Palmitoylation of the ciliary GTPase ARL13b is necessary for its stability and its role in cilia formation

Primary cilia are hairlike extensions of the plasma membrane of most mammalian cells that serve specialized signaling functions. To traffic properly to cilia, multiple cilia proteins rely on palmitoylation, the post-translational attachment of a saturated 16-carbon lipid. However, details regarding the mechanism of how palmitoylation affects cilia protein localization and function are unknown. Herein, we investigated the protein ADP-ribosylation factor-like GTPase 13b (ARL13b) as a model palmitylated ciliary protein. Using biochemical, cellular, and in vivo studies, we found that ARL13b palmitoylation occurs in vivo in mouse kidneys and that it is required for trafficking to and function within cilia. Myristoylation, a 14-carbon lipid, is shown to largely substitute for palmitoylation with regard to cilia localization of ARL13b, but not with regard to its function within cilia. The functional importance of palmitoylation results in part from a dramatic increase in ARL13b stability, which is not observed with myristoylation. Additional results show that blockade of depalmitoylation slows the degradation of ARL13b that occurs during cilia resorption, raising the possibility that the sensitivity of ARL13b stability to palmitoylation may be exploited by the cell to accelerate degradation of ARL13b by depalmitoylating it. Together, the results show that palmitoylation plays a unique and critical role in controlling the localization, stability, abundance, and thus function of ARL13b. Pharmacological manipulation of protein palmitoylation may be a strategy to alter cilia function.

Cilia are hairlike microtubule-based protrusions of the plasma membrane that are found on most mammalian cells. A wide range of human diseases arise from mutations involving genes that encode proteins that localize to cilia. Collectively known as ciliopathies, these diseases, which include Meckel-Gruber syndrome, Joubert’s syndrome, and nephronophthisis, commonly involve a diverse array of abnormalities, including renal cysts, heterotaxy, polydactyly, neurological deficits, and ultimately function. A wide variety of proteins of different functional classes and subcellular localizations are palmitoylated, including receptors, ion channels, scaffolds, and signaling proteins. Palmitoylation has several unique features that distinguish it from two other common lipid modifications, myristoylation and prenylation. First, palmitoylation is reversible. Numerous enzymes that catalyze both palmitate addition and removal (e.g. APT1 and others) have been identified. Dynamic palmitoylation has been observed for multiple proteins (4) and shown to play a role in certain signaling cascades (5) and in protein localization (6). Second, palmitoylation may occur at a variety of sites on the protein, whereas myristoylation occurs at the amino terminus and prenylation at the carboxyl terminus. Third, a single protein may have multiple palmitoyl groups either adjacent or at different locations.

Many cilia proteins require lipid modifications, such as myristoylation, prenylation, and/or palmitoylation, for proper trafficking and localization to cilia. For at least some myristoylated and prenylated proteins, the lipid modification serves to mediate binding to chaperones (UNC119b for myristoylated proteins (7) and PDE6b for prenylated proteins (8–10)), which in turn facilitate solubilization and localization to cilia. However, the mechanisms by which palmitoylation promote cilia localization are unknown. Additionally, the functional roles (apart from trafficking) for protein palmitoylation within cilia are unknown.

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Palmitoylation of ARL13b

In this paper, the cilia protein ARL13b is used as a model palmitoylprotein to begin to explore the roles of palmitoylation in mediating trafficking to and function within cilia. ARL13b is a GTPase that is highly localized to cilia. Human mutations in Arl13b lead to the disease Joubert’s syndrome (11), which is characterized by abnormal cilia function leading to neurological and other developmental deficiencies. Similarly, null mutations in Arl13b cause neurodevelopmental abnormalities (12) and kidney cysts in mouse (13, 14) and lead to kidney cysts and abnormal body curvature in zebrafish (15, 16). Palmitoylation of ARL13b has been reported in Caenorhabditis elegans and was shown to be essential for efficient trafficking of ARL13b to cilia (17, 18).

Herein, palmitoylation of ARL13b is demonstrated in mouse kidneys and in a mouse renal tubular cell line. Further, in a series of biochemical, cell biological, and in vivo experiments, palmitoylation is shown to be required both for trafficking to cilia and for function of ARL13b. Palmitoylation probably exerts these effects via mechanisms beyond simple membrane association, as substituting myristoylation or farnesylation for palmitoylation can restore membrane association but not protein function. Palmitoylation is shown to be a key regulator of ARL13b stability, a property that may contribute to its role in ARL13b function. Additional results suggest that the cell may trigger degradation of ARL13b by depalmitoylating it when cilia resorption is required.

Results

Numerous cilia proteins are palmitoylated

Several cilia proteins have been reported to be palmitoylated and to require the lipid for proper trafficking to cilia. In addition to ARL13b, calflagin (19), fibrocystin (20), and probably STRADβ (21) all require palmitoylation for cilia localization. To explore further the frequency of palmitoylation among cilia proteins, a set of cilia-localized proteins identified in a proteomic study (21) was searched in SwissPalm, a comprehensive database of palmitoylproteins, http://swisspalm.epfl.ch/ (3), 2 Of the 162 “Tier 1” (i.e. high confidence) cilia proteins, 56 (35%) were found in SwissPalm. Further, of 209 Tier 2 cilia proteins, 67 (32%) appeared in SwissPalm. These results suggest that palmitoylation of cilia proteins is widespread.

