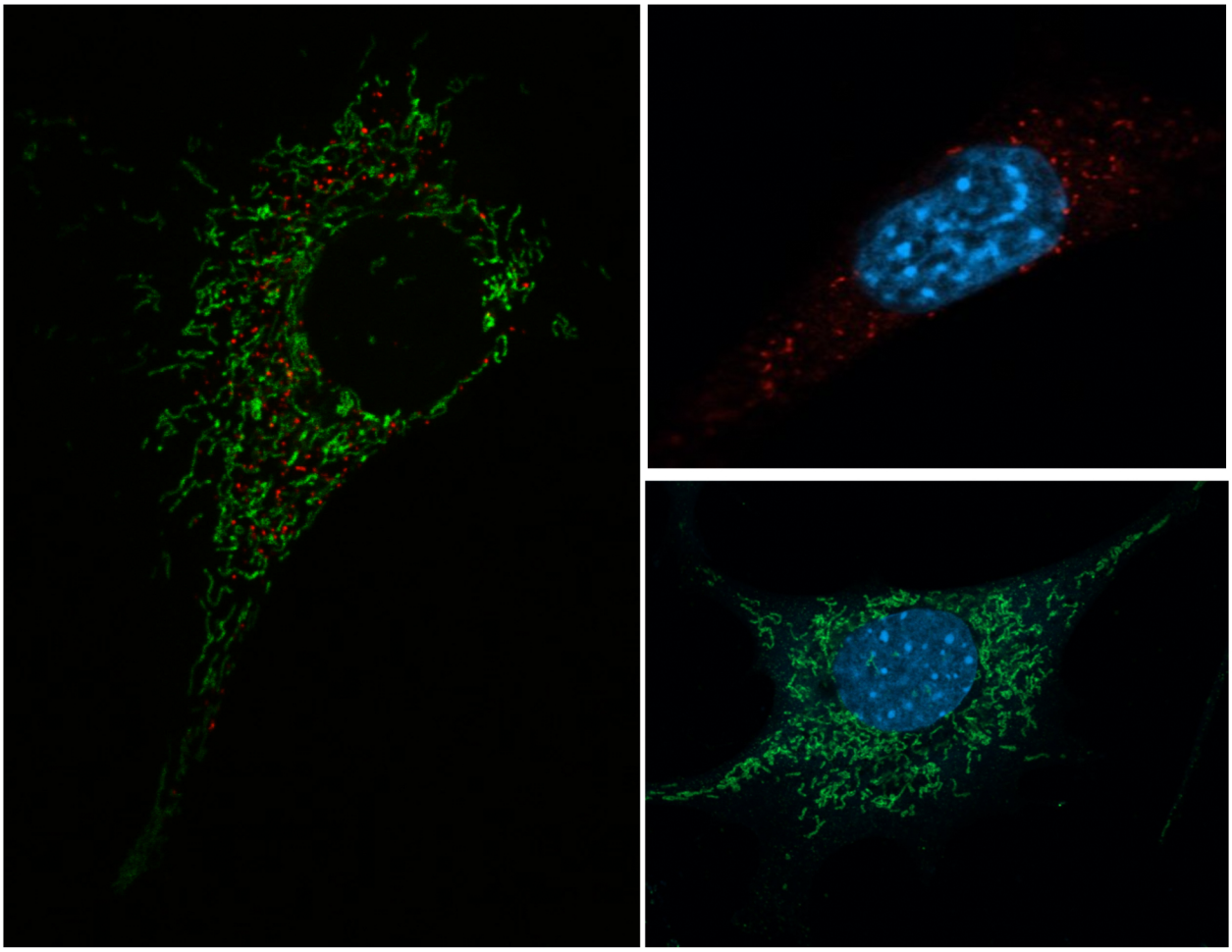

Tiago Alexandre Branco Fonseca

THE ROLE OF DYNAMINS AND DYNAMIN-RELATED PROTEIN 1 IN THE REGULATION OF MITOCHONDRIAL AND PEROXISOMAL FISSION

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 Doutor Nuno Raimundo e da Professora Anabela Rolo.

Setembro 2017

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UNIVERSIDADE DE COIMBRA

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Dissertation presented to the Faculty of Sciences and Technology of the University of Coimbra in partial fulfillment of the requirements for the degree of Master of Cellular and Molecular Biology, under supervision of Doctor Nuno Raimundo and cosupervision of Professor Doctor Anabela Rolo.

2017

The present work was performed at the facilities of the Institute of Cell Biochemistry of Medical University Gottingen and in the European Neurosciences Institute, Germany, under the guidance of Doctor Nuno Raimundo (Institute of Cellular Biochemistry, Medical University of Gottingen) and co-supervision of Doctor Ira Milosevic (European Neurosciences Institute).

Institutional supervision was provided by Professor Doctor Anabela Rolo (Department of Life Sciences, DCV, University of Coimbra).

The first page contains fluorescent images of Mouse Embryonic Fibroblasts (MEFs) expressing a mitochondrial (green) and a peroxisomal (red) marker (left) or stained for Catalase (right above) or for TOM20 (right below).

The present work was funded by the Grant 337327 from the European Research Council (ERC) and partially supported by the Association of Portuguese Post-Graduates in Germany (ASPPA).

I hereby declare that this thesis, entitled “The role of Dynamins and Dynamin-related protein 1 in mitochondrial and peroxisomal fission”, is a true copy of my thesis, which has been approved by my supervisors and submitted for a master degree to any University or Institution. I declare that the present thesis is my own work.

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•NO	Nitric Oxide
ACADs	Acyl-CoA Dehydrogenases
ACOX1	Acyl-CoA Oxidase 1
ADP	Adenine diphosphate
APs	Autophagosomes
ATP	Adenosine Triphosphate
BAPTA-AM	Cell permeant chelator
Bp	base pairs
BSA	Bovine Serum Albumin
BSE	Bundle Signalling Element
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CCP	Clatrhin-coated pit
CCV	Clatrhin-coated vesicle
CI, II, III, IV, V	Complex I, II, III, IV and V
CJ	Cristae Junction
CLC-RFP	Clathrin-light chain protein fused with mRFP
CME	Clatrhin-mediated endocytosis
CMs	Cristae Membranes
CNM	Centronuclear myopathy
CoA	Coenzyme A
Cre-ER	Cre recombinase
Cu/ZnSOD	Copper/Zinc SOD
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DHA	Docosahexanoic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNM1,2,3	Dynamin 1,2,3 gene
Drp1	Dynamin-related Protein 1
Drp1 KD	Drp1-Knockdown
DRPs	Dynamin-related proteins
dsiRNAs	Dicer-substrate short interfering RNAs
Dyn MEFs	Dynamin1/2/3-floxed MEFs
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
EtOH	Ethanol 100%
FACS	Fluorescence-activated cell sorting
$FADH_2$	Flavin Adenine Dinucleotide
FAs	Fatty Acids
FBS	Fetal Bovine Serum
Fis1	Mitochondrial Fission 1
G Domain	GTPase Domain
GaAsP	Gallium arsenide phosphide
GAP	GTPase Activating protein

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GED	GTPase Effector Domain
GS	Goat Serum
GSH	Reduced Glutathione
GTP	Guanine triphosphate
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Buffered Salt Solution
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
HPR 1,2	Heptad Repeat 1 and 2
HPRT	Hypoxanthine phosphoribosyltransferase 1
IBM	Inner Boundary Membrane
IMM	Inner Mitochondrial Membrane
l-OPA1	long-OPA1 isoforms
LC3	Microtubule-associated protein 1 light chain 3
LICOR	Odyssey infrared imaging system
MEFs	Mouse Embryonic Fibroblasts
Mff	Mitochondrial Fission Factor
Mfn 1,2	Mitofusin 1 and 2
MiCOS	Constriction of the mitochondrial inner compartments
MiD49/51	Mitochondrial Dynamic Proteins of 49 and 51 kDa
Mitoribossomes	Mitochondrial Ribosomes
MnSOD	Manganese SOD
mPTP	Mitochondrial Permeability Transition Pore
mPTS	Membrane Peroxisomal Targeting Signal
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTG	Mitotracker Green
mtNOS	Mitochondrial NOS isoform
mtROS	MitochondrialReactive Oxygen Species
MW	Molecular Weight
NAD^+	Oxidized form of NADH
NADH	Reduced Nicotinamide Adenine Dinucleotide
NBR1	Neighbour of BRCA1 Gene
nDNA	Nuclear DNA
NDP56	Nuclear Dot Protein 56
$O_2^{\bullet -}$	Superoxide
OCR	Oxygen consumption rate
$OH^{\bullet}$	Hydroxyl radical
OHT	4-hydroxitamoxifen
OMA1	Metalloendopeptidase 1
OMM	Outer Mitochondrial Membrane
OPA1	Optic Atrophy 1
OXPHOS	Oxidative Phosphorylation
P/S	Penicillin-Streptomycin
p62/SQSTM1	Ubiquitin-binding Protein p62/Sequestosome 1
PBDs	Peroxisomal Biogenesis Disorders
PBS	Phosphate-Buffered Salt

PFA	Paraformaldehyde
PH	Pleckstrin-homology domain
Pi	Inorganic Phosphate
PINK1	PTEN-induced putative kinase 1
PLL	Poly-L-Lysine
PM	Plasma Membrane
PMP70	ABC transporter peroxisomal membrane protein 70
PMPs	Peroxisomal Membrane Proteins
PMTs	PhotoMultiple Tubes
POLRMT	Mitochondrial RNA Polymerase
POLγA	Mitochondrial DNA polymerase
PRG	Proline-arginine rich domain
PTS 1, 2	Peroxisomal Targeting Signal 1 and 2
Q	Ubiquinone
QH_2	Ubiquinol
RNAi	RNA interference
RNS	Reactive Nitrogen Species
ROI	Region of interest
ROS	Reactive Oxygen Species
RPL7	60S ribosomal protein 7
rRNA	Ribosomal RNA
RT	Room Temperature
s-OPA1	short-OPA1 isoforms
S616	Drp1 Serine 616
S637	Drp1 Serine 637
SC	Scrambled-transfected cells
SKL	Serine-lysine-leucyine
TBS	Tris-Buffered saline
TBS-T	TBS with 0,1% Tween 20
TFAM	Mitochondrial Transcription Factor A
TKOs	Triple Knockout
TMRM	Tetramethylrhodamine, methyl ester
TPP+	Triphenylphosphonium
tRNA	Transfer RNA
VDAC	Voltage-dependent Anion Channel
VLCFAs	Very-long chain fatty acids
YMEL1	YME1 like 1 ATPase Protease
Δp	Proton Motive Force
ΔpH	Chemical component of the Δp
ΔΨ	Mitochondrial Membrane Potential

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Abstract

Peroxisomes and mitochondria are extremely dynamic organelles. Mitochondria, the cellular powerhouse, present ongoing cycles of fusion and fission that constantly influence and reshape the mitochondrial reticulum. Peroxisomes, in turn, proliferate through growth and division processes, being also capable of arising from different cellular compartments. These dynamic processes are regulated by membrane-remodelling proteins, some of which are shared between the two organelles, such as Dynamin-related protein 1 (Drp1), a member of the superfamily of dynamin proteins.

Drp1 is recruited and assembled in helical rings around peroxisomes or mitochondria undergoing division to fully separate their membranes. However, the diameter of Drp1 rings has been suggested to not provide the necessary constricting force in mitochondrial fission. Recently, Dynamin 2 (Dyn 2), another member of this family, seems to assume the final cleavage step in Drp1-mediated mitochondrial division, thereby resolving this conundrum in Drp1 fission. Whether this mutual Drp1-Dyn2 division is involved in peroxisome division is unknown.

Since Dyn2 is one of the three classical dynamins (Dyn1/2/3) expressed in mammalian cells, we decided to use a conditional-triple knockout model of these three dynamins to investigate their role in peroxisome division and mitochondrial division. Our results suggest that peroxisome morphology is unaltered regardless of Dyn1/2/3 deletion. Moreover, depletion of Dyn1/2/3 did not result in mitochondrial elongation, whereas knockdown of Drp1 induced the formation of a hyperfused mitochondrial network. Furthermore, in live-cell imaging of calcium- and depolarisation-induced fragmentation of mitochondria, Dyn1/2/3-depleted cells did not reveal a division blockage. However, mitochondrial metabolism in this triple knockout model is altered.

Our results shed light on the complexity of mitochondrial and peroxisomal fission and help understand in more detail how peroxisomal and mitochondrial fission are regulated, suggesting that Dyn1/2/3 are not involved in peroxisomal fission and that certain stimuli might not require Dyn2 and/or that Dyn2 is not absolutely needed for mitochondrial fission.

***Keywords**:* mitochondria, peroxisomes, classical dynamins, dynamin-related protein 1

Resumo

Peroxisomas e mitocôndrias são organelos extremamente dinâmicos presentes em quase todas as células. As mitocôndrias, fábricas energéticas, apresentam ciclos contínuos de fusão e fissão que constantemente influenciam e adaptam o retículo mitocondrial às necessidades das células. Por outro lado, peroxisomas conseguem proliferar continuamente através de processos de divisão, mas também são capazes de ser formados a partir de outros compartimentos celulares. Em ambos os casos, estes processos dinâmicos são regulados por proteínas capazes de remodelarem membranas, algumas das quais são mesmo partilhadas entre estes dois organelos, nomeadamente a Dynamin-related protein 1 (Drp1), uma proteína que pertence à superfamília das dinaminas.

Quando recrutada, a Drp1 forma estruturas em hélice em torno das membranas de um peroxisoma ou de uma mitocôndria para completar a separação das suas membranas. Contudo, foi recentemente sugerido que o diâmetro destas hélices da Drp1 parece não proporcionar a energia necessária para a divisão das mitocôndrias e que a Dinamina 2 (Dyn 2), também da superfamília das dinaminas, é capaz de resolver esta limitação da Drp1, provocando a clivagem final das membranas durante a sua divisão. No entanto, este mecanismo ainda não foi estudado para a fissão dos peroxisomas.

Dado que a Dyn2 e outras duas isoformas (Dyn1/2/3) constituem as dinaminas clássicas, nós decidimos usar um modelo condicional para eliminar todas estas isoformas de forma a estudar o seu papel na fissão dos peroxisomas e em mais detalhe na divisão mitocondrial. Os nossos resultados mostram que a morfologia dos peroxisomas não depende destas proteínas. Além disso, a eliminação das três isoformas não resulta na elongação das mitocôndrias, ao contrário do que acontece com a inibição da Drp1. Por outro lado, através de live-cell imaging observámos diversos estímulos que induziram a fragmentação mitocondrial independentemente das Dyn1/2/3. Contudo, é importante realçar que a funcionalidade da mitocôndria poderá estar alterada neste modelo de triple knockout das Dyn1/2/3.

Os nossos resultados revelam a complexidades de ambos os processos de divisão e ajudam a compreender mais a regulação dos mesmos, mostrando que provavelmente as

Dyn1/2/3 são dispensáveis tanto para a divisão dos peroxisomas como para a divisão mitocondrial em resposta a certos estímulos ou em condições basais.

***Palavras-chave**:* mitocôndria, peroxisomas, dinaminas clássicas, dynamin-related protein1

1. Introduction

1.1 Peroxisomes

Peroxisomes are no longer seen as simple, unobtrusive cellular players. After their identification in 1954 by Rhodin (Rhodin, 1954), and the subsequent discovery of enzymes producing and consuming hydrogen peroxide (H_2O_2) in the lumen of the organelle by Christian de Duve in 1965, who ultimately named these compartments as "peroxisomes" (De Duve et al., 1966), much has changed in their scientific acknowledgement and research.

The recognition of peroxisomes as important cellular organelles was not imaginable until peroxisomal defects were found to be the culprit of several human fatal diseases in the 1970s (Goldfischer et al., 1973; Vamecq et al., 2014). Further characterizations of such disorders revealed multiple metabolic dysfunctionalities, mainly in lipid metabolism. Indeed, peroxisomes are now known to be essential for the degradation and synthesis of lipids and for the control of the cellular redox state, among other functions (Singh, 1997; Wanders et al., 2006).

1.1.1 Peroxisomal Morphology

Peroxisomes are scattered throughout the cytoplasm of many different eukaryotic cells (Lüers et al., 2006). Their number, size and morphology is not uniform and is dependent on the cell and tissue type (Lazarow, 1995). Tissues active in lipid metabolism, such as the liver, have increased amounts of peroxisomes (Novikoff et al., 1982; Novikoff et al., 1980).

Structurally, peroxisomes are usually small (0.1-1μm in diameter) single-membrane bound organelles, whose matrix is densely packed with metabolic enzymes (Schrader et al., 2008; Smith et al., 2013). This matrix is, however, devoid of DNA or any translation activity and, therefore, peroxisomal proteins are encoded in the nucleus, synthesized in the cytosol and only then imported into the organelle (Lazarow et al., 1985).

Peroxisomes are extremely dynamic. In terms of shape, they can be found either as small, spherical-like compartments or rather elongated and twisted tubules (Fig. 1,

below) (Grabenbauer et al., 2000). Moreover, they can actively interact with each other, forming sometimes a sort of reticulum (Yamamoto et al., 1987). Lastly, several intracellular and extracellular stimuli can impinge on their morphology and, thus, peroxisomes are capable of readjusting their shape, and numbers, according to the cellular needs (Smith et al., 2013).

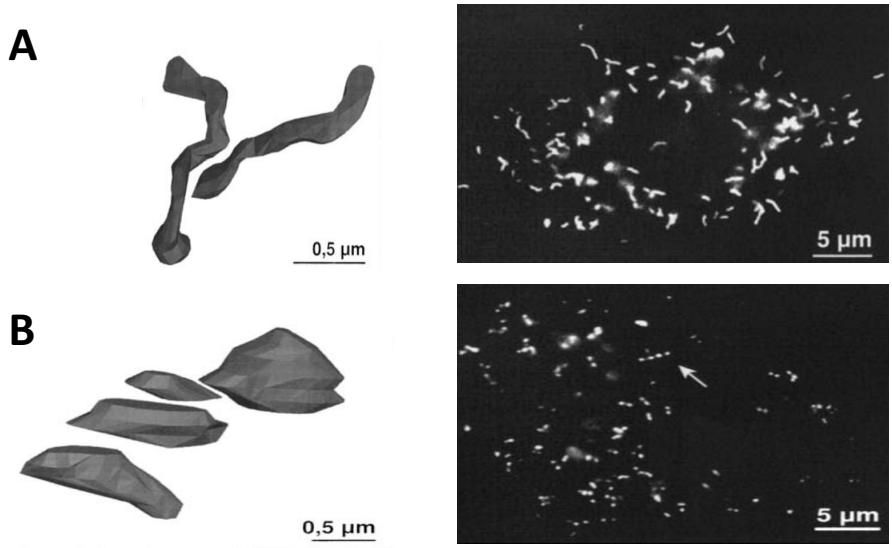


Figure 1. Morphology of peroxisomes.

Peroxisomes are heterogeneous organelles, being sometimes found as (A) elongated tubules or (B) spherical organelles. (A) and (B) represent hepatocytes stained for peroxisomal proteins (right) and 3D reconstructions (left) of some of such peroxisomes. From Fahimi et al, 2000.

1.1.2. Multipurpose organelles

After the initial suggestions implying a crucial role in the lipid metabolism, our understanding of peroxisomal functions has broadened considerably. In mammals, up to 50 different enzymatic activities are present in peroxisomes, as around 100 enzymes and other proteins are found within the organelle (Antonenkovet al., 2010; Waterham et al., 2015).

Peroxisomes perform several biochemical pathways that are adjusted to the cellular needs, being the β-oxidation of fatty acids (FAs) the most conserved across evolution (Wanders, 2014). In addition, peroxisomes are involved in the redox

metabolism and in non-metabolic functions, including cellular anti-viral immunity (Dixit et al., 2010). Overall, this emphasizes the importance of peroxisomes and explains why these intracellular compartments are considered "multipurpose organelles" (Goldfischer et al., 1996).

1.1.3 Lipid metabolism

Peroxisomes, and mitochondria, are of tremendous importance in lipid metabolism. In mammalian cells, for instance, the degradation of fatty acids by β-oxidation requires the interplay of both organelles. Peroxisomal β-oxidation started to be only appreciated with the discovery of peroxisomal biogenesis disorders (PBDs), in which no functional peroxisomes are formed to degrade lipids of difficult digestion and inherent toxicity (Schrader et al., 2006).

In fact, patients with PBDs accumulate specific substrates of the peroxisomal β-oxidation system, including branched- and very-long chain fatty acids (VLCFAs), the latter being composed of more than 24 carbon atoms (Waterham et al., 2012). These and other lipids are first decomposed by peroxisomes before being transferred to mitochondria, wherein full oxidation of FAs to CO_2 and water is possible (Camões et al., 2015). Peroxisomes, instead, are capable of only chain-shortening the FA and the electrons obtained from this oxidation are not coupled to energy conversion but transferred directly to oxygen, forming the hallmark product of peroxisomal metabolism: H_2O_2 (Fig. 2, below).

However, and despite some striking differences, peroxisomal and mitochondrial β-oxidation share some patterns: both remove two carbons from the fatty acyl-CoA, which arises from the activation step of a FA with a Coenzyme A (CoA) by acyl-CoA synthetase, through four similar sequential reactions (Fig. 2, below). First, the acyl-CoA begins to be converted into a 2-trans-enoyl-CoA by a dehydrogenation reaction; secondly, a hydration reaction forms 3-hydroacyl-CoA; another dehydrogenation gives rise to 3-ketoacyl-CoA; lastly, a thiolytic cleavage releases acetyl-CoA and, consequently, the acyl-CoA is shortened two carbons (Lodhi et al., 2014; Reddy et al., 2001).

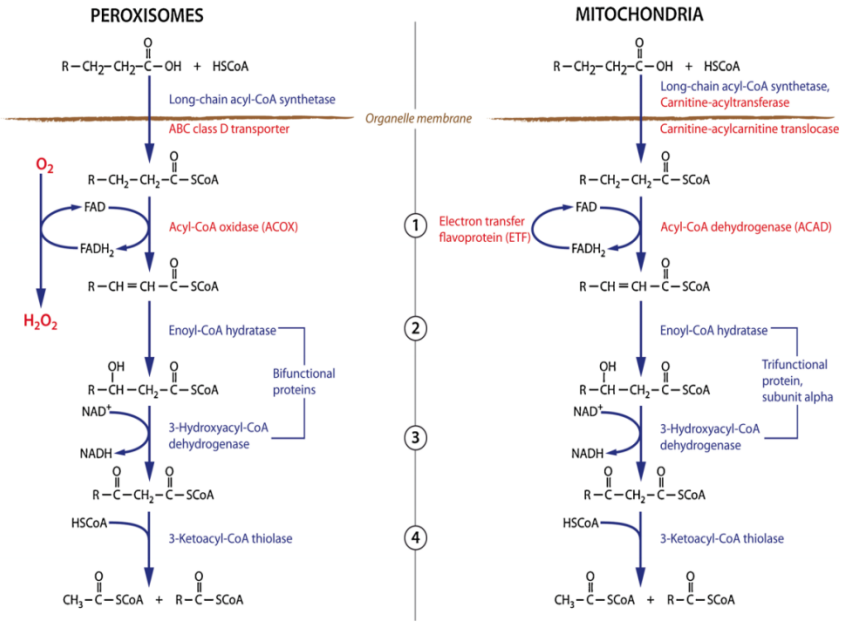


Figure 2. Beta-oxidation in mammalian cells.

