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# MOLECULAR MECHANISMS OF HUMAN MITOCHONDRIAL DYNAMICS: INSPIRATION AND CHALLENGE

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# Molecular mechanisms of human mitochondrial dynamics: Inspiration and Challenge

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*Dedicated to my beloved family*

# ABSTRACT

Mitochondria are highly dynamic organelles in cells. They frequently change the morphology via continuous fission and fusion events, referred to as *mitochondrial dynamics*, which includes the shape, size and number of mitochondria in the cell, as well as mitochondrial subcellular distribution and mitochondrial quality control. Mitochondrial dynamics plays critical roles in sustaining the physiological functions of mitochondria. Dysfunction of mitochondrial dynamics is associated with various human diseases.

Mitochondrial dynamics is mediated by a group of mitochondria-shaping proteins in yeast and in mammals. In yeast, the mitochondrial fusion mechanism consists of three central proteins, the inner membrane-anchored Mgm1p, and the outer membrane-anchored Fzo1p and Ugo1p. Correspondingly, Mfn1/2 (the homologs of Fzo1p) and OPA1 (the homolog of Mgm1p) are the core mitochondrial fusion proteins in mammals. On the mitochondrial fission side, the dynamin-related protein Dnm1p (in yeast)/Drp1 (in mammals) is a core component of the mitochondrial fission machinery, and the recruitment of cytosolic Dnm1p/Drp1 to the mitochondrial surface is the crucial step for mitochondrial division process. In yeast, Dnm1p is recruited to mitochondria by the mitochondrial receptor Fis1p via Mdv1p or Caf4p as the adaptor. However, in mammals, Fis1 (the homolog of yeast Fis1p) is not essential for the recruitment of Drp1 (the homolog of Dnm1p), although Fis1p and Fis1 proteins are evolutionarily conserved and both can induce mitochondrial fragmentation when overexpressed. In agreement with this notion, overexpression or depletion of hFis1 does not affect Drp1 distribution in cells. Furthermore, no equivalents of Caf4p and Mdv1p have been found in mammalian cells to date. The functions of Fis1 in mammalian cells remain elusive. Instead, Mff and MIEF1/2 have been identified as new mitochondrial receptors for Drp1 in mammals. While the three receptors all can recruit Drp1 to mitochondria, whether they have distinct functions and how they work together in the mitochondrial fission process need to be further evaluated. In this thesis, we investigated the functions of Drp1 receptors Fis1, MIEF1/2 and Mff in mitochondrial dynamics of human cells and provide novel insights into the molecular mechanisms of human mitochondrial dynamics.

In study I, we explored the distinct functions of MIEF1 and its paralog MIEF2. The similarities of MIEF1 and MIEF2 are: Both MIEFs share 45% amino acid identity in human cells and are highly conserved in vertebrates. They are anchored in the mitochondrial outer membrane (MOM), associate with Drp1 and recruit Drp1 from the cytosol to mitochondria, resulting in mitochondrial elongation. However, they are dissimilar in certain aspects. For example, their expression levels are different in human tissues and various cell lines, especially during organism development. Although overexpression of either MIEF1 or MIEF2 triggers mitochondrial elongation, MIEF2 overexpression induces a higher extent of elongated mitochondrial clustering and is reverted to a lower extent by hFis1 and Mff than MIEF1. Furthermore, MIEF1 and MIEF2 proteins form distinct types of oligomers in cells and contain different oligomerization domains. All of these data imply that the mitochondrial elongation factors MIEF1 and MIEF2 partly differ in their regulation of mitochondrial dynamics.

In study II, we evaluated how Mff and MIEF1/2 coordinately work together in regulating Drp1-driven mitochondrial fission. Firstly, loss of MIEFs significantly impairs the

association between Mff and Drp1, as well as the Drp1 recruitment by Mff to the mitochondrial surface, whereas knockdown of Mff does not affect the functions of MIEFs as mitochondrial Drp1 receptors. Secondly, MIEFs can bind to both Drp1 and Mff independently and serve as adaptors linking Drp1 and Mff together in a Drp1-MIEF-Mff trimeric complex, which facilitates the direct association between Drp1 and Mff. Thus, we find that MIEFs can promote the interaction of Drp1 with Mff. Furthermore, the relative amounts of MIEFs and Mff in cells govern the balance of mitochondrial dynamics. Enhanced levels of MIEFs decrease the interaction between Drp1 and Mff leading to mitochondrial elongation, while higher levels of Mff versus lower levels of MIEFs result in mitochondrial fragmentation. In sum, MIEFs and Mff work coordinately during Drp1-dependent mitochondrial fission, steering the balance between mitochondrial fission and fusion.

In study III, we addressed the role of Drp1-S637 phosphorylation in Drp1 translocation from the cytosol to mitochondria and in the regulation of mitochondrial fission. Reversible Drp1-S637 phosphorylation has been considered to regulate the Drp1-dependent mitochondrial fission and the recruitment of Drp1 to mitochondria, but the extent of this regulation is not fully understood. We confirm that Drp1 phosphorylation at S637 (Drp1-pS637) exists both in the cytosol and on mitochondria, and can be recruited and accumulated on mitochondria by MIEFs and Mff. Increased Drp1-pS637 does not affect the interaction between Drp1 and Mff whereas depletion of MIEFs decreases the binding of Mff with Drp1. Furthermore, similar to wild-type Drp1, overexpression of either phospho-deficient Drp1S637A or phosphomimic Drp1S637D mutants leads to mitochondrial fission in Drp1 deficient cells. However, Drp1S637D was less efficient than Drp1S637A and wild-type Drp1. Additionally, PKA, a kinase phosphorylating Drp1 at the S637 site, partially resides at the mitochondrial surface and is immunoprecipitated by MIEFs or Mff. However, PKA silencing does not abolish the Drp1-Mff or Drp1-MIEFs association. In brief, Drp1-S637 phosphorylation plays a fine-tuning but not a dominant role in governing Drp1 subcellular distribution and Drp1-mediated mitochondrial fission, whereas Drp1 receptors MIEFs and Mff coordinately regulate the process of Drp1 recruitment and mitochondrial fission.

In study IV, given a minor role of hFis1 in Drp1-dependent mitochondrial fission in human cells, we investigated the underlying molecular mechanism of hFis1-induced mitochondrial fragmentation and the roles of hFis1 in mitochondrial dynamics. Firstly, we observed that hFis1-induced mitochondrial fragmentation occurred both in presence and absence of Drp1 and Dyn2, indicating mitochondrial fragmentation promoted by hFis1 is independent of the Drp1/Dyn2-medicated mitochondrial fission process. Furthermore, immunoprecipitation revealed that hFis1 binds to the pro-fusion proteins Mfn1, Mfn2 and OPA1 at endogenous levels and inhibits the GTPase activities of these proteins specifically, suggesting that the function of hFis1 is probably to block the fusion machinery and thereby shifting the balance to mitochondrial fission. Consistent with these results, destruction of all the three pro-fusion proteins in Drp1 KO cells phenocopied the hFis1-induced mitochondrial fragmentation phenotype, and the actin cytoskeleton was partially involved in this process. In conclusion, we reveal a novel molecular mechanism of hFis1 in mitochondrial dynamics.

Collectively, this thesis develops novel insights into the molecular mechanisms of human mitochondrial dynamics, and provides more detailed knowledge for the studies of mitochondria-related diseases.

# LIST OF SCIENTIFIC PAPERS

I. Tong Liu, **Rong Yu**, Shao-Bo Jin, Liwei Han, Urban Lendahl, Jian Zhao and Monica Nistér. *(2013)* **The mitochondrial elongation factors MIEF1 and MIEF2 exert partially distinct functions in mitochondrial dynamics.**
*Experimental Cell Research. 319(18): 2893-2904. doi: 10.1016/j.yexcr.2013.07.010.*

II. **Rong Yu[1]**, Tong Liu[1], Shao-Bo Jin, Chenfei Ning, Urban Lendahl, Monica Nistér[2] and Jian Zhao[2]. *(2017)* **MIEF1/2 function as adaptors to recruit Drp1 to mitochondria and regulate the association of Drp1 with Mff**.
*Scientific Reports. 7(1):880 doi: 10.1038/s41598-017-00853-x.*

III. **Rong Yu[1]**, Tong Liu[1], Fei Tan, Shao-Bo Jin, Chenfei Ning, Urban Lendahl, Jian Zhao[2] and Monica Nistér[2]. **Drp1-S637 phosphorylation fine-tunes mitochondrial fission and is not a determinant of Drp1 recruitment to mitochondria.**
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IV. **Rong Yu**, Shao-Bo Jin, Urban Lendahl, Monica Nistér[2] and Jian Zhao[2]. *(2019)* **Human Fis1 regulates mitochondrial dynamics through inhibition of the fusion machinery.**
*EMBO Journal. 38(8) e99748. doi: 10.15252/embj.201899748*

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[2]These authors share senior responsibility

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Aβ | amyloid β |
| AD | Alzheimer's disease |
| ADOA | autosomal dominant optic atrophy |
| AKAP121 | A-kinase anchoring protein 121 |
| APP | amyloid precursor protein |
| ATP | adenosine triphosphate |
| GTPases | guanosine triphosphatases |
| DRP1 | Dynamin-related protein 1 |
| OXPHOS | oxidative phosphorylation |
| mtDNA | mitochondrial DNA |
| Caf4p | C-C chemokine receptor type 4-associated factor 4 |
| CMT2A | Charcot-Marie-Tooth neuropathy type 2A |
| Cos-7 cells | Cos-7 cells from African green monkey kidney |
| CSF | cerebrospinal fluid |
| Dnm1p | dynamin-related GTPase protein |
| Dyn2 | Dynamin 2 |
| Fis1p | Fission 1 protein |
| IMM | Inner mitochondrial membrane |
| MARCH5 | E3 ubiquitin-protein ligase MARCH5 |
| Mdv1p | Mitochondrial division protein 1 |
| MEF | Mouse embryo fibroblasts |
| Miro1 | Mitochondrial Rho GTPase 1 |
| MOM | Mitochondrial outer membrane |
| OMA1 | Mitochondrial metalloendopeptidase OMA1 |
| CCCP | carbonyl cyanide m-chlorophenylhydrazone |
| Parkin | E3 ubiquitin-protein ligase parkin |
| PARL | presenilin associated rhomboid like |
| PKA | cAMP-dependent Protein kinase A |
| ROS | reactive oxygen species |
| Mfns | Mitofusins |

| | |
|---|---|
| OPA1 | Optic atrophy 1 |
| TPR | tetratricopeptide repeat |
| NTE | N-terminal extension |
| siRNA | small interfering RNA |
| CaMKIα | $Ca^{2+}$/calmodulin-dependent protein kinase I α |
| PP2A | Protein phosphatase 2A |
| ER | endoplasmic reticulum |
| MAMs | mitochondria-associated membranes |
| INF2 | Inverted formin 2 |
| MOMP | mitochondrial outer membrane permeabilization |
| PINK1 | PTEN-induced putative kinase protein 1 |
| MIEF1/2 | Mitochondrial elongation factor 1/2 |
| co-IP | co-immunoprecipitation |
| Fis1 | Fission 1 |
| hFis1 | human Fis1 |
| Sept2 | Septin-2 |
| SLC25A46 | Solute carrier family 25 member 46 |
| LatB | latrunculin B |
| Tom20 | Translocase of outer membrane 20 |
| Spire1C | Protein spire homolog 1 C |
| YME1L | YME1-like 1 ATPase |

# 1 INTRODUCTION

## 1.1 THE REGULATION OF MITOCHONDRIAL DYNAMICS

Mitochondria as double membrane-bound organelles are present in most eukaryotic cells. They are traditionally considered to function as the powerhouses of the cell, generating adenosine triphosphate (ATP) through oxidative phosphorylation, used for cellular chemical energy. Mitochondria have their own genome, also known as mitochondrial DNA (mtDNA), which encodes some essential polypeptides of complexes I, III, IV and V in the oxidative phosphorylation system. Meanwhile, most mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytoplasm, and imported to mitochondria (El-Hattab et al., 2017; Sun and St John, 2016; Taanman, 1999). In addition to producing ATP, mitochondria are also involved in regulating numerous aspects of cellular activity, including the cell-cycle progression and cell proliferation, the maintenance and differentiation of cells, mitophagy and autophagy, mitochondria-involved intrinsic cell-death pathway, transduction of calcium signaling, etc. Additionally, mitochondria are the major organelles generating and detoxicating reactive oxygen species (ROS) to adjust cellular redox homeostasis (McBride et al., 2006).

Mitochondria themselves are highly dynamic and constantly alter their shape through fusion and fission events. The balance of fission and fusion events is termed *mitochondrial dynamics*, which is controlled by a number of mitochondria-shaping proteins encoded by nuclear genomic DNA. Not just related to mitochondrial morphology, mitochondrial dynamics also involves the size, number, distribution, quality control and transport of mitochondria in cells. Activated fission and/or inhibited fusion can lead to fragmented mitochondria, whereas active fusion and/or inhibited fission can cause mitochondrial elongation (Altieri, 2018; Chen and Chan, 2009; Senft and Ronai, 2016). Over the last decades, increasing data indicate that deregulation of mitochondrial dynamics causes mitochondrial dysfunction, impacting on a broad range of cellular functions, and is associated with a number of human diseases (Altieri, 2018; Chan, 2012; Chen and Chan, 2017; Mishra and Chan, 2014; Roy et al., 2015; Trotta and Chipuk, 2017; Zhao et al., 2013a).

