Uncoupling the Dynamics of Inner and Outer Mitochondrial Membrane Fission

Samantha Gumbin
Molecular, Cellular, and Developmental Biology
College of Arts and Sciences
University of Colorado at Boulder

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Thesis Advisor: Dr. Gia Voeltz
Department of Molecular, Cellular, and Developmental Biology
MCDB Department Honors Chair: Dr. Brian DeDecker
Department of Molecular, Cellular, and Developmental Biology
Honors Thesis Committee Member: Dr. Jeffrey Cameron
Department of Chemistry and Biochemistry

Abstract

Mitochondria are dynamic organelles undergoing constant changes in their morphology through two opposing processes; mitochondrial fission and fusion. The current model of mitochondria fission begins with the endoplasmic reticulum (ER) localizing to the mitochondrial outer membrane and initiating membrane constriction. Additional key molecular players, specifically a mitochondrial transmembrane protein, MFF and a cytosolic GTPase, Drp1, localize to the outer mitochondrial membrane to drive constriction and subsequent fission of the mitochondria. While several proteins have been identified to be involved in mitochondrial fission, almost all of them function at the outer mitochondrial membrane (OMM). We therefore have very little knowledge about what regulates the fission of the inner mitochondrial membrane (IMM). Using live cell fluorescent microscopy, we have quantitatively assessed the morphological state of the IMM and OMM leading up to mitochondrial fission. Our results show that IMM constriction is an early event in mitochondrial fission, often preceding OMM constriction. Furthermore, we show that IMM constriction is independent of Drp1, as membrane constriction is still prevalent even in the absence of Drp1. Together our data suggests that IMM and OMM constriction are separate events and the mechanisms driving constriction of both membranes may be independent of each other.

Introduction

Mitochondria play critical roles in the cell, performing several functions indispensable for cellular survival and homeostasis, including generating ATP through oxidative phosphorylation (OXPHOS), regulation of apoptosis, generation and control of reactive oxygen species, and calcium homeostasis (Youle et al, 2005). Mitochondria are dynamic organelles that undergo changes in their morphology through the coordinated and tightly regulated processes of mitochondrial fission and fusion (Westermann, 2010; Chan, 2012; Liesa and Shirihai, 2013; van der Bliek et al., 2013). Mitochondrial morphology is linked to its proper functioning, and disrupting the processes which regulate mitochondrial morphology can have severe consequences on the functioning of the entire cell. Fission, fusion, transportation, and degradation are all processes which regulate health of the mitochondrial population. Mitochondria cannot be generated *de novo*, and must be inherited by each daughter cell when the parent cell divides. To ensure each daughter cell inherits a portion of the mitochondrial population, the mitochondrial network undergoes a dramatic remodeling characterized by an increase in mitochondrial fragmentation that ensures an even distribution to the resulting daughter cells (Mitra, 2009). Defects in mitochondrial fission have been linked to severe cell cycle defects and subsequent cell death implicating an important role for mitochondrial dynamics in cell cycle progression and cell survival (Qian, 2013; Westrate, 2014).

Mitochondria contain many copies of their own small circular genome, which are held in protein rich complexes called nucleoids. Replication of mitochondrial DNA (mtDNA) occurs within the mitochondria and uses distinct mitochondrial DNA replication machinery, which is controlled separately from nuclear genome replication. Fusion allows for complementation between two mitochondria; a healthy mitochondrion can share its genome, RNA, and proteins with a mildly damaged mitochondrion, therefore assisting the less healthy mitochondria in

counteracting its' defects. Division of mitochondria is proposed as a mechanism to segregate newly replicated nucleoids into separate daughter mitochondria, as mitochondrial division sites are spatially linked to replicating nucleoids (Lewis et al. 2016).

Mitochondrial division has also been shown as a mechanism to sequester unhealthy mitochondria. These mitochondria may have damaged OXPHOS machinery, loss of membrane potential, or may have mutations in the mtDNA which are harmful to the health of the mitochondria (Youle and Van der Bliek, 2012). By undergoing scission, the healthy part of the mitochondria can be separated from the damaged section, therefore protecting the healthy section (Gilkerson at al. 2008). The damaged section may undergo mitophagy, where it will be broken down and its components recycled (Ding et al. 2012). Mitochondrial morphology varies across cell types, depending on the metabolic activity of the cell. Defects in both fission and fusion machinery have severe consequences at the organism level. Loss of function of inner membrane fusion protein Optic Atrophy 1 (OPA1) is embryonic lethal (Rahn et al. 2013), and loss of function of fission protein Dynamin Related Protein 1 (DRP1) is embryonic lethal as well (Wakabayashi et al. 2009).

The current model of mitochondrial fission begins with localization of the endoplasmic reticulum (ER) to the mitochondria (Figure 1). The ER is a critical part of the division machinery; 94% of mitochondrial divisions occur at an ER: mitochondria contact site (Friedman et al. 2011). MFF, MiD 49, and MiD 51 act as adaptors and recruiters on the outer membrane for Drp1, a dynamin related GTPase. Drp1 localizes to the mitochondria and oligomerizes into a ring around the mitochondria, and upon GTP hydrolysis Drp1 drives further constriction of the mitochondrion. A recent paper from Voeltz lab has identified an additional regulator of mitochondrial fission, Dynamin 2 (Dyn2), which comes onto the mitochondria after Drp1 to facilitate further constriction and subsequent fission of the mitochondrion (Figure 1).

Knockdown of any of these division proteins results in an elongated mitochondrial network due to the inability of mitochondria to divide, and the retention of the ability to fuse (Labrousse et al. 1999).

While there is significant insight into the mechanism driving fission of the outer membrane mitochondrial, an important outstanding question in the field is exactly how division of the inner mitochondrial membrane is regulated. All structures and proteins currently identified as components of the division machinery localize to and function at the outer mitochondrial membrane. Thus, it is unclear if inner mitochondrial membrane constriction and fission occurs concurrently with that of the outer membrane or if it is independent and regulated through a novel and currently unknown pathway.

