DRP1 regulates endoplasmic reticulum sheets to control mitochondrial DNA replication and segregation

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Abstract

Mitochondria are multi-faceted organelles crucial for cellular homeostasis that contain their own genome. Mitochondrial DNA (mtDNA) codes for several essential components of the electron transport chain, and mtDNA maintenance defects lead to mitochondrial diseases. mtDNA replication occurs at endoplasmic reticulum (ER)-mitochondria contact sites and is regulated by mitochondrial dynamics. Specifically, mitochondrial fusion is essential for mtDNA maintenance. In contrast, while loss of mitochondrial fission causes the aggregation of nucleoids (mtDNA-protein complexes), its role in nucleoid distribution remains unclear. Here, we show that the mitochondrial fission protein DRP1 regulates nucleoid segregation by altering ER sheets, the ER structure associated with protein synthesis. Specifically, DRP1 loss or mutation leads to altered ER sheets that enhance physical interaction with mitobulbs, mitochondrial structures containing aggregated nucleoids. Importantly, nucleoid distribution and mtDNA replication were rescued by expressing the ER sheet protein CLIMP63. Thus, our work identifies a novel fission-independent mechanism by which DRP1 regulates mtDNA replication and distribution.

Keywords: mtDNA, DRP1, fission, ER sheets, CLIMP63, mitochondria

Introduction

Mitochondria are dynamic organelles regulating an array of cellular processes including energy production, cellular metabolism, apoptosis, calcium signaling, ROS signaling, cellular differentiation and immune response against pathogens (*1-6*). These mitochondrial functions are regulated by mitochondrial dynamics, the processes of mitochondrial fusion and fission. Mitochondrial fission requires DRP1 (Dynamin-Related Protein 1) (*7, 8*), while fusion is regulated by Mitofusins 1 and 2 (MFN1 & 2) and Optic Atrophy Protein 1 (OPA1) present on mitochondrial outer and inner membrane respectively (*9, 10*). Mitochondrial dynamics are also essential for mitochondrial DNA (mtDNA) maintenance, with defects in mitochondrial fusion affecting mtDNA integrity and copy number (*11, 12*). On the other hand, while defective mitochondrial fission does not generally affect overall mtDNA content, it alters the distribution of nucleoids (mtDNA-protein complexes), leading to the formation of bulb-like mitochondrial structures termed mitobulbs (*13-16*). Importantly, both mitochondrial fission and mtDNA replication are initiated at sites of contact between mitochondria and the endoplasmic reticulum (ERMCS)(*17-19*), indicating a crucial role for the ER in the regulation of mitochondrial structure and function.

The ER is a complex web-like organelle that includes flattened cisternae/sheets around the perinuclear region and tubulated structures towards the cellular periphery (*20*). ER sheets/ rough ER (rER) are enriched with ribosomes and regulate the production and assembly of proteins for secretion (*21*). On the other hand, ER tubules / smooth ER (sER) play important roles in lipid synthesis and calcium signaling, but also in mitochondrial dynamics and mtDNA replication (*17-19, 21*). At the molecular level, the ER resident protein CLIMP63 (CKAP4) is essential for the maintenance of ER sheets, while reticulons (RTN) and DP1/Yop1p are essential for both ER tubule formation and the maintenance of ER sheet curvature (*22, 23*). The ratio of sheet to tubule proteins regulates the balance between the two ER structures (*22*). In addition, recent studies showed that the mitochondrial fission protein DRP1 associates with the ER independently of mitochondria where it helps regulate ER tubulation (*24, 25*). While this suggests a complex interplay between mitochondria-shaping proteins and ER tubules, the role of ER sheets in these processes remains poorly understood. This is an important question because some reports suggest that ER sheets also contact mitochondria (*26*).

Here, we show that DRP1 controls the interaction between ER sheets and mitochondria by regulating ER sheet structure. Specifically, primary human fibroblasts from patients with a mutation in DRP1 showed altered ER sheet structure and increased interaction with mitochondria that were associated with a reduction in mtDNA replication and distribution. Importantly, modulating ER sheets through the expression of CLIMP63 normalised ER sheets-mitochondrial interaction, thereby rescuing mtDNA replication and distribution in DRP1 mutants. Altogether, our results demonstrate that DRP1-dependent control of ER sheet structure regulates replication and distribution of mtDNA independently of mitochondrial dynamics.

Results

DRP1 associates with ER sheets

DRP1 is a key mitochondrial dynamics protein required for mitochondrial fission. (*27*). Recent evidence suggest that DRP1 also alters ER tubules in a manner that is independent of its GTPase activity (*25*) and ER shaping proteins (CLIMP63, RTN4). To perform these roles, DRP1 directly associates with the ER (*24, 25*), especially ER tubules. However, a large portion of perinuclear ER adopts a specific sheet structure that is morphologically and functionally distinct from ER tubules. To determine whether DRP1 associates with ER sheets, we marked primary human fibroblasts for mitochondria (mitotracker orange), ER sheets (endogenous CLIMP63), and endogenous DRP1 and imaged them by confocal microscopy. We found that the majority of DRP1 foci present in the perinuclear region were associated with mitochondrial tubules (**Fig. 1A**, arrows in left panel, line scan in right panel, **quantification in B**). Of these, 30% were also colocalised with ER sheets (**Fig. 1B**). In addition, we observed a sub-population of DRP1 (10%) associated with ER sheets independently of mitochondria (**Fig 1A**, arrowheads in left panel, line scan in right panel, **quantification in B**). To further define the interaction between DRP1 and ER sheets relative to mitochondria, we transfected control human fibroblasts with mCherry-CLIMP63 and GFP-DRP1 and performed live cell imaging. Consistent with our results in fixed cells, some DRP1 foci remained associated with CLIMP63 over time in the absence of mitochondrial signal (**Fig. 1C**, arrows). In addition, several DRP1 foci were associated with both ER sheets and mitotracker labelled mitochondria (**Fig. 1C**, open arrowheads). Altogether, these results indicate that DRP1 interacts with ER sheets.

As a significant fraction of DRP1 was associated with both ER sheets and mitochondria, we then determined whether this was linked to mitochondrial fission. In fact, while the majority of DRP1 foci associated with both ER sheets and mitochondria did not lead to fission, all observed DRP1-dependent fission events were marked by an ER sheet (**Fig. 1C**, arrowhead; quantification in **1D**). The ER sheet was then usually released from one of the mitochondrial ends following the segregation of the two daughter mitochondria (**Fig. 1D, average time to release 50±23 sec**), while the DRP1 foci present at the fission site remained associated with the ER (**Fig. 1C**, arrowhead; quantification in **1E**). Altogether, these results indicate that DRP1 associates with ER sheets where it regulates mitochondrial fission. In addition, the observation that DRP1 stays associated with ER sheets following fission suggests that this process could regulate ER sheet-mitochondria contact sites.

Mutation in DRP1 affects ER sheets-mitochondria interaction

To determine whether DRP1 mutation alters mitochondria-ER sheet contact sites, we measured the interaction between the two organelles in primary fibroblasts from patients with a dominant-negative mutation in the middle domain of DRP1 which is required for DRP1 oligomerisation (*25, 28*). Mitochondria-ER sheet contact sites were identified using a proximity ligation assay (PLA) for CLIMP63 (ER sheets) and TOM20 (mitochondria). To assess the specificity of the interaction, we also co-labelled the cells for CLIMP63 and TOM20. Clear PLA foci were present at sites where CLIMP63 and TOM20 signals overlapped (**Fig 2A**, IgG control in Sup. **Fig S1A**), suggesting that ER sheets interact with mitochondria (**Fig 2A**, Zoom image). We specifically measured this interaction by manually counting PLA foci at the sites where CLIMP63 and TOM20 signals

overlapped (**Fig 2A-B**). We then measured mitochondria-ER sheet interactions in DRP1 mutants. As shown in **Fig 2A-B**, DRP1 mutation significantly increased the interaction between mitochondria and ER sheets. A similar increase in PLA was also observed for the more general ER marker Calnexin (**Fig 2C, S1B**), which marks both ER tubules and sheets, further suggesting that DRP1 regulates the interaction between the two organelles.

To further confirm our results, we imaged control and patient fibroblasts by TEM (**Fig 2D**) and manually quantified the interaction between ER sheets and mitochondria (≤30 nm) near the perinuclear region. We observed that mitochondria closely interacted with ER sheets in control fibroblasts. Consistent with the PLA data, this interaction was further enhanced in DRP1 mutant fibroblasts, as shown by the increase in both the number of ERMCS per mitochondrial length and the length of these ERMCS (**Fig 2D-F**). Altogether, our data indicates that ER sheets interact with mitochondria and mutations in DRP1 alters this interaction.

Mutation or knockdown of DRP1 alters ER sheet structure

Given that our results suggest an important functional role for ER sheets-mitochondria interaction, we then hypothesised that DRP1 mutation results in altered ER sheets. To address this question, we immunolabelled control and DRP1 mutant cells for the ER sheets marker CLIMP63 and found that DRP1 mutants had altered ER sheets compared to control cells. These alterations were characterised by a punctate appearance mostly apparent towards the periphery, and sometimes thick patches of sheets in the perinuclear region (**Fig 3A**, enlarged image showing ER sheet structure; complete cell in Fig. **S2**). Similar alterations were observed in ER sheets marked with another ER sheet marker, RRBP1/p180 (**Fig. 3B**). Overall, 80% of mutant cells showed an altered ER sheet phenotype (**Fig. 3C**). Consistent with this, ER sheets were smaller in DRP1 mutant cells (**Fig. 3D**), but the overall area covered by ER sheets was expanded (**Fig. 3E**). Nevertheless, protein levels of ER proteins CLIMP63, RTN4, the general ER marker Calnexin and the mitochondrial proteins TOM20 and ATP5a were similar between control and DRP1 mutant cells, indicating that the changes induced by DRP1 mutation are not caused by changes in protein expression levels (**Fig. 3F**).

While our results indicates that loss of DRP1 function alters ER sheet structure, we wanted to eliminate the possibility of secondary effects in the human DRP1 mutants we used. We thus knocked down DRP1 (DRP1 KD) in mouse embryonic fibroblasts (MEFs) (**Fig. S3A**) and labelled them with CLIMP63 (ER sheets). Like patient fibroblasts, DRP1 KD MEFs had mostly punctate ER sheets (Fig. **S3B-C**) that covered an expanded area of the cell (**Fig. S3D**).

DRP1 has been shown to alter ER tubules in a manner that is independent of its GTPase activity (*25*). However, most disease associated DRP1 mutations (including the one used here (*25, 28*)) occur outside of the variable domain associated with ER tubulation. Consistent with this, there were no major differences between control and DRP1 mutant ER immunolabeled for endogenous RTN4 and imaged by confocal microscopy, with ER tubules clearly present in the periphery of cells from either genotype (**Fig. S2**). RTN4-labeled perinuclear ER was also similar in control and DRP1 mutant cells (**Fig. S2**). As with human fibroblasts, DRP1 KD MEFs did not show clear changes in ER tubules labelled with RTN4 (**Fig. S3E**).

To further confirm the structural alteration in ER sheets, we imaged control and DRP1 mutant fibroblasts by transmission electron microscope (TEM) (**Fig. 4A**), where ER sheets are identified as rER. ER sheets from DRP1 mutants were short with less surface area and narrower luminal width compared to control ER sheets (**Fig 4B-C**), supporting our immunofluorescence data. On the other hand, ER tubule area and luminal width (identified as sER) were similar in control and DRP1 mutants (**Fig 4D-E**). Altogether, our data suggest that DRP1 modulates ER sheet structure.