In this review, I will discuss how mitochondrial fusion and fission is regulated by the mitochondria-shaping proteins, and by other co-factors such as the endoplasmic reticulum (ER) and the actin-related cytoskeleton, as well as how mitochondrial dynamics influences a variety of cellular biological processes such as cell-cycle regulation, apoptosis, mitophagy, energy metabolism and embryonic development.

### 1.1.1 The mitochondrial fusion machinery

#### *1.1.1.1 Key proteins of the mitochondrial fusion machinery in yeast*

Mitochondrial dynamics has been studied in yeast for many years. The mitochondrial fusion machinery characterized in yeast consists mainly of three key proteins, Fzo1p and Ugo1p, which are anchored in the mitochondrial outer membrane, and Mgm1p localized in the inner

membrane (listed in Table 1). Depleting any of these proteins results in mitochondrial fragmentation (Braun and Westermann, 2011; Merz et al., 2007; Shaw and Nunnari, 2002).

Fzo1p, a large dynamin-related GTPase, is integrated in the MOM through two adjacent transmembrane sections near its C-terminus, and with a highly conserved N-terminal GTPase domain facing the cytoplasm. Fzo1p is requisite for fusion of the mitochondrial outer membrane (Hermann et al., 1998; Rapaport et al., 1998). Mgm1p is also a dynamin-related GTPase, with an N-terminal transmembrane domain anchored in the mitochondrial inner membrane, and a GTPase domain and two hydrophobic segments close to the C terminus, which are exposed in the intermembrane space. Mgm1p is required for both outer and inner mitochondrial membrane fusion, and the disruption of the MGM1 gene can result in mitochondrial fragmentation and loss of mtDNA in a Dnm1p-dependent fission (Sesaki et al., 2003b; Wong et al., 2000; Wong et al., 2003). Both Fzo1p and Mgm1p are evolutionally conserved from yeast to humans. There are two homologs of Fzo1p in mammalian cells, termed mitofusin1 (Mfn1) and mitofusin2 (Mfn2), and the mammalian homolog of Mgm1p is known as OPA1 (Alexander et al., 2000; Santel and Fuller, 2001).

Ugo1p was initially identified through the screening of yeast mutants that lost mtDNA in the Dnm1p-dependent division (Sesaki and Jensen, 2001). Ugo1p is a mitochondrial outer membrane-anchored protein and has three transmembrane domains in the middle region. The N-terminus faces the cytosol interacting with Fzo1p directly and the C-terminal domain is exposed to the intermembrane space for Mgm1p binding, thus Ugo1p has been viewed as a molecular bridge between Fzo1p and Mgm1p in mitochondrial fusion (Coonrod et al., 2007; Sesaki and Jensen, 2004). Ugo1p is required for both outer and inner mitochondrial membrane fusion (Hoppins et al., 2009), and loss of Ugo1p causes mitochondrial fragmentation (Sesaki and Jensen, 2001). In human cells, SLC25A46 has been found to have similar protein sequence with Ugo1p, but knockdown of SLC25A46 results in mitochondrial hyperfusion, indicating an opposite effect in human mitochondrial dynamics compared to Ugo1p in yeast (Abrams et al., 2015; Janer et al., 2016). Still some other proteins are also involved in the mitochondrial fusion process in yeast. For example, the F-box protein Mdm30p is essential for Fzo1p ubiquitylation and degradation after GTP hydrolysis in the late stage of outer membrane fusion for maintaining fusion-competent mitochondria in yeast, and this step requires Ugo1p (Anton et al., 2011; Cohen et al., 2011; Fritz et al., 2003). Pcp1p and Ups1p are required for the processing of Mgm1p to control mitochondrial morphology (Sesaki et al., 2006; Sesaki et al., 2003a).

#### *1.1.1.2 The mitochondrial fusion machinery in mammals*

In mammals, there are three key dynamin-like proteins controlling the mitochondrial fusion machinery, mitofusin 1 and 2 (Mfn1, Mfn2) and optic atrophy 1 (OPA1) (Table 1). All of them are large dynamin-related GTPases and their activation proceeds via a three-step mitochondrial fusion process: Two mitochondria are tethered and form a docking ring structure around the contact outer membranes, further the two outer membranes fuse together

triggered by GTP hydrolysis, then coming to the inner membrane fusion (Brandt et al., 2016; Tilokani et al., 2018) (Figure 1).

Both mammalian Mfn1 and its paralog Mfn2 reside in the outer mitochondrial membrane. They are homologs of *Drosophila fzo,* and similar to the fusion protein fzo1p in yeast. Human Mfn1 and Mfn2 share 60% identity and 77% similarity with each other in protein sequence. Both of them have a bipartite transmembrane domain, with both the N-terminal and C-terminal domains exposed towards the cytoplasm and GTPase domain close to the N-terminus (Santel and Fuller, 2001). Overexpression of either Mfn1or Mfn2 can induce perinuclear mitochondrial clustering, whereas Mfn1/2-deficient cells contain severely fragmented mitochondria. Furthermore, Mfn1 and Mfn2 can form homo- and hetero-oligomers, and these three complexes can work in concert to tether the outer mitochondrial membranes of adjacent mitochondria (Chen et al., 2003). In spite of that both proteins are essential for the maintenance of mitochondrial morphology, however, Mfn1 has much higher GTPase activity than Mfn2 (Ishihara et al., 2004). In addition to controlling mitochondrial morphology, Mfn2 has other distinct functions different from Mfn1. For instance, Mfn2 is involved in endoplasmic reticulum-mitochondria connections (de Brito and Scorrano, 2008), energy metabolism and insulin signaling (Zorzano et al., 2015), mitophagy (Chen and Dorn, 2013) and apoptosis (Perumalsamy et al., 2010).

OPA1 is the mammalian homolog of yeast Mgm1p, and is also a dynamin-related GTPase localized in the mitochondrial inner membrane acting in mitochondrial inner membrane fusion. OPA1 was originally discovered by gene mutation screening of autosomal dominant optic atrophy (Alexander et al., 2000). There are at least eight mRNA variants identified from the OPA1 gene through alternative splicing, generating long and short isoforms (Delettre et al., 2001). Further proteolytic cleavage can be performed by a group of proteases residing in the mitochondrial intermembrane space (e.g. PARL, PRELI, Yme1L, OMA1). Long forms of OPA1 undergo further processing in the matrix to produce short forms of OPA1, and both long and short forms of OPA1 complement each other to reconstitute the activity of mitochondrial fusion (Ishihara et al., 2006; Mishra et al., 2014; Song et al., 2007; Zhao et al., 2013a). OPA1 processing can also be affected by mitochondrial membrane potential and proapoptotic stimuli. For example, Caspase-3 can lead to N-terminal cleavage of OPA1 resulting in mitochondrial fragmentation (Loucks et al., 2009), and CCCP treatment is also known to induce cleavage of long OPA1 forms by OMA1 (Ehses et al., 2009; Head et al., 2009). Knockdown of OPA1 causes mitochondrial fragmentation (Griparic et al., 2004), and overexpression of OPA1 induces mitochondrial elongation (Cipolat et al., 2004). Although Mfn1, Mfn2 and OPA1 all are essential for controlling mitochondrial fusion, overexpression of OPA1 can counteract the effect of Mfn2 knockout but not the effect of Mfn1 loss on mitochondrial morphology (Cipolat et al., 2004). This also confirms that there is a functional difference between Mfn1 and Mfn2.

### 1.1.2 The mitochondrial fission machinery

#### *1.1.2.1 The mitochondrial fission machinery in yeast*

In yeast, four key proteins are involved in the mitochondrial division process: Dnm1p, Fis1p, Mdv1p and Caf4p (Table 1). The dynamin-related GTPase Dnm1p is a central component of the mitochondrial fission machinery, which was initially discovered by screening yeast mutants with defective mitochondrial morphology (Bleazard et al., 1999; Otsuga et al., 1998). The re-localization of Dnm1p from the cytosol to mitochondria is the key step in mitochondrial fission. Fis1p acts as the mitochondrial receptor to recruit Dnm1p to the surface of mitochondria via one of the adaptors Mdv1p or Caf4p, acting as a protein bridge between Fis1p and Dnm1p. Then Dnm1p self-assembles around the mitochondrial surface forming spiral-like structures at constriction sites leading to scission of mitochondria (Griffin et al., 2005; Ingerman et al., 2005; Legesse-Miller et al., 2003; Tieu and Nunnari, 2000).

| Yeast | GTPase | Mammals | GTPase |
|---|---|---|---|
| Dnm1p | + | Drp1 | + |
| Fis1p | - | Fis1 | - |
| Mdv1p | - | - | |
| Caf4p | - | - | |
| - | | MIEF1/2 | - |
| - | | Mff | - |
| Vps1p | + | Dyn2 | + |
| Fzo1p | + | Mfn1/2 | + |
| Mgm1p | + | OPA1 | + |
| Ugo1p | - | SLC25A46 (just similar) | - |

Table 1. Key mitochondria-shaping proteins in yeast and mammals

Fis1p is anchored in the MOM via the C-terminal tail and the N-terminal domain is facing the cytosol (Mozdy et al., 2000). The cytoplasmic domain of Fis1p contains a tetratricopeptide repeat (TPR) domain forming a concave surface, and a short N-terminal helix, which is required for recruiting and binding Mdv1p to the concave surface (Suzuki et al., 2005). Both Mdv1p and its paralog Caf4p are soluble cytosolic proteins containing an N-terminal extension (NTE), a middle coiled-coil domain, and a C-terminal WD repeat domain. Acting as molecular adaptors and bridge between Dnm1p and Fis1p, these proteins can bind to Dnm1p through the WD repeat domain and then associate with Fis1p through the NTE region (Griffin et al., 2005; Tieu et al., 2002).

In Dnm1p-null cells, mitochondrial fission is blocked, mitochondria form long tubular appearances and mitochondrial membranes collapse to one side of the cell (Otsuga et al., 1998). Fis1p-null mutation also causes mitochondrial reticular formation (Mozdy et al., 2000). This indicates that both Dnm1p and Fis1p are critical for mitochondrial division in yeast. As to the two adaptors, in yeast cells with single Mdv1p-null or Caf4p-null mutant, mitochondrial morphology is similar to wild type, but in cells with double Mdv1p/Caf4p-null

mutants, like in Fis1p-null mutant cells, mitochondria show elongated and net-like morphology, and most of Dnm1p stays in the cytoplasm. Interestingly, it was reported that overexpression of Mdv1p or Caf4p can inhibit mitochondrial fission as well. This may occur because overexpressed Mdv1p or Caf4p blocks the recruitment of Dnm1p to mitochondria (Cerveny and Jensen, 2003; Griffin et al., 2005). Additionally, Mdv1p and Caf4p also have distinct functions, for instance Mdv1p is more active than Caf4p in promoting fission, whereas Caf4p but not Mdv1p in association with Fis1p can determine the polarized localization of Dnm1p clusters on the mitochondrial surface after Dnm1p recruitment (Schauss et al., 2006). However, it was further reported that Fis1p is not essential for Dnm1p recruitment or mitochondrial membrane scission in yeast under a specified condition. Mdv1p contains an N-terminal extension (NTE) domain, that binds with Fis1p (Tieu et al., 2002). A truncated form of Mdv1p lacking NTE domain, fused with the transmembrane domain of Tom20, can be tethered to the outer mitochondrial membrane. This is sufficient to recruit Dnm1p to mitochondria and trigger fission in the absence of Fis1p (Koirala et al., 2013).

#### *1.1.2.2 The regulation of mitochondrial fission in mammals*

##### 1.1.2.2.1 Drp1 and Dyn2

In mammals, the regulation of mitochondrial fission is much more complicated than in yeast. The dynamin-related GTPase Drp1 is the ortholog of Dnm1p and shares 42% homology with Dnm1p (Pitts et al., 1999). Drp1-null MEFs (mouse embryo fibroblasts) or Drp1-knockdown HeLa cells show elongated mitochondrial appearance (Ishihara et al., 2009; Lee et al., 2004; Wakabayashi et al., 2009), which suggests that Drp1 is the central regulator of mitochondrial division in mammals like Dnm1p in yeast. Drp1 is primarily distributed in the cytoplasm, recruited by its four known receptors (Fis1, Mff, MIEF1/MiD51, MIEF2/MiD49, see Table 1) from the cytoplasm to the mitochondrial surface, where Drp1 is assembled into higher-order complexes that wrap around the mitochondrial surface to trigger mitochondrial fission through its GTPase activity (Otera et al., 2013). Another member of the dynamin-related family, Dynamin 2 (Dyn2) exists in cells ubiquitously, promoting the membrane remodeling of multiple organelles (Ferguson and De Camilli, 2012). Recently, it was reported that Dyn2 is recruited to mitochondrial constrictions transiently after Drp1 puncta accumulation and works in the final step of mitochondrial division (Lee et al., 2016). In the fission process, Drp1 recruitment to mitochondria is a critical step, but the mechanisms underlying this process are not fully understood. A number of issues remain to be elucidated, for instance, why multiple mitochondrial receptors of Drp1 simultaneously exist in the cell, whether these receptors work independently of each other or in a coordinated pathway, how the post-translational modifications of Drp1 impact on the process of mitochondrial division, etc.