Figure 1

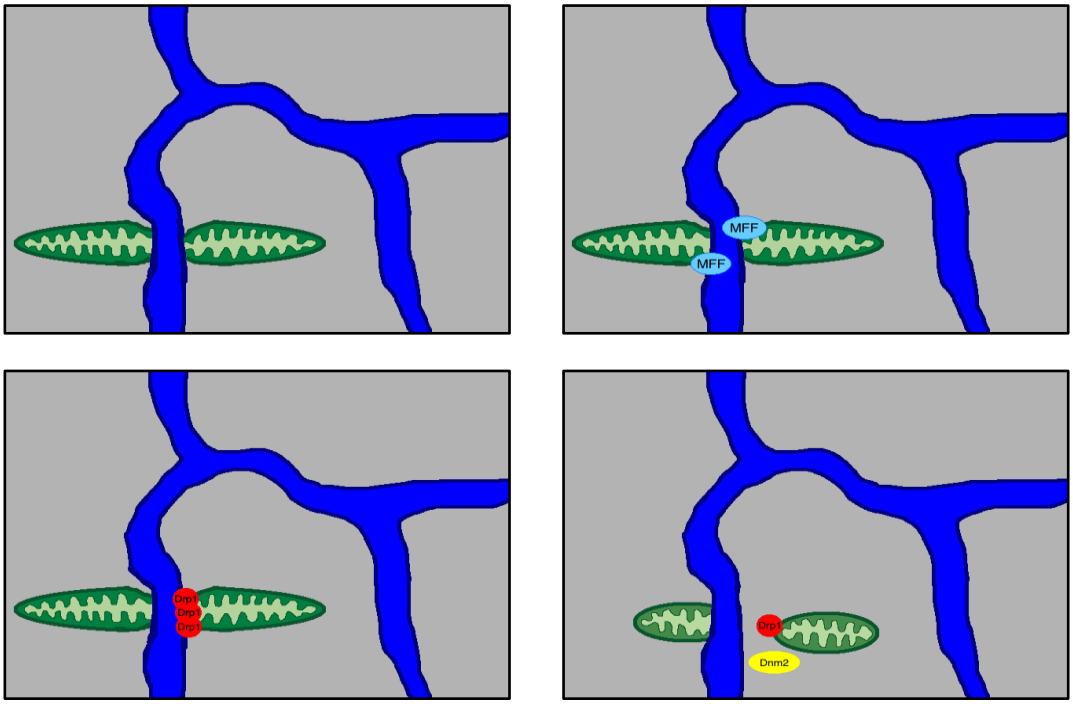

Figure 1: Current model of Mitochondrial Division

Results

Inner Mitochondrial Membrane constriction occurs prior to Outer Mitochondrial Membrane constriction leading up to Fission

The current model of mitochondrial division involves several molecular players (Figure 1), all of which localize and act at the cytoplasmic side of the outer mitochondrial membrane (OMM). However, while the last 20 years of research have largely elucidated our understanding of the mechanism of mitochondrial fission (Hales et al. 1997), it remains unclear how fission of the inner mitochondrial membrane (IMM) occurs. In fact, much of the existing literature relies heavily on mitochondrial matrix markers to track dynamic changes to mitochondrial morphology. Therefore we have very little mechanistic insight on whether constriction and fission of the IMM and OMM are coupled or regulated through independent pathways. To better understand how the inner mitochondrial membrane fits into the model of mitochondrial division, we used live cell microscopy to track dynamics of the IMM and OMM, particularly the morphological state of both membranes leading up to mitochondrial fission.

Mammalian cells were transfected with fluorescently tagged Drp1, an OMM marker Translocase of Outer Mitochondrial Membrane 20 (TOM20), and a subunit of the IMM marker Cytochrome C Oxidase IV (COX IV_su8) which has been previously show to localize to the matrix of mitochondria (Karbowski et al. 2004). Live cell microscopy of cells expressing all three constructs were used to track mitochondrial dynamics over time. We identified mitochondrial fission events in cells expressing these markers, and measured fluorescent intensity of the mitochondrial membranes to quantitatively describe both OMM and IMM membrane continuity and dynamics leading up to the fission event (Figure 2A, B, C). A dip in fluorescent intensity corresponds to constriction of the mitochondrial membrane and was used to assess the timing when membrane constriction was first observed.

To differentiate between non-division related variance in fluorescence, and division related constriction we measured the stochastic change in mitochondrial fluorescence (due to natural variance) by measuring the maximum change in fluorescence intensity along a region of mitochondrion unmarked by fluorescently tagged Drp1. We found that the average level of variance of fluorescence of the outer membrane was approximately 20%, while the variance in inner membrane fluorescence is an average of 30% (Figure 2F). The calculated variance of fluorescence of OMM and IMM were used as a threshold to mark the time point when constriction of the membrane was first detected (i.e. an OMM dip in fluorescence over 20% is considered a constriction, and a 30% dip in IMM fluorescence is considered a constriction).

Constriction analysis of the IMM and OMM prior to fission in Drp1 marked movies revealed that in nearly all cases, the IMM is constricted before the OMM (Figure 2B, C). This is unexpected, because Drp1 localizes to the cytoplasmic side of the OMM and it is unclear how IMM constriction could be induced prior to OMM constriction. If constriction of the IMM was regulated by Drp1 induced mechanical forces at the OMM, we would expect both membranes constrict at the same time (or OMM constriction would precede IMM constriction). We found that IMM constriction is observed, on average, 56 seconds before division occurs; whereas outer membrane constriction is observed around 30 seconds before division (Figure 2D, E). From this data, it appears that inner membrane constriction occurs prior to or at the time of Drp1 localization. The division machinery upstream of Drp1 may control or initiate inner membrane constriction, while it appears that outer membrane constriction occurs as a later step.

