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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biomedicina, realizada sob a orientação científica da Doutora Daniela Ribeiro, Investigadora Auxiliar no Instituto de Biomedicina e Departamento de Ciências Médicas da Universidade de Aveiro, e pelo Doutor Michael Schrader, Professor Catedrático da Universidade de Exeter, Reino Unido.

Thesis submitted at University of Aveiro to fulfil the requirements to obtain the Doctor degree in Biomedicine, held under the scientific guidance of Dr Daniela Ribeiro, Assistant Researcher at the Institute of Biomedicine and Department of Medical Sciences from the University of Aveiro, and Dr Michael Schrader, Full Professor at the University of Exeter, UK.

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Dedico este trabalho ao Pi e ao Zé

**o júri**

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*"Para ser grande, sê inteiro: nada*
*Teu exagera ou exclui.*
*Sê todo em cada coisa. Põe quanto és*
*No mínimo que fazes.*
*Assim em cada lago a lua toda*
*Brilha, porque alta vive."*

*(Ricardo Reis)*

**palavras-chave**

HCMV, peroxissomas, imunidade inata celular, MFF, DLP1, FIS1, maquinaria de divisão, MAVS, mitocôndria, vMIA, resposta antiviral

**resumo**

Os peroxissomas são organelos celulares de membrana simples que desempenham funções metabólicas cruciais. Eles foram descritos pela primeira vez nos anos 60 e, ao longo dos anos, sua importância para a homeostasia celular tem sido cada vez mais destacada. Ao nível celular, os peroxissomas e as mitocôndrias cooperam em vários mecanismos metabólicos e recentemente foi descoberto que também têm funções complementares ao nível da resposta imune antiviral. Estes organelos também partilham várias proteínas, incluindo as proteínas chave das suas maquinarias de divisão MFF, FIS1 e DLP1 bem como a proteína antiviral MAVS.

Como a correta função destes organelos depende em grande parte da capacidade de adequar a sua morfologia e localização celular de acordo com as necessidades das células e, por isso, da correta regulação dos eventos de fissão membranar, neste trabalho decidimos avaliar o papel da maquinaria de divisão peroxissomal e mitocondrial, especificamente dos adaptadores chave da DLP1, MFF e FIS1 na resposta antiviral contra um dos vírus mais disseminados na comunidade: o *citomegalovírus humano* (HCMV).

Assim, iniciámos este trabalho com a caracterização da maquinaria de divisão peroxissomal a com a distinção de diferentes funções das proteínas de fissão partilhadas com as mitocôndrias (MFF e FIS1). Seguidamente, analisámos em detalhe a função destas proteínas na sinalização antiviral peroxissomal na defesa contra o HCMV.

Os nossos resultados indicam que a MFF desempenha um papel crucial na regulação da fissão peroxissomal, enquanto que a FIS1 parece ter maior impacto na divisão mitocondrial. Além disso, foi descoberto que a MFF interage com a proteína viral vMIA, e assim parece desempenhar um papel crucial na infeção pelo HCMV.

No seu conjunto, estes resultados enfatizam a importância da maquinaria de divisão peroxissomal na defesa antiviral mediada pelos RLR e poderá levar, em última instância, à descoberta de novos mecanismos peroxissomais, que poderão ser usados com alvos terapêuticos na infeção viral.

**keywords**

HCMV, peroxisomes, cellular innate immunity, MFF, DLP1, FIS1, fission machinery, MAVS, mitochondria, vMIA, antiviral response

**abstract**

Peroxisomes are single membrane bound organelles involved in crucial cellular metabolic functions. They were noticed for the first time in the 1960s and, along the years, their importance for the cellular homeostasis has been increasingly highlighted. Peroxisomes and mitochondria cooperate in several cellular metabolic processes and more recently were found to have a complementary role in the antiviral innate immune response. These two organelles also share many proteins including the fission machinery key proteins MFF, FIS1 and DLP1 and the antiviral signaling protein MAVS.

As the proper function of these organelles strongly depends on the capacity to adequate their shape and cellular localization in accordance with the cellular needs, and thus on the correct regulation of membrane fission events, in this work we evaluated the role of the peroxisomal and mitochondrial fission machinery, specially the key DLP1 adaptors MFF and FIS1 in the antiviral immune response against one of the most spread viruses in the community: the human cytomegalovirus (HCMV). In line with this, we started this work by characterizing the peroxisomal fission machinery and by distinguishing different roles of key components shared with mitochondria (MFF e FIS1). The role of these proteins in the peroxisomal antiviral signaling against HCMV was afterwards analyzed in detail.

Our results strongly indicate that MFF plays a crucial role at the regulation of peroxisomal fission whereas FIS1 significantly impacts mitochondrial fission events. In addition, we found that MFF interacts with the HCMV viral protein vMIA and that it is essential to vMIA´s function. MFF seems to, thus, play a crucial role in HCMV´s infection.

Altogether, these results empathize the importance of peroxisomal fission machinery for the RLR-mediated antiviral defense and may lead to the discovery of novel peroxisome-dependent mechanisms, which can ultimately be used as targets for antiviral therapy.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-binding cassette |
| ABCD1 | ATP-binding cassette sub-family D member 1 |
| ACAD | acyl-CoA dehydrogenase |
| ACADL | acyl-CoA dehydrogenase long chain |
| ACBD1 | acyl coenzyme A binding domain protein 1 |
| ACBD3 | acyl coenzyme A binding domain protein 3 |
| ACBD5 | acyl coenzyme A binding domain protein 5 |
| Acetyl-CoA | acetyl coenzyme A |
| ACOX | acyl-CoA oxidase |
| ACOX1 | peroxisomal acyl-coenzyme A oxidase 1 |
| Acyl-CoA | long-chain fatty acid-Acyl-CoA |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| BAT | brown fat tissue |
| BAP31 | B-cell receptor-associated protein 31 |
| CAF4 | CCR4-associated factor 4 |
| CARDIF | CARD adaptor inducing IFN- β |
| CARDs | caspase recruitment domains |
| CD36 | cluster of differentiation 36 |
| cGAMP | cyclic-GMP-AMP |
| cGAS | cyclic GMP-AMP synthase |
| CTD | C-terminal regulatory domain |
| DAI | DNA-dependent activator of interferon regulatory factor |
| DDX41 | DEAD-Box Helicase 41 |
| DHA | docosahexaenoic acid |
| DLP1 | dynamin-like protein |
| DNA | deoxynucleic acids |
| dsDNA | double-stranded deoxyribonucleic acid |
| dsRNA | double-stranded RNA |
| E | early |
| ER | endoplasmic reticulum |
| FAD | flavin adenine dinucleotide |
| FIS1 | mitochondrial fission factor 1 |
| G0S2 | G0/G1 switch protein 2 |
| GAGs | glycosaminoglycans |

| | |
|---|---|
| GDAP1 | ganglioside-induced differentiation-associated protein 1 |
| GED | GTPase effector domain |
| GTPase | guanin triphosphate enzyme |
| HCMV | Human cytomegalovírus |
| hrs | hours |
| HSD17B4 | peroxisomal multifunctional enzyme type 2 |
| HSDL2 | hydroxysteroid dehydrogenase-like protein 2 |
| HSV | herpes simplex virus |
| IE | immediate early |
| IFNs | interferons |
| IGFBP1 | insulin-like growth factor-binding protein 1 |
| IKK | IκB kinase |
| INF2 | inverted formin-2 |
| IPS-1 | IFN-β promoter stimulator-1 |
| IRFs | interferons regulatory factors |
| ISG | interferon-stimulated genes |
| KO | knock-out |
| L | late |
| LETM1 | leucine zipper-EF-hand-containing transmembrane protein 1 |
| LC3 | microtubule-associated proteins 1A/1B light chain 3B |
| LDs | lipid droplets |
| LGP2 | laboratory of genetics and physiology 2 |
| LOMP2 | Lon protease homolog 2, peroxisomal |
| MAM | mitochondria associated membranes |
| MAPKs | mitogen-activated protein kinases |
| MAVS | mitochondrial antiviral signaling |
| MDA-5 | melanoma differentiation-associated gene-5 |
| MDV1 | mitochondrial division protein 1 |
| Mefs | mouse embryonic fibroblasts |
| MFF | mitochondrial fission factor |
| MFN1 | mitofusin-1 |
| MFN2 | mitofusin-2 |
| min | minutes |
| MID49 | mitochondrial dynamics protein MID49 |
| MID51 | mitochondrial dynamics protein MID51 |
| MIRO1 | Mitochondrial Rho GTPase 1 |

| | |
|---|---|
| MOM | mitochondrial outer membrane |
| mRNAs | mature RNAs |
| MyD88 | myeloid differentiation primary response 88 |
| NBR1 | neighbor of BRCA1 gene 1 |
| NF-κB | nuclear factor kappa-light-chain enhancer of activated B cells |
| NLR | nucleotide oligomerization domain-like receptors |
| NPC | Niemann–Pick disease type C |
| NPC1 | NPC intracellular cholesterol transporter 1 |
| OPA1 | dynamin-like 120 kDa protein, mitochondrial |
| PAMPs | pathogen-associated molecular patterns |
| PEX11α | peroxisomal membrane protein 11α |
| PEX11β | Peroxisomal membrane protein 11β |
| PEX11Y | peroxisomal membrane protein 11Y |
| PEX3 | peroxisomal biogenesis factor 3 |
| PEX5 | peroxisomal biogenesis factor 5 |
| PEX13 | peroxisomal membrane protein PEX13 |
| PEX14 | peroxisomal membrane protein PEX14 |
| PEX16 | peroxisomal membrane protein PEX16 |
| PEX19 | peroxisomal biogenesis factor 19 |
| PGC-1α | peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| PMP34 | Peroxisomal membrane protein PMP34 |
| PMP70 | 70 kDa peroxisomal membrane protein |
| PMPs | peroxisomal membrane proteins |
| PPAR | peroxisome-activated receptor |
| PPARα | peroxisome-activated receptor alpha |
| PPAR | peroxisome-activated receptor delta |
| PPARY | peroxisome-activated receptor gamma |
| PPARδ | peroxisome-activated receptor delta |
| PTS1 | Peroxisomal targeting signal 1 |
| kDA | quilodalton |
| PARP | poly(ADP-ribose) polymerase |
| PBD | peroxisome biogenesis disorders |
| PPRα | proliferator-activated receptor α |
| PRRs | pattern recognition receptors |
| RCDP1 | rhizomelic chondrodysplasia punctata type 1 |
| RIG-I | retinoic acid-inducible gene I |

| | |
|---|---|
| RLRs | RIG-I-like receptors |
| RNA | ribonucleic acids |
| RNA pol III | RNA polymerase III |
| ROS | reactive oxygen species |
| RNS | reactive nitrogen species |
| SEM | standard error mean |
| SEC16β | protein transport protein Sec16β |
| SOD1 | super oxide dismutase 1 |
| SQSTM1 | sequestosome-1 |
| STING | stimulator of interferon genes |
| SULT2A1 | bile salt sulfotransferase |
| TBC1D15 | TBC1 domain family member 15 |
| TBK1 | TRAF family member associated NF-κB activator-binding kinase 1 |
| TIR | toll/interleukin-1 receptor |
| TLRs | toll-like receptors |
| TNF | tumor necrosis factor |
| TNKS | poly [ADP-ribose] polymerase tankyrase-1 |
| TNKS2 | poly [ADP-ribose] polymerase tankyrase-1 |
| TRAF | TNF receptor-associated factor |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| VDAC1 | voltage-dependent anion-selective channel protein 1 |
| VISA | virus-induced signaling adaptor |
| VLCFA | very long-chain-fatty acids |
| vMIA | viral mitochondria-localized inhibitor of apoptosis |
| WT | wild type |
| X-ALD | X-linked adrenoleukodystrophy |
| ZSD | Zellweger spectrum disorder |

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## LEGAL CONSIDERATIONS

The author declares that part of the results presented in this thesis were published under the name of Gouveia, A., and that she has participated in the planning and execution, as well as in the preparation and interpretation of the data.

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*Shared first authorship

# 1 GENERAL INTRODUCTION

## 1.1 PEROXISOMES

Described for the first time in the 1960s, peroxisomes are ubiquitous subcellular organelles which regulate essential metabolic functions such as very long chain fatty acid β-oxidation and reactive oxygen species (ROS) and reactive nitrogen species (RNS) metabolism. In mammalian cells they are also involved in other metabolic processes including the biosynthesis of cholesterol, dolichol, bile acids and glycerophospholipids such as plasmalogens [1].

These single membrane bound organelles are highly dynamic, adjusting their number and shape, according to the cellular metabolic demands [2–4].

### 1.1.1 Peroxisome morphology and biogenesis

Morphologically, peroxisomes range from 0.1 to 1 micrometers (μm) in diameter and are bound by a single lipid bilayer membrane which surrounds a highly granular matrix devoid of DNA or transcription/translation systems. They are highly dynamic organelles that vary in number and shape, ranging from spherical or rod/bean-like structures to a more elongated morphology, in response to physiological stimuli such as fatty acids composition or temperature alterations, in order to maintain cellular homeostasis [2,3].

Peroxisomes are highly plastic and responsive organelles, adapting metabolic functions, position within the cell, shape and number in accordance to the cellular metabolic needs. Their number is tightly regulated by formation (biogenesis) and degradation (pexophagy) or inheritance (cell division) processes [2–5]. Through peroxisome biogenesis mechanisms, new fully functional peroxisomes are formed. This process involves the membrane formation and the insertion of peroxisomal membrane proteins (PMPs) which are required for the import of soluble matrix proteins [5,6]. These peroxisome-specific family of proteins required for peroxisome assembly (peroxins or PEXs) are synthetized on free polyribosomes and imported to peroxisomes upon post-translation modifications [7]. Upon protein synthesis, PMPs are recognized by their mPTS signaling sequence which determines their specificity for insertion at the peroxisomal membrane. Two classes of PMPs can be defined: the ones which, harboring a mPTS1 sequence, are transported to peroxisomal membranes via peroxisomal biogenesis factor 19 (PEX19) - class I PMPS; and the class II PMPs that do not require PEX19 for peroxisome targeting, and contain a different targeting signaling, mPTS2 [8,9]. Conversely, peroxisome matrix proteins harbor one of the two peroxisomal targeting signals (PTS), a C- terminal PTS1 or an N-terminal PTS2, and are recognized by the cytosolic

receptor-cargo protein PEX 5 (for PTS 1) or PEX7 (for PTS2), which transport them to docking sites present at the peroxisomal membrane and mediate the import into the peroxisomal matrix [9–12].

Regarding the peroxisome biogenesis, two main models are currently accepted: the "*de novo* formation" and the "growth and division" models. These two models describe the capability of peroxisomes to be formed either directly from the ER or mitochondria or by maturation of pre-existing peroxisomes. These are not conflicting models and, on the contrary, reflect complementary processes. In the absence of pre-existing peroxisomes, PMP´s membrane enriched structures budding from the endoplasmic reticulum (ER) or mitochondria form pre-peroxisomal vesicles which may fuse and mature leading to functional peroxisomes. These newborn peroxisomes can further multiply by growth and division [1,5,13].

#### 1.1.1.1 Peroxisome proliferation – The growth and division model

According to the cellular demands, when more functional peroxisomes are needed, new units can be formed from pre-existing ones. As proposed by Lazarow and Fujiki [6], mature peroxisomes undergo a multistep of processes including membrane elongation, constriction, and final scission, originating new fully functional organelles (Figure 1).

##### *1.1.1.1.1 Membrane elongation and remodeling*

Studies have shown that elongated peroxisomes are formed by incorporating new phospholipids in a membrane elongation process [3,4]. Upon extracellular stimuli, peroxisomal membrane protein peroxin 11 (PEX11) is activated and a cascade of processes are initiated, leading to membrane elongation and peroxisome proliferation. The PEX11 gene encodes three PEX11 isoforms, specifically PEX11α, PEX11β and PEX11γ, which are integral membrane proteins with their N- and C-termini facing the cytosol [14–16]. However, peroxisome proliferation has been mainly linked to the PEX11β isoform. In contrast to PEX11β, which is constitutively expressed, PEX11α and PEX11γ are more tissue-specific proteins, localizing mainly at the liver. Besides the studies that have proven that PEX11β is crucial for peroxisome proliferation, metabolic functions, and neonatal viability, it has been observed that, in mice, its absence led to serious developmental deficiencies such as Zellweger-like pathologies. In 2012 Ebberink and colleagues described for the first time a PEX11β associated disease in humans [17]. Contrarily, in knockout PEX11α mouse cells no significant effect on peroxisome proliferation or metabolism was revealed [14,18–20]. Koch and Brocard suggested that

PEX11γ is required for peroxisome membrane elongation in mammalian cells, by acting in coordination with the other PEX11 isoforms [15].

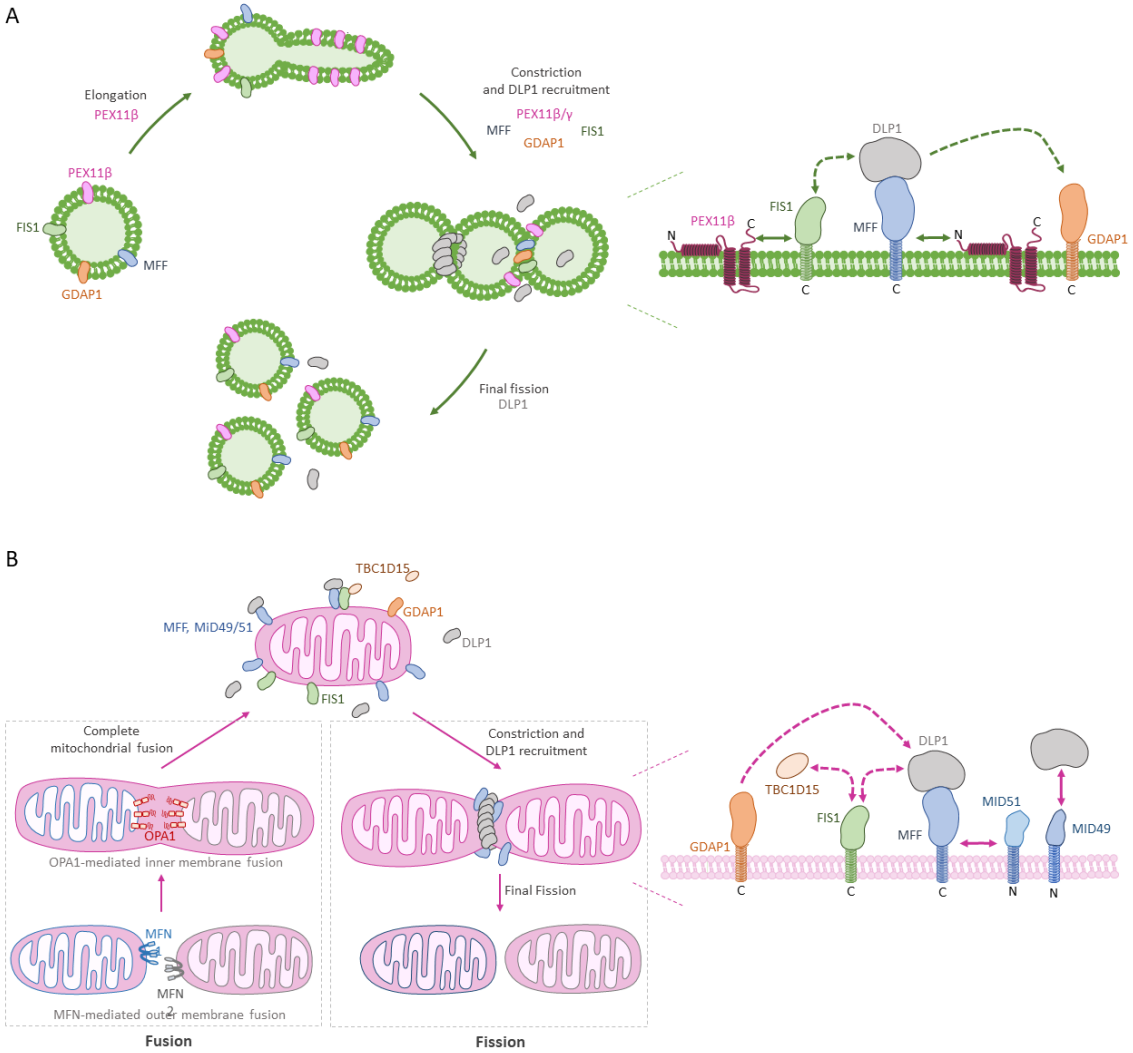


***Figure 1 - Growth and division of peroxisomes and mitochondria in mammalian cells***. A) Upon external stimuli peroxisomes undergo a multistep process including elongation of preexistent mature peroxisomes, constriction (forming beads on a string-like structures) and final scission, enabling the formation of new peroxisomes. PEX11β initiates the remodeling process and mediates the elongation, being also involved in the final constriction; DLP1 is recruited from the cytosol to the constriction sites by the adaptor proteins MFF and FIS1. GDAP1 also seems to be involved in peroxisome division regulation in a MFF and DLP1-dependent manner. DLP1 form ring like structures around the constricted membranes (fission foci) and mediates final scission upon the activation by PEX11β. B) Unlike peroxisomes, mitochondria elongation involves a fusion process mediated by the mitofusins MFN1 and MFN2 and OPA1. Peroxisomes and mitochondria share some key components of their fission machinery such as DLP1, FIS1 and MFF. GDAP1 also seems to be involved in the regulation of mitochondria division in a MFF and DLP1-dependent manner. MiD49 and MiD51 are mitochondria-specific adaptors which inhibit DLP1-mediated fission. Alternatively, fission can be regulated by FIS1 which interacts with the Rab GTPase activating protein TBC1D15.

Upon PEX11β activation, a membrane remodeling process is initiated. Although some of the stimuli that lead to peroxisome elongation were not yet identified, docosahexaenoic acid (DHA) appears

to be crucial on signaling for peroxisomal tubulation since it directly impacts on PEX11β oligomerization and is, thus, a prerequisite for subsequent fission and peroxisome division [21].

Once the elongation process is initiated, PEX11β recruits new phospholipids deriving from the ER (or other organelles) to the peroxisomal membranes and, peroxisomes start expanding in one direction, forming tubular-shaped peroxisomes. While the peroxisome tubules are formed PEX11β migrates to these protrusions, forming PEX11β-enriched tubules (Figure 1). Along with the peroxisomal membrane growth, new PMPs are recruited from the cytosol to the peroxisomal membranes in order to originate new fully functioning peroxisomes [14].

The elongation capacity of PEX11β has been mainly linked to its N-terminal amphipathic helices, which has been shown to bind to negatively charged liposomes leading to membrane tubulation [22,23]. Several studies have shown that deletion of these helices or the impairment of their function, inhibits PEX11β mediated growth of peroxisomes [22–24]. Moreover, results indicate that PEX11β oligomerization is also involved in peroxisomal elongation and it may be involved in the stabilization of peroxisome elongated membranes [16].

#### *1.1.1.1.2 Membrane constriction (fission foci)*

After elongation, peroxisome membrane is deformed, and constriction foci are formed. Membrane constriction is an imperative stage in peroxisomal and mitochondrial fission since it enables the formation of DPL1 ring like structures to encircle and cleave the organelle´s membrane [25,26].

Despite the constriction process not being yet fully understood, PEX11β seems to be involved in the formation of these narrow neck sites at PEX11β enriched locations. Specifically, PEX11β oligomerization seems to be related to this peroxisome membrane deformation. It has been suggested that PEX11β homo-dimers at the peroxisome membrane, besides being a prerequisite for peroxisome membrane elongation, are ultimately involved in the membrane constriction [16]. Although Bonekamp et al. 2013 study has pointed to a PEX11β homo-oligomerization, hetero-dimers between Pex11γ and Pex11β cannot be excluded. In line with this, Koch and Brocard, 2012 suggested a model where PEX11γ acts together with PEX11β in peroxisomal elongation and remodeling. According to this model, both PEX11β and PEX11γ are involved in peroxisome membrane protrusion and, similarly to PEX11β, the amphipathic domain of PEX11γ is involved in membrane elongation, being also necessary for interaction with PEX11β and with the mitochondrial fission 1 (FIS1) protein [15]. Results suggest that the two PEX11 isoforms may act in different stages of peroxisome proliferation. Although Koch and Borcard´s study has demonstrated that PEX11γ

heterodimers, but not homodimers, can bind FIS1, PEX11γ seems to play its role mainly at membrane elongation. On the other hand, PEX11β, additionally to elongation, seems to have a more critical role on the peroxisomal fission machinery, since it interacts with both adaptor proteins of the GTPase dynamin like protein 1 (DLP1): mitochondria fission factor (MFF) and mitochondria fission 1 protein (FIS1). This may also indicate that PEX11β is involved in the assembly of these anchor proteins at the fission sites (Figure 1A) [15].

In mitochondria, some studies have pointed to the involvement of the ER in conjunction with F-actin assembly in membrane constriction. In yeast, the ER seems to encircle mitochondria and constrict the membrane to a diameter compatible with the DLP1 ring [27,28]. An ER role in mitochondrial fission has also been addressed in mammals. Korobova and colleagues have shown that at the ER-mitochondria contact sites, actin filaments are required for constriction and its activity its facilitated by the ER protein Inverted formin-2 (INF2) [29].

No similar mechanism has yet been proven to occur in peroxisomes. However, due to the close relationship between these two organelles, an analogous mechanism cannot be ruled out [4].

#### *1.1.1.1.3 DLP1 recruitment*

As previously mentioned, in addition to the role in reshaping the peroxisomal membrane during proliferation, PEX11 proteins have also been demonstrated to recruit the tail-anchor proteins FIS1 and MFF to the constriction sites which in turn recruit DLP1 leading to the final scission of peroxisomes through GTP hydrolysis. Koch and Brocard, 2012 revealed that FIS1 interacts with both PEX11β and PEX11γ heterodimers, whereas MFF interacts preferably with PEX11β [15].

FIS1 is a 16 kDa C-terminal tail-anchor protein, inserted at peroxisomal and mitochondrial membranes exhibiting a C-terminal tail to the organelle´s lumen and the N-terminal to the cytosol. The small tail and the transmembrane domain define the correct targeting to peroxisomes and mitochondria, whereas the N-terminal, facing the cytosol, promotes organelle´s scission through the recruitment of the effector fission protein DLP1 [30–32].

FIS1 was described for the first time in yeast, localizing at mitochondrial membrane and to mediating mitochondrial fission by recruiting the DLP1 yeast homologue (DNM1) to this organelle's membrane through the yeast-specific adaptor proteins mitochondrial division protein 1 (MDV1) and/or its paralogue CCR4-associated factor 4 (CAF4) [33,34]. Years later, Yoon et al. 2003 also suggested a role for FIS1 at mitochondrial fission in mammals. Yoon´s study revealed that mammalian FIS1 directly interacts with DLP1 and is an effector of mitochondrial fission by recruiting

DLP1 from the cytosol to the mitochondrial membrane. In mammals, homologue proteins to MDV1 or CAF4 have not yet been described [31]. A similar role for hFIS1 at peroxisomes has also has been addressed [35,36].

However, how exactly FIS1 regulates fission via DLP1 in mammals, remained intriguing, primarily because in yeast, FIS1 does not bind directly DMN1 without the bridging activity of MDV1 and CAF4, and no homologues of these two adaptor proteins have been found in the mammalian genome. Secondly, although in yeast FIS1 is required for DMN1 recruitment to mitochondria, knockdown of FIS1 in mammalian cells did not reveal to importantly affect DLP1 [37].

Years later, Gandre-babbe and Van der Bliek, using an siRNA screen in *Drosophila* cells, identified a novel tail-anchored membrane protein, MFF, which has been suggested to predominantly control mitochondrial and peroxisomal fission through DLP1 interaction rather than FIS1 [37,38].

The MFF gene is conserved in metazoans and encodes at least nine different isoforms resulting from alternative splicing of exons 1, 5, 6, and 7. Such as FIS1, MFF is a C-terminally anchored protein at mitochondrial and peroxisomal membranes and has also been reported to recruit and dock DLP1 at these organelles' membranes. Its localization depends on its C-terminal whereas its N-terminal regulates the fission activity [15,37,38].

The role of MFF was initially studied the in mitochondria fission machinery and Otera´s group have shown that MFF localizes at mitochondria´s constriction sites, directly binding and recruiting DLP1 from the cytosol, and to have a stronger impact on mitochondrial morphology regulation than FIS1 [38]. Further studies focusing specifically on the role of MFF on peroxisome fission machinery, confirmed the assumptions that the previous studies have speculated, showing that MFF also localizes at peroxisome´s constriction sites [15,39] and that, in addition to FIS1, PEX11β directly interacts with MFF [15]. A ternary complex constituted of PEX11β, MFF and DLP1, where PEX11β interacts with DLP1 via MFF, was suggested by Itoyama et al. 2013 to orchestrate peroxisomal fission at the constriction sites [39] (Figure 1A). Taking this into account and since no homologue to MFF seems to exist in yeast, these studies have proven a different organelle´s fission mechanism in mammals [38,39]. In parallel, studies on the regulation of peroxisomal morphology, revealed that the mitochondrial tail-anchor protein GDAP1 also plays a role at peroxisomal fission. GDAP1 is a member of the ganglioside-induced differentiation-associated protein family, located at the mitochondrial outer membrane, known to regulate the mitochondrial fission and to play a role in a signal transduction pathway during neuronal development. Mutations in this protein have been associated with Charcot-Marie-Tooth disease [40]. Huber´s group have found evidences that GDAP1

is targeted to peroxisomes via PEX19 and to be involved in peroxisomal fission in a DLP1 and MFF dependent manner, acting upstream of this complex [41].

