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CHARACTERIZATION OF MITOCHONDRIAL MEMBRANE PROTEINS AS NOVEL COMPONENTS IN MITOCHONDRIAL DYNAMICS

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To my beloved family

ABSTRACT

Mitochondria are dynamic organelles that frequently change their shape by shifting the balance of fusion and fission in response to cellular metabolic needs. This process is termed mitochondrial dynamics. Dysregulation of mitochondrial dynamics has been described in many human diseases. Mitochondrial dynamics has been widely studied in yeast, while in mammals the proteins involved in mitochondrial fusion and fission are divergent to some extent. To better understand the mechanisms in mammals, it is imperative to discover the key proteins underlying mitochondrial dynamics. In this thesis, we characterized three novel mitochondrial membrane proteins, MIEF1, MIEF2 and MTGM (Romo1), which play important roles in mitochondrial dynamics. In **paper i**, MIEF1 is characterized as an integral mitochondrial outer membrane protein. Overexpression of MIEF1 induced extensive mitochondrial fusion, whereas depletion of MIEF1 caused mitochondrial fragmentation. MIEF1 interacts with and recruits Drp1 to mitochondria independent of hFis1, Mff and Mfn2 but this results in mitochondrial fusion. MIEF1 also interacts with hFis1 and elevated hFis1 levels partially reversed the MIEF1-induced fusion phenotype. MIEF1-induced mitochondrial fusion occurs in a manner distinct from Mfn2. In **paper ii**, we compared MIEF1 and MIEF2, which are two paralogs in human. MIEF1 and MIEF2 possess many functions in common in mitochondrial dynamics. They anchor in the mitochondrial outer membrane, recruit Drp1 to mitochondria, and impede mitochondrial fission. They interact with Drp1 and hFis1. To some extent, MIEF1 and MIEF2 may play functionally distinct roles in mitochondrial dynamics. They are differentially expressed in human tissues. Ectopic expression of MIEF2 displayed a stronger mitochondrial fusion effect than MIEF1. hFis1 and Mff partially reverted MIEF2-induced fusion in contrast to the extensive rescue of fusion induced by MIEF1. MIEF2 forms higher order oligomers, while MIEF1 mainly presents as dimers. By studying engineered deletion mutants, it was shown that MIEF1 requires the amino acid residues 109-154 and MIEF2 the amino acids 1-49 for their dimerization/oligomerization. In MIEF1, oligomerization is not required for mitochondrial localization and interaction with Drp1. In **paper iii**, gene expression profiling indicated that MTGM might be related to human brain tumors. We identified the highly conserved human MTGM gene that encodes an integral mitochondrial inner membrane protein and confirmed the upregulation of MTGM in human brain tumors. Overexpression of MTGM resulted in Drp1-dependent mitochondrial fragmentation and release of mitochondrial Smac/Diablo to the cytosol, but had no effect on apoptosis. Cell proliferation was inhibited by stalling of cells in S phase. Downregulation of MTGM induced mitochondrial elongation, an increase of cell proliferation and inhibition of cell death induced by apoptotic stimuli. In **paper iv**, we discovered that the subcellular localization of NCX3 varies with the cell cycle phases. One phenotype is distribution of NCX3 in the plasma membrane (NCX3-PM), the other in the cytoplasm/ER (NCX3-ER). Modification of NCX3 by N-linked glycosylation of a single asparagine residue, N45, is normally required for targeting of the protein to the plasma membrane. Importantly, this modification also affects the cell cycle. In sum, this thesis has unveiled novel proteins playing pivotal roles in the regulation of mitochondrial dynamics.

LIST OF SCIENTIFIC PAPERS

I. Zhao, J., **T. Liu**, S. Jin, X. Wang, M. Qu, P. Uhlen, N. Tomilin, O. Shupliakov, U. Lendahl and M. Nister (2011). "Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission." EMBO J 30(14): 2762-2778.

II. **Liu, T**., R. Yu, S. B. Jin, L. Han, U. Lendahl, J. Zhao and M. Nister (2013). "The mitochondrial elongation factors MIEF1 and MIEF2 exert partially distinct functions in mitochondrial dynamics." Exp Cell Res 319(18): 2893-2904.

III. Zhao, J., **T. Liu**, S. B. Jin, N. Tomilin, J. Castro, O. Shupliakov, U. Lendahl and M. Nister (2009). "The novel conserved mitochondrial inner-membrane protein MTGM regulates mitochondrial morphology and cell proliferation." J Cell Sci 122(Pt 13): 2252-2262.

IV. **Liu, T**., J. Zhao, C. Ibarra, P. Uhlén, M. Nister. "Glycosylation determines sodium-calcium exchanger 3 subcellular distribution during cell cycle; effects on ER Ca^{2+} handling."
Manuscript.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species
mtDNA	mitochondrial deoxyribonucleic acid
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
Fzo	fuzzy onions
DRP	dynamin-related protein
GTP	guanosine triphosphate
GED	GTP effector domain
Mfn1	mitofusin 1
Mfn2	mitofusin 2
MOM	mitochondrial outer membrane
MIM	mitochondrial inner membrane
Drp1	dynamin-related protein 1
TRP	tetratricopeptide repeat
hFis1	human Fis1
VD	variable domain
CNS	central nervous system
co-IP	co-immunoprecipitation
MEF	mouse embryonic fibroblast
ER	endoplasmic reticulum
Mff	mitochondrial fission factor
siRNA	small interfering RNA
MIEF1	mitochondrial elongation factor 1
MIEF2	mitochondrial elongation factor 2
ADP	adenosine diphosphate
PINK1	PTEN-induced putative kinase 1
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
GDAP1	ganglioside-induced differentiation-associated protein1

Endo B1	endophilin B1
MTP18	mitochondrial protein of 18 kDa
MTGM	mitochondrial targeting GxxxG motif
KO	knockout
MPP	matrix-processing peptidase
HP	heptad repeats
PARL	presenilin associated, rhomboid-like
l-OPA1	long OPA1
s-OPA1	short OPA1
SUMO	small ubiquitin-like modifier
MAPL	mitochondrial-anchored protein ligase
PKA	cAMP-dependent protein kinase A
CaMKIα	Ca^{2+}/calmodulin-dependent protein kinase Iα
CaN	Ca^{2+}/CaM-dependent protein phosphatase calcineurin
AD	Alzheimer's disease
MAM	mitochondria-associated ER membrane
ERMD	ER-associated mitochondrial division
INF2	inverted formin-2
Ins(1,4,5)P3R	inositol 1,4,5-trisphosphate receptor
VDAC1	voltage-dependent anion selective channel protein 1
MCU	mitochondrial calcium uniporter
MPTP	mitochondrial permeability transition pore
$\Delta\Psi m$	mitochondrial membrane potential
NCX	Na^+/Ca^{2+} exchanger
NCX_{mito}	mitochondrial Na^+/Ca^{2+} exchanger
MOMP	mitochondrial outer membrane permeabilization
Mdivi-1	mitochondrial division inhibitor-1
Bnip3	Bcl2/adenovirus E1B 19-kDa interacting protein 3

1 INTRODUCTION

Mitochondria are well-known for their function to produce adenosine triphosphate (ATP) through the process of oxidative phosphorylation (OXPHOS) thereby regulating cellular metabolism in all eukaryotic cells (Mitchell 1961). However, the mitochondrion is more than just a powerhouse of the cell. In the past two decades, many other roles of mitochondria have emerged. One is apoptosis mediated by the release of cytochrome c. Maintaining Ca^{2+} homeostasis is another important function of the mitochondrion. Regulation of cell signaling through reactive oxygen species (ROS) involves the cell cycle and embryonic development (McBride, Neuspiel et al. 2006). Dysfunction of mitochondria is involved in a variety of human diseases, especially in mitochondrial deoxyribonucleic acid (mtDNA) related diseases. Mitochondrial function has been widely studied for example in neurological disorders, myopathy, diabetes, encephalomyopathies, cardiomyopathies, and complex multisystem syndromes (Zeviani 2004).

1.1 MITOCHONDRIA AND THEIR ORIGIN

Mitochondria originate from bacterial progenitors which were engulfed by a host cell through endosymbiosis more than 1 billion years ago (Bhattacharya and Gross 2009). We have been aware of the presence of mitochondria in the cytoplasm of cells for over 150 years. They are the most obvious organelles in the cytoplasm (Alberts 2014). In the early 1950s, by electron microscopy, it was reported that mitochondria contain an outer membrane encasing an inner, often highly invaginated, membrane (Palade 1953). These membranes enclose two compartments: an internal space named the matrix and an intermembrane space between the outer and inner membranes. An outer membrane located transport protein, porin, forms aqueous channel which is permeable to molecules lower than 5000 daltons, while the inner membrane is only permeable to the selected small molecules that such as pyruvate and fatty acids enter into the matrix via a path formed by specific membrane transport proteins. As a result, the mitochondrial matrix contains highly specific molecules. The inner membrane is the site of OXPHOS and contains the proteins of the electron-transport chain, the proton pumps, and the ATP synthases required for ATP production. The inner membrane forms a lot of infoldings denoted as cristae projecting into the matrix space, providing a site for biochemical reactions (Alberts 2014). The human mitochondrion contains a compact circular genome, the mtDNA, comprising 16,569 bps encoding 13 polypeptides, 2 messenger ribonucleic acids (mRNAs) and 22 transfer ribonucleic acids (tRNAs). The 13 proteins are components of the OXPHOS process (Anderson, Bankier et al. 1981, Schatz 1996).

1.2 EVOLUTION OF MITOCHONDRIAL DYNAMICS

1.2.1 History of mitochondrial dynamics

It is well known that mitochondria have different morphologies. In 1994, the dynamics of mitochondria in living cells was discovered and it was widely accepted that the mitochondrion is a dynamic organelle (Bereiter-Hahn and Voth 1994). In 1997, the first gene, fuzzy onions (Fzo) was discovered in Drosophila. It was required for mitochondrial fusion and involved in mitochondrial dynamics (Hales and Fuller 1997, Lu and SpringerLink 2011). The mitochondrial network is remarkably plastic (Figure 1). For an even distribution of

mitochondria within the cell, mitochondria enter into fission and form small or short tubules, punctate forms and aggregates (Figure 1A, B). Conversely, to meet the high energy demands, mitochondria appose and connect to each other in that way forming longer tubules, tubular networks, extremely long tubules and tubular clusters (Figure 1D, E). In certain conditions, the balance completely switches to fusion and leads to the formation of compact clusters (Figure 1F). Mitochondrial dynamics is determined by the interplay of fusion, fission, movement and positional tethering (Lackner 2013, Rafelski 2013, Hoppins 2014). Mitochondrial morphology changes dramatically by shifting the balance of fusion and fission events. In the past two decades, new microscopic techniques have facilitated the investigation of mitochondrial dynamics in response to cellular events within intact cells. We now understand that mitochondrial dynamics, along with altered cell metabolism, is involved in cell cycle regulation, apoptosis, mitophagy and Ca^{2+} signaling, and that abnormal mitochondrial dynamics is associated with many human diseases (McBride, Neuspiel et al. 2006, van der Bliek, Shen et al. 2013, Hoppins 2014).

Figure 1. Mitochondrial morphology in HEK 293T cells

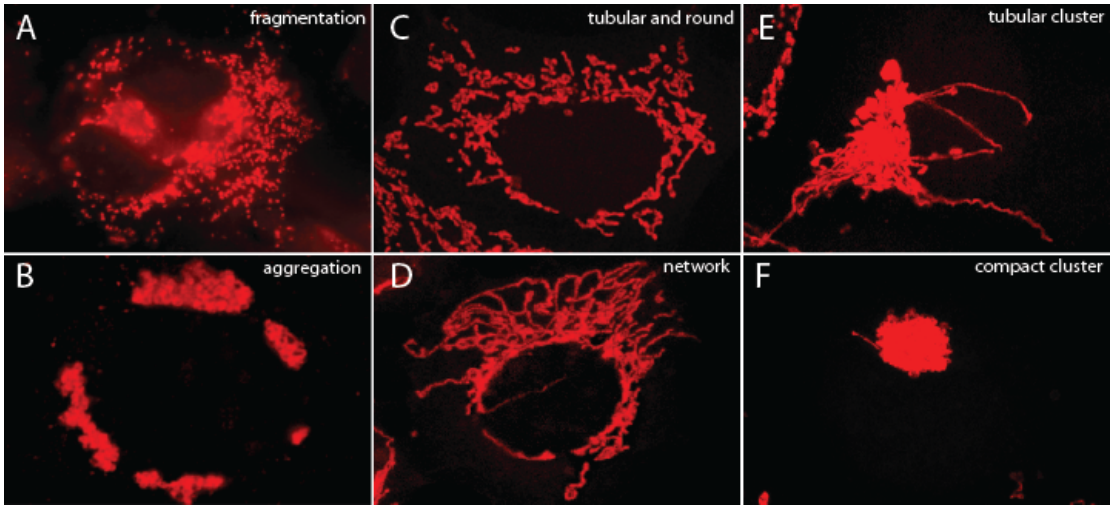


Fig. 1. Mitochondrial morphology in HEK 293T cells stained with MitoTracker. (A) Mitochondrial fragmentation. (B) Mitochondrial aggregation. (C) Normal mitochondrial morphology. (D) Mitochondrial network. (E) Mitochondrial tubular cluster. (F) Mitochondrial compact cluster.

1.2.2 The components involved in mitochondrial dynamics

The essential proteins in mitochondrial fusion and fission belong to the dynamin related protein (DRP) family (Elgass, Pakay et al. 2013, Escobar-Henriques and Anton 2013). These proteins are large self-assembling GTPases. They are capable of binding to lipids and self-stimulation of its guanosine triphosphate (GTP) hydrolysis activity. The DRPs have three highly conserved domains. The GTPase domain is involved in GTP binding and hydrolysis; the middle domain and GTP effector domain (GED) play an important role in oligomerization and self-assembly; the region in-between of those two domains serves in lipid interaction (Praefcke and McMahon 2004, Lu and SpringerLink 2011). Three DRPs, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) located in the mitochondrial outer membrane (MOM) and OPA1 in the mitochondrial inner membrane (MIM) are responsible for mitochondrial fusion. In mitochondrial fission, dynamin-related protein 1(Drp1), located both in the cytosol and on mitochondria, mediates a membrane scission event by GTP binding and

hydrolysis. Together these four mitochondrial DRPs constitute the core of the mitochondrial fusion and fission machineries, respectively and are subject to various regulatory mechanisms including proteolysis and post-translational modifications (Hoppins 2014). In mammals, in addition to the DRPs, a number of other mitochondria-shaping proteins have been identified to modulate and orchestrate mitochondrial dynamics, e.g. MIEFs, Mff, hFis1, MTP18, Endo B1 and others (Zhao, Lendahl et al. 2013).

