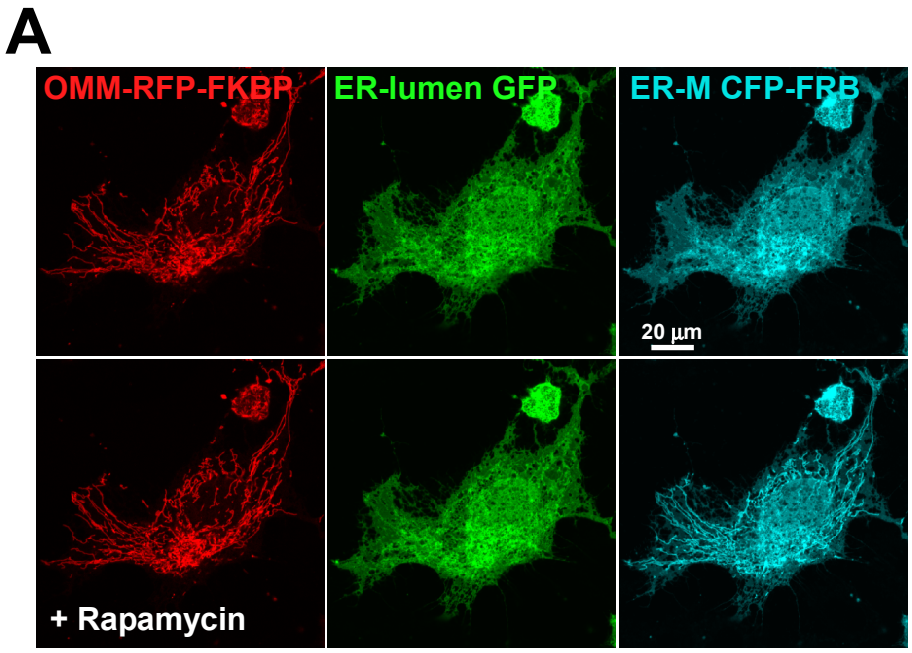
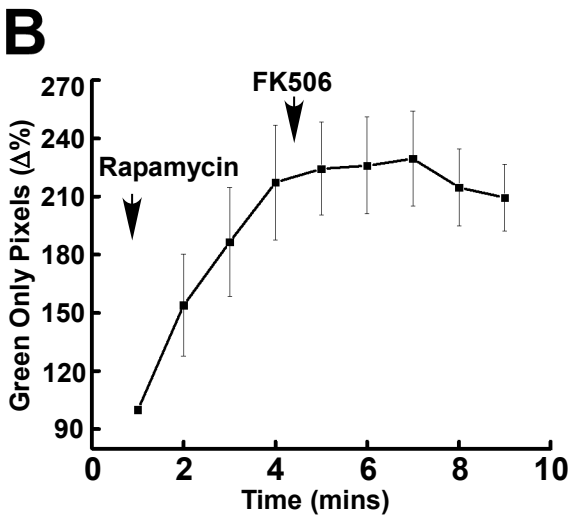
Supplemental Information

Supplemental Figures

Figure S1


A
OMM-RFP-FKBP
ER-lumen GFP
ER-M CFP-FRB
20 µm
+ Rapamycin


B
FK506
Rapamycin
Green Only Pixels (Δ%)
270
240
210
180
150
120
90
0
2
4
6
8
10
Time (mins)

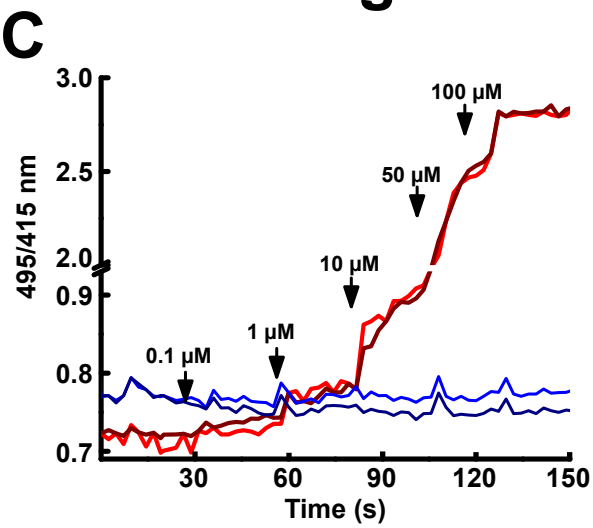

C
100 µM
50 µM
10 µM
1 µM
0.1 µM
495/415 nm
3.0
2.5
2.0
0.9
0.8
0.7
30
60
90
120
150
Time (s)

