UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

L'EMPAQUETAGE SÉLECTIF DES PROTÉINES MITOCHONDRIALES DANS DES VÉSICULES EXTRACELLULAIRES EMPÊCHE LA LIBÉRATION DE DAMPS MITOCHONDRIAUX

SELECTIVE PACKAGING OF MITOCHONDRIAL PROTEINS INTO EXTRACELLULAR VESICLES PREVENTS THE RELEASE OF MITOCHONDRIAL DAMPs

THÈSE PRÉSENTÉE
COMME EXIGENCE PARTIELLE DU
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To my family and friends who were my
strength in this journey

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RÉSUMÉ

Les mitochondries sont des organites essentiels de la cellule, impliquées dans diverses fonctions cellulaires, dont la synthèse d'ATP et l'homéostasie du Ca2 +. Par conséquent, il est essentiel d'avoir des mitochondries saines dans les cellules. La plupart des cellules sécrètent de manière constitutive de l'ADN mitochondrial et des protéines mitochondriales dans des vésicules extracellulaires (VE), de petites vésicules qui permettent le transfert de matériel entre les cellules. Il a été proposé que ce transfert mitochondrial joue un rôle dans la communication cellule-cellule, la régénération des organites et le contrôle de la qualité mitochondriale. Le contenu mitochondrial extracellulaire pourrait également participer à la régulation métabolique et à la réponse immunitaire. Cependant, le processus d'inclusion de matériel mitochondrial dans les VE et ses mécanismes de libération n'ont pas encore été identifiés. Par conséquent, le but de mon projet était de définir le contenu mitochondrial qui est incorporé dans les VE, le mécanisme par lequel cela se produit et la relation entre la libération de matériel mitochondrial et l'inflammation.

Dans cette étude, nous avons isolé les VE de diverses lignées cellulaires et analysé leur contenu mitochondrial. Nous avons trouvé un contenu mitochondrial dans les VE dans des conditions non stimulées, suggérant que les cellules sécrètent du matériel mitochondrial en conditions basales. Sur une note intéressante, nous avons trouvé une inclusion sélective de protéines mitochondriales, car certaines des protéines étaient très enrichies par rapport à d'autres. En raison de cette l'inclusion sélective, nous avons proposé que les vésicules dérivées des mitochondries (MDV) pourraient être responsables de la libération de contenu mitochondrial dans les VE. Les MDV sont de petites vésicules dérivées de mitochondries portant des protéines mitochondriales spécifiques. Les protéines SNX9 et Parkin sont impliquées dans la formation de MDV qui servent à la présentation d'antigènes mitochondriaux. Nous avons constaté que le knockdown de SNX9, qui bloque la formation de MDV, bloque la libération de ces protéines mitochondriales dans les VE. Des résultats similaires ont été observés suite à la délétion de la protéine de membrane interne mitochondriale OPA1, confirmant que les MDV jouent un rôle important dans la libération de protéines mitochondriales dans les VE.

L'activation de la protéine Parkin suite à un dommage oxydatif aux mitochondries favorise la formation de MDV, ainsi que leur transport vers les lysosomes pour être dégradés. Cependant, malgré le fait que Parkin stimula la formation de MDV, son expression inhibe la sécrétion de protéines mitochondriales dans les VE. Nos résultats indiquent que cette inhibition est le résultat de la dégradation lysosomale du contenu de ces MDV. Nous pensons donc que les cellules préviennent la sécrétion de matériel mitochondrial endommagé en envoyant ce matériel directement aux lysosomes pour dégradation.

Le contenu mitochondrial cytosolique ou extracellulaire agit comme DAMP pour induire une réponse inflammatoire. Par conséquent, notre objectif était d'étudier le rôle du contenu mitochondrial des VE dans la réponse inflammatoire. Nous avons constaté que les

mitochondries isolées et les VE activent des voies inflammatoires distinctes. De plus, alors qu'un dommage oxydatif aux mitochondries les rend plus pro-inflammatoires, ce n'était pas le cas pour les VE isolées des mêmes cellules. Nos résultats suggèrent donc qu'en régulant le processus d'inclusion de protéines mitochondriales dans les VE, les MDV permettent de protéger les cellules d'une induction inappropriée de la réponse inflammatoire.

Mots clés : vésicules dérivées des mitochondries (MDVs), SNX-9, OPA1, inflammation, contrôle qualité mitochondriale, vésicules extracellulaires

ABSTRACT

Mitochondria are essential cellular organelles involved in various cellular functions, including ATP synthesis and Ca^{2+} homeostasis. Therefore, it is essential to have healthy mitochondria to maintain cell homeostasis. Most cells constitutively secrete mitochondrial DNA and mitochondrial proteins in extracellular vesicles (EVs), small vesicles that allow the transfer of material between cells. This mitochondrial transfer has been proposed to play a role in cell-cell communication, organelle regeneration, and mitochondrial quality control. Extracellular mitochondrial content may also participate in metabolic regulation and the immune response. However, the process of inclusion of mitochondrial material in EVs and its release mechanisms have not yet been identified. Therefore, my project aimed to define the mitochondrial content incorporated into EVs, the mechanism by which this occurs, and the relationship between the release of mitochondrial material and inflammation.

In this study, we isolated EVs from various cell lines and analyzed their mitochondrial content. We found mitochondrial content in EVs under unstimulated conditions, suggesting that cells secrete mitochondrial material under basal conditions. On an interesting note, we found a selective inclusion of mitochondrial proteins as some of the proteins were highly enriched compared to others. Due to this selective inclusion, we proposed that mitochondrial-derived vesicles (MDVs) might be responsible for the release of mitochondrial content in EVs. MDVs are small vesicles derived from mitochondria carrying specific mitochondrial proteins. SNX9 and Parkin proteins are involved in the formation of MDVs, which are used for the presentation of mitochondrial antigens. We have found that SNX9 knockdown inhibits MDV formation and blocks the release of these mitochondrial proteins in EVs. Similar results were seen following the deletion of the mitochondrial inner membrane protein OPA1, confirming that MDVs play an essential role in the release of mitochondrial proteins in EVs.

Activation of the Parkin protein following oxidative damage to mitochondria promotes the formation of MDV and their transport to lysosomes to be degraded. However, despite the fact that Parkin stimulated MDV formation, Parkin overexpression inhibits the secretion of mitochondrial proteins in EVs. Our results indicate that this inhibition is the result of lysosomal degradation of the content of these MDVs. We, therefore, believe that cells prevent the secretion of damaged mitochondrial material by sending this material directly to lysosomes for degradation.

The cytosolic or extracellular mitochondrial content acts as DAMP to induce an inflammatory response. Therefore, our objective was to study the role of mitochondrial content of EVs in the inflammatory response. We found that isolated mitochondria and active EVs have distinct inflammatory pathways. Additionally, while oxidative damage to mitochondria makes them more pro-inflammatory, this was not the case for EVs isolated from the same cells. Our results, therefore, suggest that by regulating the process

of inclusion of mitochondrial proteins in EVs, MDVs protect cells from inappropriate induction of the inflammatory response.

Keywords: Mitochondria-derived vesicles (MDVs), SNX-9, OPA1, Inflammation, Mitochondrial quality control, Extracellular vesicles

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Actyl Co-A	Acetyl-Coenzyme A
ADP	Adenosine diphosphate
Alix	Apoptosis-linked gene 2 interacting protein X
APAF-1	Apoptosis activating factor
ATP	Adenosine triphosphate
cAMP	Cyclic AMP
CARD	Caspase recruitment domain
BM-MSC	Bone marrow-derived mesenchymal stem cells
Ccf-mtDNA	Circulating cell-free circulating mitochondrial DNA
cGAS	Cyclic GMP-AMP synthase
CSF	Cerebro-spinal fluid
DAMPs	Damage-associated molecular patterns
DRP1	Dynamin related protein-1
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ESCRT	Endosomal sorting complexes required for transport
ETC	Electron transport chain
EV	Extracellular vesicles
FADH	Flavin adenine dinucleotide
FIS1	Mitochondrial fission protein-1
GS	Glutamine synthetase (GS)
HIF-1	Hypoxia-inducing factor 1
H_2O_2	Hydrogen Peroxide

HUVEC	Human umbilical vein endothelial cells
IL-6	Interleukin-6
IMM	Inner mitochondrial membrane
L1CAM	L1 cellular adhesion molecule
LPS	Lipopolysaccharide
MAPL	Mitochondrial-anchored protein ligase
MAVS	Mitochondrial antiviral signalling
MDV	Mitochondria-derived vesicles
MEF	Mouse embryonic fibroblast
MFN	Mitofusin
MHC	Major histocompatibility complex
mitAP	Mitochondrial antigen presentation
mROS	Mitochondrial reactive oxygen species
MSC	Mesenchymal stem cells
mtDNA	Mitochondrial DNA
mtHSP70	Mitochondrial heat shock protein-70
MVB	Multi-vesicular bodies
MVs	Micro-vesicles
NADH	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
nDNA	Nuclear DNA
NF-kB	Nuclear factor-kB
NRF	Nuclear respiratory factor
OH	Hydroxyl radical
o-GlcNAc	O-link N-acetylglucosamine
OMM	Outer mitochondrial membrane

OPA1	Optic atrophy protein-1
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PINK1	PTEN-induced putative kinase 1
PMN	Polymorphonuclear
PPRs	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene-I
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SNARE	Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor
SNX9	Sorting nexin-9
SOD2	Superoxide dismutase
STING	Stimulator of interferon gene
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TLR	Toll-like receptor
TME	Tumor microenvironment
TOM20	Translocase Of Outer Mitochondrial Membrane 20
TRAPS	Tumor necrosis factor receptor-associated periodic syndrome
tRNA	Transfer RNA
UPS	Ubiquitin-proteasome system

CHAPTER I

INTRODUCTION

Mitochondria are known as the powerhouse of the cell as they are the primary source of adenosine triphosphate (ATP). ATP is the energy-rich compound that drives cell functions, including muscle contractions, cell division, biosynthesis, anabolic pathways, proteins folding and degradation, and the generation and maintenance of cellular membrane potential[1,2]. Mitochondria are a major metabolic hub as they are involved in the biosynthesis of amino acids (lipids, cholesterol, glucose), cardiolipins, Kreb's cycle, β-oxidation, and many cellular processes such as calcium signalling and apoptosis[3-5].

Mitochondria were once an independent prokaryote that existed around 2 billion years ago. It is believed that this prokaryote was engulfed by a large primitive eukaryotic cell. Over time, mitochondria transferred most of their genetic material to the host cell but still retain some mitochondrial DNA (mtDNA)[6,7]. mtDNA contains 16500 base pairs and is organized into discrete nucleoids in the mitochondrial matrix. mtDNA encodes 37 genes that are essential for mitochondrial function, including components of the electron transport chain (ETC) and the ATP synthase required for ATP generation and ribosomal and transfer RNA needed for protein assembly[8,9]. However, most of the mitochondrial proteins, including ETC proteins, are encoded by nuclear DNA (nDNA)[10]. Mitochondrial translation needs transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs) encoded by mtDNA but also needs translation initiation factors such as MtIF2 and MtIF3, as well as elongation factors encoded by nDNA[11-13]. nDNA encodes essential proteins for mtDNA maintenance, also proteins necessary for transcription.

1.1 Structure of mitochondria

Mitochondria have a double-membrane structure that divides mitochondria into four separate compartments, the outer mitochondrial membrane (OMM), the intermembrane space, the inner mitochondrial membrane (IMM), and the matrix (Figure 1.1). Each compartment performs different roles in mitochondrial function. The OMM has a number of pores called porins, allowing the diffusion of molecules across the membrane and an inter-membrane space. The inter-membrane space, between the OMM and IMM contains proteins such as cytochrome C, which play a role in mitochondrial energetics and apoptosis. The IMM is highly impermeable and has a large proportion (around 20%) of total mitochondrial proteins, including ETC components. The IMM is the place where oxidative phosphorylation takes place through membrane protein complexes. The IMM has infoldings known as cristae, the invagination of the mitochondrial IMM where ATP synthesis takes place[14]. The fourth compartment, the matrix, is enclosed by the IMM and contains enzymes responsible for the Tricarboxylic acid cycle (TCA cycle) lipid catabolism, oxidative phosphorylation, urea cycle and ribosomes. In tissues such as the brain, muscle, heart, which require high energy for their function, the cristae structure is closely stacked and takes up much of the mitochondrial space. On the other hand, in tissues such as the liver or kidney that require low energy, cristae are loosely packed, leaving more room for a matrix with its biosynthetic enzymes[2,15-17].

1.2 Mitochondrial bioenergetics

1.2.1 ATP production

Depending on the energy need of the cell, the number of mitochondria varies. For example, heart muscle cells have a high number of mitochondria as they demand a lot of energy and almost 40% of the cytoplasm of a heart muscle cell is occupied by mitochondria[18]. As mentioned earlier, one of the crucial functions of mitochondria is ATP production which provides energy to cells and tissues. This production process works through the TCA cycle. In the initial stage, pyruvate molecules are produced through glycolysis in the cytosol and this converted pyruvate undergoes oxidation in mitochondria.

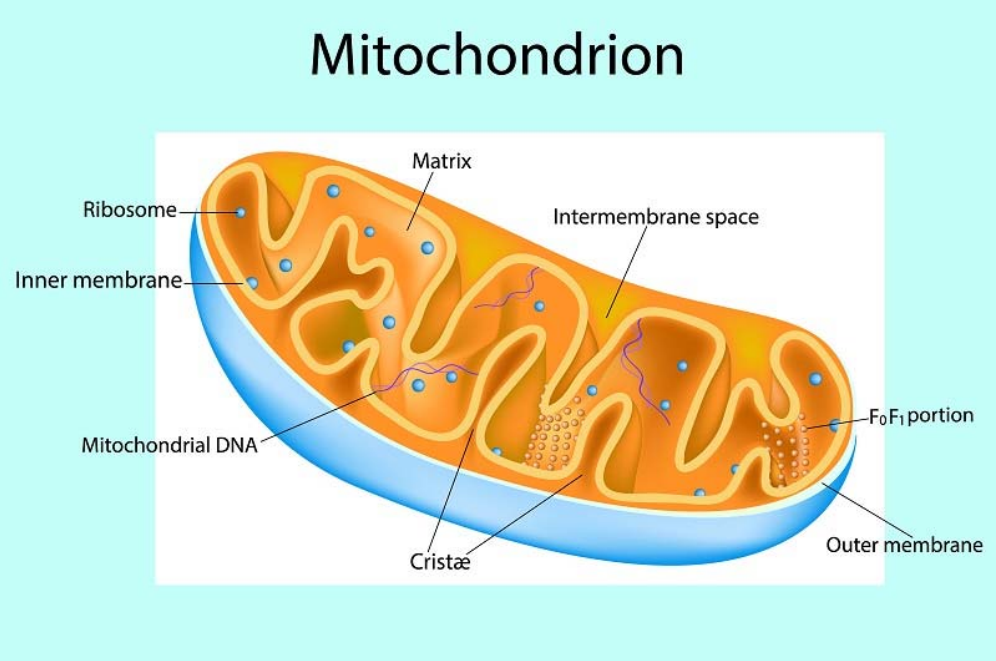

Figure 1.1 : Mitochondrial structure. Mitochondria are made up of two membranes 1) Outer membrane, which covers the organelle, and 2) Inner membrane, which folds over many times and creates layered structures called cristae. The inner membrane contains an electron transport chain surrounded by a mitochondrial matrix where the TCA cycle produces the electrons that travel from one protein complex to the next in the inner membrane. Photo by: Sakurra via Shutterstock.

Pyruvate is converted to acetyl-Coenzyme A (acetyl CoA), which further gets metabolized through the TCA cycle[19-21].

There are four complexes (I-IV) in the ETC, and they are present in multiple copies in the mitochondrial inner membrane. The energy obtained from the TCA cycle in reduced compounds (NADH/FADH2) is used to generate an electrochemical gradient across the IMM. The potential energy of the gradient is used to generate ATP, and the ATP production at the ATP synthase is known as oxidative phosphorylation (OXPHOS) (Figure 1.2).

1.2.2 Electron transport chain

Complex I accepts electrons from the soluble carrier molecule NADH into the respiratory chain and transfers them to ubiquinone in the membrane. The energy released

in the ETC is used for pumping four hydrogen ions across the membrane from the matrix into the intermembrane space, and the hydrogen ion gradient is established[22,23].

Q and Complex II: The connecting compound for the first and second complexes to the third is ubiquinone (Q). Once ubiquinone is reduced (QH_2), it delivers its electrons to complex III in the electron transport chain. Ubiquinone receives the electrons obtained from NADH of complex I and the electrons derived from $FADH_2$ of complex II, including succinate dehydrogenase. This enzyme and $FADH_2$ form a small complex and delivers electrons directly to the ETC[24].

Complex III: Complex III takes electrons from reduced quinolone and passes its electrons to cytochrome c for transport to the fourth complex of proteins and enzymes[25].

Complex IV: The fourth complex of ETC comprises cytochrome proteins c, a, and a_3. The cytochromes assist in reducing oxygen which then picks up two hydrogen ions from the surrounding medium to make water (H_2O). The removal of the hydrogen ions from the system contributes to the ion gradient. The electrochemical gradient promotes chemiosmosis, which initiates the flow of hydrogen across the membrane through the ATP synthase[26].

ATP Synthase: This complex protein acts as a tiny generator, powered by the force of the hydrogen ions diffusing through it. This reflux of hydrogen releases free energy, facilitating the addition of a phosphate to adenosine diphosphate (ADP), forming ATP, using the potential energy of the hydrogen ion gradient[27,28].

1.3 Other mitochondrial functions

In addition to ATP production, mitochondria have many fundamental metabolic functions such as providing metabolites for building macromolecules for cellular functions, apoptosis and ones that assist in differentiation[5,29,30]. In particular, during the TCA cycle, intermediates exit the cycle to be used as a precursor in the biosynthesis

of macromolecules such as nucleotides, citrate, lipids, heme, and iron-sulphur clusters[29,31-33]. These biosynthesis pathways are engaged in stress response mechanisms and are often misregulated in diseases. Lipids are energy-rich nutrients that cells use as fuel for cellular functions; also, lipids can function as building blocks for cellular structures. Mitochondria also act as a hub for amino acid synthesis, including glutamine, glutamate, proline, and aspartate. Glutamine synthetase (GS) converts glutamate and ammonia to make glutamine to be utilized as a nitrogen source for numerous reactions[5,34-36]. Glutamine is essential for the energetic needs of proliferating cells, such as in cancer cells. In addition to cancer, mitochondrial metabolic dysfunction can play a role in several diseases such as obesity, increased blood pressure, type 2 diabetes and pro-inflammatory metabolic disorders[37,38]. Defective cell metabolism is considered to be the main reason behind metabolic syndrome as this is the result of discrepancies between nutrient uptake and their utilization for energy. Mitochondrial dysfunction inhibits insulin signaling[39] as a consequence of reduced fatty acid oxidation[40] and through the generation of mitochondrial ROS (mROS)[41]. Both OXPHOS and fatty acid oxidation are essential for the regulation of anti-inflammatory function[42,43,44]. Defects in fatty acid oxidation and mROS can induce immune responses[42,43,44].

1.4 Mitochondrial reactive oxygen species (mROS)

Another essential function of mitochondria is to generate, sequester, and interconvert ROS, which are the by-product of oxidative metabolism. Overproduction of mitochondrial ROS (mROS) can be detrimental to cells; hence its production and decomposition need to be well-controlled. mROS levels can be modulated in response to hypoxia, nutrient availability, and changes in mitochondrial membrane potential. Increased mROS lead to irreversible damage to cell components and cause cellular death[45-49]. mROS is generated through an electron leak from the electron transport chain. As a by-product of electron transport, a small portion of molecular oxygen (O2) undergoes partial reduction at intermediate steps of the mitochondrial respiratory chain, which results in the production of superoxide radicals.

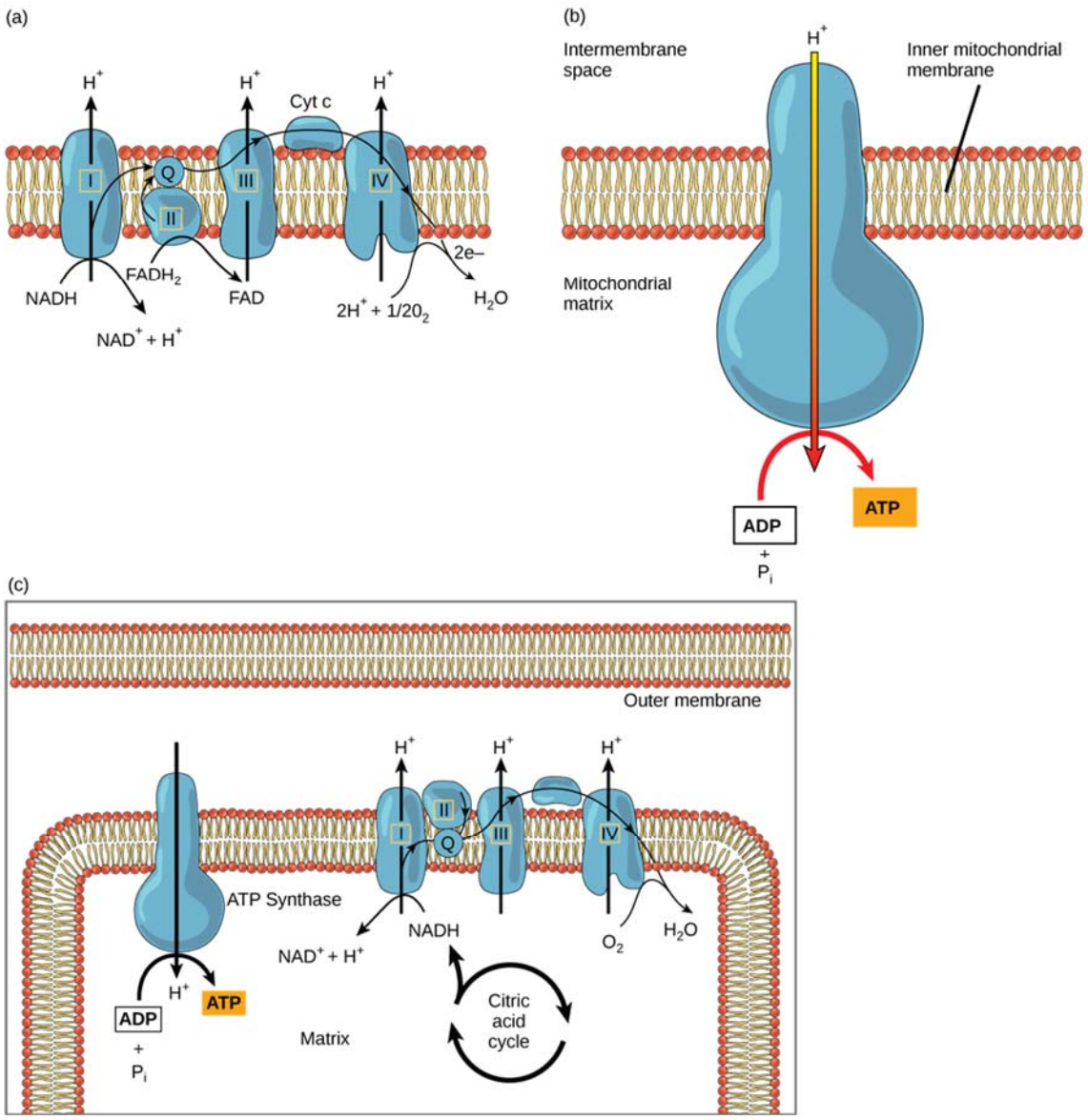

Figure 1.2 : Electron transport chain present in the inner mitochondrial membrane.
A) Complex i-IV is a set of molecules that regulate oxidation-reduction reactions b) ATP synthase is a complex mechanism that used H+ gradient to generate ATP from ADP c) overview of oxidative phosphorylation through mitochondrial complexes I-IV leading to ATP generation. (Book: Concepts of Biology, Samantha Fowler, Rebecca Roush, James Wise, OpenStax, 2013.)

mROS act as mitogen signalling agents at physiological levels that play a role in proliferation, differentiation, and migration of cancer cells[45,48,50-53]. mROS are essential for various physiological activities such as adaptation to hypoxia through hypoxia-inducing factor stabilization[54,55], although a sustained increase in mROS production can be detrimental for the cells. Cells lacking hypoxia-inducing factor-1 (HIF-1) have a constant high level of ROS under chronic hypoxia, leading to cell death[56]. Also, mROS is

involved in the regulation of autophagy. Autophagy is an essential cellular response for cellular metabolic stress such as starvation, ischemia, and pathogen infections[57,58] in which cell removes unnecessary or dysfunctional components. mROS plays dual roles as low levels may induce organelle autophagy to efficiently utilize intracellular resources, whereas in cases when survival is not possible, high levels of mROS will cause autophagic cell death. Cells lacking Atg5, an essential autophagy regulatory protein, accumulate dysfunctional mitochondria, leading to mROS induction[59]. mROS and mitochondrial autophagy (mitophagy) function as a feedback loop mechanism as mROS induces mitophagy, limiting mROS production by maintaining mitochondrial quantity.

Under normal physiological conditions, respiratory chain-generated ROS are detoxified by antioxidant systems and do not cause any mitochondrial or cellular damage. Superoxide dismutase (SOD2) converts superoxide into less reactive H_2O_2 (catalases), which can be degraded into the water by combined activities of peroxiredoxins, glutathione, thioredoxins, and thioredoxin reductase 2[60]. Under the pathological conditions or damaged mitochondrial condition, imbalances in ROS generation or detoxification system can further accumulate mitochondrial and cellular damage, leading to cell death[56]. Overproduction of mROS can cause mitochondrial oxidative damage by inducing mtDNA mutations, altering membrane potential or cell apoptosis, further causing pathologies including autoimmune diseases, cardiomyopathies, rheumatoid arthritis, multiple sclerosis, and atherosclerosis[61-63]. In addition to excessive mROS production, deficiencies in the mROS scavengers SOD2, peroxiredoxin, or thioredoxin can lead to vascular and inflammatory complications[50,60,64,65].

Various reports have suggested a role for mROS in regulating inflammatory signalling and being necessary to establish and maintain immune cell responses[66,67]. The activation of cell surface TLRs increases mROS production[66]. This TLR/mROS pathway is essential in the regulation of many diseases[66]. For example, patients with tumour necrosis factor receptor-associated periodic syndrome (TRAPS) have higher mROS levels, making them more sensitive to lipopolysaccharides (LPS), an endotoxin that initiates immune processes[68]. In addition to increased mROS levels, Atg5-/- cells also

exhibit enhanced RIG-I-like receptors (RLRs), which induce innate immunity[69]. mROS regulate proinflammatory responses through the activation of the transcription factor nuclear factor-kB (NF-kB) and the release of cytokines such as tumour-necrosis factor-α (TNF-α)[70]. NF-kB proteins are essential transcription factors in inflammation and immunity[71,72]. mROS has been shown to contribute to the induction of cytokines by LPS and enhanced responsiveness to LPS in TRAPS[68] and thrombi-induced NF-kB activation via inositol 1,4,5-triphosphate receptor and Ca^{2+} signalling. Inhibition of mROS reduces hypoxia-induced endothelial NF-kB activation and interleukin-6 (IL-6) secretion[70,73,74]. These studies show that mROS play several essential roles at physiological levels, including regulating immune responses but can be detrimental at elevated levels. Hence, it is vital to maintain the physiological levels of mROS in the cells.

