SI Appendix

Contact-ID, a tool for profiling organelle-membrane contact sites, reveals regulatory proteins of mitochondrial-associated membrane formation

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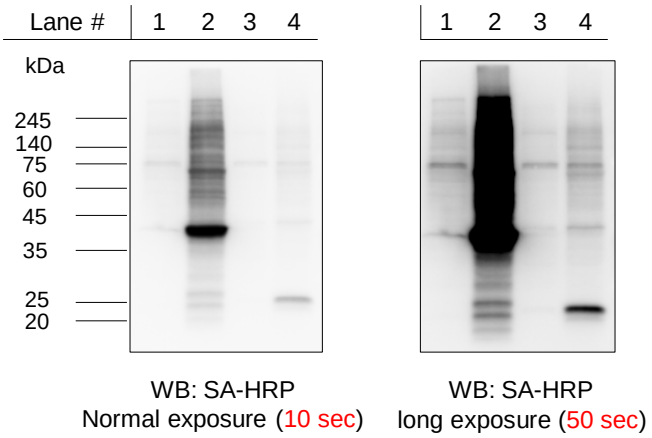
Supplementary Table 1. Construct Information

Name (expected size)	Features	Promotor/ Vector	Details
FKBP-split C-term pBirA fragment (Q65, G79, G257, S263, E284 -C)-HA	*NotI*-FKBP-*BamHI*-pBirA(Q65, G79, G257, S263, E284 -C)-HA-Stop-*XhoI*	CMV/ pCDNA3	pCDNA3.1 MCS-BirA(R118G) (= promiscuous biotin ligase, pBirA or BioID)-HA was a gift from Kyle Roux (Addgene plasmid #36047). FKBP and FRB a gift from Prof. Takanari Inoue (Johns Hopkins University, Baltimore, MD).
Flag-split N-term pBirA fragment (N-I64, G78, E256, L262, K283)-FRB	*NotI*-FLAG-pBirA (N-I64, G78, E256, L262, K283)-*BamHI*-FRB-Stop-*XhoI*	CMV/ pCDNA3	
Flag-split N-term pBirA fragment (N-G78, or N-E256)-SEC61B	*NotI*-Flag-pBirA(N-G78 or N-E256)-linker-*EcoRI*-SEC61B-Stop-*XhoI*	CMV/ pCDNA5	linker: GGASGGSGSGPVAT SEC61B (NM_006808)
TOM20-split C-term pBirA fragment (G79-C or G257-C)-HA	*KpnI*-TOM20-*BamHI*-linker-pBirA (G79-C or G257-C)-HA-Stop-*NotI*	CMV/ pCDNA5	Linker: SGGSGGSR Tom20 (NM_014765.2)
APEX2-V5-FKBP8	*HindIII*-APEX2-*KpnI*-V5-*NotI*-FKBP8-Stop-*AscI*	CMV/ pCDNA5	for APEX-EM imaging of FKBP8 expression
Mito-V5-APEX2 (Processed: 30 kDa)	*KpnI*-Mito-*BamHI*-*NheI*-V5-APEX2-Stop-*NotI*	CMV/ pCDNA5	Mito-V5-APEX2 was a gift from Prof. Alice Ting (Addgene plasmid #72480)
SCO1-V5-APEX2 (Processed: 62 kDa)	*KpnI*-SCO1-*V5*-APEX2-Stop-*NotI*	CMV/ pCDNA5	IMS marker protein for EM imaging
mcherry-Flag-KDEL (Processed: 31 kDa)	*Igk chain signal sequence-Apa1-mcherry-flag-KDEL-Stop-Not1*	CMV/ pDisplay	Red fluorescent ER marker protein for optical imaging
mCherry-BioID-HA-FKBP8 (expected MW: 108 kDa)	*HindIII*-mCherry-*BamHI*-HA-*NotI*-FKBP8-Stop-*AscI*	CMV/ pCDNA5	FKBP8-BioID (for FKBP8 interactome mapping experiment)
mCherry-BioID (full pBirA) (expected MW: 64 kDa)	*HindIII-mcherry-pBirA-Stop-Xho1*	CMV/ pCDNA5	Cytoplasm-localized BioID (for control experiment)

Figures S1-17

a

N-term fragment (flag)-FRB	B1	B1	N-E256	N-E256
C-term Fragment (HA)-FKBP	B2	B2	G257-C	G257-C
Rapamycin	-	+	-	+
biotin	+	+	+	+
Lane #	1	2	3	4



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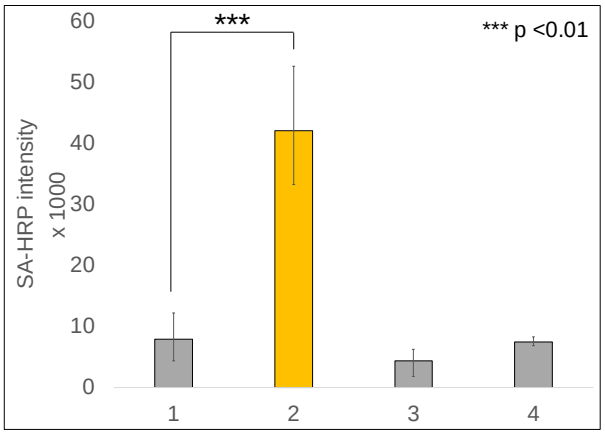

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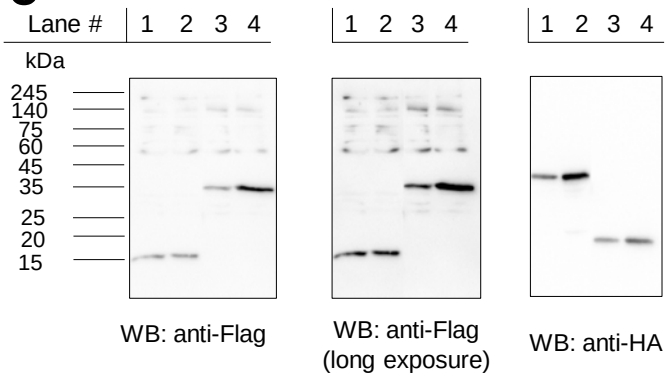
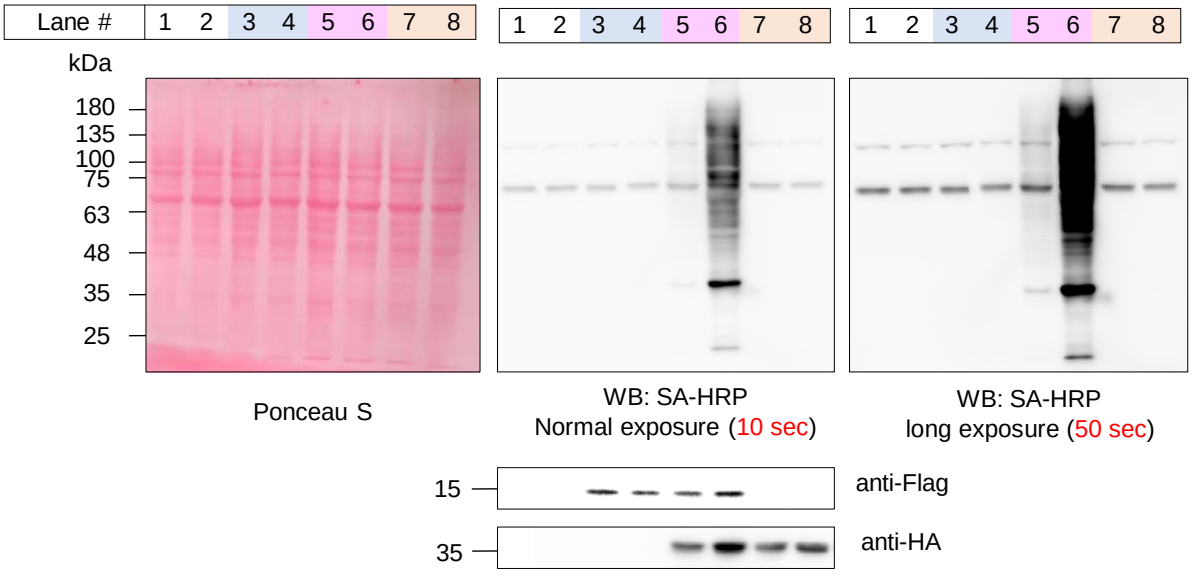


d

Flag-B1(N-G78)-FRB	-	-	+	+	+	+	-	-
FKBP12-HA-B2 (G79-C)	-	-	-	-	+	+	+	+
Rapamycin	-	+	-	+	-	+	-	+
biotin	+	+	+	+	+	+	+	+
Lane #	1	2	3	4	5	6	7	8



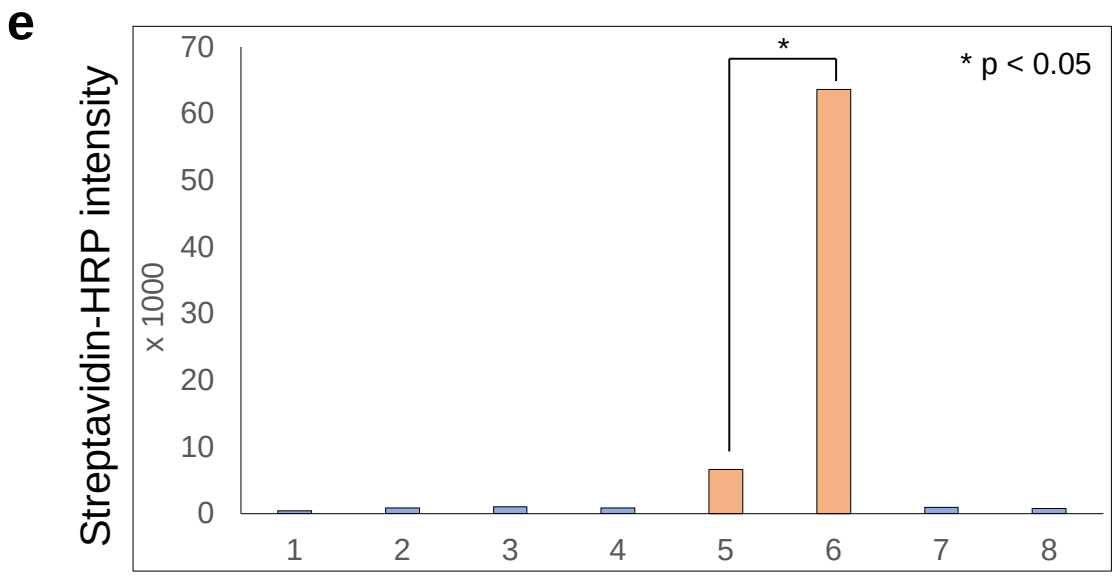


Figure S1. Comparison of the biotinylating activity of the B1/B2 pair (Contact-ID) and previously reported split-BioID pair (N-E256/G257-C)(1) in the FKBP-FRB system with or without rapamycin treatment.

(a) Streptavidin-HRP (SA-HRP) immunoblot analysis of biotinylated proteins from split-BioID pairs. For rapamycin control, rapamycin (100nM) was treated for 16 h with biotin (50uM). HEK293T cells were used for this assay

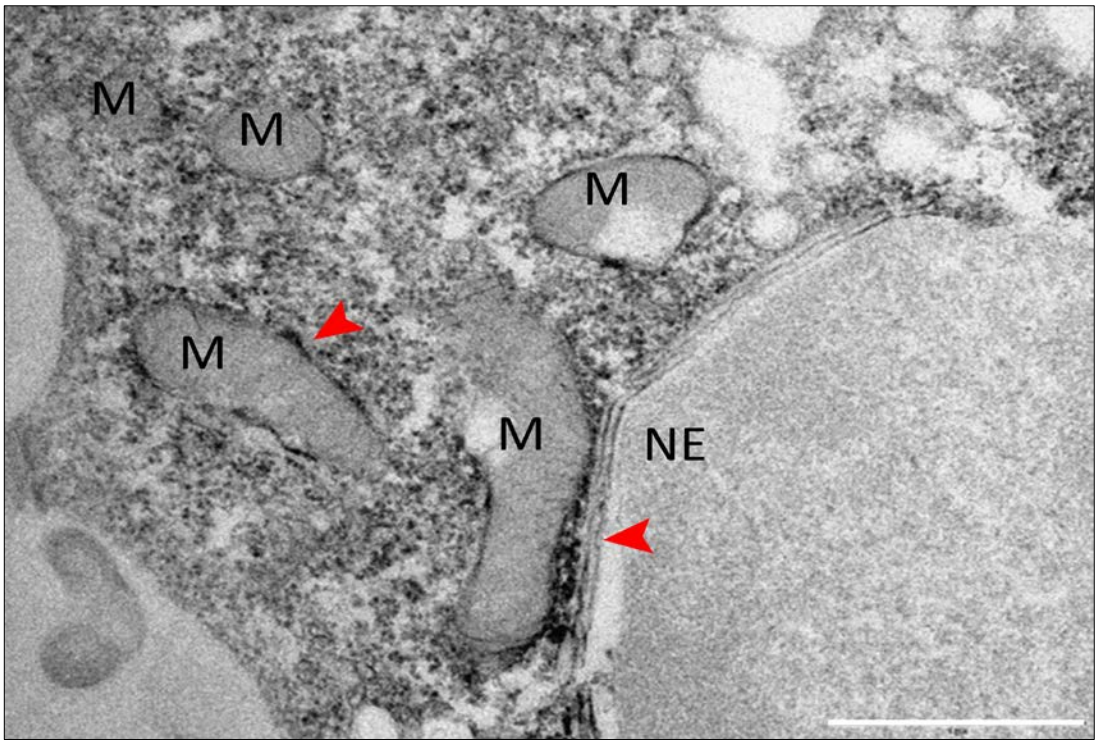
(b) Statistical analysis of the SA-HRP signal intensity of triplicate experiments of (a); ***$p < 0.01$.

(c) Anti-Flag and anti-HA immunoblots represent the expression level of each split fragment in the same sample.

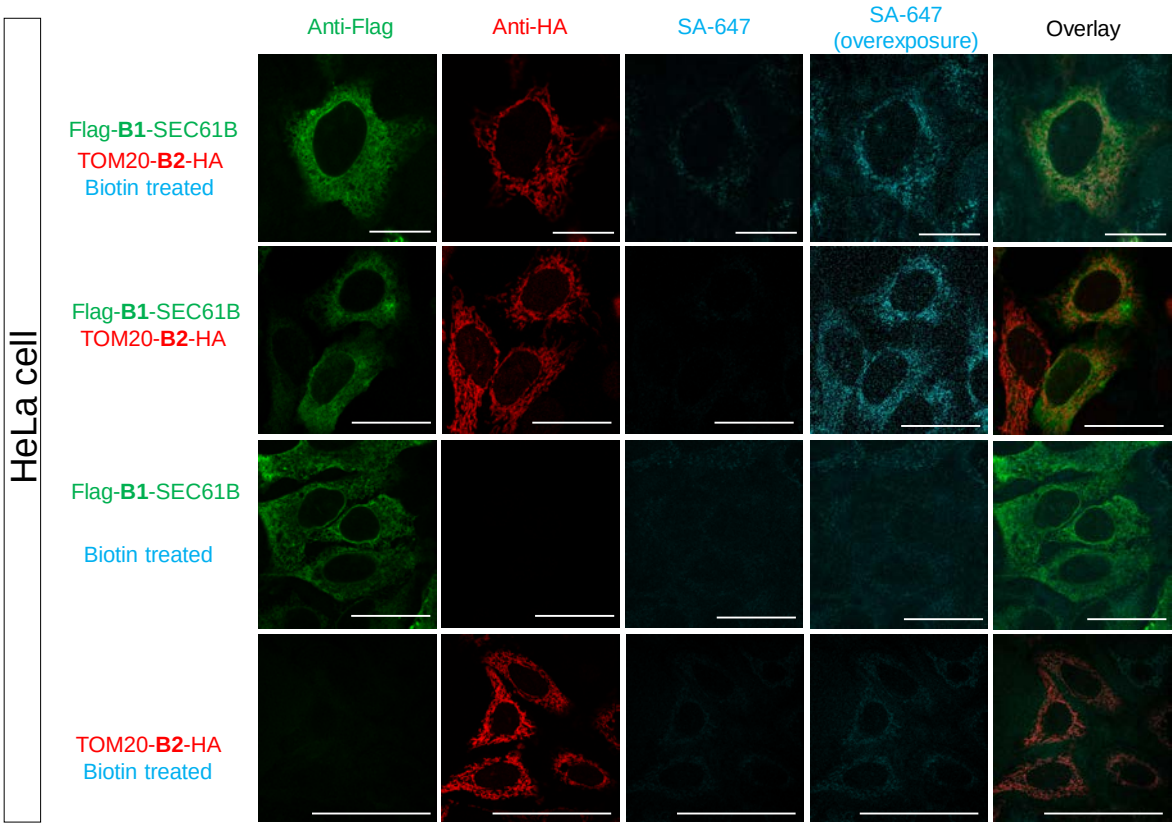
(d) SA-HRP, anti-Flag, and anti-HA immunoblot analysis of each split fragment at all with or without treatment of rapamycin from our Contact-ID pair.

(e) Statistical analysis of the SA-HRP signal intensity of triplicate experiments of (d); *$p < 0.05$.

