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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	MaxQuant (version 1.6.5.0)
Data analysis	Perseus software (version 1.5.8.5); R software (version 3.6.1); Graph Pad Prism (8.2.1); Image J (Fiji, 2.0.0); Flow jo (10.5+ & 10.6.2); Image lab (6.0.1.34); XDS/XSCALE (20180808); Phaser (2.8.2); Phenix (1.17.1); Coot (0.9.1); PyMOL (2.4.0)

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

The atomic model of K29-linked diUb in complex with sAB-K29 has been deposited in the Protein Data Bank (PDB) under the accession code 7KEO [https://www.rcsb.org/structure/unreleased/7KEO]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024428 and PSD0244425 [https://www.ebi.ac.uk/pride/].

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	All experiments involving tissue culture were performed using two or three independent culture preparations to ensure the reproducibility.
Data exclusions	No data was excluded.
Replication	Three independent biological replications were performed for the mass spectrometry analysis, with proper controls. At least three independent injections were performed in the cell cycle analysis of Hela and A549 cells in flow cytometry experiments, with non-transfected cells and empty vector transfected cells as controls. All attempts at replication were successful.
Randomization	Randomization is not relevant to this study since there is no grouped samples.
Blinding	Blinding is not relevant to this study since there is no grouped samples.

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

Antibodies Source Identifier
Anti-k29 polyubiquitin likage-specific antibody This paper N/A
Anti-k48 polyubiquitin likage-specific antibody abcam cat#ab140601
Anti-k48 polyubiquitin likage-specific antibody millipore sigma cat#05-1307/ clone Apu2
Anti-k63 polyubiquitin likage-specific antibody millipore sigma cat#05-1308/ clone Apu3
Anti-ubiquitin antibody abcam cat#ab19247
Anti-α-Tubulin antibody Genscript cat#A01410S
Anti GAPDH Santa Cruz cat#sc-32233
Anti-β-Actin antibody Santa Cruz Biotechnology cat#sc-47778
Anti-cMyc-Tag antibody Genscript cat#A00704S
Anti-cMyc-Tag antibody Thermo Fisher cat#A-21281
Anti-cMyc-Tag antibody Santa Cruz Biotechnology cat#sc-40
Anti-cMyc-conjugated PE antibody Santa Cruz Biotechnology cat#sc-40 PE
Anti-AuroraB/AIM-1 antibody Fisher scientific cat#BDB611082
Anti G3BP1 proteintech cat#13057-2-AP
Anti-eIF3B antibody Santa Cruz Biotechnology cat#sc-137214
Anti-INCENP antibody Santa Cruz Biotechnology cat#sc-376514
Anti-INCENP antibody Abcam cat#ab12183
Anti-MKLP1/Kif23 antibody Santa Cruz Biotechnology cat#sc-136473
Anti-MKLP1/Kif23 antibody abcam cat#ab9259
Anti-Proteasome 20S core subunits antibody Fisher NC0603631
Anti pTBK1 antibody cell signaling cat#5483s
Anti TBK1 antibody CST cat#38066

Anti PLK1 antibody Santa Cruz cat#sc-17783
Anti PLK1 antibody abcam cat#ab17056
Anti-VCP/p97 antibody Santa Cruz Biotechnology cat#sc-57492
Anti-HA-Tag (C29F4) Cell Signaling Rabbit mAb #3724
Peroxidase-conjugated AffiniPure F(ab`)2 Fragment Goat Anti Human IgG Jackson ImmunoResearch #134200
Peroxidase-conjugated AffiniPure Donkey Anti Mouse IgG Jackson ImmunoResearch #139035
Peroxidase-conjugated AffiniPure Goat Anti Rabbit IgG Jackson ImmunoResearch #139080
Alexa Flour 647-Conjugated AffiniPure F(ab`)2 Fragment Goat Anti Human IgG Jackson ImmunoResearch #144201
Alexa Flour 488-Conjugated AffiniPure F(ab`)2 Fragment Goat Anti Human IgG Jackson ImmunoResearch # 135112
Goat anti Chicken IgY Alexa Flour 594 invitrogen A11042
Goat anti Mouse IgG Alexa Flour 647ThermoFisher A-21235
Donkey anti mouse IgG Alexa Flour 488 ThermoFisher A-21202
Donkey anti mouse IgG Alexa Flour 594 ThermoFisher A-21203
Goat anti Rabbit IgG Alexa Flour 647 ThermoFisher A-21244
Goat anti Rabbit IgG Alexa Flour 488 invitrogen A-11008
Goat anti Rabbit IgG Alexa Flour 594ThermoFisher A-11012

