13B’s GEF activity, consistent with the notion that specific ARL13B function is affected by JS-causing point mutations (Gotthardt et al. 2015; Ivanova et al. 2017; Alkanderi et al. 2018). Most JSRD-causing ARL13B mutations cluster within the protein’s GTPase domain, although two are located in the coiled coil domains in the C terminal half of the protein (Cantagrel et al. 2008; Bachmann-Gagescu et al. 2015; Thomas et al. 2015; Shaheen et al. 2016; Rafiullah et al. 2017). ARL13B complexes with the inositol phosphatase INPP5E, which is also implicated in causing JSRD (Bielas et al. 2009; Humbert et al. 2012). ARL13B is critical for targeting INPP5E to cilia and JSRD-causing ARL13B mutations disrupt INPP5E ciliary targeting (H Humbert et al. 2012). INPP5E controls ciliary lipid composition through its phosphatase activity and most JSRD-causing mutations are within its phosphatase domain (Bielas et al. 2009; Chavez et al. 2015; Garcia-Gonzalo et al. 2015). Other proteins implicated in JSRD also affect ciliary targeting with many functioning at the transition zone, supporting the notion that abnormal ciliary traffic leading to defective signaling underlies JSRD (Arts et al. 2007; Delous et al. 2007; Garcia-Gonzalo et al. 2011; Hopp et al. 2011; Srour et al. 2012; Roberson et al. 2015).

The mechanistic connection between the cilia-related proteins implicated in JSRD and the MTS are elusive in part because distinct biological processes are at play. Abnormal proliferation may underlie the hypoplastic cerebellar vermis whereas defective axonal targeting is likely involved in the abnormal SCP tracts. One signaling pathway
potentially linked to both processes is vertebrate Hedgehog (Hh) which relies on cilia
(Huangfu et al. 2003). Sonic hedgehog (Shh) is a mitogenic cue that controls
proliferation in the developing cerebellum so its misregulation could underlie the
cerebellar hypoplasia (Dahmane and Ruiz i Altaba 1999; Wechsler-Reya and Scott
1999; Kenney and Rowitch 2000). While the SCP tracts that normally project from the
deep cerebellar nuclei to the contralateral thalamus are guided by unknown signals,
Shh is a known commissural axon guidance cue (Charron et al. 2003). JSRD patients
can also display axon guidance defects in decussation of the pyramidal tracts (Yachnis
and Rorke 1999; Poretti et al. 1997).

ARL13B and INPP5E, encoding ciliary proteins linked to JSRD, are known to
regulate vertebrate Hh signaling. In mouse models, ARL13B loss disrupts cell fate
specification in the neural tube, proliferation of the cerebellar granule precursor cells in
the cerebellum and Shh-directed guidance of commissural axons in the spinal cord
(Caspary et al. 2007; Bay et al. 2018; Ferent et al. 2019). These data support a model
whereby disruption of Shh signaling by ARL13B mutation could provide a single
mechanism underlying the MTS. This model is bolstered by the fact that additional
phenotypes exhibited by JSRD patients, such as craniofacial defects or polydactyly, can
arise from aberrant Hh signaling (Valente et al. 2008; Lan and Jiang 2009; Lipinski et al.
2010).

As attractive as a Hh-based model for JSRD may be, not all the data support that
JSRD phenotypes result from misregulation of Hh signaling. Some features of JSRD,
such as the renal and liver anomalies, are not clearly due to misregulation of Hh
signaling (Doherty 2009; Breslow et al. 2018). Additionally, of over 35 genes implicated
in JSRD, only 22 play some role in Hh pathway regulation (Davey et al. 2006; Reiter and Skarnes 2006; Caspary et al. 2007; Vierkotten et al. 2007; Huang et al. 2009; Weatherbee et al. 2009; Bimonte et al. 2011; Dowdle et al. 2011; Sang et al. 2011; Chih et al. 2011; Christopher et al. 2012; Thomas et al. 2012; Abdelhamed et al. 2013; Hynes et al. 2014; Wu et al. 2014; Chavez et al. 2015; Garcia-Gonzalo et al. 2015; Asadollahi et al. 2018; Frikstad et al. 2019; Munoz-Estrada and Ferland 2019). Some of these links are tenuous. For example, mouse Arl3 mutants mislocalize the Hh transcription factor, GLI3, in their cilia yet do not exhibit any of the phenotypes normally displayed by mutants in the Hh pathway (Schrick et al. 2006, Lai et al. 2011; Schwarz et al. 2017).

Additional signaling pathways are linked to cilia including others known to be important in cell proliferation and axon guidance. Loss of either of the JSRD-linked genes Ahi1 or Cep290 in mouse leads to a small cerebellar vermis due to aberrant Wnt signaling (Lancaster et al. 2011; Ramsbottom et al. 2020). Possible JSRD-causing mutations in Znf423 are associated with defects in Wnt, BMP and retinoic acid signaling (Hata et al. 2000, Huang et al. 2009, Casoni et al. 2020, Deshpande et al. 2020). Conditional Arl13b or Inpp5e deletion in the SCPs results in their disorganization and thickening through misregulation of ciliary PI3 kinase and AKT (Guo et al. 2019).

