Supporting information

Mass Spectrometry-compatible Subcellular Fractionation for Proteomics

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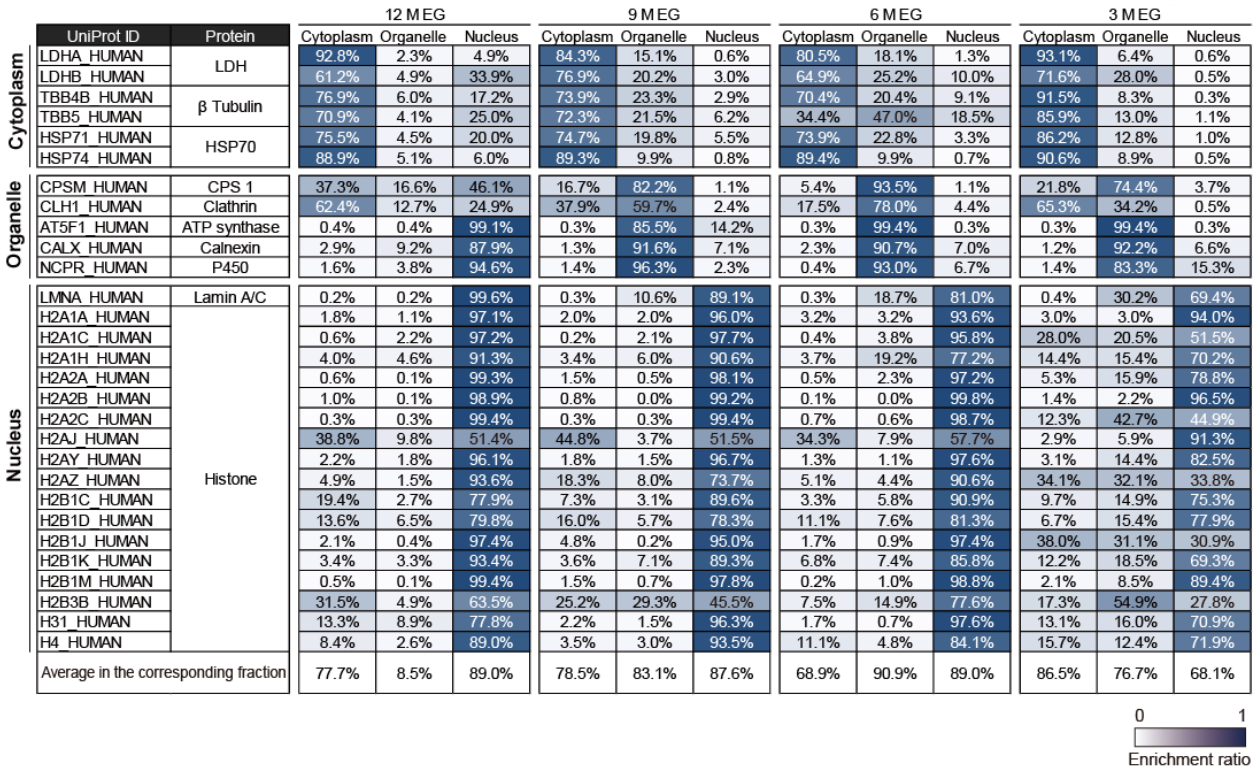
Table of Contents

Supplementary Table S1 Peak picking parameters used in Mass Navigator v1.2.

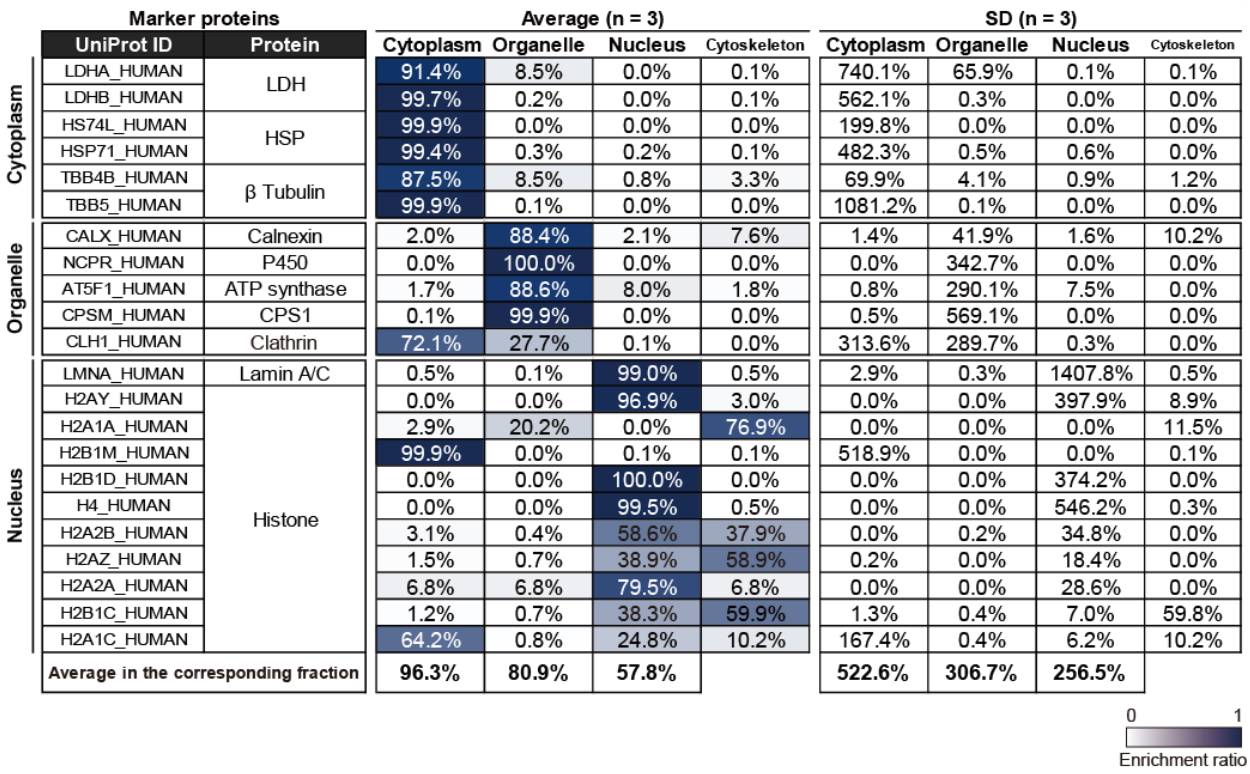
Parameters	LTQ-Orbitrap
Precursor charge state	auto
Survey scan centroid height percentage	50%
Survey scan, centroid merge distance	0.1
MSMS scan, centroid height percentage	50%
MSMS scan, centroid merge distance	0.1
Minimum peak with intensity	0.1 % of the highest peak or 10 absolute intensity
Minimum peak number in MSMS spectra	10
Precursor mass tolerance for grouping	0.1
Max number cycles between groups	10
Max number cycles between groups	1



