UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

LA POLYMÉRISATION DE L'ACTINE RÉGULE LA FUSION MITOCHONDRIALE

ACTIN POLYMERISATION REGULATES MITOCHONDRIAL FUSION

THÈSE PRÉSENTÉE COMME EXIGENCE PARTIELLE DU DOCTORAT EN BIOLOGIE CELLULAIRE ET MOLÉCULAIRE

PAR

PRIYA GATTI

AVRIL 2024

UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

DOCTORAT EN BIOLOGIE CELLULAIRE ET MOLÉCULAIRE (PH. D.)

Direction de recherche :

Marc Germain — Directeur de recherche

Jury d'évaluation de la thèse :

Marc Germain — Directeur de recherche

Geneviève Pépin — Évaluateur interne

Jean-Philippe Leduc Gaudet — Évaluateur interne

Samantha Lewis — Évaluatrice externe

Thèse soutenue le

I dedicate my thesis to my lovely family and friends

ACKNOWLEDGEMENTS

First and foremost, I extend my deepest gratitude to Prof. Marc Germain, my PhD advisor, who stands out as both an exceptional mentor and wonderful human being. He has been incredibly supportive throughout my graduate career and has helped me to progress effectively. I cherish every interaction with him, which has been a blend of learning, brainstorming, and genuine fun. Whether it is confusion about the study, troubleshooting experiments, or just surviving regular winter days, his enthusiastic positivity made everything feel okay. He has been extremely supportive throughout. In these five years, I have transformed into a critical thinker and a more confident researcher, thanks to Prof. Germain's mentorship in instilling these qualities in me. Apart from science, I have learned many life values, good virtues, and humility from him, which is shaping me as a better human being. "Thank you" feels like an understatement for given this incredible opportunity to pursue PhD and making this scientific adventure truly special. You are everything one can aspire to be; I am so blessed and honored for being guided by you. Thanks a million. 🌟

I am truly grateful for the support of my collaborators. Prof. Urrmi Chatterji from the University of Calcutta, where my PhD project originated. Special thanks to her students Pritha Mukherjee and Priyanka Talukdar, who were fundamental in shaping the project. Next, many thanks to Prof. Uri Manor (UCSD), who has been very kind and generous offering research materials for our interventions. Thank you so much for your everlasting support and for exuding incredibly motivating energy

A big shout out and a heartfelt thanks to the funding agencies that have been the financial backbone of my PhD journey – Queen Elizabeth II Scholarship (QES), Rideau Hall Foundation, and Fond de recherche Sante (FRQS), Fondation UQTR, CERMO, RISUQ, and NSERC. Your support has been invaluable. Much gratitude! 🙏

I want to express my sincere gratitude to all the labs in our department and the professors for their generosity, kindness, and collaborative spirit.

I want to express my heartfelt thanks to each colleagues/friends from our lab. Dr.Kiran Todakar, Dr. Hema Saranya Ilamathi, Sara Benhammouda, Anjali Vishwakarma and Anahita Kasmahie. You all made life in the laboratory truly beautiful and enjoyable. Your support, camaraderie, and shared passion for our work have made every day memorable. Working with such a fantastic team has been an incredible experience, and I am grateful for the positive impact you have had on both my professional and personal life. Also, previously passed

students Pamela, Lilia, Lydia and summer undergraduate students Joseph, Andre Anne, Jihane, thank you for being an integral part of this journey.

I am immensely grateful for the beautiful friendships that began here, and I want to express my deepest gratitude to each one of you. Special thanks to Amita and Hema, my constants in everyday life, who took care of me and loved me like no one else, with special memories from broken leg. While Kiran and Shwetha, filled the cravings of home, Priyanka and Lucky, added excess cherriers to every beautiful moments. Dantès true self, care and warmth are deeply appreciated. You all are incredibly beautiful souls, thank you for being more than friends. You've been a source of comfort, joy, and companionship. To all other friends including Saeed, Meraj, Catherine and friends from the university, you all have made this PhD journey a cherishing one ❤️.

I have to mention my friends Pooja and Rashika, who have been with me in every moments of life, kicking and raising each other. I am extremely grateful to them for being in my life.

A special mention to Marc`s wife Jennifer, for all the love and care showered on us. You are truly inspiring to me, thank you so much for being so kind, warm and supportive. Thanks to Miko, Anouk and Cedrick for all the good times together. 😊

Finally, I cannot wrap up this acknowledgment without mentioning my family. They have been my rock, shaping me into who I am today. I am grateful to my parents for their unconditional love and patience. I am indebted to them for their sacrifices and unwavering support throughout my journey. My siblings, Poornima and Jeevan, selflessly supported me in all my endeavors. You are blessings in my life. And not forgetting my brother-in-law Vijendra for his constant encouragement, sister-in-law Nishmitha for immense energy, and little nephew Krishank (love of my life), who have been my cheerleaders through thick and thin. Finally, my husband Dev, for wholeheartedly embracing the role of being my steadfast supporter and unwavering pillar of strength. I loye you guys a lot. Thank you so much.

I am heartily grateful to all my well-wishers, thanks a lot. 😊❤️

****

RÉSUMÉ

Les mitochondries jouent un rôle crucial dans le métabolisme et la signalisation cellulaire en répondant aux signaux internes et externes. Cependant, l'impact de la matrice extracellulaire (MEC) sur la régulation de la structure et de l'activité des mitochondries reste inexploré. De plus, la régulation de la fonction mitochondriale implique un équilibre dynamique des réseaux mitochondriaux par le biais de processus de fission et de fusion mitochondriale. La fission se produit au niveau des sites de contact mitochondries-réticulum endoplasmique (MERCS) et dépend de la formation de filaments d'actine, qui induisent une constriction mitochondriale et activent la GTPase de fission DRP1. La fusion mitochondriale, en revanche, est orchestrée par des protéines de fusion (MFN1/2 sur la membrane externe et OPA1 sur la membrane interne) et se produit également au niveau du MERCS. Cependant, le rôle de l'actine dans la fusion mitochondriale reste flou. Comme les signaux provenant de l'ECM activent la polymérisation de l'actine, mon travail de doctorat a étudié l'interaction entre l'ECM et l'actine dans la régulation de la fusion mitochondriale.

Pour analyser l'influence précise de la signalisation ECM sur la régulation mitochondriale, nous avons utilisé des sphères dérivées du cancer du sein (mammosphères) capables de proliférer en culture en suspension sans ECM. Cette approche nous a permis d'explorer l'adaptabilité métabolique des mammosphères en réponse à différents microenvironnements. Nous avons constaté que les mammosphères attachées à la MEC entraînent un allongement mitochondrial significatif, accompagné d'une respiration mitochondriale et d'une production d'ATP accrues. Notamment, cette restructuration s'est produite indépendamment du régulateur conventionnel de fission mitochondriale, DRP1, mais dépendait plutôt de la signalisation des intégrines et de la polymérisation de l'actine.

De plus, pour comprendre le rôle de la polymérisation de l'actine dans la fusion mitochondriale, des sondes d'actine spécialisées ont été utilisées pour cibler l'actine interagissant avec les mitochondries et le réticulum endoplasmique. Nous révélons pour la première fois que l'actine est présente non seulement sur les sites de fission mitochondriale, mais également sur les sites de fusion. L'inhibition de la formation de filaments d'actine a perturbé les deux processus, soulignant le rôle indispensable de l'actine dans la dynamique mitochondriale.

Au total, mes travaux de doctorat mettent en évidence le rôle central de la polymérisation de l'actine pilotée par l'ECM dans la formation des réseaux mitochondriaux et la promotion de la phosphorylation oxydative (OXPHOS), essentielle à la croissance, à l'adhésion et à la migration cellulaires. De plus, l'implication essentielle des filaments d'actine dans la fusion mitochondriale positionne l'actine comme un régulateur principal qui orchestre les réseaux mitochondriaux en réponse aux stimuli environnementaux. Ces résultats contribuent à notre compréhension de l'interaction dynamique entre la MEC, le cytosquelette et les mitochondries et ont des implications dans divers domaines, notamment la recherche sur le cancer, où le ciblage de la régulation des réseaux mitochondriaux médiés par l'actine peut offrir des avantages thérapeutiques potentiels.

Mots clés : Mitochondries, Matrice Extracellulaire, Polymérisation de l'Actine, Fusion, Mammosphères

ABSTRACT

Mitochondria play a crucial role in cellular metabolism and signaling by responding to both internal and external signals. However, the impact of the extracellular matrix (ECM) in regulating mitochondrial structure and activity remains unexplored. Moreover, the regulation of mitochondrial function involves a dynamic equilibrium of mitochondrial networks through fission and fusion processes. Mitochondrial fission occurs at mitochondria-endoplasmic reticulum contact sites (MERCS) and is dependent on actin filament formation, which induces mitochondrial constriction and activates the fission GTPase DRP1. Mitochondrial fusion, on the other hand, is orchestrated by fusion proteins (MFN1/2 on the Outer membrane and OPA1 on the inner membrane) and is also found to occur at MERCS. However, the role of actin in mitochondrial fusion remains unclear. As ECM signals trigger actin polymerization my PhD study investigated the interplay between ECM and actin in the regulation of mitochondrial fusion.

To analyze the precise influence of ECM signaling on mitochondrial regulation, we utilized breast cancer-derived spheres (mammospheres) with the ability to thrive in suspension culture without ECM. This approach allowed us to explore the metabolic adaptability of the mammospheres in response to varying microenvironments. We found that mammospheres attached to the ECM lead to significant mitochondrial elongation, accompanied by heightened mitochondrial respiration and ATP production. Notably, this restructuring occurred independently of the conventional mitochondrial fission regulator, DRP1, but rather depended on integrin signaling and actin polymerization.

Further, to comprehend the role of actin polymerization in mitochondrial fusion, specialized actin chromobody probes were employed to target actin interacting with mitochondrial and ER membranes. We reveal for the first time that actin is not only present at the sites of mitochondrial fission, but also at the fusion sites. Inhibition of actin filament formation disrupted both processes, underscoring the indispensable role of actin in mitochondrial dynamics.

Altogether, My PhD work highlights the pivotal role of ECM-driven actin polymerization in shaping mitochondrial networks and promoting oxidative phosphorylation (OXPHOS), which is essential for cell growth, adhesion, and migration. Furthermore, the essential involvement of actin filaments in mitochondrial fusion positions actin as a master regulator that orchestrates the mitochondrial networks in response to environmental stimuli. These results contribute to our understanding of the dynamic interplay between the ECM, cytoskeleton, and mitochondria and have implications for various fields, including cancer research, where targeting the regulation of actin-mediated mitochondrial networks may offer potential therapeutic benefits.

Keywords: Mitochondria, Extracellular Matrix, Actin polymerization, Fusion, Mammospheres

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

ABP	Actin Binding Proteins
AC	Actin Cgromobody
Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine Di phosphate
AIF	Apoptosis-Inducing Factor
AM	Adherent monolayer
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
Arf1	ADP-Ribosylation Factor-1
ATP	Adenosine Triphosphate
ATP5a	ATP Synthase Alpha Subunit 1
BrCSC	Breast Cancer stem cell
BSA	Bovine Serum Albumin
CAMK1	Calcium/Calmodulin-Dependent Proteinase Kinase-1
CC	Coiled Coil
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone, protonophore
CCO	Cytochrome C Oxidase
Cdc42	Cell division control protein 42 homolog, Rho family GTPase
CDK1	Cyclin-Dependent Kinase 1
CL	Cardiolipin
CMT2A	Charcot-Marie-Tooth type 2A
CO_2	Carbon Dioxide
CP	Capping Protein
Crk	CT10 regulator of kinase
CSC	Cancer stem cell
Ctrl	Control
Cyt C	Cytochrome C
Cyto D	Cytochalasiin D
Cytb5	Cytochrome B5
CuA	Bimetallic copper center
DAPI	4′,6-diamidino-2-phenylindole, fluorescent dye for DNA
DeAct	Disassembly-promoting, encodable Actin tool
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
Dnm	Dynamin
DOA	Dominant Optic Atrophy
DRP1	Dynamin Related Protein 1

ECAR	Extracellular acidification rate (mpH/min)
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERK 1/2	Extracellular-Signal-Regulated Kinase 1/2
ERMD	ER-associated mitochondrial division
ERMCS	ER Tubules-Mitochondria Contact Sites
ETC	Electron Transport Chain
$FADH_2$	Flavin adenine dinucleotide
FAK	Focal Adhesion Kinase
FAT	Focal adhesion-targeting
FBS	Fetal Bovine Serum
FIS1	Mitochondrial Fission 1 Protein
FH	Formin Homology
FHL2	Four and a Half LIM Domains 2.
FN	Fibronectin
FMN	Flavin mononucleotide
FRAP	Fluorescence Recovery After Photobleaching
Fzo1	Fuzzy onion
GAG	Glycosaminoglycan
GAP	GTPase-Accelerating Proteins
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GED	GTPase Effector Domain
GFP	Green Fluorescent Protein
GLUT	Glucose transporters
GSN	Gelsolin
GTP	Guanosine Triphosphate
HA	Hyaluronan
HC	Heavy Chain
HR	Heptad Repeat
IACs	Integrin adhesion complexes
IF	Immunofluorescence
ILK	Integrin-linked kinase
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space
INF2	Inverted Formin-2
KD	Knockdown
KO	Knockout
MAPK	mitogen-activated protein kinase
MAPL	Mitochondrial-Associated Protein Ligase also known as MULAN
MARCH5	Membrane associated ring finger 5, also known as MITOL

mCh	mCherry
MCU	Mitochondrial Ca^{2+} Uniporter
MD	Middle Domain
MERCS	Mitochondria–ER contacts
MFF	Mitochondrial Fission Factor
MFNs	Mitofusins
MICOS	Mitochondrial Contact Site and Cristae Organizing System
MiD	Mitochondrial Dynamics Protein
MMP	Mitochondrial Membrane Potential
MOMP	Mitochondrial outer membrane permeabilization
MS	Mammospheres
mtDNA	Mitochondrial DNA
MTCO1	Mitochondrially Encoded Cytochrome C Oxidase I
mTORC2	Mammalian Target of Rapamycin Complex 2
Myo	Myosin
NAD^+	Nicotinamide Adenine Dinucleotide (+)
nDNA	Nuclear DNA
NDUFA9	FADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9.
NGF	Nerve growth factor
NPF	Nucleation promoting factor
NRF2	Nuclear factor erythroid 2-related factor
OCR	Oxygen Consumption Rate
OMA1	Overlapping proteolytic activity with m-AAA protease 1
OMM	Outer Mitochondrial Membrane
OPA1	Optic Atrophy Protein 1
OXPHOS	Oxidative Phosphorylation
OSCP	Oligomycin Sensitivity Conferring Protein
PA-GFP	Photoactivable GFP
PDL	Poly D Lysine
PFA	Paraformaldehyde
PFK	Phosphofructokinase
PGAM5	Phosphoglycerate mutase family member 5
pH	Position of Hydrogen
Pi	Inorganic Phosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PMF	Proton motive force’
PP	Protein Phosphatase
PR	Proline-Rich Domain
ROI	Region of interest

