UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

IMPACT DE LA SANTÉ MITOCHONDRIALE SUR LE TRAFIC ENDOSOMAL ET LA DYNAMIQUE DES VÉSICULES EXTRACELLULAIRES

IMPACT OF MITOCHONDRIAL HEALTH ON ENDOSOMAL TRAFFICKING AND EXTRACELLULAR VESICLE DYNAMICS

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This thesis is dedicated to my family and friends……..

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RÉSUMÉ

Les mitochondries sont des organites essentiels qui soutiennent non seulement la bioénergétique cellulaire, mais régulent également un large éventail de processus, notamment la différenciation, la signalisation intracellulaire et la modulation immunitaire. Si le dysfonctionnement mitochondrial est depuis longtemps associé à une déplétion en ATP, de nouvelles données soulignent ses conséquences plus larges, telles que l'augmentation des espèces réactives de l'oxygène (ERO) et la perturbation de la communication entre les organites. Des études antérieures ont démontré que le dysfonctionnement mitochondrial était un facteur déclenchant d'une altération lysosomale ; cependant, son impact sur le réseau endocytaire au sens large, en particulier sur les endosomes précoces et de recyclage, n'a pas été bien caractérisé.

Nous avons étudié l'impact du dysfonctionnement mitochondrial sur le trafic endosomal et le positionnement des vésicules. En utilisant des approches génétiques et chimiques pour altérer la fonction mitochondriale, nous avons observé un regroupement périnucléaire sélectif et frappant des endosomes précoces, tandis que les endosomes tardifs et de recyclage maintenaient leur intégrité spatiale. Cette réorganisation spatiale a coïncidé avec un défaut de livraison de la cargaison aux lysosomes, révélant une altération du trafic intracellulaire. Fonctionnellement, nous avons identifié que ces défauts étaient médiés par des altérations induites par les ROS dans l'organisation des microtubules et le positionnement des centrosomes. Un traitement antioxydant a notamment restauré la distribution endosomale et l'efficacité du trafic, soulignant le rôle crucial des mitochondries, via la régulation des ROS, dans le maintien de la dynamique du cytosquelette et de la fonction endosomal.

Forts de ces résultats, nous avons voulu déterminer si le contenu mitochondrial des vésicules extracellulaires (VE) pouvait potentiellement remédier aux anomalies associées à un dysfonctionnement mitochondrial. Pour cela, nous avons d'abord examiné l'absorption et le trafic intracellulaire des VE dans des cellules présentant une altération de la fonction mitochondriale. Cette première étude était essentielle pour déterminer si ces cellules sont capables d'internaliser efficacement les VE et de les diriger vers les compartiments intracellulaires appropriés. Nous avons constaté que le dysfonctionnement mitochondrial, notamment dû à la perte d'OPA1, altère significativement l'absorption des VE et modifie leur dynamique de trafic. Alors que les VE des cellules sauvages étaient généralement acheminées vers les lysosomes, dans les cellules déficientes en OPA1, elles étaient associées aux mitochondries, suggérant une modification du traitement induite par le stress. Ces résultats soulignent non seulement l'influence de la santé mitochondriale sur l'internalisation des VE, mais ouvrent également la voie à de futures recherches visant à déterminer si les composants mitochondriaux délivrés via les VE peuvent restaurer l'homéostasie du trafic et l'organisation du cytosquelette dans les cellules dysfonctionnelles.

Dans l'ensemble, cette thèse présente les mitochondries comme des coordinateurs centraux du trafic intracellulaire et intercellulaire. En régulant les réseaux cytosquelettiques, la motilité des vésicules et la maturation endosomale, les mitochondries influencent la dynamique des flux de matière au sein et entre les cellules. Ces connaissances ont des implications pour divers contextes physiologiques et pathologiques, notamment la neurodégénérescence, le cancer et les maladies

métaboliques, où des perturbations de l'intégrité mitochondriale et du trafic vésiculaire sont fréquemment observées. À l'avenir, ces travaux poseront les bases pour tester si l'administration mitochondriale médiée par les VE peutrestaurer l'homéostasie fonctionnelle dans les cellules malades et apporteront des éclairages mécanistiques sur l'intersection entre la santé mitochondriale et la biologie des vésicules.

Mots clés : Mitochondries, Endocytose, Vésicules Extracellulaires, OPA1, Espèces Réactives de l'Oxygène, Microtubules, Centrosome, Cytosquelette, Trafic Cellulaire

ABSTRACT

Mitochondria are essential organelles that support not only cellular bioenergetics but also regulate a wide range of processes including differentiation, intracellular signaling and immune modulation. While mitochondrial dysfunction has long been associated with ATP depletion, emerging evidence emphasizes its broader consequences such as the increase of reactive oxygen species (ROS), perturbation of organelle crosstalk. Prior studies have demonstrated mitochondrial dysfunction as a trigger for lysosomal impairment; however, how it affects the broader endocytic network particularly early and recycling endosomes has not been well characterized.

Here, we investigated how mitochondrial dysfunction affects endocytic trafficking and vesicle positioning. Using genetic and chemical approaches to impair mitochondrial function, we observed a selective and striking perinuclear clustering of early endosomes, while late and recycling endosomes maintained their spatial integrity. This spatial reorganization coincided with defective cargo delivery to lysosomes, revealing an alteration in intracellular cargo trafficking. Mechanistically, we identified that these defects were mediated by ROS induced alterations in microtubule organization and centrosome positioning. Notably, antioxidant treatment restored endosomal distribution and trafficking efficiency, underscoring the crucial role of mitochondria via ROS regulation in maintaining cytoskeletal dynamics and endocytic function.

Building on these findings, we wanted to explore whether mitochondrial content within extracellular vesicles (EVs) could potentially rescue mitochondrial dysfunction associated defects. For this we first examined the uptake and intracellular trafficking of EVs in cells with impaired mitochondrial function. This initial investigation was essential for establishing whether such cells are capable of efficiently internalizing EVs and directing them towards appropriate intracellular compartments. We found that mitochondrial dysfunction, particularly due to OPA1 loss, significantly impaired EV uptake and altered their trafficking dynamics. While EVs in wild type cells typically routed to lysosomes, in OPA1-deficient cells they were associated with mitochondria, suggesting a stress induced shift in processing. These findings not only underscore the influence of mitochondrial health on EV internalization but also set the stage for future investigations into whether mitochondrial components delivered via EVs can restore trafficking homeostasis and cytoskeletal organization in dysfunctional cells.

Together, this thesis presents mitochondria as central coordinators of both intracellular and intercellular trafficking. By regulating cytoskeletal networks, vesicle motility, and endosomal maturation, mitochondria influence the dynamic flow of material within and between cells. These insights have implications for diverse physiological and pathological contexts, including neurodegeneration, cancer, and metabolic diseases, where disruptions in mitochondrial integrity and vesicle trafficking are frequently observed. Moving forward, this work lays the foundation for testing whether EV mediated mitochondrial delivery can restore functional homeostasis in diseased cells and provides mechanistic insights into the intersection of mitochondrial health and vesicle biology.

Keywords: Mitochondria, Endocytosis, Extracellular Vesicles, OPA1, Reactive Oxygen Species,

Microtubules, Centrosome, Cytoskeleton, Cellular Trafficking

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LIST OF ABBREVIATIONS AND ACRONYMS

ATP	Adenosine Triphosphate
ΔΨm	Mitochondrial Membrane Potential
AIFM1	Apoptosis Inducing Factor Mitochondria Associated 1
AKT	Protein Kinase B
ALIX	Apoptosis Linked Gene 2 Interacting Protein X
AMPK	AMP activated Protein Kinase
AP2	Adaptor Protein Complex 2
ATP	Adenosine Triphosphate
ApoE	Apolipoprotein E
Atg5	Autophagy Related Protein 5
BAR	Bin/Amphiphysin/Rvs
BBB	Blood Brain Barrier
CD9/CD63/CD81	Cluster of Differentiation markers (Exosome markers)
CI-M6PR	Cation Independent Mannose-6-Phosphate Receptor
CLASPs	Clathrin Associated Sorting Proteins
COPII	Coat Protein Complex II
COX-IV	Cytochrome c Oxidase Subunit IV
CoQ	Coenzyme Q / Ubiquinol
DRP1	Dynamin Related Protein 1
Drp1	Dynamin related Protein 1
EEA1	Early Endosome Antigen 1
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase

ESCRT	Endosomal Sorting Complex Required for Transport
ETC	Electron Transport Chain
EVs	Extracellular Vesicles
FADH	Flavin Adenine Dinucleotide
FasL	Fas Ligand
Fis1	Fission 1 Protein
GA	Golgi Apparatus
GAPs	GTPase Activating Proteins
GEFs	Guanine Nucleotide Exchange Factors
GJCs	Gap Junctional Channels
H2O2	Hydrogen Peroxide
HIF-1?	Hypoxia Inducible Factor 1-alpha
ICAM-1	Intercellular Adhesion Molecule 1
ILVs	Intraluminal Vesicles
KLF2	Kruppel Like Factor 2
LAMP1	Lysosomal Associated Membrane Protein 1
LHON	Leber Hereditary Optic Neuropathy
M6P	Mannose-6-Phosphate
Mφ-EVs	Macrophage Derived Extracellular Vesicles
MβCD	Methyl-β-Cyclodextrin
MAPK	Mitogen Activated Protein Kinase
MAPL	Mitochondrial Anchored Protein Ligase
MDCs	Mitochondria Derived Compartments
MDVs	Mitochondria Derived Vesicles
MFN1/2	Mitofusin 1 and 2
MHC	Major Histocompatibility Complex
MIRO	Mitochondrial Rho GTPase

MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MSC-EVs	Mesenchymal Stem Cell-Derived Extracellular Vesicles
MVBs	Multivesicular Bodies
Mff	Mitochondrial Fission Factor
MiD	Mitochondrial Dynamics Proteins
NADH	Nicotinamide Adenine Dinucleotide
NARP	Neurogenic Ataxia Retinitis Pigmentosa
NK cells	Natural Killer Cells
NSF	N-ethylmaleimide-Sensitive Factor
OPA1	Optic Atrophy 1
OPA1	Optic Atrophy Protein 1
OXPHOS	Oxidative Phosphorylation
PCr	Phosphocreatine
PDH-E1α	Pyruvate Dehydrogenase E1 Component Subunit Alpha
PI(3)P	Phosphatidylinositol 3-phosphate
PI(3,5)P2	Phosphatidylinositol 3,5-bisphosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PINK1	PTEN induced Putative Kinase 1
PLD	Phospholipase D
PTEN	Phosphatase and Tensin Homolog
POLG	DNA Polymerase Gamma
RME	Receptor Mediated Endocytosis
ROCK	Rho Associated Protein Kinase
ROS	Reactive Oxygen Species
Rab GTPases	Ras Related Protein Family (Guanosine Triphosphatases)
SGs	Secretory Granules

SNARE	Soluble NSF Attachment Protein Receptor
SNX	Sorting Nexin
SNX9	Sorting Nexin 9
SOD	Superoxide Dismutase
STX17	Syntaxin 17
TCA	Tricarboxylic Acid Cycle
TGF-β1	Transforming Growth Factor Beta 1
TGN	Trans Golgi Network
TNF-α	Tumor Necrosis Factor Alpha
TNTs	Tunneling Nanotubes
TOLLIP	Toll Interacting Protein
TOMM20	Translocase of Outer Mitochondrial Membrane 20
TRAIL	TNF Related Apoptosis Inducing Ligand
TSG101	Tumor Susceptibility Gene 101
Tf	Transferrin
VPS	Vacuolar Protein Sorting Complex
Vps	Vacuolar Protein Sorting
mPTP	Mitochondrial Permeability Transition Pore
mTOR	Mechanistic Target of Rapamycin
mtDNA	Mitochondrial DNA
OH	Hydroxyl Radical

CHAPTER I

INTRODUCTION

1.1 Mitochondria: Cell's Powerhouse

Mitochondria are highly dynamic organelle primarily known for generating ATP through oxidative phosphorylation. However, their role extends beyond energy production. They are involved in regulating cellular metabolism, controlling calcium levels, participating in apoptosis and contributing to various signalling pathways[1] [2]. To effectively carry out these diverse functions, mitochondria have established communication routes within their own network as well as with various other cellular components [3]. Through continuous fusion and fission, they adapt to changing cellular demands, maintaining structural integrity and quality control to support mitochondrial function [4]. Additionally, mitochondria closely interact with other organelles, such as the endoplasmic reticulum and lysosomes, to coordinate essential processes like calcium transfer and lipid metabolism [5].

1.1.1 Mitochondrial function

Mitochondria contain an enzymatic system that generates ATP through oxidative phosphorylation, which takes place in a series of protein complexes called electron transport chain embedded in the inner mitochondrial membrane [6]. Before this happens metabolic fuels like glucose, fatty acids, and amino acids are metabolised and enter the tricarboxylic acid cycle. Tricarboxylic cycle also called Krebs cycle extract high energy electrons from these molecules and store them in electron carriers such as NADH and $FADH_2$. These electrons are then transferred to the electron transport chain, that drives proton pumping across the inner mitochondrial membrane and facilitates ATP synthesis [7].

Mitochondrial ATP production is crucial for high energy demanding tissues like the heart, muscles and brain. Cardiomyocytes depend almost entirely on mitochondrial ATP for contraction, with over 90% of cardiac ATP generated by mitochondria [8]. Likewise, neurons require a constant ATP supply to support synaptic transmission and maintain ion gradients, underscoring the essential role of mitochondria in neuronal function and survival [9]. In addition to serving as a primary site for ATP production, mitochondria also contribute to the biosynthesis of nucleotides, fatty acids, cholesterol, amino acids, glucose, and heme. These biosynthetic pathways play a crucial role in stress responses

and are often dysregulated in disease [10 11].

Oxygen consumption and reactive oxygen species production occur universally as part of mitochondrial respiration. As electrons are transferred through the electron transport chain to drive ATP synthesis, a small fraction escapes before completing the process [12]. This makes mitochondria the primary source of reactive oxygen species within cells. Reactive oxygen species are actively involved in regulating key processes such as signaling pathways, cell proliferation, differentiation, migration and angiogenesis [13 14]. In addition to being dynamic, mitochondria are mobile. However, when intracellular calcium levels rise, mitochondrial motility decreases [15]. In high calcium regions, stationary mitochondria actively buffer excess calcium and regulates calcium level. Through this process, mitochondria function as intracellular calcium modulators, buffers, and sensors [16 17 18].

In addition to their metabolic and biosynthetic roles, mitochondria are increasingly recognized as central regulators of immune cell function. In immune cells, mitochondria not only provide ATP to fuel energy-intensive processes such as phagocytosis, cytokine production, and migration, but also serve as signaling platforms that shape immune responses [12]. Mitochondrial dynamics, including fusion and fission, influence the activation, differentiation, and survival of immune cells such as macrophages, dendritic cells, T cells, and B cells. For example, mitochondrial reactive oxygen species (ROS) act as signaling molecules that modulate the activation of the inflammasome and the production of pro-inflammatory cytokines [13]. Furthermore, mitochondria participate in the antiviral response by hosting the mitochondrial antiviral-signaling protein, which is essential for the induction of type I interferons following viral infection [15]. Mitochondrial metabolites, such as succinate and citrate, also play important roles in regulating immune cell metabolism and function, acting as signaling molecules that influence gene expression and inflammatory pathways [9]. Additionally, the release of mitochondrial DNA into the cytosol can trigger innate immune sensors and promote inflammation [17]. These multifaceted roles underscore the importance of mitochondria in orchestrating both innate and adaptive immune responses, highlighting their significance beyond classical bioenergetics and positioning them as key integrators of cellular metabolism and immunity [14].

Evolutionary stress led to the development of multiple communication pathways between mitochondria and other organelles through direct contacts. Many of these interactions are essential for maintaining cellular homeostasis and serve as key signaling hubs [3]. Mitochondria are known to interact with various organelles, including peroxisomes, the Golgi apparatus, the endoplasmic reticulum, and lysosomes [19]. Among these, the interaction between mitochondria and the ER is well

established. This connection plays a vital role in coordinating essential cellular functions, such as calcium signaling, apoptosis regulation, and the transfer of phospholipids between the ER and mitochondrial membranes [19] [20] [21]. In recent years, mitochondria and lysosome interactions have gained increasing attention due to their involvement in nutrient signaling and energy sensing [22]. One of the well known mechanisms of this interaction is mitophagy, the selective degradation of mitochondria by lysosomes [22] [3]. Beyond mitophagy, mitochondria and lysosome interactions play a crucial role in regulating the dynamics of both organelles. Mitochondrial fission occurs via two distinct modes: midzone fission (central part of mitochondria), supporting proliferation, and peripheral fission (near mitochondrial tips), linked to mitophagy [23]. Lysosomal contacts serve as key sites for mitochondrial peripheral fission, allowing lysosomes to influence mitochondrial network organization and to promote the removal of damaged mitochondria [24] [25]. In turn, mitochondria contribute in regulating lysosomal structure and function, ensuring proper coordination between the two organelles. Through these bidirectional interactions, mitochondria and lysosomes work together to maintain cellular function and homeostasis [26] [27]. To perform these essential functions, mitochondria must preserve their health by maintaining structural integrity through optimal mitochondrial fusion and fission.

1.1.2 Mitochondrial structure

Mitochondria possess a unique and highly specialized structure comprising an outer membrane, an inner membrane, an intermembrane space, and a central matrix. Each of these structural components plays a distinct role in facilitating mitochondrial activities [28] [29].

The outer membrane is porous and encapsulates the organelle, providing protection while allowing selective exchange of ions and small molecules via channel forming proteins known as porins. These porins enable the passage of metabolites such as ADP, ATP, and phosphate, essential for cellular metabolism [30]. The membrane also houses the large GTPases Mitofusin 1 and Mitofusin 2, which mediate mitochondrial fusion. In addition, proteins anchored to the outer membrane, such as Fission protein 1 (FIS1), play a critical role in recruiting the mitochondrial fission machinery [31]. In addition to mitochondrial dynamic protein outer membrane also house anti apoptotic Bcl-2 family proteins that regulate cytochrome c release by inhibiting the activation of pro apoptotic family members Bax and Bak [32].

Through the outer mitochondrial membrane, mitochondria establish functional contact sites with nearly all other cellular organelles, facilitating a wide range of physiological processes and enabling the coordination of diverse cellular functions [33]. Although the outer mitochondrial

membrane appears relatively smooth and flat, the inner mitochondrial membrane exhibits a highly folded and dynamic architecture. It consists of two distinct regions: the inner boundary membrane, which runs parallel to the outer mitochondrial membrane, and the cristae distinctive invaginations that extend into the mitochondrial matrix [33]. Despite being continuous, these two domains serve different functional roles. The cristae are primarily responsible for housing the electron transport chain and the ATP synthase complexes necessary for oxidative phosphorylation, whereas the inner boundary membrane is enriched with protein translocases, ion channels, and components of the mitochondrial dynamics machinery [34]. The extensive folding of the inner mitochondrial membrane significantly increases its surface area, allowing a high density of enzymatic complexes to be accommodated within a compact mitochondrial volume, thereby enhancing metabolic efficiency [32].

The intermembrane space of mitochondria is enclosed between the outer and inner mitochondrial membranes. As these membranes are separated by only a few nanometers, the intermembrane space occupies a compartment of very small volume [30]. Despite its limited size, it plays a crucial role in energy transduction. During electron transport, protons are pumped into this space, creating a high proton concentration relative to the matrix and establishing the electrochemical gradient essential for ATP synthesis [35]. Moreover, the intermembrane space is enriched with proteins involved in molecular translocation and apoptotic signaling pathways [36]. Since all matrix destined proteins imported from the cytoplasm must travel both the outer and inner membranes, they necessarily pass through the intermembrane space [30].

The matrix, the innermost compartment of the mitochondria, is a viscous gel like substance that contains enzymes required for the tricarboxylic acid cycle and β oxidation of fatty acids, generating electron donors for the electron transport chain [37]. It is also the crucial site for key metabolic reactions, including the decarboxylation of pyruvate and the synthesis of urea and heme groups. The matrix also houses mitochondrial DNA (mtDNA), ribosomes, and tRNAs, and is close to the electron chain complexes. mtDNA present in matrix of mitochondria encodes important proteins for electron transport chain essential for ATP synthesis [38]. The unique structure of mitochondria creates an optimal environment for numerous biochemical processes, including protein and lipid biosynthesis, as well as mtDNA replication [30]. This dynamic and highly organized architecture is crucial for efficient energy production. In particular, the extensively folded inner membrane forms the structural foundation for the electron transport chain and ATP synthesis [38 39].

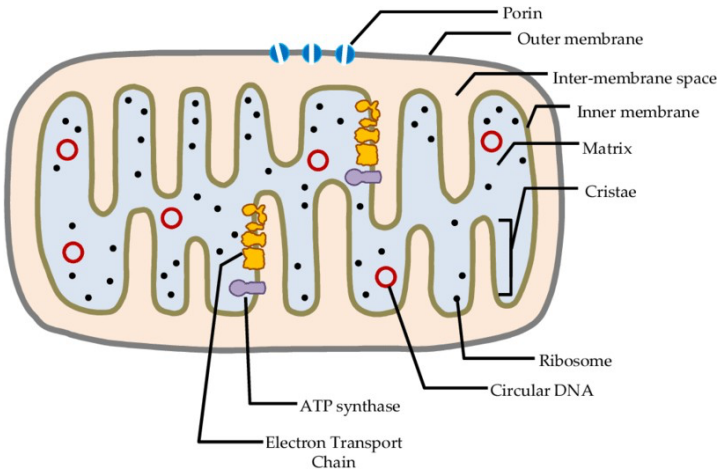

Figure 1.1.2: Mitochondrial structure

The diagram illustrates key mitochondrial components, including the outer and inner membranes, intermembrane space, matrix, and cristae. Functional elements such as porins, the electron transport chain (ETC), ATP synthase, circular mitochondrial DNA, and mitochondrial ribosomes are also shown, highlighting the organelle's roles in bioenergetics and protein synthesis [39].

1.1.3 Electron transport chain

The ETC is embedded within the inner mitochondrial membrane, positioned near the mitochondrial matrix, where the tricarboxylic acid cycle occurs. It facilitates the transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) produced in the tricarboxylic acid cycle to oxygen, driving ATP synthesis. This process occurs across Complexes I, II, III, and IV, ensuring efficient energy production while simultaneously influencing reactive oxygen species (ROS) signaling and cellular regulation [40]. Complexes I and III are the most relevant sites of reactive oxygen species production within the electron transport chain [41] [42].

Electrons enter the electron transport chain via two distinct pathways: Complex I, which receives electrons from NADH, and Complex II, which receives electrons from $FADH_2$. Both complexes transfer their electrons to ubiquinone (coenzyme Q), reducing it to ubiquinol (QH_2) [40]. Ubiquinol then carries the electrons to Complex III, where it is oxidized in a process known as the Q cycle. This step transfers electrons one at a time to cytochrome c and simultaneously pumps 4 protons into the intermembrane space for every pair of electrons processed. Cytochrome c then shuttles electrons to Complex IV, where molecular oxygen (O_2) acts as the final electron acceptor, being reduced to water (H_2O). During this reaction, 4 protons are pumped into the intermembrane space,

while 2 protons are consumed in the reduction of oxygen, resulting in a net translocation of 2 protons at Complex IV [43].

The accumulated protons in the intermembrane space generate an electrochemical proton gradient, known as the mitochondrial membrane potential ($\Delta\Psi m$). This, combined with proton concentration, forms the proton motive force, which is essential for energy generation. The proton motive force couples electron transport (Complexes I-IV) to ATP synthesis at Complex V (ATP synthase). Complex V is a multi subunit enzyme with two domains: the extramembranous F_1 and the transmembrane F_0. Protons reenter the mitochondrial matrix through F_0, dissipating the proton gradient. This movement drives the rotation of the F_0 domain, inducing conformational changes in F_1, where ADP is phosphorylated to ATP [43 44].

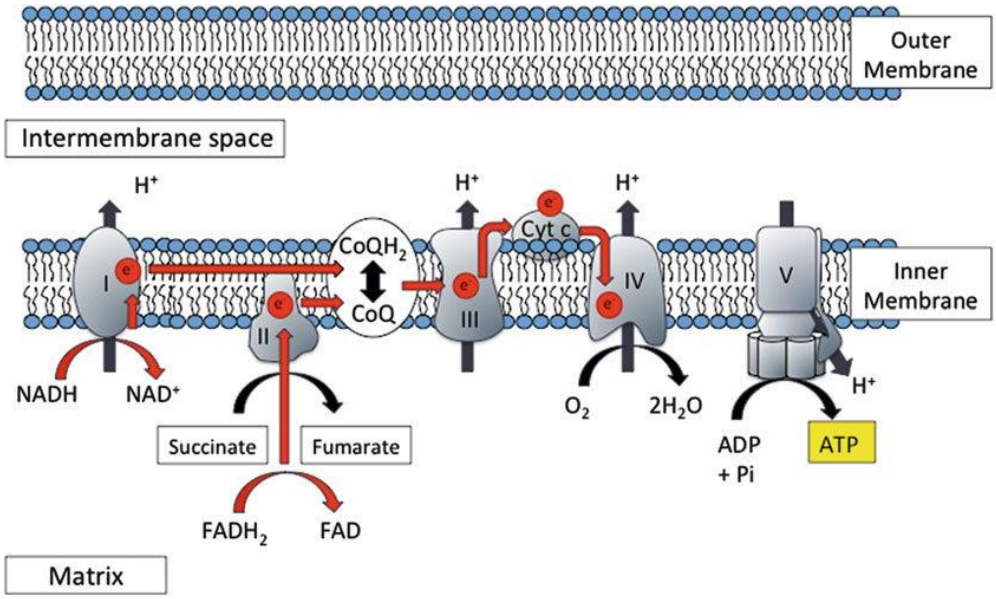

Figure 1.1.3: Electron transport chain complex

Electrons are transferred sequentially through Complexes I to IV of the electron transport chain, during which some electron leakage may occur. Ultimately, electrons are transferred to molecular oxygen (O_2), forming water. The movement of electrons drives the pumping of protons (H^+) across the inner mitochondrial membrane, generating a proton gradient. This electrochemical gradient provides the energy for ATP synthesis by Complex V (ATP synthase). In this process, CoQ (ubiquinone) is reduced to $CoQH_2$ (ubiquinol) as it shuttles electrons between complexes [44].

Maintaining the proper function of ETC complexes (I–V) is essential for mitochondrial energy production and metabolic homeostasis. However, mutations or defects in either mitochondrial encoded ETC complexes or nuclear DNA can severely disrupt this balance, leading to metabolic dysfunction and diseases [45]. Complex I deficiency is the most commonly observed mitochondrial respiratory chain disorder and is linked to a variety of clinical conditions [46]. Studies have shown that Complex I deficiency in mouse models leads to neurological and cardiac phenotypes, associated with partial functional loss of Complex I [47]. Additionally, mutations in Complex IV have been associated with Acute Myeloid Leukemia, resulting in the undifferentiated proliferation of myeloid progenitor cells [47].

To further investigate ETC dysfunction and its impact on cellular metabolism, researchers have utilized chemical inhibitors that selectively block specific ETC complexes. Commonly used inhibitors are antimycin A and rotenone. Rotenone inhibits electron transfer from Complex I to ubiquinone, disrupting ATP synthesis. The incomplete transfer of electrons results in increased ROS production, which can lead to mitochondrial component damage [48] [49]. Similarly, Antimycin A inhibits Complex III, preventing the oxidation of ubiquinol in the electron transport chain. This disruption blocks electron transfer between cytochrome b and cytochrome c, leading to the collapse

of the proton gradient and a loss of mitochondrial membrane potential [50]. Consequently, mitochondrial dysfunction occurs, marked by increased ROS production and reduced ATP levels [51].

1.1.4 Reactive oxygen species

Mitochondria continuously consume oxygen to produce ATP through oxidative phosphorylation, a process that also generates ROS as byproducts of electron transport [52]. ROS is a general term for highly reactive oxygen derived molecules. These oxidants include molecules that are free radicals and non radicals. Free radicals are derived from oxygen and include superoxide anion, its protonated form perhydroxyl radical, and hydroxyl radical. Non radical reactive oxygen species include hydrogen peroxide, singlet molecular oxygen and singlet molecule oxygen. They are produced by various cellular compartments, including cytoplasm, cell membrane, endoplasmic reticulum, and mitochondria. Among these compartments, mitochondria are the major contributor of ROS production which are produced when there is small fraction of electrons that escapes from ETC. Superoxide anions (O_2^-) are the most abundant ROS generated in mitochondria and are considered the primary form of mitochondrial ROS. During oxidative phosphorylation, an estimated 0.4–4% of the oxygen consumed is partially reduced to form superoxide. This superoxide is rapidly converted by mitochondrial superoxide dismutase into hydrogen peroxide (H_2O_2), which can further give rise to highly reactive hydroxyl radicals (•OH). Under conditions of excessive ROS production, superoxide and its derivatives can react with other cellular molecules, generating additional, more damaging species often referred to as “secondary” ROS [12].

Mitochondrial ROS generation is generally low under physiological conditions but fluctuates depending on the efficiency and state of ETC function. Different sites within the mitochondria contribute to ROS production, and each responds distinctly to cellular conditions [41]. For example, Complex I tends to generate more ROS when the $NADH/NAD^+$ ratio is high and electron flow is slowed, such as during nutrient excess or low ATP demand [12]. In contrast, Complex III produces ROS more prominently under high mitochondrial membrane potential and when the Q cycle becomes over reduced [53]. Factors like nutrient availability, ATP production rate, and membrane potential influence the redox state of these complexes, thereby modulating the rate and site of ROS generation within the ETC [54]. H_2O_2 produced by breakdown of superoxide anions is more stable and membrane permeable molecule [55]. Although often associated with oxidative damage, H_2O_2 at low physiological concentrations typically in the nanomolar range is not merely a byproduct but a regulated signaling molecule [56]. At these controlled levels, H_2O_2 acts as a key second messenger,

selectively modifying redox sensitive protein targets involved in metabolic regulation, antioxidant defence, and stress adaptation [57]. This role in intracellular signaling reflects a major shift from the historical view of ROS as purely deleterious agents to one recognizing their importance in maintaining cellular homeostasis and coordinating adaptive responses to environmental changes [58 59 60].

ROS exert their effects by oxidatively modifying different types of proteins, including receptors, kinases, phosphatases, caspases, ion channels, and transcription factors [61]. Notably, ROS generated at Complex III are essential for the stabilization of hypoxia inducible factor-1α (HIF-1α), which promotes the proliferation of vascular smooth muscle cells, endothelial cells, and erythroid progenitors [62]. Beyond their role in cell proliferation, ROS are also involved in key protein kinase signaling cascades, such as the protein kinase B (AKT), AMP activated protein kinase (AMPK), and mitogen activated protein kinase (MAPK) pathways, which regulate the balance between autophagy and apoptosis [63]. Under hypoxic conditions, ROS activate AMPK, which enhances cytoprotective autophagy by inhibiting mammalian target of rapamycin (mTOR) activity, thereby promoting cell survival [64].

While ROS play essential roles in cellular signaling, their overproduction can be detrimental. Oxidative stress is induced when excessive ROS production outpaces antioxidant defense [65]. Excess ROS can damage mitochondrial proteins, enzymes, membranes, and DNA, ultimately disrupting ATP generation and other critical mitochondrial functions [66]. To maintain balance, cells have evolved multiple defense mechanisms to counteract oxidative damage. These include strategies to reduce free radical production and scavenge excess ROS through a diverse system of enzymatic and non enzymatic antioxidants. The enzymatic defense system includes antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase, which work to neutralize harmful ROS [53].

Among these defense mechanisms, the superoxide dismutase family plays a critical role in detoxifying superoxide anions ($O_2 \cdot^-$) by catalyzing their conversion into H_2O_2. This reaction is carried out by copper and zinc SODs in the intermembrane space and manganese SOD in the mitochondrial matrix [43]. However, H_2O_2 itself can be harmful, and its breakdown requires the action of specific enzymes, including catalase, thioredoxin peroxidase, and glutathione peroxidase [67]. Catalase is primarily localized in peroxisomes and may not play a major role in eliminating mitochondrial ROS, except in heart and liver mitochondria, where it is essential for converting H_2O_2 into water [68]. On the other hand, glutathione peroxidase and thioredoxin peroxidase act directly in

mitochondria, using reduced glutathione and thioredoxin, respectively, to convert H_2O_2 into water. The resulting oxidized glutathione and thioredoxin are subsequently recycled back to their reduced forms by glutathione reductase and thioredoxin reductase. In addition to enzymatic protection, non enzymatic antioxidants, including Vitamin E, Vitamin C, glutathione, carotenoids, and flavonoids, help shield cells from oxidative damage [68].

Altogether, mitochondria are complex and highly specialized organelles whose structure is finely tuned to support their diverse functions, including ATP production, metabolic processing, and redox signaling [37]. The organization of their membranes and internal compartments ensures that these processes occur efficiently and in coordination [69]. Yet, maintaining mitochondrial health and performance goes beyond structure alone as mitochondria are highly dynamic, continuously reshaping themselves to meet changing cellular needs. This ability to fuse, divide, and reorganize is central to how mitochondria respond to stress, distribute energy, and preserve their function over time [70].

1.1.5 Mitochondrial dynamics

Mitochondria are highly dynamic organelles that continuously undergo fission, fusion, mitophagy, and transport cycles, processes that collectively regulate mitochondrial function, morphology, quality, quantity, and distribution within the cell [71]. Like other organelles, mitochondria regularly repair and replace damaged components to maintain their function. However, due to their central role in energy metabolism and constant exposure to ROS, they are particularly reliant on efficient quality control systems to preserve their integrity [22]. Through mitochondrial dynamics, damaged components can be selectively removed, or impaired mitochondria can be eliminated via mitophagy, preventing further cellular damage. Fission plays a key role in mitochondrial quality control by isolating damaged or dysfunctional mitochondria, allowing their removal through mitophagy [72]. It also contributes to apoptosis a programmed cell death when cells experience severe stress. In contrast, fusion enables the exchange and mixing of intramitochondrial contents, helping to maintain mitochondrial integrity and function [73]. Mitochondrial transport further ensures that mitochondria are positioned in cellular regions where energy demand is highest, enabling efficient ATP production and metabolic regulation [74] [1]. Maintaining a balance between these processes is crucial for optimal mitochondrial function and cell survival [75] [73].

Mitochondrial fission is a multi step process where one mitochondrion splits into two. It primarily occurs at sites where the outer mitochondrial membrane constricts, a process driven by actin polymerization at interactions site of mitochondria and endoplasmic reticulum [76]. This process

requires the recruitment of the GTPase enzyme Drp1, which is facilitated by various outer mitochondrial membrane adapter proteins, including mitochondrial fission 1 (Fis1), mitochondrial fission factor (Mff), mitochondrial dynamics protein (MiD)-49, and MiD51 [77]. Once recruited, Drp1 undergoes oligomerization and is activated by the mitochondrial specific lipid cardiolipin, forming large helical structures that enhance GTPase activity at the mitochondrial fission foci. The nucleotide driven allosteric regulation of Drp1 enables its self assembly, conformational transformation, and disassembly, ultimately allowing it to encircle mitochondria and induce mitochondrial fission [78] [79] [80].

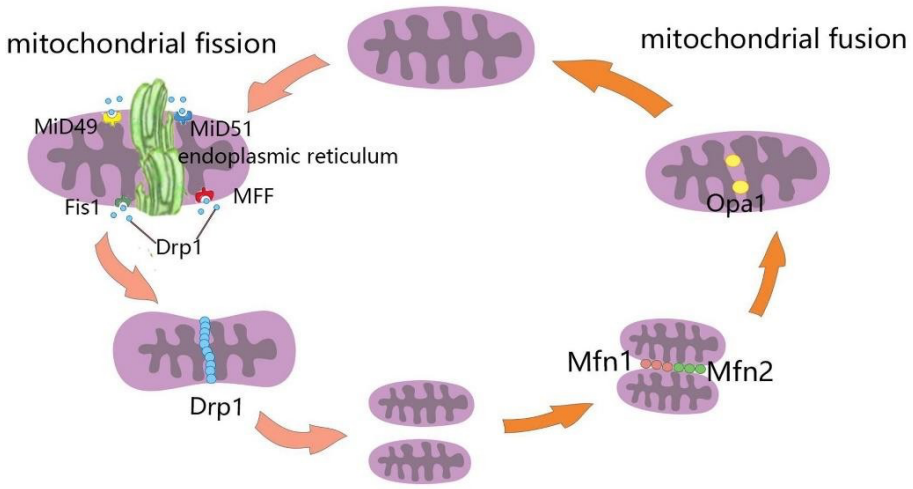

Figure 1.1.5: Mitochondrial dynamics: fission and fusion

Mitochondrial fission involves the division of a single mitochondrion into two daughter organelles and is primarily mediated by the GTPase Drp1. Drp1 is recruited from the cytosol to the outer mitochondrial membrane (OMM) by its receptors: Mff, MiD49, MiD51, and Fis1, where it oligomerizes into a constriction ring and hydrolyzes GTP to drive membrane scission. In contrast, mitochondrial fusion maintains mitochondrial network connectivity. Mfn1 and Mfn2, located on the OMM, mediate outer membrane tethering and fusion, while Opa1, located in the inner mitochondrial membrane, is responsible for inner membrane fusion and is also involved in cristae remodeling. Together, these proteins maintain mitochondrial morphology, distribution, and function [80].

Inhibiting Drp1 activity through dominant inactivation mutations leads to the formation of elongated and tangled mitochondria [78]. Moreover, genetic knockout of Drp1 in mice results in embryonic lethality, highlighting the essential role of Drp1 dependent mitochondrial division in embryogenesis [79]. Beyond its role in regulating mitochondrial morphology and function, mitochondrial fission serves several other critical functions. Fission facilitates mitochondrial transport by dividing mitochondria into smaller, separate organelles, enabling their efficient movement along the cytoskeletal network and ensuring proper mitochondrial distribution within the cellular region [81]. Mitochondrial fission facilitates apoptosis by promoting Bax (pro apoptotic

factor) mediated permeabilization of the outer mitochondrial membrane, enabling cytochrome c release and programmed cell death; supporting this, studies have shown that inhibition of mitochondrial fission through Drp1 knockdown delays cytochrome c release and subsequently reduces apoptosis [72]. Additionally Drp1 mediated fission is believed to support mitophagy by fragmenting mitochondria into sizes suitable for autophagosome engulfment [82] [83] or by isolating damaged mitochondrial regions for targeted degradation [84] [85].

Mitochondrial fusion occurs in a series of coordinated steps, beginning with the activation of dynamin related GTPases, including mitofusin (MFN) 1/2 on the outer mitochondrial membrane and optic atrophy protein 1 (Opa1) on the inner mitochondrial membrane. This process starts with outer mitochondrial membrane fusion, driven by GTP hydrolysis, followed by inner mitochondrial membrane fusion, and ultimately results in the mixing of intra mitochondrial components [86]. By allowing the exchange of mitochondrial proteins, mtDNA, and other matrix components, mitochondrial fusion helps dilute dysfunctional proteins and mutated mitochondrial DNA, thereby promoting mitochondrial homogeneity and functional stability [34].

