SUPPLEMENTAL INFORMATION

The supplemental data includes four supplemental figures with legends

Figure S1. Oxidant Exposure Exhibits Persistent MCU-Mediated $[Ca^{2+}]_m$ Uptake. Related to Figure 1.

(**A**) Mean traces for $[Ca^{2+}]_m$ (GCaMP2-mt) fluorescence measured in control, H_2O_2 and H_2O_2 + MnSOD treated HeLa cells. (**B**) Quantification of normalized GCaMP2-mt fluorescence at peak and 600 s. Bar represents Mean ± SEM; * P <0.05; n = 4. (**C**) Mean traces of $[Ca^{2+}]_c$ (Fluo-4) fluorescence measured in control, H_2O_2 and H_2O_2 + MnSOD treated HeLa cells. (**D**) Bar graph represents peak Fluo-4 fluorescence after histamine stimulation. Data indicate Mean ± SEM; ns-not significant, n=4. (**E**) Representative confocal images showing HyPer-dMito fluorescence in control and LPS treated HPMVECs (left). Scatter plot of HyPer-dMito fluorescence in HPMVECs (right). Data indicate Mean ± SEM; *** P <0.001. (**F**) Representative confocal images showing MitoSox Red fluorescence from control and H/R subjected primary adult cardiomyocytes (left). Scatter plot of MitoSox Red fluorescence in myocytes (right). Data indicate Mean ± SEM; *** P <0.001. (**G**) Representative $[Ca^{2+}]_{out}$ traces in Neonatal Rat Ventricular Myocytes (NRVM) exposed to normoxia and H/R. Permeabilized cells were loaded with extramitochondrial Ca^{2+} ($[Ca^{2+}]_{out}$) indicator Fura2FF to which a single 10 μM Ca^{2+} pulse was added to assess the $[Ca^{2+}]_{out}$ clearance rate. (**H**) Quantification of the rate of $[Ca^{2+}]_m$ uptake as a function of decrease in $[Ca^{2+}]_{out}$. Data represents Mean ± SEM; **P <0.01; n = 4. (**I**) Representative $[Ca^{2+}]_{out}$ traces in NRVMs after addition of CCCP. (**J**) Quantification of total matrix $[Ca^{2+}]_m$ released following 10 μM Ca^{2+} pulse. Data represents Mean ± SEM; **P <0.01; n = 4. (**K**) Oxidative stress-induced modification of MCU complex. HEK293T cells expressing Flag-tagged MCU,

MCUb, MICU1, MCUR1 and EMRE were exposed to menadione (10 μM) for 10 min. Lysates were prepared, incubated with mPEG5 and Western blotted with FLAG antibody. (**L** and **M**) NRVMs expressing MCU-FLAG (Ad MCU) were exposed to menadione, isoproterenol (10 μM) or H/R. Lysates were prepared, incubated with mPEG5 and Western blotted with FLAG antibody (n = 3).

Figure S2. MCU Mutants reside in the mitochondrial inner membrane and alter MCU-Mediated $[Ca^{2+}]_m$ Uptake. Related to Figure 2.

(**A**) Alignment of MCU sequence indicating the conserved cysteine residues among species. (**B**) Confocal micrographs of HeLa cells co-transfected with matrix localized GCaMP2-mt (green) and, either MCU WT-mRFP or mRFP-tagged MCU cysteine mutants. (**C**) Mean traces of $[Ca^{2+}]_c$ (Fluo-4) fluorescence in HeLa cells expressing Vector control, MCU^{WT}, MCU^{C97A}, and MCU^{CF}. After baseline recording, cells were treated with SERCA inhibitor, Thapsigargin (Tg; 2 μM) and changes in fluorescence were measured. (**D**) Quantification of $[Ca^{2+}]_c$ fluorescence after Tg addition. Data represents Mean ± SEM; n = 5-7. (**E**) Bar graph showing the mitochondrial membrane potential indicator, TMRM fluorescence in control vector, MCU, MCU^{C97A}, and MCU^{CF} HeLa cells. Data represents Mean ± SEM; n = 5. (**F**) Mean traces of $[Ca^{2+}]_m$ (GCaMP2-mt) fluorescence in HeLa cells expressing MCU^{WT}, MCU^{C67A}, and MCU^{C191A}. After baseline recording, cells were stimulated with histamine and changes in fluorescence were measured. (**G**) Quantification of normalized GCaMP2-mt peak fluorescence. Data represents Mean ± SEM; n = 5-7. (**H**) Mean traces of $[Ca^{2+}]_c$ (Fluo-4) fluorescence measured in HeLa cells expressing MCU, MCU^{C67A}, and MCU^{C191A}. (**I**) Quantification of normalized (Fluo-4) peak fluorescence. Data represents Mean ± SEM; n = 5-7. (**J**) Representative $[Ca^{2+}]_{out}$ traces before and after CCCP (3 μM) addition in HeLa cells stably expressing control vector, MCU, MCU^{C97A},

and MCU^{CF}. (**K**) Quantification of resting matrix $[Ca^{2+}]_m$ after the addition of CCCP. Data represents Mean ± SEM; **P <0.01; n = 3. (**L**) Representative $[Ca^{2+}]_{out}$ traces before and after CCCP (3 μM) addition in HPMVECs stably expressing control vector, MCU, MCU^{C97A}, and MCU^{CF}. (**M**) Quantification of resting matrix $[Ca^{2+}]_m$ after the addition of CCCP. Data represents Mean ± SEM; **P <0.01; n = 3.

Figure S3. Interaction between MCU and its regulatory components are unaffected by MCU cysteine mutation. Related to Figure 2.

(**A**) COS-7 cells were transfected with HA-tagged MCU and, either Flag-tagged MCU or MCU cysteine (MCU^{C97A}, and MCU^{CF}) mutants as indicated. Following immunoprecipitation with HA antibody, total cell lysates and immunoprecipitated materials were subjected to Western blot analysis. Samples were probed with anti-Flag (top) and anti-HA antibodies (bottom). (n=3).
(B) COS-7 cells were transfected with V5-tagged MCUR1 and, either Flag-tagged MCU or MCU cysteine (MCU^{C97A}, and MCU^{CF}) mutants as indicated. Following immunoprecipitation with V5 antibody, total cell lysates and immunoprecipitated materials were subjected to Western blot analysis. Samples were probed with anti-Flag (top) and anti-V5 antibodies (bottom). (n=3).
(C) COS-7 cells were transfected with HA-tagged EMRE and, either Flag-tagged MCU or MCU cysteine (MCU^{C97A}, and MCU^{CF}) mutants as indicated. Following immunoprecipitation with HA antibody, total cell lysates and immunoprecipitated materials were subjected to Western blot analysis. Samples were probed with anti-Flag (top) and anti-HA antibodies (bottom). (n=3).
(D) COS-7 cells were transfected with HA-tagged MCUb and, either Flag-tagged MCU or MCU cysteine (MCU^{C97A}, and MCU^{CF}) mutants as indicated. Following immunoprecipitation with HA antibody, total cell lysates and immunoprecipitated materials were subjected to Western blot analysis. Samples were probed with anti-Flag (top) and anti-HA antibodies (bottom). (n=3).

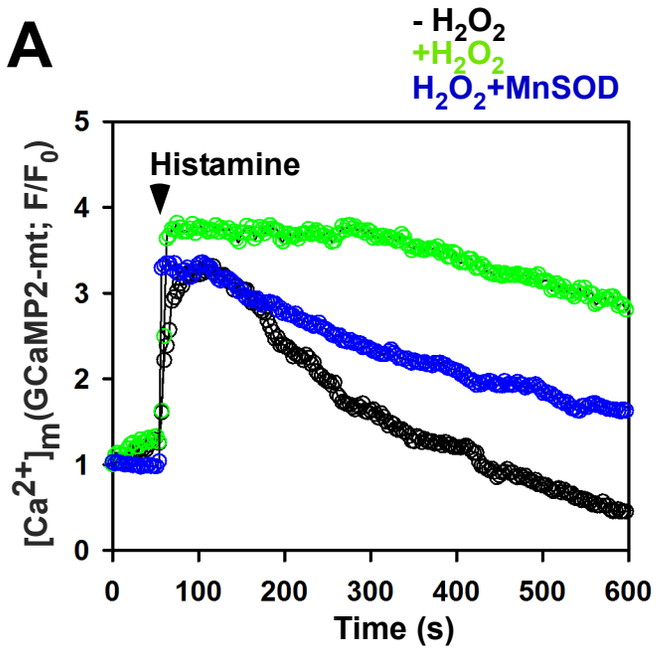
(E) MCU-MICU1 interaction is intact in MCU^{C97A} mutant. COS-7 cells were transfected with HA-tagged MICU1 and Flag-tagged MCU, MCU^{C97A}, and MCU^{CF} as indicated. Following immunoprecipitation with HA antibody, total cell lysates and immunoprecipitated materials were subjected to Western blot analysis. Cell lysates were probed with anti-Flag (top left) or anti-HA antibodies (bottom left) to serve as input. Immunoprecipitated samples were probed with anti-Flag (top right) and anti-HA antibodies (bottom right). (n=3).

Figure S4. Oxidation of MCU Enables MCU Complex Redistribution at the Inner Mitochondrial Membrane. Related to Figure 5.

(**A**) MCU-WT was tagged with a photo-switchable protein, mEOS3.2. HeLa cells were transfected, fixed, and imaged using super-resolution photoactivatable localization microscopy (PALM). The HeLa cells were treated with menadione for various time points (2, 5, 15, 20 minutes). The blue box marks the magnified area (right panels).