#### *1.1.1.1.4 Final Scission*

In mammals, final scission of peroxisomes and mitochondria is controlled by the dynamin-like/related protein DLP1 which is a large multimeric GTPase of the dynamin superfamily. In an effort to identify mammalian homologues for the yeast 85 kDa Dnm1 protein, Yoon and colleagues identified for the first time an 80 kDa protein, termed DLP1 and associated its activity to cytoplasmatic vesicle trafficking [42]. Subsequent studies have reported the DLP1 fission activity in organelles such as the ER, mitochondria or peroxisomes [31,43–45]. DLP1 present multiple alternative splicing isoforms and, as typical dynamin, four main functional structural domains: the N-terminal GTP-binding, middle GTPase domain, insert B and C-terminal GTPase effector domain (GED) [42,46]. The GTPase domain mediates peroxisomal and mitochondrial membrane fission through GTPase hydrolysis. A negative dominant mutant K38A has been revealed to impair GTP hydrolysis but not GTP binding, impairing DLP1 function and leading to an elongated organelle phenotype [25,32,43,44]. In turn, the GTPase middle has been hypothesized to be involved in the binding to adaptor proteins at organelle´s membrane, since a putative binding motif to MFF has been identified. The GED domain has been shown to interact with the N-terminal GTP-binding and middle domains and thus, in addition to the stimulation of GTPase activity, demonstrated to be involved in the regulation of DLP1 oligomerization and to play a role in a proper targeting to mitochondria and efficient mitochondria fission [46–48]. The insert B is an unstructured domain whose function is not yet well understood. However, more recently Frohlich´s results show that insert B may play a role in DLP1 oligomerization [47,49].

Furthermore, by revealing the crystal structure of DLP1, and in spite of all the structural similarities with a typical dynamin structure, a double rather than a single filament has been suggested and some particularizes in DLP1 oligomerization have been linked to the specificities of mitochondria membrane remodeling and fission [49].

DLP1 is mainly found in the cytosol and is recruited in the monomeric form to peroxisomal and mitochondrial membranes. As DLP1 has low affinity for membranes, multimeric complexes such as homo-tetramers and/or adaptor proteins such as FIS1 and MFF are needed for the DLP1 anchoring at peroxisomal and mitochondrial membranes [46,49]. At these membranes, DLP1 self assembles into higher order structures, forming ring-like complexes around constricted membranes – the fission

foci. At this stage, elongated peroxisomes assume the shape of “beads on a string”-like structures. Through GTPase hydrolysis constriction is increased (Figure 1A). DLP1 collards can decrease in diameter around 60 nm when comparing pre-mitochondrial membrane constriction and post membrane scission [50]. However, whether constriction is enough for membrane scission or other mechanisms are involved is still unclear. Once the final membrane fission occurs, new singular fully functional organelles are formed [44,45].

#### 1.1.1.2 *De novo* formation

The close association of peroxisomes with the ER, frequently revealed by electron microscopy (EM), and the observation of peroxisomes’ formation in human patient fibroblasts and yeast cells lacking peroxisomes, has raised the question whether the “Growth and division model” would be the single mechanism for peroxisome biogenesis in mammalian and yeast cells [51,52]. In fact, in addition to the first EM observations in 1972 by Novikoff and Novikoff, more recent EM techniques based on immunolabeling and three-dimensional image reconstruction have corroborated the connection of peroxisomes with the ER [53,54].

In the craving for the clarification of an alternative pathway for peroxisome biogenesis, Hoepfner´s group have deepened the studies on peroxisome regeneration, in cells barren of peroxisomes due to a mutation in PEX3 and PEX19 peroxins [55].

Peroxisomal biogenesis factor 3 (PEX3) is a an integral peroxisomal membrane protein, exposing its N- and C-terminal parts to the cytosol and docking PEX19 at the peroxisomal membrane [56–59]. Moreover, PEX3 initiates membrane assembly of peroxisomal membrane vesicles before the import of peroxisomal matrix proteins [60]. Conversely, PEX 19, with a farnesylated tail, acts both as a cytosolic chaperone and as an import receptor for class I (PTS1) peroxisomal membrane proteins (PMPs) [56,57,59].

In their study Hoepfner and colleagues observed that, in PEX3 or PEX19 deficient cells, after reintroducing the wild-type version of these genes, functional PEX3 and PEX19 proteins are firstly targeted to the ER prior to mature peroxisomes. At the ER membrane, PEX3 docks PEX19, forming PEX3/PEX 19 enriched foci. In a subsequent stage, these proteins were observed to migrate into fully mature peroxisomes, confirming that peroxisomes can be newly formed, deriving from the ER, which contributes with membrane material during peroxisome formation [55].

In addition to PEX13 and PEX19, peroxisomal membrane protein PEX16 has been also reported to be crucial for membrane assembly in peroxisome biogenesis and PMPs import. This peroxisomal

membrane protein, with both N- an C-terminus, facing the cytosol is involved in the early stages of peroxisome formation deriving from the ER (*de novo* formation) by recruiting other peroxisomal proteins such as PEX3 and peroxisomal membrane protein PMP34 [57,61]. The correct localization of this peroxin at the peroxisome membrane is determined by a cluster of positively charged amino acid residues at 66–81 and the first transmembrane segment (TM1), whereas the C-terminal has been shown to be involved in peroxisome assembly [61].

Once these peroxisomal proteins are assembled into specialized domains at the ER, these new peroxisomal compartments bud from the ER into fully peroxisomes which mature through the import of new membrane and matrix proteins [60,62,63]. The assembly of PEX3 and PEX16 proteins into the pre-peroxisomal compartments at the ER has been shown to be dependent on SEC16β pathway [64].

Besides this role of the ER on peroxisomal biogenesis, recent studies have shown that the ER is vital not only for the *de novo* formation but also to the multiplication of the pre-existing ones by providing lipid material and new PMPs [621].

Although peroxisomes are structurally simple organelles, their biogenesis in highly complex, and key mechanisms for its regulation, such as the route of PMPs to newly formed peroxisomes, remain poorly understood. Results substantiate two different pathways for PMPs integration at peroxisomes, either directly, upon protein synthesis (as defended by the growth and division model) of indirectly, via the ER (according to the *de novo* model) [9]. Additionally, more recent studies described the involvement of mitochondria, in addition to the ER, on peroxisome biogenesis, both in mammalian and yeast cells [65,66]. In these studies, once PEX3 has been restored in deficient cells, this protein was observed to be targeted to vesicle structures in mitochondria, positively marking for PEX14, PMP70 and catalase, which budded from this organelle forming new peroxisomes. However, PEX16 was positively signaled for ER, but not mitochondria, derived vesicles [66]. Taken together these results, allowed to hypothesize that, in the absence of peroxisomes, PMPs can be targeted to any endomembrane, and upon vesiculation of these PMP´s enriched membrane compartments, new peroxisomes are formed [1,5].

Primarily, it has been believed that *de novo formation*, takes place only in the absence of pre-existing peroxisomes, however, further studies have counteracted this hypothesis, since new peroxisomes deriving from the *de novo* pathway have been observed, even in cells with pre-existing peroxisomes [62]. Currently, It is widely accepted that both mechanisms coexist within the cells and collaborate in controlling peroxisome population and homeostasis [9]. However, further studies on

how peroxisome biogenesis is orchestrated within the cells, should be addressed, since no consistent results have been produced.

### 1.1.2 Peroxisome dynamics

Peroxisomes are crucial organelles for the normal metabolic function of the cell and the impairment of their proper function may lead to severe metabolic diseases. In order to face the metabolic needs of the cells, peroxisomes easily adapt their shape, number, enzyme content and location [2,4,8,67,68].

#### 1.1.2.1 Peroxisome proliferation

Peroxisome plasticity is highly influenced by microenvironmental and genetic factors which can trigger or inhibit peroxisome biogenesis and function [69]. Despite other pathways may influence peroxisome biogenesis, the peroxisome-activated receptor (PPAR)-dependent pathway has a major role on peroxisome regulation in response to nutrient changes and xenobiotic stimuli [4,13,69]. As members of the nuclear receptor superfamily, PPAR transcription factors include three PPAR isotypes: PPARα (NR1C1), PPARγ (NR1C2), and PPARδ (NR1C3), which function as lipid sensors and regulate lipid metabolism [70].

In mammals, the PPARα is the protagonist pathway for the regulation of peroxisome proliferation. Various fatty acids and fatty acid derivates and fibrates, have been described, especially in rats, to activate PPARα, leading to an increase on the PEX11β gene expression [71]. However, in Human cells the modulation of peroxisome proliferation seems to be more complex than in other mammalian cells. Studies have shown that, in human hepatocytes, PPARα pathway via fibrates activation does not have a prominent role in peroxisome proliferation [72]. and the correlation with PEX11β remains controversial. Nevertheless, several genes that code for peroxisomal enzymes such as acyl-CoA oxidase 1 (ACOX1), bile salt sulfotransferase (SULT2A1), acyl-CoA dehydrogenase long chain (ACADL) can be induced by fibrates (GW7647) via PPARα [70].

Less is known about how PPARγ and PPARδ affect proliferation. However, studies provide evidence that under certain xenobiotic agonists also PPARγ and PPARδ may induce proliferation of peroxisomes. For instance, in mice, the thiazolidinedione rosiglitazone have been linked to the expression of several PEX genes via PPARγ, in preadipocyte cells, suggesting that PPARγ promotes peroxisomal biogenesis in adipocytes [73] whereas, high fat feeding was observed to stimulate PPARγ expression, enhancing peroxisome proliferation in hypothalamus cells [74]. Additionally, in brown fat

tissue (BAT), under thermogenic stimuli, PPARγ and PPARδ but not PPARα seems to be involved in peroxisome proliferation promoted by the transcriptional coactivator PGC-1α [75].

Despite, several studies have been performed and many fundamental questions regarding the regulation of peroxisome via PPAP mechanisms have been answered, many mechanistic questions remain unclear [4,76].

#### 1.1.2.2 Peroxisome motility

With the improvement of the microscopy techniques, it had been possible to observe that peroxisomes are highly dynamic, moving constantly within the cells, not only in accordance to surrounding signals and cellular needs but also to ensure peroxisomal inheritance by daughter cells during cell division [13,69,77]. Associating to the cytoskeleton tracks, peroxisomes' motility includes small oscillations and also short and long distance trafficking throughout the cells [78,79]. However, some differences have been noticed when comparing the range of movement in yeast and plants and eukaryotic cells. In yeast and plants, peroxisomes are transported along actin filaments by myosin motor proteins, whereas in mammalian cells, for the long range and bidirectional journeys, peroxisome move through the microtubule's cytoskeleton [78]. Recently, studies have linked the Ras GTPase mitochondrial Rho GTPase 1 (MIRO1) to peroxisome microtubule-dependent motility [80,81] Castro et. al 2018 have shown that this protein localizes at peroxisomes and not only impacts on peroxisomes distribution and motility but also has a role on pulling apart the sibling peroxisomes during proliferation [80]. Similarly, to the motor complex of mitochondria, MIRO1 has been addressed to link peroxisomes to the motor proteins kinesin and dynein, which in turn move the organelle along the microtubules cytoskeleton [82].

#### 1.1.2.3 Peroxisome Degradation

Similarly to other organelles, such as mitochondria, lysosomes and ER, peroxisomes undergo degradation in order to maintain the integrity and quality of their community and also adjust their number according to the environmental conditions of the cells [83,84]. Peroxisome homeostasis can be achieved by different mechanisms including, selective autophagy (pexophagy), proteolysis by peroxisomal Lon protease 2 (LONP2), and 15-lipoxyge-nase-1 (ALOX15)-mediated autolysis. However, pexophagy represents the major mechanism for degradation and can be stimulated by several stress conditions such as oxidative stress of starvation [84].

Several studies focusing on pexophagy have been performed along the years and, in mammalian cells, three different pathways for pexophagy are generally assumed: a) via the ubiquitin-binding autophagy receptors NBR1 and sequestosome 1 (SQSTM1 or p62) which bind both to the microtubule-associated protein 1A/1B-light chain 3 (LC3) associated with the phagophore and to an ubiquitinated PMP such PEX5 or ACBD3, leading to clustering and degradation of peroxisomes [85]; b) via PEX14 interacting directing with LC3-II, the lipidated form of LC3 or the poly(ADP-ribose) polymerase (PARP) family members tankyrase 1 (TNKS) and tankyrase 2 (TNKS2) TNKS/TNKS2 [86], and c) via other ubiquitinated PMPs which leads to the recruitment of an autophagosome after interaction with LC3-II [87]. PEX 3 has been also suggested to play a role in pexophagy in a SQSTM1-dependent manner. Despite SQSTM1 being a prerequisite for pexophagy, in coordination with NBR1 it increases the efficiency of NBR1-mediated pexophagy [87,88]. Additionally, peroxisome fission seems to be involved in pexophagy. Similarly to mitophagy, DLP1 mediated fission leads to division of peroxisomes into smaller fragments facilitating autophagosome engulfment [84,89]. Nevertheless, despite several clues have demystified pexophagy, further studies are needed to fully understand the mechanism which regulate peroxisome homeostasis.

### 1.1.3 Peroxisomes and other organelles

To perform their multiple metabolic tasks, peroxisomes cooperate with several other subcellular compartments including the ER, lipid droplets, lysosomes and mitochondria. The interaction and crosstalk with other organelles occurs via direct contact, vesicular transport or diffusion of molecules or metabolites [4,90,91].

#### 1.1.3.1 Peroxisomes and the ER

As it has been previously mentioned, ER has a family relationship with peroxisomes, since peroxisomes in certain circumstances can start their life in the ER. During *de novo* biogenesis, ER can provide the formation of pre-peroxisomal compartments and intermediate the tethering of peroxins to peroxisomes. Additionally, this organelle contributes with phospholipids for the formation and elongation of peroxisomal membranes during peroxisomal proliferation [5,92]. However, the relationship between these two organelles goes further than the peroxisome biogenesis. Recently, Costello and colleagues described an intriguing association between peroxisomes and the ER. This group characterized peroxisome-ER membrane contact sites, where

the peroxisomal membrane proteins acyl-coenzyme A–binding domain protein 4/5 (ACBD4/5) interact with the ER protein vesicle-associated membrane protein-associated protein B (VAPB) [93,94,95]. This peroxisome-ER association revealed to impact on peroxisome dynamics since ACBD5 knockdown, leading to the loss of ACBD5/VAPB interaction, enhanced peroxisome mobility but perturbed membrane elongation [94]. Moreover, it has been also demonstrated that the ACBD5/VAPB interaction play a role in plasmalogen biosynthesis and cholesterol homeostasis [96]. The involvement of peroxisomes-ER interactions in apoptosis has been also suggested. In mitochondria, FIS1 was identified in a tethering complex with ER-resident B-cell receptor-associated protein 31 (BAP31), a 28-kDa integral membrane chaperone protein of the ER and ER protein-sorting factor, to establish a platform for apoptosis induction [97,98]. As FIS1 also localizes to peroxisomes, the FIS1-BAP31 tether may also contribute to peroxisome-ER membrane interactions [91].

### 1.1.3.2 Peroxisomes and the lipid droplets

Despite the puzzle behind the association of peroxisome and lipid droplets being far from solved, along years few clues have raised to understand this connection. Peroxisomes are known to play important functions in lipid metabolism and several studies have provided evidence of their association with lipid droplets [99–103]. In human glioblastoma cells, under hypoxia, the increase of lipid droplets in parallel with peroxisomes has been observed. Further studies have revealed the stimulation of PPARα with the increase of lipid droplets. Moreover, the induction of the hypoxia-inducible factor Hif1α, in hypoxic conditions, has been hypothesized to induce PPARα expression, leading, consequently, to peroxisome proliferation [104]. However, these results have raised the question why, in glioblastoma cells, PPARα is associated with the lipid droplets increase, if the PPAPα generally induces catabolic lipid metabolism [13]. The answer might be settled in the peroxisomal β-oxidation, that under hypoxic conditions might be used to produce acetyl-CoA as a substrate for *de novo* lipid synthesis [13,104]. It is known that abnormal lipid metabolism is closely related to cancer and there are growing evidences that peroxisomes have an important role in cancer development. In line with this, Valença et al. 2015 have correlated the peroxisomal localization of monocarboxylate transporter 2 (MCT2) with the increase of peroxisomal β-oxidation in prostate cancer localized tumor cells [105]. More recently, significant changes in peroxisome metabolism have been shown to occur throughout the different stages of prostate cancer and the presence of MCT2 at the peroxisomes has shown to be correlated with the cell proliferation in this

type of cancer [106]. In addition, in recent studies, the peroxisomal protein hydroxysteroid dehydrogenase-like 2 (HSDL2), a protein containing sterol carrier protein 2 (SCP2) domain and which is involved in the fatty acid metabolism, was found to be upregulated in glioblastomas and ovarian cancer cells and the expression of this protein have been reported to be associated with the malignity of cancer cells [13,107]. However, more studies are needed to understand the connection between peroxisomes, lipid metabolism and lipids droplets in the development of cancer [13].

#### 1.1.3.3 Peroxisomes and the Lysossomes

The transport of cholesterol within the animal cells is crucial for their normal function, impacting on membrane fluidity, permeability, and organization. There is growing evidence of a close cooperation between peroxisomes and lysosomes in the routing of cholesterol within the cells, however, fundamental questions remain unclear. Recently, Chu et. al, 2015 unveiled the interplay between peroxisomes and lysosomes through the binding of the integral lysosomal membrane protein synaptotagmin 7 to the lipid $PI(4,5)P_2$ on the peroxisomal membrane [108]. This study also revealed the importance of peroxisomes in cholesterol distribution since the disruption of the critical peroxisome gene ABCD1 led to cholesterol accumulation in lysosomes. The lysosomal polytopic membrane protein NPC1 also revealed to be required to the tethering of cholesterol to other organelles such as peroxisomes, and its impairment resulted in the accumulation of cholesterol in lysosomes and ultimately in the Niemann–Pick disease type C (NPC) [13,108]. Kleinecke and colleagues, demonstrated for the first time *in vivo* evidences for functional interactions between lysosomes and peroxisomes, corroborating the previous findings in cultured cells [108,109]. Their study suggests that, in peripheric nerves, peroxisomal dysfunction causes secondary impairment of lysosomes and contributes to peripheric neuropathy. Since peroxisomes are in charge of very long chain fatty acids (VLCFA) and 2-hydroxy fatty acids β-oxidation, and gangliosides are frequently esterified with VLCFA and 2-hydroxy fatty acids and must be degraded in lysosomes, peroxisomal dysfunction results in glycolipids accumulation in lysosomes, thereby impairing the turnover of gangliosides in myelin [109].

#### 1.1.3.4 Peroxisomes and mitochondria: global similarities and crucial particularities

Morphologically, peroxisomes and mitochondria are drastically different organelles: mitochondria are double membrane-bound organelles provided of their own genomes and protein synthesis machinery whereas peroxisomes are bounded by a single lipid bilayer membrane, surrounding a highly granular matrix devoid of DNA or transcription/translation systems [2,13,110,111]. In spite of these differences, they are highly interconnected not only by sharing key components of their division machinery, but also due to an intense crosstalk and shared functions at the lipid and ROS metabolism and innate immune response [67,110,112,113].

#### *1.1.3.4.1 Organelle Division*

In response to external physiological stimuli both peroxisomes and mitochondria change in number and shape, following a similar proliferation cycle of elongation, constriction and final scission (Figure 1). However, each organelle has their specificities. While mitochondrial elongated structures are formed through fusion of pre-existing ones, studies have shown that elongated peroxisomes are not formed through fusion but by incorporating new phospholipids leading to membrane elongation.

In mammals, mitochondria fusion is orchestrated by the dynamin-related GTPases MFN 1 and MFN2 anchored to the outer membrane, and by the dynamin-like 120 kDa protein (OPA1) which play a role at the fusion of the inner membrane (Figure 1B). Besides this role in mitochondrial fusion, OPA1 shaping activity also includes the control of the cristae structure and the release of cytochrome c and thus is a key player at apoptosis regulation. More recently the activity of the leucine-zipper and EF-hand-containing transmembrane protein 1 (LETM1) has been also described to be crucial for the cristae organization and morphology maintenance of mitochondria [114–116]. The activity of all these membrane-shaping proteins are strongly regulated by complex post-translational mechanisms that include proteolytic processing. In peroxisomes, a similar process to mitochondria fusion have not yet been described. Transient and long term contacts between individual and mature peroxisomes have been described, but no complete fusion, has been observed [117]. As discussed in the chapter *1.1.1.*, peroxisomal membrane elongation is controlled by the PEX11 family, specially the PEX11β protein.

Despite the elongation mechanisms being importantly different, peroxisomes and mitochondria share the key components of their fission machinery (FIS1, MFF, GDAP1 and DLP1, Figure 1B). In mitochondria, additionally to these proteins, other proteins have been described to play a role at the fission machinery. In mammalian cells, the mitochondrial dynamic proteins of 49 and 51kDa

(MiD49 and MiD51, respectively) were identified at the outer mitochondrial membrane (Figure 1B) [118]. These proteins (also called mitochondrial elongation factor 2 and 1, respectively) are amino-terminally anchored in the mitochondrial outer membrane with C-terminal cytosolic domains and were shown to directly interact with DLP1 and to recruit this protein to mitochondrial outer membrane [118–121].The question whether MiD49 and MiD51 affect positively [118] or negatively [119] has been addressed. Zhao et al. 2011, observed mitochondrial elongation upon MIEF1/MiD51 overexpression and suggested that at the mitochondrial membrane, interaction with DLP1 induces the reduction of DLP1's GTP-binding activity and impairs DLP1-mediated mitochondrial fission. It has been proposed that MFF and MIEF1 tun on and off, DLP1 activity, respectively [119] In contrast, Palmer´s group suggested a role of MiD49/51 in the stabilization of DLP1 at mitochondria [118] and, acting independently of MFF and FIS1, provide specificity to mitochondria fission [122]. This group attributed the appearance of fused mitochondrial tubules following overexpression of MiD49/51 not to a direct promotion of fusion or to fission blockage but to Mfn1/2-dependent mitochondrial elongation [122]. Nevertheless, most studies on this subject agree that MiD49/51 overexpression causes the accumulation of the inactive phosphorylated form of DLP1 at the mitochondrial membrane [121,122]. However, whether this sequestration and inhibition of DLP1 is indirectly or directly caused by MiD49/51 activity still needs to be clarified [122]. Parallel studies on mitochondria morphology regulation have described another player in mitochondrial fission machinery, the Rab GTPase activating protein TBC1D15. Onoue et al., 2013, while searching for FIS1 binding partners in Hela cells, identified TBC1D15, a 77.8 kDa member of the TBC (Tre2/ Bub2/Cdc16)-domain-containing protein family. This domain acts in the GTPase-activation of small GTPase Rab family proteins [123]. The authors revealed that this protein is recruited by FIS1 at the mitochondrial membranes and the interaction was hypothesized to be involved in the regulation of mitochondrial morphology, mediating fission through GTP hydrolysis by small GTPase proteins such as Rab7 [123–126]. In fact, in recent studies, the complex FIS1/TBC1D15/Rab7 has been also linked to mitochondria fission events such as in mitophagosome biogenesis [124,125]. This TBC1D15/Rab7 fission seems to not be mitochondria-specific as is also shared with lysosomes [127]. However, to date, MiD49/51 and TBC1D15 were not found at peroxisomes, indicating that besides peroxisomal and mitochondrial fission machineries being globally very similar, there are organelle-specific differences in the regulation their division [112,122,123].

#### *1.1.3.4.2 Lipid Metabolism*

In mammals, the capability of both peroxisomes and mitochondria to perform fatty acid β-oxidation was one of the primary evidences of their close relationship [67,90]. β-oxidation processes in both organelles are similar and, upon activation outside the organelle by conjugation to either coenzyme A (peroxisomes) or carnitine (mitochondria), fatty acids follow a reaction cascade of: a) dehydrogenation, b)hydration, c)dehydrogenation, and d) thiolytic cleavage [73,90,128]. Despite the cascade being globally similar, the first reaction is catalyzed by different enzymes. In mitochondria, lipid metabolism is catalyzed by the acyl-CoA dehydrogenases whereas in peroxisomes acyl-CoA oxidases (ACOX) assume this role. Different enzymatic activity results in different sub products. In mitochondria, the electron transfer to flavin adenine dinucleotide (FAD) generates FADH2 via Acyl-CoA dehydrogenase ACAD, which is later incorporated in the respiratory chain for ATP production, whereas in peroxisomes ACOX transfer hydrogen ions to Oxygen molecules ($O_2$) and hydrogen peroxide ($H_2O_2$) is formed [128,129]. In addition to this specific enzymatic activity they also target different substrates. While mitochondria is in charge of short to long chain fatty acids (LCFA) oxidation derived from diet, peroxisomes degrade long to very long chain fatty acids (VLCFA) and complex molecules such as long- and medium-chain dicarboxylic acids, prostaglandins, bile acid precursors, leukotrienes and mono- and poly-unsaturated fatty acids [73,130]. Peroxisomes and mitochondria β-oxidation are intricately connected since VLCFA shortened in peroxisomes, are routed to mitochondria for subsequent metabolization. Thus peroxisomes, by performing lipid metabolism to protect the cells against the toxic effect of their accumulation, fuel mitochondria for ATP production [90,128].

#### *1.1.3.4.3 ROS Metabolism*

Peroxisome and mitochondria metabolism results in the production of side products such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These have been considered to cause extensive damages within the cells, and are closely related to aging [131]. Fatty acid β-oxidation in peroxisomes leads to the production of $H_2O_2$. However, peroxisomes have an exclusive system that enables to control ROS within the cells. Through the enzyme catalase, which is located in peroxisomal matrix, $H_2O_2$ is degraded and converted into water. In turn, ROS is also a by-product of mitochondrial respiration and ATP production. Similarly to peroxisomes, mitochondria also have mechanisms to counteract and remove $H_2O_2$, specifically via super oxide dismutase 1 (SOD1) and peroxiredoxin 5 activities [90,132].

Albeit the destructive potential of ROS, it is known that it can modulate cellular signaling not only in mitochondria but throughout the cell, as there are redox-sensitive proteins whose activity is controlled by the redox state of the cell, which is largely controlled by peroxisomes. The redox state of the cell is involved in the regulation of the cellular metabolism and can modulate the switch from a proliferative state to apoptosis. Further studies have linked ROS and RNS to immune response to microbial pathogens [133,134].

#### *1.1.3.4.4 Antiviral innate immune response*

Besides their metabolic functions, studies have recently linked peroxisomes to the antiviral innate immune response. Peroxisomes and mitochondria share the antiviral adaptor protein, mitochondrial antiviral signaling protein (MAVS), which is activated by the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) upon infection by RNA viruses. Peroxisomes act in coordination and complementation with mitochondria:, peroxisomes provide a short-term reaction against viruses with a rapid expression of IFN-stimulated genes (ISGs), whereas mitochondria lead to a long-term reaction which amplifies and stabilizes the antiviral response [135,113].

### 1.1.4 Peroxisomes in health and disease

Peroxisomes's dysfunction can lead to severe disorders impacting lifespan. Peroxisome disorders can be divided in three major groups according to the impaired functions: peroxisome biogenesis disorders (PBD), single peroxisomal enzyme deficiencies (PEDs) and single peroxisomal substrate transport deficiencies [136].

Mutations in the PEX genes involved in peroxisome biogenesis generally lead to the development of autosomal recessive disorders such as the Zellweger spectrum disorders (ZSDs). Different groups among ZSDs can be distinguished: Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and Heimler syndrome. ZS leads to the most severe phenotypes whereas IRD is the less severe disorder among the Zellweger spectrum [67,137]. ZSDs clinical symptoms generally include neonatal hypotonia, craniofacial dysmorphy, adrenal atrophy, hepatic dysfunction and skeletal and neurological abnormalities with the impairment of the psychomotor development [67,137]. Besides the ZSDs other groups have been identified - the rhizomelic chondrodysplasia punctata type 1 (RCDP1) and type 5 (RCDP5) disorders [136]. Although,

PBDs are highly heterogenous, they are generally characterized by the accumulation of VLCFA phytanic acid and deficient or no plasmalogens and phospholipids synthesis [137].

In addition to these PBDs, deficiencies at the peroxisomal fission machinery have been also associated to disease. In 2007, Waterham et al. 2007 described a lethal disease in a patient caused by a heterozygous dominant-negative in DLP1 causing mitochondrial and peroxisomal dysfunction. This mutation impaired the normal mitochondrial and peroxisomal fission and thus, organelles remained elongated. Symptoms included microcephaly, neurological abnormalities, persistent lactic acidemia, and a mild increase of VLCFA, among others [138]. Later, a recessive Infantile Encephalopathy caused by DLP1 mutations, affecting both peroxisome and mitochondria morphologies was described by Nasca and colleagues [139]. Another mutation affecting peroxisomal and mitochondrial division has been presented by Shamseldin et al 2012. Mutations in the MFF gene disturbs the normal mitochondrial and peroxisomal fission causing Leigh-like encephalopathy [140]. Similarly to DLP1 disease, organelles remained mostly elongated [140–142]. Only affecting peroxisomes, a mutation in PEX11β has been linked to a pathology characterized by congenital cataracts and mild intellectual disability and ataxia in adults. Similarly to DLP1 and MFF patients, skin fibroblasts also revealed an abnormal elongated morphology of peroxisomes [17,143].

Single peroxisome enzyme deficiencies include a wide range of disorders and can be divided according to the metabolic pathway affected: a) Ether phospholipid synthesis; b) β-oxidation; c) α-oxidation; d) Glyoxylate detoxification and e) ROS metabolism [132,144]. ROS and ether phospholipids metabolism has been associated to several neurodegenerative diseases such as Parkinson's or Alzheimer's disease, and to play a role in cancer and ageing [145,146].

On the other hand, single peroxisomal substrate transport deficiencies only includes one disorder - the X-linked adrenoleukodystrophy (X-ALD) [144]. X-ALD, which is the most common inherited peroxisomal disorder, is a slowly progressive axonopathy affecting mainly males [147] and is caused by a mutation in the ATP-binding cassette (ABC) sub-family D member 1 ABCD1, which is involved in the transport of long- and VLCFA-CoA across the peroxisomal membrane for β-oxidation [148–150].

As it has been previously mentioned and it will be discussed into more detail in the next chapter, peroxisomes are also important players in the innate immune response against viruses.