Rot	Rotenone,
ROS	Reactive Oxygen Species
RR	Repeat Region
rRNA	Ribosomal RNA
RT	Room Temperature
RTN	Reticulon
SD	Standard Deviation
SDHB	Succinate Dehydrogenase Complex Iron Sulfur Subunit B
SENP5	Sentrin/ SUMO Specific Protease 5
SH2	the Src homology 2
SOX 2	SRY (Sex-Determining Region Y)-Box Transcription Factor 2.
SI-1	Src Inhibitor 1
siRNA	Small Interfering RNA
SMIFH2	formin homology 2 (FH2) domains
TCA	Tricarboxylic acid cycle
TGN	Trans-Golgi Network
TICs	Tumour-initiating cells
TM domain	Transmembrane Domain
TME	Tumor microenvironment
TMRM	Tetramethylrhodamine, Methyl Ester
TOM20	Translocase of Outer Membrane 20
TRIM21	Tripartite Motif-containing Protein 21,
tRNA	Transfer RNA
Ubc9	Ubiquitin-conjugating enzyme 9
UDP-GlcNAc	UDP-N-acetylglucosamine
UQCRC2	Ubiquinol-Cytochrome C Reductase Core Protein 2
UQ/UQH2	Ubiquinone/Ubiquinol (oxidized/reduced forms)
VD	Variable Domain
VDAC	Voltage Dependent Anion-Selective Channel
WASH	Wiskott-Aldrich syndrome protein and SCAR homolog
WHAMM	WASP homolog associated with actin, membranes, and microtubules
WASP	WASP family verprolin-homologous
YME1L	YME1-like protein

CHAPTER I

INTRODUCTION

1.1 Power Organelle: Mitochondria and its Structure

Mitochondria are double-membraned, rod-shaped organelles present in the cytoplasm of most eukaryotic cells. Often referred to as the "powerhouses of the cell," mitochondria play a crucial role in cellular respiration, a process that generates adenosine triphosphate (ATP), the primary energy currency of cells. Additionally, mitochondria are involved in regulating apoptosis (programmed cell death), production of reactive oxygen species (ROS), calcium signaling and maintaining calcium homeostasis (1-9). Mitochondria are unique among cellular organelles because they contain their own genetic material, mitochondrial DNA (mtDNA), and believed to have evolved from ancient bacteria engulfed by ancestral eukaryotic cells in a symbiotic relationship (10). This theory, known as the endosymbiotic theory, explains the presence of mitochondria's own DNA and some structural similarities to bacteria. Typically, mitochondria display a variety of shapes from fragmented round-like to the formation of an interconnected network (11). The quantity of mitochondria per cell can differ greatly; for instance, human erythrocytes (red blood cells) lack mitochondria entirely, while liver and muscle cells (high-energy demanding cells) possess hundreds or even thousands of mitochondria. Notably, the oxymonad Monocercomonoides species is the sole known eukaryotic organism that is devoid of mitochondria (12).

1.1.1 Mitochondrial Structure:

Mitochondria comprise specialized compartments crucial for diverse functions. Here is a brief description of its intricate architecture (Figure 1.1), whose organisation is vital to orchestrate cellular metabolism and energy production.

Outer Mitochondrial Membrane (OMM): This is the outermost mitochondrial membrane separating the mitochondria from the cytoplasm of the cell. It is lipid -rich, highly fluid, relatively permeable to low-molecular weight solutes (< 5kDa), contains special channels capable of transporting large molecules and provides a platform for receptors and tethering proteins (13, 14).

Inner Mitochondrial Membrane (IMM): In contrast to the OMM, the inner membrane is far less permeable, allowing only very small molecules to cross into the gel-like matrix that makes up the organelle's central mass (15). This membrane is highly folded into structures called cristae, which greatly increase its surface area and contain numerous proteins crucial for metabolism, hosting complexes of the electron transport chain (ETC) for mitochondrial respiration (ATP

synthesis) (16-18). Because the IMM is impermeable to most ions and molecules, it allows establishment and maintenance of the proton gradient across the membrane (19).

Intermembrane Space (IMS): The region between the outer and inner mitochondrial membranes is known as the intermembrane space. It contains a variety of enzymes (oxidoreductases MIA40, Cytochrome C Oxidase/ haem lyase) and proteins (TIM, SOD1, Rieske iron–sulfur protein) involved in processes such as protein import and apoptotic signaling (20).

Matrix: The matrix is the innermost compartment of the mitochondria, enclosed by the IMM. It contains a dense mixture of enzymes, mtDNA, ribosomes, and other molecules required for various metabolic processes, including Krebs cycle and fatty acid oxidation. ATP is synthesized via oxidative phosphorylation in the matrix (10, 11, 15).

Mitochondrial DNA: The mtDNA, located in the matrix is a ~16kb circular double-strand structure encoding 37 genes, 13 protein-coding genes, 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs). Most proteins (~1400 mitochondrial proteins) and other molecules that constitute the mitochondrion are encoded by nuclear genes and are imported into mitochondria (21). Each mitochondrion contains 800 to 1000 copies of mtDNA, which are maternally inherited and packaged in high-ordered nucleoprotein structures called nucleoids (22).

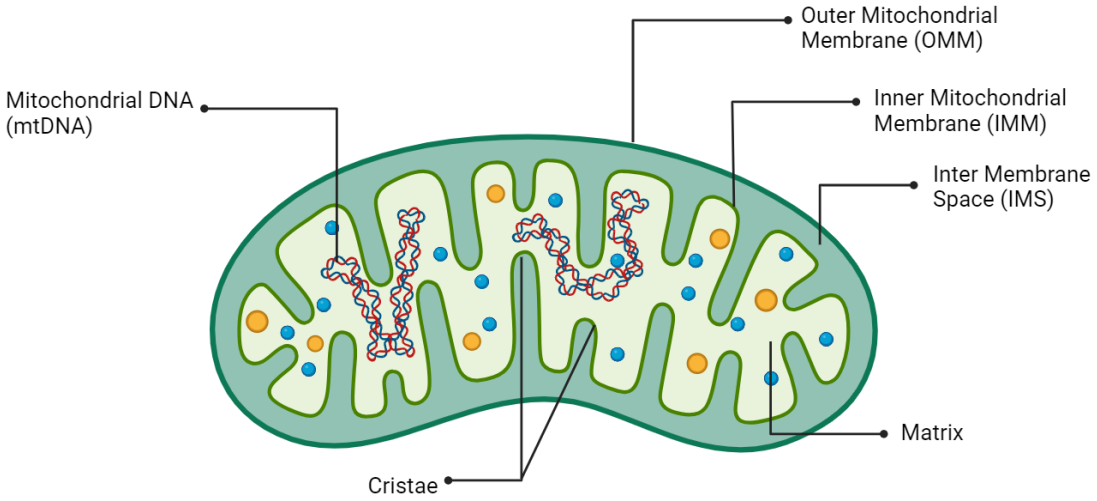


Figure 1: Mitochondria Structure
Basic structural organization of the mitochondrial membranes and compartments. OMM, outer mitochondrial membrane; IMS, inner membrane space; IMM, inner mitochondrial membrane; mtDNA, Mitochondrial DNA; Crisate, IMM invaginations. The figure was generated using biorender.

1.2 Mitochondrial Function

Mitochondria, often called the 'powerhouses' of cells, produce ATP to meet the energy needs of cells (15). They function as the main site for aerobic respiration, where pyruvate is transformed into CO_2, leading to the production of reduced cofactors that drive the electron transport chain to synthesize ATP (17, 23, 24). Oxygen serves as the final electron acceptor in this process, which explains why it is essential for most eukaryotic life forms. Research over the past five decades has revealed that mitochondria have a multi-faceted role beyond their role in respiration. They are involved in a range of biochemical processes, including protein synthesis, amino acid and nucleotide metabolism, fatty acid catabolism, lipid maintenance, regulation of apoptosis, and ion balance (25, 26).

Mitochondria play critical roles in metabolism, even in organisms that live under anaerobic conditions. Their central functions include carbon metabolism, where they participate in the oxidation of sugars, fats, and proteins, and provide the carbon skeletons for biomolecule biosynthesis (27). In addition, mitochondria have a crucial role in 1-carbon metabolism (28, 29) and nitrogen metabolism (30), participating in glutamate and glutamine metabolism (31) and the urea cycle (32). They are also essential in the synthesis of molecules like hemoglobin and iron-sulfur clusters (33, 34). Mitochondria adhere to a continuous mechanism termed mitochondrial dynamics, wherein they undergo morphological alterations crucial for regulating above mentioned mitochondrial function and to maintain cellular homeostasis (13, 14, 35). Amongst the various functions of mitochondria, this thesis will focus its discussion on the topics of metabolism and mitochondrial dynamics.

1.3 Mitochondrial Metabolism:

The cell has complex pathways for energy extraction from nutrients, biomolecule synthesis, and waste product removal. These interconnected processes make up cellular metabolism, including glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), and biosynthetic pathways. Mitochondrial metabolism focuses on oxidative phosphorylation, which produces ATP from food oxidation. In the mitochondria matrix, fuels like carbohydrates (pyruvate), fats (fatty acids), and proteins (amino acids) are oxidized through the tricarboxylic acid (TCA) cycle and OXPHOS to create ATP. (23, 24). Let us understand this in detail.

1.3.1 Glycolysis: Oxygen independent ATP production

Glycolysis is the first metabolic pathway elucidated, splitting one glucose molecule into two pyruvate molecules (36). Glucose transporters (GLUT) facilitate glucose uptake in cells, with GLUT1-4 being the most commonly expressed (37). Once inside the cell, glucose breakdown

requires ten biochemical reactions in the cytosol. ATP is initially consumed during the investment phase before the net ATP production in the payoff phase. The complete glycolysis cycle releases a net of two ATP and two NADH (nicotinamide adenine dinucleotide (NAD^+) + hydrogen (H)) reducing equivalents for every glucose molecule and occurs in the cytosol in the absence of oxygen (Figure 1.3.1). After glycolysis, pyruvate has two major fates that are largely dependent on oxygen availability, glycolytic rate, and other factors (36) (Figure 1.3.1).

1. In the presence of oxygen, pyruvate is translocated into the mitochondria and utilized in the TCA cycle (36)
2. In the absence of oxygen, pyruvate becomes an electron acceptor and is converted to lactate by lactate dehydrogenase to regenerate NAD^+ (36, 38, 39).

Cytosolic NADH can be relocated to the mitochondria through shuttle pathways, such as the malate-aspartate shuttle, and combined with the mitochondrial NAD^+/NADH pool (40). However, this mechanism may not be sufficient during rapid glycolysis, as the accumulation of intracellular NADH (reductive stress) can inhibit GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase) activity and slow down glycolysis (41).

Like NADH, lactate accumulation is undesirable, and cytosolic lactate and protons are transported into the extracellular environment through monocarboxylate transporters (MCT) (42, 43). There is a misconception that lactate in the circulation is a metabolic waste product (44), but it is actually a valuable carbon substrate used as fuel in different contexts, especially in the tumor microenvironment (TME) (45), by neurons during periods of high energy demand (46) and by cardiac muscle at all times (47). Lactate is also a key signaling molecule that regulates gene expression through histone lactylation (48).

Glycolysis also supports various biosynthesis processes, such as

1. Glucose 6-phosphate is subsequently converted into a building block for nucleotide synthesis through the pentose phosphate pathway (49).
2. NADH can be converted to NADPH for anabolic reactions via cytosolic or mitochondrial NAD kinases (50, 51).
3. Production of Fructose 6-phosphate, a precursor of UDP-N-acetylglucosamine (UDP-GlcNAc), an essential molecule for protein post-translational modification (52, 53).