Figure 2

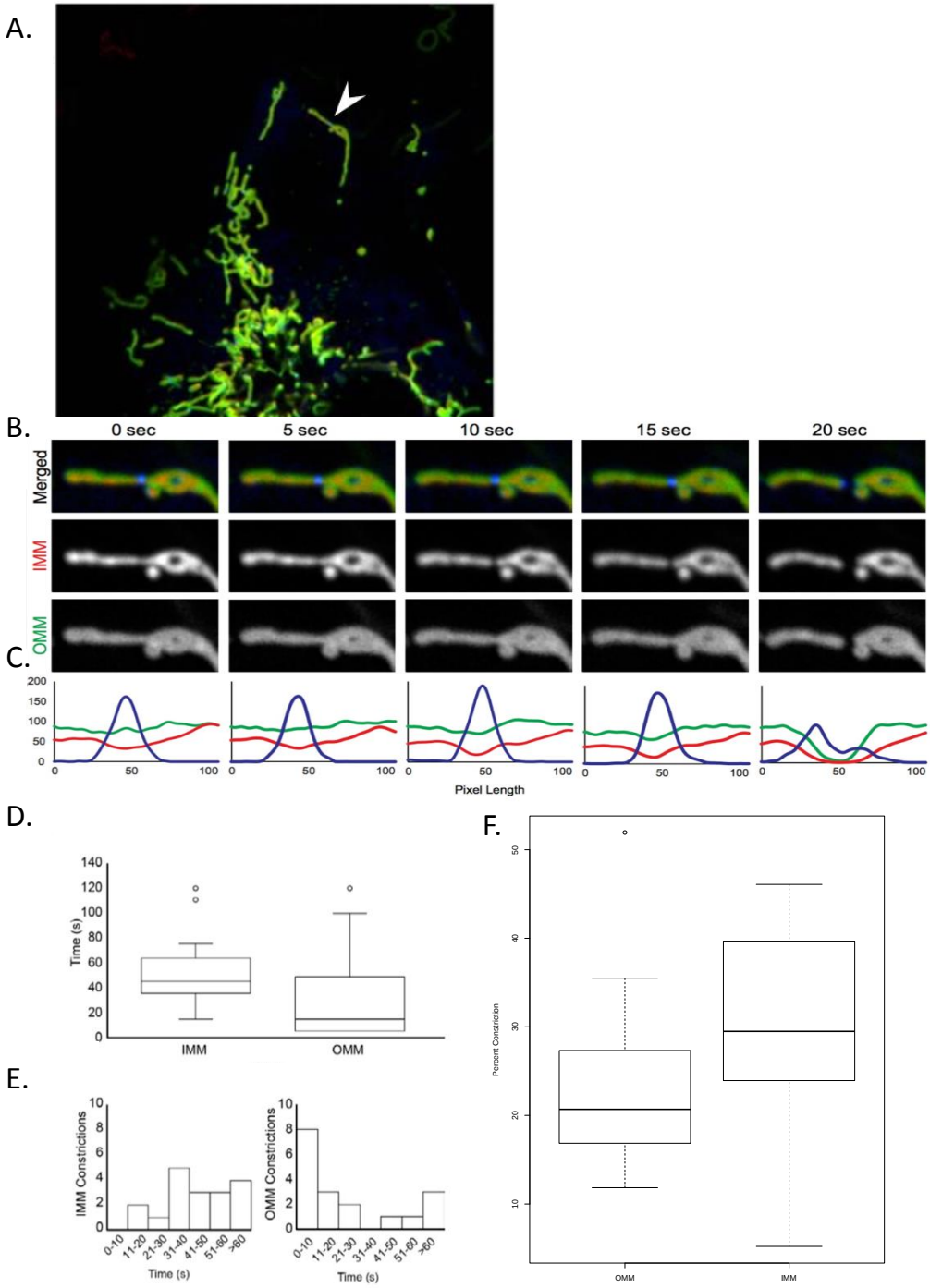

Figure 2: (A) Panel shows a representative image of a live COS7 cell transiently transfected with COX IV-BFP (IMM), Tom20-GFP (OMM), and Drp1-mCherry imaged by confocal microscopy at 37°C. **(B)** Cropped view of the mitochondria indicated in (A) with an arrowhead, shown through time leading to the division event. **(C)** The pixel intensity of COX IV-BFP, Tom20-GFP, and Drp1-mCherry from a line scan drawn along the mitochondrial tubule (green line is the Tom20 signal (OMM), red line is the COX IV signal (IMM), and the blue line is the Drp1 signal). **(D)** Box and Whisker plot of the number of seconds prior to the division event when each membrane becomes constricted. Mean constriction time for IMM: 56.6 ± 31 s and OMM:30.6 ± 36 s (N = 18 fission events) (**E**) Histograms showing the number of seconds before division when each membrane becomes constricted (**F)** Box and whisker plot of distribution of percent change in fluorescence along a section of mitochondria where no Drp1 puncta are localized to the mitochondria.

Constriction of the Inner Mitochondrial Membrane is Drp1 independent

A recent publication from Lewis et. al (2016), found that polymerase gamma 2 (POLG2), was a reliable marker of mitochondrial DNA undergoing replication. Lewis et al. found that POLG2 sites (replicating nucleoids) marked nascent sites of mitochondrial division; so that 73.3% of ER-associated mitochondrial division (ERMD) events occur within 1um of a POLG2 site. With this information, we decided to look at POLG2 sites to narrow our focus from all ER: mitochondria contact sites, which are numerous and can be transient or stable, to more stable contact sites which are most likely to undergo division.

Wildtype cells show a distribution of POLG2 sites throughout the mitochondrial network, with some mitochondria having multiple POLG2 sites, and some mitochondria having no POLG2 sites (Figure 3A). In wildtype cells, POLG2 sites are often located at tips of mitochondria, due to a recent division event near the POLG2 site, which leaves the POLG2 site at the tip of the mitochondria (Lewis et al, 2016). We looked at the distribution of POLG2 sites in wildtype cells, and cells treated with scrambled siRNA and Drp1 siRNA (Figure 3A, C). Cells depleted of Drp1 show an elongated mitochondrial phenotype, due to their inability to undergo division and retention of their fusion machinery (Labrousse et al, 1999), (Figure 3A). Our data suggests that Drp1 knockdown does not affect replication of mtDNA, as there is no significant difference in the number of POLG2 sites in scrambled siRNA treated or Drp1 siRNA treated cells (Figure 3B). Therefore, Drp1 is not necessary to license mtDNA replication. The distribution of POLG2 sites throughout the mitochondrial network appears mildly altered in Drp1 siRNA treated cells compared to wildtype or scrambled siRNA treated cells (Figure 3A). There appears to be fewer tip-localized POLG2 sites, likely due to the lack of division events which create mitochondrial tips near POLG2 sites. Lewis et al (2016) show that ER: mitochondria contact sites may be necessary to license mtDNA replication, and our data is consistent with this conclusion.

To test the hypothesis that constriction of the IMM is Drp1 independent, we looked at COS7 cells expressing GFP-POLG2, the ER marker mCherry-KDEL, and the IMM marker, BFP-COX IV (Figure 4A). We measured the width of the mitochondria at POLG2 marked sites in cells treated with scrambled siRNA and Drp1 siRNA to test whether constriction of the IMM is dependent on Drp1 (Figure 4A, D). We found that in the presence or absence of Drp1, the inner membrane could become severely constricted. In both scrambled and Drp1 siRNA treated cells, the level of constriction of the inner membrane at POLG2 sites at any random time can range from 0% constricted up to 70% constricted (Figure 4B, C). Cells in both conditions displayed the same distribution of percent constriction at POLG2 marked sites, indicating that knockdown of Drp1 does not affect inner membrane constriction. These results indicate that some division machinery upstream of Drp1 must be the regulator and initiator of IMM constriction. We found that the ER was present at all sites of inner membrane constriction, further implicating its importance in IMM constriction. We also measured the distance from each POLG2 site to the nearest ER tubule, and found that this distance was highly varied. We found no correlation between distance from ER tubule to POLG2 site, and level of constriction at that site.

It is unlikely that Mitochondrial Fission Factor (MFF) is the regulator of inner membrane constriction because it resides in the outer membrane, and because inner membrane constriction was still shown in the absence of MFF (Friedman et al, 2011). These data, taken together suggest that IMM constriction is regulated prior to accumulation of MFF or Drp1. Given that the ER recruitment is one of the first identified steps of mitochondrial fission we set out to investigate whether ER-mitochondria contact could be regulating inner membrane constriction.

