DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

The peroxisome-mitochondria connection

Identification and characterization of novel membrane proteins shared by both organelles

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Michael Schrader (Universidade de Aveiro) e do Professor Doutor Carlos Duarte (Universidade de Coimbra)

Inês Gomes de Oliveira e Castro

2011

Acknowledgments

I would like to thank Dr. Michael Schrader for the chance of working in his laboratory and thus develop this project. I am grateful for all his advice and work discussions throughout the year, and more importantly, for all the opportunities and encouragement he has given me.

I would like to thank Daniela for her guidance and confidence on me and on my work, and also for pushing me to take on new challenges and fight for my goals. And for the friendship too.

I would to thank Professor Carlos Duarte for his concern and willingness to help me.

I thank everyone who has welcomed me and helped me in the laboratory, particularly Conni for always being there, for trusting me and being my friend, and for all the silly and happy moments.

I thank all my friends who have supported me, especially Sofy for always laughing at, but sharing, my crazy ideas. I am very happy for having done this master degree together.

Finally, I would like to thank my family for all their support, especially my parents and my sister Bárbara who have always been there for me, helping me stay on the right track.

Table of contents

Abbreviations

Acetyl-CoA – acetyl coenzyme A
ALDP – adrenoleukodystrophy protein
ATP – adenosine-5'-triphosphate
A. niger – Aspergillus niger
BSA – bovine serum albumin
CLSM – confocal laser scanning microscope
DLP1 – dynamin-like protein 1
DMEM – Dulbecco's modified eagle medium
DMSO – dimethyl sulfoxide
DTT – dithiothreitol
ECL – enhanced chemiluminescence
EDTA – 2-Amino-2-(hydroxymethyl)-1,3-propanediol
E.coli – Escherischia coli
ER – endoplasmatic reticulum
ERAD – ER-associated protein degradation machinery
FBS – fetal bovine serum
Fis1 – mitochondrial fission 1 protein
Get3 – guided entry of TA proteins
GFP – green fluorescence protein
H. polymorpha – Hansenula polymorpha
HRP – horseradish peroxidase
IMF – immunofluorescence
IRD – infantile Refsum disease
ISG – interferon-stimulating gene
LCFA – long-chain fatty acids
MAO-A/B – monoamine oxidase A/B
Miro – mitochondrial Rho GTPase
MAPL – mitochondria-anchored protein ligase
MAVS – mitochondrial anti-viral signaling protein
MDA5 – melanoma differentiation-associated gene 5
MDV – mitochondria-derived vesicle
Mff – mitochondrial fission factor
MiD49/51 – mitochondrial dynamics proteins 49/51
MIPS – Munich information center for protein sequences
NALD – neonatal adrenoleukodystrophy

PAGE – polyacrylamide gel electrophoresis

PBD – peroxisomal biogenesis disorder

PBS – phosphate buffer saline

PCR – polymerase chain reaction

PED – peroxisomal single enzyme deficiency

PEI – polyethylenimine

Pex - peroxin

PMP – peroxisomal membrane protein

PPAR – peroxisome proliferator-activated receptor

PPRE – peroxisome proliferator response elements

PTS – peroxisomal targeting signal

RCDP – rhizomelic chondrodysplasia punctata

RIG-I – retinoic acid-inducible gene I

RLR – RIG-I-like receptor

ROS – reactive oxygen species

S. cerevisiae – Sacchromyces cerevisiae

SDS – sodium dodecyl sulfate

SIMAP – similarity matrix of proteins

SUMO – small ubiquitin-like modifier

TA – tail-anchored

TAE – tris-acetate-EDTA

TMD – transmembrane domain

TOM – translocase of the mitochondrial outer membrane

TPR – tetratricopeptide repeat motif

Tris – 2-Amino-2-(hydroxymethyl)-1,3-propanediol

U.maydis – Ustilago maydis

UV - ultraviolet

VLCFA – very-long-chain fatty acids

WB – western blot

ZS – Zellweger syndrome

ZSS – Zellweger syndrome spectrum

Abstract

Peroxisomes and mitochondria are known to act in concert, sharing a growing number of proteins and cellular functions. This connection includes metabolic cooperations and cross-talk (e.g. in fatty acid β-oxidation), a novel putative vesicular trafficking pathway from mitochondria to peroxisomes, an overlap in key components of their fission machinery as well as in signalling events leading to antiviral defence. These findings indicate that these organelles exhibit a closer interrelationship than previously expected. Thus, peroxisome alterations in metabolism, biogenesis, dynamics and proliferation can potentially influence mitochondrial functions, and *vice versa*, and might contribute to the onset of diseases.

There is currently great interest in the identification and characterization of other proteins shared by peroxisomes and mitochondria. The leading candidates are a class of integral membrane proteins with diverse cellular functions, known as tail-anchored (TA) proteins. The main focus of this project was thus to identify and characterize novel TA proteins that are dually targeted to peroxisomes and mitochondria. For this, an antibody/expression-based screening approach was followed which resulted in the identification of new shared components.

Our results show that Miro proteins are not only present on peroxisomes and mitochondria but also affect peroxisome motility. This effect is, in principle, due to the interaction of Miro proteins with kinesin motors, promoting anterograde transport of organelles. Furthermore, mutations in the calcium-binding domains of these proteins induce peroxisome aggregation within the cells and near the nucleus, suggesting an unknown function for calcium in the regulation of peroxisome dynamics. As organelle positioning and transport are crucial for cellular functions, especially in neurons, these findings are highly relevant for the study of peroxisome functions in health and disease.

Additionally, a bioinformatics analysis of the *Ustilago maydis* proteome was initiated in order to identify putative tail-anchored proteins. This model organism shares many important processes (e.g. long-distance microtubule transport, polarized growth) and high protein sequence similarity with human cells, while providing the technical advantages of yeast cells.

Key words: peroxisome, tail-anchored protein, mitochondria, organelle dynamics

Resumo

Os peroxisomas e as mitocôndrias partilham um número crescente de proteínas e funções celulares. As ligações entre estes dois organelos passam por uma cooperação metabólica e *crosstalk* (e.g. na β-oxidação de ácidos gordos), uma nova via de transporte de vesículas das mitocôndrias para os peroxisomas e a partilha de componentes da maquinaria de fissão e da via de sinalização que promove a defesa antiviral. Estas características mostram que os peroxisomas e as mitocôndrias exibem uma relação mais próxima do que a prevista. Assim, alterações no metabolismo, biogénese, dinâmica e proliferação dos peroxisomas poderão influenciar as funções das mitocôndrias, e vice-versa, e contribuir para o desenvolvimento de doenças.

Actualmente existe um grande interesse na identificação e caracterização de novas proteínas partilhadas pelos peroxisomas e mitocôndrias. Os principais candidatos são uma classe de proteínas membranares com diversas funções celulares conhecidas como proteínas *tail-anchored* (TA). Desta forma, o principal objectivo deste projecto foi a identificação e caracterização de novas proteínas TA presentes em ambos os organelos. Para tal, seguiu-se uma abordagem baseada na análise da localização destas proteínas com anticorpos ou por expressão destas proteínas, que resultou na identificação de novos componentes partilhados por estes dois organelos.

Os nossos resultados mostram que as proteínas Miro não só estão presentes nos peroxisomas e nas mitocôndrias, como também afectam a mobilidade dos peroxisomas. Este efeito dever-se-á, em princípio, a uma interacção entre as proteínas Miro e as cinesinas, promovendo o transporte anterógrado de organelos. Para além disso, mutações destas proteínas nos domínios de ligação a cálcio induziram a formação de agregados de peroxisomas na célula, sugerindo uma função até agora desconhecida para o cálcio na regulação da dinâmica dos peroxisomas. Tendo em conta que a posição e o transporte de organelos são cruciais para as funções celulares, especialmente em neurónios, estas descobertas são de elevada importância para o estudo dos peroxisomas na saúde e doença.

Adicionalmente, uma análise bioinformática do proteoma do fungo *Ustilago maydis* foi iniciada de modo a identificar proteínas TA putativas. Este modelo de estudo partilha vários processos importantes com as células humanas (e.g. transporte de longa distância por microtúbulos, crescimento polarizado) e elevada homologia ao nível proteico, permitindo assim tirar partido das várias vantagens técnicas de trabalhar com fungos.

Palavras-chave: peroxisoma, mitocôndria, proteína *tail-anchored*, dinâmica de organelos

Chapter 1 Introduction

1.1. Peroxisomes

General overview

Peroxisomes are virtually present in all eukaryotic cells, ranging from unicellular organisms to plants and mammals. They were initially described by Rhodin in 1954 as spherical and oval microbodies, found in mouse kidney cells (Rhodin, 1954). Only later, in 1966, they were metabolically characterized by Christian de Duve and his group, who identified several enzymes responsible for hydrogen peroxide metabolism (oxidases and catalase), therefore renaming them as peroxisomes (de Duve, 1965).

Peroxisomes harbour a wide array of metabolic functions, from lipid metabolism to reactive oxygen species (ROS) scavenging. These highly dynamic organelles are able to alter their morphology, number and metabolic functions in response to alterations in cellular environment (Kaur and Hu, 2009; Schrader and Fahimi, 2008).

Peroxisomes are delimited by a single lipid membrane that encloses a fine granular matrix, devoid of DNA and protein synthesis machinery. All peroxisomal proteins are encoded by nuclear genes and most of them are synthesized on free polyribosomes in the cytoplasm. Peroxisomes are usually found as spherical or rod-like forms (0.1 to 0.5 μm in diameter), but also as elongated tubular structures (up to 5 μm) and small tubulo-reticular networks (Schrader et al., 2000). Their number can range from just a few peroxisomes in yeast cells to up to several hundreds in mammalian hepatocytes.

The importance of peroxisomes for normal mammalian development and growth is underlined by the existence of a group of inherited peroxisomal disorders in humans, which are characterized by severe metabolic dysfunctions and neurological and developmental defects (reviewed in Baes and Aubourg, 2009; Shimozawa, 2011; Steinberg et al., 2006; Wanders et al., 2010).

1.1.1. Functions

Peroxisomes are “multipurpose” organelles whose functions vary according to species, cell type, developmental stage and environmental conditions. In most organisms they play an essential role in lipid metabolism, ROS generation and H_2O_2 metabolism. Moreover, they are also responsible for several anabolic and catabolic reactions such as purine catabolism, bile acid synthesis and glycerol synthesis (Fig.1.1) (Islinger et al., 2010; Wanders and Waterham, 2006a).

In addition to these main activities, peroxisomes carry out other functions such as photorespiration and the glyoxylate cycle in plant cells, glycolysis in trypanosomes and light emission in fireflies (Gould et al., 1987; Parsons, 2004; Reumann and Weber, 2006).

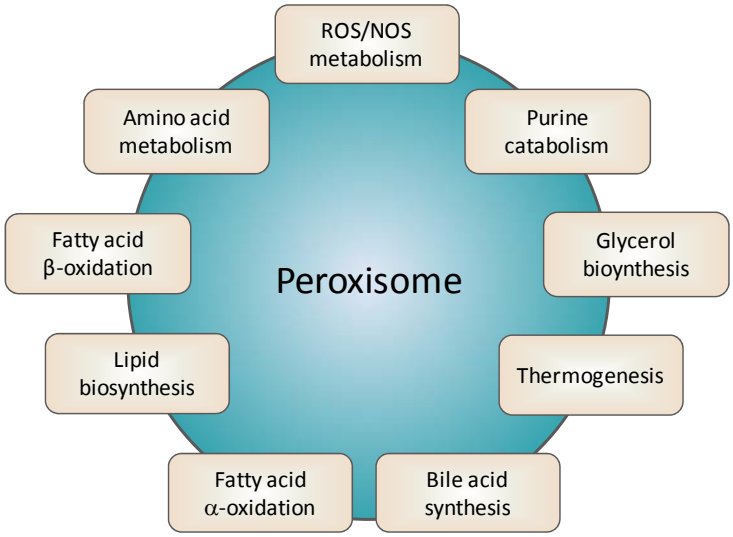

Figure 1.1 - Major metabolic functions of peroxisomes in mammals

1.1.1.1. Peroxisome lipid metabolism

Peroxisomes are responsible for three key pathways in lipid metabolism: fatty acid β-oxidation, fatty acid α-oxidation, and ether phospholipid biosynthesis.

In mammalian cells most fatty acids are degraded by means of β-oxidation. This process can be performed by both mitochondria and peroxisomes, although each organelle is responsible for specific substrates (see section 1.2.1.1) (Poirier et al., 2006).

The process of peroxisomal β-oxidation involves four consecutive reactions: (1) dehydrogenation, (2) hydration, (3) dehydrogenation again, and (4) thiolytic cleavage. After each cycle, fatty acids are shortened by two carbon atoms which are released as acetyl-CoA. In contrast to mitochondria, the peroxisomal β-oxidation system is unable to completely degrade fatty acids, shuttling its products to mitochondria for full oxidation. Due to the lack of a respiratory chain, peroxisomal β-oxidation is an obligatorily uncoupled and heat-generating process that, in cooperation with mitochondria, plays a major role in thermogenesis (Visser et al., 2007).

In higher eukaryotes, peroxisomes are the only site of α-oxidation. 3-methyl branched-chain fatty acids, such as phytanic acid, cannot undergo β-oxidation without first going through a step of α-oxidation. In this reaction, a terminal carboxyl-group is

removed as CO_2, and the resulting 2-methyl fatty acid is further degraded by β-oxidation (Jansen and Wanders, 2006).

Peroxisomes are also involved in the biosynthesis of ether-phospholipids, namely plasmalogens. Plasmalogens are the most abundant phospholipids in myelin, and are therefore essential for the development of the nervous system (Gorgas et al., 2006). Peroxisomes are responsible for the formation of the ether-bond present in these lipids, through the activity of alkyl-dihydroxyacetone phosphate synthase; further biosynthesis of these lipids is conducted in the ER.

1.1.1.2. ROS metabolism in peroxisomes

Many oxidative reactions take place in the peroxisomal lumen leading to the production of ROS (Antonenkov et al., 2010; Schrader and Fahimi, 2006b; Titorenko and Terlecky, 2011). The main by-product of peroxisomal fatty acid oxidation is H_2O_2 which is produced by the transfer of hydrogen to molecular oxygen by oxidases. This compound can then be converted into more aggressive oxygen radicals.

To balance out the production of ROS, peroxisomes also possess several antioxidant enzymes that degrade H_2O_2 to produce water and O_2. The most well known is catalase which can metabolize, in addition to H_2O_2, a variety of substrates such as ethanol, methanol, phenols and nitrites (Kirkman and Gaetani, 2007). Other enzymes, such as manganese and copper-zinc superoxide dismutases are responsible for the degradation of superoxide anions and hydroxyl radicals.

Although ROS have been shown to participate in cellular signalling (Murphy et al., 2011), an excess of these compounds can induce oxidative modifications of proteins, lipids and nucleic acids, and is associated with several pathological conditions such as cancer and neurodegenerative diseases (Bonekamp et al., 2009; Titorenko and Terlecky, 2011).

1.1.2. Peroxisome Biogenesis

Peroxisome formation and maintenance is dependent on a unique set of proteins called peroxins (Distel et al., 1996). Many of these proteins are conserved from yeast to mammals and mutations in their respective genes (PEX genes) are known to cause severe human disorders, such as Zellweger syndrome (Ebberink et al., 2011). Peroxins are usually grouped by their functions which can vary from the import of either matrix or membrane proteins, to membrane biogenesis and the regulation of peroxisome proliferation. Nevertheless, the main activity of some of these proteins is still unknown

and their proposed role in peroxisome biogenesis is based on the pleiotropic phenotypes of mutated cells and organisms.

Peroxisomes possess two distinct molecular machineries responsible for the import of matrix proteins and membrane proteins (reviewed in Ma et al., 2011; Rucktaschel et al., 2011). The complex machinery responsible for matrix protein import is substantially different from the ones present in other cellular compartments, due to its ability to import completely folded, co-factor bound and even oligomeric proteins (Meinecke et al., 2010). In this regard, this machinery has recently been compared to the ER-associated protein degradation (ERAD) machinery, giving rise to the concept of an export-driven import (Schliebs et al., 2010).

Also, in contrast to other organelles, the mechanism of peroxisome biogenesis is still a matter of debate, confronting a classical view of peroxisome generation by growth and division and a more recent view of *de novo* biogenesis from the ER (Mast et al., 2010).

1.1.2.1. Peroxisomal matrix protein import

According to the extended shuttle model, the import of matrix proteins can be divided in four steps: (1) receptor-cargo interaction; (2) docking at the peroxisome membrane; (3) translocation and release of cargo in the peroxisome lumen; and (4) receptor recycling (Rucktaschel et al., 2011).

Sorting of matrix proteins is mediated by the cytosolic receptors Pex5 and Pex7 which recognize, respectively, a PTS1 or PTS2 (Peroxisomal Targeting Signal type 1 and 2) sequence in their target proteins. The PTS1 is composed of a noncleavable tripeptide SKL (or conserved variants) located at the very C-terminus of the protein, and is used by most peroxisomal matrix proteins (Brocard and Hartig, 2006). On the other hand, the PTS2 is a nonapeptide (R/K)(L/V/I/Q)XX(L/V/I/H/Q)(L/S/G/A/K) X(H/Q)(L/A/F) near the N-terminus of a smaller subset of proteins, which can be cleaved inside the peroxisome lumen (Lazarow, 2006). Some proteins lacking these sequences can also be imported by interacting with PTS containing proteins in a “piggyback” manner (Islinger et al., 2009).

These receptors cycle between a soluble cytosolic form and an integral membrane-bound state. Upon receptor-cargo binding, the complex docks at the peroxisomal membrane with the docking complex, Pex13 and Pex14. The receptor is then thought to integrate into the membrane to form a transient cargo-translocation channel, releasing the cargo. In the end, the receptor is recycled by an export machinery that ubiquitinates the receptor (Pex4, Pex22, Pex2, Pex10 and Pex12) and removes it from the membrane (AAA-ATPase complex Pex1, Pex6 and Pex26). All the

membrane proteins involved in receptor docking, cargo translocation and receptor recycling are collectively termed the importomer (Brown and Baker, 2008; Meinecke et al., 2010).

1.1.2.2. Trafficking and insertion of peroxisomal membrane proteins (PMPs)

The mechanism by which PMPs are transported to and inserted in peroxisomes is still poorly understood. While some proteins are thought to be synthesized on free ribosomes in the cytosol and then transported to peroxisomes via Pex19 interaction, others are supposed to be targeted via ER-derived vesicles (Tabak et al., 2008).

Pex19 is a cytosolic receptor/chaperone that interacts with an internal targeting sequence of PMPs (Pex19 binding site) and delivers them to a docking complex at the peroxisome membrane, while preventing their aggregation in the cytosol (Schueller et al., 2010; Shibata et al., 2004). At the membrane, Pex19 interacts with Pex3 and, by a mechanism that is still unknown, promotes PMP insertion into the membrane (Fang et al., 2004). Pex16 is also involved in this process and it has been shown to interact with Pex3, but its function remains elusive (Matsuzaki and Fujiki, 2008). These three peroxins are essential for peroxisome membrane biogenesis in mammals, and loss of either one of them results in the complete loss of peroxisomes and peroxisomal membranes (Steinberg et al., 2006).

Transport of PMPs via the ER has been more recently debated and the transport of some peroxins by this pathway has been shown in different organisms (Hoepfner et al., 2005; Kim et al., 2006; Kragt et al., 2005). Proteins following this pathway are thought to gather in a specialized sub-domain of the ER and bud off in vesicles with the aid of Pex19. Recently, two reports have addressed this possibility in yeast (Agrawal et al., 2011; Lam et al., 2010). In a cell-free ER-budding assay using yeast cells, both articles report on the formation of vesicles containing Pex3 and either Pex15 (Lam et al., 2010) or Pex11 (Agrawal et al., 2011), which are formed in the presence of Pex19 and cytosolic factors. Nevertheless, it is still unknown how this process is regulated, which PMPs follow this pathway, and the role of Pex19 in the formation of the ER-derived vesicles.

1.1.2.3. Peroxisome formation: growth and division vs *de novo* synthesis

Peroxisomes are now regarded as semi-autonomous organelles that can multiply following a model of “growth and division”. Accordingly, peroxisomes grow by importing newly synthesized proteins from the cytosol and lipids from the ER, through close interactions with this organelle. Subsequently, peroxisomes follow a multistep pathway

that leads to their elongation, constriction and fission (Nagotu et al., 2010; Schrader and Fahimi, 2006a).

Recently, however, it has been reported that in mutant cells lacking essential peroxins and which are therefore without peroxisomes, reintroduction of these genes leads to *de novo* formation of peroxisomes from the ER (Hoepfner et al., 2005; Nagotu et al., 2010). This model of ER-dependent "*de novo* synthesis" is supported by several observations (reviewed in Mast 2010). Some peroxins, namely Pex16, have been shown to travel to peroxisomes via the ER (Yonekawa et al., 2011). Nevertheless, findings in *Saccharomyces cerevisiae* indicate that peroxisomes in wild-type cells only multiply by growth and division (Motley and Hettema, 2007).

The physiological significance of this process is still controversial and its relevance for the cellular pool of peroxisomes, when compared to the growth and division pathway, is still unknown. It might be that the *de novo* pathway only comes into play when, by defects in peroxisome segregation, a cell finds itself without peroxisomes (Mast et al., 2010). Overall, this raises the possibility of a semi-autonomous model where the ER supplies peroxisomes with some peroxins and lipids, enabling the growth and division of this organelle (Fig.1.2) (Hettema and Motley, 2009; Nagotu et al., 2010).

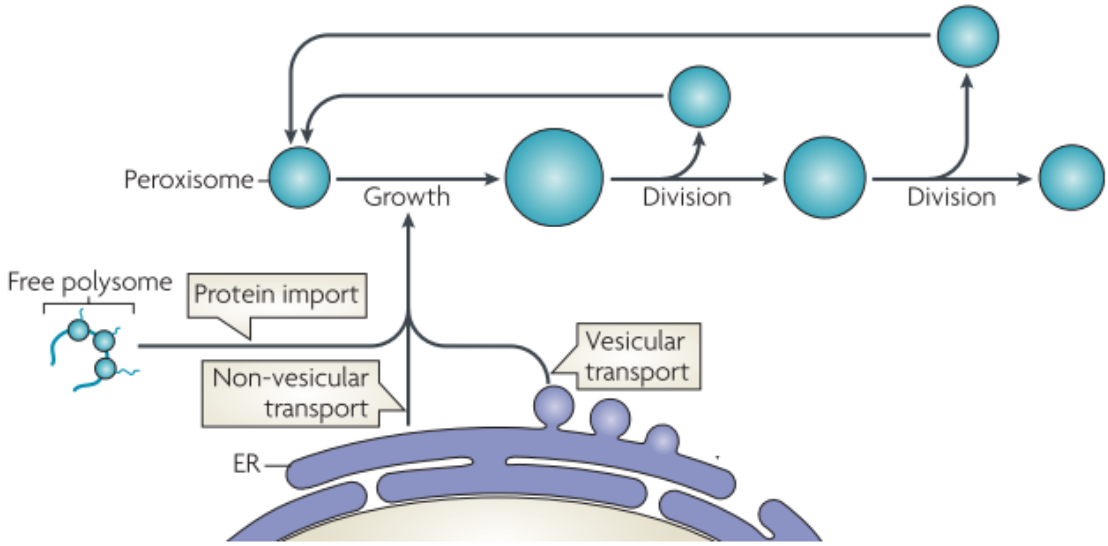

Figure 1.2 – Model of peroxisome growth and division. Peroxisome membrane expansion is achieved mainly by the import of lipids derived from ER, presumably though both vesicular and non-vesicular pathways (i.e. by close interaction of peroxisomal and ER membranes). All matrix proteins and some PMPs are acquired by post-translation import from the cytosol after synthesis on free polysomes. In contrast, some PMPs are transported from the ER via ER-derived vesicles. Adapted from (Fagarasanu et al., 2010)

1.1.3. Peroxisome dynamics

1.1.3.1. Proliferation

Peroxisomes are highly dynamic organelles that adapt their morphology, abundance and distribution in response to a wide range of stimuli (Fig.1.3) (Kaur and Hu, 2009; Platta and Erdmann, 2007).