ARL13b requires palmitoylation for cilia localization

Palmitoylation of ARL13b has previously been reported in the C. elegans ortholog ARL-13 as well as in the mammalian ARL13b when transfected in retinal pigmented epithelial cells (17, 18, 22). To investigate whether ARL13b is palmitoylated in intact mouse tissue, the resin-assisted capture (RAC) 3 protocol was used to purify palmitoylated proteins from lysates of mouse kidney (23, 24). The RAC protocol involves cleavage of the thioester-linked lipids using neutral hydroxylamine, followed by capture of the newly liberated thiol of the cysteine side chain using thiol-binding resin. ARL13b was indeed shown to be palmitoylated in mouse kidneys (Fig. 1A). Similarly, palmitoylated ARL13b was identified in cultured mouse kidney cells derived from inner medullary collecting duct cells (IMCD3) (Fig. 1A). The site of palmitoylation has been reported to involve cysteines at the amino terminus of ARL13b (17). Accordingly, Cys-8 and Cys-9 were mutated to serines, and the resulting construct (C8S/C9S) was expressed in HEK293 cells. This mutant was not palmitoylated, supporting the conclusion that Cys-8 and Cys-9 are probably sites of palmitoylation (Fig. 1B). The cellular localization of C8S/C9S mutant was investigated in transiently transfected IMCD3 cells by immunofluorescence (Fig. 1C). The WT construct localized nearly exclusively to the cilia, with virtually no expression detected in the cell body. In contrast, the C8S/C9S mutant localized to the cytoplasm (Fig. 1C), in agreement with previous reports (17, 18, 22). These data suggest the importance of palmitoylation in localization of ARL13b to cilia.

Palmitoylated ARL13b is required for cilia elongation in IMCD3 cells

To test the functional role of ARL13b palmitoylation in IMCD3 cells without the confounding effects of native ARL13b expression, the Arl13b gene was knocked out using CRISPR/Cas9. In particular, the first exon of Arl13b was deleted using two guide RNAs targeting sites flanking exon 1 (Fig. 2A). Clonal cell lines derived from IMCD3 cells transiently transfected with Cas9 and the guide RNAs were analyzed by a PCR-based genotyping strategy, and the line 2E9 was identified, which exhibited deletion alleles but no evidence of an intact WT allele (Fig. 2B). Sanger sequencing of the Arl13b gene in 2E9 revealed the presence of two distinct deletion alleles, which differed by 2 nucleotides (supplemental Fig. S1A), each of which lacked exon 1. Absence of the ARL13b protein in 2E9 was verified by Western blotting (Fig. 2C). Arl13b has been reported to be necessary for proper cilia structure and function in C. elegans (17), zebrafish (15), mice (12), and probably humans. Accordingly, 2E9 cells exhibit stunted cilia that are only a fraction of the size of those in IMCD3 cells (~0.5 versus 4.0 μm) (Fig. 2 (D and E) and supplemental Fig. S1C). The cilia appeared shortened when stained with both acetylated tubulin as well as with a second marker, IFT81, suggesting that the appearance was not merely due to loss of acetylated tubulin (supplemental Fig. S1C). The formation of elongated cilia (~6.5 μm) was rescued by stable expression of WT ARL13b in 2E9 cells via lentiviral transduction (2E9+WT) (Fig. 2D). The cilia in 2E9+WT were in fact longer than in IMCD3, probably due to overexpression of ARL13b (~10-fold greater in 2E9+WT than in IMCD3 as judged by Western blotting; supplemental Fig. S1B) as a consequence of the strong promoter used in the lentiviral construct. Cilia length has been reported to depend on expression levels of ARL13b (25). ARL13b C8S/C9S could be robustly expressed in 2E9, but it localized to cytoplasmic puncta (as was observed in transiently transfected IMCD3 cells in Fig. 1C), and it did not rescue the cilia length defect (Fig. 2, D and E). This result suggests that the nonpalmitoylated ARL13b cannot serve its usual function in support of cilia structure, probably because of its

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3 The abbreviations used are: RAC, resin-assisted capture; 2BP, 2-bromo-palmitate; CYX, cycloheximide; IMCD3, inner medullary collecting duct cell line; 17-ODYA, 17-octadecynoic acid; dpf, days postfertilization; HCQ, hydroxychloroquine; HDFP, hexadecylfluorophosphonate.
inability to localize to cilia. The importance of cilia localization in ARL13b function was demonstrated previously in zebrafish (15).

Regulation of ARL13b palmitoylation

To explore whether nucleotide binding or other features of ARL13b might affect palmitoylation, a series of mutants was studied for ability to incorporate palmitate, localize to cilia, and support cilia elongation. In particular, mutations predicted to affect nucleotide binding site (T35N and G28V) (15), mutations that cause Joubert’s syndrome in humans (R79Q and R200C) (11), and a truncation of the large carboxyl-terminal domain distal to the guanine nucleotide binding domain (residues 1–308) were prepared. They were stably expressed in the 2E9 line via lentiviral transduction. ARL13b R200C could not be characterized due to very poor expression levels. All of the remaining mutants were found to be palmitoylated to levels similar to or greater than WT when incorporation of the palmitate analog 17-octadecynoic acid (17-ODYA) (26) was normalized to total ARL13b protein levels (Fig. 3A), suggesting that neither the status of the bound nucleotide nor the presence of the carboxyl-terminal domain affects palmitoylation. Mutations predicted to affect nucleotide binding (T35N and G28V) did, however, lead to cilia that were significantly shorter than those in cells expressing WT ARL13b, suggesting that nucleotide exchange may play a role in the function of ARL13b (Fig. 3C), as was reported in zebrafish (15). Further, the truncation mutant 1–308 did not rescue the cilia length defect of the 2E9 line. A fraction of it seemed to localize to the stunted cilia present in 2E9 (Fig. 3B), but a larger fraction appeared to reside in the plasma membrane as well as in vesicular structures. These data suggest that palmitoylation occurs before entry of ARL13b into the cilia and that it is necessary but not sufficient for cilia localization.

Myristoylation, farnesylation, and monopalmitoylation are sufficient for cilia localization and partially rescue membrane binding and cilia elongation

Palmitoylation is unique relative to other common lipid modifications in that it is reversible and that more than one lipid can be attached. To explore whether other lipid modifications could substitute for palmitoylation in supporting cilia localization of ARL13b, mutants were designed to add well-characterized myristoylation (from c-Src (27)) or farnesylation (from N-Ras (28)) sequences onto the ARL13b C8S/C9S background (Fig. 4A). In addition, Cys-8 and Cys-9 were mutated individually to assess the effects of monopalmitoylation of ARL13b. Each mutant was assessed for formation of and localization to cilia (identified with acetylated tubulin) when transiently expressed in IMCD3 cells.