##### 1.1.2.2.2 Fis1 and Mff

Fis1 was the first identified receptor of Drp1 in mammals, and is the ortholog of the yeast Fis1p. hFis1 is localized to the outer mitochondrial membrane via its C-terminal TM domain, and the N-terminal region of hFis1 faces the cytosol (James et al., 2003; Yoon et al., 2003).

Overexpression of hFis1 promotes mitochondrial fission, resulting in extensively fragmented mitochondria (James et al., 2003; Yoon et al., 2003). However, several studies report that knockdown of Fis1 results in distinct mitochondrial morphologies in different cell lines. For example, in HeLa cells or in Fis1-null MEFs, absence of Fis1 can induce mitochondrial elongation (Arai et al., 2004; Lee et al., 2004; Loson et al., 2013), whereas knockdown of hFis1 in HCT116 cells does not affect mitochondrial morphology (Otera et al., 2010). Interestingly, it has been found that overexpression and knockdown of hFis1 does not affect the distribution of Drp1 in most human cells. For instance, elevated levels of hFis1 in 293T cells does not affect the subcellular distribution of Drp1 (Zhao et al., 2011). Likewise, knockdown of hFis1 in HeLa and HCT116 cells does not reduce levels of Drp1 on mitochondria (Lee et al., 2004; Otera et al., 2010). However, in Fis1-null MEFs, Drp1 puncta on mitochondria are reduced to some degree (Loson et al., 2013). Taken together, these studies suggest that Fis1 might have certain roles in some types of mammalian cells, whereas in other cell types it likely plays minor roles in Drp1-dependent mitochondrial fission. In spite of this fact, it is consistently found that overexpression of Fis1 can induce extensive mitochondrial fragmentation in all analyzed mammalian cells. This implies that Fis1-induced mitochondrial fission is likely at least under certain conditions involved in a Drp1-independent fission pathway. Recent reports show that the GTPase regulator protein TBC1D15 binding to hFis1 but not to Drp1 may play an important role in the regulation of mitochondrial morphology (Onoue et al., 2013). TBC1D15 is the GTPase-activating protein of Rab7, which is recruited to mitochondria through the interaction between Fis1 and TBC1D15, and the Rab7-TBC1D15-Fis1 complex plays a role in mitochondrial fission (Wong et al., 2018). In normal conditions, the interaction between hFis1 and Drp1 is weak, but after treatment with different apoptotic or autophagic stimuli, an increased interaction between hFis1 and Drp1 is observed in cells accompanied with mitochondrial fragmentation (Ciarlo et al., 2010; De Palma et al., 2010; Kaddour-Djebbar et al., 2010; Shen et al., 2014; Zhao et al., 2011). These data indicate that hFis1 may have important functions in stress-induced mitochondrial fission, whereas Mff is more likely to play a critical role in normal physiological Drp1-dependent mitochondrial division in mammals. Collectively, the mechanisms underlying hFis1-mediated mitochondrial fission are not fully understood so far. However, two reports suggest hFis1 has additional functions in controlling the association of MIEF1 with Drp1 via its direct interaction with MIEF1 to block MIEF1's further binding to Drp1 (Zhao et al., 2011). Further supporting this hypothesis is that elevated levels of hFis1 evidently can reduce the interaction between Drp1 and MIEF1 or MIEF2, respectively (Liu et al., 2013).

Mff was primarily identified through small interfering RNA (siRNA) screening in *Drosophila* cells, and it exists in metazoans but not in yeast (Gandre-Babbe and van der Bliek, 2008). The Mff gene generates at least nine different isoforms by alternative splicing. Mff has a C-terminal transmembrane domain, by which it is anchored in the mitochondrial outer membrane, while its N-terminal region facing to the cytosol contains three short amino acid repeats (R1-R3 motifs) and a coiled-coil domain. The 50 N-terminal residues containing

R1 and R2 motifs are essential for Drp1 recruitment, and this is also the minimal region required for Drp1-Mff interaction (Gandre-Babbe and van der Bliek, 2008; Liu and Chan, 2015; Otera et al., 2010). Knockdown of Mff in HeLa cells by siRNA (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010) or knockout of Mff in MEFs (Loson et al., 2013) severely inhibits mitochondrial fission and abrogates Drp1 recruitment to mitochondria, whereas overexpression of Mff in HeLa cells induces extensive mitochondrial fission and recruits most of Drp1 from the cytosol to mitochondria (Otera et al., 2010). These findings suggest that Mff is the major receptor for Drp1 recruitment to mitochondria and thus actively acts in the mitochondrial fission process of mammals.

#### 1.1.2.2.3 MIEF1 and MIEF2

MIEF1 and its paralog MIEF2 (also known as MiD51 and MiD49) are conserved vertebrate-specific mitochondrial proteins (Zhao et al., 2013a). They were characterized by our group and by others as novel mitochondrial receptors for Drp1 recruitment to mitochondria (Liu et al., 2013; Palmer et al., 2011; Zhao et al., 2011). MIEF1 and MIEF2 are highly similar with respect to protein sequences, sharing 45% amino acid identity in human. Both of them have an N-terminal transmembrane domain anchoring them in the mitochondrial outer membranes (Liu et al., 2013; Palmer et al., 2011).

In 293T and HeLa cells, expression of exogenous MIEF1 or MIEF2 consistently induces mitochondrial elongation. Both of these proteins can interact with and recruit Drp1 to mitochondria and distribute in a punctate manner on the mitochondrial surface (Liu et al., 2013; Zhao et al., 2011). In Cos-7 cells, expression of exogenous MIEF1 or MIEF2 also leads to mitochondrial fusion and accumulation of Drp1 on mitochondria, however, at low levels of the protein, mitochondrial morphology is relatively normal (Palmer et al., 2011). Further studies show that longer-term expression of MIEF1 results in a variety of morphological changes of mitochondria from fragmentation to a network state, suggesting that low levels of exogenous MiD51/MIEF1 increase fission events, whereas at high expression levels, fission events are inhibited, resulting in opposing fusion (Elgass et al., 2015). On the other hand, knockdown of MIEF1 and MIEF2 by siRNA leads to inconsistent effects on mitochondrial morphology. It was reported that in Cos-7 monkey cells, knockdown of both MIEF genes is required to cause a mitochondrial fusion phenotype (Palmer et al., 2011), and another group found that in MEFs (mouse embryonic fibroblasts), knockdown of either of the two MIEF genes caused a similar mitochondrial fusion phenotype. Knockdown of both genes has been reported to induce mitochondrial elongation in Cos-7 cells and MEFs (Loson et al., 2013). However, it was also reported that in human HeLa cells, down-regulation of MIEF1 by siRNA resulted in mitochondrial fission (Zhao et al., 2011), while complete knockout of either MIEF alone, or double-knockout of both MIEF1/2 in HeLa cells caused mitochondrial elongation (Otera et al., 2016). The reason for these inconsistent results is currently unclear, but one possible explanation is that the relative levels of MIEFs in the cell can control the balance of mitochondrial fission and fusion differently depending on various cell types. It can be that the different cell types used for experiments have dissimilar endogenous levels of the

fission/fusion proteins and also are differentially regulated depending on cell type and physiological state.

Furthermore, MIEF1 and MIEF2 are different in some aspects. Biochemical analysis shows that in addition to monomeric form, MIEF1 appears predominantly as dimers, whereas MIEF2 appears as oligomers. Importantly, the first 1-48 residues including the transmembrane domain are required for oligomerization of MIEF2, whereas the transmembrane domain is not crucial for dimerization of MIEF1. Additionally, MIEF1 and MIEF2 can form heterodimers (Liu et al., 2013). Moreover, their different crystal structures also indicate their distinct functions. Both proteins have a nucleotidyl transferase domain, but MIEF1 can bind nucleotide diphosphates (ADP and GDP), while MIEF2 does not, and MIEF1 binding to ADP can stimulate Drp1 oligomerization, self-assembly and its GTPase activity (Loson et al., 2014; Loson et al., 2015; Richter et al., 2014). Interestingly, treatment of MIEF1- or MIEF2- overexpressing cells with antimycin A, an inhibitor of complex III of the electron transport chain, leads to mitochondrial fragmentation in cells overexpressing MIEF1 but not in cells overexpressing MIEF2. Moreover, MIEF1-induced fission requires ADP binding to MIEF1 in this process (Loson et al., 2014; Loson et al., 2013).

Although a large amount of effort has been devoted to understand the potential functions of MIEF1/2 in the regulation of mitochondrial dynamics, there are a number of questions to be further investigated. For example: (1) Whether MIEF1 and MIEF2 work together or they are involved in different steps during the Drp1 recruitment process; (2) Whether and how MIEFs work together with Mff or hFis1 during Drp1-dependent mitochondrial fission; (3) If the relative levels of the two MIEFs may affect the balance of mitochondrial fission and fusion; (4) How the potential post-translation modifications of these Drp1 receptors impact mitochondrial fission; (5) Whether they also play roles in other aspects of cellular physiology, besides mitochondrial dynamics.

#### 1.1.2.2.4 Drp1 phosphorylation and dephosphorylation

In general, mitochondrial dynamics can be modulated by the amount, location and activity of mitochondria-shaping proteins and post-translational modifications of these proteins constitute important aspects for controlling the balance between mitochondrial fusion and fission. In a recent review, the different types of post-translational modifications occurring in the core mitochondria-shaping proteins have been presented, including phosphorylation, S-nitrosylation, sumoylation, ubiquitination, tyrosine sulfation and acetylation (Pagliuso et al., 2018; Willems et al., 2015). So far, Drp1 phosphorylation/dephosphorylation is the best studied and viewed as an important modification for the regulation of mitochondrial dynamics (Mishra and Chan, 2016; Tilokani et al., 2018). Here we will focus on how phosphorylation/dephosphorylation of Drp1 regulates mitochondrial fission.

In human cells, phosphorylation/dephosphorylation of Drp1 occurs at two serine residues, S616 and S637, located at the junction of Drp1's variable domain and GTPase effector domain (corresponding to human Drp1 isoform 1). These two sites are believed to have

opposite effects on Drp1-driven mitochondrial fission. Drp1-S616 phosphorylation is regulated by CDK1/cyclin B during mitosis and leads to mitochondrial fragmentation (Taguchi et al., 2007). In contrast, Drp1-S637 is phosphorylated by cAMP-dependent protein kinase A (PKA), which inhibits the GTPase activity of Drp1, leading to mitochondrial elongation. Mutation of the S637 site to Asp ($Drp1^{S637D}$) is believed to have similar effects as PKA stimulation. Additionally, Drp1 phosphorylated at the S637 residue can be dephosphorylated by calcineurin, promoting mitochondrial division (Chang and Blackstone, 2007; Cribbs and Strack, 2007). Interestingly, it was reported that when using a double phosphomimetic Drp1 mutant $Drp1^{S616D/S637D}$, the phosphorylation status of S637 was dominant over that of S616 in controlling the distribution of Drp1 in cells and the mitochondrial morphology (Cereghetti et al., 2008). Based on the results described above, it is believed that phosphorylation of Drp1 at S637 by PKA prevents its recruitment to mitochondria, thereby resulting in mitochondrial elongation, but a recent report shows that the Drp1 phosphomimetic mutant $Drp1^{S637D}$ is still recruited to mitochondria and interacts with Mff (Zhang et al., 2016). Moreover, a problem exists in that PKA is a multifunctional enzyme with a broad substrate specificity, therefore it cannot be excluded that PKA simultaneously affects the phosphorylation status of other mitochondria-shaping proteins, such as Mfn2 and OPA1 (Chang and Blackstone, 2010; Willems et al., 2015; Zhou et al., 2010). Furthermore, the reversible phosphorylation and dephosphorylation of endogenous Drp1 is probably a dynamic process, while the phosphomimetic substitution lacks the dynamic aspects of endogenous phosphorylation in vivo as suggested (Chang and Blackstone, 2007).

Furthermore, the mitochondrial scaffolding protein AKAP121 (A-kinase anchoring protein 121) is also involved in Drp1 phosphorylation via translocation of PKA to the MOM, and controlling mitochondrial dynamics through PKA-modulated phosphorylation of Drp1 at S637. In line with this, knockdown of AKAP121 induces mitochondrial fragmentation, whereas overexpression of AKAP121 promotes mitochondrial elongation (Dickey and Strack, 2011; Merrill et al., 2011). These results are in agreement with the described effect of PKA stimulation. In addition, under hypoxia, AKAP121 together with Siah2 were described to inhibit Drp1-Fis1 interaction independently of PKA, inducing mitochondrial fission (Kim et al., 2011).

In addition, the two serine residues S616 and S637 can be phosphorylated and dephosphorylated by different kinases and phosphatases. For example, the Drp1-S637 site can be phosphorylated by $Ca^{2+}$/calmodulin-dependent protein kinase I α (CaMKIα), and this phosphorylation can stimulate mitochondrial fragmentation and increased affinity between Drp1 and hFis1 (Han et al., 2008). In neurons, protein phosphatase 2A (PP2A) also dephosphorylates Drp1 at S637 via its neuron-specific Bbeta2 regulatory subunit to cause mitochondrial fragmentation (Dickey and Strack, 2011). Similarly, multiple kinases have been found to be involved in the phosphorylation of Drp1 at S616 in addition to CDK1/cyclin B complex, for example, ERK1/2- and PKCδ-induced phosphorylation at S616 results in mitochondrial fragmentation under hyperglycemia and oxidative stress (Qi et al., 2011; Yu et

al., 2011), whereas CDK5-mediated phosphorylation at this serine residue reduces the activity of Drp1 and leads to mitochondrial elongation (Cho et al., 2014).

