Figure S1. Cycloheximide treatment causes mitochondrial elongation in OPA1 cells expressing OPA1 isoform 1ΔS1.
(A) Representative images of OPA1-null cells expressing OPA1 isoform 3 or 1ΔS1, with and without cycloheximide. (B) Quantification of mitochondrial morphology in cells treated as in (A). Error bars indicate standard deviation from 3 experiments. (C) Western blot of cell lysates from wildtype cells or OPA1-null cells expressing the indicated OPA1 isoforms.

Figure S2. PEG cell hybrid assay at 7 hours.
(A) PEG cell hybrid assays were performed between the indicated parental cells expressing with mito-EGFP or mCherry-OMP25. The large inset is a magnified image of the small boxed area. Arrows point to mitochondria that are doubly labeled. (B) Quantification of the experiment in (A). Error bars indicate standard deviation from 3 experiments.

Figure S3. Mitochondrial fusion in wildtype MEFs monitored with matrix localized mitochondrial markers.
Mito-DsRed and mito-PA-GFP were expressed in wildtype MEFs. A subset of mitochondria were photo-activated and mitochondrial fusion was monitored by time-lapse confocal imaging. (A). The levels of GFP and DsRed fluorescence within two mitochondria (indicated by the white rectangles in B) are plotted in arbitrary units for each still frame. Between frames 4 and 5 (the region marked by a blue frame), the GFP fluorescence of mitochondrion *a* suddenly falls, and its DsRed fluorescence correspondingly increases. At the same time, the GFP fluorescence of mitochondrion *b* increases. These fluorescence changes are due to fusion of mitochondrion *a* with *b* (see B). (B). Still frames 4 and 5, showing the fusion of mitochondrion *a* with *b*. White boxes indicate areas used for fluorescence measurements in A. With photoactivation of PA-GFP, DsRed fluorescence is reduced due to photobleaching.

Figure S4. Assay for outer membrane fusion versus inner membrane fusion. Mito-DsRed and PA-GFP-OMP25 were expressed in wildtype MEFs. A subset of mitochondria were photo-activated, and the cells were imaged as in Figure S1. (A). Time course of DsRed and PA-GFP levels in two mitochondria. The region indicated by a blue frame shows the stepwise changes associated with a fusion event. (B). Still frames 3 and 4, showing full fusion of mitochondrion *a* with *b*. Note that both the outer membrane marker (PA-GFP-OMP25) as well as the matrix marker (mito-DsRed) are exchanged.

Figure S5. (A) Electron micrograph of mitochondria in wildtype cells. (B) Mitochondria in Mfn-null cells.

Figure S6. Knockdown of Phb1.
Western blot of cell lysates from wildtype cells or cells expressing shRNAi against Phb1. Anti-actin was used as a loading control.

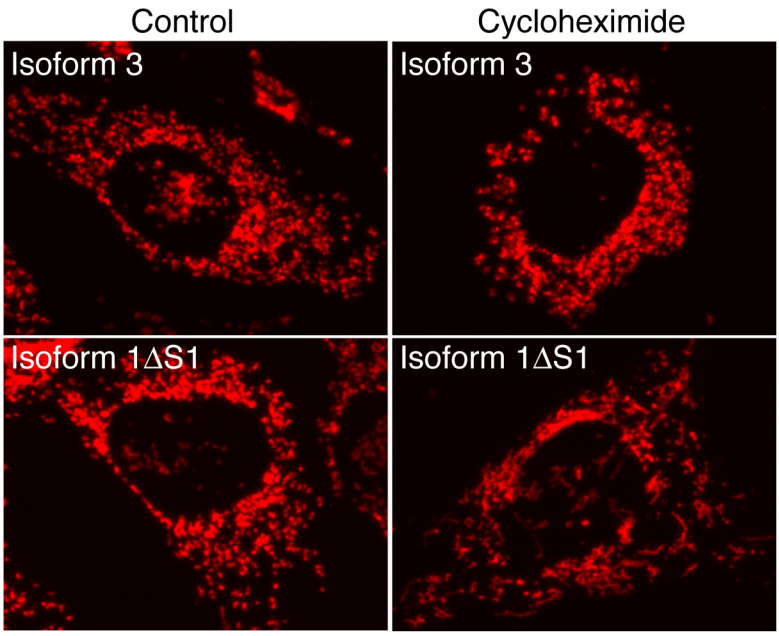
Movie S1. Time-lapse movie of mitochondrial outer membrane fusion in OPA1-null cells.
Photoactivated mitochondria in OPA1-null cells (expressing PA-GFP-OMP25 and mito-DsRed) were tracked by time-lapse confocal imaging. Some examples of mitochondrial outer membrane fusion are indicated by arrowheads. This movie shows only the green channel to facilitate