1.3.2 The tricarboxylic acid cycle (TCA) cycle

Mitochondria generate ATP through a series of redox reactions (54) using cytosolic pyruvate converted into acetyl coenzyme A (acetyl-CoA) or oxaloacetate by pyruvate dehydrogenase or pyruvate carboxylase, respectively (55). These reactions occur in the TCA cycle (also known as the citric acid cycle or Krebs cycle), first introduced by Sir Hans Krebs in the 1930s (56, 57), in

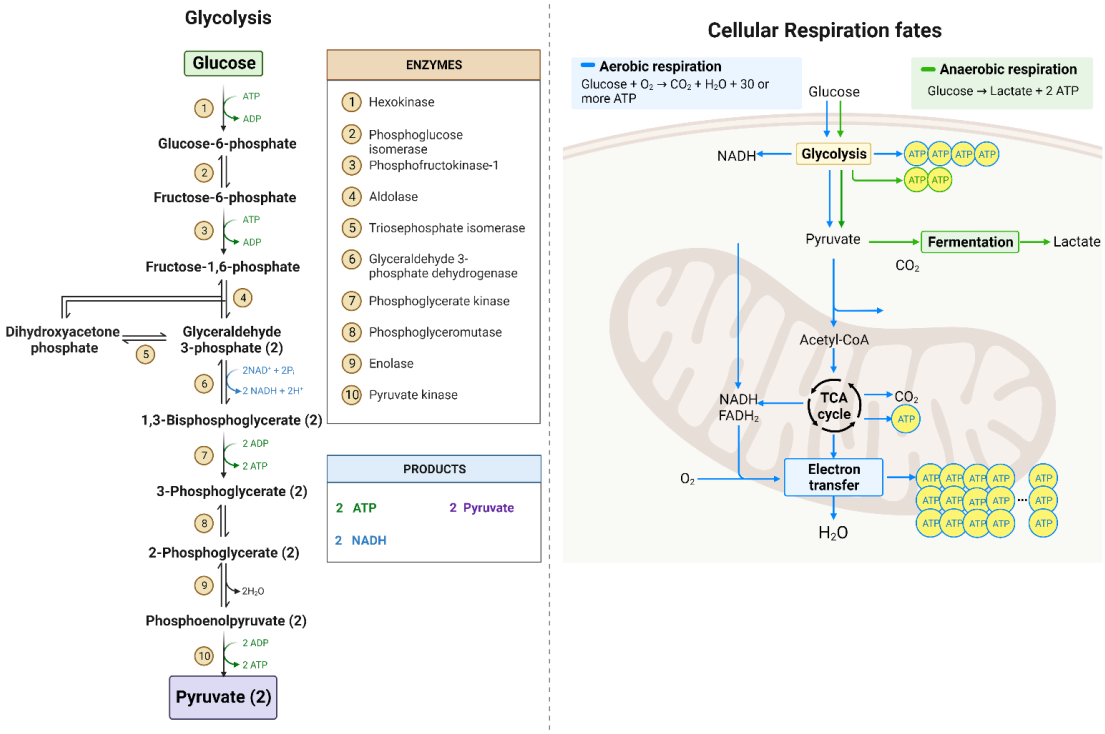


Figure 1.3.1: Glycolysis and Cellular Respiration fates
One glucose molecule generates a net product of 2 ATP, 2 reducing equivalents (NADH) and 2 pyruvate molecules. Steps 1 (hexokinase), 3 (phosphofructokinase) and 10 (pyruvate kinase) are irreversible reactions. Pyruvate after glycolysis has two fates, lactate production or import into the mitochondria for further processing in the TCA cycle. Glycolytic intermediates can be diverted for other cellular purposes. For example, as building blocks for biosynthesis or signaling molecules. To regenerate NAD^+, pyruvate can be converted to lactate. The figure was generated using biorender inspired from (36).

mitochondrial matrix. The TCA cycle consists of eight reactions in which citrate synthase combines the two carbons of acetyl-CoA with the four carbons of oxaloacetate to form citrate. The subsequent seven reactions result in catabolism of citrate, generating reducing equivalents (three NADH and one $FADH_2$ (flavin adenine dinucleotide)), along with one GTP (Guanosine Triphosphate). The cycle continues to regenerate oxaloacetate and so on (58, 59). NADH and $FADH_2$ carry electrons and H^+ ions, and function as electron sources for the ETC in the presence of oxygen. Succinate dehydrogenase, which plays a role in the TCA cycle, also serves as a component of the respiratory chain within Complex II. It directly provides electrons derived from $FADH_2$ (60, 61) (Figure 1.3.2).

Beyond ATP production, the TCA cycle generates multiple TCA intermediates that function as signaling molecules for various anabolic processes (25):

1) Oxaloacetate is involved in the synthesis of aspartate, a precursor for nucleotide base pyrimidine, and asparagine (62, 63).

2) α-Ketoglutarate is converted to glutamate (31) and utilized for the synthesis of non-essential amino acids such as proline (51).

3) Citrate is exported from mitochondria and cleaved into acetyl-CoA and oxaloacetate (59, 64, 65). While acetyl-CoA is used for fatty acid synthesis and is imported to the nucleus for histone acetylation (66, 67), oxaloacetate re-enters the mitochondria and is fed back into the TCA cycle (59).

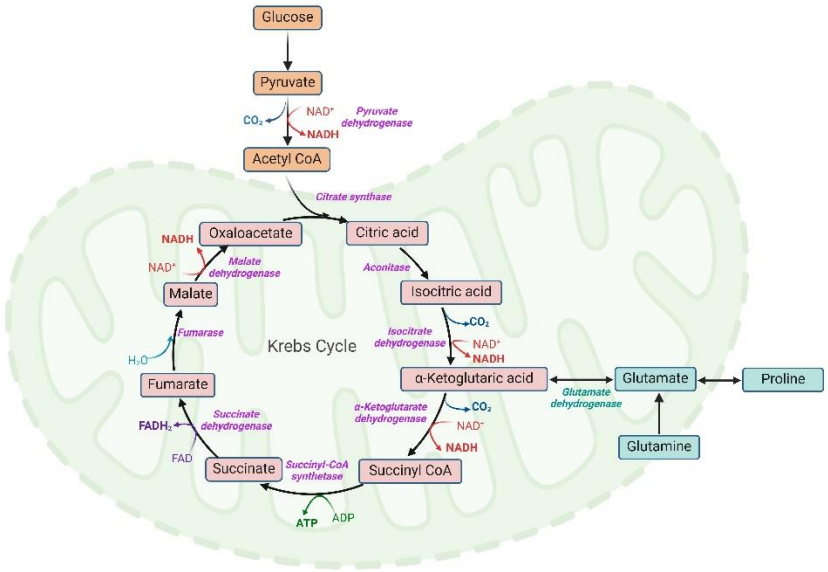


Figure 1.3.2: The TCA cycle
In the mitochondrial matrix, pyruvate is converted into acetyl-CoA before entering the eight chemical reactions of the TCA cycle, centered on the cyclic catabolism and recovery of citrate. Alternately, pyruvate can be converted directly into oxaloacetate. The reducing equivalents released are the fuel for the ETC afterwards. For a complete catalytic cycle, three NADH, 1 $FADH_2$ along with one GTP are produced. Parallelly, Glutamine can enter the TCA cycle by converting to glutamate, and subsequently to α-ketoglutarate by glutamate dehydrogenase (glutaminolysis). The figure was generated using biorender inspired from (31, 52).

1.3.3 Oxidative phosphorylation (OXPHOS): Oxygen dependent ATP production

OXPHOS is the process by which ATP is produced in mitochondria by utilizing oxygen molecules. This process occurs in the IMM and involves a sequence of reactions within the ETC, where multiple protein complexes work together to generate ATP (Figure 1.3.3). This OXPHOS-dependent ATP production in mitochondria follows a chemio-osmotic process.

The chemiosmotic theory

Peter Mitchell, a British biochemist, and Nobel laureate pioneered a paradigm shift in the field of cellular bioenergetics with his 1961 seminal paper (68). In this study, he posited that the link between oxidation and ATP synthesis is a proton gradient, which is the redox energy derived from

reducing equivalents (such as NADH) used to generate a proton electrochemical gradient across a membrane, which then drives ATP synthesis (68). This scheme is now known as the 'chemiosmotic theory.' Mitchell coined the term 'proton motive force' (pmf) to describe the proton electrochemical gradient (69, 70), despite the lack of experimental evidence at the time to support this hypothesis. The theory faced controversy but was eventually confirmed by critical studies, by Racker and Stockenius in 1974, which found that ATP synthase could utilize a bacteriorhodopsin-driven proton gradient to produce ATP (71). Mitchell's ground-breaking work was recognized with the Nobel Prize in Chemistry in 1978 (70).

Role of 'proton motive force' in OXPHOS pathway:

The proton motive force across the IMM is maintained through the ETC (explained in detail in next section), which consists of four large protein complexes I-IV. Complexes I, III, and IV have the ability to transport protons out of the matrix (72). The driving force for proton transport is the oxidation of TCA-derived NADH and $FADH_2$, with the electrons being passed between complexes (I→III→IV or II→III→IV) to the final electron acceptor, oxygen, which results in the formation of water (23, 73). The ETC complexes transporting protons or H^+ ions out of the matrix into the IMS, creates the above-mentioned proton gradient in the IMS. This gradient forces H^+ back to the mitochondrial matrix via the ATP synthase embedded in the IMM, leading to phosphorylation of ADP into ATP, a process known as 'oxidative phosphorylation' or OXPHOS (23, 60).

The pmf (measured in mV) is determined by two components: ΔpH, which is the pH gradient (74) across the mitochondrial membrane (pH_{IMS} minus the pH_{matrix}), and Δψm, the mitochondrial membrane potential (an electrical term) (72, 75). The pmf is highly dynamic, with local variations in pH and Δψm within cristae structures (74, 76).

Complexes of the electron transport chain (ETC)

The ETC is composed of four complexes (I-IV) and an ATP synthase unit, also known as complex V. There are two critical electron carriers in the ETC, ubiquinone (UQ) and Cytochrome C (Cyt C). UQ is a lipid-soluble organic molecule reduced by either complex I or complex II to form ubiquinol (UQH2). UQH2 then diffuse within the IMM to complex III, which is oxidized back to UQ. Cyt C is a protein bound peripherally to the IMS face of the IMM, and shuttles electrons from complex III to complex IV(24, 60, 77) (Figure 1.3.3).

Complex I is referred to as NADH dehydrogenase, for its role in oxidizing NADH to NAD^+. It is responsible for transferring electrons from NADH to UQ, while simultaneously moving protons (78). It has a hydrophobic domain and hydrophilic arm. NADH is oxidized at the base of the

hydrophilic arm, where a flavin mononucleotide (FMN) cofactor accepts electrons and is reduced to $FMNH_2$. The electrons then move up a series of nine iron-sulfur (Fe-S) centers before reaching the hydrophobic module and IMM (79). Rotenone, an inhibitor, prevents the transfer of electrons to the UQ and increases ROS production (80). Other inhibitors, such as mucidin or metformin, can inhibit Complex I without producing ROS (81, 82).

Complex II is referred to as succinate dehydrogenase, for its role in the oxidation of succinate to fumarate. It also converts FAD into $FADH_2$, which shuttle electrons from $FADH_2$ to UQ. However, not enough energy gets released in this process, and thus no H^+ ions are pumped into the IMS from this complex. Under hypoxic stress or inhibition of complexes III and IV, complex II can reverse its activity by oxidizing UQH_2 back to UQ (25). This reverse activity of complex II allows complex I to continue to deposit electrons into the ETC to regenerate mitochondrial NAD^+.

Complex III is referred to as Cyt C reductase, for its role in reducing Cyt C. The enzymatic action of complex III involves the transfer of electrons from UQH2 to Cyt C and subsequent pumping of 4 H^+ ions into the IMS space. Although UQH2 can deliver two electrons, Cyt C can only accept one electron. Thus, Cyt C reductase exists in a dimeric form to accommodate two Cyt C molecules. The process of Cyt C reduction via complex III is the Q cycle (73, 83). Various compounds inhibit complex III function, including antimycin A, myxothiazol, and stigmatellin. Antimycin A treatment is a potent trigger for complex III-mediated ROS generation (80).

Complex IV, the last complex in the ETC referred to as Cyt C oxidase, for its role in the oxidation of Cyt C. It reduces O_2 to H_2O with the help of two electrons from Cyt C coupled with protons pumping into the IMS. Electrons are first passed from Cyt C to a bimetallic copper center (CuA), and then to a heme a3/CuB catalytic site, which serves as the center for oxygen reduction. Eight protons were utilized from the mitochondrial matrix for each O_2, with four protons ending up in H_2O and four pumped across the IMM (24, 60, 84).

The mitochondrial F_0F_1-ATP synthase is a remarkable nanomachine consisting of two functional domains: the membrane-intrinsic F_0 sector (in IMM) and the membrane-extrinsic F_1 sector (in the matrix) (85-87). The two domains are connected by central and peripheral stalks. The F_0 domain contains a motor that generates a rotary action using the pmf, while the F_1 domain is the catalytic part of the enzyme that phosphorylates ATP from ADP (Adenosine Diphosphate) and Pi (inorganic phosphate) (87). ATP synthase dimerizes at the highly curved base of the cristae ridge in mitochondria, contributing to IMM folding and cristae formation (88). Oligomycin, a potent inhibitor of ATP synthase, binds to the F_0 domain of this complex (89) and inhibits ATP synthase activity.

The ETC complexes can function independently, however, three complexes (I, III, and IV) form a supramolecular assembly called the 'respirasome' with all the necessary electron carriers and Cyt C for efficient electron transfer (90, 91). The respirasome is found on the mitochondrial cristae in mammalian cells and affects respiratory functions. A recent study suggests that complex II can form a supercomplex in Tetrahymena thermophila, a unicellular eukaryote (92). Additionally, it has been proposed that the presence or absence of supercomplexes does not improve respiratory efficiency in *Drosophila melanogaster* but is essential for stabilizing the complexes under suboptimal biogenetic conditions (93). However, there is no experimental evidence to support this in mammalian systems (94).