Figure 3

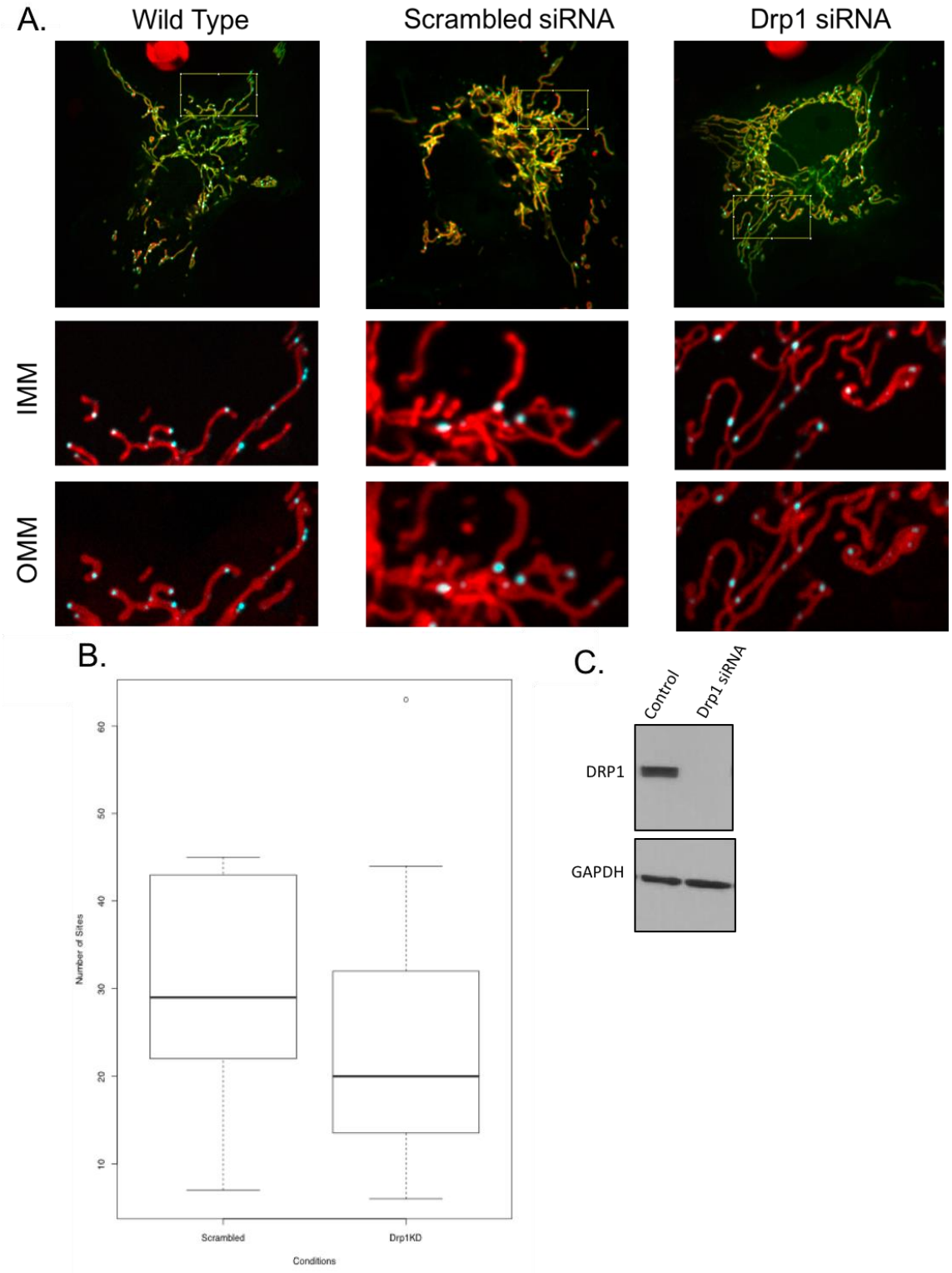

Figure 3: (A) Top - Panels showing representative images of WT, scrambled siRNA, and Drp1 siRNA treated live COS7 cells transiently transfected with COX IV-BFP (IMM), Tom20-mCherry (OMM), and POLG2-GFP (replicating nucleoids) imaged by confocal microscopy at 37°C. Bottom - Cropped view of the mitochondria indicated above by a box. **(B)** Box and Whisker plot of the number of POLG2 puncta in scrambled and Drp1 siRNA treated cells (scrambled siRNA n=9 cells, Drp1 siRNA n=12 cells). **(C)** Immunoblot showing effect of treatment with scrambled and Drp1 siRNA on protein levels.

Figure 4

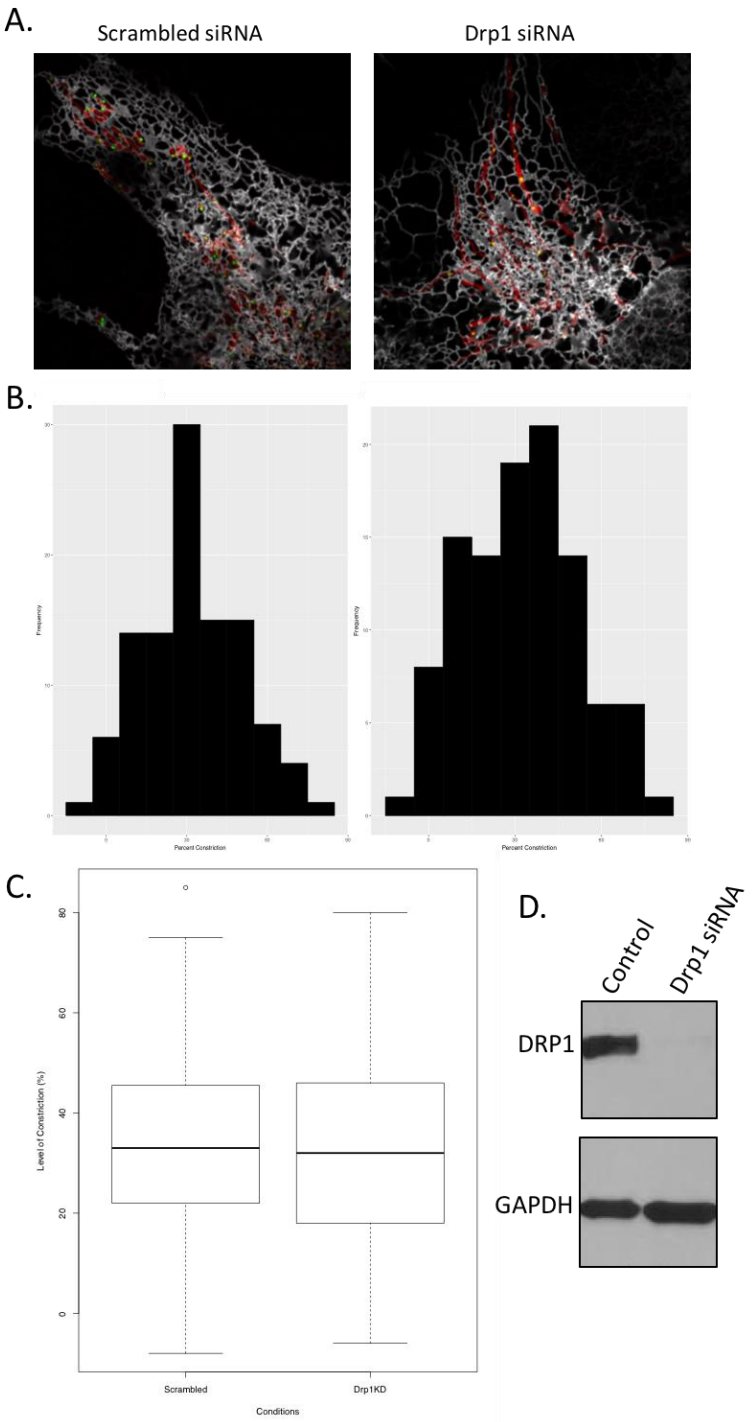

Figure 4: (A) Panels showing representative images of scrambled siRNA and Drp1 siRNA treated live COS7 cells transiently transfected with COX IV-BFP (IMM), KDEL-mCherry (ER), and POLG2-GFP (replicating nucleoids) imaged by confocal microscopy at 37°C. **(B)** Histograms showing the distribution of level of constriction of the IMM at POLG2 puncta at a random time in scrambled siRNA and Drp1 siRNA treated live COS7 cells (scrambled n=107 POLG2 sites from 18 cells, Drp1 siRNA n=105 POLG2 sites from 19 cells). P value=.996. **(C)** Box and Whisker plot showing the distribution of level of constriction of the IMM at POLG2 puncta at a random time in scrambled siRNA and Drp1 siRNA treated live COS7 cells (scrambled n=107 POLG2 sites from 18 cells, Drp1 siRNA n=105 POLG2 sites from 19 cells). **(D)** Immunoblot showing effect of treatment with scrambled and Drp1 siRNA on protein levels.

