Supplemental:

Fig. S1. Western blots of knockout cells and scatterplots for immunofluorescence analysis. (a) Western blot confirmation of knockouts in all MEF and HeLa cell lines used for IF experiments. Actin and tubulin were used as loading controls. (b) Scatter plots of immunofluorescence with Drp1 KO cells with or without PXA as an example to illustrate colocalization of mitochondrial matrix and outer membrane (Hsp60 and Tom20). Matrix and outer membrane markers largely colocalize in cells treated with DMSO, evident as a linear relation in the scatterplot. PXA treatment contracts the matrix compartment separating that from the outer membrane marker, which leads to a random distribution on the scatterplot.

Fig. S2. and Western blot to confirm NCLX knockout in HeLa cells, PXA-induced calcium release monitored with Rhod-2AM and a cartoon to illustrate how membrane potential is affected by calcium release. (a) Validation of NCLX KO in HeLa cells with a Western blot of extracts from Oma1 KO cells, flanked by extracts from WT and NCLX siRNA cells. The NCLX antibody shows cross-reacts with another protein, but a band corresponding to the predicted MW of NCLX (64 kDa, shown with an arrow) is present in WT cells, absent form Oma1 KO cells and greatly diminished in NCLX siRNA cells. (b) Time course of mitochondrial calcium concentrations in MEFs determined with Rhod-2 AM, which binds soluble mitochondrial calcium. Cells were transduced with scrambled shRNA (SCR) or shNCLX, pretreated with CGP37157, or Mfn2 Drp1 double KO MEFs (labeled $Mfn2^{-/-}$). (c) Quantification of mitochondrial calcium after PXA treatment and basal calcium levels (no PXA treatment). (d) Cartoon to illustrate how $\Delta\Psi$ is reduced by Na^+/H^+ exchange through NHE1 when Ca^{2+} efflux is induced by PXA.

Fig. S3. Verification that Mfn2-flag and Mfn2-myc tagged proteins are functional. (a) Mitochondria are clearly fragmented in Mfn2 KO MEFs. Transfection with tagged Mfn2 constructs (Mfn2-FLAG or Mfn2-myc) restores mitochondrial morphology, giving rise to filaments similar to those in wildtype cells. This shows that normal mitochondrial morphology can be restored using tagged constructs and that the tags do not interfere with this restoration. Scale bar is 10 μm. (b) Validation of NCLX band in MEFs under conditions for CETSA experiments. This was necessary because the cross-reactivity observed with the available NCLX antibody was different with the different solubilization conditions: Laemmli sample buffer to verify NCLX KO in HeLa cells (Fig. S2) versus dodecyl maltoside for the MEFs used for CETSA (Fig. 3). In both cases, identification of NCLX was confirmed by the presence or absence of a 64 kDa band, which is the predicted MW of NCLX.

Fig. S4. Effects of Oma1 on Mfn2-NCLX association in glycolytic cells and verification of Oma1 KO. (a) Western blot analysis of wild type (WT) and Oma1 KO HeLa cells. Tubulin was used as loading control. (b) Western blot analysis of wild type (WT) and Drp1-Opa1 DKO MEFs. (c) PLA of Mfn2-myc and NCLX-HA (red spots) in WT and Oma1 KO HeLa cells grown under glycolytic conditions and treated for 30 min with DMSO or 10 μM Oligomycin and 10 μM Antimycin. Mitochondria were detected by immunofluorescence with a chicken antibody against Hsp60 (green). The scale bar is 10 μm. (d) Average numbers of PLA spots per cell from 3 independent experiments with SD and results of an Student's T test. PLA spots in 35 cells were counted for each experiment.