	UniProt ID	Protein	12 MEG Cytoplasm	12 MEG Organelle	12 MEG Nucleus	9 MEG Cytoplasm	9 MEG Organelle	9 MEG Nucleus	6 MEG Cytoplasm	6 MEG Organelle	6 MEG Nucleus	3 MEG Cytoplasm	3 MEG Organelle	3 MEG Nucleus
Cytoplasm	LDHA_HUMAN	LDH	92.8%	2.3%	4.9%	84.3%	15.1%	0.6%	80.5%	18.1%	1.3%	93.1%	6.4%	0.6%
	LDHB_HUMAN		61.2%	4.9%	33.9%	76.9%	20.2%	3.0%	64.9%	25.2%	10.0%	71.6%	28.0%	0.5%
	TBB4B_HUMAN	β Tubulin	76.9%	6.0%	17.2%	73.9%	23.3%	2.9%	70.4%	20.4%	9.1%	91.5%	8.3%	0.3%
	TBB5_HUMAN		70.9%	4.1%	25.0%	72.3%	21.5%	6.2%	34.4%	47.0%	18.5%	85.9%	13.0%	1.1%
	HSP71_HUMAN	HSP70	75.5%	4.5%	20.0%	74.7%	19.8%	5.5%	73.9%	22.8%	3.3%	86.2%	12.8%	1.0%
	HSP74_HUMAN		88.9%	5.1%	6.0%	89.3%	9.9%	0.8%	89.4%	9.9%	0.7%	90.6%	8.9%	0.5%
Organelle	CPSM_HUMAN	CPS 1	37.3%	16.6%	46.1%	16.7%	82.2%	1.1%	5.4%	93.5%	1.1%	21.8%	74.4%	3.7%
	CLH1_HUMAN	Clathrin	62.4%	12.7%	24.9%	37.9%	59.7%	2.4%	17.5%	78.0%	4.4%	65.3%	34.2%	0.5%
	AT5F1_HUMAN	ATP synthase	0.4%	0.4%	99.1%	0.3%	85.5%	14.2%	0.3%	99.4%	0.3%	0.3%	99.4%	0.3%
	CALX_HUMAN	Calnexin	2.9%	9.2%	87.9%	1.3%	91.6%	7.1%	2.3%	90.7%	7.0%	1.2%	92.2%	6.6%
	NCPR_HUMAN	P450	1.6%	3.8%	94.6%	1.4%	96.3%	2.3%	0.4%	93.0%	6.7%	1.4%	83.3%	15.3%
Nucleus	LMNA_HUMAN	Lamin A/C	0.2%	0.2%	99.6%	0.3%	10.6%	89.1%	0.3%	18.7%	81.0%	0.4%	30.2%	69.4%
	H2A1A_HUMAN	Histone	1.8%	1.1%	97.1%	2.0%	2.0%	96.0%	3.2%	3.2%	93.6%	3.0%	3.0%	94.0%
	H2A1C_HUMAN		0.6%	2.2%	97.2%	0.2%	2.1%	97.7%	0.4%	3.8%	95.8%	28.0%	20.5%	51.5%
	H2A1H_HUMAN		4.0%	4.6%	91.3%	3.4%	6.0%	90.6%	3.7%	19.2%	77.2%	14.4%	15.4%	70.2%
	H2A2A_HUMAN		0.6%	0.1%	99.3%	1.5%	0.5%	98.1%	0.5%	2.3%	97.2%	5.3%	15.9%	78.8%
	H2A2B_HUMAN		1.0%	0.1%	98.9%	0.8%	0.0%	99.2%	0.1%	0.0%	99.8%	1.4%	2.2%	96.5%
	H2A2C_HUMAN		0.3%	0.3%	99.4%	0.3%	0.3%	99.4%	0.7%	0.6%	98.7%	12.3%	42.7%	44.9%
	H2AJ_HUMAN		38.8%	9.8%	51.4%	44.8%	3.7%	51.5%	34.3%	7.9%	57.7%	2.9%	5.9%	91.3%
	H2AY_HUMAN		2.2%	1.8%	96.1%	1.8%	1.5%	96.7%	1.3%	1.1%	97.6%	3.1%	14.4%	82.5%
	H2AZ_HUMAN		4.9%	1.5%	93.6%	18.3%	8.0%	73.7%	5.1%	4.4%	90.6%	34.1%	32.1%	33.8%
	H2B1C_HUMAN		19.4%	2.7%	77.9%	7.3%	3.1%	89.6%	3.3%	5.8%	90.9%	9.7%	14.9%	75.3%
	H2B1D_HUMAN		13.6%	6.5%	79.8%	16.0%	5.7%	78.3%	11.1%	7.6%	81.3%	6.7%	15.4%	77.9%
	H2B1J_HUMAN		2.1%	0.4%	97.4%	4.8%	0.2%	95.0%	1.7%	0.9%	97.4%	38.0%	31.1%	30.9%
	H2B1K_HUMAN		3.4%	3.3%	93.4%	3.6%	7.1%	89.3%	6.8%	7.4%	85.8%	12.2%	18.5%	69.3%
	H2B1M_HUMAN		0.5%	0.1%	99.4%	1.5%	0.7%	97.8%	0.2%	1.0%	98.8%	2.1%	8.5%	89.4%
	H2B3B_HUMAN		31.5%	4.9%	63.5%	25.2%	29.3%	45.5%	7.5%	14.9%	77.6%	17.3%	54.9%	27.8%
	H31_HUMAN		13.3%	8.9%	77.8%	2.2%	1.5%	96.3%	1.7%	0.7%	97.6%	13.1%	16.0%	70.9%
	H4_HUMAN		8.4%	2.6%	89.0%	3.5%	3.0%	93.5%	11.1%	4.8%	84.1%	15.7%	12.4%	71.9%
	Average in the corresponding fraction		77.7%	8.5%	89.0%	78.5%	83.1%	87.6%	68.9%	90.9%	89.0%	86.5%	76.7%	68.1%

Supplementary Figure S1. Optimization of the EG concentration in organellar fractionation.

