Figure S1

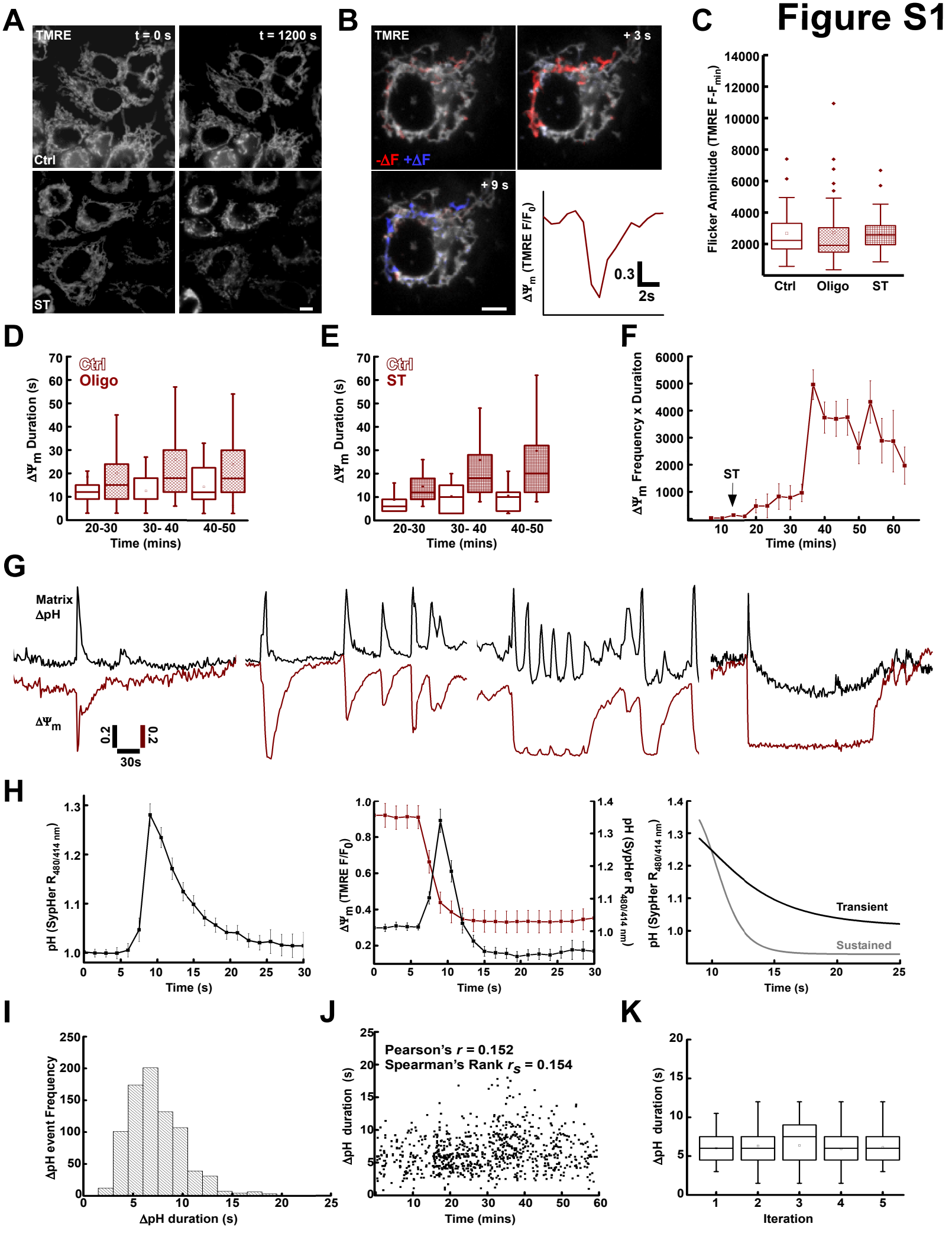
A
TMRE
t = 0 s
t = 1200 s
Ctrl
ST
B
TMRE
+ 3 s
-ΔF +ΔF
+ 9 s
ΔΨm (TMRE F/F0)
0.3
2s
C
Flicker Amplitude (TMRE F-Fmin)
Ctrl
Oligo
ST
D
Ctrl
Oligo
ΔΨm Duration (s)
20-30
30- 40
40-50
Time (mins)
E
Ctrl
ST
ΔΨm Duration (s)
20-30
30- 40
40-50
Time (mins)
F
ΔΨm Frequency x Duraiton
ST
Time (mins)
G
Matrix
ΔpH
ΔΨm
0.2
0.2
30s
H
pH (SypHer R480/414 nm)
Time (s)
ΔΨm (TMRE F/F0)
pH (SypHer R480/414 nm)
Time (s)
pH (SypHer R480/414 nm)
Transient
Sustained
Time (s)
I
ΔpH event Frequency
ΔpH duration (s)
J
Pearson's r = 0.152
Spearman's Rank rS = 0.154
ΔpH duration (s)
Time (mins)
K
ΔpH duration (s)
Iteration

Fig. S1 Related to Fig. 1: Spontaneous and stimulated permeability transition pore events underpin the intra and extra mitochondrial features of mitochondrial flickers A, Live-cell imaging of control (Ctrl, upper) and Staurosporine-treated (ST, lower) HepG2 cells labeled with TMRE. Mean time course of TMRE calculated for all the recordings is plotted in Fig 1F. **B,** Difference image analysis of individual mitochondrial flicker in ST-stimulated HepG2 cells. Loss (Red) and recovery (Blue) of TMRE fluorescence used to monitor $\Delta\psi_m$ over time. Flicker kinetic (lower right) derived from region of interest (ROI) restricted to active mitochondria. **C,** Box plot of mitochondrial flicker amplitude (Pre-flicker