DRP1-mediated mitochondrial fission is essential to maintain cristae morphology and bioenergetics

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Abstract

Mitochondria and peroxisomes are both dynamic signaling organelles that constantly undergo fission. While mitochondrial fission is known to coordinate cellular metabolism, proliferation, and apoptosis, the physiological relevance of peroxisome dynamics and the implications for cell fate are not fully understood. DRP1 (dynamin-related protein 1) is an essential GTPase that executes both mitochondrial and peroxisomal fission. Patients with *de novo* heterozygous missense mutations in the gene that encodes DRP1, *DNM1L*, present with encephalopathy due to defective mitochondrial and peroxisomal fission (EMPF1). EMPF1 is a devastating neurodevelopmental disease with no effective treatment. To interrogate the mechanisms by which DRP1 mutations cause cellular dysfunction, we utilized human-derived fibroblasts from patients with mutations in DRP1 who present with EMPF1. As expected, patient cells display elongated mitochondrial morphology and lack of fission. Patient cells display a lower coupling efficiency of the electron transport chain, increased proton leak, and upregulation of glycolysis. In addition to these metabolic abnormalities, mitochondrial hyperfusion results in aberrant cristae structure and hyperpolarized mitochondrial membrane potential, both of which are tightly linked to the changes in metabolism. Peroxisome structure is also severely elongated in patient cells and results in a potential functional compensation of fatty acid oxidation. Understanding the mechanism by which DRP1 mutations cause these metabolic changes will give insight into the role of mitochondrial dynamics in cristae maintenance and the metabolic capacity of the cell, as well as the disease mechanism underlying EMPF1.

Introduction

Mitochondrial dynamics are controlled and executed by a vast group of proteins and other organelles. The underlying morphology of the mitochondria is an important determinant for the ability of this organelle to regulate a variety of cellular processes, such as apoptosis, cell fate, lipid biosynthesis, and epigenetic modifications (Vantaggiato *et al*, 2019; Khacho *et al*, 2016; Iwata *et al*, 2020; Bayraktar *et al*, 2019; King *et al*, 2021; Prigione & Adjaye, 2011; Rasmussen *et al*, 2020). Mitochondrial fusion is primarily catalyzed by the large GTPases Mitofusin 1 and Mitofusin 2 at the outer mitochondrial membrane (Chen *et al*, 2003). The GTPase OPA1 is responsible for executing fusion of the inner mitochondrial membrane (Delettre *et al*, 2000; Alexander *et al*, 2000). Mitochondrial fission, mediated through the large GTPase DRP1 (Dynamin-related protein 1), is orchestrated by a complex sequence of events (Smirnova *et al*, 2001; Fonseca *et al*, 2019). The first steps of mitochondrial fission include contact with the endoplasmic reticulum (ER) and replication of the mitochondrial genome (Lewis *et al*, 2016; Friedman *et al*, 2011). This is followed by contact and pre-constriction by actin filaments, which are associated with the ER membrane (Li *et al*, 2015; Ji *et al*, 2015, 2017; Chakrabarti *et al*, 2018; Hatch *et al*, 2016, 2014). Fission is also regulated through adaptor proteins at the outer mitochondrial membrane, including MiD49, MiD51, MFF, and FIS1; however, their distinct roles in fission are not completely understood (Osellame *et al*, 2016; Palmer *et al*, 2013; Losó n *et al*, 2013). After the association with adaptor proteins, DRP1 then forms a multimeric ring around the mitochondria and hydrolyzes GTP to GDP, causing a conformational change in the ring that executes fission (Chang & Blackstone, 2007; Fröhlich *et al*, 2013; Nagashima *et al*, 2020; Francy *et al*, 2015; Wang *et al*, 2018; Kamerkar *et al*, 2018). DRP1 is essential for mitochondrial fission, and without functional DRP1 the mitochondrial network becomes hyperfused. In addition to the effects on mitochondrial morphology, DRP1 also mediates the fission of peroxisomes (Koch *et al*, 2003). Peroxisomes are highly specialized organelles vital for the beta oxidation of very-long-chain fatty acids,

detoxification of reactive oxygen species (ROS), production of cholesterol, bile acids, and plasmalogens, which contribute to the phospholipid content in the brain white matter (Schrader *et al*, 2020; Ravi *et al*, 2021; Ding *et al*, 2021; Lodhi & Semenkovich, 2014; Ferreira *et al*, 2021; Chandel, 2021; Sugiura *et al*, 2017). The adaptor proteins MFF and FIS1 are also involved in DRP1 recruitment to sites of peroxisome fission (Gandre-Babbe & Van Der Bliek, 2008; Itoyama *et al*, 2013; Koch & Brocard, 2012). The contacts between mitochondria and peroxisomes are essential for linking peroxisomal β -oxidation and mitochondrial ATP generation by the ETC, maintenance of redox homeostasis, and lipid transfer (Fransen *et al*, 2017; Fan *et al*, 2016; Chandel, 2021). Thus, the metabolic activity of peroxisomes and mitochondria are functionally interconnected. Consistent with this, peroxisomal disorders caused by mutations in genes involved in the biogenesis and function of peroxisomes are often associated with mitochondrial dysfunction (Tanaka *et al*, 2019). This connection may be particularly important during brain development and metabolic stress. However, the molecular mechanisms that facilitate this communication are still poorly understood.

