Stress-Regulated Translational Attenuation Adapts Mitochondrial Protein Import Through Tim17A Degradation

T. Kelly Rainbolt[1], Neli Atanassova[1], Joseph C. Genereux and R. Luke Wiseman[2]

Department of Molecular & Experimental Medicine, Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA 92037 USA

Supplemental Figure Legends

Figure S1 (Supplement to Figure 1)

- **A.** Immunoblot of lysates prepared from HEK293T cells treated with the indicated concentration of As(III) for 6 h.
- **B.** Immunoblot of lysates prepared from SHSY5Y cells treated with the indicated concentration of As(III) for 6 h.
- **C.** Immunoblot of lysates prepared from Hela cells treated with the indicated concentration of As(III) for 6 h.
- **D.** Immunoblot of lysates prepared from Huh7 cells treated with As(III) (100 μM) for the indicated time.
- **E.** Immunoblot of lysates prepared from retinoic acid differentiated SHSY5Y cells treated with As(III) (50 μM; 6 h), as indicated. Lane 3 shows a sample prepared from retinoic acid-differentiated SHSY5Y cells were retinoic acid was removed prior to As(III) addition to control for potential anti-oxidant activities of retinoic acid.
- **F.** Immunoblot of lysates prepared from MEF^{WT} cells treated with As(III) (100 μM) for the indicated time. Note the transient increase in eIF2α phosphorylation that peaks at 2 h, which reflects the negative feedback loop of ISR signaling afforded by the ISR-dependent increase in the eIF2α phosphatase regulatory subunit GADD34.
- **G.** Immunoblot of lysates prepared from HEK293 cells stably-expressing Fv2e-PERK treated for the indicated time with AP20187 (5 nM) or As(III) (50 μM), as indicated.
- **H.** Immunoblot of lysates prepared form HEK293T cells treated for the indicated time with thapsigargin (Tg; 500 nM).
- **I.** Immunoblot of lysates prepared from HEK293T cells treated with CHX (50 μg/mL) for the indicated time.

Figure S2 (Supplement to Figure 2)

- **A.** qPCR analysis of *TIM17A* and *HSP60* in MEF^{WT} treated with thapsigargin (Tg; 500 nM, 6 h), as indicated. mRNA levels were normalized to *GAPDH*. The error bars show mean +/- 95% confidence interval. Data are representative of 3 independent experiments.

B. Representative autoradiogram of immunopurified [^{35}S]-labeled Tim17A and total [^{35}S]-labeled proteome in HEK293T cells chased in the presence or absence of CHX (50 µg/mL) for 6 h. The experimental paradigm used for this analysis is identical to that shown in **Figure 2F**.

C. Quantification of autoradiograms as shown in **Figure S2B**. Error bars show SEM for n=5 ***indicates p-value < 0.005.

Figure S3 (Supplement to Figure 3)

Immunoblot of lysates prepared from HEK293T cells expressing control or *AFG3L2* shRNA treated with cycloheximide (CHX; 50 µg/mL, 6 h), as indicated.

Figure S4 (Supplement to Figure 5)

A. Immunoblot of total (T), cytosolic (C) or mitochondrial (M) fractions prepared from HEK293T cells overexpressing GFP or OTC^{HA}. The arrows highlight the cytosolic-localized precursor OTC^{HA} (pOTC) and the mitochondrial localized intermediate (iOTC) and mature (mOTC) OTC^{HA}. We did not observe significant levels of mature OTC^{HA} in any of our experiments, therefore we only quantified the iOTC fraction as the mitochondrial fraction in experiments described in **Figure 5A**.

B. Immunoblot of total (T), cytosolic (C) or mitochondrial (M) fractions prepared from HEK293T cells expressing ^{mt}M-TTR. The arrows reflect the cytosolic (Cyto) and mitochondrial (Mito) fractions of ^{mt}M-TTR. The different sizes of these proteins reflects the proteolytic removal of the CoxVIII mitochondrial targeting sequence of ^{mt}M-TTR once this protein localizes to the mitochondrial matrix.