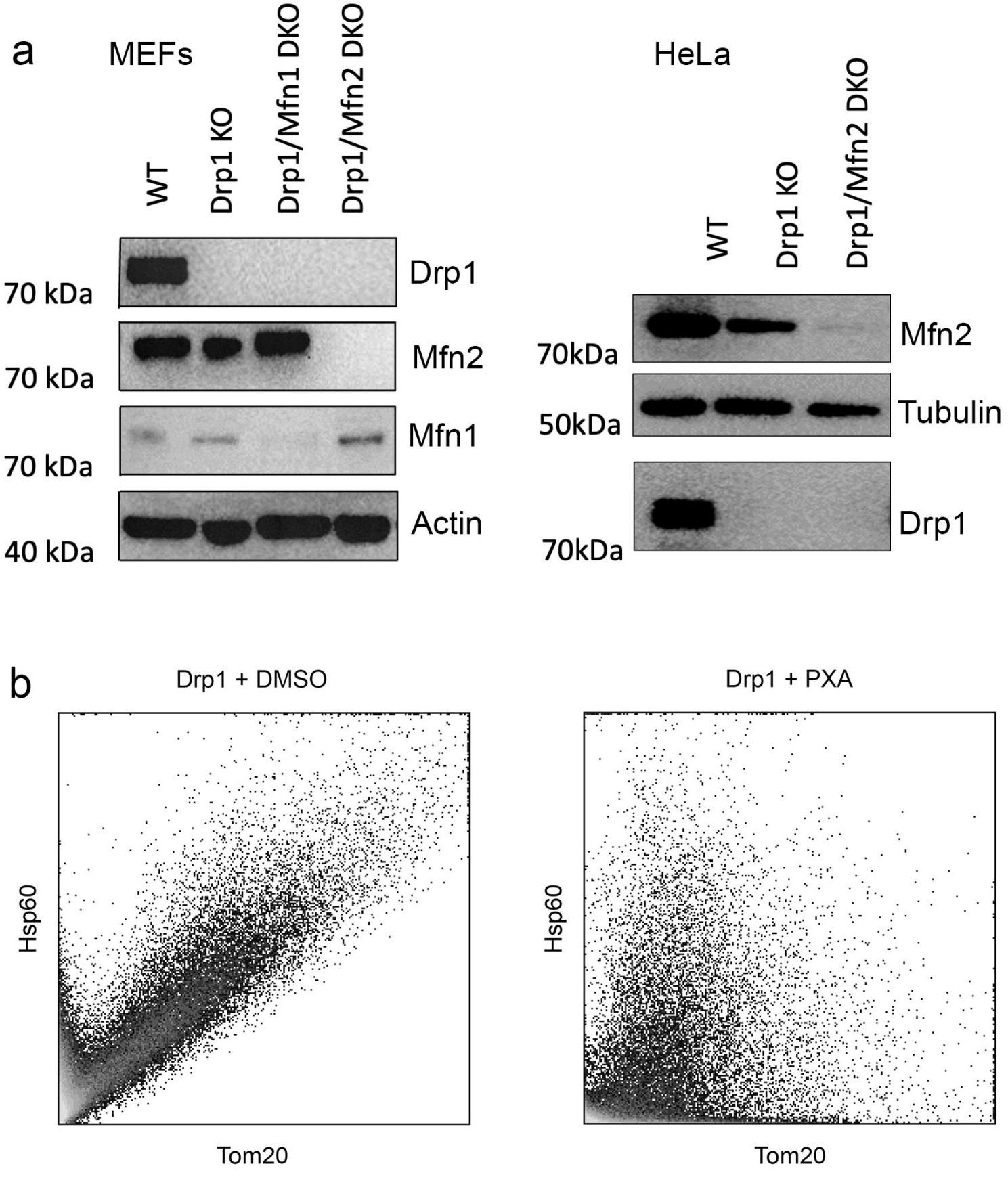
a
MEFs
WT
Drp1 KO
Drp1/Mfn1 DKO
Drp1/Mfn2 DKO
70 kDa
Drp1
70 kDa
Mfn2
70 kDa
Mfn1
40 kDa
Actin
HeLa
WT
Drp1 KO
Drp1/Mfn2 DKO
70kDa
Mfn2
50kDa
Tubulin
70kDa
Drp1
b
Drp1 + DMSO
Drp1 + PXA
Hsp60
Tom20
Hsp60
Tom20

