Supplemental Information for:

YME1L Degradation Reduces Mitochondrial Proteolytic Capacity During Oxidative Stress

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Supplemental Methods.

Cell Lines and Culture Conditions

To increase reliance on oxidative phosphorylation, HEK293T cells were seeded into glucose free DMEM supplemented with 10 mM galactose, 4 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and grown at 37°C and 5% CO_2 for at least one passage prior to experiments. For glucose depravation, media was removed, cells were washed twice in Dulbecco's phosphate buffered saline (DPBS) and glucose free DMEM supplemented with 10 mM 2-deoxy-D-glucose, 4 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin was added for the treatment time. Control cells were likewise washed and received complete DMEM for the treatment.

Antibodies and Chemicals

Primary antibodies were obtained commercially and used as follows: YME1L (1:1000, ProteinTech), AFG3L2[N1N2] (1:1000, Genetex), Hsp60[LK1] (1:1000, Neomarkers), Tubulin[B-5-1-2] (1:2000, Sigma Aldrich), OPA-1 (1:2000, BD Transduction Labs), PARP (1:1000, Cell Signaling), Cleaved caspase-3[5A1E] (1:1000, Cell Signaling), phospho-eIF2α (1:1000, Cell Signaling), eIF2α (1:1000, Abcam), Tim17A (1:1000, Genetex), Tim23 (1:1000, BD Transduction Labs), and OMA1 (1:200, Santa Cruz).

Chemical reagents were obtained commercially as follows: H_2O_2 (Fisher Scientific), Antimycin A (Sigma-Aldrich), Arsenite (JT Baker), Oligomycin A (Sigma-Aldrich), 2-deoxyglucose (Sigma-Aldrich), CCCP (Tocris), paraquat (Sigma-Aldrich), cycloheximide (Chemservice), ATP (Sigma-Aldrich), ADP (Sigma-Aldrich), AMP (Sigma-Aldrich), AMP-PNP (Sigma-Aldrich), o-phenanthroline (Sigma-Aldrich), and staurosporine (LC Laboratories). All reagents were prepared as stock solutions and added as indicated. *YME1L* shRNA and *TIM17A* shRNA were obtained from Open Biosystems in the pGIPz vector. Stable cell lines expressing these shRNA were selected using puromycin (Corning).

Lysate Preparation and Immunoblot Analysis

Cells were washed twice in cold DPBS, scraped and collected by centrifugation. Cellular lysates were prepared in Lysis Buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton, complete EDTA-free protease

inhibitors (Roche)). Total protein concentrations were normalized by Bio-Rad protein assay. Lysates were then separated by Tris-glycine SDS-PAGE using standard methods and transferred to nitrocellulose membranes for immunoblotting. Cells were washed twice in cold DPBS, scraped and collected by centrifugation. Cellular lysates were prepared in Lysis Buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton, complete EDTA-free protease inhibitors (Roche)). Total protein concentrations were normalized by Bio-Rad protein assay. Lysates were then separated by Tris-glycine SDS-PAGE using standard methods and transferred to nitrocellulose membranes for immunoblotting. After incubation with the indicated primary antibodies, membranes were probed with the appropriate 680nm and 800nm labeled secondary antibodies (Li-COR Biosciences) then analyzed and quantified using the Odyssey Infrared Imaging System (Li-COR Biosciences).

TMRE Fluorescence

Cells were plated in poly-D-lysine coated 96-well plates and treated as indicated. Control cells were treated with 20 μM CCCP for 20 mins to depolarize mitochondria. Samples were stained with 200 nM TMRE for 20 mins. Each well was washed twice in DPBS+ 0.2% BSA to remove excess dye. TMRE fluorescence was read in a Safire II plate reader (Tecan) and background corrected. All measurements were made in biologic triplicate.

Statistical Analysis

Data were analyzed using a two-tailed Student t test to determine significance. Error bars represent standard error of the mean (SEM).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (Supplement to Figure 1) - YME1L is a stress-sensitive mitochondrial protease.

A. Quantification of Tim23 levels in SHSY5Y cells treated with the indicated concentration of H_2O_2 for 6 h. Representative immunoblot is shown in **Figure 1B**. Error bars show SEM for n ≥ 3.

B. Immunoblot of lysates prepared from N2a cells incubated with the indicated concentration of hydrogen peroxide (H_2O_2) for 6 h.

C. Immunoblot of lysates prepared from HEK293T cells cultured in galactose-containing media and treated with the indicated concentration of H_2O_2 for 6 h.