Figure 1. Mouse ARL13b is palmitoylated. A, ARL13b is palmitoylated in mouse kidney and IMCD3 cells, as judged by the RAC assay. ARL13b is recovered in the presence, but not the absence, of hydroxylamine (HA). WB, Western blotting. B, mutant ARL13b (C8S/C9S), overexpressed in HEK cells, shows no palmitoylation. C, WT ARL13b, but not the C8S/C9S mutant, localizes to cilia (identified with acetylated tubulin) when transiently expressed in IMCD3 cells. The C8S/C9S mutant is found in a punctate pattern in the cytoplasm. Scale bars, 10 μm.
proteins, including NPHP3 (7, 29). In certain cases, myristoylation has been shown to promote cilia localization via mediation of binding of the chaperone UNC119b (7). The mutant ARL13b C8S/C9S Myr was stably expressed in 2E9 cells via lentiviral transduction. Immunofluorescence analysis showed that myristoylation fully restored cilia localization in 2E9 cells with minimal signal detected in the cell body (Fig. 4B). The cilia were, however, shorter than those in 2E9+WT cells (∼6.5 μm versus −5 μm, p = 0.03; Fig. 4C). The membrane-binding ability of ARL13b C8S/C9S Myr was tested by isolation of crude cellular membranes via centrifugation (Fig. 4D). As expected, ARL13b WT was found entirely in the membrane fraction (consistent with high membrane affinity), whereas ARL13b C8S/C9S was entirely in the supernatant (consistent with low membrane affinity). ARL13b C8S/C9S Myr was 50% found in the membrane fraction and 50% in the supernatant, suggesting partial rescue of membrane binding. This observation may reflect the reduced hydrophobicity of a single myristoyl group relative to two palmitoyl groups (as in ARL13b WT). These data suggest that tight membrane binding is not required for cilia localization.

ARL13b C8S/C9S Myr did not show uptake of the palmitate analog 17-ODYA (26), as would be predicted given the deletion of Cys-8 and Cys-9 (Fig. 4I). In addition, treatment of 2E9+C8S/C9S Myr cells with the palmitoylation inhibitor 2-bromopalmitate (2BP) did not show loss of ARL13b from cilia or cilia localization. In contrast, 2E9+WT cells treated with
2BP showed both loss of ARL13b from cilia and shortening of cilia (Fig. 4, F–H), further demonstrating the importance of palmitoylation to the localization and function of ARL13b WT.

A mutant was prepared in which a farnesylation site was added to the carboxyl terminus of the ARL13b C8S/C9S mutant (ARL13b C8S/C9S Far). Immunofluorescence and biochemical membrane isolation experiments showed that farnesylation could indeed promote partial membrane binding (Fig. 4D). However, the C8S/C9S Far protein did not support cilia elongation (Fig. 4B and C), although it did localize to the ciliary stubs. These results suggest that mere membrane binding is not sufficient for ARL13b to promote cilia elongation.

One difference between palmitoylation and both myristoylation and farnesylation is that the former probably involves the addition of two acyl chains at positions Cys-8 and Cys-9, whereas myristoylation and farnesylation each involve the addition of only a single lipid. To characterize singly palmitoylated ARL13b, Cys-8 and Cys-9 were mutated individually. Both single mutants could still be palmitoylated, as judged by uptake of the palmitate analog 17-ODYA (Fig. 4E), suggesting that both cysteines are targets for palmitoylation and that the native protein may indeed be modified by two palmitates. Both of the single palmitate mutants could bind to membranes only partially (Fig. 4D). Interestingly, both could nonetheless localize to cilia, although neither supported full cilia elongation (Fig. 4B and C). These data suggest that although partial membrane binding is sufficient for ARL13b to localize at cilia base, it is not sufficient to carry out cilia elongation function of WT ARL13b.

**Palmitoylation is required for ARL13b function and is incompletely rescued by myristoylation**

As discussed above, myristoylation rescues cilia localization in ARL13b C8S/C9S mutants. However, cilia length, a readout for ARL13b function, was shorter than in 2E9-WT cells. To

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**Figure 3. Palmitoylation occurs in a variety of ARL13b mutants.** A variety of mutants of ARL13b were prepared and stably expressed in 2E9 cells, including T35N and G28V, which are predicted to alter nucleotide binding; R79Q, which causes Joubert’s syndrome; and a deletion of the carboxyl-terminal tail distal to amino acid 308 (see “Results” for details). A, palmitoylation of each mutant occurred normally, as demonstrated by uptake of the palmitate analog 17-ODYA. IP, immunoprecipitation. B, immunofluorescence of ARL13b mutant proteins stably expressed in 2E9 cells. All of the mutants form cilia, although shorter than those in 2E9+ARL13b WT. All of the mutants localized predominantly to cilia, except ARL13b 1–308, which localizes extensively to sites outside cilia. Scale bars, 5 μm. C, quantification of cilia length. Data for IMDC3, 2E9, 2E9+W, and 2E9+C8S/C9S are replotted from Fig. 2 for comparison. Lengths are significantly shorter when compared with 2E9+W. **p < 0.001; ****, p < 0.0001.
Palmitoylation of ARL13b

further explore the functional competence of ARL13b C8S/C9S Myr, two assays were performed.

A functional readout for ARL13b involves its role as a guanine nucleotide exchange factor for ARL3 (30). The intensity of active (i.e. GTP-bound) ARL3 staining in cilia was assessed in 2E9+WT, 2E9+C8S/C9S, 2E9+C8S/C9S Myr, and 2E9+1–308 cell lines. WT ARL13b supports strong localization of active ARL3 in cilia (Fig. 5A). ARL13b C8S/C9S and 1–308 do

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**B.**

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**C.**

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**D.**

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**E.**

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**G.**

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**H.**

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**I.**

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not yield any appreciable staining for active ARL3, even at sites of localization of the ARL13b outside the cilia, suggesting that these mutants are unable to activate ARL3. This result is consistent with the inability of these mutants to promote cilia elongation. ARL13b C8S/C9S Myr does partially rescue formation of active ARL3, but quantitative analysis reveals ~40% less staining intensity (Fig. 5, A and B). These data again suggest that myristoylation may not functionally substitute fully for palmitoylation on ARL13b.

