A human ciliopathy reveals essential functions for NEK10 in airway mucociliary clearance

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Mucociliary clearance, the physiological process by which mammalian conducting airways expel pathogens and unwanted surface materials from the respiratory tract, depends on the coordinated function of multiple specialized cell types, including basal stem cells, mucus-secreting goblet cells, motile ciliated cells, cystic fibrosis transmembrane conductance regulator (CFTR)-rich ionocytes, and immune cells. Bronchiectasis, a syndrome of pathological airway dilation associated with impaired mucociliary clearance, may occur sporadically or as a consequence of Mendelian inheritance, for example in cystic fibrosis, primary ciliary dyskinesia (PCD), and select immunodeficiencies. Previous studies have identified mutations that affect ciliary structure and nucleation in PCD, but the regulation of mucociliary transport remains incompletely understood, and therapeutic targets for its modulation are lacking. Here we identify a bronchiectasis syndrome caused by mutations that inactivate NIMA-related kinase 10 (NEK10), a protein kinase with previously unknown in vivo functions in mammals. Genetically modified primary human airway cultures establish NEK10 as a cilium- and patient-derived HBECs at an air–liquid interface (ALI), a well-established method for the generation of airway epithelium in vitro. NEK10 WT

Unexpectedly, both control and mutant ALI samples demonstrated robust

and nasal biopsies revealed normal ciliary radial ultrastructure (Fig. 1a,b). Cystic fibrosis and immunodeficiency were ruled out after extensive clinical and genetic testing. Similar but milder findings were present in two siblings (Extended Data Fig. 1a,b and Supplementary Table 1), strongly suggestive of autosomal recessive inheritance. Whole exome sequencing of the affected individuals unexpectedly revealed homozygous intronic splice site mutations (NM_152534:c.1230+5G>C, NEK10g>5) in NEK10, which encodes a serine/threonine kinase homologous to Aspergillus nidulans NIMA (never in mitosis a) that has not been implicated previously in human disease (Fig. 1c). The functions of mammalian NIMA-related kinases remain incompletely characterized. Several, including NEK2 and NEK5/6/7/9, function similarly to their fungal ortholog in cell cycle regulation through phosphorylation of centrosome components and the mitotic spindle. Mutations in NEK1 and NEK5 cause polycystic kidney phenotypes in mice, consistent with a role in the regulation of primary cilia. Recent reports have proposed roles for NEK10 in the response of cancer cells to DNA damage and in the specification of the nervous system and the body axis in teleost fish, but to date no published work suggests any role for NEKs in the respiratory system.

To study the effects of NEK10g>5 in the lung, we isolated and cultured control and proband human bronchial epithelial cells (HBECs) obtained at the time of bilateral lung transplantation. Although NEK10 messenger RNA was robustly expressed in airway tissue, it was essentially undetectable both in NEK10g>5 and NEK10g>5 HBECs, suggesting that its expression might be restricted to mature airway cells (Fig. 1d). We therefore differentiated control and patient-derived HBECs at an air–liquid interface (ALI), a well-validated method for the generation of airway epithelium in vitro. Unexpectedly, both control and mutant ALI samples demonstrated robust NEK10 mRNA expression (Fig. 1d and Extended Data Fig. 1c) despite immunoblot evidence that NEK10g>5 encodes a
loss-of-function allele (Fig. 1e and Extended Data Fig. 1d). To elucidate the mechanism by which NEK10<sup>G>C</sup> impairs protein expression, we sequenced full-length complementary DNAs from mutant ALI samples and revealed a mutation-dependent in-frame insertion of seven amino acids, which we suspected rendered NEK10 unstable (Fig. 1f). To test this hypothesis, we expressed epitope-tagged NEK10 constructs in HEK293T cells and found, as in ALI cultures, that the mutant protein was severely under-expressed, supporting a destabilizing effect of NEK10<sup>G>C</sup> that causes loss of function (Fig. 1g).

These findings led us to ask whether NEK10 mutations might underlie other cases of unexplained bronchiectasis. Indeed, further sequencing revealed six additional patients from four families who harbored homozygous NEK10 mutations and exhibited bronchiectasis (Extended Data Fig. 1e–o). The first, an 11-year-old girl, had a homozygous c.1869dupT mutation that resulted in frameshift and premature stop (His624Serfs*4). The second, a 15-year-old girl, had a homozygous c.2243C>T mutation that caused substitution of a leucine for a highly conserved proline (Pro748Leu) within the kinase domain. The third, a 23-year-old woman, had a homozygous c.1373+1G>T mutation that caused exon skipping, frameshift, and premature stop (Cys437Thrfs*9). The fourth, fifth, and sixth were siblings with a homozygous c.2317C>T mutation that resulted in the replacement of a highly conserved arginine by a cysteine (Arg773Cys). Notably, clinical assays revealed normal nasal ciliary ultrastructure observed by electron microscopy, normal nasal nitric oxide levels, no evidence of heterotaxy, and only very subtle abnormalities observed by clinical high-speed video microscopy in all tested individuals, suggesting that such patients might escape detection during standard PCD evaluation (Supplementary Table 1). Linkage analysis that incorporated kindreds 1–3 yielded a single, highly significant genome-wide linkage signal (pLOD 5.2), defining a 7.3 Mb interval that included the NEK10 locus (Fig. 1h). By contrast, healthy individuals with biallelic inactivating NEK10 mutations are completely absent from publicly available variant databases<sup>1</sup>. Together, these data provide strong genetic and clinical evidence that NEK10 represents a novel and bona fide autosomal recessive bronchiectasis locus.