Taking these data into account, it is not fully understood how the phosphorylation status of Drp1 promotes or inhibits mitochondrial fission. Whether individual kinases and phosphatases that regulate phosphorylation and dephosphorylation of Drp1 can also simultaneously affect the phosphorylation status of other mitochondria-shaping proteins needs to be clarified.

### 1.1.3 The roles of the endoplasmic reticulum in mitochondrial dynamics

The endoplasmic reticulum (ER) communicates with mitochondria through mitochondria-associated ER membranes (MAMs) in yeast and mammals, and these connections have been shown to participate in different physiological functions, such as phospholipid synthesis, $Ca^{2+}$-mediated signal transduction, protein import, mitochondrial distribution, mitophagy, and so on (de Brito and Scorrano, 2010; Lang et al., 2015; Murley et al., 2013). It is also reported that some of the mitochondria-shaping proteins are involved in this intercommunication. The mitochondrial profusion protein Mfn2 is localized on both mitochondria and the ER, and Mfn2 on the ER associates with Mfn1/2 on mitochondria and tethers the ER and mitochondria together to maintain the efficient mitochondrial $Ca^{2+}$ uptake, which is essential for ATP production (de Brito and Scorrano, 2008).

The ER has been also shown to play an active role in mitochondrial division. ER tubules were observed to wrap around mitochondria, mark the prospective sites of mitochondrial division and reduce the mitochondrial diameter by about 30% before Drp1 recruitment, which is to be followed by mitochondrial division, and the marked ER tubules mainly occur at positions of Drp1 and Mff foci. In fact, ER tubules also mark positions of mitochondrial constriction in the absence of Mff or Drp1 (Friedman et al., 2011). In addition to Drp1 and Mff, two other receptors of Drp1, MIEF1 and MIEF2, are also observed at mitochondria-ER contact sites, and co-localized with other fission proteins, such as Drp1 and Mff. However, less than 40% of observed mitochondria-ER contacts at MIEF foci are also constriction sites, implicating MIEFs are not the essential factors to determine ER-mitochondria constriction sites (Elgass et al., 2015). Furthermore, MIEFs require the presence of Drp1 to form foci, whereas Mff can form foci in cells lacking Drp1 (Friedman et al., 2011; Richter et al., 2014). Another known Drp1 receptor Fis1, has been shown in *C. elegans* to enter into a complex containing Drp1, Mff and ER proteins at the ER-mitochondrial interface during stimulation of mitophagy (Shen et al., 2014). However, it is still unclear if to what extent ER tubules are essential for mitochondrial fission and fusion.

### 1.1.4 The roles of the actin cytoskeleton in mitochondrial dynamics

Besides the ER, the actin cytoskeleton is also involved in regulating of mitochondrial fission (Moore and Holzbaur, 2018; Tilokani et al., 2018). The cycling of actin assembly and disassembly around mitochondrial subpopulations efficiently promotes local mitochondrial fission dependent on Drp1. However, inhibiting Drp1 activity by transfected with Drp1-

K38A cannot affect actin cycling onto mitochondrial subpopulations as observed through live-cell imaging (Moore et al., 2016). Furthermore, some proteins related to the actin regulation play important roles in mitochondrial fission. An ER-localized actin regulator, INF2 (inverted formin 2) induces actin filaments to drive the initial mitochondrial constriction and then promotes Drp1 recruitment to ER-mitochondria constriction sites (Korobova et al., 2013). The isoform of the actin-nucleating protein Spire1C, localized to MOM, interacts with INF2, promoting actin assembly at the mitochondrial surface.

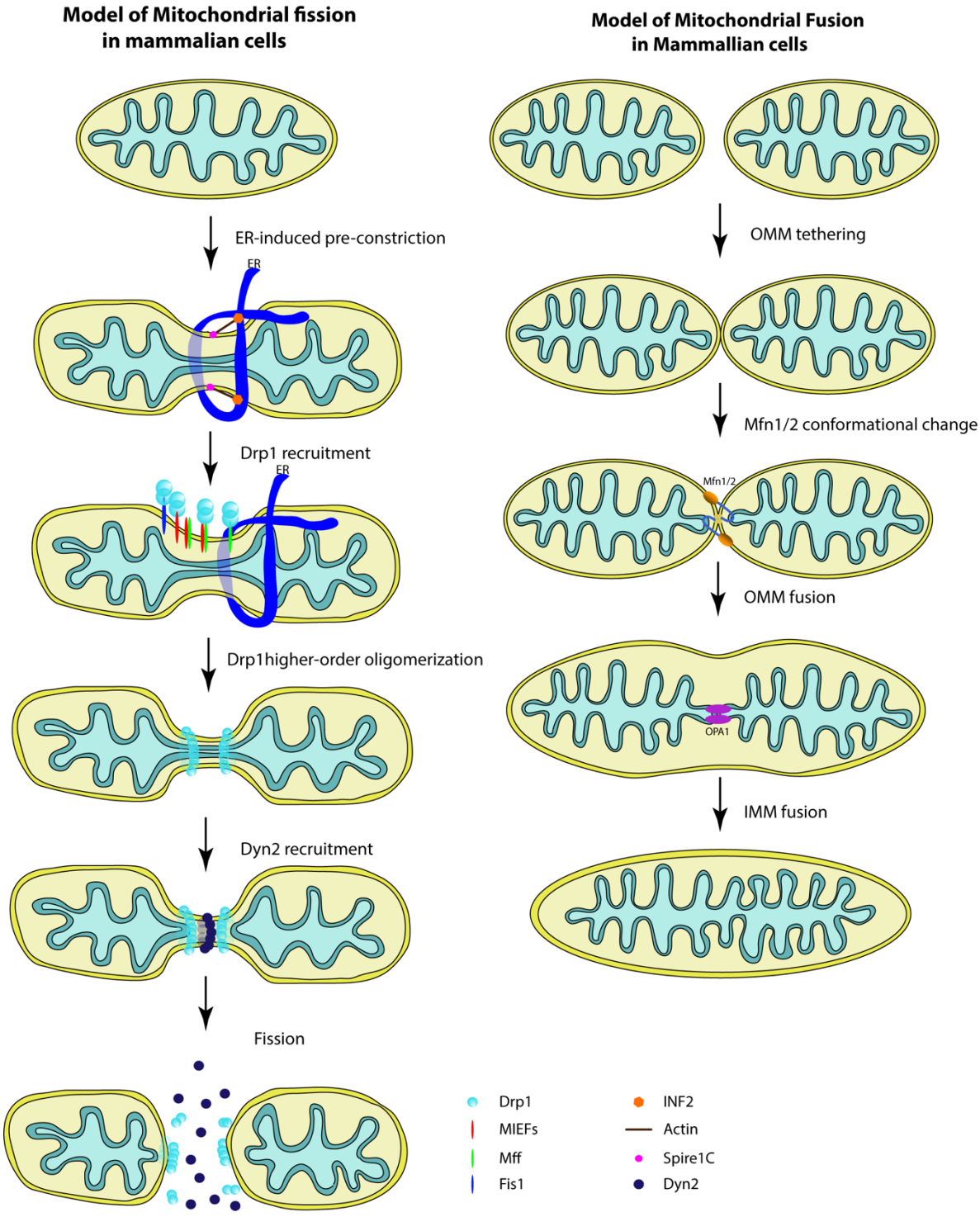


**Figure 1**. The proposed models of mitochondrial fission and fusion in mammalian cells

Disturbing Spire1C or its formin-binding activities can affect the mitochondrial constriction and further mitochondrial fission (Manor et al., 2015). Myosin II also plays similar roles in mitochondrial fission. Myosin II enriches at mitochondrial constriction sites, and deletion of Myosin II reduces Drp1 accumulation on mitochondria (Korobova et al., 2014). Interestingly, Sept2 silencing also decreases the Drp1 association with mitochondria and increases the average distance between Drp1 clusters (Pagliuso et al., 2016). In addition, knockdown of actin-regulating factors cortactin, cofilin, and Arp2/3 complexes results in elongated mitochondria and disassembled mitochondrial F-actin around mitochondrial subpopulations (Li et al., 2015; Moore et al., 2016). In sum, it is a great challenge to understand how the ER, actin cytoskeleton and Drp1 receptors interplay and coordinate their actions to mediate mitochondrial division.

Collectively, the proposed models of mitochondrial fission in mammalian cells are summarized in Figure 1. Mitochondria are pre-constricted by the ER, together with actin filaments associated with mitochondria by Spire1C and with ER by INF2. At the ER constriction site, Drp1 is recruited by its receptors to mitochondria and assembled to form higher-order oligomers around the mitochondrial surface. Then mitochondria are further constricted by Drp1 oligomers, and Dyn2 is recruited to the constricted site instantly to finish the scission of mitochondria through GTP hydrolysis.

## 1.2 THE CROSSTALK BETWEEN MITOCHONDRIAL DYNAMICS AND CELLULAR BIOLOGICAL PROCESSES

In living cells, mitochondria form a highly dynamic network, and continuously undertake fusion and fission changes. Though a very great deal of progress has been built on elucidating the molecular mechanisms that control mitochondrial morphology by mitochondria-shaping proteins in coordination with ER and actin-cytoskeleton, it is still poorly understood how mitochondrial dynamics and mitochondria-shaping proteins are mechanistically linked to mitochondrial functions and cellular biological processes.

So far, accumulating evidence indicates that mitochondrial dynamics plays a crucial role in controlling mitochondrial functions. The carefully orchestrated the balance of mitochondrial fusion and fission is critical for keeping a healthy population and normal physiological functions of mitochondria. Fission allows the exclusion of damaged mitochondria via mitophagy and mitochondrial biogenesis, whereas fusion allows mitochondria to exchange their substance including proteins and mtDNA among individual mitochondria (Knott et al., 2008). Abnormal mitochondrial dynamics is directly associated with mitochondrial dysfunction, thus impacting on a wide range of cellular processes, such as mitochondrial transport and biogenesis, cell-cycle regulation, cell proliferation and differentiation (Chen and Chan, 2009), energy metabolism, ROS production (Mishra and Chan, 2016; Willems et al., 2015), $Ca^{2+}$ signaling, mtDNA maintenance, apoptotic resistance of cells, mitochondrial quality control via autophagy (mitophagy), and ultimately programmed cell death, although the specific mechanisms are not clear (Mishra and Chan, 2014; Suen et al., 2008). Moreover, some important mitochondrial fission and fusion proteins have been established to be critical

for the embryonic development in mice (Chen et al., 2003; Chen et al., 2015; Davies et al., 2007; Ishihara et al., 2009), further emphasizing that functions of mitochondrial dynamics go far beyond the appearance of mitochondria and have important physiological consequences.

### 1.2.1 Mitochondrial dynamics and apoptosis

Emerging data indicate that mitochondrial dynamics participates in the regulation of cell death pathways and remodeling of the mitochondrial network takes place in response to cellular stresses such as hypoxia, drug treatments and various pathological conditions. During apoptosis mitochondria undergo extensive fragmentation and some proteins of the mitochondrial fusion/fission machinery are directly involved in the regulation of apoptosis (Otera and Mihara, 2012; Suen et al., 2008). In general, elongated mitochondria confer cellular resistance to apoptosis, whereas fragmented mitochondria make cells sensitive to apoptotic stimuli (Chen and Chan, 2005; Suen et al., 2008). For example, knockdown of the pro-fission proteins hFis1 or Drp1 leads to mitochondrial elongation and resistance to different apoptotic stimuli, whereas depletion of the profusion proteins Mfn1, Mfn2 or OPA1 leads to mitochondrial division and makes cells more sensitive to apoptosis (Otera and Mihara, 2012; Ugarte-Uribe and Garcia-Saez, 2014). In agreement with that mitochondrial dynamics regulates sensitivity of cells to apoptotic stimuli, it was previously reported that overexpression of MTGM/ROMO1 results in fragmented mitochondria and triggers a significant release of the death factor Smac/Diablo from mitochondria to the cytosol but no other death factors, for instance cytochrome *c*, AIF or Omi/HtrA2, and this did not lead to spontaneous apoptosis. However, down-regulation of MTGM results in elongated mitochondria and increases cell proliferation and the resistance of cells to apoptotic stimuli (Zhao et al., 2009). Furthermore, it was reported that MIEF1 and MIEF2 are also involved in the regulation of apoptosis. Expression of exogenous MIEF1 induces elevated autophagic activity and decreases the sensitivity of cells to apoptotic stimuli. Conversely, human cells depleted of MIEF1 are more sensitive to apoptotic stimuli (Zhao et al., 2011). However, loss of both MIEF1 and MIEF2 together with Mff in MEFs confers resistance to apoptosis (Osellame et al., 2016). Additionally, Drp1-dependent mitochondrial division controls cristae remodeling through MIEF1/2 during intrinsic apoptosis (Otera et al., 2016). Moreover, the OMM-associated E3 ubiquitin ligase MARCH5 coupled with MIEF2 controls Drp1-dependent mitochondrial fission and cell sensitivity to stress-induced apoptosis (Xu et al., 2016). Interestingly, MIEF1 is recently reported to regulate Bax translocation, and cells depleted of MIEF1 are sensitive to apoptotic stimuli and PINK1-Parkin-mediated mitophagy (Xian and Liou, 2019).