In mitochondria and peroxisomes, β-oxidation consists of a cycle of four sequential reactions, which is carried out by specific enzymes in each organelle. The first step reaction in peroxisomes produces H_2O_2 by acyl-CoA Oxidases (ACOXs), whilst mitochondrial Acyl-CoA dehydrogentas (ACADs) couple the FA oxidation with energy synthesis by transferring the resulting electrons into electron carriers. From Schrader et al, 2015.

Besides β-oxidation, peroxisomes are unique in decomposing branched FAs through α-oxidation, a pathway restricted to peroxisomes. On the other hand, peroxisomes are also crucial for synthesizing lipids, such as ether phospholipids, bile acids and docosahexanoic acid (DHA) (Waterham et al., 2015).

1.1.4. Peroxisomes and Oxygen Metabolism

Since Duve's findings, the peroxisomal metabolism has been intimately associated with the production of reactive oxygen species (ROS), molecules of oxygen with unpaired electrons (Nordgren et al., 2014). Indeed, a considerable amount of peroxisomal enzymes can degrade or produce ROS, including the acyl-CoA oxidase (ACOX), which produces H_2O_2 in the first step of peroxisomal β-oxidation. In addition, peroxisomes can produce other radicals, namely reactive nitrogen species (RNS) such as nitric oxide (•NO) (Corpas et al., 2001).

The peroxisomal metabolism is, however, coped with a rich repertoire of antioxidant defences. Peroxisomes contain non-enzymatic and enzymatic defences to counterbalance ROS increases, being catalase one of most important contributors in H_2O_2 breakdown (Bonekamp et al., 2009). The importance of these cellular organelles in the cellular redox state is highlighted in aging processes in which dysregulation of the control of ROS production is also related to peroxisomal dysfunctions (Manivannan et al., 2012).

1.2. Mitochondria

1.2.1 Endosymbiosis

Mitochondria, the long-depicted cellular powerhouses, are essential intracellular organelles whose origin dates to 2 billion years ago (Lang et al., 1999). Indeed, and in striking contrast with peroxisomes, mitochondria are the product of a successful endosymbiotic process between a proteobacteria and a primitive eukaryotic cell, according to the endosymbiotic theory (Margulis, 1970). During this process, the bacterial and the host genome have mutually committed to the process.

As such, mitochondria retained a trimmed version of their ancestral genetic material, the mitochondrial DNA (mtDNA), along with simple transcription and translation systems, whereas hundreds of bacterial genes have been relocated to the hots' genome (Leister, 2005). This compartmentalization, however, raised a complex mitochondria-nucleus crosstalk, as most mitochondrial proteins are now encoded in the nucleus, synthesized in the cytoplasm and thereafter imported to mitochondria (Becker et al., 2012; Chacinska et al., 2009) (Fig. 3, below).

1.2.2 Mitochondrial DNA

The mtDNA is small, approximately 16,600 kilo base pairs (bp), and presents itself as a double-stranded molecule (Falkenberg et al., 2007). It lacks introns in higher eukaryotes and is found in multiple copies per cell in the form of complexes of DNA-protein, the nucleoids (Lang et al., 1999; Pakendorf et al., 2005). Mitochondrial transcription factors A (TFAM) is one major component of these nucleoids and is highly associated with the regulation of mtDNA copy number (Wanrooij et al., 2010). In addition, mtDNA presents unique replication and transcription machineries, including a mitochondrial RNA polymerase (POLRMT) and DNA polymerase gamma (POLγA) (Falkenberg et al., 2007).

Despite having been reduced to 37 genes, the mtDNA codifies for 13 essential subunits of the of the oxidative phosphorylation system (OXPHOS), the process in which mitochondria excel in converting energy into ATP (adenine triphosphate). These proteins are translated in mitochondrial ribosomes (mitoribosomes). Besides the 11 messenger RNAs (mRNAs) that yield 13 OXPHOS subunits, mtDNA also encodes two

ribosomal RNAs (rRNAs) (12S and 16S rRNA) and 22 transfer RNAs (tRNAs) (Gustafsson et al., 2016). The nuclear DNA (nDNA) accounts for over 1000 other mitochondrial proteins, including the remaining subunits of the OXPHOS, but also proteins involved in mitochondrial biogenesis, architecture and mtDNA transcription and translation (Hock et al., 2009; Scarpulla et al., 2012) (Fig. 3).

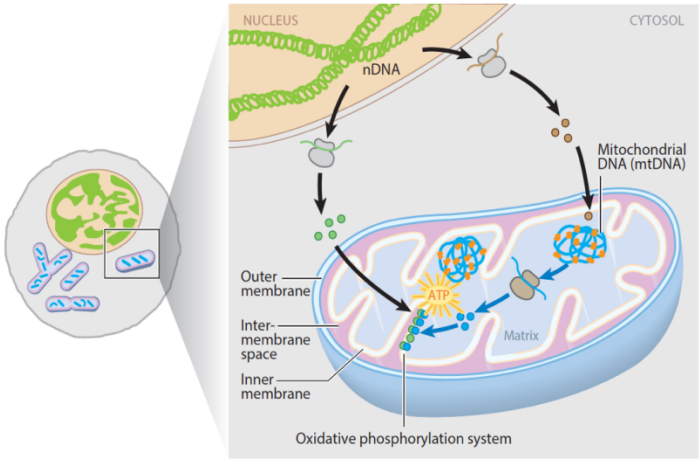


Figure 3. The Interplay of the both genomes in OXPHOS.

The mtDNA, gene-condensed, codes for 13 respiratory chain subunits, which are translated by mitoribosomes. The nDNA ships hundreds of proteins to mitochondria, complementing the OXPHOS subunits and all the other mitochondrial proteins. From Larsson et al 2016.

1.2.3 Mitochondrial structure

In the endosymbiotic process, the mitochondrion emerged as a double-membrane organelle (Fig. 4, below) (Stewart et al., 2015). The two membranes are fundamental for mitochondrial function and endowed with different functions. The Outer Mitochondrial Membrane (OMM) constitutes a free entrance of smaller molecules and ions, whereas the Inner Mitochondrial Membrane (IMM) segregates the cytosolic environment from the interior matrix content of the organelle. Nonetheless, and like peroxisomes, mitochondria need to import proteins from the cytosol and such transport is achieved by specific translocases (Kühlbrandt, 2015). Promoting also material exchanges between the matrix and the cytosol is the space between the two

membranes, the Intermembrane Space (IMS) (Herrmann et al., 2010; Rich et al., 2010) (Fig. 4, below).

Compared to the OMM, the IMM is of tremendous complexity. Biochemical analysis have actually distinguished several compartments in the IMM: the inner boundary membrane (IBM), regions of close apposition between the OMM and IMM, the cristae junctions (CJs) and the cristae membranes (CMs), the latter especially important for the OXPHOS process (Detmer et al., 2007) (Fig. 4, below).

Indeed, CMs are invaginations of the IMM that host the complexes involved in OXPHOS and are thought to create local and favourable environments for this energy-converting process. Furthermore, CMs contain several apoptotic factors imprisoned within their microdomains, which empowers mitochondria with life and death decisions (Delivani et al., 2006).

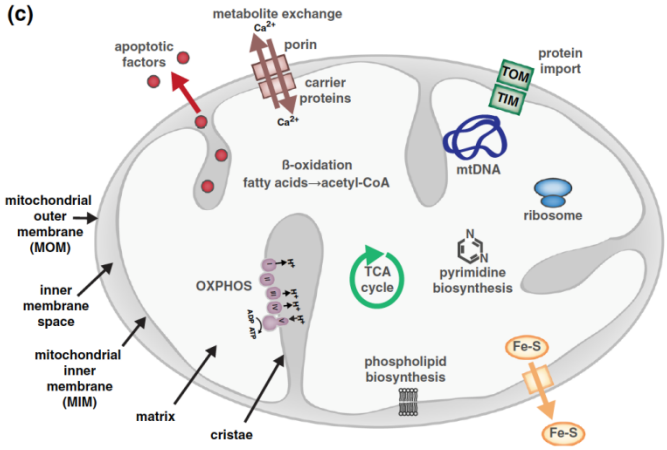


Figure 4. Mitochondrial structure.

The well-defined mitochondrial structure comprises a permeable OMM/MOM encircling a protein-enriched impermeable IMM/MIM. This membrane presents multiple local compartments within its membranes, especially the cristae, wherein the OXPHOS complexes are organised. From Lackner, 2014.

1.2.4 Oxidative Phosphorylation

Mitochondria are the cellular power plant. Cells use mostly ATP as an energy source and mitochondria make sure to provide the vast majority, more than 90%, of this

energy molecule through OPXHOS (Salin et al., 2015). First, from different processes occurring throughout the cell, including glycolysis and β-oxidation, electrons are collected by electron carriers, such as NADH (reduced nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide) (Heuvel et al., 2001). These carriers fuel the ETC, which comprises four (CI-CIV) of the five multisubunit protein complexes involved in OXPHOS, all of which are specifically organised in the CMs (Vedel et al., 1999) (Fig. 4, above).

The electrons from the NADH and $FADH_2$ are passed first onto NADH (or CI) or succinate dehydrogenase (or CII), respectively. In the ETC, these electrons continue to be transferred from groups of gradually increasing reduction potentials to the final electron acceptor, molecular oxygen (Jastroch et al., 2010). Indeed, CI and CII donate their electrons to Cytochrome c reductase (or CIII) by reducing ubiquinone (Q), via the Q-cycle. Thereafter, cytochrome c is reduced in CIII and used to reduce, in turn, oxygen to water by Cytochrome c oxidase (or CIV). Such electron transfer is exergonic and drives the pumping of protons (H+) through all the complexes, except CII, into the IMS (4 H+ are translocated by CI and CIII, but only 2 H+ from CIV) (Huttemann et al., 2007).

The accumulation of positive charges and H+ into the IMS generates, in turn, a proton motive force (Δp) constituted by a chemical (ΔpH, from the concentration of H+) and an electrical (mitochondrial membrane potential, ΔΨ, from the positive charges) component (Dzbek et al., 2008). This energy stored as a Δp is used by the last complex of the OXPHOS system, Complex V (CV), or ATP synthase, to couple the inward movement of protons down the energy gradient with the conversion of energy into ATP from ADP (adenine diphosphate) and Pi (inorganic phosphate) (Fig. 5, below). This constitutes the OXPHOS theory of Mitchel: oxidation of substrates is coupled to phosphorylation of ADP + Pi into ATP (Mitchell, 1961).

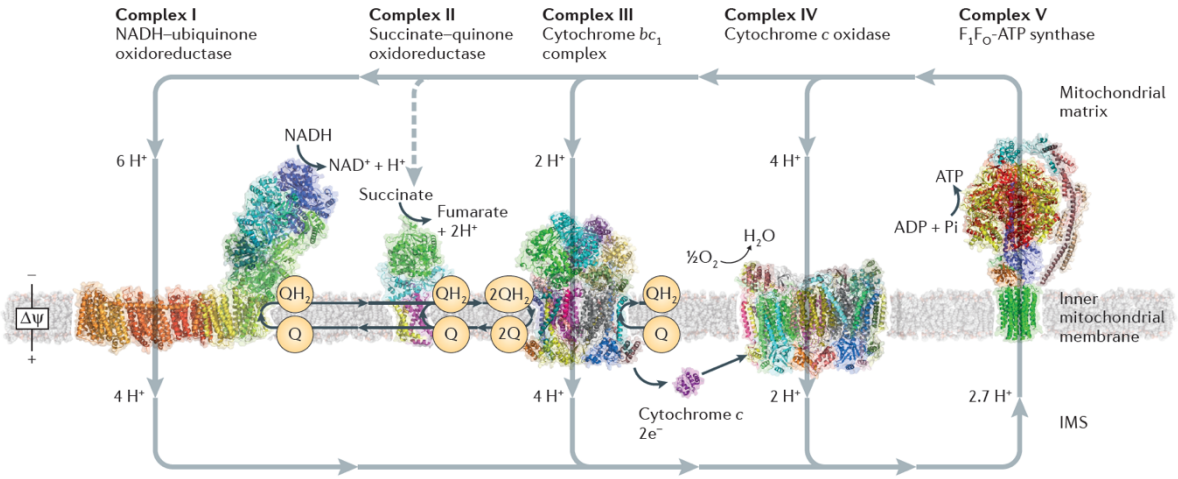


Figure 5. Oxidative Phosphorylation System.

The OXPHOS comprises the ETC and ATP synthase. NADH and $FADH_2$ fuel the nutrients-harvested electrons to CI and CII, respectively. Consequently, ubiquinone (Q) is reduced to ubiquinol (QH2), whose electrons are accepted in CIII to reduce cytochrome c. This shuttles to the CIV, in which oxygen serves as the final electron acceptor of the system. The electrochemical gradient formed is coupled, by ATP synthase, to the phosphorylation of ADP + Pi in ATP. From (Sazanov, 2015).

1.2.5 Mitochondrial Reactive Oxygen Species

The electron flow through the ETC has its predicaments: electrons lost from the electron carriers can be captured by oxygen as a one-electron reduction, thereby generating singlet oxygen, superoxide (O2• -), one of the ROS. By and large, the level of ROS produced in mitochondria respiration is thought to outnumber the remaining cytosolic sources (Kowaltowski et al., 2009).

The primary molecule formed is O2• -, the precursor of all ROS, which is mainly formed at CI and CIII (Muller, 2004; Winterbourn, 2008). O2• - can quickly give rise to H_2O_2, a molecule not only capable of diffusing across membranes, being a signalling mediator, but also of giving rise to the more reactive hydroxyl radical (OH•) via Fenton chemistry (Bonekamp et al., 2009). Besides respiration, other sources are responsible for mitochondrial ROS (mtROS) and RNS production, including the mitochondrial nitric oxide synthase (mtNOS) isoform that produces •NO (Ghafourifar et al., 1997).

In comparison to peroxisomes, mitochondria evolved strategies to tightly regulate and to protect their genome from mtROS. For instance, O2• - is quickly converted to H_2O_2 by the manganese superoxide dismutase (SOD) or the Copper/Zinc SOD (MnSOD and Cu/ZnSOD), present in the matrix and IMS, respectively (Bolisetty et al., 2013; Matsumoto et al., 2001). In addition, non-enzymatic molecules also play a role in eliminating mtROS, including the mitochondrial pool of reduced glutathione (GSH) (Orrenius et al., 2007). Nevertheless, if overwhelmingly elevated, mtROS may accumulate and irreversibly modify mitochondrial lipids, proteins and the mtDNA, which altogether impair mitochondrial function and compromise cell viability (Galloway et al., 2012; Marchi et al., 2012).

The apoptotic intrinsic pathway, for instance, is intimately associated with the mitochondrial permeability transition pore (mPTP) (Marchi et al., 2012). This pore, a megachannel of several subunits present at contact sites of the OMM with IMM, can be induced due to high levels of mtROS (Kowaltowski et al., 1996), turning mitochondrial membrane permeable. The resultant massive influx of solutes and water into mitochondria - mitochondrial permeability transition - dissipates $\Delta\Psi$, causes matrix swelling and disorganisation of the cristae, which no longer trap pro-apoptotic signals (Bernardi et al., 2006; Marchetti et al., 1996; Marchi et al., 2012; Marzo et al., 1998). Among such factors is the electron carrier cytochrome c, which diffuses to the cytosol and activates the intrinsic apoptotic pathway, emphasizing how crucial is mitochondrial ROS homeostasis to cell survival.

1.3 Organelle Dynamics

The dynamics of mitochondria and peroxisomes influence constantly their number, shape and function. In both organelles, their dynamics comprise mechanisms involved in membrane fission, intracellular movement, organelle biogenesis and selective degradation pathways, among others.

1.3.1 Peroxisome biogenesis

Peroxisomes are no longer considered to be one of the most autonomous cellular compartments (Just et al., 2016). In part, newly functional peroxisomes can arise through the division, and subsequent maturation, of parental peroxisomes, which represents the growth and division model (Smith et al., 2013). However, peroxisomes can also be formed *de novo* from other cellular organelles, including the ER (Agrawal et al., 2013; Sugiura et al., 2017).

Each step of peroxisome biogenesis, a multistep process involving protein import, maturation, proliferation and inheritance of peroxisomes, is modulated by specific, and conserved, peroxisomal proteins, the so-called peroxins (Agrawal et al., 2017). More than 30 peroxins have been found, 14 of which are absolutely essential for forming new peroxisomes (Smith et al., 2013).

1.3.2 Peroxisomal Protein Import

Peroxisomes have evolved strategic mechanisms to differently import soluble and peroxisomal membrane proteins (PMPs). Soluble proteins are imported into the peroxisomal matrix after being recognised by two major cytosolic receptors, Pex5 and Pex7 (Baker et al., 2016). PMPs, which can also be recognised in the cytosol and targeted directly to peroxisomes, are additionally shipped to peroxisomes in ER-derived vesicles (Subramani et al., 2009).

Most soluble proteins exhibit peroxisomal targeting signals (PTS), PTS1 and PTS2, for their import (Emmanouilidis et al., 2016). Proteins bearing the C-terminal PTS1, firstly identified as a tripeptide composed of serine-lysine-leucine (SKL), are recognised and transported by Pex5 (Fujiki et al., 2014). Pex7 binds to PTS2, but the resulting Pex7-cargo complex is only translocated to peroxisomes with the additional

help of Pex5 (Otera et al., 2002). Both types of cargo are targeted to the peroxisomal membrane, where a transient channel is thought to be formed with Pex5 and the two docking factors, Pex14 and Pex13 (Albertini et al., 1997; Bottger et al., 2000; Neufeld et al., 2009). This pore helps to translocate the cargo proteins into the lumen, while Pex5 and Pex7 are exported and reused in further rounds of protein import (Nagotu et al., 2012).

PMPs exhibit also targeting sequences, the membrane peroxisomal targeting signals (mPTS), and can be transported directly or indirectly to peroxisomes with the help of three major peroxins, Pex19, Pex3 and Pex16 (Cross et al., 2016). Similar to Pex5 and Pex7, Pex19 is believed to be a chaperone and cycling receptor, shuttling between the cytosol and peroxisomal membranes, wherein it delivers and inserts PMPs together with Pex3, an integral PMP that can *per se* also recruit PMPs (Eitzen et al., 1997; South et al., 1999). Pex16, which is also an integral PMP, is not present in lower eukaryotes and has been shown to traffic first to ER domains where it is believed to sort and reroute PMPs to peroxisomes via ER-derived vesicles (Aranovich et al., 2014), a pathway likely important in the two biogenesis models.

1.3.3 Peroxisomal growth and division model

Peroxisome division allows mature peroxisomes to divide into smaller peroxisomes. This growth and division process, which can be quickly stimulated, is a constitutive event that features three steps: elongation, constriction and fission (Fransen, 2012).

The most important peroxins involved in this process are peroxin 11 (Pex11) proteins, which include three isoforms in mammalian cells (Pex11α, Pex11β and Pex11γ) (Schrader et al., 2012). In contrast to Pex11α and Pex11γ, whose expressions are more restricted, Pex11β is ubiquitously expressed and considered crucial for the division of peroxisomes (Itoyama et al., 2013). Indeed, its absence is linked with PBDs, whereas its overexpression results in proliferation of peroxisomes (Marshall et al., 1995).

Pex11 proteins insert themselves in the peroxisomal membrane through an N-terminal amphipathic helix, which is used to deform the membrane and to initiate

peroxisome division (Opaliński et al., 2011). This division is asymmetrical, since the deformed, protruding membrane becomes gradually enriched in fission proteins, whereas matrix proteins are mostly retained by the parental peroxisome (Delille et al., 2010). Besides Pex11, other fission proteins include adaptor proteins that are shared with mitochondria, such as mitochondrial fission protein 1 protein (Fis1) and mitochondrial fission factor (Mff) (Kobayashi et al., 2007; Koch et al., 2012).

The clustering of Pex11 and the above adaptor proteins starts membrane constriction, but most importantly they mark the division sites, or division foci, by recruiting dynamin-related protein 1 (Drp1). Drp1 belongs to the family of dynamins, and dynamin-related proteins (discussed ahead), which are large GTPases that present membrane-scission properties (Kaur et al., 2009). As it is observed in mitochondrial fission, Drp1 forms helical structures around the fission-clustered area of peroxisomes (Fig. 6, below). These ring structures are then stimulated by GTP hydrolysis to constrict the diameter of the membranes until they are completely disrupted and separated apart (Hoppins et al., 2003). In this way, the protruding membrane gives rise to a new peroxisome, which begins immediately a maturation period that requires, among other processes, protein import mechanisms (Delille et al., 2010; Koch et al., 2011).

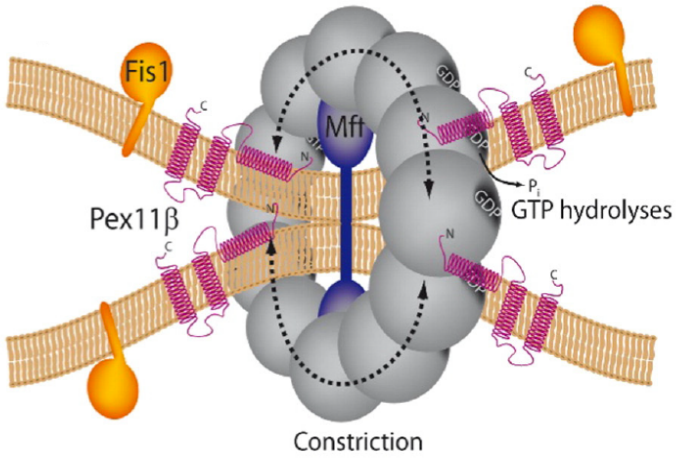


Figure 6. Pex11 proteins and peroxisome division.

Pex11 proteins project their N- and C-terminal towards the exterior of the peroxisomal matrix. Their amphipathic N-terminal domains are suitable for deforming the peroxisomal membrane, a step that precedes Drp1 assembly and fission of the organelle. From Schrader, 2015.

