Cell Reports, Volume *23*

Supplemental Information

MIRO-1 Determines Mitochondrial Shape Transition upon GPCR Activation and Ca^{2+} Stress

Neeharika Nemani, Edmund Carvalho, Dhanendra Tomar, Zhiwei Dong, Andrea Ketschek, Sarah L. Breves, Fabián Jaña, Alison M. Worth, Julie Heffler, Palaniappan Palaniappan, Aparna Tripathi, Ramasamy Subbiah, Massimo F. Riitano, Ajay Seelam, Thomas Manfred, Kie Itoh, Shuxia Meng, Hiromi Sesaki, William J. Craigen, Sudarsan Rajan, Santhanam Shanmughapriya, Jeffrey Caplan, Benjamin L. Prosser, Donald L. Gill, Peter B. Stathopulos, Gianluca Gallo, David C. Chan, Prashant Mishra, and Muniswamy Madesh

SUPPLEMENTAL INFORMATION

The supplemental data includes 7 supplemental figures with legends, 5 video legends, supplemental extended experimental procedures, and supplemental references.

Figure S1

A
MEFs
Cox-8-mRFP
DHR123
MERGE
Before stimulation
After stimulation
Ionomycin
20 µm
B
MEFs
Iono
C
HeLa
D
MEFs
Cox-8-mRFP
DHR123
MERGE
Before stimulation
After stimulation
FCCP
E
MEFs
F
HeLa
% Mitochondrial length > 5µm
Time (s)
G
Before Iono
After Iono
+ BAPTA-AM
H
Ionomycin
WT + Ionomycin
WT + BAPTA-AM + Iono
Fluo 4 (f.a.u.)
I
% Mitochondria length (> 5 µm)
J
+ EGTA
K
WT + Ionomycin
WT + EGTA + Iono
L
M
Neg shRNA
MCU #861
cccp
10 µM Ca2+
N
Before Histamine
After Histamine
WT
MCU KD
O
WT + Histamine
MCU KD + Histamine
WT + Ionomycin
MCU KD + Ionomycin
P
Histamine
Ca2+
WT
MCU KD
Q
+ Histamine
+ Thrombin
+ Ionomycin
R
Peak
100 s

Figure S1, Related to Figure 1. Ca^{2+}-Induced MiST is Upstream of $\Delta\Psi_m$.

(A) Representative confocal images of MEFs transfected with Cox8a-mRFP, and loaded with $\Delta\Psi_m$ indicator DHR123 for 30 min. After a baseline recording of 60s, MEFs were challenged with ionomycin (2.5 μM) and images were collected every 3s for a total of 700s.

(**B** and **C**) Mean traces of mitochondrial morphology > 5 μm (red) and $\Delta\Psi_m$ (black) changes from MEFs and HeLa cells treated with ionomycin. Mean ± SEM; n=4.

(**D**)Representative confocal images of MEFs transfected with Cox8a-mRFP, and loaded with $\Delta\Psi_m$ indicator DHR123 for 30 min. After a baseline recording of 60s, MEFs were challenged with FCCP (5 μM) and images were collected every 3s for a total of 400s.

(**E** and **F**) Mean traces of mitochondrial morphology > 5 μm (red) and $\Delta\Psi_m$ (black) changes from MEFs and HeLa cells treated with FCCP. Mean ± SEM; n=4.

(G and J) Representative confocal images of HeLa cells transiently transfected with cox8a-mRFP, treated with (G) BAPTA-AM (25 μM) or (J) EGTA (0.5 mM) for 30 minutes and challenged with ionomycin to observe mitochondrial shape changes. n=11.

(H and K) Mean traces of Fluo-4 ($[Ca^{2+}]_c$) fluorescence measured in (G) BAPTA-AM or (J) EGTA treated HeLa cells transfected with cox8a-mRFP. Data represent Mean ± SEM; n=11.

(I and L) Mean traces of change in mitochondrial length (> 5 μm) over time following ionomycin challenge in (G) BAPTA-AM or (J) EGTA pretreated HeLa cells. Mean ± SEM; n=3.

(**M**) Representative traces of $[Ca^{2+}]_{out}$ clearance in HeLa Neg shRNA (blue) and MCU KD (red) cells. n=3.

(**N**) Representative live confocal images of HeLa Neg shRNA (top) and MCU KD (bottom) cells transfected with cox8a-mRFP and challenged with histamine (100 μM). n=10.

(O) Mean traces of change in mitochondrial length over time from (N) WT + Histamine, WT + Ionomycin, MCU KD + Histamine, and MCU KD + Ionomycin. Mean ± SEM; n=3.

(P) Mean traces of $[Ca^{2+}]_c$ (Fluo-4) fluorescence measured in WT + Histamine and MCU KD + Histamine treated HeLa cells transfected with cox8a-mRFP. n=6.

(Q) Representative traces of $[Ca^{2+}]_i$ rise following histamine (100 μM) (blue), thrombin (5 mU/ml) (red) or ionomycin (2.5 μM) (black) stimulation. n=6.

(R) Bar graph represents $[Ca^{2+}]_i$ rise following histamine (blue), thrombin (red) or ionomycin (black) at peak (left) and at 100s (right) challenge. Mean ± SEM; n=6.

Figure S2

A
WT
Bax/Bak DKO
SPG7 KO
VDAC1/3 DKO
(Before)
(After)
20 µm
B
0.2 - 2 µm
2 - 5 µm
>5 µm
Mitochondria length (%)
67.2±0.537
86.667±3.33
35.812±1.33
53.004±1.28
79.545±3.78
51.23±10.05
44.213±8.73
59.82+-13.15
40.18±13.15
44.76±1.09
45.0±5.00
80.27±3.6
Iono
WT
BB DKO
SPG7 KO
VDAC DKO
C
Iono
GCaMP6-mt (f.a.u.)
Time (s)
WT
BB DKO
SPG7 KO
VDAC DKO

Figure S2, Related to Figure 2. Genetic Deletion of SPG7 andVDAC1/3 Did Not Limit $[Ca^{2+}]_c$-Induced MiST.

(A) Representative confocal images of WT MEF, Bax Bak DKO, SPG7 KO and $VDAC^{1/3}$ DKO MEFs transfected with a $[Ca^{2+}]_m$ marker, GCaMP6-mt. After baseline recording of 60s, cells were challenged with 2.5 μM ionomycin and MiST was recorded. n=8.

(B) Bar graph represents change in mitochondrial length before and after stimulation with ionomycin in WT, Bax Bak DKO, SPG7 KO and $VDAC^{1/3}$ DKO MEFs. Mean ± SEM; ***$p<0.001$(p value calculated for 0.2-2 μm length mitochondria). n=3.

(C) Mean traces for $[Ca^{2+}]_m$ (GCaMP6-mt) uptake in WT, Bax Bak DKO, SPG7 KO, and $VDAC^{1/3}$ DKO MEFs. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM) and changes in $[Ca^{2+}]_m$ fluorescence were measured. Mean ± SEM; n=6.

Figure S3

A
Ionomycin
Scr siRNA
Kif5b siRNA
Fluo-4; [ca2+]c (f.a.u)
Time (s)
ns
Scr siRNA
Kif5b siRNA
B
Ionomycin
Scr siRNA
Kif5b siRNA
Rhod-2; [ca2+]m (f.a.u)
Time (s)
ns
Scr siRNA
Kif5b siRNA
C
DMSO
Actin filaments
Microtubules
Colchicine
50 µm
D
Phase contrast
DMSO
Latrunculin-A
Actin filaments
25 µm
E
Before blebbstatin
After blebbstatin
20 µm
Before Lat A
After Lat A
F
+Blebbistatin
Before ionomycin
After ionomycin
G
0.2 - 2 µm
2 - 5 µm
> 5 µm
Mitochondrial length (%)
84.05 ± 2.3
92.33 ± 1.1
84.12 ± 2.04
34.09 ± 6.8
45.4 ± 4.4
88.28 ± 0.285
***
Blebbistatin
Lat A
Ionomycin

Figure S3, Related to Figure 3. KIF5B-Deficient Cells Undergo Ca^{2+}-Induced MiST.

(A) Mean traces of $[Ca^{2+}]_c$ as measured by Fluo-4 fluorescence in Scr siRNA (black) and KIF5B siRNA (red) MEFs. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). The bar graph (right panel) depicts the amplitude of Fluo-4 fluorescence after stimulation. Mean ± SEM; n=4.

(B) Mean traces of $[Ca^{2+}]_m$ as measured by rhod-2 fluorescence in Scr siRNA (black) and KIF5B siRNA (red) MEFs. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). The bar graph (right panel) depicts the amplitude of rhod-2 fluorescence after stimulation. Mean ± SEM; n=4.

(C) Representative images of fixed MEFs stained with actin filament and microtubule markers rhodamine phalloidin and anti-β-tubulin-FITC, respectively before and after colchicine treatment. n=3.

