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Review

The molecular mechanism of mitochondrial fusion

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ABSTRACT

This review is focused on mitochondrial membrane fusion, which is a highly conserved process from yeast to human cells. We present observations from both yeast and mammalian cells that have provided insights into the mechanism of mitochondrial fusion and speculate on how the key players, which are dynamin-related GTPases do the work of membrane tethering and fusion.

It is now generally accepted that mitochondria are dynamic organelles, constantly undergoing both division and fusion. This was not always the case. In science it is often that a historical and foundational discovery does not drive progression of a field over several decades. In the case of mitochondrial fusion, however, the roots of the field remain at the heart of the current questions being addressed. These roots can be attributed in large part to the pioneering work of our late colleague Dr. Ron Butow on the transmission of the mitochondrial nucleoid. Specifically, the field was born from a set of experiments by Ron Butow and others aimed at deciphering non-random mitochondrial DNA transmission in yeast zygotes. Ron and other colleagues, such as his long time collaborator Phil Perlman, observed by elegant pedigree analyses that in $rho^+ \times rho^+$ yeast crosses, there is limited mixing of parental mtDNAs in the zygote. Indeed, the mixing of mtDNA, as evidenced by either recombination or the presence of both parental mtDNA populations, is confined to middle region of zygotes where the medial bud emerges. In contrast, end buds tend to inherit a non-recombined mtDNA only from the parent that gave rise to that end of the zygote [1,2].

To determine the cellular basis for this non-random inheritance pattern, Ron's lab showed that haploid derived mitochondrial protein is efficiently redistributed from one parental end of the zygote to the other [3]. This observation directly revealed mitochondrial fusion and suggested that there was an active and specific segregation machine that restricts and directs the movement of mtDNA. This work inspired many investigators to delve deeper into this phenomenon and develop direct assays for mitochondrial fusion and division and to discover the protein components required for these events [4]. The intimate tie between mitochondrial fusion and mtDNA transmission has been validated by observations in yeast and mammalian cells. Indeed, the most fundamental function of mitochondrial fusion is to faithfully transmit mtDNA. While we are acquiring a more detailed understanding of the mechanism of mitochondrial fusion, the exact molecular basis of the relationship between fusion and mtDNA transmission is still an outstanding and fundamental question that is currently the focus of several laboratories. Thus, it seems fitting that this review and this special issue of BBA is in honor of Dr. Ron Butow and the amazing body of work that came from his laboratory that has served to drive many fields in biology, including the mitochondrial dynamics field.

Here, we focus on mitochondrial membrane fusion, which is a highly conserved process from yeast to human cells and present observations from both yeast and mammalian cells that have provided insights into the mechanism of mitochondrial fusion and speculate on how the key players, which are dynamin-related GTPases do the work of membrane tethering and fusion.

1. How is membrane fusion driven by dynamin-related proteins?

The fundamental function of the large GTPase dynamin-related protein (DRP) family is to regulate membrane dynamics in a variety of different cellular processes [5]. The canonical member of the DRP family, dynamin, and the mitochondrial division dynamin, Dnm1, have been the most extensively characterized and each likely promotes membrane scission by the use of forces generated by GTPase cycle dependent self-assembly [6,7]. Other members of the dynamin family are involved in different types of membrane remodeling events [8]. Two of these are Fzo1 and Mgm1, highly conserved mitochondrial DRPs, that are essential for outer and inner mitochondrial fusion respectively. Other cellular fusion events rely on proteins such as SNAREs, which interact in *trans*, working to overcome

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the intrinsic repulsive forces involved in bringing two bilayers into close apposition and to destabilize the membrane bilayer structure [9]. Therefore, as proteins that mediate mitochondrial membrane fusion events, Fzo1 and Mgm1 represent a novel membrane remodeling function for DRPs.

