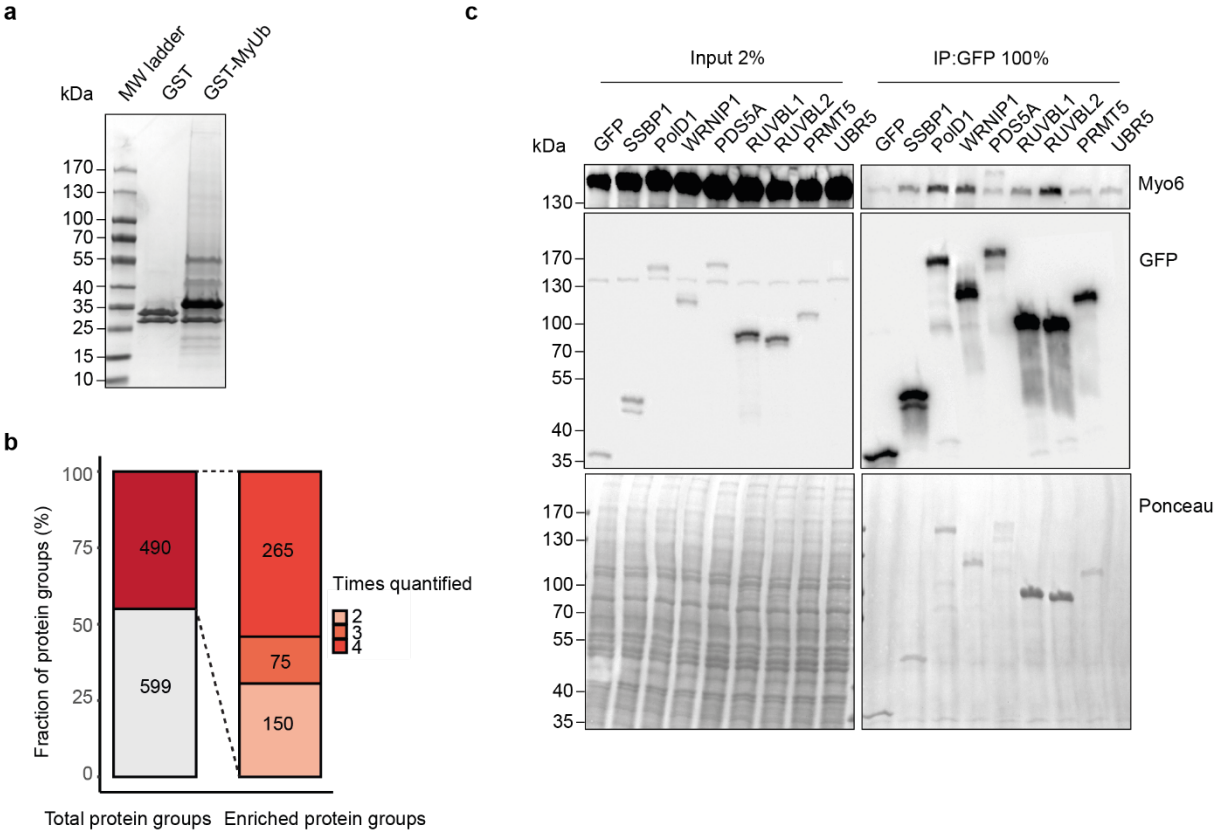
SUPPLEMENTARY INFORMATION

Nuclear myosin VI maintains replication fork stability

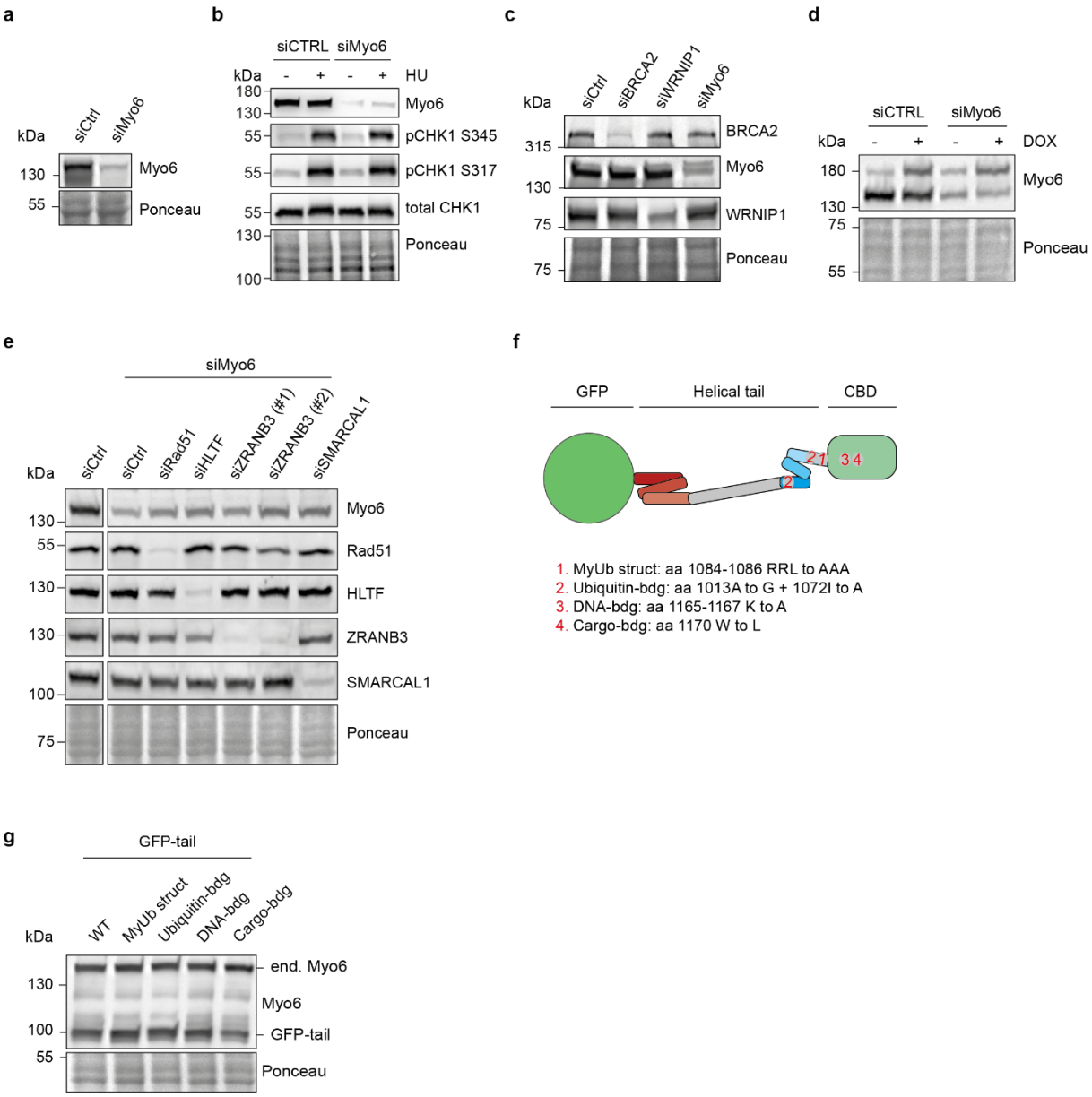


Supplementary Fig. 1 Identification of novel Myosin VI interactors

a, GST-pulldown assay using GST or GST-MyUb as bait and SILAC-labeled cellular lysates as prey. Samples were separated via SDS-PAGE and proteins were stained using a Colloidal Blue staining kit.

b, Left: Bar plot showing the number of significantly enriched protein groups in the pulldown using GST-MyUb followed by LC-MS/MS (fold change > 2, FDR < 0.05). Right: Number of replicates in which the enriched proteins were quantified.

c, Validation of novel interactors. HEK293T cells were transfected with GFP fusion constructs of the indicated proteins for 24 h. Immunoprecipitations (IPs) against GFP using GFP-trap beads (Chromotek) were performed, followed by western blotting with antibodies against myosin VI and GFP. Ponceau S staining was performed to control for equal loading. For panels **a** and **c**: Results were confirmed by at least two independent experiments. Uncropped blots are shown at the end of this file.