Given the roles of NEKs in cell cycle regulation, we initially reasoned that NEK10 is required for the specification of one or more cell types that are involved in mucociliary clearance. Quantitative PCR with reverse transcription (qRT–PCR) revealed that NEK10 is robustly induced during airway epithelial differentiation, coincident with the acquisition of markers for ciliated and secretory cells and the depletion of stem cell markers (Extended Data Fig. 2a–d). However, NEK10<sup>G>C</sup> cultures induced and repressed these markers with kinetics identical to those of control cultures and produced similar numbers of secretory, goblet, and multiciliated cells (MCCs, Extended Data Fig. 2e,f). These data suggested that NEK10 might instead function specifically within one of the specialized cells that are important for mucociliary clearance. To characterize the NEK10 expression domain, we generated ALI cultures in which enhanced green fluorescent protein (eGFP) was expressed under the control of the NEK10 promoter (NEK10:eGFP), which we found directs expression within a subset of ALI cells in a differentiation-dependent manner (Extended Data Fig. 2g,i). GFP+ cells purified by FACS from mature NEK10:eGFP ALI cultures revealed a 149-fold enrichment of the MCC marker FOXJ1 (ref. <sup>14</sup>) and reciprocal depletion of secretory and basal cell marker transcripts (Fig. 2a). Confocal imaging confirmed that GFP positivity was restricted to cells that harbored apical cilia (Fig. 2h). Conversely, FACS-purified ciliated cells from FOXJ1:eGFP ALI cultures revealed a 152-fold enrichment of NEK10 (Extended Data Fig. 2h,i and Fig. 2a). These findings establish NEK10 as a ciliated-cell-specific gene in the human airway that is induced during, but dispensable for, differentiation of this cell type.

To explore the functional roles of NEK10 in MCCs, we imaged live ALI cultures with high-frame-rate phase contrast microscopy and observed a striking reduction in overall ciliary motion in NEK10<sup>G>C</sup> cultures (Fig. 2c, Extended Data Fig. 3a and Supplementary Movie 1). Given this abnormality, we performed advanced MCC functional phenotyping using micro-optical coherence tomography (μOCT), a state-of-the-art high-resolution live imaging technique capable of quantitatively interrogating multiple ciliary parameters<sup>15</sup>. Unlike control cultures that robustly transport polylysine beads added to the apical ALI surface, NEK10<sup>G>C</sup> ALI cultures showed a near-absence of mucociliary transport (MCT) (Fig. 2d, Extended Data Fig. 3b and Supplementary Movie 2) and a reduction in the depth of the periciliary liquid layer (PCL, Fig. 2e). We were surprised to measure normal ciliary beat frequency in mutant ALI cultures (Extended Data Fig. 3c), again suggesting a mode of dysfunction distinct from that of classical PCD. Although these data suggest a causative role for NEK10 loss of function in MCC dysfunction, they do not rule out the possibility that secondary genetic or acquired changes in proband-derived cells were responsible<sup>16</sup>. We therefore generated CRISPR–Cas9-mediated NEK10 loss-of-function ALI cultures (NEK10<sup>Δ</sup>) by genetically disrupting the NEK10 locus in wild-type HBECs. Immunoblotting confirmed the efficient depletion of NEK10, and live microscopy of NEK10<sup>Δ</sup> ALI cultures revealed a dramatic reduction in ciliary motion, as observed in NEK10<sup>G>C</sup> (Fig. 2f and Extended Data Fig. 3d,e). We also subjected NEK10<sup>Δ</sup> ALI cultures to μOCT imaging and found a severe reduction in maximal particle transport velocity and a thinning of the PCL, again without a change in ciliary beat frequency (CBF) (Fig. 2g,h and Extended Data Fig. 3f).

Given a recent report of catalysis-independent roles for NEKs<sup>17</sup>, we sought to understand whether kinase activity per se is necessary for NEK10 function in MCCs. We therefore generated NEK10<sup>G>C</sup>...
ALI cultures with FOXJ1-promoter-driven re-expression of NEK10WT or, alternatively, NEK10 variants predicted to inactivate18 (NEK10K548R) or putatively hyperactivate (NEK10S684D, NEK10Y590A) activity based on prior studies of paralogous NEK kinases6,19,20. Despite sub-physiological expression of transduced NEK10 variants, live microscopy demonstrated a striking increase in motility upon expression of NEK10S684D, partial rescue with NEK10WT and NEK10Y590A, and no effect of catalytic-dead NEK10K548R (Extended Data Fig. 3g–i). Live imaging with μOCT demonstrated that CBF was again unaffected by NEK10 status but that particle transport...
**Fig. 2 | NEK10 is a ciliated-cell-specific gene required for effective mucociliary transport.**