(**B**) Nanoclustering of MCU WT-mEOS3.2 was conducted as previously described (Baumgart et al., 2016). The normalized density of (ρ/ρ_0) molecules was plotted against the relative area covered by the clusters (η). The red line fitted to the graph denotes 100% randomly distributed molecules, while true clustering has a higher density (ρ/ρ_0) of molecules along a higher percentage of the area covered by the clusters (η). MCU-WT showed increased clustering with increasing time points of menadione treatment as depicted by the higher ρ/ρ_0. (n=4-7) For each condition, we randomly quantified 5 cells for cluster analysis.

Figure S1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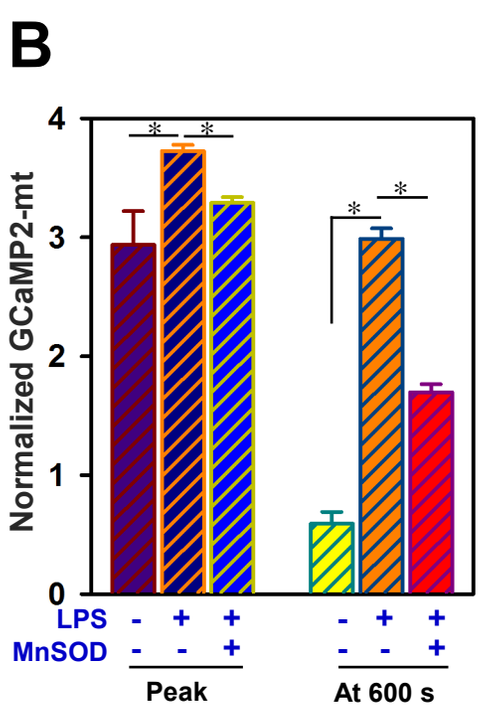
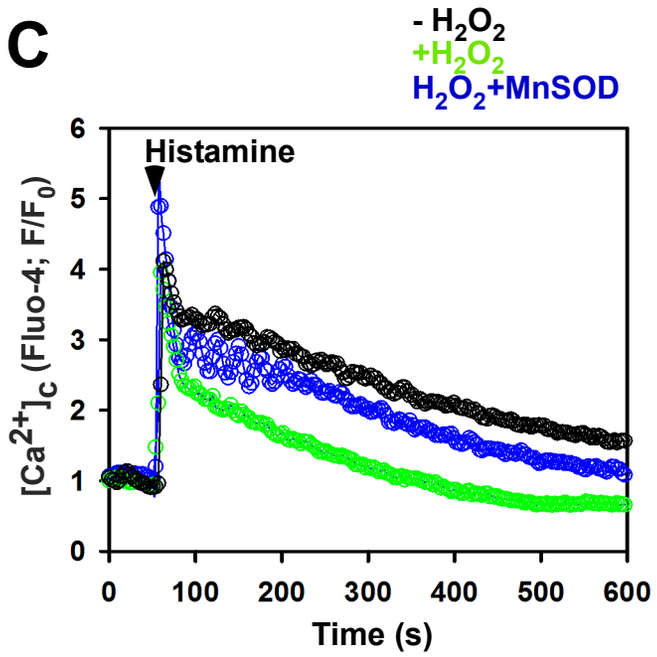
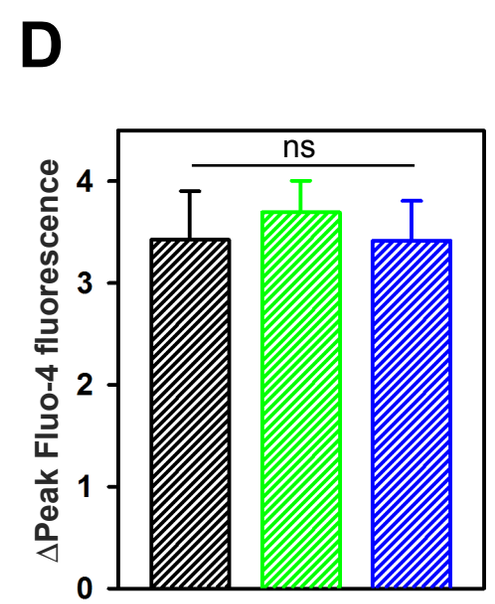
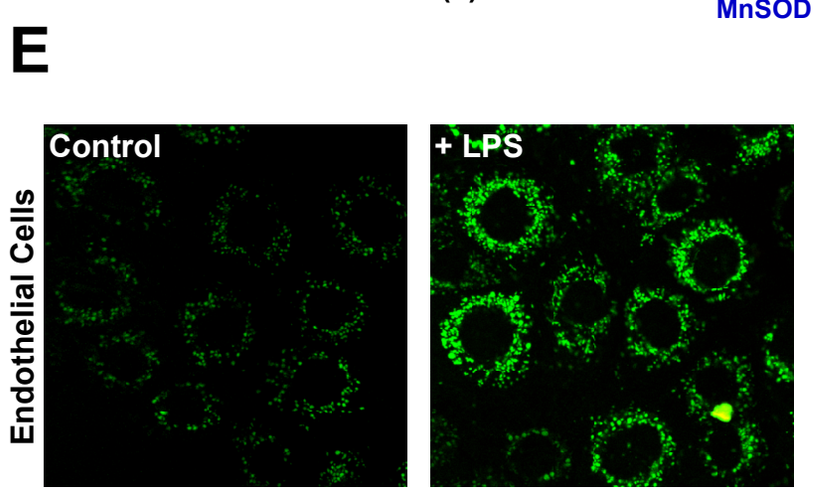
+H2O2
H2O2+MnSOD
Histamine
[Ca2+]m(GCaMP2-mt; F/F0)
Time (s)

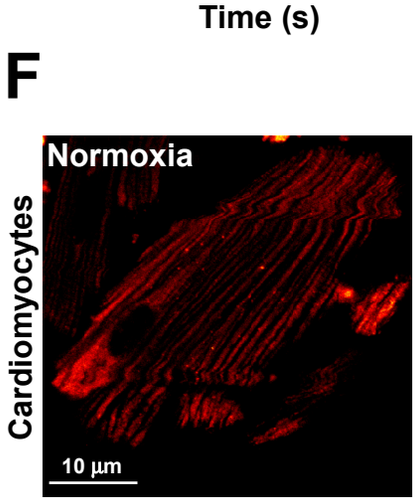

B
Normalized GCaMP2-mt
LPS
MnSOD
Peak
At 600 s


C
- H2O2
+H2O2
H2O2+MnSOD
Histamine
[Ca2+]c (Fluo-4; F/F0)
Time (s)