Constriction of the IMM is an early event in mitochondrial division

The ER has many contacts with the mitochondria, forming about 100 stable ER: mitochondria contacts per cell. The vast majority of these contacts do not mediate constriction or division of the mitochondria (87.6%), and yet 94% of mitochondria division events occur at an ER: Mitochondria contact site (Lewis et. al, 2016, Friedman et al, 2011). The role of the ER in forming functional contacts at sites of mitochondria fission led us to investigate whether the ER plays a role upstream of Drp1 to regulate dynamics of the IMM.

In cells transfected with fluorescently tagged Drp1, the inner membrane is consistently observed as being constricted prior to the outer membrane. To further investigate this phenotype, we looked farther upstream in the division process, at the ER. Mammalian cells were transfected with fluorescent markers for the ER, IMM, and OMM (Figure 5A). We measured the fluorescent intensity of the inner and outer membranes leading up to division events, and found that once again the inner membrane was constricted prior to the outer membrane, (Figure 5B, C) duplicating the result that we found in Drp1 marked fission events. While we saw no significant changes in the timing of IMM constriction before fission (56.6 ± 31s for Drp1 marked events and 61 ± 37s for ER marked events), we did observe a striking difference in the timing of OMM constriction before fission (30.6 ± 36 s for Drp1 marked events and 12.0 ± 10 s for ER marked events) (Figure 5D, E). One likely explanation for why we see variation in the timing of OMM constriction prior to fission may be because fluorescently tagged Drp1 has been previously observed by the Voeltz lab to delay/stall fission. Therefore, the increased time of OMM constriction that was observed in Drp1 marked fission events may be due to the fluorescent tag on Drp1 impacting the kinetics of mitochondrial membrane severing. Our data suggests that inner membrane constriction is an early event, occurring around the time of ER localization. It remains unclear if and how the ER could initiate constriction, perhaps through an unknown signaling mechanism. The constriction of the outer membrane occurred just 10 seconds before

Figure 5

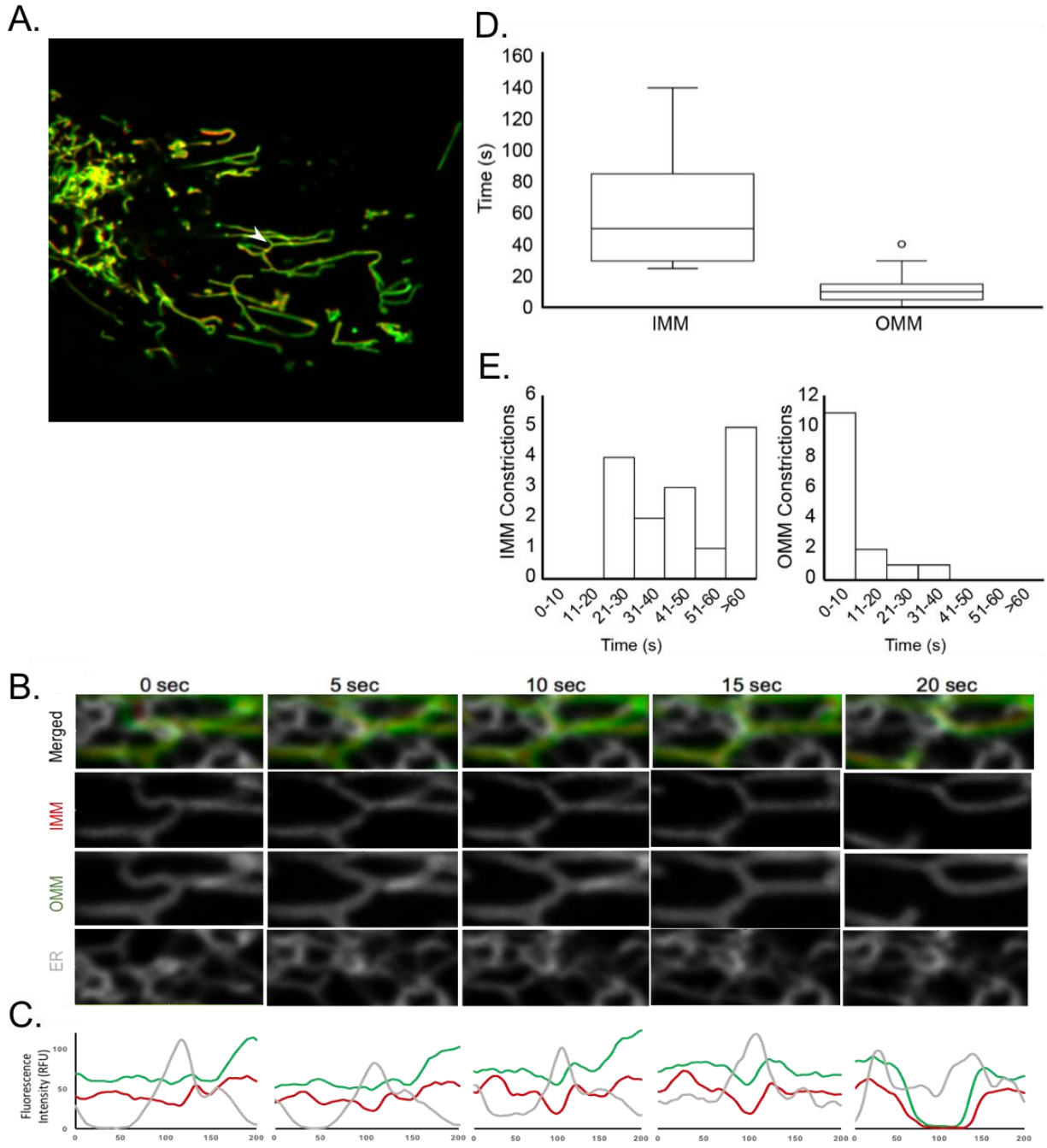

Figure 5: (A) Panel shows a representative image of a live COS7 cell transiently transfected with COX IV-BFP (IMM), Tom20-GFP (OMM), and KDEL-mCherry (ER) imaged by confocal microscopy at 37°C. **(B)** Cropped view of the mitochondria indicated in (A) with an arrowhead, shown through time leading to the division event. **(C)** The pixel intensity of COX IV-BFP, Tom20-GFP, and KDEL-mCherry from a line scan drawn along the mitochondrial tubule (green line is the Tom20 signal (OMM), red line is the COX IV signal (IMM), and gray line is KDEL signal (ER)). **(D)** Box and Whisker plot of the number of seconds prior to the division event when each membrane becomes constricted. Mean constriction time for IMM: 61 ± 37 s and OMM: 12 ± 10 s (N=15 Division events). (**E**) Histograms showing the number of seconds before division when each membrane becomes constricted (N=15 Division events).

division, indicating that the mechanisms regulating inner membrane constriction are likely independent of those regulating outer membrane constriction.