Peroxisomal proliferation can be induced by nutritional and environmental cues such as increases in free fatty acid uptake, cold exposure and treatment with fibrates (Bagattin et al., 2010; Hoivik et al., 2004; Pollera et al., 1983). These stimuli induce an increase in the number and size of peroxisomes and promote the expression of several peroxisomal enzymes. In mammalian cells this response is mediated by a family of ligand-activated transcription factors, the peroxisome proliferator activated receptors (PPARs) (Rakhshandehroo et al., 2010). There are three different PPAR subtypes: PPARα, PPARβ/δ and PPARγ, that form heterodimers with the retinoid X receptor to bind peroxisome proliferator response elements (PPRE) in target genes. These transcription factors are usually activated by lipid-ligands and regulate the expression of genes associated with lipid metabolism and adipocyte differentiation (Kliewer et al., 1992).

PPARα is a major regulator of hepatic lipid metabolism, promoting the activation of genes involved in peroxisomal and mitochondrial β-oxidation. In addition to being activated by a wide variety of fatty acids, this receptor also binds synthetic ligands, namely fibrates, which can be used for the treatment of dyslipidemia (Staels and Fruchart, 2005).

Peroxisome biogenesis can also be regulated by the PPARγ coativator-1α (PGC-1α) in brown fat tissue, in response to cold exposure. PGC-1α specifically affects enzymes responsible for β-oxidation, as well as Pex11β which promotes peroxisome proliferation. Unexpectedly, this effect is independent of PPARα and it induces genes that lack PPRE sites in their promoters (Bagattin et al., 2010).

Additionally, dysfunction of peroxisomes can potentially promote the proliferation of other organelles, such as mitochondria, as a compensatory response. For example, impairment of peroxisomal biogenesis or function induces structural and functional changes in mitochondria (Baumgart et al., 2001). Conversely, loss of mitochondrial respiratory function promotes peroxisome proliferation (Epstein et al., 2001).

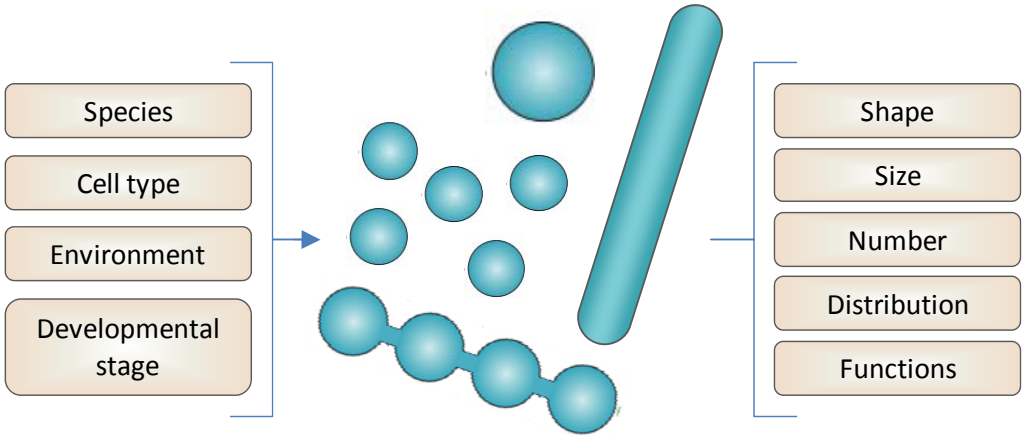

Figure 1.3 – Effect of several variables on peroxisome morphology, function and dynamics.

1.1.3.2. Division of peroxisomes

Growth and division of peroxisomes in mammalian cells is a multistep process that starts with the formation of elongated peroxisomes, constriction to a "beads-on-a-string" morphology and final fission and formation of spherical organelles (Schrader and Fahimi, 2006a).

The Pex11 family of proteins is implicated in the elongation of peroxisomal membranes in mammals, plants and yeast (Kaur and Hu, 2009; Schrader and Fahimi, 2006a; Thoms and Erdmann, 2005). In general, an increase in Pex11 protein levels induces peroxisome proliferation, while a loss of function leads to fewer, enlarged peroxisomes. The three mammalian Pex11 isoforms, designated Pex11α, Pex11β and Pex11γ, are integral PMPs and have both their amino and carboxyl termini exposed to the cytosol. All Pex11 proteins interact with themselves, and are likely to form homo-oligomers or homo-dimers (Koch et al., 2010; Thoms and Erdmann, 2005). Pex11α is the only isoform to be induced by peroxisome-proliferating agents. Pex11γ is the least studied isoform although new data suggests a role for it in the initiation of peroxisome elongation by interacting with either Pex11α or Pex11β (Koch et al., 2010).

In mammals, Pex11β induces the most prominent peroxisome proliferation. Overexpression of this protein results in peroxisome tubulation, followed by an increase in peroxisome number (Schrader et al., 1998). However, in mutant cells lacking DLP1, overexpression of Pex11β induces hypertubulation without an increase in peroxisome number, suggesting that Pex11 proteins cannot constrict and divide peroxisomes themselves, most likely functioning upstream of DLP1 by promoting peroxisome membrane tubulation (Koch et al., 2003). Mice lacking Pex11β display many of pathological features of Zellweger syndrome, which include neuronal migration defects, developmental delay, hypotonia and neonatal lethality (Li et al., 2002b). These

mice are only mildly affected in peroxisomal protein import and metabolism, suggesting that some of the pathological features of Zellweger syndrome are not caused by major alterations in metabolism but by compromising peroxisome dynamics. In contrast, mice lacking Pex11α are morphologically indistinguishable from wild-type ones (Li et al., 2002a).

The exact molecular mechanism by which these proteins promote organelle elongation is still a matter of discussion (Thoms and Erdmann, 2005). Recent data has shown that Pex11 proteins contain a conserved amphipathic helix at their N-terminus that interacts with membranes. This domain has been shown to mediate membrane curvature and elongation in vesicles with similar lipid composition to peroxisomal membranes (Opalinski et al., 2011).

Peroxisome elongation also implies a segregation mechanism prior to constriction that enables the production of proficient peroxisomes. Recent data from our laboratory has shown that, upon inhibition of peroxisome constriction and division by a Pex11β-YFP fusion protein, peroxisomal membrane and matrix proteins are segregated to different regions of peroxisomal structures by an unknown mechanism (Delille et al., 2010).

In recent years, several proteins of the molecular machinery responsible for peroxisome fission have been identified. This process is conserved from yeast to mammals and plants, and is mediated by members of the dynamin family of large GTPases. In mammals, peroxisome fission requires the dynamin-like protein DLP1 and the membrane adaptors Fis1 and Mff. DLP1 is believed to assemble into higher ordered ring-like structures in a GTP-dependent manner that wrap around membrane tubules to sever the membrane in a GTP hydrolysis-dependent process (Praefcke and McMahon, 2004). Fis1 and Mff are both C-tail anchored membrane proteins with cytosolic domains that favour protein-protein interaction. Fis1 contains an N-terminal tetratricopeptide repeat (TPR) motif, whereas Mff exposes its N-terminal part with a central coiled-coil motif into the cytosol (Gandre-Babbe and van der Bliek, 2008; Serasinghe and Yoon, 2008). Pex11β has been shown to form a heterocomplex with some of these proteins, namely Fis1 and DLP1 (Kobayashi et al., 2007), interacting indirectly with DLP1 through Fis1. Interestingly, some of these components are shared by both peroxisomes and mitochondria (see section 1.2.3) (Schrader, 2006).

1.1.3.3. Selective peroxisome degradation

Upon withdrawal of a peroxisome proliferating stimulus, this process is reversed and excess particles are removed by pexophagy. This selective process of

degradation through an autophagy-related mechanism can occur by either macropexophagy or micropexophagy (Klionsky et al., 2007).

Autophagy depends on the interplay between the organelle's proteins with the autophagic machinery. In macropexophagy, peroxisomes are selectively surrounded by autophagosomal membranes while in micropexophagy one or more peroxisomes are engulfed by invaginations of vacuoles/lysosomes. In both processes, peroxisomes are degraded by lysosomal hydrolytic enzymes and recycled as free amino acids, lipids, and carbohydrates for macromolecular synthesis and/or energy production (Yorimitsu and Klionsky, 2005). Up to 30 different genes have been shown to control pexophagy, many of which are conserved from yeast to mammals.

Pexophagy has been mainly studied in the methylotrophic yeast species *Hansenula polymorpha* and *Pichia pastoris*. In *H. polymorpha* two peroxins play a major role in the recognition of peroxisomes for macropexophagy - Pex3 and Pex14. One of the first steps in this process seems to be the removal of Pex3 from the peroxisomal membrane and its degradation, followed by recognition of peroxisomes containing Pex14 by the macroautophagic machinery (Todde et al., 2009).

Peroxisome degradation in mammalian cells is not so well characterized. A few proteins such as Atg7, Pex14 and p62 have been associated with macropexophagy (Todde et al., 2009). Curiously, a second form of peroxisome degradation has been described involving peroxisome lysis. This process is mediated by 15-lipoxygenase and induces the release of peroxisomal contents that are then degraded by cytosolic proteases (Yokota, 2003).

1.1.3.4. Peroxisome motility

Eukaryotic cells strictly regulate the movement and distribution of their organelles in order to guarantee their optimal activity and inheritance to daughter cells.

Like with other organelles, peroxisome movement through the cell is dependent on cytoskeletal tracks. In yeast and plants, peroxisomes move through the actin cytoskeleton with the aid of type-V myosins (Fagarasanu et al., 2010; Muench and Mullen, 2003), whereas in mammalian cells they have been shown to interact with microtubules both *in vivo* (Schrader et al., 1996) and *in vitro* (Schrader et al., 2000). In mammals, the majority of peroxisomes (85-90%) exhibit a slow, energy-free and microtubule-independent oscillatory movement, while a smaller population (10-15%) exhibits a fast, directional movement which is dependent on microtubules. Peroxisomes move both towards and away from the cell centre, in a dynein and kinesin dependent manner (Kural et al., 2005; Schrader et al., 2003). Strikingly, the proteins

that recruit the translocation machineries to the peroxisomal membrane are yet to be found.

Recently, Bharti et al. (2011) proposed a new role for Pex14 in the regulation of peroxisome motility. This peroxin, which is involved in the translocation of peroxisomal matrix proteins and has been associated with macropexophagy (de Vries et al., 2006), was shown to bind tubulin directly. The authors propose that this interaction is necessary to regulate peroxisome motility and suggest that this protein anchors peroxisomes to microtubules, promoting their degradation by pexophagy.

Several microtubule-active drugs have been used to study the importance of microtubule-dependent movement for peroxisome dynamics (Schrader et al., 1996; Wiemer et al., 1997). Depolymerising agents, such as nocodazole, were shown to disrupt the intracellular distribution of peroxisomes while promoting organelle elongation. Although these organelles can still constrict and divide, their distribution is affected, suggesting that microtubule-dependent movement is important for the correct segregation of peroxisomes.

In addition, cells from patients with peroxisomal disorders possess enlarged and less abundant peroxisomes, which tend to cluster and detach from microtubules. Overexpression of Pex11β in these cells promotes peroxisome proliferation and redistribution throughout the cell, suggesting that control over peroxisome dynamics is essential for peroxisome activity (Nguyen et al., 2006).

1.1.4. Peroxisomal disorders

The importance of peroxisomes for human health is demonstrated by a group of genetic disorders that promote peroxisome metabolic dysfunction and widespread organ pathology (Wanders, 2004). These disorders are characterised by severe alterations of neuronal development and by affecting several organs such as the liver and kidneys.

Peroxisomal disorders are usually divided into two groups: peroxisome biogenesis disorders (PBDs) and peroxisomal single enzyme deficiencies (PEDs).

In the case of PBDs, the affected protein is a peroxin, which is involved in the biogenesis and maintenance of peroxisomes (reviewed in Steinberg et al., 2006; Wanders and Waterham, 2005; Weller et al., 2003). As a result, cells either lack peroxisomes or only possess empty and non-functional peroxisomal membrane “ghosts”. The PBD group comprises Zellweger spectrum disorders (ZSS) (which include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease(IRD)) and rhizomelic chondrodysplasia punctata (RCDP) type I.

Patients with ZS usually die within the first year of life and suffer from severe neurological abnormalities, neonatal hypotonia, facial dysmorphism, hepatomegaly, renal cysts and adrenal atrophy. These diseases are characterized by an accumulation of very-long-chain fatty acids (VLCFA), bile acid precursors, pristanic and phytanic acid, and a decrease in plasmalogens synthesis.

Patients with PEDs carry defects in one of several enzymes involved in peroxisomal metabolism, altering β-oxidation, ether-lipid synthesis, α-oxidation, glyoxylate detoxification and ROS metabolism (reviewed in Wanders et al., 2010; Wanders and Waterham, 2006b). The most common of these disorders is X-linked Adrenoleukodstrophy where β-oxidation is affected. This disease is caused by a defect in the ABCD1 gene which encodes an ABC half-transporter responsible for VLCFA transport into peroxisomes. Mutations in this gene inhibit the oxidation of VLCFA and thus induce the accumulation of these toxic lipids in the blood.

The genetic basis of most peroxisomal disorders is now well known but the actual translation from the molecular/cellular phenotype to whole organism pathology is still a matter of discussion.

1.2. The Peroxisome-Mitochondria connection

General overview

Mitochondria and peroxisomes are ubiquitous subcellular organelles that share morphological and functional similarities. They are highly dynamic and exhibit great plasticity, adopting a variety of shapes.

Mitochondria are generally seen as the powerhouse of the cell, generating most of the energy derived from the breakdown of lipids and carbohydrates. They have an unusual structure organized by two membranes, in which the inner membrane is highly folded into cristae and is the main site of ATP synthesis (McBride et al., 2006).

Most peroxisomal and mitochondrial proteins are synthesized on free polyribosomes on the cytoplasm and are directed to each organelle by specific targeting sequences. The import of proteins to each organelle is selective and depends on the presence of targeting factors, receptors and different translocation machineries.

Peroxisomes and mitochondria have long been shown to share some metabolic functions, such as β-oxidation and ROS degradation (see section 1.2.1), but it was only recently that other interconnections became evident (Schrader and Yoon, 2007). For instance, peroxisomes and mitochondria share components of their division machinery,

coordinate antiviral response and may communicate through a mitochondria-to-peroxisome vesicle pathway (Fig.1.4) (Dixit et al., 2010; Neuspiel et al., 2008; Schrader, 2006). In line with this, as the basic components of these pathways are shared by both organelles, dysfunction of these components is likely to affect both organelles and lead to combined diseases (Camões et al., 2009).

1.2.1. Metabolic cross-functions

1.2.1.1. Beta oxidation

In animals, both peroxisomes and mitochondria perform fatty acid β-oxidation, while in plant cells and eukaryotic microorganisms, peroxisomes are thought to be the only site of fatty acid β-oxidation, which makes them essential for the use of fatty acids in these organisms (Poirier et al., 2006).

Although the steps of fatty acid β-oxidation in mitochondria and peroxisomes are identical, each organelle possesses its own set of enzymes, which differ in their molecular and catalytic properties. Furthermore, each organelle is responsible for the breakdown of different substrates, as peroxisomes oxidize more complex compounds such as VLCFA, dicarboxylic acids, bile acid precursors, prostanglandins, leukotrienes and mono- and polyunsaturated fatty acids, whereas mitochondria degrade the majority of long-chain fatty acids (LCFA) such as palmitate, oleate and linoleate, supplying acetyl-CoA for ATP production and anabolic reactions (Schrader and Yoon, 2007).

In contrast to mitochondria, the peroxisomal β-oxidation system is unable to completely degrade fatty acids, thus shuttling its products to mitochondria for full oxidation. This process involves a coordinated regulation of both organelles' lipid metabolism, as well as metabolite transfer through a mechanism that is still not well understood (Visser et al., 2007).

Furthermore, the PPARγ coactivator-1α (PGC-1α) has been recently identified as a common regulator of peroxisome and mitochondria remodelling and biogenesis, in brown fat tissue (Bagattin et al., 2010). PGC-1α plays a central role in the regulation of energy homeostasis and is strongly induced in response to cold exposure, linking this environmental stimulus to adaptive thermogenesis.

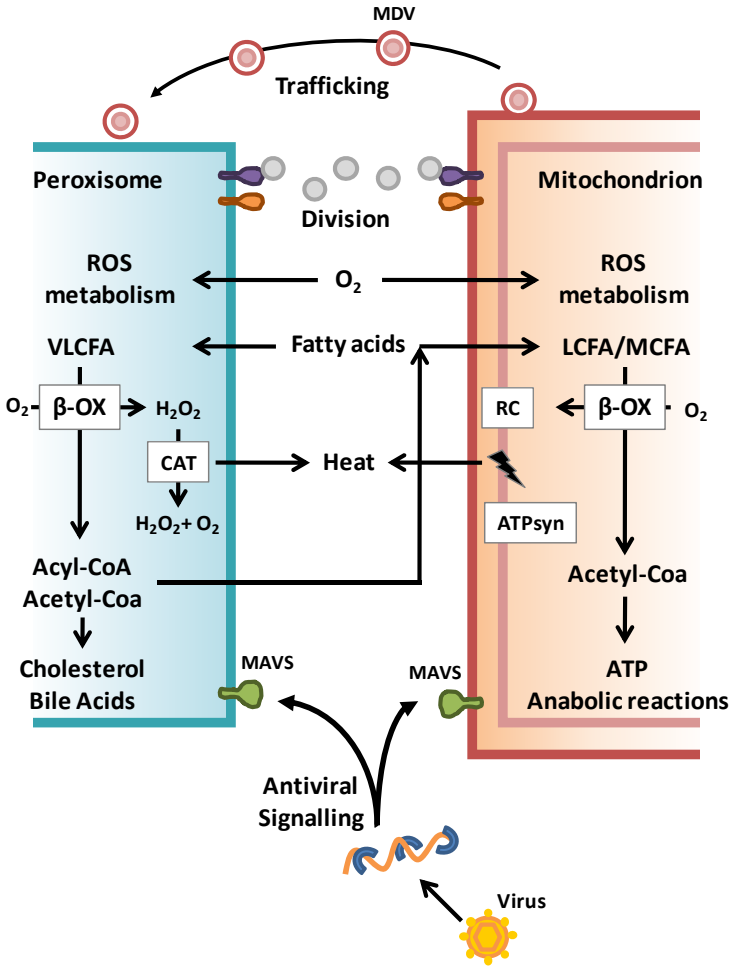

Figure 1.4 – Schematic view of peroxisomal and mitochondrial cooperation and cross-talk. MDV, mitochondrial derived vesicle; ROS, reactive oxygen species; VLCFA, very long-chain fatty acids; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; β-OX, fatty acid β-oxidation; CAT, catalase; RC, respiratory chain; ATPsyn; ATP synthetase. Adapted from (Schrader and Yoon, 2007)

1.2.1.2. ROS metabolism

ROS are on the one hand critical regulators of multiple cellular signalling pathways but on the other, they can act as negative counterparts of metabolic activity, damaging lipids, proteins and nucleic acids.

In mammalian cells, peroxisomes and mitochondria are two major sources of ROS and have a key role in the maintenance of the cellular redox balance. Thus, dysfunction in either organelle that leads to ROS generation is prone to promote pathological conditions such as cancer and neurodegenerative disordes, and has been associated with ageing (Beal, 2005; Bonekamp et al., 2009; Titorenko and Terlecky, 2011).

Peroxisomes contain several enzymes responsible for the production of H_2O_2, O^-_2 and NO, as metabolic by-products. They also contain several anti-oxidant enzymes such as catalase, Cu/Zn-superoxide dismutase and peroxiredoxin V (Schrader and Fahimi, 2006b). Mitochondria produce superoxide radicals mainly through complex I

and complex III of the respiratory chain, which can be decomposed by several enzymes such as manganese superoxide dismutase, glutathione dismutase and glutathione peroxidase.

How each organelle contributes to the cellular pool of ROS is still a matter of debate. The concept that mitochondria are the main source of cellular ROS has been recently disputed (Brown and Borutaite). Meanwhile, a recent paper regarding the effects of ROS on different cellular compartments has extended the understanding of the redox connection between peroxisomes and mitochondria (Ivashchenko et al., 2011). Interestingly, it was reported that an increase in ROS generation inside peroxisomes disturbs the mitochondrial redox state. Also, in catalase-deficient cells, peroxisomes maintain their redox balance while the mitochondrial membrane potential is significantly altered. This suggests that peroxisomes hold a mechanism to cope with alterations in their redox state, while signalling a mitochondrial stress response. In line with this, previous reports have shown that increasing peroxisomal catalase levels restore mitochondrial integrity in late-passage human cells (Koepke et al., 2007), while reducing catalase activity affects mitochondrial membrane potential and stimulates the cell's ageing process (Koepke et al., 2008; Titorenko and Terlecky, 2011).

1.2.1.3. Biotin synthesis

Biotin, also known as vitamin B_7, is a cofactor essential for many cellular carboxylation and decarboxylation reactions, fatty acid biosynthesis, gluconeogenesis and amino acid metabolism (Streit and Entcheva, 2003). In plants and fungi part of the biosynthesis of this compound takes place in mitochondria, whereas the initial steps that lead to its production remain largely unknown.

Recently, a new role for peroxisomes in the synthesis of biotin was described by Tanabe et al. (2011) in plants and fungi. The authors show that BioF, a protein responsible for one of the early steps of biotin synthesis, has a PTS1 sequence both in plants and fungi. Moreover, the targeting of this protein to peroxisomes in required for biotin biosynthesis. This finding reinforces the stance of peroxisomes as a multipurpose organelle and strengthens the metabolic connection between peroxisomes and mitochondria.

1.2.2. Cooperative antiviral signalling

The cellular antiviral response is usually triggered by the recognition of viral components in the cytoplasm. Viral genomes can be detected by soluble RNA helicases, such as RIG-I and MDA5, which interact with the mitochondrial TA protein

MAVS, triggering a signalling pathway that promotes antiviral defence (Kumar et al., 2011).

Recently, it has been shown that MAVS also localizes to peroxisomes, and that peroxisomes in concert with mitochondria serve as signalling platforms in antiviral defence. This new peroxisomal function highlights the important role of this organelle in health and disease. Both peroxisomal and mitochondrial MAVS are required for a strong antiviral response (Dixit et al., 2010): peroxisomal MAVS promotes an early response through the induction of interferon-stimulating genes (ISGs), providing short-term protection, whereas mitochondrial MAVS induces a delayed response through the induction of ISGs and type I interferons, amplifying and stabilizing the antiviral response. Interestingly, activation of the MAVS pathway induces mitochondrial elongation, which, in turn, modulates antiviral signalling downstream from MAVS (Castanier et al., 2010). In line with this, viral stimulation also induces the formation of peroxisomal tubules (Dixit et al., 2010), suggesting that a change on this organelle's morphology might also contribute to the antiviral response.