The GTPase domain of DRPs is highly conserved and composed of four motifs required for GTP binding and hydrolysis [6,10]. In addition to a GTPase domain, DRPs possess other regions that are comprised of predicted coiled-coil structures. These regions are often referred to as the middle domain and the GTPase effector domain (GED), although whether these regions actually form discreet structural domains is uncertain [11,12] (Fig. 1). While other G-proteins involved in signal transduction require factors to regulate the GTPase cycle, DRPs are built to transition through the GTP hydrolysis cycle by virtue of their intrinsic structural attributes [13]. First, unlike the small GTPases, which have a high affinity for GTP and therefore require nucleotide exchange factors (NEFs), DRPs have a relatively low affinity for GTP and consequently do not require NEFs [14,15]. Probably the most functionally important unique feature of this protein family is their ability to self assemble. The most extensively characterized DRPs, dynamin and Dnm1, are dimeric and self assembly of dimers into large oligomeric structures stimulates GTP hydrolysis, precluding the need for GTPase activating proteins [14,15]. The middle and GED regions of DRPs are important for mediating the intra- and inter-molecular interactions required for self-assembly and oligomerization-dependent GTPase activity. Although additional factors are not essential for the DRP GTPase cycle, DRPs interact and potentially co-assemble with proteins that likely function as effectors to modulate their kinetic and structural properties. DRP molecules are also often supplemented with independent domains required for membrane targeting, such as a pleckstrin-homology (PH) domain in dynamin to promote its association with lipids and a proline rich domain to promote interaction with proteins containing SH3 (SRC-homology-3) domains.

High-resolution structures of eucarytotic DRPs have not been reported, presumably due to their conformational plasticity and self-assembly activity. The exceptions to this are the dynamin-related human guanylate-binding protein and the ATPase, EHD, which shares similar kinetic and structural properties to DRPs [16]. However, a high-resolution structure of BDLP, a procaryotic GDP-bound DRP, has recently been solved and potentially provides an excellent structural model for eucaryotic DRPs [17]. One striking feature of the BDLP structure is that the regions predicted to correspond to the DRP middle and GED regions do not form discrete domains. Rather, these regions are intertwined into parallel four helix coiled-coil bundles. In addition, BDLP forms a crystallographic dimer whose interface is comprised of interactions between the two GTPase domains. A similar dimerization interface is also present in human guanylate-binding protein [18], suggesting that all members of the DRP family are dimeric by virtue of inter-molecular GTPase domain interactions.

Amongst the eucaryotic DRPs, the mitochondrial outer membrane fusion DRPs, Fzo1, Mfn1 and Mfn2 are the most closely related to BDLP. Given the procaryotic origin of mitochondria, this suggests that eucaryotic DRPs evolved as a result of endosymbiosis. Like BDLP, Fzo1 possesses coiled-coil regions that likely correspond to eucaryotic middle and GED regions, which have been shown to be important for inter-molecular interactions. Two bona fide transmembrane domains mediate the membrane association of the Fzo1 family. The predicted membrane interaction regions of BDLP that correspond to the transmembrane domains of Fzo1 are within a paddle region in the crystal structure that is predicted to have considerable conformational plasticity, raising the possibility that the structure of this membrane interaction region in Fzo1 is modulated to help do the work of membrane fusion. The membrane topology of Fzo1 places the GTPase and coiled-coil regions in the cytosol with only a short loop between the transmembrane domains in the intermembrane space [19,20] (Fig. 1). In contrast to Fzo1, Mgm1 is more closely related to eucarytotic-like dynamins in that it contains canonical GTPase, middle and GED regions. The N-terminus of Mgm1 harbors a mitochondrial targeting signal and two regions of hydrophobicity, which are all required for targeting Mgm1 to the mitochondrial inner membrane and intermembrane space [21] (Fig. 1).

Both GTP binding and hydrolysis by the mitochondrial fusion DRPs are critical for the fusion of the outer and inner mitochondrial membranes, but the exact molecular role of the GTPase cycle in fusion is not completely understood. In contrast, more is known about the role of the GTPase cycle in the function of membrane division DRPs. Data suggest that in the GTP bound state, both dynamin and Dnm1 assemble into helix-like structures with functionally specific dimensions and that these structures can constrict and tubulate spherical liposomes in vitro [22,23]. Based on this and other observations, dynamin and Dnm1 have been proposed to function through a mechanochemical mechanism where the forces required for membrane constriction and division are provided by GTP driven self-assembly together with assembly-stimulated GTP hydrolysis [6,7].