Ciliopathies are well established to be genetically complex. JSRD patients with different ARL13B mutations can display distinct phenotypes, such as obesity in an individual expressing ARL13B\textsuperscript{Y86C} and occipital encephalocele in a patient expressing ARL13B\textsuperscript{R79Q} (Cantagrel et al. 2008; Thomas et al. 2015). This is further exemplified in cases of related individuals carrying the same mutation but exhibiting different phenotypes and even diagnoses. For example, JSRD-causing mutations in TMEM67...
(R208X) and TMEM216 (R73H) can also cause the more severe disease Meckel syndrome (Consugar et al. 2007; Otto et al. 2009; Valente et al. 2010). Understanding the genetic modifiers and environmental contribution underlying the phenotypic variation will be key to understanding disease etiology as will understanding when and how relevant pathways interact. In mouse models of the JSRD- and Meckel Syndrome-linked gene Tmem67, two phenotypic categories emerged: one with cerebellar malformations resembling JSRD and another with more severe CNS defects reminiscent of Meckel syndrome (Abdelhamed et al. 2013). The two categories correlated with whether cilia were retained, with the severe Meckel-like phenotype observed in animals lacking cilia. Furthermore, the two phenotypic groups impacted Hh and Wnt signaling differently, pointing to both pathways being critical (Abdelhamed et al. 2019). Importantly, it is not simply whether cilia are present, as another JSRD mouse model, Talpid3, lacks cilia yet displays a JSRD-like small cerebellar vermis (Bashford and Subramanian 2019). Thus, mouse models are incredibly informative yet point to the enormous complexity underlying the MTS.

Here we investigate the role of ARL13B in relation to Hh signaling in two major features of the MTS: targeting of the SCPs to the thalamus and hypoplasia of the cerebellar vermis. We explore these processes using a series of mouse alleles through which we first define the roles of Hh signaling and ARL13B in SCP projections. Subsequently, we untangle the role of ARL13B from within and outside of the cilium and investigate a JS-causing patient allele. Taken together, our data illuminate the roles of ARL13B in MTS etiology and the complexity in modeling aspects of the MTS in mouse.
MATERIALS & METHODS

Mouse lines

All mice were cared for in accordance with NIH guidelines and Emory University’s Institutional Animal Care and Use Committee (IACUC). Lines used were Nex-Cre (C3H-HeJ-Neurod6\textsuperscript{tm1(cre)Kan} [MGI:2668659], Brn4-Cre (C3H/HeJ-Tg(Pou3f4-cre)32Cren) [MGI:2158470], Smo\textsuperscript{flox} (C3H/HeJ-Smo\textsuperscript{tm2AMC}) [MGI:2176256], Arl13b\textsuperscript{flox} (C3H/HeJ-Arl13b\textsuperscript{tm1Tc}) [MGI:4948239], Arl13b\textsuperscript{V358A} (C57BL/6J-Arl13b\textsuperscript{em1Tc}) [MGI:6256969], and Arl13b\textsuperscript{R79Q} (C57BL/6J-Arl13b\textsuperscript{em2Tc}) [MGI:6279301]. Note that Arl13b\textsuperscript{D} is the deletion allele resulting from germline deletion of the conditional Arl13b\textsuperscript{flox} allele. Genotyping was as previously described (Heydemann et al. 2001; Goebbels et al. 2006; Nolan-Stevaux et al. 2009; Su et al. 2012; Gigante et al. 2020).

To generate the R79Q mutation in Arl13b, a CRISPR gRNA (ATTATTATGCTGAATCCTATGGGGTAATATTTGTT; PAM sequence is italicized) targeting exon 3 of the Arl13b locus along with a 180bp donor oligo (5'-CTCCCACTGTGGCCTTTCTAAAA-TTGATCTGAGACAAGGAAAGTTCCAAGTTACCATTTGACTTAGGAGGTGAAAAAGAATTCAGGGCATATGGGAAGAATTATTATGCTGAATCCTATGGGGTAATATTTGTT GTGGATTCCAGTGAGGGAGGAAGAATATCCTGGGGTAATATTTGTT; underlined bases are engineered) were designed to generate a G-to-A change creating the R79Q point mutation as well as A-to-C and T-to-A silent changes to create a NdeI restriction site that could be used for genotyping (Millipore Sigma). The gRNA (50 ng/ul), ssDNA oligo donor (50 ng/ul) and wild type Cas9 mRNA (10 ng/ul) were injected into 1-cell C57BL/6J zygotes and subsequently transplanted at the 2-cell stage into C57BL/6J pseudopregnant females by the Emory Transgenic and Gene Targeting Core; all
reagents were purchased from Millipore Sigma. Genomic DNA from toes was amplified via PCR using primers (5'-TCACCTTGCAACAGAGCATCC-3') and (5'-ACAGCTCTGCCCCTGTTTAC-3') located upstream and downstream of the donor oligo breakpoints; products were sequenced with the forward (first) primer. A single founder animal heterozygous for both the R79Q mutation and Ndel restriction site was identified with no additional editing. Subsequent allele-specific genotyping of progeny was performed on ear punch or yolk sac using the following primers: Fwd-wt primer: 5'-GGAGGTGGAAAAAGAATaCg-3'; Fwd-mut primer: 5'-gtctatggctgGGAGGTGGAAAAAGAATTga-3'; Rev primer: 5'-AGTGCTAAGACACCCGAGGA-3'. PCR bands at 142bp (wild type) and/or 154bp (mutant) were produced, due to the addition of 12 non-templated bases to the 5' end of the Fwd-mut primer (lowercase). Note the 3' ends of the two forward primers differed in the base that codes for the R to Q change (final nucleotide of the primer) and includes a "wobble" base (lowercase) to provide allele-specific amplification after the first round of PCR (Gaudet et al. 2009). In order to breed away any potential off-target edits, the founder was backcrossed to C57BL/6J for three generations with at least two independent meiotic opportunities for recombination in each generation.

