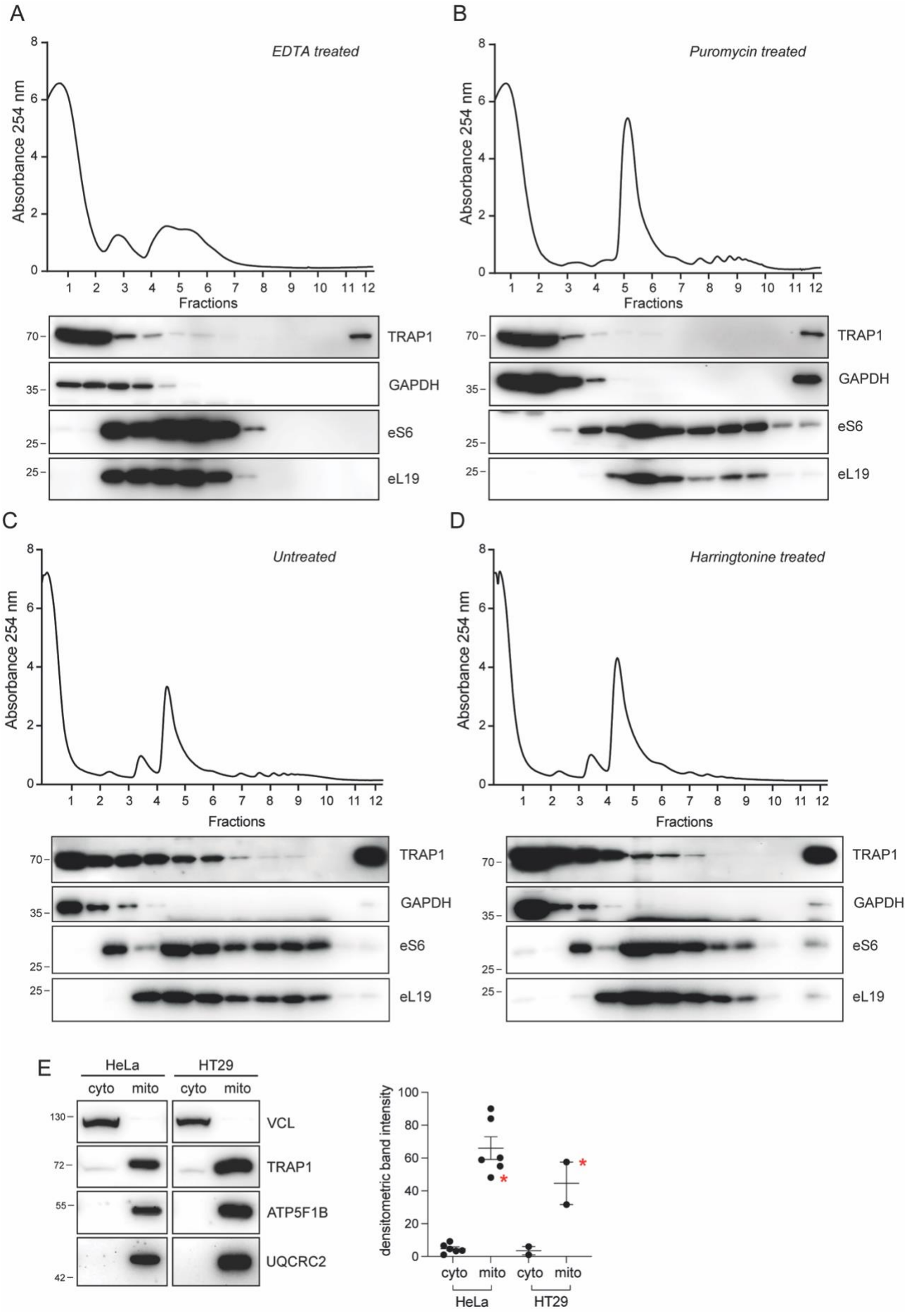
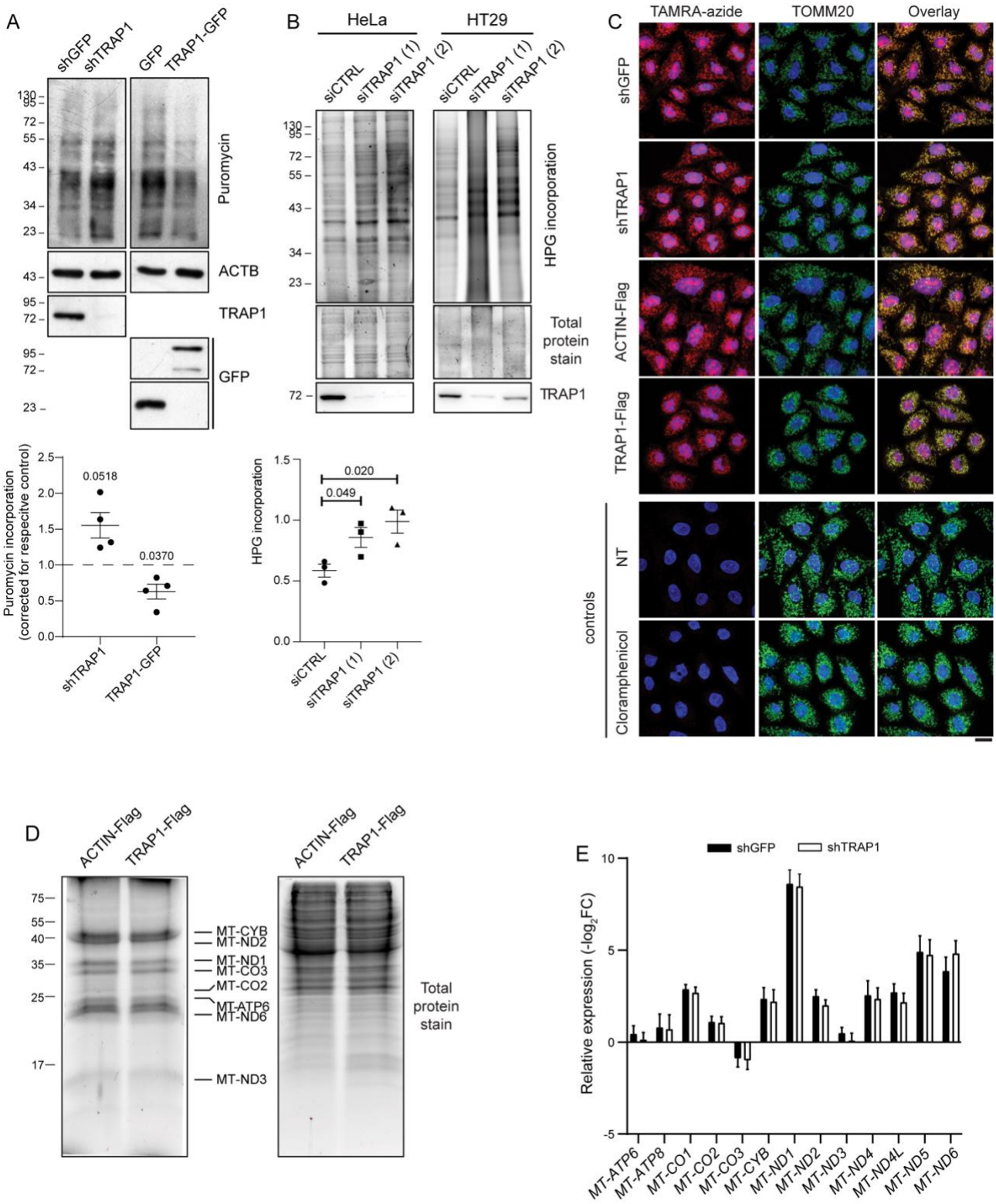
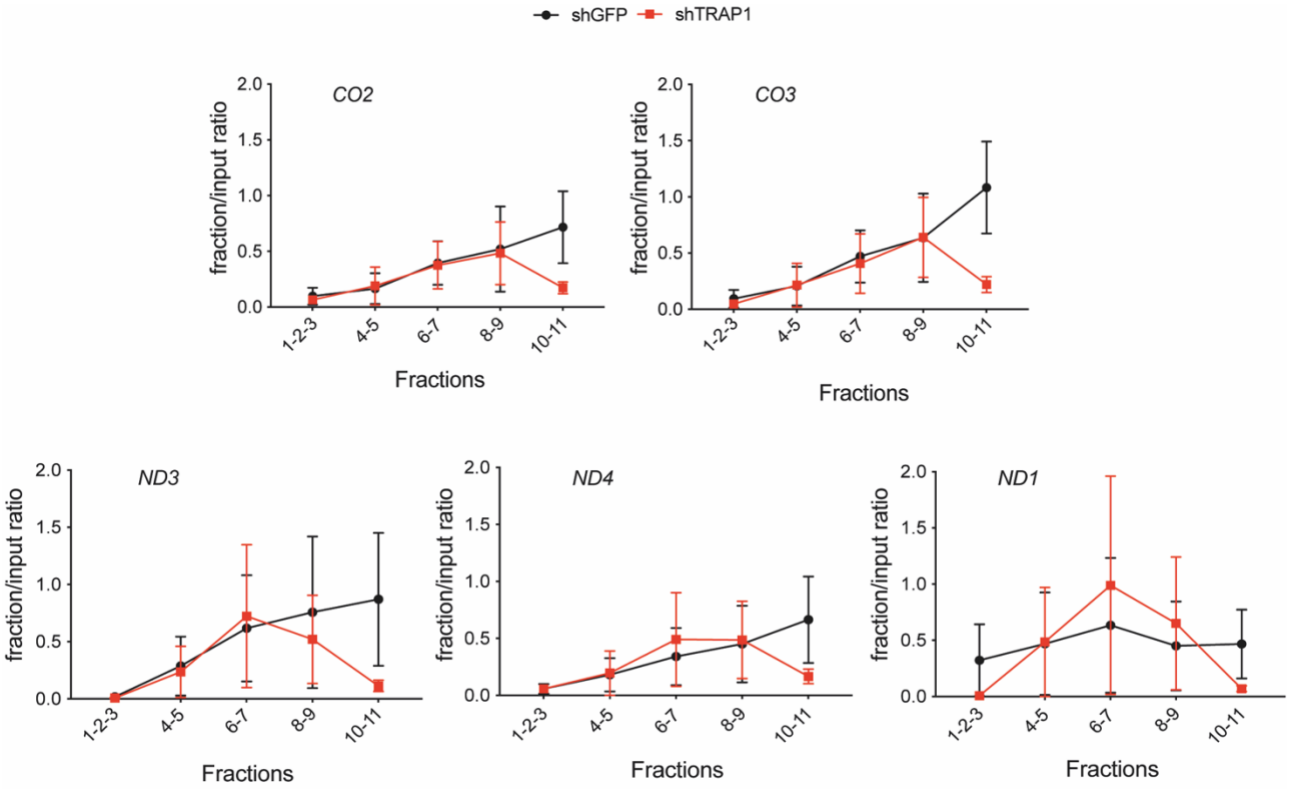

A
EDTA treated
Absorbance 254 nm
Fractions
B
Puromycin treated
C
Untreated
D
Harringtonine treated
TRAP1
GAPDH
eS6
eL19
E
HeLa
HT29
cyto
mito
VCL
TRAP1
ATP5F1B
UQCRC2
densitometric band intensity

Supplemental Figure S1: (**A-B**) Polysome profiling absorbance, measured at 254 nm, of HeLa cell extracts, in the presence of 30 mM EDTA (A) or following a 15-minute treatment with 100 µg/mL puromycin (B). Proteins from each fraction were analysed by WB with the indicated antibodies. (**C-D**) Polysome profiling absorbance, measured at 254 nm, of HT29 cell extracts, untreated (C) or following a 15-minute treatment with 2 µg/mL harringtonine (D). Proteins from each fraction were analysed by WB with the indicated antibodies. (**E**) Subcellular fractionation of HeLa and HT29 cells showing the presence of indicated proteins into cytosolic (cyto) and mitochondrial (mito) fractions (left). The amount of TRAP1 in the cytosol and mitochondria was calculated by densitometric quantification of bands (right) in six (HeLa) and two (HT29) replicates. Red asterisks indicate the bands shown in the left panel.