1.3.4 *De novo* formation: mitochondria and ER

Peroxisomes can also be formed *de novo*. Compelling evidences supporting this model were obtained from PBDs-related studies, in which a given essential peroxin is missing and later expressed in cells, in a so-called complementation study (Fujiki et al., 2012). For example, in Pex19-depleted cells, no peroxisomes are formed. However, when Pex19 is reintroduced, peroxisomes arise (Matsuzono et al., 1999). Monitoring the formation of newly formed peroxisomes in these complementation studies showed clearly that ER subdomains are behind the formation of these new peroxisomes.

More recently, mitochondria have also been suggested as a source of peroxisomes. In fact, by studying PMPs that are dually targeted to peroxisomes and mitochondria, McBride and colleagues showed mitochondrial-derived vesicles containing peroxisomal proteins, namely the essential Pex3, fusing with ER-derived vesicles, in turn transporting additional sets of peroxisomal proteins, such as Pex16 (Sugiura et al., 2017). This promptly suggests that peroxisomes may be rather hybrid organelles with ER- and mitochondrial-origin and can undoubtedly be formed *de novo* (Fig. 7, below).

Nevertheless, and even though extremely important for studying peroxisome biogenesis, these complementation studies are non-physiological and, therefore, it is still highly debatable which of the two models is more preponderant in basal conditions.

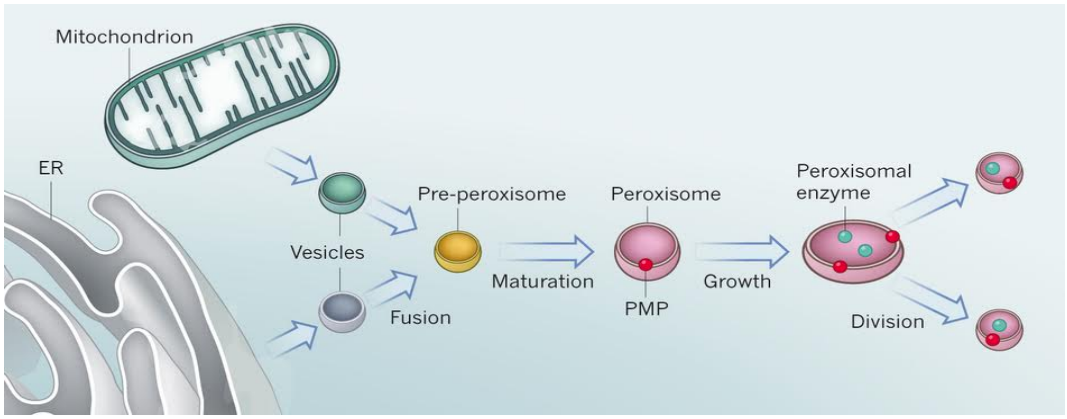


Figure 7. Peroxisome Biogenesis in mammals.

Mature peroxisomes can grow and divide into daughter peroxisomes or be formed *de novo*, in which mitochondrial- and ER-derived vesicles can fuse to form preperoxisomes that gradually mature into functional peroxisomes. From E. Hettema and S. Gould, 2017.

1.3.5 Mitochondrial Dynamics

As for peroxisomes, mitochondrial dynamics is a burgeoning field and refers to processes of fusion and fission, as well as movement, of mitochondria (Zorzano et al., 2015). Intracellularly, a mitochondrion is not an isolated organelle and fusion events between mitochondria, which have not been hitherto described for peroxisomes, may occur each 5-20 minutes (Twig et al., 2008). For this reason, mitochondria can be found in several shapes, either as small, roundish or rod-like, organelles or even as a continuous reticulum in the cytosol (Bereiter-Hahn, 1990).

Tightly regulating and balancing this network is mitochondrial fission and fusion. These processes are indispensable and, if dysregulated, mitochondrial morphology and function are affected, which can be detrimental to cell viability (Campello et al., 2010). Interestingly, the prominent proteins involved in either fission or fusion are structurally similar to the large dynamins involved in membrane remodelling (Praefcke et al., 2004), one of which, Drp1, is shared with peroxisomes.

1.3.6 Fusion proteins

Mitochondrial fusion promotes a certain homogeneity in the mitochondrial population. For instance, compromising mutations in a given mitochondrion are no longer threatening after being diluted by fusion to the rest of the mitochondrial reticulum (DiMauro & Schon, 2003). Fusion has to be divided into two steps, one for fusion of the OMM and another for IMM fusion (Bliek et al., 2013). In mammals, the GTPases Mitofusin 1 and 2 (Mfn 1, 2) oversee tethering and fusion of the OMM, whereas the GTPase Optic Atrophy 1 (Opa1) is involved in IMM fusion (Cipolat et al., 2004).

Mfn1 and 2 are structurally similar. Both are anchored in the OMM in a U-configuration, presenting a small loop embedded in the IMS, whilst both C- and N-termini protrude to the cytosol (Pernas et al., 2015). The N- and C-termini contain each one a coiled-coil heptad repeat (HR1 and 2, respectively) that are thought to interact, either in homo- or heterotypic mitofusin complexes, to tether two approaching mitochondria by their OMM just before fusion (Koshiba et al., 2004).

The following OMM fusion is then accompanied by Opa1-mediated IMM fusion, which merges the contents of the fused organelles. As mitofusins, Opa1 is extremely important and mutations in its gene, as it name suggests, causes an inherited blindness called autosomal dominant optic atrophy (Alexander et al., 2000; Delettre et al., 2000). Alternatively spliced *OPA1* gene gives rise to 8 splice variants that are imported to mitochondria, wherein they can be further processed to form long-Opa1 isoforms (l-Opa1) or shorter isoforms (s-Opa1) (Belenguer et al., 2013; Wai et al., 2016). L-Opa1 have transmembrane domains, being inserted in the IMM, whereas s-Opa1 lose those sequences and are only able to associate peripherally with the IMM, gaining, in turn, free mobility in the IMS (Belenguer et al., 2013; Chan, 2012; Satoh et al., 2003). Interestingly, both isoforms are required for IMM fusion (Ishihara et al., 2006).

IMS protease YMEL1 cleaves l-Opa1 constitutively, whereas mitochondrial depolarisation can activate the zinc-protease OMA1 to excessively cleave l-Opa1 in s-Opa1 and this inhibits fusion (Anand et al., 2014). Since l-Opa1 is also known to stabilize the mitochondrial cristae, tilting the balance in favour of s-Opa1 can be detrimental and precede apoptosis induction (Frezza et al., 2006).

1.3.7 Fission proteins

Fission is also physiologically important. For instance, by generating smaller organelles, fission facilitates mitochondrial movement and mitophagy (Ishihara et al., 2009; Twig et al., 2008; Wakabayashi et al., 2009). Until recently, Drp1 had been characterised as the sole GTPase mediating the fission of mitochondria (Oettinghaus et al., 2012).

Being mainly cytosolic, Drp1 must be efficiently assembled prior to the division event. Adaptor proteins, including Fis1 and Mff, which are present in both peroxisomes and mitochondria, assist Drp1 recruitment. On the other hand, several PTMs regulate Drp1 activity. For example, an energy deficit due to starvation is translated into lower ATP levels that lead to Protein Kinase A activation. This kinase then stimulates mitochondrial elongation by inhibiting Drp1 activity through an inhibitory phosphorylation in its Serine 637 (S637), thereby protecting mitochondria from degradation by autophagy (Gomes et al., 2011). Calcineurin, instead, is a phosphatase activated by calcium that targets and dephosphorylates this residue, stimulating Drp1 translocation to mitochondria and subsequent fragmentation (Cereghetti et al., 2008).

The division of a mitochondrion is, thus, a multi-step process with multiple intervenient partners. Firstly, ER membranes, together with actin factors and constrictions of the inner compartment (MiCOS), help constringe the mitochondrial diameter down to the range of action of Drp1 rings (Cho et al., 2017; Friedman et al., 2011; Korobova et al., 2013). This occurs at defined regions, or foci, of the dividing mitochondrion, which are also highly enriched in adaptor proteins. Afterwards, Drp1 is able to assemble in spiral structures around these mitochondrial division foci to begin cleavage of the membranes in a GTP hydrolysis-dependent mechanism (Lackner et al., 2009; Mears et al., 2011).

However, in vitro studies have reported that these Drp1 constrictions do not completely induce fission of membranes. In fact, a recent cryo-electron microscopy study suggests that the diameter of Drp1 rings are not short enough to provide the necessary force required for the spontaneous separation of the 4 mitochondrial membranes during division, and that this mechanochemical enzyme forms only intermediate, superconstricted structures (Basu et al., 2017; Lee et al., 2015; Yoon et al.,

2001). In 2016, Lee and colleagues suggested that the final cleavage of these structures is mediated by Dynamin 2 (Dyn2), a classical dynamin protein, which is explored ahead (Lee et al., 2015) (Fig. 8).

Despite these recent discoveries, mitochondrial fission is still not completely understood. For instance, peroxisome division might present the same unresolved structures caused by Drp1 constriction. It remains, thus, to be addressed whether Dyn2 or other fission factors are also recruited to facilitate Drp1-mediated peroxisomal fission.

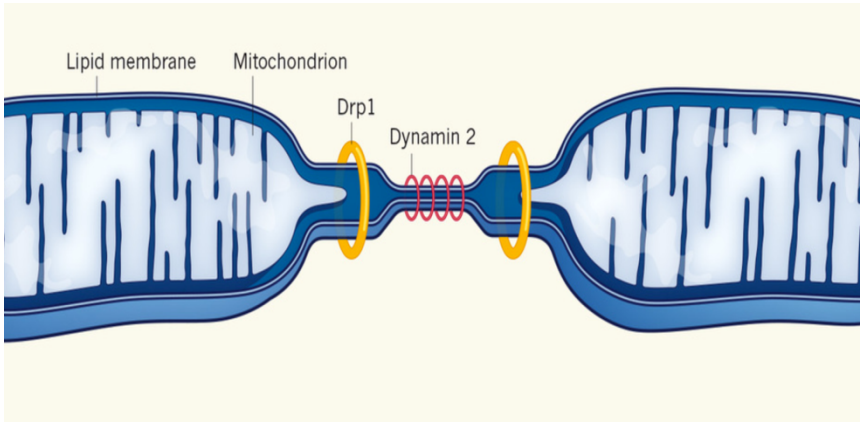


Figure 8. Dynamin 2 role in mitochondrial fission.

Mitochondrial division requires two large GTPases in a two-step process, with Dynamin-related protein 1 (Drp1) constricting the mitochondrial diameter to superconstricted sites where Dynamin 2 (Dyn 2) is recruited to perform the final membrane cleavage. From Lee, 2015.

1.3.8 Organelle Degradation

Autophagy is a conserved process among eukaryotes that ensures clearance of damaged and excessive intracellular components (Mizushima et al., 2011). Macroautophagy is the most relevant autophagic mechanism (denoted hereafter as autophagy) and consists of trapping cytoplasmic contents within autophagosomes (APs), a double membrane organelle unique to this process (Noda et al., 2015). The incorporated material in APs is delivered and degraded in autolysosomes, which arise from the fusion between an AP with a lysosome (Mizushima et al., 2011). APs can

selectively target cellular organelles for degradation, including mitochondria (mitophagy) and peroxisomes (pexophagy) (Klionsky et al., 2007).

1.3.8.1 Mitophagy

There are several mechanisms to induce mitophagy. PINK1 (PTEN-induced Putative Kinase 1)/Parkin quality control system is the best elucidated mitophagy mechanism so far (Pickrell et al., 2015). The levels of the constitutively imported kinase PINK1 are efficiently downregulated by the joined action of the IMM-protease PARL (Presenilin-associated Rhomboid-like protein) and the proteasome in healthy mitochondria (Jin et al., 2010; Matsuda et al., 2010). However, upon depolarization, both PINK1 import and subsequent degradation are halted and, consequently, the kinase accumulates on the OMM to induce mitophagy (Fig. 9, below).

Mitophagy induction is initiated by recruiting mitophagy receptor proteins to the OMM, including Optineurin and Nuclear Dot Protein 56 (NDP56), which interact with microtubule-associated protein 1 light chain 3 (LC3)-mammalian proteins, namely LC3-II, which is attached to the membrane of APs (Heo et al., 2015; Wong et al., 2014). Thereafter, the E3 ubiquitin ligase Parkin amplifies the PINK1 signal upon recruitment from the cytoplasm to the OMM, which is also stimulated by the PINK1 kinase activity (Nguyen et al., 2016). The amplified signal of PINK1 results in the selective engulfment of a given mitochondrion by an AP, which can potentially be formed nearby the PINK1-flagged mitochondrion (Nguyen et al., 2016).

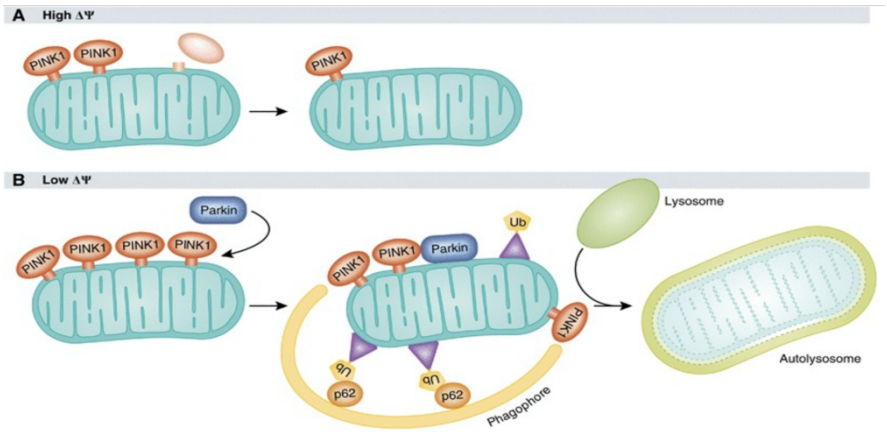


Figure 9. PINK1/Parkin-dependent mitochondrial clearance.

PINK1 is constitutively degraded in healthy mitochondria. Import-incompetent mitochondria, due to loss of mitochondrial potential, are marked for degradation, as PINK1 accumulates on the OMM, recruiting Parkin and autophagic receptors to engage the AP with the depolarised mitochondrion. From N. Exner, 2017.

1.3.8.2 Pexophagy

Peroxisomes can also be directly engulfed by APs. In several conditions, including nutrient starvation and oxidative stress, pexophagy decreases significantly the number of peroxisomes (Kuge et al., 2008; Zhang et al., 2015). Peroxins and other specific proteins are involved in pexophagy.

Initially, Pex3 and PMP34 were reported to induce pexophagy when fused with ubiquitin, whilst Pex3p overexpression *per se* was also a pexophagy inducer (Honsho et al., 2016; Yamashita et al., 2014). Moreover, so far two autophagic receptor proteins capable of binding LC3 to mark peroxisomes for degradation have been discovered in mammalian cells, p62/SQSTM1 and NBR1 (neighbour of BRCA1 gene), which are have also been reported in mitophagy mechanisms (Hollville et al., 2014; Okatsu et al., 2010).

However, despite these studies, the mechanisms driving pexophagy in mammalian cells are still largely unclear. For instance, mitochondrial division is

suggested to facilitate mitophagy, whereas associations between peroxisome division and pexophagy have been so far only reported in yeasts (Mao et al., 2014).

1.4 Dynamin superfamily of proteins

1.4.1 Atypical mechanochemical enzymes

Dynamin evolved from an initial microtubule-associated protein to an indispensable vesicle-scission GTPase that is now the funding father of the dynamin superfamily of proteins (Shpetner et al., 1989; Takei, McPherson et al., 1995). These large enzymes are associated with membrane remodelling events and are considered mechanochemical enzymes by coupling GTP (guanine triphosphate) hydrolysis with mechanical work production (Daumke et al., 2016).

Within this family there are the so-called classical dynamins, hereafter denoted simply as dynamins, and the dynamin-related proteins (DRP), in which Opa1, Mfn1,2 and Drp1 are included (Praefcke et al., 2004) (Fig. 10, below). All these members, when compared to other GTPases, have an unusual low affinity for GTP and fast nucleotide hydrolysis, as well as a predisposition to self-assemble (Song et al., 2003). In addition, structural domains are well-conserved within the family, particularly the N-terminal GTPase domain (G domain), but also a stalk (consisted by a Middle and a GTPase Effector Domain or GED) and a bundle-signalling element (BSE) (Chappie et al., 2013; Praefcke et al., 2004; Schmid et al., 2011).

1.4.2 Classical dynamins and endocytosis

Mammalian cells have three genes (*DNM1,2,3*) encoding three dynamins isoforms, Dyn1, Dyn3 and Dyn2. Dyn1 and 3 are mostly expressed in neurons, in which Dyn1 seems to be most preponderant, whereas Dyn2 is the ubiquitous isoform (Antonny et al., 2016). These proteins are distinguished within the family because of their structural composition, which includes a pleckstrin-homology domain (PH), used to bind lipids, and a proline-arginine rich domain (PRD), for protein interaction (Dar et al., 2017).

These two domains are especially important for their role in clathrin-mediated endocytosis (CME), which is a constitutive and an essential endocytic pathway for nutrient uptake and signalling cascades (Conner et al., 2003). When a given cargo-complex starts being internalised by CME, the clathrin protein rapidly covers the

invaginating membrane, forming the so-called clathrin-coated pits (CCPs). These continue to expand until they are just barely connected to the plasma membrane (PM) through a narrow neck. Dynamins are recruited and assembled around this connection to cleave it in a GTP-driven constriction, thereby forming the clathrin-coated vesicles (CCVs) (Mettlen et al., 2009).

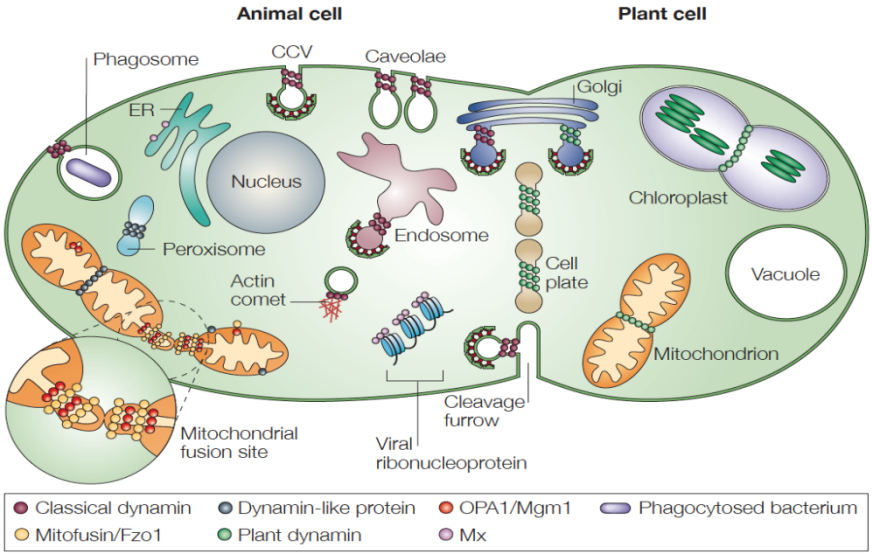


Figure 10. Dynamins and dynamin-related proteins functions.

Dynamin and dynamin-related proteins redistribute to several intracellular compartments, performing a myriad of functions including organelle division (peroxisome and mitochondrial fission), cytokenesis, endocytosis to cytoskeletal dynamics regulation. From Praefcke, 2004.

1.4.3 Pleiotropic roles of Dynamin 2

Dyn2 ubiquitous expression is also matched with housekeeping functions. In fact, Dyn2 deletion is lethal and not compensated by the other two isoforms (Ferguson et al., 2009). Moreover, Dyn2 seems to be preponderant in muscle and neuronal tissue development. For instance, Dyn2 has been shown to regulate neuronal growth cone migration, while mutations in its gene have been reported in neuronal and muscle pathologies, including the Centronuclear myopathy (CNM) (Cowling et al., 2012; Kurklinsky et al. 2011).

In many mutations, CME is impaired. However, Dyn2 has other pleiotropic roles, associating with membranes in other cellular structures, such as the Golgi compartment (Henley et al., 1996). Furthermore, and consistent with the early characterization of dynamin as a microtubule-binding protein, Dyn2 has been shown to regulate cytoskeletal dynamics. Indeed, Dyn2 is involved in cytokinesis by destabilising microtubules, but is also suggested to play a role in actin polymerisation (Jamett et al., 2017; Tanabe et al., 2009). All these functions can be compromised when *DNM2* is mutated or lost.

Overall, Dyn2 is not restricted to endocytic pathways and its functions influence directly cell homeostasis processes. Moreover, intracellular organelles might require this protein for its fission events, including mitochondria, which might suggest a Dyn2 role in peroxisomal biology.

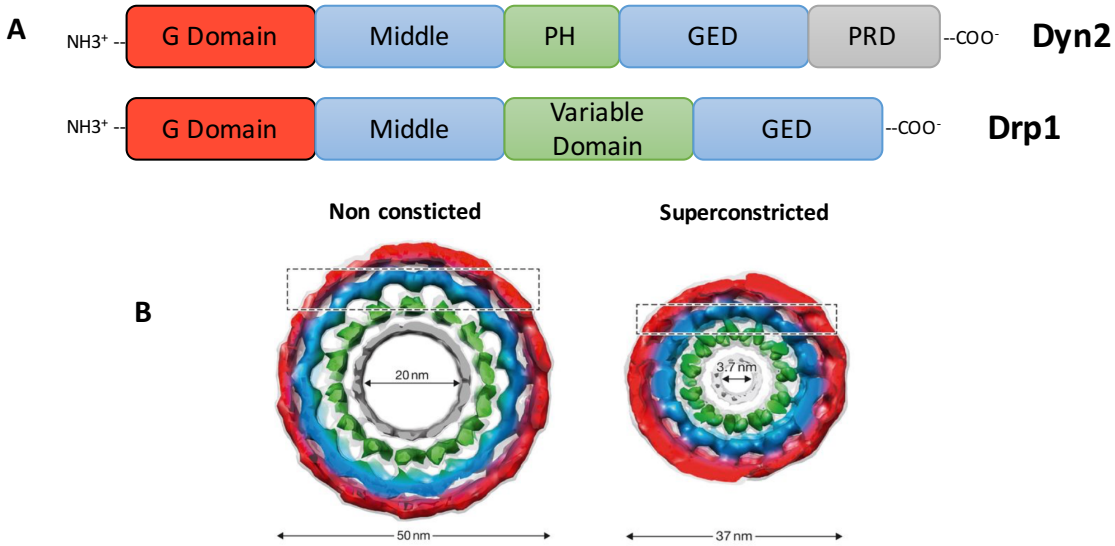


Figure 11. Schematic representation of dynamins constrictions.