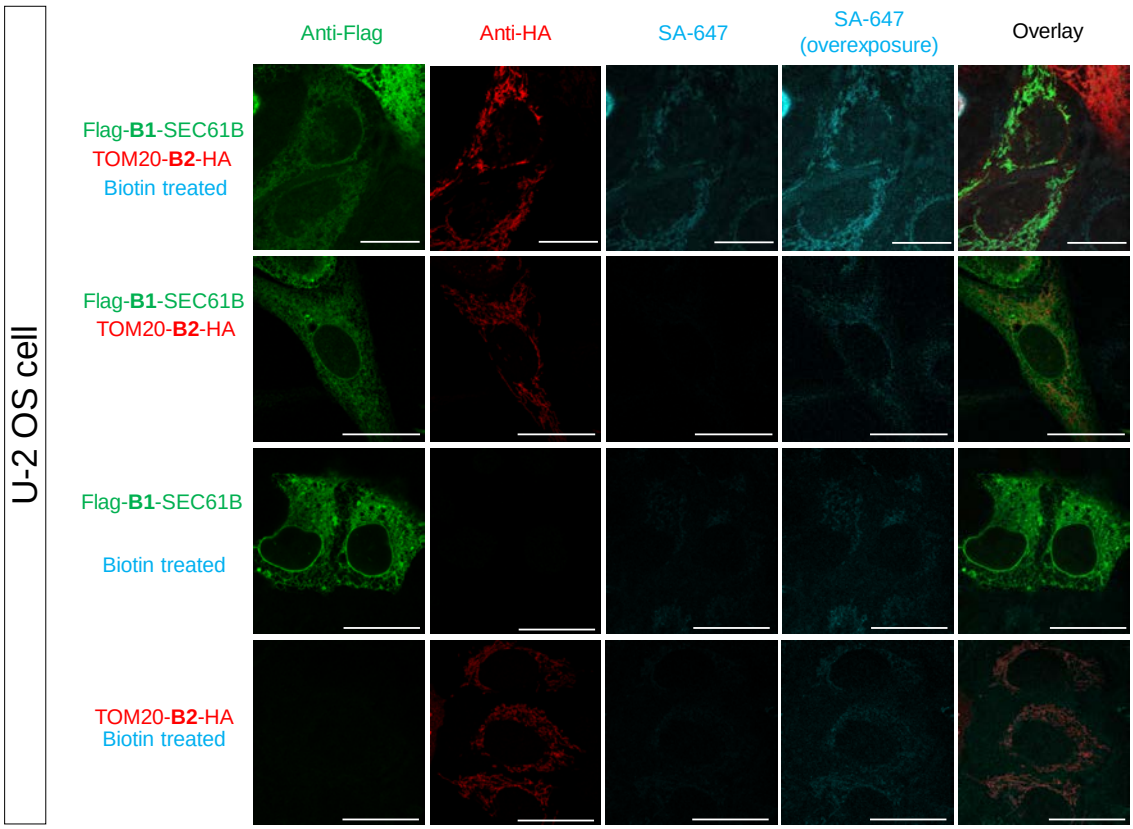
a


M
M
M
M
M
NE

b


HeLa cell
Anti-Flag
Anti-HA
SA-647
SA-647 (overexposure)
Overlay
Flag-B1-SEC61B
TOM20-B2-HA
Biotin treated
Flag-B1-SEC61B
TOM20-B2-HA
Flag-B1-SEC61B
Biotin treated
TOM20-B2-HA
Biotin treated

c (continued)



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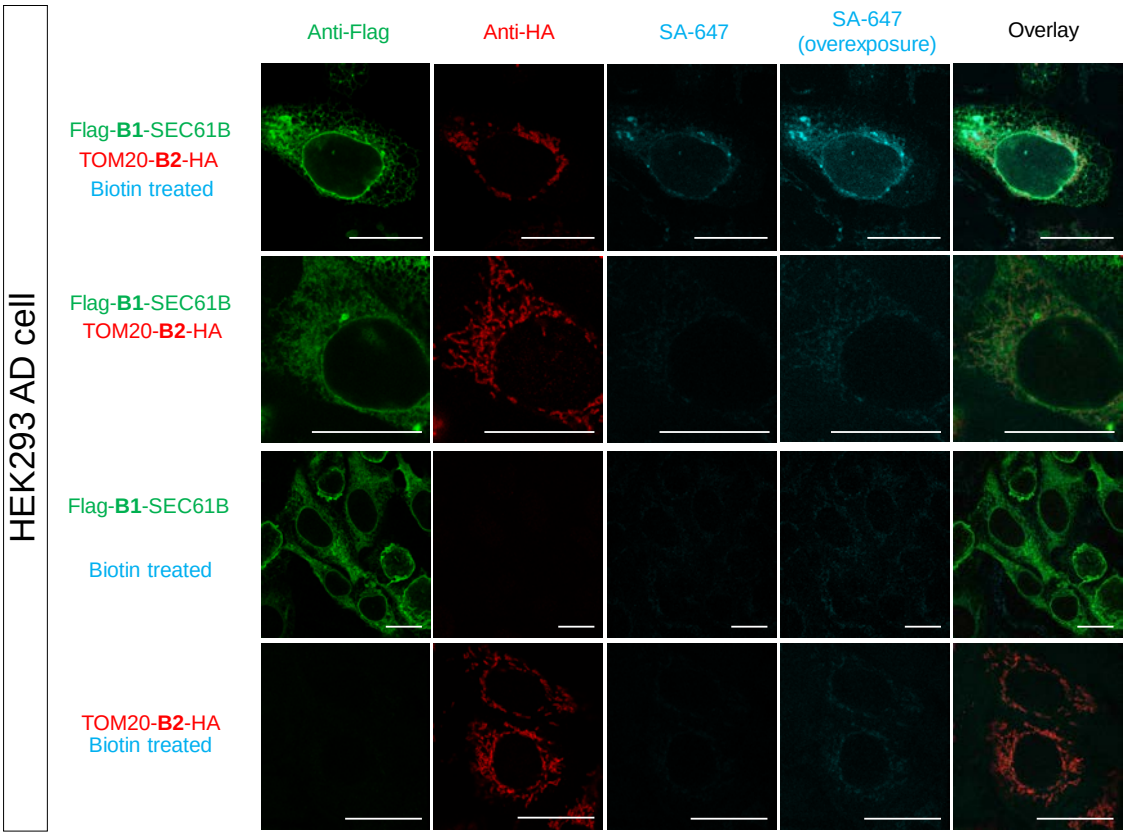

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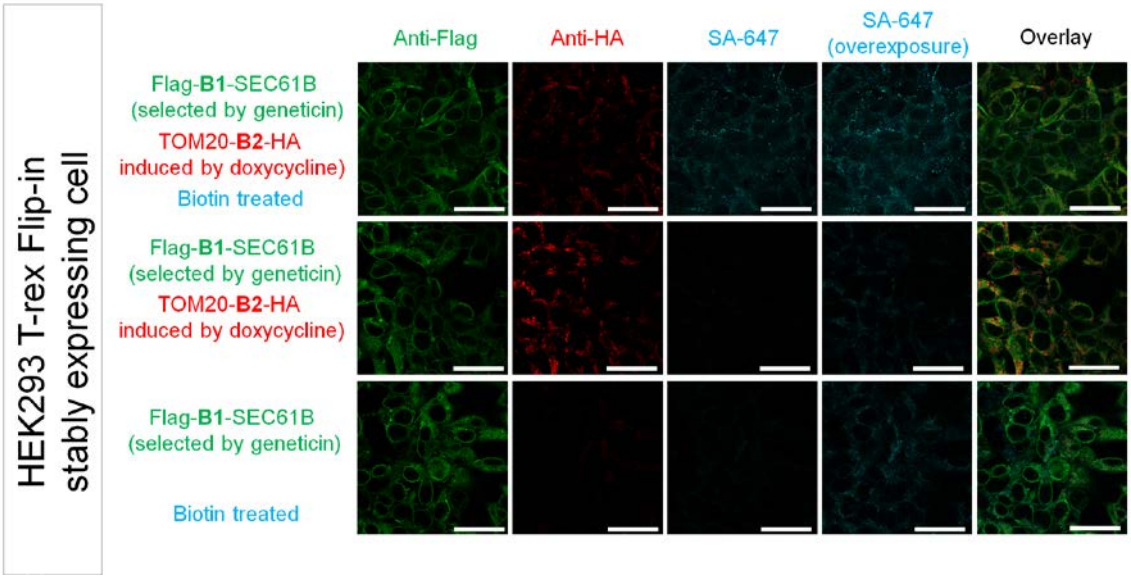
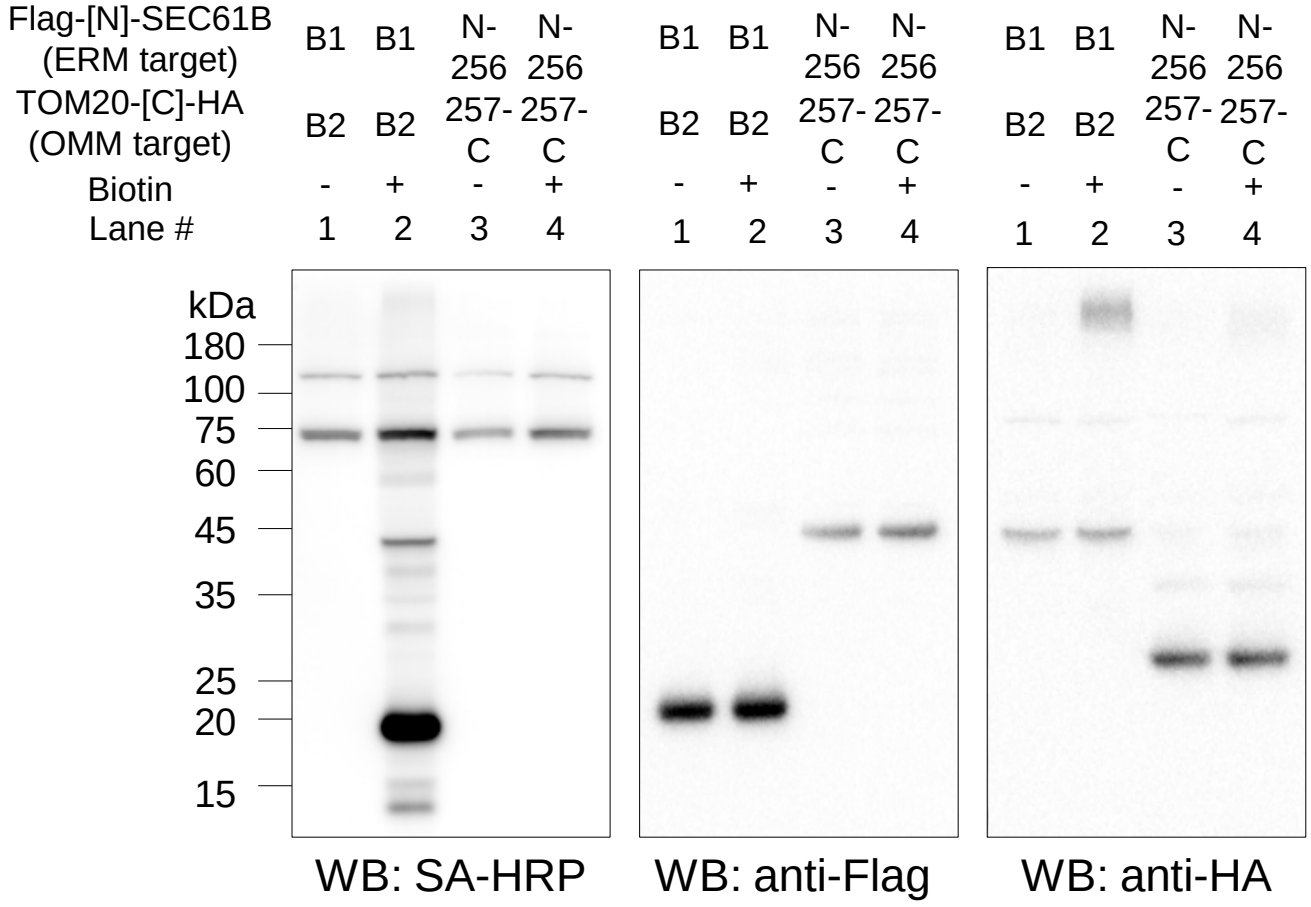


Figure S2. Transmission electron microscopy (TEM) imaging of biotinylated proteins by Contact-ID (Flag-B1-SEC61B: TOM20-B2-HA) at the MAM.

(a) Streptavidin-HRP was used for generation of the diaminobenzidine/OsO_4 stain at the biotinylated protein accumulation region (red arrows) in fixed and permeabilized cells. Detailed procedures are described in the Supplementary Information. “M” indicates mitochodnria and “NE” indicates nuclear envelope. Scale bar = 2 μm.

(b-e) Confocal microscopy imaging of MAM biotinylation by Contact-ID in HeLa (b, Scale bar: 20 μm), U-2 OS (c, Scale bar: 20 μm), HEK293AD (d, Scale bar: 15 μm) and HEK293T-Rex (e, Scale bar: 23 μm). Flag-B1-SEC61B was visualized by anti-Flag antibody (AF488-conjugated, green fluorescence channel) and TOM20-B2-HA was visualized by anti-HA antibody (AF568-conjguated, red fluorescence channel). Biotinylated proteins were visualized by AF647-conjugated streptavidin (Cy5 fluorescence channel).

a



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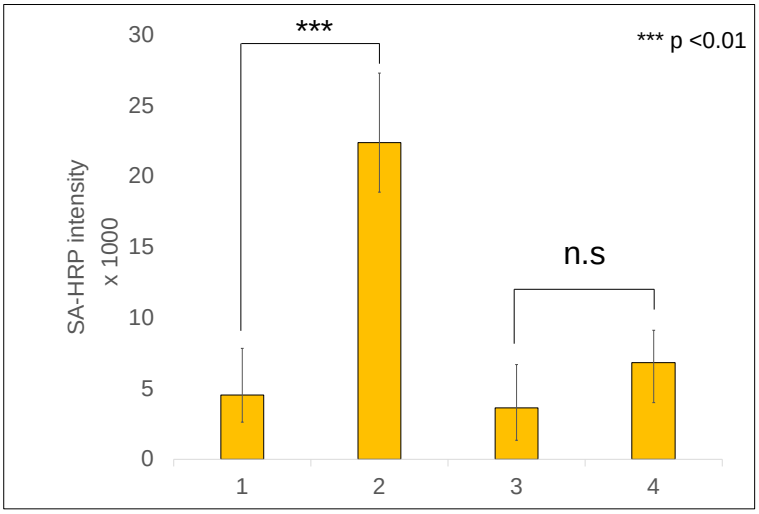


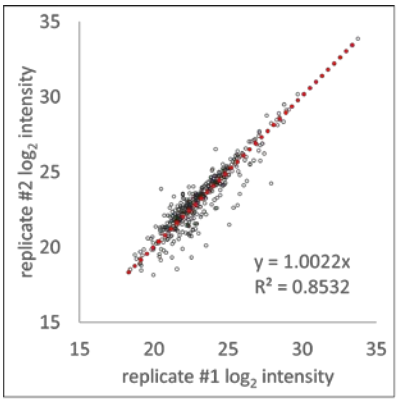
Figure S3. Comparison of the biotinylating activity of the B1/B2 pair (Contact-ID) and a previously reported split-BioID pair (N-E256/G257-C)(1) at the MAM.

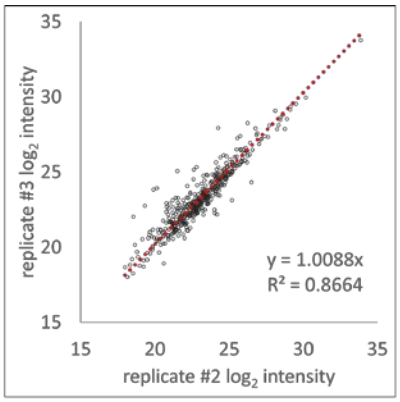
(a) SA-HRP immunoblot for biotinylated proteins from split-BioID pairs at the MAM. Anti-Flag and anti-HA immunoblots represent the expression level of each split fragment in the same sample.

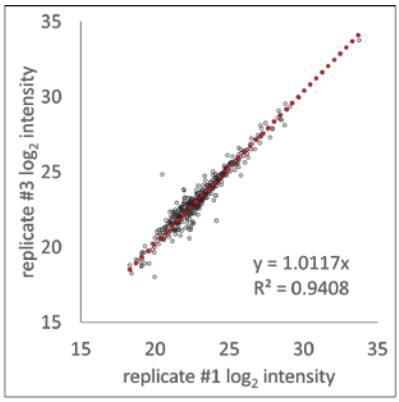
(b) Statistical analysis of the SA-HRP signal intensity of triplicate experiments of (a); ***p < 0.01.

a

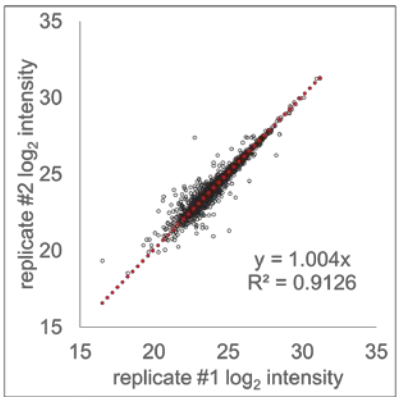
Contact-ID

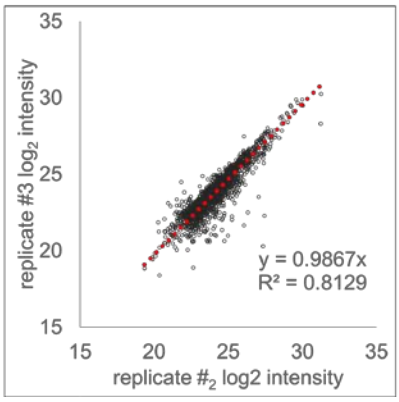


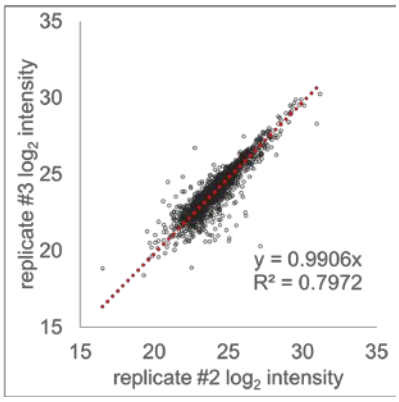




Cytosol-BioID (mcherry-BioID)







b

	Pre-enrichment (Rep 1)	Pre-enrichment (Rep 2)	Pre-enrichment (Rep 3)	Post-enrichment (Rep1)	Post-enrichment (Rep2)	Post-enrichment (Rep3)
Total PSM #	9438	9924	9870	1247	969	1116
Biotinylated PSM #	0	0	1	148	128	158
Enrichment Efficiency (%)	0%	0%	0%	12%	13%	14%
Total Peptide #	5185	5431	5339	834	663	770
Biotinylated peptide #	0	0	1	59	60	60

Figure S4. Data correlation between triplicate mass analysis results of biotinylated proteins by Contact-ID and mCherry-BioID (cytosolic control) in mammalian cells (HEK293).

(a) Pearson correlation between triplicate datasets from three biological replicates. The R^2 values were separately calculated for proteins in the upper 75% and lower 25% abundance percentiles.

(b) Mass identification result of pre- and post-streptavidin enrichment samples of Contact-ID. Significant enrichment of biotinylated peptides of stably expressed Contact-ID cell line (Flp-in HEK293T-Rex) is shown in the post-enriched sample of the Streptavidin-bead.

