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Journal Title: Molecular Biology of the Cell
Volume: Volume 27, Number 23
Publisher: American Society for Cell Biology | 2016-11-15, Pages 3780-3790
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1091/mbc.E16-03-0189
Permanent URL: https://pid.emory.edu/ark:/25593/rwp0m

Final published version: http://dx.doi.org/10.1091/mbc.E16-03-0189

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Accessed August 4, 2017 9:11 PM EDT
Arl13b regulates Shh signaling from both inside and outside the cilium

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ABSTRACT The regulatory GTPase Arl13b localizes to primary cilia, where it regulates Sonic hedgehog (Shh) signaling. Missense mutations in ARL13B can cause the ciliopathy Joubert syndrome (JS), and the mouse null allele is embryonic lethal. We used mouse embryonic fibroblasts as a system to determine the effects of Arl13b mutations on Shh signaling. We tested seven different mutants—three JS-causing variants, two point mutants predicted to alter guanine nucleotide handling, and four that disrupt palmitoylation and thus membrane binding—in assays of transcriptional and nontranscriptional Shh signaling. We found that mutations disrupting Arl13b’s palmitoylation site, cilia localization signal, or GTPase handling altered the Shh response in distinct assays of transcriptional or nontranscriptional Shh signaling. In contrast, JS-causing mutations in Arl13b did not affect Shh signaling in these same assays, suggesting that these mutations result in more subtle defects, likely affecting only a subset of signaling outputs. Finally, we show that restricting Arl13b from cilia interferes with its ability to regulate Shh-stimulated chemotaxis, despite previous evidence that cilia themselves are not required for this nontranscriptional Shh response. This points to a more complex relationship between the ciliary and nonciliary roles of this regulatory GTPase than previously envisioned.

INTRODUCTION

The Sonic hedgehog (Shh) signaling pathway regulates many biological processes by controlling the processing and consequently the activity of Gli transcription factors. This transcriptional form of Shh signaling requires the primary cilium; mutations in any of a large number of cilia-associated genes cause serious disruption or total ablation of transcriptional Shh signaling (Huangfu et al., 2003; Mariani and Caspary, 2013). Indeed, most components of the Shh pathway, including the Shh receptor Patched1 (Ptch1), the obligate Shh transducer Smoothened (Smo), and the Gli proteins themselves, are dynamically trafficked in and out of cilia in response to Shh ligand (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). In addition, components of the Shh signaling pathway can also regulate transcription-independent cellular processes. This nontranscriptional branch of the pathway regulates axon guidance (Charron et al., 2003; Sánchez-Camacho and Bovolenta, 2008; Jin et al., 2015) and fibroblast migration (Bijlsma et al., 2007, 2012). Of note, Shh-dependent chemotaxis in fibroblasts occurs even in cells completely lacking cilia, suggesting that nontranscriptional Shh signaling is cilia-independent (Bijlsma et al., 2012).

Joubert syndrome (JS) can result from mutations in >20 different cilia-associated genes (Parisi and Glass, 1993–2016; Romani et al., 2014; Thomas et al., 2014; Tuz et al., 2014), including the gene on which we focus, ARL13B. JS is diagnosed by a neuroanatomical abnormality of the hindbrain known as the molar tooth sign. JS has been described as a disorder of axon guidance (Engle, 2010) and leads to abnormal development of many white matter tracts (Yachnis and Rorke, 1999; Poretti et al., 2007). Some JS signs and symptoms,
such as cerebellar hypoplasia, are reminiscent of transcriptional Shh signaling defects, but the etiology of the axon guidance defects associated with J5 remain elusive. Whether cilia-associated genes regulate nontranscriptional Shh signaling is unknown.

The regulatory GTPase Arl13b regulates the transcriptional response to Shh in a number of ways. Loss of Arl13b misregulates ciliary traffic of Pch1, Smo, Gli2, Gli3, and other Shh-associated proteins (Larkins et al., 2011). In addition to its role in protein traffic, Arl13b regulates Shh transcriptional output downstream of Smo activation. Loss of Arl13b also results in constitutive, ligand-independent production of Gli activator (GliA) but leaves the normal regulation of Gli repressor production intact (Caspar et al., 2007). Arl13b is primarily known as a ciliary protein, and loss of Arl13b leads to abnormalities in cilia structure (Caspar et al., 2007). However, recent studies show that Arl13b is also present in early endosomes and cell surface circular dorsal ruffles (Barral et al., 2012; Casalou et al., 2014), indicating that it also functions outside of the cilium.

Arl13b is ancient, predicted to be present in the last eukaryotic common ancestor. The ARL13 gene appears to have been lost during evolution in organisms that lack cilia and duplicated (to ARL13A and ARL13B) in the urochordates, a common ancestor to all vertebrates. Intriguingly, this duplication coincides with the vertebrate-specific link between cilia and Hedgehog signaling (Logsdon and Kahn, 2003; Li et al., 2004; Kahn et al., 2008; East et al., 2012; Schlacht et al., 2013). In Caenorhabditis elegans, which lacks Hedgehog signaling, arl-13 mutations disrupt cilia structure and ciliary protein traffic (Cevik et al., 2010; Li et al., 2010; Warburton-Pitt et al., 2014). Although no functional or loss-of-function mutations have been reported for Arl13a orthologues, mutations in Arl13b cause a number of defects in humans and model organisms, such as developmental defects in zebrafish (Sun et al., 2004; Duldulao et al., 2009). Total loss of Arl13b is embryonic lethal in mammals (Caspary et al., 2008). Some point mutations in ARL13B are compatible with life and are linked to the human ciliopathy JS (Cantagrel et al., 2008). Previous studies identified JS patients with homozygous R79Q and Y86C mutations in ARL13B, as well as with an R200C mutation in combination with a truncating mutation (Cantagrel et al., 2008; Thomas et al., 2015).