1.3 MITOCHONDRIAL FUSION AND FISSION

In principle, the same type of mitochondrial fission and fusion processes occur in yeast and mammals, but the proteins involved vary and the regulation is more complex in mammals than in yeast. Due to the fact that mitochondrial dynamics has been studied mostly in yeast, several of the key mammalian proteins were described quite recently. This thesis has contributed to the identification of novel proteins involved in mitochondrial dynamics in human.

1.3.1 Mitochondrial fission

Mitochondrial fission is important for the cells ability to maintain an adequate number of mitochondria to continue the normal cellular events (Youle and van der Bliek 2012). By mitochondrial fission, the cell generates new organelles and performs a quality control of mitochondria, targeting dysfunctional mitochondria for elimination by mitophagy (da Silva, Mariotti et al. 2014). Drp1 (Dnm1p in yeast) is the major player in mitochondrial division. In yeast, the machinery of Dnm1p-dependent mitochondrial fission requires Fis1p, Mdv1p, or its paralogue, Caf4p. In mitochondrial division, Dnm1p interacts with Fis1p at the site of the tetratricopeptide-repeat (TRP) motif via adaptor proteins Mdv1p/Caf4p in the cytosol. Fis1p functions as the mitochondrial receptor for Dnm1p, aggregating the self-assembling Dnm1p at the mitochondrial fission sites and at the same time stimulating mitochondrial membrane constriction in a GTP-dependent manner (Otera, Ishihara et al. 2013). Another proposed model suggests that Mdm36 is required for the formation of protein complexes of Dnm1p and Num1p, ensuring mitochondrial fission at the cell cortex in yeast (Hammermeister, Schodel et al. 2010). Therefore, there are two pathways regulating Dnm1p-mediated mitochondrial fission in yeast: Dnm1p-Mdv1p/Caf4p-Fis1p and Dnm1p-Mdm36p-Num1p complexes. In mammals, although the homologue of Fis1 has been identified, human Fis1 (hFis1) is not required for recruitment of Drp1 to mitochondria (Lee, Jeong et al. 2004). Moreover, homologues of Mdvp1, Caf4p, Num1p, and Mdm36p have not been found in mammalian cells (Zhao, Lendahl et al. 2013). Accordingly, the mechanisms of Dnm1p- and Drp1-mediated mitochondrial fission are highly divergent between yeast and mammals. Recently several fission-related proteins have been identified in mammals, but the detailed integrated functions of the complex mitochondrial fission machinery remain unresolved.

1.3.1.1 Drp1

Drp1 (Dnm1p in yeast) is a core component of the mitochondrial fission machinery and plays a central role in the process of mitochondrial fission in both yeast and mammals. The majority of Drp1 is present in the cytosol, but the protein shuttles between the cytoplasm and the mitochondrial outer menbrane (MOM). During mitochondrial fission, Drp1 is recruited from the cytosol to the mitochondrial surface and mediates fission (Bleazard, McCaffery et

al. 1999, Smirnova, Griparic et al. 2001). The protein domain structure of Drp1 (Figure 2) (Frohlich, Grabiger et al. 2013) illuminated that Drp1 comprises a head (GTPase domain) sitting on a neck region which is known as the bundle signaling element, a stalk including the middle domain and GED, and a foot of the non-characterized variable domain (VD). The VD domain in Dnm1p is essential for binding to its adaptor Mdv1p aiming to modulate mitochondrial fission in yeast (Bui, Karren et al. 2012). However, in HeLa cells, a Drp1 mutant lacking the VD could still target to the MOM through the mitochondrial Drp1 receptor Mff, and induce mitochondrial fission. This indicates that the VD of Drp1 is dispensable for recruitment of Drp1 to the MOM and mitochondrial fission (Figure 2) (Strack and Cribbs 2012). GTP binding provides the driving force to form Drp1 helix assembly and GTP hydrolysis ensues. In the proposed model based on the Drp1 crystal structure, interface-2 in the center of the Drp1 stalks is the site for forming stable dimers. These dimers are the minimal units of Drp1 that can oligomerize further through interface-3, forming higher order assemblies. Interface-1 can show similar mode of assembling via interface-1 and -3 to form filaments. Interface-4, undescribed before, is required for assembling two neighboring Drp1filaments (Frohlich, Grabiger et al. 2013, Ugarte-Uribe and Garcia-Saez 2014). During mitochondrial fission, Drp1 assembles into helical structures, which wrap around mitochondrial tubules and constrict the bilayer membranes for fission (Legesse-Miller, Massol et al. 2003).

Drp1 constitutes the core of the mitochondrial fission machinery. Its pathophysiological roles have been highlighted in many studies. In Drp1 gene knockout models, loss of Drp1 results in embryonic lethality with developmental abnormalities: defects in forebrain and synapse development, defects in cardiac function and poor development of the liver at about embryonic day 12. Neural cell-specific (NS) Drp1 (-/-) mice died from brain hypoplasia with apoptosis after birth (Ishihara, Nomura et al. 2009, Wakabayashi, Zhang et al. 2009). Recently it has been reported that Drp1 affects human central nervous system (CNS) development. A newborn girl died 37 days after birth, carrying a Drp1dominant-negative mutation. She exhibited several neurological defects, including microencephaly, abnormal brain development and optic atrophy (Waterham, Koster et al. 2007, Westermann 2010).

Recruitment of Drp1 from the cytosol to the mitochondria is an essential step in mitochondrial fission. In mammals, four mitochondria-anchored proteins hFis1, Mff and MIEFs (MIEF1 and MIEF2) have been identified as potential mitochondrial receptors for Drp1. However, the different mechanisms by which Drp1 is recruited to mitochondria via these receptors are still incompletely understood.

1.3.1.2 Fis1

Fis1 is an integral membrane protein with its carboxyl-terminal tail anchored in the MOM. It functions as a potential receptor to recruit Drp1 to mitochondria. The N-terminus of Fis1 protrudes to the cytoplasm and its C-terminus to the intermembrane space (IMS) (Mozdy, McCaffery et al. 2000, Yoon, Krueger et al. 2003). The amino-terminus of Fis1 consists of six α-helices. The central four helices consist of two TRP motifs which are thought to be responsible for the recruitment of Drp1 to mitochondria in a TRP-dependent manner (Figure 2) (Suzuki, Neutzner et al. 2005, Zhang and Chan 2007). Conversely, in co-immunoprecipitation (co-IP) experiments little or no interaction was observed between Drp1

and Fis1 (Yu, Fox et al. 2005, Wells, Picton et al. 2007), and Chan et al. suggested that the interaction between Drp1 and Fis1 requires adaptor proteins (Lu and SpringerLink 2011).

The role of Fis1 in mitochondrial fission has been challenged recently. José Marín-García said "Although previous studies have demonstrated that overexpression of Fis1 has led to mitochondrial fragmentation and its depletion has resulted in interconnected mitochondrial networks caution should be taken interpreting these data, as these manipulations can induce nonphysiological stress causing perturbations of the mitochondrial morphology" (Marin-Garcia and SpringerLink 2013). hFis1 is evenly dispersed on the MOM, while the pool of Drp1 foci on the MOM is punctate. Depletion or exogenous expression of hFis1 neither affects Drp1 recruitment to mitochondria nor modulates mitochondrial morphology markedly (Suzuki, Jeong et al. 2003, Lee, Jeong et al. 2004, Otera, Wang et al. 2010). Recently Koirala et al. showed that Mdv1p in yeast is membrane-tethered, concomitantly recruiting Dnm1p to mitochondria independent of Fis1p (Koirala, Guo et al. 2013). Zhao et al. suggested that there are additional proteins or molecular signals potentially involved in the recruitment of Drp1 to the mitochondrial surface (Zhao, Lendahl et al. 2013). Altogether, Fis1 seems not to be a critical protein in the regulation of Drp1 recruitment in mammals.

Conversely, in hypoxia-induced mitochondrial fission, knockdown of Fis1 by shFis1 lentivirus diminished mitochondrial fragmentation in mouse embryonic fibroblasts (MEFs) (Kim, Scimia et al. 2011). Fis1 can act in sequence with Mff at the endoplasmic reticulum (ER) – mitochondrial interface to couple stress-induced mitochondrial fission with downstream degradation processes (Shen, Yamano et al. 2014). It has been reported that both the amount of Drp1 at the mitochondrial surface and the Drp1-Fis1 interaction could be increased upon apoptotic stimuli activated by anti-Fas (CD95) IgM antibody (Ciarlo, Manganelli et al. 2010). Given that, it is likely that Fis1-Drp1 mediated fission predominately occurs in cells suffering from cellular stress.

In summary, the mechanism of Fis1-mediated mitochondrial fission remains unclear and there could be other proteins cooperating with Fis1 in the regulation of Drp1 recruitment.

1.3.1.3 Mitochondrial fission factor (Mff)

Mff is a tail anchored protein, which was first identified in a small interfering RNA (siRNA) screen in *Drosophila* cells. It has N-terminal heptad repeats, coiled-coil domain, and a C-terminal transmembrane tail anchored in the MOM (Figure 2). A homologue of Mff exists in human. Through alternative splicing, the human Mff gene can generate at least nine isoforms (Gandre-Babbe and van der Bliek 2008). Knockdown of Mff by siRNA promotes mitochondrial elongation and dramatically decreases the amount of Drp1 on the MOM. On the other hand, overexpression of Mff induces mitochondrial fragmentation and enhanced Drp1 targeting to mitochondria. The N-terminus of Mff is required for recruitment of Drp1 to the MOM (Gandre-Babbe and van der Bliek 2008, Otera, Wang et al. 2010, Zhao, Liu et al. 2011, Liu, Yu et al. 2013). Otera and Mihara found that the punctate foci of Mff diffused within the MOM when Drp1 was depleted. They suggested that Drp1 affects oligomerization of Mff and that Mff functions as a Drp1 receptor to mediate fission (Otera, Ishihara et al. 2013). In addition, a recent study showed that the R376 residue in the Drp1 stalk is required

for its interaction with Mff, assembly into spirals and mitochondrial fission (Strack and Cribbs 2012).

1.3.1.4 MIEFs

Mitochondrial elongation factor 1 (MIEF1/MiD51) was discovered by searching a random subcellular localization screen of human proteins fused to green fluorescent protein (GFP) (Simpson, Wellenreuther et al. 2000). MIEF1 was identified as a mitochondrial protein, which is encoded by the MIEF1 gene on chromosome 22. This gene was previously named SMCR7L. Ectopic expression of MIEF1 induces mitochondrial elongation. Mitochondrial elongation factor 2 (MIEF2/MiD49) was subsequently discovered by protein sequence homology search, and this protein is encoded by the MIEF2 gene on chromosome 17, which was previously named SMCR7. MIEF1 and MIEF2 proteins share 45% amino acid sequence identity. Both MIEFs contain a predicted N-terminal transmembrane (TM) domain anchoring the proteins to the MOM. The C-terminus of MIEFs is facing the cytoplasm (Palmer, Osellame et al. 2011, Zhao, Liu et al. 2011, Liu, Yu et al. 2013). Recently, it has been reported that a nucleotidyl transferase domain is located in the central region of MIEF1. MIEF1 forms a dimer and binds adenosine diphosphate (ADP) in this region (Figure 2). The crystal structure study indicated that ADP is dispensable for MIEF1 in the recruitment of Drp1 to mitochondria, but it could activate Drp1 assembly and GTP hydrolysis (Loson, Liu et al. 2014).

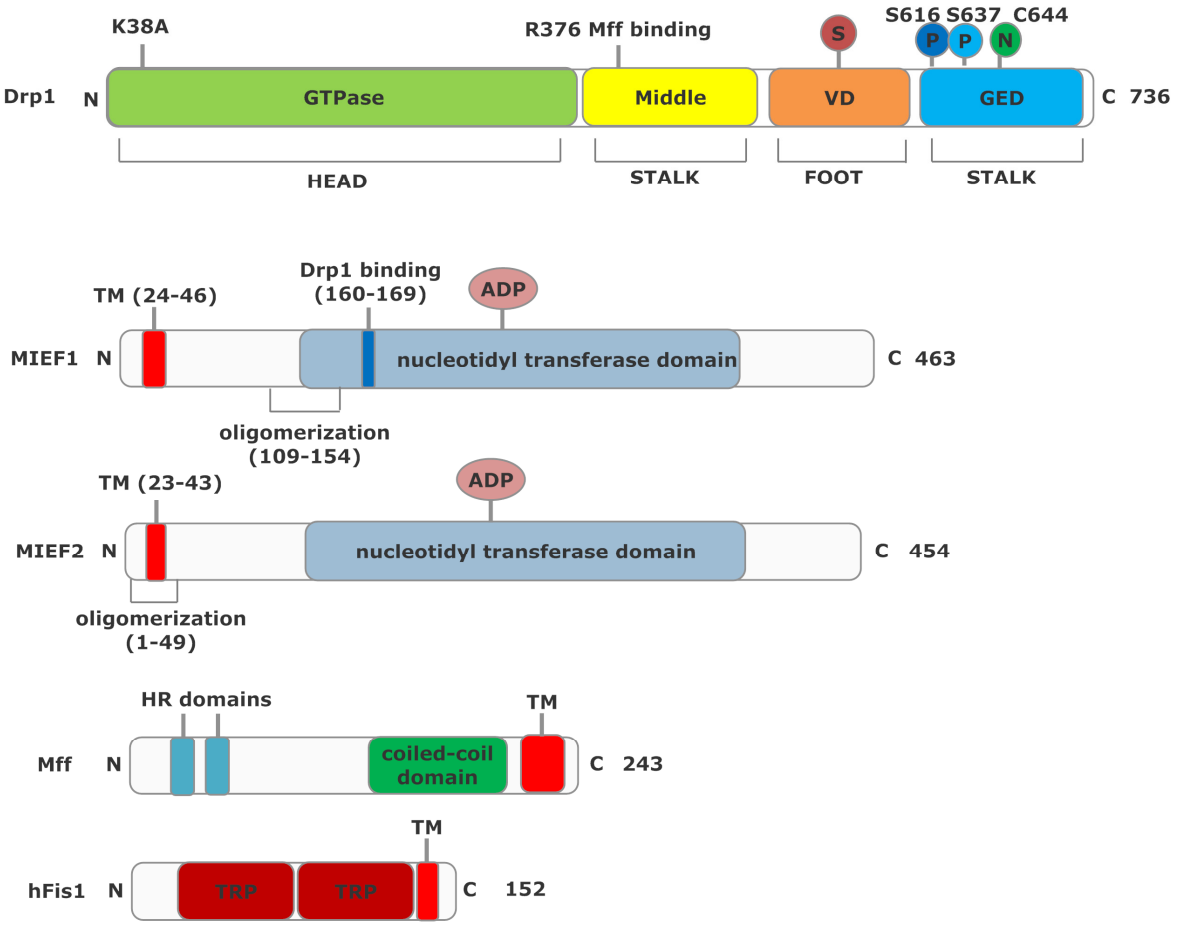
MIEF1 and MIEF2 appear to have many similar functions. They are N-terminally anchored in the MOM, recruit Drp1 to mitochondria and interact with Drp1 and hFis1. Ectopic expression of MIEF1 or MIEF2 induces extensive mitochondrial fusion. Despite sharing 45% amino acid sequence identity, to some extent, there are functional discrepancies between MIEF1 and MIEF2. When comparing the mRNA expression levels of MIEF1 and MIEF2 in human fetal and adult tissues, MIEF1 is highly expressed in fetal tissues, MIEF2 in adult tissues. MIEF2 has a higher potency than MIEF1 to promote mitochondrial fusion. Fis1 and Mff could only partially rescue MIEF2 induced fusion, while most of the fusion phenotype induced by MIEF1 was reversed by hFis1 and Mff. Oligomers of MIEF2 formed higher order assemblies requiring the N-terminal amino acid residues 1-49 (Figure 2). In the MIEF1 amino acid sequence residues 109-154 were essential for oligmerization but not for Drp1 binding (Figure 2). Dimers were largely observed for MIEF1 (Zhao, Liu et al. 2011, Liu, Yu et al. 2013). Additionally, antimycin A, an inhibitor of complex III of the electron transport chain, induced mitochondrial fragmentation in MIEF1/MiD51-expressing MEFs which were depleted of Fis1 and Mff but this did not occur in MIEF2/MiD49-expressing cells (Loson, Liu et al. 2014). In summary, this indicates that MIEF1 and MIEF2 also have divergent functions in mitochondrial fission.