To pursue a more sensitive in vivo assay of ARL13b function, a rescue experiment in zebrafish was performed (15). Zebrafish heterozygous for a null allele of Sco (Sco<sup>h4459</sup>; Sco is the zebrafish ortholog of Arl13b) were bred, and the resulting embryos were injected with mRNA encoding GFP-tagged Sco-WT, Sco-C8S/C9S, or Sco-C8S/C9S Myr. Expression of each construct was verified by GFP expression at 6 h postinjection (supplemental Fig. S2, A–H). Embryos that are homozygous for Sco<sup>h4459</sup> (expected to be 25% of offspring of mating of heterozygotes) exhibit a characteristic ventral body curvature defect (15). Embryos were evaluated for this phenotype at 3 days postfertilization (dpf) (Fig. 5C). This defect was rescued with high efficiency by injection of mRNA encoding Sco-WT (only 1 of 58 fish exhibited ventral curvature after injection; Table 1) but not by Sco-C8S/C9S or Sco-C8S/C9S Myr. Fish injected with these alleles still exhibited ~25% ventral curvature (Table 1). Sco-C8S/C9S Myr showed some activity, in that 7 out of 67 embryos exhibited ventral curvature after injection; Table 1) but not deficiency by injection of mRNA encoding Sco-WT (only 1 of 58

Figure 4. Myristoylation, farnesylation, and monopalmitoylation confer cilia localization in ARL13b but do not fully restore cilia length and membrane binding.

A, partial amino acid sequences of different acylation mutants, showing insertion–myristoylation sequence (ARL13b C8S/C9S Myr), farnesylation sequence (ARL13b C8S/C9S Far), and monopalmitoylation mutants C8S and C9S, B, immunofluorescence of 2E9 cells stably expressing the indicated mutants. All acylation mutants localized predominantly to cilia. Scale bars, 5 μm. C, all acylation mutants show shorter cilia than WT, but longer than the non-acylated C8S/C9S (data for 2E9 and 2E9 + C8S/C9S are replotted from Fig. 2 for comparison). D, fractionation study shows that monopalmitoylation, myristoylation, and farnesylation all partially restore membrane association relative to WT. L, cell lysate in detergent-free lysate buffer after 900 × g spin; P, pellet; S, supernatant obtained after 20,000 × g spin of the cell lysate. E, cadherin is shown as a control for membrane-bound proteins, and GAPDH is shown as a control for soluble proteins. F, RAC assay confirms both the cysteines at positions 8 and 9 can be palmitoylated individually. G, chemical blockade of palmitoylation with 2BP treatment reduces cilia length (quantified in G) and ARL13b density in 2E9 + WT (quantified in H) but not in 2E9 + C8S/C9S Myr cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant.
Palmitoylation of ARL13b

A.

Merged | Arl13b | Active Arl3 | Total Arl3

ZE9

ZE+E-C6S

ZE+E-C6S/Myr

ZE+E+1-388

C.

Uninjected control | Sco-WT Injected | Sco-C6S/C9S Injected | Sco-C6S/C9S Myr Injected

D.

E.

Uninjected control - straight | Uninjected control - ventral body curvature | Sco-WT injected - straight

Sco-C6S/C9S curvature | Sco-C6S/C9S Myr injected - ventral body curvature | Sco-C6S/C9S Myr - dorsal body curvature

Normalized Active Arl3 Intensity

2E9 WT | 2E9 C6S Myr

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We tested whether MG132 could promote elongation of the shortened cilia observed in ARL13b C8S/C9S and ARL13b C8S/C9S Myr. No elongation was observed (supplemental Fig. S3); however, MG132 is obviously nonspecific and probably affected levels of numerous proteins, which may have interfered with cilia elongation via cellular toxicity or other mechanisms.

For many palmitoylproteins, in particular several monomeric GTPases like ARL13b, evidence of repeated cycles of palmitoylation and depalmitoylation has been identified (4). To explore whether cycling of palmitoylation was occurring with ARL13b, the half-life of palmitoylation was estimated in pulse-chase studies. This half-life was found to be similar to the half-life of the protein itself, suggesting that ARL13b may not undergo depalmitoylation without concomitant degradation (Fig. 7, A and B). Additionally, the ability of mature (as opposed to newly synthesized) ARL13b to incorporate palmitoyl groups was assessed by treatment of cells with cycloheximide for 2 h (to block new protein synthesis) followed by metabolic labeling with [17-OYA]. This protocol reduced palmitoylation by ~90%, suggesting that the majority of ARL13b palmitoylation occurs within 2 h after protein synthesis, with little occurring on the mature protein (Fig. 7, C and D). In an effort to identify whether any ZDHHC protein acyltransferases were localized within cilia, each mouse ZDHHC isoform was transiently expressed in IMCD3 cells and evaluated by immunofluorescence. None showed localization to cilia (supplemental Fig. S4), suggesting that palmitoylation occurs before localization to the cilia. These data are consistent with the finding that the ARL13b 1–308 mutant is palmitoylated yet does not localize to the cilia (Fig. 3).

**Depalmitoylation may be a cellular mechanism to induce ARL13b degradation**

The marked acceleration of degradation observed in nonpalmitoylated mutants of ARL13b (Fig. 6, E and G) raises the question as to whether endogenous enzymatic depalmitoylation may be a cellular mechanism to induce degradation of ARL13b. Analogously, regulated palmitoylation has been proposed as a rapid means of controlling cellular concentrations of calcineurin (31). Degradation of ARL13b is presumably advantageous when the cilia is to be disassembled and resorbed, as occurs, for example, during cell division. A heat shock protocol was used to induce cilia resorption (33). After 30 min of heat shock treatment, ARL13b WT was found to have decreased in concentration ~50%, suggesting that the treatment induces rapid degradation of ARL13b, along with cilia resorption. Treatment of the cells with blockers of depalmitoylation (HDFP (4) and palmostatin B (34)) largely inhibited the degradation (Fig. 8). This result suggests that depalmitoylation of ARL13b may be a cellular mechanism to effect rapid degradation of ARL13b and facilitate cilia resorption.