a. 18S rRNA-normalized relative expression of indicated transcripts from FACS-sorted ALI cells. The dashed line indicates the expression level from unsorted mature ALI cells. b. Confocal immunofluorescence demonstrating GFP expression in ciliated cells of NEK10:eGFP ALI cultures, representative of two independent ALI differentiations. Scale bar, 10 μm. c. Pseudocolored video microscopy of ALI cultures of the indicated genotypes, representative of three independent ALI differentiations. Mean Δ inter-frame (motion) pseudocolor represents motion amplitude as color intensity. Scale bars, 50 μm. d. MCT (μOCT) of ALI cultures of the indicated genotypes. The center line of the box plot indicates the median, the box bounds the 25th and 75th percentiles, the whiskers indicate the 10th and 90th percentiles, and the open circles indicate the remaining points. n = 485 for NEK10WT and n = 180 for NEK10G>C, pooled from three independent ALI differentiations. e. PCL (μOCT) of ALI cultures of the indicated genotypes. n = 11 for NEK10WT and n = 12 for NEK10G>C, pooled from three independent ALI differentiations. Mean ± s.e.m. Mean ± s.e.m. f. Pseudocolored video microscopy of CRISPR–Cas9-edited ALI cultures. Images show representative fields from three independent ALI differentiations. Scale bars, 50 μm. g. MCT of CRISPR–Cas9-edited ALI cultures plotted as in d. n = 361, n = 131, and n = 104 for sgAAVS1, sgNEK10a, sgNEK10b, and sgNEK10c, respectively, pooled from three independent ALI differentiations. h. PCL of CRISPR–Cas9-edited ALI cultures. n = 4, n = 4, n = 5, and n = 6 for sgAAVS1, sgNEK10a, sgNEK10b, and sgNEK10c, respectively, pooled from three independent ALI differentiations. Mean ± s.e.m. i. MCT of NEK10G>C ALI cultures expressing cDNAs encoding the indicated mutants, plotted as in d. n = 71, n = 254, n = 129, and n = 1,081 for no cDNA, NEK10WT, NEK10K548R, and NEK10S684D, respectively, pooled from three independent ALI differentiations. Mean ± s.e.m. j. MCT of NEK10K548R ALI cultures expressing cDNAs encoding the indicated mutants, plotted as in d. n = 1,385, n = 1,624, n = 728, n = 401, and n = 426 for FOXJ1:NEK10WT, FOXJ1:NEK10K548R, FOXJ1:NEK10S684D, NEK10:NEK10WT, and NEK10:NEK10K548R, respectively, pooled from three independent ALI differentiations. WT, wild type. *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001.
**Fig. 3 | Morphologically abnormal ciliated cells in NEK10-deficient airway.** a, Schematic masking workflow for IFC morphological analysis. b, Histogram of ciliary zone thickness in mature ALI MCCs of the indicated genotypes. $n = 4,108$ for NEK10$^{WT}$ and $n = 3,513$ for NEK10$^{G>C}$. The shaded bars indicate medians $\pm 0.25\mu m$. c, Histogram of ciliary area in mature ALI MCCs of the indicated genotypes. $n = 4,108$ for NEK10$^{WT}$ and $n = 3,513$ for NEK10$^{G>C}$. d, Single cell images taken from the shaded regions in b. Scale bars, 7 $\mu m$. e, Confocal maximum intensity projections (MIPs) of ALI cultures of the indicated genotype and maturity after IFM against Ac-α-tubulin, representative of three independent ALI differentiations. Scale bars, 25 $\mu m$ (left four panels) and 10 $\mu m$ (right two panels). f, Confocal MIPs of mature ALI cultures after IFM against the basal body marker centrin. Dashed boxes mark full resolution regions shown in the middle panels. Scale bars, 10 $\mu m$ (left two panels) and 1 $\mu m$ (middle two panels). The column graph shows the number of centrin puncta per $\mu m^2$ of ciliated cell surface area. $n = 71$ cells and 10,855 puncta for NEK10$^{WT}$, and $n = 38$ cells and 5,369 puncta for NEK10$^{G>C}$, pooled from four independent ALI differentiations. Mean±s.d. g, Confocal MIPs of mature ALI cultures after IFM against the PCP marker Vangl1, representative of three independent ALI differentiations. The dashed boxes mark full resolution regions shown in the right panels. Scale bars, 10 $\mu m$ (left panels) and 2.5 $\mu m$ (right panels). h, H&E stained human large airway tissue. The upper three samples were taken from lung explants during transplantation for end-stage bronchiectasis owing to the indicated etiologies, and the fourth sample was taken from a patient undergoing resection for an unrelated diagnosis. Scale bars, 5 $\mu m$. WT, wild type. ****$P \leq 0.0001$. 
Fig. 4 | NEK10 regulates ciliary length through widespread effects on the ciliary proteome. a. SEMs of mature ALI cultures of the indicated genotype, representative of three independent ALI differentiations. The dashed boxes mark full resolution regions shown in the right panels. Scale bars, 10 μm (left panels) or 1 μm (right panels). b. STEMs of mature ALI cultures of the indicated genotype after embedding and sectioning orthogonal to the epithelial surface, representative of three independent ALI differentiations. Tick marks are spaced at 1 μm. c. Representative negative stain electron microscopy grids prepared from purified cilia of the indicated genotypes, representative of two independent ALI differentiations. Scale bar, 1 μm. d. Histogram of ciliary length from purified cilia of the indicated genotypes. n = 101 for NEK10WT and n = 102 for NEK10G>C, pooled from two independent ALI differentiations. The inset shows a box and whisker plot of these data, in which the center line indicates the median, the box bounds the 25th and 75th percentiles, the whiskers indicate 1.5 times the interquartile range, and the circles indicate outliers. e. Cumulative distribution of phosphopeptides by log2[fold change]. Previously identified motile ciliary proteins are shown in red, and all other detected proteins are shown in black. sgNEK10b and sgNEK10c are independently targeting guide RNAs validated in Extended Data Fig. 3d. f. Table of ciliary genes by functional class showing phosphopeptides that were depleted more than twofold upon NEK10 deletion. WT, wild type. ****P ≤ 0.0001.
was significantly increased upon expression of NEK10S684D (Fig. 2i and Extended Data Fig. 3). These data led us to ask whether ectopic activation of NEK10 signaling in wild-type ALI cultures could augment mucociliary transport to supraphysiological levels. Indeed, expression of NEK10WT or NEK10S684D under the control of the FOXJ1 promoter significantly increased transport velocity compared to catalytic-dead NEK10D488S, a phenotype that was also reproduced with the more transcriptionally active NEK10 promoter (Fig. 2). Together, these data establish (1) that cilium-cell-specific NEK10 kinase activity is required for effective airway mucociliary transport, (2) that NEK10 activity is constrained by the serine 684 activation loop residue, and (3) that the potentiation of NEK10 activity may represent a strategy for the augmentation of mucociliary transport.