**Tract tracing injections and analysis**

Tract tracing experiments were performed according to a protocol approved by Emory University's Institutional Animal Care and Use Committee (IACUC). Male and female mice at postnatal day 90 or older were used for tract tracing experiments. At least 3 mice of each genotypic group were analyzed in experiments (exact N included in
Figures 1-3, S1). Mice were anesthetized with inhaled isoflurane and maintained under anesthesia throughout the procedure. Animals were secured in the stereotax, and the scalp was opened with bregma and lambda aligned to flat skull position. Dorsal thalamus injections were targeted to the bregma (AP:-0.70, ML:+1.13, DV:-3.28, Angle:0°) and ventral thalamus injections were targeted using coordinates to the bregma (AP:-0.70, ML:-3.11, DV:-4.69, Angle:25°). Ventral injection targeting includes a 25° angle to avoid pulling dye through the dorsal thalamus upon needle removal. Then, a 5 ul Hamilton microsyringe was lowered to target and target was injected with lysine fixable dextran tetramethylRhodamine neuroanatomical tracer (fluoro-Ruby, 10,000 MW, ThermoFisher Scientific D1817). Animals received 0.05 - 0.5 ul injections of 10% dextran tetramethylRhodamine in sterile phosphate buffered saline (PBS, pH = 7.25) unilaterally at a rate of 0.1 ul/minute. The 0.5 ul injection volume initially used resulted in high background (non-specific) fluorescence. Later surgeries were conducted with 0.05 ul of dye, which resulted in lower background and retention of a strong signal in the DCN.

Seven days post-procedure, mice were perfused with 50mls of PBS followed by 30mls of 4% paraformaldehyde (PFA). Brains were kept in PFA overnight and subsequently placed in 30% sucrose in 0.1M phosphate buffer (pH 7.3) for cryoprotection for at least 48 hours. Brains were embedded in Tissue-Tek OCT compound (Sakura) for coronal cryostat sectioning. Sections were 60 microns thick and processed through 70% ethanol dehydration and 0.1% sudan black autofluorescence quencher, rehydrated in PBS and treated with DAPI to stain nuclei prior to fluorescence imaging. Images were taken on a Lionheart FX automated microscope (Biotek) or at 5x magnification on a Leica DM6000B microscope (Leica) using SimplePCI imaging.
software (Hamamatsu). The Leica images were subsequently stitched together to reveal
the entirety of the brain section in Fiji (Schindelin et al. 2012) or Photoshop (Adobe).
Surgical injection sites were assessed to ensure dye was present at the desired
injection site. If the injection was off-target or dye at the injection site was not seen,
samples were removed from analysis. Cerebellar images from injections were evaluated
for DCN staining with assessor blinded to genotype. The number of injections that
resulted in fluorescent DCN for each injection site (dorsal and ventral thalamus) were
compared between mutant and control genotypes using a two-sided Fisher’s exact test
(PRISM 8.2.0).

Phenotypic analysis of embryos
Timed matings of mice were performed to generate somite-matched embryos at
embryonic day 10.5 (E10.5). Embryos were dissected in cold PBS and processed for
immunofluorescence staining as previously described (Constable et al. 2020). Primary
antibodies used were: mouse anti-Shh (5E1, 1:10), mouse anti-Pax6 (PAX6, 1:100)
(Developmental Studies Hybridoma Bank), rabbit anti-Olig2 (AB9610, 1:500, Millipore
Sigma), and mouse anti-Arl13b (N295B/66, 1:1500, NeuroMab). Multiple sections from
three embryos of each genotype were examined.

Analysis of anatomical measures
Weanling age (P20-P24) male and female mice were sacrificed and brains were
harvested and fixed in 4% PFA overnight at 4°. Ten sex-matched pairs of mouse brains
were collected for each genotype; control animals were either wild type or heterozygous
for the Arl13b point mutation or floxed allele (so Arl13b^{R79Q/+}, Arl13b^{V358A/+} or Arl13b^{flox/+})
or lacked Brn4-Cre (for Arl13b^{Brn4-Cre}). Brains were imaged on a tilted stage to present a
surface view of the cerebellum, with a standard ruler in frame to confirm scale, using a
dissecting microscope (Leica MZFLIII). Measurements were made in FIJI (Schindelin et
al. 2012) with the investigator blind to genotype. Whole cerebellar width was measured
at the widest part of the cerebellum, coinciding with lobule CI or CII. Vermis width was
calculated by measuring the widest part of lobule VII (Deshpande et al. 2020).
Hemisphere and vermis heights were measured at the longest rostro-caudal point of the
hemisphere or at the midline of the vermis (see Figure 5). Body and brain weights were
measured on a standard lab scale. For each sex- and age-matched pair, the ratio of
mutant to control measures were calculated and graphed. A one-sample t-test was
performed after transforming the ratio data to a log(2) scale to normalize data
distribution (PRISM 8.2.0). Using the vermis width measures from our control brains
(mean = 2.6mm, SD = 0.18), a power calculation for a one-sided t-test estimated 90%
power to detect a 10% difference with 9-10 paired samples for a nominal alpha=0.05
(https://clincalc.com/stats/samplesize.aspx and
https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html).

Data availability

Mouse lines are available upon request. Supplemental material available at
figshare. Figure S1 contains diagrams illustrating the complete results of the injection
and tract tracing experiments. Figure S2 contains a schematic of the CRISPR strategy.
The authors affirm that all data necessary for confirming the conclusions of the article are present within the article and figures.