In addition to regulating inner mitochondrial membrane fusion, OPA1 plays a crucial role in maintaining cristae integrity, mitochondrial DNA stability, and mitochondrial bioenergetics. As a result, mutations or deficiencies in OPA1 can directly impact cytochrome c release and oxidative respiratory efficiency [79]. Furthermore, mutations in the OPA1 gene are identified in 60–70% of autosomal dominant optic atrophy (ADOA) cases, a condition characterised by the loss of retinal ganglion cells and early onset visual impairment [1]. Additionally, a deficiency in MFN1 leads to highly fragmented mitochondrial morphology, whereas cells lacking both MFN2 and OPA1 exhibit a high percentage (85%) of mitochondria appearing as spherical or oval shaped structures, indicating severe alterations in mitochondrial dynamics [86].

1.1.6 Mitochondrial quality control

Dynamic remodeling alone is not sufficient to preserve mitochondrial integrity over time. If damaged mitochondria or misfolded proteins accumulate, they can impair bioenergetic efficiency, disrupt cellular signaling, and contribute to oxidative stress [6]. To counteract this, cells have evolved a sophisticated mitochondrial quality control system, which detects, repairs, and eliminates impaired mitochondria [6]. Mitochondrial quality control encompasses a range of mechanisms, including mitophagy: the selective degradation of damaged mitochondria, proteostasis: the removal and replacement of dysfunctional mitochondrial proteins, and mitochondrial biogenesis, which replenishes the mitochondrial network with newly synthesized organelle.

Mitophagy, a conserved process, selectively removes dysfunctional or excess mitochondria through autophagy, playing a critical role in maintaining both mitochondrial quantity and quality control [6]. This complex and dynamic process occurs in two key steps. First, damaged or dysfunctional mitochondrial regions are identified and selectively enclosed within double membraned autophagosomes [87]. Second, these autophagosomes fuse with lysosomes, forming autolysosomes, where mitochondrial degradation is facilitated by hydrolases [88]. In mammals, the PTEN induced kinase 1 (PINK1) Parkin pathway has been identified as a key regulator of mitophagy. Under normal conditions, PINK1 is rapidly cleaved and degraded in a proteasome dependent manner within the inner mitochondrial membrane. However, in damaged mitochondria, loss of inner mitochondrial membrane potential prevents PINK1 degradation, leading to the accumulation of full length PINK1 with kinase activity at the outer mitochondrial membrane [89]. This accumulation recruits Parkin, an E3 ubiquitin ligase, to the outer mitochondrial membrane, where PINK1 phosphorylates ubiquitin, activating Parkin's ubiquitin ligase function. Parkin mediated ubiquitination facilitates the degradation of multiple outer mitochondrial membrane proteins, including MFN1 and MFN2, while also recruiting autophagy receptors such as p62 and optineurin [89]. These receptors link ubiquitinated mitochondria to LC3 positive autophagosomes, leading to their selective elimination [90]. Impaired mitophagy occurs when cells fail to effectively remove dysfunctional mitochondria, resulting in their accumulation and disruption of mitochondrial function [91].

Most mitochondrial proteins are not encoded by mitochondrial DNA but are instead imported as unfolded polypeptides through a highly selective mechanism [92]. This process ensures that proteins required for mitochondrial function are efficiently delivered and correctly assembled within the organelle. Proteins containing a mitochondrial targeting sequence are specifically directed to mitochondria, where their import is dependent on mitochondrial membrane potential ($\Delta\Psi m$). This import requires the coordinated activity of the outer membrane translocase and inner membrane translocase [93]. Together, outer membrane translocase and inner membrane translocase, along with chaperones and proteases, facilitate the translocation of pre proteins, cleavage of the mitochondrial targeting sequence, and proper folding of the proteins into their native conformation within mitochondria [92]. Inside the mitochondrial matrix, Hsp60 chaperonin, composed of Hsp60 and Hsp10 subunits, plays a crucial role in directing protein transport, assisting in folding, and ensuring proper localization of small soluble proteins [94 95]. Another key chaperone, mtHsp70, participates in protein import, folding, and complex assembly, while also being essential for iron–sulfur cluster formation [96 97 98]. Both Hsp60 and Hsp70 contribute to protein folding and ETC complex assembly,

while also preventing the aggregation of unfolded or misfolded proteins, ensuring mitochondrial protein stability and functionality [93].

Proteins that fail to fold or assemble properly within the mitochondrial matrix or intermembrane space are typically targeted for degradation by a specialized system of mitochondrial proteases. Among these, ClpXP and Lon are matrix localized AAA proteases responsible for the degradation of soluble proteins [99]. Notably, Lon protease has been shown to preferentially degrade oxidized proteins, such as aconitase, preventing the accumulation of damaged components [100]. Additionally, the matrix localized (m-AAA) protease and the inter membrane space localized (i-AAA protease) play a crucial role in eliminating misassembled or unfolded oxidative phosphorylation proteins, ensuring mitochondrial protein quality control and maintaining bioenergetic stability [101]. While mitochondrial proteases efficiently clear dysfunctional proteins within the matrix and intermembrane space, outer mitochondrial membrane proteins require additional support due to their exposure to the cytoplasm. Given its strategic position at the interface between mitochondria and the cytosol, the outer mitochondrial membrane relies on the cytoplasmic ubiquitin proteasome system for protein degradation. The ubiquitin proteasome system specifically targets and degrades ubiquitin tagged proteins, ensuring the removal of damaged or unneeded outer mitochondrial membrane components [101]. Several mitochondrial E3 ubiquitin ligases localized in the outer mitochondrial membrane have been identified as key regulators in mitochondrial dynamics, stress response, and apoptosis, further highlighting the importance of ubiquitin mediated proteostasis in maintaining mitochondrial function.

Additionally, mitochondria can eliminate damaged or potentially harmful lipids, proteins, and other cellular components through mitochondria derived vesicles (MDVs). These vesicular structures, formed from mitochondrial membranes, facilitate the transport of materials between mitochondria and other organelles under normal physiological conditions [102]. The production of MDVs is significantly upregulated following peroxide stimulation, serving as an early protective response against oxidative stress [103]. The process is initiated by microtubule associated adaptor proteins MIRO1/2, which induce the protrusion of specific mitochondrial membrane regions. While DRP1 was initially thought to be not involved in MDV formation [104], a recent study suggest that DRP1 receptors MID49, MID51, and MFF help recruit DRP1 to these membrane protrusions, leading to membrane fission and the formation of MDVs containing specific cargo [105 106]. Once formed, MDVs are directed to different cellular destinations. Some are transported to peroxisomes via the outer membrane mitochondrial anchoring protein ligase, while others are delivered to lysosomes through the Parkin pathway, where their contents are degraded and processed [102].

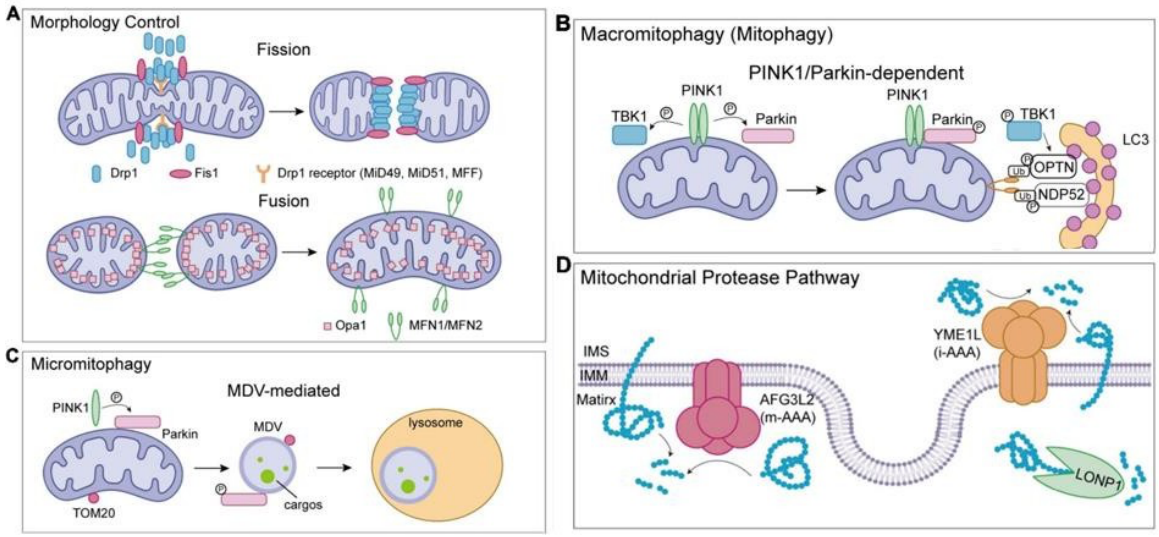

Figure 1.1.6: Mitochondrial quality control pathways

Mitochondrial morphology is regulated by a dynamic balance between fission and fusion events. A) Fission is mediated by the GTPase Drp1, which is recruited to the outer mitochondrial membrane by receptors including Fis1, MFF, and MiD49/51, while fusion involves mitofusins (MFN1 and MFN2) on the outer membrane and OPA1 on the inner membrane. B) Damaged mitochondria are selectively eliminated through macromitophagy, a process initiated by the accumulation of PINK1 on the outer membrane, which recruits and activates the E3 ligase Parkin. This leads to the ubiquitination of outer membrane proteins and the recruitment of autophagy adaptors such as OPTN and NDP52, facilitating mitochondrial engulfment by autophagosomes. C) In parallel, micromitophagy enables the removal of specific mitochondrial components via mitochondrial derived vesicles (MDVs), which deliver selected cargo to lysosomes, also regulated by PINK1 and Parkin. D) Additionally, mitochondrial protease systems contribute to MQC by degrading misfolded or damaged proteins through membrane embedded proteases such as YME1L and AFG3L2, and the matrix localized LONP1 protease [106].

1.1.7 Mitochondrial dysfunction

I have already discussed that under normal conditions, mitochondrial quality control mechanisms ensure proper function by removing defective mitochondria through mitophagy, regulating mitochondrial remodeling via fusion and fission, maintaining the systematic functioning of the ETC to prevent excess ROS production, and activating the mitochondrial unfolded protein response to preserve proteostasis [22]. However, when there is mutation in mitochondrial proteins and when the protective mechanisms fail, mitochondrial dysfunction occurs, leading to excessive ROS accumulation, which damages macromolecules, disrupts energy metabolism, and impairs mitochondrial signaling [106]. This dysfunction compromises genomic stability and cellular bioenergetics, contributing to the pathogenesis of metabolic, cardiovascular, and neurodegenerative diseases, as well as cancer [93].

1.1.7.1 Primary mitochondrial disorders

Mitochondrial disorders can arise from mutations in either mitochondrial DNA or nuclear DNA, with distinct genetic and clinical features. While mitochondrial DNA encodes only 13 essential components of the ETC and is inherited maternally, the vast majority of mitochondrial proteins are encoded by nuclear genes [107]. Diseases resulting from mtDNA mutations follow a maternal inheritance pattern, whereas those caused by mutations in nuclear genes that directly or indirectly affect the oxidative phosphorylation (OXPHOS) system are inherited according to Mendelian inheritance [108]. Disorders that arise from genetic mutations in mitochondrial DNA, such as NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa), leads to mitochondrial myopathy, encephalopathy, and progressive neurological impairment. Certain mitochondrial diseases affect a single tissue, as seen in Leber hereditary optic neuropathy (LHON), which primarily impacts the eyes, and non syndromic hearing loss, which affects the ears. In contrast, other mitochondrial disorders involve multiple tissues, often presenting with prominent neurological and myopathic features [109]. Polymerase gamma (Polγ), encoded by the nuclear genome, is the sole DNA polymerase responsible for mitochondrial DNA replication in humans. Mutations in the POLG gene disrupt mtDNA replication, leading to multiple deletions, mutations, or depletion. These defects are linked to various disorders, including developmental regression, liver failure, and mitochondrial recessive ataxia [110] [111]. Mutation in the nuclear encoded mitochondrial ETC proteins is associated clinically as Leigh syndrome, encephalopathy, lactic acidosis, myopathy, hypotonia, and fatal infantile disorders, depending on the specific complex and mutation involved [112].

1.1.7.2 Secondary mitochondrial disorders

Mutations disrupting mitochondrial proteins define classical mitochondrial diseases, primarily affecting energy production. However, beyond these inherited disorders, mitochondria are central to a much broader spectrum of conditions such as type 2 diabetes, neurodegeneration, cardiovascular disease, and aging [113]. In these cases, dysfunction arises not from mutations but from accumulated damage, impaired signaling, and declining mitochondrial efficiency, highlighting the broader relevance of mitochondrial health in human disease [114]. Clinical and in vivo studies highlight that insulin resistance, commonly seen in obesity and type 2 diabetes, is linked to mitochondrial respiratory dysfunction and decreased ATP production in muscle mitochondria [115]. This impairment is also evident in the pancreatic β cells of type 2 diabetes patients, where it is accompanied by an increase in mitochondrial membrane potential [116]. Additionally, in the adipocytes of obese patients, a reduction in mitochondrial oxidative capacity

is observed, which is further associated with decreased mitochondrial biogenesis [116].

1.1.7.3 Mitochondria in Cancer

Mutations in mtDNA have been identified in various types of human cancer [117]. A number of cancer related mitochondrial defects have been identified which includes altered expression and activity of respiratory chain subunits and glycolytic enzymes, decreased oxidation of NADH linked substrates, as well as mtDNA mutations [117]. Thus, various tumor cells display a high rate of mutations in mitochondrial DNA and alterations of the mitochondrial DNA replication leading to mitochondrial dysfunction and in return, to the production of ROS, as a vicious cycle. In some cancer cells, high levels of mitochondrial fission activity are associated with high proliferation and invasiveness [118]. Deregulated mitochondrial dynamics are observed differentially, depending on the oncogenic pathway. Thus, the transformation of fibroblasts with Ras oncogene induces an overexpression of DRP1 and mitochondrial fragmentation in association with decreased mitochondrial respiration and ATP production [119]. DRP1 overexpression is observed in human breast carcinomas and cancer cells in lymph nodes, comparatively to that of non metastatic carcinomas or adjacent normal tissue, suggesting that fragmentation is associated with cancer progression
[120].

1.1.7.4 Mitochondria in cardiovascular health

Mitochondrial function is crucial for cardiovascular health, as mitochondria occupy ~30% of cardiac cell volume and provide the ATP needed for heart function [121]. However, mitochondrial dysfunction contributes to heart disease, impairing bioenergetics by reducing ATP production and phosphocreatine (PCr) levels, as seen in heart failure and ischemia reperfusion injury [122]. Disruptions in mitochondrial calcium transport further exacerbate ROS generation and mitochondrial permeability transition pore opening, leading to cardiac damage. Mitochondrial quality control mechanisms are directly implicated in cardiac disorders [123]. Loss of Dars2, a mitochondrial aspartyl tRNA synthetase, disrupts proteostasis and OXPHOS, inducing the mitochondrial unfolded protein response this promotes cell survival under mitochondrial stress and has been shown to have cardioprotective effect in cardiac stress [124]. Impaired mitochondrial dynamics also contribute to cardiomyopathy, as MFN1/2 or OPA1 deficiencies lead to dilated cardiomyopathy and heart failure. Additionally, a dominant negative DRP1 mutation, which inhibits mitochondrial fission, prevents left ventricular hypertrophy. In this senario, inhibition of

DRP1 preserves the mitochondrial network, reducing fragmentation and thereby limiting cardiomyocyte death. This protective effect is further associated with reduced opening of the mitochondrial permeability transition pore (mPTP) and preservation of mitochondrial membrane potential, underscoring the role of mitochondrial dynamics in cardiac resilience [125]. In diabetic cardiomyopathy, mitochondrial dysfunction is evident through increased ROS production, altered mitochondrial morphology, and reduced MFN1 levels. Mouse models further suggest defective mitophagy plays a role in diabetic cardiomyopathy pathogenesis by downregulation of mitophagy regulator Parkin, emphasizing the importance of mitochondrial QC in maintaining cardiac function [126].

1.1.7.5 Mitochondria in neurological disorders

Neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) are marked by the progressive deterioration of neuronal structure and function, often manifesting in late adulthood [127]. These conditions are age associated and characterized by multiple pathological hallmarks, including the accumulation of misfolded or aggregated proteins, synaptic loss, neuroinflammation, impaired proteostasis, and dysregulation of intracellular trafficking and organelle homeostasis [128]. A central and increasingly recognized feature of these disorders is the disruption of mitochondrial homeostasis, which contributes to neuronal vulnerability long before overt clinical symptoms arise [129].

PD is a complex neurodegenerative disorder shaped by a complex interplay of genetic predispositions and environmental exposures. Among the primary non mitochondrial contributors are the pathological accumulation and aggregation of α synuclein into Lewy bodies, which interfere with vesicular trafficking, synaptic signaling, and proteostasis [130]. Dysfunctions in protein degradation pathways, such as the ubiquitin proteasome system and autophagy lysosome pathway, further compromise the clearance of misfolded proteins, exacerbating intracellular stress [131]. Neuroinflammation, driven by activated microglia and elevated pro inflammatory cytokines, contributes to a hostile microenvironment, while dopamine metabolism, calcium imbalance, and glutamate excitotoxicity increase the vulnerability of dopaminergic neurons especially in the substantia nigra [132] [133].

Amidst these overlapping pathologies, mitochondrial dysfunction emerges as both a primary driver and a secondary amplifier of neurodegeneration in PD. It plays a central role in both familial and sporadic forms of the disease. Mutations in PINK1 and Parkin, key regulators of mitochondrial quality control, impair mitophagy and result in the accumulation of dysfunctional mitochondria,

elevated ROS, and neuronal apoptosis [134] [135]. Additionally, other PD associated proteins such as DJ-1, a redox-sensitive chaperone, protect mitochondrial function under oxidative stress, while LRRK2 mutations are linked to excessive mitochondrial fragmentation via disrupted fission and fusion dynamics [136]. Misfolded or overexpressed α synuclein has also been shown to localize to mitochondrial membranes, where it disrupts complex I activity, induces membrane permeabilization, and promotes oxidative damage [137]. Furthermore, a reduction in transcriptional coactivators like PGC1α compromises mitochondrial biogenesis, weakening neuronal energy capacity [138].

AD is the most prevalent age related neurodegenerative disorder, characterized by the gradual decline in cognitive function and memory due to the progressive loss of neuronal structure and connectivity [139]. The pathological hallmarks of AD include the extracellular deposition of amyloid β (Aβ) plaques and the intracellular accumulation of hyperphosphorylated tau protein, forming neurofibrillary tangles [139]. These aggregates disrupt synaptic integrity and neuronal signaling, contributing to widespread neurodegeneration [140]. In parallel, impairments in proteostasis, chronic neuroinflammation, and synaptic dysfunction amplify neuronal stress [141]. Activated microglia release pro inflammatory cytokines and fail to clear protein aggregates efficiently, further exacerbating neuronal injury [142]. Additionally, altered calcium homeostasis, lipid dysregulation, and oxidative stress also contribute to the deterioration of neuronal networks [143].

Within the broader perspective of these intersecting pathological processes, mitochondrial dysfunction has gained recognition as a pivotal and early contributor to the pathogenesis of AD. Accumulating evidence suggests that mitochondrial abnormalities precede the overt deposition of Aβ and tau pathology [144]. Mitochondria in AD brains are often fragmented and bioenergetically impaired, showing reduced ATP production, increased ROS generation, and compromised calcium buffering capacity [145] [146]. Aβ peptides have been shown to translocate into mitochondria, where they inhibit key enzymes such as cytochrome c oxidase (complex IV) and α-ketoglutarate dehydrogenase, disrupt ETC function, and interact with mitochondrial proteins like ABAD (Aβ-binding alcohol dehydrogenase), promoting mitochondrial stress and triggering apoptosis [147] [148]. In this case, mitochondrial dysfunction acts as a key amplifier of the pathological cascade, not merely as a consequence of Aβ and tau accumulation, but as an early driver that synergizes with other disease mechanisms [146].

1.1.7.6 Environmental factors in mitochondrial disorders

In addition to genetic factors, environmental influences significantly contribute to mitochondrial

dysfunction. Various toxins, including volatile organic compounds (VOCs) and heavy metals, have been shown to impair mitochondrial integrity and function, limiting the organelle's ability to adapt to metabolic stress. Exposure to VOCs alters mitochondrial morphology, making them more spherical, while toxic metals such as lead, mercury, and cadmium damage mitochondrial complexes, ultimately reducing energy production [149] [150]. Among these environmental toxins, cyanide is a particularly potent mitochondrial inhibitor. It specifically targets complex IV of the electron transport chain (ETC) and reduces ATP production [151]. Exposure of rats to the pesticide and mitochondrial complex I inhibitor rotenone replicates key pathological features of Parkinson's disease, including systemic mitochondrial impairment, oxidative damage, selective degeneration of nigrostriatal dopaminergic neurons and the aggregation of Lewy body like inclusions [152] [153]. Vitamins B and D and essential minerals like iron and calcium play a crucial role in mitochondrial function. Deficiencies in these micronutrients are commonly associated with symptoms frequently reported in individuals with mitochondrial disorders, such as fatigue and muscle pain [154].

The complex structure, dynamic behavior, and diverse functions of mitochondria including ATP production via the ETC, regulation of ROS, and organelle quality control are fundamental to maintaining metabolic stability, redox balance, and intracellular signaling networks [155]. When these processes are disrupted, mitochondrial dysfunction can propagate stress signals across cellular compartments, thereby influencing broader homeostatic and adaptive responses [5].

Given the central role of mitochondria in regulating intracellular signalling, increasing attention has turned to how other trafficking systems particularly endocytic pathways contribute to maintaining cellular organization and responding to metabolic cues. To understand this interplay, it is first essential to consider the fundamental principles and mechanisms of endocytosis.

1.2 Endocytic pathway

Endocytosis enables cells to internalize macromolecules and surface proteins, playing a vital role in intracellular signaling, cell migration, and antigen presentation. This process occurs through receptor independent and receptor dependent pathways [156]. Receptor independent endocytosis includes phagocytosis and pinocytosis, both are nonselective mechanisms [157] [158] [159]. In phagocytosis, the plasma membrane engulfs large particles (>0.5 μm), forming intracellular vesicles called phagosomes. In contrast, pinocytosis, or "cellular drinking," involves the uptake of fluids and nutrients through smaller vesicles (0.5–5 μm) as the membrane pinches inward [157]. Receptor mediated endocytosis (RME), on the other hand, is a selective process that allows cells to

internalize specific molecules via receptor interaction. This can occur through distinct pathways such as clathrin dependent and independent endocytosis and caveolae mediated endocytosis, ensuring precise cargo uptake to meet cellular demands [158] [159].

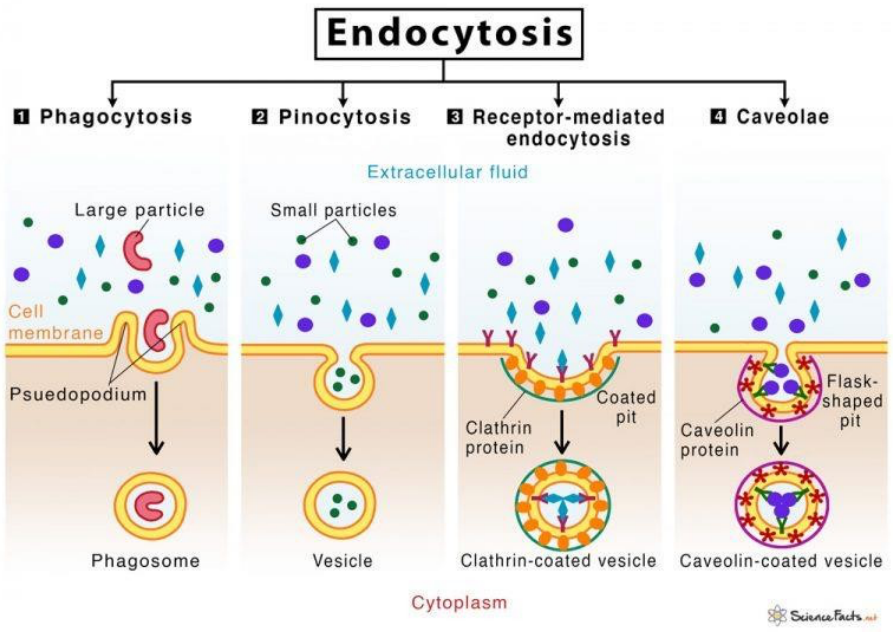

Figure 1.2: Major types of endocytosis mechanisms in eukaryotic cells

Phagocytosis, pinocytosis, receptor mediated endocytosis: clathrin dependent, and caveolae mediated endocytosis [159].

1.2.1 Types of endocytosis:

1.2.1.1 Clathrin mediated endocytosis

It is the best characterized endocytic pathway. It involves clathrin coated pits and vesicles. It plays a key role in low density lipoprotein and transferrin uptake by binding to their receptors [160]. Clathrin coated vesicles consist of two principal components: heavy and light chains and four subunits of the heterotetrameric adaptor protein 2 (AP2) complex. The AP2 is the principal component of the cargo recognition molecule and links coated vesicles to the membrane bilayer [161]. Other adaptor proteins, called CLASPs, recognise specific cargo receptor domains. Clathrin mediated endocytosis proceeds first with clathrin coated pit assembly, which is initiated by AP2 complex recruited to plasma membrane enriched phosphatidylinositol lipid, $PI(4,5)P_2$ [162]. AP2 rapidly recruits clathrin. Other scaffolding molecules are also involved in assembly and play a role in nucleating or stabilizing clathrin coated pits [163]. Besides AP2, other adaptor proteins like CALM, Epsin, and Dab2 help clathrin mediated endocytosis by recognizing different cargo and assisting in vesicle assembly [164] [165].

For efficient budding, clathrin has been shown to assemble into closed cages in vitro, along with other curvature generating proteins by recruiting to nascent clathrin coated pits [166]. For example, intrinsically curved Bin-Amphiphysin-Rvs (BAR) domain containing proteins create deeper curvature, helping in the progression of clathrin coated pits [167]. As the nascent pit grows, adaptor protein 2 and other cargo specific adaptor proteins recruit and concentrate cargo. Clathrin polymerization stabilizes the curvature of the pit. The release of mature clathrin coated vesicles from the plasma membrane depends on the large GTPase dynamin. Dynamin assembles into collar like structures encircling the neck of clathrin coated pits and undergoes GTP hydrolysis to drive membrane fission [167]. Finally, the vesicle is detached from the plasma membrane, and the clathrin coat is disassembled by ATPase chaperone Hsc70 which is recruited and activated by the co chaperone auxilin [168 169]. This allows the uncoated vesicle to traffic inside the cell and fuse with its target endosome. Bacteria and large particles, up to 1 μm in diameter, can hijack clathrin mediated endocytosis by forming actin rich structures that aid their uptake. Similarly, rod shaped viruses and nanoparticles, which exceed the typical clathrin coated vesicle size, may enter cells through clathrin mediated endocytosis by actin driven elongation of clathrin coated pits [170 158 171].

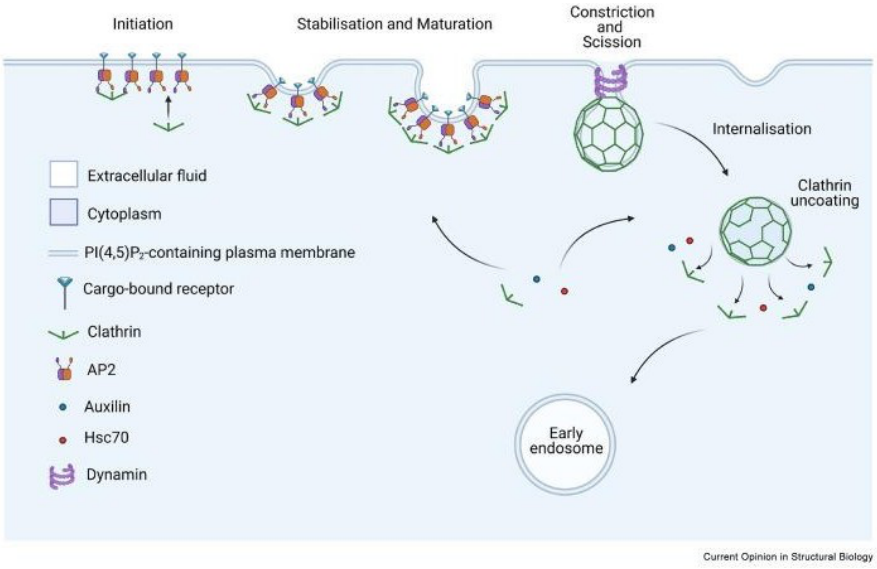

Figure 1.2.1.1: Mechanism of clathrin mediated endocytosis

Adaptor proteins and clathrin assemble at the plasma membrane to form coated pits. After vesicle scission by dynamin, auxilin recruits Hsc70 to mediate ATP dependent clathrin uncoating, allowing fusion with early endosomes [171].

1.2.1.2 Caveolae mediated endocytosis

It is the second most known and the most studied endocytic pathway. It has been found to play a

role in transcytosis trafficking across endothelial cells as well as in lipid regulation. Caveolae, the site of caveolae mediated endocytosis, are flask or omega shaped plasma membrane invaginations with a diameter of 50- 100 nm [170]. Caveolae mediated endocytosis is highly regulated and triggered by the binding of ligands to cargo receptors concentrated in caveolae. Caveolae are abundant in some cell types but absent in neurons and many blood cells [171]. The major structural proteins in caveolae are members of the caveolin protein family, particularly caveolin 1, along with cytosolic coat proteins known as cavins. Caveolin 1 is a small integral membrane protein that is inserted into the inner leaflet of the plasma membrane bilayer. The cytosolic N terminal domain of caveolin 1 binds to cholesterol and functions as a scaffolding domain that interacts with signaling molecules [172]. The steps involved in caveolin mediated endocytosis are not well understood. However, the budding of caveolae from the plasma membrane is known to be regulated by kinases and phosphatases. Similar to clathrin mediated endocytosis, in caveolin mediated endocytosis, caveolae are pinched off from the plasma membrane by dynamin [172].

1.2.1.3 CLIC/GEEC (Clathrin-Independent Carrier/GPI-AP Enriched Early Endosomal Compartment)

This pathway is a major form of clathrin-independent endocytosis (CIE) in mammalian cells [5]. Unlike clathrin-mediated endocytosis, the CLIC/GEEC pathway operates independently of clathrin and dynamin and is characterized by the formation of uncoated, tubulovesicular carriers called CLICs, which bud directly from the plasma membrane [6]. These carriers subsequently mature into early endosomal compartments enriched in glycosylphosphatidylinositol-anchored proteins (GEECs) and can fuse with sorting endosomes in a Rab5- and PI3K-dependent manner [7]. The CLIC/GEEC pathway selectively internalizes various GPI-anchored proteins, certain transmembrane proteins (such as CD44), and is also responsible for the uptake of bulk fluid-phase markers and specific toxins like cholera toxin and aerolysin [8]. Regulation of this pathway involves small GTPases, particularly CDC42, and the GTPase-activating factor GRAF1, which coordinate membrane deformation and carrier formation [8]. The CLIC/GEEC pathway is thought to play important roles in membrane homeostasis, cellular signaling, and the regulation of plasma membrane tension [9].

1.2.1.4 Macropinocytosis

This involves the actin driven extension of plasma membrane sheets, which enclose extracellular fluid as they fold back. Lattice light sheet microscopy has revealed tentpole like actin extensions forming with membrane sheets between them. As these tentpoles twist, fluid gets trapped at the

base, leading to macropinosome formation [172]. After closure, the macropinosome undergoes tubulation and membrane recycling, shrinking into an early endosomal compartment. Cellular macropinocytosis capacity varies by cell type and can be constitutive or inducible [172]. Macrophages and dendritic cells perform constitutive macropinocytosis to sample extracellular pathogens, while induced macropinocytosis can be triggered by growth factors, receptor tyrosine kinases (e.g., EGFR), proteoglycans, or G protein coupled receptors [173]. Neutrophils activate macropinocytosis in response to viral infections, and dendritic cells upregulate it upon lipopolysaccharide exposure. It has been shown that in breast and prostate cancer cells use it to enhance ErbB3 (tyrosine kinase receptor) trafficking into the nucleus, promoting proliferation [174]. RAS activation is a well-established driver of increased macropinocytosis, particularly in pancreatic cancer cells. This process involves actin-driven extension of plasma membrane sheets that fold back and enclose extracellular fluid, forming macropinosomes [174].

1.2.1.5 Phagocytosis

Phagocytosis is the process by which neutrophils, monocytes, and macrophages engulf particulate material. While traditionally associated with the uptake of particles larger than 0.5 μm, its lower size limits remain unclear. This process is essential for clearing cellular debris, removing dead cells, and eliminating pathogens as part of the innate immune response [175]. Particles or microorganisms are surrounded by a tight fitting membrane, which extends and fully encloses them before vesicle scission from the plasma membrane. The resulting intracellular vacuole, the early phagosome, matures into a degradative compartment enriched with microbicidal agents [176]. Phagocytosis begins when particles bind to surface receptors, such as scavenger receptors, which recognize a diverse range of materials, or through specific receptor interactions. Among phagocytic processes, the uptake of pathogens by macrophages is one of the most extensively studied [176] [174].

1.2.2 Endosomal trafficking

Once internalized through any of the endocytic pathways, receptors and ligands merge into a common endosomal network. The endosomal network is an interconnected highway-like network that facilitates intracellular trafficking and cargo transfer between distinct membrane compartments called endosomes. The primary function of the endosomal network is to internalize cargo, sort it, and disseminate it to its final destination [156].

After entering the cell, vesicle containing cargo undergoes multiple rounds of homotypic fusion, forming early endosomes [177]. Within these early endosomes, initial sorting decisions are made,

determining the fate of cargo and internalized receptors. Eventually, cargo can be recycled back to the plasma membrane by recycling endosomes, transported to the trans Golgi network via retrograde trafficking for cargo modification and redistribution, or delivered to lysosomes for degradation [177]. Spatial and temporal mechanisms tightly control the regulation of traffic and cargo sorting to ensure the proper identity of endosomes [178 179].

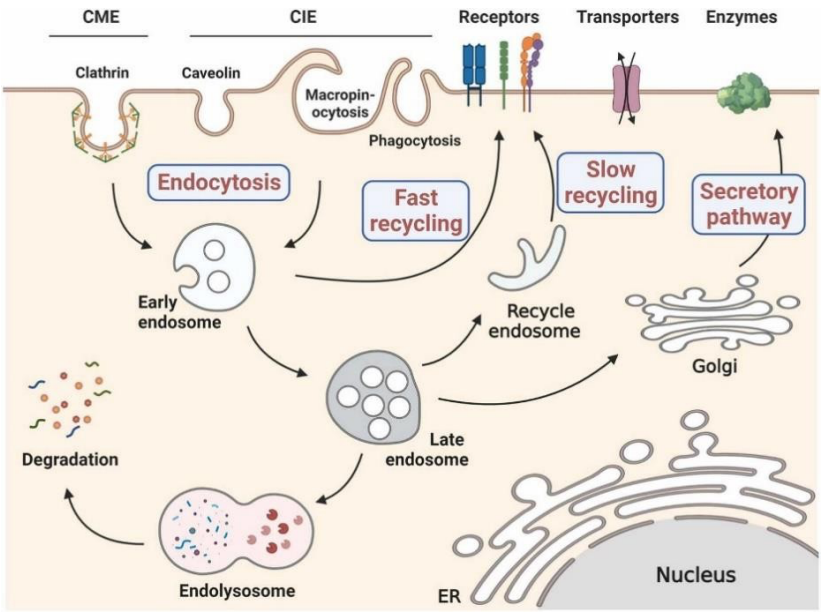

Figure 1.2.2: Endocytic cargo trafficking pathway

Endocytosed cargo enters early endosomes via various pathways including clathrin mediated, caveolin mediated, and macropinocytosis. Cargo is either directed toward degradation via late endosomes and endolysosomes or recycled back to the membrane through fast or slow recycling routes. The Golgi and ER participate in the secretory pathway, maintaining membrane and protein homeostasis [179].

1.2.3 Endosome identity

Each endosomal compartment within the endosomal network has a unique identity, which can change as cargo is trafficked and exchanged between endosomes. This process, known as endosomal maturation, leads early endosomes to transition into late endosomes through various mechanisms [180]. One of the most notable changes during maturation is the progressive acidification of the endosomal lumen, which occurs as V type vacuolar H^+ ATPase in the membrane bilayer actively pumps hydrogen ions into the lumen, decreasing the pH [181]. Other regulatory mechanisms influence the directional flow of membrane trafficking, such as alterations in phosphatidylinositol phospholipids (PIPs) and the differential recruitment and activation of Rab family GTPases [181].

Each endosomal compartment is characterized by specific PIPs and Rab family GTPases. PIPs, which are phosphorylated derivatives of phosphatidylinositol, are synthesized in the endoplasmic

reticulum and subsequently delivered to endosomal compartments through membrane trafficking [182]. The segregation of PIPs on distinct membranes is crucial for the directional movement of cargo. On different endosomal membranes, PIPs are regulated by lipid kinases and phosphatases, which mediate reversible phosphorylation and dephosphorylation [182]. Studies suggest that lipid distribution plays an essential role in recruiting proteins involved in trafficking to specific endosomal membranes through PIP specific binding domains [183]. For instance, AP2, which is involved in clathrin mediated endocytosis, selectively binds to $PI(4,5)P_2$, which is enriched at the plasma membrane [183].

There is a close relationship between PIP modifying enzymes and Rab GTPases. PIPs recruit Rab proteins, including guanine nucleotide exchange factors (GEFs), which activate Rabs, and GTPase activating proteins (GAPs), which inactivate Rabs [184]. This activation enables membrane targeting through the association of lipid binding domains. Furthermore, PIP kinases and phosphatases often act as Rab effectors. A notable example is Rab5 dependent PI(3)P synthesis on early endosomes, which is regulated by Class III PI3-kinase VPS34, a known Rab effector [184]. Additionally, many Rab effectors contain PI(3)P binding motifs, such as Early Endosome Antigen 1 (EEA1), a well established marker of early endosomes that associates with PI(3)P. Thus, phosphoinositides and Rab GTPases coordinate membrane identity through positive and negative feedback mechanisms, ensuring efficient endosomal trafficking and cargo sorting [158].

1.2.4 Endosomal sorting mechanism

As cargo enters the endosomal network, they are sorted in the early endosome towards their final destination [185]. Sorting of cargo in the early endosome depends on endosomal acidification and ligand dissociation from the receptor. Internalized ligand receptor complexes exhibit varying pH sensitivities that influence receptor ligand dissociation [180]. This allows differential recycling of cell surface receptors. For example, receptors that need to recycle to the plasma membrane release their ligands in the early endosome, where the pH is approximately 6.5. This allows rapid recycling of receptors such as transferrin or low density lipoprotein receptors. Cargoes that are destined for the trans Golgi network release ligands in the late endosome, with a pH range of approximately 5.5 (e.g., mannose-6-phosphate receptor) [185].