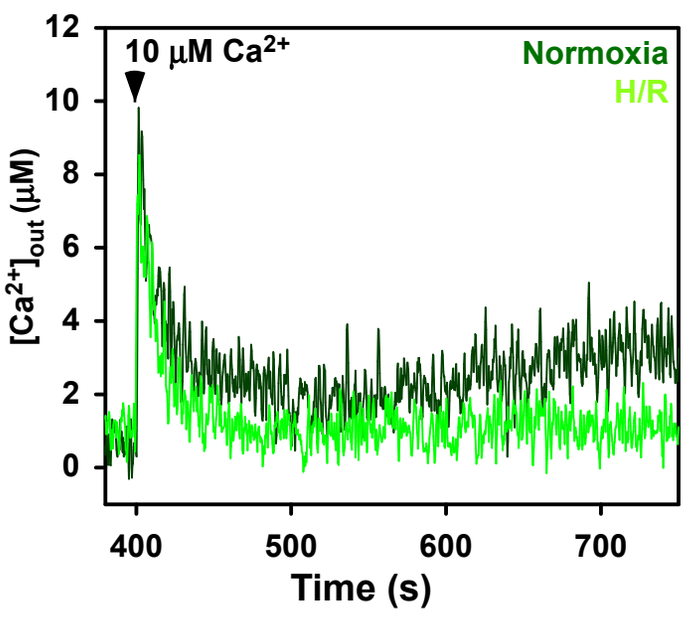

D
ns
ΔPeak Fluo-4 fluorescence

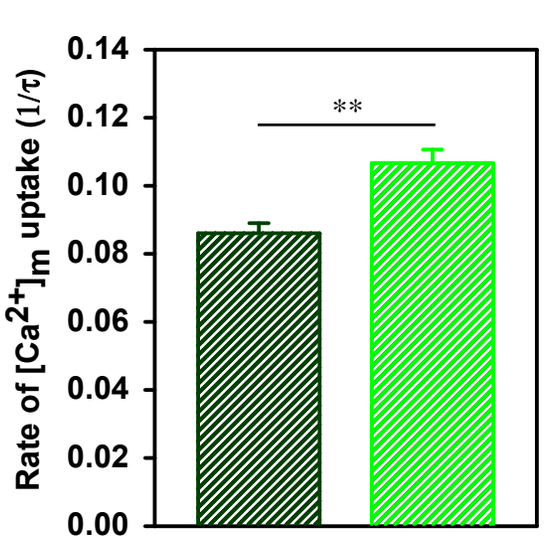

E
Control
+ LPS
Endothelial Cells

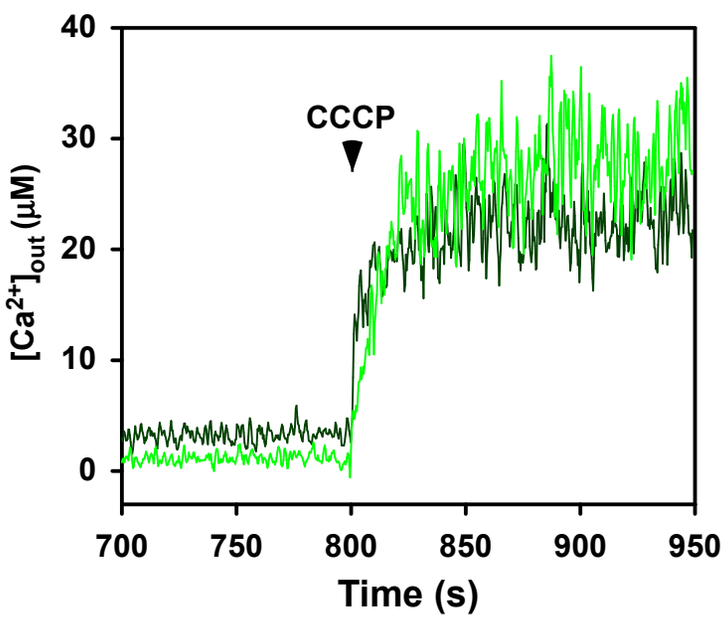
***
HyPer-dMito (f.a.u.)
Control
+ LPS

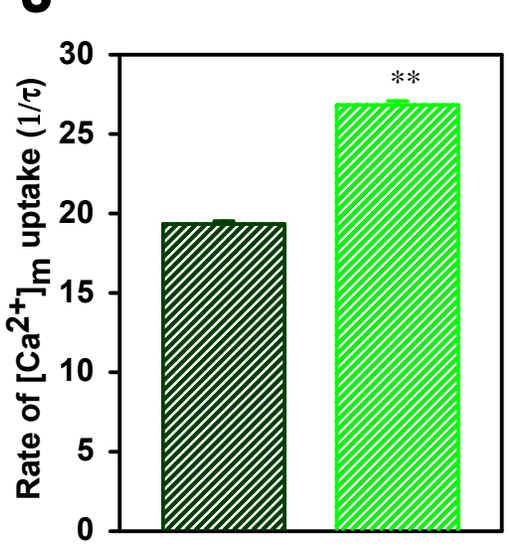

F
Normoxia
H/R
Cardiomyocytes
10 μm

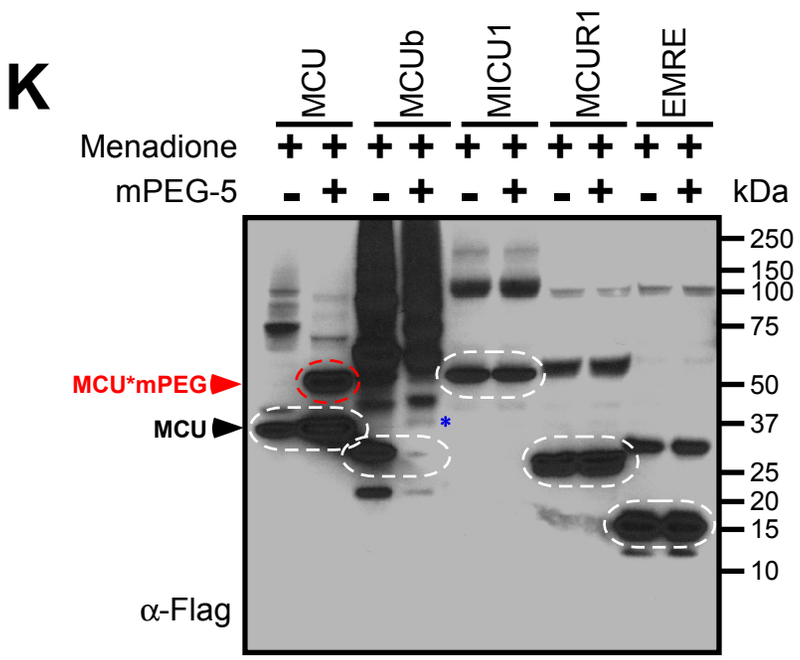
***
MitoSox (f.a.u.)
Normoxia
H/R


G
10 μM Ca2+
Normoxia
H/R
[Ca2+]out (μM)
Time (s)


H
**
Rate of [Ca2+]m uptake (1/τ)


I
CCCP
[Ca2+]out (μM)
Time (s)


J
**
Rate of [Ca2+]m uptake (1/τ)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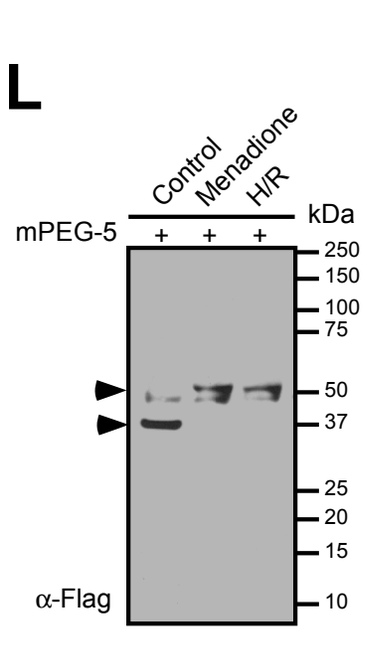
MCU
MCUb
MICU1
MCUR1
EMRE
Menadione
mPEG-5
kDa
MCU*mPEG
MCU
α-Flag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Control
Menadione
H/R
mPEG-5
kDa
α-Flag

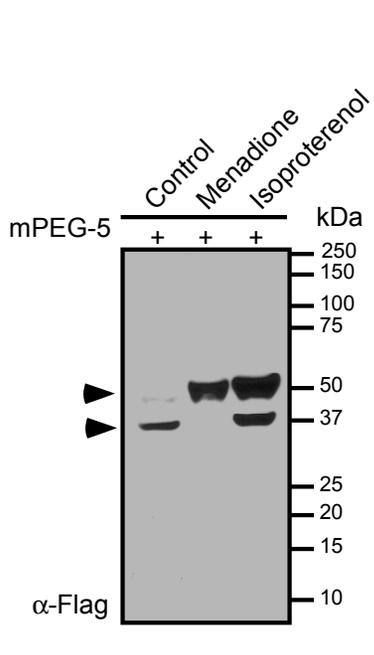
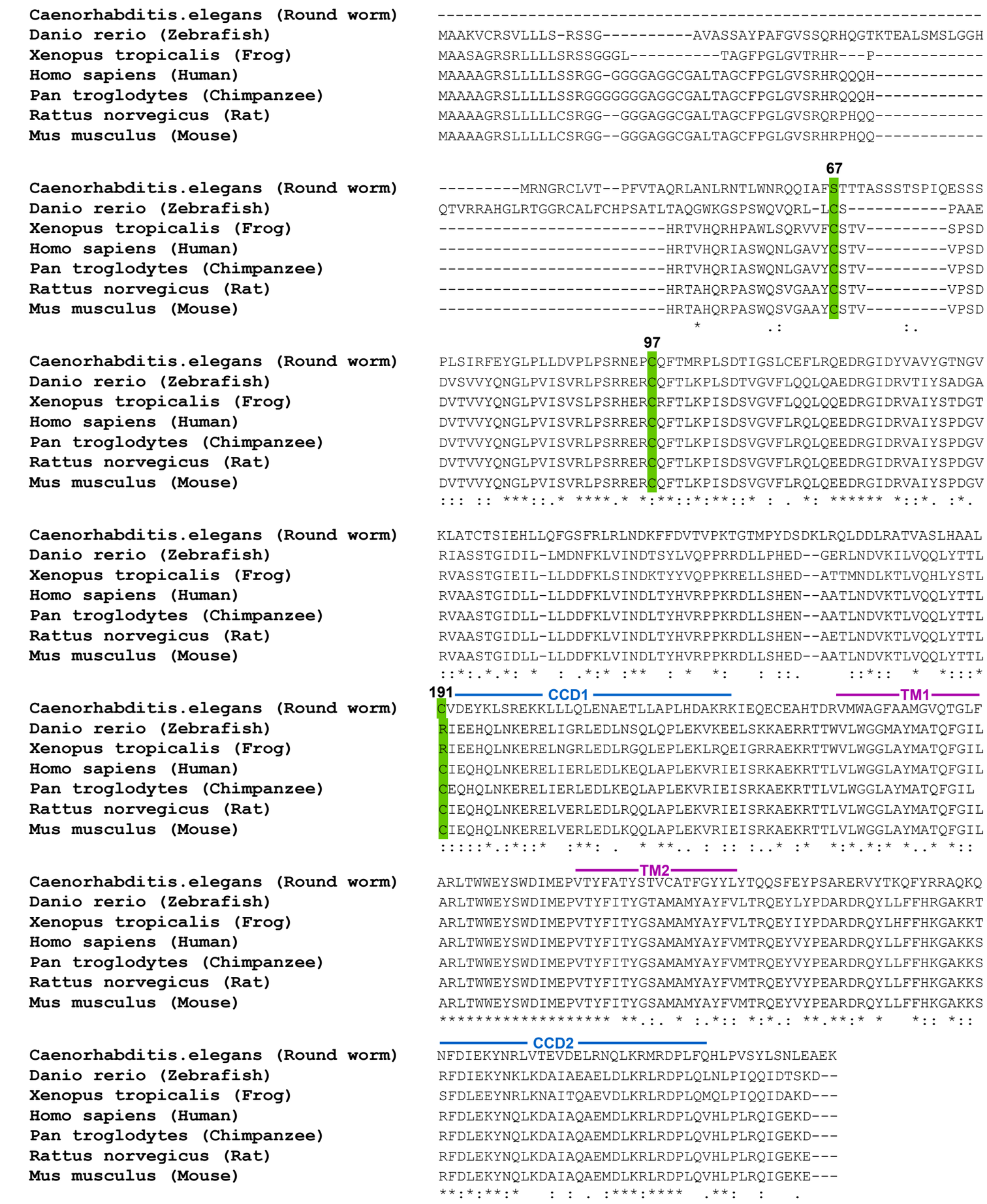

