ELUCIDATING MECHANISMS OF HOMOTYPIC MITOCHONDRIAL FUSION

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Abstract

Mitochondrial fusion occurs in many eukaryotes including animals, plants, and fungi. It is essential for cellular homeostasis, and yet, the underlying mechanisms remain elusive. Fusion in all systems requires core fusion GTPases that reside in the outer mitochondrial membrane, called Fzo1 in yeast or Mfn1 and Mfn2 in mammalian systems. While post-translational ubiquitination of yeast Fzo1 was shown to drive fusion in yeast, a cell-free mitochondrial fusion assay using mitochondria from HeLa cells revealed that this modification is not required for fusion. Comparative analyses and phylogenetic reconstructions revealed further critical distinctions between the fungal Fzo1 and mammalian Mfns. These GTPases are highly diverged from one another, and lack strong sequence similarity. Bioinformatics analysis showed that fungal Fzo1 proteins exhibit two predicted transmembrane domains, whereas metazoan Mitofusins contain only a single transmembrane domain. This prediction contradicts the current models suggesting both animal and fungal proteins share one topology. This newly predicted topology of MFN1 and MFN2 was demonstrated biochemically, confirming that the C-terminal, redox-sensitive cysteine residues reside within the intermembrane space (IMS). Serial truncation mutants revealed that the ~15kDa C-terminus was required for targeting of MFN2. However the heptad repeat 2 (HR2) domains that reside within the intermembrane space of MFN1 and MFN2 were shown to be regulatory, but non-essential for mitochondrial fusion, since MFNs lacking HR2 partially rescued mitochondrial fragmentation morphology in cells lacking these GTPases. Functional experiments further established that redox-mediated disulfide modifications within the IMS domain are key modulators of reversible MFN oligomerization that drives fusion. Together, these results lead to a revised understanding of MFNs as single-spanning outer membrane proteins with an N_{out}-C_{in} orientation, providing functional insight into the IMS contribution to redox-regulated fusion events.

Résumé

La fusion mitochondriale se produit chez de nombreux eucaryotes, notamment les animaux, les plantes et les champignons. C'est essentiel pour l'homéostasie cellulaire, et pourtant, les mécanismes sous-jacents restent insaisissables. La fusion dans tous les systèmes nécessite des GTPases de fusion de base qui résident dans la membrane mitochondriale externe, appelée Fzo1 dans la levure ou Mfn1 et Mfn2 chez les mammifères. Alors qu'il a été démontré que l'ubiquitination post-traductionnelle de la Fzo1 chez les levures entraînait la fusion dans la levure, un test de fusion mitochondriale sans cellules utilisant des mitochondries de cellules HeLa a révélé que cette modification n'était pas nécessaire pour la fusion. Les analyses comparatives et les reconstitutions phylogénétiques ont révélé d'autres distinctions critiques entre le Fzo1 des levures et les Mfns des mammifères. Ces GTPases sont très différentes les unes des autres et n'ont pas de similarité de séquence. Une analyse bioinformatique a montré que les protéines fongiques Fzo1 présentaient deux domaines transmembranaires prédits, alors que les Mitofusins metazoans ne contiennent qu'un seul domaine transmembranaire. Cette prédiction contredit les modèles actuels suggérant que les protéines animales et fongiques partagent la même topologie. La topologie nouvellement prédite de MFN1 et MFN2 a été démontrée de manière biochimique, confirmant que les résidus de cystéine C-terminal sensibles au redox se trouvent dans l'espace intermembranaire (IMS). Des mutants de troncature en série ont révélé que l'extrémité C-terminale ~15 kDa était nécessaire pour cibler MFN2. Cependant, les domaines heptad repeat 2 (HR2) situés dans l'espace intermembranaire de MFN1 et MFN2 se sont avérés régulateurs, mais non essentiels pour la fusion mitochondriale, car les MFN dépourvues de HR2 ont partiellement sauvé la morphologie de la fragmentation mitochondriale dans les cellules dépourvues de ces GTPases. Des expériences fonctionnelles ont également démontré que les modifications au disulfure induites par l'oxydo-réduction dans le domaine IMS sont des modulateurs essentiels de l'oligomérisation réversible de MFN qui conduit à la fusion. Ensemble, ces résultats ont permis de mieux comprendre les MFNs en

tant que protéines membranaires externes à orientation unique orientées vers Nout-Cin, offrant ainsi un aperçu fonctionnel de la contribution du système IMS aux événements de fusion régulés par l'oxydo-réduction.

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To my family, you know how much I appreciate your unconditional love and support even though I am truly unhinged, unstuck.

To Fr Michael Todary, thank you. Thank you. Thank you.

To GOD,

I will praise You, for I am fearfully and wonderfully made;

Marvelous are Your works,

And *that* my soul knows very well.

Contribution of Authors

In accordance with the guidelines for thesis preparation and under advisement of the thesis supervisor, the candidate has opted to present this thesis in standard format. A general introduction is presented in Chapter 1 followed by a hypothesis and rationale for the thesis project. A detailed description of the experimental procedures used to address the specific aims of the thesis is presented in Chapter 2. Results are described in Chapter 3, some of it published in the following article:

Mattie S., Riemer J., Wideman, J.G., McBride, H.M., A new mitofusin topology places the redox-regulated C terminus in the mitochondrial intermembrane space. Journal of Cell Biology. 217(2): 507-515, 2018.

Following the Results chapter, Discussion and Concluding remarks follow in Chapter 4. The latter is followed by bibliography.

I planned and performed experiments presented under the supervision of Dr McBride and under advisement of my advisory committee members, Dr Hekimi and Dr McPherson.

All bioinformatics analyses presented in Figure 7 was performed by Dr Wideman (Wissenschaftskolleg zu Berlin, Berlin, Germany). I acknowledge the contribution of Dr Riemer (Institut für Biochemie, University of Cologne, Köln, Germany) for his advice on the design of redox experiments and calculation of glutathione redox potential (Figure 20-22).

Contribution to Original Knowledge

Mitochondrial fusion is considered of great importance owing primarily to the reports that mutations in core fusion genes lead to severe neurodegenerative disorders. The mechanism and regulation of mitochondrial fusion remain weakly understood, however. In this thesis, we are pursuing an answer to the following questions:

1. Does ubiquitination/de-ubiquitylation play a role in mammalian mitochondrial fusion?
    - ATP hydrolysis is required for fusion, using a cell free fusion assay. The role of ATP in fusion has been controversial, with opposite observations having been published.
    - Using a cell free mitochondrial fusion assay, ubiquitination and de-ubiquitylation do not significantly alter mitochondrial fusion.
    - p97, an ATPase which has a role in the extraction of ubiquitinated MFNs, has no role in the mechanism of fusion, determined by drug inhibition of p97 in cell free fusion assay.
    - UBR4, an E3 ubiquitin ligase, which was detected as a hit in MFN2 BioID, is not involved in the turnover of MFN2 in cells.
2. What is the evolutionary history of mitochondrial outer membrane fusogenic GTPases?
    - Bioinformatics analyses, performed in collaboration with Dr Wideman, revealed that metazoan Mfns and fungal Fzos are distantly related, and surprisingly are as close to each other as each is close to bacterial GTPases.
    - Bioinformatics analysis predicted a difference in topology between metazoan clade and the fungal clade.
3. What is the topology of MFN1 and MFN2?
    - I experimentally determined the membrane topology of MFN1 and MFN2. The new topology consisting in a single transmembrane domain, N_{out}-C_{in}, with the C-terminus

residing in the intermembrane space, which prompted a re-evaluation of the role of the C-terminus, including HR2, in fusion.

4. The role of the C-terminus in targeting of MFN2 to mitochondria?
    - We found that the C-terminus is required for targeting MFN2 to mitochondria, as was previously reported by Rojo et. Al. (2002).
    - We narrowed down the region in the C-terminus required for targeting MFN2 to mitochondria to be the region between the TMD and HR2.
    - While the C-terminus was required for targeting MFN2 to mitochondria, the C-terminus alone was not sufficient for targeting to mitochondria, implying a contribution of the transmembrane domain as a signal anchor sequence, and/or roles for additional domains within the cytosolic-exposed portion of MFNs.
5. Is HR2 domain in the C-terminus required for fusion?
    - To this end we used MFN1 and MFN2 mutants lacking HR2. ΔHR2 MFNs partially rescued the mitochondrial fragmentation morphology in Mfn1-null or Mfn2-null MEFs, suggesting that HR2 is not required for fusion but might play a regulatory role.
6. How does oxidative stress activate fusion?
    - Oxidized glutathione was previously shown to activate mitochondrial fusion (T. Shutt et al. 2012). We determined the values of the activating glutathione redox potential, which fall within physiological ranges.
    - Reactive oxygen species, both mitochondrial and extra-mitochondrial, activated fusion in a GSH-dependent manner.
    - GSSG promotes the formation of disulfide-linked MFN2 oligomers. We show that these oligomers were dynamic and were recycled by GSH.

List of Abbreviations

ActA: Actin assembly-inducing protein

ALR: FAD-linked sulfhydryl oxidase ALR or Augmenter of liver regeneration

AntA: antimycin A

ATP: Adenosine triphosphate

ATPγS: ATP-γ-S, Adenosine 5'-(3-thiotriphosphate)

Bax: BCL2 Associated X Protein

BDLP: Bacterial dynamin-like proteins

BioID: Proximity-dependent biotin identification

BirA: Bifunctional ligase/repressor

cAMP: Cyclic adenosine monophosphate

CCCP: Carbonyl cyanide m-chlorophenyl hydrazone

CDK1: Cyclin-dependent kinase 1

CHX: Cycloheximide

C-terminus: Carboxy terminus

DAPI: 4',6-diamidino-2-phenylindole

DAG: Diacylglycerol

Drp1: Dynamin-related protein 1

DUBs: deubiquitinating enzymes

ER: Endoplasmic reticulum

ETC: Electron transport chain

Fis1: Mitochondrial fission 1 protein

Fzo1: Fuzzy onion 1

GDP-AlF_4^-: Guanosine 5′-diphosphate tetrafluoroalumanuide

GDP-BeF_3^-: Guanosine 5′-diphosphate beryllium fluoride

GMP-PNP: Guanosine 5′-[β,γ-imido]triphosphate

GSH: Reduced glutathione

GSSG: Glutathione disulfide

GST: Glutathione S-transferase

GTP: Guanosine-5'-triphosphate

GTPγS: Guanosine 5′-[γ-thio]triphosphate

HR: heptad repeat

IMS: intermembrane space

March5: Membrane-associated RING finger protein 5

Mdm30: Mitochondrial distribution and morphology

MEFs: Mouse Embryonic Fibroblasts

MFF: Mitochondrial fission factor

MFN1: Mitofusin 1

MFN2: Mitofusin 2

Mia40: Mitochondrial intermembrane space import and assembly protein 40

MiD49/**MiD51**: Mitochondrial dynamics proteins 49/51

MIGA1: Mitoguardin 1

MIGA2: Mitoguardin 2

MitoPLD: Mitochondrial phospholipase D

MTFP1: Mitochondrial fission process protein 1

mTORC1: Mechanistic target of rapamycin complex 1

Myx: Myxothiazol

NEM: N-ethylmaleimide

N-terminus: amino terminus

OPA1: Optic atrophy protein 1

P97 (VCP): Valosin-containing protein

PA: Phosphatidic acids

PA-PLA1: Phosphatidic Acid-preferring Phospholipase A1

PEG: Polyethylene glycol

mPEG-mal: polyethyleneglycol-maleimide

PINK1: PTEN-induced kinase 1

PK: Proteinase K

PKA: Protein kinase A

PMSF: Phenylmethanesulfonyl fluoride

PRDX3: Thioredoxin-dependent peroxide reductase, mitochondrial

PYR-41: 4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester

RNAi: RNA interference

ROS: Reactive oxygen species

SDHA: Succinate dehydrogenase complex, subunit A

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SLC25A46: Solute carrier family 25 member 46

SNARE: SNAP receptor

TCA cycle: Tricarboxcylic acid cycle

TMD: Transmembrane domain

TOM20: Mitochondrial import receptor subunit TOM20 homolog

Tx100: Triton X-100

Ub: Ubiquitin

Ubal: Ubiquitin aldehyde

Ubp2: Ubiquitin carboxyl-terminal hydrolase 2

Ubp12: Ubiquitin carboxyl-terminal hydrolase 12

UBR4: N-recognin-4

Ub-VS: Ubiquitin vinyl sulfone

VAMP1B: Vesicle-associated membrane protein 1 B

VDAC: Voltage-dependent anion-selective channel

Chapter 1: Introduction

Mitochondria are dynamic organelles, consisting of two membranes, an outer membrane encasing a larger inner membrane that inwardly invaginates into structures called cristae. Historically, mitochondria were considered as mere energy production compartments in the cell. This simplistic understanding of mitochondria cannot be more remote from actual mitochondrial functions in the cell. Mitochondria play a central role in the generation of essential metabolites, amino acids, purines and pyrimidines, in phospholipid and bile acid synthesis, in apoptosis, in innate and adaptive immunity (Russell 2003; Vakifahmetoglu-Norberg, Ouchida, and Norberg 2017; Weinberg, Sena, and Chandel 2015; West, Shadel, and Ghosh 2011). Therefore, understandably, defects in mitochondrial functions caused by mutations in mitochondrial genes have been implicated in the pathogenesis of diverse diseases (Liesa, Palacín, and Zorzano 2009; Koopman et al. 2012; Vyas, Zaganjor, and Haigis 2016).

Not only do mitochondria adapt to different cellular needs and stresses by altering their proteomic and enzymatic landscape, mitochondria change shape as well. Individual mitochondria fuse together into an extensive tubular network, or divide into small rod-shaped mitochondria again. The goal of fusion, the "why," has been difficult to answer definitively, especially because a great deal remains unknown about the mechanism of homotypic mitochondrial fusion, the "how." In broad terms, mitochondrial fusion is primarily thought to be cytoprotective , however chronic hyperfusion, in diseases where mitochondrial fission is lost, is also lethal (T. E. Shutt and McBride 2013). Therefore it is a complex process, where mitochondrial fusion is regulated by multiple physiological triggers for which little is known.

Mitochondrial fission

Mitochondrial fission is the opposing process to mitochondria fusion (**Figure 1 A, B**). The cytosolic large GTPase Drp1 is required for mitochondrial fission, and loss of Drp1 is embryonic lethal in

mice (Smirnova et al. 1998, 2001; Frank et al. 2001; Ishihara et al. 2009). Drp1 is recruited to the mitochondrial outer membrane by mitochondria-anchored receptors, MFF, Mid49, Mid51, and Fis1 (Gandre-Babbe and van der Bliek 2008; Otera et al. 2010; Palmer et al. 2011, 2013; James et al. 2003; Yoon et al. 2003; Losón et al. 2013). To pinch mitochondria, Drp1 oligomerizes into a ring that is proposed to provide the mechanical force (Ingerman et al. 2005; Kalia et al. 2018). The recruitment of Drp1 to mitochondria is regulated by the phosphorylation status of multiple sites. Drp1 phosphorylation at Ser616 by the kinase cyclin B/Cdk1 promotes mitochondrial fragmentation during mitosis (Taguchi et al. 2007; Kashatus et al. 2011). In contrast, Drp1 phosphorylation at Ser637 by the cAMP-dependent kinase PKA, which is proposed to be induced during starvation, prevents Drp1 translocation to mitochondria, thus inhibiting mitochondrial fission (Chang and Blackstone 2007; Cribbs and Strack 2007; Rambold et al. 2011). The S637 phosphorylation is removed by the phosphatase Calcineurin, thereby opposing the PKA-dependent inhibition of fission (Cribbs and Strack 2007; Cereghetti et al. 2008). In addition to phosphorylation, Drp1 was shown to be SUMOylated, a modification that stabilizes the membrane associated oligomer at sites of ER contact (Wasiak, Zunino, and McBride 2007; Prudent et al. 2015). Apart from Drp1, the GTPases dynamins are proposed to play a role in mitochondrial fission, but this role of dynamins has been recently challenged as being nonessential for fission (J. E. Lee et al. 2016; Kamerkar et al. 2018).

Lipid involvement in mitochondrial dynamics

The essential role for the enzymatic modification of lipids that regulate and facilitate membrane fusion and fission has been demonstrated in numerous pathways such as endocytosis, vesicle fusion, and homotypic vacuole fusion (Puchkov and Haucke 2013; Wickner 2010). While the contribution of lipids to mitochondrial dynamics is significantly less understood, a requirement for phosphatidic acid (PA) in mitochondrial fusion has been demonstrated. PA is generated in the mitochondrial outer membrane by the outer membrane anchored protein mitoPLD (also called PLD6), an enzyme that

hydrolyzes cardiolipin to PA (Choi et al. 2006). Loss of mitoPLD led to mitochondrial fragmentation and inhibition of mitochondrial fusion as quantified in a whole-cell fusion assay monitoring the matrix content mixing of labelled mitochondria from each cell (Choi et al. 2006; H. Huang et al. 2011). The pro-fusogenic role of mitoPLD is proposed to be regulated by the outer membrane proteins Mitoguardin 1 and 2 (MIGA1 and MIGA2), which regulate dimerization of mitoPLD (Zhang et al. 2016). Interestingly, mitoPLD was also reported to inhibit fission through the binding of Drp1 to PA (Adachi et al. 2016). This demonstrated dual function in fusion and fission suggests that PA and mitoPLD may act as a link between the seemingly independent processes, thereby providing a common mode of regulation to block fission and activate fusion through the use of the same machinery. MitoPLD may have a more complex role in fission than inhibition of Drp1 by PA. The catalytically-active MitoPLD was shown to recruit the lipid phosphatase Lipin 1b to mitochondria (H. Huang et al. 2011). This Lipin 1b was reported to convert PA to diacylglycerol (DAG) on mitochondria, to fragment mitochondria when overexpressed, and to reverse the mitoPLD-induced hyperfusion morphology in co-overexpression (H. Huang et al. 2011; Adachi et al. 2016). Another phospholipase that cleaves PA (PA-PLA1) has also been implicated in mitochondrial dynamics (Baba et al. 2014). This suggests that lipids play an important role in mitochondrial dynamics. Nevertheless, future work is required to dissect the precise mechanism of PA/DAG and mitoPLD and Lipin 1b in fusion and fission.

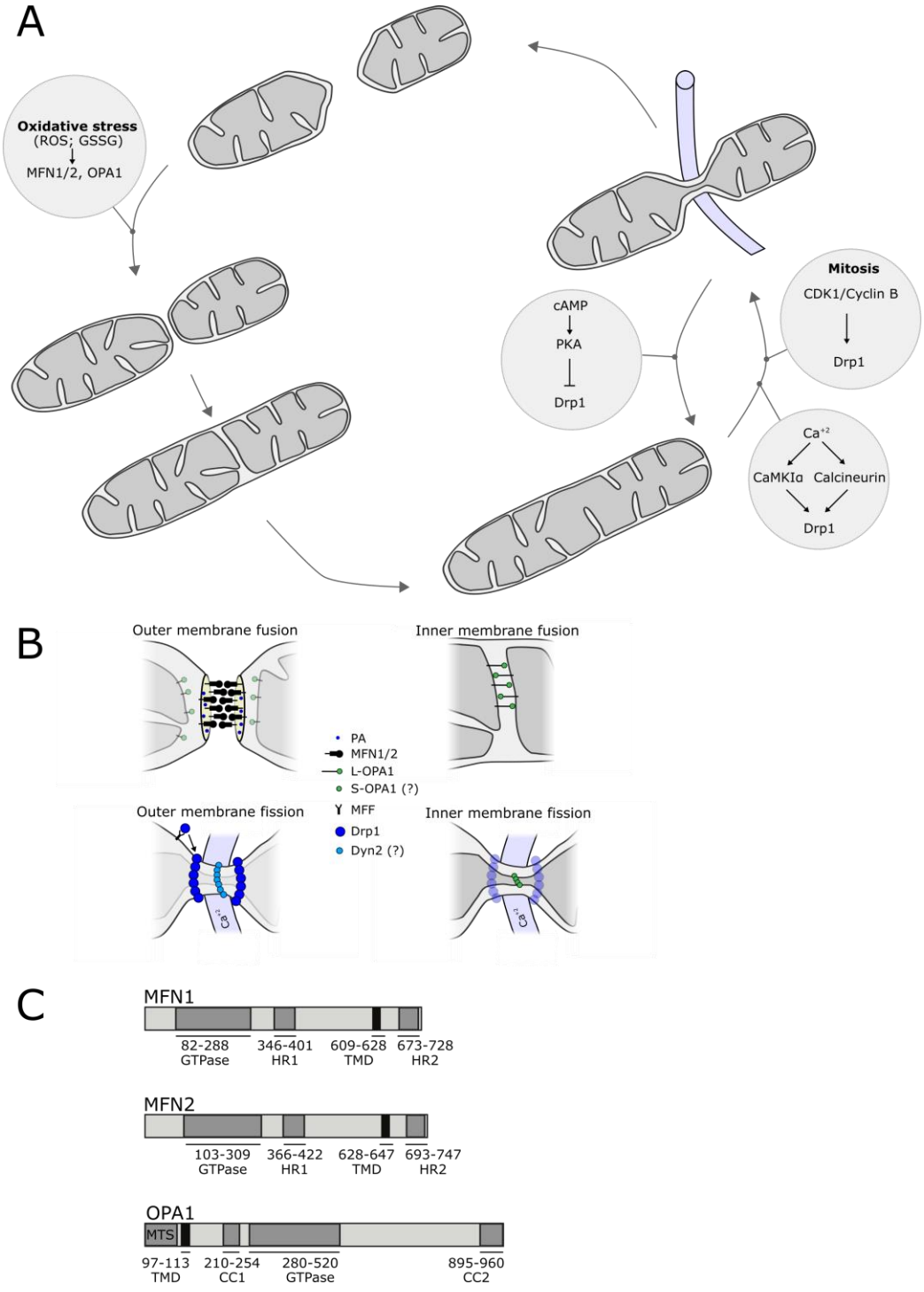

Figure 1. Illustration of mitochondrial dynamics.

A, an overview cartoon depicting mitochondrial fusion and fission processes. Highlighted are the activators and inhibitors of these processes. B, factors involved in mitochondrial fusion and fission. C, domains of human mitochondrial fusion proteins (Liesa, Palacín, and Zorzano 2009).

Mitochondrial fusion

In general, the mechanism of membrane fusion in cells involves tethering factors, small GTPases, and helical proteins called SNAREs, the latter to provide the mechanical force needed to fuse the apposing membranes together (Martens and McMahon 2008). SNARE-mediated fusion has been shown to be required for both vesicular fusion and organellar fusion (Mattie et al. 2017; Gao, Reggiori, and Ungermann 2018; Wickner 2010), and recently has been implicated in endoplasmic reticulum (ER) fusion (M. Lee et al. 2015). However, homotypic mitochondrial fusion has been proposed to be accomplished through a SNARE-independent mechanism which requires large GTPases of the Dynamin family to provide the mechanical force.

Having two membranes that separate three distinct compartments, mitochondria are placed in a unique position where two distinct membrane fusion events must occur for the matrix content to mix (**Figure 1 A, B**). This raises many questions, mechanistic and functional. Are outer and inner membrane fusion events synchronized, inter-linked? Are similar basic mechanisms employed for outer and inner membrane fusion? Are there communal activators of outer and inner fusion events? Do intra-mitochondrial triggers activate outer membrane fusion, and do cytosolic factors influence inner membrane fusion? In answering these questions we may arrive at a functional meaning for mitochondrial fusion.

Mammalian mitochondrial outer membrane fusion is mediated by Mitofusin 1 (MFN1) and Mitofusin 2 (MFN2), two large GTPases. Human MFN1 and MFN2, 63% identical in sequence, are 741 and 757 amino acid long, respectively, and consist of an N-terminal GTPase domain and two coiled-coil domains (heptad repeat 1 (HR1) and heptad repeat 2 (HR2)) flanking a transmembrane domain (TMD) (**Figure 1 C**) (Liesa, Palacín, and Zorzano 2009). It has been broadly assumed that MFNs contain two very short TMDs, thought to cross the bilayer twice in a “hairpin”, reminiscent of caveolin or atlastins. This is

highly distinct from the fungal orthologue, Fzo1, whose TMDs are separated by a functionally important loop of about 10 amino acids that extend within the intermembrane space (Rojo et al. 2002; Fritz et al. 2001). In all clades, the bulk of the Mitofusin protein is cytosolic, including the GTPase domain, HR1 and, presumably, the very C-terminal HR2 domain. The existence of two proteins, MFN1/2, in mammals is not conserved in other distant organisms. All fungi, including *S. cerevisiae*, contain a single Mfn-orthologous protein in the outer membrane, Fzo1. The presence of two fusion GTPases inserted in the outer membrane raises the question of why mammalian cells require two similar outer membrane GTPases. Both are ubiquitously expressed in all cell types, although the levels of each can vary, perhaps hinting at differential regulation in distinct cellular contexts (Santel et al. 2003).

