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KDM3A coordinates actin dynamics with intraflagellar transport to regulate cilia stability

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Cilia assembly and disassembly are coupled to actin dynamics, ensuring a coherent cellular response during environmental change. How these processes are integrated remains undefined. The histone lysine demethylase KDM3A plays important roles in organismal homeostasis. Loss-of-function mouse models of Kdm3a phenocopy features associated with human ciliopathies, whereas human somatic mutations correlate with poor cancer prognosis. We demonstrate that absence of KDM3A facilitates ciliogenesis, but these resulting cilia have an abnormally wide range of axonemal lengths, delaying disassembly and accumulating intraflagellar transport (IFT) proteins. KDM3A plays a dual role by regulating actin gene expression and binding to the actin cytoskeleton, creating a responsive “actin gate” that involves ARP2/3 activity and IFT. Promoting actin filament formation rescues KDM3A mutant ciliary defects. Conversely, the simultaneous depolymerization of actin networks and IFT overexpression mimics the abnormal ciliary traits of KDM3A mutants. KDM3A is thus a negative regulator of ciliogenesis required for the controlled recruitment of IFT proteins into cilia through the modulation of actin dynamics.

Introduction

Primary cilia are homeostatic sensory organelles driven by intraflagellar transport (IFT) that integrate multiple and diverse cellular inputs critical to normal development and organ homeostasis. Diverse environmental cues affect their assembly and disassembly in a manner that is tightly coupled to the actin cytoskeleton and changes in gene expression. How cells coordinate these processes to effect a coherent cellular response remains unclear.

Actin network architecture is increasingly recognized as a major driver of ciliogenesis in cycling cells (Pitaval et al., 2010). Chemical and genetic perturbations of multiple actin remodeling factors have been found to promote ciliogenesis or affect ciliary length (Kim et al., 2010, 2015; Cao et al., 2012; Hernandez-Hernandez et al., 2013; Kang et al., 2015) and intriguingly rescue ciliogenesis defects in Ift88 (Kim et al., 2010) and IFT121 (Fu et al., 2016) mutant cells. Actin-myosin-mediated contraction participates in ciliary abscission as the neuronal differentiation program is triggered (Das and Storey, 2014). Leptin elongates hypothalamic cilia via transcriptional regulation and actin destabilization (Kang et al., 2015), whereas the microRNA mir-129-3p regulates cilia assembly through the concomitant transcriptional silencing of centrosomal proteins and repression of actin filament formation (Cao et al., 2012). Altogether, these findings highlight the existence of concurrent regulatory mechanisms coordinating ciliogenesis, actin dynamics, and transcription.

Interestingly, mouse mutants for the histone N-lysine demethylase Kdm3a (Jmjd1a, Tsga, or Jhdm2a) are obese, predisposed to diabetes (Inagaki et al., 2009; Tateishi et al., 2009), and have male infertility phenotypes (Okada et al., 2007) overlapping with mouse models of dysfunctional cilia. Structural abnormalities of the acrosome and manchette were observed in mutant Kdm3a mouse spermatids (Kasioulis et al., 2014) similar to those reported for conditional inactivation of Ift88 (Kierszenbaum, 2002; San Agustin et al., 2015). KDM3A localizes to the acrosome-like acroplaxome region, which is altered in Kdm3a mutant spermatids (Kasioulis et al., 2014). In spite of these phenotypic and functional overlaps, a link between KDM3A and ciliogenesis has not been investigated.

KDM3A is part of the large family of Jumonji C (JmjC) domain-containing proteins (Clissold and Ponting, 2001; Klose et al., 2006; Markolvic et al., 2016). Deregulated expression of KDM3A is associated with colorectal cancer (Uemura et al.,...
of KDM3A (Fig. 1E, KDM3Ai2-EGFP; Kasioulis et al., 2014). Overall, these findings indicate that KDM3A plays fundamental roles in organismal homeostasis, integrating multiple environmental cues with physiological states.

KDM3A presents different regulatory modalities and subcellular distributions. Through the catalytic removal of monomethyl and N-dimethyl groups from lysine 9 of histone H3, KDM3A acts as a transcriptional activator (Yamane et al., 2006) of multiple genes involved in spermatogenesis (Okada et al., 2007), fat storage (Okada et al., 2010), and energy expenditure (Inagaki et al., 2009) as well as of diverse types of cancers (Ohguchi et al., 2016; Zhao et al., 2016). More recently, scaffolding and noncatalytic modes of transcriptional regulation by KDM3A have emerged wherein KDM3A binds to the SWI–SNF (Switch-sucrose nonfermentable) chromatin-remodeling complex (Abe et al., 2015) or Hedgehog-responsive transcription factor GLI1 (Schneider et al., 2015), regulating expression of target genes. Although these functions relate to the role of KDM3A in the nucleus, KDM3A exits the nucleus in response to mechanosensitive stimuli (Kaukonen et al., 2016) and is found at various cytoplasmic sites of somatic cells and during germ cell development (Okada et al., 2007; Yang et al., 2009; Yamada et al., 2012; Kasioulis et al., 2014). Altogether, these findings indicate that KDM3A is a multifunctional protein with highly regulated subcellular distributions and nontranscriptional roles that remain to be explored.

Here, we present evidence on the regulation of actin dynamics by KDM3A and the subsequent impact on IFT and ciliogenesis. KDM3A is required for the stabilization of primary cilia and regulates ciliary responsiveness to environmental cues. RNA-sequencing, proteomic, and two-hybrid studies reveal a dual role for KDM3A in this integrated response modulating ciliogenesis. On a global level, it senses extracellular signaling and transcriptionally regulates the free pool of actin available. On a very local level, KDM3A directly binds to the actin cytoskeleton, creating a responsive “actin gate” and regulating IFT within cilia and periciliary compartments. Importantly, deregulated expression of an IFT protein, IFT81, coupled with actin depolymerization phenocopies ciliary defects of KDM3A mutants, demonstrating that KDM3A-mediated cytoskeletal changes tightly regulate IFT during ciliary growth.