1.2.3. Organelle division by DLP1, Fis1 and Mff

It is now evident that mitochondria and peroxisomes share components of their fission machinery, following a similar division strategy for mammals, yeast and plants (Fig.1.5) (Delille et al., 2009). The first protein discovered to be involved in this process in both organelles was the dynamin-like protein DLP1 (Dnm1 in yeast), a large GTPase that is involved in the tubulation and fission of cellular membranes (Li and Gould, 2003). Although DLP1 is cytosolic, fractions of this protein are recruited to the organelle membrane at sites of constriction. DLP1 oligomers form a ring-like structure that wraps around constricted membranes, inducing final membrane fission in a GTP hydrolysis-dependent manner. In line with this, inhibition of DLP1 function leads to the formation of highly elongated peroxisomes and mitochondria (Koch et al., 2003). Even though they are unable to divide, these peroxisomes still display a constricted morphology, which indicates that DLP1 is necessary for fission but not for constriction (Koch et al., 2004).

Recruitment of DLP1 to mitochondria and peroxisomes requires adaptor proteins present at the organelle membrane. The TA adaptor protein Fis1 is present on both organelles, and in yeast it interacts with Mdv1 and Caf4 in order to anchor Dnm1 to the membrane (Koch et al., 2005; Motley et al., 2008). In mammals, Fis1 has been shown to affect organelle division: inhibition of Fis1 results in the elongation of mitochondria and peroxisomes while its overexpression promotes organelle division/fragmentation

(Koch et al., 2005). However, the role of this protein in DLP1-dependent fission in mammalian cells is still a matter of discussion. Fis1 localizes throughout the membrane in contrast to the punctate localization of DLP1, and the downregulation of Fis1 only partially inhibits the recruitment of DLP1 to the membrane. Also, knockout of this gene in a carcinoma cell culture model did not hamper mitochondrial fission (Otera et al., 2010). Accordingly, another protein has been proposed to anchor DLP1 to the mitochondrial membrane. Mff (mitochondrial fission factor) is the most recent component of the fission machinery to be found on both organelles (Gandre-Babbe and van der Bliek, 2008). This protein has been shown to recruit DLP1 to mitochondria and promote mitochondrial fission. Furthermore, knockdown of Mff promotes peroxisome and mitochondrion elongation, similar to that observed after DLP1 silencing.

It is probable that more components of the fission machinery, which are yet to be identified, are shared by both organelles.

As a matter of fact, two new proteins have recently been identified as part of the mitochondrial division machinery (Fig.1.5). The N-tail anchored membrane proteins Mid49 and Mid51 were shown to form foci and rings at the mitochondrial membrane, suggesting a role for these proteins in the recruitment of DLP1 for mitochondrial fission (Palmer et al., 2011). Interestingly, Zhao et al. (2011) has also shown that Mid51 recruits DLP1 to the mitochondrial membrane but proposes that this protein sequesters DLP1, inhibiting mitochondrial fission. Furthermore, this report also shows an interaction between Fis1 and Mid51, that it is mutually exclusive from its interaction with DLP1. Subsequently, a new model for the regulation of mitochondrial fission/fusion has been proposed (Dikov and Reichert, 2011)

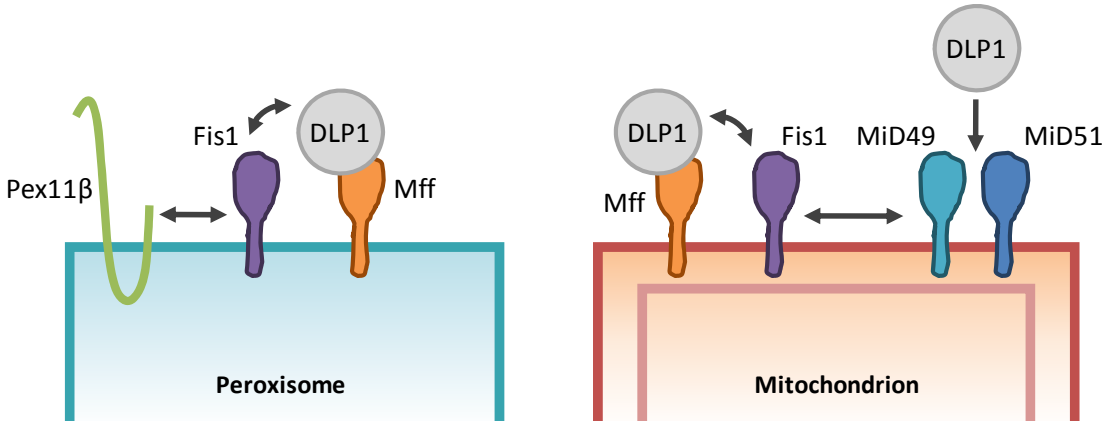

Figure 1.5 – Peroxisomal and mitochondrial fission. Organelle division requires the TA protein Mff, which is thought to recruit DLP1 to the membrane to promote organelle fission. Fis1 also contributes to organelle fission although its function in mammals is still a matter of discussion. In peroxisomes, Fis1 has been shown to interact with the elongation factor Pex11β. In contrast, in mitochondria two new factors – MiD49 and MiD51 – have also been associated with the regulation of mitochondrial fission/fusion. The interaction of these proteins with Fis1 suggests a regulatory function for Fis1 in mitochondria.

1.2.4. Peroxisome to mitochondria vesicular trafficking

Recently, McBride and colleagues proposed a novel vesicular transport pathway from mitochondria to peroxisomes in mammalian cells (Neuspiel et al., 2008). The authors identified a new mitochondrial-anchored protein ligase (MAPL), which is a small ubiquitin-like modifier (SUMO) E3 ligase that positively regulates the fission GTPase DLP1. This protein is incorporated into a set of mitochondria-derived vesicles (MDVs) that form by lateral segregation of the mitochondrial membrane in a DLP1-independent manner. Through live-cell imaging experiments, MAPL-positive MDVs were observed to fuse with a small subpopulation of peroxisomes. It was also demonstrated that MAPL is found in a complex with Vps35 and Vps26 (Braschi et al., 2010). These two proteins are part of the retromer complex which is responsible for the vesicle transport from endosomes to the Golgi apparatus. Silencing of these two proteins inhibited the formation of MAPL-positive MDVs and their delivery to the peroxisome, confirming the function of the retromer complex in the modulation of MDV formation and transport to the peroxisome.

Although the physiological function of this pathway is still unknown, it may act as a new means for the transport of proteins, lipids or metabolites to a subpopulation of peroxisomes. This pathway might also retrieve peroxisomal proteins which have been mistargeted to mitochondria due to some overlap in the targeting sequences of peroxisomal and mitochondrial proteins (Schrader and Fahimi, 2008).

1.2.5. Organelle dynamics and disease

Due to their important roles in cellular metabolism, peroxisomal and mitochondrial dysfunction can lead to severe neurological and developmental disorders (Camoes et al., 2009).

Mitochondrial disorders are a heterogeneous group of diseases associated with severe muscular and neuronal dysfunction (DiMauro, 2004). Defects in mitochondrial fission/fusion proteins affect the number, shape and distribution of mitochondria in the cell, altering several pathways such as calcium signalling, neuronal plasticity and apoptosis. Mitochondrial networks are thought to promote rapid membrane potential transmission and offer a pathway for defective mitochondria to regain their lacking components through fusion with other mitochondria (Detmer and Chan, 2007). Furthermore, elongated mitochondria are able to escape autophagic degradation and maintain ATP production, sustaining cell viability (Gomes et al., 2011). The advantages of fission are not yet clear, but this process enables the transport of mitochondria to

different cellular locations and is thought to allow mitophagy by segregation of defective mitochondria.

Owing to the importance of mitochondrial dynamics in cell function and its effect on disease, alterations in peroxisomal dynamics may as well have a major role in cellular dysfunction. Since the basic components of the fission machinery are shared by both organelles, it is likely that defects in these proteins affect both organelles and lead to combined diseases.

In line with this, the first case of a newborn with a defect in the division machinery of both organelles has been recently reported (Waterham et al., 2007). The patient, who died few weeks after birth, showed signs of both mitochondrial and peroxisomal dysfunction, such as defects in lipid metabolism and mitochondrial respiration. A close analysis of the patient's genotype revealed a dominant-negative mutation in the middle domain of the DLP1 protein, which interfered with the ability of DLP1 to form oligomers (Chang et al., 2010). Furthermore, skin fibroblasts from the patient showed elongated peroxisomes and mitochondria, which indicate a block in DLP1-dependent fission.

Moreover, a DLP1 knockout mouse has been generated which shows developmental abnormalities that result on embryonic lethality (Ishihara et al., 2009). Cells from these mice show elongated peroxisomes and mitochondria, confirming the results obtained with siRNA and mutational studies in cell culture.

At present, it is unclear to what extent defects in mitochondrial or in peroxisomal function contribute to the clinical phenotype of the patient and to the pathological alterations observed in the knockout models.

Moreover, since peroxisomes and mitochondria are involved in ROS metabolism, alterations in the production and scavenging of ROS are thought to promote the onset of several disorders such as Type 2 diabetes, inflammation, cancer and neurodegenerative conditions such as Parkinson's disease and Alzheimer's disease (Kou et al., 2011). Additionally, they have also been associated with ageing (Beal, 2005; Titorenko and Terlecky, 2011).

1.3. Dual targeting of TA proteins to peroxisomes and mitochondria

General overview

As mentioned above, peroxisomes and mitochondria share some components of their fission machinery and antiviral responsive elements. Surprisingly, all known membrane anchored proteins that are shared by these organelles are TA proteins

(Fis1, Mff and MAVS). This feature is quite interesting and it allows us to investigate the mechanism through which these proteins are selectively transported to their organelles, how they are inserted and, on a broader view, the evolutionary process that favoured their function on both organelles.

Tail-anchored (TA) proteins constitute a heterogeneous group of integral membrane proteins that mediate several essential biochemical activities, such as vesicular trafficking, apoptosis, signal transduction and redox reactions (Borgese and Fasana, 2011). In eukaryotes, these proteins can be found in the mitochondrial and chloroplast outer membrane, peroxisomes, and intracellular compartments that are connected to the secretory and endocytic pathway.

TA proteins are characterized by a N-terminal cytosolic region that usually contains the functional domains, a single hydrophobic transmembrane domain (TMD) located near the C-terminus, that anchors them to the lipid bilayer, and a short C-terminal polar tail with no more than 30 amino acids, that protrudes into the organelle matrix (Fig.1.6) (Borgese et al., 2003). Due to the proximity of the TMD to the C-terminus, this tail only emerges from the ribosome after complete translation, and thus, these proteins are only inserted into membranes post-translationally.

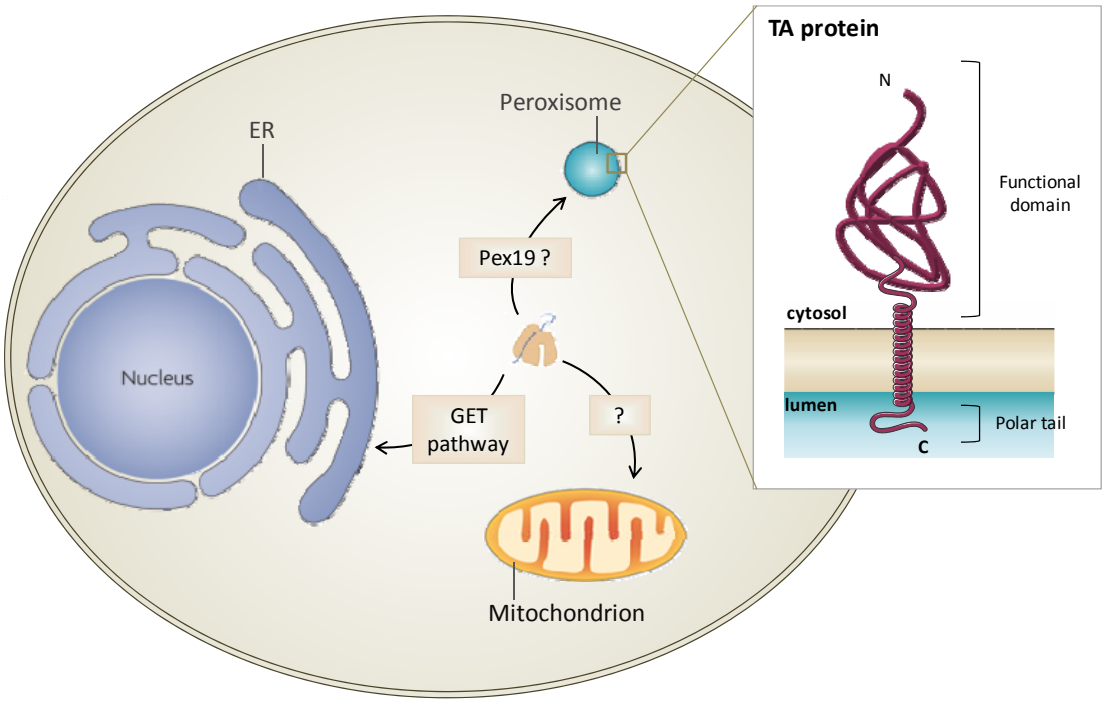

Figure 1.6 – Pathways of TA protein targeting and insertion in cellular membranes. After being released from a ribosome, a TA protein can insert into the ER, the mitochondrial outer membrane or the peroxisomal membrane. Targeting and transport to the ER is promoted by the GET pathway, whereas transport and insertion into the peroxisomal membrane is thought to depend on the cytosolic peroxin Pex19. No mechanism has yet been unveiled for transport and insertion of mitochondrial TA proteins. The structure of a typical TA protein is depicted in the cut out.

Upon being released from ribosomes, new proteins are immediately targeted and inserted into their respective membrane (Borgese et al., 2007; Rabu et al., 2009). The signalling for targeting is usually within the tail region, containing the TMD and flanking residues. For example, the TMD and C-terminus of the mammalian TA protein Fis1 are sufficient for its targeting to both organelles, while the N-terminal region is necessary for its function but not for targeting (Delille and Schrader, 2008).

1.3.1. Targeting and insertion of TA proteins

For some years several mechanisms of targeting and insertion of TA proteins to their respective membrane have been debated (reviewed in Borgese and Fasana, 2011). Early theories proposed a spontaneous insertion of TA proteins into the lipid bilayer in the absence of a translocation machinery. However, it is unlikely that all TA proteins are inserted by this mechanism, which would inevitably induce the mistargeting to several membranes and complicate the regulation of their function. *In vitro* studies revealed that, in fact, some proteins follow an unassisted pathway by which proteins with weakly hydrophobic TMDs spontaneously integrate into membranes. An example of this is mammalian cytochrome b5, which, *in vitro*, is inserted in the lipid bilayer in the absence of a translocation machinery or cytosolic factors (Brambillasca et al., 2005; Colombo et al., 2009). Nevertheless, most TA proteins are thought to follow a chaperone-mediated targeting pathway, and assisted insertion in the membrane (Fig.1.6).

Several mechanisms have been proposed for the biogenesis of ER-targeted TA proteins (reviewed in Rabu et al., 2009). The best described is the TRC40 or Get3 pathways in mammals and yeast, respectively (Schuldiner et al., 2008). These proteins recognize and selectively bind the TMD of TA proteins in the cytosol and target them to ER receptors. There, the protein is released for insertion into the lipid bilayer. This pathway is regulated by ATP binding and hydrolysis by the TRC40 and Get3 proteins.

The pathway followed by mitochondrial and peroxisomal TA proteins is still mainly unknown. Mitochondrial TA proteins have a TMD of moderate hydrophobicity that can be flanked by positive charges on one or both sides (in mammals). Although recent work has excluded the TOM complex for the insertion of some of these proteins (Setoguchi et al., 2006), TOM20 and TOM22 have been implicated in the import of Bcl-2 and Bax, respectively (Bellot et al., 2007). On the other hand, peroxisomal TA proteins also possess moderately hydrophobic TMDs and have a positively charged C-terminal polar region. Studies on the targeting of peroxisomal proteins are more complicated since there are few known TA proteins in this organelle (i.e. Pex26, Fis1,

Mff and MAVS), and there are so far three different mechanisms through which peroxisomal membrane proteins are targeted to peroxisomes (Camoes et al., 2009). The most likely pathway for these proteins is the Pex19 pathway used by PMPs. In fact, Pex26 and Fis1 are both delivered to peroxisomes by a Pex19-dependent mechanism (Delille and Schrader, 2008; Halbach et al., 2006), and it is probable that other peroxisomal TA proteins are also targeted by this pathway.

1.3.2. Other TA proteins on mitochondria

In this study the intracellular localization and function of the TA proteins Miro and monoamine oxidase (up to now identified solely on mitochondria) will be addressed.

1.3.2.1. Monoamine oxidases

Monoamine oxidases (MAOs) A and B are mitochondrial TA proteins responsible for the oxidative deamination of dietary amines and monoamine neurotransmitters such as serotonin, norepinephrine and dopamine (Shih et al., 1999). Both proteins are present in most mammalian tissues, but the proportions of each vary among tissues. In peripheral tissues such as the intestine and liver, these proteins seem to protect the body by oxidizing amines from the blood or preventing their entry into the circulation. In the nervous system, MAOs are responsible for the rapid degradation of neurotransmitters, ensuring the proper function of synaptic neurotransmission, regulating emotional behaviour and other brain functions (Youdim et al., 2006). Due to their importance in brain development, several studies have focused on the genetic variability that determines the activity of these proteins and their relation to personality and addictive behaviours (Shih et al., 1999). In fact, inhibitors of these proteins were the first drugs developed to treat depression due to their mood-enhancing activity, based on their ability to increase the levels of serotonin and dopamine (Zeller and Barsky, 1952). Also, since the by-products of MAO activity (e.g. hydrogen peroxide and ammonia) have neurotoxic potential, their excessive activity is associated with mitochondrial damage and neurodegenerative conditions. Therefore, MAO inhibitors have also been tested in the treatment of several neurodegenerative diseases, with positive results in the treatment of Parkinson's disease (Bortolato et al., 2008; Youdim et al., 2006).

1.3.2.2. Miro proteins

Miro proteins are a subclass of Ras GTPases with key importance for the distribution of mitochondria in yeast, plants and animals (Reis et al., 2009). Human

Miro1 and Miro2 share 60% homology and contain two GTPase domains flanked by two calcium-binding EF-hand motifs (see Fig.3.3). Miro proteins are known to regulate mitochondrial transport along microtubules by linking this organelle to kinesin motors in a complex with Grif-1 or OIP106 (Liu and Hajnoczky, 2009). Recent studies have shown that Miro1 functions as a calcium sensor, promoting mitochondrial anchoring in active synapses where glutamate signalling induces high intracellular calcium concentrations (Macaskill et al., 2009b). This function is of critical importance for synapse formation and neuronal development due to the high energy demands of these processes.

Several reports have shown that the expression of Miro proteins with mutations, either in their first GTPase domain or in the calcium binding domains, promotes alterations in mitochondrial morphology and distribution (reviewed in Liu and Hajnoczky, 2009). Curiously, mutations of the yeast homolog Gem1p and the plant homologs of Miro also affect mitochondrial distribution and morphology, even though mitochondria are transported via the actin cytoskeleton in these organisms (Koshiba et al., 2011).

Besides forming a complex with Grif-1/OIP106 and kinesin motors, Miro proteins have also been shown to interact with Pink1, a kinase associated with Parkinson's disease and HUMMR, a protein sensitive to hypoxic conditions (Li et al., 2009; Weihofen et al., 2009). Although the mechanism and functional importance of these interactions is still unknown, this data combined with Miro's activity in yeast and plants, reveals a broader activity of this protein in regulating mitochondrial dynamics than simply linking this organelle to motor proteins.

1.4. Objectives

It is now evident that peroxisomes and mitochondria exhibit a closer interrelationship than previously appreciated. This connection includes metabolic cooperation/crosstalk, as well as an overlap in key components of their division and antiviral machinery. As both organelles are indispensable for human health and development, there is currently great interest in the identification and functional characterization of novel proteins targeted to both organelles.

The main objective of this project is thus to identify and characterize novel proteins that are dually targeted to peroxisomes and mitochondria. For this, I will study the role of TA proteins on the molecular connection between peroxisomes and mitochondria, following two mains approaches.

First, I will perform an antibody/expression-based screening approach to study the cellular localization of known mitochondrial TA proteins, specifically of MAO-B, Miro1 and Miro2. This will be followed by a functional characterization of the proteins which are found to be dually targeted to peroxisomes and mitochondria, through the analysis of phenotypic alterations in peroxisome morphology and dynamics.

Additionally, a bioinformatics analysis of the *Ustilago maydis* proteome will be performed to identify putative TA proteins. This model system, which has been recently introduced in our laboratory, shares many important processes (e.g. long-distance microtubule transport, polarized growth) with human cells and provides the technical advantages of yeast cells. Moreover, a genome-wide comparison of the predicted *U. maydis, S. cerevisiae* and human proteomes revealed that *U. maydis* shares more protein sequence similarity with humans than with yeast (Munsterkotter and Steinberg, 2007). More importantly, several of the shared proteins have been related to serious human diseases and a large portion is of unknown function. This model system is therefore a potentially important tool to understand the abnormal developmental processes that occur in certain human peroxisome and mitochondrial metabolic disorders.

Chapter 2 Methods

2.1. Materials

2.1.1. Reagents

HEPES sodium salt, para-Formaldehyde, Triton X-100, sodium chloride (NaCl), potassium chloride (KCl), sodium phosphatase dibasic (Na_2HPO_4), potassium phosphate dibasic (KH_2PO_4), ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), bromophenol blue, dithiothreitol (DTT), β-mercaptoethanol, glycine and kanamycin disulphate salt were purchased from Sigma-Aldrich (Steinheim, Germany). Ethidium bromide, dextrose and n-propyl-gallate were acquired from Fluka (Steinheim, Germany). Acetic acid, sodium hydroxide (NaOH), ethanol and methanol were purchased from Merck-Schuchardt (Darmstadt, Germany). Agarose NEEO, bovine serum albumin (BSA) fraction V, Tris, potassium acetate (KAc) and glycerol were purchased from Roth (Karlsruhe, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum Gold (FBS), penicillin/streptomycin and trypsin-EDTA were all purchased from PAA (Pasching, Austria). Trypsin was obtained from Promega (Wisconsin, USA) and polyethylenimine (PEI) was acquired from Sigma-Aldrich (Missouri, USA). Mowiol 4-88 reagent was purchased from Calbiochem/Merck (Darmstadt, Germany). Immersion oil type F was obtained from Olympus (Japan). LB broth was purchased from Formedine (Norfolk, UK). AgeI, BglII, Antarctic phosphatase, T4-ligase, KOD DNA polymerase and RNase were all purchased from New England Biolabs (United Kingdom). TurboFect, O'Gene Ruler DNA ladder mix and 6x Orange loading dye were purchased from Fermentas (France). Amersham ECL Advance solutions were obtained from GE Healthcare (Buckinghamshire, UK) and Precision Plus Protein Dual Color Standart was obtained from Bio-Rad (Munich, Germany).

2.1.2. Antibodies

Table 2.1. Primary and Secondary antibodies.

Primary Antibodies	Dilution		Source
	IMF	WB	
Monoamine oxidase B (pc mouse)	1:100	-	Abcam Cambridge, England
RhoT1 (Miro1) (pc rabbit)	-	1:250	Sigma-Aldrich Schnelldorf, Germany
Myc (mc mouse)	1:200	-	Santa Cruz Biotechonology, Santa Cruz, USA
Myc (mc rabbit)	1:200	-	Cell Signaling Technology Massachusetts, USA

TOM20 (mc mouse)	1:200	-	BD Transduction Laboratories San Diego, USA
Pex14 (pc rabbit)	1:1400	1:4000	Kind gift from D.Crane Griffith University, Brisbane, Australia
Porin (mc mouse)	-	1:500	Abcam Cambridge, England

Secondary Antibodies	Dilution		Source
	IMF	WB	
AlexaFluor 488 Donkey anti Rabbit IgG	1:400	-	Molecular Probes, as part of Invitrogen Life Techonologies Eugene, USA
AlexaFluor 488 Donkey anti Mouse IgG	1:400	-	Molecular Probes, as part of Invitrogen Life Techonologies Eugene, USA
TRITC Donkey anti Mouse IgG	1:100	-	Dianova Hamburg, Germany
TRITC Donkey anti Rabbit IgG	1:400	-	Dianova Hamburg, Germany
HRP Goat anti Mouse IgG	-	1:2000	Bio-Rad Munich, Germany
HRP Goat anti Rabbit IgG	-	1:2000	Bio-Rad Munich, Germany
Hoechst dye	1:2000	-	Polysciences, Inc. Eppelheim, Germany

Abbreviations: IMF, immunofluorescence; WB, western blot; mc, monoclonal; pc, polyclonal; HRP, horseradish peroxidase.

