Supplementary Figure S1 | Presence of Opa1 forms per MEF cell line. (a) (Top) Western blot detection of Opa1 forms in indicated MEF cell lines using Opa1 antibody. (Bottom) Actin was used as loading control. **(c)** Genetic schematic and cartoon depictions of Opa1 forms present in MEF cell lines used in this study.

Supplementary Figure S2 | Gallery of cryo-ET data. (a) Summed, projected central slices of cryo-electron tomograms visualizing mitochondria in wild-type, Opa1-OE, l-Opa1*, s-Opa1* and Opa1-KO MEF. White arrowheads indicate calcium deposits, blue arrowheads indicate ellipsoidal mitochondria and purple arrowheads indicate round mitochondria. **(b)** Mitochondria size (μm^2) broken down by shape per cell line. Scatter plots show data distribution, the mean is shown by a bold black line. Significance of difference is tested relative to wild type using Mann Whitney; ****p<0.0001. For b: N refers to number of mitochondria: wild-type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12. Scale bar = 200 nm.

Supplementary Figure S3 | Mitochondrial subcompartment volumes. (a) Three-dimensional renderings of segmented inter-membrane space (IMS, pink surface), cristae lumen (CL, magenta surface), and matrix (translucent grey surface) volumes. **(b)** Total mitochondrial volume across indicated cell lines. **(c)** Quantification of IMS volume, **(d)** CL volume and **(e)** matrix volume relative to total volume of each mitochondrion indicated in (b). **(f)** CL to matrix ratio and **(g)** normalized grey scale mitochondrial matrix value across cell lines. (**h**) Graph bar representing percentage of cells with detected calcium deposits in cryo-electron tomograms. Scatter plots show data distribution, the mean is shown by a bold black line. Significance of difference is tested relative to wild type using Mann Whitney test in b, d, e, g; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; and unpaired t test in (c): **p<0.01; N refers to number of cells, for b-g: N = 5 for all cell lines. For h: N refers to the number of mitochondria: wild-type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO =12. Scale bar = 200 nm.

Supplementary Figure S4 | Cristae analysis. (a) Cristae density (cristae per μm^2) and **(b)** Number of cristae per mitochondria represented as scatter plots. **(c)** (Top) Summed, projected central slices of cryo-electron tomograms visualizing mitochondria with stacking crista characteristics, supported by 3D representations consisting of their sub compartments (bottom) in indicated MEF lines. **(d)** Graph bar representing percentage of mitochondria with stacking crista formation in each MEF line. For a: N refers to number of cells, N: wild-type = 33, Opa1-OE = 7, l-Opa1* = 21, s-Opa1* = 28, Opa1-KO = 11. For b:

N wild-type = 51, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12. For (c) and (d): N wild-type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12. Scale bar = 200 nm.

Supplementary Figure S5 | Mitochondrial network morphology in MEF lines by fluorescence microscopy. Representative images of mitochondrial morphology in indicated MEF lines labeled with MitoTracker™ Deep Red FM. Insets show magnified view of regions indicated with dashed boxes. Scale bar = 10 µm. Inset scale bar = 5 µm. (**b**) Graph bar representing mitochondrial network morphology scored in indicated MEF lines. N = 100 cells analyzed per cell line.

Supplementary Figure S6 | Unusual cristae morphology. (a) Graph bar representing the relative proportion of unusual cristae morphology observed in indicated MEF lines. Unusual cristae were categorized into vesicular, zipped, ring, split, amorphous, straight-across, pinched and loop. N refers to number of cristae analyzed, N: wild-type = 222, Opa1-OE = 430, l-Opa1* = 323, s-Opa1* = 653, Opa1-KO = 243. **(b)** Summed, projected central slices of cryo-electron tomograms showing examples of unusual cristae in mitochondria across cell lines in 2D (top) and 3D (bottom). Loop, ring, straight-across, pinched, vesicular, and amorphous cristae are shown. Scale bar = 200 nm.

Supplementary Figure S7 | Cristae length, width quantification, junction width, angle. **(a)** Cartoon schematics representing sub-tomogram averaging (STA) approach for measuring crista length and **(b)** width in 3D. **(c)** Cartoon schematic for measurement of cristae junction width and **(d)** angle. See Methods for details.

Supplementary Figure S8 | **Multijunction cristae. (a)** Scatter plot showing the percentage of multijunction cristae per mitochondrion in indicated MEF lines. **(b)** Graph bar representing percentage of multijunction cristae categorized into straight-across and loop morphology in each MEF line. Scatter plot shows data distribution, the mean is marked by a bold black line. Significance of difference is tested relative to wild type using Mann Whitney; ****p<0.0001. N refers to number of cristae, for (a), N: WT = 18, Opa1-OE =5, l-Opa1* = 30, s-Opa1* = 16, Opa1-KO = 3. For (b), N: WT = 26, Opa1-OE = 9, l-Opa1* = 79, s-Opa1* = 29, Opa1-KO = 4.

Supplementary Figure S9 | **Cell viability following apoptotic priming.** Assessment of cell viability by Annexin V staining in MEF cell lines after treatment with the indicated compounds for **(a)** 48 hours and **(b)** 72 hours. N = minimum 4 biological replicates.

Supplementary Figure S10 | **Oma1$^{-/-}$ cell functional characterization. (a)** BH3 profiling of WT and Oma1$^{-/-}$ MEF for sensitizer BIM BH3 and PUMA. N = 3 biological replicates. **(b)** Representative traces of mitochondrial calcium retention capacity assays done in indicated MEF lines. **(c)** OCR plotted against time for indicated MEF lines. **(d)** Aspects of mitochondrial respiration; basal respiration rates, the amount of respiration used for ATP production, maximum respiration, and spare capacity, are extracted by the data plotted in (c). N = 3 biological replicates. Significance of difference between l-Opa1* and Oma1$^{-/-}$ is tested using Welch's t-test; *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure S11 | mtDNA maintenance characterization. (a) Representative live iSIM images of mitochondrial network (PKmito Orange, in green) and nucleoid signal, (SYBR Gold, in magenta). **(b)** Quantification of mean nucleoid area and **(c)** total nucleoid number per cell, normalized to mitochondrial area and relative to the experimental controls (median of WT cells imaged on the same day). Significance of difference is tested relative to wild type using Mann Whitney; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N refers to the number of quantified cells per MEF line, WT = 83, Opa1-OE =51, l-Opa1* = 31, s-Opa1* = 55, Opa1-KO = 58. **(d)** qPCR-based determination of mtDNA (RNR2 and ND1 probes) copy number relative to nuclear genome copies (HK2 probe), normalized to WT cells. Significance of difference is tested relative to wild type using Welch's t-test; **p<0.001. N refers to 3 biological replicates. Scale bar = 10 µm.

Movie 1: 3D renderings of WT mitochondrial membranes (OMM in green and IMM in yellow) and subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar = 200 nm.

Movie 2: 3D renderings of Opa1-OE mitochondrial membranes (OMM in green and IMM in yellow) and subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar = 200 nm.

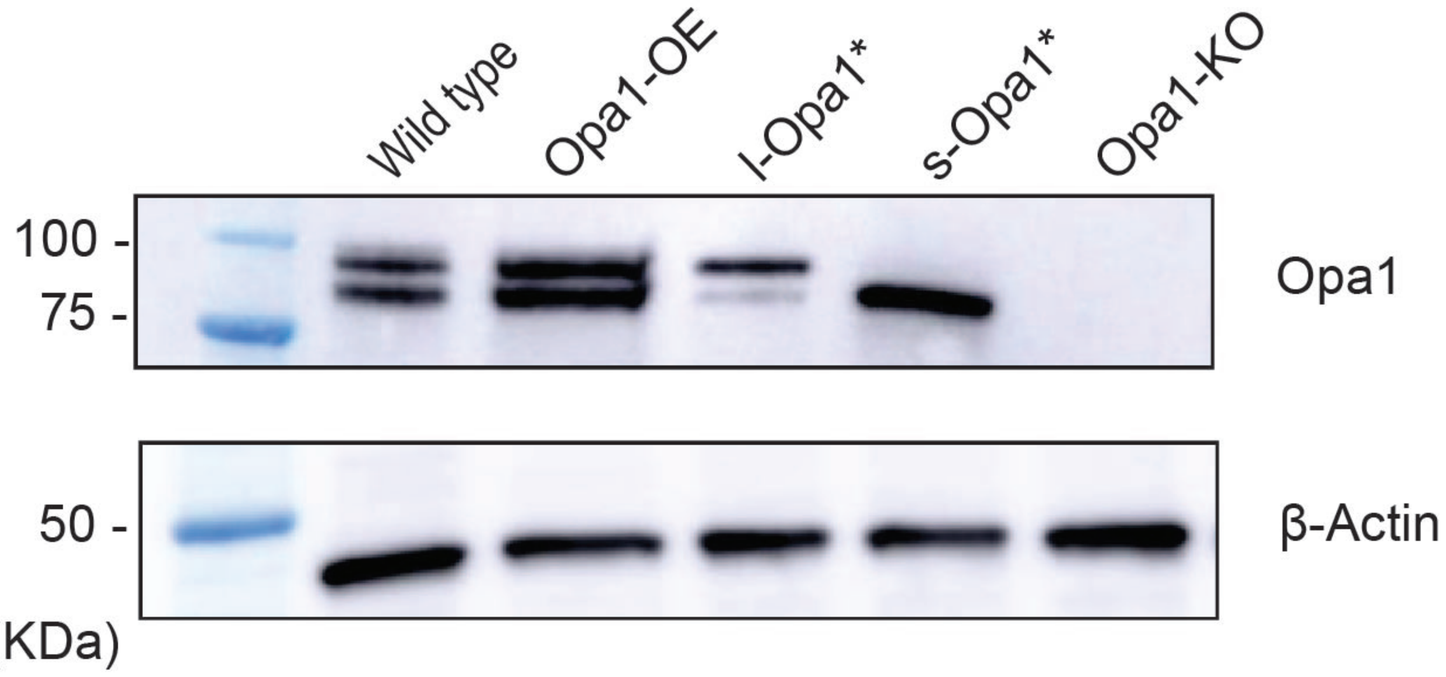
Movie 3: 3D renderings of l-Opa1* mitochondrial membranes (OMM in green and IMM in yellow) and subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar = 200 nm.