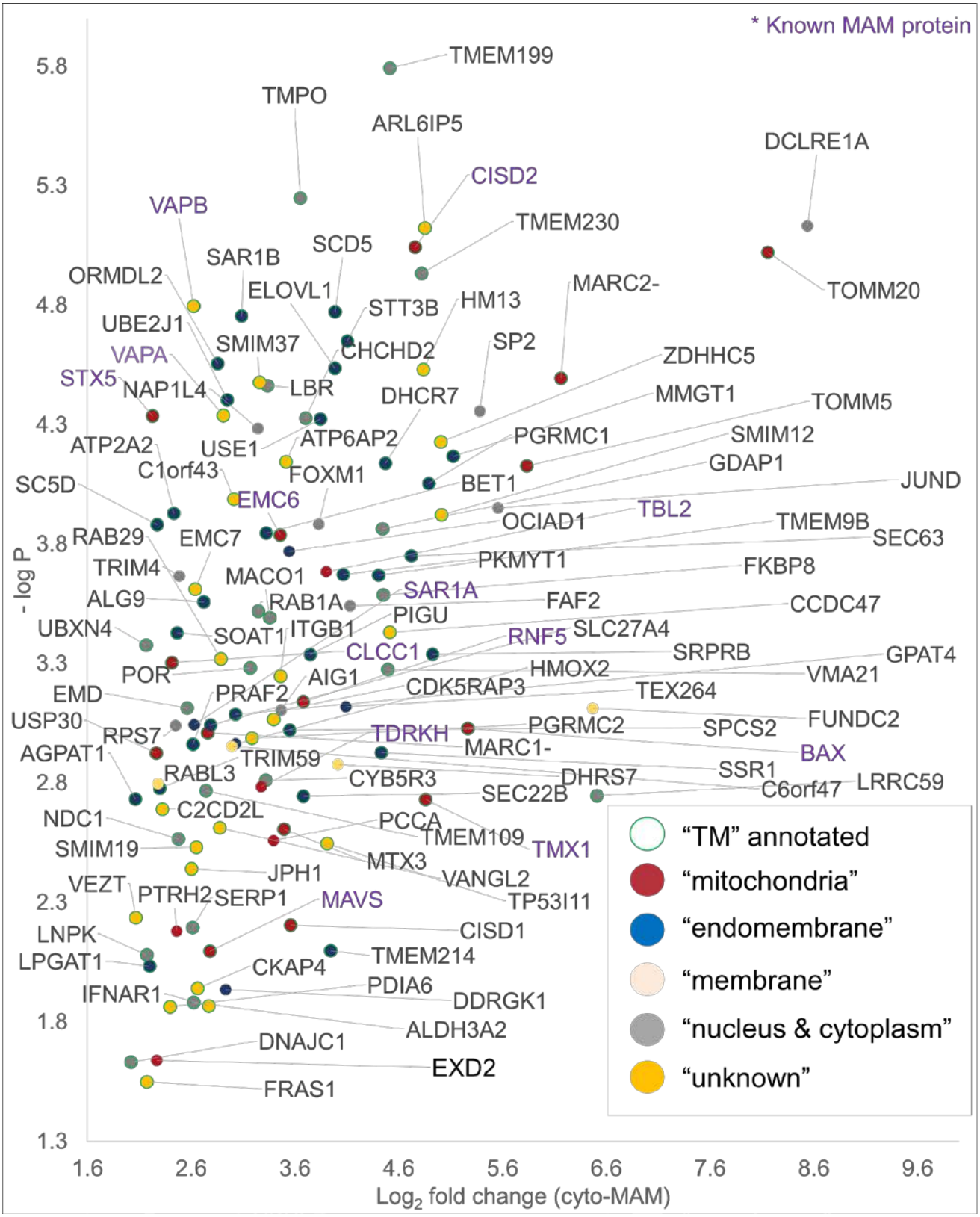
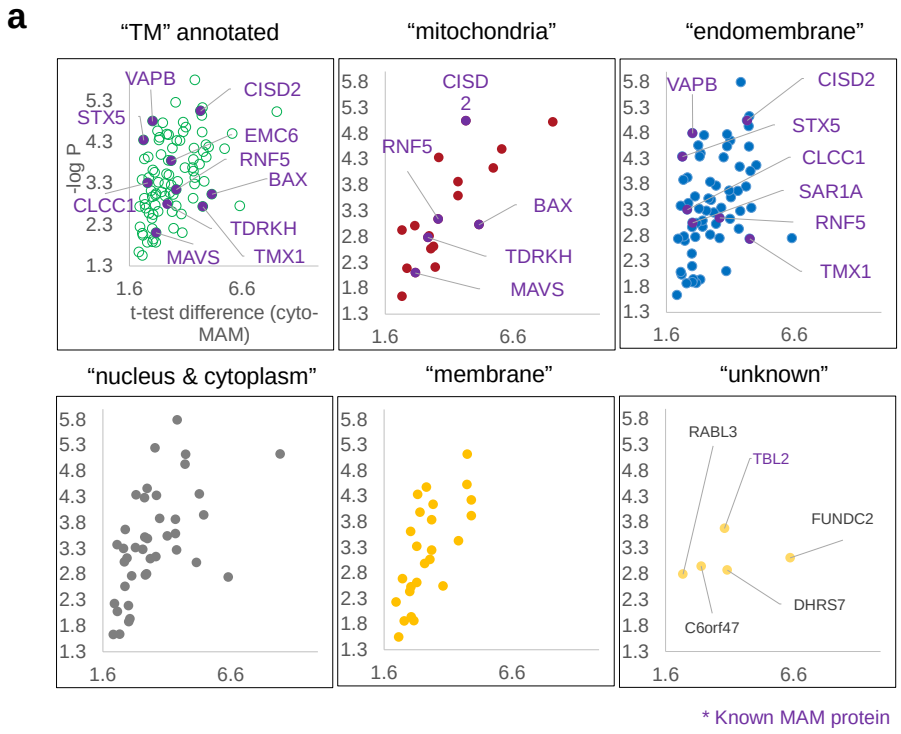


Figure S5. Expanded view of group-MAM proteins with gene names in the volcano plot of Figure 2c. Subcellular localization information (e.g., mitochondrial, endomembrane, membrane, nucleus, and cytoplasm) marked in different colors. Transmembrane (TM) proteins are circled with a green line. Gene names of previously characterized MAM proteins are shown in purple.



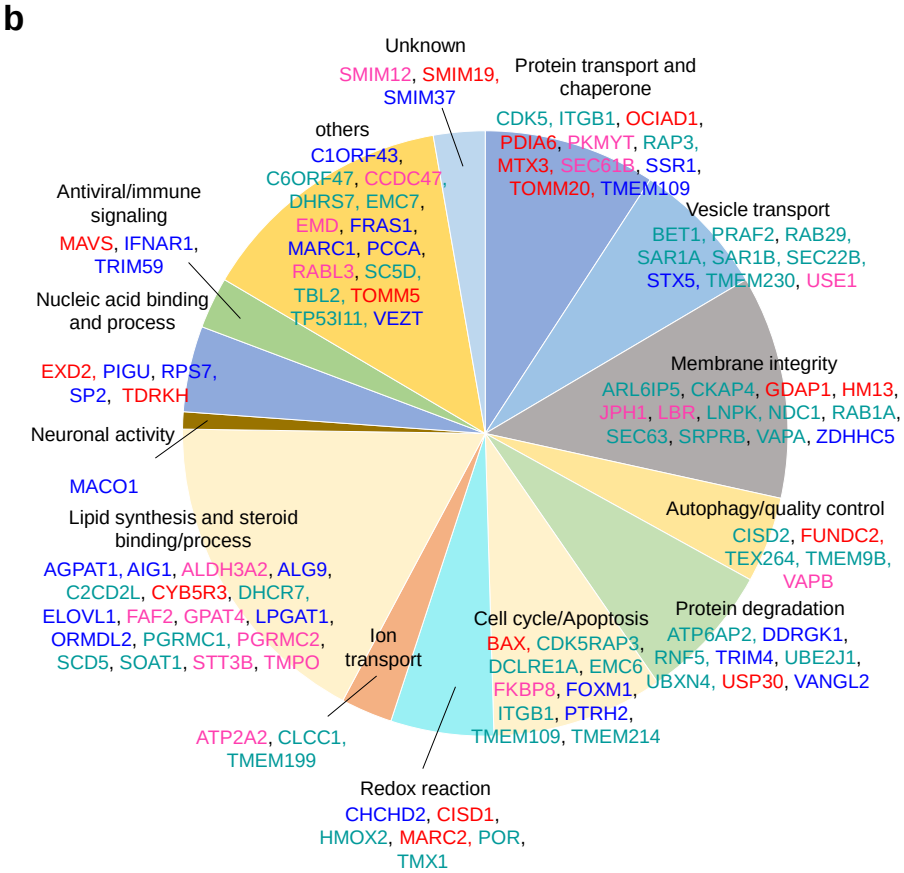
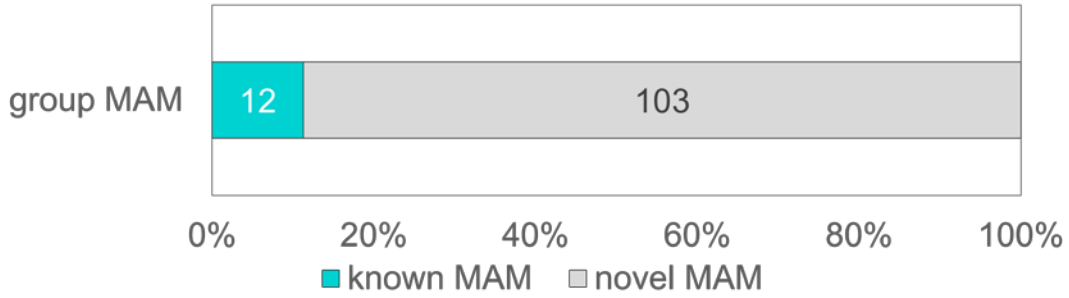


Figure S6. Clustering of group-MAM proteins (115 proteins)

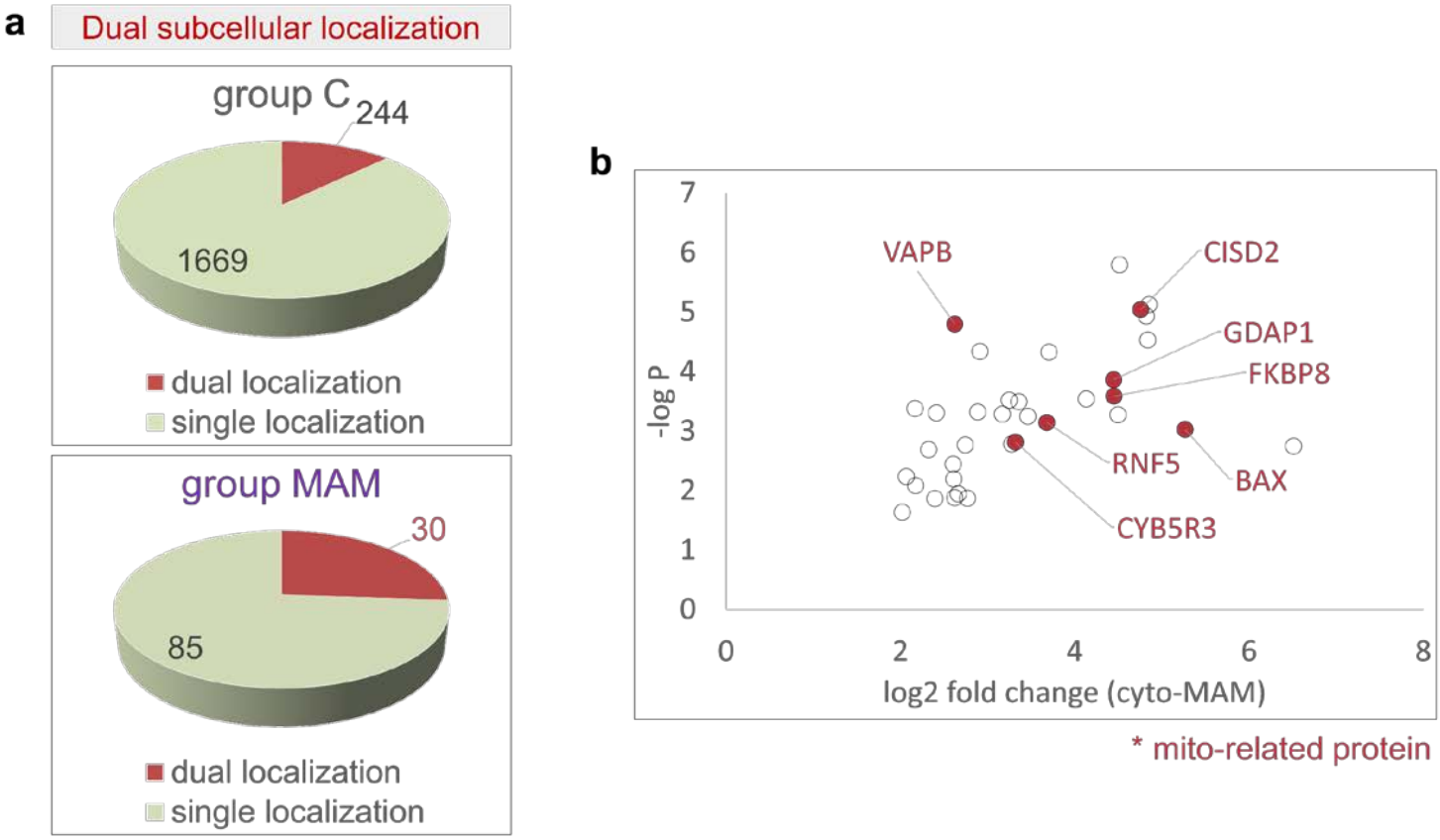
(a) Subcellular clustering of group-MAM proteins in a volcano plot of Figure 2c.

(b) Functional clustering of group MAM proteins according to the annotated function in Uniprot. See Supplementary dataset 1 & 2 table for details.



No.	Known MAM proteins	Verification method	References (PMID)
1	**VAPB**	• Immunofluorescence imaging • Calcium measurement assay in knockdown (KD) and overexpression (OE)	Gomez-Suaga et al.Curr Biol. 2017 (PMID:28132811)
2	**TDRKH**	• Subcellular Fractionation • Immunofluorescence imaging	Wang et al. Proteomics. 2018 (PMID: 29785746)
3	**TMX1**	• Electron microscopy imaging of MAM Calcium measurement assay in knockout (KO) and OE cell line	Raturi et al. J Cell Biol. 2016 (PMID: 27502484)
4	**CISD2**	• Electron microscopy imaging of MAM • Calcium measurement assay in knockout (KO) and OE cell line	Yeh et al. PLOS Biology, 2019 (PMID: 24833725)
5	**MAVS**	• Biochemical assay to identify the protein interaction with a known MAM protein, Gp78	Jacobs et al. J Biol Chem. 2014 (PMID: 24285545)
6	**CLCC1**	• Proximity labeling method and Split-GFP assay to identify the protein interaction with an OMM protein, PIGBOS.	Chu et al. Nat Commun. 2019 (PMID: 31653868)
7	**STX5**	• Cholesterol measurement from ER to mitochondria in KD cell	Lin et al. Mol Endocrinol. 2016 (PMID: 26771535)
8	**TBL2**	• Subcellular Fractionation assay • co-IP assay	Tsukumo et al. PLOS One, 2014 (PMID: 25393282)
9	**SAR1A**	• Immunofluorescence imaging • Electron microscopy in yeast	Ackema et al., PLOS One, 2016 (PMID 27101143)
10	**BAX**	• Immunofluorescence • Immunoelectron microscopy • Subcellular fractionation	Scorrano et al. Science. 2003 (PMID: 12624178)
11	**EMC6**	• Measurement of Mitochondrial Lipid Content • ERMES complex formation • Electron microscopy imaging of MAM in knockout cell line	Lahiri et al., PLoS Biol. 2014 (PMID: 25313861)
12	**RNF5**	• Subcellular fractionation • Fluorescence image of FP-tagged protein	Zhong et al. Immunity. 2009 (PMID: 19285439)

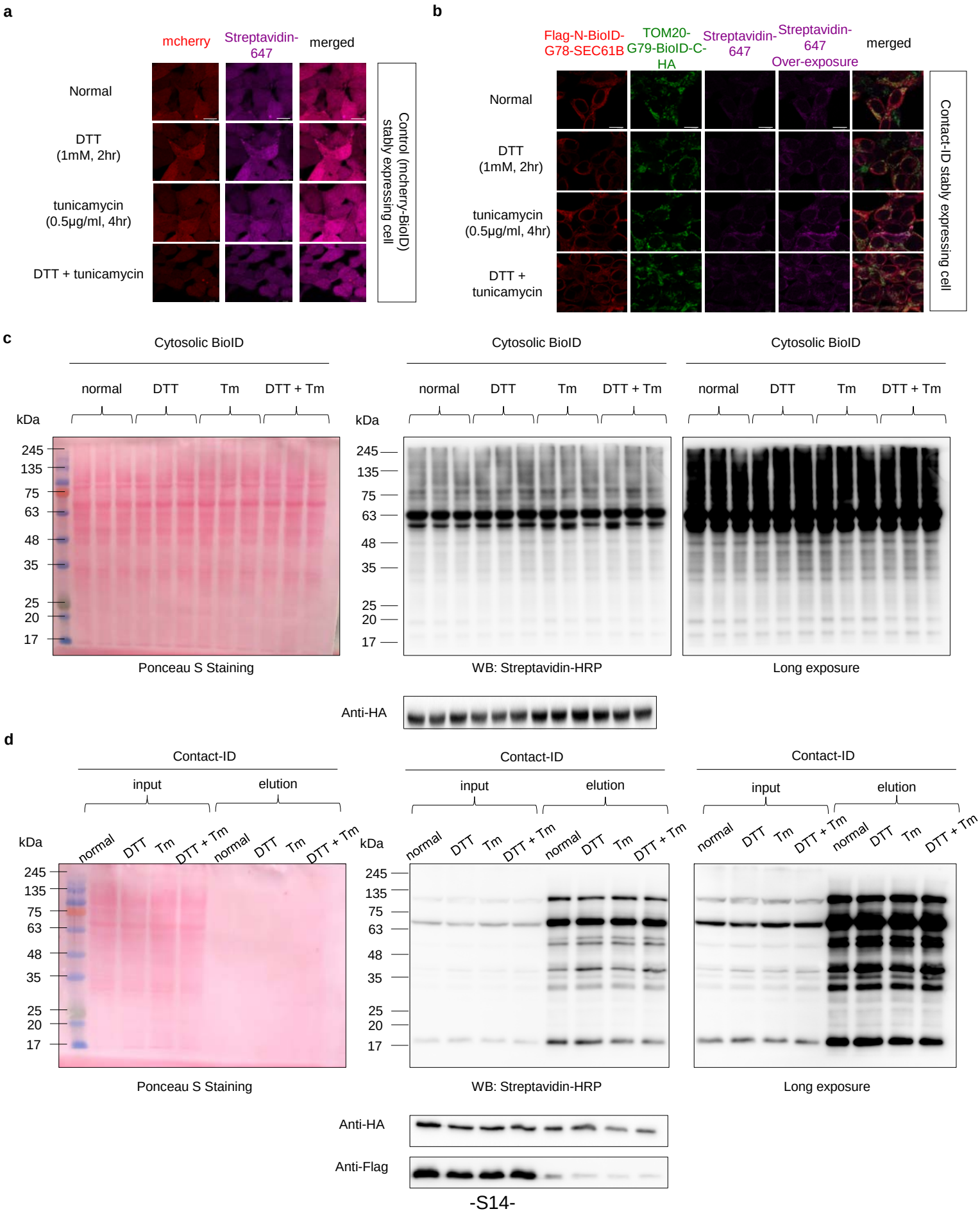
Figure S7. Previously characterized MAM proteins in group-MAM and verification methods with the reference.



