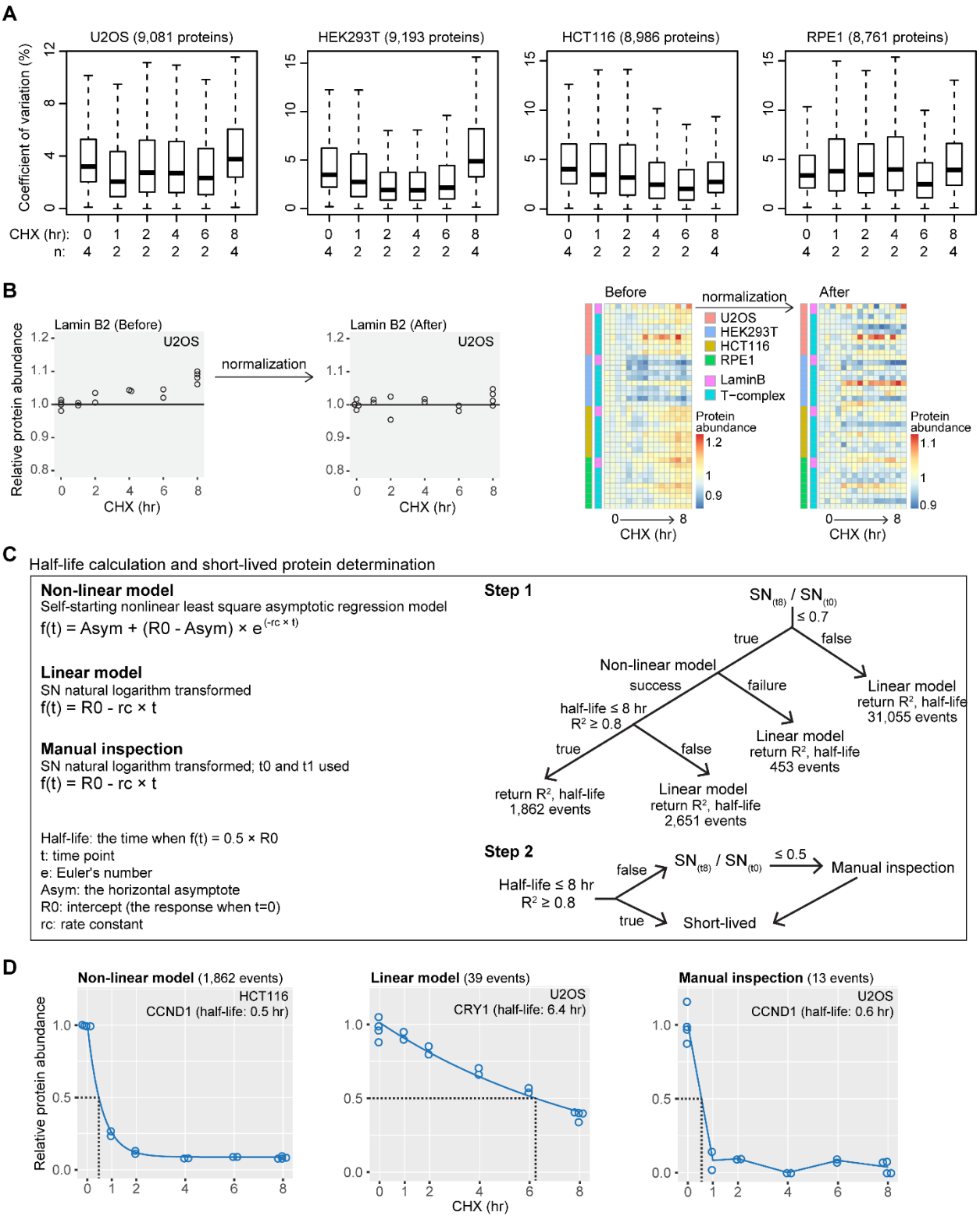


Figure S1. Data quality control and determination of short-lived proteins under translational inhibition. Related to Figure 1. (**A**) Protein coefficient of variation (CV) of replicates. A median CV of less than 5% was observed for all time points, demonstrating low variance within replicates. Data are presented as box plots (center line: median; box limits: the first and third quartiles; whiskers: 1.5x

interquartile range; outliers not shown). (**B**) Data normalization by long-lived proteins. Lamin B2 was one of the reported long-lived proteins and its quantification before and after the normalization is shown in the left panel. An overview of all long-lived proteins used is shown in the right panel. Overall normalization factors were small and within a range of 0.9-1.1. See Methods for details. (**C**) A non-linear or linear model was fitted to the time course of TMTpro signal-to-noise (SN) or natural logarithm transformed TMTpro SN. Proteins with half-lives shorter than 8 hr (≥ 50% loss in protein abundance at the last time point) and coefficients of determination (R^2) greater than 0.8 were defined as short-lived proteins. See Methods for details. (**D**) Examples showing protein half-lives calculated via a non-linear model, linear model and manual inspection. The vast majority of the short-lived protein events was captured by non-linear models (1,862 events). Note that the y-axis indicates untransformed relative protein abundance, and the linear model uses natural logarithm transformed relative protein abundance, therefore the fitted line in the linear model panel in the middle is a curve, not a straight line.