Movie 4: 3D renderings of s-Opa1* mitochondrial membranes (OMM in green and IMM in yellow) and subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar = 200 nm.

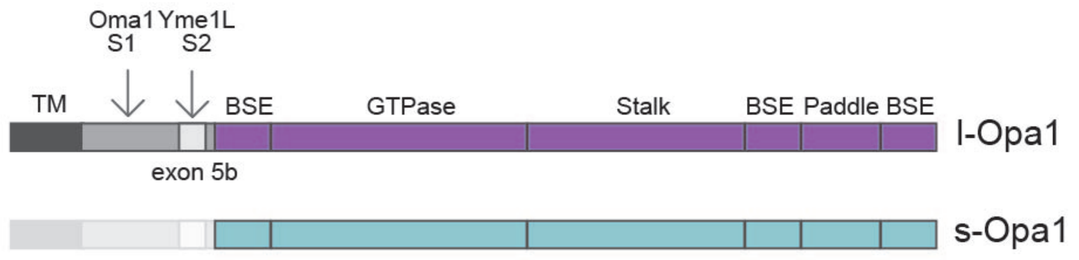
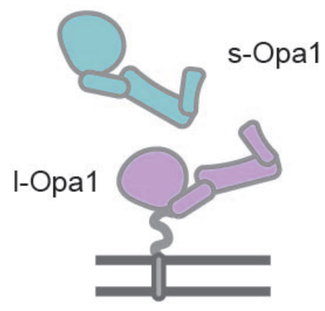
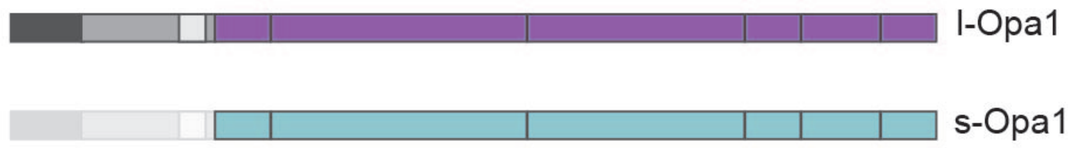
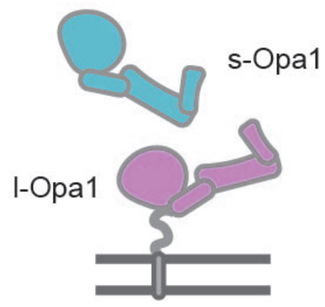
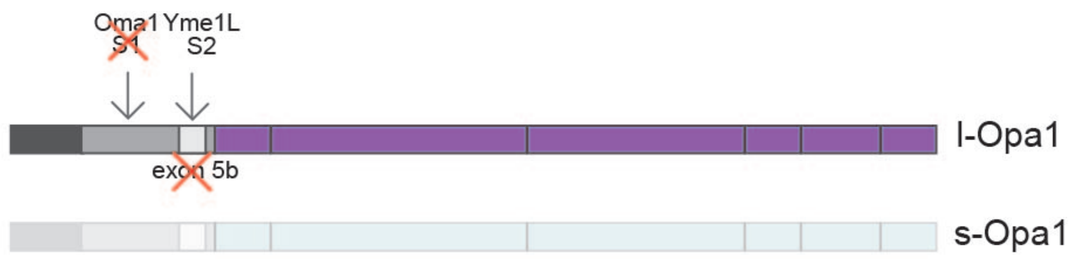
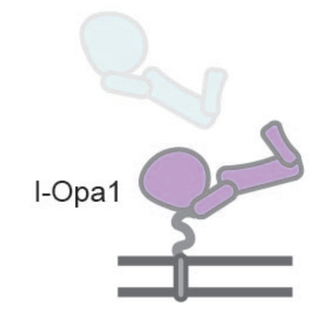
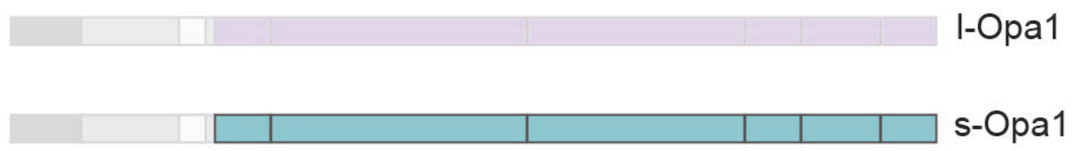
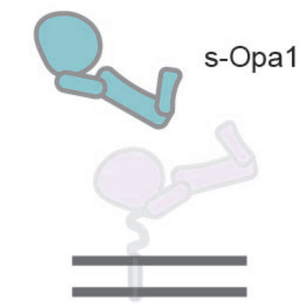
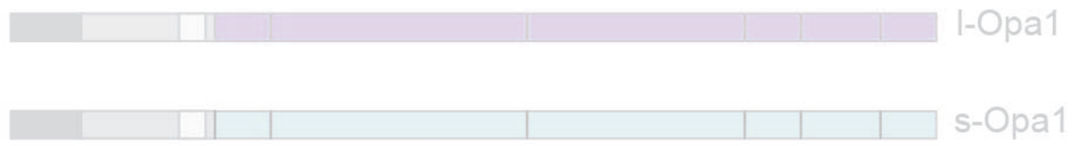
Movie 5: 3D renderings of Opa1-KO mitochondrial membranes (OMM in green and IMM in yellow) and subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar = 200 nm.

Movie 6: Live-cell fluorescence microscopy of MitoTracker™ Deep Red FM-stained mitochondria in indicated MEF cell lines. Movies were taken at 30 seconds per frame for 5 mins. Playback at 2 frames per second (60x real-time). Scale bar = 10 µm.

a



b

Name of line:	*Referred to in this study as:*	*Linear scheme for protein products produced:*	*Cartoon depiction of Opa1 form(s) present:*
WT MEF	WT		
Stable Opa1-overexpressing MEF	Opa1-OE		
MEF CRISPR Δexon5b in Oma1$^{-/-}$ background	l-Opa1*		
Opa1-KO MEF transfected with Opa1 isoform 5 only	s-Opa1*		
Opa1-KO MEF	Opa1-KO		