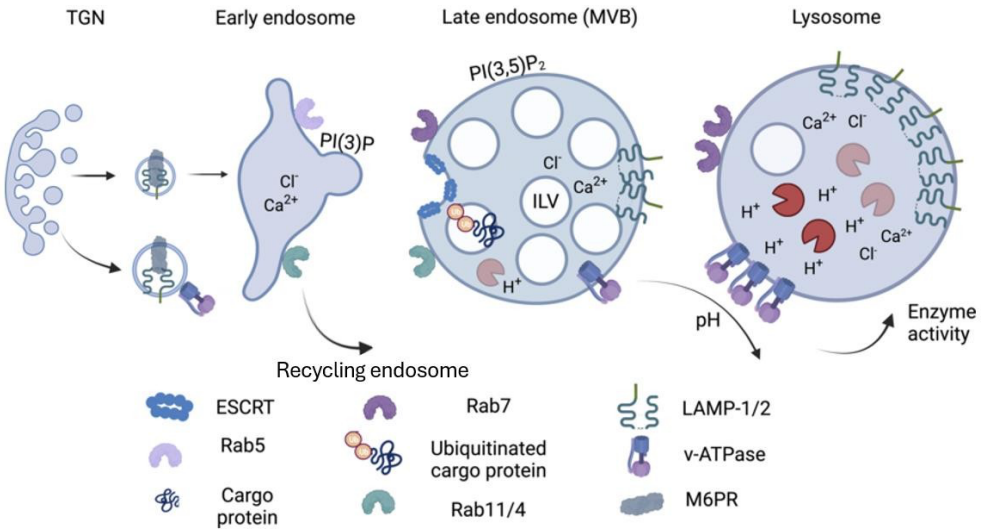

Figure 1.2.4: Endosomal maturation

During endosomal maturation, Rab5 and PI(3)P on early endosomes are replaced by Rab7 and PI(3,5)P_2, signaling their transition into late endosomes (LEs). These enlarging endosomes receive LAMPs and V- ATPase from the trans Golgi network via the mannose-6-phosphate receptor (M6PR). V-ATPase drives acidification, enabling lysosomal enzyme activity. ESCRT complexes are recruited to the endosomal membrane to generate intraluminal vesicles (ILVs) by sorting ubiquitinated cargo into MVBs. These ILVs are either secreted as exosomes or delivered to lysosomes for degradation. Alternatively, Rab4 or Rab11 positive recycling endosomes are generated. Ion concentration and acidity increase steadily throughout this process, preparing vesicles for lysosomal fusion [186].

In addition to endosomal pH, endosome geometry is essential for sorting internalized cargo. Early endosomes are highly dynamic and pleomorphic structures. As early endosomes mature, they tubulate, which facilitates sorting and recycling of internalised cargo This geometry maximizes the surface area, favoring the accumulation of ligands in the lumen of early endosomes [187]. The tubulated structures formed by early endosomes allow the accumulation of transmembrane receptors destined for recycling. In some cells, the tubules released from early endosomes accumulate in the perinuclear region before returning to the cell surface, forming recycling endosomes [186 187].

In addition to endosomal pH and membrane geometry, endocytic cargo recycling relies on the active recruitment and coordination of specific sorting machinery. The sorting nexin (SNX) protein family plays a key role in this process, particularly by regulating membrane tubulation at the endosome [188]. A prominent subgroup, known as the SNX-BAR proteins, is characterized by the presence of a BAR (Bin/Amphiphysin/Rvs) domain, that function as dimers facilitating the formation and stabilization of tubular membrane carriers that are essential for cargo sorting [189]. These proteins often associate with the retromer complex, forming a functional unit that mediates

the retrograde trafficking of specific cargo from endosomes to the trans Golgi network [190].

The retromer complex is divided into two sub complexes: the membrane recognition complex, containing nexins (SNX1/2 or SNX5/6), and the cargo selective complex, containing Vps26, Vps29, and Vps35. The retromer complex is recruited to the early endosome through SNX mediated PI(3)P binding and/or associates with Rab7 positive late endosomes through Vps35-Rab7 interaction [191]. Once assembled the canonical retromer complex, composed of VPS35, VPS29, and VPS26, is partially required for retrograde trafficking of cargo, such as the cation independent mannose-6-phosphate receptor (CI-M6PR) and sortilin from endosomes back to the trans Golgi network [192]. Thus, the retromer complex protects cargo receptors from the degradative lysosomal pathway, by retrieving them from endosomes and facilitating their recycling to the trans Golgi network [193] [194].

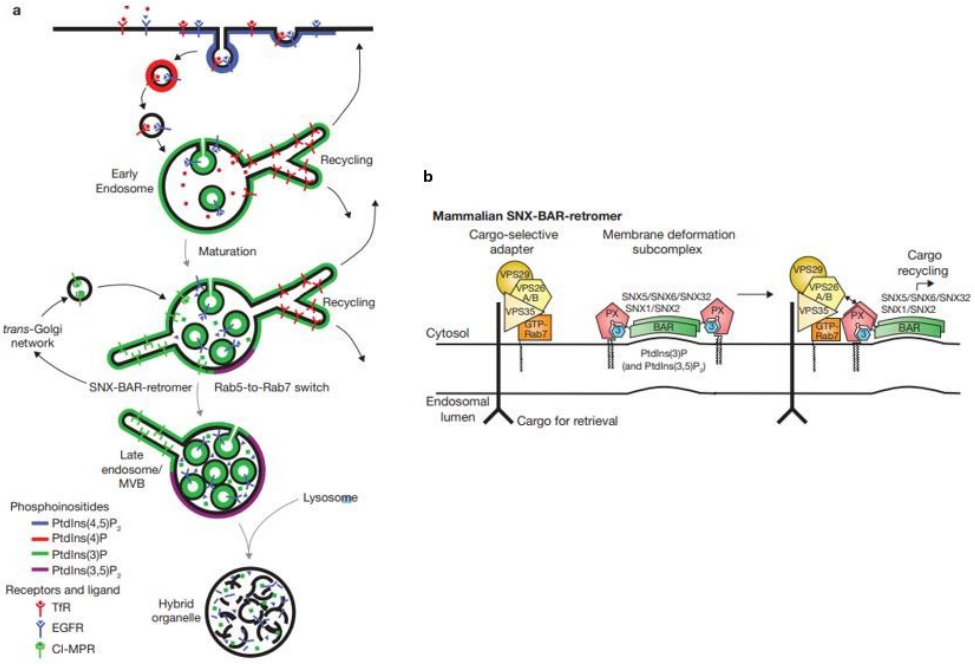

Figure 1.2.4: Sorting itineraries in endosomal network and SNX-BAR-retromer

(a) Internalized cargo destined for degradation, such as EGFR, is sorted into intraluminal vesicles (ILVs) and trafficked to late endosomes/multivesicular bodies (MVBs). Fusion of MVBs with lysosomes leads to cargo degradation. In contrast, receptors like the transferrin receptor (TfR) are recycled back to the plasma membrane via fast or slow recycling routes, while the cation independent mannose-6-phosphate receptor (CI-MPR) is retrieved to the trans Golgi network (TGN) via retrograde transport. This includes Rab9 and SNX-BAR-retromer dependent pathways, with phosphoinositide composition helping to define compartment identity, such as PI(3)P in early endosomes. (b) The SNX-BAR-retromer consists of cargo selective and membrane deforming subcomplexes, this includes two isoforms of VPS26 and BAR domain–containing sorting nexins (SNXs) that help mediate retrograde cargo sorting [194].

A critical mechanism for sorting transmembrane proteins within the endosomal system involves their incorporation into intraluminal vesicles (ILVs) for degradation in lysosome [195]. This process is regulated by post translational modification, especially mono ubiquitination, which serves as a molecular signal directing selected membrane proteins toward the degradative pathway. For instance, activated receptors such as the epidermal growth factor receptor (EGFR) are tagged with mono ubiquitin to mark them for internalization and lysosomal degradation following signaling [196]. As early endosomes mature, they begin to generate ILVs via inward budding of the limiting membrane, ultimately forming multivesicular bodies (MVBs) specialized endosomal compartments that sequester cargo within internal vesicles [197]. The ESCRT (Endosomal Sorting Complex Required for Transport) machinery orchestrates this process through four sequentially acting complexes ESCRT 0, I, II, and III which collectively recognize mono ubiquitinated proteins, cluster them in endosomal membrane domains, and mediate the formation of ILVs. The ATPase Vps4 subsequently disassembles the ESCRT complexes to complete vesicle formation and recycle the machinery [198]. Once multivesicular bodies fuse with lysosomes, the internalized ILVs and their cargo are degraded by lysosomal enzymes, including proteases and lipases, thereby terminating signaling activity and ensuring turnover of membrane proteins [195].

1.2.5 Cross talk between endocytic pathways and mitochondria

Traditionally, endocytic pathways have been recognized for transporting internalized cargo from the plasma membrane to early endosomes, where cargo is sorted for recycling, lysosomal degradation, or, in specific cases, retrograde transport to the trans Golgi network [156]. Although mitochondria are not traditionally viewed as direct components of endocytic trafficking, emerging evidence suggests bidirectional communication and functional interdependence between endocytic systems and mitochondria, particularly under stress conditions and during organelle quality control [27 199].

One known example of this cross talk involves direct endosome–mitochondria interactions to facilitate iron transfer, particularly in erythroid cells. Using super resolution imaging (3D dSTORM) and a mitochondria specific iron sensor dye called rhodamine B-[(2,2-bipyridin-4-yl) aminocarbonyl] benzyl ester, transferrin (Tf) positive endosomes were observed to engage in transient "kiss and run" contacts with mitochondria, enabling targeted iron delivery [200]. These interactions are influenced by iron release, as endosomes carrying iron release defective transferrin mutants (lock hTf) displayed altered motility and prolonged mitochondrial contact, suggesting that iron mediated alterations in cargo conformation or intraluminal conditions modulate endosome–

mitochondria interactions [199].

In addition to iron transfer, non canonical endocytic routing has revealed further dimensions of this cross talk. For example, studies using FM dyes, that fluorescent in lipid rich environments. These dyes were found to enter cells via clathrin mediated endocytosis, but instead of trafficking through classical endosomal compartments, the internalized vesicles appeared to bypass early endosomes and interact directly with mitochondria [201]. This suggests the existence of a non canonical endocytic pathway in which mitochondria act as trafficking targets, possibly for the delivery of specific lipid components or signaling molecules [201].

Conversely, mitochondria can also become cargo within the endosomal system. Under conditions of acute mitochondrial stress, damaged mitochondria are selectively internalized into Rab5 positive early endosomes via the ESCRT complex in a Parkin dependent manner. Unlike conventional mitophagy, this pathway is autophagy independent and precedes autophagic activation [202]. The autophagy regulator Beclin1 has been shown to activate Rab5, mechanistically linking endosome mediated mitochondrial degradation with autophagic signaling pathways [202]. Importantly, impairment of Rab5 or ESCRT function disrupts this early response, leading to accumulation of defective mitochondria and increased susceptibility to cell death in systems such as embryonic fibroblasts and cardiomyocytes [202].

During acute mitochondrial stress, the cell activates quality control pathways such as mitophagy and lysosomal degradation to eliminate damaged mitochondria and preserve cellular homeostasis. However, when mitochondrial stress becomes chronic, the sustained activation of these pathways may lead to pathological accumulation of lysosomal structures [203]. Mosquera et al. (2017) demonstrated that in response to prolonged mitochondrial dysfunction, cells initiate a feedback suppression of lysosomal biogenesis, acting as a protective mechanism to prevent lysosomal overload [203]. This suppression is mediated, in part, by transcriptional regulation and serves to differentiate the response to acute versus chronic mitochondrial stress, modulating lysosomal capacity accordingly [203].

This stress adaptive lysosomal suppression is further studied by transcriptional crosstalk between lysosomes and mitochondria. As demonstrated by Yambire et al. (2019), who showed that in the lysosomal storage disorders, a group of hereditary disease that affects the function of lysosomes, chronic lysosomal stress activates the transcription factor KLF2, which plays a central role in repressing mitochondrial biogenesis. This transcriptional response affects mitochondrial respiratory chain complex [204]. Altogether, these findings highlight a reciprocal relationship

between mitochondria and lysosomes, where not only does mitochondrial dysfunction impair lysosomal dynamics, but lysosomal stress, in turn, actively represses mitochondrial regeneration, amplifying organelle dysfunction in chronic disease settings [204].

Further supporting this interorganelle connection, studies have shown that mitochondrial dysfunction such as that caused by the loss of AIFM1 (apoptosis inducing factor mitochondria associated 1), OPA1 involved in mitochondrial fusion, or PINK1 involved in mitochondrial quality control can lead to lysosomal impairments, including the vacuolated lysosomal structures with reduced hydrolytic activity. These effects occur independently of ATP depletion, indicating that mitochondria exert regulatory control over lysosomal structure and function through mechanisms beyond energy supply [27].

Altogether, these findings emphasize a reciprocal and self reinforcing relationship between mitochondria and lysosomes, particularly under chronic stress conditions. Mitochondrial damage disrupts lysosomal integrity and signaling, while lysosomal dysfunction feeds back to inhibit mitochondrial biogenesis and turnover [204] [205]. This maladaptive loop intensifies organelle dysfunction and may contribute to the progression of metabolic and neurodegenerative diseases where both mitochondrial and lysosomal pathways are impaired [206].

While endocytosis facilitates the internalization of cargo, exocytosis enables the outward transport of cellular components. This outward flow is not only essential for membrane turnover and secretion but also underlies the release of extracellular vesicles (EVs). EVs, which often originate from endosomal pathways, represent a crucial link between endocytic processing and intercellular communication, highlighting the dynamic reciprocity between endocytosis and exocytosis.

1.3 Exocytosis and extracellular vesicles

Exocytosis is a fundamental process by which eukaryotic cells deliver intracellularly synthesized materials including proteins, lipids, and signaling molecules to the plasma membrane or the extracellular space [15]. It plays a central role in maintaining cellular homeostasis, regulating membrane composition, and facilitating intercellular communication. As a core component of the secretory pathway, exocytosis involves the formation of secretory granules (SGs) or transport vesicles, their maturation, and eventual fusion with the plasma membrane, resulting in the release of vesicle contents outside the cell [207]. Simultaneously, vesicle membranes contribute lipids and proteins to the plasma membrane, supporting its remodeling and expansion. Exocytosis is essential for a wide range of physiological functions, including hormone secretion, neurotransmission,

immune responses, and tissue development, reflecting its integral role in cell to cell communication and systemic regulation [208]. The steady state composition of the plasma membrane results from the balance between endocytosis and exocytosis. Macromolecules destined for secretion are packaged into intracellular storage vesicles, which are delivered to the plasma membrane in response to specific cellular signals [209]. Exocytosis occurs via two main modes: regulated and constitutive. In regulated exocytosis, vesicles remain stored in the cytoplasm and fuse with the plasma membrane only upon receiving specific stimuli, such as hormones or neurotransmitters [209]. In contrast, constitutive exocytosis occurs continuously, transporting newly synthesized proteins and lipids to the cell surface without the need for an external signal [210].

These exocytic processes are part of the broader secretory pathway, a highly coordinated intracellular transport system that begins in the endoplasmic reticulum (ER) [211]. Soluble proteins delivered to this pathway include ER resident chaperones, lysosomal hydrolases, and various secretory proteins. Most of these proteins contain a signal sequence that directs them to the ER, where they undergo folding and glycosylation [211]. Correctly folded proteins are then packaged into COPII coated vesicles for transport to the Golgi apparatus [212] [213]. Within the Golgi, proteins are further modified and sorted at the trans Golgi network (TGN) for delivery to specific destinations such as the plasma membrane, endosomes, lysosomes, or secretory storage granules [214].

Among the cargo sorted through this pathway are lysosomal hydrolases such as cathepsin D and β- hexosaminidase A, which are directed to lysosomes via M6P tagging to facilitate the degradation of macromolecules [215]. Simultaneously, the secretory pathway also supports plasma membrane remodeling by delivering key proteins like SNAREs and dynamin, which mediate vesicle fusion and membrane scission. These processes are essential not only for maintaining membrane integrity but also for ensuring precise trafficking and communication at the cell surface [216] [217].

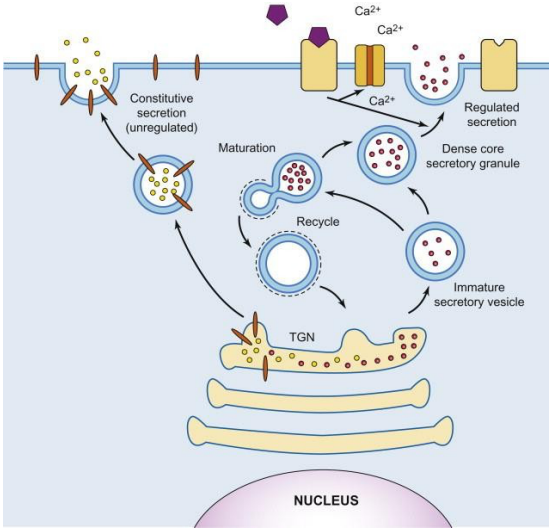

Figure 1.3: The constitutive and regulatory secretory pathway: Exocytosis

Eukaryotic cells direct newly synthesized proteins and lipids to the plasma membrane through two main exocytic pathways: the constitutive and regulated secretory pathways. In the constitutive pathway, cargo lacking specific sorting signals such as lipids, extracellular matrix proteins, and integral membrane proteins is continuously packaged at the trans Golgi network (TGN) and delivered to the plasma membrane. This default route maintains membrane composition and supports general secretion. In contrast, the regulated secretory pathway, prominent in specialized cells like neurons and endocrine cells, requires extracellular stimuli to trigger secretion. Selected proteins such as hormones, peptides, and neurotransmitters are sorted at the TGN into immature secretory vesicles, which undergo maturation to form secretion competent dense core granules. This process involves cargo concentration, clathrin mediated membrane remodeling, and recycling of components. Upon stimulation typically involving ligand receptor binding and Ca^{2+} influx mature granules fuse with the plasma membrane to release their contents. A highly specialized, rapid form of this regulated secretion occurs at neuronal synapses [217].

1.3.1 Extracellular vesicles (EVs)

In addition to the conventional secretory pathways, cells can export proteins, lipids, and nucleic acids through EVs. EVs are membrane bound carriers that are released into the extracellular space through exocytic processes. They originate either from the endosomal pathway (as in exosomes) or via direct budding from the plasma membrane (as in microvesicles) [218]. They facilitate the targeted delivery of bioactive molecules to recipient cells, playing a critical role in intercellular communication and signaling regulation. Initially identified as vehicles for genetic exchange and the disposal of cellular debris [219 220], EVs are now recognized for their diverse and essential roles in development, immune response, cell migration, tissue remodeling, and tumor progression [220].

EVs carry a wide array of molecular cargo including proteins, lipids, mRNAs, non coding RNAs, and metabolites which can influence recipient cell behavior and signaling networks [220 221]. Due to their biocompatibility, and ability to cross physiological barriers like the blood brain

barrier, EVs are increasingly being studied as therapeutic delivery systems, especially in gene and RNA therapies [222]. Notably, EVs can also transport mitochondrial components, such as mitochondrial DNA (mtDNA) and proteins, and emerging evidence suggests that mitochondria actively regulate EV release, pointing to a bidirectional interaction between mitochondrial function and vesicle mediated communication [223].

EVs are classified into three main types based on their size and origin: exosomes (30-200 nm), microvesicles (100-1000 nm), and apoptotic bodies (>1000 nm). Exosomes are derived from the endocytic pathway, microvesicles form through budding and blebbing of the plasma membrane, and apoptotic bodies are released by cells undergoing programmed cell death, signalling their removal by other cells [224] [225] [226].

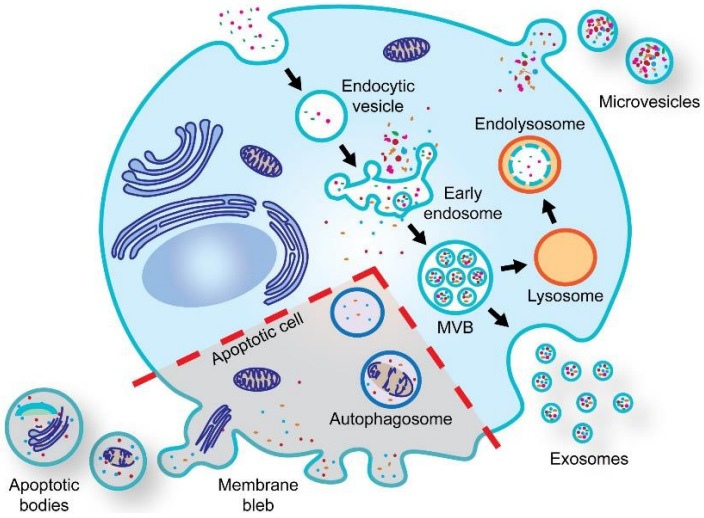

Figure 1.3.1: Biogenesis and types of EVs

EVs are produced by virtually all cell types and are broadly classified into three main subtypes based on their origin. Exosomes are generated through the endosomal pathway and released upon fusion of multivesicular bodies with the plasma membrane. Microvesicles form by outward budding directly from the plasma membrane, while apoptotic bodies are released as membrane blebs during the late stages of apoptosis [226].

1.3.1.1 Exosome and microvesicle biogenesis:

Exosome biogenesis

MVBs and late endosomes are specialized endosomal compartments characterized by the accumulation of ILVs, which are formed by the inward budding of the endosomal membrane [227]. Canonically, ILVs function to sequester membrane bound receptors and cytosolic proteins, targeting them for degradation upon fusion of MVBs with lysosomes a key process in receptor

downregulation and endocytic cargo turnover. This pathway is essential for regulating signaling pathways, such as the EGFR degradation route [228] [229].

However, not all MVBs are destined for degradation. A subset is directed to the plasma membrane, where they undergo exocytosis and release ILVs into the extracellular space as exosomes. This alternative, non canonical fate of ILVs enables them to act as EVs involved in intercellular communication [230]. Thus, MVBs serve as a central decision point, they either fuse with lysosomes for cargo degradation or with the plasma membrane to release exosomes, reflecting a functional divergence that is tightly regulated by cellular state and signaling cues [231]. The concept of exosome release was first recognized in studies on transferrin receptor secretion by reticulocytes, where functional ILVs were secreted rather than degraded [228]. The transport of MVBs toward the plasma membrane is facilitated by the microtubule and cytoskeletal network [230]. The Ras related protein GTPase Rab, Sytenin 1, TSG101 (tumor susceptibility gene 101), ALIX (apoptosis linked gene 2 interacting protein X), syndecan-1, ESCRT (endosomal sorting complexes required for transport) proteins, phospholipids, tetraspanins, ceramides, sphingomyelinases, and SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) complex proteins are involved in the origin and biogenesis process of exosomes [230]. Moreover, several molecules are frequently used as exosomal markers, including CD9, CD81, CD63, flotillin, TSG101, ceramide, and ALIX. However, the specific rate limiting steps and in vivo functions of these regulators remain incompletely defined and warrant further investigation [230].

As previously discussed in endocytic maturation, ILVs are formed through inward budding of the endosomal membrane within multivesicular bodies (MVBs), a process largely driven by the ESCRT machinery [195]. While the ESCRT machinery is a well characterized driver of ILV formation and exosome biogenesis, alternative ESCRT independent mechanisms have also been proposed [228]. Among these, tetraspanins, a family of conserved four pass transmembrane proteins have gained attention for their roles in organizing membrane microdomains and influencing cargo selection, even though their direct involvement in vesicle budding remains less well defined [232].

Tetraspanins such as CD9, CD63, and CD81 are widely used as exosomal markers and appear to influence the composition of exosomal cargo [233]. For instance, CD63 (also known as LAMP 3) protects certain proteins, such as the Epstein–Barr virus oncoprotein LMP1, from lysosomal degradation by facilitating their incorporation into exosomes [234]. CD63 also mediates ESCRT independent sorting through its interaction with apolipoprotein E (ApoE), a process shown to be essential in melanogenesis [235]. Loss of CD63 shifts this sorting pathway toward ESCRT dependent

lysosomal degradation of premelanosomes, suggesting a protective, sorting specific role [236]. CD63 is commonly utilized in engineered exosomes to enhance the loading efficiency of cargo molecules and reporter proteins [233].

Other tetraspanins also contribute to selective cargo recruitment. CD9, for example, enhances exosomal loading of metalloproteinase CD10, while CD81 enriched membrane domains appear to support the sorting of specific membrane proteins. Moreover, tetraspanins can interact with lipid domains, as shown by the role of CD9 and CD82 in ceramide dependent β-catenin secretion via exosomes [237].

Beyond their roles in cargo sorting and membrane organization, tetraspanins located in the plasma membrane have also been implicated in the formation of specialized microdomains and have been detected in shedding vesicles and viral budding [238]. Their ability to cluster specific proteins and lipids has been linked to the organization of membrane regions involved in viral budding [99] suggesting a broader role in membrane remodeling. Moreover, tetraspanin interactions with the cytoskeletal network may influence the vesicle fission process, although this remains to be conclusively demonstrated. Collectively, these observations suggest that tetraspanins might contribute not only to cargo selection but also to membrane shaping and vesicle release, although further experimental validation is needed to establish these mechanisms [232].

In addition to ESCRT components and tetraspanins, which shape exosome content and vesicle formation, Rab GTPases and SNARE complex protein play a distinct and essential role in regulating the spatiotemporal dynamics of MVB trafficking and exosome release [239]. Although their broader involvement in vesicular transport has been previously noted in endocytic part, certain Rab isoforms exhibit specific and direct roles in exosomal secretion that depends on cell type and different cellular conditions [239].

Specific members of the Rab GTPase family play pivotal roles at different stages of MVB trafficking and exosome release. For instance, Rab27a facilitates the docking of MVBs at the plasma membrane, a critical step in the final exocytic fusion, and has been shown to influence exosome size and release in HeLa cells, neurons, and carcinoma cells. Its closely related isoform, Rab27b, is primarily involved in the intracellular positioning and distribution of MVBs, which determines their accessibility to the release machinery [228].

Upstream in the pathway, Rab11 and Rab35, which are traditionally associated with endosomal recycling, also contribute to cargo loading and secretion into exosomes. Notably, Rab35 facilitates

the release of proteolipid protein rich exosomes in oligodendrocytes, and disruption of either Rab11 or Rab35 leads to intracellular accumulation of exosomal cargo, suggesting a failure in vesicle maturation or exit [240]. In contrast, Rab7, known for regulating MVB fusion with lysosomes, modulates the balance between degradation and secretion. Interestingly, its impact on exosome release appears to be cell type specific, suggesting a finely tuned control mechanism depending on cellular conditions [177]. Altogether Rab GTPases not only direct vesicle transport and positioning but also determine the fate of MVBs whether they fuse with lysosomes for degradation or the plasma membrane for exosome release [240] [241]. Their activities function in concert with ESCRT components, tetraspanins, and lipid based sorting mechanisms, forming an integrated regulatory network that ensures the spatial precision and molecular specificity of exosomal secretion [228].

Microvesicle biogenesis

Microvesicle biogenesis occurs through the outward blebbing and pinching of the plasma membrane, releasing newly formed microvesicles into the extracellular space [242]. This process is accompanied by specific, localized modifications in plasma membrane proteins and lipids, which regulate membrane curvature and rigidity [243]. Additionally, these changes in membrane composition are complemented by the selective enrichment and redistribution of cargo to the sites of microvesicle biogenesis at cell surface [243]. Microvesicle biogenesis shares mechanisms with exosome formation, involving ESCRT I, II, III, and accessory proteins [244]. Among them, arrestin domain containing protein 1 drives plasma membrane budding by recruiting TSG101, while VPS4 also plays a role [245]. Microvesicle formation arises from the coordinated interplay between phospholipid redistribution and cytoskeletal remodeling. The plasma membrane is not a homogeneous structure; instead, it contains microdomains with asymmetrical distributions of lipids and proteins, tightly regulated by a family of enzymes known as aminophospholipid translocases [246]. These include flippases, which transfer phospholipids such as phosphatidylserine from the outer to the inner leaflet, and floppases, which move lipids in the opposite direction from the inner to the outer leaflet. A critical trigger for membrane budding is the externalization of phosphatidylserine to the outer membrane leaflet, disrupting lipid asymmetry and promoting membrane curvature [247]. This process is further facilitated by the actin myosin cytoskeletal network, where contractile forces drive membrane constriction and eventual vesicle scission, completing the budding of microvesicles [247]. While the general steps described above are widely observed, cell specific pathways often modulate microvesicle formation through lipid metabolism and signaling enzymes. In astrocytes, for example, acid sphingomyelinase generates ceramide, a

bioactive lipid that induces negative membrane curvature, thereby promoting microvesicle shedding in a ceramide dependent manner [248]. In contrast, cholesterol has been shown to be essential for microvesicle formation in neutrophils, as cholesterol depletion impairs their ability to form microvesicles [249].

The release of microvesicles from the cell surface is believed to involve a coordinated interplay between the actin cytoskeleton and myosin motor proteins, followed by ATP dependent contraction to facilitate membrane fission [250]. One proposed regulator of this process is the small GTPase ADP ribosylation factor 6 (ARF6), which has been associated with actin remodeling and endocytic trafficking. ARF6 is involved by activating extracellular signal regulated kinase (ERK) through phospholipase D (PLD), potentially leading to localized stimulation of myosin light chain kinase (MLCK) and subsequent phosphorylation of myosin light chain (MLC) a step that may contribute to contractile activity at budding sites [245]. In parallel, RhoA, another small GTPase, could participate in myosin light chain phosphorylation via the ROCK (Rho associated kinase) pathway, and also influence actin filament dynamics through the RhoA cofilin pathway, possibly supporting membrane remodeling during vesicle formation [251]. These observations suggest that cytoskeletal dynamics and localized contractile signaling may play supportive roles in the regulation of microvesicle release, particularly under conditions that promote active remodeling of the plasma membrane [252] .

Additional support for the role of the actin myosin system comes from observations in other cellular processes. For instance, actin polymerization, which powers membrane protrusions during cellular movement, may plausibly provide the force needed to initiate membrane curvature and bud formation [253]. Similarly, myosin motor proteins are known to assist in both cargo delivery and membrane scission, as seen in cytokinesis, where myosin II functions alongside ESCRT components. Notably, both conventional myosin II and ARF6 have been detected in osteoclast derived EVs, further suggesting their potential involvement in vesicle formation [254]. Collectively, these observations suggest that cytoskeletal dynamics and localized contractile signaling may play supportive and conditions dependent roles in the regulation of microvesicle release, particularly under conditions that promote active remodeling of the plasma membrane [255 256].

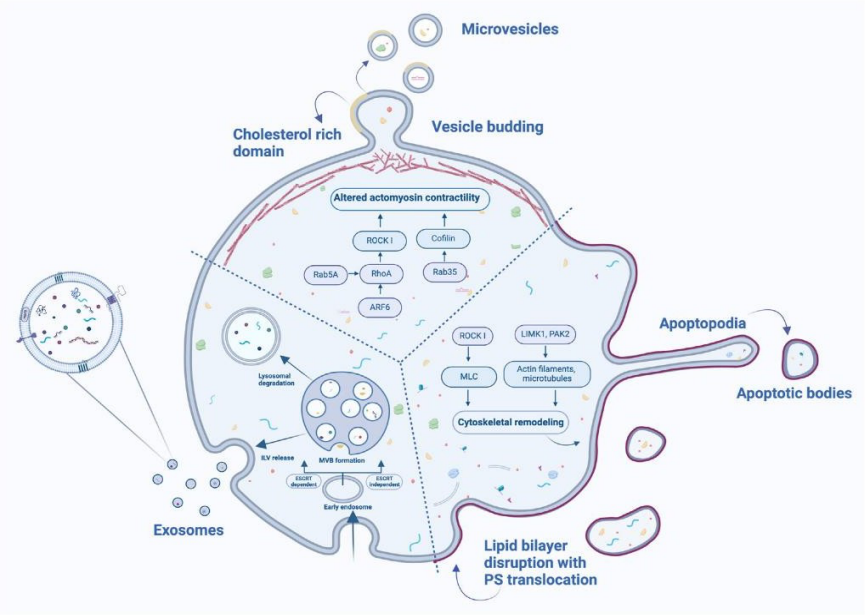

Figure 1.3.1.1: Microvesicle, exosome and apoptotic bodies biogenesis

The figure illustrates the formation of three major types of EV: exosomes, microvesicles (MVs), and apoptotic bodies (ApoBDs). Exosomes are formed through the endosomal pathway, where intraluminal vesicles (ILVs) are generated within multivesicular bodies (MVBs) via endosomal sorting complex required for transport (ESCRT) dependent or ESCRT independent mechanisms. MVs are produced through outward budding of the plasma membrane, a process regulated by the interplay between small guanosine triphosphatases (GTPases), including Rab5A, Rab35, and ARF6, which impact downstream effectors such as Rho associated protein kinase I (ROCK I) and cofilin. These pathways collectively regulate actomyosin contractility and drive cytoskeletal remodeling required for MVs budding. ApoBDs release is characterized by extensive cytoskeletal reorganization driven by ROCK I, which phosphorylates and activates myosin light chain (MLC), enhancing actomyosin contractility and facilitating membrane blebbing. This reorganization is further supported by the actions of Lim kinase 1 (LIMK1) and p21- activated kinase 2 (PAK2), which regulate actin filament stabilization and dynamics. Translocation of phosphatidylserine (PS) to the outer leaflet of the lipid bilayer signals ApoBDs recognition and clearance [256].

1.3.1.2 Extracellular uptake and intracellular trafficking

Effective cellular communication via EVs depends on their successful uptake by recipient cells [219]. EVs can enter recipient cells through multiple pathways, including macropinocytosis, lipid raft mediated uptake, phagocytosis, and membrane fusion [257] [258], with some studies also suggesting tunnelling nanotubes as a potential transfer mechanism [258] [259]. However, based on existing literature, clathrin and caveolin mediated endocytosis appears to be the primary route of EV uptake [257]. EVs can be rapidly taken by endosomal compartments, with vesicles detected inside cells as early as 15 minutes after exposure [259]. Several studies have demonstrated that when cells are incubated at 4°C, their ability to internalize EVs is significantly reduced, indicating that uptake is

an energy dependent process [260] [261]. Additional evidence supporting the active nature of this process comes from observations that EVs are not taken up by cells fixed with paraformaldehyde [262]. Cytochalasin D, a potent inhibitor known to disrupt the actin cytoskeleton and inhibit endocytic pathways [263], has been shown across multiple studies and cell types to significantly reduce, though not entirely block, EV uptake in a dose dependent manner [264]. Collectively, these findings suggest that EV internalization relies on energy and an intact cytoskeleton, both characteristic of endocytic pathways [264]. However, the inability of single inhibitors to fully prevent uptake implies that EVs enter cells through multiple mechanisms [264].

EV uptake is mediated by protein interactions that promote subsequent endocytosis [257]. Evidence for the role of proteins in this process comes from studies showing that Proteinase K treatment of EVs significantly reduces their uptake by ovarian cancer cells [265]. Additionally, numerous EV associated proteins have been identified as interacting with membrane receptors on target cells [266]. Therefore, EV uptake is likely influenced by both the signaling status of recipient cells and the specific protein composition of the vesicle [266].

Due to the presence of tretraspanins on the EV surface and their role in cell adhesion, they could be involved in EV uptake. This was demonstrated by blocking the tetraspanins CD81 or CD9 with antibodies. And this has shown has shown to reduce EV uptake by dendritic cells [261]. Tspan8, another tetraspanin, is known to interact with integrins acting as cell adhesion molecules [257]. When cells overexpress Tspan8, they release EVs carrying a Tspan8-CD49d complex, which helps these vesicles enter rat aortic endothelial cells. Further studies suggest that CD106 may enhance this interaction [257]. Additionally, EVs displaying the Tspan8- CD49d complex on their surface are easily taken up by endothelial and pancreatic cells, where intercellular adhesion molecule 1 (ICAM-1, also known as CD54) serves as the main binding partner [267].

Multiple studies suggest that clathrin mediated endocytosis plays a role in EV uptake. Chlorpromazine, which blocks the formation of clathrin coated pits at the plasma membrane, has been shown to reduce EV uptake in both ovarian cancer cells and phagocytic cells [257]. Dynamin2, a GTPase essential for clathrin mediated endocytosis [268], is recruited to newly forming clathrin coated pits, where it assembles a collar like structure around the neck of deeply invaginated pits [269]. Through GTP hydrolysis, dynamin2 undergoes conformational changes that drive membrane fission, leading to the release of clathrin coated vesicles [268]. Additionally, dynamin2 contributes to membrane binding and curvature during clathrin mediated endocytosis [270]. Dynamin2, can be blocked by the specific inhibitor dynasore [270]. Blocking dynamin2 has been shown to

significantly reduced internalization of exosomes or larger microvesicles, suggesting a role for caveolae mediated endocytosis in vesicular uptake [271].

Lipid rafts are microdomains within the plasma membrane that are enriched with protein receptors and sphingolipids, such as sphingomyelin. Sphingolipids within EVs play a key role in binding and endocytosis, potentially by interacting with cholesterol rich microdomains in dendritic cells [257]. EV uptake was inhibited when recipient cells were pre treated with cholesterol reducing agents such as MβCD, filipin, and simvastatin [257]. These treatments disrupt lipid raft mediated endocytosis but may also impact EV membrane integrity, contributing to the observed reduction in EV uptake. These findings support the hypothesis that lipid rafts play a role in the EV uptake mechanism [257].

After internalization, exosomes follow the typical endosomal pathway, transitioning from early endosomes, which act as sorting compartments, to acidic vesicles such as late endosomes and multivesicular bodies [228]. These compartments eventually fuse with lysosomes, leading to degradation [272]. Lysosomal targeting requires active transport along the cytoskeleton, a process influenced by lipid composition, SNARE proteins, and intracellular pH [272].

However, recent evidence indicates that endosomal acidification while associated with degradation, may also be a prerequisite for EV to escape lysosomal trafficking which would otherwise lead to cargo degradation [273 274]. Therefore, escaping the endosomal pathway is a crucial step for functional delivery [275]. For successful delivery, exosomal cargo must escape the endosome before full maturation into lysosomes. In this regard, late endosomes enriched with anionic lipids may facilitate membrane fusion or pore formation, creating an environment conducive to cargo release into the cytoplasm [228 275]. For example, in dendritic cells, exosomes can be redirected to a CD81 positive, LAMP1 negative compartment, bypassing the lysosomal route entirely and gaining direct access to the cell surface [261].