c

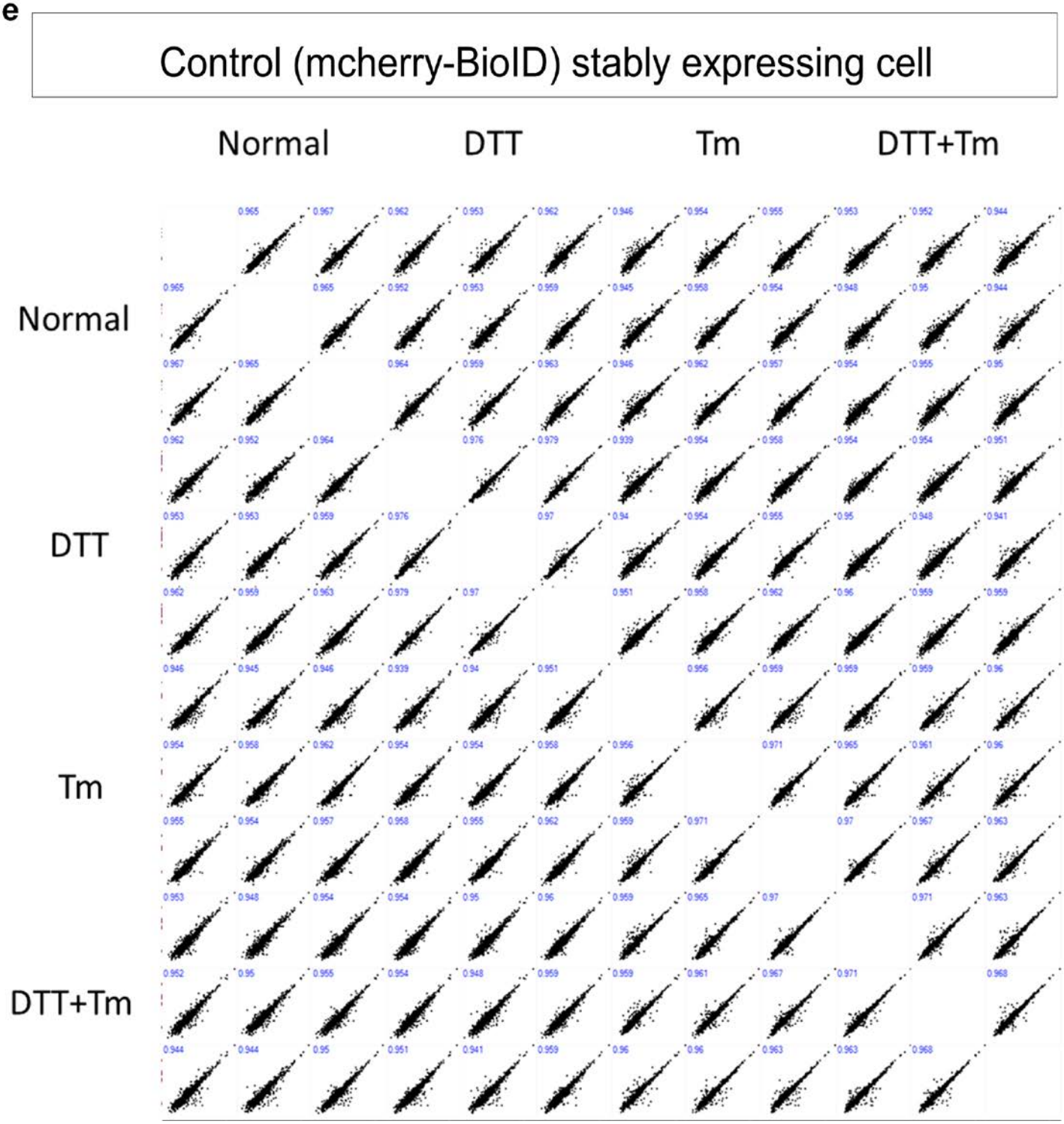
Protein name	Verification method of dual localization	reference
BAX	FP-tagged protein expression (Localized at mitochondria and ER)	Nutt et al., J Biol Chem., 2002 (PMID: 11741880)
VAPB	Immunofluorescence; Proximity ligation assay (Localized at mitochondria and ER)	KJ et al., Hum Mol Genet., 2012 (PMID: 22131369)
CISD2	Immunofluorescence (Localized at mitochondria and ER)	Chang et al., EMBO, 2010 (PMID: 20010695)
RNF5	Immunofluorescence, immunoblotting after mitochondrial isolation, and proteinase K assay (Localized at mitochondria); Immunofluorescence and immunoprecipitation (Localized at ER)	Tang et al., PLoS One, 2011 (PMID: 21931693); Khouri et al., JBC, 2013 (PMID: 24019521)
CYB5R3	Immunofluorescence (Localized at ER); Mitochondrial functional assay (Localized at mitochondria)	Uhlen et al, Mol. Cell. Proteomics, 2005 (PMID 16127175) ; Neve et al, JBC, 2011 (PMID 22203676)
FKBP8	Immunofluorescence (Localized at mitochondria and ER)	Zambarlal Bhujabal et al., EMBO Reports, 2017 (PMID: 28381481)
GDAP1	Immunofluorescence (Localized at mitochondria and cytosol)	Uhlen et al, Mol. Cell. Proteomics, 2005 (PMID 16127175)

Figure S8. Dual-localized "mobile" proteins in group-MAM.
(a) Group portion of dual localization annotated proteins in group-Cyto and group-MAM.
(b) Gene names of dual-localized mitochondrial proteins in group-MAM on the volcano plot of Figure 4b. (c) Reference list of (b).


a
mcherry
Streptavidin-647
merged
Normal
DTT (1mM, 2hr)
tunicamycin (0.5μg/ml, 4hr)
DTT + tunicamycin
Control (mcherry-BioID) stably expressing cell
b
Flag-N-BioID-G78-SEC61B
TOM20-G79-BioID-C-HA
Streptavidin-647
Streptavidin-647 Over-exposure
merged
Normal
DTT (1mM, 2hr)
tunicamycin (0.5μg/ml, 4hr)
DTT + tunicamycin
Contact-ID stably expressing cell
c
Cytosolic BioID
normal
DTT
Tm
DTT + Tm
kDa
245
135
75
63
48
35
25
20
17
Ponceau S Staining
WB: Streptavidin-HRP
Long exposure
Anti-HA
d
Contact-ID
input
elution
Ponceau S Staining
WB: Streptavidin-HRP
Long exposure
Anti-HA
Anti-Flag

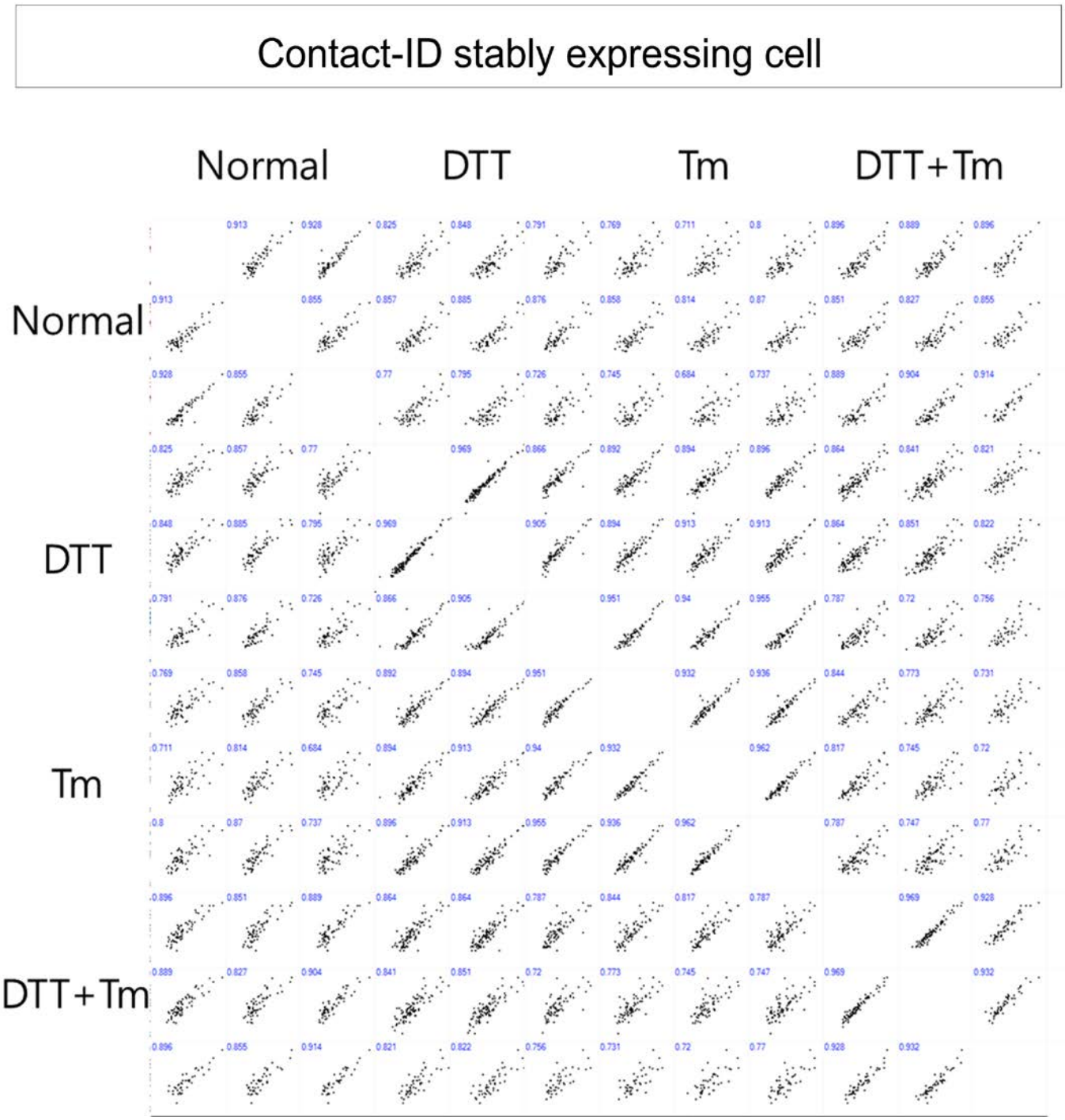
(continued)

e


Control (mcherry-BioID) stably expressing cell
Normal
DTT
Tm
DTT+Tm
Normal
DTT
Tm
DTT+Tm

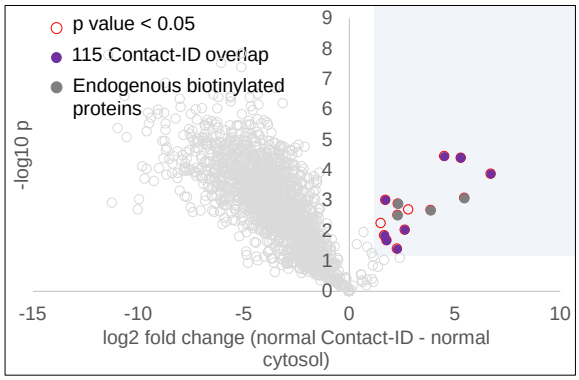
(continued)

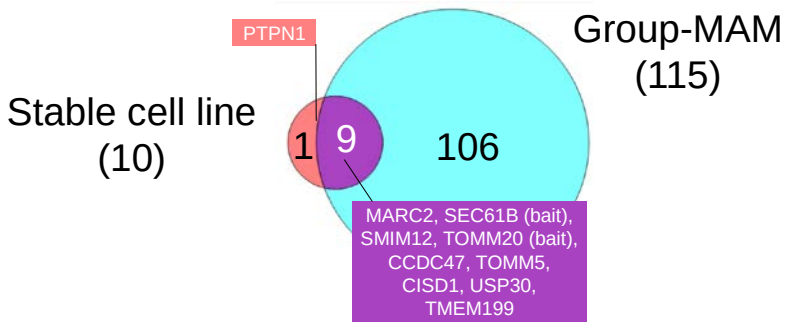
f


Contact-ID stably expressing cell
Normal
DTT
Tm
DTT+Tm
Normal
DTT
Tm
DTT+Tm

(continued)

g





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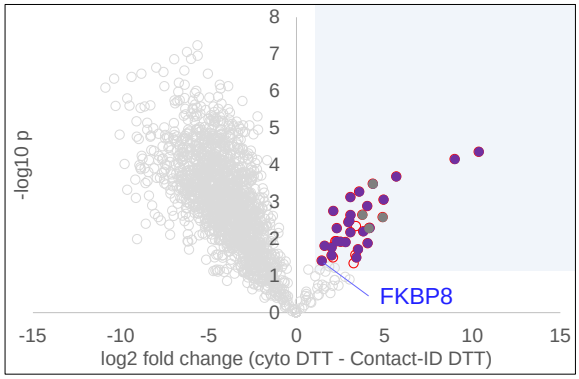
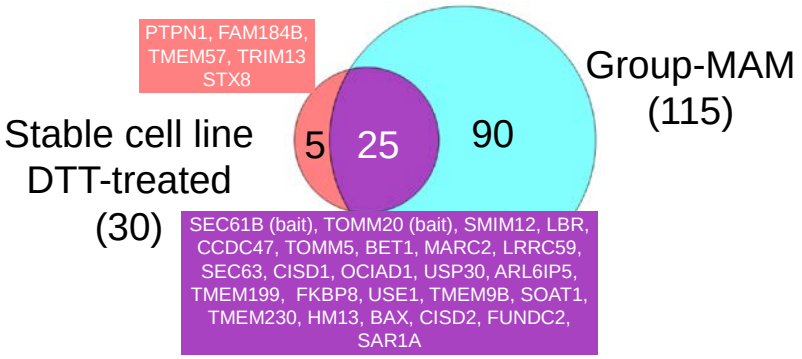
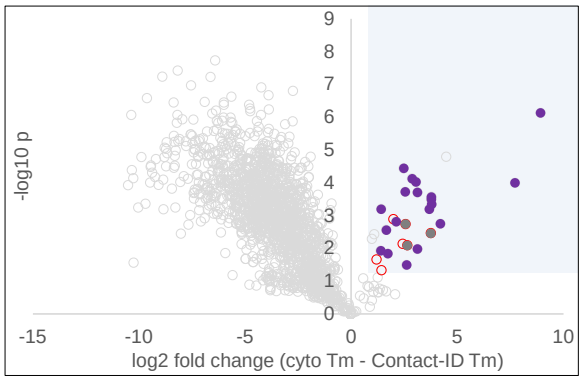
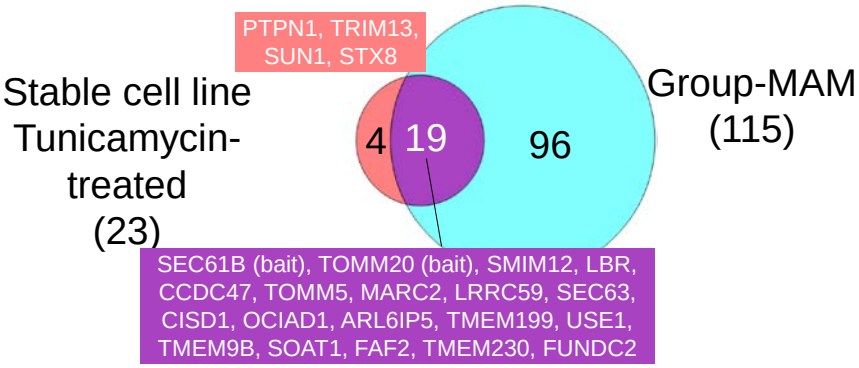




i





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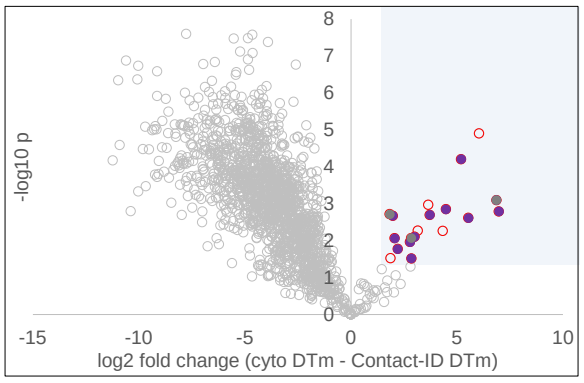