Indeed, absence of Mfn1 or Mfn2 is embryonic lethal in mice, with embryos not surviving past midgestation (Chen et al. 2003; Chen, McCaffery, and Chan 2007). The Chan group reported that lethality was due to placental defects (Chen, McCaffery, and Chan 2007). Consistent with this, the generation of specific knock-out lines where loss of the Mitofusins occurs post-implantation resulted in live pups born with Mendelian ratios (Chen, McCaffery, and Chan 2007). Surprisingly, Mfn1-null mice were healthy and fertile, however Mfn2-null mice died between day 1-17 after birth, partially due to an underlying neurodegeneration in the cerebellum (Chen, McCaffery, and Chan 2007). The lack of a strong phenotype in Mfn1-null mice raises major questions as to the role of Mfn1, especially because Mfn1 is widely considered to be of more importance for fusion than Mfn2 (Cipolat et al. 2004). Chen et al (2007) argued that Mfn2 perhaps sufficiently compensates for the loss of Mfn1 (but not, obviously, the other way around), thereby Mfn1-null mice are healthy and fertile. This argument of full complementation of Mfn1 loss by Mfn2 is not consistent with cultured mouse embryonic fibroblast cells (MEF) lacking either of the two Mfn proteins, since Mfn1 or Mfn2 null MEFs contain highly fragmented mitochondrial morphology (Chen et al. 2003). Interestingly, the fragmentated mitochondria present in Mfn1- or Mfn2-null MEFs was rescued with exogenous expression of either Mfn1 or Mfn2, demonstrating some

functional redundancy in fusion (Chen et al. 2003). These results clearly illustrate the need for a better understanding of the function, and regulation of the mitochondrial fusion machinery.

Do MFN1 and MFN2 form complexes together? A definitive conclusion cannot be reached from current knowledge. Knowledge about a possible collaboration between MFN1 and MFN2 comes from two types of experiments, biochemical and functional. Using co-immunoprecipitation experiments, Chan group reported that tagged Mfn1 and Mfn2 formed homo- and hetero-complexes (Mfn1:Mfn1, Mfn1:Mfn2 and Mfn2:Mfn2) (Chen et al. 2003). In contrast, the Mihara group reported that Mfn1:Mfn1 complexes were the primary complexes formed under fusion conditions, also using co-IP of tagged Mfn1 and Mfn2 (Ishihara, Eura, and Mihara 2004). Furthermore, The Mfn1 complexes migrated differently through sucrose density gradients than Mfn2 in the presence of GTP, suggesting that the Mfn1 and Mfn2 complexes are distinct from each other (Ishihara, Eura, and Mihara 2004). Therefore, biochemical analysis of Mfn1 and Mfn2 complexes is inconclusive. Functional analysis of the roles of Mfn1 and Mfn2 has been confusing as well. MEFs lacking either Mfn1 or Mfn2 show fragmented morphology, suggesting that both Mfn1 and Mfn2 are required (Chen et al. 2003). Nevertheless, overexpression of Mfn1 or Mfn2 in Mfn2-null or Mfn1-null MEFs, respectively, rescues mitochondrial fusion defects, suggesting that Mfn1 or Mfn2 are individually sufficient to fuse mitochondria, without the need of hetero-complexes (Chen et al. 2003). Nunnari group used a two-color cell-free fusion assay to answer the question of role of homo- and hetero-complexes. Mitochondria, which were labeled with dsRED or EGFP, were isolated from Mfn1-null or Mfn2-null cells, followed by mixing the two populations of mitochondria under fusion conditions (Hoppins, Edlich, Cleland, Banerjee, Mccaffery, et al. 2011). Mitochondria isolated from Mfn1-null fused significantly more efficiently with mitochondria isolated from Mfn2-null than with Mfn1-null mitochondria, suggesting that hetero-complexes form the main fusion machinery (Hoppins, Edlich, Cleland, Banerjee, Mccaffery, et al. 2011). In direct opposition to the cell-free fusion assay, Chan group did not find any distinguishable differences between the fusogenic

function of homo- and hetero-complexes using whole-cell PEG assay of Mfn1-null and Mfn2-null MEFs (Chen, Chomyn, and Chan 2005). Therefore, it is safe to conclude that Mfn1:Mfn2 hetero-complexes are not necessary for mitochondrial fusion, and that Mfn1 or Mfn2 homo-complexes are sufficient in fusing mitochondria.

Mitochondrial inner membrane fusion requires another GTPase, OPA1 (**Figure 1**) (Olichon et al. 2003; Cipolat et al. 2004). OPA1 is anchored to the inner membrane in its long form, but is proteolytically cleaved into a soluble form in the IMS (Satoh et al. 2003; Olichon et al. 2002). OPA1 consists of eight splice variants and two post-translational proteolytically-processed forms (Song et al. 2007). Functionally, OPA1 has been implicated not only in fusion, but fission and cristae remodelling (Frezza et al. 2006; Cipolat et al. 2006; Tadato et al. 2010; Anand et al. 2014). As to its fusion role however, a recent study using liposomes reconstituted with recombinant full-length OPA1 revealed a surprising mechanism of action. OPA1 reconstituted on one set of liposomes was sufficient in fusing the membranes with "naked" liposomes (without OPA1), only when the second set of liposomes contained cardiolipin, an important lipid of the inner membrane (Ban et al. 2017). This suggested that OPA1 does not interact in *trans* to tether the apposing membranes as was previously thought, but that OPA1 on one membrane interacts directly with cardiolipin-containing second membrane, leading to full fusion. Surprisingly, soluble short OPA1 (S-OPA1) was also shown to fuse cardiolipin-containing liposomes in the absence of L-OPA1 (Ban et al. 2018). Future work is needed to dissect the mechanism in further detail and elucidate the physiological implications of the fusogenic ability of S-OPA1.

Does outer membrane fusion follow a similar mechanism as that of the inner membrane? Early studies using cell-free mitochondrial fusion assays concluded that either Fzo1 (in yeast) or Mfns were required on both mitochondria for fusion to occur (Meeusen, McCaffery, and Nunnari 2004; Hoppins, Edlich, Cleland, Banerjee, Mccaffery, et al. 2011). However, full reductionistic systems of Mfn-

reconstituted liposomes have not been reported for outer membrane fusion which would help dissect such details as was published about OPA1-mediated membrane fusion.

Mitochondrial fusion in disease

Mutations in the fusion GTPases OPA1 and MFN2 cause human diseases, dominant optic atrophy and Charcot-Marie Tooth type 2 (CMT2), respectively (Liesa, Palacín, and Zorzano 2009). Mutations in MFN2 are the most frequent cause of axonal CMT2 neurodegenerative disorder. Most mutations, which have been identified in many populations, are located in the GTPase, HR1 and HR2 domains (Calvo et al. 2009; Feely et al. 2011; Brožková et al. 2013; Stuppia et al. 2015; Di Meglio et al. 2016; Ando et al. 2017). Patients manifest broad clinical features even patients carrying the same mutation. Phenotypes range from severe early onset to mild late onset. Some MFN2 mutations cause CMT2 associated with optic atrophy, suggesting the presence of a functional overlap with OPA1 mutations (Züchner et al. 2006; Di Meglio et al. 2016; Ando et al. 2017). Mechanistically, disease-causing MFN2 mutations have variable phenotypes on mitochondrial morphology and mitochondrial function. The MFN2 Arg94Gln (R94Q) missense mutation, which falls in the GTPase domain, causes mitochondrial clumping in MEFs, and rat and fly neurons (Baloh et al. 2007; El Fissi et al. 2018). MFN2 R94Q was non-functional, failing to rescue the fragmented mitochondrial morphology in Mfn1/Mfn2 double KO MEFs (dKO MEFs) and failing to fuse mitochondria by whole cell PEG fusion assay (Detmer and Chan 2007; El Fissi et al. 2018). However, GTP binding of R94Q was unaltered and MEFs cultured from knock-in mice showed tubular mitochondria, suggesting a more complex phenotype of this mutation that requires future work to elucidate (Baloh et al. 2007; Detmer and Chan 2007). Another mutation Trp740Ser, which is located in HR2, showed no defects in GTP binding (Baloh et al. 2007). Mfn2 W740S rescued mitochondrial fusion in primary neurons and dKO MEFs, suggesting that the pathogenesis might be at least partially due to non-fusion MFN2 function (Baloh et al. 2007; Detmer and Chan 2007). Future work

will have to take into account the expression levels of these mutants to eliminate overexpression phenotype observed by Detmer and Chan (2007).

Function of mitochondrial fusion

The question of why mitochondria fuse together into an intricate tubular network remains unanswered. Important insights into the function of mitochondrial fusion have been gained by determining the physiological conditions where mitochondrial hyperfusion occurs, and those where fusion is inhibited (**Figure 2**). The first known trigger for hyperfusion was in conditions of mild oxidative stress (Tondera et al. 2009). Oxidative stress has been shown to activate mitochondrial fusion using an in vitro fusion assay and mitochondrial morphology assessment in cells. Specifically, oxidized glutathione activated mitochondrial fusion in vitro (T. Shutt et al. 2012). Glutathione, a tripeptide, exists in a reduced (GSH) or an oxidized form (GSSG), in the latter a disulfide bond is formed between two glutathione molecules (Calabrese, Morgan, and Riemer 2017; Deponte 2013). Glutathione in cells constitutes one of the essential antioxidant systems, existing mainly as GSH (Calabrese, Morgan, and Riemer 2017). In case of oxidative stress, reactive oxygen species (ROS) is neutralized with GSH, yielding GSSG as a product (Deponte 2013). GSSG plays a role as a signaling molecule in cells and can be enzymatically converted back to two reduced GSH molecules (Deponte 2013). Not only was GSSG shown to activate fusion in vitro, increasing GSSG in cells, by drug-induced GSH oxidation, increased mitochondrial fusion as well (T. Shutt et al. 2012). GSSG was reported to induce oligomerization of MFNs, by forming new intermolecular disulfide bonds in the proteins (T. Shutt et al. 2012). The mechanism of action of GSSG remains to be further elucidated. Does GSSG affect GTP hydrolysis? Does it increase tethering capability of MFNs, due to MFNs oligomerization? Or does GSSG provide mechanical force that promotes fusion?

The activation of mitochondrial fusion under oxidative stress raises an interesting idea about a possible function of fusion in cells. Mitochondria are one of the major sources of ROS in cells. They produce ROS not only as a side effect to their metabolic function of ATP production, but also as a deliberate signaling molecule in diverse pathways (Brand 2016). The increase of the rate of ROS production might reflect a problem in the physiology of mitochondria: lack of efficiency or effectiveness in electron handling by the electron transport chain and other pathways, either due to protein damage or due to problems in stoichiometry of different components within mitochondria. Therefore, mitochondrial fusion might be an attempt to alleviate such deficiencies, by shuffling and mixing the protein components and metabolites, thereby forming a more homogenate mitochondrial population. This might dilute any problems experienced by individual mitochondria. Further, a mitochondrial network might be more resistant to oxidative damage when all the antioxidant pathways are contained within a single compartment.

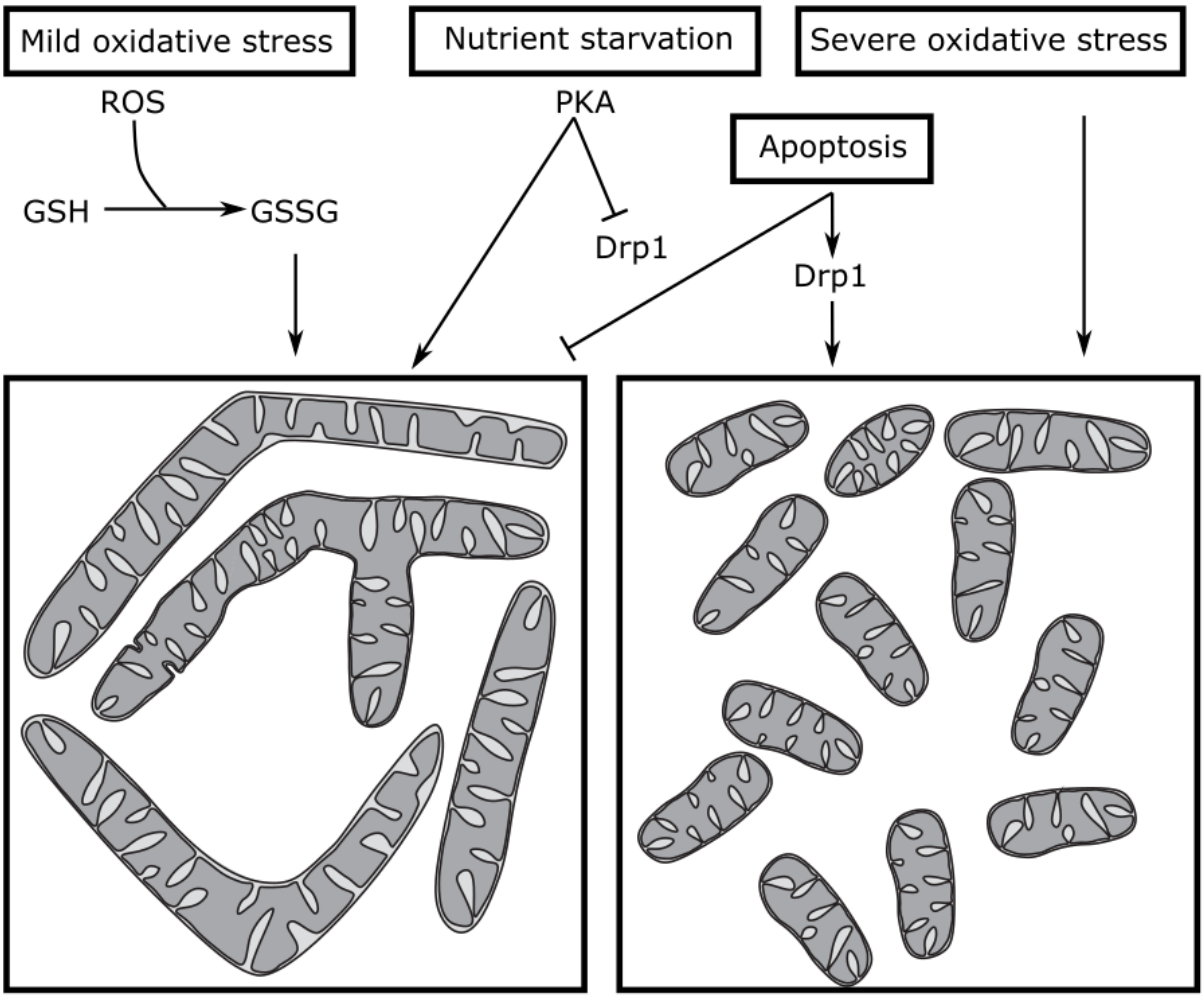

Figure 2. Physiological conditions that alter mitochondrial morphology.

A second important stimulator of mitochondrial hyperfusion is under conditions of nutrient deprivation. Under starvation mitochondria have hyperfused morphology in cultured cells and in vivo (Gomes, Di Benedetto, and Scorrano 2011; Rambold et al. 2011). In this case, the mechanisms are primarily linked to an inhibition of mitochondrial fission. Starvation leads to phosphorylation of Drp1, which is the GTPase required for fission, at Ser637 by PKA, ultimately leading to dissociation of Drp1 from mitochondria (Gomes, Di Benedetto, and Scorrano 2011; Rambold et al. 2011; Cribbs and Strack 2007; Cereghetti et al. 2008). This phosphorylation is proposed to inhibit fission. Although the starvation phenotype is thought to induce hyperfusion through Drp1 inhibition, cytosol from forskolin-treated

cells, an activator of PKA, has been reported to promote fusion in an in vitro fusion assay which only measures fusion (Schauss et al. 2010). This suggests that either PKA has targets affecting fusion other than Drp1, or that Drp1 might have a role in regulating fusion itself. From the phenotype accompanying starvation, it was proposed that mitochondrial fusion is protective against mitochondrial degradation by autophagy. Chemical inhibition of mTORC1, an inhibition which activates autophagy, was recently shown to promote mitochondrial hyperfusion, along with an increase of Drp1 Ser637 phosphorylation (Morita et al. 2017). Starvation conditions are known to inhibit mTORC1 (Demetriades, Doumpas, and Teleman 2014). This suggests that the metabolic state of the cell sensed by mTORC1 is translated into mitochondrial morphology. However, the mTORC1 regulation of mitochondrial morphology might be more complex, acting through translational regulation of the inner membrane fission factor MTFP1 which in turn affects Drp1 phosphorylation and recruitment to mitochondria (Morita et al. 2017; Aung et al. 2017).

In conditions of cell death, mitochondrial fusion is actively inhibited, and Drp1 mediated fragmentation dominates (Breckenridge et al. 2003; Karbowski et al. 2004; Sugioka, Shimizu, and Tsujimoto 2004). Apoptotic stimuli were shown to induce translocation of Drp1 to mitochondria, suggesting the increase of fission as the cause of the fragmentation morphology under these conditions (Breckenridge et al. 2003; Sugioka, Shimizu, and Tsujimoto 2004; Prudent et al. 2015). Inhibition of fusion under cell death was also observed using photoactivatable fluorescent mitochondrial marker (Karbowski et al. 2004). This suggests a dual mechanism of activating fission and inhibiting fusion during an apoptotic trigger. Increasing fission might be mainly due to increase in translocation of Drp1 to mitochondria and stabilization of Drp1 complexes by Sumo post-translational modification of Drp1 (Prudent et al. 2015). On the other hand, overexpression of MFN or the expression of constitutively active MFN2 (GTP hydrolysis deficient) inhibited stimuli-induced cell death, confirming a cyto-protective role of fusion (Sugioka, Shimizu, and Tsujimoto 2004; Neuspiel et al. 2005). Mfn2 was shown to be

degraded by the proteasome following cell death stimuli, highlighting a relationship between fission and fusion (Leboucher et al. 2012). What is the role of fusion in cell death? The inhibition of fusion seems to be required to maximize fission. This is supported by the proposed requirement of Drp1 to provide membrane curvature for Bax insertion into mitochondrial membrane, where both Drp1 and active Bax were shown to colocalize on the mitochondrial membrane (Prudent et al. 2015). MFN2 was shown to interact with Drp1 through its HR1 domain (P. Huang, Galloway, and Yoon 2011). Therefore, MFN overexpression might sequester Drp1, thereby preventing it from providing the proper environment for Bax insertion and cytochrome c release. The observation that cell death is inhibited by overexpression of MFN or constitutively active MFN2 led primarily to the hypothesis that fusion is protective from cell death.

An important cellular function for mitochondrial fusion was in the maintenance of mitochondrial DNA (mtDNA). The loss of DAPI-stained mtDNA nucleoids in the absence of Fzo1 was observed in *S. cerevisiae* (Hermann et al. 1998). Consistent with this work in yeast, in the absence of fusion, in Mfn-null MEFs and muscles, loss of mitochondrial DNA (mtDNA) and an increase of mtDNA mutation rate were observed, leading to the hypothesis that perhaps fusion plays a role in the maintenance of the mtDNA fidelity (Chen et al. 2010). Furthermore, shuffling of respiratory chain complexes was shown to proceed mitochondrial fusion (Muster et al. 2010; Wilkens, Kohl, and Busch 2013). It was predicted that several rounds of fusion and fission events are required for proper mixing of complexes of the fusing mitochondria (Wilkens, Kohl, and Busch 2013; Busch, Kowald, and Spelbrink 2014). Such shuffling of complexes would ensure not only efficient ATP production and minimization of ROS production, but perhaps this would help sort and isolate damaged complexes for degradation. Apart from the mixing of mtDNA and proteins, mitochondrial fusion may sequester small metabolites together in one connected compartment, from amino acids to lipids to TCA cycle substrates, thereby facilitating production in the various pathways within mitochondria. While the physiological consequences in cells lacking

mitochondrial fusion have been described, the true mechanisms by which fusion ensures the retention of mtDNA remains unclear.

What we know: functional domains of MFNs

Starting at the amino-terminus, within the first 300 amino acids, MFN1 and MFN2 contain a GTPase domain. The GTPase domain of the two MFN proteins are not functionally identical, however. Mfn1 GTPase domain has low affinity for GTP with a high hydrolysis activity, which is the opposite of Mfn2, having a high GTP affinity with low hydrolysis activity (Ishihara, Eura, and Mihara 2004; Neuspiel et al. 2005). What does the difference in GTP binding affinity and hydrolysis mean with respect to the Mfn1/Mfn2 role in fusion? Though we do not have direct experimental evidence to answer this question, we may make a meaningful inference from other observations. Not only is GTP required for fusion, unsurprisingly, GTP hydrolysis is also required. The addition of non-hydrolysable GTP analogs, GTPγS or GMP-PNP, inhibits fusion as observed by in vitro fusion assays (Schauss et al. 2010; Meeusen, McCaffery, and Nunnari 2004; Brandt et al. 2016). This has been assigned to the requirement for MFNs as the essential GTPase, however it cannot be formally excluded that unknown GTPases are also contributing. According to Brandt et al (2016), GTP hydrolysis seems to play a role in extending membrane contact area between fusing yeast mitochondria in vitro. How does non-hydrolysable GTP analogs affect Mfns? On the protein level, the addition of GTPγS inhibited the binding of Mfn1 proteins together, by co-immunoprecipitation, suggesting that GTP hydrolysis is required for efficient Mfn1-Mfn1 binding (Ishihara, Eura, and Mihara 2004). The requirement for GTP hydrolysis for protein dimerization was confirmed in crystallographic analysis of mini-MFN1, consisting of amino acids 1-364 linked to residues 694-741. In this work, MFN1 was only dimeric in the presence of transition state GTP analogs (GDP-BeF_3^- and GDP-AlF_4^-), remaining monomeric in the presence of GTPγS (Yan et al. 2018). The crystal structures of mini-MFN1 also revealed that the dimerization of MFN1 occurs through the GTPase domain, whereby, upon GTP binding and hydrolysis a conformational change in the GTPase domain

permits binding of two domains in trans (**Figure 3, model 3**) (Cao et al. 2017; Yan et al. 2018). The MFN1 GTPase was also shown to tether membranes using a liposome tethering assay, which required GTP hydrolysis (Cao et al. 2017), and such a tethering role of the GTPase domain has also been observed in atlastins, which are GTPases required for homotypic ER fusion (Byrnes and Sondermann 2011; Byrnes et al. 2013). As stated earlier, Mfn1 has an opposite GTP binding and hydrolysis activity to that of Mfn2. Mfn2, having low hydrolysis activity, might tether membranes, without being able to expand the contact area to form a "protein ring" observed in isolated yeast mitochondria by Brandt et al (2016). The opposite is true for Mfn1, which, having a high hydrolysis activity, might form a large contact area. The difference in GTP hydrolysis by Mfn1 and Mfn2 is intriguing and might be a good start for future work to tease out a meaning for mammalian cells having two fusogenic GTPases in the outer membrane.

The second conserved structural feature of MFNs is the heptad repeat domains (HR; also called coiled-coil). Coiled-coils are alpha helices that are amphipathic, and have alternating hydrophilic and hydrophobic amino acids every seven residues (heptad repeats) (A. Lupas, Van Dyke, and Stock 1991). Coiled-coil proteins play diverse roles in cells, of which an example is the fusogenic SNARE complex forming a four-helix coiled-coil. HR1 is about 30 residues in length, located in the middle of MFN proteins, as predicted by COILS algorithm (A. Lupas, Van Dyke, and Stock 1991), while HR2 is about 50 residues long, located at the very C-terminal end of the MFN proteins. Mfn2 mutations in and near HR domains have been reported in patients diagnosed with neurodegenerative diseases, suggesting functional importance of HR1 and HR2 (Liesa, Palacín, and Zorzano 2009). Published work on HR1 is scarcer than HR2. Consequently conclusions on the physiological function of HR domains are mainly, speculative. What is known about HR1 and HR2?

One proposed function is that HR1 binds HR2, acting in some way to drive conformational changes that mediate bilayer fusion and/or tethering between apposing mitochondria (**Figure 3, model 1**). Do HR1 and HR2 bind together? This idea that HR1 directly binds HR2 comes from a Yeast-Two-

Hybrid (Y2H) screen in which Huang et al reported that the domain between the GTPase and the TM domains (264-603; the authors termed HR1) binds the region C-terminal of the TMD (648-757; the authors termed HR2) (P. Huang, Galloway, and Yoon 2011). These domains, named HR1 and HR2 by the authors, should not be confused with the predicted HR1/HR2 structural domains. Interestingly, in the same work, also using Y2H, the authors found that the more precisely defined HR2 domain (684-757) did not actually bind HR1, a critical finding which the authors dismissed, concluding instead that the whole of the C-terminus (648-757) is required to bind HR1 (P. Huang, Galloway, and Yoon 2011). Therefore, it is important to consider that the direct evidence to support a direct interaction between HR1 and HR2 remains rather weak and inconclusive. Even so, the field has generally accepted this idea, including it in models, and have even used this potential interaction as a basis for drug development to disrupt such binding (Franco et al. 2016; Rocha et al. 2018). Nevertheless an important discovery came out of the yeast two hybrid studies. Huang et al found that the expression of a small peptide of HR1 in cells activated mitochondrial fusion, a finding that was later used by the Dorn lab for drug development (P. Huang, Galloway, and Yoon 2011; Franco et al. 2016; Rocha et al. 2018). Dorn reasoned that this peptide would disrupt the HR1/HR2 interaction, opening the structure to promote fusion (**Figure 3, model 1**). However, this proposed mechanism of action of HR1 peptide as an activator remains to be experimentally proven.

