Arl13b regulates Sonic Hedgehog signaling from outside primary cilia

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Abstract

ARL13B is a regulatory GTPase highly enriched in cilia. Complete loss of *Arl13b* disrupts cilia architecture, protein trafficking and Sonic hedgehog signaling. To determine whether ARL13B is required within cilia, we knocked in a cilia-excluded variant of ARL13B (V358A) and showed it retains all known biochemical function. We found that ARL13B^{V358A} protein was expressed but could not be detected in cilia, even when retrograde ciliary transport was blocked. We showed *Arl13b*$^{V358A/V358A}$ mice are viable and fertile with normal Shh signal transduction. However, in contrast to wild type cilia, *Arl13b*$^{V358A/V358A}$ cells displayed short cilia and lacked ciliary ARL3 and Inpp5e. These data indicate that ARL13B's role within cilia can be uncoupled from its function outside of cilia. Furthermore, these data imply that the cilia defects upon complete absence of ARL13B do not underlie the alterations in Shh transduction, which is unexpected given the requirement of cilia for Shh transduction.

Keywords:

ARL13B; Sonic Hedgehog signaling; primary cilia; mouse development

Introduction

The Hedgehog (Hh) signaling pathway is essential for embryogenesis in a wide variety of organisms. Initially discovered in *Drosophila* where there is a single Hh ligand, the core components of the Hh pathway are conserved in vertebrates (Nusslein-Volhard and Wieschaus 1980). These include the vertebrate Hh receptor Patched1 (Ptch1), the obligate transducer of the pathway Smoothened (Smo), as well as the Gli transcription factors (Ci in *Drosophila*) that act as both activators (GliA) and repressors (GliR) to control target gene transcription (Briscoe and Therond 2013). There are three Hh ligands in vertebrates, Sonic (Shh), Indian (Ihh) and Desert (Dhh), that regulate a multitude of developmental processes including formation of the limbs and digits, the bones of the skull and face, and the patterning of the neural tube (Placzek and Briscoe 2018). Diminished Hh signaling during embryogenesis results in birth defects whereas increased Hh signaling leads to tumors, highlighting the importance of the pathway and its regulation (Raleigh and Reiter 2019).

Given the deep homology between invertebrate and vertebrate Hh signaling, the discovery that primary cilia are required for vertebrate, but not invertebrate, Hh signaling was unexpected (Huangfu et al. 2003; Huangfu and Anderson 2006). Vertebrate Hh components dynamically traffic within cilia in response to ligand (Corbit et al. 2005; Haycraft et al. 2005; Rohatgi et al. 2007). In the absence of ligand, Ptch1 is enriched on the ciliary membrane and Smo is barely detectable (Corbit et al. 2005; Rohatgi et al. 2007). Furthermore, full length Gli proteins traffic to the ciliary tip and back to the cytoplasm before being cleaved to their repressor form, which actively shuts down Hh target gene transcription in the nucleus (Kim et al. 2009; Liu et al. 2005; Wen et al.

2010; Humke et al. 2010). In contrast, upon ligand stimulation (Shh, Ihh or Dhh), the Ptch1 receptor binds the ligand and shuttles out of cilia (Rohatgi et al. 2007). Subsequently, Smo is enriched in the cilium and is subsequently activated (Kong et al. 2019; Corbit et al. 2005). Activated Smo promotes the processing of full-length Gli transcription factors into GliA, which turns on target genes (Aza-Blanc et al. 2000; Ruiz i Altaba 1998). Ablation of cilia results in an absence of GliA and GliR production, rendering the pathway inert and leading to an absence of transcriptional response (Huangfu and Anderson 2005). The dynamic ciliary movement of Shh components appears to be critical to pathway function, as alterations to cilia disrupt pathway output (Caspary et al. 2007; Huangfu and Anderson 2006; Liem et al. 2012; Liem et al. 2009; Goetz et al. 2009; Murdoch and Copp 2010; Tuz et al. 2014; Cortellino et al. 2009; Houde et al. 2006; Liu et al. 2005; Tran et al. 2008; Taylor et al. 2015).

Given that the fundamental logic of the pathway is conserved from flies through vertebrates, and that flies transduce Hh signals without relying on cilia, both evolutionary and mechanistic questions are raised as to how vertebrate cells co-opted the cilium for Hh signal transduction. One distinction lies in the fact that vertebrates use Hh signaling over a longer distance than flies, leading to the proposal that the cilium is a critical part of a mechanism underlying long range signaling (Bangs et al. 2015). For example, in neural patterning Shh is initially expressed in the notochord and is secreted to specify fates more than 20 cells away (Chiang et al. 1996; Briscoe et al. 2001; Roelink et al. 1994). At the evolutionary level, comparisons among organisms with cilia and or Hh have provided some clues. The round worm *C. elegans* have cilia yet do not possess Hh signaling as they don't have most of the genes encoding the core

components of Hh signal transduction (Consortium 1998; Roy 2012). Curiously, a few components of Hh signaling such as fused and costal 2 are in the *C. elegans* genome where they are functionally important for ciliogenesis (Ingham et al. 2011). Additionally, *C. elegans* retained a Ptch1 homolog important for development and pattern formation, but no Hh or Smo (Zugasti et al. 2005; Kuwabara et al. 2000). In contrast, planaria flatworms possess both cilia and Hh signaling but the cilia are not required to transduce Hh signaling (Rink et al. 2009). These data suggest that the mechanistic link of cilia and Hh is limited to vertebrates and originated in the last common ancestor of vertebrates, the urochordates.

ARL13B is a member of the ARF family of regulatory GTPases and is highly enriched on the ciliary membrane (Caspary et al. 2007). In mice, a null mutation of *Arl13b* leads to short cilia and to alterations in Shh signal transduction (Caspary et al. 2007; Larkins et al. 2011). ARL13 is ancient, predicted to be present in the last common eukaryotic ancestor. ARL13 appears to have been lost during evolution in organisms that lack cilia and duplicated to ARL13A and ARL13B in the urochordates, thus ARL13B is proposed to hold important clues in deciphering the links between cilia and vertebrate Hh signaling (Schlacht et al. 2013; Li et al. 2004; Kahn et al. 2008; East et al. 2012; Logsdon and Kahn 2004).

ARF regulatory GTPases, like ARL13B, are best known to play roles in membrane trafficking (D'Souza-Schorey and Chavrier 2006). As is true for a large number of regulatory GTPases, ARL13B is functionally diverse (Sztul et al. 2019). It regulates endocytic traffic (Barral et al. 2012) as well as the phospholipid composition of the ciliary membrane through recruitment of the lipid phosphatase Inpp5e to the ciliary

membrane (Humbert et al. 2012). ARL13B also has a conserved role as a guanine nucleotide exchange factor (GEF) for ARL3, another ciliary ARF-like (ARL) protein (Gotthardt et al. 2015; Zhang et al. 2016; Hanke-Gogokhia et al. 2016; Ivanova et al. 2017). ARL13B regulates intraflagellar transport (IFT), the process that builds and maintains cilia (Cevik et al. 2010; Li et al. 2010; Nozaki et al. 2017). It is known to interact with several proteins associated with cilia, including the exocyst, tubulin and UNC119 (Seixas et al. 2016; Zhang et al. 2016; Larkins et al. 2011; Revenkova et al. 2018). Critical to this work, loss of ARL13B disrupts Shh signal transduction in at least two distinct ways: Smo enrichment in cilia occurs even in the absence of ligand and Gli activator production is diminished, although Gli repressor is made normally (Caspary et al. 2007; Larkins et al. 2011).

Due to the high enrichment of ARL13B on the ciliary membrane, ARL13B is assumed to function in its diverse roles from within the cilium. However, ARL13B is present in early endosomes and circular dorsal ruffles on the cell surface (Barral et al. 2012; Casalou et al. 2014). We previously showed that a V358A variant of ARL13B does not localize to cilia as it disrupts a known VxPx cilia localization sequence (Higginbotham et al. 2012; Mariani et al. 2016). Exogenous overexpression of a ARL13B^{V358A} construct in *Arl13b* null cells does not rescue ARL13B-dependent phenotypes such as cilia length as well as interneuron migration and connectivity, consistent with ciliary ARL13B mediating these processes (Higginbotham et al. 2012; Mariani et al. 2016). In contrast, we found that overexpressed ARL13B^{V358A} does rescue the Shh-dependent ciliary enrichment of Smo in mouse embryonic fibroblasts, arguing

that ARL13B may function outside the cilium to regulate Smo traffic (Mariani et al. 2016). Together, these results raise the question of where ARL13B functions.

To define where ARL13B functions in relation to cilia, we wanted an *in vivo* model so generated mice carrying the ARL13B^{V358A} point mutation using CRISPR/Cas9. Here we demonstrate that ARL13B^{V358A} protein was undetectable in *Arl13b*$^{V358A/V358A}$ cilia in both neural tube and mouse embryonic fibroblast cilia, even after blocking retrograde ciliary traffic. We report that *Arl13b*$^{V358A/V358A}$ mice were viable, fertile, and transduced Shh signal normally. We found ARL3 and Inpp5e did not localize to the short cilia present in/on *Arl13b*$^{V358A/V358A}$ cells. These data indicate that ARL13B's roles within and outside cilia can be uncoupled; ARL13B's role in regulating cilia length is from within cilia, whereas its control of Shh signaling appears to be from outside the cilium. Thus, these data imply that the cilia defects seen in the complete absence of ARL13B do not underlie the alterations in Shh transduction, which is unexpected given the requirement of cilia for Shh signal transduction.