## 1.2 CELLULAR ANTIVIRAL SIGNALING

The innate immunity is comprised of inherited strategies which enable the host to recognize the self- from the non-self-molecules, and triggers complex signaling cascades in order to eliminate the pathogens and protect the host. In this protective journey, the innate immune system can also stimulate the adaptive immune system for a specific response. All the innate immunity responses start with the recognition of specific molecules of the pathogens, namely the pathogen-associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs) spread through the intra- and extracellular spaces. PRRs can be divided according to their location; they can be either cytosolic or be bound to a membrane. In the antiviral immune response cytosolic PRRs include the RIG-I-like receptors (RLRs) nucleotide oligomerization domain-like receptors (NLRs) and DNA cytosolic sensors which specifically recognize viral DNA. On the other hand, Toll-like receptors (TLRs) are the most well studied and characterized class of membrane PRRs [151,152].

Despite the different locations and types of PRRs, upon microbial infection, they trigger similar signaling cascades which culminate with the production of proinflammatory cytokines and type I interferons (IFN-α and IFN-β), or ISGs which allow the elimination of the pathogen by autophagy, phagocytosis and cell death [153].

### 1.2.1 Toll-like receptors (TLRs)

Toll like receptors (TLRs) are type I transmembrane proteins which play a central role in pathogen recognition and response against microbial agents. They have specific ectodomains containing leucine-rich repeats for the recognition of molecular structures that are broadly shared by pathogens, known as pathogen-associated molecular patterns (PAMPs) and IL-1 intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domains required for downstream immune signaling. TLRs can be found bound to organelles such endosomes, lysosomes and endolysosomes or plasmatic membranes [154].

Upon viral infection, TLR-mediated response is triggered by the recognition of viral PAMSs and six different TLRs might be involved. Localized at the plasma membrane TLR2 and TLR4 recognize viral structural proteins, whereas at the endosomal membranes TLR3, TLR7, TLR8 and TLR9 recognize viral ribonucleic acids (RNA) (in the case of the first three) or deoxyribonucleic acid (DNA) (in the case of TLR9) [155].

TLR activation may lead to two different signaling pathways: via the protein myeloid differentiation primary response 88 (MyD88) or via Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) [156]. MyD88 is the more expressive pathway, since TRIF pathway is only

activated exclusively by TLR3 or TLR4 which can activate both pathways. These pathways leads to different signaling cascades ending with the induction of inflammatory cytokines mediated by activation of NF-κB and mitogen-activated protein kinases (MAPKs) via MyD88 whereas TRIF-dependent signaling pathway leads to the production of type I IFNs and inflammatory cytokines via the transcription factors IRF3 and NF-κB activation [157,158].

### 1.2.2 RIG-I- like receptors

The RLR family is composed by three proteins which exhibit a central ATPase containing DExD/H box helicase: retinoic acid-inducible gene I protein (RIG-I), melanoma differentiation associated gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2). These cytosolic proteins sense viral nucleic acids, mostly RNA, and share a central helicase and a C-terminal regulatory domain (CTDs). RIG-I and MDA5 share homology at their N-terminal with two caspase recruitment domains (CARD) whereas LGP2 lack the CARD domains. CARD activation triggers a downstream signaling leading to the activation of type I IFN genes. Both RIG-I and LGP2, but not MDA5, have a repressor domain (RD) in their CTD. Thus, while RIG-I and MDA5 generate an antiviral response via the CARD domains, LGP2 seems to have more regulatory functions by acting as a dominant-negative regulator of RIG-I and MDA5 [153,159]. However, LGP2 role remains controversial, and some authors defend that LGP2 act positively in the immune response mediated by RIG-I- and MDA5 [160,161].

Despite RIG-I and MDA5 mediated response being mostly associated with RNA viruses, there are growing evidences that they also play a role in antiviral signaling against DNA viruses: a) some viral proteins in DNA viruses target and activate RIG-I- and/or MDA5; [162,163] b) viral dsDNA may be transcribed via RNA polymerase III (RNA pol III) leading to RIG-I activation [164,165], although there are also evidences of RNA pol III- independent responses [166]; c) MDA5 was described to be involved in herpes simplex virus (HSV) antiviral signaling in a RNA pol III-independent pathway [165].

Upon activation, RIG-I and MDA5 interact with the mitochondrial antiviral-signaling protein (MAVS) through the CARD motif and trigger a signaling cascade leading to expression of IFNs, cytokines and IFN-stimulated genes (ISGs) via IR3 (Figure 3) [167–170]. MAVS, also known as IFN-β promoter stimulator-1 (IPS-1), CARD adaptor inducing IFN- β (CARDIF) and virus-induced signaling adaptor (VISA), localizes at the mitochondrial outer membrane, the mitochondrial associated membranes (MAMs) and peroxisomes. MAVS is a C-terminal tail anchor protein composed of a cytosolic CARD domain and a central proline-rich [135,168–171]. MAVS activation at mitochondria triggers a signaling cascade which includes: a) the activation of the TRAF family member associated NF-κB activator-

binding kinase 1 (TBK1) protein and the IκB kinase (IKK) complex; b) these kinases mediate the phosphorylation of IRF3 and NF-κB proteins, respectively; c) activated IRF3 translocates to the nucleus where type I IFNs and type III IFNs are expressed; d) IFNs activate the JAK/STAT pathway leading to the production of ISGs [135,153,159]. The signaling cascade downstream MAVS activation at peroxisomes is not yet fully unraveled.

Although the MAVS pathway culminates with the production of ISGs, studies have provided evidence that there are significant differences in the responses mediated by peroxisomal or mitochondrial MAVS. Peroxisomal MAVS, leads to a faster response with a short-term expression of ISGs, whereas mitochondrial MAVS activation, results in a delayed but long-term ISGs production [135,172]. However, further studies are needed to better comprehend the role of each organelle in antiviral response via MAVS pathway.

### 1.2.3 Cytosolic DNA sensors

Cytosolic DNA sensors include many sensor proteins such as DNA-dependent activator of IFN regulatory factor (DAI), DEAD-Box Helicase 41 (DDX41), Gamma-interferon-inducible protein (IFI16 and), and cyclic GMP-AMP synthase (cGAS). In turn, cGASs leads to the production of cyclic-di-GMP-AMP (cGAMP) [173–176]. These sensors recognize viral PAMs in the cytosol and mediate a signaling cascade generally with IRF3, TBK-1 and NF-κB activation, stimulating the expression of type I IFNs. Localized at the ER, the stimulator of interferon genes protein (STING) is the adaptor protein of these sensors and upon activation, STING translocate to the Golgi complex and activates TBK-1 leading to the phosphorylation of IRF3 [36,174,176,177].

In addition to DNA viruses, cGAS has been also reported to be involved in the antiviral response against certain RNA viruses. Some authors defend the STING pathway activating by cGAMP, [178] while others state that STING is not required for IFN expression [179]. In RNA viruses' infections STING has been also reported to interact with MAVS [174,176,177].

### 1.2.4 Human Cytomegalovirus

Human cytomegalovirus (HCMV) is an enveloped dsDNA virus which belongs to the *Herpesviridae* family. With a symmetric icosahedral capsid, HCMV has a lipid bilayer envelope with glycoprotein

complexes attached. The tegument layer separates the envelop from the capsid and is formed by viral phosphoproteins which are attached to the capsid and viral mature RNAs (mRNAs). The capsid encloses a ~240kb viral genome which is estimated to be composed between 165-252 open reading frames, that encode a vast number of proteins and micro RNAs [180].

HCMV virus is highly complex and has developed several virulence factors which allow it to infect different cell types such as epithelial and immune cells and to escape from the innate immune system, resulting in a high infection rate [152,163,181]. This herpesvirus is highly disseminated among humans prevailing lifelong latent in most adult individuals. Depending on the geographical and socioeconomic conditions, prevalence of HCMV viral may range from 40%-50% up >90% in less developed countries [182–184].

HCMV establishes latency in myeloid cells of the bone marrow but can periodically reactivate, with shedding of the infectious virions which allows an efficient transmission [185]. Although the infection is mostly asymptomatic in adult individuals, it may lead to serious diseases in immunosuppressed individuals, such as AIDS or transplant recipients [186].

Additionally, during pregnancy, HCMV primary infection reactivation or reinfection may cause significant congenital defects such as hearing loss and neurological impairment [183]. Due to the substantial risk of infection during pregnancy, several studies have been performed in order to unravel the virulence strategies of HCMV, and ultimately to possibly develop a vaccine [183,184].

#### 1.2.4.1 HCMV Life Cycle

HCMV has a specialized infection machinery which enables the infection of a wide range of cells. The glycoproteins B and H (gB and gH, respectively) present in the viral envelope allow the attachment to the host cells through the interaction with membrane proteoglycans, and the internalization of the virus either by fusion or endocytosis [186,187]. Once HCMV enters the cell, the nucleocapsid is released to the cytosol and the tegument proteins, attached to capsid, interact with the microtubule transport systems and are directed the nucleus. Reaching the nucleus, the nucleocapsid fuses with nuclear membrane and the viral genome is released (Figure 2) [188,189]. Through the host translation and transcription system, viral dsDNA is translated and replicated, and new viral particles are formed. Viral replication involves four stages: a) the “immediate early” (IE) gene expression, leading to IE proteins which stimulate the transcription of “Early” (E) genes; b) In the “Early” (E) stage, E genes control viral replication and modulate mechanisms to evade the infected host innate immune system; c) the “late” (L) gene expression leads to production of the

capsids, in the factories of the cells, and assembly; d) viral egress, with the release of new viral particles into the cytosol [190].

The maturation process of the non-enveloped capsids occurs in a perinuclear body, called the viral cytoplasmic assembly compartment (AC). Infected cells exhibit an enlarged kidney-shaped nucleus surrounding this assembly compartment which consists of the ER, Golgi apparatus and endosomes. [191] At the AC, the new non-enveloped capsids acquire the tegument proteins and the envelope (Figure 2). Ultimately, new viruses are released by exocytosis leading to death of the host cell [192,193].

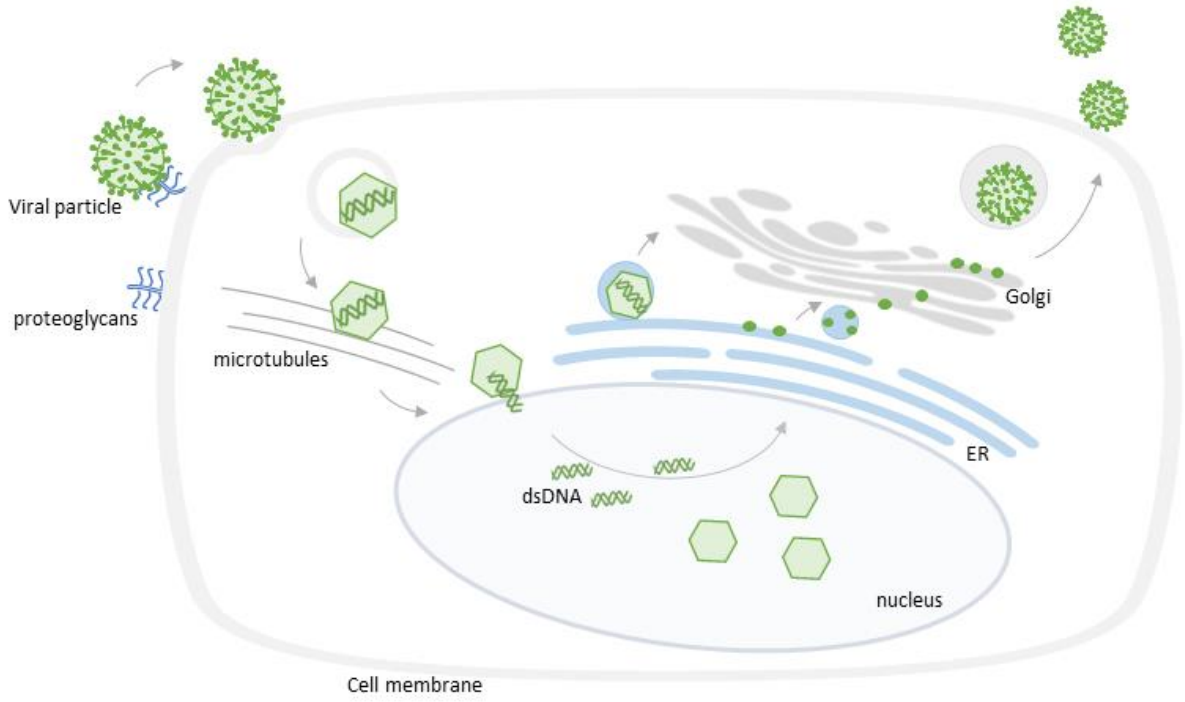


***Figure 2 - The human cytomegalovirus life cycle.*** HCMV enters the cells by fusion with cell membrane or endocytosis. In the cytosol, viral particles release the nucleocapsids and, making use of the microtubules "highways", the tegument proteins move towards the nucleus. In the nucleus viral DNA is replicated and translated and new capsids are assembled. Leaving the nucleus, new viral particles follow a maturation processes at the ER-Golgi compartments and new enveloped and fully functional viruses are formed and released via exocytosis to infect other cells.

One major characteristic of HCMV is its latency and the ability of periodically reactivate. Latent viral genome acquires the form of closed circular episomes and dormant viruses remain in infected myeloid cells but maintaining the expression of a small number of viral genes. Reactivation of the virus has been linked to the differentiation of myeloid cells into macrophages and dendritic cells (DCs). Latent viruses keep all the replication mechanisms during latency, therefore, can rapidly replicate upon reactivation [192,194].

### 1.2.4.2 vMIA and the MAVS pathway

To escape the innate immune response, HCMV has developed multiple mechanisms to suppress cell death, enabling viral replication. Although HCMV is a dsDNA virus, the RLR pathway has been demonstrated to play a central role in the antiviral immune response against this *herpesvirus.* In other to escape from the antiviral sentinels, HCMV developed strategies to block the MAVS pathway, either by acting upstream MAVS, through the degradation of RIG-I [195] or by acting downstream MAVS, via the viral mitochondria inhibitor of apoptosis (vMIA) protein that targets the MAVS-mediated signaling, inhibiting IFN-β production. [196–198]. vMIA is encoded by the CMV "Immediate Early" gene UL37 (pUL37x1) and has been initially reported to localize at mitochondria and to abolish apoptosis either by disrupting the mitochondrial transition pore formation or by blocking the permeabilization of Mitochondrial Outer Membrane (MOM) [196,197]. Regarding its structure/antiapoptotic function, vMIA is composed of two main domains: a mitochondrial localization domain at the N-terminal and a BAX-binding domain at the C-terminal domain. At mitochondria, vMIA recruits the proapoptotic Bcl-2 family member BAX and neutralizes it by inducing its oligomerization and membrane sequestering [199].

Furthermore, vMIA has been also reported to impact on the modulation of mitochondrial fission/fusion process and thus, lead to the mitochondrial network's disruption. While some authors [200] have associated the perturbation of mitochondrial fission with the vMIA´s antiapoptotic function, others defend that mitochondrial network disruption disturbs the RIG-I/MDA-5 antiviral pathway [201]. These authors observed that the abnormal mitochondrial dynamics imposed by HCMV infection impaired the mitochondrial MAVS downstream signaling, inhibiting the production of IFNs and ISGs (Figure 3) [162].

Additionally, vMIA has been also shown to have a proactive function in HCMV infection via viperin. Although viperin is an interferon-inducible protein with antiviral activity, vMIA recruits viperin from the ER to mitochondria, with the purpose to disrupt cellular metabolism, such as fatty acids β-oxidation and ATP generation, and thus, enhancing the infectious process [202].

Recently, Magalhães et. al have demonstrated that peroxisomes are strategically used by some viruses to escape the cellular antiviral armies. vMIA has been shown to interact with peroxisomal MAVS and specifically impair this antiviral pathway. Furthermore, vMIA induces peroxisomal morphological changes and interact with the chaperone PEX19 in order to reach this organelle [203].

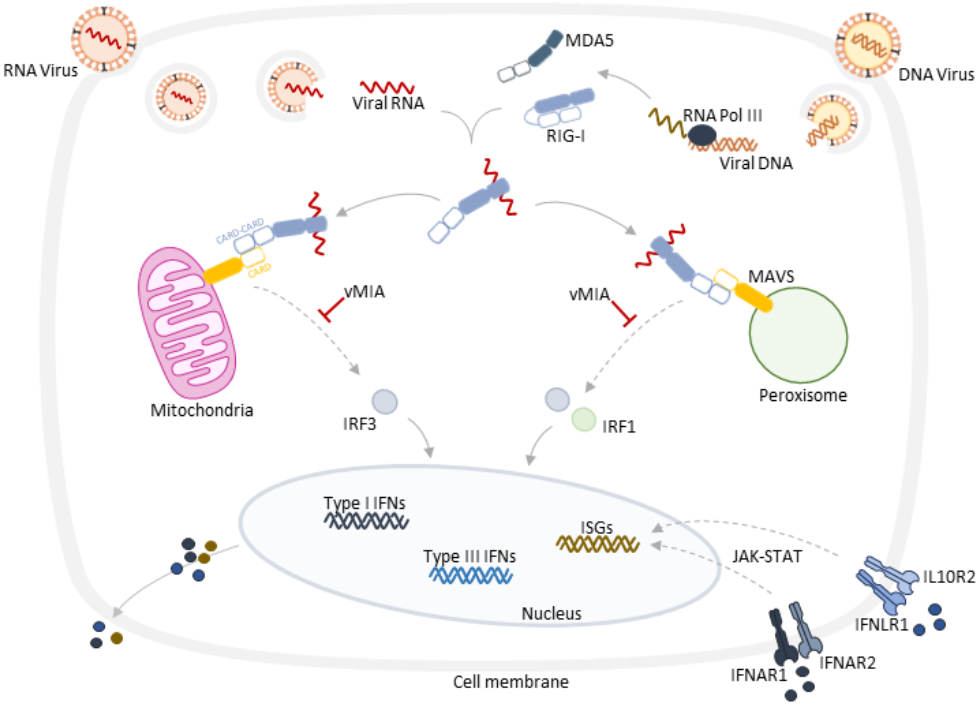


***Figure 3 - The RLRs antiviral signaling pathway in the immune response against HCMV.*** In the cytosol, RIG-I Like receptors MDA5 and/or RIG-I sense viral RNA and activate mitochondrial and peroxisomal MAVS through CARD-CARD domain interactions. The MAVS pathway culminates with the expression of IFNs, cytokines and IFN-stimulated genes (ISGs) via IR3. Peroxisomes and mitochondria provide a complementary response against viruses since peroxisomes provide a short-term and rapid response whereas mitochondria lead to a long-term response which amplifies and stabilizes the antiviral peroxisomal response. The Human cytomegalovirus has developed strategies to escape from the innate immune system such has the pro-infection viral protein vMIA which impairs the MAVS pathway.

# 2 OBJECTIVES

Since their very first discovery in 1966 by DeDuve and coworkers, peroxisomes have inspired a growing scientific interest. If initially they were described as microbodies containing hydrogen peroxide-generating oxidases, peroxisomes have been proven to have further key functions and to closely cooperate with several other subcellular compartments acting as a well-orchestrated team in order to maintain cellular homeostasis.

Peroxisomes are highly dynamic organelles which operationality depends on the correct orchestration of their fission machinery. They adapt in number and size/shape to correctly perform their functions and to contribute to the cellular defense and homeostasis [13,203]. Despite most of the mechanistic of their fission machinery being already described, important questions remain, including the importance of the peroxisome morphology and peroxisomal fission machinery in the antiviral immune response.

In line with this, this work encompasses two main objectives:

1. Further characterize peroxisomal fission machinery and distinguish different roles of key components shared with mitochondria (Section 3.1)
2. Characterize the role of peroxisomal fission proteins MFF and FIS1 in the peroxisomal antiviral signaling against viruses, more specifically HCMV (Section 3.2).

# 3 RESULTS

## 3.1 MFF AND FIS1 ASSUME DISTINCT ROLES AND IMPORTANCE AT PEROXISOMES AND MITOCHONDRIA

**Part of the results presented in this section are included in a manuscript which will soon be submitted for publication as:**

Ana Rita Ferreira[1,2]*, **Ana Gouveia[1]*,** Ana Cristina Magalhães[1], Isabel Valença[1], Mariana Marques[1], Jonathan C. Kagan[2] and Daniela Ribeiro[1], "Human cytomegalovirus' vMIA modulates peroxisome morphology and antiviral defense via MAVS and MFF"

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### 3.1.1 Abstract

Peroxisomes and mitochondria are highly dynamic subcellular organelles whose morphology control has important consequences for cellular physiology. Peroxisome and mitochondria fission depend on the recruitment of the dynamin-like GTPase DLP1 that mediates final scission. The search for the adaptor proteins for DLP1 at these organelle's membranes has been the subject of many controversial studies. Earlier results pointed out FIS1 as the main DLP1-anchor at the peroxisomal and mitochondrial membranes and, more recently, MFF has been favored to play this role in both organelles. In this study we have performed a detailed comparison analysis on the localization and function of these two proteins in two different cell lines. Our results strengthen previous studies which have pointed MFF to be the preferential DLP1-adaptor at the peroxisomal membrane, whether FIS1 seems to be the most relevant protein within mitochondrial membrane fission. In addition, we have confirmed that the most recent players described to have a function in the regulation of mitochondria morphology, MiD49/51 and TBC1D15 do not play a similar role at peroxisomes. Our results suggest that, although peroxisomes and mitochondria share the main components of their fission machinery, they adopt distinct mechanisms of action in each organelle and also suggest the presence of another DLP1 -like protein that may contribute to the final fission of both organelles.

### 3.1.2 Introduction

Peroxisomes and mitochondria are essential and highly dynamic subcellular organelles whose protein composition, morphology and abundance are tightly regulated upon external stimuli in order to maintain cellular homeostasis [3,4,67,111,204]. Several studies have shown that mitochondrial morphology plays an important role in cell physiology, influencing neurodegeneration, calcium signaling, lifespan, cell death and even the immune response [204–208]. Similarly, peroxisome dynamics and morphology have been shown to alter in certain disease conditions such as peroxisome biogenesis disorders, carcinogenesis, liver cirrhosis and viral infections [20,105,135,143,209,210]

Mitochondrial morphology is controlled by a set of proteins that orchestrate continuous fusion and fission of this organelle. Mitochondrial fusion in mammals is mainly regulated by the two dynamin-related GTPases MFN1 and MFN2 [211] at the outer membrane and OPA1 [212] and LETM1 [114–116] at the inner membrane, through a mechanism that is not yet fully understood. Mitochondrial fission depends on the recruitment of the dynamin-like GTPase DLP1 (DNM1, in yeast) that forms ring-like structures around constricted membranes to mediate scission through GTP hydrolysis [43,213–215]. The search for the adaptor proteins for DLP1 at the mitochondrial membrane has been the subject of many controversial studies. In yeast, the tail-anchored protein FIS1 was shown to play an essential role in the recruitment of DNM1 to mitochondrial fission foci through the adaptor proteins MDV1 and CAF4, whose mammalian homologues have not yet been identified [216–218]. Likewise, in mammalian cells FIS1 was the first candidate to be the DLP1-receptor at mitochondria membrane. Studies have shown that its exogenous overexpression induced mitochondrial fragmentation and its knockdown caused mitochondrial elongation [31,32,219,220]. However, its absolute prerequisite for DLP1 mediated fission remained controversial. In more recent years It has been suggested that FIS1 mediates mitochondrial fission by recruiting the cytoplasmic protein TBC1D15 independently from DLP1. TBC1D15 is member of the Ras-like proteins that share the TBC (Tre-2/Bub2/Cdc16) domain, conserved in the GTPase-activating proteins [123]. TBC1D15 seems to regulate mitochondrial fission through the small GTPase Rab7, however, the regulation of mitochondrial morphology via TBC1D15 activity remains to be fully elucidated [123–125,127,221]. Complementary studies provided evidence that FIS1 not only displays a role at organelle division but is also involved in other mechanisms such as the regulation of apoptosis and autophagy [222–226].

Later, the discovery of the tail-anchored protein MFF as DLP1 anchor, has contributed to the unpuzzling of the mitochondrial fission mechanism, as this protein has been pointed to have a major role in the recruitment of DLP1 to the mitochondrial membranes [37,38,48,121] The authors claim that MFF, but not FIS1, is an essential factor for mitochondrial recruitment of DLP1 during mitochondrial

fission in mammalian cells [38]. More recently, two other adaptor proteins, MiD49 and MiD51, were described to play a role as DLP1 anchors, negatively promoting fission by inhibiting DLP1 activity [120–122,227].

The regulation of peroxisome morphology has also been extensively studied and it has been shown that, although peroxisome fusion does not seem to occur [117], several components of the fission machinery are shared with mitochondria. Mammalian peroxisomes can proliferate though a process of growth and division [4,6,228]. According to this model, peroxisomes grow and multiply by taking up newly synthesized proteins from the cytosol [3,6]. This organelle's division occurs in three steps: elongation, constriction, and final fission [3,21,45,229,230]. PEX11β, a peroxisome-specific protein that is conserved from yeast to humans, is a key player in peroxisomal elongation [16,229,231]. Overexpression and deletion of this protein cause, respectively, the increase and reduction in peroxisome proliferation [14,16,232]. The process of peroxisome constriction is not yet well understood although PEX11β seems to be involved [16]. Nevertheless, other components and mechanisms that have not yet been identified may be involved. On the other hand, the fission machinery, has been the subject of many studies and, similarly to mitochondria, FIS1 was initially assumed as the main DLP1-adaptor at the peroxisomal membrane [35,36]. Likewise, MFF has also been shown to play an important role in the recruitment of DLP1 to the peroxisomal membrane prior to final scission [15,37,39,233] and also to be able to interact and regulate an interaction between DLP1 and PEX11β [39].

In order to better understand the similarities and specificities of peroxisomal and mitochondrial fission machineries, especially the roles of the adaptor proteins MFF and FIS1 in these organelle´s division, we have performed a detailed comparison study on the localization and function of these two proteins in the two organelles in two different cell lines. Overexpression, silencing and phenotype-recovery experiments have been performed and strengthen previous results showing that, although sharing the main division machinery components, DLP1-anchors MFF and FIS1 impact differently in the peroxisome and mitochondria fission: FIS1 seems to be the most relevant protein within mitochondrial membrane fission whether MFF displays a more prominent role in peroxisomes. Additionally, we confirm that, unlike mitochondria, DLP1 anchors MiD49/51 are not involved in peroxisomal fission neither TBC1D15 plays a role in the regulation of peroxisomal morphology. Our results also suggest the presence of another DLP1-like protein that may contribute to the final scission of peroxisomes.

## 3.1.3 Results

### 3.1.3.1 MFF and FIS1 overexpression have distinct effects on peroxisome and mitochondria morphology

To better understand the relative importance of FIS1 and MFF proteins within the mitochondrial and peroxisomal division, we have performed a series of detailed studies on the influence of the overexpression of these two proteins on peroxisomal and mitochondrial morphology in HepG2 cells. MFF or FIS1 overexpression is expected to induce the recruitment of a higher number of DLP1 proteins to the peroxisomal membrane, triggering the organelles' fragmentation [35,233,234]. To compare the effects of the overexpression of MFF and FIS1 on peroxisomal morphology, HepG2 cells were transfected with either flag-MFF or myc-FIS1 and subjected to immunolocalization with antibodies against flag or myc and the peroxisomal protein catalase. Peroxisome morphology was observed by confocal microscopy and statistical analyses were performed. Cells were analyzed for each condition, considering the size/shape and number of their peroxisomes (examples for the different peroxisome morphologies are shown in Figure 4). As shown in Figure 4c, we considered cells containing "fragmented peroxisomes" as those whose peroxisomes were smaller and in higher number when compared to the control cells (Figure 4a).

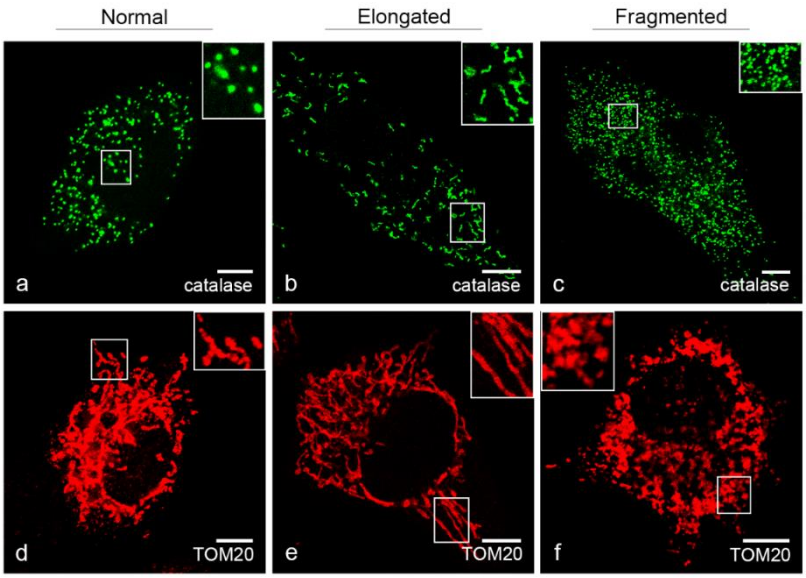


***Figure 4 - Control HepG2 cells for the different peroxisomal and mitochondrial morphologies.*** (a) Cell with normal round/rod-shaped peroxisomes; (b) an example of elongated peroxisomes; (c) cell exhibiting fragmented peroxisomes; (d) cell with normal mitochondria cell exhibiting elongated mitochondria and (f) an example of fragmented mitochondria. Bars represent 10 µm.

As shown in Figure 5A a-c, where one can observe a transfected and a non-transfected cell, MFF overexpression induced a significant peroxisomal fragmentation and these results were further

confirmed by statistical analysis (Figure 5B), showing an increase from 8% (control cells) to 44% of cells containing mainly fragmented peroxisomes. Using the Spot Detector plug-in from Icy Bioimage Analysis Software, we confirmed that, upon MFF overexpression, there was an increase in the number of peroxisomes (Figure 5C). Surprisingly, although the same phenomenon was observed upon FIS1 overexpression (Figure 5 d-f), the fragmentation occurred in a much lower level (Figure 5B), increasing to only about 19%. Furthermore, this FIS1-related fragmentation originated smaller and more peroxisomes than in control cells, but bigger and less than upon MFF overexpression (Figure 5 A d-f and 1C). These results suggest that, according to previous studies, MFF have a more prominent role than FIS1 on the peroxisomal division mechanism.