a

WT

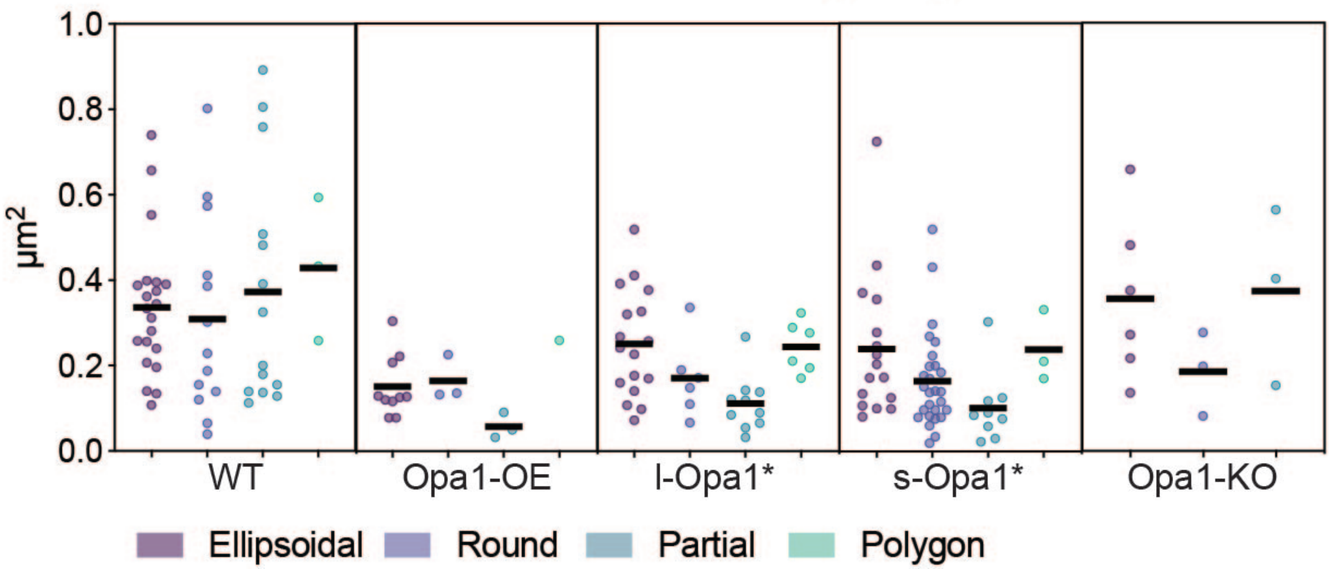
Opa1-OE

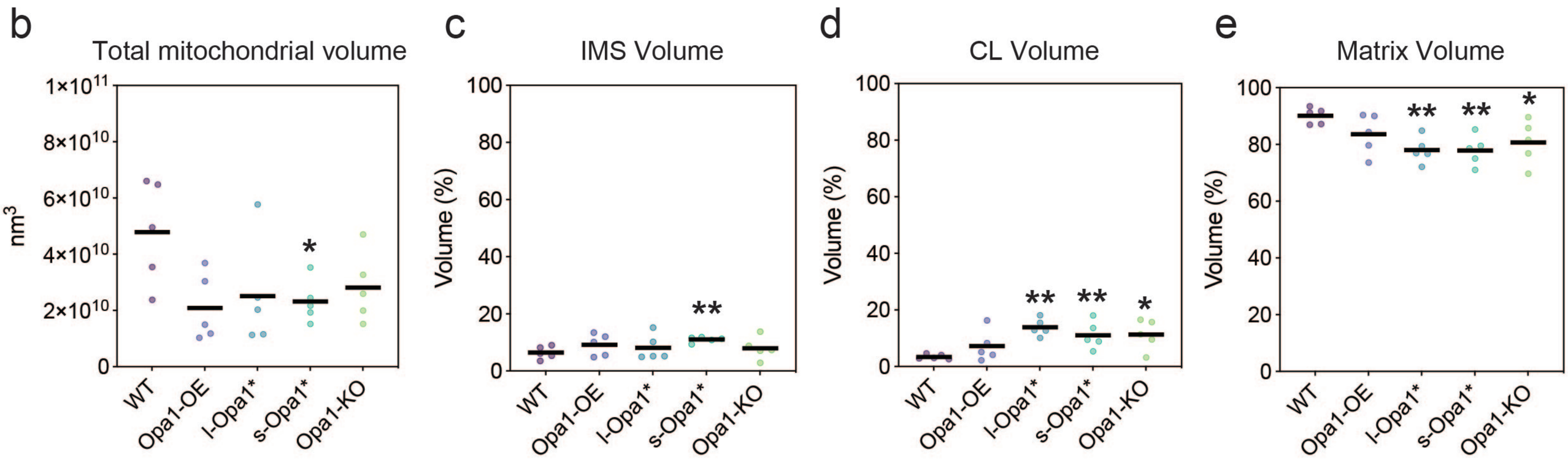
l-Opa1*

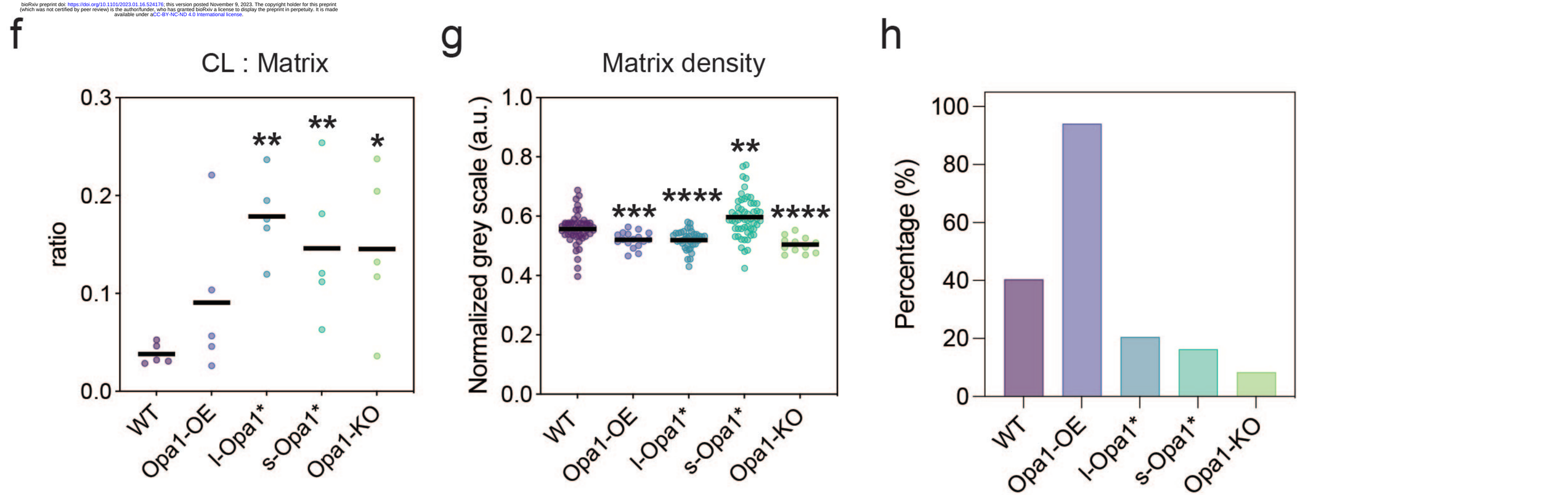
s-Opa1*

Opa1-KO

b







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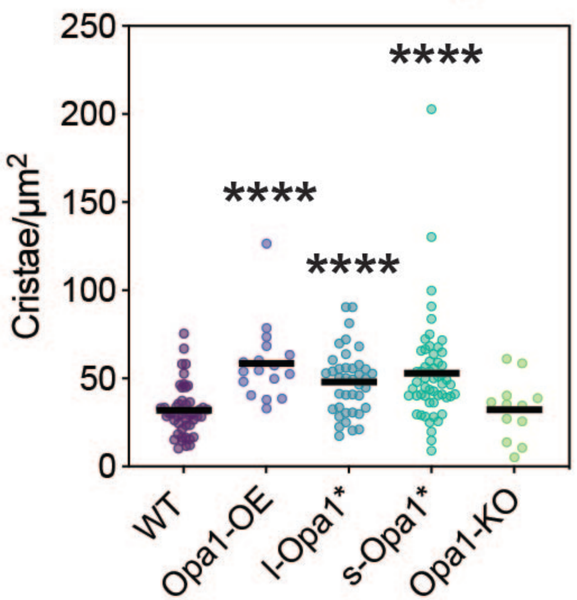

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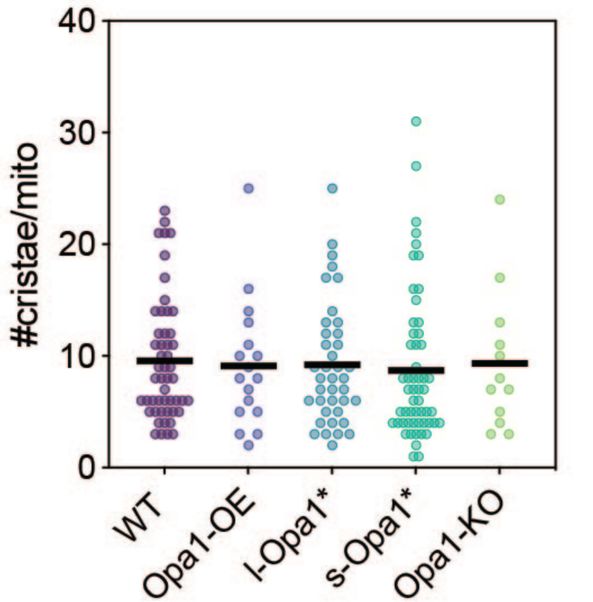

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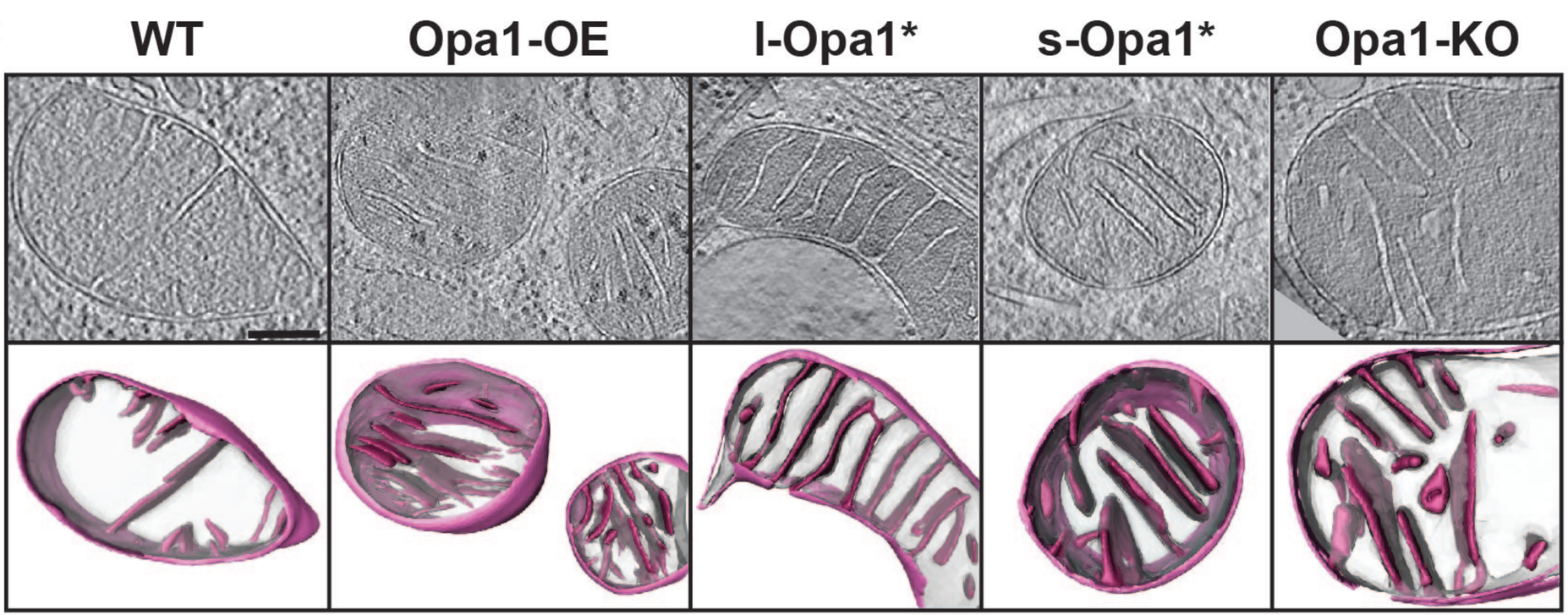