1.5 Mitochondrial dynamics

Changes in mitochondrial structure regulate mitochondrial function, including ATP production and the generation of mROS. To perform their functions, mitochondria continually fuse (fusion) and divide (fission), the overall processes termed mitochondrial dynamics. These processes control the morphology (mitochondrial length and branching), allow and regulate content exchange between mitochondria to adapt to metabolic demands of the cell and fuel availability[75,76]. This process also facilitates the release of intermembrane space proteins during apoptosis. In general, mitochondria are depicted as round, bean-like structures; however, they are usually observed as interconnected tubules with connected branching points in living cells. These interconnected structures are critical to the mitochondrial bioenergetic flux of metabolites such as ATP, lipids, proteins, mtDNA, ions throughout the mitochondrial network[77-79]. These mitochondrial dynamics have a significant impact on mitochondrial functions. Over the last decade, several reports have indicated that deregulation of mitochondrial dynamics leads to mitochondrial dysfunction. These alterations affect a broad range of cellular functions leading to the onset of human diseases[80-84,85]. Mitochondrial fusion can be divided into two different sequential processes: first, the fusion of the mitochondrial outer membrane, followed by the fusion of the inner membrane. Outer membrane fusion requires mitofusins (Mfn1 and

Mfn2), whereas inner membrane fusion mainly involves optic atrophy protein-1 (OPA1). Mitochondrial fusion can increase OXPHOS in case of higher energy needs[85-88]. On the other hand, mitochondrial fission results in the division of one mitochondrion into two daughter mitochondria. This process requires the dynamin GTPase dynamin-related protein 1 (DRP1) and its receptors, tail-anchored proteins mitochondrial fission factor (MFF), and mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51). These proteins recruit DRP1 to mitochondria from the cytosol. DRP1 then forms spirals around mitochondria to constrict the membrane at the fission site and split the mitochondrial tubule (Figure 1.3)[89-95]. Endoplasmic reticulum (ER) and mitochondria interaction play an important role in mitochondrial dynamics. ER and mitochondria join together at multiple contact sites to form specific domains known as mitochondria-ER associated membranes (MAM) or mitochondria-ER contacts (MERC). MAM fractions are significantly enriched with proteins involved in mitochondrial fusion and fission[96]. The ER plays an early role in mitochondrial fission by wrapping around mitochondria and initiating its constriction with the help of actin and myosins. MERCs also facilitate DRP1 recruitment to the mitochondrial outer membrane[97,98].

Several essential mitochondrial dynamic proteins, including MFN1/2, OPA1, and DRP1, are the target of post-translational modifications. These modifications are common mechanisms to modify the activity of mitochondrial dynamic regulators. These modifications, such as phosphorylation and ubiquitination, coordinate mitochondrial dynamics with physiological demands[85]. For example, nutrient flux or high glucose mediates O-link N-acetylglucosamine (o-GlcNAc) modification of DRP1, enhancing DRP1-mediated fission activity[99,100]. Similarly, various signalling molecules can modulate mitochondrial functions through retrograde signalling. Cyclic AMP (cAMP) is an intracellular second messenger produced during nutrient stresses such as starvation and nutrient deprivation that mediates various cellular and mitochondrial functions. cAMP signalling enhances mitochondrial fusion by increasing the expression of MFN1[101,102] and through preventing proteolytic processing of OPA1[102]. Besides, the cAMP/ protein kinase A axis phosphorylates DRP1, inhibiting its mitochondrial fission capacity[102,103]. In addition, mROS can serve as a retrograde signal for mitochondrial functional status and

modulate mitochondrial dynamics to maintain mitochondrial quality control. Higher mROS levels can promote hyperfusion of the mitochondrial network, reducing ROS production to protect mitochondria from mitophagy[104]. Increased antioxidant glutathione (GSSG) levels induce disulphide-mediated cis-oligomerization of MFNs to increase mitochondrial fusion activity[105]. Similar to mROS, mitochondrial lipid composition can alter mitochondrial dynamics. However, the function of mitochondrial lipids can vary based on their localization in mitochondria. For example, when predominantly found in the inner membrane, cardiolipin can interact with OPA1 and promote fusion[106-108]. However, when cardiolipin is localized to the outer membrane under apoptotic conditions, it can recruit DRP1 and activate its GTPase activity to induce mitochondrial fission[109].

Mitochondrial fusion promotes the exchange of membranes and content such as mtDNA between mitochondria. It has been shown that mitochondrial fusion removes the detrimental effects of mtDNA mutations and maintains mtDNA and OXPHOS activity[110,111]. Mitochondrial hyperfusion serves different functions, including supporting the high energy demand of cells during starvation and nutrient deprivation and acting as a stress response (UV irradiation, mRNA translation inhibition) mechanism[112]. However, in neurons, hyper-fused long mitochondria are difficult to transport long distances; hence the transport of short mitochondria to nerve terminals is more efficient than hyper-fused mitochondria[113]. Therefore, proper balancing of mitochondrial fusion and fission is more important than the absolute level of either one of them. Emerging evidence suggests crosstalk between fusion and fission processes. For example, DRP1 colocalizes with Mfn2[114] and can interact on the mitochondrial surface, while overexpression of DRP1 in Mfn1/2 deficient cells facilitates mitochondrial fusion[115]. Anand et al. have shown that a short form of OPA1 can trigger mitochondrial fragmentation and colocalize with the fission machinery[116]. Thus, interaction between fusion and fission machineries plays an important role in mitochondrial dynamics and regulates mitochondrial morphology.

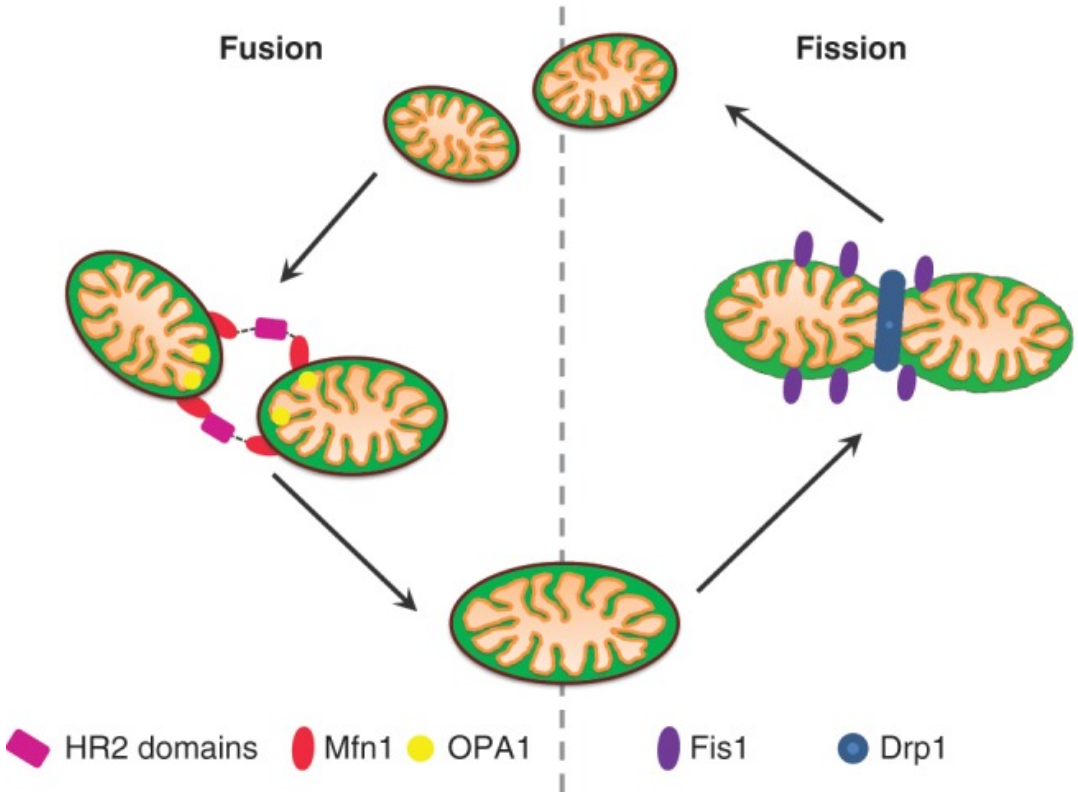

Figure 1.3 : Mitochondrial dynamics.
Mitochondria are dynamic organelles that constantly undergo the fusion and fission process. Molecular machinery involving several proteins such as MFN1/2, OPA1, DRP1 is necessary for these processes to occur. (DOI: 10.1177/1073858411385469.)

1.6 Mitochondrial quality control

Maintenance of healthy and functional mitochondria in response to stress is essential during development and during the entire duration of life. Considering their role in energy production and constant exposure to ROS, mitochondria are vulnerable to mtDNA mutations and protein misfolding. Mitochondria require mitochondrial quality control processes to degrade damaged or misfolded proteins. In addition to ubiquitin-mediated degradation, induction of mitophagy by mitochondrial kinase PINK1/cytosolic E3 ubiquitin ligase parkin removes damaged mitochondria from the cell. Mutations in these genes lead to the early onset of Parkinson's disease (PD)[117-119]. Certain proteins involved in mitochondrial quality control also regulate mitochondrial biogenesis. Examples include mitochondrial transcription factor A (TFAM), nuclear respiratory factor 1 (NRF1) and NRF2, and peroxisome proliferator-activated receptor-gamma coactivator 1α[120].

Considering the important functions of mitochondria, it is not surprising that mitochondrial dysfunction is involved in the onset and/or progression of many diseases such as diabetes mellitus, cancer, and hearing loss[121,122]. Mutations in either mtDNA or nDNA can lead to human mitochondrial diseases, a genetically heterogeneous group of disorders characterized by neuronal and muscular pathologies. mtDNA mutations can occur through point mutations and/or through mtDNA deletions which can occur through mROS-mediated damage or from aberrations in the mtDNA replication process[123]. High levels of mtDNA deletions have been reported in neuronal populations from substantia nigra, hippocampus, striatum, and spinal cord in various neurodegenerative diseases, including Alzheimer's disease (AD) and PD[124-127]. The accumulation of these mtDNA mutations correlated with Lewy body formation, leading to PD[128,129]. In muscular pathologies, loss of mitochondrial fusion protein MSTO1 causes mtDNA depletion and leads to muscular dystrophy[130]. In addition to those diseases, Leigh syndrome and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) are other common diseases caused by mtDNA mutations[131,132]. mtDNA mutations are responsible for almost 20% of cases of Leigh syndrome[131]. Currently, there is no treatment for mitochondrial diseases, and available treatments are directed to relieve symptoms[122]. The diagnosis of the mitochondrial disease remains complicated as some of the mtDNA mutations can give rise to different clinical symptoms[133].

Given the harmful effects of mitochondrial dysfunction, cells must maintain healthy mitochondria. Cells have thus established mechanisms to repair damaged mitochondria (Figure 1.4). Various mitochondrial quality control mechanisms are in place to maintain healthy mitochondria and keep control on the onset of mitochondrial diseases. These mitochondrial quality control mechanisms involve the degradation of damaged mitochondria and mitochondrial dynamics, and mitochondrial homeostasis.

1.6.1 Protease regulation of mitochondrial quality control

Mitochondrial quality control includes the use of antioxidants to detoxify ROS and the use of chaperones, proteases, and the ubiquitin-proteasome system (UPS) to maintain

mitochondrial proteostasis[119,134]. Chaperones and proteases collaborate to fine-tune the proteome of mitochondria. Most of these proteases are members of the AAA+ superfamily and are located in all mitochondrial compartments to regulate the fate of damaged, unfolded proteins[135,136]. Two mitochondrial AAA+ proteases, i-AAA and m-AAA, are anchored to IMM and degrade damaged mitochondrial membrane proteins[137,138 135]. In addition to AAA+ proteases, Lon is a major soluble protease in mitochondria involved in protein degradation and prevention of aggregate formation[139]. Disruption of Lon function has been shown to affect mtDNA integrity[140]. In addition to these proteases, the cytosolic UPS ensures that functional cytosolic quality control of proteins delivered to mitochondria as the majority of the mitochondrial proteins are encoded by the nucleus and translated in the cytosol[141-143].

The UPS system is also involved in outer mitochondrial membrane protein degradation with downstream effects on mitochondrial morphology and death. For example, UPS-mediated degradation of anti-apoptotic protein MCL-1 promotes apoptosis[144] while the degradation of the mitochondrial fusion protein MFN-2 augments mitochondrial fragmentation[145]. Hence, defects in UPS will result in the accumulation of ubiquitinated mitochondrial protein aggregates in both cytosolic and mitochondrial compartments of the cell, highlighting the importance of proper protein trafficking for mitochondrial function and maintenance. It has been shown that excessive uptake of misfolded mitochondrial proteins causes mitochondrial damage and can lead to cell death[101,146].

1.6.2 Mitophagy

Damaged mitochondria can be sent to lysosomal degradation if they cannot be repaired by other mitochondrial quality control mechanisms described above, a process is known as mitochondrial autophagy (mitophagy). Two major pathways are involved in mitophagy; the first one is PTEN-induced putative kinase 1 (PINK1)/Parkin-dependent, and the second functions through receptors on the OMM. In the PINK1/Parkin pathway, PINK1 is constitutively imported to healthy mitochondria, gets cleaved by proteases, and

returns to the cytosol for degradation[147-149]. However, when there is irreversible mitochondrial damage, the affected mitochondrion loses its membrane potential, leading to PINK1 accumulation on the OMM[150,151]. Once accumulated on the OMM, PINK1 recruits the E3 ubiquitin ligase Parkin[150]. This leads to the ubiquitination of several OMM proteins[152], the recruitment of the autophagy machinery, and the sequestration of the entire damaged mitochondrion in an autophagosome which will be subsequently delivered to a lysosome for degradation. The PINK1/Parkin pathway can also interact with the UPS, and UPS-dependent degradation of some mitochondrial proteins is essential for mitophagy initiation. In healthy mitochondria, PINK1 gets degraded by proteases; however, with mitochondrial damage, ubiquitylation of mitochondrial proteins can proceed the autophagic clearance of mitochondria [149,153]. The PINK1/Parkin pathway and the UPS can also compensate mechanisms for each other. For example, chemical inhibition of the UPS (e.g. MG132) upregulates autophagic activity[154], while impairment of autophagy activates the UPS[155].

Another form of mitophagy is receptor-mediated mitophagy, where specific mitochondrial proteins act as receptors in sensing damaged mitochondria, targeting them to autophagosomes for subsequent lysosomal degradation[157]. Several mitophagy receptors have been identified in mammalian cells, including NIX/BNIP3, FUNDC1 and cardiolipins. FUNDC1 interacts with microtubule-associated proteins 1A/1B light chain 3A (LC3) and mediates hypoxia-induced mitophagy[158,159]. NIX/BINP3 is localized on the outer mitochondrial membrane, which harbours LC3 interacting receptors and mediates damaged mitochondrial clearance in reticulocytes[160,161]. Cardiolipin, a membrane lipid in the IMM gets transported to OMM upon external toxic signals. Cardiolipin on OMM interacts with LC3 to initiate autophagy process[162].

As describes above, whole mitochondria get degraded during mitophagy. However, some organs (heart, brain) can not afford to lose mitochondria as they are very much dependent on their mitochondria as their primary energy source. Hence, other mitochondrial Quality control pathways can target damaged mitochondrial content to the lysosome for degradation while leaving intact the rest of the unaffected mitochondrion.

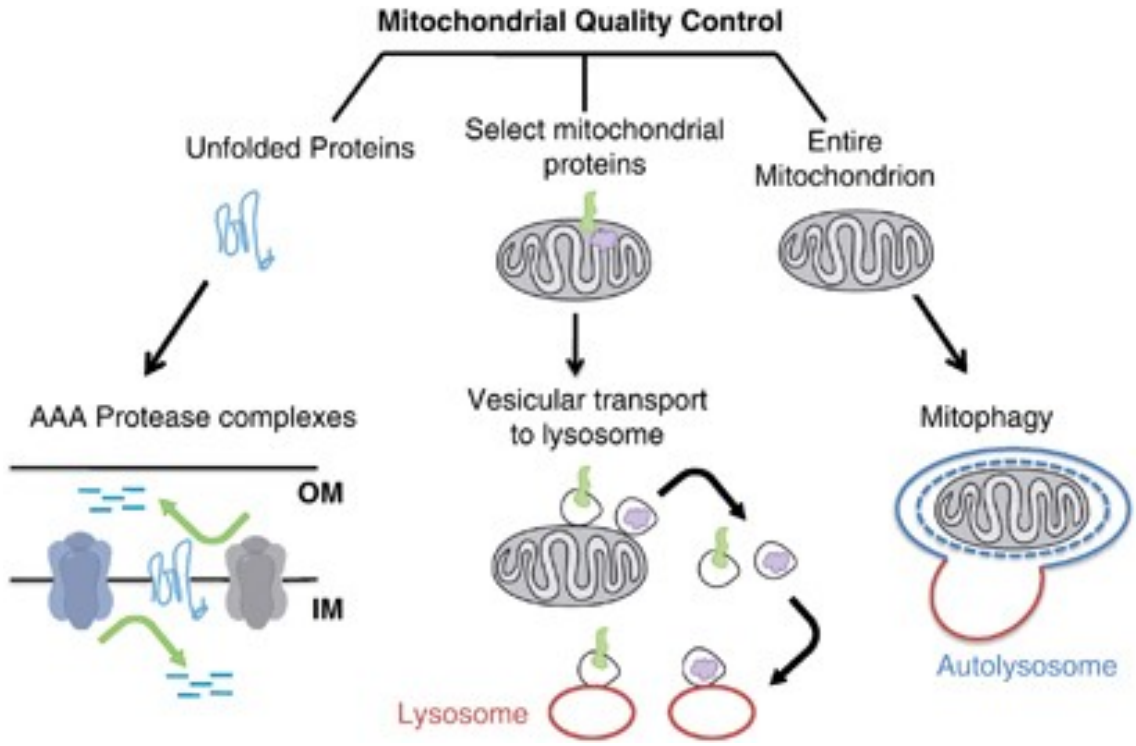

Figure 1.4 : Major pathways of mitochondrial quality control.
Depending on the mitochondrial damage,1) misfolded mitochondrial proteins can be degraded by AAA protease complexes 2) Vesicles containing mitochondrial content will be transported to the lysosome for degradation 3) Through mitophagy, where whole mitochondria get transported to the lysosome for degradation[156].

1.6.3 Mitochondria-derived vesicles

Recent studies have shown that another form of mitochondrial quality control mechanism can deliver damaged parts of a mitochondrion to lysosomes for degradation through small vesicles termed mitochondria-derived vesicles (MDVs). MDVs are approximately 70-150 nm in diameter, and they can be either single-membrane vesicles containing outer-membrane proteins or double-membrane vesicles containing mitochondrial matrix proteins (Figure 1.5)[163]. MDV formation comprises the incorporation of mitochondrial components from various mitochondrial sub-compartments into small vesicles. These vesicles are transported to peroxisomes[164], to the lysosome for degradation[163,165,166] or to endosome for antigen presentation[167]. One of the known characteristics of MDVs is they contain selective mitochondrial content from either the mitochondrial matrix or the OMM[163].

Mitophagy-associated proteins PINK1/Parkin can regulate mitochondrial clearance depending on the intensity of mitochondrial damage. If mitochondrial damage is within limits to repair, it will drive the damaged portion of mitochondrial content into specific MDVs targeted to the lysosome for degradation. However, if the damage is beyond repair, PINK1/Parkin will drive the whole mitochondrial to the lysosome for degradation (mitophagy). Initial studies showed that MDVs which are positive for the mitochondrial-anchored protein ligase (MAPL) get transported to peroxisomes, whereas a second MDV population (containing the mitochondrial matrix protein pyruvate dehydrogenase (PDH)) transports damaged oxidized cargo to lysosomes for degradation[164,168-170]. PINK1/parkin mediates the formation of PDH E2/E3 -containing MDVs and their transport to the lysosome. However, PINK1/parkin does not play a role in the formation of MDVs containing the mitochondrial outer membrane protein TOM20. McLelland et al. have shown that Parkinson's disease-associated point mutations in parkin (C431F, K211N) impair MDV formation[169]. This impairment in MDV formation might affect the degradation of damaged mitochondrial cargo and may be harmful for familial PD patients with these point mutations[169]. These studies show that PINK1/Parkin plays a role in the formation of MDVs involved in the clearance of oxidative stress and mitochondrial antigen presentation (mitAP). However, the physiological and functional role of peroxisome targeted MDVs is still unknown. It is plausible that these MDVs are involved in peroxisome biogenesis. Recently, it has been shown that in human patient fibroblasts lacking peroxisomes, MDVs containing mitochondrial E3 ubiquitin-protein ligase (MUL)-1 fuse with ER-derived pre-peroxisomes, which subsequently mature into fully functional peroxisomes[171].

Along with the transport of mitochondrial content to either lysosome or peroxisomes, MDVs also play a role in immune system regulation. Recently, Matheoud *et al.* have shown that MDVs can play an important role in the regulation of autoimmunity through mitochondrial antigen presentation[167]. Antigen presentation is essential in both establishing immune tolerance and limiting autoimmunity. The presence of sorting nexin 9 (Snx9) and Rab9 is required to form antigen-presenting MDVs, and their recruitment to mitochondria is inhibited by Parkin[167]. These MDVs contain high levels of

mitochondrial antigen, which are subsequently presented in both macrophages and dendritic cells on major histocompatibility factor (MHC) class I molecules to initiate an immune response. However, these mito-globulin containing antigen-presenting MDVs can be implicated in autoimmune diseases. Defects in PINK1 and Parkin can lead to aberrant activation of mitAP containing OGDH, triggering autoimmune diseases in Parkinson's disease[167]. Selective inclusion of mitochondrial proteins in MDVs suggests that MDVs play a diverse role in addition to transporting mitochondrial proteins for degradation. However, limited information is available about this, and further studies need to be conducted to address various plausible roles of MDVs[167].

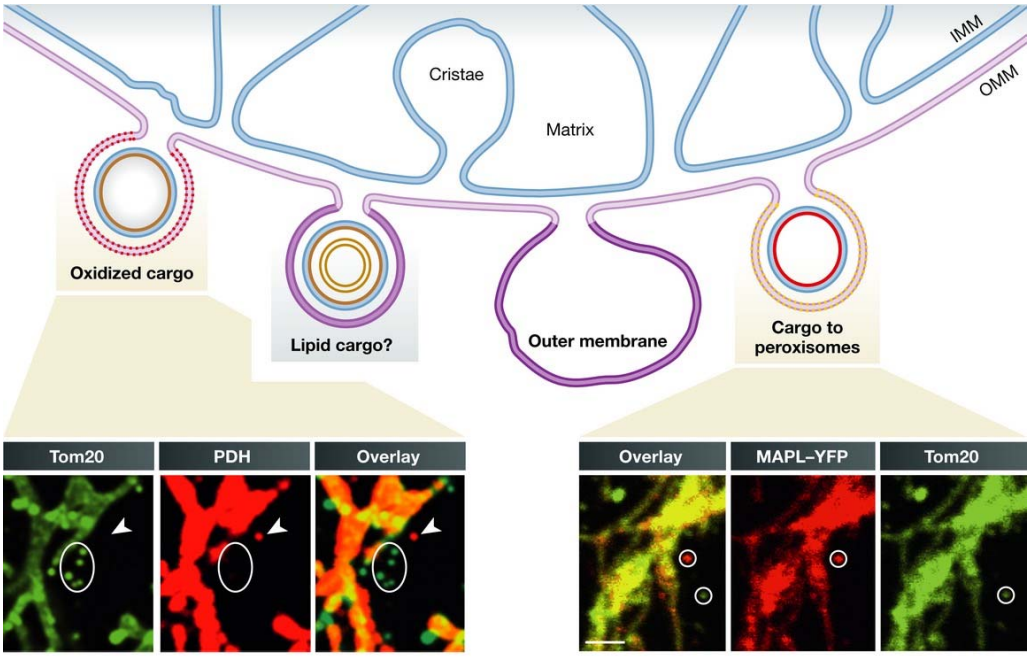

Figure 1.5 : Mitochondria-derived vesicles. MDVs are small vesicles carrying either outer mitochondrial membrane, inner membrane, or matrix proteins directed towards peroxisomes and lysosomes. The destination is based on physiological conditions and MDV content[163].

1.7 Extracellular Vesicles

In the last 20 years, research advancement in the field of intercellular communication that led to the discovery of new cell communication mechanisms and functional roles. We now know that cells exchange signals through metabolic or organelle transfer or modulate the functions of recipient cells. Extracellular vesicles (EVs) are small

vesicles released by almost all cell types and contain DNA, mRNA, miRNA, protein (including mitochondrial proteins), and lipids[172-175]. However, the composition of EVs can vary depending on cell type and the physiological status of the cell. For example, the composition of EV cargo can be different in oxidatively stressed cells or diseased conditions such as sepsis or malignancy[176-180]. Nevertheless, the precise mechanisms that govern EVs formation, cargo loading, trafficking, and release in extracellular space remain incompletely understood. EVs can be distinguished based on their surface markers, size distribution, and origin. Based on these factors, EVs are usually classified into two different categories, exosome and microvesicles[179].

1.7.1 Exosomes

Exosomes sizes range from 10-120 μm and contain a double membrane structure[181,182]. In the early 1990s, studies revealed that exosomes might be involved in intercellular communication. Initial studies showed the release of EVs from blood reticulocytes[183,184]. Since then, many reports have shown that different cell types secrete exosomes *in vitro,* including stem cells[185-187], epithelial cells[188], neurons[189], Schwann cells[190], and tumour cells[191]. Exosomes are involved in various physiological processes, such as removing excess/unnecessary constituents from cells to maintain cellular homeostasis[192,193]. They can also carry functional, targeted, mechanism-driven cellular material, which plays a role in intercellular communication[193-195].

Exosomes are generated by the fusion of multi-vesicular bodies (MVB) with the plasma membrane, releasing its content (intraluminal vesicles) into the extracellular space in the form of exosomes. MVBs are a late endosome/intermediate compartment that contains intraluminal vesicles. The intraluminal vesicles carry proteins from early endosomal and cytoplasmic content. Most of these MVBs fuse with a lysosome for degradation, but a certain number of MVBs fuse with the plasma membrane to release their content in the extracellular milieu in the form of exosomes (Figure 1.6). Various protein complexes, TSG101, ALIX (apoptosis-linked gene 2 interacting protein X), ESCRT (endosomal sorting complexes required for transport), SNARE proteins

(Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor), are involved in the origin and biogenesis of exosomes[181,182,192,195-197].

1.7.2 Microvesicles

Microvesicles constitute the second type of extracellular vesicles. In contrast to exosomes, where an MVB fuses with the plasma membrane to release its content, microvesicles are generated directly through the outward budding of the plasma membrane. Microvesicles are much bigger than exosomes, ranging from 120-1000 nm, and can carry larger organelles such as the whole mitochondria[182,198,199]. The function of microvesicles depends on the cargo they have and also on cell status. For example, neutrophilic granulocytes, polymorphonuclear neutrophils (PMN) derived microvesicles contain antibacterial proteins such as PMN granule proteins contributing to the immune response[200]. Tumour microvesicles are involved in the horizontal transfer of oncogenic growth factor receptors such as epidermal growth factor receptor variant III (EGFRvIII) from glioblastoma to naïve non-aggressive cells[201]. The limiting membrane of the microvesicle contains surface receptors and transmembrane proteins, allowing the identification and targeting of recipient cells by microvesicles[181,202-204]. Until now, it is still unclear how the protein selection process works and how cargo is targeted to microvesicles. Because of these limitations, it remains difficult to know the exact roles of microvesicles in physiological conditions.