Results

ARL13B^{V358A} *displays normal GEF activity*

We previously showed that mouse ARL13B^{V358A} protein retained normal intrinsic and GAP-stimulated GTP hydrolysis activities by analyzing GST-ARL13B^{V358A} purified from human embryonic kidney (HEK) cells (Mariani et al. 2016). In order to test ARL13B^{V358A} GEF activity for ARL3, we used the same GST-ARL13B^{V358A} protein preparation and measured the rates of spontaneous or GEF-stimulated GDP dissociation from ARL3 in the presence and absence of ARL13B or ARL13B^{V358A}. ARL3

spontaneously releases pre-bound GDP quite slowly under the conditions in the assay, though release is linear with time, and by extrapolation requires just under 30 min for 50% of pre-bound [^{3}H]GDP to dissociate (**Figure 1**). In marked contrast, addition of ARL13B under the same conditions caused the release of 50% of the GDP within ~10 seconds and close to 100% in one minute. We detected no differences between the wild type and mutant ARL13B^{V358A} proteins in this assay, consistent with this point mutation having no effect on ARL13B GEF function (**Figure 1**). This result is consistent with GEF activity being conserved within the protein's GTPase domain while the V358A mutation is located in the C-terminal domain (Gotthardt et al. 2015). These data indicate that ARL13B^{V358A} retains all known ARL13B biochemical activities, suggesting that the V358A mutation only disrupts ARL13B localization.

CRISPR engineered* Arl13b$^{V358A/V358A}$ *mice express cilia-excluded ARL13B protein.

To determine the consequences of ARL13B^{V358A} expression *in vivo*, we used CRISPR/Cas9 editing to change residue 358 from valine to alanine (**Figure 2A**). We performed sequencing of the region that flanked exon 8, using primers outside the region of the donor oligonucleotide. We confirmed the heterozygous T to C base pair change for a valine to alanine change at residue 358 (**Figure 2B**). We backcrossed heterozygous F1 progeny to FVB/NJ for three generations before analysis to minimize any off target confounds (see details in Methods).

To determine whether ARL13B^{V358A} was detectable in neural tube cilia, we performed immunofluorescence using antibodies directed against ARL13B and the cilia marker IFT88. At embryonic day (E)10.5, we saw IFT88 staining, indicating the

147 presence of cilia. In neural tube cilia of *Arl13b*$^{+/+}$ and *Arl13b*$^{V358A/+}$ embryos, we
148 observed equivalent ARL13B staining but could not see ARL13B signal in neural tube
149 cilia of *Arl13b*$^{V358A/V358A}$ embryos, indicating the ARL13B^{V358A} protein is undetectable in
150 cilia *in vivo* (**Figure 2C**).
151 One possible explanation for the absence of ciliary ARL13B in the
152 *Arl13b*$^{V358A/V358A}$ embryos is that the ARL13B^{V358A} protein is not expressed or is
153 unstable. To determine whether ARL13B protein expression was affected by the V358A
154 mutation, we performed Western blots on E10.5 whole embryo lysates (**Figure 2D**). We
155 found a ~7% (±20%) reduction of ARL13B levels in *Arl13b*$^{V358A/+}$ embryos and a ~37%
156 (±6%) decrease of ARL13B levels in *Arl13b*$^{V358A/V358A}$ embryos, compared to WT
157 (**Figure 2E**). This decrease may reflect a change in ARL13B^{V358A} stability compared to
158 ciliary ARL13B or could signify ARL13B^{V358A} protein having a distinct half-life when
159 localizing to different cellular compartments. Regardless, these data indicate that
160 ARL13B^{V358A} is expressed in *Arl13b*$^{V358A/V358A}$ embryos.
161
162 ***ARL13B^{V358A} protein is undetectable in cilia in mouse embryonic fibroblasts.***
163 To more closely investigate whether any ARL13B^{V358A} protein could localize to
164 cilia, we generated immortalized mouse embryonic fibroblasts (MEFs). We performed
165 double labeling to first identify cilia using acetylated α-tubulin or IFT88 antibodies and
166 subsequently determined whether ARL13B is present in cilia. We used five distinct
167 antibodies against ARL13B: 4 antibodies against amino acids 208-427 of the mouse
168 ARL13B protein (1 mouse monoclonal (NeuroMab), 3 rabbit polyclonal antibodies
169 (Caspary et al. 2007)), as well as 1 antibody against the full-length human ARL13B

protein (rabbit polyclonal (Protein Tech)). By eye, we observed strong ciliary staining of ARL13B with each of the antibodies in *Arl13b*$^{+/+}$ and *Arl13b*$^{V358A/+}$ cells, but we were unable to identify any evidence of ARL13B staining above background within cilia in *Arl13b*$^{V358A/V358A}$ cells with any of these five antibodies (**Figure 3A**). Taken together, these data support our conclusion that ARL13B^{V358A} is not detectable in cilia using the currently available, validated ARL13B antibodies.

While we observed no evidence of detectable ciliary ARL13B in *Arl13b*$^{V358A/V358A}$ MEFs, it is possible that a small amount of ARL13B^{V358A} is constantly trafficking in and out of cilia, at steady-state levels that remain below the limits of detection. To address this possibility, we blocked retrograde ciliary transport, reasoning that any ARL13B undergoing trafficking in and out of cilia would accumulate. We treated cells with ciliobrevin-D, which blocks the retrograde motor protein dynein (Firestone et al. 2012). As a positive control, we examined IFT88, a ciliary protein known to accumulate at ciliary tips upon blocking of retrograde transport. We found IFT88 enrichment at ciliary tips in *Arl13b*$^{+/+}$, *Arl13b*$^{V358A/+}$ and *Arl13b*$^{V358A/V358A}$ cells upon ciliobrevin-D treatment relative to the respective DMSO-treated controls with no difference in IFT88 staining among the 3 genotypes (**Figure 3B**). To determine whether ARL13B accumulated in cilia of ciliobrevin-D-treated MEFs, we examined cells co-stained for acetylated α-tubulin and ARL13B. In *Arl13b*$^{+/+}$ and *Arl13b*$^{V358A/+}$ cells, we saw that about 90% of acetylated α-tubulin-positive cilia also stained for ARL13B in DMSO-treated control or ciliobrevin-D treated MEFs (**Figure 3C**). In *Arl13b*$^{V358A/V358A}$ MEFs, we saw no ciliary ARL13B staining in DMSO-treated control or ciliobrevin-D-treated cells (**Figure 3C**).

Thus, even when retrograde traffic out of cilia is blocked, we were unable to detect ARL13B protein in cilia in *Arl13b*$^{V358A/V358A}$ MEFs.

We re-examined these data and repeated our analyses using over-exposed images. After defining the region of interest using the acetylated α-tubulin staining, we subsequently overexposed the ARL13B channel five-fold relative to the images in Figure 3 and acquired measurements at the cilium and the cell body (**Supplemental Figure 1A**). As a control, we used *Arl13b*$^{hnn/hnn}$ MEFs, which are devoid of any ARL13B, and obtained a ratio of 1.0, with or without ciliobrevin-D treatment. We found the same ratio when we analyzed *Arl13b*$^{V358A/V358A}$ MEFs consistent with ARL13B being absent from the cilia (**Supplemental Figure 1B, C**). We observed a few instances of ARL13B appearing to co-localize with acetylated α-tubulin, but these were rare (<5%, 2/49) and occurred in both *Arl13b*$^{V358A/V358A}$ and *Arl13b*$^{hnn/hnn}$ (null) cells indicating this is the background staining of the primary antibody (**Supplemental Figure 1D**). We extended our analysis of overexposed images in *Arl13b*$^{V358A/V358A}$ and *Arl13b*$^{hnn/hnn}$ neural tube sections four-fold relative to the images in Figure 2; we identified no overlap of overexposed ARL13B with cilia marker IFT88 (**Supplemental Figure 1E**). While it is formally possible that an extremely small amount of ARL13B^{V358A} remains in cilia, we are not able to find evidence of it; thus, we designate ARL13B^{V358A} protein as cilia-excluded ARL13B.

Arl13b$^{V358A/V358A}$ *mice are viable and fertile.*

In order to determine the phenotypic consequence of the *Arl13b*^{V358A} allele, we intercrossed *Arl13b*$^{V358A/+}$ mice. We observed progeny in Mendelian proportions with an

average of 7.3 pups per litter, consistent with the reported FVB/NJ litter size of 7-9 (Murray et al. 2010) (**Table 1**). To test whether homozygous mice breed normally, we crossed *Arl13b*$^{V358A/V358A}$ males to heterozygous or homozygous females. We found the *Arl13b*^{V358A} allele segregated in Mendelian proportions and the litter sizes were normal indicating that *Arl13b*^{V358A} does not impact viability, fertility or fecundity.

ARL13B*^{V358A} *permits normal embryonic development and Shh signaling.

Loss of *Arl13b* leads to morphologically abnormal embryos, lethality and neural patterning defects (Caspary et al. 2007). As ARL13B^{V358A} overcame the embryonic lethality, we examined overall embryo morphology at E9.5, E10.5 and E12.5. We found the overall morphology of *Arl13b*$^{V358A/V358A}$ embryos resembled those of *Arl13b*$^{+/+}$ and *Arl13b*$^{V358A/+}$ embryos indicating that ARL13B^{V358A} did not lead to gross morphological defects. (**Figure 4A-I**).

At E9.5, Shh is normally expressed in the notochord as well as the floor plate of the ventral neural tube. Moving dorsally, the adjacent domains express Nkx2.2 and subsequently Olig2. In the *Arl13b*$^{hnn/hnn}$ (null) neural tube, the Shh-positive columnar cells of the floor plate are absent resulting in both ventral and dorsal expansion of intermediate Shh-dependent cell fates such as Olig2 (Caspary et al. 2007). To determine whether ARL13B^{V358A} disrupts Shh signaling, we examined neural tube patterning in *Arl13b*$^{+/+}$, *Arl13b*$^{V358A/+}$, and *Arl13b*$^{V358A/V358A}$ embryos at E9.5, E10.5, and E12.5. At E9.5 we observed no differences in Shh, Nkx2.2 or Olig2 among *Arl13b*$^{+/+}$, *Arl13b*$^{V358A/+}$, and *Arl13b*$^{V358A/V358A}$ embryos (**Figure 4A-C**). Cell fates in the neural tube are specified by both the concentration and duration of Shh signaling so we examined

neural patterning at subsequent stages (Dessaud et al. 2007). By E10.5, some Olig2 precursors have differentiated to HB9+ motor neurons and all Shh-responsive cells express Nkx6.1. We found that Olig2, HB9 and Nkx6.1 positive cells are normally distributed in all 3 genotypes at E10.5 and E12.5 (**Figure 4D-I**). These data indicate that ARL13B^{V358A} mediates normal Shh signaling.