A) Classical dynamins (Dyn1-3) present 5 domains, whereas Drp1 lacks a PH and PRD domain, being recruited to peroxisomal and mitochondrial membranes by adaptor proteins.

B) The non-constricted helical structures of dynamins can be stimulated by GTP hydrolysis to a constricted state that reduces the membranes diameters to the point of favouring its separation and cleavage. From B. Anthony, 2016.

2. Materials and Methods

2.0 Materials

2.0.1 Plasmids

Pex11β-myc was kindly provided by Dr. Schrader (University of Exeter, Exeter, UK) and used to drive peroxisomal fission. Mitochondrial-targeted GFP protein (Mito-GFP), a kind offer from Pietro di Camilli (Yale University, New Haven, USA), was used to label mitochondria for live-cell imaging and morphology analysis. The plasmid Ub-RFP-SKL was used for labelling peroxisomes in live-cell imaging, which is an ubiquitin moiety fused with a mRFP with a peroxisomal targeting sequence, namely PTS1, as explained in (Aranovich et al., 2014).

2.0.2 Antibodies

Table 1. List of primary antibodies

PRIMARY ANTIBODIES

ANTIBODY	Supplier	Cat. No.	Species
HPRT	Abcam	ab10479	rabbit
RPL7	Abcam	ab72550	rabbit
ACTIN	Merck	MAB1501	Mouse
OPA1	BD Biosciences	612606	Mouse
MFN1	Santa Cruz Biotechnology	sc-166644	mouse
MFN2	Abcam	ab56889	mouse
DRP1	Cell Signaling Tecnhology	8570	Rabbit
PHOSPHO-DRP1 (SER616)	Cell Signaling Tecnhology	3455	rabbit
PHOSPHO-DRP1 (SER637)	Cell Signaling Tecnhology	4867	rabbit
MFF	Proteintech	17090-1-AP	rabbit
PEX11B	Abcam	ab74507	rabbit
PEX5	Merck	HPA039259	rabbit
CATALASE	Santa Cruz Biotechnology	sc-271803	mouse
PMP70	Merck	SAB4200181	Mouse
PORIN/VDAC	Abcam	ab110326	mouse
DYNAMIN PAN	BD Transductions Laboratories	610245	mouse

GAPDH	Merck	G9545	rabbit

Table 2. List of secondary antibodies.

SECONDARY ANTIBODIES	COMPANY
ALEXA-488	Invitrogen
ALEXA-568	Invitrogen
IRDYE 680/800	LI-COR

2.0.3 Reagents and instruments

The following reagents were obtained from Thermo Fisher Scientific (Waltham, MA, United States): high-glucose Dulbecco's Modified Eagle Medium (DMEM, Cat 10567014), Trypsin-EDTA (0.25%) (Cat 25200056), Penicillin-Streptomycin (10,000 U/mL) (P/S, Cat 15140122), Fetal Bovine Serum (FBS, Cat 16000036), Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (Inhibitors Cocktail, Cat 78440), Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM, Cat T668), PageRuler™ Prestained Protein Ladder (PageRuler, Cat 26616), Mr. FrostyTM Container (Mr. Frosty, Cat 5100-0001), Pierce BCA Protein Assay Kit (BSA, Cat 23225i) and Incubator Heracell 150i (Incubator).

From Life Technologies (Carlsbad, CA, United States) the following materials were obtained: Mitotracker Green FM (MTG, Cat M7514), MitoSOX™ Red (MitoSOX, Cat M36008) and Goat Serum (GS, Cat 10000C); all the reagents related with electrophoresis and western blotting were purchased from BioRad (Hercules, Ca, United States). SeaHorse materials and softwares were obtained from Agilent (Santa Clara, Ca, United States). From Carl Roth (Karlsruhe, Germany) the following materials were obtained: DAPI (Cat C6335.1), 2-mercaptoethanol (Cat 4227.3), HEPES (Cat HN77.2) and N-dodecylmaltoside (Cat CN26.2).

Gelatin from cold water fish skin (Gelatin, Cat G7041), Bovine Serum Albumine (BSA, Cat A7906), Dimethyl sulfoxide (DMSO, Cat D8418), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, Cat C2920), Soldium Dodecyl Sulfate (SDS, Cat L4509-500G), Tetramethylethylenediamine (TEMED, Cat T7024),

Phosphate-Buffered Salt (PBS, Cat P44177-100TAB), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Cat 2759), Cyclosporin H (Cyclo H, Cat SML1575), BAPTA-AM (Cat A1076), Bunsen Burner (Cat Z270288-1EA), Mowiol-488 (Mowiol, Cat 81381), 4-Hydroxytamoxifen (4-OHT, Cat H6278) and Poly-L-lysine (PLL, Cat P4707) were obtained from Sigma-Aldrich/Merck (Saint Louis, MO, Germany).

2.0.4 Software

Table 3. List of Software.

Imaris 8	Bitplane
Fiji	https://fiji.sc
Prim 6	Graphpad Software
Office	Windows
Zen	Zeiss
Volocity	PerkinElmer
Illustrator CS3	Adobe
FACS DIVA	BD Biosciences
Odysseia CLx	LI-COR
Nucleofector^{TM} Device	Lonza

2.1 Mammalian Cell Culture

2.1.1.1 Culture of Dynamin floxed cells (Dyn MEFs)

The Mouse Embryonic Fibroblasts (MEFs) with the three dynamin genes flanked by loxP recognition sites (floxed) and transgenic for 4-hydroxitamoxifen (OHT)-inducible Cre recombinase (Cre-ER) were a kind gift from Pietro de Camilli and have been intensively studied in endocytic studies (Park et al., 2013). These Dynamin MEFs were cultured in DMEM, supplemented with 1% P/S and 10% FBS and maintained at 37ºC in a humidified atmosphere with 5% CO2. Upon reaching 80% confluency, cells were washed once with PBS, detached with Trypsin-EDTA for 3 min and counted on a Neubauer Chamber (Celeromics, Grenoble, France) before being collected by centrifugation at 1100rpm for 3min and subcultured again.

2.1.1.2 Induction of Triple Knockout

To induce the excision of the three dynamin genes, 80% confluent Dyn MEFs grown in T75 flasks (Cat CC7682-3617, CytoOne, Orlando FL, USA) were diluted in 1/4 and subcultured in new T75 flasks for 48h in the presence of 3μM OHT, being new medium with OHT replenished after 24h. Thereafter, cells were passaged once again and cultured in fresh medium containing 10% of the old, OHT-containing medium to ensure full recombination of the dynamin genes. As the Cre recombinase is fused to a human estrogen receptor responsive only to OHT, the enzyme remains inactive in the cytosol unless OHT is administrated. Upon addition of OHT, the enzyme is translocated to the nucleus wherein it will efficiently excise the three dynamin genes. In parallel, Dyn MEFs were treated with ethanol (EtOH, vehicle) as a negative control. Every single experiment was done after 6 days of the OHT/EtOH treatment.

2.1.1.3 Total Protein Isolation

For western blot studies, Dyn MEFs treated with EtOH or OHT grown in T75 flasks were harvested by trypsinization when 80% confluent, as in 3.1.1.1. Cell pellets were washed once with PBS, centrifuged again at 1100rpm and, after supernatant removal, stored at -20°C before being processed for western blot.

2.1.1.3 Cryopreservation and Cell Retrieval

Cryopreservation was done to preserve the Dyn MEFs cell line with the earliest passage. Pellets from cells growing in T75 flasks were collected by trypsinization and resuspended in freezing medium (complete medium with 10% DMSO), aliquoted into cryogenic storage vials and immediately placed in Mr. Frosty to be frozen at -80°C for 24h. DMSO acts as a cryoprotectant, whereas the Mr. Frosty filled with isopropanol guarantees a 1°C/minute cooling rate to preserve cell viability. After 24h, the vials were transferred to liquid nitrogen (-196°C) containers for longer storage. For thawing cell lines, cryogenic vials were removed from the liquid nitrogen racks and defrosted in the

water bath at 37°C for 1 minute to be finally diluted in pre-warmed medium and cultured in T25 flasks or 10cm dishes.

2.1.2 Poly-L-Lysine Coating

Coverslips were treated with the cationic polymer of Poly-L-Lysine (PLL) to attract the negatively charged cell surface membranes, thereby facilitating cell adhesion. First, coverslips were thoroughly washed and immersed in absolute ethanol before being flamed quickly in a Bunsen Burner, placed in multi-well plates and incubated with 0.1 mg.mL-1 of PLL in deionized water for at least 30 min at 37°C. PLL was then removed and the coverslips were washed twice with deionized water and dried under the hood, being preserved at 4°C or used immediately.

2.2 Transient Gene Expression

2.2.1 Amaxa Electroporation Protocol

Electroporation, through brief electric pulses, changes transiently the properties of cellular membranes, including the nuclear envelope, to create pores that facilitate the transport and incorporation of extracellular macromolecules, namely plasmids. Thus, after 5 days of the beginning of the treatment, both OHT- and EtOH-treated cells were transfected by electroporation with the nucleofection protocol of Amaxa (Lonza, Basel, Switzerland) according to the manufacturer standard protocol. Briefly, 80% confluent cells were detached by trypsinization and counted as in 3.1.1. Thereafter, $200x10^3$ up to 1 million cells were resuspended in PBS, harvested once again by centrifugation and the cell pellets were resuspended in 20μL of Nucleofection reagent with the appropriate quantity of plasmid DNA (1-5ug). An electric pulse was then applied to the small nucleofection cuvettes using the Primary 4 (P4) cell line MEFs program of Lonza Nucleofection Device. As soon as the reaction was completed, cells were brought to the hood and allowed to recover in the cuvettes for 10 minutes, being finally diluted in complete, fresh medium and seeded at the desired density either in Petri Dishes or multi-well plates coated with poly-lysine.

2.2.2 Silencing of Dynamin-related protein 1

To specifically suppress (silence) the Drp1 gene by RNA interference, Dyn MEFs were transfected with dicer-substrate short interfering (dsiRNA) against the mouse Drp1. As in 3.2.1, electroporation was used to transfect cells with 200nM of Drp1 dsiRNA ordered from Integrated DNA technologies (Coralville, Iowa, United States). After 48h, cells were electroporated again for Mito-GFP, seeded on 12MW plates and imaged 26-30h after electroporation. Drp1 silencing was analysed by comparing Drp1 levels of 200nM scrambled-transfected cells with Drp1-dsiRNA-transfected cells 72h after transfection.

2.3. Molecular Biology Techniques

2.3.1 Protein Extraction, Quantification and Sample Preparation

For protein extraction, cells' pellets were diluted with 50µL or less in 1,5% N-dodecylmaltoside, supplemented with complete Inhibitors Cocktail and incubated for 20 minutes with constant rotation in a tube rotator at 4°C. Thereafter the cell debris was removed by centrifugation at 13200rpm for 20 minutes, at 4°C, and the supernatant collected in new eppendorfs. Protein concentration was quantified using the Pierce BCA Protein Assay kit, according to the manufacturer's manual. Equal amounts of protein were diluted in Laemmli buffer 6x supplemented with Mercaptoethanol 5% and denatured at 95°C for 5 min.

2.3.2 Western Blot Analysis

20µg to 40µg of protein loaded in polyacrylamide gels were separated based on their molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel electrophoresis was firstly conducted at 80mV, whereby all the proteins reached the resolving gel at a similar rate, and then at 100mV. Separated proteins were blotted onto nitrocellulose and transferred using a wet electroblotting system of BIO-RAD, during 1h30min, at 4°C. Thereafter, membranes were incubated in 5% of non-fat milk powder or 5% Bovine Serum Albumin (BSA)-containing Tris-Buffered saline (TBS) with 0,1% Tween 20 (TBS-T) for 1h at RT. This step blocks nonspecific binding sites of the membranes before the use of specific antibodies. Thus,

and following three washing steps of 10 min with TBS-T, primary antibodies (list) were incubated overnight at 4°C, with constant shaking. Thereafter, membranes were washed three times for 10 min to remove any traces of the primary antibodies and only then incubated with Alexa Fluor secondary antibodies diluted 1:7500 in 5% milk in TBS-T for 1h at RT, with shaking. The fluorescent signal of the respective antibodies was acquired using the Odyssey infrared imaging system (LICOR) and the protein bands were normalised with the corresponding loading controls and compared between samples using Fiji.

2.4. Flow Cytometry and Microscopy Studies

2.4.1 Flow cytometry

Flow cytometry can be extremely powerful to characterise several cells' parameters. In the cytometer, a cell suspension is filtered so that one cell at a time is interrogated by lasers at a specific measuring point during their flow. By combining different lasers and fluorescent detectors, the cytometer can collect information not only regarding cell size and granularity, but also intensity of a given fluorophore. In each of the following experiments, at least 10000 events were recorded per sample and the average of three different sets was used for comparison studies.

2.4.2 Mitochondrial Membrane Potential

Mitochondria sustain an electric and chemical gradient across their inner mitochondrial membrane. Tetramethylrhodamine, methyl ester (TMRM) is a cationic, lipophilic dye that redistributes easily in mitochondria according to the mitochondrial potential; highly polarised mitochondria, in which the mitochondrial matrix is more negative, will accumulate more TMRM than depolarised ones. As described in Bolletta and colleagues, TMRM fluorescence was measured by flow cytometry to access mitochondrial potential (Rowe et al., 2013). Briefly, 3000 cells in suspension were incubated with 10nM TMRM and 1μM Cyclosporine H in Hank's Buffered Salt Solution (HBSS), containing 10mM HEPES, for 30 min at 37°C in the dark. After incubation, cells were collected by centrifugation, washed once in HBSS and resuspended in cold PBS before being analysed in the cytometer. Cyclosporine H was

used to inhibit the activity of the multidrug resistant pump, thereby avoiding unwanted oscillations in the TMRM signal, and negative controls were treated with 10μM CCCP to normalise the TMRM fluorescence, which was analysed with a 585/42nm long-band filer. In parallel, representative images of the TMRM fluorescence were obtained by live-cell imaging, as explained in 2.4.4.

2.4.3 Mitochondrial Superoxide Levels

Mitochondrial ROS levels were estimated using MitoSOX fluorescence by flow cytometry. This dye is cationic due to the triphenylphosphonium (TPP+) group and can also react specifically with superoxide, being used as a mitochondrial superoxide sensor. Thus, $10x10^3$ cells cm-2 were washed twice with HBSS and incubated with 5μM MitoSOX in HBSS supplemented with 10mM HEPES and placed again in the cell incubator for 15 min. MitoSOX-containing medium was then removed and cells were washed twice with pre-warmed PBS before being trypsinized and centrifuged in 15mL falcons. Cell pellets were washed once with cold PBS and then diluted in 400μL cold-PBS in FACS tubes. MitoSOX fluorescence from samples and positive control tubes treated with 100mM Antimycin A were analysed with 585/42nm long-band filer. As for the mitochondrial potential, representative images of cells incubated with MitoSOX were also obtained, as explained in 2.4.4

2.5 Microscopy and Imaging Analysis

2.5.1 Immunocytochemistry

Cells grown on PLL-coated coverslips were washed twice with pre-warmed PBS before being fixed for 20min at RT with 4% paraformaldehyde (PFA) (AppliChem GmbH, Darmstadt, Germany, Cat A3813), a cross-linking reagent that preserves cellular structures. After (3x) washes in PBS, coverslips were incubated with blocking buffer (3% BSA in PBS, 0,1% Triton x100, 1% GS and 0,01% Gelatin) for 1h at RT with shaking, to reduce nonspecific antibody binding, and washed again (3x). Primary antibodies, PMP70 peroxisomal morphological studies, were diluted in blocking buffer and then incubated for 2h at RT with constant shaking or overnight at 4°C. Three more washing steps removed any traces of primary antibodies before incubation with Alexa

flour-conjugated secondary antibodies, diluted in blocking buffer, for 1h at RT without shaking. Finally, coverslips were washed with PBS, incubated for 10min with 1:5000 DAPI in PBS, washed (1x) and dipped in de-ionised water before being mounted with Mowiol.

2.5.2 Live-cell Imaging

Cells seeded on PLL-coated 12 Well plates (12MW, CytoOne, Cat CC7672-7512) were briefly dipped in PBS, for washing off the cellular medium, mounted carefully in live-cell imaging chambers and incubated in imaging buffer (16mM NaCl, 2,5mM KCl, 2mM CaCl2, 1mM MgCl2, 10nM HEPES, pH 7.4). Cells were then immediately placed on the stage of Nikon/PerkinElmer Ultra VIEW VoX system (Perkin Elmer, Waltham, Massachusetts, USA) with a spinning disk confocal scan head (CSU-XI, Yokogawa) and Nikon Eclipse Ti-E microscope with a Plan Fluor 40x/1.3 Oil DIC N2 / 0,17 WD 0.24 OFN 25, Plan APO λ 60x / 1.40 Oil DIC N2 / 0,17 0.13 OFN 25 and APO TIRF 100x/ 1.49 DIC N2 / 0,13-0,20 WD 0.12 objectives, operated by Volocity. Cells were imaged at a constant temperature, 37˚C.

2.5.3 Mitochondrial-division studies

For studying organelle division, $10x10^3$ cells cm-2 Dyn MEFs, control and TKO, were treated with either Ionomycin, BAPTA-AM, CCCP or DMSO (vehicle). Ionomycin, a calcium ionophore, raises intracellular calcium levels, which is a stimulus for mitochondrial division (Ji, Hatch, Merrill, Strack, & Higgs, 2015). Thus, Mito-GFP-expressing Dyn MEFs were imaged for 7 min to study basal mitochondrial morphology before addition of Ionomycin (4μM). BAPTA-AM (10μM), which is a cell-permeant calcium chelator, and DMSO were also added as control experiments. Mitochondrial dynamics were recorded for 8 more minutes after each treatment. In parallel, CCCP, an oxidative phosphorylation uncoupler, was also used to stimulate Drp1-dependent mitochondrial fragmentation (Cereghetti et al., 2008). In this case, basal mitochondrial dynamics were imaged for 4 min prior to incubation of 10μM of CCCP or DMSO and recorded for more 15 min after addition of each drug. The time required for each

compound to induce mitochondrial fragmentation was compared between control and TKO cells.

The effect of Ionomycin in peroxisomes was evaluated as explained above but with cells expressing Ub-RFP-SKL-expressing. Representative kymographs of the peroxisomal movement were then obtained in Fiji.

2.5.4 Mitochondrial Characterization by Live-cell imaging

Representative images of the flow cytometry studies were obtained by live-cell imaging. For TMRM fluorescence, $10x10^3$ cells cm-2 were incubated with 10nM TMRM + 1μM Cyclosporine H in HBSS, supplemented with 10mM HEPES and each coverslip was mounted in the imaging chamber and incubated with the TMRM-containing HBSS, to allow equilibration of the dye. On the other hand, $15x10^3$ cells cm-2 were incubated with 2.5μM MitoSOX + 200nM Mitotracker Green (MTG) in HBSS, supplemented with 10mM HEPES. MTG, which is widely used to study mitochondrial mass, was used to co-label mitochondria, as this probe becomes fluorescent upon binding to mitochondrial proteins regardless of the mitochondrial potential. Using the 40x objective, around 25 cells were snapped in the two experiments.

3.5.5 Confocal Microscope

Zeiss laser scanning confocal microscope 800 (LSM800) with an inverted stand AxiObserverZ1 was used for image acquisition from fixed samples. Plan NEUOFLUAR, 40x/1,3 Oil and Plan Apo-Chromat DIC 63x/1,4 Oil objectives were used for imaging. Fluorophores were excited 405 nm, 488 nm and 568 nm solid state lasers. An Airyscan array and 2 Gallium arsenide phosphide (GaAsP) PhotoMultiple Tubes (PMTs) were used for detection of fluorophores. The microscope was operated by Zen software Blue 2.1. version.

3.5.5.1 Mitochondrial morphology analysis

For 3D reconstructions of the mitochondrial network, the Airyscan detector was used. Firstly, 63x Z-stacks with a thickness of <.2μm apart of Mito-GFP transfected cells were used to span the entire mitochondrial network and resolved by Airyscan

processing, thereby increasing the signal-to-noise ratio that is lost with increased resolution. Thereafter, Z-stacks were compressed into a collapsed 2D image by using the maximum intensity projection in Fiji, in which a region of interest (ROI) was designed around the cells‘ boundaries and an automated Otsu-thresholding was applied to 8 bit-images before the Mito-Morphology macro was run, a public available algorithm used to measure several mitochondrial parameters, namely the interconnectivity and elongation indexes (Dagda et al., 2009). Results from at least 25 cells of two different trials were loaded into an excel sheet and used for analysis.

2.5.5.2 IMARIS Surfaces studies of peroxisomal morphology

Peroxisomal morphology was studied by the Surfaces wizard of the IMARIS software. Firstly, Z-stacks 3µm apart were obtained from PMP70-stained peroxisomes and subjected to background subtraction in IMARIS. Then, using the Surface wizard, a region of interest was used to mask each cell into a new channel, from which the volume was also calculated. The masked channel was then subjected to another Surface wizard, with the region growing enabled, for peroxisome analysis. The sphericity index, as well as the number of surfaces, were studied in at least 15 cells per condition.

2.5.5.3 Peroxisomal Proliferation studies

After transfection with Pex11β-myc, cells were seeded in PLL-coated 12 MW overexpressing Pex11β for 48h. Then cells were fixed and immunostained for PMP70 and 40x snaps of peroxisomes were then taken with the LSM800 and analysed for peroxisomal numbers and morphology in Fiji. Firstly, optimised background subtraction and 8bit-autothreshold was applied. Thereafter, peroxisomes within a given ROI were analysed by the Analyse Particles plugin of Fiji. Results were inserted into an excel sheet and the density and perimeter of peroxisomes were compared with cells transfected without any plasmid, negative control, or with Pex11β.

2.6. Oxygen Consumption Rate analysis by Seahorse platform

Oxygen is the last electron acceptor in the ETC. The XF96 Mito Stress characterises how much active the mitochondrial network is by studying, not

exclusively, the oxygen consumption rate (OCR) of adherent, cultured cells. Briefly, prior to the assay, $20x10^3$ cells were seeded on a SeaHorse 96MW culture plate for 24h and 200μL of XF medium incubated into each sensor cartridge well for hydration overnight, at 37°C, in a non- CO2 incubator. In the day of the assay, XF assay medium was prepared with 2mM glutamine, 1mM pyruvate and 5,5mM glucose, and the pH calibrated to 7.4 with 1M NaOH. After medium preparation, cells were washed once with XF medium and incubated with the respective medium for 1h, at 37°C, in a non-CO2 incubator. Meanwhile, Oligomycin (1μM), FCCCP (4μM) and a mixture of Antimycin A (1μM)/Rotenone (1μM) were loaded into Port A, B and C, respectively, of the hydrated cartridge to calibrate the instrument before replacing the calibration plate with the 96MW culture plate. Protein quantification was done by Bradford and read on a microplate reader to normalise OCR levels, according to the manufacturers' instructions.