ER sheets are associated with mitobulbs in DRP1 mutants

Loss of DRP1 results in mitochondrial elongation with the formation of bulb-like mitochondrial structures containing enlarged nucleoids (mitobulbs)(*13, 15, 16*). We observed a similar phenotype in DRP1 mutant fibroblasts stained for mitochondria (TMRM) and nucleoids (picogreen) and imaged by confocal microscopy (**Fig. 5A**). Mitobulbs are present in the perinuclear region of DRP1 mutant cells (*14*) where ER sheets are present, but absent from the peripheral region characterised by RTN4-positive ER tubules (**Fig. S2**). These results thus suggest that the enhanced interaction between ER sheets and mitochondria leads to mitobulb formation. To test this, we analysed the interaction between ER sheets and mitobulbs. We first immunolabelled primary fibroblasts with CLIMP63 (ER sheets) and ATP5a (mitochondria) and imaged by confocal microscopy (**Fig. 5B**). Line scan analysis showed that mitobulbs are in close association with ER sheets in DRP1 mutants. (**Fig. 5B-C**). Further, we manually quantified the number of mitobulbs in close association with ER sheets. We found that the vast majority of mitobulbs are in close proximity to ER sheets (**Fig. 5C**), supporting the idea that ER sheets interact with mitobulbs. Stable association between ER and mitobulbs was also evident in live cell imaging of DRP1 mutants (ER, mCherry-Cyb5; mitochondria, mitotracker; Fig. **5D, arrows; arrowheads indicate sites of ER interacting with mitochondrial tubules**).

To further confirm the presence of a physical interaction between mitobulbs and ER sheets, we performed PLA for CLIMP63 (ER sheets) and TOM20 (mitochondria). PLA foci were clearly visible at sites where ER sheets were in close proximity to mitobulbs (**Fig 5E**, quantification in **5F**), consistent with an interaction between the two structures. Similar results were observed when Calnexin was used instead of CLIMP63 for the PLA (**Fig 5F**, **S1C**). Altogether, our results indicate that the altered ER sheets present in DRP1 mutants interact with mitobulbs.

Modulation of ER sheets recovers the nucleoid defects present in DRP1 mutants

To directly determine whether ER sheets affect nucleoid distribution, we modulated ER sheet structure by expressing mCherry-tagged CLIMP63 under conditions that did not overtly alter RTN4-positive ER tubules in control cells (**Fig. S4A**) to avoid nucleoid defects caused by the loss of ER tubules (*19*). Importantly, CLIMP63 expression did not rescue the fused mitochondrial phenotype of DRP1 mutant (**Fig. S4B**). Nevertheless, CLIMP63 expression altered ER sheet structure resulting in expanded web-like ER sheet network in both control and DRP1 mutant fibroblasts (**Fig. S5**). Importantly, this was accompanied by a loss of the punctate structures typically observed in mutant cells transfected with mCherry alone (**Fig. S5**), suggesting that CLIMP63 expression can at least partially rescue the ER sheet defects observed in DRP1 mutants. To determine if this was associated with a change in ER sheets-mitochondria interactions, we performed PLA for CLIMP63 (ER sheets) and TOM20 (mitochondria). CLIMP63 expression did not affect mitochondria-ER sheet interaction in control cells (**Fig. 6A**) but rescued the excessive contact sites found in DRP1 mutant cells (**Fig. 6A**). Similarly, the number of mitobulbs associated

with PLA foci was reduced in DRP1 mutant cells expressing CLIMP63 (**Fig. 6B**), indicating that recovery of ER sheet structure in DRP1 mutants normalizes its interaction with mitochondria.

Loss of DRP1 results in enlarged nucleoids (**Fig. 6C**) that are likely caused by nucleoid aggregation (*14-16*). To confirm the presence of multiple mtDNA copies in the enlarged nucleoids present within mitobulbs, we labelled dividing nucleoids with the nucleotide analogue EdU and observed multiple EdU-positive foci within mitobulbs (**Fig. 6D-E**). To determine the effect of modulating ER sheets on this nucleoid aggregation, we transiently transfected control and DRP1 mutant fibroblasts with mCherry or mCherry-CLIMP63 and immunolabelled them for mitochondria (TOM20) and nucleoids (TFAM) (**Fig. S**6). We first determined the effect of mCherry-CLIMP63 expression on nucleoid aggregation by measuring nucleoid size. Nucleoid size was significantly reduced in DRP1 mutants expressing mCherry-CLIMP63 compared to cells expressing only mCherry (**Fig. 6C, Fig. S6**), consistent with CLIMP63 expression stimulating nucleoid distribution out of mitobulbs. We then reasoned that if CLIMP63 rescues nucleoid distribution, it should also rescue the decreased nucleoid numbers present in DRP1 mutant cells (**Fig. 6F**)(*14*). Indeed, while mCherry-CLIMP63 expression did not significantly affect nucleoid numbers in controls cells, it caused a significant increase in overall nucleoid content in DRP1 mutants (**Fig 6F, S6**). Altogether, these results indicate that modulating ER sheet structure rescues nucleoid aggregation in DRP1 mutants. As the enlarged nucleoids present in DRP1 mutant cells are found within mitobulbs, we also determined the effect of mCherry-CLIMP63 expression on the presence of mitobulbs. While almost all mitobulbs present in mCherry-transfected DRP1 mutant fibroblasts contained TFAM-positive nucleoids, mCherry-CLIMP63 expression drastically reduced this number (**Fig. 6G**). On the other hand, the total number of enlarged, bulb-like mitochondrial structures was unchanged (**Fig. 6H**), suggesting that DRP1 is still required to maintain mitochondrial structure in these conditions. Altogether, our data indicates that nucleoid segregation is regulated by ER sheet-mitochondria interaction independently of DRP1-dependent fission.

Altering ER sheets in DRP1 mutant promotes mtDNA replication and distribution

While our results are consistent with CLIMP63 expression promoting nucleoid distribution away from mitobulbs, it remained possible that it also altered mtDNA replication. Thus, we measured the effect of CLIMP63 expression on mtDNA replication. Replicating DNA was labelled with EdU, after which cells were directly fixed, immunolabelled for mitochondria (TOM20) and imaged by confocal microscopy. In cells expressing mCherry, DRP1 mutants had fewer EdU-positive nucleoids compared to control cells (**Fig. 7A**), suggesting that DRP1 is required for proper mtDNA replication. Importantly, expression of CLIMP63 rescued the number of EdU foci in DRP1 mutant cells (**Fig. 7A**), suggesting that the effect of DRP1 on nucleoid replication depends on its ability to modulate ER structure. On the other hand, CLIMP63 expression did not significantly alter the number of EdU foci in control cells (**Fig. 7A**), indicating that modulating ER sheets does not directly affect mtDNA replication.

As clusters of EdU-positive mtDNA are found within mitobulbs (**Fig. 6D-E**), we then specifically addressed the effect of CLIMP63 expression on mtDNA replication within mitobulbs. Consistent with the total EdU counts, expression of CLIMP63 in DRP1 mutant fibroblasts caused a large increase in EdU incorporation in mtDNA present within mitobulbs (**Fig. 7B**). We then took advantage of the accumulation of EdU foci within mitobulbs to determine whether the reduction in nucleoid size that we observed in CLIMP63-transfected DRP1 mutant cells (**Fig. 6C**) is due to

the rescue of nucleoid segregation from mitobulbs towards the rest of the mitochondrial network. To do that, we chased EdU for 24 hours before fixing the cells. Consistent with our observation that the number of nucleoid-containing mitobulbs was decreased in CLIMP63-expressing cells (**Fig. 6G**), the number of EdU-positive mitobulbs was decreased in CLIMP63-expressing DRP1 mutant cells, but not the mCherry-expressing cells at 24 hours (**Fig. 7B**). Importantly, this was not due to the loss of overall EdU staining in mutant cells (**Fig. 7C**). Overall, our data indicates that modulating ER sheet structure through CLIMP63 expression reactivates both mtDNA replication and nucleoid distribution in DRP1 mutant cells even in the absence of mitochondrial fission. Thus, proper DRP1-mediated regulation of ER sheet structure is essential to modulate mtDNA replication and nucleoid segregation.

Discussion

mtDNA replication is intimately associated with mitochondrial dynamics, especially mitochondrial fusion which is required for mtDNA replication and integrity (*11, 12*). On the other hand, mitochondrial fission has been suggested to be associated with nucleoid segregation on the basis of the fact that defects in mitochondrial fission result in nucleoid aggregation within mitobulbs (*13, 15, 16*). With the help of Mitomate Tracker, an automated tool developed in our lab, we have shown that this alters overall nucleoid distribution along mitochondrial networks (*14*). Nevertheless, the mechanism by which DRP1 affects nucleoid distribution remains unclear. Here, we demonstrate that DRP1 regulates mtDNA replication and distribution along mitochondrial network through modulating ER sheet structure, independently of mitochondrial fission.

It is well known that ER tubules regulate mtDNA replication (*19*). However, whether ER sheets also play a role in this process remains unclear. Nevertheless, our data shows that ER sheets directly interact with mitochondria and are present at the site of mitochondrial fission in the perinuclear region (**Fig. 5**). This is in consistent with the previous observation that ER sheets interact with mitochondria and regulate lipid metabolism in mouse liver cells (*26*), suggesting an important physiological role for this interaction. As we previously showed that, in DRP1 mutants, nucleoids aggregate around the perinuclear region where ER sheets are found (*14*), we further defined how DRP1 impacts this process. Our data demonstrates that DRP1 associates with ER sheets independently of mitochondria and that loss of DRP1 function alters ER sheet structure. Furthermore, DRP1 mutant cells showed increased interaction between ER sheets and mitochondria. Of note, we did not observe distinct changes in ER tubules in our cell models, possibly because ER tubular formation requires the variable domain of DRP1 (*25*) which is still intact in our mutant lines (mutation in the middle domain (G362D))(*28*). Overall, these results indicate that DRP1 regulates ER sheet structure and their interaction with mitochondria.

The altered ER sheets present in DRP1 mutant cells also lead to the accumulation of mtDNA within mitobulbs. Importantly, modulating ER sheets by expressing CLIMP63 recovered nucleoid aggregation and nucleoid number in DRP1 mutants, supporting the idea that ER sheets regulate nucleoid distribution. In addition, our EdU data demonstrate that proper ER sheet structure is necessary to actively regulate mtDNA replication. Altogether, our data show that DRP1-regulated ER sheet structure is essential for both mtDNA replication and distribution, and that this occurs independently of DRP1-dependent mitochondrial fission.

While we demonstrated that ER sheets play an important role in mtDNA replication and distribution, ER tubules are still required. This was shown in a previous study overexpressing CLIMP63 under conditions that caused the complete loss of ER tubules, which significantly reduced mitochondrial DNA replication in cells with wild-type DRP1 (*19*). In contrast, when we expressed CLIMP63 under conditions that do not affect tubular ER structure, it did not impact cells with wild-type DRP1.

Our study also provides novel insights into the defects present in DRP1 mutant patients. Reported human DRP1 mutations are mostly found in the middle and GTPase domains of DRP1, leading to peripheral neuropathy, epilepsy, optic atrophy, and encephalopathy in these patients (*28, 29*). Here,

we have used patient fibroblasts with a dominant negative mutation in the middle domain of DRP1 (G362D), leading to refractory epilepsy(*28*). At the cellular level, conditional deletion of DRP1 in mice affects the movement of mitochondria to the nerve terminal, leading to the loss of dopamine neurons (*30*), while its postnatal deletion in CA1 hippocampal neurons results in functionally impaired mitochondria at the nerve terminals (*31*). Based on our key findings here, we speculate that the altered mtDNA replication and distribution could severely hamper the movement of functional mitochondria towards the synaptic nerve terminal in the patient neurons and thereby impairing neuronal signaling.

Altogether, our results demonstrate the importance of ER sheets in mtDNA maintenance and distribution and identify DRP1 as key regulator of ER sheets and their interaction with mitochondria. Alteration of this regulation following DRP1 mutation leads the perinuclear accumulation of mtDNA, which could explain the defects observed in these cells in the absence of overt metabolic alterations (*28*).

Materials and Methods

Reagents

Cell culture reagents were obtained from Wisent. Other chemicals were purchased from Sigma-Aldrich, except where indicated.