(D) Representative images of fixed HeLa cells stained with actin marker Alexa488-phalloidin, before and after Latranculin A (1 μM) treatment. n=3

(E) Real time visualization of mitochondrial shape changes in HeLa transfected with cox8a-mRFP and treated with blebbistatin (100 μM) (top panels) and LatrunculinA (1 μM) (bottom panels). n=3-6.

(F) Real time visualization of mitochondrial shape changes in HeLa transfected with cox8a-mRFP and treated with blebbistatin (100 μM) for 10 min before and after stimulation with ionomycin (2.5 μM). n=3-6.

(G) Bar graph representing quantification of mitochondrial length from control, blebbistatin, and LatA treated MEFs before and after stimulation with ionomycin. Mean ± SEM; ***$p<0.001$(p value calculated for 0.2-2 μm length mitochondria). n = 3-6.

Figure S4

A
+ FK506
Before Iono
After Iono
20 µm
B
% Mitochondrial length (> 5 µm)
Time (s)
WT
+ FK506
C
Iono
[Ca2+]c
Fluo-4 (f.a.u.)
Time (s)
WT
+ FK506
D
Vector
Drp1-S579D
Drp1-S579A
Drp1-S600D
Drp1-S600A
Drp1
βActin
E
Drp1 WT
Drp1 S579A
Drp1 S579D
Drp1 S600A
Drp1 S600D
NH2
GTPase
Middle domain
B domain
GED domain
COOH
S579A
S579D
S600A
S600D
1
349 350
495 523
615 617
706
742
712
F
Ionomycin
Fluo-4; [Ca2+]c (f.a.u)
Time (s)
ns
Control
Drp1 S579A
Drp1 S579D
Drp1 S600A
Drp1 S600D
G
Ionomycin
Rho-2; [Ca2+]m (f.a.u)
Time (s)
ns
Control
Drp1 S579A
Drp1 S579D
Drp1 S600A
Drp1 S600D
H
Before stimulation
After stiumation
Before stimulation
After stimulation
Drp1 S579A
Drp1 S579D
Drp1 S600A
Drp1 S600D
10 µm
I
0.2 - 2 µm
2 - 5 µm
> 5 µm
Control
Drp1S579A
Drp1S579D
Drp1S600A
Drp1S600D
% Mitochondria length
89.05 ± 0.46
99.05 ± 0.76
77.64 ± 3.12
95.15 ± 0.93
83.9 ± 3.56
96.01 ± 2.11
87.74 ± 4.07
89.79 ± 2.04
80.56 ± 1.01
89.05 ± 0.46
Ionomycin
J
Relative mRNA expression
Scr siRNA
Dyn2 siRNA
Mid 51 siRNA
Mid 49 siRNA
***
**
**

Figure S4, Related to Figure 4. Fission Deficient Mitochondria also Elicit $[Ca^{2+}]_c$-Induced MiST.

(A) Representative confocal images of HeLa cells transfected with cox8a-mRFP and pretreated with FK506 (1 μM) for 30 minutes and challenged with ionomycin (2.5 μM).n=5

(B) Mean traces of change in mitochondrial length (> 5 μm) over time in HeLa cells with or without FK506 pretreatment following ionomycin stimulation. Mean ± SEM; n=3.

(C) Mean traces of Fluo-4 ($[Ca^{2+}]_c$) fluorescence in control and FK506 pretreated cells. Mean ± SEM; n=3.

(D) Stable ectopic expression of Drp1 mutants as confirmed by immunoblotting. Lysates were prepared from vector control and Drp1 mutants (S579D, S579A, S600D, S600A) expressing MEFs and probed with Drp1 antibody. β-actin served as loading control.

(E) Schematic representing wild-type Drp1 and Drp1 mutants.

(F) Mean traces of $[Ca^{2+}]_c$ as measured by Fluo-4 fluorescence in control, and Drp1 mutants expressing MEFs. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). The bar graph (right panel) depicts the amplitude of Fluo-4 fluorescence after stimulation. Mean ± SEM; n=3.

(G) Mean traces of $[Ca^{2+}]_m$ as measured by rhod-2 fluorescence in control, and Drp1 mutants expressing MEFs. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). The bar graph (right panel) depicts the amplitude of rhod-2 fluorescence after stimulation. Mean ± SEM; n=3.

(H) Representative confocal images of control and Drp1 mutants expressing MEFs transfected with cox8a-mRFP. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (2.5 μM). n=8

(I) Bar graph represents change in mitochondrial length before and after stimulation with ionomycin in control and Drp1 mutants expressing MEFs. Mean ± SEM; ***$p<0.001$ (p value calculated for 0.2-2 μm length mitochondria). n=6.

(J) mRNA abundance of Dyn2, Mid51, and Mid49 from MEFs by qRT-PCR after siRNA treatment. Mean ± SEM; ***$p<0.001$, **$p<0.01$

Figure S5

B

Protein	EF-hand	Sequence
		X*Y*ZGyhx**z
mMiro-1	EF1	DQDNDGTLNDAE
mMiro-2	EF1	DQDRDHGLSDEE
mMiro-1	EF2	DLDRDCALSPDE
mMiro-2	EF2	DQDHDGVLSPTE
hMiro-1	EF1	DQDNDGTLNDAE
hMiro-2	EF1	DQDLDGALSDEE
hMiro-1	EF2	DLDRDCALSPDE
hMiro-2	EF2	DQDRDEALSPTE
most prevalent:		DKDGDGKISFEE

Figure S5, Related to Figure 5. Miro Canonical EF-Hand Ca^{2+} Binding Loop Structural Analysis; GTPase hands of Miro1 are dispensable for MiST and MiST occurrence in primary neurons and cardiomyocytes.

(A) Representative confocal images of stably expressing M2WT, M2EF1, M2EF2 and M2EF1/2 mutant MEFs transfected with cox8a-mRFP. After a baseline recording of 60s, cells were challenged with ionomycin. n=10

(B) Canonical EF-hand Ca^{2+} binding loop sequence alignment for mouse and human Miro-1 and Miro-2 homologues. Mouse Miro-1 (mMiro-1), mouse Miro-2 (mMiro-2), human Miro-1 (hMiro1) and human Miro-2 (hMiro-2) sequences were from NCBI accession numbers NP_067511.4, NP_666111.1, NP_001028740.1 and NP_620124.1, respectively. The EF1 and EF2 residues not conserved between human Miro-1 and Miro-2 are shaded green. The Ca^{2+} coordinating residues (*X, Y, Z, y, x, z*) are indicated above the alignment. *G* denotes the highly invariant Gly at position 6 and *h* denotes the invariant hydrophobic amino acid position 8. The most prevalent residue type at each of the 12 loop positions are shown below (red text) the alignment (Gifford et al., 2007; Marsden et al., 1990; Zhou et al., 2009).

(C) Structural alignment of Miro-1 and homology modelled Miro-2 EF1 Ca^{2+} binding loops. The Miro-1 and Miro-2 loops are colored brown and pink, respectively, with the oxygen and nitrogen atoms colored red and blue, respectively. The Ca^{2+} atom is represented by a yellow sphere and the H_2O involved in the Ca^{2+} coordination is a red sphere. The H-bond between Asn205 and Glu208 observed in Miro-1, but not in the Miro-2 homology structure is indicated by a dashed red line and ellipse. The Gln202 of Miro-2 which does not adopt the most favorable Ramachandran main chain dihedral angles in contrast to Gly202 of Miro-1 is also indicated by a dashed ellipse.

(D) Structural alignment of the Miro-1 and homology modelled Miro-2 EF2 Ca^{2+} binding loops with the coloring and labeling profile as described for panel (F). The residue colors in (E), (F) and (G) are consistent, and the residue positions with respect to the loop in (F) and (G) are indicated in parentheses beside the residue number. M1, Miro-1; M2, Miro-2. The homology models were generated using Modeler v9.17 (Webb and Sali, 2014) after alignment of the Miro-1 and Miro-2 sequences (NP_001028740.1 and NP_620124.1, respectively) in Clustal Omega (Sievers and Higgins, 2014). The Ca^{2+}-loaded human Miro-1 structure (5KTY.pdb) was used as the template structure in Modeller.

(E) Mean traces for $[Ca^{2+}]_c$ as measured by Fluo-4 in WT (black) and M1EF1 mutant (red) MEFs. After measurement of baseline fluorescence, cells were stimulated with thrombin (5 mU/ml). Mean ± SEM; n=3.

(**F**) Mean traces of $[Ca^{2+}]_m$ as measured by rhod-2 fluorescence WT (black) and M1EF1 mutant (red) MEF cells. After measurement of baseline fluorescence, cells were stimulated with thrombin (5 mU/ml).Mean ± SEM; n=3.

