ISCI, Volume *19*

Supplemental Information

A Cancer-Associated Missense Mutation in PP2A-Aα Increases Centrosome Clustering during Mitosis

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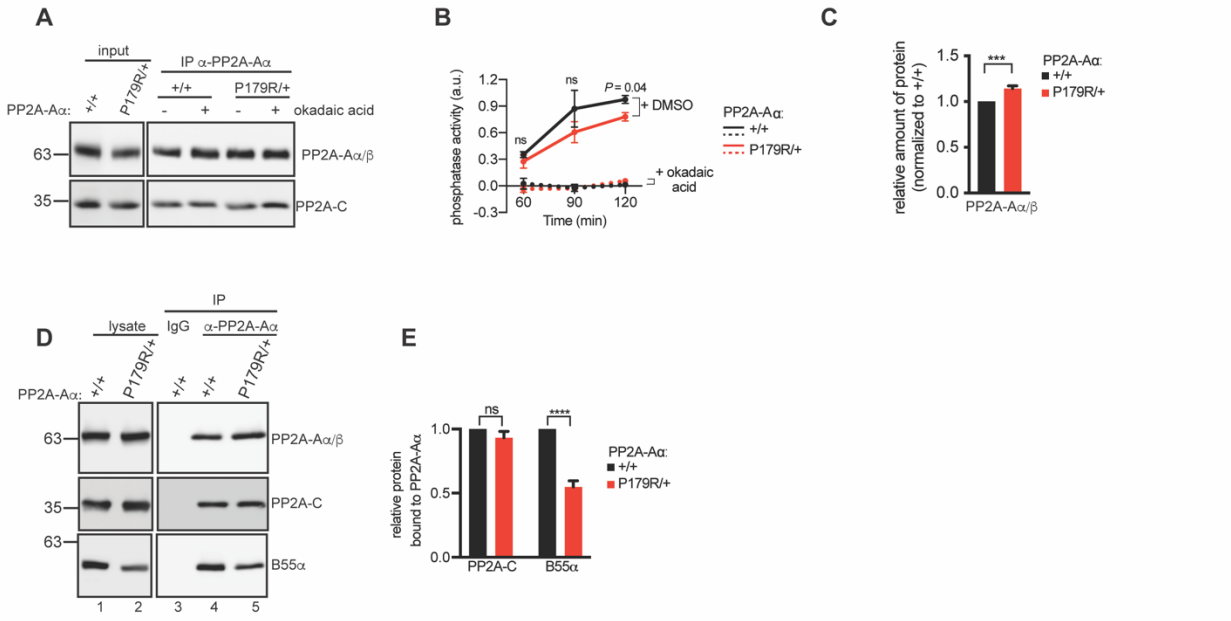
SUPPLEMENTAL INFORMATION TITLES AND LEGENDS