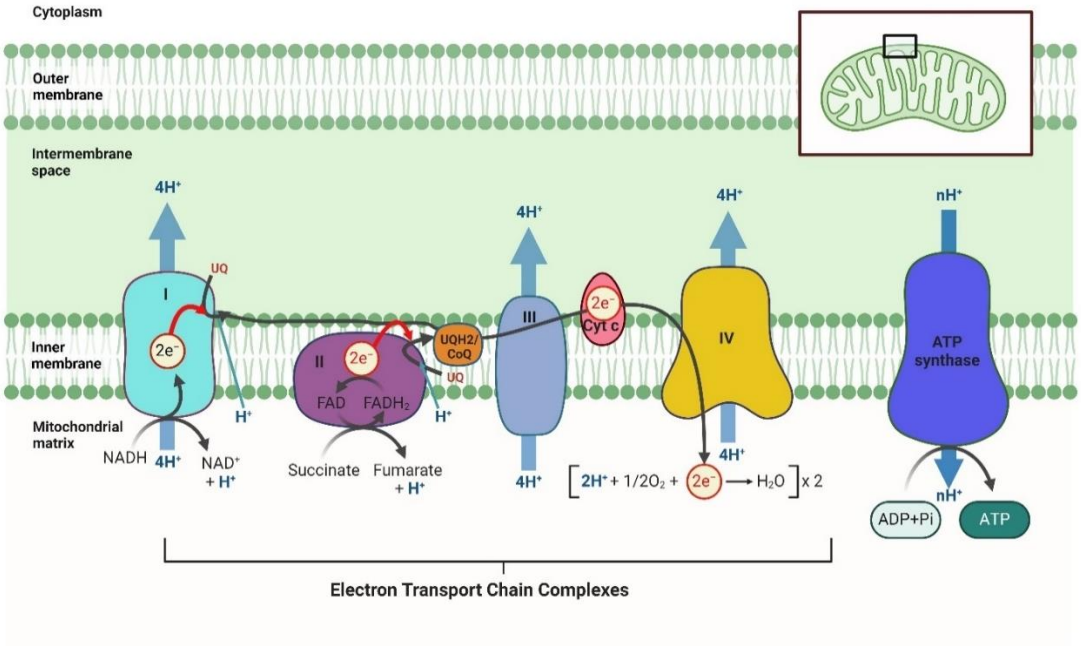


Figure 1.3.3: The mitochondrial ETC complexes
Complex I translocates four protons for two electrons donated from NADH oxidation. Electrons from Complex I are carried by the ubiquinone (UQ)/ubiquinol (UQH2) pool at the hydrophobic core of the IMM. Electrons at Complex III are transferred to Cyt C. Each Cyt C shuttles one electron from complex III to IV, where oxygen is the terminal acceptor electrons. pmf across the IMM drives ATP production through ATP synthase. Complex I and Complex II have similar roles, Complex II converting succinate to fumarate instead of NADH with the transfer of electrons. Also, complex II is incapable of transporting protons across the IMM to IMS. The figure was generated using biorender inspired from (95)

1.3.4 Glycolysis and/or oxidative phosphorylation: A mystery shift

The Pasteur effect:

Louis Pasteur (in 19th century), demonstrated that baker's yeast ferments simple sugars, such as glucose, to produce ethanol in the absence of oxygen. However, the presence of oxygen suppresses

fermentation. Therefore, they proposed that cells convert glucose to ethanol or lactate in response to hypoxia. This phenomenon, where metabolic switches shift from respiration to fermentation or glycolysis during hypoxia, and high oxygen tension inhibits glycolysis, is collectively referred to as the "Pasteur effect."(96)

It is now widely acknowledged that the inhibition of the ETC or the loss of mitochondrial membrane potential ($\Delta\psi m$) leads to an increase in glycolysis (36, 97, 98). The tumour microenvironment (TME) represents a prime example where metabolic switching is crucial, as immune cells must survive in this hostile environment (99, 100).

These findings form the basis for Otto Warburg's ground-breaking theory regarding the distinctive metabolism observed in cancer cells.

The Warburg effect:

In the 1920s, Warburg observed that cultured tumor tissues had high abnormal rates of glucose utilization and lactate secretion, even in the presence of abundant oxygen (101). This phenomenon, where cancer cells preferentially perform anaerobic metabolism, is known as the 'Warburg effect,' (97) or 'aerobic glycolysis' (102). Warburg concluded that mitochondria in cancer cells were 'impaired' since oxygen could no longer suppress glycolysis (103-105). He suggested that the transformation of a normal cell to a cancerous cell occurs in two stages: 1. When normal cells encounter irreversible respiratory injury, they become cancerous and 2. carcinogenesis or the period of adaptation for injured cells to maintain their viability (106). Today, we understand that mitochondria are not dysfunctional in cancer cells, rather it can be considered as a 'damage' to the regulation of glycolysis or cytosolic oxygen-sensing pathways (107-109).

Metabolic Plasticity: an ongoing scenario

Metabolic flexibility refers to the ability to adapt to changes in metabolic demand and has roots in the early research by Harris and Benedict. Their work in the 1920s and the 1930s on basal metabolism, exercise responses, and metabolic studies in individuals with conditions, such as diabetes, remain influential. Individuals with type 2 diabetes and obesity exhibit metabolic inflexibility during the post-meal period, which affects glucose and fat oxidation (110, 111). Metabolic plasticity allows cells to dynamically adjust their metabolic programs, switch between different metabolic states, and optimize energy production and nutrient utilization to support their diverse functions in response to changing conditions. It plays a significant role in various physiological and pathological processes, including cancer progression, immune cell function, and stem cell homeostasis (112-114). Additionally, mutations in nuclear-encoded mitochondrial genes

may disrupt mitochondrial bioenergetics, resulting in metabolic reprogramming characterized by reduced OXPHOS and increased glycolysis, potentially contributing to carcinogenesis (98, 115). Cancer cells in specific, exploit metabolic plasticity to adapt easily to gain survival advantages. In some cancers, cells use glycolytic metabolism even under high oxygen tension, as observed in lung tumors (116). In some other cancers, active mitochondria operate together with accelerated glycolysis, whereas slow-growing cancer cells prefer OXPHOS (117). Altogether, this adaptability enables cells to adjust their metabolic processes to meet specific cellular requirements and maintain overall cellular health and function.

1.4 Mitochondrial Dynamics

To govern mitochondrial metabolism and various cellular functions, mitochondria employ a distinctive characteristic known as Mitochondrial dynamics. Mitochondrial dynamics refers to the continuous and coordinated processes of mitochondrial fission, fusion and cristae arrangements, which regulate the morphology, distribution, and connectivity of mitochondria within the cell (118). Through this dynamic feature, mitochondria adapt to changes in energy requirements, repair or eliminate damaged components, and preserve the optimal functionality of mitochondria within a cell.

Mitochondrial dynamics involves three fundamental processes: fission, fusion and cristae remodeling. Fission splits one mitochondrion into two; while fusion does the opposite and combines mitochondria together. Both division and fusion have distinct molecular mechanisms and signaling cues. The constant remodelling and delicate balance between the 2 processes regulate mitochondrial morphology, behavior, and movement within the cellular environment (119, 120). Cristae remodeling refers to the dynamic alteration of the IMM structures where cristae invaginations undergo changes in the shape, size, and arrangement. This process is essential for regulating mitochondrial functions, optimizing energy production, and adapting to the changing metabolic needs of the cell (121, 122).

Mechanism of Mitochondrial Dynamics

The dynamic events are mediated by a series of molecular machinery and signaling pathways. The intricate regulatory mechanisms governing these events have been gradually unveiled over the past 2 decades, including the identification of specific proteins and their roles in organizing this process.

1.4.1 Mitochondrial Fission

The main, central protein facilitating fission or division is a dynamin-related GTPase protein, DRP1 (123), that causes division of a single mitochondrion into two daughter mitochondria.

DRP1

DRP1's involvement in mitochondrial division was initially observed in *Caenorhabditis elegans* (124) and yeast (125), and has been extensively studied in mammals (123). DRP1 is a cytosolic protein having 1. a GTPase domain at the N-terminus, 2. a middle domain (MD), 3. a variable domain (VD), and 4. a GTPase effector domain (GED) at the C-terminus (Figure 1.4.1). The association of these domains via intra- and inter-molecular interactions promotes the self-assembly of DRP1 into higher order filamentous and spiral-like structures (126) mediating mitochondrial fission upon GTP hydrolysis.

The GTPase domain regulates the self-assembly of DRP1 and stimulates the polymerization of DRP1 by hydrolyzing GTP (127). The middle domain is important for the self-assembly of DRP1 into dimers, tetramers, and even high-order oligomers (128). The C-terminal GED domain stimulates GTPase activity and maintain the stability of higher order complexes. The GED domain folds back to interact with the GTP-binding domain, the middle domain, and the GED domains of other DRP1 proteins (129). The variable domain is located between the MD and GED, in which 8 lysines within 2 clusters in the VD function as conjugation sites for SUMOylation (130). The MD and the GED harbor α-helix-containing stalk domains which mediate DRP1 dimerization (131).

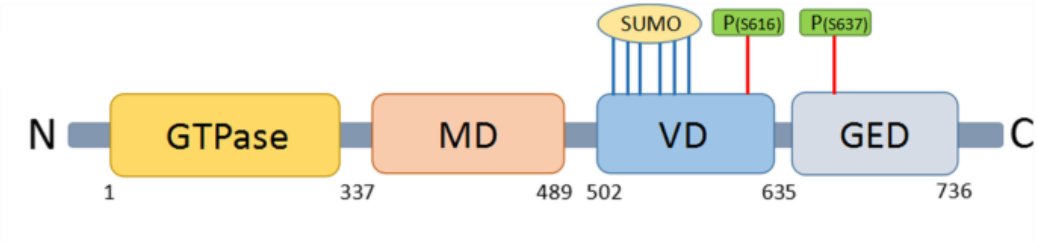


Figure 1.4.1: The structure of DRP 1
DRP1 contains an N-terminal GTPase domain, a middle domain (MD), a variable domain (VD, inset B), and a GTPase effector domain (GED). Adapted from (132)

1.4.1.1 OMM Fission: Mitochondrial fission articulates in three steps (Figure 1.4.2):

(I) The selection and marking of a prospective fission site

The marking of the fission site initiates the division process by ensuring that the mitochondrial tubule, normally 300-500 nm in diameter, constrict to an extent compatible with the 120-nm DRP1 helix. This is accomplished by the endoplasmic reticulum (ER) wrapping around prospective fission sites on mitochondria (133) in a process termed ER-associated mitochondrial division

(ERMD). Then, The ER-bound inverted formin (INF2) and OMM-bound Spire1C form a scaffold for the assembly of actin cables. These actin cables along with Myosin II provide the mechanical force required to induce further membrane constriction, marking sites for the assembly of division complex (134, 135).

(II) Recruitment and Activation of DRP1

The decrease in mitochondrial diameter facilitates the recruitment of DRP1 and formation of oligomeric rings. However, under physiological conditions, DRP1 exists in a dynamic equilibrium between cytosolic and membrane-bound forms, transitioning through a dimeric intermediate (136). Moreover, DRP1 lacks a membrane-anchoring domain and requires activation for recruitment to the OMM. Recruitment of DRP1 from the cytosol to mitochondria is mediated by the mitochondria-anchored DRP1 adaptor proteins mitochondrial fission factor (MFF), mitochondrial dynamic proteins of 49 and 51 kDa (MiD49 and MiD51), and hFis1 (137-139).

Parallel to its recruitment to the OMM, post-translational modifications regulate DRP1 fission capacity. These include phosphorylation, S-nitrosylation, ubiquitylation, and SUMOylation. (SUMO: small ubiquitin-like modifier).

For instance, two distinct phosphorylated serine residue sites have been described, with contrasting effects on fission activity. **Ser616 phosphorylation** promotes DRP1 activation, whereas Ser637 phosphorylation inhibits DRP1 fission activity. Several kinases have been identified as inducers of Ser616 phosphorylation, including phosphoglycerate mutase family member 5 (PGAM5)(140), AMP-activated protein kinase (AMPK), calcium/calmodulin-dependent proteinase kinase-1 (CaMK1), ERK 1/2 (extracellular-signal-regulated kinase 1/2) protein kinase C (PKC)(141), mitogen-activated protein kinase (MAPK), cyclin-dependent kinases/cyclin B1 (CDK1/Cyclin B1), and Aurora kinase A. Likewise, phosphorylation of Ser637 by protein kinase A (PKA) activated by cyclic adenosine monophosphate (cAMP) (129) or CaMKI; (142) inhibits DRP1 fission activity. Conversely, calcineurin and protein phosphatase 2A (PP2A) lead to the dephosphorylation of Ser637, enhancing DRP1 fission activity (143).

DRP1 can also undergo **S-nitrosylation** on a conserved cysteine residue in the GTPase effector domain. This leads to an increase in mitochondrial fission, possibly by enhancing the effects of DRP1-activating phosphorylation (144).

In addition, **ubiquitylation** results in DRP1 degradation. The Ubiquitin E3 ligase MARCH5 (membrane associated ring finger 5, also known as MITOL) can ubiquitylate DRP1 and MiD49, modulating mitochondrial morphology in a pro- or anti-fusion fashion in a manner that is still controversial (145, 146).

Finally, SUMO can be covalently attached to DRP1, rendering it more stable at the OMM. MAPL (Mitochondrial-Associated Protein Ligase also known as MULAN), Ubc9 (Ubiquitin-conjugating enzyme 9) and SUMO1 have all been implicated in DRP1 **SUMOylation** (147), while the removal of the SUMO moieties is performed by SUMO protease SenP5 (Sentrin/SUMO-specific protease 5) (148). Of note, DRP1 stabilization by MAPL-mediated SUMOylation has recently been implicated as an apoptosis-promoting mechanism downstream of Bax/Bak activation, stabilizing ER-mitochondria contacts that generate mitochondrial constriction and Cyt C release (149).

(III) GTPase driven constriction.

Following recruitment and phosphorylation, DRP1 assembles into a higher-order multimeric complex with a ring-like structure encircling mitochondrion, ultimately inducing fission through its GTPase activity. DRP1 oligomerization followed by mitochondrial constriction leads to the recruitment of dynamin 2 to terminate membrane scission (150). However, it was later shown that DRP1 could induce mitochondrial fission in the absence of dynamin 2 (151).

1.4.1.2 ***IMM fission***

The constriction of the IMM is proposed to be an independent process regulated by calcium influx. It is the initial step in the fission process after stimulation by ER-induced calcium release to mitochondria, potentially before DRP1 recruitment, and can be independent of OMM constriction (152, 153). In certain cell types, IMM constriction is attributed to mitochondria–ER contacts (MERCS) and INF2-mediated actin polymerization (152), while in neurons, it is regulated by IMM fusion protein OPA1 (Optic atrophy1) processing (154). Calcium entry into mitochondria reduces the mitochondrial membrane potential, thus activating OMA1 (overlapping proteolytic activity with m-AAA protease 1) in the IMM leading to the processing of OPA1 into S-OPA1 (Short OPA1). The accumulation of S-OPA1 disrupts the capacity of the MICOS (mitochondrial contact site and cristae organizing system) complex to stabilize OMM–IMM tethering, causing IMM untethering and constriction (153). S-OPA1 localizes at MERCS within the OMM fission machinery and enhances mitochondrial fission (155). However, more research is needed to fully understand the regulators of IMM constriction and division, and their integration into the overall mitochondrial division process.

1.4.1.3 Types of Fission

Two functionally and mechanistically distinct types of fission have been identified leading to distinct mitochondrial fates (Figure 1.4.2).

Midzone fission is DRP1-dependent and regulated by MFF. Most fission events are midzone divisions that occur at MERCs (156, 157). These events are mostly associated with healthy

mitochondria and leads to the proliferation of mitochondria. Additionally, during midzone division, mtDNA synthesis occurs at the same MERCs prior to division (158).