In mouse models, global DRP1 knockout is embryonic lethal (Wakabayashi *et al*, 2009; Ishihara *et al*, 2009). With the advent of exome sequencing, patients with *de novo* mutations in the gene that encodes DRP1, *DNM1L* (*Dynamin 1 Like*), have been identified (Waterham *et al*, 2007; Chao *et al*, 2016; Fahrner *et al*, 2016; Sheffer *et al*, 2016; Ryan *et al*, 2018; Liu *et al*, 2021; Nascia *et al*, 2016; Vandeleur *et al*, 2019; Nolan *et al*, 2019; Keller *et al*, 2021; Longo *et al*, 2020). The disease associated with mutations in *DNM1L* is known as encephalopathy due to defective mitochondrial and peroxisomal fission-1 (EMPF1). Most reported mutations are heterozygous missense, and patients present with heterogeneous symptoms including neurodevelopmental delay, seizures, muscle abnormalities, and ataxia. Patient fibroblasts show very elongated mitochondrial networks and elevated lactate levels, suggesting an impairment in oxidative phosphorylation (Liu *et al*, 2021; Whitley *et al*, 2018). Currently, there is no effective treatment or cure for patients effected

by DRP1 mutations, and the life expectancy for most patients with DRP1 mutations is childhood to early adolescence (Ryan *et al*, 2018). Although other proteins are involved in fission, DRP1 has been shown to be indispensable for mitochondrial fission (Smirnova *et al*, 2001; Kamerkar *et al*, 2018; Fonseca *et al*, 2019; Nagashima *et al*, 2020; Csordás *et al*, 2006; Li *et al*, 2015), yet the cellular consequences of DRP1 dysfunction are not well understood. In this study, we aimed to characterize the effects of two mutations in DRP1 identified in patients with EMPF1: the G32A mutation, which lies in the catalytic GTPase domain of DRP1, and the R403C mutation, which lies in the stalk domain (Fröhlich *et al*, 2013). We resolved and quantified mitochondrial and peroxisomal morphological features associated with EMPF1-associated mutations and reveal their impact on cellular metabolism to put into perspective previously described clinical findings (Ryan *et al*, 2018; Fahrner *et al*, 2016; Liu *et al*, 2021) and gain fundamental insight into the metabolic adaptations to dysfunctional organelle fission. We found that the inability of DRP1 to fragment the mitochondria in patient-derived fibroblasts leads not only to the hyperfusion phenotype previously described, but also to delayed cell death in response to apoptotic signals, aberrant mitochondrial cristae structure, and hyperpolarized mitochondrial membrane potential. Metabolically, the changes in mitochondrial and peroxisomal morphology result in inefficiency of the electron transport chain coupling oxygen consumption with ATP production and upregulation of glycolysis. Altogether, these data indicate that mitochondrial and peroxisomal fission influence dynamics of the inner mitochondrial membrane and the metabolic state of the cell.

Results

DRP1 variants found in patients have dominant negative effects that alter the expression of critical proteins at the mitochondria

Light microscopy studies have shown profound alterations of mitochondrial fission in cells from patients harboring mutations in DRP1 (Ryan *et al*, 2018; Whitley *et al*, 2018). These studies highlighted the clinical relevance of mitochondrial fission in neural development. We quantified the morphology of the mitochondria in patient-derived primary fibroblasts at super resolution level using structured illumination microscopy (SIM). Both G32A and R403C patient fibroblast lines showed increased mitochondrial volume, surface area and major axis length consistent with a hyperfused mitochondrial morphology as reported for DRP1 null cells (Smirnova *et al*, 2001; Kamerkar *et al*, 2018; Fonseca *et al*, 2019) (Fig. 1A-B). Live-cell imaging of mitochondria stained with MitoTracker confirmed that mitochondrial fission events are rare in patient cells compared to control fibroblasts (Supplementary Fig. 1A). High resolution three-dimensional images of control and patient fibroblasts were obtained using serial block facing-scanning electron microscopy (SBF-SEM) (Garza-Lopez *et al*, 2021). Manual segmentation and stack reconstruction of the mitochondrial networks validated the increased length and volume of the mitochondrial networks as well as increased mitochondrial complexity index (MCI) in patient fibroblasts (Fig. 1C-D). MCI is a quantitative measurement of network complexity, which takes into account both volume and surface area of the mitochondria ($MCI = SA^3 / 16\pi^2 V^2$) (Vincent *et al*, 2019; Faitg *et al*, 2021). Both mutations in DRP1 result in significant alterations of the mitochondrial network with G32A fibroblasts showing slightly more severe elongation than R403C (Fig. 1D and Supplementary Fig. 1B).

Both mutations in DRP1 are found to be heterozygous in patients, suggesting that mutant DRP1 has a dominant-effect that can interfere with the ability of wild-type DRP1 to fragment the mitochondria. Previous studies have demonstrated that the GFP can interfere with the activity of

DRP1 (Montecinos-Franjola *et al*, 2020). Thus, we generated mCherry tagged- constructs containing the patient derived mutations and expressed them in human fibroblasts homozygous for endogenous wild-type DRP1. Overexpression of mCherry-DRP1-G32A and mCherry-DRP1-R403C by transient transfection in wild-type fibroblasts demonstrated that, as previously described (Ryan *et al*, 2018; Fahrner *et al*, 2016; Whitley *et al*, 2018), the mitochondrial hyperfusion seen in patient cells is not due to the genetic background and that these mutations act in a dominant negative manner (Fig. 1E-F). While both mutations of DRP1 significantly impaired DRP1 function in human fibroblasts, the G32A show a slightly more severe phenotype than R403C.

DRP1 sequestration and inactivation is thought to lead to unopposed fusion events, which increase the severity of mitochondrial elongation (Palmer *et al.*, 2013). We examined the effects of DRP1 mutations on the protein expression levels of mitochondrial dynamics proteins. Both Mitofusin 1 and Mitofusin 2 were expressed at lower levels in patient cells than in control cells. However, there was no significant difference in the expression of OPA1 (Fig. 2A). These data show that there is a reduction in the expression of mitochondrial outer membrane fusion proteins in patient cells, suggesting that active mitochondrial fission promotes protein stability at the outer mitochondrial membrane. These data also indicate that aberrant fission caused by DRP1 mutations may not alter fusion dynamics at the inner mitochondrial membrane.