**Discussion**

Palmitoylation is a common post-translational modification that involves ~10% of mammalian proteins (3). It possesses multiple unique features that distinguish it from other common lipid modifications, such as reversibility and a diversity of involved enzymes. A number of cilia proteins have been reported to require palmitoylation for proper trafficking to cilia, and, as reported herein, a much larger number (~30% of total) may be palmitoylated, based on cross-referencing of proteomic data sets of cilia-localized and palmitoylated proteins. The cilia membrane has been reported to be enriched in cholesterol and sphingolipids, components of membranes in which palmitoyl groups preferentially localize (35). Together, these observations suggest that palmitoylation probably plays a key role in cilia biology. To explore this hypothesis, ARL13b was studied as a model palmitoylprotein. Here, palmitoylation of ARL13b has been confirmed to occur in intact mouse kidneys and to be required for localization to cilia in cultured mouse renal tubular cells. Further, as has been observed in other proteins, palmitoylation confers dramatic additional stability to ARL13b and greatly extends its half-life (Fig. 6). Interestingly, we show for the first time that myristoylation can replace palmitoylation to mediate cilia localization. Further, myristoylated ARL13b is at least partially active in cilia as judged by its ability to mediate cilia elongation, ARL3 activation, and partial activity in zebrafish. Myristoylation, however, does not rescue the stability of nonpalmitoylated ARL13b. These data suggest that palmitoylation possesses unique properties relative to other lipids that are relevant to its stability. Additionally, we find that the cell may exploit the sensitivity of ARL13b to palmitoylation by removing the palmitoyl group to facilitate ARL13b degradation and cilia resorption (Fig. 8).

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**Table 1**

| Phenotype of injected and un.injected embryos |
|---------------------------------------------|
| | Uninjected, n (%) | Injected, n (%) |
| | Straight | Ventral curvature | Straight | Ventral curvature | p* |
| Sco-WT | 41 (75) | 14 (25) | 57 (98) | 1 (2) | <0.01 |
| Sco-C8S/C9S | 42 (78) | 12 (22) | 24 (83) | 5 (17) | 0.77 |
| Sco-C8S/C9S Myr | 46 (71) | 19 (29) | 48* (72) | 19 (28) | 1.00 |

*Comparison of injected versus uninjected by Fisher’s exact test.

**Figure 5.** Myristoylation incompletely replaces functional role of dual palmitoylation despite restoring cilia localization. A, myristoylation only partially restores activation of ARL3. ARL3 is a guanine nucleotide exchange factor for ARL3. Active ARL3 is observed in the cilia of Zebrafish (Zebrafish) cells, whereas it is absent in Zebrafish (Zebrafish) cells. This demonstrates a loss of functional activity in the palmitoylation-deficient ARL13b mutant. Active ARL3 is restored in Zebrafish (Zebrafish) cells, albeit at lower intensity. No active ARL3 is observed in Zebrafish (Zebrafish) cells. The truncation mutant cannot activate ARL3. Scale bars, 5 µm. B, quantification of active ARL3 staining, normalized to level of ARL13b staining. C, zebrafish heterozygous for Sco-C8S/C9S Myr null allelic line of the zebrafish ortholog of ARL13b were mated. Homozygous null progeny display ventral body curvature, whereas heterozygous and homozygous WT allelic line display straight body. Injection of mRNA encoding Sco-WT rescues body curvature defect (Table 1), whereas Sco-C8S/C9S and Sco-C8S/C9S Myr do not. Scoring Cilia (Fig. 5) Cilia demonstrates some abnormal activity, as judged by the presence of optional dorsal-curving fish. D, genotyping of Sco-C8S/C9S Myr injected dorsal curving fishes. Reference fishes were as follows: heterozygous fish (straight) (lanes 1), wild-type fish (straight) (lanes 1), and homozygous mutant fishes (ventral body curvature) and test fishes (lanes 3 and 4), and homozygous mutant fishes (dorsal body curvature) (lanes 5–11). E, embryos 3 dpf were stained with antibody against acetylated tubulin to evaluate the pronuclear cilia phenotype. In wild-type (uninjected control, straight) and rescued (Sco-WT–injected) embryos, bundles of cilia in the duct appear as a tight line (arrow). In the mouse embryos with ventral body curvature, only ragged discontinuous staining could be detected (arrow), suggestive of abnormal cilia morphology. In the Sco-C8S/C9S Myr–injected dorsal body curvature embryos, there is partial rescue of cilia formation morphology. *, p < 0.05.
Palmitoylation of ARL13b

Role of palmitoylation in ARL13b trafficking

The mechanism by which palmitoylation promotes trafficking of ARL13b to cilia is unclear. Localization to cilia does not require dual palmitoylation, because myristoylation, farnesylation, and monopalmitoylation alone were able to rescue cilia localization (Fig. 4). Thus, these data suggest that the effect of acylation with regard to cilia localization is merely to promote at least a modest amount of membrane affinity. It is interesting that myristoylation, farnesylation, and monopalmitoylation seem to fully rescue cilia localization while only displaying a fraction of the membrane binding of ARL13b WT (Fig. 4D). Perhaps even low membrane affinity is sufficient for cilia localization. However, mere membrane attachment is not sufficient for complete ARL13b function of cilia elongation and maintenance.

An alternate possibility is that the mechanism by which acylation supports cilia localization of ARL13b (and possibly other palmitoylated cilia proteins) involves binding to an unknown chaperone. This hypothesis is based on data showing that the mechanism by which myristoylation and prenylation promote cilia localization involves lipid-specific binding of the chaperones UNC119 (for myristoylated proteins (9)) and PED6d (for prenylated proteins (8)). An analogous chaperone with affinity for palmitoylated proteins has not been identified thus far but may exist. Of note, it is possible that the myristoylated and farnesylated ARL13b mutants may bind UNC119 and PED6d, respectively, and utilize those interactions for trafficking.