Discussion

Identifying the factors that control constriction and fission of the mitochondrial inner membrane will provide a better understanding of how mitochondrial dynamics are regulated at the molecular level. This may further elucidate the relationship between structure and function of mitochondria, as well as give insight into how different cellular conditions affect mitochondrial dynamics. Taken together our data suggests that some mechanism, independent of outer membrane constriction initiates constriction of the inner membrane. First, we demonstrated that in Drp1 marked fission events, the inner membrane consistently constricts well before outer mitochondrial membrane constriction is observed prior to division. Next, we looked at POLG2 as a marker of future division sites. We found that the number and distribution of POLG2 sites throughout the mitochondrial network was mostly unchanged in the absence of Drp1. We then looked at the level of constriction of the inner membrane at ER crossings adjacent to POLG2 sites. We found that the level of constriction could vary significantly, from no measurable constriction to some sites measuring 70% constriction. The distribution of constriction severity observed at these sites was unchanged in the absence of Drp1, giving further evidence that the mechanism which controls inner membrane constriction does not depend on the presence or action of Drp1. Finally, we showed that in ER marked fission events the IMM becomes constricted significantly earlier than the OMM prior to division.

Our data suggests that constriction of the inner membrane is an early step in the mitochondrial division process. It is therefore intriguing to hypothesize that constriction of the IMM may play a regulatory role in initiating the cascade of division machinery assembly at that site. Perhaps constriction of the inner membrane determines where a division will occur. If this is

the case, then the signaling mechanism which initiates constriction of the inner membrane determines where the mitochondria will divide. Mitochondrial division has been shown to play a critical role in regulating the health of the mitochondrial population; perhaps the mechanism which initiates constriction of the inner membrane is a sensor of mitochondrial health.

If constriction of the inner membrane is occurring via factors acting on the outer membrane, then we would expect to see the inner membrane becoming constricted either at the same time or after the outer membrane becomes constricted. For the inner membrane to become constricted prior to the outer membrane there must be other factors acting at the inner membrane that are not acting on the outer membrane. The ER is the source of intracellular calcium, and can release calcium onto the mitochondria, which can be imported into the mitochondrial matrix though calcium channels in the outer and inner membrane. Perhaps calcium release at ER: mitochondria contact sites initiates constriction of the inner mitochondrial membrane. The ER is also a major site of lipid biogenesis (Philips and Voeltz, 2015), so perhaps the transfer of lipids to or from the mitochondrial membrane initiates the mitochondrial division pathway, beginning with constriction of the inner membrane.

Another possible inner membrane constriction regulator is Optic Atrophy Protein 1 (OPA1). This protein has 8 isoforms, which are differentially expressed in different tissue types (Delettre et al. 2001). The long isoforms of OPA1 (L-OPA1) are crucial for fusion of the mitochondrial inner membrane, and have a transmembrane domain which anchors it in the IMM. OPA1 can undergo cleavage by two proteases, YME1L and OMA1 which cleave OPA1 into short forms (S-OPA1) which are free floating in the inner membrane space (Macvicar and Langer, 2016; Anand et al. 2014). L-OPA1 is necessary for fusion of the inner membrane and its cleavage blocks IMM fusion. Overexpression of S-OPA1 has also been shown to stimulate mitochondrial fragmentation, and S-OPA1 co-localizes with mitochondrial division machinery

(Anand et al. 2014). Depolarization of mitochondria stimulates cleavage of OPA1 by OMA1, blocking fusion and preventing an unhealthy mitochondrion from spreading its depolarization to rest of the mitochondrial population (Suen et al. 2008). Cleavage by OMA1 occurs under stress conditions, so perhaps the accumulation of S-OPA1 is the senor of mitochondrial health, whose accumulation initiates mitochondrial division.

There is also evidence that OPA1 is required for maintenance and replication of mtDNA (Elachouri et al. 2010). Perhaps in healthy mitochondria the site where mtDNA is replicating and OPA1 is localized are the sites where the inner membrane becomes constricted, initiating division at that site to allow segregation of the recently replicated mtDNA into each daughter mitochondria. This is supported by the finding that replicating nucleoids mark the future sites of division (Lewis et al. 2016). Under stress conditions perhaps the inner membrane becomes constricted at sites where OPA1 has been cleaved, to allow the unhealthy or depolarized portion of the mitochondria to be separated from the healthy portion.

Materials and Methods

Cloning and Constructs

For COX IV-BFP, the COX IV gene was PCR-amplified from Hela cDNA and ligated into the pAcBFP-N1 backbone (Clontech) using the Xho1 and BamH1 restriction sites at the 5′ and 3′ ends, respectively. For Tom20, the mitochondrial targeting sequence of Tom20 was PCR-amplified from Hela cDNA and ligated into the pAcBFP-N1 and pAcmCherry-N1 backbone (Clontech) using the Xho1 and BamH1 restriction sites at the 5′ and 3′ ends, respectively. For POLG2-GFP, the mitochondrial targeting sequence of POLG2 was PCR-amplified from Hela cDNA and ligated into the pAcGFP-N1 backbone (Clontech) using the Kpn1 and BamH1 restriction sites at the 5′ and 3′ ends, respectively. The Drp1-mCherry construct was generated as previously described in Friedman et al. 2011 and the KDEL-mCherry construct was generated as previously described in Zurek et al. 2011.

Cell Culture and Transfection of COS-7 cells:

COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were seeded at a concentration of 1.0x10^5 cells/mL in a 2mL well of a 6-well plate. Transient transfections were performed in 1mL of DMEM (with 10% FBS and no penicillin/streptomycin) with 5μL of Lipofectamine 2000 reagent (Invitrogen) for 5 hours with the following amounts of vector DNA. .1μg mCherry-KDEL, .1μg mCherry-Drp1, .1μg COX IV-BFP, .1μg Tom20-GFP, .1μg Tom20-mCherry, .1μg POLG2-GFP. After 5 hour incubation period, cells were treated with .5mL of tripsin and moved into a 2mL, 35mm glass bottom microscope dish and quenched with 1.5mL of DMEM with 10% FBS and 1% penicillin/streptomycin. After approximately 18-24 hours the cells were placed in 2mL of FluoroBrite (DMEM based) with 10% FBS for live cell imaging.

Confocal microscopy

Live cell images were acquired using an inverted fluorescence microscope (TE2000-U; Nikon) equipped with Photometrics Cascade II EM-CCD or a Andor iXon Ultra camera and Yokagowa Spinning disc Confocal (CSU-Xm2) (Nikon Instruments, Inc.). Images were taken in a live cell chamber heated to 37°C with a 100x numerical aperture 1.4 oil objective lens. Images were acquired using NIS-elements Confocal (Nikon Instruments, Inc.) and contrasted, merged, and analyzed using Image-J.