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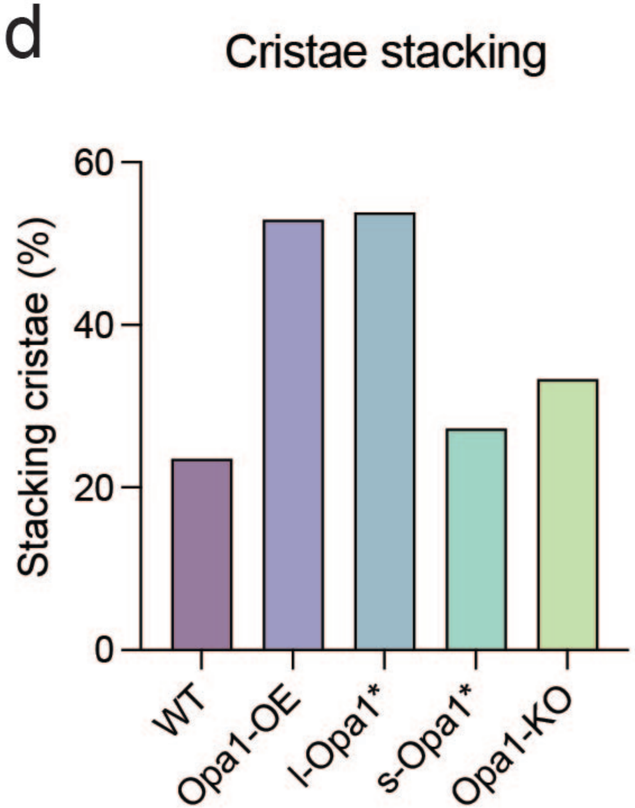

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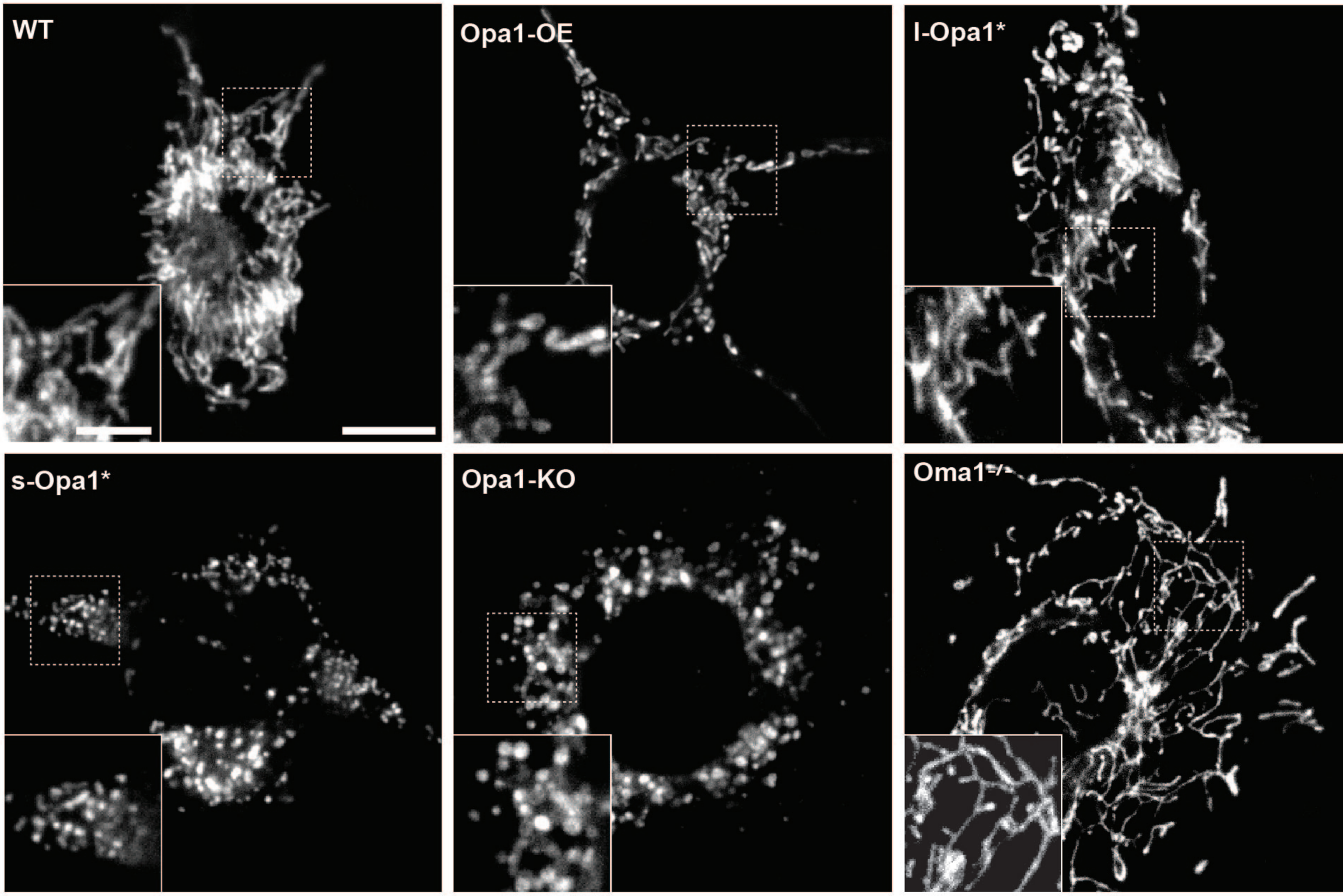

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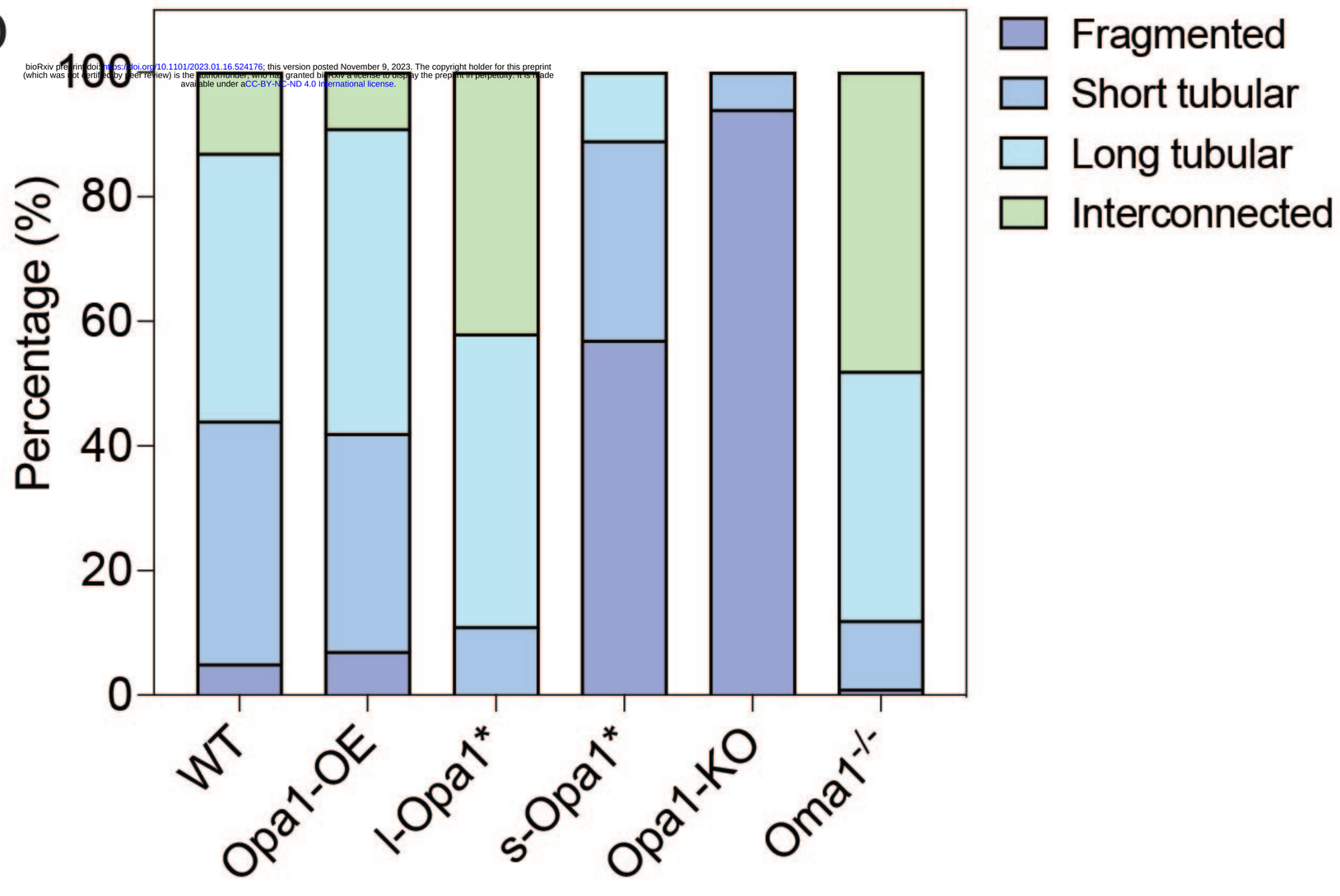