Cell culture and live cell imaging

Primary human fibroblasts (controls and DRP1 mutants) were generated from skin biopsies, collected as part of an approved research protocol (Research Ethics Board of the Children's Hospital of Eastern Ontario (DRP1 mutants)), and written informed consent from participants was obtained. This study was performed in accordance with the Declaration of Helsinki. Biopsy samples were processed as described and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with Penicillin/Streptomycin (100 IU/ml/100µL/mL)(*28*). Immortalized Mouse Embryonic Fibroblasts (MEFs) were cultured in DMEM supplemented with 10% fetal bovine serum. For live cell imaging, cells were seeded onto glass bottom dishes and allowed to adhere to the plate. Then cells were stained for 30 minutes with 250 nM TMRM (Thermo fisher Scientific, T668) and the DNA dye PicoGreen (Thermo Fisher Scientific, P11495) (3 µL/mL). After staining, cells were washed 3 times with pre-warmed 1X phosphate buffered saline (PBS), and normal growth media was added prior to imaging.

Microscopy

Images were acquired with a Leica TSC SP8 confocal microscope fitted with a 63x/1.40 oil objective using the optimal resolution for the wavelength (determined using the Leica software).

siRNA treatment

MEFs were seeded onto 24 well dish and transfected with 15nM of DRP1 siRNA (Thermo Fisher Scientific, Silencer Select, 4390771) and negative siRNA (Thermo Fisher Scientific, Silencer Select, 4390843) using siLenFect lipid reagent (Bio-Rad,1703361). After 48 hrs, cells were treated again with siRNA for another 48 hours. The cells were collected for either western blotting or seeded onto the coverslips for immunofluorescence.

Transient transfection of primary cells

Primary cells were trypsinized and centrifuged at 577 X g for 10 minutes. Cell pellets were suspended in 10µl of the Neon transfection buffer R (ThermoFisher Scientific, MPK1096). Cells were transiently transfected with mCherry (gift from Michael Davidson, Addgene, 54517), mCherry-tagged CLIMP63 (gift from Gia Voeltz (*32*), Addgene, 136293), DRP1-GFP (gift from Thomas Cribbs (*33*)), mCherry-Cytochrome b5 (gift from Uri Manor) using the Neon transfection system (ThermoFisher Scientific, MPK5000) according to the manufacturer's protocol. To minimize effects on ER tubules, minimal concentrations (1µg) of mCherry and mCherry-CLIMP63 plasmids were used.

Immunofluorescence

Cells were seeded onto glass coverslips (Fisherbrand, 1254580) and allowed to adhere overnight. Mitochondria was stained using 50 nM Mitotracker Orange (Thermo fisher scientific, M7510) prior to fixing for certain experiment. Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). For antigen retrieval, cells were incubated in sodium citrate buffer (pH 6.0) for 10 minutes at 95°C. Then, cells were permeabilized with 1% BSA / 0.2% Triton X-100 in PBS followed by blocking with 1% BSA / 0.1% Triton X-100 in PBS. The following antibodies were used: CLIMP63 (Rb, Abcam, ab84712, 1:150), CLIMP63 (Mo, ENZO, ENZ-ABS669, 1: 200), RRBP1 (Rb, Novus Biologicals, NBP1-32813, 1:200), DRP1 (Mo, BD Transduction Laboratories, 611112, 1:100), TOM20 (Rb, Abcam, ab186735, 1:250), mtTFAM (Mo, Novusbio, NBP1-71648, 1:150), Calnexin (Mo, Millipore, MABF2067, 1:200), RTN4/NOGOA (Rb, Bio-Rad, AHP1799, 1:200), ATP5a (Mo, Abcam, ab14748, 1:150). Next, cells were incubated with fluorescent tagged secondary antibody (Jackson Immunoresearch, 1:500). To verify the interaction of DRP1 with organelles (ER sheets, mitochondria) or to detect the interaction between organelles (bulb-like mitochondria and ER sheets), line scan analysis was performed using the *RGB profiler* in Image J. To avoid bias during manually classification of ER structure, images were renamed to random numbers and both control and mutant images were shuffled together. The blindfolded ER sheet images were manually classified as structured or altered using reference images. In addition, we segmented the ER sheet images in ImageJ (Filter/minimum (0.5), Filter/Median (1.0), then thresholding and adjusting the resulting image using Binary/Erode) and measured total area and the median size of individual ER sheets using the Analyse Particle function. Mitochondrial structures were manually quantified by binning them into the indicated categories (short, intermediate, elongated). To measure DRP1 association with mitochondria and ER sheets in fixed cell, DRP1 foci were identified in individual cell using ImageJ. The DRP1 channel was first segmented as for ER sheets. Total DRP1 foci were then measured using the Analyse Particle function. Selected DRP1 foci were then applied onto mitochondria and ER sheet channels and DRP1 distribution on each organelle was manually counted. For live cell experiment, the images were manually analyzed for the association of DRP1 with mitochondria, ER sheets or both at the fission sites. Further, ER sheets association with mitochondrial ends was tracked following fission event.

Proximity Ligation Assay (PLA)

Cells were grown on glass coverslips and fixed with 4% paraformaldehyde. Following antigen retrieval, cells were permeabilized with 1% BSA/0.2% Triton X-100 in PBS for 15 minutes at RT. The ER was marked with an antibody recognizing CLIMP63 or calnexin and mitochondria was marked with an antibody recognizing TOM20. The PLA assays were performed using Duolink In Situ green kit Mouse/ Rabbit (Sigma Aldrich) following the manufacturer's protocol. The primary antibodies were then labelled using fluorescent-tagged secondary antibodies. Total and mitobulb associated PLA foci were manually counted at the site of CLIMP63-TOM20 signal while overall calnexin-TOM20 foci were quantified using the *analyze particles* function in ImageJ.

Transmission Electron Microscopy (TEM)

Primary fibroblasts were seeded on Nunc Lab-Tek chamber slides (Thermo Fisher Scientific, 177437) and allowed to adhere overnight. Cells were washed with 0.1M sodium cacodylate buffer (pH 7.3) and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3), overnight at 4°C. Fixed cells were further processed in McGill's facility for electron microscopy research (FEMR). Images were acquired using a EMS208S electron microscope (Philips) by an independent trained operator in University of Quebec in Trois-Rivières' TEM facility. ER sheet-mitochondrial contact sites in the perinuclear region was manual quantified, considering only contact sites ≤ 30 nm based on a previous study (*34*). To measure changes in ER structure we considered rough ER as 'sheets' and smooth ER as 'tubules'. We manually measured ER surface area and luminal width for 10 randomly chosen ER sheets/tubules in each of the four quadrants per image of the cell. The width was measured across each individual ER sheets/tubules and averaged to obtain the luminal width of each individual ER tubules /sheets.

Western Blot

Cells were lysed in 10 mM Tris-HCl, pH 7.4, 1mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM sodium fluoride, complemented with a protease inhibitor cocktail (Sigma-Aldrich), centrifuged at 15890 X g for 5 minutes and protein supernatants collected. Protein concentrations were estimated calorimetrically by DC protein assay kit (BioRad). For SDS-PAGE, 20 µg (siRNA treatment in MEFs)/25 µg (Mitochondrial and ER proteins in human fibroblasts) of proteins were mixed with 1X Lammeli buffer containing β-mercaptoethanol, then subjected to SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibodies (DRP1 (BD Transduction Laboratories, 611112, 1:1000), CLIMP63 (Mo, ENZO, ENZ-ABS669, 1: 500), TOM20 (Rb, Abcam, ab186735, 1:1000), Calnexin (Mo, Millipore, MABF2067, 1:1000), RTN4/NOGOA (Rb, Bio-Rad, AHP1799, 1:1000), ATP5a (Mo, Abcam, ab14748, 1:1000), HRP-tagged Actin (1:10,000). Membranes were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and visualized by enhanced chemiluminescence (Thermo Fisher scientific) using a Bio-Rad imaging system.

EdU Labeling

Primary fibroblasts (Control and DRP1 mutants) were incubated with 60µM EdU for 2 hours at 37°C. For the chase experiments, the EdU containing media was replaced with fresh media and incubated further for 24 hours. Cells were then fixed with 4% paraformaldehyde for 15 minutes at RT, permeabilized and EdU detected using Click-iT EdU imaging kit (Thermo Fisher Scientific, C10337). Cells were then immunolabelled for TOM20 (Abcam, ab186735, 1:250). EdU foci in mitobulbs and total EdU foci were manually counted.

Data analysis and statistics

All graphs and statistical analysis were done using R. Immunofluorescence data were quantified and images representative of at least three independent experiments shown (exact n are in the

quantification figures). Data are represented as average ± SD as specified in figure legends. Statistical significance was determined using Student's t test (between 2 groups) or one-way ANOVA with a tukey post hoc test (multiple comparisons).

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Author contribution

HSI performed the cell biology experiments. HSI and MG designed the experiments. HSI, JDG, SB and MG analysed the data. MAL provided the clinical samples. HSI and MG wrote the paper. All authors reviewed and discussed the manuscript.

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Figure Legends

Figure 1. DRP1 associates with ER sheets. (A) DRP1 associates with ER sheets. Representative image of control human fibroblasts showing DRP1 (Green), mitochondria (Mitotracker orange, Blue) and ER sheets (CLIMP63, Red). Colocalization of DRP1 with mitochondria (arrows) and ER sheets (arrowheads). Line scan analysis confirmed the colocalization (right panel). (B) Quantification of the association of DRP1 with mitochondria (mitotracker orange) and ER sheets (RRBP1). Each data point represents one cell. Bars represent the average of 41 cells in 3 independent experiments ± SD (C) Live cell imaging of control human fibroblasts transfected with DRP1-GFP (Green) and mCherry-CLIMP63 (Red). Mitochondria were labelled with Mitotracker Deep Red (Blue). DRP1 foci associated with ER sheets (arrow), ER sheets and mitochondria (open arrowheads) and ER sheets at the site of mitochondrial fission (filled arrowheads) are indicated. (D-E) Quantification of mitochondrial fission from time lapse videos as in (C). A total of 37 fission events were observed in 15 cells (5 minutes total/video). Fission events occurring in the last two frames of the videos were excluded from the calculation of the number of fission events where ER sheets dissociated from mitochondria within 2 minutes of the fission event (D).

Figure 2. DRP1 regulates the interaction between ER sheets and mitochondria. (A) Representative images of control and DRP1 mutant fibroblasts showing the PLA for CLIMP63 and TOM20 (White), along with CLIMP63 (red), TOM20 (Green) and nuclei (Hoechst, Blue). Scale bar 10 µm, 2 µm for the zoomed images. (B) Quantification of CLIMP63-TOM20 PLA. Each data point represents one cell. Bars represent the average of 50 control and 48 mutant cells in 3 independent experiments ± SD *** $p<0.001$ two-sided t-test (C) Quantification of Calnexin-TOM20 PLA. Each data point represents one cell. Bars represent the average of 40 cells per genotype in 3 independent experiments ± SD ** $p<0.01$ two-sided t-test (D) Representative TEM images of control and DRP1 mutant fibroblasts. Scale bar 500 nm (E) Quantification of the number of ERMCS per mitochondrial length in the TEM images. Each data point represents one mitochondrion. Bars represent the average of 103 control and 97 mutant mitochondria ± SD ** $p<0.01$ two-sided t-test (F) Quantification of ERMCs length (µm) per mitochondria in the TEM images. Each data point represents one mitochondrion. Bars represent the average of 103 control and 97 mutant mitochondria ± SD *** $p<0.001$ two-sided t-test.

Figure 3. Loss of DRP1 function alters ER sheet structure. (A-B) Representative images of CLIMP63 (A) and RRBP1 (B) staining in control and DRP1 mutant fibroblasts. Scale bars 10 µm. (C) Quantification of ER sheet structure as Structured (Blue) or Altered (red; presence of punctate structures and thick ER sheet patches). Each point represents one independent experiment, with at least 20 cells quantified per experiment. Bars show the average ± SD. * $p<0.05$, ** $p<0.01$ One-way ANOVA using the data for Structured ER. (D-E) Quantification of the median size of individual ER sheets (D) and the total area covered by ER sheets (E). Each data point represents one cell. Bars represent the average of 41 cells in 3 independent experiments ± SD *** $p<0.001$ two-sided t-test. (F) WB showing ER (CLIMP63, RTN4, calnexin) and mitochondrial proteins (TOM20, ATP5a) in control and DRP1 mutant human fibroblasts.