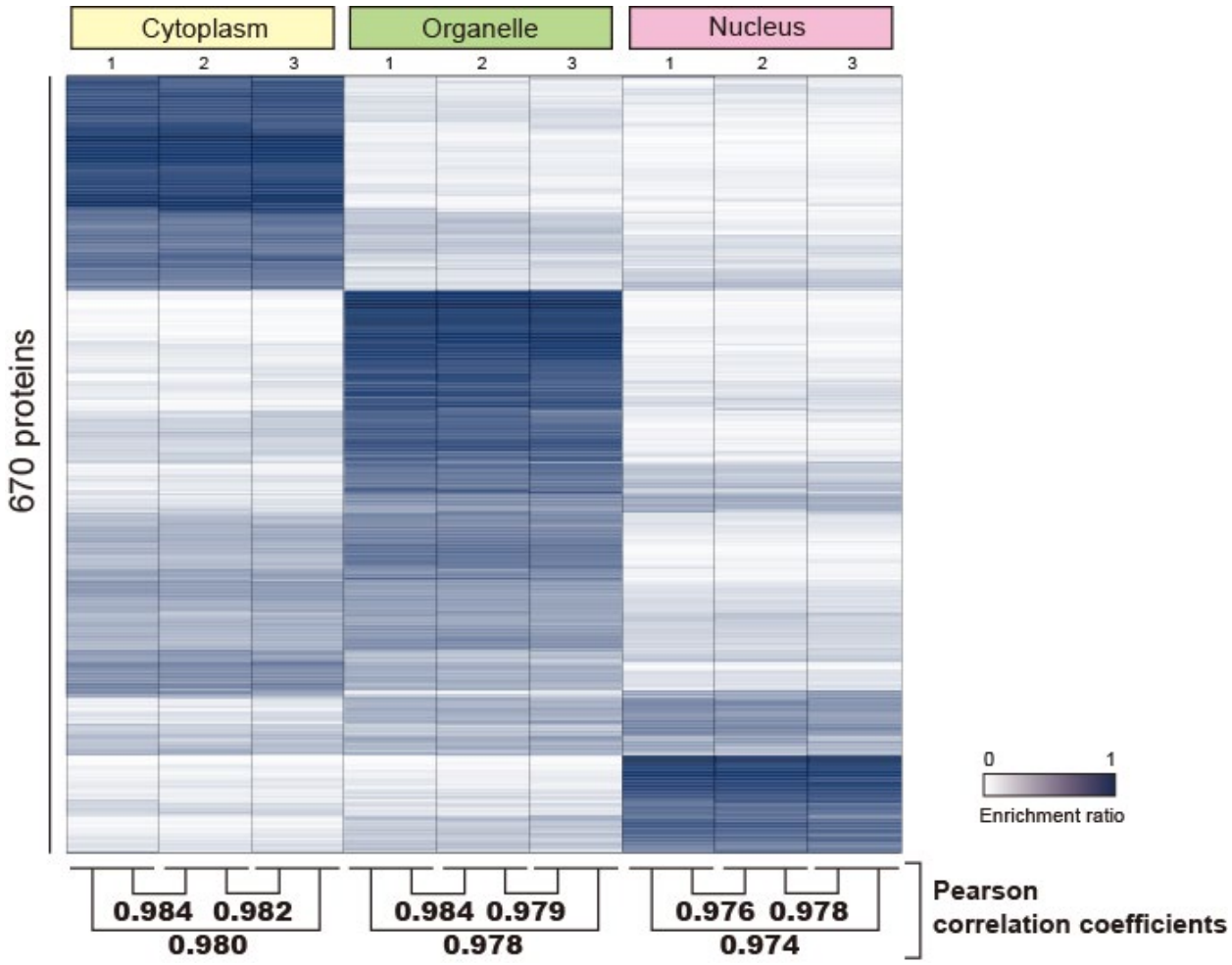
(A) Subcellular fractionation was performed with 3.5×10^6 HeLa cells. Four different MSC-RIPA buffers with concentrations of 12, 9, 6, and 3 M were used to extract the organellar fractions. After fractionation, SILAC-labeled whole cell lysate proteins (L-D_4-lysine and L-$^{13}C_6{}^{15}N_4$-arginine) were added to each fraction prior to shotgun proteomics. A total of 29 marker proteins identified in this examination, consisting of six cytoplasmic, five organellar, and 18 nuclear markers, were used to evaluate the developed protocol. Enrichment ratios in the appropriate fractions were averaged. The enrichment ratios in each appropriate fraction were shown by a heat map ranging from white to blue for regions with low to high enrichment ratios.



	Marker proteins		Average (n = 3)				SD (n = 3)			
	UniProt ID	Protein	Cytoplasm	Organelle	Nucleus	Cytoskeleton	Cytoplasm	Organelle	Nucleus	Cytoskeleton
Cytoplasm	LDHA_HUMAN	LDH	91.4%	8.5%	0.0%	0.1%	740.1%	65.9%	0.1%	0.1%
	LDHB_HUMAN		99.7%	0.2%	0.0%	0.1%	562.1%	0.3%	0.0%	0.0%
	HS74L_HUMAN	HSP	99.9%	0.0%	0.0%	0.0%	199.8%	0.0%	0.0%	0.0%
	HSP71_HUMAN		99.4%	0.3%	0.2%	0.1%	482.3%	0.5%	0.6%	0.0%
	TBB4B_HUMAN	β Tubulin	87.5%	8.5%	0.8%	3.3%	69.9%	4.1%	0.9%	1.2%
	TBB5_HUMAN		99.9%	0.1%	0.0%	0.0%	1081.2%	0.1%	0.0%	0.0%
Organelle	CALX_HUMAN	Calnexin	2.0%	88.4%	2.1%	7.6%	1.4%	41.9%	1.6%	10.2%
	NCPR_HUMAN	P450	0.0%	100.0%	0.0%	0.0%	0.0%	342.7%	0.0%	0.0%
	AT5F1_HUMAN	ATP synthase	1.7%	88.6%	8.0%	1.8%	0.8%	290.1%	7.5%	0.0%
	CPSM_HUMAN	CPS1	0.1%	99.9%	0.0%	0.0%	0.5%	569.1%	0.0%	0.0%
	CLH1_HUMAN	Clathrin	72.1%	27.7%	0.1%	0.0%	313.6%	289.7%	0.3%	0.0%
Nucleus	LMNA_HUMAN	Lamin A/C	0.5%	0.1%	99.0%	0.5%	2.9%	0.3%	1407.8%	0.5%
	H2AY_HUMAN	Histone	0.0%	0.0%	96.9%	3.0%	0.0%	0.0%	397.9%	8.9%
	H2A1A_HUMAN		2.9%	20.2%	0.0%	76.9%	0.0%	0.0%	0.0%	11.5%
	H2B1M_HUMAN		99.9%	0.0%	0.1%	0.1%	518.9%	0.0%	0.0%	0.1%
	H2B1D_HUMAN		0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	374.2%	0.0%
	H4_HUMAN		0.0%	0.0%	99.5%	0.5%	0.0%	0.0%	546.2%	0.3%
	H2A2B_HUMAN		3.1%	0.4%	58.6%	37.9%	0.0%	0.2%	34.8%	0.0%
	H2AZ_HUMAN		1.5%	0.7%	38.9%	58.9%	0.2%	0.0%	18.4%	0.0%
	H2A2A_HUMAN		6.8%	6.8%	79.5%	6.8%	0.0%	0.0%	28.6%	0.0%
	H2B1C_HUMAN		1.2%	0.7%	38.3%	59.9%	1.3%	0.4%	7.0%	59.8%
	H2A1C_HUMAN		64.2%	0.8%	24.8%	10.2%	167.4%	0.4%	6.2%	10.2%
	Average in the corresponding fraction		96.3%	80.9%	57.8%		522.6%	306.7%	256.5%	

Supplementary Figure S2. Enrichment ratios of marker proteins in S-PEK.

Subcellular fractionation was performed with S-PEK according to the instruction manual using 3.5 × 10^6 HeLa cells in triplicate. Acetone precipitation was conducted to remove incompatible surfactants prior to sample preparation for proteomics. SILAC-labeled whole cell lysate proteins (L-D_4-lysine and L-$^{13}C_6{}^{15}N_4$-arginine) were added to each fraction before reduction with DTT. A total of 23 marker proteins identified in this examination, consisting of six cytoplasmic, five organellar, and 12 nuclear markers, were used to evaluate the developed protocol. Their enrichment ratios were calculated as percentages by dividing the SILAC ratio by the total SILAC ratios over all fractions. To validate the accuracy of subcellular fractionation, the enrichment ratios in the corresponding fractions were averaged. The enrichment ratios were shown by a heat map ranging from white to blue for regions with low to high enrichment ratios.



Supplementary Figure S3. Reproducibility of the developed protocol.

