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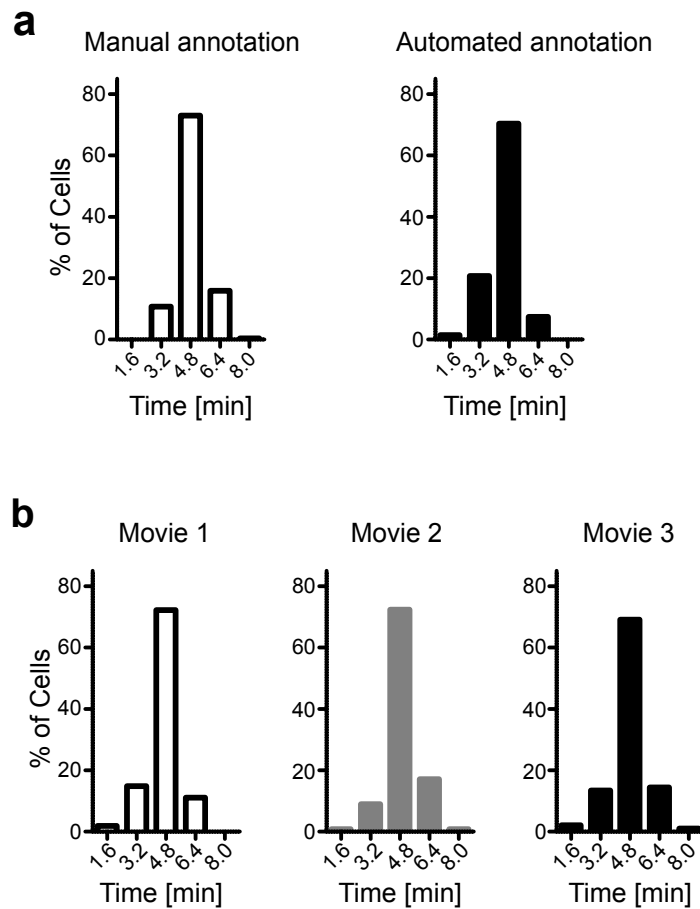


Figure S1 Validation of automated image analysis. **(a)** Histograms of mitotic exit timing based on IBB-import after anaphase onset ($t = 0$) of the same dataset analysed manually (left, white bars), or automatically (right, black bars). **(b)** Automated annotation is highly reproducibly between movies (three independent movies shown).