Small interfering RNA oligonucleotides

Drp1 small Interfering RNA oligonucleotides were generated as previously described in Friedman et al. 2011. As a control, Silencer Negative Control #1 siRNA (Ambion) was used. Cells were seeded at a concentration of 0.8x10^5 cells/mL in a 2mL well of a 6-well plate. On day 2 cells a transient transfection was performed in 1mL of DMEM (with 10% FBS and no penicillin/streptomycin) with 50ng of scrambled or Drp1 siRNA with 5μL of Dharmafect reagent (Dharmacon) for 5 hours. Cells were then returned to DMEM with 10% FBS and 1% penicillin/streptomycin, and rested for the third day. On the fourth day cells were transiently transfected with vector DNA at the concentrations listed above, along with 50ng of scrambled or Drp1 siRNA using Lipofectamine 2000 reagent (Invitrogen) for 5 hours. Cells were then treated with .5mL of tripsin and moved into a 2mL, 35mm glass bottom microscope dish and quenched with 1.5mL of DMEM with 10% FBS and 1% penicillin/streptomycin. After approximately 18-24 hours the cells were placed in 2mL of FluoroBrite (DMEM based) with 10% FBS for live cell imaging. One 2mL well of scrambled siRNA treated and one 2mL well of Drp1 siRNA treated cells were collected using a cell scraper and suspended in 50 μL 2X protein sample buffer with

5% ß-mercaptoethanol to be run on a SDS-Page gel. Samples were analyzed using standard SDS-PAGE and Western blotting protocols.

Capturing mitochondrial division and analyzing mitochondrial constrictions.

Dynamic events and interactions, such as mitochondrial division and constriction, were imaged live with fluorescently tagged proteins. Time-lapse videos were acquired over the course of 2 min with each channel captured every 5 s. Exposure times ranged between 200–500 ms in each channel. Tom20–GFP, COX-IV-BFP, mCherry–Drp1, and mCherry-KDEL were imaged in mitochondrial division studies. Division events were verified by line-scan analysis using ImageJ. A segmented line was drawn along the length of the mitochondria, and then the fluorescence intensity of all channels was measured along the length of the line. COS-7 cells expressing COX IV–BFP, mCherry-KDEL, and POLG2-GFP were used to investigate mitochondrial constrictions in Drp1 siRNA treated cells. The background fluorescence of each channel was controlled for using the rolling-ball background subtraction method (radius, 50 pixels). Endoplasmic-reticulum-marked constrictions were identified by decreases in COX IV-BFP fluorescence that were greater than 30% lower than the fluorescence of the non-constricted portion of the same mitochondria. Percent constriction at POLG2 sites was quantified by measuring fluorescence intensity of the mitochondria where no ER tubule was crossing and no POLG2 site was present. This fluorescence intensity was compared with the lowest fluorescence intensity of the mitochondria within 1 μm of the POLG2 site, by this formula:

((Non-constricted fluorescence intensity – lowest fluorescence intensity of area within 1 μm of POLG2 site)/ Non-constricted fluorescence intensity) * 100 = Percent Constricted

Analyses of POLG2 puncta quantity

POLG2 sites in COS-7 cells treated with either Drp1 siRNA or Scrambled siRNA were quantified by using the 'Colocalization Highlighter' tool in ImageJ (National Institutes of Health). This was followed by automated 8-bit Otsu Thresholding, and then thresholded images were evaluated using the 'Analyze Particles' function in ImageJ to obtain the number of POLG2 sites within that image.

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References

Anand, Ruchika, Timothy Wai, Michael J. Baker, Nikolay Kladt, Astrid C. Schauss, Elena Rugarli, and Thomas Langer. "Thei-AAA Protease YME1L and OMA1 Cleave OPA1 to Balance Mitochondrial Fusion and Fission." *The Journal of Cell Biology* 204.6 (2014): 919-29. Web. Available at: http://jcb.rupress.org/content/204/6/919?ijkey=87100f1f2f68064f90da56e5394e221c6ec6e11b&keytype2=tf_ipsecsha

Chan, D. C. (2012). Fusion and Fission: Interlinked Processes Critical for Mitochondrial Health. *Annu. Rev. Genet. Annual Review of Genetics, 46*(1), 265-287. Available at: http://www.annualreviews.org/doi/abs/10.1146/annurev-genet-110410-132529

Delettre, C., Jean-Michel Griffoin, Josseline Kaplan, H. Dollfus, Birgit Lorenz, Laurence Faivre, Guy Lenaers, Pascale Belenguer, and Christian P. Hamel. "Mutation Spectrum and Splicing Variants in the OPA1 Gene." *Human Genetics* 109.6 (2001): 584-91. Web. Available at: https://www.ncbi.nlm.nih.gov/pubmed/11810270

Ding, Wen-Xing, and Xiao-Ming Yin. "Mitophagy: Mechanisms, Pathophysiological Roles, and Analysis." *Biological Chemistry* 393.7 (2012): n. pag. Web. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3630798/

Elachouri, G., S. Vidoni, C. Zanna, A. Pattyn, H. Boukhaddaoui, K. Gaget, P. Yu-Wai-Man, G. Gasparre, E. Sarzi, C. Delettre, A. Olichon, D. Loiseau, P. Reynier, P. F. Chinnery, A.

Rotig, V. Carelli, C. P. Hamel, M. Rugolo, and G. Lenaers. "OPA1 Links Human Mitochondrial Genome Maintenance to MtDNA Replication and Distribution." *Genome Research* 21.1 (2010): 12-20. Web. Available at: https://www.ncbi.nlm.nih.gov/pubmed/20974897

Ernster, L., and G. Schatz. "Mitochondria: A Historical Review." *The Journal of Cell Biology* 91.3 (1981): n. pag. Web. Available at: http://jcb.rupress.org/content/jcb/91/3/227s.full.pdf

Friedman, J. R., Lackner, L. L., West, M., Dibenedetto, J. R., Nunnari, J., & Voeltz, G. K. (2011). ER Tubules Mark Sites of Mitochondrial Division. *Science,334*(6054), 358-362. Available at: http://science.sciencemag.org/content/334/6054/358.full?rss=1

Gandre-Babbe, S., & Bliek, A. M. (2008). The Novel Tail-anchored Membrane Protein Mff Controls Mitochondrial and Peroxisomal Fission in Mammalian Cells. *Molecular Biology of the Cell, 19*(6), 2402-2412. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2397315/

Gilkerson, Robert W., Eric A. Schon, Evelyn Hernandez, and Mercy M. Davidson. "Mitochondrial Nucleoids Maintain Genetic Autonomy but Allow for Functional Complementation." *The Journal of Cell Biology* 181.7 (2008): 1117-128. Web. Available at: http://jcb.rupress.org/content/181/7/1117.full

Hales, Karen G., and Margaret T. Fuller. "Developmentally Regulated Mitochondrial Fusion Mediated by a Conserved, Novel, Predicted GTPase." *Cell* 90.1 (1997): 121-29. Web. Available at: https://www.ncbi.nlm.nih.gov/pubmed/9230308

Karbowski, Mariusz, Damien Arnoult, Hsiuchen Chen, David C. Chan, Carolyn L. Smith, and Richard J. Youle. "Quantitation of Mitochondrial Dynamics by Photolabeling of Individual Organelles Shows That Mitochondrial Fusion Is Blocked during the Bax Activation Phase of Apoptosis." *The Journal of Cell Biology* 164.4 (2004): 493-99. Web. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2172000/

Korobova, F., Ramabhadran, V., & Higgs, H. N. (2013). An Actin-Dependent Step in Mitochondrial Fission Mediated by the ER-Associated Formin INF2. *Science, 339*(6118), 464-467. Available at: https://www.ncbi.nlm.nih.gov/pubmed/23349293