Figure 4. EM analysis of ER sheets and tubules in DRP1 mutant cells. (A) Representative EM images. Asterisks denote ER sheets (Top) or ER tubules (Bottom). Scale bar 500 nm. (B-C) Quantification of the average ER sheet area (B) and luminal width (C). Each data point represents one cell. Bars represent the average of 10 cells ± SD ** $p<0.01$ two-sided t-test. (D-E) Quantification of the average ER tubule area (D) and luminal width (E). Each data point represents one cell. Bars represent the average of 10 cells ± SD. ns, not significant. two-sided t-test.

Figure 5. Modified ER sheets are associated with mitobulbs. (A) Representative live cell images showing the mitochondrial marker, TMRM and picogreen-stained DNA in control and DRP1 mutant human fibroblasts. The zoomed images show the enlarged nucleoids present in mitobulbs (arrowheads). (B-D) Colocalisation between ER sheets and mitobulbs. Colocalisation was measured in cells immunolabelled for CLIMP63 (ER sheets) and ATP5a (mitochondria). (B) representative image (Top) and Line scan analysis (Bottom) along the line shown the image. (C) Quantification of the percent of mitobulbs that are associated with CLIMP63-positive ER sheets in two independent DRP1 mutant lines (P1 and P2). Each data point represents one cell. Bars represent the average of 45 control and 45 mutant mitochondria ± SD. (D) Live cell imaging of DRP1 mutant cells showing the interaction between ER sheets (mCherry-Cyb5, Red) and mitochondria (arrowheads) (Mitotracker, Green), including mitobulbs (arrow). (E-F) Interaction between ER sheets and mitobulbs as measured by PLA for TOM20 and CLIMP63 or Calnexin. (E) Representative image of TOM20-CLIMP63 PLA. Arrowheads denote PLA foci (White) on mitobulbs (TOM20, Green) at sites where they contact ER sheets (CLIMP63, Red). (F) Quantification of mitobulbs associated with PLA foci for TOM20-CLIMP63 and TOM20-Calnexin. Each data point represents one cell. Bars represent the average of 48 (CLIMP63 PLA) and 40 cells (Calnexin PLA) in 3 independent experiments ± SD. Scale bars 2 µm, 10 µm for (A), left panel.

Figure 6. CLIMP63 expression rescues nucleoid distribution in DRP1 mutant fibroblasts. (A-B) Quantification of PLA foci (CLIMP63-TOM20) in mCherry and mCherrry-CLIMP63 expressing control and DRP1 mutant cells. Total PLA foci (A) and mitobulbs associated with PLA foci (B) were quantified. Each point represents one cell, with at least 43 cells quantified per condition in 3 independent experiments. Bars show the average ± SD. ** $p<0.01$, *** $p<0.001$, ns not significant. One-way ANOVA (C) Quantification of nucleoid area in mCherry and mCherry-CLIMP63 expressing control and DRP1 mutants from images in Figure S6. Each point represents one cell, with at least 32 cells quantified per condition in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$, ns not significant. One-way ANOVA. (D-E) Mitobulbs are mtDNA clusters. (D) Representative images of mCherry-expressing DRP1 mutants labeled for EdU (green) and TOM20 (mitochondria). Arrowheads indicate EdU-positive mtDNA in mitobulbs. Scale bar 10µm (E). Quantification of number of EdU foci/mitobulb. Each point represents an individual cell, with 45 cells quantified in 3 independent experiments. Bars show the average ± SD. (F-H) Rescue of nucleoid numbers in CLIMP63-expressing DRP1 mutant cells. Quantification of total nucleoids (TFAM-positive, F), mitobulbs containing nucleoids (TFAM-positive, G) and mitochondrial bulb-like structures (independently of the presence of nucleoids, H) in mCherry and mCherrry-CLIMP63 expressing control and DRP1 mutant cells. Each point represents one cell, with 40 mCherry (mch) and 47 mCherry-CLIMP63 cells quantified in 3

independent experiments. Bars show the average ± SD. One way ANOVA (F), two-sided t-test (G, H). * $p<0.05$, *** $p<0.001$, ns, not significant.

Figure 7. CLIMP63 expression rescues mtDNA replication and distribution in DRP1 mutant fibroblasts. (A) Quantification of EdU foci in control and DRP1 mutants expressing mCherry or mCherry-CLIMP63 in cells labelled with EdU for 4 hours. Each point represents one cell, with at least 43 cells quantified in 3 independent experiments. Bars show the average ± SD. One way ANOVA. * $p<0.05$, *** $p<0.001$, ns not significant. (B) Quantification of EdU-positive mitobulbs in EdU-labelled DRP1 mutants expressing mCherry or mCherry-CLIMP63. Cells were pulsed with EdU as in (A) then the EdU was chased for 24 hours where indicated. Each point represents one cell, with at least 44 cells quantified in 3 independent experiments. Bars show the average ± SD. One way ANOVA. *** $p<0.001$. (C) EdU foci ratio (chase/no chase) from the experiments in (A-B). Each point represents one independent experiment (n=3). Bars show the average ± SD. Two-sided t-test. ns not significant.

Supplementary Figure 1. Calnexin-TOM20 PLA. (A) Representative image of PLA (IgG and TOM20; white) for the antibody control (IgG), along with IgG (red), TOM20 (green) and nuclei (Hoechst, Blue) in control fibroblasts. (B) Representative images of control and DRP1 mutant fibroblasts showing the PLA for Calnexin and TOM20 (white), along with Calnexin (red), TOM20 (Green) and nuclei (Hoechst, Blue). (C) Zoomed in image of DRP1 mutant. Arrowheads denote PLA foci on mitobulbs. Scale bar 10 µm, 2 µm for the zoomed images.

Supplementary Figure 2. ER structure in DRP1 mutant fibroblasts. Representative images of control (Top) and DRP1 mutant (Bottom) human fibroblasts showing CLIMP63 (ER sheets, Red), RTN4 (ER, Green) and mitochondria (Mitotracker orange, Blue). The enlarged boxed areas show ER structure in the perinuclear area (CLIMP63-positive) and periphery of the cells. Scale bar 10 µm, 2 µm for the enlarged images.

Supplementary Figure 3. ER Structure following DRP1 knockdown in MEFs. (A) WB showing DRP1 expression in WT MEFs transfected with a control siRNA (siCtrl) or a siRNA against DRP1 (siDRP1). (B) Representative images showing CLIMP63 staining in MEFs transfected with control and DRP1 siRNAs. White lines represent the cell edge. Scale bar 10 µm (C) Quantification of ER sheet structure as Structured (Blue) or Altered (red; presence of punctate structures and thick ER sheet patches). Each point represents one independent experiment, with at least 20 cells quantified per experiment. Bars show the average ± SD. * $p<0.05$, Two-sided t-test using the data for Structured ER. (D) Quantification of ER sheet surface area relative to the total cell area (determined by DIC). Each data point represents one cell. Bars represent the average of 54 cells in 3 independent experiments ± SD. ** $p<0.01$ two-sided t-test. (E) Representative images showing RTN4 in control and DRP1 siRNAs . Scale bar 10 µm, 2 µm for the zoomed images.

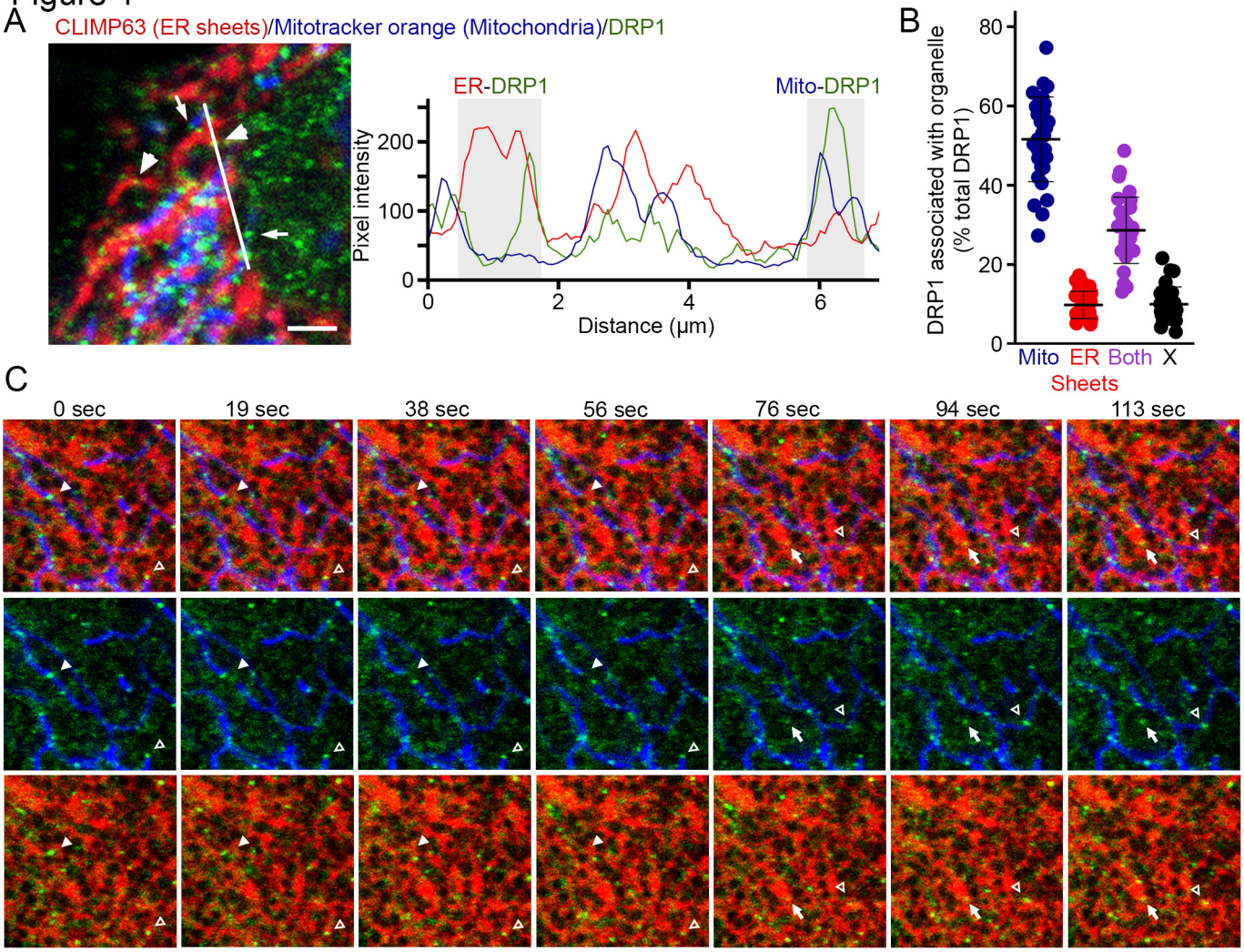
Supplementary Figure 4. ER tubules are not affected by CLIMP63 expression. (A) Representative images of Control cells expressing mCherry and mCherry-CLIMP63 (Magenta) and immunolabelled for RTN4 (green). Scale bar 10 µm, 2 µm for the zoomed images. (B)

Quantification of mitochondrial phenotypes (short (green), intermediate (red), elongated (blue)) in mCherry and mCherry-CLIMP63 expressing control and DRP1 mutant fibroblasts. Each point represents an independent experiment. Bars show the average ± SD. Two-way ANOVA. *** $p<0.001$, ns not significant.

Supplementary Figure 5. CLIMP63 expression alters ER sheet structure in control and DRP1 mutants. Representative images of control and DRP1 mutant fibroblasts expressing mCherry or mCherry-CLIMP63 (Magenta) and immunolabelled for CLIMP63 (ER sheets, green). Scale bar 10 µm.

Supplementary Figure 6. CLIMP63 expression rescues nucleoid aggregation in DRP1 mutants. Representative images of control and DRP1 mutant fibroblasts expressing mCherry or mCherry-CLIMP63 (blue) immunolabeled for TFAM (nucleoids, Green) and TOM20 (mitochondria, Red). Scale bar 10 µm.