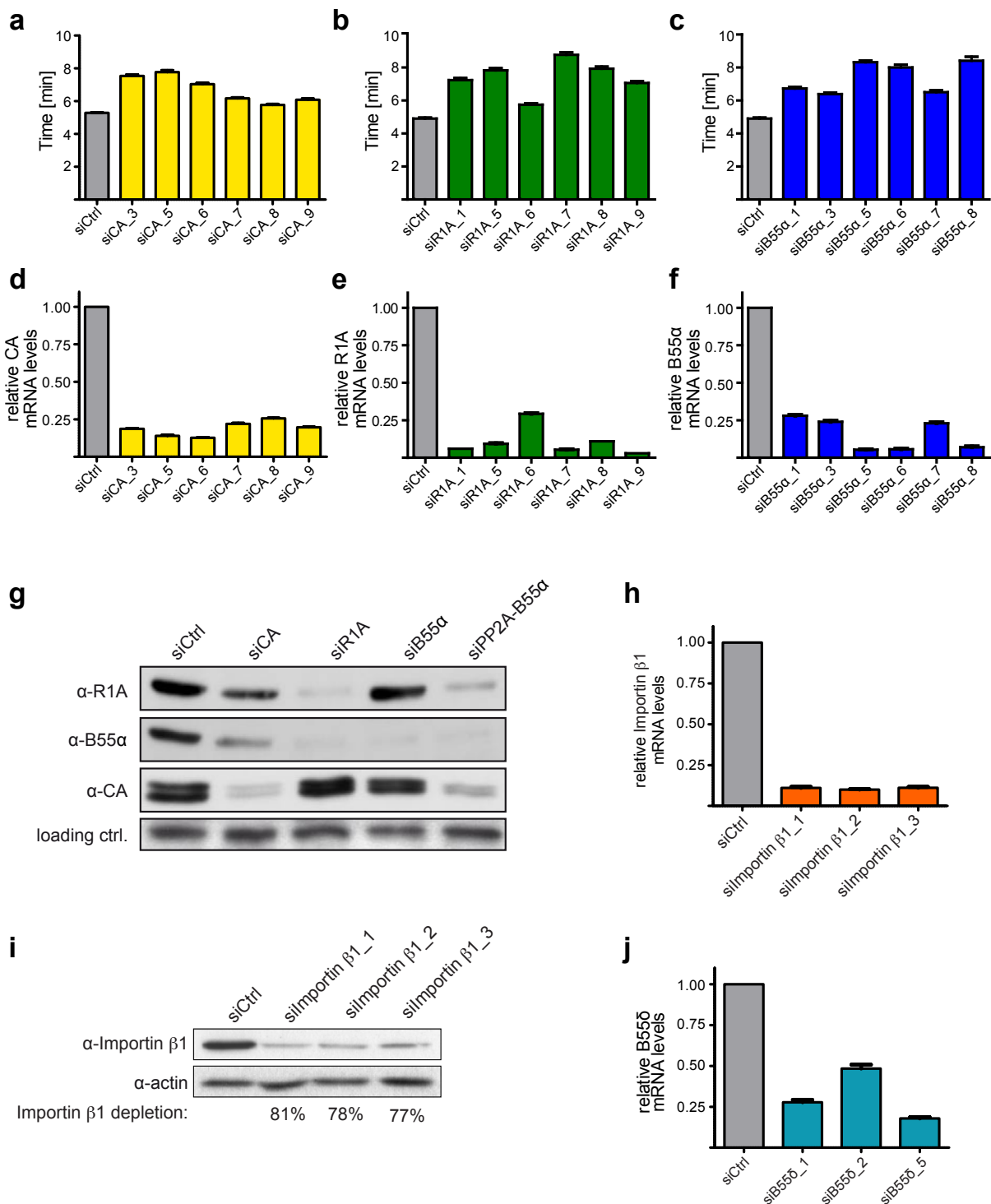


Figure S2 Validation of RNAi efficiency. (a-c) Mitotic exit timing measured as in Figure 1, for six different siRNAs targeting CA ($n \geq 382$ for each siRNA condition; mean \pm s.d) (a), R1A ($n \geq 175$ for each siRNA condition) (b), or B55 α ($n \geq 108$ for each siRNA condition) (c). (d-f) Quantification of mRNA knockdown 40 h post transfection by real-time PCR for the same siRNAs as in (a-c), targeting CA (d), R1A (e), or B55 α (f), normalized against GAPDH ($n = 3$ for each condition; mean \pm s.d). See Supplementary Information, Table 2 for

siRNA sequences. (g) Protein depletion levels of CA, R1A, and B55 α , detected by Western blotting in cells depleted for the indicated siRNAs 60 h post-transfection. Note that depletion of CA or R1A co-depletes other subunits of the PP2A-B55 α complex, consistent with previous reports^{39,40}. (h) Quantification of Importin β 1 mRNA knockdown 48 h post transfection. (i) Importin β 1 protein levels, detected by Western blotting 64 h after siRNA transfection. (j) Quantification of B55 δ mRNA knockdown 48 h post transfection.

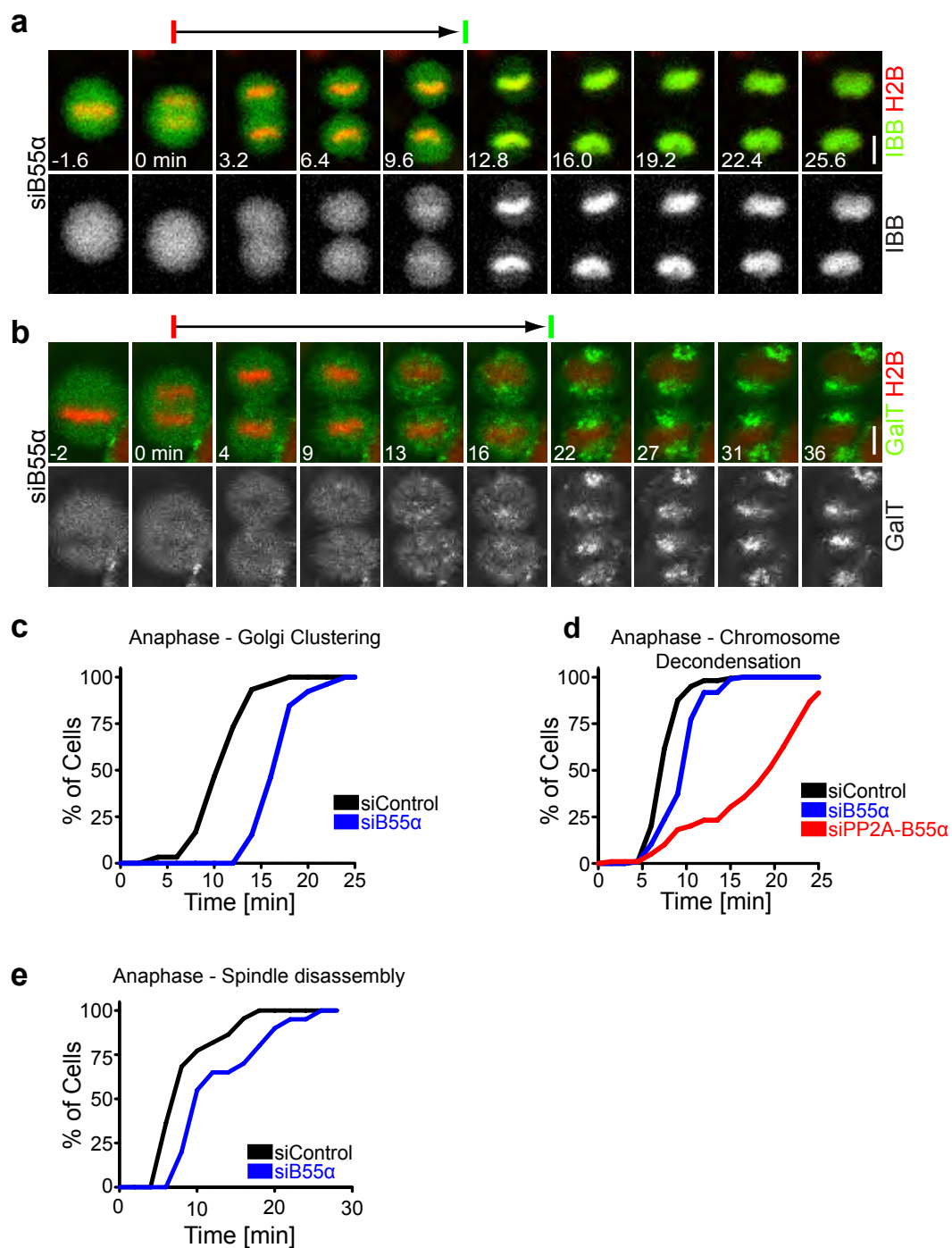


Figure S3 Phenotypes of B55 α single depletions. **(a)** Nuclear reassembly timing in a B55 α -depleted cell, see also Supplementary Information, Movie S11. **(b)** Golgi reassembly timing in a B55 α -depleted cell, see also

Supplementary Information, Movie S12. **(c-e)** Cumulative histograms of postmitotic Golgi clustering **(c)**, chromosome decondensation **(d)**, and spindle disassembly **(e)** relative to anaphase onset (0 min). Scale bars: 10 μ m.