1.3.2 EV mediated mitochondrial transfer

1.3.2.1 Mitochondrial Quality Control beyond mitophagy

Mitochondria are capable of generating MDVs and mitochondrial derived compartments (MDCs), which may play roles in the selective sequestration and trafficking of mitochondrial contents [276]. Emerging evidence suggests that MDVs can carry lipids and mitochondrial proteins to various destinations, including peroxisomes, endosomes, and lysosomes [277]. This targeted transfer has been proposed as a complementary mechanism within the broader mitochondrial quality control

network, alongside established pathways such as mitochondrial protease activation, ubiquitin mediated proteasomal degradation, and mitophagy [278]. By facilitating the selective removal of specific mitochondrial components, MDVs may help maintain mitochondrial function and integrity under stress conditions [277]. In parallel, the formation of MDCs has been implicated in the degradation of misfolded mitochondrial proteins, potentially contributing to protein quality control within mitochondria. While these pathways remain under active investigation, they represent promising components of the intricate system cells use to manage mitochondrial health and homeostasis [279].

1.3.2.2 Emerging pathways for intercellular mitochondrial transfer

Intercellular mitochondrial transfer is thought to occur through multiple mechanisms, including microvesicle formation, tunneling nanotubes (TNTs), gap junctional channels (GJCs), and cell fusion [280]. Among these, TNTs transient, actin based filamentous structures have been observed to connect cells across distances of up to several hundred microns [281]. These structures, with diameters ranging from approximately 50 to 700 nm, may serve as conduits for the transport of various cellular components, including mitochondria [282]. Studies in co culture systems have shown that TNTs can form between different cell types, both within and across species, and may support unidirectional or bidirectional mitochondrial exchange [283]. Similarly, gap junctional channels, composed of paired hemichannels formed by connexin subunits, have been proposed as another route for intercellular transfer [284]. In particular, heterotypic GJCs formed by connexin 43 and connexin 32 have been associated with mitochondrial transfer from bone marrow stromal cells (BMSCs) to motor neurons, potentially offering protection under stress conditions such as oxygen glucose deprivation [221]. Additionally, the internalization of gap junctions by one of the adjacent cells can lead to the formation of double membrane vesicles known as connexosomes or annular gap junctions, which have been suggested to encapsulate and transport organelles, including mitochondria [285]. While these findings are still under investigation, they point to a growing range of potential intercellular pathways through which mitochondrial exchange may occur, contributing to tissue homeostasis and stress adaptation [283].

1.3.2.3 Extracellular Vesicles as vehicles for mitochondrial transport

Emerging research suggests that EVs may act as important mediators of mitochondrial transport [286]. Mitochondrial components encapsulated within EVs appear to be more resistant to extracellular stressors such as high calcium concentrations, potentially due to the protective properties of the

EV lipid bilayer, which limits calcium diffusion and shields cargo from enzymatic degradation [221]. Compared to isolated mitochondria, mitochondrial material transferred through EVs as well as via tunneling nanotubes (TNTs), gap junctional channels (GJCs), and cell fusion may be better protected from the complexity of the extracellular environment [221]. These contact dependent mechanisms are typically associated with local, proximal mitochondrial transfer, whereas EVs have been proposed to support longer range delivery. Some studies have indicated that EV associated mitochondrial content can reach distant tissues and may even cross biological barriers such as the blood brain barrier (BBB), although the extent and physiological relevance of such transport are still under investigation [287]. Engineered EVs, designed with enhanced membrane targeting features, have also shown potential for more directed mitochondrial delivery in experimental settings [288]. While these findings highlight a promising role for EVs in supporting intercellular mitochondrial communication, further research is needed to fully understand their mechanisms, targeting specificity, and functional outcomes in recipient cells.

1.3.2.4 Heterogeneity of mitochondrial rich extracellular vesicles

Mitochondrial rich extracellular vesicles exhibit considerable heterogeneity in terms of size, mitochondrial content, and functional potential. Small EVs, such as exosomes (<200 nm), often carry mitochondrial fragments including mitochondrial DNA (mtDNA) and mitochondrial proteins [287]. Increased mitochondrial fission under certain conditions may lead to the formation of smaller mitochondria, which could potentially be incorporated into these small vesicles [289]. In contrast, microvesicles formed through plasma membrane budding are generally larger and may carry both damaged and functional mitochondrial components [290]. Some large microvesicles (>500 nm) have been reported to contain more intact mitochondria, although the functional state of these organelles can vary [291]. Additionally, certain catabolic mitochondrial proteins such as COX-IV and pyruvate dehydrogenase E1 component subunit alpha (PDH-E1α) have been selectively identified in specialized vesicles referred to as mitovesicles. Under mild mitochondrial stress, damaged mitochondria may be packaged into migrasomes vesicles formed during cell migration in a process termed migracytosis, which is thought to contribute to mitochondrial quality control [292]. Other larger vesicle types, such as apoptotic bodies and exophers, have also been observed to carry mitochondrial material, including whole mitochondria [293] [294].

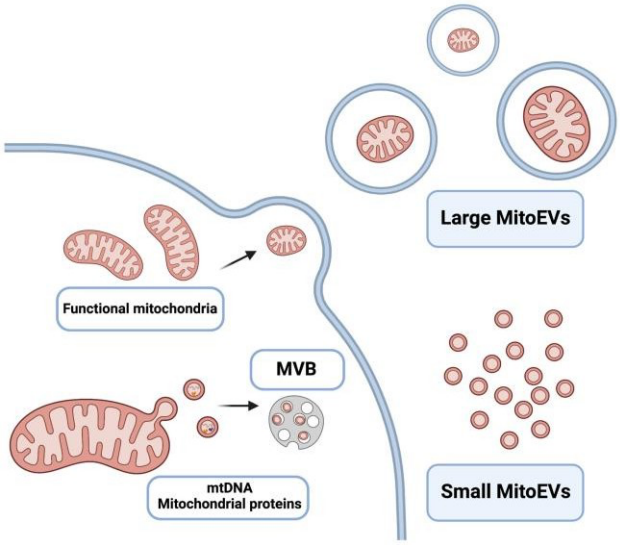

Figure 1.3.2: Biogenesis and classification of mitochondria containing extracellular vesicles (MitoEVs)

This diagram illustrates the two major types of MitoEVs based on their origin and content. Large MitoEVs are shed directly from the plasma membrane and may contain intact, functional mitochondria, making them promising tools for mitochondrial transfer. Small MitoEVs, formed within multivesicular bodies (MVBs), are released as exosomes and typically carry mitochondrial components such as mtDNA and mitochondrial proteins, serving as potential biomarkers in diagnostics [294].

1.3.2.5 Fate and Function of Transferred Mitochondria in Recipient Cells

Following uptake by recipient cells, exogenous mitochondria may follow different intracellular fates [295]. While some are targeted to lysosomes for degradation, others appear capable of escaping these pathways and integrating into the host mitochondrial network. This process may be regulated in part by fusion related proteins such as MFN1, MFN2, and OPA1 [114]. In some experimental systems, mitochondrial transfer has been associated with increases in oxygen consumption, mitochondrial mass, and expression of fusion related proteins in recipient cells. These observations support the possibility that mitochondrial EVs could play a role in modulating cellular metabolism and stress responses [296] [297]. However, further research is needed to fully elucidate the mechanisms, efficiency, and physiological relevance of mitochondrial EV mediated transfer. As such, while mitochondrial EVs are being explored for their potential in intercellular communication and regenerative applications, their therapeutic value remains an area of ongoing investigation [294].

1.3.3 Biogenesis mechanism

MDVs are small, membrane bound structures that bud off from mitochondria, typically ranging from 70 to 150 nm in diameter. Unlike bulk mitochondrial degradation pathways such as mitophagy, MDVs represent a more refined, cargo selective, and compartmentalized response to various cellular stressors [277]. Initially discovered in mitochondrial quality control, MDVs are now recognized as multifunctional vesicles involved in inter organelle communication, immune surveillance, signaling, and disease progression [279]. Importantly, MDVs are not limited to intracellular roles they can also be secreted into the extracellular environment via both microvesicles and exosomes, though a majority of them are reported to be abundant in microvesicles, thereby facilitating intercellular communication and systemic stress responses. Several studies have shown that mitochondrial proteins and mitochondrial DNA are packaged into EVs released by cells such as dendritic cells [221].

MDV biogenesis involves a complex interplay of molecular regulators and stress responsive mechanisms. One of the well known pathways is the PINK1/Parkin axis, which is activated under mitochondrial stress [298]. When mitochondrial membrane potential is lost, PINK1 accumulates on the outer mitochondrial membrane, recruiting the E3 ubiquitin ligase Parkin, which in turn promotes ubiquitination of damaged mitochondrial proteins and facilitates MDV formation particularly for vesicles targeted to lysosomal degradation [299].

Beyond this canonical pathway, additional regulators such as Sorting Nexin 9 (SNX9) and the inner membrane GTPase OPA1 have been shown to selectively drive the formation of matrix positive MDVs [161]. These MDVs are distinct from those carrying only outer mitochondrial membrane proteins such as TOMM20, indicating that separate pathways govern the inclusion of different mitochondrial cargos [300]. Other structural proteins like ARRDC1 and TSG101 of ESCRT complex are implicated in plasma membrane budding of mitochondria containing microvesicles [301]. Cytoskeletal elements such as KIF5B and Myosin19 play a role in directing mitochondria toward the cell periphery during mitocytosis a process in which mildly damaged mitochondria are extruded from the cell within vesicular structures known as migrasomes. This mechanism is observed particularly in migrating cells that serves as an alternative to intracellular degradation [302].

Once formed, MDVs follow distinct intracellular trafficking routes. Some are directed to lysosomes via endolysosomal proteins like STX17, TOLLIP, involved in SNARE mediated fusion and endosomal sorting respectively, and associated SNARE complexes for degradation [303] [304]. While others, particularly MAPL positive MDVs, are shuttled to peroxisomes with the help of the

VPS26/35 complex [279]. MDVs also play roles in immune surveillance; matrix derived MDVs can deliver mitochondrial antigens to MHC-I molecules for presentation, a process dependent on SNX9, RAB7, and RAB9 [305].

Under conditions where lysosomal degradation is impaired or overwhelmed, MDVs may be incorporated into MVBs and released as a subset of EVs, particularly exosomes [306]. This route enables cells to offload mitochondrial material into the extracellular space, contributing to intercellular communication [306]. Indeed, studies have shown that mitochondrial proteins, mitochondrial DNA, and even components with ATP producing capacity can be found in EVs, especially under stress conditions such as hypoxia or cardiotoxic injury [307]. Proteomic overlaps between MDVs and EVs further support their shared lineage, particularly in stress induced conditions where mitochondrial matrix components are preferentially loaded [308]. The inclusion of mitochondrial content in EVs has been shown to depend on SNX9 and OPA1, which regulate the packaging of matrix components, while outer membrane proteins may follow distinct mechanisms [300]. Notably, stress exposure can shift MDV fate toward lysosomal degradation or extracellular release, depending on the nature and localization of the mitochondrial damage. Altogether, MDV biogenesis reflects a finely tuned and adaptive response to cellular stress, operating through multiple molecular pathways to maintain mitochondrial and cellular integrity [309].

While the mitochondrial fission protein DRP1 has been implicated in MDV formation interacting with its receptors MID49, MID51, and mitochondrial dynamics proteins it is not universally required [91]. MDV formation has been observed even in the absence of key autophagy proteins such as Atg5 and Beclin, and independently of Rab9, a marker of late endosomes. This has been further supported by studies in yeast, where MDVs can still form in Dnm1 knockout strains [103]. Interestingly, recent work by König et al. demonstrated that TOM20 positive MDVs may be generated through a process where microtubule associated motor proteins (MIROs) pull on mitochondrial protrusions, which are then cleaved by DRP1 mediated fission. These findings underscore that while DRP1 contributes to certain MDV subtypes, its involvement varies across different vesicle populations [298].

1.3.4 Functional role of extracellular vesicle

EVs are increasingly being explored for their potential in therapeutic and diagnostic applications, owing to their ability to transport proteins, lipids, and nucleic acids between cells, including across complex barriers like the blood brain barrier [310]. Their stability in circulation, biocompatibility, and capacity for tissue specific targeting either naturally or through surface modification

position them as promising candidates for future therapeutic strategies [172] [311]. Several studies have investigated the possible utility of EVs in diverse disease, such as cancer, neurological disorders, cardiovascular conditions, and inflammatory diseases [312]. In addition to their therapeutic potential, EVs are also being explored as non invasive biomarkers, as their cargo can reflect the physiological or pathological state of their cell of origin, offering valuable insights for early diagnosis, prognosis, and disease monitoring [313].

1.3.4.1 In cancer

EVs may contribute to cancer progression through their dynamic exchange between tumor cells and surrounding stromal or immune cells. Studies suggest that EVs from cancer cells can potentially influence the tumor microenvironment by reprogramming neighboring non malignant cells. For instance, pancreatic cancer cell derived EVs have been shown to induce endoplasmic reticulum stress in recipient cells, which may promote transformation [314]. Conversely, EVs from non cancerous cells carrying tumor suppressors such as PTEN have been proposed to inhibit cancer cell proliferation by modulating Akt signaling [315]. The secretion and uptake of EVs within the tumor microenvironment appears to be a finely tuned process. Cancer cells often exhibit higher EV release rates in vitro, though whether this holds true in vivo remains to be fully determined [316].

EVs may also carry immunomodulatory cargo. For example, EVs derived from Natural killer (NK) cells have been shown to contain cytotoxic proteins with potential anti tumor effects, while cancer cell derived EVs have been linked to immune evasion [316]. Multiple myeloma EVs initially activate NK cells but may later impair their function through downregulation of NKG2D receptors [317]. Similarly, EVs have been implicated in modulating T cells, dendritic cells, macrophages, and myeloid derived suppressor cells in ways that may promote tumor immune escape [318]. Interestingly, not all EV interactions support tumor progression. Certain macrophage populations, such as $CD169^{+}$ subcapsular sinus macrophages, have been reported to internalize tumor EVs and potentially prevent their immunosuppressive effects on other immune cells [319]. However, the determinants guiding EV cargo fate whether toward degradation or functional engagement remain poorly understood [318].

1.3.4.2 In neurological disorders

Neurodegenerative disorders are frequently characterized by the misfolding, aggregation, and accumulation of pathological amyloids in or around neurons [320]. The detection of such protein aggregates in body fluids has been proposed as a potential tool for early diagnosis. However,

effective therapies capable of delaying or preventing the progression of these diseases remain limited [321].

Emerging research suggests that EVs may participate in intercellular communication within the central nervous system (CNS) by transporting proteins, lipids, and nucleic acids between cells [322]. Various CNS resident cell types, including neurons, oligodendrocytes, microglia, and astrocytes, have been reported to secrete EVs. These EVs are proposed to play potential roles in regulating neuronal activity and maintaining brain homeostasis [323]. For instance, it has been reported that microglia derived EVs may deliver miR-124 to astrocytes, influencing the expression of glutamate transporters. This EV mediated communication may represent a potential pathway by which microglia help regulate synaptic activity and maintain neuronal balance [323]. Additionally, EVs carrying neuroactive molecules such as Hsp70 and synaptophysin I have been shown to be associated with neurite growth and neuronal support in in vitro and in vivo models [269]. EV associated insulin degrading enzymes released from microglia under serotonin stimulation have also been suggested to contribute to amyloid β (Aβ) degradation [324].

Furthermore, studies have explored the possible involvement of EVs in the spread of neurodegenerative pathology. For example, EVs isolated from AD brains have been reported to contain elevated levels of Aβ, raising the possibility that they may facilitate its intercellular transmission [325]. Similarly, microglial EVs have been implicated in the potential transfer of α synuclein, a key protein associated with Parkinson's disease pathology [326]. These findings collectively point to a potential dual role of EVs in CNS physiology and disease progression. However, further research is needed to clarify the mechanisms involved and to assess their relevance in clinical settings [326].

1.3.4.3 EVs in Inflammation and Immune Regulation

EVs have been increasingly studied for their potential involvement in modulating immune responses and inflammatory processes [327]. While it remains unclear whether EVs act as initiators or consequences of immune activation, current research suggests that they may participate in both immune stimulation and suppression depending on the physiological or pathological condition [230].

In terms of immune activation, EVs have been proposed to facilitate communication among immune cells and modulate inflammatory signaling [328]. For instance, a study showed that EVs derived from platelets and granulocytes have been associated with enhanced inflammation in disorders such as rheumatoid arthritis and inflammatory bowel disease [329]. It has also been

suggested that EVs may present self antigens to autoreactive T cells, possibly contributing to autoimmune responses observed in conditions like systemic lupus erythematosus and type 1 diabetes [330]. Additionally, EVs carrying major histocompatibility complex (MHC) molecules released from immune cells have been shown to potentially support antigen specific immune interactions [328].

On the other hand, EVs have also been seen to be implicated in immunosuppressive processes, particularly in the tumor microenvironment. Tumor derived EVs have been studied for their role in reducing cytotoxic T cell and NK cell activity [331]. Some reports have shown that these vesicles carry molecules such as Fas ligand (FasL), TRAIL, and transforming growth factor-β1 (TGF-β1), which may influence immune cell activity by promoting T cell apoptosis or enhancing regulatory T cell responses [332]. These effects have been proposed to contribute to immune evasion and tumor progression [332]. In certain settings, EV uptake by immune cells such as macrophages may functionally sequester their cargo, limiting delivery to other target cell populations [331].

A growing body of literature has also examined the role of EVs secreted by mesenchymal stem cells (MSCs), macrophages, dendritic cells (DCs), endothelial progenitor cells (EPCs), and astrocytes in immune modulation [333]. These vesicles are believed to carry bioactive molecules that influence immune effector cells and regulate inflammation [333]. For example, MSC derived EVs have been shown to attenuate inflammation and alleviate cognitive deficits in a murine model of epilepsy, possibly by targeting astrocytes. The cargo of MSC EVs has also been associated with cardioprotective effects, showing potential anti inflammatory, immunomodulatory, and anti angiogenic activities [334].

Macrophage derived EVs (Mφ EVs), in particular, have received attention for their potential roles in inflammatory resolution, immune cell modulation, and tissue repair [335]. Depending on the phenotype of the parent macrophage whether M1 or M2 these EVs may exert different effects. For instance, M1 EVs have been reported to carry microRNAs like miR-222 and miR-155 involved in regulating inflammation and vascular remodeling, while M2 EVs have been linked to anti apoptotic signaling in cardiac repair [336 337]. In case of insulin resistance and diabetic complications, EVs from both M1 and M2 macrophages have been suggested to play roles in wound healing, osteogenesis modulation, and inflammation control, although their effects may vary with disease stage and phenotype heterogeneity [336].

Moreover, EVs have been studied as potential therapeutic carriers. Engineered EVs modified to display specific surface markers or encapsulate targeted therapeutic molecules have shown promise

in delivering anti inflammatory agents to inflamed or diseased tissues [338]. For example, Mφ EVs loaded with berberine or baicalin have demonstrated enhanced targeting and neuroprotection in experimental models of stroke and inflammation [339] [340].

1.3.4.4 EV in mitochondrial function

Emerging evidence suggests that EVs carry mitochondrial components including mitochondrial proteins, mtDNA, and sometimes whole mitochondrial fragments and may participate in cellular communication pathways that influence both metabolic and immune responses [341]. While the functional consequences of such mitochondrial transfer remain an active area of investigation, studies across various biological systems have begun to outline scenarios in which mitochondrial EVs could contribute to homeostasis or pathology, depending on the condition in which they are released and the state of the recipient cell [342].

Mitochondrial transfer via EVs has been proposed to support metabolic function in stressed or deficient cells. For example, MSCs have long been studied for their capacity to donate mitochondria, with studies showing that MSC derived EVs can enhance oxidative phosphorylation and rescue metabolic activity in recipient cells lacking mitochondrial function [330]. This has been observed in models of myocardial infarction and ischemia reperfusion injury, where administration of mitochondrial rich EVs was associated with improved tissue repair and reduced cellular damage [343]. Although these findings are encouraging, the precise mechanisms through which these improvements occur whether through direct mitochondrial integration or by modulating signaling pathways remain to be fully clarified [221].

Furthermore, mitochondrial delivery through EVs has been associated with immunomodulatory effects in certain inflammatory conditions. For instance, in an acute respiratory distress model, Morrison et al. observed that alveolar macrophages receiving mitochondrial cargo from MSC derived EVs showed increased OXPHOS activity [344]. This metabolic shift was accompanied by features consistent with an anti inflammatory M2 like phenotype, along with a reduction in tumor necrosis factor alpha (TNF-α) secretion and signs of pulmonary inflammation [344]. These findings suggest a potential role for mitochondrial EVs in modulating immune responses during inflammation [344]. Similarly, exosomes derived from adipose tissue derived MSCs have been reported to transfer mitochondrial content to alveolar macrophages, potentially enhancing mitochondrial integrity and OXPHOS activity [345]. This mitochondrial support may contribute to the restoration of metabolic and immune balance in airway macrophages and help mitigate pulmonary inflammation [345]. In another study, Hough et al. described a mechanism in which

airway myeloid derived regulatory cells released mitochondrial EVs that were taken up by T cells, leading to increased MitoSOX fluorescence, indicting increased mitochondrial ROS production. This points to a potential mode of mitochondrial EV mediated immune regulation in inflammatory airway diseases such as asthma [346].

Interestingly, mitochondrial material within EVs does not always elicit a protective effect. Under certain stress conditions such as exposure to bacterial lipopolysaccharide (LPS) or expression of neurotoxic proteins immune cells have been shown to release mitochondrial content that may instead exacerbate inflammation [347] . In these settings, EV mediated release of damaged or oxidized mitochondria has been associated with immune activation, recruitment of inflammatory cells, and in some cases, neuronal toxicity [347 348]. These contrasting roles suggest that the biological effects of mitochondrial EVs are highly dependent on the state of the donor cell and the physiological condition of the recipient [342]. Taken together, these observations point toward a multifaceted role for mitochondrial content in EVs, which may range from metabolic support and immune regulation and suppression. While much of the current evidence is based on in vitro models or limited in vivo systems, ongoing research continues to refine our understanding of how mitochondrial EVs operate across physiological and pathological states [221].

Endocytosis, exocytosis, and extracellular vesicle trafficking are coordinated by the cytoskeletal network, which acts as the main transport system guiding the spatial organization and directed movement of vesicular cargo within the cell [349 350].

1.4 Cytoskeletal network

The cytoskeleton plays a fundamental role in various cellular processes, including mitosis, cell division, mechanical stiffness, polarity, and extracellular matrix organization. These functions collectively influence development and tissue differentiation. Acting as a dynamic framework, the cytoskeleton processes and transmits both intracellular and extracellular signals, coordinating essential physiological responses [351]. There are three main types of cytoskeletons in eukaryotic cells: microfilaments (actin filaments), microtubules, and intermediate filaments each playing distinct roles in maintaining cellular structure, transport, and dynamics [352]. Actin filaments rapidly assemble into dense, branching networks, such as in the lamellipodium, where they generate the forces needed for cell movement. Microtubules, made of tubulin, act as internal highways, guiding the transport of proteins, organelles, and other cellular components [353]. Intermediate filaments provide mechanical strength, stabilizing the cell against stress and regulating cell movement [352 354].

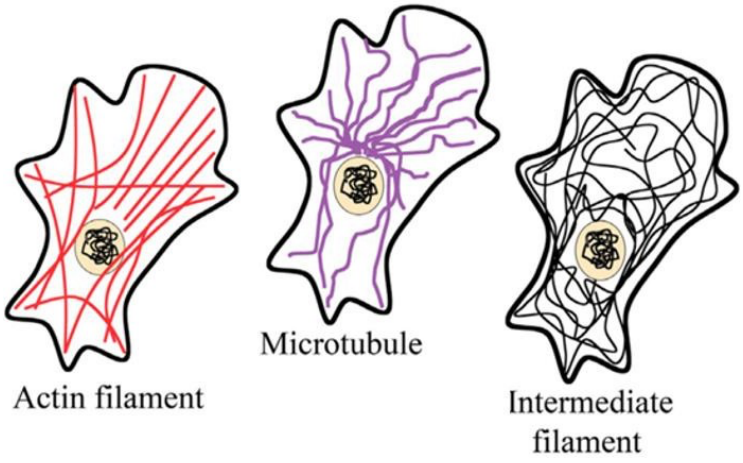

Figure 1.4: Cytoskeletal network

Actin filaments are composed of actin monomers and provide structural support while enabling cell movement. Microtubules are hollow cylinders made of tubulin dimers and are involved in processes such as mitosis and intracellular vesicle transport. Intermediate filaments, formed from various filamentous proteins, contribute to cellular integrity and help maintain cohesion among epithelial cells
[354].

1.4.1 Microtubules

Microtubules are organized into one or multiple arrays of polarized tracks, varying in arrangement depending on the cell type, and play a key role in establishing intracellular polarity. They are composed of α and β tubulin heterodimers, with α-tubulin exposed at the minus end and β-tubulin at the plus end [355]. Both subunits bind GTP, but only β tubulin hydrolyzes its GTP during normal microtubule polymerization. The proper folding of α and β tubulin requires the assistance of specialized chaperones [356]. In addition to these core components, γ-tubulin plays a crucial role in microtubule nucleation, initiating the growth of new microtubule structures [357]. Like α and β tubulin, γ-tubulin has been identified in all free living eukaryotic organisms, suggesting that the combination of these three proteins represents the essential set of tubulins required for microtubule function in eukaryotic cells [358]. Microtubules have inherent structural polarity, with a dynamic plus end and a comparatively stable and slow growing minus end allowing them to explore the cytoplasm and perform multiple cellular functions [359]. These characteristics of microtubule minus ends are, in part, a function of microtubule structure, but can be influenced *in vivo* by an association with a microtubule organizing centre (MTOC). MTOCs can be broadly defined as sites that localize microtubule minus ends, with functions that include microtubule nucleation, stabilization, and or anchoring [360]. A diverse set of microtubule associated proteins (MAPs) exists [361], playing a crucial role in regulating microtubule structure, dynamics, and functions in various cellular

processes [361] [362].

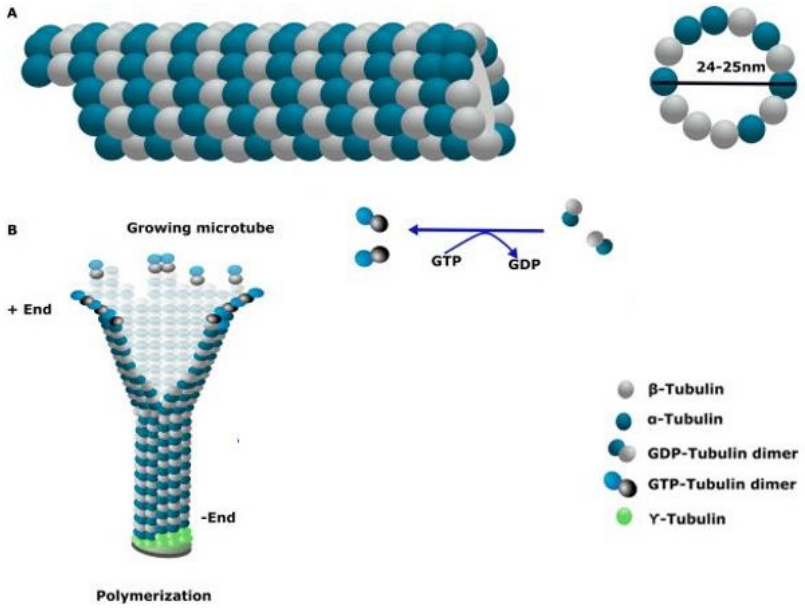

Figure 1.4.1: Microtubule structure

A) This figure represents the structure and growth of a microtubule, a hollow tube formed by α and β tubulin dimers arranged into protofilaments. B) Microtubules have inherent polarity, with growth primarily occurring at the plus end, where GTP bound tubulin adds to the structure. The minus end is typically anchored to γ-tubulin at microtubule organizing centers (MTOCs). GTP hydrolysis on β-tubulin drives dynamic instability, allowing microtubules to alternate between growth and shrinkage key for cellular processes like intracellular transport and mitosis [362].

1.4.2 Centrosome

The best characterized MTOC in animal cells is the centrosome, a non membranous organelle composed of a pair of centrioles surrounded by a protein dense matrix called the pericentriolar material (PCM). This structure plays a pivotal role in organizing a radial microtubule array, essential for proper spatial arrangement of organelles, vesicle trafficking, and chromosome segregation during mitosis [360]. The centrosome serves as the primary MTOC in many cell types, acting as a central hub from which microtubules are nucleated and anchored [363]. During interphase, it directs microtubule organization to support cell shape, polarity, and intracellular transport [364]. Anchored microtubules extend outward, forming a radial array that centers the centrosome through balanced mechanical forces. Experimental depolymerization using nocodazole has demonstrated that microtubule generated pushing and pulling forces are critical for centrosome positioning [365]. Dynein, a minus end directed motor protein, contributes to stabilizing this array; its inhibition leads

to microtubule alteration and centrosome displacement [366].

Microtubule nucleation primarily depends on γ-tubulin and its associated proteins (GCP2–GCP6). In higher eukaryotes, these proteins assemble into ring shaped complexes called γ TuRCs, which act as scaffolds for tubulin dimers, promoting microtubule polymerization [367]. However, efficient γ-tubulin mediated nucleation requires additional regulatory factors that either activate γ TuRCs or target them to MTOCs [368]. The centrosomal binding of γ TuRCs involves multiple factors, including four mammalian proteins: pericentrin (PCNT) and its paralog AKAP450 (also known as AKAP350 or CG-NAP), along with CDK5Rap2 (CEP215) and its paralog myomegalin [369]. These proteins contain conserved yet degenerate motifs for γ-tubulin binding and have been shown to interact with γ-tubulin in vitro. Due to these characteristics, they are considered γ TuRC receptors, playing a presumed role in microtubule nucleation at the centrosome [370 371].

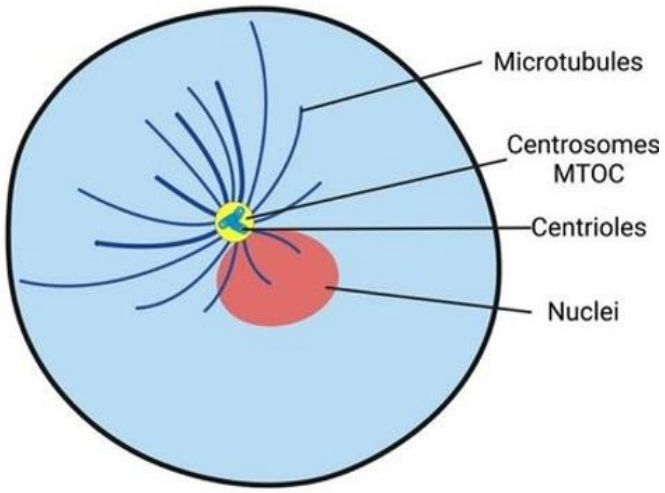

Figure 1.4.2: Centrosome as the primary microtubule organizing center (MTOC)

Depicts organization of microtubules in a typical animal cell. The centrosome, depicted in yellow, serves as the primary MTOC and is located near the nucleus (red). It comprises a pair of centrioles surrounded by pericentriolar material and anchors the minus ends of microtubules (blue lines), which radiate outward to establish intracellular polarity and facilitate cargo transport and structural organization [371].

1.4.3 Molecular motor proteins

Molecular motors facilitate cargo transport along microtubule tracks by directly binding to cargo or interacting with adaptor or scaffold proteins for linkage. These motors harness ATP hydrolysis to generate energy, enabling movement with varying directionality and speed. Dynein functions as a minus end directed motor, while the kinesin family includes both plus end and minus end directed motors [372]. Although these molecular motors perform complementary functions on microtubules, they differ in structure and mechanism. Kinesin 1 features a globular motor domain that binds

to microtubules and hydrolyzes ATP to generate movement. Two identical motor domains are linked by a short neck linker to a shared tail [372]. In vitro studies have demonstrated that kinesin moves through a coordinated stepping mechanism, resembling the motion of human walking [373].

Unlike kinesin, dynein features large heterohexameric rings of AAA+ ATPase subunits, which connect to microtubules through a coiled coil stalk [374]. Unlike kinesin's tightly coordinated movement, dynein's motor domains step independently, with either monomer advancing while the other remains attached as an anchor [375]. Dynein also exhibits a diffusional stepping behavior, frequently taking sideways and backward steps [376]. The net displacement of organelles is influenced by the relative activity of multiple motors attached to each organelle type. Kinesins and dyneins function in opposition, much like a tug of war, where changes in transport direction occur when one motor gains a numerical advantage over the other [358]. In the absence of cellular or environmental signals, directional control mechanisms remain inactive, resulting in minimal net organelle movement. However, during stimulated transport, physiological signals such as hormones or growth factors trigger cargo motility. This regulation is often cargo specific and typically involves post translational modifications that fine tune motor activity [377 378].

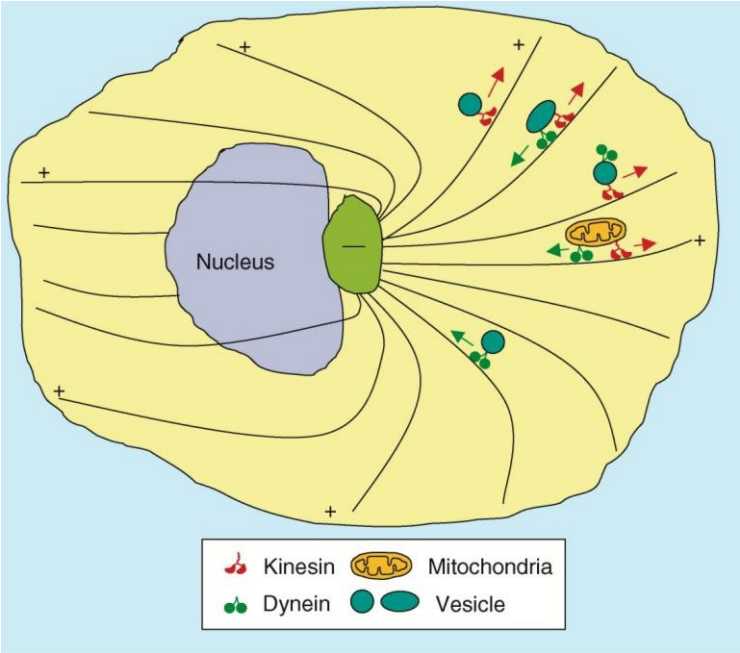

Figure 1.4.3: Microtubule based intracellular transport mediated by motor proteins

This diagram shows how intracellular cargo such as vesicles and mitochondria are transported along microtubules using motor proteins. The microtubules radiate from the microtubule organizing center (MTOC) located near the nucleus, with minus ends anchored at the center and plus ends extending toward the cell periphery. Kinesins (red arrows) move cargo such as vesicles and mitochondria toward the plus ends (anterograde transport), while dyneins (green arrows) direct them toward the minus ends (retrograde transport), enabling spatial distribution and organelle

positioning within the cell [378].

1.4.4 Role of microtubule motor proteins in endocytic pathway

It is well known that all types of endosomes move actively along the microtubule network, a process essential for cargo sorting, maturation, fusion, and long distance intracellular transport. This motility is particularly evident in neurons, but also occurs in cultured cells [379]. While dynein, a minus end directed motor protein, plays a predominant role in inward movement of all endocytic compartments, including early endosomes, late endosomes, and lysosomes [380], kinesins, which generally move toward the microtubule plus end, also contribute critically to bidirectional transport and peripheral redistribution.

Rab5 positive early endosomes predominantly undergo dynein mediated inward long range transport from the cell periphery toward the MTOC. However, they also exhibit outward and bidirectional motility, especially when influenced by cortical actin zones to travel intracellular short distances or during receptor recycling [381]. Their motility pattern is characterized by long range directed movement interspersed with pauses and occasional reversal, generating tension that can drive cargo sorting and membrane remodeling [382]. Most of the time, early endosomes travel through long range inward movements, followed by non directed resting phases and occasional changes in direction [382]. These abrupt transitions in motility are thought to generate membrane tension, which may play a regulatory role in cargo sorting and membrane fission. Altogether, the highly dynamic nature of early endosome transport driven by coordinated cycles of motility, fission, and fusion facilitates efficient cargo exchange and redistribution within the cell [382].

Crucially, early endosome transport is regulated by both dynein and kinesin motors in a coordinated manner. Multiple kinesins particularly kinesin-1, kinesin-2, and kinesin-3 family members have been implicated in early endosomal dynamics [383]. For example, the kinesin-3 motor KIF16B interacts with the early endosomal lipid PIP(3)P via its PX domain to direct Rab5 positive endosomes to the cell periphery. This action supports receptor recycling and transcytosis of transferrin receptor, highlighting kinesin's role in balancing degradation and recycling pathways [384]. Late endosome and lysosome transport also involve multiple kinesins [380]. Disrupting KIF3A (kinesin-2) causes clustering of both late endosomes and lysosomes, and KIF3A moves late endosomes in vitro [385]. The kinesin-1 motor KIF5B is essential for lysosome redistribution under cytoplasmic acidification, with SKIP (SifA and kinesin interacting protein) linking kinesin 1 motor KIF5B to lysosomes via Arl8, Arf like GTPase. Additionally, KIF1Bβ (kinesin-3) and KIF2β (kinesin-13) contribute to plus end directed lysosomal transport [386].

Dynein plays a key role in sorting recycling cargo like transferrin away from degradative cargo such as EGF [383]. This process may involve endosomal membrane domain segregation through a tug of war between dynein and kinesin [387]. Additionally, dynein facilitates the sorting of recycling cargo into tubules that emerge from endosomes during their maturation into late endosomes. These tubules are formed by SNXs, membrane deforming proteins that transport cargo to the plasma membrane or TGN, often in coordination with the Vps26/29/35 retromer complex [193]. Different SNXs specialize in distinct sorting pathways. For example, mannose-6-phosphate receptor recycling from late endosomes to the TGN requires retromer, SNX1/2, and SNX5/6, with SNX5/6 recruiting dynactin for dynein dependent tubule elongation and scission [388]. Meanwhile, SNX4 directs transferrin receptor sorting from early endosomes to Rab11 positive recycling endosomes [209]. Altogether microtubules and motor proteins work together to drive membrane tubulation and endosomal maturation. The mechanical forces they generate contribute to membrane tension, facilitating the formation, elongation, and scission of membrane tubules at key stages of vesicle trafficking process [379].

1.5 Summary

From the current literature, it is increasingly evident that mitochondria function beyond their conventional role as energy generators, influencing a broad range of intracellular processes, including organelle communication and redox balance [18]. Among these, the interplay between mitochondrial integrity and vesicle trafficking is emerging as a key area of interest.

This study aimed to understand how mitochondrial dysfunction impacts the spatial organization and function of endocytic compartments beyond the well-established effects on lysosomes. Additionally, since our lab has already demonstrated a lysosomal defect under mitochondrial stress [27], we aimed to determine whether such an alteration affects the trafficking of functional EV cargo. Understanding this will help us know the potential of EV mediated rescue of mitochondrial function. By analysing both the intracellular consequences of mitochondrial dysfunction and its influence on EV uptake and routing, this work aims to uncover the interconnected mechanisms that govern vesicle dynamics and intracellular vesicle trafficking.