D. Immunoblot of lysates prepared from Hela cells incubated with H_2O_2 (1 mM) for the indicated time.

E. Immunoblot of lysates prepared from SHSY5Y cells incubated with paraquat (10 mM) for the indicated time.

F. Immunoblot of lysates prepared from HEK293T cells cultured in glucose-containing media and treated with the indicated concentration of antimycin A (AntA) for 6 h.

Figure S2 (Supplement to Figure 2) – YME1L degradation involves the activity of the ATP-independent mitochondrial protease OMA1

A. Immunoblot of mitochondria isolated from mouse livers incubated for 0 or 6 h in the presence of ATP (5 mM) or o-phenanthroline (o-phe; 1 mM), as indicated.

B. Immunoblot of mitochondria isolated from SHSY5Y cells incubated for 0 or 6 h in the presence of ATP (5 mM), o-phenanthroline (1 mM), and hydrogen peroxide (H_2O_2; 100μM), as indicated.

C. Immunoblot of mitochondria isolated from SHSY5Y cells incubated for 0 or 6 h in the presence of ATP (5 mM), ADP (5 mM), AMP (5 mM), AMP-PNP (5 mM), and oligomycin A (5 nM), as indicated.

D. Immunoblot of mitochondria isolated from SHSY5Y cells incubated for 0 or 6 h in the presence of PMSF (a serine protease inhibitor; 1 mM), bortezomib (Bor; a serine protease inhibitor; 5 μM), pepstatin A (PepA; an aspartic acid protease inhibitor; 50 μM) or E64 (a cysteine protease inhibitor; 50 μM).

E. Immunoblot of lysates from *OMA1*$^{+/+}$ and *OMA1*$^{-/-}$ MEFs treated with the indicated concentrations of H_2O_2 for 6 hours.

Figure S3 (Supplement to Figure 3) – YME1L degradation is initiated by reductions in intracellular ATP and OMA1 activation

- **A.** Immunoblot of mitochondria isolated from SHSY5Y cells incubated for 0 or 6 h in the presence of ATP (5 mM) and *o*-phenanthroline (o-phe; 1 mM), as indicated.
- **B.** Immunoblot of lysates prepared from HEK293T cells cultured in galactose-supplemented media incubated in the presence of 100 nM antimycin A (AntA) for the indicated time. Full-length (FL) OMA1 and a cleaved, proteolytically-active OMA1 fragment are shown by the arrows.
- **C.** Plot showing the TMRE fluorescence of HEK293T cells cultured in galactose-supplemented media incubated in the presence of 100 nM antimycin A (AntA) for the indicated time. The uncoupler CCCP (20 μM; 20 mins) was included as a control for mitochondria membrane depolarization.

Figure S4 (Supplement to Figure 4) – Loss of YME1L decreases cellular capacity to regulate mitochondrial proteostasis and sensitizes cells to oxidative insult

- **A.** Immunoblot of lysates prepared from SHSY5Y cells treated with As(III) (100 μM) for the indicated time. The arrows show active caspase-3, full-length (FL) PARP, and cleaved PARP, as indicated.
- **B.** Immunoblot of lysates from SHSY5Y cells expressing NS or *TIM17A* shRNA treated with the indicated concentrations of H_2O_2 for 6 hours.
- **C.** Immunoblot of mitochondria isolated from HEK293T cells incubated for 0 or 6 h in the presence of ATP (5 mM) and/or o-phenanthroline (o-phe; 1 mM), as indicated. H_2O_2 (100 μM) was added to mitochondria as indicated. *indicates a non-specific band detected by the Tim17A antibody in isolated mitochondria.
- **D.** Immunoblot of lysates from SHSY5Y cells expressing NS or *YME1L* shRNA treated with staurosporine (STS) (1 μM) for the indicated times. Full-length (FL) and cleaved PARP are shown by the arrows.