Figure 1



D

	ER sheets associated	ER release (at 2 min)
Fission events	37/37 (100%)	23/27 (85%)

E

	Mitochondria	ER sheets	Both
DRP1 foci after fission	4/37 (11%)	16/37 (43%)	17/37 (46%)

Figure 2

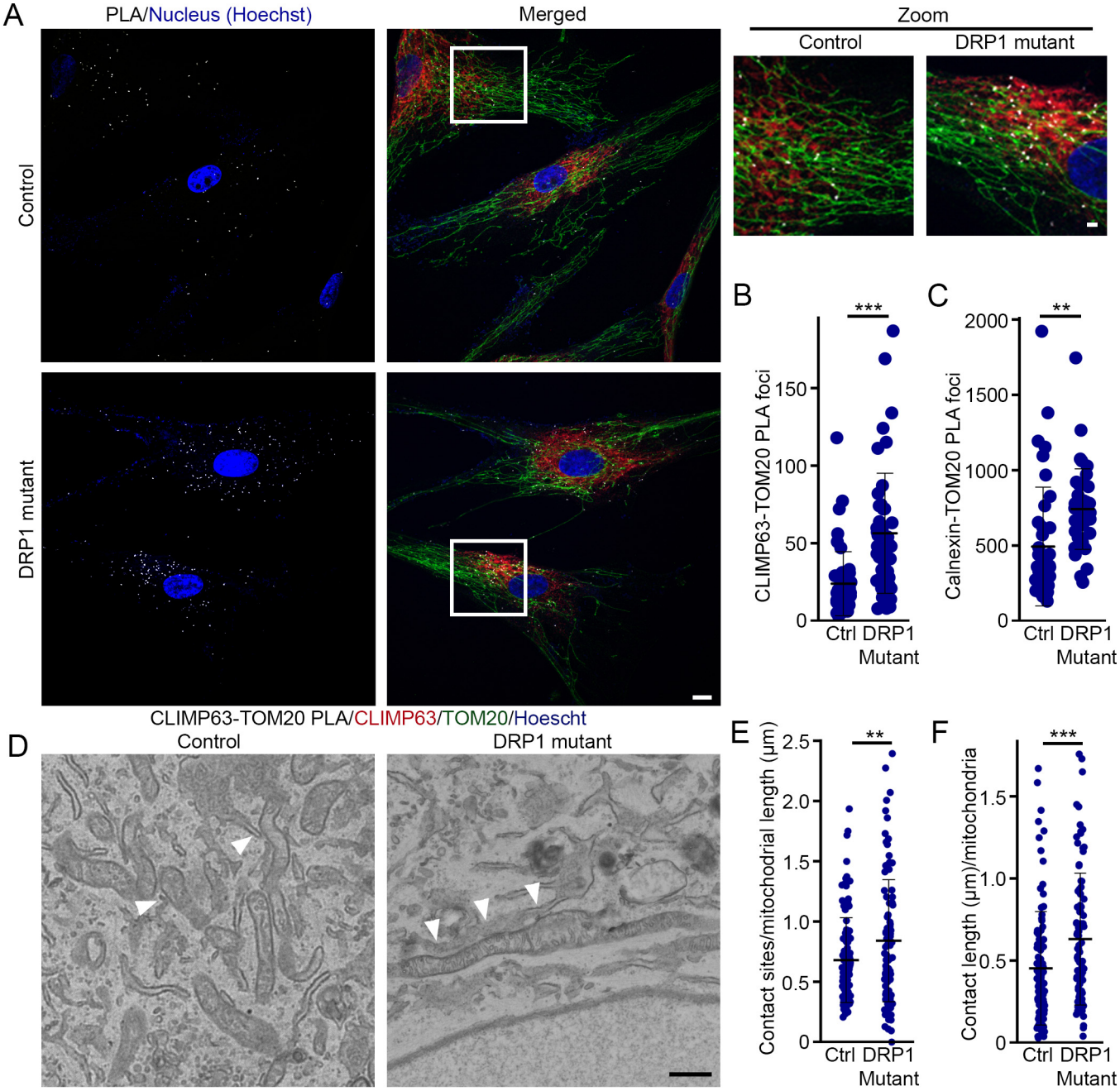

A
PLA/Nucleus (Hoechst)
Merged
Zoom
Control
DRP1 mutant
Control
DRP1 mutant
B
***
CLIMP63-TOM20 PLA foci
150
100
50
0
Ctrl
DRP1 Mutant
C
**
Calnexin-TOM20 PLA foci
2000
1500
1000
500
0
Ctrl
DRP1 Mutant
CLIMP63-TOM20 PLA/CLIMP63/TOM20/Hoescht
D
Control
DRP1 mutant
E
**
Contact sites/mitochodrial length (µm)
2.5
2.0
1.5
1.0
0.5
0
Ctrl
DRP1 Mutant
F
***
Contact length (µm)/mitochondria
1.5
1.0
0.5
0
Ctrl
DRP1 Mutant

Figure 3

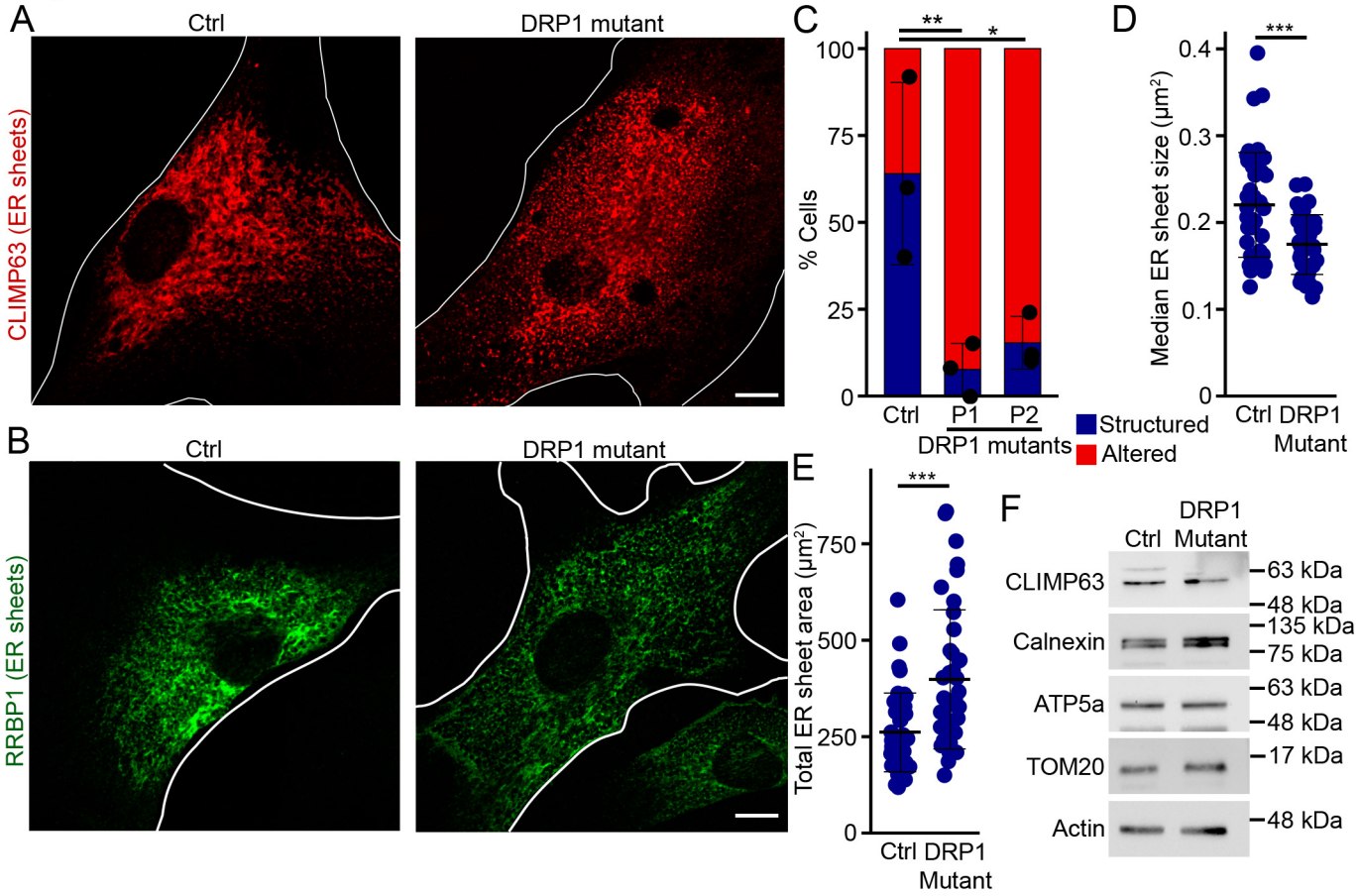

A
Ctrl
DRP1 mutant
CLIMP63 (ER sheets)
B
Ctrl
DRP1 mutant
RRBP1 (ER sheets)
C
**
*
100
75
50
25
0
% Cells
Ctrl
P1
P2
DRP1 mutants
Structured
Altered
D
***
0.4
0.3
0.2
0.1
0
Median ER sheet size (µm²)
Ctrl
DRP1 Mutant
E
***
750
500
250
0
Total ER sheet area (µm²)
Ctrl
DRP1 Mutant
F
Ctrl
DRP1 Mutant
CLIMP63
63 kDa
48 kDa
Calnexin
135 kDa
75 kDa
ATP5a
63 kDa
48 kDa
TOM20
17 kDa
Actin
48 kDa

Figure 4

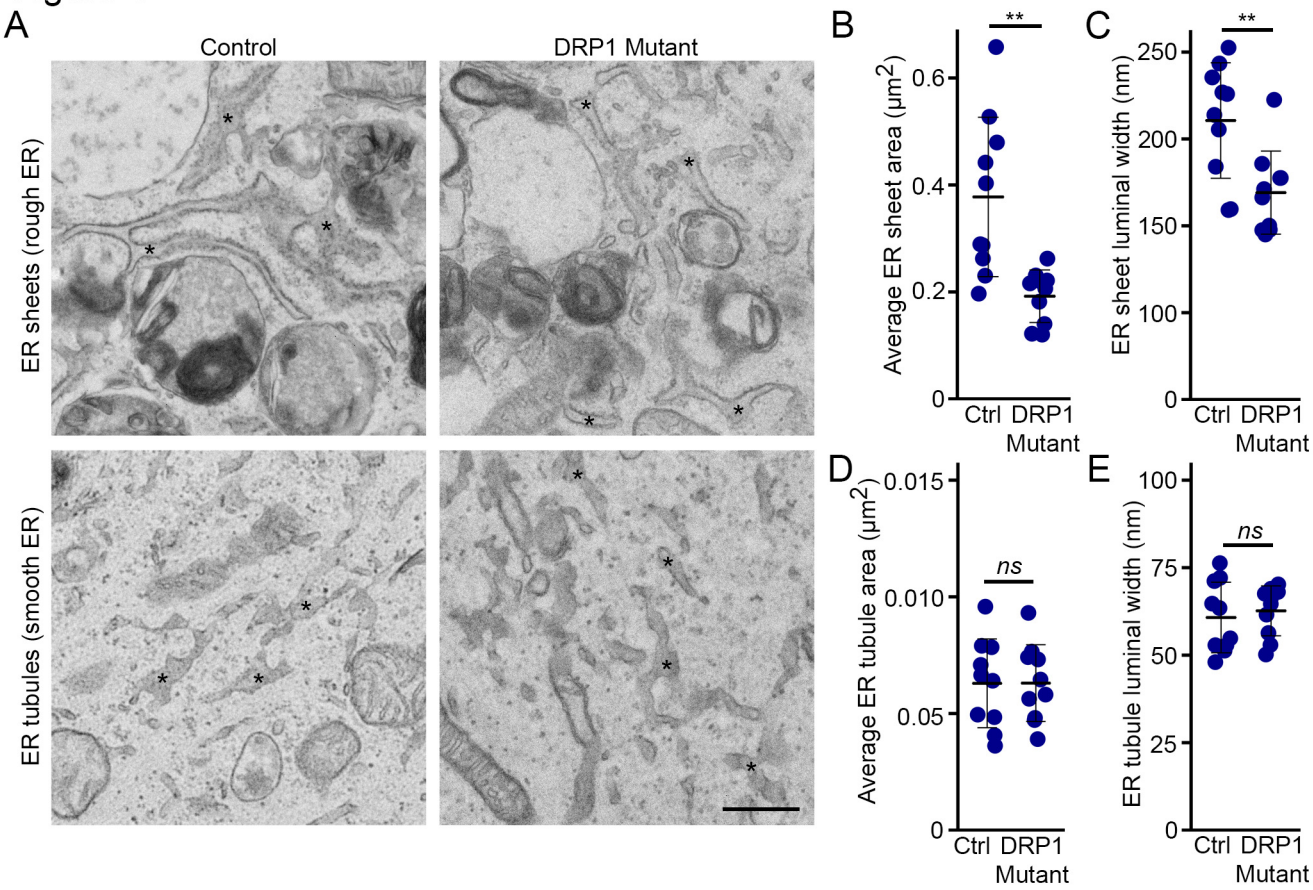

A
Control
DRP1 Mutant
ER sheets (rough ER)
ER tubules (smooth ER)
B
Average ER sheet area (μm2)
0
0.2
0.4
0.6
**
Ctrl
DRP1 Mutant
C
ER sheet luminal width (nm)
0
100
150
200
250
**
Ctrl
DRP1 Mutant
D
Average ER tubule area (μm2)
0
0.05
0.010
0.015
ns
Ctrl
DRP1 Mutant
E
ER tubule luminal width (nm)
0
25
50
75
100
ns
Ctrl
DRP1 Mutant