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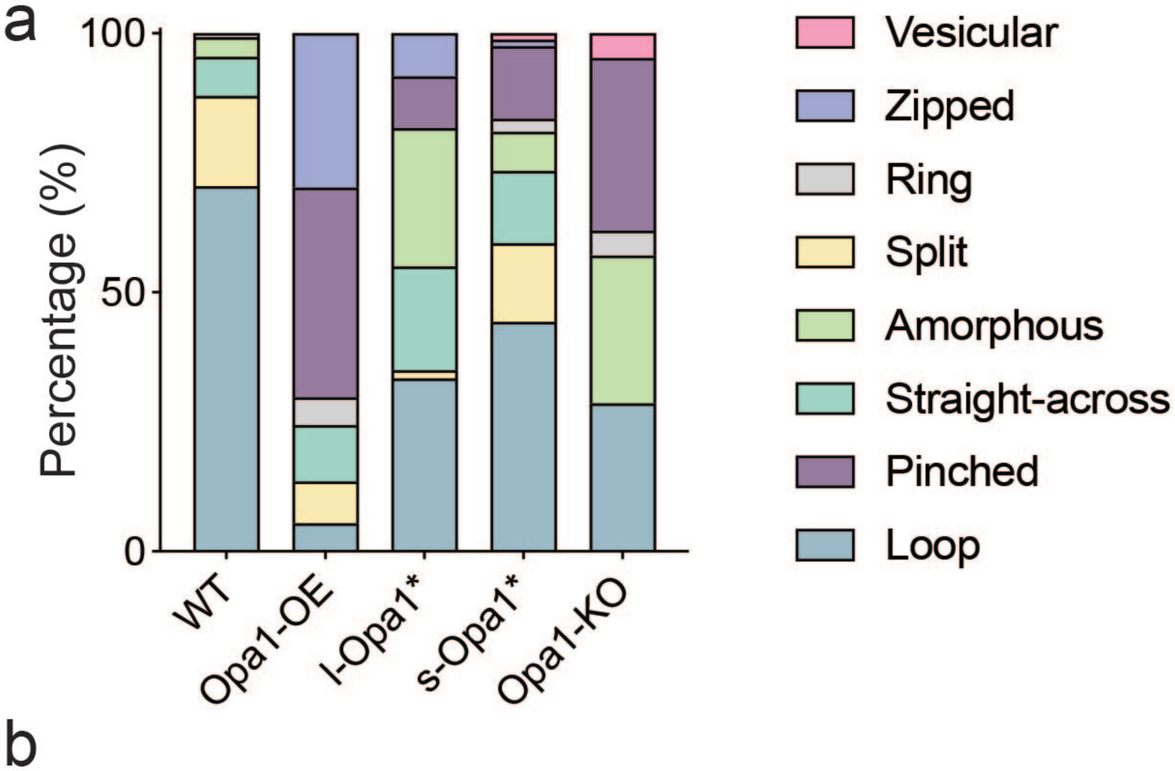

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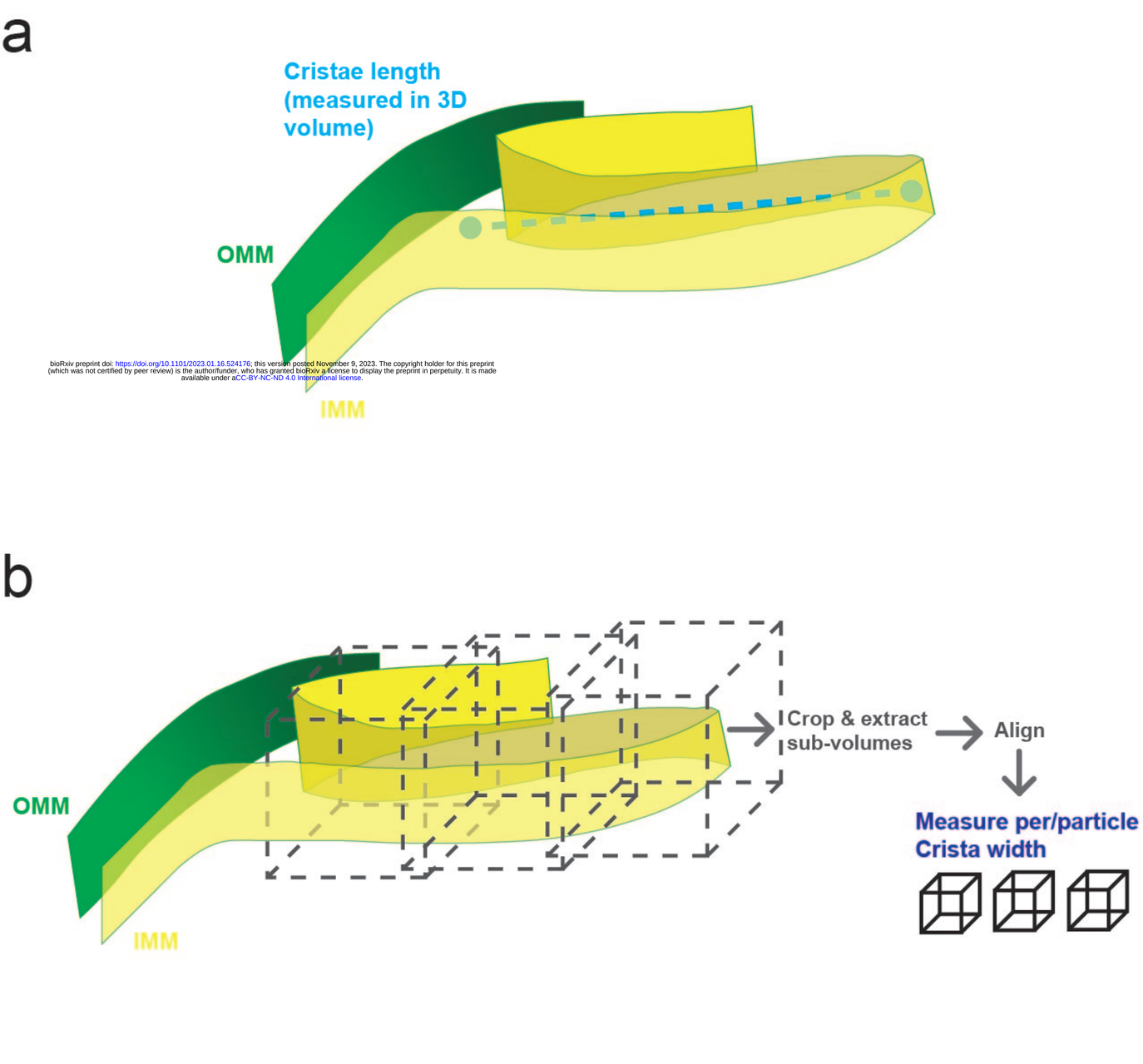

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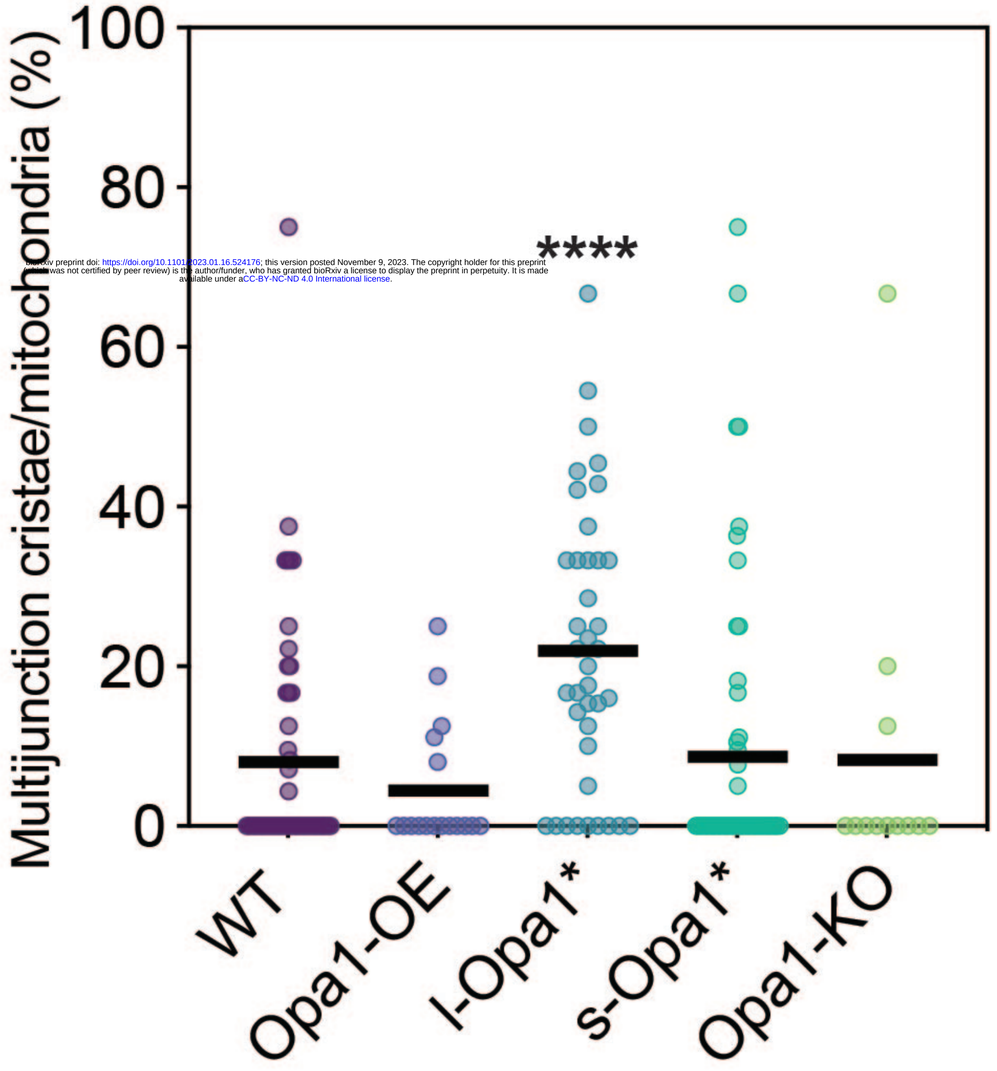