2.1.3. Constructs

Myc-tagged human Miro1 and Miro2 constructs – wild-type, V13, N18, EF (K208K328) and ΔTM – have been previously described (Fransson et al., 2006) and were generously offered by Dr. Pontus Aspenström (Karolinska Institute, Sweden). The V13 and N18 constructs are mutated in the first GTPase domain: the former is constitutively active whereas the latter is dominant negative. The mutations change amino acid residue 13 for a valine (V) and 18 for an asparagine (N). The EF construct is mutated in the two calcium binding EF-hand domains and is therefore unable to bind calcium. This mutation changes amino acid residues 208 and 328 to lysines (K). The ΔTM mutant lacks the TMD and C-terminal tail. Plasmid DNA was amplified following the protocol for *E.coli* transformation (see section 2.2.3) and subsequently amplified by midi preparation (see section 2.2.4).

For the cloning of Myc-tagged Miro proteins directed to peroxisomes, the pAH26 vector was used (Halbach et al., 2006), which was a kind gift from Dr. Ralf Erdmann (University of Bochum, Germany). The pAH26 vector comprises a GFP protein with an artificial tail that directs it to the peroxisomal matrix (Fig.2.1). This tail is composed by

the Pex26 tail region, which contains a Pex19 binding site, and two TMDs from the adrenoleukodystrophy protein (ALDP). These TMDs are part of the peroxisomal membrane protein targeting signal but lack the Pex19 binding site.

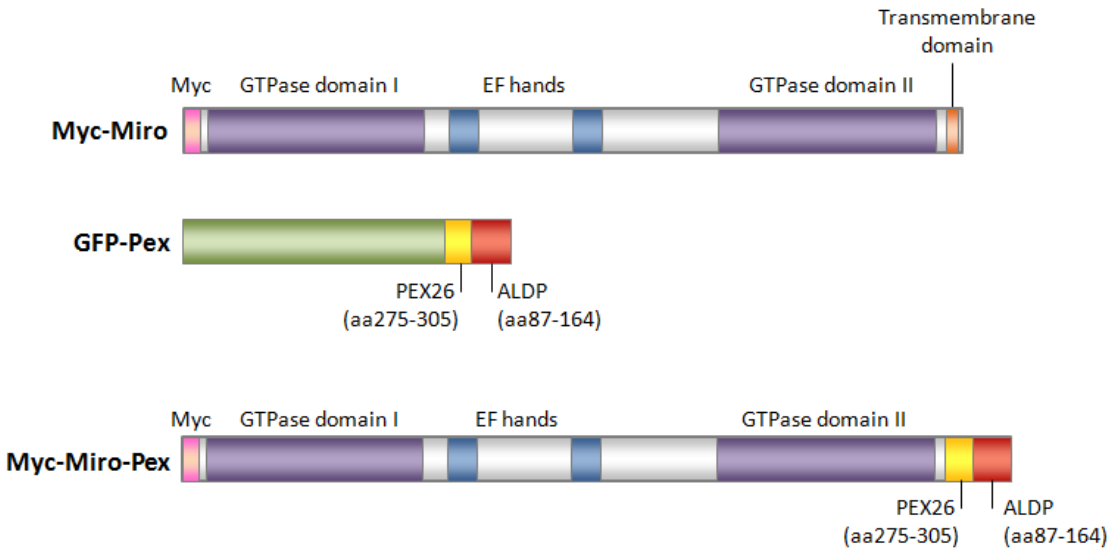

Figure 2.1 – Structure of Myc-Miro constructs.

2.2. Cloning of peroxisome targeted Miro proteins

2.2.1. Amplification and isolation

Myc-Miro1 and Myc-Miro2, wild-type and EF mutant, were amplified by PCR from the pRK5-Myc-Miro constructs described before (Fransson et al., 2006). The following primers were used: Myc-forward 5' GGAACCGGTCACCATGGAGCAGAAGC TGATC 3', Miro1-ΔTM-reverse 5' GGAAGATCTAAACGTGGAGCTCTTGGGGTC 3' and Miro2-ΔTM-reverse 5' GAAGATCTCCGGAGCCAGAAGGAAGAGGG 3'. KOD DNA polymerase (*Thermococcus kodakaraensis*) was used due to its high efficiency proof-reading activity, therefore minimizing mutation probability. The same program was followed for the 4 PCR reactions: 95°C for 30 seconds (1 cycle); 95°C for 30 seconds, 64°C for 20 seconds and 70°C for 2 minutes (25 cycles); and 70°C for 10 minutes (1 cycle).

DNA electrophoresis was performed in 1x TAE buffer (0.8 mM Tris, 0.4 mM acetic acid, 0.02 mM EDTA, in ddH_2O, diluted from a 50x stock solution) in an agarose gel (0.8-1% agarose in TAE buffer) stained with ethidium bromide (0.5 µg/ml). Separation was performed at maximal 5 V per cm electrode distance (60 to 75 V) for 45 to 90 minutes. DNA samples were mixed with 6x Orange loading dye buffer and loaded to individual wells. A DNA ladder was also loaded to one well to compare band

sizes. Gel images were taken using the AlphaImager HP system and processed with the AlphaImager Software 1.01.10 (Cell Biosciences, USA). Specific DNA bands were excised with a scalpel under UV light and transferred to eppendorf tubes. DNA isolation and purification was done with the NucleoSpin Extract II kit (Macherey-Nagel, Germany), following the manufacturer's protocol up to the eluting step, in which 30 µl of milliQ water were used.

2.2.2. DNA restriction, dephosphorylation and ligation

Myc-Miro (insert) and pAH26 vector DNAs were digested with the restriction enzymes AgeI and BglII (in NEBuffer 2) at 37°C overnight.

Upon digestion, the vector DNA was desphosphorylated to avoid re-ligation. For this, the vector was incubated for 30 minutes at 37°C with Antarctic phosphatase in its appropriate buffer, followed by enzyme inactivation for 10 minutes at 65°C.

Both vector and insert DNAs were subjected to eletrophoresis in an agarose gel and the DNA was isolated and purified as described above.

DNA concentration was determined using the Qubit fluorometer (Invitrogen, USA) following the manufacturer's protocol.

For ligation, the molecule ratios between vector and insert were 1:1 and 1:3. To calculate the exact amount of DNA the following formula was used:

Mass insert [ng] = (5 x Mass vector [ng] x length insert [kb]) / (length vector [kb])

Control ligation was performed in parallel only with the dephosphorylated vector. Ligation was performed with the enzyme T4 ligase at 16°C overnight.

2.2.3. Bacterial transformation by heat shock

For each plasmid, 50 µl of competent XL1-Blue *E.coli* bacteria were mixed with 2 µl of ligation mixture and incubated for 30 minutes on ice, followed by a 90 seconds heat shock at 42°C, and a short incubation on ice. For recovery, bacteria were incubated with 950 µl of LB medium for 45 minutes at 37°C with agitation. Cells were centrifuged for 1 minute at 17000 rpm and the pellets were resuspended in 50 µl of LB medium. Cells were spread with the aid of glass beads on LB agar plates containing kanamycin (30 µg/ml). Plates were incubated overnight at 37°C.

2.2.4. Colony selection

Test colonies were inoculated with 5 ml of LB medium (containing kanamycin) overnight. Plasmid isolation was done by a mini preparation protocol based on alkaline lysis. 1.5 ml of bacterial cell culture were centrifuged for 2 minutes at 12000 g and the supernatant was removed. The pellet was completely resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA, autoclaved) and lysed with 200 µl of solution II (0.2 M NaOH, 1% SDS) by inverting the tube a few times leaving it on ice for 2 minutes. After, 150 µl of cold solution III (3 M KAc, 11.5% (v/v) glacial acetic acid) were added and mixed by inverting the tube a few times, incubating for 5 minutes on ice. The mix was centrifuged at 17000 g for 10 minutes at 4°C and the supernatant was transferred to a new tube. DNA was precipitated by mixing the supernatant with 200 µl of cold 100% ethanol and keeping it on ice for 2 minutes followed by centrifugation at 17000 g for 5 minutes at 4°C. The supernatant was removed and the pellet was washed with 1 ml of 70% ethanol at 17000 g for 5 minutes at 4°C. The supernatant was discarded and after air-drying the pellet, the DNA was resuspended in 50 µl of water containing RNase (20 µg/ml).

DNA testing was initially done with one restriction enzyme (either AgeI or BglII). After analysis of DNA bands in an agarose gel, one clone for each plasmid was selected and digested with both enzymes to confirm the insertion of the correct sized-insert.

Selected clones were grown overnight in LB medium and mini preparations were performed using the NucleoSpin Plasmid kit (Macherey-Nagel, Germany) following the manufacturer's protocol. DNA concentration was determined using the Qubit fluorometer (Invitrogen, USA) following the manufacturer's instructions.

Plasmid DNA was sent for sequencing at Eurofins MWG Operon (Germany). For each plasmid, three sequencing reactions were performed with the following primers: CMVfor 5' CGCAAATGGGCGGTAGGCGTG 3', pEGFPC1rev 5' CATTTTATGTTT CAGGTTCAGGG 3', and either Miro1-mid-for 5' GATGGTGTGGCTGACAGTGGG 3' or Miro2-mid-for 5' GCTCAACGCTTTCCAGAAATCC 3'. Sequencing results were analysed with the FinchTV 1.4.0 program (Geospiza) and compared with original sequences using the BLAST tool from NCBI.

DNA from positive clones was isolated from midi preparations (for higher yield) using the NucleoBond Xtra Midi kit (Macherey-Nagel, Germany) following the manufacturer's protocol.

2.3. Cell Culture and Transfection

2.3.1. Cell Culture

All transfection and immunofluorescence procedures were performed using either COS-7 or HepG2 cell lines. COS-7 cells are derived from kidney cells of *Ceropithecus aethiops* (African green monkey) that were transformed with a mutant SV40 to produce large T antigen. This cells are adherent and have a fibroblast morphology. HepG2 cells are derived from a human hepatocellular carcinoma (hepatoblastoma) and have an adherent epithelial morphology. These cells can form bile canaliculi-like structures in culture and secrete most of the serum proteins.

COS-7 and HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) high glucose (4.5 g/L) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2 and 95% humidity.

Passaging and splitting of cells was carried out twice per week, after the cells reached confluence. Cells were washed once with phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4*$2H_2O$, 1.5 mM KH_2PO_4, in dH_2O, pH 7.35) and incubated for 5 minutes with 2.5 ml trypsin-EDTA at 37°C. Upon resuspension in 7.5 ml of medium, cells were centrifuged for 3 minutes at 1000 rpm to remove dead cells and debris. The pellet was resuspended in 10 ml medium and cells were seeded at 1:10 dilution (≈10^4 cells/ml). Cells were routinely grown on 10ø cm dishes. For immunofluorescence, cells were seeded on round 18ø mm glass coverslips in 6ø cm dishes 24 hours prior to transfection. To ensure reproducibility between experiments, cell number was determined using a Fuchs-Rosenthal counting chamber.

2.3.2. Cell freezing and thawing

Stocks for each cell line were kept through cryopreservation in liquid nitrogen. Cell pellets from confluent dishes were prepared as described above (section 2.3.1) and resuspended in freezing medium (DMEM supplemented with 20% FBS and 10% DMSO). 1 ml aliquots were prepared in cryovials and frozen overnight at -80°C, before being stored in a liquid nitrogen tank.

For thawing, cells were quickly resuspended with pre-warmed culture medium and seeded in a 10ø cm dish. The culture medium was changed after a few hours (after cell adhesion) to remove debris and DMSO.

2.3.3. Transfection methods

2.3.3.1. PEI (Polyethylenimine)

PEI solution (0.9-1 mg/ml in water) was prepared fresh for each transfection and sterilized by filtration. 10 µg of DNA were diluted in 750 µl of 150 mM NaCl solution, while 100 µl of PEI solution were diluted in 650 µl of 150 mM NaCl solution. After 15 minutes of incubation at room temperature the PEI dilution was added drop-wise to the DNA solution and incubated for an additional 15 minutes. During incubation, cell dishes were washed with PBS and new medium (without FBS and antibiotics) was added. 500 µl of the final mixture were added drop-wise to the 6ø cm culture dishes containing coverslips. Cells were incubated for 3 to 6 hours at 37 °C, after which they were washed with PBS and incubated with fresh complete medium for 24-48 hours before fixation for immunofluorescence.

2.3.3.2. TurboFect

4 µg of DNA and 6 µl of TurboFect were diluted in 400 µl of DMEM medium without FBS and antibiotics and incubated for 20 minutes. During incubation, the culture dish was washed once with PBS and fresh medium (without FBS or antibiotics) was added. The DNA/TurboFect mixture was added drop-wise to the dish and incubated for 3 to 6 hours at 37 °C after which the cells were washed with PBS and incubated in fresh complete medium for 24-48 hours before fixation for immunofluorescence.

2.4. Microscopic techniques

2.4.1. Immunofluorescence (IMF)

Immunofluorescence is a technique that enables the labelling of a specific protein in order to examine its localization in the cell by fluorescence or confocal microscopy. For this, a fluorescente dye (fluorophore) is conjugated to either a primary antibody (direct IMF) or to a secondary antibody (indirect IMF), which labels the protein of interest. The primary antibody binds directly to one (monoclonal) or more (polyclonal) epitopes of a specific protein, and can be produced in several different animals. The secondary antibody is raised against the Fc domain of the primary antibody and is coupled to the fluorophore. The advantages of an indirect IMF lie in the fact that several secondary antibodies can bind one single primary antibody, resulting in signal amplification, and that it is cost-effective since a small number of fluorophore-coupled

secondary antibodies can be used for several different experiments. If no antibody against a protein is available, a tagged version (e.g. YFP, Myc, HA) can be introduced in the cell by transfection and labelled with an antibody against the tag-region.

Cells must undergo a series of steps before being labelled with antibodies. First, a fixation step is performed in order to preserve the cell's structure and protein conformation. For this, a cross-linking agent such as formaldehyde can be used, since it enables the formation of methylene bridges between primary amino groups in proteins and nearby nitrogen atoms. Following this, cells must be permeabilized to enable the access of antibodies to different cellular compartments and binding to their target protein. Several detergents with different strengths and specificities can be used to permeabilize cells. The most commonly used one is Triton X-100, which creates pores in all cell membranes by removing lipids. Prior to staining, a final blocking step is performed to prevent non-specific binding of the primary antibody. For this, a protein solution of bovine serum albumin (BSA) can be used to block excessive protein epitopes and free aldehyde groups.

Cells grown on glass coverslips were processed for IMF 24-48 hours after seeding or transfection. Cells were fixed for 20 minutes with 4% (w/v) para-Formaldehyde (pH 7.4), permeabilized with 0.2% (w/v) Triton X-100 for 10 minutes and blocked with 1% (w/v) BSA for 10 minutes. Cells were incubated with 30 µl of primary antibody for 1 hour in a humid chamber. This step was repeated for the secondary antibody, protected from light. Regularly, two coverslips per condition were processed and one was stained with 30 µl of Hoechst dye for 2 minutes. Coverslips were washed with ddH_2O to remove PBS and mounted with Mowiol medium on glass slides. Mowiol mounting medium was prepared from a 3 to 1 mixture of Mowiol solution (12 g of Mowiol 4-88, 20 ml glycerol, 40 ml PBS) and n-propyl-gallate (2.5% (w/v) n-propyl-gallate, 50% glycerol, in PBS), which is an antifading agent. All immunofluorescence steps were performed at room temperature and cells were washed three times with PBS in between each individual step.

2.4.2. Fluorescence microscopy

Fluorescence techniques are based on the use of a fluorescent dye which absorbs high energy light (short wavelength), leading to the emission of a lower energy light (long wavelength). Fluorophores with different excitation and emission spectra can be used in the same preparation to label several proteins.

In fluorescence microscopy, light passes through an excitation filter that selects light of the wavelength that excites the fluorophore. In return, a different light of a lower

wavelength is emitted that passes through the dichroic mirror to a second filter (barrier filter) which blocks all wavelengths except the one emitted by the fluorophore. This is then directed to an emission filter and focused in the ocular. The light source is usually a mercury-vapor arc lamp which provides a mixture of wavelength from UV to red.

For these experiments, cells were observed with an Olympus IX81 microscope, 100x/1.40 oil objective (Olympus Optical, Hamburg, Germany). Digital images were taken with a CCD camera F-View II and selected and adjusted for contrast and brightness using the Olympus Soft Imaging Viewer software (Olympus Soft Imaging Solutions GmbH) and the Microsoft Power Point Software.

2.4.3. Confocal microscopy

The convencional fluorescence microscope has some optical limitations that affect, in particular, the results obtained for co-localization studies. In this case, the signal from above and below the focal plane can lead to false-positive results. To overcome this, a confocal laser scanning microscope (CLSM) can be used, which decreases the blurring and background of the images taken.

The key difference in confocal microscopy is the restriction of emitted fluorescence light that has to pass a pinhole before being detected. The illumination is achieved using a laser beam that scans point-by-point the specimen. The sequences of points of light from the specimen are detected by a photomultiplier through a pinhole, and the result is assembled into a digital image. This technique can also be used to create 3D images, by stacking consecutive focal planes.

Cells were observed with a Zeiss LSM 510 confocal microscope, 100x/1.40 oil objective (Carl Zeiss, Oberkochen, Germany), using the Zeiss LSM Image Browser software (Carl Zeiss MircroImaging GmbH). Two lasers were used: the Argon-ion laser (488 nm) for samples stained with TRITC dye and DPSS laser (561 nm) for samples stained with Alexa Fluor 488 dye.

2.5. Electrophoresis and Immunoblotting

In order to study Miro1 localization in cells, subcellular fractions of rat liver cells were analysed by western blot. The following fractions were used: highly purified peroxisomal fraction (PO), heavy mitochondrial fraction (HM) and a microsomal and cytosolic fraction (MC) (kindly provided by Dr.Markus Islinger). 20 µg of protein from each fraction were diluted in SDS-containing loading buffer (60 mM Tris pH-6.8, 2%

(w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 20 mM DTT, 5% (v/v) β-Mercaptoethanol), and denatured at 95°C for 5 minutes.

Standard 1D-SDS PolyAcrylamide Gel-Elecrophoresis (SDS-PAGE) was performed with 12.5% separating gels and 4% stacking gels (Tab.2.2). Gel electrophoresis in mini slap gel chambers was conducted for approximately 30 minutes at 80 V until the proteins entered the separating gel and continued at 130 V for approximately 90 minutes. The gel chambers were filled with 1x SDS running buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS). To mark protein size a pre-stained molecular weight marker (Precision Plus) was used and the sample running front was visualized by bromophenol blue added to the loading buffer.

Protein transfer to the membrane was performed by semi-dry Western blotting. The nitrocellulose membrane and two Whatman filter papers (3 mm) were soaked with semidry blotting buffer (48 mM Tris, 39 mM glycine, 0.4% (w/v) SDS, 20% (v/v) methanol) and a stack of Whatman filter, membrane, gel, and Whatman filter was formed. Air bubbles were removed to guarantee complete transfer. The stack was put into a semidry transfer chamber and the proteins were blotted for 50 minutes at 12 V.

After protein transfer, the membrane was blocked by incubation with 5% low fat powdered milk in PBS for 1 hour. Membrane incubation with the primary antibody was performed in a sealed plastic bag with the respective antibody dilution in PBS and incubated with shaking overnight at 4°C. After incubation, the membrane was washed three times for 10 minutes with PBS. Incubation with the secondary antibody was performed for 90 minutes at room temperature, after which the membrane was washed three times for 10 minutes with PBS.

For the enhanced chemiluminescence (ECL) reaction, ECL 1 (containing luminol) and ECL 2 (phenol-containing enhancer) solutions were mixed (ratio 1:1) and the membrane was incubated for 2 minutes. Film exposition (2 to 15 minutes), development and fixation were performed in a light protected environment. For presentation the films were scanned with a Bio-Rad GS-710 Calibrated Imaging Densitometer.

Table 2.2 – Gel solutions for SDS-PAGE

	12.5% Separating Gel	4% Stacking Gel
30% Polyacrylamide	3.33 ml	0.83 ml
2 M Tris pH 8.8	1.86 ml	-
1 M Tris pH 6.8	-	0.63 ml
20% SDS (0.1%)	50 µl	25 µl
dH_2O	4.73 ml	3.43 ml
10% APS	30 µl	40 µl
TEMED	5 µl	5 µl
Total volume	10 ml	5 ml

2.6. Statistics

For the quantitive analysis of the effect of Myc-Miro expression on peroxisome morphology and distribution, 150 cells were examined at the fluorescence microscope, in 3 independent experiments (50 cells per coverslip). For each cell, peroxisome distribution (scattered or centered) and aggregation (in the cell periphery or in the cytoplasm) were accounted. Statistical analyses were performed using Microsoft Excel 2007 and GraphPad Prism 5 software. Data are presented as means ± standard deviation (SD). An unpaired t-test was used to determine statistical differences between experimental groups. P values < 0.05 are considered as significant and P values < 0.01 are considered as highly significant.

2.7. Bioinformatics

Identification of putative TA proteins from the *U. maydis* model system was made with the help of several bioinformatic tools available online.

Selection of proteins with a single predicted TMD was done via the Pedant 3 database (Walter et al., 2009), which automatically categorizes all know protein sequences from an organism according to several characteristics, namely, protein structure. A single file containing all *U.maydis* protein sequences with one TMD was exported in FASTA (467 proteins).

Subsequently, this file was fed to the TMHMM server v. 2.0 (Krogh et al., 2001) to identify the sequences with a TMD near the C-terminus of the protein and a tail with no more than 30 amino acids. A new file with the sequences that followed these criteria was created, containing 85 proteins. This list was further filtered with the Signal P program (Bendtsen et al., 2004) to exclude those proteins predicted to have a signal sequence for import through the ER translocon. The hidden Markov model was used and all sequences were automatically truncated to their first 70 amino acids. Proteins with a Sprob higher than 0.750 were excluded, which reduced the list to 65 proteins. A FASTA file with only the first 70 amino acids of each protein was created and fed to the MitoProt program (Claros and Vincens, 1996), where the probability of import to mitochondria was calculated. Proteins with a probability higher than 0.850 were excluded and the final list contained 58 proteins.

Each one of the 58 predicted TA proteins was analysed for its putative function and homology to other organisms. A first analysis was carried out using the MIPS *Ustilago maydis* Database (Mewes et al., 2004) that automatically searches for

U.maydis homologs using the SIMAP server (Rattei et al., 2010). A file was created containing information on predicted human homologs for each protein and the percentage of homology. Each protein was also analysed using the PhyloBuilder program (Glanville et al., 2007) that gathers several bioinformatic tools to identify protein homologs and create phylogenetic trees. Protein function was predicted, when possible, by frequency of similar hits and sequence alignment comparison.