Fig. S1

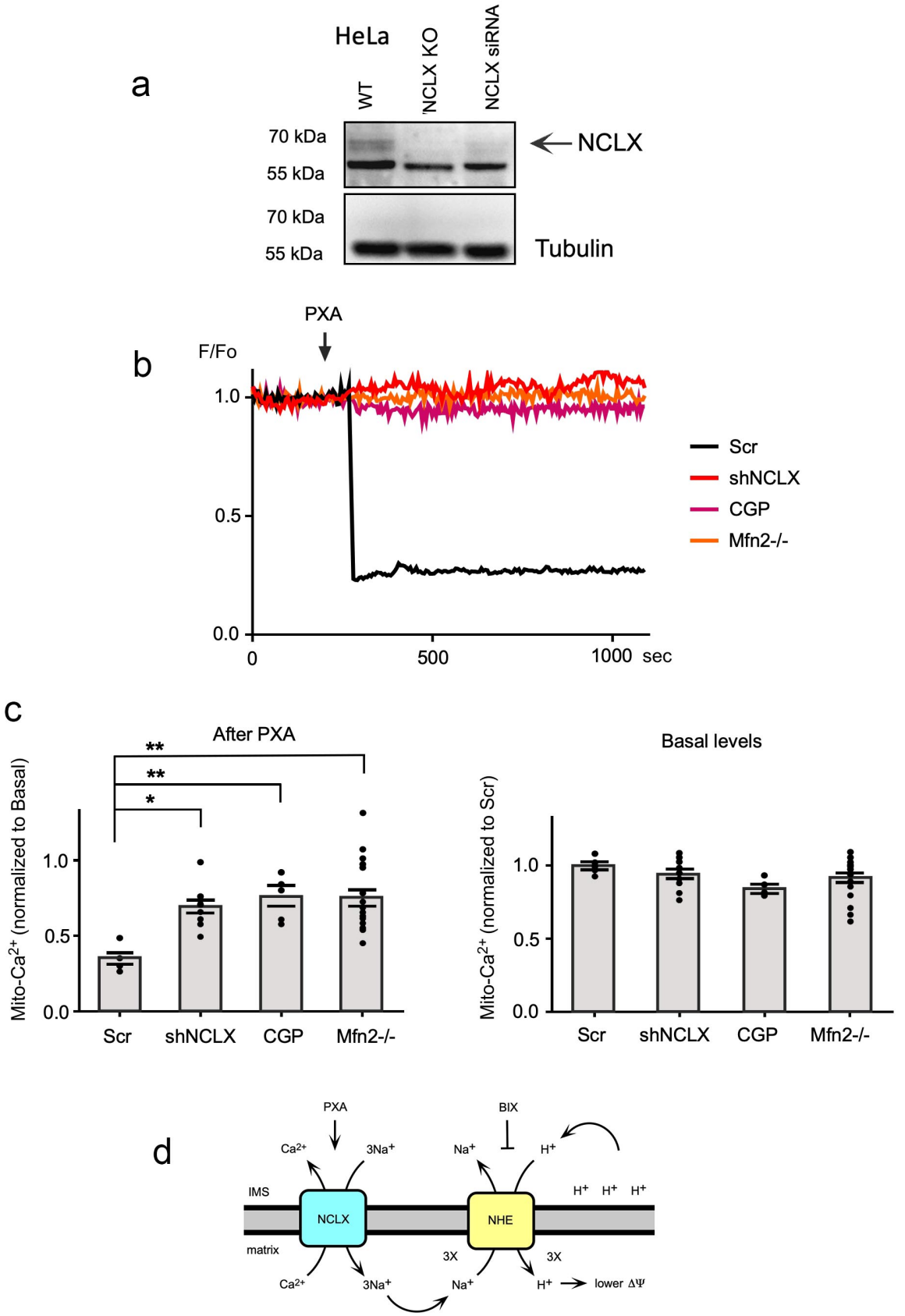
a
HeLa
WT
NCLX KO
NCLX siRNA
70 kDa
55 kDa
NCLX
70 kDa
55 kDa
Tubulin
b
PXA
F/Fo
1.0
0.5
0.0
0
500
1000 sec
Scr
shNCLX
CGP
Mfn2-/-
c
After PXA
Mito-Ca2+ (normalized to Basal)
**
**
*
Scr
shNCLX
CGP
Mfn2-/-
Basal levels
Mito-Ca2+ (normalized to Scr)
d
PXA
BIX
Ca2+
3Na+
Na+
H+
IMS
matrix
NCLX
NHE
3X
3X
lower ΔΨ