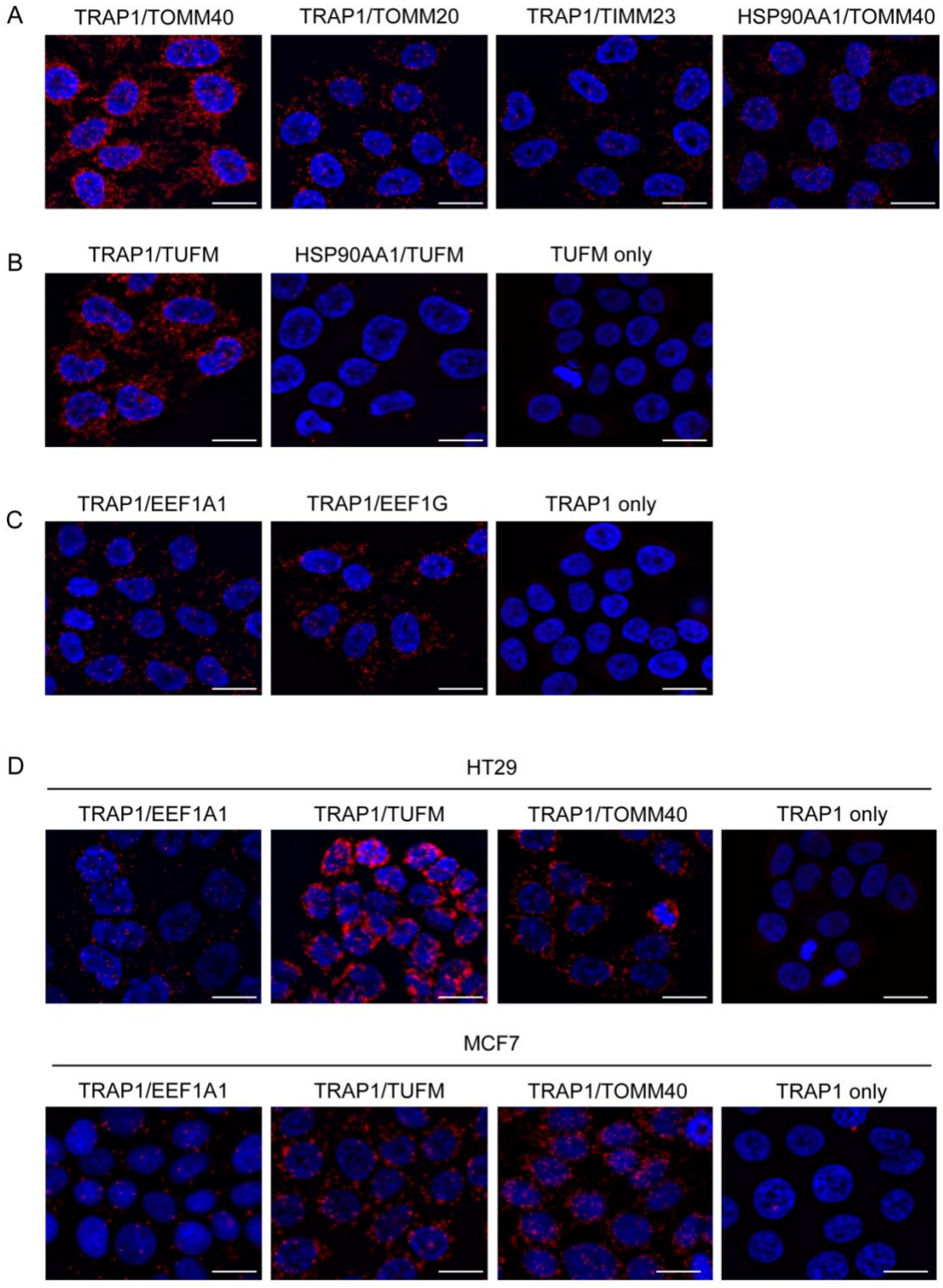


Supplemental Figure S2: (**A**) Following tetracycline-induced induction of TRAP1-directed shRNA and nontargeting control shRNA (72 hrs) or of TRAP1-GFP and unfused control GFP (24 hrs), HeLa cells were treated with puromycin (1 µg/mL, 15 min). Representative immunoblots of total cellular lysates with indicated antibodies are shown (upper panel), with relative densitometric band intensities and analysis (lower panel). The p-value in the graph indicate the statistical significance based the Student's *t*-test (n=4). (**B**) FUNCAT-gel. HeLa and HT29 cells were transfected with two different TRAP1-directed siRNA for 72 hours before labeling

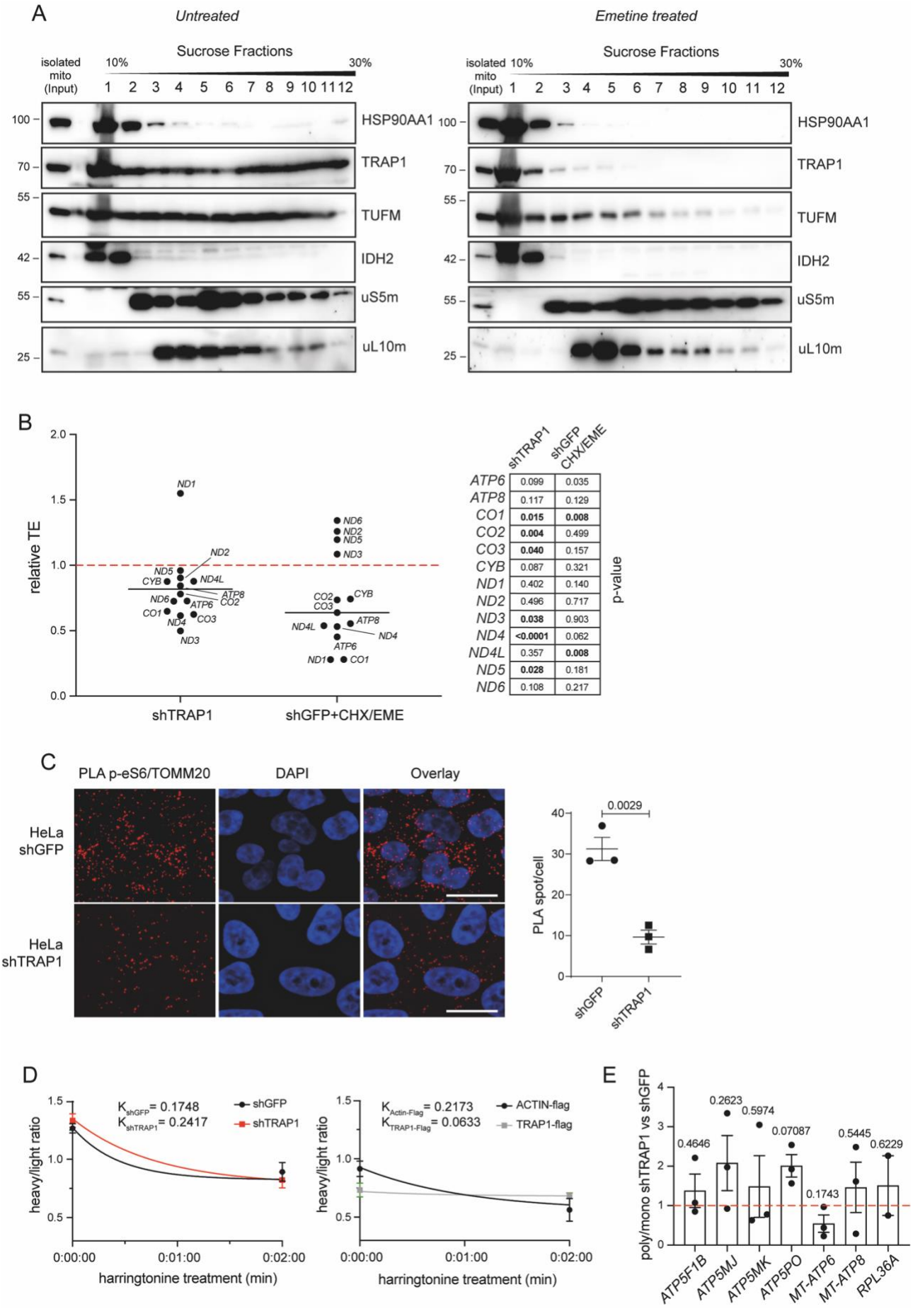
with 100 µM HPG-alkyne for 1 hour. The resulting lysates were subjected to a click reaction with a TAMRA-azide, loaded for SDS-PAGE and detected at 550 nm. The gel was then stained for total protein quantification and transferred onto a membrane for western blot. The lower panel shows densitometric band intensities and analysis of fluorescence normalized to total protein stain. The p-value in