The cytoplasmic, organellar, and nuclear fractions were collected from 3.5×10^6 HeLa cells in triplicate according to the developed protocol. SILAC-labeled whole cell lysate proteins (L-D_4-lysine and L-$^{13}C_6{}^{15}N_4$-arginine) were added to each fraction prior to reduction with DTT. Proteins with a minimum of two peptides with at least seven amino acid residues were employed for this evaluation. The enrichment ratios of 670 proteins quantified in all fractions were shown by a heat map ranging from white to blue for regions with low to high enrichment ratios. The Pearson correlation coefficient was calculated for each fraction.

A

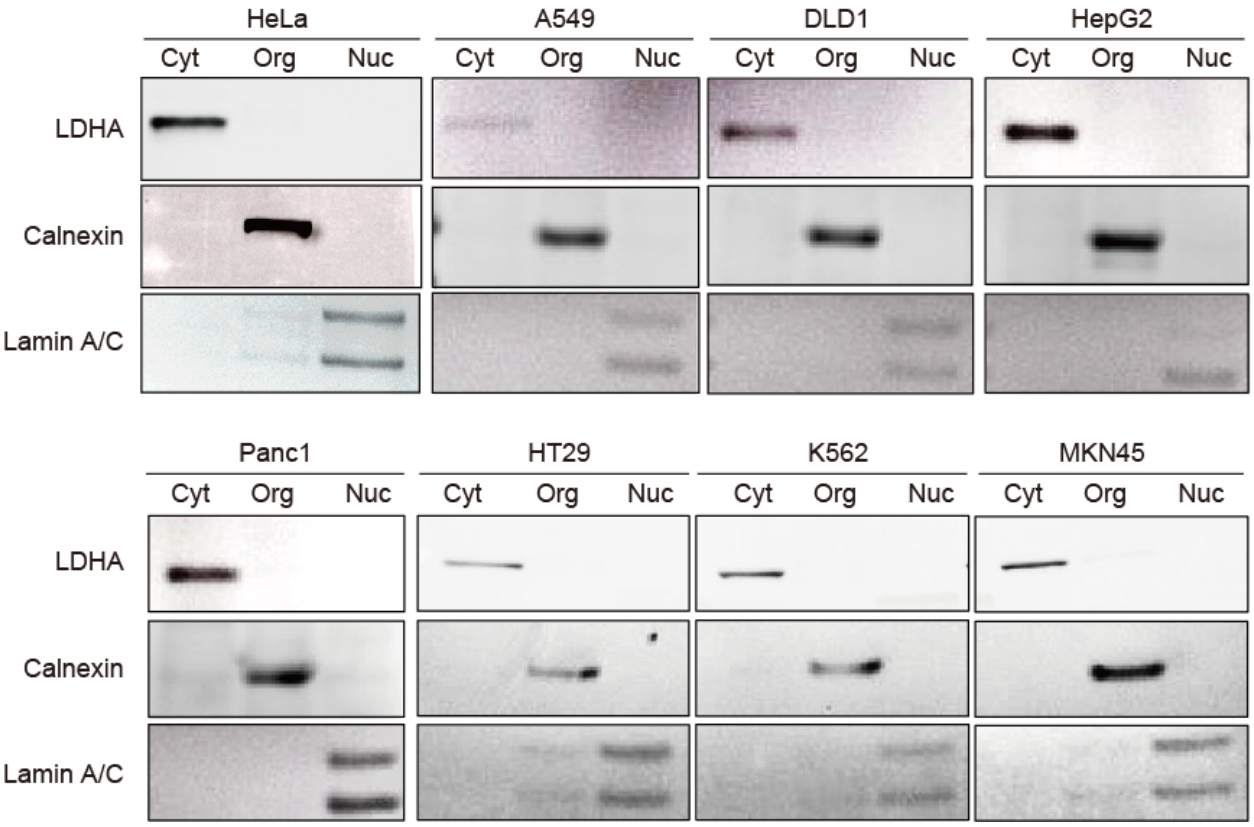
	Marker proteins		Developed protocol (n = 3)					
			Average			S.D.		
	UniProt ID	Protein	Cyt	Org	Nuc	Cyt	Org	Nuc
Cytoplasm	LDHA_HUMAN	LDH	80.0%	18.6%	1.4%	1.1%	1.6%	1.8%
	LDHB_HUMAN		79.1%	19.1%	1.8%	4.7%	2.8%	2.8%
	HSP74_HUMAN	HSP	49.5%	48.4%	2.1%	1.5%	2.2%	3.4%
	HSP71_HUMAN		40.6%	48.3%	11.2%	3.5%	4.4%	1.1%
	TBB4B_HUMAN	β Tubulin	69.0%	27.9%	3.1%	1.7%	2.0%	3.4%
	TBB5_HUMAN		64.0%	31.5%	4.5%	3.3%	0.7%	4.0%
Organelle	CALX_HUMAN	Calnexin	0.3%	96.8%	2.9%	0.0%	2.8%	2.8%
	NCPR_HUMAN	P450	2.1%	95.3%	2.6%	0.8%	3.4%	4.1%
	AT5F1_HUMAN	ATP synthase	6.5%	66.6%	26.9%	3.2%	2.4%	4.8%
	CPSM_HUMAN	CPS1	9.3%	50.9%	39.8%	1.4%	2.6%	2.4%
	CLH1_HUMAN	Clathrin	26.4%	70.5%	3.1%	0.9%	2.7%	3.6%
Nucleus	LMNA_HUMAN	Lamin A/C	4.4%	12.3%	83.4%	0.2%	1.7%	1.5%
	H2AY_HUMAN	Histone	0.3%	0.3%	99.4%	0.1%	0.1%	0.2%
	H2A1A_HUMAN		1.4%	1.1%	97.5%	0.3%	0.2%	0.4%
	H2B1M_HUMAN		2.0%	1.9%	96.1%	0.5%	0.5%	0.9%
	H2B1D_HUMAN		1.4%	1.4%	97.1%	0.4%	0.4%	0.8%
	H4_HUMAN		1.1%	1.1%	97.8%	0.3%	0.6%	0.9%
	H2AZ_HUMAN		4.4%	1.9%	93.7%	0.5%	0.2%	0.7%
	H31_HUMAN		5.1%	5.3%	89.6%	0.3%	1.6%	1.5%
	Average in the corresponding fraction		**63.7%**	**76.0%**	**94.3%**	**2.6%**	**2.8%**	**0.9%**