In a recent study using liposomes reconstituted with MFN1 HR1, Daste et al revealed that HR1 interacts with liposomes, and is, surprisingly, sufficient to fuse HR1-reconstituted liposomes with protein-free liposomes (**Figure 3, model 4**) (Daste et al. 2018). It might be tempting to envision how HR1 lipid destabilizing feature might fit in the context of the full-length protein, with the GTPase and TMD and HR2 in place. Does HR1 interact with the membrane in *cis* or *trans*? Does the GTPase activity affect HR1 accessibility to a bilayer? A liposome reconstituted with full-length protein might be required to answer such mechanistic details.

The second heptad repeat region in MFNs is at the very C-terminal end of the protein. HR2 is thought to play a role as a tether of mitochondria (**Figure 3, model 2**). The basis of this is the observation that recombinant HR2, containing mutations engineered to facilitate crystallization, formed two antiparallel dimeric helices, prompting the conclusion that this could tether mitochondria in *trans*. That study also revealed that Myc-tagged HR2 and HA-tagged HR2 co-immunoprecipitated (Koshiba et al. 2004). Qi et al (2016) could not, however, reproduce this HR2-HR2 binding by co-immunoprecipitation. Further, a recent study, using liposomes reconstituted with either HR1 or HR2, showed that HR1 has a stronger tethering character than HR2 (Daste et al. 2018). These inconsistent findings bring into question whether or not HR2/HR2 binding in *trans* does act as a tether to drive fusion in vivo. Recent crystallographic work further challenged this proposed model of HR2-HR2 binding. Crystal structures of "mini-MFN1", where HR2 (residues 694-741) was artificially linked to the N-terminal region 1-364 (or 1-365), showed that HR2 formed a helix in a 4-helix bundle with three N-terminal helices, thereby making it unavailable to bind to another HR2 in *trans* (**Figure 3, model 3**) (Qi et al. 2016; Cao et al. 2017). The authors who reported the structures argued that HR2 forms a stable structural element in the 4-helix bundle and because the binding of the helices is strong, HR2 is unlikely to unwind. On the other hand it was argued that the structure of "mini-MFN1," heavily truncated and artificially linking the N-terminal region with a small region of the C-terminus, might not reflect the architecture of full-length protein (Dorn 2019).

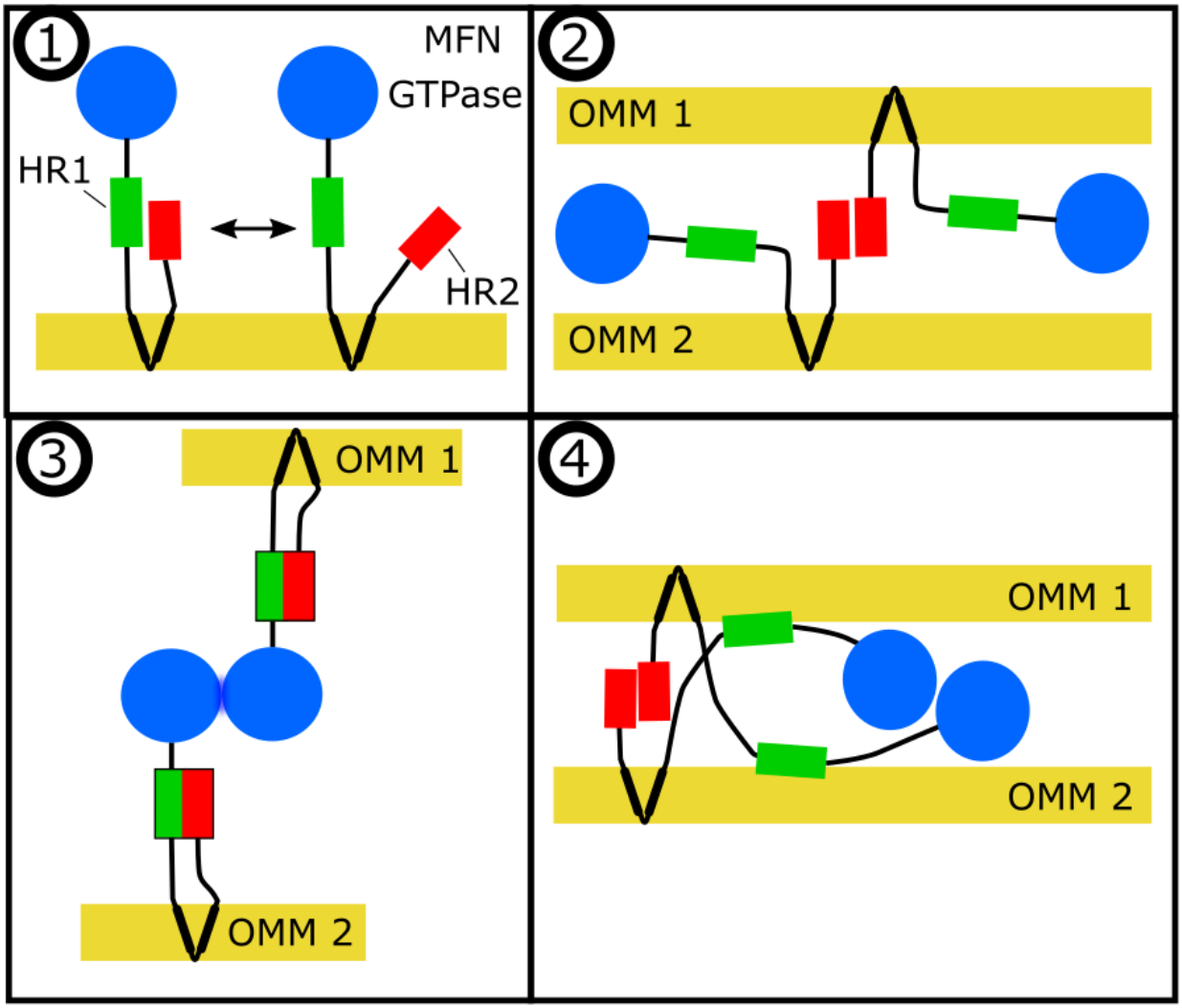

Figure 3. Schematic depicting the mechanism of MFN domains in mitochondrial fusion.

1, The Dorn model of HR1-HR2 binding. 2, Chan model of HR2-HR2 binding in trans. 3, Gao model from mini-MFN1 crystal structure showing tethering through the GTPase domain. 4, Tareste model where recombinant HR1 was shown to tether and fuse liposomes.

To summarize what has been most recently demonstrated about the structure/function of the Mitofusins, the structural insights gained from the "Mini-MFN1" showed the GTPase domain of MFN proteins interacting in *trans* to tether membranes, a process which requires GTP hydrolysis. Second, HR1 has a membrane binding ability, and in an HR1-reconstituted liposome fusion assay HR1 was shown to possess both a tethering and fusogenic functions. This characteristic is interesting but needs to be

further studied in the context of full-length MFNs. Third, it is important to highlight the fact that there is no direct evidence that HR1 and HR2 interact within MFNs; the inference is mainly based on structural homology prediction of MFN folding from solved crystal structure of the bacterial GTPase BDLP. This BDLP-based predicted MFN2 structure was employed to propose a mechanism for the activation of mitochondrial fusion observed when cells were treated with HR1 minipeptide. Fourth, HR2 dimerizing and binding in *trans* remains controversial and inconclusive. Work described within this thesis directly addresses these structural points through the experimental demonstration of a revised topology of MFNs in the metazoan clade.

Regulation of mitochondrial fusion by ubiquitination

In *S. cerevisiae*, ubiquitination has been implicated in the regulation of mitochondrial fusion. One of the proteins involved in this pathway is Mdm30, which is a cytosolic E3 ubiquitin ligase. In the absence of Mdm30, mitochondria were shown to aggregate and fusion was inhibited as assessed by mating cells whose mitochondria were labeled with two fluorescent colors (Fritz, S; Weinbach, N; Westermann 2003). The Mdm30 deletion morphology phenotype was rescued by deleting Dnm1, the yeast orthologue of mammalian DRP1, at the same time (Fritz, S; Weinbach, N; Westermann 2003). However, in the absence of Mdm30, Fzo1 levels were shown to be elevated, inferring that Mdm30 has a role in the turnover of Fzo1 in a proteasome-dependent manner (Fritz, S; Weinbach, N; Westermann 2003; Escobar-Henriques, Westermann, and Langer 2006; M. M. J. Cohen et al. 2008). The observation that Mdm30 did not efficiently degrade GTPase-dead Fzo1, led to the hypothesis that Mdm30 ubiquitination occurs after GTP hydrolysis (Anton et al. 2011; M. M. Cohen et al. 2011). The role of ubiquitination gained a deeper, more complex, meaning when two deubiquitylases (DUBs), Ubp2 and Ubp12, were identified to remove ubiquitin chains from Fzo1 (Anton et al. 2013). The absence of Ubp2 revealed an opposite mitochondrial morphology to that observed in the absence of Ubp12 (Anton et al. 2013). This raises questions as to what is the mechanistic contribution of ubiquitination in mitochondrial

fusion. A cell free fusion assay is perhaps needed to dissect the role of ubiquitination in fusion, and to remove a possible indirect effect of removing Mdm30 or Ubp2/12 in cells, especially these factors have other essential functions in cells (Ho, MacGurn, and Emr 2017; Gödderz et al. 2017). Also, is the ubiquitination regulation of fusion a yeast-specific pathway? No known mammalian orthologues of Mdm30, Ubp2, or Ubp12 have been identified. This does not eliminate the possibility that there exists other mammalian proteins performing the functions of Mdm30p and Ubp2/12. Parkin, which is a cytosolic E3 ubiquitin ligase, was reported to be involved in the turnover of MFNs under mitochondrial depolarization with CCCP (Tanaka et al. 2010), thought to inhibit depolarized mitochondria from fusing. March5 (also named Mitol), an E3 ubiquitin ligase which is anchored to mitochondrial outer membrane, was implicated in mitochondrial morphology. March5 was shown to ubiquitinate Drp1, Fis1, and Mfn2 (Nakamura et al. 2006; Yonashiro et al. 2006; Sugiura et al. 2013). Contradicting observations have been reported regarding the role of March5 in morphology. The loss of March5 function was reported to lead to fragmentation on the one hand (Yonashiro et al. 2006), or on the other, hyperfusion (Karbowski, Neutzner, and Youle 2007). Therefore, the role March5 plays in regulating morphology seems to be more complex, perhaps acting at the cross-section between fusion and fission, thus determining the final morphological outcome based on its level. March5 has also been reported to be involved in the turnover of some mitochondrial proteins, including the Drp1 receptor Mid49 and SLC25A46 (Cherok et al. 2016; Steffen et al. 2017), and involved in regulating ER-mitochondria contact sites (Sugiura et al. 2013). The mechanism of how March5 affects mitochondrial morphology remains to be determined. It remains also to be determined whether mammalian mitochondrial fusion require ubiquitination in its mechanism. In each of these cases, the use of experimental systems that allow the precise staging of the reactions (fusion or fission) are important for the field to move forward.

Rationale for the studies presented in this thesis

The work described in this thesis is aimed to elucidate the molecular mechanisms and regulation of mitochondrial fusion using a cell-free mitochondrial fusion assay. The first question we tested addresses the potential role for ubiquitination in driving mitochondrial fusion. Apart from protein turnover, ubiquitination has been proposed as being a part of the mechanism of mitochondrial fusion in *S. cerevisiae*. As just described, the E3 ubiquitin ligase Mdm30 and two DUBs, Ubp2 and Ubp12, have been implicated in mitochondrial fusion, perhaps through ubiquitination/deubiquitinylation cycles, of Fzo1 (Escobar-Henriques, Westermann, and Langer 2006; M. M. J. Cohen et al. 2008; M. M. Cohen et al. 2011; Anton et al. 2011, 2013). Most of the work was performed by mitochondrial morphology assessment in cells, making it difficult to dissect the mechanism of action or to untangle the ubiquitination role from general protein turnover. A role of ubiquitination in mitochondrial fusion has not been reported in mammalian mitochondrial fusion, and orthologues of the yeast E3 ligase and DUBs have not been identified yet. Therefore, we set out to investigate a role for ubiquitination and deubiquitylation and the ATPase p97 in mitochondrial fusion. We used a cell-free mitochondrial fusion assay which specifically measures matrix-content mixing. We also investigated a role of a novel E3 ubiquitin ligase, UBR4, which was identified through the BioID of MFN2.

The second major question addressed was how the structure and topology of the mitofusins may mechanistically drive mitochondrial fusion. The C-terminal region of MFNs has been controversial, both functionally and structurally. Several ideas had been proposed as to the function of the C-terminus: 1) the C-terminus, specifically HR2, is a tether, linking two mitochondria together (Koshiba et al. 2004; Daste et al. 2018); 2) HR2 binds HR1, acting possibly as a switch in an unknown fashion (Franco et al. 2016; P. Huang, Galloway, and Yoon 2011); and 3) HR2 binding a region in the N-terminus, acting as a structural stabilizer of the GTPase domain, as speculated from the mini-MFN1 crystal structures (Qi et al.

2016; Cao et al. 2017). We set out to characterize the C-terminus of MFNs through bioinformatics, biochemical and functional approaches.

Chapter 2: Materials and Methods

Homology searching, phylogenetic reconstruction, and other bioinformatic methods

Characterized human Mitofusin and yeast Fzo1 sequences were used as BLAST (Altschul et al. 1997) queries to identify closely related animal and fungal sequences. Surprisingly, the top non-holozoan hits for Mfns and non-fungal hits for Fzo were BDLPs. This prompted us to entertain the hypothesis that Mfns and Fzos may have arisen independently from horizontal gene transfers of different BDLPs. To test this hypothesis, Hidden Markov models (HMMs) based on Mfn and Fzo sequences obtained from diverse animal and fungal genomes were used to search for Mfn- and Fzo-related sequences using HMMer v3.0 (Eddy 2011) in diverse prokaryote, holozoan, and fungal genomes. Sequences retrieved with an evalue score <1e-10 for either HMM were retained for phylogenetic analysis. Sequences were aligned by MUSCLE (Edgar 2004) and manually adjusted and trimmed. Phylogenetic reconstructions were performed using MrBayes v3.2 (posterior probability) (Ronquist and Huelsenbeck 2003) and RaxML v8.2 (maximum likelihood) (Stamatakis 2006). Mfn and Fzo proteins were subjected to bioinformatic analysis using TMHMM2.0 (Krogh et al. 2001) and COILS (Andrei Lupas 1996) to predict transmembrane domains and coiled-coil domains, respectively. In the case of TMHMM2.0, each protein was analyzed individually. Only one animal sequence (Caenorhabditis elegans) was strongly predicted to have two TMDs. Since TMHMM2.0 is trained on more canonical membrane proteins than mitochondrial outer membrane proteins, it likely incorrectly predicted some of the more divergent sequences (e.g. Homo sapiens Mfn1, see Fig. 1B). Manual inspection of aligned sequences suggests that all fungal Fzo1 proteins have two TMDs whereas all animal Mfn proteins have a single TMD (including the aberrantly predicted C. elegans Mfn protein). TMD prediction results for representative proteins are depicted (Fig 1B grey lines). In the case of COILS, alignments consisting of all Fzo1 proteins and all Mfn

proteins were subjected to analysis. Results from the 28-residue window with a score >0.7 were depicted against the respective sequences (Fig. 1B orange lines).

Materials

Antibodies were obtained as follows: rabbit anti-Mfn2 (Cell Signaling; #11925 (CS)); mouse anti-Mfn2 (Abnova; H00009927-M01 and H00009927-M03); rabbit anti-Mfn1 (Richard Youle); mouse anti-OPA1 (BD transduction laboratories; 612607); rabbit anti-TOM20 (Santa Cruz; sc-11415); mouse anti-FLAG (Sigma; F1804); rabbit anti-UBR4 (Abcam; ab86738). Triton-X100 (cat# X100), trypsin (cat# T4549), soybean trypsin inhibitor (cat# T9003), reduced glutathione (cat# G6529), oxidized glutathione (cat# G4376), proteinase K (cat# P8044; cat# P2308), and creatine kinase (cat# C3755; C7886) were obtained from Sigma. Methoxy polyethylene glycol maleimide 10kDa was obtained from JenKem Technology (cat# M-MAL-10K). Phenyl-Methyl Sulfonyl Fluoride (PMSF) was obtained from Calbiochem (cat# S2332). For immunofluoresence, goat anti-mouse and goat anti-rabbit IgG Alexa Fluor were used as secondary antibodies (Molecular Probes). For transient transfection, cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Ubiquitin and ubiquitin mutants were obtained from Boston Biochem: His6-Ubiquitin Mutant R74 (cat# UM-HR74), Ubiquitin Mutant G76A (cat# UM-G76A), HA-Ubiquitin aldehyde (cat# U-211), HA-Ubiquitin-Vinyl Sulfone (cat# U-212), and FLAG-Ubiquitin (cat# U-120). NMS-859 (ATPase VCP/p97 Inhibitor) was obtained from Xcessbio Biosciences (cat# M60148-2s). siRNA were obtained from GE Healthcare Biosciences Company: SMART pool ON-TARGET Plus human UBR-4 (L-014021-01-0005; Dharmacon) and ON-TARGET Plus Non-Targeting Pool (D-001810- 10; Dharmacon). ATPγS (Cat# NU-406-50) and GTPγS (Cat# NU-412-20) were obtained from Jena Bioscience.

Previously described cDNA encoding Mfn2-16xMyc1xHis was obtained from Addgene (plasmid # 23213; deposited by David Chan).

Cloning, expression and purification of MFN2 565-757 and MFN2 648-757

MFN2 fragments were amplified by PCR from a plasmid containing full-length MFN2 gene. The following primers were used containing desired restriction sites:

5’ 648, BamHI: AAAGGATCCGAGCGTCTGACCTGGACCAC

5’ 565, BamHI: AAAGGATCCGCCTTGATGGGCTACAATGAC

3’ 648 and 565, EcoRI: AAAGAATTCTCTGCTGGGCTGCAGGTAC

Purified PCR products and pGEX 4T1 empty vector were digested with BamHI-HF and EcoRI-HF. Following gel extraction, the digested construct and vector were ligated and transformed into BL21 competent cells and then plated on ampicillin LB agar plates. Bacterial colonies were tested for insert. Then bacterial preculture containing either GST-MFN2(565-757) or GST-MFN2(648-757) were grown in LB (100µg/ml ampicillin) at 37^{o}C and when OD600 was around 0.5, IPTG was added to induce expression for 2-3 hours. Cells were harvested by centrifugation and broken by sonication on ice. Triton X-100 was added to 1% and lysate was precleared by centrifugation. Supernatant was added to glutathione sepharose 4B slurry to purify GST tagged MFN2 fragments, and incubated at room temperature for 10 minutes. After washing the beads 3 times with 1% Triton X-100/PBS, batch thrombin cleavage was done overnight. The supernatant containing the cleaved product was saved and flash frozen at -80C.

Cloning of truncated MFNs

The following primers were used for the cloning of MFN2(1-627)-VAMP1b(96-116) into pcDNA3.1+:

5’ BamHI-Mfn2: AAA GGATCC ATGTCCCTGCTCTTCTCTCGATG

3’ EcoRI-VAMP1bTM-Mfn2: AAA GAATTC

TCAGTCCCGCCTTACAATAACTACCACGATGATGGCACAGATGGCTCCCAGCATGATCATCATCTT

CCACACCACTCCTCCAACAAC

The following primers were used for the cloning of MFN2(1-647)-RRD into pcDNA3.1+:

5’ BamHI-MFN2: AAA GGATCC ATGTCCCTGCTCTTCTCTCGATG

3’ EcoRI-RRD-MFN2(1-647): AAA GAATTC TCAGTCCCGCCT ATAGACGTAGAGGAGGCCATAGAGC

The following primers were used for the cloning of MFN2(1-627)-ActA(TMD) into pcDNA3.1+:

5’ BamHI-MFN2: AAA GGATCC ATGTCCCTGCTCTTCTCTCGATG

3’ EcoRI-ActA-MFN2: AAA GAATTC

ttaattatttttttcttaattgaataattttgataaacgcccctaaagagaacacgccaatagctaacattgcaagaattaa

CTTCCACACCACTCCTCCAAC

The following primers were used for the cloning of MFN2(1-693) into pcDNA3.1+:

5’ BamHI-MFN2: AAA GGATCC ATGTCCCTGCTCTTCTCTCGATG

3’ EcoRI-STOP-MFN2(693): AAA GAATTC CTA AGACAGTTCCTGCTGGACTTGG

The following primers were used for the cloning of FLAG-MFN1(1-672) into pcDNA 3.1+

5’ BamHI-ATG-FLAG-MFN1: AAA GGATCC ATG GATTACAAGGATGACGACGATAAG ATGGCAGAACCTGTTTCTCCAC

3’ EcoRI-STOP-MFN1(672): AAA GAATTC TTATTGTTGTTTTACTTGGTGACTGCAGTT

The following primers were used for the cloning of MFN2(648-757) into pcDNA3.1+:

5’ 3xFLAG-MFN2(648): AAA GGATCC ATG

GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAG

GAGCGTCTGACCTGGACC

3’ EcoRI-MFN2: AAA GAATTC CTATCTGCTGGGCTGCAGGTAC

Mitochondrial isolation

Mitochondria were isolated from suspension HeLa stably expressing either N-MitoVZL or C-MitoLZV as described previously (Schauss et al. 2010). Briefly, sHeLa were grown in 2L flasks in sMEM, and were harvested by centrifugation for 20min at 4^{o}C at 3000 xg. Cells were broken using a dounce homogenizer. Nuclei were centrifuged at 600xg for 10min at 4^{o}C and the post-nuclear supernatant was centrifuged for 15min at 8000 xg at 4^{o}C. The pellet was washed in isolation buffer (220 mM Mannitol, 68 mM sucrose, 80 mM KCl, 0.5 mM EGTA, 2 mM MgAc2, 10 mM HEPES pH 7.4), then resuspended in isolation buffer containing 10% glycerol and snap frozen in liquid nitrogen for storage at -80^{o}C.

Mitochondrial fusion assay

The in vitro fusion assay was carried out in a 96-well plate, which was modified from Schauss et al (2010). Briefly, 10 µg of each mitochondrial population (total 20 µg) was added per reaction (25µL) containing 15 mM HEPES pH 7.4, 110 mM Mannitol, 68 mM sucrose, 80 mM KCL, 0.5 mM EGTA, 2 mM $Mg(CH_3COO)_2$, 0.5 mM GTP, 2 mM K_2HPO_4, 1 mM ATP(K+), 0.08 mM ADP. The ATP regeneration system was driven by the addition of 5 mM Na succinate, or in the presence of creatine kinase/phosphate (20 U/ml creatine kinase and 20 mM creatine phosphate), as indicated. Reactions were assembled on ice in a 96-well plate. A previously described initial centrifugation step to concentrate mitochondria was removed (Hoppins et al., 2011; Meeusen et al., 2004; Schauss et al., 2010). After assembling the reactions, samples were incubated for 30min at 37^{o}C, followed by immediate solubilization and addition of substrate to quantify luciferase activity (reflecting assembly of the complementary split-luciferase polypeptides) using the Renilla luciferase assay kit (Promega, WI, USA).

Role of ubiquitination in mitochondrial fusion in vitro

We added the specified drugs or ubiquitin mutants that interfered with ubiquitination or de-ubiquitylation to our in vitro fusion assay prior to incubation at 37^{o}C for 30 minutes. This was followed by quantification of luciferase activity as a measure of fusion. For Western blotting analysis, 20uM recombinant FLAG-Ub was added to all reactions, to detect the level of new ubiquitination within the 30 minutes duration.

siRNA knock down

40pmol of siRNA was reverse transfected into HeLa or HEK293 cells, using Lipofectamine RNAiMAX Transfection Reagent, following the manufacturer's protocol. Cells were incubated for 72hr before a second round of siRNA transfection and incubation for 48hr. After the 48hr incubation, cells were treated with DMSO or 20ug/ml CHX or 20uM CCCP for 5hr. Cells were harvested for Western blotting.

Trypsin and proteinase K digestion protection experiment

Mitochondria (1mg/ml) were isolated from HEK293 and incubated with specified trypsin concentrations for 20 min on ice. Soybean trypsin inhibitor (SBTI) was added in excess (5mg/ml) and incubated for another 20 min on ice. Laemmli sample buffer was added, samples were boiled for 5 min, and processed for standard Western blotting analysis. For proteinase K experiment, 1mg/ml freshly isolated mitochondria were incubated with specified concentration of PK for 30 min on ice, followed by addition of 4mM PMSF and incubation for 20 min. For hypotonic shock control, mitochondrial pellet was resuspended in 20 mM Hepes pH 7.4 to cause inner membrane swelling, which was treated with PK. Samples were separated on 12% SDS-PAGE gels (unless otherwise indicated in the figure legends) and processed for Western blotting similar to trypsin experiment.

