A CRISPR-based screen for Hedgehog signaling provides insights into ciliary function and ciliopathies

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Primary cilia organize Hedgehog signaling and shape embryonic development, and their dysregulation is the unifying cause of ciliopathies. We conducted a functional genomic screen for Hedgehog signaling by engineering antibiotic-based selection of Hedgehog-responsive cells and applying genome-wide CRISPR-mediated gene disruption. The screen can robustly identify factors required for ciliary signaling with few false positives or false negatives. Characterization of hit genes uncovered novel components of several ciliary structures, including a protein complex that contains δ-tubulin and ε-tubulin and is required for cilium maintenance. The screen also provides an unbiased tool for classifying ciliopathies and showed that many congenital heart disorders are caused by loss of ciliary signaling. Collectively, our study enables a systematic analysis of ciliary function and of ciliopathies, and also defines a versatile platform for dissecting signaling pathways through CRISPR-based screening.

The primary cilium is a surface-exposed microtubule-based compartment that serves as an organizing center for diverse signaling pathways. Mutations affecting cilia cause ciliopathies, a group of developmental disorders including Joubert syndrome, Meckel syndrome (MKS), nephronophthisis (NPHP), and Bardet–Biedl Syndrome. The defining symptoms of ciliopathies include skeletal malformations, mental retardation, sensory defects, obesity, and kidney cysts, and are thought to arise from misregulation of ciliary signaling pathways. Advances in human genetics have led to the identification of more than 90 ciliopathy-associated genes. However, the molecular basis for many ciliopathy cases remains undiagnosed, and critical aspects of cilium assembly and function remain poorly understood.

A leading paradigm for ciliary signaling is the vertebrate Hedgehog (Hh) pathway, which plays key roles in embryonic development and in cancers such as medulloblastoma and basal cell carcinoma. Primary cilia are required for Hh-signaling output, and all core components of the Hh-signaling machinery—from the receptor PTCH1 to the GLI transcriptional effectors—dynamically localize to cilia during signal transduction.

Efforts to systematically identify genes needed for ciliary assembly or Hh signaling have been reported, but those studies have relied on arrayed short interfering RNA (siRNA) libraries and hence exhibit the high rates of false positives and false negatives characteristic of such screens. Recently, genome-wide screening using CRISPR–Cas9 for gene disruption has emerged as a powerful tool for functional genomics. However, the pooled screening format used in those studies requires a means to select for/against, or otherwise isolate, cells exhibiting the desired phenotype, a requirement that has limited the scope of biological applications amenable to this approach. Indeed, most studies to date have searched for genes that either intrinsically affect cell growth or affect sensitivity to applied perturbations.

Here, we engineered a Hh-pathway-sensitive reporter to enable an antibiotic-based selection platform. Combining this reporter with a single guide RNA (sgRNA) lentiviral library targeting the mouse genome, we conducted a CRISPR-based screen that systematically identified ciliary components, Hh-signaling machinery, and ciliopathy genes with few false positives or false negatives. We further show that previously uncharacterized hits encode new components of cilia and centrioles and also include novel ciliopathy genes.

Results

Development of a Hh-pathway reporter for pooled screening. Pooled functional screening requires the ability to enrich or deplete mutants that exhibit a desired phenotype. Because ciliary signaling is not intrinsically linked to such a selectable phenotype, we engineered a reporter that converts Hh signaling into antibiotic resistance. To validate our reporter cell line, we virally introduced sgRNAs targeting regulators of the Hh pathway (Supplementary Table 1). The transmembrane receptor SMO and intraflagellar transport (IFT) complex subunit IFT88 are required for Hh signaling, whereas SUFU and GLI3 restrain Hh-pathway activity (Fig. 1c, left). As expected, sgRNAs targeting Smo or Ift88 severely decreased Sonic Hedgehog N-terminal domain (ShhN)-induced blasticidin resistance, whereas deleting GlI3 potentiated blasticidin resistance, and targeting Sufu led...
to ligand-independent blasticidin resistance (Fig. 1c, right). These effects on blasticidin resistance were paralleled by concordant changes in endogenous-pathway outputs, including GLI1 expression and changes in GLI3 processing (Supplementary Fig. 1a). Additionally, western blotting confirmed a loss of target-protein expression for Gli3, Ift88, and Sufu sgRNAs (Supplementary Fig. 1a,b).

We next tested the suitability of our reporter cells for pooled screening, which involves quantifying sgRNAs in blasticidin-selected and unselected cell pools to identify sgRNAs that confer a selective advantage or disadvantage (Fig. 1d). We mimicked screening conditions by mixing GFP-marked cells expressing a Smo sgRNA with mCherry-marked cells expressing a portion of the 8× Gli-BS Pmin BlastR sgRNA library (10 sgRNAs/gene). sgRNA lentivirus sgRNA-infected cells Unselected Two rounds signaling & blasticidin selection

Gene A (positive Hh-pathway regulator) sgRNAs Gene B (negative Hh-pathway regulator) sgRNAs Gene C (noncilium/Hh-pathway gene) sgRNAs Control sgRNAs

sgRNA library (10 sgRNAs/gene)

3T3-[Shh-BlastR;Cas9] cells sgRNA-infected cells Unselected

Fig. 1 | Development of a Hedgehog-pathway reporter-based screening strategy. a, A transcriptional reporter combining eight copies of the GLI binding sequence (8×Gli-BS) with a minimal promoter (Pmin) to convert Hh signals into blasticidin resistance. b, Blasticidin resistance was assayed across a range of concentrations in stimulated (+ShhN) and unstimulated (−ShhN) 3T3-[Shh-BlastR;Cas9] cells. DMSO, dimethylsulfoxide control. Representative curves of 5 independent experiments performed in duplicate. c, Overview of the Hh pathway, with key negative and positive regulators shown in red and green, respectively (left). Effects of control sgRNAs on blasticidin resistance in stimulated and unstimulated 3T3-[Shh-BlastR;Cas9] cells (right). Bars show mean half-maximal inhibitory concentration (IC50) values, and circles show IC50 values from n = 2 (for gene-targeting sgRNAs) or 5 (for no sgRNA and negative-control (Ctrl) sgRNA) independent experiments performed in duplicate. d, Overview of the screening strategy. Cells receiving a negative-control sgRNA, a positive-regulator-targeting sgRNA, and a negative-regulator-targeting sgRNA are gray, green, and red, respectively. e, Flow cytometry histograms of cell mixtures showing the fraction of GFP-positive (Smo sgRNA-2, green) cells relative to cells expressing other library sgRNAs (blue) either in the absence of selection (left) or after two rounds of signaling and selection (right). Representative results from three independent experiments. f, Quantification of cell depletion in e.
our genome-wide sgRNA library. Flow cytometry showed that the fraction of Smo sgRNA-transduced cells decreased by >12-fold and by >50-fold after one and two rounds of signaling and selection, respectively, thus indicating that our strategy is suitable for pooled screening (Fig. 1e,f).

**Genome-wide screening.** We conducted our genome-wide screen by using a newly developed mouse sgRNA library. Key features of this library are the use of ten sgRNAs per gene and the inclusion of >10,000 negative-control sgRNAs that are either nontargeting or that target ‘safe’ sites with no predicted functional role (Supplementary Fig. 2a). We lentivirally transduced 3T3-[Shh-BlastR;Cas9] cells with this library at a low multiplicity of infection and maintained sufficient cell numbers to ensure ~1,000× coverage of the library. Cells were next exposed to ShHn for 24 h to fully stimulate Hh signaling, split into separate blasticidin-selected and unselected pools, and then subjected to a second cycle of signaling and selection before sgRNA quantification by deep sequencing (Fig. 1d). Genes affecting ciliary signaling were identified by comparing sgRNAs in the blastidicin-selected versus unselected cell pools, whereas genes affecting proliferation were identified by comparing the plasmid sgRNA library to the sgRNA population after 15 d of growth in the absence of blastidicin. For statistical analysis, a maximum-likehood method termed casTLE was used to determine a P value for each gene on the basis of the changes in sgRNA abundance. In addition, the casTLE method estimates the apparent strength of the phenotype (effect size) caused by knockout of a given gene.