We next turned our attention to the mechanisms by which NEK10 regulates MCC function. To evaluate whether mutant MCCs harbor some physical abnormality, we used imaging flow cytometry (IFC), which enables the capture of thousands of single cells for statistically robust morphology analysis. After gating singlet MCCs from NEK10:eGFP ALI cultures (Extended Data Fig. 4a,b), we measured per-cell ciliary area and ciliary zone thickness, revealing a clear and statistically significant reduction in both parameters in the mutant MCCs (Fig. 3a–c). Representative single cells corroborated this finding, demonstrating a hypoplastic ciliary layer in NEK10−/− MCCs (Fig. 3d). To validate this result using an orthogonal method, we subjected NEK10−/− MCCs to confocal immunofluorescence microscopy (IFM) against acetylated α-tubulin (Ac-α-tubulin). We again found that mutant ALI cultures harbored strikingly abnormal cilia with a hypoplastic appearance (Fig. 3e). NEK10WT ALI cultures phenocopied this morphology, and NEK10WT-complemented NEK10−/− ALI cultures reversed it (Extended Data Fig. 4c,d), indicating that this phenotype is specifically attributable to NEK10 kinase activity. Previously described human mutations24,25 are known to impair the nucleation and density of motile cilia. We therefore evaluated MCC basal body density by IFM but found no reduction that would account for the NEK10−/− phenotype (Fig. 3f). In light of the links between planar cell polarity (PCP) and ciliogenesis36–38, we also confirmed that NEK10 activity is dispensable for MCC planar polarization (Fig. 3g). Finally, to validate ALI culture findings in human patients, we compared airway tissue from the explanted lungs of the proband with airway tissue from subjects without bronchiectasis and patients with end-stage cystic fibrosis. As in ALI cultures (Extended Data Fig. 4e), histological analysis revealed ciliary hypoplasia only in the airway of the patient with the NEK10 mutation (Fig. 3h), indicating that this phenotype does not reflect a non-specific consequence of severe bronchiectasis and that NEK10 deficiency produces short motile cilia in vivo, a previously undescribed human genetic phenotype.

As the size of motile cilia limits their structural analysis by light microscopy, we next subjected ALI preparations to three complementary modes of electron microscopy analysis. Scanning electron microscopy (SEM) of intact ALI, like IFM, revealed that NEK10−/− and NEK10WT ALI cultures harbor morphologically abnormal cilia of heterogeneous length with excess mucus accumulation (Fig. 4a and Extended Data Fig. 5a). Electron microscopy imaging of sectioned ALI cultures revealed a decrease in ciliary length in the NEK10−/− cultures (Fig. 4b) but did not show the undocked centrioles seen in ciliary aplasia syndromes or the ciliary tip ‘ballooning’ reported with certain intraflagellar transport defects22,23. To quantify this length defect precisely, we biochemically isolated axonomes from ALI cultures (Extended Data Fig. 5b) and subjected these preparations to negative stain electron microscopy followed by measurement of individual cilia. Isolated NEK10−/− cilia were indeed shorter than NEK10WT cilia (Fig. 4c,d, 6.24 ± 1.26 μm versus 7.86 ± 1.06 μm), providing at least a partial basis for ciliary transport failure based on biophysical models of mucociliary transport that include ciliary length as a critical parameter in force generation26–28.

To explore the biochemical functions of NEK10 in airway epithelium, we next performed iron-enrichment proteomics77 (LC–IMAC MS) to identify a set of phosphopeptides that were depleted upon NEK10 inactivation in ALI cultures (Extended Data Fig. 5c). Gene ontology analysis30,31 revealed that these depleted peptides were highly enriched in ciliary motility and axonemal assembly genes, suggesting that a large complement of ciliary proteins is dysregulated upon NEK10 loss (Extended Data Fig. 5d). To test this hypothesis directly, we analyzed the effects of NEK10 deletion on a set of proteins previously identified by proteomics in airway cilia39 and found a striking and highly significant depletion of such ciliary phosphopeptides (median log[fold change] −1.06 (sgNEK10b), −0.771 (sgNEK10c), Fig. 4e). Notably, although we observed similar findings upon analysis of peptides that mapped to annotated PCD genes, we saw little to no depletion of peptides from non-PCD ciliopathy genes, consistent with a specific role for NEK10 in the regulation of motile ciliogenesis (Extended Data Fig. 5e,f). Peptides from virtually all classes of motile ciliary genes were depleted in NEK10KO ALI cultures (Fig. 4f), including axonemal motors, intraflagellar transport components, central pair constituents, and ciliary length control proteins, indicating that diverse members of the ciliary proteome are directly or indirectly dysregulated upon NEK10 loss and providing a data set for future efforts to dissect its target network.

In summary, we describe a novel human disease caused by NEK10 deficiency and characterized by pathologically short motile cilia, as evidenced by multiple orthogonal assays in physiologically-relevant human airway preparations. In light of the highly cell-type-restricted expression of NEK10 in our own data and those of recently generated cell atlas34–36, as well as the absence of extra-respiratory phenotypes in our patients, this gene does not appear to be essential for primary ciliogenesis in humans, as has been proposed previously31, but instead probably specifically controls MCC function. Indeed, as the lengths of protozoan beating cilia40 and flagella11,46 are regulated by distant NEK homologs, analogous pathways likely operate in metazoan MCCs to tune ciliary function to physiological needs. Although the central role of NEK10 in the potentiation of mucociliary clearance is clear from our study, the mechanistic basis for this activity remains to be fully explained because our current proteomic data cannot distinguish unambiguously between direct phosphorylation effects and secondary protein abundance changes. We are optimistic that future work will clarify the full repertoire of direct and indirect NEK10 targets in ciliated cells.