The data in this report refute two other hypotheses regarding the role that palmitoylation plays in ARL13b localization. One mechanism by which palmitoylation has been shown to promote specific localization of particular proteins is via cycling between palmitoylated and unpalmitoylated states. For example, cycling has been shown to be important for H- and N-Ras (36). However, we did not find evidence for cycling of palmitoylated ARL13b (Fig. 7). Another proposed mechanism by which palmitoylation may affect protein trafficking involves kinetic trapping, a process in which a protein is “trapped” at a membrane site following the localized addition of palmitoyl groups (37). However, in the case of ARL13b, palmitoylation does not seem to be operating strictly through a “trapping” mechanism. Palmitoylation appears to be occurring before ARL13b enters the cilia based on the fact that no ZDHHC isoform could be identified in cilia (supplemental Fig. S4) and that the ARL13b 1–308 deletion mutant, which does not localize to cilia, is nonetheless robustly palmitoylated (Fig. 3).

Palmitoylation as a dynamic means to regulate protein stability and abundance

The effect of palmitoylation on the stability and abundance of ARL13b is striking. Interestingly, myristoylation does not rescue the stability despite its ability to rescue cilia localization. A strong connection between palmitoylation and protein stability has been observed in a variety of different proteins (38). For example, several investigators have used quantitative palmitoylproteomics in an effort to identify proteins exhibiting altered levels of palmitoylation in cells or tissues lacking a particular DHHC protein acyltransferase isoform (39) (32). The results of these studies showed that although the palmitoylated version of a number of proteins was indeed reduced in the knock-out cells or tissues, the total amount of each protein was typically also reduced by a similar degree (32, 39). One explanation for these results is that the nonpalmitoylated version of the protein is rapidly degraded. Because this was observed for a variety of proteins identified in these proteomic studies, it may be a widespread phenomenon.

An intriguing question is whether cells actively exploit palmitoylation-dependent stability of many proteins as a means of dynamically controlling protein abundance. For example, a recent study of the synthesis, degradation, and palmitoylation dynamics of the endoplasmic reticulum protein calnexin suggests that regulated palmitoylation of a pool of unpalmitoylated protein is a means of rapidly stabilizing the protein and increasing its concentration (31). In a similar vein, the degradation of ARL13b, which is induced by a heat shock protocol was attenuated by blockers of depalmitoylation (Fig. 8). These data are consistent with a scenario in which rapid ARL13b degradation is facilitated via depalmitoylation. Further experiments are necessary to fully evaluate this phenomenon. The mechanism by which palmitoylation protects ARL13b from rapid degradation is unclear.

Delivery of ARL13b to the cilia is not sufficient to protect it from degradation, as the ARL13b C8S/C9S Myr mutant is localized to cilia. It may be that soluble protein is degraded, whereas membrane associated is protected. In this situation, the reduced membrane affinity observed in myristoylated ARL13b would contribute to its reduced stability without interfering with cilia localization.

Future questions

Many unanswered questions remain regarding palmitoylation of cilia proteins, including the identification of relevant enzymes, the subcellular sites of palmitoylation, and the delineation of situations in which dynamic palmitoylation occurs to serve signaling or other functions. Ultimately, exploration of the role of palmitoylation may allow for the use of drugs that target the palmitoylation machinery to alter the course of diseases associated with cilia dysfunction.
Experimental procedures

Chemicals and antibodies

Palmitic acid (P0500), hydroxylamine hydrochloride (159417), cycloheximide (66-81-9), MG132 (M7449), 2-bromopalmitate (21604), S-methyl methanethiosulfonate (2949-92-0), and hydroxychloroquine sulfate (H0915) were obtained from Sigma-Aldrich. 17-ODYA (0443296-19) was from Cayman. Azide IRDye 800CW (929-60000) was from LI-COR. PureProteome Protein G magnetic beads and palmostatin B (508738) were from Millipore. HDFP was a gift from Dr. B. Martin (University of California).
Palmitoylation of ARL13b

A. IP: Arl13b

| Time (in min) | 0 | 30 | 30 |
|--------------|---|----|----|
| HDFP         | - | -  | +  |
| Palm B       | - | -  | +  |
| Arl13b       | - | -  | +  |
| GAPDH        | - | -  | +  |
| 17-ODYA      | - | -  | +  |

B. Normalized 17-ODYA intensity

| Time (in min) | 0 | 30 | 30 |
|--------------|---|----|----|
| HDFP         | - | -  | +  |
| Palm B       | - | -  | +  |

C. IP: Arl13b

| Time (in min) | 0 | 30 | 30 |
|--------------|---|----|----|
| HDFP         | - | -  | +  |
| Palm B       | - | -  | +  |
| Arl13b       | - | -  | +  |
| GAPDH        | - | -  | +  |
| 17-ODYA      | - | -  | +  |

D. Normalized 17-ODYA intensity

| Time (in min) | 0 | 30 | 30 |
|--------------|---|----|----|
| HDFP         | - | -  | +  |
| Palm B       | - | -  | +  |

Figure 7. ARL13b palmitoylation is stable with little or no cycling. A, 2E9 WT cells were pulsed with 17-ODYA for 2 h and washed, and chased with palmitic acid-rich media containing cycloheximide (CYX) for 4 h. B, graph showing that the drop in normalized 17-ODYA intensity is comparable with ARL13b protein loss over 4 h (Fig. 6). C, most palmitoylation of ARL13b occurs within 2 h of synthesis. 2E9 WT cells were treated with cycloheximide + MG132 for 2 h, followed by metabolic labeling with 17-ODYA, to assess palmitoylation of ARL13b that was >2 h postsynthesis. Minimal palmitoylation occurs under these conditions. D, graph shows normalized 17-ODYA intensity. IP, immunoprecipitation. **, p < 0.01; ***, p < 0.001.