1.7.3 EV regulation

All cell types secrete EVs under physiological conditions. However, high levels of circulating EVs can be observed in pathological conditions such as in patients suffering from cardiovascular diseases and obesity, type 2 diabetes mellitus, insulin resistance, or atherosclerosis[202,205]. EVs are associated with the onset and/or progression of many diseases. EV production and shedding are different between noncancerous and cancerous breast epithelial cells. Some cancerous breast cell lines were shown to shed a lower number of EVs compared to noncancerous tissues. However, other cancer cells secrete

more EVs compared to normal cells[206,207]. These differences can be associated with cell culture conditions, pathological conditions, stimulus, and the isolation method. EVs can transmit signals through endocrine pathways, although some EVs can only be transferred to recipient cells in a paracrine way as they can not get into the circulatory system. For example, EVs released from astrocytes get transported to nearby neurons, while in cardiomyocytes, EVs transport functional mitochondria to neighbouring cells. On the other hand, EVs released from platelets can get transported to long distances as they enter into blood circulation[208].

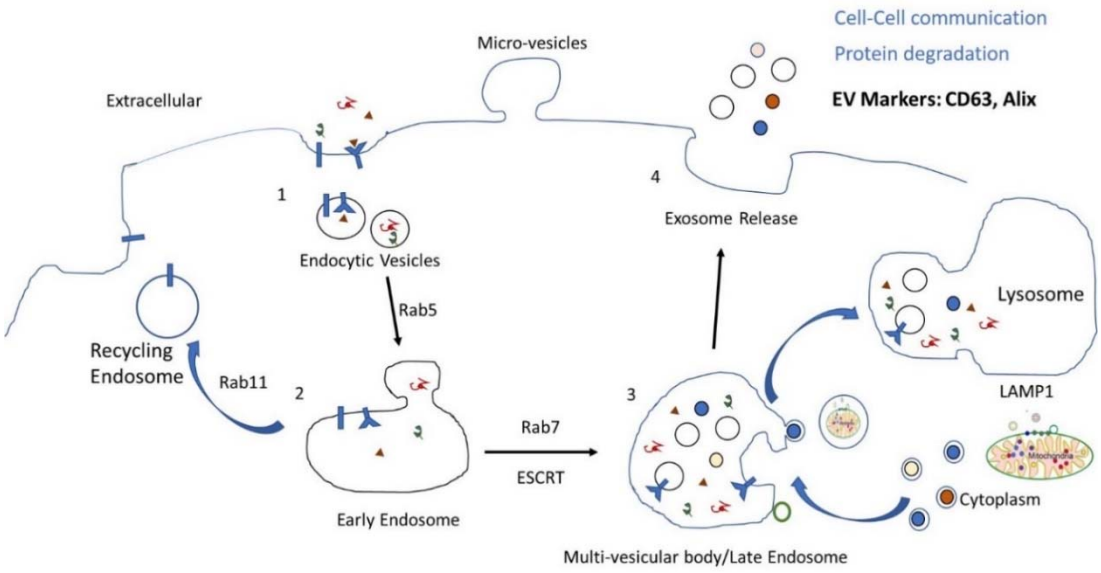

Figure 1.6 : Classical endocytic pathway. 1) transmembrane proteins get endocytosed inside the cells 2) After early sorting, some of the content goes to recycling endosomes and gets recycled with the help of Rab11 3) early endosome gets mature to form late endosome/MVB through sorting process 4) MVB can be transported to the lysosome for degradation or fuse with the plasma membrane and release the content in the form of exosomes.

1.7.4 EV fusion with target cells

By enclosing EV cargoes inside a lipid bilayer, EVs protect them from degradative proteases and RNAses so they can be transferred to cells situated at either short or long distances. The binding of EVs to an external surface of recipient cells can be mediated through membrane receptors on the plasma membrane of recipient cells which recognizes proteins, glycoconjugates, or lipids in the EV membrane or associated with EV

membranes[209-214]. Transfer of EVs from donor cells to recipient cells occurs through endocytic uptake or through direct fusion with the plasma membrane. In the first case, EVs are taken up by cells through an endocytic pathways such as clathrin-mediated endocytosis, caveolin-mediated uptake, phagocytosis, or micropinocytosis[215,216]. On the other hand, the fusion between an EV and the plasma membrane requires various processes such as lipid reorganization, protein restructuring, and membrane dimpling[217-219]. For example, EVs released from leukemia cells were efficiently taken up by macrophages but not from other cells and required the activation of PI3K to facilitate membrane insertion into forming phagosome[218,220]. The mechanisms for EV uptake may depend on proteins and glycoproteins present on the EV surface and the target cell surface. Hence, EV uptake is most likely dependent on EV composition, their cell of origin, and the recipient cells status.

1.8 Functional roles of EVs transfer between cells

One of the important functional roles of EVs is to cause molecular alterations in recipient cells upon uptake. The intercellular communication via EVs highlights the importance of functional cargoes transferred between cells in physiological and pathological processes. Tumor-derived EVs may promote the transfer of receptors, active proteins, or genetic information between tumour and stromal cells. For example, tumor-derived EVs enhance tumour invasion by delivering interleukin-8 and chemokines[221] or matrix metalloproteases[222] to neighbouring cells[223]. Also, tumour-derived EVs transfer genetic information (mRNA, oncogenes) to neighbouring cells, inducing cell migration and angiogenesis[224]. In the brain, cross-talk between glia and neurons is crucial for various biological functions, including brain development, homeostasis maintenance. Microglia-derived EVs regulate synaptic transmission by producing ceramide and sphingosine, which positively affect excitatory neurotransmission[225]. Also, glia-derived EVs play a role in inflammatory responses as ATP-induced microglial EVs contain IL-1β, promoting the regulation of neuroinflammatory responses in the brain[226]. Similarly, NSC-34 motor neuron-derived EVs have been shown to transfer inflammatory-related microRNA to neighbouring cells[227].

Along with functional changes in recipient cells, EVs can transfer material that is destined for degradation to neighbouring cells. This functional role is particularly essential in donor cells that have lysosomal defects. Internalized EV content can also be targeted to lysosomes for degradation. Joshi *et al.* have shown that internalized GFP-CD63 tagged EVs were transported to lysosomes[211]. Similarly, oligodendrocyte-derived EVs are transferred to microglia and transported to lysosomes for degradation[193,228].

1.8.1 EVs as diagnostic tools

According to the NIH Biomarkers Definitions working group, biomarkers are a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention"[229]. As the definition suggests, a biomarker can be used as a diagnostic tool for identifying a disease or abnormal condition. It can help clinicians know the disease status and make a reliable diagnosis and assist in assessing clinical trials progress and evaluating responses in the trials. Hence, EVs could be used as biomarkers for detecting diseases as most cell types secrete them and they can be isolated from urine[230,231], plasma[232], semen[233], cerebrospinal fluid[234]. According to biomarker type and disease, biofluid should be selected to collect EVs depending on biofluid's density, sensitivity, and accessibility. For example, the serum-derived EVs from 7 out of 25 glioblastoma patients contained EGFRvIII, suggesting that glioblastoma-derived EVs containing EGFRvIII could be used as a metastasis biomarker[201,224]. In biological fluids, the vast majority of EVs originate from healthy cells; hence, it is essential to distinguish and monitor changes in EV composition between healthy and disease samples to identify differences according to cellular status. Taylor and colleagues have shown that the sensitivity of the analysis can be higher by isolating EVs using antibody-based capturing (anti-epithelial cell adhesion molecule (EPCAM)) and subsequently performing miRNA quantification[235,236]. A similar approach was used to detect α-synuclein levels in healthy versus PD patients. α-synuclein levels in plasma EVs positive for the L1 cellular adhesion molecule (L1CAM) were analyzed to distinguish between healthy and PD patients. L1CAM-positive EVs from PD patients showed increased levels of α-synuclein, suggesting that L1CAM-positive EVs

can be used to assess the severity of PD[237]. However, there are still various limiting factors in using EVs as biomarkers. The first one is a lack of standardized purification protocols, which hampers reproducibility and biomarker identification. In addition to purification protocols, sample collection procedure, processing time, and sample storage conditions can affect the outcome. Hence, specific guidelines have to be defined for using EVs as biomarkers as we still don't have a standardized protocol for EV isolation.

1.8.2 Roles of EVs in disease

1.8.2.1 Neurodegenerative diseases

EVs functional responses depend on the content inside EVs, physiological condition of donor cells and recipient cells. EVs can be generated by various cell types in the brain, including neurons, astrocytes, and glial cells[193,194,238-240]. Central-nervous system (CNS)-derived EVs can be found in cerebrospinal fluids (CSF) and serum[232,234,241]. In the CNS, interaction between neurons and glia is essential for brain development and neuronal circuit maturation. Neuronal interaction with glial cells is in part mediated by EVs transferring proteins, mRNAs, and miRNA between the two cell types[193,194]. For example, EVs derived from mouse primary cortical neurons containing miR-124a have been shown to be taken up by astrocytes, resulting in the upregulation of excitatory amino acid transporter 2, the mediator of glutamate uptake in the brain[242]. A second example involves oligodendrocytes, the cells that produce myelin sheath, which increases impulse conduction. EVs released from oligodendrocytes include myelin proteins such as proteolipid protein, 2',3'-Cyclic nucleotide 3'-phosphodiesterase, Myelin-associated glycoprotein, tetraspanins, and heat shock proteins[243]. These EVs were shown to be taken up by a subset of MHC class II negative microglia and subsequently degraded[228], suggesting that they negatively regulate myelin synthesis in an autocrine fashion[244].

EVs might be a key component in neuronal communication under physiological conditions, including neuronal development, immune surveillance, and pathogen response. However, in pathological conditions, EVs can transfer disease-associated nucleic acids and proteins from affected neurons to normal neurons to alter their

physiological function. One of the putative roles of the neuronal stress response is to alert the immune system via activation of heat-shock protein (Hsp) through exosome release of Hsp70 from astrocytes[245]. Also, ATP-stimulated microglia induce EV release through $P2X_7$ receptor activation, which is important for antigen presentation[226]. In the case of neurodegenerative diseases, EVs can transfer aggregate-prone proteins that can lead to the progression of diseases. For example, one of the hallmarks of PD is the presence of Lewy bodies (LBs), mainly composed of misfolded filamentous α-synuclein[246]. EVs can transport oligomeric α-synuclein, a toxic form of α-synuclein, to neighbouring neurons, which is more easily taken up than free α-synuclein by neuron and cause aggregation[247-250]. In another neurodegenerative disease, AD, β-amyloid (Aβ) plaque accumulation causes impaired neuronal signalling and neuronal death[251]. Aβ is a c-terminal cleavage product of the transmembrane protein amyloid precursor protein (APP). EVs isolated from neuronal cell lines with AD mutation and AD patients showed a significant increase in soluble Aβ and soluble APP levels[252-257]. The development of disease-modifying treatments for neurodegenerative diseases such as PD and AD is blocked by a lack of specific biomarkers of disease status and its progression. These roles of EVs in the advancement of neurodegenerative diseases suggest that neuron-derived EVs can be used as biomarkers of disease progression and evaluate treatment response and prognosis.

1.8.2.2 Cancer

A tumour microenvironment (TME) is an interactive cellular environment surrounding the tumour which contributes to tumour progression, invasion, and metastasis. Tumour cells have been shown to modulate the tumour microenvironment[258]. Tumour-released EVs contain oncoproteins, signalling molecules, and RNAs and can get transferred to the TME to enhance tumour progression[201,224,259]. Human lung or colorectal cancer-derived EVs carry oncoproteins (EGFRvIII) and transfer them to cultured endothelial cells and promote tumour invasion. EVs secreted from tumour cells have self-promoting effects, such as EVs isolated from glioblastoma and multiple myeloma have been shown to enhance cultured cell proliferation. Additionally, it has been shown

that these EVs suppress immune cell activation and prepare other organs and tissues for metastasis[133,260,261]. For example, colorectal cancer derived-EVs transfer miR-21 to liver tissue and drive liver macrophage polarization towards an interleukin-6 secreting pro-inflammatory phenotype and induce liver metastasis[262]. Because of EV's nature and their role in tumour progression, they are considered a promising source of biomarkers and possibly non-invasive or minimally invasive diagnostic and/or therapeutic targets as removal of tumour circulating EVs is proposed to inhibit tumour growth[263].

1.9 EVs isolation

As mentioned earlier, EVs are a heterogeneous group of vesicles distinguished based on size and shape. EVs have been found in all biological fluids of the body, including blood, urine, saliva, semen, bile, and cerebrospinal fluid. EVs can also be isolated from cells in culture [179, 264-266].

Various techniques have been used to isolate EVs for analysis purposes. The traditional methods (ultracentrifugation, microfiltration, and gel filtration) are based on EV properties such as size and buoyant density, whereas techniques such as precipitation with polyethylene glycol or sodium acetate were based on its solubility and/or aggregation properties[267]. Ultracentrifugation is the quickest and classical robust method. At the first stage, samples are centrifuged at low speed to collect dead cells and/or apoptotic bodies and then processed at high speed to collect small-size EVs. There are advantages and disadvantages of ultracentrifugation methods. Disadvantages include longer EV collection duration as repeated centrifugation is needed to reduce non-EV particle contamination. Ultracentrifugation also results in lower yield that hinders its application in clinical studies. However, there are certainly advantages of using this technique, such as it is a very cheap and straightforward technique to perform and can be used to isolate EVs from large sample volumes. Several studies have also suggested using a sucrose gradient combined with ultracentrifugation to improve the purity of collected EVs[268]. Besides density and size-based EV isolation techniques, affinity-based isolation is also widely accepted. This technique is based on the interaction between highly

selective and specific proteins found on EVs and their corresponding ligands. These ligands, such as antibodies, are conjugated with a variety of solid media (e.g. magnetic beads and monolithic columns). The most commonly used ligand antibodies are tetraspanin proteins such as CD9 or CD63, which are abundantly present on EV surfaces. For example, most cancer EVs overexpress EpCAM; hence EpCAM conjugated magnetic beads are used to isolate tumour-derived EVs. This technique has various advantages, including fast process, high specificity, and selectivity. The drawbacks of this technique are expensive antibody ligands, lower yield as it isolates selective EVs. To analyze and characterize isolated EVs, dynamic light scatters measurement, flow cytometric analysis, micro spectrometry, and electron microscopy can be used[269]. Other imaging techniques can be implemented for EV analysis, including micronuclear magnetic resonance, x-ray scattering[270].

1.10 Intercellular Mitochondrial Transfer

Along with different organelles and cellular content, whole mitochondrion, or mitochondrial content, including mtDNA, can be found in EVs. Extracellular mitochondria can have effects on cellular activities of short or long-distance cells[204,271-274]. Recent studies have shown that functional mitochondria can be transferred to cells with damaged mitochondria. These distantly transported mitochondria can be taken up by a cell to repair its damaged mitochondria, or to degrade the endocytosed mitochondria, inducing distant mitophagy. These processes assist in maintaining healthy and functional mitochondria and play a role in mitochondrial quality control. In intercellular mitochondrial, whole mitochondria or mtDNA can be transported from one cell to another[204,271,275]. Inter-cellular mitochondrial transfer can take place through various ways, including EVs, nanotubes between neighbouring cells or through free mitochondrial content circulating in body fluids[272,275-278]. While here, I focus on mitochondrial release from cells as the theme of my thesis; it is part of a larger emerging relationship between mitochondria and the endosomal compartment, which is discussed in the review I wrote in the journal Mitochondrion and have attached to this thesis as an appendix.

1.11 Mechanisms of mitochondrial transfer

1.11.1 Extracellular vesicles (EVs)

A number of studies have shown that EVs can carry mitochondrial content, including mtDNA and mitochondrial proteins[273,279]. Some EVs are big enough to carry whole mitochondria, while others might carry specific mitochondrial components[203,215,274,280,281]. However, the exact nature of mitochondrial EV content and the mechanism for mtDNA or mitochondrial inclusion in EVs are still poorly understood. Also, the mechanism through which EVs deliver mitochondrial content to distant cells and their role remains unclear.

One of the most studied cell types for mitochondrial transfer is mesenchymal stem cells (MSCs). MSCs have been shown to transfer functional mitochondria in co-cultures with cells with dysfunctional mitochondria and with stem-cell therapy in animal models, and the therapeutic effects of MSCs depend on their capacity to secrete EVs. Astrocytes and MSCs can secrete larger EVs, which can carry mitochondria and get transported by paracrine signalling to cells such as epithelial cells, neurons, or immune cells[282,283]. This transfer of mitochondria or mtDNA has been seen to repair cellular damage and regenerate ATP production and overall bioenergetics[282]. For example, in a transient focal cerebral ischemia mouse model, astrocytes released EV-bound mitochondria were taken up by neurons as a protective mechanism[283]. In addition, damaged mitochondria were transferred from the optic nerve head of retinal ganglion cell axons to nearby astrocytes to undergo lysosomal degradation[279,283]. Thus, the release and transfer of mitochondria through EVs play an important role in cell maintenance and repair. Astrocytes have been shown to release EVs containing functional mitochondria and transported to stroke-affected neurons through a calcium-dependent mechanism involving CD38/cyclic ADP-ribose signalling to promote neuro recovery[283]. It has also been shown that MSCs can transfer functional mitochondria to damaged cells to recover from functional loss and stress[284]. In human bone marrow-derived MSC (BM-MSC), damaged mitochondria get exported through EVs and captured by neighbouring macrophages to recycle them to

increase bioenergetics[284]. However, we have limited information available about how mitochondrial cargo selection for EVs works to introduce communication between cells.

1.11.2 Nanotubes

A second mechanism for mitochondrial transfer between donor and recipient cells is through the formation of nanotubes between cells. Nanotubes are filopodial protrusions originating from donor cells that get extended to make contact with the recipient cell membrane and transfer cargo to the recipient cell's cytosol. Liu et al. used human MSCs (hMSCs) with human umbilical vein endothelial cells (HUVECs) in an *in-vitro* ischemia-reperfusion injury model[285] and found an occurrence of mitochondrial transfer from hMSCs to HUVEC cells through nanotubes and rescued aerobic respiration in HUVECs. This nanotube formation between hMSCs and HUVECs was inhibited by inhibiting F-actin polymerization and protecting phosphatidyl serines on apoptotic HUVECs[285]. One of the interesting things that Liu *et al.* discovered was that this mitochondrial transfer was mostly uni-directional between healthy hMSCs and HUVECs; this transfer only occurred from hMSCs to HUVEC following injury[285]. This study suggests that in physiological conditions, cells can have a bi-directional mitochondrial exchange. However, when there is an injury or mitochondrial defect in the cell, there is the unidirectional transfer of healthy mitochondria from a donor cell to a recipient cell with mitochondrial damage[51,286,287]. This suggests the possible sensing signal mechanism to initiate the repair mechanism. Ahmad et al. have shown that the mitochondrial Rho GTPase protein Miro1, a calcium adapter protein involved in mitochondrial transport along microtubules, is involved in mitochondrial transfer between cells[185].

1.11.3 Cell-free circulating mitochondria and mitochondrial content

In addition to the presence of mitochondrial content (fractions or whole mitochondria) within EVs, fragments of mtDNA can be released outside of the cell and can be found in extracellular fluids as circulating, cell-free mtDNA (ccf-mtDNA). Along with mtDNA, cell-free mitochondria can be found in human blood as a respiratory

competent[288] or can be released by platelets to promote inflammation[280]. Ccf-mtDNA can be found in healthy[288] as well as in diseased patients[289,290]. The double-stranded mtDNA structure is more stable and resistant to nuclease degradation. It makes them stable in the extracellular milieu as it can be found in plasma and cerebrospinal fluid (CSF). In particular, reduced levels of ccf-mtDNA can be found in CSF of both AD and PD patients[291-294]. The differences could be influenced by inflammation, body mass index (BMI), and psychological and physical stress[290]. Also, in the acute phase of relapsing-remitting multiple sclerosis (RRMS), elevated levels of ccf-mtDNA were reported[295]. Changes in levels can be used as biomarkers for the onset and progression of neurodegenerative diseases[289]. It can be used as a biomarker for various diseases associated with mitochondrial dysfunction, such as cancer progression[296,297], cardiac arrest survival[298], diabetes mellitus[299]. Although the origin of ccf-mtDNA in serum is not known, it is possibly released into the circulation during cellular injuries such as apoptosis, necrosis, or trauma[300,301].

1.12 Roles of mitochondrial transfer

In 1982, Clark and Shay performed the first artificial mitochondrial transfer using the co-incubation step between recipient cell and exogenous mitochondria, leading to functional changes[302]. Since then, many studies have been performed to observe its effects on recipient cells and possibilities to develop new therapies. Most of the mitochondrial transfer studies have used MSCs as a primary tool as these cells can promote tissue repair through cell-cell communication[303]. It has been suggested that MSCs are less dependent on their own mitochondria as an energy source. Hence, they are more prone to transfer functional mitochondria to other cells. More research articles suggest mitochondrial transfer and regeneration of mitochondrial function in cells where it is compromised or has become dysfunctional[215,277,281,283]. Most of these studies with mitochondrial transfer were mainly performed by co-culture between healthy donor cells and recipient cells with mitochondrial dysfunction. Initial studies have shown the presence of mitochondrial transfer between A549 lung cancer cells and BM-MSCs. This transfer rescued aerobic respiration in A549 lung cancer cells[304]. Similar results were observed by using transgenic

mice expressing the red fluorescent protein in their mitochondria and visualizing whole mitochondrial transfer from host murine tissues to lung cancer cells[305].

Some studies have shown that the regeneration of functional mitochondria helps to improve cellular conditions and can have therapeutic effects on damaged cells[275,283]. MSCs, when activated by inflammatory signals, promote inter-cellular mitochondrial transfer to stressed or damaged cells, rescuing their viability and function[284,306]. However, some studies have suggested that inter-cellular mitochondrial transfer can induce an inflammatory response[286]. For example, activated platelets release functional mitochondria encapsulated in microparticles or as free organelle promoting leukocyte activation. Islam et al. have shown that *in vivo* mitochondrial transfer between BM-MSCs and alveolar epithelium of endotoxin-treated lungs and the transfer protects against high mortality of endotoxin-induced acute lung injury[199]. Similar to lung injury conditions, a cell line from different diseased conditions such as in asthma[185], cultured human mesothelioma cells[307], human laryngeal squamous carcinoma cells[308], and the breast cancer cell line MCF7[309] were also used to study the functional significance of mitochondrial transfer. In breast cancer cells, mitochondrial transfer elicited a chemo-resistant phenotype in MCF7 cells. Intercellular transfer and interaction of extracellular mitochondria with other cells induce several responses in a neighbouring cell or distant cells, such as functional mitochondrial regeneration, rescuing aerobic respiration. Understanding these roles of extracellular mitochondria will help to develop new therapies for various pathologies.

The release of extracellular mitochondria has also been proposed to act as an alternative quality control mechanism. Davis O et al. reported that a large number of mitochondria get released from neurons to be degraded by the lysosomes of adjoining glial cells[310]. Similarly, MSCs secrete mitochondria to be degraded in macrophages[311]. In PC12 cells, mitochondrial damage induced by carbonyl cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, and rotenone, a mitochondrial electron transport chain complex I inhibitor, promoted the release of extracellular mitochondria. However, Parkin overexpression reduced extracellular mitochondrial level[215]. These studies suggest

that extracellular mitochondrial release functions as a remote form of mitophagy to maintain mitochondrial homeostasis. However, overall, less number studies have analyzed the functional role of EVs in *in vivo* conditions, and further work is needed to establish the role of EVs in quality control and biogenesis process *in vivo*.

1.13 Mitochondria and immunity

The immune system refers to a collection of cells, chemicals, and processes that functions to protect various organs from foreign antigens such as microbes, viruses, cancer cells, and toxins. To do so, various secreted mediators play a role in coordinating defence and repair to avoid further damage.

1.13.1 Immunity and cytokines

There are two types of immune responses, innate and adaptive immune responses. An innate immune response is an antigen-nonspecific defence mechanism that the host uses immediately or within several hours as the first response to pathogens. This response takes place through the release of cytokines and chemokines and does not retain a memory of the response. If the pathogen persists, the adaptive immune system gets activated as it is composed of highly specialized, systemic cells that engage pathogens with specificity and memory[312]. Adaptive immunity recognizes specific 'non-self' antigens from 'self' antigens. This recognition generates pathogen-specific immunogenic effectors that eliminate specific pathogen or pathogen-associated cells and develop immunogenic memory to eliminate specific pathogen if subsequent infections occur[312,313]. Innate and adaptive immunity are key factors in the control of infections or chronic diseases.

Cytokines are released by immune cells in response to infection or produced and released from a wide variety of cells in response to cellular damage. Cytokines are responsible for maintaining a balance between these two types of immunities. Hence, cytokines are critical mediators of immune communication and essential for host defence against pathogens[314,315]. These cytokines bind to cellular receptors to regulate the

development and repair of tissues, inflammation and maintain the healthy status of the cell. Cytokines can act on their target cells in an autocrine, paracrine, and/or endocrine manner to induce systemic and/or localized immune responses. Cytokines can be classified into two distinct categories 1) cytokines that induce inflammatory response (proinflammatory cytokines), which includes interleukin (IL)-1, IL-6, chemokines, and tumour necrosis factor (TNF) and 2) cytokines that reduce inflammation and promote healing (anti-inflammatory cytokines) include IL-4, IL-10, IL-11, IL-13, transforming growth factor β.

1.13.2 PAMPs and DAMPs

Innate immunity to pathogens mainly relies on pattern recognition receptors (PPRs), allowing immune cells to recognize and respond rapidly against pathogens that share specific pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are LPS, porins, bacterial lipoproteins[316,317]. The primary function of PPRs is to mediate inflammation by sensing pathogens and molecules from damaged cells[316-318]. Upon detecting PAMPs, PRRs can initiate different signaling pathways, leading to the up-regulation of various type I interferons, pro-inflammatory cytokines, and chemokines.

Damage-associated molecular patterns (DAMPs) are cellular immunogenic triggers released upon cellular stress or tissue damage that activate an innate immune response[318-320]. DAMPs can originate from different sources, including extracellular matrix, intracellular proteins such as heat shock proteins, uric acid, histones, and mitochondrial contents (mtDNA, mROS)[320]. It has been shown that mitochondria and mitochondrial content act as DAMPs to induce immune response[321-325], consistent with the bacterial origin of mitochondria. The hypomethylated CpG motifs present in mtDNA are thought to be the remnant of engulfed bacteria and thus are highly immunogenic[326]. Hence, it is pertinent to know the role of mitochondrial content in regulating immune responses.

1.13.2.1 Mitochondria as a source of DAMPs

Mitochondria regulate inflammation either by regulating the immune pathways or by producing DAMPs. Damaged mitochondria releasing danger signals result in inflammasome activation, leading to caspase-1 dependent secretion of inflammatory cytokines IL-1β and IL-18[327]. Mitochondrial damage can lead to cytosolic exposure of DAMPs, which get recognized by PRRs, leading to an inflammatory response[324]. Along with mtDNA, various mitochondrial components can act as DAMPs. These components include Ccf-mtDNA, N-formyl peptides, cytochrome C, ATP, cardiolipins, Carbamoyl Phosphate Synthase 1 (CPS1), mROS (Figure 1.7)[274,275].