Chapter 3 Results

3.1. Screening of known TA proteins for dual localization to peroxisomes and mitochondria

3.1.1. Miro proteins are dually targeted to peroxisomes and mitochondria

Miro proteins have been previously shown to localize to the outer mitochondrial membrane and to regulate mitochondrial transport, functioning as a linker to motor proteins. To verify if Miro1 and Miro2 are targeted to peroxisomes, Myc-tagged wild-type and mutant proteins were expressed in COS-7 cells. For each protein (Miro1 and Miro2) five different constructs were expressed: wild-type, V13, N18, EF and ΔTM. The V13 mutant is constitutively active at the first GTPase domain whereas the N18 mutant is dominant negative for the same domain. The EF mutant as two mutations at the calcium-binding EF-hands is unable to bind calcium. Lastly, the ΔTM mutant lacks the TMD and the C-terminal tail. The cell transfections were performed using polyethylenimine (PEI), unless stated otherwise, and cells were subjected to immunofluorescence using antibodies raised against Myc and Pex14.

As previously described by Fransson et al. (2006), the expression of Myc-Miro1-wt and Myc-Miro2-wt resulted in the formation of perinuclear aggregations of mitochondria. Furthermore, expression of both constructs induced the formation of interconnected thread-like mitochondrial networks (Fig.3.1). This last phenotype had only been described for the expression of Myc-Miro1 constructs and therefore, the different results might be due to the transfection method used and the DNA concentration.

Overexpression of Myc-Miro1-wt and Myc-Miro2-wt induced a strong mitochondrial signal, hampering the visualization of their potential localization to smaller organelles such as peroxisomes. In order to circumvent this problem, zoomed images where taken where the signal was amplified; thus it was possible to observe a clear co-localization between Myc-Miro1-wt and Myc-Miro2-wt and the peroxisome marker Pex14 (Fig.3.1 D,H). A preliminary statistical analysis of the morphology and distribution of peroxisomes in transfected cells showed that up to 45 ± 5.0% of COS-7 cells expressing Myc-Miro1-wt exhibit peroxisome aggregates in the cell periphery (Fig.3.1B, arrows; Fig.3.4). Furthermore, in cells with aggregated mitochondrial networks, peroxisomes were frequently seen closer to the cell nucleus (Fig.3.3B; Fig.3.4). No significant differences were seen in peroxisome morphology.

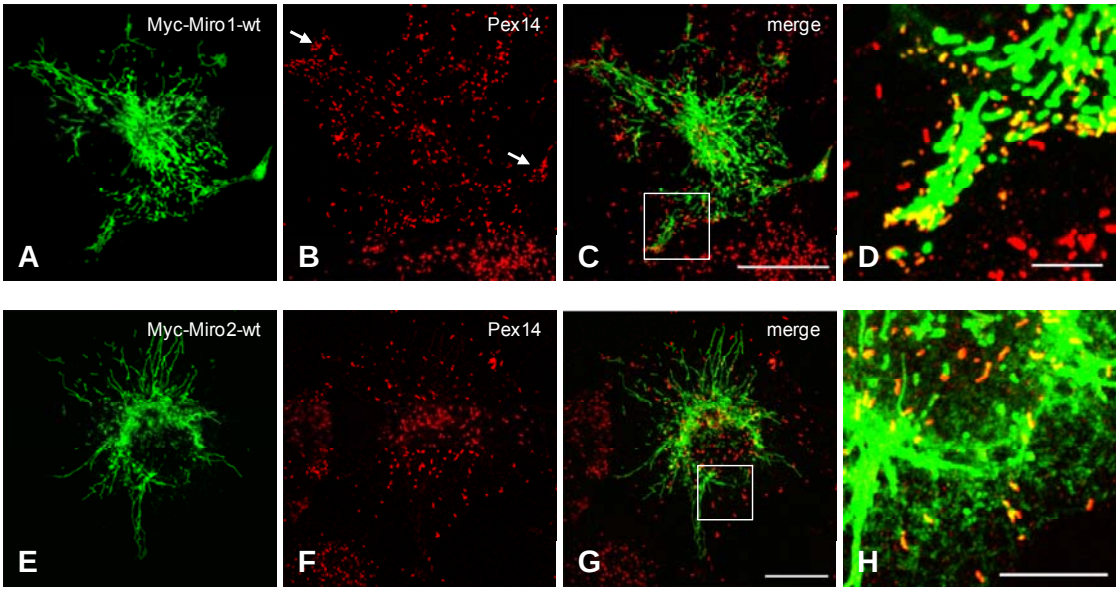

Figure 3.1 – Myc-Miro1-wt and Myc-Miro2-wt are targeted to both peroxisomes and mitochondria in COS-7 cells. COS-7 cells were transfected with Myc-Miro1-wt (A-D) and Myc-Miro2-wt (E-H), using PEI. Fixed cells were labeled with anti-Pex14 and anti-Myc. Magnifications from C and G are shown in D and H, respectively. Arrows – aggregated peroxisomes. The images were obtained by confocal microscopy. Scale bars: 10 µm (A-C, E-G), 5 µm (D, H).

In order to confirm that the peroxisomal localization of Miro proteins is not an artifact induced by protein overexpression, the endogenous expression of Miro1 was analysed by immunobloting of highly purified peroxisome fractions from rat liver (kindly provided by Dr.Markus Islinger). The results presented in Fig.3.2 show a high level of Miro1 in the mitochondrial fraction (HM), as well as a lower but significant level of Miro1 in the peroxisomal fraction (PO). Low levels of Miro1 were also present in the microsomal and cytosolic fraction (MC) due to a probable contamination with mitochondria. To assess the level of purity of each sample, the membrane was reprobed with antibodies against the proteins Pex14 (peroxisome membrane marker) and Porin (mitochondria outer membrane marker). As we can, Miro1 is enriched in the peroxisomes when compared with Porin, excluding the possibility of a contamination of the highly purified peroxisomal fraction with mitochondrial membranes.

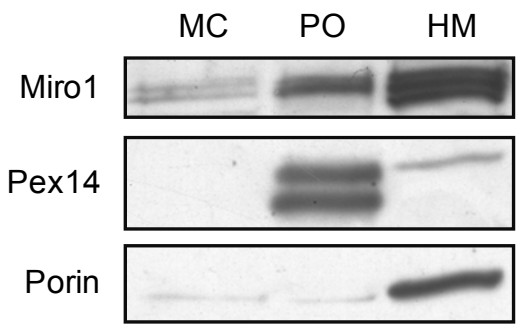

Figure 3.2 – Miro1 localises to highly purified peroxisome fractions from rat liver. Subcellular fractions of rat liver cells were analysed by Western Blot. The membrane was probed for endogenous Miro1 and reprobed with antibodies against Pex14 (peroxisome marker) and Porin (mitochondrion marker). All pictures were obtained from the same blot. Three different fractions were analysed: microsomal and cytosolic fraction (MC), highly purified peroxisomal fraction (PO) and heavy mitochondrial fraction (HM) Purified fractions from rat liver were kindly provided by Dr.Markus Islinger.

Miro1 and Miro2 share 60% sequence identity and have an identical domain structure, with two GTPase domains flanked by two calcium binding EF-hands, and a C-terminal TMD. To determine if peroxisomal dynamics are also disturbed by mutations in the functional domains of Miro, four mutants of Miro1 and Miro2 were expressed in COS-7 cells (Fig.3.3). As previously described (Fransson et al., 2006), the expression of Myc-Miro1-V13, Myc-Miro1-N18 and Myc-Miro1-EF induced the formation of perinuclear aggregations of mitochondria (Fig.3.3 A, I), albeit to a higher degree than in cells expressing Myc-Miro1-wt. Additionally, expression of the constitutively active Myc-Miro1-V13 mutant induced the formation of thread-like mitochondrial networks (Fig.3.3A, arrow). This effect was not seem in cells expressing either the Myc-Miro1-N18 mutant (Fig.3.3E-H) or the Myc-Miro1-EF mutant (Fig.3.3I-L). Expression of Myc-Miro2 mutants induced the formation of clustered mitochondria to a similar extent as that seen with the Myc-Miro2-wt (not shown). Cells expressing Myc-Miro1-ΔTM and Myc-Miro2-ΔTM showed a cytoplasmic distribution of this protein and had no effect on the mitochondrial network (not shown).

Surprisingly, even though all mutated proteins co-localized with peroxisomes, expression of Myc-Miro mutants had no substantial effect on peroxisome morphology and distribution. Nevertheless, a careful examination showed that in cells with a collapsed mitochondrial network, peroxisomes tended to concentrate together with mitochondria near the nucleus (Fig.3.4 A-D).

3.1.2. Expression of peroxisome targeted Miro alters peroxisome distribution

As shown before, wild-type and mutant forms of Myc-Miro1 and Myc-Miro2 are dually targeted to mitochondria and peroxisomes. Moreover, the expression of wild-type proteins revealed the formation of some peroxisome aggregates in the cell periphery, whereas the formation of collapsed mitochondrial networks induced alterations in peroxisome distribution. To clarify the effect of Myc-Miro proteins on peroxisomes we created a fusion protein that is solely directed to peroxisomes. For this, the tail and TMD of Miro were replaced with a previously described fusion Pex26/ALDP construct (Halbach et al., 2006). This strategy was used to create four fusion proteins: Myc-Miro1-wt-Pex, Myc-Miro1-EF-Pex, Myc-Miro2-wt-Pex and Myc-Miro2-wt-EF-Pex, corresponding to the respective previously described plasmids. While the first three constructs were successfully created, the last had mutations and the cloning process is currently being repeated.

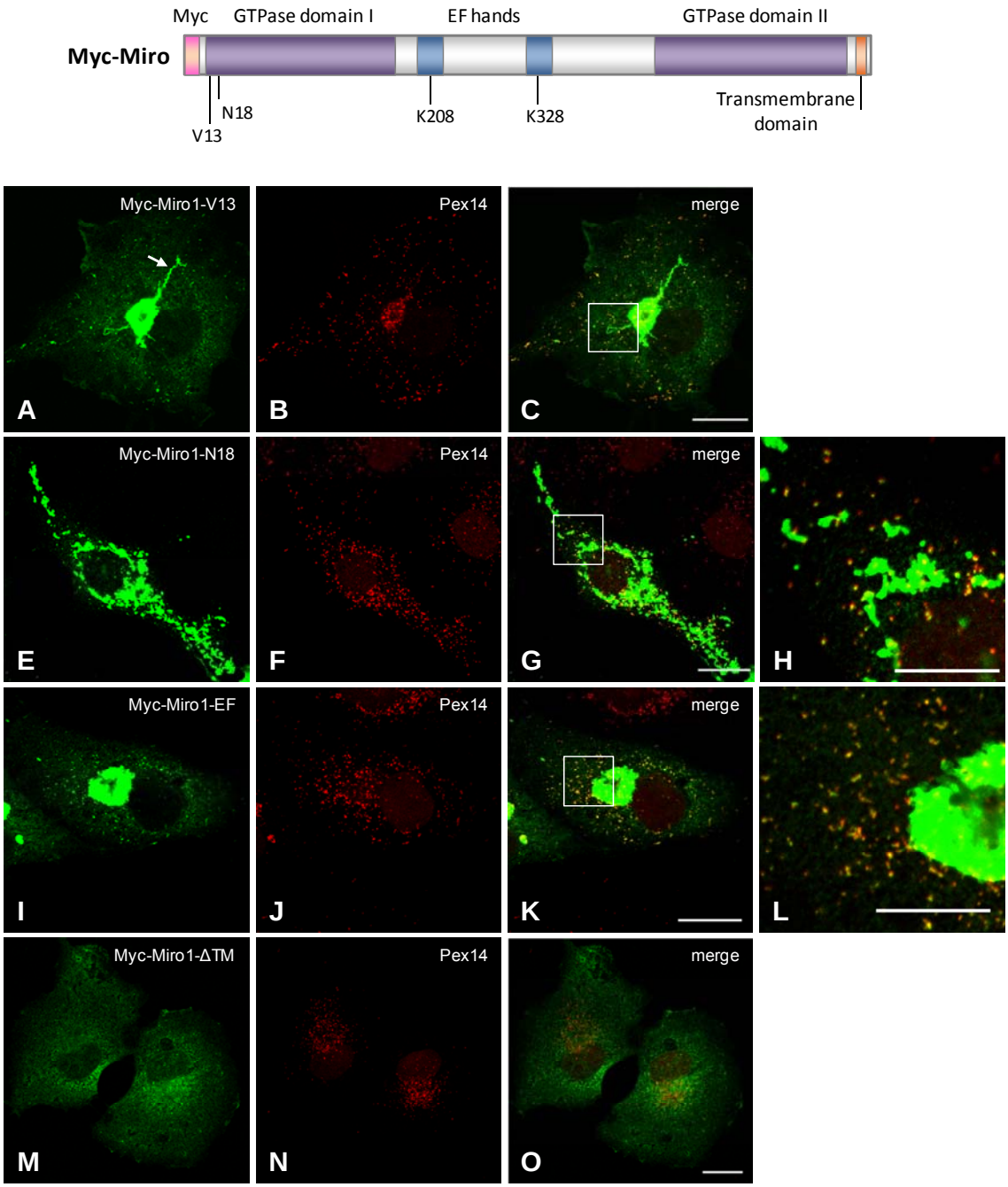

Figure 3.3 – Myc-Miro1 mutants co-localize with peroxisomes. Schematic representation of Miro and its mutants. COS-7 cells were transfected using PEI with Myc-Miro1-V13 (A-D), Myc–Miro1-N18 (E-H), Myc-Miro1-EF (I-J) and Myc-Miro1-ΔTM (M-O). Fixed cells were labeled with anti-Pex14 and anti-Myc. Magnifications from C, G and K are shown in D, H and L, respectively. Arrow – elongated mitochondria. Images were obtained by confocal microscopy. Scale bars: 10 µm (A-C, E-G, I-K, M-O), 5 µm (D, H, L).

Expression of the Myc-Miro1-wt-Pex and Myc-Miro1-EF-Pex constructs in COS-7 cells revealed an exclusively peroxisomal localisation thus confirming the success of the cloning strategy (Fig.3.5). Similarly to the cells expressing Myc-Miro1-wt, observation of cells transfected with Myc-Miro1-wt-Pex at the fluorescence microscope showed no alterations on peroxisome morphology. A preliminary analysis of the cells demonstrated that up to 52 ± 2% of cells exhibit peroxisome aggregates in the cell

periphery (Fig.3.4; Fig.3.5A-D). The magnified area shown in Fig.3.5 D reveals the presence of several spherical and rod-shaped peroxisomes in these aggregates. Furthermore, there were less cells with peroxisomes closer to the nucleus in those transfected with Myc-Miro1-wt-Pex than in cells expressing Myc-Miro1-wt and untransfected cells (10 ± 2,3% to 45 ± 5,0% and 27 ± 3,1%, respectively) (Fig.3.4). The expression of Myc-Miro1-wt-Pex had no obvious effect on mitochondrial morphology and distribution (Fig.3.5 H-J).

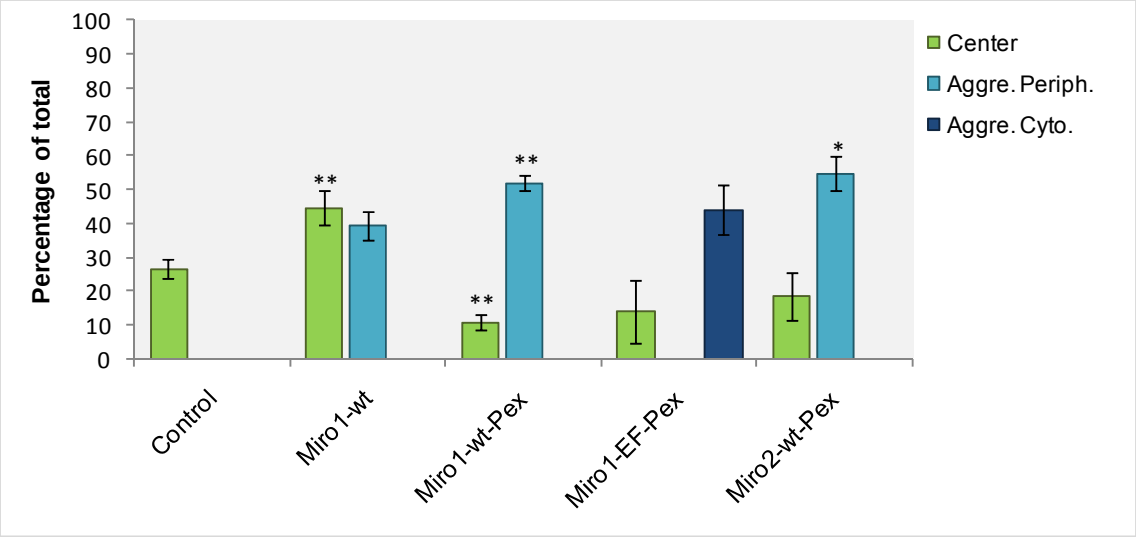

Figure 3.4 – Quantitative analysis of peroxisomal distribution in cells expressing different Myc-Miro contructs. COS-7 cells were transfected with Myc-Miro1-wt, Myc-Miro1-wt-Pex, Myc-Miro1-EF-Pex, Myc-Miro2-wt-Pex or not transfected (Control) using PEI. Cells were fixed at 24h and categorized as cell with scattered or centered peroxisomes (only centered is shown since both categories sum to 100%). The formation of peroxisome aggregates was also analysed in each cell (in the periphery or distributed in the cytoplasm). Data are expressed as means ± SD (** $p<0.01$, * $p<0.05$; distribution compared to control and aggregates to Myc-Miro1-wt)

Expression of Myc-Miro1-EF-Pex revealed a mixed phenotype of peroxisomes in cells of the same preparations. Whereas many cells had a peroxisome distribution as that seen in the control cells (Fig.3.6 E-G), others had peroxisome aggregates not on the cell periphery, but dispersed through the cytoplasm or closer to the nucleus (Fig.3.6 A-D). These aggregates were present in 44 ± 7,2% of the cells (Fig.3.4) and were only seem upon the expression of this construct.

Similar to what was seen with Myc-Miro1-wt-Pex, expression of Myc-Miro2-wt-Pex also induced the formation of peroxisome aggregates in the cell periphery (Fig.3.6 H-K). Surprisingly, even though the Myc-Miro1-Pex proteins were targeted exclusively to peroxisomes, the Myc-Miro2-wt-Pex was frequently targeted to the ER (Fig.3.6 L-N) and, very rarely, even to mitochondria (Fig.3.6 O-Q).

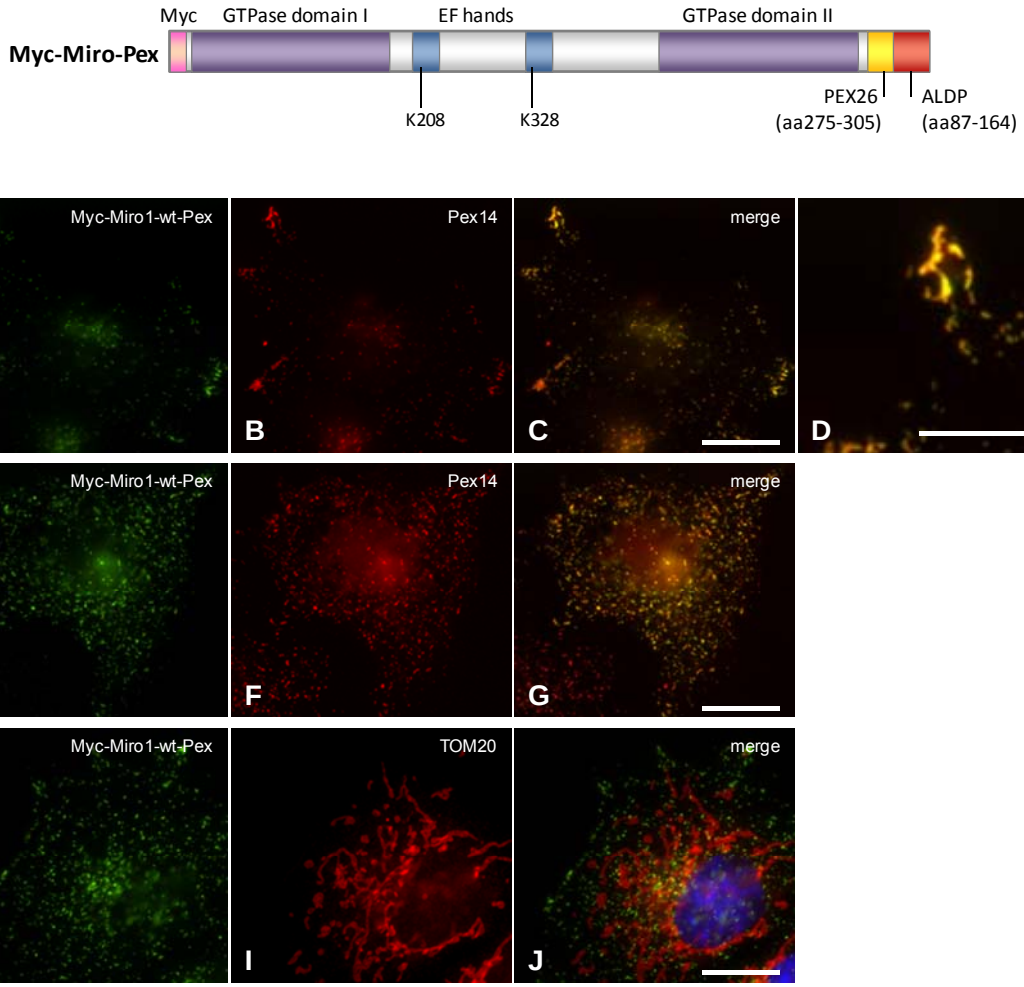

Figure 3.5 – Myc-Miro1-wt-Pex mutant is exclusively targeted to peroxisomes and alters peroxisome distribution. Schematic representation of the construct expressed. COS-7 cells were transfected using PEI with Myc-Miro1-wt-Pex(A-D). Fixed cells were labeled with anti-Pex14 (B, F) or anti-TOM20 (I) and anti-Myc. Magnification from C is shown in D. The images were obtained by fluorescence microscopy. Scale bars: 20 µm (A-C, E-J), 10 µm (D).

3.1.3. Monoamine oxidase B is only localized to mitochondria

Monoamine oxidases have a crucial role in brain function and development. Although they have been previously shown to localize to the outer mitochondrial membrane, a report from (Mann et al., 1992) described their presence on mouse liver peroxisomes. Furthermore, studies in lower eukaryotes such as the fungus *Aspergillus niger* propose a peroxisomal localization of the homologs protein MAO-N (Schilling and Lerch, 1995). To investigate the cellular localization of MAO-B, HepG2 cells were subjected to immunofluorescence with antibodies raised against MAO-B and Pex14 (used as a peroxisomal marker) and visualized at the confocal microscope. As demonstrated in Fig.3.7, MAO-B localizes to worm-like structures that correspond to the mitochondrial network, similar to those observed in control cells stained with an anti-TOM20 antibody (used as a mitochondrial marker). A higher magnification view

(Fig.3.7D) clearly does not show any co-localisation indicating absence of MAO-B on peroxisomes. Similar results were obtained in HeLa cells (not shown).