M
Control
Menadione
Isoproterenol
mPEG-5
kDa
α-Flag

Figure S2


A
Caenorhabditis.elegans (Round worm)
Danio rerio (Zebrafish)
Xenopus tropicalis (Frog)
Homo sapiens (Human)
Pan troglodytes (Chimpanzee)
Rattus norvegicus (Rat)
Mus musculus (Mouse)
MAAKVCRSVLLLS-RSSG----------AVASSAYPAFGVSSQRHQGTKTEALSMSLGGH
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67
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----------HRTVHQRHPAWLSQRVVFCSTV---------SPSD
----------HRTVHQRIASWQNLGAVYCSTV---------VPSD
----------HRTVHQRIASWQNLGAVYCSTV---------VPSD
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----------HRTAHQRPASWQSVGAAYCSTV---------VPSD
97
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RVAASTGIDLL-LLDDFKLVINDLTYHVRPPKRDLLSHED--AATLNDVKTLVQQLYTTL
191
CCD1
TM1
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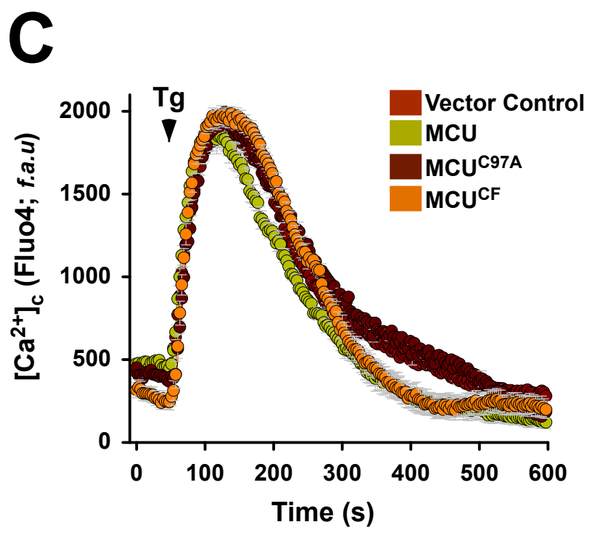
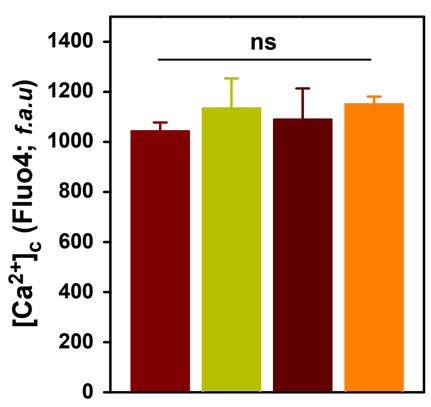
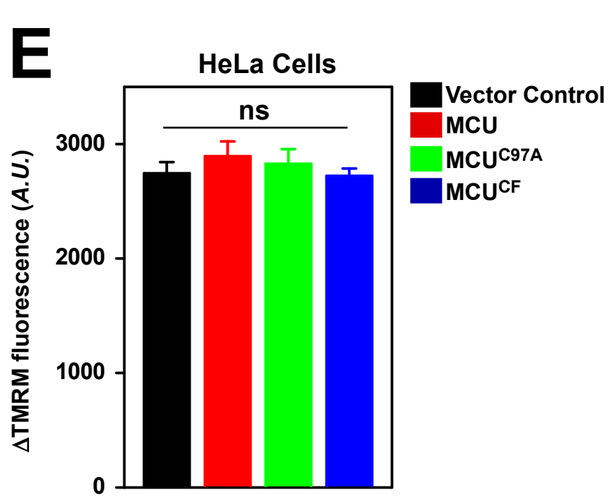
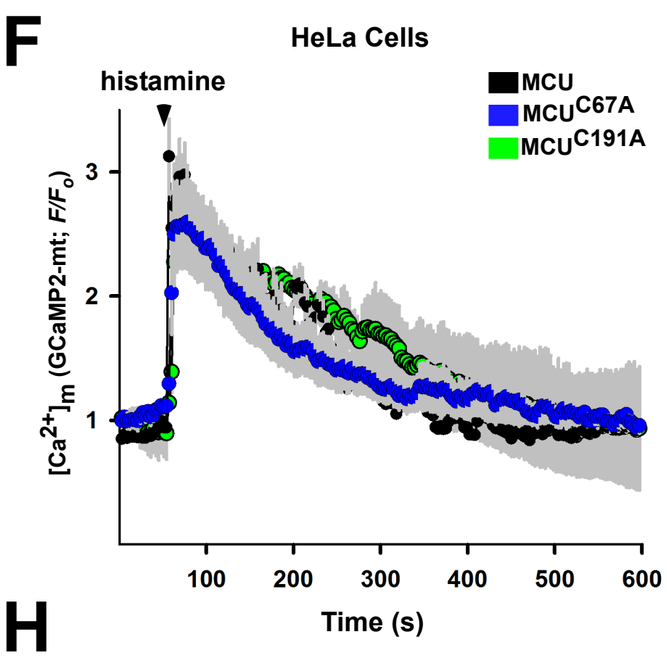
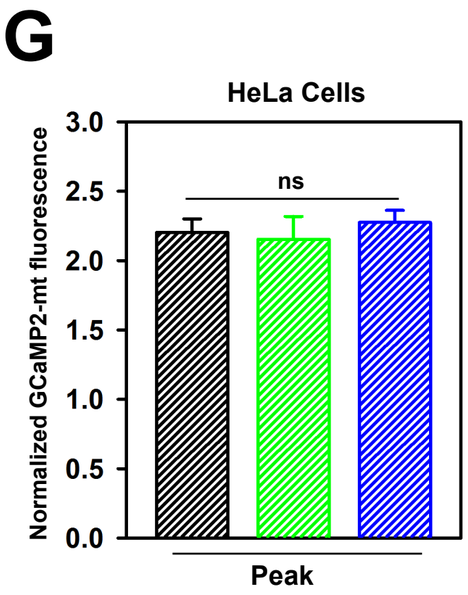
B
GCaMP2-mt
MCU-RFP
MERGE
MCU
MCUC67A
MCUC97A
MCUC191A
MCUCF
MCUC97M
20 μm

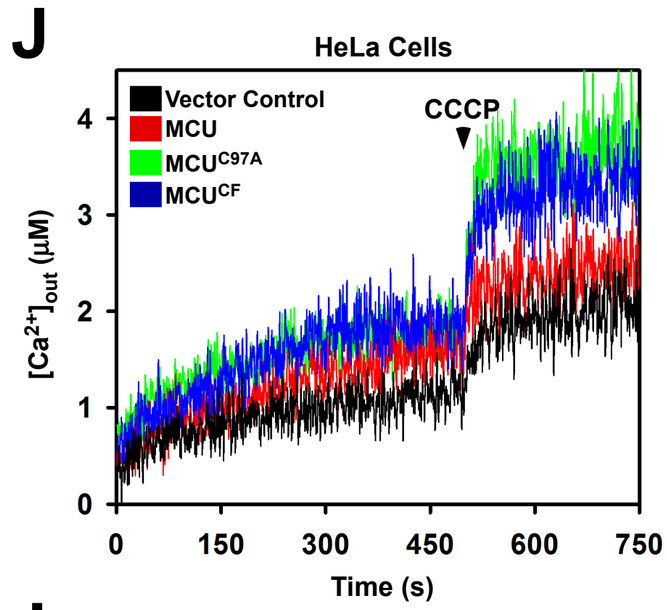

C
Tg
Vector Control
MCU
MCUC97A
MCUCF
[Ca2+]c (Fluo4; f.a.u)
Time (s)

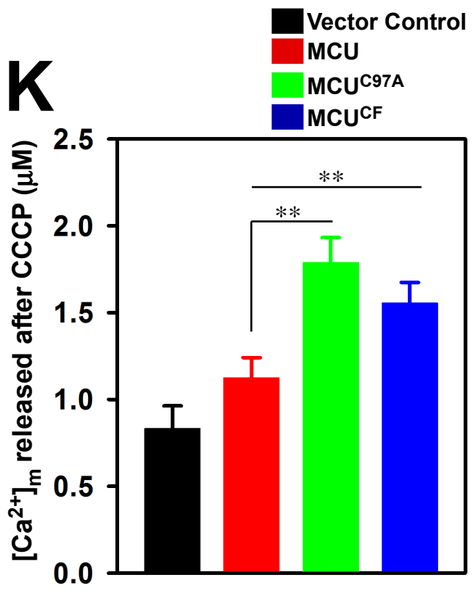

D
ns
[Ca2+]c (Fluo4; f.a.u)

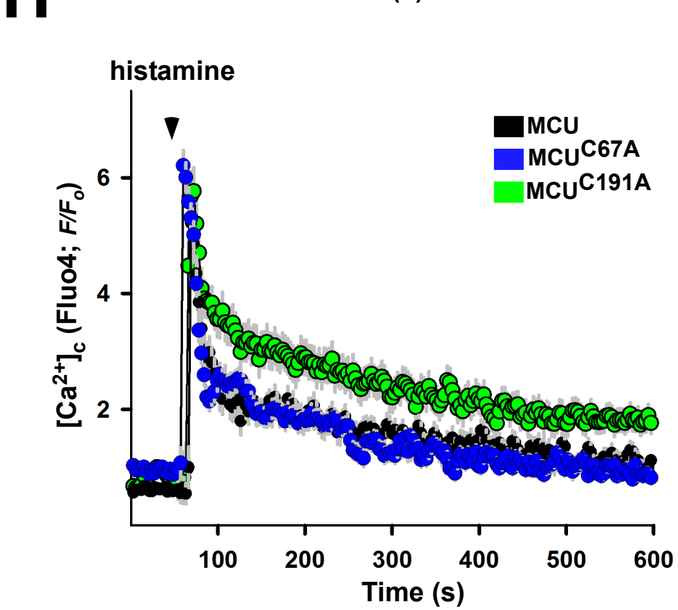

E
HeLa Cells
ns
Vector Control
MCU
MCUC97A
MCUCF
ΔTMRM fluorescence (A.U.)