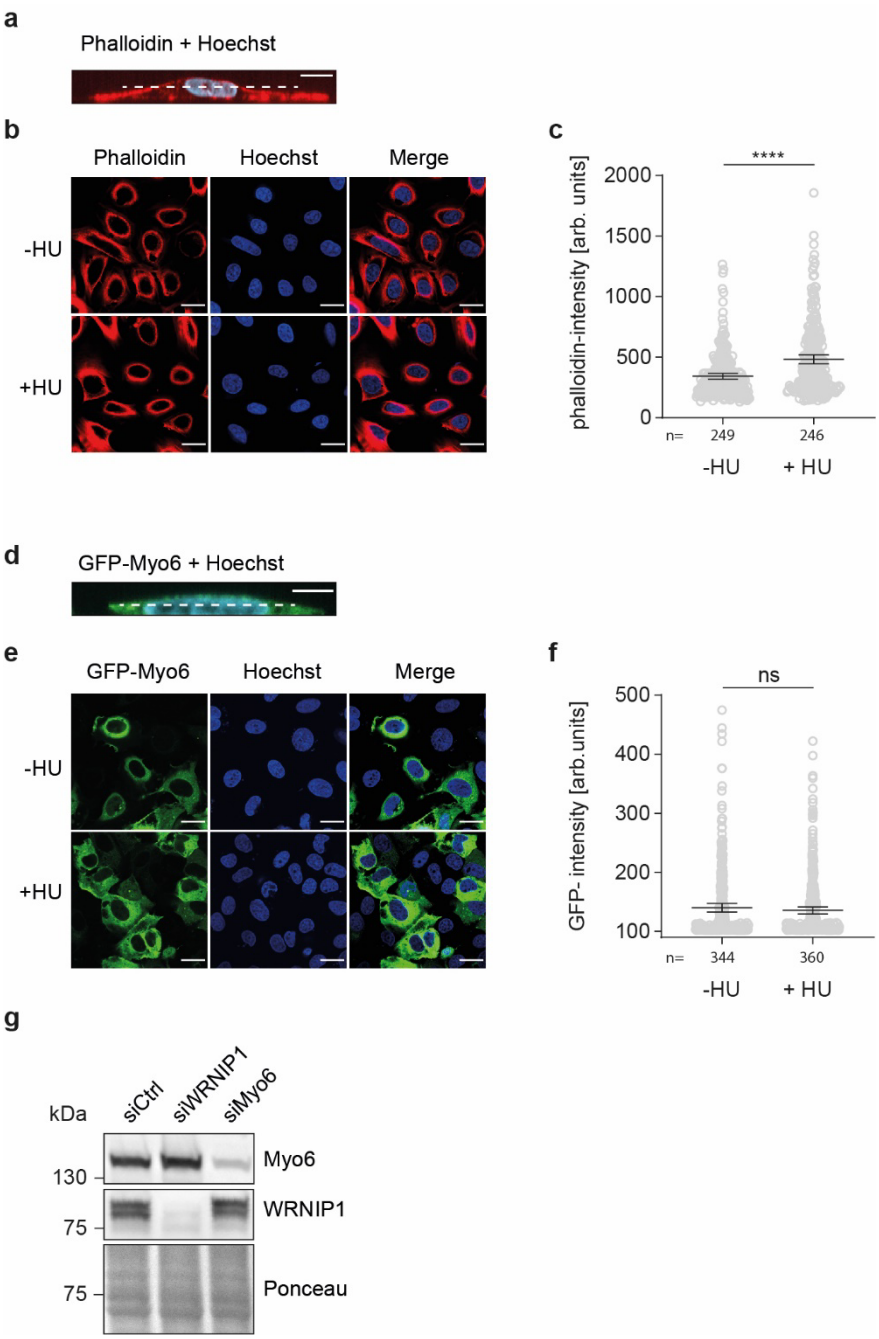
Supplementary Fig. 2 Knockdown efficiencies in fiber assay experiments shown in Fig. 2

a,**c**,**d**,**e**,**g**, Western blots to monitor knockdown efficiencies and expression levels for the fiber assays shown in **Fig. 2**. U2OS cells were transfected with the indicated siRNAs for 72 h or with the indicated overexpression constructs for 48 h, followed by western blotting using the indicated antibodies. Ponceau S staining was performed to control for equal loading. Panel **a** relates to Fig. **2a**, panel **c** relates to Fig. **2b**, panel **d** relates to Fig. **2c**, panel **e** relates to Fig. **2d**, panel **g** relates to Fig. **2e**.

b, Depletion of myosin VI does not induce ATR activation. U2OS cells were transfected with the indicated siRNAs for 72 h and treated with 4 mM HU for 1 h as indicated, followed by western blotting using the indicated antibodies. Ponceau S staining was performed to control for equal loading.

f, Schematic representation of the respective GFP-tail mutants according to Fig. **1a** used in panel **g**.

For panels **a-e** and **g:** Results were confirmed by at least three independent experiments. Uncropped blots are shown at the end of this file.



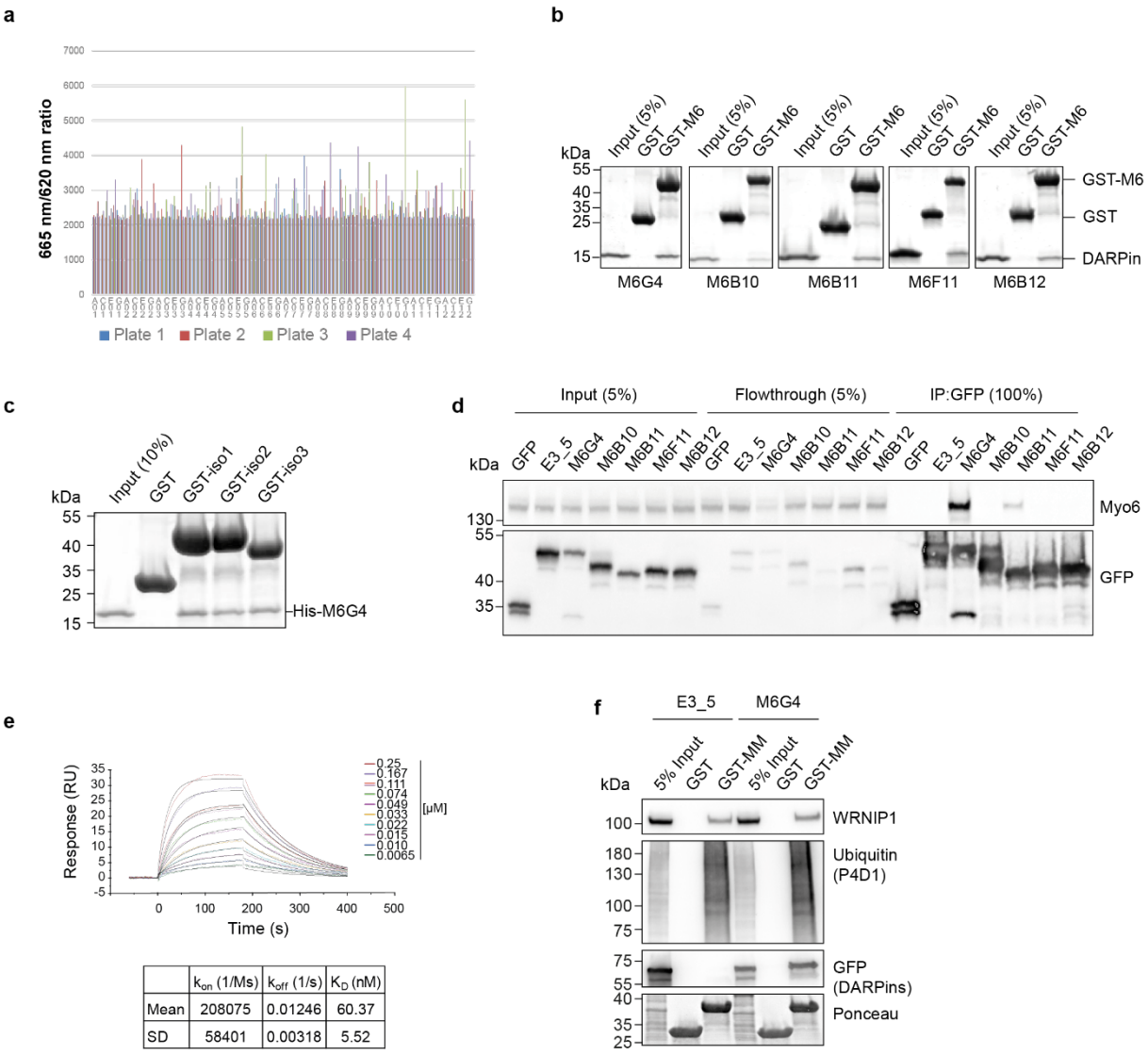
Supplementary Fig. 3 Nuclear actin and myosin VI levels upon HU treatment

a-c, Confocal microscopy shows an increase in nuclear F-actin upon HU-induced replication stress. U2OS cells were treated for 5 h with 4 mM HU where indicated, followed by staining with Hoechst and Alexa Fluor 647 Phalloidin. A central plane (dashed line) was chosen for quantification of nuclear F-actin **(a)**, scale bar 50 µm. Example images are shown in panel **b**, scale bar 25 µm. Relative nuclear intensities from the phalloidin channel were quantified from the indicated number of cells using Image J and plotted with mean values -/+ 95% confidence intervals. Significance levels in panel **c** were calculated using the two-tailed Mann-Whitney test (****: $p<0.0001$).

d-f, Confocal microscopy shows no difference in nuclear myosin VI levels upon HU-induced replication stress. Note that the detection of endogenous nuclear myosin VI via confocal microscopy was not possible with the available antibodies. Instead, we visualized ectopically expressed myosin VI. U2OS cells were transfected with GFP-Myo6 for 24 h and treated for 5 h with 4 mM HU where indicated, followed by staining with Hoechst. A central plane (dashed line) was chosen for quantification of nuclear GFP-Myo6 **(d)**, scale bar 50 µm. Example

images are shown in panel **e**, scale bar 25 µm. Relative nuclear intensities from the GFP channel (488 nm) were quantified from the indicated number of cells using Image J and plotted with mean values -/+ 95% confidence intervals. Significance levels in panel **f** were calculated using the two-tailed Mann-Whitney test (ns: non-significant).

g, Western blots to monitor knockdown efficiencies for the fiber assay shown in Fig. **3h**. U2OS cells were transfected with the indicated siRNAs for 72 h, followed by western blotting using the indicated antibodies. Ponceau S staining was performed to control for equal loading. Uncropped blots are shown at the end of this file. For panels **a**-**g**: Results were confirmed by at least three independent experiments.



	k_{on} (1/Ms)	k_{off} (1/s)	K_D (nM)
Mean	208075	0.01246	60.37
SD	58401	0.00318	5.52

Supplementary Fig. 4 Selection and characterization of the myosin VI-specific DARPin M6G4

a, HTRF screening of bacterial crude extracts of expressed DARPin clones. 380 single DARPin clones encoded in the expression plasmid pQIq were picked from agar selection plates and expression was induced in four 96-well deep well plates (plate 1 to 4). After detergent lysis using B-PER (Pierce), HTRF was performed to detect binding of the FLAG-tagged DARPins to the biotinylated target myosin VI isoform 1. The ratio of readings at 665 nm divided by reading at 620 nm multiplied by 10,000 reflects the degree of specific binding. x-axis: position of the well of the 96 well expression plate.

b, DARPin screening via GST-pulldown assay. GST-pulldown assay using GST or GST-MyUb (labeled GST-M6) as bait and purified DARPin candidates as prey. Proteins were separated via SDS-PAGE and stained using Instant Blue.