In 2004, Collins et al. first discovered that injecting mtDNA into joints of mice induced localized inflammation and arthritis, which opened up a new perspective about the role of mtDNA in potential immune responses[328]. mtDNA released from mitochondria can activate the Cyclic GMP-AMP synthase (cGAS)-STING pathway[323,329,330]. cGAS detects and binds dsDNA to form dimers undergoing conformational changes which facilitate the conversion of ATP and GTP into 2'3'-cyclic GMP-AMP (cGAMP). cGAMP is another messenger that binds to the endoplasmic reticulum (ER)-resident protein stimulator of interferon gene (STING)[331-334]. Cell death and mitochondrial transcription factor A (TFAM) deficiency have been shown to induce mitochondrial stress and mis-packaging mtDNA, resulting in its cytoplasmic release and activating the cGAS pathway to induce type I interferon response. This can be seen in various viral infections, including Herpes-simplex virus-1, Dengue virus, Mycobacterium tuberculosis[335-337].

The release of mtDNA from mitochondria and its subsequent recognition by PRRs occurs during many cellular processes such as infection, cell death, cellular inflammation, and neuroinflammation[335,338]. Damaged neurons release DAMPs such as mtDNA in the extracellular space by increasing inflammasome activation and IL-1β secretion and also inducing TNFα and nitric oxide (NO) production[339,340]. mtDNA has been shown to mediate an inflammatory response and at least in part through caspase-1 activation[341,342].

Cardiolipin is a mitochondria-specific lipid required for functional roles of mitochondrial metabolism and mitochondrial biogenesis that has also been shown to act as a DAMP[343]. Cardiolipin binds to nucleotide-binding oligomerization domain NOD-like receptors (NLRs), leading to inflammasome activation[344]. Another biologically active mitochondrial component that can act as a DAMP is ATP, which once released binds to plasma membrane family receptors P2X. This binding activates P2X receptors to trigger procaspase-1 cleavage and IL-1β activation[345]. High extracellular ATP concentrations induce a pro-inflammatory response in human microvascular endothelial cells releasing Il-6 and IL-8 cytokines[346,347]. These studies show that various mitochondrial components can act as DAMPs and play an important role in immune activation.

1.13.3 Mitochondria regulating immunity by means other than DAMPs

In addition to DAMPs, a number of other pathways are involved in regulating immune responses. Mitochondrial antiviral signalling protein (MAVS) has three functional domains, an N-terminal caspase recruitment domain (CARD), a proline-rich region, and a c-terminal transmembrane segment. The CARD of MAVS interacts with the CARDs of the retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-association gene 5 (MDA5). This interaction activates downstream NF-kB and IRF signaling pathways to stimulate pro-inflammatory cytokines and type I IFN production[348-351]. Immune suppression by viruses is associated with mitochondrial dynamics and mitochondria-associated membranes (MAMs). In the case of Severe Acute Respiratory Syndrome-associated Coronavirus (SARS CoV), ORF-9b causes mitochondrial elongation in addition to MAVS degradation[352]. Dengue virus infection downregulated fission protein DRP1 expression, inhibiting the IFN-dependent immune response while promoting mitochondrial fission by MFN2 knockdown enhances the immune response in dengue infection[353-355]. This suggests that viruses selectively target mitochondria to evade the immune response. Contrarily, in mouse bone-marrow-derived macrophages, mitochondrial elongation by knocking down DRP1 leads to NOD-like receptor protein 3 (NLRP3)-dependent caspase-3 activation and IL-1β, while activation of mitochondrial fission inhibited NLRP3 inflammasome activation[356]. These studies

provide strong support for the connection between mitochondrial dynamics and immune responses.

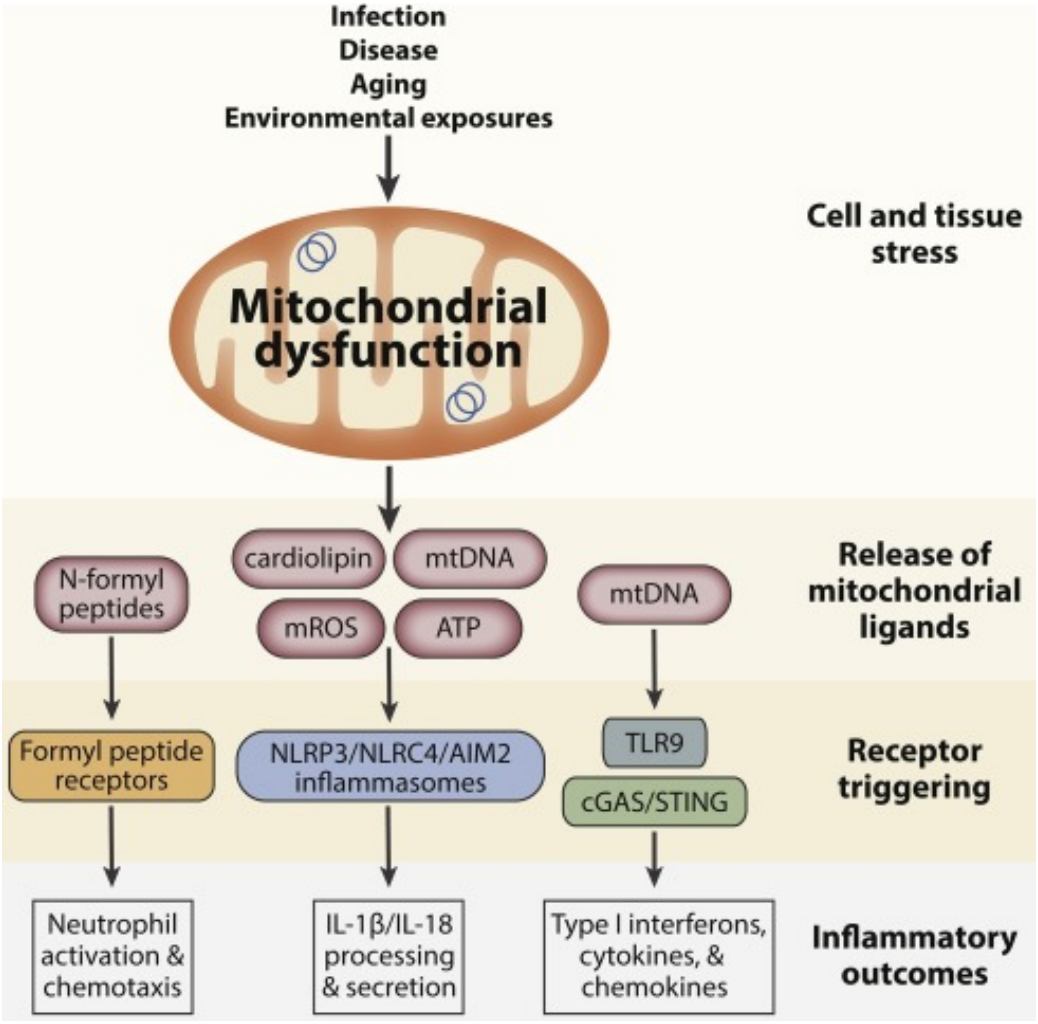

Figure 1.7 : Damaged mitochondria act as DAMPs.
Mitochondrial stress as a source for innate immune response and inflammation Upon mitochondrial stress or damage by infection or diseased condition, DAMPs are released to activate inflammatory responses. (West P. Toxicology 2017.)

Along with other functional roles, mROS are also involved in regulating immune responses. In systemic lupus erythematosus (SLE) patients, mROS in peripheral blood mononuclear cells induced MAVS oligomerization, resulting in induced type I IFN[357]. Increased ROS activates the NLRP3 inflammasome and the release of proinflammatory cytokines such as IL-1β[358]. In the brain, dysregulated activation of microglia and astrocytes produces and releases ROS resulting in persistent inflammasome activation,

leads to low-level chronic inflammation and the development of age-related pathological processes such as cognitive decline the onset of chronic neurodegenerative diseases[321]. Along with ROS, activated microglia also release proinflammatory cytokines, complement proteins, and proteinases that, if uncontrolled, can lead to chronic inflammation and neurodegeneration[359,360]. Hence, inhibition of microglia-activated inflammation can be considered as a therapeutic target for treating neurodegenerative diseases.

CHAPTER II

HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Mitochondrial proteins and mtDNA have been found in EVs[272,279,284,301], but the extent to which this occurs, and their exact role(s) remain unclear. One important unknown is the nature of mitochondrial content itself: in some cases, whole mitochondria have been reported to be released[280,361], but this does not necessarily extend to all cell types. The role(s) of the released mitochondrial content is also poorly understood. It has been proposed to be involved in various functions including cell-cell communication, mitochondrial regeneration, and mROS homeostasis. However, extracellular mitochondria can also enhance inflammation under conditions that are already pro-inflammatory[301,325,362]. In that regard, the role of extracellular mitochondrial content in the absence of pro-inflammatory signals remains unknown. In addition, how mitochondrial proteins are sorted and transported to EVs is unclear. Given that a number of mechanisms regulate mitochondrial quality control[119,135,141,215] and that damaged mitochondrial elicit an inflammatory response[68,321,330,335,356,363-366], I hypothesize that the sorting mechanism regulating the export of mitochondrial proteins into EVs is related to the known mitochondria quality control mechanisms.

2.2 Objectives

1) What is the actual mitochondrial content of EVs, and does it vary following mitochondrial stress?

Mitochondrial content (whole mitochondria, mitochondrial proteins, and DNA) can be found in EVs[277,284,288,362,367,368]. However, the nature and extent of the inclusion of mitochondrial content in EVs in unstimulated conditions are still unclear. In addition,

while cellular stress can alter the composition of EVs[180,238,245], the effect of mitochondrial stress on the inclusion of mitochondrial components into EVs is not known. Hence, my first objective is to determine the mitochondrial content of EVs under control conditions and following mitochondrial stress. To address the question, I will isolate EVs from different cell types by ultracentrifugation and determine the levels of mitochondrial (IMM, matrix, and OMM) proteins in EVs.

2) What are the mechanisms involved in mitochondrial release in EVs?

Mitochondria and mitochondrial components get released in EVs[277,284,288,362,367,368]. The intercellular mitochondrial transfer can occur through nanotubes and EVs[281,283,288,309]. However, how the sorting and transport of mitochondrial components occurs is not clear yet. MDVs are a form of mitochondrial quality control that participates in the presentation of mitochondrial antigens[167]. Given that MDVs can transport mitochondrial material to multivesicular bodies for their lysosomal degradation[163,369], it has also been suggested that MDVs could participate in the release of mitochondrial content in the extracellular space[166]. Hence, we hypothesize that MDVs play a role in defining mitochondrial components to be released in EVs. I will address the question by studying MDV formation and its mitochondrial cargo content upon different stimuli and mitochondrial damage.

3) What is the effect of extracellular mitochondria on immune responses in the absence of pro-inflammatory stimulation?

Upon mitochondrial damage in various pathological conditions, mitochondrial proteins can act as DAMPs to induce an inflammatory response[68,317,321,323]. In addition, pro-inflammatory stimuli can further promote the release of mitochondrial content, acting as a feed-forward mechanism for inflammation[198,301,362,370]. However, the role of mitochondrial proteins in EVs under normal, non-pro-inflammatory conditions remains unknown. We will thus determine mitochondrial damage promote the pro-inflammatory role of extracellular mitochondrial content. This will give insight into the relation between mitochondrial quality control processes and inflammatory response and how this process

is regulated in the absence of inflammation stimulus. I will address this objective by collecting mitochondrial fractions and EVs from control and mitochondrial damaged cells and treat macrophages with collected samples. We will evaluate the cytokine release from macrophages by performing ELISA assays.

CHAPTER III

SELECTIVE PACKAGING OF MITOCHONDRIAL PROTEINS INTO EXTRACELLULAR VESICLES PREVENTS THE RELEASE OF MITOCHONDRIAL DAMPS

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Preface

This project was initiated from our lab's previous publication linking the functional relationship between mitochondria and lysosome, to which I participated[371]. As we observed altered lysosomes[371], as well as changes in the release of lysosomal enzymes upon mitochondrial dysfunction (my own unpublished data), I set out to study the relationship between mitochondria and EVs. Answering the three questions I set out to address, we realized that together, it would provide a compelling story that constitutes the body of my thesis and is now accepted in Nature Communications.

Author Contributions

In this article, I did all the major experiments. Lilia Chikhi performed some of the western blots for the SNX9 siRNA experiments, which were used in the analysis. Veronique Desjardins provided the WB for CD9, Alix shown in Figure 2A. Both Lilia and Veronique were summer interns that I directly trained and supervised. Firas El-Mortada, an undergraduate student in Geneviève Pépin's laboratory helped me with the ELISA experiments. Experiments were designed and data analyzed by me, Geneviève Pépin, and Marc Germain. Me and Marc Germain wrote the manuscript, which was critically reviewed by all authors.

Abstract

Most cells constitutively secrete mitochondrial DNA and proteins in extracellular vesicles (EVs). While EVs are small vesicles that transfer material between cells, Mitochondria-Derived Vesicles (MDVs) carry material specifically between mitochondria and other organelles. Mitochondrial content can enhance inflammation under pro-inflammatory conditions, though its role in the absence of inflammation remains elusive. Here, we demonstrate that cells actively prevent the packaging of pro-inflammatory, oxidized mitochondrial proteins that would act as damage-associated molecular patterns (DAMPs) into EVs. Importantly, we find that the distinction between the material to be included into EVs and the damaged mitochondrial content to be excluded is dependent on selective targeting to one of two distinct MDV pathways. We show that Optic atrophy gene 1 (OPA1) and sorting nexin 9 (Snx9)-dependent MDVs are required to target mitochondrial proteins to EVs, while the Parkinson's disease-related protein Parkin blocks this process by directing damaged mitochondrial content to lysosomes. Our results provide insight into the interplay between mitochondrial quality control mechanisms and mitochondria-driven immune responses.

Introduction

Most cells secrete a range of extracellular vesicles (EVs) that act as communication devices by carrying proteins and nucleic acids between cells[281,372,373]. EVs can be broadly divided in two classes: exosomes, small vesicles arising from the fusion of a multivesicular body with the plasma membrane, and the larger microvesicles, which are thought to arise from the direct budding of vesicles from the plasma membrane[374]. Intriguingly, a number of studies have identified mitochondrial proteins as EV cargo[199,284,304,310,375,376].

Mitochondria are essential organelles that act as a central metabolic hub. As a consequence, mitochondria regulate a number of key cellular processes, ranging from the production of cellular energy to the induction of apoptosis and cellular differentiation[377-379]. Impaired mitochondrial function has thus major impacts on both cells and the organism, which are compounded by the production of toxic reactive oxygen species (ROS) by damaged mitochondria[380,381]. Importantly, damaged mitochondria, or the release from mitochondria of N-formyl peptides and mitochondrial DNA can act as damage-associated molecular patterns (DAMPs) that activate the innate immune system[325,382-387]. In fact, mitochondrial cargo within EVs and free mitochondria released by some cell types following pro-inflammatory stimulation like exposure to lipopolysaccharide (LPS) have been shown to stimulate the production of proinflammatory cytokines, further enhancing LPS-induced inflammation[198,280,386,388-390].

While inflammation promotes the secretion of mitochondrial content, mitochondrial proteins are also clearly present in EVs under unstimulated conditions[375,391]. The mechanism by which they are secreted and whether they also participate in immune activation remain unknown. Here we show that cells selectively target damaged mitochondrial components for lysosomal degradation to prevent the release of this pro-inflammatory content into EVs. This process is dependent on Mitochondria-Derived Vesicles (MDVs), small vesicles that carry mitochondrial proteins to other organelles. Specifically, this accurate sorting of mitochondrial components requires two distinct

MDV pathways. First, delivery of mitochondrial proteins to EVs requires Snx9-dependent MDVs, a subset of MDVs that were previously shown to regulate mitochondrial antigen presentation[167]. Second, MDVs carrying damaged mitochondrial components are instead targeted for lysosomal degradation in a process that depends on the Parkinson's disease related protein Parkin. Altogether, our results demonstrate that cells selectively regulate the packaging of mitochondrial protein into EVs to prevent the release of damaged components that would otherwise act as pro-inflammatory DAMPs.

Results

Mitochondria, not EVs stimulate a strong IL6 response

Mitochondrial content, especially oxidized components, can act as DAMPs that activate an inflammatory response when present in the cytosol or released from cells[325,382-387]. This can be demonstrated by exposing immune cells, such as the RAW264.7 macrophage cell line, to mitochondria isolated from other cells (here Mouse Embryonic Fibroblasts (MEFs), Supplementary Figure 1A) and monitoring two different inflammatory pathways (Interferon using IP10, and NF-κB using IL6). Exposure of RAW cells to isolated mitochondria caused a dramatic, dose-dependent increase in IL6 (Figure 1A) but no significant change in IP10 (Figure 1B), as measured in the cell culture media. Mitochondrial content could readily be found in EVs isolated from different cell lines under conditions where cell death was kept below 5% to avoid the presence of apoptotic bodies (Protein yield in Supplementary Figure 1B; Cell death in Supplementary Figure 1C; Mitochondrial content in Figure 1C) but was absent from non-conditioned media (Supplementary Figure 1D). Therefore, we then tested the ability of EVs to stimulate inflammation using EVs isolated from MEFs, as MEFs provided more consistent EV yields than the other non-immune cells we tested. Compared to mitochondria, EVs caused a significant increase in IP10 (Figure 1B; $10x10^6$ cells yield 120 µg mitochondria and 12 µg EVs) but did not stimulate IL6 production (Figure 1A). This suggests that while EVs stimulate IP10 production, they do not activate the strong IL6 response associated with mitochondrial DAMPs.

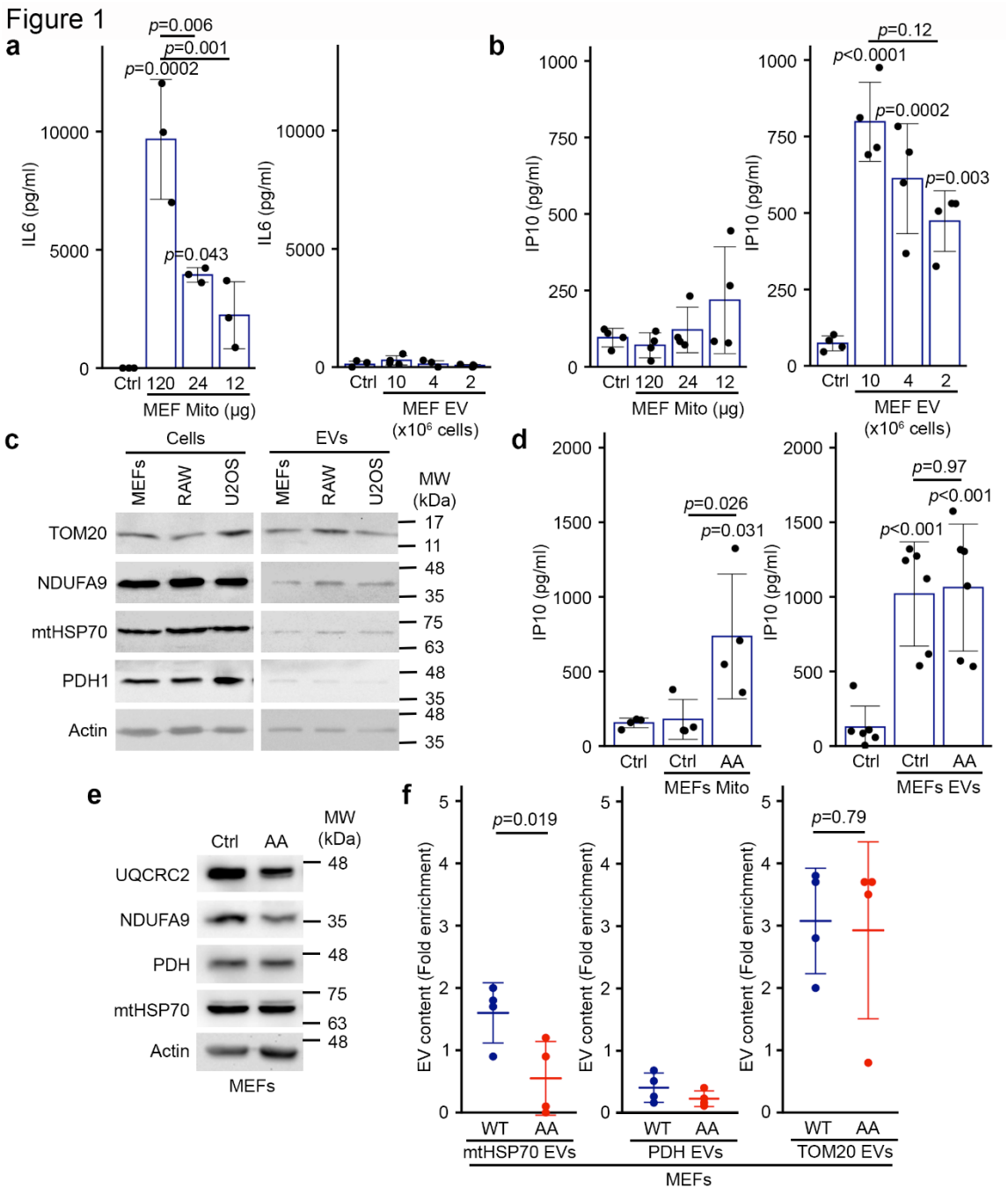

Figure 1: Mitochondria and EVs activate distinct pro-inflammatory cytokines.
(A-B) Extracellular mitochondria induce the secretion of pro-inflammatory cytokines. Mitochondria (Mito) (isolated from MEFs) or EVs (isolated by differential centrifugation from the media of MEFs grown for 24 hours in EV-depleted media) were added to RAW cells in their culture media. Release of IL6 (A) and IP10 (B) into culture media was measured by ELISA 24 hours. Individual points represent independent experiments (A, n=3; B, n=4). Bars show the average ± SD. One-way ANOVA. (C) Representative western blot showing the amount of the specified mitochondrial proteins in the indicated cell types (20 µg) vs their EVs (5 µg). TOM20, outer membrane protein; NDUFA9, Complex I subunit; mtHSP70 matrix protein associated with

the IM; PDH, matrix protein. Actin is used as a control, as it has been shown to associate with EVs. (D) AA-treated MEFs mitochondria stimulate IP10 production. RAW cells were treated as above with EVs (from $10x10^6$ cells) and mitochondria (12 µg) isolated from Control or AA treated MEFs, and IP10 release in culture media measured by ELISA. Individual points represent independent experiments (mitochondria, n=4; EVs, n=6). Bars show the average ± SD. One-way ANOVA. I AA treatment of WT MEFs causes the selective degradation of mitochondrial proteins. MEFs were treated with AA for 24 hours and the indicated mitochondrial proteins analysed by western blot. (F) Enrichment of the indicated mitochondrial proteins in EVs was measured by western blot in Control (Blue) and AA-treated (24 hours, Red) WT MEFs. Individual points represent independent experiments (n=4). Bars show the average ± SD. Two-sided t-test.

We then determined whether inducing oxidative damage to mitochondria further stimulated the IP10 response. For this, MEFs were treated for 24 hours with Antimycin A (AA), a complex III inhibitor that stimulates the production of ROS[371,392,393] but causes minimal cell death (Supplementary Figure 1E). RAW cells were then exposed to EVs or mitochondria collected from these cells. For these experiments, we used an equivalent amount of proteins from mitochondria and EVs (12 µg mitochondria, corresponding to protein yield of EVs isolated from $10x10^6$ cells), limiting the very strong IL6 response to mitochondria while allowing the detection of IP10 responses in EVs. Consistent with oxidized mitochondrial components being more inflammatory, mitochondria isolated from AA-treated cells, but not control mitochondria, induced significant IP10 secretion (Figure 1D). In contrast, EVs isolated from AA-treated cells did not further stimulate IP10 secretion (Figure 1D), suggesting that these mitochondrial DAMPs are prevented from being incorporated into EVs. In fact, an examination of mitochondrial content revealed that the Complex I subunit NDUFA9 and the Complex III subunit UQCRC2 were selectively degraded following AA treatment (Figure 1E). In contrast the matrix proteins mtHSP70 and PDH were not affected (Figure 1E). Nevertheless, mtHSP70 levels within EVs were significantly decreased (Figure 1F), while PDH levels were very low in control EVs and further decreased following AA treatment (Figure 1F). In contrast, the amount of the mitochondrial outer membrane protein TOM20 found in EVs was not affected by AA treatment (Figure 1F), consistent with outer membrane proteins not being affected by AA treatment[163]. Interestingly, addition of the antioxidant N-Acetyl-Cysteine (NAC) to the AA-treated cells rescued the mtHSP70 incorporation into EVs (Supplementary Figure 1F), supporting the role of oxidative damage in this process. Altogether, these

results suggest that cells selectively regulate the incorporation of mitochondrial components within EVs to prevent the secretion of pro-inflammatory DAMPs.

Mitochondrial proteins are selectively enriched in EVs

To identify the mechanism through which cells regulate the inclusion of mitochondrial proteins into EVs, we first characterised MEFs EVs and their mitochondrial content. EV preparations were enriched with the exosomal markers Alix and CD9 but excluded the nuclear protein Lamin B1 and the recycling endosome protein Rab11 (Figure 2A). Protein quantification by normalizing the amount present in EVs to the cellular content confirmed that the exosome marker Alix was enriched in our EV preparations, while different endosomal markers were not (Rab11, Rab9 (late endosome), Snx9 (endocytosis); Figure 2B), consistent with the selective inclusion of proteins within EVs. Having demonstrated the selectivity of EV content isolated from MEFs, we quantified the presence of a number of mitochondrial proteins in these EVs and found striking differences in the enrichment of individual mitochondrial proteins (Figure 2B). Among the proteins tested, TOM20 was present at the highest levels in unstimulated EVs, while PDH and the intermembrane space protein Cytochrome c (Cyt C) were present at very low levels (Figure 2B). Proteins associated with the inner membrane (mtHSP70, NDUFA9 and the mitochondrial fusion protein OPA1) were present at intermediate levels (Figure 2B). In addition to mitochondrial proteins, mitochondrial DNA (mtDNA) has been reported to be released from different cell types[199,389,394]. Consistent with this, we found that mtDNA was enriched in unstimulated MEF EV preparations (Figure 2B).