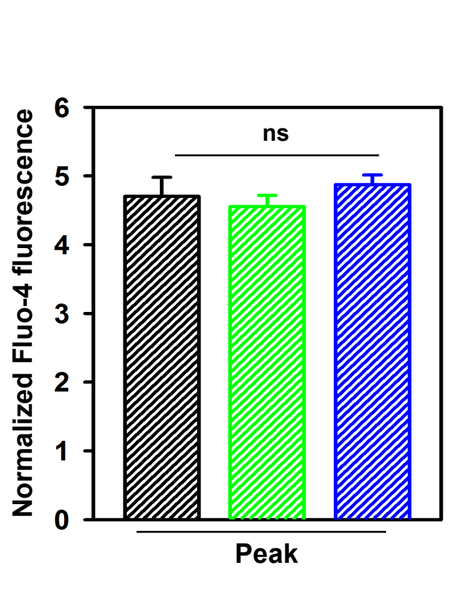

F
HeLa Cells
histamine
MCU
MCUC67A
MCUC191A
[Ca2+]m (GCaMP2-mt; F/Fo)
Time (s)

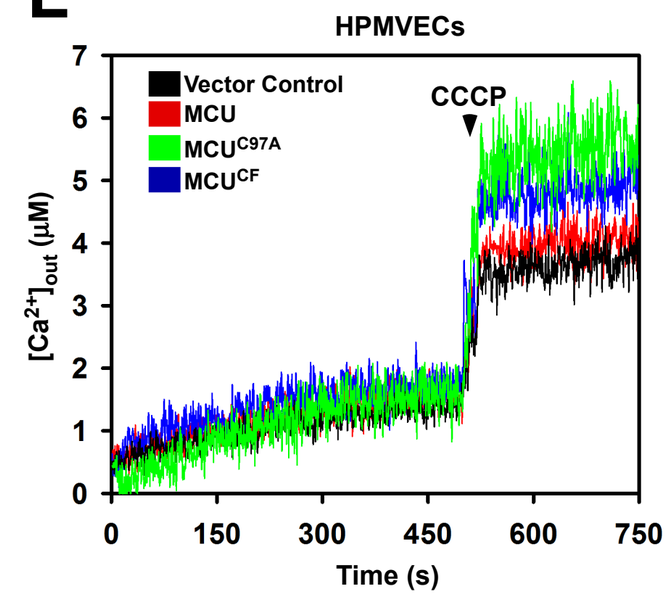

G
HeLa Cells
ns
Normalized GCaMP2-mt fluorescence
Peak


J
HeLa Cells
Vector Control
MCU
MCUC97A
MCUCF
CCCP
[Ca2+]out (μM)
Time (s)


K
Vector Control
MCU
MCUC97A
MCUCF
**
**
[Ca2+]m released after CCCP (μM)


H
histamine
MCU
MCUC67A
MCUC191A
[Ca2+]c (Fluo4; F/Fo)
Time (s)


I
ns
Normalized Fluo-4 fluorescence
Peak


L
HPMVECs
Vector Control
MCU
MCUC97A
MCUCF
CCCP
[Ca2+]out (μM)
Time (s)

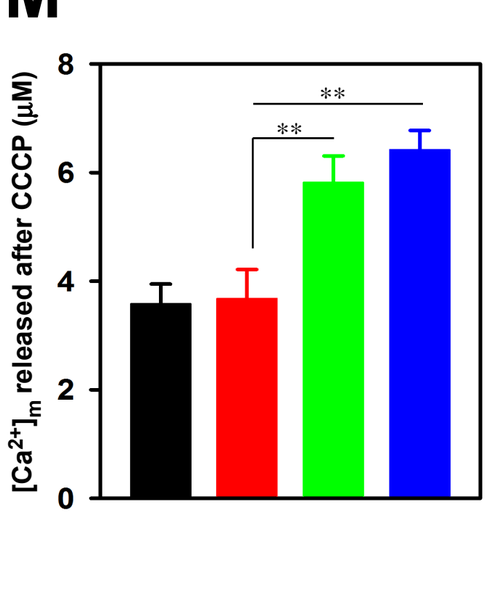
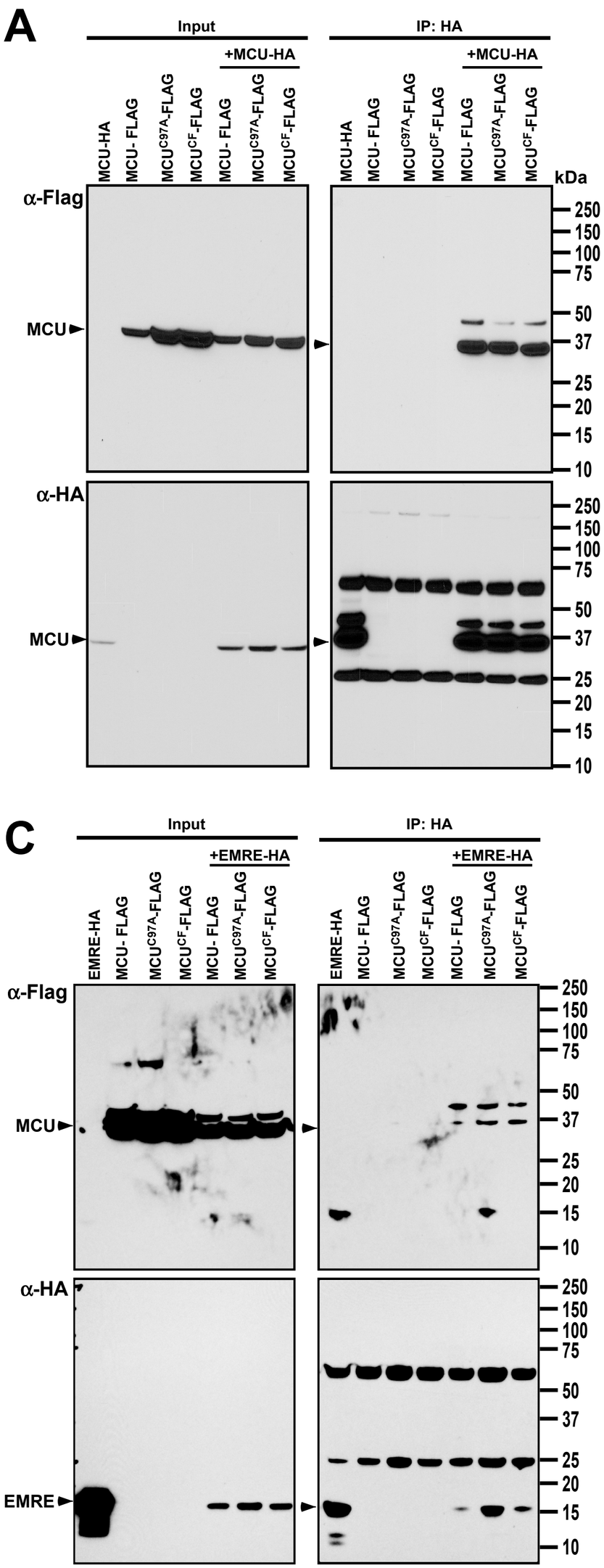
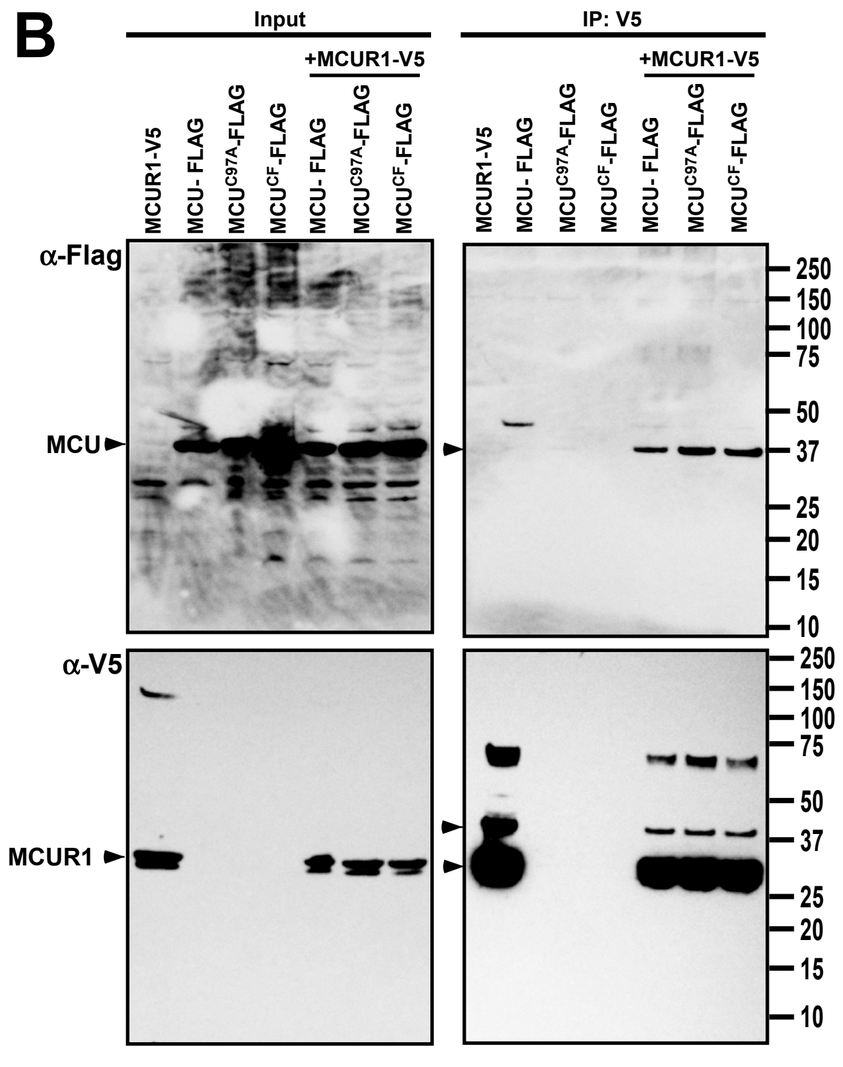
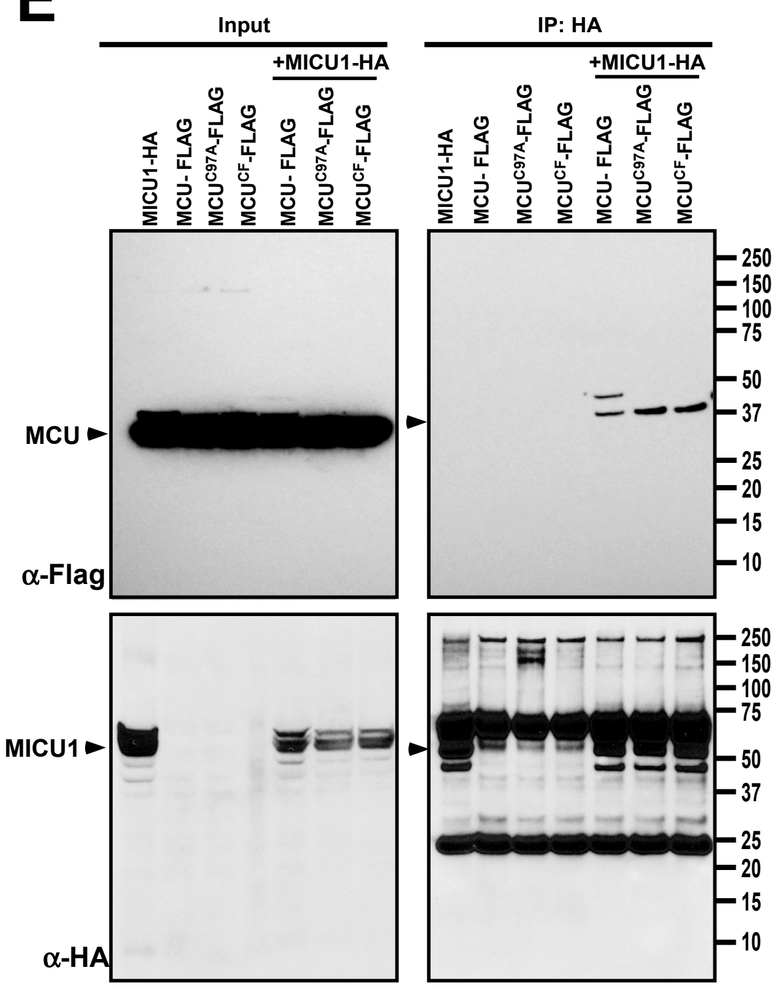