If the structural and kinetic properties of dynamin and Dnm1 are shared amongst all DRPs, this raises the question of how they are harnessed to promote membrane tethering and fusion events. As we outline in this review, data indicate that membrane tethering is accomplished via the self-assembly of the mitochondrial fusion DRPs in *trans* across two lipid bilayers. Insights into how the subsequent lipid fusion event is mediated by mitochondrial fusion DRPs may be provided by the recent characterization of the ATPase EHD2 [16]. EHD2 is a member of the EH domain containing proteins, such as Eps15, that function in clathrin independent endocytosis and endocytic recycling. Like DRPs, EHD2 is a dimer that can self assemble into spiral-like structures that tubulate membranes in vitro. However, one significant difference between the membrane division DRPs, Dnm1 and dynamin, and EHD2 lies in their kinetic properties.

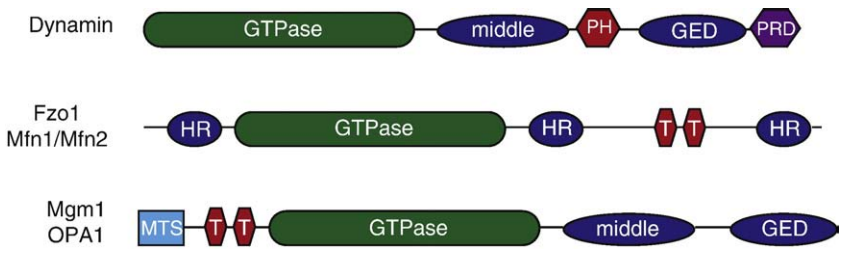

Fig. 1. Domain structure of dynamin and the mitochondrial fusion dynamin-related proteins. Domains are represented by different symbols. The GTPase domain found in all dynamin-related proteins binds and hydrolyzes GTP. The middle, GTPase effector domains (GED) and heptad repeats (HR) are involved in DRP oligomerization. The pleckstrin-homology (PH) domain and the proline rich domain (PRD) are not conserved in the fusion DRPs. Both mitochondrial fusion DRPs, Fzo1/Mfn1,2 and Mgm1/OPA1, contain transmembrane domains (T) to anchor the proteins to the outer and inner mitochondrial membrane respectively. In addition, Mgm1/OPA1 has an N-terminal mitochondrial targeting sequence (MTS) required for its targeting and import into mitochondria.

Although EHD2 possess a G-domain structurally, it hydrolyzes ATP. More importantly, the rate of ATP hydrolysis is much slower than the hydrolysis rates for dynamin and Dnm1 [16]. The slower rate of EHD2 hydrolysis is correlated with extensive tubule formation in vivo, suggesting that ATP hydrolysis promotes the disassembly of EHD2 spirals [16]. Our model for DRP function in mitochondrial fusion is that DRP driven membrane tubulation mediates lipid bilayer destabilization and mixing. Thus, it is possible that the mitochondrial fusion DRPS have been kinetically modified to be more like EHD2 with a relatively slow hydrolysis rate compared to other DRPs such as dynamin and Dnm1. The slow rate of hydrolysis would favor self-assembly over disassembly and thus promote membrane tubulation, which in turn would promote membrane fusion. This model for fusion DRP function is in contrast to high rates of assembly-stimulated GTP hydrolysis that drive the structural changes in dynamin and Dnm1 spirals that are likely required for membrane scission [23,24]. Consistent with this idea is that EHD2 tubules form an extensive connected network in vitro, suggesting that EHD2 mediates membrane fusion.

2. Measuring mitochondrial fusion

The development of both in vivo and in vitro mitochondrial fusion assays has been essential for the analysis of the requirements and mechanism of outer and inner membrane fusion. In both assays, mitochondrial fusion is measured by content mixing of mitochondrial targeted fluorophores. The in vivo mitochondrial fusion assay was developed in yeast where, during mating, mitochondria from each haploid fuse and content mixing of distinguishable fluorophores targeted to various mitochondrial compartments indicates that mitochondrial fusion is active [4]. This assay has been recapitulated in mammalian cells by making PEG-fused polykaryons and observing mitochondrial content mixing [25]. More recently, the utilization of photo-activatable GFP allows for fusion rates to be observed in a single cell [26]. These assays are essential for the characterization of the energetic requirements and the identification of components that are essential for fusion.

Recapitulating mitochondrial fusion in vitro using yeast mitochondria allowed further dissection of mitochondrial fusion. For the mitochondrial in vitro fusion assay, the establishment of proximity mediated in vivo by cytoskeletal dependent mitochondrial movement is accomplished by centrifugation followed by incubation. In agreement with the requirement of DRPs, GTP is required for both outer and inner membrane fusion in vitro [27]. Further, different non-hydrolyzable variants of GTP inhibit fusion indicating that both GTP binding and hydrolysis are required for mitochondrial fusion.