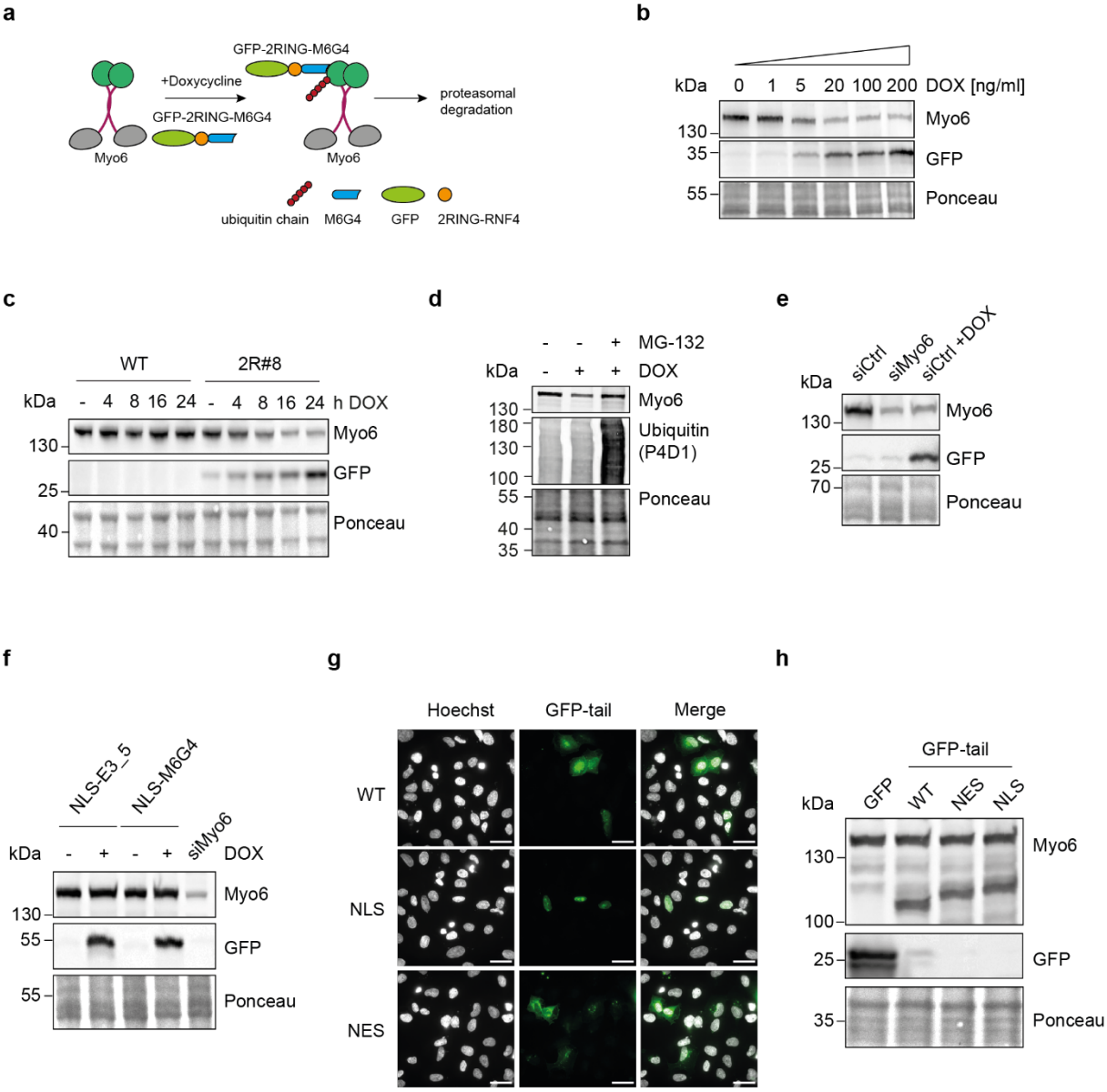
c, M6G4 is a pan-isoform-specific binder. GST-pulldown assay was performed using GST or the indicated GST-MIUMyUb isoforms as bait and purified His-M6G4 as prey. Proteins were separated via SDS-PAGE and stained using Instant Blue.

d, DARPin M6G4 depletes myosin VI from cellular lysates. HeLa cells were transfected with the indicated GFP-tagged DARPins for 24 h. Immunoprecipitations (IPs) against GFP using GFP-trap beads (Chromotek) were performed, followed by western blotting with antibodies against myosin VI and GFP.

e, M6G4 binds the MIUMyUb domains with a KD of 60 nM. Surface Plasmon Resonance measurements were performed on a Biacore X100 instrument. Mean values and standard deviation (SD) from four experiments are listed below a graph showing one representative assay.

f, M6G4 does not compete with WRNIP1- and ubiquitin-binding to myosin VI. HEK293 cells were transfected with GFP-tagged DARPin constructs as indicated for 24 h. Cells were lysed and GST-pulldown assays were performed

using GST or GST-MIUMyUb as bait. Proteins were separated via SDS-PAGE and analyzed by western blotting using the indicated antibodies. Ponceau S staining was performed to control for equal loading.
For panels **b-f:** Results were confirmed by at least two independent experiments. Uncropped blots are shown at the end of this file.



Supplementary Fig. 5 Establishment of DARPin-based tools and dominant-negative constructs to study contributions of cytoplasmic versus nuclear pools of myosin VI

a, Schematic representation of the DARPin-based myosin VI-degradation system adapted from Ibrahim et al.[1].

b,**c**, A U2OS Flp-In T-REx single cell clone harboring DOX-inducible GFP-M6G4-2RING fusion construct (2R#8) was treated with increasing concentrations of DOX for 24 h **(b)** or with 20 ng/ml DOX for the indicated times **(c)**, followed by western blotting using antibodies against myosin VI and GFP. Ponceau S staining was performed to control for equal loading.

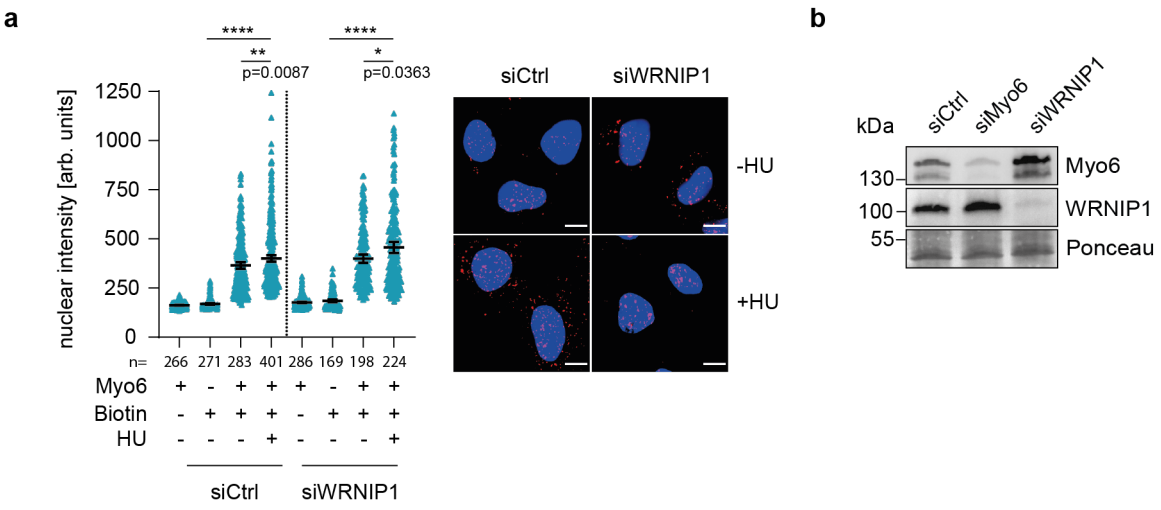
d, The M6G5-2RING fusion targets endogenous myosin VI for proteasomal degradation. A U2OS Flp-In T-REx single-cell clone harboring a DOX-inducible GFP-M6G4-2RING fusion construct (2R#8) was treated with 2 µg/ml DOX for 24 h and 5 µM proteasome inhibitor MG-132 as indicated. Cellular lysates were analyzed via western blotting using antibodies against myosin VI and ubiquitin. Ponceau S staining was used to compare loading between samples.

e,**f**,**h**, Western blots to monitor knockdown efficiencies and expression levels for the fiber assays shown in Fig. **4**. Panel **e** relates to **Fig. 4c**: U2OS Flp-In T-REx cells harboring DOX-inducible GFP-M6G4-2RING fusion construct (2R#8) were transfected with siRNAs for 72 h and treated with DOX for 24 h as indicated, followed by western blotting using antibodies against myosin VI and GFP. Panel **f** relates to **Fig. 4e**: U2OS Flp-In T-REx cells harboring DOX-inducible GFP-M6G4-NLS or GFP-E3_5-NLS (control) fusion constructs were transfected with siRNAs against

myosin VI and treated with DOX as indicated, followed by western blotting using antibodies against myosin VI and GFP. Panel **h** relates to **Fig. 4f**: U2OS cells were transfected with GFP-tail constructs for 24 h as indicated, followed by western blotting using antibodies against myosin VI and GFP. Ponceau S staining was performed to control for equal loading.

g, Compartment-specific localization of tagged myosin VI-tails. U2OS cells were transfected with GFP or GFP-tail constructs as indicated. Nuclei were stained with Hoechst (white signal) (scale bar = 40 µm).

For panels **b-h:** Results were confirmed by at least two independent experiments. Uncropped blots are shown at the end of this file.



Supplementary Fig. 6 WRNIP1 depletion has no effect on the association of myosin VI with replication forks

a, U2OS cells were transfected with siRNAs for 72 h as indicated, followed by SIRF assay. Left: dot plots of PLA signal intensities with mean values -/+ 95 % confidence interval. Significance levels were calculated using the two-tailed Mann-Whitney test from the indicated number of nuclei per sample (ns, non-significant, ****: p<0.0001, ***: p<0.001, *: p<0.05). Right: representative images with Hoechst staining in blue and PLA signals in magenta, scale bar = 10 µm.

b, Western blot analysis to monitor knockdown efficiencies for SIRF assays shown in Fig. **5c** and panel **a**. Uncropped blots are shown at the end of this file.