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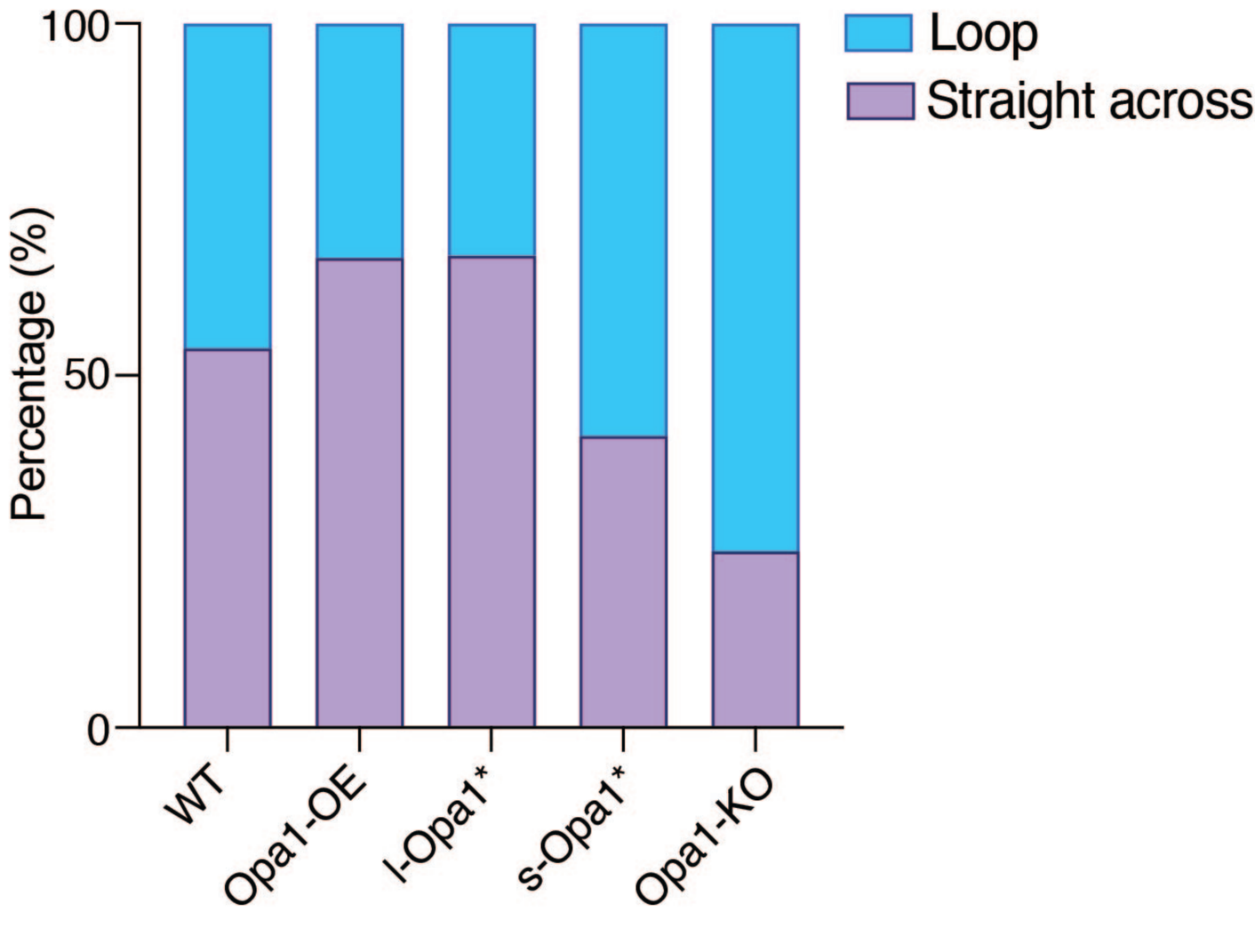