Mitochondrial inner membrane potential is also important for mitochondrial fusion in vivo and in vitro. Dissipation of membrane potential causes mitochondria tubules to fragment into smaller organelles and, despite retaining the ability to move and establish proximity, mitochondrial fusion is attenuated in cells treated with protonophores [25]. Interestingly, work in vitro has shown that outer membrane fusion requires an inner membrane proton gradient while inner membrane fusion requires the electrical component of the inner membrane potential [27]. The mechanistic role of the inner membrane potential in outer and inner membrane fusion is not currently known. One possibility is that changes in this gradient could result in remodeling of fusion competent protein complexes. Alternatively, it is also possible that membrane potential may drive membrane fusion through an electrophoretic or a pH-dependent step. Regardless of the exact mechanistic role of membrane potential, it serves to functionally link both outer and inner membrane fusion events to the energetic status of the cells and also serves as a quality control mechanism, where non-functional mitochondria that lose potential become fusion incompetent and become substrates for the autophagic pathway.

3. Outer membrane fusion

The essential mitochondrial outer membrane fusion component, Fzo1, is highly conserved with two mammalian orthologs, Mfn1 and Mfn2. Like other DRPs, GTPase activity and complex assembly are essential for Fzo1 function in mitochondrial fusion. Mutations in the GTPase domain or in the predicted coiled-coil regions of the heptad repeat (HR) domains attenuate fusion resulting in mitochondrial fragmentation, loss of mtDNA and consequent loss of the ability to respire [19,28–30]. Consistent with the importance of Fzo1 homo-oligomerization for function, intragenic complementation was observed between specific pairs of GTPase domain mutants and coiled-coil mutants suggesting that the job of Fzo1 can be performed if the function of the GTPase domain and coiled-coil regions is provided by different molecules in a complex [29]. Fzo1 function is also sustained if the protein is expressed in non-overlapping halves, which separate the GTPase domain from the C-terminal HR domains [29]. This is also consistent with a DRP-like function as these domains are known to support inter-molecular interactions and suggest that the HR domains on the membrane anchored C-terminal half of Fzo1 can recruit the GTPase domain to the membrane and build fusion competent complexes.

Several lines of evidence support the hypothesis that Fzo1–Fzo1 interactions are not only important in the same membrane, but also function to tether outer membranes through complex formation of Fzo1 complexes in *trans*. When tested in vitro, Mfn1 and to a lesser extent Mfn2, can establish interactions in *trans* effectively tethering isolated organelles [28]. In addition, mutations in the HR domains of Mfn1 that block fusion lead to the accumulation of mitochondrial intermediates in vivo that appear to be tethered. In these cells, mitochondria are aggregated with uniform gaps of 160 Å between adjacent mitochondrial outer membranes, a distance that could correspond to stalled tethered protein complexes [31]. Structural analysis of the second C-terminal HR region of Mfn1 also supports a role for *trans* interactions in mitochondrial tethering. This region is capable of forming a dimeric, anti-parallel coiled-coil structure via inter-molecular interactions [31]. Together, this suggests that interactions of the HR regions in *trans* are involved in the ability of mitofusins to tether adjacent mitochondrial outer membranes.

Also in support of this hypothesis is the requirement for functional Fzo1/Mfn1,2 on both mitochondrial membranes for efficient mitochondrial fusion in vitro and in vivo [27,28]. Fusion of wild type cells with Mfn1 or Mfn2 null cells does not support mitochondrial fusion, demonstrating that the presence of functional Mfn1/2 on one mitochondrial membrane is not sufficient to support fusion [31,32]. In yeast, the fusion mutant *fzo1-1* is a temperature sensitive allele of Fzo1 with mutations in an HR domain that attenuates fusion at elevated temperatures [19]. Characterization of the fusion defect in vitro revealed that this mutation blocks outer membrane fusion [27]. Interestingly, when testing for fusion activity in combination with wild type mitochondria in vitro, fusion activity was not greatly increased. This suggests that functional Fzo1, and in this case a functional HR domain specifically, is required on both mitochondrial partners for fusion in vitro, consistent with this domain playing a role in outer membrane tethering events.