F_{TMRE}-F_{min}) in control or ST-challenged cells (Ctrl, open. ST, Red hatching). **D,** Box plot of mitochondrial flicker duration in control cells (Ctrl, open) or oligomycin-treated cells (Oligo, 2.5µg/ml. Red hatching). **E,** Box plot of mitochondrial flicker duration (time of 1/2 max depolarization) in control or ST-challenged cells (Ctrl, open. ST, Red hatching) **F,** Frequency (mHz/cell) x Duration (time ½ max fluorescence) plot of individual mitochondrial flickers following ST addition (arrow). Mean ± SEM. **G,** Examples of matrix pH (SypHer, Black) fluctuations during single, repeat, burst and sustained mitochondrial flickers (TMRE, dark red). **H,** Average trace of matrix pH within individual mitochondria during transient (left) and sustained (center) flickers (Mean ± SEM, events synchronized to ½ max depolarization). Recovery kinetics of pH synchronized to peak were used to create best fit curves for transient (right, black exponential) and sustained (right, grey logistic) mitochondrial flickers. **I,** Histogram plot of matrix pH response duration (full width at half-maximum, FWHM). **J,** XY scatter plot of pHlash duration (at half-maximum) vs. Time. Pearson's and Spearman's rank correlations computed, (r = correlation coefficient). **K,** Box plot of pH increase duration vs iteration (number of pH events in a single mitochondrion within 600 s). Scale bars 5µm.