For panels **a**-**b**: Results were confirmed by at least two independent experiments.

Nucleotide sequence of the lead DARPin M6G4

GACCTGGGTAAGAAACTGCTGGAAGCTGCTTGGTACGGTCAGGACGACGAAGTTCGTATCCTGATGGCAAACGGTGCTGACGTTAACG
CTACTGACGACTACGGTGCTACTCCGCTGCACCTGGCTGCTCATCAGGGTCACCTGGAAATCGTTGAAGTTCTGCTGAAGGCCGGCGCTG
ACGTTAACGCTGACGACCAGATGGGTTTCACTCCGCTGCACCTGGCTGCTTACCATGGTCACCTGGAAATCGTTGAAGTTCTGCTGAAAA
CCGGTGCTGACGTTAACGCTGAAGACATCGTTGGTTTCACTCCGCTGCACCTGGCTGCTTGGTTCGGTCACCTGGAAATCGTTGAAGTTC
TGCTGAAGCACGGCGCCGACGTTAACGCTCAGGACATCGTTGGTAAAACTCCGTTCGACCTGGCTATCGACAACGGTAACGAGGACATC
GCTGAAGTTCTGCAGAAATCTGCTAAG

Amino acid sequence of the lead DARPin M6G4

N-cap	1st repeat
DLGKKLLEAAWYGQDDEVRILMANGADVNA	TDDYGATPLHLAAHQGHLEIVEVLLKAGADVNA

2nd repeat	3rd repeat
DDQMGFTPLHLAAYHGHLEIVEVLLKTGADVN	AEDIVGFTPLHLAAWFGHLEIVEVLLKHGADVNA

C-cap
QDIVGKTPFDLAIDNGNEDIAEVLQKSAK

Supplementary Fig. 7 Nucleotide and amino acid sequence of the myosin VI-specific DARPin M6G4

Supplementary Tables

Supplementary Table 1. Plasmids used in this study

Plasmids	Source	ID, Cat#
pGEX6P1	Merck	Cytiva 28-9546-48
pGEX6P1-MyUb	He et al.[2]	2893
pBirAcm	Avidity	AVB99
pAC4	Avidity	pAC4
pET30-MIUMyUb-iso1-AVI-His	This study	3392
pET30-MIUMyUb-iso2-AVI-His	This study	3393
pET30-MIUMyUb-iso3-AVI-His	This study	3394
pENTR4-myo6-iso2	This study	4382
pDEST-TO-YFP-FRT-myo6	This study	4478
pEGFP-C1	Clontech	2535
pEGFP-C1 E3_5 (DARPin)	This study, derived from Binz et al.[3]	4008
pEGFP-C1 M6B10 (DARPin)	This study	4007
pEGFP-C1 M6G4 (DARPin)	This study	4009
pEGFP-C1 M6B11 (DARPin)	This study	4057
pEGFP-C1 M6F11 (DARPin)	This study	4058
pEGFP-C1 M6B12 (DARPin)	This study	4059
pCDNA5 FRT TO GNb-2xRING	Ibrahim et al.[1]	4758
pCDNA5-FRT-TO-EGFP-M6G4-2xRING	This study	4912
pCDNA5-FRT-TO-EGFP-E3_5-2xRING	This study	5056
pENTR4-3xNLS-M6G4	This study	5053
pDEST-FRT-TO-YFP-3xNLS-M6G4	This study	5055
pENTR4-3xNLS-E3_5	This study	5052
pDEST-FRT-TO-YFP-3xNLS-E3_5	This study	5054
pOG44 –Flp-Recombinase	Thermo Fisher Scientific	V600520
pEGFP-C1-myo6 (fl)	Wollscheid et al.[4]	2905
pEGFP-C1-myo6-tail (aa732-1262)	Wollscheid et al.[4]	2908
pEGFP-C1-myo6-tail RRL→AAA	This study	5356
pEGFP-C1-myo6-tail ubiquitin binding mutant (A1013G+I1072A*)	This study	5358
pEGFP-C1-myo6-tail DNA binding mutant (K1165-1167A)	This study	5498
pEGFP-C1-myo6-tail lipid binding mutant (K1093A, K1095A)	This study	5384
pEGFP-C1-myo6-tail cargo binding mutant (WLY)	This study	5383
p3xFlag-CMV-YFP-3xNLS-myo6-tail	This study	5463
p3xFlag-CMV-YFP-NES-myo6-tail	This study	5453
pENTR221-SSBP1	Orfeome OCAA	3183
pENTR221-PolD1	Orfeome OCAA	2925
pENTR221-PDS5A	Orfeome OCAA	3185
pENTR221-RUVBL1	Orfeome OCAA	3187
pENTR221-RUVBL2	Orfeome OCAA	3188
pENTR221-WRNIP1	Orfeome OCAA	3184
pENTR221-PRMT5	Orfeome OCAA	3193

pDEST-EGFP-SSBP1	This study	42340
pDEST-EGFP-PolD1	This study	39024
pDEST-EGFP-PDS5A	This study	49948
pDEST-EGFP-RUVBL1	This study	38500
pDEST-EGFP-RUVBL2	This study	34352
pDEST-EGFP-WRNIP1	This study	5313
pDEST-EGFP-PRMT5	This study	37484
pcDNA6.2 N-EmGFP-UBR5-V5	Addgene (#52050)	2689
pET15b-TEVs-F-USP2cat-A	This study	3239
pET3a-Ubi(K63R)	Parker et al.[5]	684
pET21(+)-His6-Ubc13	Renz et al.[6]	3010
pET30-His6-TEV-Mms2	Renz et al.[6]	2807
pET30-His6-TEV-Pib1RING+100aa	Renz et al.[6]	3019
pET28-mE1 (ubiquitin activating enzyme E1)	Carvalho et al.[7]	2448

*: I1072 in this construct (isoform 2) corresponds to I1104 in the long isoform (3). Mutation to alanine abrogates ubiquitin binding of the MyUb domain.

Supplementary Table 2. Antibodies used in this study

Antibody	Source	Identifier
Mouse monoclonal anti-GFP (clone 7.1/13.1)	Roche	Cat#11814460001; RRID: AB_390913
Mouse monoclonal anti-Myosin VI (clone MUD19)	Merck	Cat#M0691 RRID:AB_369989
Rabbit polyclonal anti-Myosin VI	Wollscheid et al.[4] (obtained from Simona Polo, IFOM in Milan, Italy)	n/a
Rabbit polyclonal anti- WRNIP1	Bethyl laboratories	Cat#A301-389A-T RRID:AB_938087
Mouse monoclonal anti-WHIP (clone A-8)	Santa Cruz Biotechnology	Cat#sc-376438 RRID:AB_11149006
Rabbit polyclonal anti-DNA-PKcs	Cell Signaling Technology	Cat#4602 RRID:AB_10692482
Rabbit polyclonal anti-53BP1	Novus Biologicals	Cat#NB100-304 RRID:AB_10003037
Rabbit polyclonal anti-SMC1	Bethyl Laboratories	Cat#A300-055A RRID:AB_66638
Rabbit monoclonal anti-VCP (clone 7F3)	Cell Signaling Technology	Cat#2649 RRID:AB_2214629
Rabbit polyclonal anti-MCM7 (clone D10A11)	Cell Signaling Technology	Cat#3735 RRID:AB_2142705
Mouse monoclonal anti-Rad18 (clone 79B1048)	Abcam	Cat#ab12007 RRID:AB_298783
Rabbit monoclonal anti-Rad21 (clone D5Y8S)	Cell Signaling Technology	Cat#12673 RRID:AB_2797988
Mouse polyclonal anti-RFC4	Abcam	Cat#ab2627 RRID:AB_303218
Mouse monoclonal anti-PCNA (clone PC10)	David P. Lane[8] (obtained from Bořivoj Vojtěšek, Masaryk Memorial Cancer Institute in Brno, Czech Republic)	n/a
Rabbit polyclonal anti-PCNA	Abcam	Cat#ab18197 RRID:AB_444313
Mouse monoclonal anti-BRCA2 (clone 2B)	Merck	Cat#OP95 RRID:AB_213443
Rabbit monoclonal anti-Rad51 (clone D4B10)	Cell Signaling Technology	Cat#8875 RRID:AB_2721109
Rabbit polyclonal anti-HLTF (clone ART2)	Alexandra Belayew[9] (obtained from Alexandra Belayew , UMONS in Mons, Belgium)	n/a
Mouse monoclonal anti-SMARCAL1 (clone A-2)	Santa Cruz Biotechnology	Cat#sc-376377 RRID:AB_10987841
Rabbit polyclonal anti-ZRANB3	Bethyl Laboratories	Cat#A303-033A
Mouse monoclonal anti-Biotin (clone Hyb-8)	Abcam	Cat#ab201341 RRID:AB_2861249
Mouse monoclonal anti-BrdU (clone B44) (IdU)	BD Biosciences	Cat#347580 RRID:AB_400326