ARL13B regulates ciliary enrichment of Shh components from outside cilia.

In *Arl13b$^{hnn/hnn}$* cells, Smo is enriched in cilia in a Shh-independent manner, which may be due to defective trafficking of Smo, as many ARL family members regulate protein trafficking (Lim et al. 2011; Larkins et al. 2011). To assess Smo enrichment in *Arl13b$^{V358A/V358A}$* embryos, we stained for the cilia marker acetylated α-tubulin and Smo in E10.5 embryos. Smo appeared enriched normally in ventral floor plate cilia and the dorsal boundary of Smo ciliary enrichment in ventral neural progenitors was identical in *Arl13b$^{+/+}$*, *Arl13b$^{V358A/+}$*, and *Arl13b$^{V358A/V358A}$* samples indicating ARL13B^{V358A} mediates normal ciliary Smo enrichment (**Figure 5A)**.

To examine Smo traffic in response to Shh stimulation, we treated MEFs with 0.5% FBS or Shh-conditioned media for 24 hours and stained for Smo. As expected in control *Arl13b$^{hnn/hnn}$* cells, we saw ciliary Smo in unstimulated MEFs which increased upon stimulation with Shh-conditioned media (Larkins et al. 2011) (**Figure 5B**). In *Arl13b$^{+/+}$*, *Arl13b$^{V358A/+}$*, and *Arl13b$^{V358A/V358A}$* MEF cilia, we saw ciliary enrichment of Smo upon Shh stimulation over their respective unstimulated controls (**Figure 5B**, left). We found no difference in Smo enrichment among these cell lines (**Figure 5B**, right). Thus, despite ARL13B being critical for Shh-dependent Smo ciliary enrichment,

ARL13B^{V358A} regulates Smo localization normally, arguing this function of ARL13B can occur when the protein is not in cilia.

In addition to aberrations in Smo trafficking, loss of *Arl13b* leads to changes in the cilia localization of other Shh components in MEFs (Larkins et al. 2011). To determine whether these components are disrupted by ARL13B^{V358A}, we examined Gli2, Gli3, Sufu and Ptch1 in MEFs. We observed no differences in distribution of Gli2 or Ptch1 among any of the examined genotypes (**Figure 5C, F**). In contrast, as we previously reported, we observed more Gli3 at the ciliary tip of *Arl13b$^{hnn/hnn}$* cells and increased Sufu in *Arl13b$^{hnn/hnn}$* cilia compared to wild type controls (Larkins et al. 2011)(**Figure 5D, E**). Thus, Shh components are normally localized in *Arl13b$^{V358A/V358A}$* MEFs consistent with the normal Shh signal transduction observed in *Arl13b$^{V358A/V358A}$* embryos (**Figure 4**).

ARL13B regulates ciliary enrichment of ARL3 and Inpp5e from within cilia.

We next examined the role of ARL13B^{V358A} in the localization of other ciliary proteins. ARL13B is the GEF for ARL3, and we showed that ARL13B^{V358A} GEF activity for ARL3 is normal (**Figure 1**). ARL13B is also essential for cilia localization of Inpp5e, as the proteins are in a common complex (Humbert et al. 2012). To determine whether the ciliary localization of either ARL3 or Inpp5e was affected by ARL13B^{V358A}, we performed immunofluorescence on MEFs. Ciliary ARL3 staining appeared the same in *Arl13b$^{+/+}$* and *Arl13b$^{V358A/+}$* MEFs, however ARL3 was not detectable in *Arl13b$^{V358A/V358A}$* or *Arl13b$^{hnn/hnn}$* cilia (**Figure 5G**). Inpp5e was normally detectable in *Arl13b$^{+/+}$* and *Arl13b$^{V358A/+}$* MEF cilia, but not in *Arl13b$^{V358A/V358A}$* and *Arl13b$^{hnn/hnn}$* MEF cilia (**Figure**

5H). These results indicate that ARL13B is required in cilia for the proper localization of ARL3 and Inpp5e to the cilium.

Ciliary ARL13B is required for normal cell ciliation and ciliary length

Loss of *Arl13b* leads to defects in the percentage of ciliated MEFs and in cilia length (Caspary et al. 2007; Larkins et al. 2011). To test whether ARL13B^{V358A} impacted cell ciliation, we examined immortalized MEFs and counted the number of ciliated cells 24 hours after induction of ciliation by growth in low serum (0.5% FBS). We found 73% (±11%) of *Arl13b*$^{+/+}$ and 75%, (±9.1%) of *Arl13b*$^{V358A/+}$ MEFs are ciliated, consistent with published results. In contrast, we found 45% (±8.7%) of *Arl13b*$^{V358A/V358A}$ cells and 53% (±6.4%) of *Arl13b*$^{hnn/hnn}$ cells had cilia (**Figure 6A**). Thus, *Arl13b*$^{V358A/V358A}$ cells exhibit a similar deficit in percentage of ciliated MEFs as complete loss of ARL13B function.

Arl13b$^{hnn/hnn}$ cilia are about half as long as wild type in both embryos and MEFs (Caspary et al. 2007; Larkins et al. 2011). To determine whether cilia length was affected by ARL13B^{V358A}, we stained MEFs with acetylated α–tubulin and measured the length of the axoneme. We found the average cilia length in *Arl13b*$^{+/+}$ and Arl13b$^{V358A/+}$ MEFs was similar, 2.7 ± 0.8 μm and 2.6 ± 0.9 μm, respectively. However, we found *Arl13b*$^{V358A/V358A}$ MEF cilia were shorter, 1.9 ± 0.7 μm, similar to *Arl13b*$^{hnn/hnn}$ MEF cilia which were 2.15 ± 0.8 μm (**Figure 6B**). These results indicate that ARL13B^{V358A} phenocopies complete loss of ARL13B for ciliation and cilia length, indicating these ARL13B functions require ARL13B in cilia. Furthermore, these data show that ARL13B function within cilia is distinct from ARL13B function outside of cilia in a subset of

304 activities, and that the cilia defects and Shh defects in the complete absence of ARL13B
305 can be uncoupled.

Discussion

306 Here we show that ARL13B's role(s) in cell ciliation and cilia length, along with
307 the ciliary enrichment of a subset of proteins, can be uncoupled from ARL13B's function
308 in regulating Shh signal transduction (**Figure 7**). Furthermore, we show this functional
309 distinction correlates with ARL13B spatial localization to the cilium. By generating a
310 mouse expressing a cilia-excluded variant of ARL13B from the endogenous locus, we
311 showed ARL13B^{V358A} protein is not detectable in cilia of embryonic neural tube or
312 MEFs, even upon retrograde ciliary traffic blockade. Furthermore, we detected 30% less
313 ARL13B protein overall in *Arl13b*$^{V358A/V358A}$ embryos compared to control embryos.
314 While this reduction is statistically significant, it is unclear whether it is biologically
315 significant given that *Arl13b*$^{hnn/hnn}$ null mutations are recessive and no *Arl13b*$^{hnn/+}$
316 heterozygous phenotype is reported (Caspary et al. 2007; Larkins et al. 2011).
317 In contrast to the E13.5 lethality of *Arl13b*$^{hnn/hnn}$ embryos, we found
318 *Arl13b*$^{V358A/V358A}$ mice were viable and fertile with correct patterning of the neural tube,
319 indicating normal Shh signal transduction. These results are consistent with our data
320 showing that ARL13B^{V358A} protein retains all known biochemical function including GEF
321 activity (Mariani et al. 2016; Ivanova et al. 2017). However, we observed several
322 *Arl13b*$^{hnn/hnn}$ phenotypes in *Arl13b*$^{V358A/V358A}$ cells, including loss of ARL3 and Inpp5e
323 ciliary enrichment, along with low percentage of ciliated cells and shorter average cilia
324 length. Taken together, our data show that despite the normal high ciliary enrichment of

wild type ARL13B and the requirement of cilia for Shh signaling, cilia-excluded ARL13B^{V358A} is sufficient for Shh signal transduction.

We regard ARL13B^{V358A} as cilia-excluded based on several lines of evidence. We did not detect ciliary ARL13B^{V358A} *in vivo* or *in vitro* using any of five validated ARL13B antibodies. These antibodies are against two antigens, the full-length protein or residues 208-427, and were independently generated so are likely to recognize a number of epitopes. Given that 4 of 5 antibodies are polyclonal and ARL13B^{V358A} displays normal GTP binding, intrinsic and GAP-stimulated GTP hydrolysis, and ARL3 GEF activity, it is likely that the ARL13B^{V358A} protein retains the wild type structure enabling antibody recognition. Indeed, we observed little or no loss in sensitivity in detecting protein in the immunoblot assays. Furthermore, we could not forcibly enrich ciliary ARL13B^{V358A} through retrograde transport blockade with ciliobrevin-D nor could we detect any ciliary enrichment of ARL13B^{V358A} upon overexposure of the relevant fluorescent channel. The fact that we observed ciliary phenotypes, namely short cilia and abnormal ciliary ARL3 and Inpp5e localization in ARL13B^{V358A} cells, argue that ARL13B normally performs these functions from within cilia (Caspary et al. 2007; Larkins et al. 2011; Zhang et al. 2016; Humbert et al. 2012; Nozaki et al. 2017). While we cannot exclude the possibility that sub-detectable levels of ARL13B^{V358A} are present and functional in cilia, we note such a level would need to be sufficient for Shh signaling yet insufficient for ARL3 or Inpp5e ciliary localization along with proper regulation of cilia length.