The mechanistic significance of two Fzo-like proteins, Mfn1 and Mfn2, in mammalian cells for mitochondrial fusion is unclear. Interestingly, while both are required for mitochondrial fusion, Mfn1 and Mfn2 appear to possess functional distinctions. For instance, the formation of tethered structures in vitro occurs more readily when mitochondria are isolated from cells overexpressing Mfn1 than Mfn2 [28]. In addition, Mfn2 specifically has been shown to associate with Bax and Bak resulting in altered Mfn2 activity [33]. Further indicating that the mitofusins possess unique functional characteristics, mutations in Mfn2 rather than Mfn1 result in the neurological disorder Charcot-Marie-Tooth syndrome. Surprisingly, these mutations can be

complemented in cells by the formation of Mfn1–Mfn2^{CMT2A} hetero-oligomers but not homo-oligomers of Mfn2^{+}–Mfn2^{CMT2A} [34]. This suggests that within the Mfn1–Mfn2 hetero-oligomeric complex, each molecule is functionally distinct. This observation provides insight into the tissue specificity of Charcot-Marie-Tooth Syndrome as higher levels of Mfn1 expression in some tissues would provide complementation while low levels in other tissues culminates in altered morphology and disease. Together, this suggests that control of the expression levels of each protein likely represents the most basic form of regulation to alter mitochondrial dynamics in mammalian tissues. Indeed, the expression level of Mfn1 and Mfn2 varies according to cell or tissue type as does the mitochondrial morphology [35].

4. Inner membrane fusion

Mgm1 is also a highly conserved protein essential for mitochondrial fusion. The mammalian ortholog is OPA1 and mutations in this protein lead to the neurological disorder Dominant Optic Atrophy. Multiple isoforms of Mgm1 and OPA1 exist at steady state in yeast and mammalian cells respectively. While alternative splicing is uniquely involved in the generation of these isoforms in mammalian cells, generation of isoforms by proteolytic processing is common to both proteins.

Mgm1 is targeted to mitochondria by an N-terminal matrix targeting signal which is followed by two hydrophobic stretches. These hydrophobic regions can function as stop-transfer signals during import of the precursor protein, resulting in the movement of the peptide from the translocation pore into the mitochondrial inner membrane following removal of the targeting sequence by the matrix processing peptidase [21,36,37]. If the translocation stops at the first hydrophobic region, the resulting isoform is a transmembrane protein and is referred to as Mgm1-long. The second hydrophobic region contains a motif recognized by an inner membrane rhomboid protease PCP1 [21,38]. Therefore, if this region is inserted into the inner membrane by the stop-transfer mechanism, PCP1 cleaves in the hydrophobic region, causing the release of this isoform from the inner membrane into the intermembrane space, generating Mgm1-short. This unique process, resulting in two distinct isoforms of Mgm1, is referred to as alternative topogenesis [39]. Interestingly, both isoforms expressed at nearly equimolar amounts, are required for normal mitochondrial dynamics [21,39].

Mgm1 alternative topogenesis could represent a form of regulation of mitochondrial fusion. It was shown that low levels of ATP result in lower levels of Mgm1-short production, probably due to a lower activity of the import motor in the mitochondrial matrix [39]. This also correlates with mitochondrial fragmentation and therefore could represent a link between the quality of mitochondrial function and mitochondrial dynamics, providing a mechanism to segregate non-functional organelles in the cell [39]. Ups1 was also recently identified as a regulator of Mgm1 processing to Mgm1-short, particularly in cells grown on fermentable carbon sources [40].

The mammalian ortholog OPA1 is also subject to processing events that are similar to alternative topogenesis, but the situation is complicated by the expression of eight splice variants of OPA1 which contain one or both of two proteolytic processing sites in addition to the matrix processing peptidase site following the N-terminal targeting sequence. Therefore, each splice variant can form OPA1-long and one or two OPA1-short isoforms. There is evidence that several mitochondrial proteases contribute to OPA1 processing, including the rhomboid protease PARL and the mitochondrial matrix AAA-protease [41–44]. However, recent evidence suggests that the proteolytic processing of OPA1 is primarily dependent on the intermembrane space AAA-protease Yme1 and processing of long OPA1 isoforms to generate short isoforms is affected by membrane potential [45,46]. The large ring-forming prohibitin complexes in the mitochondrial inner membrane have also been implicated in regulating OPA1 processing, in particular in maintenance of OPA1-long isoforms [47]. Interestingly, dissipation of membrane potential is associated with increased proteolysis of OPA1 in mammalian cells, leading to conversion of OPA1-long to OPA1-short and ultimately defective mitochondrial fusion [45,48]. This mechanism also links mitochondrial function with mitochondrial fusion to facilitate the separation of dysfunctional organelles as occurs with Mgm1.