In parallel with these scientific insights, our work has potential implications for the diagnosis and treatment of bronchiectasis. In particular, the identification of multiple kindreds with NEK10 mutations in whom ciliary electron microscopy, nasal nitric oxide, and high-speed video microscopy findings are all essentially normal suggests that standard algorithms for ruling out PCD may fail to capture such patients and others with mechanistically similar motile ciliopathies. Indeed, because prior studies have suggested that ciliary shortening (for example, owing to smoking26,49) may have a role in impaired mucociliary clearance in vivo, our results suggest that this relatively subtle histological abnormality should be more regularly evaluated for patients with otherwise unexplained mucus clearance deficiencies, as it may be more common than is currently appreciated. Finally, because this report directly implicates a kinase mutation in cilary dyskinesia to our knowledge for the first time, it opens the door to understanding new regulatory networks in ciliated cells and, based on this understanding, potentially to targeting this signaling axis in more common diseases of mucociliary clearance where promotion of mucociliary transport may be therapeutically beneficial26,40.
Online content
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Methods

Whole exome sequencing and clinical phenotyping. Clinical whole exome sequencing was performed on kindreds 1–3 by the Molecular Diagnostics Laboratory of the King Faisal Specialist Hospital and Research Centre (KFSHRC). Exome enrichment was performed using the Agilent SureSelect Target Enrichment workflow before high-throughput sequencing on the Illumina HiSeq 2500 system. Greater than 30× coverage of 95% of the target regions was obtained for all samples. Exome sequences were mapped to the UCSC hg19 reference sequence with a custom pipeline and interrogated for variants using databases customized to Arab populations. Sequencing of proband siblings and additional kindred was performed under a protocol approved by the KFSHRC Institutional Review Board (REC 2121053). Sequencing and analysis of kindred 4 were performed under a protocol approved by the University Children’s Hospital Muenster Institutional Review Board (AZ 2015-104-1-S). Sequencing and analysis of kindred 5 were performed under protocols approved by the UNC Chapel Hill Institutional Review Board (05-2979 and 13-2348).

HBECS and ALI tissue culture. Control human samples were obtained from discarded lung allografts under a protocol approved by the Partners Human Research Committee (IRB 2012P001079). Samples from the proband were obtained at the time of bilateral lung transplantation under a protocol approved by the Partners Human Research Committee (IRB 2013P002332) and informed consent was obtained before organ explantation. Airway cells were obtained as described previously40. In brief, bronchial tube sections were rinsed in MEM supplemented with dithiothreitol and DNase I before overnight incubation in MEM supplemented with pronase, DNAse I, antibiotics, and antifungals. Epithelial sheets were further dissociated with Accutase (Innovative Cell Technologies, AT104) and plated into PneumoCult-Ex Plus expansion medium (StemCell Technologies 05040). All plates were pre-coated with sterile-filtered, laminin-rich conditioned medium (DMEM + 10% FBS) of the 804G rat bladder cell line to promote HBECC adhesion. HBECCs were used between passages 2 and 5 for experiments and were dissociated for sub-culturing with TrypLE Select (Gibco 12536011). All cultures were established using 24-well (Corning 3470) or 6-well (Corning 3412) plates coated with 804G-conditioned medium. At confluence, apical medium was removed and basolateral medium was changed to PneumoCult-ALI (StemCell Technologies 05001) for 4 to 6 weeks of differentiation (“mature ALI”), except where stated otherwise in the text. Medium was changed every 48 h, and cultures were washed weekly with PBS buffer beginning on ALI day 14 on a plate shaker at 600 r.p.m. ×2.

Immunoblotting. Protein lysates were prepared in lysis buffer that contained 1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM HEPES pH 7.4, 2.5 mM MgCl2, and one mini tablet of EDTA-free protease inhibitor (Roche cOmplete Mini, EDTA-free, product 1183617001) per 10 mL. Lysates were subjected to SDS–PAGE electrophoresis and transferred to polyvinylidene difluoride membranes before immunoblotting with the indicated antibodies. Primary antibodies and working dilutions were rabbit anti-NEK10 (Sigma HPA038941, lot R35857, 1:1,000), mouse anti-NEK10 (Sigma WH0152110M1, lot 09058-1C9, 1:1,000), rabbit anti-GAPDH (Abcam ab9485, 1:2,500), mouse anti-FLAG M2 (Sigma F1804, lot SBL53530V, 1:1,000), rabbit anti-Raptor (Millipore 09-217, lot 3263533, 1:1,000), mouse anti-β-actin (Santa Cruz sc-47778, lot K1718, 1:1,000), and mouse anti-Ac-α-tubulin (Sigma T7431, 1:1,000). Secondary antibodies and dilutions were HRP-conjugated anti-rabbit IgG (Cell Signaling Technologies 7074, 1:3,000) and HRP-conjugated anti-mouse IgG (Cell Signaling Technologies 7076, 1:3,000).

NEK10 cDNA cloning. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s protocol before first strand cDNA synthesis with the SuperScript IV system (Invitrogen). qRT–PCR was performed using the PowerUp SYBR Green reagent (ABI) on an ABI QuantStudio 6 instrument. qRT–PCR primer sequences are available in Supplementary Table 2. Expression was validated by cloning 3′UTR sequences from each genotype were used for further experiments as indicated in the text. Cloning primers are available in Supplementary Table 2.