(G) Mean traces of $[Ca^{2+}]_c$ as measured by GCaMP6 in WT (black) and M1EF1 mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with histamine (100 μM). Mean ± SEM; n=3.

(**H**) Mean traces for $[Ca^{2+}]_m$ uptake as measured by mito-R-GECO1 in WT (black) and M1EF1 mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with histamine (100 μM). Mean ± SEM; n=3.

(I) Representative confocal images of Miro1 T18N mutant expressing HeLa cells transfected with cox8a-mRFP. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (2.5 μM). n=4.

(J) Mean traces of $[Ca^{2+}]_c$ as measured by Fluo-4 in WT (black) and M1T18N mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). Mean ± SEM; n=4.

(**K**) Mean traces of change in mitochondrial length over time from WT (black) and M1T18N mutant (red). Mean ± SEM; n=4.

(L) Representative images of Miro1 P13V mutant expressing HeLa cells transfected with cox8a-mRFP. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (2.5 μM). n=5.

Figure S5, Related to Figure 5. Miro Canonical EF-Hand Ca^{2+} Binding Loop Structural Analysis; GTPase hands of Miro1 are dispensable for MiST and MiST occurrence in primary neurons and cardiomyocytes.

(A) Representative confocal images of stably expressing M2WT, M2EF1, M2EF2 and M2EF1/2 mutant MEFs transfected with cox8a-mRFP. After a baseline recording of 60s, cells were challenged with ionomycin. n=10

(B) Canonical EF-hand Ca^{2+} binding loop sequence alignment for mouse and human Miro-1 and Miro-2 homologues. Mouse Miro-1 (mMiro-1), mouse Miro-2 (mMiro-2), human Miro-1 (hMiro1) and human Miro-2 (hMiro-2) sequences were from NCBI accession numbers NP_067511.4, NP_666111.1, NP_001028740.1 and NP_620124.1, respectively. The EF1 and EF2 residues not conserved between human Miro-1 and Miro-2 are shaded green. The Ca^{2+} coordinating residues (*X, Y, Z, y, x, z*) are indicated above the alignment. *G* denotes the highly invariant Gly at position 6 and *h* denotes the invariant hydrophobic amino acid position 8. The most prevalent residue type at each of the 12 loop positions are shown below (red text) the alignment (Gifford et al., 2007; Marsden et al., 1990; Zhou et al., 2009).

(C) Structural alignment of Miro-1 and homology modelled Miro-2 EF1 Ca^{2+} binding loops. The Miro-1 and Miro-2 loops are colored brown and pink, respectively, with the oxygen and nitrogen atoms colored red and blue, respectively. The Ca^{2+} atom is represented by a yellow sphere and the H_2O involved in the Ca^{2+} coordination is a red sphere. The H-bond between Asn205 and Glu208 observed in Miro-1, but not in the Miro-2 homology structure is indicated by a dashed red line and ellipse. The Gln202 of Miro-2 which does not adopt the most favorable Ramachandran main chain dihedral angles in contrast to Gly202 of Miro-1 is also indicated by a dashed ellipse.

(D) Structural alignment of the Miro-1 and homology modelled Miro-2 EF2 Ca^{2+} binding loops with the coloring and labeling profile as described for panel (F). The residue colors in (E), (F) and (G) are consistent, and the residue positions with respect to the loop in (F) and (G) are indicated in parentheses beside the residue number. M1, Miro-1; M2, Miro-2. The homology models were generated using Modeler v9.17 (Webb and Sali, 2014) after alignment of the Miro-1 and Miro-2 sequences (NP_001028740.1 and NP_620124.1, respectively) in Clustal Omega (Sievers and Higgins, 2014). The Ca^{2+}-loaded human Miro-1 structure (5KTY.pdb) was used as the template structure in Modeller.

(E) Mean traces for $[Ca^{2+}]_c$ as measured by Fluo-4 in WT (black) and M1EF1 mutant (red) MEFs. After measurement of baseline fluorescence, cells were stimulated with thrombin (5 mU/ml). Mean ± SEM; n=3.

(**F**) Mean traces of $[Ca^{2+}]_m$ as measured by rhod-2 fluorescence WT (black) and M1EF1 mutant (red) MEF cells. After measurement of baseline fluorescence, cells were stimulated with thrombin (5 mU/ml).Mean ± SEM; n=3.

(G) Mean traces of $[Ca^{2+}]_c$ as measured by GCaMP6 in WT (black) and M1EF1 mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with histamine (100 μM). Mean ± SEM; n=3.

(**H**) Mean traces for $[Ca^{2+}]_m$ uptake as measured by mito-R-GECO1 in WT (black) and M1EF1 mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with histamine (100 μM). Mean ± SEM; n=3.

(I) Representative confocal images of Miro1 T18N mutant expressing HeLa cells transfected with cox8a-mRFP. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (2.5 μM). n=4.

(J) Mean traces of $[Ca^{2+}]_c$ as measured by Fluo-4 in WT (black) and M1T18N mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). Mean ± SEM; n=4.

(**K**) Mean traces of change in mitochondrial length over time from WT (black) and M1T18N mutant (red). Mean ± SEM; n=4.

(L) Representative images of Miro1 P13V mutant expressing HeLa cells transfected with cox8a-mRFP. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (2.5 μM). n=5.

(M) Mean traces of $[Ca^{2+}]_c$ as measured by Fluo-4 in WT (black) and M1P13V mutant (red) HeLa cells. After measurement of baseline fluorescence, cells were stimulated with ionomycin (2.5 μM). Mean ± SEM; n=4.

(**N**) Mean traces of change in mitochondrial length over time from WT (black) and M1P13V mutant (red). Mean ± SEM; n=5.

(O) Miro1EF1 mutant is resistant to higher dose of ionomycin-induced MiST. Representative images of Miro1EF1 mutant expressing MEFs transfected with cox8a-mRFP. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (5 μM).

(P) Mean traces of $[Ca^{2+}]_c$ as measured by Fluo-4 in WT (black) and M1EF1 mutant (red) MEFs. After measurement of baseline fluorescence, cells were stimulated with ionomycin (5 μM). Mean ± SEM; n=4.

(**Q**) Mean traces of change in mitochondrial length over time from WT (black) and M1EF1 mutant (red). Mean ± SEM; n=4.

(R) Miro1 EF1 mutant expressing cells undergo Ca^{2+} independent fragmentation. Representative confocal images of Miro1WT and EF1 mutant MEFs transfected with cox8a-mRFP and treated with oligomycin (100 μM) + antimycin (10 μM) for 4 hours. **Right panel** Bar graph represents change in mitochondrial length before and after treatment with oligomycin + antimycin in Miro1 WT and M1EF1 mutant MEFs. Mean ± SEM; ***$p<0.001$ (p value calculated for 0.2-2 μm length mitochondria). n=3.

(S) Representative images of the axons of primary sensory neurons loaded with Mitotracker Green. After acquisition of baseline pre-treatment images, cells were challenged with ionomycin (5 μM, 10 min post-treatment shown). n = 6.

(T) Representative images of primary sensory neurons transfected with M1WT (left) and M1EF1 mutant (right) tagged with mRFP and loaded with Mitotracker Green. After a baseline recording, cells were challenged with ionomycin (5 μM, 10 min). The blue arrowhead in the M1EF1 panels denotes the rounding of mitochondria in a non-transfected nearby gial Scwhann cell.

(U) Graph showing measurements of mitochondrial length pre and at 10 min post ionomycin treatment. Mean ± SEM, medians (black lines) and the results of Dunn's Multiple Comparisons Test are presented (n=number of mitochondria shown in bars).

(V) Mean traces of $[Ca^{2+}]_i$ as measured by Fluo-4 in embryonic rat hippocampal neurons. After measurement of baseline fluorescence, cells were stimulated with glutamate (200 μM and 1mM). Mean ± SEM; n=4. **Right panel** represents quantification of normalized Fluo-4 fluorescence at peak. Data represents Mean ± SEM; n=4.

(W) Representative images of the mitochondria in the processes of hippocampal rat embryonic neurons transfected with M1WT and M1EF1 mutant tagged with mRFP and loaded with Mitotracker Green. After acquisition of baseline pretreatment images, cells were challenged with glutamate (1 mM) and imaged at 10, 20, 30 and 45 min. **Right panel** represents measurement of mitochondrial length pre and at 20 and 45 min post treatment with glutamate. Within treatment across times comparisons performed using Dunn's Multiple Comparisons Test. Time-matched comparisons between M1WT and M1EF1 mutant performed using the Mann-Whitney test.

(X) Representative images of the mitochondria in adult rat ventricular myocytes infected with Ad-cox8a-mRFP. After 72hrs of infection, blebbistatin (10 μM) pretreated myocytes were stimulated with Ionomycin (10 μM) and imaged. n=6. **Right panel** represents change in mitochondrial length over time in cardiomyocytes. Mean ± SEM; n=6.