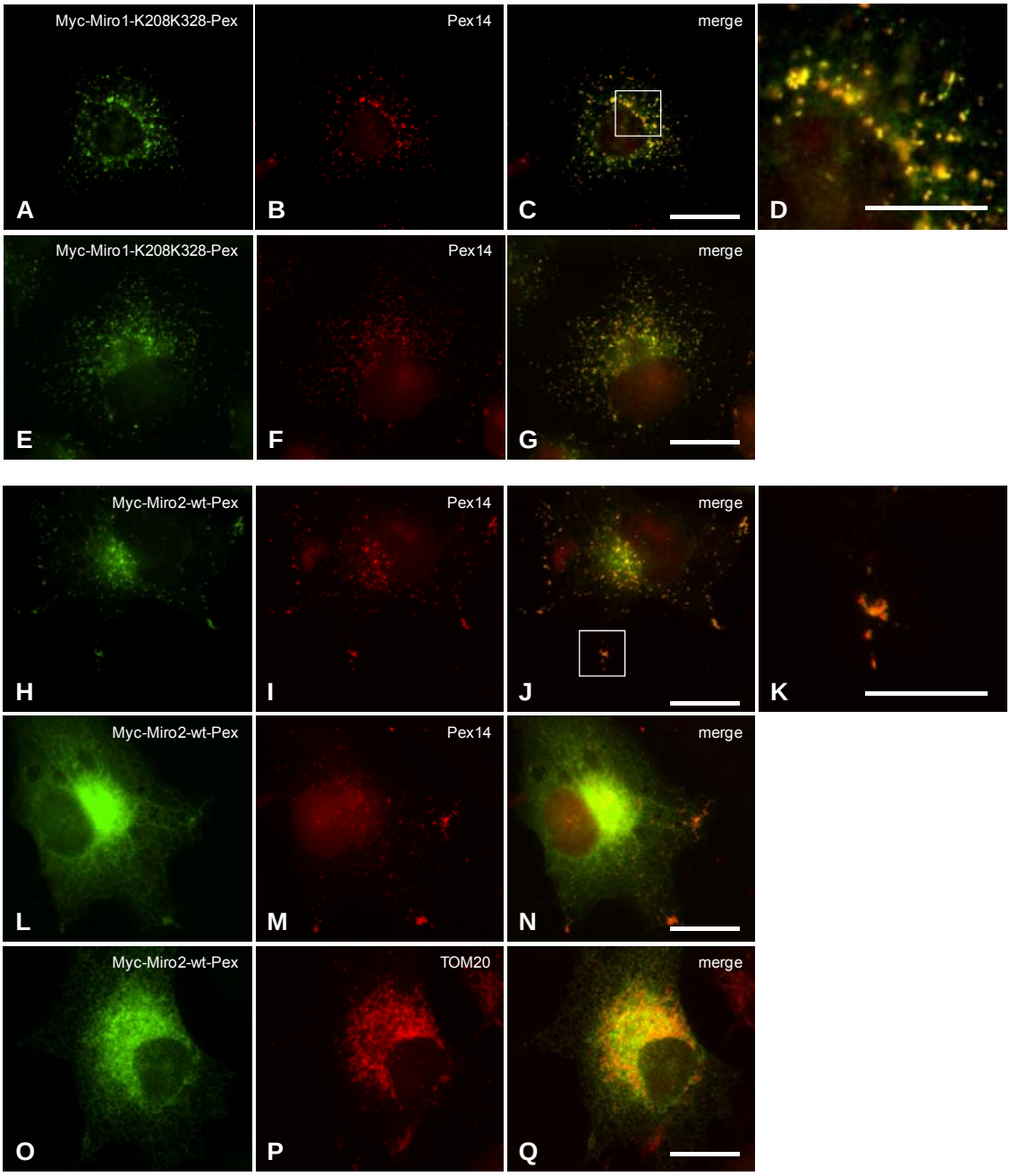

Figure 3.6 – Myc-Miro1-EF-Pex and Myc-Miro2-wt-Pex mutants are targeted to peroxisomes and, exceptionally to the ER and mitochondria. COS-7 cells were transfected with Myc-Miro1-EF-Pex(A-G) and Myc-Miro2-wt-Pex (H-Q) using PEI. Fixed cells were labeled with anti-Pex14 (B, F, I, M) or anti-TOM20 (P) and anti-Myc. Magnification from C and J are shown in D and K, respectively. The images were obtained by fluorescence microscopy. Scale bars: 20 µm (A-C, E-J, L-Q), 10 µm (D, K).

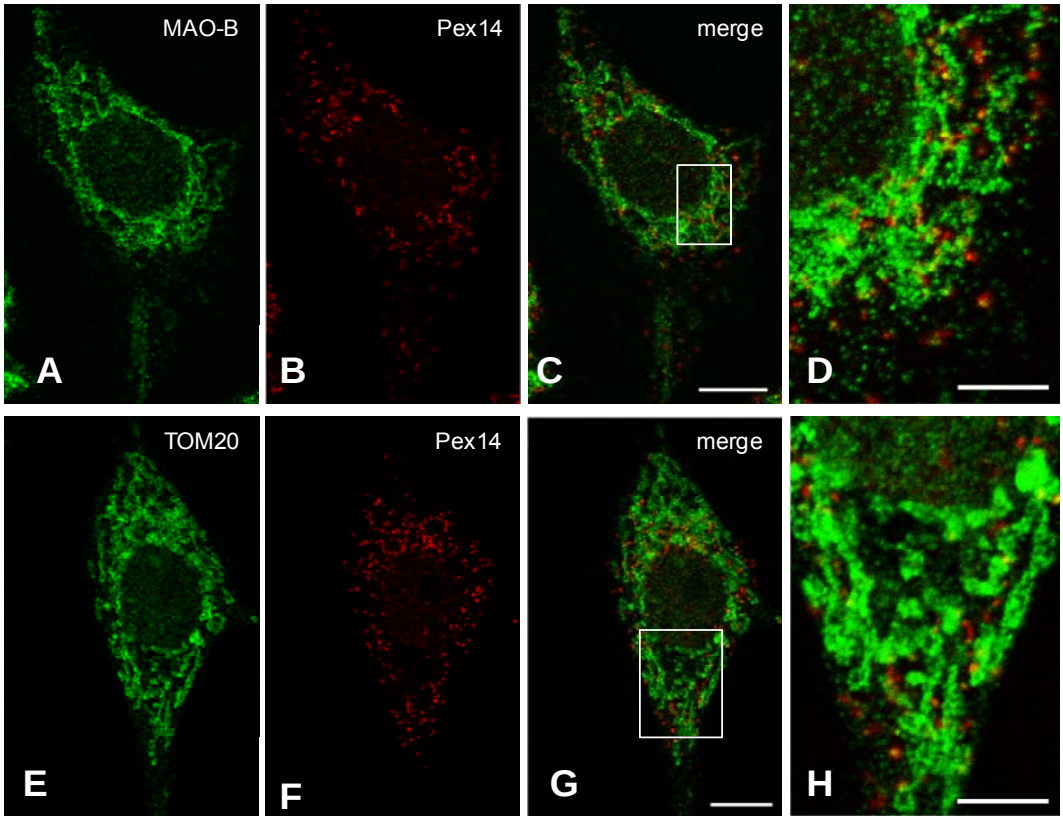

Figure 3.7 – MAO-B does not localise to peroxisomes. Fixed HepG2 cells were subjected to immunofluorescence with anti-MAO-B (A) or anti-TOM20 (E), and anti-Pex14 (B, F). Magnifications from C and G are shown in D and H, respectively. The images were obtained by confocal microscopy. Scale bars: 10 µm (A-C, E-G), 5 µm (D, H).

3.2. *Ustilago maydis* – a new model system to study peroxisomes

Recently, the basidomycete *Ustilago maydis* has been introduced as a new model for the study of cell biological processes (Steinberg and Perez-Martin, 2008). This model brings numerous advantages for the study of the peroxisome-mitochondria relationship. There is strong evidence that, as in humans, *U. maydis* possesses fatty acid β-oxidation in both organelles and, as a result, metabolic cooperation in lipid metabolism (unpublished data). Moreover, *U. maydis* is capable of organelle long-distance transport through microtubules and polarized growth, which are valuable features for the study of peroxisome and mitochondrion transport in neurons. Furthermore, a genome-wide comparison of the proteomes of *U. maydis, S. cerevisiae* and *H. sapiens* has shown that *U. maydis* is more closely related to *H. sapiens* than to *S. cerevisiae (Munsterkotter and Steinberg, 2007)*.

With the objective to identify putative TA proteins and analyzing *U.maydis* homologs of human TA proteins, a bioinformatics analysis of the *U. maydis* proteome was performed.

3.2.1. Identification of putative tail-anchored proteins in the *Ustilago maydis* model system

The identification of putative TA proteins from the *U.maydis* proteome was performed initially by pre-selection of proteins with a single TMD, using the Pedant 3 database. This list was further filtered by removing proteins with N-terminal TMDs, luminal tails with more than thirty amino acids, and signal peptides (both for the ER and mitochondria) (Fig.3.8). The final list obtained contained a total of 58 proteins.

A first analysis of the 58 sequences was performed by searching the MIPS *U.maydis* database for functions already attributed to each protein (Fig.3.9). As a result, one protein was identified as dolichyl-phosphate beta-D-mannosyltransferase. Although thirty three proteins were of unknown function/homology (i.e. hypothetical and putative proteins), twenty-four were identified as “probable” or “related” to known proteins (Tab.3.1). Of these, fourteen are related to TA proteins already described in mammals, of which ten are members of the SNARE family of proteins, two are part of the ER translocon and two are homologs of Fis1 and cytochrome b5. Of the remaining ten proteins, a further comparison was made with a list of bioinformatically predicted human TA proteins (Kalbfleisch et al., 2007), which resulted in the identification of cytochrome-c oxidase subunit VII as a homolog to a human TA protein.

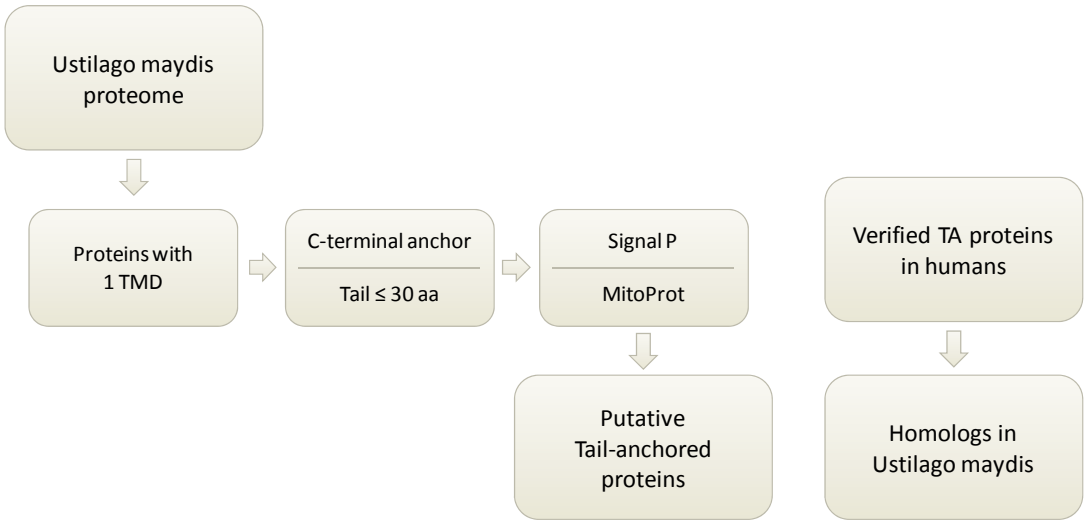

Figure 3.8 – Schematic view of the bioinformatics approach followed for the identification of TA proteins in *U. maydis*.

Table 3.1 – Putative TA proteins identified in *U.maydis* with identified or probable function.

	Known function		H. Hs
	um06329	dolichyl-phosphate beta-D-mannosyltransferase	28.5%
Known TA proteins	**SNARE family**		
	um00284	related to vesicle-associated membrane protein 7	30.5%
	um00340	related to syntaxin 18	18.6%
	um12108	probable syntaxin, vesicular transport protein	39.4%
	um11669	related to syntaxin family member TLG1	27%
	um02338	related to PEP12 syntaxin (T-SNARE), vacuolar	27.3%
	um11485	related to SNARE protein of Golgi compartment	30.6%
	um10441	probable synaptobrevin (v-SNARE) homolog present on post-Golgi vesicles	30.9%
	um05645	related to VTI1 - v-SNARE: involved in Golgi retrograde protein traffic	34.4%
	um11027	probable SEC22 - synaptobrevin (V-SNARE)	42,1%
	um11219	related to TLG2 - member of the syntaxin family of t-SNAREs	26.4%
	ER translocon		
	um10035	probable protein transport protein sec61 beta subunit	38.6%
	um11624.2	related to SSS1 - ER protein-translocase complex subunit	51.4%
	Other		
	um10232	probable cytochrome b5	34.9%
	um03919	related to FIS1 - protein involved in mitochondrial division	30.2%
	Other proteins		
	um00053	probable 3-dehydroquinate dehydratase	17.9%
	um11614	related to TOM5 - mitochondrial outer membrane protein	2.6%
	um01150	related to COX7 - cytochrome-c oxidase, subunit VII	20.9%
	um11696	related to SCS2 - required for inositol metabolism	20.7%
	um01869	related to NADH oxidase	10.5%
	um06284	related to Cutl1 or CASP protein	30.2%
	um11097	related to QCR10 - ubiquinol--cytochrome-c reductase 8.5 kDa subunit	2.8%
	um03933	related to WWM1 - WW domain containing protein interacting with Metacaspase	11.9%
	um04210	probable phytoene dehydrogenase	21.1%
	um05065	related to SPT23 - suppressor of TY retrotransposon	10.2%

Abbreviations: H. Hs, Homology to *Homo sapiens* proteins.

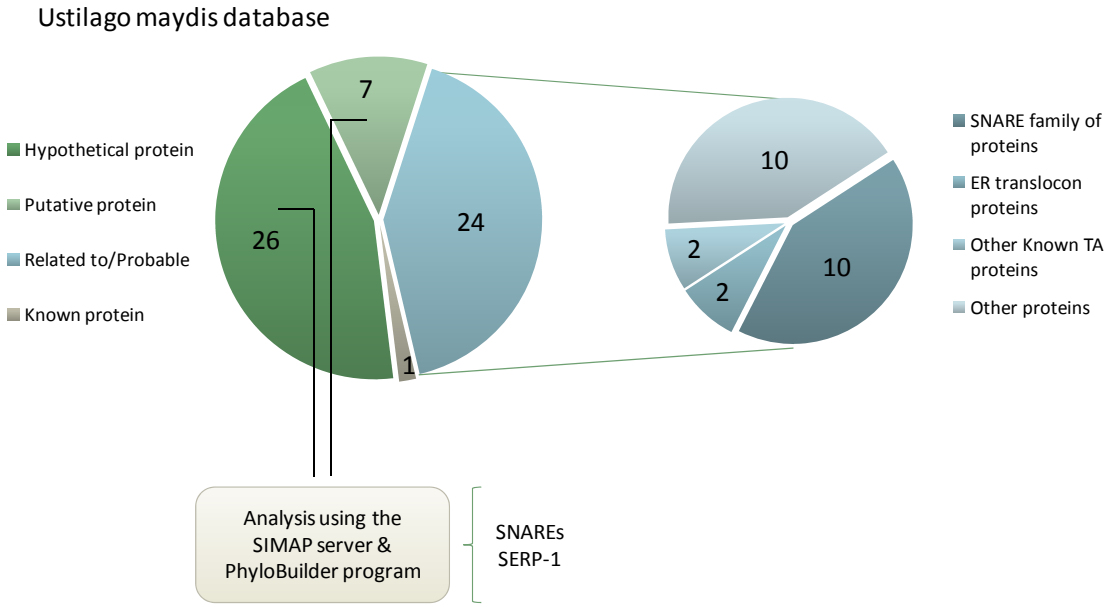

Figure 3.9 – Graphical representation of putative TA proteins identified in *U.maydis*.

A further examination of the hypothetical/putative proteins was performed using the MIPS *U.maydis* database which automatically searches the SIMAP database for homology sequences in several organisms. In parallel, each sequence was run through the PhyloBuilder software to create phylogenetic trees. Analysis of the results revealed the probable function of 4 proteins. Of these, three were classified as possible SNAREs and one is a probable homolog of the stress-associated ER protein 1 (SERP-1) (Fig.3.9).

Furthermore, a list of verified human TA proteins (excluding SNAREs) was created from an existing list (Kalbfleisch, 2007) to which data from our laboratory was added. These proteins were individually fed to the WUBLAST search tool, available at the MIPS *U.maydis* database to search for possible *U.maydis* homologs. Since many proteins are yet to be characterized in this organism, it was difficult to find credible homologs for many of the human TA proteins. Nevertheless, eleven possible homologs were identified (Tab.3.2). Of these, three were already identified in the first bioinformatics analysis (i.e. Fis1, cytochrome b5 and Sec61). The remaining eight were analysed following the same steps described for the selection of putative TA proteins. Six proteins were found to lack predictable TMDs, one had a tail which goes beyond the defined size limit and one possesses a signal peptide for mitochondria.

Table 3.2 – *U.maydis* homologs of verified human TA proteins

Human protein	U.maydis probable homolog		
MAO-A/MAO-B	um05423	related to Monoamine oxidase A [flavin-containing]	Lacks detectable TMD
Cytochrome b5	um10232	probable cytochrome b5	Identified
TOM7	um10037	probable mitochondrial import receptor subunit tom7	Lacks detectable TMD
ACBD5	um02959	conserved hypothetical protein	Lacks detectable TMD
Miro1/Miro2	um02638	conserved hypothetical protein	Longer tail – 56a.a.
Fis1	um03919	related to FIS1 - protein involved in mitochondrial division	Identified
HMOX-1	um00783	related to Heme oxygenase	Lacks detectable TMD
Sec61	um10035	probable protein transport protein sec61 beta subunit	Identified
ALDH3A2	um11241	related to aldehyde dehydrogenase [NAD(P)]	Lacks detectable TMD
UBE2J2	um11635	probable UBC6 - E2 ubiquitin-conjugating enzyme	Predicted signal peptide for mitochondria
PTPN1	um10534	related to Protein-tyrosine phosphatase, receptor type 1	Lacks detectable TMD

Chapter 4 Discussion

4.1. The peroxisome-mitochondria connection – the role of TA proteins

Peroxisomes and mitochondria are a perfect example of intracellular cooperation due to their ability to perform similar and, at the same time, complementary functions, from the β-oxidation of several lipids to the metabolism of ROS. The recent discovery that these organelles share components of their division machinery and act in concert on antiviral defence has led to a closer examination of their interrelationship.

Most of the membrane proteins known to be shared by these organelles belong to the class of TA proteins (e.g. Fis1, Mff and MAVS). This has led us to investigate the mechanisms by which these proteins are targeted to different membranes (Delille and Schrader, 2008) and the possibility that other proteins are dually targeted to peroxisomes and mitochondria. Hence, a list of verified mammalian TA proteins was created containing data published by Kalbfleisch et al. (2007) and unpublished data from our laboratory.

In this work I present the results obtained for three of these proteins: monoamine oxidase B (MAO-B), Miro1 and Miro2.

4.1.1. Monoamine oxidase – a new role for peroxisomes in the brain?

Monoamine oxidases are mitochondrial TA proteins which are essential for normal brain development due to their ability to degrade several neurotransmiters such as serotonin, histamine and catecholamines. Genetic variance that affects the activity of these proteins has been related to several personality traits, more specifically the development of aggressive and addictive behaviours. Moreover, MAO inhibitors have been used for several years for the treatment of depression and, more recently, Parkinson's disease (Youdim et al., 2006). Although MAOs are commonly regarded as mitochondrial proteins, a report by Mann et al. (1992) showed that liver peroxisome fractions from genetically obese mice (*ob/ob* and *db/db*) contain monoamine oxidases. However, our analysis of the cellular localization of MAO-B by immunofluorescence has shown that this protein is only localized to mitochondria in HepG2 and HeLa cells.

How is it then that these proteins were identified in liver peroxisome fractions of obese mice? One of the proposed explanations in the aforementioned article was that the peroxisomal membrane would be altered due to variations in the proportion of several fatty acids in obese mice and that this could promote protein mistargeting. Whereas at the time the authors considered this hypothesis unlikely, it becomes a possible scenario if we consider that the insertion of TA proteins in their target

membranes is affected by the composition of the lipid bilayer. It is then possible that MAO is not usually targeted to peroxisomes but is instead mistargeted if the lipid composition of their membrane is altered. In fact, this could have a physiological role in the determination of protein localization and function in different cellular conditions.

Moreover, we have only addressed the localization of MAO-B due to antibody availability. It is possible that MAO-A has a peroxisomal localization due to some variation in the TMD of the protein. Additionally, although it is unlikely that the localization of MAOs differs between humans and rodents, we cannot reject the fact that initial evidence of peroxisomal MAOs was described in mice. To examine this hypothesis, an immuno-localization analysis of this protein was attempted in rat liver cell lines but the antibody available did not recognize rat MAOs.

Curiously, in contrast to the human MAO-A and MAO-B, the MAO-N protein from *Aspergillus niger* is proposed to have a peroxisomal localization. This protein contains a conserved PTS1 sequence at the very C-terminus and lacks the helical membrane anchor seen in the human counterparts. There has been considerable interest in MAO-N due to its sequence homology to the two forms of human MAO and due to its response to highly selective inhibitors of MAO-A and MAO-B. Moreover, analysis of the X-ray structure of these proteins suggests that MAO-N could be an evolutionary precursor of vertebrate MAOs. From an evolutionary point of view, it will be interesting to see if the *U.maydis* monoamine oxidase is localised to peroxisomes, the mitochondrial outer membrane or to other cellular compartment. The *U.maydis* MAO protein lacks any predicted peroxisome targeting signals but it also lacks a transmembrane domain.

4.1.2. Miro proteins – the missing link to peroxisome motility?

Organelles are transported and actively positioned inside cells to maintain correct cellular organization and effective cell functioning. In yeast and plants, peroxisomes are known to move through actin filaments whereas in mammalian cells peroxisomes bind and move along microtubules (Fagarasanu et al., 2010; Muench and Mullen, 2003).

Several studies in *S.cerevisiae* have enabled the identification and characterization of the molecular machinery responsible for actin-based movement of peroxisomes (reviewed in Fagarasanu et al., 2007; Fagarasanu et al., 2010). In this organism, peroxisomes of unbudded cells are static and distributed over the cell cortex. As soon as the bud starts to form from the mother cell, peroxisomes are transported to the new cell by the class V myosin motor Myo2. This motor interacts with the peroxisomal membrane receptor protein Inp2. Conversely, the Inp1 protein anchors

peroxisomes to the cell periphery of the mother cell and the bud, enabling an equal distribution of this organelle.

The first reports on microtubule-dependent movement of mammalian peroxisomes were published in 1996 (Rapp, 1996; Schrader, 1996; Wiemer et al., 1997). Since then, however, only a few groups have succeeded in revealing new insights in the molecular machinery responsible for peroxisome transport in mammalian cells (Bharti et al., 2011; Schrader et al., 2003).

Peroxisomes move both towards and away from the cell centre, by a mechanism that is likely dependent on kinesin and dynein motors. Although the involvement of dynein and its activator complex dynactin has been demonstrated (Schrader et al., 2000), an interaction of this organelle with kinesins in mammalian cells remains circumstantial. Nonetheless, (Kural et al., 2005) showed that peroxisome motility in cultured *Drosophila* S2 cells is also microtubule-dependent and that several motors, from kinesins to dyneins, coordinate their actions to promote organelle transport.

In an attempt to identify the molecular link between peroxisomes and motor proteins, in particular with kinesins, we decided to test if the mitochondrial Miro proteins could also be responsible for the anterograde transport of peroxisomes.

Miro proteins are a family of RasGTPases which were initially associated with the regulation of mitochondrial homeostasis and apoptosis (Fransson et al., 2003). Further investigation in *Drosophila* connected this protein with the anterograde transport of mitochondria, in association with another protein – Milton – which is required to link Miro and the kinesin heavy chain (KHC) (Guo et al., 2005). More recently, a number of publications have reported on the importance of these proteins in the regulation of mitochondrial movement in neuronal cells and the importance of their calcium-binding domains for the anchoring of mitochondria in active synapses (reviewed in Liu and Hajnoczky, 2009; Reis et al., 2009).