Triton X-100, alkaline carbonate, and urea extractions

25 µg of mitochondria isolated from non-transfected or Mfn2-16xMyc-transfected HEK293 cells were centrifuged at 8000 xg for 10 min. Mitochondrial pellet was either resuspended in 100 µl 1% Triton X-100 (prepared in isolation buffer), or 100 µl 100 mM alkaline carbonate (pH 12), or 100 µl urea extraction buffer (4.5 M urea, 150 mM KCl, 1 mM DTT, 20 mM Hepes (pH 7.4)). Samples were incubated on ice for 30 min. Alkaline carbonate samples were vortex mixed briefly for few times. Samples were then centrifuged at 200,000 xg, at 4 °C for 15 min. Supernatants were transferred to new microfuge tubes and pellets were resuspended in buffers corresponding to type of extraction. Sample buffer was added and samples were processed for Western blotting.

Polyethylene glycol maleimide experiment

We used a 10kDa PEG-mal to avoid leakage through VDAC (Colombini 1980) and ensure only protein regions facing the cytosol could be conjugated. Samples were kept on ice throughout the experiment. 25µg of mitochondria (1mg/ml) isolated from HEK293 were incubated with 1mM 10kDa PEG maleimide (PEG-mal) for 1hr (or isolation buffer for controls). Excess PEG-mal was neutralized by adding 25mM DTT. Samples were then centrifuged for 10min at 8000xg at 4°C. Pellets were resuspended in isolation buffer and trypsin (0.5mg/ml) where indicated, followed by inhibition of trypsin with 2mM PMSF and 1mM SBTI, for 20 min on ice.

For solubilization of mitochondria, Triton X-100 was added to a final concentration of 1%. Additional treatments performed on trypsinized mitochondria were done following complete inactivation of the trypsin with 1mg/ml SBTI. Samples were boiled for 3 min after addition of sample buffer, and processed for Western blotting.

Glutathione redox potential determination that activates fusion

The GSH:GSSG ratios were used to calculate the glutathione redox potential at pH 7.4 that promotes fusion using a modified Nernst equation as shown:

$$\Delta E=\{-240mV+(-61.5mV/2e-)\times(\text{actual pH}-7.0)\}-(61.5mV/2e-)\times\log([GSH]2/[GSSG])$$

GSH was added to a concentration of 4mM, while increasing concentrations of GSSG were added, to create a GSH:GSSG ratio. The concentrations of GSH and GSSG with the calculated ΔE are reported in the following table:

[GSH] (mM)	[GSSG] (mM)	*ΔE* (mV)
4	0.01	-258
4	0.1	-228
4	0.25	-216
4	0.5	-206
4	1	-197

Western blotting under denaturing non-reducing conditions

To visualize disulfide-linked MFN2 oligomers, sample buffer containing 50mM iodoacetamide (with no reducing agents) was added to samples after 30min fusion assay. Iodoacetamide will react with free cysteine residues, blocking against non-specific reactions during sample preparation (Wrobel et al. 2016). Samples were boiled for 3 min and processed for Western blotting analysis.

Acrylamide gel purification of MFN2 (565-757)

To remove contaminating GST and GST-MFN2(565-757), and isolate only the pure, cleaved fragment of MFN2 (565-757), we separated the recombinant preparation on a 4-16% SDS-PAGE with denaturing/reducing conditions and the region between molecular marker 25 and 15 was excised and chopped into small pieces. These pieces were washed three times, five minutes each at room temperature, with PBS, then with water. The gel was further minced with a razor and placed in an Eppendorf tube. 300 µl PBS with 0.1% SDS was added. It was sonicated on ice for three times, thirty seconds each with rests in between. The gel was then centrifuged and the supernatant collected. This contained the purified fragment.

Immunofluorescence microscopy

Mfn2-null MEFs were seeded into 24-well plates upon glass coverslips. Mfn2-3xFLAG was transfected into Mfn2-null cells. For immunofluorescence, cells were fixed in 5 % paraformaldehyde (PFA) in PBS, at 37^{o}C for 15 minutes, then washed 3 times with PBS, followed by quenching with 50 mM ammonium chloride in PBS. After 3 washes in PBS cells were permeabilized in 0.1 % Triton X-100 in PBS, followed by 3 washes in PBS. Then the cells were blocked with 10 % fetal bovine serum (FBS) in PBS, followed by incubation with primary antibodies in 5 % FBS in PBS, for 1 hour, at RT. After 3 washes with 5 % FBS in PBS, cells were incubated with appropriate secondary antibodies (1:1000) for 0.5-1 hour at RT. After 3 washes in PBS, coverslips were mounted onto slides using Dako fluorescence mounting medium (Dako). Cells were imaged using a 100X objective NA1.4 on an Olympus IX83 inverted microscope with appropriate lasers using an Yokogawa spinning disc system microscope coupled to a Neo camera (Andor).

Statistical analysis

Errors bars displayed on graphs represent the means ± S.D (or ± SEM when specified) of at least three independent biological replicates. Statistical significance was analyzed using unpaired Student's t-test. *: $P<0.05$, **: $P<0.01$.

Chapter 3: Results

Chapter 3.1: Role of ubiquitination in mammalian mitochondrial fusion

To establish a role of ubiquitination in mammalian mitochondrial fusion we used an in vitro fusion assay, which is based on biomolecular complementation of a functional luciferase enzyme (Schauss et al. 2010). The assay consists of mixing two populations of mitochondria, which are isolated from two HeLa cell lines expressing one of the two parts of matrix-targeted luciferase. Upon full fusion, matrix mixing, luciferase is complemented and its activity is measured as a reflection of fusion. ATP hydrolysis has been shown to be required for mitochondrial fusion using in vitro fusion assays (Schauss et al. 2010; Mishra et al. 2014), or to be not required for fusion (Hoppins, Edlich, Cleland, Banerjee, McCaffery, et al. 2011). Ubiquitin conjugation requires ATP hydrolysis; therefore, we first sought to confirm that ATP hydrolysis was required for fusion. Adding nonhydrolyzable ATP analog, ATPγS, to the mitochondrial fusion assay inhibited fusion (**Figure 4**). A sample lacking GTP or treated with GTPγS were used as controls, showing as previously reported that GTP hydrolysis is required for fusion (**Figure 4**). Therefore, mitochondrial fusion requires both ATP and GTP hydrolysis.

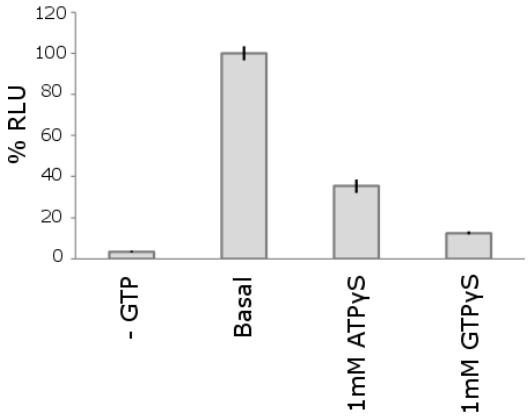

Figure 4. ATP hydrolysis is required for mitochondrial fusion.

Mitochondria containing each half of the matrix-targeted split-luciferase probes were incubated within the cell-free fusion assay system with nonhydrolyzable ATP or GTP analogs. Mitochondrial fusion under these conditions was quantified by

measuring luciferase activity. Luciferase counts were normalized to standard condition. Reported values are the mean at least two biological replicates, each performed in duplicate. Error bars are means ± SEM

The role of ubiquitination and deubiquitylation in mammalian mitochondrial fusion

To test a role of ubiquitination or deubiquitylation on our fusion assay, we added ubiquitination inhibitors (UbG76A and UbR74) or DUB inhibitors (Ubal and Ub-VS), which are recombinant mutant ubiquitin. Neither ubiquitination nor DUB inhibitors significantly affected mitochondria fusion (**Figure 5 A**). The fusion assay was also performed in the presence of recombinant FLAG-Ub to help track ubiquitination within the 30-minute incubation, followed by Western blot analysis. This confirmed that ubiquitination and deubiquitylation were inhibited by the drugs as was revealed by the decrease and increase of FLAG-Ub conjugation, respectively (**Figure 5 B**). We also used PYR-41, an E1 inhibitor (Yang et al. 2007). However, PYR-41 did not inhibit ubiquitination at concentrations we tested; therefore, it was not useful for our purposes. We treated one sample with oxidized glutathione (GSSG), which is an oxidative stress product, as a positive control, because GSSG is a known activator of fusion. As previously reported, GSSG activated fusion (**Figure 5 A**) while, at the same time, inhibited ubiquitination (**Figure 5 B**). Oxidative stress has been reported to inhibit ubiquitination by inhibiting E1 and E2 enzymes, through GSSG (Jahngen-Hodge et al. 1997; Obin et al. 1998). We concluded that neither ubiquitination nor deubiquitylation have a role in mammalian mitochondrial fusion, as determined by a cell-free fusion assay.

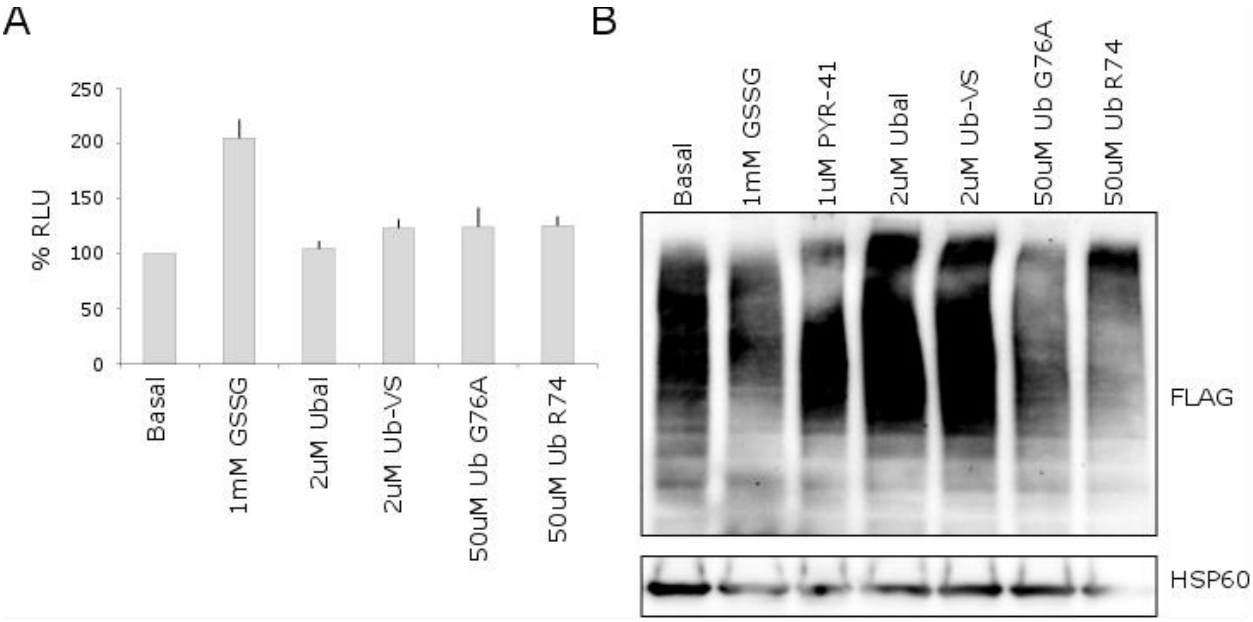

Figure 5. The role of ubiquitination and deubiquitylation in mammalian mitochondrial fusion.

A. Ubiquitination and DUB inhibitors were added to the in vitro fusion assay, followed by luciferase activity measurement, which is normalized to the basal condition. Reported values are the mean at least three biological replicates, each performed in duplicate. Error bars are means ± SEM

B. FLAG-Ub was added to the fusion assay along with the indicated drugs. The 30-minute incubation was followed by processing for Western blotting and immunoblotting with FLAG antibody to detect the ubiquitination levels. HSP60 was used as a control.

The role of UBR4 and p97 in fusion

BioID, a proximity biotinylation assay, of MFN2 was performed (by Eric Shoubridge lab; data not published). MFN2 was N-terminally tagged with BirA, an enzyme producing activated biotin which covalently reacts with exposed lysine residues on proteins in the vicinity of the tagged protein. Many of the main BioID hits were proteins involved in proteasomal degradation pathway, surprisingly. Two proteins captured our interest, UBR4 and p97. UBR4 is a 600-kDa cytosolic ubiquitin E3 ligase, which may play a role in autophagy (Tasaki et al. 2013). UBR4, along with UBR1 and UBR2, was reported to degrade cleaved PINK1, a mitochondrial kinase involved in mitochondrial quality control (Yamano and Youle 2013). The PINK1 partner Parkin, which is a cytosolic E3 ubiquitin ligase that was previously shown

to degrade MFN2 under mitochondrial depolarization, was not detected in the Mfn2 BioID. We knocked-down UBR4 in two cell lines, HEK293 and HeLa. The knock-down of UBR4 was efficient at the protein level as detected by an antibody recognizing UBR4 (**Figure 6 A**). We noticed no apparent difference in the levels of MFN2. Neither was there any detectable effect under cycloheximide treatment, which inhibits new protein synthesis to follow turnover, nor under treatment with CCCP, which is an uncoupler that depolarises mitochondria (**Figure 6 A**). CCCP depolarisation was shown to recruit Parkin which ubiquitinates and degrades MFN2 (Tanaka et al. 2010). HEK293 contains endogenous Parkin (Narendra et al. 2008) while HeLa cells lack Parkin (Denison et al. 2003). Therefore, under the conditions we tested, endogenous Parkin did not degrade Mfn2 as was previously reported in cells exogenously-expressing Parkin (Tanaka et al. 2010). Because the absence of UBR4 did not lead to a significant effect on MFN2 levels in HEK293 and HeLa cells, we therefore conclude that UBR4 does not have a role in MFN2 turnover.

The second hit we investigated from MFN2 BioID is p97 (also called VCP), which is an ATPase that plays a role in many pathways including membrane protein degradation and membrane fusion (Stach and Freemont 2017). p97 has been shown to specifically play a role in mitochondrial outer membrane protein turnover, including MFN1 and MFN2 (Tanaka et al. 2010; Xu et al. 2011; Kim et al. 2013). As ATP hydrolysis is required for mitochondrial fusion (**Fig. 4 and 6 B**), we therefore thought that this ATP requirement is for the function of the ATPase p97. We used our in vitro fusion assay, to specifically uncouple a possible role of p97 in fusion from its other functions in the cell. We used NMS-859 (named here p97i) a chemical drug that covalently inhibits p97, by reacting with its active site cysteine 522, with an IC_{50} of 370 nM (Magnaghi et al. 2013). We titrated the drug in our fusion assay, from 100-1000 nM concentrations, and found no significant effect (**Fig. 6 B**), suggesting that while p97 is known to have a role in mitochondrial biology in cells, it does not have a role in cell free fusion assay.

A summary of chapter one: 1) A requirement of ATP hydrolysis was confirmed in mitochondrial fusion with no known protein target for this ATP; 2) we found that ubiquitination and deubiquitylation do not have a role in mammalian mitochondrial fusion using a cell-free assay; 3) UBR4, an E3 ubiquitin ligase, which was identified in MFN2 BioID, did not seem to have a role in MFN2 turnover, both in presence or absence of Parkin, with or without CHX and CCCP treatments; 4) p97, a known ATPase that has a role in MFNs turnover, which was also identified by Mfn2 BioID, has no role in our cell-free mitochondrial fusion assay.

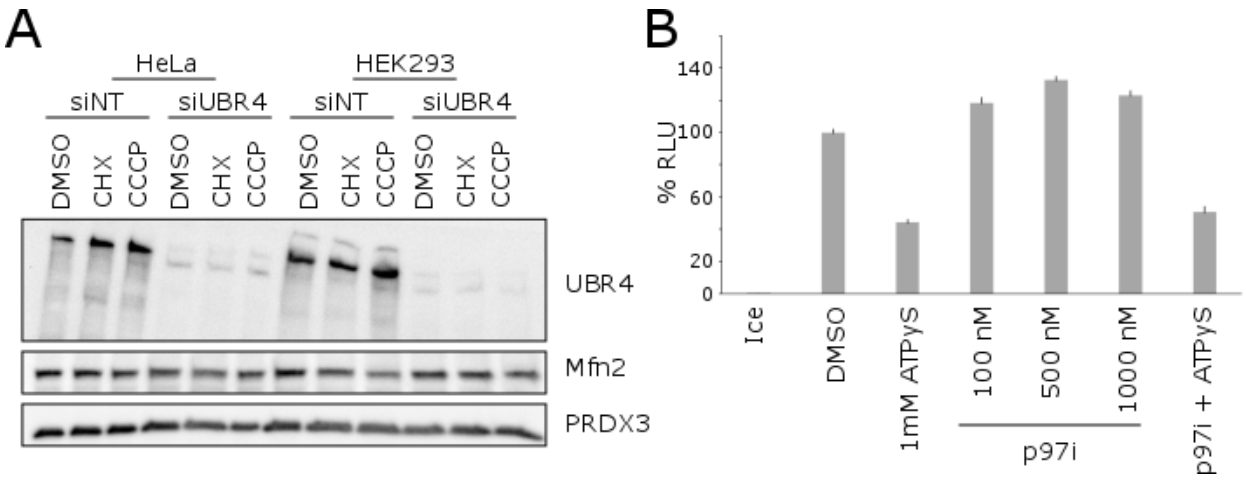

Figure 6. The role of E3 Ub ligase UBR4 in MFN2 turnover and the role of p97 in fusion.

A. siRNA knockdown of UBR4 for 5 days, followed by treatment with 20 ug/ml CHX or 20 uM CCCP for 5h, before processing for Western blot analysis.

B. ATPγS or p97 inhibitor were added to the in vitro fusion assay, followed by luciferase activity measurement, which is normalized to the basal condition. Fusion reaction on ice were used as a control. Reported values are the mean of a single representative experiment performed in duplicate.

Chapter 3.2: Revising MFNs membrane topology and refining the redox regulation of mitochondrial fusion

Metazoan Mfn and Fungal Fzo are evolutionarily divergent GTPases

To better understand mitochondrial outer membrane GTPases involved in mitochondrial fusion, we set out to reconstruct the evolutionary history of these GTPases using homologous protein sequences. Phylogenetic analysis of the GTPase domains of metazoan, fungal and bacterial GTPases (bacterial dynamin-like proteins, BDLPs) suggested that there is no clear relationship between metazoan Mfns and fungal Fzos, nor a relationship between Mfns or Fzos with BDLPs (**Figure 7 A**). This suggests that metazoan and fungal GTPases have diverged to such an extent that the evolutionary history cannot be reliably traced back.

The second observation gleaned from bioinformatics analysis is that metazoan and fungal outer membrane GTPases do share structural features, as was previously described: GTPase domain, HR1, TMD, and HR2. However, metazoan Mfns are predicted to contain a single TMD, as opposed to two TMDs for fungal Fzos (**Figure 7 B**). Mfns contain two C-terminal cysteines which have been reported to have a role in redox regulation of mitochondrial fusion (T. Shutt et al. 2012). Therefore, we performed a manual inspection to determine whether these cysteine residues are conserved. We could not find conservation of the C-terminal cysteines in fungal Fzos. We found a striking correlation between the emergence of multicellular metazoans and the appearance of the cysteines (**Figure 7 C**). This suggests that the appearance of the cysteines perhaps co-insides with a need for redox regulation of fusion in animals.

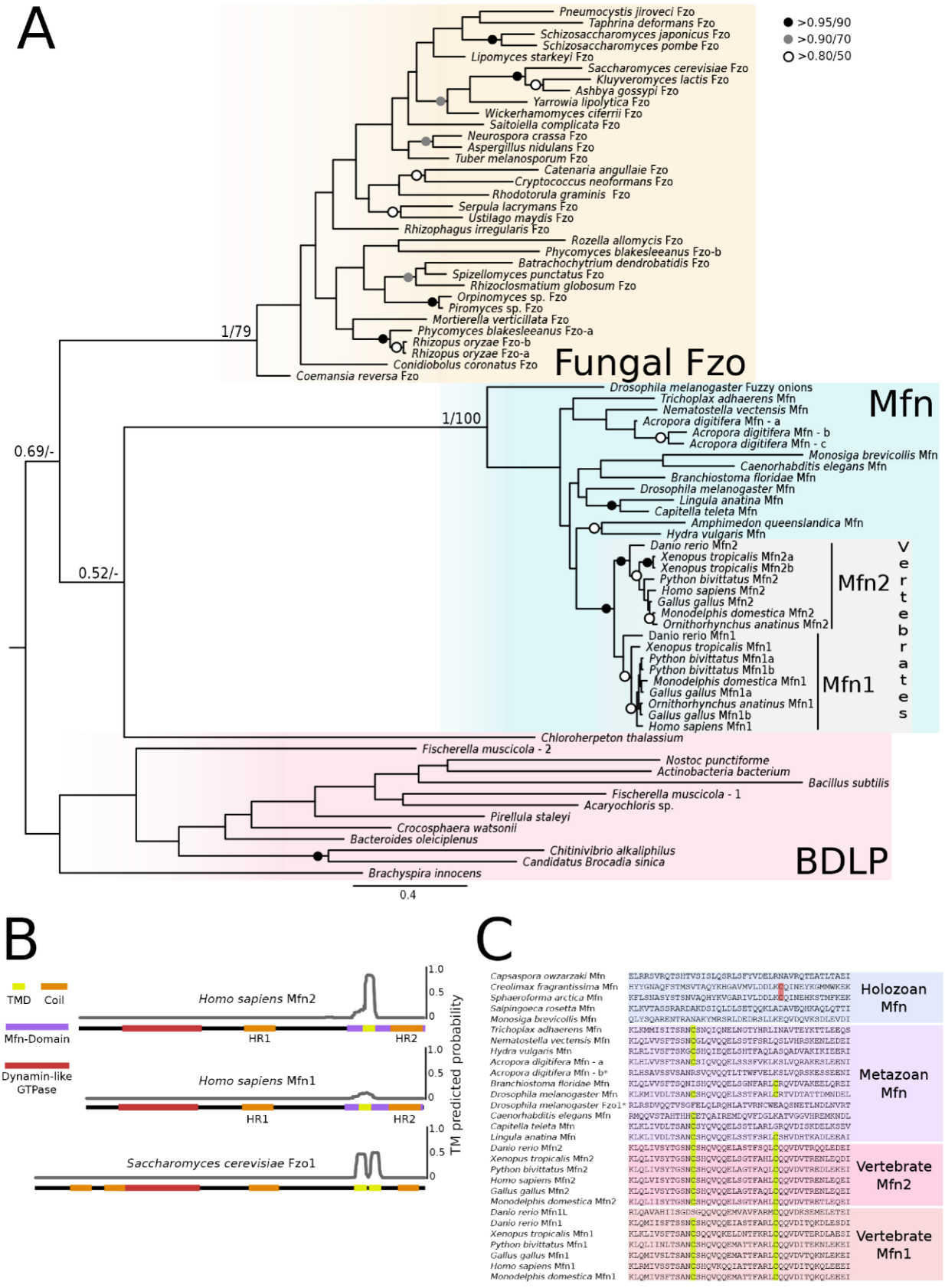
A
>0.95/90
>0.90/70
>0.80/50
Pneumocystis jiroveci Fzo
Taphrina deformans Fzo
Schizosaccharomyces japonicus Fzo
Schizosaccharomyces pombe Fzo
Lipomyces starkeyi Fzo
Saccharomyces cerevisiae Fzo
Kluyveromyces lactis Fzo
Ashbya gossypi Fzo
Yarrowia lipolytica Fzo
Wickerhamomyces ciferrii Fzo
Saitoiella complicata Fzo
Neurospora crassa Fzo
Aspergillus nidulans Fzo
Tuber melanosporum Fzo
Catenaria anguillaie Fzo
Cryptococcus neoformans Fzo
Rhodotorula graminis Fzo
Serpula lacrymans Fzo
Ustilago maydis Fzo
Rhizophagus irregularis Fzo
Rozella allomycis Fzo
Phycomyces blakesleeanus Fzo-b
Batrachochytrium dendrobatidis Fzo
Spizellomyces punctatus Fzo
Rhizoclosmatium globosum Fzo
Orpinomyces sp. Fzo
Piromyces sp. Fzo
Mortierella verticillata Fzo
Phycomyces blakesleeanus Fzo-a
Rhizopus oryzae Fzo-b
Rhizopus oryzae Fzo-a
Conidiobolus coronatus Fzo
Coemansia reversa Fzo
Fungal Fzo
1/79
Drosophila melanogaster Fuzzy onions
Trichoplax adhaerens Mfn
Nematostella vectensis Mfn
Acropora digitifera Mfn - a
Acropora digitifera Mfn - b
Acropora digitifera Mfn - c
Monosiga brevicollis Mfn
Caenorhabditis elegans Mfn
Branchiostoma floridae Mfn
Drosophila melanogaster Mfn
Lingula anatina Mfn
Capitella teleta Mfn
Amphimedon queenslandica Mfn
Hydra vulgaris Mfn
Mfn
1/100
0.69/-
Danio rerio Mfn2
Xenopus tropicalis Mfn2a
Xenopus tropicalis Mfn2b
Python bivittatus Mfn2
Homo sapiens Mfn2
Gallus gallus Mfn2
Monodelphis domestica Mfn2
Ornithorhynchus anatinus Mfn2
Mfn2
Danio rerio Mfn1
Xenopus tropicalis Mfn1
Python bivittatus Mfn1a
Python bivittatus Mfn1b
Monodelphis domestica Mfn1
Gallus gallus Mfn1a
Ornithorhynchus anatinus Mfn1
Gallus gallus Mfn1b
Homo sapiens Mfn1
Mfn1
Vertebrates
0.52/-
Chloroherpeton thalassium
Fischerella muscicola - 2
Nostoc punctiforme
Actinobacteria bacterium
Bacillus subtilis
Fischerella muscicola - 1
Acaryochloris sp.
Pirellula staleyi
Crocosphaera watsonii
Bacteroides oleiciplenus
Chitinivibrio alkaliphilus
Candidatus Brocadia sinica
Brachyspira innocens
BDLP
0.4
B
TMD
Coil
Mfn-Domain
Dynamin-like GTPase
Homo sapiens Mfn2
HR1
HR2
Homo sapiens Mfn1
HR1
HR2
Saccharomyces cerevisiae Fzo1
1.0
0.5
0
TM predicted probability
C
Capsaspora owzarzaki Mfn
Creolimax fragrantissima Mfn
Sphaeroforma arctica Mfn
Salpingoeca rosetta Mfn
Monosiga brevicollis Mfn
Trichoplax adhaerens Mfn
Nematostella vectensis Mfn
Hydra vulgaris Mfn
Acropora digitifera Mfn - a
Acropora digitifera Mfn - b*
Branchiostoma floridae Mfn
Drosophila melanogaster Mfn
Drosophila melanogaster Fzo1*
Caenorhabditis elegans Mfn
Capitella teleta Mfn
Lingula anatina Mfn
Danio rerio Mfn2
Xenopus tropicalis Mfn2
Python bivittatus Mfn2
Homo sapiens Mfn2
Gallus gallus Mfn2
Monodelphis domestica Mfn2
Danio rerio Mfn1L
Danio rerio Mfn1
Xenopus tropicalis Mfn1
Python bivittatus Mfn1
Gallus gallus Mfn1
Homo sapiens Mfn1
Monodelphis domestica Mfn1
Holozoan Mfn
Metazoan Mfn
Vertebrate Mfn2
Vertebrate Mfn1

Figure 7. Bioinformatic analysis of Fzo and Mfn.