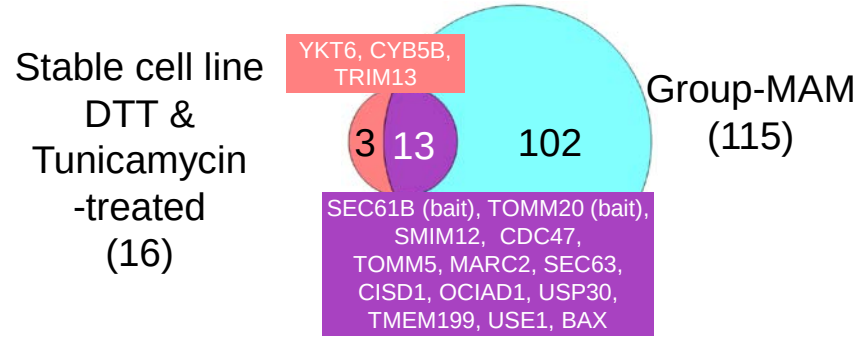

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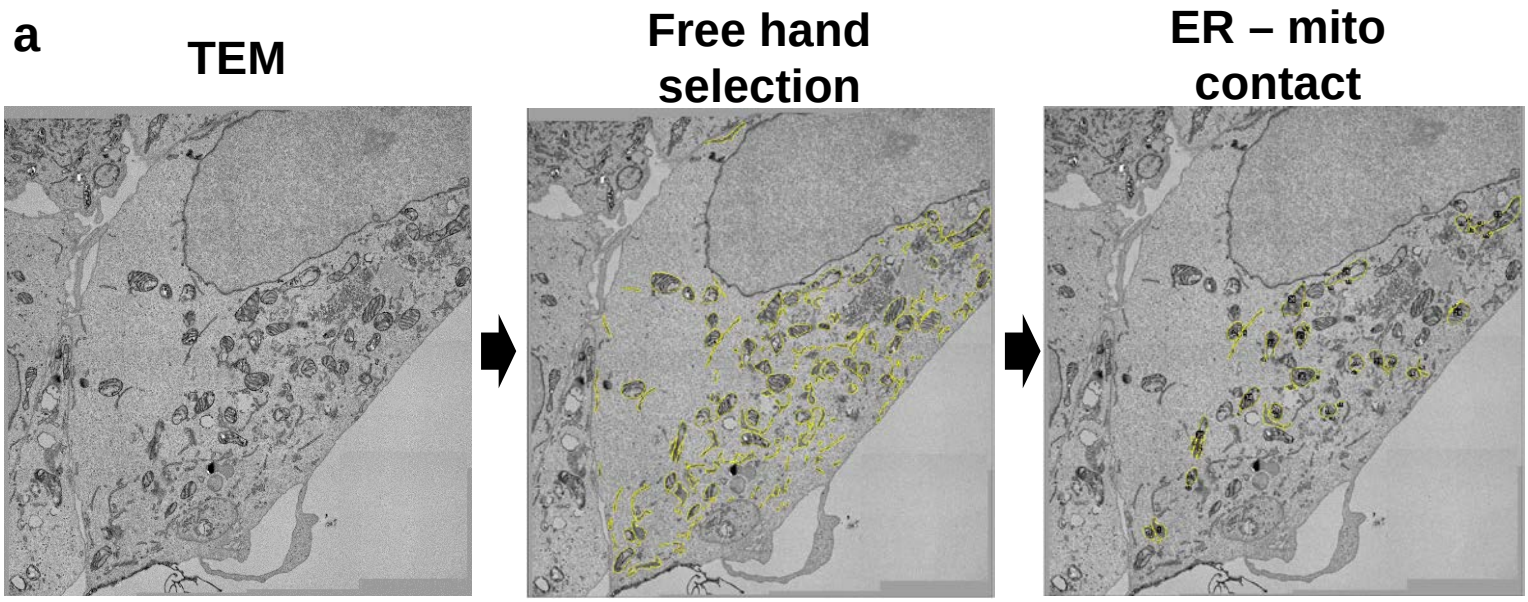
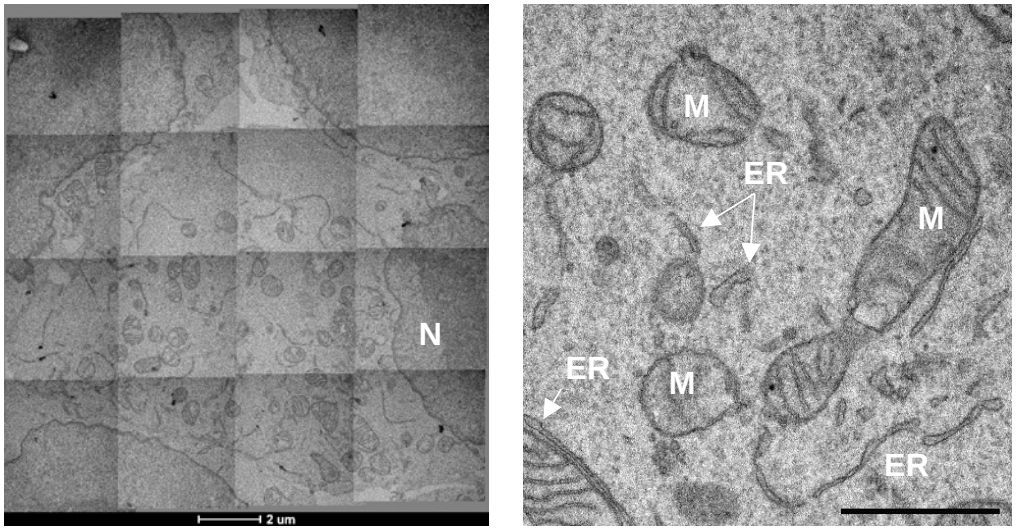
Figure S9. MAM proteome is remodeled under the stress condition (a, b) imaging analysis of biotinylating activity of cytosol-BioID (mCherry-BioID) and Contact-ID under the normal, DTT, tunicamycin and DTT plus tunicamycin treatment conditions (**c,d**) western blot analysis of cytosol-BioID (c) and Contact-ID (d). These results (a-d) implies that DTT and tunicamycin treatment do not compromise the labeling activity of BioID and Contact-ID. **(e,f)** Reproducibility test of the mass analysis of the replicates samples of cytosol-BioID (e) and Contact-ID (f) samples under the various conditions. **(g-j)** Volcano plot analysis of stably expressed Contact-ID versus stably expressed cytosol-BioID under the normal (g), DTT treated (h), Tunicamycin (Tm)-treated (i) and DTT and Tunicamycin-treated condition (j). The Venn-diagram analysis showed the overlap between the identified proteins of stably expressed Contact-ID proteins and those of group-MAM proteins (115 proteins). Although fewer proteins (10 proteins) were identified from the stably expressed Contact-ID cells due to the low expression level of Contact-ID constructs, most of the MAM proteins (9 of 10) from the stably expressed Contact-ID cells overlapped with group-MAM proteins from the transfected Contact-ID cells (g). FAM184B and TMEM57 were outstandingly enriched at the MAM upon DTT-treatment (h) while SUN1 and FAF2 are enriched upon tunicamycin treatment (i). See **Supplementary Dataset 5** for details.

a

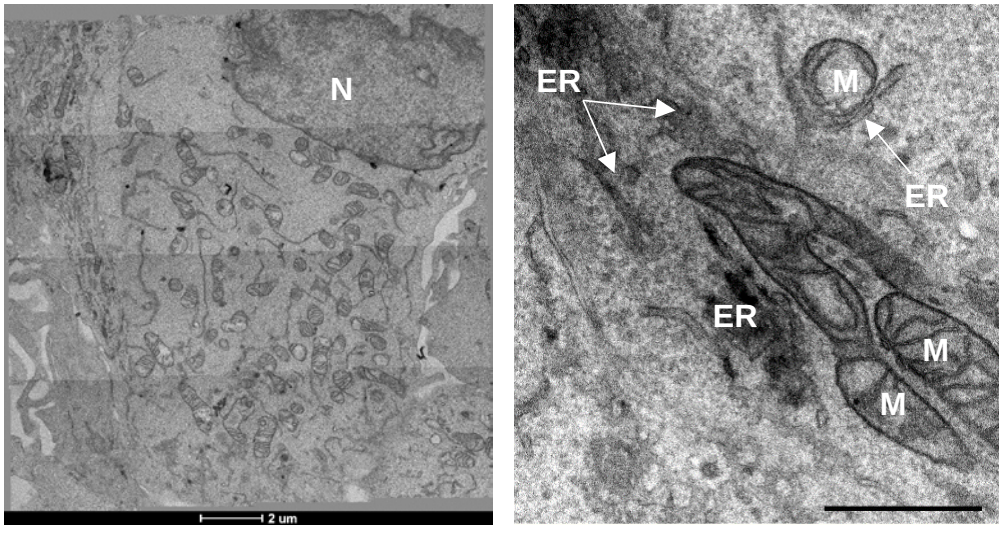


b (FKBP8 OE)

Untransfected cell



APEX2-FKBP8



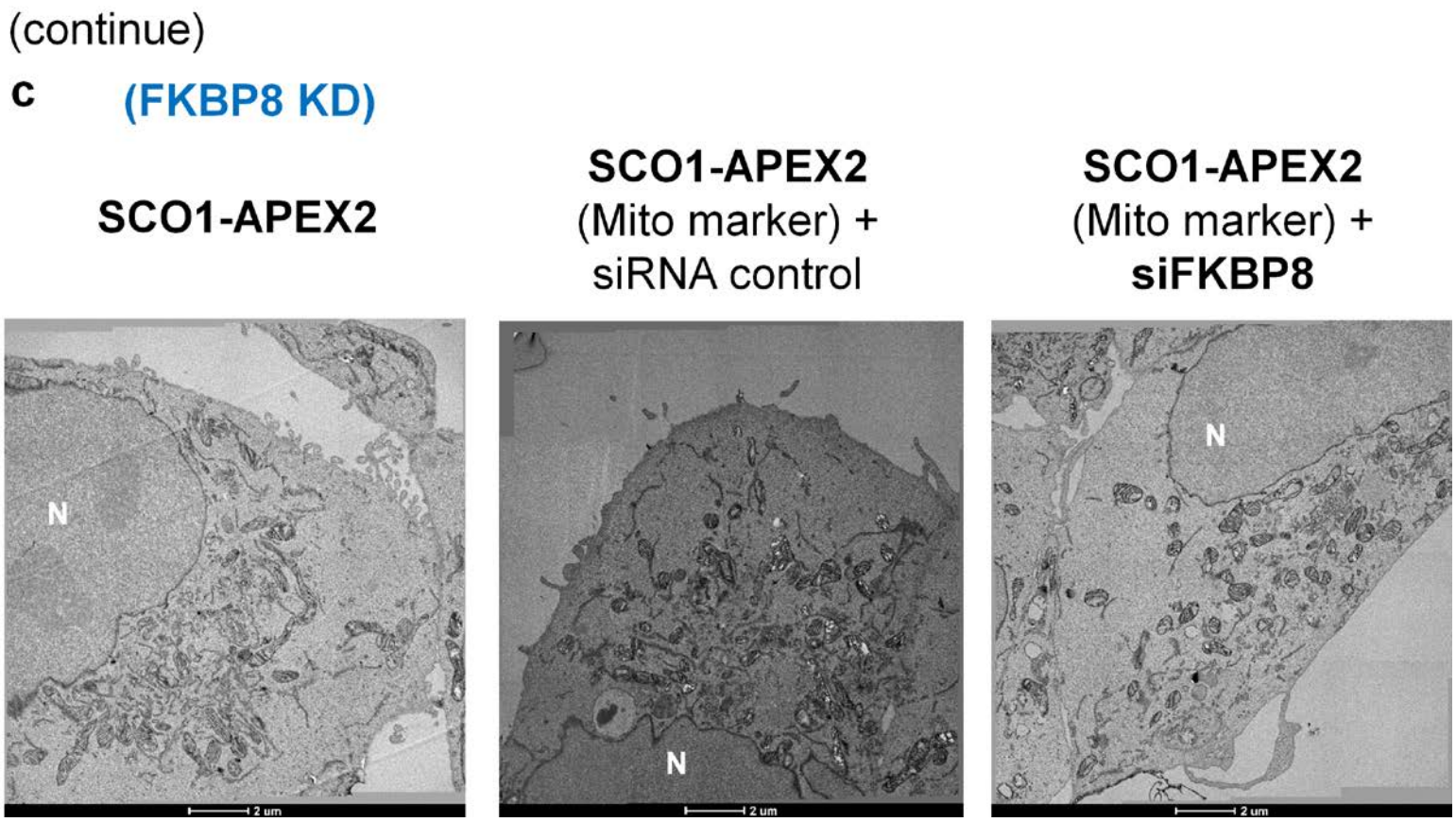
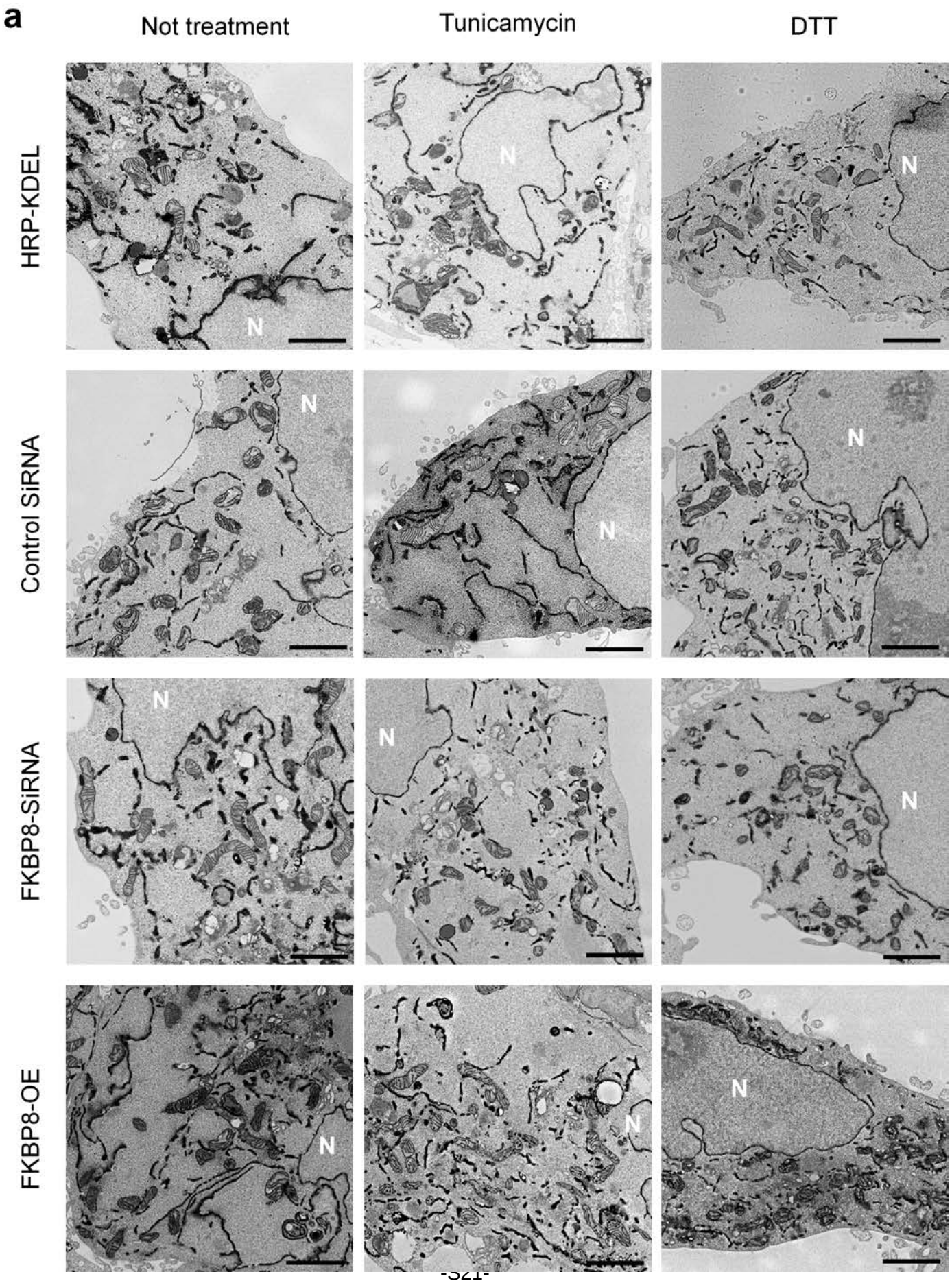


Figure S10. Raw data of EM imaging result for Figures 5.
(a) Workflow of freehand selection of ER–mitochondrial contact on the EM images.
(b) Raw image data of APEX2-FKBP8-overexpressing cells and untransfected cells.
(c) Raw image data of siFKBP8-treated SCO1-APEX2 stable cell line and control samples.


a
Not treatment
Tunicamycin
DTT
HRP-KDEL
Control SiRNA
FKBP8-SiRNA
FKBP8-OE
N

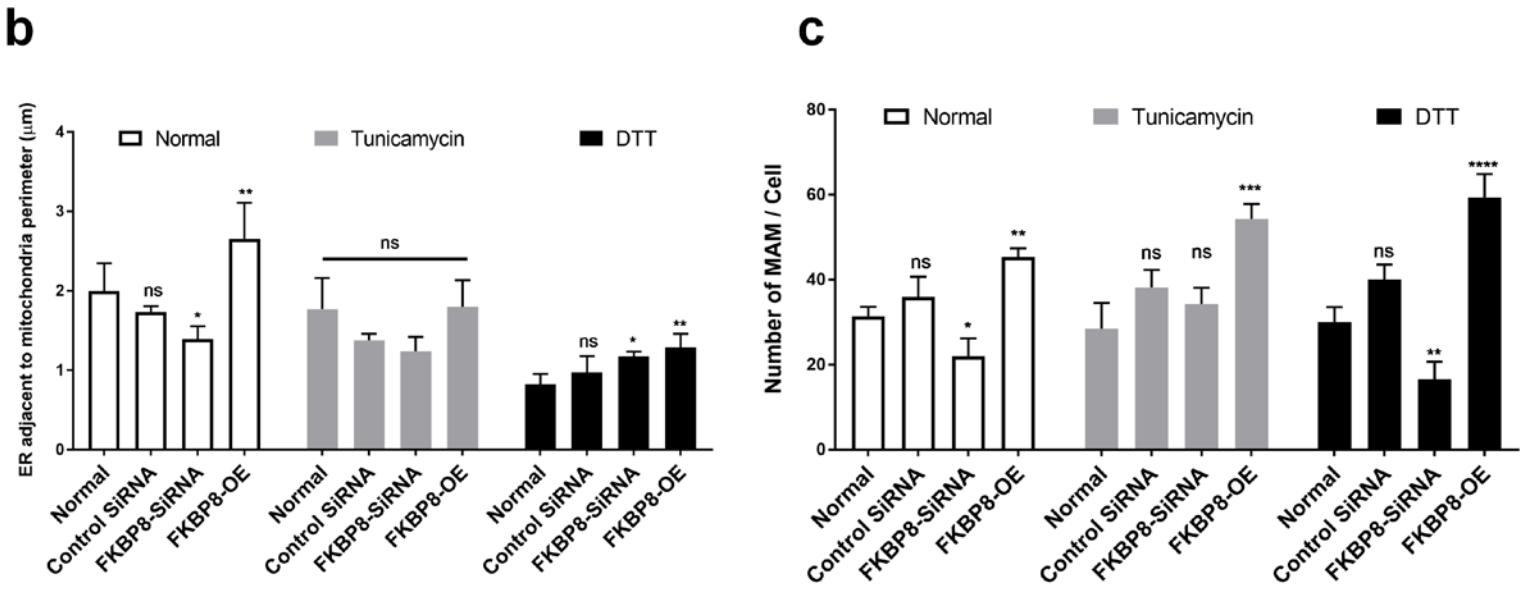


Figure S11. EM imaging results of MAM formation under the stress conditions. (a) Normal, FKBP8 siRNA-treated, and FKBP-overexpression (OE) HEK293T cells were subjected to no treatment, DTT treatment, and tunicamycin treatment. HRP-KDEL was used as the ER marker protein in EM imaging. Scale bar: 2 μm. Under either stress condition of DTT or tunicamycin treatment, the number of contact sites was steadily maintained in FKBP8-overexpressed cells compared to the wild-type cells. However, in FKBP8-knocked down cells, MAM formation was significantly perturbed under the DTT-treatment. (b,c) Quantification results of ER adjacent to mitochondria perimeter (b) and Number of MAM per cell (c). * $p<0.05$; **$p<0.01$; ***$p<0.005$; ****$p<0.001$