**Assessment of screen performance.** We first assessed our ability to detect genes affecting growth. This readout was independent of our reporter-based selection strategy and enabled comparisons to other proliferation-based screens. Using reference positive and negative essential gene sets, we found that our screen identified >90% of essential genes with a 5% false discovery rate (FDR) (Supplementary Fig. 2b and Supplementary Tables 2 and 3). This performance validated the design of our sgRNA library and is comparable to that seen with other recently described libraries.

We next evaluated the ability of our screen to identify genes known to participate in ciliary Hh signaling. Initial inspection of screen results for Smo, Ift88, Gli1, Gli3, and Sufu identified several sgRNAs targeting each gene that were depleted or enriched as expected after blastidicin selection (Fig. 2a). Virtually all known Hh-signaling components were among the top hits, including the positive regulators Smo, Gsk2, Kif7, Prkar1a, Gli1, and Gli2 and the negative regulators Ptc1, Apc, Gsk3b, Sufu, and Gli3 (Fig. 2b and Supplementary Table 4). Our screen also recovered hits that encompassed nearly all functional and structural elements of cilia, thus highlighting the diverse features of cilia needed for signaling (Fig. 2c). For example, several hits encoded components of the basal body, which nucleates the cilium; the transition fibers, which anchor the basal body to the cell surface; the transition zone, which gates protein entry into the cilium; the motors that mediate intra-ciliary transport; and the IFT complexes, which traffic ciliary cargos (Fig. 2c and Supplementary Table 4). We observed no apparent correlation between growth and signaling phenotypes, thus indicating that our antibiotic selection strategy was not biased by general effects on proliferation (Supplementary Fig. 2c).

In total, we obtained 472 hits at a 10% FDR and 969 hits at a 20% FDR, and 92% of these hits led to decreased rather than increased signaling (Fig. 2b). This asymmetry indicates that, under the saturating level of ShHn used here, many genes are required to sustain high-level signaling, whereas fewer genes act to restrain pathway output. Gene ontology (GO)-term analysis with the Database for Annotation, Visualization and Integrated Discovery (DAVID) showed that the top 472 hit genes were enriched in expected functional categories (for example, cilium morphogenesis, with P=9.6×10⁻⁴, and the Smoothened signaling pathway, with P<3.6×10⁻¹²) as well as some novel categories, thereby indicating new avenues for future investigation (Fig. 2d and Supplementary Table 5). In some cases, corroborating reports supported these new connections: mouse mutants for two hit genes that enable diphthamide modification have been found to exhibit Hh-pathway-related phenotypes such as polydactyly and DPH1 mutations likewise cause a syndrome with ciliopathy-like features.

We next sought to use reference sets of expected hit and non-hit genes to quantitatively assess screen performance. We curated a set of ciliogenesis reference genes to generate a list of 130 expected hits; for expected nonhits, we used 1,386 olfactory-receptor and vomeronasal-receptor genes (Supplementary Table 3). We then calculated precision-recall and receiver operating characteristic (ROC) curves (Fig. 3a) from the P values generated by casTLE. Both performance metrics showed a high area under the curve (0.802 for precision recall, 0.892 for ROC), thus demonstrating that our screen detects hits with high sensitivity and precision (Fig. 3a).

Using a second means of evaluation, we compared the ability of our screen and of three related screens to detect expected hit genes. These studies used arrayed, siRNA-based screening to study either Hh signaling, by using a luciferase reporter, or ciliogenesis, by using microscopy-based measures of ciliary markers. Although there were notable differences among the screens (for example, Roosing et al. incorporated gene expression data to score hits), they each defined a number of hit genes similar to that defined by our screen. Overall, we detected most expected hits across functional categories ranging from Hh-pathway components to ciliopathy genes. Furthermore, even though our screen was focused on Hh signaling, we detected a greater fraction of ciliary hits than did the ciliogenesis screens across categories including IFT subunits, ciliary motors, and nearly all classes of ciliopathy genes (Fig. 3b,c, Supplementary Fig. 3a and Supplementary Table 4). Indeed, among the 88 genes encompassed by the categories shown in Fig. 3b (except for NPHP-specific genes; described below), we detected 65 as hits, thus indicating that our screen approached saturation (Fig. 3c). Interestingly, few hits were found for genes mutated exclusively in NPHP, thereby raising the possibility that the pathophysiology of NPHP may be distinct from that of other ciliopathies (Supplementary Note).

In a final assessment of our screening platform, we evaluated reproducibility across replicate screens. We observed high concordance among hits for the 95 genes measured in two different batches of the screen, such that 50 of 54 screen hits also scored as hits in the second batch (Supplementary Fig. 3b). Similarly, strong overlap in hits was found for 263 genes that were screened in parallel using two similar but distinct activators of Hh signaling: PTC1 ligand (ShhN) and SMO agonist (SAG) (Supplementary Fig. 3c). This reproducibility made it possible to pinpoint genes acting at specific steps in Hh signal transduction. For example, Gas1 was a hit in the ShHn screen but not in the SAG screen, a result in agreement with GAS1's known function as a Shh co-receptor.

**Identification of new ciliary components.** To further establish the value of our screen, we next set out to characterize six previously unstudied hit genes. We first focused on Fam92a and Ttc23 because their gene products contain domains associated with membrane trafficking. For Fam92a, we generated mutant cell pools by using individually cloned sgRNAs, then confirmed by sequencing that most cells contained likely null alleles. Indeed, a high rate of mutagenesis was observed for all genes characterized here and below (Supplementary Fig. 4a). Fam92a disruption caused a strong defect in inducible blastidicin resistance (Supplementary Fig. 4b). This defect was also seen for induction of luciferase from a GLI-binding-site reporter and was rescued by sgRNA-resistant Fam92a (Fig. 4a), thus indicating that the phenotype is specific and independent of...
the blasticidin-based readout. Notably, ciliogenesis was severely decreased in Fam92a-knockout cell pools (Fig. 4b). To gain further insight into Fam92a function, we identified FAM92A-assOCIated proteins from cells expressing FAM92A-LAP (S-tag-HR3C-GFP localization and affinity-purification tag). The FAM92A-LAP purification specifically recovered CBY1 and D2ZIP1, which are components of the transition zone35–37 (Fig. 4c and Supplementary Table 6). In agreement with this finding, we detected FAM92A localization at the transition zone by using a validated antibody (Supplementary Fig. 4c,d) and the FAM92A-LAP cell line (Fig. 4d). While this work was in progress, another group independently established FAM92A as a transition-zone protein that interacts with CBY1 and promotes ciliogenesis38.

To characterize the TPR-domain-containing protein TTC23, we identified TTC23-interacting proteins through affinity purification and mass spectrometry. Notably, the most prominent TTC23-associated proteins were IQCE and EFCAB7, which localize to a proximal region of the cilium known as the Ellis–van Creveld (EvC) zone39,40 (Fig. 4e and Supplementary Table 6). Although the four known EvC-zone proteins are dispensable for cilium assembly, they...
might be mutated in ciliopathies of previously unknown etiology. Because most ciliopathy genes affect the EvC-zone proteins EVC and EVC2 cause the ciliopathy Ellis–van Creveld syndrome39–41. In agreement with TTC23 being a new EvC-zone component, TTC23-LAP colocalized with EVC and IQCE at the EvC zone (Fig. 4f and Supplementary Fig. 4e). Although Ttc23 knockout had no effect on cilia assembly, mutant cells, compared with control cells, exhibited lower blasticidin resistance and less localization of IQCE and EVC to the EvC zone (Fig. 4g and Supplementary Fig. 4b–f–h). Similarly, Iqce RNA interference led to decreased localization of TTC23-LAP to the EvC zone (Fig. 4h).

Together these results establish TTC23 as a novel EvC-zone component that participates in Hh signaling.

