Supporting Information

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SI Materials and Methods

Label-Free Quantification Using Mass Spectrometry. Accession numbers of proteins identified in the combined searches were submitted to Protein Prospector for MS1 quantification of precursor ions in "multisample" mode. Extracted peptide ion chromatographs were generated using a retention time window of −10/+20 s from when the precursor was selected for MS/MS, and average ion intensities and peak areas for all quantified peptides were reported. In these datasets, ion intensity values were used for quantification. Shared peptides in the Protein Prospector output file were assigned uniquely to the highest-ranked protein containing their sequence and after this filtering, all remaining unique peptides (in all charge states) were included in the quantitative analysis. In the ESRE dataset with two biological replicates, protein intensities were calculated from the sum of the individual unique peptide MS1 precursor ion intensities, using an in-house script, and intensities of undetected proteins were set to 1 (low nonzero value to permit calculation of log ratios).

Protein abundance quantification in the K562 dataset with three biological replicates was performed using the MS1 filtering approach implemented in Skyline v. 3.7.0.11317 (1), which allows more consistent quantification of peptides not identified by the search engine in all tested conditions. Raw data files were imported into Skyline, along with a spectral library file for our filtered hits (BiblioSpec output file generated in Protein Prospector from our database search), and the corresponding SwissProt human database. Using MS1 filtering in Skyline (2), extracted ion chromatograms for peptide precursor ions across all datasets were aligned within a 10-min time window for peak picking and quantification. Peak assignments in all runs were manually inspected to confirm extracted ion chromatogram quality and check for any misassignments based on incorrect retention times (>±0.5 min), mass errors (>±20 ppm), or missing isotope peaks (at least two required). Areas for precursor ion isotopes M, M + 1, and M + 2 were summed in this analysis, and the final Skyline output file was filtered to include only quantified peptides with an isotopic dot product score of 0.8 or higher. The peptide quantification report was parsed using an in-house script to calculate total protein abundances by summing corresponding peptide areas. The total protein areas were then converted to log2 values for the final fold change calculations.

Filtering of MS Results. Protein identification datasets were assembled including unique number of peptide identifications and summed peptide intensity values per protein, in all replicates of the four conditions of each experiment. Stringent filtering for identification of OPP-detected nascent proteins was applied. First, proteins with greater than 1 identified peptide in any replicate in either non-OPP treated control were removed to filter out sticky proteins. Second, to be included, identified proteins were required to have two or greater unique identified peptides in two replicates in either OPP-labeled sample. Next, for each identified protein, intensity ratios in the OPP-treated conditions were calculated. Nascent protein synthetic abundance was calculated by fold change (FC) = log2 (Control/Intervention) in each replicate, followed by averaging across replicates. Candidates were pursued with log2FC > 1 for both datasets and for the K562 dataset, P values were calculated for the log2FC ratios using a two-tailed, one-sample t test against the assumed mean of 0, with P value of <0.05 as the significance cutoff (3). Log2 intensity ratios measured by LC-MS/MS are approximately normally distributed and are often compared using various modifications of the t test, including Student's t test (4). Histograms and scatter plots were made using GraphPad Prism software. Gene Ontology networks were classified using DAVID Bioinformatics Database (david.ncifcrf.gov). Graphical representations of data were performed using Prism 6 (GraphPad Software). Alignment of protein identifications for similarity was performed using Venny 2.1.0 (bioinfogp.cnb.csic.es/tools/venny/) and visualized for proportionality using the Whitehead Institute for Biomedical Research Venn Diagram Generator (jura.wi.mit.edu/bioc/tools/venn.php).

To aid in evaluation of background proteins in our streptavidin affinity purification experiments, we downloaded a subset of 119 relevant control experiments from the online Contaminant Repository for Affinity Purification (www.crapome.org/), v1.1, to compare with our datasets. We selected for human proteins and filtered by "Strep-HA" for Epitope tag, "total cell lysate" for Subcellular fractionation, and "Streptactin" for Affinity approach (119/411 total experiments referred to here as "SA-CRAPome"). Proteins from these controls were compared directly with our K562 human proteins. To enable use of the database with our ESRE experiment, we searched against human orthologs of our mouse proteins (where possible).