Peripheral divisions are also DRP1 dependent, is preceded by lysosomal contact and regulated by FIS1. Peripheral division is driven by a stress response, such as the accumulation of ROS and loss of membrane potential. Peripheral division enables damaged material to be shed into smaller mitochondria which is subjected for mitophagy (156, 157).

Other cellular organelles in Mitochondrial fission

During mitophagy, lysosomes are recruited to mitochondrial fission sites to eliminate damaged mitochondria (159). Lysosomes/late endosomes are tethered to mitochondria by the GTPase protein Rab7, which is hydrolyzed by TBC1D15 (Rab7 GTP) to untether them during fission (160). The functional role of lysosomal-mitochondrial contacts during fission is poorly understood, but it has been postulated that lysosomes are recruited before IMM fission and modulate fission process through calcium (Ca^{2+}) signaling (161, 162).

Recent studies have demonstrated the involvement of trans-Golgi network (TGN) vesicles in mitochondrial membrane scission. These vesicles are recruited to the fission site after DRP1-mediated constriction and carry essential cargo, such as Arf1 and PI4KIIIâ, on their membrane. Loss of these cargos impairs fission and results in hyperconnected mitochondrial networks (163). However, the mechanism of Arf1-PI4KIIIâ containing TGN vesicles in mitochondrial membrane scission is unknown. It is suggested that these vesicles might regulate the lipid composition of constricted membranes to control mitochondrial membrane scission (164, 165).

More research is necessary to understand this complex process involving various organelles working together to regulate mitochondrial division.

1.4.2 Mitochondrial Fusion

Mitochondrial fusion is a two-step process that involves the fusion of the OMM, followed by the fusion of the IMM (166, 167). These two steps occur almost simultaneously and are tightly coordinated. The fusion of OMM and IMM is predominantly mediated by dynamin-related GTPases with three key players being mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy 1 (OPA1). Recent research indicates that mitochondrial fusion occurs at MERCs, although their specific function has not yet been determined (168-170).

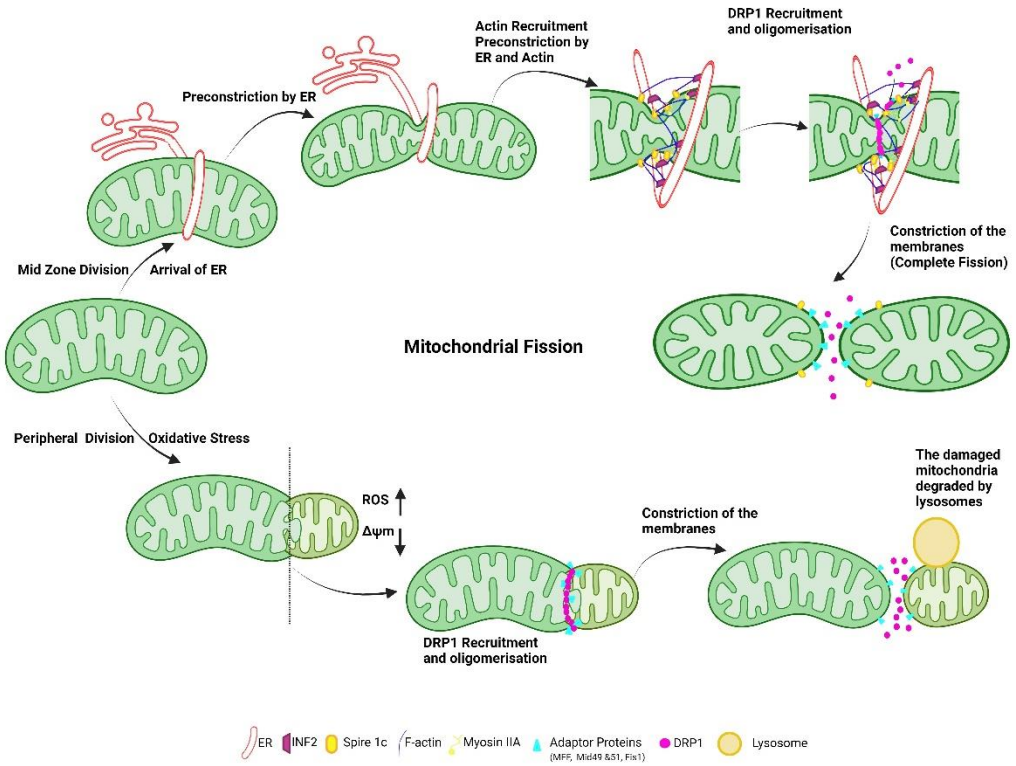


Figure 1.4.2: DRP1-dependent mitochondrial division
Fission comes in at least two forms. Midzone and Peripheral division. For Midzone, the event is associated with the ER and actin filaments. Actin polymerized by ER-bound INF2 and mitochondrially-bound Spire 1C leads to enhanced ER-mitochondrial contact in a Myosin II-dependent manner leading to "pre-constriction". The DRP1 adaptors on the OMM recruit DRP1 to the constriction site, enhancing its oligomerization and GTPase activity forming full ring assembly around OMM leading to constriction. For peripheral division, mitochondrial stressors like a drop in mitochondrial membrane potential (ψm) trigger the asymmetrical division which is assisted by lysosome recruitment. After division, the smaller unit is targeted for organelle clearance at lysosomes. The figure was generated using biorender inspired from (156)

1.4.2.1 OMM fusion

In mammals, OMM fusion is regulated by two large GTPases, MFN1 and MFN2, which share approximately 80% sequence similarity. These proteins accumulate at the contact points between adjacent mitochondria and form homo- or heterotypic complexes (171), bringing them to close proximity facilitating mitochondrial fusion (172). The ortholog of MFNs, known as the fuzzy onion (Fzo1), was initially identified in *Drosophila melanogaster* and is evolutionarily conserved from yeast to humans (173-175).

The structure of MFNs:

Mitofusins contain an amino-terminal GTPase domain, which is exposed to the cytosol, and two heptad repeat domains (HR1 and HR2), also referred to as coiled-coil domains, separated by two

transmembrane domains that are suggested to anchor the proteins in the OMM (Figure 1.4.2.1), although the actual topology of the protein is debated (176). The overall configuration of MFN1 and MFN2 is expected to be similar, with slight divergences in MFN2 arising from specific sequence variations in its GTPase domain, leading to diminished GTPase activity. However, MFN2 proteins display a robust tethering effect, as demonstrated at the sites of ER-mitochondria contacts (177).

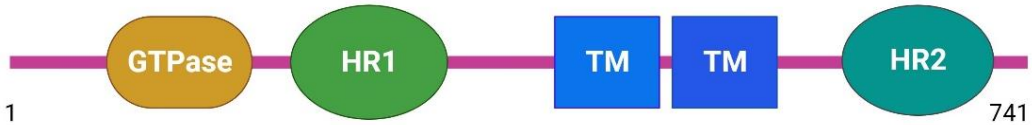


Figure 1.4.2.1: The structure of MFN
MFNs contain an N-terminal GTPase domain, a middle domain (MD), a variable domain (VD, inset B), and a GTPase effector domain (GED). The figure was generated using biorender inspired from (132).

There are several models proposed for MFN mediated OMM fusion (Figure 1.4.2.2):

1. ***Trans-dimerization model:*** According to this model, MFN contains two transmembrane domains that are inserted into the OMM with the heptad repeat (HR1 and HR2) domains facing the cytosol, along with the GTPase domain. The dimerization of HR2 domains on opposing mitochondria brings them close to each other, and GTP hydrolysis causes conformational changes in MFNs, resulting in the merging of the OMM (178). In vitro studies suggest that GTPase-dependent activity of coiled coil HR1 domains regulates mitochondrial membrane tethering and lipid mixing (179). GTP hydrolysis by MFN1 requires K^+ as a charge-compensating factor, which makes the nucleotide-binding pocket highly selective (176). The binding of guanine nucleotide regulates G domain dimerization, inducing a conformational change from the 'closed' tethering-constrained state to the 'open' tethering-permissive state allowing efficient tethering of two OMMs. The latter process is also termed as nucleotide-triggered dimerization (180)

2. ***ROS-based dimerization:*** The proposed model suggests that MFNs have a single transmembrane domain, with the HR2 domain facing the IMS, and the GTPase and HR1 domains facing the cytoplasm. It is hypothesized that an oxidative milieu in the IMS would result in the dimerization of HR2 via disulfide bond formation, hereby causing two mitochondria to be tethered by the GTPase-dependent HR1 domain followed by the fusion of membranes (181, 182). It has been proposed that under such topology, it may facilitate coordinated fusion of both the membranes by interacting with the IMM fusion machinery (183).

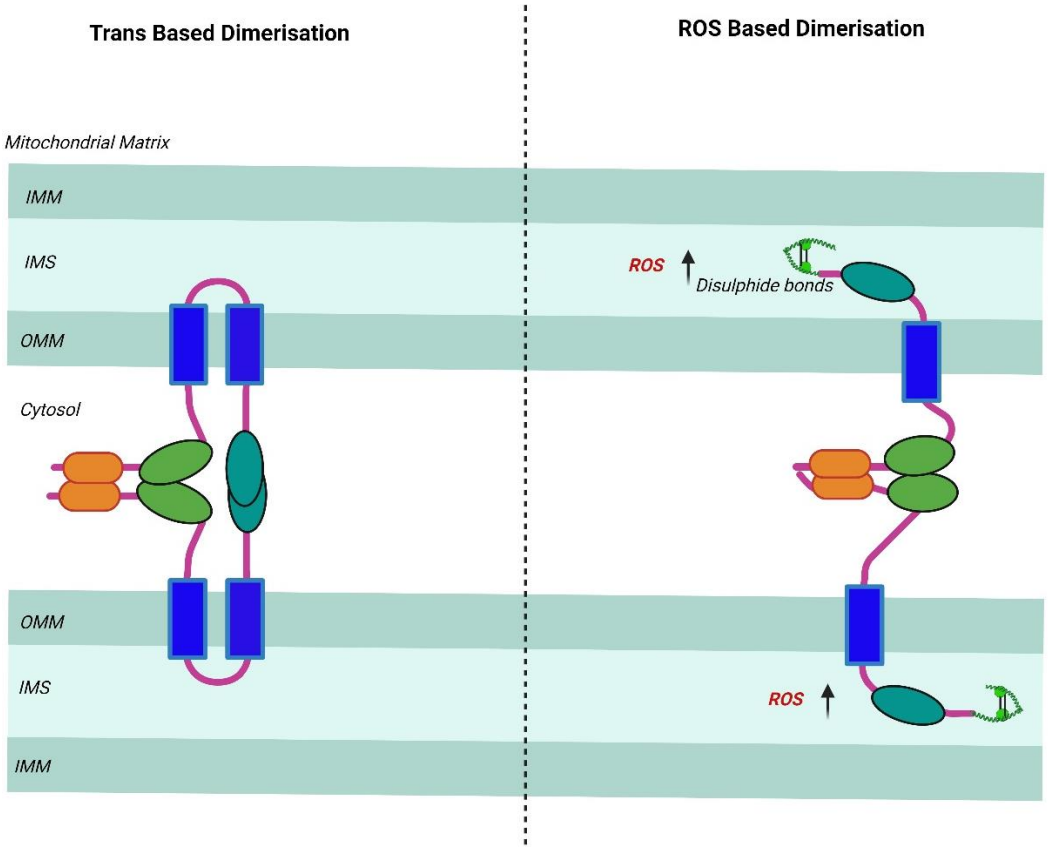


Figure 1.4.2.2: Dimerization models proposed for MFN-mediated OMM fusion
Trans-based dimerization where MFNs from opposing mitochondrial membranes can physically interact in trans, forming antiparallel dimers between HR2 domains. The HR2-HR2 interaction will tether two opposing mitochondria further forming dimers of the GTPase domains. In ROS mediated dimerization, the HR2 and disulfide cysteine residues residing within the IMS lead to conformational changes that promote tethering or changes in GTPase activity. The GTPase loading promotes GTP hydrolysis activity facilitating membrane fusion. The figure was generated using biorender inspired from (181)

1.4.2.2 IMM fusion

IMM fusion is governed by the large GTPase OPA1 in conjunction with the IMM-specific lipid component Cardiolipin (CL). OPA1, initially identified in yeast (as Mgm1p)(184), is evolutionarily conserved and is characterized by eight splice variants. It is a dynamin-like GTPase anchored to the IMM via its N-terminal transmembrane domain, with GTP-binding and GTPase effector domains exposed in the intermembrane space (185). OPA1 can be cleaved at two sites, S1 and S2, by two membrane-bound metalloproteases, OMA1(Overlapping Activity with m-AAA protease 1) and YME1L (YME1-like protein), generating at least five OPA1 fragments, with two higher molecular weight forms referred as L-OPA1 and the three shorter as S-OPA1 variants (186, 187). The abundance of these OPA1 isoforms varies in different cellular contexts, thereby

influencing the regulation of mitochondrial dynamics. Interestingly, OPA1 localization to just one of the two opposing mitochondria is sufficient to drive the fusion of both IMM membranes (188). CL is a mitochondria-specific negatively charged lipid primarily found in the IMM that is essential for the assembly and stability of large protein complexes, such as the cristae organizing system (MICOS), and OXPHOS complexes (16). Studies have shown that when recombinant L-OPA1 is exposed to CL-containing liposomes, it can stimulate heterotypic interactions between L-OPA1 and CL, leading to membrane fusion (189). Likewise, S-OPA1 has the capacity to enhance OPA1 binding to CL, facilitating IMM fusion. These findings align with yeast models that highlight the role of CL and both L-OPA1 and S-OPA1 in IMM fusion (190, 191).

1.4.2.3 Cristae modeling

Along with the fusion process, OPA1 controls cristae morphology and remodeling. The two processes are independent, as interference with OMM fusion does not affect cristae shape (192). The current model proposes that oligomers of L-OPA1 line the length of the cristae, where they tether opposite-facing membranes. The cleavage of L-OPA1 results in disruption of the cristae structure and the release of proteins from the intracristae space (193). In contrast, S-OPA1 forms are believed to be less important for steady-state cristae structures but are more relevant for dynamic remodeling of cristae morphology following stress challenges (193). Loss of OPA1 completely disrupts cristae structure and affects mitochondrial OXPHOS activity (194, 195).