FIGURE S1

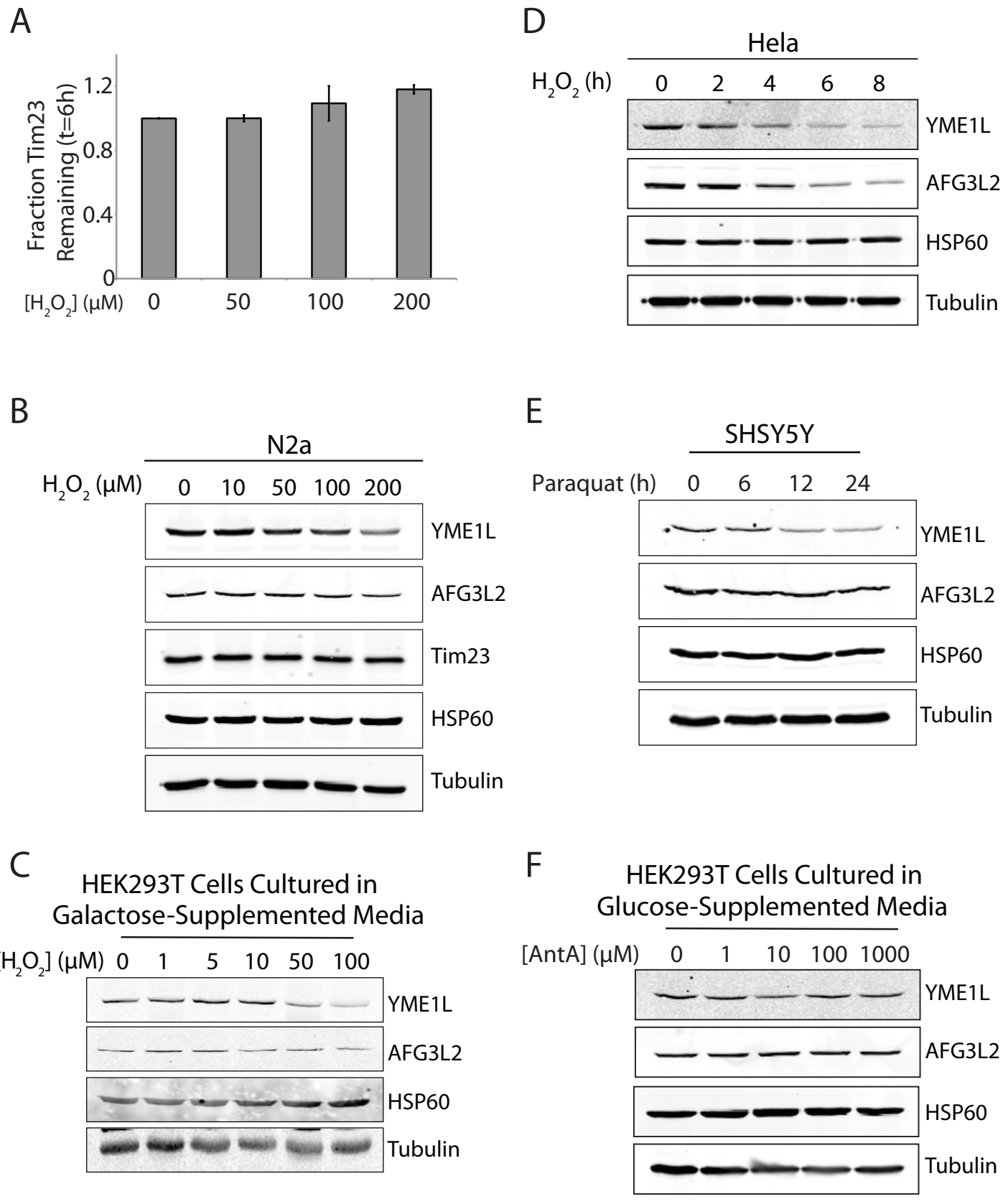

A
Fraction Tim23 Remaining (t=6h)
1.2
0.8
0.4
0
[H2O2] (μM) 0 50 100 200
B
N2a
H2O2 (μM) 0 10 50 100 200
YME1L
AFG3L2
Tim23
HSP60
Tubulin
C
HEK293T Cells Cultured in Galactose-Supplemented Media
[H2O2] (μM) 0 1 5 10 50 100
YME1L
AFG3L2
HSP60
Tubulin
D
Hela
H2O2 (h) 0 2 4 6 8
YME1L
AFG3L2
HSP60
Tubulin
E
SHSY5Y
Paraquat (h) 0 6 12 24
YME1L
AFG3L2
HSP60
Tubulin
F
HEK293T Cells Cultured in Glucose-Supplemented Media
[AntA] (μM) 0 1 10 100 1000
YME1L
AFG3L2
HSP60
Tubulin