Phosphorylation of DRP1 at Serine 616 has been proposed to be a critical determinant of DRP1 recruitment and activity (Koch & Brocard, 2012; Taguchi *et al*, 2007; Valera-Alberni *et al*, 2021). Whether mutations in DRP1 found in patients with EMPF1 affect the levels of phosphorylation at serine 616 (pS616-DRP1) has not been explored. We examined DRP1 levels in patient derived fibroblasts. Although there was no significant difference in total DRP1 expression, G32A patient cells expressed significantly less pS616-DRP1 (Fig. 2B). Since G32A mutation impairs DRP1 phosphorylation to a greater extent than the R403C mutation, the GTPase domain of DRP1 may

be structurally or functionally involved in interactions with kinases responsible for DRP1 phosphorylation. We wondered whether the expression of one copy of mutant DRP1 in patient fibroblasts affected the recruitment of endogenous pS616-DRP1 to the mitochondria. To test this, we quantified the recruitment of phosphorylated DRP1 to the outer mitochondrial membrane using super-resolution microscopy. On average, there was no significant difference in the recruitment of pS616-DRP1 to the mitochondria in patient cells and control cells (Fig. 2C-D). While in all cell lines, pS616-DRP1 was found in close contact to the mitochondrial membrane, in the patient fibroblasts this interaction appeared less transient. These results point to generalized stalling of the fission events in patient fibroblasts.

The onset of apoptosis is accompanied by mitochondrial remodeling, stable association of DRP1 to the mitochondrial membrane, and increased fragmentation of the mitochondrial membrane (Martinou & Youle, 2006; Wasiak *et al*, 2007). In DRP1 deficient cells, this remodeling of mitochondria is significantly reduced and the onset of cell death is delayed (Lee *et al*, 2004). To determine the effects of mitochondrial stress on mitochondrial fragmentation in patient fibroblasts, we quantified changes in mitochondrial fission in response to carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment. As previously shown using RNAi experiments in HeLa cells (Lee *et al*, 2004), patient fibroblasts showed reduced mitochondrial fragmentation induced by CCCP compared to control cells (Fig. 2E-F). Although some mitochondrial fragmentation may occur, mitochondrial volume and major axis length are significantly larger in patient cells treated with CCCP than control cells. During apoptosis, BAX translocates to the mitochondria and cytochrome c is released to the cytosol (Smalli *et al*, 2001). When BAX is recruited to the mitochondria in DRP1-depleted cells, cytochrome c release is delayed. Consistent with this, patient fibroblasts show higher cell survival when treated with CCCP and etoposide as well as lower caspase-3 activity than control fibroblasts when treated with STS (Supplementary Fig. 2A-B). Interestingly, basal levels of both BAX and BAK are significantly reduced in patient fibroblasts

with mutations in DRP1 (Supplementary Fig. 2C). Levels of the anti-apoptotic protein MCL-1 were significantly reduced in G32A fibroblasts but not in R403C fibroblasts, while BCL-XL expression levels remained relatively consistent across all cell lines. Thus, patient fibroblasts may display a compensatory adaptation to reduce the basal levels of BAX/BAK activation. Whether this reduction in the ability to undergo apoptosis in response to mitochondrial stress is a widespread phenomenon in all tissues of EMPF1 patients and/or whether apoptosis inhibition results in deleterious accumulation of mitochondrial and nuclear DNA encoded mutations remains unexplored. No significant alteration in the protein expression of DRP1 adaptors was detected (Supplementary Fig. 2D)

Mitochondrial hyperfusion in DRP1 mutant fibroblasts leads to higher glycolytic rate and increased mitochondrial membrane potential

Mitochondrial morphology is tightly linked to metabolic function (Wai & Langer, 2016; Dorn, 2015). Thus, we assessed the effects of DRP1 patient mutations on cellular metabolism by measuring rates of glycolysis and oxidative phosphorylation (OXPHOS) in cells using a Seahorse XF Analyzer. The Seahorse analyzer measures extracellular oxygen consumption as a readout of OXPHOS and extracellular acidification as a readout of glycolysis. Basal and maximal oxygen consumption was not significantly different in mutant cells compared to control, suggesting no major defects in ATP production (Fig. 3A). However, proton leak was significantly higher in the G32A patient cells and coupling efficiency was significantly lower in both patient lines (Fig. 3B). Therefore, although basal ATP production is not perturbed in the mutant cells, the electron transport chain is not effectively coupling substrate oxidation and ATP synthesis. Extracellular acidification rate was significantly increased in both patient lines, consistent with an upregulation of glycolysis (Fig. 3A) and the hyperlactacidemia observed in more than 50% of patients with mutations in DRP1 (Liu *et al*, 2021). A glycolytic rate assay confirmed that both basal and compensatory glycolysis are higher in both patient lines (Fig. 3C). Thus, although the cells are

capable of producing ATP, *DRP1* mutations cause a defect in OXPHOS efficiency and a metabolic dependency on glycolysis (Fig. 3D).

The upregulation of glycolysis with no accompanied increase in oxidative phosphorylation led us to interrogate a potential impairment in pyruvate transport. Two proteins are essential for mitochondrial pyruvate transport in humans, Mitochondrial Pyruvate Carrier 1 and 2 (*MPC1* and *MPC2*) (Herzig *et al*, 2012; Bricker *et al*, 2012). *MPC1* and *MPC2* associate to form an ~150-kilodalton complex in the inner mitochondrial membrane. We examined the expression of *MPC1* and *MPC2* using Western blot, which revealed a significant decrease in *MPC2* expression in the R403C cells (Fig. 3E). Real time quantitative PCR (RT-qPCR) gene expression analysis showed a significant downregulation of *MPC2* expression in R403C cells and *MPC1* in both patient lines (Fig. 3F). Taken together these data suggest that the upregulation in glycolysis seen in *DRP1* mutant patient fibroblasts is a compensatory mechanism for impaired pyruvate import into the mitochondria and/or decreased coupling efficiency of the ETC.