Figure S2

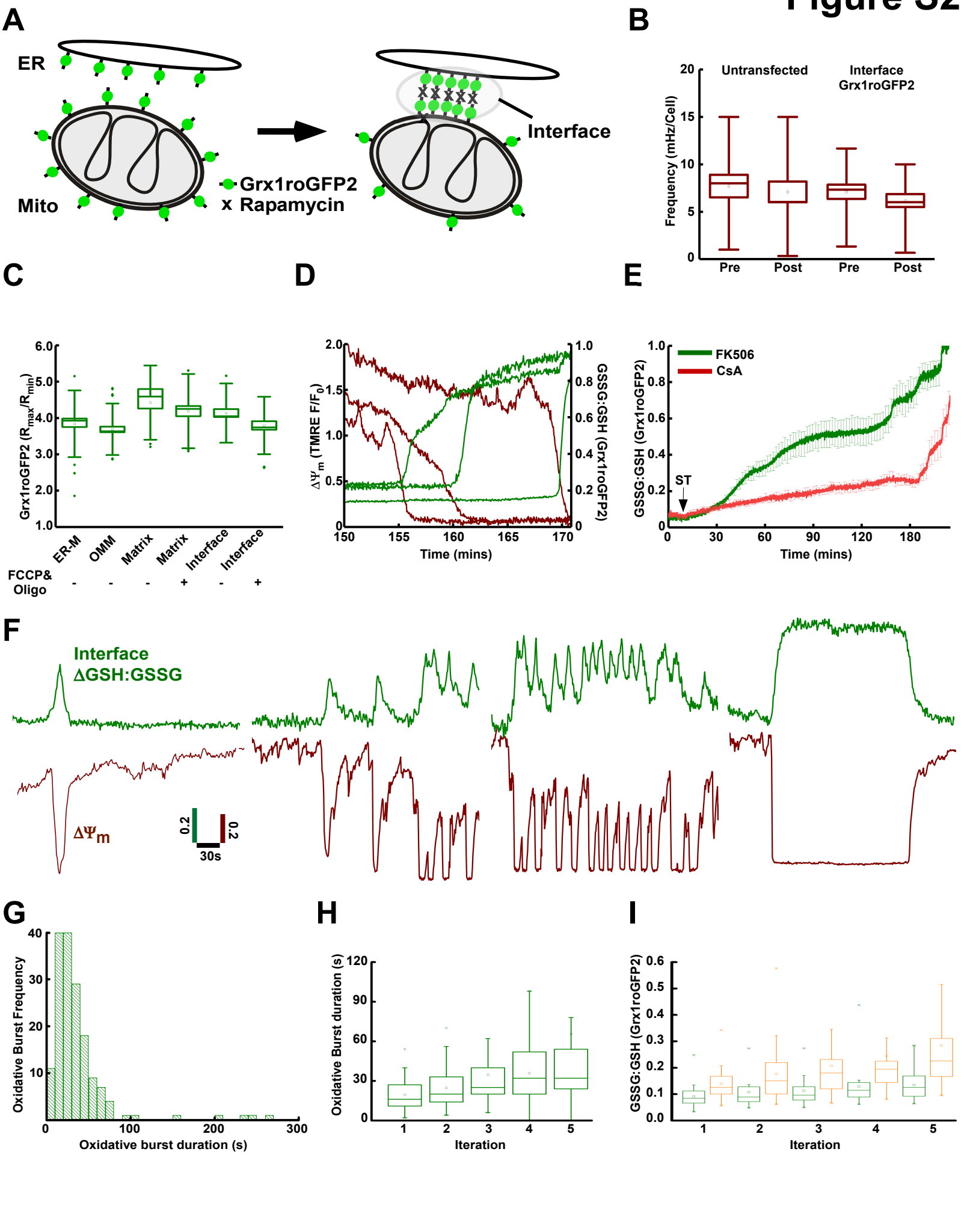
A
ER
Mito
Grx1roGFP2
x Rapamycin
Interface
B
Untransfected
Interface
Grx1roGFP2
Frequency (mHz/Cell)
Pre
Post
Pre
Post
C
Grx1roGFP2 (Rmax/Rmin)
ER-M
OMM
Matrix
Matrix
Interface
Interface
FCCP&
Oligo
D
ΔΨm (TMRE F/F0)
GSSG:GSH (Grx1roGFP2)
Time (mins)
E
FK506
CsA
ST
GSSG:GSH (Grx1roGFP2)
Time (mins)
F
Interface
ΔGSH:GSSG
ΔΨm
0.2
0.2
30s
G
Oxidative Burst Frequency
Oxidative burst duration (s)
H
Oxidative Burst duration (s)
Iteration
I
GSSG:GSH (Grx1roGFP2)
Iteration

Fig. S2 Related to Fig. 2: Mitochondrial flickers generate dynamic redox signals at the ER-mitochondrial interface. A, Schematic representation of the mechanism used to target Grx1roGFP2 (green) to the ER-mitochondrial interface. We coupled the OMM and ER targeting sequences with the two components of the FKBP-FRB heterodimerization system and Grx1roGFP2 to each. Addition of rapamycin causes heterodimerization between adjacent FKBP and FRB domains to rapidly connect the ER-and OMM targeted anchors. Induction of the bridge formation is initially confined to the areas where the ER and OMM were naturally close. Thus, the probe-containing fusion proteins are concentrated at the ER-mitochondrial contacts via heterodimerization induced by a pulse of rapamycin (black). **B,** Box plot of flicker frequency in control (Untransfected) conditions or in cells expressing ER-M Grx1roGFP2 and OMM Grx1roGFP2 (Interface Grx1roGFP). Both Untranfected and Interface Grx1roGFP2 were subject to rapamycin pulse/FK506 protocol. **C,** Box plot of the dynamic range (Ratio maximum; oxidized with 200µM H_2O_2/Ratio minimum; fully reduced with 5mM DTT) of Grx1roGFP2 when targeted to subcellular compartments. Cytosolic face of ER: ER-M. Cytosolic face of the outer mitochondrial membrane (OMM), mitochondrial matrix (Matrix) and ER-M & OMM pairs targeted to the interface with rapamycin pulse/FK506 protocol. Both matrix and interface Grx1roGFP2 were measured with mitochondria polarized of depolarized with FCCP & Oligo (FCCP; 10µM & Oligomycin; 2.5µg/ml). **D,** Example traces of whole-cell oxidation of Interface Grx1roGFP2 following global depolarization of the mitochondrial network in 3 cells. **E, C,** plots of redox poise (GSSG:GSH) measured with Interface-targeted Grx1roGFP1 during the application of ST, following pre incubation with cyclosporine A (CsA; 5µM (Red)) or FK506 (FK506; 5µM (Green)) Plots are Mean ± SEM. **F,** Examples of interface oxidative burst (Grx1roGFP2, green) activity during single, repeat, burst and sustained mitochondrial flickers stimulated by ST, respectively (TMRE, dark red). **G,** Histogram plot of interface oxidative burst