(A) Phylogenetic reconstruction of dynamin domain-containing proteins from bacteria, fungi, and animals. Fzo1- and Mfn-like sequences collected by BLAST (Altschul et al., 1997) and HMMer (Eddy, 2011) searching were aligned and subjected to phylogenetic reconstruction using MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) and RaxML v8.2 (Stamatakis, 2006). Support values are as inset. The topology shown is from the MrBayes analysis.

(B) Domain organization *of Saccharomyces cerevisiae* Fzo1 and *Homo sapiens* Mfn1 and Mfn2. TMDs and coiled-coil domains were predicted using TMHMM2.0 (Krogh et al., 2001) and COILS (Lupas, 1996), respectively.

(C) Alignment of Mfn proteins highlighting conserved cysteine residues putatively involved in oligomerization. Mfn proteins were aligned using MUSCLE (Edgar, 2004) and manually inspected to identify conserved cysteine residues.

The C-terminus of human MFN1 and MFN2 resides in the intermembrane space

To experimentally investigate the topology of endogenous MFN2, we performed protease protection experiments on isolated mitochondria from HEK293 cells. We used two MFN2 antibodies, anti-MFN2 (Cell Signaling) recognizing a region just N-terminal of the predicted TMD, and anti-MFN2 (Abnova) recognizing the region C-terminal of the TMD (**Figure 8 A & B**).

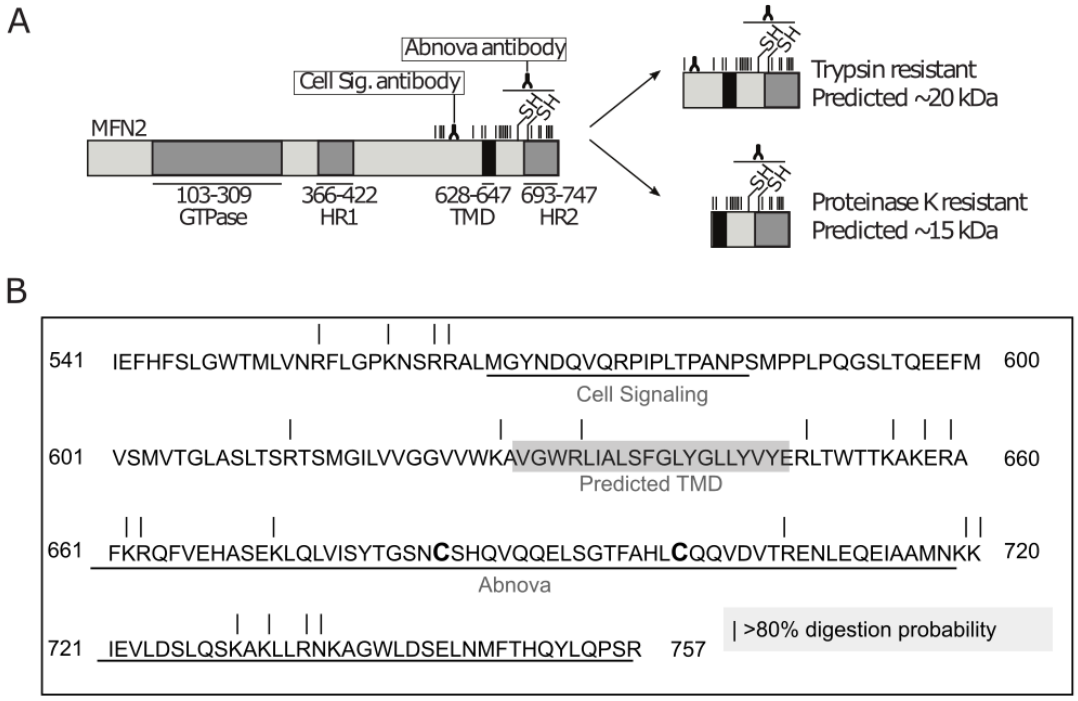

Figure 8. MFN2 domains and predicted digestion fragments.

(A) Scheme showing predicted MFN2 domain structures including the GTPase, heptad repeat 1 (HR1), HR2 and the predicted transmembrane domain (TMD) (Liesa et al., 2009). Trypsin cut sites within the C-terminal region (predicted by PeptideCutter) are shown by vertical lines above MFN2 scheme. The antigenic regions recognized by the two antibodies are indicated. Predicted molecular weights of protected fragments following the two protease treatments are shown, along with the antibody epitopes. Only a single predicted trypsin site resides between the Cell Signaling epitope and the TMD.

(B) Amino acid sequence of MFN2 541-757 showing predicted trypsin digestion sites, anti-MFN2 (Cell Signaling) and (Abnova) antibody binding sites, and predicted transmembrane domain (TMD).

We confirmed the antibodies' recognition regions by using recombinant MFN2 fragments (MFN2 565-757 or MFN2 648-757) (**Figure 9**). To confirm the binding sites of the two anti-MFN2 antibodies, two GST-tagged MFN2 truncated forms were expressed in *E. coli* and purified using glutathione sepharose beads. Then the GST tag was cleaved with thrombin. The thrombin digestion was not complete; therefore, both the GST-tagged (top bands indicated) and the cleaved truncated MFN2 (bottom bands indicated) appear in the blot. One of the constructs consisted of amino acids from 565-757, predicted molecular weight of ~20 kDa. MFN2 565-757 is recognized by both antibodies (**Figure 9, lane 4 both panels**). MFN2 648-757 includes the amino acid residues just following the predicted transmembrane domain, which has a predicted molecular weight of ~14kDa. This recombinant fragment was recognized by the anti-MFN2 Abnova antibody as expected but not the Cell Signaling antibody (**Figure 9, lane 5 both panels**). Isolated mitochondria which were digested with trypsin or proteinase K, were run as controls for antibodies. The Cell Signaling antibody recognized a ~20kDa fragment upon digestion with trypsin, which was lost upon digestion with proteinase K (**Figure 9, left panel lane 2 vs 3**). On the other hand, the Abnova antibody binding site located C-terminal to the TMD was not affected by proteinase K digestion, and as expected the protected fragment is smaller than the trypsin cleaved fragment (**Figure 9, right panel lane 2 vs 3**).

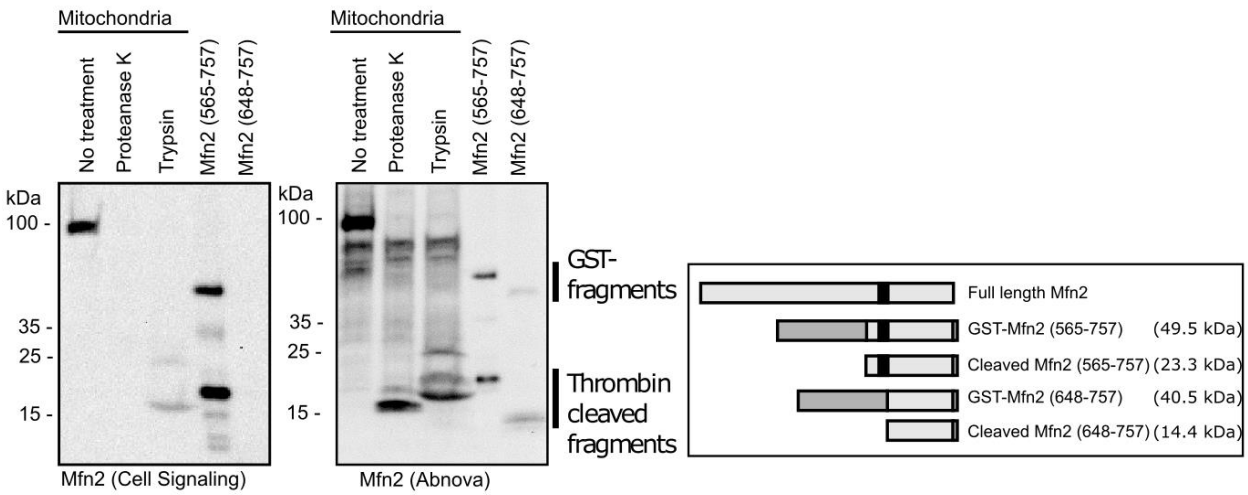

Figure 9. Confirmation of MFN2 antibodies' binding sites.

Two GST-tagged MFN2 truncated forms (565-757 or 648-757) were used to confirm the binding regions of the two commercial antibodies we used to test MFN2 topology. Isolated mitochondria with no treatment or digested with trypsin or proteinase K were processed for Western blotting as well, to confirm specificity. The panel to the right shows schemes of constructs used before and after thrombin cleavage with the predicted molecular weight of each recombinant protein.

Incubating isolated mitochondria with increasing amounts of trypsin, we saw disappearance of the full length MFN2 and accumulation of a protected fragment (~20 kDa) that was detected by MFN2 (Cell Signaling) (**Figure 10 A**) and MFN2 (Abnova) (**Figure 9**) antibodies. However, the protected fragment was digested in the presence of detergent, suggesting that the fragment is not resistant to trypsin (**Figure 10 A**). The MFN2 (Cell Signaling) binding region is predicted to remain "protected" because of lack of high probability trypsin restriction sites between the antibody's recognition site and the TMD (**Figure 8**). OPA1 and TOM20 are controls for IMS and outer membrane protein controls, respectively (**Figure 10 A**). For the next protease protection experiment, we used proteinase K (PK), which is a broad spectrum protease. When we incubated mitochondria with increasing amounts of PK, a protected fragment (~15 kDa) was detected by MFN2 (Abnova) antibody which recognized the C-terminal tail (**Figure 10 B**). This fragment was digested under hypotonic shock condition, which ruptures

the outer membrane while keeping the inner membrane intact, and was digested in the presence of detergent which solubilizes both outer and inner membranes (**Figure 10 B**). Interestingly, the epitope of the MFN2 (Cell Signaling) antibody, which is N-terminal of the TMD, was digested by PK (**Figure 10 B**). The use of the two antibodies helped us predict more accurately the PK-protected fragment sequence detected by the Abnova antibody as being C-terminal of the Cell Signaling recognition site. We used OPA1 and Mia40, as IMS controls, which were protected from PK, unless mitochondria were treated with hypotonic shock or detergent solubilisation (**Figure 10 B**). The matrix control PRDX3 was protected under hypotonic shock as predicted, while digested under detergent solubilisation (**Figure 10 B**).

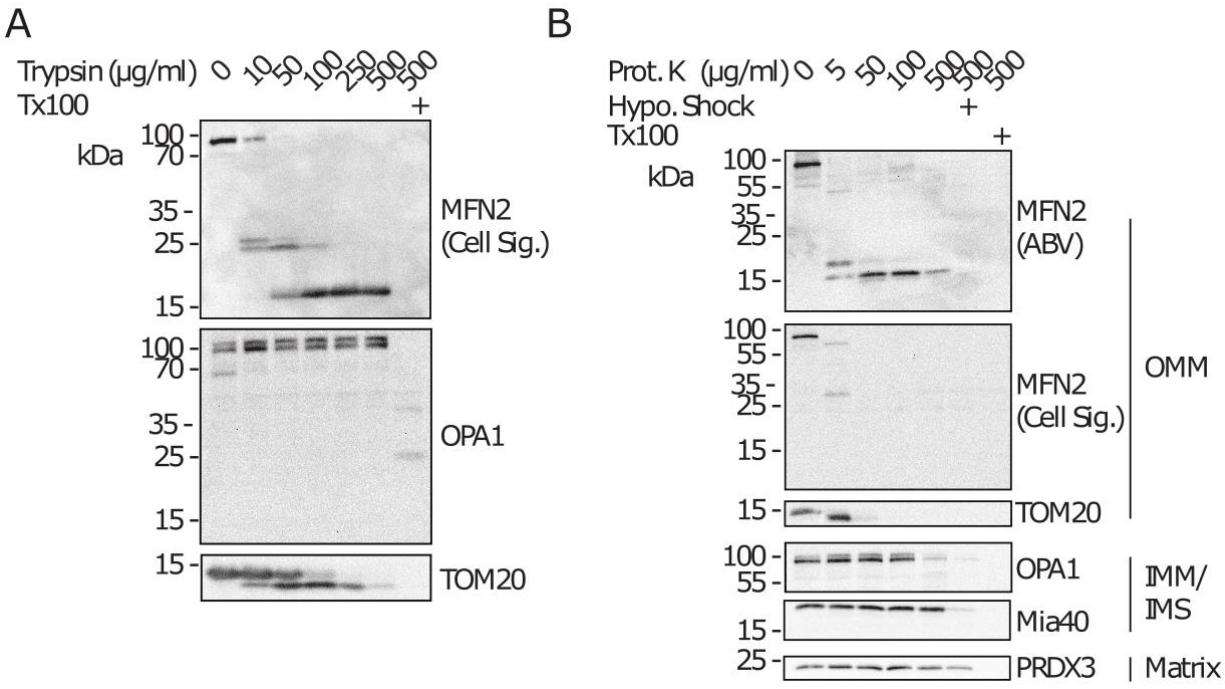

Figure 10. Investigating MFN2 topology using protease protection experiments.

(A) Trypsin protease protection experiment. Isolated mitochondria from HEK293 were incubated with specified trypsin concentration for 20 min on ice. As a control 1% Triton X-100 was added with trypsin (last lane). Trypsin was inhibited with soybean trypsin inhibitor (SBTI) and samples were processed for SDS-PAGE and immunoblotted with anti-MFN2 (Cell Signaling), anti-OPA1 (intermembrane space) and anti-TOM20 (outer membrane).

(B) Proteinase K protection experiment examining MFN2 topology. Isolated mitochondria from HEK293 were incubated with increasing amounts of Proteinase K (PK) on ice. As controls, mitochondria were treated with PK under hypotonic shock which disrupts outer membrane while keeping inner membrane intact, or in the presence of 1% Triton X-100. PK was inhibited by PMSF, before samples were processed for Western blotting and immunoblotted with specified antibodies.

To investigate the whether the topology of MFN1 is similar to that MFN2, protease-protection experiment was performed using an antibody that recognized the C-terminus of MFN1 from Youle lab (Santel et al. 2003). A C-terminal fragment was also detected in the PK experiment, suggesting that MFN1 has a protected C-terminal tail in the IMS as well (**Figure 11**).

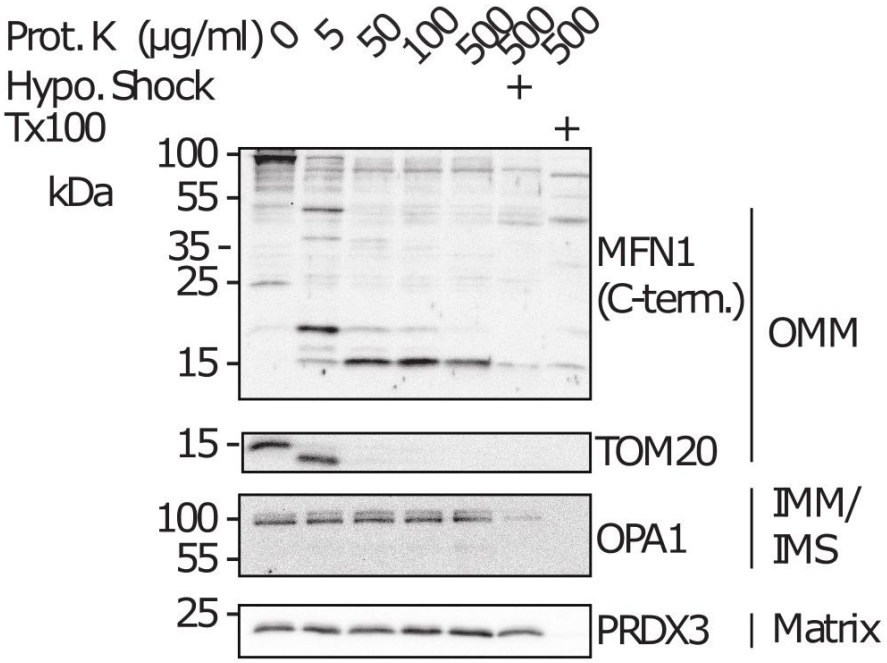

Figure 11. Investigating MFN1 topology using protease protection experiments.

Proteinase K protection experiment examining MFN1 topology. Isolated mitochondria from HEK293 were incubated with increasing amounts of Proteinase K (PK) on ice. As controls, mitochondria were treated with PK under hypotonic shock which disrupts outer membrane while keeping inner membrane intact, or in the presence of 1% Triton X-100. PK was inhibited by PMSF, before samples were processed for Western blotting and immunoblotted with specified antibodies.

To independently confirm the topology of MFN2 we used a cysteine alkylation experiment. In this approach mitochondria are incubated with polyethyleneglycol-maleimide (mPEG-mal) which covalently reacts with accessible cysteines, thereby altering protein migration rate during electrophoresis. MFN2 has two cysteine residues in the C-terminal tail (C684 and C700). Therefore, we asked whether these two cysteines were exposed to the cytosolic or IMS environment. If the cysteines are in the IMS, they will not be modified with the mPEG-mal (**Figure 12**).

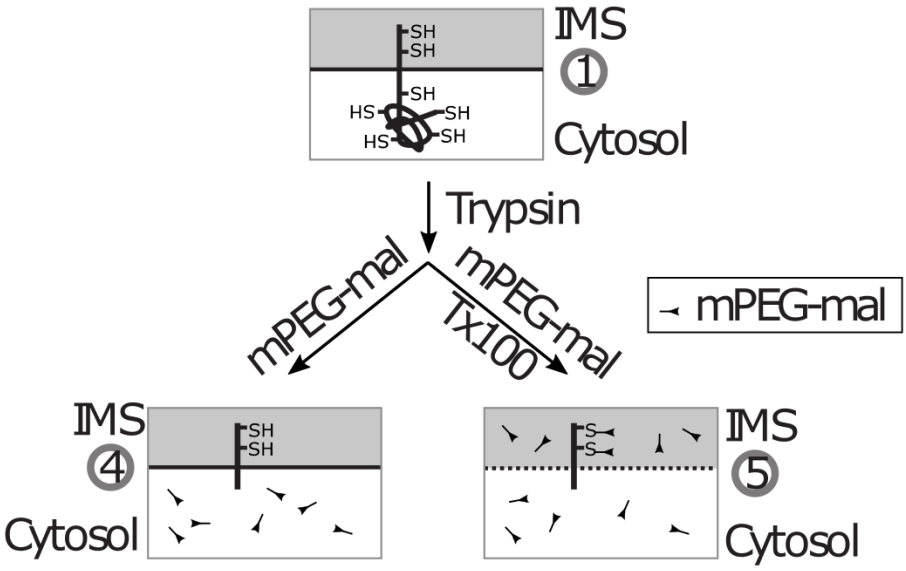

Figure 12. Model to illustrate the treatments used in the mPEG-mal experiment.

Following trypsin treatment, mPEG-mal (indicated in the legend) can only conjugate to free cysteine residues exposed on the cytosolic face of the mitochondrial outer membrane (shown as –SH). Upon detergent solubilization, all cysteine residues become exposed.

We incubated intact mitochondria with 10 kDa mPEG-mal, which cannot cross the outer membrane through VDAC (Colombini 1980). In intact mitochondria treated with mPEG-mal, full length MFN2 shifts in molecular weight, migrating at a slower rate on SDS-PAGE, due to full-length MFN2 containing many cysteine residues in the N-terminus (**Figure 13 A, lane 1 vs 2**). Digesting mitochondria with trypsin, we again detected the protected C-terminal fragment (**Figure 13 A, lane 3**). We then

treated mitochondria with mPEG-mal, followed by trypsin digestion. The protected fragment remained unconjugated with mPEG-mal (**Figure 13 A, lane 4**), migrating at the same rate as mPEG-mal-untreated trypsin-digested mitochondria (lane 3), suggesting that the two cysteines are inaccessible to the mPEG-mal. To confirm that the two cysteines do react with mPEG-mal when made accessible, mitochondria were trypsin digested, followed by treating the mitochondria with mPEG-mal in the presence of detergent. The protected fragment was conjugated with mPEG-mal in the presence of detergent and its migration shifted on the SDS-PAGE (**Figure 13 A, lane 5**). This reaction with mPEG-mal in the presence of detergent was prevented when intact mitochondria, prior to mPEG-mal treatment, were pre-treated with membrane-permeable N-ethylmaleimide (NEM), which irreversibly reacts with free cysteines, thereby making them unavailable for mPEG-mal (**Figure 13 A, lane 6**). The 20kDa fragment is not trypsin resistant, as it is digested when made accessible in the presence of detergent (**Figure 13 A, lane 7**). The cysteine alkylation experiment was done with MFN2 (Cell Signaling) antibody (**Figure 13 A**) and MFN2 (Abnova) antibody (**Figure 13 B**). We saw a reduction in the strength of the antibodies' recognition of the mPEG-mal conjugated MFN2 C-terminal fragment (**Figure 13 A & B, lane 5**), especially with the MFN2 (Abnova) antibody which binds where the alkylated cysteines are.

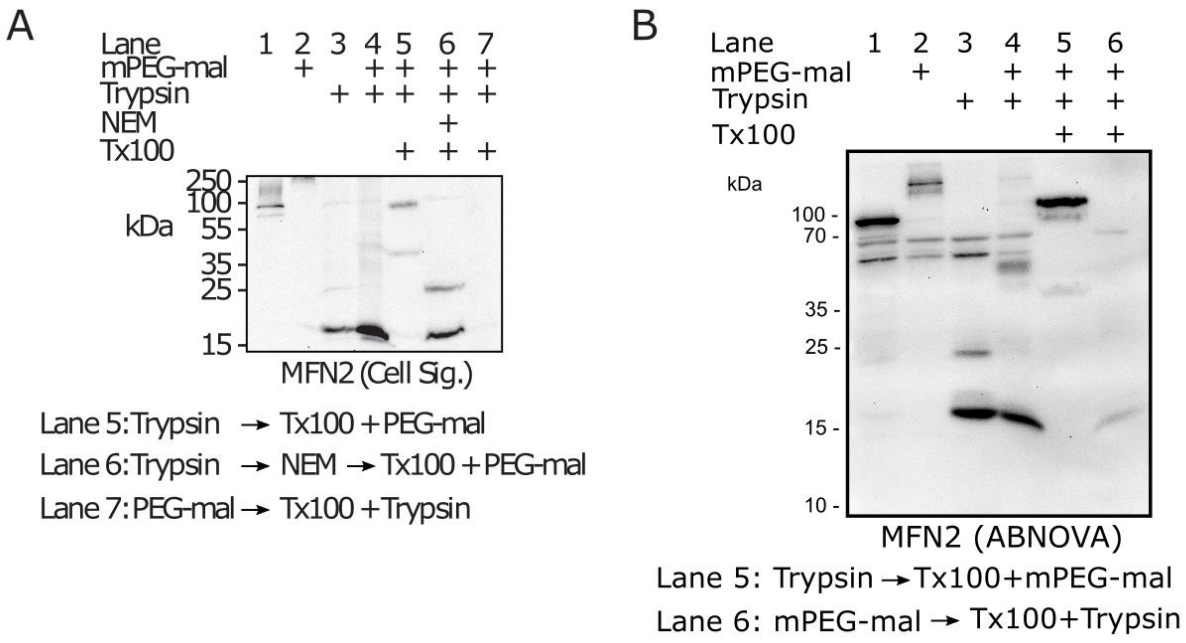

Figure 13. Validation of MFN2 topology using cysteine alkylation, PEGylation experiment.