MIEFs have been discovered to recruit Drp1 to the mitochondrial surface resulting in extensive mitochondrial fusion by inhibition of Drp1 activity. The process is independent of hFis1, Mff, and Mfn2 (Zhao, Liu et al. 2011, Loson, Song et al. 2013, Palmer, Elgass et al. 2013). Nevertheless, the function of MIEF1/2 in mitochondrial dynamics is still under debate. Zhao et al. showed that MIEF1 recruits Drp1 to mitochondria but promotes fusion by sequestering Drp1 in an inactive state on mitochondria. Depletion of MIEF1 by siRNA in HeLa cells induced mitochondrial fragmentation (Zhao, Liu et al. 2011). On the other hand,

Palmer et al. reported that double knockdown of MIEF1 and MIEF2 in COS7 cells results in an irregular distribution of the mitochondrial network and fused mitochondria. At the same time the pool of Drp1 foci was decreased on mitochondria. Palmer et al. therefore proposed that MIEF1and MIEF2 are mediators of mitochondrial fission (Palmer, Osellame et al. 2011). Recently, Palmer et al. clarified that overexpression of MIEF1/2 caused unopposed mitochondrial fusion events by blocking Drp1 activity along with elongation of peroxisomes. By PEG cell fusion assay, they elaborated that Mfn1 and Mfn 2 were required for MIEF-mediated fusion. In addition, MIEF appeared to have a higher capacity to recruit Drp1 than Mff or Fis1 have (Loson, Song et al. 2013, Palmer, Elgass et al. 2013). Nowadays, it has been reported that Mff, MIEF1 and MIEF2 recruit Drp1 in proximity of PTEN-induced putative kinase 1 (PINK1) and Parkin on depolarized mitochondria. But, only depletion of MIEF1/MiD51 and MIEF2/MiD49 attenuated mitochondrial fragmentation and mitochondrial clearance after 24 h of Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment. Remarkably, downregulation of MIEF1, functioning upstream of mitophagy, suppressed Parkin-dependent mitochondrial loss and mitophagy (Buhlman, Damiano et al. 2014).

The main debate has dealt with the potentially different roles of MIEFs in fusion or fission. There are several possible explanations to the different observations after knockdown of MIEFs. Firstly, in these studies, different cell lines were used for the experiments, and differences between cell lines could result in divergent conclusions. Secondly, different siRNA products and siRNA side effects on e.g. endogenous microRNAs could be another reason for the inconsistent results. As the MIEFs are recently discovered, we still know very little about their potential functions in mitochondrial dynamics. The detailed mechanisms by which MIEFs, Mff, and hFis1orchestrate Drp1 recruitment and regulate mitochondrial fission are still unknown. From my point of view, MIEFs could work on both sides affecting both mitochondrial fission and fusion. They could fine tune the balance of fission and fusion by interactions with different regulatory proteins in mitochondrial dynamics. The mechanisms of MIEF-related fusion and fission events are still an open question and need to be studied in depth in the future.

Figure 2. Domain structure of key mitochondrial outer membrane fission proteins in human.



1.3.1.5 Additional mitochondrial outer membrane and inner membrane fission proteins

Ganglioside-induced differentiation-associated protein1 (GDAP1), endophilin B1 (Endo B1), mitochondrial protein of 18 kDa (MTP18), and mitochondrial targeting GxxxG motif (MTGM) protein are additional regulatory factors involved in mitochondrial fission. GDAP1 is anchored in the MOM by its C-terminal TM region. Overexpression of GDAP1 induces Drp1-mediated mitochondrial fission, while depletion of GDAP1 promotes mitochondrial fusion. GDAP1 mutations have been discovered in peripheral neuropathy Charcot-Marie-Tooth (Niemann, Ruegg et al. 2005, Pedrola, Espert et al. 2005). Endo B1 is primarily distributed in the cytosol and involved in lipid remodeling of the MOM. It can be recruited to the MOM resulting in mitochondrial fragmentation. Endo B1 can be activated by Bax, and is involved in remodeling of the mitochondrial membrane in apoptosis (Pedrola, Espert et al. 2005). The MTP18 is a MIM-associated protein. Depletion of MTP18 results in fusion, while fission is reduced upon overexpression (Tondera, Czauderna et al. 2005).

MTGM (also termed Romo1) is an integral membrane protein of the MIM. The protein is highly conserved from yeast to human with the GxxxG motif which presumably mediates protein-protein interaction. In mammals, MTGM orthologs share 100% amino acid identity. Knockdown of MTGM results in mitochondrial elongation, whereas overexpression of

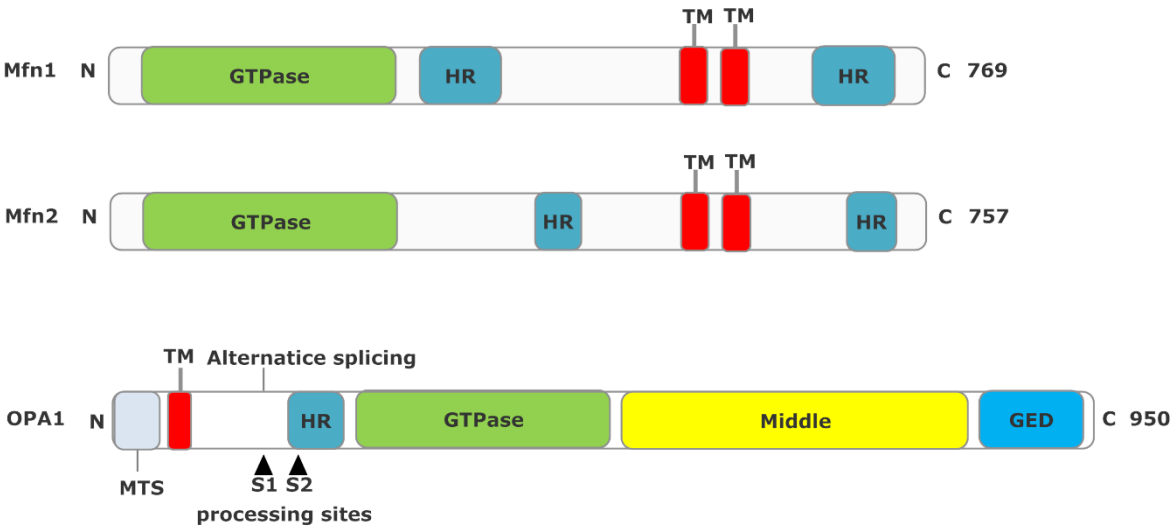
MTGM induces fragmentation in a Drp1-dependent manner (Zhao, Liu et al. 2009). This was the first time that MTGM was identified as a potential fission regulator localized in the MIM. As MTGM is anchored in the MIM, accordingly, it is conceivable that MTGM could be a candidate protein bridging the outer and inner membranes in the process of fission. MTGM is highly abundant in brain tumor cells and tumor tissues. In glioblastoma, knockdown of MTGM by siRNA resulted in a decrease of mitochondrial ROS level, while overexpression of MTGM increased the ROS production (Zhao, Liu et al. 2009, Yu, Song et al. 2014). In addition, it has been reported that MTGM is a potential diagnostic biomarker for non-small cell lung cancer (Lee, Lee et al. 2014). It is compelling that MTGM bridges the mechanisms of mitochondrial dynamics and ROS production in multiple types of cancer. More recently, it was shown that knockdown of MTGM led to mitochondrial fusion in HeLa cells, while overexpression of MTGM cDNA restored the normal mitochondrial network morphology in MTGM depleted U2OS cells (Norton, Ng et al. 2014). That finding is the opposite of our finding reported in paper iii (Zhao, Liu et al. 2009) of this thesis. In adenocarcinoma A549 cells (p53 wild type), upregulation of endogenous Romo1 (MTGM) resulted in partial cell death and cell growth inhibition (Kim, Kim et al. 2014), which is consistent with our finding that overexpression of MTGM results in mitochondrial fission and stalls the cells in S phase. We postulate that the divergent findings are due to the use of different cells for the experiments or to side effects from the siRNA treatment. Further studies are required to characterize the MTGM functions in mitochondrial dynamics.

1.3.2 Mitochondrial fusion

Three dynamin-related GTPases constitute the core components of the mitochondrial fusion machinery in mammals, including Mfn1, Mfn2, and OPA1. Mfn1 and Mfn2 are required for fusion of the MOM, and OPA1 for fusion of the MIM. These proteins coordinate the fusion process in both the mitochondrial outer and inner membranes (Rojo, Legros et al. 2002).

Fusion events are required to facilitate maximal ATP production especially in the nervous system which has the highest energy demand (Rugarli and Langer 2012). Mitochondrial fusion helps the cells to cope with stress stimuli, maintaining normal OXPHOS and the mitochondrial membrane potential. By fusion, the healthy mitochondria can complement the dysfunctional ones with mtDNAs, solutes, metabolites, and proteins. Additionally, mitochondrial fusion is crucial for embryonic development (Chen and Chan 2010, Youle and van der Bliek 2012). The mechanisms by which Mfns and OPA1 coordinately work to promote fusion of the MOM and MIM are still unclear.

Figure 3. Domain structure of key fusion proteins in mammals.



1.3.2.1 Mitofusins (Mfns)

Mfns (Fzo1p in yeast), termed Mfn1 and Mfn2 in mammals, are large GTPases localized in mitochondria where they regulate mitochondrial fusion. Mfns are anchored to the MOM with two TM domains, and their N- and C-termini are both opposing the cytoplasm (Escobar-Henriques and Anton 2013). The GTPase domain is located in the N-terminus, followed by a coiled-coil heptad repeat (HR1). The C-terminus has another coiled-coil heptad repeat (HR2) (Figure 3). In yeast, Fzo1p (Mfns) has an additional heptad repeat (HPN) in front of the GTPase domain but this is not present in mammals (Rojo, Legros et al. 2002, Honda, Aihara et al. 2005, Griffin and Chan 2006). All the HRs are important for Mfn's function. Like dynamin, mammalian Mfns can execute oligomerization by GTP-binding (Santel, Frank et al. 2003, Ishihara, Eura et al. 2004). Within Mfns, the interaction between HR1 and HR2 is dependent on GTPase activity and an intact N-terminal domain of Mfns (Rojo, Legros et al. 2002, Honda, Aihara et al. 2005).

Mouse knockout (KO) studies have provided clear evidence that these proteins are essential for mitochondrial fusion. In mice either Mfn1 or Mfn2 knockout results in embryonic lethality because of functional placental defect (Chen, Detmer et al. 2003). When Mfn1 was depleted after placental formation, mice appeared normal, whereas when Mfn2 was depleted after placental formation, mice displayed impaired cerebellar development and lethality at day 1 after birth (Chen, McCaffery et al. 2007). Mfn2 depletion results in impairment of the mitochondrial membrane potential (Bach, Pich et al. 2003). Isolated MEF cells lacking both Mfn1 and Mfn2 showed a series of cellular dysfunctions, including impairment of mitochondrial membrane potential, delayed cell growth and decreased cellular respiration, while cells lacking either Mfn1 or Mfn2 maintain mitochondrial fusion in low levels and survive from severer cellular dysfunction. This indicates that both homotypic and heterotypic interactions of Mfns can inducing mitochondrial fusion (Chen, Chomyn et al. 2005, Escobar-Henriques and Anton 2013) and suggests that Mfn1 and Mfn2 possess redundant functions to compensate for potential defects in fusion functions if either protein becomes inactivated.

Although Mfn1 and Mfn2 share 80% similarity, they still have distinct functions. Purified recombinant Mfn1 exhibits eightfold higher GTPase activity than Mfn2, and Mfn1 is mainly responsible for GTP-dependent membrane tethering (Ishihara, Eura et al. 2004). The *trans* interactions for apposing mitochondria are mainly dependent on Mfn1 (Benard and Karbowski 2009). The pro-apoptotic proteins Bax and Bak preferentially associate with Mfn2 (Lu and SpringerLink 2011). These findings indicate that the two mitochondrial fusion proteins Mfn1 and Mfn2 are functionally divergent to some extent.