Arl13b is so named because of its sequence homology to the Arf and Arf-like (Arl) family of small GTPases, although this homology is limited to the N-terminal GTPase domain of Arl13b, which comprises only about half of the protein. Arl13b’s N-terminal Arf domain includes all four of the consensus nucleotide-binding motifs, as well as the canonical switch 1 and switch 2 loops that mediate GTPase interactions with effectors and modulators (Joneson et al., 1996; Kuai and Kahn, 2000). However, Arl13b lacks the highly conserved glutamine in the second nucleotide-binding motif: the amino acid sequence is DVGGQ in Arfs and heterotrimeric G proteins but is DLGGG in Arl13b. This change is predicted to alter Arl13b’s biochemical activities because mutation of this glutamine in other GTPases, including Arfs (Zhang et al., 1994), Arls (Van Valkenburgh et al., 2001; Zhou et al., 2006), and Ras (Cox and Der, 2010), leads to a dominant-activating phenotype with loss of both intrinsic and GTPase-activating protein (GAP)-stimulated GTP hydrolysis. Another key feature of Arfs is that they have a conserved glycine residue at their N-terminus that is the site of N-myristoylation (Kahn et al., 1988), which facilitates their association with membranes (reviewed in Donaldson and Jackson, 2011). Many Arls, including Arl13b, lack this conserved glycine. Uniquely among Arls, Arl13b contains near its N-terminus a pair of cysteines that become palmitoylated and are essential for its membrane association and cilia localization (Cevik et al., 2013). Also unique within the Arf family, Arl13b contains a cilia localization signal (CLLS) motif that is homologous to the VxP-containing CLLS in rhodopsin, polycystin-1, and polycystin-2 (Deretic et al., 2005; Geng et al., 2006; Ward et al., 2011). This motif in Arl13b is required for its cilia localization (Higginbotham et al., 2012; Cevik et al., 2013).

We investigated the effects of disease-causing and other predicted functional mutations in murine Arl13b on cilia morphology and Shh signaling. To establish a model for Shh signaling and advance the process of determining the specific roles and mechanisms of action of Arl13b in multiple, distinct pathways, we generated a series of point mutations and examined their abilities to rescue transcriptional and nontranscriptional responses to Shh in mouse embryofibroblasts deleted for the endogenous protein. Previous studies of Arl13b’s function in Shh signaling relied on constitutive or conditional null alleles of Arl13b (Caspar et al., 2007; Horner and Caspary, 2011; Larkins et al., 2011; Su et al., 2012). Furthermore, these studies examined only transcriptional Shh signaling. Our goals include a molecular understanding of Arl13b actions as a regulatory GTPase in Shh signaling in both normal and disease states.

RESULTS
Model system used in our studies
We engineered mutations in murine Arl13b at residues that are conserved across species and predicted to affect protein function (Figure 1A). We generated a G75Q mutation, which restores the homologous glutamine, to determine the significance of Arl13b’s departure from other Arf/Arl proteins at this residue. We also generated a predicted dominant-negative T53N mutation, which is homologous to the RasT53N and Arf1T31N mutations that disrupt guanine nucleotide binding in those proteins (Feig and Cooper, 1988; Dascher and Balch, 1994). Of note, Arl13bT35N was analyzed in other studies, but its effect on Shh signaling was not measured (Hori et al., 2008; Duldulao et al., 2009; Humbert et al., 2012). To study the functional effects of disrupting Arl13b palmitoylation, we used the CBS,CYS mutation, which was previously found to affect membrane and cilia localization of Arl13b. To test the effects of reduced cilia localization on Arl13b functionality, we generated a V358A mutation, which disrupts the CLLS motif. Finally, we generated the R79Q, Y86C, and R200C point mutations in the mouse Arl13b protein, homologous to the JS-causing mutations in humans, to assess their effects on Shh signaling.

To test the effects of Arl13b mutations in the absence of wild-type Arl13b, we used protein null Arl13bfl/fl mouse embryonic fibroblasts (MEFs; Caspary et al., 2007). We infected the immortalized MEFs with lentiviruses driving the expression of wild-type or mutant Arl13b with C-terminal green fluorescent protein (GFP) fusion proteins or GFP alone as a negative control. We previously showed that fusion of GFP to Arl13b’s C-terminus does not interfere with Arl13b function (Larkins et al., 2011). To enrich for transduced cells, we used fluorescence-activated cell sorting (FACS) to purify GFP-positive cells and performed all assays on the resulting heterogeneous cell populations. By analyzing heterogeneous populations, we effectively increase our sample size within a single experiment, as opposed to analyzing multiple clonal populations separately. Our cell populations are composed of MEFs with different insertion sites for the lentiviral expression vector and variable Arl13b-GFP expression levels. Thus the average level of expression within each population may shift, depending on environmental factors such as growth conditions and passage number. Western blot analysis provides an unbiased estimate of the true mean expression level in the population (Supplemental Figure S1). We addressed concerns over the effects
of levels of protein expression by performing Western blots on the cell populations at different times. This enabled us to monitor changes in population-level averages. In fact, we observed consistent results in assays done at a variety of passage numbers, suggesting that even if population average protein expression levels fluctuated between assays, this did not affect the overall results.