1. MacLean B, et al. (2010) Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 26:966–968.
2. Schilling B, et al. (2012) Platform-independent and label-free quantitation of proteomic data using MS1 extracted ion chromatograms in skyline: Application to protein acetylation and phosphorylation. *Mol Cell Proteomics* 11:202–214.
3. Doll S, Urisman A, Oses-Prieto JA, Arnott D, Burlingame AL (2017) Quantitative proteomics reveals fundamental regulatory differences in oncogenic HRAS and isocitrate dehydrogenase (IDH1) driven astrocytoma. *Mol Cell Proteomics* 16:39–56.
4. Ting L, et al. (2009) Normalization and statistical analysis of quantitative proteomics data generated by metabolic labeling. *Mol Cell Proteomics* 8:2227–2242.

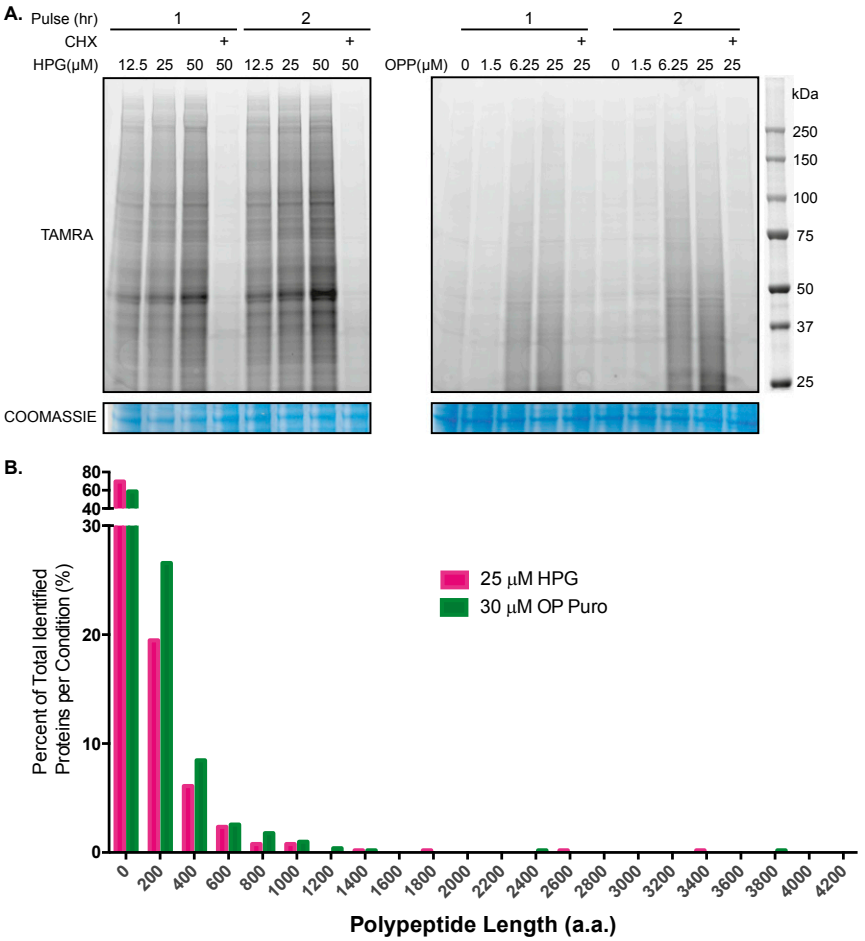

Fig. S1. (*A*) Dose and time pulse titration of HPG and OPP in K562 cells. K562 cells of symmetric confluency were pulsed with specified doses of HPG (grown in methionine-depleted RPMI media) or OPP (grown in standard RPMI media) in the presence or absence of cycloheximide (CHX) over the course of 1 or 2 h. Isolated protein from cell lysates was conjugated to TAMRA-azide by cycloaddition and analyzed for labeling on SDS/PAGE gels by fluorescence scan. Coomassie-stained lanes demonstrate equivalent loading. (*B*) Estimation of minimal polypeptide length in labeled proteins. Isolated peptides identified by LC-MS/MS from each treatment strategy were positionally aligned to their respective protein as an indirect measure of polypeptide length before digestion. Identified proteins were subdivided into polypeptide-length bins and divided by total isolated proteins per labeling condition to yield percentage of total identified proteins per condition.