Fig. S2

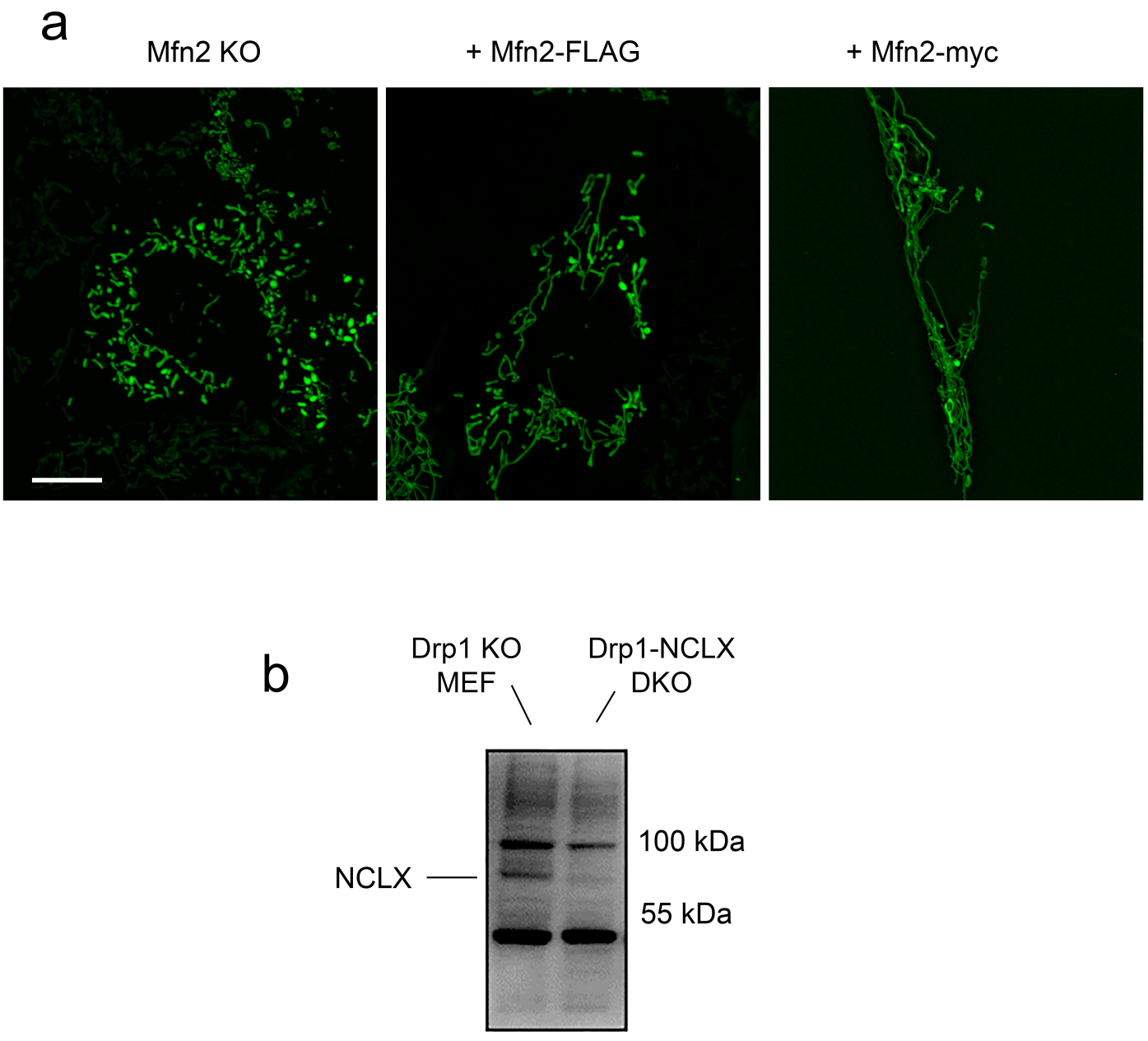
a
Mfn2 KO
+ Mfn2-FLAG
+ Mfn2-myc
b
Drp1 KO MEF
Drp1-NCLX DKO
NCLX
100 kDa
55 kDa