While the exact significance of each molecular OPA1 species is not clear, like Mgm1, OPA1 requires both the longer membrane associated form and shorter forms of OPA1 for full fusion function. Although both clearly function together in fusion, their roles may be subtly different. Of note, without the N-terminal transmembrane domain, Mgm1 and OPA1 do not have a region that is predicted to promote association of the protein with the inner membrane bilayer. Thus, it is conceivable that both isoforms are required for the DRP function of Mgm1/OPA1 in fusion in that the membrane tethered isoform is required to target the short isoforms to membranes via self-assembly interactions.

In support of the importance of DRP activity in Mgm1 function, both GTPase and GED mutants attenuate fusion in vivo [49–51]. Interestingly, similar to Fzo1 mutations, intragenic complementation is observed upon co-expression of Mgm1 GTPase mutants with GED mutants in vivo, indicating that Mgm1 oligomerizes [49]. While loss of either OPA1 or Mgm1 function ultimately leads to mitochondrial fragmentation [50,52], time lapse analysis of mitochondria in temperature sensitive *mgm1* cells reveals the early accumulation of multi-matrix structures following the shift to non-permissive temperature. This indicates that the primary defect associated with loss of Mgm1 function is in inner membrane fusion and that the outer membrane defect is secondary [51]. Confirming that the primary defect is inner membrane fusion in *mgm1* mutants, characterization of the in vitro fusion activity of mitochondria isolated from temperature sensitive *mgm1* mutants reveals that outer membrane fusion occurs at wild type levels, while there is a severe block for inner membrane fusion at non-permissive temperatures [51]. This is demonstrated by the accumulation of outer membrane fused intermediates under conditions that support both outer and inner membrane fusion in wild type samples. In fact, several rounds of outer membrane fusion occur successively, resulting in structures with multiple matrices surrounded by a single outer membrane. Intriguingly, some mutants exhibited a higher propensity to form these structures suggesting that Mgm1 may also play a role in negative regulation of outer membrane fusion.

Mgm1 function has been further dissected into the two distinct steps of (1) inner membrane tethering and (2) lipid mixing. The outer membrane fused intermediates formed in the in vitro fusion assay highlight the tethered nature of the mitochondrial inner membrane by the formation of tight interfaces in wild type samples. In contrast, outer membrane fused intermediates from some *mgm1* temperature sensitive mutants lack a tight interface between the adjacent matrix compartments, suggesting that Mgm1 plays a role in its formation [51]. The most logical explanation for this activity would be via the formation of Mgm1 containing complexes in *trans* to physically connect the inner membranes. Both cytological and biochemical data suggest that Mgm1 is in the right place at the right time to do this job. Localization of Mgm1 in outer membrane fused structures by immuno-EM and fluorescence indicates that it is found at the interface in large complexes [51]. Also, co-immunoprecipitation of differentially tagged versions of Mgm1 indicates that Mgm1 interacts in *trans* in outer membrane fused intermediates [51].

Complex formation in *trans* is also supported by complementation analysis of the *mgm1* alleles. Complex formation in *trans* is mimicked in the in vitro fusion assay when mitochondria isolated from different strains are combined and tested for fusion activity. The in vitro fusion activity of the Mgm1 GTPase mutant *mgm1-6* and the Mgm1 GED mutant *mgm1-7* are both increased from the levels obtained when in a homoallelic reaction (e.g. *mgm1-6*+*mgm1-6*) when they are combined

in a heteroallelic reaction (*mgm1-6+mgm1-7*) [51]. Interestingly, in homoallelic reactions these mutants lack tight inner membrane interfaces in outer membrane fused intermediates suggesting that the fusion defect is in formation of tethered complexes. Therefore, the complementation observed is due to restoration of GTPase and GED activity in these *trans* complexes.