the graph indicate the statistical significance based the Student's *t*-test (HeLa n=3, HT29 n=2). (**C**) Mitochondrial translation products were labeled using HPG in both shTRAP1 and TRAP1-Flag HeLa cells and in control cells (shGFP and Actin-Flag, respectively). TOM20 immunostaining was used to confirm the mitochondrial localization of the HPG signal visualized by a copper-catalyzed cycloaddition reaction (click) to TAMRA-azide. We used cells that were not incubated with HPG (NT) or those treated with chloramphenicol as negative controls. Scale bar 10 µm. (**D**) MITOFUNCAT-gel. Expression of Actin-Flag and TRAP1-Flag was induced in HeLa cells with Tetracycline 24 hours before labeling with 100 µM HPG-alkyne for 2 hours. The resulting lysates were subjected to a click reaction with a TAMRA-azide, loaded for SDS-PAGE and detected at 550 nm. The same gel was then stained for total protein quantification. (**E**) RT-qPCR of the 13 mitochondrial-encoded protein coding-RNAs upon induction (72 hours) of GFP-directed (control) and TRAP1-directed shRNAs in HeLa cells. Total RNAs were extracted from isolated mitochondria. Data are expressed as mean ± S.E.M. from four independent experiments with technical triplicate each. The 12S rRNA was used as the internal control.



Supplemental Figure S3: RT-qPCR performed on RNAs extracted from mitoribosomal fractions pooled as indicated on the x-axis, isolated from HeLa cells 72 hrs after induction of shGFP/shTRAP1. Graphs show the ratio between mRNAs amplified from pooled fraction and respective total RNAs (input), both normalized on 12S rRNA. Data are expressed as mean **±** SEM (n=3).



Supplemental Figure S4: (A-B-C) Representative image of PLA showing the interaction, in HeLa cells, between TRAP1 and TOMM40, TOMM20 and TIMM23 (A), between TRAP1 or HSP90AA1 and TUFM (B) and between TRAP1 and EEF1A1 and EEF1G (D). Positive signals of interaction are shown as red dots, nuclei are stained with DAPI (blue). Scale bar = 20 µm. **(D)** Representative image of PLA showing the interaction, in HT29 (upper panel) and MCF7 (lower panel) between TRAP1 and TOMM40, TUFM or EEF1A1. Positive signals of interaction are shown as red dots, nuclei are stained with DAPI (blue). Scale bar = 20 µm.