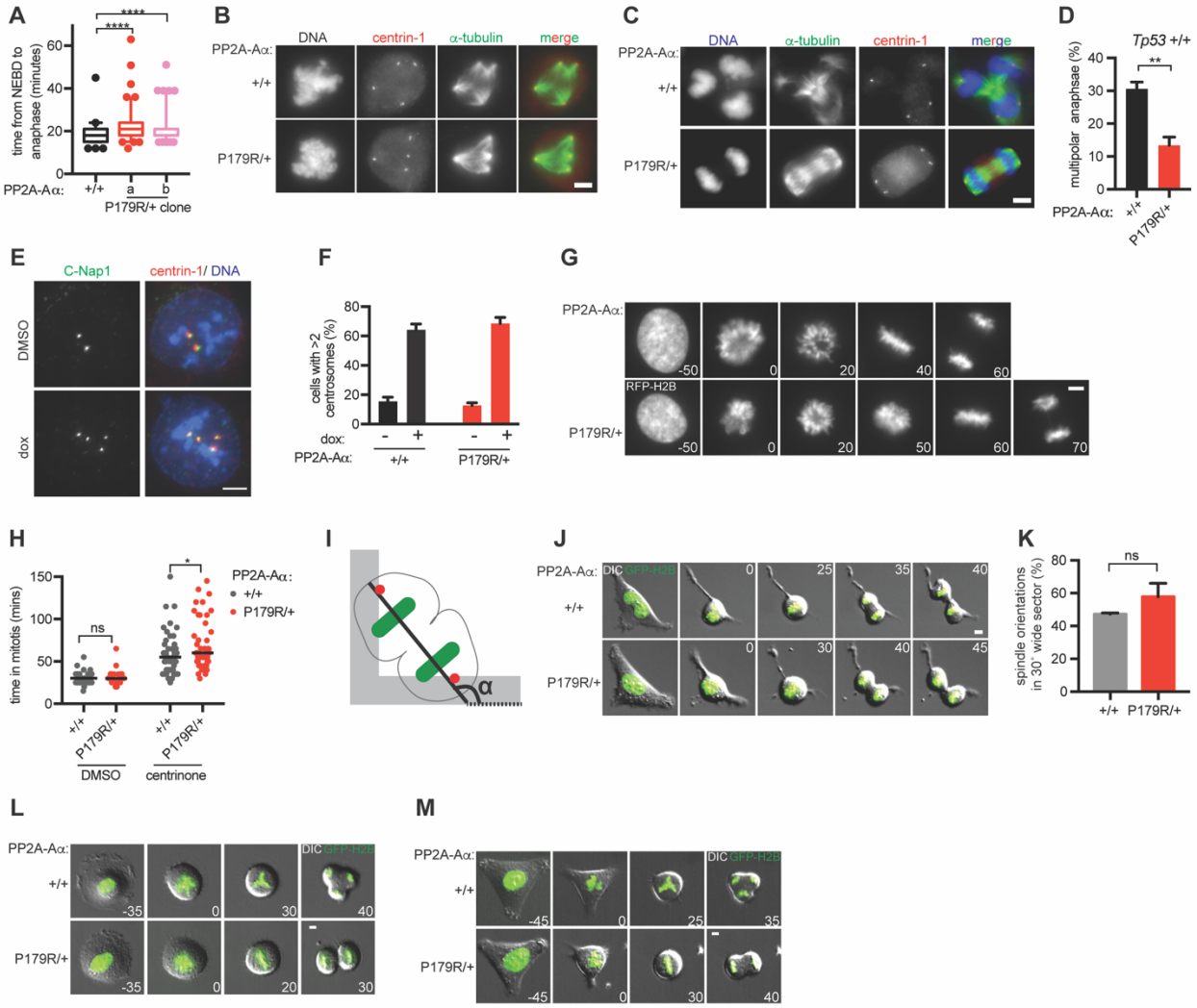
Figure S1. Analysis of PP2A activity and holoenzyme assembly in PP2A-Aα$^{P179R/+}$ cells, Related to Figure 2. (A-B) PP2A-Aα IPs in mitotic lysates from WT (+/+) or PP2A-Aα$^{P179R/+}$ (P179R/+). Okadaic acid was included in wash steps as indicated. The reaction was split and **(A)** associated proteins were analyzed by western blot or **(B)** incubated with a phosphopeptide substrate. Phosphate release was quantified using a molybdate dye-based spectrophotometric assay. Absorbance values were normalized to the maximum value per experiment. Mean ± s.d. from three experiments is plotted. **(C)** Plotted is the normalized mean ± s.e.m. of the amount of bait protein (PP2A-Aα) IP'd in the experiment in Figure 2D performed at least three times. **(D)** Western blot analysis of lysates (lanes 1-2) and IPs of control IgG (lane 3) or PP2A-Aα IgG (lane 4-5) in indicated cell lines **(E)** Plotted is the normalized mean ± s.e.m. of the experiment in (D) performed three times. a.u., arbitrary units. ****, $P < 0.00005$, ***, $P < 0.0005$, ns, not significant ($P > 0.05$) Student's *t*-test