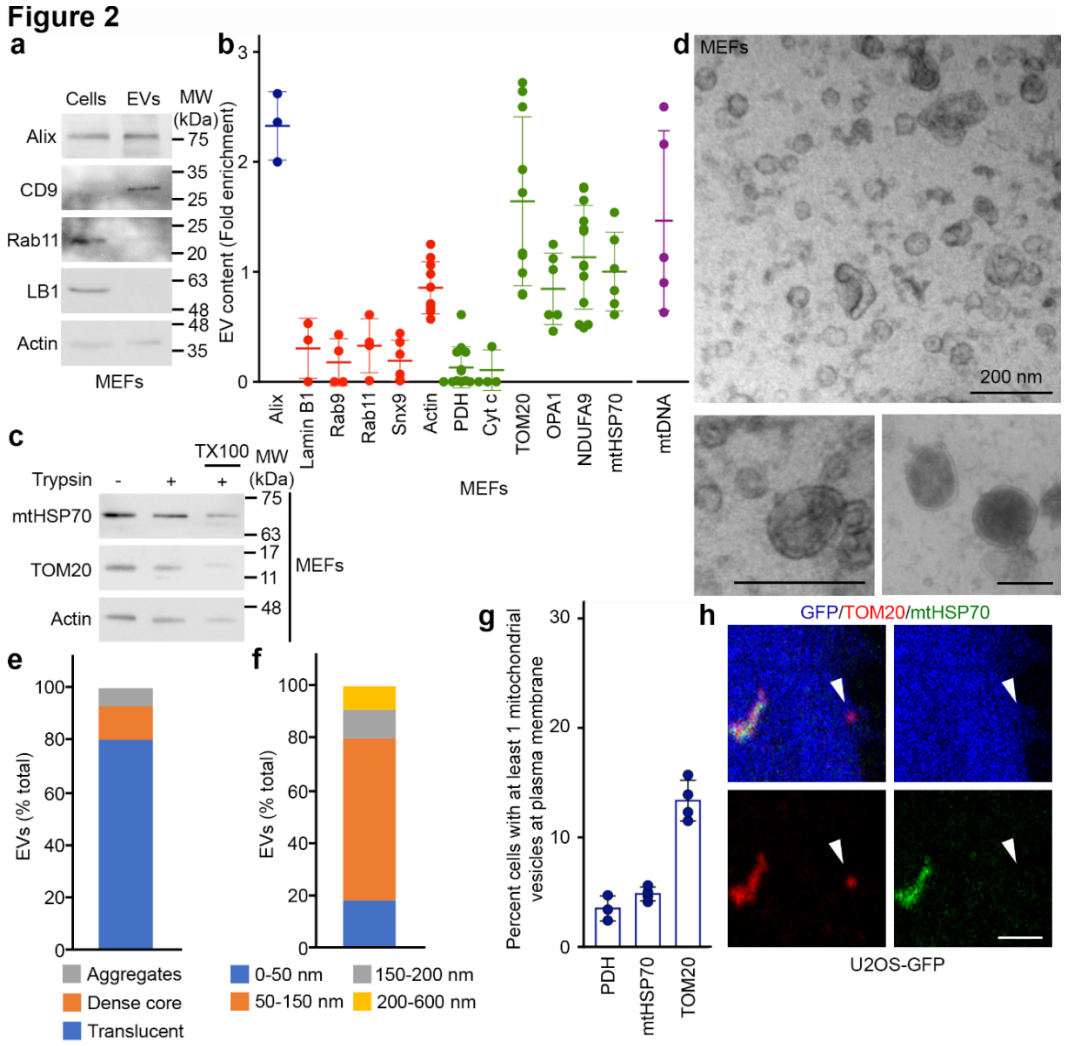

Figure 2: **Selective inclusion of mitochondrial proteins in EVs.**
(A) EV markers were analysed by western blot in 20 µg cell extracts and 5 µg MEFs EVs isolated as in Figure 1. (B) Quantification of protein inclusion in MEF EVs. The amount of the indicated protein present in EVs was normalised to its cellular content. Individual points represent independent experiments. Bars show the average ± SD. Alix, Exosome marker (Blue); Lamin B1, nuclear protein; Rab9, Rab11, Snx9, endosomal proteins; Cyt c, IMS protein, OPA1, IM protein. Mitochondrial proteins (Green), non-mitochondrial proteins (Red), mtDNA (Purple) (C) MEF EVs isolated as above were treated with Trypsin in the absence or the presence of detergent (TX100) and analysed by western blot for the presence of the indicated proteins. (D-F) MEF EV ultrastructure was analysed by EM and quantified based on structure I and size (F). Representative images are shown in (D). Scale bars, 200 nm. (G-H) Vesicles containing selective mitochondrial cargo are found in proximity to the plasma membrane (≤1 µm) but away from the main mitochondrial network (>1 µm). The number of TOM20-positive, mtHSP70-positive or PDH-positive vesicles are quantified in (G) with individual points representing the fraction of cells with plasma membrane associated vesicles in 4 independent experiments. Each positive cell typically contained one vesicle. Points show independent experiments and bars show the average ± SD. A representative image is shown in (H) with GFP (Blue) used as a cytosolic marker to identify cellular boundaries. The arrowhead denotes a TOM20-positive vesicle close to the plasma membrane. Scale bar, 2 µm.

Some proteins are peripherally associated with EVs rather than being sequestered inside these vesicles[175]. We thus tested whether mitochondrial proteins found in EVs were sensitive to trypsin digestion. As shown in Figure 2C, mitochondrial proteins were protected from trypsin digestion in EV preparations, unless the membranes were solubilised with Triton X-100 (Figure 2C; TX100), indicating that mitochondrial proteins are found inside EVs. Overall, the distinct pattern we observed for the inclusion of mitochondrial content within unstimulated EVs suggest that this is a selective process distinct from bulk mitochondrial export. To confirm this, we examined our EV preparations by electron microscopy (EM). The majority of the vesicles were small, electron translucent and often cup-shaped (Figure 2D, Bottom Left panel, quantified in 2E), consistent with exosomes[175,391]. A second population of vesicles had a distinct structure, containing a dense core (Figure 2D Bottom Right panel, quantified in 2E), while a small amount of material seemed aggregated and did not have a clear ultrastructure (Figure 2D-E). These vesicular structures were not observed in material isolated from non-conditioned media (Supplementary Figure 1G). Quantification of vesicle size indicated that the majority of the isolated EVs were less than 150 nm in diameter, while the few larger vesicles were smaller than 600 nm (Figure 2F). Importantly, we did not find vesicles with a structure or size consistent with whole mitochondria. Altogether, these results are consistent with the reported characteristics of isolated EVs (including exosomes and microvesicles)[175,391], not extracellular mitochondria.

Snx9 regulates the inclusion of mitochondrial IM/Matrix proteins in EVs

To determine if this process is associated with the presence of intracellular vesicles accumulating specific mitochondrial proteins, we used U2OS cells, large flat cells that are easy to visualise by confocal microscopy. We stained U2OS cells stably expressing GFP (to mark cytosol) for TOM20 and mtHSP70, two mitochondrial proteins enriched in our EV preparations (Figure 2B). Structures positive for one mitochondrial marker but not the other were present close to the plasma membrane of these cells (Figure 2G-H). In addition, there were more TOM20-positive structures than mtHSP70-positive structures (Figure 2G), correlating with the amount of each protein present in EVs (Figure 2B).

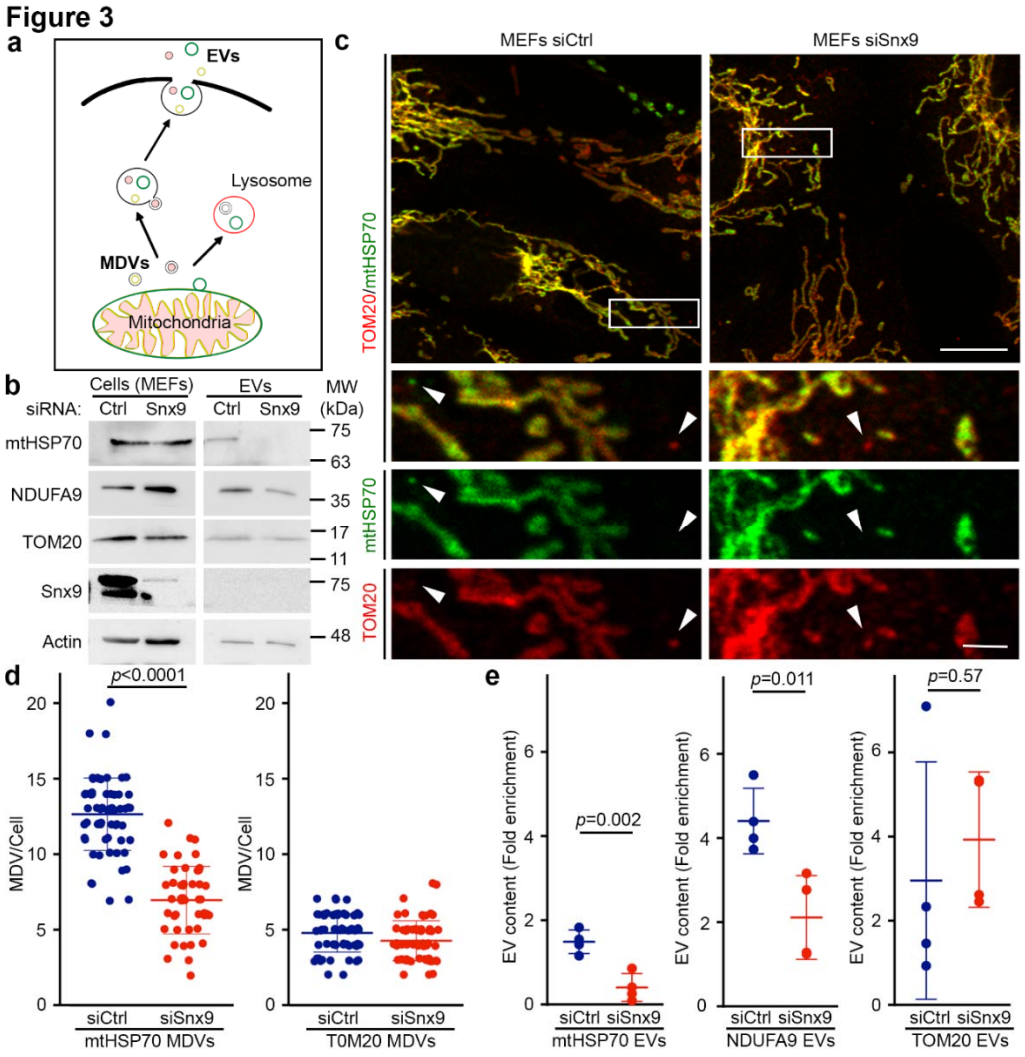

Figure 3: Snx9-dependent MDVs contribute to the inclusion of IM/matrix proteins into EVs. (A) Schematic representation of the two distinct MDV pathways leading to EVs and lysosomes, respectively. (B) MEFs were treated for 24 hours with a control siRNA (siCtrl, Blue) or a siRNA against Snx9 (siSnx9, Red). Mitochondrial proteins were then measured by western blot, with Actin serving as a loading control. (C) Representative images showing TOM20 and mtHSP70 MDVs in MEFs treated with a control siRNA (siCtrl) or a siRNA against Snx9 (siSnx9). Arrowheads denote MDVs (positive for one marker but negative for the other). Scale bars: top panels, 10 µm; enlargements in bottom panels, 2 µm. (D) MDV quantification from images as in (C). Each data point represents one cell. Bars represent the average of 40 cells in 3 independent experiments ± SD. Two-sided t-test. I Enrichment of the indicated mitochondrial proteins was measured by western blot as in Figure 1. Individual points represent independent experiments (n=4. Bars are shown as average ± SD. Two-sided t-test.

Similarly, few vesicles positive for PDH (present at low levels in EVs) were present close to the plasma membrane (Figure 2G). These results are thus consistent with the presence of an intracellular vesicular pathway regulating the selective inclusion of

mitochondrial proteins into EVs. The cargo-selective structures we observed are highly reminiscent of MDVs, small cargo-selective vesicles budding off mitochondria that are used to transport specific mitochondrial proteins to other organelles, including lysosomes where the material is degraded[166,369]. As this suggests that MDVs could target mitochondrial proteins to EVs, we addressed their role in EV cargo selection of mitochondrial proteins (general hypothesis in Figure 3A).

A recent study demonstrated that Snx9, a dynamin-binding protein essential for clathrin-mediated endocytosis, is required for the production of MDVs positive for the mitochondrial matrix protein PDH[167]. To confirm that Snx9 could be used to manipulate IM/Matrix MDV formation, we knocked down Snx9 using siRNA (Figure 3B, Supplementary Figure 2A) in MEFs. Snx9 knockdown did not affect intracellular mitochondrial content or overall mitochondrial morphology (Figure 3B-C, Supplementary Figure 2A). We thus measured the presence of MDVs in these cells. MDVs are defined by their cargo selectivity, and distinct mechanisms are thought to regulate the formation of MDVs containing outer membrane proteins and IM/matrix proteins, the latter forming double-membrane vesicles lacking outer membrane markers[166,369]. We thus labelled cells with TOM20 (outer membrane) and mtHSP70 (matrix) and counted the total number of small vesicles positive for one marker but not the other (Figure 3C). Loss of Snx9 caused a significant reduction in MDVs positive for mtHSP70 and negative for TOM20 (mtHSP70 MDVs), but did not affect MDVs positive for TOM20 and negative for mtHSP70 (TOM20 MDVs) (Figure 3D). This indicates that Snx9 regulates the formation of IM/matrix MDVs but not outer membrane MDVs.

Having confirmed that Snx9 knockdown can be used as a tool to define the role of MDVs in the inclusion of IM/Matrix proteins into EVs, we isolated EVs from siCtrl and siSnx9 cells media and measured their mitochondrial EV content. Consistent with the hypothesis that MDVs are required to target mitochondrial proteins to EVs, mtHSP70 and NDUFA9 were dramatically reduced in siSnx9 EVs (Figure 3E, Supplementary Figure 2B). In contrast, but consistent with the MDV data (Figure 3D), knockdown of Snx9 did not affect the cellular levels of TOM20 (Figure 3B, Supplementary Figure 2B)

or decrease its inclusion into EVs (Figure 3E, Supplementary Figure 2B). The selective loss of MDV formation and EV incorporation of mitochondrial proteins upon Snx9 knockdown thus suggest that MDVs regulate the inclusion into EVs of mitochondrial proteins associated with the mitochondrial IM/matrix.

Loss of OPA1 inhibits MDV formation and inclusion of mitochondrial proteins in EVs

To confirm our hypothesis, we sought for an independent way to prevent MDV formation without directly affecting the endosomal compartment. OPA1 is a mitochondrial IM protein required for mitochondrial IM fusion and maintenance of cristae structure[14,395-397]. Given that OPA1 was one of the mitochondrial proteins enriched in our EV preparation (Figure 2B) and that its Dynamin-like activity could promote the budding of IM/Matrix MDVs from the IM, we reasoned that genetic deletion of OPA1 should abrogate the formation of IM/Matrix-derived MDVs, but not of outer membrane MDVs. To determine whether this was the case, we measure MDV formation in WT and OPA1 KO MEFs (Figure 4, representative images shown in Supplementary Figure 3).

We first measured PDH MDVs (PDH-positive, TOM20-negative), as these MDVs have been extensively studied[165,167,369,398]. As shown in Figure 4A, the number of PDH MDVs was greatly reduced in OPA1 KO MEFs. NDUFA9 and mtHSP70 MDVs were similarly reduced in OPA1 KO MEFs (Figure 4B-C), further supporting a role for OPA1 in the generation of IM/Matrix MDVs. Importantly, TOM20 MDVs were not affected by OPA1 deletion (Figure 4D).

Figure 4

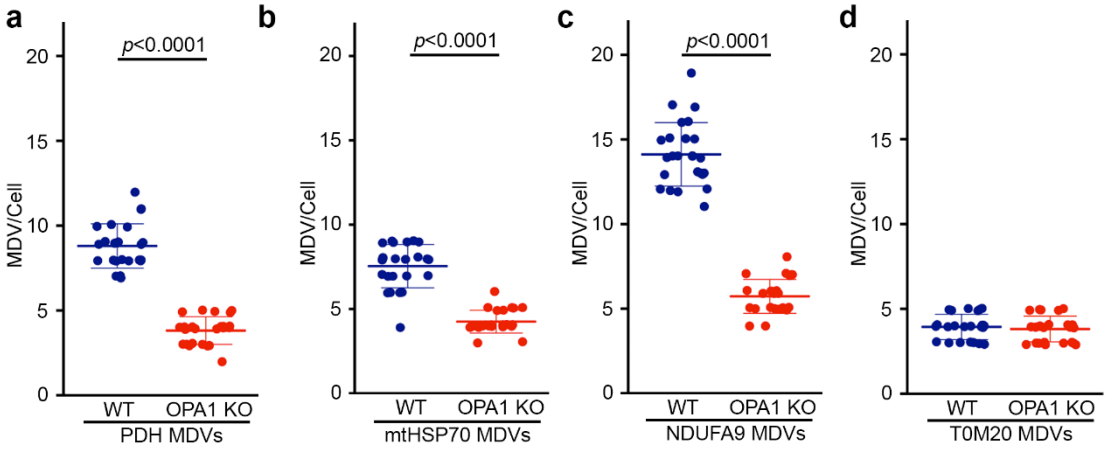

Figure 4: OPA1 deletion inhibits the formation of IM/matrix MDVs.
Quantification of MDVs positive for PDH (A), mtHSP70 (B), NDUFA9 (TOM20-negative) (C), as well as TOM20-positive (mtHSP70-negative) (D) in immunofluorescence images of WT (Blue) and OPA1 KO (Red) MEFs. Each data point represents one cell. Bars represent the average of 23 cells (except for PDH where n=20) in 3 independent experiments ± SD. Two-sided t-test.

As these results demonstrate that OPA1 deletion selectively affects the formation of MDVs containing IM/Matrix proteins, we used OPA1 KO cells to test our hypothesis that MDVs are required to package IM/Matrix proteins into EVs. Size, overall protein content and Alix expression were similar between WT and OPA1 KO EVs (Figure 5A-B; Supplementary Figure 2C), and these contained similar amounts of cytoplasmic proteins (Figure 5C, ACC1 and DJ1 quantified in 5D). As these results indicate that overall EV formation is not disrupted by the loss of OPA1, we measured the incorporation of mitochondrial proteins in WT and OPA1 KO EVs. At the cellular level, none of the mitochondrial proteins examined were affected by OPA1 deletion (Figure 5E). However, consistent with MDVs being required for the inclusion of IM/Matrix proteins into EVs, NDUFA9 and mtHSP70 were almost completely absent from OPA1 KO EVs (Figure 5E-F). Other electron transport chain components (SDHA (Complex II), UQCRC2 (Complex III) were also affected (Figure 5E). In contrast, TOM20 was still incorporated into EVs in OPA1 KO MEFs (Figure 5E, quantification in 5F), further supporting the selectivity of this process. Importantly, mitochondrial EV content was rescued by re-expressing OPA1 in OPA1 KO cells (Supplementary Figure 2D-F).

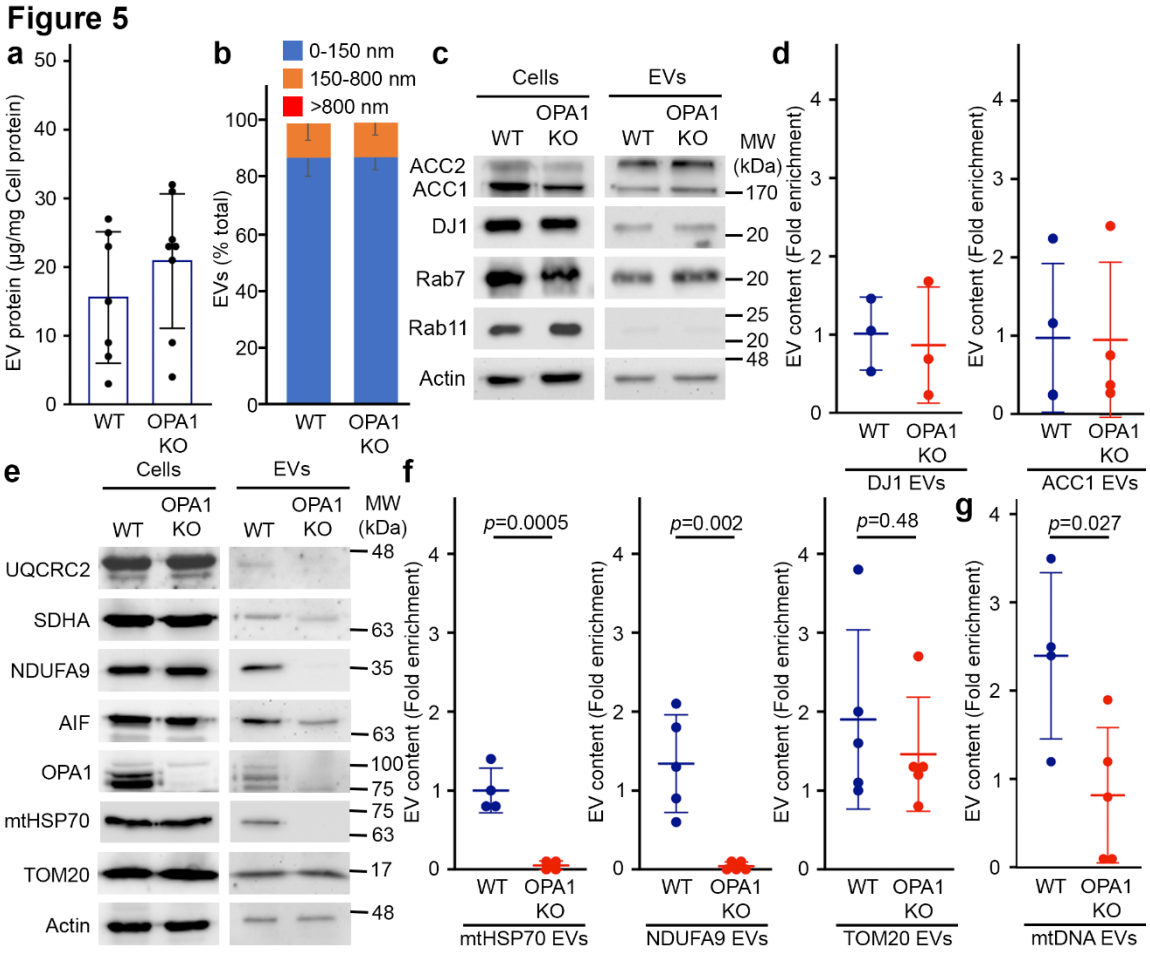

Figure 5: OPA1 regulates the inclusion of IM/matrix proteins and mtDNA into EVs. (A) Quantification of EV protein yields relative to cellular protein content in WT and OPA1 KO EVs isolated as in Figure 1. Each point represents one experiment (n=7). Bars show the average ± SD. (B) OPA1 deletion does not alter overall EV size. WT and OPA1 KO EV size was analyzed by EM and binned as indicated. The average percent of EVs in each category ± SD is shown (n=3). No EVs larger than 800 nm were detected. (C-D) The inclusion of cytoplasmic proteins into EVs is not altered following OPA1 deletion. Representative western blot (C) and quantification (D) as in Figure 1. Individual points represent independent experiments (n=3). Bars show the average ± SD. (E-G) The inclusion of IM/matrix proteins, but not TOM20, into EVs is prevented by OPA1 deletion. Representative western I and quantification as in Figure 1 (F). mtDNA was quantified by PCR (G) Individual points represent independent experiments. Bars show the average ± SD. Two-sided t-test. WT (Blue), OPA1 KO (Red).

As mtDNA was present in WT EVs, we also determined the effect of OPA1 deletion on mtDNA incorporation into EVs. Similar to IM/Matrix proteins, mtDNA content was decreased in EVs isolated from OPA1 KO MEFs (Figure 5G; relative to cellular levels to take into account mtDNA differences between WT and OPA1 KO MEFs[14]). Altogether,

our results indicate that OPA1 regulates the formation of IM/matrix MDVs, which are then required for the selective inclusion of mitochondrial content into EVs.

OPA1 KO mitochondria, but not EVs enhance IP10 secretion

Overall, our results indicate that MDVs are required to package IM/Matrix proteins into EVs. As we observed that increased mitochondrial ROS (AA treatment) prevents the inclusion of mitochondrial proteins into EVs (Figure 1F, Supplementary Figure 1F), our results suggest that this pathway is inhibited following oxidative damage to mitochondria. OPA1 deletion prevents MDV formation and packaging of mitochondrial proteins into EVs, but also leads to oxidative damage to mitochondria[366,371], allowing us to directly test if inhibiting MDV formation alters the release of pro-inflammatory cytokines caused by oxidized mitochondrial components. We thus measured the response of RAW cells to mitochondria and EVs isolated from WT and OPA1 KO cells. Consistent with our previous results (Figure 1), IP10 levels in media from cells exposed to WT mitochondria was not significantly different to that of control (media alone) (Figure 6A). On the other hand, OPA1 KO mitochondria caused a significant increase in IP10 secretion (Figure 6A), consistent with OPA1 KO mitochondria being oxidised. This contrasts with the response to EVs, where OPA1 KO EVs did not further increase IP10 secretion (Figure 6B). In fact, IP10 levels were lower with OPA1 KO EVs in each of our experiments (Figure 6B), suggesting that OPA1 KO EVs are actually less pro-inflammatory than their WT counterpart. This is also supported by the observation that the mRNA expression of the two IFN-dependent genes *Rsad2* and *mIfit1* was significantly lower in cells exposed to OPA1 KO EVs compared to those exposed to WT EVs (Figure 6C). In contrast, the strong IL6 secretion caused by WT mitochondria was not further stimulated by OPA1 KO mitochondria while neither EV types caused significant IL6 secretion (Figure 6D-E), suggesting that the response to oxidized mitochondrial content is IP10-selective. Altogether, these results indicate that inhibiting the release of oxidised mitochondrial proteins is sufficient to reduce the capacity of EV to stimulate IP-0 production.

Figure 6

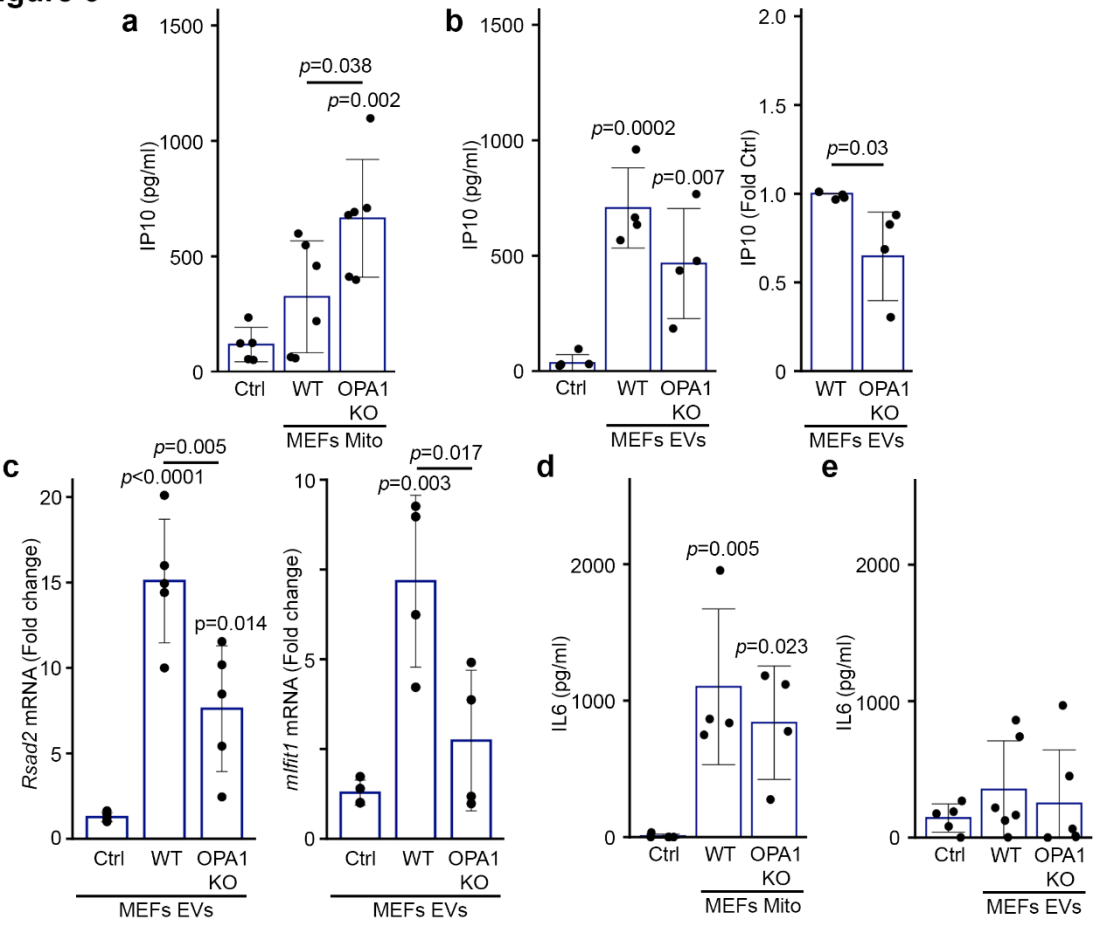

Figure 6: OPA1 KO mitochondria but not EVs isolated from the same cells induce an IP10 inflammatory response.
(A-B) RAW cells were treated as in Figure 1 with mitochondria (Mito, 12 µg) (A) or EVs (from 10 x 10^6 cells) (B) isolated from WT or OPA1 KO MEFs and IP10 release in culture media measured by ELISA. Individual points represent actual concentrations (or normalised to WT for B, right panel) in independent experiments (Mito, n=6; EVs, n=4). Bars show the average ± SD. One-way ANOVA, two-sided t-test for B, right panel. (C) OPA1 KO EVs show reduced expression of IFN-dependent genes. RAW cells were treated as in (A-B) and mRNA levels of the IFN-dependent genes Rsad2 (Left) and mIfit1 (Right) measured by qPCR. Individual points represent independent experiments (Rsad2, n=6; mIfit1, n=4). Bars show the average ± SD. One-way ANOVA. (D-E) RAW cells were treated as in (A-B) and IL6 was measured in the culture media by ELISA. Individual points represent independent experiments (Mito, n=4, EVs, n=5). Bars show the average ± SD. One-way ANOVA.