Identification of novel disease genes. Because most ciliopathy genes were hits in the screen, we asked whether uncharacterized hit genes might be mutated in ciliopathies of previously unknown etiology. We first examined Txndc15, which encodes a thioredoxin-domain-containing transmembrane protein. A previous analysis of patients with MKS identified a family with a TXNDC15 mutation; however, a coincident EXOC4 variant was favored as the causative mutation44. We analyzed Txndc15-knockout cells by using luciferase reporter assays and found a clear defect in Hh signaling. Furthermore, wild-type Txndc15 rescued this defect, whereas the mutant allele found in people with MKS behaved like a null allele (Fig. 5a). We also found that cilia in Txndc15-knockout cells, as compared with control cells, exhibited greater variability in length and lower levels of the ciliary GTPase ARL13B (Fig. 5b and Supplementary Fig. 5a,b). Thus, TXNDC15 is likely to be a novel MKS gene. In agreement with our findings, a recent follow-up study has identified TXNDC15 mutations in additional families with MKS and has characterized ciliary defects resulting from TXNDC15 disruption44.

Our observation of Armc9 as a screen hit raises the possibility that it may also be a ciliopathy gene. Recently, Kar et al. reported that individuals with a homozygous mutation in ARMC9 exhibit mental retardation, polydactyly, and ptosis, but a diagnosis of Bardet–Biedl syndrome was disfavored44,45. We found that cilia from Armc9-mutant cells were short and exhibited lower levels of acetylated and polyglutamylated tubulin than did control cells (Fig. 5c and Supplementary Fig. 5c). Furthermore, triple-FLAG-tagged (3×FLAG) ARMC9 localized to the proximal region in cilia when it was stably expressed in IMCD3 or NIH-3T3 cells (Fig. 5d,e and Supplementary Fig. 5d). Notably, stimulation of Hh signaling in IMCD3 cells led to redistribution of ARMC9 toward the ciliary tip within 6 h, then a gradual return to its original proximal location, thus suggesting that ARMC9 might become ectocytosed from the ciliary tip at later time points46,47 (Fig. 5d,e). This change in localization was due to Hh signaling, because it was blocked by the SMO inhibitor vismodegib (Supplementary Fig. 5e). Furthermore, Armc9-mutant NIH-3T3 cells, compared with control cells, exhibited less ciliary accumulation of GLI2 and GLI3 (but not SMO) after pathway activation, thus suggesting that ARMC9 participates in the trafficking and/or retention of GLI proteins at the ciliary tip (Fig. 5f and Supplementary Fig. 5f–h). Collectively, these findings demonstrate that ARMC9 is a ciliary signaling factor and suggest that ARMC9 is a novel ciliopathy gene. Indeed, a recent study has confirmed that ARMC9 is mutated in Joubert syndrome and has reported that loss of the zebrafish ortholog disrupts ciliogenesis and function48. That study has further demonstrated that ARMC9 localizes to centrioles in RPE cells, whereas we found that ARMC9-FLAG localized to cilia (Fig. 5d,e and Supplementary Fig. 5d) and was present in the ciliary proctome (D. Mick and M.V.N., unpublished data). Although the basis for these differing observations warrants further investigation, these data together show an important role for ARMC9 in ciliary signaling.

Our evidence that syndromes based on ARMC9 (and DPH1) probably represent unrecognized ciliopathies led us to ask whether our screen might help classify other genetic disorders as ciliopathies. In support of this possibility, CW2C27, which encodes a peptidylprolyl isomerase, and INT51 and INT58, which encode subunits of the Integrator, are orthologs of screen hits for which patient mutations have recently been described48. Some canonical ciliopathy symptoms are present in these patients, and thus these disorders may stem from altered ciliary signaling (Supplementary Note).

These cases of individual disorders that can now be classified as likely ciliopathies led us to ask whether systematic efforts to map disease genes might show broader commonalities with our screen hits. We found that human orthologs of the screen hits were all identified in an exome–sequencing study of patients with congenital heart defects (CHD)49,50. The significant overlap in these two unbiased datasets (P = 6.11 × 10−4) indicates that defective ciliary signaling may be a prevalent cause of CHDs. Moreover, mutations in these genes
Fig. 4 | FAM92A and TTC23 as transition-zone and EvC-zone components. a, Induction of Hh-pathway luciferase reporter, shown for cells transduced with the indicated sgRNAs, transfected with plasmids encoding Fam92a-3×FLAG (Fam92a) or GFP-FKBP (GFP). Cells were untreated or stimulated with SAG. Bars show means of 4 replicate measurements (circles); one of two representative experiments. b, Analysis of cilia in 3T3-[Shh-BlastR;Cas9] cells transduced with the indicated sgRNAs. Bars show mean percentage of ciliated cells; dots show ciliated percentage in each of two independent experiments (>200 cells analyzed per data point). Ac, acetylated. Scale bars, 5 μm. c, Mass spectrometry analysis of FAM92A-associated proteins purified from IMCD3 cells. Normalized spectral abundance factor (NSAF), the percentage of each protein covered by identified peptides, and the percentile rank of corresponding genes in the screen are indicated. d, FAM92A localizes to the transition zone of IMCD3 cells, distal to centrioles (γ-tubulin) and proximal to the ciliary membrane (ARL13B). One of two independent experiments (five fields of view each). Scale bars, 5 μm and 1 μm (insets). e, Mass spectrometry analysis of TTC23-associated proteins purified from IMCD3 cells. f, TTC23-LAP colocalizes with IQCE, distal to FAM92A, in IMCD3 cells. Line plots show normalized intensity along the length of the cilium; tick marks are 1-μm intervals. Representative images shown from two independent experiments (five fields of view each). Scale bars, 5 μm and 1 μm (insets). g, Median ± interquartile range of ciliary IQCE levels, shown for cells transduced with the indicated sgRNAs; one of two independent experiments. AU, arbitrary units. h, Ciliary TTC23-LAP and IQCE signals, analyzed after introduction of Iqce-targeting or control (Ctrl) siRNAs. The median (center line), interquartile range (box boundaries), 10–90% percentile range (whiskers), and outliers are plotted for n = 390 (Ctrl) and n = 300 (Iqce) cilia. One of two (IQCE) or four (GFP) replicate experiments. Scale bars, 1 μm.
Fig. 5 | Insights into ciliopathies from previously uncharacterized screen hits. a, TXNDC15, with transmembrane domain (TMD), thioredoxin domain, and MKS-associated mutation indicated (top). Luciferase reporter levels measured for cells transduced with the indicated sgRNAs and transfected with plasmids encoding GFP-FKBP (GFP), wild-type Txndc15-3xFLAG (Tx-WT), or mutant Txndc15-3xFLAG (Tx-mut). Bars show means of 3 replicates (circles); one of two representative experiments. b, Cilia were analyzed in 3T3-[Shh-BlastR;Cas9] cells transduced with the indicated sgRNAs. Bars in graph show percentages of cells with normal, distorted, or absent cilia. Each bar per condition represents an independent experiment with >200 cells counted. Scale bars, 5 μm and 1 μm (insets). c, Cilia analyzed in 3T3-[Shh-BlastR;Cas9] cells transduced with the indicated sgRNAs. Representative images are shown at top. At bottom, median cilium length (center line), interquartile range (box boundaries), 10–90% percentile range (whiskers), and outliers are plotted. One of three independent experiments. Scale bars, 5 μm. d, Analysis of ARMC9-3xFLAG localization relative to centrioles (ninein) and ciliary membrane (ARL13B), shown for IMCD3 cells treated as indicated. Scale bar, 1 μm. e, ARMC-3xFLAG intensity along the length of the cilium from base (position 0) to tip (position 1.0), measured for IMCD3 cells treated as indicated. The mean ± s.d. were plotted after the total intensity in each cilium was normalized to 1.0; one of three representative experiments. f, Fluorescence intensity of GLI3 at the cilium tip, measured for the indicated cells in the presence or absence of ShhN. Mean ± s.e.m. are shown for each of n = 3 independent experiments (at least 250 cilia analyzed per condition). g, Table showing select clinical features in canonical ciliopathies and their observation in the context of specific mutations and syndromes. Colors indicate high (red), moderate (orange), and low (yellow) prevalence.
and communoprecipitation, and moreover we found that TEDC1 and TEDC2 mutually stabilized each other’s expression (Fig. 6c and Supplementary Fig. 6b). In further support of our data, two large-scale proteomic datasets have also identified interactions among TED-complex proteins \(^{62,63}\).