Our data support ARL13B regulating different biological processes from its distinct subcellular localizations. ARL13B^{V358A} disrupts cilia localization of Inpp5e and

ARL3, but not Shh components. It is surprising that ARL13B^{V358A} is sufficient to regulate Shh signaling because cilia are required for Shh signal transduction and because ARL13B is highly enriched in cilia (Caspary et al. 2007; Huangfu et al. 2003). These observations suggested that the ciliary defects observed in *Arl13b*$^{hnn/hnn}$ mutants caused the Shh defects. However, our data with ARL13B^{V358A} indicate that ARL13B regulates cilia length and Shh signaling through distinct localization and effectors.

Based on the normal cilia trafficking of Shh components in *ARL13B*$^{V358A/V358A}$ mutants and the abnormal cilia trafficking of Shh components in the complete absence of ARL13B (Larkins et al. 2011), ARL13B likely regulates Shh signaling from the cell body by controlling Shh component traffic to and/or from the cilium. Both Smo and ARL13B traffic is linked to endosomes providing one possible non-ciliary organelle (Barral et al. 2012; Wang et al. 2009; Milenkovic et al. 2009). *Arl13b*$^{hnn/hnn}$ cells display constitutive Smo ciliary enrichment along with little enrichment of Gli proteins at the ciliary tip (Larkins et al. 2011). Our data do not distinguish between ARL13B playing direct roles in traffic of multiple Shh components or whether the normal Smo ciliary enrichment with ARL13B^{V358A} subsequently causes normal cilia traffic of downstream components. ARL13B regulates a step downstream of activated Smo that controls transcription factor Gli activation, but not repression (Caspary et al. 2007; Bay et al. 2018). We argue from our results that this step is also intact in the presence of ARL13B^{V358A}.

The fact that ARL13B^{V358A} can mediate normal ciliary Smo enrichment is especially interesting given that ARL3 and Inpp5e localization to cilia is compromised by this mutation. This suggests that ARL13B controls cilia enrichment via multiple

localizations and effectors. This is consistent with our understanding of ARF family members, as they are known to perform multiple tasks from different sites within a cell (Sztul et al. 2019). We speculate that ARL3 residence in cilia may require that ARL3 be in (or at least cycle through) its activated, GTP-bound conformation as ARL13B^{V358A} retains its ARL3 GEF activity, but not its cilia localization. ARL13B is in a common protein complex with Inpp5e so absence of ARL13B from cilia may disrupt formation or maintenance of the complex there (Humbert et al. 2012; Nozaki et al. 2017). Inpp5e regulates Shh signaling through regulation of the phosphoinositol composition of the ciliary membrane. Inpp5e loss results in increased ciliary PIP_2 and enrichment of Shh repressors in cilia thus resulting in lowered Shh response (Garcia-Gonzalo et al. 2015; Chavez et al. 2015; Dyson et al. 2017). It is not clear why the absence of ciliary Inpp5e in ARL13B^{V358A} cells does not lead to aberrant Shh signal transduction.

In *Arl13b$^{hnn/hnn}$* embryos and MEFs, cilia are shorter than normal and have a microtubule defect where the B-tubule of the microtubule outer doublet is open (Caspary et al. 2007; Larkins et al. 2011). Consistent with this, we observed shorter cilia in *Arl13b$^{V358A/V358A}$* MEFs. Similarly, we observed a comparable reduction in the percent of cilia in *Arl13b$^{hnn/hnn}$* and *Arl13b$^{V358A/V358A}$* MEFs compared to wild type MEFs. While loss of ARL13B results in short cilia, increased expression of ARL13B promotes cilia elongation so it is not yet clear what ARL13B's role is in controlling cilia length (Lu et al. 2015; Hori et al. 2008; Caspary et al. 2007; Larkins et al. 2011). Additionally, it is unclear whether the mechanism from within cilia through which ARL13B controls length or percent of ciliated cells is the same, or distinct from, the mechanism through which ARL13B regulates ARL3 or Inpp5e residence in cilia.

Perhaps the most intriguing implication of our data pertains to understanding the evolution of cilia and Hh signaling. ARL13 functions in cilia formation and maintenance in *Chlamydomonas* and *C. elegans*, neither of which have Hh signaling, consistent with the ancient role of Arl13b controlling ciliation and cilia length (Cevik et al. 2010; Cevik et al. 2013; Stolc et al. 2005; Miertzschke et al. 2014). Our data support ARL13B retaining ARL13's ancient role in ciliogenesis. However, our data show that ARL13B does not work from within the cilium to regulate Shh component ciliary traffic or Shh signal transduction. As an ARL protein involved in membrane traffic, we speculate that ARL13B may have linked Shh component trafficking to the cilium, albeit from outside the cilium. That the ARL13 duplication in the urochordates coincides with when ARL13B adopted new cellular roles in Hh signaling that linked the pathway to cilia fits well with this model.

Methods

Protein purification and ARL3 GEF assay

Plasmids directing the expression of mouse GST-ARL13B or GST-ARL13B^{V358A} proteins were transiently transfected into HEK cells and the recombinant proteins were later purified by affinity chromatography using glutathione-Sepharose, as described previously (Cavenagh et al. 1994; Ivanova et al. 2017). Human ARL3 (98.4% identical to mouse ARL3) was expressed in BL21 bacteria and purified as previously described (Van Valkenburgh et al. 2001). The ARL3 GEF assay was performed as previously described (Ivanova et al. 2017). Briefly, ARL3 (10μM) was pre-incubated with [^{3}H]GDP (1 μM; PerkinElmer Life Sciences, specific activity ~3,000 cpm/pmol) for 1 h at 30°C in

25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$. In contrast, pre-loading of ARL13B (10μM) was achieved by incubation in 100 μM GTPγS for 1 min at room temperature, due to its much more rapid exchange kinetics. The GEF assay was initiated upon addition of ARL3 (final = 1μM), ARL13B (final = 0 or 1μM), 10 μM GTPγS, and 100 μM GDP (to prevent re-binding of any released [^{3}H]GDP), in a final volume of 100 μL. The intrinsic rate of GDP dissociation from ARL3 was determined in parallel as that released in the absence of any added ARL13B. The reactions were stopped at different times (0–15 min) by dilution of 10μL of the reaction mixture into 2 ml of ice-cold buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). The amount of ARL3-bound [^{3}H]GDP was determined by rapid filtration through BA85 nitrocellulose filters (0.45 μm, 25 mm, Whatman), with 3 x 2 mL washes, and quantified using liquid scintillation counting. Time points are routinely collected in at least duplicate and each experiment reported was repeated at least twice, yielding very similar results. Data were analyzed in GraphPad Prism 7 software.

Mouse allele generation and identification

All mice were cared for in accordance with NIH guidelines and Emory's Institutional Animal Care and Use Committee (IACUC). Lines used were *Arl13b*^{V358A} (*Arl13b*^{em1Tc}) [MGI: 6256969], *Arl13b*^{hnn} [MGI: 3578151] and FVB/NJ (Jackson Laboratory). To generate the V358A point mutation in the mouse, a CRISPR gRNA (CCAGTCAATACAGACGAGTCTA) targeting exon 8 of the *Arl13b* locus along with a donor oligo (5'-CCTATATTCTTCTAGAAAACAGTAAGAAGAAAACCAAGAAACTACGAATGAAAAGGA

GTCATCGGGCAGAACCAGTGAATACAGACGAGTCTACTCCAAAGAGTCCCACGCC TCCCCAAC-3'; underlined bases are engineered) were designed to generate a T-to-C change creating the V358A point mutation and C-to-G change creating a TspRI restriction site that could be used for genotyping (Millipore Sigma). The gRNA (50 ng/μl), oligo donor (50 ng/μl) and CRISPR protein (100 ng/μL) were injected by the Emory Transgenic and Gene Targeting core into one-cell C57Bl/6J zygotes. Zygotes were cultured to 2-cell before being transferred to pseudopregnant females. Genomic tail DNA from resulting offspring was amplified using PCR primers (5'-GAAGCAGGCATGGTGGTAAT-3' & 5'-TGAACCGCTAATGGGAAATC-3') located upstream and downstream of the donor oligo breakpoints. The products were sequenced (5'-GAAGCAGGCATGGTGGTAAT-3') and 2 animals were identified heterozygous for the desired change and with no additional editing. One line (#173) was bred to FVB/NJ for three generations prior to performing any phenotypic analysis. Males from at least two distinct meiotic recombination opportunities were used in each generation that to minimize potential confounds associated with off-target CRISPR/Cas9 editing.

Genotyping was performed on DNA extracted from ear punch or yolk sac via PCR using the following primers: Fwd: 5'- AAGAATGAAAAGGAGTCAGCG -3', Rev: 5'-TGAACCGCTAATGGGAAACT -3'; a SNP was engineered in the forward primer that, in combination with the V358A mutation, created a Cac8I restriction site. Thus, the PCR product was digested with Cac8I enzyme, run out on a 4% agarose gel and the relevant bands were detected: undigested wild type (~192bp) and digested mutant bands (~171bp).

461

462 *Phenotypic analysis of embryos*

463 Timed matings of heterozygous intercrosses were performed to generate embryos of
464 the indicated stage, with somite-matched pairs examined at each stage when
465 appropriate. Embryos were dissected in cold phosphate-buffered saline and processed
466 for immunofluorescence staining? as previously described (Horner and Caspary 2011).