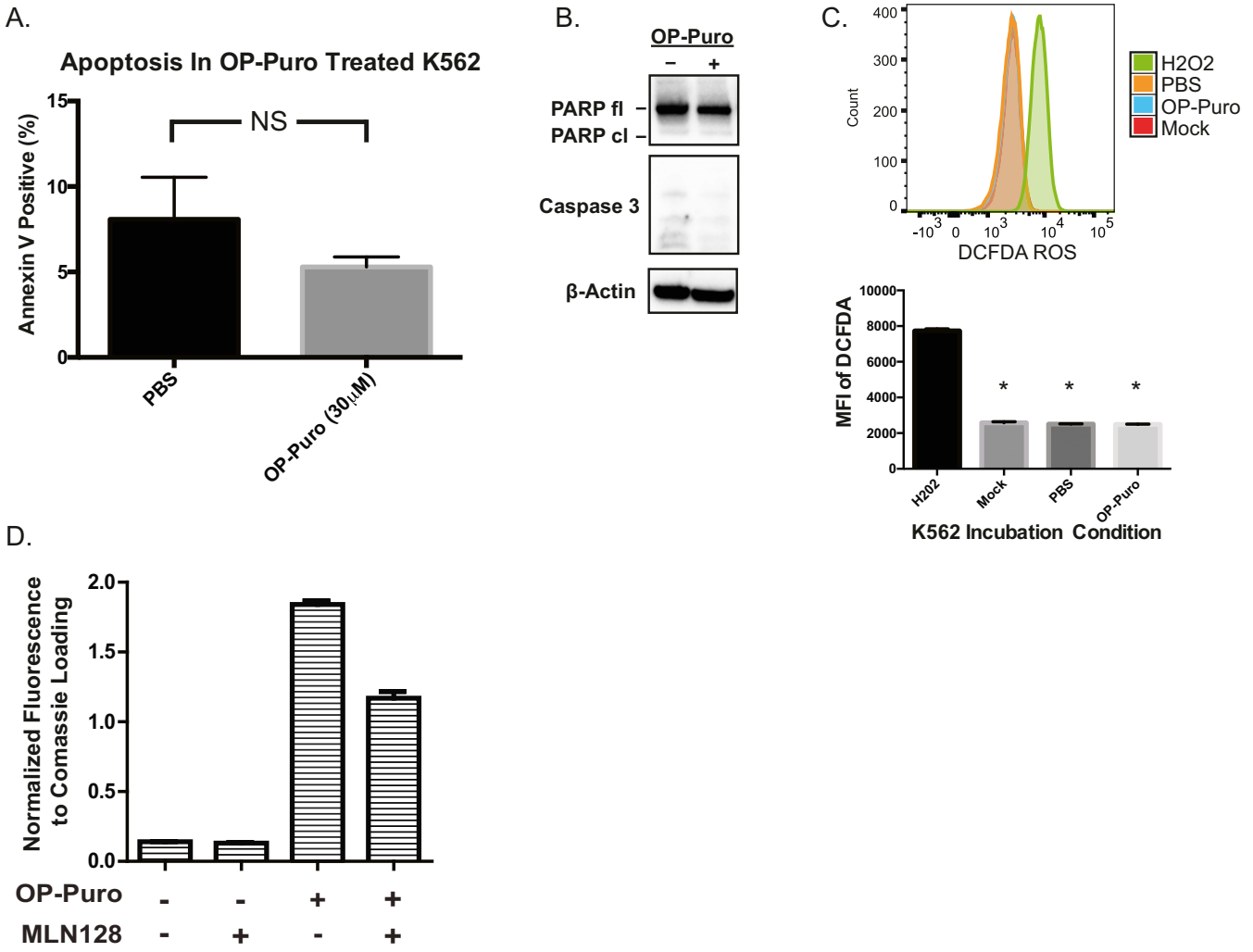

Fig. S2. Pulse treatment with OPP does not alter cell apoptosis or markers of cellular stress. (*A*) K562 cells were treated with either PBS vehicle or OPP (30 μM) for 2 h and then harvested and stained with Annexin V and 7-AAD. Apoptosis represented as percentage (%) of cells staining positive for both Annexin V and 7-AAD. (*B*) Western blot of K562 cell lysate showing cleavage of PARP and Caspase 3. (*C*) ROS generation as measured by 2′,7′-dichlorofluorescein diacetate in H_2O_2 (positive control), PBS, OP-Puro, and Mock-treated cells. (*D*) Normalization of the TAMRA fluorescence signal to Coomassie-stained total protein loading corresponding to Fig. 3*B*. NS, not significant ($P > 0.05$). *$P < 0.05$.