Figure 8. Depalmitoylation inhibitors slow degradation of ARL13b. A, 2E9 WT cells were heat-shocked at 42 ºC to induce cilia resorption, which is accompanied by reduced ARL13b at 30 min. The addition of blockers of deplamitoylation HDFP and palmostatin B (Palm B) mitigate the degradation of ARL13b. B, quantification of data. *, p < 0.05; ***, p < 0.001; ns, not significant.

of Michigan) (4). Thiopropyl-Sepharose 6B was from GE Healthcare (17-0420-01). Primary antibodies used were ARL13b (Protein Tech, 17711-1-AP), α-tubulin (Rockland, 200-301-880), E-cadherin (Fisher, BDB 610182) GAPDH (Rockland, 600-401-A33), Myc tag (Cell Signaling, 2278 and 2276), GFP (Sigma, G1544), acetylated α-tubulin (Sigma, T7451), β-tubulin (Abcam, ab6046), IFT 81 (Protein Tech, 11744-1-AP), ARL3 and active ARL3 mouse antibodies (Neweastbio ARL3 activation assay kit; active ARL3-GTP monoclonal antibody 26925; anti-ARL3 mouse monoclonal antibody 26070).

Figure 6. Palmitoylation of ARL13b increases stability and protects it from proteosomal degradation. A, steady-state levels of ARL13b C8S/C9S are reduced relative to WT, and levels are not increased by the addition of myristoylation. Expression levels of ARL13b were studied by Western blotting of lysates from 2E9 cell lines stably expressing the indicated protein. B, quantification of ARL13b expression levels. C, Sco-C8S/C9S expression in zebrafish is reduced relative to Sco-WT and is not rescued in Sco-C8S/C9S Myr. The blot shows levels of the GFP-tagged Sco-WT, Sco-C8S/C9S, and Sco-C8S/C9S Myr protein expression at 24 h postinjection. D, degradation rates of native ARL13b in IMCD3 cells are similar to rates of ARL13b-WT stably expressed in 2E9 cells. Rates were determined by cycloheximide (CYX) treatment, which blocked new protein synthesis for the indicated times, followed by Western blotting, and quantified in F. E, degradation rates of ARL13b C8S/C9S are much faster than ARL13b WT and are not rescued by myristoylation. Western blots are quantified in G. H, blockade of proteasome with MG-132 inhibited degradation of both ARL13b C8S/C9S and ARL13b C8S/C9S Myr, whereas blockade of lysosomal degradation with HCQ had only a minor effect. I, quantification of blots in H. CYX + HCQ and CYX + MG-132 were compared with CYX alone at 6 h. J, ARL13b expression level and cilia length are correlated. Data for expression level (derived from densitometry of Western blots from cells lysates and normalized to WT levels) are plotted with cilia lengths derived from analyses of fluorescent micrographs. When mutants that do not localize to cilia are excluded (i.e. ARL13b 1–308 and ARL13b C8S/C9S), a general correlation between expression level and cilia length is observed. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant.
Cells and culture conditions
IMCD3 and HEK293 cells (obtained originally from the American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO₂. Knock-out and stable cell lines were prepared using IMCD3 cells. If not specified otherwise for the cilia growth study, confluent cells were used, which were serum-starved for 24 h. When indicated, cells were transfected with Transfectamine 2000 (Invitrogen) as per the manufacturer’s instructions. In general, cells were grown in 10-cm dishes to 70–80% confluence and transfected with the indicated 1 μg of DNA and 3 μl of Transfectamine 2000. Stable cells were generated using a third-generation lentiviral system (Addgene plasmids 19067, 12251, 12253, and 12259). For selection neomycin sulfate (BP266925) was used at a concentration of 0.5 mg/ml.

Zebrafish
Standard protocols were used for maintaining zebrafish colonies (41). Previously published protocols (15) were followed for zebrafish assays. All procedures were approved by the Yale institutional animal care and use committee. Briefly, zebrafish heterozygous for the Sco null allele hi459 were crossed. Embryos were injected with 100 pg of GFP-tagged Sco-WT, Sco-C8S/C9S, and Sco-C8S/C9S Myr mRNA at the 1–4-cell stage. GFP expression was checked 6 h postinjection with a fluorescent microscope. Protein extraction for Western analysis was done at 24 h postinjection. Embryos were scored for body curvature at 3 dpf. Afterward, cilia were detected by immunostaining with anti-acetylated tubulin, followed by HRP-conjugated anti-mouse IgG. Signal was detected via a color reaction using 3,3-diaminobenzidine (Sigma-Aldrich) as the chromogenic substrate. For mRNA injection experiments, appropriate plasmids (pCS2+scoeGFP and described mutants) were linearized with HindIII enzyme, and mRNA was prepared using the mMessage mMachine kit, following the manufacturer’s protocol.

Acyll-RAC
The acyl-RAC assay was carried out as described previously (23, 24). In short, following treatments, cells were collected and washed with PBS. Cells were then taken in blocking buffer (100 mM HEPES, 1.0 mM EDTA, 2.5% SDS, 0.1% S-methyl methanesulfonate, pH 7.5) containing protease inhibitor mixture (Roche Applied Science), sonicated, and then centrifuged. The lysate was incubated for 15 min at 42 °C with frequent vortexing. Three volumes of cold acetone were added, and proteins were allowed to precipitate at −20 °C overnight. The lysate was centrifuged, and the pellet was washed three times with 70% acetone solution at 5,000 × g for 10 mins. Finally, the pellet was resuspended in 600 μl of binding buffer (100 mM HEPES, 1.0 mM EDTA, 1% SDS, pH 7.5), and a 20-μl aliquot of the suspension was saved as the “lysate.” The remaining suspension was split into two samples and combined with 2 μl hydroxylamine (final concentration 235 μM; freshly prepared in H₂O and adjusted to pH 7.5) or 2 μl NaCl as control. To both, 40 μl of prewashed thiopropyl-Sepharose was added. Binding reactions were carried out on arotator at room temperature for 2–4 h. Resins were washed extensively with binding buffer. For immunoblot analysis, 2× Laemmlili loading buffer containing 100 mM DTT was added, heated to 95 °C for 5 min, and then separated via SDS-PAGE on a Mini-Gel apparatus (Bio-Rad). Proteins were electrophoretically transferred to nitrocellulose, analyzed with the indicated primary antibody and a fluorophore-conjugated secondary antibody (LI-COR), and imaged with a LI-COR Odyssey Fc instrument.