Fig. S1, related to Figure 1: Targeting of an H_2O_2 reporter to the ER-mitochondrial interface while preserving organelle morphology

(A) Whole cell images for the subcellular areas displayed in Figure 1B showing that heterodimerization of linker halves progressively changes the distribution of the ER-surface targeted probe (ER-M CFP-FRB; cyan) to co-localize with OMM targeted probes (OMM-RFP-FKBP; red) in response to rapamycin (lower row vs upper row). However, the total ER-structure visualized with ER-lumen targeted GFP (ER-lumen GFP; green) remains unaltered. To limit the long term effects of rapamycin it was washed out after 4 incubation and FK506 (5 µM) was added.

(B) Percentage change in the number of green (ER lumen GFP) only pixels in images following treatment with rapamycin and FK506. The increase shows that redistribution of ER-M CFP in the ER to the close associations with mitochondria results in a progressive increase in the ER-specific pixels that lack the ER-M linker half (*n* = 16 cells).

(C) Sample traces of single HepG2 cells expressing OMM-targeted HyPer (red, dark red; *n* =13), and SypHer (blue, dark blue; *n* =14) permeabilized (Saponin, 40 µg/ml) and exposed to H_2O_2 (stepwise, 0.1, 1, 10, 50&100 µM respectively).

Figure S2

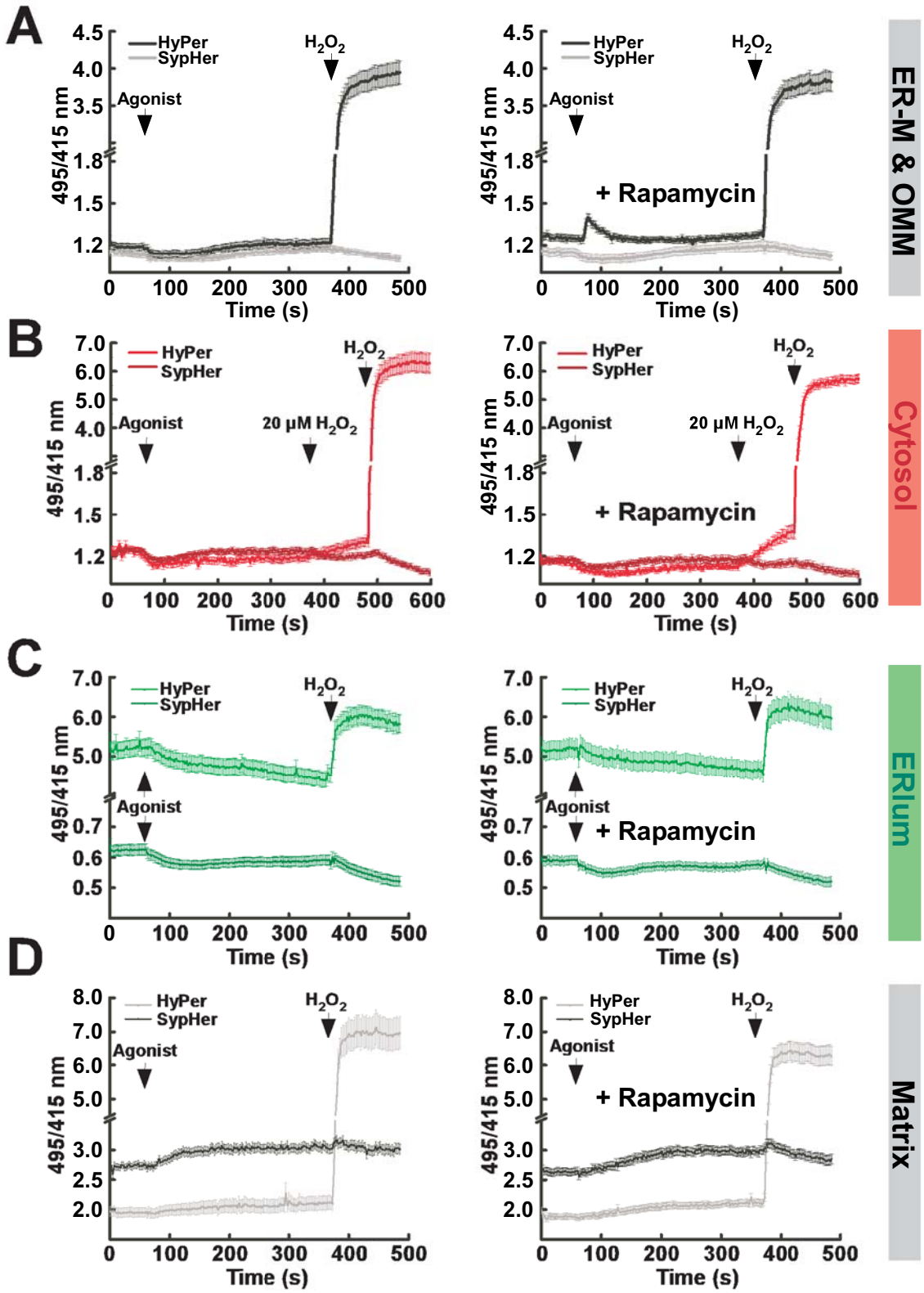

A
B
C
D
HyPer
SypHer
H_2O_2
Agonist
20 µM H_2O_2
+ Rapamycin
495/415 nm
Time (s)
ER-M & OMM
Cytosol
ERlum
Matrix