duration (full width at half-maximum, FWHM). **H,** Box plots of individual interface GSSG:GSH peak durations vs iteration (number of GSSG:GSH peak events within 600 s) Relationship between iteration and oxidative burst duration significant (Chi Square 4-degrees of freedom, P = < 0.001). **I,** Box plots of interface GSSG:GSH (Grx1roGFP2, R-R_{min}/R_{max}) at baseline (Grx1roGFP2, green) and peak (orange). Relationship between iteration vs. baseline, iteration vs. peak and iteration vs. ΔGSSG:GSH (iteration peak-iteration baseline) all significant. (Chi square, 4-degrees of freedom, P = < 0.001).

Figure S3

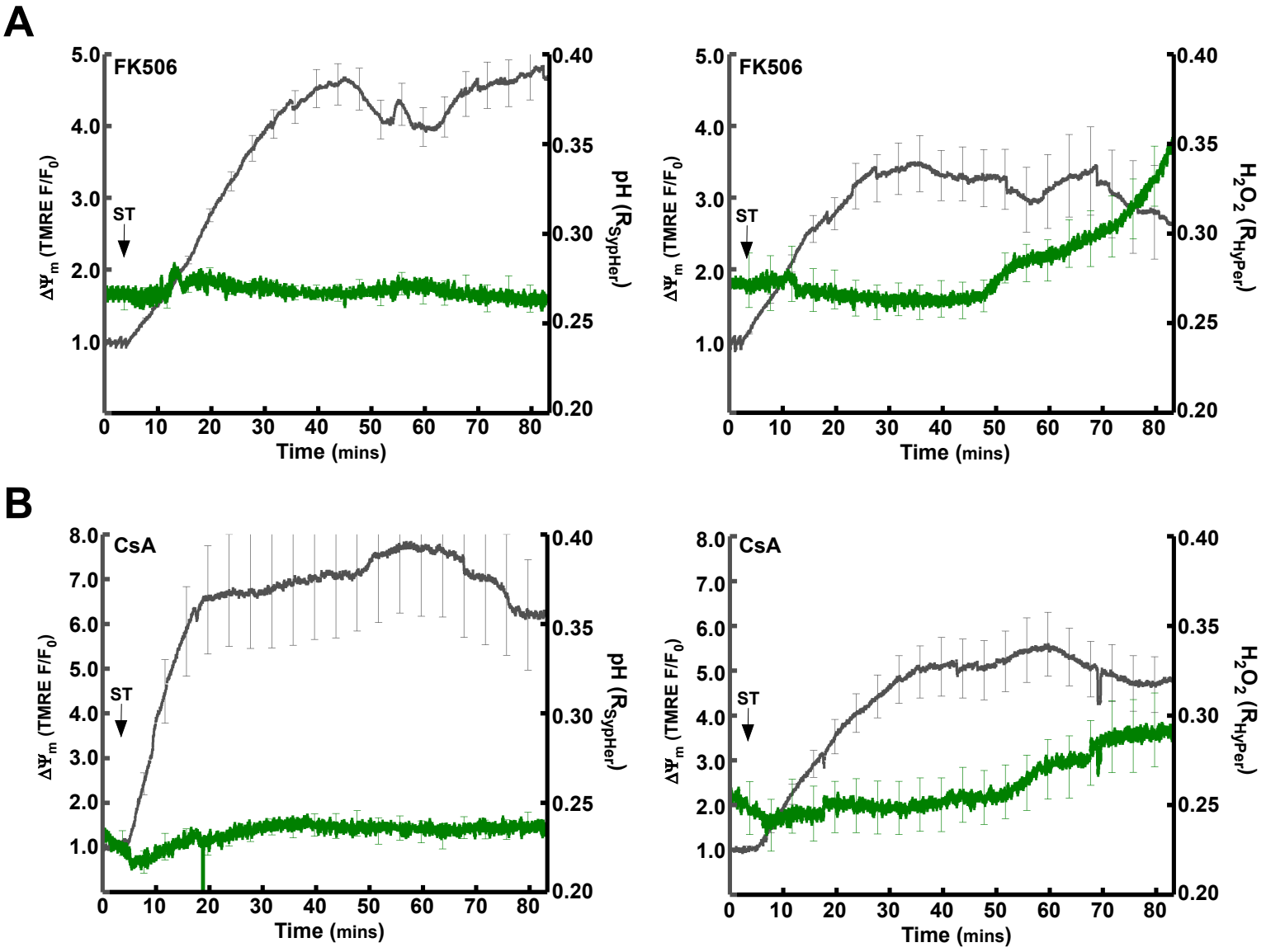
A
FK506
ST
ΔΨm (TMRE F/F0)
pH (RSypHer)
H2O2 (RHyPer)
Time (mins)
B
CsA
ST
ΔΨm (TMRE F/F0)
pH (RSypHer)
H2O2 (RHyPer)
Time (mins)

Fig S3 Related to Fig. 3 Mitochondrial flickers promote local Ca^{2+} signals via oxidation of the IP_3R. A, Normalized recordings of $\Delta\psi_m$ (TMRE; Black) and IP_3R pH (Left, SypHer-IP_3R1) and H_2O_2 (Right, HyPer-IP_3R1) levels during induction of flickers with ST following pre-incubation with 5µM FK506. **B,** Normalized recordings of $\Delta\psi_m$ (TMRE; Black) and IP_3R pH (Left, SypHer-IP_3R1) and H_2O_2 (Right, HyPer-IP_3R1) levels during induction of flickers with ST following pre-incubation with 5µM cyclosporine A (CsA; 5µM).