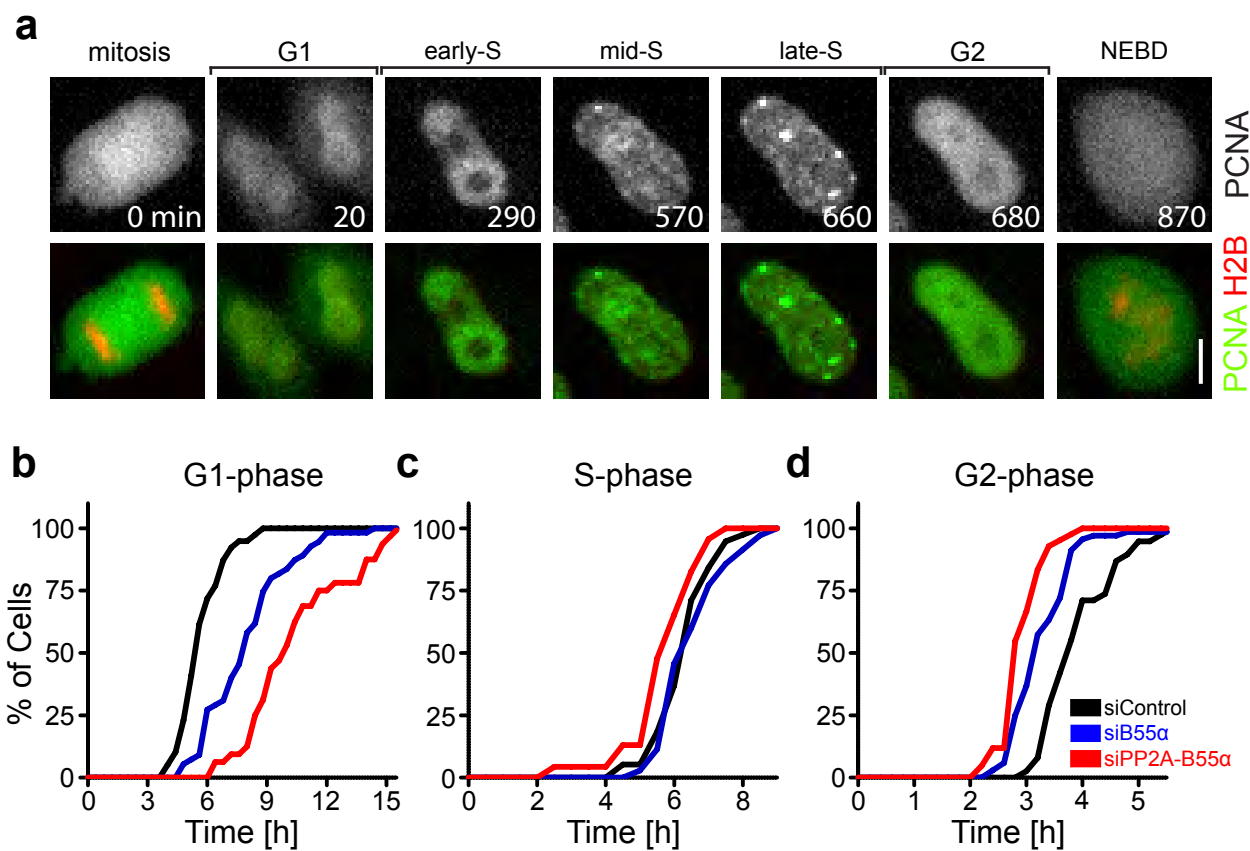


Figure S4 Effect of PP2A-B55 α -depletion on cell cycle progression. **(a)** Cell cycle staging by DNA replication factor pattern of EGFP-PCNA. **(b, c,**

d) Cumulative histograms of G1-phase **(b)**, S-phase **(c)**, and G2-phase **(d)** duration. Scale bars: 10 μ m.