Fig. S2, related to Figure 2: Agonist-induced H_2O_2 elevation is detectible only when HyPer is targeted to the ER-mitochondrial interface, whereas a pH change occurs throughout the cytoplasm.

(A) HepG2 cells expressing both components of the interface targeting system, OMM and ER-M (HyPer, black or SypHer, gray) stimulated with ATP 100 µM+Tg 2 µM and positive control (H_2O_2, 200 µM) following pretreatment with DMSO (left, *n* =115) or rapamycin (100 nM, 3 mins) and FK506 wash (5µM, 1 min) (right, *n* =93).

(B) HepG2 cells expressing Hyper and SypHer targeted to the cytosol (HyPer, red SypHer, dark red) were stimulated with ATP 100 µM+Tg 2 µM and positive control (H_2O_2, 20 µM then 200 µM) following pretreatment with DMSO (left, *n* =149) or rapamycin (100 nM, 3 mins) and FK506 wash (5µM, 1 min) (right, *n* =162).

(C) HepG2 cells expressing HyPer and SypHer targeted to the ER lumen (HyPer, green, SypHer, dark green) stimulated with ATP 100 µM+Tg 2 µM and positive control (H_2O_2, 200 µM) following pretreatment with DMSO (left, *n* =77) or rapamycin (100 nM, 3 mins) and FK506 wash (5µM, 1 min) (right, *n* =95).

(D) HepG2 cells expressing HyPer and SypHer targeted to the mitochondrial matrix (HyPer, gray, SypHer, black) stimulated with ATP 100 µM+Tg 2 µM and positive control (H_2O_2, 200 µM) following pretreatment with DMSO (left, *n =70*) or rapamycin (100 nM, 3 mins) and FK506 wash (5µM, 1 min) (right, *n =75*).