467

468 *Mouse embryonic fibroblasts*

469 Mouse embryonic fibroblasts (MEFs) were isolated and immortalized as previously
470 described (Mariani et al. 2016). MEFs were maintained in DMEM with 10% fetal bovine
471 serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO_2. For experimental use,
472 *Arl13b*$^{+/+}$, *Arl13b*$^{V358A/+}$, and *Arl13b*$^{V358A/V358A}$ and *Arl13b*$^{hnn/hnn}$ MEFs were grown on
473 coverslips at a density of 0.5 x 10^6 cells/mL and treated for 24 h with 0.5% FBS Shh
474 conditioned media or 0.5% FBS media to induce ciliogenesis (Larkins et al. 2011).

475

476 *Antibodies*

477 Primary antibodies used were: mouse anti-Shh (5E1), mouse anti-Nkx2.2 (74.5A5),
478 mouse anti-HB9 (81.5C10) (1:5 Developmental Studies Hybridoma Bank); rabbit anti-
479 Olig2 (1:300 Millipore AB9610); mouse anti-acetylated α-tubulin (1:2500 Millipore
480 Sigma; T6793), mouse anti-Arl13b (1:1000 NeuroMab N295B/66), rabbit anti-Arl13b
481 (1:1000, Protein Tech 17711-1-AP); rabbit anti-Arl13b sera from 3 distinct rabbits (503,
482 504 and 505) (Caspary Lab (Caspary et al. 2007)), rabbit anti-Smoothened (1:1000,
483 kindly provided by K. Anderson); rabbit anti-IFT88 (1:1000, kindly provided by B.

Yoder); rabbit anti-ARL3 (1:1000, (Cavenagh et al. 1994)); rabbit anti-Inpp5e (1:150, Protein Tech 17797-1-AP); guinea pig anti-Gli2 (1:200, kindly provided by J. Eggenschwiler); rabbit anti-Ptch1 (1:150, kindly provided by R. Rohatgi), goat anti-Sufu (1:100, SC10933, Santa Cruz); goat anti-Gli3 (1:200, R&D AF3690) Alexa Fluor 488 and Alexa Fluor 568 (1:300, ThermoFisher); and Hoechst nuclear stain (1:3000).

Image Quantification

Fluorescent intensities were measured in ImageJ software. Cilia were first identified by positive acetylated α-tubulin and an outline was hand drawn around the length of the cilium. The channel was switched to the protein of interest and a measurement of the average fluorescent intensity was acquired. The same outline was then used to acquire a background reading of the cell-body that most closely matched the background at the cilium. In all cases immunofluorescent averages of proteins of interest in cilia were normalized to cell-body intensities (Supplemental Figure 1A). For samples with antibodies targeting Gli2, Gli3, and Sufu the ciliary tip was isolated and measured. The cilia tip was identified by weak acetylated α-tubulin staining or the cilium base was identified by the presence of the acetylated α-tubulin positive fibrils in the cell body that cluster at the base of the cilium (Larkins et al. 2011). For samples with antibodies targeting Smo, Ptch1, ARL3, and Inpp5e the entire cilium was measured. We plotted the ratio of fluorescence intensity of the protein of interest to the cell body background. The number of cilia examined per genotype and per condition are listed below their respective violin plot. Data were analyzed by one-way ANOVA, except for Smo intensity where a two-way ANOVA was used with Sidak's multiple comparison. In the event of a

507 significant ANOVA, Tukey's multiple comparisons were employed to determine
508 significance. Data were analyzed in GraphPad Prism 7 software.
509 In a similar experiment, retrograde IFT was inhibited by the addition of 30 μM
510 ciliobrevin-D (Millipore Sigma; 250401) or 0.1% DMSO control in low-serum media for
511 60 min after ciliation was induced by serum starvation for 24 h prior with 0.5% FBS
512 media. Cilia were identified by staining with antibodies directed against acetylated α-
513 tubulin, IFT88, and ARL13B. Fluorescent intensities were measured in ImageJ software.
514 Again, cilia were first identified by positive acetylated α-tubulin and an outline was hand
515 drawn. The channel was switched to the protein of interest and a measurement of the
516 average fluorescent intensity was acquired. The same outline was then used to acquire
517 a background reading of the cell-body. To measure IFT88, the cilia tip was identified by
518 weak acetylated α-tubulin staining or the cilium base was identified by the presence of
519 acetylated α-tubulin fibrils in the cell body that cluster at the base of the cilium (Larkins
520 et al. 2011). Data were analyzed by two-way ANOVA with Sidak's multiple comparison.
521
522 *Western Blots*
523 Western blotting was performed as previously described (Mariani et al. 2016), with
524 antibody against ARL13B (1:1000 Neuromab N295B/66). Lysates were prepared with
525 RIPA buffer and SigmaFast protease inhibitors (Nachtergaele et al. 2013). Values
526 presented as volume intensity measured by chemiluminescence detected on ChemiDoc
527 Touch Imaging System were normalized to total protein as measured on a stain-free
528 gel. Normalized values were analyzed by one-way ANOVA and Tukey's multiple
529 comparisons. Data were analyzed in GraphPad Prism 7 software.

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Competing Interests

The authors have no competing interests to disclose.

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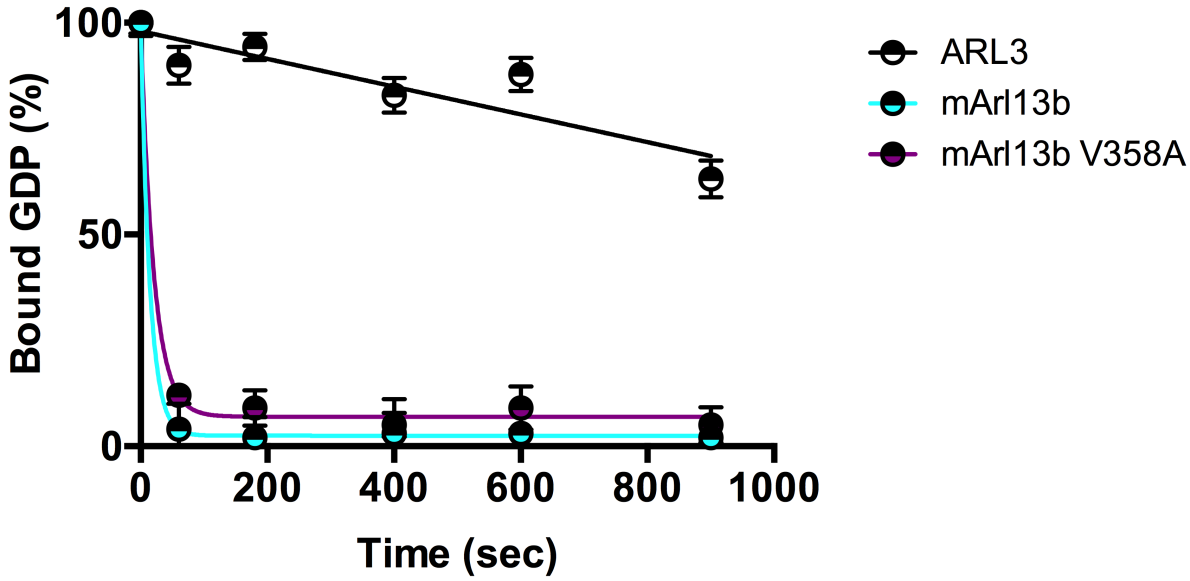


Figure 1.

ARL3 GEF activity is retained in the ARL13B^{V358A} mutant. Time course of the release of pre-bound [^{3}H]GDP from purified, recombinant ARL3 in the absence (ARL3) or presence of mouse wild type ARL13B or ARL13B^{V358A} (mARL13B) are shown. See Methods for details. Error bars ± standard deviation.

Figure 2.

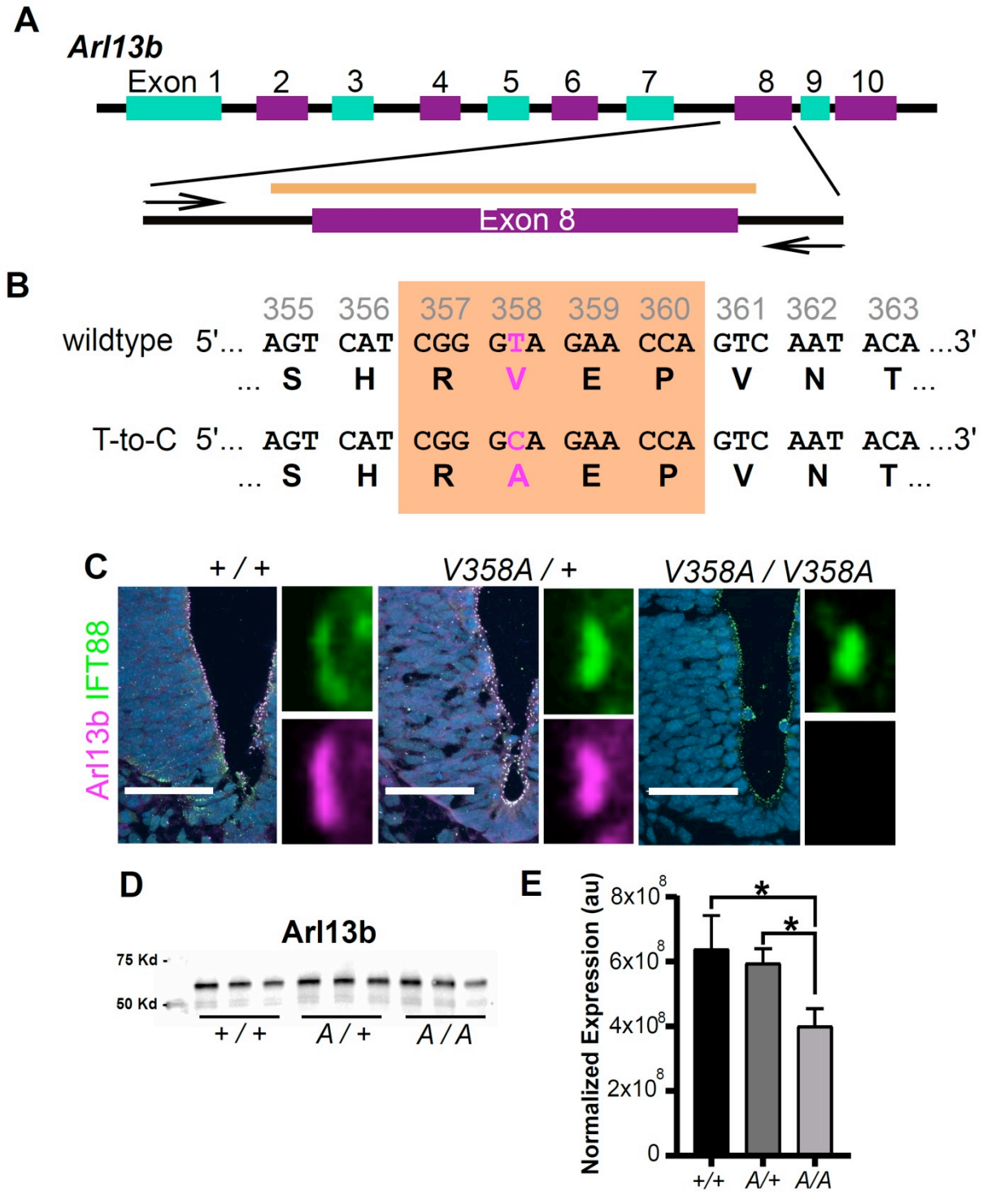


Figure 2.