Figure S2. Analysis of mitotic duration in PP2A-Aα$^{P179R/+}$ cells and centrosome amplification efficiency in Plk4-inducible cells, Related to Figure 3 and 4 (A) WT (+/+) and PP2A-Aα$^{P179R/+}$ (P179R/+) cells were imaged live and the time from nuclear envelope breakdown to anaphase onset was measured. Clones a and b are independently derived cell lines. A box-and-whisker plot is shown. Whiskers indicate the 5-95 percentile range and circles indicate cells outside of this range. Result is representative of two experiments. **(B)** Maximum intensity projections of WT (+/+) and PP2A-Aα$^{P179R/+}$ (P179R/+) cells treated with cytochalasin D and then briefly with nocodazole. Cells were analyzed for microtubule re-growth by immunofluorescence upon nocodazole removal. **(C-D)** Cells were treated with cytochalasin D, fixed and analyzed by immunofluorescence. **(C)** Representative maximum intensity projection. **(D)** Multipolar anaphase incidence in cells with four centrin-1 foci is plotted (mean ± s.e.m. of the experiment in (C) performed three times). **(E-F)** *Tp53*$^{-/-}$ WT (+/+) and PP2A-Aα$^{P179R/+}$ (P179R/+) cells with tet-inducible Plk4 expression were treated with dox or DMSO for 30 h, arrested in G2 with RO-3306 and dox or DMSO for 18 h, fixed and processed for

immunofluorescence. **(E)** Maximum intensity projections of C-Nap1 and a merge with centrin-1 and DNA. **(F)** Fraction of cells with >2 centrosomes (mean ± s.d. from two experiments with 200 cells scored per condition, per experiment). **(G-H)** Cells were imaged live after DMSO or centrinone treatment. **(G)** RFP-H2B montage of centrinone treated cells. Time (min) relative to nuclear envelope breakdown is indicated. **(H)** Mitotic duration is plotted. Line, median; circle, cell. Result is representative of three experiments **(I-K)** Cell division on L-shaped fibronectin micro-patterns in cells with two centrosomes. **(I)** Schematic of cell division on an L-shaped fibronectin pattern (grey). The angle (α) of chromosome segregation axis (solid line) relative to the reference X-axis (dashed line) is measured. Chromatin, green; Centrosome, red. **(J)** GFP-H2B and DIC merge time-lapse images. Time (min) relative to nuclear envelope breakdown is indicated. **(K)** Percentage of cells dividing within 30 degrees of the median angle is plotted (mean ± s.d. of two experiments). **(L-M)** GFP-H2B and DIC merge time-lapse images of Plk4-inducible cells treated with dox and imaged on (L) O-shaped and (M) Y-shaped fibronectin micro-patterns. Time (min) relative to nuclear envelope breakdown is indicated. Scale bars, 5 μm. ****, $P < 0.00005$, **, $P < 0.005$, *, $P < 0.05$, ns, not significant ($P > 0.05$) Student's *t*-test.

Antao et al. Figure S1, Related to Figure 2




A
time from NEBD to anaphase (minutes)
PP2A-Aα:
+/+
a
b
P179R/+ clone
B
DNA
centrin-1
α-tubulin
merge
PP2A-Aα:
+/+
P179R/+
C
DNA
α-tubulin
centrin-1
merge
PP2A-Aα:
+/+
P179R/+
D
Tp53 +/+
multipolar anaphsae (%)
PP2A-Aα:
+/+
P179R/+
E
C-Nap1
centrin-1/ DNA
DMSO
dox
F
cells with >2 centrosomes (%)
dox:
PP2A-Aα:
+/+
P179R/+
G
PP2A-Aα:
RFP-H2B
P179R/+
H
time in mitotis (mins)
PP2A-Aα:
+/+
P179R/+
ns
DMSO
centrinone
I
α
J
PP2A-Aα:
DIC GFP-H2B
+/+
P179R/+
K
spindle orientations in 30˚ wide sector (%)
ns
+/+
P179R/+
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PP2A-Aα:
DIC GFP-H2B
+/+
P179R/+
M
PP2A-Aα:
DIC GFP-H2B
+/+
P179R/+

TRANSPARENT METHODS

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Emily A Foley (emilyafoley@gmail.com).

Experimental model and subject details

RPE-1 hTERT (WT) cells (a gift from Alexey Khodjakov) and derivative cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2 in DMEM:F12 growth medium. Human embryonic kidney 293-T cells (ATCC) and Human embryonic kidney 293 cells (ATCC) were grown in DMEM growth medium. All media were supplemented with 10% fetal bovine serum (VWR Life Science), penicillin-streptomycin (Gemini Bio-Products), non-essential amino acids (Life Technologies), and L-glutamine (Sigma-Aldrich). Cell lines were authenticated upon initial receipt and upon initial generation by Short Tandem Repeat analysis at the Integrated Genomics Operations at Memorial Sloan Kettering Cancer Center. Cell lines were also tested for mycoplasma contamination by the Antibody and Bioresource core facility at Memorial Sloan Kettering Cancer Center.