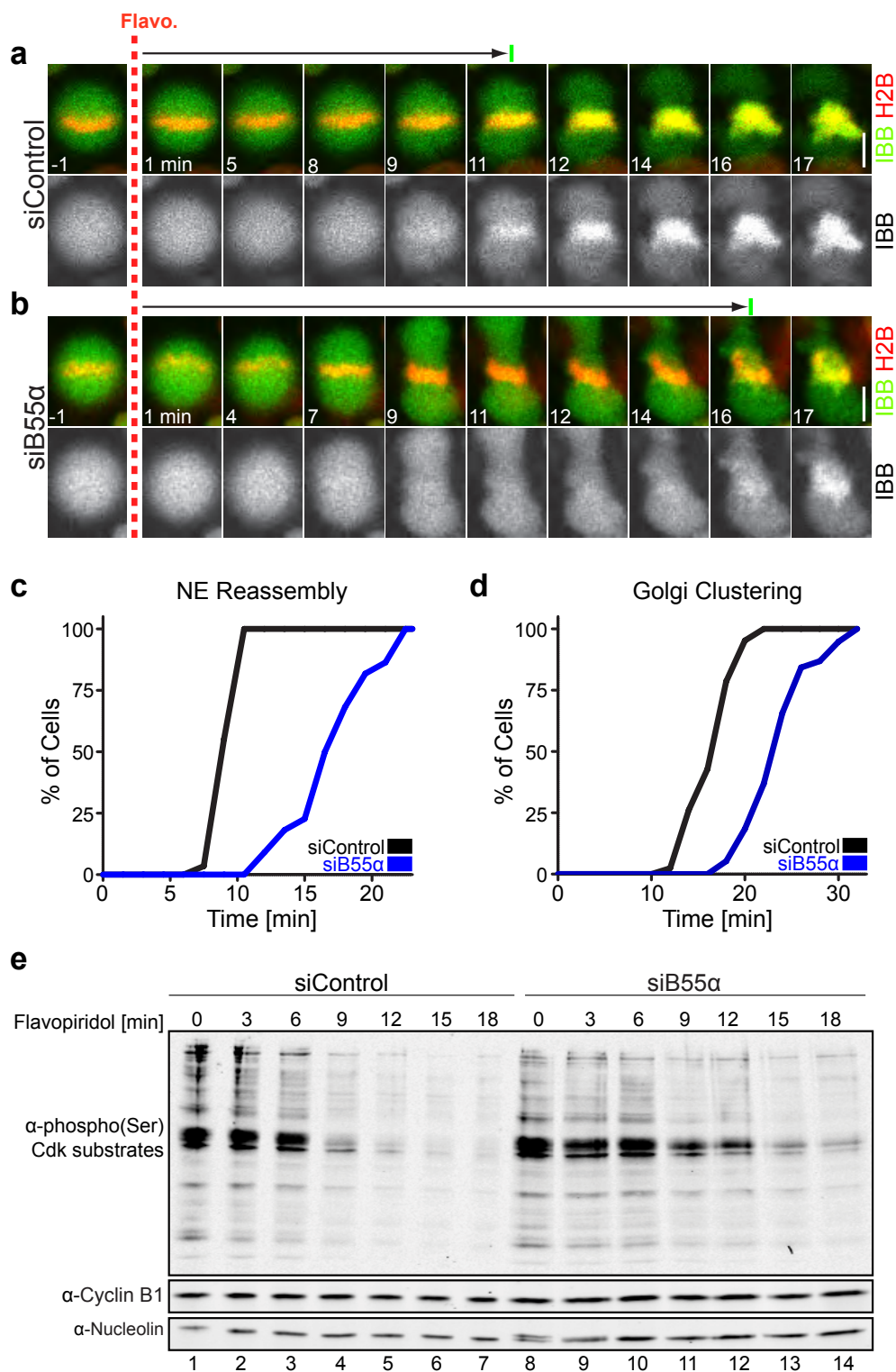


Figure S5 B55 α functions downstream of Cdk1-cyclin B inactivation. **(a)** Movie of a cell transfected with non-silencing control siRNA, following the protocol indicated in Figure 4a. Arrowhead indicates onset of nuclear import of IBB-EGFP. **(b)** Time-lapse images of a cell transfected with siRNA targeting B55 α , see also Supplementary Information, Movie S13. **(c, d)** Cumulative histograms of nuclear envelope assembly

(c) and Golgi clustering **(d)** timing based on the data shown in (a-b, and data not shown). **(e)** Chemical induction of mitotic exit in presence of proteasome inhibitor in nocodazole arrested mitotic cells results in rapid dephosphorylation of Cdk substrates (siControl, lanes 1-7). Cells depleted for B55 α show delayed dephosphorylation (siB55 α , lanes 8-14). Scale bars: 10 μ m.

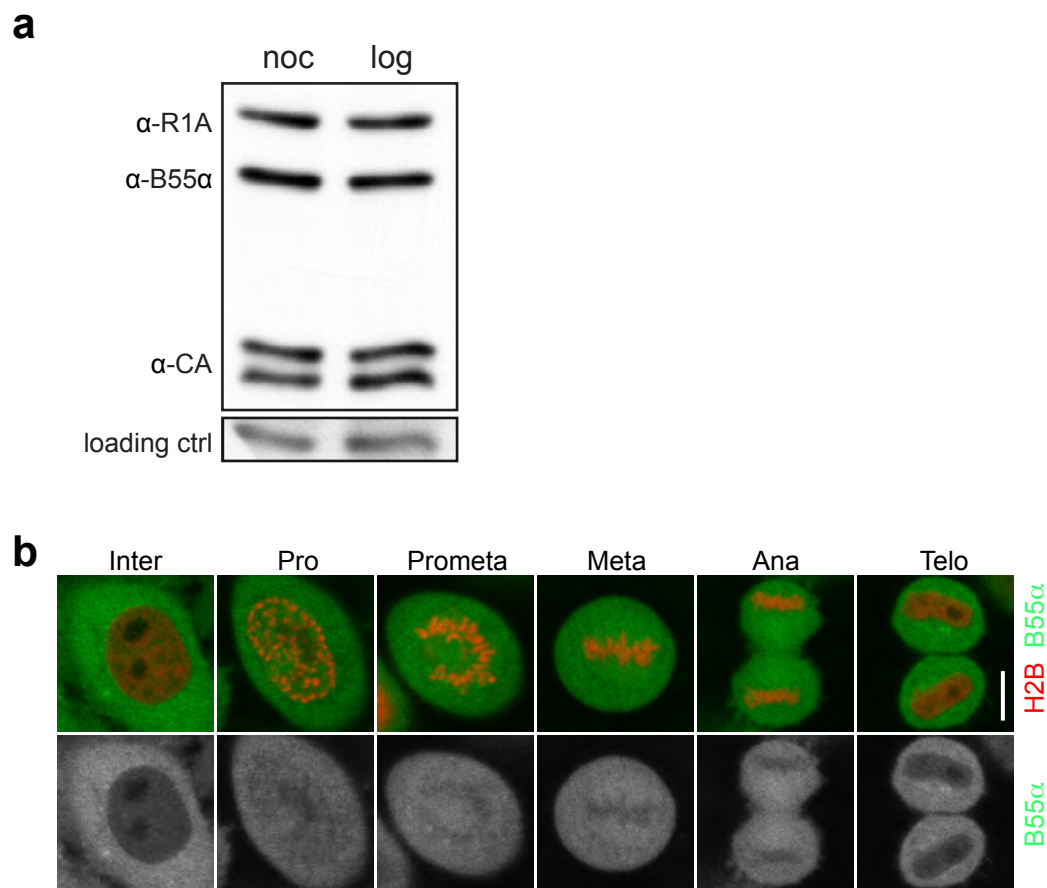


Figure S6 Expression levels and localization of PP2A-B55 α . **(a)** PP2A-B55 α subunits are expressed at constant levels during the cell cycle. Whole cell lysates were probed by Western Blotting using the antibodies against CA, R1A, and B55 α . Left lane: 17 h nocodazole arrested cells (noc), right lane:

unsynchronized interphase cells (log). Note that CA migrates as a doublet band (see also Supplementary Information, Figure S2g). **(b)** Localization of B55 α . Confocal live images of cells expressing EGFP-mouse-B55 α from endogenous promoter (see Fig. 2b). Scale bar: 10 μ m.