visualization of PA-GFP-OMP25 transfer. Movie S2 shows both the green and red channels. The movie represents 7 minutes of recording.

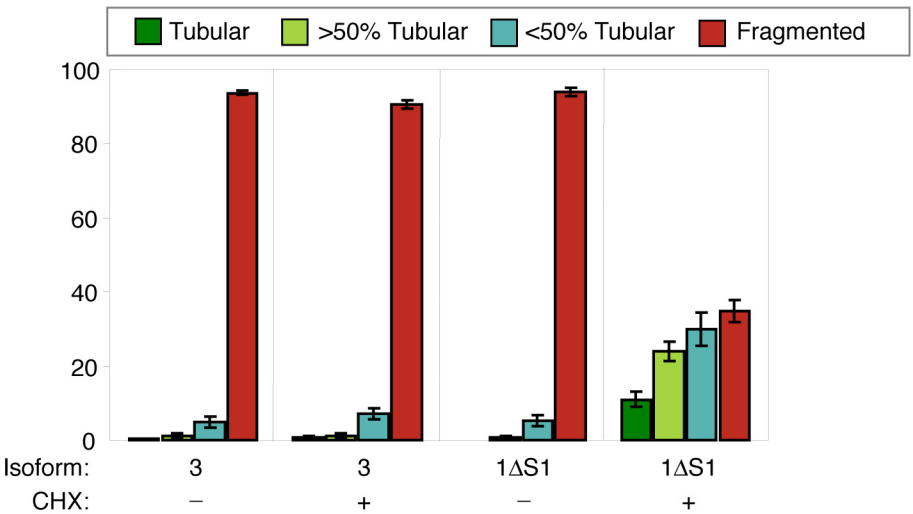
Movie S2. Time-lapse movie of mitochondrial outer membrane fusion in OPA1-null cells. Same as Movie S1, except that both green and red channels are shown. Note that no mito-DsRed transfer occurs in the indicated outer membrane fusion events.

Fig. S1

A


Control
Cycloheximide
Isoform 3
Isoform 3
Isoform 1ΔS1
Isoform 1ΔS1

B


Tubular
>50% Tubular
<50% Tubular
Fragmented
100
80
60
40
20
0
Isoform:
3
3
1ΔS1
1ΔS1
CHX:
–
+
–
+