In addition, mitochondrial outer membrane permeabilization (MOMP) is an important phenomenon closely coupled with pro-apoptotic stimuli. MOMP leads to the release of cytochrome *c* from mitochondria to the cytosol, in turn leading to activation of caspases and subsequent cell death. Although the precise mechanisms of MOMP remain to be elucidated, it is accepted that Bax and Bak are indispensable for this process (Wei et al., 2001). In healthy cells Bax is normally distributed predominantly in the cytosol, and during apoptosis,

Bax is translocated from the cytosol to the surface of mitochondria (Wolter et al., 1997), where Bax in coordination with Bak results in MOMP (Nechushtan et al., 2001). Several lines of evidence show that some mitochondria-shaping proteins participate in the Bax translocation. For example, hFis1 is required for translocation of Bax from the cytosol to the surface of mitochondria (Lee et al., 2004), Drp1 is required for activation and oligomerization of Bax (Montessuit et al., 2010; Wang et al., 2015), and OPA1 regulates cristae remodeling and triggers the release of cytochrome *c* to the cytosol (Mopert et al., 2009; Saita et al., 2016).

It is generally accepted that one challenge of cancer therapy is the development of cancer resistance to chemotherapy and evading apoptosis constitutes one of the essential hallmarks of cancer, however, the machinery by which the resistance is developed in cancer still need be fully understood (Hanahan and Weinberg, 2000). Given the importance of mitochondrial dynamics in the regulation of cells resistance to apoptotic stimuli/ anticancer drugs, it is an important challenge to elucidate whether cancer cells can modify mitochondrial dynamics to thereby acquire resistance to anticancer drug treatment (Grandemange et al., 2009). Cumulative evidence is beginning to uncover a link between apoptotic resistance of cancer cells and abnormal mitochondrial dynamics. For instance, dysregulation of mitochondrial dynamics in lung cancer cells contributes to apoptotic resistance (Kawada et al., 2013; Rehman et al., 2012; Thomas and Jacobson, 2012). Treatments with different anticancer drugs can modify mitochondrial dynamics by regulating levels of mitochondria-shaping proteins in cancer (Lee et al., 2012; Tailor et al., 2013). The published data highlights the importance of mitochondrial dynamics in apoptotic resistance of cancer cells and in controlling life and death signals in cancer cells.

In summary, mitochondrial fragmentation occurs in most forms of apoptosis through activation of the mitochondrial fission machinery and/or inhibition of the fusion machinery in various physiological and pathophysiological conditions. However, how this process is regulated and the precise mechanisms by which the mitochondria-shaping proteins involved in the apoptotic progression are largely unclear.

### 1.2.2 Mitochondrial dynamics and mitophagy

The mitochondrial quality control is executed through mitophagy, which selectively removes senescent or damaged mitochondria and balances the overall mitochondrial mass between biogenesis and degradation. Mitophagy is known to be regulated by Parkin and PINK1 (PTEN-induced putative kinase protein 1) in mammals, and mutations in the genes encoding these proteins are associated with Parkinson's disease (Gomes and Scorrano, 2012; Youle and Narendra, 2011). While Parkin normally exists in the cytosol, when cells lose the mitochondrial membrane potential, such as by CCCP treatment, PINK1 accumulates at the surface of impaired mitochondria and recruits Parkin from the cytosol to mitochondria. At the mitochondrial surface, Parkin, recruited by PINK1 to damaged mitochondria, ubiquitylates some mitochondrial proteins (e.g. Mfn1/2) and promotes damaged mitochondria to be engulfed by lysosomes (Sugiura et al., 2014; Youle and Narendra, 2011). There are a number

of Parkin substrates on the mitochondrial outer membrane and in the cytosol, which are ubiquitinated by Parkin (Martinez et al., 2017; Sarraf et al., 2013). The mitochondrial outer membrane protein Miro1 (Mitochondrial Rho GTPase 1), essential for mitochondrial transport (Schwarz, 2013), interacts with Parkin and functions as calcium-sensitive docking site of Parkin during mitochondrial damage. Knockdown of Miro1 reduces Parkin translocation to mitochondria and suppresses mitophagy (Safiulina et al., 2018).

Growing evidence links mitochondrial dynamics with the regulation of mitophagy. The mitochondrial fusion factor Mfn2 was reported to mediate Parkin recruitment to damaged mitochondria. PINK1 can phosphorylate Mfn2, thereby promoting the translocation of Parkin to mitochondria and Parkin-mediated ubiquitylation of mitochondrial proteins. Loss of Mfn2 prevents the translocation of Parkin to mitochondria (Chen and Dorn, 2013), and high levels of Fis1 are associated with mitochondrial dysfunction and trigger autophagy (Gomes and Scorrano, 2008). In addition, CCCP treatment induces mitochondrial fragmentation in general, however, cells lacking Fis1 and Mff are highly resistant to CCCP-induced mitochondrial fragmentation. Nevertheless, this resistance can be reversed through overexpression of MIEF1 or MIEF2 (Loson et al., 2013; Osellame et al., 2016; Xian and Liou, 2019; Zhao et al., 2011). These results indicate that mitochondria-shaping proteins play important roles in regulating mitophagy.

### 1.2.3 Mitochondrial dynamics and cellular metabolism

The biological impacts of mitochondrial dynamics are not restricted to the regulation of cell death pathways and mitophagy, but also involved in other cellular biological processes. The most common view is that mitochondria play important roles in cellular energy production, and it is believed that elongated mitochondria are more active and produce more energy, whereas short and fragmented mitochondria are weak and have reduced ATP production for cellular processes. This indicates that mitochondrial energy metabolism is coupled to mitochondrial dynamics. Accumulating evidence show that the mitochondria-shaping proteins, besides controlling mitochondrial fusion and fission events, are involved in cellular metabolism (Mishra and Chan, 2016; Roy et al., 2015). For example, Mfn2 was recently discovered to regulate cell metabolism and insulin signaling by limiting the production of ROS, and Mfn2 is considered to be a more important factor for energy metabolism than Mfn1 (Zorzano et al., 2015). Another group also shows that ablation of both Mfn1 and Mfn2 in MEFs impairs ATP production and increases ROS production (Song et al., 2015). Furthermore, it was reported that Mfn2 regulates the expression of subunits that participate in OXPHOS complexes. Loss-of-function of Mfn2 reduces nuclear-encoded subunits of oxidative phosphorylation (OXPHOS) complexes I, II, III and V, whereas Mfn2 overexpression increases the subunits of complexes I, IV and V (Pich et al., 2005). In addition to mitofusins, also OPA1 is involved in the regulation of mitochondrial energy status. The level of OXPHOS can regulate mitochondrial inner membrane fusion through Yme1L, which cleaves OPA1 more efficiently under high OXPHOS conditions to promote inner membrane fusion, while the mitochondrial outer membrane is insensitive to OXPHOS

levels (Mishra et al., 2014). As to fission proteins, Drp1 is the central factor for mitochondrial division, and it was discovered that knockdown of Drp1 by siRNA in HeLa cells decreases cellular ATP content and elevates ROS levels. In MEFs deleted of Drp1, however, the intracellular ATP levels and ROS production are normal (Ishihara et al., 2009; Song et al., 2015; Wakabayashi et al., 2009). Additionally, MTGM/ROMO1 was previously discovered as a regulator of cellular ROS generation (Chung et al., 2006; Chung et al., 2008).

In addition, an increasing amount of studies show that dysregulation of mitochondrial dynamics is involved in the shift towards ATP generation through glycolysis during cancer development (Corrado et al., 2012), and abnormal mitochondrial fission or fusion regulates migration and invasion of breast cancer cells (Zhao et al., 2013b). Moreover, the pathophysiology of metabolic diseases such as type 2 diabetes and obesity has been found to be associated with abnormalities in mitochondrial division and fusion. Several core mitochondria-shaping proteins, such as Drp1, OPA1 and Mfn2, have been discovered to be involved in these metabolic diseases (Roy et al., 2015).

### 1.2.4 Mitochondrial dynamics and embryonic development

During the development of embryos to adult individuals, mitochondria undergo a series of changes in quantity, size and morphology as they mature from an immature state. It has been reported that few mitochondria exist with poorly developed cristae and lower mitochondrial mass in blastocysts and embryonic stem cells, and after one-week differentiation, the resulting cells contain numerous larger tubular mitochondria with distinct cristae (Bavister, 2006; Lonergan et al., 2007; St John et al., 2005).

Mitochondria-shaping proteins have been shown to play essential roles in embryonic development of mice and human, emphasizing the significance of mitochondrial dynamics in embryonic development. For instance, mice deficient in the fusion-promoting proteins Mfn1/Mfn2 die in midgestation (Chen et al., 2003) and embryonic lethality occurs by 13.5 days post coitum when lacking OPA1 (Davies et al., 2007). The fission-promoting factor Drp1 is essential for embryonic and brain development, and Drp1-null mice die at an early embryonic stage (Ishihara et al., 2009; Wakabayashi et al., 2009). Moreover, inhibition of Drp1-dependent mitochondrial division can impede myogenic differentiation and impair somatic cell reprogramming to pluripotent stem cells (Kim et al., 2013; Vazquez-Martin et al., 2012). In the two studies, a chemical inhibitor of Drp1, mdivi-1, has been used to block the functions of Drp1. These data imply that Drp1-dependent mitochondrial division is critical for myogenic differentiation and somatic cell reprogramming. Furthermore, it has been shown that MIEF1 and MIEF2, are differentially expressed in human fetal and adult tissues. MIEF1 mRNA was more abundant than MIEF2 in fetal tissues, while the MIEF2 mRNA level was higher than the MIEF1 in adult tissues (Liu et al., 2013). Additionally, Mff-deficient mice dye at 13 weeks by heart failure (Chen et al., 2015). Moreover, mitochondrial fusion is also necessary for cardiomyocyte differentiation. After ablation of Mfn1/2 or OPA1 in embryonic mouse heart or mouse embryonic stem cells, heart development and

cardiomyocyte differentiation are arrested. Meanwhile, calcineurin-induced $Ca^{2+}$ activity and Notch1 signaling activity are increased (Kasahara et al., 2013). Interestingly, Mfn2 is discovered to be specifically required for the maintenance of haematopoietic stem cells with extensive lymphoid potential based on the $Ca^{2+}$ transmission through Mfn2 tethering of ER and mitochondria (Luchsinger et al., 2016). Based on the data mentioned above, it is suggested that both mitochondrial fusion and fission, as well as the respective regulatory proteins are likely important for the maintenance and differentiation of stem cells and for embryonic development. Therefore, it will be crucial for future research in regenerative medicine to elucidate how to control the differentiation of stem cells and embryonic development through manipulating mitochondrial dynamics.

## 1.3 MITOCHONDRIAL DYNAMICS AND NEURODEGENERATIVE DISEASES

Mitochondrial dysfunction leads to many pathological problems, and a number of studies are focusing on the relationship between mitochondrial dynamics and neurodegenerative diseases, such as Alzheimer's disease (AD) (Corrado et al., 2012; Gao et al., 2017; Polanco et al., 2018; Qi et al., 2019; Wilson et al., 2013; Yoo and Jung, 2018). Neurodegenerative diseases are a group of disorders resulting in gradually progressive degeneration or death of neurons. In some hereditary neurodegenerative diseases, mitochondria-shaping proteins have been reported to play a critical role. For example, Charcot-Marie-Tooth neuropathy type 2A (CMT2A) is one type of peripheral neuropathies, which is associated with missense mutation of Mfn2 (Zuchner et al., 2004). Mutations of the OPA1 gene is found in a prevalent subset of autosomal dominant optic atrophy (ADOA) (Alexander et al., 2000). Till now, the treatment for neurodegenerative diseases is still a big challenge.

AD is a chronic irreversible neurodegenerative disorder, characterized by the extracellular amyloid β (Aβ) plaques and intraneuronal deposits of neurofibrillary tangles formed by protein tau aggregation. Aβ is distributed normally in plasma and cerebrospinal fluid (CSF) (Shoji et al., 1992), and derived through secondary proteolytic cleavage by γ-secretase complex from a 99aa C-terminal fragment (known as APP-C99) of amyloid precursor protein (APP), which is initially cleaved by β-secretase from the full-length APP (Portelius et al., 2011; Takami et al., 2009). Abnormal ER-mitochondria associated membranes (MAM) have received considerable attention in AD, as γ-secretase is highly active in the ER-mitochondria associated membranes, and Aβ is produced at these sites including the mitochondrial outer membrane and mitochondria-associated ER membranes (Area-Gomez et al., 2009; Schreiner et al., 2015). There Aβ aggregation increases the number of MAM points and mitochondrial calcium concentration (Hedskog et al., 2013). Additionally, the unprocessed APP-C99 is also enriched in ER-MAM in cells from AD patients, affecting the sphingolipid synthesis and hydrolysis (Pera et al., 2017). However, knockdown of Mfn2 decreases the production of Aβ (Leal et al., 2016). Furthermore, the contact sites between lysosomes and mitochondria are reported under physiological condition (Wong et al., 2018), but Aβ oligomers, the precursors of amyloid plaques, are found to disrupt the crosstalk between mitochondria and lysosomes in the early stage of Alzheimer's disease (Norambuena et al., 2018).