Generation of the *Arl13b*$^{V358A/V358A}$ mouse.

(A) Schematic of *Arl13b* gene and donor oligo (orange bar) at exon 8 used to generate the V358A causing point mutation. Arrows indicate primers used for allele validation. (B) *Arl13b* DNA and relevant amino acid sequence with the RVEP sequence in the orange box and the T-to-C mutation highlighted in pink. (C) Confocal images of cilia marker IFT88 (green) and ARL13B (magenta) staining in neural tube of E10.5 somite-matched embryos. ARL13B-positive cilia are visible in *Arl13b*$^{+/+}$ and *Arl13b*$^{V358A/+}$, but not in *Arl1b*$^{V358A/V358A}$ embryos. At least 5 embryos per genotype across five litters were examined. Scale bars are 50 μm. (D) ARL13B Western blot of E10.5 whole embryo lysates, wild type (+/+), *Arl13b*$^{V358A/+}$ (*A*/+) and *Arl13b*$^{V358A/V358A}$ (*A/A*) and (E) quantification presented as average intensity normalized to total protein ± standard deviation. Representative blot of whole embryo lysates (n = 3 embryos per genotype with technical duplicate of each). * $p < 0.05$, one-way ANOVA and Tukey's multiple comparison.

Figure 3.

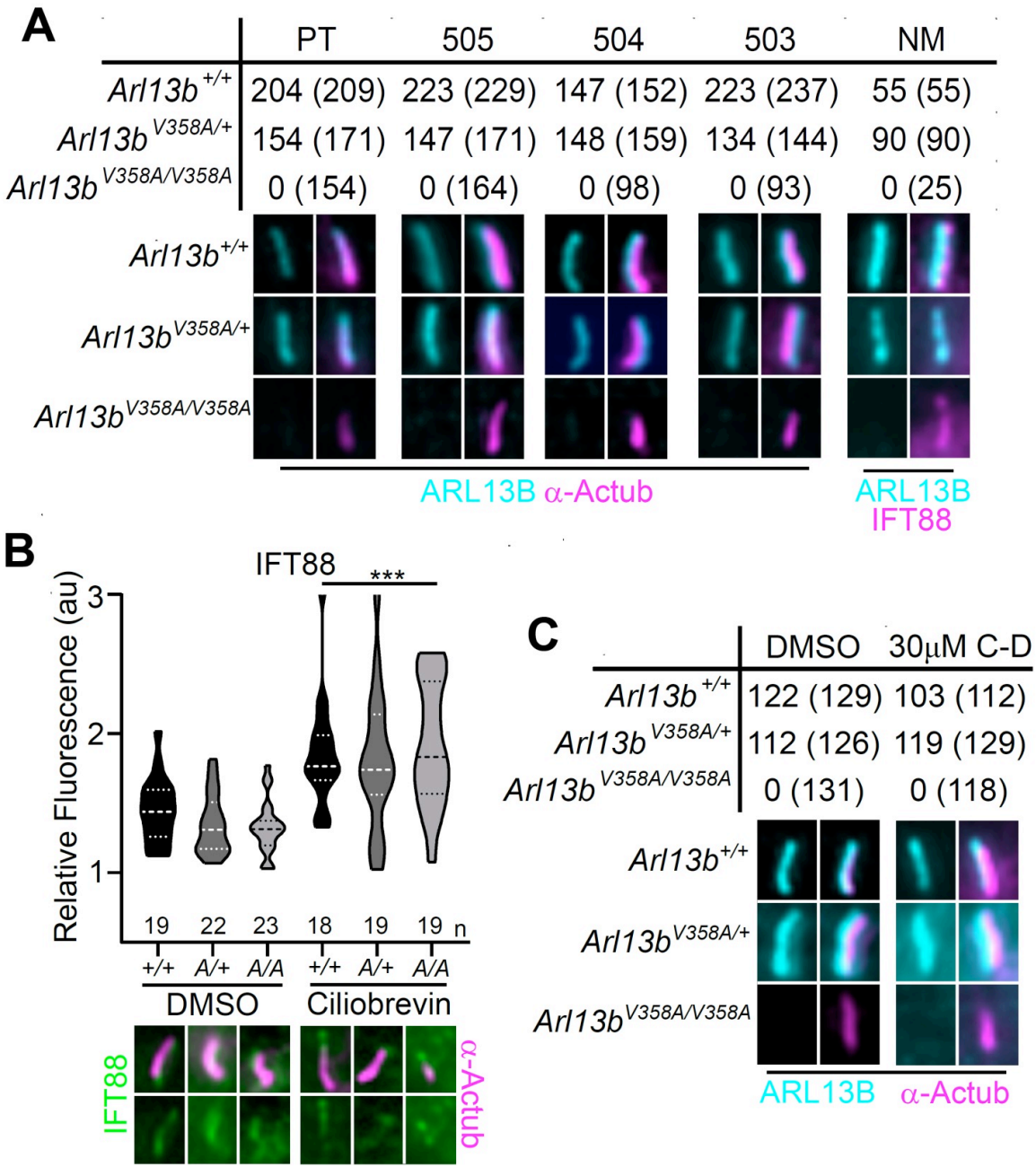


Figure 3.

ARL13B^{V358A} is undetectable in cilia and cannot be enriched by inhibition of retrograde transport.

(A) Antibodies against ciliary markers acetylated α-tubulin or IFT88 (magenta) and ARL13B (cyan) in *Arl13b*$^{V358A/V358A}$ MEFs. Representative images show staining for five indicated ARL13B antibodies: (PT) polyclonal rabbit antibody against full-length human ARL13B from ProteinTech, (503, 504, 505) polyclonal rabbit sera from three distinct rabbits raised against C-terminus of mouse ARL13B (amino acids 208-427) (Caspary et al. 2007), and (NM) monoclonal mouse antibody against C terminus of mouse ARL13B from NeuroMab. *Arl13b*$^{+/+}$ and *Arl13b*$^{V358A/+}$ show ciliary ARL13B staining. Table lists ARL13B-positive cilia and the total number of cilia identified by acetylated α-tubulin or IFT88 antibody in parentheses. Cilia appear shorter in *Arl13b*$^{V358A/V358A}$ cells (see Fig. 6). (B) IFT88 (green) is enriched in the tips of cilia in *Arl13b*$^{+/+}$ (+/+), *Arl13b*$^{V358A/+}$ (*A*/+) and *Arl13b*$^{V358A/V358A}$ (*A/A*) cells following ciliobrevin-D treatment. Violin plots depict relative fluorescence of IFT88 at cilia tip to cell body with number of cilia measured (n) listed beneath each plot. (C) Table lists ARL13B (cyan) positive cilia (rabbit anti-ARL13B; ProteinTech) and the total number of cilia (acetylated α-tubulin: magenta) examined in control (DMSO) and ciliobrevin-D treated (30μM CB) cell lines. Representative images show staining for cilia and ARL13B. Two-way ANOVA and Sidak's multiple comparisons. (*** $p < 0.001$).

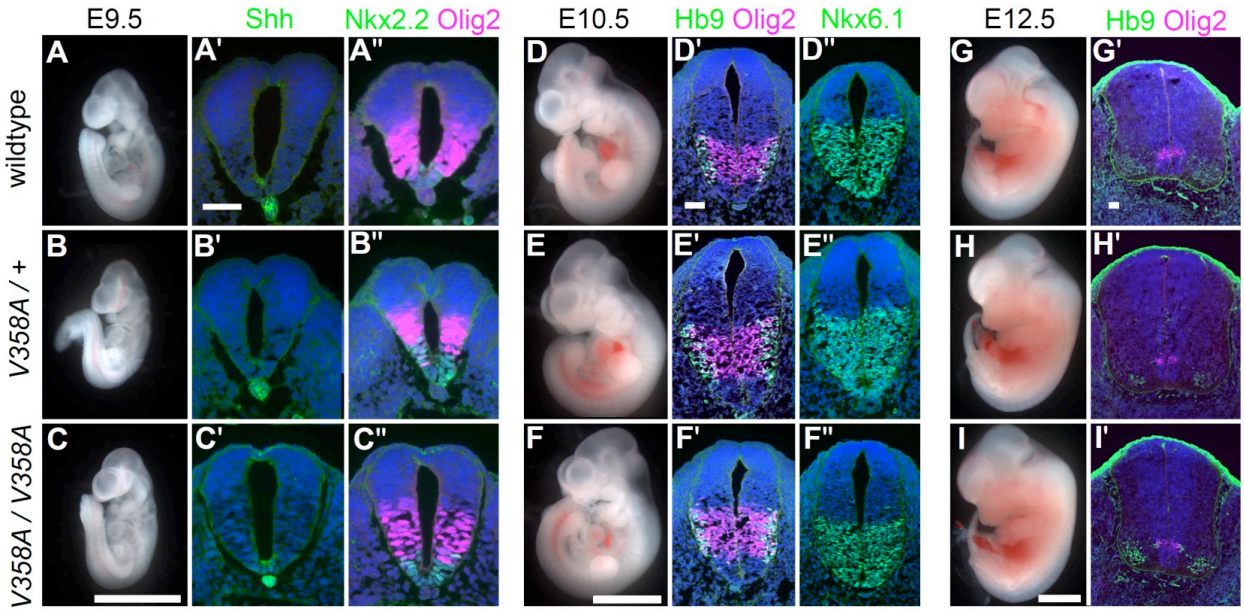


Figure 4.