Figure 5

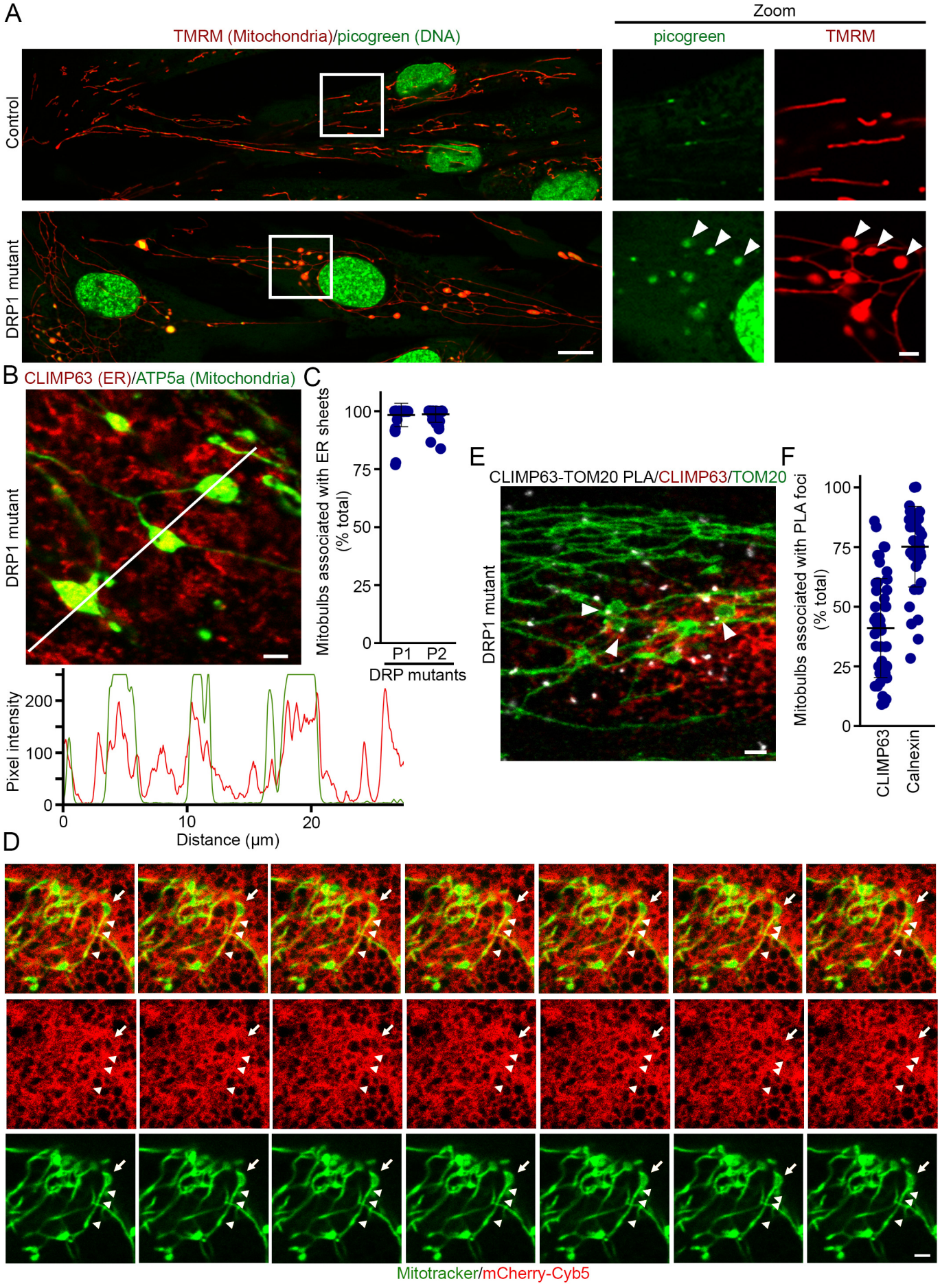

A
Zoom
TMRM (Mitochondria)/picogreen (DNA)
picogreen
TMRM
Control
DRP1 mutant
B
CLIMP63 (ER)/ATP5a (Mitochondria)
DRP1 mutant
Pixel intensity
200
100
0
0
10
20
Distance (μm)
C
Mitobulbs associated with ER sheets (% total)
100
75
50
25
0
P1
P2
DRP mutants
E
CLIMP63-TOM20 PLA/CLIMP63/TOM20
DRP1 mutant
F
Mitobulbs associated with PLA foci (% total)
100
75
50
25
0
CLIMP63
Calnexin
D
Mitotracker/mCherry-Cyb5

Figure 6

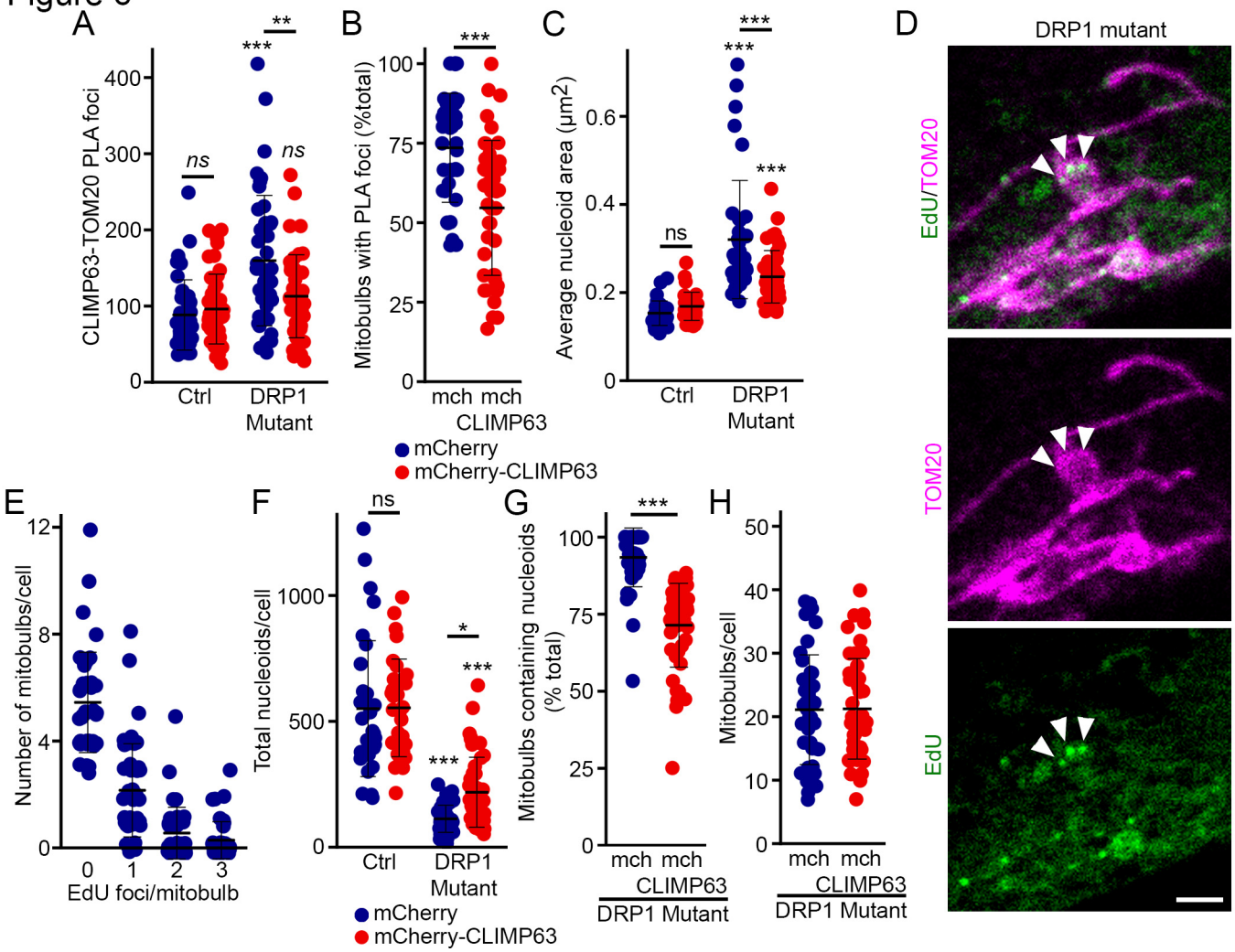
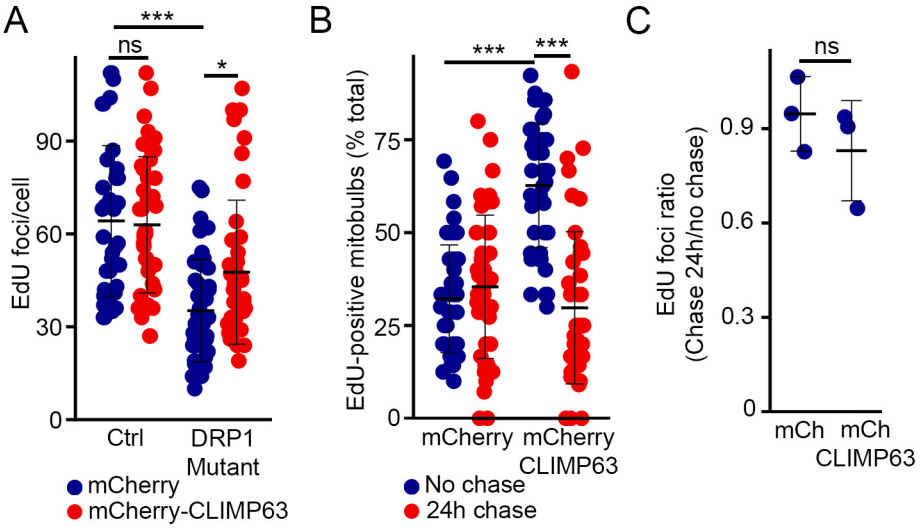
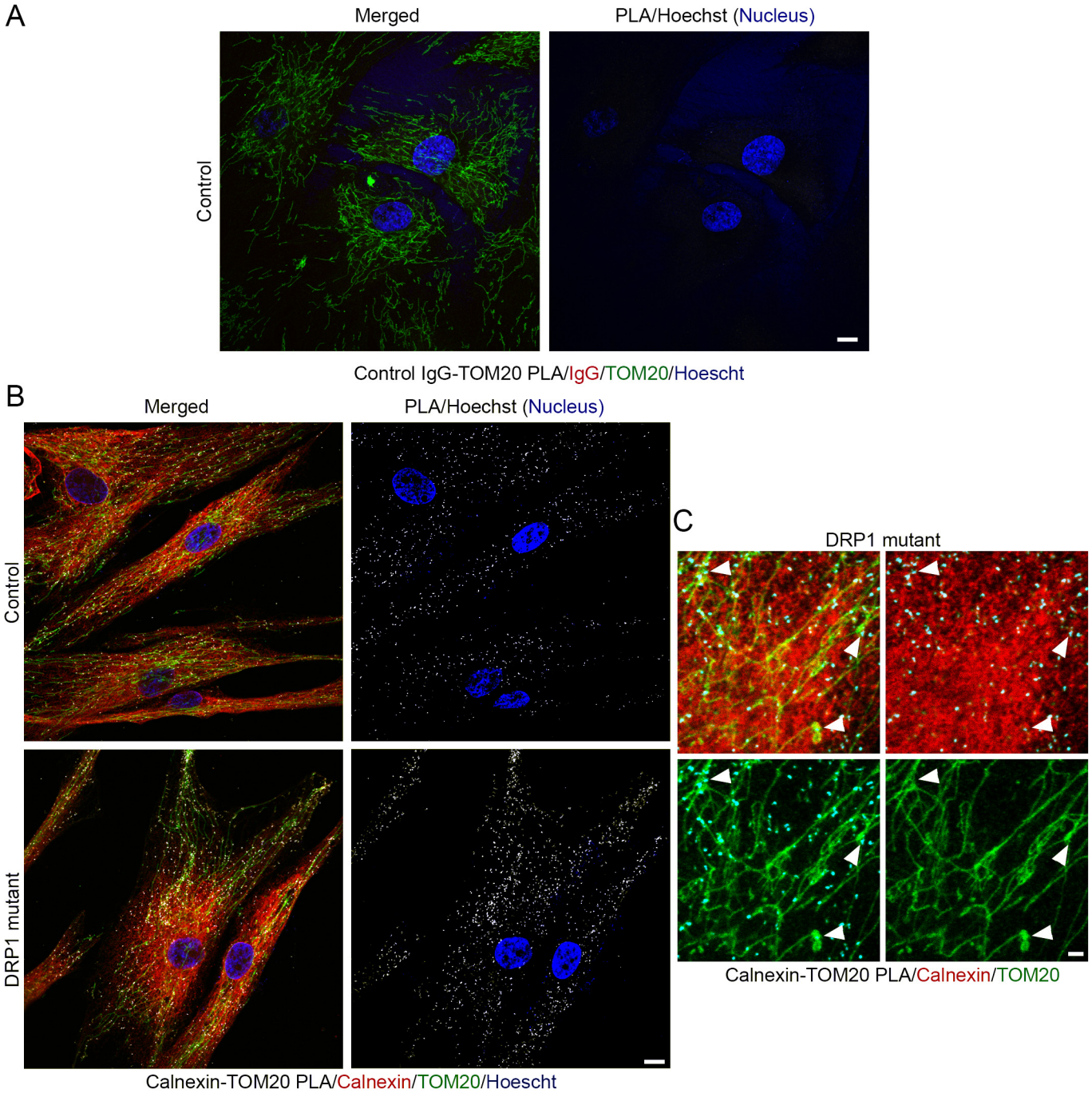