A.

Number Identified	DMSO			MLN128			DMSO + OPP			MLN128 + OPP		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
Total Peptides	448	326	483	376	244	220	1975	1426	3073	1310	942	2422
Total Proteins	154	103	148	123	83	81	549	415	789	370	271	704

Number Identified	All Conditions	DMSO	MLN128	DMSO + OPP	MLN128 + OPP
Total unique proteins in combined replicates	1001	223	173	919	765

Filtering Strategy	Initial Dataset	Meeting Inclusion/ Exclusion Criteria
Total Unique Proteins	1001	217

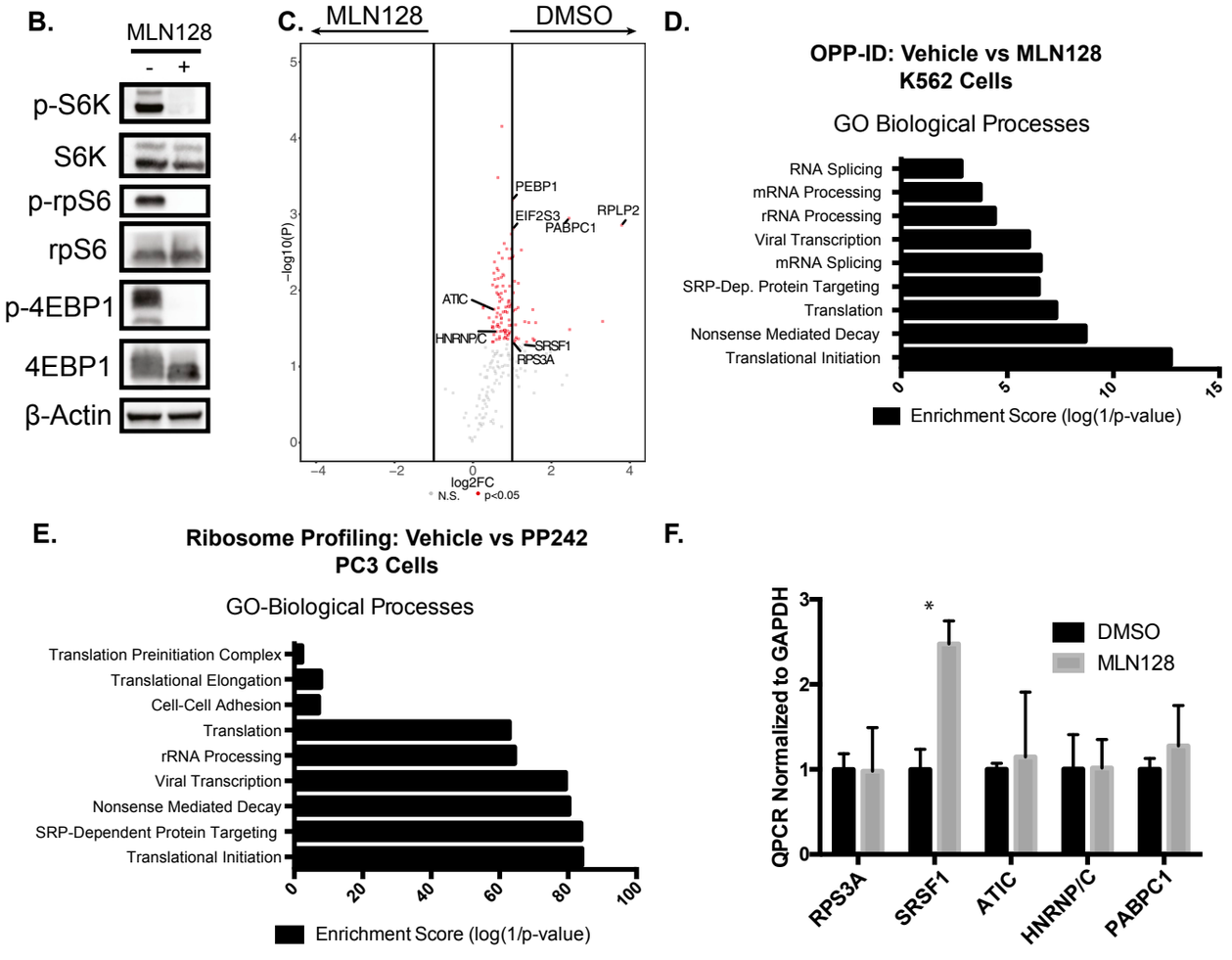

Fig. S3. Identification of specific OPP-labeled nascent proteome in MLN128-treated K562 cells. (*A*) Table showing number of total peptides and identified proteins per replicate after LC-MS/MS analysis on an LTQ-Orbitrap Velos. Replicates were then analyzed for total number of unique proteins in combined replicates per condition and filtered for inclusion/exclusion criteria before quantitative analysis. (*B*) Western blot verification of efficacy of MLN128 treatment during a 3-h time frame on canonical mTORC1 targets. (*C*) Volcano plot of −log10(*P* value) vs. log2(FC) for proteins identified in DMSO- vs. MLN128-treated cells meeting our inclusion/exclusion criteria. Gray dots indicate *P* value >0.05. Red dots indicate *P* value <0.05. (*D*) GO analysis of proteins with log2 FC >1× in DMSO- vs. MLN128-treated cells detected by OPP-ID using DAVID for GO-Biological Processes with attributed enrichment scores. (*E*) GO analysis of proteins with log2 FC >1× in vehicle vs. PP242-treated cells detected by ribosomal profiling (1), using DAVID for GO-biological processes with attributed enrichment scores. (*F*) qPCR of cDNA extracted from DMSO- or MLN128-treated K562 cells. **P* < 0.05.

1. Hsieh AC, et al. (2012) The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* 485:55–61.

A.

Number Identified	Expansion Replicate 1	Expansion Replicate 2	Maturation Replicate 1	Maturation Replicate 2	Expansion +OPP Replicate 1	Expansion +OPP Replicate 2	Maturation +OPP Replicate 1	Maturation +OPP Replicate 2
Total Peptides	2740	4130	3981	5020	7616	9496	6655	8616
Total Proteins	615	881	841	1096	1527	1778	1305	1708

Number Identified	All Conditions	Expansion	Maturation	Expansion + OPP	Maturation + OPP
Total Unique Proteins in Combined Replicates	2194	929	1166	1939	1808

Filtering Strategy	Initial Dataset	Meeting Inclusion/ Exclusion Criteria
Total Unique Proteins	2194	290

B.