Arl13b^{V358A} mediates normal Shh signaling and neural tube patterning.

(A-I) Whole embryo and neural tube sections of somite-matched littermates at E9.5, E10.5, and E12.5 stained with indicated markers of cell fate. Whole embryo scale bars: 2 mm. (A'-I' & A"-F") Shh-dependent neural tube patterning at three separate time points. Cell fate markers are listed above each image. All neural tube scale bars are 50 μm. Neural tube patterning was examined in five embryos for each embryonic stage. E, embryonic day.

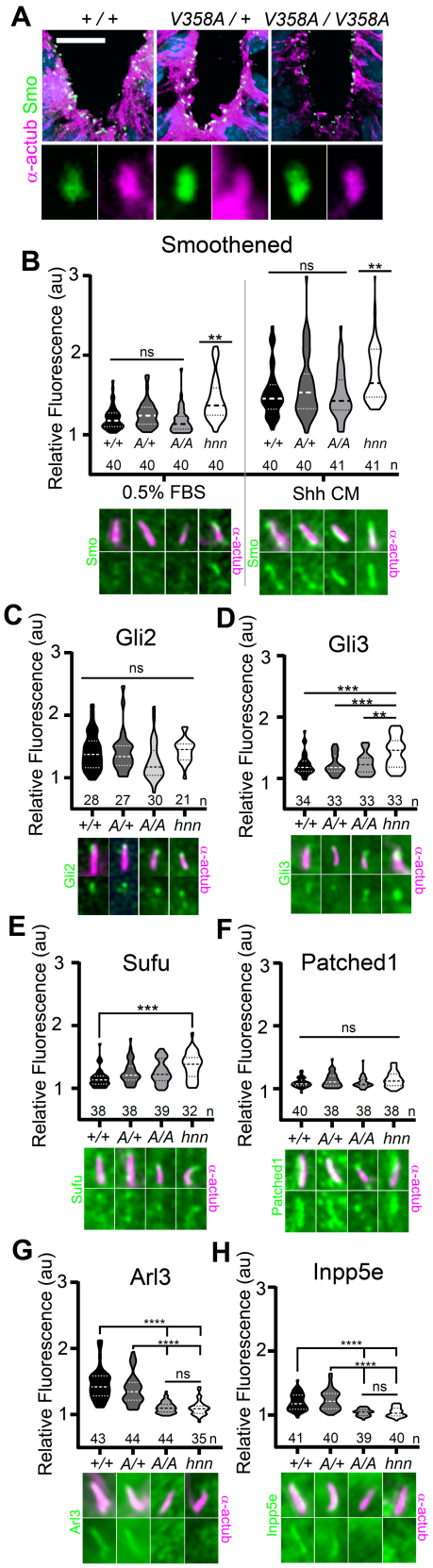


Figure 5.

ARL13B^{V358A} mediates normal ciliary enrichment of Shh components, but not ARL3 or Inpp5e.

(A) Smo (green) enrichment in ventral neural tube cilia (acetylated α-tubulin: magenta) is normal in E10.5 embryos. Images are confocal projections. Scale bar is 25 μm. (B-H, Top) Quantification of average fluorescence intensity in the tip of the cilium (Gli2, Gli3, and Sufu) or the entire cilium (Ptch1, Smo, ARL3, and Inpp5e) relative to background level. Violin plots depict relative fluorescent intensity per cilium with number of cilia examined below each plot. (B-H, Bottom) Representative images for each condition and cell type with the cilia marker acetylated α-tubulin (magenta) and indicated protein (green). Wild type (+/+), heterozygous (*A/*+), homozygous (*A/A)* and *hennin* (*hnn)*. Data analyzed by one-way ANOVA and Tukey's multiple comparisons, except Smo data analyzed by two-way ANOVA and 16 comparisons, corrected $p < 0.003$ deemed significant. (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

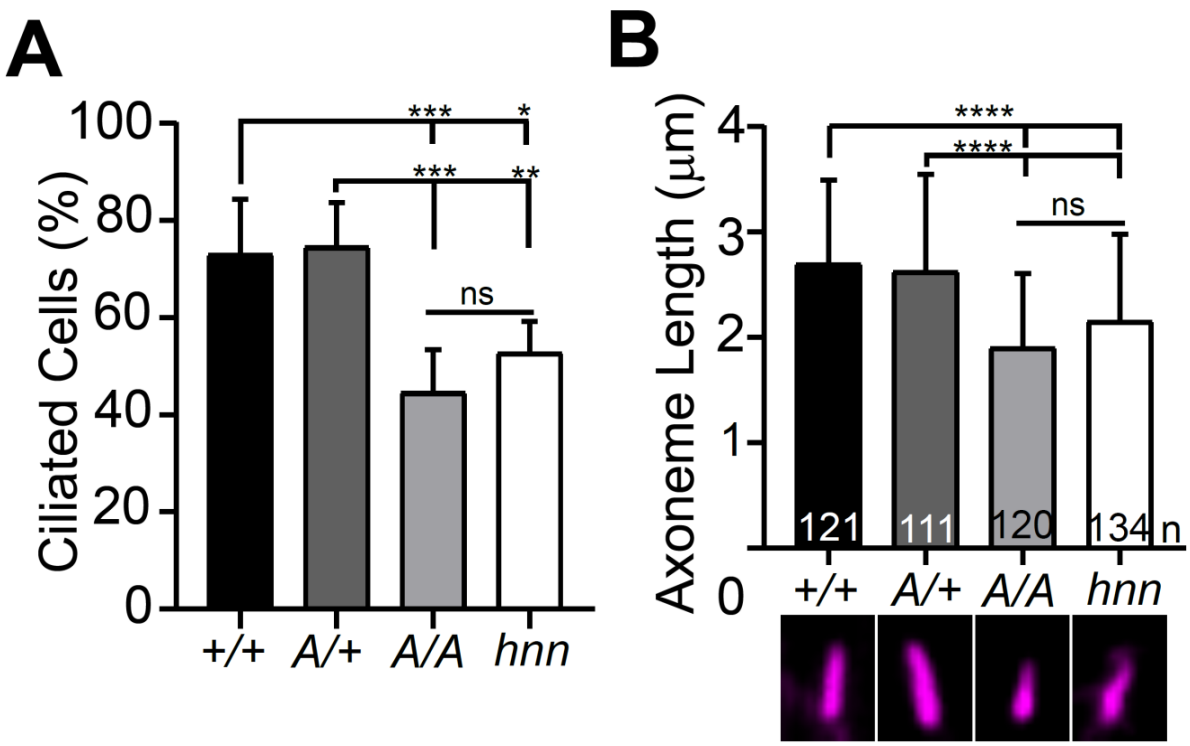


Figure 6.

ARL13B^{V358A} results in decreased ciliation rates and short cilia.

(A) Quantification of ciliation rates in all cell types; wild type (+/+), heterozygous (*A/+*), homozygous (*A/A)* and *hennin* (*hnn)*. Fewer *Arl13b$^{V358A/V358A}$* and *Arl13b$^{hnn/hnn}$* MEFs form cilia compared to *Arl13b$^{+/+}$* or *Arl13b$^{V358A/+}$* cells. (B) Quantification of axoneme length as labeled by acetylated α-tubulin (magenta) in indicated MEFs. Data are presented as mean (μm) ± standard deviation with the number of cilia measured per genotype depicted at the base of each bar. Data analyzed by one-way ANOVA and Tukey's multiple comparisons. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