1.6 Hypothesis

Mitochondrial dysfunction is known to impair lysosomal structure and function, and this impairment is associated with increased ROS. Given that endosomal maturation is a stepwise process leading toward lysosomes, it is reasonable to speculate that mitochondrial impairment might also affect upstream compartments of the endocytic pathway, particularly early and recycling endosomes. However, this remains poorly understood. Based on this, we hypothesize that mitochondrial dysfunction may influence the structure and functional dynamics of these earlier endosomal compartments. If such alterations exist, we aim to determine whether they are mediated by ROS, and further investigate whether these effects are direct or occur through mitochondrial regulation of other organelles or intracellular networks. Understanding this relationship will offer mechanistic insights into how mitochondrial dysfunction contributes to endosomal misregulation and defects in vesicular trafficking.

Furthermore, as trafficking defects may underlie broader physiological consequences, we seek to determine whether lysosomal alteration is associated with intracellular trafficking function of cargo. In the latter part of this study, we investigate whether EVs which are known carriers of mitochondrial components can restore alterations observed in cells with mitochondrial dysfunction. To explore this rescue potential, it is first essential to understand how mitochondrial dysfunction affects the uptake and intracellular routing of EVs. Together, these investigations will aim to elucidate the mechanistic link between mitochondrial status and endosomal dynamics, and to investigate how mitochondrial dysfunction influences the uptake, intracellular trafficking, and fate of EVs, particularly in their potential to deliver mitochondrial components and interact with altered cellular networks.

To address these questions, we employed both genetic and chemical models. Genetically, we utilized mouse embryonic fibroblasts (MEFs) with a targeted knockout of OPA1, a key regulator of mitochondrial inner membrane fusion, as well as primary cells carrying mutations in DRP1, a protein critical for mitochondrial fission. In parallel, we chemically altered mitochondrial function in wild type MEFs by inhibiting complex I by Rotenone and complex III by Antimycin A of the ETC, thereby inducing mitochondrial dysfunction through impaired oxidative phosphorylation and elevated ROS production. Using these models, we aim to address the following objectives:

Objective 1: Does mitochondrial dysfunction have an impact on endocytic pathway?

Although mitochondrial dysfunction is known to impair lysosomal function, its impact on upstream endocytic compartments like early and recycling endosomes is not well understood. Since these compartments play critical roles in cargo sorting and trafficking, studying them can reveal whether mitochondrial impairment affects the broader endocytic pathway. By examining structural and functional changes in these organelles, we aim to uncover whether mitochondrial health is intricately linked to endocytic organization and trafficking efficiency.

Objective 2: Is increased oxidative stress resulting from mitochondrial dysfunction responsible for altering the endocytic pathway?

Mitochondrial dysfunction is a significant source of intracellular ROS, which are implicated in lysosomal alterations. Although the association between mitochondrial derived ROS and lysosomal impairment is well established, it is not yet known whether ROS similarly affect upstream components of the endocytic pathway, such as early and recycling endosomes. Exploring the role of oxidative stress in modulating the organization and function of these compartments will provide deeper insights into how mitochondrial dysfunction disrupts endocytic trafficking and overall cellular organization.

Objective 3: Does mitochondrial dysfunction alter the uptake and fate of internalised EVs?

Since EV internalisation often relies on endocytic pathways and is known to be destined to lysosomes, we aimed to investigate whether this process is altered under mitochondrial dysfunction. Particularly, we sought to determine whether lysosomal defects or potential disruptions in other endosomal compartments, which will be examined in Objective 1, affect the efficiency and intracellular routing of EVs within recipient cells. Understanding whether mitochondrial dysfunction alters the uptake and intracellular fate of EVs will shed light on how intercellular communication is affected under stress conditions and whether diseased cells retain the capacity to respond to EV mediated signals or therapeutic cargo.

CHAPTER II

MITOCHONDRIAL DYSFUNCTION ALTERS EARLY ENDOSOMES ROS-MEDIATED MICROTUBULE REORGANIZATION

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Abstract

Mitochondria are essential for bioenergetics and key processes including differentiation and immunity, their dysfunction leading to several pathologies. While these pathologies have traditionally been associated with ATP deficits, mitochondrial dysfunction also leads to reactive oxygen species (ROS) generation, inflammation, and alterations in the function of other organelles. While the negative impact of mitochondrial dysfunction on lysosomal activity has been established, the relationship between mitochondria and the rest of the endocytic compartment remains poorly understood. Here, we show that inhibiting mitochondrial activity through genetic and chemical approaches causes early endosome (EE) perinuclear aggregation and impairs cargo delivery to lysosomes. This impairment is due to altered microtubule architecture and centrosome dynamics, mediated by ROS. Antioxidants can rescue these EE defects, underlying the pivotal role of mitochondria in maintaining cellular activities through ROS regulation of microtubule networks. Our findings highlight the significance of mitochondria beyond ATP production, emphasizing their critical involvement in endocytic trafficking and cellular homeostasis. These insights emphasize mitochondria's critical involvement in cellular activities and suggest novel targets for therapies to mitigate the effects of mitochondrial dysfunction.

Introduction

Mitochondria are key bioenergetic organelles that play crucial roles in not only metabolite generation and exchange, but also in a range of cellular processes including cell differentiation and immunity (1,2). As a consequence, alterations in mitochondrial functions cause a wide range of muscular and neurological pathologies (3). While these pathologies were historically thought to result from ATP deficits, it is now clear that other mechanisms are also involved, including enhanced inflammation and the generation of reactive oxygen species (ROS) (4,5). In fact, the ATP-independent roles of mitochondria are crucial for the control of cellular differentiation during development, inflammation and cancer (6).

At the cellular level, altered mitochondrial structure and function are linked to defects in other organelles. For example, loss of mitochondrial function leads to defects in lysosomal activity (7,8). As lysosomes are the main degradative organelles, this affects protein turnover and causes the accumulation of aggregated material within the affected cells. Lysosomes receive their material to degrade through autophagy, which is known to be affected by mitochondrial dysfunction, and

following endocytosis of extracellular material (9,10). In the endocytic pathway, extracellular material first accumulates in early endosomes (EEs) that sorts this material towards recycling to the cell surface or targets them to late endosomes and lysosomes for degradation (11). The sorting and transfer of material to recycling and late endosomes requires the action of small GTPases of the Rab family. Rabs coordinate all the steps required for cargo sorting and transfer to other endocytic organelles, including the transport along microtubules that allow fusion between vesicles and transfer to lysosomes (12).

While alterations in mitochondrial activity impact lysosomes, the consequences on other components of the endocytic pathway remain poorly understood. Here, we show that mitochondrial dysfunction disrupts EE distribution and cargo trafficking, revealing a previously unrecognized link between mitochondrial function and endocytic transport mechanisms. Specifically, the presence of genetic mutations in mitochondrial proteins or the chemical inhibition of the electron transport chain results in the perinuclear aggregation of EEs and impairs their ability to deliver cargo to lysosomes. These alterations in EE distribution are caused by changes in microtubule architecture and centrosome dynamics, which then facilitates EE transport and aggregation in the perinuclear region of the cell. We found that this is mediated by ROS, which affect microtubules and centrosome organization and thus EE distribution and function. Consequently, antioxidants rescue EE defects present in cells with mitochondrial dysfunction. Overall, our results demonstrate that ROS generated by defective mitochondria actively disrupt endocytic trafficking through the reorganisation of microtubule networks, underscoring the critical role of mitochondria in the maintenance of cellular activities.

Results

Mitochondrial dysfunction causes early endosome aggregation

Mitochondrial dysfunction impairs lysosomal activity, which is accompanied by the presence of enlarged and vacuolated lysosomes and late endosomes (7,8,13). We thus determined whether this extended to the rest of the endocytic compartment. For this, we first used mouse embryonic fibroblasts (MEFs) in which the essential mitochondrial protein OPA1 is deleted as a model for mitochondrial dysfunction (8,14). As we previously reported (8), these cells contained large vacuolated structures marked with the lysosomal marker LAMP1 (Figure 1A-B). As early endosomes (EEs) are required to deliver extracellular material to lysosomes, we determined whether they become vacuolated similar to lysosomes by staining cells with an antibody against the

EE marker Rab5. However, in contrast to LAMP1-positive lysosomes, Rab5-positive EEs did not appear vacuolated in OPA1 KO MEFs (Figure 1A-B). Nevertheless, there was a clear shift in EE distribution in OPA1 KO cells. Whereas EEs were equally dispersed throughout in control cells, we observed perinuclear clustering of EEs in the mutant cells, which was seen as large aggregates of Rab5- positive structures (Figure 1A, C-D). On the other hand, LAMP1-positive lysosomes did not accumulate in the perinuclear region (Figure 1E), although their distribution was somewhat altered (with overall decreased amounts in the middle of the cytoplasm relative to the perinuclear and plasma membrane regions, Sup. Figure 1A). Rab11-positive recycling endosomes were present in the perinuclear region irrespective of the genotype (Figure 1F, Sup. Figure 1B-C), as previously reported for WT cells (15).

To confirm that the alterations we observed in EEs are caused by the impairment of mitochondrial function, not some other defect associated with mutant fibroblasts, we chemically inhibited subunits of the electron transport chain (ETC) in WT MEFs. Similar to OPA1 KO MEFs, rotenone (Complex I inhibitor) or Antimycin A (AA, Complex III inhibitor) caused the perinuclear aggregation of Rab5- positive EEs (Figure 1G). AA treatment also caused EE perinuclear aggregation in HeLa cells (Figure 1H). Interestingly, a similar aggregation of rab5-positive EEs was observed in primary human fibroblasts mutant for the mitochondrial fission protein DRP1 (Figure 1I). To confirm that the changes are the consequence of altered EEs, not simply a change in Rab5 distribution, we measured EE distribution using EEA1 as a second EE marker. Similar to Rab5-positive EEs, we found that EEA1-positive early endosomes aggregated in the perinuclear region of OPA1 KO MEFs, WT MEFs treated with ETC inhibitors and DRP1 mutant fibroblasts (Figure 1J-L, Sup. Figure 1D), further indicating that EEs are altered in these cells.

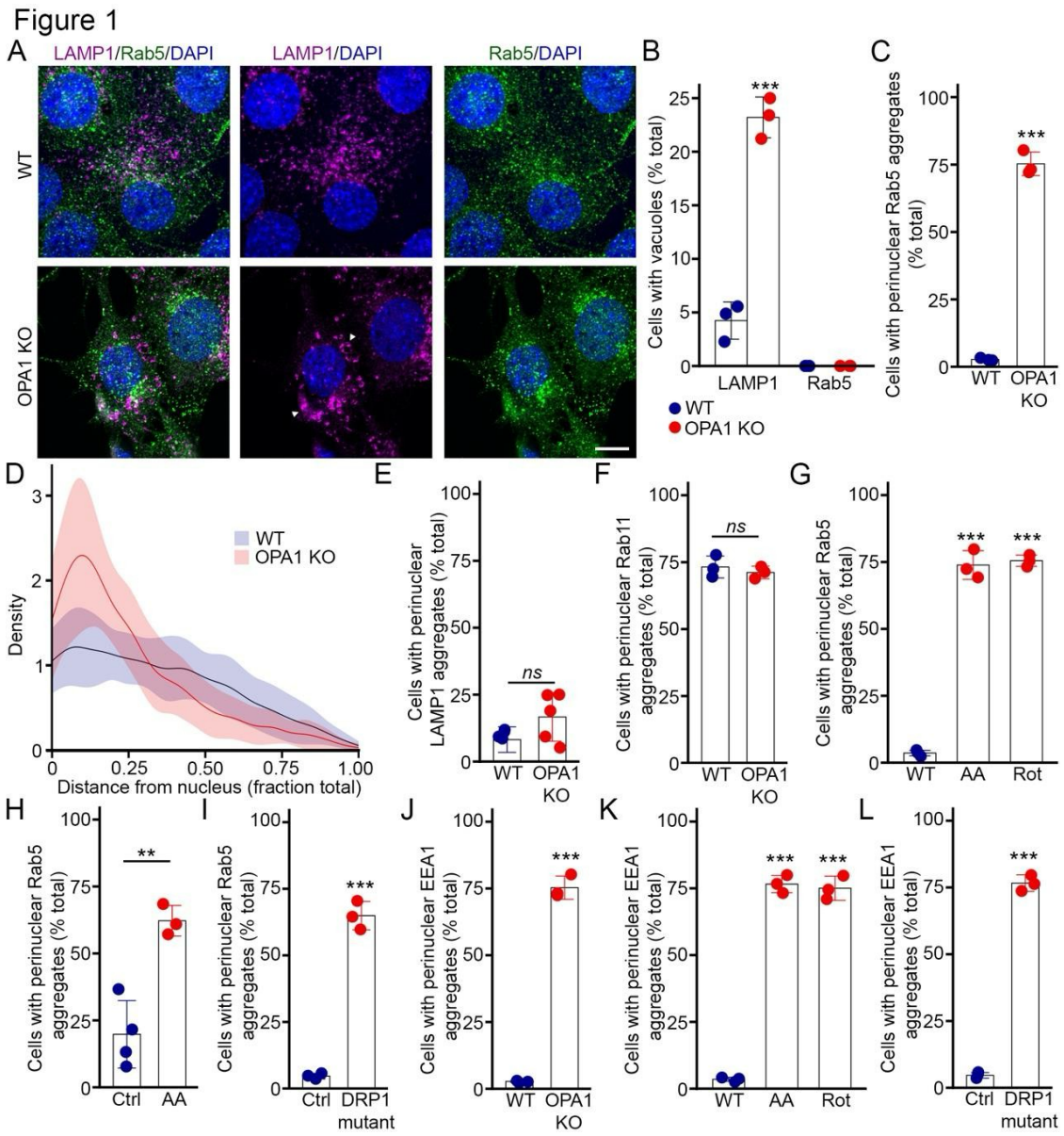

Figure 1. Mitochondrial dysfunction alters the distribution of early endosomes. (A) Representative confocal images of WT and OPA1 KO MEFs stained for the lysosomal marker LAMP1 (magenta) and the EE marker Rab5 (green), along with DAPI to mark nuclei (blue). Arrowheads denote enlarged vacuolated lysosomes. Scale bar 10 µm. (B) Quantification of WT and OPA1 KO cells with LAMP1- positive and Rab5-positive vacuoles from images in (A). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, two-sided t-test. (C-F) Quantification of perinuclear clustering of the endosomal markers Rab5 (C), LAMP1 (E) and Rab11
(F) in WT and OPA1 KO MEFs from confocal images as in (A). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition (5 for LAMP1). *** $p<0.001$, two-sided t-test. ns, not significant. The density of Rab5-positive vesicles relative to their localisation was also measured for Rab5 from the same images (D). The data shows the quantification of 30 cells per condition in 3 independent experiments ± SD. (G) Quantification of perinuclear clustering of Rab5 in WT MEFs treated with the Complex III inhibitor Antimycin A (AA) or the Complex I inhibitor Rotenone (Rot). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, One-way

ANOVA. (H) Quantification of Rab5 perinuclear clustering in AA- treated HeLa cells. Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. ** $p<0.01$, two-sided t-test. (I) Quantification of Rab5 perinuclear clustering in Control and DRP1 mutant primary fibroblasts. Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, two-sided t-test. (J-L) Quantification of perinuclear clustering of the EE marker EEA1 in OPA1 KO MEFs (J), WT MEFs treated with AA or Rotenone (K) and DRP1 mutant primary fibroblasts (L). Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, two-sided t-test, one-way ANOVA for (K).

EE aggregation in cells with mitochondrial dysfunction is associated with impaired EE cargo trafficking

The altered EE distribution in cells with mitochondrial dysfunction prompted us to address its consequences on EE function. EEs serve as sorting organelles to transfer cargo to late endosomes/lysosomes for degradation, or recycling endosomes for material returning to the cell surface (11). We first tracked the transfer of EE cargo towards lysosomes using fluorescently labelled dextran. Cells were first pulsed for 5 minutes with dextran, after which dextran uptake was evaluated by immunofluorescence and quantified by measuring the number of dextran-positive vesicles present in each cell. As cells with mitochondrial dysfunction showed similar uptake of dextran than control cells (Figure 2A), we then performed a chase experiment where the fate of the dextran is followed over time after the initial pulse. We investigated the trafficking of dextran in these endosomes by measuring the association of dextran with Rab5 and the lysosomal marker LAMP1 by confocal microscopy. As expected, dextran colocalized with Rab5-positive EEs immediately after the initial pulse, and gradually transferred to LAMP1-positive late endosomes/lysosomes over time in control cells (Figure 2B-C). We also confirmed this manual evaluation of colocalization by measuring Pearson correlation coefficients in the same images. While the overall low amount of colocalization between dextran and Rab5 caused low Pearson coefficient values, there was a clear increase in Pearson coefficient between dextran and LAMP1 over time, confirming our colocalization data (Sup. Figure 2A-B).

Having validated the assay, we then measured dextran distribution in the endosomes of OPA1 KO MEFs. In contrast to WT cells, dextran colocalized with Rab5-positive EEs to a much lower extent in OPA1 KO MEFs. Consistent with this, dextran transferred to LAMP1-positive lysosomes at a lower rate in OPA1 KO MEFs (Figure 2B-C, Sup. Figure 2A-B), demonstrating a defect in cargo delivery from the EEs to lysosomes in these cells. Similar results were obtained when WT MEFs were treated with AA (Figure 2B- C, Sup. Figure 2A-B).

We then examined the recycling function of EEs by tracking the delivery of cargo from EEs to recycling endosomes using fluorescently labelled transferrin (Tf). Tf binds to the Tf receptor at the

cell surface, liberates its associated iron within EEs and is then recycled to the cell surface in recycling endosomes (16). As with dextran, an initial pulse of Tf led to the endocytosis of a similar amount of Tf in control, OPA1 KO and AA-treated MEFs, as judged by the similar number of Tf-positive vesicles present in these cells (Figure 2D). In control cells, the total number of transferrin spots then dropped, as expected because it is recycled to the cell surface (Figure 2D). Tf foci also decreased in OPA1 KO cells and WT cells treated with AA, although at a somewhat reduced rate (% left at 30 min: WT 12.5±4.05%; KO 28.9±8.24%, p=0.022; AA 30.5±1.62%, p=0.015). Interestingly, the colocalization with Rab5-positive EEs was increased in cells with mitochondrial dysfunction (Fig. 2E, Sup. Figure 2C). In addition, the colocalization between Tf and the recycling endosome marker Rab11 was increased at the beginning of the chase time but not at later time points (Fig. 2F, Sup. Figure 2D). Overall, these results indicate that both lysosome delivery and endosomal recycling are affected by impaired mitochondrial activity, although in a distinct manner.

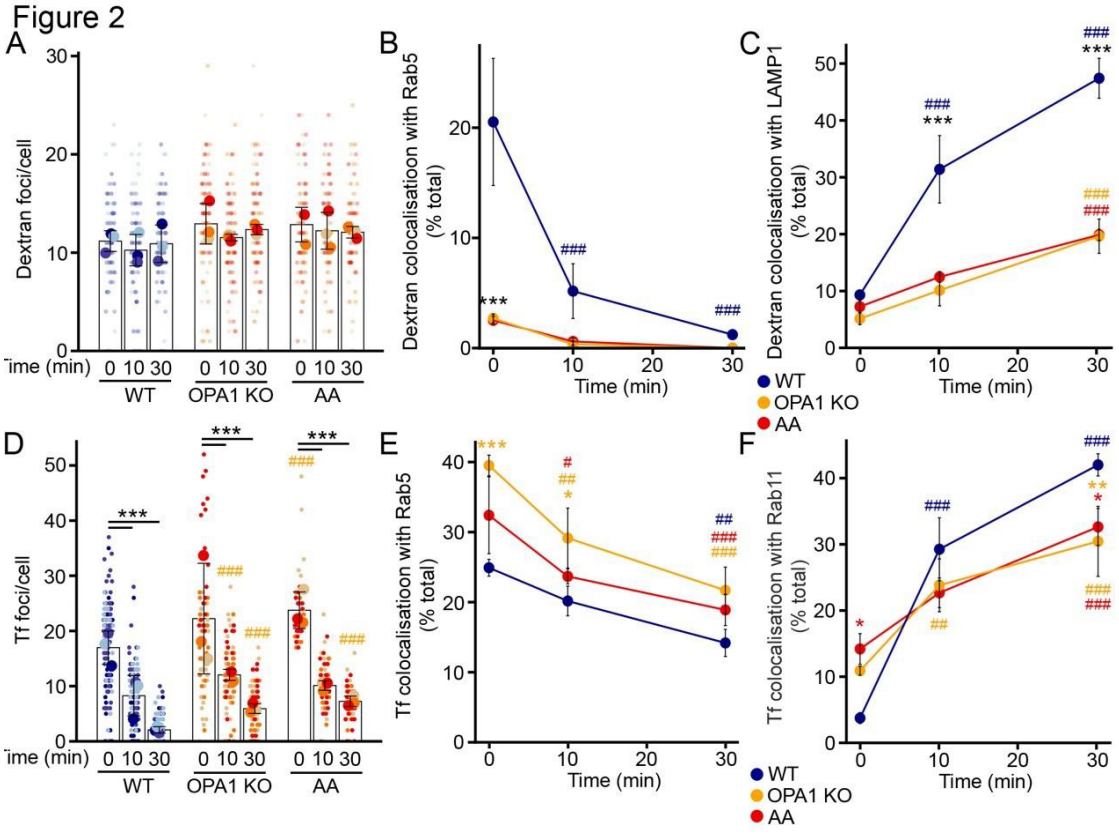

Figure 2. Mitochondrial dysfunction impairs proper EE cargo trafficking. (A-C) Dextran trafficking in OPA1 KO MEFs and AA-treated WT MEFs. Cells were pulsed with dextran for 5 minutes then chased for the indicated times and total dextran (A) and its colocalization with Rab5 (B) and LAMP1 (C) quantified by immunofluorescence. Each point represents an independent experiment, with small points in (A) representing individual cells (number of cells: 97 (WT), 101 (KO), 92 (AA)). Bars show the average ± SD for 3 experiments per condition. *** p<0.001 vs WT, ### p<0.001 vs 0 min, two-way ANOVA. (D- F) Transferrin (Tf) trafficking in OPA1 KO MEFs and AA-treated WT MEFs. Cells were pulsed with Tf for 5 minutes then chased for the indicated times and total Tf (D) and its colocalization with

Rab5 (E) and Rab11 (F) quantified by immunofluorescence. Each point represents an independent experiment, with small points in (D) representing individual cells (number of cells: 118 (WT), 115 (KO), 62 (AA)). Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$ vs WT, ### $p<0.001$ vs 0 min, two- way ANOVA. All stats are calculated from experiment averages, not cell averages.

EE clustering is driven by microtubule-dependent retrograde transport

Mitochondrial dysfunction induces perinuclear aggregation of EEs, resulting in altered EE spatial distribution that subsequently impacts cargo trafficking towards lysosomes. In order to elucidate the underlying cause, we initially determined the behaviour of EEs by live cell imaging. WT and OPA1 KO cells were transfected with RFP-Rab5 and imaged over time using a confocal microscope. We then analysed the movement of RFP-Rab5-positive EEs in these cells by measuring the radial (towards/away from the nucleus) and angular (side-to-side) components of vesicle speed (Figure 3A). This revealed that while the angular velocity of the EEs was not significantly affected by OPA1 deletion, their radial velocity was significantly increased (Figure 3B-C). Specifically, and consistent with the perinuclear aggregation of EEs we observed, mutant cells exhibited an augmented velocity of EEs directed towards the nucleus relative to control cells (Figure 3D). We then determined if these alterations were selective for EEs by repeating the experiment in cells in which lysosomes were tagged using GFP-LAMP1. In contrast to Rab5-positive vesicles, the radial velocity of LAMP1-positive vesicles was not altered in OPA1 KO MEFs (Figure 3B, D), although a subset of cells showed increased angular velocity (Figure 3C). Altogether, these results indicate that EE movements are increased in OPA1 KO cells.

As EE transport occurs along microtubules, we next investigated the relationship between EE aggregation and microtubule transport in cells with mitochondrial dysfunction. For this, we first stained WT and OPA1 KO cells for Rab5, the microtubule protein ß-tubulin and the centrosome marker g-tubulin. In control cells, where EEs were dispersed throughout the cell, no association between EEs and centrosomes was observed (Figure 3E-F). Conversely, in mutant cells characterized by the aggregation of early endosomes in the perinuclear region, we identified the minus end of microtubules and centrosomes associating with the aggregated early endosomes (Figure 3E-F). This triple association substantiates the involvement of microtubules and centrosomes in the aggregation of early endosomes.

To investigate the causal relationship between microtubules and the altered distribution of early endosomes, we employed the microtubule depolymerizing agent nocodazole as an experimental tool. A short pulse of nocodazole (15 min) had no impact on the distribution of Rab5-positive EEs in control cells (Figure 3G). In contrast, nocodazole treatment dispersed aggregated EEs from the

perinuclear region in mutant cells (Figure 3G). A similar redistribution of Rab5 upon nocodazole treatment was observed in WT cells treated with AA (Figure 3G), consistent with microtubule-dependent transport being required for perinuclear EE aggregation. The motor protein Dynein plays a key role in the retrograde transport of EE (17). Thus, we then determined the effect of the Dynein inhibitor Dynarestin on Rab5 clustering in cells with mitochondrial dysfunction. Similar to the disruption of microtubules with nocodazole, Dynarestin treatment caused a redistribution of Rab5-positive EEs in OPA1 KO cells and in AA-treated WT cells (Figure 3H). Altogether, these results indicate that microtubule-based transport promotes perinuclear EE aggregation in our cellular models of mitochondrial dysfunction.

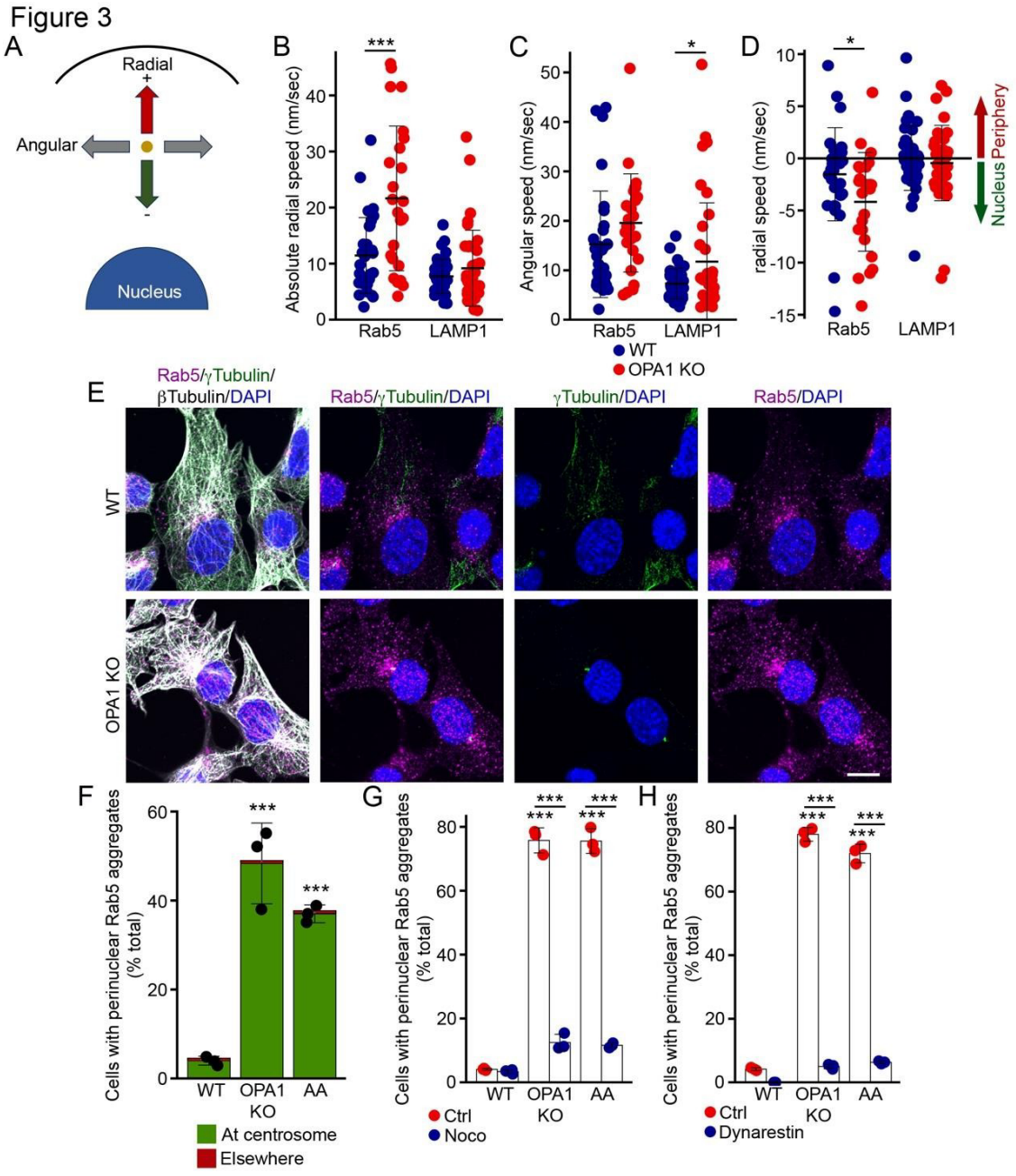

Figure 3. Microtubules drive EE perinuclear clustering. (A-D) Quantification of EE and lysosome velocity in OPA1 KO MEFs transfected with either Rab5-RFP (EE) or LAMP1-GFP (lysosome). (A) schematic representation of radial and angular velocity. (B) Absolute radial speed (independent of direction), (C) Angular speed, (D) Radial speed according to its direction (negative towards the nucleus, positive towards the cell membrane). Each point represents an individual endosome, with endosomes analysed in a minimum of 4 cells per condition in at least 3 independent experiments. Bars show the average ± SD. *** $p<0.001$, * $p<0.05$, two-sided t-test. (E-F) Rab5-positive EEs cluster at centrosomes. (E) Representative confocal images of WT and OPA1 KO MEFs stained for the EE marker Rab5 (Magenta), along with the microtubule marker ß-tubulin (White) and the centrosome marker g-tubulin (Green). Scale bar 10 µm. (F) Quantification of images in (E). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, one-way ANOVA. (G-H) Inhibition of microtubule-dependent transport rescues Rab5-positive EE distribution in OPA1 KO MEFs.

WT and OPA1 KO MEFs were treated with the microtubule inhibitor nocodazole (noco, G) or the the Dynein inhibitor Dynarestin (H) in the absence or the presence of AA as indicated. Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, one-way ANOVA.

Mitochondrial dysfunction leads to centrosome alterations

To determine the reason why microtubule-based transport specifically altered EE localisation in cells with mitochondrial dysfunction, we first assessed potential alterations in microtubule organisation. Most interphase cells contain one centrosome from where microtubules are organised, while dividing cells have duplicated their centrosome in prevision of chromosome segregation and cytokinesis (18). Consistent with this, almost all WT MEFs contained either one (57%) or two (41%) centrosomes (Figure 4A-B), as detected by immunofluorescence using the centrosomal marker γ-tubulin. In contrast, OPA1 KO cells and AA-treated WT cells displayed a decrease in cells with one centrosome, and a significant proportion of cells with more than two centrosomes (Figure 4A-B). In addition, while the two centrosomes were apart from each other in WT cells, as would be expected from cells preparing to divide, the distance between the two centrosomes decreased as the number of centrosomes increased in OPA1 KO and AA-treated cells (Figure 4C), suggesting that duplicated centrosomes fail to segregate in these cells.

In osteoclasts, centrosome clustering facilitates microtubule bundling, thereby enhancing microtubule- based transport (19). A similar bundling was present in OPA1 KO MEFs and AA-treated WT MEFs, as seen by the presence of much thicker microtubule fibres in cells with closely apposed duplicated centrosomes, compared to control cells (Figure 4D-E). Importantly, the presence of these dense microtubules correlated with EE perinuclear aggregation (Figure 3E, 4E). We also assessed microtubule bundling using microtubule directionality, a measure based on the dispersion of the microtubule signal. In this assay, a decrease in the dispersion of the fluorescent signal of microtubules (i.e. bundling) is seen as a decrease in the standard deviation (SD) of the signal, which is exactly what we observed in OPA1 KO cells and AA-treated cells (Figure 4F). Collectively, our results suggest that alterations in centrosomes lead to changes in the organization of microtubules, promoting rapid dynein-driven EE movement and their perinuclear aggregation.

To test this more directly, we manipulated centrosome numbers by overexpressing PLK4, a kinase regulating centrosome duplication that promotes the presence of supernumerary centrosomes when overexpressed (20,21). Expression of GFP-tagged PLK4 resulted in the presence of either several centrosomes dispersed throughout the cell (69% of transfected cells; Dispersed (D), Sup. Figure 3A) or two closely juxtaposed centrosomes (31% of transfected cells; Cluster of 2 (C2),

Sup. Figure 3A). Consistent with our results, cells with a cluster of 2 centrosomes, but not cells with dispersed centrosomes, had bundled microtubules (Sup. Figure 3B-C) and aggregated Rab5 vesicles (Figure 4G). These results thus support the idea that centrosome alterations drive the endosomal phenotype present in cells with impaired mitochondrial activity.

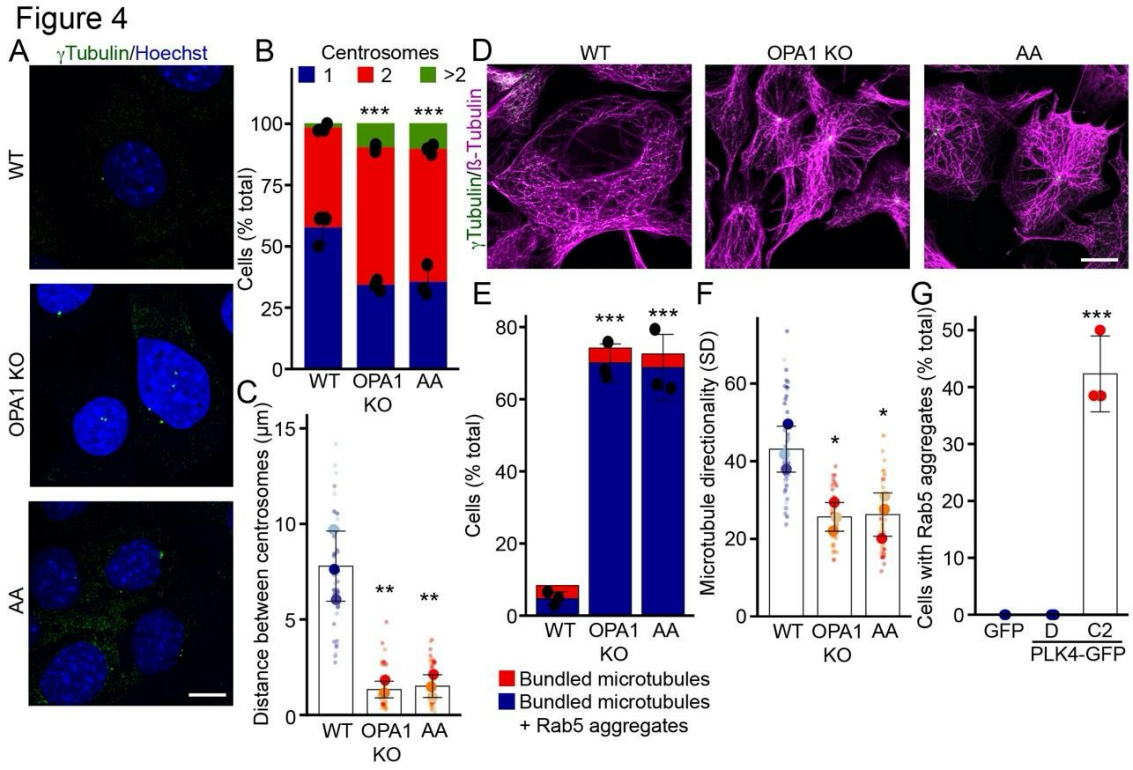

Figure 4. Mitochondrial dysfunction leads to aberrant centrosome duplication. (A) Representative confocal images of WT (control and AA-treated) and OPA1 KO MEFs stained for the centrosome marker g-tubulin (green) along with DAPI (blue) to mark nuclei. Scale bar 10 μm. (B) Quantification of the number of centrosomes per cell from images in (A). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, two-way ANOVA. (C) Quantification of the distance between centrosomes from images in (A). Each point represents an independent experiment, with smaller points representing individual cells. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, one-way ANOVA. (D) Representative confocal images of WT (control and AA-treated) and OPA1 KO MEFs stained for the centrosome marker g-tubulin (green) and the microtubule marker ß-tubulin (magenta). Scale bar 10 μm. (E) Quantification of the correlation between the presence of bundled centrosomes and Rab5 aggregates in OPA1 KO and AA-treated cells. Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, two-way ANOVA. (F) Microtubule bundling was also quantified by measuring the variation in microtubule directionality expressed as the standard deviation (SD). Each point represents an independent experiment, with smaller points representing individual cells. Bars show the average ± SD for 3 experiments per condition. * $p<0.05$, one-way ANOVA. (G) PLK4 expression causes EE aggregation. WT MEFs were transfected with the indicated plasmids and Rab5 aggregates were quantified by immunofluorescence. D refers to PLK4-transfected cells with dispersed centrosomes, while C2 refers to transfected cells with 2 clustered centrosomes (see Sup. Figure 3). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, one-way ANOVA. All stats are calculated from

experiment averages, not cell averages.

Oxidative stress promotes centrosome duplication and EE aggregation

Mitochondrial dysfunction often leads to an increase in reactive oxygen species (ROS)(22,23) and we previously showed that this was associated with the impaired lysosomal structure and function found in these cell (8). Consistent with this, OPA1 KO MEFs and AA-treated cells showed an increase in oxidized proteins as detected using oxyblots (Figure 5A, Sup. Figure 4A). The level of oxidized proteins nevertheless remained low compared with the exposure of cells to H_2O_2, which causes a large and rapid increase in oxidative stress (Figure 5A).

Oxidative stress can also cause aberrant centrosome duplication, leading to the presence of supernumerary centrosomes and aberrant cell division (24). To directly test whether this is leads to EE aggregation, we sought to induce a low but sustained level of exogenous ROS akin to what is found in our models of mitochondrial dysfunction. We thus added glucose oxidase (GO) to cell media, to generate a controlled amount of ROS in WT cells (8). GO caused a significant increase in the number of centrosomes, leading to the appearance of clustered centrosomes similar to OPA1 KO and AA-treated cells (Figure 5B-D). This was associated with an increase in the number of cells with bundled microtubules as quantified directly (Figure 5E) and by measuring microtubule directionality (Figure 5F). Consistent with ROS-driven microtubule alterations being responsible for Rab5 aggregation, exposure of WT cells to GO also caused the perinuclear aggregation of Rab5-positive EEs (Figure 5G), similar to what we observed in our cellular models of mitochondrial dysfunction. Importantly, a single large burst of oxidative stress caused by exposing cells to H_2O_2 did not cause microtubule alterations or Rab5 aggregation (Sup. Figure 4B-E), consistent with a previous report (25). Overall, our data indicates that a small increase in ROS phenocopies the defects present in OPA1 KO MEFs, but that this is not the consequence of non-specific endosomal damage caused by high H_2O_2 levels.