1.4.2.4 Types of mitochondrial fusion

Mitochondrial fusion can be classified into two distinct forms: transient and complete. In the case of complete fusion, both membranes merge, whereas transient fusions involve the merging of both membranes and sometimes only the OMM. Transient fusion is a brief or partial fusion event that may separate within seconds. In contrast, complete fusion maintains its fused state for a more extended period. The IMM protein OPA1 is necessary for both complete and transient fusion, however, a lower level of OPA1 is sufficient to facilitate transient fusion (196, 197).

Transient fusion events occur when mitochondria come into close contact, facilitating the quick exchange of soluble proteins within the IMS and matrix. After this exchange, the two mitochondria separate, retaining their original morphology. These transient fusion events exhibit rapid kinetics and allow only partial exchange of the integral membrane proteins. Transient fusion acts as a "pit stop" for mitochondria, efficiently recharging them to rapidly restore movement and metabolic activity. This process serves as a quality control mechanism to ensure mitochondrial stability and adaptability for optimal metabolic function (196, 197).

A **complete fusion event** is a rapid diffusion of soluble mitochondrial components, followed by a more gradual mixing of integral membrane elements, including mtDNA. Complete fusion results in a change in the morphology, leading to a more connected or elongated structure. Complete fusion is believed to confer complementation between individual mitochondrial units, thereby promoting increased homogeneity throughout the mitochondrial network. Complementation serves as a crucial mechanism by which mitochondria can rescue damaged units within the network (196, 197).

1.4.3 Importance of fission and Fusion

Fission is essential for the removal of damaged or dysfunctional mitochondria and the distribution of mitochondria throughout the cell. Fission occurs in response to nutrient excess, cellular dysfunction, during cell division, to controls calcium homeostasis and as autophagic response (14, 120, 198).

The process of mitochondrial fusion allows for the mixing of various mitochondrial contents between neighboring mitochondria (135, 198). The complementation of fusion between damaged and healthy mitochondria helps buffer transient stresses or defects within a mitochondrion by diluting toxins.

Any imbalance in fission or fusion during cell division can impair mtDNA segregation (199). Dysregulation of the fission-fusion process leads to defects in the recalibration of cellular responses and the transmission of diffusible signals within mitochondria, which can cause mitochondria-associated diseases. Mutations in MFN2 cause Charcot-Marie-Tooth type 2A (CMT2A), a peripheral neuropathy affecting sensory and motor neurons of the distal extremities (200). Mutations in OPA1 are the predominant cause of autosomal dominant optic atrophy (DOA), a degeneration of retinal ganglia cells that leads to optic nerve atrophy (201). The defect in the fission protein DRP1 leads to encephalopathy due to defective mitochondrial and peroxisomal fission-1 EMPF1 and optic atrophy 5 (autosomal dominant form of nonsyndromic optic atrophy). (202)

1.4.4 Fusion-Fission Balance

Mitochondrial fission and fusion play pivotal roles in maintaining optimal mitochondrial function. Under normal physiological conditions, the equilibrium between these processes regulates the shape of the mitochondrial network. The state of balance between the four dynamin-related proteins controlling fusion and fission determines mitochondrial morphology. Complete network fragmentation can result from the increased expression or activation of fission proteins. However, the mitochondrial network will also fragment if the fusion activity is inhibited (203, 204).

Similarly, elongated tubular networks occur with enhanced fusion activity as well as through blockage of fission (123, 205, 206). These extremes are reminders that antagonism between counteracting enzymatic forces sets the shape of mitochondria and, therefore, must always be considered simultaneously when deciphering the network morphology.

This morphological adaptation of mitochondrial networks appears to align with the cellular metabolic requirements. For example, during periods of starvation, mitochondria adopt an elongated network structure to meet increased energy demands (206). Some studies have observed hyperfusion of mitochondria in response to acute stress-inducing agents, such as actinomycin D, cycloheximide, or oxidized glutathione, a phenomenon known as stress-induced hyperfusion. Conversely, excessive stress, such as ROS or exposure to fatty acids, triggers mitochondrial fragmentation, compromises mitochondrial structure, and potentially leads to the release of pro-apoptotic proteins, such as Cyt C and Smac/Diablo (207-209). However, it is important to note that fragmented mitochondrial networks do not always correlate directly with reduced mitochondrial bioenergetics (210).

Altogether, the structure of the mitochondrial network is dynamically adjusted depending on the balance between the fission and fusion proteins. It has been suggested that mitochondrial network integrity remains undisturbed when fission and fusion proteins are simultaneously depleted. Nonetheless, research has shown that the genetic alteration of fission (DRP1) and fusion (MFN1/2) proteins leads to premature death and are associated with various diseases (211). This emphasizes the significance of mitochondrial dynamics for cellular health and well-being.

1.4.5 The role of Mitochondria-ER contact sites in Mitochondrial Dynamics

The ER communicates with mitochondria through (MERCs) in yeast and mammals, and these connections have been shown to participate in different physiological functions, such as phospholipid synthesis, Ca^{2+}-mediated signal transduction, protein import, mitochondrial distribution, mitophagy, and so on (212-214). It is also reported that some of the mitochondria-shaping proteins are involved in this intercommunication. The mitochondrial profusion protein MFN2 is localized on both mitochondria and the ER, and MFN2 on the ER associates with MFN1/2 on mitochondria and tethers the ER and mitochondria together to maintain the efficient mitochondrial Ca^{2+} uptake, which is essential for ATP production (215). Nagashima et al. presented a novel hypothesis that MARCH5 regulates ER and mitochondria tethering by ubiquitination of mitochondrial MFN2 and activation of MFN2 GTP binding ability. The activated MFN2 oligomerizes with the ER-localized MFN2 and apposes the mitochondria to the ER (216).

As described previously, the ER has been also shown to play an active role in mitochondrial dynamics. During fission, ER tubules were observed to wrap around mitochondria, marking the prospective sites of mitochondrial division and reduce the mitochondrial diameter by about 30% before DRP1 recruitment, which is to be followed by mitochondrial division (133). Recently, similar to fission, the ER is also observed at the sites of fusion (168, 169). However, it is still unclear to what extent ER tubules are essential for mitochondrial fusion.

Besides the ER, the actin cytoskeleton is also involved in regulating of mitochondrial fission (217, 218). The cycling of actin assembly and disassembly around mitochondrial subpopulations efficiently promotes local mitochondrial fission dependent on DRP1. The elaborated understanding of Actin and its regulatory role on mitochondrial dynamics and its function is provided in the following sections.

1.4.6 Mitochondrial Dynamics on Apoptosis

Mitochondrial dynamics are crucial for regulating apoptosis and determining the balance between cell survival and programmed cell death. The equilibrium between mitochondrial fusion and fission processes impacts cellular response to apoptosis, where interconnected mitochondrial networks resist apoptosis, while fragmented mitochondria increase susceptibility to apoptotic stimuli (219, 220). The interaction with internal apoptotic pathway releases apoptotic factors, such as Cyt C, Smac/Diablo, and Omi, from the IMS to the cytosol and subsequent activation of caspases leading to the activation of cell death pathways (209, 221-223).

Mitochondrial fusion prevents apoptosis by preserving cristae integrity (192), while a shift to mitochondrial fission signals activation of the apoptotic pathway. Bax and Bak, crucial BCL-2 family proteins, regulate mitochondrial outer membrane permeabilization (MOMP) triggering the formation of the apoptosome and subsequent activation of caspases, which execute the apoptotic program (149, 209, 224, 225)

Furthermore, the mitochondrial fission protein Fis1 physically interacts with ER-located Bap31, inducing Bap31 cleavage into pro-apoptotic p20Bap31. The Fis1-Bap31 complex bridges the mitochondria and the ER and signals for apoptosis. This signalling pathway establishes a feedback loop by releasing Ca^{2+} from the ER that activates the mitochondria for apoptosis. Additionally, the dysregulation of key regulatory proteins such as MFF, MFN1, MFN2, OPA1, and DRP1 during apoptosis has been reported, highlighting the intricate relationship between mitochondrial dynamics and the apoptotic pathway (226).

The crosstalk between mitochondrial dynamics and apoptosis is a complex and multifaceted process, where dysregulation of mitochondrial dynamics is closely correlated with apoptosis, and the equilibrium of mitochondrial dynamics is critical in the regulation of apoptosis.

1.4.7 Mitochondrial dynamics and cellular metabolism

Mitochondrial dynamics affect not only cell death pathways but also influence cellular processes. It is believed that elongated mitochondria are more active and produce more energy (ATP), whereas short and fragmented mitochondria are weak and have reduced ATP production, suggesting that mitochondrial energy metabolism is linked to mitochondrial dynamics (227, 228). Research suggests that mitochondria-shaping proteins affect cellular metabolism, with MFNs regulating ROS production and energy metabolism (118, 229). MFN2 also regulates the expression of subunits in OXPHOS complexes, with loss-of-function reducing subunits in complexes I, II, III, and V and overexpression of subunits in complexes I, IV, and V. In addition to MFNs, OPA1 is involved in the regulation of the mitochondrial energy status. The level of OXPHOS can regulate IMM fusion through Yme1L, which cleaves OPA1 more efficiently under high OXPHOS conditions to promote IMM fusion, whereas the OMM is insensitive to OXPHOS levels (227).

On the other hand, knockdown of the fission protein DRP1 in HeLa cells decreased cellular ATP content and elevates ROS levels (229, 230). ROS production simultaneously regulates both DRP1-mediated fission and fusion events in mitochondria. Metabolic diseases, such as type 2 diabetes and obesity, are associated with abnormalities in mitochondrial division and fusion, involving core mitochondria-shaping proteins such as DRP1, OPA1, and MFN2. High blood glucose levels increase DRP1 activity in mitochondria, leading to more fission and ROS production as a by-product (231). Dysregulation of mitochondrial dynamics and shift in metabolic pathways are well reported as a common phenomenon in various cancer studies (67, 99). Altogether, mitochondrial dynamics exert a profound influence on cellular metabolism to maintain cellular homeostasis.

1.4.8 Mitochondrial dynamics in cellular nutrient sensing.

Another important role of mitochondrial dynamics is in cellular nutrient sensing and providing a suitable metabolic response. These changes were, to a large extent, interpreted in the context of quality control activation (232) Nonetheless, it is now suggested as an adjustment to variations in ATP demand and supply. When cells are grown under nutrient deprived conditions like starvation, they exhibit an elongated mitochondrial network and maximize energy production efficiency (16, 205, 233). Conversely, exposing cells to excess nutrients caused mitochondrial fragmentation with a decrease in ATP synthesis efficiency (less ATP per molecule of nutrient oxidized) (205, 233,

234). This comparison strengthens the theory that mitochondrial dynamics are influenced by cellular nutrient availability and play an active role in changing the mitochondrial bioenergetic efficiency and capacity.

1.5 Extracellular Matrix

As described in the previous section, regulation of mitochondrial bioenergetics is largely affected by changes in the cellular microenvironment. The cellular microenvironment forms a primary niche where cells rely for their support and survival. The microenvironment refers to the surroundings of a cell, including neighboring cells, the extracellular matrix (ECM), and chemical signals.

A major component of the cell microenvironment, the ECM, provides structural support to cells and helps regulate various cellular processes including cell adhesion, migration, and tissue development. The key role of the ECM is to provide survival signals under anchorage-dependent conditions. It offers stability to the cells by withstanding many forces acting upon it including gravity, external mechanical forces and internal cell tension, while remaining elastic enough to provide tissue integrity and support for cell movement (156).

1.5.1 ECM Composition

There is an intricate network of macromolecules that surrounds cells and anchors them in tissues. This matrix is composed of a variety of highly glycosylated proteins that are secreted locally and assembled into an organized meshwork in direct association with the surface of the cells that produced them. Proteins in the ECM are normally grouped as glycoproteins, proteoglycans and collagens (235) (Figure 1.6.1).

Collagens are he most abundant protein in the ECM and this protein gives the matrix its strength. Collagens are characterized by their unique triple helix structure, composed of three polypeptide chains known as alpha chains known as collogen domains. Collagens are commonly associated with glycoproteins or proteoglycans that contain one or more collagenous domains. Dependent on the requirement of the tissue, collagens can be aligned as bundles to bear tensile stress (e.g.in tendons) or be arranged as meshwork with incorporated proteoglycans acting as water cushion (e.g. in cartilage). ***Glycoproteins*** occur in the ECM that confer functionality to the matrix in different tissues. Glycoproteins contain domains that interact with other ECM components during ECM assembly, they also possess domains that interact with cells. Major glycoproteins include elastin, laminin, fibronectin and tenascins. Elastin allows tissue to stretch and reform after movement, an important property in the ECM of veins and arteries (236). Laminin and fibronectin

are major components of the basal lamina. These glycoproteins make the ECM adhesive; binding to other ECM proteins such as collagen, and interacting with cells via integrins (237, 238).

Proteoglycans are glycoproteins with unbranched highly charged sulphated glycosaminoglycan (GAG) side chains (235). These chains are strongly hydrophilic and hence are most abundant in those tissues where the ECM is highly hydrated with collagen fibres. GAGs are linear, charged polysaccharides consisting of repeating pairs of sugars, one of which is an amino sugar. The negatively charged GAGs act as space fillers within the ECM. Around 40 proteoglycans are identified, which are divided into families based on their structure. Certain proteoglycans, such as decorin, consist of only one GAG chain, while others, like aggrecan, can have over 100 chains.

Nonsulphated GAG: In contrast to the GAGs found in proteoglycans, these GAGs are not bound to a protein core. Hyaluronic acid, the most prevalent unsulfated GAG, is a linear polysaccharide consisting of as many as 10,000 disaccharides. It possesses a high anionic charge and is a critical factor in tissue hydration. It is found in the ECM, on the cell surface, and within the cell (239, 240).

1.5.2 Cell-ECM interactions

The cell-ECM interactions are mediated via cell surface receptors either directly or indirectly with co-operative molecules. (235, 237, 238, 241).