RESULTS

SMO is required for normal SCP projection to the dorsal thalamus

In order to test whether proper projection of SCPs requires Hh signaling, we compared the SCP tracts in mice in which we deleted the gene encoding the obligate Hh transducer, Smoothened (Smo), to control animals. As Smo null embryos die during embryogenesis, we deleted Smo specifically in the projection neurons by generating Nex-Cre;Smo<sub>fl/fl</sub> mice, which we refer to as Smo<sup>Nex-Cre</sup> (Zhang et al. 2001; Caspary et al. 2002). Nex-Cre initiates CRE recombinase expression at E11.5, as the precursor cells of the deep cerebellar nuclei (DCN) begin to migrate and become specified (Fink et al. 2006; Goebbels et al. 2006). In the mature cerebellum, the SCPs project rostrally from the DCN (illustrated in Figure 1A). After entering the midbrain, the SCPs cross the midline and again turn rostrally to project to two positions in the rostral thalamus: one tract takes a slight dorsal path and the other tract remains in the same plane; for simplicity here, we term these projection sites the dorsal and ventral thalamus, respectively (Bohne et al. 2019). To examine the SCP tracts, we used retrograde tract tracing in which we performed stereotaxic injections of a lipophilic dextran dye into either the dorsal (Figure 1B) or ventral (Figure 1C) thalamus and allowed the dye to diffuse through the axons to the associated neuron’s cell body (~7 days); we then sacrificed the animal and examined the cerebellum for evidence of the lipophilic dye indicating tracing.
We found that both dorsal and ventral thalamus injections resulted in visible clusters of dye-stained cells in the contralateral DCN, and not the ipsilateral DCN, in control animals indicating the retrograde tract tracing reliably labelled the SCPs in our hands (Figures 1D-E, S1A-B, dorsal: 6/8; ventral: 8/11). In the Smo\textsuperscript{Nex-Cre} mice, the results differed depending on whether we injected in the dorsal or ventral thalamus (Figures 1F-G, S1C-D). In the ventral thalamus injections, we detected dye-stained clusters of cells in the contralateral DCN but not the ipsilateral DCN, indicating normal SCP projection to the ventral thalamus (Figure 1G, 4/4 injections). This indicates that at least some SCPs cross the midline. In the dorsal thalamus injections, we could not detect dye-stained clusters of cells in either the contralateral or ipsilateral DCN (Figure 1F, 0/6 injections) suggesting that SCPs lacking SMO do not project to the dorsal thalamus. These data implicate SMO as critical for proper projection of the SCPs to the dorsal thalamus.

ARL13B is required for normal SCP projection to the dorsal thalamus

Given that ARL13B regulates vertebrate Hh signaling in a variety of contexts, we next assessed ARL13B’s role in proper SCP projection. In order to delete ARL13B specifically in projection neurons, we generated Nex-Cre;\textit{Arl13b}\textsuperscript{flo/flo} mice, which we refer to as \textit{Arl13b}\textsuperscript{Nex-Cre}. We performed dorsal and ventral thalamus injections for retrograde tract tracing to examine the SCPs (Figures 1H-I, S1C-D). In the ventral thalamus injections of \textit{Arl13b}\textsuperscript{Nex-Cre} mice, we found dye-stained clusters of cells in the contralateral DCN consistent with normal SCP projections crossing the midline and projecting to the ventral thalamus (Figure 1I, 4/4 injections). In contrast, in the dorsal
thalamus injections of Arl13b\textsuperscript{Nex-Cre} mice, we generally did not detect dye-stained clusters of cells in either the contralateral or ipsilateral DCN, suggesting that the SCPs lacking ARL13B do not project to the dorsal thalamus (Figure 1H, 1/7 injections). These data link ARL13B function to normal SCP projection. Furthermore, they reveal a similar phenotype in Smo\textsuperscript{Nex-Cre} and Arl13b\textsuperscript{Nex-Cre} mice.

**ARL13B does not function from within cilia to mediate SCP guidance**

ARL13B and the other 35 genes implicated in Joubert syndrome associate with the cilium or centrosome leading to the assumption that protein dysfunction from these locales underlies JSRD phenotypes (Parisi 2019). To directly ask whether ARL13B mediates SCP guidance to the dorsal thalamus from within cilia, we examined a mouse expressing a cilia-excluded variant of ARL13B, ARL13B\textsuperscript{V358A} (Figure 2) (Gigante et al. 2020). We previously demonstrated that ARL13B\textsuperscript{V358A} retains all known ARL13B biochemical activity, is undetectable in cilia yet transduces vertebrate Hh signaling normally (Mariani et al. 2016; Gigante et al. 2020). We found that either dorsal or ventral thalamus injections resulted in visible clusters of dye-stained cells in the contralateral DCN in control (Figures 2A-B, S1G-H, dorsal: 3/3; ventral: 4/4) and Arl13b\textsuperscript{V358A/V358A} (Figure 2C-D, dorsal: 4/4; ventral: 3/3) animals. In the context of the previous result showing that Arl13b\textsuperscript{Nex-Cre} mice display abnormal SCP projections to the dorsal thalamus, these data demonstrate that ARL13B does not function from within cilia to regulate SCP projections.

**SCP projection is normal in mice expressing a Joubert-causing allele, Arl13b\textsuperscript{R79Q}**
In order to understand the relationship between ARL13B and MTS formation in Joubert syndrome, we generated a mouse expressing the JSRD-causing R79Q mutation (Figure 3A). We used CRISPR/Cas9 editing to change the conserved residue in the mouse genome (Figures 3B, S2). This amino acid change disrupts ARL13B's GEF activity for ARL3 (Gotthardt et al. 2015; Ivanova et al. 2017). We found 

Arl13b<sup>R79Q/R79Q</sup> mice were viable and fertile. We bred the Arl13b<sup>R79Q</sup> allele to the null Arl13b<sup>Δ/Δ</sup> allele to make Arl13b<sup>R79Q/Δ</sup> animals, which we found survived to adulthood. As Arl13b<sup>Δ/Δ</sup> are embryonic lethal, this genetically demonstrates that Arl13b<sup>R79Q</sup> is a hypomorphic allele of ARL13B (Su et al. 2012).