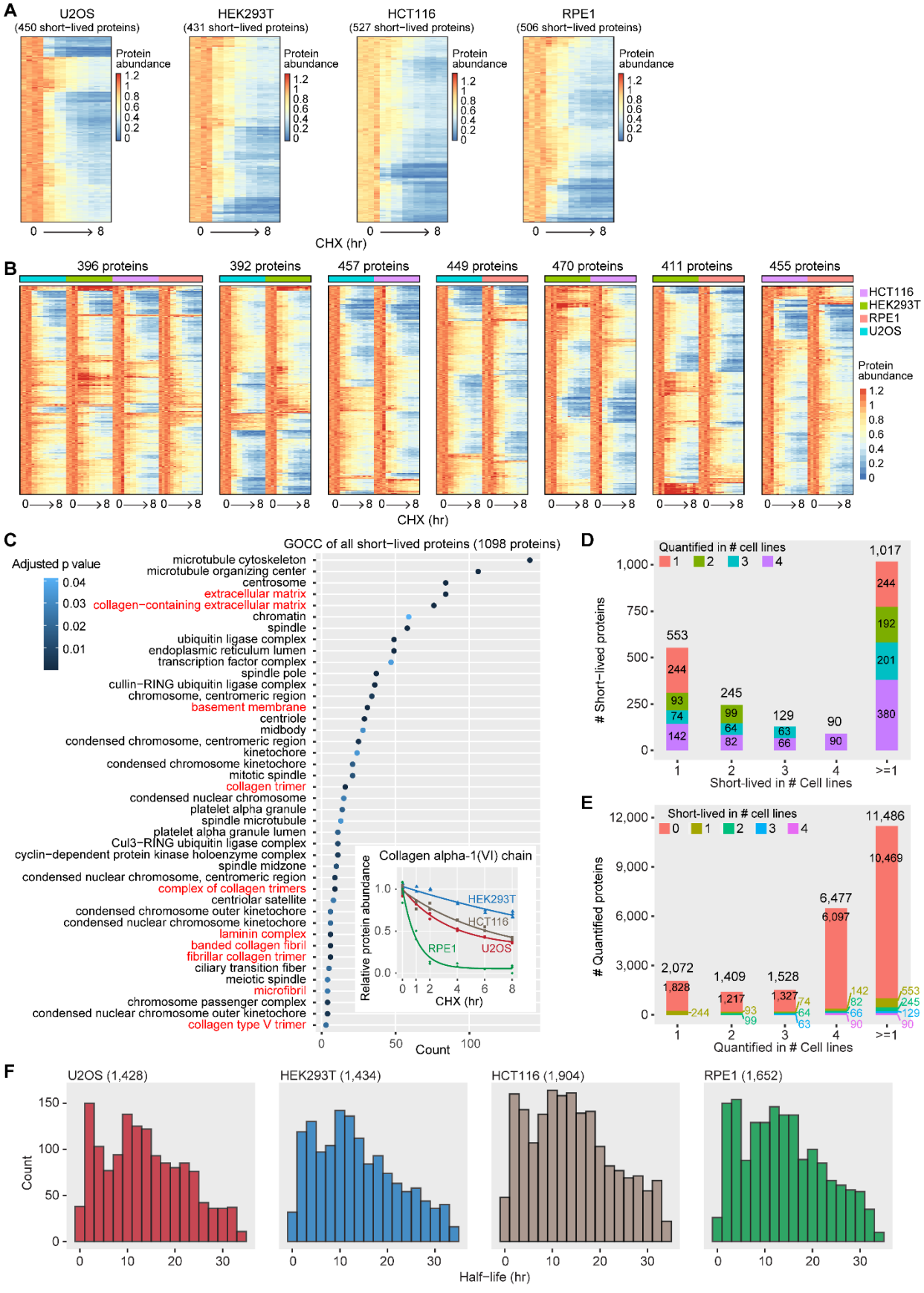

A
U2OS
(450 short−lived proteins)
HEK293T
(431 short−lived proteins)
HCT116
(527 short−lived proteins)
RPE1
(506 short−lived proteins)
Protein abundance
CHX (hr)
B
396 proteins
392 proteins
457 proteins
449 proteins
470 proteins
411 proteins
455 proteins
HCT116
HEK293T
RPE1
U2OS
Protein abundance
CHX (hr)
C
GOCC of all short−lived proteins (1098 proteins)
Adjusted p value
microtubule cytoskeleton
microtubule organizing center
centrosome
extracellular matrix
collagen−containing extracellular matrix
chromatin
spindle
ubiquitin ligase complex
endoplasmic reticulum lumen
transcription factor complex
spindle pole
cullin−RING ubiquitin ligase complex
chromosome, centromeric region
basement membrane
centriole
midbody
condensed chromosome, centromeric region
kinetochore
condensed chromosome kinetochore
mitotic spindle
collagen trimer
condensed nuclear chromosome
platelet alpha granule
spindle microtubule
platelet alpha granule lumen
Cul3−RING ubiquitin ligase complex
cyclin−dependent protein kinase holoenzyme complex
spindle midzone
condensed nuclear chromosome, centromeric region
complex of collagen trimers
centriolar satellite
condensed chromosome outer kinetochore
condensed nuclear chromosome kinetochore
laminin complex
banded collagen fibril
fibrillar collagen trimer
ciliary transition fiber
meiotic spindle
microfibril
chromosome passenger complex
condensed nuclear chromosome outer kinetochore
collagen type V trimer
Count
Collagen alpha−1(VI) chain
Relative protein abundance
HEK293T
HCT116
RPE1
U2OS
CHX (hr)
D
Quantified in # cell lines
Short−lived proteins
Short−lived in # Cell lines
E
Short−lived in # cell lines
Quantified proteins
Quantified in # Cell lines
F
U2OS (1,428)
HEK293T (1,434)
HCT116 (1,904)
RPE1 (1,652)
Count
Half−life (hr)

Figure S2. An overview of short-lived proteins under translational arrest. Related to Figure 2. (**A**) Heatmaps of short-lived proteins detected in each cell line. Replicates are shown individually in the heatmaps. Protein abundance is TMTpro signal-to-noise normalized to the mean of the first time point. (**B**) Heatmaps for all short-lived proteins stratified by their cell line detection. Each short-lived protein was examined for its degradation profile across the other cell lines. The first heatmap shows 396 short-lived proteins detected in all four cell lines. There was a visible trend for most proteins to be short-lived across multiple cell lines. To better examine the trends and to account for proteins not expressed or not detected in a cell line, all potential pairs of cell lines are shown highlighting proteins that were detected in both lines. Protein abundance is TMTpro signal-to-noise normalized to the mean of the first time point. (**C**) Subcellular components enriched among proteins that were short-lived in at least one cell line. Adjusted p-values (Benjamini-Hochberg) were filtered at 0.05. Categories highlighted in red are extracellular matrix proteins. Extracellular matrix protein collagen alpha-1 (VI) chain, a known long-lived protein, is highlighted. (**D**) Short-lived proteins shared by different cell lines. Extracellular matrix proteins were excluded from this analysis. For example, the third bar means that 129 proteins were short-lived in three cell lines. Among the 129 proteins, 63 were found in the same three cell lines and 66 were found in all four cell lines. (**E**) Quantified proteins shared by different cell lines. Extracellular matrix proteins were excluded from this analysis. For example, the second bar means that 1,409 protein were detected in two cell lines. Among the 1,409 proteins, 99 proteins were short-lived in both cell lines, 93 proteins were short-lived in only one of the two cell lines, and 1,217 were short-lived in neither cell lines. (**F**) Distributions of all measured protein half-lives under translational arrest in the four cell lines (data in Table S2). Values in parentheses are numbers of proteins. Bin size is 2 hr.