(A) Isolated mitochondria from HEK293 were treated with 10kDa mPEG-mal, which reacts with cysteine residues, and shifts full-length MFN2 into higher molecular weight conjugates (lane 2). Digestion with 500 µg/ml trypsin is shown in lane 3, revealing the protected fragment. Mitochondria treated with mPEG-mal prior to trypsin digestion did not show any shift in the protected fragment (lane 4). However, the trypsin protected fragment was conjugated when mPEG-mal was added in the presence of Triton X-100 to solubilize the membranes (lane 5). Treating trypsin digested mitochondria with NEM, which reacts with cysteines before the treatment with mPEG-mal, prevents the binding of mPEG-mal and the shift of the trypsin protected fragment (lane 6). The addition of trypsin in the presence of detergent lead to digestion of the fragment, confirming that the fragment is protease sensitive in solubilized lysates (lane 7).

(B) As in (A), except MFN2 (Abnova) antibody was used to reveal conjugated MFN2.

To further investigate the effect of mPEG-mal conjugation on the two C-terminal cysteines in the absence of trypsin digestion, we treated intact mitochondria with mPEG-mal in the absence or presence of detergent. In the absence of detergent, MFN2 shifted in molecular weight, suggesting mPEG-mal conjugation of full-length MFN2 (**Figure 14 A & B, lane 1 vs 2**). However, the signal was greatly reduced when mitochondria were treated with mPEG-mal in the presence of detergent, exposing the C-terminal

cysteines to mPEG-mal (**Figure 14 A & B, lane 2 vs 3**), again validating without trypsin digestion that the C-terminal cysteines are not exposed to the cytosolic side. The effect of PEGylation on the antigenicity was confirmed by treating purified recombinant C-terminal fragment with mPEG-mal (**Figure 14 C**).

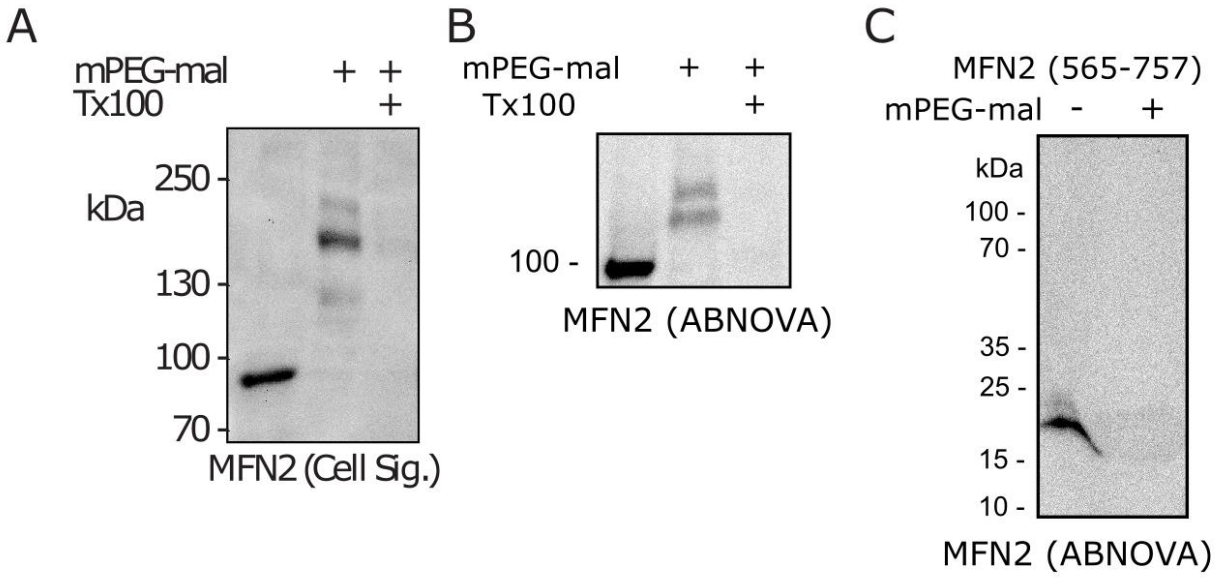

Figure 14. Effect of PEGylation of C-terminal cysteines on binding of MFN2 antibodies

(A) and (B) To test whether PEGylation of the C-terminal domain results in a loss of antigenicity in the absence of trypsin, intact mitochondria were treated with mPEG-mal in the absence (land 2) or presence (lane 3) of detergent, followed by processing for SDS-PAGE, and blotting with (A) MFN2 (Cell Signaling) or (B) MFN2 (Abnova) antibodies. Full-length Mfn2 shifts up upon PEGylation (lane 2). In the presence of detergent the antibody loses antigenicity and MFN2 PEGylated bands disappear (lane 3).

(C) PEGylation of recombinant MFN2 (565-757) again reveals a loss in antigenicity of MFN2 (Abnova) antibody.

We concluded that MFNs contain a single membrane spanning domain, a topology different to the previously predicted one, from the following: 1) the trypsin and PK digestion experiments, with the use of two different antibodies permitting us to predict the sequence of the protected fragment; 2) cysteine alkylation experiments, independently confirming the localization of the two C-terminal cysteine residues within the IMS.

Having a single TMD near the C-terminal end raises the question of tagging MFNs. Does tagging the C-terminus interfere with the targeting, membrane insertion, or function of the protein? MFNs have been tagged at both ends by different groups. Mfns tagged at the C-terminus with 16xMyc-1x6His tag has been reported to rescue mitochondrial morphology in either Mfn1-null or Mfn2-null MEFs (Chen et al. 2003). We acquired the Mfn2-16xMyc-1x6His construct from Addgene and transiently transfected it into HEK293 cells. We performed protease protection experiments on mitochondria isolated from these cells and found that the C-terminus of the tagged protein was protease accessible as detected by Myc antibody (**Figure 15 A**). Endogenous MFN2 C-terminus was protected as seen by MFN2 (Abnova) antibody (**Figure 15 A**). To investigate membrane insertion of tagged Mfn2, we then performed alkaline carbonate, Triton X-100, and urea extraction experiments on the Mfn2-16xMyc-1x6His as compared with endogenous MFN2 from non-transfected cells. Both tagged Mfn2 and endogenous MFN2 were efficiently extracted by Triton X-100, suggesting that protein aggregation is not a problem (**Figure 15 B**). With the alkaline extraction, a small portion of the tagged Mfn2 was detected in the soluble fraction; however, almost half of Mfn2-16xMyx-1x6His was solubilized with urea extraction (**Figure 15 B, bottom panel**), suggesting that the tagged Mfn2 was not properly inserted in the membrane and seems to be associated with mitochondrial membranes primarily through hydrophobic interactions and to a lesser extent through hydrophilic interactions. We used SDHA, a protein that loosely associates with the inner membrane, and Porin, which spans the outer membrane, as controls. We also used mitochondria from non-transfected cells as a control for the behavior of the endogenous MFN2 under the same experimental conditions, which was not extracted with alkaline carbonate or urea, remaining mainly in the membrane fraction (**Figure 15 B, top panel**).

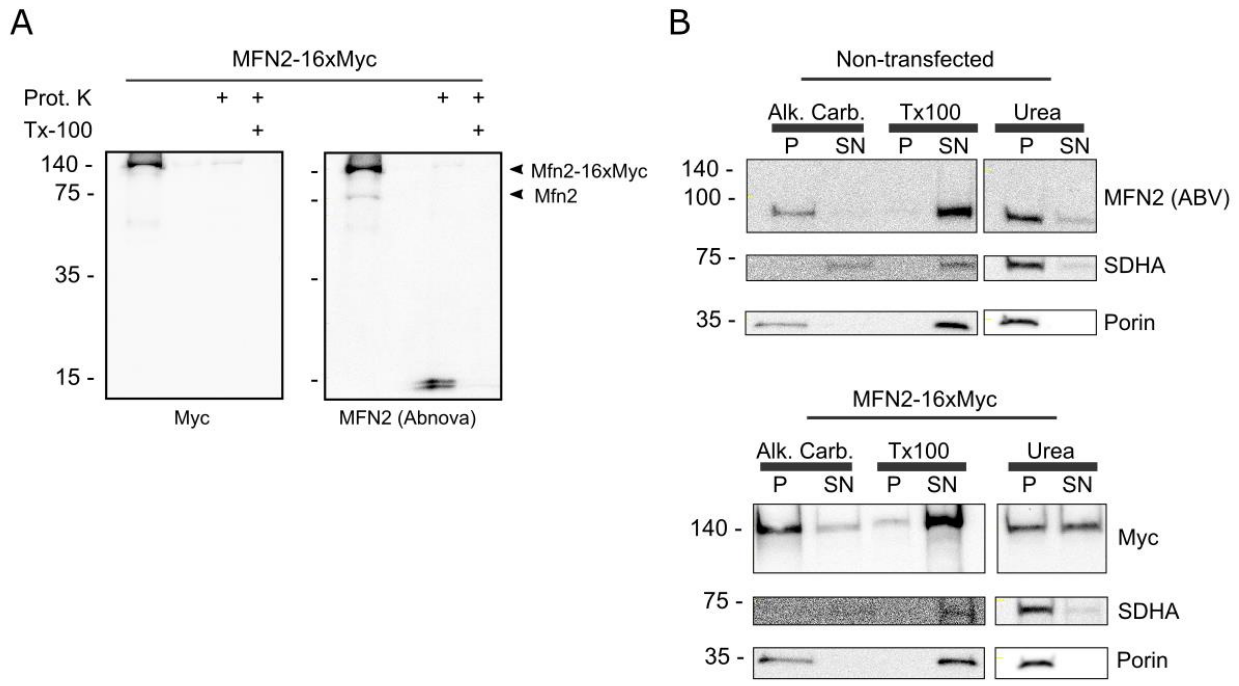

Figure 15. Topology of the C-terminally tagged Mfn2-16xMyx-1x6His

(A) Proteinase K digestion of isolated mitochondria from Mfn2-16xMyc-1xHis-transfected HEK293 in the presence or absence of detergent. Samples were processed for Western blotting, and immunoblotted with Myc antibody followed by reblotting with Mfn2 (Abnova) antibody without stripping the membrane. This revealed that the Myc epitope tags were completely protease accessible, unlike endogenous MFN2.

(B) To determine whether the tagged Mfn2 was properly inserted into the outer membrane, we performed alkaline carbonate and urea extraction and Triton X-100 solubilization. After extraction the samples were processed for Western blotting. SDHA and Porin were used as controls.

This tagged Mfn2 construct was previously reported to be functional (Chen et al. 2003). Therefore, we transfected untagged MFN2 or Mfn2-16xMyc-1x6His into Mfn2-null MEFs. 69% of cells in Mfn2-null MEFs reveal a fragmented mitochondrial morphology (n = 191 cells), while the remaining cells had in the intermediate and tubular morphologies (**Figure 16**). Transfected cells with untagged Mfn2 rescued the fragmented morphology (>90% tubular, n = 153), regardless on the level of Mfn2 expression (**Figure 16**). Mfn2-16xMyc-1x6His partially rescued the Mfn2-null morphology (55% tubular, n = 171

cells); however, 24% of cells still showed a fragmented morphology (**Figure 16**). The ability to rescue appeared to correlate with very high expression of the Mfn2-16xMyc-1x6His protein, with tubular mitochondrial morphology in cells possessing high protein expression and fragmented morphology in low protein expression cells, respectively (**Figure 16, left panel, bottom image**). The above analysis of Mfn2 tagging reveals that although the C-terminally tagged Mfn2 retained some function, it was not inserted properly in the membrane. This suggests that the localization of the C-terminal domain within the IMS is not absolutely essential to drive fusion, rather may play a regulatory function in the activation of fusion, presumably through redox regulated oligomeric assemblies.

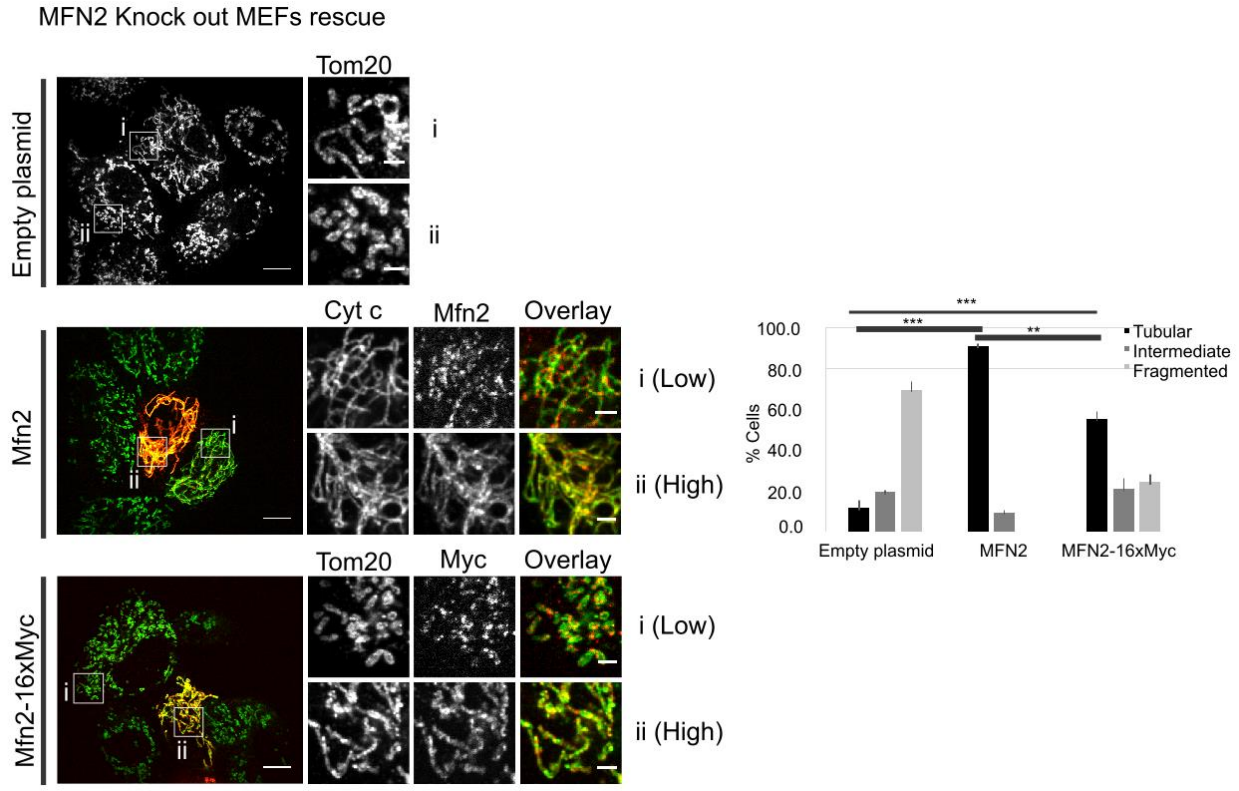

Figure 16. Functional analysis of C-terminally tagged Mfn2-16xMyx-1x6His.

Mfn2-null MEFs were transfected with either empty plasmid, or untagged MFN2, or Mfn2-16xMyc-1xHis. After 17 hrs, cells were fixed and processed for immunofluorescence. Transfected cells were imaged and representative images are shown. Mitochondrial morphology was quantified into “tubular,” “intermediate,” or “fragmented” categories. The plot presents

quantification of three independent experiments. The total number of cells counted is 191, 153, and 171 cells, for empty plasmid, MFN2, and Mfn2-16xMyc-1x6His, respectively. Scale bar is 10 µm; scale bar for insets is 2 µm. Statistical significance was analyzed using unpaired Student's t-test. **: P<0.01, ***: P<0.005.

The C-terminus of MFN2 is required, but not sufficient, for targeting to mitochondria

To answer the simple question of whether the C-terminus is required for fusion, we made a MFN2 mutant lacking the C-terminus after the TMD, removing residues 648 to the end. Because we knew that the C-terminus plays a role in targeting the protein, we replaced the C-terminus of MFN2 with the C-terminus of the mitochondrial tail-anchored protein VAMP1B, which consists of three amino acid residues RRD, which was previously reported to dually localize to mitochondria and ER (Rojo et al. 2002). This mutant MFN2 (1-647)-RRD failed to target to mitochondria as tested by immunofluorescence. We then removed the endogenous TMD with the C-terminus, replacing it with TMD and C-terminus of other proteins that have been used to target proteins to mitochondria, the mitochondria SNARE VAMP1B or the insertion sequence of the bacterial ActA which was previously used to target BCL2 and full-length Mfn2 to mitochondria (Isenmann et al. 1998; Lan, Isenmann, and Wattenberg 2000; Zhu et al. 1996; Sugiura et al. 2013). These truncated MFN2 mutants failed to efficiently target to mitochondria as well (**Figure 17, table of mutants and localization**). This shows that the C-terminus is required for mitochondrial targeting of MFN2. Therefore, we decided to create a MFN2 mutant lacking HR2 only (consisting of residues 1-693), HR2 being the known functional feature of the C-terminus. This ΔHR2 MFN2 targeted efficiently to mitochondria as determined by immunofluorescence, suggesting that the mitochondrial targeting information is within the stretch of amino acids between the TMD (ending at residue 647) and HR2 (starting at residue 694). We tested whether the C-terminus was alone sufficient to target to mitochondria. We found that while the C-terminus was required for targeting to mitochondria in MFN2, the C-terminus alone (648-757) was not sufficient to target to mitochondria but remained cytosolic in cells (**Figure 17**).

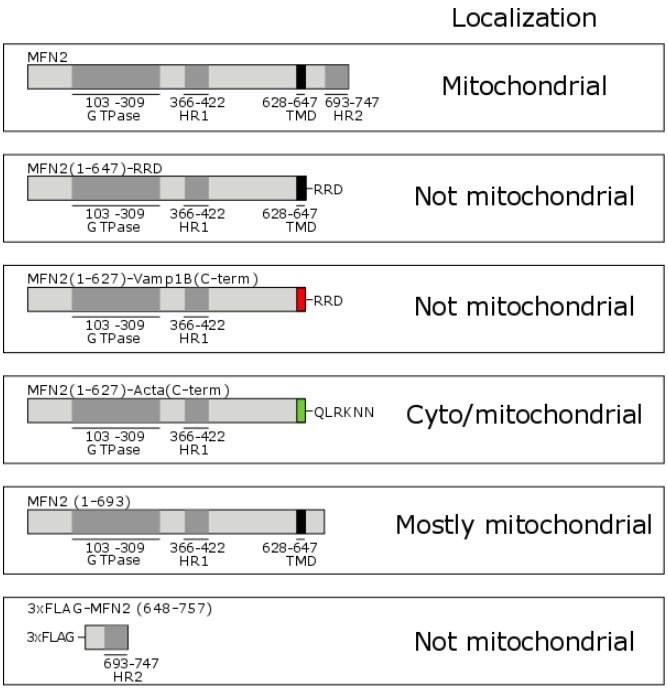

Figure 17: List of MFN2 mutants and their localization

Truncations of MFN2 C-terminus were created and transiently transfected into Mfn2-null MEFs or HeLa cells. Localization was determined by immunofluorescence, which is listed as “mitochondrial” or “Not mitochondrial.”

HR2 of MFNs is not required for fusion

We used this ΔHR2 MFN2, which efficiently targeted to mitochondria, to test whether HR2 is required for mitochondrial fusion. ΔHR2 MFN2 was transfected into Mfn2-null MEFs, the cells which have been used in previous studies. Over 60% of these Mfn2-null MEFs have a fragmented mitochondrial morphology, as was previously reported, which can be rescued with the expression of untagged full-length MFN2 (>90% of cells with tubular morphology) (**Figure 18**). Expression of ΔHR2 MFN2 partially rescued the mitochondrial fusion defect, with 53.2% of cells with tubular morphology (**Figure 18**); however the rescue of ΔHR2 MFN2 was not as effective as of full-length MFN2 (>90% of cells with tubular morphology). This argues that HR2 is not necessary for fusion, because MFN2 lacking HR2 was still capable of rescuing mitochondrial fusion defects in Mfn2-null MEFs. However, the partial rescue

in comparison to full-length MFN2 could be due to: 1) HR2 having a regulatory function, which impairs the robustness of fusion when absent; 2) MFN2 lacking HR2 might be less stable, leading to a lower amount of protein; 3) the observation that targeting of ΔHR2 MFN2 is less efficient than full-length MFN2, as can be seen by immunofluorescence; 4) it is also likely that fusion is done by endogenous Mfn1 as cells we used are only lacking Mfn2. It is more likely that it is a combination of these, as can be inferred from our results and from previous work. Nevertheless, ΔHR2 MFN2 does rescue mitochondrial morphology defects in Mfn2-null MEFs.

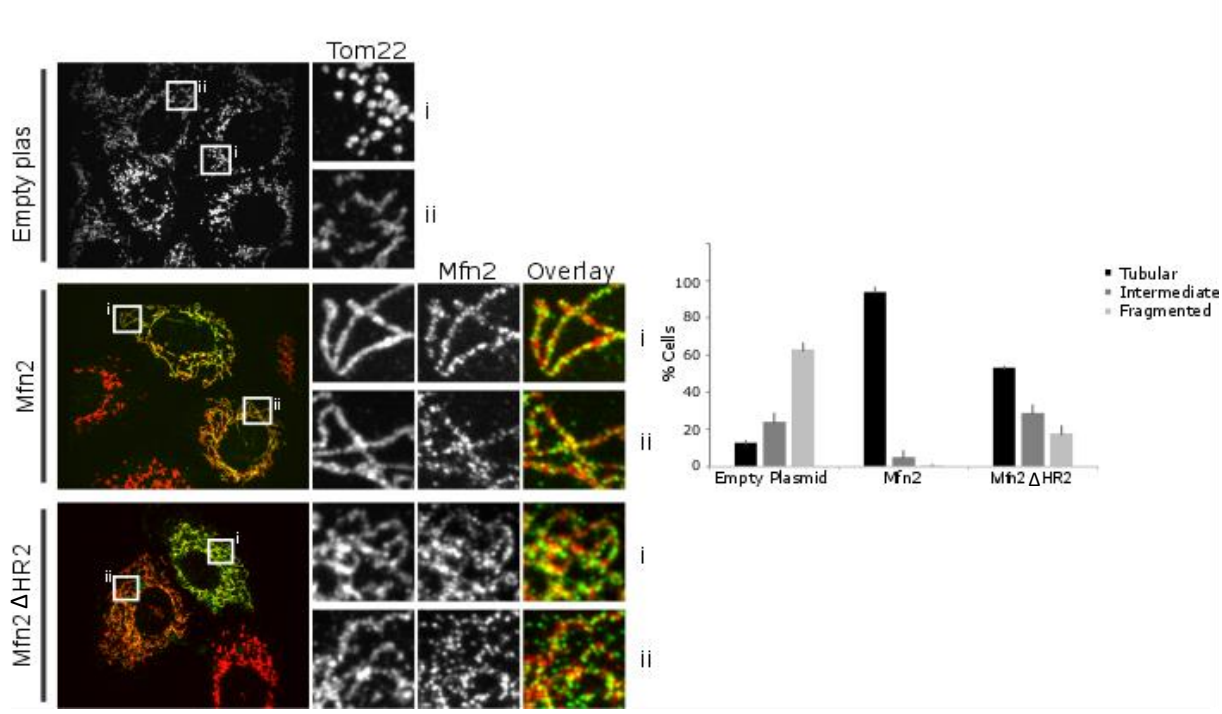

Figure 18: HR2 of MFN2 is not required for mitochondrial fusion.

Empty plasmid, full-length MFN2, or ΔHR2 MFN2 were transfected into Mfn2-null cells, followed by immunofluorescence analysis, staining with Tom22 and MFN2. Samples were imaged and mitochondrial morphology was quantified into three categories (see methods for details). The total number of cells counted is 209 (n = 3), 121 (n =2), and 137 (n = 3) cells, for empty plasmid, MFN2, and ΔHR2 MFN2, respectively.