B

	Marker proteins		S-PEK (n = 3)							
			Average				S.D.			
	UniProt ID	Protein	Cyt	Org	Nuc	CSK	Cyt	Org	Nuc	CSK
Cytoplasm	LDHA_HUMAN	LDH	46.2%	17.0%	11.3%	25.5%	5.1%	2.3%	1.3%	6.1%
	LDHB_HUMAN		45.8%	16.4%	13.2%	24.6%	3.9%	2.1%	1.9%	4.1%
	HSP74_HUMAN	HSP	39.5%	15.3%	16.0%	29.2%	8.8%	3.1%	3.7%	8.5%
	HSP71_HUMAN		30.3%	15.1%	16.7%	38.0%	7.4%	3.1%	2.1%	8.9%
	TBB4B_HUMAN	β Tubulin	45.8%	15.8%	10.0%	28.4%	7.2%	1.9%	1.1%	7.7%
	TBB5_HUMAN		45.2%	17.2%	10.8%	26.8%	5.2%	0.5%	2.1%	4.7%
Organelle	CALX_HUMAN	Calnexin	2.4%	44.6%	18.1%	34.9%	1.2%	13.2%	4.8%	8.3%
	NCPR_HUMAN	P450	4.0%	49.4%	15.5%	31.1%	4.2%	8.2%	7.2%	0.5%
	AT5F1_HUMAN	ATP synthase	6.4%	18.7%	27.0%	48.0%	3.0%	6.0%	6.1%	2.7%
	CPSM_HUMAN	CPS1	11.9%	30.7%	14.5%	42.9%	4.3%	9.3%	4.0%	16.7%
	CLH1_HUMAN	Clathrin	25.2%	17.5%	13.2%	44.1%	4.0%	4.5%	2.2%	6.6%
Nucleus	LMNA_HUMAN	Lamin A/C	5.3%	1.8%	19.8%	73.2%	2.0%	0.4%	2.4%	1.6%
	H2AY_HUMAN	Histone	2.3%	1.1%	35.7%	60.9%	2.4%	0.8%	5.6%	5.0%
	H2A1A_HUMAN		3.9%	1.8%	28.7%	65.6%	1.8%	0.2%	5.4%	5.5%
	H2B1M_HUMAN		4.8%	3.0%	30.0%	62.1%	1.4%	0.1%	5.4%	5.2%
	H2B1D_HUMAN		4.5%	2.1%	31.6%	61.7%	2.3%	0.2%	3.6%	3.2%
	H4_HUMAN		4.0%	3.4%	31.0%	61.7%	1.3%	0.6%	6.4%	6.2%
	H2AZ_HUMAN		4.9%	1.0%	30.9%	63.2%	1.0%	0.1%	7.3%	7.1%
	H31_HUMAN		7.1%	5.4%	29.8%	57.6%	1.7%	1.4%	3.5%	3.0%
	Average in the corresponding fraction		**42.1%**	**32.2%**	**29.7%**		**6.3%**	**8.2%**	**4.9%**	

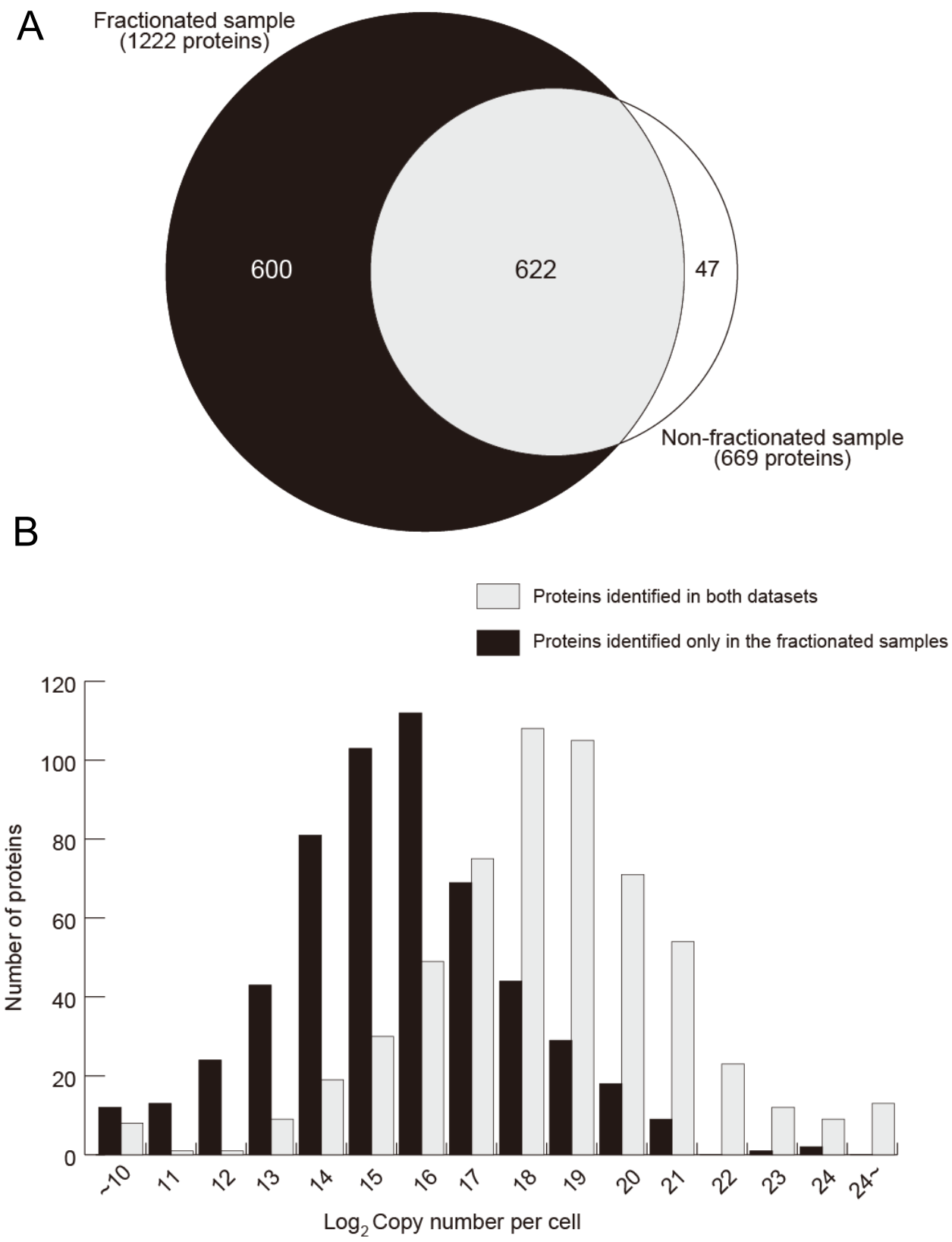
Supplementary Figure S4. Enrichment ratios of marker proteins from 3.5×10^5 HeLa cells.

HeLa cells were cultured in DMEM supplemented with 10% FCS. The number of cells was counted with a hemocytometer. (A) A 20 μL-scale subcellular fractionation was conducted in triplicate using 3.5×10^5 HeLa cells. SILAC-labeled proteins (L-D_4-lysine and L-$^{13}C_6{}^{15}N_4$-arginine) were added to each fraction prior to shotgun proteomics. A total of 19 marker proteins identified in this examination, consisting of six cytoplasmic, five organellar, and eight nuclear markers, were used to evaluate the developed protocol. The averages and standard deviations of the enrichment ratios were obtained from triplicate analyses. The enrichment ratios in each fraction were shown by a heat map ranging from white to blue for regions with low to high enrichment ratios. (B) Subcellular fractionation using S-PEK was conducted in triplicate as per the instruction manual with minor modifications. We reduced the buffer volume to one-tenth. After separating into cytoplasmic, organellar, nuclear, and cytoskeletal (CSK) fractions, the incompatible surfactants were removed by acetone precipitation. The averages and standard deviations of the enrichment ratios were obtained from triplicate analyses.