To functionally characterize Ted1 and Ted2, we examined mutant cell pools and found that they were almost completely devoid of centrioles, as assessed by staining with antibodies to centrin, ninein, polyglutamylated tubulin or \(\gamma\)-tubulin (Fig. 7a,c,d). Mutant cells also lacked cilia (Fig. 7a) and had strong defects in Hh signaling (Supplementary Fig. 6c,d). Notably, centrioles and cilia were restored in Tedc1-mutant cells after introduction of sgRNA-resistant TEDC1-3xFLAG (Supplementary Fig. 6e,f), which also localized to centrioles (Supplementary Fig. 6g).

We noted that Ted1 and Ted2 mutants exhibited a mild growth defect (Supplementary Table 3), which is consistent with recent evidence that NHI-3T3 cells lacking centrioles proliferate at a reduced rate \(^{61}\). In contrast, in other cell types, a p53-dependent arrest prevents growth in the absence of centrioles. These observations prompted us to investigate whether the varying effects of Ted1 or Ted2 disruption on proliferation across different cell types could enable predictive identification of genes with similar function. We therefore examined a collection of CRISPR-based growth screens conducted in 33 different human cell lines and used hierarchical clustering to group genes on the basis of their cell-type-specific growth phenotypes \(^{61}\). This unbiased approach placed TEDC1, TEDC2, and TUBE1 in a single cluster (Fig. 7b and Supplementary Table 7), thus suggesting that they share highly similar functions. Although TUBD1 was not found in this cluster, this absence was probably due to ineffective targeting of TUBD1 by the sgRNA library used. Indeed, although other CRISPR screens have reported pronounced growth defects for TUBD1 mutants \(^{62,63}\), ref. \(^{64}\) did not.

To better understand the basis for centriole loss in Ted1 and Ted2 mutants, we examined cells at different stages of the cell cycle. Whereas mutant cells typically had zero or one centriole in interphase, nearly all mitotic cells had excess centrioles (more than four), a result suggestive of de novo centriole formation before mitotic entry (Fig. 7c,d). As expected, centriole loss was rescued by overexpression of TEDC1 or TEDC2 (Supplementary Fig. 6g). In contrast, cells exiting mitosis showed significantly fewer centrioles than did control cells (Fig. 7d). Together, these observations suggest that Ted1 and Ted2 are dispensable for centriole biogenesis but are required for centriole stability, and newly generated centrioles are rapidly lost as cells exit mitosis. In further support of a shared function of TED-complex components, TUBE1- and TUBD1-deficient human cells have recently been shown to have a similar phenotype \(^{61}\).

**Discussion**

Here we presented a functional screening platform that pairs a pathway-specific selectable reporter with genome-wide CRISPR-based gene disruption. Applying these technologies to cilium-dependent Hh signaling, we obtained a comprehensive portrait of cilium biology that identified hit genes with high sensitivity and specificity. Several factors probably contributed to the quality of our screen, including the pooled screening format, the use of CRISPR for gene disruption, and a newly designed sgRNA library (Supplementary Note). Furthermore, our use of a selectable pathway reporter makes this technology applicable to virtually any process with a well-defined transcriptional response.

Our pooled CRISPR-based screening approach enabled us to generate a rich dataset that should be a valuable resource for dissecting ciliary signaling, defining ciliopathy genes, and discovering potential therapeutic targets in Hh-driven cancers. Although siRNA-based screens have contributed to understanding of cilia and Hh signaling, these datasets have drawbacks of false positives or false negatives that limit their utility. Roosing et al. \(^{48}\) improved their ciliogenesis-screen results by using reference gene sets to help
classify hits and by integrating gene expression datasets with their microscopy-based screen data. Our approach achieved high performance without dependence on other data sources, which may not always be available, or on a priori definition of hits, which could bias discovery of new hit classes. Our screens are also highly reproducible, thereby enabling comparative screening approaches that
should be instrumental in uncovering novel factors acting at specific steps in Hh signaling. Modifications to our screening strategy, such as performing screens in other cell lines or in unstimulated (or weakly stimulated) cells, may better replicate certain in vivo signaling modalities and may have improved sensitivity for identifying negative regulators of Hh signaling.

The value of our screen was demonstrated by the discovery of new genes that participate in ciliary signaling and novel candidate ciliopathy genes. Although the precise roles of FAM92A at the transition zone and TTC23 at the EvC zone will require further study, our screen demonstrates that new components remain to be identified, even for well-studied ciliary structures. Similarly, our analyses of TXNDC15, ARM9, CWC27, DPH1, INTS6, INTS10, ANKRD11, CDK13, CHD4, FOXP1, PRKD1, and KTM2D illustrated how screen hits can help to identify a ciliopathy-causing gene from among a short list of variants, as is frequently the case in studies involving small pedigrees, and to classify new genetic syndromes as disorders of ciliary signaling. With the exception of TXNDC15, all of the aforementioned genes had been previously linked to disease without a potential role for cilia described (for example, KMT2D and Kabuki syndrome, MIM 147920; ANKRD11 and KBG syndrome, MIM 148050; and DPH1 and Louches–Innes syndrome, MIM 616901). Among the syndromes caused by mutations in these genes, it is striking that the most prevalent feature was CHD. Our screen thus provides unbiased evidence that several CHD cases are ciliopathies, building upon similar connections observed in mice and providing motivation for future investigations by human geneticists and developmental biologists.

Notably, few of the ciliopathy genes primarily linked to kidney pathology were found as screen hits, thus suggesting that these renal diseases are mechanistically distinct (Supplementary Note). By capturing an unbiased picture of cillum-based signaling, our screen refines the classification of ciliopathies. More broadly, as genome sequencing reveals disease-associated variants at ever-growing rates, genome-wide functional studies such as that presented here should become a powerful resource to distinguish disease-causing mutations from innocuous variants and to gain insight into underlying disease mechanisms.

Our screen identifies hit genes with diverse roles in cilium function, Hh signaling, and centriole biology. For hits Tedc1, Tedc2, Tubel1, and Tubel1, we found that these genes ensure centriole stability by acting in concert, as evidenced by the association of their gene products in a stoichiometric complex. This finding identifies a new direct link between α-tubulin and β-tubulin and raises the possibility that they may form a heterodimer analogous to α/β-tubulin. In addition to the physical association of TED-complex components, deficiency in TED-complex-encoding genes produced a remarkably similar pattern of growth phenotypes across cell lines. Components, deficiency in TED-complex-encoding genes produced

Because CENPJ is a microcephaly gene, TED-complex components are potential candidate genes for this neurodevelopmental disorder.

In summary, we developed a functional screening platform that provides a resource for investigating long-standing questions in Hh signaling and primary cilia biology. By further applying these tools, it may become possible to systematically define vulnerabilities in Hh-pathway-driven cancers, to identify modifiers of Hh-pathway-inhibiting chemotherapeutics, and to search for suppressors of ciliopathies that may inform treatment. Integrating this functional genomie approach with complementary insights from proteomics and human genetics promises a rich tool kit for understanding ciliary signaling in health and disease.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0054-7.