C

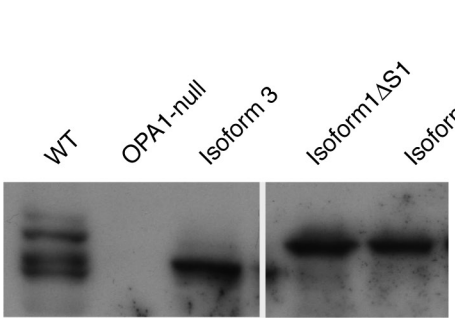

WT
OPA1-null
Isoform 3
Isoform1ΔS1
Isoform1ΔS1+CHX

Fig. S2

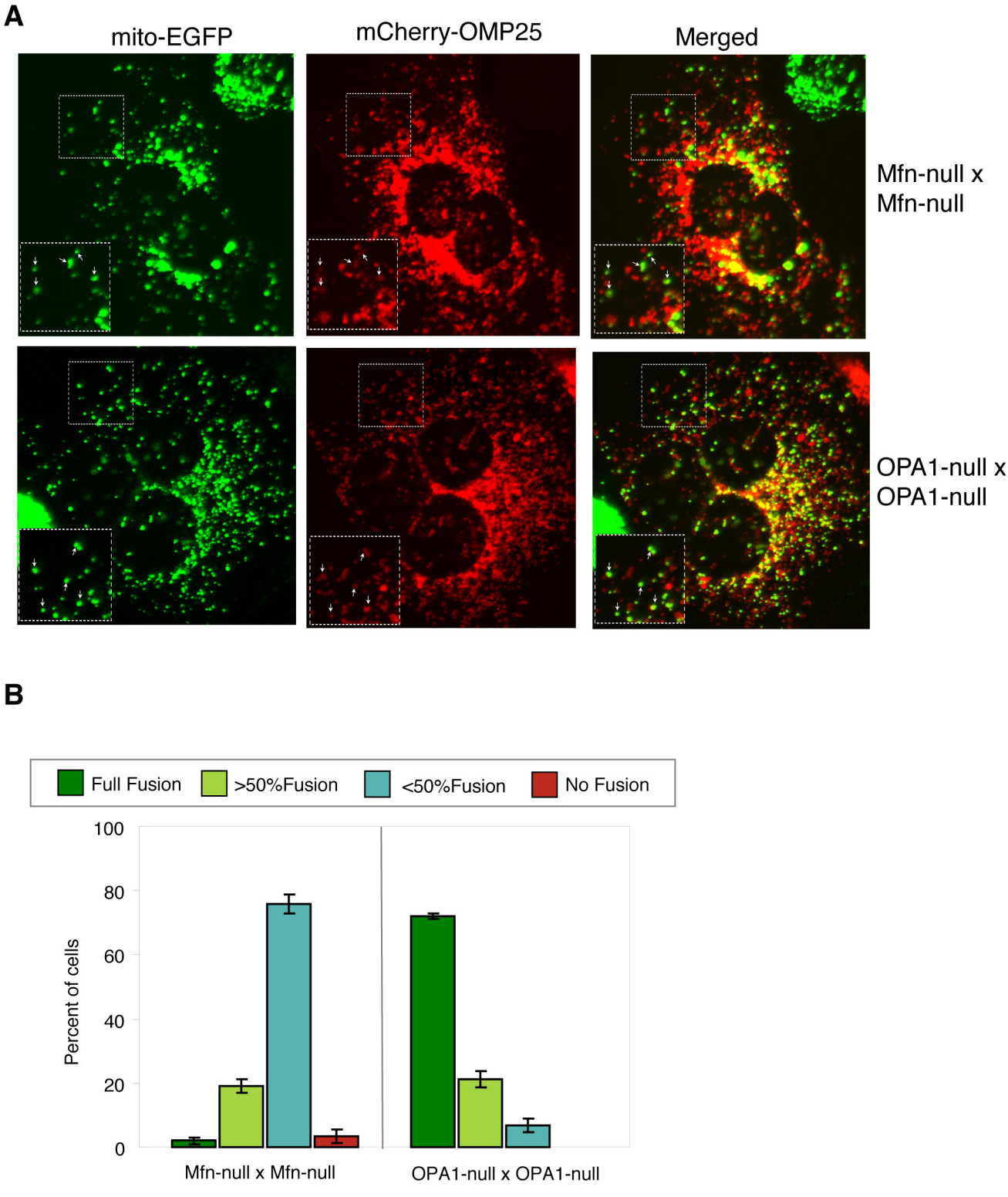
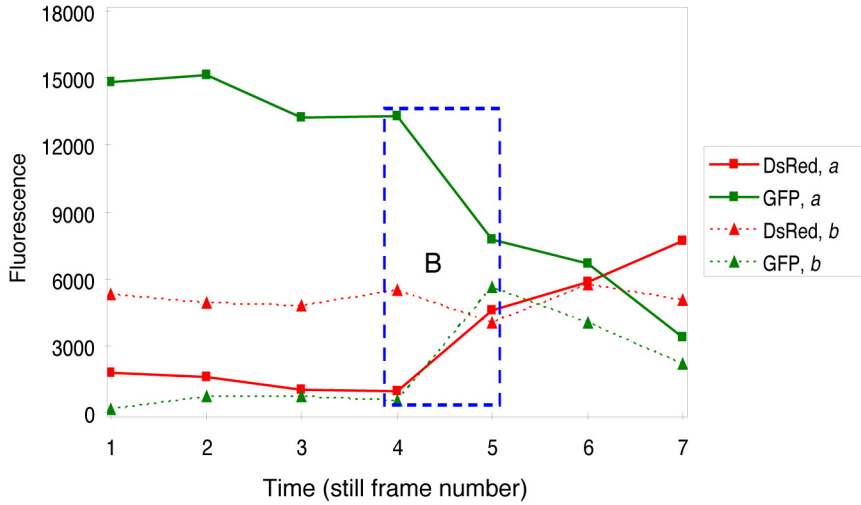