C. Plots showing the TMRE fluorescence of HEK293T cells treated with As(III) (50 µM; 6 h). Cells treated with CCCP (50 µM; 30 min) are shown as a control. TMRE fluorescence was measured by flow cytometry. Error bars show SEM for n=6.

D. Graph depicting the normalized total amount of [^{35}S]-labeled ^{mt}M-TTR (mitochondrial + cytosolic fractions) in the experiments described in **Figure 5D**. Error bars show SEM from n=6.

E. Plots showing the TMRE fluorescence of HEK293T-REX cells expressing control or *TIM17A* shRNA. Cells treated with CCCP (50 µM; 30 min) are shown as a control. TMRE fluorescence was measured by flow cytometry.

Figure S5 (Supplement to Figure 6)

qPCR analysis of *HSP60* and *YME1L* in SHSY5Y cells stably-expressing control or *TIM17A* shRNA. mRNA levels were normalized to *GAPDH*. (n=8). The error bars show the mean +/- 95% confidence interval.

Supplemental Table Legends

Table S1 (Supplement to Figure 5). – TMT-MuDPIT analysis of mitochondrial proteins in HEK293T cells expressing control or *TIM17A* shRNA.

Table S2 (Supplement to Supplemental Experimental Procedures). Table showing the specific MW of the TMT mass tags used for the TMT-MuDPIT analysis of mitochondria isolated from HEK293T cells expressing non-silencing (NS) shRNA or *TIM17A* shRNA. Three different preparations for each cell line are shown.

Sample	Mass Tag
NS shRNA #1	126
NS shRNA #2	127
NS shRNA #3	128
TIM17A shRNA #1	129
TIM17A shRNA #2	130
TIM17A shRNA #3	131

Supplemental Materials and Methods

Plasmids

^{mt}M-TTR was cloned into the Gateway pENTR1A plasmid (Invitrogen) and recombined into the pT-REx-DEST30 destination vector (Invitrogen). Human OTC was cloned into the pCI vector containing three C-terminal HA sequences. *TIM17A*, *TIM17B*, *YME1L*, and the control non-targeting shRNA in the pGIPz vector were purchased from Open Biosystems. *AFG3L2* shRNA in the pLKO.1 vector was obtained from Sigma Aldrich.

Antibodies and Drugs

Professor J.W. Kelly (TSRI) generously provided polyclonal antibodies against TTR. Other antibodies were obtained commercially as follows: Tim17A (Genetex), Tim17A [H-1] (Santa Cruz), Tim17B (Protein Tech), Tim23 (BD Transduction Laboratories), Hsp60 [LK1] (Neomarkers), Hsp90 (Genetex), Tubulin (Sigma Aldrich), eIF2α (Abcam), eIF2α pS51 (Cell Signaling), YME1L (Genetex), AFG3L2 [N1N2] (Genetex), HA [16B12] (Covance). AP20187 was obtained from Ariad Pharmaceuticals.

TMT-MuDPIT Analysis of Mitochondrial Proteomes

Only MS grade organic solvents were used during sample preparation for mass spectrometry. 75 ug of each mitochondrial isolate was lysed in 40 μL RIPA (150 mM NaCl, 50 mM Tris pH 7.5, 1% Triton X100, 0.5% sodium deoxycholate, 0.1 % SDS) on ice and centrifuged at 13,000 x *g* for 15 minutes to remove cellular debris. Lysates were brought to 90 μL, 50 mM triethylammonium bicarbonate (Sigma), reduced in 10 mM TCEP (Pierce) for 1 hour at 55 ^{O}C, and alkylated with 17 mM iodoacetamide (Sigma) for 30 min at ambient temperature and in the dark. Reduced and alkylated proteins were $CHCl_3$/MeOH precipitated and the pellets washed in methanol prior to resuspension in 100 mM TEAB. Proteins were digested overnight at 37 ^{O}C with agitation in the presence of 1.7 μg sequencing grade trypsin (Promega, 2.5 μg per 100 μg protein) and 1 mM $CaCl_2$.