Cell line generation

To generate the PP2A-A$\alpha^{P179R/+}$ cell lines, we used adeno-associated virus-mediated gene targeting. Q5 High-Fidelity DNA polymerase (New England Biolabs) was used to amplify two homology arms spanning exons 5 and 6 of *PPP2R1A* and cloned into a bacterial artificial chromosome (Life Technologies). The homology arms were cloned into vector pNX (Papi et al., 2005) flanking a *loxP*-Neo^R-*loxP* cassette. Site-directed mutagenesis was used to introduce a C to G mutation at nucleotide 23280 in exon 5 of *PPP2R1A*. The *PPP2R1A* homology fragment was then cloned into pAAV (Berdougo et al., 2009) by NotI restriction enzyme digestion. Isolation of AAV particles, selection of stable transductants, and PCR screening were carried out as previously described (Berdougo et al., 2009). We determined the *PPP2R1A* genotype in isolated clones by genomic PCR amplification of *PPP2R1A* followed by Sanger sequencing. We isolated two clones (termed a and b), each with a heterozygous *PPP2R1A* P179R mutation. For

each clone, the neomycin cassette was excised by infecting cells with adenovirus expressing Cre recombinase (Vector Development Laboratory, Baylor College of Medicine) at a multiplicity of infection of 80 plaque-forming units/cell. We identified single clones that were negative for the neomycin cassette but positive for the remnant loxP site by PCR amplification of endogenous *PPP2R1A*. Clone a was used for all experiments and, where indicated, clone b was also examined.

To generate *Tp53* knockout cell lines, codon-optimized Cas9 (Addgene 41815) and a guide RNA targeting *Tp53* (Wang et al., 2015) were transfected into cells using a Nucleofector 2b Device (Lonza) with the setting T023. Colonies were screened for p53 loss by western blot using an antibody for the N-terminus of p53 (Santa Cruz Biotechnology SC-126, clone DO-1). To generate cell lines expressing tetracycline-inducible Plk4, we used the lentiviral pLVX-Tight-Puro system (Clontech). Lentiviruses were generated in HEK 293T cells transfected with psPAX2 (Addgene plasmid 12260), pCMV-VSV-G (Addgene plasmid 8454) and Plk4 cloned into pLVX-Tight-puro (Clontech) using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's instructions. 48 h after transfection, the virus containing suspension from HEK293T cells was filtered with a 0.45 μM filter (EMD Millipore), supplemented with polybrene (4 μg/mL, Sigma) and applied to *Tp53*-knockout WT cells or PP2A-Aα$^{P179R/+}$ cells. Cells were first transduced with Plk4 in pLVX-tight-puro lentivirus and selected with puromycin (15 μg/mL, Thermo Fisher Scientific) for 1 week. Puromycin-resistant cells were then grown in Tetracycline-free serum (Clontech) and transduced with pLVX-Tet-On-Advanced lentivirus followed by G418 selection (500 μg/mL, Thermo Fisher Scientific) for 1 week. For each PP2A-Aα genotype, we chose a clone that yielded centrosome amplification in 60% of cells after 48 h doxycycline treatment (1 μg/mL).

We generated cells with fluorescent H2B by lentiviral transduction. H2B-RFP (Addgene plasmid 26001) or H2B-GFP (Addgene plasmid 25999) plasmids were co-transfected with psPAX2 and pCMV-VSV-G into HEK 293T cells. Supernatants were filtered, mixed with polybrene (4 μg/mL) and applied to target cells. We generated cells expressing GFP-PP2Aα by retroviral transduction as in (Foley et al., 2011) using WT PP2A-Aα (Foley et

al., 2011) or P179R and R183W mutants, which were cloned via site-directed mutagenesis.