A
mito-EGFP
mCherry-OMP25
Merged
Mfn-null x Mfn-null
OPA1-null x OPA1-null
B
Full Fusion
>50%Fusion
<50%Fusion
No Fusion
Percent of cells
100
80
60
40
20
0
Mfn-null x Mfn-null
OPA1-null x OPA1-null

Fig. S3

A


18000
15000
12000
9000
6000
3000
0
Fluorescence
1
2
3
4
5
6
7
Time (still frame number)
B
DsRed, a
GFP, a
DsRed, b
GFP, b

B

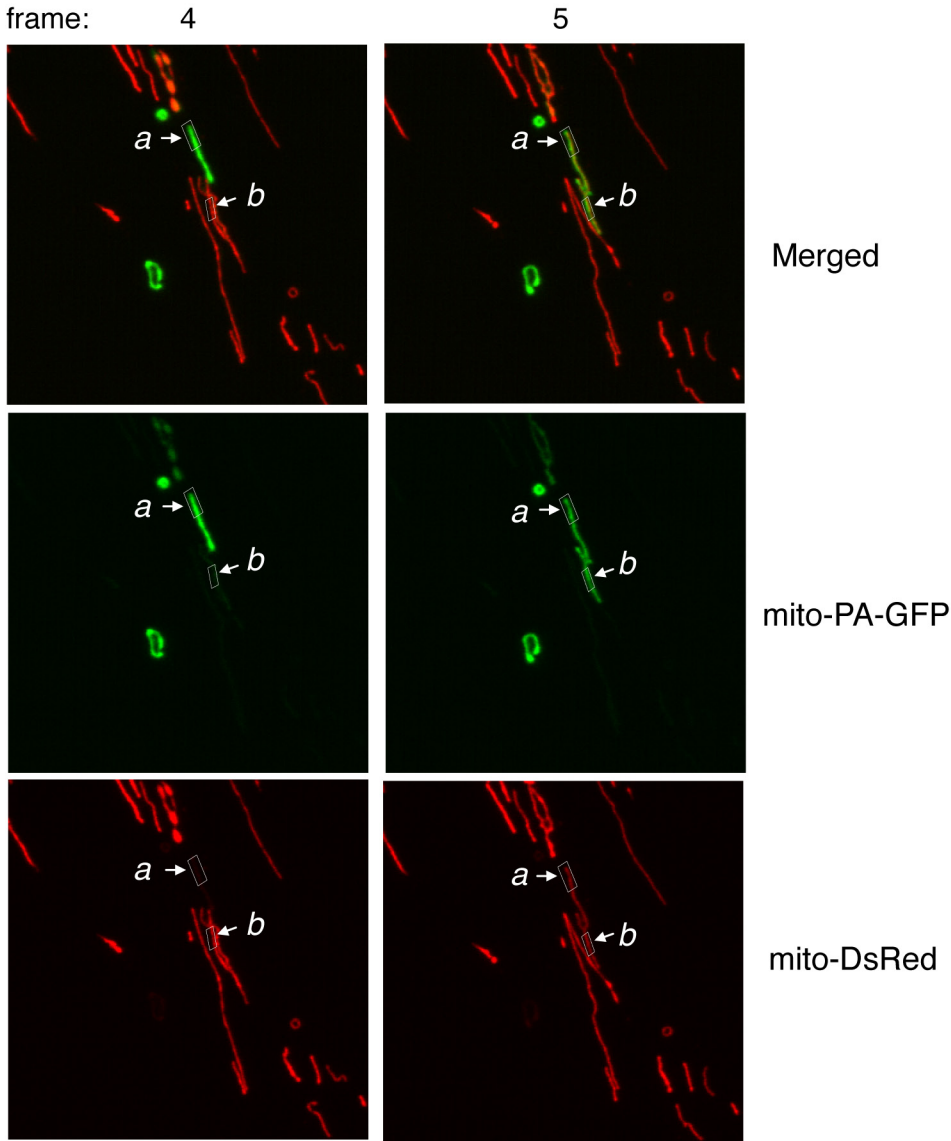
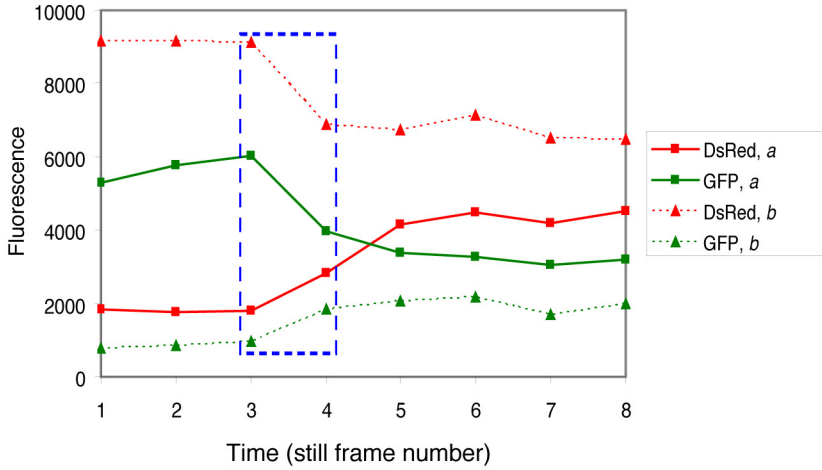