(Y) Mean traces of $[Ca^{2+}]_i$ as measured by Fluo-4 in cardiomyocytes. After measurement of baseline fluorescence, myocytes were stimulated with ionomycin (10 μM). Mean ± SEM; n=6.

Figure S6

A
Before Ionomycin
GFP-Sec61β
Mito-Red
After Ionomycin
M1-WT
M1-EF1 mut
10µm
B
GFP-Sec61β
Mito-Red
M1-WT
Before Ionomycin
After Ionomycin
M1-EF1 mut
Fluorescence intensity
Distance (pixels)
C
M1WT
M1EF1
% Mitochondrial length > 5 µm
Time (s)
D
pBSD
M1-WT
M1-EF1
Flag
β actin
F
15 min
4 hours
Control
M1-WT
M1-EF1
Thapsigargin
Ionomycin
XBP1s
ATF6α
βActin
G
XBP1s/actin ratio
ATF6α/actin ratio
ns
Control
M1-WT
M1-EF1
Untreated
Ionomycin
Thapsigargin
15 min
4 hours
E
Untreated
Ionomycin
M1WT
M1EF1
0.2 µm

Figure S6, Related to Figure 6. $[Ca^{2+}]_c$-Induced MiST does not disrupt ER network

(A) Representative confocal images of COS7 cells stably expressing M1WT and M1EF1 mutant transfected with GFP-Sec61β and Mito-Red to visualize ER-mito contact sites. n=3 (cells 15-20).

(B) Line plot for the ER-mito contact sites before and after ionomycin challenge from Figure S8A.

(C) Mean traces of change in mitochondrial length over time from COS7 cells stably expressing M1WT (black) and M1EF1 mutant (red) after ionomycin challenge. Mean ± SEM; n=3.

(D) Immunoblot analysis of COS7 cells stably expressing Flag-tagged M1WT and M1EF1 mutant. Cell lysates were probed with anti-Flag antibody and β-actin served as protein loading control.

(E) Representative high resolution EM images depicting the retention of ER-Mitochondrial contact and the change in mitochondrial morphology in M1WT but not M1EF1 MEFs after treatment with ionomycin (5 μM). n=3.

(F) Immunoblot analysis for ER stress in MEFs stably expressing M1WT and M1EF1 mutant after challenge with ionomycin (2.5 μM) and thapsigargin (2 μM) for indicated time points. Cell lysates were probed with anti-XBP1s, ATF6α antibodies and β–actin served as a protein loading control. n=2

(G) Bar graphs represent quantification of XBP1s and ATF6α abundance from M1WT and M1EF1 expressing MEFs after ionomycin and thapsigargin challenge. Mean ± SEM; ns (not-significant).

Figure S7

A
OCR (pmol/min/mg protein)
Time (s)
B
Basal OCR (pmol/min/mg protein)
MEF WT
M1 WT
M1EF1
M1 EF2
C
Maximal OCR (pmol/min/mg protein)
MEF
M1 WT
M1EF1 mut
M1EF2 mut
D
M1 WT
M1 EF1
M1 EF2
M2 WT
M2 EF1
M2 EF2
LC3-I
LC3-II
p62
β–Actin
Control
Starvation
Ionomycin
E
LC3-I/LC3-I+LC3-II
Normal
Starvation
Ionomycin
F
p62
G
Normal
Starvation
Ionomycin
GFP
mRFP
Merge
H
Noramalized LC3 puncta/cell
M1 WT
M1 EF1
M1 EF2
M2 wt
M2 EF1
M2 EF2
I
M1WT
M1EF1
FCCP
10 µm
Parkin-mCherry
Mito-EGFP
Ratio (Parkin-mCherry/Mito-EGFP)
J
pBSD
M1WT
M1EF1
K
Before stimulation
After stimulation
L
% Mitochondrial length > 5 µm
control
+3-MA
Time (s)
M
OCR (pMoles/min)
Time (s)
N
Basal OCR (pMoles/min)
- thrombin
+ thrombin
O
Maximal OCR (pMoles/min)
P
ATP luminescence/mg protein
-thrombin
+ thrombin
Q
Mitosox Red (f.a.u.)
Thrombin
R
FCCP
NADH/NAD+ (f.a.u.)
Time (s)
S
VE-Cre
MCU-/-
% PI positive cells
Unt
H2O2
C2
Iono
Tg
T
- Ionomycin
+ Ionomycin
WT
BB DKO
U
Ctl
M1WT
M1EF1
M1EF2
M2WT
M2EF1
M2EF2
V
MCU KD + pBSD
MCU KD + M1WT
MCU KD + M1EF1

Figure S7, Related to Figure 7. Miro1 EF1 Mutant confers resistance to autophgic flux and stress-induced cell death.

(A) Measurement of oxygen consumption rate (OCR) in WT (black), M1WT (red), M1EF1mutant (blue) and M1EF2 mutant (green) MEFs. After basal OCR, oligomycin (1 μM) (A), FCCP (1 μM) (B), and rotenone (100 μM) + Antimycin A (100 μM) (C) were added as indicated.

(B and C) Bar represents mean basal OCR, and maximal OCR. Data indicate Mean ± SEM; **p<0.01; *p <0.05; ns= non-significant; n=4.

(D) Western blot analysis of cell lysates from MEFs stably expressing M1WT, M1EF1-, M1EF2-, M2WT, M2EF1- and M2EF2 mutants either treated with ionomycin (5 μM) for 4h or starved for 24h. The cell lysates were probed for LC3 and p62 antibodies. β–actin served as loading control n=3.

(E and F) Bar graph represents quantification of LC3-I/LC3-I+LC3-II and p62. Data indicate Mean ± SEM; **p<0.01; *p <0.05; ns= non-significant; n=3.

(G) Representative confocal images of mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3) in M1WT, M1EF1, M1EF2, M2WT, M2EF1 and M2EF2 mutant MEFs before and after starvation (24 hours) or ionomycin treatment (5 μM, 4 hours). n=4.

(H) Quantification of autophagy performed as normalized LC3 puncta. Mean ± SEM; ***p < 0.001; n=4.

(I) Representative confocal images of MEFs stably expressing M1WT and M1EF1 mutant transfected with Parkin-mCherry and mito-EGFP and treated with 10 μM FCCP for 1 hour. (n=3; 20-30 cells) (Left panel). Quantification of Parkin-mCherry/mito-EGFP puncta. Data indicate Mean ± SEM, *** $p < 0.001$ (n=3; 20-30 cells) (Right panel).

(J) Concentration of mitochondrial matrix Ca^{2+} release from control, M1WT, and M1EF1 mutant MEFs after FCCP challenge. Mean ± SEM, ns=not significant; n=3.

(K) Blockade of autophagy does not prevent $[Ca^{2+}]_c$-induced MiST. Representative confocal images of cox8-a-mRFP transfected MEFs pretreated with 3MA (10 mM) for 2 hours. Real time visualization of mitochondrial shape changes in cells challenged with ionomycin (2.5 μM). n=4.

(L) Mean traces of change in mitochondrial length over time from control (black) and 3MA treated MEFs (red). Mean ± SEM; n=4.

(M) Measurement of oxygen consumption rate (OCR) in M1WT (black), M1EF1 mutant (red) and M1EF2 mutant (blue) MEFs. After basal OCR, thrombin (5mU/ml) (A), oligomycin (1 μM) (B) FCCP (1 μM) (C), and rotenone (5 μM) + Antimycin A (5 μM) (D) were added as indicated.

(N and O) Bar represents mean basal OCR, and maximal OCR. Data indicate Mean ± SEM; ns = non-significant; n=8.

(P) Bar graph represents cellular ATP levels in M1WT, M1EF1 and M1EF2 mutant MEFs before and after stimulation with thrombin (5 mU/ml). Data indicate Mean ± SEM. ns= not significant; n=4.

(Q) Quantification of MitoSOX Red fluorescence in M1WT and M1EF1 mutant MEFs before and after stimulation with thrombin (5 mU/ml). Data indicate mean ± SEM. *p < 0.05, ns= not significant; n=4.

(R) Mean traces of cellular $NADH/NAD^+$ fluorescence in M1WT and M1EF1 mutant MEFs. Inset: Peak $NADH/NAD^+$ fluorescence. Data indicate Mean ± SEM; ns=not significant; n=3.

(S) Quantification of PI-positive cells by FACS analysis in VE-Cre and $MCU^{-/-}$ primary endothelial cells treated with H_2O_2 (2 mM), ceramide (C2) (100 μM), ionomycin (Iono) (5 μM) and thapsigargin (Tg) (10 μM) for 18 h. Data represents Mean ± SEM; ns = not significant; n=3.

(T) Quantification of PI-positive cells by FACS analysis in WT and Bax Bak DKO MEFs treated with (grey) or without (black) ionomycin (5 μM) for 18 h. Data represents Mean ± SEM; ns = non-significant; n=3.

