miR-34/449 miRNAs are required for motile ciliogenesis by repressing cp110

Rui Song1*, Peter Walentek2*, Nicole Sponer1*, Alexander Klimke1, Joon Sub Lee1, Gary Dixon1, Richard Harland2, Ying Wan4, Polina Lishko1, Muriel Lize5, Michael Kessel3 & Lin He1

The mir-34/449 family consists of six homologous miRNAs at three genomic loci. Redundancy of miR-34/449 miRNAs and their dominant expression in multilayered epithelia suggest a functional significance in ciliogenesis. Here we report that mice deficient for all mir-34/449 miRNAs exhibited postnatal mortality, infertility and strong respiratory dysfunction caused by defective mucociliary clearance. In both mouse and Xenopus, mir-34/449-deficient multiciliated cells (MCCs) exhibited a significant decrease in cilia length and number, due to defective basal body maturation and apical docking. The effect of mir-34/449 on ciliogenesis was mediated, at least in part, by post-transcriptional repression of Cpi110, a centriolar protein suppressing cilia assembly. Consistent with this, cp110 knockdown in mir-34/449-deficient MCCs restored ciliogenesis by rescuing basal body maturation and docking. Altogether, our findings elucidate conserved cellular and molecular mechanisms through which miR-34/449 regulate motile ciliogenesis.

MicroRNAs (miRNAs) encode a class of small, non-coding RNAs that regulate gene expression through post-transcriptional repression1–4. Although the initial discovery of miRNAs was made through classic forward genetics in worm development5,6, loss-of-function studies on most individual miRNAs yield no overt developmental defects in multiple organisms, suggesting strong functional redundancy among homologous miRNAs7. Redundant miRNAs can be generated from multiple genomic loci or transcribed from a single polycistronic precursor. Collectively, these redundant miRNAs could constitute the majority of expressed miRNAs in specific cell types8. Such extensive homo- and dominant cell-type specific expression of a single miRNA family could confer a robust functional readout that can only be revealed by complete removal of redundant miRNAs.

mir-34/449 miRNAs constitute a conserved family in vertebrates9–11, comprising three genomic loci, mir-34a, mir-34b/34c and mir-449c/449b/449a (mir-449), which encode six homologous miRNAs (mir-34a, 34b, 34c, 449a, 449b and 449c)12,13 (Fig. 1a and Extended Data Fig. 1a). Sequence homology among mir-34/449 miRNAs, particularly at the seed region, predicts robust functional redundancy. mir-34/449 miRNAs are highly enriched in mucociliary epithelia that contain motile cilia10, which beat cilia, including lung, brain, testis and female reproductive organs (Extended Data Fig. 1). Mature miR-34/449 miRNAs were enriched in organs containing motile cilia, including lung, brain, testis and female reproductive organs (Extended Data Fig. 3a). We specifically detected and quantified individual miR-34/449

PCD-like phenotype in mir-34/449 TKO mice

To characterize mir-34/449 functions, we generated triple knockout (TKO) mice deficient for all mir-34/449 loci (mir-34a, mir-34b/34c and mir-449)14 (Extended Data Fig. 1b, c). Although mir-449 resides in intron 2 of cdc20b15, mir-449 deletion in mice does not negatively affect cdc20b expression (data not shown). TKO mice were born at a Mendelian ratio with normal body weight (Fig. 1b and Extended Data Fig. 1d); yet exhibited frequent postnatal mortality with only ~40% surviving to adulthood (Fig. 1b). TKO mice also exhibited growth attenuation with ~50% lower body weight than littermate-controlled double knockout (DKO) mice (mir-34a−/−; mir-34b/34c−/− or mir-34a−/−; mir-449−/−; mir-34a−/−; mir-449−/−, Fig. 1c and Extended Data Fig. 1d).

Surviving TKO mice showed severe respiratory distress characterized by frequent coughing and sneezing (Extended Data Fig. 1e and Supplementary Video 1). Dying and surviving TKO mice displayed respiratory dysfunction, with excessive mucus accumulation in the paranasal cavities and increased susceptibility to respiratory infections (Fig. 1d and Extended Data Fig. 1f). Littermate-controlled mir-34a−/−; mir-34b/34c−/− or mir-34a−/−; mir-449−/− DKO mice phenotypically resembled wild-type mice, without obvious developmental or respiratory defects (Fig. 1d and Extended Data Fig. 1f, g).

Unlike phenotypically normal DKO controls (mir-34a−/−; mir-449−/− or mir-34a−/−; mir-34b/34c−/−), surviving TKO males and females were infertile, generating no pregnancies when mated with wild-type animals. TKO males exhibited defective spermatogenesis during differentiation from elongating spermatids to spermatozoa (Fig. 1e and Extended Data Fig. 2a, b), when flagella formation occurs17. TKO females exhibited a decrease in epithelial ciliation of the fallopian tube, presumably causing defects in oocyte transport17 (Fig. 1e and Extended Data Fig. 2a, c). Altogether, the TKO mouse phenotype resembled symptoms of a subset of PCD patients, exhibiting predominant respiratory and fertility defects without hydrocephaly or left-right asymmetry defects18,19 (Extended Data Fig. 2d).

Ciliogenesis defects in mir-34/449 TKO mice

Mature miR-34/449 miRNAs were enriched in organs containing motile cilia, including lung, brain, testis and female reproductive organs (Extended Data Fig. 3a). We specifically detected and quantified individual miR-34/449

©2014 Macmillan Publishers Limited. All rights reserved
miRNAs using single knockout and TKO controls (Fig. 2a and Extended Data Fig. 3b, c). In situ hybridization revealed high-level miR-34/449 expression in respiratory epithelia, with miR-34a being expressed broadly in multiple cell types, and mir-34c or mir-449c being enriched specifically in airway MCCs (Fig. 2a and Extended Data Fig. 3d).

A major symptom of PCD is dysfunctional airway clearance\textsuperscript{16,17}. Defective mucociliary clearance in mir-34/449 TKO mice, along with the MCC-specific mir-34/449 expression, prompted us to examine the roles of miR-34/449 in airway MCCs. High-speed imaging revealed a slow and limited fluid movement in TKO tracheal explants, accompanied by a significant reduction of visibly ciliated MCCs (Fig. 2b and Supplementary Video 2). This contrasts the effective anteriorward fluid flow in wild-type and DKO tracheal explants (Fig. 2b and Supplementary Videos 2 and 3).

The decrease of visible MCCs in TKO tracheas could reflect defective cell fate specification or ciliogenesis. We analysed mir-34a\textsuperscript{–/–}; mir-449\textsuperscript{–/–} DKO and TKO tracheas for Foxj1, a master regulator of motile ciliogenesis\textsuperscript{24}, and acetylated α-tubulin (Ac-α-tub), a cilia marker. Both DKO and TKO tracheas had Foxj1 immunofluorescence staining and Foxj1 mRNA levels comparable to wild-type controls (Fig. 2c and Extended Data Fig. 3e). Nevertheless, a portion of Foxj1-positive cells lacked cilia in TKO tracheas, yet most Foxj1-positive cells were fully ciliated in DKO and wild-type tracheas (Fig. 2c, and data not shown). This suggests normal cell fate specification with defective ciliation in mir-34/449-deficient MCCs. Scanning electron microscopy supported this finding, revealing a significant reduction in cilia length and number per MCC in TKO, but not in DKO and wild-type tracheal epithelia (Fig. 2d and Extended Data Fig. 3f). Notably, TKO MCCs displayed a spectrum of ciliation phenotypes (non-ciliated, partially or fully ciliated; Fig. 2d), possibly due to the mixed genetic background.

MCC ciliogenesis is characterized by the multiplication of basal bodies, which, after docking to the apical membrane, act as microtubule-organizing centres to assemble motile axonemes\textsuperscript{25,26}. We stained TKO tracheas with antibodies against Ac-α-tub and γ-tubulin (γ-tub) to visualize (top) and surviving adult TKO mice (bottom). Arrow, infection; arrowhead, mucus accumulation; n = 15. e, Adult TKO males and females are infertile. Although early spermatids (Sd) are developed, few intact spermatozoa (Sz) are generated (top, n = 3). A significant MCC reduction is observed in TKO fallopian tubes (bottom, n = 3).
Figure 3 | miR-34/449 deficiency causes defective basal body docking in mouse airway MCCs. a, TKO tracheas exhibit ciliation defects, shown by immunofluorescence staining for Ac-α-tub (cilia) and γ-tubulin (basal bodies). Numbers on the image indicate fully (1), partially (2) or non-ciliated (3) MCCs; n = 3. b–d, Basal bodies fail to dock to the apical membrane of TKO MCCs. cilia and basal bodies of MCCs, respectively. Consistent with previous observations, Ac-α-tub staining was greatly decreased in TKO tracheas; yet the percentage of γ-tub-enriched MCCs remained normal (Fig. 3a and Extended Data Fig. 4a, b). Air–liquid interface (ALI) culture of primary TKO tracheal epithelia yielded a similar observation (Extended Data Fig. 4c). Thus, basal body multiplication occurred normally in miR-34/449-deficient MCCs following MCC cell fate specification, yet cilia formation was impaired.