Figure S3

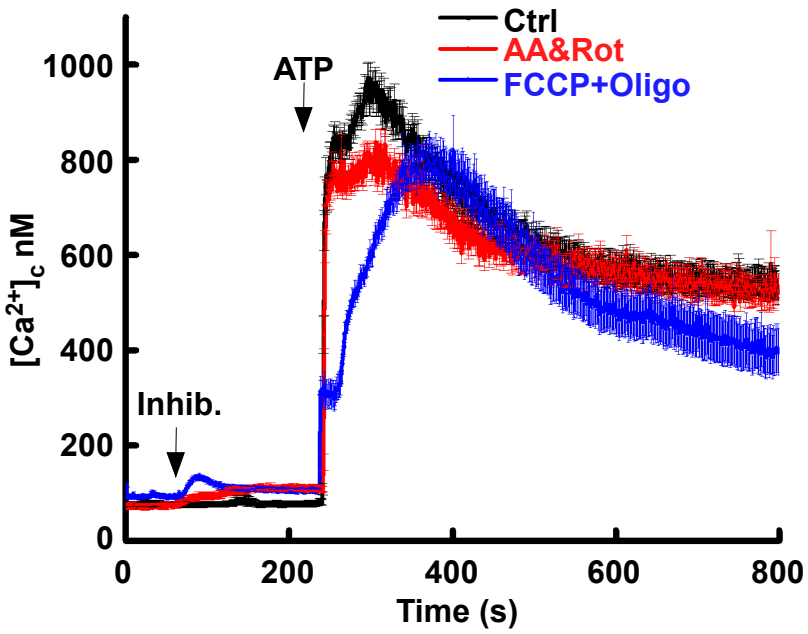

Ctrl
AA&Rot
FCCP+Oligo
ATP
Inhib.
1000
800
600
400
200
0
[Ca2+]c nM
0
200
400
600
800
Time (s)

Fig. S3, related to Figure 3: The agonist induced cytoplasmic [Ca^{2+}] signal is maintained when mitochondrial respiratory chain activity is disabled. HepG2 cells preincubated with inhibitors (Inhib.) of complex III and I (red; antimycin A, 5 µM & rotenone, 5 µM *n* =204) or protonophore and ATP synthase inhibitor (blue; FCCP, 5 µM & oligomycin, 10 µM *n* =211) compared with control (black; *n* =229).