Linkage analysis. Genomic DNA was extracted from whole blood using a standard protocol. Genome-wide genotypes were obtained using an Affymetrix Axiom GWH-96 SNP Chip platform following the manufacturer’s instructions. Blocks of homozygosity were identified using AutoSNPA41. Linkage analysis was performed on the single nucleotide polymorphism (SNP) genotypes using the Allegro component of easyLINKAGE software42. Statistical significance was assessed using a genome-wide logarithm of odds (LOD) score threshold of 3.3, which corresponded to a type I error rate of 5%.

Immunofluorescence microscopy. ALI samples were washed in PBS, fixed for 15 min in 4% paraformaldehyde (PFA), washed three times in PBS, and cut from their plastic supports. For centrin staining only, samples were fixed in ice-cold methanol for 15 min at −20°C but otherwise processed identically. ALI membranes were then blocked (5% donkey serum + 0.3% Triton X-100) for 1 h at room temperature (22°C) before incubation with the indicated primary antibodies overnight at 37°C in dilution buffer (1% BSA + 0.3% Triton X-100). Membranes were washed four times in wash buffer (PBS + 0.1% Triton X-100) before probing with fluorophore-conjugated secondary antibodies for 1 h at 37°C in dilution buffer. DAPI was added to the secondary antibody solution for nuclear counterstaining. Membranes were washed four additional times in wash buffer and once in PBS before mounting in Fluoromount-G (Southern Biotech). Confocal images were obtained with an Olympus FV10i confocal laser-scanning microscope with a x60 oil objective.

The following primary antibodies were used: mouse anti-Ac-α-tubulin, (gift from J. Rajagopal, Sigma T6793, 1:1000), mouse anti-MUC5AC, (gift from J. Rajagopal, Thermo Fisher MS-145, lot 14SP1709C, 1:500), goat anti-CCSP (gift from B. Stripp, no lot data (non-commercial), 1:5000), chicken anti-KRT5, (gift from J. Rajagopal, Bioregion 905901, 1:500), rabbit anti-CETN1, (gift from I. Cheeseman, no lot data (non-commercial), 1:5000), and rabbit anti-VANG1L1, (gift from J. Rajagopal, Siggena 08025325, lot cl01064, 1:300). All secondary antibodies were Alexa Fluor conjugates used at 1:500 dilution (Life Technologies): goat anti-chicken 488 (A-11093, lot 1599396), donkey anti-mouse 488 (A-21202, lot TF271737), donkey anti-rabbit 488 (A-21206, lot T2173141), donkey anti-mouse 594 (R37115), and donkey anti-rabbit 594 (R37119, lot T217728).

Assessment of SCGB1A1, MUC5AC, and Ac-α-tubulin-positive areas was performed using objects automated script in ImageJ (http://rsb.info.nih.gov/ij). The KRT5-positive area could not be quantified, as basal cells form an essentially contiguous layer. Centrin puncta were quantified using automated scripts in ImageJ/FIJI implementation43, and null hypothesis testing was performed using the two-tailed Student’s t-test. The KRT5-positive area could not be quantified, as basal cells form an essentially contiguous layer.

Lentivirus cloning, production, and HBECC infection. NEK10eGFP and FOXJ1eGFP vectors were generated by amplifying the respective promoter regions from human genomic DNA and replacing the existing cytomegalovirus promoter of the plJ1c2 construct (Addgene 19319) using NEBuilder Gibson assembly (New England Biolabs). The putative NEK10 promoter was identified as a conserved 1.6 kb region bounding the NEK10 transcription start site and harboring epigenetic marks consistent with promoter function. The FOXJ1 promoter sequence was cloned using a previously described40 region as a guide. Gibson assembly of FOXY1 also used to generate FOXY1–FOXJ1–eGFP vectors. Lentiviral vectors expressing Cas9–sgRNA (single guide RNA) were generated using the pLentiCRISPRv2 vector (Addgene 52961). In brief, guide RNAs that targeted NEK10 or the AAVS1 control locus were selected from our previously published45 guide library (Addgene 1000000100), synthesized from annealed DNA oligonucleotides, cloned into Bsmbl-digested pLentiCRISPRv2 vector, screened, and sequenced to confirm identity. Cloning primers are available in Supplementary Table 2.

Lentiviruses were produced as follows: HEK293T cells were transfected with lentiviral constructs and viral packaging plasmids psPAX2 (Addgene 12260) and pCMV-VSVG (Addgene 8454) using the X-tremeGene HP reagent (Sigma). Viral supernatants were collected and concentrated using the Lenti-X Concentrator system (Takara) before use for HBECC infection. Low-passage HBECs were infected with concentrated lentivirus and selected 48 h later with 1 μg/mL puromycin for an additional 48 h before ALI culture seeding.

FACS. Mature ALI cultures were washed with PBS and incubated in TrypLE Select (Gibco) for 1 h at 37°C to liberate single cells. Cells were stained through a 70 μm cell strainer to remove clumps and debris, washed in 1X PBS, and resuspended in a buffer that contained 1% FBS, 1 mM EDTA, and 25 mM HEPS. Sorting was performed using a BD FACSaria instrument (BD Biosciences) running FACS Diva software, and analysis was performed using FlowJo (version 10) software. GFP+ cells were sorted after gating (Extended Data Fig. 2J) on viable (by exclusion of vital dye) singlets (by measurements of forward scatter and side scatter) and collected for RNA isolation in Trizol.

ALI live phase contrast imaging. Mature ALI transwells were moved from their media to glass-bottomed tissue culture dishes and imaged under x40 objective magnification with phase contrast optics at 30 frames per s for a total of 300
frames using a Zeiss AxiosObserver Z1 inverted microscope. The resulting 8-bit monochrome videos were processed in ImageJ/FIJI as follows: each 300-frame stack was duplicated, and a new 299-frame stack (the ‘difference stack’) was generated by subtracting the (n + 1)th frame from the nth frame. The mean intensity of every pixel in the difference stack was then calculated, and the resultant data were output to a single TIFF file that visually represented the average pixel intensity change over the course of video, which was a surrogate for motion. This TIFF file was pseudocolored using the ‘fire’ lookup table to yield the final processed images.