We decided to test whether MFN1 lacking HR2 behaves in the same manner as ΔHR2 MFN2. We created MFN1 (1-672), named hereafter ΔHR2 MFN1 and tested it for its ability to rescue mitochondrial morphology in Mfn1-null MEFs. Mfn1-null MEFs show a fragmented mitochondrial morphology (>80% of cells) (**Figure 19**). Though both Mfn1-null and Mfn2-null MEFs reveal fragmented mitochondria, mitochondrial morphology of Mfn1-null cells is discernably different from that of Mfn2-null cell, the latter being larger and swollen-looking (**Figure 18 versus 19**). Transfection of ΔHR2 MFN1 partially rescued the morphology (20.9% tubular and 53.6% intermediate) (**Figure 19**). This again suggests that HR2 is not required for fusion.

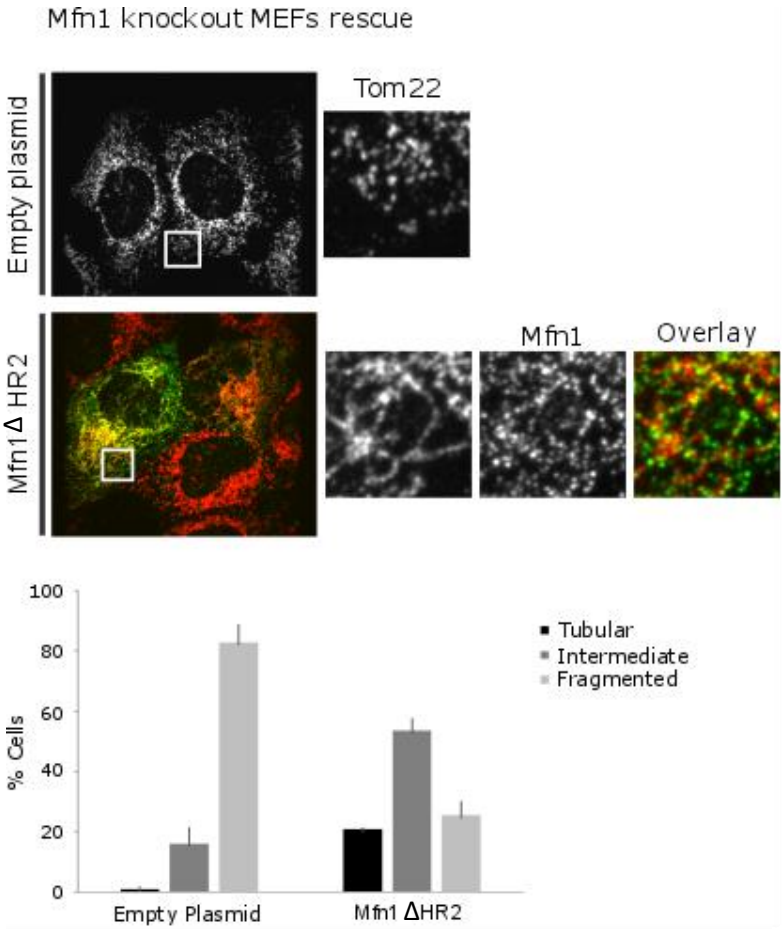

Figure 19: HR2 of MFN1 is not required for mitochondrial fusion.

Empty plasmid or ΔHR2 MFN1 were transfected into Mfn1-null MEFs, followed by immunofluorescence analysis, staining with Tom22 and MFN1. Samples were imaged and mitochondrial morphology was quantified into three categories (see methods for details). The total number of cells counted from three biological replicates is 95 and 100 cells, for empty plasmid and ΔHR2 MFN1, respectively.

A redox sensing role of the IMS-localized C-terminus

The C-terminal cysteine residues have been previously implicated in oxidative stress induced fusion, specifically responding to oxidized glutathione (T. Shutt et al. 2012). Glutathione is present in millimolar amounts in cells and is usually expressed as a ratio of reduced-to-oxidized glutathione or as glutathione redox potential. Therefore we sought to determine the glutathione redox potential that activates mitochondrial fusion in vitro. This was achieved by adding varying amounts of reduced and oxidized glutathione to our cell-free fusion assay and quantified the effect on fusion. We found that mitochondrial fusion activation was proportional to how oxidizing the redox potential is (**Figure 20 A**). The more oxidizing the potential, the higher the activation of fusion, the activation plateauing at redox potential -206 and -197 (**Figure 20 A**). We processed the samples for Western blotting under denaturing nonreducing conditions, conditions used to detect newly formed disulfide bonds. We observed accumulation of high molecular weight MFN2 oligomers at the oxidizing redox potential, which correlates with the activation of fusion under the same conditions (**Figure 20 B**). These complexes confirm the previously observed MFN2 oligomers formed in response to GSSG (T. Shutt et al. 2012). Glutathione redox potentials have been measured in cells using several fluorescence probes in yeast and mammalian cells (Hu, Dong, and Outten 2008; Kojer et al. 2012; Bilan and Belousov 2017). While the measured potentials vary depending on the probes and cell types used in the studies, the redox potentials activating fusion which we observed, fall within physiological reported ranges.

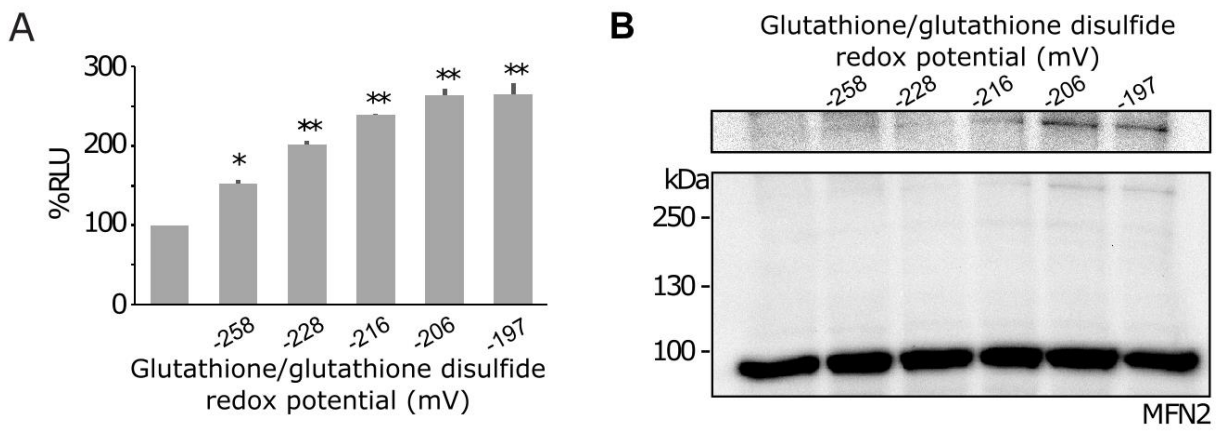

Figure 20: Physiological glutathione redox potential activates mitochondrial fusion.

(A) Mitochondria containing each half of the matrix-targeted split-luciferase probes were incubated within the cell-free fusion assay system with different ratios of GSH:GSSG, corresponding to increasing glutathione redox potential. Mitochondrial fusion under these conditions was quantified by measuring luciferase activity. Luciferase counts were normalized to standard condition. Fusion is activated as the potential become oxidizing. Reported values are the mean of three biological replicates, each performed in duplicate. Error bars are means ± SEM. Statistical significance was analyzed using unpaired Student's t-test. *: $P<0.05$, **: $P<0.01$.

(B) Fusion samples (from A) were analyzed under denaturing non-reducing SDS-PAGE followed by immunoblotting MFN2 (Abnova) antibody. Top panel shows higher exposure the high molecular weight MFN2 complexes.

Disturbances in the glutathione ratio from reduced to oxidized (i.e. increased formation of the GSSG) occurs under oxidative stress, when ROS is produced in excess. There are two possible sources of ROS, mitochondrial and extra-mitochondrial. Mitochondria, as a side effect of their metabolic functions, produce ROS, especially the ROS produced from the electron transport chain and TCA cycle complexes (Brand 2016). As a source of mitochondrial ROS, we used inhibitors of Complex III, Antimycin A and myxothiazol (Starkov and Fiskum 2001; Muller, Liu, and Van Remmen 2004). The addition of Antimycin A and myxothiazol to our mitochondrial fusion assay inhibited fusion, when the ATP regeneration system in the fusion assay was mitochondrial, by feeding succinate through the electron transport chain (**Figure**

21 A and B, Succinate). Complex III inhibitors inhibit succinate-dependent ATP production. This inhibition of fusion by electron transport chain inhibitors has been previously reported (Mishra et al. 2014). Therefore to bypass this problem, we added an extra-mitochondrial ATP regeneration system, by enzymatically maintaining ATP levels by creatine kinase and creatine phosphate. This extra-mitochondrial ATP regeneration system rescued fusion in the presence of Complex III inhibitors (**Figure 21 A and B**). This again confirms that ATP is required for mitochondrial fusion. Under creatine kinase/creatine phosphate, both antimycin A and myxothiazol activated mitochondrial fusion, in a GSH-dependent manner (**Figure 21 A and B**). Myxothiazol activated fusion to a higher degree than antimycin A (**Figure 21 A, creatine kinase/phosphate**), suggesting that either myxothiazol produces more ROS than antimycin A, or the orientation of the ROS produced by these inhibitors is different. Antimycin A blocks Complex III at the Q_i site on the matrix side while myxothiazol block at the Q_o site at the IMS side of the membrane, suggesting the ROS production the two drugs promote is likely to be directed to the matrix or the IMS, respectively (Starkov and Fiskum 2001).

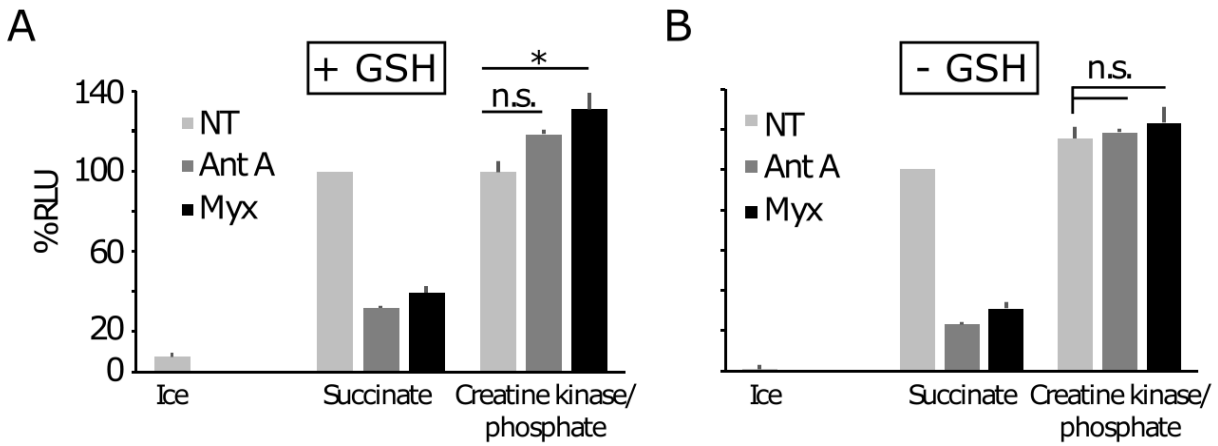

Figure 21: Mitochondrial ROS activates mitochondrial fusion in a GSH-dependent manner.

(A) and (B) Mitochondrial fusion assays were carried out with succinate to drive respiration. This reaction for 30 minutes at 37 ^{o}C was set as the basal 100%, against which the other reactions were normalized. Reactions carried out with succinate at 4^{o}C

are shown (Ice). Alternatively, an exogenous ATP regenerating system creatine kinase and its substrate creatine phosphate was added in place of succinate, which was sufficient for fusion (NT grey bars, succinate vs. creatine kinase/phosphate). Mitochondria under each condition were treated with complex III inhibitors (1 µM antimycin A or 0.4 µM myxothiazol) to produce ROS in the presence (A) or absence (B) of 4mM GSH to drive the formation of GSSG. When respiration and ATP production was driven by succinate, fusion was inhibited under these conditions. The addition of exogenous ATP regeneration system, creatine kinase and its substrate creatine phosphate, rescued the inhibition of fusion inhibition caused by blocking Complex III by Ant A and Myx. In the presence of exogenous ATP, fusion was activated upon complex III inhibition, as long as GSH was present (A). Reported values are the mean of three biological replicates, each performed in duplicate. Error bars are means ± SEM. Statistical significance was analyzed using unpaired Student's t-test. *: P<0.05.

To bypass this ambiguity, we decided to provide a controllable ROS source, both in localization and amount. We added xanthine oxidase, which produced ROS enzymatically, to our fusion assay. Increasing the amount of xanthine oxidase did not significantly activate fusion (**Figure 22**). However, in the presence of GSH, mitochondrial fusion was activated as the amount of xanthine oxidase increased (**Figure 22**), suggesting that GSH is converted to GSSG in the presence of ROS.

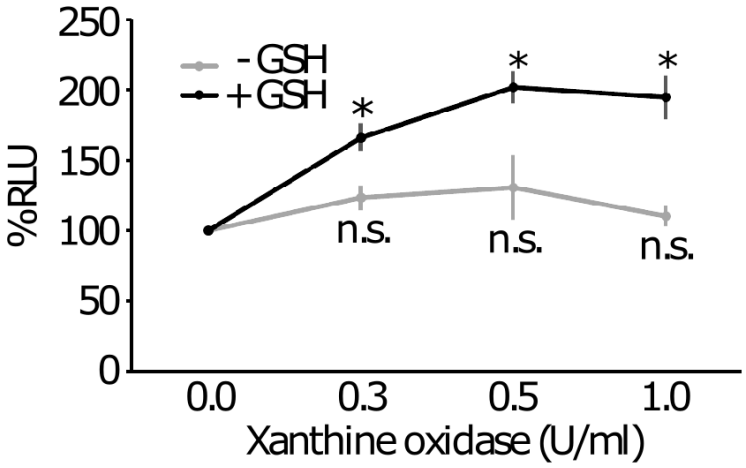

Figure 22: Extra-mitochondrial ROS activates mitochondrial fusion in a GSH-dependent manner.

Mitochondria were incubated in the presence of increasing amounts of xanthine oxidase/xanthine, which produces ROS enzymatically. In the absence of GSH, mitochondrial fusion was not activated significantly by ROS (light gray). In the presence of

4mM GSH, fusion was activated gradually corresponding to increasing specific activity of xanthine oxidase (black). Reported values are the mean of three biological replicates, each performed in duplicate. Error bars are means ± SEM. Statistical significance was analyzed using unpaired Student's t-test. *: P<0.05.

GSSG is known to induce disulfide-based MFN2 oligomerization (**Figure 23 A, last lane**). Therefore, we decided to look at MFN2 oligomerization under xanthine oxidase treatment. In the absence of GSH, MFN2 oligomers accumulated with the increase of the xanthine oxidase, when the samples we processed under denaturing, non-reducing conditions (**Figure 23 A**); however, surprisingly, no detectable MFN2 oligomers were observed in the presence of GSH (**Figure 23 A**), under which fusion was activated (**Figure 22**). We thought that this might be due to a destabilizing effect of GSH on produced MFN2 disulfide-linked oligomers. To test this, we first incubated isolated mitochondria, under fusion conditions, with GSSG for 20 minutes. After 20 minutes, GSH was added and samples were incubated for several durations. In the absence of GSH, we observed the formation of MFN2 oligomers which did not form in the absence of GSSG (**Figure 23 B, lane 1 vs 2**). The addition of GSH after GSSG incubation caused a gradual reduction of the MFN2 oligomers, suggesting a reaction of GSH with the formed intermolecular disulfide bonds, to perhaps recycle the disulfide conjugated oligomers into a monomeric form (**Figure 23 B**).

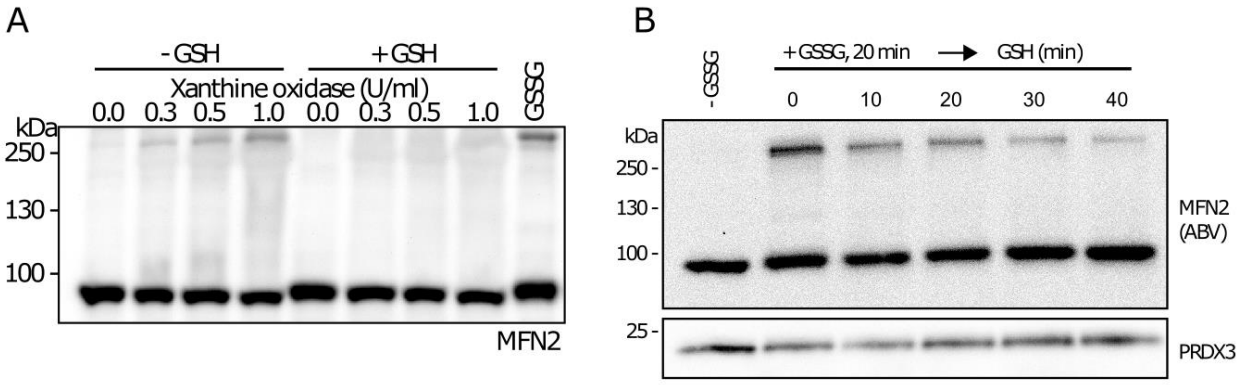

Figure 23: The dynamic redox regulated MFN2 disulfide oligomers.

(A) Extra-mitochondrial ROS affects MFN2 oligomers in a GSH-dependent manner. Fusion samples from “figure 22” were processed for denaturing, non-reducing SDS-PAGE, followed by immunoblotting with MFN2 (Abnova) antibody. One additional sample was included where mitochondria were incubated with 1mM GSSG as positive control for the formation of Mfn2 disulfide oligomers.

(B) Reduced glutathione recycles back MFN2 disulfide-conjugated complexes to monomeric form. Isolated mitochondria were incubated at 37^{o}C in the absence or presence of GSSG, followed by the addition of GSH and incubation for the specified duration. The samples were processed for Western blotting under denaturing, non-reducing conditions and immunoblotting with MFN2. PRDX3 was immunoblotted as a control.

We have demonstrated that MFNs have a single TMD, leaving a C-terminal tail in the IMS. Cysteine residues in the C-terminal tail have been reported to respond to oxidative stress in the form of GSSG (T. Shutt et al. 2012). Here we determined the glutathione redox potential at which fusion is activated, to be physiological. Further, reactive oxygen species produced by the mitochondrial electron transport chain or extra-mitochondrial sources activate fusion in a GSH-dependent manner. We confirmed that GSSG also leads to the formation of MFN2 disulfide-linked oligomers. These oligomers are recycled by reduced glutathione to the monomeric form. This might reflect a scenario in which oxidative stress leads to increase in GSSG, followed by activation of fusion and oligomerization of MFN2. When the oxidative stress ceases, GSH:GSSG ratio becomes reducing again, leading to recycling of MFN2 oligomers again to the monomeric form (**Figure 24**).

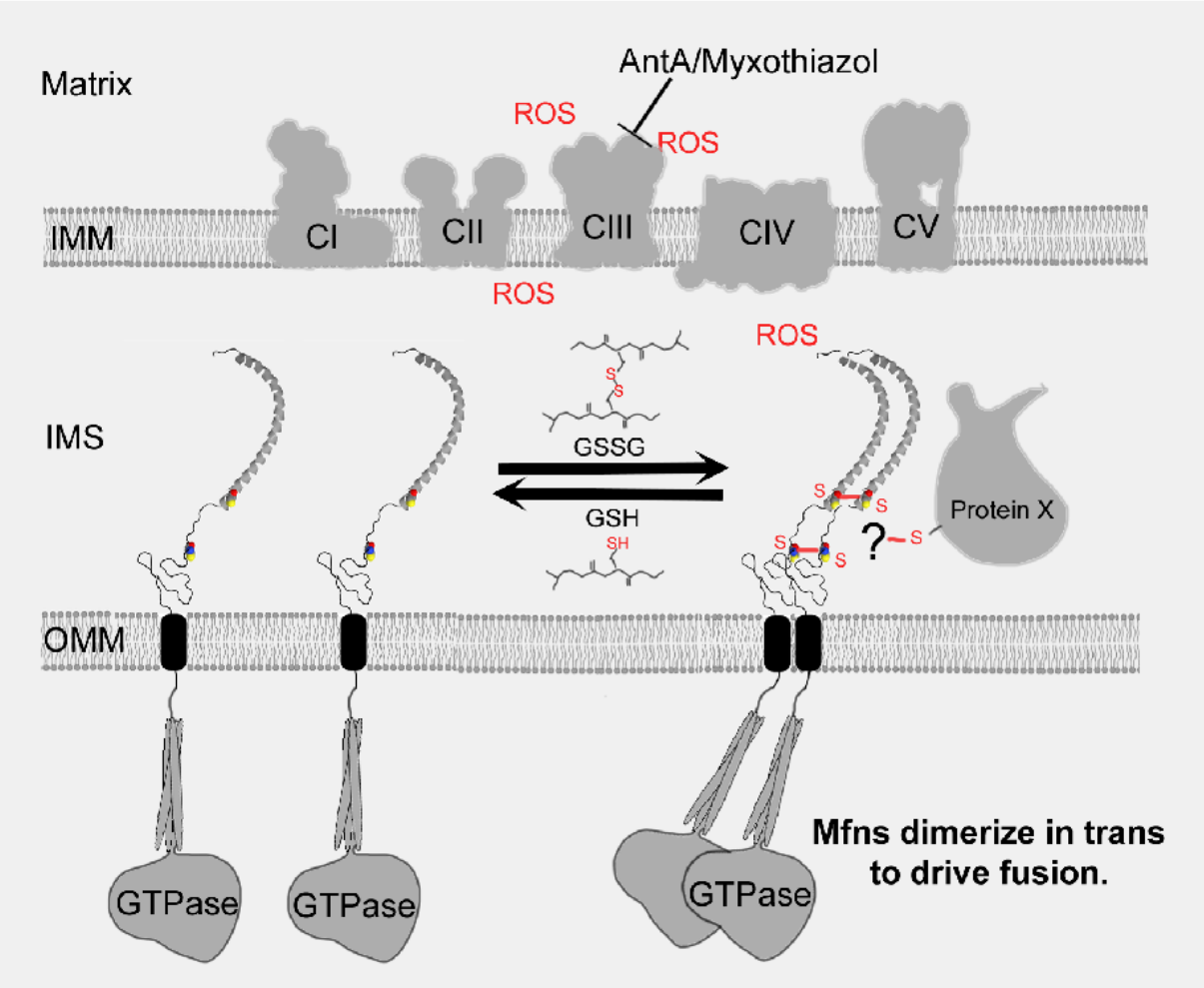

Figure 24: Proposed model of mitochondrial fusion.

Reactive oxygen species (ROS), produced from within or without the mitochondria, activate fusion in the presence of GSH. GSSG activates fusion. In the presence of GSSG, fusion protein MFN2 forms disulfide-conjugated oligomers. The identity of the protein(s) MFN2 is conjugated to remains to be shown. However, Mfn2 is either conjugated to itself, forming homo-oligomers, or it may be conjugated to another protein in the IMS. Nonetheless, the disulfide conjugation may lead to conformational changes that promote tethering or changes in GTPase activity. Notably, MFN2 is recycled back from the GSSG-induced high molecular weight complexes by GSH, making the process dynamic. This recycling may prime mitochondria to go through another fusion event. The structure of C-terminal tail of MFN2 was predicted using Protein Structure Prediction Server [(PS)2] version 3.0 (T. T. Huang et al. 2015) and the two C-terminal cysteines are presented in space-fill scheme using Jmol (www.jmol.org).

Chapter 4: Discussion

Mitochondria are dynamic, fuse into an intricate tubular network and divide into small rod-shaped mitochondria. Mutations in genes required for mitochondrial fusion have been reported to cause neurodegenerative disorders; therefore, mitochondrial fusion has been inferred to play an essential role in cell physiology. What that role of mitochondrial fusion is has remained primarily speculative, perhaps partially due to the sparsity of information of how fusion occurs, the regulation and mechanism.