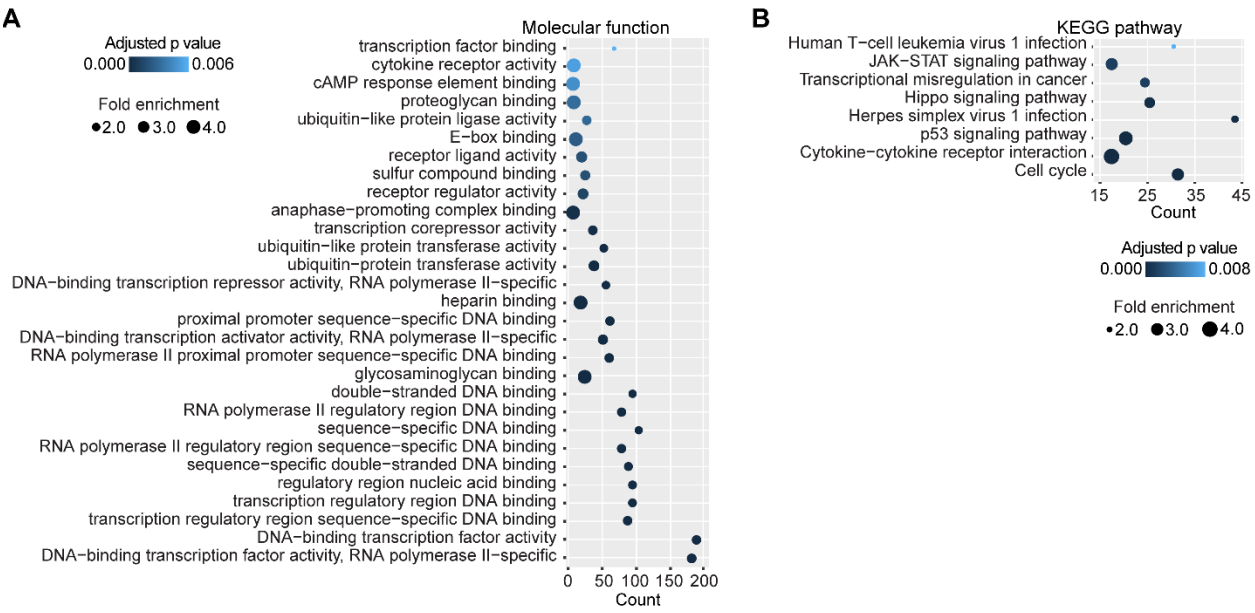


Figure S3. Gene Ontology analysis of short-lived proteins. Related to Figure 3. Proteins that were short-lived in at least one cell line were used as the foreground. All quantified proteins were used as the background. (**A**) Molecular functions enriched among short-lived proteins. (**B**) KEGG pathways enriched among short-lived proteins.

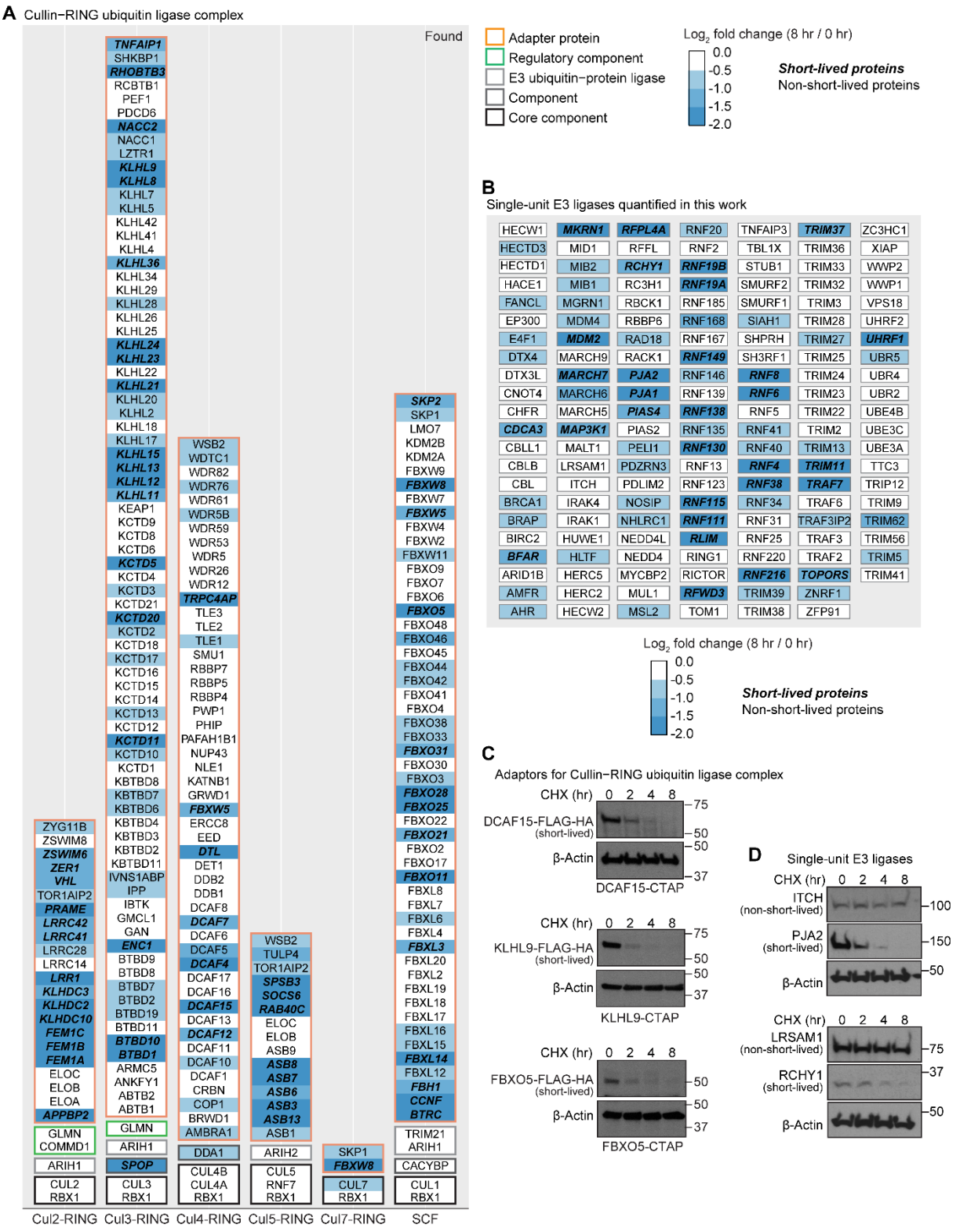


Figure S4. Cullin−RING ubiquitin ligase complex members and single-unit E3 ligases found in this study. Related to Figure 4. (**A**) Core components of cullin-RING ubiquitin ligase complexes were stable, and substrate recognition subunits tended to be short-lived. The hand-curated list was kindly provided by Dr. Wade Harper's laboratory at Harvard Medical School as an alternative to the GO category gene set in