1.3.2.2 OPA1

OPA1 (Mgm1p in yeast) is widely expressed and is localized in the MIM with discrete foci (Escobar-Henriques and Anton 2013). OPA1 is a nuclear-encoded protein that is a key protein in fusion of the MIM (Wong, Wagner et al. 2000, Wong, Wagner et al. 2003). It has an N-terminal mitochondrial targeting motifs by which OPA1 is targeted to the MIM. The mitochondrial targeting sequence is cleaved by matrix-processing peptidase (MPP) (Westermann 2010). OPA1 comprises the GTPase domain, middle domain, GED with a coiled-coil motif, and multiple heptad repeats (HR) (Figure 3). Eight OPA1 isoforms derive from alternative splicing and alternative processing at two sites in the N-terminus (Delettre, Griffoin et al. 2001, Song, Chen et al. 2007, Marin-Garcia and SpringerLink 2013). In mammals, the presenilin associated, rhomboid-like (PARL), the IMS and matrix AAA proteases, and the MIM peptidase OMA1 have been identified to catalyze the alternative processing of OPA1. Consequently long and short isoforms are generated. The long OPA1(l-OPA1) isoforms possess an N-terminal TM domain anchored in the MIM, while the short OPA1(s-OPA1) isoforms lack the TM and are targeted to the MIM through associating with l-OPA1(Cipolat, Rudka et al. 2006, Griparic, Kanazawa et al. 2007, Ehses, Raschke et al. 2009, Head, Griparic et al. 2009). It is reported that in OPA1-null cells, re-introduced l-OPA1 and s-OPA1 work together to facilitate MIM fusion. On their own, l-OPA1 or s-OPA1 is insufficient for fusion (Song, Chen et al. 2007). On the other hand, Ruchika et al. reported that l-OPA1 is adequate to maintain fusion in MEF cells. The proteolysis process of OPA1 is dispensable for mitochondrial fusion but crucial for mitochondrial integrity and quality control (Anand, Wai et al. 2014). In vitro studies of OPA1 mutants with GTP hydrolysis, oligomerization and lipid interaction deficiencies, all presented similar phenotypes to that of OPA1 deletion (Meeusen, DeVay et al. 2006, Rujiviphat, Meglei et al. 2009, Zick, Duvezin-Caubet et al. 2009). Hence, GTP hydrolysis, oligomerization and lipid interactions are essential for OPA1 functions in mitochondrial fusion.

OPA1 KO in mice is embryonic lethal. Repression of OPA1 in mammals slows down cell growth and decreases oxygen consumption (Escobar-Henriques and Anton 2013). Mutations in OPA1 cause autosomal dominant optic atrophy connected with a number of secondary neurological defects such as ataxia, deafness, sensory motor neuropathy and myopathy (Amati-Bonneau, Milea et al. 2009). Dysregulation of OPA1 results in alteration of cristae morphology, damage of mtDNA replication, mtDNA deletions, and even loss of mtDNA (Olichon, Baricault et al. 2003, Hudson, Amati-Bonneau et al. 2008). The function of OPA1 in cristae formation arises by oligomeric self-interactions instead of fusion processes (Sesaki, Southard et al. 2003). Moreover, OPA1 could stabilize the mitochondrial respiratory chain complexes and regulate OXPHOS by interacting with respiratory complexes I, II and III. In

summary, MIM fusion is critical for the maintenance of mtDNA and metabolic function of mitochondria (Chen, Chomyn et al. 2005, Zanna, Ghelli et al. 2008, Chen and Chan 2010).

1.3.3 Regulation of mitochondrial dynamics

Mitochondria keep changing their morphology by controlling the ratio of two opposing processes, fusion and fission. As changes in the balance between these opposing processes can occur rapidly, many studies focus on post-translational modifications of the proteins involved in mitochondrial dynamics. A number of investigations focus on modifications of Drp1 in fission and of OPA1 in fusion.

1.3.3.1 Regulation of mitochondrial fission

Drp1 SUMOylation

The modification of proteins by small ubiquitin-like modifier (SUMO) regulates many cellular processes. SUMOylation plays an important role in mitochondrial fission (Figure 4). SUMO1 belongs to the SUMO protein family. It is involved in nuclear transport, transcription, cell cycle transition, and protein stability (Marchiani, Tamburrino et al. 2011). Ectopic expression of SUMO1 stabilizes Drp1 on the mitochondrial membrane associating with Bax and inducing mitochondrial fission. Apoptosis induces SUMO1 conjugation to Drp1 in a Bax/Bak-dependent manner, increasing Drp1's mitochondrial association (Wasiak, Zunino et al. 2007, Zunino, Schauss et al. 2007). The conjugation of SUMO1 to its substrates requires a SUMO E3 ligase. The mitochondrial-anchored protein ligase (MAPL) is the first mitochondria-anchored SUMO E3 ligase identified to sumoylate Drp1. Overexpression of MAPL results in mitochondrial fragmentation (Braschi, Zunino et al. 2009). SENP5 is a SUMO protease. Overexpression of SENP5 promotes removal of SUMO1 from Drp1 and Drp1 degradation, subsequently inhibits the SUMO1-induced mitochondrial fission. In contrast, knockdown of SENP5 increases Drp1 sumoylation and promotes fission (Zunino, Schauss et al. 2007). Additionally, SUMO2/3-mediated Drp1 sumoylation decreases Drp1 on mitochondria and hinders Drp1-mediated apoptosis, which could be reversed by increase of SENP3 (Guo, Hildick et al. 2013). All these data indicate that SUMOylation of Drp1 by different proteins is important for the regulation of Drp1-mediated mitochondrial fission.

Drp1 Phosphorylation

There are two Drp1 phosphorylation sites that have undergone extensive study, amino acids Ser616 (Ser585 in rat Drp1) and Ser637 (numbering corresponds to human Drp1, isoform 1) (Figure 4). During mitosis, phosphorylation of Drp1-Ser616 by Cdk1/cyclin B results in mitochondrial fission and ensures the proper distribution of mitochondria into daughter cells (Taguchi, Ishihara et al. 2007). The mitotic kinase Aurora A phosphorylates a small Ras-like GTPase, RALA at Ser194 and increases the pool of RALA and its effector RALBP1 on the mitochondria. The RALA/RALBP1 complex stimulates phosphorylation of Drp1 by Cdk1/cyclin B resulting in mitochondrial fission. Knockdown of Mff by RNAi impaired the recruitment of Drp1 to mitochondria in mitosis (Kashatus, Lim et al. 2011).

cAMP-dependent protein kinase A (PKA) phosphorylates human Drp1 at Ser637 in response to different stimuli (Cribbs and Strack 2007). Mitochondrial fission is inhibited by this

phosphorylation through blocking the intra-molecular interaction between the middle domain and GED domain, GTP hydrolysis and Drp1 recruitment of mitochondria (Chang and Blackstone 2007, Cribbs and Strack 2007, Otera, Ishihara et al. 2013). Ca^{2+}/calmodulin-dependent protein kinase Iα (CaMKIα) regulates Drp1 phosphorylation at Ser637 that can be activated by high K^+ treatment. Although PKA-mediated phosphorylation inhibits Drp1-mediated fission, phosphorylation by CaMKIα increases Drp1 recruitment to mitochondria and induces fission (Han, Lu et al. 2008).

The Ser637 (Ser656 in rat) phosphorylation can be dephosphorylated by two phosphatases, Ca^{2+}/CaM-dependent protein phosphatase calcineurin (CaN) and PP2A/Bβ2, which activates Drp1 and mitochondrial fission. Phosphorylation of the mitochondrial targeting sequence of Bβ2 regulates the recruitment of PP2A to the MOM. Subsequently PP2A/Bβ2 dephosphorylates Drp1 at Ser656 (rat) (Merrill, Slupe et al. 2013). CaN-induced dephosphorylation of Drp1 at Ser637 accompanies mitochondrial depolarization and results in mitochondrial fragmentation. However, the Drp1S637D phosphomimetic mutant is distributed in the cytosol, while the Drp1S637A dephosphomimetic mutant is localized to mitochondria. The data indicate that dephosphorylation of Drp1 at Ser637 is required for Drp1 recruitment to mitochondria and inducing mitochondrial fragmentation (Cereghetti, Stangherlin et al. 2008).

Drp1 S-Nitrosylation

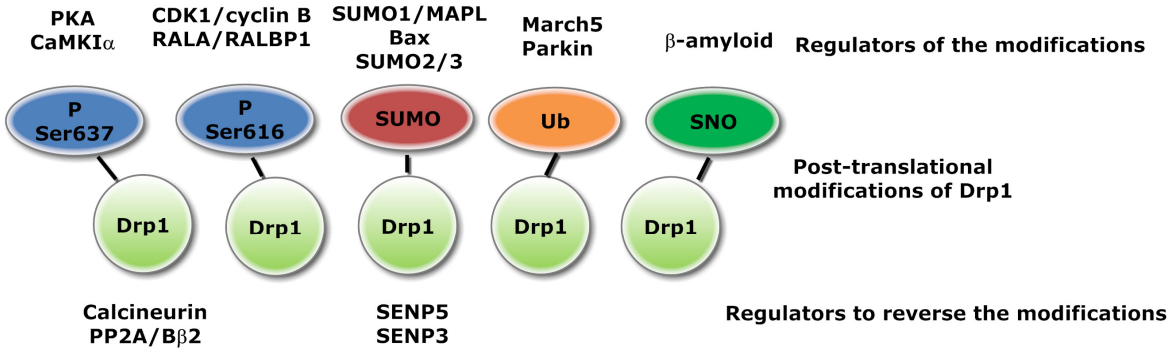
S-nitrosylation is also involved in the regulation of mitochondrial fission. In Alzheimer's disease (AD), nitric oxide (NO), which is produced in response to β-amyloid protein, S-nitrosylates Drp1 and forms S-nitrosylated Drp1 (Figure 4). This activates mitochondrial fission with resulting synaptic loss and neuronal damage (Cho, Nakamura et al. 2009). While another report showed that the mechanism underlying NO-induced mitochondrial fragmentation in AD is not regulated by Drp1 S-nitrosylation because the S-nitrosylated Drp1 levels in brains of age-matched normal and AD patients were not significantly different. Instead, the NO-induced mitochondrial fragmentation in AD was found to be regulated by Drp1 phosphorylation at Ser616 (Bossy, Petrilli et al. 2010). It is obvious that the regulation of Drp1 by S-nitrosylation is still controversial and needs to be determined in further studies.

Drp1 Ubiquitination

In concert with SUMOylation, ubiquitination regulates Drp1 function via March5 (also known as MITOL) (Figure 4). March5 is a mitochondria-associated N-terminal RING-finger motif E3 ubiquitin ligase which is located in the MOM (Otera, Ishihara et al. 2013). March5 knockdown results in mitochondrial fragmentation. Likewise, overexpression of a March5 mutant with deficient ubiquitin ligase activity induces fission (Nakamura, Kimura et al. 2006, Yonashiro, Ishido et al. 2006). On the contrary, Karbowski et al. showed that overexpression of the March5 RING mutants and knockdown of March5 by siRNA induced an abnormal mitochondrial elongation (Karbowski, Neutzner et al. 2007, Park, Lee et al. 2010). Recently, it has been shown that Drp1 and MAP1B are substrates for March5. March5-knockout MEFs are sensitive to NO-induced stress. It is suggested that March5 can protect the MEFs from NO-induced dysfunction of mitochondrial dynamics (Nagashima,

Tokuyama et al. 2014). Recently, Wang et al. reported that Parkin promoted mitochondrial fusion by directly ubiquitinating Drp1 and reducing Drp1 levels (Wang, Song et al. 2011), where the opposite opinion is that Drp1 is not a direct target of the PINK/Parkin pathway (Gegg, Cooper et al. 2010, Tanaka, Cleland et al. 2010). The effect of March5- and Parkin-mediated ubiquitination of Drp1 on mitochondrial dynamics therefore remains controversial.

Figure 4. Drp1 post-translational modifications.



1.3.3.2 Regulation of mitochondrial fusion

PARL

In mammals, PARL (Pcp1 in yeast, Rho-7 in fly) is a mitochondrial rhomboid protease with seven membrane-spanning regions. It is located in the intermembrane space (IM) (Yaffe 2003). PARL is conserved throughout eukaryotes. Expression of mammalian PARL in yeast can substitute the function of its yeast ortholog (McQuibban, Saurya et al. 2003). In apoptosis, PARL has been reported to involve in the heat shock response, release of s-OPA1 isoforms from mitochondria and accumulation of a soluble intermembrane space form of OPA1 (Cipolat, Rudka et al. 2006, Sanjuan Szklarz and Scorrano 2012, Escobar-Henriques and Anton 2013).

YME1L and OMA1

YME1L is an i-AAA protease which is responsible for the recognition and degradation of mis-folded proteins in mitochondria. YME1L is anchored in the MIM and its protease domain facing the intermembrane space (Song, Chen et al. 2007, Escobar-Henriques and Anton 2013). OMA1, located in the inner membrane of mitochondria, is required for the stress-induced cleavage of OPA1 and mitochondrial fragmentation. OMA1 and YME1L cleave OPA1 at S1 and S2 respectively (Figure 3), by proteolysis. Thus there are two s-OPA1 isoforms, c and e generated by OMA1 processing. Another s-OPA1, d, is generated by YME1L processing. Depletion of YME1L impairs the processing of OPA1 at S2 and causes mitochondrial fragmentation, perturbs cristae morphogenesis, and renders cells susceptible to apoptosis (Anand, Wai et al. 2014).

Bax/Bak

Many apoptosis-related proteins have been suggested to be regulators of mitochondrial dynamics. Bcl-2 family members, especially Bax/Bak, were widely studied in the regulation of mitochondrial morphology. These proteins play special roles via their interaction with mitochondrial dynamics proteins (Autret and Martin, 2009; Martinou and Youle, 2011).

In healthy cells Bax and Bak are required for the normal level of mitochondrial fusion through activation of Mfn2 and altering its distribution and mobility on mitochondria (Karbowski, Norris et al. 2006). Bak is a MOM-associated protein, which is capable of binding both Mfn1 and Mfn2, and shuttles between the cytosol and mitochondria (Brooks and Dong 2007, Hoppins, Edlich et al. 2011). In normal cells, Bax and Bak activate Mfn2, leading to its altered distribution and mobility to possible fusion sites and assembly. Bak has been shown to inhibit mitochondrial fission (Karbowski, Norris et al. 2006, Brooks and Dong 2007). In contrast, Hoppins and coworkers showed in an in vitro mammalian mitochondrial fusion assay that the soluble form of Bax can promote mitochondrial fusion when only Mfn2 is present on two tips of the fusion sites. It indicates that Bax is required for the particular homotypic Mfn2 complexes to induce fusion (Hoppins, Edlich et al. 2011, Ugarte-Uribe and Garcia-Saez 2014). When the shuttling of Bax from mitochondria to the cytosol is blocked, Bax ability to stimulate fusion is impaired (Hoppins, Edlich et al. 2011). In summary, Bax works like a cofactor in the regulation of mitochondrial fusion (Ugarte-Uribe and Garcia-Saez 2014).