Results

KDM3A plays a conserved role in the regulation of ciliary dynamics

During the analysis of a deletion mutant mouse model of Kdm3a lacking the catalytic domain JmJC (ΔJC; Kasioulis et al., 2014), we observed that flagella of mutant spermatids were consistently shorter than those of control littermates (Fig. 1, A–C). We observed that flagella of mutant spermatids were consistently shorter than those of control littermates (Fig. 1, A–C). We observe mutant mitotic cells with cilia, we conclude that mutant cilia must be eventually resorbed albeit with altered kinetics.

Both Kdm3aΔJC deletion and Kdm3aΔJC mutant models indicate that KDM3A plays a role in axonemal dynamics, yet the deletion mutants have on average shorter cilia (Fig. 1 D) rather than the wider range of lengths observed in Kdm3aΔJC cells (Fig. 2 B). These contrasting outcomes may be caused by a gain of function of the truncated KDM3A protein encoded by Kdm3aΔJC mutants, as MEFs derived from a second

Kdm3aΔJCΔJC MEFs also displayed accumulations of IFT88 at the ciliary base and tip compared with the uniform distribution of IFT88 in wild-type cilia (Fig. 1, F and G). These observations suggested that KDM3A could play a role in axonemal dynamics and IFT in primary cilia. Because Kdm3aΔJC mutant cells encode a truncated KDM3A protein, albeit at very low levels (Kasioulis et al., 2014), we generated KDM3AΔJC cells in the immortalized human retinal epithelium hTERT RPE1 cell line. RPE1 cells have been thoroughly characterized to study ciliary growth, resorption, and axoneme length in response to cell density and serum content of the culture media (Pugacheva et al., 2007; Kim et al., 2015).

Using CRISPR/Cas9 gene editing directed to exon 22 of KDM3A, we generated independent RPE1 cell lines null for KDM3A. Lines 1, 2, and 8 (Kdm3aΔJC) did not express KDM3A protein (Fig. 2 A) and were found to bear compound KDM3A mutations by genotyping (Fig. S1, A and B). As controls, we used the parental RPE1 line (RPE1WT) as well as an RPE1 sister line that has undergone CRISPR/Cas9 editing but was wild type for KDM3A (RPE1ΔJC). Cells were plated at equal densities and promoted either to (a) ciliate, by serum withdrawal (−FCS), or (b) resorb cilia, by serum replenishment (+FCS), with or without temperature stress. No differences were observed in the ciliary length between controls and KDM3A null cultures after 3 or 24 h without serum (Fig. 2 B, 3 h and 24 h −FCS). In contrast, under resorption conditions, KDM3AΔJC cilia remained significantly longer than those of controls (Fig. 2 B, resorption +FCS), suggesting that KDM3AΔJC cells failed to efficiently resorb cilia. Moreover, the proportion of KDM3AΔJC cells that remained ciliated was unchanged after serum replenishment, in contrast to the efficient resorption of ciliated control cells (Fig. 2 C).

It was noted during these studies that KDM3AΔJC cilia displayed a wider range of lengths than RPE1WT under all conditions (Fig. 2 B, analysis of variance F). Also 3 h after serum withdrawal, KDM3AΔJC cultures had a higher proportion of ciliated cells than controls (Fig. 2 D). Together, these results suggest that mammalian KDM3A negatively regulates ciliogenesis.

Ciliogenesis is usually promoted through serum withdrawal, but cycling RPE1 cells also form cilia in the presence of serum, albeit at lower frequencies (Cao et al., 2012). To confirm the propensity of KDM3AΔJC cells to ciliate, we examined ciliogenesis in exponentially growing cultures. In the presence of serum, some extremely long cilia with abnormal bulges were observed in KDM3AΔJC cells (Fig. 2 E, arrow). Importantly, higher proportions of ciliated cells were consistently observed in mutants (Fig. 2 F), in spite of comparable cell density and mitotic index to controls (RPE1WT; 4.05%, SD = 1.90; KDM3AΔJC 6%, SD = 1.63; N = 3 biological replicates, n > 300 cells scored/condition). A higher proportion of KDM3AΔJC cells retained cilia into late G2 (Fig. 2, G and H), whereas most wild-type cilia were already resorbed (Spalluto et al., 2013). However, these changes were neither caused by nor associated with alterations in cell cycle progression (Fig. 2 I). As we did not observe mutant mitotic cells with cilia, we conclude that mutant cilia must be eventually resorbed albeit with altered kinetics.
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hypomorphic mouse model of Kdm3a (Kdm3aΔJC; Kasioulis et al., 2014) present, as observed in KDM3A-null RPE1 cells, a wider range of cilial lengths (Fig. S1, C and D). Ciliary resorption in response to serum replenishment and temperature stress was similarly delayed in Kdm3aGT/GT MEFs (Fig. S1 E).

In summary, given the increased proportion of ciliated cells, abnormal range of cilial lengths, and delayed cilial resorption kinetics, our loss-of-function mutants suggest that in the absence of KDM3A, ciliogenesis is facilitated and may be less tightly regulated. Conversely, overexpressing KDM3A reduces cilial formation in the parental RPE1WT line (Fig. S1, F and G).

KDM3A regulates actin expression and binds actin in vivo and in vitro

Given KDM3A’s localization to actin-rich structures and altered biochemical properties of actin in Kdm3a mutant spermatids (Kasioulis et al., 2014), we hypothesized that the effect of KDM3A depletion on ciliogenesis is a consequence of actin dysregulation. As KDM3A had been shown to bind the promoter and regulate the expression of smooth muscle α-actin (Acta2; Lockman et al., 2007), these effects could be caused by altered transcription or protein–protein interactions or both. Indeed, phalloidin staining of KDM3A-null cells (Fig. 3 A) showed consistently decreased abundance of actin filaments compared with RPE1WT (Fig. 3 B), and this was also evident in Kdm3aΔJCΔJC MEFs (Fig. S2 A).