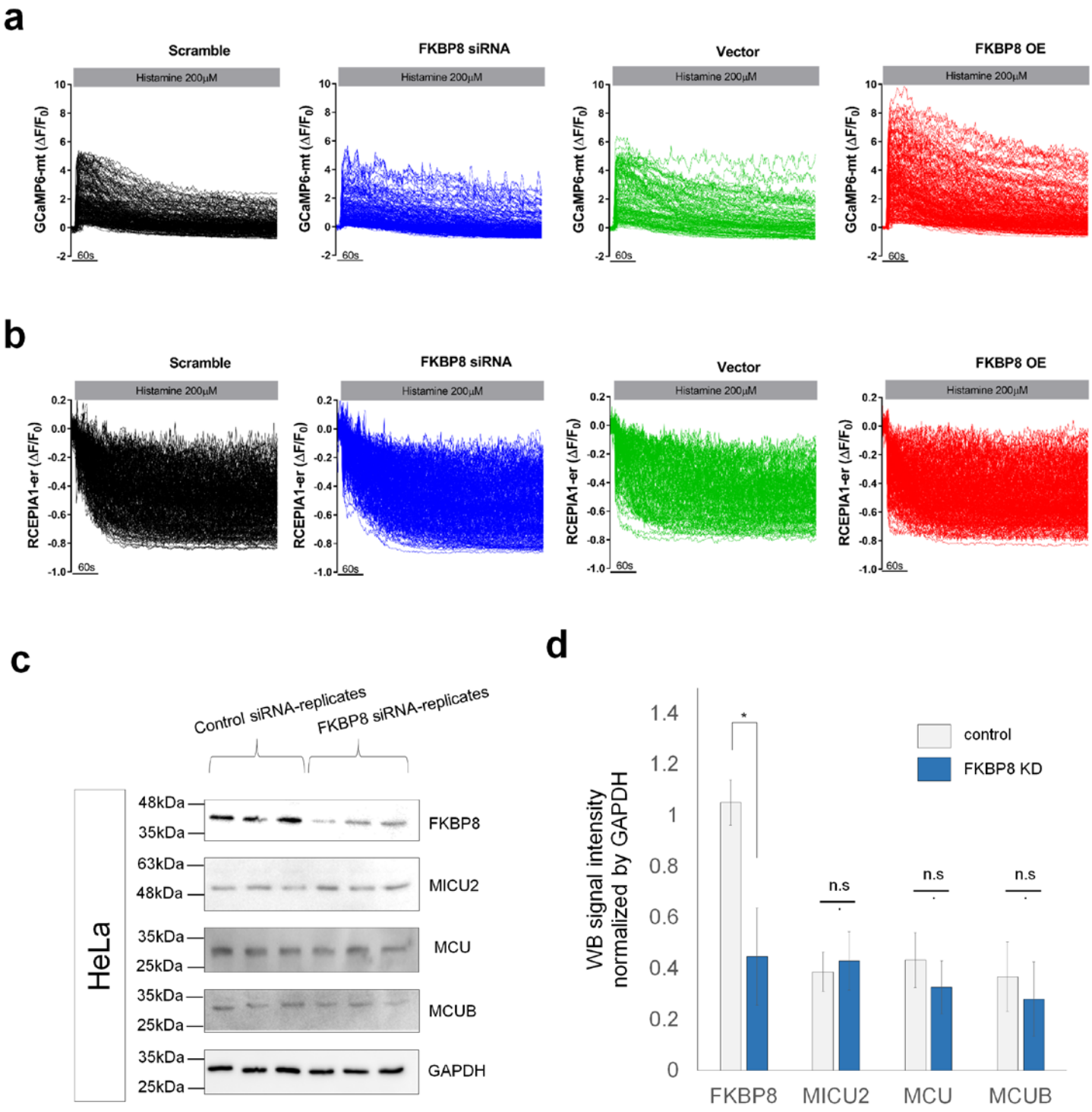


Figure S12. ER and mitochondrial calcium measurements in siFKBP8-treated cells, FKBP8-overexpressing cells, and control cells (raw data related to Figure 6).

(a) Mitochondrial matrix calcium measurement by GcaMP6-mt.

(b) ER luminal calcium measurement by RCEPIA-er.

(c) Western blot analysis of the protein component of MCU complex under the FKBP8 siRNA treatment (right) and control siRNA treatment (left). Antibodies for MICU2, MCU, MCUB and GAPDH (control) were used for these experiments. The information of the antibodies is described in the Supplementary Information (d) Quantification of the signals of the western blot of (c). The expression level of MCU, MICU2 and MCUB did not significantly change under FKBP8 silencing.

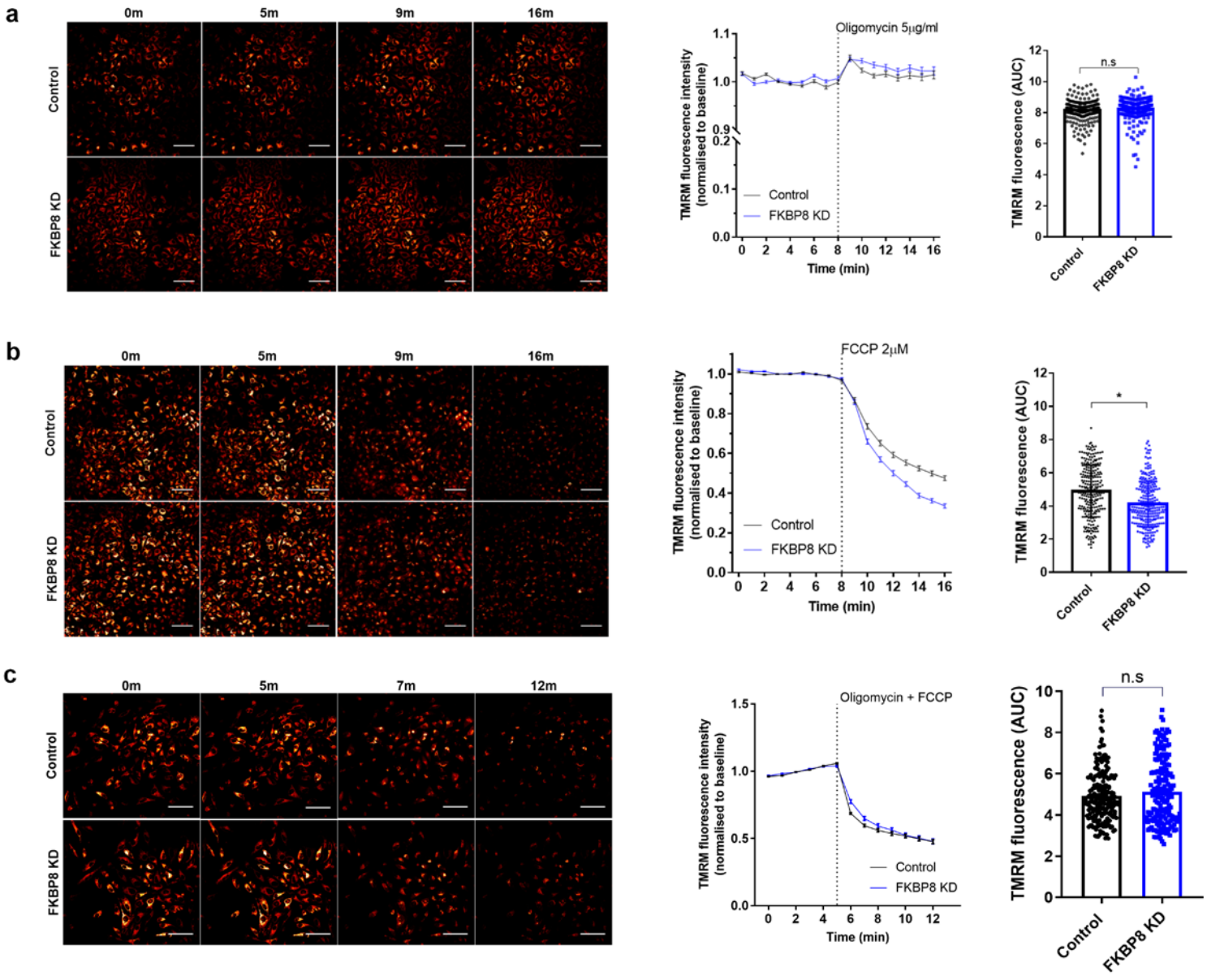


Figure S13. Mitochondrial membrane potential is not perturbed by FKBP silencing

(a) Fluorescence microscopy imaging analysis of Tetramethylrhodamine methyl ester (TMRM) of the FKBP8-KD sample and control sample (HeLa cells) under the normal and oligomycin treatment. n = 256 for control and n = 241 for FKBP8-KD.

(b) Fluorescence microscopy imaging analysis of TMRM of the FKBP8-KD sample and control sample (HeLa cells) under the normal and FCCP treatment. n = 256 for both control and FKBP8-KD.

(c) Fluorescence microscopy imaging analysis of TMRM of the FKBP8-KD sample and control sample (HeLa cells) under the normal and oligomycin and FCCP treatment. n = 212 for control and 188 for FKBP8-KD. Statistical significance was determined by unpaired t-test (n.s.; not significant, *; $p < 0.05$). Under oligomycin or FCCP treatment, the membrane potential of FKBP8-silenced samples responded in a similar pattern as that of control samples except that FKBP8-silenced samples showed a slightly stronger response to FCCP treatment, which can also be a consequence of defects ER-mitochondria calcium transfer. These results support the notion that FKBP8 silencing may not perturb the mitochondrial physiology, including oxidative phosphorylation, which can be reflected by the mitochondrial membrane potential.

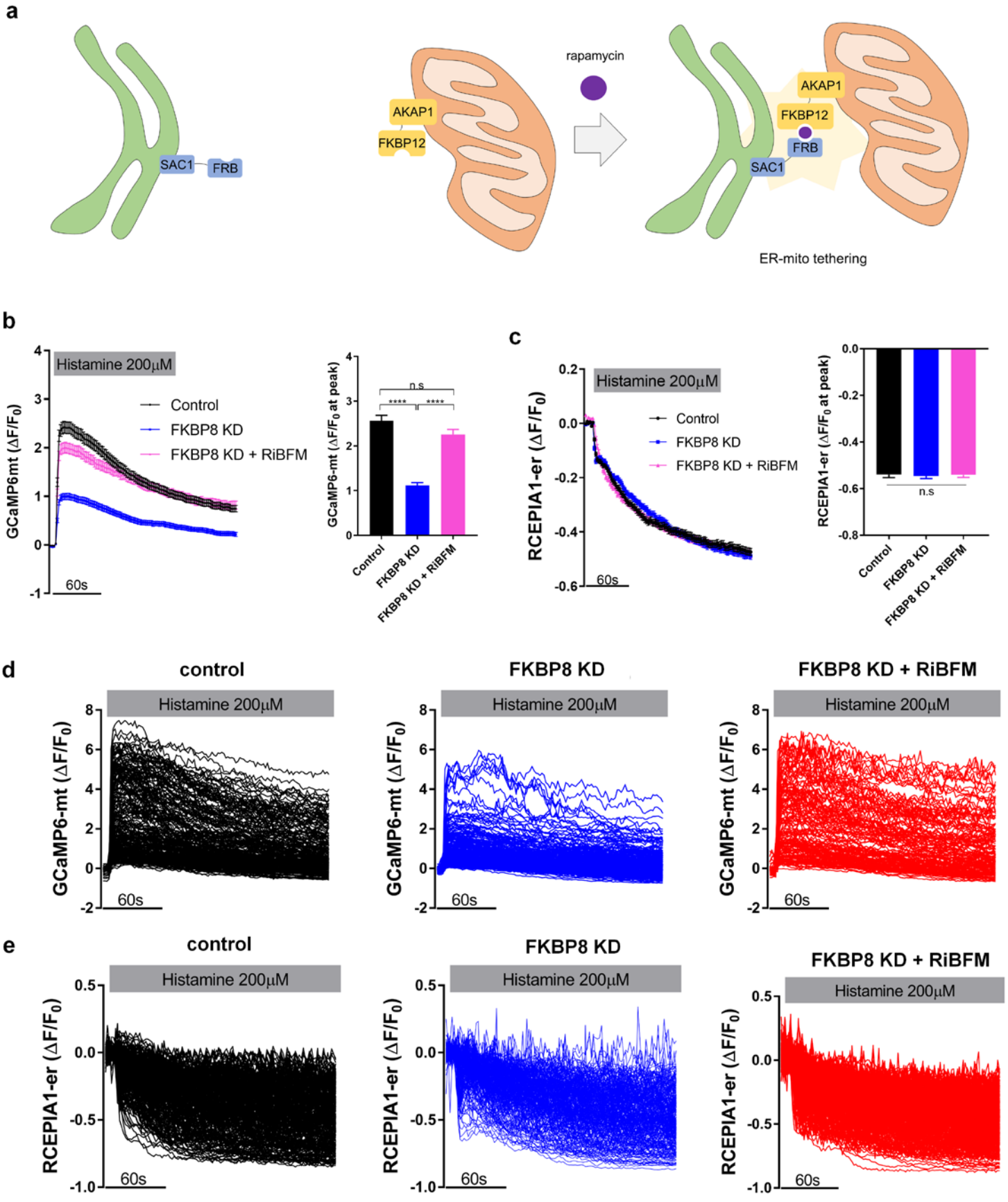


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Figure S14. Rescue of ER-mitochondria calcium transport by artificial ER-mitochondrial tethering in FKBP8-knockdown cells (HeLa) (a) Scheme of mitochondria–ER tethering by a rapamycin-induced bridge-forming module (RiBFM,(2)). (b-c) Mitochondrial calcium and ER calcium levels were measured by GCaMP6mt and R-CEPIA1er, respectively. These constructs were co-transfected with scramble siRNA (grey), and FKBP8 siRNA (blue) and RiBFM constructs, respectively; 200 μM histamine was utilized to stimulate ER Ca^{2+} release, and changes of GCaMP6mt and R-CEPIA1er fluorescence were recorded simultaneously and normalized to the basal signals (F0). Bar graphs represent the peak amplitude of ΔF/F0 in the mitochondria and ER. The data were assembled and analyzed from three independent sets of experiments. Lines and bars represent the mean ± SEM. n.s., not significant; *$p < 0.05$; ****$p < 0.0001$ determined by unpaired t-tests (cell numbers = 256 for scrambled siRNA, FKBP8 siRNA, and RiBFM, and 245 for the vector control). (d, e) GCaMP6mt and R-CEPIA1er fluorescence signal traces of individual cells for the data in (b) and (c). Mitochondrial calcium uptake level is recovered only when RiBFM construct expression and transient rapamycin treatment in FKBP8 knockdown cells

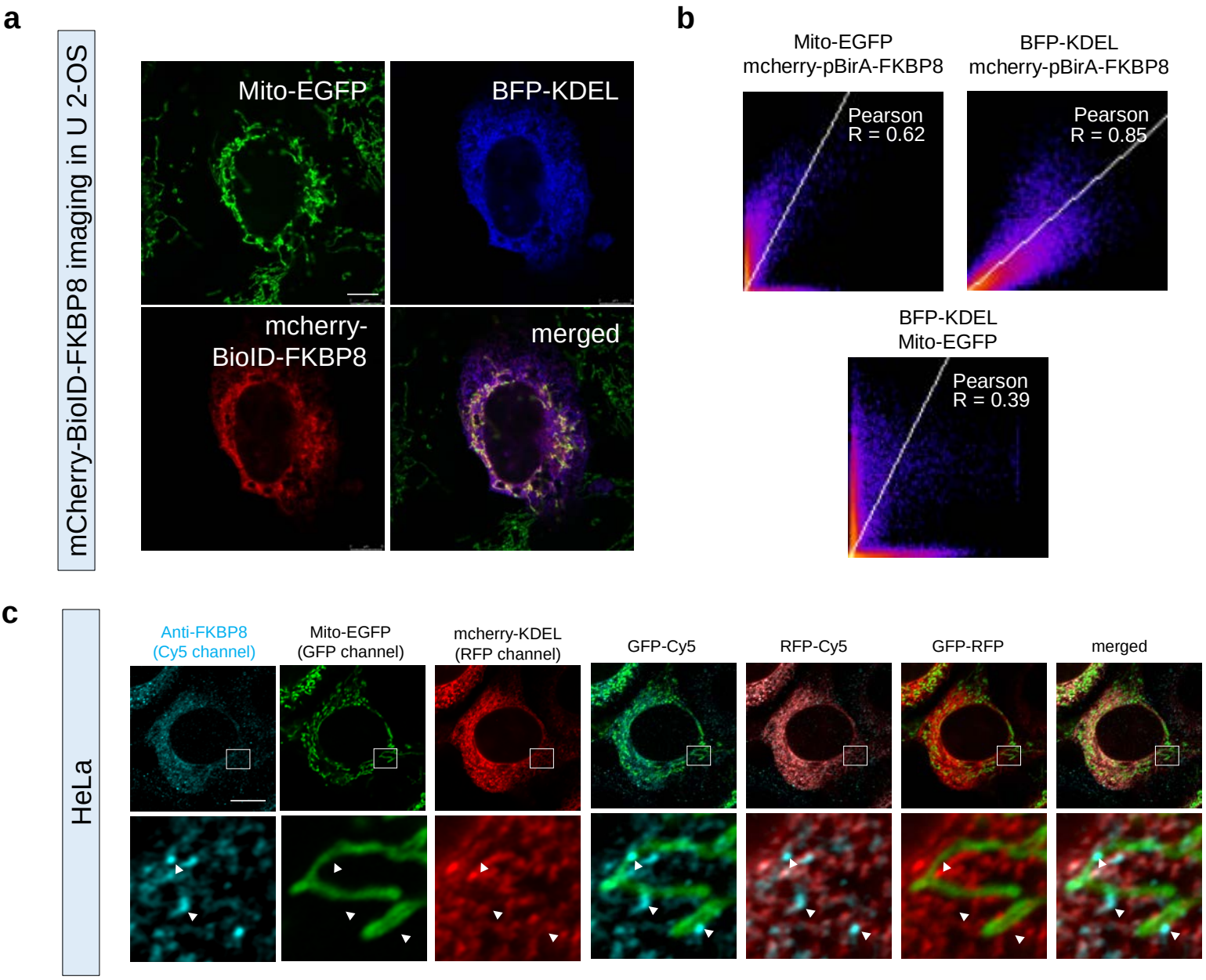
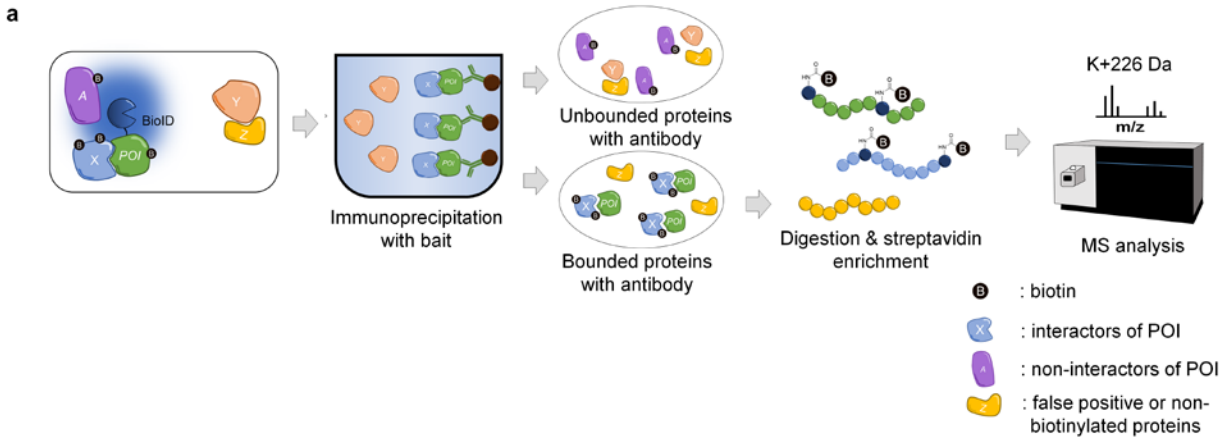