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References

1. Goetz, S. C. & Anderson, K. V. The primary cilium: a signalling centre during vertebrate development. Nat. Rev. Genet. 11, 331–344 (2010).
2. Nachury, M. V. How do cilia organize signalling cascades? Phil. Trans. R. Soc. Lond. B 369, 20130465 (2014).
3. Bangs, F. & Anderson, K. V. Primary cilia and mammalian Hedgehog signaling. Cold Spring Harbor Perspect. Biol. 9, a021875 (2017).
4. Braun, D. A. & Hildebrandt, F. Ciliopathies. Cold Spring Harbor Perspect. Biol. 9, a028191 (2017).
5. Akizu, N. et al. Mutations in CSP1 lead to classical joubert syndrome. Am. J. Hum. Genet. 94, 80–86 (2014).
6. Wu, F., Zhang, Y., Sun, B., McMahon, A. P. & Wang, Y. Hedgehog signaling: from basic biology to cancer therapy. Cell Chem. Biol. 24, 252–280 (2017).
7. Pak, E. & Segal, R. A. Hedgehog signal transduction: key players, oncogenic drivers, and cancer therapy. Dev. Cell 38, 333–344 (2016).
8. Roosig, S. et al. Functional genome-wide siRNA screen identifies KIAA0586 as mutated in joubert syndrome. eLife 4, e06602 (2015).
9. Wheway, G. et al. An siRNA-based functional genomics screen for the identification of regulators of ciliogenesis and ciliopathy genes. Nat. Cell Biol. 17, 1074–1087 (2015).
10. Jacob, L. S. et al. Genome-wide RNAi screen reveals disease-associated genes that are common to Hedgehog and Wnt signaling. Sci. Signal. 4, ra4 (2011).
11. Kim, J. et al. Functional genomic screen for modulators of ciliogenesis and ciliarch length. Nature 464, 1049–1051 (2010).
12. Shalem, O. et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343, 84–87 (2014).
13. Koike-Yusa, H., Li, Y., Tan, E. P., Velasco-Herrera Mdel, C. & Yusa, K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat. Biotechnol. 32, 267–273 (2014).
14. Wang, T., Wei, J. J., Sabatini, D. M. & Lander, E. S. Genetic screens in human cells using the CRISPR-Cas9 system. Science 343, 80–84 (2014).
15. Zhou, Y. et al. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. Nature 509, 487–491 (2014).
16. Deans, R. M. et al. Parallel shRNA and CRISPR-Cas9 screens enable antiviral drug target identification. Nat. Chem. Biol. 12, 361–366 (2016).
17. Wang, T. et al. Identification and characterization of essential genes in the human genome. Science 350, 1096–1101 (2015).
18. Wang, T. et al. Gene essentiality profiling reveals gene networks and synthetic lethal interactions with oncogenic Ras. Cell 168, 890–903.e15 (2017).
19. Hart, T. et al. High-resolution CRISPR screens reveal fitness genes and genotype-specific cancer liabilities. Cell 163, 1515–1526 (2015).
20. Zelepis, K. et al. A CRISPR dropout screen identifies genetic vulnerabilities and therapeutic targets in acute myeloid leukemia. Cell Rep. 17, 1193–1205 (2016).
21. Orchard, R. C. et al. Discovery of a proteinaceous cellular receptor for a norovirus. Science 335, 933–936 (2012).
22. Park, R. J. et al. A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. Nat. Genet. 49, 193–203 (2017).
23. Arroyo, J. D. et al. A genome-wide CRISPR death screen identifies genes essential for oxidative phosphorylation. Cell Metab. 24, 875–885 (2016).
24. Taipale, J. et al. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 406, 1005–1009 (2000).
25. Morgens, D. W. et al. Genome-scale measurement of off-target activity using Cas9 toxicity in high-throughput screens. Nat. Commun. 8, 15178 (2017).
26. Morgens, D. W., Deans, R. M., Li, A. & Bassik, M. C. Systematic comparison of CRISPR/Cas9 and RNAi screens for essential genes. *Nat Biotechnol.*, 34, 634–636 (2016).

27. Hart, T., Brown, K. R., Sircoloumb, F., Rottapel, R. & Moffat, J. Measuring error rates in genomic perturbation screens: gold standards for human functional genomics. *Mol. Biol. Cell.*, 73 (2014).

28. Huang, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.*, 4, 44–57 (2009).

29. Webb, T. R. et al. Diphtalmide modification of EF2 requires a J-domain protein and is essential for normal development. *J. Cell Sci.*, 121, 3104–3115 (2008).

30. Chen, C. M. & Behringer, R. R. Ovca1 regulates cell proliferation, embryonic development, and tumorigenesis. *Genes Dev.*, 18, 320–332 (2004).

31. Loucks, C. M. et al. Establishing two independent cohorts. Validates DPH1 as a gene responsible for autosomal recessive intellectual disability with short stature, craniofacial, and ectodermal anomalies. *Hum. Mutat.*, 36, 1015–1019 (2015).

32. Allen, B. L. et al. Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev. Cell.*, 20, 775–787 (2011).

33. Izzii, L. et al. Boc and Gas1 each form distinct Shh receptor complexes with Ptc1 and are required for Shh-mediated cell proliferation. *Dev. Cell.*, 20, 788–801 (2011).

34. Oegema, R. et al. CEP120 interacts with CPAP and positively regulates centriole elongation. *Nat. Genet.*, 44, 1360–1365 (2012).

35. Mahjoub, M. R., Xie, Z. & Stearns, T. Cep120 is asymmetrically localized to the daughter centriole and is essential for centriole assembly. *J. Cell Biol.*, 191, 331–346 (2010).

36. Wang, T. et al. The CEP19-RABL2 GTPase complex binds IFT-B to initiate intraflagellar transport at the ciliary base. *Dev. Cell.*, 22, 36–42 (2012).

37. Kanie, T. et al. The CEP19-RABL2 GTPase complex binds IFT-B to initiate intraflagellar transport at the ciliary base. *J. Cell Biol.*, 191, 823–832 (2011).

38. Li, F. G. et al. BAR domain-containing FAM92 proteins interact with ChIBby1 to facilitate ciliogenesis. *Mol. Cell. Biol.*, 36, 2668–2680 (2016).

39. Dorn, K. V., Hughes, C. E. & Rohtagi, R. A. Smoothed-EvC2 complex transduces the Hedgehog signal at primary cilia. *Dev. Cell.*, 23, 821–832 (2012).

40. Pusapat, G. V. et al. EFCAB7 and IQCE regulate hedgehog signaling by promoting hedgehog function. *Mol. Cell. Biol.*, 28, 243–254 (2010).

41. Ruiz-Perez, V. L. & Goodship, J. A. Ellison-van Crefeld syndrome and Weyers acrocentric dysostosis are caused by cilia-mediated diminished response to hedgehog ligands. *Am. J. Med. Genet. C, Semin. Med. Genet.*, 151C, 341–351 (2009).

42. Shaheen, R. et al. Genomic analysis of Meckel-Gruber syndrome in Arabs reveals marked genomic heterogeneity and novel candidate genes. *Eur. J. Hum. Genet.*, 21, 762–768 (2013).

43. Shaheen, R. et al. Characterizing the morbid genome of ciliopathies. *Genome Biol.*, 17, 242 (2016).

44. Kar, A., Phadke, S. R., Das Bhawmik, A. & Dalal, U. Whole exome sequencing reveals mutation in ARMC9 as a cause of mental retardation, ptosis and polydactyly. Preprint at https://www.biorxiv.org/content/early/2017/02/17/109124 (2017).

45. Wang, Y. L. et al. ChIBby1 promotes Aih1 recruitment to a ring-shaped domain at the centriole–cilium interface and facilitates proper ciliation formation and function. *Mol. Biol. Cell.*, 25, 2919–2933 (2014).

46. Burke, M. C. et al. ChIBby2 promotes ciliolar vesicle formation and basal body docking during airway cell differentiation. *J. Cell Biol.*, 207, 125–137 (2014).

47. Glazer, A. M. et al. The Zn finger protein Iguana impacts Hedgehog signaling by promoting ciliogenesis. *Dev. Biol.*, 337, 148–156 (2010).

48. Li, F. G. et al. Domain-containing FAM92 proteins interact with ChIBby1 to facilitate ciliogenesis. *Mol. Cell. Biol.*, 36, 2668–2680 (2016).

49. Dorn, K. V., Hughes, C. E. & Rohtagi, R. A. Smoothed-EvC2 complex transduces the Hedgehog signal at primary cilia. *Dev. Cell.*, 23, 821–832 (2012).

50. Pusapat, G. V. et al. EFCAB7 and IQCE regulate hedgehog signaling by tethering the EVC-EVCC2 complex to the base of primary cilium. *Dev. Cell.*, 28, 483–496 (2010).

51. Panigrahi, I., Phadke, S. R. & Agarwal, S. S. Mental retardation, ptosis and polydactyly: a new autosomal recessive syndrome? *Clin. Dysmorphol.*, 319, 289–292 (2002).

52. Nager, A. R. et al. An actin network-dispatches ciliary GPCRs into extracellular vesicles to modulate signaling. *Cell.*, 168, 252–263.e14 (2017).

53. Phsa, S. C. et al. Dynamic remodeling of membrane composition drives cell cycle through primary cilia excision. *Cell.*, 168, 254–279.e15 (2017).