Oxidative damage to mitochondria blocks the inclusion of mitochondrial proteins within EVs

We next determined how oxidative damage to mitochondria inhibits the constitutive, MDV-dependent secretion of mitochondrial proteins in EVs. Different

classes of MDV have been identified based on cargo selectivity and destination. These include Snx9-dependent MDVs that deliver mitochondrial antigens to endosome for antigen presentation[167], and ROS-induced MDVs that deliver oxidized mitochondrial cargo to lysosomes for degradation[369]. We thus determined whether activation of the ROS-induced pathway targeted mitochondrial content to lysosomes at the expense of the EV pathway (Figure 3A) in MEFs. Treatment of WT MEFs with AA induced the formation of mtHSP70 and TOM20 MDVs (Figure 7A) and, consistent with MDVs containing oxidised proteins being delivered to lysosomes for degradation, AA treatment also increased the proportion of mtHSP70 MDVs associating with the lysosomal marker LAMP1 (in the presence of lysosomal inhibitors E64 and Pepstatin A to prevent cargo degradation; Figure 7B, Supplementary Figure 4A). Similarly, while the total number of mtHSP70 MDVs was lower in OPA1 KO cells (Figure 4D), they were positive for LAMP1 to a larger extend than WT mtHSP70 MDVs (Figure 7C), indicating that the few IM/Matrix MDVs remaining in OPA1 KO MEFs are preferentially targeted to lysosomes for degradation. Overall, as mtHSP70 inclusion into EVs is blocked under these conditions (Figure 1F, 5F), our results suggest that the EV-targeted MDV pool is inhibited by the activation of the damage-induced pathway.

We thus next determined whether directly stimulating the lysosomal pathway in the absence of mitochondrial damage is sufficient to inhibit the inclusion of IM/Matrix proteins into EVs. For this, we took advantage of the Parkinson's Disease related protein Parkin, which stimulates the formation of lysosome-targeted MDVs and, importantly, inhibits the Snx9-dependent pathway in the context of antigen presentation[167]. As the cell lines used in our experiments express very low or undetectable levels of Parkin (Supplementary Figure 4B), we used U2OS cells stably expressing GFP-Parkin to drive the formation of lysosome-directed MDVs, as previously done by us and others to study Parkin-Dependent MDVs[169,371]. Consistent with its role in the formation of MDVs destined for lysosomes[369], increased Parkin expression stimulated MDV formation under both basal and AA-stimulated conditions (Figure 7D) but did not alter the intracellular levels of mitochondrial proteins (Supplementary Figure 4C). In addition, GFP-Parkin partially associated with mtHSP70 MDVs in AA-treated U2OS cells stably expressing

GFP-Parkin (Figure 7E, Supplementary Figure 4D), similar to other matrix cargos[169]). We then measured the inclusion of mitochondrial proteins into EVs. Consistent with Parkin stimulating the delivery of mitochondrial proteins to lysosomes at the expense of EVs, mtHSP70 and NDUFA9 were significantly decreased in EVs from GFP-Parkin compared to GFP-expressing cells (Figure 7F, Supplementary Figure 4C). However, TOM20 inclusion into EVs was not significantly affected (Figure 7F, Supplementary Figure 4C). Altogether, these results indicate that Parkin inhibits the recruitment of mitochondrial proteins to EVs by promoting instead their lysosomal degradation.

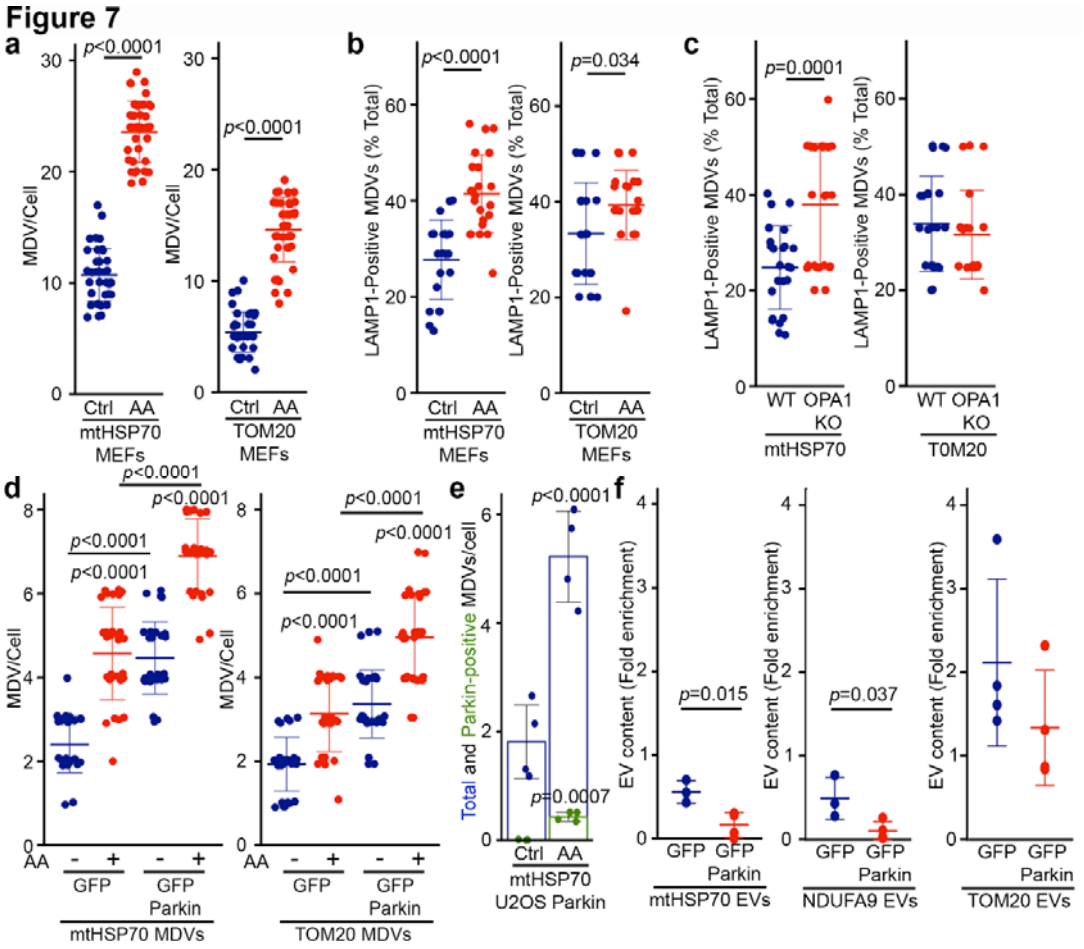

Figure 7: Parkin activation inhibits the inclusion of mitochondrial proteins within EVs. (A) AA treatment induces MDV formation. WT MEFs grown on coverslips were incubated in the absence (Blue) or presence of AA for 1 hour (Red). mtHSP70 and TOM20 MDVs were then quantified. Each data point represents one cell. Bars represent the average of at least 30 cells in a minimum of 3 independent experiments ± SD. Two-sided t-test. (B-C) Damage-induced MDVs are targeted to lysosomes. (B) WT MEFs were treated as in (A) in the presence of E64/Pepstatin and MDV association with the lysosomal marker LAMP1 quantified as the fraction of total MDVs associated with LAMP1. Alternatively (C) LAMP1-positive MDVs were quantified from OPA1 KO MEFs. Each data point represents one cell. Bars represent the average of at least 20 cells in a minimum of 3 independent experiments ± SD. Two-sided t-test). (D) Stable expression of GFP-Parkin increase MDV formation. U2OS cells stably expressing GFP (Blue) or GFP-Parkin (Red) were treated with AA for 4 hours and MDVs quantified. Each data point represents one cell. Bars represent the average of at least 20 cells in a minimum of 3 independent experiments ± SD One-way ANOVA. I mtHSP70 MDVs association with Parkin. U2OS cells stably transfected with GFP-Parkin were treated as in (D) and the association of mtHSP70 MDVs quantified by immunofluorescence. Individual points represent independent experiments (n=4). Bars show the average percent of total (blue) and Parkin-positive (green) mtHSP70 MDVs ± SD. Two-sided t-test. (F) GFP-Parkin expression impairs the inclusion of IM/matrix proteins into EVs. EVs were isolated from U2OS cells stably expressing GFP (Blue) or GFP-Parkin (Red) and the indicated mitochondrial proteins measured as in Figure 1. Individual points represent independent experiments (n=3, except for TOM20 where n=4). Bars show the average ± SD. Two-sided t-test.

Discussion

Cells secrete mitochondrial proteins and mtDNA into their environment. This has been proposed to serve many functions, including serving as a form of quality control, participating in long-range metabolic regulation or stimulating the immune system[187,198,277,280,365,376,383,386,394]. However, the underlying mechanisms have remained for the most part elusive. Here we show that mitochondria-derived vesicles are required for release of mitochondrial proteins in EVs. This is blocked upon mitochondrial damage, preventing the release of pro-inflammatory oxidised mitochondrial content.

A number of mitochondria-secreted factors behave as DAMPs that are associated with inflammatory cell activation during various pathological conditions. In these conditions, mtDNA and other mitochondrial components (cardiolipin, formyl peptides) can be released in extracellular media and act as an activator for immune cells to stimulate clearance of damaged cells and activate proinflammatory responses[367,399]. Importantly, these studies used LPS stimulation to trigger the release of mitochondrial DAMPs. For example, Joshi et al showed that damaged (low ATP, low membrane potential) extracellular mitochondria released from LPS-activated microglia acts as effectors of the innate immune system by targeting adjacent neurons and astrocytes[365]. Similarly, a number of studies have demonstrated that extracellular DAMPs act as a feedback loop for neutrophil activation[325,368,400]. On the other hand, several studies including this report have shown that mitochondrial proteins can be secreted in EVs in the absence of pro-inflammatory signals[375,391], but the role or mechanism of release remains poorly understood.

To study this question, we first quantified a number of mitochondrial proteins present in EVs. Consistent with a previous report showing selective enrichment of mitochondrial proteins in cancer cell lines[375], our results demonstrated that mitochondrial proteins are selectively incorporated into EVs under non-stimulated conditions. This process is dependent on MDVs, small cargo-selective vesicles that target mitochondrial proteins to other intracellular locations, including lysosomes where they

are degraded[166,369]. Specifically, inhibition of IM/Matrix MDV formation by either knocking down Snx9 or deleting OPA1 inhibited the inclusion of these proteins in EVs.

While MDV cargo is selected based on target destination and the nature of mitochondrial stress, the mechanisms defining MDV mitochondrial cargo selection are still unclear. Our results show that the same protein cargo (mtHSP70) can be targeted to EVs or lysosomal degradation depending on the stimulus. Specifically, we observed that upon mitochondrial damage, a greater number of MDVs get transported to lysosome which in turn blocks the secretion of mitochondrial proteins within EVs. Formation of MDVs containing damaged oxidized proteins is stimulated by the PINK/Parkin pathway[169]. Consistent with this, Parkin expression inhibited the secretion of IM/Matrix protein in EVs, further demonstrating that ROS-induced MDVs prevent the secretion of oxidised mitochondrial proteins by targeting them to lysosomes for degradation.

This dual targeting mechanism requiring Snx9 but blocked by Parkin is similar to the mechanism by which professional antigen presenting cells present mitochondrial antigen presentation (mitAP)[167]. In these studies, stimulation of immune cells (heat shock, LPS or bacteria) stimulates Snx9-dependent mitAP. This was exacerbated by the loss of the PINK1/Parkin pathway, leading to increased inflammation and Parkinsonian symptoms[401-403]. Here, we found that in cells that are not professional antigen presenting cells (fibroblasts, epithelial cells), Snx9 is required for the basal secretion of mitochondrial proteins in EVs, and that this is not associated with the strong IL6 response elicited by extracellular mitochondria. Rather, EVs selectively activate an IP10 response that is decreased following mitochondrial oxidative stress. In this context, the role of the PINK1/Parkin pathway would thus be to prevent the release and pro-inflammatory role of oxidised mitochondrial components. This could be particularly important given that oxidised mitochondrial components are more immunogenic and trigger a larger IP10 response (AA, Figure 1D; OPA1 KO, Figure 6A-B)[364,386,404] and suggests that, as with bacterial infection[322], mutations in the PINK1/Parkin pathway could lead to increased inflammation following oxidative damage to mitochondria. Nevertheless, other ROS-induced, lysosome-targeted MDV pathways must exist as these MDVs can be

induced in a number of cell lines with undetectable Parkin levels (MEFs (Figure 7A), U2OS (Figure 7D), HeLa cells[369].

Altogether, our results demonstrate that cells constitutively incorporate mitochondrial DNA and proteins in their EVs in the absence of an IL6-dependent inflammatory response. One remaining question concerns their physiological role. Previous work suggested that secretion of mitochondrial proteins could be a form of quality control where cells export their damaged mitochondria destined for degradation in distant cells[187,310,365]. However, given that lysosome-targeted MDVs prevent the release of oxidised mitochondrial components, this is unlikely to be a widespread phenomenon. On the other hand, an increased number of studies have demonstrated transfer of mitochondrial content (including mtDNA) between cells, affecting the metabolic output of the recipient cells and promoting tumour growth[273,309,405]. In this context, mitochondrial quality control mechanism identified here would serve the dual role of preventing inflammation and ensuring that only functional mitochondrial components are transferred.

Methods

Cell culture and treatments

The following cell lines were used: WT and OPA1 KO MEFs (gift from Dr. Luca Scorrano, University of Padua) including a line with OPA1 reintroduced that we previously described[38], U2OS cells stably transfected with pcDNA3-GFP and GFP-Parkin (gift from Edward Fon) and the mouse macrophage cell line Raw 264.7 (ATCC). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent) supplemented with 10% fetal bovine serum. Where indicated, cells were treated with Antimycin A (AA) (50 μM) for 24 hours and samples were collected for Immunofluorescence and western blots. For lysosomal inhibition experiments, cells were treated with combination of E64 (10 μM) and Pepstatin-A (20 μM) with or without AA for 1 hour and processed for immunofluorescence. Cell viability was measured by trypan blue exclusion. For suppression of endogenous Snx9 levels in WT MEFs, Snx9 siRNA (Silencer™ Select ID s83577) and negative control siRNA (Silencer™ Select Negative Control No. 2

siRNA) were used (Thermo life technology). Cells were allowed to adhere on plate/coverslips for 24 hours and then transfected with 20 nm of siRNA using siLentFect™ lipid reagent (Bio-Rad) for 24 hours.

EV collection and mitochondria isolation

24 hours after seeding cells in 150mm plates, media was replaced with DMEM supplemented with FBS that had been heat-inactivated and ultracentrifuged at 100,000 x g speed for 9 hrs to remove extracellular vesicle content. Media (15 ml) and cells (20 x 10^6) were collected 24 hours later for EV isolation. To isolate EVs, media was centrifuged at 400 x g for 5 mins to remove dead cells. EVs were then pelleted by ultracentrifugation at 100,000 x g for 1 h (Rotor 70.1Ti). The pellet was washed once with PBS. For western blot analysis, EVs were stored at -20 °C before use. For ELISAs and mtDNA quantification, EVs were used immediately. For trypsin digestion, 5 µg of Evs were digested with trypsin (0.01µg/ µl) in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) for 20 minutes on ice. The reaction was then stopped with soybean trypsin inhibitor.

Mitochondria were isolated as previously described[406]. Briefly, cells were harvested, resuspended in 200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM EGTA then broken by passing them 24 times through a 25-gauge needle. Nuclei and cell debris were removed by centrifugation at 1000 x g and the mitochondrial fraction isolated by centrifugation at 9000 x g. The mitochondrial pellet was washed once with the same buffer before use.

Electron microscopy

EVs collected from ultracentrifugation were fixed in 2% paraformaldehyde at room temperature. The pellets were then shipped in fixative to Mount Sinai Hospital (Toronto) for processing. There, samples were first incubated in 1% glutaraldehyde for 5 min, then contrasted in a solution of uranyl oxalate (pH 7) before contrasting and embedding in a mixture of 4% uranyl acetate and 2% methyl cellulose in a ratio of 1:9. Images were

acquired using EMS 208S (Philips) by an operator that was blinded to the experiment (4-5 fields per grid for a total of 50-60 EVs/grid). EVs size analysis (diameter at widest point) was performed using ImageJ software.

Antibodies and immunoblots

The following antibodies were used: mouse anti-actin (A-5316, Sigma-Aldrich); mouse anti-Cytochrome C (SC-13156), rat anti-LAMP1 (SC-19992), goat anti-AIF (SC-9416), rabbit anti-TOM20 (SC-11415), mouse anti-SDHA (SC-390381) and mouse anti-CD9 (SC-13118) (Santa Cruz Biotechnology, Inc.); mouse anti-NDUFA9 (ab14713), rabbit anti-DJ1 (ab18257), mouse anti PDH1 (ab110330), mouse anti-UQCRC2 (ab14745), mouse anti-Parkin (ab77924), rabbit anti-tubulin (ab52866), rabbit anti-Lamin B1 (ab133741) and mouse anti-Alix (ab117600) (Abcam); rabbit anti-ACC (3676-P), rabbit anti-Rab7 (9367-S), and rabbit anti-Rab11 (5589-P) (Cell Signaling Technologies); mouse anti-mtHSP70 (MA3-028) (Thermo scientific); mouse anti-OPA1 (612607) (BD Biosciences); rabbit anti-SNX9 (15721-1-AP) (proteintech). Secondary antibodies were from Jackson Immunoresearch (Alexa Fluor® 488 Donkey Anti-Mouse (715-545-150), Alexa Fluor® Cy-3 Donkey Anti-Mouse (715-165-150), Alexa Fluor® Cy-3 Donkey Anti-Rabbit (711-165-152), Alexa Fluor® 647 Donkey Anti-Rabbit (711-605-152), Alexa Fluor® 647 Donkey Anti-Rat (712-605-153), HRP Goat Anti-Rabbit (111-035-003), HRP Donkey Anti-Goat (705-035-003)), except HRP Anti-mouse that was from Cell Signalling Technology (7076S).

EVs and whole cell lysates were lysed in 10 mm Tris-HCl, pH 7.9, 150 mm NaCl, 1 mm EDTA, 1% Triton X-100, 50 mm sodium fluoride. Triton-insoluble material was then pelleted at 15890 x g for 10 min and protein concentration measured with the DC assay (Bio-rad). For immunoblot analysis, 5 µg EVs and 20 µg whole cell lysates were diluted in 1x Laemmli buffer supplemented with ß-mercaptoethanol, then subjected to SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibodies (1/1000, except actin at 1/10,000). Membranes were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies (Jackson

Immunoresearch) and visualized by enhanced chemiluminescence (Thermo scientific) using a Bio-Rad imaging system. Samples were quantified using ImageJ (National Institutes of Health). Uncropped blots can be found in the source data file.

Immunofluorescence

Cells were seeded overnight onto glass coverslips (Fischer Scientific) in 24 well plates, then treated as indicated and fixed with 4% paraformaldehyde for 10 mins at room temperature (RT). Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA in 0.1% Triton X-100 in PBS. Coverslips were incubated with primary antibodies (1/200, except for NDUFA9 at 1/100), followed by incubation with fluorescent conjugated secondary antibodies (1/250, Jackson Immunoresearch). Images were acquired with a Leica SP8 laser scanning fluorescence confocal microscope equipped with a 63x oil immersion objective using accompanying software (LAS AF). Images were quantified using ImageJ. MDVs were manually defined as circular vesicles significantly smaller in size than mitochondria (typically <150 nm vs 400-500 nm diameter for mitochondria) that were one mitochondrial marker was present but a second one completely absent as judged by the absence of fluorescence signal. These MDVs were considered to be associated with the lysosomal marker LAMP1 if they were clearly within a LAMP1-positive vesicle. Similarly, mtHSP70 MDVs were considered to be associated with Parkin if they were clearly within a GFP-Parkin-positive vesicle. Mitochondrial vesicles close to the plasma membrane were defined as for MDVs, except that only vesicles within 1 μm of the plasma membrane (as defined by the edge of the GFP staining) and 1 μm away from the main mitochondrial network were counted.

Mitochondrial DNA Quantification

Total DNA from cells and EVs was isolated using a silica-based column DNA purification kit (Purelink DNA isolation kit) following the manufacturer's instructions. Relative mitochondrial DNA levels was measured by quantitative PCR using PowerUp™ SYBR™ Green (Applied biosystems™). Independent reactions were performed for the

mtDNA gene cytochrome *c* oxidase 1[389] and the nuclear gene coding for the 18S ribosomal RNA. Primer sequences are provided in Supplemental Table 1. mtDNA levels were calculated using the CFX Manager™ software in a Bio-Rad CFX Real-Time PCR system.

Quantification of inflammatory responses

The proinflammatory effects of extracellular mitochondrial components was tested by adding isolated EVs (EVs from 10 x 10^6 cells) or whole mitochondria (12 µg, except where indicated) to 0.2 x 10^6 RAW264.7 cells for 16 hours. Media and cells were then collected. The presence of IP10 in the media was then determined by ELISA using the Mouse CXCL10/IP-10/CRG2 DuoSet ELISA from R&D system (cat. DY466) according to the manufacturer's instructions. The presence of IL-6 in the media was then determined by ELISA using the Mouse IL-6 OptEIA™ ELISA set from BD biosciences (cat. 555240) according to the manufacturer's instructions. For mRNA quantification, cellular RNA was extracted using the GENEzolTM TriRNA pure kit (Geneaid, Cat No. GZX100), from which cDNAs were generated using a high-capacity cDNA reverse transcript kit (Applied biosystems, Cat No. LS4368814). qPCRs were done using the Sensifast SYBR No-ROX kit (Bioline Cat no. BIO-98005) for the following genes (primer sequences in Supplementary Table 1): *Rsad2*, *mIfit1*, and actin as a loading control.

Statistics and reproducibility

All immunofluorescence data was quantified and images representative of at least three independent experiments shown (exact n are in the quantification figures). For western blots, most experiments are quantified as described in the appropriate figures. For the few figures that were not quantified (i.e. control experiments showing enrichment of EV markers, mitochondrial fractionation, efficiency of siRNAs), the images shown are representative of at least three independent experiments. For western blots and mtDNA quantification, EV content was calculated by measuring the ratio of EV content to cellular content normalized to the equivalent amount of total material for each fraction. For experiments where different treatments/cells were compared, the amount of each

tested protein/DNA was first normalized in each fraction (cells, EVs) to that of the control (Actin for proteins, 18S for DNA) before calculating the ratios. Individual experiments are shown along with the average and SD. For MDV quantification, quantification is shown for at least 20 individual cells within three experiments, along with the average and SD. Statistical significance was determined using Student's t test (between two groups) or one-way ANOVA with a tukey post hoc test (multiple comparisons). Complete data and statistics for all quantifications can be found in the Source Data file.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Source data for all western blot images and quantifications are provided in the Source data file.

Acknowledgements

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Author Contributions

Experiments were designed and data analysed by KT, GP and MG. KT, LC, VD and FEM performed experiments. KT and MG wrote the manuscript, which was critically reviewed by all authors.

Competing interests

"The authors declare no competing interests."

Supplementary information

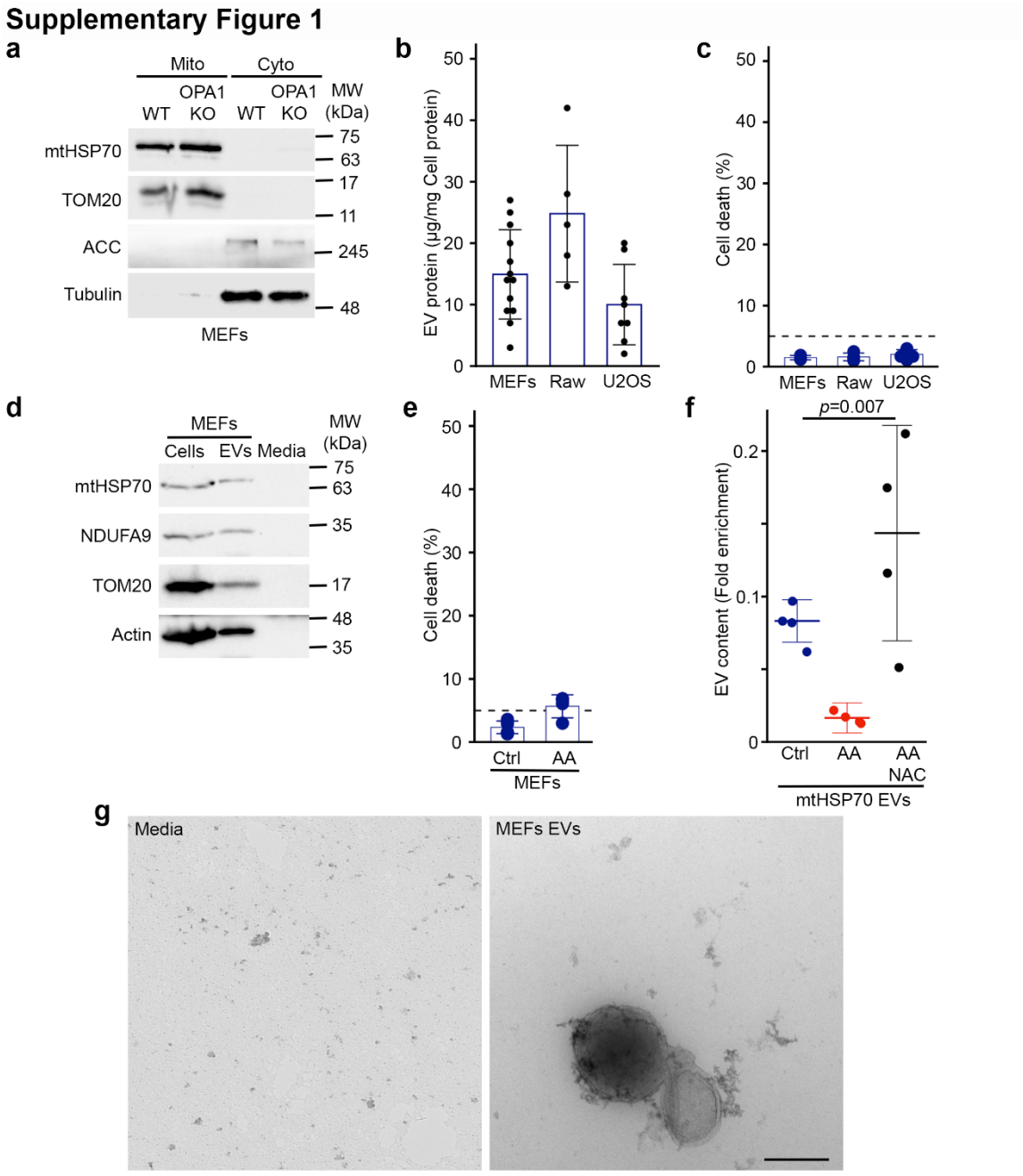
Selective packaging of mitochondrial proteins into extracellular vesicles prevents the release of mitochondrial DAMPs

Todkar *et al.*

This file contains

Supplementary Figures (1-4).