Alterations of mitochondrial structure are found in the brain tissues of AD patients, (Baloyannis, 2006; Hirai et al., 2001), and many sporadic mutations of the mtDNA occur in

AD patients (Coskun et al., 2004; Hirai et al., 2001). Drp1, as the key mitochondria-shaping GTPase, is reported to interact with Aβ (Manczak et al., 2011) and phosphorylated tau (Manczak and Reddy, 2012). Now, Drp1 has been considered as one of the targets for the AD treatment. Mdivi-1 (Drp1 small molecular inhibitor) treatment decreases the extracellular Aβ deposition, increases the production of ATP and rescues the dysfunction of mitochondria in AD cybrids (Gan et al., 2014; Wang et al., 2017). Furthermore, the levels of the mitochondrial fission proteins Drp1 and Fis1 are increased and the levels of the mitochondrial fusion proteins Mfn1, Mfn2, OPA1 are decreased in AD patients or cells compared with control (Manczak et al., 2011; Reddy et al., 2018). However, the roles of mitochondria-shaping proteins in AD need to be further studied. Treatment targeting mitochondrial dysfunction in AD is the aim for the future.

## 1.4 IN SUMMARY

In the past twenty years, a great deal of progress has been built on identifying core components and regulatory factors of the mitochondrial fission/fusion machineries in mammals, and a considerable effort has been made in attempting to elucidate the potential molecular mechanisms that control and regulate mitochondrial fission and fusion events. This has provided us a number of new insights into the mechanisms of mitochondrial dynamics, and crosstalk with various cellular biological processes, embryonic development, numerous mitochondria-associated human diseases, as well as tumorigenesis and tumor progression. Although early studies believed that the mitochondrial fission and fusion machineries are evolutionarily conserved from yeast to human, it has become increasingly clear that these processes are much more complicated in mammalian cells than in single-cell yeast. The precise mechanisms and regulation of mitochondrial fission and fusion events are still largely unclear, and the relationship between mitochondrial dynamics and cellular biological processes as well as different human diseases needs to be further investigated.

# 2 PRESENT INVESTIGATION

## AIM OF THE STUDY

The overall aim in this thesis was to investigate deeply the functions of the Drp1 receptors Fis1, MIEF1/2 and Mff in mitochondrial dynamics of human cells and further understand the molecular mechanisms of human mitochondrial dynamics. The four specific aims are to:

1. Comparing the similarity and divergence of the human paralogs MIEF1 and MIEF2 in protein structures and functions.
2. Investigating how MIEF1 and MIEF2 regulate mitochondrial fission and whether Mff and MIEFs have distinctive functions in the Drp1-mediated mitochondrial fission process.
3. Elucidating the roles of Drp1 S637 phosphorylation status in regulating mitochondrial recruitment of Drp1 by MIEFs and Mff, and in Drp1-modulated mitochondrial fission.
4. Investigating the underlying molecular mechanism of hFis1-triggered mitochondrial fragmentation in human cells.

# 3 RESULTS AND DISCUSSION

## 3.1 STUDY I

***The mitochondrial elongation factors MIEF1 and MIEF2 play distinct roles in mitochondrial dynamics to some extent***

Dynamin-related protein 1 (Drp1) is a central GTPase in mitochondrial fission, and shifted from the cytosol to mitochondria, further resulting in mitochondrial fission. MIEFs (MIEF1 and MIEF2) have been described as the major receptors of Drp1 in previous reports (Palmer et al., 2011; Zhao et al., 2011). Here we compare the functions of these two paralogs MIEF1 and MIEF2 in mitochondrial dynamics.

### 3.1.1 Protein sequences of MIEFs and their expressions in human cell lines and tissues

**Similarities:** 1) MIEF1 and MIEF2 are highly conserved in protein sequences, both contain C-terminal cytosolic part and a predicted N-terminal transmembrane domain localized in the mitochondrial outer membrane. 2) MIEF1 shares 45% sequence identity with MIEF2. 3) Both paralogs exist only in vertebrates and there are no homologs in yeast and invertebrates.

**Divergences:** 1) The molecular masses of MIEF1 and MIEF2 are ~52 and ~49kDa separately determined from Western blotting. 2) They show distinct expression patterns in different human cancer cell lines, human fetal and adult tissues. In detail, expression of MIEF1 is relatively higher than MIEF2 in fetal tissues. In contrast, its expression is relatively lower than MIEF2 in adult tissues.

### 3.1.2 The effects of wild-type MIEFs and their cytosolic mutants on mitochondrial morphology and interaction with Drp1

**Similarities:** 1) Both the full-length MIEFs and their cytosolic mutants lacking transmembrane domains can bind to Drp1. However, the binding abilities of the cytosolic mutants are relatively lower than that of the full-length proteins. 2) Like wild-type MIEFs, the cytosolic mutants of MIEFs can also induce mitochondrial elongation when overexpressed.

**Divergences:** Overexpression of MIEF1 results in large Drp1 aggregates on clustered mitochondria, while MIEF2 overexpression often leads to punctate accumulation of Drp1 along elongated mitochondria. Moreover, MIEF2 induces a stronger fusion phenotype than MIEF1 at similar expression levels.

### 3.1.3 Oligomer formation of MIEFs and the potential domains for oligomerization

**Similarities:** MIEF1 and MIEF2 both can form homodimers and higher-order oligomers and they also interact with each other and form heterodimers.

**Divergences:** 1) MIEF1 and MIEF2 have different domains that are responsible for the formation of their oligomers. The MIEF1 mutant lacking amino acids 109-154 is incapable of dimerization and higher-order oligomerization, whereas the first 49 amino acids of MIEF2 are essential for oligomerization. 2) Overexpression of either of these mutants without essential domains of oligomerization still induces mitochondrial elongation, and the MIEF1 mutant Δ109-154 interacts with Drp1 at the similar level as the wild-type control.

### 3.1.4 Roles of MIEFs in mitochondrial dynamics related to other Drp1 receptors

**Similarities:** hFis1 and Mff have been recognized as Drp1 receptors also, and overexpression of either one causes the mitochondrial fission phenotype (Chan, 2012; Zhao et al., 2013a). Our data showed that both MIEF1 and MIEF2 have the ability to interact with hFis1.

**Divergences:** Both hFis1 and Mff can reverse MIEF1- or MIEF2-induced mitochondrial fusion phenotype, but they are more efficient to counteract mitochondrial fusion induced by MIEF1 compared to MIEF2.

So far, no homologs of MIEF1 and MIEF2 have been identified in yeast, which provides evidence of divergent evolution of functions in Drp1-dependent fission between yeast and vertebrates. Even though both MIEFs participate in Drp1-driven mitochondrial fission, they still have distinct functions to some extent, implicating that they may have divergent functions in cells. Furthermore, whether both MIEFs can coordinately work together for Drp1 recruitment needs to be further elucidated.

## 3.2 STUDY II

***MIEF1/2 function as adaptors for the initial Drp1 recruitment to mitochondria and regulate the association of Drp1 with Mff***

In mammalian cells, there are four mitochondrial outer membrane anchored proteins, Fis1, Mff, MIEF1 and MIEF2, which are responsible for recruitment of cytosolic Drp1 to mitochondria. Overexpression of hFis1 or Mff results in severe mitochondrial fragmentation (Gandre-Babbe and van der Bliek, 2008; James et al., 2003; Yoon et al., 2003), whereas overexpression of MIEF1 or MIEF2 leads to mitochondrial elongation, and further to the perinuclear mitochondrial clustering as described above. However, whether these mitochondrial receptors work together in a Drp1-dependent mitochondrial fission process is poorly understood. Here we investigated the underlying molecular mechanism of Drp1-dependent mitochondrial fission controlled by MIEFs and Mff.

### 3.2.1 MIEF1/2 in coordination with Mff act at distinct steps in the mitochondrial recruitment of Drp1

**Firstly, MIEFs have important roles in the initial recruitment of Drp1 to mitochondria and affect Mff-mediated recruitment of Drp1 to mitochondria.**

Immunofluorescence showed that depletion of MIEFs or Mff obviously decreased the Drp1 co-localization with mitochondria and induced mitochondrial elongation. However, simultaneous knockdown or knockout of both MIEFs, the Drp1 recruitment to mitochondria induced by Mff overexpression is significantly decreased. In contrast, knockdown of Mff by siRNA affected neither MIEFs-mediated recruitment of Drp1 to mitochondria nor the interaction between Drp1 and MIEFs. Additionally, cells without all of MIEFs and Mff exhibited further lower Drp1 co-localization with mitochondria. These data implied that even though both MIEFs and Mff have the ability to translocate Drp1 from the cytosol to mitochondria, the Drp1 recruitment through Mff largely relied on the

existence of MIEFs. In contrast, Mff was dispensable for the MIEF-mediated Drp1 recruitment. According to these results, we have suggested a model for this initial step of Drp1 recruitment (Figure 2a), in which MIEFs play a key role in the initial recruitment of Drp1 and depletion of MIEFs reduces Mff-driven recruitment of Drp1.

**Secondly, MIEF1/2 work as adaptors connecting Drp1 and Mff to form a Drp1-MIEF-Mff triplex**

Confocal images combined with surface rendering revealed that Drp1, MIEF and Mff formed four patterns of co-localization at the mitochondrial surface: Drp1-MIEF, Drp1-Mff, Drp1-MIEF-Mff and MIEF-Mff. Co-immunoprecipitation (co-IP) experiments and a sequential co-IP experiment proved the existence of these complexes. Using different Drp1 binding-deficient MIEFs (MIEF1Δ160-169, MIEF2Δ151-160) and Mff (MffΔ1-50) mutants subjected to co-IP experiments, we further revealed that MIEF functions as a molecular bridge linking Drp1 and Mff together in the trimeric complex, as exhibited in the schematic diagram Figure 2b.

**Next, endogenous MIEF1/2 promotes the direct association between Drp1 and Mff**

To compare the levels of the Drp1-Mff complex in wild-type and MIEF1&2 double-knockout (MIEF-DKO) cells, we designed a set of experiments as follows. First, we immunodepleted endogenous MIEFs from wild-type 293T cells to remove three of the MIEF-related complexes (Drp1-MIEF, Drp1-MIEF-Mff and MIEF-Mff), then the supernatant together with the cell lysate of MIEF-DKO cells were subjected to co-IP with Mff antibody. Western blotting showed that the amount of Drp1-Mff complex in MIEF-DKO cells was much less than in wild-type cells. This data implicates that endogenous MIEFs in wild-type cells facilitate the direct Mff-Drp1 interaction, as the model displays (Figure 2c).

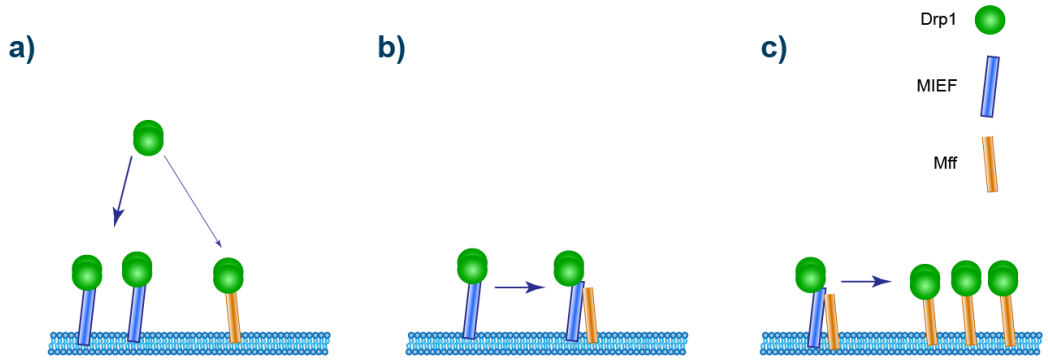


**Figure 2.** Separate schematic diagrams for the steps of Drp1 recruitment from the cytosol to mitochondria. 1a, MIEFs have important roles in the initial recruitment of Drp1 to mitochondria. 1b, MIEF1/2 work as adaptors to form a Drp1-MIEF-Mff triplex. 1c, the existence of endogenous MIEF1/2 promotes the direct association between Drp1 and Mff.

### 3.2.2 The relative levels of MIEFs mediate the balance of mitochondrial fusion and fission

We next tested whether the different levels of Mff or MIEFs affect the association of Drp1 with MIEFs or Mff. Our results revealed that increased levels of MIEFs decreased

the association between Drp1 and Mff, whereas elevated expression of Mff reduced the interaction of MIEFs with Drp1. These data implied that Mff and MIEF competed the interaction with Drp1.

Then we transfected MIEF-DKO cells with exogenous MIEF1 or MIEF2 to further explore the effect of different levels of MIEFs on mitochondrial morphology. We found that lower levels of exogenous MIEFs induced fragmented mitochondria, while cells with elongated mitochondria always had higher expressions of exogenous MIEFs. To further confirm these results, we knocked down endogenous MIEF1/2 by siRNA treatment in 293T cells and re-introduced the mouse Mief1 or Mief2 cDNA. The similar results were observed as described in the above experiments. However, when we re-introduced mouse Mff cDNA to human 293T cells treated with Mff siRNA, mitochondrial fragmentation always occurs no matter of the expression levels of mouse Mff were high or low. These data suggested that higher levels of MIEFs prevent mitochondrial fission, whereas appropriate levels of MIEFs facilitate fission.