Figure S4

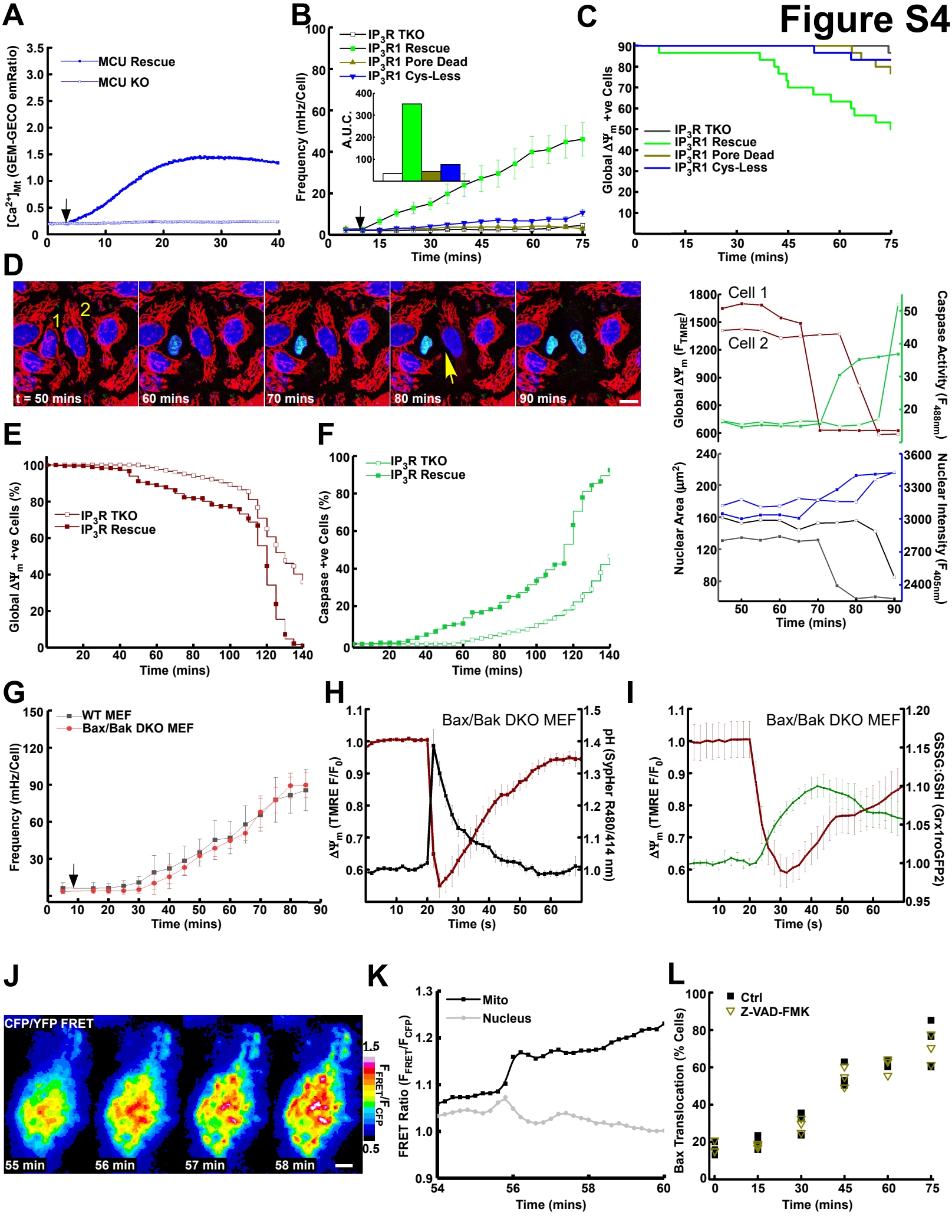
A
MCU Rescue
MCU KO
[Ca2+]Mt (GEM-GECO emRatio)
B
IP3R TKO
IP3R1 Rescue
IP3R1 Pore Dead
IP3R1 Cys-Less
A.U.C.