Direct interaction: the ECM macromolecules interact via integrins or certain other types of cell receptors such as a receptor for GAG hyaluronan (HA), namely CD44, and syndecans.

Indirect interaction: ECM macromolecules co-operate concurrently with several receptor molecules and growth factors.

Cell-ECM interactions are mediated directly or indirectly via cell surface receptors by binding to the specific ligands of the ECM. Integrins are the major class of ECM receptors in humans. Upon binding of a ligand to the extracellular portion of integrins, a structural rearrangement occurs, leading to the rapid assembly of integrin adhesion complexes (IACs) to the receptor's cytosolic region (242, 243). The maturation of the multimolecular complex involves the recruitment of proteins that physically link the integrin to actin, like talin and vinculin, but also signaling molecules, like focal adhesion kinase (FAK), extracellular signal regulated kinase (ERK), proto-oncogene tyrosine kinase Src (c-Src) and Rho family GTPases (244), enabling downstream signaling. Integrin signaling is bidirectional, involving both outside-in and inside-out signaling.

Further, the reciprocal nature of the cell-matrix interaction also allows cells to reorganize the ECM. For instance, cellular traction forces exerted on the ECM can lead to the organization of collagen fibrils into sheets and cables, influencing the alignment and tensile strength of the matrix

(245). Therefore cell-ECM interaction is a crucial aspect of cellular function, mediating various processes such as adhesion, signaling, and mechanotransduction. Various pathways can be activated during cellular interaction with the ECM. In this context, I will brief on the Integrins and their associated signaling pathway, focusing on actin polymerization to remain within the confines of my study.

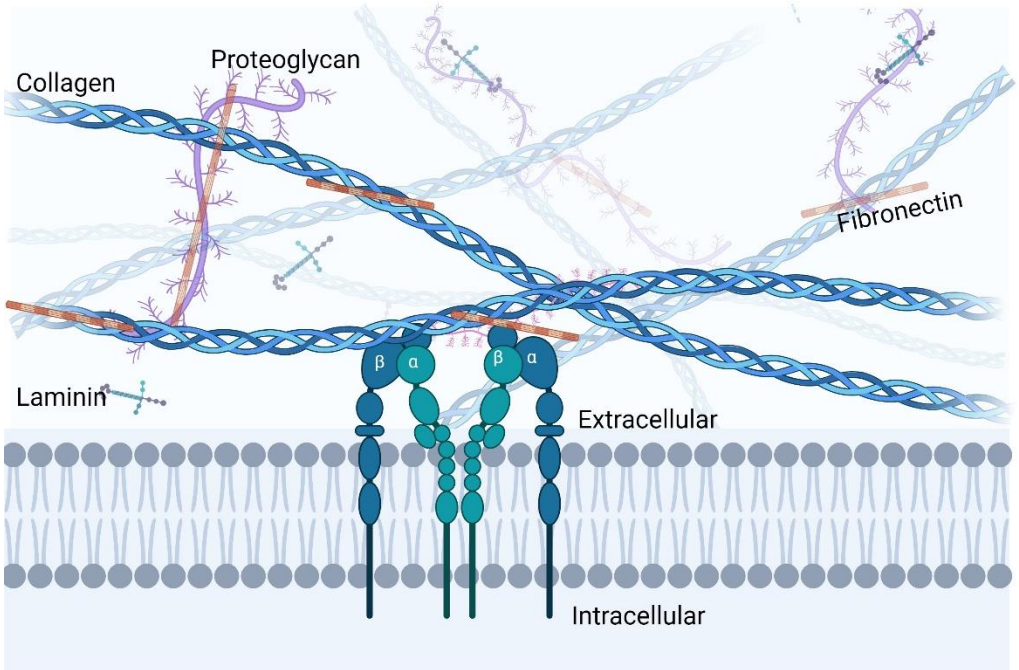


Figure 1.5.1: Overview of the extracellular matrix
The proteins fibronectin, collagen, and laminin contain binding sites for one another, as well as binding sites for cell surface receptors such as integrins. The figure was generated using biorender inspired from (245).

1.5.3 Integrins

Integrins are transmembrane heterodimers made of α and β subunits containing large extracellular domains that bind to the ECM and an intracellular domain which interacts with cytoskeleton proteins. In humans, integrins family consists of 24 heterodimers formed from combinations of 18 α and 8 β subunits, each with a specific but somewhat overlapping ligand binding specificity (247). After the cell receives mechanical information from the outside, the integrin tails undergo conformational changes that facilitate binding to focal adhesion complexes (Talin, vinuculin, FAK, etc.) and promote the active conformation of the integrin. After activation, the cytoplasmic domain of integrins binds IACs and stimulates the formation of new adhesions. The new focal complexes are formed at the leading edge and some of them eventually mature into focal adhesions by recruiting additional proteins to site. This transition depends on formation of actin bundles and their contractility, which applies force at cell-matrix adhesions. Thus, focal adhesions can only

form on a mechanically stable (stiff) substrate. Integrins can also be activated from inside the cell. This is achieved via the interactions between different cytoplasmic factors and the cytoplasmic domain of integrins. The phenomenon is called inside-out signaling (248) (Figure 1.5.2). For example, Talin can transduce signals from the cytoplasm, activating integrins, that increases the adhesion potential of the cells.

Most integrins are known to bind to several different ECM macromolecules. The integrin receptors always contain one α chain and one β chain and each α β combination has its own binding specificity and signaling properties, such as α2β1 integrin designated as the major receptor for fibrillar type I collagen, while α5β1 is a typical Fibronectin (FN) receptor (238, 241).

As the external domain of the integrins bind to their ligand, they change from an inactive to an active configuration and become clustered in the plane of the cell membrane. This is accompanied by the transduction of signals into intracellular signal transduction pathways that mediate a number of intracellular events.

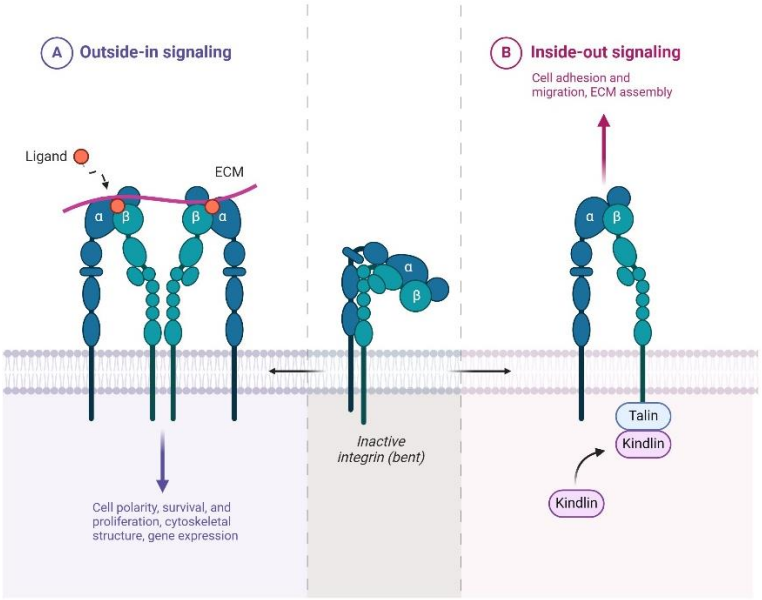


Figure 1.5.2: Integrin Activation and Bidirectional signaling

The inactive Integrins have a bent conformation and low affinity. The open conformation with separated cytoplasmic leg domains having high affinity, is an activated integrin. A) Extracellular domains of resting integrins open to bind extracellular matrix ligands. This leads to "outside-in" signaling of integrins, resulting in cytoskeletal remodeling and downstream signaling cascade. B) Agonist stimulation, signals from cytoplasmic proteins receptors can lead to "inside-out" signaling of integrins, for example, Talin can transduce signals from the cytoplasm, activating integrins, that increases the adhesion potential of the cells. The figure was generated using biorender inspired from (248).

1.5.4 The FAK/Src Pathway

Integrins activate various protein tyrosine kinases, including focal adhesion kinase (FAK), Src family kinases, Abl, and a serine-threonine kinase, integrin-linked kinase (ILK) etc. In this section, I provide an overview of integrin-dependent pathways concerning FAK and Src-family kinases, pertinent to my research.

FAK is a protein tyrosine kinase that is composed of a N-terminal FERM domain, a central kinase domain and a C-terminal focal adhesion-targeting (FAT) domain. Upon ligand binding to the integrin receptors, clustered integrins initially promote recruitment of FAK to adhesion site via interaction with integrin-associated proteins such as talin and paxillin. Ligand-induced integrin clustering thus brings FAK molecules together and allows FAK trans-autophosphorylation at Tyr397, which becomes a high affinity binding site for the Src homology 2 (SH2) domain of Src tyrosine kinase. The now activated Src can in turn phosphorylate FAK at the kinase domain (tyrosines 576 and 577) and C-terminal domain (tyrosines 861 and 925). By these phosphorylations, FAK becomes both an active kinase and an adaptor protein for recruitment of additional proteins (237, 248). The activated FAK-Src activates downstream proteins. For example, p130CAS activated by FAK-Src complex through phosphorylation binds to Crk (CT10 regulator of kinase), leading to Rac-1 activation and lamellipodia formation (249) (Figure 1.6.3). These changes influence cytoskeletal re-organisation, gene expression, cell proliferation, and adhesion (237, 238).

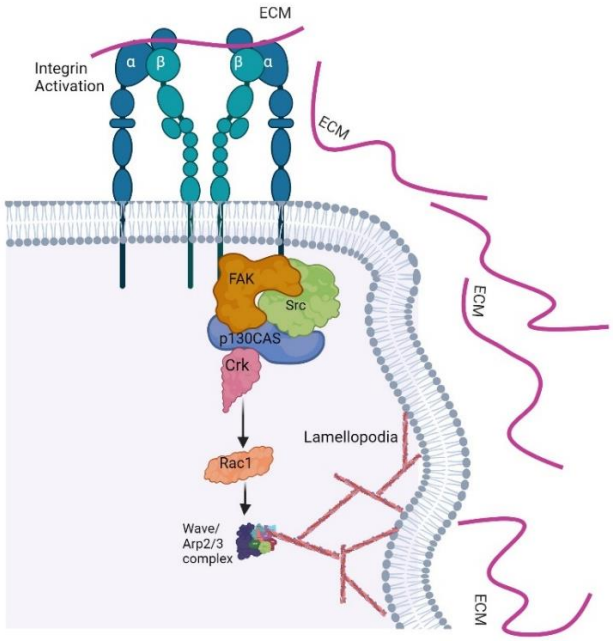


Figure 1.5.3: Integrin signalling cascade

Upon extracellular matrix binding to integrins, they obtain open conformation, activating the outside-in signalling cascade. Integrin stimulates phosphorylation FAK at Tyr39 and in a Src-dependent manner, further activating p130Cas which has been known to be activated during cellular invasion and migration in ovarian cancer cell. p130Cas recruits' proteins that activate downstream pathways (Rac1, Wave-Arp2/3 complex), resulting in actin cytoskeleton re-organization promoting lamellipodia formation assisting in cell migration The figure was generated using biorender inspired from (249, 250)

1.5.6 Mechanotransduction: Factors affecting Cell-ECM Interactions:

The initial interaction between integrins and the ECM is independent of force (251) but from then onwards, the formation of IACs and the resulting signaling is force dependent. The mechanical properties of the matrix affect integrins in multiple ways. It affects integrin-ligand binding kinetics; conformation and activation; clustering and diffusion; and trafficking and subcellular localization (252). The maturation of nascent adhesions into larger focal adhesions is force-dependent (251, 253) and different integrin heterodimers are differentially regulated by force (254, 255). This means that integrin-mediated mechanotransduction is influenced by integrin expression patterns as well as by the ECM composition. Alongside the force dependent strengthening of ECM-integrin bonds and adhesion complex maturation, the cytoskeleton is also stiffening in proportion to the applied force (256). The actin filament system contributes the most to cell tension, maintained by actomyosin filaments and balanced by microtubules and the ECM-connected IACs (257). The cellular response to mechanical stress depends on the global structural alterations in the cell's cytoskeleton. The factors affecting these responses are:

ECM Stiffness: Cells sense the substrate elasticity by exerting traction forces at the anchorage points (focal adhesions) and sensing their displacement as a function of the applied force. If the substrate is stiff, it may not move or be deformed; therefore, all the generated tension will remain within the cell and promote cell spreading. In contrast, a soft substrate can be easily deformed, which causes cells to generate much less internal tension within the cytoskeleton and less polymerisation in stress fibres which provokes cells to appear round and small (219).

Mechanical stress: Cells exert force on the materials they adhere to and sense the resulting displacement in a process known as mechanosensing. The mechanisms by which cells identify and process this mechanical information into chemical signals depend on its nature and the structures involved (ion channels, G-protein coupled receptors, integrins and protein kinases). For instance, In the bone, the osteocyte is the mechanosensor that transforms mechanical information (shear stress and stretch, modifications in extracellular pressure and strains) into biochemical signals, directing osteogenesis; osteocytes detect microcracks and promote bone remodelling (258).

1.5.7 Impact of the ECM on Mitochondrial function

As previously described, the focal adhesion sites pair intracellular actin fibers to the ECM via integrin activation during cell-ECM interactions. At these sites, mitochondria has emerged as a critical factor, by conducting localized ATP generation to allow formation and maturation of focal adhesions (259). Furthermore, a link between the mechanical properties of the cell microenvironment and mitochondrial dynamics has been recently reported, suggesting ECM stiffness and composition affecting mitochondrial morphology (219). Despite evidence linking mitochondrial function and the ECM, the mechanism by which ECM properties affect mitochondrial function remains elusive. Investigating specific ECM proteins involved in regulating mitochondrial function can elucidate cellular mechanisms behind this link.

1.6 The Actin Cytoskeleton

We have now studied that ECM-driven Integrin-dependent pathway promotes actin cytoskeleton-mediated intracellular signaling. *So, what is Actin?* Actin constitutes one of the three filamentous components that make up the cytoskeleton. The other two, intermediate filaments and microtubules, will not be discussed in the context of this thesis.