The turnover of the actin cytoskeleton is both transcriptionally and posttranscriptionally regulated (Olson and Norden, 2010), requiring a pool of actin monomers (G-actin) and multiple actin-remodeling factors that determine F-actin formation (Blanchoin et al., 2014). We therefore investigated both transcriptional changes and protein–protein interactions that could contribute to KDM3A ciliary traits. To address the global transcriptional changes that occur in the absence of KDM3A during ciliary growth and resorption, we used a combined approach of RNA sequencing and quantitative label-free mass spectrometric analysis (Tables S2 [raw datasets] and S3 [thresholds]). Principal-component analysis of RPE1WT and KDM3A-null proteomes first showed that differences between samples primarily reflected serum content of the culture media as opposed to genotypes of the cell lines (Fig. 3 C). This supports previous studies reporting that KDM3A does not cause large global transcriptional changes but is instead a fine regulator of gene expression (Okada et al., 2007). Subsequently,
considering that KDM3A is a transcriptional activator (Yamane et al., 2006), we identified genes for which protein or RNA abundance were reduced in KDM3Anull cells compared with RPE1WT controls (Table S3). GO (Gene Ontology) analysis suggests that the transcriptional activity of KDM3A is required to sense the extracellular environment (Fig. 3D and Table S4). In agreement with previous findings (Lockman et al., 2007), direct interrogation of actin expression in these datasets reveals that smooth α-actin ACTA2 expression (Table S3) as well as protein levels (Fig. 3E) failed to be up-regulated in response to serum replenishment. A reduced abundance of ACTA2 in mutants could potentially compromise actin polymerization by limiting available actin monomers during ciliary resorption (+FCS). However, as actin levels are normal in KDM3Anull cells in the absence of serum, transcriptional control alone cannot explain perturbations of actin dynamics observed in mutant cells during ciliary growth (−FCS).

Next, we investigated a potentially direct effect of KDM3A on the cytoskeleton through protein interactions using KDM3A immunopurification of total cell extracts followed by...
mass spectrometry. In parallel, we performed a subsaturation two-hybrid screen using the JMJC domain of KDM3A as bait. The KDM3A antibody significantly enriched 166 proteins from RPE1WT and KDM3Anull cells during ciliary growth and resorption compared with RPE1WT in the transcriptomic and proteomic datasets during ciliary growth and resorption. Dysregulation of extracellular environment is the most enriched term by GO analysis (Table S4). (E) Label-Free quantification (LFQ) intensities for peptides specific to ACTA2 and ACTG2 (nonseparable because of sequence homology) across the indicated growth conditions obtained from total cell proteomes of control (RPE1WT and RPE1CrWT) and KDM3Anull (line 2) lines. **, P < 0.001; ns, not significant. (F) Proteomic-based identification of endogenous components significantly enriched in KDM3A pull-downs during ciliary growth (3 h − FCS). P-values are from t tests of label-free quantifications from RPE1WT relative to KDM3Anull from three experimental replicates. Select isolated actin-binding proteins are highlighted. (G) Schematic of the KDM3A region used as bait (JMJC domain in red) and ACTA2 prey identified in the two-hybrid screen. (H) Independent colonies (one to three) of rescued bait/prey plasmid cotransformed onto two-hybrid Gold yeast and grown in double selection (Leu/Trp) were resuspended and assessed for aureobasidin A resistance and α-Gal activity. Only yeast cotransformed with JmjC and ACTA2 grew in the stringent conditions indicating a binary interaction. (I) Pure smooth muscle actin resolved in 4–12% acrylamide gel (three serial dilutions) transferred to nitrocellulose is bound by GST-JMJC, but not GST.
KDM3A modulates actin dynamics

To characterize the functional interaction between KDM3A and actin, we further investigated cellular phenotypes known to depend on actin dynamics.

It was recently shown that KDM3A expression is upregulated when cells are in suspension (Pedanou et al., 2016) and that its cytoplasmic localization depends on mechanosensitive signals (Kaukonen et al., 2016). Indeed, Halo-tagged KDM3A is cytoplasmic and enriched in cortical actin filaments and cellular edges immediately after attachment of suspended cells (Fig. 4 A). We reasoned that the fast wave of actin polymerization (Fig. 4 B) that follows early spreading over solid substrates (Wolfenson et al., 2014) would thus provide functional evidence on the impact of cytoplasmic KDM3A on actin dynamics. Wild-type (RPE1WT and RPE1CWTS) and KDM3Anull (lines 1, 2, and 8) cultures were trypsinized and maintained in suspension in serum-free media for 2 h before plating (Fig. 4 C) or plated directly (Fig. 4 D) onto plastic plates. We found that in both cases, a greater proportion of KDM3Anull cells remained unspread compared with control cultures (Fig. 4, C and D; P0/P1 ratio χ² = 0.001), indicating that loss of KDM3A interferes with the early stages of cell spreading that require fast actin polymerization.

In the presence of serum, KDM3Anull cells consistently have rounder cellular edges compared with RPE1WT (Fig. 4 D). Branching or elongation of actin fibers is dynamically driven by the antagonism between ARP2/3 nucleating activity and capping proteins, ultimately determining the length of the actin fiber (Edwards et al., 2014). At the cellular edge, this interplay is manifested as the extension of lamellipodia to filopodia formation (Fig. 4 E), where the underlying actin-remodeling events steer cell migration (Pollard and Borisy, 2003; Koestler et al., 2013).

Consistent with these findings, KDM3Anull cells presented reduced cell motility (Fig. 4 F) and prominent leading edges (Fig. S2 D). Conversely, overexpressing KDM3A increases the proportion of cellular membrane ending in spike/filopodia-like protrusions (Fig. 4, H–J; and Fig. S2 E).

Together, the morphological and behavioral features found in the absence or overexpression of KDM3A demonstrate that KDM3A modulates global actin dynamics and suggest that KDM3A binding to actin may compete with or facilitate the binding of different actin-remodeling proteins.

Altered actin dynamics underlies the ciliary traits of KDM3A-null cells

To confirm that a deregulated actin cytoskeleton in the absence of KDM3A underlies the abnormal ciliary phenotypes observed in mutants, we aimed to rescue these phenotypes using chemical modulators of F-actin formation. Depolymerization of F-actin promotes ciliary growth (Kim et al., 2010, 2015). Conversely, actin polymerization is thought to be required for ciliary resorption (Liang et al., 2016).