Taken all together, in this study, we presented that Drp1 is recruited by MIEF together with Mff in a sequential and coordinated manner for Drp1-mediated mitochondrial fission, and the relative levels of MIEFs mediate the balance of mitochondrial fusion and fission. According to our results, we propose three models to explain how MIEFs and Mff mediate the balance of mitochondrial dynamics (Figure 3). When higher levels of MIEFs exist in cells, Drp1 is sequestered in Drp1-MIEF-Mff and/or Drp1-MIEF complexes, inhibiting the direct Drp1-Mff

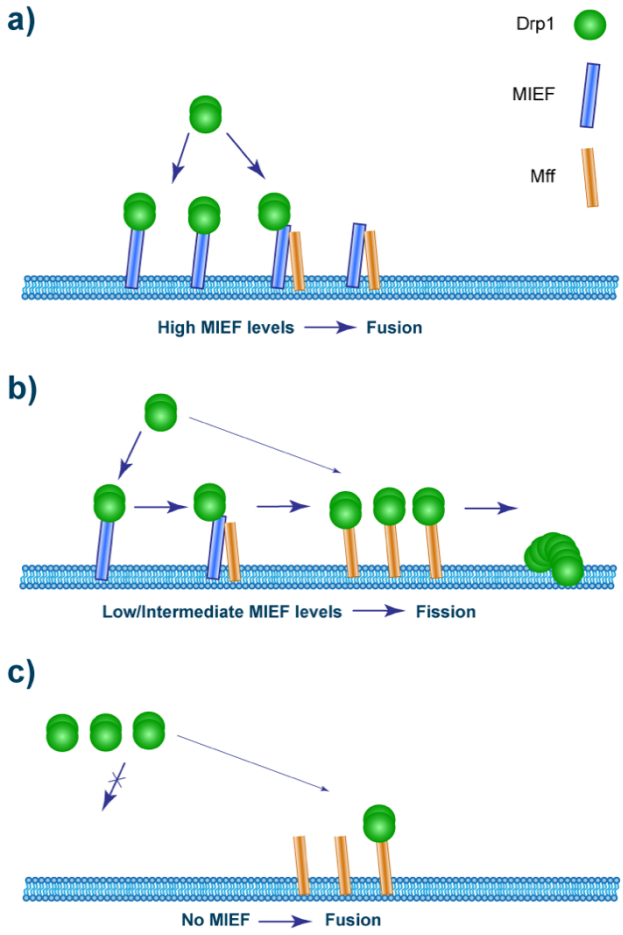


**Figure 3.** Schematic diagrams for different levels of MIEFs regulating mitochondrial dynamics

binding and resulting in mitochondrial fusion (Figure 3a). When MIEFs are maintained at moderate levels in cells, Mff can receive sufficient Drp1 via MIEFs action as a molecular bridge facilitating a direct interaction between Mff and Drp1 to maintain normal and balanced mitochondrial morphology (Figure 3b). However, in the absence of MIEFs, Mff cannot directly capture sufficient Drp1 from the cytosol by itself, thus the balance shifts to mitochondrial fusion, resulting in mitochondrial elongation (Figure 3c).

## 3.3 STUDY III

***The Drp1-S637 phosphorylation status is not a determinant of Drp1 recruitment to mitochondria but fine-tunes mitochondrial fission***

Reversible phosphorylation of Drp1 has been implicated in the regulation of Drp1 recruitment to mitochondria, but it is still poorly understood whether the phosphorylation status at S637 decides the subcellular localization and the fission activity of Drp1. In this study, we explore the potential roles of phosphorylation status at Drp1-S637 in the Drp1-mediated fission activity, subcellular distribution of Drp1 and interactions of Drp1 with MIEFs or Mff.

### 3.3.1 Phosphorylated Drp1-S637 (Drp1-pS637) is present both in the cytosol and on mitochondria

The basal level of Drp1-S637 phosphorylation was low in 293T cells. We treated 293T cells with forskolin (PKA activator) or forskolin combined with FK506 (calcineurin inhibitor) to increase the relative level of Drp1-pS637. Confocal microscopy and subcellular fractionation analysis indicated that Drp1-pS637 was present both in the cytoplasm and at the mitochondrial surface.

### 3.3.2 MIEFs and Mff recruit both Drp1-pS637 and non-phospho-Drp1-S637 to the surface of mitochondria

Results from confocal imaging and subcellular fractionation analysis showed that, cells transfected with either MIEFs or Mff facilitate the obvious Drp1-pS637 accumulation on mitochondria.

### 3.3.3 MIEFs and Mff bind to both Drp1-pS637 and non-phospho-Drp1-S637

Co-IP results revealed that exogenous MIEFs or Mff interacted with Drp1-pS637. Similarly, endogenous Drp1-pS637 could also immunoprecipitate with endogenous MIEFs and Mff. In support of the results in the Study II, depletion of both MIEF1/2 through siRNA largely diminished Mff interaction with Drp1-pS637 and total Drp1.

### 3.3.4 Phosphorylation or dephosphorylation at S637 slightly affects but is not essential for governing the localization of Drp1 on mitochondria

To further elucidate the potential role of Drp1 phosphorylation, we generated Drp1 knockout 293T cells using CRISPR/Cas9-based genome editing technique and constructed untagged plasmids with wild-type Drp1 or with Drp1-S637D, Drp1-S637A mutants. Immunofluorescence showed that, like WT Drp1, the Drp1-S637D and Drp1-

S637A mutants were localized to the cytosol and mitochondria. Overexpression of either Drp1-S637D or Drp1-S637A reversed the superfused mitochondria in Drp1-deficient cells to normal morphology. However, a significant difference was observed also: The phosphomimetic Drp1-S637D weakened Drp1-induced mitochondrial division. Furthermore, both Drp1-S637A and Drp1-S637D interact with MIEFs and Mff by co-IP experiments in Drp1-knockout and wild-type 293T cells. No obvious difference was found in interactions of MIEFs with exogenous WT Drp1, Drp1-S637A or Drp1-S637D in Drp1-knockout cells, but there was a slightly higher amount of the phospho-deficient Drp1-S637A and a lower amount of the phosphomimetic Drp1-S637D associating with Mff compared to WT Drp1.

### 3.3.5 PKA also interacts with Mff and MIEFs, but is not a key regulator for the subcellular localization of Drp1

Subcellular localization analysis and confocal imaging showed that PKA was present in both mitochondria and the cytosol. Co-IP showed that endogenous PKA was immunoprecipitated with MIEFs and Mff regardless the presence or absence of Drp1. Moreover, the absence of PKA did not affect the association of Drp1 with MIEFs and Mff, indicating that PKA is not a key modulator of Drp1 binding to MIEFs and Mff in cells.

This study addressed the question whether the phosphorylation state of Drp1-S637 determines Drp1 recruitment to mitochondria through its receptors Mff and MIEFs. Several previous publications consider that Drp1-S637 phosphorylation blocks mitochondrial recruitment of Drp1 and keeps it in the cytosol (Chang and Blackstone, 2007; Cribbs and Strack, 2007; Tilokani et al., 2018). In this study, our data indicated that phosphorylation or dephosphorylation of Drp1-S637 has a minor effect on mitochondrial recruitment of Drp1, and plays only a fine-tuning role for the fission activity of Drp1.

## 3.4 STUDY IV

***An unanticipated function of human Fis1: inhibiting the mitochondrial fusion machinery***

Fis1 is a highly conserved MOM protein from yeast to humans. This protein was originally identified on mitochondria in yeast as Fis1p, where Fis1p functions as the Dnm1p receptor to captures cytosolic Dnm1p to mitochondria with the help of adaptors Mdv1p or Caf4p. In mammals, owing to the fact that overexpression of Fis1 promotes mitochondrial fragmentation, Fis1 was initially considered to be a mitochondrial fission factor and to work as the Drp1 receptor on mitochondria (James et al., 2003; Yoon et al., 2003). However, increasing evidence suggests that Fis1 is dispensable for Drp1 recruitment and Drp1-triggered mitochondrial division (Chan, 2012; Koirala et al., 2013; Shen et al., 2014). The molecular mechanism of Fis1-induced mitochondrial fragmentation is therefore poorly understood. Here we investigated the underlying mechanism of hFis1-induced mitochondrial fragmentation and the function of hFis1 in mitochondrial dynamics.

### 3.4.1 Mitochondrial fragmentation triggered by hFis1 overexpression is independent of Drp1 and Dyn2

Both wild type and Drp1-knockout 293T cells transfected with hFis1 displayed mitochondrial fission morphology, this phenotype was also observed in HeLa cells with or without endogenous Drp1. Furthermore, depletion of Dyn2 by siRNA in wild type or Drp1-knockout 293T cells did not block hFis1-triggered mitochondrial fragmentation, implying that overexpression of hFis1 led to mitochondrial fragmentation in a way independent of fission proteins Drp1 and Dyn2. Moreover, the average size of punctate mitochondria induced by hFis1 overexpression in Drp1-knockout cells was larger and the total number of mitochondria was lower than in wild-type cells. This phenomenon is probably due to that additional mitochondrial fission still occurs by a Drp1-dependent manner in wild-type cells.

### 3.4.2 hFis1 mainly interact with fusion proteins Mfn1/2 and OPA1

Next, we examined whether hFis1 affected the mitochondrial fusion pathway instead of the fission machinery. First, loss of hFis1 in Drp1 knockout 293T cells resulted in superfused tubular clusters of mitochondria, located beside the nucleus. This was quite similar with the morphology observed in Drp1-knockout cells overexpressing any one of Mfn1/2 and OPA1. Furthermore, endogenous hFis1 is strongly associated with endogenous Mfn1/2 and OPA1 but no Drp1 and Dyn2. These results suggested that hFis1 has a functional relationship with the mitochondrial fusion pathway. Moreover, we tested the specific regions of hFis1 protein that associated with Mfn1/2 and OPA1. Co-IP results revealed that the N-terminal part (containing TPR1 domain) is dispensable, while other parts of the hFis1 protein, including the C-terminal tail, TM and TPR2 domain, are required for binding to Mfn1/2 and OPA1.

### 3.4.3 Human Fis1 disturbs the activity of mitochondrial fusion

The PEG-induced cell fusion assay and a photoactivatable mtPA-GFP-based mitochondrial fusion assay were used to explore the extent and rate of mitochondrial fusion. Wild-type cells overexpressing hFis1 displayed decreased mitochondrial fusion activity compared with empty-vector transfected cells, whereas depletion of endogenous hFis1 by siRNA led to increased mitochondrial fusion when compared to control siRNA treatment. And similar results were discovered in Drp1-knockout 293T cells. These data suggest that overexpression of hFis1 impedes and knockdown of hFis1 promotes the activity of the mitochondrial fusion machinery irrespective of the presence or absence of Drp1.

### 3.4.4 hFis1 impairs the activities of pro-fusion GTPases

We tested the in vitro GTPase activity of Mfn1/2, OPA1, Drp1 and Dyn2 pre-incubated with or without hFis1 recombinant protein. The results from the GTPase activity assay showed that the presence of hFis1 recombinant protein significantly decreased the capacity of Mfn1/2 or OPA1 for GTP hydrolysis, but did not affect the GTPase activity of Drp1 and Dyn2, indicating that hFis1 primarily regulates the GTPase activity of the pro-fusion proteins Mfn1/2 and OPA1 but not the pro-fission proteins Drp1 and Dyn2.

### 3.4.5 Destroying the fusion machinery phenocopies the hFis1-mediated mitochondrial fragmentation phenotype in Drp1 KO cells

To further address the relationship between the fusion machinery and hFis1, we developed Drp1/OPA1 double-knockout cells using the CRISPR/Cas9 system, then knocked down either Mfn1, Mfn2, Mfn1&2, Dyn2, or Dyn2&Mfn1 by siRNA silencing, and combined with empty vector or Myc-hFis1 overexpression.

Down-regulation of Mfn1 in Drp1/OPA1 double-knockout cells induced extensively fragmented mitochondria compared with control cells. After knockdown of both Mfn1 and Mfn2, the number of cells with a fragmented mitochondrial phenotype was further increased, which is similar to that observed in the Drp1-knockout and Drp1/OPA1 double-knockout cells overexpressing hFis1. These results suggest that destruction of the mitochondrial fusion machinery phenotypes mitochondrial fragmentation triggered by hFis1 overexpression.

### 3.4.6 F-actin is involved in mitochondrial fission triggered by hFis1-overexpression in a Drp1-independent manner

Drp1-knockout cells were transfected with exogenous hFis1 for 4h, followed by treatment with latrunculin B (LatB) for F-actin depolymerization or DMSO (control group). The number of cells with fragmented mitochondria in the LatB-treated group was reduced compared to the control group. These results implied that F-actin plays an important role in regulating mitochondrial fission in Drp1-KO cells overexpressing hFis1.

In this study, we revealed novel functions of hFis1 in mitochondrial dynamics: hFis1-triggered mitochondrial fragmentation is not due to hFis1 participating in the mitochondrial fission machinery but due to hFis1 inhibiting the activity of the mitochondrial fusion process, uncovering an unanticipated divergence in functional evolution from yeast to mammals. A schematic diagram is showed in Figure 4.