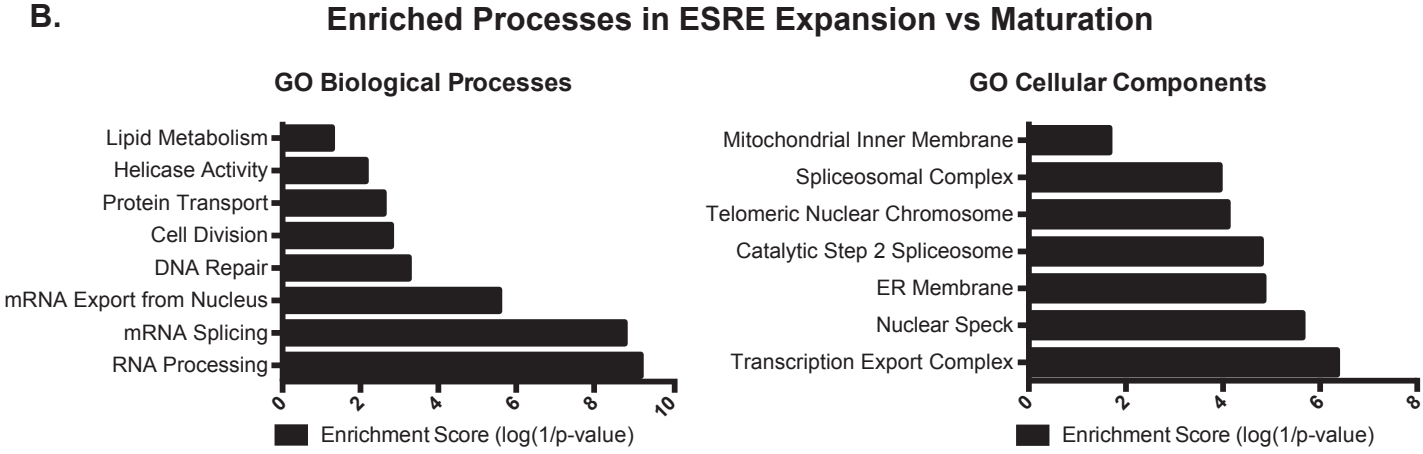

Fig. S4. Identification of specific OPP-labeled nascent proteome in expansion vs. maturation media-treated ESRE cells. (*A*) Table showing number of total peptides and identified proteins per replicate after LC-MS/MS analysis on a Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer. Replicates were then analyzed for total number of unique proteins in combined replicates per condition and filtered for inclusion/exclusion criteria before quantitative analysis. (*B*) Proteins with log2 FC >1× in expansion vs. maturation media-treated cells were subjected to gene ontology analysis using DAVID for GO biological processes and GO cellular components with attributed enrichment scores.