Interfering with actin dynamics through cytochalasin D (CytoD) treatment promotes ciliogenesis in cycling RPE1 cells without the requirement of serum withdrawal (Cao et al., 2012). Indeed, CytoD treatment alone is sufficient to rescue ciliogenesis in genetic mutants affecting IFT machinery (Kim et al., 2010; Fu et al., 2016). Surprisingly, we instead observed that KDM3Anull cells accumulated abnormal phalloidin-stained foci (Fig. 5 A, 2 µM CytoD) and have decreased proportion of ciliated cells (Fig. 5 B). Live-cell imaging with a ciliary marker (5-HT6-GFP; Berbari et al., 2008) suggests that the decreased proportion of KDM3Anull ciliated cells in 0.5 µM CytoD is likely caused by reduced ciliary stability, as cilia break off the cell body (Fig. 5 C), without the net increase in ciliary length observed in control cultures (Fig. 5 D). CytoD treatment of cycling RPE1WT cultures thus phenocopies the increased proportion of ciliated cells found in KDM3Anull cells in the presence of serum (Fig. 2 E). The low abundance of actin fibers of KDM3A mutants likely facilitates ciliogenesis, but further perturbations in actin polymerization are not tolerated by KDM3Anull cells (Fig. 5 E).

Critically, the converse experiment, where we promoted actin polymerization using jasplakinolide treatment (Kustermans et al., 2008) in the presence of serum, rescued the abnormal resorption of KDM3Anull cilia (Fig. 5, F and G). This suggests that failure of KDM3Anull cells to polymerize actin in response to serum contributes to the abnormal resorption kinetics of mutant cilia (Fig. 5 H).

These pharmacogenetic results support a functionally relevant association between KDM3A and the actin cytoskeleton during ciliogenesis. In the absence of KDM3A, the formation of actin filaments is perturbed and underlies the ciliary defects of KDM3Anull cells.

A requirement for KDM3A in ciliary stability and IFT

Live imaging suggested that KDM3Anull cilia were unstable. We investigated whether this instability was caused by the drug treatment or whether it is an inherent property of mutant cilia. Live imaging of cell during ciliary growth (∼FCS) revealed that RPE1WT cilia remained fairly stable, whereas KDM3Anull cilia underwent excessive elongation and fragmentation (Videos 1 and 2 and Fig. 6 A). Similar ciliary instability was observed in Kdm3aKc mutant MEFs (Video 3). These results suggest that instability of mutant axonemes in the absence of KDM3A reflects an inability to regulate axonemal length. These findings are supported by the unresponsiveness of KDM3Anull cilia to resorb (Video 4) and the wider range of lengths observed in fixed KDM3A mutant cilia (Fig. 2).

The presence of ciliary bulges (Fig. 2 E) and their shedding observed during live imaging of KDM3Anull cells suggest that the instability of KDM3A mutant cilia results from perturbation of IFT. Indeed, IFT staining of MEFs (Fig. 1 F) and RPE1 null for KDM3A (Fig. 6 B) showed abnormal distributions of IFT88 and IFT81. The anomalies were most evident at the ciliary tip (Fig. 6 C) but could also be observed at the ciliary base (Fig. 1 F).

In Chlamydomonas reinhardtii, actin is required for the recruitment of IFT particles to the basal body, where it controls flagellar entry of IFT trains (Avasthi et al., 2014). Accordingly, the abnormal distribution of IFTs observed in KDM3Anull cells could be caused by the permissive and depolymerized state of the actin cytoskeleton in these mutants. If this was true, then perturbing actin dynamics in RPE1WT would phenocopy the IFT
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anomalies observed in KDM3A mutants. To test this, we treated cells with CytoD or CK-869, a small molecule that specifically inhibits ARP2/3 complex components (Hetrick et al., 2013) copurifying with KDM3A (Fig. 3 F). Perturbations of ARP2/3 activity could contribute to thomorphological phenotype of KDM3A null cells (Fig. 4, E–G). RPE1 WT cells treated with either drug in the presence or absence of serum showed large accumulations of IFT81 at the ciliary tip of RPE1 WT and KDM3A null cells (Fig. 6, C–E) accompanied by ciliary elongation (Fig. 6 F). The effect of CK-869 was particularly interesting, as KDM3A null cells were extremely responsive to ARP2/3 inhibition, showing ciliary elongation from doses as low as 2 μM (Fig. 6 G), whereas RPE1 WT cells only respond from 50 μM (Fig. 6 H). This functional synergism between KDM3A mutants and CK-869 treatment indicates a common underlying mechanism by which KDM3A influences IFT through actin polymerization involving the ARP2/3–actin assembly pathway.

In summary, live imaging studies provide evidence that in the absence of KDM3A, cilia become unstable. Antibody staining showed abnormal distributions of endogenous IFTs in mutant cilia, and this trait can be phenocopied in wild-type cells by perturbing actin dynamics. Using two different drugs interfering with actin nucleation, we demonstrate that in mammalian primary cilia, actin dynamics also modulates IFT. Altogether, these findings provide evidence that in the absence of KDM3A, actin dynamics are perturbed, disrupting IFT and destabilizing cilia.

KDM3A null cilia reveal an actin-driven constraint for IFT in wild-type cilia

The finding that inhibiting actin polymerization in mammalian control cells phenocopies the abnormal IFT loaded bulges found in KDM3A null cells suggests that wild-type cilia have an actin-dependent constraint that modulates ciliary IFT. To test this further, we increased IFT protein levels by transfecting

Figure 4. KDM3A is required for actin polymerization. (A) 60 min after plating on glass coverslips, 3D reconstruction reveals cytoplasmic distribution of Halo-KDM3A with strong presence at cellular edges and apical cap. (B) Schematic of stages requiring fast actin polymerization occurring from initial attachment (Phase 0 [P0]) to early spreading (P1). P2 represents fully spread cells. (C) Bright-field image and number of RPE1 WT and KDM3A null cells that remain round (P0) or began spreading (P1) over plastic plates after suspension in the absence of serum for 2 h. Red arrow and green encircling indicate cells at P0, defined by birefringence at edges, and P1, defined by flattened edges, respectively. (D) Proportion of cells that remain in P0 when cultures are plated immediately after trypsinization (N = 3, n > 50 cells scored/genotype). Error bars: mean ± SD. *P < 0.0001, from χ². (E) Wide-field images illustrate differences in cellular edges between RPE1 WT and KDM3A null. (F) Schematic of actin filament capping or branching leading to changes in cell morphology. As KDM3A binds actin, the rounder cellular edges of KDM3A cells suggest an imbalance between capping (yellow) and ARP2/3 mediated branching (green). (G) Median and range of the distance migrated by attached RPE1 WT cells and KDM3A null (lines 1, 2, and 8; 4 h after plating). Dots represent measurement of distance moved by a single cell. (H) Confocal images illustrate the distinct cellular edges of RPE1 WT cells transfected with Halo vector or Halo-KDM3A. (I) Hyperfilopodia-like projections are most prominent at the apical pole of Halo-KDM3A transfected cells (plane 2 in diagram). (J) Median and range of membrane lengths at cellular edge of Halo transfected cells measured as described in Fig. S2 D. N = 2 transfections. Statistics from G and J are from unpaired t tests.
tagged IFT81 into RPE1wt and KDM3A-null cells, reasoning that if an actin gate does exist and is dysfunctional in the absence of KDM3A, IFT overexpression would have opposing effects in wild-type and KDM3A mutants.