The major contributor of the proton motive force of the ETC is the mitochondrial membrane potential, which drives oxidative phosphorylation. Thus, changes in OXPHOS coupling efficiency can point to perturbation of the mitochondrial membrane potential and the maintenance of key ion gradients. We investigated this possibility using the fluorescent sensor Tetramethylrhodamine, ethyl ester (TMRE). TMRE is a cell-permeant, cationic, red-orange fluorescent dye that is sequestered by polarized mitochondria. We found that mitochondrial membrane potential as reported by TMRE was significantly increased in patient fibroblasts (Fig. 4), indicative of hyperpolarization of the mitochondria.

***DRP1* mutations alter mitochondrial cristae morphology**

Cristae topology readjustments participate in energy balance control (Eramo *et al*, 2020; Kondadi *et al*, 2020). The hyperpolarization of the mitochondrial membrane detected in patient fibroblasts

lead us to assess the effects of DRP1 mutations on cristae morphology. We used a previously optimized method to quantify and analyze cristae morphology changes from transmission electron microscopy (TEM) (Eisner *et al*, 2017; Lam *et al*, 2021). In this method, cristae abundance is calculated by manually quantifying the number of cristae per mitochondrion, and a score between 0 (worst) and 4 (best) is assigned based on the morphology: (0—no well-defined cristae, 1—no cristae in more than ~75% of the mitochondrial area, 2—no cristae in approximately ~25% of mitochondrial area, 3—many cristae (over 75% of area) but irregular, 4—many regular cristae) (Supplementary. Fig 3A). TEM of patient fibroblasts confirmed the mitochondrial elongation observed in SIM (Supplementary Fig. 3B-D) and revealed differences in the cristae structure (Fig. 5A). The quantification of cristae morphology demonstrated a reduced cristae score and decreased cristae number relative to mitochondrial length in DRP1-deficient cells compared with the control cells (Fig. 5B). These data link aberrant outer mitochondrial membrane fission caused by DRP1 mutations to generalized cristae structural changes. The structure and respiratory function of mitochondrial cristae is maintained by the mitochondrial contact sites and cristae organizing system (MICOS) complex – a large complex of scaffolding and membrane shaping proteins localized at the inner mitochondrial membrane. Given the reduced cristae score, we examined expression of some key components of the MICOS complex. *MIC19*, *MIC25*, and *MIC60* are all reduced in either one or both patient lines at the mRNA level; however, protein expression is not significantly changed (Supplementary. Fig. 4). The data suggest that the structural abnormalities seen in patient fibroblasts are not due to changes in the expression of proteins of the MICOS complex. Additional biochemical assays are needed to assess the functionality of these proteins in the context of EMPF1.

Patient fibroblasts display hyperfused peroxisomes

In addition to catalyzing mitochondrial fission, DRP1 also participates in peroxisomal fission (Kamerkar *et al*, 2018). While fission of peroxisomes has been demonstrated to be involved in *de*

novo peroxisomal biogenesis, the functional consequences of peroxisomal fission are not completely understood. Previous reports using EMPF1 patient fibroblasts have not reported elongation of peroxisomes, but DRP1 null cells have been reported to have hyperfused peroxisomes (Kamerkar *et al*, 2018). To interrogate the effects of DRP1 mutations on peroxisome morphology, we performed super-resolution microscopy. SIM revealed that both mutations in DRP1 result in significant elongation of peroxisomes (Fig. 6A). Quantification of the peroxisome structure shows an increase in volume, surface area, and length of peroxisomes (Fig. 6B). These data confirm that DRP1 mutations found in patients with EMPF1 result in impaired peroxisome fission.

Since peroxisomes and mitochondria are hubs for the regulation of fatty acid metabolism, we first tested whether the dramatic changes in morphology would alter the expression of key genes involved in fatty acid oxidation in patient fibroblasts. The expression of these genes have previously been reported to increase in a DRP1 phosphomimetic mouse model (Valera-Alberni *et al*, 2021). RT-qPCR analysis showed that the expression of carnitine palmitoyltransferase I, *CPT1*, and acyl-coA dehydrogenase medium chain, *ACADM*, are significantly lower in patient cells compared to control (Fig. 6C). *CPT1* is associated with the outer mitochondrial membrane and is responsible for catalyzing the transfer of the acyl group of acyl-CoA to L-carnitine. Fatty acids are then able to move from the cytosol into the inner membrane space of the mitochondria and the matrix, where fatty acid oxidation occurs. *ACADM* encodes for the medium-chain-acyl-CoA dehydrogenase (MCAD) protein, which catalyzes the first step of mitochondrial fatty acid beta-oxidation. Thus, the reduction in *CPT1* and *ACADM* expression may hinder the ability of the mitochondria to carry out fatty acid oxidation.

To examine the ability of patient fibroblasts to perform fatty acid oxidation, we used the Seahorse analyzer to quantify oxygen consumption rates of patient fibroblasts treated with either vehicle or etomoxir, a *CPT1* inhibitor. The cells were cultured with minimal glucose and glutamine and high

298 L-carnitine and palmitate to promote fatty acid oxidation over glycolysis or glutaminolysis. Control
 299 fibroblasts treated with etomoxir show a reduction in maximal respiration following FCCP injection.
 300 In contrast, the G32A patient-derived cells show no change in OCR with etomoxir treatment and
 301 the R403C patient-derived cells show an increase in maximal respiration following FCCP injection
 302 (Fig. 6D and Supplementary Fig. 5). These data indicate that, in contrast to the reliance of control
 303 cells on long chain fatty acid oxidation under conditions of maximal respiration, both patient lines
 304 have lost this dependency and adapted to the inhibition of long-chain fatty acid import into
 305 mitochondria. These data show that fibroblasts with DRP1 mutations appear to have
 306 compensatory adaptations to alterations in long-chain fatty acid oxidation.