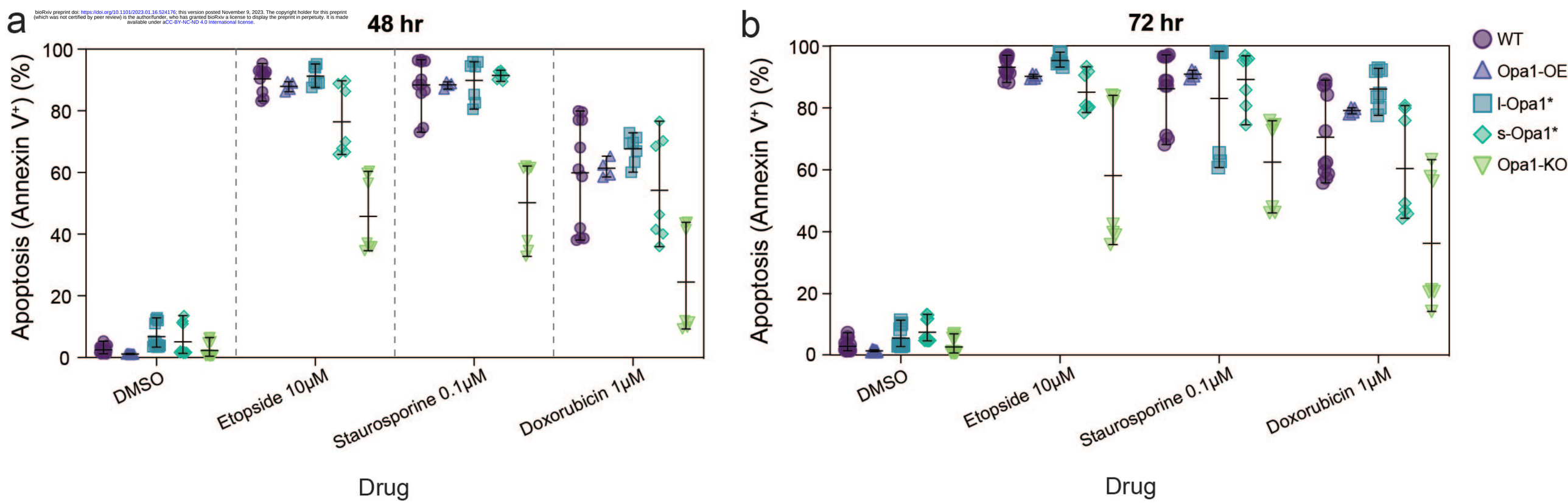

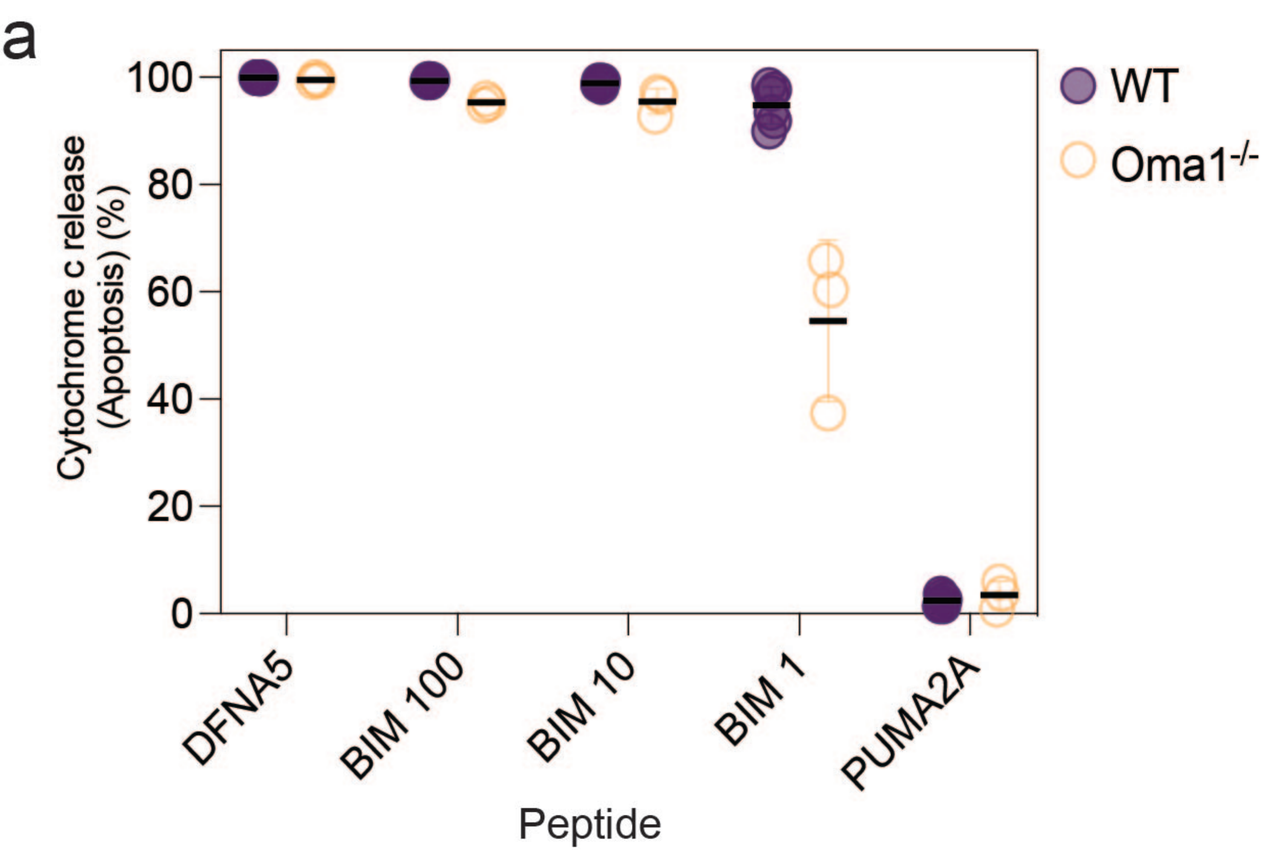

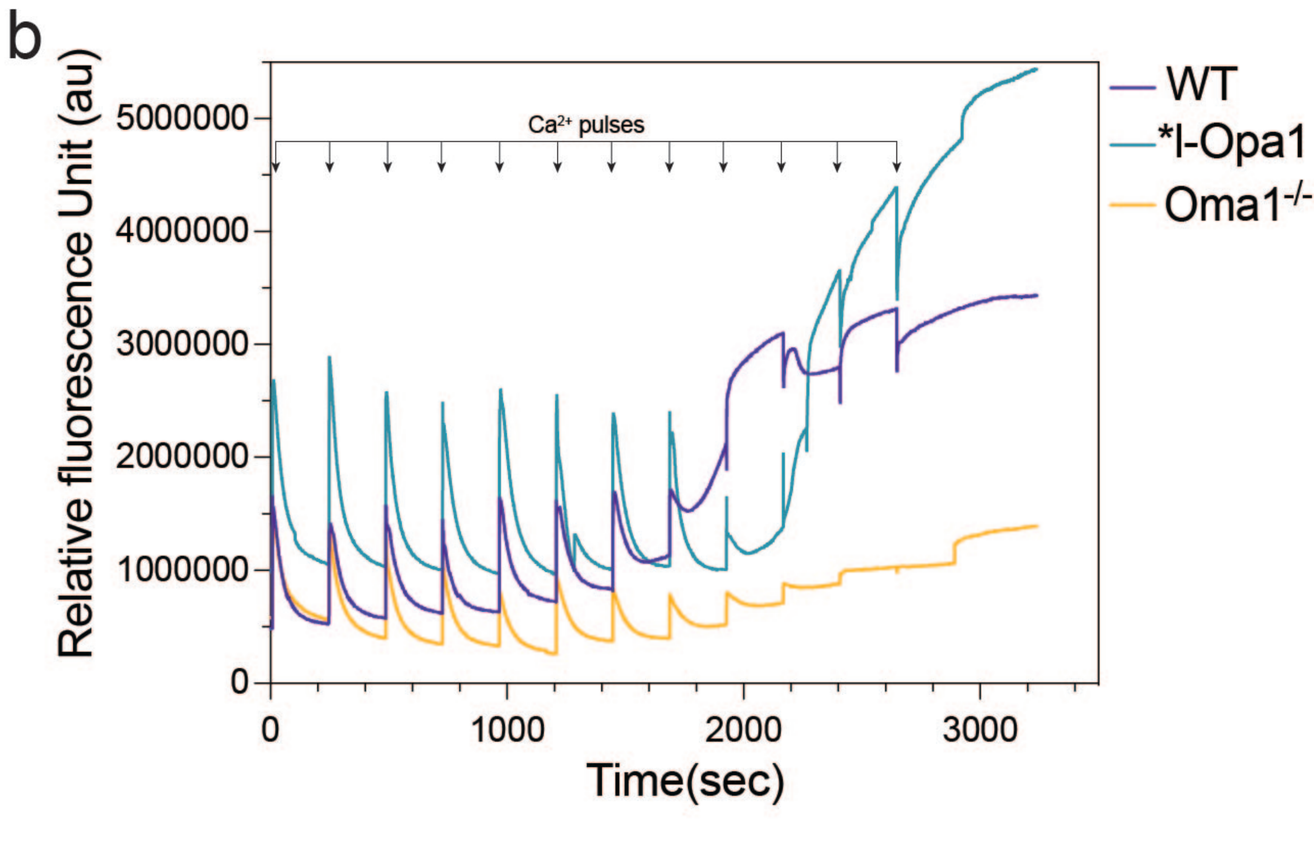

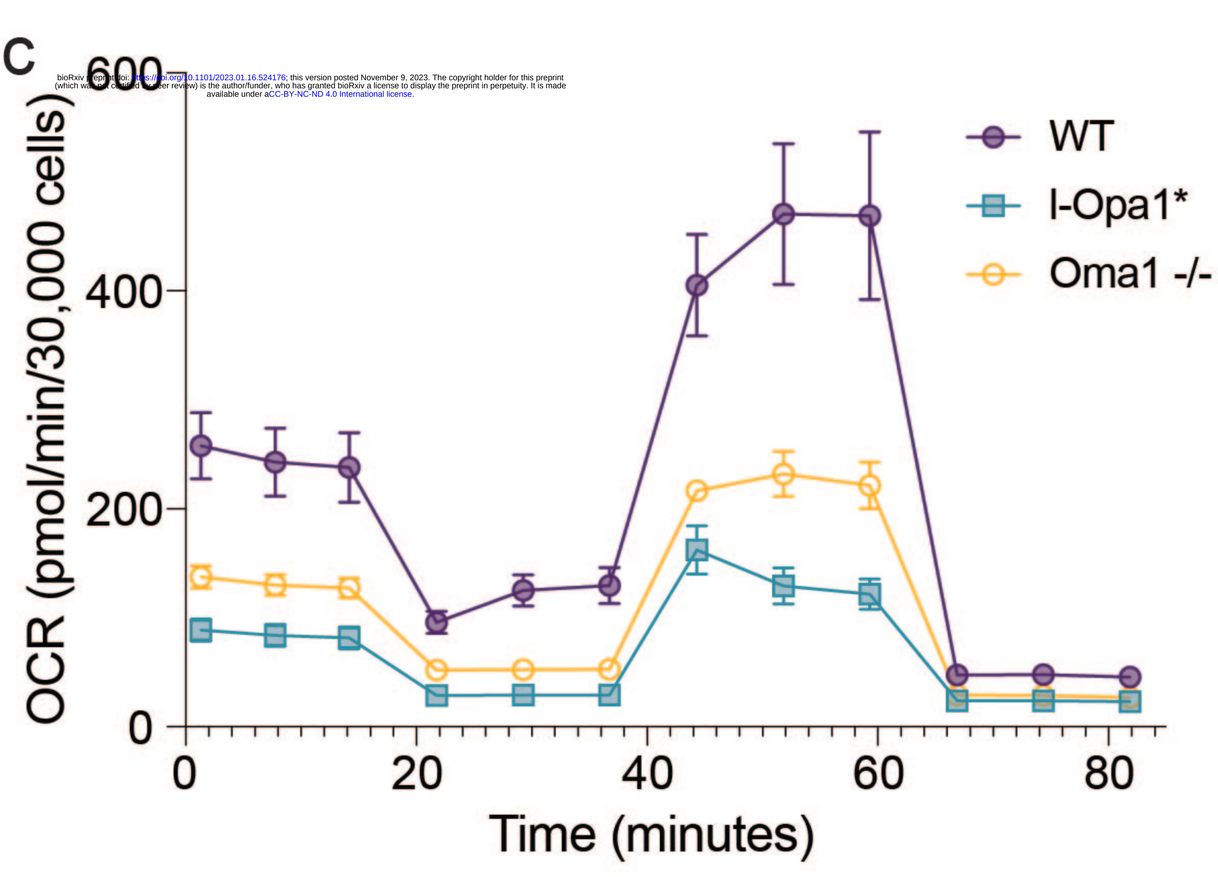

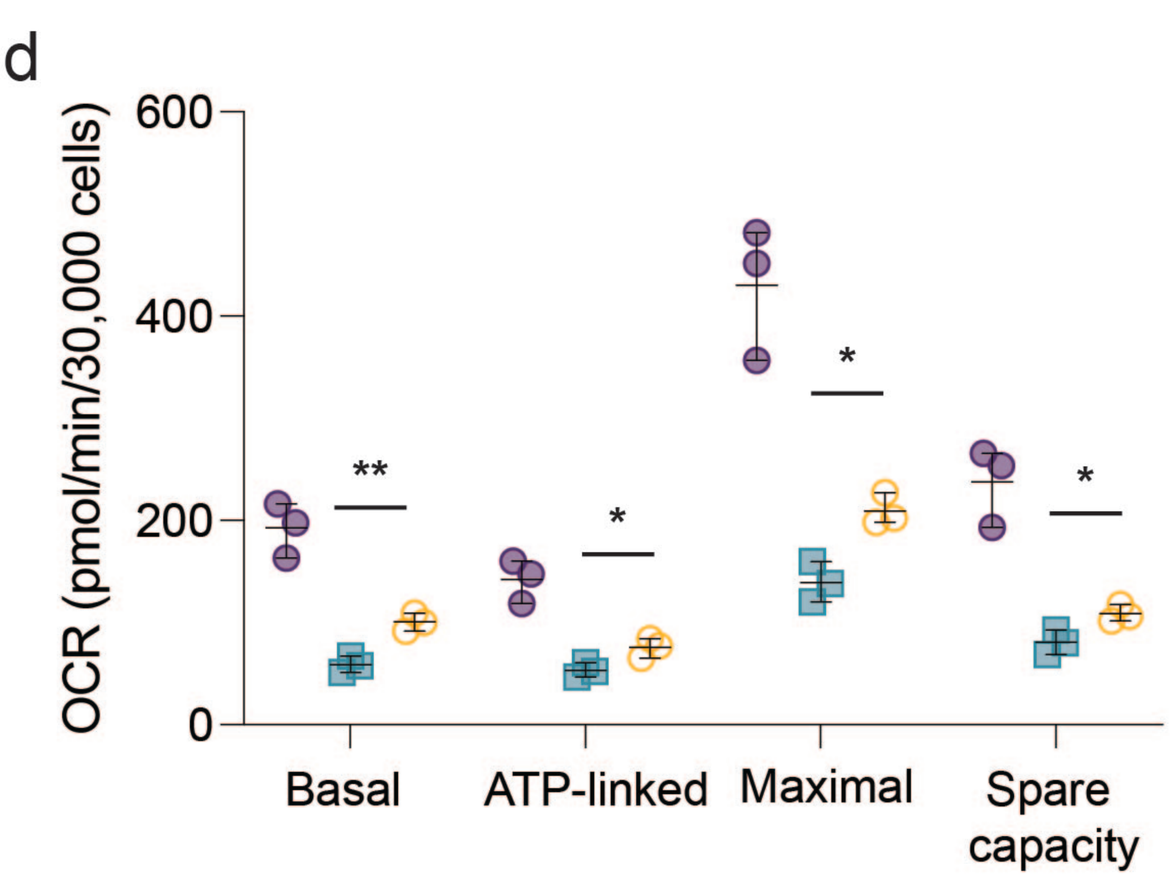

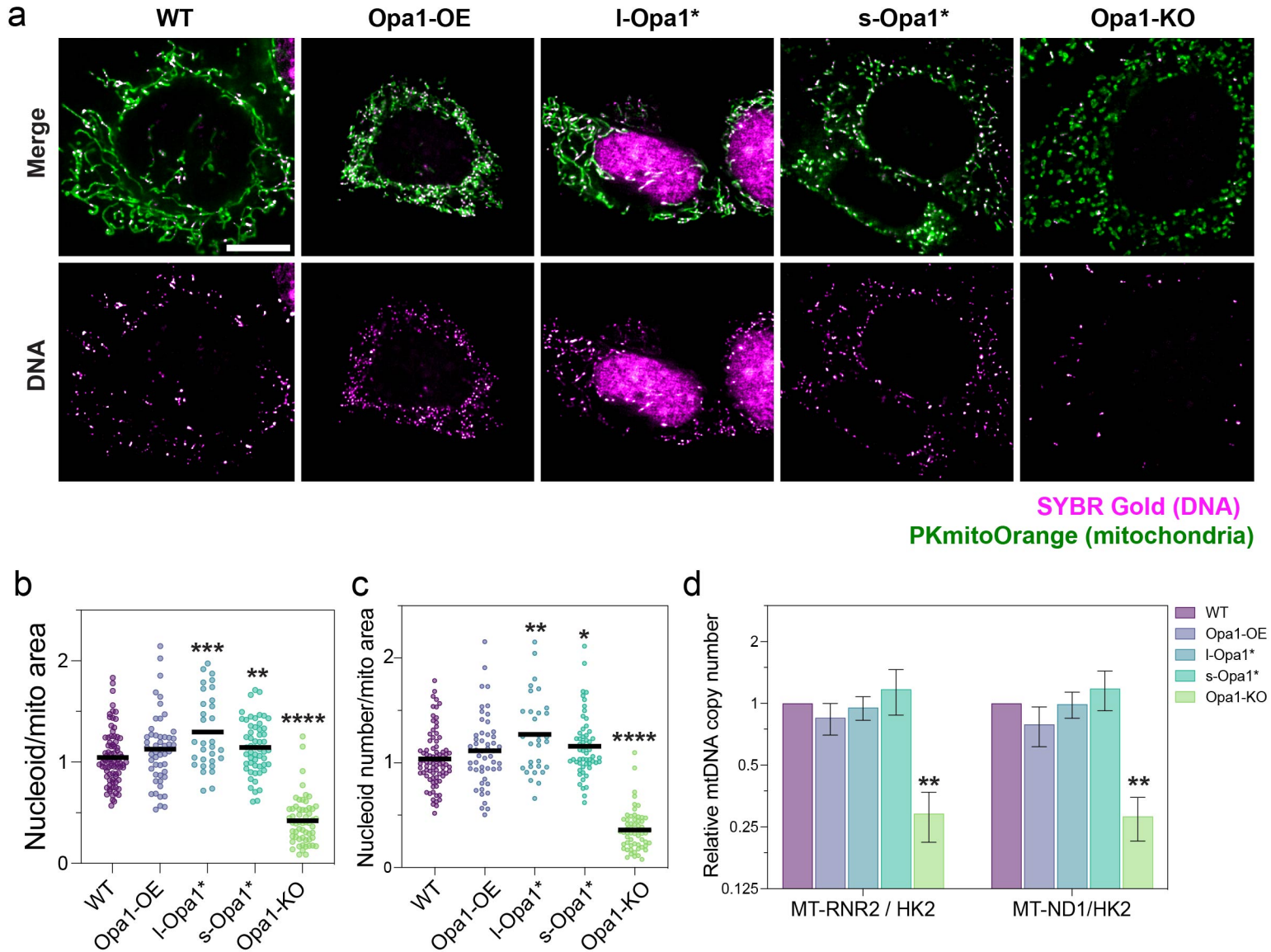
a
WT
Opa1-OE
l-Opa1*
s-Opa1*
Opa1-KO
Merge
DNA
SYBR Gold (DNA)
PKmitoOrange (mitochondria)
b
Nucleoid/mito area
0
1
2
***
**
****
WT
Opa1-OE
l-Opa1*
s-Opa1*
Opa1-KO
c
Nucleoid number/mito area
0
1
2
**
*
****
WT
Opa1-OE
l-Opa1*
s-Opa1*
Opa1-KO
d
Relative mtDNA copy number
0.125
0.25
0.5
1
2
**
**
MT-RNR2 / HK2
MT-ND1/HK2
WT
Opa1-OE
l-Opa1*
s-Opa1*
Opa1-KO

Supplementary Table 1. Summary of data acquisition and image processing for cryo-ET data in this study.

Sample		wt	Opa1-OE	l-Opa1*	s-Opa1*	Opa1-KO
Cryo-FIB milling	**Microscope**	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific
Acquisition settings	**Microscope**	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific
	Voltage (KeV)	300	300	300	300	300