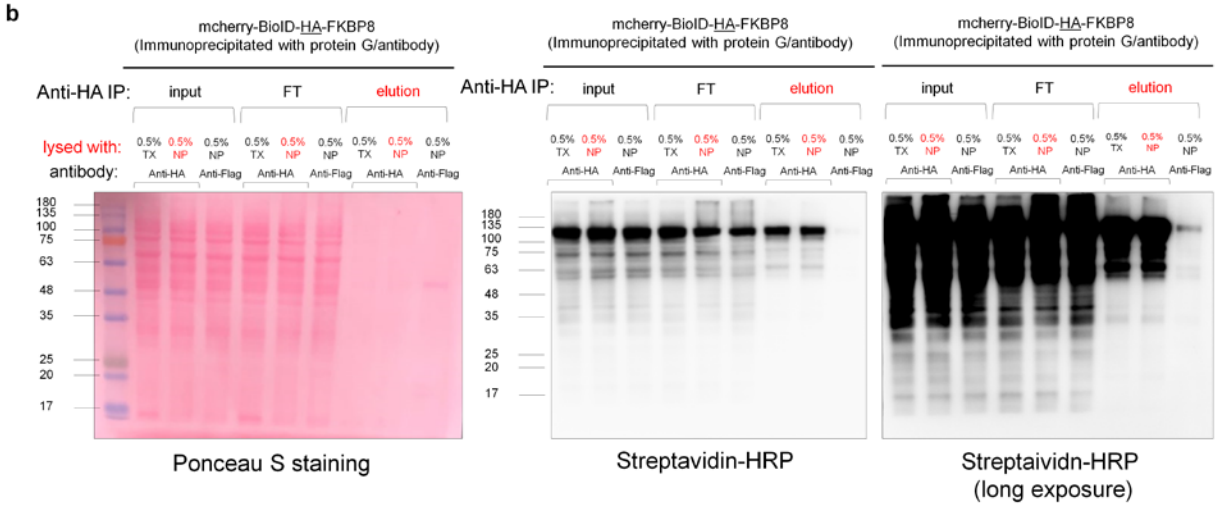
Figure S15. Confocal microscope imaging of BioID-FKBP8 and endogenous FKBP8

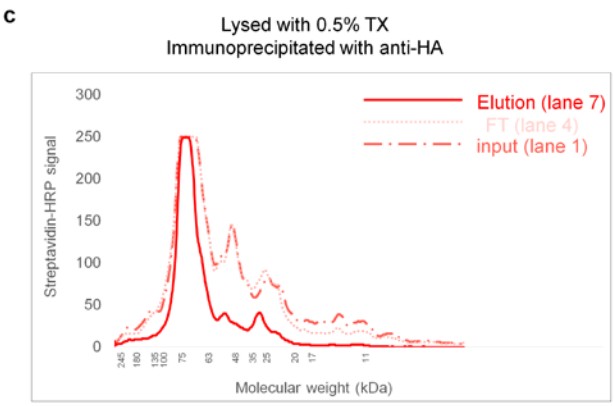
(a) Transiently expressed mCherry-BioID-FKBP8 in U2-OS cells was imaged in The RFP channel, the GFP channel was used for imaging the mitochondrial marker (Mito-EGFP), and the BFP channel was used for imaging the ER marker (BFP-KDEL). Scale bar = 8 μm.

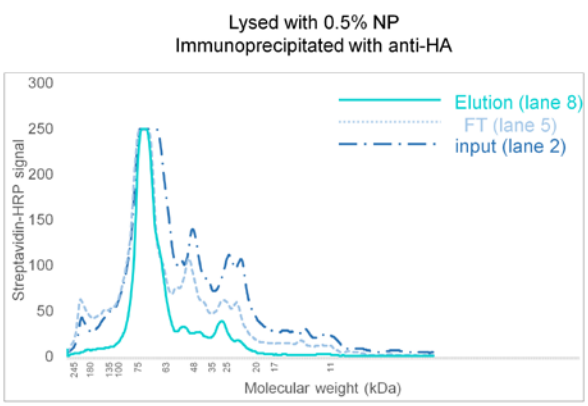
(b) Pearson correlation results between fluorescence signals at each channel shown on the right.

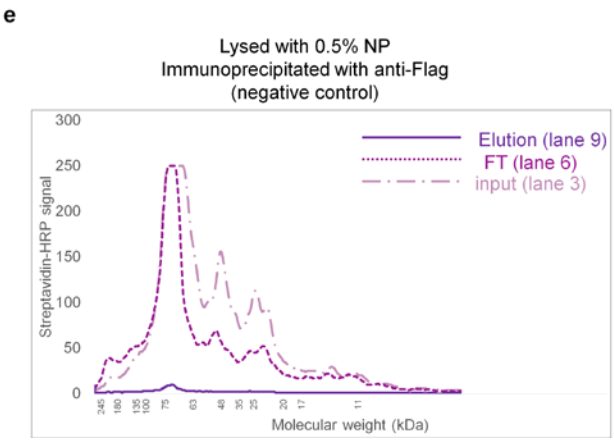
(c) Airy scan confocal microscope (Zeiss) imaging of anti-FKBP8 immunofluorescence. Immunofluorescence was imaged by Cy5 fluorescence (pseudo-colored in cyan), and the mitochondrial marker protein (Mito-EGFP) and ER marker protein (mCherry-KDEL) were imaged in the GFP (green) and RFP (red) fluorescence channels, respectively. The punctate localizations of FKBP8 at the interfaces of mitochondria and ER tubules are marked by arrows in the digitally magnified (zoom-in) images. Scale bar = 10 μm

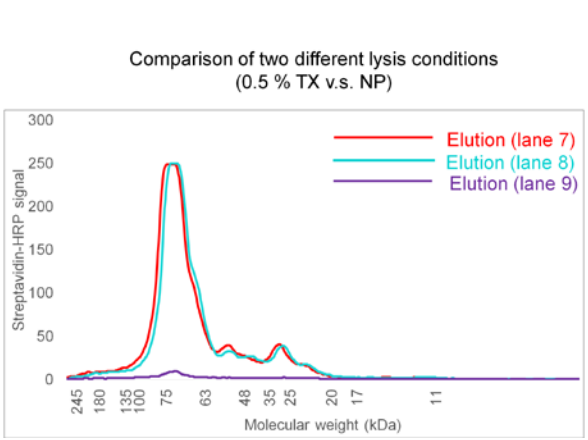

a
BioID
POI
Immunoprecipitation with bait
Unbounded proteins with antibody
Bounded proteins with antibody
Digestion & streptavidin enrichment
K+226 Da
m/z
MS analysis
: biotin
: interactors of POI
: non-interactors of POI
: false positive or non-biotinylated proteins


b
mcherry-BioID-HA-FKBP8 (Immunoprecipitated with protein G/antibody)
Anti-HA IP:
input
FT
elution
lysed with:
antibody:
Anti-HA
Anti-Flag
Ponceau S staining
Streptavidin-HRP
Streptaividn-HRP (long exposure)


c
Lysed with 0.5% TX
Immunoprecipitated with anti-HA
Elution (lane 7)
FT (lane 4)
input (lane 1)
Streptavidin-HRP signal
Molecular weight (kDa)


d
Lysed with 0.5% NP
Immunoprecipitated with anti-HA
Elution (lane 8)
FT (lane 5)
input (lane 2)
Streptavidin-HRP signal
Molecular weight (kDa)


e
Lysed with 0.5% NP
Immunoprecipitated with anti-Flag
(negative control)
Elution (lane 9)
FT (lane 6)
input (lane 3)
Streptavidin-HRP signal
Molecular weight (kDa)


f
Comparison of two different lysis conditions
(0.5 % TX v.s. NP)
Elution (lane 7)
Elution (lane 8)
Elution (lane 9)
Streptavidin-HRP signal
Molecular weight (kDa)

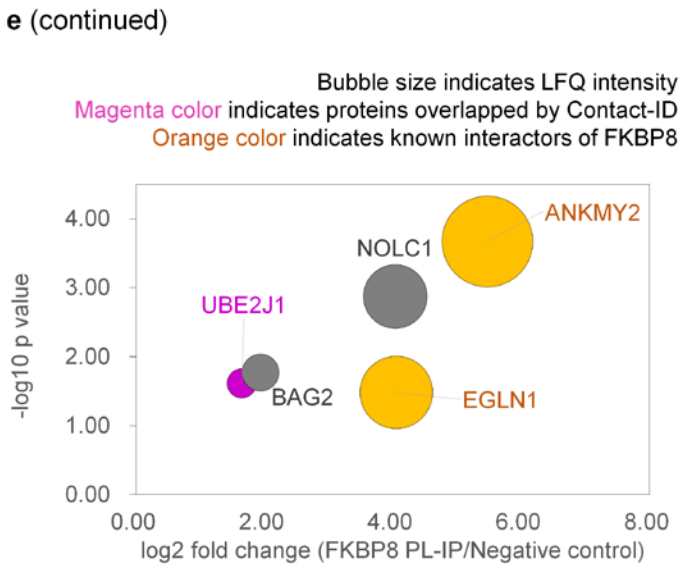


Figure S16. Mass identification of physical interaction partners of FKBP8 through co-immunoprecipitation after proximity labeling (PL-IP). (a) Scheme of the co-immunoprecipitation (PL-IP) experiment for the BioID-FKBP8-HA sample. (b) Streptavidin-HRP western blot result of PL-IP by using anti-HA for co-immunoprecipitation under two different lysate buffer conditions (NP40 and Triton X); anti-Flag was used as the negative control for the enrichment. Several biotinylated proteins were observed in the elution fraction of anti-HA immunoprecipitation (IP) while negligible biotinylated bands were detected in elution fraction of anti-Flag IP. (c–e) Line scan analysis of Streptavidin-HRP western blot of the input, flow-through (FT), and elution samples with anti-HA antibody pull-down under Triton-X lysis (c) and NP-40 lysis (d), and samples of anti-Flag (negative control) pull-down with NP40 lysis (e). The changing pattern throughout the immunoprecipitation experiment was reproducible in two biological replicate experiments using different lysis buffer solutions (Triton-X and NP40). (f) Comparison of biotinylated protein populations in the three pulled-down samples of each condition in (c–e). (g) FKBP8's physical interaction partners. Volcano plot showing statistically significant enrichment of biotinylated proteins of BioID-FKBP8 in the anti-HA pull-down sample over the anti-Flag pull-down sample. Mass-identified pulled-down protein information is shown in **Dataset S4 (Tab2)**. Detailed experimental procedures are described in the Supplementary Information.

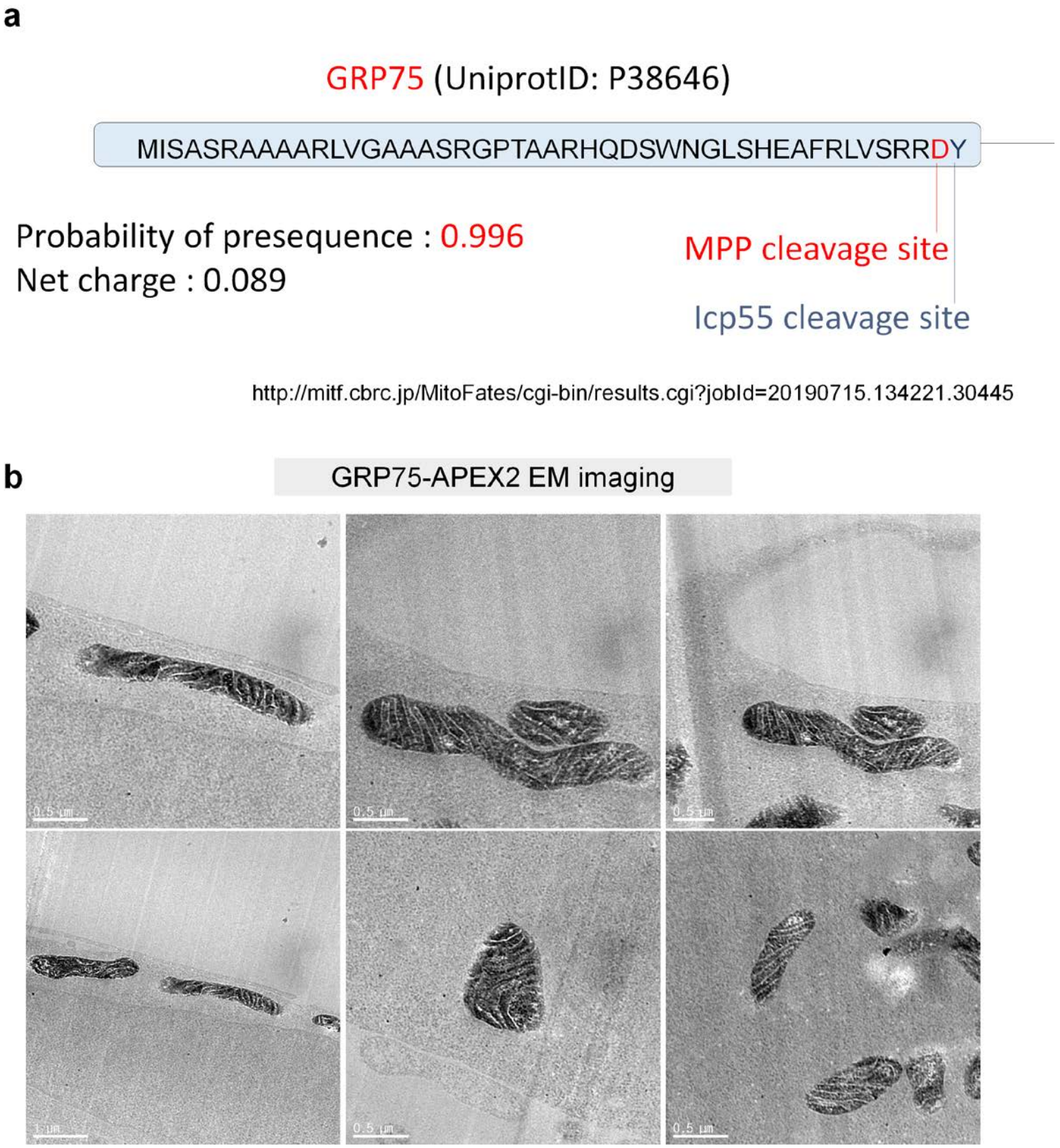


Figure S17. GRP75 is localized in the mitochondrial matrix.

(a) Predicted mitochondrial matrix-targeting sequence of GRP75 by Mitofates: (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi)(3) (b) TEM imaging of mitochondrial matrix-targeted GRP75-APEX2. Scale bar = 0.5 µm.

Movie S1. ER and mitochondrial calcium measurements by GcaMP6-mt and RCEPIA-er in live cells (raw data related to Figure 9 and Figure S10).

1a. real-time movement of organelle calcium concentration in scrambled siRNA treatment.

1b. real-time movement in siFKBP8 treatment.

1c. real-time movement in transiently expression of empty vector.

1d. real-time movement in transiently overexpression of FKBP8.

Dataset S1. Contact-ID proteome list (115 MAM proteins)

Tab 1. List of 115 MAM proteins predominantly labeled by Contact-ID over cytosolic control (mCherry-BioID). Statically curated from Student`s t-test. Used for Volcano Plot analyses in **Figure 2c**.

Tab 2. List of 1634 proteins labeled by mCherry-BioID (cytosolic control). Statically curated from Student`s t-test. Used for Volcano Plot analyses in **Figure 2c**.

Dataset S2. Functional annotation of filtered 115 Contact-ID proteins.

Dataset S3. Biotinylated peptide information of Contact-ID.

Tab 1. Total 327 biotin-labeled peptide information by Contact-ID.

Tab 2. Membrane topology Information of 85 proteins.

Dataset S4. Proteome list of mCherry-BioID-HA-FKBP8 (BioID-FKBP8)

Tab1. Predominately biotin-labeled proteins over cytoplasm.

Tab2. Affinity purified & biotinylated proteins from anti-HA pull-down experiment

Dataset S5. Proteome list of stably expressed Contact-ID under the ER stress conditions: DTT- and Tunicamycin (Tm)-treated conditions.

SI Materials and Methods

Expression plasmids and antibodies

Genes were cloned into the specified vectors using standard enzymatic restriction digest and ligation with T4 DNA ligase. To generate constructs where short tags (e.g., HA or Flag epitope tag) or signal sequences were appended to the protein, the tag was included in the primers used to PCR-amplify the gene. PCR products were digested with restriction enzymes and ligated into cut vectors (e.g., pcDNA3, pcDNA5, and pDisplay). In all cases, the CMV promoter was used for expression in mammalian cells. **Table S1** (see ***SI Appendix***) below summarizes the genetic constructs cloned and used for this study.