To further demonstrate that ROS cause the centrosome alterations leading to EE clustering, we quenched ROS in our cellular models of mitochondrial dysfunction using antioxidants. Consistent with our GO data, N-Acetyl-cysteine (NAC) decreased the number of OPA1 KO and AA-treated cells that contained more than 2 centrosomes and increased the distance between centrosomes (Figure 5H-I, total centrosome numbers in Sup. Figure 5A). Similarly, we observed a rescue of microtubule bundling and directionality in antioxidant-treated cells (Figure 5J-K). This rescue of microtubule structure caused the dispersion of EEs from the perinuclear region towards the entire cell (Figure 5L), supporting a key role for ROS in this process.

We then determined whether mitochondrial ROS were required for this process. For this, we first used the mitochondria-targeted antioxidant MitoQ. Similar to NAC, MitoQ rescued centrosomes, microtubules and Rab5 distribution in both OPA1 KO and AA-treated cells (Figure 5H and K, Sup. Figure 5A). Mitochondria generate ROS in the matrix and in the intermembrane space in the form of superoxide, which is then converted to H_2O_2 by superoxide dismutase. To further support the role of mitochondria- derived ROS in Rab5 aggregation and to determine whether H_2O_2 is specifically involved in this process, we expressed catalase specifically in the mitochondrial matrix using a previously described construct (26). In our hands, this construct targeted catalase to mitochondria in about 50% of the cells, the other transfected cells showing high cytosolic expression (Sup. Figure 5B; catalase was detected using an antibody that also recognizes the peroxisome-localised endogenous protein as seen in the top panel). Consistent with H_2O_2 being the main ROS responsible for the EE phenotype, cytosolic catalase fully rescued Rab5 localisation and microtubule structure (Sup. Figure 5C-D). Similarly, matrix-targeted catalase partially rescued EEs and microtubules (Sup. Figure 5C-D), further indicating that mitochondria- derived H_2O_2 promotes microtubule-dependent EE aggregation. The partial rescue in this context is likely because matrix-targeted calatase quenches matrix-generated H_2O_2 but not the H_2O_2 generated in the intermembrane space that can still escape to the cytosol. Collectively, our data demonstrates that oxidative stress-induced changes in microtubule organization and centrosomes contribute to the altered EE distribution.

To further define the link between ROS and microtubule organisation, we then assessed the effect of centrosome amplification on ROS production. While AA treatment led to an increase in ROS as measured using MitoSOX, PLK4 transfection did not (Sup. Figure 5E), supporting the idea that ROS act upstream of centrosome alterations. The kinase ROCK1 was previously shown to promote centrosomal amplification in response to ROS (27). Consistent with this, treatment of OPA1 KO cells with a ROCK1 inhibitor (Y-27632) rescued both microtubule structure and EE distribution (Sup. Figure 5F-G), suggesting that mitochondria-generated ROS alter EE distribution by causing ROCK1-dependent microtubule alterations.

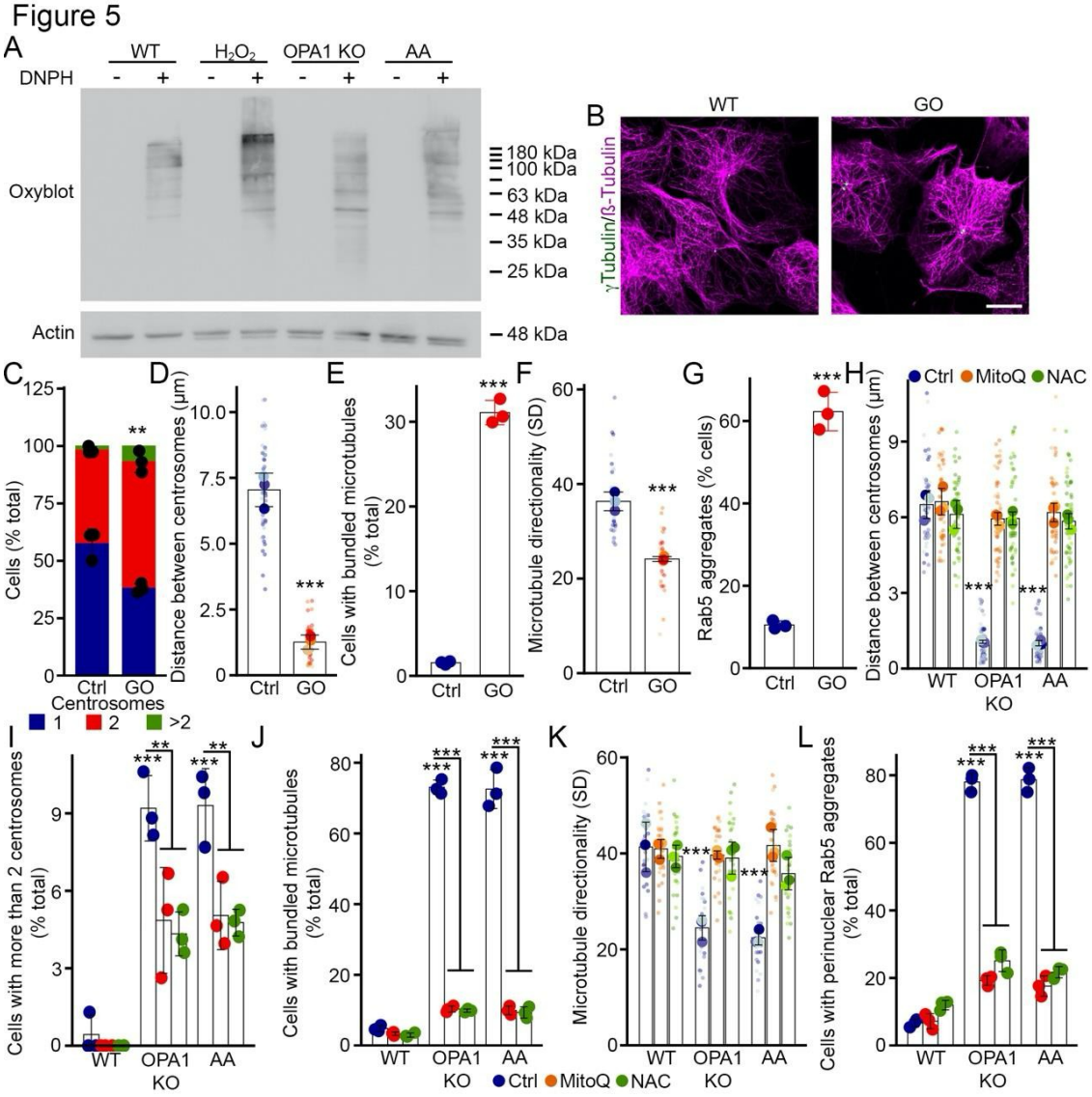

Figure 5. Oxidative stress promotes centrosome alterations leading to EE perinuclear clustering.

(A) Oxidative stress increases the levels of oxidized proteins. Oxyblot showing the amount of oxidized proteins in the indicated condition in the absence (DNPH -) or the presence (DNPH +) of the labelling chemical. Actin was used as a loading control. (B-G) Glucose oxidase (GO) promotes centrosome alterations and EE clustering. (B) Representative confocal images of control and GO-treated WT MEFs stained for the microtubule marker ß-tubulin (Magenta) and the centrosome marker g-tubulin (Green). Scale bar 10 µm. The number of centrosomes (C), the distance between them (D), as well as the number of cells with bundled microtubules (E) and their directionality (F) was quantified from these images. The presence of Rab5 aggregates was also quantified (G). Each point represents an independent experiment, with smaller points in (D) and (F) representing individual cells. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, ** $p<0.01$, two-sided t-test. (H-L) Antioxidants rescue both centrosome defects and EE aggregation. WT and OPA1 KO MEFs were treated as indicated. The distance between centrosomes (H) and the number of cells with more than 2 centrosomes (I, total distribution of centrosomes in Sup. Figure 5A) was quantified as well as the number of cells with bundled microtubules

(J) and their directionality (K). Rab5 aggregates were also quantified (L) were quantified from confocal images as in (B-G). Each point represents an independent experiment, with smaller points in (H) and (K) representing individual cells. Bars show the average ± SD for 3 experiments per

condition. *** $p<0.001$, ** $p<0.01$, two-way ANOVA. All stats are calculated from experiment averages, not cell averages.

Oxidative stress causes a functional loss of cargo trafficking

As ROS promote EE aggregation, we then investigated the impact of oxidative stress on the trafficking capacity of EEs. We first exposed control cells to GO to induce an increase in ROS production. Consistent with our models of mitochondrial dysfunction, GO did not decrease the overall uptake of dextran (Figure 6A). However, GO reduced the colocalization between dextran and Rab5-positive EEs and reduced trafficking towards LAMP1-positive lysosomes (Figure 6B-E), similar to cells with mitochondrial dysfunction (Figure 2B-C). We then exposed OPA1 KO and AA-treated cells to the antioxidant MitoQ, which significantly reduced the microtubule alterations in these cells (Figure 5H and K). As we previously observed (Figure 2), MitoQ treatment had no impact on total dextran uptake (Figure 6A). However, it rescued dextran transport to Rab5-positive EEs and LAMP1-positive lysosomes (Figure 6D-G), consistent with oxidative stress playing a key role in the EE alterations present in cells with mitochondrial dysfunction. Altogether, our results demonstrate that ROS alter EE distribution and subsequent cargo trafficking to lysosomes.

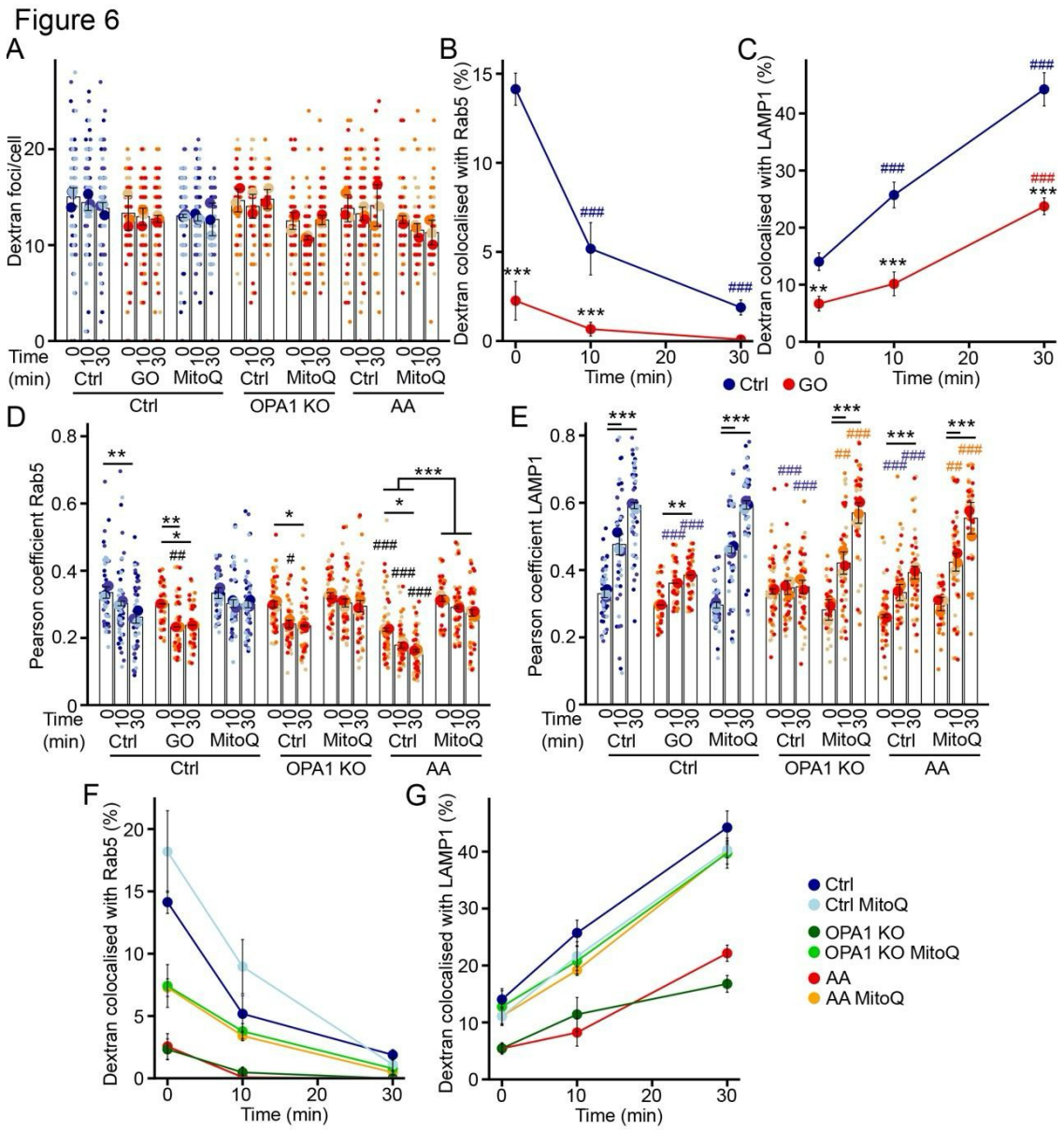

Figure 6. Antioxidants rescue EE function in cells with mitochondrial dysfunction.

(A-C) Dextran trafficking in WT MEFs treated with glucose oxidase (GO). Cells were pulsed with dextran for 5 minutes then chased for the indicated times and total dextran (A) and its colocalization with Rab5 (B) and LAMP1 (C) quantified by immunofluorescence. Each point represents an independent experiment, with small points in (A) representing individual cells (cells: 82 (Ctrl), 92 (GO), 74 (MitoQ), 73 (KO), 79 (KO+MitoQ), 81 (AA), 76 (AA+MitoQ)). Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$ vs WT, ### $p<0.001$ vs 0 min, two-way ANOVA. (D-G) Antioxidants rescue dextran trafficking in OPA1 KO MEFs and AA-treated WT MEFs. Cells were pulsed with dextran for 5 minutes then chased for the indicated times and total dextran (A) and its colocalization with Rab5 (F) and LAMP1 (G) quantified by immunofluorescence. Each point represents an independent experiment, with small points in (D-E) representing individual cells (51 cells/condition). Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$ vs WT, ### $p<0.001$ vs 0 min, two-way ANOVA. All stats are calculated from experiment averages, not cell averages.

Discussion

Genetic or chemical alteration in mitochondrial functions lead to muscular and neurological pathologies (3) that were historically associated with ATP deficits. It is now clear that the consequences of mitochondrial defects extend beyond ATP maintenance and include enhanced inflammation and the generation of reactive oxygen species (ROS) (4,5). At the cellular level, mitochondria interact with various organelles to maintain cellular homeostasis. While functional interactions with the endoplasmic reticulum and lysosomes are well established (28), the influence of mitochondria on other cellular components is less understood. Here, we addressed this question using genetic and chemical models of mitochondrial dysfunction. Our findings revealed that types of mitochondrial dysfunction associated with increase ROS production induce ROS-dependent alterations in centrosomes and microtubules that affect EE distribution and their ability to efficiently deliver cargo to lysosomes.

In physiological conditions, mitochondrial ROS play essential roles in cellular signalling (22). Nevertheless, an imbalance in ROS production and removal can lead to oxidative stress (23,29). Alterations in mitochondrial structure and function caused by mutations or inhibition of the electron transport chain, as well as the accumulation of damaged mitochondria following the inhibition of mitophagy, increase the production of mitochondrial ROS (30). These ROS can cause cellular damage and contribute to the development of neurodegenerative, metabolic, cardiovascular, and inflammatory diseases (24,31,32,33,34). Nevertheless, the relationship between ROS signalling and ROS-induced damage is complex and depends on the nature of ROS as well as its spatiotemporal distribution (23). For example, while nuclear H_2O_2 lead to nuclear DNA damage, excess mitochondrial H_2O_2 does not (35). Similarly, exposure of cells to high concentrations of H_2O_2 causes microtubule depolymerization and loss of architectural stability (36,37) while the lower levels of endogenous ROS produced in our models of mitochondrial dysfunction or GO treatment (8), did not cause microtubule depolymerization.

On the other hand, the elevated ROS present in our models of mitochondrial dysfunction was associated with the presence of supernumerary centrosomes, consistent with a previous report indicating that ROS promote centrosomes amplification (24). These centrosomal clusters increase microtubule nucleation and alter intracellular trafficking under physiological conditions in osteoclasts (19). We similarly found that the ROS-dependent centrosome amplification present in cells with mitochondrial dysfunction altered EE trafficking, causing their accumulation around

these centrosomes. Importantly, Rab5 aggregation was recapitulated by PLK4-dependent aberrant centrosome duplication and was blocked by inhibiting ROCK1, a kinase that has previously been shown to induce aberrant centrosome duplication in the presence of elevated ROS (27). Altogether, these results suggest that mitochondrial ROS trigger ROCK1- driven centrosome amplification, leading to microtubule alteration and EE clustering. It nevertheless remains possible that other ROS-driven cellular alterations also contribute to this phenotype, including oxidative modifications of centrosomal proteins or microtubules.

Interestingly, the distribution of late endosomes/lysosomes was much less affected by the centrosomal amplification found in our models of mitochondrial dysfunction. This is likely a consequence of the more complex trafficking of these organelles, both anterograde and retrograde, in contrast to the dominant retrograde transport of EEs from the plasma membrane. Consistent with this, while only Rab5-positive vesicles showed perinuclear aggregation and changes in radial speed, LAMP1-positive lysosomes were less present in the center of the cytoplasm and had increased angular speed. Overall, these changes in lysosomal behaviour could contribute to the previously reported alterations present in lysosomal structure and function caused by mitochondrial dysfunction (7,8).

Pathological conditions due to mitochondrial defects were originally explained by defects in ATP production. However, more recent work has highlighted the multifaceted aspects of mitochondria-related diseases, including several metabolic alterations not directly related to ATP production, ROS production and alterations in organelle contact sites (38,39,40). This complex interplay between metabolism and organelle distribution and activity likely plays a major role in the etiology of mitochondria related diseases. For example, neurodegenerative diseases are associated with defects in both mitochondria and the endolysosomal compartment, with oxidative stress playing an important role (38,41). In this context, it is noteworthy that we identified microtubule transport as an important target of ROS that impact EE function and could explain some of the features of these diseases. Nevertheless, as mitochondrial diseases encompass a large array of mutations that affect mitochondrial activities and ROS production in distinct manners, the specific contribution of this ROS-dependent mechanism likely varies across mutations/diseases.

Overall, we propose that the elevated ROS generated by damaged mitochondria cause aberrant centrosome duplication, leading to microtubule alterations that alter EE trafficking and impair their ability to transfer their cargo to lysosomes. Our model is supported by the fact that EE and microtubule defects are recapitulated in healthy cells by promoting aberrant centrosome

duplication or by adding a controlled source of ROS (GO), while being rescued by antioxidants. Our study highlights that mitochondrial dysfunction not only impacts lysosomes but also influences the function and distribution of EEs, likely contributing to the neuronal impairment caused by mitochondrial dysfunction in neurodegenerative diseases.

Material and Methods

Cell culture reagents were bought from Wisent. Other chemicals were purchased from Sigma-Aldrich, except where indicated.

Cell culture:

WT and OPA1 KO MEFs (gift from Dr. Luca Scorrano, University of Padua), Control and DRP1 mutant primary human fibroblasts (42), and HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were maintained in an incubator with 5% CO2 until they reached 70-80% confluency before commencing the experiments. Cells were treated as follow: mitochondrial inhibitors antimycin A (50 μM) or rotenone (5 μM) for 4 hours; antioxidants N- acetylcysteine (NAC) (10 mM) or MitoQ (100 μM, Focus Biomolecules # 10-1363) for 4 hours; induction of oxidative stress with 50 milliunits/ml of glucose oxidase for 1 hour; microtubule depolymerisation with nocodazole (5 μM) for 15 minutes; inhibition of dynein with dynarrestin (2 μM) for 30 minutes, H2O2 treatment (250 μM) 30 min, ROCK1 inhibition with Y-27632 (10 μM, Medchem Express) for 3 hours.

Dextran and transferrin uptake experiments:

MEFs were grown on coverslips until they reached 70% confluency. Cells were then exposed to 0.5 mg/ml of Dextran, Tetramethylrhodamine, 10,000 MW, Lysine Fixable (fluoro-Ruby) (Thermofischer # D1868) for 5 minutes. The media containing dextran was then removed, and fresh media was added. The cells were then chased for 0, 10, and 30 minutes before being fixed with 4% paraformaldehyde (PFA). For the Tf experiments, the cells were kept in serum-free media on ice for 30 minutes, then incubated with 0.25 mg/ml of Transferrin from Human Serum (Tetramethylrhodamine Conjugate, Thermofisher #T2872) for 5 minutes. Afterward, the media containing transferrin was replaced with fresh media, and the cells were chased for 0, 10, and 30 minutes before being fixed 10 minutes with 4% PFA at 25° C. To investigate the endosomal/lysosomal trafficking function under oxidative stress conditions, the cells were first treated with glucose oxidase or MitoQ as above. The media was then changed, and cells were exposed to dextran as above in the present of GO or mitoQ.

ROS detection:

Protein oxidation was quantified using Oxyblots (Sigma #S7150) according to the manufacturer's instructions. Briefly, cells were lysed in 10 mM Tris-HCl, pH 7.4, 1mM EDTA, 150 mM NaCl, 1% Triton X-100, complemented with a protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich), kept on ice for 10 min and centrifuged at 13000 rpm for 10 minutes. Protein supernatants were collected, and protein concentration was estimated by DC protein assay kit (BioRad). Oxyblot samples (15 µg) were prepared by adding 5 µL of 12% SDS (6% final) and treating them with 2,4- dinitrophenylhydrazine (DNPH; +DNPH) or the negative control solution (-DNPH), for 15 min at room temperature according to the manual. A neutralization solution was then added followed by 1X Laemmli buffer supplemented with ß-mercaptoethanol. Samples were then run on a SDS-PAGE, transferred to nitrocellulose membranes and blotted with an antibody against the DNP (2,4-dinitrophenylhydrazone) moiety of the proteins. Membranes were then incubated with a 1:5000 dilution of horseradish peroxidase- conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) and visualized by enhanced chemiluminescence (Thermo scientific) using a Bio-Rad imaging system. For MitoSOX, cells were incubated with 5 µM MitoSO Red (Life Technologies) for 20 min at 37 °C, after which fluorescence was measured at a wavelength of 610 nm in FGP-positive cells using a CytoFLEX (Beckman Coulter).

Immunofluorescence and cell imaging:

Cells were grown on glass coverslips for 24 hours before the experiments, then treated as indicated and fixed 10 min with 4% PFA at 25° C. Cells were permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA / 0.1% Triton X-100 in PBS. Cells were then incubated with primary antibodies followed by fluorescent tagged secondary antibodies (Jackson Immunoresearch, 1:500) and DAPI (Invitrogen, Thermo Fisher, D1306, 1:100). The following primary antibodies were used: mouse Anti-β-Tubulin (Sigma-Aldrich # T5293), rat Anti-β-Tubulin (Abcam #6161), mouse anti-gamma tubulin (Sigma-Aldrich #T5326), rat anti-LAMP1 (SCBT #19992), rabbit anti-Rab5 (Cell signalling Technologies #3547S, 1:200), rabbit anti-EEA1 (Cell signalling Technologies #2411, 1:200), and rabbit anti-Rab11 (Cell Signaling Technologies #3539S, 1:200), mouse anti-Catalase (Proteintech #66765-1-Ig, 1:200). Imaging was performed using a Leica TSC SP8 confocal microscope fitted with a 63×/1.40 oil objective.

Transfections were done in WT and OPA1 KO MEFs using Metafecten Pro (Biontex). The following plasmids were used: mRFP-Rab5 (Addgene plasmid # 14437), LAMP1-GFP (Addgene plasmid # 16290), GFP (pcDNA3-EGFP; Addgene Plasmid #13031), GFP-PLK4 (pEGFP-C3-PLK4-3xFLAG; Addgene Plasmid #69837) and catalase targeted to the mitochondrial matrix by

replacing the C-terminal peroxisomal targeting sequence by the N-terminal targeting sequence of ornithine transcarbamylase (gift from Peter S. Rabinovitch, U. Washington (26)). Cells were fixed 24 hours later for immunofluorescence. For live cell imaging, cells were plated after 24 hours on glass bottom dishes in complete medium and grown for another 24h. The plates were then mounted onto Leica TSC SP8 confocal microscope fitted with a 63×/1.40 oil objective. Time-lapse images were acquired at a speed of (0.05-0.125 frames/s) for 10 minutes.

Image processing and analysis:

All image manipulation and analysis were done in Image J/Fiji. The images shown are from single focal planes unless stated otherwise. Image analysis was done as follows: 1. Images were segmented in ImageJ using Filter/Median (1.0 (in pixels), to reduce noise), then thresholded and adjusted using Binary/Erode. 2. The position of each foci was then identified using the Analyse particle function with size of 10 px- infinity. Rab5 foci were considered an aggregate when greater than 0.75 μm^2 (3 SD above the average size of WT cells). For the analysis of Rab5 aggregates, cells were considered positive for Rab5 aggregates if they contained at least two particles larger than 0.75 μm^2 that were within the perinuclear area (defined as ≤30 % of the distance between the nuclear membrane and the plasma membrane). For density distributions, the position of all particles (10 px-infinity) was compiled, along with the position of the nucleus and the plasma membrane was also identified. The data was then fed to a custom R script to calculate the relative distance to the nucleus and the distribution calculated using the Density function. Radial and Angular speeds were similarly calculated using a custom R script. Directionality was calculated using the Fiji plugin *Directionality* with Fourier component analysis.

Data analysis and statistics:

Data analysis and statistical procedures were conducted using R. Quantification of immunofluorescence data was performed, and representative images from a minimum of three independent experiments were presented (specific sample sizes are indicated in the respective quantification figures). For experiments where individual cells were quantified, the smaller points represent individual cells in each independent experiment while the larger points represent the average of each experiment. Data is expressed as the mean ± standard deviation (SD) per experiment (not cells) as indicated in the figure legends. To assess statistical significance, Student's t-test was utilized for comparisons between two groups, while one-way ANOVA with a Tukey post hoc test was employed for multiple comparisons.

Acknowledgements

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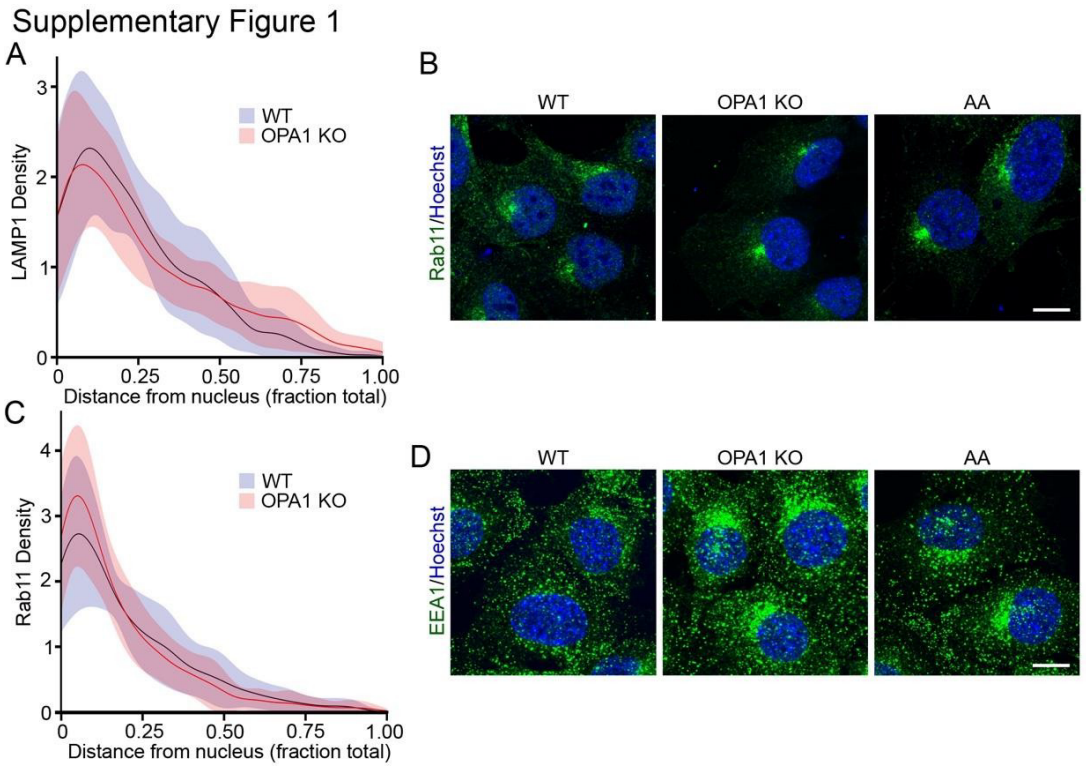
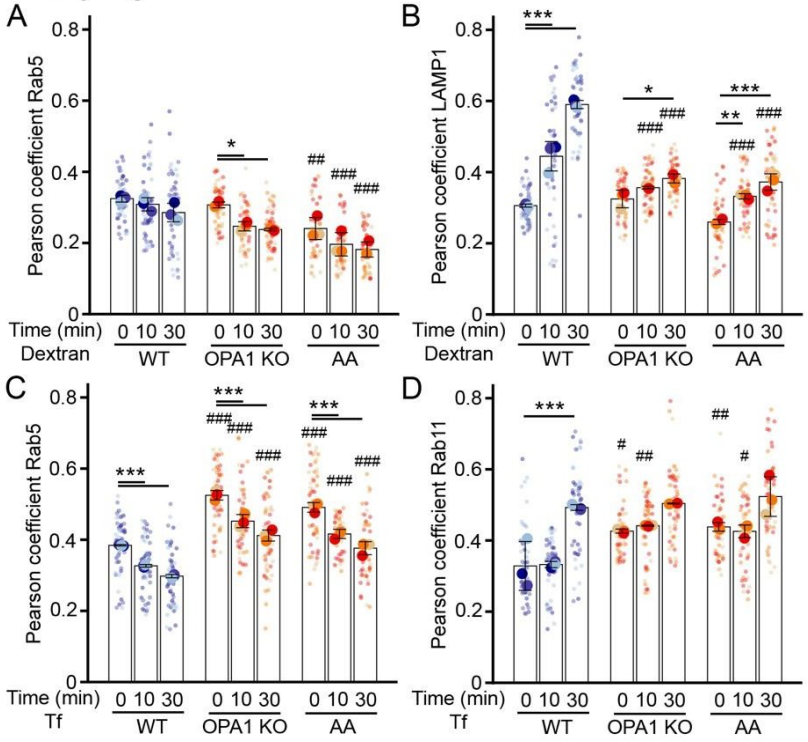
Supplementary Figures



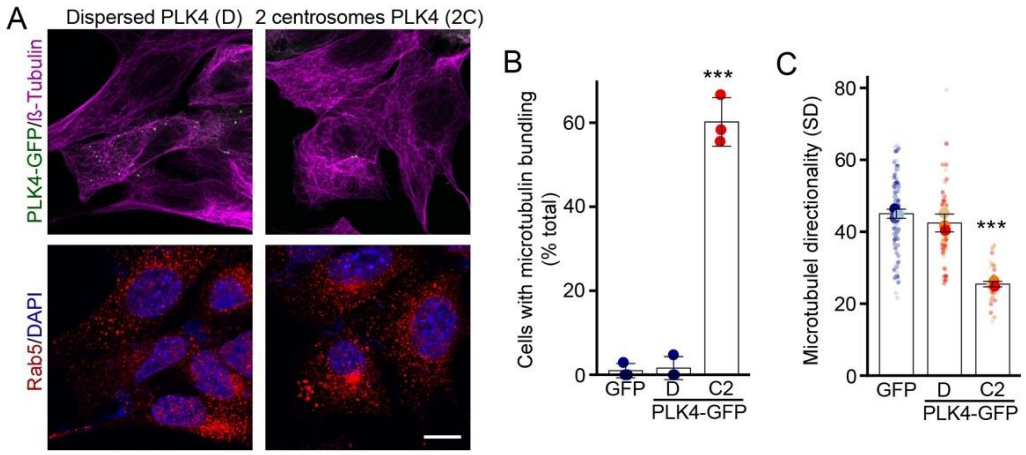
Supplementary Figure 1. Distribution of endosomal markers in cells with mitochondrial dysfunction. (A) Density of LAMP1-positive vesicles relative to their localisation as measured from confocal images in Figure 1A. The data shows the quantification of 30 cells per condition in 3 independent experiments ± SD. (B) Representative confocal images of WT, OPA1 KO and AA-treated WT MEFs stained for the recycling endosome marker Rab11 (green), along with DAPI to mark nuclei (blue). Scale bar 10 µm. (C) Density of Rab11-positive vesicles relative to their localisation as measured from confocal images in (B). The data shows the quantification of 30 cells per condition in 3 independent experiments ± SD. (D) Representative confocal images of WT, OPA1 KO and AA-treated WT MEFs stained for the EE marker EEA1 (green), along with DAPI to mark nuclei (blue). Scale bar 10 µm.

Supplementary Figure 2



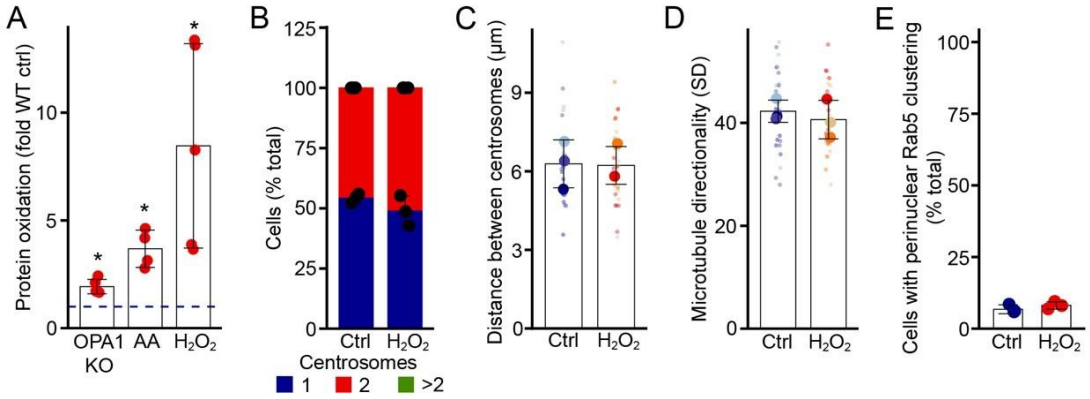
Supplementary Figure 2. Loss of colocalisation between dextran and lysosomes in cells with mitochondrial dysfunction. (A-B) Dextran trafficking in OPA1 KO MEFs and AA-treated WT MEFs. Cells were pulsed with dextran for 5 minutes then chased for the indicated times and Pearson coefficients were calculated between Dextran and Rab5 (A) or LAMP1 (B) from the same images as in Figure 2. Each point represents an independent experiment, with small points representing individual cells (51 cells/condition). Bars show the average ± SD for 3 experiments per condition. *** p<0.001, ** p<0.01, * p<0.05 vs WT, ### p<0.001, ## p<0.01 vs 0 min, two-way ANOVA. (C-D) Transferrin (Tf) trafficking in OPA1 KO MEFs and AA-treated WT MEFs. Cells were pulsed with Tf for 5 minutes then chased for the indicated times and Pearson coefficients were calculated betewwn Tf and Rab5 (C) or Rab11 (D) from the same images as in Figure 2. Each point represents an independent experiment, with small points representing individual cells (54 cells/condition). Bars show the average ± SD for 3 experiments per condition. *** p<0.001 vs WT, ### p<0.001, ## p<0.01, # p<0.05 vs 0 min, two-way ANOVA. All stats are calculated from experiment averages, not cell averages.

Supplementary Figure 3



Supplementary Figure 3. PLK4 overexpression induces centrosomal amplification and microtubule bundling. (A) Representative confocal images of WT MEFs transfected with PLK4-GFP (Green) and stained for the microtubule marker ß-tubulin (magenta) and EE marker Rab5 (Red) along with DAPI to mark nuclei (blue). Transfected cells show either multiple dispersed PLK4-labelled centrosomes (Left, labelled D in panels B-C and Figure 4G) or two clustered centrosomes (Right, labelled 2C in panels B-C and Figure 4G). Scale bar 10 µm. (B-C) Quantification of microtubule bundling (B) and directionality (C) in transfected cells. GFP was used as a control for these experiments. Each point represents an independent experiment, with smaller points in (C) representing individual cells. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, one-way ANOVA. All stats are calculated from experiment averages, not cell averages.