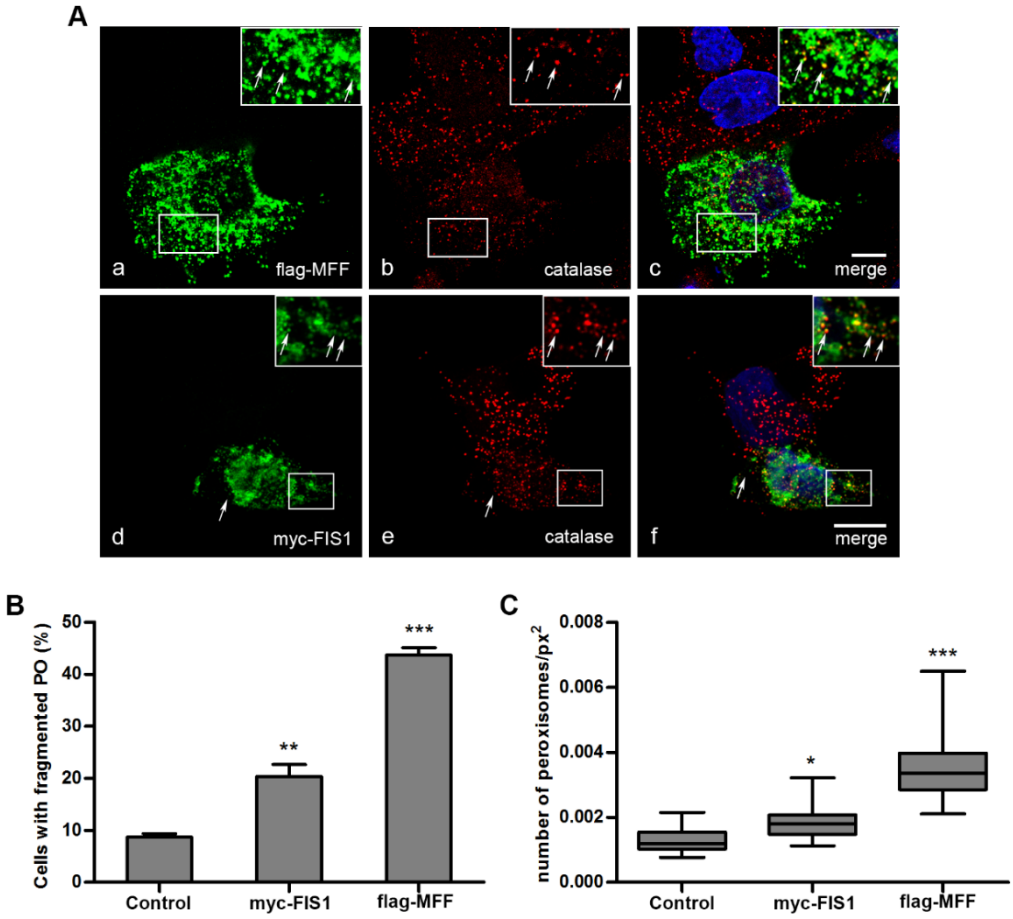


***Figure 5 - Effect of MFF and FIS 1 overexpression on peroxisomal morphology in HepG2 cells.*** (A) Immunofluorescence analyses. (a-c) Overexpression of flag-MFF in HepG2 cells: (a) anti-flag, (b) anti-catalase, (c) merge image of a and b. (d-f) Overexpression of myc-FIS1 in HepG2 cells: (d) anti-myc, (e) anti-catalase, (f) merge image of d and e. Nucleus are shown in blue. Arrows indicate co-localization loci. Bars represent 10 µm. (B) Statistical analysis on peroxisomal morphology upon MFF or FIS1 overexpression in HepG2 cells. (C) Box-plot shows peroxisomal fragmentation upon MFF or FIS1 overexpression in HepG2 cells. Number of peroxisomes in each control and transfected cells was quantified using the Spot Detector plug-in from Icy Bioimage Analysis Software. Data represent means ± SD (*, p< 0.01, **, p< 0.01; ***, p<0,001, compared with control).

Similarly to peroxisomes, also mitochondria fragment upon MFF or FIS1 overexpression [31,38]. To compare the mitochondria morphology upon overexpression of the two proteins, HepG2 cells were transfected with either flag-MFF or myc-FIS1 and subjected to immunolocalization with antibodies

against flag or myc and the mitochondrial protein TOM20. Similarly to the peroxisomal analysis, mitochondrial morphology (examples for the different mitochondrial morphologies are shown in Figure 4) was observed by confocal microscopy and statistical analyses were performed. As shown in Figure 4 f, we considered cells containing "fragmented mitochondria" as those whose mitochondria were significantly smaller and rounder then the ones from control cells (Figure 4 d).

Our results show that FIS1 overexpression caused a higher mitochondrial fragmentation than the overexpression of MFF (Figure 6 a-f and B). In fact, FIS1 overexpression induced fragmentation in about 82% of the cells (with only 10% in the control cells), whereas only about 52% of the cells overexpressing MFF showed fragmented mitochondria (Figure 6B).

Intriguingly, opposite results were obtained when analyzing mitochondrial and peroxisomal morphologies. Our results suggest that (unlike for peroxisomes), FIS1 seems to be the main DLP1-adaptor at the mitochondrial membrane. However, the fission effect mediated by the FIS1/TBC1D15/Rab complex cannot be ruled out.

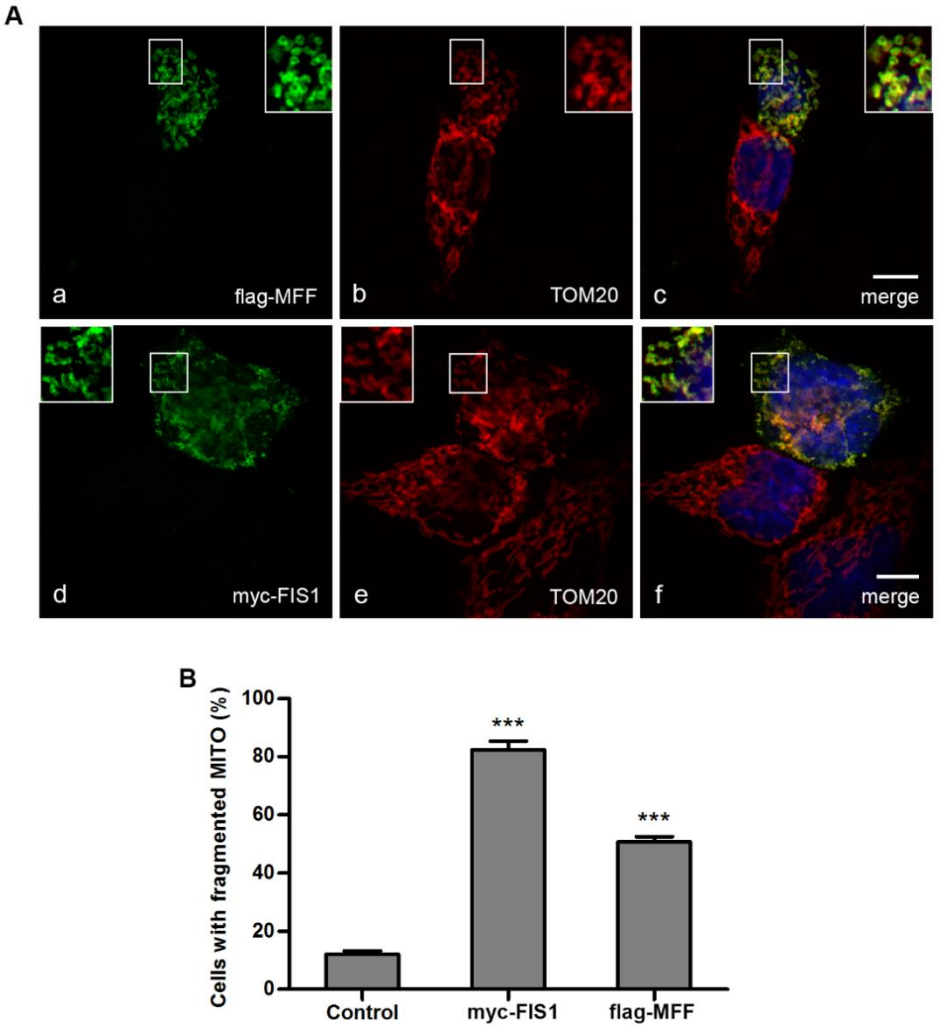


***Figure 6 - Effect of MFF and FIS 1 overexpression on mitochondrial morphology in HepG2 cells.*** (A) Immunofluorescence analyses. (a-c) Overexpression of flag-MFF in HepG2 cells: (a) anti-flag, (b) anti-TOM20, (c) merge image of a and b. (d-f)

Overexpression of myc-FIS1 in HepG2 cells: (d) anti-myc, (e) anti-TOM20, (f) merge image of d and e. Nucleus are shown in blue. Bars represent 10 μm. (B) Statistical analysis on mitochondrial morphology upon MFF or FIS overexpression in HepG2 cells. Data represent means ± SD (***, p<0,001, compared with control).

#### 3.1.3.2 Peroxisomes and mitochondria react differently to MFF and FIS1 absence

With the intriguing results obtained upon MFF and FIS1 overexpression, we deepened our studies with the analysis of the effect of MFF or FIS1 knock-down on peroxisomal morphology, using specific siRNAs. In parallel and for comparison purposes, DLP1 was also silenced, as this is known to cause significant peroxisomal elongation [44]. Upon immunolocalization with antibodies against the silenced proteins and the peroxisomal proteins catalase or PEX14, peroxisome morphology was observed by confocal microscopy and statistical analyses were performed. Transfection of HepG2 cells with an siRNA specific for the knock-down of MFF (siMFF, Figure 7A a-c, and B) resulted in the elongation of peroxisomes to a similar size and at a similar level as upon transfection with a siRNA specific for the knock-down of DLP1 (siDLP1, Figure 7A g-i, D and E). We considered cells containing "elongated peroxisomes" as those whose peroxisomes were hypertubulated and constricted (Figure 4b). 77% of the cells upon siMFF treatment displayed hypertubulated and constricted peroxisomes compared with about 12% in control cells (Figure 7E). As shown in Figure 8B, silencing of MFF did not alter the concentration of either DLP or FIS1 in the cells. On the other hand, cells transfected with a siRNA specific for the knock-down of FIS1 (siFIS1, Figure 7A d-f and C) showed longer peroxisomes than in control cells, but in about 25%, a much lower level than upon MFF or DLP1 silencing (Figure 7A and E). Furthermore, the elongation degree was also lower upon FIS1 silencing as the peroxisomes appear less long than upon MFF or DLP1 silencing (Figure 7A). Silencing of FIS1, also did not interfere with endogenous expression of both MFF and DLP1 proteins (Figure 8C).

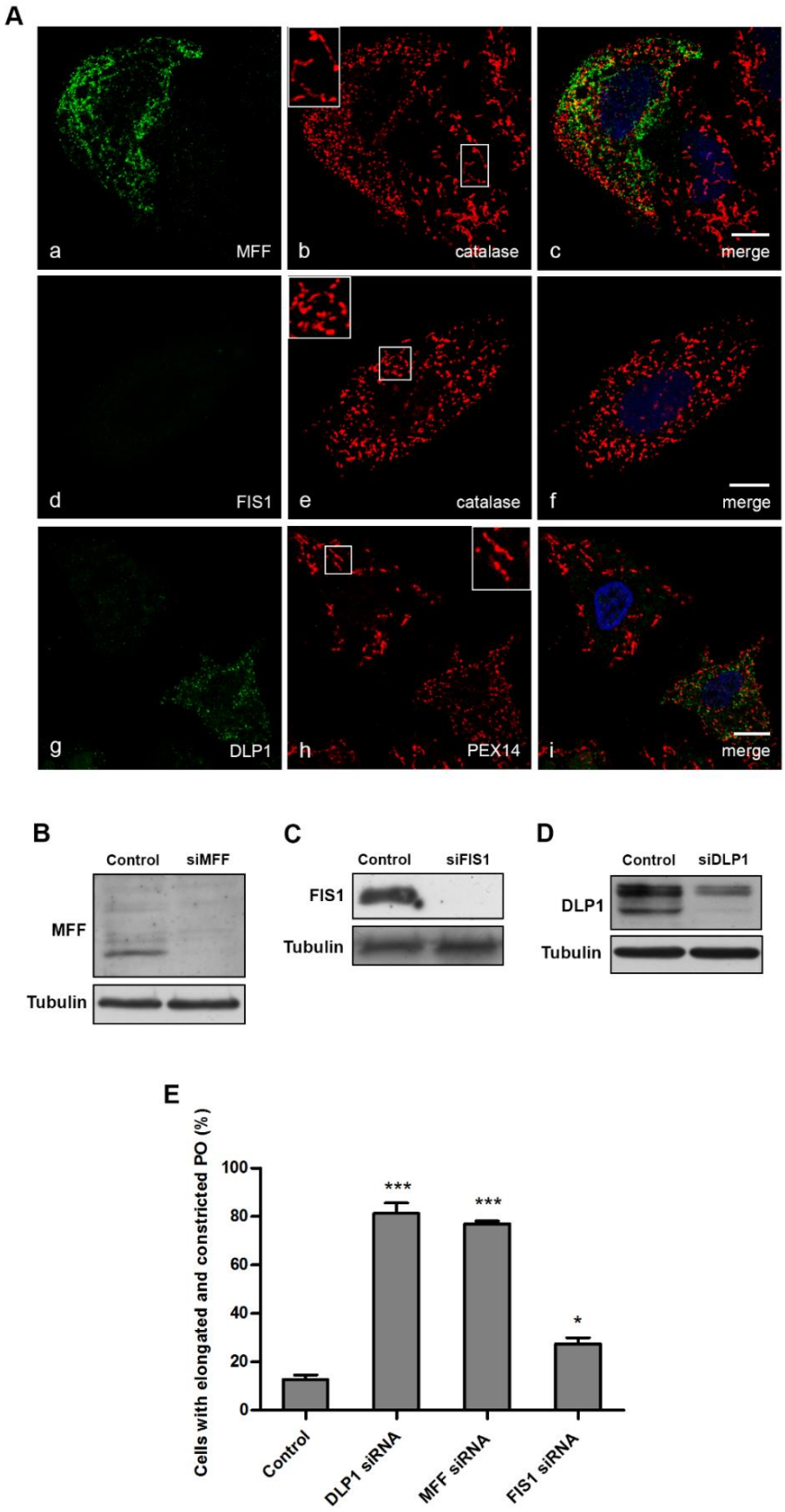


***Figure 7 - Effect of DLP1, MFF and FIS 1 silencing on peroxisomal morphology in HepG2 cells***. (A) Immunofluorescence analyses. (a-c) Silencing of MFF in HepG2 cells: (a) anti-Mff, (b) anti-catalase, (c) merge image of a and b. (d-f) Silencing of FIS1 in HepG2 cells: (d) anti-Fis1, (e) anti-catalase, (f) merge image of d and e. (g-i) Silencing of DLP1 in HepG2 cells: (g) anti-DLP1, (h) anti-Pex14, (i) merge image of g and h. Nucleus are shown in blue. Bars represent 10 µm. (B) Immunoblot showing MFF silencing. (C) Immunoblot showing FIS1 silencing. (D) Immunoblot showing DLP1 silencing (E) Statistical analysis on peroxisomal morphology upon MFF or FIS silencing in HepG2 cells. Data represent means ± SD (*, p< 0,1; ***, p<0,001, compared with control).

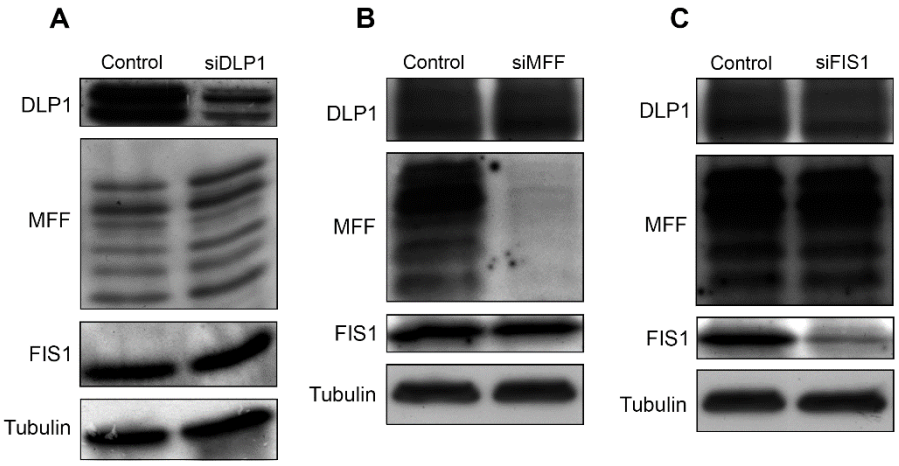


***Figure 8 - Effect of siDLP1, siMFF and siFIS1 on endogenous expression levels of DLP1, MFF and FIS1 proteins, in HepG2 cells.*** Cells were transfected with DLP1 siRNA, MFF siRNA or FIS1 siRNA and assayed for silencing 72 h after transfection. (A) Endogenous MFF and FIS1 expression upon DLP1 silencing (B) Endogenous DLP1 and FIS1 expression upon MFF silencing (C) Endogenous DLP1 and MFF expression upon FIS1 silencing. Equal amounts of protein (50µg/lane) were run on 12,5% acrylamide gels, blotted onto nitrocellulose membranes, and incubated with antibodies to DLP1, MFF and FIS1. Anti-tubulin was used as loading control.

These results complement the previous findings and substantiate the idea that MFF plays a more important role than FIS1 on the peroxisomal division mechanism.

As before, we performed similar experiments to analyze the changes in mitochondrial morphology upon silencing of MFF or FIS1, in comparison with DLP1 silencing. Upon silencing and immunolocalization with antibodies against the silenced proteins and the mitochondrial proteins TOM20 or VDAC1, mitochondrial morphology was observed by confocal microscopy and statistical analyses were performed. As shown in Figure 9 A and B, silencing of MFF, FIS1 and DLP1 resulted in elongated mitochondria, in a similar level for the three conditions.

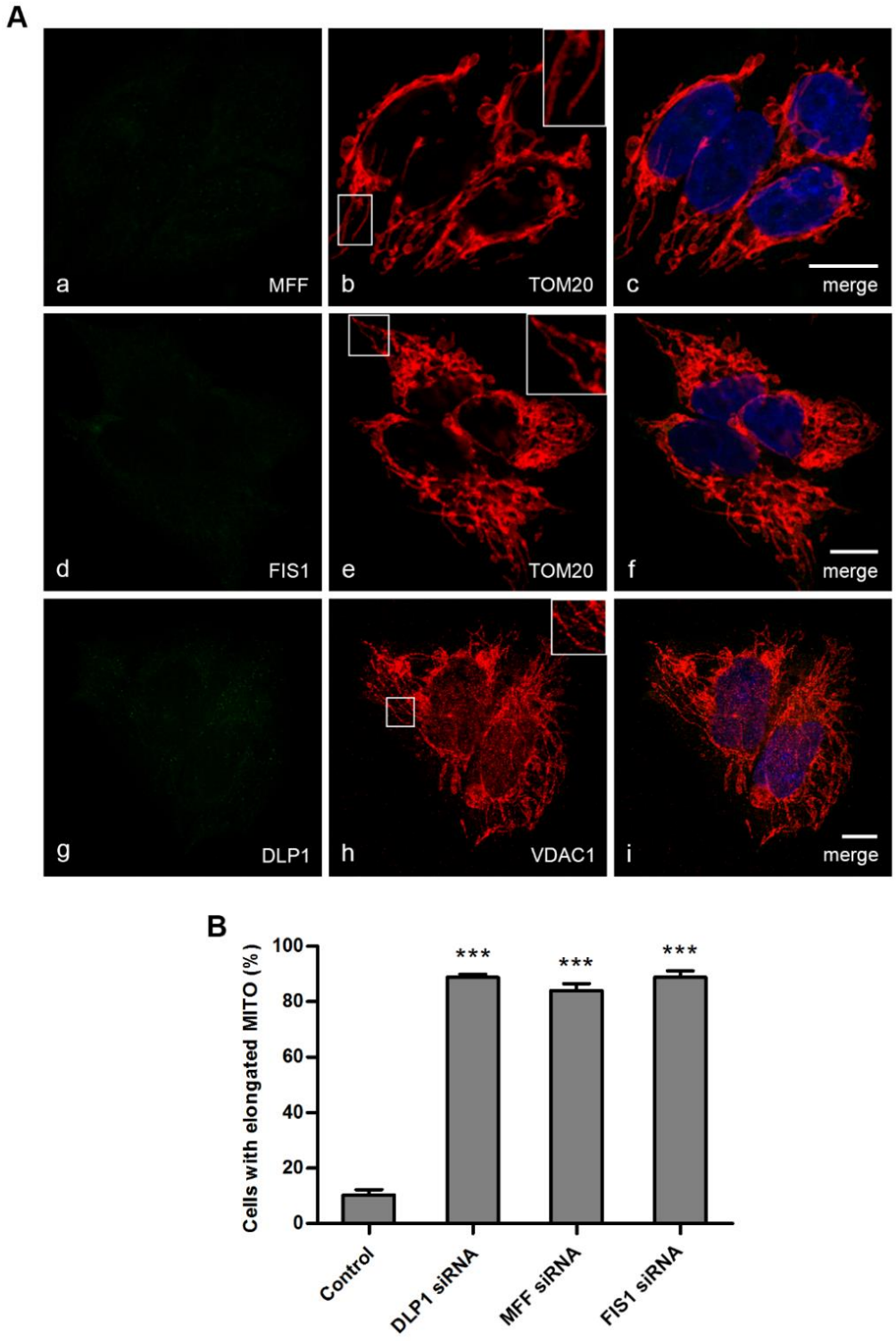


***Figure 9 - Effect of DLP1, MFF and FIS1 silencing on mitochondrial morphology in HepG2 cells.*** (A) Immunofluorescence analyses. (a-c) Silencing of MFF in HepG2 cells: (a) anti-MFF, (b) anti-TOM20, (c) merge image of a and b. (d-f) Silencing of FIS1 in HepG2 cells: (d) anti-FIS1, (e) anti-TOM20, (f) merge image of d and e. (g-i) Silencing of DLP1 in HepG2 cells: (g) anti-DLP1, (h) anti-VDAC1, (i) merge image of g and h. Nucleus are shown in blue. Bars represent 10 µm. (B) Statistical analysis on peroxisomal morphology upon MFF or FIS1 silencing in HepG2 cells. Data represent means ± SD (***, p<0,001, compared with control).

#### 3.1.3.3 MFF and FIS1 assume different localization patterns at the peroxisomal and mitochondrial membranes

To deepen our studies, we have analyzed the specific localization of MFF and FIS1 at the peroxisomal and mitochondrial membranes using cells that present mainly hypertubulated and constricted peroxisomes and mitochondria (Figure 10 a-c). These cells were isolated from a patient with a defect on the fission of both organelles, caused by an heterozygous dominant-negative mutation in the DLP1 gene [138]. The elongated morphology of the organelles allows a more specific and clearer establishment of the proteins' localization pattern. These cells were transfected with flag-MFF or myc-FIS1 constructs and subjected to immunolocalization analyses with antibodies against flag or myc and proteins that localize at peroxisomes (catalase) or mitochondria (TOM20). Upon observation and imaging by confocal microscopy, the images were treated with a deconvolution software (Huygens Deconvolution). As shown in Figure 10 d-f, MFF concentrates in specific spots within the hypertubulated peroxisomal membrane that mostly, but not only, represent the organelle's constriction sites where DLP1-mediated fission occurs. On the other hand, MFF localizes throughout the whole mitochondrial membrane (Figure 10 g-i), not concentrating in any specific loci.

FIS1, on the other hand, assumes a very similar localization pattern at the peroxisomal and mitochondrial membrane: at both organelles, this protein localizes in a spot-like pattern (Figure 10 j-o), although only some of these spots seem to correspond to the constriction sites.

To complete this study, we have also analyzed the localization of endogenous MFF at both organelles. As shown in Figure 11 a-c, results corroborate the overexpression studies, as in peroxisomes endogenous MFF also concentrates mainly, but not only, at the constriction sites. Along with this, in mitochondria, although endogenous MFF localizes in some specific constriction loci, it has also a pronounced localization throughout the membrane (Figure 10 d-e). Unfortunately, we were not able to evaluate endogenous FIS1 localization pattern due to the lack of an antibody anti-FIS1 able to recognize clearly and specifically the endogenous protein. However, we are convinced that results for the endogenous localization would not differ significantly from the overexpression studies, since overexpression of FIS1 already results in a very punctate staining.

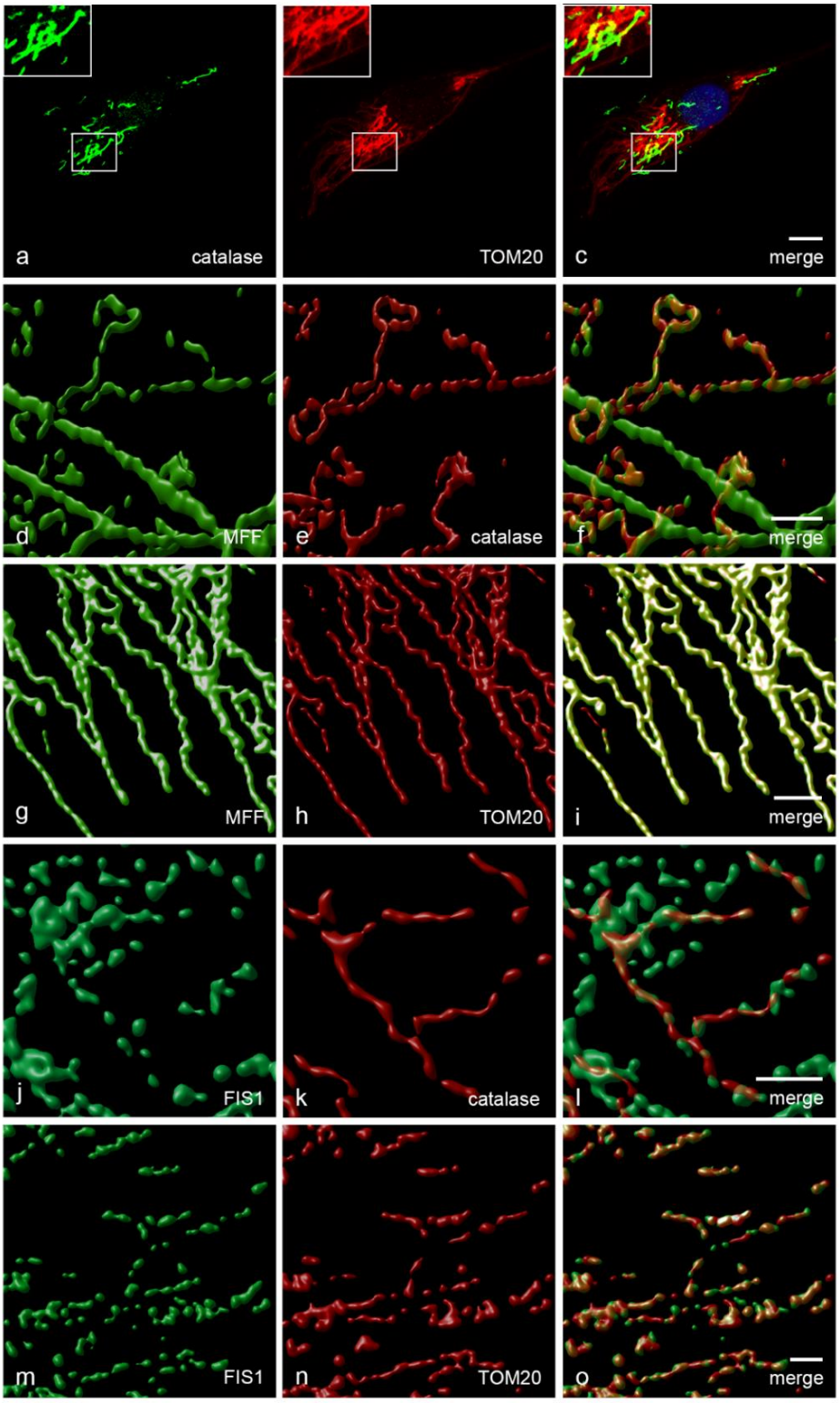


***Figure 10 - Localization pattern of MFF and FIS1 at the peroxisomal and mitochondrial membranes in a DLP1-patient cell line.*** (a-c) Immunofluorescence analysis of peroxisomes and mitochondria in control DLP1 patient cells: (a) anti-catalase, (b) anti-TOM20, (c) merge image of a and b. (d-f) Overexpression of flag-MFF: (d) anti-flag, (e) anti-catalase, (f) merge image of d and e. (g-i) Overexpression of flag-MFF: (g) anti-F, (h) anti-TOM20, (i) merge. (j-l) Overexpression of myc-FIS1: (j) anti-myc, (k) anti-catalase, (l) merge image of j and k. (m-o) Overexpression of myc-FIS1: (m) anti-myc, (n) anti-TOM20, (o) merge image of m and n. The images were treated with a deconvolution software. Nucleus are shown in blue. Bars represent 10 µm in (c) and 2 µm in (f)(i)(l)(o).