Impaired basal body docking in miR-34/449 TKO MCCs

Basal body docking to the apical MCC membrane is essential for proper ciliogenesis. In mir-34a c−/−; mir-34b/34c−/− DKO MCCs, γ-tub staining was apically localized, indicating normal basal body docking (Fig. 3b). In contrast, γ-tub staining was diffuse in TKO tracheal MCCs and ALI culture, suggesting defective basal body docking to or stabilization at the apical membrane (Fig. 3b and Extended Data Fig. 4d). Transmission electron microscopy revealed well-aligned basal bodies at the apical membrane of wild-type and DKO MCCs (Extended Data Fig. 4e). Yet in TKO MCCs, a significant percentage of basal bodies were mislocalized to the cytoplasm and unable to grow cilia, and those apically docked generally formed shorter cilia (Fig. 3c, d). The extent of ciliation defects correlated well with basal body docking defects, suggesting aberrant basal body docking as a key mechanism for impaired ciliation (Fig. 3d). Defective ciliation and basal body docking in TKO MCCs also correlated with a disturbed apical actin organization (Extended Data Fig. 4f).

Despite defective basal body docking in TKO MCCs, the structural components of basal bodies, either apically docked or mislocalized, remained largely intact (Extended Data Fig. 5a, b). Although axoneme structure was unaffected in TKO MCCs, basal body orientation and ciliary axoneme directionality exhibited mild defects (Extended Data Fig. 5c, d), which, in combination with the ciliation defects, probably evoked a strong mucociliary clearance phenotype.

miR-34/449 functions in Xenopus MCCs

Mammalian and Xenopus miR-34/449 miRNAs are not only conserved in sequence, but also in MCC-specific expression and ciliogenesis.

As a mucociliary epithelium, the Xenopus embryonic epidermis resembles mammalian airway epithelia in MCC development and function. As in mouse, knockdown of Xenopus mir-34a/34b/449a by morpholino (MO) injection (miR-34/449 MOs) reduced cilia number and length in tadpole epidermal MCCs (Fig. 4a, b). No obvious defects in embryonic development, hydrocephalus, MCC cell fate specification or other cell type specification were observed (Extended Data Fig. 6a–f). In miR-34/449 morphants, a significant portion of MCCs were either partially ciliated (>50%), or devoid of cilia (29% ± 17%), with frequent, unorganized subapical Ac-α-tub enrichment indicating defective basal body docking (Fig. 4b). Consistently, basal bodies detected by γ-tub or Sas6–GFP exhibited irregular distribution and frequently failed to form cilia in miR-34/449-deficient MCCs (Fig. 4c and Extended Data Fig. 6g).

miR-34/449 miRNAs directly repress cp110 in MCCs

The miR-34/449 increase during MCC differentiation predicts a decrease of functionally important targets, which invariably contain miR-34/449 binding sites (miR-34/449-Binding sites) in the 3′ untranslated region (3′ UTR). We analysed published gene expression profiles of tracheal MCCs during differentiation, then selected 57 potential miR-34/449 targets for quantitative polymerase chain reaction with reverse transcription (qRT–PCR) validation in DKO and TKO tracheas, and finally narrowed down to those with important ciliogenesis functions (Extended Data Table 1). Cpi110 emerged as a strong candidate, containing two miR-34/449 binding sites (Extended Data Fig. 7a) and exhibiting miR-34/449-dependent repression in vivo and in luciferase assays (Fig. 5a, b and Extended Data Fig. 7b, c).

Cpi110 is a distal centriolar protein suppressing primary cilia assembly. Aberrant Cpi110 retention in mother centrioles is correlated with impaired basal body docking. In addition to its well-characterized roles in primary cilia, Cpi110 is also implicated as an important regulator of motile ciliogenesis.

miR-34/449 and cp110 are conserved in mice and frogs. Xenopus cp110 contains one predicted miR-34/449 binding site (Extended Data Fig. 7d), suggesting a selective pressure to preserve miR-34/449–cp110 regulation.
Causes a PCD-like airway and fertility phenotype. miR-34 miRNAs have miR-34/449 represent the first non-coding RNAs whose deficiency whose loss-of-function causes a PCD-like phenotype38. Although the localization of Centrin4 (ref. 37), an important basal body component whose derepression during vertebrate MCC ciliogenesis (Fig. 5e). Data Fig. 8a, b). We subsequently examined, in control and miR-34/449 morphants, the localization of Centrin4 (ref. 37), an important basal body component whose loss-of-function causes a PCD-like phenotype38. Although strong Centrin4 foci were enriched apically in controls, these foci significantly decreased in intensity and failed to localize to the apical membrane of miR-34/449-deficient MCCs (Fig. 3c and Extended Data Fig. 8c). These findings demonstrated defective basal body docking and decreased basal body incorporation of Centrin4 in miR-34/449-deficient MCCs, both of which were rescued by co-injection of cp110 MO (Fig. 3d).

Consistently, cp110 overexpression generally phenocopied miR-34/449 knockdown, causing impaired ciliation and decreased Centrin4 incorporation into basal bodies, without affecting MCC cell fate specification or apical actin organization (Fig. 3c and Extended Data Fig. 8b, d, e and 9a). cp110 with a 3’ UTR deletion exhibited a stronger phenotype than full-length cp110, suggesting cp110 repression by miR-34/449 even during overexpression (Extended Data Fig. 7d and 8d). Surprisingly, cp110 MO injection alone also gave rise to reduced cilia number and length, and aberrant basal body agglomeration in MCCs (Fig. 3c and Extended Data Fig. 8a, b and 9b). Thus, miR-34/449 miRNAs mediate precise Cp110 regulation during vertebrate MCC ciliogenesis (Fig. 3e).

**Discussion**

miR-34/449 represent the first non-coding RNAs whose deficiency causes a PCD-like airway and fertility phenotype. miR-34 miRNAs have been mostly characterized as p53 targets that elicit multiple tumour suppressor effects20,39; yet the MCC-specific miR-34/449 expression and functions are likely p53-independent (data not shown). Although most characterized PCD mutations affect structural components of the ciliary axoneme37,40 or basal body structure41, miR-34/449 miRNAs regulate ciliogenesis by promoting basal body maturation and docking without affecting overall basal body structure. Interestingly, redundant miR-34/449 miRNAs are not functionally equivalent in mice. One mir-34b/34c allele is sufficient for normal MCC ciliation; two intact mir-34a alleles in mir-34b/34c−/−; mir-449−/− DKO mice still yield clear respiratory and fertility phenotypes (data not shown). Distinct roles of miR-34/449 miRNAs could reflect differential expression rather than target specificities.

In a previous study, miR-449 inhibition alone caused defective ciliogenesis by derepressing Notch1 and Dll1 (ref. 10). Yet normal MCC specification in miR-34/449-deficient MCCs suggest that Notch pathway components, with well-characterized roles in regulating MCC specification42,43, may not act as key miR-34/449 targets in motile ciliogenesis. Here we provide molecular and functional evidence demonstrating cp110 as a major miR-34/449 target. Cp110 levels have to be tightly regulated spatially and temporally during ciliogenesis; and proper Cp110 removal from mother centrioles is essential for ciliation19. Previous studies mostly focused on ubiquitin-mediated proteasomal degradation of Cp110 (refs 44, 45). Our study reveals the important post-transcriptional regulation of Cp110 by miR-34/449. Although MCC-enriched miR-34/449 miRNAs repress cp110 to facilitate ciliation, other miRNAs (for example, miR-129) repress cp110 expression in other cell types to regulate ciliogenesis of primary or motile mono-cilia36. Thus, ciliogenesis differentiation in Xenopus; and cp110 mRNA was derepressed in miR-34/449 morphants (Extended Data Fig. 7e, f, g). Strikingly, cp110 knockdown in miR-34/449-deficient MCCs significantly rescued ciliation defects (Fig. 5c and Extended Data Fig. 8a, b).