Primary antibodies were used for western blotting and immunofluorescence imaging: anti-FKBP8 (1:200 dilutions, Atlas, cat. HPA045177), anti-MCU (1:200 dilutions, Invitrogen, cat. MA5-24702), anti-MICU2 (1:200 dilutions, Abcam cat. Ab101405), anti-MCUb (1:200 dilutions, Abcepta, cat. AP12355B), Alexa Fluor 647 conjugated Streptavidin (1:3000 dilutions, Invitrogen, cat no. S-21374), HRP conjugated Streptavidin (1:10000 dilutions, Invitrogen, cat no. S-21126), rabbit anti-Flag (1:3000 dilutions, Sigma Aldrich, cat no. F3165), mouse anti-Flag (1:3000 dilutions, Sigma Aldrich, cat no. F7425), and rabbit anti-HA (1:1000 dilution, Invitrogen, cat no. 71-5500). Secondary antibodies including anti-mouse, rabbit-HRP and Alexa Fluor-conjugated anti-mouse, rabbit is purchased from Invitrogen.

Cell culture and transfections

HEK293 were obtained from ATCC (Manassas, VA, USA) (passages < 20), HEK293 Flip-in T-rex were obtained from Thermo Fisher Scientific (Cat. No. R78007), HEK293FT were a gift from Professor Jung-Weon Lee (Seoul National University), and COS-7 cells and U-2 OS cells were obtained from the Korean Cell Line Bank. The cell lines were frequently checked and tested for morphology under a microscope and mycoplasma contamination but were not authenticated. All cell lines were maintained in high glucose DMEM medium with 10% fetal bovine serum at 37°C in 5% CO2 (v/v). All cell lines were transiently transfected at 60–80% confluence using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and turbofect (Invitrogen, R0531).

Construction of stably expressed Contact-ID cell line (Flip-in HEK293T-Rex)

Flp-In™ T-REx™ 293 cells (Life Technologies) were cultured in DMEM (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/ml streptomycin at 37°C under 5% CO2. Cells were grown in a T25 flask. Stable cell lines were firstly generated by transfection with the pcDNA™3 expression construct plasmid expressing FLAG-B1 (split fragment)-SEC61B. Cells were transfected at 60–80% confluence using TurboFect (Invitrogen), typically with 6 μL of TurboFect transfection reagent and 2,000 ng plasmid per 6 well cell culture plate. After 24 h, cells were split into a 90 mm cell culture dish (SPL, 11090) with the proper concentration of Geneticin (G418) (500 μg/mL). Media containing Geneticin (G418) were changed every 3–4 days. After 2–3 weeks, 3–4 colonies were selected and transferred to a 24-well plate. Cells were continuously split into larger plates, and a cell stock was made. After splitting the cells into a 6-well plate, separate samples were prepared for expression testing. And then, for second expression, half of Contact-ID stable cell lines co-transfected pcDNA™5/FRT/TO expression construct and the pOG44 plasmid using TurboFect (Invitrogen), typically with 12 μL TurboFect and 4,000 ng plasmid (9:1 = pOG44:pcDNA5) per T25 flask. After 24 h, cells were split into a 90 mm cell culture dish (SPL, 11090) with the proper concentration of hygromycin B (100 μg/mL). Media containing hygromycin B were changed every 3–4 days. After 2–3 weeks, 3–4 colonies were selected and transferred to a 24-well plate. Cells were continuously split into larger plates, and cell stock of complete Contact-ID stable cell line was made. After splitting the cells into a 6-well plate, separate samples were prepared for expression testing. Unlike ERM-Contact-

ID stably expression, OMM-Contact-ID expression was induced by 5 ng/mL doxycycline (Sigma Aldrich), and co-treated with biotin for biotinylation as well as doxycycline.

In situ biotinylation and immunoblotting

All cell lines such as HEK293, HEK293FT, HeLa, U2-OS, and HEK293 Flip-in T-rex cells for this experiments were transfected from Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), biotinylated with 50 μM of biotin (Alfa aesar, A14207) or/and 100 nM of rapamycin (Selleck, S1039) for 16-18 h and lysed with RIPA buffer (ELPISBIO, EBA-1149) containing 1× protease cocktail (Invitrogen, 78438) for 30 min at 4°C. Lysates were transferred to e-tube and vortexed for 2 min. And lysates were clarified by centrifugation at 15,000g for 10 min at 4°C. The lysates were loaded by boiling at 95°C for 10 min after 1x SDS-PAGE loading buffer. After that, the proteins were resolved by SDS–PAGE (Invitrogen) and subjected to immunoblotting with the indicated antibodies.

Immunofluorescence and confocal microscopy

To visualize the subcellular localization of the transiently expressing POI, cells were plated on coverslips (thickness no. 1.5 and radius: 18 mm). For fixed cell imaging, cells were fixed by 4% paraformaldehyde and permeabilized with cold methanol for 5 min at -20°C. Next, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and blocked for 1 h with 2% BSA in DPBS at room temperature. Immunolabeling was conducted in blocking solution with proper diluted antibodies—anti-HA antibodies and anti-FLAG antibodies — and Alexa-labelled secondary antibodies (anti-streptavidin Alexa 647 antibodies, mouse anti-Alexa 488 antibodies, rabbit anti-Alexa 568 antibodies, and rabbit anti-Alexa 647 antibodies with extensive washes. Immunofluorescence images were obtained and analyzed using LSM 880 (IBS, Center for Genomic Integrity, Korea), LSM780 (UOBC in UNIST, Korea) with alpha Plan-Apochromat 100x/ NA: 1.46 and PMT detectors, controlled by Zen software, and SP8 X, Leica (NICEM in Seoul national university, korea) with objective lens (HC PL APO 100x/1.40 OIL), White Light Laser (WLL, 470–670 nm, 1 nm tunable laser), HyD detector, and controlled by LAS X software. And Airyscan was carried out for high-resolution imaging using a confocal laser scanning microscope (Carl Zeiss LSM780, Germany) with 63X/1.43NA objective. The Airyscan confocal images were obtained at UNIST optical biomed imaging center. The laser and emission filter were properly applied according to dye (488 nm laser and BP420-480 + BP495-550 filter for EGFP; 561 nm laser and BP495-550 + LP570 filter for mCherry; and 647 nm laser and BP570-620 + LP645 filter for Alexa 647 dye).

siRNA-mediated RNA interference

For siRNA transfection in HEK293T cells, cells were transfected with scrambled negative control siRNA (SN-1001, Bionner) or FKBP8-specific siRNA (custom sequence [AAGAGUGGCUGGACAUUCUGG], Thermo Fisher Scientific) using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were incubated for 48 h before lysis.

Correlative light and electron microscopy (CLEM)

HEK293T cells was grown in 35-mm glass grid-bottomed culture dishes to 50%−60% confluency. SCO1-APEX2 stable cells with and without FKBP8 RNAi treatment, FKBP8–APEX2 overexpressed cells, control cells were fixed with 1 % glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate solution (pH 7.0). After washing, 20 mM glycine solution was used for blocking. DAB staining was done for approximately 5−45 min until a light brown stain is visible under an inverted light microscope. DAB stained cells were post-fixed with 2% osmium tetroxide in distilled water for 1 h at 4°C

and dehydrated with a graded ethanol series. Before dehydration, some samples were stained with TCH, reduced osmium, uranyl acetate, and lead following NCMIR method to enhance contrast. Selected stained cells as DAB signal under light microscopy were then embedded into epoxy medium using flat embedding method (PMID: 25981764) (EMS, USA). 70-nm sections were cut horizontally to the plane of the block (UC7; Leica Microsystems, Germany) and were mounted on copper grids. Sections without TCH en-block staining were stained with uranyl acetate and lead citrate. The apex signals and cellular organelles were observed at 200kV in Tecnai G2 (Thermo Fisher, Waltham, MA, USA).

Quantification of mitochondria and ER contacts from EM images

The 16 mosaic images per one cell were analyzed using ImageJ (National Institutes of Health, USA). Mitochondria and ER were delimitated using the free hand selection tool of ImageJ. The selected areas were converted to masks corresponding object identification. The perimeter of ER, rate of ER adjacent to mitochondria, and the perimeter of ER adjacent to mitochondria to total ER perimeter were measured using 5 cells in each group. In addition, number and area of mitochondria were calculated.

Measurement of calcium concentration in ER and mitochondria

Hela cells were plated on 18-mm coverslips at 150,000 cells/well (12-well plate) and transfected with plasmids using Lipofectamin 2000 (Invitrogen). Four hours after transfection, cells were maintained in DMEM (Hyclone) supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific) and 1% penicillin/streptomycin. Live calcium imaging was performed 72 hours after transfection. For calcium imaging, the medium was substituted to Hank's buffered salt solution (HBSS) supplemented with HEPES (pH7.4; 2.5mM). Cells were stimulated with 200 μM histamine (Sigma Aldrich) under an inverted microscope (model IX71; OLYMPUS) with a UPLSAPO 20x / 0.75 NA objective and a CCD camera (model C9100-13; Hamamatsu Photonics).

To specify the effect of FKBP8 at MAM, we transfected rapamycin-inducible bridge-forming module (RiBFM) (eBFP-FRB-Sac1-IRES-AKAP1-FKBP12) (2). The fluorescence intensities were recorded by Cellsense software at an interval of 2s. Background fluorescence was subtracted, and the amplitude (ΔF/F0) of each cell was calculated as (F−F0)/F0, where F0 is the baseline GCaMP6-mt or RCEPIA1-er fluorescence signal averaged over a five-second period ahead of histamine stimulation, and the peak intensity of fluorescence in response to histamine was designated as Fmax.

Measurement of mitochondrial membrane potentials

Hela cells were plated and transfected with pCMV-BFP and either scramble siRNA or FKBP8 siRNA. After transfection 72 hours, cells were incubated with 1nM Tetramethylrhodamine methyl ester (TMRM, Merck) for 45min, the medium was substituted to Tyrode's buffer (145 mM NaCl, 5 mM KCl, 10 mM glucose, 1.5 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES pH 7.4) and 1nM TMRM. Cells were imaged 20x (0.75 NA) objective on an inverted confocal microscope (model IX71; OLYMPUS equipped with a CCD camera (model C9100-13; Hamamatsu Photonics). Time-lapse imaging was performed for 12 min and images were captured (1 image/min for 5 min) to establish a baseline. To depolarize mitochondrial membrane potential, cells were treated with 2μM FCCP (Sigma) and 5μg/ml Oligomycin.

Proteome digestion and enrichment of biotinylated peptides

Cells were grown in three T75 flasks for triplicate mass analysis per one condition. For the transiently expressed Contact-ID in HEK293T, cells were induced at 60–80% confluence and transfected with plasmids using Lipofectamin 2000. 50 μM of biotin was added for biotinylation. After overnight (16 hrs) labeling, the cells were washed three times with DPBS and then lysed with 1.5 mL 2% SDS in 1× TBS (25 mM Tris, 0.15 M NaCl, pH 7.2, Thermoscientific, 28358), 1 × protease inhibitor cocktail. Lysates were clarified by ultrasonication (Bioruptor, diagenode) for 15 min with cold water bath. Then, the two T75 flask samples were combined (~3 mL). For removal of free biotin, 6 times the sample volume of cold acetone

(-20°C, Sigma-Aldrich, 650501) was added to each lysate and kept at -20°C. After at least two hours, samples were centrifuged at 13,000 × g for 10 min at 4°C. Supernatant was removed gently. 6.3 mL of cold acetone and 700 µL of 1× TBS were added to pellet. Samples were vortexed vigorously and kept at -20°C. After two hours to overnight, samples were centrifuged at 13,000 × g for 10 min at 4°C. Supernatant was removed gently. Allow pellet to air dry 3–5min. Pellet was resolubilized with 1 mL of 8 M urea (Sigma-Aldrich, U5378) in 50 mM ammonium bicarbonate (ABC, Sigma-Aldrich, A6141). Concentration of protein was measured by BCA assay. Samples were denatured at 650 rpm for 1 h at 37°C using Thermomixer (Eppendorf). The samples were reduced by adding dithiothreitol (Sigma-Aldrich, 43816) to 10 mM final concentration and incubated at 650 rpm for 1 h at 37°C using Thermomixer. The samples were alkylated by adding iodoacetamide (Sigma-Aldrich, I1149) of 40 mM to final concentration and mixed at 650 rpm for 1 h at 37°C using Thermomixer. The samples were diluted eight times using 50 mM ABC. CaCl2 (Alfa aesar, 12312) was added to 1 mM final concentration. Trypsin (Thermoscientific, 20233) was added to each sample (50:1 w/w). Samples were incubated at 650 rpm for 6–18 h at 37°C using Thermomixer. Samples were centrifuged at 10,000 × g for 3 min to remove insoluble material. Then, 300 µL of streptavidin beads (Pierce, 88817) was washed with 2 M urea in 1× TBS for four times and added to the sample. The samples were rotated for 1 h at room temperature. The flow-through fraction was kept, and the beads were washed twice with 2 M urea in 50 mM ABC. After removing the supernatant, the beads were washed with pure water in new tubes. Biotinylated peptides were heated at 60°C and mixed at 650 rpm after adding 500 µL 80% acetonitrile (Sigma-Aldrich, 900667), 0.2% TFA (Sigma-Aldrich, T6508), and 0.1% formic acid (Thermoscientific, 28905). Each supernatant was transferred to new tubes. Repeat elution step four more times. Combined elution fractions were dried using Speed vac (Eppendorf). Samples can be stored -20°C or injected to mass spectrometry directly.

Tunicamycin and DTT treatment for proteomics analysis and EM imaging experiment.

Contact-ID was used for profiling dynamically changed MAM proteome components under pharmacologically stressed conditions using tunicamycin and/or reducing agents that have been reported to modulate MAM physiology (4, 5). For the sample preparation of mass analysis under the ER stress condition, tunicamycin (Sigma T7765, 0.5 µg/ml for 4 hrs prior to lysis) or/and dithiothreitol (DTT, 1mM for 2 hrs prior to lysis) was treated in the culture media of the stably expressed Contact-ID cell line (Flip-in Hek293T-Rex) during the biotin (50 µM) treatment for 16 hrs. The tunicamycin and DTT treatment was followed by the previous reports. After cell lysis, mass sample preparation was followed by the protocol written in "**Proteome digestion and enrichment of biotinylated peptides**." For the EM imaging experiment, tunicamycin 0.5 µg/ml for 4 hrs) or/and DTT (1mM for 2 hrs) was treated after siFKBP8 (for FKBP8 silencing) or mCherry-FKBP8 transfection (for the overexpression of FKBP8) for 48 hrs onto HEK293T cells. HRP-KDEL was expressed for ER tubule staining. Quantification of mitochondria-ER contact sites was followed by the protocol written in "Measurement of mitochondria and ER from EM images".

LC-MS/MS analysis of enriched peptide samples.

Analytical capillary columns (100 cm x 75 µm i.d.) and trap columns (2 cm x 150 µm i.d) were packed in-house with 3 µm Jupiter C18 particles (Phenomenex, Torrance). The long analytical column was placed in a column heater (Analytical Sales and Services) regulated to a temperature of 45°C. NanoAcquity UPLC system (Waters, Milford) was operated at a flow rate of 300 nL/min over 2 h with linear gradient ranging from 95% solvent A (H2O with 0.1% formic acid) to 40% of solvent B (acetonitrile with 0.1% formic acid). The enriched samples were analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) equipped with an in-house customized nanoelectrospray ion source. Precursor ions were acquired (m/z 300-1500)at 120 K resolving power and the isolation of precursor for MS/MS analysis was performed with a 1.4 Th. Higher-energy collisional dissociation (HCD) with 30% collision energy was used for sequencing with auto gain

control (AGC) target of 1e5 . Resolving power for acquired MS2 spectra was set to 30 k at with 200 ms maximum injection time.

FKBP8 interactome analysis by proximity labeling and immunoprecipitation

Stably expressed cell line of mCherry-BioID-HA-FKBP8 (BioID-FKBP8) was constructed in Flip-in T-rex cell line. Cells were grown in thee T75 flasks and biotin (50 μM) was treated for ## hrs and cells were lysed. For the direct biotinylated peptide detection, soluble proteins were digested to the peptide level by trypsin incubation and biotinylated peptides were enriched by streptavidin magnetic beads and analysed by the mass spectrometry (see the detail protocol in "Proteome digestion and enrichment of biotinylated peptides"). The obtained biotinylated proteome information was analysed with cytosol-BioID (mCherry-BioID) to exclude abundant cytosolic proteins in the list. The filtered protein list of BioID-FKBP8 was regarded as proximal proteins nearby FKBP8 in live cells.

To obtain the information of physically interacting proteins with FKBP8, stable cell line of mCherry-BioID-HA-FKBP8 (BioID-FKBP8) was grown in three T75 flaks and biotin (50 μM), doxycycline (195 nM) was treated for 16 hrs and cells were lysed in Triton-X (0.5 %) or 0.5 % NP40 in PBS buffer solution (pH 7.4). The lysate was mixed with anti-HA (Santacruz, sc-7392) coated Protein G magnetic bead (Thermo Scientific, 88847) for 16 hrs at 4°C. Using the same sample, we also conducted anti-Flag immunoprecipitation for the negative control experiment because bait protein (BioID-FKBP8) has no Flag epitope tag. After the collection of flow-through (FT) fraction from the each experiments, beads were washed with each lysis buffer for 2 times and 500 μl of 8M urea was added for protein elution from the bead. The small fraction of FT and elution samples were used for western blot analysis and majority of the samples were trypsin digested and biotinylated peptides were enriched by streptavidin magnetic bead (see the detail protocol of the biotinylated peptide enrichment in "**Proteome digestion and enrichment of biotinylated peptides**"). Enriched biotinylated peptides from anti-HA or anti-Flag immunoprecipitation elution sample was analysed by the mass spectrometry. The obtained biotinylated protein information from the anti-HA pull-down experiment was compared with that of anti-Flag pull-down experiment (**Figure S16e**). The filtered protein list (**Dataset S4, Tab2**) was regarded as physical interactome of FKBP8.

MS data processing and protein identification.

All MS/MS data were searched by MaxQuant (version 1.5.3.30) with Andromeda search engine at 10 ppm precursor ion mass tolerance against the SwissProt Homo sapiens proteome database (20,199 entries, UniProt (http://www.uniprot.org/)). The label free quantification (LFQ) and Match Between Runs were used with the following search parameters: semi-trypic digestion, fixed carbaminomethylation on cysteine, dynamic oxidation of methionine, protein N-terminal acetylation with biotin labels of lysine residue. Less than 1% of false discovery rate (FDR) was obtained for unique labeled peptide and as well as unique labeled protein. LFQ intensity values were log-transformed for further analysis and missing values were filled by imputed values representing a normal distribution around the detection limit. To impute the missing value, first, the intensity distribution of mean and standard deviation was determined, then for imputation values, new distribution based on Gaussian distribution with a downshift of 1.8 and width of 0.3 standard deviations was created for total matrix.
Raw file of our proteomics analysis data is publically available on ProteomeXchange consortivum via MassIVE (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp) repository (accession no. PDX015534/MSV000084362) (Download Link: ftp://massive.ucsd.edu/MSV000084362/).

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