FIGURE S2

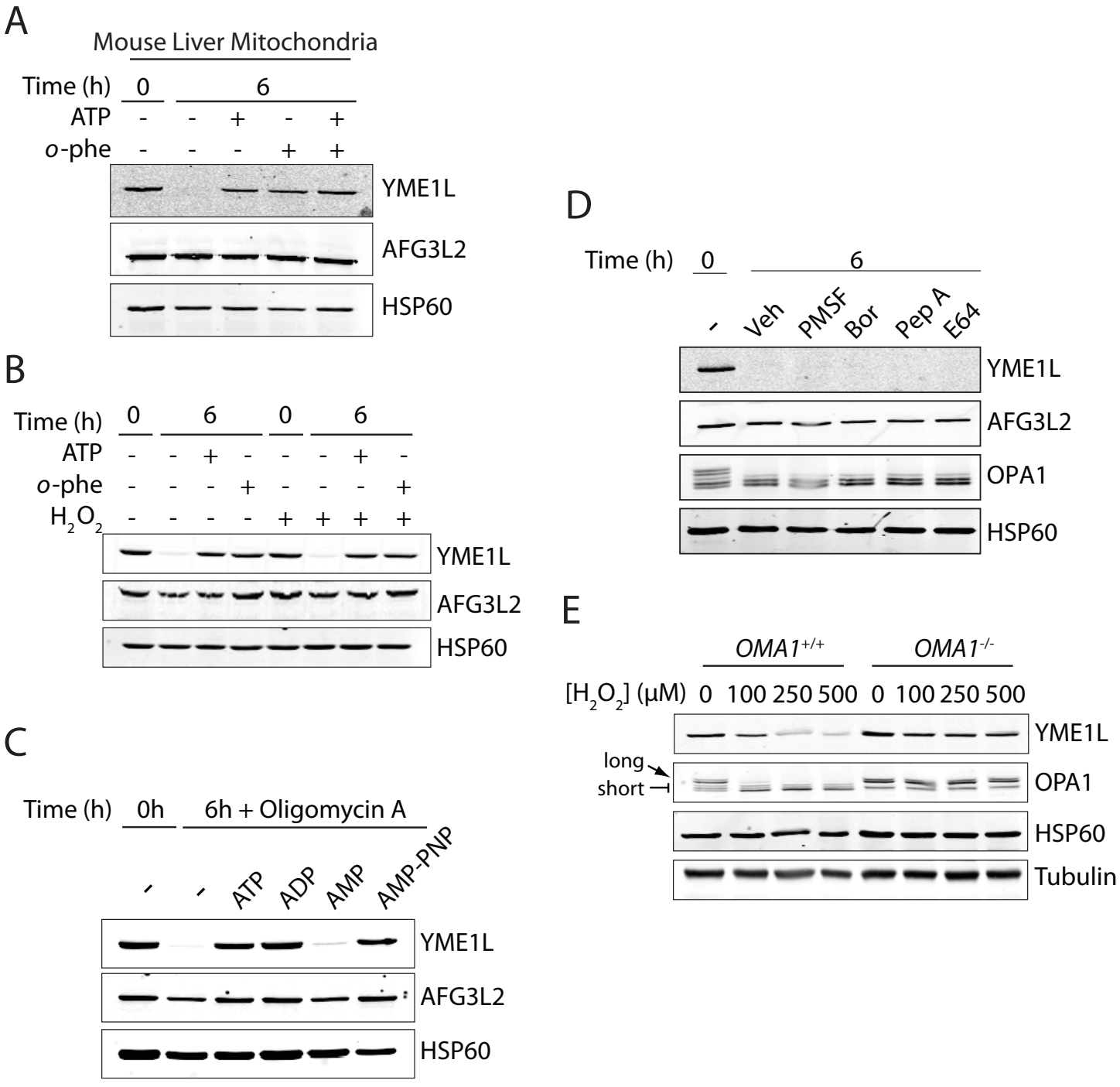

A
Mouse Liver Mitochondria
Time (h) 0 6
ATP - - + - +
o-phe - - - + +
YME1L
AFG3L2
HSP60
B
Time (h) 0 6 0 6
ATP - - + - - - + -
o-phe - - - + - - - +
H2O2 - - - - + + + +
YME1L
AFG3L2
HSP60
C
Time (h) 0h 6h + Oligomycin A
- - ATP ADP AMP AMP-PNP
YME1L
AFG3L2
HSP60
D
Time (h) 0 6
- Veh PMSF Bor Pep A E64
YME1L
AFG3L2
OPA1
HSP60
E
OMA1+/+ OMA1-/-
[H2O2] (μM) 0 100 250 500 0 100 250 500
long
short
YME1L
OPA1
HSP60
Tubulin