Figure 4 | miR-34/449 deficiency causes defective ciliogenesis in the Xenopus embryonic epidermis. a, MCCs in miR-34/449 morphants show reduced cilia length and number, demonstrated by immunofluorescence for Ac-α-tub (cilia) and phalloidin-488 (actin). b, Quantification of MCC ciliation in a. P < 0.001 from a χ²-test. c, Co-staining of Ac-α-tub (cilia) and γ-tub (basal bodies) in miR-34/449 morphants reveals uneven/aggregated distribution of basal bodies, which frequently fail to form cilia. Total numbers of embryos/total number of cells analysed were un.injected (4/14; a total of 14 cells from a total of 4 injected embryos) and miR-34/449 MOs (5/30). Embryos were derived from at least two females and independent fertilizations per experiment.
is controlled by downregulation of \( cp110 \) through distinct miRNAs in distinct cell types.

For miRNAs, their small size and imperfect target recognition facilitate regulation of multiple mRNA targets. Here, \( cp110 \) knockdown restored basal body maturation/docking and ciliogenesis in miR-34/449-deficient MCCs; however, additional miR-34/449 targets probably exist to regulate the organization of the apical actin cytoskeleton in MCCs.

Mucociliary epithelia are morphologically and functionally conserved among vertebrates. Our findings demonstrated a conserved mechanism that regulates basal body maturation and docking in MCCs by miR-34/449-dependent fine-tuning of \( Cp110 \) levels. This mechanism could have profound implications for the underlying mechanisms disrupted in patients with PCD-like syndromes.

**METHODS SUMMARY**

Detailed information on the following methods can be found in the full Methods: mouse breeding and monitoring, histological analyses, qRT–PCR, in situ hybridization (ISH), visualization of ciliary beating and mucociliary transport, immunofluorescence staining, scanning and transmission electron microscopy (SEM and TEM), western blotting and manipulation of *Xenopus* embryos.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 14 January; accepted 29 April 2014.**

1. Ambros, V. The functions of animal microRNAs. *Nature* **431**, 350–355 (2004).
2. He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nature Rev. Genet.* **5**, 522–531 (2004).
3. Kim, V. N. Small RNAs: classification, biogenesis, and function. *Mol. Cell* **19**, 1–15 (2005).
4. Du, T. & Zamore, P. D. microPrimer: the biogenesis and function of microRNA. *Development* **132**, 4645–4652 (2005).
5. Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Nature* **383**, 843–854 (1993).
6. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862 (1993).
7. Miska, E. A. et al. Most Caenorhabditis elegans microRNAs are individually not essential for development or viability. *PLoS Genet.* **3**, e215 (2007).
8. Park, C. Y. et al. A resource for the conditional ablation of microRNAs in the mouse. *Cell Rep.* **1**, 385–391 (2012).
9. Marson, A. et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **134**, 521–533 (2008).
10. Marce, B. et al. Control of vertebrate multiciliogenesis by miR-449 through direct repression of the Delta/Notch pathway. *Nature Cell Biol.* **13**, 693–699 (2011).
11. He, L. et al. A microRNA component of the p53 tumour suppressor network. *Nature* **447**, 1130–1134 (2007).
12. Chang, T. C. et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol. Cell* **26**, 745–752 (2007).
13. Ravew-Shapira, N. et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol. Cell* **26**, 731–743 (2007).
14. He, L., He, X., Lowe, S. W. & Hannon, G. J. microRNAs join the p53 network—another piece in the tumour-suppression puzzle. *Nature Rev. Cancer* **7**, 819–822 (2007).
15. Hermeking, H. p53 enters the microRNA world. *Cancer Cell* **12**, 414–418 (2007).
16. Sato, P. & Christensen, S. T. Overview of structure and function of mammalian cilia. *Annu. Rev. Physiol.* **69**, 377–400 (2007).
17. Fliegauf, M., Benzing, T. & Örn, H. When cilia go bad: cilia defects and ciliopathies. *Nature Rev. Mol. Cell Biol.* **8**, 880–893 (2007).
18. Spektor, A., Tsang, W. Y., Khoo, D. & Dynlacht, B. D. Cep97 and CP110 suppress a cilia assembly program. *Cell* **130**, 678–690 (2007).
19. Tsang, W. Y. & Dynlacht, B. D. CP110 and its network of partners coordinately regulate cilium assembly. Cilia 2, 9 (2013).
20. Choi, Y. J. et al. miR-34 miRNAs provide a barrier for somatic cell reprogramming. Nature Cell Biol. 13, 1353–1360 (2011).
21. Lin, Y., Klimke, A. & Dobbelstein, M. MicroRNA-449 in cell fate determination. Cell Cycle 10, 2874–2882 (2011).
22. Loges, N. T. et al. DNA2 mutations cause primary ciliary dyskinesia with defects in the outer dynein arm. Am. J. Hum. Genet. 83, 547–558 (2008).
23. Castleman, V. H. et al. Mutations in radial spoke head protein genes RSPH9 and RSPH4A cause primary ciliary dyskinesia with central-microtubular-pair abnormalities. Am. J. Hum. Genet. 84, 197–209 (2009).
24. Stubbs, J. L., Oishi, I., Izpisúa Belmonte, J. C., Kintner, C. & Izpisúa, J. C. The forkhead protein FoxJ1 specifies node-like cilia in Xenopus and zebrafish embryos. Nature Genet. 40, 1454–1460 (2008).
25. Marshall, W. F. Basal bodies: platforms for building cilia. Curr. Top. Dev. Biol. 85, 1–22 (2008).
26. Avasthi, P. & Marshall, W. F. Stages of ciliogenesis and regulation of cilium length. Differentiation 83, S30–S42 (2012).
27. Werner, M. E. & Mitchell, J. B. Understanding ciliated epithelia: the power of Xenopus. Genesis 50, 176–185 (2012).
28. Gomperts, B. N., Gong-Cooper, X. & Hackett, B. P. FoxJ1 regulates basal body anchoring to the cytoskeleton of ciliated pulmonary epithelial cells. J. Cell Sci. 117, 1329–1337 (2004).
29. Klos Dehring, D. A. et al. Foxf1 controls cilia number and makes a critical contribution to ciliogenesis in the kidney. Dev. Cell 27, 103–112 (2013).
30. Martinez-Anton, A. et al. Changes in microRNA and mRNA expression with differentiation of human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 49, 384–395 (2013).
31. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20 (2005).
32. Miranda, K. C. et al. A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. Cell 126, 1203–1217 (2006).
33. Hoh, R. A., Stowe, T. R., Turk, E. & Stearns, T. Transcriptional program of ciliated epithelial cells reveals new cilium and centrosome components and links to human disease. PLoS ONE 7, e52166 (2012).
34. Tanos, B. E., Yang, H. & Soni, R. Centriole distal appendages promote membrane docking, leading to cilia initiation. Genes Dev. 27, 163–168 (2013).
35. Ili, Y. et al. Inflammation-mediated upregulation of centrosomal protein 110, a negative modulator of ciliogenesis, in patients with chronic rhinosinusitis. J. Allergy Clin. Immunol. 128, 1207–1215.e1 (2011).
36. Cao, J. et al. miR-129–3p controls cilia assembly by regulating CP110 and actin dynamics. Nature Cell Biol. 14, 697–706 (2012).
37. Park, T. J., Mitchell, B. J., Abitua, P. B., Kintner, C. & Wallingford, J. B. Dishevelled controls apical docking and planar polarization of basal bodies in ciliated epithelial cells. Nature Genet. 40, 871–879 (2008).
38. Delaval, B., Covassin, L., Lawson, N. D. & Doxsey, S. Centrin depletion causes cyst formation and other ciliopathy-related phenotypes in zebrafish. Cell Cycle 10, 3964–3972 (2011).
39. Okada, N. et al. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. Genes Dev. (2014).
40. Kott, E. et al. Loss-of-function mutations in RSPH1 cause primary ciliary dyskinesia with central-complex and radial-spoke defects. Am. J. Hum. Genet. 93, 561–570 (2013).
41. Kimura, K. et al. Coordinated ciliary beating requires Odf2-mediated polarization of basal bodies via basal feet. Cell 148, 189–200 (2012).
42. Deblander, G. A., Wettstein, D. A., Koyano-Nakagawa, N. & Kintner, C. A two-step mechanism generates the spacing pattern of the ciliated cells in the skin of Xenopus embryos. Development 126, 4715–4728 (1999).
43. Tsao, P. N. et al. Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. Development 136, 2297–2307 (2009).
44. D’Angiolla, V. et al. SFCYclin f controls centrosome homeostasis and mitotic fidelity through CP110 degradation. Nature 466, 138–142 (2010).
45. Li, J. et al. USP33 regulates centrosome biogenesis via deubiquitination of the centriolar protein CP110. Nature 495, 255–259 (2013).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank M. J. Bennett, M. Butler, B. Dynlacht, W. Finkbeiner, P. Kysar, B. Lee, T. Machen, B. Mitchell and J. Wallingford for constructs, technical assistance, stimulating discussions and helpful input. We also thank P. Margolis for careful reading of our manuscript. L.H. acknowledges an R01 and an R21 grant from NCI (R01 CA139067, R21CA175560-01), a CIRM new faculty award (RN2-00923-1), a TRDRP research grant (21RT-0133), and a research scholar award from American Cancer Society (ACS, 123339-RSO-12-265-01-RMC). R.S. acknowledges the support of Siebel postdoctoral fellowship and CIRM postdoctoral fellowship. P.W. was funded by the Deutsche Forschungsgemeinschaft (DFG, Wa 3365/1-1), and frog work in the Harland laboratory was funded by NIH grant GM42341. M.L. would like to thank M. Dobbelstein for support and discussions, and was financed by a Dorothée Schloemer Fellowship.