Figure S4

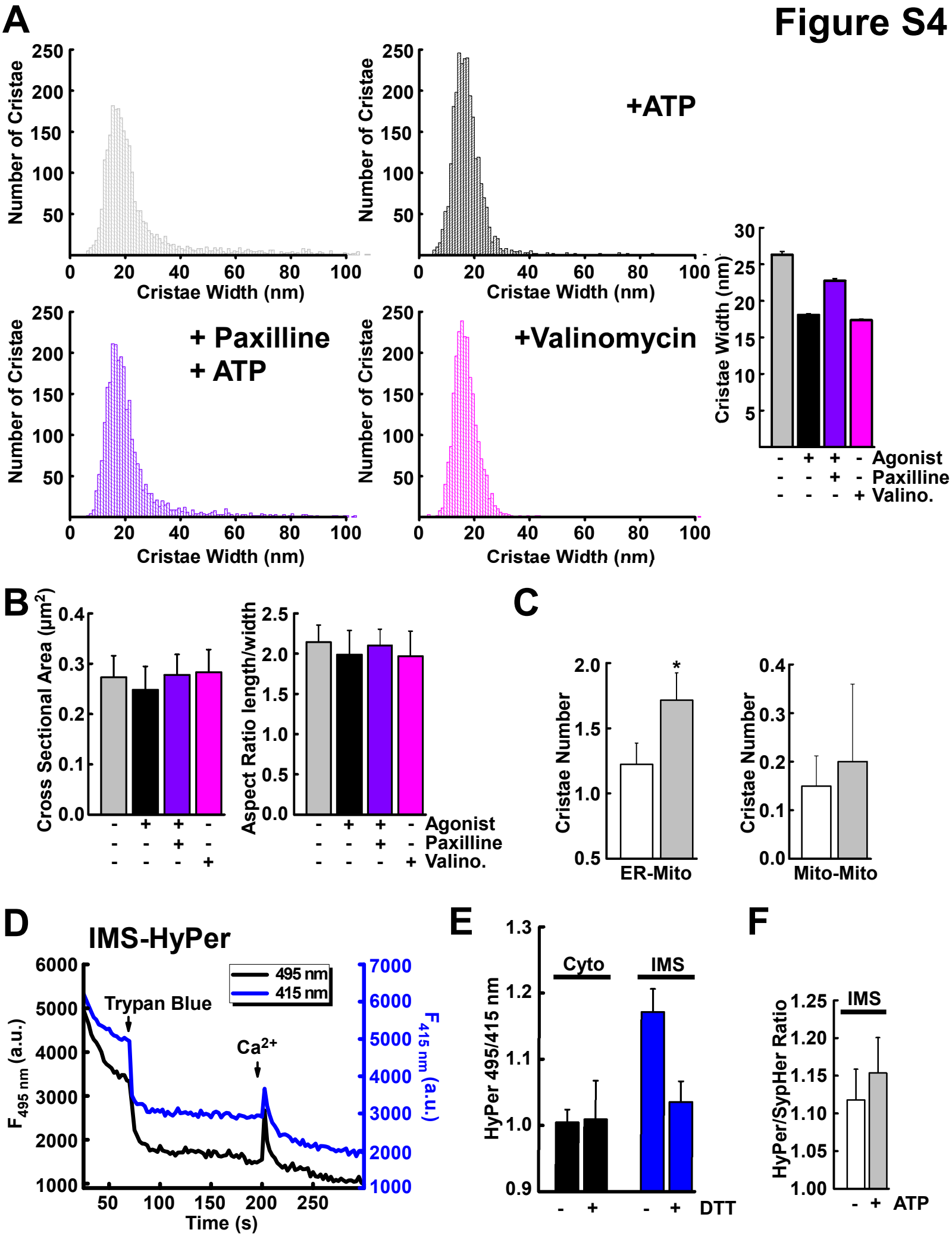

A
Number of Cristae
Cristae Width (nm)
+ Paxilline
+ ATP
+ATP
+Valinomycin
Cristae Width (nm)
Agonist
Paxilline
Valino.
B
Cross Sectional Area (μm²)
Aspect Ratio length/width
C
Cristae Number
ER-Mito
Mito-Mito
D
IMS-HyPer
495 nm
415 nm
Trypan Blue
Ca2+
F495 nm (a.u.)
F415 nm (a.u.)
Time (s)
E
Cyto
IMS
HyPer 495/415 nm
DTT
F
IMS
HyPer/SypHer Ratio
ATP

Fig. S4 related to Figure 4: Mitochondrial cristae have oxidized content and change volume in response to matrix Ca^{2+} uptake

(A) Histograms of mitochondrial cristae width derived from transmission electron microscopy images of HepG2 cells under control conditions (gray, *n* =43 cells), agonist stimulated (black, *n* =45), agonist stimulated following pre-incubation with mitoBK_{Ca} inhibitor (paxilline; 25µM; purple, *n* =33) or incubated with a K^+ ionophore (valinomycin 0.5 nM; pink, *n* =37) alone. Right, combined and normalized (Mean ±SEM) cristae width for all conditions.

(B) Mitochondrial cross sectional area (μm^2, left) and aspect ratio (length/width, right) measured in HepG2 cells in solvent (gray, *n* =381), agonist-treated (black, *n* =454), paxilline pretreatment+agonist (purple, *n* = *402*) and valinomycin pretreatment+agonist (*n* = 411) treatment conditions.

(C) Quantification of cristae junctions within ER-mitochondrial (ER-Mito; left) or mitochondrial-mitochondrial (Mito-Mito; right) interaction zones. Predicted (white; total cristae junctions/fraction of mitochondrial perimeter interacting (< 100 nm) with ER) vs. observed (gray) cristae junction within 100nM of ER (left) or mito (right) surface *n* =43 cells. * $p<0.01$.

(D) Sample traces of trypan blue (10mg/ml) induced quench of the IMS-targeted HyPer (Cox8a-HyPer) in permeabilized HepG2 cells at both excitation wavelengths (black: 495 nm, blue: 415 nm, *n* =15). Trypan blue does not permeate the IMM therefore quenching indicates that the probe is located outside the matrix. Addition of Ca^{2+} indicates increased access of trypan to the probe (Giacomello et al., 2010), which might result from changes to IMS/cristae morphology.

(E) Mean (±SEM) 495/415 nm ratios of HyPer targeted to the cytosol (black, *n* =41) or the IMS (blue, *n* =25) before and after treatment with DTT.

(F) Hyper/SypHer ratios of IMS targeted probes before and after agonist (ATP; 100 µM) treatment, (Mean±SEM, *n* =22).