Fusion model

Mitochondrial fusion might be a more complex process than fission. It requires mitofusins working on the MOM and OPA1 working on the MIM harmoniously. In fusion of the MOM, mitofusins in *cis* dimerize triggered by GTP binding. Physical in *trans* interactions among mitofusins facilitate bringing two mitochondria closer for the fusion process (Ishihara, Eura et al. 2004). GTP hydrolysis allows mitofusin ubiquitylation, remodels the membrane structure, and facilitates the MOM merging (Low, Sachse et al. 2009). In fusion of the MIM, at the beginning, the fusion process requires cleavage of the long OPA1 isoforms into short isoforms by YME1L-mediated proteolysis (Song, Chen et al. 2007). The ratio of l-OPA1 and s-OPA1 is modulated by proteolysis that produces the proper combination of l- and s- OPA1 facilitating inner membrane fusion. The l-OPA1 forms act on membrane tethering, while the s-OPA1 contributes to GTPase activity. As soon as MOM fusion has been activated, l- and s-OPA1 can form homo- and hetero-oligomers, which promote the contact between apposing inner membranes and achieve inner membrane fusion (Ishihara, Eura et al. 2004, Meeusen, DeVay et al. 2006, DeVay, Dominguez-Ramirez et al. 2009, Escobar-Henriques and Anton 2013).

1.4 TETHERING OF MITOCHONDRIA AND ER

The region of mitochondria and ER interaction is called mitochondria-associated ER membrane (MAM), which is also called ER-associated mitochondrial division (ERMD) site. This association is required for proper mitochondrial distribution, maintaining Ca^{2+} homeostasis and phospholipid biosynthesis. Mfn2 is localized both in mitochondria and in ER, and it connects these organelles to each other (da Silva, Mariotti et al. 2014). Nagashima et al. presented a novel hypothesis that March5 regulates ER and mitochondria

tethering by ubiquitination of mitochondrial Mfn2 and activation of Mfn2 GTP binding ability. The activated Mfn2 oligomerizes with the ER-localized Mfn2 and apposes the mitochondria to the ER (Nagashima, Tokuyama et al. 2014). At the site of ERMD, ER tubules encircle mitochondria, before Drp1 foci form in mitochondria, and mark the regions where the division events will predominantly occur. Drp1 and Mff are found at ERMD site. Mff is localized there in a Drp1-independent manner. The potential mechanism of ER regulating mitochondrial fission was suggested. Inverted formin-2 (INF2) is an inverted formin protein regulating F-actin nucleation and depolymerization. Both INF2 and actin have been found to localize at ERMD sites. INF2 marks the fission sites by facilitating Drp1 localization to mitochondria and promotes actin polymerization. This actin polymerization induced by INF2 generates a force that constricts mitochondria to a diameter compatible with the size of the Drp1 helical structure. Drp1 assembles in spirals at these sites, ultimately leading to mitochondrial fission (da Silva, Mariotti et al. 2014).

1.5 CALCIUM SIGNALING FROM ER TO THE MITOCHONDRION

Ca^{2+} transfer occurs from the ER to mitochondria at MAM. Inositol 1,4,5-trisphosphate receptor (Ins(1,4,5)P3R) on the ER membrane and the voltage-dependent anion selective channel protein 1 (VDAC1) on the MOM orchestrate the Ca^{2+} uptake in the MOM. In the MIM, the mitochondrial calcium uniporter (MCU) is a regulator of Ca^{2+} uptake (Rowland and Voeltz 2012). As a result, firstly, mitochondrial membrane proteins acquire Ca^{2+} for their functions. Secondly, Ca^{2+} oscillations can stimulate mitochondrial fission in a Drp1-dependent manner, e.g. MIRO-mediated mitochondrial fission requires local Ca^{2+} influx, which has been found at the ERMD sites. Thirdly, local Ca^{2+} flux can stimulate apoptosis inducing cytochrome c release by opening the mitochondrial permeability transition pore (MPTP) (Rowland and Voeltz 2012).

In addition to the Ca^{2+} transfer from the ER lumen to mitochondria, mitochondria are key players in the buffering of cytosolic Ca^{2+} overload and excitotoxicity (Szydlowska, Krowczynski et al. 2010). The MOM functions as a permeability barrier for both Ca^{2+} influx and efflux (Crompton, Barksby et al. 2002). Calcium enters mitochondrial matrix passively via the MCU that is driven by the mitochondrial membrane potential ($\Delta\Psi m$) in the MIM (Baughman, Perocchi et al. 2011). In excitable cells, Ca^{2+} fluxes back into the cytoplasm via the mitochondrial Na^+/Ca^{2+} exchanger (NCX_{mito}) (Wood-Kaczmar, Deas et al. 2013). Wood-Kaczmer et al. first reported that plasma membrane NCX2 and NCX3 present in human nerve cell mitochondria and work downstream of PINK1 protecting the cell from neurodegeneration induced by mitochondrial Ca^{2+} accumulation (Wood-Kaczmar, Deas et al. 2013). The NCX_{mito} buffering activity is regulated by A-Kinase anchoring protein AKAP121, which controls the mitochondrial membrane potential and oxidative phosphorylation (Scorziello, Savoia et al. 2013). Increasing AKAP121 levels can also control mitochondrial dynamics through the PKA- and AKAP121-depenent phosphorylation of Drp1 at Ser637 and attenuate hypoxia-induced mitochondrial fission (Kim, Scimia et al. 2011). Given that, it is conceivable that the NCX proteins may play a role in mitochondrial quality control and mitochondrial dynamics through association with PINK1 and AKAP121. Although the plasma membrane NCX2 and NCX3 proteins have been identified also in mitochondria, in addition to Ca^{2+} fluxes, other potential roles of the

NCXs in mitochondria remain unclear. Thus, it is compelling to uncover the mystery of NCX functions in different subcellular compartments.

1.6 MITOCHONDRIAL DYNAMICS AND APOPTOSIS

The Bcl-2 family is constituted by three subgroups. First, Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1, which belong to the antiapoptotic Bcl-2 proteins, interact with proapoptotic proteins directly. Second, the intrinsic proapoptotic pathway including Bax and Bak, are involved in MOM permeabilization. Third, the BH3-only proteins, which share a common BH3 domain, are responsible to sense cell stress and initiate apoptosis (Garcia-Saez 2012).

Mitochondria are involved in the intrinsic Bax/Bak apoptotic pathway. The fission and fusion machineries are related to apoptosis in mammals (Elgass, Pakay et al. 2013, Escobar-Henriques and Anton 2013). The balance of mitochondrial fusion and fission alters the cells response to apoptosis and cells with fragmented mitochondria are more sensitive than cells with an interconnected network of mitochondria. A pivotal role in apoptosis is played by mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome c, Smac/Diablo, and Omi from the intermembrane space into the cytosol to activate downstream cell death pathways (Martinou and Youle 2011, Garcia-Saez 2012).

Initially, apoptosis stimuli induced cell stress results in a highly interconnected mitochondrial network of mitochondria. Mitochondrial fusion can increase in response to the cellular accumulation of oxidized glutathione which modifies the oligomerization of Mfn2. Simultaneously, the l-OPA1 isoforms can be stabilized by a MIM protein, SLP-2, facilitating the fusion process. In this case, cells display a tubular mitochondrial network and are less prone to die in response to the cell stress (Friedman and Nunnari 2014). On basis of current studies, mitochondrial fusion inhibits apoptosis by sustaining cytochrome c in mitochondria and keeping cristae integrity mediated by OPA1 (Frezza, Cipolat et al. 2006).

With the progression of apoptosis, the balance of mitochondrial dynamics shifts from fusion to fission and MOMP occurs based on the formation of outer membrane homo-oligomeric pores composed of Bak and Bax that facilitates cytochrome c to leak out and activates the cascade of the intrinsic apoptosis pathway (Wasiak, Zunino et al. 2007, Youle and Strasser 2008, Westphal, Kluck et al. 2014). Bax/Bak and Drp1 mutually regulate their accumulation on mitochondria. Drp1 stimulates Bax oligomerization for enhancing MOMP on the mitochondrial membrane (Rowland and Voeltz 2012). In addition, Fis1 has been reported to participate in ER–mitochondrial communication in apoptosis. Fis1 interacts with the ER-located Bap31 at the MAM and induces Bap31 cleavage into the pro-apoptotic p20Bap31. Simultaneously, Fis1 transfers the apoptotic signal, procaspase-8, from mitochondria to Fis1-Bap31 complex and activates the release of Ca^{2+} from the ER. The Ca^{2+} efflux from the ER to the mitochondrial matrix induces apoptosis by interaction with cyclophilin D, then helps to form the MPTP and release cytochrome c (Iwasawa, Mahul-Mellier et al. 2011, Elgass, Pakay et al. 2013).

According to the current knowledge, mitochondrial dynamics and the apoptosis machinery possess a complex relationship and the ultimate regulatory mechanism in apoptosis still remains to be elucidated.

1.7 MITOCHONDRIAL DYNAMICS AND MITOPHAGY

Autophagy is a process of self-degradation of cellular components that are dysfunctional or residual within the cell. The organelles or portions of the cytosol targeted to undergo autophagy are sequestered by double-membrane autophagosomes that fuse with lysosomes or vacuoles and are broken down by resident hydrolases (He and Klionsky 2009, Westermann 2010). Mitophagy is the specific autophagic elimination of dysfunctional mitochondria and mediates regulation of mitochondrial number to meet cellular demands (Youle and Narendra 2011, Gomes and Scorrano 2013). Mitochondrial division generates two uneven daughter mitochondria, one with high membrane potential and high possibility for fusion and the other with decreased membrane potential, no or less mtDNA, low respiration activity, and reduced OPA1 levels. The latter loses the fusion capability and is prone to removal by mitophagy, in order to ensure mitochondrial quality (Twig, Elorza et al. 2008, Mouli, Twig et al. 2009, Westermann 2010).

Mitophagy is regulated by Parkin and PINK1 in different cells and mutations of Parkin and PINK1 have been discovered in Parkinson's disease (Youle and Narendra 2011). PINK1 is a serine/threonine kinase positioned in mitochondria. In healthy mitochondria, newly translated PINK1 is progressively targeted and degraded on the MIM. Parkin is an E3 ubiquitin ligase (Jin and Youle 2012). Upon mitochondrial depolarization, PINK1 is activated by phosphorylation and recruits cytosolic Parkin to mitochondria on the MOM, and subsequently ubiquitinates and degrades Mfns and Drp1in damaged mitochondria before the formation of autophagosomes (Narendra, Tanaka et al. 2008, Jin, Lazarou et al. 2010, Rakovic, Grunewald et al. 2011, Okatsu, Oka et al. 2012). In addition, Parkin is involved in proteasome-dependent degradation of MOM proteins, facilitating breakdown of the MOM (Yoshii, Kishi et al. 2011).

Several mitochondrial fission and fusion proteins contribute to the process of mitophagy. Drp1 is required for Bcl2/adenovirus E1B 19-kDa interacting protein 3 (Bnip3) induced mitophagy. Overexpression of Drp1K38E, a dominant negative mutant of Drp1 blocked mitochondrial fission and Bnip3-induced mitophagy (Lee, Lee et al. 2011). It has been shown that modest levels of ROS specifically induce mitophagy dependent on Drp1 (Frank, Duvezin-Caubet et al. 2012). High levels of hFis1 enhance mitophagy more than it triggers mitochondrial fragmentation (Gomes and Scorrano 2008). In contrast, knockdown of hFis1 in pancreatic β-cells by siRNA attenuates mitophagy and results in reduced respiration and repressed insulin secretion (Twig, Elorza et al. 2008). On the fusion side, upon membrane depolarization Parkin induces the ubiquitination of Mfn1 and Mfn2 leading to their degradation in a proteasome- and p97-dependent manner and blocking mitochondrial re-fusion (Tanaka, Cleland et al. 2010).

1.8 MITOCHONDRIAL DYNAMICS IN CANCER

Losing control of cell proliferation and resistance to apoptosis are both well-known characteristics of cancer. Both of these characteristics are accompanied by mitochondrial dysfunction. The Warburg effect shifts the cancer cells' metabolism from oxidative metabolism to aerobic glycolysis (Evan and Vousden 2001, Bonnet, Archer et al. 2007). Recently, Hanahan and Weinberg published the update of the review paper "Hallmarks of

Cancer" and emphasized the new hallmarks, in which deregulating cellular energetics has been included. Altered energy metabolism works in concert with other well-established core hallmarks in cancer (Hanahan and Weinberg 2011). Mitochondrial fusion and fission are involved in regulating mitochondrial energy production. Mitochondria with the fusion phenotype maintain high membrane potential, generate more ATP, and inhibit mitophagy. Conversely, mitochondria with the fission phenotype have low membrane potential, low ATP production and facilitate apoptosis and mitophagy. Some direct connections between mitochondrial dynamics and cancer have been uncovered gradually. It has emerged that mitochondrial fusion and fission events are related to lung cancer. Inhibition of fission negatively affects cell cycle progression in cancer cells (Rehman, Zhang et al. 2012). In lung cancer cells and tumors without treatment, the phosphorylation of Drp1at Ser616 via cyclin E/CDK1 was activated and the phosphorylation ratio of Ser616 to Ser637 was increased. Mitochondria showed fragmentation. Seen from another point, Mfn2 was downregulated in the lung cancer compared with the adjacent healthy lung (Rehman, Zhang et al. 2012). In addition, high Drp1 expression potentially predicts recurrence and a greater cisplatin resistance (Chiang, Chen et al. 2009). Inhibition of Drp1 or activation of Mfn2 results in regression of human lung tumors in a xenotransplantation model (Rehman, Zhang et al. 2012, Archer 2013). More recently, the mitochondrial division inhibitor-1 (Mdivi-1), an inhibitor of Drp1, was found to regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human cancer cells. Mdivi-1 could induce cell death in malignant melanoma, lung cancer and osteosarcoma cells, while normal cells survive. Both mdivi-1 treatment and knockdown of Drp1 could sensitize cancer cells to TRAIL-induced apoptosis. It was suggested that cancer cells are more vulnerable to changes in mitochondrial fusion and fission (Akita, Suzuki-Karasaki et al. 2014). Accordingly, both mitochondrial fusion and mitochondrial fission are involved in tumor development although the precise mechanisms are still unclear. At least, these findings can shed new light on mitochondrial dynamics in the field of cancer research, and manipulation of mitochondrial fusion and fission dynamics is promising novel therapeutic targets.