Imaging flow cytometry. Single cells were generated from mature ALI cultures as described above, fixed in 2% PFA, and resuspended in PBS before analysis on an ImageStreamx MkII instrument (Amnis). The gating strategy was as follows: starting with unclipped events using a centroid X gate, we obtained in-focus cells using a gradient root mean square gate. We next used a liberal area versus aspect ratio gate (R1) to exclude clumps, followed by an area versus GFP intensity plot to exclude clumps, followed by an area versus aspect ratio gate (R3) to exclude GFP doublets, followed by a final doublet removal step again gated on bright-field (R4). We finally gated on cells with a minimum mean pixel value of 45 in the GFP channel (R5). This strategy yielded 4,108 imaged single cells from NEK10+/− and 3,513 imaged single cells from NEK10−/− ALI samples. From this parent population of unclipped, singlet, in-focus GFP+ cells, we defined masks of whole cells and GFP+ cell bodies. The major axis difference was calculated by subtracting the major axis of the GFP mask from the whole-cell mask, and the area difference was calculated by subtracting the GFP mask area from the whole-cell mask area. Null hypothesis testing was performed using a two-tailed Student’s t-test.

**Phosphoproteomics and analysis.** ALI cultures were grown to maturity, washed in PBS, and lysed in urea lysis buffer that contained 20 mM HEPES (pH 8.0), 9.0 M urea, 50 mM NaCl, 10 mM CaCl2, and 1 mM β-mercaptoethanol. 1x Roche Complete Mini Protease Inhibitor Cocktail) and 1 min of vigorous rocking at 4°C. Supernatants, which contained cilia, were removed to pre-chilled 1.5 ml tubes, and a second aliquot of deciliation buffer was applied for 1 min. Supernatants were then pooled and centrifuged for 1 min at 1,000g to pellet debris. Supernatants were removed carefully and centrifuged for 5 min at 12,000g to pellet axonemes. Ciliary preparations were resuspended in chilled resuspension buffer (300 mM HEPES pH 7.3, 250 mM NaCl, 50 mM MgSO4, 10 mM EGTA, 1 mM EDTA, 10 mM dithiothreitol, 1x Roche Complete Mini Protease Inhibitor Cocktail) before experimental imaging session for use in electron microscopy or immunoblots. De-ciliated ALI cultures were collected in lys buffer (composition as above) and used for immunoblots.
statistical tests (.xlsx files) and uncropped immunoblots that correspond to Figs. 1–4 and Extended Data Figs. 2,3,5 (.pdf files) are provided.

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Author contributions
R.R.C. initiated the project, phenotyped the index proband, designed and performed all the experiments except as noted, analyzed the data, prepared the figures, and wrote the manuscript. D.T.M. assisted with the design and performance of the cell culture, IFM, and FACS experiments, analyzed the data, and edited the manuscript. H.M.L. performed the pJCT experiments, M. Manion performed the figures. J.X assisted with molecular cloning, site-directed mutagenesis, and cell culture, and edited the manuscript. H.E.S. performed the whole exome sequencing and linkage analysis on kindreds 1–3. M.S.T. acquired the clinical histopathology images and prepared the figures. G.W.D. performed the sequencing, molecular biology, and high-speed video microscopy (HSV M) analysis on kindred 4. M.A.Z. led the molecular analysis of kindred 5. J.C. performed and interpreted the kindred 5 ciliary electron microscopy and HSV M. M.L.D. identified the kindred 5 patients and provided clinical data. P.R.S. performed the HSV M. K.E.B. and L.P.H. assisted with the acquisition of proband 1 clinical samples. I.A. identified bromodeoxyuridine kindreds 2 and 3. E.M.F. assisted with the analysis of the phospho-proteomics data. V.V. assisted with the IFM experiments, analyzed the data, and edited the manuscript. M.R.K. supervised and led the kindred 4 analyses. M.R.K. supervised and led the kindred 5 patients and provided clinical data. D.M.S. supervised the project, designed the experiments, and edited the manuscript.