Plasmids and primers
Arl13b-pDEST40 (mouse) plasmid was obtained from Addgene (plasmid 40874). Arl13b C8S/C9S mutant was prepared using the primers GATGGCCAACCTCCTCAACTTTGGTTCAAG and AGACTGAACATGGTGAAGG with the help of the Q5 site-directed mutagenesis kit (New England Biolabs, E0554S). To generate the Arl13b C8S and C9S single mutants, the primers used were GATGGCCAACCTCCTCAACTTTGGTTCAAG and AGACTGAACATGGTGAAGG. Previously published protocols (15) were followed where two guide RNAs were used to delete the first exon of the Arl13b gene in the IMCD3 cells. pSpCas9(BB)-2A-GFP (PX458) plasmid was obtained from Addgene (plasmid 48138). The guides CTCTTTCCGCCCCCTTGAAGCAG and GAGAAAAACAACTCTATAGTT were inserted in the PX458 plasmid as

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CRISPR/Cas9
Arl13b KO was generated using the CRISPR/Cas9 system, where two guide RNAs were used to delete the first exon of the Arl13b gene in the IMCD3 cells. pSpCas9(BB)-2A-GFP (PX458) plasmid was obtained from Addgene (plasmid 48138). The guides CTCTTTCCGCCCCCTTGAAGCAG and GAGAAAAACAACTCTATAGTT were inserted in the PX458 plasmid as
described (40). The guides were then transiently transfected in the cell, and after 48 h of transfection, the GFP-positive cells were selected using FACS (MoFlo; Yale central facility) and sorted at 1 cell/well into 96-well plates. The clones were expanded and then screened using PCR with two sets of primers: one set flanking the zone within the deletion (internal primers, GTCTCACATGCACTTCTTCTTAAG and TCTTTTCCGTCCACACTAGG) and another set outside the guide recognition sites (external primers, AGCTCAAAGACCAGCTGT and GACGCTCAGTTGGCTAGTT). Colonies with homogeneous deletion showed a single smaller PCR product described (26) as visualized on a LI-COR Odyssey Fc fluorescent scanner.

Confocal images were analyzed using NIS-Elements Analysis version 4.3 software. At least 150 cilia were considered in aggregate. All experiments were done three times (n = 3) or more, unless otherwise described, and data are reported (and graphed) as means ± S.E. The statistical calculations were done with the help of Graph Pad Prism version 6. Unless noted otherwise, statistical analyses were done by t test, or, in the case of multiple comparisons, by one-way analysis of variance with Dunnett test for multiple comparisons. p values < 0.05 were considered to be significant. Test results are indicated in figures as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Author contributions—E. P. M. conceived and coordinated the study and wrote the manuscript. K. R. performed most experiments, contributed to the conception of the study, and contributed to writing of the manuscript. S. J. and Z. S. designed and performed zebrafish-related experiments and helped with interpretation of studies. T. M. and G. O. helped to perform experiments in Figs. 3 and 4.

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**Palmitoylation of ARL13b**

For palmitoylation studies, cell treatment with the palmitate analog 17-ODYA (26) was followed by immunoprecipitation of ARL13b using anti-ARL13b rabbit (Protein Tech, 17711-1-AP). The immunoprecipitation and click chemistry steps are briefly mentioned. The treated cells were washed with cold PBS, scraped with lysis buffer (PBS + 1% Triton X-100) containing complete protease inhibitor mixture, and rotated at 4 °C for 15 min. This was followed by centrifugation, and supernatant was collected. The protein was measured by a Bradford assay. An equal amount of protein was taken, and volume was made up to 1 ml for immunoprecipitation. A small aliquot of supernatant was kept aside as lysate. 4 ml for immunoprecipitation. A small aliquot of supernatant was kept aside as lysate. 4 ml of ARL13b antibody was added and rotated at 4 °C overnight. The next day, 20 ml of Protein G magnetic beads (PureProteome, Millipore) were added and rotated at 4 °C for 1 h. Next, the beads were separated from the supernatant and washed three times with PBS. This was followed by incubation of the beads with click reagents for 1 h as described (26) to attach a fluorophore. The beads were washed, boiled for 3 min in 2× loading buffer containing TCEP, and run on SDS-PAGE. The gel was transferred on a nitrocellulose membrane and assayed with appropriate antibody. Palmitoylated proteins were identified by attachment of the fluorophore-coupled 17-ODYA, as visualized on a LI-COR Odyssey Fc fluorescent scanner.

**Subcellular fractionation study**

Confluent cells from a 30-cm dish were washed twice with ice-cold PBS and scrapped in 100 ml of detergent-free homogenization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM MgCl2) containing protease inhibitor. The cells were passed through a 20-gauge syringe, sonicated, and then centrifuged for 10 min at 900 × g. The pellet was (nuclei and unbroken cells) discarded, and the cell lysate (L) was again centrifuged for 30 min at 20,000 × g. The resulting pellet (P) and supernatant (S) were stored. The pellet was dissolved in 100 ml of lysis buffer (PBS + 1% Triton X-100). 30 ml of all of the samples were used for immunoblotting.

**Immunocytochemistry**

Cells were washed with PBS and fixed with 4% paraformaldehyde (EMS 15710) for 15 min at room temperature. This was followed by permeabilization and blocking with 5% FBS and 0.25% Triton X-100 in 0.5× PBS. The cells were then stained overnight with specific antibodies, washed three times for 5 min each with PBS, and stained with secondary antibody for 2 h. Cells were then washed with PBS three times and stained with DAPI (R37606). Afterward, the cells were imaged with a spinning disc confocal microscope (Nikon Eclipse Ti-E).
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