1.9 MITOCHONDRIAL DYNAMICS IN OTHER HUMAN DISEASES

Control of the balance between fusion and fission is very important in high energy-demanding cells, such as cardiac, skeletal muscle, and neuronal cells. Disorders of mitochondrial dynamics are implicated in neurodegenerative, endocrine, and cardiovascular diseases. Mutations in key mitochondrial dynamics proteins have been identified in a variety of human diseases (Zuchner, Mersiyanova et al. 2004, Cho, Nakamura et al. 2009, Romanello, Guadagnin et al. 2010, Wang, Song et al. 2011, Ong, Hall et al. 2013). As neuronal cells are the most energy demanding to execute a number of cellular events, they are very sensitive to alterations of mitochondrial morphology. Several nervous system diseases are associated with dysregulation of mitochondrial fusion and fission, including Parkinson's disease (Chinta and Andersen 2008), Charcot-Marie-Tooth neuropathy type 2A and 4A (Baxter, Ben Othmane et al. 2002, Cuesta, Pedrola et al. 2002, Zuchner, Mersiyanova et al. 2004), autosomal dominant optic atrophy (Alexander, Votruba et al. 2000, Delettre, Lenaers et al. 2000, Olichon, Guillou et al. 2006), abnormal brain development (Waterham, Koster et al. 2007), Huntington's disease (Wang, Lin et al. 2009, Haun, Nakamura et al. 2013), amyotrophic lateral sclerosis (Song, Song et al. 2013), and Alzheimer's disease (Cho,

Nakamura et al. 2009). In the cardiovascular system, ischemia/reperfusion injuries, cardiomyopathy, and the diabetic heart have been associated with abnormal mitochondrial dynamics (Brooks, Wei et al. 2009, Ashrafian, Docherty et al. 2010, Makino, Scott et al. 2010, Ong, Subrayan et al. 2010, Sharp, Fang et al. 2014). Diabetes is also related to impairment of mitochondrial fusion (Twig, Hyde et al. 2008, Sebastian, Hernandez-Alvarez et al. 2012). In addition, many other diseases have emerged as more or less connected with changes in mitochondrial dynamics and further studies of mitochondrial dynamics in human disease will be compelling. Here I just briefly go through the above diseases and list the involved genes controlling mitochondrial fusion and fission, mitochondrial phenotypes and the pathological effects in the following table 1.

Table 1. Mitochondrial dynamics in human diseases

Disease	Affected gene	Mitochondrial phenotype	Effect	Reference
Parkinson's disease	Mutations of PINK1 or Parkin	Accumulation of damaged mitochondria	Increase of ROS production and degeneration of dopaminergic neurons in substantia nigra	(Chinta and Andersen 2008)
Charcot-Marie-Tooth neuropathy type 2A	Mutations of Mfn2	Deficient fusion	Degeneration of peripheral nerves	(Zuchner, Mersiyanova et al. 2004)
Charcot-Marie-Tooth neuropathy type 4A	Mutations of GDAP1	Deficient fusion	Degeneration of peripheral nerves	(Baxter, Ben Othmane et al. 2002, Cuesta, Pedrola et al. 2002)
Autosomal dominant optic atrophy	Mutations of OPA1	Deficient fusion	Degeneration of retinal ganglion cells and atrophy of optic nerve	(Alexander, Votruba et al. 2000, Delettre, Lenaers et al. 2000, Olichon, Guillou et al. 2006)
Abnormal brain development	Mutations of Drp1	Deficient fission	Microcephaly, abnormal brain development and optic atrophy	(Waterham, Koster et al. 2007)
Huntington's Disease	Mutation of Huntingtin, activates Drp1	Increased fission	Choreoathetosis, dementia, and premature death	(Wang, Lin et al. 2009, Haun,

				Nakamura et al. 2013)
Amyotrophic Lateral Sclerosis	Mutation of superoxide dismutase 1, activates Drp1	Increased fission	Motor neuron degeneration	(Song, Song et al. 2013)
Alzheimer's Disease	β-amyloid increases NO, S-nitrosylation of Drp1	Increased fission	Neuronal damage	(Cho, Nakamura et al. 2009)
Ischemia/Reper-fusion Injury	Drp1	Increased fission	Drp1 targeting to mitochondria, increased production of ROS and elevated Ca^{2+} promoting diastolic cardiac dysfunction	(Ong, Subrayan et al. 2010, Sharp, Fang et al. 2014)
Cardiomyo-pathy	Drp1, Mfns, and OPA1	Disturbed mitochondrial morphology	Drp1 Python mutation mice suffer mitochondrial dynamics and ATP depletion	(Ashrafian, Docherty et al. 2010)
Diabetic Heart	Drp1 and OPA1	Increased fission	Elevated level of Drp1, reduced levels of OPA1	(Brooks, Wei et al. 2009, Makino, Scott et al. 2010)
Diabetes	Mfn2 and OPA1	Impaired fusion	Loss of OPA1/Mfn2, reduced mitochondrial biogenesis and oxygen consumption	(Twig, Hyde et al. 2008, Sebastian, Hernandez-Alvarez et al. 2012)

2 AIM

The aims of this thesis are:

- Identification of novel mitochondrial proteins involved in the regulation of mitochondrial dynamics in human
- Molecular and functional characterization of MIEF1, MIEF2 and MTGM in mitochondrial dynamics
- Comparison of the molecular and functional characteristics between MIEF1 and MIEF2 in mitochondrial dynamics
- Elucidation of human NCX3 subcellular distribution and its function as a Na^{+}/Ca^{2+} transporter in cellular events

3 MATERIALS AND METHODS

Cell cultures and transfection

293T, HeLa and MCF-7 cells were grown in Dulbecco's modified Eagle's medium with 10% FCS. The three clonal human malignant glioma cell lines, U-343 MG, U-343 MGa Cl2:6 (Cl2:6) and U-87MG, were grown in Eagle's minimum essential medium with 10% FCS. The neuroblastoma cell line SH-SY5Y was grown in DMEM/F-12 medium, the U2OS osteosarcoma cells in McCoy's 5A modified medium, and the HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ colon carcinoma cell lines in RPMI-1640 medium. All media contained 10% fetal bovine serum and 1% Penicillin/Streptomycin, and the cultured cells were maintained at 37 °C in 5% CO_2 at 95% humidity.

Generation of expression constructs

To study the genes' functions in cells, the cDNAs with the full-length ORF of MIEF1, MIEF2 and MTGM were amplified by PCR using the Human Fetal Brain BD™ Marathon-Ready cDNA (Clontech) as template, and cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) to produce a C-terminally V5-His-tagged MIEF1, MIEF2 and MTGM. The generated plasmid were verified by sequencing. We also generated an N-terminally HA-tagged MIEF1 plasmid in pcDNA3.1 (HA-MIEF1). Deletion mutations of MIEF1 and MIEF2 and NCX3.2-EmGFP were constructed in the pcDNA3.1/V5-His-TOPO vector (Invitrogen) using standard PCR technique. Internal deletion mutants of MIEF1 and MIEF2 were constructed in the same expression plasmid using a previously described method (Ho, Hunt et al. 1989). All deletion constructs were verified by sequencing. Site directed mutagenesis of NCX3-N45D and NCX3-N67D was carried out using a protocol based on the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) with the plasmid (pcDNA3.1-NCX3.2/V5-His).

Real-time PCR analysis

To monitor the normal tissue distribution of MIEF1, MIEF2, and MTGM expression, the Human Adult MTC™ Panel I and Human Fetal MTC™ Panel (Clontech) containing cDNAs from multiple tissues were used as templates for standard real-time PCR, and the results were normalized against β-actin.

Western blotting

Protein extracts were separated by electrophoresis and transferred to PVDF membranes (Millipore). After blocking with 10% nonfat dry milk in PBS, the membranes were incubated with primary antibodies and the peroxidase-conjugated secondary antibody (Amersham), and immunocomplexes were detected with an enhanced chemiluminescence (ECL) kit (Amersham). Densitometric analysis for quantifying Western blots was performed using the ImageJ gel analysis software.

Indirect immunofluorescence and confocal microscopy

To investigate the protein subcellular localization, mitochondrial morphology, proteins colocalization and cell fusion assay, the cells were cultured on coverslips, washed with PBS, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, incubated in blocking buffer with 2% BSA (bovine serum albumin) in PBS and subsequently with the primary antibodies and secondary antibodies conjugated with different fluorophores. After a final PBS wash, the coverslips were mounted with the Mounting Medium containing DAPI (Vector Laboratories). For mitochondrial staining, the MitoTracker Red CMXRos (500 nM, Molecular Probes) was added to cultures for 15 minutes before fixation. Specimens were examined by fluorescence microscopy system (Zeiss) or by SP5 confocal microscopy system (Leica).

Transmission electron microscopy

Transmission electron microscopy was used to visualize the ultrastructure of mitochondria. Transfected cells were prepared for the experiment. Series of sections were counterstained with uranyl acetate and lead citrate and analyzed in a Tecnai 12 (FEI) electron microscope.

Subcellular fractionation

Subcellular fractionation is a classic method to study the protein distribution in different cellular compartments. MIEF1 and MTGM have been identified in the MOM and the MIM respectively. The mitochondrial fraction was isolated as described (Parone, James et al. 2006). Cells were collected in ice-cold mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5) with protease inhibitors (Roche). For proteinase K digestion, mitochondrial pellets were suspended in mitochondrial buffer (MB) with 50 µg/ml proteinase K and incubated for 30 minutes at room temperature. For membrane protein analysis, mitochondrial pellets were resuspended in 100 µl of MB buffer, or MB buffer containing 0.1 M Na_2CO_3 (pH 11.5) and incubated on ice for 30 minutes. The insoluble membrane fractions were centrifuged at 16,000 × *g* for 15 minutes, and the supernatants were precipitated with 10% (v/v) trichloroacetic acid.

In vivo protein-protein cross-linking and co-immunoprecipitation

Protein-protein cross-linking is required for the study of oligomerization. The cells were kept in PBS buffer containing 1% formaldehyde (FA) for 20 min at room temperature as described (Hajek, Chomyn et al. 2007). The crosslinking reaction was terminated by PBS containing 100 mM glycine. The crosslinked cells were dissolved in SDS-sample buffer and the whole cell extracts were analyzed by Western blotting.

To measure protein-protein interaction, co-immunoprecipitation experiments were carried out according to the method described (Hajek, Chomyn et al. 2007) with a slight modification. The captured immune complexes were dissolved in SDS-sample buffer and analyzed by SDS-PAGE and immunoblotting.

PEG cell fusion assay

To visualize the MIEF1 function in mitochondrial fusion, PEG-mediated cell fusion experiments were performed as previously described (Liesa, Borda-d'Agua et al. 2008,

Kamp, Exner et al. 2010) with slight modifications. Cells were transiently transfected with either mito-GFP or mito-DsRed. After 24 h, cells carrying differently labeled mitochondria were mixed (1:1) and then the mixed cells were transfected with either MIEF1-V5 or empty vector. Cell fusion was induced by a treatment with polyethylene glycol (PEG) and fixed with 4% paraformaldehyde in PBS. After immunofluorescence staining with anti V5-tag antibody (for MIEF1-V5 positive cells), polykaryons were analyzed by confocal microscopy using the Leica Colocalization Software. Mitochondrial fusion was evaluated as the percent of mito-GFP and mito-DsRed colocalization in mitochondria.

GTP-agarose pull-down assay

To study the effect of MIEF1 on Drp1 GTP-binding capability, GTP-binding assays were performed as described (De Palma, Falcone et al. 2010) with slight modifications. The proteins bound to the GTP-agarose beads were visualized by performing immunoblot analysis.

Bioinformatics

Serial Analysis of Gene Expression (SAGE) libraries (www.cgap.nci.nih.gov) were used for analysis of gene expression profiles. LOCtree (http://cubic.bioc.columbia.edu) and Lokero (http://www-bs.informatik.uni-tuebingen.de) were used for prediction of subcellular localization. The TMHMM was used for prediction of transmembrane regions. Multiple alignments were generated with the CLUSTALW (http://npsa-pbil.ibcp.fr/). SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) and TargetP 1.1 (www.cbs.dtu.dk/services /TargetP/) were used for prediction of a mitochondrial targeting sequence.

BrdU incorporation

BrdU can be incorporated into the synthesized DNA during the S phase of the cell cycle. To uncover the expressed proteins' function in cell proliferation, cells were incubated with BrdU (20 μM/ml) in medium for 4 hours before harvest, fixed in 1% formalin in PBS for 8 minutes, and followed by 1% formalin, 0.3% Triton X-100 in PBS for 18 hours at 4°C. BrdU immunolabeling was performed with anti-BrdU antibody and detected with secondary antibodies conjugated with fluorophore.

Cell cycle analysis by flow cytometry (FACS)

The cell cycle distribution was determined by FACS analysis of DNA content. Briefly, 293T cells were harvested at indicated times post-transfection, fixed in 70% ethanol and stained with DAPI. DNA content was analyzed using a BD LSR II flow cytometer.

Measurement of $[Ca^{2+}]_i$ in HeLa cells using Fura-2

To better understand the effect of NCX3 on the intracellular Ca^{2+} concentration, HeLa cells were cultured in 35 mm Petri dishes and transfected with plasmids. After 24 hours, cells were rinsed with Ca^{2+}-free Krebs buffer and loaded with 5 μM Fura-2/AM in 2 mL Ca^{2+} free Krebs buffer for 30 min at 37°C in the dark, then subjected to measurement of $[Ca^{2+}]_i$.

Live cell imaging

To study the dynamic subcellular distribution of NCX3 during the cell cycle, we performed live cell imaging with HeLa cells expressing the NCX3-EmGFP fusion protein. Leica TCS SP5 II confocal system (Leica) and INU2 incubation system for microscopes (TOKAI HIT) constituted the integrated live cell imaging system.

4 RESULTS AND DISCUSSION

Mitochondrial dynamics is a highly organized process in keeping with normal cellular demands. Mitochondrial fusion and fission are two opposing processes for the maintenance of mitochondrial morphology. The balance between fusion and fission determines the outcome of mitochondrial morphology. Several large self-assembling GTPases, belonging to the DRP family, constitute the key players in mitochondrial dynamics. In fission, the function of Drp1 has been widely studied. However, the mechanisms of Drp1 recruitment from the cytosol to the mitochondrial surface are highly divergent between vertebrates and yeast (Zhao, Lendahl et al. 2013). This thesis has identified key, previously unknown genes involved in the regulation of mitochondrial morphology and in interplay with DRPs in mammals.