2.7 Data analysis

All data analysis was conducted in Microsoft Excel 2013. Unless clearly stated, data are presented as Mean + standard deviation (SD) and the unpaired Student's test assuming normal distribution between samples used for studying statistical significance. P-values lower than 0.5 were considered significant. P-values smaller <0.5, 0.1 and 0.00 were denoted as *, ** and ***, respectively.

3. Aims of the study

Mitochondrial and peroxisomal fission are regulated by the same division machinery, including adaptor proteins, such as Mff and Fis1, and the large GTPase Drp1 (Schrader, 2006). The division of both organelles is essential for their functions and homeostasis and the multitude of proteins regulating these processes is most likely still bound to increase. Indeed, endocytic proteins have been recently shown to control mitochondrial fission and homeostasis (Farmer et al., 2017; Lee et al., 2015).

Dyn2, one of the three isoforms of the superfamily of dynamins, is one of the newest additions to the repertoire of mitochondrial fission proteins (Lee et al., 2015). In this new paradigm, Drp1 is responsible to create superconstricted structures that coincide with Dyn2 recruitment and final fission of the organelle (Lee et al., 2015). However, it is still not understood whether Drp1-dependent peroxisomal fission requires Dyn2 or other classical dynamins.

By using a cell line that presents all the three dynamin genes (*DNM1,2,3*) floxed and that has been genetically modified to express a mutant version of a Cre recombinase responsive to OHT (Cre-ER), we have decided to deplete all the Dyn genes and, thus, investigate in more detail how important are these classical dynamins for peroxisomal fission, but also for mitochondrial division.

All the work presented in the next two chapters were performed by me, except for Figure 12 (Fig. 12B and C) that is integrated in "Fiuza et al 2017" (The Journal of Cell Biology) paper of our laboratory (Synaptic Vesicle Dynamics), as part of the PhD project of Christine Rostosky (second author).

4. Mitochondria in Dynamins Triple knockout

4.1. Absence of Dyn1-3 impairs endocytosis

Conditional-knockout models are especially important when a given gene to be deleted, such as *DNM2*, is essential (Ferguson et al., 2009). In this chosen cell line, here designated as dynamin MEFs (Dyn MEFs), a tamoxifen-inducible Cre-loxP system has been used not only to circumvent the embryonic lethality associated with Dyn2 depletion, but also to exclude any overlapping effects between the three dynamins. As observed by western blot, the dynamin expression levels are already dramatically reduced after 4 days of tamoxifen (OHT) treatment (results not shown) and by the 5th /6th day no remaining bands are visible (Figure 12A). On the other hand, only two dynamin bands were recognised by the pan-dynamin antibody (designed to recognise equally well all isoforms) in EtOH-treated cells. These two bands correspond most likely to Dyn1 and Dyn2, since Dyn3 expression is almost negligible in fibroblasts (Ferguson et al., 2009).

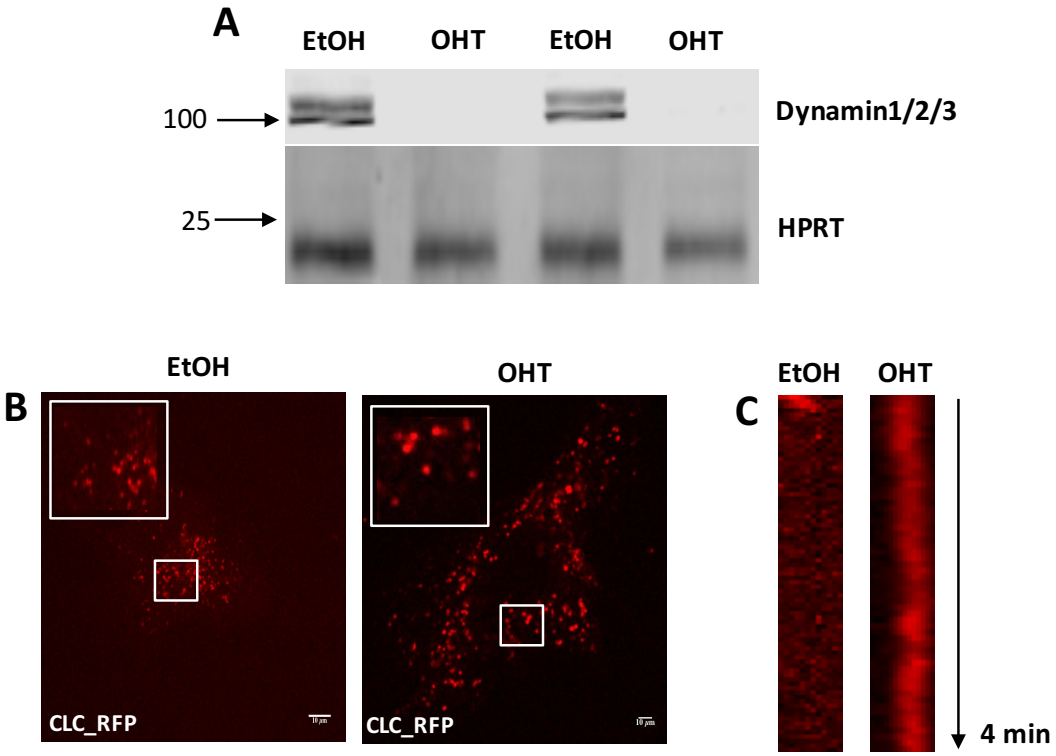


Figure 12. Cre-induced triple knockout of dynamins impairs endocytosis.

A) Dynamin 1/2 expression levels in control (EtOH-treated cells) and OHT-treated cells after 6 days of treatment. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was used as loading control.
B) Representative live-cell images of EtOH- and OHT-treated cells expressing clathrin-light chain fused with mRFP (CLC-RFP) and respective kymographs (C). 60x, scale bar, 10μm.

Secondly, the proliferation of Dyn MEFs exposed to OHT, but not EtOH, was reduced in the first 72h of treatment and strongly affected starting from day 4, as it has been reported elsewhere (Ferguson et al., 2009; Ishida, Nakamura, Tanabe, Li, & Takei, 2011). A third and final characterization of this model was achieved by studying endocytosis, specifically by transfecting EtOH/OHT-treated Dyn MEFs with a human clathrin-light chain protein fused with mRFP (CLC-RFP) (Fig. 12B). By live-cell imaging, clathrin seems to not be removed from CCPs in OHT-treated cells, whereas in EtOH-treated cells clathrin presents most of the times a short residence time at the PM (Fig. 12C). Overall, this reveals the well-characterised blockage in this endocytic pathway after dynamin depletion (Ferguson et al., 2009; Park et al., 2013). Thus, and after this initial validation of the model and of the treatment, EtOH-treated and OHT-treated Dyn MEFs, hereafter denoted as Control and TKOs, respectively, were always used after day 6-8 days of OHT treatment to study peroxisomes and mitochondria.

4.2. Dynamin TKOs present normal mitochondrial morphology

Mitochondrial morphology and dynamics are constantly fine-tuned. Recently, Dyn2 has been associated with the mitochondrial fission process, specifically by helping Drp1 constricting fully a dividing mitochondrion (Lee et al., 2015). To study in more detail how dynamins influence mitochondrial morphology, we decided to transfect cells with the Mito-GFP construct, a GFP protein with a mitochondrial targeting sequence. Mito-GFP-expressing TKOs and control cells were fixed and imaged by Airyscan microscopy to create 3D reconstructions of the mitochondrial network, which were quantified semi-automatically, in a blind fashion, with the Mito-Morphology macro of Fiji (Fig. 11A) (Dagda et al., 2009). Two mitochondrial features analysed by this macro consist on the number of mitochondria per cell and the correspondent mitochondrial content, defined as the area of cytoplasm occupied by mitochondria. Mitochondrial

content and number of mitochondria were similar in TKO and control cells (Fig. 11B). Furthermore, mitochondrial morphology, calculated by the ratio between Area and Perimeter (Area/Perimeter), and designated as index of interconnectivity, was not changed in TKO cells (Fig. 11C).

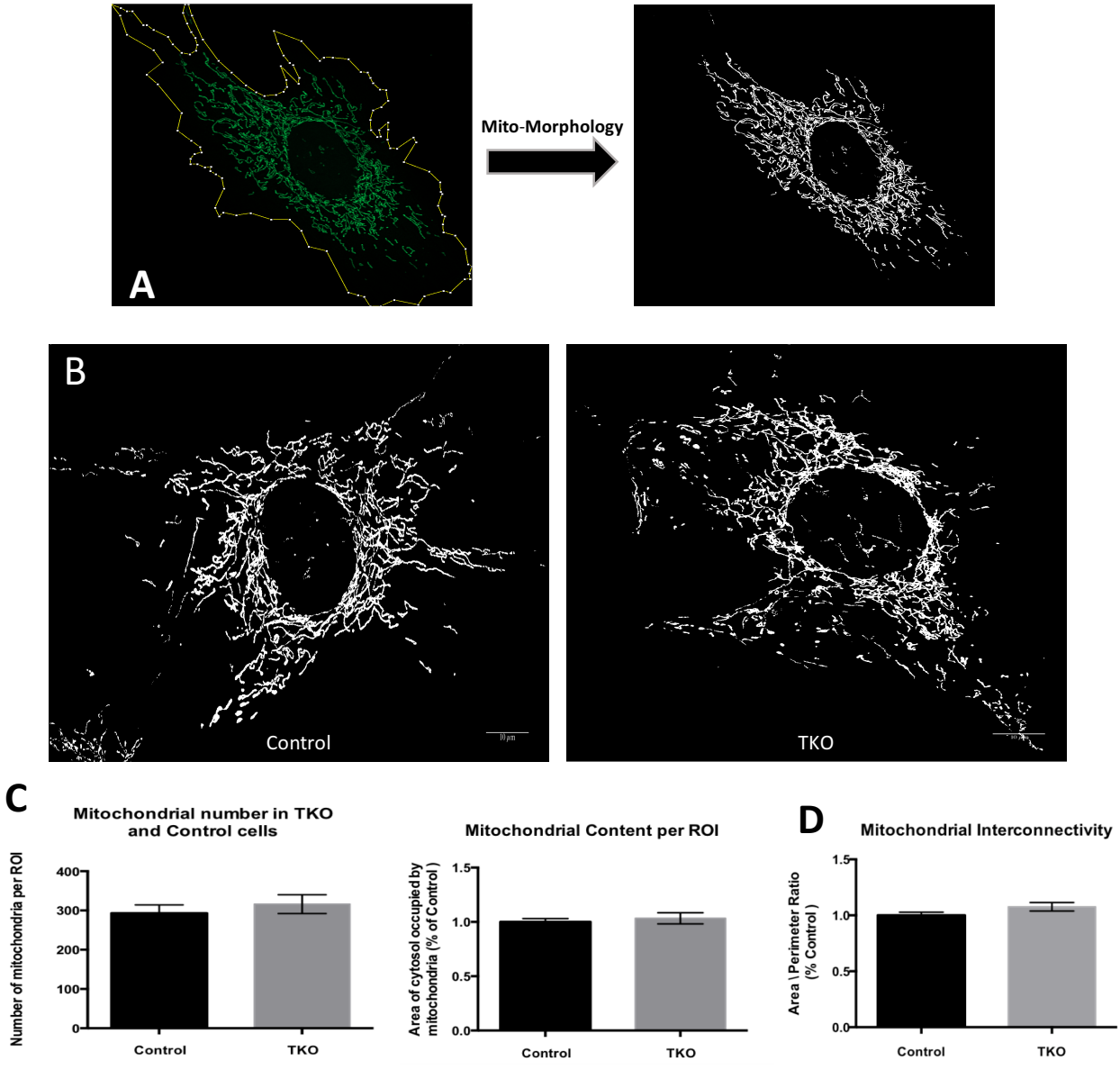


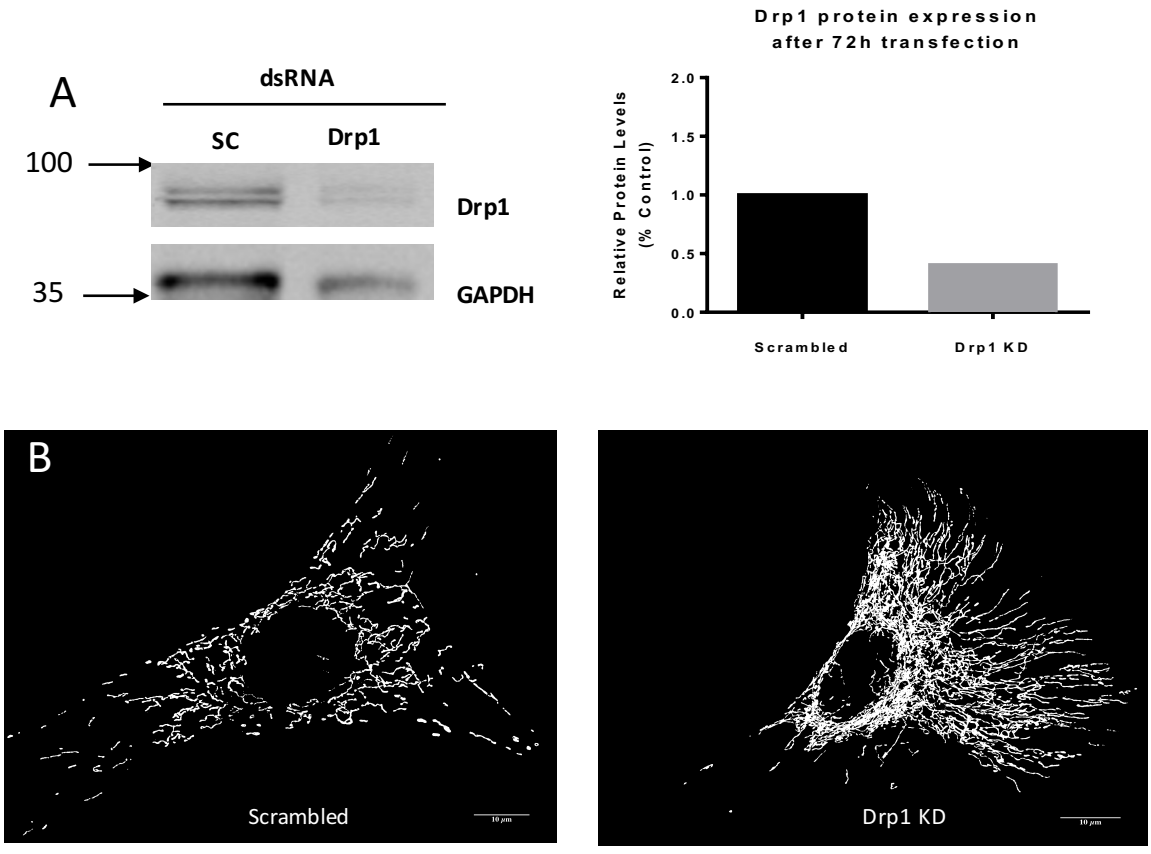
Figure 13. Characterization of mitochondrial morphology using Mito-Morphology macro in Fiji.

A) Representative analysis in Fiji, including the region of interest (ROI) and its respective thresholding, and representative image of a control and TKO cell (below). 63x, scaler bar, 10µm.
B) Quantification of mitochondrial number and content (area of cytosol occupied by mitochondria) per ROI, n=30.
C) Quantification of the interconnectivity index (area/perimeter ratio) of control and TKO cells, n=30.

In this cell model, Drp1 might be able to drive mitochondrial fission independently of Dyn2 or other dynamins in basal conditions.

4.3. Inhibition of Dynamin-related protein 1 results in mitochondrial elongation

Ablation of Drp1, hitherto the main identified mitochondrial fission protein, has been shown to cause mitochondrial elongation (Smirnova et al., 2001). To confirm that mitochondrial fission is largely governed by this protein in our Dyn MEFs, we decided to silence Drp1 by RNA interference (RNAi). Immunoblotting for Drp1 shows a 60% reduction in the expression levels of this protein in cells transfected with a Dicer-substrate short interfering RNAs (dsiRNA) designed against Drp1 compared to its scrambled sequence (Fig. 14A). Airyscan microscopy (as explained above, further details in methods) showed that mitochondrial morphology from scrambled-transfected cells (SC) resembled those of control cells, whereas silencing Drp1 induced the formation of a hyperfused mitochondrial reticulum (Fig. 14AB).



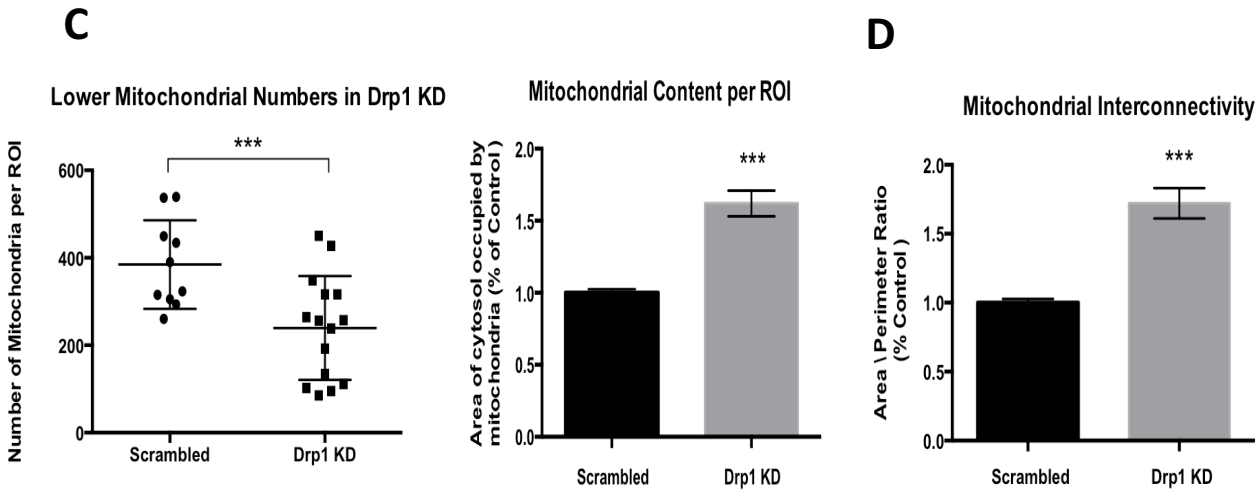


Figure 14. Effect of Drp1 silencing in mitochondrial morphology of Dyn MEFs.

A) (Left) Representative blot and (B) quantification of Drp1 expression levels in cells transfected with scrambled or Drp1 dsiRNA (Drp1). GAPDH was used as loading control.
B) Representative images of scrambled and Drp1 KD cells (below). 63x, scaler bar, 10μm.
C) Quantification of mitochondrial number and content (area of cytosol occupied by mitochondria) per ROI in scrambled and Drp1 KD cells, n=15 for Drp1 KD and n=9 for SC.
D) Quantification of the interconnectivity index (area/perimeter ratio) of scrambled and Drp1 KD cells, n=15 for Drp1 KD and n=9 for SC.

Quantification of mitochondrial morphology revealed that Drp1-knockdown (Drp1 KD) cells present a lower number of mitochondria and an increased mitochondrial content in comparison to SC (Fig. 14C). Moreover, and as expected, the index of mitochondrial interconnectivity is remarkably higher in cells silenced for Drp1 (Fig. 14D). On one hand, these results prove the sensitivity of the Mito-morphology macro (Dagda et al., 2009). On the other, Dyn MEFs seem to rely mostly, or even solely, on Drp1 for mitochondrial fission in basal conditions, since Drp1 silencing, but not dynamin triple-knockout, induces mitochondrial elongation.

4.4. Mitochondrial proteins are not altered in TKO cells

The equilibrium between mitochondrial fusion and fission is regulated by multiple proteins. The levels of adaptor proteins, for instance, are important to help recruiting Drp1 into the division foci on the OMM (Otera et al., 2010). Regulation of Drp1 activity, in turn, by PTMs, namely by phosphorylation, is another important determinant of mitochondrial fission (Chang et al., 2010). Indeed, two widely studied

regulatory amino acids of Drp1 correspond to S637 and S616; phosphorylated S637 is associated with decreased Drp1 activity, whereas phosphorylation of S616 increases mitochondrial fission (Hu et al., 2017). The ratio of these phosphorylation sites and Mff expression levels do not seem to be altered between control and TKOs (Fig. 15).

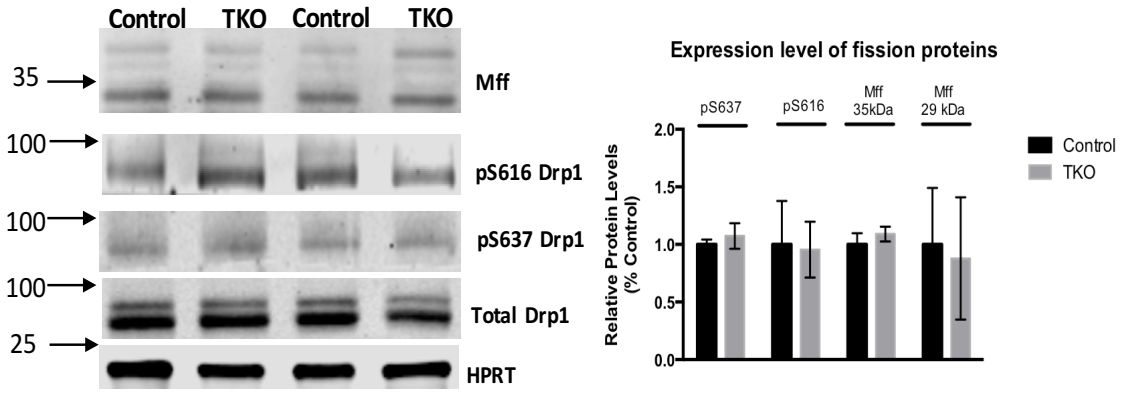


Figure 15. Expression levels of mitochondrial fission proteins in TKO and control cells.

(left) Representative blot and (right) quantification of respective bands. pS637 and pS616 levels were normalised per total Drp1 levels and HPRT was used as loading control.

In general, this seems to indirectly exclude the possibility that the fission machinery, upon dynamin absence, is dysregulated or increased to overcome the putative defect in mitochondrial fission and that TKOs are not under energy starvation, since the ratio of phosphorylated S637, which has been shown to be upregulated during starvation (Gomes et al., 2011), is unaltered compared with control cells.