TMT isotopic labels (Thermo Scientific) were resuspended (0.8 g/150 μL acetonitrile) and 33 μL of label added to each 100 μL sample of digested peptides as indicated in the **Table S2**. Labeling was allowed to proceed for 1 hour at ambient temperature, followed by quenching with 0.3% hydroxyamine for 15 min. at

ambient temperature. Samples were pooled, acidified, centrifuged for 30 min. at 13,000 x *g* to remove any insoluble debris, and 50 µg total protein used for MudPIT analysis.

Samples were analyzed using two dimensional LC/MS/MS on an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific, San Jose, CA) with an HP 1200 series HPLC pump (Agilent Technology, Santa Clara, CA) according to standard MuDPIT protocols (Washburn et al., 2001). Biphasic loading columns were prepared by polymerizing a Kasil 1624 frit into a 250 µm diameter fused silica capillary (Agilent) and then packing with 2.5 cm 5 µm strong cation exchange resin (Partisphere, GE Healthcare, UK) and 2.5 cm reversed-phase 5 µm C18 resin (Aqua, Phenomenex, Torrance, CA). Analytical columns were prepared by pulling on 100 µm diameter fused silica columns (Agilent) with a P-2000 laser (Sutter Instrument Co., Novato, CA), followed by packing with 15 cm reversed-phase 5 µm C18 resin. Analysis was performed using an eleven cycle standard chromatographic run, with a progressively more concentrated ammonium acetate salt bump beginning each cycle. Each cycle has a linearly increasing acetonitrile gradient, and eluted peptides were scanned from 300 to 1600 m/z with resolution 30000 and the mass spectrometer in a data dependent acquisition mode. The top ten peaks for each full scan were fragmented by HCD using a normalized collision energy of 45%, a 100 ms activation time, and a resolution of 7500. Dynamic exclusion parameters were 1 repeat count, 30 ms repeat duration, 500 exclusion list size, 120 s exclusion duration, and exclusion width between 0.51 and 1.51.

MS/MS spectra were extracted using RAW Xtractor (version 1.9.1), and searched using ProLuCID against a custom database containing 20245 human sequences (longest entry for the IPI database for each protein) and reverse sequences for each entry as the decoy set. ProLuCID searches allowed for static modification of cysteine residues (57.02146 Da, acetylation) N-termini, and lysine residues (229.1629 Da, TMT-tagging), half tryptic peptidolysis specificity, and mass tolerance of 600 ppm for precursor mass and 10 ppm for product ion masses. Spectra matches were assembled and filtered by DTASelect2 (version 2.0.27) (Tabb et al., 2002). The stringency of spectral matching was chosen such that <1% of identified peptides were from the decoy database. Quantitation in Census (Park et al., 2008) was performed by averaging TMT reporter ion intensities for all spectra associated with an individual peptide, followed by averaging at the protein level, deconvolution of isotopic impurity as reported in the lot analysis supplied by Thermo Fisher, and finally correction of ratio values to the mode for mitochondrial proteins.

TMRE Fluorescence measurement of mitochondrial membrane potential

HEK293T or HEK293T-Rex cells were cultured using standard conditions and treated as indicated. For membrane poteneial measurements, cells were stained for 1 hour with 25 nM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen) at 37°C. Cells were washed twice in 1x DPBS then rinsed off plates and resuspended into 1x TBS supplemented with 1 mM EDTA. Fluorescence-activated cell sorter (FACS) data were collected on a Calibur 2 flow cyctometer (BD Biosience). Acquired data were analyzed using FlowJo software (Tree Star, Inc.).

C. elegans Strains and Stress Sensitivity Assays

Strains containing the mtUPR reporter *hsp-60*$_{pr}$*::gfp(zcls9) V*, have been previously described (Yoneda et al., 2004). The *haf-1(ok705) and atfs-1(tm4525)* mutant worms were generously provided by C. Haynes (MSKCC) and are described in (Haynes et al., 2010; Nargund et al., 2012).