Author Contributions R.S. identified and characterized PCD-like motile cilia defects in miR-34/449 TKO mice, defined miR-34/449 expression in ciliated epithelia, identified and validated cp110 as a key miR-34/449 target in mice. P.W. contributed all Xenopus data, in particular providing functional data validating cp110 as a key miR-34/449 target. N.S. performed all immunofluorescence experiments in mice, and contributed to target validation experiments. R.S. and P.W. both made significant contribution to experimental planning and result interpretation. M.L., A.K. and M.K. generated and characterized mir-449 KO mice, defined miR-449 expression patterns in mouse embryos and contributed to the revision of the manuscript. J.S.L. and G.D. contributed to histology analyses and qRT–PCR analyses. P.L. contributed to the high-speed imaging experiments. Y.W. characterized miR-34/449 expression in human respiratory epithelia. R.H. contributed to the interpretation of data and to manuscript preparation. L.H. generated mir-34a and mir-34b/34c KO mice, interpreted the data and coordinated with different groups to complete this study. R.S., P.W., N.S. and L.H. were the major contributors to the preparation of this manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to L.H. (lhe@berkeley.edu) and R.S. (ruisong@berkeley.edu).
METHODS

Mouse breeding, genotyping and miRNA detection. Primer sequences were as follows: for mir-34a, Common-R, ACTCGTGTACCC TGCTGCTT, with mir-34a WT-F, GTACCGAGCTATGGAACTT (wild-type band, 400 bp), or mir-34a KO-F, GCAGAGGCACCTGGATATTC (KO band, 263 bp); mir-34b/34c Common-R, GAGATTTCCGTGAGCTGTTT, with mir-34b/34c WT-F, GCTCCTGTGAAATGGTCT (wild-type band, 264 bp), or mir-34b/34c KO-F, GCAGGCGGATCTCGTTAT (KO band, 155 bp); mir-449 Common-R, AC ATCCCAGATAGATCCA, with mir-449 WT-F, GTATGCGCCAAACCACA (wild-type band, 724 bp), or mir-449 KO-F, GAGTTTCTGCGGCTTGCC (KO band, 406 bp). Litters were monitored daily for the first 60 days for survival, and their body weight was measured every other day for the first 30 days.

The following primers were used for genotyping, with parenthetical values indicating the size of the diagnostic PCR product: mir-34a-Common-F, CTCTCACCTGCCTTCA; miR-34c, AGGCAGTGTAGTTAGCTGATTGC; miR-449, AGGCAG. For mir-34a, primer sequences were as follows: mir-34a-Common-F, CTCTCACCTGCCTTCA; mir-34a-Common-R, ACTCGTGTACCC TGCTGCTT; mir-34a WT-F, GTACCGAGCTATGGAACTT (wild-type band, 400 bp), or mir-34a KO-F, GCAGAGGCACCTGGATATTC (KO band, 263 bp); mir-34b/34c Common-R, GAGATTTCCGTGAGCTGTTT, with mir-34b/34c WT-F, GCTCCTGTGAAATGGTCT (wild-type band, 264 bp), or mir-34b/34c KO-F, GCAGGCGGATCTCGTTAT (KO band, 155 bp); mir-449 Common-R, AC ATCCCAGATAGATCCA, with mir-449 WT-F, GTATGCGCCAAACCACA (wild-type band, 724 bp), or mir-449 KO-F, GAGTTTCTGCGGCTTGCC (KO band, 406 bp). Litters were monitored daily for the first 60 days for survival, and their body weight was measured every other day for the first 30 days.

The sound wave analyses were performed using audios that recorded the TKO phenotypes (Supplementary Video 1), and respiratory sound was analysed using Audacity.

Histological analyses. Tissues were dissected and fixed overnight in 10% neutral buffered formalin (pH 7.4) (NBF) (Fisher Scientific, #S100-04), processed by standard procedures, and embedded in paraffin blocks. All the blocks were sectioned at 10 μm, and slides were stained by hematoxylin and eosin (H & E). In this analysis, lungs were inflated via trachea with 10% NBF before fixation, and sinususes were processed by post-fixation decalcification for 5 to 10 days in 10% EDTA, pH 7.0.

PCR. Total RNA was isolated by TRizol (Invitrogen, #15596) from tracheal epithelium per the manufacturer's protocol, and treated with DNAs (Invitrogen, #18066) to remove DNA contamination. For quantification of miRNAs, TRizol prepared RNA was reversely transcribed into cDNA using SuperScript III reverse transcriptase (Invi- trogen, #18080) with random primers. SYBR Green-based qPCR was subsequently performed on a 7900HT real-time PCR system (Applied Biosystems) using cDNA as template. The Gapdh-encoding transcript was used as an endogenous control in each qPCR. The following qPCR primers were used in this study, with parenthetical values indicating the size of the diagnostic PCR product: For mir-34a, -WT-F, CTCTCACCTGCCTTCA; -WT-R, CTTGCTTCTTTTCAGCAGTC; -KO-F, GAGTTTTCTGGGCTTGCC, (KO band, 400 bp), or -KO-R, GAGATTTCCGTGAGCTGTTT. Statistical evaluation was performed using a paired t-test.

In situ hybridization (ISH). Standard histology protocols were used to prepare P25 lung and trachea section for miRNA ISH using diethylpyrocarbonate (DEPC) treated water for all procedures. After deparaffinization and rehydration, slides were fixed with 4% paraformaldehyde (PFA), treated with proteinase K, and fixed again with 4% PFA. Slides were incubated first with pre-hybridization solution (3 to 4 h at 60°C), and then with hybridization solution mixed with digoxigenin (DIG)-labelled LNA probes against each mir-34a/34b/miRNA (16 h at 60 °C). Post hybridization, slides were washed for 10 min at 60 °C in a graded series of SSC solutions (2×, 1.5×, 0.2×), then incubated with alkaline phosphatase (AP)-conjugated anti-DIG antibody in blocking solution. After washing with PBS and alkaline phosphatase (AP) buffer, the slides were incubated with NBT/BCIP in AP buffer to visualize blue ISH signals. Nuclear fast red (Sigma, #N3020) was used for nuclear counter-staining. Slides were then dehydrated and mounted with Permount (Fisher Scientific, #SP15-100).

Solutions for ISH (BioChain #K291020) as described above. DIG-labelled miR-34a (AACACACGCTAAGCAGCAGAGC), miR-34b (GCAATGCAGTAACTACGTGCTT), and miR-449 (CCAGCTAGAATCTGGCTT) probes were purchased from Exiqon (#38487-01, #38542-01, and #39641-01 respectively). For Xenopus laevis embryos, the ISH clusters were fixed in MEMFA at the indicated stages, and standard protocols were used for ISH and staining of embryos. Foxj1 antisense probe was synthesized using SP6 polymerase (Promega, #P1085). Whole mount ISH was performed on groups of 25 control and manipulated specimens per time point and batch, which were derived from two different mothers.