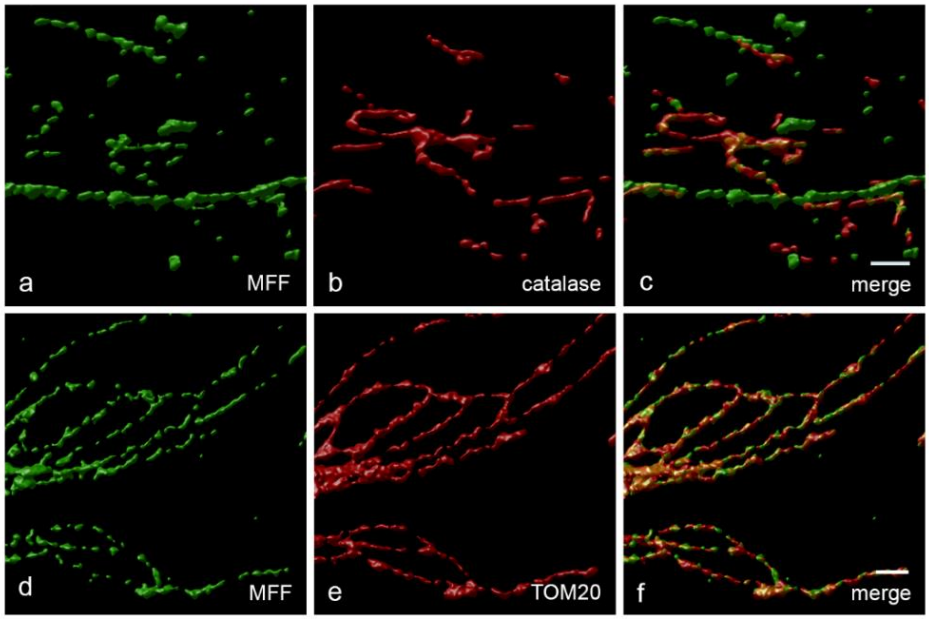


***Figure 11 - Endogenous Localization pattern of MFF at the peroxisomal and mitochondrial membranes in a DLP1-patient cell line***. (a-c) Immunofluorescence analysis showing endogenous localization of MFF at peroxisomal membrane: (a) anti-MFF, (b) anti-catalase, (c) merge image of a and b. (d-f) Endogenous localization of MFF at mitochondrial membrane: (d) anti-MFF, (e) anti-TOM20, (f) merge image of d and e. The images were treated with a deconvolution software. Nucleus are shown in blue. Bars represent 2 µm.

#### 3.1.3.4 MFF overexpression compensates the lack of DLP1 (upon silencing) at the peroxisomal but not at the mitochondrial membranes

Our results indicate that MFF may play a more important role than FIS1 during peroxisomal division. To further extend our studies, we tested the effect of MFF overexpression in cells previously silenced with DLP1. If, indeed, MFF is the main DLP1-adaptor at the peroxisomal membrane, its overexpression could recruit the remaining DLP1 present in the cell upon DLP1 silencing and still be able to, at some degree, fragment the peroxisomes. On the other hand, as MFF seems to have a less prominent role than FIS1 on mitochondria division, this effect may be less significant than in peroxisomes. To perform these experiments, HepG2 cells were transfected with siDLP1 and, after 48h, were again transfected with flag-MFF. Upon immunolocalization with antibodies against flag and a peroxisomal protein (catalase) or a mitochondrial protein (TOM20), the organelles morphology was observed by confocal microscopy and statistical analyses were performed. As shown in Figure 12A a-c and D, the overexpression of MFF caused an almost complete recovery of the peroxisomal phenotype induced by DLP1-silencing: most of the peroxisomes were fragmented upon MFF overexpression. On the other hand, there was almost no difference observed on mitochondrial morphology (Figure 12A d-f and E). These results corroborate our previous

assumptions, constituting one more strong indication that MFF exhibits a key role at peroxisomes division, assuming a more modest role on mitochondria.

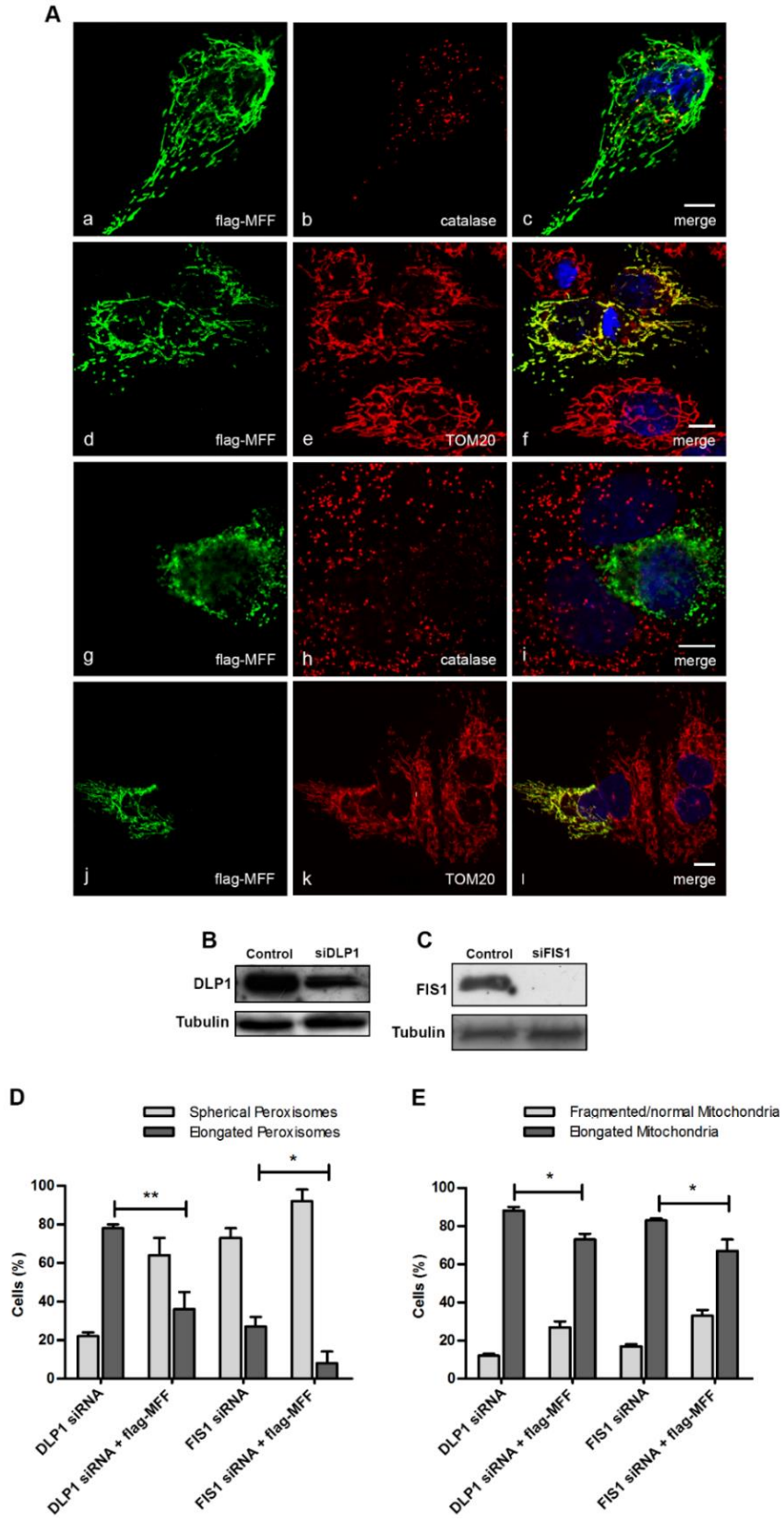


***Figure 12 - Effect of MFF overexpression upon DLP1-silencing or FIS1-silencing on peroxisomal and mitochondrial morphology in HepG2 cells.*** (A) Immunofluorescence analyses. (a-f) Overexpression of MFF in DLP1-silenced HepG2 cells: (a) anti-flag, (b) anti-catalase, (c) merge image of a and b, (d) anti-flag, (e) anti-TOM20, (f) merge image of d and e. (g-l)

Overexpression of MFF in FIS1-silenced HepG2 cells: (g) anti-flag, (h) anti-catalase, (i) merge image of g and h, (j) anti-flag, (k) anti-TOM20, (l) merge image of j and k. Nucleus are shown in blue. Bars represent 10 µm. (B) and (C) Immunoblots confirming DLP1 and FIS1 silencing, respectively. (D) and (E) Statistical analyses on peroxisomal and mitochondrial morphologies upon overexpression of MFF on DLP1-silenced or FIS1-silenced HepG2 cells, respectively. Data represent means ± SD (*, p< 0,1; **, p<0,01).

However, given the fact that the silencing of DLP1 was very efficient throughout our experiments (Figure 12B), this phenotype reversal might reflect not only the recruitment of the few DLP1 molecules still existing in the cells, but, more importantly, suggest the existence of another (yet-unknown) protein that assumes the role of DLP1 and interacts with MFF at the peroxisomal membrane but not at the mitochondrial membrane.

To check whether MFF overexpression would overcome the effect of FIS1-silencing on peroxisomal and mitochondrial morphology, we performed similar experiments and analysis in HepG2 cells where MFF was overexpressed in FIS1 silenced HepG2 cells. As shown in Figure 12 A g-i and D, MFF overexpression causes a strong reduction in the number of cells with elongated peroxisomes induced by FIS1 silencing. As previously demonstrated, the effect of FIS1 silencing on peroxisomes was not nearly as strong as DLP1 or MFF-silencing. However, these results suggest that MFF can almost overcome whatever role FIS1 may have as a DLP1-adaptor at the peroxisomes membranes. On the other hand, and somewhat expected in correlation with our previous results, the results obtained were different when analyzing mitochondria morphology (Figure 12 j-l and E). As previously shown, FIS1-silencing has, by itself, a dramatic effect on mitochondria morphology, causing their elongation in about 90% of the cells. The overexpression of MFF was able to revert this effect but only down to about 75% (Figure 12 E), demonstrating that FIS1 assumes a more prominent role than MFF at mitochondria fission.

#### 3.1.3.5 FIS1 overexpression reverses the mitochondrial and not the peroxisomal elongation induced by DLP1-silencing

Pondering our previous results suggesting a more important role for FIS1 than MFF on mitochondrial division (the opposite for peroxisomes), we wanted to test whether FIS1 could revert the DLP1-silencing-induced mitochondrial and/or peroxisomal phenotype. Upon FIS1 overexpression in siDLP1 HEPG2, immunolocalization with specific antibodies allowed to interestingly observe that once more the opposite results from the ones obtained with MFF overexpression upon DLP1-silencing: on the one hand, overexpressed FIS1 caused almost no recovery of the peroxisomal phenotype induced by DLP1-silencing (Figure 13A a-c and D); on the other hand, there was an almost complete recovery from the mitochondrial phenotype (Figure 13 A d-f and E). These results may reflect a recruitment of the remaining DLP1 to the mitochondrial membrane or an alternative fission mechanism such as the TBC1D15/Rab mediated fission. These

results once again strengthen our hypothesis of a more important role of FIS1 on mitochondria than on peroxisomal division.

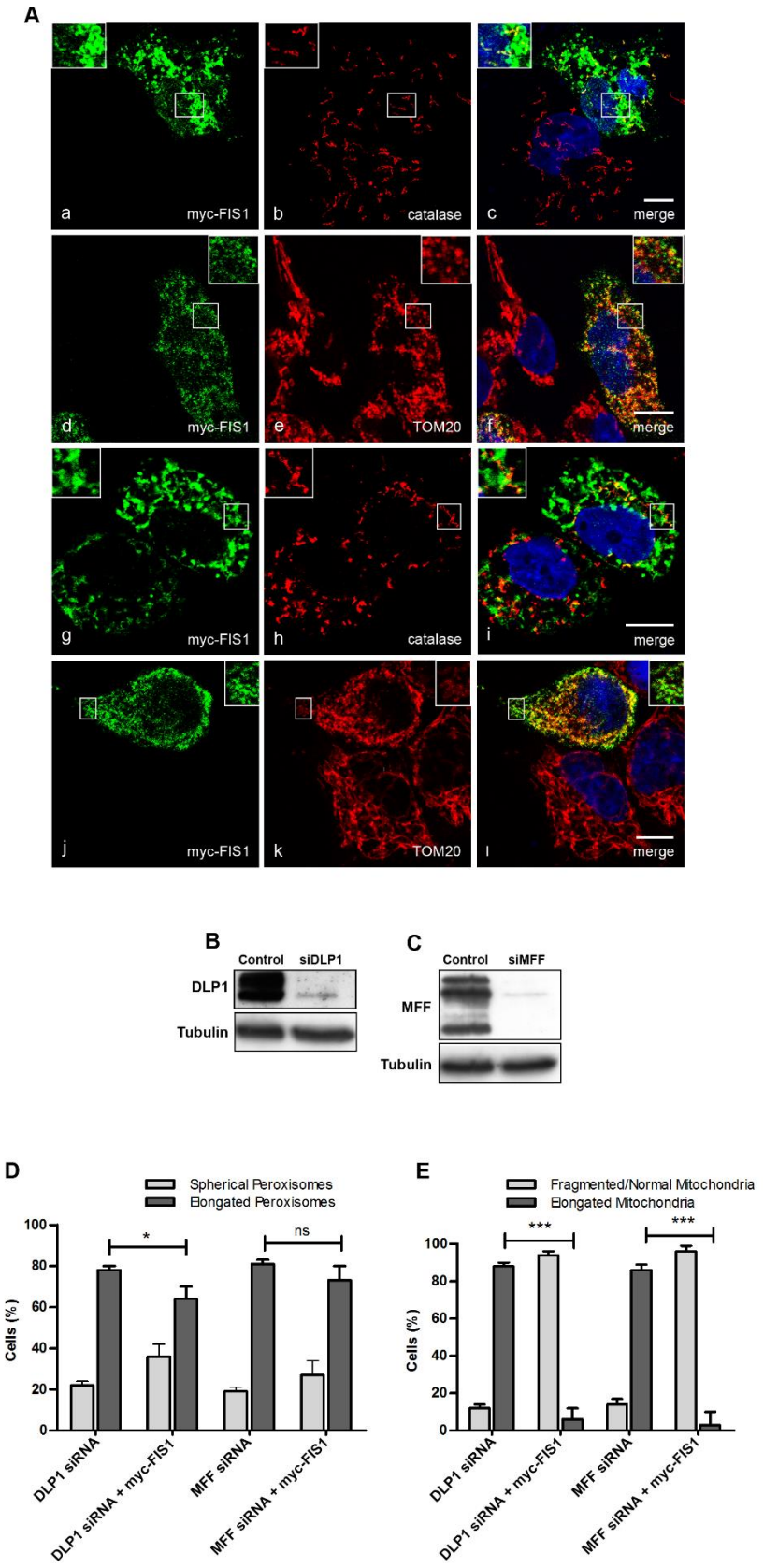


***Figure 13 - Effect of FIS1 overexpression upon DLP1-silencing or MFF-silencing on peroxisomal and mitochondrial morphology in HepG2 cells.*** (A) Immunofluorescence analyses. (a-f) Overexpression of FIS1 in DLP1-silenced HepG2 cells:

(a) anti-myc, (b) anti-catalase, (c) merge image of a and b, (d) anti-myc, (e) anti-TOM20, (f) merge image of d and e. (g-l) Overexpression of FIS1 in MFF-silenced HepG2 cells: (g) anti-myc, (h) anti-catalase, (i) merge image of g and h, (j) anti-myc, (k) anti-TOM20, (l) merge image of j and k. Nucleus are shown in blue. Bars represent 10 µm. (B) and (C) Immunoblots confirming DLP1 and FIS1 silencing, respectively. (D) and (E) Statistical analyses on peroxisomal and mitochondrial morphologies upon overexpression of MFF on DLP1-silenced or FIS1-silenced HepG2 cells, respectively. Data represent means ± SD (ns = non-significant, *, p< 0,1; ***, p<0,001).

We also investigated whether the overexpression of FIS1 could compensate for the lack of MFF (upon silencing) on peroxisomal and mitochondrial morphology. We performed similar experiments where myc-FIS1 was transfected upon 48h of siMFF transfection in HepG2 cells. As shown in Figure 13A g-i and D, FIS1 overexpression did not cause a significant reduction on the number of cells with elongated peroxisomes induced by MFF-silencing. On the other hand, the results obtained were quite different when analyzing mitochondria morphology (Figure 13A j-l and E): there was an almost complete recovery of the phenotype induced by MFF-silencing. These results clearly demonstrate FIS1 can substitute MFF as DLP1-adaptor at the mitochondria membrane.

#### 3.1.3.6 MFF overexpression reverses abnormal peroxisomal but not mitochondrial elongation in DLP1-patient cells

Taking into account the previous results that show that MFF is able to revert the peroxisomal elongation caused by DLP1-silencing, we deepened our studies and wondered whether the same would be observed upon MFF overexpression in DLP1-patient cells. As previously explained, these cells were isolated from a patient with an heterozygous, dominant-negative mutation in the DLP1 gene [138] and present a dramatic defect on peroxisomal and mitochondrial fission, exhibiting mainly hypertubulated organelles (Figure 10 a-c). These cells were transfected with flag-MFF and subjected to immunolocalization with antibodies against flag and a peroxisomal protein (catalase) or a mitochondrial protein (TOM20) and the organelles' morphology was observed by confocal microscopy. Surprisingly, MFF overexpression was able to cause the fragmentation of the elongated peroxisomes but not of the mitochondria (Figure 14 A a-f, B and C). These results strengthen the previous observations on the different importance of MFF in both organelles' fission machinery. Although these patient cells are mostly disabled of a normal DLP1 activity, cells can express both the normal and the mutated protein at different levels in individual cells, which can explain partially the results observed. Nevertheless, these results raise interesting and important questions, corroborating the hypothetic existence of another protein alternative to DLP1 in peroxisomal fission machinery. This and other hypotheses will be further addressed in the Discussion section.

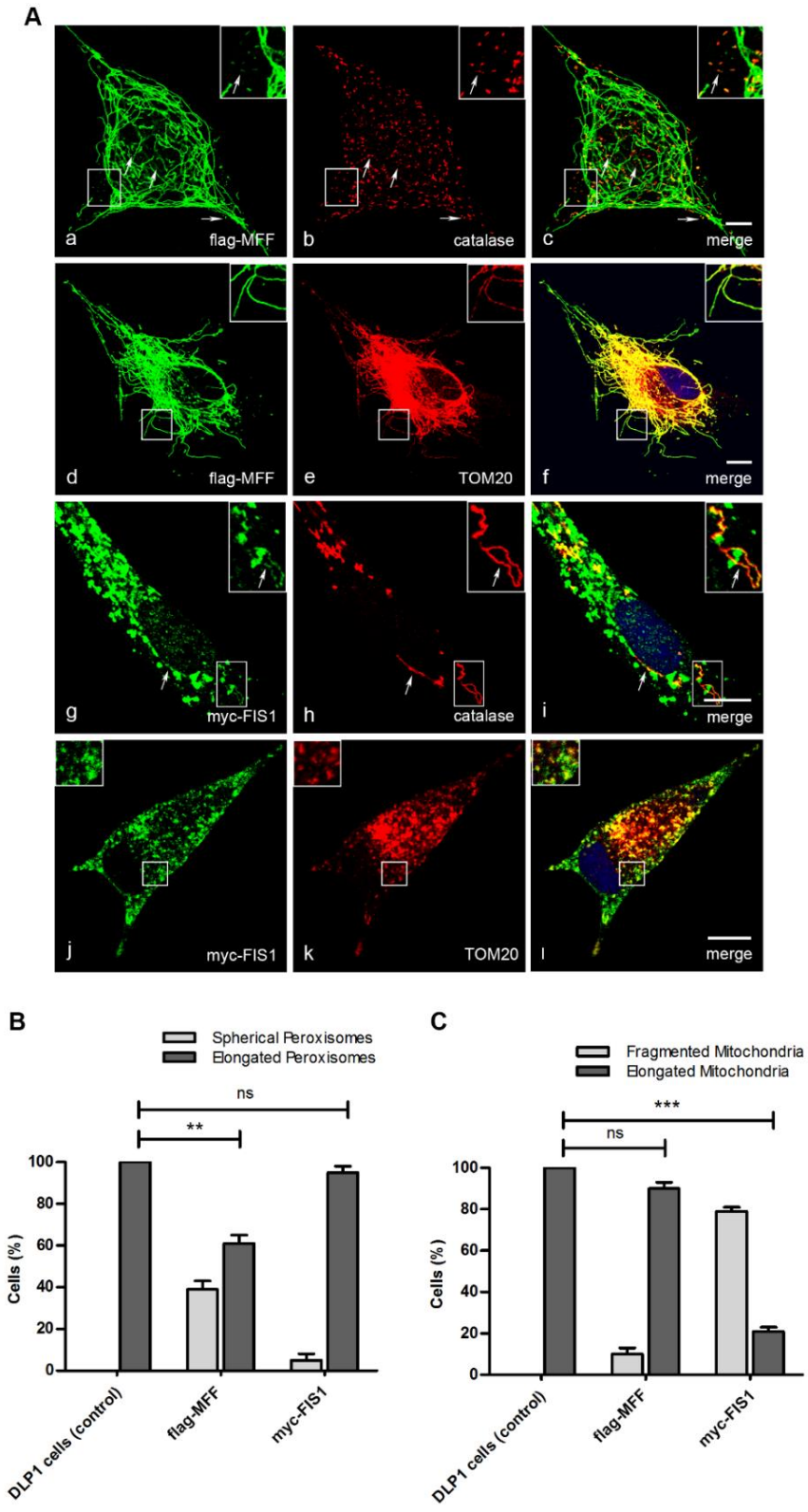


**Figure 14 - Effect of MFF or FIS1 overexpression on peroxisomal and mitochondrial morphology in DLP1-patient cells.** *(A) Immunofluorescence analyses. (a-f) Overexpression of MFF in DLP1-patient cells: (a) anti-flag, (b) anti-catalase, (c) merge image of a and b, (d) anti-flag, (e) anti-TOM20, (f) merge image of d and e. (g-l) Overexpression of FIS1 in DLP1-patient cells: (g) anti-myc, (h) anti-catalase, (i) merge image of g and h, (j) anti-myc, (k) anti-TOM20, (l) merge image of j and k. Nucleus are shown in blue. Arrows indicate co-localization loci. Bars represent 10 µm. (B) Statistical analysis on peroxisomal morphology upon overexpression of MFF or FIS1 on DLP1-patient cells. (C) Statistical analyses on mitochondrial morphology upon overexpression of MFF or FIS1 on DLP1-patient cells. Data represent means ± SD (ns = non-significant, **, p< 0,01, ***, p< 0,001).*

#### 3.1.3.7 FIS1 overexpression reverses the mitochondrial and not the peroxisomal elongation in DLP-patient cells

In order to analyze the effect of overexpressed FIS1 on the hypertubulated peroxisomes and mitochondria from the DLP1-patient cells, these cells were transfected with myc-Fis1 and subjected to immunolocalization with antibodies against myc and a peroxisomal protein (PEX14) or a mitochondrial protein (TOM20). The organelles' morphology was observed by confocal microscopy and statistical analyses were performed. Interestingly, FIS1 overexpression was able to cause the fragmentation of the elongated mitochondria but not of the peroxisomes (Figure 14 A g-l, B and C). These results corroborate previous observations and allows to strongly infer a more prominent role in mitochondria rather than in peroxisomes machineries. However, it is important to stress that the elongated morphology reversion may be explained by some residual activity of normal DLP1 molecules but also to a fragmentation induced via TBC1D15/Rab pathway.

#### 3.1.3.8 TBC1D15 has no influence on peroxisomal morphology

Although, up to now, peroxisomes and mitochondria seemed to assume a very similar division mechanism, our results show that this similarity may rely only on some common components of the division machinery and not on its regulation or the mechanism by which they act. Besides FIS1, MFF and DLP1, other proteins have been shown to interfere with the mitochondrial division machinery. While searching for FIS1-binding proteins in mammalian cells, Onoue and colleagues [123] have identified TBC1D15 and concluded that this protein, together with FIS1, is involved in mitochondrial morphology regulation in a DLP1-independent manner. FIS1 may lead to mitochondria fission through the activation of small Rab GTPases such as Rab7 via TBC1D15. [124–127] In order to check whether this protein is also localized at peroxisomes and whether it has any effect on their morphology regulation, HA-TBC1D15 was transfected into DLP1-patient cells that were further subject to immunolocalization with antibodies against HA and the peroxisomal protein Pex14. These cells were chosen due to their elongated mitochondria and peroxisomes, facilitating the visualization of colocalization between the two proteins. As shown in Figure 15A a-c, TBC1D15 does not co-localize with the peroxisomes. As expected, upon immunolocalization with antibodies against HA and the mitochondrial protein VDAC1 (Figure 15A d-f), it is possible to observe some degree of co-localization between TBC1D15 and the mitochondria, although the majority of the protein is localized at the cytoplasm.

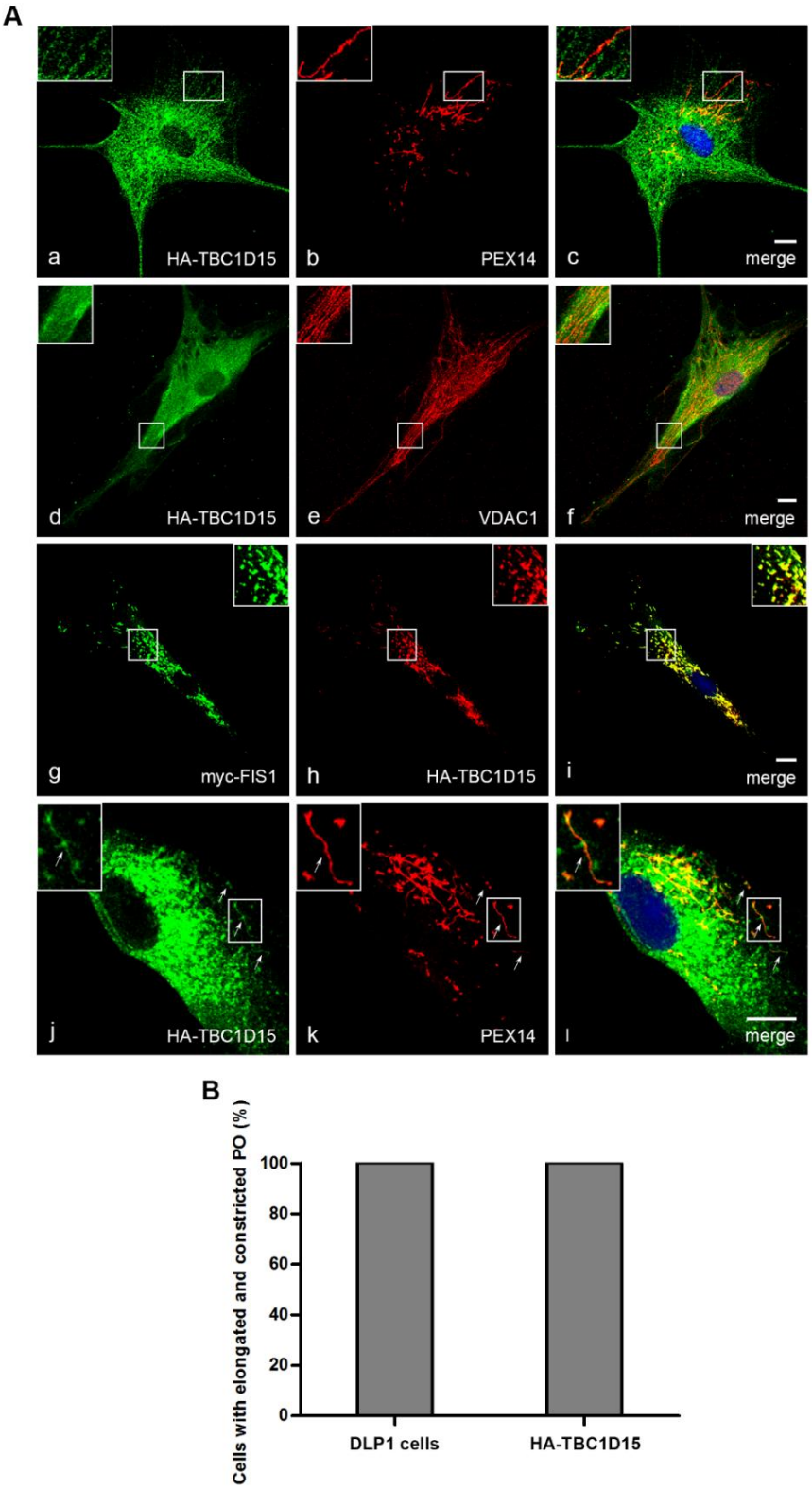


***Figure 15 - Effect of the overexpression of HA-TBC1D15 and co-expression with FIS1 on peroxisomal and mitochondrial morphology in DLP1-patient cells.*** (A) Immunofluorescence analyses. (a-c) Overexpression of HA-TBC1D15 in DLP1-patient cells: (a) anti-HA, (b) anti-PEX14, (c) merge image of a and b. (d-f) Overexpression of HA-TBC1D15 in DLP1-patient cells : (d) anti-HA, (e) anti-VDAC1, (f) merge image of d and e. (g-i) Overexpression of HA-TBC1D15 and FIS1 in DLP1-patient cells : (g) anti-myc, (h) anti-HA, (i) merge image of g and h. (j-l) Overexpression of HA-TBC1D15 and FIS1 in DLP1-patient cells : (j) anti-HA, (k) anti-PEX14, (l) merge image of j and k. Nucleus are shown in blue. Arrows indicate co-localization loci. Bars represent 10 µm. (B) Statistical analysis on peroxisomal morphology upon overexpression of HA-TBC1D15 on DLP1-patient cells.

We have also performed statistical analyses of the effect of HA-TBC1D15 overexpression on the peroxisome morphology in these cells. As shown in Figure 15B, there is no change in the peroxisomal phenotype upon TBC1D15 overexpression. These results indicate that, at least in the absence of DLP1 (from which TBC1D15 was shown to act independently) this protein has no influence on the peroxisomal morphology regulation. To further substantiate these results and check whether, when forced to localize at the peroxisomes, this protein influences their morphology, we transfected DLP1-patient cells with both myc-Fis1 and HA-TBC1D15. These cells were subject to immunolocalization analyses with antibodies against myc, HA and the peroxisomal protein Pex14. As shown in Figure 15A g-i, TBC1D15 and FIS1 almost completely colocalize, mainly at fragmented mitochondria. Due to the overload of FIS1, TBC1D15 is recruited to the hypertubulated peroxisomes (Figure 15 A j-l, FIS1 is not shown but its presence in the cell is proven by the localization of TBC1D15 at peroxisomes) but causes no change on the organelle's morphology.