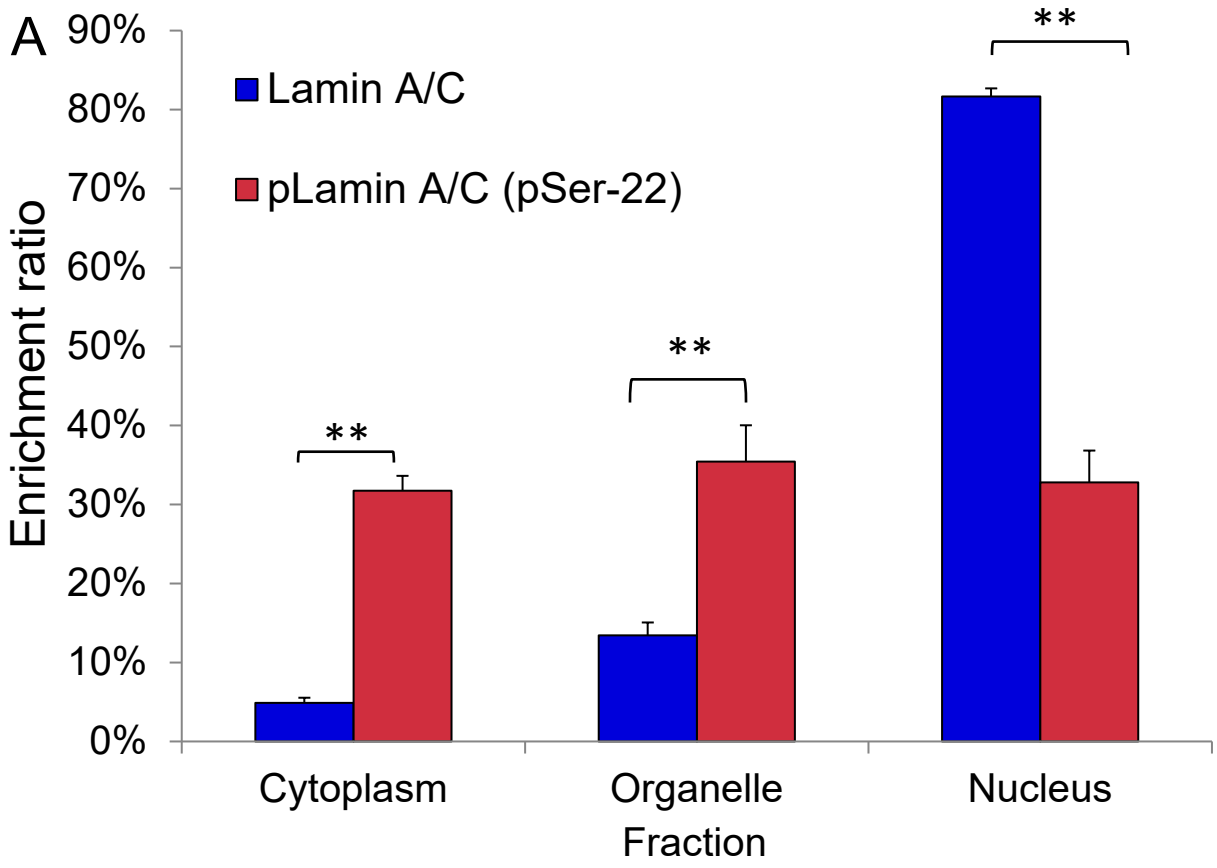
Supplementary Figure S5. Application of the developed protocol to several cell lines.

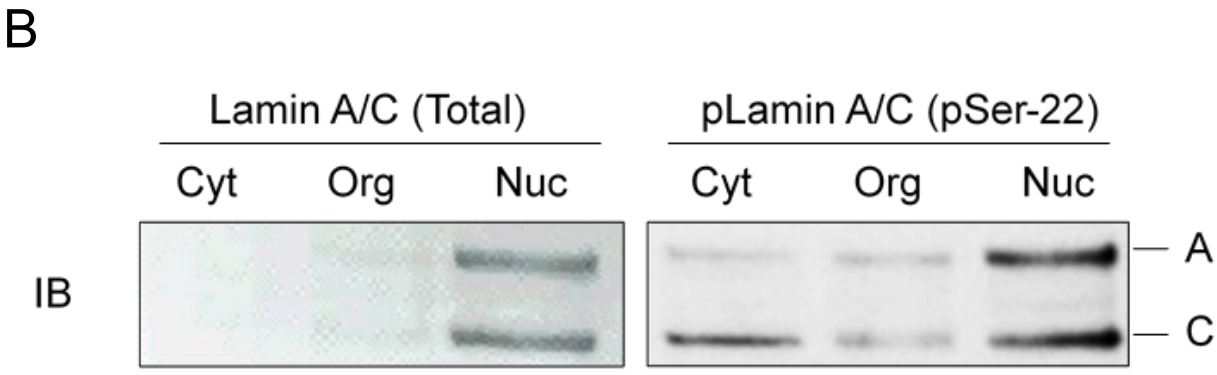
HeLa, A549, DLD1, HepG2, Panc1, HT29, K562, and MKN45 cells were used in this experiment. Cells were grown in DMEM or RPMI supplemented with 10% FCS. Cell numbers were counted with a hemocytometer. The developed protocol was applied to 3.5×10^6 cells. One-fiftieth by volume of each fraction was subjected to 10%–20% SDS-PAGE, and LDHA, calnexin, and lamin A/C were detected by immunoblotting using the appropriate antibody. Full blots are shown in Supplementary Figure S9.


A
Fractionated sample
(1222 proteins)
600
622
47
Non-fractionated sample
(669 proteins)
B
Proteins identified in both datasets
Proteins identified only in the fractionated samples
Number of proteins
120
100
80
60
40
20
0
~10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
24~
Log2 Copy number per cell

Supplementary Figure S6. Effect of subcellular fractionation on the identification efficiency.

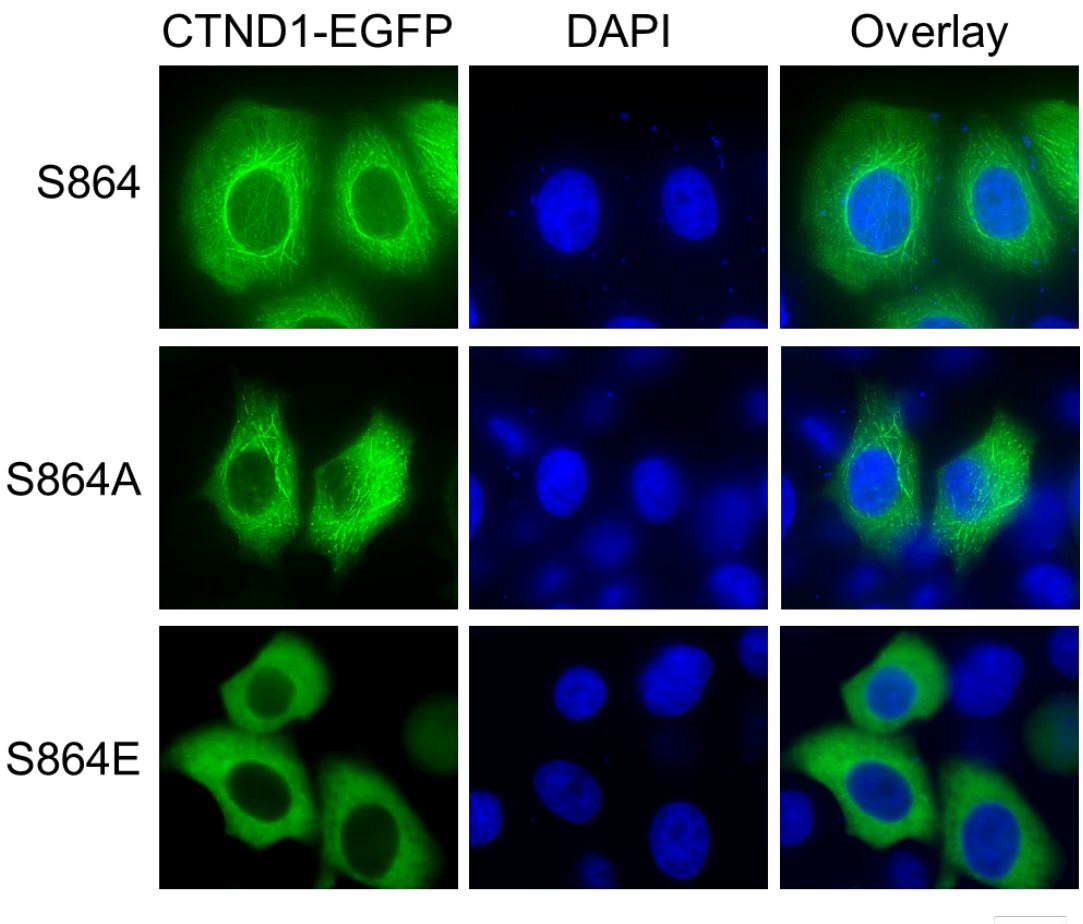
For details, see legend to Figure 5. (A) Venn diagram showing the number of identified proteins in the fractionated and unfractionated samples. The numbers of proteins with a minimum of two peptides with at least seven amino acid residues identified in each fraction are shown. (B) The distribution of the copy number of proteins. The copy numbers referred to a previous paper reported by Beck et al[19]