4.1 PAPER I

Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission

In this paper, we firstly searched for proteins potentially involved in controlling mitochondrial morphology by querying the LIFEdb functional genomics resource which provides a lot of information about the proteins' subcellular localization (Simpson, Wellenreuther et al. 2000). We found a vertebrate-specific nuclear gene SMCR7L, which encodes a mitochondrial protein that we functionally characterized and named mitochondrial elongation factor 1 (MIEF1), as overexpression of the protein in cells induced extensive mitochondrial elongation. MIEF1 contains 463 amino acids and has an N-terminal TM domain. Endogenous MIEF1, ~ 52 kDa, is expressed relatively high in human heart, skeletal muscle, pancreas and kidney. By isolation of mitochondrial fractions from 293T cells overexpressing MIEF1 and different treatments of the fractions, we described that MIEF1 is anchored in the mitochondrial outer membrane with its C-terminus exposed in the cytoplasm. Functionally, MIEF1 could affect the cellular sensitivity to apoptotic stimuli and the autophagic activity.

MIEF1 induces mitochondrial fusion by interacting with Drp1 and inhibiting Drp1 activity

The recruitment of Drp1 to the mitochondrial surface was still a mystery, although Mff and hFis1 had been identified as Drp1 receptors on the MOM. MIEF1, however, was found to be a new receptor or adaptor able to aggregate Drp1 on mitochondria.

Overexpression of MIEF1 recruited the bulk of endogenous Drp1 from the cytoplasm to the mitochondria where Drp1 was colocalized with MIEF1. As a result, in the isolated mitochondrial fractions the Drp1 level increased remarkably, concomitant with a reduction of the Drp1 level in the cytosolic fraction. Co-IP confirmed that ectopically expressed MIEF1 could co-precipitate with Drp1 in 293T cells. In addition, we found that Mff, hFis1 and Mfn2 were not required for the MIEF1-induced recruitment of Drp1 to mitochondria. $Drp1^{K38A}$, a Drp1 GTPase deficient mutant, shows cytosolic distribution. It could still be co-precipitated by MIEF1 and was recruited to the mitochondrial surface when coexpressed with MIEF1. Overexpression of MIEF1 affected neither Drp1 oliogmerization nor Drp1 phosphorylation at

Ser637, whereas the detection of an interaction between MIEF1 and phosphomimetic Drp1-Ser637 mutants allowed us to conclude that the MIEF1-Drp1 interaction did not depend on the phosphorylation status of Ser637. Taken together, our data showed that the MIEF1-mediated recruitment of Drp1 to mitochondria is an active process that is independent of Mff, hFis1, Mfn2, Drp1 GTPase activity, and Drp1 phosphorylation status.

Intriguingly, just like overexpression of wildtype MIEF1, overexpression of an engineered deletion mutant (MIEF1$^{\Delta 1-48}$), lacking the TM domain and therefore distributed in the cytoplasm, could also induce a substantial tubular cluster phenotype of mitochondria, which is similar to phenotypes induced by blocking Drp1-dependent fission pathways, e.g. by expression of the Drp1^{K38A} mutant or by knockdown of Drp1 or Mff. Furthermore, knockdown of Drp1 could not perturb this mitochondrial morphology promoted by MIEF1 and MIEF1$^{\Delta 1-48}$ expression. We also identified that overexpression of MIEF1 attenuated Drp1's GTP-binding activity. Together, the findings confirmed that Drp1 was inhibited by MIEF1, independently of MIEF1's mitochondrial localization.

Engineered deletion mutants of MIEF1 facilitated our study of the interaction between Drp1 and MIEF1. The MIEF1 mutant with deletion of amino-acid residues 160-169 (MIEF1$^{\Delta 160-169}$) lost Drp1-binding ability, and overexpression of MIEF1$^{\Delta 160-169}$ did not affect the mitochondrial morphology. These data indicated that the MIEF1-Drp1 interaction is required for sequestering Drp1 by MIEF1 and impairment of mitochondrial fission.

In sum, MIEF1 plays a crucial role in the recruitment of Drp1 to mitochondria and inhibition of Drp1-induced mitochondrial fission. Moreover, the data provided new information on how Drp1 shuttles from the cytosol to mitochondria, subsequently regulated by the cofactors in mitochondrial dynamics.

hFis1 interacts with MIEF1 and partially reverses MIEF1 induced fusion

By using engineered MIEF1 deletion mutants, and co-immunoprecipitations, we elaborated that the interaction between MIEF1 and hFis1 did not require Drp1 or MIEF1 localization on mitochondria. Conversely, hFis1 was not required for the MIEF1-Drp1 interaction. With non-denaturing (native blue) gel electrophoresis, it was confirmed that MIEF1-Drp1 and MIEF1-hFis1 formed distinct protein complexes. This suggested that MIEF1's interaction with Drp1 was independent of MIEF1's interaction with hFis1. The MIEF1-hFis1 interaction seemed much stronger than the hFis-Drp1 interaction. It is consistent with the hypothesis that MIEF1 is a potential receptor or adaptor that can increase the pool of Drp1 on mitochondria. However co-expression of hFis1 with MIEF1 could partially revert the MIEF1 induced mitochondrial fusion effect, indicating that there is a balance between the two types of MIEF1 containing protein complexes.

MIEF1 promoted mitochondrial fusion is to some extent independent of Mfn2

When we had confirmed that MIEF1 is a mitochondrial protein, we checked to what extent MIEF1 affected mitochondrial morphology. Overexpression of MIEF1 resulted in mitochondrial elongation compared to the non-transfected cells as well as compared to the cells transfected with empty vector. ~ 90% of the MIEF1 positive cells displayed extensive

perinuclear distribution of mitochondrial tubular clusters associated with extremely long tubules or compact clusters. The latter phenotype became more marked with an increased MIEF1 expression level.

The transmission electron microscopy results confirmed these findings. Both tubular networks and compact clusters were observed. Even more, reconstruction images from serial ultrathin sections displayed extremely long mitochondria and in the compact clusters the fusion process could have been complemented by membrane fusions between two tethering mitochondria. In addition, knockdown of endogenous MIEF1 with siRNAs switched the balance to mitochondrial fission and dramatically increased the number of cells with divided mitochondria. In the polyethylene glycol (PEG) induced *in vivo* cell fusion assay (Liesa, Borda-d'Agua et al. 2008, Kamp, Exner et al. 2010), mito-GFP and mito-DsRED were transfected into two sets of 293T cells, respectively. The two sets of cells were co-cultured under MIEF1 overexpression or empty vector conditions, and the co-cultures were subsequently treated with PEG inducing cell fusion. The mitochondrial fusion incidence doubled in MIEF1 expressing cells compared to the control group.

It has been reported that Mfns can oligomerize, facilitating mitochondrial elongation. We investigated if MIEF1 was capable of oligomerization. By chemical crosslinking and co-immunoprecipitation (co-IP), self-association (dimerization) of MIEF1 was confirmed. By using engineered MIEF1 deletion mutants, the region from residues 49 to 195 was found to be required for dimer formation. In addition, overexpression of MIEF1 induced unique mitochondrial compact clusters which were likely related to MIEF1 oligomerization. MIEF1 appeared as dots at the tips of two adjacent mitochondrial heads in low level MIEF1 expressing cells. As the fusion evolved, MIEF1 became evenly distributed in the MOM.

The MIEF1-mediated mitochondrial elongation was different from that induced by Mfn2. It is conceivable that the two proteins function in different pathways. Knockdown of Mfn2 by siRNAs resulted in mitochondrial fragmentation, whilst overexpression of MIEF1 could rescue Mfn2 knockdown-induced fission, concomitantly increasing fusion. This suggests that Mfn2 is dispensable for the MIEF1-induced fusion. Additionally, knockdown of endogenous MIEF1 by siRNAs switched the balance to fission and dramatically increased the number of cells with divided mitochondria. Those results indicated that MIEF1 has the ability to enhance mitochondrial apposition by oligomerization so as to facilitate fusion.

Drp1, however, does not affect the mitochondrial morphology when overexpressed in cells (Smirnova, Shurland et al. 1998). Coexpression of MIEF1 and Drp1 could reverse the MIEF1 induced fusion phenotype to a limited extent. Mitochondrial fragmentation was detectable in a small portion of cells with ectopic expression of both MIEF1 and Drp1. This indicated that an increased ratio of Drp1 to MIEF1 to a moderate level could induce fission. More importantly, it hinted the potential role of MIEF1 in mitochondrial fission.

Main findings:

- MIEF1 has a dual role in inhibiting mitochondrial fission and promoting fusion
- MIEF1 interacts with and recruits Drp1 to the mitochondrial surface but inhibits Drp1 activity, resulting in mitochondrial fusion.

- MIEF1-mediated recruitment of Drp1 to mitochondria is independent of Mff, hFis1, Mfn2, Drp1 GTPase activity, and Drp1 phosphorylation status.
- MIEF1 interacts with hFis1 and overexpression of hFis1 attenuates MIEF1-mediated fusion.
- MIEF1 may also actively promote mitochondrial fusion in a way that does not require Mfn2.

4.2 PAPER II

The mitochondrial elongation factors MIEF1 and MIEF2 exert partially distinct functions in mitochondrial dynamics

MIEF2 (also named Mid49) is a paralog of MIEF1, which is located on chromosome 17 in human. Recently, Palmer and co-workers reported that double knockdown of MIEF1 and MIEF2 led to mitochondrial fusion in COS7 cells (Palmer, Osellame et al. 2011), whereas in our previous paper, we suggested that knockdown of MIEF1 by siRNA resulted in mitochondrial fragmentation in HeLa cells (Zhao, Liu et al. 2011). Thus, the function of MIEFs in mitochondrial dynamics was controversial. In paper II we cloned and expressed the MIEF2 cDNA and focused on the functional similarities and divergences between MIEF1 and MIEF2.

Functional similarities between MIEF1 and MIEF2

By protein sequence homology analysis, we found that MIEF1 and MIEF2 share 45% amino acid identity. Like MIEF1, MIEF2 has a predicted N-terminal TM domain from amino acid residue 23 to 43 and the C-terminus faces the cytosol. MIEF2 is also a vertebrate-specific protein. In line with MIEF1, overexpression of MIEF2 leads to mitochondrial fusion. MIEF1-mediated fusion does not require the localization of MIEF1 to mitochondria (Zhao, Liu et al. 2011). To perform this investigation in MIEF2, a TM deletion mutant, $MIEF2^{\Delta 1\text{-}49}$, was generated. It was localized in the cytoplasm similar to $MIEF1^{\Delta 1\text{-}48}$. Overexpression of $MIEF2^{\Delta 1\text{-}49}$ sequestered Drp1 in the cytosol and promoted a mitochondrial tubular cluster phenotype, which were the same effects as seen by expressing $MIEF1^{\Delta 1\text{-}48}$. By co-IP, both $MIEF1^{\Delta 1\text{-}48}$ and $MIEF2^{\Delta 1\text{-}49}$ could interact with Drp1 but the binding efficiency was lower than with the wild-type MIEF1 and MIEF2 proteins. Overexpression of either MIEF1 or MIEF2 increased the pool of Drp1 foci on mitochondria. Co-IP indicated that MIEF2, like MIEF1, was capable of interacting with both hFis1 and Drp1. With increased expression levels of hFis1, interactions between Drp1 and either MIEF1 or MIEF2 declined, which indicated that hFis1 could affect the Drp1-MIEF1/MIEF2 interactions. Taken together, MIEF1 and MIEF2 possess certain common characteristics in controlling mitochondrial fission and fusion. If either protein were lost, these redundant functions could be compensated for mutually in the regulation of mitochondrial dynamics.

Functional divergences between MIEF1 and MIEF2

Nevertheless, there are still some dissimilarities between MIEF1 and MIEF2. We monitored the differential expression levels of MIEF1 and MIEF2 in different cell lines, including a variety of tumor cell lines. MIEF2 was more ubiquitously expressed relative to MIEF1.

Screening of MIEF1 and MIEF2 mRNA by qPCR indicated that both of them are widely expressed in different human fetal and adult tissues. Relatively, MIEF1 mRNA was more abundant in human fetal tissues and was high in fetal skeletal muscle, brain, kidney, liver and heart, whereas MIEF1 mRNA was decreased in those tissues in the adult. On the other hand MIEF2 mRNA was higher in human adult tissues, especially in adult skeletal muscle and heart. This hints that MIEF1 and MIEF2 function at distinct stages of development. When it came to their functions in mitochondrial dynamics, overexpression of either MIEF1 or MIEF2 resulted in the mitochondrial fusion phenotypes with either tubular or compact cluster morphologies in a dose-dependent manner. By increasing the dose of either MIEF1 or MIEF2 expression, the phenotype with compact clusters became more severe. Statistical analysis unveiled that the MIEF2-induced fusion phenotype was much stronger than that induced by MIEF1. Consistently, co-expression of hFis1 and MIEF1 indicated that hFis1 could negatively modulate MIEF1induced fusion and decreased the percentage of cells with the fusion phenotype from 90% to 48%, while co-expression of hFis1 and MIEF2 showed that hFis1 had little effect on MIEF2-induced fusion. When substituting Mff for hFis1, Mff could reverse MIEF1-induced fusion and significantly increased the cells with fragmented and aggregated mitochondria to 79%. In contrast, Mff could only rescue the MIEF2-induced fusion slightly. Only 7.3% of the cell co-expressing MIEF2 and Mff exhibited mitochondrial fragmentation. Taken together, MIEF2 is more potent to induce mitochondrial fusion than MIEF1.

In paper I we demonstrated that MIEF1 could form both dimers and higher order oligomers (Zhao, Liu et al. 2011). In this paper, we narrowed down the region required for MIEF1 dimerization to amino acid residues 109 to 154. By chemical cross-linking and western blot, we found that MIEF2 mostly formed higher order oligomers instead of the dimers that were mainly seen with MIEF1. This finding is a plausible reason why MIEF2 possesses a higher fusion-promoting capability than MIEF1. By engineered MIEF2 deletion mutants, we defined the N-terminus, the first 49 amino acid residues, as required for MIEF2 oligomerization. This is different from the region defined in MIEF1.