#### 3.1.3.9 The mitochondrial DLP1 adaptor proteins MiD49/51 are not involved in peroxisome fission

In mitochondria, two other adaptor proteins alternatively to MFF and FIS1 have been proven to regulate DLP1 activity. Whereas MFF switch on the DLP1 GTPase activity, MiD49/51 blocks DLP1 fission activity. Previous studies ruled out the localization of these proteins of peroxisomes [122] conferring specificity to mitochondria. We decided to confirm these results (Figure 16) in our setup. Indeed, upon MiD49/51 exogenous expression in HepG2 cells, a co-localization with the peroxisome's marker was not observed (Figure 16), corroborating Palmer´s and colleague's observations. Additionally, in accordance with the previous observations reported by [122] although MiD49/51 seems to not influence directly peroxisome morphology, their overexpression leads to the elongation of peroxisomes. This indirect elongation may be explained by a massive to recruitment of nonfunctional DLP1 to mitochondria, which induces a similar phenotype to that observed following Drp1 knock-out or knockdown (Figure 16 c and f) [122].

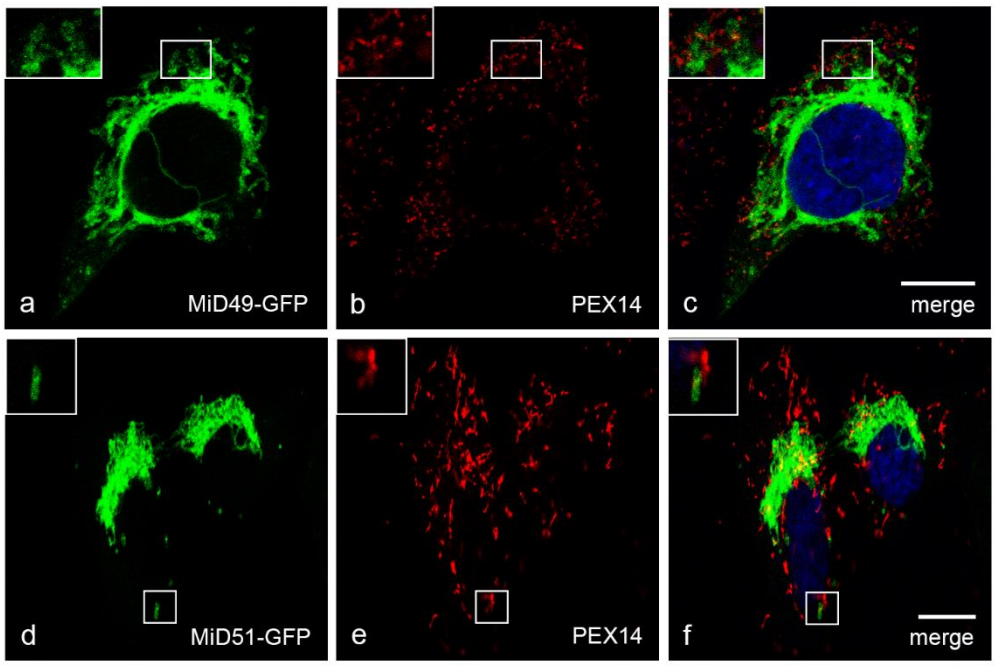


***Figure 16 - Co-localization analysis of MiD49/51 with peroxisomes***.(a-c) Overexpression of Mid49-GFP in HepG2 cells: (a) GFP, (b) anti-PEX14, (c) merge image of a and b. (d-f) Overexpression of MiD51-GFP in HepG2 cells: (d) GFP, (e) anti-PEX14, (f) merge image of d and e. Nucleus are shown in blue. Bars represent 10 µm.

### 3.1.4 Discussion

FIS1 and MFF have been shown to play an important role in the recruitment of DLP1 to the mitochondrial and peroxisomal membrane prior to final scission [31,32,36–38,219,220]. However, the results concerning the mechanisms involved and the specific roles of each of these proteins in each of the organelles are still controversial. In this study we have performed a detailed comparative analysis on the relative significances of these two proteins for DLP1 recruitment and peroxisome and mitochondria morphology control in two cell lines.

Our results clearly show that MFF has a more prominent role than FIS1 on the peroxisomal division mechanism. Overexpression of MFF resulted in a higher peroxisome fragmentation (with smaller and more peroxisomes) than the one observed upon FIS1 overexpression. Furthermore, the lack of MFF (upon silencing) had a much stronger effect on peroxisome morphology than FIS1-silencing, resulting in a higher number of cells with elongated peroxisomes. Moreover, the specific MFF localization pattern at the peroxisomal membrane upon overexpression shows that a large part of this protein is present at the constriction sites. Although also presenting a spot-like localization pattern, FIS1 is rarely present at the peroxisomal constriction sites. Altogether, these data strongly indicate that MFF is the main responsible for DLP1-anchoring at the peroxisomal membrane. On the other hand, our studies show opposite results for the role of these proteins in mitochondria

morphology: FIS1 is more relevant than MFF for mitochondrial division. Furthermore, while MFF is present throughout the mitochondrial membrane, FIS1 localizes in spots, some of which co-localize with the constriction sites.

We have also demonstrated that, upon overexpressing MFF in cells with elongated peroxisomes resulting from a previous DLP1-silencing, the peroxisomes were still able to fragment. On the other hand, there was almost no mitochondrial fragmentation observed. This may indicate that the overexpressed peroxisomal MFF has recruited most of the remaining DLP1, demonstrating once again that this protein assumes a more important role at peroxisomes than at mitochondria. The results obtained upon FIS1 overexpression following DLP1-silencing showed the opposite effect: almost no recovery from the peroxisomal phenotype induced by DLP1-silencing and an almost complete recovery of the mitochondrial phenotype. These results strengthen our hypothesis of a more important role of FIS1 on mitochondria than on peroxisomal division mechanisms.

Our conclusions were further strengthened with the finding that MFF is able to overcome the role of FIS1 in the fission of peroxisomes membranes but not at the mitochondrial membranes. Accordingly, FIS1 can overcome the loss of MFF on mitochondrial but not on peroxisomal morphology control.

Thus, to confirm the previous results we extended our studies by overexpressing MFF or FIS1 in cells that were isolated from a patient with an heterozygous, dominant-negative mutation in the DLP1 gene [138]. These cells present a dramatic defect on peroxisomal and mitochondrial fission, exhibiting mainly hypertubulated and constricted organelles. We observed that, even in these cells, the presence of overexpressed MFF was enough to induce a dramatic peroxisomal fragmentation, while it had almost no effect on mitochondria morphology. Overexpressed FIS1 was able to induce mitochondrial fragmentation and, once again, not able to revert the peroxisomal phenotype. Besides corroborating very clearly our previous theory, these results point out to a possible existence of another fission protein, which acts independently of DLP1. In fact, in mitochondria the Rab GTPase activating protein (Rab-GAP) TBC1D15, as proven to, in coordination with FIS1, impact in mitochondria fission independently of DLP1 via small Rab GTPases activity such as Rab7 [123–125,127,221]. The FIS1/TBC1D15 pathway has been shown to be involved not only at mitophagosomes biogenesis but also is a shared fission mechanism with lysosomes [124,125,127,235]. In peroxisomes, not such a pathway has been described. In fact, when TBC1D15 is forced to localize at peroxisomes due to a FIS1 overload, no influence on peroxisome morphology was observed. Thus, other unknown and alternative mechanism may play a role in peroxisomal fission.

It is, however, important to notice that these DLP1-patient cells have an heterozygous mutation, and different amounts of normal and mutated DLP1 are produced in every cell. One cannot, thus, exclude the possibility that the remaining functional DLP1 may be recruited by the overexpressed

MFF or FIS1. However, this is unlikely to strongly impact the results since the level of peroxisomal and mitochondrial elongation (hypertubulation) in these cells is much higher than the one obtained in cells where DLP1 had been very efficiently silenced. Nevertheless, a clearer result would be obtained by performing similar experiments in DLP1 KO cells, which were not available at the time these experiments were performed. The existence of another protein or a DLP1-independent mechanism that would be responsible for the mitochondrial cleavage had been previously suggested to occur upon infection by Listeria monocytogenes [236,237]. However, up to now, no data has ever been shown that suggests that this possibility would also be valid for peroxisomes. Further experiments would have to be performed in order to discover the regulation and specific machinery involved in this new unraveled mechanism.

The higher relevance of MFF for peroxisome morphology control had already been suggested [15,37,39] and our results clearly confirm this theory. Although the roles of MFF and FIS1 on mitochondrial fission have been the subject of more controversial studies, our results seem to corroborate with the earlier reports that also indicate a more prominent role of FIS1 in this mechanism at least in the studied cell lines [31,32,219,220]. We cannot, however, exclude the possibility that a part of the effects we observed may be due to other parallel mechanisms that may be induced by the overexpression or silencing of FIS1 or MFF. FIS1 has been implicated in several fission-dependent processes, such as apoptosis and autophagy [223]. It has been also suggested that during stress-induced mitochondrial fission, DLP1 is recruited by MFF at mitochondrial membranes and afterwards delivered into an ER-mitochondria complex which includes FIS1, leading to downstream degradation processes [224]. Nevertheless, if MFF would be the main DLP1-anchor at the mitochondrial membranes, we would not expect such a strong complementation of the lack of MFF upon silencing by FIS1 overexpression, as show in this study. Furthermore, we would expect stronger effects upon overexpression/silencing of MFF on mitochondrial morphology, similarly as what was observed for peroxisomes.

Other proteins such as MiD49, MiD51 [118,121,216] which play a role in the regulation of DLP1-mediated fission were not found to localize at peroxisomes [122].

Our results demonstrate that, although peroxisomes and mitochondria share the main components of their fission machinery, they adopt distinct mechanisms of action in each organelle.

### 3.1.5 Materials and Methods

**Antibodies and cDNAs**

Rabbit polyclonal antibodies directed to PEX14 (a kind gift from D. Crane, Griffith University, Brisbane, Australia) was used for morphological studies. Rabbit anti-MFF (kindly provided by A. van der Bliek, University of California, Los Angeles) [37] and anti-hFIS1 (Alexis Biochemicals, Grunberg, Germany) polyclonal antibodies were used for immunofluorescence and immunoblotting. Rabbit monoclonal antibodies directed to myc-tag (71D10) (Cell Signaling Technology, Beverly, MA, USA) and Flag epitope (Sigma-Aldrich, St. Louis, USA); mouse monoclonal antibodies directed to catalase, TOM20 and myc epitope (9E10) (Santa Cruz Biotechnology, USA) were used for immunofluorescence. Anti-α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) monoclonal mouse antibody was used for immunoblotting as loading control. Species-specific anti-IgG antibodies conjugated to HRP (BioRad, Hercules, California, USA), IRDye 800CW and IRDye 680RD secondary antibodies (LI-COR Biotechonology, Cambridge, UK) or to the fluorophores TRITC (Jackson Immunoresearch, West Grove, Pennsylvania, USA) and Alexa 488 (Invitrogen, Waltham, Massachusetts, USA) were used.

The cDNAs flag-MFF (kindly provided by H. Otera Kyushu University, Japan) [38] myc-hFIS1 [3135], HA-TBC1D15 (a gift from Naotada Ishihara, Rikkyo University, Tokyo, Japan) [123] and MiD49-GFP and MiD51-GFP (gently provided by Michael T. Ryan La Trobe University, Melbourne, Victoria Australia) [118] were used for mammalian expression.

**Cell culture, transfection, and RNA interference experiments**

HepG2 (obtained from American Type Culture Collection, HB-8065) and DLP1-patient cell lines (gently provided by H. Waterham, Department of pediatrics, Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam)[138] were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (all from PAA Laboratories GmbH, Germany) at 37°C in a humidified atmosphere of 5% CO2. For morphological studies HepG2 cells were transfected with DNA constructs by incubation with TurboFect (Thermo Scientific) according to the manufacturer's instructions. Cells were fixed from 24 to 48 h after transfection. DLP1-patient cells were microporated with DNA encoding for flag-MFF of myc-hFIS1 and HA-TBC1D15 using the Neon® Transfection System (Invitrogen, Carlsbad, CA)

(1700V, Width:20 1 pulse), according to the manufacturer's instructions. Cells were assayed for organelle morphology 24 h after transfection.

To knock down the expression of MFF, FIS1 and DLP1 by RNA interference, 21-nucleotide small interfering RNA (siRNA) duplexes were transfected in HepG2 cells by incubation with Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions. Control cells were treated with transfection mix without siRNAs complexes. In co-transfection experiments both silenced and control cells were transfected with DNA encoding for myc-hFIS1 or flag-MFF by TurboFect 48h after transfection with siRNAs and assayed for silencing and organelle morphology 72h after seeding. siRNA oligonucleotides were obtained as pre-designed siRNAs from Ambion (Austin, TX) as follows: MFF (sense strand: 5-CGCUGACCUGGAACAAGGAdTdT-3 for exon 2) [37]; DLP1 (sense strand: 5´- UCCGUGAUGAGUAUGCUUUdTdT - 3´ [25]. To knock down the expression of hFIS1 (accession no. AF151893) by siRNA (sense strand, 5'-CGAGCUGGUGUCUGUGGAGdTdT-3') (Dharma- con, Lafayette, CO) was used.

**Immunofluorescence and microscopy**

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4, permeabilized with 0.2% Triton X-100 or 2,5 µg/ml digitonin, for 10 min, blocked with 1% BSA solution, for 10 min and incubated with the indicated primary and secondary antibodies, for 1 h. Before mounting the coverslips on the glass slides, cells were incubated with Hoechst (1:2000) for 3 min to stain the nucleus. Transfected cells were processed for immunofluorescence 24-72h after transfection. Fixed samples were examined using an Olympus IX-81 inverted microscope (Olympus Optical Co. GmbH, Hamburg, Germany) equipped with the appropriate filter combinations and a 100x objective (Plan-Neofluar, 100x/1.35 oil objective). Confocal images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) and a Leica SP5 confocal microscope ( (Leica Microsystems, Wetzlar, Germany ) using a Plan- Apochromat 63× and 100×/1.4 NA oil objectives, , a 561 nm DPSS laser and the argon laser line 488 nm (BP 505-550 and 595-750 nm filters). Images were processed using LSM 510 software (Carl Zeiss MicroImaging, Inc.) and Leica Application Suite Advanced Fluorescence software (Leica Microsystems). Digital images were optimized for contrast and brightness using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

To generate high resolution images of the MFF and FIS1 localization at the peroxisomal membrane, deconvolution microscopy was performed. Fixed cells were examined by confocal microscopy. Using the 488 and 543 nm laser lines, z-stacks of transfected cells were generated (8x zoom) using the optimal number of slices suggested by the program (Leica Confocal Software). Oversaturation of signals was avoided by adjusting of respective photomultipliers. Image deconvolution was

performed using Huygens Professional Software (Scientific Volume Imaging, Hilversum, The Netherlands). Using the 2D and 3D images generated by the program the precise MFF and FIS1 localization at the peroxisomal membrane was assessed.

**Gel Electrophoresis and Immunoblotting**

Cells were lysed with specific lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100 and a protease-inhibitor mix). To improve protein extraction, samples were passed 20 times through a 26-gauge syringe needle and then incubated on a rotary mixer at for 30 min at 4$^{o}$C. After clearing by centrifugation (17000 x g, 15 min), protein concentrations were determined using the Bradford assay (BioRad, Hercules, California, USA). Protein samples were separated by SDS-PAGE on 10% or 12.5% polyacrylamide gels, transferred to nitrocellulose (PROTAN®, Whatman®, Dassel, Germany) using a semidry apparatus (BioRad, Hercules, CA, USA), and analyzed by immunoblotting. Immunoblots were processed using specific primary antibodies, HRP-conjugated secondary antibodies and enhanced chemiluminescence reagents (GE Healthcare, Waukesha, WI, USA).

**Quantification and statistical analysis**

For the evaluation of peroxisome and mitochondria morphology, six hundred cells from three independent experiments were counted for each condition, considering the size/shape and number of their peroxisomes or mitochondria. For these experiments we considered cells containing “fragmented peroxisomes” or “fragmented mitochondria” as those whose organelles were significantly smaller and rounder than the ones from the control cells. The increase in peroxisome number was also taken as a prerequisite for peroxisome fragmentation. On the other hand, considered cells containing “elongated peroxisomes” and “elongated mitochondria” as those whose organelles were tubular-shaped and significantly longer when compared to the control cells. Statistical analysis was performed in Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, California, USA). Data are presented as mean ± standard deviation (SD). Differences among groups were analyzed by one-way ANOVA, followed by Bonferroni’s multiple comparison test; comparisons between two groups were made by Student’s t test. P values of ≤0.05 were considered as significant, ***P<0.001, **P<0.01, and *P<0.05.

Boxplots for quantitative evaluation of peroxisome fragmentation represent ≥30 control and transfected cells from three independent experiments. In each cell the number of peroxisomes was

quantified using the Spot Detector plug-in from Icy Bioimage Analysis Software created by the Quantitative Image Analysis Unit at Institute Pasteur (Paris, France).

## 3.2 THE ROLE OF PEROXISOMAL FISSION IN THE ANTIVIRAL IMMUNE RESPONSE AGAINST HCMV

**Part of the results presented in this section are included in a manuscript which will soon be submitted for publication as:**

Ana Rita Ferreira[1,2]*, **Ana Gouveia[1]***, Ana Cristina Magalhães[1], Isabel Valença[1], Mariana Marques[1], Jonathan C. Kagan[2] and Daniela Ribeiro[1], "Human cytomegalovirus' vMIA modulates peroxisome morphology and antiviral defense via MAVS and MFF"

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### 3.2.1 Abstract

Peroxisomes and mitochondria are ubiquitous subcellular organelles which, despite their global differences in morphology and physiology, share different components of key machineries and co-orchestrate crucial cellular functions such as the antiviral immune response. Upon viral infection, these organelles mediate complementary responses: while mitochondria trigger a more delayed and long-lasting response, peroxisomes are in the first battlefield and lead to a more rapid but short-term retaliation. The human cytomegalovirus (HCMV) is one of the most disseminated viruses among the community, remaining latent in most individuals, but resulting in serious disease in more vulnerable patients and fetuses. This virus has developed effective strategies to evade the cellular antiviral mechanisms and to potentiate its slow replication cycle resulting in a high infectiousness. One of these strategies involves the viral protein vMIA which targets mitochondria impairing BAX function and inhibiting apoptosis. In addition to its antiapoptotic function, vMIA also impacts on the modulation of mitochondrial dynamics and leads to the mitochondrial network's disruption. It has been further shown that vMIA-mediated mitochondria fission is crucial to impair mitochondria mediated immunity thought the disruption of the MAVS pathway. vMIA also localizes at peroxisomes and induces the inhibition of the antiviral signaling originating from this organelle. However, peroxisomal fission arising from vMIA expression has been shown not to be essential to the inhibition of the peroxisome-dependent antiviral response.

To better understand the mechanism by which vMIA inhibits the peroxisomal MAVS pathway, in this study we explore the function of the key fission-related proteins shared by peroxisomes and mitochondria - DLP1, MFF and FIS1 – on the action of vMIA towards the immune response. We demonstrate that, although peroxisomal fragmentation seems to not directly impact at the antiviral peroxisomal pathway, vMIA interacts with MFF at the peroxisomal membrane and this protein is shown to be essential for the inhibition of the antiviral immune response. Furthermore, in line with the chapter 3.1 we addressed the role of peroxisomes´ and mitochondria´s morphologies in the vMIA-dependent inhibition of the antiviral immune response. Surprisingly, we have also observed that, in contrast to mitochondria, peroxisome morphology does not impact the peroxisome dependent antiviral response.

### 3.2.2 Introduction

The Human cytomegalovirus (HCMV) is highly disseminated among humans prevailing lifelong latent in myeloid cells of the bone marrow in most adult individuals. Although the infection caused by this enveloped dsDNA virus belonging to the *Herpesviridae* family is generally asymptomatic for most of individuals, it may be particularly serious in pregnant women as it may cause significant congenital defects and, in immunosuppressed individuals, such as AIDS or transplant recipients, this virus can cause serious disease conditions [183,185,186,238]. Due to it slow replication cycle, the human HCMV has evolved strong mechanisms to escape from the cellular antiviral response. This virus encodes the viral mitochondrial inhibitor of apoptosis (vMIA) which suppresses programmed cell death of infected cells by blocking apoptotic signaling pathways and therefore plays a crucial role in viral replication [239,240]. vMIA localizes at the mitochondrial outer membrane and has been proven to act upstream the mitochondrial permeabilization by inhibiting the pro-apoptotic protein BAX [241,242].

Upon viral infection, the intracellular retinoic acid-inducible gene I-like receptors (RLRs) such as the retinoic acid inducible gene-I (RIG-I) and the melanoma differentiation-associated gene-5 (MDA-5), sense the viral genome and, after a conformational change, interact with MAVS at the peroxisomal and mitochondrial membranes through their CARD domain [162]. In turn, MAVS activates two cytosolic protein kinase complexes, TBK1 and IKK, leading to the production of type I interferons and pro-inflammatory cytokines [204]. Mitochondrial morphology has been reported to be crucial to the MAVS-mediated antiviral response. Studies have shown that mitochondrial elongation is required to enhance the interaction between MAVS and cytoplasmic DNA sensor STING (stimulator of interferon genes) and the downstream antiviral signaling propagation [201,204]. HCMV vMIA has been shown to inhibits mitochondrial-ER tethering and reduces MAVS-STING association by inducing mitochondrial fragmentation [201].

Peroxisomes and mitochondria cooperate in a multiplicity of functions. Both are membrane-bound and highly dynamic organelles which are involved in ROS metabolism, fatty acids $\beta$-oxidation and share key proteins of their fission machinery such as DLP1, MFF and FIS1 [90,110].

Magalhães et al.[203] have demonstrated that HCMV vMIA also localizes at peroxisomes, inhibits the peroxisomal MAVS-signaling pathway and induces peroxisome fragmentation. However, opposite to mitochondria, this fragmentation has been shown not to be essential to the inhibition of the peroxisome-dependent antiviral response. Importantly, vMIA can specifically interact with the peroxisomal MAVS. Thus, the mechanisms by which vMIA abolishes peroxisomal MAVS pathway seem distinct from the ones occurring in mitochondria [203].

In order to better understand the vMIA impairment of the peroxisomal MAVS antiviral pathway, in this study we explore the mechanisms used by vMIA to mediate peroxisomal fragmentation and the role of the key proteins FIS1, MFF and DLP1 in the evasion of the cellular immune response mediated by peroxisomes. Our results clearly demonstrate that despite organelle fragmentation not being crucial to the peroxisomal antiviral pathway, vMIA interacts directly with the fission protein MFF. Furthermore, MFF seems to play an essential role in vMIA-mediated inhibition of peroxisomal MAVS. Our results further unravel key differences between role of peroxisomes' and mitochondria´s morphology in the impairment of the antiviral immune response mediated by vMIA.

### 3.2.3 Results

#### 3.2.3.1 vMIA-induces DLP1-dependent peroxisomal fragmentation but DLP1-independent inhibition of the peroxisomal antiviral response.

Mitochondria morphology regulation has been shown to play a key role in the MAVS-dependent antiviral pathway. Upon infection, the RLR activation results in mitochondrial membrane elongation which facilitates the interaction of mitochondrial MAVS with STING at the ER. Conversely, HCMV´s vMIA induces mitochondria fragmentation and consequently reduces MAVS-STING interaction enabling viral infection [201,242]. In contrast, Magalhães and colleagues have proven that, although vMIA induces fragmentation of peroxisomes, it does not impair the peroxisomal-dependent antiviral signaling [203].

To better understand the importance of peroxisome dynamics to the antiviral response against HCMV, we overexpressed a myc-tagged construct of vMIA in mouse embryonic fibroblasts (Mefs) that contain MAVS solely at peroxisomes (Mefs MAVS-PEX cells as in [135] and [203]) and analyzed peroxisome and mitochondria morphology (Figure 17). We performed statistical analyses where six hundred cells from three independent experiments were analyzed, considering the size/shape and number of peroxisomes and mitochondria. We considered cells containing "fragmented peroxisomes" or "fragmented mitochondria" as those whose organelles were significantly smaller and rounder than the ones from the control cells (differences in fluorescence intensities were considered for each cell and experiment) The increase in peroxisome number was also taken as a prerequisite for peroxisome fragmentation. As shown in Figure 17 C, in Mefs MAVS-PEX cells expressing vMIA, fragmented peroxisomes were observed in 61% of the cells and fragmented mitochondria was massively observed in 93% of the cells.

We followed our studies by independently analyzing the effect of the key fission proteins DLP1, MFF and FIS1 on vMIA-mediated organelle fission and inhibition of the immune response. As DLP1

is assumed to be unequivocally the main responsible for the final fission of both peroxisomes and mitochondria, we initially evaluated the effect of vMIA on organelle's morphology in the absence of DLP1. To that end, 24h upon DLP1 silencing (via RNAi), we overexpressed vMIA-myc in Mefs MAVS-PEX cells and assessed peroxisome and mitochondrial morphology (Figure 18). As expected, DLP1 silencing led to a strong peroxisomal elongation and vMIA was not able to induce peroxisome fragmentation (Figure 18 A).

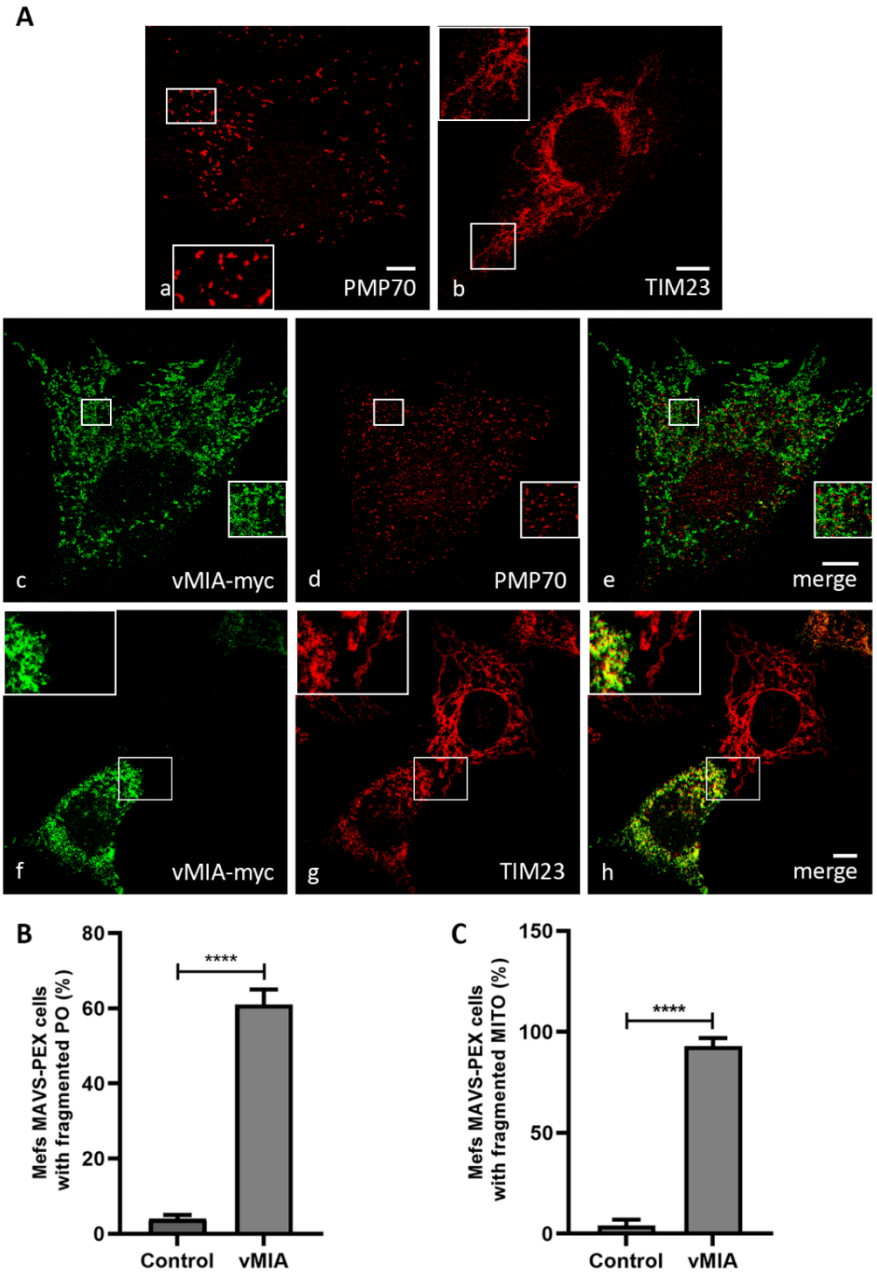


***Figure 17 - Effect of vMIA overexpression in peroxisome and mitochondria morphologies in Mefs MAVS-PEX cells.*** (A) Immunofluorescence analyses: (a-b) Control cells with normal peroxisomal and mitochondrial morphologies: (a) anti-PMP70, (b) TIM23. (c-e) Peroxisomal morphology upon overexpression of vMIA-myc: (c) anti-myc, (e) anti-PMP70, (f) merge image of c and d; f-h) Mitochondrial morphology upon overexpression of vMIA-myc: (c) anti-myc, (e) anti-TIM23, (f) merge image of c and d. Bars represent 10 µm. Zoom images are 2x digital zooms. (B) Statistical analysis on peroxisomal and mitochondrial morphologies upon overexpression of vMIA-myc in Mefs MAVS-pex cells. Data represents the means ± SD of three independent experiments. Error bars represent SD (****$p < 0.0001$).