By expressing Miro1 and Miro2 in COS-7 cells, we were able to show that these proteins are dually targeted to peroxisomes and mitochondria. Furthermore, analysis of highly purified peroxisomal fractions from rat liver confirmed that endogenous Miro1 protein is in fact present in peroxisomes. Like with other TA proteins that are targeted to peroxisomes and mitochondria this raises the question of how these proteins are specifically targeted and inserted into each organelle. Interestingly, peroxisomal TA proteins appear to use the same sorting machinery as PMPs to target peroxisomes. Our group has shown that the targeting of the human TA protein Fis1 is mediated by Pex19 (Delille and Schrader, 2008) and similar results have been obtained for the targeting of Pex26 and its yeast homolog Pex15 (Halbach et al., 2006). It will be

interesting to see if other TA proteins, including Miro1 and Miro2, follow a similar pathway.

The expression of Myc-Miro proteins induced significant alterations of the mitochondrial network (Fransson et al., 2006). Curiously, only minor changes were observed on peroxisome morphology and distribution. This could be explained by the low proportion of peroxisomes in a cell that exhibit microtubule-dependent movement (only 10-15%). Although peroxisomes change their motility state with time, a large number of peroxisomes is anchored either to the actin or microtubule cytoskeleton at any given time. Therefore, overexpression of Miro may not be sufficient to substantially increase the microtubule-dependent movement of peroxisomes. Furthermore, peroxisome motility has been shown to be tightly regulated in yeast (Fagarasanu et al., 2010) and, although less studied, some signalling pathways have been associated with the regulation of peroxisome motility in mammals (Huber et al., 2000).

The most prominent alterations in peroxisome distribution were seen in cells transfected with either Myc-Miro1-wt or Myc-Miro2-wt, which presented aggregates of peroxisomes in the cell periphery. These aggregates are likely the result of an increase in the number of binding sites for kinesin motors, favouring therefore anterograde transport of peroxisomes. Moreover, in cells with completely aggregated mitochondrial networks, peroxisomes were usually found closer to the nucleus. This result is most likely an indirect effect of the altered distribution of the mitochondrial network.

Curiously, no major alterations were seen in peroxisomes of cells transfected with either one of the Myc-Miro mutants, except for the concentration of peroxisomes in the perinuclear area in cells with collapsed mitochondrial networks. Since most of the cells expressing Myc-Miro mutants had an altered mitochondrial network, many times with aggregated mitochondria, a possible effect in peroxisome distribution might have been masked by the disturbance of the mitochondrial network.

Overall, the results obtained for cells transfected with either the wild-type or mutants forms of both Miro proteins were not conclusive due to the severe alterations observed in the mitochondrial network. Owing to this, we created a fusion protein exclusively directed to peroxisomes by exchanging Miro's TMD and tail with an artificial Pex26/ALDP construct. This tail, which has been used previously to direct GFP to peroxisomes, contains a Pex19 binding site that enables the transport of PMPs to the peroxisomal membrane.

Transfected cells expressed Myc-tagged Miro proteins which were exclusively targeted to peroxisomes. Moreover, although expression of these proteins promoted alterations in peroxisome distribution in several cells, transfected cells with normal peroxisome morphology and distribution were also detected. This could be due to the

expression level of the transfected proteins but also to the intrinsic mechanisms that regulate peroxisome motility. Althought little is known about the proteins that regulate peroxisome movement in mammalian cells, in *S.cerevisiae* peroxisome distribution in tightly regulated in cell division (Fagarasanu et al., 2010).

Expression of Myc-Miro1-wt-Pex and Myc-Miro2-wt-Pex induced the formation of aggregates in the cell periphery as seen with the regular proteins, but in a higher number of cells. This shows that Miro does in fact promote anterograde transport of peroxisomes, very likely following the same mechanism used for the transport of mitochondria. Additionally, peroxisome morphology was not altered in these cells, suggesting that peroxisome motility is not essential for organelle elongation.

Unexpectedly, even though expressed Myc-Miro2-wt-Pex was targeted to peroxisomes, some cells also showed an ER targeting of this protein and, less frequently, a mitochondrial targeting. Mislocalization of this protein to the ER was also seen in cells transfected with Myc-Miro2-wt, indicating that there might be some type of targeting information in the cytoplasmic domain. It is also possible that Miro2 interacts with other proteins present at the ER membrane and can therefore be recruited and inserted in this membrane. In these cells, unlike those transfected with Myc-Miro1-Pex constructs, mitochondrial morphology was frequently altered, although with less severe phenotypes when compared to Myc-Miro2-wt transfection.

In addition to the wild-type Miro-Pex proteins, we cloned the calcium mutants of Miro. Although the effect of calcium on the regulation of mitochondrial distribution is well described, to the best of our knowledge no effects on peroxisome dynamics have yet been reported.

As seen in the results, expression of Myc-Miro1-EF-Pex induced the formation of peroxisome aggregates scattered throughout the cell or closer to the nucleus. This result is very interesting if we consider that in neurons, expression of Miro1-EF keeps mitochondria from docking at active synapses. It appears that peroxisome distribution and transport to the cell periphery might as well be dependent on the capacity of Miro to bind calcium. Consequently, it is possible that under normal conditions where Miro is able to bind calcium, peroxisome motility might be regulated by shifts in calcium concentration. This might have implications for the control of peroxisome function and motility in mitosis, as well as in stress conditions. In fact, a plant calcium-dependent protein kinase has been shown to target peroxisomes, providing a potential mechanism for calcium to regulate several metabolic pathways in this organelle (Dammann et al., 2003).

Overall, we can propose a mechanism for the transport of peroxisomes in mammalian cells (Fig.4.1), where Miro1 and Miro2 bridge the interaction between

peroxisomes and kinesin motors. This interaction is likely regulated by shifts in intracellular calcium concentrations, with potential implications for peroxisome function and motility, as well as possible new roles for peroxisomes in calcium signalling. Furthermore, proteins which have been shown to interact with Miro in mitochondria are likely to also form a complex with peroxisomal Miro and regulate peroxisome motility. Additionally, peroxisomal proteins such as Pex14 which have been shown to affect this organelle's motility might as well interact with Miro proteins forming a protein complex.

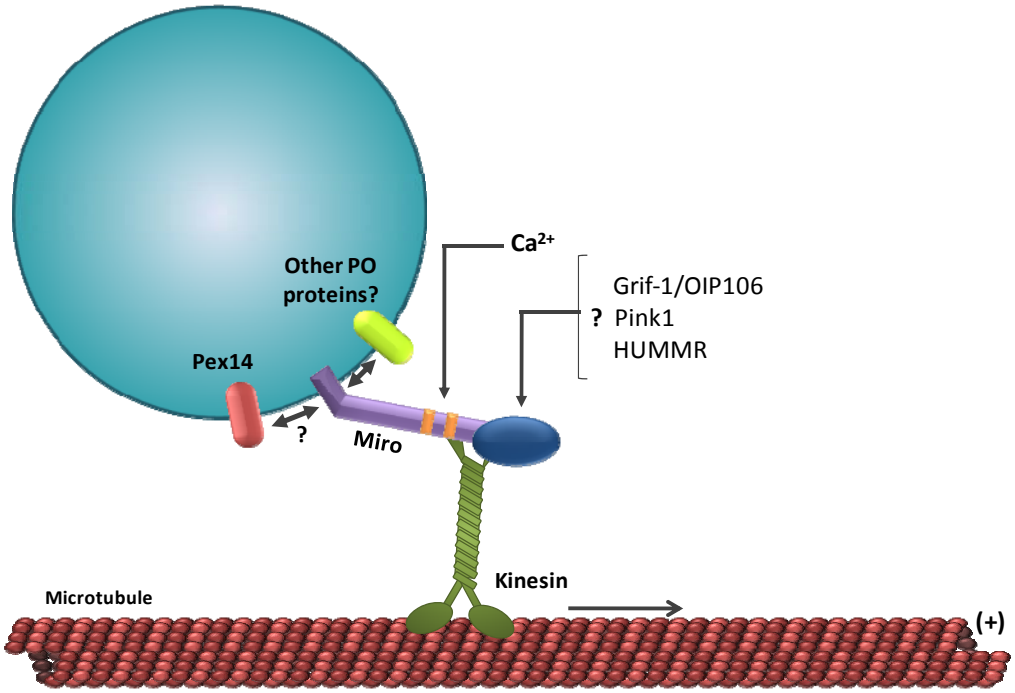

Figure 4.1 – Model for microtubule-dependent transport of peroxisomes in mammalian cells.

4.2. *Ustilago maydis* – a new model to study peroxisomes

The use of unicellular model systems for the study of cell and molecular biology has several advantages, such as the ability to easily manipulate genetic information, short generation times, simple cultivation methods and the existence of sophisticated molecular tools. Nevertheless, some models like the commonly used yeast *Saccharomyces cerevisiae* have their limitations and lack some structures and functions present in mammalian cells.

Recently, the basidomycete *Ustilago maydis* has been introduced as a new model for the study of cell biology processes (Steinberg and Perez-Martin, 2008). This organism shares several features common to higher eukaryotes such as long-distance organelle transport through microtubules, polarized growth and the removal of the

nuclear envelope during mitosis. Moreover, a genome-wide comparison of the proteomes of *U. maydis, S. cerevisiae* and humans revealed that *U. maydis* shares more protein sequence similarity with humans than with *S. cerevisiae (Munsterkotter and Steinberg, 2007)*. Indeed, many proteins shared by humans and *U.maydis* but not present in *S.cerevisiae*, are functionally characterized and can be associated with several cellular processes. Also, a set of 42 proteins is related to serious human disorders.

This cell model also brings numerous advantages for the study of the peroxisome-mitochondria relationship. There is strong evidence that *U. maydis* possesses β-oxidation in both organelles and, as a result, metabolic cooperation in lipid metabolism, like humans (unpublished data). Even more, *U. maydis* is capable of organelle long-distance transport through microtubules and polarized growth, which are valuable features for the study of peroxisome and mitochondria transport across neurons.

Given the advantages of this system, the establishment of *U. maydis* in our laboratory will surely prove valuable for the study of peroxisomal functions and dynamics, and its interactions with other organelles, namely mitochondria.

In line with this, a bioinformatics analysis of the *U. maydis* proteome was performed following two main objectives: the identification of putative TA proteins, and the analysis of *U.maydis* homologs of known human TA proteins.

As described in the results section, a first analysis of the *U.maydis* proteome enabled the identification of 58 putative TA proteins from a total of 6.925 sequences.

Using several bioinfomatic tools it was possible to identify the function of some of these proteins, as well as their human homologs. As expected, members of the conserved SNARE family of proteins were found in this list, as well as some members of the ER translocon. Nevertheless, many proteins remained as hypothetical/putative due to lack or very low homology to other sequences on the used databases.

Furthermore, the inverse search of the *U.maydis* proteome using the list of human TA proteins shows an interesting result, since many of the identified proteins lack predicted TMDs. In these cases, it will be interesting to see if these proteins are in fact cytosolic and to compare each sequence with several homologs from different organisms and complexities to see where and when they became tail-anchored.

Chapter 5 Final Remarks

5.1. Conclusion

In this study we have focused on the role of TA proteins in the peroxisome-mitochondria connection. In order to extend our knowledge about this interesting interrelationship, we aimed at identifying new proteins which are dually targeted to both peroxisomes and mitochondria and to unveil their functions in peroxisome biology. We found that (1) MAO-B is only localized to mitochondria; (2) Miro proteins are targeted to both peroxisomes and mitochondria, where they are likely involved in the regulation of organelle transport/motility through kinesin motors and the microtubule cytoskeleton; (3) peroxisome motility is dependent on the ability of Miro proteins to bind calcium and might, therefore, be regulated by shifts in intracellular calcium concentrations.

Taken together, we were able to identify the missing molecular link between peroxisomes and kinesin motors, shedding light on the mechanism that regulates anterograde transport of peroxisomes in mammalian cells. Moreover, we also suggest a new function for calcium in the regulation of peroxisome motility. Further investigation of Miro proteins will certainly lead to a better understanding of peroxisome dynamics and its importance for peroxisome biology and cellular vitality.

Furthermore, we have created new tools (i.e. the Miro-Pex constructs) to study peroxisome movement in mammalian cells. These will prove valuable to study the importance of motility for peroxisome elongation, division and proliferation, as well as its effect on pexophagy.

We have also started a bioinformatics analysis of the *U. maydis* proteome. This model system will be used, in parallel, to study peroxisome motility as it shows microtubule-dependent long distance transport of organelles. Additionally, there is strong evidence that, as in humans, *U.maydis* possesses fatty acid β-oxidation not only in peroxisomes but also in mitochondria (unpublished data). Taken together, the use of this model system will aid us in the study of peroxisome biology, as well as its connection to mitochondria.

Overall, the discovery of new proteins and functions of peroxisomes will not only promote a better understanding of this organelle *per se* but will also have implications in the diagnosis and treatment of patients with peroxisomal disorders.

5.2. Future perspectives

Although we have identified the potential molecular link between peroxisomes and kinesin motors, we have yet to identify the regulatory mechanisms involved in this process. A first step to continue this work would be to confirm that the peroxisome distribution phenotypes observed in cells transfected with Myc-Miro-Pex proteins are the result of alterations in the microtubule-dependent transport of peroxisomes. In order to do this, we will study the effect of depolymerising drugs on peroxisome distribution.

We will also continue our cloning approach to study the effect of mutations in the GTPase domains of Miro on peroxisome motility. It will be interesting to see if the constituvely active mutant of Miro (V13) has a similar or enhanced effect compared to the wild-type protein.

Additionally, we will study the effects of Miro expression on peroxisome motility by live-cell imaging analysis of the direction and velocity of peroxisomes in transfected and non-trasnfected cells. In these studies we will also examine the influence of different calcium concentrations on peroxisome movement and the importance of Miro's calcium-binding domains in this mechanism.

Furthermore, several binding partners of Miro have been identified in mitochondria. Specifically, Grif-1 and OIP106 have a key function in linking Miro proteins to the kinesin heavy chain, and have been shown to significantly alter mitochondrial morphology (MacAskill et al., 2009a). Expression of these proteins and analysis of their localization will provide us with more information on the regulation of peroxisome motility. Also, it will be interesting to test if Pink1 and HUMMR, two proteins which have been found to interact with Miro proteins, are also associated with peroxisomal Miro (Li et al., 2009; Weihofen et al., 2009). Pink1 is a kinase that is usually targeted to mitochondria and has been associated with Parkinson's disease, whereas HUMMR is a newly identified hypoxia up-regulated mitochondrial movement regulator.

We will also study a possible interaction of Miro with peroxisomal proteins such as Pex14. This peroxin has been recently shown to interact directly with tubulin and to affect peroxisome movement in mammalian cells, by possibly anchoring this organelle to microtubules. Since peroxisomal mutants in ΔPex14 cells are immotile, it would be interesting to see if the expression of Miro proteins in these cells affects their movement and distribution.

With the aim of identifying the mechanisms of targeting and insertion of Miro proteins into peroxisomes, we will also analyse the possible interaction with Pex19, a peroxisomal chaperone that has been shown to target Fis1 to the peroxisomal membrane (Delille and Schrader, 2008).

5.3. Publications resulting from this work

Islinger M., Castro I., Almeida M., Bonekamp N. and Schrader M.: Targeting of tail-anchored proteins to peroxisomes (2011, in preparation).

Castro I., Ribeiro D. and Schrader M.: A role for Miro1 in peroxisomal motility (2011, in preparation).

Chapter 6 References

Agrawal, G., Joshi, S., and Subramani, S. (2011). Cell-free sorting of peroxisomal membrane proteins from the endoplasmic reticulum. Proc Natl Acad Sci U S A *108*, 9113-9118.

Antonenkov, V.D., Grunau, S., Ohlmeier, S., and Hiltunen, J.K. (2010). Peroxisomes are oxidative organelles. Antioxid Redox Signal *13*, 525-537.

Baes, M., and Aubourg, P. (2009). Peroxisomes, myelination, and axonal integrity in the CNS. Neuroscientist *15*, 367-379.

Bagattin, A., Hugendubler, L., and Mueller, E. (2010). Transcriptional coactivator PGC-1alpha promotes peroxisomal remodeling and biogenesis. Proc Natl Acad Sci U S A *107*, 20376-20381.

Baumgart, E., Vanhorebeek, I., Grabenbauer, M., Borgers, M., Declercq, P.E., Fahimi, H.D., and Baes, M. (2001). Mitochondrial alterations caused by defective peroxisomal biogenesis in a mouse model for Zellweger syndrome (PEX5 knockout mouse). Am J Pathol *159*, 1477-1494.

Beal, M.F. (2005). Mitochondria take center stage in aging and neurodegeneration. Ann Neurol *58*, 495-505.

Bellot, G., Cartron, P.F., Er, E., Oliver, L., Juin, P., Armstrong, L.C., Bornstein, P., Mihara, K., Manon, S., and Vallette, F.M. (2007). TOM22, a core component of the mitochondria outer membrane protein translocation pore, is a mitochondrial receptor for the proapoptotic protein Bax. Cell Death Differ *14*, 785-794.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. J Mol Biol *340*, 783-795.

Bharti, P., Schliebs, W., Schievelbusch, T., Neuhaus, A., David, C., Kock, K., Herrmann, C., Meyer, H.E., Wiese, S., Warscheid, B.*, et al.* (2011). PEX14 is required for microtubule-based peroxisome motility in human cells. J Cell Sci *124*, 1759-1768.

Bonekamp, N.A., Volkl, A., Fahimi, H.D., and Schrader, M. (2009). Reactive oxygen species and peroxisomes: struggling for balance. BioFactors *35*, 346-355.

Borgese, N., Brambillasca, S., and Colombo, S. (2007). How tails guide tail-anchored proteins to their destinations. Curr Opin Cell Biol *19*, 368-375.

Borgese, N., Colombo, S., and Pedrazzini, E. (2003). The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. J Cell Biol *161*, 1013-1019.

Borgese, N., and Fasana, E. (2011). Targeting pathways of C-tail-anchored proteins. Biochim Biophys Acta *1808*, 937-946.

Bortolato, M., Chen, K., and Shih, J.C. (2008). Monoamine oxidase inactivation: from pathophysiology to therapeutics. Adv Drug Deliv Rev *60*, 1527-1533.

Brambillasca, S., Yabal, M., Soffientini, P., Stefanovic, S., Makarow, M., Hegde, R.S., and Borgese, N. (2005). Transmembrane topogenesis of a tail-anchored protein is modulated by membrane lipid composition. EMBO J *24*, 2533-2542.

Braschi, E., Goyon, V., Zunino, R., Mohanty, A., Xu, L., and McBride, H.M. (2010). Vps35 mediates vesicle transport between the mitochondria and peroxisomes. Curr Biol *20*, 1310-1315.

Brocard, C., and Hartig, A. (2006). Peroxisome targeting signal 1: is it really a simple tripeptide? Biochim Biophys Acta *1763*, 1565-1573.

Brown, G.C., and Borutaite, V. There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. Mitochondrion *In Press, Corrected Proof*.

Brown, L.A., and Baker, A. (2008). Shuttles and cycles: transport of proteins into the peroxisome matrix (review). Mol Membr Biol *25*, 363-375.

Camões, F., Bonekamp, N.A., Delille, H.K., and Schrader, M. (2009). Organelle dynamics and dysfunction: A closer link between peroxisomes and mitochondria. J Inherit Metab Dis *32*, 163-180.

Castanier, C., Garcin, D., Vazquez, A., and Arnoult, D. (2010). Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. EMBO Rep *11*, 133-138.

Chang, C.R., Manlandro, C.M., Arnoult, D., Stadler, J., Posey, A.E., Hill, R.B., and Blackstone, C. (2010). A lethal de novo mutation in the middle domain of the dynamin-related GTPase Drp1 impairs higher order assembly and mitochondrial division. J Biol Chem *285*, 32494-32503.

Claros, M.G., and Vincens, P. (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem *241*, 779-786.

Colombo, S.F., Longhi, R., and Borgese, N. (2009). The role of cytosolic proteins in the insertion of tail-anchored proteins into phospholipid bilayers. J Cell Sci *122*, 2383-2392.

Dammann, C., Ichida, A., Hong, B., Romanowsky, S.M., Hrabak, E.M., Harmon, A.C., Pickard, B.G., and Harper, J.F. (2003). Subcellular targeting of nine calcium-dependent protein kinase isoforms from Arabidopsis. Plant Physiol *132*, 1840-1848.

de Duve, C. (1965). Function of microbodies (peroxisomes). The Journal of Cell Biology *27*, 25A-26A.

de Vries, B., Todde, V., Stevens, P., Salomons, F., van der Klei, I.J., and Veenhuis, M. (2006). Pex14p is not required for N-starvation induced microautophagy and in catalytic amounts for macropexophagy in Hansenula polymorpha. Autophagy *2*, 183-188.

Delille, H.K., Agricola, B., Guimarães, S.C., Borta, H., Luers, G.H., Fransen, M., and Schrader, M. (2010). Pex11pbeta-mediated growth and division of mammalian peroxisomes follows a maturation pathway. J Cell Sci *123*, 2750-2762.

Delille, H.K., Alves, R., and Schrader, M. (2009). Biogenesis of peroxisomes and mitochondria: linked by division. Histochem Cell Biol *131*, 441-446.

Delille, H.K., and Schrader, M. (2008). Targeting of hFis1 to peroxisomes is mediated by Pex19p. J Biol Chem *283*, 31107-31115.

Detmer, S.A., and Chan, D.C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol *8*, 870-879.

Dikov, D., and Reichert, A.S. (2011). How to split up: lessons from mitochondria. EMBO J *30*, 2751-2753.

DiMauro, S. (2004). Mitochondrial diseases. Biochim Biophys Acta *1658*, 80-88.

Distel, B., Erdmann, R., Gould, S.J., Blobel, G., Crane, D.I., Cregg, J.M., Dodt, G., Fujiki, Y., Goodman, J.M., Just, W.W*., et al.* (1996). A unified nomenclature for peroxisome biogenesis factors. J Cell Biol *135*, 1-3.

Dixit, E., Boulant, S., Zhang, Y., Lee, A.S., Odendall, C., Shum, B., Hacohen, N., Chen, Z.J., Whelan, S.P., Fransen, M*., et al.* (2010). Peroxisomes are signaling platforms for antiviral innate immunity. Cell *141*, 668-681.

Ebberink, M.S., Mooijer, P.A., Gootjes, J., Koster, J., Wanders, R.J., and Waterham, H.R. (2011). Genetic classification and mutational spectrum of more than 600 patients with a Zellweger syndrome spectrum disorder. Hum Mutat *32*, 59-69.

Epstein, C.B., Waddle, J.A., Hale, W.t., Dave, V., Thornton, J., Macatee, T.L., Garner, H.R., and Butow, R.A. (2001). Genome-wide responses to mitochondrial dysfunction. Mol Biol Cell *12*, 297-308.

Fagarasanu, A., Fagarasanu, M., and Rachubinski, R.A. (2007). Maintaining peroxisome populations: a story of division and inheritance. Annu Rev Cell Dev Biol *23*, 321-344.

Fagarasanu, A., Mast, F.D., Knoblach, B., and Rachubinski, R.A. (2010). Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast. Nat Rev Mol Cell Biol *11*, 644-654.

Fang, Y., Morrell, J.C., Jones, J.M., and Gould, S.J. (2004). PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. J Cell Biol *164*, 863-875.

Fransson, A., Ruusala, A., and Aspenstrom, P. (2003). Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis. J Biol Chem *278*, 6495-6502.

Fransson, S., Ruusala, A., and Aspenstrom, P. (2006). The atypical Rho GTPases Miro-1 and Miro-2 have essential roles in mitochondrial trafficking. Biochem Biophys Res Commun *344*, 500-510.

Gandre-Babbe, S., and van der Bliek, A.M. (2008). The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell *19*, 2402-2412.