We generated a functional IFT81-DDK construct capable of rescuing ciliogenesis in an IFT81-null RPE1 line (Fig. S3, A–D). Comparable transfection levels of IFT81-DDK onto RPE1wt and KDM3A-null cells were confirmed by immunoblotting (Fig. 7 A). Live imaging revealed that overexpressing IFT81 enabled formation of more cilia and with faster kinetics in KDM3A-null mutants compared with RPE1 WT cells (Fig. 7 B, slopes; and Fig. S3, E and F). Importantly, expression of 5HT6-GFP alone did not recapitulate this increased rate of ciliogenesis in KDM3A-null cells (Fig. 7 C), demonstrating that this difference is not simply caused by an increased propensity to ciliate but is dependent on ectopic IFT81. In the presence of IFT81-DDK, KDM3A-null cilia are also longer than controls (Fig. 7 D). These results did not reflect differences in cell density (Fig. 7 E) or levels of endogenous IFT-B components (Fig. 7 F). This indicates that increasing the abundance of IFT promotes ciliogenesis in KDM3A mutants, but not wild-type controls.

Because CytoD treatment of control cells phenocopied the ciliary traits of KDM3A-null mutants, we simultaneously induced actin depolymerization along with IFT81 overexpression in RPE1wt cultures. Indeed, this dual treatment allows wild-type cells to become sensitive to IFT-B overexpression when actin is depolymerized (Fig. 7 G).

Together, these findings provide further and independent evidence of deregulated IFT in KDM3A mutant cilia that result from perturbations in actin dynamics. When actin is unperturbed, increasing IFT pools in control cells makes no difference to ciliary growth, indicating that actin allows wild-type cells to maintain tight regulation of IFT entry into the ciliary compartment (Fig. 8 A). In contrast, when actin nucleation is perturbed either genetically (KDM3A mutants) or chemically (CytoD treatment ARP2/3 inhibition), this constraint is lifted.

Discussion

Our results show that KDM3A plays a role in mammalian ciliogenesis through the modulation of actin dynamics. In the absence of KDM3A, the assembly and growth of primary cilia is enhanced. However, ciliary stability is compromised such that the length of mutant axonemes and presence of IFT proteins within cilia become unregulated. We find that KDM3A-null cells have reduced abundance of actin filaments. This is because KDM3A plays a transcriptional role regulating actin gene expression and
binds directly to actin protein, influencing local actin networks. We demonstrate that the ciliary traits uncovered in KDM3A mutants can be chemically reversed or phenocopied in wild-type cultures by altering actin dynamics. Through independent approaches involving genetics, small-molecule inhibitors, and IFT overexpression, we uncover an actin-mediated constraint on IFT within mammalian cilia. Our findings uncover a functional interaction between the actin cytoskeleton and IFT that is perturbed in the absence of KDM3A.

Figure 6. Perturbing actin polymerization phenocopies IFT abnormalities of KDM3A mutants. [A] Single time frames from Videos 1 and 2 of live RPE1WT and KDM3A\textsuperscript{null} (line 2) cultures. Arrows point to bulges forming along cilia of KDM3A\textsuperscript{null} cells. [B] Confocal images after 48 h –FCS indicate low levels of IFT81 at tip of RPE1WT cilia (green arrow) but conspicuous accumulations in KDM3A\textsuperscript{null} mutant cilia, frequently accompanied by IFT81-containing “vesicles” (yellow arrows). [C] Wide-field images of RPE1WT cultures in the presence of serum show accumulations of endogenous IFT81 in CytoD treated cells that appear sequentially as ciliary buds (cb; without acetylated α-tubulin–stained axonemes, most frequent with 0.2 μM CytoD) and progress toward ciliary tip (ct; 2 μM CytoD). [D] Representative images of IFT81 distribution in cilia formed after 3 h with solvent control or 100 μM CK-869 –FCS. [E] Quantification of cilia with or without IFT81 bulges among treated (2 μM CytoD and 100 μM CK-869) and solvent controls. Numbers in bars indicate number of cilia scored. Error bar represents SD. P-value is from χ\textsuperscript{2} test. [F] Frequency of length ranges illustrate increased proportion of long cilia in 100 μM CK-869–treated cultures. [G] Differential sensitivity of RPE1WT and KDM3A\textsuperscript{null} to ARP2/3 inhibition as illustrated by the length of cilia in subconfluent cultures treated for 3 h with 2 and 10 μM CK-869 –FCS. Mean ± 5th–95th percentile (N = 3 replicates, n > 30 cilia measured/condition). [H] Mean ± 5th–95th percentile of ciliary lengths indicate that RPE1WT cultures begin to show significant cilial elongation in ≥50 μM CK-869 [N = 3 replicates, n > 30 cilia measured/condition]. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (paired t test); ns, not significant. Bars, 5 μm.
interaction of KDM3A with actin locally creates an actin gate at the ciliary base (Fig. 8, B and C; and Video 5) to regulate entry of IFT. In the absence of KDM3A, access to cilia is unrestricted and balance and/or kinetics of IFT are perturbed.

The fact that KDM3A is an enzyme that binds actin through its catalytic domain, together with previous observations that actin proteins could be abnormally methylated in Kdm3a mutant testes (Kasioulis et al., 2014), prompted us to test the demethylase activity of KDM3A in lysine-methylated actin peptides found in this study. However, as yet we have failed to detect changes in methylation by KDM3A in any tested peptide in vitro (unpublished data). Although it remains to be seen whether KDM3A can posttranslationally modify actin in vivo, the binding of KDM3A to actin itself is likely to modulate binding of other actin modifiers, including ARP2/3 and the capping components, to directly tune actin networks.