M
**
**
[Ca2+]m released after CCCP (μM)

Figure S3


A
Input
IP: HA
+MCU-HA
MCU-HA
MCU- FLAG
MCUC97A-FLAG
MCUCF-FLAG
kDa
α-Flag
MCU
α-HA
250
150
100
75
50
37
25
20
15
10


B
Input
IP: V5
+MCUR1-V5
MCUR1-V5
MCU- FLAG
MCUC97A-FLAG
MCUCF-FLAG
α-Flag
MCU
α-V5
MCUR1
250
150
100
75
50
37
25
20
15
10


E
Input
IP: HA
+MICU1-HA
MICU1-HA
MCU- FLAG
MCUC97A-FLAG
MCUCF-FLAG
MCU
α-Flag
MICU1
α-HA
250
150
100
75
50
37
25
20
15
10

C
Input
IP: HA
+EMRE-HA
EMRE-HA
MCU- FLAG
MCUC97A-FLAG
MCUCF-FLAG
α-Flag
MCU
α-HA
EMRE
250
150
100
75
50
37
25
20
15
10

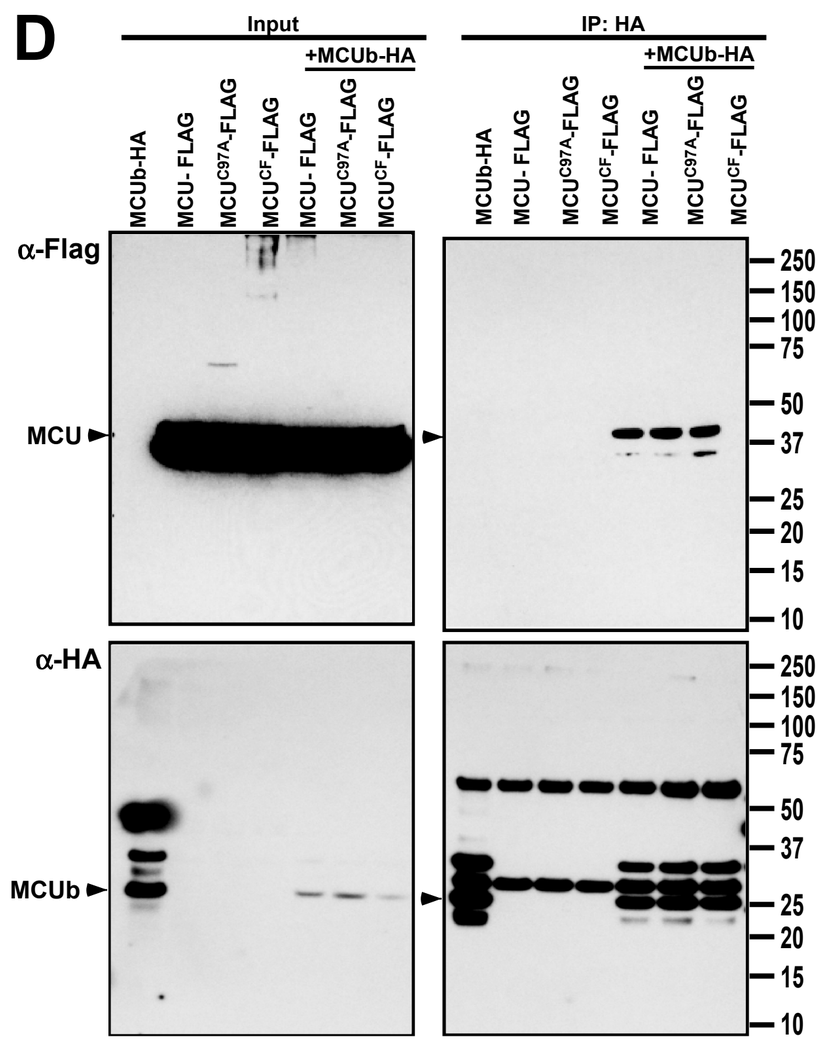
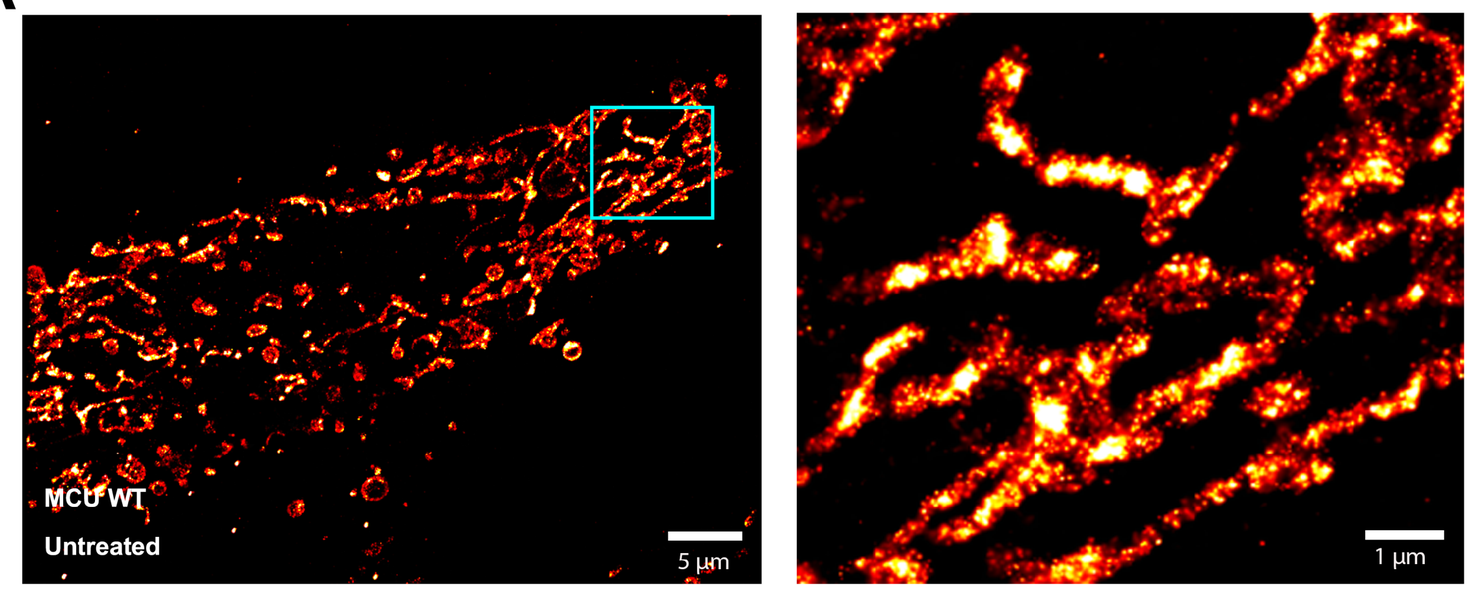
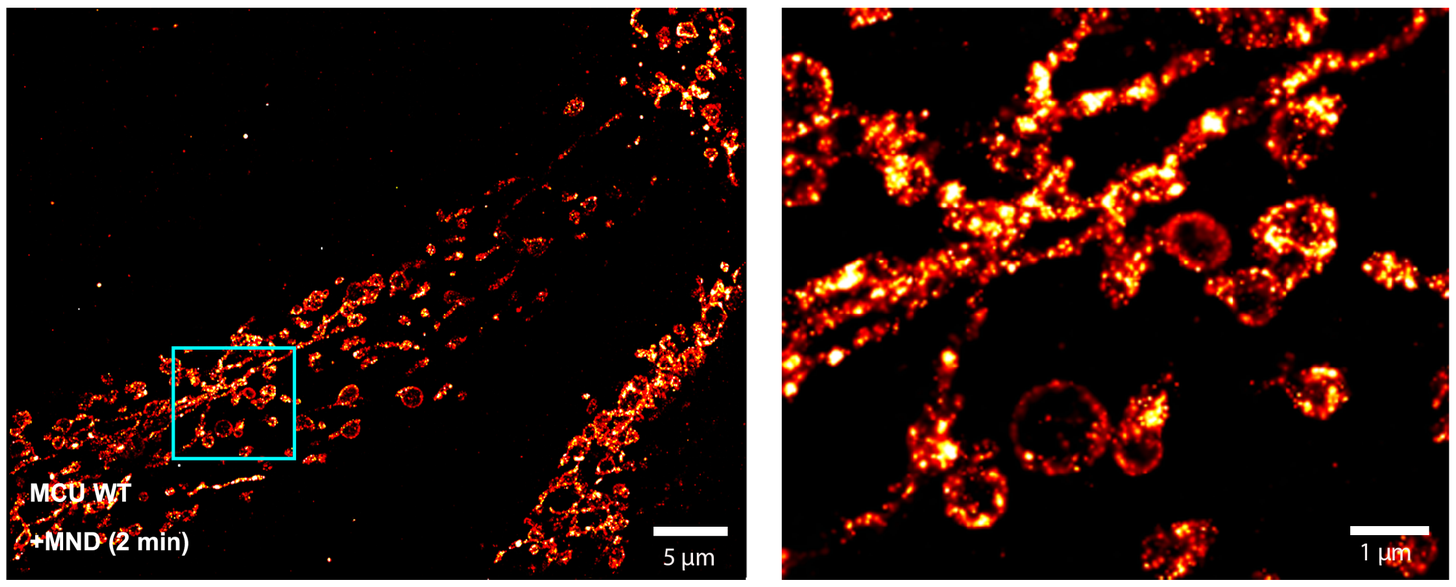
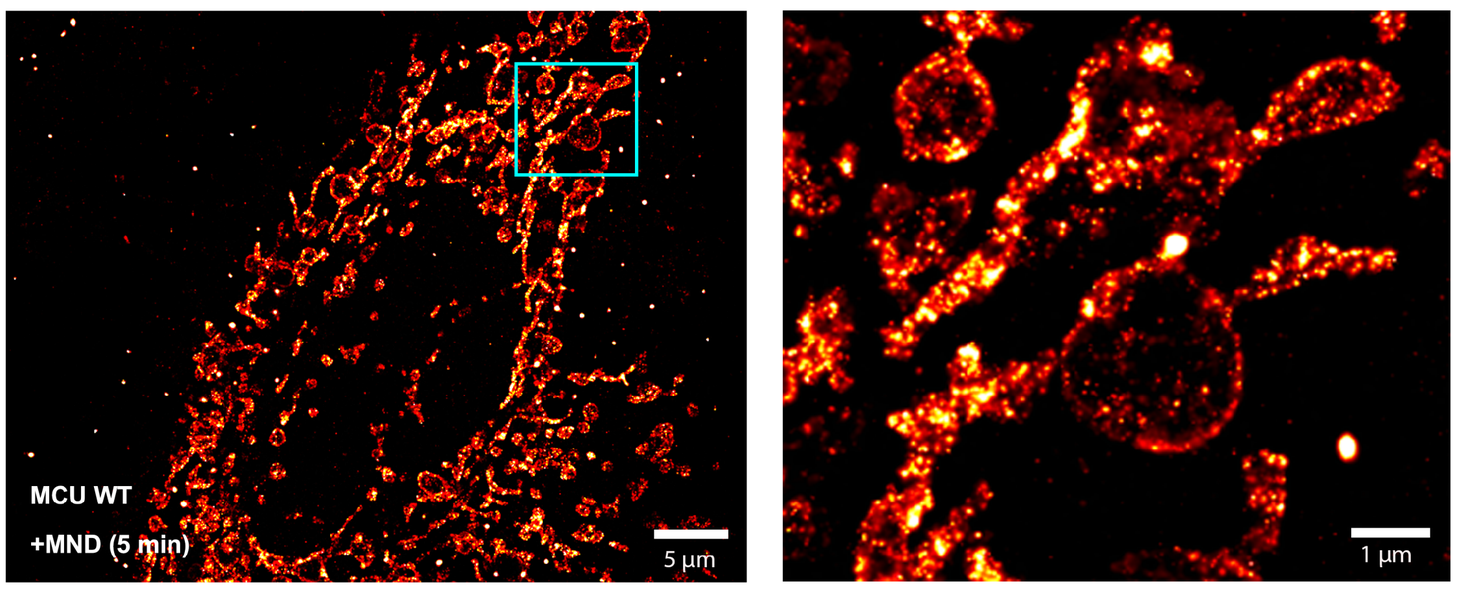