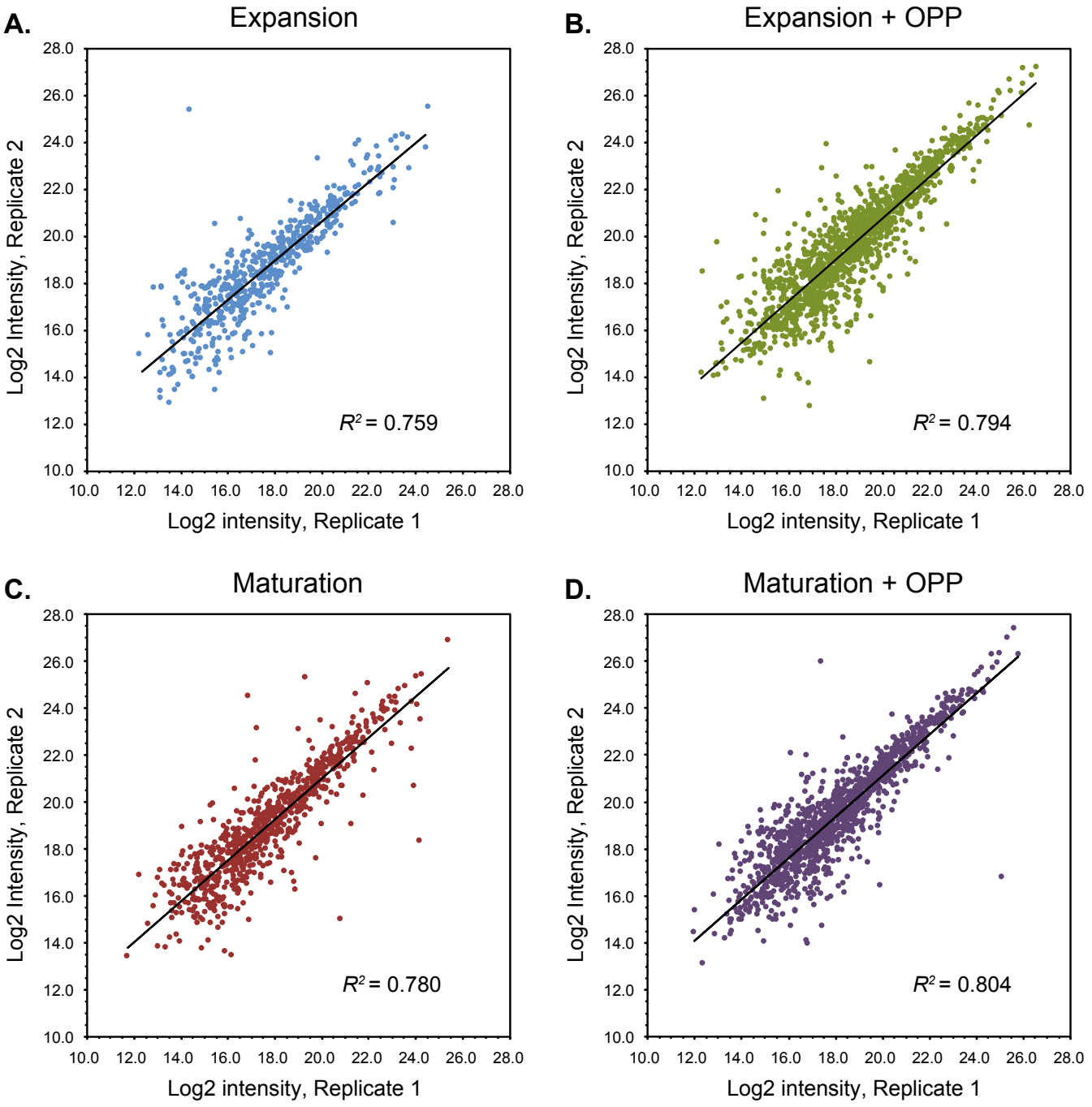

Fig. S5. Scatter plots comparing protein intensities (log2) between the first and second biological replicates of the four treatment conditions of our ESRE experiment: (*A*) expansion media, (*B*) expansion media +OPP, (*C*) maturation media, and (*D*) maturation media +OPP. Linear regressions were performed on the datasets, and trend lines and correlation coefficients (R^2 values) are indicated on the plots. The numbers of data points (N) per dataset are as follows: (*A*) 567 proteins, (*B*) 1,366 proteins, (*C*) 771 proteins, and (*D*) 1,205 proteins.