Although perhaps unlikely, decreased fusion could explain why mitochondrial elongation is not verified in TKOs. In this process, apposition and fusion of two OMMs are regulated by mitofusins, Mfn1 and Mfn2, while Opa1 is responsible for fusion of IMMs (Cipolat et al., 2004; Santel et al., 2001). Immunoblotting of Mfn1 and 2 did not reveal any change in the levels of the OMM fusion proteins (Fig. 16). VDAC, an OMM pore protein that functions as an entry point of metabolites in mitochondria and that is used as a mitochondrial marker, is unchanged, in agreement with the data of mitochondrial morphology presented above (Fig. 13B, above). In addition,

immunoblotting for mitochondrial Opa1, whose levels as cleaved l-OPA1 or s-OPA1 isoforms are dependent on the mitochondrial membrane potential $\Delta\Psi$, are also unchanged (Fig. 16).

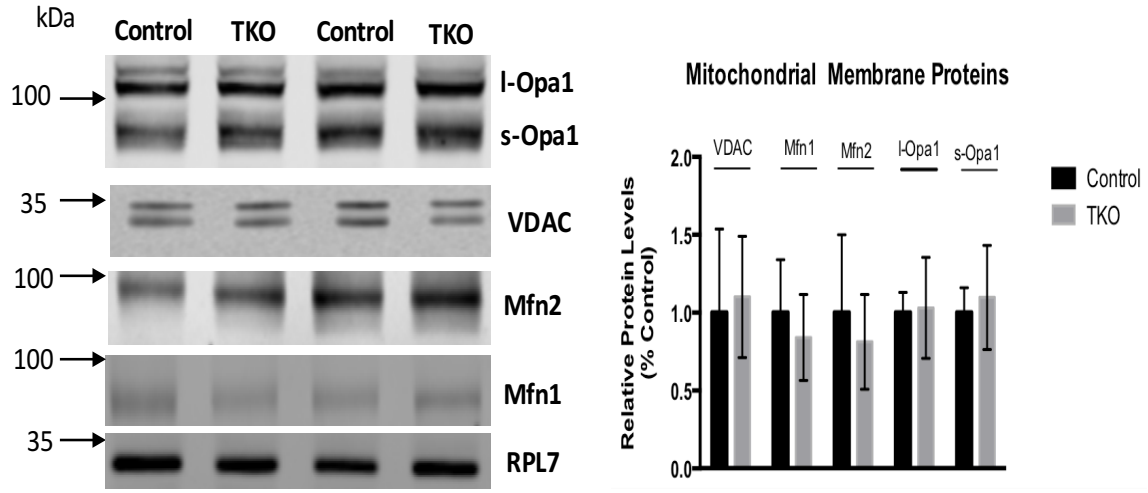


Figure 16. Expression levels of mitochondrial membrane proteins in TKO and control cells.

(left) Representative blot and (right) quantification of respective bands. RPL7 (60S ribosomal protein 7) was used as loading control.

These results do not point out a defect in mitochondrial fusion proteins and, albeit indirectly, suggest that fusion is not affected. Moreover, and even though more mitochondrial markers are needed, mitochondrial mass does not seem to be changed, which is consistent with the mitochondrial content analysis obtained above (Fig. 13,above). This could also indicate that mitochondrial turnover is not compromised upon dynamins depletion. Lastly, mitochondrial potential from TKOs does not seem to be altered in the absence of dynamins, since dysfunction at the mitochondrial potential would induce a dramatic dysregulation of Opa1 cleavage (Head et al., 2009; Jones et al., 2017).

4.5 Calcium-induced mitochondrial division is not blocked in TKOs

The mitochondrial reticulum integrates different cues. In fact, depending on the stimulus, mitochondrial fission, fusion and mobility can be quickly adjusted according

to the cells' needs (Gottlieb et al., 2016; Wai et al., 2016). Despite not having seen an abnormal mitochondrial morphology in TKOs (Fig. 13,above), Drp1-mediated fission was reported to be stalled Dyn2-depleted cells (Lee et al., 2015). Therefore, to understand more this relationship between dynamins and mitochondrial division we decided to stimulate mitochondrial division with a given stimulus.

Calcium, for instance, is a known activator of mitochondria fission (Cribbs et al., 2007; Hom et al., 2007). Using Ionomycin, a calcium ionophore known to induce Drp1-dependent mitochondrial division, we observed the expected fragmentation of the mitochondrial network (Fig. 17). Mitochondrial fission was immediately induced upon drug addition, with some fission events being observed within the first minute – mostly in periphery-located mitochondria - followed by a more synchronized and complete mitochondrial fragmentation that was approximately completed by the third minute of the treatment (Fig. 18B, below). Furthermore, during our time frame, mitochondrial fragmentation did not seem to be reversible upon Ionomycin treatment. On the other hand, treatment with DMSO (vehicle) or a cell-permeant calcium-chelator, BAPTA-AM, did not seem to disturb mitochondrial dynamics (Cereghetti et al., 2008) (Fig. 17), probably illustrating how rises in intracellular calcium levels might shift mitochondrial dynamics towards fission.

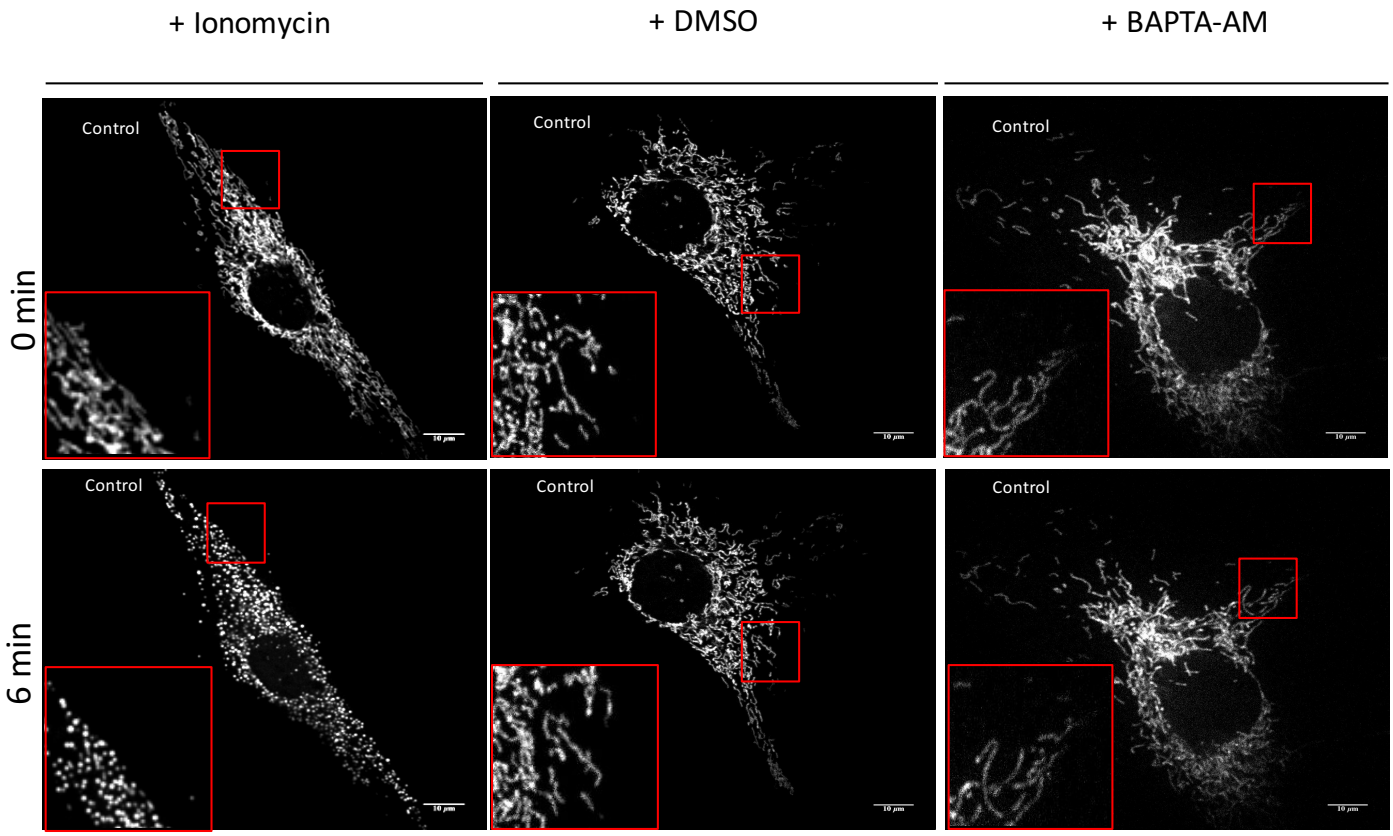
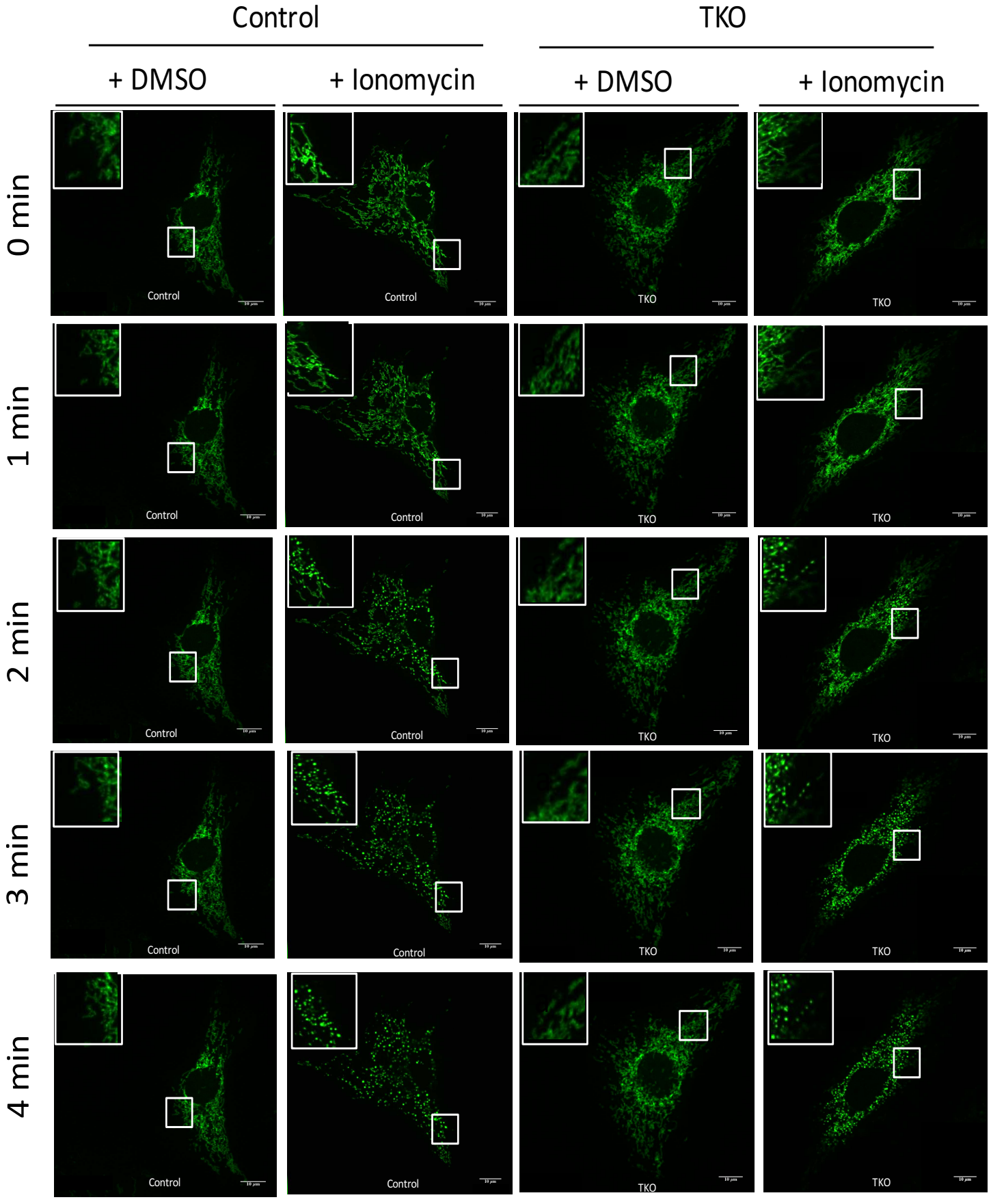


Figure 17. Effects of intracellular calcium elevation in mitochondrial morphology.

Time-lapses of mitochondrial network of Mito-GFP-transfected control cells treated with the 4μM of Ionomycin, a calcium ionophore, 10μM BAPTA-AM, a calcium-chelator, or DMSO (vehicle). Frames depict the morphology of mitochondria before and 6 min after addition of the respective drug. 60x, scale bar, 10μm.

Since this Ionomycin effect has been shown to depend on functional Drp1, we compared the fission kinetics between control and TKOs to understand if mitochondrial division upon calcium signalling is partially inhibited or completely blocked without dynamins. Surprisingly, mitochondrial fission in TKOs is not blocked upon Ionomycin treatment and it does not seem partially delayed or inhibited (Fig. 18A, B). Like control cells, mitochondrial division started as soon as Ionomycin was added in peripheral mitochondria, whereas the remaining mitochondria required more time to divide synchronously, being the mitochondrial network completely punctiform within approximately 3 minutes (Fig. 18B, below).

A


Control
TKO
+ DMSO
+ Ionomycin
+ DMSO
+ Ionomycin
0 min
1 min
2 min
3 min
4 min
Control
Control
TKO
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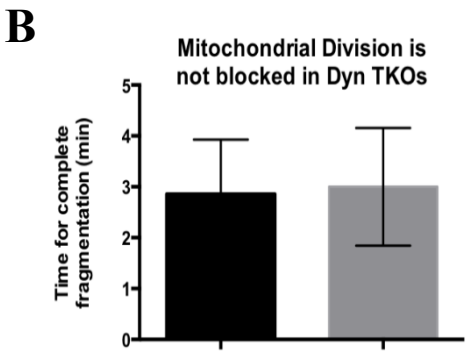


Figure 18. Mitochondrial fission kinetics upon Ionomycin treatment.

A) Time-lapses of mitochondrial network of Mito-GFP-transfected control and TKO cells treated with the 4μM of Ionomycin, a calcium ionophore, or DMSO (vehicle). Frames depict the morphology of mitochondria before and 1, 2, 3 or 4 min after addition of the respective drug. 60x, scale bar, 10μm.
B) Time for Ionomycin-mediated full mitochondrial fragmentation, n=7. Error bars correspond to standard-error of the mean (SEM).

Overall, permeabilization to calcium sources might activate signalling cascades that culminate in Drp1 activation and subsequent mitochondrial fragmentation (Cereghetti et al., 2008; Cribbs et al., 2007), which seems to not depend on dynamins since TKO are still able to form fragmented mitochondria.

4.6 Depolarization induces formation of mitochondrial doughnuts

Mitochondrial fragmentation can also be induced by uncoupling oxidative phosphorylation (Mishra et al., 2016). CCCP, for instance, has been shown to shift the balance of mitochondrial dynamics towards fission by inhibiting the fusion protein Opa1 (Ishihara et al., 2003). Treating cells with CCCP induced a robust mitochondrial fragmentation in both control and TKO cells (Fig. 19A). This mitochondrial fragmentation with CCCP is slower than the mitochondrial fragmentation observed during Ionomycin treatment and is preceded by formation of mitochondrial "doughnuts" (Fig. 19B) (Cereghetti et al., 2008).

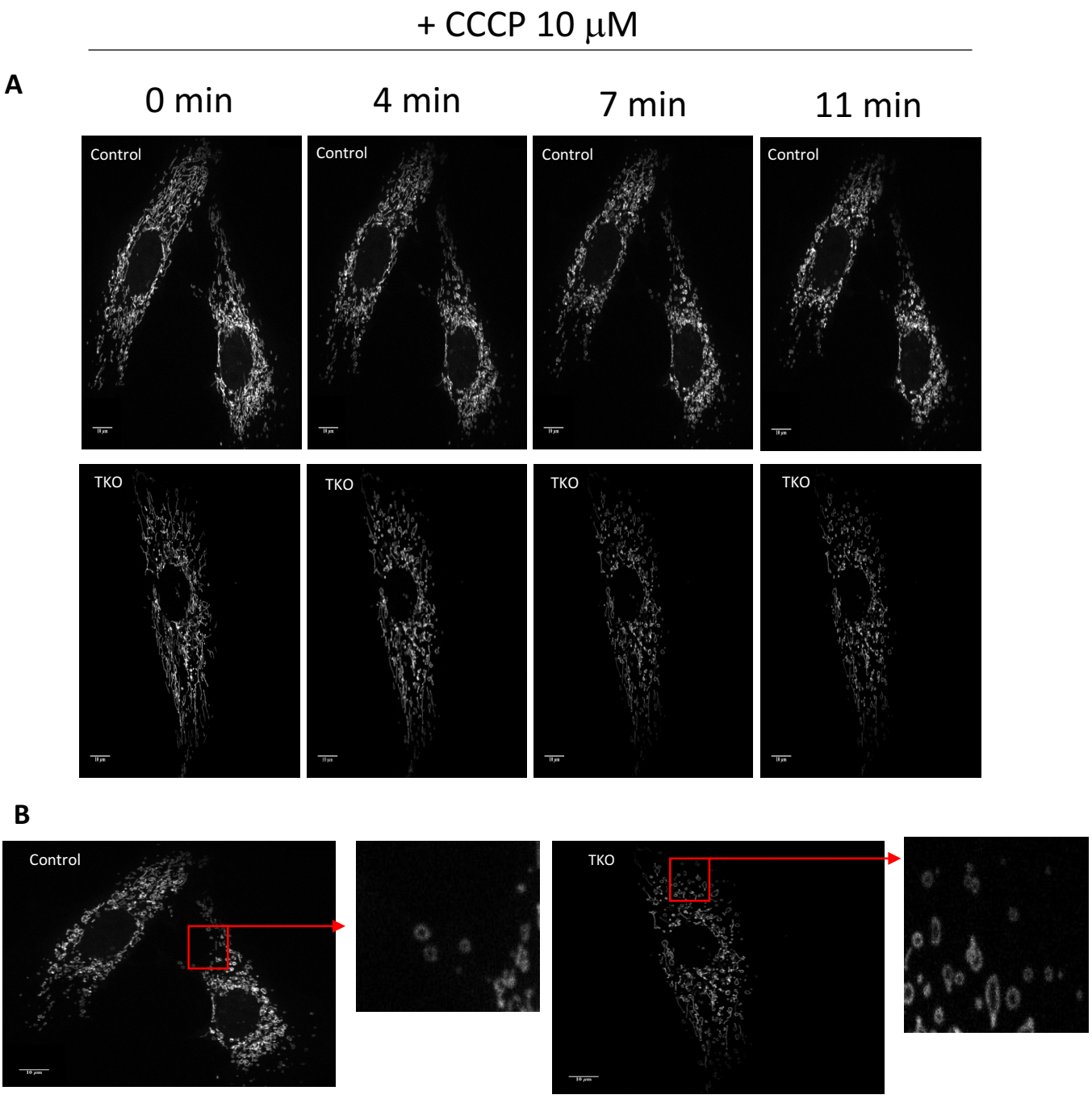


Figure 19. Mitochondrial fragmentation and doughnuts formation by CCCP treatment.

A) Time-lapses of mitochondrial network of Mito-GFP-transfected control and TKO cells treated with the 10µM of CCCP, mitochondria depolarizing agent. Frames depict the morphology of mitochondria before and 4, 7 or 11 min after addition of the respective drug. 60x, scale bar, 10µm.

B) Formation of doughnut-like mitochondria in TKO and Control cells.

Even though fragmentation triggered by uncouplers has been shown to depend on Drp1 (Cereghetti et al., 2008; Losón et al., 2013), this fragmentation is clearly distinct as the one observed upon Ionomycin treatment (Fig. 18, above), as mitochondria upon CCCP treatment encircle, forming this so-called doughnut-like mitochondria (Ding et al., 2012). Nonetheless, and even though a time point for full CCCP-mediated mitochondrial fragmentation was not studied, both TKO and control cells seemed to respond equally to this treatment (Fig. 19A, B, above), suggesting that dynamins do not prevent or delay this CCCP-induced fragmentation.

4.7 Mitochondrial respiration, but not potential, is decreased in Dyn TKOs

The relationship between clathrin-mediated endocytosis defects or inhibition of classical dynamins and mitochondrial fitness has not been intensively studied. Given the plasticity of mitochondria to readjust to extracellular and intracellular stimuli, we decided to characterize the mitochondrial function in TKOs. By measuring oxygen consumption with the Seahorse platform, basal mitochondrial respiration was shown to be lowered in TKOs when compared to control cells (Fig. 20). Moreover, collapsing the mitochondrial potential with FCCP increased dramatically OCR in control cells, whereas this FCCP-induced maximal respiration in TKOs did not even surpass the basal OCR of control cells (Fig. 20).

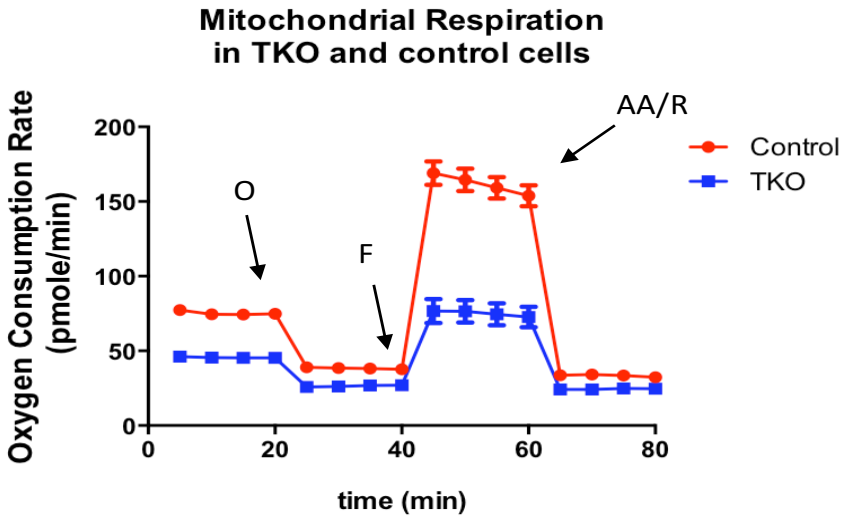


Figure 20. Mitochondrial respiration upon depletion of dynamin.

Oxygen consumption rate, measured over time (min), and mitochondrial bioenergetics parameters were studied using the XF Cell Mito Stress Test assay with the Seahorse extracellular analyser (Seahorse, Agilent). Injection of Oligomycin (0, 1μM), FCCP (F, 4μM) and Antimycin A/Rotenone (AA/R, 1μM) were used to study ATP-linked OCR, maximal OCR and non-mitochondrial OCR (respectively).