To assess the role of ARL13B<sup>R79Q</sup> in SCP guidance, we performed dorsal and ventral thalamus dye injections in control and Arl13b<sup>R79Q/R79Q</sup> mice. We identified visible clusters of dye-stained cells in the contralateral DCN in control (Figures 3C-D, S1E-F, dorsal: 4/4; ventral: 3/3) and Arl13b<sup>R79Q/R79Q</sup> (Figure 3E-F, dorsal: 3/3; ventral: 3/4) animals. Thus, despite the constitutive expression of the JSRD-causing allele throughout development, we did not detect a SCP projection defect in the Arl13b<sup>R79Q/R79Q</sup> mouse model. In the context of the abnormal SCP projections to the dorsal thalamus that we identified in the Smo<sup>Nex-Cre</sup> and Arl13b<sup>Nex-Cre</sup> mice, this result indicates that the Arl13b<sup>R79Q</sup> allele does not disrupt SMO function or any ARL13B function that regulates SMO.

Arl13b<sup>R79Q/R79Q</sup> mice display normal Shh signal transduction in neural tube patterning

To further investigate the role of the Arl13b<sup>R79Q</sup> allele in Hh signaling, we examined embryonic neural patterning as it is exquisitely sensitive to alterations in Shh.
activity (Chiang et al. 1996; Briscoe and Ericson 1999). We generated E10.5 embryos and stained neural tube sections with antibodies against Shh, Olig2 and Pax6 (Figure 4). As expected in wild type embryos, we observed Shh expression in the ventral-most cells (the floorplate), Olig2 expression in lateral cells and Pax6 expression dorsally. We also saw the established abnormal cell fates in null Arl13b<sup>V/A</sup> embryos: loss of Shh staining in the floorplate (Figure 4D), dorsal and ventral expansion of Olig2 expression (Figure 4H), and a dorsal shift in Pax6 expression (Figure 4L) (Su et al. 2012). We found both Arl13b<sup>R79Q/R79Q</sup> (Figure 4B, F, J) and Arl13b<sup>R79Q/A</sup> (Figure 4C, G, K) embryos displayed neural patterning indistinguishable from wild type embryos, indicating the Arl13b<sup>R79Q</sup> allele is not dosage-sensitive and does not disrupt Shh signaling in determining neural cell fate. In addition, ARL13B<sup>R79Q</sup> protein localized to cilia (Figure 4N), consistent with previous results (Li et al. 2010; Humbert et al. 2012; Li et al. 2016; Mariani et al. 2016).

Arl13b<sup>R79Q/R79Q</sup> mice display normal cerebellar width

The lack of a SCP projection phenotype in the Arl13b<sup>R79Q/R79Q</sup> mice surprised us since JS patients display the MTS. In addition to defects in the SCPs, the MTS is due to an underdeveloped cerebellar vermis, so we examined the width of the cerebellum and the cerebellar vermis (Figure 5) (Aguilar et al. 2012). To quantify cerebellar width, we performed analysis of surface-facing anatomical measurements validated to be sufficiently sensitive to detect small differences in vermis width (Deshpande et al. 2020). Briefly, we measured cerebellar width as well as cerebellar vermis width (widest part of lobule VII) of fixed whole mount dissected brains. For each sex- and age-matched pair,
we calculated the ratio of the width measurements from mutant to control, which we compared to a hypothetical value of 1 (indicating no difference between groups). We detected no differences in the overall cerebellar width or the cerebellar vermis width between control and $\text{Arl13b}^{R79Q/R79Q}$ mice of either sex (Figure 5B). Furthermore, we found no difference in body or brain weight for $\text{Arl13b}^{R79Q/R79Q}$ mutants compared to controls (Figure 5B). Thus, unlike patients carrying $\text{ARL13B}^{R79Q/R79Q}$, $\text{Arl13b}^{R79Q/R79Q}$ mice do not display a detectable growth deficit in the cerebellar vermis.

**Global cerebellar hypoplasia is observed in mice lacking Ar13b in all neurons**

Cerebellar size is well established to be regulated, in part, via Shh signaling which controls proliferation of the cerebellar granule precursor cells (Kenney and Rowitch 2000; Chizhikov et al. 2007). In order to better understand the vermis hypoplasia phenotype seen in JBTS patients in relation to ARL13B, we wanted to investigate how ARL13B regulates cerebellar width. To do so, we crossed the $\text{Brn4-Cre}$ allele into the conditional null $\text{Arl13b}^{fl/fl}$ background, called $\text{Arl13b}^{Brn4-Cre}$ (Figure 6). $\text{Brn4-Cre}$ initiates expression at E8.5 throughout the neuroectoderm so the cerebellum develops in the absence of ARL13B (Heydemann et al. 2001; Hazen et al. 2012). We again calculated width ratios using surface-facing anatomical measurements and found the overall width of the cerebellum was 6% reduced in both females and males lacking ARL13B compared to control littermates at weaning (Figure 6B, $p<0.1$). More striking, in the cerebellar vermis we detected a 27% reduction in width in female and a 33% reduction in male mutants compared to controls (Figure 6B, $p<0.0001$). $\text{Arl13b}^{Brn4-Cre}$ mice develop hydrocephaly just after weaning which often leads to death. This is
reflected in the body and brain weight ratios: while the mutant mice had slightly lower
body weights, they had comparatively heavier brains (Figure 6B). From these data, we
conclude that loss of ARL13B leads to a modest global cerebellar width deficit and a
more pronounced cerebellar vermis width reduction.