(U) Quantification of PI-positive cells by FACS analysis in WT, MEFs stably expressing M1WT, M1EF1-, M1EF2-, M2WT, M2EF1- and M2EF2 mutants were treated with (grey) or without (black) ionomycin (5 μM) for 18 h. Data represents Mean ± SEM; *$p < 0.05$; n=3.

(V) Quantification of PI-positive cells by FACS analysis in MCU KD HeLa cells stably expressing M1WT and M1EF1 mutant were treated with or without ionomycin (5 μM) for 18 h. Data represents Mean ± SEM; *$p < 0.05$; n=3.

EXTENDED EXPERIMENTAL PROCEDURES

Cell Culture

HeLa (ATCC# CCL-2), HEK-293 (ATCC# CRL-1573), COS7 (ATCC# CRL-1651) and mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37ºC and 5% CO_2.

Generation of Hepatocyte-Specific Mice and Isolation of Primary Hepatocytes

Hepatocyte-specific Mcu knockouts were generated by a Cre-LoxP approach using hepatocyte-specific Cre-recombinase transgenic mice (B6.Cg-Tg(Alb-cre)21Mgn/J, The Jackson Laboratory, USA) and $MCU^{fl/fl}$ mice. All animal experiments were approved by Temple University's IACUC and followed AAALAC guidelines.
Primary adult mouse hepatocytes were isolated using a two-step collagenase perfusion technique with slight modifications (Li et al., 2010). In brief, mice liver was sequentially perfused with perfusion medium- I (DPBS containing 10mM HEPES, 0.05% w/v KCl, 5mM Glucose, 200µM EDTA, pH 7.4) and perfusion medium-II (DPBS containing 30mM HEPES, 0.05% w/v KCL, 5mM Glucose, 1mM $CaCl_2$, pH 7.4) containing collagenase D (400µg/ml). Liver lobes were dissected, dissociated, and crude hepatocyte preparation passed through the gauze mesh filter (100µM diameter). The crude hepatocyte preparation was centrifuged at the speed of 50g for 2 min to pellet down parenchymal hepatocytes. The hepatocytes were washed five times with perfusion medium-II. Following the wash, cells were plated in culture dishes in hepatocyte attachment medium (Williams E medium containing 1% (v/v) antibiotic-antimycotic solution (Gibco), 1% (v/v) 200mM L-glutamine, 1% (v/v) non-essential amino acids, and 10% heat-inactivated fetal bovine serum). The next day, attachment media was replaced with hepatocyte culture medium (Williams E medium containing 1% (v/v) antibiotic-antimycotic solution (Gibco), 1% (v/v) 200mM L-glutamine, and 1% (v/v) non-essential amino acids).

Plasmids and Antibodies

Plasmids GCaMP6-mt (Ref), peredox-mCherry (Hung et al., 2011), GFP-Sec61β (Addgene plasmid# 15108, Parkin-mCherry (Addgene plasmid # 23956), , Tubulin-GFP, GFP-LC3 and mCherry-LC3 were purchased. Mito-EGFP Mito-Red (pDsRed2-Mito Vector) purchased from Clontech Laboratories. COX8A-C-mRFP (pCMV6-AC-RFP), KIF5B-Flag and Peroxiredoxin 3 was purchased from Origene Technologies. Mito-BFP from Dr. Gia Voeltz (Friedman et al., 2011) and Drp1-WT plasmid described earlier (Shanmughapriya et al., 2015a). The full-length wild-type and EF-hand mutant Miro1 (M1EF1 with point mutations in D210A and E221K; M1EF2 with point mutations in D330A and E341K), Miro2 (M2EF1 with point mutations in D197A and E208K; M2EF2 with point mutations in D317A and E328K), and constitutively active human MIRO1 GTPase mutant P13V, and dominant negative mutant T18N constructs were custom synthesized as gBlock gene fragments from IDT Inc. and subcloned into appropriate vectors for further use as described previously (Dong et al., 2017). Lentiviral constructs expressing Drp1 mutants S600A, S600D, S579A and S579D were generated and transduced for stable expression. All the plasmids were confirmed by sequencing before use. Antibodies specific for LC3 (Sigma, Cat# L7543; 1:10000), p62 (Cell Signaling Technology, Cat# 5114; 1:1000), β-actin (Santa Cruz Biotechnology Inc, Cat# sc-47778 HRP; 1:10000), KIF5B (Santa Cruz Biotechnology Inc, Cat# sc-376246; 1:200), Drp1 (Santa Cruz Biotechnology Inc, Cat# sc-32898; 1:200), Mfn1 (Santa Cruz Biotechnology Inc, Cat# sc-50330; 1:500), Mfn2 (Santa Cruz Biotechnology Inc, Cat# sc-100560; 1:500), Miro1(Novus Biologicals, cat# NBP1-59021; 1:1000), Tubulin (Santa Cruz Biotechnology Inc, Cat# sc-8035; 1:1000), cytochrome c (Santa Cruz Biotechnology Inc, Cat# sc-13156; 1:1000), Tom 20 (Santa Cruz Biotechnology Inc, Cat# sc-11415), MCU (Sigma-Aldrich, cat# HPA016480-100UL; 1:500), Xbp1s (Cell Signaling Technologies, cat# CST-12782), ATF6a (Santa Cruz Biotechnology Inc, Cat# sc-166659), Flag (Monoclonal anti-flag M2, Sigma, Cat# F1804, 1:500 for IP), Flag-HRP (Monoclonal anti-flag M2, Sigma, Cat# A8592), HA (Sigma, Cat# H6908, 1:500 for IP) and HA-Peroxidase (Roche, Cat# 12013819001, 1:10000) were used.

siRNA knockdown

Transient knockdown of Mid49, Mid51, Dynamin2 and KIF5B was achieved by using siRNA pool of four oligos (SMARTpool, Dharmacon, USA) (5 nM) for **Dnm2**
(ACCAUGAGCUGCUGGCUUA,GAAGAGGGCCAUACCCAAU,
AAAGUUCGGUGCUCGAGAA,GGAGCCCGCAUCAAUCGUA),

MID51 (GAUAGUAUGAGAAACGUGA, CUCAAGAAAUAGACGAGUU, GAGCUAGGCUAUAUAUUUA, GAAGAGUGAUUGCAUGUUU),
MID49 (CUCUAUGGGCCACGAAUUA, GUAAAGCGGCUCAUCGACA, CCUCUUGGCUAAUGCUCGA, AGAUUGAUGACAUUGGCUA),
KIF5b (GAGCUAAACCGUUGGCGUA, GCAAGAAGUAGACCGGAUA, CAACAGACAUGUCGCAGUU, CAGGACAGAUGAAGUAUAA), with scrambled siRNA as control at a final concentration of 20nM using Lipofectamine® RNAiMAX transfection reagent.

Generation of Stable Cells Expressing M1WT, M1EF1, M1EF2, M2WT, M2EF1, M2EF2 and M2EF12

MEFs were transfected with M1WT, M1EF1, M1EF2, M2WT, M2EF1, M2EF2 and M2EF12 and 48 h post-transfection, the cells were selected with blasticidin (2 μg/ml) for 6–10 days and expanded. COS7 and HeLa cells were transfected with M1WT and M1EF1 mutant plasmid and after 48 h cells were selected with blasticidin (2 μg/ml) for 6–10 days.

Simultaneous Measurement of Mitochondrial Shape (MiST) and $[Ca^{2+}]_c$ or Mitochondrial Swelling or Mitochondrial Membrane Potential ($\Delta\Psi_m$) or cytoskeleton

HeLa WT cells were grown on 25-mm glass coverslips and transfected with COX8-mRFP. 48 h post transfection, cells were treated for 30 minutes ± CsA (5 μM) and stained with PTP opening indicator Calcein-AM. Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°◦C and imaged. After 1 min of baseline recording, ionomycin (2.5 μM) was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a ×63 oil objective. The reduction in fluorescence and change in mitochondrial morphology following stimulation with 2.5 μM ionomycin was assessed and compared with before ionomycin stimulation. Mitochondrial swelling was measured as a decrease in Calcein fluorescence (Kroemer et al., 2007; Petronilli et al., 1999; Shanmughapriya et al., 2015b). The length of individual mitochondria from each image was measured using image J and represented as a percentage of mitochondria in 0.2-2 μm, 2-5 μm and > 5 μm.

HeLa WT/ MEF WT/ MEFs stably expressing M1WT, M1EF1, M1EF2, M2WT, M2EF1 and M2EF2 mutants or HeLa cells expressing M1P13V/ M1T18N were grown on 25-mm glass coverslips and transfected with COX8-mRFP. To monitor $[Ca^{2+}]_c$, 48 h post transfection, cells were loaded with 5 μM Fluo-4AM (30 min) in the extracellular medium as described previously (Mallilankaraman et al., 2012). Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37 °C and imaged. After 1 min of baseline recording, agonist (histamine (100 μM); ionomycin (2.5 μM); thrombin (5 mU)) was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a 63x oil objective. Images were analyzed and quantified using ImageJ (NIH) and custom-made software (Spectralyzer). The length of individual mitochondria from each image was measured using image J and represented as a percentage of mitochondria in 0.2-2 μm, 2-5 μm and > 5 μm.