Glanville, J.G., Kirshner, D., Krishnamurthy, N., and Sjolander, K. (2007). Berkeley Phylogenomics Group web servers: resources for structural phylogenomic analysis. Nucleic Acids Res *35*, W27-32.

Gomes, L.C., Di Benedetto, G., and Scorrano, L. (2011). During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol *13*, 589-598.

Gorgas, K., Teigler, A., Komljenovic, D., and Just, W.W. (2006). The ether lipid-deficient mouse: tracking down plasmalogen functions. Biochim Biophys Acta *1763*, 1511-1526.

Gould, S.G., Keller, G.A., and Subramani, S. (1987). Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J Cell Biol *105*, 2923-2931.

Guo, X., Macleod, G.T., Wellington, A., Hu, F., Panchumarthi, S., Schoenfield, M., Marin, L., Charlton, M.P., Atwood, H.L., and Zinsmaier, K.E. (2005). The GTPase dMiro is required for axonal transport of mitochondria to Drosophila synapses. Neuron *47*, 379-393.

Halbach, A., Landgraf, C., Lorenzen, S., Rosenkranz, K., Volkmer-Engert, R., Erdmann, R., and Rottensteiner, H. (2006). Targeting of the tail-anchored peroxisomal membrane proteins PEX26 and PEX15 occurs through C-terminal PEX19-binding sites. J Cell Sci *119*, 2508-2517.

Hettema, E.H., and Motley, A.M. (2009). How peroxisomes multiply. J Cell Sci *122*, 2331-2336.

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., and Tabak, H.F. (2005). Contribution of the endoplasmic reticulum to peroxisome formation. Cell *122*, 85-95.

Hoivik, D.J., Qualls, C.W., Jr., Mirabile, R.C., Cariello, N.F., Kimbrough, C.L., Colton, H.M., Anderson, S.P., Santostefano, M.J., Morgan, R.J., Dahl, R.R.*, et al.* (2004). Fibrates induce hepatic peroxisome and mitochondrial proliferation without overt evidence of cellular proliferation and oxidative stress in cynomolgus monkeys. Carcinogenesis *25*, 1757-1769.

Huber, C.M., Saffrich, R., Gorgas, K., and Just, W.W. (2000). Organelle motility regulated by the cell's environment: dissection of signaling pathways regulating movements of peroxisomes. Protoplasma *213*, 18-27.

Ishihara, N., Nomura, M., Jofuku, A., Kato, H., Suzuki, S.O., Masuda, K., Otera, H., Nakanishi, Y., Nonaka, I., Goto, Y.*, et al.* (2009). Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol *11*, 958-966.

Islinger, M., Cardoso, M.J., and Schrader, M. (2010). Be different--the diversity of peroxisomes in the animal kingdom. Biochim Biophys Acta *1803*, 881-897.

Islinger, M., Li, K.W., Seitz, J., Volkl, A., and Luers, G.H. (2009). Hitchhiking of Cu/Zn superoxide dismutase to peroxisomes--evidence for a natural piggyback import mechanism in mammals. Traffic *10*, 1711-1721.

Ivashchenko, O., Van Veldhoven, P.P., Brees, C., Ho, Y.S., Terlecky, S.R., and Fransen, M. (2011). Intraperoxisomal redox balance in mammalian cells: oxidative stress and interorganellar cross-talk. Mol Biol Cell *22*, 1440-1451.

Jansen, G.A., and Wanders, R.J. (2006). Alpha-oxidation. Biochim Biophys Acta *1763*, 1403-1412.

Kalbfleisch, T., Cambon, A., and Wattenberg, B.W. (2007). A bioinformatics approach to identifying tail-anchored proteins in the human genome. Traffic *8*, 1687-1694.

Kaur, N., and Hu, J. (2009). Dynamics of peroxisome abundance: a tale of division and proliferation. Curr Opin Plant Biol *12*, 781-788.

Kim, P.K., Mullen, R.T., Schumann, U., and Lippincott-Schwartz, J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. J Cell Biol *173*, 521-532.

Kirkman, H.N., and Gaetani, G.F. (2007). Mammalian catalase: a venerable enzyme with new mysteries. Trends Biochem Sci *32*, 44-50.

Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., and Evans, R.M. (1992). Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature *358*, 771-774.

Klionsky, D.J., Cuervo, A.M., Dunn, W.A., Jr., Levine, B., van der Klei, I., and Seglen, P.O. (2007). How shall I eat thee? Autophagy *3*, 413-416.

Kobayashi, S., Tanaka, A., and Fujiki, Y. (2007). Fis1, DLP1, and Pex11p coordinately regulate peroxisome morphogenesis. Exp Cell Res *313*, 1675-1686.

Koch, A., Schneider, G., Luers, G.H., and Schrader, M. (2004). Peroxisome elongation and constriction but not fission can occur independently of dynamin-like protein 1. J Cell Sci *117*, 3995-4006.

Koch, A., Thiemann, M., Grabenbauer, M., Yoon, Y., McNiven, M.A., and Schrader, M. (2003). Dynamin-like protein 1 is involved in peroxisomal fission. J Biol Chem *278*, 8597-8605.

Koch, A., Yoon, Y., Bonekamp, N.A., McNiven, M.A., and Schrader, M. (2005). A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell *16*, 5077-5086.

Koch, J., Pranjic, K., Huber, A., Ellinger, A., Hartig, A., Kragler, F., and Brocard, C. (2010). PEX11 family members are membrane elongation factors that coordinate peroxisome proliferation and maintenance. J Cell Sci *123*, 3389-3400.

Koepke, J.I., Nakrieko, K.A., Wood, C.S., Boucher, K.K., Terlecky, L.J., Walton, P.A., and Terlecky, S.R. (2007). Restoration of peroxisomal catalase import in a model of human cellular aging. Traffic *8*, 1590-1600.

Koepke, J.I., Wood, C.S., Terlecky, L.J., Walton, P.A., and Terlecky, S.R. (2008). Progeric effects of catalase inactivation in human cells. Toxicol Appl Pharmacol *232*, 99-108.

Koshiba, T., Holman, H.A., Kubara, K., Yasukawa, K., Kawabata, S., Okamoto, K., MacFarlane, J., and Shaw, J.M. (2011). Structure-function analysis of the yeast mitochondrial Rho GTPase, Gem1p: implications for mitochondrial inheritance. J Biol Chem *286*, 354-362.

Kou, J., Kovacs, G.G., Hoftberger, R., Kulik, W., Brodde, A., Forss-Petter, S., Honigschnabl, S., Gleiss, A., Brugger, B., Wanders, R.*, et al.* (2011). Peroxisomal alterations in Alzheimer's disease. Acta Neuropathol.

Kragt, A., Voorn-Brouwer, T., van den Berg, M., and Distel, B. (2005). Endoplasmic reticulum-directed Pex3p routes to peroxisomes and restores peroxisome formation in a Saccharomyces cerevisiae pex3Delta strain. J Biol Chem *280*, 34350-34357.

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol *305*, 567-580.

Kumar, H., Kawai, T., and Akira, S. (2011). Pathogen recognition by the innate immune system. Int Rev Immunol *30*, 16-34.

Kural, C., Kim, H., Syed, S., Goshima, G., Gelfand, V.I., and Selvin, P.R. (2005). Kinesin and dynein move a peroxisome in vivo: a tug-of-war or coordinated movement? Science *308*, 1469-1472.

Lam, S.K., Yoda, N., and Schekman, R. (2010). A vesicle carrier that mediates peroxisome protein traffic from the endoplasmic reticulum. Proc Natl Acad Sci U S A *107*, 21523-21528.

Lazarow, P.B. (2006). The import receptor Pex7p and the PTS2 targeting sequence. Biochim Biophys Acta *1763*, 1599-1604.

Li, X., Baumgart, E., Dong, G.X., Morrell, J.C., Jimenez-Sanchez, G., Valle, D., Smith, K.D., and Gould, S.J. (2002a). PEX11alpha is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor alpha-mediated peroxisome proliferation. Mol Cell Biol *22*, 8226-8240.

Li, X., Baumgart, E., Morrell, J.C., Jimenez-Sanchez, G., Valle, D., and Gould, S.J. (2002b). PEX11 beta deficiency is lethal and impairs neuronal migration but does not abrogate peroxisome function. Mol Cell Biol *22*, 4358-4365.

Li, X., and Gould, S.J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. J Biol Chem *278*, 17012-17020.

Li, Y., Lim, S., Hoffman, D., Aspenstrom, P., Federoff, H.J., and Rempe, D.A. (2009). HUMMR, a hypoxia- and HIF-1alpha-inducible protein, alters mitochondrial distribution and transport. J Cell Biol *185*, 1065-1081.

Liu, X., and Hajnoczky, G. (2009). Ca2+-dependent regulation of mitochondrial dynamics by the Miro-Milton complex. Int J Biochem Cell Biol *41*, 1972-1976.

Ma, C., Agrawal, G., and Subramani, S. (2011). Peroxisome assembly: matrix and membrane protein biogenesis. J Cell Biol *193*, 7-16.

MacAskill, A.F., Brickley, K., Stephenson, F.A., and Kittler, J.T. (2009a). GTPase dependent recruitment of Grif-1 by Miro1 regulates mitochondrial trafficking in hippocampal neurons. Mol Cell Neurosci *40*, 301-312.

Macaskill, A.F., Rinholm, J.E., Twelvetrees, A.E., Arancibia-Carcamo, I.L., Muir, J., Fransson, A., Aspenstrom, P., Attwell, D., and Kittler, J.T. (2009b). Miro1 is a calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses. Neuron *61*, 541-555.

Mann, V.M., Nwosu, V.U., Silcox, A., Jones, C.J., Burdett, K., and Connock, M.J. (1992). Association of monoamine oxidase and malate dehydrogenase with liver peroxisomes of genetically obese (ob/ob and db/db) mice. Comp Biochem Physiol B *102*, 561-571.

Mast, F.D., Fagarasanu, A., Knoblach, B., and Rachubinski, R.A. (2010). Peroxisome biogenesis: something old, something new, something borrowed. Physiology (Bethesda) *25*, 347-356.

Matsuzaki, T., and Fujiki, Y. (2008). The peroxisomal membrane protein import receptor Pex3p is directly transported to peroxisomes by a novel Pex19p- and Pex16p-dependent pathway. J Cell Biol *183*, 1275-1286.

McBride, H.M., Neuspiel, M., and Wasiak, S. (2006). Mitochondria: more than just a powerhouse. Curr Biol *16*, R551-560.

Meinecke, M., Cizmowski, C., Schliebs, W., Kruger, V., Beck, S., Wagner, R., and Erdmann, R. (2010). The peroxisomal importomer constitutes a large and highly dynamic pore. Nat Cell Biol *12*, 273-277.

Mewes, H.W., Amid, C., Arnold, R., Frishman, D., Guldener, U., Mannhaupt, G., Munsterkotter, M., Pagel, P., Strack, N., Stumpflen, V.*, et al.* (2004). MIPS: analysis and annotation of proteins from whole genomes. Nucleic Acids Res *32*, D41-44.

Motley, A.M., and Hettema, E.H. (2007). Yeast peroxisomes multiply by growth and division. J Cell Biol *178*, 399-410.

Motley, A.M., Ward, G.P., and Hettema, E.H. (2008). Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p. J Cell Sci *121*, 1633-1640.

Muench, D.G., and Mullen, R.T. (2003). Peroxisome dynamics in plant cells: a role for the cytoskeleton. Plant Science *164*, 307-315.

Munsterkotter, M., and Steinberg, G. (2007). The fungus Ustilago maydis and humans share disease-related proteins that are not found in Saccharomyces cerevisiae. BMC Genomics *8*, 473.

Murphy, M.P., Holmgren, A., Larsson, N.G., Halliwell, B., Chang, C.J., Kalyanaraman, B., Rhee, S.G., Thornalley, P.J., Partridge, L., Gems, D.*, et al.* (2011). Unraveling the biological roles of reactive oxygen species. Cell Metab *13*, 361-366.

Nagotu, S., Veenhuis, M., and van der Klei, I.J. (2010). Divide et impera: the dictum of peroxisomes. Traffic *11*, 175-184.

Neuspiel, M., Schauss, A.C., Braschi, E., Zunino, R., Rippstein, P., Rachubinski, R.A., Andrade-Navarro, M.A., and McBride, H.M. (2008). Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. Curr Biol *18*, 102-108.

Nguyen, T., Bjorkman, J., Paton, B.C., and Crane, D.I. (2006). Failure of microtubule-mediated peroxisome division and trafficking in disorders with reduced peroxisome abundance. J Cell Sci *119*, 636-645.

Opalinski, L., Kiel, J.A., Williams, C., Veenhuis, M., and van der Klei, I.J. (2011). Membrane curvature during peroxisome fission requires Pex11. EMBO J *30*, 5-16.

Otera, H., Wang, C., Cleland, M.M., Setoguchi, K., Yokota, S., Youle, R.J., and Mihara, K. (2010). Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol *191*, 1141-1158.

Palmer, C.S., Osellame, L.D., Laine, D., Koutsopoulos, O.S., Frazier, A.E., and Ryan, M.T. (2011). MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep *12*, 565-573.

Parsons, M. (2004). Glycosomes: parasites and the divergence of peroxisomal purpose. Mol Microbiol *53*, 717-724.

Platta, H.W., and Erdmann, R. (2007). Peroxisomal dynamics. Trends Cell Biol *17*, 474-484.

Poirier, Y., Antonenkov, V.D., Glumoff, T., and Hiltunen, J.K. (2006). Peroxisomal beta-oxidation--a metabolic pathway with multiple functions. Biochim Biophys Acta *1763*, 1413-1426.

Pollera, M., Locci-Cubeddu, T., and Bergamini, E. (1983). Effect of cold adaptation on liver peroxisomes and peroxisomal oxidative activities of rat. A morphometric/stereologic and biochemical study. Arch Int Physiol Biochim *91*, 35-42.

Praefcke, G.J., and McMahon, H.T. (2004). The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol *5*, 133-147.

Rabu, C., Schmid, V., Schwappach, B., and High, S. (2009). Biogenesis of tail-anchored proteins: the beginning for the end? J Cell Sci *122*, 3605-3612.

Rakhshandehroo, M., Knoch, B., Muller, M., and Kersten, S. (2010). Peroxisome proliferator-activated receptor alpha target genes. PPAR Res *2010*.

Rattei, T., Tischler, P., Gotz, S., Jehl, M.A., Hoser, J., Arnold, R., Conesa, A., and Mewes, H.W. (2010). SIMAP--a comprehensive database of pre-calculated protein sequence similarities, domains, annotations and clusters. Nucleic Acids Res *38*, D223-226.

Reis, K., Fransson, A., and Aspenstrom, P. (2009). The Miro GTPases: at the heart of the mitochondrial transport machinery. FEBS Lett *583*, 1391-1398.

Reumann, S., and Weber, A.P. (2006). Plant peroxisomes respire in the light: some gaps of the photorespiratory C2 cycle have become filled--others remain. Biochim Biophys Acta *1763*, 1496-1510.

Rhodin, J. (1954). Correlation of ultrastructural organization and function in normal experimentally changed convoluted tubule cells of the mouse kidney. In Karolinska Institutet (Stockholm, Aktiebolaget Godvil).

Rucktaschel, R., Girzalsky, W., and Erdmann, R. (2011). Protein import machineries of peroxisomes. Biochim Biophys Acta *1808*, 892-900.

Schilling, B., and Lerch, K. (1995). Cloning, sequencing and heterologous expression of the monoamine oxidase gene from Aspergillus niger. Mol Gen Genet *247*, 430-438.

Schliebs, W., Girzalsky, W., and Erdmann, R. (2010). Peroxisomal protein import and ERAD: variations on a common theme. Nat Rev Mol Cell Biol *11*, 885-890.

Schrader, M. (2006). Shared components of mitochondrial and peroxisomal division. Biochim Biophys Acta *1763*, 531-541.

Schrader, M., Burkhardt, J.K., Baumgart, E., Luers, G., Spring, H., Volkl, A., and Fahimi, H.D. (1996). Interaction of microtubules with peroxisomes. Tubular and spherical peroxisomes in HepG2 cells and their alterations induced by microtubule-active drugs. Eur J Cell Biol *69*, 24-35.

Schrader, M., and Fahimi, H.D. (2006a). Growth and division of peroxisomes. Int Rev Cytol *255*, 237-290.

Schrader, M., and Fahimi, H.D. (2006b). Peroxisomes and oxidative stress. Biochim Biophys Acta *1763*, 1755-1766.

Schrader, M., and Fahimi, H.D. (2008). The peroxisome: still a mysterious organelle. Histochem Cell Biol *129*, 421-440.

Schrader, M., King, S.J., Stroh, T.A., and Schroer, T.A. (2000). Real time imaging reveals a peroxisomal reticulum in living cells. J Cell Sci *113 (Pt 20)*, 3663-3671.

Schrader, M., Reuber, B.E., Morrell, J.C., Jimenez-Sanchez, G., Obie, C., Stroh, T.A., Valle, D., Schroer, T.A., and Gould, S.J. (1998). Expression of PEX11beta mediates peroxisome proliferation in the absence of extracellular stimuli. J Biol Chem *273*, 29607-29614.

Schrader, M., Thiemann, M., and Fahimi, H.D. (2003). Peroxisomal motility and interaction with microtubules. Microsc Res Tech *61*, 171-178.

Schrader, M., and Yoon, Y. (2007). Mitochondria and peroxisomes: are the 'big brother' and the 'little sister' closer than assumed? BioEssays *29*, 1105-1114.

Schueller, N., Holton, S.J., Fodor, K., Milewski, M., Konarev, P., Stanley, W.A., Wolf, J., Erdmann, R., Schliebs, W., Song, Y.H.*, et al.* (2010). The peroxisomal receptor Pex19p forms a helical mPTS recognition domain. EMBO J *29*, 2491-2500.

Schuldiner, M., Metz, J., Schmid, V., Denic, V., Rakwalska, M., Schmitt, H.D., Schwappach, B., and Weissman, J.S. (2008). The GET complex mediates insertion of tail-anchored proteins into the ER membrane. Cell *134*, 634-645.

Serasinghe, M.N., and Yoon, Y. (2008). The mitochondrial outer membrane protein hFis1 regulates mitochondrial morphology and fission through self-interaction. Exp Cell Res *314*, 3494-3507.

Setoguchi, K., Otera, H., and Mihara, K. (2006). Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins. EMBO J *25*, 5635-5647.

Shibata, H., Kashiwayama, Y., Imanaka, T., and Kato, H. (2004). Domain architecture and activity of human Pex19p, a chaperone-like protein for intracellular trafficking of peroxisomal membrane proteins. J Biol Chem *279*, 38486-38494.

Shih, J.C., Chen, K., and Ridd, M.J. (1999). Monoamine oxidase: from genes to behavior. Annu Rev Neurosci *22*, 197-217.

Shimozawa, N. (2011). Molecular and clinical findings and diagnostic flowchart of peroxisomal diseases. Brain Dev.

Staels, B., and Fruchart, J.C. (2005). Therapeutic roles of peroxisome proliferator-activated receptor agonists. Diabetes *54*, 2460-2470.

Steinberg, G., and Perez-Martin, J. (2008). Ustilago maydis, a new fungal model system for cell biology. Trends Cell Biol *18*, 61-67.

Steinberg, S.J., Dodt, G., Raymond, G.V., Braverman, N.E., Moser, A.B., and Moser, H.W. (2006). Peroxisome biogenesis disorders. Biochim Biophys Acta *1763*, 1733-1748.

Streit, W.R., and Entcheva, P. (2003). Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. Appl Microbiol Biotechnol *61*, 21-31.

Tabak, H.F., van der Zand, A., and Braakman, I. (2008). Peroxisomes: minted by the ER. Curr Opin Cell Biol *20*, 393-400.

Tanabe, Y., Maruyama, J.I., Yamaoka, S., Yahagi, D., Matsuo, I., Tsutsumi, N., and Kitamoto, K. (2011). Peroxisomes are involved in biotin biosynthesis in Aspergillus and Arabidopsis. J Biol Chem.

Thoms, S., and Erdmann, R. (2005). Dynamin-related proteins and Pex11 proteins in peroxisome division and proliferation. FEBS J *272*, 5169-5181.

Titorenko, V.I., and Terlecky, S.R. (2011). Peroxisome metabolism and cellular aging. Traffic *12*, 252-259.

Todde, V., Veenhuis, M., and van der Klei, I.J. (2009). Autophagy: principles and significance in health and disease. Biochim Biophys Acta *1792*, 3-13.

Visser, W.F., van Roermund, C.W., Ijlst, L., Waterham, H.R., and Wanders, R.J. (2007). Metabolite transport across the peroxisomal membrane. Biochem J *401*, 365-375.

Walter, M.C., Rattei, T., Arnold, R., Guldener, U., Munsterkotter, M., Nenova, K., Kastenmuller, G., Tischler, P., Wolling, A., Volz, A.*, et al.* (2009). PEDANT covers all complete RefSeq genomes. Nucleic Acids Res *37*, D408-411.

Wanders, R.J. (2004). Metabolic and molecular basis of peroxisomal disorders: a review. Am J Med Genet A *126A*, 355-375.

Wanders, R.J., Ferdinandusse, S., Brites, P., and Kemp, S. (2010). Peroxisomes, lipid metabolism and lipotoxicity. Biochim Biophys Acta *1801*, 272-280.

Wanders, R.J., and Waterham, H.R. (2005). Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders. Clin Genet *67*, 107-133.

Wanders, R.J., and Waterham, H.R. (2006a). Biochemistry of mammalian peroxisomes revisited. Annu Rev Biochem *75*, 295-332.

Wanders, R.J., and Waterham, H.R. (2006b). Peroxisomal disorders: the single peroxisomal enzyme deficiencies. Biochim Biophys Acta *1763*, 1707-1720.

Waterham, H.R., Koster, J., van Roermund, C.W., Mooyer, P.A., Wanders, R.J., and Leonard, J.V. (2007). A lethal defect of mitochondrial and peroxisomal fission. N Engl J Med *356*, 1736-1741.

Weihofen, A., Thomas, K.J., Ostaszewski, B.L., Cookson, M.R., and Selkoe, D.J. (2009). Pink1 forms a multiprotein complex with Miro and Milton, linking Pink1 function to mitochondrial trafficking. Biochemistry *48*, 2045-2052.

Weller, S., Gould, S.J., and Valle, D. (2003). Peroxisome biogenesis disorders. Annu Rev Genomics Hum Genet *4*, 165-211.

Wiemer, E.A., Wenzel, T., Deerinck, T.J., Ellisman, M.H., and Subramani, S. (1997). Visualization of the peroxisomal compartment in living mammalian cells: dynamic behavior and association with microtubules. J Cell Biol *136*, 71-80.

Yokota, S. (2003). Degradation of normal and proliferated peroxisomes in rat hepatocytes: regulation of peroxisomes quantity in cells. Microsc Res Tech *61*, 151-160.

Yonekawa, S., Furuno, A., Baba, T., Fujiki, Y., Ogasawara, Y., Yamamoto, A., Tagaya, M., and Tani, K. (2011). Sec16B is involved in the endoplasmic reticulum export of the peroxisomal membrane biogenesis factor peroxin 16 (Pex16) in mammalian cells. Proc Natl Acad Sci U S A *108*, 12746-12751.

Yorimitsu, T., and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. Cell Death Differ *12 Suppl 2*, 1542-1552.

Youdim, M.B., Edmondson, D., and Tipton, K.F. (2006). The therapeutic potential of monoamine oxidase inhibitors. Nat Rev Neurosci *7*, 295-309.

Zeller, E.A., and Barsky, J. (1952). In vivo inhibition of liver and brain monoamine oxidase by 1-Isonicotinyl-2-isopropyl hydrazine. Proc Soc Exp Biol Med *81*, 459-461.

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