307

Discussion

DRP1 is an essential gene for embryonic development (Wakabayashi *et al*, 2009; Ishihara *et al*, 2009). RNAi based approaches in human cell culture models have been used to explore the impact of DRP1 loss on mitochondrial fission and downstream processes such as apoptosis and mitophagy (Otera *et al*, 2016; Kleele *et al*, 2021; Smirnova *et al*, 2001). Although loss of DRP1 results in mitochondrial hyperfusion, it's not fully understood how mutations in DRP1 can impact cellular metabolic function, cristae structure, and/or peroxisomal function. The analysis of organelle morphology in fibroblast lines derived from patients with EMPF1 that harbor one wild-type copy of DRP1 and one missense copy of DRP1, demonstrate a dominant negative effect of mutant DRP1 that results in stalling of the fission machinery that has a wide impact on cellular function.

Mitochondrial fission is essential for cellular processes such as apoptosis, mitophagy, and mitochondrial biogenesis. However, how mitochondrial fission helps maintain cristae structure and/or cellular metabolism has not been thoroughly explored. Our studies revealed that while DRP1 is recruited to the mitochondria in EMPF1 patient lines, the fission event is incomplete, and thus, mitochondrial fission is paused. This stalling of mitochondrial fission results in dramatic changes to cristae morphology, notably a decrease in cristae score and number, indicative of immature and poorly formed cristae. Changes in redox signaling can lead to cysteine oxidation of MIC60 (Wasilewski & Chacinska, 2021; Li *et al*, 2021), an inner mitochondrial membrane protein responsible for cristae structure integrity. Thus, the lack of proper mitochondrial fission in patient cells may disrupt redox signaling, which may in turn be responsible for the aberrant cristae morphology detected in patient cells.

Detailed metabolic analysis of patient fibroblasts showed that these cells have decreased coupling efficiency and upregulation of glycolysis, suggesting that the functional impact of stalled fission is electron transport chain inefficiency. This inefficiency may be due to the changes in

cristae structure, since cristae structure is essential for F_0F_1 -ATPase to function effectively (Wilkens *et al*, 2013; Afzal *et al*, 2021; Davies *et al*, 2011; Cogliati *et al*, 2013). Previous studies revealed that there are distinct fission signatures that are key determinants of the decision of mitochondria to proliferate or degrade (Kleele *et al*, 2021). Peripheral fission events were shown to be preceded by a decrease in membrane potential and proton motive force. We found that the elongated mitochondria from patient fibroblasts have a hyperpolarized membrane potential, which could result from inefficient function of the F_0F_1 -ATPase. Under certain conditions, the F_0F_1 -ATPase can pump protons out of the mitochondrial matrix into the intermembrane space in a reverse-mode action, causing $\Delta\Psi_m$ hyperpolarization. Alternatively, hyperpolarization may be due to complex I inhibition or VDAC (voltage-dependent anion channel) opening, which can cause hyperpolarization independently of F_0F_1 -ATPase reverse-mode action (Forkink *et al*, 2014). A comprehensive analysis of the functionality of the ETC complexes would be needed to elucidate the connection between fission stalling events and mitochondrial hyperpolarization. Upregulation of glycolysis in these patient fibroblasts could result in subsequent glucose depletion locally within the cells, which could in turn cause $\Delta\Psi_m$ hyperpolarization. Carbon-tracing could help reveal whether all of the products of glycolysis are efficiently shuttled into the TCA cycle or if intermediates are lost in the process. Investigating the function of uncoupling proteins such as UCP2 and UCP3 may elucidate whether the decreased efficiency is a byproduct or cause of mitochondrial dysfunction, such as increased $\Delta\Psi_m$, in these cells. Our finding that cells with DRP1 mutations have profound alterations in cristae morphology but apparent maintenance of overall ATP production, raises the interesting possibility that DRP1 mutant cells have evolved specialized mechanisms to cope with electron leakage and damaging reactive oxygen species. A landmark study from Spinelli and collaborators demonstrated the ability of complex II to work in reverse in some tissues with the intrinsic capacity to use fumarate as a terminal electron acceptor (Spinelli *et al*, 2021). It is plausible that complex II reversal has evolved as a mechanism to maintain mitochondrial function in cells with alterations in fission caused by DRP1 mutations.

The existence of two mechanistically and functionally distinct types of fission at the mitochondria and the peroxisomes have not been clearly elucidated. In our study, we demonstrate that patient fibroblasts with mutations in DRP1 also have elongated peroxisomal morphology. Previous studies in mammalian cell culture revealed that peroxisome membrane elongation precedes membrane constriction and final membrane fission (Delille *et al*, 2010; Schrader *et al*, 2016). Whether peroxisomes have a specialized fusion machinery is still under debate. However, we speculate that under particular metabolic conditions peroxisomes, particularly those in contact with mitochondria, may be able to fuse to respond to cellular metabolic needs. Elongation of the peroxisomes in various human disorders has been associated with alterations in fatty acid oxidation (Waterham *et al*, 2016). However; in these cells, peroxisomal function cannot be fully dissociated from changes in mitochondrial morphology. Our model (Figure 7) suggests that the mechanistic and functional connection between peroxisome remodeling and metabolic homeostasis could be dysregulated in mitochondrial diseases such EMPF1. We propose that in this context, human diseases could provide insight into the causal mechanisms underlying basic cellular processes, such as organelle fission. The detailed elucidation of DRP1-centric mechanisms will reveal the connection between organelle dynamics and cell fate. Several questions remain: Does stalling of mitochondrial fission affect the association/dissociation of other proteins at point of fission? Is peripheral fission also inhibited in patient fibroblasts? If that is the case, what are the consequences for mitophagy and mitochondrial DNA integrity? What are the effects of peroxisomal elongation on fatty acid metabolism? How is cell division and mitochondrial segregation affected by DRP1 mutations? How are cell fate transitions affected by DRP1 mutations? Answering these fundamental questions would provide insight into the mechanisms of mitochondrial fission under the lens of this rare disease caused by dysregulated mitochondrial and peroxisomal homeostasis, and would inform the field on more rational and specific therapeutic targets for these devastating neurodevelopmental disorders.