**Arl13b mutations affecting its localization to cilia**

We found no significant differences between the percentage of ciliated cells in MEFs expressing GFP alone, Arl13bWT-GFP, or any of the variants (Figure 1C). In primary MEFs, loss of Arl13b results in fewer ciliated cells, but a significant fraction of cells (∼20%) retain their cilia (Larkins et al., 2011). In contrast, our immortalized Arl13bhnn MEFs, expressing Arl13b-GFP or GFP alone, were ciliated at rates ranging from 40 to 55% (Figure 1C). These data raise the possibility that the immortalization process may have altered other aspects that control ciliogenesis—for example, a cell-cycle checkpoint.

**Arl13b mutations affect nontranscriptional Shh signaling in MEF migration**

To determine whether Arl13b regulates nontranscriptional Shh signaling, we tested the effect of Arl13b mutations on Shh-dependent chemotaxis (Bijlsma et al., 2007, 2012). We found that Arl13bhnn MEFs expressing GFP alone showed significant impairment in migration toward either recombinant Shh ligand (rShhN, Figure 2A) or the Smo agonist purmorphamine (Figure 2D) compared with Arl13bWT-GFP expressing MEFs rescued with Arl13bWT-GFP. This supports the conclusion that Arl13b does indeed play a role in mediating Shh-dependent chemotaxis in MEFs. As a control for general cell motility defects, we demonstrated that Arl13bhnn MEFs expressing GFP alone or Arl13bWT-GFP migrate similarly toward fetal calf serum, a nonspecific chemoattractant (Figure 2D).

We next tested several Arl13b mutants—the JS-causing Arl13bR79Q-GFP, Arl13bY86C-GFP, and Arl13bV358A-GFP (Figure 1, B and D; summarized in Table 1). Previous data suggested that Arl13bV358A-GFP and Arl13bhnn-GFP would fail to localize to cilia due to the disruption of Arl13b's palmitoylation site and cilia localization signal, respectively. The behavior of Arl13bK400C-GFP was more surprising, especially given the fact that other JS-causing mutants had no effect on cilia localization, suggesting that different JS-associated ARL13B mutations likely act through distinct mechanisms to cause disease.
as were the other mutants that rescued chemotaxis (Supplemental Figure 1B).

One explanation of these results is that JS-causing Arl13b mutations have only subtle effects on Shh-related Arl13b functions, whereas mutating Arl13b’s CiLS domain ablates or severely disrupts overall protein function. However, on the basis of previous work involving Arl13b mutants (Higginbotham et al., 2012; Humbert et al., 2012; Gotthardt et al., 2015) and Arl13b functioning outside of cilia (Barral et al., 2012; Casalou et al., 2014), we believe it most likely that Arl13b outside of the cilium retains its biochemical properties and functions. To test this, we generated purified recombinant preparations of mouse glutathione S-transferase (GST)–Arl13b and GST-Arl13bV358A after expression in human embryonic kidney 293T (HEK293T) cells. The two proteins expressed to comparable levels, remained soluble throughout purification, and yielded similar levels of purified protein. To assess their biochemical properties and abilities to interact with other biologically relevant partners, we determined the intrinsic and GAP-stimulated rates of GTP hydrolysis, using an assay described previously to purify ARL2 GAPs (Bowzard et al., 2005, 2007). We found strong (∼20-fold) GAP-stimulated activity using a detergent-solubilized preparation from bovine brain as the source of Arl13b GAP. The intrinsic rates of GTP hydrolysis were 0.018 ± 0.019 and 0.013 ± 0.009 pmol of GTP hydrolyzed/min/pmol Arl13b for GST-Arl13b and GST-Arl13bV358A, respectively. The GAP-stimulated rates of hydrolysis for these same two preparations were 0.366 ± 0.020 and 0.316 ± 0.010 pmol GTP hydrolyzed/min/pmol Arl13b. Thus there were no differences between the abilities of GST-Arl13b and GST-Arl13bV358A to serve as substrates in this reaction (Figure 2B).

Chlamydomonas Arl13 was recently shown to have exchange factor activity toward Arl3 (Gotthardt et al., 2015), and so we tested for Arl3 guanine nucleotide exchange factor (GEF) activity of murine Arl13b using a comparable assay, as described in Materials and Methods. The rate-limiting step in activation of regulatory GTPases is typically the release of bound GDP, so we measured the ability of purified Arl13b proteins to increase the rate of dissociation of prebound [35S]GTPγS from purified human Arl3. We used N-terminal truncations, termed Δ19-Arl13b or Δ19-Arl13bV358A, as used previously (Zhang et al., 1994). We found that the rate of dissociation of GDP from Arl3 (1 μM) alone under these conditions was 3.2 × 10⁴ s⁻¹, yielding a half-life of the bound species of ∼37 min. Addition of GST-Δ19-Arl13b or GST-Δ19-Arl13bV358A (2 μM) resulted in dramatic increases in this rate to 3.4 × 10⁻³ and 3.6 × 10⁻³ s⁻¹, respectively, yielding half-lives of only 3 min for Arl3-GDP in the presence of either Arl13b protein. We separately measured the rate of [35S]GTPγS binding in the same assay because the binding of the activating ligand is limited by the release of bound GDP and found comparable effects of added Arl13b proteins in this assay (unpublished data). Cleavage of the GST tag did not alter these rates, suggesting that the tag did not interfere with any of these activities. We interpret these biochemical data as strong evidence that the V358A mutation does not cause defects in protein folding or the ability to bind to either of these two binding partners, including effectors. Instead, this mutation is expected to interfere with a different protein–protein interaction that is required for cilia localization.