Rat monoclonal anti-BrdU (clone BU1/75 (ICR1)) (CldU)	Abcam	Cat#ab6326 RRID:AB_305426
Rabbit polyclonal anti-phospho-Chk1 (Ser345)	Cell Signaling Technology	Cat#2341 RRID:AB_330023
Mouse monoclonal anti-Chk1 (clone 2G1D5)	Cell Signaling Technology	Cat#2360 RRID:AB_2080320
Rabbit polyclonal anti-phospho-Chk 1 (Ser317)	Cell Signaling Technology	Cat#2344 RRID:AB_331488
Mouse monoclonal anti-polyHistidine (clone HIS-1)	Merck	Cat#H1029 RRID:AB_260015
Mouse monoclonal anti-β-actin (clone AC-74)	Merck	Cat#A2228 RRID:AB_476697
Mouse monoclonal anti-ubiquitin (clone P4D1)	Cell Signaling Technology	Cat#3936 RRID:AB_331292
IRDye® 680LT donkey anti-rabbit IgG secondary Antibody	LICOR	Cat#926-68023; RRID: AB_10706167
IRDye® 680LT donkey anti-mouse IgG secondary antibody	LICOR	Cat#926-68072; RRID: AB_10953628
IRDye® 800CW goat anti-rabbit IgG secondary antibody	LICOR	Cat#926-32211; RRID: AB_621843
IRDye® 800CW donkey anti-mouse IgG secondary antibody	LICOR	Cat#926-32212; RRID: AB_621847
Polyclonal goat anti-goat HRP secondary antibody	Dako	Cat# P044901-2 RRID:AB_2617143
Polyclonal goat anti-mouse HRP secondary antibody	Dako	Cat#P044701-2 RRID:AB_2617137
Polyclonal goat anti-rabbit HRP secondary antibody	Dako	Cat#P044801-2 RRID:AB_2617138
Goat anti-Rat IgG (H+L) Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11006 RRID:AB_2534074
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific	Cat#A-21236 RRID:AB_2535805
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed, Alexa Fluor 647	Thermo Fisher Scientific	Cat#A-21244 RRID:AB_2535812

Supplementary Table 3. siRNAs used in this study

siRNA	Source	Identifier
Allstars Negative control siRNA	Qiagen	Cat#SI03650318
Hs_MYO6_5 FlexiTube siRNA	Qiagen	Cat#SI03142692
Hs_MYO6_7 FlexiTube siRNA	Qiagen	Cat#SI04243351
Hs_MYO6_8 FlexiTube siRNA	Qiagen	Cat#SI04370737
Hs_MYO6_10 FlexiTube siRNA	Qiagen	Cat#SI04998749
FlexiTube GeneSolution GS56897 for WRNIP1	Qiagen	Cat# GS56897
FlexiTube GeneSolution GS675 for BRCA2	Qiagen	Cat#GS675
SiRNA Silencer Select [Hs] RAD51	Thermo Fisher Scientific	Cat#s531930
SiRNA Silencer Select [Hs] RAD51	Thermo Fisher Scientific	Cat#s11734
siRNA Silencer Select [Hs] HLTF	Thermo Fisher Scientific	Cat#s13137
SiRNA Silencer Select [Hs] ZRANB3	Thermo Fisher Scientific	Cat#s38488
SiRNA Silencer Select [Hs] ZRANB3	Thermo Fisher Scientific	Cat#s224929
Hs_SMARCAL1_3 FlexiTube siRNA	Qiagen	Cat#SI00103194
Hs_SMARCAL1_1 FlexiTube siRNA	Qiagen	Cat#SI00103180

Supplementary Table 4. Oligonucleotides used in this study

Oligonucleotides for mutagenesis	Sequence (5´- 3´)	Construct	ID
Myo6 RRL→ AAA For	GGCAGCTTGCAGAGAAGAATTTCATGCGGCAGCAAAAGTGTATCATGCTTGGAAATCTAAG	pEGFP-C1-myo6-tail RRL→AAA	4898
Myo6 RRL→ AAA Rev	CTTAGATTTCCAAGCATGATACACTTTTGCTGCCGCATGAAATTCTTCTCTGCAAGCTGCC		4899
Myo6 A1013G For	CGGCTTCACTCTGGGCAATCCTCAGGCCCAGCTCCCGGTCCCTGCGCTCC	pEGFP-C1-myo6-tail ubiquitin binding mutant (A1013G+I1072A)	4896
Myo6 A1013G Rev	CGGCTTCACTCTGGGCAATCCTCAGGCCCAGCTCCCGGTCCCTGCGCTCC		4897
Myo6 I1072A* For	CGTGATACCATCAATACTTCTTGTGATGCTGAGCTCCTGGCAGCTTGCAGAGAAGAATTTCATAGG		5250
Myo6 I1072A* Rev	CCTATGAAATTCTTCTCTGCAAGCTGCCAGGAGCTCAGCATCACAAGAAGTATTGATGGTATCACG		5251
Myo6 DNA bdg mut For	GCCGACCAGTACAAAGACCCTCAGGCTGCGGCAGCAGGCTGGTGGTATGCCC	pEGFP-C1-myo6-tail DNA binding mutant (K1165-1167A)	6223
Myo6 DNA bdg mut Rev	GGGCATACCACCAGCCTGCTGCCGCAGCCTGAGGGTCTTTGTACTGGTCGGC		6224
Myo6 lipid bdg mut For	GGAGACTAAAAGTGTATCATGCTTGGGCATCTGCGAACAAGAAGAGAAATACTGAAACAGAGCAACGTGCTCC	pEGFP-C1-myo6-tail lipid binding mutant (K1093A, K1095A)	6068
Myo6 lipid bdg mut Rev	GGAGCACGTTGCTCTGTTTCAGTATTTCTCTTCTTGTTCGCAGATGCCCAAGCATGATACACTTTTAGTCTCC		6069
Myo6 WWY→WLY For	CCCTCAGAGTAAGAAAAAAGGCTGGTTGTATGCCCATTTTGATGGACCATGGATTGCCCGG	pEGFP-C1-myo6-tail cargo binding mutant (WLY)	6064
Myo6 WWY→WLY Rev	CCGGGCAATCCATGGTCCATCAAAATGGGCATACAACCAGCCTTTTTTCTTACTCTGAGGG		6065
Oligonucleotide for amplification	Sequence (5´- 3´)	Construct	ID
MIU FOR BamHI	CGGGATCCCAACAGCAAGCAGTTCTGGAGC	pET30-MIUMyUb-iso2-AVI-His	3837
MyUb Rev no STOP EcoRI	GGAATTCTCTCTTCTTGTTCTTAGATTTCCAAGCATG		3838
Myo6 KpnI FWD	GGGGTACCATGGAGGATGGAAAGCCCGTTTGGG	pENTR4-myo6-iso2	5055
Myo6 Xba REV	GCTCTAGACTACTTTAACAGACTCTGCAGCATGGC		5056
EGFP for_HINDIII	CCCAAGCTTATGGTGAGCAAGGGCGAGGA	pCDNA5-FRT-TO-EGFP-M6G4-2xRING	5597
M6G4 rev_NHEI	CTAGCTAGCTAGCAGATTTCTGCAGAACTTCAGCG		5594
E3_5_rev_NHEI	CTAGCTAGCTTGCAGGATTTCAGCCAGGTC	pCDNA5-FRT-TO-EGFP-E3_5-2xRING	5688
3xNLS for_KpnI	GGGGTACCCCATGGGGGCCCAG	pENTR4-NLS-G4	5641
M6G4 rev_XhoI	CCGCTCGAGTAGCAGATTTCTGCAGAACTTCAGCG		5640
E3_5 XhoI REV	CGGCTCGAGTTGCAGGATTTCAGCCAGGTCCTCG	pENTR4-NLS-E3_5	5360
GFP For KpnI	GATCGGTACCGATGGTGAGCAAGGGCG	p3xFlag-CMV-YFP-NES-myo6-tail	6189
Myo6 Xba REV	GCTCTAGACTACTTTAACAGACTCTGCAGCATGGC		5056

*: I1072 in this construct (isoform 2) corresponds to I1104 in the long isoform (3).

Supplementary Table 5. Reagents used in this study

Chemical	Source	Identifier
Doxycycline hydrochloride	Merck	Cat#D3447
5-Chloro-2′-deoxyuridine	Merck	Cat#C6891
5-iodo-2′-desoxyuridin	Merck	Cat#I7125
5-ethynyl-2′-deoxyuridine	Merck	Cat#900584
Azide-PEG3-biotin conjugate	Merck	Cat#762024
Mirin	Merck	Cat#M9948
DNA2 inhibitor C5	AOBIOUS	Cat#AOB9082
IPTG	Generon	Cat#GEN-S-02122
Ni-NTA agarose	Qiagen	Cat#30250
Imidazole	Merck	Cat#I2399
Glutathione	Merck	Cat#G4251
IPTG	Generon	Cat#GEN-S-02122
Paraformaldehyde	Merck	Cat#P6148
Formaldehyde solution, 36.5-38% in H2O	Merck	Cat#F8775
Glycine	Merck	Cat#G7126
Methanol	Thermo Fisher Scientific	Cat#15654570
Acetic acid	Merck	Cat#A6283
SIGMAFAST protease inhibitor cocktail	Merck	Cat#S8830
cOmplete Protease Inhibitor Cocktail	Roche	Cat#5056489001
Hygromycin B Gold	Invivogen	Cat#ant-hg-1
Pfu Turbo DNA Polymerase	Agilent	Cat#600250
DpnI	New England Biolabs	Cat#R0176L
Hydroxyurea	Merck	Cat#H8627
Streptavidin Agarose Resin	Thermo Fisher Scientific	Cat#11846764
Polyethyleneimine	Polysciences	Cat#23966-2
Lipofectamine® 2000	Thermo Fisher Scientific	Cat#11668019
Lipofectamine® RNAiMAX	Thermo Fisher Scientific	Cat#13778150
FuGENE® HD Transfection Reagent	Promega	Cat#E2311
ProLong™ Diamond Antifade Mountant	Thermo Fisher Scientific	Cat#15205739
DMEM, high glucose, pyruvate, no glutamine	Thermo Fisher Scientific	Cat#21969035
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	Cat#25300054
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15140122
L-Glutamine (200 mM)	Thermo Fisher Scientific	
Blasticidin	Invivogen	Cat#ant-bl-1
Triton X-100	Merck	Cat#T9284
Albumin (bovine serum albumin, BSA)	Merck	Cat#A7906
Copper (II) sulfate	Merck	Cat#469130
Sodium deoxycholate	Merck	Cat#D6750