Inhibitor treatments

Commercially available small molecules and chemical inhibitors were used as follows - cytochalasin D (0.2 μM), cytochalasin B (4 μM), blebbistatin (100 μM), SB203580 (10 μM), doxycycline (1 μg/mL), nocodazole (3.3 μM), centrinone (200 nM), and Okadaic acid (200 nM). To induce whole genome doubling, cells were plated in a glass bottom dish (Cellvis) or on coverslips (Fisher) and, 24 h later, treated with cytochalasin D, cytochalasin B, or blebbistatin for 20 h. To wash out the inhibitor, the media was replaced six times over 1 h. *Tp53*$^{+/+}$ cells were released into SB203580, and imaged 20 h later. This inhibitor was omitted in *Tp53*$^{-/-}$ cells. Cells were then fixed and processed for immunofluorescence microscopy or imaged live by DIC and fluorescence microscopy. Plk4 induction with doxycycline was performed for 48 h. Unless otherwise indicated, ≥50 cells were analyzed per condition, per experiment and at least three experiments were analyzed per treatment condition. Where indicated, mitotic cell enrichment was performed using a 14 h treatment with nocodazole followed by manual detachment from the tissue culture dish.

Live-cell imaging

Live imaging was performed on a Nikon Eclipse TiE inverted microscope equipped with a PI Piezo stage controller and Nikon Perfect Focus. Cells were maintained at 37 °C and 5% CO_2 with a Stage Top Incubator with Flow Control and a Sub Stage Environmental Enclosure (Tokai Hit). Cells were imaged by DIC and fluorescence microscopy (GFP-Ex470 nm; Em522 nm or DsRed-Ex508 nm; Em620 nm). Images were acquired with a 20X or 40X objective and recorded on a deep-cooled, ultra-low noise sCMOS camera (Andor) at 16-bit depth using Elements software (Nikon). Image cropping was performed in Fiji (Schindelin et al., 2012).

Immunofluorescence microscopy

For immunofluorescence microscopy of α-tubulin, C-Nap 1, and centrin-1, cells on coverslips were fixed in methanol at −20 °C for 10 min. For PP2A-Aα and B56α

immunofluorescence, cells on coverslips were pre-extracted for 40 s at 37 °C in PEM buffer (100 mM K-PIPES pH 6.9, 10 mM EGTA, 1 mM $MgCl_2$) with 0.5% Triton X-100 and 4 M glycerol. Coverslips were then fixed in PEM buffer with 3.7% formaldehyde and 0.2% Triton X-100 for 5 min at 37 °C. Coverslips were washed in TBS + 0.1% Triton X-100, blocked in same buffer with 2% donkey serum (Jackson Immunoresearch Laboratories) for 30 min, and stained with primary antibody for 3 h at room temperature. Species-specific secondary antibodies conjugated to Alexa Fluor 488, Rhodamine, Alexa Fluor 647 (Jackson Immunoresearch Laboratories), or IgG2a Alexa Fluor 594 (Thermo Fisher Scientific) were applied for 30 min at 0.6 µg/mL. Coverslips were mounted onto slides with Prolong Gold with DAPI (Thermo Fisher Scientific) and sealed. Imaging was performed on a DeltaVision Elite microscope running softWoRx software (GE Life Sciences). Images were acquired at 60X or 100X magnification with a PCO Edge CMOS Camera. Z-stacks were acquired with 0.2 µm spacing. Maximum intensity projection images were created in softWoRx. Pseudo coloring and cropping was performed in Fiji (Schindelin et al., 2012) and images were assembled in Illustrator (Adobe, San Jose, CA). Kinetochore/centromere intensity was calculated in Fiji using published analysis macros (Nijenhuis et al., 2014). 20 cells were analyzed per condition per experiment.