Besides exhibiting a downregulation of the basal and maximal respiration, TKOs reveal also a lowered spared respiratory capacity from these cells, a parameter that can be calculated by the difference between maximal and basal mitochondrial respiration (Rose et al., 2014).

To understand if this decreased oxygen consumption rate was accompanied by a general decrease in ΔΨ, we decided to study by flow cytometry TMRM fluorescence, a cationic dye that accumulates in polarised mitochondria (Rowe et al., 2013). TMRM fluorescence was found to be similar in both TKO and control cells (Fig. 21A, B).

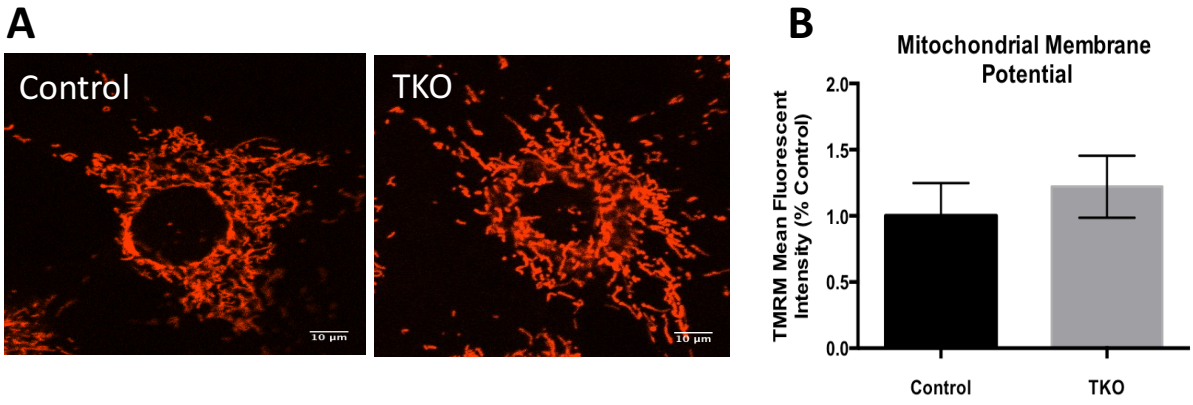


Figure 21. Effect of blocking clathrin-mediated endocytosis in mitochondrial membrane potential.

A) Representative of images of control and TKO cells loaded with 10nM TMRM. 40x, scale bar, 10μm.
B) Quantification of Mean Fluorescent Intensity (MFI) of TMRM between control and TKO cells by flow cytometry.

Since this cell line had lower respiration, energy synthesis is probably provided mostly by glycolysis, suggesting that TKOs became more glycolytic in comparison to

control cells. On the other hand, the lower respiration could be due to impairment in the electron transport chain (Wu et al., 2007), which warrants further studies. In any case, mitochondrial metabolism seems to be regulated differently in these TKO and control cells.

A lower mitochondrial respiration in these cells might be the result of inefficient flow of electrons and increased mitoROS production. By flow cytometry, MitoSOX fluorescence, a probe that measures mitochondrial superoxide levels, is upregulated in TKOs (Fig. 22B). However, a careful interpretation of such results must be taken. Indeed, by live cell-imaging almost all control cells produced negligible or small amounts of mitochondrial superoxide, which was co-localised with Mitotracker Green (MTG), a mitochondrial probe that accumulates in mitochondria irrespective of $\Delta\Psi$ (Fig. 22A). On the other hand, and even though TKOs were by and large observed with a stronger MitoSOX fluorescence, in many cases MitoSOX signal did not co-localise with MTG and was cytosolic.

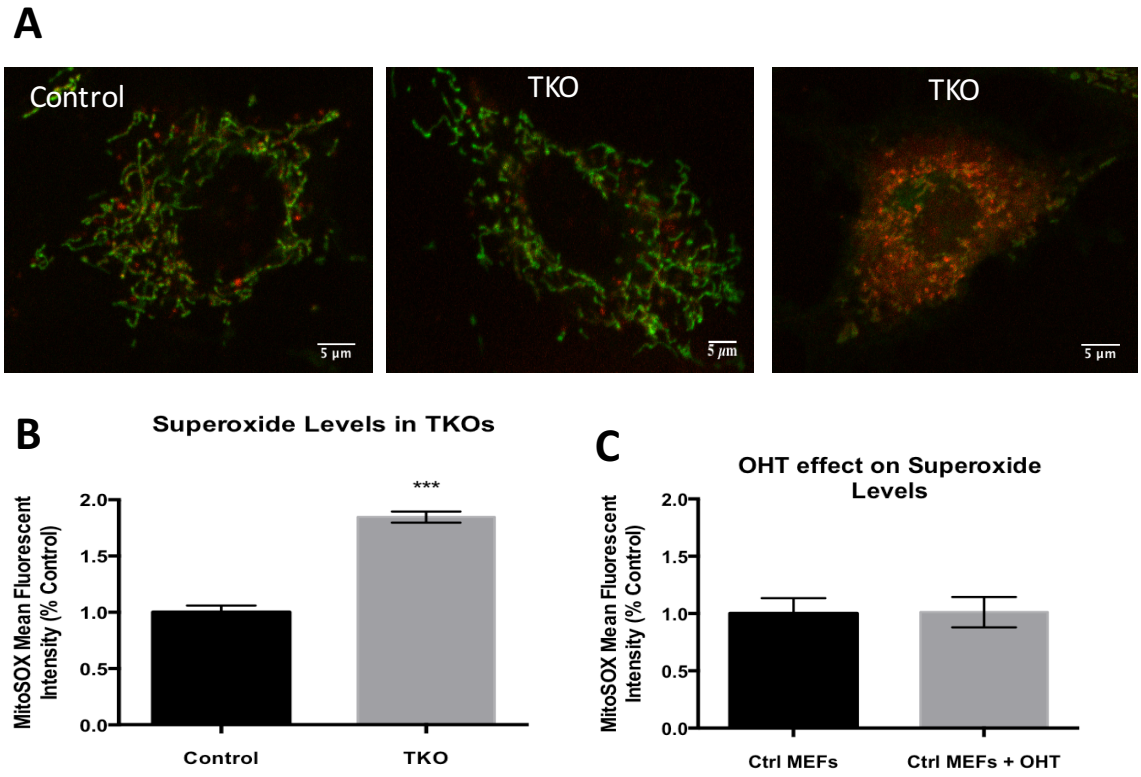


Figure 22. Mitochondrial superoxide production due to endocytic defects and tamoxifen treatment.

A) Representative images of control and TKO cells loaded with 2,5μM of MitoSOX and 200nM of Mitotracker Green. 40x, Scaler bar, 5μm.
B) Mean Fluorescent Intensity of MitoSOX in TKO and control cells loaded with 5μM MitoSOX.
C) Mean Fluorescent Intensity of MitoSOX in Wt MEFs untreated (control) or treated with tamoxifen (OHT).

Moreover, this effect in superoxide levels is not due to OHT treatment, since treatment of Wt MEFs (Bl6 immortalised fibroblasts, denoted as control MEFs) with this compound does not affect MitoSOX fluorescence levels, as assessed by flow cytometry (Fig. 22C, above).

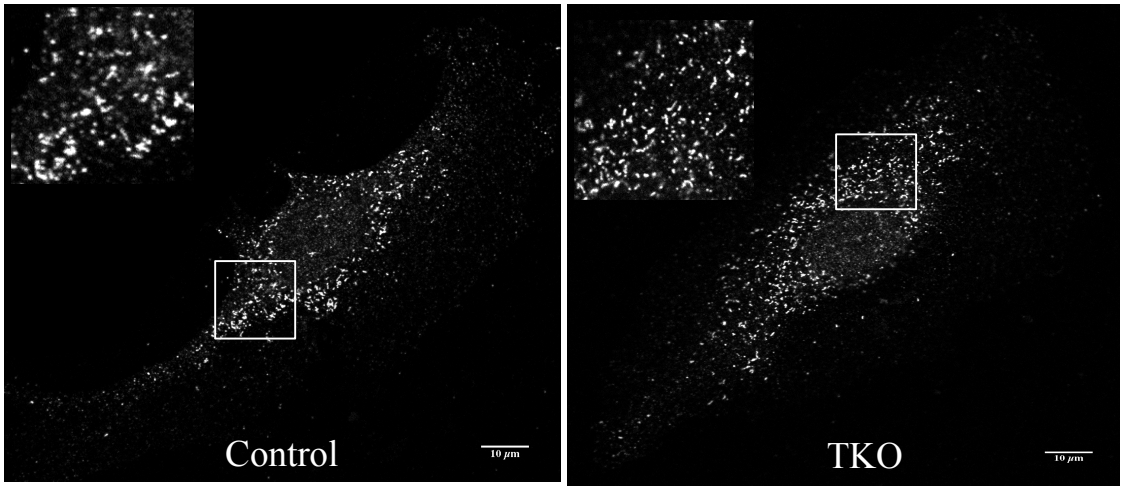
Collectively, these results suggest that mitochondrial metabolism is changed. The unspecific staining of MitoSOX warrants further studies of the intracellular redox state of TKOs, because in some cases MitoSOX can react in the cytosol (Dikalov et al., 2014). Whether indirect or not, dynamins depletion does result in changed mitochondrial metabolism and, possibly, in a higher rate of glycolytic flux.

5. Peroxisomes in Dynamins Triple-knockout

5.1. Depletion of dynamins does not elongate peroxisomes

Peroxisomes are ubiquitous single-membrane bound organelles (Demarquoy et al., 2015). Depending on the cell type, peroxisomes are highly heterogeneous, being found not only as spherical but also as elongated organelles whose diameter can range from 0.1-1μm (Lodhi et al., 2014; Schrader, 2006). To investigate peroxisomal morphology in TKO cells, fixed cells were immunostained for the integral ABC transporter peroxisomal membrane protein 70 (PMP70) and imaged by confocal microscopy. In control cells, peroxisomes were found as vesicle-like or elongated organelles (Fig. 23A). In the triple-knockout model, peroxisomes were equally found as heterogeneous organelles, some being spherical and others more tubular (Fig. 23A). Indeed, quantification of their sphericity index, using the Surface wizard of IMARIS software, revealed that peroxisomes from TKO and control cells present a similar sphericity index (Fig. 23B, C). Indeed, most peroxisomes are quite spherical-like in both cells. Moreover, peroxisomal numbers per cell were not changed (Fig. 23C).

A



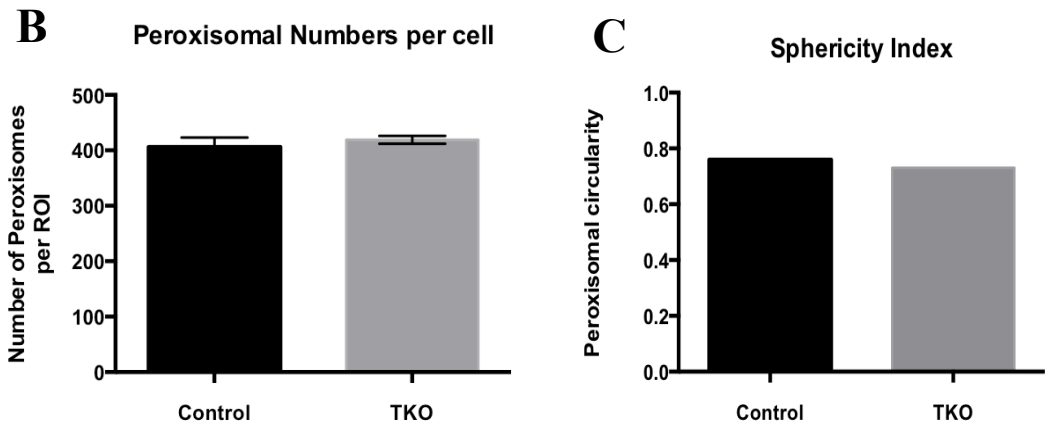


Figure 23. Peroxisomal morphology in dynamin TKO and control cells.

A) Representative images of control and TKO cells immunostained for PMP70. 63x, scaler bar, 10μm.

B) Surface analysis and (C) quantification of the number of peroxisomes (C) and the sphericity index by the Surface wizard of Imaris software; n=17 for Control and TKO

The fact that peroxisomal number per cell is not distinct might suggest that processes related with of peroxisome biogenesis or with peroxisomal turnover mechanisms are not impaired. Indeed, immunoblotting for Pex11 and Pex5, peroxisomal proteins invoved in peroxisome biogenesis, and Catalase and PMP70, markers of peroxisomal numbers, did not reveal changes between control and TKO cells (Fig. 24). Nonetheless, peroxisomal functionality has yet to be addressed in this cell model.

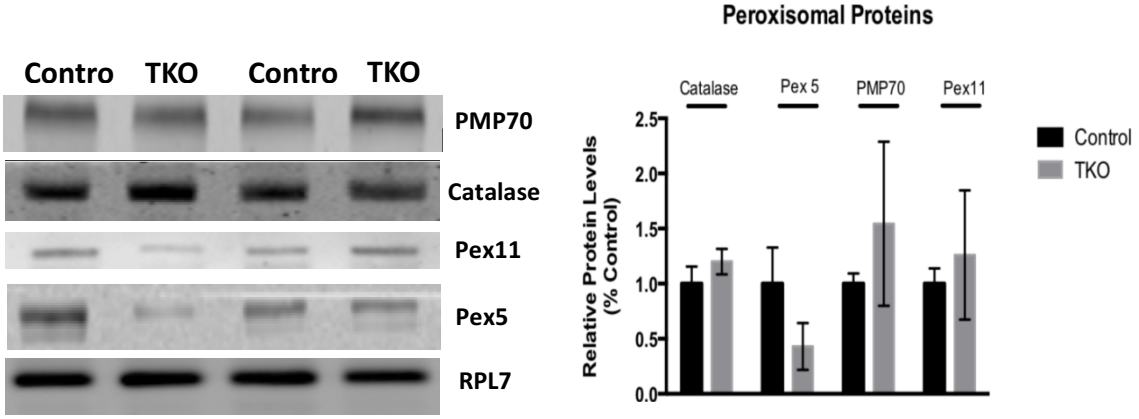


Figure 24. Expression levels of peroxisomal proteins in TKO and control cells.

(left) Representative blot and (right) Quantification of respective bands. RPL7 was used as loading control.

5.2. Peroxisomes are constantly moving in both control and TKO cells

Peroxisomes are extremely plastic organelles, readjusting even their matrix contents, according to the cellular needs and extracellular stimuli (Demarquoy et al., 2015). Even though the above morphological analysis did not suggest a defect in peroxisomal division, we studied in more detail whether peroxisomes from TKOs are dynamic and able to divide upon dynamin depletion. Transfected cells for Ub-RFP-SKL, a ubiquitin moiety fused to a mRFP protein bearing a peroxisomal targeting sequence 1 (SKL are the three amino acids that constitute the PTS1) (Fig. 25A), and which is a construct used to label peroxisomes (Aranovich et al., 2014), showed several spherical-like organelles constantly moving in both control and TKO cells (Fig. 25B). In fact, in the triple knockout model the movement of peroxisomes seemed to resemble roughly those of control cells, thereby suggesting that peroxisomes are mobile and dynamic despite dynamins depletion.

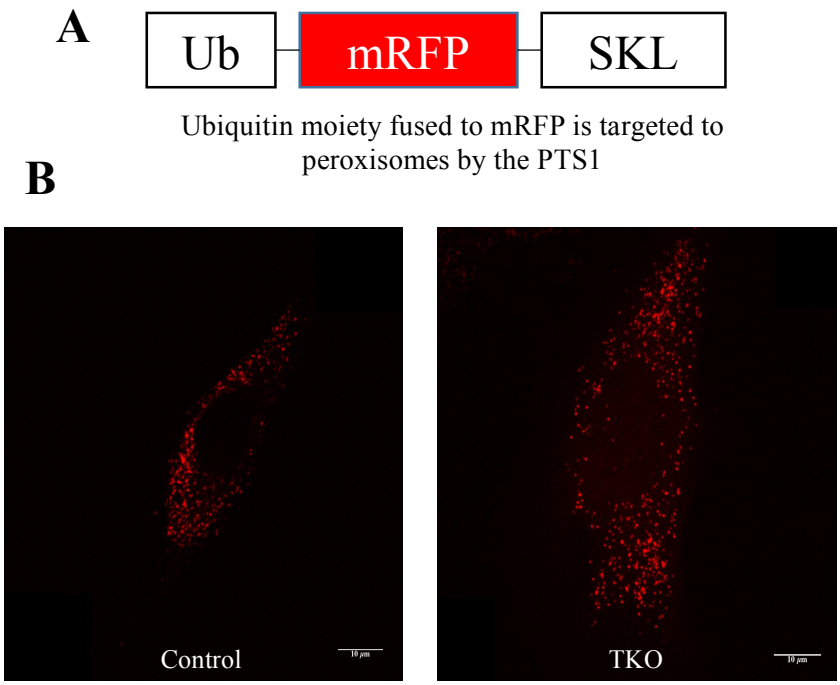


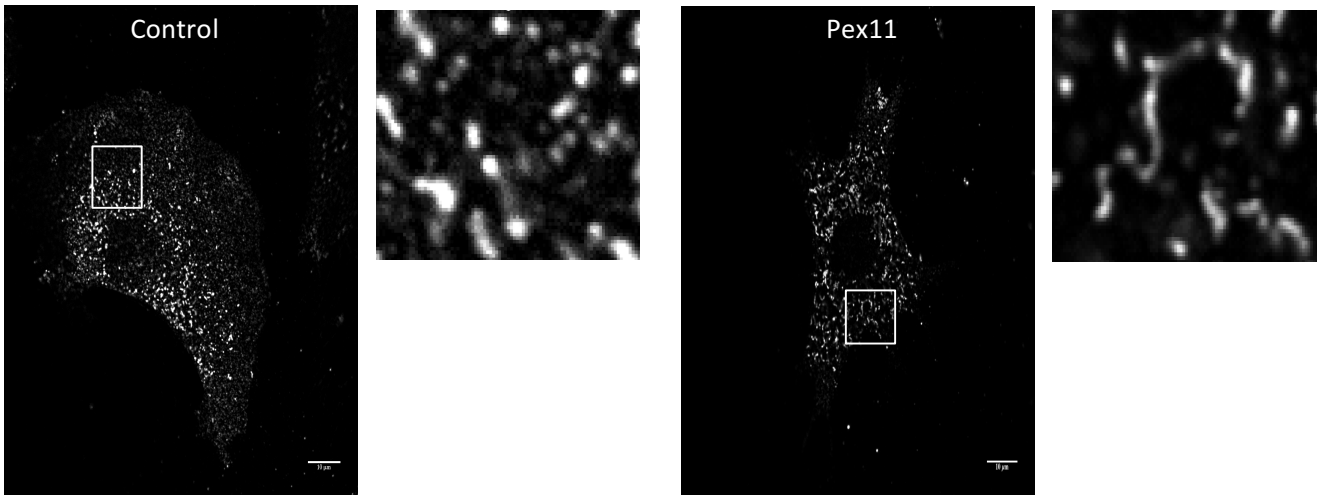
Figure 25. **Live-cell imaging of Ub-RFP-SKL-labelled peroxisomes.**

A) Depiction of the Ub-RFP-SKL construct.
B) Representative images of labelled peroxisomes imaged by live-cell imaging in control and TKOs. 60x, scale bar 10µm.

The movement of peroxisomes is mostly restricted and confined. In addition, during our time frame no peroxisomal division events were recorded or identified; unlike these organelles, mitochondria were observed to divide and fuse multiple times in the same period.

Nevertheless, to bypass this limitation one can force peroxisomal fission. Pex11β overexpression, for example, is a widely-used approach to increase the number of peroxisomal division events, since increasing Pex11β levels in peroxisomal membranes will induce peroxisomal elongation that precedes the recruitment and assembly of the fission machinery to force pre/existing peroxisomes to divide (Schrader et al., 1998, 2012). This approach is still being optimised in our hands, but so far processing for PMP70 in Pex11β-overexpressing fixed cells (denoted as Pex11) revealed several highly-elongated peroxisomes (Fig. 26A). Using the Fiji Particle Analysis, peroxisomal density was confirmed to be similar in both cells and the length to be strikingly increased, which means that the overexpression was yet not sufficient to drive peroxisomal proliferation compared to cells that were simply electroporated (without any plasmid) and used as a negative control (denoted as Control) (Fig. 26B, C).

A



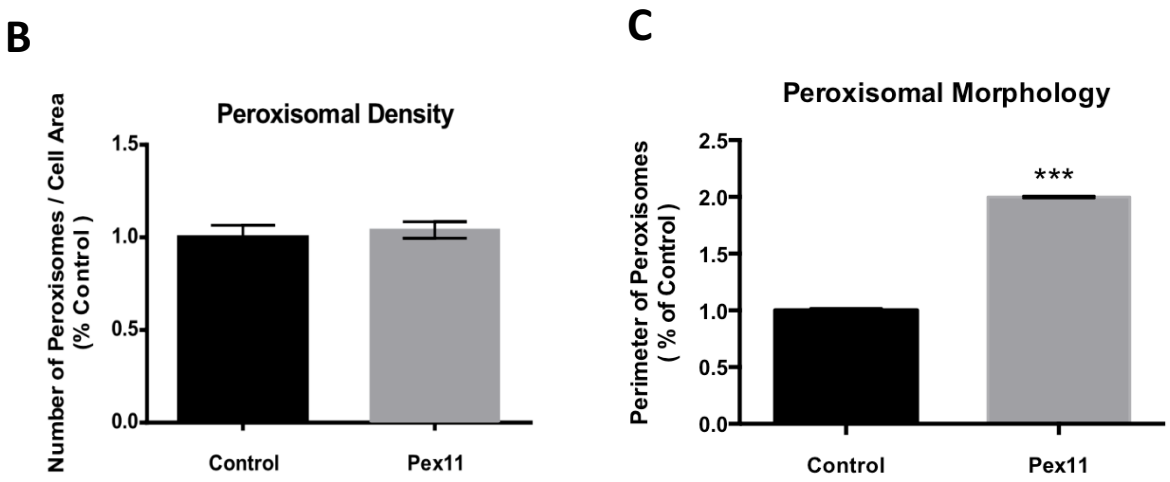


Figure 26. Overexpression of Pex11β increases peroxisomal fission.

A) Representative image of an electroported cell without (left) or with Pex11β (right) and respective amplifications. 63x, scale bar 10μm.
B) Peroxisomal density and (C) Peroxisomal Perimeter analysed by the Particle analysis plugin of Fiji.