Freshly isolated rat ventricular myocytes infected with 2 μl/ml of Ad-Cox8a-mRFP. 72 h post infection cells were loaded with 5 μM Fluo-4 to monitor calcium and blebbistatin 10 μM to prevent hypercontraction. Ionomycin (10 μM) was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation.

To further validate that MiST is independent of PTP opening, MCU KO and $MCU^{fl/fl}$ hepatocytes isolated from $MCU^{fl/fl}$Alb-Cre^{+} mice were coated on 25-mm glass coverslips and loaded with matrix-localized $\Delta\Psi_m$ indicator dihydrorhodamine 123 (DHR123 10μM) for 30 min (Shanmughapriya et al., 2015b). Confocal images were recorded every 30 s (510 Meta; Carl Zeiss) at 488 nm excitation using a ×40 oil objective. Images were analyzed and quantified using ImageJ (NIH) and custom-made software (Spectralyzer). The length of individual mitochondria from each image was measured using image J and represented as a percentage of mitochondria in 0.2-2 μm, 2-5 μm and > 5 μm.

To confirm $[Ca^{2+}]_c$-mediated MiST phenomenon, HeLa WT cells were transfected with COX8a-mRFP. 48 h post transfection, cells were loaded with 5 μM Fluo-4/AM (30 min) and treated for 30 minutes with $[Ca^{2+}]_c$ chelator BAPTA-AM (25 μM) or EGTA (0.5 mM) confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a ×63 oil objective. Images were analyzed and quantified using ImageJ (NIH) and

custom-made software (Spectralyzer). The length of individual mitochondria from each image was measured using image J and represented as percentage of mitochondria in 0.2-2 μm, 2-5 μm and > 5 μm.
For simultaneous measurement of $\Delta\Psi_m$ and MiST, HeLa WT/ MEF WT/ M1EF1 KI MEF cells were grown on 0.2% gelatin-coated 25-mm glass coverslips and transfected with COX8a-mRFP. 48 h post transfection, cells were loaded with 10 μM DHR123 (30 min) in the extracellular medium as described previously (Shanmughapriya et al., 2015a). Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37 °C and imaged. After 1 min of baseline recording FCCP (10 μM); ionomycin (2.5 μM); was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a ×63 oil objective. Images were analyzed and quantified using ImageJ (NIH) and custom-made software (Spectralyzer). The length of individual mitochondria from each image was measured using image J and represented as percentage of mitochondria in 0.2-2 μm, 2-5 μm and > 5 μm. Loss of $\Delta\Psi_m$, was measured over time as the dissipation of the DHR123 from the mitochondria or increase in DHR123 fluorescence in the cytosol and plotted.

HeLa WT cells grown on 25-mm glass coverslips were co-transfected with COX8-mRFP and Tubulin-GFP. 48 h post transfection, cells were treated with 5 mM N-acetyl cysteine (NAC) or 10 mM GSH (reduced glutathione) for 30 minutes. Additionally, HeLa WT cells grown on 25-mm glass coverslips were transfected with a combination of cox8a-mRFP, Ad-MnSOD and peroxiredoxin (Prdx3) and 48 h post transfection, imaged using confocal microscopy. After a baseline recording of 60s, cells were stimulated with 2.5 μM ionomycin to observe ionomycin-dependent ROS-mediated change in morphology using a confocal microscope. MiST was measured as a decrease in mitochondrial length as above.

To evaluate MiST in the presence of cytoskeletal inhibitors, HeLa / MEF WT cells grown on 25-mm glass coverslips were transfected with COX8a-mRFP and Tubulin-GFP, a marker that labels microtubules. 48 h post transfection, cells were imaged using confocal microscope. After a baseline recording of 60s, cells were stimulated with 1 μM colchicine or Blebbistatin (100μM) (Sigma #B0560) or latrunculin (1 μM) (Enzo Life Sciences #BML-T119) and images were recorded every two minutes (colchicine) or 3 s (Lat A/Blebbistatin) to observe changes in mitochondrial morphology on microtubule de-polymerization. The length of individual mitochondria were measured as above.

To validate that MiST is independent of mitochondrial fission, Drp1 KO (Kageyama et al., 2014), MFF/Fis1 DKO (Loson et al., 2013), lentiviral constructs expressing Drp1 mutants S600A, S600D, S579A and S579D transduced MEFs and Dyn2 KD/ Mid51 KD and Mid49 KD MEFs were grown on 25-mm glass coverslips and transfected with COX8a-mRFP. 48 h post transfection, cells were analyzed for MiST using a confocal microscope. Additionally, HeLa cells transfected with COX8a-mRFP were treated with 1 μm FK506 (Sigma-Aldrich; F4679-5MG) for 30 minutes, a Ca^{2+}-dependent phosphatase, calcineurin inhibitor. After a baseline recording of 60s, cells were stimulated with 2.5 μM ionomycin and images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a ×63 oil objective. The length of individual mitochondria were measured as above.

CypD KO (Baines et al., 2005), SPG7 KO (Shanmughapriya et al., 2015b), VDAC1/3 DKO (Weeber et al., 2002) and Bax/Bak DKO (Lindsten et al., 2000) MEFs were transiently transfected with GCaMP6-mt. 48 h post transfection, cells were analyzed for MiST using confocal microscope upon ionomycin stimulation as described above.

Generation of M1EF1 KI MEFs

The candidate sgRNAs were selected based on the CRISPR Design platform. The selected gRNAs targeting the M1EF1 site and the CRISPR-Cas9 nickase mRNA were synthesized in vitro. A 200 bp single-stranded oligodeoxynucleotide (ssODN) was used as a template for HDR-mediated gene repair. The synthesized sgRNAs, ssODN, and nickase mRNA were transfected in MEFs. 48 hours post-transfection, cells were passaged and genomic DNA was subjected for PCR-RFLP genotyping. For genotyping, the region corresponding to the EF1 site (exon 9; 793 bp) was amplified. The purified PCR product was subjected for RFLP with Bcc1 restriction enzyme. The recognition site for Bcc1 is 5'CCATCNNNN3', and therefore M1EF1 targeted allele was not cleaved while the wild type was into two fragments (414 and 379 bp). The mutation was confirmed by DNA sequencing. We screened ~450 clones and found two clones which expressed M1EF1 KI.

Mitochondrial Oxygen Consumption Rate

MEFs stably expressing M1WT, M1EF1 and M1EF2 mutant plasmids were subjected to oxygen consumption rate (OCR) measurement at 37°C in an XF96 extracellular flux analyzer (Seahorse Bioscience). MEFs (1.5 x 10^4) were sequentially challenged with oligomycin, FCCP, and rotenone plus antimycin A (Doonan et al., 2014).

Measurement of Mitochondrial Superoxide

Mitochondrial superoxide was measured by using the mitochondrial oxygen free radical indicator MitoSOX Red (molecular probes; Invitrogen) as described previously (Mukhopadhyay et al., 2007). Briefly, cells grown on 0.2% gelatin coated glass coverslips were loaded with 5 μM MitoSOX Red for 30 min, and coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°C and imaged. Confocal (510 Meta; Carl Zeiss, Inc.) images were obtained at 561 nm excitation by using a 63 x oil objective. Images were analyzed, and the mean MitoSOX Red fluorescence was quantified by using Image J software (NIH).

ATP Measurement

Total ATP abundance was assessed using CellTiter-Glo® luminescent assay, and the luminescence was measured using a plate reader (Infinite M1000 PRO, Tecan).

NADH Measurement

MEFs stably expressing M1WT and M1EF1 mutant (1×10^5 cells) were transiently transfected with NADH-NAD+ redox sensor, peredox m-cherry (Hung et al., 2011). Briefly, cells were imaged 48 hr post transfection. Confocal images were acquired at 405 nm and 561 nm excitation every 3s using Carl Zeiss 710 Meta NLO. Images were analyzed using ZEN 2010 software and plotted with SigmaPlot. (Shanmughapriya et al., 2015). The experiments were performed at 37°C.

Flow Cytometry and PI Staining

Cells were treated with H_2O_2 (2 μM), ceramide (C2) (100 μM), ionomycin (Iono) (5 μM) and thapsigargin (Tg) (10 μM) for 18 h. For necrotic cell death measurement, cells were stained with propidium iodide. The cells were analyzed with a flow cytometer (BD Calibur). Relative PI staining was plotted on a logarithmic scale using Flow-Jo software (Irrinki et al., 2011).