**Table 1: Summary of experimental results testing the effects of expressing Arl13b mutants in Arl13bhnn MEFs.**

<table>
<thead>
<tr>
<th>Arl13b variant (in hnn MEFs)</th>
<th>Mutation type</th>
<th>GFP in cilia</th>
<th>Cilia number</th>
<th>Smo in cilia</th>
<th>qPCR Shh response</th>
<th>Shh migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-GFP</td>
<td></td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>GFP only</td>
<td>Null</td>
<td>No</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Reduced</td>
<td>Impaired</td>
</tr>
<tr>
<td>R79Q-GFP</td>
<td>JS</td>
<td>Yes</td>
<td>Varied</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Y86C-GFP</td>
<td>JS</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>R200C-GFP</td>
<td>JS</td>
<td>No</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>T35N-GFP</td>
<td>GTase</td>
<td>Yes</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>G75Q-GFP</td>
<td>GTase</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>V358A-GFP</td>
<td>CiLS</td>
<td>No</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>C85,C95-GFP</td>
<td>Palm.</td>
<td>No</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

Statistically significant differences between Arl13bWT-GFP and other mutants are shown in bold.
pathway components, including Smo itself (Larkins et al., 2007), so the effects we observed on transcriptional signaling could be due to a Smo-independent mechanism. Therefore we assayed for Smo enrichment in the cilia of Arl13b\textsuperscript{V358A} MEFs expressing Arl13b-GFP mutants at baseline and under Shh-stimulated conditions. Because a small amount of Smo is present in cilia even in the absence of Shh signaling (Ocbinia and Anderson, 2008; Milenkovic et al., 2015) and we can detect low levels of Smo in almost all cilia under control conditions, we refer to “Smo-enriched” cilia rather than “Smo-positive” cilia when describing the accumulation of ciliary Smo that normally reflects active Shh signaling.

Consistent with previous reports, in Arl13b\textsuperscript{V358A} MEFs expressing Arl13b\textsuperscript{WT}-GFP, Smo enrichment in cilia occurred upon Shh stimulation, whereas in MEFS expressing GFP alone, Smo enrichment in cilia was evident regardless of Shh stimulation (Figure 4). We observed elevated baseline Smo enrichment, consistent with a deficiency in Arl13b activity, in MEFS expressing Arl13b\textsuperscript{T35N}-GFP and Arl13b\textsuperscript{C8S,C9S}-GFP. Even in cell populations with abnormally high ciliary enrichment of Smo at baseline, we saw a trend toward further Smo accumulation in cilia upon Shh stimulation, suggesting that Arl13b is not the sole determinant of Smo’s Shh-dependent ciliary traffic. Still, results from these studies suggest that Arl13b plays an important role in regulating Smo enrichment in cilia that is sensitive to Arl13b’s presence on cell membranes and its normal regulation of guanine nucleotide cycling. Of interest, this regulation of Smo does not explicitly require Arl13b to be present in cilia: Arl13b\textsuperscript{R200C}-GFP and Arl13b\textsuperscript{V358A}-GFP, each of which failed to localize to cilia, show normal patterns of Smo ciliary enrichment under control and Shh-stimulated conditions. This suggests that Arl13b functions outside the cilium to regulate ligand-dependent Smo enrichment.

**DISCUSSION**

We set out to assign function to particular amino acid residues in the Arl13b protein to see whether we could deconvolve the distinct phenotypes we observe in Arl13b\textsuperscript{hnn} cells and better understand the cellular defects associated with the disease-linked mutations. We examined several classes of mutants, including GTP-handling mutants (T35N and G75Q), JS-causing mutants (R79Q, Y86C, and R200C), a palmitoylation-deficient mutant (C8S,C9S), and a nonciliary mutant (V358A). We found that the mutants affected ciliary localization of Arl13b protein, the transcriptional response to Shh, and Shh-dependent ciliary Smo enrichment in distinct ways. In addition, we extended Arl13b’s functions to include regulation of nontranscriptional, Shh-dependent MEF migration. Because nontranscriptional, Shh-dependent MEF migration occurs in the absence of cilia, we were surprised to find that Arl13b\textsuperscript{V358A}, which was not evident in cilia, was unable to mediate MEF migration (summarized in Table 1). Taken together, our data support Arl13b functioning as a GTPase, albeit an atypical one, and operating both in and out of the cilium in a complex, and potentially interdependent, manner.

Both of the GTase-handling mutants, T35N and G75Q, localized to cilia, yet neither could rescue the decreased Shh transcriptional response of Arl13b\textsuperscript{hnn} MEFs, consistent with alterations in their biochemical activity. However, the mutations affect Arl13b function distinctly: only the T35N mutant, with its presumptive decrease in binding of guanine nucleotides, failed to rescue ligand-independent ciliary Smo enrichment in Arl13b\textsuperscript{hnn} MEFs. In contrast, restoration of the homologous Q in the G75Q mutant was functional in this assay. The palmitoylation-deficient mutant (C8S,C9S) behaved similarly to the T35N mutant, although the C8S,C9S mutant did not localize to cilia. Association of a variety of regulatory GTases with membranes is fundamental to their actions, and so the profound effects of removing sites of palmitoylation are readily understood. We interpret these data as evidence that both membrane association and nucleotide binding are critical components in Arl13b cellular actions. Thus, despite Arl13b being an atypical member of the Arf family with regard to

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**Figure 2:** Nonciliary Arl13b\textsuperscript{V358A} mutant is defective in Shh-stimulated MEF migration despite Arl13b\textsuperscript{V358A} being biochemically indistinguishable from wild-type Arl13b protein. (A) Average total migration for Arl13b\textsuperscript{hnn} MEFs expressing Arl13b-GFP mutants, normalized to wild-type Arl13b-GFP, migrating toward rShhN. N = 12. Analysis described in Materials and Methods. (B) Recombinant mouse Arl13b and Arl13b\textsuperscript{V358A} display indistinguishable intrinsic and GAP-stimulated rates of GTP hydrolysis. (C) Arl13b\textsuperscript{hnn} MEFS expressing GFP or Arl13b\textsuperscript{WT}-GFP normalized to the GFP-only control, migrate similarly toward fetal calf serum (FCS). (D) Arl13b\textsuperscript{hnn} MEFS expressing GFP show impaired migration toward the Smo agonist purmorphamine, which can be rescued by Arl13b\textsuperscript{WT}-GFP.