D
Input
IP: HA
+MCUb-HA
MCUb-HA
MCU- FLAG
MCUC97A-FLAG
MCUCF-FLAG
α-Flag
MCU
α-HA
MCUb
250
150
100
75
50
37
25
20
15
10

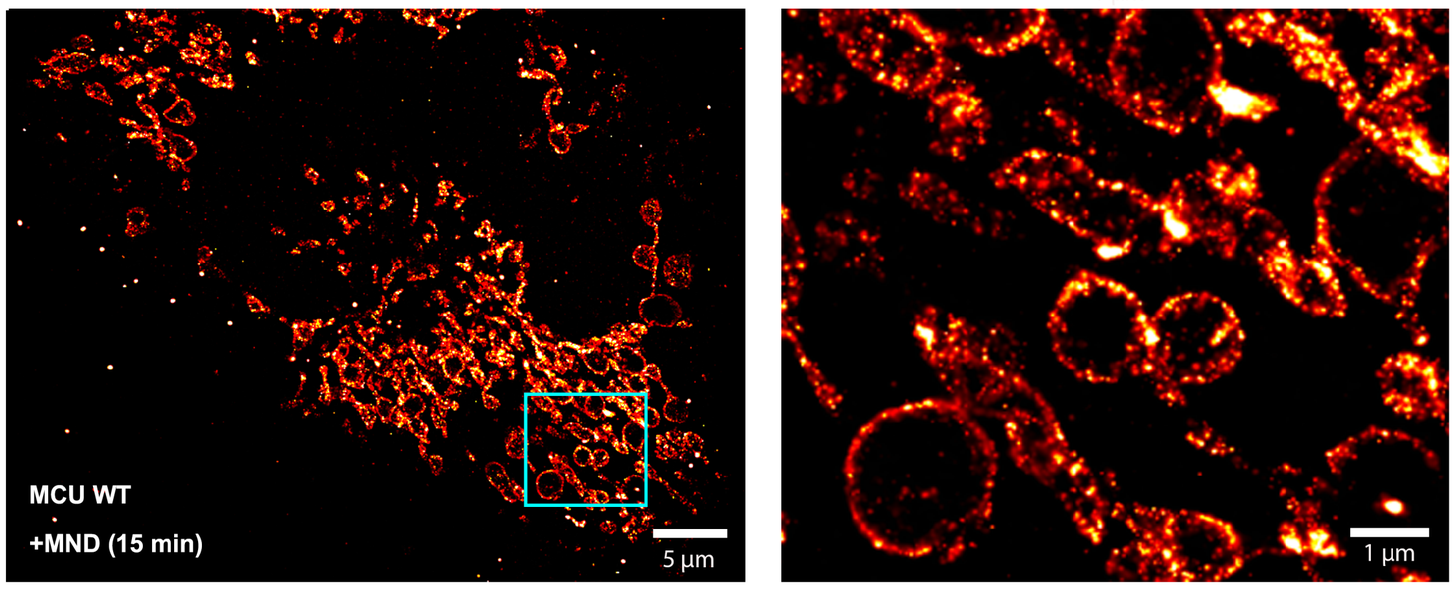
Figure S4

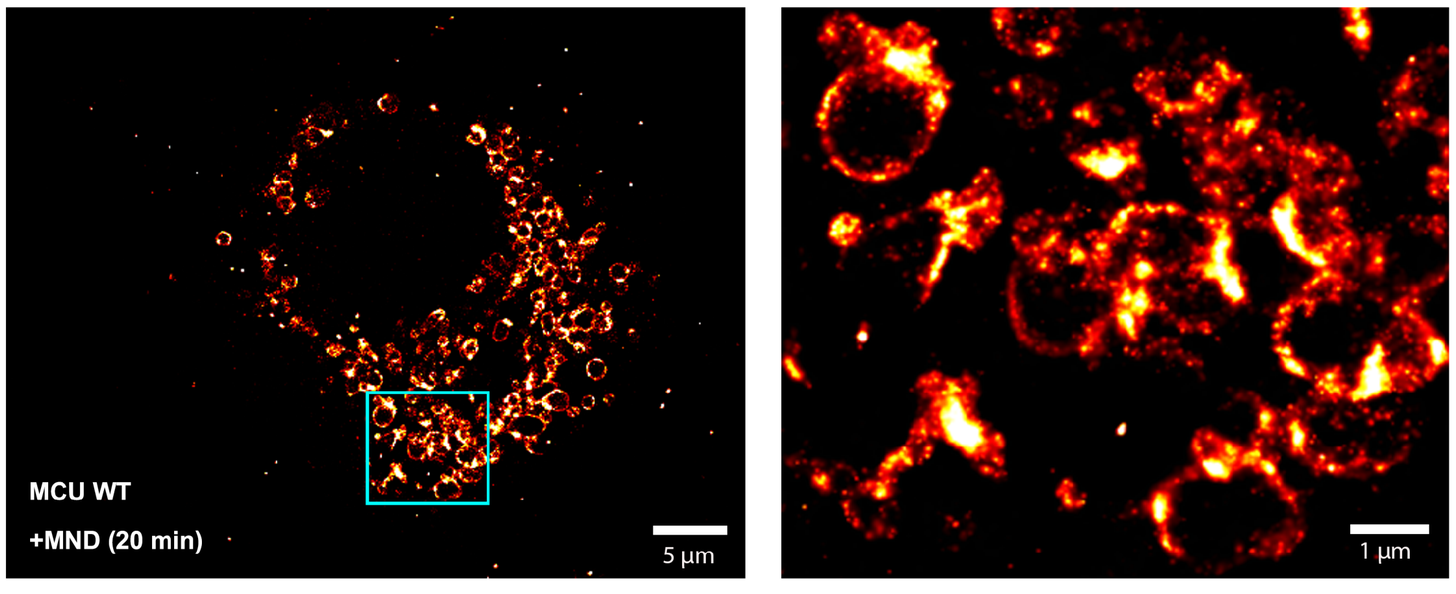
A


MCU WT
Untreated
5 µm
1 µm

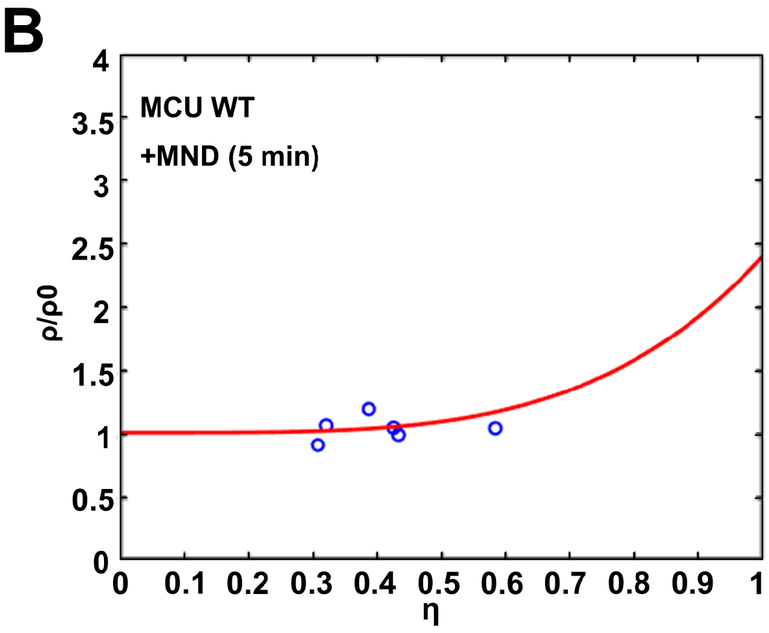

MCU WT
+MND (2 min)
5 µm
1 µm

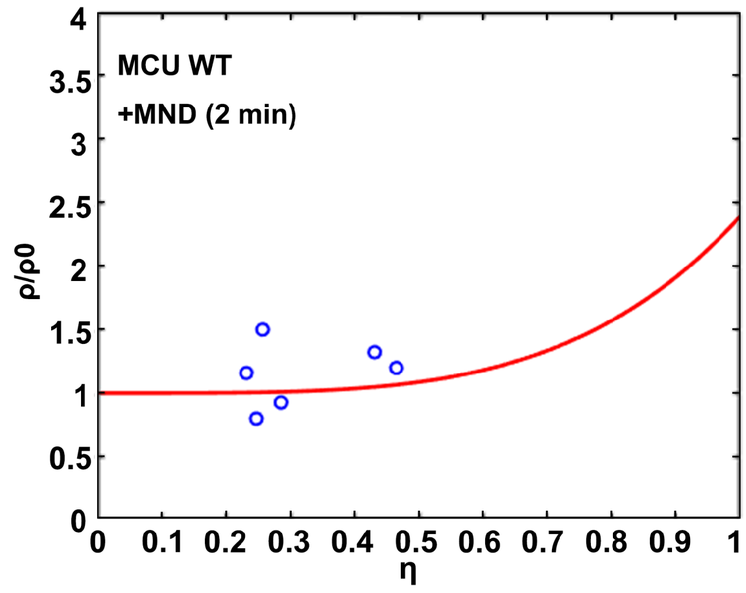

MCU WT
+MND (5 min)
5 µm
1 µm