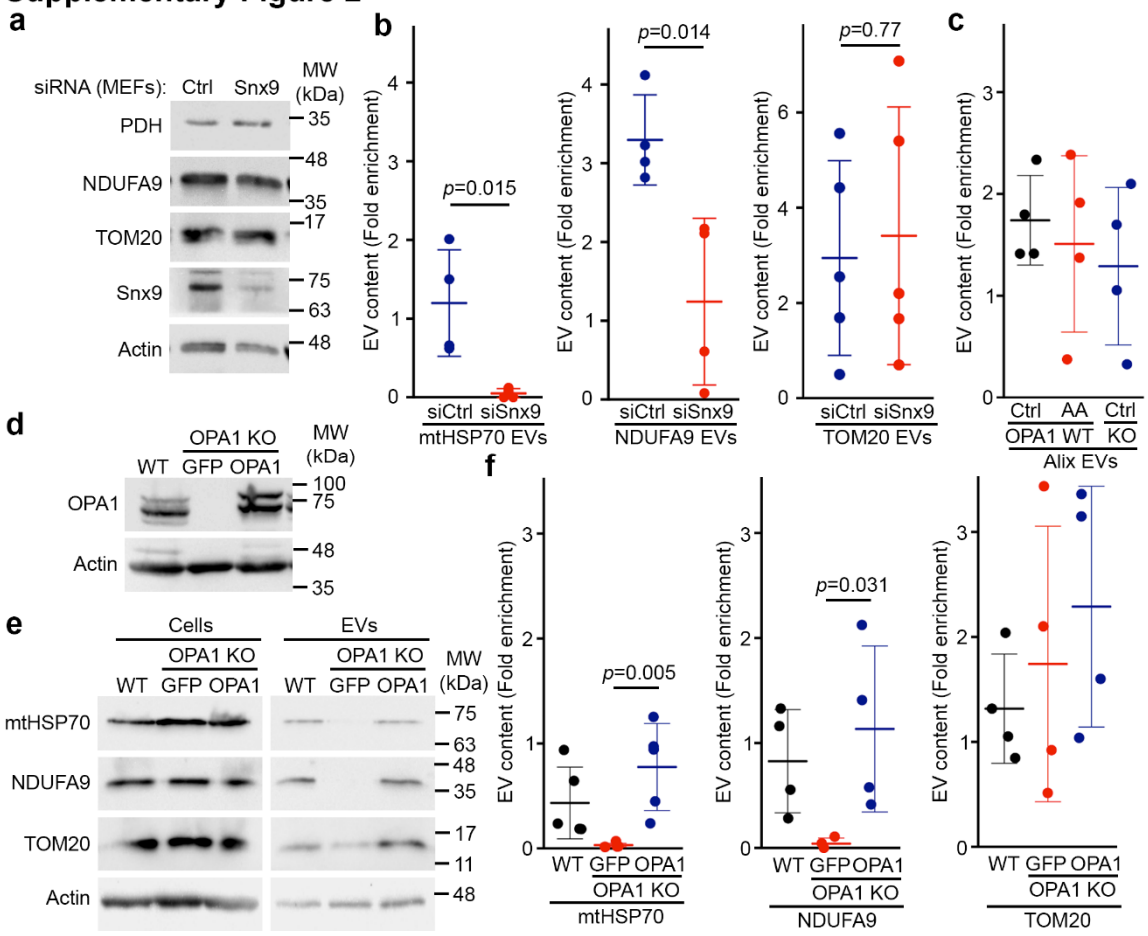
Supplementary Table 1.



Supplementary Figure 1: Isolation of mitochondria and EVs.
(A) Isolation of MEFs mitochondria. Mitochondrial and post-mitochondrial fractions were analysed by western blot for mitochondrial (mtHSP70, TOM20) and cytosolic (ACC, Tubulin) markers. (B) Quantification of EV protein yields relative to cellular protein content. Each point represents one experiment (MEFs, n=13; Raw, n=5; U2OS, n=8). Bars show the average ± SD. (C) Cell viability was measured by Trypan Blue (dashed line represents 5% cell death). Individual points represent independent experiments (n=4). Bars show the average ± SD. (D) Non-conditioned media does not contain mitochondrial proteins. EVs were isolated from non-conditioned media or media exposed to cells and analysed for the presence of mitochondrial

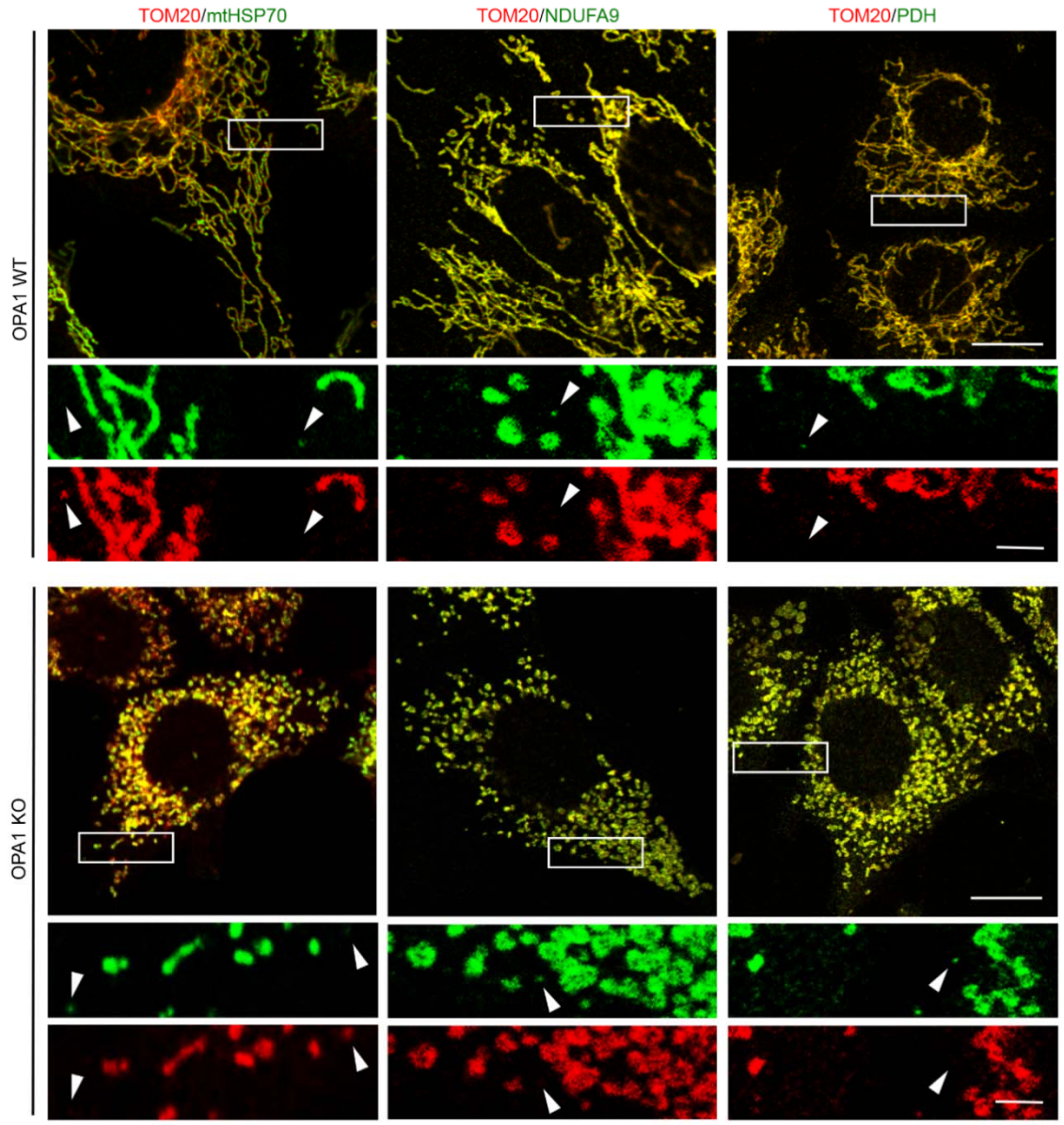
proteins and actin. I Cell viability (measured by Trypan Blue) following 24 hours treatment of WT MEFs with AA. Individual points represent independent experiments (n=3). The dashed line represents 5% death. Bars show the average ± SD. (F) NAC rescues the inclusion of IM/matrix proteins into EVs of AA-treated cells. EVs were isolated from WT MEFs treated with AA in the absence (Red) or the presence of 10 mM NAC (Black) and the presence of mtHSP70 in EVs measured as in Figure 1. Individual points represent independent experiments (n=4). Bars show the average ± SD. One-way ANOVA. (G) EVs isolated from non-conditioned media as in (D) were analysed by EM. Images representative of 3 independent experiments are shown. Scale bars, 200 nm.

Supplementary Figure 2



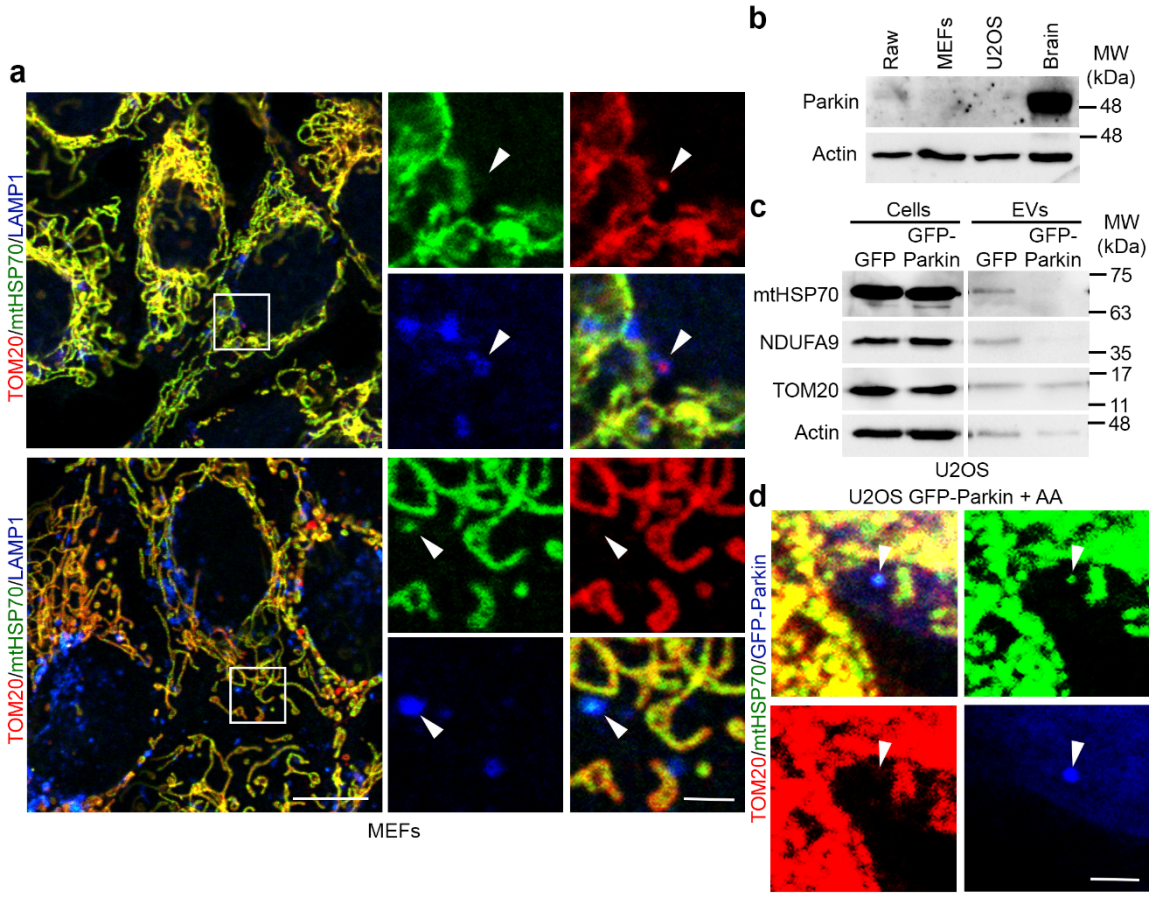
Supplementary Figure 2: Regulation of mitochondrial EV content by Snx9 and OPA1. (A) Knockdown of Snx9 (B) Enrichment of the indicated mitochondrial proteins in siCtrl (Blue) or siSnx9 (Red) EVs was measured by western blot as in Figure 1. Individual points represent independent experiments (n=4, except for TOM20 where n=5)). Bars show the average ± SD. Two-sided t-test. (C) The inclusion of the exosomal marker Alix into EVs is not altered following AA treatment or OPA1 deletion. Quantification was done as in as in Figure 1. Individual points represent independent experiments (n=4). Bars show the average ± SD. (D-F) Reintroduction of OPA1 in OPA1 KO MEFs rescues the inclusion of mitochondrial proteins into EVs. EVs from WT MEFs (Blue) and OPA1 KO MEFs stably expressing GFP (Red) or OPA1(Black) were analysed by western blot (OPA1 levels in (D), representative western blot in I. Data was analysed as in Figure 1 (F). Individual points represent independent experiments (n=4). Bars show the average ± SD. One-way ANOVA.

Supplementary Figure 3



Supplementary Figure 3: Immunofluorescence data for OPA1 KO MDVs.
WT and OPA1 KO cells were stained with the indicated antibodies and imaged. Representative images are shown. Scale bar, 10 µm for the full image, 2 µm for the insets. Arrows denote MDVs.

Supplementary Figure 4



Supplementary Figure 4: Activation of the lysosomal MDV pathway.
(A) WT cells were stained with antibodies against mitochondrial proteins (TOM20, mtHSP70) and the lysosomal marker LAMP1 and imaged. (A) TOM20-positive MDV (Red) colocalized with LAMP1 (Blue). Scale bar, 10 µm; 2 µm for insets (B) Representative western blot showing the Parkin expression in mouse brain and the cell lines used in this study. (C) Representative western blot showing the decreased inclusion of IM/matrix proteins into EVs of GFP-Parkin expressing U2OS cells. (D) mtHSP70-positive MDV (Green) colocalized with Parkin (Blue). U2OS cells stably transfected with GFP-Parkin were treated with AA and stained for TOM20 (Red) and mtHSP70 (Green). Arrowhead denote a mtHSP70 MDV colocalizing with GFP-Parkin (Blue). Scale bar, 2 µm. Representative images are shown.

Supplementary Table 1: List of primers

Gene	Forward Primer	Reverse Primer
cytochrome *c* oxidase 1 (mouse)	5'-GCCCCAGATATAGCATTCCC-3'	5'-GTTCATCCTGTTCCTGCTCC-3'
18S ribosomal RNA (mouse)	5'-TAGAGGGACAAGTGGCGTTC-3'	5'-CGCTGAGCCAGTCAGTGT-3'
RSAD2 (mouse)	5-CTGTGCGCTGGAAGGTTT-3	5-ATTCAGGCACCAAACAGGAC-3
Actin (mouse)	5-GCCTTCCTTCTTGGGTATGG-3	5-AGCACTGTGTTGGCATAGAG-3
mIFit1 (mouse)	5-GAGAGTCAAGGCAGGTTTCT-3	5-TCTCACTTCCAAATCAGGTATGT-3

CHAPTER IV

DISCUSSION

Cell-cell communication through EVs has been a recent focus of research interest because of both their functional roles and potential use as a biomarker. The release of these components has been proposed to exhibit a range of functions, including serving as a form of quality control, participating in metabolic regulation through paracrine signalling, or stimulating the immune system[187,198,277,280,365,376,383,386,394]. Because of their impact on near or distant cells as well as in disease progression, EVs are being considered for developing therapeutic treatment and diagnostic tools. With the help of EVs, cells have been shown to release proteins, RNAs, lipids, mRNA as well as mitochondrial components (including mtDNA) into their environment [175,198,199,209,215,217,223,225,242,272,288,399,400]. Nevertheless, the mechanisms governing the inclusion of specific material within EVs, including mitochondrial content, remain elusive. During my Ph.D., I set to address some of these questions by defining the mitochondrial content that gets incorporated in EVs, the mechanism through which this occurs, as well the relationship between mitochondrial EV content and inflammation.

4.1 Selective inclusion of mitochondrial proteins in EVs

To understand the mechanisms behind the inclusion of mitochondrial content into EVs, the first objective was to study mitochondrial proteins and DNA present in EVs from different cell lines. Until now, most of the cell lines used for EV studies were either stimulated to promote inflammation (with LPS for example)[198,199,401], or in diseased conditions[198,271,275,288,324,386,407]. Cells release heterogeneous vesicles of different intracellular origins and of different sizes comprising small EVs formed inside endosomal compartments and larger EVs budding from the plasma membrane[391]. We found that EVs collected from MEFs were in the range of 10-600 nm, which confirms the presence of

small and larger size vesicles. These EVs carried various exosomal markers but were devoid of cytosolic markers. Thus, size analysis and protein content of EV confirm the presence of EVs isolated with ultracentrifugation technique.

Mitochondrial proteins can be released in response to oxidative stress, cellular damage, or mitochondrial damage. Activated mast cells[362], T cells[370], neutrophil[368], platelets[280,408-410] and damaged organs or tissues[408-410] release extracellular mitochondria. Hurwitz *et al.* suggested that in cancer cell lines, mitochondrial ETC complexes can be released in EVs[280,375]. Our results demonstrated that mitochondrial proteins are incorporated into EVs at basal levels in MEFs, Raw, and U2OS cells, and this inclusion process is specific, with differential enrichment of a number of the mitochondrial matrix, IMM, and OMM proteins. According to the current cellular EV biogenesis model, cytosolic proteins remain inside the vesicles, and plasma membrane proteins reside outside of the vesicles[411]. However, little information is available about the packaging and localization of the proteins in EV. Using trypsin and triton X-100, we could confirm that mitochondrial proteins are localized inside these vesicles and get digested with triton X-100, confirming the localization of mitochondrial proteins inside EVs.

Previous studies have shown that whole mitochondrial structures were observed in blood[288], and platelets activated with immunoglobulins trigger the release of free mitochondria[280]. Similarly, astrocytes and MSCs also release the whole mitochondria[284]. Most of these studies have analyzed mitochondrial release with mitochondrial stress and with physiological conditions[280,284,288]. However, little is known about mitochondrial release in non-stimulated conditions. We did not observe whole mitochondrial structures with electron microscopy of EVs. There are few possible reasons for not detecting whole mitochondria in EVs. First, this might be because we collected EVs, including small and larger vesicles and the proportion of smaller EVs was higher in sample preparation to detect whole mitochondria. Therefore, we hypothesized that cells release fractions of mitochondria in EVs. We found that there was selective inclusion of mitochondrial proteins which confirmed that mitochondrial fractions are getting released in EVs in non-stimulated conditions. The difference might be cell-line specific effect as we have

fibroblast cells and the other researchers have found whole mitochondrial structures in cardiomyocytes, astrocytes, neurons and cancer lines. Another possibility is that those whole mitochondria do not get exocytosed in non-stimulated conditions. In most studies, mitochondrial release was experimentally triggered by calcium signaling[283], thrombin[280], or transfer through nanotubes[187]. The only study where the release of whole mitochondria without trigger was observed in a study by Dache *et al.* using colon cancer cell lines[288]. However, in our project, we mainly used fibroblasts, and we could see mitochondrial fractions in EVs. This might lead to the differences in mitochondrial content in EVs as we have studied extracellular mitochondria in different conditions. We further confirmed the selective release of mitochondrial proteins in different conditions such as fibroblasts treated with mitochondrial complex III inhibitor antimycin A, with siRNA against SNX9, as well as in OPA1 KO MEFs. This selectivity could be due to the release of mitochondrial fractions instead of whole mitochondria. However, further validation needs to be done by collecting EVs by differential ultracentrifugation and collecting fractions with larger vesicles and perform electron microscopy to confirm if whole mitochondria is getting exocytosed in fibroblasts.

4.2 MDVs are required for the selective inclusion of mitochondrial content into EVs

As we observed the selective inclusion of mitochondrial proteins in EVs, our second objective was to identify the mechanism by which the selectivity occurs. We discovered that MDVs are involved in specific mitochondrial protein inclusion in EVs.

MDVs are small vesicles derived from mitochondria and carry specific mitochondrial proteins[166]. However, the molecular events involved in MDV formation are still unclear, and limited information is available on proteins or mechanisms involved in the process. Nevertheless, recent studies have shown that SNX9 and Parkin are involved in mROS-induced MDV formation[167,407]. While SNX9 is well known for its role in clathrin-mediated endocytosis[412], is also required for the formation of oxidative stress-induced MDVs and to present mitAP MDVs[167].

It has also been proposed that upon oxidative stress, mitochondrial protein aggregation occurs at the proximity of the mitochondrial membrane[163]. This aggregation, along with cardiolipin oxidation, would generate curvatures in mitochondrial membrane structure. The curvature is thought to be followed by accumulation of PINK1 at OMM, followed by ubiquitination and Parkin recruitment. Parkin localization on mitochondria will promote the cleavage of vesicles containing mitochondrial proteins[166,407]. As OPA1 interacts with cardiolipin for cristae remodelling and mitochondrial fusion[106,107], we also identified OPA1 as essential for the formation of IM/matrix proteins containing MDVs. Thus, in this project, we focused on the role of OPA1, SNX9, and Parkin in MDV formation and its correlation with extracellular mitochondrial content.

We found that in fibroblasts, inhibiting IMM/matrix MDV formation through OPA1 KO or Snx9 knockdown prevented the release of IMM/matrix proteins into EVs, confirming that MDVs are required to release mitochondrial proteins within EVs. MDV formation can be modulated by various factors such as oxidative stress or mitochondrial metabolic activity (with galactose-containing media)[163,165]. *In vivo* production of MDVs occurs not only at the basal level but also at a higher rate upon oxidative stress[165,169]. Induced mROS levels promote mitochondrial quality control mechanisms and induce transport of oxidized proteins to the lysosome[165,367,369]. Upon oxidative stress, Parkin-dependent MDVs carry PDH1 but not TOM20[169] to lysosomes for degradation, whereas MDVs targeted to peroxisomes carry the mitochondrial outer membrane protein MAPL[164]. We found that antimycin A-induced oxidative stress stimulated the formation of MDVs but that these MDVs were transported to the lysosome for degradation at the expense of transporting mitochondrial content into EVs. Similar targeting of MDVs to lysosomes was observed in OPA1 KO MEFs, supporting the idea that the lysosomal pathway is activated at the expense of the EV pathway. This was supported by the observation that the antioxidant NAC rescued EV mitochondrial content.

It has been shown that Parkin colocalizes with MDVs in a PINK1-dependent manner, and antimycin A stimulates Parkin-dependent MDV formation[163]. We showed

that oxidative stress (antimycin A treatment) and Parkin overexpression induced the formation of MDVs that were targeted to lysosomes for degradation rather than being released in EVs. It has been shown that PINK1/Parkin are essential proteins in mitophagy[151]. Deletion or mutation of these proteins causes the accumulation of damaged mitochondria and leads to the onset of PD[118,119,246]. This shows the importance of PINK1/Parkin in mitochondrial quality control and also in MDV formation. However, many cell lines, including ones that have been used in MDV studies, have very low or undetectable levels of Parkin[67,167,169,170,413]. We have shown that MDVs can be induced in a number of cell lines (MEFs and U2OS cells) with undetectable Parkin levels by western blot. Several other studies have shown that Parkin-null HeLa cells could produce MDVs upon oxidative stress[67,167,169,170,413]. In the absence of PINK1/Parkin, pro-inflammatory LPS activates MDV formation and the presentation of mitochondrial antigens at the surface of antigen-presenting cells[167]. Altogether, these results suggest that there must be the existence of other Parkin-independent ROS-induced MDV pathways. Till now, Parkin-independent MVD pathways have not been identified, but there are Parkin independent mitophagy pathways involving FUNDC1 or the E3 ubiquitin ligase HUWE1[158,414,415]. In fact, a recent study has shown that while mitochondrial release into EVs was modulated by Parkin expression, overexpression of BNIP3L/NIX also decreased mitochondrial EV content, supporting the presence of Parkin independent mitochondrial quality control mechanisms[215].

Another possible mechanism for Parkin-independent MDV formation is through interaction between FUNDC1 and OPA1. This interaction is important to coordinate mitochondrial dynamics and mitophagy[416]. Under stress conditions, the dissociation between FUNDC1 and OPA1 leads to mitophagy[416]. However, it might be possible that under a limited stress level, the coordination might induce Parkin-independent MDV formation to drive damaged mitochondria to lysosome rather than promoting mitophagy. However, more studies need to be done to discover the mechanisms associated with Parkin-independent pathways.

4.3 Inflammatory responses to mitochondrial proteins

A mitochondrion is an organelle of endosymbiotic origin that still caries its bacterial DNA characteristics. It has been shown that extracellular mitochondria and mitochondrial content can act as DAMPs which are associated with inflammatory cell activation during many inflammation stimulus conditions[277,283,321,338,417]. However, the role of extracellular mitochondrial proteins in regulating immune responses is not clear yet. Hence, my third objective was to address the actual role of EVs with normal mitochondrial content and determine how the selective inclusion of mitochondrial proteins in EVs regulates immune responses. Here, we show that the release of mitochondrial proteins regulates inflammatory responses, and cells with oxidized mitochondria have a mechanism to protect distant cells by blocking the release of this damaged pro-inflammatory content.

Extracellular mitochondria have been observed in many pathological conditions, including trauma[325], brain injury[239], cancer[418], rheumatoid arthritis[419], systemic lupus erythematosus (SLE)[419,420]. Extracellular mitochondria play a role in pro-inflammatory responses through the activation of TLRs, cytokine release, and cytosolic pathogen recognition receptors, which are all key components of the innate immune system [321,325,338,363]. Importantly, most of the previous studies addressing the relationship between mitochondria and immune activation used a pro-inflammatory stimulation (e.g. LPS) to trigger the release of mitochondrial DAMPs[68,198,417]. For example, Joshi *et al.* showed that damaged (low ATP, low membrane potential) extracellular mitochondria released from LPS-activated microglia act as effectors of the innate immune system by targeting adjacent neurons and astrocytes[365]. On the other hand, several studies, including our own, have shown that mitochondrial proteins can be secreted in EVs in the absence of pro-inflammatory signals[375,391].