the main text. (**B**) Single-unit E3 ligases found in this work. There were 154 single-unit E3 ligases in the list from the literature (Li et al., 2017). We detected 152 of them. SYVN1 and RNF170 were not found. (**C**) Western blotting showed that three adapter proteins for E3 ubiquitin ligase complexes (DCAF15, KLHL9, and FBXO5) were short-lived in U2OS cells. The three proteins were blotted with anti-FLAG. (**D**) Western blotting showed that two single-unit E3 ligases (PJA2 and RCHY1) were short-lived, and two other single-unit E3 ligases (ITCH and LRSAM1) were stable in U2OS cells.

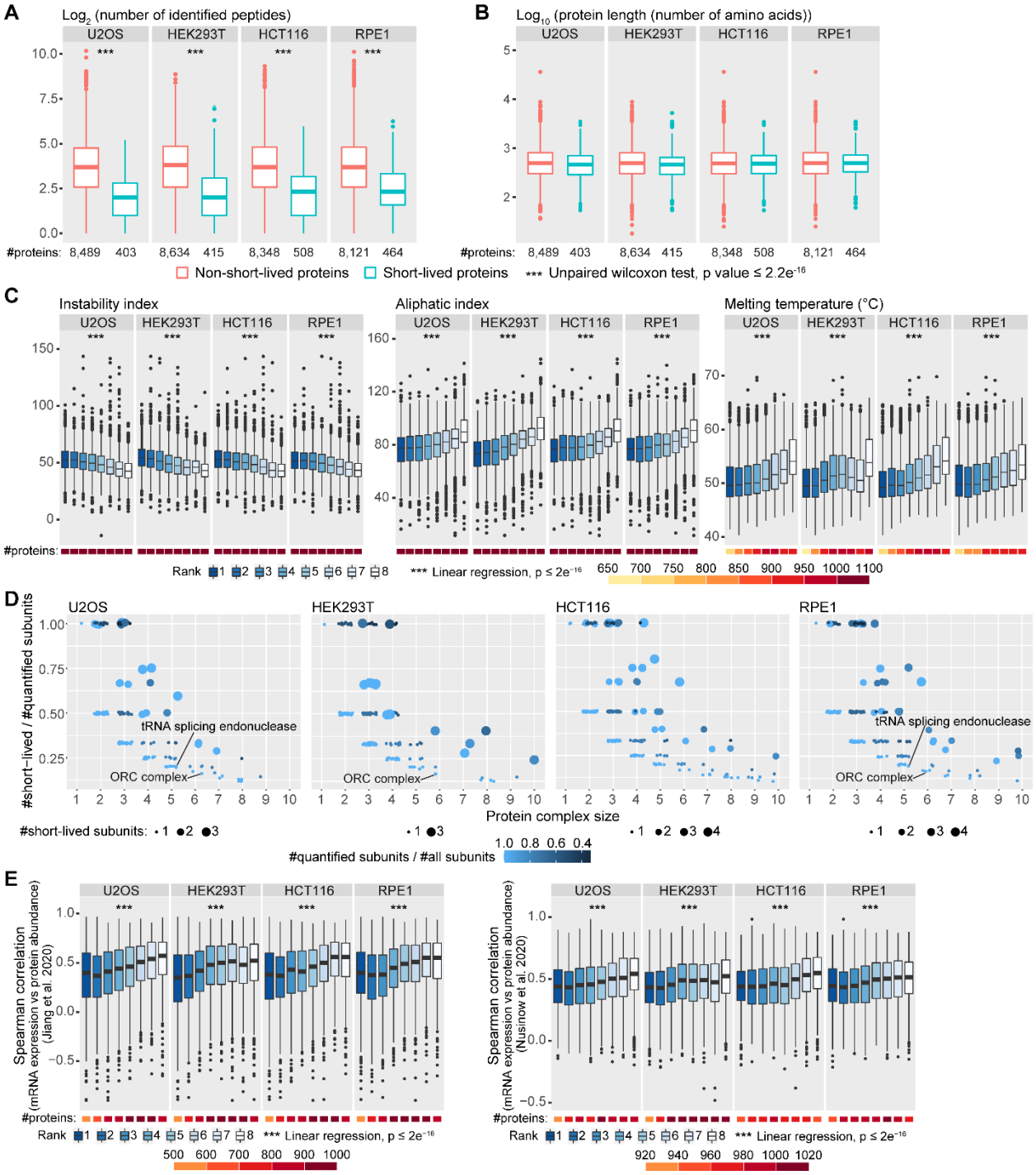


Figure S5. Properties of short-lived proteins under translational inhibition. Related to Figure 5. (**A**) Fewer peptides were identified for short-lived proteins compared to non-short-lived proteins. (**B**) Short-lived proteins and non-short-lived proteins showed similar protein length distributions. (**C**) The least stable proteins (Rank 1) showed higher instability index, lower aliphatic index and lower melting temperatures compared to the most stable proteins (Rank 8). (**D**) Diverse fractions of subunits were short-lived in small protein complexes in the CORUM database. Only protein complexes with less than ten subunits were included. (**E**) The least stable proteins (Rank 1) showed lower mRNA expression and protein abundance

correlation across human tissues (left) and cancer cell lines (right) compared to the most stable proteins (Rank 8). Data are presented as box plots (center line: median; box limits: the first and third quartiles; whiskers: 1.5x interquartile range) in panel A-C and E.

