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☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

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 form ATCC, except for IMCD3 FlpIn cells, provided by Peter Jackson (originally obtained form Invitrogen), HEKT293-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

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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 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. ☐