Paraquat survival assays were performed in liquid culture based on the method in (Solis and Petrascheck, 2011), with minor modification. Briefly, cultures of HT115 *E. coli* carrying pL4440 plasmids, empty vector, *tim-17*, *or tim-23* were grown to OD_{600}=0.6 at 37°C and then dsRNA expression was induced with 1 mM IPTG. Cultures were incubated at 20°C following induction for 6 hours, and harvested as described with the addition of 1 mM IPTG to the final resuspension in S-complete. Plates containing starved N2, *haf-1*, or *atfs-1* mutant gravid adults were bleached and eggs incubated in S-complete overnight to generate synchronous L1 larval populations. Larva were plated in S-complete containing 6 mg/ml of the indicated HT115 *E. coli* with 1 mM IPTG into 96-well plates and incubated at 20°C. At the L4 developmental stage the larva were sterilized by the addition of FUDR. On day 1 of adulthood, 10 µL of paraquat stock solutions were added to the indicated final concentrations and the worms were incubated for 24 hours prior to visually scoring viability.

SUPPLEMENTAL REFERENCES

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Tabb, D.L., McDonald, W.H., and Yates, J.R., 3rd (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J Proteome Res *1*, 21-26.
Washburn, M.P., Wolters, D., and Yates, J.R., 3rd (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol *19*, 242-247.
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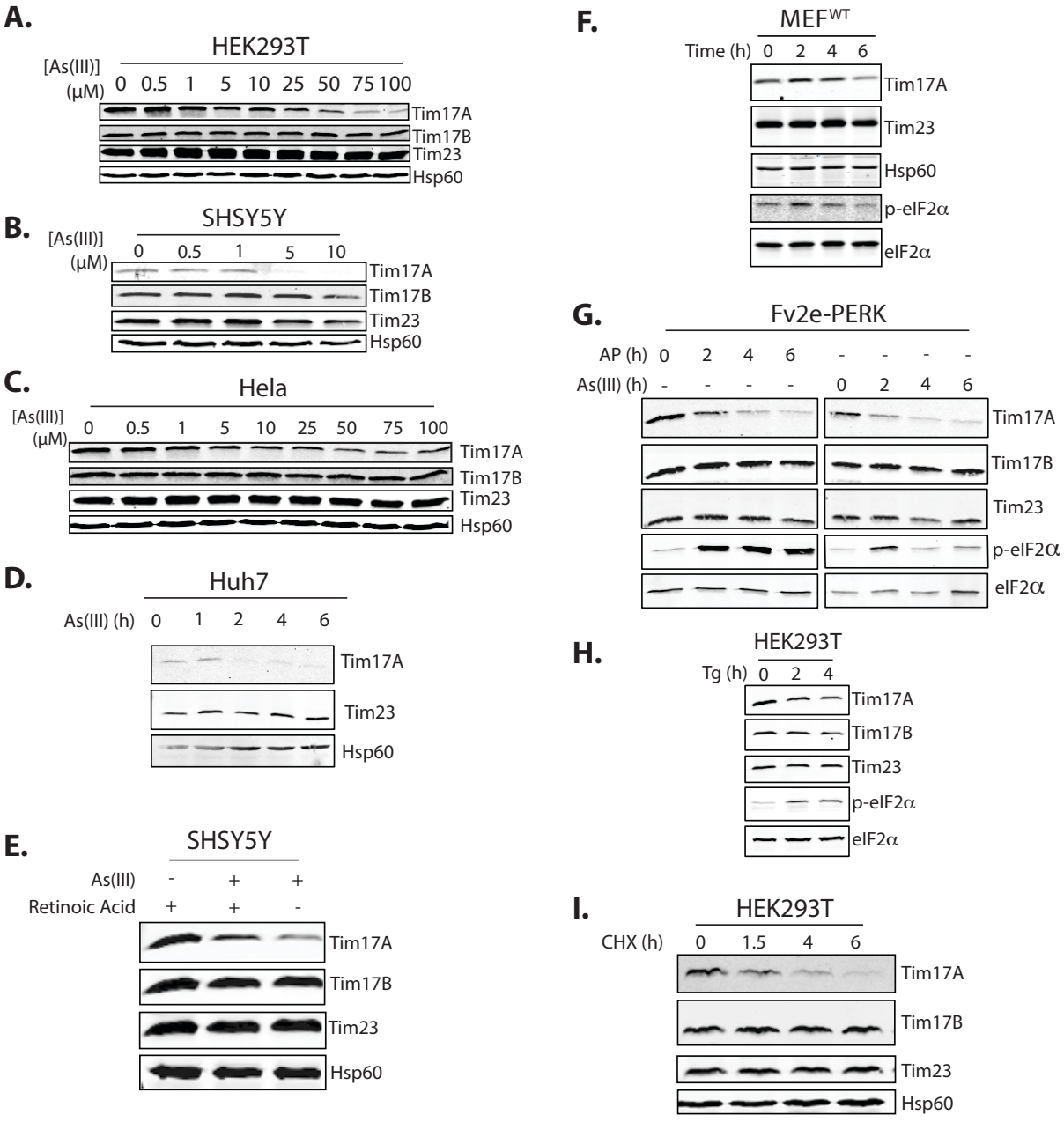
Figure S1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HEK293T
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Tim17A
Tim17B
Tim23
Hsp60
B.
SHSY5Y
[As(III)] (μM) 0 0.5 1 5 10
Tim17A
Tim17B
Tim23
Hsp60
C.
Hela
[As(III)] (μM) 0 0.5 1 5 10 25 50 75 100
Tim17A
Tim17B
Tim23
Hsp60
D.
Huh7
As(III) (h) 0 1 2 4 6
Tim17A
Tim23
Hsp60
E.
SHSY5Y
As(III) - + +
Retinoic Acid + + -
Tim17A
Tim17B
Tim23
Hsp60
F.
MEF^WT
Time (h) 0 2 4 6
Tim17A
Tim23
Hsp60
p-eIF2α
eIF2α
G.
Fv2e-PERK
AP (h) 0 2 4 6 - - - -
As(III) (h) - - - - 0 2 4 6
Tim17A
Tim17B
Tim23
p-eIF2α
eIF2α
H.
HEK293T
Tg (h) 0 2 4
Tim17A
Tim17B
Tim23
p-eIF2α
eIF2α
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HEK293T
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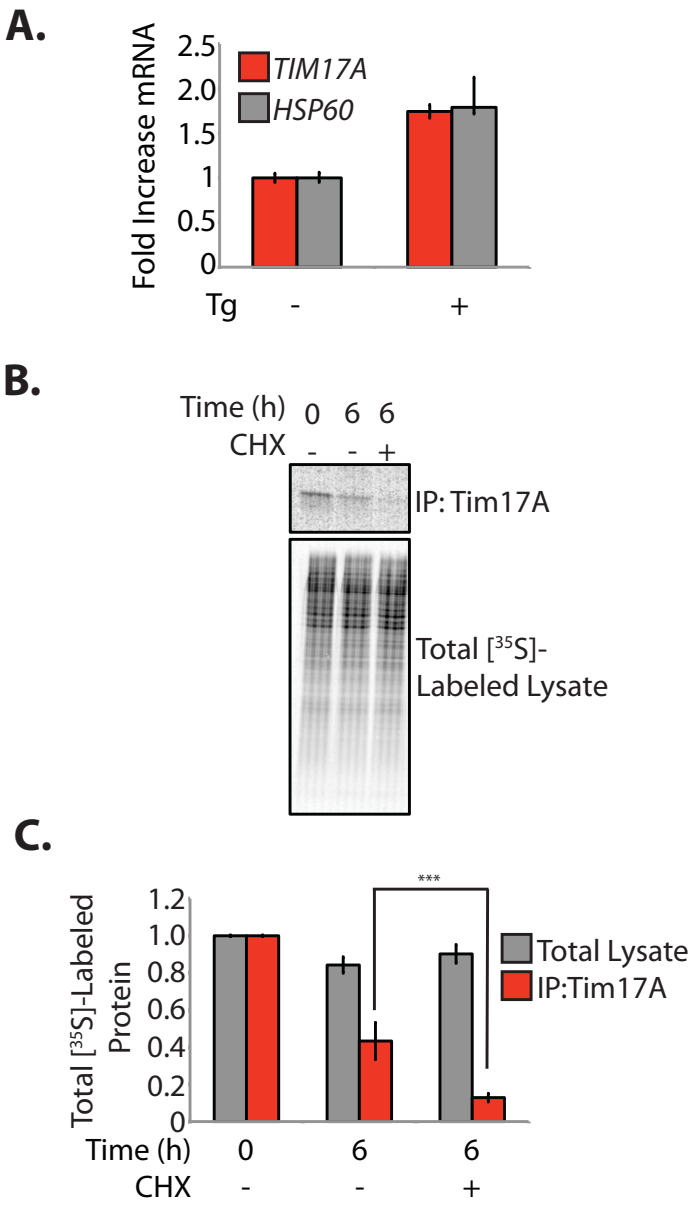
Tim17A
Tim17B
Tim23
Hsp60