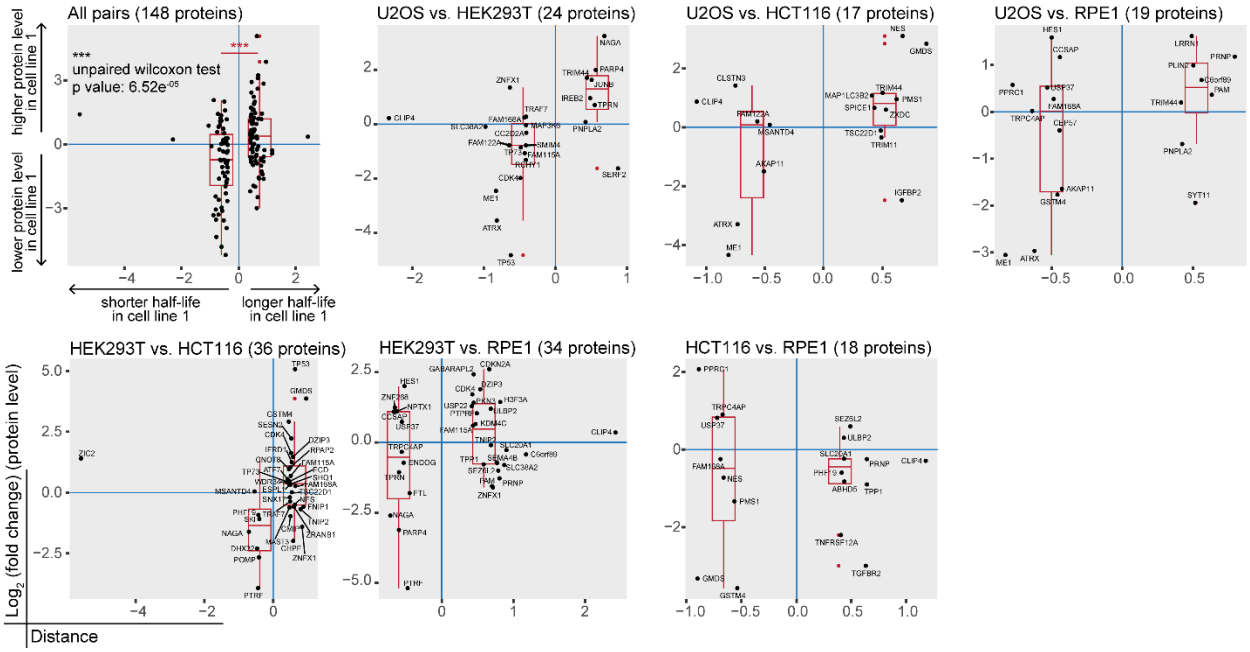
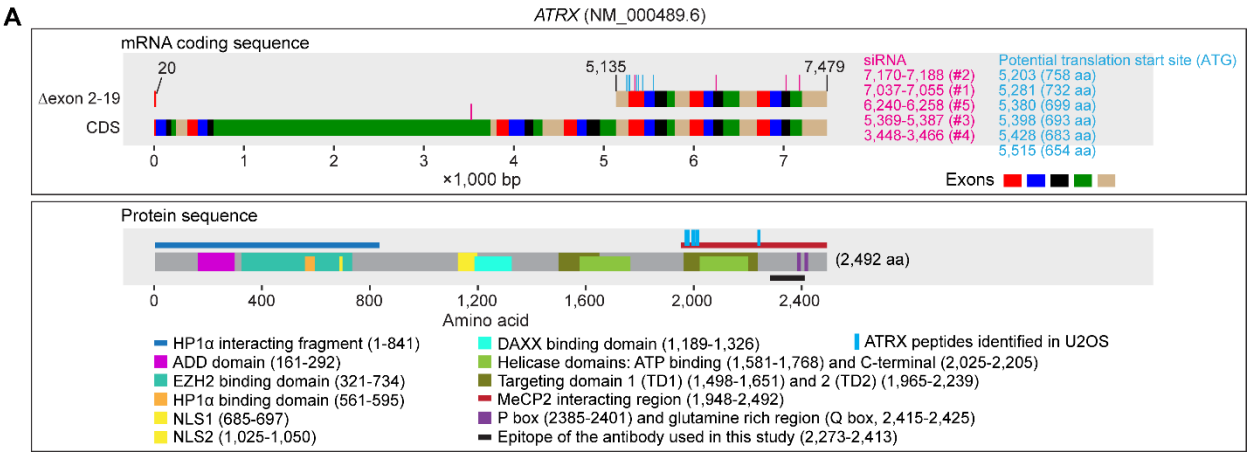
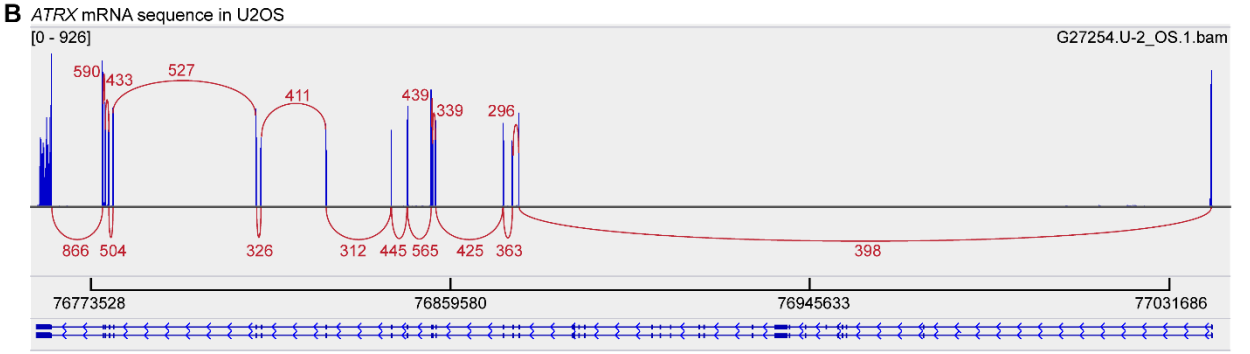


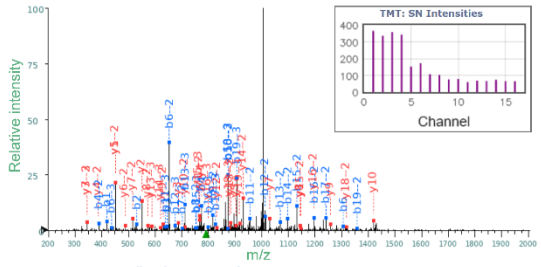
Figure S6. Differences in protein degradation rates correlated to differences in protein expression levels between cell lines. Related to Figure 6. X-axes represent distance defined in the differential protein half-life analysis (see Methods for details). Y-axes represent $\log_2$ fold changes of protein abundance levels between cell lines. A greater distance means a longer half-life in the first cell line. A larger $\log_2$ fold change indicates a higher protein expression level in the first cell line. Proteins with shorter half-lives in one cell line tended to show lower protein abundance in the same cell line. For example, ATRX showed a shorter half-life in U2OS cells, and its protein abundance was also low in U2OS cells.