5. Coordinating outer and inner membrane fusion

While outer and inner membrane fusion events are separable in vitro, they are temporally linked in vivo, indicating that a mechanism exists to synchronize the outer and inner membrane fusion machines. The best candidate for this role is Ugo1, an essential fusion component that is a member of the mitochondrial transport family. Sequence analysis of Ugo1 suggests that it may contain as many as six transmembrane domains [53] but experimental evidence shows that the N-terminus faces the cytosol while the C-terminus is localized to the intermembrane space, requiring an odd number of transmembrane domains [54]. A recent report indicates that that Ugo1 is a multi-spanning membrane protein with 3 or 5 transmembrane domains [55]. As a member of the mitochondrial transport family, Ugo1 has energy transfer motifs each containing one positively and one negatively charged residue. While the role of these motifs in Ugo1 function is not known, charge reversal of both residues in the second motif reduces the fusion activity in the mutant strain [55].

Ugo1 is proposed to function as an adaptor, creating a two membrane spanning complex with Fzo1 and Mgm1 [49,54,56]. This complex is likely dynamic as stoichiometric amounts of all proteins are not found in immunoprecipitates using antibodies directed against any of these components. Ugo1 is important for the interaction of Fzo1 and Mgm1 as this association is significantly reduced in the absence of Ugo1 [56]. However, abolishing the interaction of Ugo1 with Fzo1 does not diminish the interaction of Ugo1 with Mgm1 and vice versa, suggesting that Ugo1 interacts with each mitochondrial fusion DRP independently of the other [49,56]. The interaction of Ugo1 with Fzo1 is likely important for efficient mitochondrial fusion given that truncation of the C-terminus of Fzo1 by 30 amino acids diminished the interaction of Fzo1 with Ugo1 and results in mitochondrial fragmentation and a growth defect on glycerol [56].

The role of this two membrane spanning complex in mitochondrial fusion is not known. It is unlikely that the GTPase function of either Fzo1 or Mgm1 is required for the assembly of the complex because a truncated version of Fzo1 lacking the GTPase domain and an Mgm1 GTPase mutant both interact with Ugo1 [49,56]. Interestingly, Fzo1 appears to be destabilized in lysates from cells lacking Mgm1, suggesting that a signal may be transduced from the inner membrane to Fzo1 through Ugo1. There are at least two possible models for Ugo1 function in mitochondrial fusion. First, the fusion DRP complex may promote a specific conformation or oligomeric structure of Fzo1 required to initiate fusion events in the outer membrane and in this scenario Ugo1 would serve in a structural capacity to facilitate the formation of this initiation complex. Alternatively, it is possible that Ugo1 has additional post-initiation roles in both mitochondrial outer and inner membrane fusion events, beyond acting as a structural component of the Fzo1/Mgm1 complex.

A mammalian molecule responsible for coordination of outer and inner membrane fusion has not been identified. Despite this, it is likely that a molecular adaptor exists. Mitochondrial outer and inner membrane fusion are coordinated in mammalian cells as they are in yeast cells and, as is the case for Fzo1, the intermembrane space region linking the transmembrane domains of Mfn1 and Mfn2 is less than ten residues, likely too short to facilitate an interaction efficiently with a OPA1 which is associated with the inner membrane.

6. The role of ubiquitination and protein turnover in fusion

Protein turnover by the 26S proteasome is involved in regulation of many cellular pathways, including mitochondrial morphology. A significant fraction of ubiquitin or proteasome mutants have aberrant mitochondrial morphology, typically causing fragmentation or aggregation [57–59]. A more direct link to mitochondrial dynamics was made when it was found that multiple systems exists to regulate the levels of Fzo1 in yeast in order to maintain a reticular morphology [60,61]. Arresting the yeast cell cycle by treatment with the alpha-factor pheromone results in Fzo1 degradation via the proteasome and corresponding mitochondrial fragmentation due to the lack of fusion and ongoing division [61]. The ubiquitin conjugating enzyme responsible for this cell cycle specific degradation of Fzo1 remains unidentified.