Arl13b mutations affect Shh-dependent Smo enrichment in cilia

After observing the effects of Arl13b mutants on transcriptional Shh signaling, we investigated whether any of these effects could be related to abnormal traffic of Smo. Our previous work showed that Arl13b\textsuperscript{hnn} MEFS exhibit abnormal Shh-dependent traffic of Shh pathway components, including Smo itself (Larkins et al., 2011). In wild-type cells, Smo is enriched in the cilium only after Shh stimulation. In Arl13b\textsuperscript{hnn} cells, however, Smo is constitutively enriched in cilia and forms abnormal puncta along the length of the cilium, suggesting that Arl13b plays a critical role in the Shh-dependent enrichment of Smo in cilia. It remains unclear whether Arl13b regulates the entry and/or the exit of Smo. However, Arl13b also acts downstream of Smo to regulate Shh signaling (Casparry et al., 2007), so the effects we observed on transcriptional signaling could be due to a Smo-independent mechanism. Therefore we assayed for Smo enrichment in the cilia of Arl13b\textsuperscript{hnn} MEFS expressing Arl13b-GFP mutants at baseline and under Shh-stimulated conditions.
the absence of the key glutamine that is directly involved in GTP hydrolysis in other family members, it retains both intrinsic and GAP-stimulated GTPase activities, suggesting that it uses a distinctive mechanism of nucleotide hydrolysis.

With Arl13b shown to function in key aspects like other members of the Arf family, we are increasingly confident that we can draw on analogies with previous results to predict mechanistic consequences of at least two of the three JS mutations. The R79Q and Y86C mutations are each within the guanine nucleotide binding-sensitive switch 2 and, by analogy, should not alter peptide bonding or nucleotide handling but instead alter one or a subset of specific GTPase-effector interactions(s). Consistent with this, each mutant localizes to cilia and rescued Shh-dependent ciliary Smo enrichment, as well as both transcriptional and nontranscriptional Shh signaling. In contrast, the R200C mutation, in the nonhomologous C-terminal domain, rarely localized to cilia and yet behaved like the wild-type protein in all other regards assayed here. Although this might be unexpected, given that these are pathogenic mutations, the fact that loss of Arl13b is embryonic lethal would be consistent with subtle hypomorphic alleles being compatible with life. Cells in vivo respond to both the amount and duration of Shh signaling (Dessaud et al., 2007, 2008). Because several of the phenotypes in JS patients are consistent with a loss in Shh signaling, it is important to note that our assays were performed at a single time point, and follow-up studies of these mutations in vivo might yet reveal a role for the JS-causing mutations in Shh signaling. Identifying binding partners whose interactions are affected by the switch 2 mutations is also predicted to reveal novel mechanisms of Arl13b actions relevant to JS disease processes.

Regulation of nontranscriptional Shh signaling
Arl13b acts at multiple steps in the transcriptional Shh pathway: it regulates the ligand-dependent ciliary enrichment of Smo and also acts downstream of Smo to regulate Glia (Casparay et al., 2007; Larkins et al., 2011). We found that the Arl13bV358A mutant is deficient in localization to cilia and disrupts Shh-dependent chemotaxis but has no effect on Shh-dependent ciliary enrichment of Smo, suggesting that Arl13b's role in nontranscriptional Shh signaling lies downstream of Smo ciliary enrichment. Little is known about the downstream mechanisms of Shh signaling in chemotaxis, but Arl13b’s common role in both the transcriptional and nontranscriptional pathways suggests that other components may be shared between the two.

The C-terminal domain of Arl13b is not homologous to any other domain, and we lack any structural information about it. Thus, given our observation that the V358A mutation is defective in cilia localization, as well as in Shh-stimulated cell migration, we tested its integrity in biochemical assays. The facts that this point mutant expressed to the same levels in HEK cells, purified the same, remained soluble throughout purification and storage, and displayed indistinguishable intrinsic and GAP-stimulated GTPase activity with the wild-type protein are all consistent with retention of protein folding and some biologically important protein–protein interactions. Of course, this mutant is predicted to be deficient in at least one protein–protein interaction: that involved in sorting of its traffic to cilia and involving the mutated VxP motif.

Decoupling Arl13b function from cilia
One of the most surprising results of this study was the fact that Arl13b mutants that are undetectable in cilia retain some functions. Arl13b is a ciliary protein, and JS, like other diseases resulting from cilia-related genetic mutations, is considered a ciliopathy. Transcriptional Shh signaling is tightly linked to cilia, and yet, nonciliary Arl13bV358A-GFP, as well as nonciliary, Shh-causing Arl13bR200C-GFP, exhibit normal induction of Shh target genes upon ligand stimulation in Arl13bV358A MEFs. Meanwhile, in assays of nontranscriptional Shh signaling, Arl13bV358A-GFP MEFs show significant impairment in their Shh response, despite the fact that the cilium itself is not necessary for Shh-dependent cell migration. This suggests distinct roles for Arl13b in transcriptional versus nontranscriptional Shh signaling, one of which requires its CiLS motif and one of which does not. In contrast, Arl13bR200C-GFP did rescue the nontranscriptional Shh signaling, which could be due to it being undetectable in cilia for a reason distinct from Arl13bV358A-GFP.
Because the cilium itself is not required for Shh-dependent chemotaxis, it is difficult to understand why Arl13b’s CiLS would be required for this process. One model to explain our results is that Arl13b must interact with a binding partner found only in cilia in order to potentiate nontranscriptional Shh signaling, and Arl13bV358A and this binding partner never encounter each other in ciliated cells. Alternatively, the CiLS in Arl13b may regulate more than just cilium localization. Arf and Arl proteins are major regulators of protein traffic at the Golgi and trans-Golgi network, where VxF motifs target rhodopsin, polycystin1, and polycystin2 to cilia in a process that requires Arf4 and Arl3 (Kahn, 2009; Mazelova et al., 2009; Kim et al., 2014). Recent work shows that Arl13b acts as an Arf GEF, and our data argue that Arl13bV358A retains this activity (Gotthardt et al., 2015). Thus mutation of Arl13b’s CiLS may prolong its ability to interact with Arl3 or other effectors at the Golgi/trans-Golgi network, which could affect traffic of proteins to nonciliary regions of the membrane that are important for regulating chemotaxis.