C

HCT116; GMDS peptide: IINEVKPTEIYNLGAQSHVK; [M+4H]⁴⁺: 792.2166 m/z; MS1 isolation purity: 1.0



Predicted Fragmentation Pattern

+1

Seq	#	b: Δ Error	b	y	y: Δ Error	+1
I	1	66.001	418.298	---	---	20
I	2	-2.865	531.383	2748.554	---	19
N	3	299.942	645.425	2635.470	---	18
E	4	-68.420	774.468	2521.427	---	17
V	5	-165.343	873.536	2392.384	---	16
K	6	-25.615	1305.839	2293.316	---	15
P	7	---	1402.891	1861.014	---	14
T	8	---	1503.939	1763.961	---	13
E	9	---	1632.982	1662.913	---	12
I	10	---	1746.066	1533.871	---	11
Y	11	---	1909.129	1420.787	169.837	10
N	12	---	2023.172	1257.723	-46.408	9
L	13	---	2136.256	1143.680	-266.318	8
G	14	---	2193.277	1030.596	104.480	7
A	15	---	2264.314	973.575	---	6
Q	16	---	2392.373	902.538	36.793	5
S	17	---	2479.405	774.479	-82.927	4
H	18	---	2616.464	687.447	11.327	3
V	19	---	2715.532	550.388	81.256	2
K	20	---	---	451.320	-17.421	1

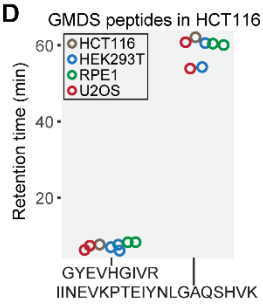
+2

Seq	#	b: Δ Error	b	y	y: Δ Error	+1
I	1	---	209.653	---	---	20
I	2	---	266.195	1374.781	---	19
N	3	---	323.216	1318.239	23.869	18
E	4	560.367	387.738	1261.217	---	17
V	5	---	437.272	1196.696	121.379	16
K	6	160.953	653.423	1147.162	-104.263	15
P	7	385.579	701.949	931.011	70.344	14
T	8	252.324	752.473	882.484	-212.077	13
E	9	134.204	816.994	831.960	182.266	12
I	10	-165.343	873.536	767.439	97.720	11
Y	11	361.019	955.068	710.897	-68.821	10
N	12	-186.348	1012.090	629.365	-496.462	9
L	13	134.197	1068.632	572.344	400.267	8
G	14	82.664	1097.142	515.802	300.774	7
A	15	219.918	1132.661	487.291	483.860	6
Q	16	126.072	1196.690	451.773	-1020.389	5
S	17	196.609	1240.206	387.743	545.889	4
H	18	---	1308.736	344.227	-6.528	3
V	19	195.196	1358.270	275.698	---	2
K	20	---	---	226.164	---	1

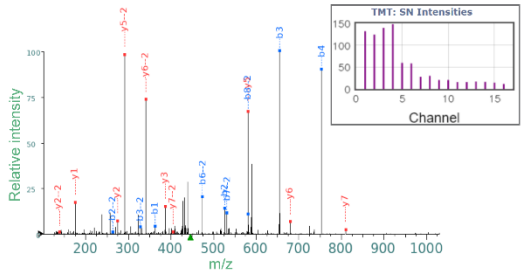
+3

Seq	#	b: Δ Error	b	y	y: Δ Error	+1
I	1	---	140.104	---	---	20
I	2	---	177.799	916.856	241.981	19
N	3	---	215.813	879.161	-427.168	18
E	4	---	258.828	841.147	---	17
V	5	---	291.850	798.133	179.195	16
K	6	-1031.002	435.951	765.110	638.519	15
P	7	---	468.302	621.009	-485.661	14
T	8	---	501.984	588.659	277.745	13
E	9	---	544.999	554.976	---	12
I	10	---	582.693	511.962	---	11
Y	11	-293.320	637.048	474.267	---	10
N	12	343.452	675.062	419.913	---	9
L	13	266.776	712.757	381.898	---	8
G	14	---	731.764	344.204	62.026	7
A	15	494.909	755.443	325.197	---	6
Q	16	183.886	798.129	301.517	---	5
S	17	102.936	827.140	258.831	---	4
H	18	-470.158	872.826	229.821	---	3
V	19	184.320	905.849	184.134	---	2
K	20	---	---	151.111	---	1

D



HCT116; GMDS peptide: GYEVHGIVR; [M+3H]³⁺: 445.2561 m/z; MS1 isolation purity: 1.0



Predicted Fragmentation Pattern

+1

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Y	2	104.495	525.299	972.526	---	8
E	3	78.401	654.342	809.463	305.306	7
V	4	124.555	753.410	680.420	-23.761	6
H	5	---	890.469	581.352	-107.939	5
G	6	---	947.491	444.293	---	4
I	7	---	1060.575	387.271	215.930	3
V	8	---	1159.643	274.187	-168.943	2
R	9	---	---	175.119	-199.286	1

+2

Seq	#	b: Δ Error	b	y	y: Δ Error	+1
G	1	---	181.622	---	---	9
Y	2	872.393	263.153	486.767	---	8
E	3	321.802	327.675	405.235	98.649	7
V	4	---	377.209	340.714	47.820	6
H	5	---	445.738	291.180	-169.942	5
G	6	-65.087	474.249	222.650	---	4
I	7	-29.996	530.791	194.139	---	3
V	8	-208.740	580.325	137.597	3575.490	2
R	9	---	---	88.063	---	1

Figure S7. U2OS and HCT116 cells express truncated forms of ATRX and GMDS, respectively. Related to Figure 7. (**A**) An illustration of *ATRX* mRNA sequence, domains, identified peptides, and the antibody epitope and siRNAs used in this work. (**B**) Sashimi plot of mapped reads to *ATRX* mRNA expression in U2O cells. Exon 2-19 were missing, leading to novel truncated isoform connecting exon 1 directly to exon 20. (**C**) Two GMDS peptides were identified in HCT116 cells in this study. The high-quality MS2 spectra, MS3 spectra and matched product ions (in blue and red) showed the high confidence of the identification and quantification for the truncated GMDS in HCT116 cells. (**D**) The retention times of the two GMDS peptides identified in HCT116 cells were consistent with the same two peptides found in other cell lines, validating further the identification of the two GMDS peptides in HCT116 cells.