The functional interplay between KDM3A-ARP2/3 is interesting: KDM3Anull cells respond to lower CK-869 doses than RPEWT cells, suggesting that the ciliary traits of KDM3A mutants have already compromised ARP2/3 function. A role for ARP2/3 at the cilium/centrosome has been further implications. Understanding how cancer cells can subvert actin bundling during metastasis is an emerging and exciting theme in research. Although the brunt of the current focus of overexpression of KDM3A in various types of cancer has been on its transcriptional targets (Krieg et al., 2010; Uemura et al., 2010; Cho et al., 2012; Osawa et al., 2013; Tee et al., 2014; Parrish

Figure 7. KDM3A uncovers an actin-mediated control on IFT that precludes IFT81 overexpression. (A) Immunoblots with DDK antibody of total cell extracts harvested 24 h after transfection with IFT81-DDK plasmid. IFT81null cells transfected with 5HT6-EGFP used as negative control for DDK signal (red arrow). Ponceau red is a loading control. (B) Line graph showing the number of 5HT6-EGFP–positive cells with a cilium at the indicated time points. Error bars represent SEM between fields of view. Linear regression and p-values from comparing the individual slopes by paired t test. See also Fig. S3 (E and F). (C) Median and range of the percentage of 5HT6-EGFP–positive cells that contain a cilium under each experimental condition. Each dot is the percentage of one independent experiment (N = 4 transfections, n > 100 GFP+ cells scored/condition). **, P < 0.01 (paired t test). [D] Mean ± 10th–90th percentile of lengths of 5HT6-EGFP–positive cilia from the two last cotransfections with IFT81-DDK presented in C. ***, P < 0.001 (paired t test). (E) Plates fixed after live imaging confirm equal cell densities in RPE1WT and KDM3Anull cultures. (F) Immunoblots of total cell extracts from the indicated wild-type and mutant lines after 3 h −FCS (ciliary growth) indicate comparable total levels of endogenous IFT components. (G) Mean ± 10th–90th percentile of percentage of 5HT6-EGFP–positive cells containing a cilium under each experimental condition. Each dot is the percentage of one transfection in the presence or absence of 0.5 µM CytoD. Each dot is the percentage of one field of view taken (N = 2 transfections, n = 4, 28, 12, and 20 fields scored/condition). Analysis of variance: P < 0.004; t test for the indicated paired comparisons: *, P < 0.05; ***, P < 0.0005. (H) Graphical summary illustrates the insensitivity of RPE1WT to IFT81 overexpression unless actin is depolymerized or KDM3A is absent. ns, not significant.
Figure 8. Proposed mechanism for KDM3A in ciliary regulation. (A) Collapsed time frames (3 h) of live RPE1 WT and KDM3A null cultures transiently transfected with ARL13B-mKate2 and IFT81-mGFP showing consistently (arrows) increased accumulations and/recruitment of IFT81-EGFP at the mutant basal body. (B) Enrichment of Halo-KDM3A in centrosomes in ∼2% of transfected cells. (C) Centrosomal localization of KDM3A confirmed by construct containing its zinc-finger and nuclear receptor–binding domains fused to GFP, colocalizing with IFT81. See also Video S. These findings support KDM3A regulating both global and local actin dynamics at specific subcellular compartments with a direct impact on ciliogenesis. (D) Summary of KDM3A mutant cellular phenotype: In the absence of KDM3A, the actin cytoskeleton contains reduced abundance of actin filaments, wide lamellipodia at all cellular edges and reduced migration. Without an actin gate, ciliogenesis is facilitated, and these cilia are long but unstable. (E) Summary of serum (+FCS or low serum [LS]) and pharmacogenetic modulation of actin at the pericentriolar periciliary compartment (PPC) illustrates that promoting actin polymerization either phenocopies ciliary growth in wild-type cells or rescues the abnormal resorption of KDM3A mutant cilia. CK-869 treatment of cells pinpoints the actin nucleation activity of ARP2/3 to be necessary to maintain normal IFT within cilia. IFT81 accumulations suggest unbalanced anterograde-retrograde transport (represented by different arrow size) when actin is perturbed. Together, these results indicate that the ciliary traits of KDM3A mutants stem from perturbations of actin dynamics, which upset IFT.
et al., 2015), our work suggests that cytoplasmic KDM3A may act as an important cytoskeletal rheostat integrating mechanosensation (Kaukonen et al., 2016; Pedanou et al., 2016) with cell adhesion and signaling through cilia. Aberrant length ranges and resorption of KDM3A mutant cilia in response to environmental cues could also contribute to metabolic imbalance in Kdm3a mouse models (Okada et al., 2010), as ciliary length is tightly regulated in response to leptin levels (Han et al., 2014).

Our findings that KDM3A has roles beyond the “direct” regulation of transcription (i.e., acting as an H3K9 demethylase; Yamane et al., 2006) links cytoplasmic sensing of actin polymerization, ciliogenesis, and transcriptional activity in response to serum. KDM3A is a versatile protein that is tightly regulated in response to environmental inputs (Pollard et al., 2008; Krieg et al., 2010; Cheng et al., 2014; Kasioulis et al., 2014; Abe et al., 2015) with both catalytic and structural roles (Abe et al., 2015; Schneider et al., 2015) involving binding to histone (Yamane et al., 2006) and nonhistone proteins (Ramadoss et al., 2017) within nuclear and cytoplasmic compartments (Okada et al., 2007; Yang et al., 2009; Yamada et al., 2012; Kasioulis et al., 2014; Kaukonen et al., 2016). KDM3A thus appears to be capable of integrating epigenetic and cytoskeletal regulation with ciliary IFT in response to extracellular cues.

While our manuscript was in press, actin-mediated scissors of the tips of mammalian primary cilia was uncovered as a mechanism to clear activated ciliary receptors (Nager et al., 2017) or to promote ciliary disassembly (Phua et al., 2017), independent of retrograde IFT involvement. The behavior of KDM3Amut cilia is consistent with these findings; through its role modulating actin dynamics, KDM3A would also modulate ciliary ectocytosis at the tip. The relative contribution of unrestricted IFT recruitment to cilia or reduced IFT clearance through ectocytosis in KDM3A mutants is an exciting topic for future research.