frame:
4
5
a
b
Merged
mito-PA-GFP
mito-DsRed

Fig. S4

A


10000
8000
6000
4000
2000
0
Fluorescence
1
2
3
4
5
6
7
8
Time (still frame number)
DsRed, a
GFP, a
DsRed, b
GFP, b

B

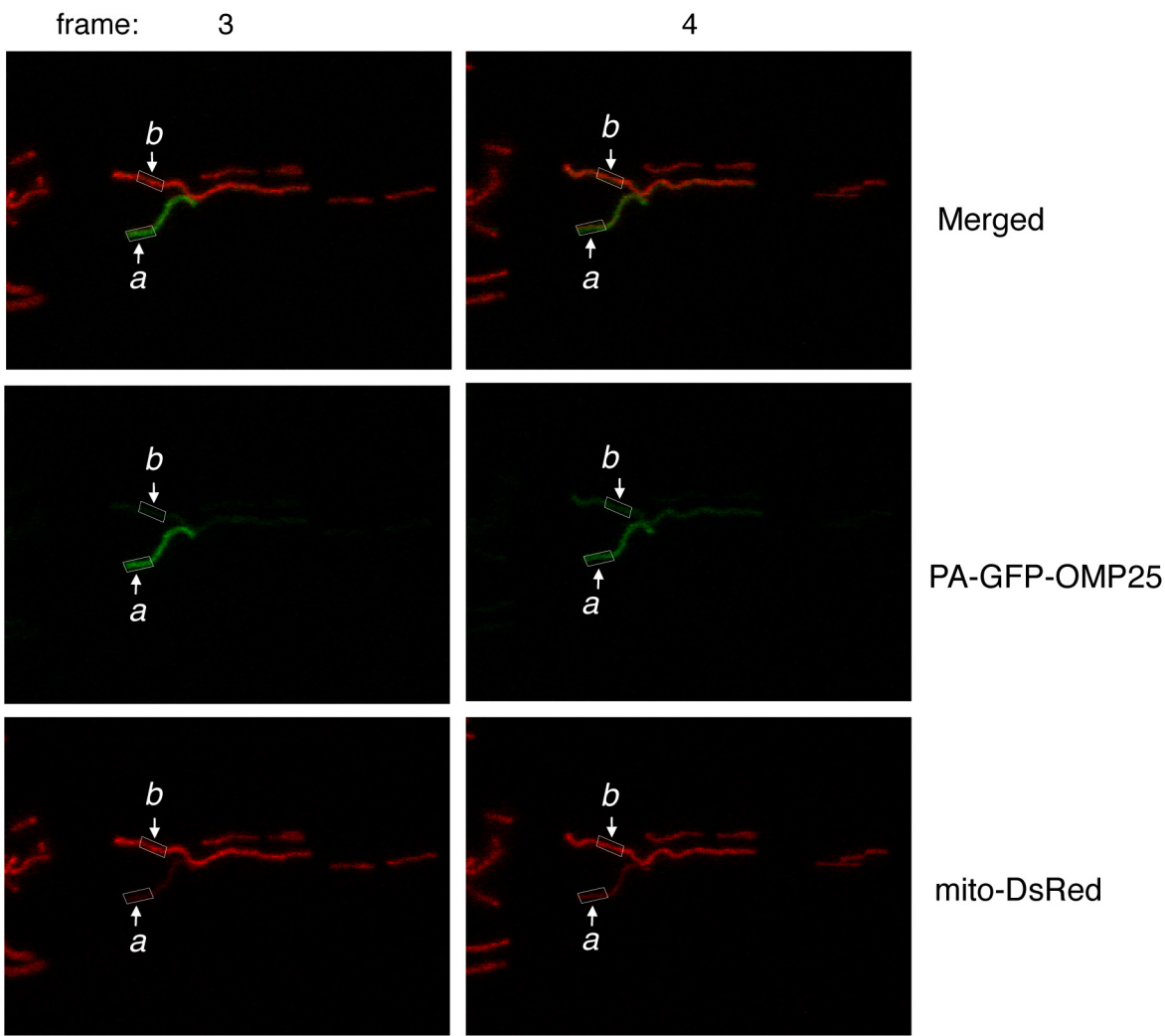

frame:
3
4
b
a
Merged
PA-GFP-OMP25
mito-DsRed

Fig. S5

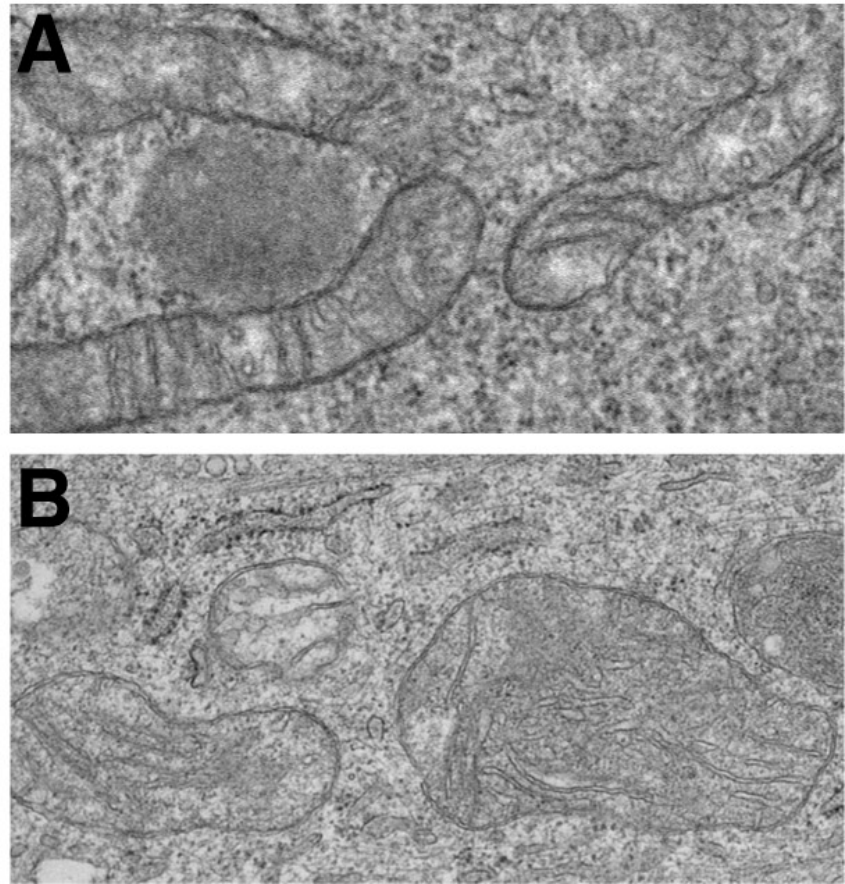


Fig. S6