Table S4. A comparison of this work and previous protein turnover studies. Related to Figure 2.

	Method	Timepoints (#)	Timepoints (hr)	# Replicates	Cell lines	# Proteins (identified or quantified)	# Proteins used	Min. # timepoints and # replicates requirement for half-lives or 50% turnover time	Half-lives or 50% turnover time (< 8 hr)	Half-lives or 50% turnover time (< 4 hr)	Half-lives or 50% turnover time (< 2 hr)
This work	Cycloheximide chase assay	6	0, 1, 2, 4, 6, 8	4, 2, 2, 2, 2, 4	HEK293T; U2OS; HCT116; RPE1	8,761-9,081	8,761-9,081	all timepoints; all replicates	403-508	223-303	81-116
Boisvert et al., 2012, MCP	pluse-chase metabolic labeling	6	0.5, 4, 7, 11, 27, 48	Not available	HeLa	8,041	6,841	Not available	136	66	43
Jovanovic et al., 2015, Science	pluse-chase metabolic labeling	10	0, 0.5, 1, 2, 3, 4, 5, 6, 9, 12	2	Dendritic cells	6,079	3,147	all timepoints; all replicates (replicates analyzed separately)	64-65	19-25	3-6
Schwanhausser et al., 2011, Nature	pluse-chase metabolic labeling	3	1.5, 4.5, 13.5	2	NIH3T3	6,445	4,203-5,028	all timepoints (replicates analyzed separately)	208-245	36-56	15-31
Mathieson et al., 2018, Nature Commun.	pluse-chase metabolic labeling	5	0, 6, 12, 24, 36	2	B cell; NK cell; Hepatocytes; Monocytes; Neurons	Not available	3,985-6,888	≥4 timepoints (replicates analyzed separately)	0-8	0	0
Doherty et al., 2009, JPR	pluse-chase metabolic labeling	7	0, 0.25, 0.5, 1, 2, 4, 8	1	A549	Not available	576	≥3 timepoints	104	46	11
Cambridge et al., 2011, JPR	pluse-chase metabolic labeling	2	0, 24	3	Hela; C2C12	Not available	3,528-4,106	all timepoints	0	0	0
McShane et al., 2016, Cell	AHA labeling	7	0, 1, 2, 4, 8, 16, 32	3	NIH3T3; RPE1; trisomic RPE1	3,086-3,292	334-557	all timepoints; all replicates	0	0	0
McShane et al., 2016, Cell	AHA labeling	7	0, 1, 2, 4, 8, 16, 32	3	NIH3T3; RPE1; trisomic RPE1	3,086-3,292	1,338-2,227	≥4 timepoints; ≥2 replicates (replicate analyzed separately)	52-109	15-29	0-1

We chose to combine TMTpro-based protein quantification and cycloheximide treatment to characterize short-lived proteins under translational arrest in this work. We note that MS-based pulse-chase metabolic labeling has also been used to determine 50% turnover times or half-lives for proteins, especially medium- and long-lived proteins. Protein abundance is monitored at multiple time points during the chase period (Ross et al., 2020). The calculation of 50% turnover times (determined by protein synthesis rates and degradation rates) requires ratios between heavy and light pairs. Half-life (determined by protein degradation rate) calculation requires signals from existing proteins and corrections to account for cell division and the recycling of endogenous amino acids (Boisvert et al., 2012). Some pulse-chase metabolic labeling studies did not differentiate between "50% turnover time" and "half-life", and some reported half-lives are actually 50% turnover times (Martin-Perez and Villen, 2017).

Long labeling times are usually employed in pulse-chase metabolic labeling studies to ensure that sufficient labeled amino acids are incorporated into newly synthesized proteins, and thus sufficient heavy and light pairs are available for calculation. However, long labeling times do not facilitate the detection of short-lived proteins. For example, almost all quantified proteins showed half-lives or 50% turnover times longer than 8 hr in studies using long labeling times (0 hr, 24 hr; 0 hr, 6 hr, 12 hr, 24 hr, 36 hr) (Cambridge et al., 2011; Mathieson et al., 2018). Missing values across time points become a considerable issue when short labeling times are used, as it takes time for new proteins to be synthesized and the detection of low abundant signals is more stochastic. Short-lived proteins being inherently low abundant also makes the detection more difficult and missing value issue severer. For example, over 6,000 proteins were identified in Jovanovic et al., 2015, however, only ~50% proteins were quantified across all time points and replicates. Although some short-lived proteins are still detectable via short labeling times, the depth is adversely affected by the missing value issue and they are still an under-examined dimension of the proteome. Only 100-200 proteins have shown half-lives or 50% turnover

times shorter than 8 hr in studies using short labeling times (Boisvert et al., 2012; Doherty et al., 2009; Schwanhausser et al., 2011). Even fewer proteins with half-lives shorter than 4 hr or 2 hr were identified in these studies compared to this work.

Metabolic labeling with the artificial amino acid azidohomoalanine (AHA, a methionine analog) is an alternative to alleviate the requirement for long labeling times (McShane et al., 2016). However, proteome coverage is also compromised as only newly synthesized proteins are retained for analysis. Moreover, up to 30% of the newly synthesized proteins have been shown to undergo immediate degradation right after synthesis (Schubert et al., 2000). The different degradation kinetics of newly synthesized and existing proteins can skew the determination of short-lived proteins in AHA labeling (McShane et al., 2016; Schubert et al., 2000). Similar to traditional pulse-chase metabolic labeling, missing values across time points and replicates are also an issue in AHA labeling. For example, over 3,000 proteins were identified in McShane et al., 2016, only ~400 proteins were quantified in all time points and replicates.