A
CLIMP63-TOM20 PLA foci
400
300
200
100
0
ns
***
ns
**
Ctrl
DRP1 Mutant
B
Mitobulbs with PLA foci (%total)
100
75
50
25
0
***
mch mch
CLIMP63
C
Average nucleoid area (μm2)
0.6
0.4
0.2
0
ns
***
***
***
Ctrl
DRP1 Mutant
mCherry
mCherry-CLIMP63
D
DRP1 mutant
EdU/TOM20
TOM20
EdU
E
Number of mitobulbs/cell
12
8
4
0
0 1 2 3
EdU foci/mitobulb
F
Total nucleoids/cell
1000
500
0
ns
*
***
***
Ctrl
DRP1 Mutant
mCherry
mCherry-CLIMP63
G
Mitobulbs containing nucleoids (% total)
100
75
50
25
0
***
mch mch
CLIMP63
DRP1 Mutant
H
Mitobulbs/cell
50
40
30
20
10
0
mch mch
CLIMP63
DRP1 Mutant

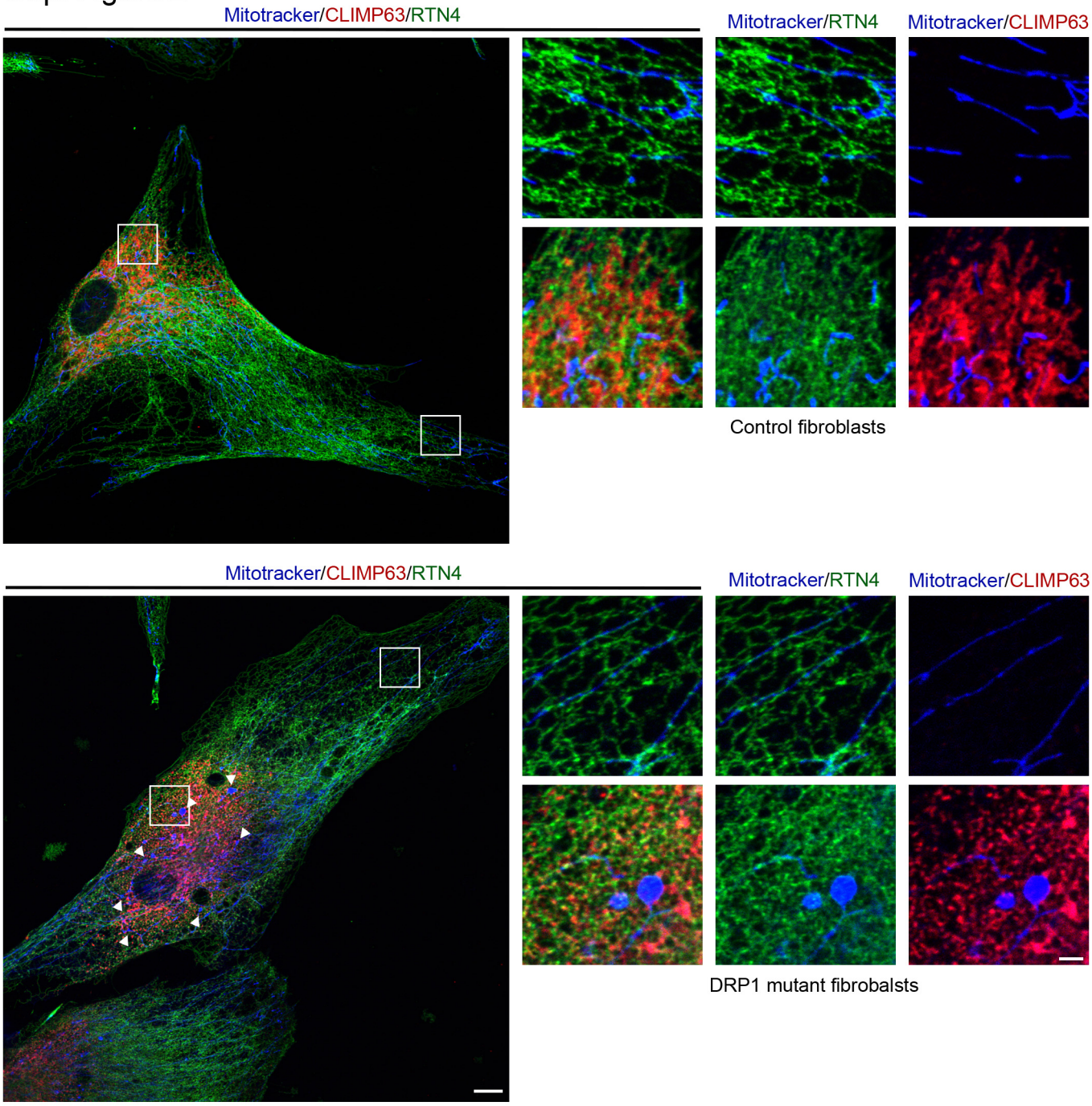
Figure 7


A
***
ns
*
EdU foci/cell
90
60
30
0
Ctrl
DRP1
Mutant
mCherry
mCherry-CLIMP63
B
***
***
EdU-positive mitobulbs (% total)
75
50
25
0
mCherry
mCherry
CLIMP63
No chase
24h chase
C
ns
EdU foci ratio
(Chase 24h/no chase)
0.9
0.6
0.3
0
mCh
mCh
CLIMP63