5.3 Ionomycin treatment interrupts peroxisomal mobility

Mitochondria and peroxisomes share components for their division processes. One interesting question that is yet to be fully explored concerns the regulation of such events. In fact, PTMs have been shown to modulate Drp1 activity and mitochondrial fission (Chang et al., 2010). Instead, how is Drp1 regulated and which signalling cascades participate in peroxisome biology is still largely unknown (Michael et al., 2016). Are both processes constantly and differently regulated? Do peroxisomes divide when mitochondrial fission is triggered?

Previously, mitochondrial fission was induced by Ionomycin treatment, a calcium ionophore that is thought to activate signalling cascades responsible for Drp1 activation and translocation to mitochondria (Fig. 17, above). To understand if such treatment could stimulate peroxisomal fission, Ub-RFP-SKL-expressing cells were imaged by live-cell imaging and treated with Ionomycin to monitor peroxisome dynamics. Peroxisomes did not seem to divide upon the putative calcium influx caused by Ionomycin (Fig. 27). However, peroxisomal movement was immediately inhibited and slowed down (Fig. 27). DMSO did not change peroxisome dynamics.

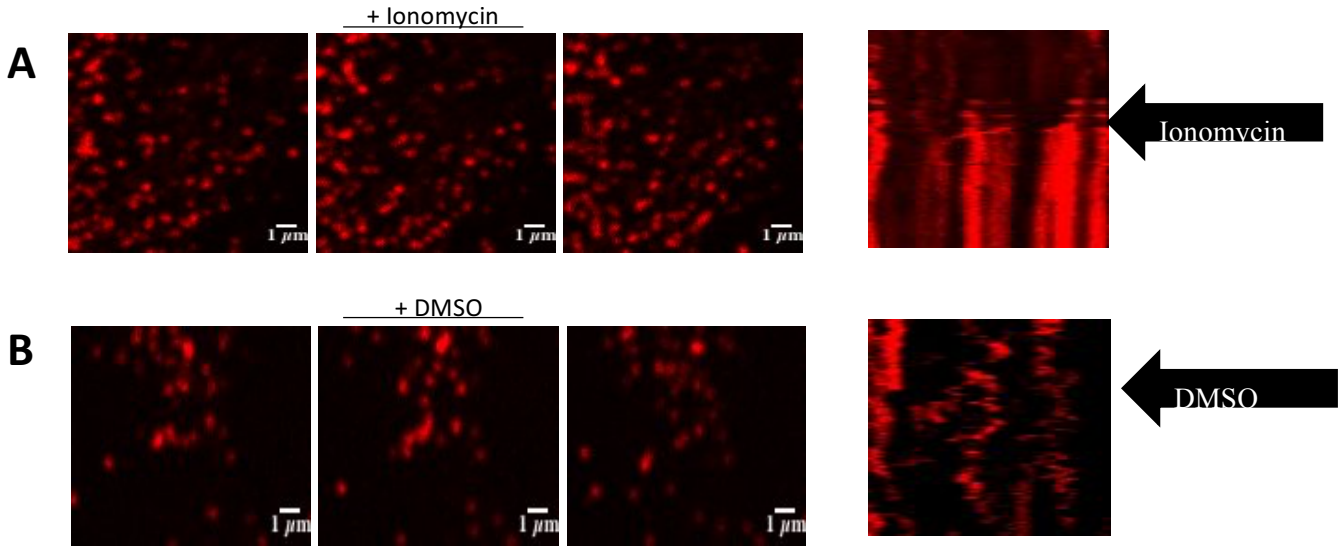


Figure 27. Ionomycin treatment inhibits peroxisomal movement.

Time lapse of Ub-RFP-SKL-transfected cells treated with Ionomycin (A) and DMSO (B) and respective kymographs (arrows indicate addition of Ionomycin or DMSO). 60x, scale bar 1µm.
Note the bidirectional, and restricted movement, of some peroxisomes in the kymograph of cells treated with DMSO. The white bigger bars correspond to the position of the section used to obtain the kymograph.

Overall, this might illustrate that Drp1 is differently regulated to drive the division of each organelle and that calcium-mediated Drp1 activation with Ionomycin might not be a stimulus for peroxisomal fission.

6. Discussion

Mitochondria and peroxisomes are two ubiquitous and dynamic organelles (Demarquoy et al., 2015). Mitochondria, long depicted as simple cellular powerhouses, emerged as incredibly essential compartments capable of controlling, among other aspects, cellular survival (Suen et al., 2008). Peroxisomes, which were in turn early recognised for their role in lipid metabolism, continue also to affirm themselves as cellular entities with pleiotropic functions, namely in cellular signalling and anti-viral defence (Dixit et al., 2010; Zhang et al., 2013). Interestingly, and despite presenting clearly distinct features, peroxisomes and mitochondria are more than ever considered as closely related organelles (Schrader et al., 2007).

One remarkable example of such connection lies on their dynamics, specifically in their division events. Indeed, mitochondrial and peroxisomal fission are regulated by the same division machinery (Schrader et al., 2012). Dyn2, one of the most recent proteins to be involved in mitochondrial fission, has been reported to work in tandem with Drp1 (Lee et al., 2015). The aim of this project was to expand this current state of knowledge on Dyn2 and its roles in organelles dynamics. Using a conditional triple knockout model of the dynamin genes, *DNM1/2/3*, we explored in more detail how dynamins influence mitochondrial division and whether this recent two-step fission process proposed for mitochondrial fission is also crucial for peroxisomal fission, thereby exploring further the connection between the two organelles.

6.1 Clathrin-mediated endocytosis and cell division defects in TKOs

The classical dynamins have been intensively studied in CME (Park et al., 2013). As expected, tamoxifen-induced excision of all the dynamin genes impaired CCVs from being formed, as it was evidently shown by the presence of arrested and stalked CCPs at the plasma membrane (Fig. 12A, B, above). Nonetheless, these large GTPases are far from being restrictedly recruited or localized to the invaginating CCPs, being found scattered throughout the cytosol wherein they play additional roles (Henley et al., 1996; Schnitzer et al., 1996). One of such functions concerns the initial identification of dynamin as a microtubule-binding protein (Shpetner et al., 1989). Indeed, we observed

that TKOs showed a defect in cell proliferation. This has been attributed to the capacity of Dyn2 in destabilizing microtubule dynamics during cytokinesis (Tanabe et al., 2009). Despite being clearly a disadvantage of this cell line, TKOs are viable, consistent with the dispensable role of dynamins in cell viability (Park et al., 2013).

6.2 Silencing Drp1, but not dynamins, results in mitochondrial elongation

Dynamins' scission has been expanded beyond endocytosis. Indeed, mitochondrial division, which had been previously attributed to Drp1 fission, is now believed to be a dual and concerted responsibility of Drp1 and Dyn2 (Lee et al., 2015). This is extremely interesting has it sheds lights not only on the multiple functions of this dynamin isoform, but also on the complexity of mitochondrial dynamics. However, in this triple knockout model mitochondrial morphology is not grossly altered from control cells; in terms of numbers, content and interconnectivity of the mitochondrial reticulum, mitochondria from TKOs resemble those of control cells (Fig. 13, above). This suggests that mitochondrial fission can occur independently of Dyn2, as well as Dyn1/3.

Instead, a 60% reduction in Drp1 levels results in a hyperfused mitochondrial network (Fig. 14, above). In fact, Drp1 KD cells had few number and higher content of mitochondria, as it has been previously described (Lee et al., 2015; Song et al., 2015). The unexpected normal mitochondrial morphology of TKOs was not due to changes in mitochondrial fission or fusion proteins, suggesting that depletion of the dynamins is not being indirectly compensated by other dynamic shaping proteins (Fig. 15 and 16, above). Even though we cannot exclude that this apparent Dyn2-independent mitochondrial fission might be a cell line specific effect, these results question the universal role of Dyn2 in mitochondrial division.

Some reports do show that *DNM2* mutations are associated with abnormal mitochondrial morphologies (Cowling et al., 2011; Durieux et al., 2010; Tinelli et al., 2013). For instance, overexpression of a Dyn2 protein with centronuclear myopathy (CNM) disease-associated mutations, a congenital myopathy, was found to globally affect mouse skeletal muscle tissue, being, among the other observed disturbed compartments, mitochondria elongated (Durieux et al., 2010). Nonetheless, an association of mitochondrial fission problems with *DNM2* loss or gain-of-function mutations is of difficult interpretation, since impairing dynamins will inevitably result

in endocytic- and membrane trafficking-related deficits. As a matter of fact, *DNM2* mutations in CNN were recently described to disturb the actin cytoskeleton, which is of great importance for muscle cells, compromising completely muscle cell development and survival (Jamett et al., 2017).

A prolonged inhibition of dynamins might, thus, destabilise the cellular cytoskeleton and endocytic pathways that are vital for the cellular homeostasis, thereby indirectly jeopardizing other cellular processes that impinge on mitochondrial morphology and function. Despite not observed, we cannot rule out that a continuous inhibition of dynamins or blockage on endocytosis might indirectly induce mitochondrial elongation in this cell model. However, after excision of all the dynamin genes, mitochondria did not present a fission problem and this suggests that Drp1 efficiently divides mitochondria independently of Dyn2, or Dyn1 and 3.

6.3 Mitochondrial division occurs independently of dynamins

The ultimate understanding of the potential role of dynamins in mitochondrial fission can be extrapolated by inducing mitochondrial fission. By taking advantage of the fact that intracellular rises of calcium or enhanced calcium accumulation in mitochondria induce Drp1-mediated mitochondrial fragmentation (Hom et al., 2007; I. et al., 2010; Ji et al., 2015), the calcium ionophore, Ionomycin, was used to monitor by live-cell imaging mitochondrial dynamics upon calcium influx. In our results, Ionomycin induced a remarkable fragmentation of the mitochondrial network of both control and TKO cells, in a similar synchronised and time-dependent fashion (Fig. 18, above). This might clearly suggest that mitochondrial division is not blocked in TKOs, a result consistent with our initial morphological studies but that does not correlate with the described Dyn2 involvement in mitochondrial fission (Lee et al., 2015).

Even though the reported Drp1 translocation to mitochondria upon Ionomycin treatment is yet to be recapitulated in our studies, Drp1 activity has been shown to be modulated by intracellular calcium sensors, including the phosphatase calcineurin (Cereghetti et al., 2008). Calcineurin, a two-subunit protein, is constituted by a catalytic calcineurin as subunit A (calcineurin A) and a regulatory calcineurin as subunit B (calcineurin B) (Li et al., 2011). Calcium binding to calcineurin B enables the subsequent calcium/calmodulin-mediated activation of calcineurin A (Jeyaraju et al.,

2009). A target of calcineurin is Drp1, specifically the S637, and, upon increased calcium levels, calcineurin dephosphorylates Drp1 to induce mitochondrial fission (Cereghetti et al., 2008).

Supporting our results, calcium chelation, by using the cell-permeable chelator BAPTA-AM, did not interfere with mitochondrial fission (Fig. 17, above). In fact, BAPTA-AM has been shown to even decrease constrictions of the IMM, a recent phenomenon preceding Drp1 fission that is actually enhanced by Ionomycin, and to inhibit mitochondrial fragmentation induced by Drp1 overexpression (Cho et al., 2017; Huang et al., 2017). Nonetheless, BAPTA-AM has also been reported to induce mitochondrial fission (Friedman et al., 2011). However, as we used a calcium-containing imaging medium, its effects on mitochondrial fission were probably not observed because BAPTA-AM could have reacted extracellularly before buffering the intracellular calcium levels, thereby not interfering with mitochondrial fission. Altogether, our data is consistent with previous results and emphasizes the link between intracellular calcium increase and mitochondrial fission.

Uncouplers of the oxidative phosphorylation also interfere with mitochondrial dynamics. Early studies with CCCP showed that depolarising active mitochondria induces fragmentation of the mitochondrial network by inhibiting Opa1 and by activating Drp1 (Ishihara et al., 2003). Indeed, CCCP enhances Drp1 translocation to mitochondria, whereas expression of a Drp1 inactive variant reduced mitochondrial susceptibility to CCCP-induced fragmentation (Buhlman et al., 2014; Losón et al., 2013). In this study, and despite not having studied exactly the time point in which full mitochondrial fragmentation is observed with CCCP, TKOs were not more protected against this uncoupler (Fig. 19, above). Drp1 KD cells, instead, have been shown to have a delayed fragmentation with CCCP treatment (Otera et al., 2016), thereby suggesting once again that dynamins absence is not impairing Drp1 function and mitochondrial division.

Nevertheless, CCCP-mediated mitochondrial fragmentation is remarkably different from the one observed with Ionomycin treatment. In our case, CCCP also rapidly fragments mitochondria, but rather than dividing, mitochondria were observed to retract, circling into doughnut-like structures (Fig. 19B, above). These doughnuts have been observed with treatments with other uncouplers, such as FCCP, but also in

situations of oxidative stress and osmotic dysregulation (Alavian et al., 2014; Cereghetti et al., 2008; Liu et al., 2011; Menges et al., 2017). Indeed, we also observed the same structures by treating MEFs with H_2O_2 (results not shown).

Interestingly, all these conditions, and Ionomycin as well, have been related to the opening of the PTP (Assaly et al., 2012; Cereghetti et al., 2008; Liu et al., 2011). Indeed, FCCP has been shown to trigger PTP opening, leading to calcium-induced calcineurin activation and, consequently, Drp1-mediated fragmentation, which is prevented by pre-treatment with PTP inhibitor Cyclosporin A (Cereghetti et al., 2008). In general, our studies seem to imply that Drp1 fission does drive mitochondrial fragmentation regardless of the absence of dynamins in our model. Calcium and uncouplers might, however, be mitochondrial fission stimulators that do not require Dyn2.

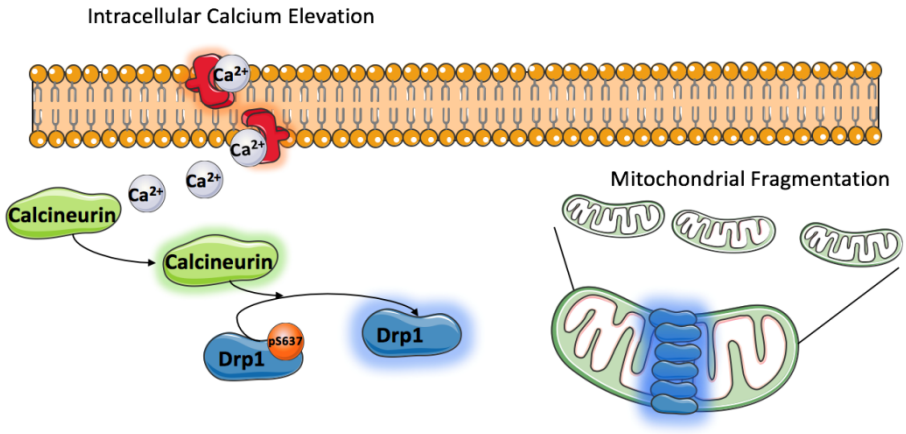


Figure 28. Schematic representation of the possible Ionomycin-induced mitochondrial fragmentation.

Ionomycin works as a calcium ionophore, permeabilizing cells to calcium sources, namely from the extracellular medium. The calcium rise is sensed by calcineurin, a phosphatase that when activated dephosphorylates the S637 residue of Drp1. This phosphorylation is inhibitory and, thus, calcineurin activates Drp1 and the concomitant mitochondrial fragmentation. Image created with Servier Medical Art templates.

Finally, it would be interesting to study whether mitochondria treated with CCCP or Ionomycin for longer periods, and with higher concentrations, are targeted for degradation and/or capable of triggering apoptosis in TKOs, since this could induce mitophagy and/or apoptosis, all of which have been shown to depend on Drp1-dependent fission (Cereghetti et al., 2010; Gomes et al., 2011; Lee et al., 2015).

6.4 Mitochondrial metabolism is altered in triple knockout cells

Mitochondrial homeostasis is influenced by the cellular state. In our study, depletion of all the classical dynamins results in an altered mitochondrial metabolism, since mitochondrial respiration is strikingly downregulated in TKOs (Fig. 20, above). As OXPHOS offers a higher yield of ATP production over glycolysis, this could potentially pinpoint functional problems at the level of the OXPHOS complexes and/or suggest an energy deficit in these cells. However, and has it has been described almost hundred years ago, cells cultured with glucose-containing mediums favour glycolysis, over OXPHOS, for energy conversion (Crabtree, 1929). As a matter of fact, an effect of starvation has been excluded in this triple knockout model, since the ratio of pS637 normalised for the total Drp1 levels is unaltered (Fig. 16, above); pathways associated with starvation, such as AMPK activation and mTOR inhibition, are unchanged, as well (results not shown). Therefore, this decreased mitochondrial respiration does not translate to an energy deficit in TKOs.

On the other hand, the mitochondrial potential of TKOs seems to be intact when compared to control cells, an observation that does not correlate with the observed oxygen consumption rates (Fig. 21, above). Interestingly, when ATP levels are not limiting but the mitochondrial potential is down, the mitochondrial ATPase can work in a reverse mode, by consuming ATP for pumping protons into the IMS, restoring the mitochondrial potential (Suzuki et al., 2003). Such scenario is not excluded here but levels of mitochondrial fusion proteins, such as Opa1, whose processing and degradation depend on mitochondrial potential, were not changed in this model.

It is worth bearing in mind that, given the importance in endocytosis and membrane trafficking for the normal influx of nutrients and ions and for intracellular pathways, including the endosomal-lysosomal system, the absence of dynamins might jeopardise cellular homeostasis and increase the intracellular ROS levels, which could explain the

observed strange nonlocalization of MitoSOX in some TKOs (Fig. 22, above). Further studies will be needed to study this hypothesis.

6.5 Peroxisomal fission does not require the classical dynamins

Peroxisomes are more than ever related to mitochondria. In fact, MacBride and colleagues have recently shown that mitochondrial- and ER-derived vesicles can contribute to peroxisome biogenesis (Sugiura et al., 2017). Even though unclear whether such process is of paramount importance in physiological conditions, peroxisomes and mitochondria resemble each other in many aspects (Demarquoy et al., 2015).

The division of the two organelles, for example, is regulated by a common fission machinery (Schrader, 2006). However, our studies suggest that the recent proposed Drp1/Dyn2 mechanism in mitochondrial fission is not important for these single membrane-bound organelles. Indeed, investigation of the peroxisomal numbers did not reveal any differences in TKOs compared to control cells. Moreover, peroxisomes were not more elongated, being the sphericity index in this cell line not distinct from the counterpart control cells (Fig. 24, above). In turn, inhibiting Drp1 or Pex11, a specific peroxin involved in peroxisomal fission, has been shown to result in fewer, but highly elongated peroxisomes (Erdmann et al., 1995; Koch et al., 2003).

Peroxisomal fission is nothing but simple. However, compared to mitochondria, the single membrane of peroxisomes might be an easier challenge for Drp1 fission, in great part due to Pex11. Firstly, this peroxin remodels the peroxisomal membrane to promote its tubulation - our Pex11β experiments clearly demonstrates such effect on peroxisomal morphology (Fig. 26, above). In a second step, the protruding membrane is thought to become enriched in fission factors, including Pex11, which help to constrict already the membranes for division (Itoyama et al., 2012; Koch, et al., 2004). Finally, Drp1 is recruited and the organelle is asymmetrically split into two smaller compartments (Huybrechts et al., 2009).

Interestingly, Pex11 might also participate in this last step of peroxisomal fission. In fact, a recent study in yeast has suggested that Pex11 does interact directly with Drp1 and that such interaction is important for Pex11 to work not only as a GTPase Activating protein (GAP) for Drp1, but also as a GTPase itself (Williams et al., 2015). Therefore, Pex11 seems to initially create a permissive environment for membrane

fission, remodelling the membrane and recruiting other factors, and later to recruit, activate and even assist Drp1 in the final fission step (Williams et al., 2015; Yoshida et al., 2015). Nevertheless, this peroxin cannot drive peroxisomal fission independently of Drp1 and, therefore, the division of this organelle might be achieved by these two co-workers: Drp1 and Pex11. Overall, this *per se* does not completely rule out a role of dynamins in peroxisomal fission, but in our knockout model such seems to be the case.

6.6 Highly dynamic peroxisomes are slowed down by Ionomycin treatment

In striking contrast to the mitochondrial division, little is known regarding the regulation of peroxisomal fission (Honsho et al., 2016; Schrader et al., 2016). As several peroxisomal proteins are now suggested to be controlled by PTMs, being Pex11β possibly regulated by phosphorylation, this is bound to change in the upcoming years.

When examined by live-cell imaging, some peroxisomes were endowed with fast, bidirectional mobility, but many were also somehow more constricted in their action, which resembles sometimes a Brownian movement. The same Brownian movement has also been reported in plant and mammalian cells (Barton et al., 2013; Wali et al., 2016). Interestingly, whereas treatment with Ionomycin rapidly fragmented the mitochondrial network, peroxisomal fission seems to not be induced. Nonetheless, peroxisomal movement was immediately inhibited (Fig. 27, above). In part, this might confirm a selective and different Drp1 regulation in peroxisome and mitochondrial fission.

In addition, this may also shed light on how peroxisomes might respond to calcium. Indeed, after being described as calcium-containing organelles in 2006 (Raychaudhury et al., 2006), the peroxisomal-calcium connection has not yet been properly explored. However, and despite some contrasting points, peroxisomes do uptake higher calcium levels upon treatments with calcium-releasing drugs (Drago et al., 2008; Lasorsa et al., 2008). Moreover, and as we observed, Ionomycin did not affect peroxisomal size (Lasorsa et al., 2008). Further studies will address whether this calcium inhibition is due to inhibition of calcium motor proteins, as it is the case of the Miro isoforms in mitochondria (MacAskill et al., 2009; Wang et al., 2009).

7. Future perspectives

Mitochondria and peroxisomes are intracellularly connected organelles with tremendous implications in cellular physiology and organism survival. The basic understanding of these two compartments helps to ignite their intracellular and physiological functions. In this study, we decided to study in more detail the potential role of the large GTPases dynamins, intensively linked with endocytic and membrane remodelling events, in mitochondrial and peroxisomal fission, thereby also unravelling further the interplay exhibited by these two organelles.

Dynamins were found to be dispensable for peroxisomal and mitochondrial morphology in basal conditions. Moreover, Drp1-mediated mitochondrial fragmentation was not blocked upon treatment with a calcium-releasing agent or a depolarization signal when all the three dynamins were deleted. In turn, and even though peroxisomal fission was not stimulated with intracellular rises in calcium, we discovered that the movement of these organelles was dramatically affected in this situation.

Besides increasing the experiment trials (obtaining n=3 for all the morphology studies) of the above experiments, it will be important to efficiently stimulate peroxisomal fission, study the impact of dynamins in autophagy processes, namely pexophagy and mitophagy, and how peroxisomal and mitochondrial fission are differently regulated.

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