Immunoblotting

Cell extracts were prepared from cells of indicated genotypes using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease inhibitor cocktail (Complete, Roche and 1 mM PMSF)). Equal amounts of protein were separated on 4-12% Bis-Tris polyacrylamide gel, transferred to a PVDF membrane, and probed with corresponding antibodies as specified.

Co-immunoprecipitation

Cell extracts were prepared from transiently transfected MEFs cells using RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease inhibitor cocktail (Complete: Roche and 1 mM PMSF). To study the interaction of M1WT with M1 WT, HA tagged M1 WT was co-transfected with Flag tagged M1WT and M1EF1 mutant plasmids. Following immunoprecipitation with HA antibody (Sigma-aldrich, USA), total cell lysates and immunoprecipitated materials were subjected to Western blot analysis. 10% of cell lysates were probed with Flag (Sigma-Aldrich) and HA antibodies to serve as inputs, and similarly, immunoprecipitated samples were probed with their corresponding antibodies to assess protein binding. To study the interaction of M1WT, M2WT, M1EF1 mutant with Mfn1 and Mfn2, MEFs transfected with Flag-tagged M1WT, M2WT and M1EF1 were used to co-immunoprecipitate the Mfn1 and 2. To study the effect of Ca^{2+} on Miro and KIF5B interaction, Flag-KIF5B and HA-Miro plasmids were co-transfected in COS7 cells. After 48 hours of transfection, cells were collected and lysed in RIPA buffer with (1 mM) or without Ca^{2+}. Co-immunoprecipitation was performed as described earlier (Tomar et al., 2016).

Simultaneous Assessment of MiST and Autophagy

Stable M1WT and M1EF1 mutant MEFs were transiently transfected with Cox8a-mRFP and GFP-LC3. Briefly, cells were imaged 48 h post transfection. After a baseline recording of 60s, cells were stimulated either with thrombin (15 mU/ml) or ionomycin (1 μM) Confocal images were acquired at 405 nm and 561 nm excitation every 3 minutes for a total of 60 minutes using Carl Zeiss 510. Images were analyzed using ZEN 2010 software and image J and plotted with SigmaPlot. M1WT, M2WT and mutant MEFs were plated on 60mm tissue culture treated dishes

and either starved in Earle's salt solution for 6h or treated with ionomycin (5 μM) for 2 h. Cell lysates were collected for immunoblotting with autophagy markers. The role of autophagy induction in MiST is evaluated by the pre-incubation of cells with 3-Methyladenine (3MA) (10mM) for 2 hours to block autophagy.

To monitor the transfer of lipid from mitochondria to autophagosome during starvation induced autophagy, M1WT and M1EF1 mutant MEFs were co-transfected with Mito-BFP and mCherry-LC3. The chloroform was removed from NBD-PS (18:1 NBD-PS Cat#810198, Avanti Polar Lipids, Inc) and reconstituted to a 2.2mM solution in DPBS and 0.22mM solution was added to cells for 30 min. After incubation, culture media is replaced with whole media or EBSS to induce the starvation. Confocal images were recorded at indicated time points using LSM510 confocal microscope at 405, 488- and 561-nm excitation using a 100x oil objective.

Mitochondrial translocation of Parkin

Mitochondrial translocation of Parkin was monitored by confocal microscopy of M1WT and M1EF1 MEFs co-transfected with Parkin-mCherry and Mito-EGFP. Cells were imaged 48 h post transfection. After a baseline recording of 60s, cells were stimulated with either ionomycin (2.5 μM) or pre-treated with FCCP (10 μM) for 1 or 4 h. Confocal images were acquired at 405 nm and 561 nm excitation every 3 minutes for a total of 60 minutes using Carl Zeiss 510. Images were analyzed using ZEN 2010 software and image J and plotted with SigmaPlot.

The translocation of autophagy adaptor protein p62 to mitochondria is monitored by immunoblotting of mitochondrial and cytosolic fractions collected from stably expressing control pBSD, M1WT and M1EF1 mutant MEFs. MEFs were challenged with ionomycin (2.5μM, 4hrs), FCCP (10μM, 4hrs) and mitochondria was isolated as described earlier (Shanmughapriya et al., 2015a). Briefly, cells were homogenized in ice-cold mitochondrial isolation buffer (10 mM sucrose, 200 mM mannitol, 5 mM HEPES, and 1 mM EGTA, pH 7.4) containing 1 mg/ml fatty acid-free bovine serum albumin. The homogenate was centrifuged for 10 min at 1000 x g, and the supernatant was centrifuged again at 14,000 x g for 10 min. The supernatant collected as cytosolic fraction and mitochondrial pellets were washed twice and centrifuged at 11,200 x g. Mitochondrial pellet were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease inhibitor cocktail (Complete, Roche and 1 mM PMSF)). Equal amounts of cytosolic fraction and mitochondrial lysate were subjected to immunoblotting.

Simultaneous Measurement of ER-mitochondrial network and MiST

COS7 cells stably expressing M1WT and M1EF1 mutant were grown on 25-mm glass coverslips and transfected with GFP-Sec61β and Mito-Red to visualize the ER-mitochondrial contacts. After 48 h of transfection, coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37 °C and imaged. After 1 min of baseline recording, ionomycin (2.5 μM) was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a 100x oil objective. Images were analyzed and quantified using ImageJ (NIH) and custom-made software (Spectralyzer). The length of individual mitochondria from each image was measured using image J and represented as a percentage of mitochondria in 0.2-2 μm, 2-5 μm and > 5 μm.
MEFs stably expressing M1WT and M1EF1 mutant plasmids were plated on 10cm dishes and treated with ionomycin 5 μM for 10 minutes and fixed EM-grade 2% glutaraldehyde (Electron Microscopy Sciences, Hartfield, PA) and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 2mM calcium chloride. Cells were fixed for 1h at room temperature. Images were acquired using Zeiss LIBRA120 TEM equipped with Gatan UltraScan, 1000 2k x 2k CCD EFTEM, energy filtering.

Assessment of ER Stress

To assess the ER stress in M1WT and M1EF1 mutant expressing MEFs, cells were challenged with ionomycin (2.5μM for 15 min and 4h) and thapsigargin (2μM for 4h). ER stress marker proteins XBP1s and ATF6α expression were monitored by immunoblotting of cell lysates. The expression of XBP1s and ATF6α is normalized with β-actin expression.

Size Exclusion Chromatographic Analysis of Miro1 Complex

Gel filtration of Miro1 complex was performed at 4°C by fast protein liquid chromatography (ÄKTA Pure FPLC; GE Healthcare), using Superdex 200 10/300 column equilibrated with PBS. The cleared cell lysates prepared from MEFs stably expressing M1WT and MiEF1 mutant were directly loaded onto a Superdex 200 FPLC column at a flow rate of 0.5 ml/min. The 0.5mL fractions were collected, concentrated and used to assay complexes by immunoblotting.

Ca^{2+} Uptake and ΔΨm Measurement in Permeabilized Cell System

Cells were washed in Ca^{2+} free PBS, pH 7.4. An equal amount of cells ($10x10^6$ cells) were resuspended and permeabilized with 40 µg/ml digitonin in 1.5 ml of intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH_2PO4, 20 mM Hepes- Tris, pH 7.2 and 2 µM thapsigargin to block the SERCA pump. All the measurements were performed in the presence of 5 mM succinate. The simultaneous measurement of ΔΨm and extramitochondrial Ca^{2+} ($[Ca^{2+}]_{out}$) clearance as an indicator of $[Ca^{2+}]_m$ retention was achieved by loading the permeabilized cells with JC-1 (800 nM) and FuraFF (0.5 µM), respectively. Fluorescence was monitored in a multiwavelength excitation dual-wavelength emission fluorimeter (Delta RAM, PTI). $[Ca^{2+}]_{out}$ is shown as the excitation ratio (340 nm/380 nm) of Fura-FF/FA fluorescence and ΔΨm as the ratio of the fluorescence of J-aggregate (570 nm excitation/595 nm emission) and monomer (490 nm excitation/535 nm emission) forms of JC-1 (Mallilankaraman et al., 2012). A series of Ca^{2+} boluses (10 µM) and mitochondrial uncoupler, CCCP (2 µM), were added at the indicated time points. All the experiments were performed at 37°C with constant stirring.

Simultaneous measurement of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in an intact cell system.

HeLa WT cells grown on 25-mm glass coverslips were co-transfected with GCaMP6 and mito-R-GECO1. MEF grown on 25-mm glass coverslips were loaded with 2 µM rhod-2/AM (50 min) and 5 µM Fluo-4/AM (30 min) in the extracellular medium as described (Hawkins et al., 2007). Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°C and imaged. After 1 min of baseline recording, agonist (histamine (100µM); thrombin (500 mU)) was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss, Inc.) at 488- and 561-nm excitation using a 63x oil objective. Images were analyzed and quantitated using ImageJ (NIH) and custom-made software (Spectralyzer).