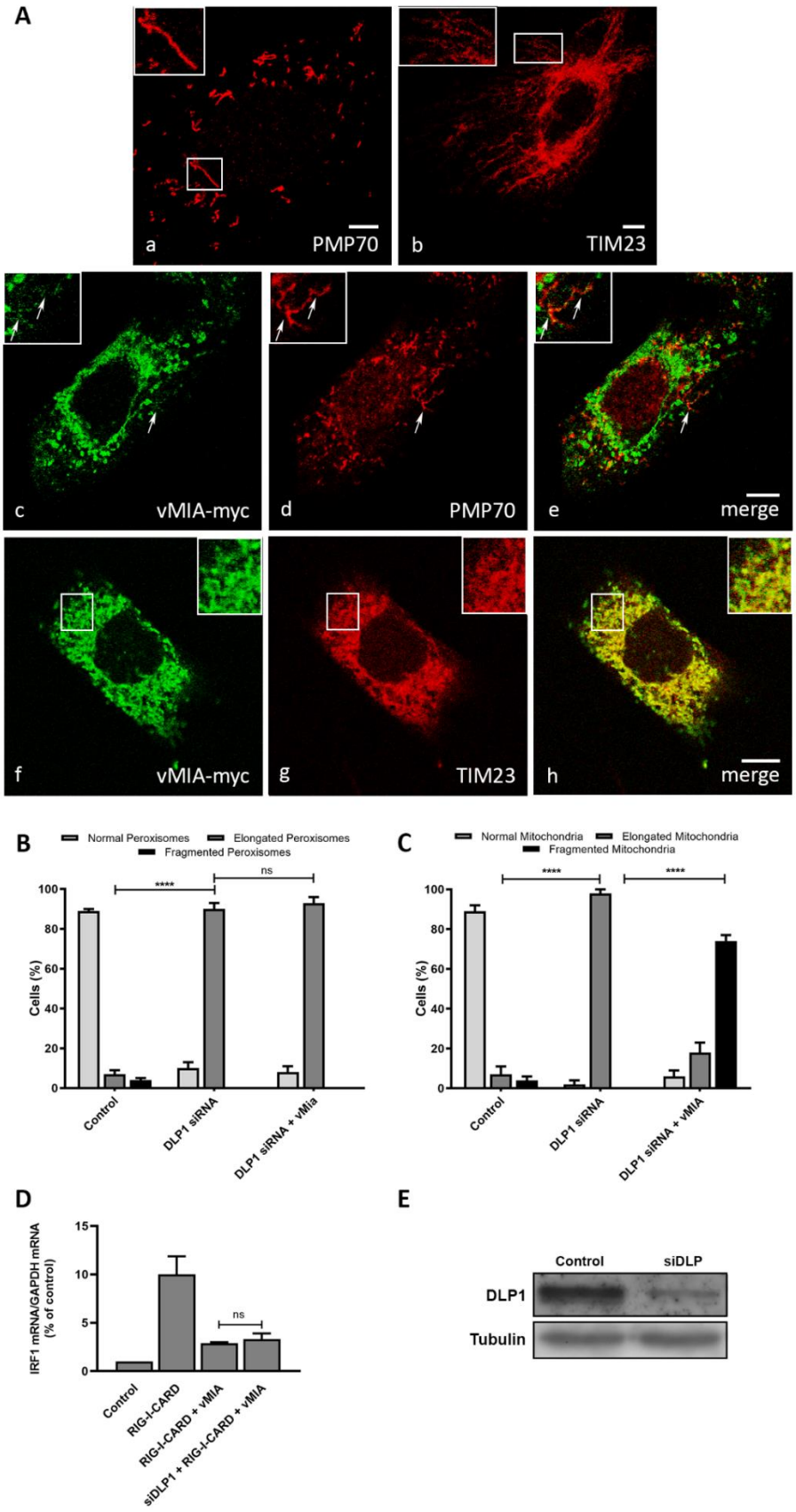


***Figure 18 - Effect of vMIA overexpression in peroxisome´s and mitochondria´s morphologies in Mefs MAVS-PEX cells upon DLP1 silencing.*** (A) Immunofluorescence analyses: (a-b) Control cells with elongated peroxisomal and mitochondrial morphology in DLP1 silenced Mefs MAVS-pex cells: (a) anti-PMP70, (b) TIM23. (c-e) Peroxisomal morphologies upon overexpression of vMIA-myc in siDLP1 Mefs MAVS-pex cells: (c) anti-myc, (d) anti-PMP70, (e) merge image of c and d. (f-h) Mitochondrial morphology upon overexpression of vMIA-myc in siDLP1 Mefs MAVS-pex cells: (f) anti-myc, (g) anti-

PMP70, (h) merge image of c and d. Bars represent 10 µm. Zoom images are 2x digital zooms. (B and C) Statistical analysis on peroxisomal and mitochondrial morphologies upon overexpression of vMIA-myc in siDLP1 Mefs MAVS-pex cells, respectively. Data represents the means ± SD of three independent experiments. Error bars represent SD (***p < 0.001) (D) RT-qPCR analysis of the expression of IRF1 mRNA in Mefs MAVS-Pex cells stimulated with GFP-RIG-I-CARD in the presence of vMIA-myc and upon silencing of DLP1. Non-silenced cells, as well as cells not expressing vMIA-myc were used as controls. GAPDH was measured as control. Data represents the means ± SEM of three independent experiments. Error bars represent SEM (***p < 0.001). (E) Western blot analysis of the silencing of DLP1 in Mefs MAVS-Pex cells. Representative image of three independent experiments.

Interestingly, vMIA was still able to fragment mitochondria in the absence of DLP1. Statistical analysis was performed and confirmed that peroxisomes remained elongated in 93% of the cells, (Figure 18 B) while only 18% of the cells contained elongated mitochondria.

In addition to these morphological studies we also evaluated the peroxisome-dependent antiviral response upon DLP1 silencing. Mefs MAVS-PEX DLP1-silenced cells were transfected with GFP-RIG-I-CARD to stimulate the antiviral response as in Magalhães et al. 2016 and, after 6 h, the amount of interferon stimulated gene (ISG) IRF1 mRNA was analyzed by RT-qPCR. As shown in Figure 18 D, upon DLP1 silencing, the presence of vMIA does not lead to an increase of the IRF1 mRNA. These results are in line with the previous findings reported by Magalhães et al. 2016 and demonstrate that vMIA impairs the peroxisomal-MAVS signaling pathway independently of peroxisome fragmentation.

#### 3.2.3.2 MFF is essential for the vMIA-dependent inhibition of the peroxisomal antiviral signaling

We continued our studies with the analysis on how the DLP1 adaptor MFF impacts on the vMIA-mediated evasion of the antiviral immune response. Following the approach of the previous experiments, we overexpressed vMIA-myc, 24h after MFF silencing, and analyzed organelle morphology by confocal microscopy. As expected, MFF silencing resulted in a high elongation of both peroxisomes and mitochondria (Figure 19 A). Statistical analysis was performed and revealed that upon MFF silencing 86% and 94% of the Mefs MAVS-PEX cells presented elongated peroxisomes and mitochondria, respectively. When expressing vMIA in these silenced cells, similarly to the DLP1 results, we observed that mitochondria but not peroxisomes were able to fragment (Figure 19 A): peroxisomes remained elongated in 84% of these cells, in contrast to mitochondria whose fragmentation was observed in 74% of the cells (Figure 19 B-C). These results are in consonance with the ones described in the previous chapter and corroborate that the fission mechanism of peroxisomes is based on MFF and DLP1-mediated scission. On the other hand, in mitochondria, when DLP1 or MFF mediated scission is impaired, an alternative scission mechanism seems to take place to (at least partially) compensate the DLP1 or MFF depletion.

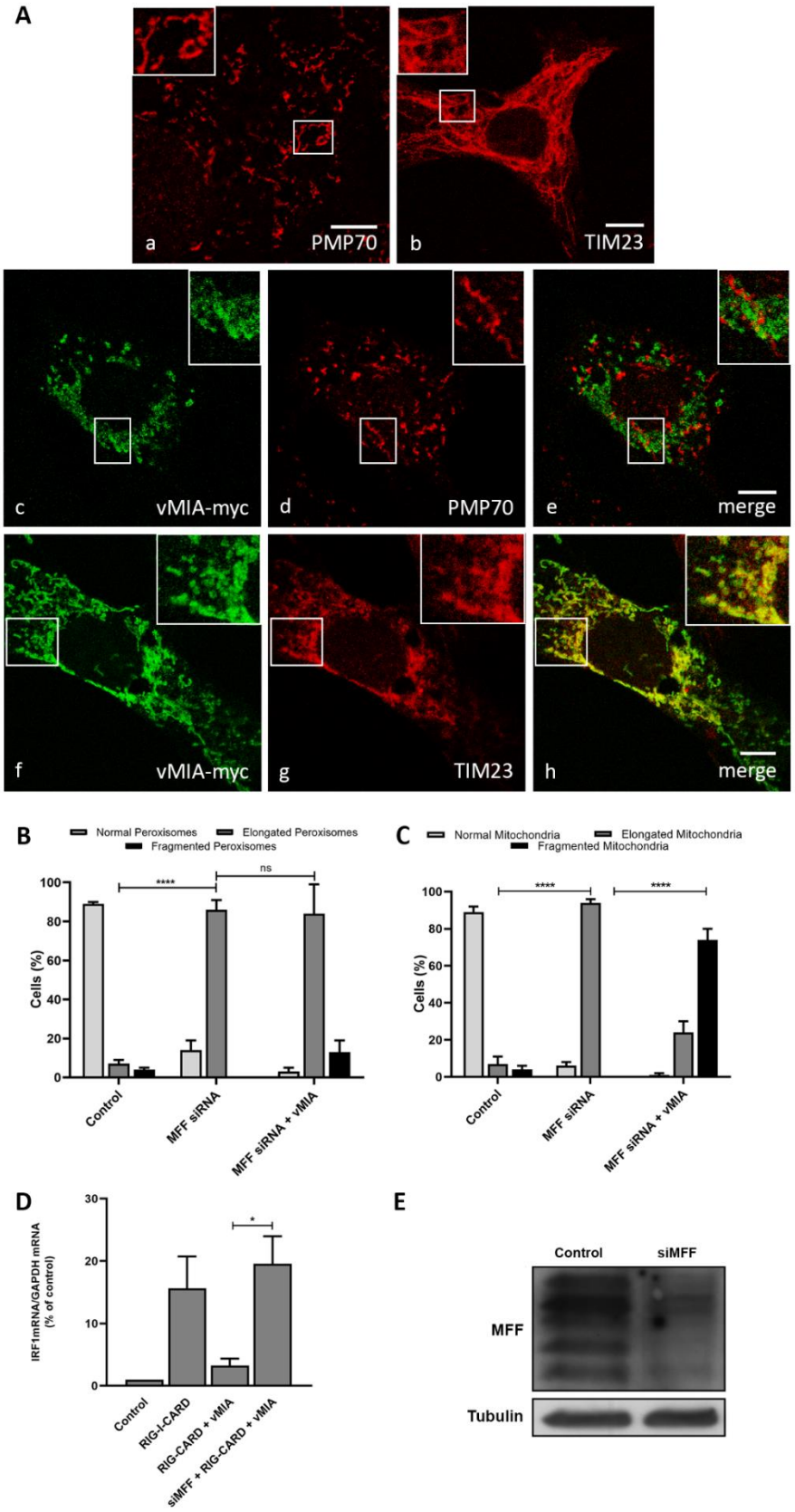


***Figure 19 - Effect of vMIA overexpression in peroxisome´s and mitochondria´s morphologies in Mefs MAVS-PEX cells upon MFF silencing.*** (A) Immunofluorescence analyses: (a-b) Control cells with elongated peroxisomal and mitochondrial morphology in MFF silenced Mefs MAVS-pex cells: (a) anti-PMP70, (b) TIM23. (c-e) Peroxisomal morphologies upon overexpression of vMIA-myc in siMFF Mefs MAVS-pex cells: (c) anti-myc, (d) anti-PMP70, (e) merge image of c and d. (f-h) Mitochondrial morphology upon overexpression of vMIA-myc in siMFF Mefs MAVS-pex cells: (f) anti-myc, (g) anti-

PMP70, (h) merge image of f and g. Bars represent 10 µm. Zoom images are 2x digital zooms. (B and C) Statistical analysis on peroxisomal and mitochondrial morphologies upon overexpression of vMIA-myc in siMFF Mefs MAVS-pex cells, respectively. Data represents the means ± SD of three independent experiments. Error bars represent SD (ns = non-significant, ****p < 0.0001) (D) RT-qPCR analysis of the expression of IRF1 mRNA in Mefs MAVS-Pex cells stimulated with GFP-RIG-I-CARD in the presence of vMIA-myc and upon silencing of MFF. Non-silenced cells, as well as cells not expressing vMIA-myc were used as controls. GAPDH was measured as control. Data represents the means ± SEM of three independent experiments. Error bars represent SEM (*p < 0.01). (E) Western blot analysis of the silencing of MFF in Mefs MAVS-Pex cells. Representative image of three independent experiments.

To evaluate the role of MFF on the peroxisomal MAVS-mediated antiviral response upon MFF silencing and/or vMIA overexpression, cells were transfected GFP-RIG-I-CARD and IRF1 stimulation was assessed by RT-qPCR (Figure 19 D). Surprisingly, the results revealed that the absence of MFF strongly impaired the capability of vMIA to inhibit the expression of IRF1. These results indicate that vMIA depends on MFF for its role on the evasion of the peroxisome-dependent antiviral immune response.

#### 3.2.3.3 FIS1 plays an important role in vMIA-mediated mitochondrial fission

We decided to further explore the organelle's fission machinery by analyzing the role of FIS1 on vMIA's effect towards the innate immune response. Following the methodology used in the previous studies with DLP1 and MFF, upon FIS1 silencing in Mefs MAVS-PEX cells, vMIA-myc was overexpressed and peroxisomal and mitochondrial morphologies were assessed (Figure 20). As shown in Figure 20 A, FIS1 silencing results in peroxisomal and mitochondrial elongation. However, as it has been previously shown in the chapter 3.1, FIS1 silencing results in a less pronounced peroxisome elongation when compared to DLP1- or MFF-silencing conditions. In contrast to the observations in DLP1 and MFF, the effect of FIS1 knockdown in the peroxisomal fragmentation mediated by VMIA was not totally clear since 40% of peroxisomal fragmentation was observed but peroxisomes remained elongated in 15% of the cells. In 45% of the cells, peroxisomes appear less elongated than in the silenced cells but longer that the fragmented peroxisomes usually observed in Mefs MAVS-PEX cells. Peroxisomes' sizes were similar to the ones in the control cells and thus were categorized as cells with normal peroxisomes. It should be noted that in this cell line peroxisomes are not as round as the ones found in other cell lines such as HepG2. In turn, and according with the findings described in the previous chapter, in the absence of FIS1, mitochondrial fission mediated by vMIA was compromised (Figure 20 C).

Upon GFP-RIG-I-CARD stimulation in the presence of vMIA, it was possible to conclude that, in the absence of FIS1, vMIA remained able to impair the IRF1 stimulation mediated by RIG-I-CARD.

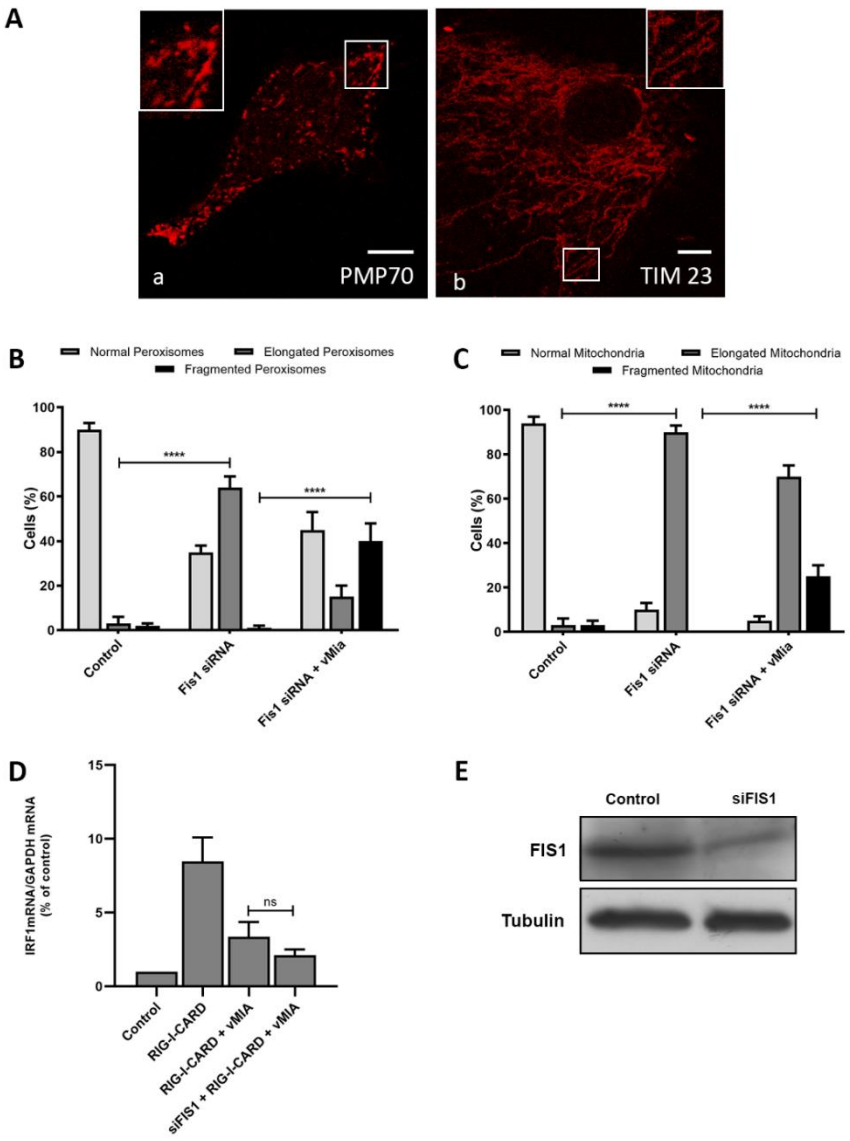


***Figure 20 - Effect of vMIA overexpression in peroxisome´s and mitochondria´s morphologies in Mefs MAVS-PEX cells upon FIS1 silencing.*** (A) Immunofluorescence analyses: (a-b) Mefs MAVS-pex cells showing elongated peroxisomal and mitochondrial morphology upon FIS1 silencing: (a) anti-PMP70, (b) TIM23. Bars represent 10 µm. Zoom images are 2x digital zooms. (B and C) Statistical analysis on peroxisomal and mitochondrial morphologies upon overexpression of vMIA-myc in siFIS1 Mefs MAVS-pex cells, respectively. Data represents the means ± SD of three independent experiments. Error bars represent SD (****p < 0.0001) (D) RT-qPCR analysis of the expression of IRF1 mRNA in Mefs MAVS-Pex cells stimulated with GFP-RIG-I-CARD in the presence of vMIA-myc and upon silencing of FIS1. Non-silenced cells, as well as cells not expressing vMIA-myc were used as controls. GAPDH was measured as control. Data represents the means ± SEM of three independent experiments. Error bars represent SEM (ns = non-significant). (E) Western blot analysis of the silencing of FIS1 in Mefs MAVS-Pex cells. Representative image of three independent experiments.

### 3.2.3.4 vMIA interacts with MFF but not with FIS1 or DLP1

As vMIA triggers peroxisomal fragmentation and has been shown to interact with MAVS [203] at peroxisomal membranes, we questioned whether the key components of peroxisomal fission machinery, DLP1, FIS1 and specially MFF, which we have shown to be essential for vMIA's role

on the antiviral response, would also interact with vMIA and be part of this protein complex. To test this, HepG2 cells were transfected with vMIA-myc and, 24h later, cells were subjected to co-immunoprecipitation studies. The pull-down with an antibody against the myc-tag revealed that vMIA interacts with endogenous MFF but not FIS1 or DLP1 (Figure 21 A). As vMIA interacts with MAVS and with MFF we wondered whether MAVS would also interact with MFF, reflecting the presence of a three-protein complex. To investigate the interaction between MAVS and MFF, HepG2 cells were transfected with GFP-MAVS-PEX plasmid and a co-immunoprecipitation was performed through pull-down of endogenous MFF. Figure 21 B suggests that, in fact, MFF interacts with the peroxisomal MAVS. Whether this is a direct interaction or a consequence of a tight complex between MAVS, MFF and vMIA remains to be further investigated. Nevertheless, these results clearly suggest that vMIA, MFF and MAVS are present in a protein complex and that vMIA interacts with MFF to inhibit the antiviral response.

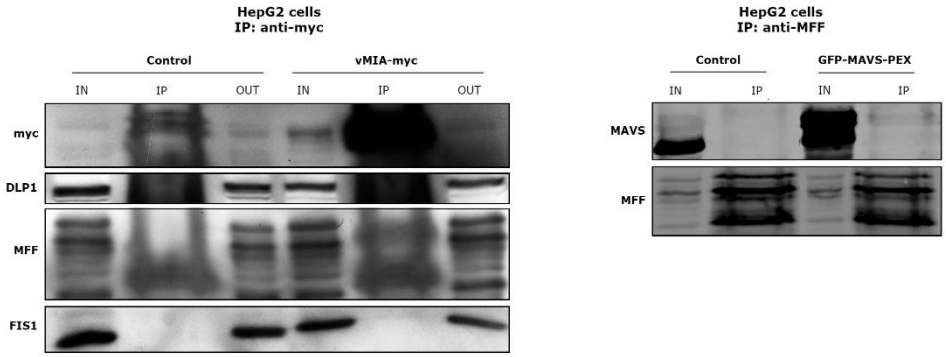


***Figure 21 – MFF interacts with vMIA and peroxisomal MAVS. (1) Co-immunoprecipitation analysis of the interaction between overexpressed vMIA-myc and endogenous DLP1, MFF and FIS1 in HepG2 cells.*** The pull-down was performed using an antibody against myc. Western blot was performed with antibodies against myc-tag and DLP1, MFF or FIS1. (B) Co-immunoprecipitation analysis of the interaction between overexpressed GFP-MAVS-PEX and endogenous MFF in HepG2 cells. The pull-down was performed using an antibody against endogenous MFF. Western blot was performed with antibodies against MAVS and MFF. Negative control was performed by immunoprecipitating non-transfected cells. IN represents total cell lysate (input), IP represents immunoprecipitation and OUT represents the cell lysate extracted after incubation with the antibody (output).

### 3.2.3.5 vMIA-induced peroxisome and mitochondrial fission does not depend on MAVS

HCMV vMIA has been shown to induce the fragmentation of both peroxisomes and mitochondria [201,243,203]. However, while mitochondrial fragmentation was shown to be essential for vMIA-mediated inhibition of the antiviral response [201], peroxisomal fragmentation induced by vMIA is not necessary for the antiviral signaling inhibition at this organelle [203]. Together, these results suggest a straighter interconnection between organelle morphology and vMIA signaling inhibition at mitochondria than at peroxisomes.

vMIA seems to act on peroxisomes via two distinct and independent mechanisms: one with the final goal of evading the antiviral immune response and one other involving organelle morphology changes with a yet unknown purpose. As these two processes seem to be unrelated, we hypothesized that the antiviral signaling itself would exert no influence on the vMIA-mediated organelle's morphology changes. To test this hypothesis, we specifically analyzed whether the presence of MAVS at peroxisomes and mitochondria would be essential for vMIA induction of peroxisomal and mitochondrial fragmentation. To this end, we transfected Mefs MAVS-KO cells with vMIA-myc and, 24 hours after, immunofluorescence was performed to analyze the organelles' morphology by confocal microscopy. As shown in Figure 22, even in the absence of MAVS, vMIA was able to induce a strong peroxisome and mitochondrial fragmentation.

#### 3.2.3.6 Contrarily to mitochondria, peroxisome elongation does not enhance antiviral signaling

Although the contribution of peroxisomes, in concert with mitochondria, to the cellular antiviral response has been established, the main differences between the two signaling pathways, which lead to the distinct kinetics and end products, remains to be unveiled. Our results suggest that HCMV vMIA acts via two seemingly different mechanisms in these two organelles, and key differences were uncovered between the relevance of the organelles' morphology in both antiviral responses: contrarily to mitochondria [201], peroxisome elongation, upon inhibition of DLP1-mediated fission, did not seem to affect the antiviral response [203]. These data led us to take a step back and further investigate the relevance of the peroxisome morphology for the establishment of the antiviral signaling originating from this organelle. To that end, we analyzed the production of the ISG IRF1 in Mefs MAVS-PEX cells upon silencing of the main key payers at the peroxisomal fission machinery: DLP1 MFF and FIS1. Forty-eight hours after siRNA transfection, antiviral signaling was stimulated by overexpressing GFP-RIG-I-CARD. Six hours after transfection, IRF1 mRNA was quantified by RT-qPCR. As shown in Figure 23, the knock-down of DLP1 (siDLP1), MFF (siMFF) and FIS1(siFIS1) which led to peroxisome elongation (Figures 18, 19 and 20), did not altered the production of IRF1 mRNA when compared to stimulated control cells. These results indicate that peroxisome elongation does not enhance peroxisomal-dependent antiviral signaling. As it has been shown that DLP1 depletion enhances signaling downstream to mitochondrial MAVS [201], these results uncover major core differences between the relevance of these organelles' morphology to these two signaling pathways.

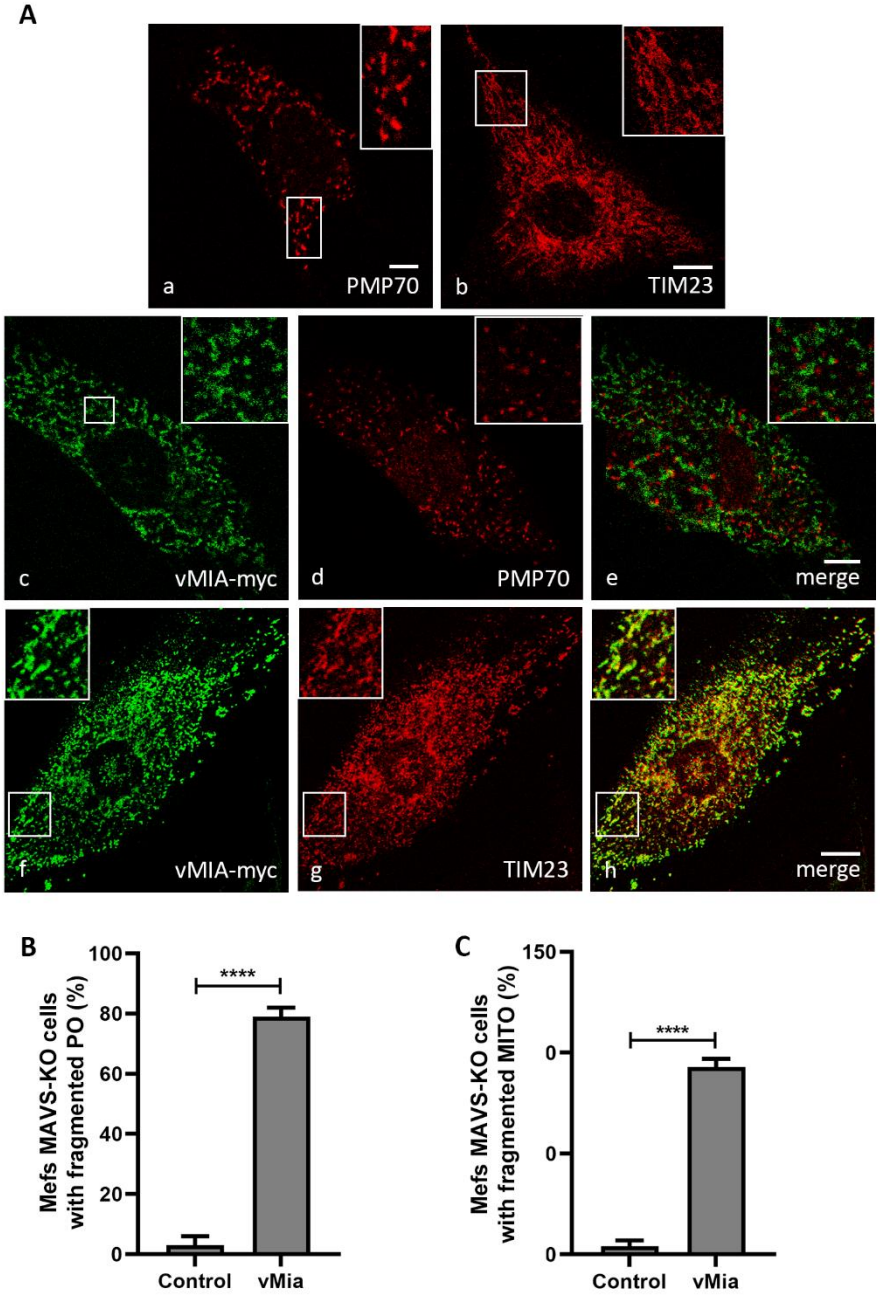


***Figure 22 - vMIA does not depend on MAVS, to induce peroxisome and mitochondria fragmentation***. (A) Immunofluorescence analyses of transfected vMIA-myc in Mefs MAVS-KO cells: (a-b) Control cells with normal peroxisomal and mitochondrial morphologies: (a) anti-PMP70, (b) TIM23. (c-e) Peroxisomal morphologies upon overexpression of vMIA-myc Mefs MAVS-KO cells: (c) anti-myc, (d) anti-PMP70, (e) merge image of c and d. (f-h) Mitochondrial morphology upon overexpression of vMIA-myc in Mefs MAVS-KO cells: (f) anti-myc, (g) anti-PMP70, (h) merge image of c and d. Bars represent 10 µm. Zoom images correspond to 2x digital zooms. (B and C) Statistical analysis on peroxisomal and mitochondrial morphologies upon overexpression of vMIA-myc in Mefs MAVS-KO cells, respectively. Data represents the means ± SD of three independent experiments. Error bars represent SD (ns = non-significant, ****p < 0.0001

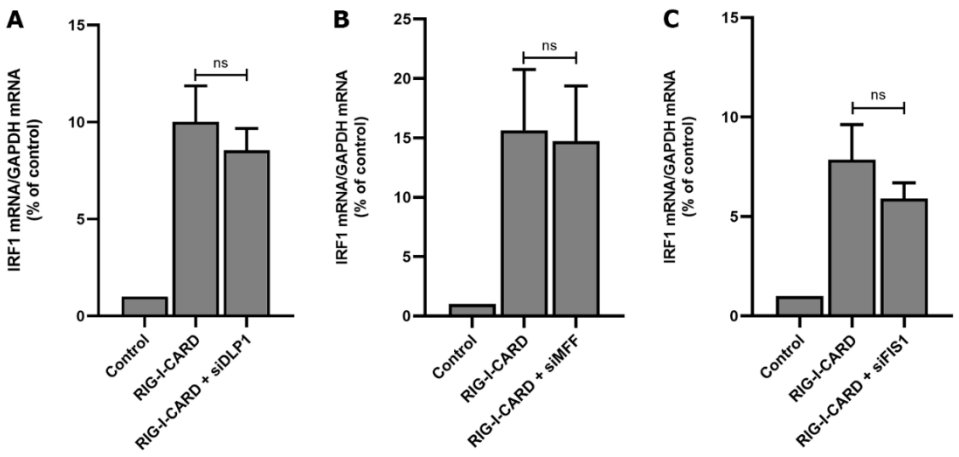


***Figure 23 - Peroxisome elongation does not enhance antiviral signaling*.** RT-qPCR analysis of IRF1 mRNA expression in Mefs MAVS-PEX cells stimulated with GFP-RIG-I-CARD, upon silencing of (A) DLP1, (B) MFF and (C) FIS1. GAPDH was used as normalizer gene and graphs depict % of control in relation to untreated samples. Data represents the means ± SEM of three independent experiments (ns = non-significant).