Visualization of ciliary beating and mucociliary transport. Tracheas from adult mice was cut into 2 mm × 2 mm pieces under dissection microscope. Trachea pieces were then transferred into the chamber on a glass slide, which was made by placing a 0.5-mm sticky spacer (Bio-Rad, #SIF-1201) on the slide surface. The chamber was filled with 100 μl M199 Hank's balanced salts medium (Invitrogen, #12350-039) mixed with 1 μl red fluorescent 0.5-μm microspheres (Invitrogen, # F-8812); and a coverglass was placed on the sticky spacer to seal the chamber. Live images of the tracheal epithelium were recorded with a high-speed GX-1 Memrecam camera (NAC Image Technology) attached to an Olympus IX71 microscope. DIC channel was used to record multiciliary beating, and the red fluorescent channel was used to record mucociliary transport. Videos were recorded at 250 frames per second (FPS) for 8 s, and are played at 250 FPS in Supplementary Videos 2 and 3. ImageJ was used to process and analyze raw images (Supplementary Videos 2 and 3).

Immunofluorescence staining and confocal imaging. For immunofluorescence staining on cryosections, whole tracheas of adult mice were fixed overnight in ice-cold acetone, and then processed through a graded series of sucrose solutions (from 5% to 20%, Fisher Scientific, #S5-500). Tracheas were embedded in (1:1) 20% sucrose and O.C.T. compound (Tissue-Tek, #4583) and sectioned with MICROM HM 550 (Fisher Scientific) at ~21 °C at thickness of 6 μm. Slides were washed in PBS (3×, 15 min), blocked (1 h at room temperature) in PBSTB (0.1% Triton X-100, 1% bovine serum albumin in PBS), and incubated (overnight at 4 °C) with primary antibody (1:400, anti-Foxj1, Sigma HPAA05714; 1:1000 anti-acetylated-α-tubulin, Sigma T6793). Slides were then washed three times in PBS (0.1% Triton X-100 in PBS) and incubated (1 h at room temperature) with secondary antibody (1:1000, Cy3 goat-anti-mouse, Molecular Probes A10521 or 1:500 Alexa Fluor 488 goat anti-rabbit, Molecular Probes A1034). Slides were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, #H-1200). Images were taken with a Zeiss LSM 710 AxioObserver inverted 34-channel confocal microscope and analysed with Zeiss Zen software.

For whole trachea staining, tracheas were cut longitudinally into two pieces, which were blocked in either 80% methanol (EMD, #MX0485-P) with 20% DMSO (Fisher Scientific, #BP231-100) overnight at ~20 °C, or in 4% paraformaldehyde (PFA) (overnight at ~4 °C), respectively. Fixed tissues were washed and blocked as described above, and then incubated (overnight at 4 °C) with primary antibodies (1:500 anti-α-tubulin, Sigma T5192 for the methanol fixed tissue; 1:400 anti-Foxj1, Sigma HPAA05714 and 1:1000 anti-acetylated-α-tubulin for the PFA fixed tissue). Tissue was then washed, incubated with secondary antibody, counterstained with DAPI, and imaged as described above.

For Xenopus laevis MCC staining, immunofluorescence was performed on whole mount embryos and skin explants in embryo medium (E3) at 30–33 (unless specified otherwise) in 4% paraformaldehyde for 1–2 h at room temperature or in Dent's fixative (80% methanol, 20% dimethylsulfoxide) for 48 h at ~20 °C. Embryos were processed according to standard procedures48. Morphological analysis of cell types was performed as previously described49. Primary antibodies were as follows: mouse monoclonal anti-acetylated-α-tubulin (1:700, Sigma T6793); rabbit polyclonal anti-γ-tubulin (1:500; Sigma T5192). Secondary antibodies (1:250) were as follows: Alexa Fluor 488-labelled goat anti-mouse antibody (Molecular Probes A11001), Alexa Fluor 555-labelled goat anti-mouse antibody (Molecular Probes A21422), Alexa Fluor 555-labelled goat anti-rabbit antibody (Molecular Probes A21428) and Alexa Fluor 555-labelled goat anti-mouse antibody (Molecular Probes A31553). Actin staining was performed by incubation (30–60 min at room temperature with AlexaFluor 488-labelled Phalloidin (1:10; Molecular Probes A12379). Z-stack analysis and processing were performed using ImageJ and Zeiss ZEN software. All confocal imaging was performed using a Zeiss LSM700.
Scanning electron microscopy (SEM). Adult trachea tissue was fixed using Karnovsky’s fixative in 0.1 M sodium phosphate buffer (Sorenson’s), washed with Sorenson’s sodium phosphate buffer and post-fixed using 1% OsO4 in Sorenson’s for 1 h. Tissue was dehydrated by passing through a graded series of ethanol solutions, then critical point dried using a Tousimis 931 super critical point dryer. The tissue was mounted on aluminum stubs and sputter coated with gold using a PELCO SC-7 coater. The samples were viewed on an FEI XL30 TEM SEM and digital images were collected. SEM was performed in the electron microscopy facility of the University of California at Davis.

Transmission electron microscopy (TEM). Adult trachea tissue was fixed, washed, and post-fixed as for SEM. After rinsing in double-distilled water (DDW), the tissue was incubated (30 min at room temperature) in 0.1% tannic acid, rinsed again in DDW, and incubated (1 h) in 1% uranyl acetate in DDW. Tissue was dehydrated by passing through a graded series of acetone solutions, then infiltrated and embedded in an epoxy resin mixture. Survey thick sections were performed in the electron microscopy facility of the University of California at Davis. ImageJ was used to measure the distance between basal bodies and apical surface in TEM pictures, and Oriana was used to analyse the directionality of ciliary axonemes. The following criteria were used to determine multi-ciliated cells (MCCs) and their apical surface in longitudinal TEM. Cells containing basal bodies in tracheal epithelium were defined as MCCs. For ciliated MCCs, the surface with intact view from ciliary axoneme to basal body was determined as the apical surface; and for non-ciliated MCCs, the surface with microvilli was determined as the apical surface.

Air–liquid interface (ALI) culture of primary tracheal epithelia. Primary tracheal epithelial cells were cultured as described previously49. In short, tracheas from three adult mir-34/449 mutant mice of the same genotype (~P60) were dissected and cut longitudinally in ice-cold Ham’s F-12 medium (Life Technologies, #11765-054) with penicillin/streptomycin (Life Technologies, #15140-163). To isolate epithelial cells, tracheas were incubated in 1.5 mg ml-1 pronase (Roche Diagnostics, #101659 21001) in Ham’s F-12 medium overnight at 4 °C. We stopped the tracheal pronase digestion by adding fetal bovine serum (FBS, Omega Scientific, #FB-01) to a final concentration of 10%. The tracheal epithelial cells were then pelleted by pooling the pronase digestion and washed with centrifugation at 400 g for 10 min at 4 °C. The pelleted cells were treated by DNase I (Sigma, #DN25), resuspended in 1 ml FBS, then plated in 9 ml pre-warmed basic medium (BM), which contained 1:1 DMEM:modified Barth’s saline from stage 9 embryos, which were either un-injected (time course experiment) or injected with Ctrl MO or mir-34/449 MOs (for quantification of cp110 and foxl1 expression). Experiments were cultured in 0.5× modified Barth’s saline until unin-manipulated control embryos reached appropriate stage. In the time course experiment, stage 10, 26 and 32 explants represented ciliation state of MCCs for quantification of cp110, foxl1 and mir-34/449 miRNAs. In addition, cp110 and foxl1 expression levels in Ctrl MO and mir-34/449 MOs injected embryos (injected 4× into the animal hemisphere at the 4-cell stage) were assessed at onset of ciliation (stage 26 explants) to examine the effect of mir-34/449 on foxl1 and cp110 levels.

Luciferase assay. A fragment of the Cp110 mRNA 3′ UTR containing two predicted miR-34/449 sites was cloned into the Firefly luciferase vector (Promega, #E1910). We amplified a fragment of Cp110 3′ UTR using PCR with Cp110 3′-UTR-F, AAGGC CGCGGGAGAACAGACTCAGTGGG, and Cp110 3′-UTR-R, GTGGCGCC CTTCTCTCTGATCCCGATGTC. NIH/3T3 cells were cultured in 10% bovine serum in DMEM (Life Technologies, # 11995-073) in a 12-well plate at a density of 1×105 cells per well. We co-transfected each well of NIH/3T3 cells with 10 ng of pG3 construct, 100 ng of pRL–TK Renilla vector (Promega, #E2211R), and 3′ 4b miRNA mimics (Integrated DNA Technologies, 5′-AGGGAGAGUGU GAAGCUUGAUUG-3′ and 5′-UAUGAUCUAUGAGCUUGCUUG-3′) and siGFP (Integrated DNA Technologies) using TransIT-TKO Transfection Reagent (Mirus Bio, # MIR 2150). At 24 h after transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega, #E1910). The luciferase activity was normalized as the ratio of firefly/Renilla luciferase activities.