A
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Enrichment ratio
Lamin A/C
pLamin A/C (pSer-22)
**
**
**
Cytoplasm
Organelle
Fraction
Nucleus


B
Lamin A/C (Total)
pLamin A/C (pSer-22)
Cyt
Org
Nuc
Cyt
Org
Nuc
IB
A
C

Supplementary Figure S7. Changes in lamin A/C subcellular localization depending on the phosphorylation state.

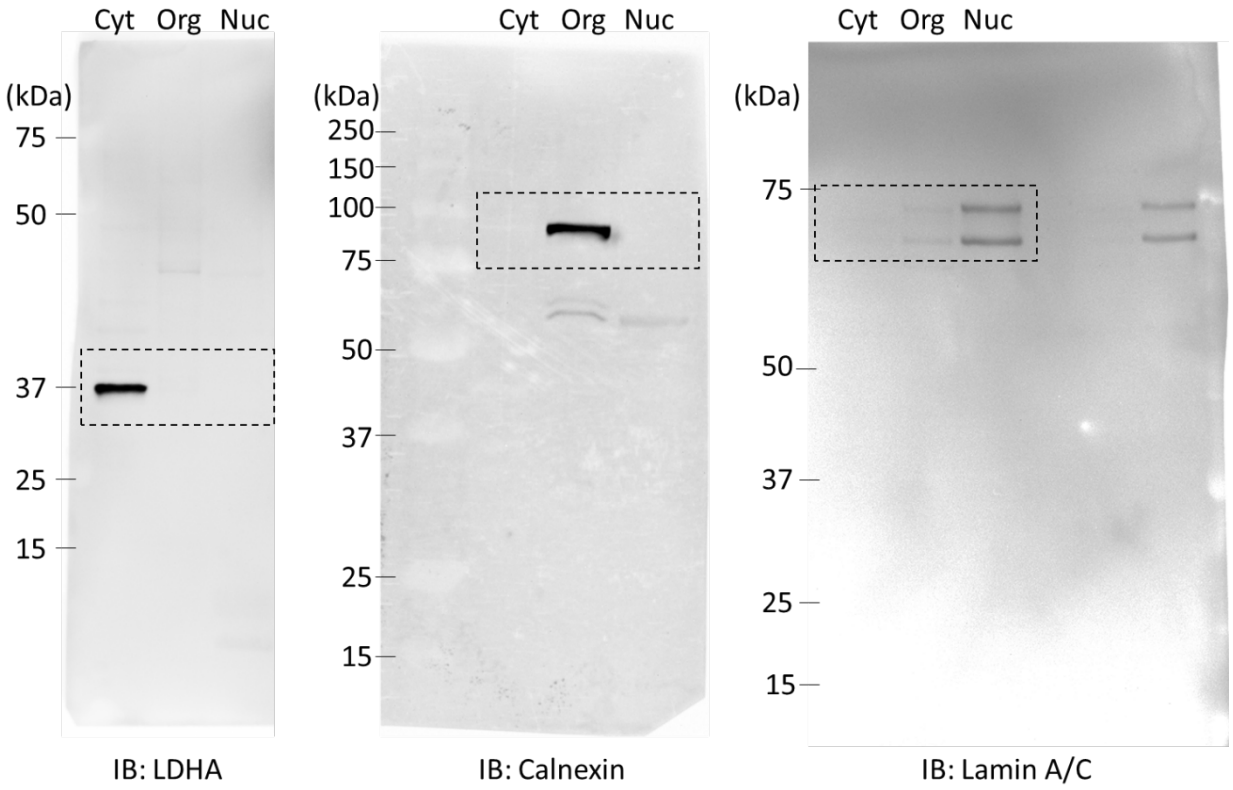
(A) Average enrichment ratios were compared for lamin A/C protein and Ser-22 phosphopeptides in each fraction. Significant differences (*t*-test) in enrichment ratios were observed for all three fractions. For details, see the legend to Figure 6. Error bars represent the standard deviation. ** $p < 0.01$. (B) After separation using the developed protocol, one-fiftieth of the fractionated samples was subjected to 10%–20% SDS-PAGE, and lamin A/C and Ser-22 phosphorylated lamin A/C were detected by immunoblotting (IB). Full blots are shown in Supplementary Figure S9.



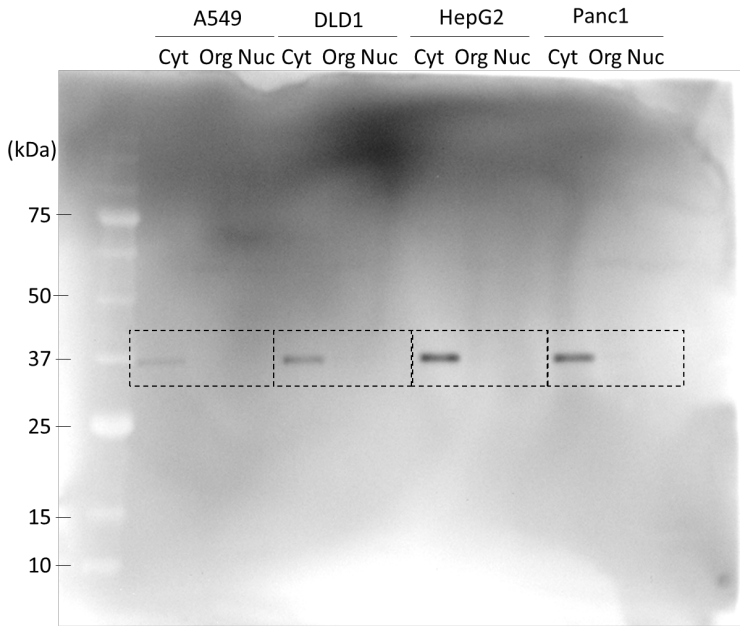
Supplementary Figure S8. Subcellular localization of CTND1 with phosphor-null (S864A) and phosphomimetic (S864E) mutations.