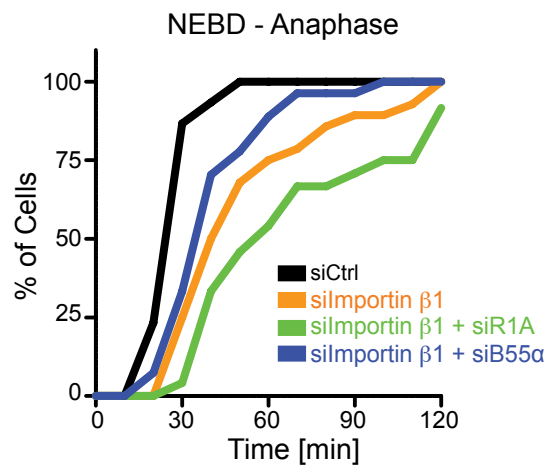


Figure S7 Early mitotic progression in Importin β 1-depleted cells. Timing from prometaphase until anaphase was measured for the same cells shown in Fig. 5e.

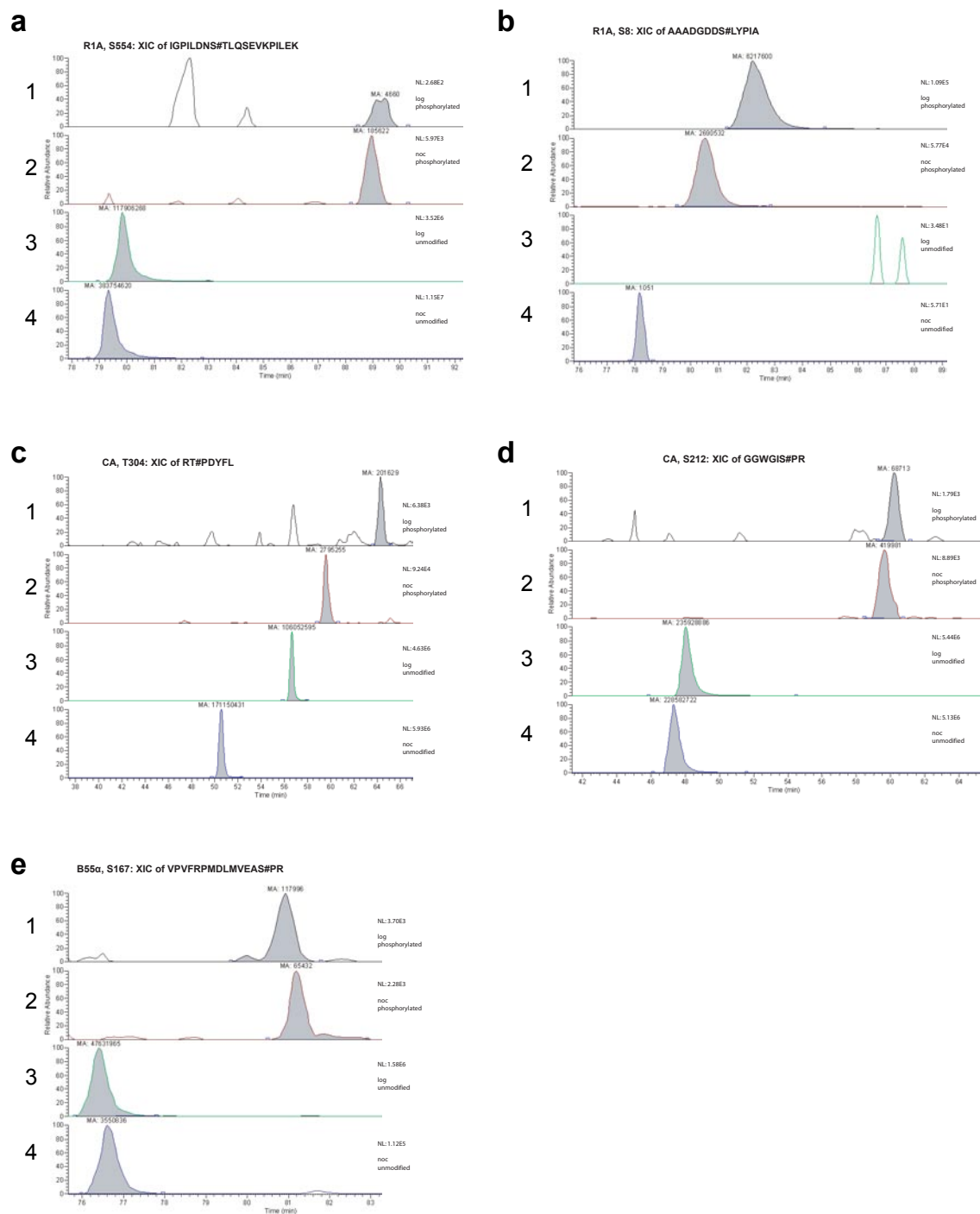


Figure S8 Quantification of phosphorylation levels by extracted ion chromatograms. Extracted ion chromatograms (XICs) of phosphopeptides identified (hash indicates the phosphorylated amino acid), and their unmodified counterparts, from purified PP2A complexes. Peak-area values for the phosphopeptides were used for quantification of the mitotic increase in phosphorylation abundance, whereas those of the unmodified peptides were used for normalization, as described in the legend for Fig. 5f. The panels show: 1. XIC featuring the phosphorylated

peptide identified from the PP2A complex purified from interphase (log) cells. 2. XIC featuring the same phosphorylated peptide identified from the mitotic (noc) cell state. 3. XIC featuring the unmodified counterpart peptide from interphase. 4. XIC featuring the unmodified counterpart peptide from the mitotic (noc) cell state. **(a)** Quantitation of S554 phosphorylation on R1A. **(b)** S8 phosphorylation on R1A. **(c)** T304 phosphorylation on CA. **(d)** S212 phosphorylation on CA. **(e)** S167 phosphorylation on B55 α .