Materials and Methods:

Chemicals

Standard chemicals were purchased from Fisher Scientific or Sigma-Aldrich. Thapsigargin, Ebselen and Paxilline were purchased from Enzo Life Sciences (Plymouth Meeting, PA). Fluorescent Ca^{2+}-indicator dyes, and Pluronic F-127 were from Teflabs (Austin, TX).

DNA Constructs

For targeting the FKBP12 protein to the cytoplasmic surface of the OMM, the N-terminal sequence of the mAKAP1 (34–63) was used, and it was fused to the N terminus of the human FKBP12 protein through a linker (DPTRSANSGAGAGAGAILSR). The fusion proteins were tagged with mRFP, HyPer (Belousov et al., 2006), SypHer (Poburko et al., 2011) or Killer Red (Bulina et al., 2006) in the pEGFP-N1 plasmid backbone. The constructs used for targeting the mRFP-tagged FRB protein to the cytoplasmic surface of the ER were described elsewhere (Varnai and Balla, 2007) and were adapted by replacing mRFP with HyPer, SypHer or Killer Red (Bulina et al., 2006). HyPer and SypHer derivatives were targeted to the cytosol, ER lumen, and mitochondrial matrix as described (Enyedi et al., 2010), Killer Red-3NLS was constructed by substituting Killer Red for HyPer-3NLS used in the same study. The constitutive OMM-PM linker was derived from the OMM-ER linker described previously (Csordas et al., 2006) where the ER-target sequence was replaced with CAAX motif. RCaMP, a red Ca^{2+} indicator was targeted to the mitochondrial matrix as described (Akerboom et al., 2013). IMS targeted HyPer and SypHer were created by replacing GFP to form Complex IV subunit 8a-HyPer/SypHer (Cox8a-HyPer, Cox8a-SypHer, derived from "CIV-G", gift, Karin Busch, Münster).

Cell Culture

HepG2 cells obtained from ATCC were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin in humidified air (CO_2 5%) at 37°C. For imaging experiments cells were plated onto poly-D lysine coated glass coverslips at a density of 30-35,000 cells/cm^2 and cultured for 24-72 hrs. Genetically encoded probes were transiently transfected with X-tremeGENE 9 (Roche) according to the manufacturer's instructions.

Statistics

Experiments were carried out with ≥3 different cell preparations, and with each preparation the measurements were done at least in triplicates. Data are presented as means ±SEM. Significance of differences from the relevant controls was calculated by Student's t test or ANOVA where appropriate using Origin 8.5 software (OriginLab, Northampton, MA.).

Supplementary References:

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Bulina, M.E., Chudakov, D.M., Britanova, O.V., Yanushevich, Y.G., Staroverov, D.B., Chepurnykh, T.V., Merzlyak, E.M., Shkrob, M.A., Lukyanov, S., and Lukyanov, K.A. (2006). A genetically encoded photosensitizer. Nature biotechnology *24*, 95-99.

Csordas, G., Renken, C., Varnai, P., Walter, L., Weaver, D., Buttle, K.F., Balla, T., Mannella, C.A., and Hajnoczky, G. (2006). Structural and functional features and significance of the physical linkage between ER and mitochondria. The Journal of cell biology *174*, 915-921.

Enyedi, B., Varnai, P., and Geiszt, M. (2010). Redox state of the endoplasmic reticulum is controlled by Ero1L-alpha and intraluminal calcium. Antioxidants & redox signaling *13*, 721-729.

Giacomello, M., Drago, I., Bortolozzi, M., Scorzeto, M., Gianelle, A., Pizzo, P., and Pozzan, T. (2010). Ca2+ hot spots on the mitochondrial surface are generated by Ca2+ mobilization from stores, but not by activation of store-operated Ca2+ channels. Molecular cell *38*, 280-290.

Poburko, D., Santo-Domingo, J., and Demaurex, N. (2011). Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. The Journal of biological chemistry *286*, 11672-11684.

Varnai, P., and Balla, T. (2007). Visualization and manipulation of phosphoinositide dynamics in live cells using engineered protein domains. Pflugers Archiv : European journal of physiology *455*, 69-82.