	Detector	Gatan K3 IS	Gatan K3 IS	Gatan K3 IS	Gatan K3 IS	Gatan K3 IS
	Energy filter	Gatan BioQuantum K3	Gatan BioQuantum K3	Gatan BioQuantum K3	Gatan BioQuantum K3	Gatan BioQuantum K3
	Slit width (eV)	20	20	20	20	20
	Super-resolution mode	Yes	Yes	Yes	Yes	Yes
	Å/pixel	2.076	2.076	2.076	2.076	2.076
	Defocus (µm)	-3.5 to -5.0	-3.5 to -5.0	-3.5 to -5.0	-3.5 to -5.0	-3.5 to -5.0
	Acquisition scheme	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric
	Total dose	~90 - 120	~90 - 180	~90 - 180	~90 - 120	~90 - 120
	Dose rate (e-/Å/sec)	~ 2.5 – 3.5	~ 2.5 – 3.5	~ 2.5 – 3.5	~ 2.5 – 3.5	~ 2.5 – 3.5
	Frame number	6	6	6	6	6
	Number of tomograms	33	7	27	22	11
Image processing	**Frame alignment and dose weighting**	*framealign*, IMOD	*framealign*, IMOD	*framealign*, IMOD	*framealign*, IMOD[7]	*framealign*, IMOD[7]
	Tilt series alignment	IMOD	IMOD	IMOD	IMOD	IMOD
	WBP	IMOD	IMOD	IMOD	IMOD	IMOD
	Denoising	Topaz	Topaz	Topaz	Topaz	Topaz
	3D-segmentation	Amira	Amira	Amira	Amira	Amira
	3D-rendering	Amira	Amira	Amira	Amira	Amira