A
Untreated
Emetine treated
Sucrose Fractions
isolated mito (Input)
10%
30%
1 2 3 4 5 6 7 8 9 10 11 12
HSP90AA1
TRAP1
TUFM
IDH2
uS5m
uL10m
100
70
55
42
25
B
relative TE
shTRAP1
shGFP+CHX/EME
ND1
ND2
ND3
ND4
ND4L
ND5
ND6
CO1
CO2
CO3
ATP6
ATP8
CYB
shTRAP1
shGFP CHX/EME
p-value
ATP6 0.099 0.035
ATP8 0.117 0.129
CO1 0.015 0.008
CO2 0.004 0.499
CO3 0.040 0.157
CYB 0.087 0.321
ND1 0.402 0.140
ND2 0.496 0.717
ND3 0.038 0.903
ND4 <0.0001 0.062
ND4L 0.357 0.008
ND5 0.028 0.181
ND6 0.108 0.217
C
PLA p-eS6/TOMM20
DAPI
Overlay
HeLa shGFP
HeLa shTRAP1
PLA spot/cell
0.0029
shGFP
shTRAP1
D
heavy/light ratio
harringtonine treatment (min)
K_shGFP = 0.1748
K_shTRAP1 = 0.2417
shGFP
shTRAP1
K_Actin-Flag = 0.2173
K_TRAP1-Flag = 0.0633
ACTIN-flag
TRAP1-flag
E
poly/mono shTRAP1 vs shGFP
0.4646
0.2623
0.5974
0.07087
0.1743
0.5445
0.6229
ATP5F1B
ATP5MJ
ATP5MK
ATP5PO
MT-ATP6
MT-ATP8
RPL36A

Supplemental Figure S5: (A) HeLa cell mitochondria were isolated after no treatment (left panel) or following a 15-minute treatment with 100 µg/mL emetine (right panel), then lysed and loaded onto a 10-30% linear sucrose gradient, followed by fractionation. Proteins were precipitated from the resulting fractions and subjected to western blot with indicated antibodies. (**B**) RT-qPCR performed on mRNAs extracted from mitoribosomal fractions (4-11) isolated from HeLa cells 72 hrs after induction of shGFP/shTRAP1, with no treatment or following 100 µg/mL emetine and 100 µg/mL cycloheximide for 1 hour. The amount of mitoribosome-associated mRNA in the two samples has been normalized on 12S rRNA and corrected for its total expression level. Data are expressed as median (n=3). Statistical significance (p-value) based on one-sample *t*-test for each gene is reported in the table on the right. P-values<0.05 are in bold. (**C**) Representative image of PLA showing the interaction of TOMM20 with phosphorylated (active) ribosomal protein eS6 in HeLa cells following 72 hrs induction of TRAP1 and GFP (control)-directed shRNAs. Positive signals of interaction are shown as red dots, nuclei are stained with DAPI (blue). Scale bar = 20 µm. The graph shows the average number of PLA spot/cell, with a p-value representing the statistical significance based on the Student's *t*-test (n=3). (**D**) Quantification of heavy/light polysomes area from profiles shown in Fig. 5A. Data are represented as mean **±** SEM from 5 (HeLa shGFP/shTRAP1) or 3 (HeLa Actin-Flag/TRAP1-Flag) independent experiments, with trend lines showing exponential one-phase decay analysis. K-values on the graph represents the decay rate constant of the two curves. (**E**) Ratio between polysome-associated and monosome-associated mRNAs in shTRAP1 vs shGFP cells. The amount of the associated transcripts was measured by RT-qPCR performed on RNAs extracted from pooled monosomal and polysomal fractions, both corrected for an external reference spike-in RNA (luciferase). Data are represented as mean **±** SEM from 3 independent experiments. Number above bars represent the statistical significance (p-value) calculated by a multiple *t*-test.