HeLa cells were transfected with plasmids expressing EGFP-fused CTND1 along with S864, S864A, or S864E. At 24 h after transfection, fluorescence images were acquired using BZ-X800.

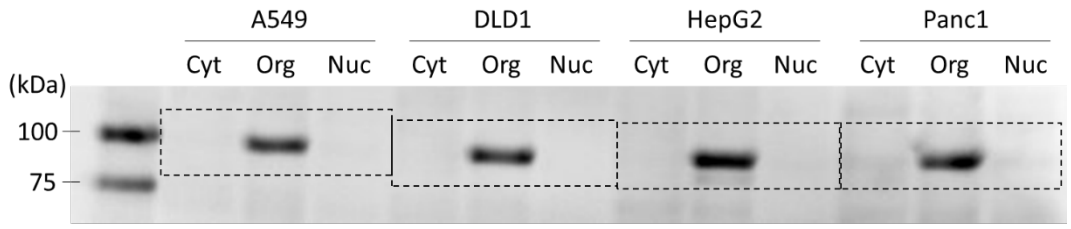
A


Cyt Org Nuc
(kDa)
75
50
37
25
15
IB: LDHA
Cyt Org Nuc
(kDa)
250
150
100
75
50
37
25
15
IB: Calnexin
Cyt Org Nuc
(kDa)
75
50
37
25
15
IB: Lamin A/C

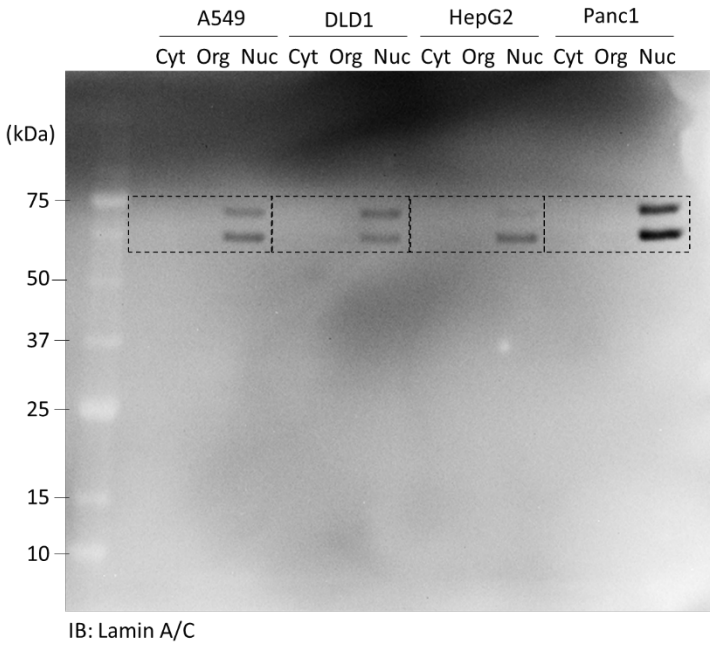
B



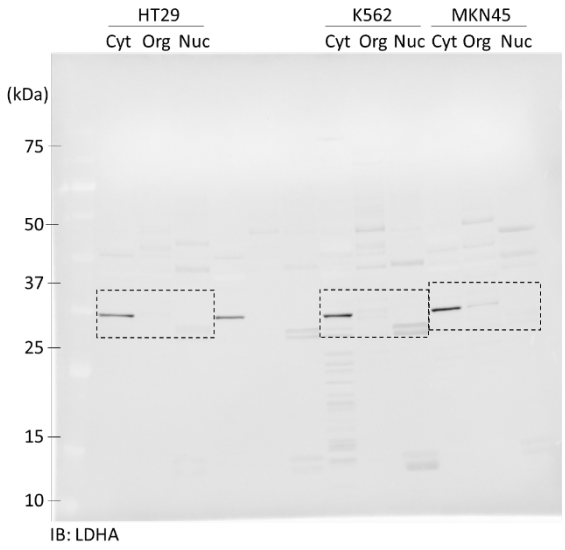
IB: LDHA



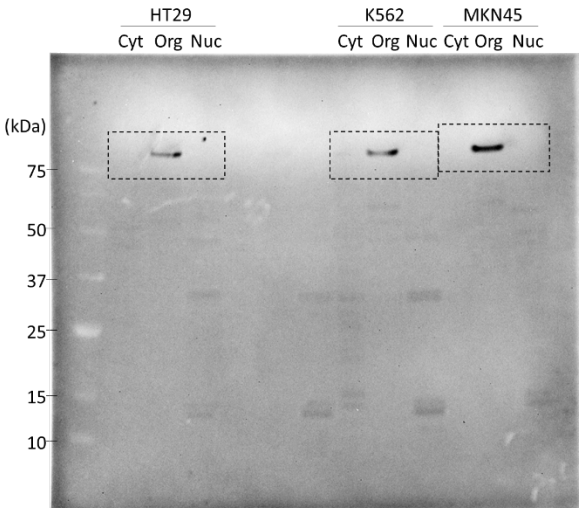
IB: Calnexin



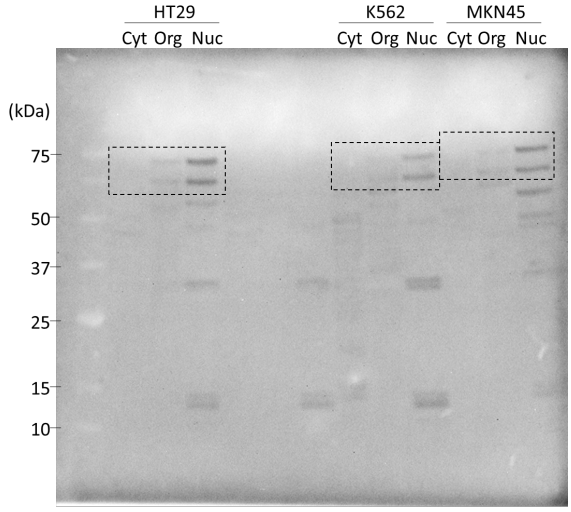
IB: Lamin A/C



IB: LDHA

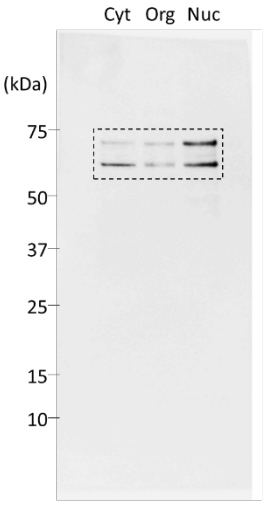


IB: Calnexin



IB: Lamin A/C

C



IB: pLamin A/C (Ser-22)

Supplementary Figure S9. Full blots of all immunoblotting results.

Subcellular fractionation was performed using 3.5×10^6 cells. For details, see the legends of Figure 3 or Supplementary Figure S5. Full blots used in Figure 3, Supplementary Figure S5, and Supplementary Figure S7 are shown in (A), (B), and (C), respectively. The blots of HeLa cells (A) are also used in Supplementary Figure S5 and Supplementary Figure S7.