Western blotting. Protein was collected from mouse tracheal epithelium by incubating resuspended and cleaned trachea in radioimmunoprecipitation assay (RIPA) buffer with complete mini protease inhibitor cocktail tablet (Roche, #11836153001) for 30 min on ice. Western blot followed the standard protocols. Mouse anti-f–b-actin (Sigma, #A5441, ~46 kDa) was used as loading control at 1:40,000 dilution. Rabbit anti-Cp110 (Thermo Scientific, #SP6, 6–34390, ~120 kDa) was used at 1:3,000 dilution in 5% non-fat dry milk in TBS-T (20 mM Tris pH 7.6, 150 mM NaCl and 0.1% Tween-20). Horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz Biotechnology, #sc-2004 and #sc-2005) were used at 1:5,000 dilution. Blots were analysed using ImageJ. Band intensities were normalized against corresponding actin and compared to wild-type controls for ratio calculation.

Manipulation of Xenopus laevis embryos and skin explants. X. laevis eggs were collected and in vitro fertilized, then cultured and microinjected by standard procedures50. Embryos were injected with morpholino nucleotides (MOs, Gene Tools) at the two- to four-cell stage using a Picospritzer setup in 1/3 modified frog Ringer’s solution (MR) with 2.5% Ficoll PM 400 (GE Healthcare 17-0300-50), and then were transferred into 1/3 MR containing gentamycin51 after injection. Drop size was calibrated to about 7–8 nl per injection. Rhodamine-B dextran (0.5–1.0 mg ml−1; Invitrogen D1841) was co-injected and used as lineage tracer. MO (Gene Tools) doses were administered as follows: a total dose of 30 ng miR-34/449 MOs: 10 ng each for mir-34a MO (5′-CAAACAGCTAAGACGCTCAA-3′), mir-34b MO (5′-ACATCGACTAATCAGCTGCTGTA-3′), and miR-449a MO (5′-AACAGCTAATACGCTAGCTCT-3′), or 30 ng of a miR-control MO (5′-TGCAGTCTTACAGACGCTGCT-3′), or 17 ng of cp110 MO (5′-ATCTCT CATAAGCTCATCTGACC-3′). An mRNA encoding Centrin–4–RFP or Centrin–GFP52 and Sase–GFP53 was prepared using the Ambion message kit using SP6 (AM1340) and diluted to 50–100 ng ml−1 for injection into the embryos (0.8–1.6 ng total per embryo). Xenopus tropicalis cp110 cDNA (PureYield Midiprep; Promega, #A2495) was derived from a clone matching BC167469 obtained from Thermo Scientific (#MX7167-202517511). Cp110A3 UTR was generated from the same clone, which was digested with SphI (New England Biolabs, #R0182S) and re-ligated to remove most of the 3′ UTR. Analysis of morphant tadpole brains for signs of hydrocephalus was performed as previously described54.

Xenopus skin explants were generated from animal caps49, dissected in 1× modified Barth’s saline from stage 9 embryos, which were either uninjected (time course experiment) or injected with Ctrl MO or miR-34/449 MOs (for quantification of cp110 and foxl1 expression). Experiments were cultured in 0.5× modified Barth’s saline until unin-manipulated control embryos reached appropriate stage. In the time course experiment, stage 10, 26 and 32 explants represented ciliation state of MCCs for quantification of cp110, foxl1 and mir-34/449 miRNAs. In addition, cp110 and foxl1 expression levels in Ctrl MO and miR-34/449 MOs injected embryos (injected 4× into the animal hemisphere at the 4-cell stage) were assessed at onset of ciliation (stage 26 explants) to examine the effect of miR-34/449 on foxl1 and cp110 levels.

Blots were analysed using ImageJ. Band intensities were normalized against corresponding actin and compared to wild-type controls for ratio calculation.

Sample size and analysis. Sample sizes for all experiments were chosen based on previous experiences. No randomization or blinding was applied for all our studies.

Ethics statement on animal experiments. This work was done with approval of University of California, Berkeley’s Animal Care and Use Committee. University of California, Berkeley’s assurance number is A3604-01, and is on file at the National Institutes of Health Office of Laboratory Animal Welfare.