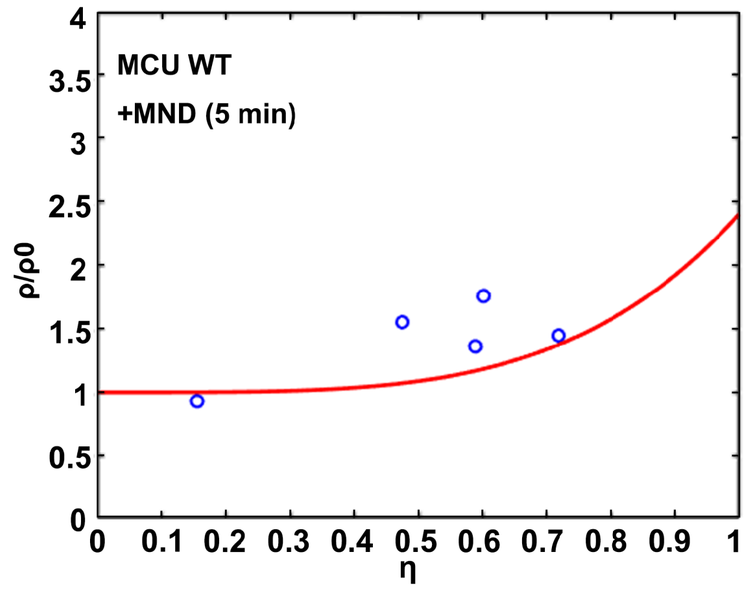

MCU WT
+MND (15 min)
5 µm
1 µm

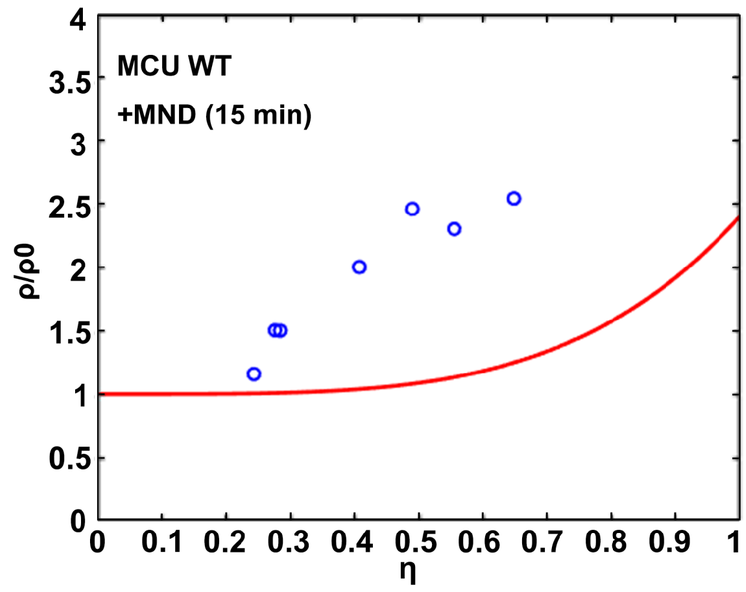

MCU WT
+MND (20 min)
5 µm
1 µm

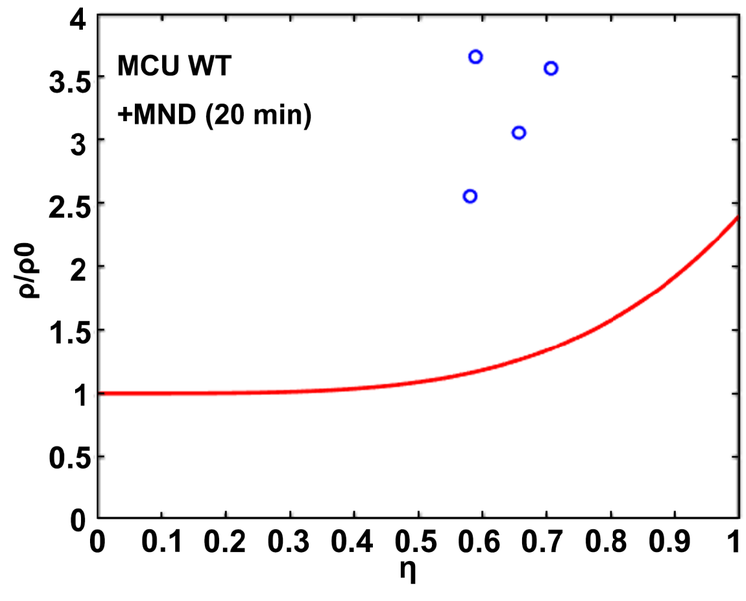
B


MCU WT
+MND (5 min)
ρ/ρ0
η
0
0.5
1
1.5
2
2.5
3
3.5
4
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9


MCU WT
+MND (2 min)
ρ/ρ0
η


MCU WT
+MND (5 min)
ρ/ρ0
η


MCU WT
+MND (15 min)
ρ/ρ0
η


MCU WT
+MND (20 min)
ρ/ρ0
η