Materials and methods

Plasmids

Plasmids encoding the serotonin receptor pEGFPN3-5HT6 (Berbari et al., 2008), IFT81-DDK, and Halo-KDM3A were purchased from Addgene (35624), OriGene (RC216437), and Kazusa DNA Res Institute (HC05559), respectively. IFT81-mGFP was made by transferring Sgf1-MluI restriction fragment from clone RC216437 into mTagGFP vector PS100048 (OriGene). The KDM3A deletion mutant GFP-ZFNR was cloned using PCR primers containing Bgl2 and mTagGFP vector PS100048 (OriGene). The KDM3A deletion mutant (Kdm3aΔC) and hypomorphic gene-tarp (Kdm3aΔGT) mouse models were described previously (Tateishi et al., 2009; Kasioulis et al., 2014). Genotyping was done with PCR primers as in Table S1.

Kdm3a mouse model

The deletion mutant (Kdm3aΔC) and hypomorphic gene-tarp (Kdm3aΔGT) mouse models were described previously (Tateishi et al., 2009; Kasioulis et al., 2014). Genotyping was done with PCR primers as in Table S1.

Cell lines and CRISPR/Cas9 mutants

Primary MEF cultures were established by mincing embryonic day 11.5–13.5 embryos in DMEM supplemented with antibiotics, FCS, and β-mercaptoethanol and grown in 3% O2. Telomerase immortalized human retinal pigment epithelial cells (hTER-T-RPE1, referred to here as RPE1WT) were from Takara Bio Inc. and maintained in DMEM-F-12 (1:1: Thermo Fisher Scientific). RPE1WT cells were engineered with a double nicking strategy using CRISPR/Cas9 nickases following reported guidelines (Ran et al., 2013). Oligonucleotides to produce guide RNAs (listed in Table S1) were designed with CRISPR Design Tool (http://tools.genome-engineering.org). In brief, guides cloned onto px461 or px462 (Addgene) mammalian expression plasmids were cotransfected onto RPE1WT cells followed by 24 h of 3 µg/ml puromycin selection and individual GFP-positive cells sorted by FACS onto 96-well plates to establish the RPE1 lines KDM3Amut and IFT81mut. RPE1WT derivative (KDM3A) (IFT81mut) derives from noneventful CRISPR targeting of IFT81, subsequent sequencing confirmed wild-type alleles for both IFT81 and KDM3A. Mutations were confirmed by immunoblotting with KDM3A and IFT81 antibodies and sequencing of genomic PCR (primers are listed in Table S1) cloned into pGEM-T Easy (Promega) using T7 and SP6 primers.

Transfections

Parental and engineered RPE1 lines were transfected with Lipofectamine2000 (Thermo Fisher Scientific) following the manufacturer’s instructions, grown without antibiotic the day before, and transfected in the absence of serum for 5 h. All cotransfections were done mixing the DNA of the relevant cotransfected plasmids before the addition of transfection reagents.

Antibodies

Antibodies were from the following sources: N-KDM3A (12835; Proteintech), C-KDM3A (NB100-77282; Novus Biologicals), anti-acetylated α-tubulin (T7451; Sigma-Aldrich), γ-tubulin (GTU-88; Sigma-Aldrich), IFT81 (13967-1-AP; Proteintech), IFT81 (11744-1-AP; Proteintech), GABDH (5019A-2; ImGENEX), ARL13B (17711-1-AP; Proteintech), DDK tag (TAS0011; OriGene), and Halo (G9281; Promega). Secondary antibodies for immunofluorescence and immunoblotting are Fab’2 IgG Alexa Fluor from Molecular Probes and HRP conjugated from EMD Millipore and Sigma-Aldrich.

Drug treatments

CytoD (25023; EMD Millipore), CK-869 (C9124; Sigma-Aldrich), and jasplakinolide (1689-05; BioVision) were dissolved as recommended by the manufacturers and used at the indicated concentrations. Controls for each drug treatment contained the relevant solvent only.

Ciliary phases

For the study of cilia dynamics, cells were plated 24 h in advance. In all cases, stocks of all cell lines were maintained at comparable cell densities. For the studies of ciliogenesis in exponentially growing cultures, cells were not allowed to reach confluency during two consecutive passages before assaying ensuring a consistent number of cycling cells. For cilia resorption studies, cells were plated between 40% and 50% confluency followed by a further 24, 48, or 72 h culture without serum (−FCS, as indicated in each panel); for ciliary growth assays (3–24 h), cells were plated at 60–70% confluency and floating the tissue culture plates on a water bath at 37°C for the indicated times. Temperature-induced resorption was done by adding 10% FCS and floating the tissue culture plates on a water bath maintained at 42°C for the indicated times.

Cell spreading assays

Actively growing cultures were trypsinized and resuspended in complete media, replated immediately, or spun down and placed in suspension without serum for 90 min in a cell incubator. Replated cells were imaged 90–120 min after replating on uncoated plastic plates.
Yeast two-hybrid studies
The initial yeast two-hybrid screen was performed using an embryonic eye mouse cDNA library (Kasidoulis et al., 2014) with the JMJC domain of KDM3A as bait cloned through partial EcoRI digestion into the DNA-binding domain encoding plasmid pGBK7T7. Bait and prey plasmids from blue colonies growing in quadruple selection (Leu/Trp/Ade/ His) were rescued by cell lysis and bacterial transformation in ampicillin (pGADT7) or kanamycin (pGBK7T7) plates and sequenced. Plasmids encoding CAPZB and ACTA2 were cotransformed back into the yeast two-hybrid Gold strain (Takara Bio Inc.) along with JMJC-pGBK7T7, as described in the Matchmaker two-hybrid system protocol. Independent colonies grown in double selection (Leu/Trp) were resuspended, and a 1/100 dilution was replaced in 0.2 µg/ml aureobasidin A double selection plates in the presence of Xα-Gal (Takara Bio Inc.).