Figure S2


A.
Fold Increase mRNA
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2.0
1.5
1
0.5
0
TIM17A
HSP60
Tg
-
+
B.
Time (h)
0
6
6
CHX
-
-
+
IP: Tim17A
Total [35S]-
Labeled Lysate
C.
Total [35S]-Labeled Protein
1.2
1.0
0.8
0.6
0.4
0.2
0
***
Total Lysate
IP:Tim17A
Time (h)
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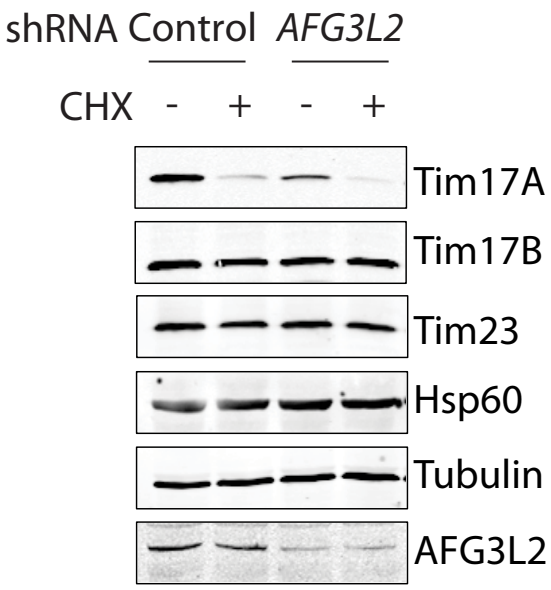
6
6
CHX
-
-
+

Figure S3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CHX - + - +
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Tim17B
Tim23
Hsp60
Tubulin
AFG3L2