STAR Methods

Cell Culture

Human fibroblasts were thawed and plated directly in maintenance medium consisting of MEM (Gibco #11095098) with 10% FBS (Sigma-Aldrich #F2442) and non-essential amino acids (Gibco #11-140-050). Cells were maintained at 37°C and 5% CO₂ with media changes every other day. Cells were passaged as needed using TrypLE (Thermo Fisher #12-064-013). For imaging experiments, cells were re-plated onto #1.5 glass-bottom 35 mm dishes (Cellvis #D35C4-20-1.5-N).

Cell Treatments

All treatments were added directly to fibroblasts in maintenance medium. The electron transport chain decoupler CCCP (Sigma-Aldrich #C2759-250MG) was added to cells at a concentration of 1 µM. Cells were treated for 2 hours prior to fixation and imaging. All stock solutions were prepared in DMSO.

Plasmid Transfection

Plasmid encoding mCherry-DRP1 (Addgene #49152) was transfected using FuGENE (Promega #E2311) as described in the manufacturer protocol. Patient mutations were introduced to plasmids using the In Fusion cloning kit (Takara Bio #638909) as described in the manufacturer protocol. Cells were maintained until optimal transfection efficiency was reached before cells were imaged.

Immunofluorescence

For immunofluorescence, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized in 1% Triton-X-100 for 10 min at room temperature. After blocking in 10% BSA, cells were treated with primary and secondary antibodies using standard methods. Cells were mounted in Fluoromount-G (Electron Microscopy Sciences #17984-25) prior to imaging. Primary

antibodies used include mouse anti-Mitochondria (Abcam #ab92824), rabbit anti-Tom20 (Cell Signaling Technology #42406S), rabbit anti-DRP1 (Cell Signaling Technology #8570S), rabbit anti-HSP60 (Cell Signaling Technologies # 12165S), mouse anti-PMP70 (Thermo Fisher # MA5-31368), and rabbit anti-pDRP1 S616 (Cell Signaling Technology #3455S). Secondary antibodies used include donkey anti-mouse Alexa Fluor-488 (Thermo Fisher Scientific #A21202), donkey anti-rabbit Alexa Fluor-488 (Thermo Fisher Scientific #A21206), and donkey anti-mouse Alexa Fluor-546 (Thermo Fisher Scientific #A10036), and donkey anti-rabbit Alexa Fluor-546 (Thermo Fisher Scientific #A10040).

Western Blot

Gel samples were prepared by mixing cell lysates with LDS sample buffer (Life Technologies, #NP0007) and 2-Mercaptoethanol (BioRad #1610710) and boiled at 95°C for 5 minutes. Samples were resolved on 4-20% Mini-PROTEAN TGX precast gels (BioRad #4561096) and transferred onto PVDF membrane (BioRad #1620177). Antibodies used for Western blotting are as follows: rabbit anti-DRP-1 (Cell Signaling Technologies #8570S), rabbit anti-pDRP-1 S616 (Cell Signaling Technologies #3455S), rabbit anti-OPA1 (Cell Signaling Technologies #67589S), mouse anti-MFN1 (Abcam #ab57602, mouse anti-MFN2 (Abcam #ab56889), rabbit anti-MFF (Proteintech #17090-1-AP), rabbit anti-MiD49 (Proteintech #16413-1-AP), rabbit anti-MiD51 (Proteintech #20164-1-AP), and rabbit anti-FIS1 (Proteintech #10956-1-AP), and mouse anti-HSP70 (Invitrogen #MA3-006).

Seahorse XF Analysis

Fibroblasts were plated onto Seahorse XF96 V3 PS cell culture microplates 2 days before the assay at 50,000 cells per well. One hour prior to the assay, media was switched to XF DMEM media containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. For the Seahorse XF Mito Stress Test, oxygen consumption rate (OCR) was measured sequentially after addition of 1.0 μ M oligomycin, 0.5 μ M FCCP, and 0.5 μ M rotenone plus Antimycin A. Protein leak and

coupling efficiency were calculated using OCR. For the Seahorse XF Glycolytic Rate Assay, basal glycolytic rate was measured by extracellular acidification, and compensatory glycolysis was measured after addition of 0.5 μ M rotenone plus antimycin A. Treatment with 50 mM 2-DG was used as a negative control. For the Seahorse XF Fuel Flex Test, glutamine dependency was measured by sequential addition of 3.0 μ M BPTES followed by 4.0 μ M Etomoxir plus 2.0 μ M UK5099. Fatty acid dependency was measured by sequential addition of 4.0 μ M Etomoxir followed by 3.0 μ M BPTES plus 2.0 μ M UK5099. Glucose dependency was measured by sequential addition of 2.0 μ M UK5099 followed by 3.0 μ M BPTES plus 4.0 μ M Etomoxir. For the Seahorse XF Long Chain Fatty Acid Oxidation Stress Test, 24 hours prior to assay cells were switched to base media containing 0.5 mM glucose, 1.0 mM glutamine, 0.5 mM XF L-carnitine, and 1.0 % fetal bovine serum. One hour prior to the assay, media was switched to XF DMEM media containing 2 mM glucose, 0.5 mM L-Carnitine, and Palmitate-BSA. Oxygen consumption rate (OCR) was measured sequentially after addition of 4.0 μ M etomoxir, 1.5 μ M oligomycin, 2.0 μ M FCCP, and 0.5 μ M rotenone plus Antimycin A.