Arl13b<sup>V358A/V358A</sup> mice display normal cerebellar width

JS is classified as a ciliopathy due to the majority of causative genes encoding
proteins that, like ARL13B, are associated with cilia. In order to better understand the
role of ciliary ARL13B in cerebellar size, we examined cerebellar width in the mice
expressing the cilia-excluded variant ARL13B<sup>V358A</sup> (Figure 7) (Gigante et al. 2020). We
detected no difference in the overall cerebellar width or that of the cerebellar vermis
between control and Arl13b<sup>V358A/V358A</sup> mice (Figure 7B). Thus, ARL13B does not control
cerebellar width from within cilia.

DISCUSSION

Here we demonstrate that complete loss of Arl13b function in mouse can account
for two aspects of the MTS: aberrant SCP thalamic targeting and cerebellar vermis
hypoplasia. We expand the role of Hh signaling as a critical guidance cue by showing it
is required for proper SCP projection to the dorsal thalamus. Our finding that the SCP
phenotype is similar in Smo<sup>Nex-Cre</sup> and Arl13b<sup>Nex-Cre</sup> mice is consistent with a model
whereby ARL13B regulates SCP projections to the dorsal thalamus via a SMO-
dependent mechanism. In line with previous work showing that ARL13B does not
function from within cilia to regulate Shh-guided axon guidance, we found normal SCP
thalamic targeting in mice expressing only a cilia-excluded ARL13B variant (Ferent et al. 2019). By mutating a conserved arginine to glutamine, we generated a mouse expressing a mutation linked to JSRD in humans and observed no change in vertebrate Hh signaling (Cantagrel et al. 2008). Additionally, we identified no defects in 

\[ \text{Arl}13b^{R79Q/R79Q} \] SCP projections. Whereas complete ARL13B deletion (\(\text{Arl}13b^{Bm4-Cre}\)) in the cerebellum led to global hypoplasia, we show the cerebellum of \(\text{Arl}13b^{R79Q/R79Q}\) mice is not significantly different in width compared to controls.

Overall, our data indicate ARL13B function is critical for both SCP targeting and controlling cerebellar vermis width. At one level, our data implicate Hh signaling in the etiology of the MTS since we show that SCP targeting requires SMO. However, at another level, our data indicate that Hh-independent pathways are at play as we don’t observe Hh-dependent neural tube patterning defects in the presence of the JSRD-causing \(\text{Arl}13b^{R79Q}\) allele. In other mouse models of JSRD where the hypoplasia is specific to the cerebellar vermis, Wnt signaling is affected (Lancaster et al. 2011). Thus, the MTS could be due to disruption of different pathways in the SCPs and the vermis. This would imply that the 35 JSRD implicated genes all affect the distinct pathways in a similar manner (Parisi 2019). Alternatively, the MTS may form due to alterations in any of a few pathways – and it is even possible that alterations in one pathway could impact other pathways – or the ability of cells to respond to those other pathways. Such a model is hinted at by previous work showing interplay between the Hh and Wnt pathways underlying the severity of hindbrain phenotypes (Hagemann and Scholpp 2012; Bashford and Subramanian 2019). While we haven’t detected any changes in Wnt response in the absence of ARL13B function, we may have not examined the
relevant biological process or used a sensitive enough readout (Horner and Caspary 2011). Parallel reasoning would thus suggest that while *Arl13b*<sup>R79Q/R79Q</sup> mice clearly transduce Hh reasonably well, there may be subtle changes in Shh signaling or changes that influence Wnt signaling. Our data are consistent with the complexity exhibited by other JSRD mouse models examined to date (Delous *et al.* 2007; Garcia-Gonzalo *et al.* 2011; Roberson *et al.* 2015; Bashford and Subramanian 2019).

In patients, the SCP targeting deficit appears more severe than what we observed in the mice. Available methods for live-imaging and examination of post-mortem tissues suggest a range of SCP decussation defects, with some tracts appearing thickened on the ipsilateral side relative to their DCN (Yachnis and Rorke 1999; Poretti *et al.* 2007). Surprisingly, in the mutant mice we infer midline crossing of the SCPs. In the case of the *Smo*<sup>Nex-Cre</sup> conditional mice, it is formally possible that the SCPs do not rely on SMO for midline crossing but only for subsequent targeting to the dorsal thalamus. The fact that the *Arl13b*<sup>Nex-Cre</sup> conditional mice display a highly similar phenotype to the *Smo*<sup>Nex-Cre</sup> conditional mice makes this less likely, since ARL13B is directly implicated in JSRD and regulates SMO-dependent axon guidance in other contexts (Cantagrel *et al.* 2008; Ferent *et al.* 2019). It is also plausible that the protein turnover driven by *Nex-Cre* completed after midline crossing occurred. *Nex-Cre* expression initiates at E11.5 in the cells on the rhombic lip of the cerebellar anlage as they start to migrate and be specified before occupying the deep cerebellar nuclei (Fink *et al.* 2006; Goebbelts *et al.* 2006). We expect deletion would occur in the precursors and therefore the neurons of the DCN would not express protein. Finally, it is possible that the mouse is not a valid system in which to model the SCP midline crossing defect.
This might explain why we saw no defects in the SCP targeting of the \textit{Arl13b}^{R79Q/R79Q} mice, as this is a constitutive mutation that requires no protein turnover, yet homozygous expression of \textit{ARL13B}^{R79Q} in patients results in the molar tooth sign, which has been associated with failed decussation of white matter tracts (Quisling \textit{et al.} 1999). Indeed, other mouse mutants such as \textit{Cep290} and \textit{Ahi1} which recapitulate the cerebellar vermis hypoplasia, also do not display midline crossing defects in the SCPs (Lancaster \textit{et al.} 2011). Whether this is due to anatomical distinctions between the cerebellum in mouse and human or the genetic background on which these models were examined are open questions. Recent work highlights clear molecular and temporal differences between mouse and human cerebellar development (Haldipur \textit{et al.} 2019; Behesti \textit{et al.} 2021).