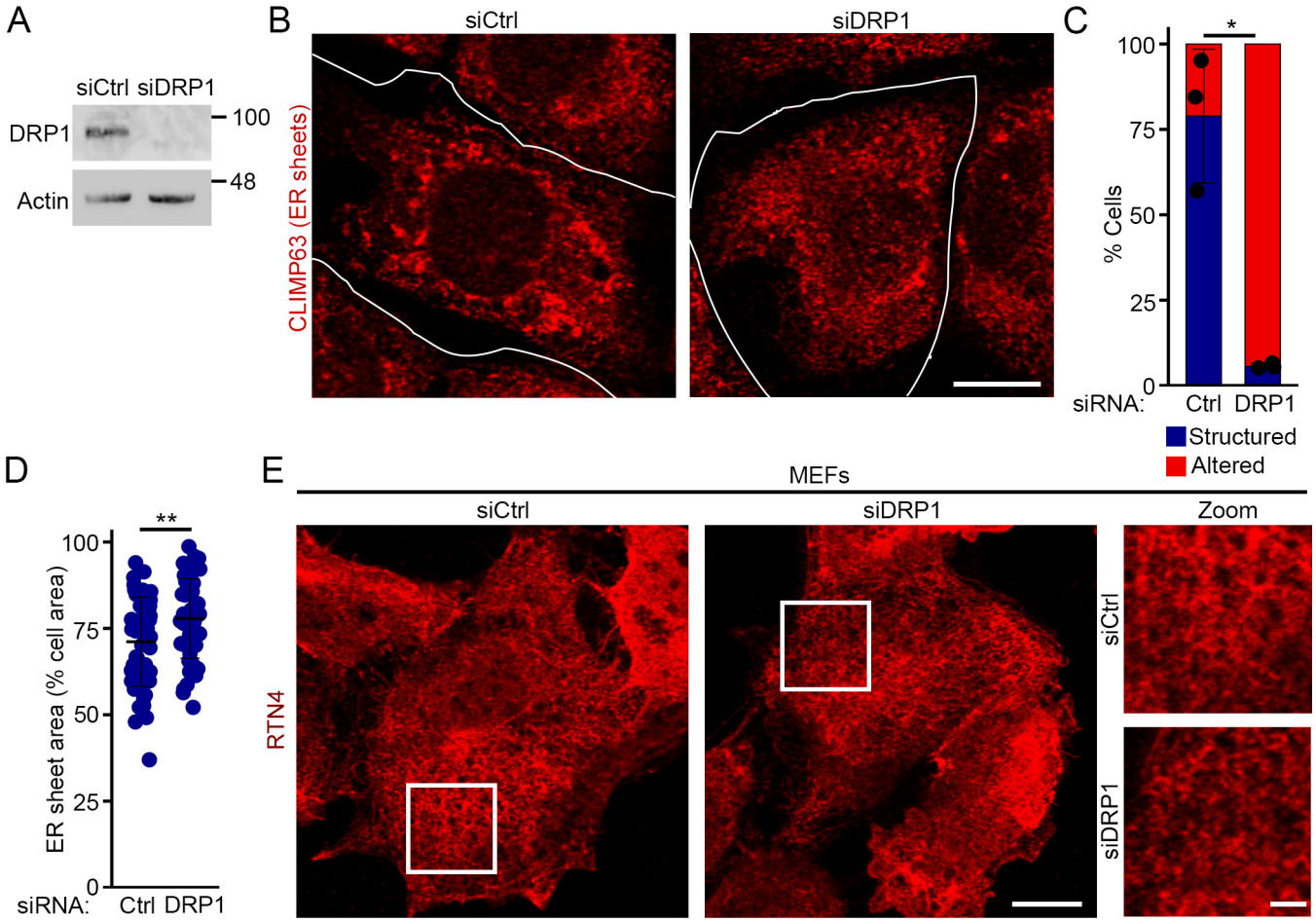
Supplementary Figure 1



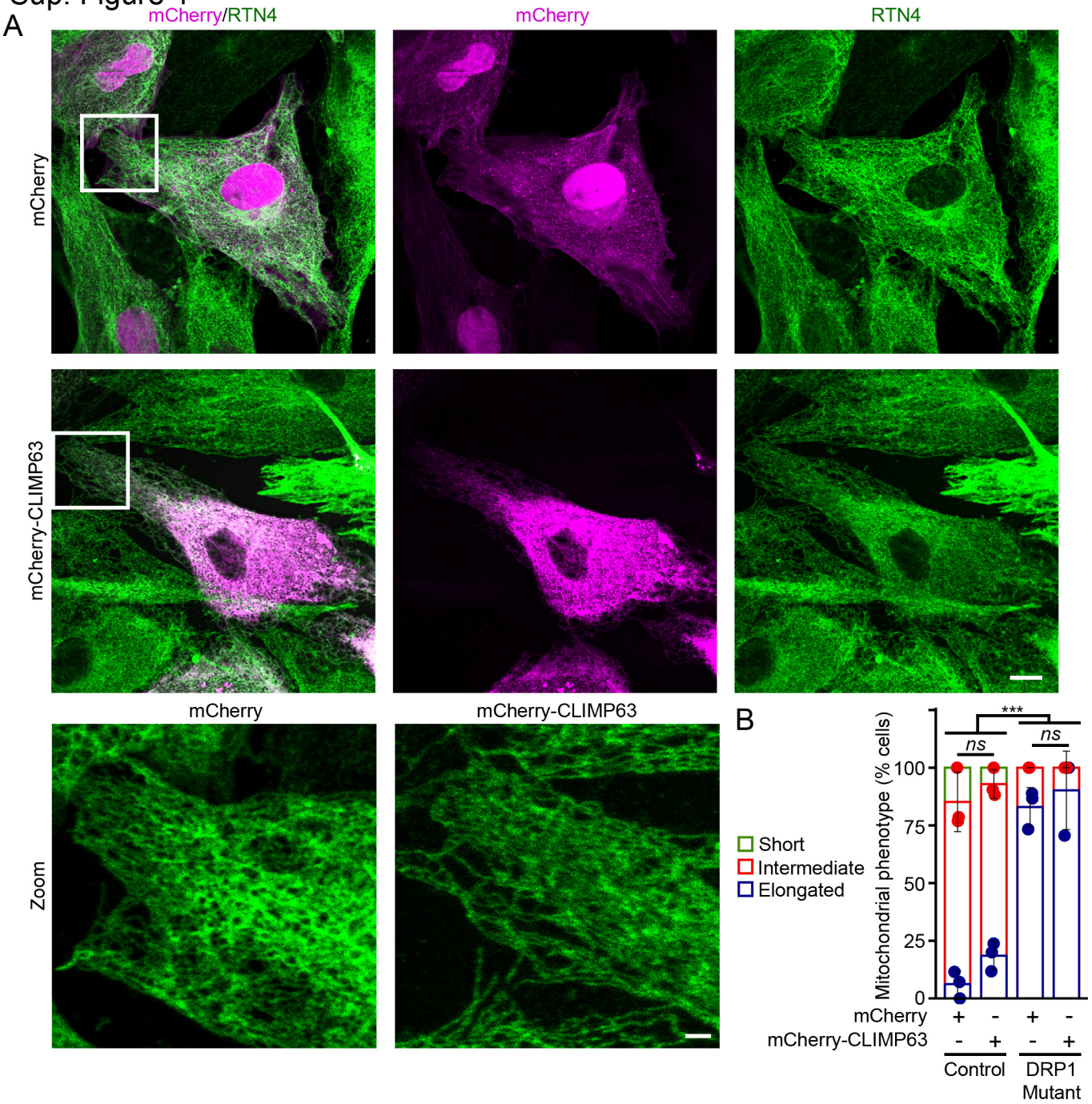
Sup. Figure 2


Mitotracker/CLIMP63/RTN4
Mitotracker/RTN4
Mitotracker/CLIMP63
Control fibroblasts
Mitotracker/CLIMP63/RTN4
Mitotracker/RTN4
Mitotracker/CLIMP63
DRP1 mutant fibrobalsts

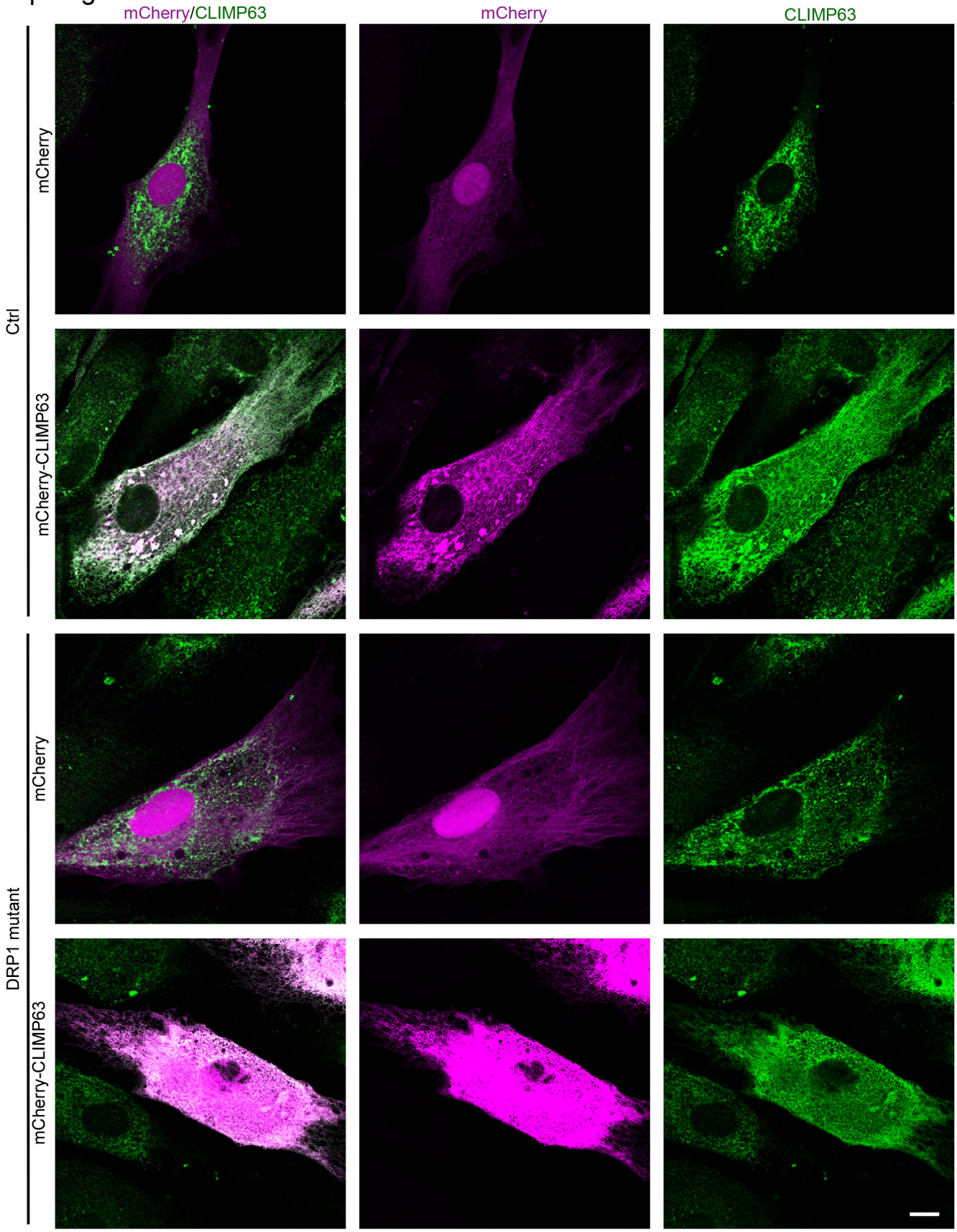
Supplementary Figure 3



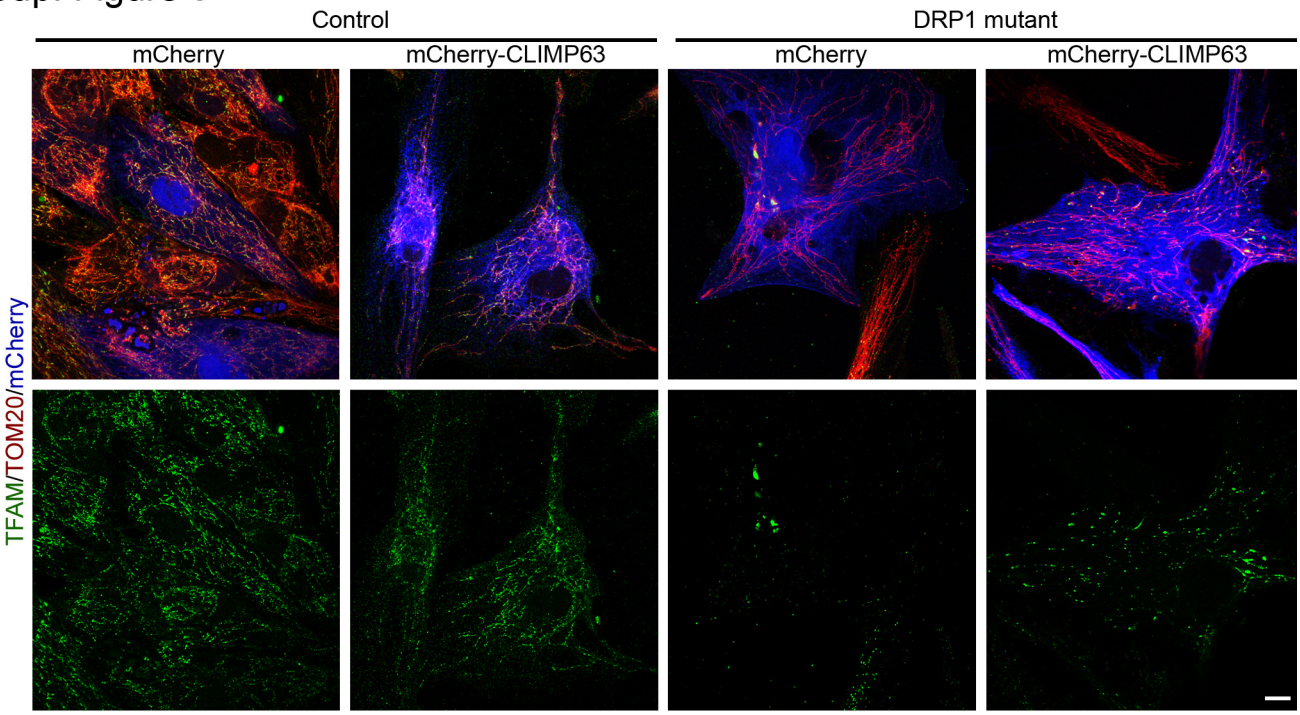
Sup. Figure 4


A
mCherry/RTN4
mCherry
RTN4
mCherry
mCherry-CLIMP63
mCherry
mCherry-CLIMP63
Zoom
B
Short
Intermediate
Elongated
Mitochondrial phenotype (% cells)
100
75
50
25
0
***
ns
ns
mCherry
mCherry-CLIMP63
Control
DRP1 Mutant

Sup. Figure 5


mCherry/CLIMP63
mCherry
CLIMP63
Ctrl
mCherry
mCherry-CLIMP63
DRP1 mutant
mCherry
mCherry-CLIMP63

Sup. Figure 6


Control
DRP1 mutant
mCherry
mCherry-CLIMP63
mCherry
mCherry-CLIMP63
TFAM/TOM20/mCherry