Fig. S3

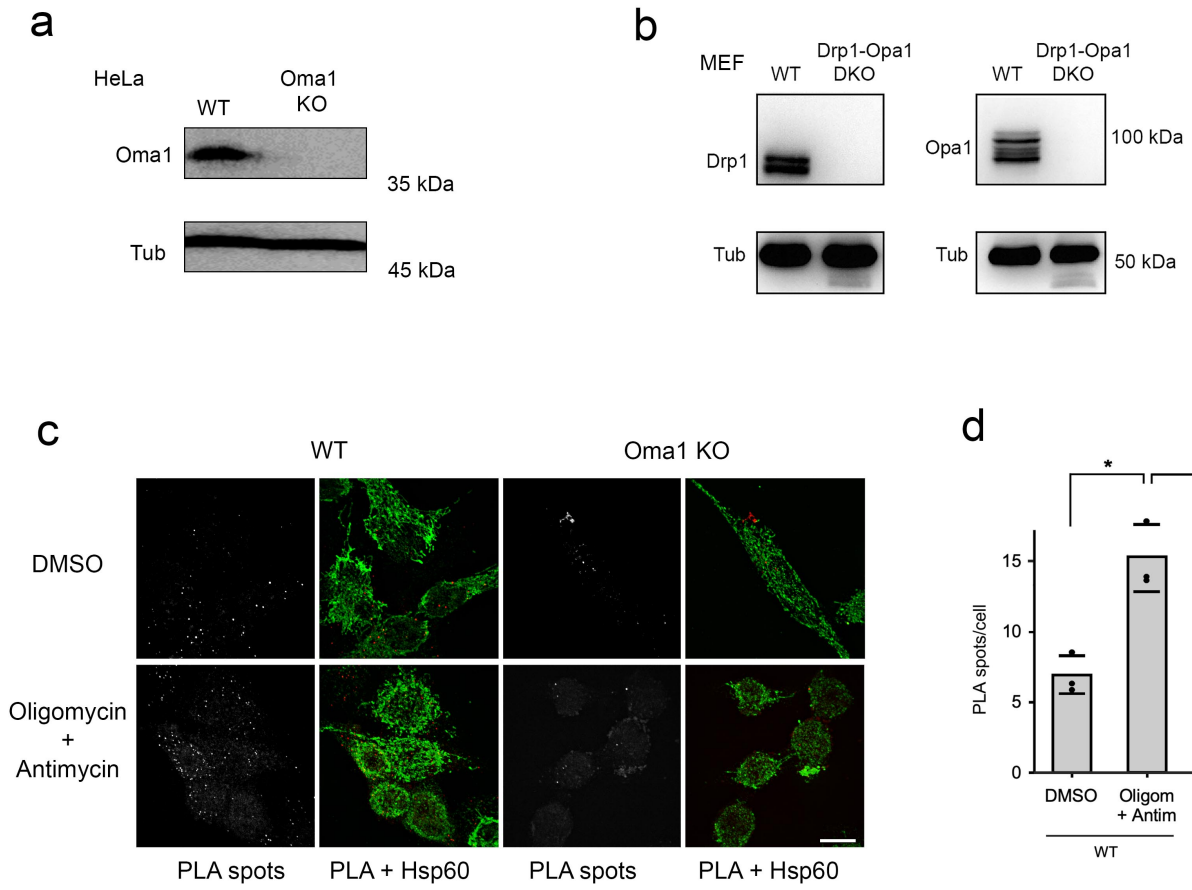

Fig. S4