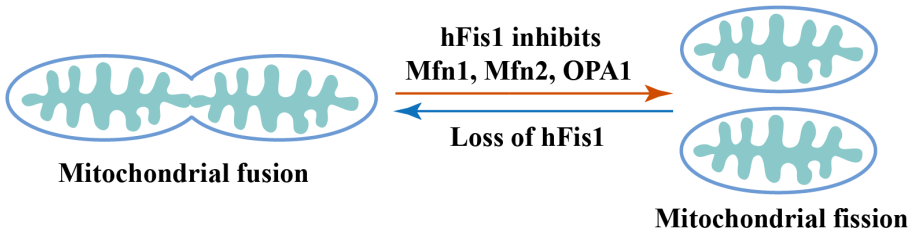


**Figure 4.** The schematic diagram of human Fis1 inhibitory influence on the mitochondrial fusion machinery to regulate mitochondrial dynamics

# 4 CONCLUSION AND SIGNIFICANCE

Drp1 recruitment from the cytosol to mitochondria is the central step of mitochondrial fission. In mammals, there are four receptors on mitochondria for Drp1 recruitment, Fis1, MIEF1/2 and Mff. In this thesis, we have investigated potential functions of these receptors in mitochondrial dynamics and the major findings are summarized as follows.

**Study I**

We demonstrated the refined functions of MIEF1 and MIEF2 in regulating mitochondrial dynamics.

***Similarities:***

- Overexpression of MIEF1 or MIEF2 captures Drp1 to the mitochondrial outer membrane and induces mitochondrial elongation.
- Both MIEF1 and MIEF2 interact with Drp1 and hFis1.
- Both MIEFs can form higher-order oligomers.

***Differences:***

- MIEF2 has a more efficient effect on mitochondrial fusion and clustering than MIEF1.
- MIEF2 has the opposite expression to MIEF1 in human fetal and adult tissues.
- The essential domain of MIEF1 for oligomerization is amino acids 109-154, while MIEF2 requires amino acid residues 1-49.

Consistent with our data, other researchers also reported the divergences of MIEF1 and MIEF2 in the crystal structures and functional analysis. In brief, MIEF1 uniquely binds ADP at the nucleotidyltransferase domain but MIEF2 does not, which is critical for Drp1 assembly and GTP hydrolysis (Loson et al., 2014; Loson et al., 2015; Richter et al., 2014). However, the reason for these functional divergences needs to be further analyzed. Furthermore, our results also suggest that MIEF1 and MIEF2 may have additional functions besides in modulating mitochondrial dynamics, such as involving in mitochondrial diseases and embryonic developmental processes, which need to be investigated in the future.

**Study II**

The roles of MIEF1/2 and Mff in Drp1 recruitment to mitochondria were presented in detail:

- MIEF1/2 collaborate with Mff step by step to mediate Drp1 recruitment to mitochondria and balance mitochondrial morphology.
- MIEF1/2 serve as the adaptors connecting Drp1 with Mff, and promotes the formation of a dimeric Drp1-Mff complex.

The paradoxical phenomenon that both overexpression and depletion of MIEFs induced mitochondrial fusion while overexpression of Mff led to mitochondrial fission is explained. Owing to the existence of a Drp1-MIEF-Mff triple complex, the relative expression levels of MIEFs control the balance between mitochondrial fusion and fission.

- Higher levels of MIEFs sequester Drp1 in Drp1-MIEF and Drp1-MIEF-Mff complexes, preventing the direct Drp1-Mff binding, and resulting in mitochondrial elongation. Consistent with this, another study also showed that MIEF1 substantially inhibits Drp1 GTPase activity with or without the presence of Mff (Osellame et al., 2016).
- When the levels of MIEFs or the relative levels between MIEFs and Mff are moderate, the two proteins work cooperatively for Drp1-dependent mitochondrial division, then the mitochondrial phenotype is normal or fragmented.
- In the absence of MIEFs, endogenous Mff does not have the capacity to capture sufficient Drp1 by itself, resulting in the balance shifting to mitochondrial fusion. This is the reason why mitochondria present an elongated morphology in the absence of MIEFs.

**Study III**

We refined the roles of Drp1 phosphorylation in Drp1-driven mitochondrial division.

- Phosphorylation or dephosphorylation of Drp1-S637 is not a major factor determining the subcellular distribution and fission activity of Drp1. Regardless whether Drp1 is phosphorylated or not, both of them can be recruited to mitochondria by MIEFs and Mff.
- The phosphorylation status of Drp1-S637 is involved in the fine-tuning of Drp1-dependent mitochondrial division although it is not important for regulating the subcellular localization of Drp1.

Post-transcriptional modifications, especially phosphorylation and dephosphorylation of Drp1 have attracted a lot of attention for their potential roles in the regulation of mitochondrial dynamics in the past years. Although it has been reported that phosphorylation levels of Drp1 S637 are increased in some diseases (Park et al., 2019; Xie et al., 2015), the potential mechanisms of diseases related to these aspects of mitochondrial dynamics need to be further elucidated.

**Study IV**

This study, as it is highlighted recently (Liesa et al., 2019), uncovers the molecular mechanisms of Fis1-triggered mitochondrial fragmentation by inhibiting mitochondrial fusion and open an exciting new area of research in mammals, and also highlights the limitation of categorizing mitochondrial dynamics proteins, given the general tendency to associate fragmentation with activation of fission.

- Surprisingly, instead of its association with the pro-fission GTPases in the mitochondrial fission pathway, hFis1 mainly associates with pro-fusion GTPases.
- hFis1-triggered mitochondrial fragmentation occurred by inhibiting the functions of the pro-fusion GTPases resulting in a shift of balance to mitochondrial fission.
- Human Fis1 plays multiple roles in mitochondrial dynamics, which is significantly divergent from the functions of its homolog Fis1p in yeast.

Finally, according to the published literature and our results, I suggest that there are multiple potential pathways to drive mitochondrial fission in mammals (Figure 5):

***Drp1-dependent fission pathway:***

- It is generally considered that Drp1 plays a critical role in mitochondrial fission. Controlling its recruitment from the cytosol to mitochondria is a key step in this process. Thus, Drp1 and its mitochondrial receptors/adapters including Mff, MIEF1/2 and Fis1 are the major components of the canonical mitochondrial fission pathway, in which Drp1 is recruited to the ER/actin constriction sites on mitochondria by one of these mitochondrial receptors/adapters or in a way by which MIEFs and Mff coordinately work to govern Drp1 recruitment, and subsequently Drp1 assembles into higher-order oligomers to wrap around mitochondria, and ultimately the Drp1-mediated mitochondrial membrane scission occurs with the help of Dyn2 recruited transiently to the division sites (Described also in Figure 1).

***Drp1-independent fission pathways:***

Accumulating data suggest that apart from the canonical Drp1-dependent pathways, there are likely additional pathways that also contribute to mitochondrial fission in mammalian cells.

- As presented in the Study IV, Fis1 binds to Mfn1/2 or OPA1 and inhibits the mitochondrial fusion machinery in Drp1-deficient cells, shifting mitochondrial morphology to a fission phenotype. Especially, the size of the punctiform mitochondria induced by hFis1 overexpression in Drp1 KO cells was larger than in wild-type cells, which we believe is attributed to the ongoing Drp1-triggered mitochondrial fission in wild-type cells. These data suggest that the Drp1-dependent mitochondrial fission pathway is probably parallel with the Fis1-mediated inhibition to the mitochondrial fusion machinery, and they work together to shift mitochondrial dynamics to fission.
- In addition to the function we reported, other studies also found that Fis1 together with TBC1D15 and Rab7 is involved in mitochondrial fission. In detail, overexpression of Fis1 and TBC1D15 (a Rab GTPase regulator protein) induced mitochondrial fission in Drp1-deficient MEFs, and Fis1 binds to TBC1D15 (Onoue et al., 2013). Recently it is found that the small GTPase Rab7 is associated with the Fis1-TBC1D15 complex via mitochondria-lysosome contacts, which regulates both mitochondrial and lysosomal dynamics (Wong et al., 2018). Furthermore, increasing evidence related to TBC1D15 and Rab7 implicates that this complex may play important roles in mitophagy (Rojansky et al., 2016; Tan and Tang, 2019; Yamano et al., 2018). In this aspect, the exact molecular mechanism is unknown.
- The ER and actin cytoskeleton are also reported to be involved in mitochondrial dynamics (Friedman et al., 2011; Korobova et al., 2013; Moore and Holzbaur, 2018). In the Study IV, we have presented that F-actin depolymerization significantly prevented hFis1 overexpression-induced mitochondrial fragmentation in a Drp1-independent manner, implying that actin plays an important role in regulating mitochondrial fission independent of Drp1. Furthermore, even now the ER is considered as the first step of Drp1-mediated mitochondrial fission, mediating mitochondrial constriction before Drp1 recruitment (Friedman et al., 2011), whether the ER induces mitochondrial scission directly independent of Drp1 needs to be further studied.

- Although Dyn2 has been reported to work at the final step of Drp1-dependent mitochondrial division (Lee et al., 2016), mitochondrial division still occurs in Dyn2-deficient cells (Kamerkar et al., 2018). In the Study IV, we found that Dyn2 knockdown reversed the mitochondrial division phenotype that was induced when both fission and fusion machineries had simultaneously been destroyed, indicating that Dyn2 may have own pathways for mitochondrial fission when the canonical Drp1-mediated pathway is blocked.

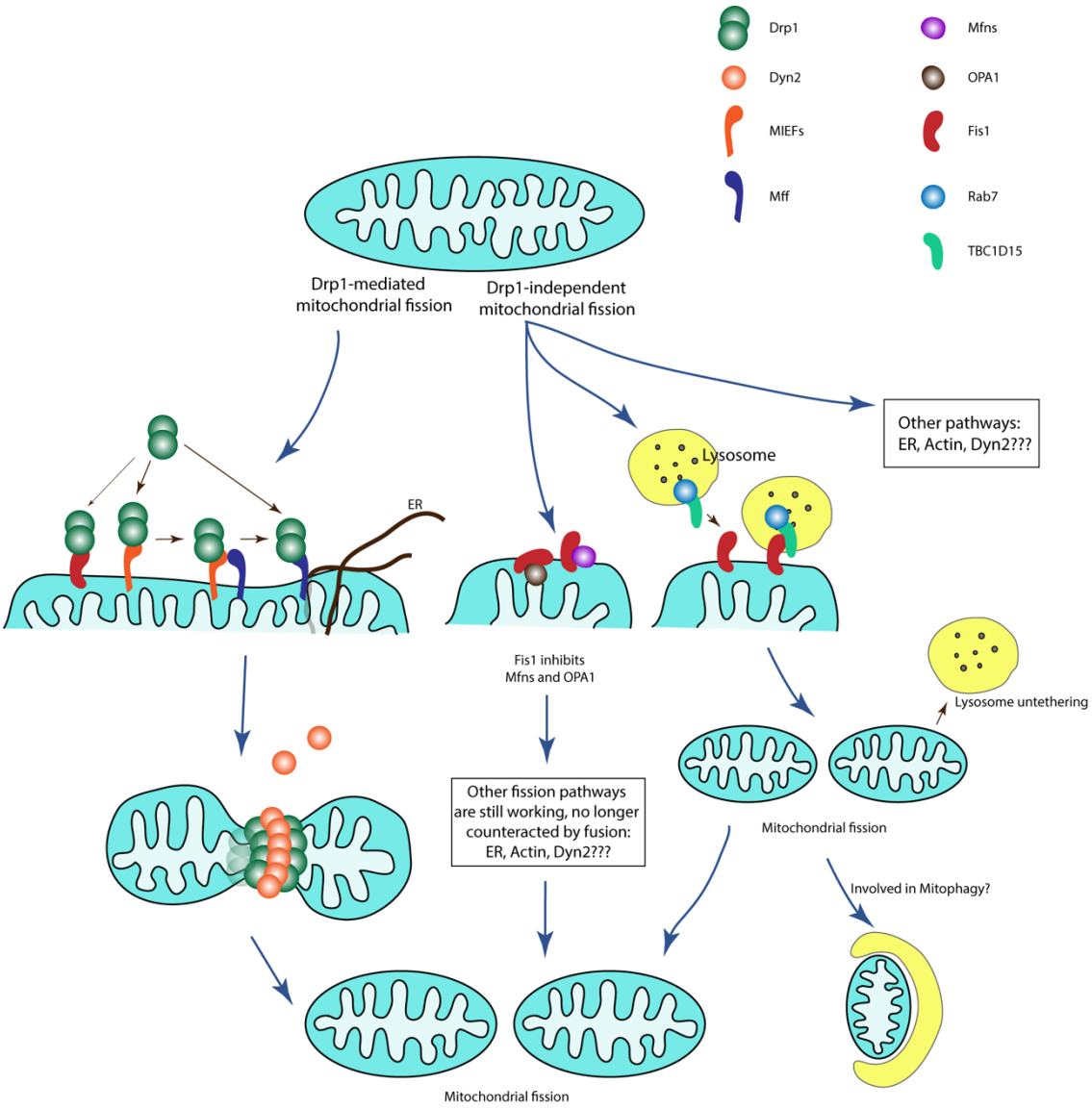


**Figure 5**. The supposed molecular mechanisms in mitochondrial fission

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