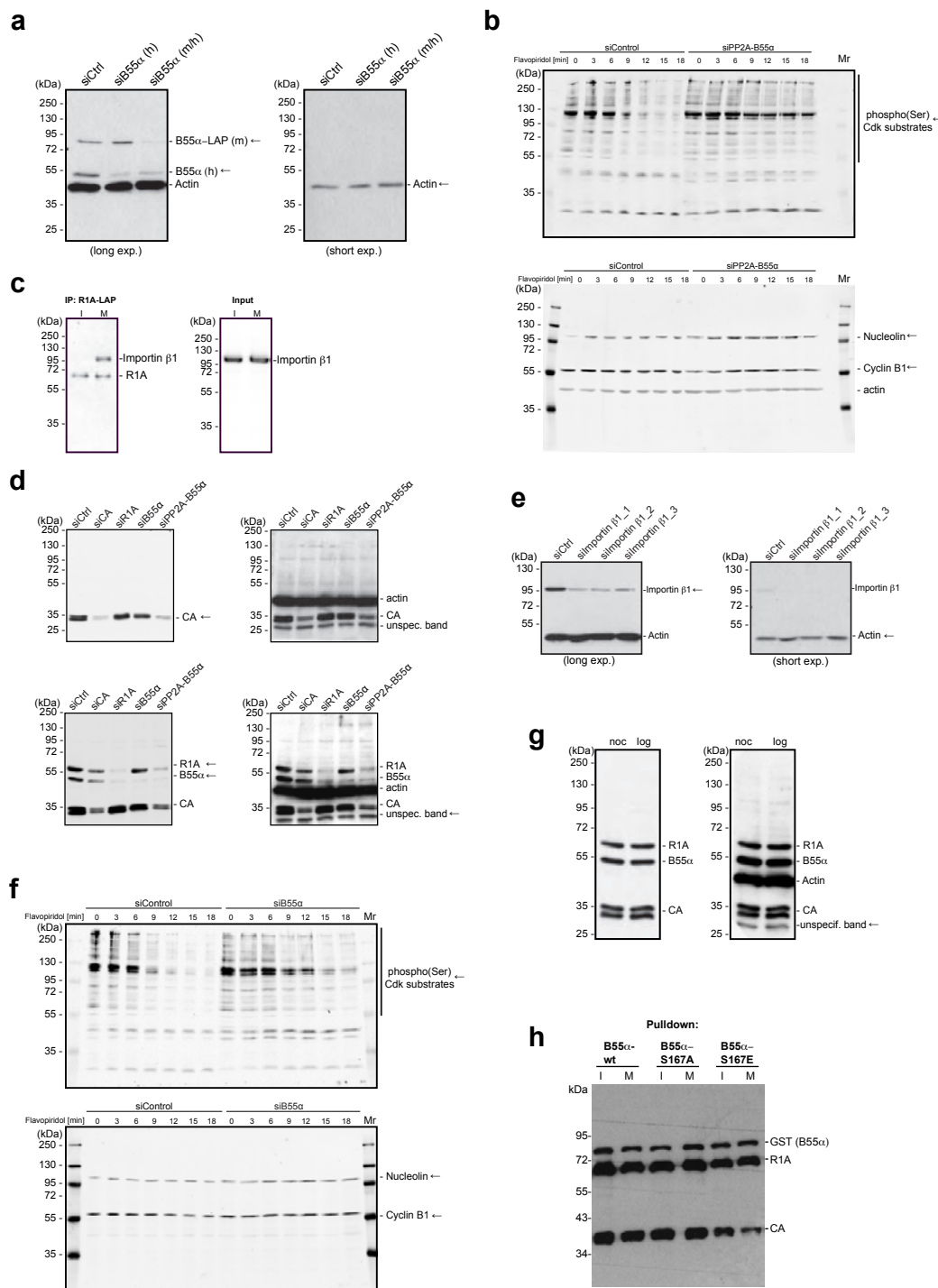


Figure S9 Full scans of blots. **(a)** Full scan for B55α and Actin immunoblot of Figure 2b. **(b)** Full scan for phospho(Ser)Cdk substrates (upper) and Cyclin B1, Nucleolin, and Actin (lower) immunoblot of Figure 4f. **(c)** Full scan for Importin B1 and R1A IP-blot (left) and Importin B1 Input-blot (right) of Figure 5d. **(d)** Full scan for CA (upper left), R1A and B55α (lower left), and loading control blots (right panels) of Figure S2g. **(e)** Full scan

for Importin B1 and Actin immunoblot of Figure S2i. **(f)** Full scan for phospho(Ser)Cdk substrates (upper) and Cyclin B1, and Nucleolin (lower) immunoblot of Figure S5e. **(g)** Full scan for CA, R1A, and B55α (right) and Actin (left) immunoblot of Figure S6a. (Sliced bands are indicated with arrows). **(h)** Relevance of B55α-S167 phosphorylation for PP2A complex assembly. Independent replica of the experiment shown in Fig. 5g.

Supplementary Movie Legends

Movie S1 Monoclonal fluorescent HeLa cell line expressing chromatin marker H2B-mCherry and the nuclear import substrate IBB-EGFP. The movie shows 13% of the total image size and represent a typical overnight screening movie. Time-lapse covers a period of ~21 hours.

Movie S2 H2B-mCherry / IBB-EGFP reporter cell that has been automatically detected, tracked, and segmented over time. Movie shows original images (left); mitotic stage classification with color label as in Fig. 1c (middle), and regions of IBB measurements (right). Time-lapse covers a period of 216 minutes.

Movie S3 H2B-mCherry / IBB-EGFP cell treated with siControl. This movie shows mitotic progression and nuclear envelope reassembly in a control cell. Time-lapse covers a period of 192 minutes.