### 3.2.4 Discussion

Despite both peroxisomes and mitochondria being involved in the MAVS-dependent antiviral immune response, they assume different but complementing roles [201,135,203]. There is growing evidence of peroxisomes' prominence for the establishment of the cellular antiviral signaling, including the demonstration that distinct viruses, such as HCMV via vMIA, have developed specific strategies to target and evade the peroxisomal antiviral signaling [113,244–250].

The peroxisomal and mitochondrial antiviral immune signaling pathways present different kinetics and lead to distinct end products [135,172]. These differences may be due to organelle-specific metabolic or morphological dissimilarities. Mitochondrial antiviral signaling has been shown to be dependent on the organelle's morphology, being upregulated upon mitochondria elongation [201]. This elongation is believed to promote the interaction between mitochondrial MAVS and STING at the ER, promoting the immune response. Accordingly, HCMV vMIA leads to mitochondrial fragmentation and the consequent impairment of the MAVS pathway [162]. In peroxisomes, although vMIA has been shown to trigger peroxisome fragmentation and its importance for HCMV's evasion of the peroxisomal antiviral signaling has been demonstrated [203], the precise mechanism of action towards this pathway had not yet been established. In this study we further elucidate this mechanism by investigating the interplay between vMIA and the key fission proteins DLP1, MFF and FIS1. Additionally, as we have previously demonstrated that MFF and FIS1 impact differently on peroxisome and mitochondria fragmentation (Chapter 3.1), we

completed these studies with a detailed analysis on the involvement of the fission machinery of both organelles on the vMIA-mediated evasion of the immune response.

In line with the findings described in the previous chapter, in contrast to FIS1, MFF has shown to play a prominent role at peroxisomes but not at mitochondria, since cells lacking MFF present a vMIA-mediated mitochondrial, but not peroxisomal, fragmentation. Together, these results corroborate the previous hypothesis that predicts a peroxisomal fission machinery mostly dependent on MFF and DLP1, whereas in mitochondria alternative fission mechanisms independent of DLP1 can take place. Considering the work of Onoue et al. 2013 we may extrapolate that the mitochondrial fission observed upon vMIA overexpression in DLP1 and MFF silenced cells may be coordinated by the FIS1 via the Rab TBC1D15 [123].

As vMIA results in organelles' fragmentation and it has been shown to interact with peroxisomal MAVS [203] we speculated whether DLP1, FIS1 and specially MFF would also interact with vMIA and MAVS and be part of this protein complex. Here, we demonstrated that in fact vMIA interacts with MFF, and not with FIS1 or DLP1, highlighting the role of this protein in the antiviral immune response. We also found that MFF interact with peroxisomal MAVS. Altogether, these results suggest that MFF mediates the interaction between vMIA and MAVS at peroxisomes. However, as this interaction has been demonstrated in cells where MAVS is present at both mitochondria and peroxisomes, it is highly likely that this interaction also occurs at mitochondria. Coherently, MFF was recently found to play a central role in MAVS-mediated innate immune response in mitochondria by sensing mitochondrial energy metabolism via AMPK signaling [251].

vMIA-induced mitochondrial fragmentation is closely related to its role as an anti-apoptotic protein, interfering with BAX to prevent mitochondrial outer-membrane permeabilization, and mediating the release of ER $Ca^{2+}$ stores into the cytosol [242,252,253]. It is, then, tempting to suggest that the interaction between vMIA and MFF may lead to the activation of the mitochondrial protein, modifying the balance between fission and fusion that normally controls mitochondrial structure, and consequently inhibiting apoptosis and antiviral signaling. At peroxisomes, as the organelle's fission machinery does not seem to play a role on the immune signaling, vMIA-induced MFF-dependent fragmentation may be a side effect from the interaction between vMIA and MFF in the process of MAVS oligomerization inhibition. On the other hand, as HCMV has been shown to alter peroxisome morphology and metabolism to its own benefit [254], this may also represent one other specific molecular mechanism involved in peroxisome manipulation in the context of viral infection.

Our results also suggest that peroxisome morphology does not impact the peroxisome-dependent antiviral pathway and that the vMIA-induced organelle fragmentation is totally independent of MAVS's presence at the organelle's membranes. These results strengthen the idea that vMIA-induced peroxisome fragmentation and inhibition of the immune response are

two distinct and independent processes. Here we demonstrated that peroxisome-dependent signaling is specifically independent from DLP1, MFF and FIS1.These results are one of the first to explicitly pinpoint specific differences between the antiviral signaling pathways arising from these two organelles and are totally in line with what is here observed in the presence of HCMV vMIA. Indeed, although vMIA depends on the mitochondrial fission machinery to inhibit the mitochondria-dependent antiviral response, this dependency is not observed at the level of peroxisomes. Hence, it is tempting to infer that these observed differences may be due to intrinsic dissimilarities among these organelles and respective antiviral signaling, emphasizing the virus capability to adapt vMIA's mechanism of action to the particular characteristics of the two pathways it inhibits.

In conclusion, in this study we found some important clues concerning the molecular mechanism by which HCMV vMIA evades the peroxisomal antiviral response and also demonstrated that the peroxisomal fission is not involved in the immune signaling at peroxisomes, highlighting important differences between peroxisomes and mitochondria on the establishment of the cellular antiviral response. Importantly, we once more emphasize the relevance of peroxisomes as platforms for antiviral signaling against HCMV and uncover molecular mechanisms that may be explored in the future as targets for antiviral therapy.

### 3.2.5 Materials and Methods

**Antibodies and cDNAs**

Mouse antibodies against PMP70 (SAB4200181, Sigma-Aldrich, St. Louis, Missouri, USA), and to TIM23 (BD Bioscience, San Jose, California, USA) and rabbit antibody directed to myc epitope (71D10, Cell Signaling Technology, Beverly, Massachusetts, USA), used to detect vMIA-myc in transfected cells, were used for immunofluorescence in morphological studies. Mouse antibody directed to myc epitope (9E10, Santa Cruz Biotechnology, Dallas, Texas, USA) to detect vMIA-myc, mouse antibodies anti-MAVS (E-3, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-DLP1 (BD Bioscience, San Jose, California, USA) and rabbit antibodies anti-MFF (kindly provided by A. van der Bliek, University of California, Los Angeles) [37] and anti-hFIS1 (Alexis Biochemicals, Grunberg, Germany) were used for immunoblotting. Species-specific anti-IgG antibodies conjugated to HRP (BioRad, Hercules, California, USA), IRDye 800CW and IRDye 680RD secondary antibodies (LI-COR Biotechonology, Cambridge, UK) or to the fluorophores TRITC (Jackson Immunoresearch, West Grove, Pennsylvania, USA) and Alexa 488 (Invitrogen, Waltham, Massachusetts, USA) were used.

The plasmid vMIA-myc was a gift from Dr. Goldmacher (ImmunoGen Inc., Cambridge, Massachusetts, USA) and GFP-RIG-I-CARD was kindly provided by Dr F. Weber, Justus-Liebig Universität Giessen, Germany. GFP-MAVS511-PEX, developed by our group, is a construct encoding a version of MAVS directed specifically to peroxisomes and was generated by replacing the localization motif of MAVS, as described by [168] with the localization motif of the peroxisomal protein Pex13 [255] and by adding a GFP tag. This was performed with the MAVS-WT and MAVS500-PEX sequences (MAVS500-PEX was based on the construct previously described by Dixit et l. 2010 where it was named MAVS-PEX [135] as templates and cloning into the EGFP–C1 (BD Biosciences Clontech) vector.

**Cell culture, transfection, and RNA interference experiments**

HepG2 (obtained from American Type Culture Collection, HB-8065), Mefs MAVS-Pex cells (described in [135]) and Mefs MAVS-KO cells (described in [135]) were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (all from GIBCO, Thermo Scientific, Waltham, Massachusetts, USA ) at 37°C in a humidified atmosphere of 5% CO2. HepG2 cells were transfected with the DNA constructs by incubation with TurboFect (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Mefs MAVS-Pex cells and Mefs MAVS-KO cells were transfected with Lipofectamine 3000 (Invitrogen, Waltham, Masschusetts, USA) or microporated with Neon® Transfection System (Invitrogen, Carlsbad, CA) (1700V, Width: 20 1 pulse), following manufacturer's instructions. Cells were fixed for organelle morphology or harvested for western blot or co-immunoprecipitation assays, 24 h to 72 h after transfection.

To knock-down the expression of MFF, FIS1 and DLP1 by RNA interference, 21-nucleotide small interfering RNA (siRNA) duplexes were transfected into HepG2 cells and Mefs MAVS-PEX cells by incubation with Lipofectamine RNAiMax (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Control cells were treated with transfection mix without siRNAs complexes. Cells were assayed for silencing and organelle morphology 72h after seeding. siRNA oligonucleotides were obtained as pre-designed siRNAs from Ambion (Austin, TX) as follows: MFF - sense strand: 5'-CGCUGACCUGGAACAAGGAdTdT-3' for exon 2 [37]; DLP1 - sense strand: 5´-UCCGUGAUGAGUAUGCUUUdTdT-3´ [35]; human FIS1 (accession no. AF151893) - sense strand: 5'-CGAGCUGGUGUCUGUGGAGdTdT-3' (Dharmacon, Lafayette, CO).

**Immunofluorescence and microscopy**

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4, permeabilized with 0.2% Triton X-100, for 10 min, blocked with 1% BSA solution, for 10 min, and incubated with the indicated primary and secondary antibodies, for 1h. Nucleus were stained Hoechst (1:2000) for 3 min, before mounting the glass coverslips on the glass slides. Transfected cells were processed for immunofluorescence 24h after transfection. Fixed samples were examined using an Olympus IX-81 inverted microscope (Olympus Optical Co. GmbH, Hamburg, Germany) equipped with the appropriate filter combinations and a 100x objective (Plan-Neofluar, 100x/1.35 oil objective). Confocal images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) and a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using a Plan-Apochromat 63× and 100×/1.4 NA oil objectives, a 561 nm DPSS laser and the argon laser line 488 nm (BP 505-550 and 595-750 nm filters). Images were processed using LSM 510 software (Carl Zeiss MicroImaging, Inc.) and Leica Application Suite Advanced Fluorescence software (Leica Microsystems). Digital images were optimized for contrast and brightness using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

**Quantification and statistical analysis**

For the evaluation of peroxisome and mitochondria morphology, two hundred cells from three independent experiments were counted for each condition, considering the size/shape and number of their peroxisomes or mitochondria. For these experiments we considered cells containing “fragmented peroxisomes” or “fragmented mitochondria” as those whose organelles were significantly smaller and rounder than the ones from the control cells. The increase in peroxisome number was also taken as a prerequisite for peroxisome fragmentation. On the other hand, considered cells containing “elongated peroxisomes” and “elongated mitochondria” as those whose these organelles were tubular-shaped and significantly longer when compared to the control cells. Statistical analysis was performed in Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, California, USA). Data are presented as mean ± standard deviation (SD). Differences among groups were analyzed by one-way ANOVA, followed by Bonferroni’s multiple comparison test; comparisons between two groups were made by Student’s t test. P values of ≤0.05 were considered as significant, ****P<0.0001, ***P<0.001, **P<0.01, and *P<0.05.

**Gel Electrophoresis and Immunoblotting**

Cells were lysed with specific lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100 and a protease-inhibitor mix). To improve protein extraction, samples were passed 20 times through a 26-gauge syringe needle and then incubated on a rotary mixer at for 30 min at 4$^{o}$C. After clearing by centrifugation (17000 x g, 15 min), protein concentrations were determined using the Bradford assay (BioRad, Hercules, California, USA). Protein samples were separated by SDS-PAGE on 10% or 12.5% polyacrylamide gels, transferred to nitrocellulose (PROTAN®, Whatman®, Dassel, Germany) using a semidry apparatus (BioRad, Hercules, CA, USA), and analyzed by immunoblotting. Immunoblots were processed using specific primary antibodies, HRP-conjugated secondary antibodies and enhanced chemiluminescence reagents (GE Healthcare, Waukesha, WI, USA).

**Immunoprecipitation analyses**

To investigate the interaction between MFF, FIS1, DLP1 and vMIA, HepG2 cells were transfected with vMIA-myc by Turbofect. 24 h after transfection, cells were harvested and lysed as described above. Protein concentration was quantified by Bradford assay. Lysates were incubated overnight with anti-myc antibody, at 4°C, on a rotary mixer. In the next day, beads (Dynabeads Protein G Magnetic beads, Invitrogen, Waltham, Massachusetts, USA) were added to the mixture and rotated for 2 h at 4°C. The complex was washed 3 times with PBS, supplemented with 0.1% Tween20 and then resuspended in 3x SDS-sample buffer and boiled for 10 min to elute bound proteins. To explore the interactions between MFF, peroxisomal MAVS and vMIA, HepG2 cells were co-transfected with vMIA-myc and/or GFP-MAVS511-PEX by Lipofectamine 3000. 24 h after transfection, cells were harvested and lysed as described previously. Lysates were incubated with the antibody against endogenous MFF overnight at 4°C on a rotary mixer. Then, beads were added to the mixture and rotated for 2 at 4°C. The complex was washed 3 times with PBS containing 0.1% Tween 20 and then resuspended in 3x SDS-sample buffer and boiled for 10 min to elute bound proteins. For untransfected control, HepG2 cells were also used as negative control for each immunoprecipitation.

**RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction**

24h upon Mefs MAVS-Pex cells transfection, total RNA was isolated using TriFast reagent (Peqlab, VWR International GmbH, Erlangen, Germany). After quantifying RNA with NanoDrop 1000

(Thermo Scientific, Waltham, Massachusetts, USA), 1 µg of total RNA was used to produce cDNA using M-MuLV reverse transcriptase (New England Biolabs, Ipswich, Massachusetts, USA). For real-time polymerase chain reaction, 2 µL of 1:10 diluted cDNA was added to 10 µL of 2x SYBR Green qPCR Master Mix (Low Rox) (Bimake, Houston, USA). The final concentration of each primer was 250 nM in 20 µL total volume. Duplicates of each sample were done, and reactions were run on Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA). Primer sequences were designed using Beacon Designer™ 7 (Premier Biosoft, Palo Alto, California, USA) for the IRF1 and GAPDH mouse genes. The oligonucleotides used for IRF1 were 5'-GGTCAGGACTTGGATATGGAA-3' and 5'-AGTGGTGCTATCTGGTATAATGT-3'; for mouse GAPDH were 5'-AGTATGTCGTGGAGTCTA-3' and 5'-CAATCTTGAGTGAGTTGTC-3'; GAPDH was used as a reference gene. The thermocycling reaction was done by heating at 95°C during 3 min, followed by 40 cycles of a 12 s denaturation step at 95°C and a 30 s annealing/elongation step at 60 °C. The fluorescence was measured after the extension step using the Applied Biosystems software (Applied Biosystems, Waltham, Massachusetts, USA). After the thermocycling reaction, the melting step was performed with slow heating, starting at 60°C and with a rate of 1%, up to 95°C, with continuous measurement of fluorescence. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method.

# 4 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Since their discovery in the 1960s by C. De Duve and colleagues, several studies have emphasized the role of peroxisomes in crucial cellular functions which strongly impact health and disease conditions, Implicated in, among others, metabolic diseases, cancer and host cell defense against viruses, there is a growing interest on unraveling peroxisomal pathways which may be used as therapeutic targets [13,68,256].

Although peroxisomes have specific characteristics and functions, they have been found to be strongly linked to other organelles such as the ER, lipid droplets, lysosomes and particularly mitochondria [91,103,130]. Peroxisomes and mitochondria display a close interrelationship and crosstalk: they not only share the key components of their fission and antiviral RLR machinery, but they also assume coordinated functions in lipid and ROS metabolism and in the antiviral immune response [90]. Since a proper organelle dynamics is crucial to the organelle's function, in lipid and ROS metabolism and in the antiviral immune response [257].

In the present work we aimed to contribute to a better understanding of the relationship between peroxisomes and mitochondria by preforming an in-depth study on the organelles' fission machinery. This comparative study was extended to the analysis on how a viral protein that strongly affects peroxisome and mitochondria morphology is able to adapt to the dissimilarities between these organelles in order to inhibit the cellular antiviral response. Additionally, as organelle morphology seems to be strongly related to the RLR antiviral pathway we decided to study the role of these fission proteins in the antiviral machinery against one of the most globally spread viruses, the human Cytomegalovirus.

As described in section 3.1, we started our studies by overexpression and gene silencing experiments to verify the state of the art and to evaluate how the fission proteins DLP1, FIS1 and MFF affect organelle morphology. The earliest studies on peroxisome and mitochondria fission pointed FIS1 as the main DLP1 adaptor at the organelle´s membrane [35,220,258]. However, few years later in a siRNA screen using *Drosophila* cells, a new protein involved in the regulation of mitochondrial fission was described and the human homologue of this protein was named MFF [37]. Subsequent studies have corroborated the assumption of MFF as the main adaptor for DLP1 not only at mitochondria but also at peroxisomal membranes [15,38,39,233]. In fact, and in line with the previous studies, our results clearly shown that MFF has a crucial role in the regulation of peroxisomal dynamics since MFF overexpression resulted in a strong peroxisomal fragmentation and MFF silencing conducted to a strong elongation of peroxisomes, whereas FIS1 overexpression and silencing produced less effect on peroxisomal morphology. Additionally, although FIS1 silencing resulted in elongated peroxisomes, the lack of FIS1 did not impede MFF to induce peroxisomal

fission. We have also shown that MFF is mainly concentrated at the constriction sites in elongated peroxisomes. On the other hand, although exhibiting a spot-like staining, FIS1 localization did not consistently correspond to the constriction sites.

To further explore the role of these DLP1 adaptors in organelle fission, we overexpressed MFF and FIS1 in DLP1 silenced cells. As DLP1 is assumed to be the main final scission factor, leading to the final membrane fission, in theory, we would not expect the overexpression of the protein adaptors to overcome the absence of DLP1. Surprisingly upon DLP1 silencing which resulted in the formation of high elongated and constricted peroxisomes, MFF but not FIS1 overexpression resulted in peroxisomal fragmentation. This suggested a fission mechanism mediated by MFF and independent of DLP1. Since the gene silencing experiments do not lead to a complete protein knockout, we complemented these results by performing the same experiments in DLP1-patient cells. These results confirmed the previous ones, and peroxisomes were observed to fragment in the absence of DLP1 upon MFF overexpression. Nevertheless, these patient cells also present some limitations as they contain an heterozygous mutation on DLP1, and residual amounts of normal DLP1 are still differently expressed by each cell. Although we have used all the available tools to perform this study at the time of the experimental period, other methodologies have been developed in the meantime which lead to a complete protein depletion. For instance, the generation of DLP1 -/-, MFF -/-, FIS1 -/- cell lines using the CRISPR/Cas9 gene-editing system [259] would allow us to explore with more certainty the role of each protein at mitochondrial and peroxisomal fission machineries. However, the possibility of existing an alternative protein to DLP1 cannot be excluded. In fact, the existence of another fission protein or a DLP1-independent mechanism that would be responsible for the mitochondrial cleavage has been previously suggested to occur upon infection by Listeria monocytogenes [236,237]. However, this issue is still very controversial. While some groups are in favor to the involvement of dynamins in organelle fission, others remain very skeptical. Recently, Lee and colleagues [260] demonstrated that mitochondrial division is a result of a collaboration of multiple Dynamin family members and is not only dependent on DLP1 activity [260–263]. These authors revealed that the mammalian ubiquitously expressed classical dynamin-2 (DNM2) works in concert with DLP1 to orchestrate sequential constriction events leading up to membrane fission which is ultimately executed by DNM2 and not by DLP1. This protein was previously known for its role in driving endocytosis at the plasma membrane and in facilitating membrane fission events at multiple organelles, while also mediating the final stages of cytokinesis [260]. Nevertheless, others have excluded the involvement of DNM2 in organelle fission. Recent studies have proven that DLP1 is sufficient to drive peroxisomal and mitochondrial fission and do not require other dynamins [264,265].

In line with this, and since peroxisomes and mitochondria share many key proteins of their fission machinery it would be interesting to better explore the role of other dynamins in peroxisomal fission. Although earlier studies have not find a role for DMN2 in peroxisomal fission [44] as other fission proteins, such as MFF, have been identified since then, complementary studies could be performed to rule out the role of this dynamin at peroxisomal fission. We would suggest to evaluate if in the absence of DLP1 (in DLP1-/- cells) DMN2 can compensate the lack of DLP1. In addition, it would be interesting to evaluate whether DMN2 localizes at peroxisomes upon overexpression of MFF, at highly elongated peroxisomal membranes in DLP1-/- cells and to assess the effect of the endogenous expression levels upon DLP1 knockout.

Since in mitochondria, other DLP1 adaptor proteins, MiD49/51, that switch off DLP1 activity have been described, we also decided to evaluate whether these proteins localize and have similar functions at peroxisomes. In line with previous results, we have shown that these proteins do not localize at peroxisomes [122]. In mitochondria an alternative fission independent of DLP1/ mechanism, involving FIS1 and the Rab protein TBC1D15, has been also revealed [123]. To confirm whether similar fission pathway is also present at peroxisomes we overexpressed TBC1D15 in HepG2 cells and assessed for colocalization with peroxisomes. A colocalization of TBC1D15 with peroxisomes was not observed and, upon FIS1 overexpression and consequent TBC1D15 recruitment to peroxisomes, this FIS1/TBC1D15 machinery did not mediate peroxisomal fission. These results provide evidence that although peroxisomes and mitochondria share key fission proteins, each organelle has distinct morphology regulation mechanisms and the peroxisomal fission machinery seems to be less complex than the one from mitochondria.

Analogously to peroxisomes, we evaluated the role of MFF and FIS1 at mitochondrial fission. In contrast to the studies which defended MFF as the major DLP1 adaptor at both peroxisomes and mitochondria membranes, our results revealed a stronger impact of FIS1 in mitochondrial morphology rather than MFF. Although the silencing of both proteins results in a strong mitochondrial elongation, overexpression of FIS1 induced mitochondrial fragmentation in a higher extent than MFF. Additionally, both in DLP1 silenced cells and DLP1-patient cells, FIS1 but not MFF reverted the mitochondrial elongation. These results point to a fission mechanism regulated by FIS1 independently on DLP1. As reported by [123] this fission mechanism may be the result of the coordination of FIS1 with the Rab protein TBC1D15 mediating fission via small Rab GTPases activity such as Rab7. [123–125,127,221]. Moreover, as it has been previously referred, some authors suggest that organelle´s fission may be the result of a collaboration of multiple dynamin proteins, including DMN2 [260] which may compensate in certain conditions and at a some extent the lack of DLP1.

Whether MFF or FIS1 is the major DLP1 adaptor or which specific mechanisms are controlled by FIS1 or MFF remains controversial. Most recent studies are in the position to defend that MFF [37] and mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51) [121,227] are the DLP1 receptors to control mitochondrial dynamics in basal conditions, whereas the FIS1 mainly regulates other fission events such as in mitophagy [125,222,261]. FIS1/DLP1 pathway seems to be involved in certain pathological conditions such as amyotrophic lateral sclerosis [266] Alzheimer´s and Huntington's diseases [267]. Thus, to elucidate whether fission mediated by MFF and FIS1 is associated with basal organelle´s dynamics or with autophagic events, it would be interesting to assess for autophagic markers such as LC3 upon MFF and FIS1 overexpression [268,269]. To independently evaluate pexophagy and mitophagy, MFF and FIS1 specifically targeted to peroxisomes or mitochondria could be used.

HCMV´s protein vMIA leads to mitochondrial network disruption in order to escape from the antiviral immune response [201]. In the case of peroxisomes, the observed organelle's fragmentation does not seem to be crucial for vMIA-dependent inhibition of the antiviral response [203]. To further explore the role of peroxisomal fission in the host defense against HCMV, in this work, as described in section 3.2, we performed a series of overexpression, silencing and protein interaction studies to evaluate the importance of the key fission proteins FIS1, MFF and DLP1 for vMIA-mediated evasion from the peroxisomal antiviral response. Our results demonstrated that MFF plays a central role on the vMIA-induced mechanisms towards peroxisomes. Indeed, the absence of MFF impairs the vMIA-mediated fragmentation of peroxisomes in contrast to FIS1 which has stronger effect in the regulation of mitochondrial fragmentation. Furthermore, we have shown that the inhibition of peroxisomal MAVS-dependent signaling by vMIA is dependent of MFF but not of FIS1 or DLP1. Additionally, we have demonstrated that vMIA and peroxisomal MAVS interact with MFF and, hence, suggest that MFF mediates the interaction between vMIA and MAVS at peroxisomes. schematic representation of our proposed model for the action of vMIA towards peroxisomes is presented in Figure 24.

Our results also show that peroxisomal and mitochondrial fragmentation mediated by vMIA does not depend on MAVS. In line with the observations reported in section 3.1, our results also strongly indicate that in the absence of DLP1, vMIA mediates mitochondrial but not peroxisomal fragmentation, pointing to an alternative fission pathway independent on DLP1 in mitochondria. It would be interesting to assess whether FIS1/TBC1D15/Rab7 pathway is involved in this fragmentation. Since this fragmentation process is not present at peroxisomes [123] the activation of FIS1/TBC1D15/Rab7 pathway may explain the opposite results regarding peroxisomes and

mitochondria. In addition, as FIS1/TBC1D15/Rab7 have been reported to regulate mitophagy [125] it would be also important to evaluate whether this fission process is associated with mitophagy by evaluating LC3 expression.

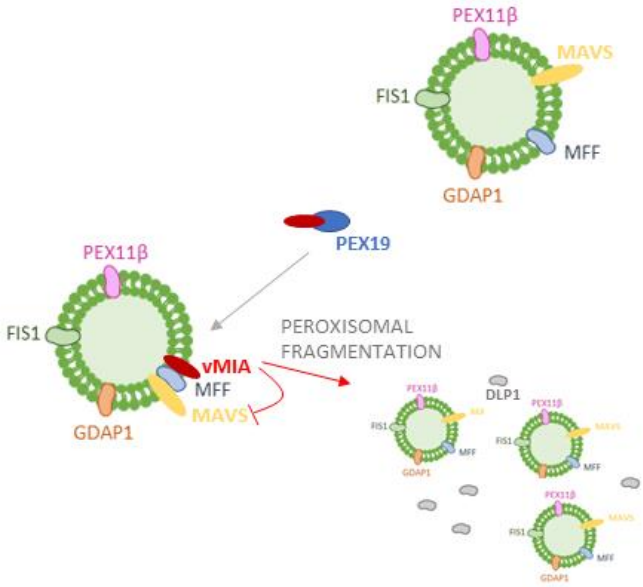


***Figure 24 - Proposed model for vMIA targeting to peroxisomes and interaction with peroxisomal membrane proteins.*** Upon HCMV infection vMIA is targeted to peroxisome´s membrane via PEX19. At peroxisomal membrane vMIA interacts with MFF which is required for vMIA to interact and to inhibit peroxisomal MAVS. In addition to the impairment of peroxisomal MAVS-dependent antiviral signaling vMIA leads to peroxisomal fragmentation which seems to not be correlation to this vMIA action.

The importance of peroxisomes as platforms for RLR immune signaling of both RNA and DNA viruses has been strengthened along the last years. With this work, we were able to expose specificities of peroxisomal and mitochondrial dynamics in HCMV evasion of the cellular antiviral response. Many questions have been solved but also many questions have been raised. Although vMIA induces fragmentation of both peroxisomes and mitochondria, peroxisomal fragmentation does not seem to be essential for vMIA to impair the peroxisomal MAVS-downstream signaling. Furthermore, our results indicate that MFF, interacts with vMIA and peroxisomal MAVS and is essential to vMIA´s function. We may hence speculate whether vMIA modulates peroxisomal morphology with the propose of controlling peroxisomal functions to enhance viral replication and dissemination. To further unravel this theory, the quantification of peroxisomal enzymes and/or products of peroxisomal metabolism after transfection of vMIA and during HCMV infection should be performed. A detailed mutagenesis analysis of the domains of vMIA that are responsible for the peroxisome's morphology change and/or the inhibition of the peroxisome-dependent antiviral

response may also help unravelling the real mechanisms by which the virus interacts with this organelle for its own benefit.

# 5 FINAL REMARKS

## 5.1 Concluding Remarks

With the work presented in this thesis we established specific differences between the peroxisomal and mitochondrial fission machineries by unravelling distinct roles of MFF and FIS1 in organelle fission and in HCMV evasion of the cellular antiviral response. Our results strongly indicate that MFF plays a crucial role at the regulation of peroxisomal fission whereas FIS1 significantly impacts mitochondrial fission events. Additionally, MFF was shown to interact with peroxisomal MAVS and vMIA and to play a crucial role in HCMV´s infection.

Altogether, these results empathize the importance of peroxisomal fission machinery for the RLR-mediated antiviral defense and may lead to the discovery of novel peroxisome-dependent mechanisms, which can ultimately be used as targets for antiviral therapy.

## 5.2 Publications Resulting from this work

Ana Rita Ferreira[1,2]*, **Ana Gouveia[1]***, Ana Cristina Magalhães[1], Isabel Valença[1], Mariana Marques[1], Jonathan C. Kagan[2] and Daniela Ribeiro[1], "Human cytomegalovirus' vMIA modulates peroxisome morphology and antiviral defense via MAVS and MFF" (soon to be submitted)

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