RNA Extraction and cDNA Synthesis

Cells were washed with PBS and scraped into 1000 μ l Trizol reagent. 200 μ l of chloroform was added and the samples were incubated at room temperature for 5 minutes. The samples were spun down at 12,000 g and the aqueous phase was collected. 500 μ l of isopropanol was added to precipitate RNA and the sample was incubated for 25 minutes at room temperature, followed by centrifugation at 12,000 g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol, semi-dried, and resuspended in 30 μ l of DEPC water. After quantification and adjusting the volume of all the samples to 1 μ g/ μ l, the samples were treated with DNase (New England Biolabs, #M0303). 10 μ l of this volume was used to generate cDNA using the manufacturer's protocol (Thermo Fisher, #4368814).

Quantitative RT PCR (RT-qPCR)

1ug of cDNA sample was used to run RT-qPCR for the primers mentioned in the table. QuantStudio 3 Real-Time PCR machine, SYBR green master mix (Thermo Fisher, #4364346), and manufacturer instructions were used to set up the assay.

Image Acquisition

Super-resolution images were acquired using a Nikon SIM microscope equipped with a 1.49 NA 100x Oil objective and an Andor DU-897 EMCCD camera. Live-imaging experiments were acquired on a Nikon Eclipse Ti-E spinning disk confocal microscope equipped with a 1.40 NA 60X Oil objective and an Andor DU-897 EMCCD camera. Image processing and quantification was performed using Fiji.

Sample Preparation for Transmission Electron Microscope (TEM) Analysis

Human fibroblasts were immediately washed in ice-cold saline. Samples were processed as described by (Lam et. al., 2021, Pereira et al., 2017). Cells were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer for 1 hour at 37 °C, embedded in 2% agarose, postfixed in buffered 1% osmium tetroxide, stained in 2% uranyl acetate, and dehydrated with an ethanol graded series. After embedding in EMbed-812 resin, 80 nm sections were cut on an ultramicrotome and stained with 2% uranyl acetate and lead citrate. Images were acquired on a JEOL JEM-1230 Transmission Electron Microscope, operating at 120 kV. Random images of human fibroblasts were obtained.

TEM Analysis

Measurements of mitochondrial area, circularity, and length were performed using the multi-measure region of interest (ROI) tool in ImageJ (Lam et. al., 2021, Parra et al., 2014). To measure cristae morphology, we used ROIs in ImageJ to determine the cristae area, circulatory index, number, volume, and cristae score. The NIH ImageJ software was used to manually trace and analyze all mitochondria or cristae using the freehand tool in the ImageJ application (Lam et. al.,

2021, Parra et al., 2014). To assign a mitochondrion a cristae score, cristae abundance and form were evaluated. A score between 0 and 4 (0 – no sharply defined crista, 1 – greater than 50% of mitochondrial area without cristae, 2 – greater than 25% of mitochondrial area without cristae, 3 – many cristae but irregular, 4 – many regular cristae) was assigned (Eisner *et al*, 2017).

Serial Block Facing-Scanning Electron Microscopy (SBF-SEM) Processing

Human fibroblasts were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and processed using a heavy metal protocol adapted from a previously published protocol (Courson et al., 2021; Mustafi et al., 2014). Human fibroblasts were immersed in 3% potassium ferrocyanide and 2% osmium tetroxide (1 hour at 4 °C), followed by filtered 0.1% thiocarbohydrazide (20 min), 2% osmium tetroxide (30 min), and left overnight in 1% uranyl acetate at 4 °C (several de-ionized H₂O washes were performed between each step). The next day samples were immersed in a 0.6% lead aspartate solution (30 min at 60 °C and dehydrated in graded acetone (as described for TEM). Human fibroblasts were impregnated with epoxy Taab 812 hard resin, embedded in fresh resin, and polymerized at 60 °C for 36–48 hours. After polymerization, the block was sectioned for TEM to identify the area of interest, then trimmed to 0.5 mm × 0.5 mm and glued to an aluminum pin. The pin was placed into an FEI/Thermo Scientific Volumescope 2 scanning electron microscope (SEM). The scope is a state-of-the-art SBF imaging system. For 3D EM reconstruction, thin (0.09 µm) serial sections, 300 to 400 per block, were obtained from the blocks that were processed for conventional TEM. Serial sections were collected onto formvar coated slot grids (Pella, Redding CA), stained, and imaged, as described above. The segmentation of SBF-SEM reconstructions was performed by manually tracing structural features through sequential slices of the micrograph block. Images were collected from 30–50 serial sections, which were then stacked, aligned, and visualized using Amira to make videos and quantify volumetric structures.

Statistical Analysis

All experiments were performed with a minimum of 3 biological replicates. Statistical significance was determined by one-way or repeated measures ANOVA as appropriate for each experiment. GraphPad Prism v8.1.2 was used for all statistical analysis and data visualization. Error bars in all bar graphs represent standard error of the mean unless otherwise noted in the figure. No outliers were removed from the analyses. Quantification of mitochondrial morphology was performed in NIS-Elements (Nikon); briefly, we segmented mitochondria in 3D and performed skeletonization of the resulting 3D mask. Skeleton major axis length, volume, and surface area measurements were exported into Excel. For EM, the results are presented as the mean $\pm$ standard error of the mean. Data were analyzed using an unpaired T-test. If more than two groups were compared, one-way analysis of variance (ANOVA) was performed, and significance was assessed using Fisher's protected least significance difference test. GraphPad PRISM and Statplus software package was used for t-tests and ANOVA analyses (SAS Institute: Cary, NC, USA). For all statistical analyses, a significant difference was accepted when $P < 0.05$.