54. Van De Weghe, J. C. et al. Mutations in ARMC9, which encodes a basal body protein, cause Joubert syndrome in humans and ciliopathy phenotypes in Chlamydomonas. *J. Cell Sci.*, 110, 23–37 (2017).

55. Wang, Y. L. et al. Reversible ciliopathy depletion with an inhibitor of Polo-like kinase 4. *Science.*, 348, 1155–1160 (2015).

56. Aguirre, A. J. et al. Genomic copy number dictates a gene-independent cell cycle control. *Cancer Discov.*, 6, 914–926 (2016).

57. Bond, J. et al. A ciliastellar mechanism involving DOK5RAP2 and CENPJ controls brain size. *Nat. Genet.*, 37, 353–355 (2005).

58. Gilmore, E. C. & Walsh, C. A. Genetic causes of microcephaly and lessons for neuronal development. *Wiley Interdiscip. Rev. Dev. Biol.*, 2, 461–478 (2013).

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Author contributions
D.K.B., S.H., J.K.C., and M.V.N. conceived the project with advice from M.C.B.; D.K.B. and S.H. developed the Hh-pathway reporter screening strategy with assistance from B.K.V.; and D.W.M., K.H., A.L., G.T.H., and M.C.B. provided functional genomics expertise, the genome-wide sgRNA library, and software for screen data analysis. D.K.B. conducted the genome-wide screen and screen data analysis with assistance from S.H. and A.R.K.; D.K.B., S.H., A.R.K., and M.C.K. functionally characterized hit genes of interest, analyzed data, and prepared figures. D.K.B., S.H., J.K.C., and M.V.N. wrote the manuscript with assistance from M.C.B.; D.K.B., S.H., G.T.H., M.C.B., J.K.C. and M.V.N. provided funding for the project.

Competing interests
The authors declare no competing interests.

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Methods

Experimental models and subject details. Cell lines and cell culture. NIH-3T3 and HEK293T cells were grown in high-glucose, pyruvate-supplemented DMEM (Gibco) with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 U/mL penicillin, and 100 μg/mL streptomycin (Gemini Bio-Products). NIH-3T3 FlpIn cells (gift from R. Rohatgi) were grown in the same medium supplemented with nonessential amino acids (Gibco). Light-II NIH-3T3 cells34 were grown in the same medium except with 10% bovine calf serum (ATCC), and IMCD3 cells were grown in DMEM/F12 medium (Gibco) with FBS, glutamine, penicillin, and streptomycin additives. Serum starvation was done with medium with 0.5% FBS for NIH-3T3 cells, 0.5% calf serum for Light-II NIH-3T3 cells, and 0.2% FBS for IMCD3 cells. IMCD3 FlpIn cells were provided by P. Jackson. NIH-3T3 cells were obtained from ATCC. HEK293T-ER-ShN cells were provided by P. Beachy. Cells were confirmed to be mycoplasma free with the MycoAlert system (Lonza).

Method details. DNA cloning. Individual sgRNAs were cloned by ligating annealed oligonucleotide pairs into pMCB306 or pMCB320 digested with BstXI and BspMoiI (Agilent) as previously described25. Briefly, oligonucleotides were amplified with annealed oligonucleotides into pMCB306 or pMCB320 digested with BstXI and Bpu1102I (New England Biolabs) and this was followed by three rounds of FACS sorting for high-level SAG-induced blastidicin resistance. Pgk-Cas9-BFP was introduced lentivirally, and this was followed by three rounds of FACS sorting for high-level BFP expression.

Cell lines and cell culture. NIH-3T3 cells were grown in high-glucose, pyruvate-supplemented DMEM (Gibco) with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 U/mL penicillin, and 100 μg/mL streptomycin (Gemini Bio-Products). NIH-3T3 FlpIn cells (gift from R. Rohatgi) were grown in the same medium supplemented with nonessential amino acids (Gibco). Light-II NIH-3T3 cells34 were grown in the same medium except with 10% bovine calf serum (ATCC), and IMCD3 cells were grown in DMEM/F12 medium (Gibco) with FBS, glutamine, penicillin, and streptomycin additives. Serum starvation was done with medium with 0.5% FBS for NIH-3T3 cells, 0.5% calf serum for Light-II NIH-3T3 cells, and 0.2% FBS for IMCD3 cells. IMCD3 FlpIn cells were provided by P. Jackson. NIH-3T3 cells were obtained from ATCC. HEK293T-ER-ShN cells were provided by P. Beachy. Cells were confirmed to be mycoplasma free with the MycoAlert system (Lonza).

Cilia-focused sgRNA libraries were cloned from oligonucleotide pools (Agilent) as previously described35. Briefly, oligonucleotides were amplified with annealed oligonucleotides into pMCB306 or pMCB320 digested with BstXI and Bpu1102I (New England Biolabs) and this was followed by three rounds of FACS sorting for high-level SAG-induced blastidicin resistance. Pgk-Cas9-BFP was introduced lentivirally, and this was followed by three rounds of FACS sorting for high-level BFP expression.

Virus production and cell transduction. VSVG-pseudotyped lentiviral particles were produced by co-transfection of HEK293T cells with a lentivector and appropriate packaging plasmids (pMD2.G, psVSV-Rev, and pMD1.G/RRE for sgRNAs expressed in pMCB320 or pMCB306; pCMV-ΔR-8.91 and pCMV-ΔVSVG for Pgk-Cas9-BFP). After transfection with polyethyleneimine (linear, 1 μg/ml polybrene (Sigma Aldrich). Appropriate titer in growth medium containing 4 μg/ml puromycin for 5 d, grown for 3 d without puromycin, and then plated for signaling, and a ~1,000:1 ratio of cells to sgRNAs was maintained during these subsequent steps. After cells reached confluence, signaling was initiated by addition of serum-starvation medium containing ShhN. After 24 h, cells were passaged, allowed to adhere, and then subjected to blastidicin selection for 4 d at 5 μg/ml, a concentration that we found sufficient to achieve strong enrichment/deletion of hits without causing sgRNA library bottlenecks due to excess cell death. After passing cells to blastidicin-free medium, a ‘T1’ sample was harvested (1,000-fold more cells than sgRNAs), and the remaining cells were passaged once more before seeding for a second round of signaling and selection. The final ‘T2’ sample was collected after a 4-d blastidicin selection and one additional passage in the absence of blastidicin. Unselected control cells were also propagated through the entire experiment and harvested at equivalent time points.

Screens with the cilia/Hh pathway-focused library were conducted as above except that a variant blastidicin reporter cell line was used, in which Cas9-BFP was expressed with the shortened EF1α promoter. Because some Cas9-negative cells accumulated during the experiment, the final blastidicin-selected and unselected cells were FACS-sorted for BFP expression.

To process cell samples for sgRNA sequencing, genomic DNA was isolated with the QiAamp DNA Blood Max kit or QiAamp DNA mini kit (Qiagen). Genomic DNA was then amplified with Hercule II polymerase (Agilent) as previously described36, first with outer primers to amplify the sgRNA cassette, then with inner primers to amplify a portion of the initial PCR product while introducing sample-specific barcodes and adapters for Illumina sequencing (Supplementary Table 9). Created PCR products were then amplified with a Qubit 2.0 fluorometer with the specific barcodes and adapters for Illumina sequencing (Supplementary Table 9).

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Articles

Immunofluorescence and localization studies. IMCD3 FlpIn or 3T3-[Shh-BlastR;Cas9] cells were plated on acid-washed 13-mm round #1.5 coverslips and incubated with Fugene 6 (Promega). Where indicated, cells were serum-starved for 24 h, and then incubated with Shh or 1 μM vismodegib (Chemietek) before fixation with 4% paraformaldehyde, ice-cold methanol, or both in succession. For GLI2/GLI3/SMO trafficking assays, cells were serum-starved for 20 h, then incubated 5–6 h in the presence or absence of ShhN-conditioned medium. For analysis of TDC2-LAP localization, cells were preextracted before methanol fixation via a 1-min exposure to PHEM buffer (60 mM PIPES, 25 mM HEPES, 4 mM MgSO4, and 10 mM EGTA, pH 7.0) with 0.2% Triton X-100.