Competing interests
Massachusetts General Hospital, the Whitehead Institute for Biomedical Research, and the Massachusetts Institute of Technology are in the process of filing a provisional patent application covering the therapeutic augmentation of NEK10 signaling in disorders of mucociliary clearance (R.R.C. and D.M.S., inventors). All other authors have no competing interests.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Recurrent NEK10 mutations in familial bronchiectasis. a, Pedigree indicating affected siblings (filled), proband (‘p’), and subjects from whom genomic DNA was available for analysis (asterisks). b, Chest CT of siblings ‘a’ and ‘b’ from panel (a), with arrows indicating regions of bronchiectatic lung. c, RefSeq-annotated NEK10 variants annotated with transcription start sites, transcript sizes, predicted protein molecular weights, and exon-exon junctions assayed by qRT-PCR in Fig. 1d. d, Immunoblotting against indicated NEK10 epitopes, representative of three experiments. HBEC bands are non-specific. Full-length 133kDa NEK10 protein is indicated with a dashed box. e, Pedigree of kindred 2. Asterisks denote family members from whom genomic DNA was available, the dashed line indicates consanguinity by shared tribal ancestry, and the Sanger sequencing trace confirms c.1869dupT. f, Chest radiograph of proband 2, with arrow highlighting bronchiectasis. g, Pedigree of kindred 3. The dashed line indicates consanguinity by shared geographical ancestry, and the Sanger sequencing trace confirms c.2243C>T. h, CT from proband 3 demonstrating cystic (green arrow) and cylindrical (red arrow) bronchiectasis. i, Pedigree of kindred 4. The Sanger sequencing trace confirms c.1371+1G>T. j, CT from proband 4 indicating right middle lobe (red arrow) and left lower lobe (green arrow) bronchiectasis. k, Proband 4 nasal biopsy TEM demonstrating normal radial ciliary ultrastructure. Scale bar, 200 nm. l, Pedigree of kindred 5. The dashed line indicates consanguinity by shared tribal ancestry, and the Sanger sequencing trace confirms c.2317C>T. m, CTs of affected siblings in (l), demonstrating bronchiectasis. n-o, Nasal biopsy TEM of affected siblings in (l). Scale bars, 1 μm (n) and 200 nm (o).
Extended Data Fig. 2 | NEK10 loss does not detectably alter airway epithelial differentiation. a, 18S rRNA-normalized relative NEK10 expression during ALI differentiation. n = 1 ALI culture per time point. b–d, 18S rRNA-normalized relative expression of ciliated cell markers FOXJ1 and DNAHS (b), secretory cell marker SCGB1A1 (c), and basal cell marker KRT5 (d). n = 1 ALI culture per time point. e,f, Whole-mount immunofluorescence microscopy against SCGB1A1 (e, upper panel), goblet cell marker MUC5AC (e, lower panel), KRT5 (f, upper panel), and ciliated cell marker Ac-α-tubulin (f, lower panel). Scale bars, 100 μm. Bar graphs indicate the fraction of the surface epithelium occupied by marker-positive cells. n = 4 per condition, representative of 6 ALI differentiations. Mean ± s.d. g, Schematic depiction of bioinformatic NEK10 promoter (red) identification using the indicated UCSC genome browser hg19 tracks: CpG islands, H3K27-Ac, DNAse I hypersensitivity clusters, and transcription factor chromatin immunoprecipitation sequencing (ChIP-seq). h, Live GFP imaging of ALI cultures of the indicated genotypes and maturity, representative of three independent ALI differentiations. Scale bars, 200 μm. i, Gating strategy for FACS sorting of GFP-labeled ALI cultures. Numbers indicate the percentage of gated cells per population.
Extended Data Fig. 3  | Functional consequences of NEK10 activity manipulation. a, Quantification of analysis in Fig. 2c. Mean ± s.d. b, Kymographs of μOCT-based particle tracking from mature ALI cultures, representative of three independent ALI differentiations. c, CBF (μOCT) of mature ALI cultures of the indicated genotypes. n = 27 for NEK10WT and n = 22 for NEK10G>C, pooled from three independent ALI differentiations. Mean ± s.e.m. d, Immunoblotting of mature ALI lysates after CRISPR–Cas9-mediated gene editing with the indicated sgRNAs, representative of two experiments. Short (S) versus long (L) exposures are indicated. e, Quantification of analysis in Fig. 2f. Mean ± s.d. f, CBF of mature ALI cultures edited with the indicated sgRNAs, n = 8 per condition pooled from three independent ALI differentiations. Mean ± s.e.m. g, Immunoblotting of mature ALI lysates transduced with the indicated cDNAs, representative of 2 experiments. Short (S) versus long (L) exposures are indicated. h, Quantification of analysis in Fig. 2i. Mean ± s.d. i, Pseudocolored video microscopy of mature ALI cultures transduced with the indicated cDNAs. Representative fields from three independent ALI differentiations are shown. Scale bars, 50 μm. j, CBF of mature ALI cultures transduced with the indicated cDNAs. n = 4 per condition, pooled from three independent ALI differentiations. Mean ± s.e.m. *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001.
Extended Data Fig. 4 | Experimental manipulation of NEK10 activity alters ciliated cell morphology. a, Gating strategy for IFC analysis of MCCs. b, Representative images and masking data of cells in (a), demonstrating the ability to generate single NEK10:eGFP+ ciliated cells for analysis. c, Confocal MIPs of mature ALI cultures edited with the indicated sgRNAs after IFM against Ac-α-tubulin, representative of two independent ALI differentiations. Scale bars, 25 μm. d, Confocal MIPs of mature ALI cultures transduced with the indicated cDNAs after IFM against Ac-α-tubulin, representative of two independent ALI differentiations. Scale bars, 25 μm. e, H&E stained mature ALI samples of the indicated genotypes after sectioning orthogonal to the epithelial surface, representative of three independent ALI differentiations.
Extended Data Fig. 5 | Structural and proteomic abnormalities in NEK10-deficient airway epithelium. a, SEMs of mature ALI cultures edited with the indicated sgRNAs, representative of two independent ALI differentiations. Scale bars, 100 μm (upper panels) and 1μm (lower panels). b, Immunoblotting against the indicated proteins from lysates generated from purified cilia (lanes 2 and 4) or remaining de-ciliated mature ALI cultures (lanes 1 and 3), representative of two experiments. c, Cumulative distribution of phosphopeptides by log2[fold change] between indicated conditions. The solid (sgNeK10b) and dashed (sgNeK10c) red lines illustrate the population of depleted phosphopeptides upon NEK10 deletion. d, Table of GO classes enriched among genes (n = 395) whose peptides are depleted >1.5 fold log2[fold change] after targeting with sgNeK10b. The enrichment levels, P values, and false discovery rates are indicated. e, Cumulative distribution of phosphopeptides by log2[fold change]. Previously validated PCD proteins are in red and all other detected proteins are in black, as in Fig. 4e. f, Cumulative distribution of phosphopeptides by log2[fold change]. Previously validated non-PCD ciliopathy proteins are in red and all other detected proteins are in black, as in Fig. 4e.