Fzo1 is also turned over in vegetatively growing cells in a pathway involving the F-box protein Mdm30, although the mechanism is controversial. Several pieces of evidence suggest that the role of Mdm30 is in quality control based turnover of Fzo1. First, F-box proteins are typically known as components of SCF E3 ubiquitin ligase complexes (*S*kp *C*dc53/Cullin *F*-box) which facilitate interactions between substrates and ubiquitin conjugating enzymes. Mdm30 has been shown to interact with Skp1 and Cdc53 by 2-hybrid and in reconstituted SCF complexes using purified protein components suggesting that it is a genuine component of an SCF complex [62,63]. As is true for other SCF complexes, Mdm30 is thought to have multiple mitochondrial outer membrane targets as it remains associated with mitochondria in the absence of Fzo1 [60]. Also in support of an SCF-dependent role for Mdm30 in regulating mitochondrial dynamics, mitochondria fragment in a *cdc53* temperature sensitive mutant at non-permissive temperature [59,64] and Fzo1 is ubiquitinated via lysine 48 ubiquitin chains, which are typically associated with proteasome dependent turnover, in an Mdm30 dependent manner [65,66]. However, there is conflicting evidence regarding the direct involvement of the 26S proteasome in Mdm30-dependent Fzo1 degradation and in the essentiality of the F-box in Mdm30 for Fzo1 turnover [64,66,67]. Therefore, it remains unclear whether Mdm30 functions as a general quality control mechanism in turnover of mitochondrial outer membrane proteins or if it directly regulates Fzo1 fusion activity. It is conceivable that Mdm30 plays two roles in mitochondrial morphology, one that is F-box and SCF complex dependent and a second that is SCF complex independent. Although there is no mammalian ortholog of Mdm30, it seems likely that mitofusins will also be subjected to ubiquitination or proteasome dependent degradation at least as a part of an outer membrane quality control mechanism.

7. Fusion and its connection to cristae morphology

Cristae are distinct structures of the mitochondrial inner membrane and represent a fourth and functionally distinct compartment in the mitochondria [68]. There is great diversity in cristae morphology that varies not only in length but also in general shape exhibiting a range from tubular to lamellar. The intermembrane space of the cristae compartment is boundaried through small, tubular segments called cristae junctions. In addition to its role in mitochondrial inner membrane fusion, Mgm1 and OPA1 have also been implicated in maintenance of cristae morphology.

Both Mgm1 and OPA1 have been localized to cristae by immuno-EM [52,68–70]. Downregulation of OPA1 causes cristae to become disorganized and cristae junctions to widen while overexpression leads to narrowing of cristae junctions and cristae themselves [69,71,72]. Changing the steady state levels of OPA1-long and short variants also leads to abnormal cristae morphology [47]. Interestingly, shifting mitochondria isolated from *mgm1* temperature sensitive alleles with mutations in the GED domain to non-permissive

temperatures resulted in loss of cristae compared to wild type controls, suggesting that Mgm1 also plays a role in maintenance of cristae and that the GED domain is particularly important for this role [51]. This would support a model in which Mgm1 participates in cristae maintenance by the formation of complexes in *trans* mediated by the GED domain.

In this context, it is interesting to raise the possibility that cristae may also be a direct product of inner membrane fusion. Following outer membrane fusion in vitro, inner membranes extensively align in a manner dependent on *trans* Mgm1 interactions and likely other as yet to be determined interactions across the membranes. The structure and maintenance of these inner membrane interfaces coupled with fusion pore placement and incomplete lateral propagation could generate structures with topologies similar to cristae. A similar phenomenon is found in vacuolar fusion where propagation of a fusion pore instead gives rise to internalized membranes [73]. The extent to which the role of Mgm1/OPA1 in fusion overlaps with its role in cristae morphology is not known. It has been proposed that the role of OPA1 in mitochondrial fusion is separable from its role in cristae morphology [72]. It is known that Mgm1 interacts in *trans* at inner membrane interfaces of the matrices connected following outer membrane fusion. It is likely that Mgm1 can also interact in *trans* on apposing mitochondrial inner membranes in cristae. Switching from its role in cristae morphology maintenance to a role in mitochondrial fusion could be triggered by interaction with outer membrane fusion components such as Ugo1 or Fzo1 and result in dynamic changes in the Mgm1 complex, such as stimulating formation of Mgm1 complexes in *cis* which are required for its role in fusion.

8. Concluding remarks

Over the past decade, our understanding of mitochondrial fusion has transitioned from the descriptive to the mechanistic. However, there is still much to be learned. Several fundamental questions remain, such as the exact role of the fusion DRPs and how mitochondrial outer and inner membrane fusion is coordinated. Fundamental questions regarding the cellular roles of mitochondrial fusion are also unanswered and include the role of fusion in the maintenance of mtDNA and how fusion is integrated into other functions such as mitochondrial motility and cellular signaling pathways.

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