SDS, 20%, Sodium dodecyl sulfate	Merck	Cat#05030
NuPAGE LDS Sample Buffer (4X)	Thermo Fisher Scientific	Cat#11559166
GFP-Trap magnetic agarose beads	Chromotek	Cat#gtma-100
Milk powder, skim milk	Merck	Cat#70166
Tween-20	Merck	Cat#P7949
Amersham ECL Select Western Blotting Detection Reagent	Thermo Fisher Scientific	Cat#RPN2235
Amersham ECL Prime Western Blotting Detection Reagent	Thermo Fisher Scientific	Cat#12994780
Ponceau S	Merck	Cat#P3504
Hoechst 33342	Thermo Fisher Scientific	Cat#11534886
Biotin	Merck	Cat#B4501
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific	Cat#11822124
4-15% Criterion™ TGX Stain-Free™ Protein Gel, 26 well, 15 µl	Bio-Rad Laboratories	Cat#567-8085
Mini-PROTEAN TGX Stain Free Gels, 4-15%, 15-well	Bio-Rad Laboratories	Cat#456-8086
SILAC light isotopes: L-Arg(0) and L-Lys(0)	Merck	Cat#A6969 Cat#L8662
SILAC medium isotopes: L-Arg(6) and L-Lys(4)	Cambridge Isotope Laboratories	Cat#CLM-2265-H-1 Cat#DLM-2640-1
Trypsin, MS-approved	Serva	Cat# 37286
Glutathione Sepharose High Performance resin (GSH)	Cytiva	Cat#17527901
Recombinant ubiquitin activating enzyme (E1)	In house protein production core facility	Carvalho et al[6]
Ubiquitin from bovine erythrocytes	Merck	Cat#U6253-25MG
MG-132, 25 mg (Z-Leu-Leu-Leu-CHO)	Enzo Life Sciences	Cat#BML-PI102-0025
Alexa Fluor™ 647 Phalloidin	Thermo Fisher Scientific	Cat#A22287
µ-Slide 8 Well Chamber Slides	Ibidi	Cat#80806
Critical commercial assays		
Duolink® In Situ Red Starter Kit Mouse/Rabbit	Merck	Cat#DUO92101-1KT
InstantBlue, 1L Protein Stain	Biozol	Cat#EXP-ISB01L
Trans-Blot® Turbo™ RTA Midi Nitrocellulose Transfer Kit	Bio Rad laboratories	Cat#1704271
Gateway® LR Clonase® II enzyme mix	Thermo Fisher Scientific	Cat#10134992
Colloidal Blue staining kit	Thermo Fisher Scientific	Cat#LC6025
Biotin CAPture kit	GE Healthcare	Cat#28920233

Supplementary References

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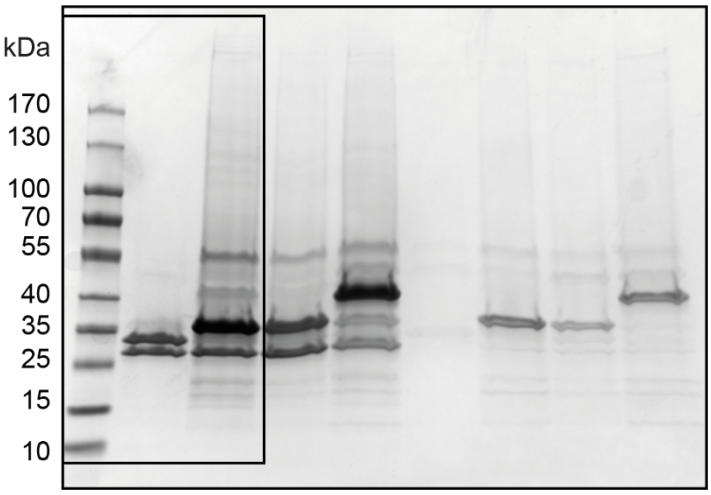
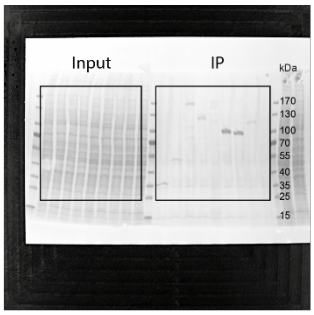
Uncropped images for Supplementary Figures

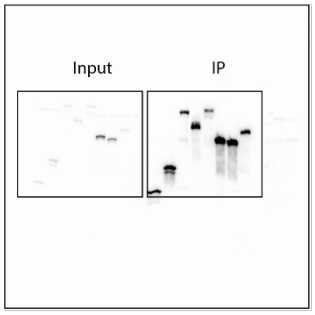
Supplementary Fig. 1a



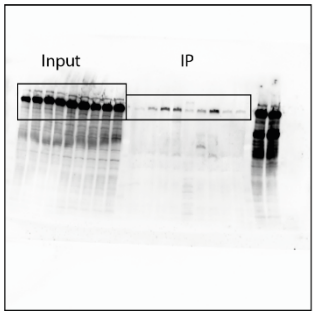
Supplementary Fig. 1c



Ponceau

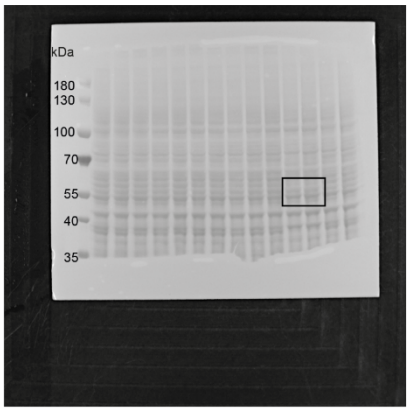


GFP



Myo6

Supplementary Fig. 2a



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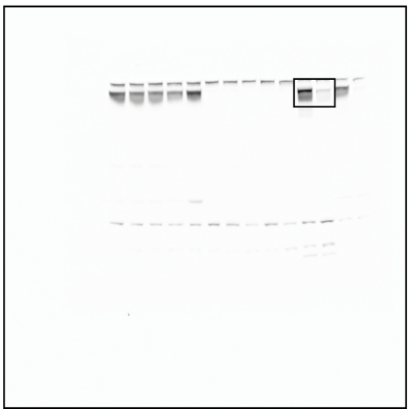

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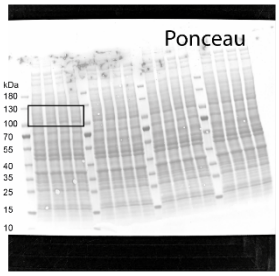
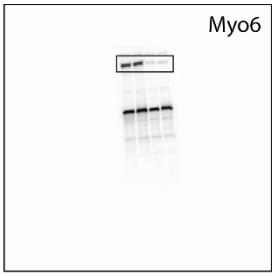
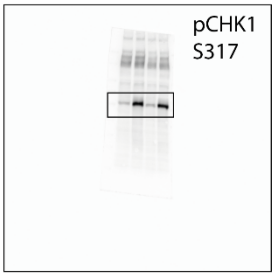
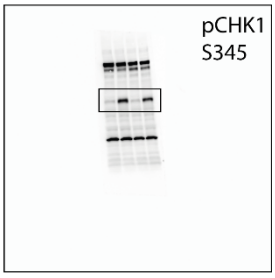
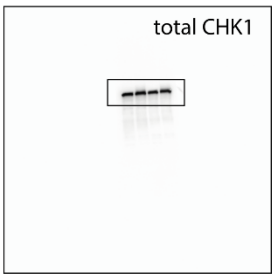
Supplementary Fig. 2b