Figure S4

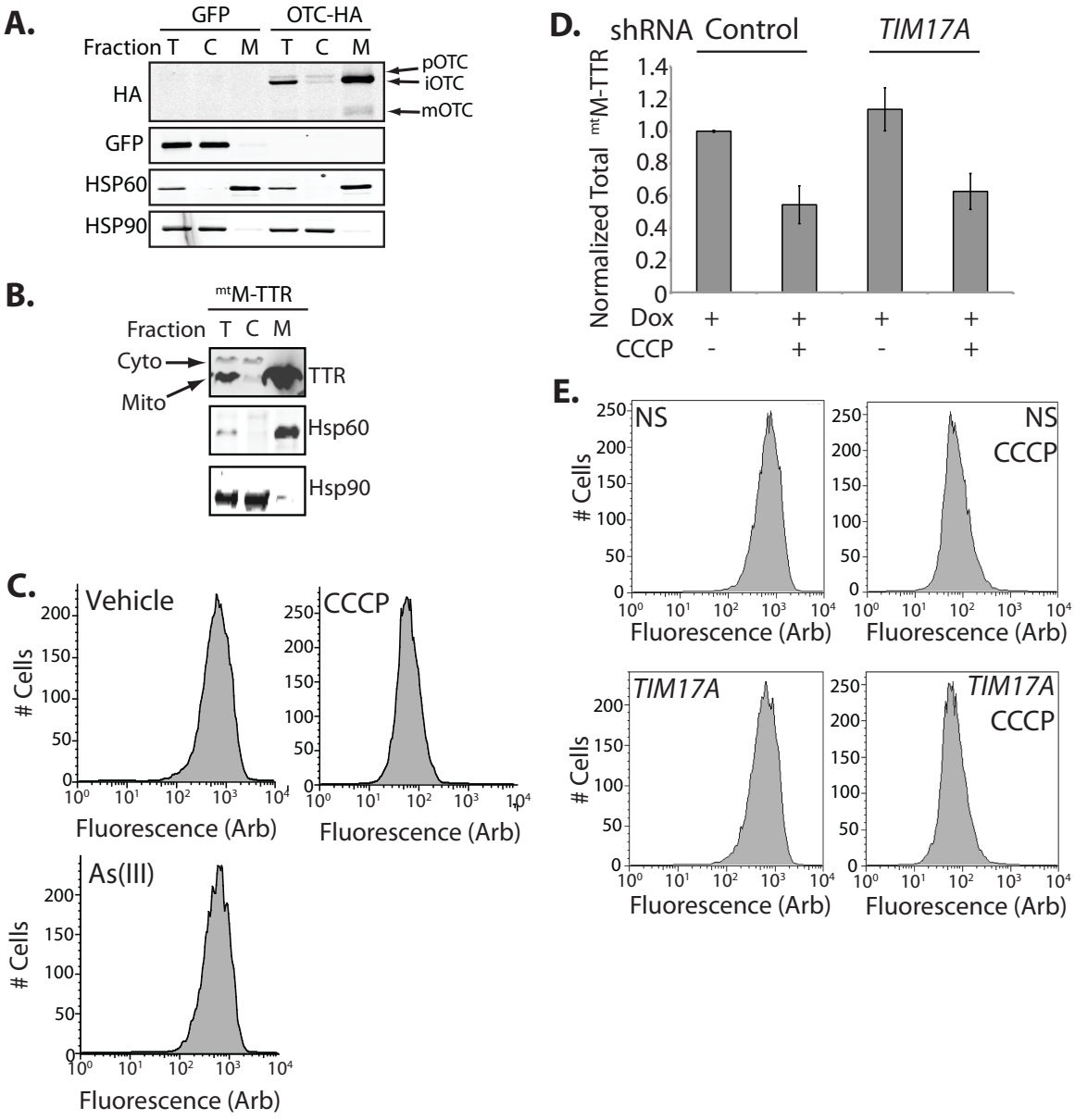
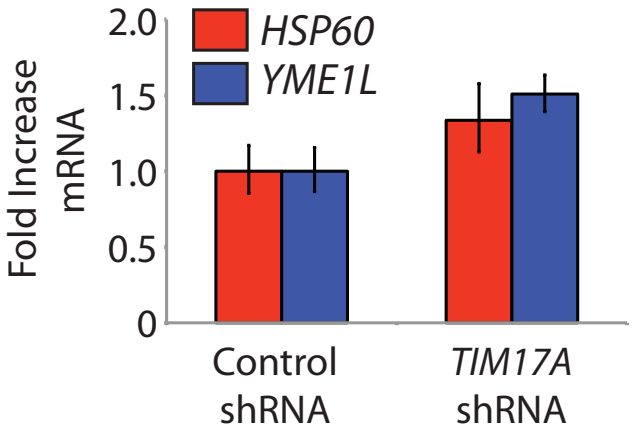

A.
GFP
OTC-HA
Fraction T C M T C M
pOTC
iOTC
mOTC
HA
GFP
HSP60
HSP90
B.
mtM-TTR
Fraction T C M
Cyto
Mito
TTR
Hsp60
Hsp90
C.
Vehicle
CCCP
As(III)
# Cells
Fluorescence (Arb)
D.
shRNA Control
TIM17A
Normalized Total mtM-TTR
Dox + + + +
CCCP - + - +
E.
NS
NS CCCP
TIM17A
TIM17A CCCP
Cells
Fluorescence (Arb)

Figure S5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0.5
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Fold Increase mRNA
HSP60
YME1L
Control shRNA
TIM17A shRNA