FIGURE S3

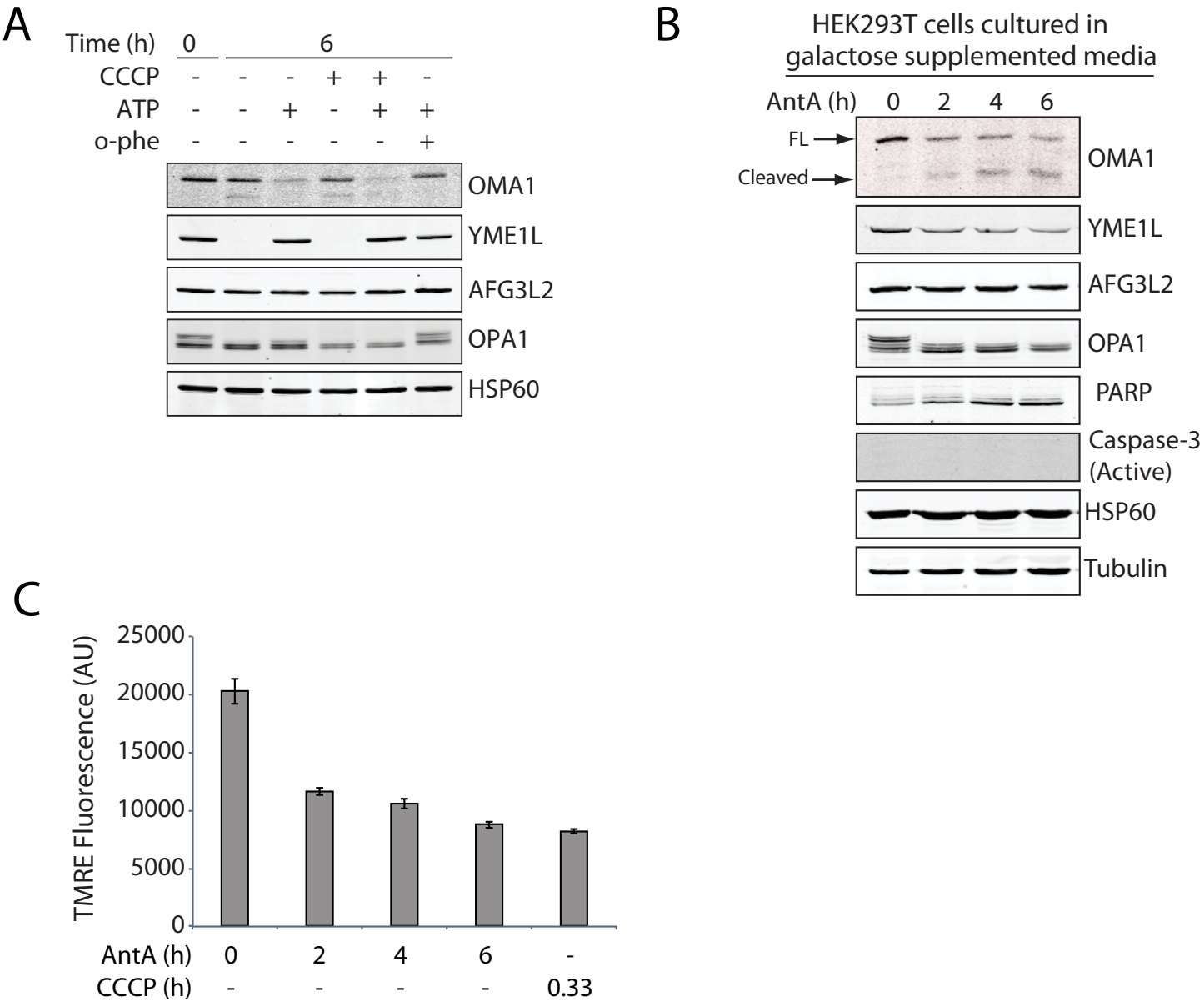
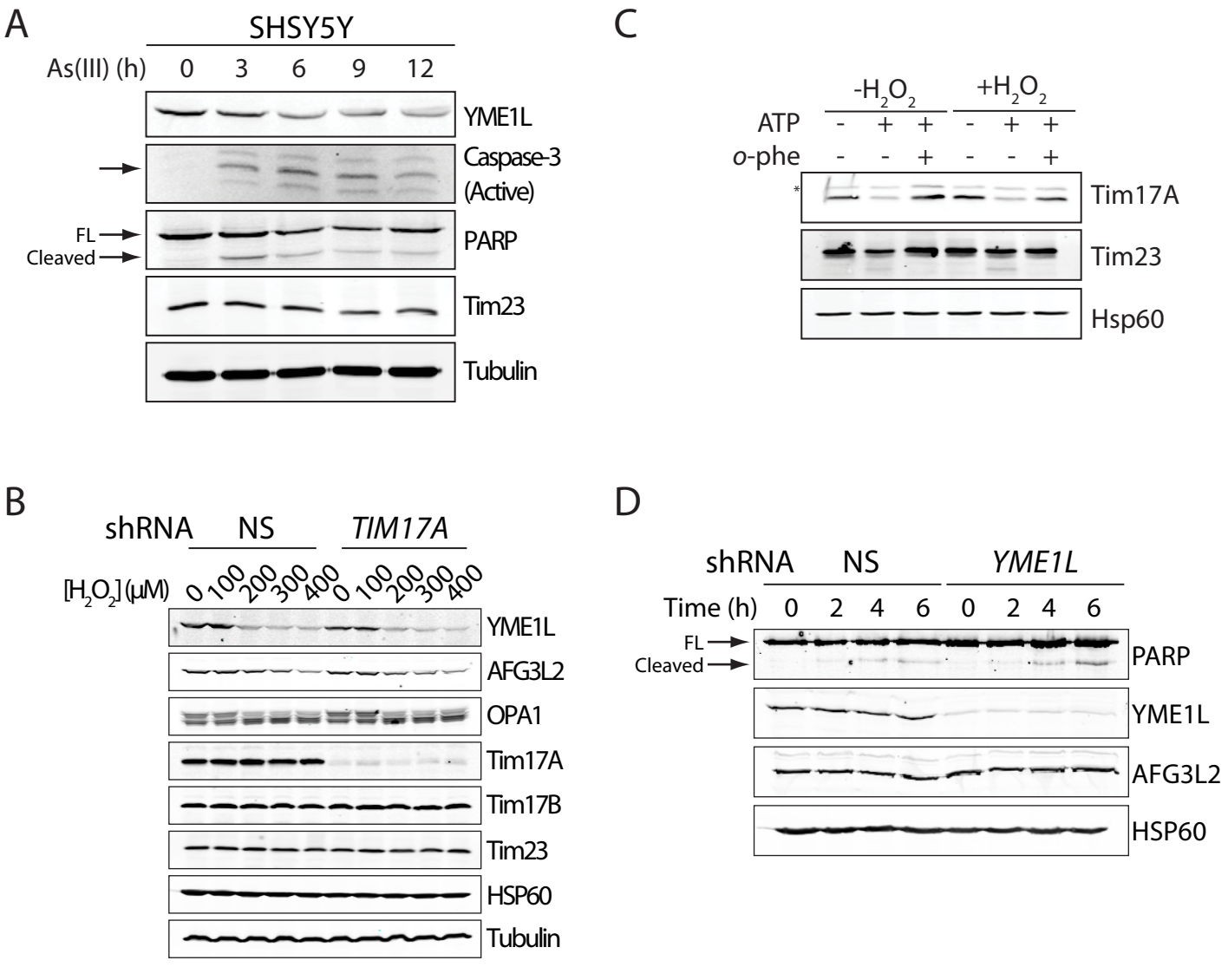