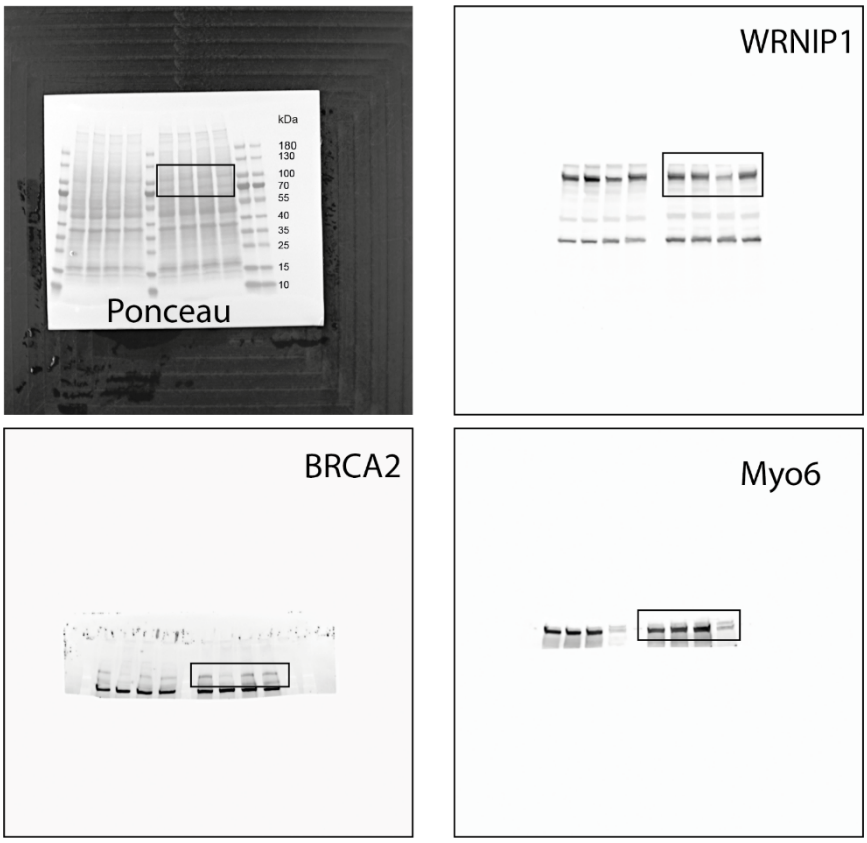




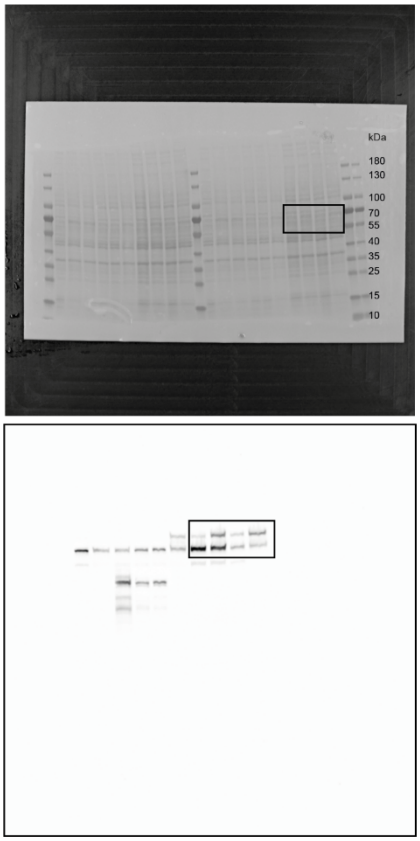




Supplementary Fig. 2c



Supplementary Fig. 2d



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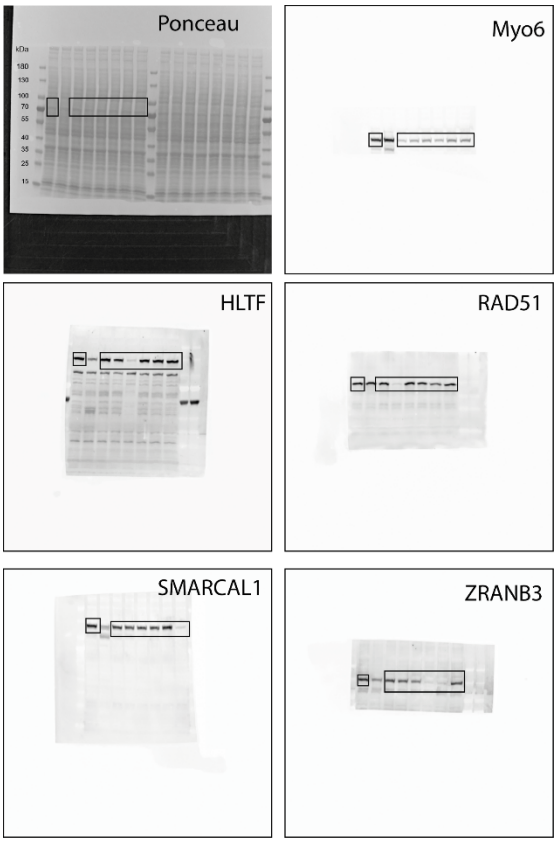
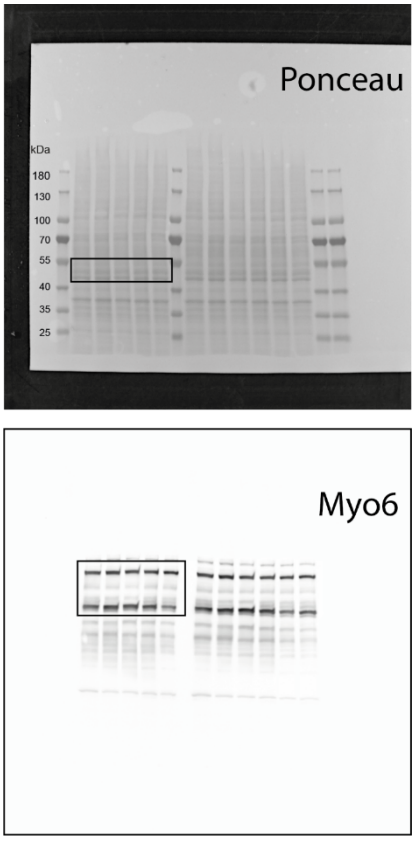
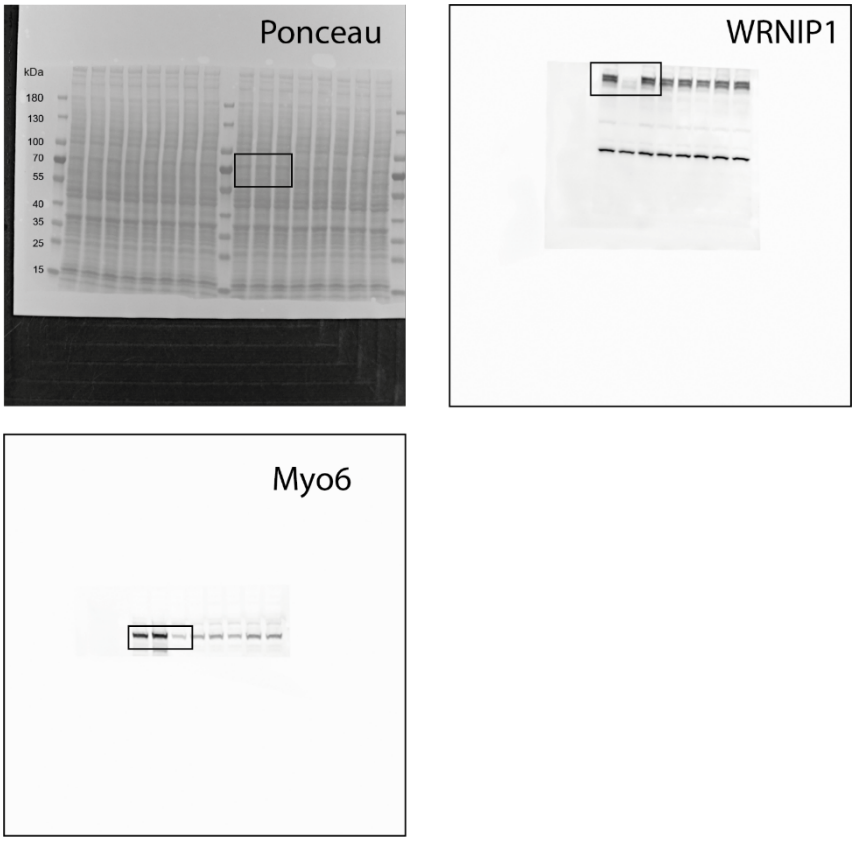


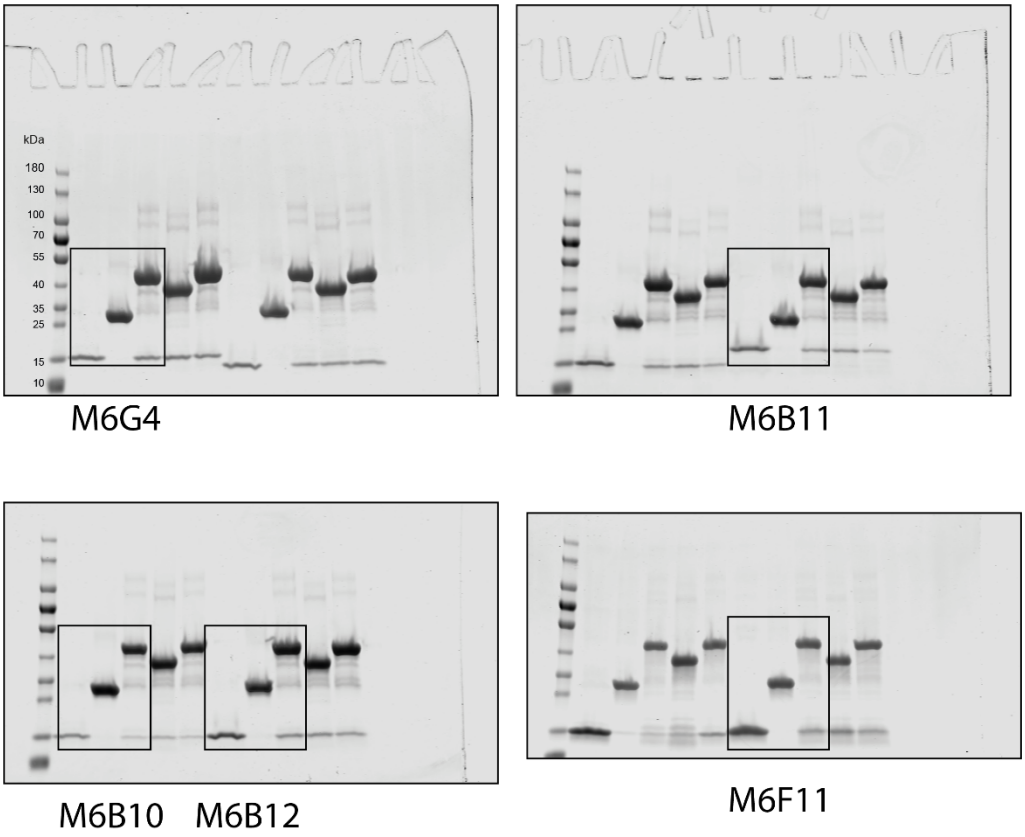
Supplementary Fig. 2g



Supplementary Fig. 3g



Supplementary Fig. 4b



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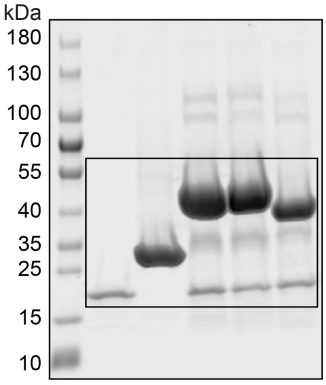

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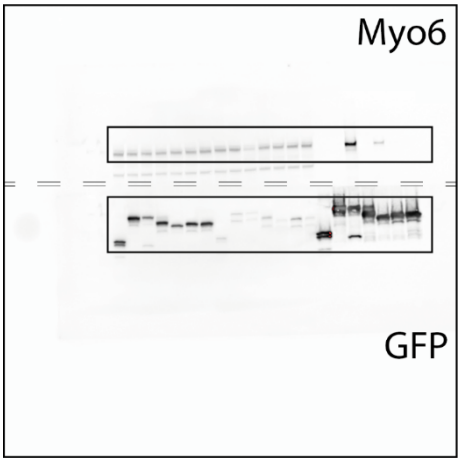