Fixed coverslips were blocked with PBS with 3% BSA and 5% normal donkey serum, permeabilized with PBS with 0.1% Triton X-100, and then incubated with the appropriate primary antibodies (Supplementary Table 10) and secondary antibodies (Jackson ImmunoResearch). Coverslips were either stained with Hoechst DNA dye and mounted on slides with Fluoromount-G mounting medium (Electron Microscopy Sciences) or directly mounted with ProLong Gold antifade reagent (Life Technologies).

Coverslips were imaged at 60x or 63x magnification with one of the following microscopy systems: an Axio Imager.M1 (Carl Zeiss) equipped with SlideBook software, an LED light source (Intelligent Imaging Innovations), and a Prime 95b sCMOS camera (Photometrics); an Axio Imager.M1 (Carl Zeiss) equipped with a CoolSNAP HQ2 CCD camera (Photometrics); an Axio Imager.M2 equipped with ZEN software, an X-Cite 120 LED light source (Excitex), and an AxioCam 503 mono camera (Carl Zeiss); or a DeltaVision Elite imaging system equipped with SoftWoRx software, an LED light source, and an sCMOS camera (Applied Precision). Z stacks were acquired at intervals of 250–500 nm and deconvolved as needed with SlideBook 6.0 or SoftWoRx software.

Cotransfection and coimmunoprecipitation. HEK293T cells were cotransfected with Teda1, Teda2 or TegRFP plasmids with Fugene 6, collected after 48 h, and lysed on ice in Co-IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM DTT) supplemented with protease inhibitors. Lysates were centrifuged at 20,000 × g for 20 min, and FLAG-tagged proteins were captured by incubation for 2 h with anti-FLAG M2 antibody (Sigma Aldrich) and Protein G Sepharose 4 Fast Flow (GE Healthcare). After four washes of resin with Co-IP buffer, protein-bound proteins were eluted by incubation at 95°C in lithium dodecyl sulfate–based gel loading buffer.

Western blotting. Lysates from 3T3-[Shh-BlastR;Cas9] cells were prepared in SDS sample buffer (50 mM Tris HCl, pH 6.8, 8% (vol/vol) glycerol, 2% (wt/vol) SDS, 100 mM DTT, and 0.1 mg/ml bromophenol blue), boiled and sonicated. Samples were loaded onto a 4–15% Criterion TGX Stainfree gel (Bio-Rad), run for 25 min at 300 V in Tris–glycine–SDS buffer (Bio-Rad), and transferred onto a PVDF membrane with a Transblot Turbo system (Bio-Rad). Membranes were blocked in 1:1 PBS/SeaBlock (Thermo Scientific) for 1 h at room temperature, then incubated with the indicated primary antibody (Supplementary Table 10) for 16 h at 4°C. After incubation with HRP-conjugated secondary antibody or HRP–Protein A (Jackson ImmunoResearch), blots were developed with Supersignal West Femto Maximum sensitivity substrate (Thermo Scientific) and imaged on a Chemidoc MP (Bio-Rad). Membranes were stripped with Restore Western Blot stripping buffer (Thermo-Fisher) and reprobed as described.

For analysis of immunoprecipitations, western blotting was performed as described above, except samples were separated in 4–12% Bis-Tris PAGE gels (Invitrogen) with MOPS running buffer, transferred to PVDF membranes with a Criterion Blottor system (Bio-Rad), developed with ECL or ECL 2 chemiluminescence detection kits (Pierce), and imaged on a Chemidoc Touch system (Bio-Rad).

Large-scale affinity purification and mass spectrometry. Affinity purifications were conducted as previously described. Briefly, an ~300–600 μl packed cell volume was lysed in LAP purification buffer containing 0.3% NP-40. Lysate was cleared sequentially at 16,000 g and 100,000 g before incubation with anti-FLAG antibody coupled to Protein A resin. After protein capture and washes, bound LAP-tagged proteins were eluted by incubation with HRV3C protease. For mass spectrometry analysis X (Supplementary Table 6), eluted proteins were further purified by capture on S-Protein agarose and eluted at 95°C in lithium dodecyl sulfate–based gel loading buffer.

For protein analysis by mass spectrometry, gel slices containing affinity-purified proteins were washed with 50 mM ammonium bicarbonate, then reduced with DTT (5 mM) and alkylated with propionamide (10 mM). Gel slices were further washed with 1% acetonitrile–ammonium bicarbonate buffer until all stain was removed. 120 ng of trypsin/LysC (Promega) reconstituted in 0.1% ProteaseMAX (Promega) with 50 mM ammonium bicarbonate was added to each gel band; after 30 min, 20 μl of additional 50 mM ammonium bicarbonate in 0.1% ProteaseMAX was added. Digestion was then allowed to occur overnight at 37°C. Peptides were extracted from the gels in duplicate, then dried in a SpeedVac concentrator. Peptide pools were reconstituted and injected onto a C18 reversed-phase analytical column, ~20 cm in length, pulled and packed in house. The UPLC was a NanoAcuity or M-Class column (Waters), operated at 450 nl/min with a linear gradient from 4% mobile phase B to 45% B. Mobile phase A consisted of 0.2% formic acid in water; mobile phase B consisted of 0.2% acetic acid in acetonitrile. The mass spectrometer was an Orbitrap Elite or Fusion (Thermo Fisher) set for data-dependent acquisition, selecting and fragmenting the 15 most intense precursor ions in the ion trap, where the exclusion window was set at 45 s, and multiple charge states of the same ion were allowed.

Quantification and statistical analysis. Analysis of CRISPR-based screens. CRISPR-based screens were analyzed as previously described, and data from each screen batch were processed separately. To determine the sgRNA counts in each sample, raw sequencing reads were trimmed to the 3′-most 17 nt of each protospacer and aligned to expected sgRNA sequences. This alignment was carried out with the makeCounts script in the casTLE software package, which uses Bowtie to perform alignment with zero mismatches tolerated. The analyzeCounts script (v0.7 and v1.0) of casTLE was then used to identify genes exhibiting significant enrichment or deple- tion and to estimate the phenotypic effect size for each gene. This method uses an empirical Bayesian approach to score genes according to the log-likelihood ratio that a gene’s observed change in sgRNA counts is drawn from a model of gene effect versus the distribution of negative-control sgRNAs. An expected negative score distribution is obtained by random permutation of gene-targeting sgRNA fold-change values and used to determine a p value. Notably, the use of more stringent criteria leads to a minimum reported P value of 1 × 10−6 (200,000 permutations were used for comparative screening in Supplementary Fig. 3c, with minimum P value of 5 × 10−6). For each gene, the casTLE algorithm also estimates the magnitude of the phenotype resulting from complete gene inactivation. This value is output as the effect size and is accompanied by an estimated range of effect sizes compatible with each gene’s sgRNA data.

Genes targeted by our sgRNA library that lacked an NCBI identifier or that severely affected growth (casTLE effect size < 2.5 and casTLE P value < 0.005) were not considered for further analysis but are included in Supplementary Table 3. Negative and positive reference genes were defined for growth and signaling phenotypes with previously defined gene sets (Supplementary Table 3). Precision-recall and ROC curves were computed in Matlab (Mathworks). Hit genes at 10% and 20% FDR cutoffs were defined with the precision-recall threshold values at precision of 0.9 and 0.8, thus yielding P-value cutoffs of 0.0163 and 0.0338, respectively. Ciliopathy-associated genes were defined from OMM. Functional category enrichment analysis for 10% FDR hits was performed with the DAVID website’s functional annotation chart tool with all mouse genes as the background. A second analysis was performed with human homologs of 10% FDR hits with all human genes as the background. The significance of overlap between the top 15 genes associated with CHD reported by Sfrim et al. and P values from functional screening was assessed with the Kolmogorov–Smirnov test.

Quantification of Hh signaling assays. Blastcandin-based inhibition of cell growth was determined by normalizing raw CellTiter-Blue fluorescence such that growth in the absence of blastcadin corresponded to 100% growth. The IC50 for blastcadin was determined with Prism 7 (Graphpad) software.