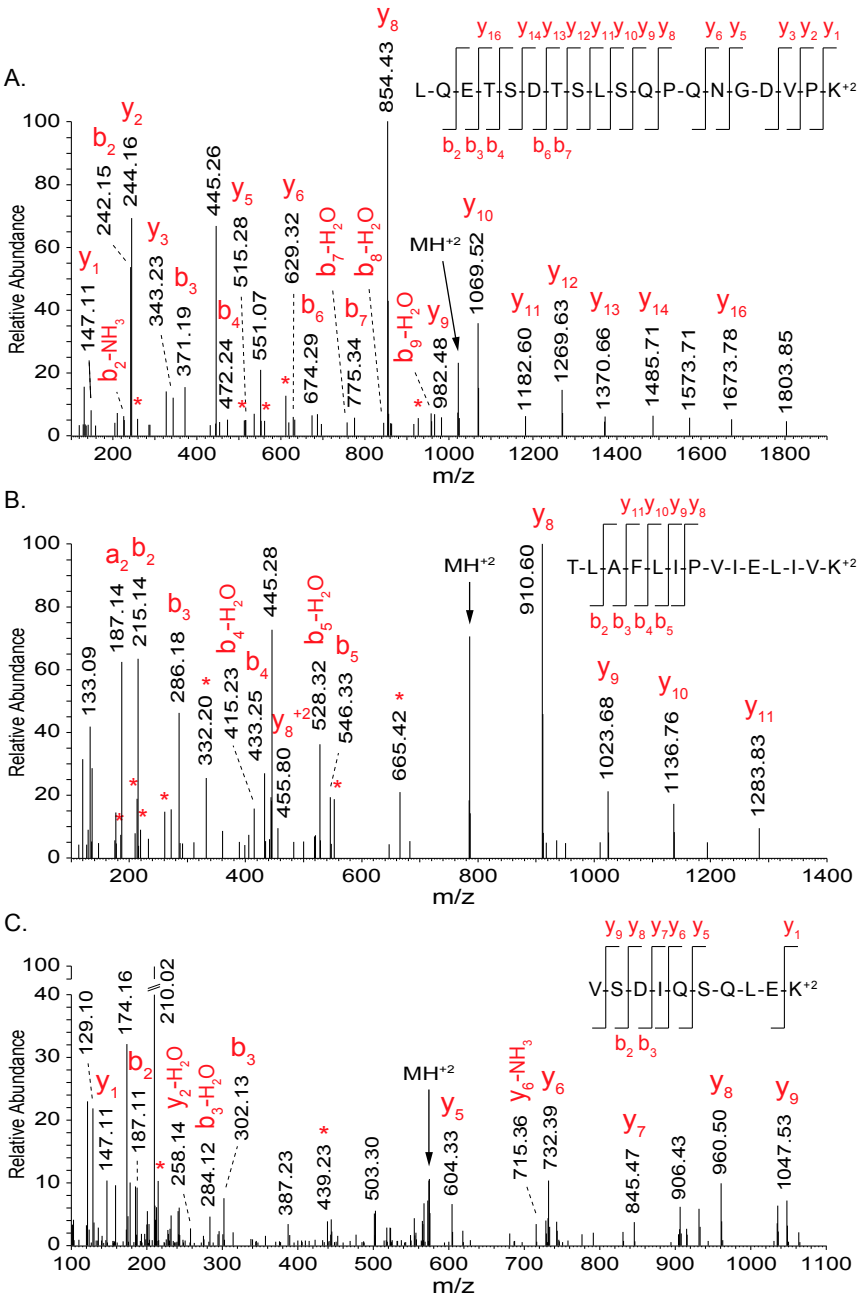

Fig. S6. HCD MS/MS spectra of three peptides from mouse ATP-dependent RNA helicase Ddx18, a protein detected as highly up-regulated in ESRE expansion media. The peptides derive from distinct regions of the 74,181.5 Da (660 amino acid) protein: (*A*) peptide ^{27}LQETSDTSLSQPQNGDVPK^{45} (MH^{+2} 1022.4939), (*B*) peptide ^{220}TLAFLIPVIELIVK^{233} (MH^{+2} 785.0052), and (*C*) peptide ^{548}VSDIQSQLEK^{557} (MH^{+2} 573.8016). Main sequence ions are labeled on the spectra and indicated on the structures. Internal fragment ions are marked with asterisks.

Gene	Primers
GAPDH	FWD: GTCGGAGTCAACGGATTTGG REV: TTGCCATGGGTGGAATCATA
RPS3A	FWD: AGCCCAAGTTTGAATTGGGAAA REV: TTTAGCACCTGTCTCGTCCC
SRSF1	FWD: ATCTCATGAGGGAGAAACTGCC REV: GTAACTGCGACTCCTGCTGT
ATIC	FWD: CTAAAAGGAGTGGTGTGGCG REV: ATGATTCCCAGTTCGTCGCA
HNRNP/C	FWD: AGACGAAGACTGAGCGGTTG REV: AGCCGAAAACAAGAAGGGGA
PABPC1	FWD: AGTCACTCCGTTCTAAGGTTGA REV: GCACAAGTTTCTTTTCATGGTCC

Fig. S7. List of primers used in qPCR identification of mRNA abundance in K562 cells treated with DMSO vehicle or MLN128.

Dataset S1. Table of identified proteins isolated by using HPG or OPP at indicated concentrations for a direct comparison between methods

Dataset S1

Protein intensities were calculated only on the basis of unique peptides.

Dataset S2. Table of identified proteins isolated from K562 cells analyzing effect of MLN128 on nascent protein synthesis

Dataset S2

Listed are individual protein gene names, identified unique peptides in each replicate, and summed peptide intensities. Data are separated into the full report of all isolated proteins and filtered hits category of proteins that filtered through our exclusion/inclusion