Labrousse, A.M., M. D. Zappaterra, D. A. Rube, A. M. van der Bliek, C. elegans dynaminrelated protein DRP-1 controls severing of the mitochondrial outer membrane. Mol. Cell 4, 815 (1999). Available at: https://www.ncbi.nlm.nih.gov/pubmed/10619028

Legesse-Miller, A. (2003). Constriction and Dnm1p Recruitment Are Distinct Processes in Mitochondrial Fission. *Molecular Biology of the Cell, 14*(5), 1953-1963. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC165089/

Lewis, S. C., Uchiyama, L. F., & Nunnari, J. (2016). ER-mitochondria contacts couple mtDNA synthesis with mitochondrial division in human cells. *Science, 353*(6296). Available at: http://science.sciencemag.org/content/353/6296/aaf5549

Li, S., Xu, S., Roelofs, B. A., Boyman, L., Lederer, W. J., Sesaki, H., & Karbowski, M. (2014). Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission. *J Cell Biol The Journal of Cell Biology,* 208(1), 109-123. Available at: http://jcb.rupress.org/content/208/1/109

Liesa, Marc, and OrianÂ S. Shirihai. "Mitochondrial Dynamics in the Regulation of Nutrient Utilization and Energy Expenditure." *Cell Metabolism* 17.4 (2013): 491-506. Web. Available at: http://www.cell.com/cell-metabolism/abstract/S1550-4131(13)00104-6

Macvicar, Thomas, and Thomas Langer. "OPA1 Processing in Cell Death and Disease, the Long and Short of It." *Journal of Cell Science* 129.12 (2016): 2297-306. Web. Available at: http://jcs.biologists.org/content/129/12/2297

Mitra, K., Wunder C., Roysam B., Lin G., Lippincott-Schwartz J. (2009) A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildups and entry into S phase. *Proceedings of the National Academy of Sciences of the United States of America.* 106: 11960-11965. Web. Available at: http://www.pnas.org/content/106/29/11960

Murley, Andrew, Laura L. Lackner, Christof Osman, Matthew West, Gia K. Voeltz, Peter Walter, and Jodi Nunnari. "ER-associated Mitochondrial Division Links the Distribution of Mitochondria and Mitochondrial DNA in Yeast." *ELife* 2 (2013): n. pag. Web. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3654481/pdf/elife00422.pdf

Phillips, Melissa J., and Gia K. Voeltz. "Structure and Function of ER Membrane Contact Sites with Other Organelles." *Nature Reviews Molecular Cell Biology* 17.2 (2015): 69-82. Web. Available at: http://www.nature.com/nrm/journal/v17/n2/full/nrm.2015.8.html#mcss-regulate-organelle-dynamics

Qian, W., Choi, S., Giboson, GA., Watkins, SC., Bakkenist, CJ et al. (2012) Mitochondrial hyperfusion induced by loss of the fission protein Drp1 causes ATM-dependent G2/M arrest and aneuploidy through DNA replication stress. *Journal of Cell Science.* 125:5745-5757. Web. Available at: http://jcs.biologists.org/content/125/23/5745

Rahn, Jennifer J., Krista D. Stackley, and Sherine S. L. Chan. "Opa1 Is Required for Proper Mitochondrial Metabolism in Early Development." *PLoS ONE* 8.3 (2013): n. pag. Web. Available at: https://www.ncbi.nlm.nih.gov/pubmed/23516612

Rowland, A. A., & Voeltz, G. K. (2012). Endoplasmic reticulum–mitochondria contacts: Function of the junction. *Nature Reviews Molecular Cell Biology Nat Rev Mol Cell Biol, 13*(10), 607-625. Available at: http://www.nature.com/nrm/journal/v13/n10/full/nrm3440.html

Smirnova, E., Griparic, L., Shurland, D., & Bliek, A. M. (2001). Dynamin-related Protein Drp1 Is Required for Mitochondrial Division in Mammalian Cells. *Molecular Biology of the Cell, 12*(8), 2245-2256. Available at: https://www.ncbi.nlm.nih.gov/pubmed/11514614

Song, Z., Ghochani, M., Mccaffery, J. M., Frey, T. G., & Chan, D. C. (2009). Mitofusins and OPA1 Mediate Sequential Steps in Mitochondrial Membrane Fusion. *Molecular Biology of the Cell, 20*(15), 3525-3532. Available at: https://www.ncbi.nlm.nih.gov/pubmed/19477917

Suen, D.-F., K. L. Norris, and R. J. Youle. "Mitochondrial Dynamics and Apoptosis." *Genes & Development* 22.12 (2008): 1577-590. Web. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2732420/

Tondera, D. (2005). The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells. *Journal of Cell Science, 118*(14), 3049-3059. Available at: https://www.researchgate.net/publication/7759398_The_mitochondrial_protein_MTP18_contributes_to_mitochondrial_fission_in_mammalian_cells

Van der Bliek, A. M., Q. Shen, and S. Kawajiri. "Mechanisms of Mitochondrial Fission and Fusion." *Cold Spring Harbor Perspectives in Biology* 5.6 (2013): n. pag. Web. Available at: https://www.ncbi.nlm.nih.gov/pubmed/23732471

Wai, Timothy, and Thomas Langer. "Mitochondrial Dynamics and Metabolic Regulation."*Trends in Endocrinology & Metabolism* 27.2 (2016): 105-17. Web. Available at: http://www.cell.com/trends/endocrinology-metabolism/fulltext/S1043-2760(15)00236-2

Wakabayashi, Junko, Zhongyan Zhang, Nobunao Wakabayashi, Yasushi Tamura, Masahiro Fukaya, Thomas W. Kensler, Miho Iijima, and Hiromi Sesaki. "The Dynamin-related GTPase Drp1 Is Required for Embryonic and Brain Development in Mice." *The Journal of Experimental Medicine* 206.10 (2009): I23. Web. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2753156/

Westermann, Benedikt. "Mitochondrial Fusion and Fission in Cell Life and Death."*Nature Reviews Molecular Cell Biology* 11.12 (2010): 872-84. Web. Available at: http://www.nature.com/nrm/journal/v11/n12/abs/nrm3013.html

Westrate, LM., Sayfie, AD., Burgenske, DM., MacKeigan, JP. (2014) Persistent Mitochondrial Hyperfusion Promotes G2/M Accumulation and Caspase-Dependent Cell Death. *PLoS One.* 9(3). Web. Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0091911#pone.0091911-Mitra1

Youle, Richard J., and Mariusz Karbowski. "Mitochondrial Fission in Apoptosis." *Nature Reviews Molecular Cell Biology* 6.8 (2005): 657-63. Web. Available at: http://www.nature.com/nrm/journal/v6/n8/full/nrm1697.html

Youle, Richard J., and Alexander M. Van Der Bliek. "Mitochondrial Fission, Fusion, and Stress." *Science* 337.6098 (2012): 1062-065. Print. Available at: http://science.sciencemag.org/content/337/6098/1062.full

Zurek, Nesia, Lenore Sparks, and Gia Voeltz. "Reticulon Short Hairpin Transmembrane Domains Are Used to Shape ER Tubules." *Traffic* 12.1 (2011): 28-41. Web. Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0854.2010.01134.x/full