Future studies investigating Arl13b’s role in nontranscriptional Shh signaling are needed to dissect the function of the CiLS motif in regulating Arl13b’s cilium localization and its role in potential cilia-independent functions. Because cells lacking cilia are capable of Shh-mediated chemotaxis, it would be informative to test whether nonciliated cells that also lack Arl13b can migrate toward a Shh source. It may be that Shh-dependent chemotaxis in nonciliated cells depends on interactions between proteins normally found in the cilium that are also capable of interacting when forced into nonciliary compartments. If Arl13b mediates these interactions, one would predict that loss of Arl13b should disrupt Shh-dependent chemotaxis in nonciliated cells, which could be rescued by Arl13bV358A. If the CiLS motif is involved in the formation of protein complexes in nonciliary compartments, however, Arl13bV358A may not be capable of restoring Shh-dependent chemotaxis. Alternatively, because the loss of the cilium results in dysregulation of the many molecules that make up the ciliary proteome, it is possible that Shh-dependent chemotaxis in nonciliated cells occurs via a different mechanism than in ciliated cells. Indeed, previous data suggest that there is some gain of function in Shh-dependent fibroblast migration in nonciliated cells (Bijlsma et al., 2012). Therefore it is possible that Arl13b is involved only in the ciliary Shh signaling cascade and that nonciliated cells would be capable of Shh-dependent chemotaxis even without Arl13b. Our data indicate that the relationship between Arl13b, cilia, and Shh signaling is more nuanced than previously believed and provide a foundation for exploring these questions.

In sum, we found that Arl13b plays multiple roles in the regulation of Shh signaling both within and outside of cilia and separated these functions using point mutations in Arl13b. Our results also showed that JS-causing Arl13b mutants disrupt Arl13b function through multiple mechanisms and yet give rise to the same disease phenotypes. This work emphasizes the importance of investigating the diverse functions of ciliary gene products as we continue to unravel the molecular etiology of JS and other ciliopathies.

MATERIALS AND METHODS
Mice and genotyping
All mouse work was performed at Emory University under approved Institutional Animal Care and Use Committee protocols. Arl13b WT mice were generated in an N-ethyl, N-nitrosourea screen (Caspy et al., 2007) on a C57/B16 background and maintained on a C3H background. Genotyping for the Arl13b V358A allele uses strain-specific linked markers to detect whether mice are B6 or C3H both upstream and downstream of the Arl13b locus. Arl13b V358A genotyping primers were hnn147, forward, AAT GCC TCA AGT GCC TCT TT, and reverse, GGG ACT CAT CTT TGG GAA CA; and hnn174, forward, TGT GGG TGG CAT ATG TAG GA, and reverse, GCT AGC TAT TTT CTG TTT CTG GA.

Generating MEFs
Pregnant dams at embryonic day 12.5 (E12.5; determined by the presence of a vaginal plug at E0.5) were killed by cervical dislocation and embryos dissected into cold phosphate-buffered saline (PBS). After removal of the head and visceral organs, embryos were mechanically dissociated by being passed several times through an 18-gauge needle in warm MEF medium (DMEM plus 10% fetal bovine serum [FBS] and 1x penicillin/streptomycin). Dissociated MEFs were cultured in gelatin-coated tissue culture dishes (0.1% gelatin in water, autoclaved and filtered, was placed on dishes for at least 1 h before use and then aspirated off before plating MEFs).

Primary MEFs at passage two or three were cultured until 50–75% confluent and then transfected with a SV40 large T antigen construct to induce immortalization. Immortalizing MEFs were cultured until confluent and split 1:10 six times (a final dilution factor of 1:1,000,000 from the original primary culture) before being considered immortalized. Immortalizing MEFs that took >14 d to become confluent after a 1:10 split were discarded.

Generating lentiviruses
Arl13b WT-GFP and Arl13b V358A-GFP lentiviruses were produced by the Emory University Center for Neurodegenerative Disease Viral Vector Core. All other lentiviruses used were generated in the Caspary lab by cotransfecting HEK293FT cells with envelope (pMD2.G), packaging (psPAX2) and lentiviral Arl13b expression (generated from L13-Arl13b-GFP) plasmids and collecting virus-containing supernatant from the cells. At 24 h after transfection, medium was changed to 10 ml of DMEM plus 10% FBS. This medium was left on the virus-producing transfected cells for 24 h and then passed through a 0.45-μm filter and stored at 4°C. This collection procedure was repeated the following day.