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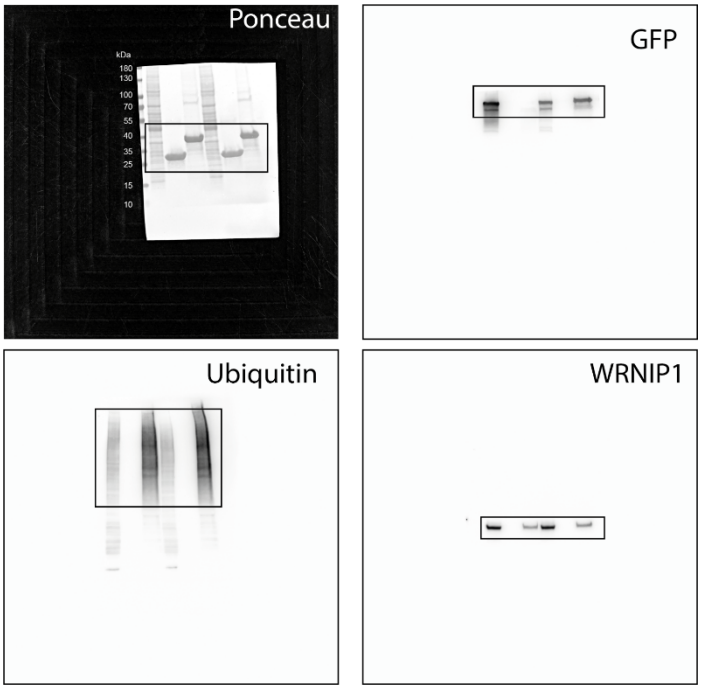

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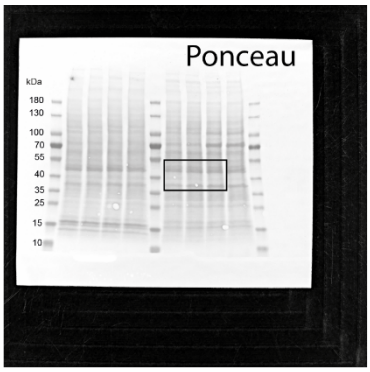

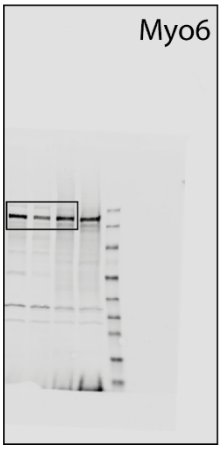

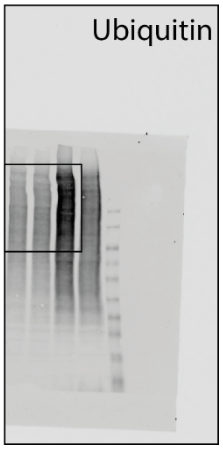

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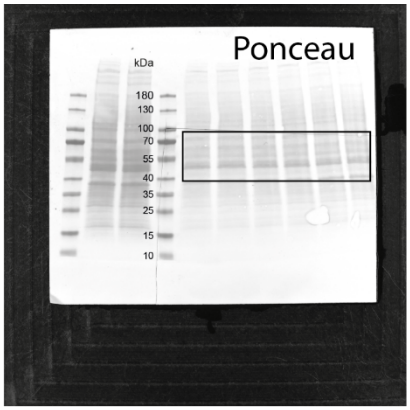

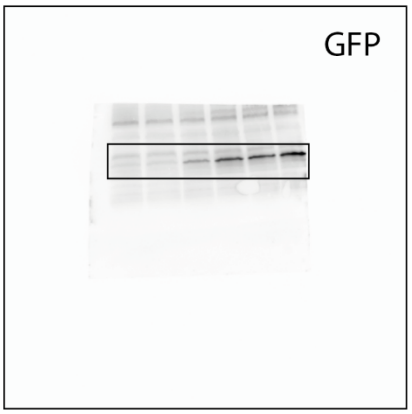

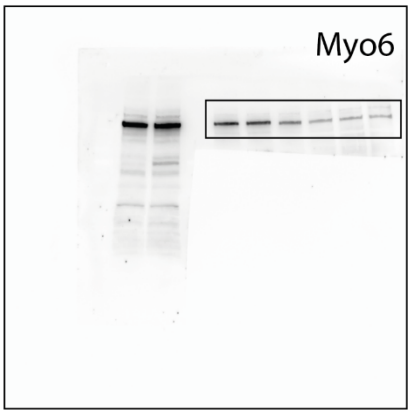

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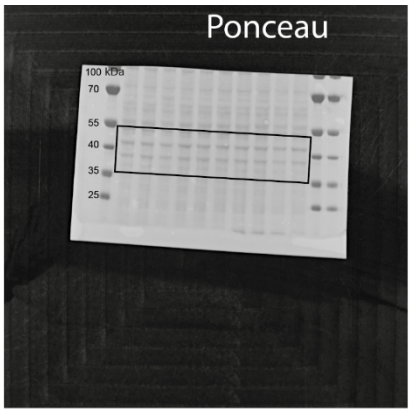

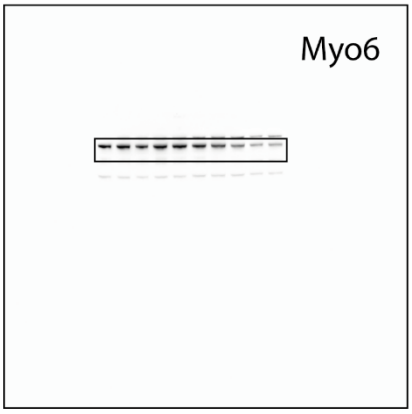

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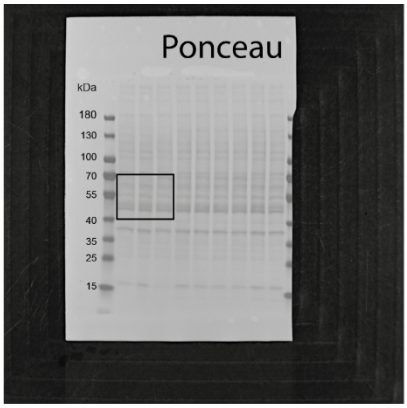

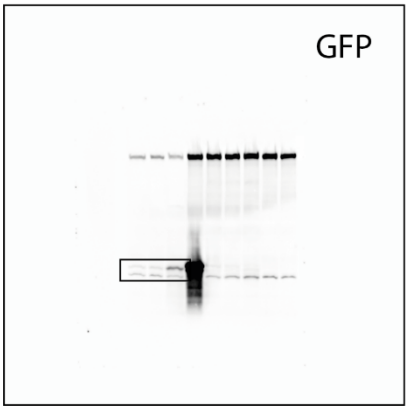

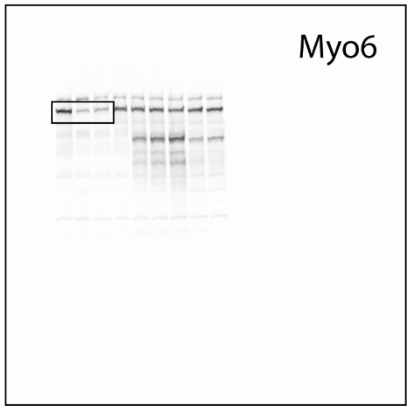

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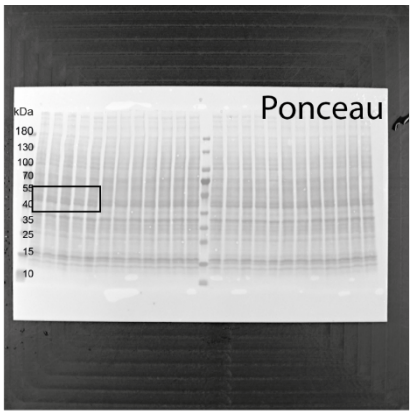

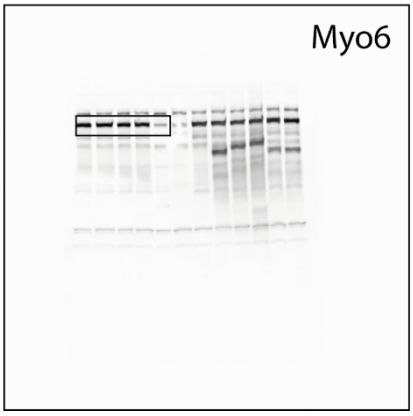

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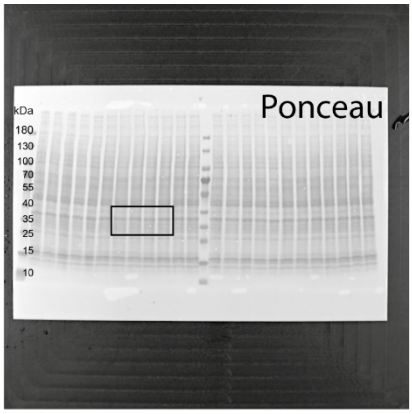

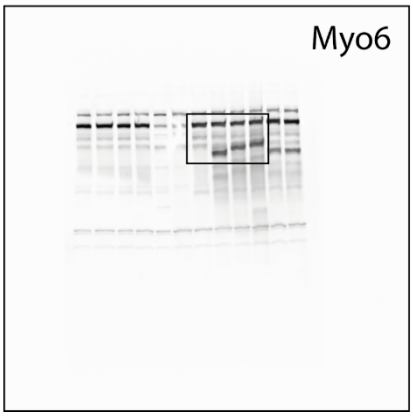

Supplementary Fig. 6b