Movie S4 H2B-mCherry / IBB-EGFP cell treated with siPP2A-B55 α . This movie shows mitotic progression and delayed nuclear envelope reassembly in a cell depleted for PP2A-B55 α . Time-lapse covers a period of 192 minutes.

Movie S5 H2B-mCherry / GalT-EGFP cell treated with siControl. This movie shows mitotic progression and Golgi reassembly in a control cell. Time-lapse covers a period of 132 minutes.

Movie S6 H2B-mCherry / GalT-EGFP cell treated with siPP2A-B55 α . This movie shows mitotic progression and delayed Golgi reassembly in a cell depleted for PP2A-B55 α . Time-lapse covers a period of 161 minutes.

Movie S7 H2B-mCherry / mEGFP- α -tubulin cell treated with siControl. This movie shows mitotic progression and mitotic spindle dynamics in a control cell. Time-lapse covers a period of 108 minutes.

Movie S8 H2B-mCherry / mEGFP- α -tubulin cell treated with siPP2A-B55 α . This movie shows mitotic progression and delayed mitotic spindle disassembly in a cell depleted for PP2A-B55 α . Time-lapse covers a period of 108 minutes.

Movie S9 H2B-mCherry / IBB-EGFP cell treated with siControl. This movie shows forced mitotic exit in presence of MG132 by addition of the Cdk inhibitor flavopiridol in a control cell. Time-lapse covers a period of 47 minutes.

Movie S10 H2B-mCherry / IBB-EGFP cell treated with siPP2A-B55 α . This movie shows delayed mitotic exit in presence of MG132 by addition of the Cdk inhibitor flavopiridol in a cell depleted for PP2A-B55 α . Time-lapse covers a period of 47 minutes.

179954	KIAA1949	Hs KIAA1949_4	NM_133474 XM_928867 XM_934935 XM	S02824423	KIAA1949	TCGGTTGGAGGCGGAGAGAAA	UUUUCUUCGCGGCGGACGCGg	CGUUCGAGGCGGAGAGAAA#
201562	PTPLB	Hs PTPLB_5	NM_198402	S02659454	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b	CACGGCGTACTGGTCATCTA	UAGAUAACACGAGUAGCGCCGg	CGCGUACUUGGUCACUCU#
201562	PTPLB	Hs PTPLB_6	NM_198402	S02659461	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b	CACCGAGAAATAGTTGCTA	UACCAACUUAUUCUUCGCGg	CGCAAGAAUAGUUCGUA#
201562	PTPLB	Hs PTPLB_3	NM_198402	S00164171	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b	TACGCTTAAC1TCTATGAT	AUGCAUAGAAAGUUAAGCG#	CGCUUAACUUCUUAUGAU#
286262	C8orf75	Hs C8orf75_1	NM_173891	S00335965	chromosome 9 open reading frame 75	CTCCTCAAGAAAGAAGATGA	UUCAUCUUCUUCUUGAGGg	CCUCAAGAAAGAAGUAGA#
286262	C8orf75	Hs C8orf75_2	NM_173891	S00335972	chromosome 9 open reading frame 75	CACAGTGGTGCCCAAGAGAA	UUUCUUCUUGGCACCAUUGg	CAGUUGUCCCAAGAGAA#
286262	C8orf75	Hs C8orf75_3	NM_173891	S00335976	chromosome 9 open reading frame 75	TCGAAATCTTTCACTGAT	AUAGCCAUAAAGAAUUCg	GAUAUUCUUCUUCALGCA#
391025	LOC391025	Hs LOC391025_5	KM_001131228 XM_372775	S04380859	similar to protein tyrosine phosphatase, receptor type, U isoform 2 precursor	CCGCGAGAGCCGATGACGA	UCCGUUCALUGCCUCCUGGg	GCAGAAAGCCGAGUAGCA#
391025	LOC391025	Hs LOC391025_6	KM_001131228 XM_372775	S04380866	similar to protein tyrosine phosphatase, receptor type, U isoform 2 precursor	CCACATGATGAGCCACATGGA	UCCAUUGUUCUUCALUGGg	ACAUGAUGAGCCACUAGG#
391025	LOC391025	Hs LOC391025_7	KM_001131228 XM_372775	S04380873	similar to protein tyrosine phosphatase, receptor type, U isoform 2 precursor	CCTGTGCTTATAGACACTCA	UGAGUUGUUAUAGAGACAGg	UGUUCUUCALUAGACUCU#
40927	LOC40927	Hs LOC40927_1	KM_376010	S00548660	TPTE and PTEN homologous inositol lipid phosphatase pseudogene	TCGAGTGTCTGAACTGGA	UUUAGUUCAGAAUACGUGg	CAGUGUUCUUGAGCGG#
40927	LOC40927	Hs LOC40927_2	NM_001034843 NR_002821 XM_376010	S00548667	TPTE and PTEN homologous inositol lipid phosphatase pseudogene	ATGGATGT1TCTCTGASTA	UACUCGAAAGAAACALCC#	GGAUUCUUCUUCUGAGUA#
40927	LOC40927	Hs LOC40927_3	NM_001034843 NR_002821 XM_376010	S00548674	TPTE and PTEN homologous inositol lipid phosphatase pseudogene	TCCACAGCAAAACGATTTAA	UUAAAUCUUCUUCUGUGg	CACAGCAAAAGAAUUA#

Supplementary Information Table 2. siRNA oligos targeting PP2A-B55 α , B55 δ , and Importin β 1.