46. Sive, H. L., Grainger, R. M. & Harland, R. M. Early Development of Xenopus laevis (Cold Spring Harbor Laboratory Press, 2000).
47. Walentek, P., Beyer, T., Thumbreger, T., Schweickert, A. & Blum, M. ATP4a is required for Wnt-dependent foxl1 expression and leftward flow in Xenopus left-right development. Cell Rep. 1, 516–527 (2012).
48. Walentek, P. et al. A novel serotonin-secreting cell type regulates ciliary motility in the mucociliary epithelium of Xenopus tadpoles. Development 141, 1526–1533 (2014).
49. Vladr, E. K. & Brody, S. L. Analysis of ciliogenesis in primary culture mouse tracheal epithelial cells. Methods Enzymol. 525, 285–309 (Elsevier, 2013).
50. Hagenlocher, C., Walentek, P., Müller, C., Thumbreger, T. & Festel, K. Ciliogenesis and cerebrospinal fluid flow in the developing Xenopus brain are regulated by foxl1. Cilia 2, 12 (2013).
Extended Data Figure 1 | The generation and phenotypic characterization of mir-34/449 TKO mice. a, miR-34/449 miRNAs are evolutionarily conserved with extensive sequence homology across many species. miR-34a has a more ancient evolutionary history compared to the rest of miR-34/449 miRNAs. miR-34a is conserved in Deuterostome, Ecdysozoa and Lophotrochozoa, yet the rest of miR-34/449 miRNAs have only vertebrate homologues. b, Diagrams of the targeted deletion strategy to generate mir-449 knockout mice. Since all mir-449 miRNAs are within intron 2 of their host gene, cdc20b, we deleted mir-449 with a minimally predicted impact on cdc20b. c, miR-449 expression is absent in mir-449 knockout animals, as demonstrated by real-time PCR analyses in lung tissues from littermate-controlled wild-type and mir-449–/– mice at postnatal day 35 (n = 3). miR-449a real-time PCR primers exhibit a modest cross-reaction with miR-34 miRNAs. d, mir-34/449 TKO mice have a significant postnatal attenuation in body weight. Littermate-controlled mir-34a–/–; mir-34b/34c–/–, mir-34a–/–; mir-34b/34c–/–; mir-449–/– and TKO mice were monitored for their body weight every other day for 30 days after birth. Paired t-test, ***P < 0.001. e, Surviving mir-34/449 TKO mice exhibit coughing/sneezing-like phenotype. The respiratory noise of littermate-controlled mir-34a–/–; mir-449–/– DKO and TKO mice was shown by sound wave analysis at postnatal day 30 (n = 14). f, Pulmonary inflammation occurs in a subset of mir-34/449 TKO mice. A representative H&E analysis of lung tissues from an adult TKO mouse indicates an increased infiltration by inflammatory cells. A total of 3 out of 15 TKO mice examined exhibit lung infection. g, mir-34a–/–; mir-34b/34c–/– DKO mice resemble wild-type mice, exhibiting no obvious respiratory defects in paranasal sinus or lung (n = 3). All error bars represent s.e.m.
Extended Data Figure 2 | Phenotypic characterization of reproductive organs and brain in mir-34/449 TKO mice. a, Adult male and female mir-34/449 TKO mice are infertile. Male (left) and female (right) reproductive organs from littermate-controlled DKO and TKO mice were subjected to H&E staining (n = 3). Boxes indicate areas depicted in Fig. 1e. b, Adult mir-34a−/−; mir-449−/− DKO male mice exhibit no defects in spermatogenesis. c, Adult mir-34a−/−; mir-34b/34c−/− DKO female mice display no defects in reproductive organs. d, The adult mir-34/449 TKO brains do not exhibit hydrocephalus, yet they are smaller in size than wild-type and DKO controls. The a/b markings on the images indicate the coronal to horizontal ratios. n = 3 for b, c and d.
miR-34/449 miRNAs are enriched in airway MCCs. a, Most miR-34/449 miRNAs are enriched in tissues with motile cilia. Using real-time PCR, the expression of miR-34a, miR-34c and miR-449c was measured in multiple tissues from newborn, P10, P20 and adult wild-type mice. Both miR-34c and miR-449c are exclusively expressed in tissues with motile cilia, whereas miR-34a exhibits a broader expression pattern (n = 3). b, The real-time PCR assay for each miR-34/449 miRNA specifically detects the corresponding miRNA. The specificity of each miRNA real-time PCR assay was validated using testis RNA from wild-type (WT), mir-34a/34b/34c−/−, mir-34b/34c−/−, mir-449−/−, and TKO mice at postnatal day 35. The miR-449a assay shows a slight cross reaction with homologous miRNAs (n = 3). c, In situ hybridization of each miR-34/449 miRNA exhibits specific detection. No measurable miR-34/449 in situ signal is detected in TKO lung sections at postnatal day 25 (n = 2). d, miR-34/449 miRNAs are enriched in tracheal MCCs. In situ hybridization analyses demonstrate that miR-34c and miR-449c are specifically expressed in the tracheal MCCs, whereas miR-34a is expressed in both tracheal MCCs and the surrounding cell types (n = 2). e, mir-34/449 TKO mice do not exhibit significant alterations in Foxj1 expression. Quantification of Foxj1 positive cells (left, n = 3) and Foxj1 mRNA (right, n = 4) was performed for well-controlled wild-type, DKO and TKO tracheas, using immunofluorescence and real-time PCR, respectively. Paired t-test; ns, P > 0.05. f, mir-34a−/−; mir-449−/− DKO tracheal epithelia are morphologically indistinguishable from wild-type controls in scanning electron microscopy (SEM) analyses (n = 3). All error bars represent s.e.m.
Extended Data Figure 4 | miR-34/449 deficiency causes defective ciliation and basal body docking in mouse airway MCCs. a, mir-34/449 TKO trachea exhibit reduced MCC ciliation. Quantification of fully ciliated MCCs (γ-tub and Ac-α-tub double-positive) and partially/non-ciliated MCCs, Ac-α-tub weak/negative) was performed in littermate controlled DKO and TKO mouse tracheas, using data from all three experiments in Fig. 3a. The number of cells with MCC identity (γ-tub positive) is unaffected in TKO tracheas, yet one-third of the TKO MCCs display aberrant Ac-α-tub staining, indicating ciliation defects. Paired t-test; ns, P > 0.05, ***P < 0.001. b, The mir-34a−/−; mir-34b/34c−/− DKO mice exhibit normal ciliogenesis in tracheal MCCs. Whole tracheas from age-matched adult wild-type and mir-34a−/−; mir-34b/34c−/− DKO mice were analysed by immunofluorescence staining for Ac-α-tub (cilia) and γ-tubulin (basal bodies) (n = 3). c, mir-34/449 TKO primary tracheal epithelial cells exhibit ciliation defects in air liquid interface (ALI) culture. ALI culture of MCCs were derived from tracheas of littermate-controlled mir-34a−/−; mir-34b/34c−/−; mir-449−/− TKO and DKO mice, and subjected to immunofluorescence staining for Ac-α-tub (cilia) and γ-tub (basal bodies). In TKO and control ALI culture, comparable levels of γ-tub positive cells are observed, however a large portion of TKO γ-tub positive cells displayed a partial or complete loss of Ac-α-tub staining. The letters on the image indicate fully (a), partially (b) or non-ciliated (c) MCCs (n = 2). d, Basal bodies fail to dock to the apical membrane of mir-34/449 TKO MCCs in ALI culture. Lateral projections of confocal micrographs described in (c) show impaired apical localization of γ-tub staining in TKO MCCs from ALI cultures, suggesting a defective basal body docking to the apical membrane. e, mir-34a−/−; mir-449−/− DKO trachea exhibit no defects in basal body docking using transmission electron microscopy (TEM) analyses (n = 3). f, TKO tracheal MCCs exhibit a defective subapical actin network. Whole tracheas from adult wild-type, mir-34a−/−; mir-34b/34c−/− DKO and TKO mice were analysed by immunofluorescence staining for Ac-α-tub (cilia) and phalloidin-488 (actin) (n = 2). All error bars represent s.e.m.
Extended Data Figure 5 | Major basal body structural components are intact in mir-34/449 TKO MCCs revealed by transmission electron microscopy. a, b, Apically docked (a) and undocked (b) basal bodies in mir-34/449 TKO MCCs have intact structural components. Basal body transition fibres (top), basal feet (middle) and striated rootlets (bottom) have comparable morphology among WT, DKO and TKO MCCs. For the top panel an arrow indicates a representative transverse view of transition fibres. For the middle panel an arrow indicates a representative transverse view of nine microtubule triplets with basal feet and arrowhead indicates a representative transverse view collected from a different height of a basal body, containing nine microtubule triplets without basal feet. For the bottom panel, an arrow indicates the longitudinal view of basal feet and arrowhead indicates the striated rootlet structure. c, Directionality of basal bodies (top) and axonemes (middle) is moderately affected in mir-34/449 TKO MCCs. In the top panel arrows point to the directions indicated by basal foot. In the middle panel red lines connecting the central pair of axonemes indicate the rotational polarity of each ciliary axoneme. In the bottom panel the angles of the axoneme directionality were statistically analysed as bidirectional circular data. The average angle was set from 0° to 180° axis. mir-34/449TKO ciliary axonemes have moderately un-coordinated directionality compared to WT and DKO controls. d, mir-34/449 TKO axonemes exhibit intact structures, including nine outer microtubule doublets, two central microtubule singlets and dynein arms (n = 3).
Extended Data Figure 6 | miR-34/449 deficiency in frog MCCs causes defective ciliogenesis without affecting cell fate specification. **a**, Injection of Ctrl or miR-34/449 MOs does not affect general embryonic development or neural tube closure. *Xenopus laevis* embryos were injected unilaterally with MOs at the 2–4-cell stage and analysed at neurula stages (18–20). Targeting of the skin ectoderm was confirmed by co-injection of fluorescent rhodamine dextran. **b**, Frog miR-34/449 morphants do not exhibit hydrocephalus. Embryos were injected animaly with control or miR-34/449 MOs into both dorsal blastomeres at the 4-cell stage to target the neural tube and brain regions. Subsequently, the whole brains were dissected and analysed at stage 45/46. The lack of hydrocephalus in miR-34/449 morphants argues against a role of miR-34/449 in ependymal ciliation. **c**, Quantification of fully ciliated, partially ciliated or non-ciliated MCCs reveals no significant change in total number of MCC-fated cells in miR-34/449 morphants. Error bars represent s.d. **d**, Embryos were unilaterally injected with Ctrl or miR-34/449 MOs to the right side at the 2–4-cell stage, cultured until stage 21 or 32 and processed for *in situ* hybridization to monitor *foxj1* expression in the mucociliary epithelium of the skin. No change in *foxj1* expression can be detected. **e**, Real-time PCR analysis in Ctrl or miR-34/449 MOs injected skin explants at stage 26 (onset of ciliation) does not indicate reduced expression levels of *foxj1*. Error bars represent s.e.m. **f**, miR-34/449 deficient frog embryos exhibit normal development of mucociliary cell types. Detailed analysis of the embryonic skin at stage 30–32 reveals the presence (specification and intercalation) of all cell types in miR-34/449 morphants, including large goblet cells, small secretory cells (SSC), *Ac-α*-tub positive ciliated cells (MCC) and non-tubulin enriched ion secreting cells (ISC). **g**, miR-34/449 morphant MCCs exhibit an uneven distribution of basal bodies. Sas6-gfp mRNA was injected at the 2–4-cell stage to visualize basal bodies at stage 30–32. In control embryos Sas6-GFP foci are evenly distributed in fully ciliated MCCs, whereas miR-34/449 morphant MCCs exhibit an uneven distribution of basal bodies. Total numbers of embryos/cells analysed were Ctrl MO (4/7) and miR-34/449 MOs (6/10).
Extended Data Figure 7 | cp110 is a direct target of miR-34/449 miRNAs.

a, A schematic representation of two predicted miR-34/449 binding sites in the mouse Cp110 3' UTR and in the luciferase reporter construct that contains Cp110 3' UTR. b, Cp110 protein levels at postnatal day 23 are elevated in mir-34/449 TKO tracheal epithelia. c, The expression of Luc-Cp110-3'UTR exhibits miR-34b-dependent repression in NIH/3T3 cells. Error bars represent s.e.m., n = 3. Paired t-test, *P < 0.05.