Validation

k29 polyubiquitin likage-specific antibody was validated in this paper.
k48 polyubiquitin likage-specific antibody abcam was validated by WB: mouse and rat cell lines and induced tissues.
k48 polyubiquitin likage-specific antibody millipore was validated Inhibition Assay (PIA), Immunocytochemistry (ICC), Immunoprecipitation (IP), Immunohistochemistry (IHC) and Western Blotting (WB).
k63 polyubiquitin likage-specific antibody millipore was validated by WB: Inhibition Assay (PIA), Immunocytochemistry (ICC), Immunoprecipitation (IP), Immunohistochemistry (IHC) and Western Blotting (WB).
ubiquitin antibody abcam was validated by WB: HeLa cell lysate
α-Tubulin antibody Genscript was validated by WB: HeLa cell lysate, NHI/3T3 cell lysate, CHO cell lysate
GAPDH antibody Santa Cruz was validated by WB: HeLa cell lysate, A549 cell lysate, KNRK cell lysate
β-Actin antibody Santa Cruz was validated by WB: HeLa cell lysate, Sol8 cell lysate, C32 cell lysate and NIH/3T3 cell lysate
cMyc-Tag antibody Genscript was validated by WB: Hela cell lysates
cMyc-Tag antibody Thermo Fisher was validated by WB: HEK-293E cell lysates
cMyc-Tag antibody Santa Cruz was validated by WB: HeLa cell lysates, Jurkat cell lysates and K-562 cell lysates
cMyc-conjugated PE antibody Santa Cruz was validated by WB: HeLa cell lysates, Jurkat cell lysates and K-562 cell lysates
AuroraB/AIM-1 antibody Fisher was validated by WB: Jurkat cell lysates
G3BP1 proteintech was validated by WB: HEK-293 cell lysates
eIF3B antibody Santa Cruz was validated by WB: Jurkat cell lysate and HeLa cell lysate
INCENP antibody Santa Cruz was validated by WB: Jurkat cell lysate and TK-1 cell lysate
INCENP antibody Abcam was validated by WB: HeLa cell lysate, NHI/3T3 cell lysate, PC12 cell lysate
MKLP1/Kif23 antibody Santa Cruz was validated by WB: K-562 cell lysate, Jurkat cell lysate, HEL 92.1.7 cell lysate and TK-1 cell lysate
MKLP1/Kif23 antibody Abcam was validated by WB: HeLa cell lysate
Proteasome 20S core subunits antibody Fisher was validated by WB: HeLa cell lysate, RSV 3T3 cell lysate
pTBK1 antibody CST was validated by WB: THP-1 cell lysate
TBK1 antibody CST was validated by WB: HCT 116 cell lysate
PLK1 antibody Santa Cruz was validated by WB: HeLa cell lysate, K-562 cell lysate, HEK293T cell lysate and Jurkat cell lysate. U2OS cell lysate
PLK1 antibody Abcam was validated by WB: U2OS cell lysate
VCP/p97 antibody Santa Cruz was validated by WB: A549 cell lysate, SH-SY5Y cell lysate, 3T3-L1 cell lysate, KNRK cell lysate and L8 cell lysate
HA-Tag (C29F4) CST was validated by WB: HeLa cell lysate
The secondary antibody
Peroxidase-conjugated AffiniPure F(ab`)2 Fragment Goat Anti Human IgG Jackson; Peroxidase-conjugated AffiniPure Donkey Anti Mouse IgG Jackson; Peroxidase-conjugated AffiniPure Goat Anti Rabbit IgG Jackson; Alexa Flour 647-Conjugated AffiniPure F(ab`)2 Fragment Goat Anti Human IgG Jackson; Alexa Flour 488-Conjugated AffiniPure F(ab`)2 Fragment Goat Anti Human IgG Jackson; Goat anti Chicken IgY Alexa Flour 594 invitrogen; Goat anti Mouse IgG Alexa Flour 647 ThermoFisher; Donkey anti mouse IgG Alexa Flour 488 ThermoFisher; Donkey anti mouse IgG Alexa Flour 594 ThermoFisher; Goat anti Rabbit IgG Alexa Flour 647; ThermoFisher A-21244 Goat anti Rabbit IgG Alexa Flour 488 invitrogen; Goat anti Rabbit IgG Alexa Flour 594 ThermoFisher have been widely valudated by many references, such as Cell Rep. 2019, 27(11):3117-3123/Cell 2020, 183(4):1058-1069/ Nat Commun. 2021, 12(1):250./ Cell. 2018, 174(4):938-952/ J Cell Biol. 2017, 216(12):4313-4330./ J Cell Biol. 2018, 217(2):715-730./ Cell Rep. 2019, 29(5):1113-1129/ Cell Death Dis. 2016, 7(11):e2443/ Front Cell Neurosci. 2019, 13:115./ Nat Cell Biol. 2015, 17(4):500-10./ Nucleic Acids Res. 2013, 41(19):e182.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Hela cells (ATCC CCL-2) were kindly provided by Dr. Chuan He at University of Chicago and originally from ATCC.
A549 cells (ATCC CCL-185) were a gift from Dr. Eileen Dolan at University of Chicago and originally from ATCC.

Authentication

None of the cell lines used were authenticated.

Mycoplasma contamination

Mycoplasma were tested by PCR-based method and no mycoplasma contamination were detected in all cell lines used.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified lines were used in the study.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 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).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Cells were transfected with the Tra construct (human TRABID, 245-697) or the pCMV vector using lipofectamine 3000 for 27 h before harvesting and washing with PBS. The cells were fixed with either 1% or 4% PFA for 15 min at room temperature, followed by washing with PBS and permeabilization with 70% ethanol at 4 °C overnight. Nonspecific binding was blocked with 2% BSA. The cells were then incubated with Anti-c-myc antibody conjugated with PE (sc-40, Santa Cruz Biotechnology) and, either sAB-K29 as primary antibody (3 ug/mL), and Alexa Fluor 647 conjugated $F(ab')_2$ fragment goat anti-human IgG, F(ab')2 fragment specific (Jackson ImmunoResearch) as secondary antibody, or sAB-K29 directly labeled with Alexa Fluor 647 for 1h at room temperature, followed by washing with PBS. Finally, the cells were incubated with Hoechst dye at room temperature for 15 min, followed by washing with PBS.
Instrument	LSRFortessa X-20 cell analyzer (BD)
Software	FLOWJO 10.5+; FLOWJO 10.6.2
Cell population abundance	The cell population abundance of live HeLa and A549 cells was > 90% .
Gating strategy	VOTAGE GATE FSC/SSC/355nm/561nm/640nm 253/144/286/434/341 or FSC/SSC/355nm/561nm/640nm 253/144/264/341/341

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