Ubiquitination in the mechanism of mammalian mitochondrial fusion

In *S. cerevisiae*, ubiquitination has been proposed to play a role in the mechanism of mitochondrial fusion. Early ideas implicated Mdm30, a cytosolic E3 ubiquitin ligase, in the process. Mdm30 is required for the proteasome-dependent turnover of Fzo1, the orthologous protein to the mammalian Mfns (Escobar-Henriques, Westermann, and Langer 2006; M. M. J. Cohen et al. 2008). The observation that the Mdm30-specific turnover of Fzo1 is GTPase dependent, led to the hypothesis that ubiquitination is followed by protease cleavage of the GTPase domain to allow the completion of fusion (Anton et al. 2011; M. M. Cohen et al. 2011). Furthermore, the DUBs Ubp2 and Ubp12 have been proposed to have opposite roles in mitochondrial fusion in yeast, inhibiting and activating fusion, respectively (Anton et al. 2013). Most of the work in yeast has been performed in cells, which has proved challenging in dissecting the mechanism of ubiquitination, leaving many unanswered questions for future work. An in vitro fusion assay might help determine the role of ubiquitination in yeast. Such an assay has been developed and previously used in the mammalian system (Schauss et al. 2010; T. Shutt et al. 2012). Using the mammalian in vitro fusion assay we tested whether ubiquitination was required for fusion. To this end, we added ubiquitination inhibitors or DUB inhibitors followed by quantification of mitochondrial fusion (**Figure 5**). We did not detect any significant effect of the

ubiquitination and deubiquitylation inhibitors on mitochondrial fusion, suggesting that ubiquitination does not have a role in the mechanism of mammalian mitochondrial fusion in vitro. This does not, however, exclude a role of ubiquitination in regulating fusion in cells under specific conditions. Parkin, a cytosolic E3 Ubiquitin ligase, was reported to ubiquitinate MFN2 under mitochondrial membrane depolarization conditions (Tanaka et al. 2010). The role of this Parkin-dependent ubiquitination is thought to inhibit fusion of depolarized mitochondria with other mitochondria, thereby priming the depolarized mitochondrion for degradation by mitophagy, the mitochondria-specific autophagic pathway (Tanaka et al. 2010). Another E3 Ubiquitin ligase that was implicated in mitochondrial morphology is March5 (also named Mitol). The role of March5 in mitochondrial morphology remains controversial: a) Loss of March5 reveals either fragmentation or hyperfusion morphologies as reported by different groups (Yonashiro et al. 2006; Karbowski, Neutzner, and Youle 2007); b) March5 has been implicated in the ubiquitination of both fission and fusion factors, Fis1, Drp1 and MFN2 (Nakamura et al. 2006; Yonashiro et al. 2006; Sugiura et al. 2013); c) March5 ubiquitinates the fission factor Mid49 and SLC25A46 for degradation (Cherok et al. 2016; Steffen et al. 2017); d) March5 regulates ER-mitochondria contact sites by ubiquitinating MFN2 (Sugiura et al. 2013). While it is clear that March5 plays a role in mitochondrial morphology, it is difficult to conclude what the mechanism is. It is likely that March5 acts at the intersection of fusion and fission processes, which might explain the morphological discrepancy observed by different groups when March5 was knocked down by RNAi.

In order to identify MFN2 interacting partners, a proximity-based interactome (BioID) was performed (data not shown; Eric Shoubridge). MFN2 was tagged at the N-terminus with mutated BirA, the latter enzymatically producing activated biotin that reacts with lysine residues in nearby proteins. From this BioID, two proteins peaked our interest. One of which is an E3 Ubiquitin ligase, UBR4. UBR4, though a poorly studied protein, was reported to degrade cleaved PINK1, in conjunction with UBR1 and UBR2 (Yamano and Youle 2013). We reasoned that UBR4 might be required for MFN2 turnover under

physiological conditions as it was detected in MFN2 BioID without any stimulation. Nevertheless, the siRNA knock down of UBR4, though efficient as revealed by Western blotting, did not affect the levels of MFN2. Neither was there any difference in MFN2 levels when new protein translation was inhibited by cycloheximide nor when mitochondria were depolarised with CCCP (**Figure 6 A**). CCCP has been previously used to induce rapid turnover of MFN2 (Tanaka et al. 2010). We tested the effect of the loss of UBR4 in two cell lines, one containing endogenous Parkin (HEK293) and another lacking endogenous Parkin (HeLa) (Narendra et al. 2008; Denison et al. 2003). Therefore, we concluded that UBR4 has no significant role in the turnover of MFN2.

Another protein detected by MFN2 BioID is p97. p97, a hexameric ATPase, has a role in many pathways in cells, including extraction of ER and mitochondrial membrane proteins for degradation, and homotypic fusion of Golgi apparatus (Stach and Freemont 2017). It was not surprising to detect p97 in the BioID of MFN2, as p97 has been reported to be required for MFN2 proteasomal degradation (Tanaka et al. 2010). What was surprising, however, was observation that p97 was one of the main BioID hits. And considering that p97 is known to have a role in homotypic Golgi fusion (Kondo et al. 1997; Uchiyama et al. 2002; Yangzhuang Wang et al. 2004; S. Huang, Tang, and Wang 2016), apart from its role in protein degradation, we set out to test a possible role of p97 in mitochondrial fusion. We used our in vitro fusion assay, to avoid indirect effects in cells, and a specific p97 inhibitor. We titrated the drug over 100-fold range with no significant effect on fusion (**Figure 6 B**). This suggests that p97, while involved in MFN2 turnover in cells, does not have a direct role in fusion, and is not the protein whose ATPase activity is required for fusion. Recently, the yeast orthologue of p97, cdc48, was implicated in mitochondrial fusion (Simões et al. 2018). In this work, cdc48 was proposed to affect mitochondrial morphology by altering the stability of Ubp12, a yeast specific DUB. This raises the question of whether ubiquitination is a yeast-specific regulation mechanism of fusion, as opposed to oxidative-based regulation in mammalian cells. The proteins involved in yeast ubiquitination pathway (Mdm30, Ubp2,

and Ubp12) do not have identified orthologues in mammalian cells. However, it may be argued that other mammalian proteins could perform the functional equivalence of the yeast Mdm30, Ubp2, and Ubp12. It is likely that other mechanisms may have evolved in multicellular organisms to regulate mitochondrial fusion, to accommodate to different needs and stresses.

Revising the membrane topology of MFNs

We have established the topology of MFNs, experimentally proving that MFNs contain a single TMD (**Figure 7 – 14**). The N-terminal region of MFNs, including the dynamin-like GTPase domain and HR1, faces the cytosolic side of the membrane. We have demonstrated that the HR2-containing C-terminal end resides within the IMS. So what does this mean in the context of previous knowledge?

Yeast Fzo1 is reported to contain two TMDs, thereby diverging from mammalian Mfns. The phylogenetic analysis revealed that the metazoan Mfns are distantly related to the fungal Fzos. Mfns and Fzos are as related to each other as they are each to bacterial DLP, suggesting that the fungal and metazoan GTPases may have evolved independently of each other, or may have diverged to such an extent as to be unrecognizable with our current bioinformatics methodology (**Figure 7**). With this in mind, it might not be surprising that the proteins have different topologies and different regulation responding to the living-condition pressures the organisms experience. However, the fungal and metazoan clades are predicted to share functional principles, both containing basic structural features. Furthermore, the IMS C-terminal region of Mfns might be partially replaced with Fzo1 loop between the two TMDs. The loop of Fzo1 was shown to be important for function in fusion and was predicted to have a role in linking the outer membrane with the inner membrane (Fritz et al. 2001). The Fzo1 loop is not conserved in Mfns, however.

The phylogenetic analysis also shows that Mfns are only distantly related to the bacterial DLP from *Nostoc punctiforme*. The *N. punctiforme* BDLP, a GTPase, was isolated in a detergent-free solution

and was shown to tubulate liposomes, similar to Dynamin 1, and its crystal structure was solved (Low and Löwe 2006; Low et al. 2009). Bioinformatics analysis revealed that this BDLP was closest in sequence to the chloroplast FZL (Low and Löwe 2006). The function of BDLP remains completely unknown, the only functional information being that GFP-BDLP forms puncta in bacteria (Low and Löwe 2006). The authors speculated that BDLP could have a role in fusion or fission (Low et al. 2009). There is no evidence at all, however, that *N. punctiforme* BDLP is structurally or functionally comparable to Mfns. Due to the scarcity of solved crystal structures of dynamin-like proteins, *N. punctiforme* BDLP crystal structure was used to predict the 3D structure of Mfns. This was done with hardly any reserve to the possibility that Mfns might have a different structure. In the work of Franco et al (2016), the predicted structure of MFN2 was used as the basis and as the mechanism in hope of finding a drug that disrupted the binding of HR1 and HR2, a binding which was inferred from this predicted structure. In this work, a peptide drug corresponding to a portion of HR1 was reported to activate fusion in cells, which was followed by the synthesis of a small compound that retained the physical features of the peptide (Franco et al. 2016; Rocha et al. 2018). In these works, surprisingly, HR1 and HR2 binding was not tested. Nor was the concluded role of the drugs in disrupting such a binding confirmed experimentally. Of course, the authors were interested in the characterization of the drugs, their effect on mitochondrial morphology and their "correction" of mitochondrial damage (Franco et al. 2016; Rocha et al. 2018). The idea that HR1 and HR2 bind together originates from a yeast 2-hybrid (Y2H) screen (P. Huang, Galloway, and Yoon 2011), where the authors suggest that this binding prevents mitochondrial fusion. In this work, the region spanning between the GTPase domain and the TMD (264-603), which the authors termed "HR1", was reported to bind the region between the TMD and the end of the protein (648-757), which the authors termed "HR2" (P. Huang, Galloway, and Yoon 2011). What is called "HR1" and "HR2" in this work does not refer to the coiled-coil domains HR1 and HR2, but to large regions of the protein containing the coiled-coil domains HR1 and HR2. In an attempt to narrow down the region responsible

for the interaction, the authors divided the "HR2" (648-757) into two smaller regions: 648-724 and 684-757 (the latter containing coiled-coil domain HR2), however either of these failed to bind "HR1" (Huang, Galloway, and Yoon 2011). Also, overexpression of Myc-tagged Mfn2-"HR1" or Myc-tagged Mfn2-(360-448) in cells resulted in elongated mitochondrial morphology, indicating that "HR1" region does not bind endogenous "HR2" domain in vivo. Taken together, the evidence that "HR1" and "HR2" bind together is based on a single observation using Y2H system, without further validation through other experiments or by other groups.

Another idea, challenged by the new MFNs topology, is HR2 as a mitochondrial tethering domain in *trans*. This idea, being proposed early on, has grown deep roots in the field's consciousness. Two findings led mainly to this conclusion. First, HR2 co-immunoprecipitated together, tagged with two different tags (Koshiba et al. 2004). Second, HR2 crystallized as two antiparallel helices (Koshiba et al. 2004). Cell-free tethering assays have not been done to confirm HR2's proposed function as a tether, until recently, where recombinant HR2 attached to liposomes revealed a weaker tethering capacity in contrast to HR1 (Daste et al. 2018). Further, Qi et al could not reproduce the co-immunoprecipitation of HR2 with itself, suggesting a need for future work to resolve whether HR2 binds to itself (Qi et al. 2016). The new topology of MFNs does not eliminate the possibility that two HR2 domains may bind together in *cis* within the IMS, but excludes a binding in *trans* as a tether. We also showed that MFN2 lacking HR2 was capable of partially rescuing the fragmented morphology in Mfn2-null MEFs, suggesting that HR2 is not required for fusion.

What is the role of the C-terminus in the IMS?

As HR2 does not have a core function in fusion, does it play a regulatory role? Our results suggest that the C-terminus might act as a redox sensor in the IMS, responding to the redox status of mitochondria. The IMS has a unique redox environment, where the disulfide relay system functions. The

disulfide relay system consists primarily of Mia40 and ALR (Erv1), two IMS proteins which catalyzes disulfide bonds formation on substrate proteins, facilitating their import into mitochondria (Mesecke et al. 2005; Hell 2008). Mia40 has also been shown to introduce inter-molecular disulfide bonds to regulate the function of IMS proteins (Petrungaro et al. 2015). The C-terminal tail of MFNs, which contains two cysteine residues, resides in the IMS. These two cysteine residues were previously reported to be active and to react with oxidized glutathione (GSSG) forming disulfide-bridged oligomers (T. Shutt et al. 2012). Therefore, it is not farfetched to envision that the C-terminal tail of MFNs senses the redox status of mitochondria in the IMS, manifested by the formation of the observed disulfide-conjugated oligomers. These new disulfide bonds in MFNs may be regulated, or formed by Mia40 under oxidative stress, a reaction known to be performed by Mia40 for other IMS proteins.

Another proposed role for the C-terminal tail of MFNs in the IMS, though currently speculative, is acting as a link between outer and inner membrane fusion events (Giacomello and Scorrano 2018). OPA1 was shown to activate mitochondrial fusion in an MFN1-dependent manner, which hinted at a possible interaction, indirect at that time because MFN1 was considered to possess no domains in the IMS (Cipolat et al. 2004). Furthermore, MFNs have been previously reported to specifically and reciprocally interact with OPA1 using immunoprecipitation, suggesting that such an interaction is of functional physiological value in the context of mitochondrial fusion (Guillery et al. 2008; Janer et al. 2016). However, the interacting regions of MFNs and OPA1 were not identified. The possibility of MFN-OPA1 binding raises a very interesting question. Is the IMS tail of MFNs required for efficient inner membrane fusion? If the tail of MFNs had such a role in the initiation of inner membrane fusion, the loss of this tail would cause delay, or desynchronization, in inner membrane fusion following outer membrane fusion. Considering that both MFNs C-terminus and OPA1 reside within the IMS, it is of interest to speculate the conditions under which the interaction is promoted. Oxidative stress conditions seem to be the most plausible candidate to stimulate such an interaction between MFNs and OPA1,

especially because we have shown that GSSG, an oxidative stress product, induces disulfide-dependent oligomerization of MFNs (**Figure 23**). GSSG could either promote a conformational change that permits MFNs-OPA1 binding, or, because the identity of the proteins of the disulfide oligomers remains unknown apart from MFN2, OPA1 might be directly linked to MFNs with a GSSG-induced disulfide bond. However, future work is required to elucidate the validity of these speculations.

Redox regulation of fusion

One of the only known activators of mitochondrial fusion is oxidative stress. Early on in the field of mitochondrial dynamics, ROS and ROS-inducing drugs were reported to lead to the formation of giant mitochondria, named megamitochondria, in cultured cells and tissues (Matsuhashi et al. 1997; Karbowski et al. 1999; T Wakabayashi 2002; T Wakabayashi et al. 2000). This ROS-induced formation of megamitochondria, proposed to be due to activation of fusion, was preventable with antioxidants treatment (Takashi Wakabayashi et al. 1997; T Wakabayashi 2002). How does ROS activate mitochondrial fusion? ROS is not thought to directly activate fusion. ROS is rapidly eliminated by the antioxidant systems in cells, primarily the glutathione antioxidant pathway. To dissect the mechanism of action of ROS, we have employed an in vitro mitochondrial fusion assay. Shutt et al. demonstrated that GSSG, the byproduct of ROS removal by the glutathione pathway, was responsible for activating fusion in vitro. The activating role of GSSG was confirmed in cells by chemically converting GSH to GSSG with diamide, followed by assessment of mitochondrial morphology (T. Shutt et al. 2012). Redpath et al. also confirmed that the manipulation of the glutathione pathway activated mitochondrial fusion in C2C12 myotubes, by inhibiting GSH synthesis or increasing GSSG with diamide treatment (Redpath et al. 2013). Glutathione exists in cells as a ratio of GSH and GSSG, where this ratio shifts during oxidative stress. Therefore, we have determined the glutathione ratio (expressed as potential) that activates mitochondrial fusion in vitro, the activating conditions falling within the reported cellular range of glutathione potential (**Figure 20**). In cells, mitochondria are immersed in millimolar amounts of GSH, and

only under oxidative stress does the amount of GSSG increase. The source of ROS could be mitochondrial, from the electron transport chain and TCA cycle, or could be extra-mitochondrial. We found that regardless of the source, ROS activated mitochondrial fusion in a GSH-dependent manner, providing proof that ROS specifically acts through the glutathione pathway to activate fusion (**Figure 21 & 22**).

How does GSSG, the product of the reaction of ROS with two molecules of GSH, activate fusion? Shutt et al. (2012) have reported that GSSG leads to the formation of disulfide-conjugated oligomers of the fusion GTPases MFN1, MFN2 and OPA1. GSSG-treated isolated mitochondria were processed for Western blotting under denaturing non-reducing conditions, conditions that preserve disulfide bonds while breaking any weak protein-protein interactions. We have demonstrated that the GSSG-induced 250-kDa MFN oligomers can be recycled back to the monomeric form by GSH (**Figure 23**).

Several questions need to be further elucidated. First the identity of the proteins in the GSSG-induced oligomers remains to be identified. The oligomers might consist of only MFN2 molecules conjugated together by disulfide bonds. Or GSSG might induce disulfide bond formation between MFN2 and other proteins. Second, what effect does GSSG have on MFN2 function? Does it lead to a conformational change that might expose an active domain? Does GSSG alter the GTPase activity of MFN2? Future work is needed to resolve these questions.

ROS has also been reported to lead to mitochondrial fragmentation (Jendrach et al. 2008; Fan, Hussien, and Brooks 2010). How do we reconcile the observations that ROS both promotes fusion and causes fission? In the many works using ROS, we need to consider four experimental parameters that will help us arrive at the meaning of the observed outcomes and reached conclusions: 1) the amount of ROS used; 2) source of ROS, pure hydrogen peroxide vs. enzymatically produced; 3) duration of treatment; 4) localization of ROS, general cell-wide vs. mitochondria specific. The reports that ROS

treatment causes mitochondrial fragmentation are undoubtedly not false. We agree that mitochondria fragment at high doses of ROS and for long duration exposure. What we have demonstrated however is a role for low doses of ROS as a signaling molecule to promote mitochondrial fusion.

We propose a model for oxidative stress regulation of mitochondrial fusion (**Figure 25**). Mitochondria are the main source of ROS in cells, as part of their role in substrate catabolism and electron transport (Brand 2016). ROS, damaging to proteins and lipids, is quickly removed by antioxidant pathways, the main being the glutathione pathway. The end product of the latter pathway, in which the enzyme glutathione peroxidase removes ROS using GSH, is GSSG. GSSG, as we have reported, activates mitochondrial fusion, both in vitro and in cells. GSSG, thus, converts ROS, which encodes information about the health and function of a mitochondrion, into form, i.e. tubulation of mitochondria. How does GSSG activate fusion? Though a detailed mechanism has not been elucidated, GSSG induces the formation of disulfide-conjugated oligomers of the fusion machinery, MFN1, MFN2 and OPA1. Speculatively, this oligomerization might prime the fusion machinery, by a conformational change or by conjugating them with other factors. These GSSG-induced oligomers are dynamic and can be de-stabilized with GSH, suggesting a recycling of the oligomeric MFN2 into monomers capable of another round of fusion.

What does the redox regulation of mitochondrial dynamics reveal about the function of mitochondrial fusion? Redox status of mitochondria may reflect ROS generation and handling by mitochondria. Mitochondrial ROS has been reported to play an important role in signalling, implicated in pro-survival functions in immunity and ageing, but also in apoptotic cell death and pathology (West et al. 2011; Yee, Yang, and Hekimi 2014; Schaar et al. 2015; Ying Wang et al. 2018; Circu and Aw 2010; Brand 2016). Because mitochondria generate ROS as a side effect of their function in substrate catabolism and electron transport for energy production, the amount of ROS produced is of significance. A mild increase of ROS production would perturb the redox homeostasis of mitochondria, but can be

handled by the glutathione antioxidant system with little damage to proteins and lipids. The increase in ROS production could be due to several proposed reasons. 1) An inefficiency of electron handling due to stoichiometric imbalance of the proteins in TCA cycle and ETC. This imbalance in the numerous inter-connected players could arise from the continual arbitrary damage of proteins, or due to proteins' distinct rates of synthesis and turnover. Therefore fusion of mitochondria helps eliminate such imbalance by mixing and homogenizing the protein content of mitochondria. 2) Imbalance of a mitochondrion's content of substrates and metabolites might also affect the amount of ROS produced. Such imbalance interferes with the kinetics of protein activity and efficiency of electron handling, especially because the matrix consists of a network of pathways consuming and producing different metabolites. Imbalance of enzymes or metabolite transporters within a mitochondrion might contribute to the imbalance of metabolites; however, fusion of mitochondria would mix the matrixes and protein content, alleviating such a problem. 3) The antioxidant capacity of an individual mitochondrion might contribute to the amount of ROS produced and to the damage a mitochondrion experiences. An isolated mitochondrion is more prone to oxidative fluctuations and might be at a higher risk of severe damage, which is a danger to a cell's fate. Therefore, fusion helps maintain a healthy homogenous mitochondrial population in cells, reducing the risk of irreversible damage and cell death. This idea is consistent with the proposal that mitochondrial fusion is protective against cell death. In this model mitochondrial fusion, in response to mild oxidative stress, ensures the maintenance of a healthy mitochondrial population that is homogenous and efficient in energy production and metabolite synthesis and catabolism.

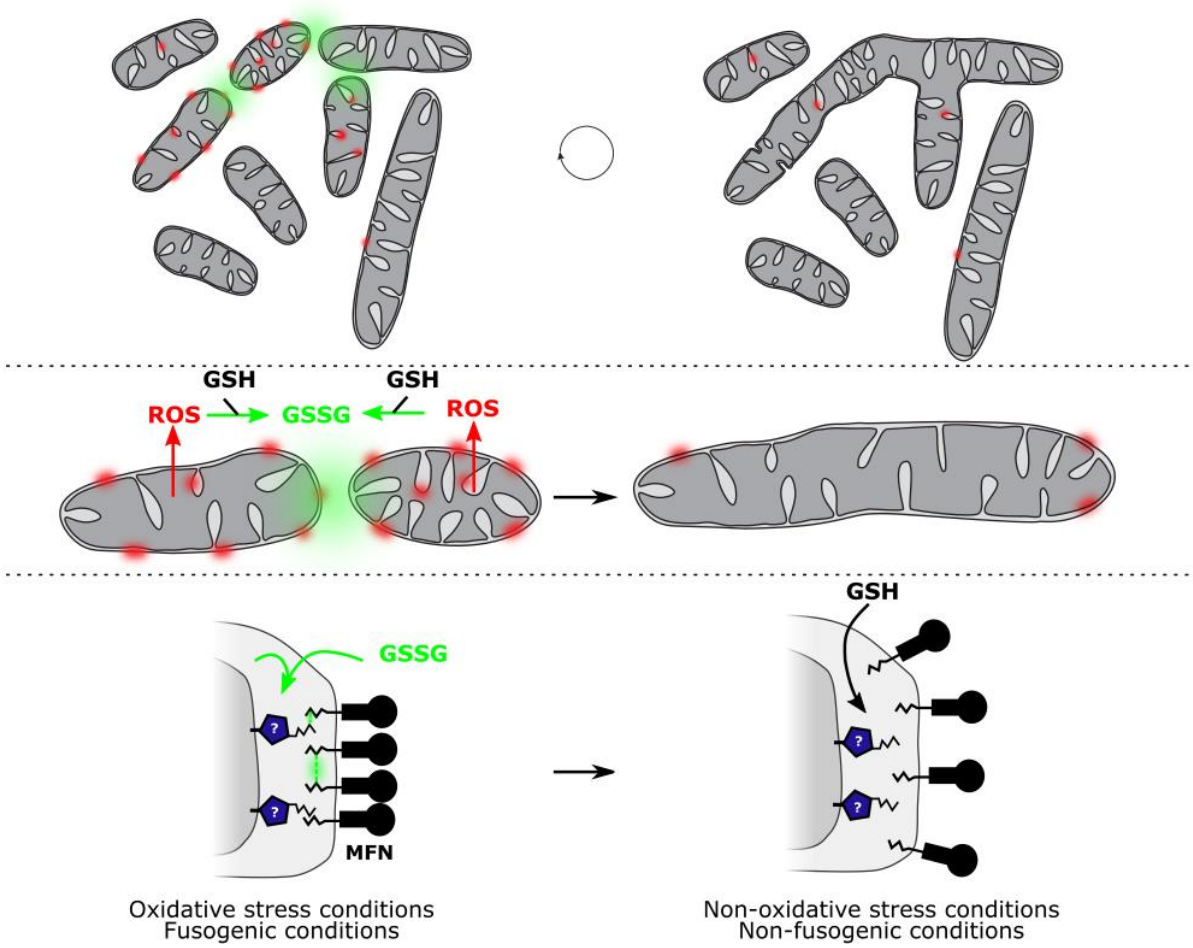

Figure 25: Proposed model for redox regulated mitochondrial fusion.

Concluding Remarks

Mitochondrial fusion is essential for cell function and survival, and mutations in the core machinery of fusion cause human diseases. The mechanism and regulation of fusion are still unclear. We demonstrated that ubiquitination and deubiquitylation do not have a significant role in mammalian mitochondrial fusion, contrary to the reported role in yeast mitochondrial fusion. Bioinformatics analysis of mitochondrial outer membrane GTPases revealed that the fungal Fzos were distantly related to metazoan Mfns, as close to each other as they are to bacterial GTPases, suggesting divergence in regulation between the fungal and the metazoan outer membrane fusion. We experimentally established the topology of mammalian MFNs as possessing a single transmembrane domain, leaving

the C-terminal tail in the IMS. This new topology prompts the rethinking of the currently proposed models of fusion. The C-terminus, which includes HR2, could function in synchronizing the outer and inner membranes fusion events. And it could act as a redox sensor, reacting to the oxidative state within the IMS. We have shown that mitochondrial fusion is activated by GSSG and by ROS in a GSH-dependent manner, through the formation of disulfide-linked MFN2 oligomers which are dynamic and recycled by GSH. Our current work has advanced our understanding of the basic principles of mitochondrial fusion. This opens new possibilities to study whether mutations in MFN2 interfere with its ability to respond to oxidative stress and how the redox regulation of fusion may be targeted for novel drug discovery.

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