Supplementary Figure 4

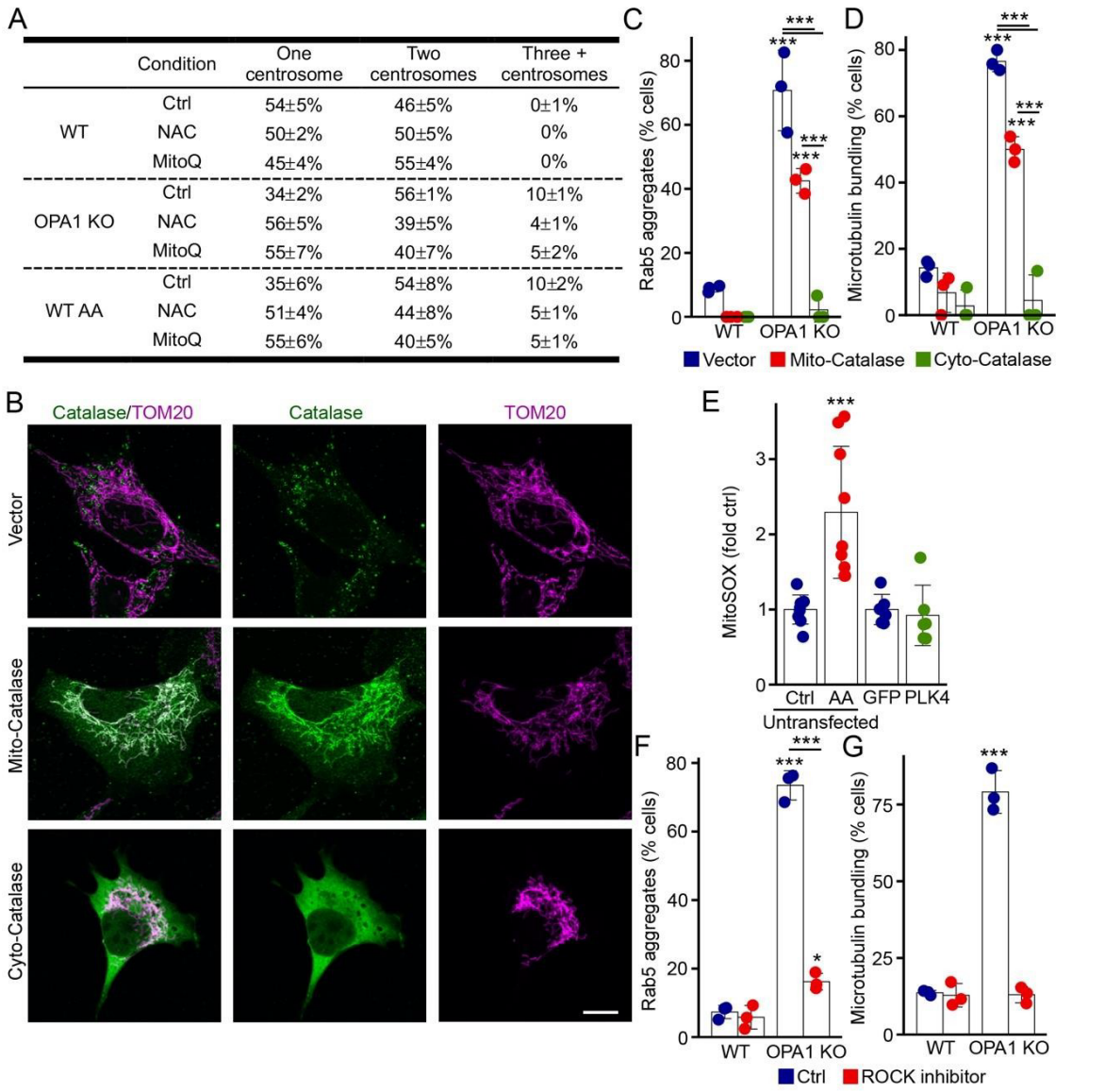


Supplementary Figure 4. H2O2 treatment does not cause microtubule bundling or Rab5 aggregation. (A) Quantification of the Oxyblots shown in Figure 5A. Each point represents an independent experiment normalised to actin. Bars show the average ± SD for 3 experiments per condition. * $p<0.05$, Pairwise t-test with FDR correction for multiple comparisons. (B-E) WT MEFs were treated with H_2O_2 and the number of centrosomes (B), the distance between centrosomes (C), microtubules directionality (D), and Rab5 aggregation (E) were quantified from confocal images as in Figure 4. Each point represents an independent experiment, with smaller points in (C-D) representing individual cells. Bars show the average ± SD for 3 experiments per condition. All stats are calculated from experiment averages, not cell averages.

Supplementary Figure 5

A

	Condition	One centrosome	Two centrosomes	Three + centrosomes
WT	Ctrl	54±5%	46±5%	0±1%
	NAC	50±2%	50±5%	0%
	MitoQ	45±4%	55±4%	0%
OPA1 KO	Ctrl	34±2%	56±1%	10±1%
	NAC	56±5%	39±5%	4±1%
	MitoQ	55±7%	40±7%	5±2%
WT AA	Ctrl	35±6%	54±8%	10±2%
	NAC	51±4%	44±8%	5±1%
	MitoQ	55±6%	40±5%	5±1%



Supplementary Figure 5. ROS-dependent centrosome alterations promote EE perinuclear clustering. (A) Quantification of centrosome numbers in WT and OPA1 KO MEFs treated as indicated. Average ± SD for 3 experiments per condition. Related to Figure 5I. (B-D) Catalase expression rescues microtubule bundling and Rab5 aggregation. (B) Representative confocal images of WT MEFs transfected with catalase targeted to the mitochondrial matrix showing the two observed localisations for the transfected catalase: mitochondrial (labelled Mito-catalase in C-D; 49 ± 2% of the transfected cells; 43 ± 2% in OPA1 KO cells) or cytosolic (labelled Cyto-catalase in C-D). Cells were stained for catalase (Green; the endogenous peroxisomal catalase is seen in Vector-transfected cells) and the mitochondrial marker TOM20 (Magenta). Scale bar 10 µm. Rab5 aggregates (C) and microtubule bundling (D) were then quantified from confocal images. Results were segregated based on the localisation of the catalase. Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition.

*** $p<0.001$, two-way ANOVA. (E) ROS quantification in cells transfected with PLK4-GFP. ROS were measured using MitoSOX in GFP-positive cells. As a control, untransfected MEFs were

treated with AA before ROS measurement. Each point represents an independent measurement. Bars show the average ± SD for 6 measurements per condition. *** $p<0.001$, one-way ANOVA. (F-G) ROCK1 inhibition rescues microtubule bundling and Rab5 aggregation. WT and OPA1 KO MEFs were treated with the ROCK1 inhibitor Y-27632 and Rab5 aggregation (F) and microtubule bundling (G) were measured as in (C-D). Each point represents an independent experiment. Bars show the average ± SD for 3 experiments per condition. *** $p<0.001$, *<0.05, two-way ANOVA

CHAPTER III

CELLULAR UPTAKE AND PROCESSING OF EXTRACELLULAR VESICLES: THE REGULATORY ROLE OF MITOCHONDRIAL HEALTH

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(In preparation)

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Abstract

Extracellular vesicles (EVs) play a pivotal role in intercellular communication by delivering diverse molecular cargo, including mitochondrial components. While EV uptake mechanisms have been studied, the dynamics of their internalization, trafficking, and cargo fate especially under conditions of mitochondrial dysfunction remain poorly understood. Here, we investigated EV uptake kinetics, endocytic routes, and intracellular fate in wild type (WT) and OPA1 knockout (KO) cells. Using pulse chase experiment, we compared EV accumulation and uptake rates in different cell types and conditions. Inhibition with dynasore revealed dynamin dependent uptake, while cointernalization with dextran indicated partial involvement of fluid phase endocytosis. Colocalization studies demonstrated that EVs progressively traffic to lysosomes, with a subset colocalizing with dextran. In OPA1 KO cells, EVs exhibited reduced lysosomal targeting and increased association with mitochondria. Furthermore, GFP tagged mitochondrial cargo enriched EVs formed a rare subpopulation that bypassed lysosomes. These findings highlight distinct EV processing routes and suggest a specialized role for mitochondria associated EVs in cellular adaptation to mitochondrial dysfunction.

Introduction

EVs are lipid bound particles secreted by virtually all cell types and play critical roles in intercellular communication by transferring proteins, lipids, and nucleic acids to recipient cells [1] [2]. A growing body of evidence suggests that EVs can also carry mitochondrial components, including mitochondrial DNA, proteins, and RNA, raising the possibility that EVs contribute to mitochondrial homeostasis or metabolic regulation in recipient cells [3]. EV uptake is known to occur via multiple mechanisms, including clathrin and caveolin mediated endocytosis, macropinocytosis, and fluid phase uptake [4]. Once internalized, EVs are typically trafficked through endosomal pathways, often culminating in lysosomal degradation [5]. Understanding how mitochondrial cargo containing EVs are trafficked and processed intracellularly is therefore essential, particularly under conditions of mitochondrial stress.

Mitochondria are highly dynamic organelles critical for maintaining cellular energy homeostasis, redox balance, and metabolic signaling [6]. In addition to their canonical role in ATP production, mitochondria form physical contact sites with other organelles including endosomes and lysosomes facilitating inter organelle communication, membrane remodeling, and cargo exchange [7]. These interactions place mitochondria at a central node in regulating organelle communication

and trafficking efficiency [7].

Mitochondrial dynamics are tightly regulated by fusion and fission events, with the inner membrane fusion protein OPA1 playing a key role in maintaining cristae structure, bioenergetic efficiency, and mitochondrial DNA integrity [8]. Loss of OPA1 leads to mitochondrial fragmentation, altered membrane potential, and increased reactive oxygen species (ROS), collectively contributing to mitochondrial dysfunction [9]. Such mitochondrial dysfunction has been reported to have profound physiological and pathological consequences, including impaired energy metabolism, altered cytoskeletal organization, and disruptions in intracellular transport [10 11].

Given these roles, the rationale for investigating EV fate under mitochondrial dysfunction stems from two core considerations: first, if EVs are to support mitochondrial recovery or intercellular stress signaling, their intracellular routing and degradation must be tightly regulated. Second, endocytosis and vesicle trafficking are energy intensive processes intimately tied to mitochondrial status. Therefore, any disruption in mitochondrial function could have downstream effects on how EVs are internalized, sorted, and processed. In this study, we investigated EV uptake dynamics, trafficking routes, and intracellular fate in both wild type and OPA1 knockout cells.

It is well established that EVs enter the cells through endocytic pathways and are destined to lysosomes which is considered canonical route of EVs. In our study we observed that OPA1 knockout cells showed reduced lysosomal localization and increased association with mitochondria. Notably, a rare subpopulation of EVs enriched in mitochondrial cargo appeared to escape lysosomal degradation. This divergence from the canonical degradative pathway highlights the potential for selective EV routing in response to cellular stress. In addition to trafficking, we observed cell type specific differences in EV uptake dynamics. Together, these findings indicate that mitochondrial integrity may play a critical role in shaping endocytic processing and intracellular fate of EVs, particularly those carrying mitochondrial content by redirecting their trafficking away from lysosomes and potentially toward mitochondria. We highlight a potentially adaptive mechanism of intracellular stress coordination.

Materials and Methods

Cell culture

Wild type mouse embryonic fibroblasts (MEFs) and RAW 264.7 macrophage cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco) at 37°C in a humidified 5%

CO_2 incubator. OPA1 knockout (KO) MEFs were maintained under identical conditions as mitochondrial dysfunctional cells. Cells were passaged at 70% confluence using 0.05% trypsin-EDTA (Gibco) and seeded for further experiments as required.

EV isolation and characterization

EVs were isolated from conditioned media (media depleted of FBS serum) of confluent WT MEFs using differential ultracentrifugation. Culture supernatants were first centrifuged at 2,000 × g for 15 min to remove debris and apoptotic bodies, followed by ultracentrifugation at 100,000 × g for 90 min at 4°C (Beckman Coulter, 70Ti rotor). Pellets were resuspended in PBS, washed once at 100,000 × g for 90 min, and finally resuspended in sterile PBS. Protein concentration was measured. Isolated EVs were used immediately for downstream applications.

EV labeling and pulse chase experiments

Isolated EVs were labeled with PKH26 (Sigma-Aldrich) or PKH67 fluorescent dye following the manufacturer's instructions. For pulse–chase experiments, labeled EVs were added to WT, OPA1 KO, or RAW cells at a final concentration of 20 μg/mL and incubated for 30 minutes pulse. Cells were either fixed immediately 0 h or washed three times with PBS and incubated in fresh medium for additional chase times of 1 and 6 hours.

Dynasore inhibition assay

To inhibit dynamin mediated endocytosis, cells were pre treated with 80 μM dynasore (Sigma-Aldrich) for 30 minutes prior to EV addition. Dynasore remained in the medium throughout the 30 minute EV pulse period. Cells were washed and fixed immediately after the pulse.

Dextran uptake assay

For cointernalization studies, recipient cells were simultaneously incubated with 10 kDa FITC dextran (Thermo Fisher, 1 mg/mL) and PKH labeled EVs for 30 minutes, followed by washing and chase incubation in fresh medium for 0, 1, or 6 hours. In parallel, EV only control condition was included to assess individual uptake dynamics.

Mitochondria enriched EV generation

To generate EVs containing labelled mitochondrial cargo, donor WT MEFs were stably transfected with a GFP tagged mitochondrial matrix protein plasmid (COX8-GFP) using Metafectene (Biontex) according to the manufacturer’s protocol. After 24 hours, conditioned media were collected and EVs were isolated using differential ultracentrifugation. EVs from untransfected cells were processed in parallel and used as negative controls.

Immunofluorescence and confocal microscopy

After treatments, cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 3 min, and blocked in 5% BSA in PBS for 1 hour. Cells were incubated with the following primary antibodies for 1 hour: rat anti-LAMP1 (1:100, SCBT

#19992), rab anti-TOM20 (1:250, ab186735), and anti-Rab5 (1:200, Cell Signaling 3547). After washing, cells were incubated with Alexa Fluor conjugated secondary antibodies for 1 hour at room temperature. Nuclei were counterstained with DAPI. Imaging was performed on a confocal microscope using a 63× oil immersion objective.

Image processing and quantification

Confocal images were analyzed using FIJI/ImageJ. Colocalization was manually analysed.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) from at three biologically independent replicates. Statistical analyses were performed using GraphPad Prism 9. For comparisons between the two groups, unpaired two-tailed Student's t-tests were used. For comparisons across multiple groups or time points, one-way or two-way ANOVA was used, followed by Tukey's post hoc test.

Result and discussion

EV uptake is time dependent and cell type specific under pulse chase conditions

To determine EV uptake dynamics, we conducted a pulse chase experiment using PKH labeled EVs isolated from WT MEFs. Recipient WT MEF cells were exposed to EVs for 30 minutes pulse, followed by fixation at 0, 1, and 6 hours post pulse to assess the dynamics of EV uptake over time.

In the initial set of experiments, EVs were removed after the 30 minutes pulse but we did not perform washing step with PBS. Meaning after 30 minutes pulse when we removed EV media, we did not wash the cells with PBS to remove excess or bound EVs. Under these conditions, we observed a steady and marked increase in intracellular EV puncta over time, from 0 to 6 hours post pulse (Figure1 A and B). This suggest that MEFs readily internalize EVs could be loosely associated with cells or the surface of the coverslip, and we see a gradual increase in their uptake.

To explore whether EV internalization is cell type specific, we extended our analysis to RAW cells, a macrophage line with phagocytic nature. Using the same 30 minute pulse protocol with PKH labeled EVs, we compared uptake dynamics in RAW cells over the 0, 1, and 6 hour chase period. Interestingly, although RAW cells did internalize EVs, the total number of intracellular EV puncta was markedly lower in RAW cells than MEFs (Figure 1B). This suggests that RAW cells may degrade internalized EVs more rapidly than MEFs, leading to reduced detectable signal over time [12]. The difference in EV accumulation profiles likely reflects variations in intracellular processing and degradation capacities rather than differences in uptake efficiency per se.

To further dissect this, we quantified the rate of EV internalization during the chase period by measuring the increase in EV puncta between 0 to 1 hour (1hour) and 1 to 6 hours (6 hours) post pulse. Surprisingly, RAW cells showed a higher initial uptake rate than MEFs in the first hour, indicating a more rapid endocytic response immediately after EV exposure (Figure1 B and 1C). However, despite this faster uptake, the total number of internalized EVs remained lower in RAW cells, reinforcing the idea that faster degradation or processing of EVs limits their accumulation [13]. In contrast, MEFs, although slower in initial uptake, exhibit more sustained EV accumulation, possibly due to slower degradation or trafficking kinetics.

To further probe this, we repeated the experiment under a washed condition, in which extracellular EVs were removed by washing with PBS after the 30-minute pulse. Washing serves to eliminate any EVs remaining in the extracellular medium or loosely associated with the cell membrane, thereby ensuring that only internalized vesicles are quantified during the chase period. This step is crucial for distinguishing between true intracellular accumulation and surface bound fluorescence and allows for a clearer interpretation of post uptake trafficking kinetics.

MEFs showed a reduction in EV spot under the washed condition (bottom panel) compared to the unwashed setup, confirming that some EVs extracellular presence facilitates ongoing uptake in these cells (Figure 1A and B). In contrast, RAW cells did not exhibit a noticeable difference in total EV puncta between washed and unwashed conditions, suggesting that most EV internalization in RAW cells occurred rapidly during the pulse window. These observations underscore cell type specific differences in EV uptake kinetics and intracellular fate, highlighting the importance of considering both pulse exposure time and washing conditions when evaluating EV internalization dynamics.

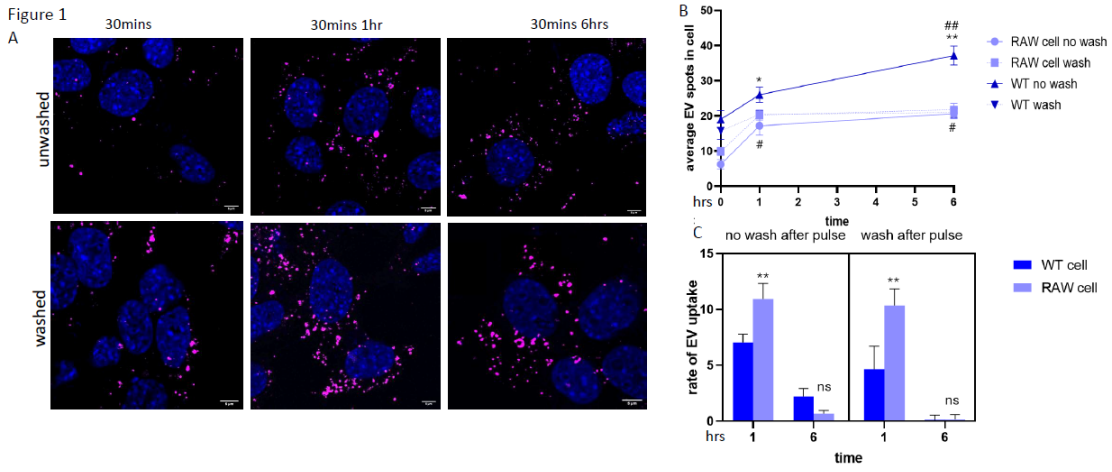

Figure 1. Time dependent uptake and accumulation of EVs in WT and RAW cells under pulse chase conditions

(A) Representative confocal images of WT recipient cells treated with PKH labeled EVs (magenta)

isolated from WT donor cells. Nuclei were stained with DAPI (blue). Cells were pulsed with EVs for 30 minutes and fixed at 0, 1, or 6 hours post pulse. (A) Top panel: Unwashed condition: Some EV may loosely attach to the cell plasma membrane and may still be there in the medium. Bottom panel: Washed condition: EVs were removed immediately after the pulse. (B) Quantification of average intracellular EV spots per cell over time from three independent experiments (n = 10 cells/experiment). ** $p < 0.01$, * $p < 0.05$, MEFs vs RAW cells; ##$p < 0.01$, #$p < 0.05$, between times in each cell line. Two-way ANOVA with Tukey's post hoc test. (C) Rate of EV uptake calculated during the chase period (0–1 h and 1–6 h) for WT and RAW cells (15 cells per condition per experiment) under washed and unwashed conditions. ** $p < 0.01$; ns, not significant. Two-way ANOVA.

Extracellular vesicles are internalized via dynamin and fluid phase dependent endocytosis and trafficked to lysosomes

Having established that EVs are taken up by recipient cells, we determined whether EV uptake occurs via an endocytic mechanism. We treated cells with dynasore, a pharmacological inhibitor of dynamin dependent endocytosis [14]. Cells were incubated with EVs under washed conditions, involving a 30 minute pulse followed by a 1 hour chase period in fresh medium. As shown in Figure 2A-B, dynasore treatment resulted in a significant reduction in the number of intracellular EV spots compared to the control. Quantification of EV internalization revealed a marked decrease in uptake, although not a complete block in EV uptake following dynamin inhibition. This indicates that EV entry into cells is at least partially dependent on dynamin mediated endocytosis. These results support the involvement of classical endocytic pathways, such as clathrin or caveolin mediated uptake, in EV internalization under these experimental conditions [15].

To further investigate the intracellular fate of EVs following internalization, we examined their colocalization with early and lysosomal compartment markers over time. Specifically, cells were stained for Rab5, an early endosome marker, and LAMP1, at 0, 1, and 6 hours post EV uptake. As shown in Figure 2C and D, EVs initially exhibited low but detectable colocalization with Rab5. However, this decrease was not statistically significant across time, suggesting that EVs may transiently pass through early endosomes without robust retention.

In contrast, colocalization with LAMP1 increased progressively over time and was significantly higher than Rab5 at all time points. This finding indicates that a substantial proportion of internalized EVs are ultimately trafficked to lysosomal compartments for degradation. These results align with our previous observations using fluorescent dextran, where over time cargo showed early endosomal localization followed by accumulation in LAMP1 positive lysosomes [11]. Together, these findings suggest that although EVs may briefly associate with early endosomes,

they are predominantly trafficked toward lysosomes for degradation under normal conditions.

To explore whether EVs exhibit internalization dynamics similar to fluid phase cargoes, we conducted parallel uptake assays using fluorescently labeled EVs and dextran under unwashed conditions. Cells were cotreated with EVs and dextran for a 30 minute pulse, followed by fixation at 0, 1, and 6 hours post pulse to monitor internalization kinetics. As shown in Figure 2E–F, although uptake rates varied across time points, the internalization profiles of EVs and dextran were closely aligned at each interval, with no significant differences observed between the two cargo types when coadministered.

Notably, the EV uptake rate remained comparable regardless of whether dextran was present, indicating that dextran does not alter EV internalization dynamics (Figure 2E). Moreover, total intracellular puncta count for EV and dextran remained stable over time in cotreatment conditions (Figure 2G), further suggesting that both cargoes accumulate at a similar and steady rate under continuous exposure. While this experimental design does not conclusively demonstrate that EVs are internalized via fluid phase endocytosis, the overlapping uptake patterns and lack of mutual interference support the idea that EVs may follow fluid phase cargo route. Future studies involving pathway specific inhibitors, receptor blockade, or live cell imaging will be necessary to dissect the precise entry routes and regulatory factors that guide EV uptake into recipient cells.

To further examine the intracellular fate of internalized EVs and their relationship to dextran trafficking, we analyzed the colocalization of each cargo with LAMP1 (Figure 2H), a lysosomal marker, at 0, 1, and 6 hours post pulse. As shown in Figure 2I, both EVs and dextran exhibited progressively increased colocalization with LAMP1 over time, indicating that a substantial proportion of each cargo is ultimately delivered to lysosomes following uptake. To specifically evaluate whether EVs internalized alongside dextran share the same fate, we assessed the triple colocalization of EVs, dextran, and LAMP1. Approximately 45% of EV-dextran double positive vesicles also colocalized with LAMP1, suggesting that majority of EVs that colocalized with dextran follows a lysosomal degradation route (Figure 2J). While these findings do not definitively identify the endocytic pathway responsible for EV uptake, they support the idea that EVs and dextran can partially converge within the endocytic pathway and be sorted to common downstream compartments.

Our findings provide an important insight into the diverse mechanisms of EV uptake and intracellular trafficking. Using a combination of dynamin inhibition, dextran uptake, and lysosomal colocalization, we demonstrate that EVs are internalized through both dynamin dependent and may

follow fluid phase endocytic pathway. Together, the data highlight the heterogeneity of EV uptake mechanisms and reinforce the central role of lysosomal targeting in determining EV intracellular fate.

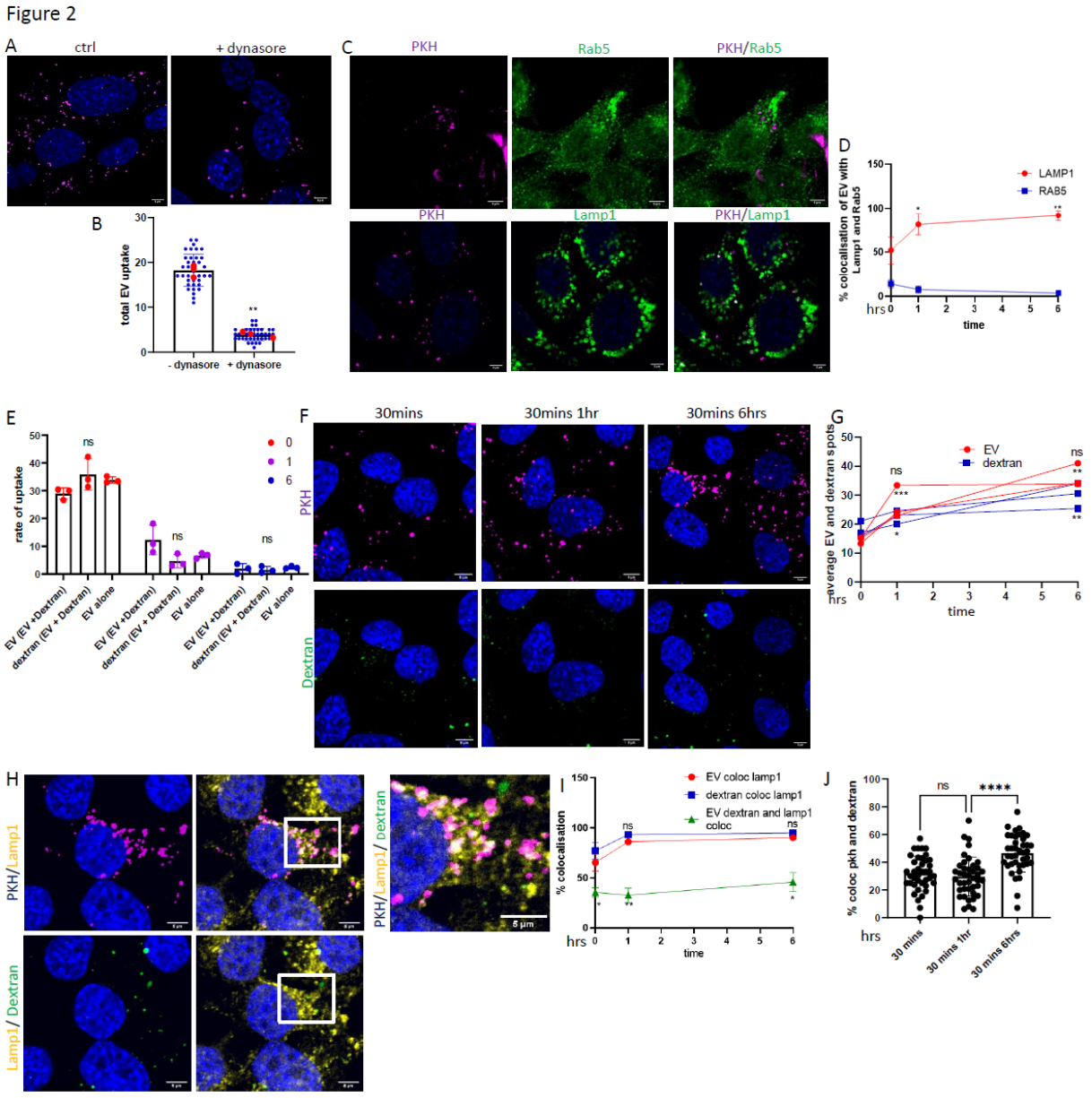

Figure 2. Dynamin dependent endocytosis regulates EV uptake and intracellular trafficking toward lysosomes

(A) Confocal images of WT cells pulsed with PKH labeled EVs for 30 minutes and with 1 hour chase, with or without dynasore (80 µM), a dynamin dependent endocytosis inhibitor, are shown in panel (magenta: EVs, scale bar: 5 µm). Quantification in panel (B) ($n = 3$; $p < 0.01$). Panel (C) shows colocalization of internalized EVs with early endosome marker Rab5 and lysosomal marker LAMP1 at 1 hour post pulse. Panel (D) presents time course quantification of EV colocalization with Rab5 and LAMP1. Statistical differences between colocalizations are indicated (*$p < 0.05$,

**p < 0.01). In panel (E), the rate of EV uptake with or without dextran cotreatment is quantified at 0, 1, and 6 hours. Panel (F) shows representative confocal images of EV (magenta) and dextran (green) accumulation at 30 minutes, 1 hour, and 6 hours, with DAPI marking the nuclei (blue). Panel (G) quantifies the average intracellular EV and dextran spots per cell over time. Finally, panel (H) presents colocalization of EVs and dextran with LAMP1, and panel (I) Quantification of EV-LAMP1, dextran-LAMP1, and triple colocalization (EV-dextran-LAMP1). (J) Quantification of dextran and PKH colocalization (15 cells per condition per experiment). (p < 0.05, **p < 0.01; ns = not significant).

Comparison of EV uptake and intracellular levels between WT and cells with mitochondrial dysfunction

Having established the intracellular trafficking pattern of EVs in WT cells, we next investigated whether mitochondrial dysfunction affects EV uptake. Since mitochondrial integrity is known to influence intracellular trafficking and endosomal dynamics [11], we used OPA1 knockout cells, which display disrupted mitochondrial structure and function, as recipient cells.

EVs were isolated from WT donor cells and added to both WT and OPA1 KO recipient cells. We first quantified the average number of intracellular EV spots at 0, 1 and 6 hours post treatment. As shown in Figure 3A and B, OPA1 knockout cells exhibited a significant reduction in the number of EV spots compared to WT cells, suggesting impaired EV uptake. However, comparison of EV uptake rates at 30 minutes pulse that corresponds to 0 hour, 0-1 hour (1 hour), and 1-6 hours (6 hours) revealed no significant differences between WT and OPA1 KO cells (Figure 3C), indicating that mitochondrial dysfunction does not affect the initial kinetics of EV internalization. These results suggest that while mitochondrial dysfunction does not impair the early uptake of EVs, it may affect post entry trafficking, retention, or vesicle processing, leading to reduced intracellular EV levels over time in OPA1 knockout cells [11]. Therefore, the reduced intracellular EV levels observed in OPA1 knockout cells could likely reflect defective vesicle progression through endosomal system, rather than a defect in EV uptake at the plasma membrane. Importantly, expanding these experiments to include a larger number of cells and additional replicates may help to clarify these trends will further elucidate the potential role of mitochondrial integrity in regulating the intracellular fate and turnover of EVs after internalization.

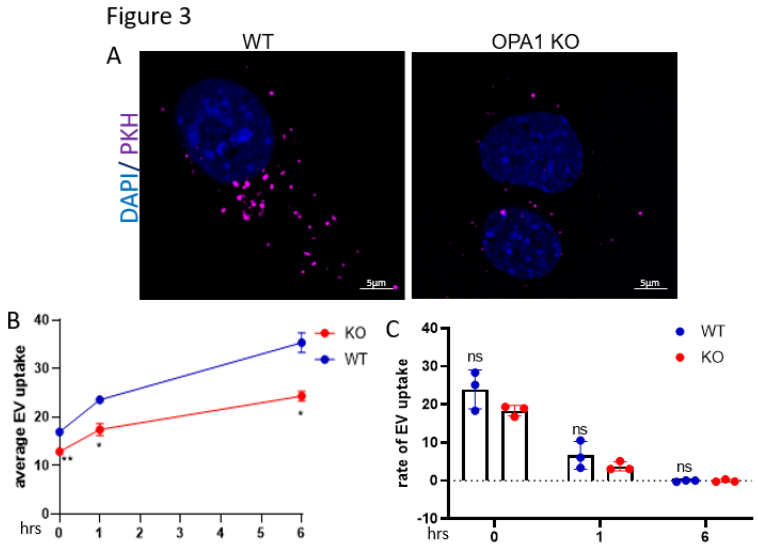

Figure 3. OPA1 knockout reduces EV accumulation without altering uptake rate
(A) Representative confocal images of WT and OPA1 knockout cells following treatment with PKH labeled EVs (magenta). Cells were pulsed with EVs for 30 minutes and chased for 1 hour in fresh medium prior to fixation. Nuclei are stained with DAPI (blue). Scale bars, 5 µm. (B) Time course quantification of intracellular EV puncta per cell in WT and OPA1 knockout cells at 0, 1, and 6 hours post EV pulse. At all the three time points KO cells. * Indicate statistically significant differences between WT and KO at each respective time point. (C) Rate of EV uptake in WT and KO cells at 30 mins pulse (0 hour), 0-1 hour (1 hour), and 1-6 hours (6 hours), calculated from time course quantification of EV spots. ns = not significant by two-way ANOVA; n = 3 experiments (15 cells per condition per experiment).

Mitochondrial dysfunction redirects EV localization toward mitochondria and reduces lysosomal targeting

Given that OPA1 KO cells exhibit alterations in EE trafficking to lysosomes, we investigated whether mitochondrial dysfunction affects the intracellular localization of internalized EVs. Since EE dysfunction is known to delay endo lysosomal maturation, we first assessed the extent to which EVs are delivered to lysosomes in OPA1 knockout cells. As shown in (Figure 4 A and B), EV colocalization with lysosomal marker LAMP1 was markedly reduced in OPA1 KO cells compared to WT controls, suggesting a disruption in the canonical degradative routing of EVs. After confirming a significant reduction in EV lysosome colocalization in these cells, we next examined whether EVs might be rerouted to alternative intracellular compartments. Since OPA1 KO is associated with mitochondrial fragmentation and dysfunction, and EVs are known to carry mitochondrial proteins or components [16], we specifically investigated their colocalization with mitochondria using the outer membrane marker TOM20. Notably, as shown in Figure 4C, association between EVs and mitochondria was significantly increased in OPA1 KO cells. These

results indicate that mitochondrial dysfunction not only impairs lysosomal delivery of EVs but also redirects them toward mitochondrial compartments.

These findings suggest that mitochondrial dysfunction caused by OPA1 knockout not only disrupts vesicular trafficking through the endolysosomal system but also alters the intracellular fate of EVs. The increased association of EVs with mitochondria in OPA1 KO cells, alongside decreased lysosomal targeting, indicates a potential redirection of EVs toward sites of mitochondrial damage. This shift may represent an adaptive cellular mechanism whereby EVs possibly carrying mitochondrial or regulatory components are recruited to dysfunctional mitochondria to support repair, fusion, or metabolic compensation. Given that EVs have been shown to carry mitochondrial proteins, lipids, and RNAs, their preferential interaction with fragmented or impaired mitochondria may help maintain mitochondrial integrity in stressed or damaged cells [17]. These observations add to the emerging view that EVs not only participate in intercellular communication but can also play a functional role in intracellular organelle homeostasis, particularly under conditions of mitochondrial stress.

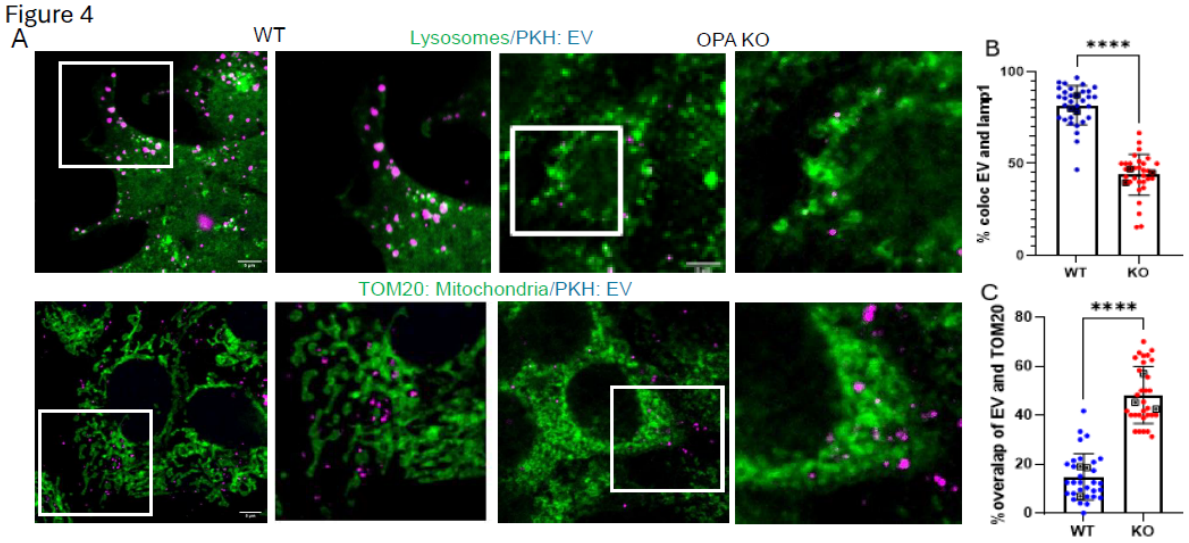

Figure 4. Mitochondrial dysfunction redirects EV localization from lysosomes to mitochondria

(A) Representative confocal image showing colocalization of PKH labeled EVs, (magenta) with lysosomes (LAMP1, green; top panels) and mitochondria (TOM20, green; bottom panels) in WT and OPA1 knockout cells. White boxes highlight representative regions of colocalization. Scale bars, 5 µm. (B) Percentage of EVs colocalizing with the lysosomal marker LAMP1. (C) Percentage of EVs overlapping with the mitochondrial marker TOM20. Each dot represents one cell; data are pooled from three independent experiments. Bars show mean ± SEM. ****$p < 0.0001$ by t test (15 cells per condition per experiment).

EVs carrying mitochondrial cargo are processed differently from the general EV population

Since the majority of EVs are typically directed to lysosomes following uptake [18], we next examined whether EVs carrying mitochondrial components are similarly targeted for degradation. To address this, we assessed the colocalization of GFP labeled mitochondrial EVs with LAMP1, in order to determine whether these cargo enriched vesicles follow the canonical degradative route or may be processed differently within recipient cells. To do this, we used stable donor cells expressing GFP tagged mitochondrial matrix protein and isolated EVs (mitoGFP EVs) from these cells. EVs isolated from untransfected donor cells (non-mitoGFP EVs) were used as controls. Both transfected and untransfected EVs contained mitochondrial material. However, in the transfected cells, the mitochondrial content was specifically tagged, allowing its transfer to recipient cells to be tracked, unlike the untransfected EVs, which were used as a reference.

When we examined the intracellular localization of GFP signal in recipient cells, we observed that cells treated with non-mitoGFP EVs displayed faint green autofluorescence, which showed low colocalization with PKH-labeled EVs but significant overlap with LAMP1, suggesting this may represent background signal or nonspecific uptake (Figure 5A). In contrast, cells treated with mitoGFP EVs exhibited distinct GFP puncta that strongly colocalized with PKH dye and showed reduced overlap with LAMP1 (Figure 5B). Analysis of the isolated EVs showed that approximately 2% of the total EV population was mitoGFP-positive (Figure 5C), indicating a small, traceable subpopulation. Quantitative analysis revealed that while about 80% of total PKH-labeled EVs colocalized with LAMP1, only around 40% of mitoGFP EVs did so (Figure 5D). These observations suggest that mito GFP-tagged EVs may follow a different intracellular route compared to the bulk EV population, but further experiments are needed to clarify whether this difference is due to the presence of mitochondrial cargo, the GFP tag itself, or other factors. Further studies will be required to clarify whether these EVs contribute to signaling, organelle homeostasis, or metabolic regulation within recipient cells.

