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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Microscopy images were collected using MetaMorph software (Molecular Devices; version 7.8.10) and NIS Elements (Nikon, version 5.30.01).
Data analysis	Image analysis was completed using MetaMorph (Molecular Devices; version 7), NIS Elements (version 5), and ImageJ/ FIGI software (version 2.1.0/1.53c). Statistical calculations were performed with GraphPad Prism [GraphPad Software Inc; version 8] and Microsoft Excel (version 16.52). Protein identification/quantification and analysis were performed with Integrated Proteomics Pipeline - IP2 [Integrated Proteomics Applications, Inc] using ProLuCID and DTASelect2.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

A data availability statement covering all of these points is included in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Details of all sample sizes are in the methods sections and/or figure legends. Sample sizes from in vitro are all equal to or exceed 90 from multiple biological replicates supporting the conclusions of this study. For instances the computational analysis pipeline was used (described in detail in methods sections), samples sizes in the thousands were recorded in a user-independent fashion. In vitro experiments with cultured cells were performed at least three times (biological replicates including at least 3 technical replicates for each experiment) to confirm reproducibility. In vivo experiments included at 5 animals per group to allow statistical analysis to support the conclusions of this study.
Data exclusions	Data were not excluded from analysis.
Replication	Independent biological replicates were performed to support the conclusions of this study. All findings described in this study were reliably reproduced.
Randomization	Animals were assigned randomly to experimental and control groups. (e.g., 10 animals were randomly assigned to two groups, one control or one experimental group with 5 animals in each group). Animals were age and sex matched. In vitro experiments were acquired and analyzed equally. For in vitro experiments, images of cells included in our analysis were acquired randomly from multiple, non-overlapping regions of the cover-slip chosen in an unbiased fashion.
Blinding	Blinding was not possible with in vitro experiments as sample groups were evident during data acquisition and analysis. When possible, quantifications were performed using a computational pipeline (described in detail in methods sections) and applied equally to all conditions and across all replicates.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Western blot: Primary antibodies were used as follows: 1:1,000 dilution of anti-GFP [Invitrogen, A-6455], mouse monoclonal anti-GFP [SantaCruz, B2 sc-9996], anti-HA [ABM, G036], anti-p150glued [BD Biosciences, 610473], H2 anti-kinesin heavy chain, 63-90 anti-kinesin light chain, anti-Kif5A [Abcam, ab5628], anti-Kif5B [Abcam, ab5629], and anti- Kif5C [Abcam, ab5630]. A horseradish peroxidase-conjugated goat anti-mouse antibody was used at a 1:10,000 dilution [Jackson ImmunoResearch, 115-035-146] or goat anti-mouse IRDye 800CW (LI-COR Biosciences, 926-32210] at 1:10,000 and donkey anti-rabbit IRDye 680RD [LI-COR Biosciences, 926-68073] at 1:10,000. Immunofluorescence: Primary antibodies used were rat anti-tyr-tubulin (1:400) [obtained from Gregg Gundersen], rabbit anti-pericentrin (1:200) [Bethyl Laboratories, IHC-00264], mouse anti-Kif5 heavy chain (1:100) [Millipore, H2], mouse anti-KLC (1:1000) [63-90; provided by Scott Brady], and mouse anti-HSV-1 VP5 (1:400) [Virusys, HA018]. Secondary antibodies used were anti-mouse Alexa-488, anti-rabbit Alexa-568, and anti-rat Alexa-647 (1:400) [ThermoFisher Scientific, A-11001, A-11011, A- 21247] and goat anti-mouse Alexa-568 (1:250) [ThermoFisher Scientific, A- 11019].
Validation	anti-GFP (size confirmed in this study and by manufacturer via western blot(WB); anti-HA (size confirmed in this study and by manufacturer via western blot(WB); anti-p150glued (size confirmed in this study and by manufacturer via western blot(WB); H2 anti-

kinesin heavy chain [(size confirmed in this study and by manufacturer via western blot(WB), specificity confirmed in this study by immunofluorescence (IF)]; 63-90 anti-kinesin light chain (size confirmed in this study and by Scott Brady via western blot(WB); anti-Kif5A [(size confirmed in this study and by manufacturer via western blot(WB), specificity confirmed in this study by siRNA depletion and WB and by the manufacturer via immunofluorescence (IF)]; anti-Kif5B [(size confirmed in this study and by manufacturer via western blot(WB), specificity confirmed in this study by siRNA depletion and WB and by the manufacturer via immunofluorescence (IF)]; anti- Kif5C [(size confirmed in this study and by manufacturer via western blot(WB), specificity confirmed in this study by siRNA depletion and WB and by the manufacturer via immunofluorescence (IF)]; anti-tyr-tubulin [(size confirmed in this study and by Gregg Gundersen via western blot(WB) specificity confirmed in this study by immunofluorescence (IF)]; anti-pericentrin [(size confirmed by manufacturer via western blot(WB), specificity confirmed in this study and by manufacturer via immunofluorescence (IF)]; anti-HSV-1 VP5 [(size confirmed by manufacturer via western blot(WB), specificity confirmed in this study and by manufacturer via by immunofluorescence (IF)]

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Vero (African green monkey kidney epithelial), were obtained from ATCC; HEK293 (human embryonic kidney), were obtained from Dane Chetkovich, Northwestern University; PK15 (pig kidney epithelial), were obtained from ATCC; hTERT-RPE (immortalized human retinal pigmented epithelial) were obtained from Vladimir Gelfand, Northwestern University; SK-N-SH (human neuroblastoma) were obtained from Richard Longnecker, Northwestern University; NHDF (normal human dermal fibroblast) were isolated from human male neonatal foreskin (Lonza Bioscience: CC-2509).
Authentication	Vero and PK15 cell lines obtained from ATCC were authenticated from the supplier. Remaining cell lines have not been authenticated.
Mycoplasma contamination	All cell lines were tested regularly for mycobacterium contamination using the PlasmoTest kit (InvivoGen) and authenticated by the source.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	male BALB/c mice (9weeks old; Charles River Breeding Laboratories)
Wild animals	No wild animals were used in this study.
Field-collected samples	No field collected samples were used in this study.
Ethics oversight	All procedures conformed to NIH guidelines for work with laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Nebraska, Lincoln (Protocol: 1086).

Note that full information on the approval of the study protocol must also be provided in the manuscript.