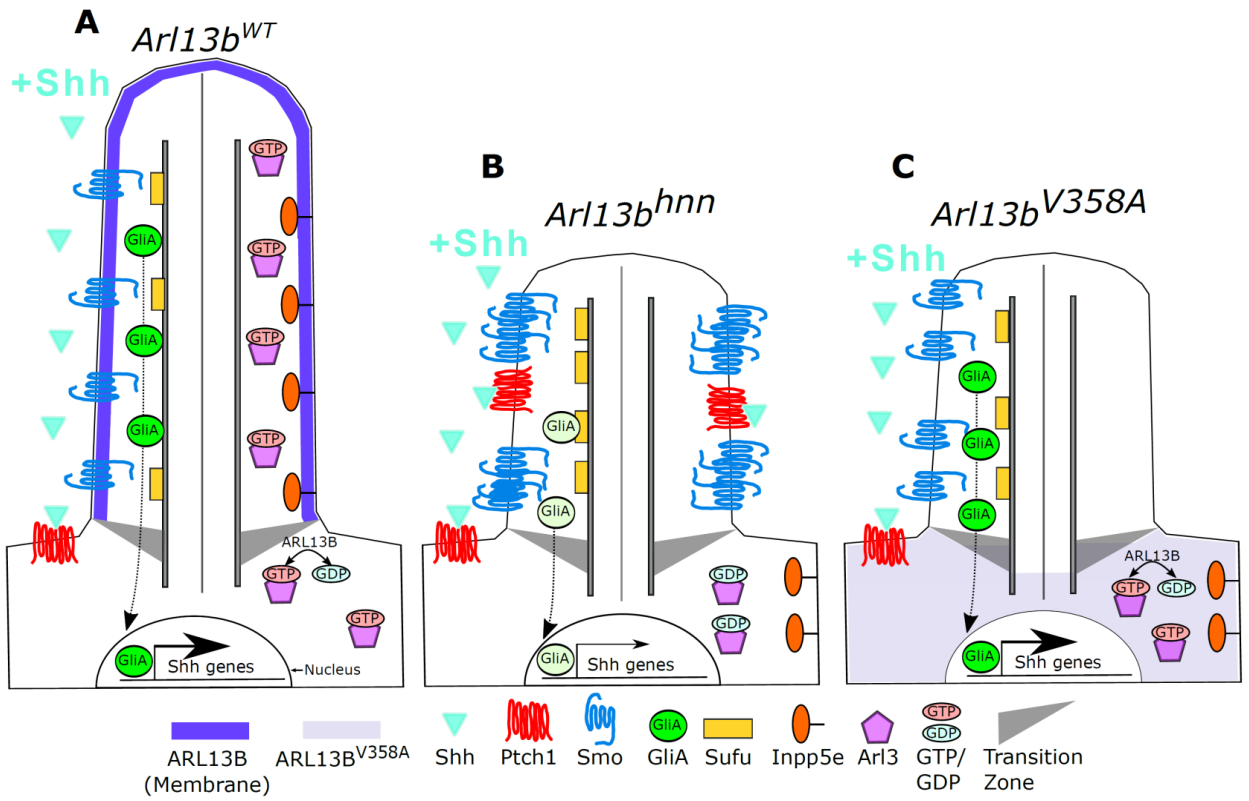


Figure 7.

Model comparing complete loss of ARL13B function to ciliary loss of ARL13B function

Wildtype (left), *Arl13b*^{hnnhnn} (middle), and *Arl13b*$^{V358A/V358A}$ (right) cilia represented as two halves. On the left half is the organization of Shh components in the presence of Shh ligand and on the right half is the organization of ARL13B interactors ARL3 and Inpp5e. (A) ARL13B associates with the ciliary membrane. In the presence of Shh, Ptch1 is removed from cilia and Smo is visibly enriched in cilia. Smo is activated which promotes the processing of full-length Gli transcription factors into their activator forms (GliA), that are shuttled out of the cilium to promote Shh-dependent gene transcription. In addition, cilia proteins ARL3 and Inpp5e are localized to the primary cilium. (B) In *Arl13b*$^{hnn/hnn}$ cells which are null for ARL13B, cilia are shorter than normal. Ciliary Ptch1 and Smo are

visible, although Smo appears punctate instead of diffuse. In addition, loss of ARL13B decreases transcription of Shh-dependent genes due to lowered GliA. *Arl13b*$^{hnn/hnn}$ cilia also display a failure of Inpp5e and ARL3 to localize to the cilium. Because ARL13B is the GEF for ARL3, we speculate in this schematic that ARL3 remains GDP bound in *Arl13b*$^{hnn/hnn}$ cells. (C) In *Arl13b*$^{V358A/V358A}$ cells, ARL13B^{V358A} is not detectable in cilia and appears diffuse within the cell body. *Arl13b*$^{V358A/V358A}$ cilia, like *Arl13b*$^{hnn/hnn}$ cilia, are shorter than wildtype. We observe normal Shh-dependent ciliary Smo enrichment and normal Shh transcriptional output. However, ARL3 and Inpp5e are absent from cilia, indicating that ciliary ARL13B is required for ciliary residence of these proteins.

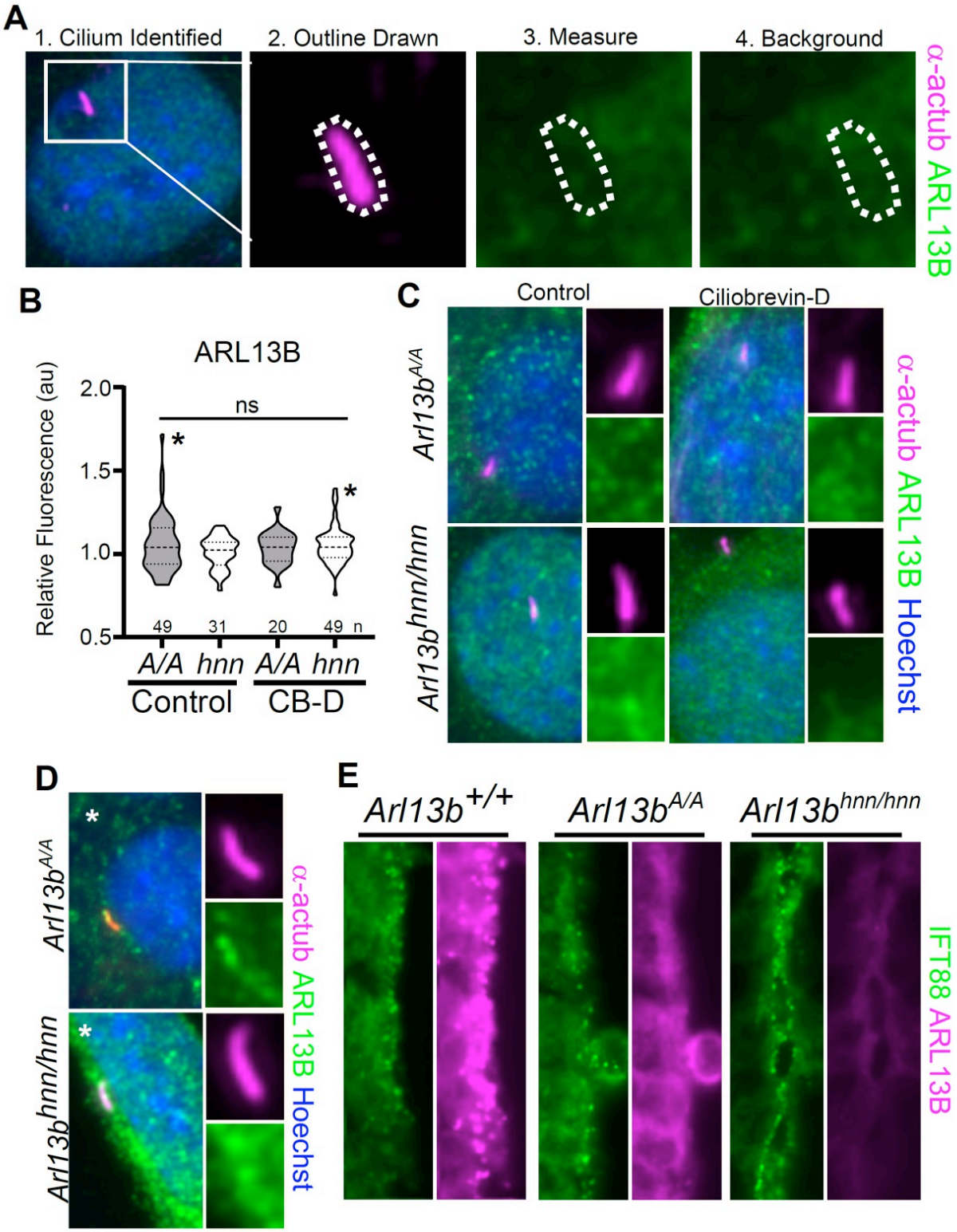
MICE	Sex	wildtype	*V358A/+*	*V358A/V358A*	
Het x Het	M (46.5%)	3	11	6	
Avg. Litter	F (53.5%)	6	8	9	
7.3	% of Total	20.9	44.2	34.9	$\chi^2 = 0.60$
Het x Hom	M (60%)	-	10	11	
Avg. Litter	F (40%)	-	6	8	
7.5	% of Total	-	45.7	54.3	$\chi^2 = 0.69$
Hom x Hom	M (47.4%)	-	-	9	
Avg. Litter	F (52.6%)	-	-	10	
9.5					

Table 1.

852 Genotype of mice born to heterozygous and/or homozygous carrier parents. Data

853 analyzed by chi-squared test.

Supplemental Figure 1.



Supplemental Figure 1.

Overexposure of *Arl13b*$^{V358A/V358A}$ cilia in E10.5 neural tube and MEFs reveals no clear ARL13B^{V358A} presence in cilia.

(A) We identified acetylated α-tubulin positive cilia and used the marker to outline the area of the cilium. We quantified ARL13B immunofluorescence detected by Caspary lab polyclonal anti-ARL13B antibody (Caspary et al. 2007) and used the same trace to measure background levels in a different area of the cell body. (B) *Arl13b*$^{V358A/V358A}$ (*A/A*) and *Arl13b*$^{hnn/hnn}$ (*hnn*) MEFs treated with control or ciliobrevin-D 0.5% FBS Media. Samples were overexposed five times longer than normal, saturating the detector in the ARL13B channel. We measured no difference in ciliary ARL13B immunofluorescence between control and ciliobrevin-D treated *Arl13b*$^{V358A/V358A}$ and *Arl13b*$^{hnn/hnn}$ MEFs. Data presented as violin plots and analyzed by one-way ANOVA. Respective number of cilia listed below each plot. Cilia that had ARL13B immunofluorescent readings above background (*) are shown in panel D. (C) Representative images of over exposed *Arl13b*$^{V358A/V358A}$ and *Arl13b*$^{hnn/hnn}$ MEFs in control and ciliobrevin-D treatment conditions. (D) Representative images of cilia with ARL13B levels above background, marked by * in panel B. (E) Overexposure of cilia in E10.5 *Arl13b*$^{+/+}$, *Arl13b*$^{V358A/V358A}$, and *Arl13b*$^{hnn/hnn}$ embryonic neural tube, stained for IFT88 and ARL13B (NeuroMab). The ARL13B channel was overexposed for four seconds instead of one second.