A
Time (h) 0 6
CCCP - - - + + -
ATP - - + - + +
o-phe - - - - - +
OMA1
YME1L
AFG3L2
OPA1
HSP60
B
HEK293T cells cultured in galactose supplemented media
AntA (h) 0 2 4 6
FL
Cleaved
OMA1
YME1L
AFG3L2
OPA1
PARP
Caspase-3 (Active)
HSP60
Tubulin
C
TMRE Fluorescence (AU)
25000
20000
15000
10000
5000
0
AntA (h) 0 2 4 6 -
CCCP (h) - - - - 0.33

FIGURE S4


A
SHSY5Y
As(III) (h) 0 3 6 9 12
YME1L
Caspase-3
(Active)
FL
Cleaved
PARP
Tim23
Tubulin
C
-H2O2
+H2O2
ATP - + + - + +
o-phe - - + - - +
*
Tim17A
Tim23
Hsp60
B
shRNA NS TIM17A
[H2O2] (μM) 0 100 200 300 400 0 100 200 300 400
YME1L
AFG3L2
OPA1
Tim17A
Tim17B
Tim23
HSP60
Tubulin
D
shRNA NS YME1L
Time (h) 0 2 4 6 0 2 4 6
FL
Cleaved
PARP
YME1L
AFG3L2
HSP60