d, A schematic representation of one predicted miR-34/449 binding site in the frog cp110 3' UTR. A truncated cp110 construct, cp110−D3'UTR, was made to generate a cp110 cDNA without the miR-34/449 target site.

e, Real-time PCR monitoring cp110 reveals elevated miRNA expression levels of cp110 in mir-34/449 morphant frog skin explants as compared to Ctrl MO injected specimens. **P<0.01. Paired t-test. Error bars, s.e.m.

f, Timeline of MCC ciliation and recapitulation of ciliation defects in skin explants (animal caps). Representative confocal images from staged whole embryos and skin explants injected with either Ctrl MO or miR-34/449 MOs show the onset of ciliation at stage 26 and fully ciliated skin ectoderm at stage 32 in whole embryos and Ctrl MO injected skin explants. miR-34/449 MOs injected skin explants develop MCC ciliogenesis defects comparable to whole embryo treatment. Cilia: Ac-tub (red), Actin: Phalloidin-488 (green) and nuclei: DAPI (blue).

g, Expression of cp110, foxj1 and miR-34/449 RNAs during time course of ciliation in skin explants. Explants at stage 10 represent unciliated MCC precursors, explants at stage 26 represent MCCs at the onset of ciliation, and stage 32 explants represent fully ciliated ectodermal epithelium. cp110 mRNA levels decrease over the time course of ciliation, with the strongest decrease between stage 10 and 26, while foxj1 mRNA levels rapidly increase during this time. mir-34a, -34b and -449c levels strongly increase between stage 10 and stage 26; and only a moderate increase or even decrease can be observed between stage 26 and 32, similar to foxj1 expression levels. Error bars represent s.e.m., n = 2, technical replicates on pools of 30 skin explants for each time point.
Extended Data Figure 8 | miR-34/449 miRNAs promote ciliogenesis by repressing cp110. a, Representative examples of confocal images used for quantification of MCC ciliation in (b). Embryos were stained for Ac-\(\alpha\)-tub (cilia) and phalloidin-488 (actin). White boxes indicate areas depicted in Fig. 5c. b, Quantification of MCC ciliation in a, d and Fig. 5c. \(\chi^2\)-test, ns \(P > 0.05\), ***\(P < 0.001\). c, Centrin4–GFP incorporation into basal bodies is affected in miR-34/449 deficient embryos. The centrin4-gfp mRNA was injected at the 2–4-cell stage to visualize basal bodies in MCCs at stage 32, and centrosomes in neighbouring epithelial cells. In Ctrl morphant embryos, Centrin4–GFP staining in basal bodies (smaller foci in ciliated cells) and centrosomes (bigger foci in non-ciliated cells, green arrowheads) are equally strong. In contrast, Centrin4–GFP staining in basal bodies is greatly reduced in miR-34/449 morphants, without alteration of fluorescent intensity in centrosomes of neighbouring cells. Total numbers of embryos/cells analysed were Ctrl MO (6/17), miR-34/449 MOs (7/23). d, Representative examples of confocal images from cp110 overexpression experiments used for quantification of MCC ciliation in b. White boxes indicate areas depicted in Fig. 5c. e, The number of MCC-fated cells in miR-34/449 or cp110 morphants, and embryos injected with cp110 DNA constructs is not reduced. Quantification of total MCC numbers (fully ciliated, partially ciliated or non-ciliated MCCs) is shown for frog embryos injected with various MOs/DNAs (corresponding to a, b, d and Fig. 5c). Error bars represent s.d.
Extended Data Figure 9 | Gain and loss of *cp110* affects MCC basal bodies, but not the apical actin meshwork. a, *cp110* overexpression phenocopies miR-34/449 knockdown. Centrin4–RFP enrichment is strongly reduced in *cp110 Δ3'UTR* overexpressing MCCs. It is noteworthy, that whereas ciliation and incorporation of Centrin4 are strongly affected in *cp110Δ3'UTR* injected embryos, formation of the apical actin meshwork appears largely unaffected. Together with the lack of *cp110* knockdown to rescue the apical actin meshwork in miR-34/449 morphants, these data indicate an additional effect of miR-34/449 miRNAs on Actin formation/organization, which is *cp110* independent. Cilia: Ac-α-tub, basal bodies: Centrin4–RFP, actin: phalloidin-488. Embryos/cells analysed: uninjected (4/7), *cp110Δ3'UTR* (6/10). b, The cellular basis for ciliation defects in *cp110* morphants is probably due to the atypical failure of basal bodies to separate from each other, thus they appear to be aggregated in clusters of *cp110*-deficient MCCs. Nevertheless, Centrin4–RFP incorporation or apical localization of basal bodies is not affected in *cp110* morphants. Basal bodies, Centrin4–RFP, actin: phalloidin-488. Total numbers of embryos/cells analysed: uninjected (2/3), *cp110 MO* (5/8). Embryos were derived from at least two females and independent fertilizations per *Xenopus* experiment.
## Extended Data Table 1 | Candidate miR-34/449 targets with potential roles in MCC differentiation and/or ciliation in respiratory epithelia

| Gene Symbol | Gene Name |
|-------------|-----------|
| Ank3        | ankyrin 3, epithelial |
| Alp2b4      | ATPase, Ca++ transporting, plasma membrane 4 |
| Aurka       | aurora kinase A |
| Aurkb       | aurora kinase B |
| Ccdo39      | coiled-coil domain containing 39 |
| Ccdo40      | coiled-coil domain containing 40 |
| Ccna2       | cyclin A2 |
| Ccnb1       | cyclin B1 |
| Ccnb2       | cyclin D2 |
| Ccne2       | cyclin E2 |
| Ccpp110     | centriolar coiled coil protein 110 |
| Cdc25a      | cell division cycle 25A |
| Cdc6        | cell division cycle 6 |
| Cdc7i       | cell division cycle associated 7 like |
| Cdk5rap2    | CDK5 regulatory subunit associated protein 2 |
| Cep152      | centrosomal protein 152 |
| Cep63       | centrosomal protein 63 |
| Cep97       | centrosomal protein 97 |
| Chek1       | checkpoint kinase 1 |
| Dda1m1      | dishevelled associated activator of morphogenesis 1 |
| Dnah5       | dynein, axonomal, heavy chain 5 |
| Dnah1       | dynein, axonomal, light intermediate polypeptide 1 |
| Dtl         | denticelloless homolog (Drosophila) |
| Dazp1       | DAZ interacting protein 1 |
| Fat4        | FAT tumor suppressor homolog 4 (Drosophila) |
| Fgfr1       | fibroblast growth factor receptor |
| Foxg1       | forkhead box G1 |
| Foxj1       | forkhead box J1 |
| Hdac6       | histone deacetylase 6 |
| Hook3       | hook homolog 3 (Drosophila) |
| Itt27       | intraflagellar transport 27 |
| Itch        | itchy, E3 ubiquitin protein ligase |
| Jag1        | jagged 1 |
| Kif24       | kinesin family member 24 |
| Lef1        | lymphoid enhancer binding factor 1 |
| Mapt        | microtubule-associated protein tau |
| Met         | met proto-oncogene |
| Myb         | myeloblastosis oncogene |
| Myr9        | myosin, heavy polypeptide 9, non-muscle |
| Pacs1       | phosphofurin acidic cluster sorting protein 1 |
| Pdgfra      | platelet derived growth factor receptor, alpha polypeptide |
| Pofut1      | protein O-fucosyltransferase 1 |
| Retn11      | retinol dehydrogenase 11 |
| Rfx3        | regulator X, 3 |
| Rrm2        | ribonucleotide reductase M2 |
| Shank3      | SH3/ankyrin domain gene 3 |
| S6x3        | sine oculis-related homeobox 3 |
| Skp2        | S-phase kinase-associated protein 2 (p45) |
| Stat6       | signal transducer and activator of transcription 6 |
| Stil        | Scl/Tal1 interrupting locus |
| Strk35      | serine/threonine kinase 35 |
| Tmem107     | transmembrane protein 107 |
| Tppp        | tubulin polymerization promoting protein |
| Tac2        | tuberous sclerosis 2 |
| Ttc26       | tetratricopeptide repeat domain 26 |
| Tlf3        | tubulin tyrosine ligase-like family, member 3 |
| Xrnp3p3     | X-prolyl aminopeptidase (aminopeptidase P) 3, putative |