FACS purification of lentiviral-infected MEF lines
Immortalized MEFs were plated at low density in 50% MEF medium plus 50% lentiviral supernatant. MEFs were cultured for 48–72 h to allow time for lentiviral infection and virus expression before FACS. On the day of sorting, MEFs were detached using 0.25% trypsin and resuspended in sort buffer (Ca2+/Mg2+-free PBS with 1% FBS, 1 mM EDTA, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.0, sterilized through a 0.22-μm filter).

Sorting was performed by the Emory University School of Medicine Core Facility for Flow Cytometry or the Emory Children’s Pediatric Research Center Flow Cytometry Core using a BD FACS Aria II Cell Sorter. A 100-μm nozzle was used to accommodate the relatively large size of the MEFs. Before sorting lentivirus-infected MEFs for GFP, an uninfected negative control was used to define gates for single cells using forward scatter and side scatter. GFP-positive infected cells were purified through a two-step process: a presort enrichment to isolate GFP-positive MEFs, followed by single-cell sorting of the GFP-enriched population into a 24-well plate. Depending on the number of infected cells in a given sample, from one to four wells were filled with 750–5000 GFP-positive MEFs. This process creates a genetically heterogeneous cell population produced from multiple founder cells representing a variety of genomic insertion sites for the lentiviral construct with a variety of GFP expression levels.
Immunofluorescence staining

Arl13b-GFP MEF lines were labeled with primary antibodies for acetylated α-tubulin (1:2500; T6793; Sigma-Aldrich, St. Louis, MO), GFP (1:1000; ab13970; Abcam, Cambridge, UK), and/or Smo (1:500; a gift from the Kathryn Anderson lab, Memorial Sloan Kettering Cancer Center, New York, NY), fluorescent secondary antibodies (Alexa Fluor 488 or 568 for the appropriate species, 1:500; Life Technologies, Carlsbad, CA), and Hoechst nuclear stain (1:3000).

Image analysis

Fluorescent micrographs were acquired with a 40x objective on a Leica CTR6000 microscope using SimplePCI software. For experiments quantifying the number of cilia, microscopy fields for imaging were chosen based only on Hoechst staining to ensure unbiased sampling of ciliated versus nonciliated cells. For experiments quantifying the number of GFP- or Smo-positive cilia, microscopy fields for imaging were chosen based on the acetylated α-tubulin staining to ensure that an appreciable number of cilia were present in each image.

Before analysis, images were coded using blind analysis (Salter, 2016), a script that replaces the original file names with a random alphanumeric string and creates a .csv file keypairing original and coded file names to be used for later decoding. All image analysis was done while blinded to Arl13b mutant and (where applicable) control versus Shh treatment conditions.

Images were analyzed using the Fiji distribution of ImageJ software (Schindelin et al., 2012). For experiments testing the ciliogenesis rate of different MEF lines, nuclei were counted based on Hoechst staining, and cilia were counted based on acetylated α-tubulin staining. All other measurements analyzed only ciliated cells. For experiments measuring the presence or enrichment of GFP or Smo in cilia, cilia were defined using acetylated α-tubulin staining in one color channel, and then the color channel representing GFP or Smo was examined to see whether staining colocalized with acetylated α-tubulin (Figure 1B).

Migration assays

MEF migration assays were performed using the FluoroBlok Transwell system (BD Falcon, Corning, NY) as in Bijlsma et al. (2012). Before beginning the migration assay, MEFs were washed with PBS and incubated with 10 μM CellTracker Green in serum-free DMEM for 1 h (cells were equilibrated in serum-free DMEM for 1 h before use). Cells were detached using 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS, resuspended and washed in serum-free DMEM, and transferred to a Transwell insert (8-μm pore size) in 100 μl of culture medium. Before beginning the migration assay, MEFs were washed with PBS and incubated with 10 μM CellTracker Green in serum-free DMEM for 1 h (cells were equilibrated in serum-free DMEM for 1 h before use). Cells were detached using 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS, resuspended and washed in serum-free DMEM, and transferred to a Transwell insert (8-μm pore size) in 100 μl of medium at a concentration of 50,000 cells/well. The lower portion of the Transwell plate contained 600 μl of control or attractant (0.1 μg/ml rShhN or 2 μM purmorphamine) medium. During migration, the plate reader measured fluorescence in the lower portion of the Transwell plate once every 2 min for ~3 h. For each cell population, 12 different wells were analyzed using this method.

To analyze migration data, several corrections were applied. Background fluorescence was controlled by subtracting the average value of plate reads from a blank well (containing medium but no cells) over the course of the entire experiment from each individual plate read for the cell-containing wells. Nonspecific migration of each cell line was controlled by subtracting the average fluorescence of cells migrating toward control medium from each individual plate read for the corresponding cells in the attractant condition. Finally, migration curves for each cell line were made more directly comparable by setting the starting point of each normalized migration curve to zero. This was done by subtracting a normalizing value from every point on the migration curve such that the value of the first point on the curve was equal to zero. Pooling of data was done using these normalized curves expressed relative to the Arl13bWT-GFP control from each experiment (Figure 2A) or relative to the GFP-only control (Figure 2C).