Entrez Gene ID	NCBI gene symbol	Symbol used this study	Gene Description	mRNA Accessions	siRNA Target Sequence	Qiagen Product ID	Product Name	mRNA knockdown this study
5515	PPP2CA	CA_3	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_002715	ACACCTCGTGAATACAATTTA	SI00041853	Hs_PPP2CA_3	81%
5515	PPP2CA	CA_5	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_002715	ATGGAACTTGACGATACTCTA	SI02225763	Hs_PPP2CA_5	86%
5515	PPP2CA	CA_6	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_002715	CAAACAATCATTGGAGCTTAA	SI02225790	Hs_PPP2CA_6	87%
5515	PPP2CA	CA_7	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_002715	TAAGACGATGTGACTGCACAA	SI04436453	Hs_PPP2CA_7	78%
5515	PPP2CA	CA_8	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_002715	ATGGTGGTCTCTCGCCATCTA	SI04436460	Hs_PPP2CA_8	74%
5515	PPP2CA	CA_9	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_002715	TACAAAGCCTCTTGTATCAA	SI04436467	Hs_PPP2CA_9	80%
5518	PPP2R1A	R1A_1	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	NM_014225	TCCCATCTTGGCAAAGACAA	SI00103733	Hs_PPP2R1A_1	94%
5518	PPP2R1A	R1A_5	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	NM_014225	CTGGTGTCCGATGCCAACCAA	SI02225811	Hs_PPP2R1A_5	91%
5518	PPP2R1A	R1A_6	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	NM_014225	ACGGCTGAACATCATCTCTAA	SI02225818	Hs_PPP2R1A_6	71%
5518	PPP2R1A	R1A_7	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	NM_014225	GACCAGGATGTGGAGGTGAAA	SI04436495	Hs_PPP2R1A_7	95%
5518	PPP2R1A	R1A_8	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	NM_014225	ATCGGGTGTCTATAGACGAA	SI04436502	Hs_PPP2R1A_8	89%
5518	PPP2R1A	R1A_9	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	NM_014225	CACCTTGCAGAGTGGAAGTCAA	SI04436509	Hs_PPP2R1A_9	97%
5520	PPP2R2A	R2A_1	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	NM_002717	CTCGCCGTGTGGCACTGAA	SI00041895	Hs_PPP2R2A_1	72%
5520	PPP2R2A	R2A_3	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	NM_002717	AAGCGAGACATAACCCCTAGAA	SI00041909	Hs_PPP2R2A_3	76%
5520	PPP2R2A	R2A_5	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	NM_002717	CTGCAGATGATTTGCCGGATA	SI02225825	Hs_PPP2R2A_5	95%
5520	PPP2R2A	R2A_6	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	NM_002717	ATGGAAGGTATAGAGATCCTA	SI02225832	Hs_PPP2R2A_6	94%
5520	PPP2R2A	R2A_7	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	NM_002717	CCCGTCTTGGTGGTGGTATA	SI04436516	Hs_PPP2R2A_7	77%
5520	PPP2R2A	R2A_8	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	NM_002717	CAGTCTCATAGCAGAGGAGAA	SI04436523	Hs_PPP2R2A_8	93%
55844	PPP2R2D	B55 δ	protein phosphatase 2, regulatory subunit B, delta isoform	NM_001003656 NM_018461	CAGAGACTACCTGTCCGTGAA	SI00691523	Hs_PPP2R2D_1	72%
55844	PPP2R2D	B55 δ	protein phosphatase 2, regulatory subunit B, delta isoform	NM_001003656 NM_018461	CCGCTCCATTAAAGAACAGTGA	SI00691530	Hs_PPP2R2D_2	52%
55844	PPP2R2D	B55 δ	protein phosphatase 2, regulatory subunit B, delta isoform	NM_001003656 NM_018461	TTCATCCATATCCGATGTAAA	SI02759148	Hs_PPP2R2D_5	82%
3837	KPNB1	Importin β 1	karyopherin (importin) beta 1	NM_002265	TCGGTTATATTTGCCAAGATA	SI00035490	Hs_KPNB1_1	89%
3837	KPNB1	Importin β 1	karyopherin (importin) beta 1	NM_002265	CAAGAACTCTTTGACATCTAA	SI00035497	Hs_KPNB1_2	90%
3837	KPNB1	Importin β 1	karyopherin (importin) beta 1	NM_002265	AAGGGCGGAGATCGAAGACTA	SI00035504	Hs_KPNB1_3	89%

red = used for single or triple knockdown, this study.

Supplementary Information Table 3. Plasmids generated for this study

For efficient generation of cell lines stably expressing fluorescently tagged marker proteins, the genes were subcloned into pIRES-puro2 and pIRES-neo3 vectors (Clontech) that allow expression of resistance genes and tagged proteins from a single transcript.

Table 3. Generated plasmids

Plasmid name	Tag	Source plasmids	Vector backbone	Backbone cloning sites
pIBB-mEGFP-IRES-puro2b	mEGFP ¹	pIBB-mEGFP ²	pIRES-puro2b	EcoRI/NotI
pGalT-GFP-IRES-puro2b	GFP	pGalT-GFP (MluI blunted/EcoRI) ³	pIRES-puro2b	(NotI blunted/EcoRI)
pEGFP-PCNA-IRES-puro2b	mEGFP ¹	pNLS-EGFP-PCNA ⁴	pIRES-puro2b	(NdeI, XbaI)

mEGFP indicates monomeric EGFP.

Supplementary Information Table 4. Monoclonal fluorescent reporter cell lines

Stable cell lines were generated as described in ⁵.

Table 4. HeLa Kyoto monoclonal cell lines

Background	Cell line name	Plasmid 1	Plasmid 2
HeLa 'Kyoto'	H2B-mCherry	pH2B-mCherry-IRES-neo3	-
HeLa 'Kyoto'	H2B-mCherry and IBB-mEGFP	pH2B-mCherry-IRES-neo3	pIBB-mEGFP-IRES-puro2b
HeLa 'Kyoto'	H2B-mCherry and GalT-GFP	pH2B-mCherry-IRES-neo3	pGalT-GFP-IRES-puro2b
HeLa 'Kyoto'	H2B-mCherry and PCNA-mEGFP	pH2B-mCherry-IRES-neo3	pPCNA-mEGFP-IRES-puro2b
HeLa 'Kyoto'	H2B-mCherry and mEGFP- α -tubulin ⁶	pH2B-mCherry-IRES-neo3	pmEGFP- α -tubulin-IRES-puro2b

Supplementary references

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