Additionally, metabolic labeling requires medium swap and the use of dialyzed serum, which may induce cellular stress and convolute the readout in some cell lines (Ross et al., 2020). In this work we intended to measure half-lives for short-lived proteins under translational arrest by cycloheximide, which is a broadly used assay in targeted studies. We noted that some half-lives under translational inhibition may be skewed compared with those under more steady-state conditions. However, the rapid removal of these proteins suggests the existence of mechanisms for immediate protein elimination, which represent potential entry points for therapeutic interventions through regulating half-lives. Cycloheximide chase assay has been widely used in a number of targeted studies and they can also benefit from our deep coverage dataset. Importantly, combined with TMTpro-based protein quantification, we were able to overcome the missing value issue and achieved an excellent protein coverage.

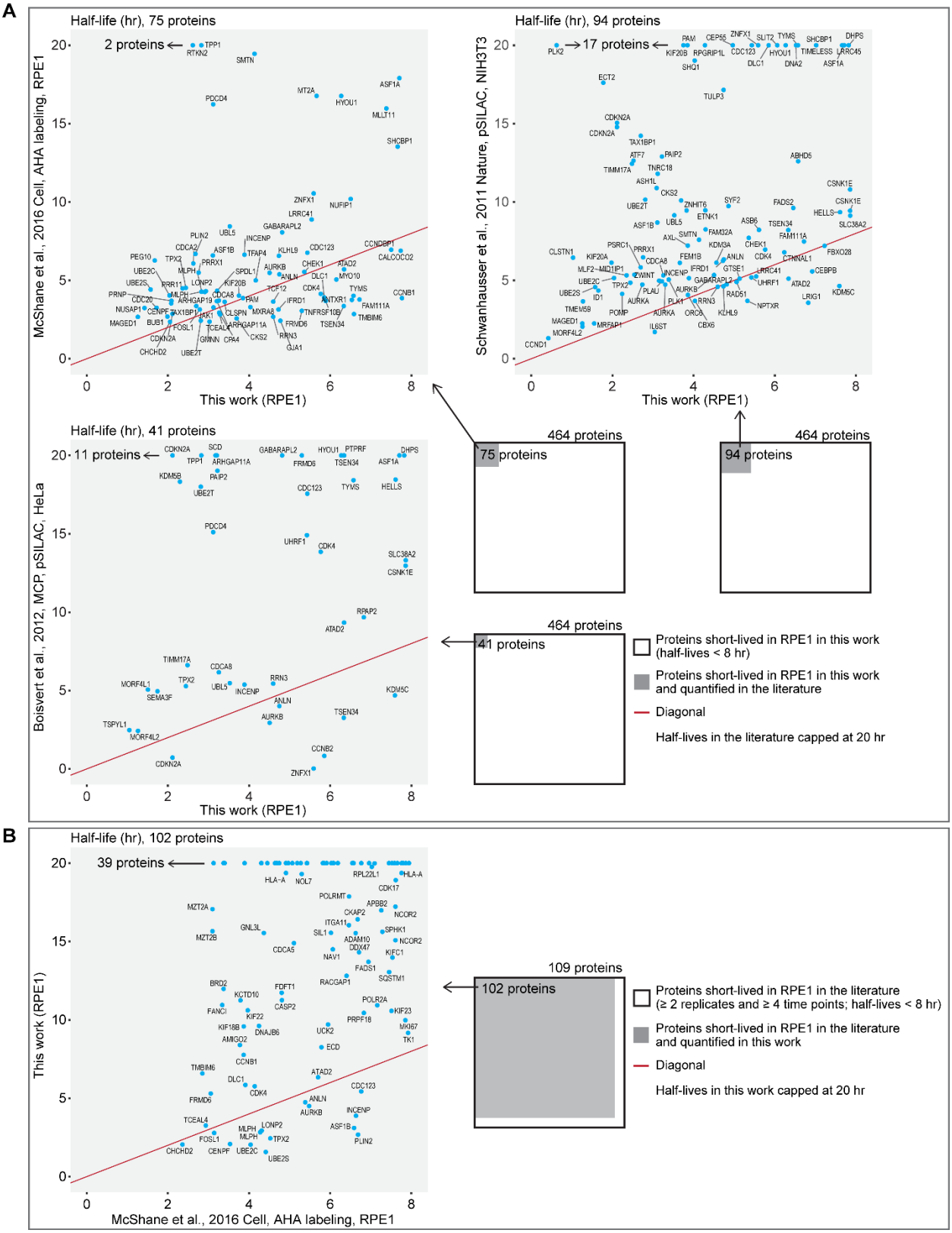

Figure S8. Comparisons of half-lives in this work and in the literature. Related to Figure 2. We chose three studies in Table S4 that have high numbers of proteins with half-lives or 50% turnover times shorter

than 8 hr (Boisvert et al., 2012; McShane et al., 2016; Schwanhausser et al., 2011). (**A**) We first focused on proteins that are short-lived in RPE1 in this work. As mentioned in Table S4 that proteome coverage is adversely affected in studies using short labeling times, only a small fraction (10%-20%) of these proteins were quantified in former studies. The overlapping proteins, however, mostly showed consistently short half-lives in previous studies, especially the study using AHA labeling in the same cell line (RPE1) (McShane et al., 2016). This suggests that the half-lives under translational arrest still reflect those under more steady-state conditions to a large extent. Red lines are diagonal lines with unit slope and zero intercept. Half-lives in the literature are capped at 20 hr. The numbers in the plots indicate the number of short-lived proteins in RPE1 in this work that showed half-lives ≥ 20 hr in the literature. (**B**) We next focused on proteins that are short-lived in RPE1 in AHA labeling in the previous study (≥ 4 time points and ≥ 2 replicates) (McShane et al., 2016). We quantified almost all these proteins in RPE1 in this work. Approximately 60% proteins showed half-lives shorter than 20 hr (≥ 25% protein loss at 8 hr) in this work. The other 40% did not present evident loss at 8 hr, which agrees with the conclusions that >10% newly synthesized proteins have different degradation kinetics with existing proteins (McShane et al., 2016), and ~30% newly synthesized proteins are rapidly degraded right after synthesis (Schubert et al., 2000). Red line is the diagonal line with unit slope and zero intercept. Half-lives in this work are capped at 20 hr. The number in the plot indicates the number of short-lived proteins in AHA labeling that showed half-lives ≥ 20 hr in this work.