Dual-luciferase data were analyzed by first subtracting background signal such that cells without luciferase gave readings of zero. Firefly/Renilla (8xGli/ constitutive) ratios were then calculated and normalized such that unstimulated control cells had a value equal to 1.

Quantification of fluorescence microscopy images. Microscopy images were analyzed with Fiji Image (software) (National Institutes of Health) and a custom Matlab (Mathworks) script. Local background subtraction was performed on all images before analysis. To determine ciliary frequency, cells were manually scored for the presence or absence of a cilium with ARL13B and acetylated tubulin as cilary markers. Analyses of cilium localization and multiple charge states of the same ion were allowed.

Differences in cilium length distribution were tested for significance with the basis of DNA morphology, and statistical significance was determined with two-sided Fisher’s exact test calculated in Stata 14.2 (StataCorp). Cell counts for each of the five centriole-number categories were used for statistical comparisons between genotypes.

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Analysis of mass spectrometry data. MS/MS data were analyzed with both Preview and Byonic v2.10.5 (ProteinMetrics). All data were first analyzed in Preview to provide recalibration criteria if necessary and were then reformatted to MGF format before full analysis with Byonic. Data were searched at 12-p.p.m. mass tolerances for precursors, with 0.4-Da fragment mass tolerances assuming up to two missed cleavages and allowing for fully specific and ragged tryptic peptides. The database used was UniProt for *Mus musculus*, downloaded on 25 October 2016. These data were validated at a 1% FDR with typical reverse-decoy techniques87. The resulting identified peptide spectral matches and assigned proteins were then exported for further analysis with Matlab (MathWorks) to provide visualization and statistical characterization.

Analysis of CRISPR growth-screen datasets. Gene-level growth phenotype data74 were downloaded from the Achilles website. Hierarchical clustering with uncentered correlation and average linkage settings was performed with Cluster 3.0 software88, and clustered data were visualized in Java Treeview89.

Phylogenetic analysis. Homologs for *Tubd1* and *Tubel* were either previously described90 or identified with protein BLAST. Homologs for *Tedc1* and *Tedc2* were identified with iterative searches with PSI-BLAST91. Phylogenetic trees were generated via neighbor joining with distance correction with Simple Phylogeny92 and visualized with Unrooted93.

Code availability. Software used for casTLE analysis can be found at http://bitbucket.org/dmorgens/castle/. Matlab scripts for quantification of cilium intensities and length are available upon request.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The data supporting the findings of this study are available within the paper and its Supplementary Information files, with the exception of raw Illumina sequencing data and sequencing-based analysis of CRISPR-induced mutations, which are available upon request.

References
82. Liew, G. M. et al. The intraflagellar transport protein IFT27 promotes BBSome exit from cilia through the GTPase ARL6/BBS3. *Dev. Cell* 31, 265–278 (2014).
83. Frank-Kamenetsky, M. et al. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J. Biol.* 1, 10 (2002).
84. Chen, J. K., Taipale, J., Young, K. E., Maiti, T. & Beachy, P. A. Small molecule modulation of Smoothened activity. *Proc. Natl. Acad. Sci. USA* 99, 14071–14076 (2002).
85. Bredlow, D. K., Koulouris, E. F., Seydel, F., Spakowitz, A. J. & Nachury, M. V. An in vitro assay for entry into cilia reveals unique properties of the soluble diffusion barrier. *J. Cell Biol.* 203, 129–147 (2013).
86. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10, R25 (2009).
87. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* 4, 207–214 (2007).
88. de Hoon, M. J., Imoto, S., Nolan, J. & Miyano, S. Open source clustering software. *Bioinformatics* 20, 1453–1454 (2004).
89. Saldanha, A. J. Java Treeview: extensible visualization of microarray data. *Bioinformatics* 20, 3246–3248 (2004).
90. Turk, E. et al. Zeta-tubulin is a member of a conserved tubulin module and is a component of the centriolar basal foot in multiciliated cells. *Curr. Biol.* 25, 2177–2183 (2015).
91. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402 (1997).
92. Li, W. et al. The EMBL-EBI bioinformatics web and programmatic tools framework. *Nucleic Acids Res.* 43, W580–W584 (2015).
93. Perrière, G. & Gouy, M. WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie* 78, 364–369 (1996).
Experimental design

1. Sample size
   Describe how sample size was determined.
   The main sample size consideration was the number of cells used for our pooled screen. This was determined based on the level of variation and statistical power seen in other pooled screens. The 1000-fold excess of cells to library sgRNAs led to strong statistical significance for hit genes and matches or exceeds that in many other pooled screens.

2. Data exclusions
   Describe any data exclusions.
   A subset of genes were excluded from part of our analyses because they likely represent mis-annotated genes (no NCBI identifier found) or because strong deleterious effects on growth precluded robust measurement of signaling phenotypes. This issue is explained further in the Methods; the full data are also included in Supp. Table 3.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   No attempts at replication failed. All replication experiments confirmed initial findings.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Randomization was not performed because it was not relevant to our experimental design. Statistical significance of screen results was determined by scoring 10-sgRNA artificial genes created by randomization of our data.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Blinding was not practical or relevant to our study. The pooled screen is effectively blinded; follow-up experiments led to sufficiently strong and consistent results that blinding was effectively precluded.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- **n/a**
- **Confirmed**

- The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

- A statement indicating how many times each experiment was replicated

- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

- A description of any assumptions or corrections, such as an adjustment for multiple comparisons

- The test results (e.g. $P$ values) given as exact values whenever possible and with confidence intervals noted

- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

- Clearly defined error bars

> See the web collection on statistics for biologists for further resources and guidance.

### Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Software used for analysis of screen sequencing data includes Bowtie (ref. 87) and the casTLE algorithm (https://bitbucket.org/dmorgens/castle, also ref. 26). Custom Matlab scripts for cilia image analysis are available upon request.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

### Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions on availability of materials.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Antibody sources are in Supplementary Table 10. FAM92A Ab was validated in Supplementary Fig. 4d. SUFU, GLI3, GLI1, and IFT88 antibodies were validated using WB analysis of KO cells (Supp. Fig. 1 and data not shown). Antibodies to TUBD1 and TUBE1 were validated by the Human Protein Atlas using Western blotting and protein arrays, and we detect a band of expected MW specifically in samples confirmed to contain the antigen by mass spectrometry. IQCE and EVC specificity were validated by RNAi in Fig. 4h and in refs. 39-40. CBY1 8-2 antibody was validated in PMID 21529289 and 25103236. SMO Ab was validated for immunofluorescence in PMID 21552265. GLI2 Ab was validated in KO cells in PMID 26193634. All other antibodies are extensively used in the field and have been used in dozens or more publications.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. All cell lines are described in the Methods. All were obtained from ATCC, except for IMCD3 FlpIn cells, provided by Peter Jackson (originally obtained from Invitrogen), HEK293-EcR-ShhN cells from Philip Beachy, LightII NIH-3T3 cells generated by the Chen lab, and NIH-3T3 FlpIn cells provided by Rajat Rohatgi.
   b. Describe the method of cell line authentication used. None of the cell lines used have been authenticated. Nearly all cell lines used were murine in origin and few tests are available for authentication of mouse cell lines.
   c. Report whether the cell lines were tested for mycoplasma contamination. All cell lines were confirmed negative for mycoplasma.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. None of the cell lines used are commonly misidentified.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study. N/A

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants. N/A
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

- **Data presentation**

  For all flow cytometry data, confirm that:

  - 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - 3. All plots are contour plots with outliers or pseudocolor plots.
  - 4. A numerical value for number of cells or percentage (with statistics) is provided.

- **Methodological details**

  5. Describe the sample preparation. Adherent cultured cell lines were analyzed following trypsinization and resuspension in PBS.

  6. Identify the instrument used for data collection. A FACScan cytometer was used for collection of flow cytometry data.

  7. Describe the software used to collect and analyze the flow cytometry data. Flowjo (Treestar) was used for data collection and analysis.

  8. Describe the abundance of the relevant cell populations within post-sort fractions. No sorting data is included.

  9. Describe the gating strategy used. The only gating performed was using FSC/SSC data to select live single cells (for Fig. 1e). A figure depicting this standard gating is not included but will be provided if needed.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☐