Measurement of basal matrix $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ release

MEFs cells stably expressing control vector, M1WT and M1EF1 were resuspended and permeabilized with digitonin (40 mg/ml) in 1.5 mL of intracellular medium composed of 120mMKCl, 10mMNaCl, 1mMKH2PO4, 20 mM HEPES-tris (pH 7.2), 5 mM succinate, bath Ca^{2+} indicator Fura-2FF (0.5 mM), MCU blocker Ru360 (1 mM) and NCLX blocker, CGP (1 mM), and 2 µM thapsigargin to block the SERCA pump. The experiments were performed at 37 °C with constant stirring. To assess the resting $[Ca^{2+}]_m$, after baseline recording of bath Ca^{2+} ($[Ca^{2+}]_{out}$), FCCP (10 µM mM) was added to release matrix mitochondrial Ca^{2+}. Fluorescence was monitored in a multiwavelength excitation, dual-wavelength emission fluorimeter (DeltaRAM, PTI). $[Ca^{2+}]_{out}$ was recorded as an excitation ratio (340 nm/380 nm) and emission at 510 nm of Fura2FF fluorescence Cells were washed in Ca^{2+} free PBS, pH 7.4.

To measure $[Ca^{2+}]_i$ release, an equal amount of cells ($10x10^6$ cells) were re-suspended in Hank's Buffered Salt Solution and loaded with 1µM Fura-2 AM for 30 minutes. Fluorescence was monitored in a multi-wavelength excitation dual-wavelength emission fluorimeter (Delta RAM, PTI). $[Ca^{2+}]_i$ is shown as the excitation ratio (340 nm/380 nm) of Fura-2 AM fluorescence. Agonist (histamine (100 µM); ionomycin (2.5 µM); thrombin (5 mU)) was added at the indicated time point. All the experiments were performed at 37°C with constant stirring.

Assessment of Mitochondrial Fragmentation

M1WT and M1EF1 mutant MEFs were transiently transfected with cox8a-mRFP. 48h post transfection cells were subjected to 10 µM oligomycin and 10 µM antimycin A for 4 hours and imaged for mitochondrial fragmentation. The length of mitochondria were measured from every image as above.

Neuronal Cultures, Transfection, Labeling and Imaging

Dissociated primary postnatal day 6 mouse dorsal root ganglion neurons were cultured in tissue culture plastic dishes with glass coverslips affixed at the bottom of a hole drilled in the center of the dish and coated with polylysine (100 µg/mL) and laminin (25 µg/mL) coated. Neurons were cultured in F12H medium with 1:50 B27 and 50 ng/mL nerve growth factor. Primary rat hippocampal embryonic day 18 neurons were prepared from hippocampi obtained from BrainBits Inc (Springfield, IL). The dissociation and culture of these neurons followed the manufacturer's protocols and reagents. On the day in vitro (DIV) 3 the medium was switched to mbactiv1 medium (BrainBits), with subsequent changes of medium every 4 days. Mouse sensory neurons were transfected prior to plating using Amaxa nucleofection (program G013) and 15 µg/mL of the plasmid. Hippocampal neurons were transfected on DIV 12 using Lipofectamine (20 µL + 2 µL plasmid in a 300 µL medium volume) for one hr, followed by replacement of the transfection solution with the medium. Mitochondria were labeled with MitoTracker

Green (25 nM for 30 min followed by medium wash). Prior to imaging in all cases, the medium was replaced with F12H without B27. All imaging was performed on an Axio Observer Z1 inverted fully motorized microscope equipped with a stage heater, Hammamatsu Orca R2 CCD camera, 63x objective. Microscope protocols and acquisition were run using Zeiss AxioVision software. Measurements of mitochondrial length were performed using Zeiss AxioVision measurement functions.

Statistical Analysis

Data were expressed as the mean ± SE. Statistical significance was evaluated via Student's unpaired t test, one-way and two-way ANOVA. $P < 0.05$ was considered statistically significant. All experiments were conducted at least three times unless specified. Data were plotted either with Sigma Plot 11.0 software or GraphPad Prism version 6 software. Data obtained from neurons was analyzed using GraphPad Instat software (v 3.10).

SUPPLEMENTAL REFERENCES

Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H., Hambleton, M.A., Brunskill, E.W., Sayen, M.R., Gottlieb, R.A., Dorn, G.W., *et al.* (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature *434*, 658-662.

Dong, Z., Shanmughapriya, S., Tomar, D., Siddiqui, N., Lynch, S., Nemani, N., Breves, S.L., Zhang, X., Tripathi, A., Palaniappan, P., *et al.* (2017). Mitochondrial Ca2+ Uniporter Is a Mitochondrial Luminal Redox Sensor that Augments MCU Channel Activity. Molecular Cell.

Doonan, P.J., Chandramoorthy, H.C., Hoffman, N.E., Zhang, X., Cardenas, C., Shanmughapriya, S., Rajan, S., Vallem, S., Chen, X., Foskett, J.K., *et al.* (2014). LETM1-dependent mitochondrial Ca2+ flux modulates cellular bioenergetics and proliferation. The FASEB Journal *28*, 4936-4949.

Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J., and Voeltz, G.K. (2011). ER tubules mark sites of mitochondrial division. Science *334*, 358-362.

Gifford, J.L., Walsh, M.P., and Vogel, H.J. (2007). Structures and metal-ion-binding properties of the Ca2+-binding helix-loop-helix EF-hand motifs. The Biochemical journal *405*, 199-221.

Hawkins, B.J., Solt, L.A., Chowdhury, I., Kazi, A.S., Abid, M.R., Aird, W.C., May, M.J., Foskett, J.K., and Madesh, M. (2007). G protein-coupled receptor Ca2+-linked mitochondrial reactive oxygen species are essential for endothelial/leukocyte adherence. Mol Cell Biol *27*, 7582-7593.

Kageyama, Y., Hoshijima, M., Seo, K., Bedja, D., Sysa-Shah, P., Andrabi, S.A., Chen, W., Hoke, A., Dawson, V.L., Dawson, T.M., *et al.* (2014). Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. The EMBO journal *33*, 2798-2813.

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial Membrane Permeabilization in Cell Death. Physiological reviews *87*, 99-163.

Li, W.C., Ralphs, K.L., and Tosh, D. (2010). Isolation and culture of adult mouse hepatocytes. Methods in molecular biology *633*, 185-196.

Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., *et al.* (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell *6*, 1389-1399.

Loson, O.C., Song, Z., Chen, H., and Chan, D.C. (2013). Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Molecular biology of the cell *24*, 659-667.

Mallilankaraman, K., Cárdenas, C., Doonan, P.J., Chandramoorthy, H.C., Irrinki, K.M., Golenár, T., Csordás, G., Madireddi, P., Yang, J., Müller, M., *et al.* (2012). MCUR1 is an essential component of mitochondrial Ca2+ uptake that regulates cellular metabolism. Nature cell biology *15*, 123.

Marsden, B.J., Shaw, G.S., and Sykes, B.D. (1990). Calcium binding proteins. Elucidating the contributions to calcium affinity from an analysis of species variants and peptide fragments. Biochemistry and cell biology = Biochimie et biologie cellulaire *68*, 587-601.

Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P., and Di Lisa, F. (1999). Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Biophys J *76*, 725-734.

Shanmughapriya, S., Rajan, S., Hoffman, N.E., Higgins, A.M., Tomar, D., Nemani, N., Hines, K.J., Smith, D.J., Eguchi, A., Vallem, S., *et al.* (2015a). SPG7 Is an Essential and Conserved Component of the Mitochondrial Permeability Transition Pore. Mol Cell *60*, 47-62.

Sievers, F., and Higgins, D.G. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. Methods Mol Biol *1079*, 105-116.

Tomar, D., Dong, Z., Shanmughapriya, S., Koch, Diana A., Thomas, T., Hoffman, Nicholas E., Timbalia, Shrishiv A., Goldman, Samuel J., Breves, Sarah L., Corbally, Daniel P., *et al.* (2016). MCUR1 Is a Scaffold Factor for the MCU Complex Function and Promotes Mitochondrial Bioenergetics. Cell reports *15*, 1673-1685.

Webb, B., and Sali, A. (2014). Protein structure modeling with MODELLER. Methods Mol Biol *1137*, 1-15.

Weeber, E.J., Levy, M., Sampson, M.J., Anflous, K., Armstrong, D.L., Brown, S.E., Sweatt, J.D., and Craigen, W.J. (2002). The role of mitochondrial porins and the permeability transition pore in learning and synaptic plasticity. The Journal of biological chemistry *277*, 18891-18897.

Zhou, Y., Frey, T.K., and Yang, J.J. (2009). Viral calciomics: interplays between Ca2+ and virus. Cell Calcium *46*, 1-17.