In addition, we discovered that dimerization of MIEF1 is not necessary for binding Drp1 and recruiting Drp1 to mitochondria. Expression of the MIEF1$^{\Delta 109\text{-}154}$, oligomerization-deficient mutant, could increase the pool of Drp1 foci at the mitochondrial surface and induce a mitochondrial fusion phenotype which was slightly weaker than the phenotype induced by wildtype MIEF1 expression. Through co-IP, MIEF1 and MIEF1$^{\Delta 109\text{-}154}$ displayed similar Drp1 binding efficiency. We also found that MIEF1 and MIEF2 can form heterodimers.

Main findings:

- Both MIEF1 and MIEF2 recruit Drp1 to mitochondria and cause mitochondrial fusion.
- MIEF2 has the same capacity as MIEF1 to interact with Drp1 and hFis1.
- MIEF1 and MIEF2 are differentially expressed in human tissues during development and in the adult.
- MIEF2 exerts a stronger fusion effect than MIEF1.
- MIEF2 forms higher order oligomers, while MIEF1 largely presents as dimers.

- MIEF2 oligomerization is dependent on the first 49 amino acid residues, while MIEF1 dimerization is dependent on the region from amino acid residues 109 to 154.
- Dimerization of MIEF1 is not required for its mitochondrial localization and interaction with Drp1.

4.3 PAPER III

The novel conserved mitochondrial inner-membrane protein MTGM regulates mitochondrial morphology and cell proliferation

In mammals, the mitochondrial fission machinery is mainly constituted by Drp1, MIEFs, Mff, hFis and the post-translational modifications on Drp1. Those fission processing events occur in the MOM. In this paper, we characterized a unique MIM protein which we named mitochondrial targeting GxxxG motif (MTGM) protein and found to be involved in mitochondrial fission.

Using SAGE libraries, we compared the gene expression patterns between human brain tumors and the corresponding normal tissues. We discovered a gene ubiquitously expressed in human adult tissues and analyzed the gene product. The gene was designated MTGM (also known as C20orf52 and Romo1).

By RACE-PCR and bioinformatics analysis, we found that the MTGM gene possesses three exons and it generates at least three transcript variants in the human brain via alternative splicing, but all the splice variants encode the same MTGM protein with 79 amino acids and a predicted molecular mass of 8.2 kDa. The protein contains a TM domain positioned from amino acid residue 22 to 44. In addition, MTGM is highly conserved from yeast to human and the homologs have a tetrad of the GxxxG motif for protein-protein interactions within the TM region that indicates MTGM is crucial for life during evolution and cellular events. By RT-PCR analysis, on the basis of MTGM expression in normal adult brain, we found that MTGM is upregulated in two human clonal glioma cell lines and one neuroblastoma cell line and in several patient samples of medulloblastoma and astrocytoma.

Overexpression of MTGM in cultured cells led to punctuated foci in the cytoplasm of transfected cells analyzed by immunofluorescence microscopy and the foci co-localized with mitochondria stained with MitoTracker. Endogenous MTGM was detected with an MTGM polyclonal antibody and also those foci co-localized with mitochondria. Moreover, we isolated the mitochondrial fraction from cells overexpressing MTGM and western blotting revealed that MTGM accumulated in the mitochondrial fraction. Accordingly, MTGM is a mitochondrial targeting protein. Isolation of the mitochondrial fraction from these cells allowed us to further study the nature of MTGM in mitochondria. By treatment of the mitochondrial fraction with different reagents, we could determine that MTGM is an integral mitochondrial inner-membrane protein.

Intriguingly, we found that overexpression of MTGM induced mitochondrial fission, whilst knockdown of MTGM resulted in interconnection of mitochondria which was confirmed with two pairs of MTGM siRNAs in both 293T and HeLa cell lines. These findings indicated that MTGM is involved in the regulation of mitochondrial morphology. It is known that

mitochondrial fission is involved in the mitochondrial quality control, impedes cell proliferation, sensitizes the cells to apoptosis, and facilitates the cells to remove the dysfunctional mitochondria by mitophagy.

Therefore we investigated if MTGM affects those functions. Firstly, we discovered that MTGM affects cell proliferation. By BrdU incorporation assay and immunofluorescence staining, we found that overexpression of MTGM dramatically decreased the cells incorporation of BrdU. The result indicated that MTGM severely impairs DNA synthesis, and it was revealed there were almost no MTGM positive cells in mitosis, identified by Ki67 and DAPI staining. Flow cytometry analysis of DNA content was performed by two different methods. One was to compare the MTGM expressing group with a GFP expressing control group, the other was to compare two subgroups, MTGM positive and negative, within the same population of cells transfected with MTGM. The results confirmed that overexpression of MTGM stalled the cells in S phase. Consistently, cyclin B1 was increased slightly by MTGM expression. Conversely, knockdown of MTGM led to robust cell proliferation and significantly promoted the cells incorporation of BrdU compared to cells treated with negative control siRNA. The level of cyclin D1 and cyclin-dependent kinase inhibitor p21 declined and cyclin B1 slightly increased in MTGM depleted cells.

Secondly, we analysed the connections between MTGM protein and the release of proapoptotic factors from mitochondria. By immunofluorescence microscopy, we found that cytochrome c remained in mitochondria when MTGM was ectopically expressed in 293T cells, while more than 50% of the cells expressing MTGM lost the pool of Smac/Diablo foci in mitochondria. Furthermore, western blotting indicated that the level of Smac in the mitochondrial fraction decreased, while it was increased in the cytosol of MTGM expressing cells. As for cytochrome c, it was kept in the mitochondrial fraction and the level in the cytosol increased slightly when expressing MTGM in 293T cells.

Intriguingly, a time course study of MTGM-induced fission and release of Smac revealed that overexpression of MTGM activated extensive mitochondrial division at 8 hours post-transfection, at which time only about 5% of the cells with mitochondrial fragmentation released Smac into the cytosol. At 24 hours post-transfection, there was a higher number of cells with Smac release. It was concluded that MTGM-induced mitochondrial fission occurred ahead of Smac release.

Drp1 is the core player in the mitochondrial fission machinery. It prompted us to unveil the connection between Drp1 and MTGM. Co-expression of MTGM and Drp1 did not affect the MTGM-induced fission. Co-expression of MTGM and Drp1^{K38A} inactive mutant substantially reversed the MTGM-induced mitochondrial fission. About 82% of the transfected cells appeared to have an interconnected tubular network of mitochondria. Moreover, MTGM-mediated Smac release was severely attenuated at the same time. Those data indicated that both MTGM-induced mitochondrial fission and Smac/Diablo release occurred in a Drp1 dependent manner.

In addition, we analyzed caspase 3, polyADP-ribose polymerase (PARP), and LC3B in cells overexpressing MTGM. There were no obvious changes in those apoptotic and autophagy

indicators, implying that overexpression of MTGM neither promoted apoptosis nor autophagy.

On the other hand, depletion of MTGM by siRNAs protected PARP from cleavage in staurosporine (STS) induced apoptosis. This indicated that mitochondrial fusion resulting from knockdown of MTGM can repress caspase 3 activity and promote resistance to apoptotic stimuli. As well, this result was confirmed by flow cytometry analysis with M30 CytoDeath antibody staining. The M30-positive cells were about 7.6% in the cell cultures treated with MTGM siRNA relative to 17.5% in the cultures treated with control siRNA when exposed to STS induced apoptotic stimuli.

Taken together, MTGM is a unique integral mitochondrial inner membrane protein cooperating with Drp1 to regulate mitochondrial dynamics and cell proliferation. MTGM strongly promotes mitochondrial fission and is unique among MIM proteins in this respect.

Main findings:

- MTGM is a novel integral mitochondrial inner-membrane protein with a tetrad of the GxxxG motif that is highly conserved from yeast to human.
- MTGM is highly expressed in human brain tumors.
- Overexpression of MTGM results in mitochondrial fission and release of Smac/Diablo from mitochondria to the cytosol in a Drp1-dependent manner.
- Overexpression of MTGM inhibits cell proliferation and sequesters the cells in S phase.
- Knockdown of MTGM induces mitochondrial fusion and promotes cell proliferation and resistance to apoptosis.

4.4 PAPER IV

Glycosylation determines sodium-calcium exchanger 3 subcellular distribution during cell cycle; effects on ER Ca^{2+} handling

Sodium-calcium exchanger (NCX), as a plasma membrane antiporter, plays an important role in the maintenance of intracellular Ca^{2+} homeostasis. Although NCXs have been extensively studied in mammals, the subcellular localization of different NCXs is still undetermined besides the fact that they are destined to be plasma membrane proteins. In this study, we tested the hypothesis that the subcellular localization of the NCX3 protein varies with the cell cycle in mammalian cells.

To dissect the biological functions of human NCX3, we overexpressed NCX3 in HEK 293T cells. By immunofluorescence microscopy, we found that two main phenotypes of NCX3 subcellular localization emerged, one localized to the plasma membrane and another associated with the ER and the nuclear envelope, which were termed NCX3-PM and NCX3-ER phenotypes respectively. By performing time-lapse ratiometric Ca^{2+} imaging in HeLa cells, we found that overexpression of NCX3 attenuated the ER-Ca^{2+} release capacity.

Using BrdU incorporation assay, we discovered that only 5.1% BrdU positive cells appeared among cells with NCX3-ER localization, while 62% among cells with NCX3-PM localization, and 44.7% in control cell cultures. By immunofluorescence staining, we found that the NCX3-ER phenotype was present mainly in interphase cells, whilst the NCX3-PM phenotype was present in mitotic cells. Moreover, NCX3-PM is the dominant phenotype in mitotic cells. Upon these findings, we concluded that the NCX3-ER phenotype is present in interphase cells that do not actively synthesize DNA, while the NCX3-PM phenotype exists in cells undergoing DNA synthesis and mitosis. To visualize how the NCX3 movements varied with the cell cycle phases, we constructed an NCX3 recombinant with green fluorescent protein (GFP) and performed live cell imaging of cells transfected with NCX3-GFP. In cells approaching mitosis, NCX3 accumulated in the plasma membrane gradually. When cells had entered mitosis, NCX3 was mostly located in the plasma membrane, i.e. the cells displayed the NCX3-PM phenotype. During telophase, NCX3 maintained the NCX3-PM phenotype and when reaching cytokinesis, the phenotype changed moderately from NCX3-PM to NCX3-ER. In the two daughter cells, NCX3-ER was the predominant phenotype. Taken together, the regulation of NCX3 subcellular localization is a dynamic process dependent on the cell cycle phases and the NCX3-ER phenotype cells were partially delayed in their entering into S phase and mitosis.

Intriguingly, by studying the biochemical properties of NCX3, we discovered that NCX3's subcellular localization potentially was related to N-glycosylation. Bioinformatics analysis revealed predicted glycosylation sites at residue N45 and N67 of NCX3. We constructed two potentially glycosylation deficient mutants, named NCX3 N45D and NCX3 N67D. The residue N45 of NCX3 was found to affect NCX3 subcellular localization when analyzed by immunoblotting and immunofluorescence microscopy. NCX3 N45D was restrained in the cytosol (NCX3-ER phenotype) and less NCX3 N45D foci could be detected in the plasma membrane. This indicated that *N*-glycosylation of asparagine residue 45 is required for the distribution of NCX3 to the plasma membrane. In addition, overexpression of NCX3 N45D, like NCX3 wild type, could largely attenuate the Ca^{2+} release from the ER. This hints that restraining NCX3 in the ER facilitates NCX3 actively pumping Ca^{2+} from the cytosol into the ER. Using flow cytometry, we found that overexpression of the NCX3-N45D mutant significantly increased the number of cells in G1/G0 phase and decreased the number of cells in S phase, which was comparable with overexpression of NCX3 wild type. The result was consistent with the above findings.

In summary, NCX3's distribution within the cell is a dynamic process associated with the cell cycle phases, and requires the dynamic regulation of post-translational N-glycosylation. Forced expression of a protein with the NCX3-ER phenotype can delay the cells entering into S phase, subsequently cells accumulate in the G1/G0 phase.

Main findings:

- Normally, NCX3 exists both in the plasma membrane and in the ER.
- The distribution of NCX3 is a dynamic process associtated with the cell cycle phases.
- Forced localization of NCX3 in the ER delays the cells entering into S phase, and consequently cells accumulate in the G1/G0 phase.

- N-glycosylaton at residue N45 of NCX3 is required for targeting the protein to the plasma membrane.

5 CONCLUSION AND PERSPECTIVES

Along with hFis1 and Mff, MIEF1 and MIEF2 are the latest characterized MOM proteins and these proteins play a pivotal role by interacting with and recruiting Drp1 to mitochondria in mammals. More importantly, MIEF1 functions differently from hFis1 and Mff. By interaction with Drp1, MIEF1 inhibits Drp1's activity in mitochondrial fission and results in a mitochondrial fusion phenotype. On the other hand, MIEF1 promotes fusion in an Mfn2 independent manner, but the fusion effect is modulated by hFis1 modestly. Paper i was highlighted by Schuldt in Nature Reviews Molecular Cell Biology (Schuldt 2011), and it was emphasized that MIEF1 seemed to provide a means of regulating mitochondrial dynamics that is unique to vertebrates. In paper ii, we dissected the similarities and differences between MIEF1 and MIEF2 on basis of their functions in mitochondrial dynamics. A better understanding of the functions of MIEF1 and MIEF2 facilitates future studies on how the two proteins coordinate their actions with other factors in recruiting Drp1, modulating Drp1 activity, and regulating mitochondrial dynamics. In paper iii, we presented the first report of MTGM regulating mitochondrial dynamics. MTGM is still the only identified integral MIM protein which regulates mitochondrial fission in a Drp1 dependent manner. More recently, MTGM-mediated ROS production has been studied in tumors by others (Shin, Chung et al. 2013, Kim, Kim et al. 2014, Yu, Song et al. 2014). Since MTGM is also involved in the regulation of mitochondrial dynamics, it is a potential factor to bridge mitochondrial dynamics, ROS production and cancer, aspects to be addressed in future studies. Taken together, in this thesis, three novel mitochondrial membrane proteins were introduced. All these proteins constitute main components of mitochondrial dynamics machinery and the discoveries shed new light on the mechanisms of mitochondrial dynamics in human.

In human, hFis1, Mff and MIEFs are regarded as mitochondrial receptors which recruit Drp1 to mitochondria and regulate mitochondrial fission. Why are there so many Drp1 receptors on mitochondria and how do they work together? Do they possess redundant functions or work with Drp1 hierarchically? Are they activated by different physiological events and signalling pathways in cells? Those questions open a large and fascinating field of research. We believe that the identification and functional characterization of the human specific proteins is a prerequisite for the proper understanding of the roles of mitochondrial dynamics in human diseases.

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