Examining SCP projections is labor intensive and it has not been done systematically among the JSRD mouse models (Bashford and Subramanian 2019; Guo \textit{et al.} 2019). While previous work showed that \textit{Arl13b}^{Nex-Cre} and \textit{Inpp5e}^{Nex-Cre} mice exhibit SCP targeting deficits, here we pinpoint the \textit{Arl13b}^{Nex-Cre} defect as specific to the projection to the dorsal thalamus (Guo \textit{et al.} 2019). The projection to the ventral thalamus remains intact, suggesting there is not a generalized deficit in axon outgrowth within the tract. The work on the \textit{Arl13b}^{Nex-Cre} and \textit{Inpp5e}^{Nex-Cre} SCP targeting deficits argue that PI3K/Akt signaling from within cilia led to the tract defects (Guo \textit{et al.} 2019). However, we found that cilia-excluded \textit{ARL13B} mediated SCP targeting normally. These conflicting results could be explained by differences in the experimental details as the data supporting ciliary \textit{ARL13B} function used a human \textit{ARL13B} viral expression construct to rescue conditionally-deleted mice whereas we used genetic mutations.
engineered at the endogenous locus in this study. Alternatively, these data could indicate that ARL13B plays an important cellular role in the ciliary trafficking of key components needed for the PI3K/Akt pathways.

JSRD-causing mutations in ARL13B are generally restricted to the GTPase domain of the protein, although two residues outside that domain are implicated in disease (Cantagrel et al. 2008; Bachmann-Gagescu et al. 2015; Thomas et al. 2015; Shaheen et al. 2016; Rafiullah et al. 2017). Based on other ARL proteins, ARL13B likely assumes distinct conformations upon the binding either GDP or GTP, permitting different binding partners or altering affinities for binding partners (Pasqualato et al. 2002; Miertzschke et al. 2014). None of the tested JSRD-causing mutations (R79Q, Y86C or R200C) disrupt GTP binding or hydrolysis, however, all three mutations disrupt ARL13B function as an ARL3 GEF (Ivanova et al. 2017). The arginine at this position is located within the Switch 2 region of ARL13B and is conserved in humans, mice, zebrafish and Chlamydomonas (Figure 3B). We were surprised that we were unable to detect any defects in our mouse, as we expected to model some aspect of Joubert syndrome. Humans with homozygous R79Q mutation exhibit motor and ocular defects (among other symptoms), whereas arl13b-null zebrafish injected with human ARL13B-R79Q mRNA only partially rescue the ciliopathy phenotypes of curved body axis and cystic kidney (Cantagrel et al. 2008). These species-dependent differences emphasize the importance of analyzing mutations expressed from the endogenous promoter over the course of normal development. A recent study linked arl13b disruption during zebrafish development to reduced granule and Purkinje cells through a down-regulation of Wnt signaling (Zhu et al. 2020). Given that complete deletion of ARL13B impacts
broader biological processes in the cerebellum than the R79Q mutation and that the null
mutant misregulates Hh signaling whereas R79Q does not, we conclude that a subset
of ARL13B function is disrupted in JSRD.

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Conflicts of Interest
The authors have no competing interests to declare.

**Author contributions statement**


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*Figure legends*
**Figure 1: SCPs lacking Arl13b or Smo fail to project to the dorsal thalamus.** (A-C)

Schematics of injections and fluorescent tracer diffusion shown horizontally (A) or sagittally (B-C). (A) Red dashed arrow depicts dye path in a successful injection from injection site (red X) caudal through the brain and across the midline and into the contralateral cerebellar DCN (red arrowhead). Grey background boxes indicate area of subsequent images: the injection site (INJ) and cerebellum (DCN). (D-I) Representative images of dorsal (D, F, H) or ventral (E, G, I) thalamus injection site (top panel) and cerebellum (middle panel) with the DCN in hatched white circle and magnified (bottom panel) with recoloring to black and white to aid visualization. The retrograde fluorescent tracer is pink-red and sections are stained with DAPI (blue). Numbers indicate the number of positively stained DCN clusters (DCN traced) out of the total number of injected animals. Note that no tracing was observed on ipsilateral side to injection. (D, E) Fluorescent tracer injection in Smo^{fl/+}; Arl13b^{fl/+}; Nex-Cre control animals resulted in contralateral DCN staining in (D) 6/8 dorsal thalamus injections and (E) 8/11 ventral thalamus injections. (F, G) Fluorescent tracer injection in Smo^{Nex-Cre} animals resulted in contralateral DCN staining in (F) 0/6 dorsal thalamus injections and (G) 4/4 ventral thalamus injections. (H, I) Fluorescent tracer injection in Arl13b^{Nex-Cre} animals resulted in contralateral DCN staining in (H) 1/7 dorsal thalamus injections and (I) 4/4 ventral thalamus injections. Two-tailed Fisher’s exact test was performed; only the dorsal injection results were significantly different from the controls (F: p<0.01; H: p<0.05)

**Figure 2: SCPs expressing cilia-excluded ARL13B^{V358A} project normally to both the dorsal and ventral thalamus.** (A-D) Representative images of dorsal (A, C) or
ventral (B, D) thalamus injection site (top panel) and cerebellum (middle panel) with the
DCN in hatched white circle and magnified (bottom panel) with recoloring to black and
white to aid visualization. Numbers indicate the number of positively stained DCN
clusters out of the total number of injected animals. Note that no tracing was observed
on the injection’s ipsilateral side. (A, B) Fluorescent tracer injection in Arl13b^{V358A/+}
control animals resulted in contralateral DCN staining in (A) 3/3 dorsal thalamus
injections and (B) 4/4 ventral thalamus injections. (C-D) Fluorescent tracer injection in
Arl13b^{V358A/V358A} animals resulted in contralateral DCN staining in (C) 4/4 dorsal
thalamus injections (Fisher’s exact test, two-tailed, ns, p>0.9) and (D) 3/3 ventral
thalamus injections (ns, p>0.9).