Antibodies

Primary antibodies were used at 1 µg/mL for immunofluorescence incubations and 0.1 µg/mL for western blotting incubations. 2-4 µg of IgG was used for immunoprecipitation. Custom PP2A-Aα antibody was generated by immunizing rabbits with the peptide sequence of MAAADGDDSLY conjugated to matriculture Keyhole Limpet Hemocyanin (Thermo Scientific). Antigens were injected into New Zealand White rabbits using an institutionally approved protocol and animal-care facility (Pocono Rabbit Farm and Laboratory, Canadensis, PA). To purify peptide antibodies, antisera were diluted with 0.1 vol 10X PBS and incubated overnight with immunizing peptide covalently coupled to sulfo-link resin (Thermo Fisher Scientific). After washing with PBS, antibodies were eluted with 0.2 M glycine, pH 2.5 and subsequently neutralized with Tris pH 8.0, followed by dialysis into PBS. Custom GFP antibodies were raised against bacterially expressed GST-GFP. The serum was loaded onto a HiTrap NHS-activated HP column (GE Life

Sciences) coupled to GST-GFP. Commercial and published antibodies used in this study include: B56α (immunofluorescence: BD Biosciences, 610615; western (Lee et al., 2017)), B56γ (Bethyl Laboratories, A303-814A), B56δ (Bethyl Laboratories, A301-100A), B56ε (Lee et al., 2017), B55α (Santa Cruz Biotechnology, SC-81606), PP2A-Aα/β (Santa Cruz Biotechnology, SC-6112), PP2A-C (BD Biosciences, 610555), centrin-1 (EMD Millipore, 04-1624), CREST Serum (Immunovision, HCT-0100; used at 1:5,000 dilution), α-tubulin (mouse DM1α; Abcam, ab7291), α-tubulin FITC conjugated (Sigma F2168), β-actin (Santa Cruz Biotechnology, SC-47778), p53 (Santa Cruz Biotechnology SC-126, clone DO-1), and C-Nap 1(a gift from Bryan Tsou).

Mutation analysis in *PPP2R1A*

PPP2R1A mutations were quantified from publicly available studies on cBioPortal (Cerami et al., 2012; Gao et al., 2013) as of June 2019.

Cell lysis, immunoprecipitation and phosphatase assays

Frozen cell pellets were suspended in buffer B (30 mM HEPES pH 7.8, 140 mM NaCl, 6 mM $MgCl_2$, 5% glycerol) supplemented with 2 mM DTT, ProBlock Gold Mammalian Protease Inhibitor Cocktail (GoldBio Technology) and PhosSTOP (Roche) and maintained at 4 °C. Samples were lysed by nitrogen cavitation (Parr Instruments) for 5 min at 2,000 psi, and then centrifuged at 20,000 x *g* for 15 min. Proteins were immunoprecipitated with antibody bound to Protein A Dynabeads (ThermoFisher Scientific) for 1 h, washed three times with buffer B, and analyzed by western blot via chemiluminescence using an ImageQuant LAS500 (GE Life Sciences). Image cropping and intensity measurements were performed in Fiji (Schindelin et al., 2012) and images were assembled in Illustrator (Adobe, San Jose, CA). For phosphatase assays, cell pellets were suspended in buffer B with protease inhibitor and DTT. Lysates were mixed with protein G sepharose beads (GE Healthcare) bound to PP2A-Aα IgG or control IgG. After washing in buffer B, samples were split and incubated with 200 nM Okadaic acid or DMSO. A portion was reserved for western blot analysis. The remainder was used in the Ser/Thr phosphatase assay (Promega). Final assay conditions were: 80 μM phosphopeptide, 50 mM imidazole pH 7.2, 0.2mM EGTA, 0.02% β-mercaptoethanol, 0.1

mg/mL BSA ± 144 nM okadaic acid. Phosphatase activity was quantified by absorbance measured at 630 nm on a spectrophotometer.