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Author Contributions

G. Robertson and V. Gama conceived the study, designed experiments, interpreted data, and wrote the manuscript. G. Robertson designed and carried out all the cell biology experiments, with technical assistance from M. Patel and S. Riffle. J. Mears provided technical feedback and insight into the conception and execution of study. A. Marshall, H. Beasley, Z. Vue, J. Shao, and E. Garza Lopez performed the TEM, 3D EM and sbf-SEM experiments, quantification, and analysis, supervised by A. Hinton.

Figure Legends

Figure 1. DRP1 patient-derived fibroblasts have elongated mitochondrial morphology. (A) Representative images of mitochondrial morphology in patient-derived fibroblasts using structured illumination microscopy (n=3, 7 cells per replicate). Scale bar: 5 μ m. (B) Quantification of mitochondrial volume, surface area, and major axis length. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (C) Representative sbf-SEM 3D reconstructed images. Each mitochondrion is colored a unique color. (D) Quantification of sbf-SEM for 3D mitochondrial length, volume, and complexity index. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (E) Representative images of mitochondrial morphology in control fibroblasts transfected with either DRP1^{WT}-mCherry, DRP1^{G32A}-mCherry, or DRP1^{R403C}-mCherry using structured illumination microscopy (n=3, 7 cells per replicate). Top scale bar: 1 μ m. Bottom scale bar: 5 μ m. (F) Quantification of mitochondrial volume, surface area, and major axis length. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 2. Mutant G32A and R403C DRP1 are recruited to mitochondria. i. (A) Western blot of total protein lysate isolated from patient fibroblasts and probed for mitochondrial fusion proteins. Representative image of three independent biological replicates. (B) Western blot of total protein lysate isolated from patient fibroblasts and probed for mitochondrial fission proteins. Band density is normalized to loading control (HSP70) and relative to control cells. Representative image of

three independent biological replicates. (C) Representative images of mitochondrial and DRP1 in patient-derived fibroblasts using structured illumination microscopy (n=3, 7 cells per replicate). Scale bar: 5 μ m. Top image is a zoom of white box from bottom image. Scale bar: 1 μ m. (D) Quantification of DRP1 puncta that overlap with mitochondria and volume of DRP1 puncta. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (E) Representative images of mitochondrial morphology in patient-derived fibroblasts treated with either DMSO or CCCP for 1 hours using structured illumination microscopy (n=3, 7 cells per replicate). Scale bar: 5 μ m. (F) Quantification of mitochondrial volume, surface area, and major axis length. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 3. Mitochondrial defects lead to inefficient coupling of the electron transport chain and higher glycolytic rate.

(A) Oxygen consumption rate and extracellular acidification rate in patient fibroblasts as measured using Seahorse Mitochondrial stress test (n=3, 20 wells per replicate). Oligomycin applied at 20 minutes, FCCP applied at 40 minutes, and rotenone/Antimycin A applied at 60 minutes. Analyzed using two-way ANOVA followed by Šídák's multiple comparisons test. (B) Proton leak and coupling efficiency calculated using oxygen consumption rate. (C) Basal glycolysis of patient fibroblasts measure using Seahorse Glycolytic Rate Assay. Compensatory glycolysis measured following application of rotenone/Antimycin A. (D) Glucose, glutamine, and fatty acid dependency of the electron transport chain measured using Seahorse Fuel Flex Assay. Glucose dependency measured after application of UK5099, glutamine dependency measured after application of BPTES, and fatty acid dependency measure after application of Etomoxir. (E) qRT-PCR analysis of gene expression in fibroblasts relative to control and normalized to two housekeeping genes (*GPI* and *GAPDH*). Quantifications are shown for three independent biological replicates. (F) Western blot of total protein lysate isolated from patient fibroblasts and probed for mitochondrial pyruvate carriers. Band density is normalized to

loading control (HSP70) and relative to control cells. Representative image of three independent biological replicates.

Figure 4. DRP1 patient fibroblasts have more hyperpolarized mitochondrial membrane potential. (A) Representative live-cell imaging of patient-derived fibroblasts treated with 500nM TMRE for 10 minutes. Cells were imaged every 15 seconds for 15 minutes using spinning disk confocal (n=3, 7 cells per replicate). Scale bar: 5 μ m.

Figure 5. Cristae morphology is altered in patient cells. (A) Transmission electron microscopy of patient fibroblasts. 3 representative images per line shown. (B) Quantification of cristae from TEM images for cristae score and cristae number relative to mitochondrial length. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 6. Patient fibroblasts have elongated peroxisomes. (A) Representative images of peroxisomal morphology in patient-derived fibroblasts using structured illumination microscopy (n=3, 7 cells per replicate). Scale bar: 5 μ m. Bottom image is a zoom from top image. (B) Quantification of peroxisomal volume, surface area, and major axis length. Analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (C) qRT-PCR analysis of gene expression in fibroblasts relative to control and normalized to two housekeeping genes (*GPI* and *GAPDH*). Quantifications are shown for three independent biological replicates. (D) Oxygen consumption rate in patient fibroblasts as measured using Seahorse Analyzer XFe96 (n=3, 20 wells per replicate). Etomoxir applied after 20 minutes, oligomycin applied at 40 minutes, FCCP applied at 60 minutes, and rotenone/Antimycin A applied at 80 minutes. Analyzed using two-way ANOVA followed by Šídák's multiple comparisons test.

Figure 7: Current model of the cellular responses to EMPF1 associated DRP1 mutations. EMPF1- associated mutations do not appear to alter the capacity of DRP1 to be recruited to points of fission, but rather cause stalling of the fission events. Pausing peroxisomal and mitochondrial

605 fission results in alterations of cristae structure, changes in mitochondrial membrane potential,
606 decreased mitochondrial coupling efficiency, delayed cell death response, and overall metabolic
607 adaptations.

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Figure 1

Figure 2

Figure 3

Figure 4

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Figure 5

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B

Figure 6

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