Protein expression and purification

Human embryonic kidney 293T (HEK; American Type Culture Collection) cells were grown in 10-cm plates in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals) at 37°C in a humidified environment gassed with 5% CO2. The medium was switched to DMEM with 2% FBS, and cells were transfected with 1 μg of DNA/ml of medium at a cell density of ~90% using 3 μg of polyethyleneimine (PEI Max; 24765-2; Polysciences)/ml of medium. All Arl13b protein expression was achieved using the pLEXm-GST parent plasmid, a gift from James Hurley (National Institutes of Health, Bethesda, MD), which uses a chicken β-actin promoter to drive expression and includes a TEV protease cleavage site after the GST and immediately upstream of the inserted open reading frame (Ivanova et al., 2014). Testing found that 2 d was optimal for protein expression. Thus cells were harvested 48 h after transfection, pelleted by centrifugation at 3000 × g, frozen in liquid nitrogen, and stored at −80°C until used for protein purification. Cells were thawed on ice and lysed in five volumes of lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1% 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate [CHAPS], protease inhibitor mixture [S8830; Sigma-Aldrich], and 10 μg/ml deoxyribonuclease I [D4263; Sigma-Aldrich]). The cells were maintained on ice for 30 min before cell debris was removed by centrifugation at 14,000 × g for 10 min at 4°C. The proteins were purified with GST-affinity chromatography. Specifically, 500 μl of glutathione-Sepharose 4B (17-0756-01; GE Healthcare, Little Chalfont, UK) beads was added to 120 mg of proteolytic lysate and incubated at 4°C for 2 h. The beads were then loaded into a disposable column and washed three times with five column volumes of lysis buffer. The proteins were eluted from the beads with three column volumes of elution buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM glutathione) and stored at −80°C.

Arl13b GAP assay

The intrinsic and GAP-stimulated GTPase activity of purified recombinant murine Arl13b proteins was determined using the GAP assay described previously for ARL2 (Bowzard et al., 2005, 2007), with minor modifications. The source of Arl13b GAP was bovine brain, which was homogenized before preparing a detergent (1% CHAPS) extract and clarifying by centrifugation at 14,000 × g. Briefly, GST-Arl13b or GST-Arl13bV358A was preloaded with [γ-32P]GTP at 30°C for 1–3 h in loading buffer (25 mM HEPES, pH 7.4, 10 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 5 mM ATP, 0.1% cholate/3 mM dimyristoyl phosphatidylcholine). The GTPase reaction, performed in a total volume of 50 μl in the assay buffer (25 mM HEPES, pH 7.4, 2.5 mM MgCl2, 1 mM dithiothreitol [DTT], 2.5 mM GTP), was initiated by the addition of 5 μl of preloaded Arl13b. The reactions were incubated for 4 min at 30°C before being stopped by the addition of 750 μl of ice-cold charcoal suspension (5% Norita charcoal in 50 mM NaH2PO4, pH 80°C).

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4°C. The amount of 32P, released during GTP hydrolysis was measured by liquid scintillation counting. The experiments were repeated at least twice with at least two different preparations of each protein performed in triplicate.

**Arl3 GEF assay**

The ability of purified recombinant murine GST-Δ19-Arl13b or GST-Δ19-Arl13bΔ236A to serve as a GEF for Arl3 was determined using a modification of the assay described in Gotthardt et al. (2015). Human Arl3 was expressed in and purified from bacteria, as previously described (Van Valkenburgh et al., 2001). Briefly, purified recombinant human Arl3 (final concentration 1 μM) was incubated at 30°C in 25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl2, and 1 μM [3H]GDP (specific activity 3000 cpm/pmol; PerkinElmer Life Sciences, Waltham, MA), along with the indicated concentration of purified Arl13b preparations. Radioligand binding/release was performed in triplicate. Start time points were chosen after previously determined that the rate of GDP dissociation was linear with time for at least 10 min under these conditions. In addition, the GEF activity of Arl13b toward Arl3 was found to increase linearly with Arl13b concentration in the range of 0.1–5 μM Arl13b, approaching saturation at >10 μM, with a half-maximal rate seen at 1–2 μM Arl13b.

**Quantitative real-time PCR**

MEFs were plated in six-well plates at densities of 3 × 105 cells/well and were treated with 0.5% serum control medium or Shh-conditioned medium (Taipale et al., 2000) 24 h after plating. After 24 h, MEFs were detached using 0.25% trypsin, spun down, and flash-frozen on dry ice. Cell pellets were kept at −80°C until RNA extraction. Cell pellets were lysed with RLT lysis buffer and QiAshredder (79656) homogenizer columns (Qiagen, Hilden, Germany). RNA was extracted using the Qiagen RNeasy kit (74104), including the on-column DNase digest to minimize contamination with genomic DNA.

First-strand cDNA synthesis was performed using the iScript Reverse Transcription Supermix (1725270), 3 μl of 1:100 forward primer, 3 μl of 1:100 reverse primer, 2 μl of diluted cDNA, 10 μl of Bio-Rad SsoAdvanced Universal SYBR Green Master Mix (1725270), and 0.5 μl of 50 mM sodium-Tris, pH 7.4, 150 mM NaCl, 2% (vol/vol) NP-40, 0.5% (wt/vol) deoxycholate, 0.1% (wt/vol) SDS, and 1 mM DTT. Ly-sates were clarified by centrifugation at 20,000 × g at 4°C for 45 min. A 20-μg amount of protein was separated on 10% Bio-Rad MiniPROTEAN TGX Stain-Free Precast Gels (4568034). Gels were imaged after activation using the Bio-Rad ChemiDoc Touch Imaging System and transferred to a 0.2-μm nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer System with the Bio-Rad defined High Molecular Weight setting. Blots were imaged again to ensure proper transfer before being blocked for 30 min with Pierce Superblock T20 (PBS; 37516). Primary antibodies were diluted in T20, and blots were incubated in primary antibody overnight at 4°C.

**Statistical analysis**

Data were analyzed for statistical significance using Prism 6 (GraphPad Software, San Diego, CA). One-way or two-way analysis of variance (factors: Arl13b genotype; control/Shh treatment) was performed using Dunnett’s multiple comparison test for simple effects of Arl13b genotype, with the mean of each Arl13b mutant compared with a control mean and an overall α level of 0.05.
REFERENCES