Figure 3: SCPs expressing JS allele Arl13b^{R79Q} project normally to both the dorsal
and ventral thalamus. (A) Schematic showing the protein domain structure of Arl13b.
The R79Q mutation occurs in a highly conserved subregion of the GTPase domain,
Switch 2 (red). (B) Alignment of protein sequence surrounding amino acid 79 of mouse
Arl13b (red highlight) showing that arginine is conserved across multiple species.
Sequences used in protein alignment: Homo sapiens NP_878899.1, Mus musculus
NP_080853.3, Danio rerio NP_775379.1, Chlamydomonas reinhardtii XP_001691430.1.
(C-F) Representative images of dorsal (C, E) or ventral (D, F) thalamus injection site
(top panel) and cerebellum (middle panel) with the DCN in hatched white circle and
magnified (bottom panel) with recoloring to black and white to aid visualization.
Numbers indicate the number of positively stained DCN clusters out of the total number
of injected animals. Note that no tracing was observed on the injection’s ipsilateral side.
Fluorescent tracer injection in Arl13bR79Q/+ control animals resulted in contralateral DCN staining in (C) 4/4 dorsal thalamus injections and (D) 3/3 ventral thalamus injections. (E-F) Fluorescent tracer injection in Arl13bR79Q/R79Q animals resulted in contralateral DCN staining in (E) 3/3 dorsal thalamus injections (Fisher’s exact test, two-tailed, ns, p>0.9) and (F) 3/4 ventral thalamus injections (ns, p>0.9).

**Figure 4:** Mouse embryos expressing JS allele Arl13bR79Q display normal cell patterning in the neural tube. Shh, Olig2, Pax6 and Arl13b staining of E10.5 Arl13b+/+(n=3), Arl13bR79Q/R79Q (n=3), Arl13bR79Q/Δ (n=3), and Arl13bΔ/Δ (n=3) mouse neural tubes. Scale bar = 100 micrometers in A-L or 10 micrometers in M-P. (A-D) Shh is visible in the notochord and floorplate of (A) Arl13b+/+, (B) Arl13bR79Q/R79Q and (C) Arl13bR79Q/Δ neural tubes but absent from the floorplate of (D) Arl13bΔ/Δ embryos. (E-H) Olig2 stains the motor neuron precursor domain in (E) Arl13b+/+, (F) Arl13bR79Q/R79Q and (G) Arl13bR79Q/Δ embryos and stains an expanded domain in (H) Arl13bΔ/Δ embryos. (I-L) Pax6 expression is visible in the dorsal neural tube in (I) Arl13b+/+, (J) Arl13bR79Q/R79Q and (K) Arl13bR79Q/Δ neural tubes but shifted dorsally in (L) Arl13bΔ/Δ neural tubes. (M-P) Arl13b is localized to cilia visible in the ventral lumen of (M) Arl13b+/+, (N) Arl13bR79Q/R79Q and (O) Arl13bR79Q/Δ neural tubes but absent from (P) Arl13bΔ/Δ embryos.

**Figure 5:** Arl13bR79Q mutation does not affect cerebellar width. (A) Representative surface-facing images of cerebella from control and Arl13bR79Q/R79Q mutant mice. Scalebar = 1mm, black line indicates where cerebellar width was measured, red line indicates where vermis width was measured, gray line indicates where cerebellar
hemisphere height was measured, blue line indicates where vermis height was measured. (B) Ratios of Arl13b<sup>R79Q/R79Q</sup> mutant to control measurements of sex- and age-matched pairs of mice showed no significant difference. Cerebellar and vermis measurements were determined from surface views (symbol colors match the lines in A), body and dissected brain weights were determined using a standard lab scale. Each symbol represents a pair of sex- and age-matched animals: females are represented as circles and males are represented as triangles.

**Figure 6: Pan-neuronal deletion of Arl13b results in a small cerebellum.** (A) Representative images of cerebella from control and Arl13b<sup>Brn4-Cre</sup> mutant mice. Scalebar = 1mm. (B) The ratio of Arl13b<sup>Brn4-Cre</sup> mutant to control cerebellar width was reduced in both females and males (one-sample t-test: p<0.1, single asterisk). The ratio of Arl13b<sup>Brn4-Cre</sup> mutant to control vermis width was reduced in female and male age matched pairs (one-sample t-test: p<0.0001, four asterisks). While Arl13b<sup>Brn4-Cre</sup> mutants had moderately lower body weights than controls (one-sample t-test: p<0.1, single asterisk), their brains were slightly heavier, likely due to hydrocephaly. Each symbol represents a pair of sex- and age-matched animals: females are represented as circles and males are represented as triangles.

**Figure 7: Mice expressing cilia-excluded Arl13b<sup>V358A</sup> have cerebella of normal width.** (A) Representative images of cerebella from control and Arl13b<sup>V358A/V358A</sup> mutant mice. Scalebar = 1mm. (B) Ratios of Arl13b<sup>V358A/V358A</sup> mutant to control measurements of sex- and age-matched pairs of mice showed no significant differences (one-sample t-
test). Each dot represents a pair of sex- and age-matched animals: females are represented as circles and males are represented as triangles.
A  Control  

B  \( \text{Arl13b}^{V358A} \)  

<table>
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<th>Ratio (mutant/control)</th>
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- Cerebellar width
- Vermis width
- Hemisphere height
- Vermis height
- Body weight
- Brain weight

Circle = female
Triangle = male