Frequency (mHz/Cell)
Time (mins)
C
Global ΔΨm +ve Cells
IP3R TKO
IP3R1 Rescue
IP3R1 Pore Dead
IP3R1 Cys-Less
Time (mins)
D
1
2
t = 50 mins
60 mins
70 mins
80 mins
90 mins
Cell 1
Cell 2
Global ΔΨm (FTMRE)
Caspase Activity (F488nm)
Nuclear Area (μm2)
Nuclear Intensity (F405nm)
Time (mins)
E
IP3R TKO
IP3R Rescue
Global ΔΨm +ve Cells (%)
Time (mins)
F
IP3R TKO
IP3R Rescue
Caspase +ve Cells (%)
Time (mins)
G
WT MEF
Bax/Bak DKO MEF
Frequency (mHz/Cell)
Time (mins)
H
Bax/Bak DKO MEF
ΔΨm (TMRE F/F0)
pH (SypHer R480/414 nm)
Time (s)
I
Bax/Bak DKO MEF
ΔΨm (TMRE F/F0)
GSSG:GSH (Grx1roGFP2)
Time (s)
J
CFP/YFP FRET
FFRET/FCFP
55 min
56 min
57 min
58 min
K
Mito
Nucleus
FRET Ratio (FFRET/FCFP)
Time (mins)
L
Ctrl
Z-VAD-FMK
Bax Translocation (% Cells)
Time (mins)

Fig. S4 Related to Fig. 4: Mitochondrial flicker activity interacts with ER-mitochondrial Ca^{2+} transport. A, Representative traces showing changes in $[Ca^{2+}]_{Mt}$ assessed with expression of the emission ratiometric Ca^{2+} sensor GEM-GECO targeted to the mitochondrial matrix ($[Ca^{2+}]_{Mt}$: GEM-GECO Emission Ratio) in MCU KO (empty) and MCU Rescue (filled) cells. **B,** Flicker frequency **(**Mean ±SEM) in HEK293T cells deficient in all 3 isoforms of the IP_3 Receptor (IP_3R-TKO; Black) Vs. Cells rescued with IP_3R1 variants, WT IP_3R1-mCherry (Green; IP_3R1-Rescue), IP_3R1 mutated to prevent Ca^{2+}-flux (IP3R1-Pore Dead; Green), or IP_3R1 deficient in cytosolic cysteine residues (IP_3R1-CysLess; Blue). **C,** Kaplan-Meier plots of cell survival indicated by permanent global depolarization of mitochondrial ψ_m in IP_3R-TKO (Black), IP_3R1-Rescue (Green), IP_3R1-Pore Dead (Gold) and IP_3R-Cys-Less (Blue). D, Image sequence of apoptotic sequence in HepG2 cells treated with Staurosporine. $\Delta\psi_m$ stained with TMRE; Red. Nucleus, Hoechst 33342; Blue and caspase 3/7 activation, Cell Event; Green. Cells labelled, #1 & 2 show sequential depolarization of $\Delta\psi_m$, (Red, traces: upper, Red) condensation and brightening of the nucleus, (Blue, traces: lower Black/Blue) and activation of executioner caspases 3/7 (Green, traces: upper Green). Global wave of $\Delta\psi_m$ depolarization illustrated in image 4 with arrow indicating direction of wave. **E,** Kaplan-Meier plots of cell survival indicated by permanent global depolarization of mitochondrial ψ_m in IP_3R-TKO (Open), IP_3R1-Rescue (Closed). **F,** Plots of cell population in (E) assayed for executioner caspase activation (Caspase 3/7, Cell Event +ve nuclei). G, Flicker frequency in wild-type (WT-MEF; Black) and Bax/Bak double KO (Bax/Bak DKO MEF; Red) mouse embryonic fibroblasts during stimulation with Sstaurosporine (ST; 2µM). **H,** Average traces of transient mitochondrial flickers (Mean ±SEM, dark red) and matrix pH rises (Mean ±SEM, black) in mouse embryonic fibroblasts with double genetic targeting of bax & bak (Bax/Bak DKO MEF). **I,** Average traces of transient mitochondrial flickers (Mean ±SEM, dark red) and matrix redox bursts (Mean ±SEM, green) in Bax/Bak DKO MEF cells. Recordings synchronized to ½ max $\Delta\psi_m$ depolarization. **J,** FRET images (CFP-YFP) of ST-treated HepG2 cells expressing both CFP-Bax & YFP-Bax during terminal $\Delta\psi_m$ depolarization. **K,** FRET traces of two regions of interest, nucleus (Nucleus, light grey) and mitochondrial region (Mito, black) of the cell depicted in D. **L,** Plot of Bax oligomerization/translocation assay of ST treated HepG2 cells in the presence (empty yellow triangles) and absence (filled black squares) of a pan-caspase inhibitor (Z-VAD-FMK). Data derived from three independent experiments plotted for each time point. Scale bars 5µm.