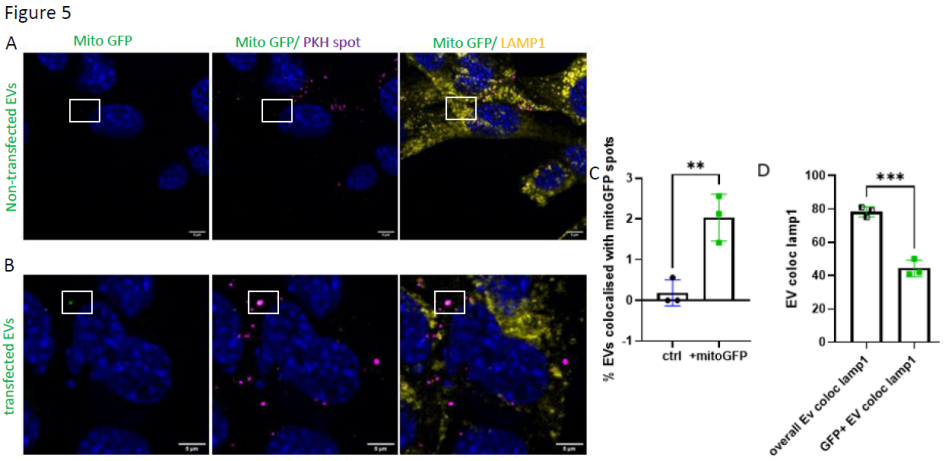

Figure 5. Mitochondria enriched EVs display selective colocalization with lysosomes and PKH labeled vesicles

Representative confocal images showing the distribution and colocalization of non-mitoGFP EVs with PKH labelled EVs and the lysosomal marker LAMP1 (A) and mitoGFP EVs (B) and (C). (A) Non- mitoGFP EV has faint autofluorescence signal in green channel (B) In transfected cells, clear GFP positive EVs were detected (left panel), Notably, most mitoGFP-PKH double positive EVs did not colocalize with LAMP1 (right panel). (C) Percentage of mitoGFP EVs detected in preparations from non-mitoGFP and mitoGFP donor cells. $p < 0.01$ by unpaired t-test. (D) Comparison of EV colocalization with lysosomes (LAMP1) in mitoGFP cells between the overall PKH labelled EV population and the mitoGFP EV subpopulation. $p < 0.001$ by unpaired t-test. Data are represented as mean ± SEM from n = 3 independent experiments with 10 cells per experiment.

Conclusion

This study reveals that EV uptake is a dynamic, cell type specific process governed by multiple endocytic mechanisms, including dynamin dependent and fluid phase pathways. Our findings suggest that while RAW cells internalize EVs more rapidly, they also degrade them more efficiently, highlighting the need to distinguish uptake kinetics from net accumulation. Importantly, we show that mitochondrial dysfunction modelled by OPA1 knockout alters EV fate, enhancing mitochondrial association while reducing lysosomal targeting. Moreover, mitochondrial component enriched EVs form a rare subpopulation with distinct trafficking behaviour, avoiding classical lysosomal degradation. These results provide new insight into the diversity of EV intracellular routes and suggest that a subset of EVs may function in mitochondrial maintenance or organelle communication, particularly under conditions of mitochondrial stress. This work supports the emerging view of EVs as regulators of not only intercellular signaling but also intracellular organelle communication.

Acknowledgements

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CHAPTER IV

DISCUSSION AND CONCLUSION

Mitochondria are traditionally known for their role in energy production and redox regulation [389]. Disruptions in both mitochondrial function and vesicle trafficking are individually implicated in diseases ranging from neurodegeneration to cancer [390] [391] [392]. However, how mitochondrial dysfunction might reshape endocytic architecture and extracellular vesicle handling remains unclear. While mitochondrial interactions with organelles like the endoplasmic reticulum and lysosomes are well established, their contribution to the organization of intracellular vesicle systems has been less clearly defined [393] [394]. This thesis investigates the consequences of mitochondrial dysfunction on vesicle architecture and trafficking, revealing new insights into how impaired mitochondrial activity reshapes the endocytic pathway. It further demonstrates that mitochondrial dysfunction impairs the uptake and intracellular routing of EVs, indicating a broader impact on endocytic system dynamics. Importantly, we uncover that these vesicular changes are accompanied by ROS dependent cytoskeletal remodeling, including microtubule bundling and centrosome amplification, both of which contribute to altered endosomal positioning and trafficking efficiency.

The study began with the observation that disrupting mitochondrial function leads to notable changes in lysosomal structure and activity, including vacuolization and loss of degradative capacity [395]. These early findings raised the possibility that mitochondrial status might influence endocytic processing more broadly. To investigate this, we examined upstream compartments of the endocytic pathway, including early endosomes and recycling endosomes, using both genetic (OPA1 knockout and DRP1 mutant cells) and chemical (electron transport chain inhibition) models. Strikingly, only early endosomes exhibited major changes in distribution and trafficking, including perinuclear clustering and impaired cargo progression to lysosomes. These effects were closely tied to the ROS induced cytoskeletal alterations, suggesting that mitochondrial redox imbalance disrupts endocytic organization not only metabolically but structurally. Recycling pathway, by contrast, was affected to a lesser extent than the degradative pathway, pointing to selective disruption within the endocytic system.

Taken together, the findings presented in this thesis uncover a role for mitochondria in organizing

vesicle trafficking pathways, particularly in the regulation of early endosomal positioning and lysosomal engagement. They also raise the intriguing possibility that EVs may serve as dynamic contributors to intracellular organelle communication, especially under stress. In the following discussion, I will explore these relationships in more detail, assess the contribution of ROS and cytoskeletal changes to endocytic remodeling, and reflect on the potential relevance of these mechanisms in broader cellular conditions. I will also address limitations of this study and outline questions that arise from these observations, particularly regarding the fate and function of EVs during mitochondrial dysfunction.

Mitochondrial Dysfunction and endocytic pathway remodelling

A striking phenotype in cells with mitochondrial dysfunction was the clear morphological and functional alteration of lysosomes, characterized by vacuolization and a noticeable reduction in degradative capacity [395] [396]. In parallel, we observed a spatial alteration of early endosomes, which aggregated prominently in the perinuclear region, while recycling endosomes maintained a distribution and morphology indistinguishable from that of wild type cells. This endosomal compartment specific sensitivity suggests that early endosomes are more vulnerable to mitochondrial perturbation, while recycling routes may be preserved, possibly as a cellular adaptation to maintain membrane turnover and receptor recycling under stress conditions.

One mechanistic explanation for this endosome specific vulnerability may lie in the differential reliance of endosomal subtypes on microtubule based transport [381] [397]. Early endosomes predominantly traffic along microtubules via dynein mediated retrograde movement towards the perinuclear region [382] [398]. This directional transport is highly sensitive to cytoskeletal organization, which is known to be disrupted under mitochondrial dysfunction due to elevated ROS [399] [400]. ROS are known to influence microtubule associated proteins and can alter microtubule dynamics, potentially impairing the spatial organization and intracellular transport of organelles [401] [402]. However, in our study, we observed an increased velocity of Rab5 positive endosomes moving towards the perinuclear region. This observation suggests a distinct mode of cytoskeletal alteration under mitochondrial dysfunction.

Contrary to previous reports suggesting that oxidative stress leads to microtubule depolymerization or network destabilization [399], our findings reveal a distinct phenotype characterized by microtubule bundling and clustering around MTOCs. Similar to observations made by Philip et al. (2022) in osteoclasts where centrosome clustering supports increased microtubule nucleation and radial

array formation we find that centrosome amplification and spatial reorganization likely drive this bundling effect in cells with mitochondrial dysfunction [403]. Given the critical role of centrosomes as microtubule nucleation sites, their remodeling under oxidative stress may represent a key mechanism underlying the cytoskeletal phenotype we observe.

Thus, rather than a direct destabilizing effect of ROS on microtubules, our findings support an alternative mechanism in which ROS induced centrosome remodeling drives localized microtubule bundling near the MTOC, consequently influencing endosomal positioning. This framework provides a potential explanation for the enhanced perinuclear accumulation of Rab5 and EEA1, canonical early endosome markers, observed in our experiments. Although we did not directly assess their colocalization or activation states, their prominent spatial clustering supports the interpretation that early endosomal organization is disrupted as a downstream consequence of ROS mediated centrosomal alterations, rather than generalized microtubule destabilization.

In contrast, lysosomes did not show spatial redistribution, although they exhibited vacuolated morphology and diminished degradative function [395]. This may reflect their reliance on both retrograde and anterograde transport mechanisms, allowing for more dynamic positioning even under cytoskeletal stress [404 405]. Early endosomes, being more reliant on retrograde dynein driven movement, may therefore be more susceptible to ROS induced cytoskeletal alterations, explaining their selective spatial disorganization in mitochondrial dysfunction [398].

These findings raise important mechanistic questions. Rab5, a GTPase critical for early endosome membrane fusion and maturation, could be dysregulated in mitochondrial dysfunction either hyperactivated or rendered functionally unstable [406 407]. Previous studies in neurodegenerative disease models have shown that Rab5 hyperactivation correlates with early endosome enlargement and perinuclear clustering, and we speculate that similar mechanisms may be at play here [408].

Notably, we observed that Rab5 positive endosomes exhibited accelerated trafficking toward the nucleus, whereas other endosomal populations maintained their typical spatial distribution. This selective inward movement suggests that mitochondrial dysfunction does not cause a global trafficking defect, but rather reprograms early endosome dynamics. Although we did not directly assess Rab5 activation status, the observed phenotype rapid perinuclear trafficking and clustering of Rab5 positive vesicles closely resemble that of Rab5 hyperactivation models, raising the possibility of altered Rab5 regulation under mitochondrial stress. Additionally, ROS induced microtubule bundling, and centrosome amplification may facilitate more directed and efficient transport toward the nucleus, enhancing perinuclear endosome accumulation. Together, these

mechanisms may account for the spatial enrichment of Rab5 near the MTOC, distinguishing this phenotype from generalized microtubule destabilization.

Finally, although this study primarily focused on endosomes and lysosomes, it is important to consider that mitochondrial dysfunction may influence the organization and function of other organelles, particularly those closely integrated into endocytic and trafficking networks. One such organelle is the Golgi apparatus, which serves as a central hub in the secretory and endocytic systems [409]. Proteins and lipids are delivered from the endoplasmic reticulum to the cis Golgi via anterograde transport, processed through the medial and trans Golgi cisternae, and subsequently sorted for delivery to the plasma membrane, lysosomes, or endosomes [410]. To maintain homeostasis and receptor recycling, the Golgi also relies on retrograde trafficking, particularly from endosomes and the trans Golgi to the ER, with the retromer complex playing a critical role in this pathway [410].

Both anterograde and retrograde vesicle trafficking to and from the Golgi are tightly regulated by the microtubule cytoskeleton, which governs not only cargo movement but also the spatial positioning of the Golgi itself [411]. The Golgi's characteristic perinuclear ribbon like structure is maintained by minus end directed dynein motors, which transport Golgi membranes along microtubules toward the centrosome, while plus end directed motors such as kinesins mediate dispersion and peripheral localization [412 413]. These dynamic movements are essential for Golgi function, directional trafficking, and cell polarization [414].

Given our observations of significant microtubule bundling and centrosome amplification under mitochondrial dysfunction, it is plausible that such cytoskeletal alterations could impair both vesicle transport to the Golgi and Golgi positioning itself. Disruption of microtubule architecture may interfere with the directional delivery of cargo or mislocalize the Golgi away from its functional niche near the MTOC. While this study did not directly assess Golgi morphology or trafficking, the structural vulnerability of the Golgi to microtubule reorganization highlights it as a compelling target for future investigation particularly in mitochondrial stress and its broader influence on intracellular organelle positioning and dynamics.

Role of mitochondrial ROS in endosomal reorganization, cytoskeletal alteration, and centrosome amplification

Endosomal reorganization

To dissect the mechanistic basis of EE aggregation and microtubule remodeling observed under mitochondrial dysfunction, we explored the role of ROS. We first validated elevated oxidative

stress in OPA1knockout cells using oxyblot analysis, which revealed increased protein oxidation. To test causality, we treated cells with exogenous H_2O_2 at an optimized, non toxic concentration. Surprisingly, this did not recapitulate EE aggregation or microtubule bundling, despite causing elevated protein oxidation. This suggests that sudden random exogenous ROS input may be qualitatively different from the gradual and localized ROS production seen with mitochondrial dysfunction. High concentration H_2O_2 treatments in other systems are reported to cause dramatic cell alterations and centrosome amplification [415].

In our model, however, exogenous H_2O_2 treatment induced a rapid and non localized surge in oxidative stress, which, despite causing significant protein oxidation, did not replicate the spatially confined phenotypic changes observed under mitochondrial dysfunction. In contrast, mitochondrial stress whether through OPA1 knockout or antimycin A treatment appeared to trigger a more gradual and compartmentalized increase in oxidative stress, likely enabling targeted remodeling events such as centrosome amplification and microtubule bundling. This distinction was further supported by oxyBlot analysis, which revealed significantly higher levels of global protein oxidation in H_2O_2 treated cells compared to the mitochondrial stress conditions. Together, these findings suggest that the phenotypic outcomes associated with mitochondrial ROS may depend not just on ROS levels, but on the spatial and temporal regulation of oxidative signaling within the cell.

To directly investigate the contribution of mitochondrial derived H_2O_2 to the phenotypes observed, we employed both pharmacological and genetic approaches. First, we used MitoQ, a mitochondria targeted antioxidant, and observed a clear rescue of endosomal aggregation and microtubule bundling. This indicates that mitochondrial ROS is a key upstream driver of these cellular changes.

To further localize the source of ROS, we utilized a mitochondrial targeted catalase construct, which was designed to detoxify H_2O_2 specifically within the mitochondrial matrix. Interestingly, the construct localized to the mitochondrial matrix in approximately 50% of cells, while the remaining population exhibited cytosolic expression, giving us a unique opportunity to compare the effects of ROS detoxification in both compartments.

We found that cytosolic catalase expression led to a complete rescue of the phenotypes, including endosomal aggregation and microtubule bundling. In contrast, cells expressing matrix targeted catalase showed only partial restoration of normal morphology. This pattern suggests that matrix derived H_2O_2 plays a significant role but is likely not the only contributor to the observed changes.

One plausible explanation is that H_2O_2 produced in the mitochondrial matrix diffuses into the cytosol, where it affects redox sensitive targets involved in cytoskeletal organization and endosome positioning. In this case, cytosolic catalase can neutralize the H_2O_2 that escapes the mitochondria, thus offering a more effective rescue. Therefore, the combined findings support a model in which both matrix localized and cytosol accessible mitochondrial ROS contribute to the phenotypes, with ROS diffusion from mitochondria to the cytosol acting as a key intermediate step in the pathway.

Cytoskeletal alteration

We further considered the role of microtubule architecture in supporting the spatial organsiation of endosome as highlighted by Granger et al. (2014) [381]. In our study nocodazole induced microtubule disruption led to the dispersal of aggregated EEs. A similar observation was reported by Etoh and Fukuda (2019), who showed that microtubule disruption causes the dispersion of Rab10 positive endosomal tubules [416]. Rab10 plays a key role in the formation and extension of recycling endosomal tubules and relies on intact microtubule tracks for its spatial positioning and cargo transport. These findings reinforce the idea that EE positioning and tubular endosomal integrity are microtubule dependent [416]. However, whether this redistribution translates into a functional rescue of endosomal trafficking remains unclear. While disaggregation of EEs might relieve spatial clustering, complete microtubule depolymerization may itself impair vesicle transport, which is crucial for proper endosomal maturation and cargo progression.

Tau is a microtubule associated protein known to support microtubule assembly and stability, with its function tightly regulated by post translational modifications, particularly phosphorylation [417]. In Alzheimer's disease, tau is frequently found in a hyperphosphorylated state, which is believed to reduce its binding affinity to microtubules, potentially contributing to microtubule destabilization and impaired axonal transport [418] [419]. This pathological form of tau has been suggested to interfere with the function of motor proteins such as kinesin, thereby affecting the trafficking of essential cargoes, including mitochondria and amyloid precursor protein [420] [421]. Mitochondrial mislocalization, including perinuclear clustering and morphological abnormalities, has been reported with tau dysfunction [422].

One proposed mechanism is that hyperphosphorylated tau may sequester JIP1, a kinesin adaptor protein, in the soma, thereby disrupting kinesin motor complex assembly and anterograde mitochondrial transport [423] [424] [425]. Moreover, tau induced microtubule disassembly may exacerbate

trafficking defects by destabilizing the cytoskeletal tracks needed for cargo movement. Together, these observations suggest a multifaceted role for pathological tau in impairing intracellular transport, which may contribute to neurodegenerative progression in AD [426] [427].

This reinforces the concept that cytoskeletal integrity is not merely a downstream consequence but also a determinant of mitochondrial health [428]. In our system, we primarily explored how mitochondrial ROS alters microtubules, but it is plausible that these microtubule changes could feedback to further impair mitochondrial mobility, distribution, or bioenergetic output thereby contributing to a positive feedback loop of dysfunction. Exploring this reciprocal relationship could provide important mechanistic insights into organelle communication during cellular stress.

In addition to directly impacting microtubule stability, mitochondrial ROS may also influence cytoskeletal organization through activation of signaling pathways such as RhoA/ROCK1, which are known to regulate centrosome positioning and structural remodeling. Building on this, our findings together with previous work by Muliyil and Narasimha (2014) and Wang et al. (2018) supports a model in which mitochondrial ROS acts upstream of the RhoA/ROCK1 pathway to mediate centrosome amplification and cytoskeletal remodeling under mitochondrial stress [429] [430]. Although we did not directly assess RhoA activation in our system, prior studies have established that ROS can activate this pathway. For instance, Jin et al. (2004) demonstrated that ROS mediate vascular contraction through Rho signalling pathway activation in the rat aorta [431]. And more recently, Wang et al. (2022) showed that tacrolimus, a pharmacological agent known to induce oxidative stress, enhances RhoA/ROCK pathway activity through ROS, thereby contributing to hypertension [432].

Mechanistically, RhoA is a small GTPase that becomes active upon binding GTP a process regulated by GEFs. Its cycling between active and inactive forms is controlled by GEFs, GTPase activating proteins (GAPs), and Rho GDP Dissociation Inhibitors (RhoGDIs) [433]. Activation requires the release of RhoA from RhoGDI, typically mediated by GDI displacement factors (GDFs), allowing translocation to membranes and activation of downstream effectors like ROCK1 and ROCK2 [434].

Once activated, ROCK1 is reported to initiate structural remodeling events such as increased microtubule bundling, actin myosin contractility, and changes in centrosome positioning [435] [436]. Given these roles, we hypothesized that ROCK1 might also contribute to the structural phenotypes observed under mitochondrial stress. In our model, inhibiting ROCK1 under mitochondrial stress significantly rescued centrosome amplification, microtubule bundling, and perinuclear clustering

of early endosomes. These findings provide strong functional evidence that ROCK1 mediates ROS induced architectural disruptions, including centrosome related abnormalities and broader trafficking defects.

Taken together, these results outline a pathway in which mitochondrial ROS activates the RhoA/ROCK axis, leading to centrosome amplification and cytoskeletal remodeling. Although we did not measure RhoA directly, this mechanistic framework, supported by established literature, offers a biologically plausible explanation for the structural phenotypes observed in our mitochondrial dysfunction model.

Centrosome amplification:

To determine whether centrosome amplification is a shared consequence of mitochondrial dysfunction, we assessed centrosomal changes in both genetic and pharmacological models. Inhibition of Complex III using antimycin A led to pronounced centrosome amplification, consistent with the phenotype observed in OPA1 knockout cells, highlighting a broader consequence of mitochondrial dysfunction on centrosome homeostasis. Although we did not directly assess centrosome number in rotenone treated cells (Complex I inhibition), we did observe marked EE aggregation and microtubule bundling, suggesting that Complex I disruption similarly perturbs intracellular architecture. These findings are in line with the study by Donthamsetty et al. (2014), who demonstrated centrosome amplification in both mtDNA-depleted (Rho0) cells and rotenone treated parental cells, attributing these effects to mitochondrial respiratory chain defects and the downstream activation of centrosomal kinases [437]. By showing that antimycin A induced Complex III inhibition leads to centrosome amplification and concurrent endosomal mispositioning, our study expands on this model and provides new insight into how ETC impairment reshapes vesicle systems. Importantly, our data suggest that mitochondrial ETC dysfunction does not only lead to centrosome amplification, but also initiates broader structural reorganization that affects vesicle positioning and trafficking. This supports a model in which mitochondrial stress coordinately affects both centrosomal and endosomal systems through cytoskeletal remodeling, ultimately impairing organelle positioning and intracellular trafficking.

Previous studies have established that PLK4 overexpression is a key driver of centrosome amplification and that its upregulation is demonstrated to be linked to elevated ROS levels [438] [439] [440]. Our aim was to test whether enforced PLK4 overexpression could recapitulate centrosome amplification observed under mitochondrial stress conditions. In alignment with reported data, we

found that overexpression of PLK4 induced centrosome amplification. However, when we assessed oxidative stress levels in PLK4 overexpressing cells using MitoSOX staining, we did not observe any significant increase in mitochondrial superoxide production. This suggests that PLK4 does not independently elevate ROS, supporting a model in which ROS acts upstream to induce PLK4 expression under mitochondrial stress [438 441].

Together, these findings reinforce the idea that mitochondrial dysfunction promotes centrosome amplification via mitochondrial ROS, with PLK4 serving as a downstream effector Importantly, while PLK4 overexpression was sufficient to increase centrosome number, only cells with closely spaced amplified centrosomes exhibited endosomal aggregation and microtubule bundling. This indicates that centrosome amplification alone is not sufficient to generate the endosomal phenotype; rather, spatial clustering of centrosomes, along with the resulting microtubule reorganization, appears essential. These results collectively suggest that in mitochondrial dysfunction cells, the centrosome amplification phenotype likely arises from ROS driven PLK4 overexpression and contributes to subsequent microtubule remodeling and endosomal mispositioning.

Collectively, our results support a model in which mitochondrial dysfunction promotes centrosome amplification leading to altered microtubule phenotype ultimately causing EE aggregation via a ROS- ROCK1 cytoskeleton axis, with. These changes disrupt endosomal positioning and function, impairing lysosomal delivery while sparing endocytic uptake and recycling. Our data further suggest that ROS acts locally and gradually to reprogram organelle positioning, rather than inducing rapid, global stress responses. Targeting ROS sensitive pathways may therefore provide strategies to restore intracellular organization in mitochondrial stress conditions.

Intracellular trafficking of endocytic cargo

Importantly, our data show that the altered spatial distribution of early endosomes has functional consequences. In our study, mitochondrial dysfunction resulted in perinuclear aggregation of early endosomes and delayed dextran trafficking to lysosomes, consistent with impaired endosomal maturation or cargo progression. Interestingly, while dextran uptake remained unchanged, its gradual transit to lysosomes was markedly slowed, and colocalization with Rab5 positive compartments was less. This suggests that trafficking was not completely halted but instead delayed due to altered endosome distribution. We suggest that the aggregation of early endosomes near the centrosome reduces their functional presence in peripheral regions, impairing efficient

cargo sorting and delivery. Although there were no significant changes in transferrin uptake between control cells and those with mitochondrial dysfunction, the alteration in the recycling pathway was comparatively less delayed than the impairment observed in dextran trafficking. Together, these findings highlight that the spatial mislocalization of early endosomes under mitochondrial dysfunction selectively impairs trafficking efficiency most notably in the lysosomal pathway where dextran delivery is markedly delayed, while the recycling of transferrin remains comparatively less affected. This underscores that endosome positioning, rather than uptake efficiency, plays a crucial role in regulating downstream sorting and maturation events.

Our findings demonstrate that mitochondrial dysfunction alters early endosome positioning and impairs downstream trafficking, leading to spatial clustering near the centrosome and delayed cargo progression. While these observations were made in fibroblast cells, they have relevance in highly polarized cells like neurons, where the precise spatial organization of endosomes is essential for long range cargo transport and synaptic function. In neurons, disruptions in endosomal sorting and recycling pathways are increasingly recognized as contributors to neurodegenerative disease pathogenesis, including Alzheimer's disease [442] [443].

For instance, BACE1 (Beta-site APP-Cleaving Enzyme 1) a protease involved in amyloidogenic processing of amyloid precursor protein, relies on early endosomal exit and proper recycling to avoid excessive Aβ generation [444]. Ubelmann et al. (2016) showed that depletion of Amphiphysin 2 (BIN1), an AD risk gene, leads to the retention of BACE1 within early endosomal tubules and impairs its axonal recycling, thereby increasing Aβ levels [445].

Similarly, loss of function mutations in BIN1 and CD2-associated protein (CDAP2) both late onset AD risk genes have been shown to cause endosomal enlargement and promote Aβ accumulation [445] [446]. Mechanistically, previous work has studied that BIN1 facilitates severing of BACE1 containing tubules, while CD2AP helps sort APP into intraluminal vesicles, limiting its surface processing [445]. These studies underscore the broader implication that spatial misorganization of early endosomes, as observed in our model, can impair the fidelity of cargo sorting and trafficking a mechanism that may be particularly detrimental in neurons and potentially relevant to neurodegenerative disease progression.

Importantly, antioxidant treatment rescued multiple phenotypes associated with mitochondrial dysfunction. Specifically, it reversed centrosome amplification and reduced Rab5 clustering, which coincided with the normalization of endosomal trafficking to lysosomes. This indicates that the observed phenotypic alterations are dependent on elevated ROS levels in the mitochondrial

dysfunction model.

Uptake and intracellular trafficking of EVs

Moreover, from a broader perspective, we were interested in exploring whether the mitochondrial content within EVs could potentially rescue mitochondrial function. However, given the observed defects in endocytic trafficking in OPA1 knockout cells, our first objective was to determine whether EV uptake itself is impaired under conditions of mitochondrial dysfunction.

To investigate this, we characterized the uptake dynamics of EVs and compared them with classical endocytic cargoes such as dextran and transferrin. While dextran and EVs both utilize fluid phase and receptor mediated endocytic pathways respectively, EVs exhibit greater structural complexity and rely more heavily on regulated internalization routes including clathrin mediated endocytosis, macropinocytosis, and lipid raft associated mechanisms [447] [448].

Interestingly, we found no significant difference in the total uptake of dextran or transferrin between WT and OPA1 knockout cells, suggesting that core endocytic entry pathways remain intact under mitochondrial dysfunction. In contrast, EV uptake was significantly lower in OPA1 knockout cells when compared with WT cells, pointing toward a selective defect in EV handling. Despite this reduction in EV levels, the rate of EV uptake over time was comparable between WT and KO cells, suggesting that initial internalization occurs efficiently, but subsequent intracellular retention or stability may be impaired.

One possible explanation is that in OPA1 knockout cells, internalized EVs may undergo faster recycling or exocytosis, thereby reducing their intracellular accumulation. This interpretation is supported by our observation that lysosomal trafficking defects were more pronounced than recycling pathway alterations in this model. Moreover, it's important to consider the timing of our uptake assays. EV internalization was assessed using a 30 minute pulse followed by 1 hour and 6 hour chase periods, which likely captured both early trafficking and longer term retention dynamics. In contrast, dextran and transferrin uptake were measured at earlier time points, potentially before intracellular level changes could become evident. It is therefore possible that differences in endosomal retention or routing of dextran and transferrin might emerge at later stages, similar to what we observed with EVs.

Collectively, these findings suggest that while initial EV internalization is not impaired by mitochondrial dysfunction, their post endocytic fate, including trafficking, retention, or recycling

could be affected. This highlights the broader impact of mitochondrial integrity on selective cargo handling within the endosomal system.

Reprogrammed fate of EVs in cells with mitochondrial dysfunction

We found that the post uptake fate of EVs was altered under mitochondrial dysfunction. EVs are commonly internalized through canonical endocytic routes, and lysosomal degradation representing a major intracellular fate. Building on our earlier observations of reduced number of intracellular EV in OPA1 knockout cells, we next examined their intracellular fate. In wild type cells, EVs primarily trafficked to lysosomes, consistent with canonical endosomal maturation. In contrast, OPA1 knockout cells exhibited a marked decrease in lysosomal delivery and a notable increase in EV association with mitochondria. These findings suggest that mitochondrial dysfunction alters vesicular sorting fidelity, diverting EVs from the degradative pathway toward alternative destinations, potentially as part of a compensatory or stress adaptive response.

These observations are supported by prior work showing that lipopolysaccharide treated macrophages preferentially internalized mitochondrial cargo containing EVs from neural stem cells via clathrin and dynamin mediated endocytosis, bypassing degradation in lysosomes and instead integrating with the mitochondrial network [449]. Similarly, Hough et al. (2018) reported that mitochondrial EVs could evade lysosomal processing and exert functional effects on recipient mitochondria [346].

In line with these studies, we found that EVs carrying mitochondrial content, when taken up by wild type cells, did not consistently follow canonical degradative pathways. Although the precise fate of these EVs remains to be determined, our findings raise the possibility that mitochondrial EVs may engage alternative intracellular destinations, such as direct mitochondrial fusion, cytosolic retention, or involvement in signaling networks. Further investigation will be required to delineate the molecular determinants guiding EV sorting under mitochondrial stress conditions.

One intriguing hypothesis is that EVs may act as compensatory vehicles, shuttling bioactive lipids, proteins, or mitochondrial components to mitigate organelle stress [449]. Our model suggest that mitochondrial dysfunction not only impairs endosome to lysosome trafficking, but also drives a broader reprogramming of vesicular routing. Whether EVs contribute to organelle rescue, stress buffering, or metabolic rewiring remains to be investigated. Nevertheless, these results suggest that EV trafficking is not passive, but dynamically adjusted based on the cell's metabolic and

organelle state.

Cell type specific response of EVs

To further examine the relationship between cell type and EV uptake, we compared EV internalization between MEFs and RAW cells. Interestingly, although RAW cells exhibited a higher rate of EV uptake consistent with their phagocytic nature the total number of EV puncta retained intracellularly was significantly lower than in MEFs [450]. This suggests that in RAW cells, rapid post uptake degradation likely outpaces vesicle accumulation. RAW cells are known for their elevated lysosomal activity, which may contribute to accelerated breakdown of internalized EVs [451]. Supporting this, we observed no significant difference in EV signal between wash and no wash conditions, indicating that reduced signal was not due to loss during processing but likely due to intracellular degradation. In contrast, MEFs showed slower but more sustained vesicle accumulation, potentially reflecting less aggressive lysosomal turnover and prolonged vesicle residence time. These findings highlight how both uptake kinetics and downstream trafficking dynamics shape EV accumulation in a cell type dependent manner.

Mechanism governing EV uptake

To further characterize the route of EV entry, we compared EV trafficking to that of dextran, a classical marker of fluid phase endocytosis. Notably, EVs were observed to partially colocalize with dextran, which itself traffics to lysosomes. This suggested that EVs may share a common trafficking route with dextran, although not necessarily the same mechanism of uptake. To test whether this involved clathrin mediated endocytosis, we treated cells with dynasore, a dynamin inhibitor. We observed reduction in EV uptake, though not completely blocked, indicating that the majority of EVs enter via dynamin dependent, clathrin mediated mechanisms, but a smaller fraction may utilize alternative pathways such as direct fusion with the plasma membrane or fluid phase endocytosis.

Our findings are further supported by a study in which the authors observed increased colocalization of small EVs with dextran, indicating that a subset of EVs enters the endocytic pathways rather than the fusion with plasma membrane [452]. Consistent with our observations, they also reported that dynamin inhibition reduced EV uptake, suggesting that clathrin mediated endocytosis plays a major role [452]. These findings are in line with our dual labeling experiments

using dextran and the dynamin inhibitor dynasore, which revealed partial colocalization between EVs and dextran and partial inhibition of EV uptake, supporting the conclusion that EVs utilize multiple endocytic entry routes within the same cellular system.

Influence of EV Size and Composition on Internalization Routes

The heterogeneity in EV uptake mechanisms observed in our study may also be explained by the presence of distinct EV subtypes within our preparations. As we did not fractionate vesicles based on size or density, our samples likely contained a mixture of exosome and microvesicle enriched populations. These subtypes differ not only in their size and surface markers, but also based on reported study they may differ in their preferred uptake routes [453] [454] [447]. While uptake mechanisms are not exclusively determined by size, several studies suggest that smaller exosome like vesicles (~30–150 nm) are internalized via clathrin mediated or lipid raft dependent endocytosis [452] [447] , whereas larger microvesicles (~100–1000 nm) may commonly follow micropinocytosis entry route [455]. Additionally, vesicle specific surface proteins, lipids, and glycosylation patterns likely contribute to selective engagement with endocytic compartments or receptors on recipient cells [456] [457].

This concept is further supported by recent work from Hirosawa et al. (2025), who used super resolution live cell imaging to show that even tumor derived small EV subtypes can follow distinct and non overlapping internalization routes [452]. In their study, all small EV subtypes were internalized via clathrin independent endocytosis, while a subset additionally utilized caveolae mediated pathways. These findings highlight the role of EV intrinsic features such as cargo identity, membrane composition, and cellular origin in dictating entry mechanisms.

Given the heterogeneous nature of our EV samples and the diversity of endocytic pathways observed, it is likely that distinct vesicle subtypes contribute differentially to the trafficking behaviour we report. Future studies employing techniques such as size exclusion, immunoaffinity based isolation, or multiplexed fluorescent labeling will be important to dissect how individual EV populations engage different internalization pathways, and how these are modulated under cellular stress conditions such as mitochondrial dysfunction.

Taken together, the findings of this thesis reveal that mitochondrial dysfunction leads to profound remodeling of endocytic architecture and vesicle trafficking, primarily through ROS-dependent cytoskeletal changes and centrosome amplification, which selectively disrupt early endosome

positioning and lysosomal function, insights have important implications for human disease, as similar alterations in mitochondrial and vesicular pathways are observed in neurodegenerative disorders like Alzheimer's disease. Where endosomal dysfunction and impaired mitochondrial dynamics contribute to pathology, as well as in cancer, where centrosome amplification and disrupted trafficking can promote genomic instability and tumor progression [10]. Furthermore, the interplay between mitochondrial ROS and cytoskeletal remodeling uncovered here may underlie shared features of organelle dysfunction in lysosomal storage diseases and other metabolic conditions, establishing a direct link between mitochondrial health and the organization of vesicle trafficking systems, this work highlights new avenues for therapeutic intervention targeting mitochondrial redox balance and cytoskeletal regulation to restore cellular homeostasis in a range of human diseases [11].

Future directions

The work presented in my thesis highlights how mitochondrial dysfunction, through altered redox signaling, leads to selective reorganization of the endocytic pathway, particularly affecting early endosomes distribution and trafficking function. However, several key mechanistic questions remain and provide clear directions for future research.

A major observation in our study was the perinuclear clustering of EEs under mitochondrial stress. Although we implicated microtubule bundling and centrosome amplification in driving this phenotype, the precise molecular cascade linking mitochondrial ROS to spatial endosome reorganization remains unresolved. Future work should aim to define the upstream signals possibly involving Rab5 regulation, microtubule post translational modifications, or motor protein recruitment that drive EE aggregation. Overexpression of Rab5 or EEA1 mutants, or use of live cell tracking of endosome movement in the presence of cytoskeletal inhibitors, could further reveal how endosomal motility is tuned by mitochondrial signals.

We also observed significant alterations in lysosomal morphology and degradative function, including vacuolization and delayed dextran trafficking [395]. These phenotypes suggest a breakdown in EE to lysosome transition or lysosomal overload, and possibly could be due to increased autophagic demand, potentially stemming from mitochondrial dysfunction. Previous studies, including Lamarche et al. (2016), have already demonstrated that mitochondrial dysfunction impairs autophagic flux, as evidenced by elevated levels of p62, indicating defective autophagosome clearance [395]. In this condition, lysosomes may become functionally saturated,

unable to efficiently process both endocytic and autophagic cargo. This scenario aligns with our observed delays in dextran trafficking, which likely reflect a congestion within the vesicular degradation pathway. These findings support the notion that mitochondrial dysfunction exerts significant stress on the degradative machinery particularly lysosomes whose altered morphology and function correlate with impaired autophagic flux. This convergence of lysosomal overload and defective autophagosome clearance could likely contribute to the observed delays in cargo trafficking, reflecting broader disruptions in vesicle turnover and intracellular transport dynamics.

Finally, our observation that dextran and transferrin uptake were unaffected, but trafficking was delayed, supports the idea that mitochondrial dysfunction specifically impairs post endocytic sorting and maturation. To further explore whether these trafficking impairments extend beyond the early endocytic pathway, future studies should examine retrograde transport routes, particularly those involving the trans Golgi network. In this case, markers such as TGN46, an integral membrane protein of the trans Golgi network, and CI-MPR (cation-independent mannose-6-phosphate receptor), which is responsible for shuttling lysosomal hydrolases from the Golgi to endosomes and recycling back via retrograde transport, offer promising tools [458] [459]. Disrupted localization or trafficking of these markers would indicate compromised retrograde transport, a pathway crucial for maintaining Golgi homeostasis, lysosomal enzyme delivery, and overall vesicular equilibrium. Moreover, CI-MPR function is closely tied to retromer mediated trafficking from early and recycling endosomes to the trans Golgi network [388]. Given the structural reorganization of the microtubule network and centrosome clustering observed in our system, it is plausible that vesicle carriers fail to navigate efficiently along microtubule tracks or are misrouted due to altered spatial dynamics.

Thus, assessing the distribution and trafficking kinetics of TGN46 and CI-MPR could not only validate the impact of mitochondrial dysfunction on retrograde pathways but also help clarify whether Golgi directed membrane trafficking is an additional node of vulnerability. This line of investigation would contribute to a broader understanding of how organellar crosstalk, specifically between mitochondria, endosomes, and the Golgi, is orchestrated under physiological versus stress conditions.

While this study provides important insights into how mitochondrial dysfunction reshapes vesicle organization and EV fate, several mechanistic questions remain open for future exploration. One of the key observations was that EV uptake could have occurred through multiple endocytic pathways, with partial sensitivity to dynamin inhibition and partial colocalization with dextran.

This would suggest that EVs are internalized via a combination of clathrin mediated and fluid phase endocytosis, possibly influenced by cell type. A combination of endocytic pathway inhibitors, when used alongside live cell imaging, could help delineate the precise routes of EV entry under both normal and mitochondrial stress conditions.

The role of the cytoskeleton, particularly microtubules and actin, also warrants further study. Our data suggest that ROS dependent microtubule bundling contributes to early endosome aggregation, but we have yet to explore how this influences EV movement, sorting, or degradation. Prior studies using microtubule targeting agents such as eribulin and paclitaxel have shown that microtubule disruption impairs EV cargo loading and endosomal sorting, leading to intracellular CD63 accumulation [460]. Similarly, others have reported reduced EV uptake upon actin depolymerization with cytochalasin D [452]. Applying these perturbations in our model could help clarify how mitochondrial ROS indirectly governs EV fate by altering the cytoskeletal network.

Moreover, while we observed that EVs associate more with mitochondria in OPA1 knockout cells, we did not test whether these EVs have any functional capacity to modulate mitochondrial stress responses. Several studies suggest that EVs carry mitochondrial proteins, lipids, or DNA, potentially supporting mitochondrial metabolism or signaling in recipient cells [461] [462] [463]. It remains an open and intriguing question whether EVs derived from healthy donor cells could restore mitochondrial membrane potential, bioenergetics, or trafficking functions in mitochondrial deficient cells. Experimental strategies such as EV transfer assays, functional mitochondrial readouts, or labeling of mitochondrial components within EVs would be essential to explore this possibility. Our current data suggest that mitochondrial dysfunction not only changes how EVs are handled intracellularly but may also alter the functional aspect in which EVs operate, shifting their role from cargo carriers to possible regulators of organelle adaptation.

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