PP2A-Aα immunoprecipitation for SILAC mass spectrometry analysis

Wild type RPE-1 cells expressing GFP-PP2A-Aα (WT, P179R, or R183W) were grown in DMEM:F-12 (1:1) media for SILAC (ThermoFisher Scientific), supplemented with 10% dialyzed FBS (ThermoFisher Scientific), 1X penicillin-streptomycin (Gemini Bio-Products), 2.5 mM L-Glutamine (Sigma-Aldrich), 0.175 mM Arginine and 0.25 mM Lysine. Arg^0 and Lys^0 amino acids were purchased from Sigma. Arg^{10} ($^{13}C_6/^{15}N_4$ arginine) and Lys^8 ($^{13}C_6/^{15}N_2$ lysine) amino acids were purchased from Cambridge Isotopes. After two weeks of passaging, incorporation of heavy amino acids was measured by mass spectrometry. Samples with 95% or greater incorporation of Lys^8 and Arg^{10} were used in analyses. For immunoprecipitation, asynchronously growing cells were trypsinized, pelleted, and frozen. Cells were thawed and re-suspended in lysis buffer (180 mM NaCl, 50 mM sodium phosphate pH 7.4, 2 mM $MgCl_2$, 0.1% Tween-20, 1X ProBlock Gold Mammalian Protease Inhibitor Cocktail (GoldBio Technology) and 1X Simple Stop 3 phosphatase inhibitor (GoldBio Technology)). All further steps were carried out at 4 °C. Cells were lysed by nitrogen cavitation (Parr Instruments) at 2,000 psi for 10 min, followed by centrifugation at 20,000 x *g* for 20 min. The supernatant was centrifuged once more. 5 mg protein was incubated with 125 µg α-GFP antibody covalently coupled to M270 magnetic resin (ThermoFisher Scientific) for 1 h with rotation. The resin was washed five times with lysis buffer. Bound proteins were eluted into 0.5 N NH_4OH, 0.5 mM EDTA and dehydrated in a vacufuge (Eppendorf).

Dehydrated samples were suspended in 20 µL of NuPAGE LDS sample buffer (ThermoFisher Scientific). SILAC pairs were mixed, denatured at 90 °C for 2 min and separated on a 10% NuPAGE gel (ThermoFisher Scientific) at 120 V for 20 min. The gel was washed and silver stained (Pierce Silver Stain kit). Each lane was excised and destained using 30 mM potassium hexa-cyanoferrate (III) /100 mM sodium thiosulfate, washed and de-hydrated using a vacuum centrifuge. Proteins in the gel slabs were reduced, alkylated with iodoacetamide, dehydrated in a vacuum centrifuge. Gel slabs

were then re-hydrated using 50 mM ammonium bicarbonate pH 8.4 (containing 0.04 µg sequencing grade modified porcine trypsin (Promega)) and incubated for 16 h at 37 ◦C. After proteolysis inhibition and tryptic peptide elution from the polyacrylamide slabs, eluates from each lane were pooled, lyophilized, and stored at −80 ◦C until analysis. The lyophilized peptide pellets were resuspended in 0.1% formic acid/ 3% acetonitrile and 5% of the solution was analyzed by LC/MS. The LC system consisted of a vented trap-elute setup (EasynLC1000, Thermo Fisher scientific) coupled to the Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) via a nano electro-spray DPV-565 PicoView ion source (New Objective). The trap column was fabricated with a 5 cm × 150 µm internal diameter silica capillary with a 2 mm silicate frit, and pressure loaded with Poros R2-C18 10 µm particles (Life Technologies). The analytical column consisted of a 25 cm × 75 µm internal diameter column with an integrated electrospray emitter (New Objective), packed with ReproSil-Pur C18-AQ 1.9 µm particles (Dr. Maisch). Peptides were resolved over 90 min using a 3%–45% gradient acetonitrile/ 0.1% formic acid (buffer B) gradient in a water/0.1% formic acid (buffer A) at 250 nL/minute. Precursor ion scans were recorded from 400–2000 m/z in the Orbitrap (240,000 resolution at m/z 200) with an automatic gain control target set at 10^5 ions and a maximum injection time of 50 ms. We used data-dependent mass spectral acquisition with monoisotopic precursor selection, ion charge selection (2–7), dynamic precursor exclusion (60 s, 20 ppm tolerance) and HCD fragmentation (normalized collision energy 35, isolation window 0.8 Th) using the top speed algorithm with a duty cycle of 2 s. Product ion spectra were recorded in the linear ion trap (“normal” scan rate, automatic gain control = 5000 ions, maximum injection time = 150 ms). Spectra were analyzed using MaxQuant Version1.5.2.8, searching within the human UniProt database (version 01/27-2016) with FDR <0.01. Intensity measurements were normalized to the PP2A-Aα intensity and then SILAC ratios were calculated. Four biological replicates were analyzed. P-values were calculated from a two-tailed student’s *t*-test for proteins identified in three or more experiments. We focused on proteins with $\geq$ 2-fold change in binding and $P < 0.05$. Raw files are openly accessible via PRIDE accession number PXD010709.

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