Immunofluorescence
Cells were grown on glass coverslips and fixed (10 min, 4% methanol-free formaldehyde), except for those shown in Fig. 6 (C and D), which were fixed with ice-cold methanol for 5 min. Fixed cells were permeabilized (15 min in 0.25% Triton X-100) and blocked in 2% BSA before incubation with antibodies and DAPI and mounted in ProLong Gold (Thermo Fisher Scientific). Rhodamine–phalloidin (R415; Thermo Fisher Scientific) added to the secondary antibody incubation was used to visualize F-actin of fixed cells.

Imaging
Live-cell microscopy was done using glass-bottom plates (662892; Greiner Bio-one) in a heated chamber with a source of nitrogen using a 40x plan Fluor 0.75 NA dry lens in an A1R confocal microscope (Nikon) in the relevant culture media for each growth condition. Movies shown were acquired as to minimize photobleaching. Postacquisition analyses were done with NIS-Elements (Nikon) on maximum-intensity projections using Fiji (ImageJ) and IMARIS 8.4 (Bitplane). Ciliary length studies on fixed cells were done with wide-field microscopy or confocal imaging as indicated in each panel using a 40x dry lens and Fiji software for measurements.

Cell lysis and immunopurifications
Total extracts for immunoblots were obtained by lysing cells in 150 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% IGEPEAL, 1% Triton X-100, 1 mM EDTA, 0.5% deoxycholate, Benzoase DNase (EMD Milipore), and Halt protease inhibitor (1860932; Thermo Fisher Scientific). Total cell extracts for total cell proteome were done in PBS and 1% SDS in the presence of protease inhibitor, sonicated, and cleared by 10-min centrifugation at 14,000 rpm. For KDM3A interactome, cells were plated the day before and grown in the absence of serum for 3 h, lysed in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM EDTA, 0.5% IGEPEAL, and protease inhibitors and incubated with N-KDM3A antibody (12835; Proteintech) for 2 h at 4°C followed by 15-min incubation with PureProteome magnetic beads (EMD Millipore) to concentrate the immunoglobulin complexes. Three successive washes were performed to reduce the IGEPEAL concentration.

Far-Western blot
Serial dilutions (50–200 ng/µl) of pure actin derived from rabbit skeletal muscle (AKL99; Cytoskeleton, Inc.) resolved in 4–12% acrylamide were transferred onto nitrocellulose and stained with Poncéeau red to visualize loadings. Bacterially expressed GST or GST-JMJC immobilized in glutathione magnetic beads (8821; Proteintech) were washed and eluted in glutathione buffer. Membrane blocked with 5% BSA was incubated with eluted GST proteins in 10 mM Tris, pH 7.6, 100 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.1% (vol/vol) Tween-20, and 2% BSA for 1 h. Subsequent steps followed standard immunoblotting protocol using GST antibody to detect GST proteins retained on membrane.

Mass spectrometry
On-bead purified complexes were digested with trypsin, reduced, alkylated, and desalted, and two runs of each sample were analyzed by liquid chromatography tandem mass spectrometry as described previously (Turriziani et al., 2014). Datasets can be found in ProteomeXchange identifier PXD004334. Data were analyzed using the MaxQuant software suite by searching against the UniProt HUMAN database with protein N terminus (acyetylated), M (oxidation). Label-free quantification values were determined by MaxQuant as described previously (Cox et al., 2014). In the search for posttranslational modifications, databases were also searched for peptides with mass modifications as follows: mono-, di-, and trimethylation (delta mass of 14.0157, 28.0313, and 42.04695 D, respectively).

RNA sequencing
RNA was isolated from RPE1 WT and KDM3Anull (line 2) cells in triplicate cultures under ciliary growing or resorption conditions (Fig. 3 A) using RNeasy Plus (QIAGEN) according to the manufacturer’s instructions. RNA integrity score was calculated with RNA 6000 Nano reagent (Agilent Technologies) in a 2100 Bioanalyzer (Agilent Technologies). All samples used for the preparation of the RNA-sequencing library had a RIN (RNA Integrity Value) score above 9. Library construction used poly(A) RNA selection, and all 100-bp reads were obtained using Illumina HiSeq2000. Cufflinks, CummeRbund, and Picard were used for alignments to reference genome, transcript assembly, quantification, and statistical analysis, respectively, done at Oxford Gene Technology (The Molecular Genetics Company). The mean read depth for each sample was of 13 × 10⁶.

GO term analyses
Genes or proteins differentially enriched in proteome or interactome datasets were analyzed using DAVID (the Database for Annotation Visualization and Integrated Discovery).

Statistics
Statistics were done using GraphPad Prism 5 software. One-way analysis of variance was used for the initial assessment of multiple comparisons. In those cases where a significant difference was observed (P < 0.01), a pairwise two-tailed Students t test was applied to compare individual conditions. χ² test was applied to compare percentages as indicated in each panel. Asterisks indicate p-values (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001) calculated through the statistical analysis indicated in each case. N represents the number of experimental or biological replicates, and n represents the number of events measured in all experimental or biological replicates as indicate.

Online supplemental material
Table S1 shows sequences of primers used to clone KDM3A expression plasmids, CRISPR/Cas9 editing, and all genotyping. Table S2 contains raw values of RNA sequencing reads and proteomic studies. Table S3 shows thresholding criteria and the resulting lists of genes that were subsequently used for GO term analysis. Table S4 shows enriched GO terms. Fig. S1 shows genotypes of KDM3Anull cells, ciliary anomalies of Kdm3aΔ/ΔΔ MEFs, and effect of overexpressing KDM3A in the percentage of ciliated cells. Fig. S2 shows phalloidin staining of Kdm3aΔ/ΔΔ MEFs, assessment of KDM3A interaction with actin by two-hybrid screening and far-Western blotting, and...
detailed measurement criteria used to quantify the extent of filopodia in transfected cells. Fig. S3 shows genotyping of IFT81<sup>wt</sup> cells, functionality of IFT81-DDK construct, and identical transfection efficiencies of 5HT6-EGFP+IFT81-DDK onto RPE1WT and KDM3A<sup>wt</sup> cells. Videos 1 and 2 show instability of KDM3A<sup>wt</sup> cilia. Video 3 shows instability of Kdm3a<sup>ΔJC/ΔJC</sup> cilia. Video 4 illustrates the unresponsive behavior of KDM3A<sup>ΔJC</sup> cilia to serum-induced resorption. Video 5 illustrates that KDM3A<sub>2</sub>-EGFP can be found in cilia.

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