MDVs are shown to be involved in mitAP[167]. Antigen presentation is a proteolytic process, presenting endogenous antigens on both MHC class I and class II molecules inducing an immune response. Bonifaz *et al.* showed that mitochondrial function plays a role in antigen presentation by endogenously expressing hen egg-white lysozyme as a

model system for antigen presentation[421]. Matheoud *et al.* have shown that MDVs regulated mitAP, and both PINK1/Parkin and SNX9 regulate MDV formation and mitAP[167]. The role of Parkin in immune responses was further evaluated in Parkin knockout mice. In these experiments, these animals were injected with gram-negative bacteria, and these bacteria induced the establishment of cytotoxic mitochondrial CD8+ T cells in the brain, inducing neuronal damage that leads to PD symptoms[403]. Our results indicate that both SNX9 and Parkin are involved in IMM and matrix MDV regulation. We found that SNX9 is involved in both oxidative stress-induced, and mitAP MDV formation as loss of SNX9 affected both types of MDVs. From our results and Matheoud *et al.* article, we observe that Parkin mitigates inflammatory response by targeting two independent MDV pathways[167]. Matheoud *et al.* showed that Parkin activation blocks the mitAP MDV formation. However, in our studies, Parkin activated IMM MDV formation and directs those MDVs to the lysosome, blocking their release in EVs and thus inhibiting the inflammatory response. The differential targeting of MDV populations could be the consequence of the specific trigger used for mitAP MDVs, such as the use of LPS or heat shock inflammatory compared to our non-stimulated cells. However, in both cases, Parkin overexpression blocked the inflammatory pathway. Considering the role of Parkin mutations in Parkinson's disease, this pathway provides a possible mechanism for targeting oxidized mitochondrial proteins in EV and the transfer of damaged mitochondria to neighbouring neurons. It has been shown that α-synuclein can bind to the mitochondrial outer membrane [422] . Hence, it is possible that α-synuclein, along with mitochondrial proteins, get transported to neighbouring neurons and form aggregates. The targeting of oxidative stress-induced MDVs to lysosomes might be the cell's protective response against inflammation activation and against the onset of diseases. This protective mechanism can also be Parkin-independent as MDVs from OPA1 KO MEFs were also more targeted to lysosomes for degradation.

To study the inflammatory mechanisms regulated with mitochondrial fraction, we studied two different pro-inflammatory pathways, IP-10 and IL-6 response. We found that mitochondrial fractions and EVs activated distinct immune pathways, which is consistent with previous research. It has been shown that overexpression of neuronal

Mfn-2 specifically suppressed LPS-induced IL-1β, not IL-6, which elicit microglial activation but not astrocyte activation[423]. Similarly, intratracheal injection of cardiolipin increased IL-10 cytokine levels and decreased basal levels of IL-2 and IFN-γ[322]. These studies, including our results, indicate that the activation of specific pro-inflammatory pathways is selective and can be modulated according to the initial trigger. We found that upon oxidative stress, oxidized mitochondrial fraction activates distinct immune pathways. However, EVs from stressed cells did not activate an immune response. This is possibly due to a selective loss of inclusion of oxidized mitochondrial content in EVs. Puhm *et al.* have shown that the inflammatory response is similar between EVs with or without sonication [198]. This rule out the possibility that the absence of inflammatory response in EVs from mitochondrial damaged cells is because the immunogens are shielded within EVs. It has been shown that mitochondria functional loss in alveolar carcinoma cell line A549 Rho-0 cells result in an increased pro-inflammatory response in lung epithelial cells[364]. Uncontrolled release of mtDNA and subsequent PPRs recognition can lead to increased inflammasome activation and the onset of various diseases[273,275,362,405,424]. However, there is no previous report on the selective release of mitochondrial content in EVs and its correlation with inflammatory responses. We observed that MDVs regulate mitochondrial content and mtDNA levels in EVs. Decreased release of oxidized mitochondrial content in EVs, controls DAMPs in EVs, and inflammatory response. Hence, this is the first report to show a link between selective mitochondrial proteins in EV and its regulation of distinct inflammatory pathways.

The intercellular mitochondrial transfer has been shown to play an important role in mitochondrial regeneration. Along with mitochondrial transfer through nanotubes, we show here that MDVs play an important role in mitochondrial transfer in EVs. However, the mechanisms behind mitochondrial selection and transport of MDVs are still unclear. Also, the molecular cues in cell-cell communications and how cells recognize the need for functional mitochondria are not well-known. These cues might be essential in developing therapeutic treatments for diseases as they can identify the cells with the need of functional mitochondria. In addition, extracellular oxidized mitochondrial content can act as DAMPs to activate inflammatory response and play a critical role in chronic

inflammation and the onset of diseases. In line with our results, controlling the release of oxidized mitochondrial release by transporting the MDVs to the lysosome for degradation can serve a therapeutic purpose. Some studies are proposing to detect MDVs as a signature in Parkinson's disease[407,425]. Thus, identifying the molecular mechanisms for MDVs generation and its role in cell-cell communication will assist in developing therapeutic treatment in various diseases.

CHAPTER V

CONCLUSION

Our results demonstrate that cells constitutively incorporate mitochondrial DNA and proteins in EVs. MDVs play an important role in the selective release of mitochondrial proteins in EVs. We identified OPA1 and SNX9 as essential proteins for Parkin-independent MDV formation. Previous work has suggested that secretion of mitochondrial proteins could be a form of mitochondrial quality control where cells export functional mitochondria to damaged cells to regenerate mitochondria[199,279,283,376] or export their damaged mitochondria destined for degradation in distant cells[187,310,365]. However, extracellular oxidized mitochondrial components (mtDNA) can act as DAMPs to trigger inflammatory responses. We showed that cells transport oxidized mitochondrial fractions through MDVs to the lysosome for degradation and prevent an induction of inflammatory response. In this context, the mitochondrial quality control mechanism identified here would serve the dual role of preventing inflammation and ensuring that only functional mitochondrial components are transferred. In this context, it will be important to elucidate the uptake mechanism for extracellular mitochondria in recipient cells. In addition to its protective mechanism against inflammation, other functional roles of extracellular mitochondria in recipient cells should be identified to better understand these cellular processes. In addition, it will be interesting to study the putative Parkin-independent MDV pathways and identify the role of those pathways in mitochondrial release in EVs.

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APPENDIX: REVIEW ARTICLE

MITOCHONDRIAL INTERACTION WITH THE ENDOSOMAL COMPARTMENT IN ENDOCYTOSIS AND MITOCHONDRIAL TRANSFER

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Authors contribution

In this article, I was the Mitochondria quality control, transfer and immunity section whereas Lilia was responsible for the mitochondrial endocytosis section. I also wrote the conclusion and took care of the revisions. Me, Lilia and Marc Germain wrote the manuscript, which was critically reviewed by all authors.

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Abstract

Mitochondria are essential organelles required for cellular processes ranging from energy production to cellular differentiation. To perform these functions, mitochondria physically and functionally interact with other organelles such as the endoplasmic reticulum (ER) and endosomes. While the role of ER-mitochondria contact sites is well established, the interaction between mitochondria and endosomes has only recently been reported. These interactions are involved in lipid and ion transfer and potentially play a crucial role in mitochondria quality control and the release of mitochondrial components within extracellular vesicles. Here, we will discuss the current view of mitochondria-endosome interaction, both physically and functionally.

Keywords: Mitochondrial dynamics; Lysosome; Metabolism; Endocytosis; Mitochondria-derived vesicles; Extracellular vesicles

Abbreviations: ER, Endoplasmic reticulum; MDV,Mitochondria-derived vesicles; MSC, mesenchymal stem cells, SLC,Solute carrier; LDL, Low density lipid; mtDNA, Mitochondrial DNA; vCLAMP;Vacuole and mitochondria patch; VPS; Vacuolar protein sorting; PVM, Parasitophorus vacuole membrane; DAMP, Damage-associated molecular pattern; EV, Extracellular vesicles; HUVEC, Human umbilical vein endothelial cells

Introduction

Mitochondria play a key role in metabolic regulation including, but not limited to, ATP generation. Moreover, work over the last decades has demonstrated a variety of crucial roles for this organelle, from Ca^{2+} homeostasis to apoptosis and stem cell maintenance (Friedman and Nunnari, 2014; Zhang *et al.*, 2018). It has also become clear that mitochondria physically and functionally interact with several other organelles (Daniele and Schiaffino, 2014; Todkar *et al.*, 2017). These interactions establish the connection between organelles essential for homeostatic control of cellular processes. For example, ER-mitochondria contact sites regulate mitochondrial division, Ca^{2+} homeostasis, as well as lipid transfer between the two organelles (Daniele and Schiaffino, 2014; Elbaz and Schuldiner, 2011). More recently, mitochondria have been shown to interact with the endosomal compartment, from early endosomes containing endocytosed material (Charman *et al.*, 2010; Das *et al.*, 2016; Hamdi *et al.*, 2016; Sheftel *et al.*, 2007), to lysosomes that break down this material for subsequent usage by the cell (Aston *et al.*, 2017; Elbaz-Alon *et al.*, 2014; Han *et al.*, 2017; Hönscher *et al.*, 2014; Wong *et al.*, 2018). Two main roles are emerging for these interactions. First, consistent with the degradative function of lysosomes, mitochondria-endosomes interactions are involved in stress-responses and mitochondria quality control (McBride, 2018). Second, these interactions are emerging as part of a larger array of transport mechanisms that shuttle ions and metabolites across cytoplasmic organelles (Soto-Heredero *et al.*, 2017; Todkar *et al.*, 2017). In addition, mitochondrial activity is required for proper functioning of lysosomes (Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016). In this review, we will discuss the roles and potential mechanisms underlying the interaction between mitochondria and the endosomal compartment, as well as their relationship with mitochondrial transfer as a cell-to cell communication device.

Mitochondria and endosomes

Mitochondria produce ATP and important metabolic intermediates such as Acetyl-CoA, from soluble molecules such as glucose and amino acids that are taken up

by cells and distributed to organelles through members of the Solute Carriers (SLC) family of transporters (Lin *et al.*, 2015). Mitochondria also perform key steps in the synthesis of steroid hormones (Miller, 2013) and Iron-Sulfur clusters, inorganic cofactors required for a large array of biochemical reactions (Braymer and Lill, 2017). Mitochondria must thus specifically import the cholesterol and iron required for these synthesis pathways. Importantly, iron and cholesterol are not taken up by cells through SLC transporters. Rather, they enter cells by receptor-mediated endocytosis and accumulate within the endosomal system. This system is divided into a series of vesicles with distinct properties and markers: internalized material accumulates within early endosomes, where it is sorted into recycling endosomes (returning receptors the cell surface) or late endosomes (directing cargo towards lysosomal degradation) (Grant and Donaldson, 2009). As a consequence of the vesicular nature of this system, endocytosed material such as iron or cholesterol cannot be directly imported into mitochondria from the cytosol. However, recent evidence indicates that physical contacts between the two organelles allow direct transfer of iron and cholesterol from one to the other.

Iron was first proposed to reach mitochondria through a transfer across the cytosol. As free iron within the cytosol can lead to the generation of toxic reactive oxygen species (ROS), this cytosolic labile pool was proposed to be tightly regulated (Dixon and Stockwell, 2014). Nevertheless, recent studies have demonstrated that iron is instead directly transferred from endosomes to mitochondria through a physical interaction between the two organelles (Sheftel *et al.*, 2007). This process occurs through a transient "kiss and run" interaction that depends on the endosomal iron concentration, although the underlying mechanism remains to be defined (Das *et al.*, 2016; Hamdi *et al.*, 2016).

Cholesterol is another essential molecule that enters cells by receptor-mediated endocytosis. Cholesterol is required for mitochondrial DNA (mtDNA) maintenance, proper mitochondrial activity (Desai *et al.*, 2017) and the synthesis of steroid hormones for which the rate limiting step occurs within mitochondria (Arenas *et al.*, 2017; Rone *et al.*, 2012). As it cannot freely diffuse across the cytosol, endocytosed cholesterol cannot directly reach mitochondria following its release from low density lipoproteins (LDLs)

within lysosomes (Arenas *et al.*, 2017; Rone *et al.*, 2012). Recent evidence indicate that this transfer occurs through direct contact between the two organelles in a process that requires the late endosome protein MLN64 (Charman *et al.*, 2010).

In addition to interactions between mitochondria and the endosomal compartment, mitochondria are also recruited to the parasitophorous vacuole membrane (PVM) of a range of intracellular parasites including Leishmania and Toxoplasma. The PVM is formed by the invagination of the plasma membrane during parasite entry and serves to protect it from host responses. Interestingly, a recent study showed that the recruitment of mitochondria to the PVM allows the host cell to redirect nutrients, including fatty acids, towards mitochondria, preventing their uptake by the parasite and thus preventing parasite growth (Pernas *et al.*, 2018). Mitochondria can also directly promote pathogen killing by delivering mitochondrial-derived ROS (mROS) to bacteria-containing phagosomes using Mitochondria-Derived Vesicles (MDVs) (Abuaita *et al.*, 2018). MDVs are small vesicles that carry various mitochondria cargo to peroxisomes and lysosomes (Sugiura *et al.*, 2014). Bacterial killing requires a specific sub-population of MDVs containing the superoxide dismutase enzyme SOD2 that are delivered directly to the bacteria-containing phagosome (Abuaita *et al.*, 2018).

Recently, the concept of direct physical interactions between mitochondria and endosomes has been supported by studies in yeast and in mammalian cells. In yeast, direct interaction between mitochondria and the vacuole (the yeast equivalent of lysosomes) is regulated by two distinct tethers. First, the vacuole and mitochondria patch (vCLAMP) requires the endosomal protein Vps39 and TOM40, a component of the mitochondrial import machinery (González Montoro *et al.*, 2018). The second tether requires Vps13, a highly conserved protein that is thought to regulate lipid metabolism (Park *et al.*, 2016). Vps13 is recruited to mitochondria through Mcp1 (a mitochondrial protein of previously unknown function), where it promotes mitochondria-vacuole interaction (John Peter *et al.*, 2017). Consistent with the proposed role of mitochondria-vacuole interaction in the shuttling of lipids across organelles, those tethers are regulated in a nutrient-dependent fashion (Hönscher *et al.*, 2014) and required for survival under starvation conditions

(González Montoro *et al.*, 2018). Interestingly, while transfer of lipids between the endoplasmic reticulum (ER) and mitochondria was previously thought to require direct ER-mitochondria contact sites, Vps39 and Vps13 have recently been shown to provide an alternative route through vesicular transport and vacuole-mitochondria interaction (González Montoro *et al.*, 2018; Lang *et al.*, 2015). In fact, Vps39 and Vps13 can compensate for defects in the ER-mitochondrial encounter structure (ERMES), a protein complex physically bridging the two organelles. Specifically, yeast defective for ERMES can maintain survival in a Vps39 and Vps13-dependant manner by providing an alternative route for lipids synthesised in the ER, and potentially other important metabolites, to reach mitochondria (John Peter *et al.*, 2017).

Direct, but transient interactions between mitochondria and endosomes/lysosomes have also been reported in mammalian cells (Aston *et al.*, 2017; Han *et al.*, 2017; Wong *et al.*, 2018). However, the nature of the mammalian molecular tethers remains to be elucidated. Interestingly, in melanocytes, the lysosome-related organelle melanosome associates with mitochondria in a manner that depends on MFN2 (Daniele *et al.*, 2014), a mitochondrial fusion protein that also regulates ER-mitochondria contact sites (de Brito and Scorrano, 2008; Naon *et al.*, 2016). Nevertheless, recent studies suggest an important role for mammalian orthologs of Vps13, VPS13A and VPS13C. These proteins localize at contact sites between mitochondria and different organelles, including ER and endosomes (Kumar *et al.*, 2018), and have been suggested to transport lipids between these organelles (Kumar *et al.*, 2018). Interestingly, VPS13A interacts with Rab7, a key regulator of late endosome maturation (Muñoz-Braceras *et al.*, 2019), and its deletion impairs lysosomal function. This is of particular interest in the context of the recently discovered role of mitochondria in the regulation of lysosomal activity. Indeed, genetic alterations of mitochondria (TFAM, Pink1, OPA1 KO) or chemical inhibition of mitochondrial activity impairs lysosomal acidification and activity, resulting in the accumulation of enlarged endosomal/lysosomal structures (Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016). In this context, VPS13A-dependent alterations in Rab7 activity following the loss of mitochondrial function could affect endosomal maturation, contributing to the accumulation of large defective lysosomal structures present under

these conditions. It should be noted, however, that other mechanisms likely play a role (Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016; Fernandez-Mosquera *et al.*, 2019). Altogether, these results indicate a previously unrecognized level of cross-talk between mitochondria and endosomes that creates a robust network for exchange of metabolites between the ER, endosomes and mitochondria. In yeast, this network is required for efficient cell growth and response to nutrient stress. However, while it is likely that this network is functionally conserved in mammals (although molecular tethers could differ), this remains to be formally demonstrated.

Endocytosis and mitochondrial dynamics

In healthy cells, mitochondria exist as a highly interconnected network that is regulated through mitochondrial dynamics (Friedman and Nunnari, 2014). Mitochondrial dynamics, which includes mitochondrial fission and fusion, is required for the proper maintenance and segregation of mtDNA, and regulates key cellular processes such as cellular differentiation and apoptosis (Friedman and Nunnari, 2014). In addition, mitochondrial degradation through mitophagy requires mitochondrial fission, where the Dynamin-related protein DRP1 pinches off individual mitochondria from the network (Twig *et al.*, 2008). Intriguingly, several proteins required for endocytosis are also involved in the regulation of mitochondrial fission. DRP1-dependent fission occurs at ER-mitochondria contact sites in a process that is reminiscent of vesicle pinching during endocytosis. For example, both processes require the formation of actin bundles around the scission site prior the severing activity of a Dynamin (Klecker *et al.*, 2014). Interestingly, while DRP1 has been thought to be the main Dynamin responsible for mitochondrial fission, a recent study indicated that Dynamin 2, classically known for its role in clathrin-mediated endocytosis, also plays a role (Lee *et al.*, 2016). The extent of this contribution however remains unclear (Kamerkar *et al.*, 2018).

Mitochondrial fission has also been proposed to be regulated by the endosomal GTPase Rab7, in a process that requires transient mitochondria-lysosome interactions at mitochondrial fission sites (Wong *et al.*, 2018). In parallel to this, recent studies indicate

that mitochondrial fission is regulated by Vps35, a core component of the retromer, the retrograde transport system from endosomes/lysosomes back to the Golgi (Burd and Cullen, 2014). Two mechanisms have been proposed for the observed regulation of mitochondrial fission by Vps35. First, Vps35 has been shown to promote the degradation of the mitochondrial fusion protein Mfn2 (Tang *et al.*, 2015). Second, it causes the removal of inactive DRP1 from mitochondria, stimulating the activity of active DRP1 and thus mitochondrial fission (Wang *et al.*, 2017; Wang *et al.*, 2016). This Vps35-dependent regulation of mitochondrial dynamics also requires the endocytic fission protein and ATPase EHD1 (Farmer *et al.*, 2017).

The involvement of endocytosis proteins in the regulation of mitochondrial dynamics raises the question as to whether the two processes are linked, for example by competing for the same, limited pool of regulators. The studies that have measured the consequences of mitochondrial inhibition on endocytosis have shown diverging results depending on the cellular system and type of endocytosis studied (Dejonghe *et al.*, 2016; Hilgemann *et al.*, 2013; Marland *et al.*, 2016; Zimin *et al.*, 2018). Early studies have shown that mitochondrial ATP is required for endocytosis in some systems, but other aspects of mitochondrial function such as ROS production could also play a role (Hilgemann *et al.*, 2013). Interestingly, exposing cells to hydrogen peroxide, a prominent cellular ROS, inhibited receptor-mediated endocytosis of transferrin (Hsu *et al.*, 2018), but stimulated the process of massive endocytosis in BHK cells (Hilgemann *et al.*, 2013). While both studies support a role for mitochondria-derived ROS in the regulation of endocytosis, the discrepancies could be due to the fact that ROS are potent signalling molecules but generate cellular damage when in excess.

Mitochondria quality control, transfer and immunity

Given the above, it is not surprising that accumulation of dysfunctional mitochondria leads to impaired metabolism, increased ROS production and altered cellular functions. In addition, defective mitochondrial components can directly activate an inflammatory response under some circumstances, likely as a consequence of their

bacterial origin. For example, mitochondrial damage leads to the release of mtDNA to the cytosol, where it acts as a Damage-Associated Molecular Pattern (DAMP) that activates a STING-dependent type-I Interferon response (West *et al.*, 2015). Several mitochondria quality control pathways thus exist to efficiently clear damaged mitochondrial components from the cell before they elicit cellular damage or an inflammatory response. These quality control mechanisms include mitophagy (Hamacher-Brady and Brady, 2016; Lemasters, 2014), sequestration into endosomes (Hammerling *et al.*, 2017) and the degradation of selective mitochondrial content using MDVs (Soubannier *et al.*, 2012; Sugiura *et al.*, 2014). In some circumstances, mitochondria can also be exported for degradation to other cells through extracellular vesicles (Phinney *et al.*, 2015).

Both mitophagy and MDV formation depend at least in part on the activation of PINK1 and Parkin, two proteins mutated in early-onset Parkinson's Disease (Pickrell and Youle, 2015). Specifically, deletion of either gene results in a STING-dependent activation of an inflammatory response as a consequence of the loss of autophagic removal of damaged mitochondria (Sliter *et al.*, 2018). Interestingly, Parkin also regulates adaptive immunity by controlling the delivery of mitochondrial antigens to endosomes where they are loaded on MHC Class I molecules (Matheoud *et al.*, 2016). In this setting, PINK1 and Parkin supress the formation of specific MDVs that transport mitochondrial proteins to endosomes for antigen presentation, while stimulating MDVs that shuttle damaged oxidized cargo from mitochondria to lysosome for degradation (Matheoud *et al.*, 2016). Thus, PINK1 and Parkin control immune activation through the regulation of mitochondria quality control processes dependent on lysosomal degradation of defective mitochondria. Impairment of either mitochondria or lysosomes can therefore lead to dramatic consequences, both for individual cells and the organism (Audano *et al.*, 2018; Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016; Hughes and Gottschling, 2012).

Because of the intimate relationship between mitochondria and lysosomes, extensive mitochondrial damage could create a feed-forward loop where the accumulation of damaged mitochondria impairs lysosomes, leading to further mitochondrial damage (Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016). While this hypothesis could explain

the progressive impairment of both mitochondria and autophagy/lysosomes observed in neurodegeneration (Audano *et al.*, 2018; Plotegher and Duchen, 2017), this is probably not the only factor involved as experimental models where mitochondrial dysfunction is the primary driver of the pathology show little accumulation of dysfunctional mitochondria within lysosomes (Audano *et al.*, 2018; Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016). This could be the consequence of existing feedback mechanisms that coordinate mitochondria and lysosomes, such as TFEB activation (Baixauli *et al.*, 2015; Fernández-Mosquera *et al.*, 2017). A second possibility that has emerged in recent years is that, at least in some cases, damaged mitochondria are exported to distant cells for their degradation (Hayakawa *et al.*, 2016; Phinney *et al.*, 2015), circumventing the need for efficient lysosomal degradation.

Cells could potentially use several mechanisms to release mitochondrial content, depending on whether this content is present or not within the endosomal compartment. First, release of lysosomal material in the extracellular milieu can occur through lysosomal exocytosis, a process where a subset of lysosomes fuse with the plasma membrane (Appelqvist *et al.*, 2013). Interestingly, lysosomal exocytosis is stimulated by TFEB (Medina *et al.*, 2011), a transcription factor regulating lysosomal biogenesis in response to lysosomal or mitochondrial defects (Fernandez-Mosquera *et al.*, 2019; Settembre *et al.*, 2012). Material contained in intraluminal vesicles within multivesicular bodies (a type of late endosome) can also be secreted from the cell as small vesicles known as exosomes, while cytoplasmic material can directly bud off the plasma membrane to create larger vesicles named microvesicles (Akers *et al.*, 2013). These vesicles (collectively termed extracellular vesicles (EVs)) carry a variety of proteins, RNAs and DNA to distant cells where they are endocytosed to be degraded within lysosomes or modulate cellular activities (Torralba *et al.*, 2016). Interestingly, several studies have found that mitochondrial proteins are secreted in EVs (Burke *et al.*, 2014; Kowal *et al.*, 2016; Sugiura *et al.*, 2014). For example, a recent study showed that damaged mitochondria are exported by bone marrow-derived mesenchymal stem cells (MSCs) through EVs and captured by neighboring macrophages to degrade them (Phinney *et al.*, 2015). Similarly, neurons can shed mitochondria that are then degraded by neighboring astrocytes (Davis *et al.*, 2014).

However, a more intriguing possibility is that mitochondrial transfer serves a functional role. In fact, several studies have suggested that functional mitochondria can be taken up by cells and rescue aerobic respiration, both in cell culture and *in vivo* (Dong *et al.*, 2017; Islam *et al.*, 2012; Spees *et al.*, 2006). The uptake of mitochondrial proteins has been observed in a large number of cell types, but, interestingly, this occurs more readily in cells with mitochondrial defects (Griessinger *et al.*, 2017), suggesting an intimate relationship between mitochondrial defects and endocytosis of EVs. On the other hand, only a handful of cell types have been studied as donors in this system. The most studied of these cells are MSCs, supporting cells found in various tissues and that can differentiate into several cell types, mostly of mesodermal lineages. More importantly, MSCs are emerging as a crucial cell type promoting tissue repair through cell-to-cell communication (Fitzsimmons *et al.*, 2018). Consistent with this role, they secrete a large array of extracellular vesicles that are an essential part of the MSCs communication toolkit. Interestingly, many cells types release extracellular vesicles with mitochondrial protein content (Burke *et al.*, 2014; Kowal *et al.*, 2016), which suggests that long range mitochondrial transport could be a general phenomenon. However, it remains to be determined whether these similarly contribute to the metabolic regulation of distant cells, or if this is a MSC-specific role.

A second important question stemming from these observations is the mechanism through which this occurs. While some studies have shown that MSCs can transfer whole mitochondria (Dong *et al.*, 2017), either within vesicles or as free-floating entities, it remains to be determined whether this is a general phenomenon or if most EVs carry mitochondrial components rather than whole mitochondria. If the latter is the case, this would suggest the presence of a selective packaging system such as delivery of selective MDVs to multivesicular bodies. It will also be important to determine whether the transfer of specific mitochondrial components such as mtDNA could be sufficient to alter mitochondrial bioenergetic of a distant cell.

Overall, recent studies indicate that alterations in mitochondrial function not only cause metabolic stress and ROS production, but also regulates innate immunity with

possibly dramatic consequences in neurodegenerative diseases and cancer. Thus, damaged mitochondria must be eliminated through their delivery to lysosomes by MDVs or mitophagy. Interestingly, degradation of mitochondrial components could potentially be used as a signal to immune cells or other cells through antigen presentation or EV-mediated mitochondrial transfer. Alteration in these inter-cellular communication systems could also contribute to disease progression under some circumstances (Matheoud *et al.*, 2016).

Conclusion

While mitochondria are classically seen as the powerhouse of the cell, work over the last decades has revealed that they in fact perform a large number of functions. Many of the recently identified roles of mitochondria require intimate communication with other organelles, including the endosomal compartment. Mitochondrial and lysosomal dysfunction are individually associated with a wide range of diseases but our understanding of how the interaction between the two organelles participates in the pathogenicity is still limited. For example, while some studies have demonstrated a functional interaction between mitochondria and lysosomes, particularly in mitochondrial diseases and neurodegeneration, the underlying mechanisms remain elusive. In addition, the mitochondrial quality control mechanisms that are thought to limit the detrimental consequences of mitochondrial dysfunction have been linked to immune regulation and cell-to-cell communication, adding a new twist to mitochondria-endosome interactions. Mitochondria-endosome interactions are emerging as an exciting new area of research with several key questions that need to be addressed in priority. These include identifying the mechanisms through which mitochondria-endosome interactions occur in mammalian cells, and defining their relevance in normal physiology and in a disease context. In addition, as the identification of disease-specific changes in EV content could provide new diagnostic tools for these diseases, the physiological relevance of mitochondrial transfers, as well as the underlying mechanisms need to be defined. Answers to these key questions will provide crucial insights into diseases ranging from neurodegeneration to immunological disorders.

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