criteria. The column entitled "frequency in SA-CRAPome" indicates percentage of 119 control experiments downloaded from the CRAPome website (www.crapome.org/), in which protein was observed as a reflection of nonspecific binding to streptavidin. Asterisk (*) denotes proteins that were observed in the filtered hits with >30% frequency in the SA-CRAPome dataset. A third tab entitled "fold change calculations" is included, showing the summed peptide peak areas for each of the filtered hits obtained by MS1 filtering in Skyline. *P* values for these measurements were calculated using a *t* test comparing the log2 ratios of the DMSO to MLN0128-treated proteins against the assumed mean of 0.

Dataset S3. Table of identified proteins isolated from ESRE cells analyzing the effect of switching from expansion to maturation media on nascent protein synthesis

Dataset S3

Listed are individual protein gene names and identified unique peptides in each replicate with concordant summed peptide intensity. Data are separated into the "full report" of all isolated proteins and "filtered hits" category of proteins that filtered through our exclusion/inclusion criteria. The column entitled "frequency in SA-CRAPome" indicates percentage of 119 control experiments downloaded from the CRAPome website (www.crapome.org/), in which protein was observed as a reflection of nonspecific binding to streptavidin. Asterisk (*) denotes proteins that were observed in the filtered hits with >30% frequency in the SA-CRAPome dataset. A third tab entitled "fold change calculations" is included that converts missing intensity values for undetected proteins from "0" to "1" (denoted in blue) for final calculations of log2 FC across replicates.