Actin, a monomeric protein with a molecular weight of approximately 42 kDa, is abundantly present in all eukaryotic cells, with concentrations ranging from 50μM to 200μM in mammalian cells (3, 260, 261). Initially discovered in muscle and purified in 1942, actin is considered one of the most prevalent proteins in eukaryotic cells, exhibiting evolutionary conservation within the cytoskeleton of diverse taxonomic groups (262-265). There is even a suggestion that actin, tubulin, and histones may share a common ancestral protein, based on their shared behavioral characteristics (266).

The ability of cells to migrate as a whole, or move subcellular components, is essential for cell growth and proliferation. Forces generated by the actin cytoskeleton power these diverse motility processes. To generate a pushing force for protrusion, the cell uses the energy of actin polymerization. Polymerization of individual actin filaments produces piconewton forces (263, 267), and filaments are organized into parallel bundles in filopodia or branched networks in lamellipodia to produce large forces. Hence it can produce pushing (protrusive) forces through coordinated polymerization of multiple actin filaments or pulling (contractile) forces through sliding actin filaments along bipolar filaments (268). Moreover, the actin cytoskeleton defines the cell shape and mechanical properties and serves as a scaffold for intracellular protein and cargo transport (269).

In humans, there are a total of six actin genes, with four of them belonging to the α-actin group found in various muscle types, while the remaining two, β-actin and γ-actin, are non-muscle variants (263, 268).

Actin exists in two distinct forms: *monomeric actin,* known as globular actin or G-actin, and ***filamentous actin***, referred to as F-actin. Maintaining a precise balance between these two actin forms is crucial to meticulously control cytoskeletal dynamics and responding to extracellular signals effectively (270). Actin as a monomer exhibits limited ATPase activity, its efficiency in ATP hydrolysis significantly increases upon polymerization into filaments (271). Actin monomers form tight interactions with ATP and ADP, provided there are calcium ions (Ca^{2+}) or magnesium ions (Mg^{2+}) present (272).

The actin filament is produced with a process termed actin treadmilling, the kinetics of which are thermodynamically unfavorable (273). Nonetheless, once oligomers are established, spontaneous actin assembly becomes feasible, as the ATPase activity of actin is enhanced during polymerization (274). In this process, actin monomers predominantly attach to the barbed (+) end of the filament in the ATP-bound state and tend to dissociate from the pointed (−) end, primarily in the ADP state (see Figure 1.6.1). More specifically, the newly formed segment of the filament predominantly contains ADP-Pi-bound actin with an ATP-bound unit cap at the tip, while older filaments consist mainly of ADP-bound actin. The ADP-Pi actin is structurally similar to ATP-actin, and both are more stable than ADP-bound F-actin (274, 275).

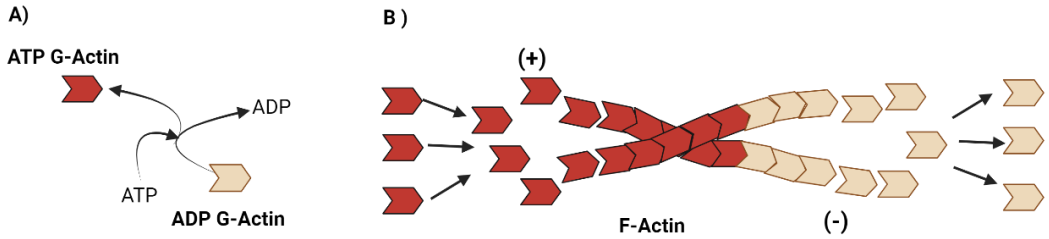


Figure 1 .6.1: Monomeric and filamentous Actin
A. Actin has ATPase activity and the conversion of G-actin from ATP- to ADP-bound and vice versa is a dynamic process in the cell. B. Actin treadmilling. The conversion of the ADP- to ATP-bound actin ensures the formation of actin filaments at the barbed (+) end of the filament. This occurs in a speedy manner. The conversion of the ATP- to the ADP-bound actin leads to the monomers separating from the filament at the pointed (-) end. This process is much slower. The figure was generated using biorender inspired from (276)

Within cells, actin filaments constitute the structural framework of the actin cytoskeleton. These filaments exhibit polarity and take the form of double-stranded helical polymers (7nm in diameter)

(272), and each actin subunit contributes an additional 2.7 nm in length to the filament (263). Actin polymerization involves two distinct phases: nucleation and elongation. Nucleation necessitates the sequential dimerization and trimerization of actin monomers, with both steps being energetically unfavorable. Once the trimeric actin "seed" forms, it can undergo rapid elongation to create the actin filament. Consequently, actin filament assembly is primarily constrained by the nucleation phase, with dimerization being the least favorable step (277). During the elongation phase, the barbed end of the filament displays higher dynamism, with on-rates and off-rates approximately ten times faster than the pointed end (268). Many cellular actin filaments turn over constantly to remodel actin-based structures according to changing needs. In here, regulatory proteins control all aspects of actin filament dynamics. Actin-binding proteins (ABPs) assemble most actin filaments into networks and bundles adapted to specific tasks and accessory proteins allow actin filaments to act in association with cellular membranes (263).

A noteworthy characteristic of actin is its ability to swiftly switch between monomeric and filamentous states, enabling the rapid assembly and disassembly of extensive filament networks within seconds to minutes (152, 278, 279). Consequently, to achieve rapid and precise mobilization of the actin cytoskeleton for specific functions, cells employ a multitude of actin-associated proteins that govern the location and structure of actin networks. Furthermore, there are additional GTPases and G-and F-actin binding proteins (ABPs), playing pivotal roles in governing the precise manner in which actin filaments are formed (discussed later).

1.6.1 Actin cytoskeleton structures

The polymerized actin filaments can further organize into different types of architectures: branched and crosslinked networks, parallel bundles, and anti-parallel contractile structures (Figure 1.6.2). These structures can act as mechanical elements to drive cell shape changes and motility.

Branched Actin Networks represent self-assembling cytoskeletal structures (280) facilitated by the coordinated interactions of various key components, including nucleation promoting factor (NPF), the Arp2/3 complex, capping protein (CP), filamentous actin, and monomeric actin bound to profilin (281-283). These networks play an indispensable role in generating compressive forces required for numerous cellular processes, such as the protrusion of leading edge membranes in migrating cells (137), the repair of cell ruptures (284), endocytosis (short actin patches) (285), phagocytosis (241) and the formation of tight cell adhesions (286).

Crosslinked actin networks are formed when crosslinker proteins connect pre-existing polymerized actin filaments to create complex macroscopic structures (267, 287, 288). Small crosslinkers like fimbrin or fascin create close-packed filament bundles, while larger ones like filamin or α-actinin form bundles, networks (288-290) or long filaments (267). These networks maintain cell shape and mechanical integrity (287, 291, 292). Crosslinked actin networks, along with branched ones, are present in extensive, sheet-like lamellipodial structures (137) essential for two-dimensional cell motility.

Parallel actin networks consist of actin filaments oriented with their barbed ends aligned in the same direction, typically facing the cell membrane, forming finger-like structures. Fimbrin, fascin, and α-actinin are the crosslinking proteins responsible for organizing these parallel networks and keeping the actin filaments in close proximity (293, 294). Two mechanisms have been proposed for initiating bundle formation: one involves the Arp2/3 complex, and the other utilizes proteins such as formins or Ena/VASP (281, 295, 296). These networks are found in structures like filopodia and microvilli (292, 295, 296)

Antiparallel actin networks play a significant role in processes like cytokinesis and the formation of stress fibers, which connect the cell's cytoskeleton to the extracellular matrix through focal adhesion sites (297-300). These networks involve myosin-driven contraction and can be stabilized in their antiparallel arrangement by crosslinking proteins like fimbrin and α-actinin (299).

As important as the construction of actin filaments and networks is, their controlled disassembly also holds equal importance. It's crucial to distinguish between disassembly and depolymerization; depolymerization occurs at the filament ends (301), while disassembly essentially disrupts the structural integrity of the actin networks (302, 303). Cofilin employs fragmentation or severing techniques to dismantle actin structures and can also bind to actin monomers, inhibiting nucleotide exchange (301, 303). Additionally, myosin can play a role in filament severing, with actin bundles either sliding along myosin-coated surfaces at low myosin density or undergoing disassembly at high myosin density (304).

1.6.2 Proteins controlling actin dynamics.

To regulate the processes of actin polymerization and depolymerization, cells depend on a variety of actin-binding proteins (277, 305). They include nucleation factors (enable filament creation), elongation factors (promote the extension of filaments), and depolymerization factors (accelerate filament disassembly). Furthermore, several of these proteins can essentially prevent spontaneous nucleation (e.g., profilin, thymosin) and elongation (capping protein).

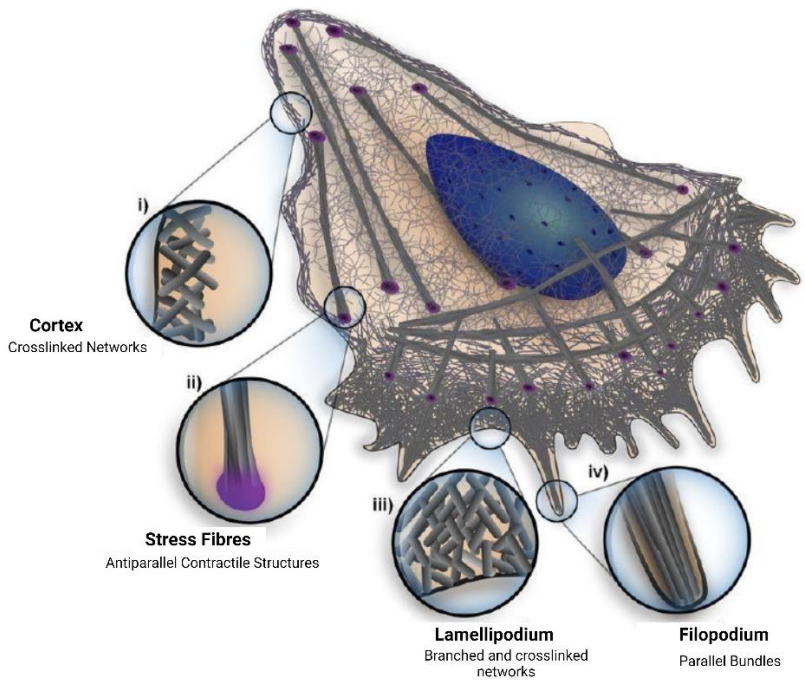


Figure 1 .6.2: Actin networks
i) crosslinked structures in cortical actin, ii) antiparallel contractile structures in stress fibers, iii) branched and crosslinked structures in lamellipodia and iv) parallel structures in filopodia. The figure was generated using biorender inspired from (291).

1) ***Nucleation factors***, initiate the formation of new filaments from actin monomers. Cells rely on regulatory proteins to initiate actin filament polymerization in a controlled manner. These Nucleation factors comprise of 3 important classes:

a**. Arp2/3 complex:** "Arps" are actin related proteins which produces branched actin filaments. Arps, originated early in the evolution of eukaryotes when the ancestral actin gene underwent repeated duplication and diversification, resulting in the emergence of multiple protein families (306). These Arps exhibit sequence similarity to actin, ranging from 17% to 52%, and are numbered Arp1 to Arp11 based on their divergence from actin. Arp2 and Arp3 are part of the Arp2/3 complex, along with five additional subunits (ArpC1-5). The complex itself is inactive and requires two activators: 1) a NPF, and 2) the side of a pre-existing filament.

In the presence of NPF, the Arp2/3 complex binds to the side of an actin filament, which results in a conformational change, with Arp2 and Arp3 moving closer together, forming the base for the growth of a branch (307). The free barbed end of the new filament elongates, whereas the Arp2/3 complex anchors the pointed end of the filament firmly to the side of the existing filament, creating characteristic 70° actin filament branches (308, 309). This actin network is termed as the dendritic nucleation model. An alternative mechanism for branch formation, known as the 'barbed-end

nucleation' model, has been proposed. In this model, the Arp2/3 complex binds to the free-barbed end of a filament, and two filaments subsequently sprout from the branch (310, 311). Regardless of the method of formation, actin filament branches exhibit substantial rigidity and stability for several seconds. Cortactin is another Arp2/3 complex-binding protein that reinforces the branches (312) (Figure 1.6.3).

b. **Formins,** represents a diverse family of 15 proteins in mammals (313) that promote nucleation and remain at the barbed end during elongation (314) Structurally, formins are characterized by their formin homology (FH) domains 1 and 2. Most formins exist as dimers, with the FH2 domains governing nucleation and moving persistently along the elongating barbed end of an actin filament. FH1 domains bind profilin-bound actin monomers, facilitating their transfer onto the growing filament. A significant distinction between formin-mediated actin assembly and Arp2/3 complex-mediated assembly lies in the fact that formins generate linear actin filaments (Figure 1.6.3).

c. **Tandem WH2 domain-containing proteins**. The precise mechanism underlying actin assembly for this category of actin-polymerizing factors remains inadequately elucidated, but their function involves the promotion of nucleation and elongation (315). Some examples of such factors include Spire1/2, Cordon Bleu (Cobl), and leiomodin (Lmod) (Figure 1.7.3).
It's worth noting that a mitochondrially-localized isoform of Spire1, Spire1C, has been documented to participate in calcium-triggered mitochondrial division in conjunction with the formin INF2 (134).

2) ***Elongation factors*** either facilitate or inhibit barbed end growth of an actin filament (268). Barbed ends grow much faster than pointed ends, with a critical concentration for polymerization of about 0.1 μm, while pointed ends exhibit slower association and dissociation rates for ATP–actin. The proteins regulating elongation are capping proteins, Formins and Ena/VASP proteins.
a. **Capping proteins** bind tightly to barbed ends and stops elongation. The capping protein (CP) is a hetero-dimer and is expressed in all eukaryotes (263). Capping proteins can be counter-acted by the next two protein classes.
b. **Formins**. After nucleating a filament, the formin remains at the actively growing barbed end, permitting elongation even when capping proteins are present.
c. **Ena/VASP proteins.** Like formins, these proteins bind near the barbed end, antagonizing the effects of capping proteins. In mammals, there are three such proteins: VASP, Mena, and EVL. Ena/VASP proteins are distributed in focal adhesions, stress fibers, lamellipodia, and filopodia (281, 316).

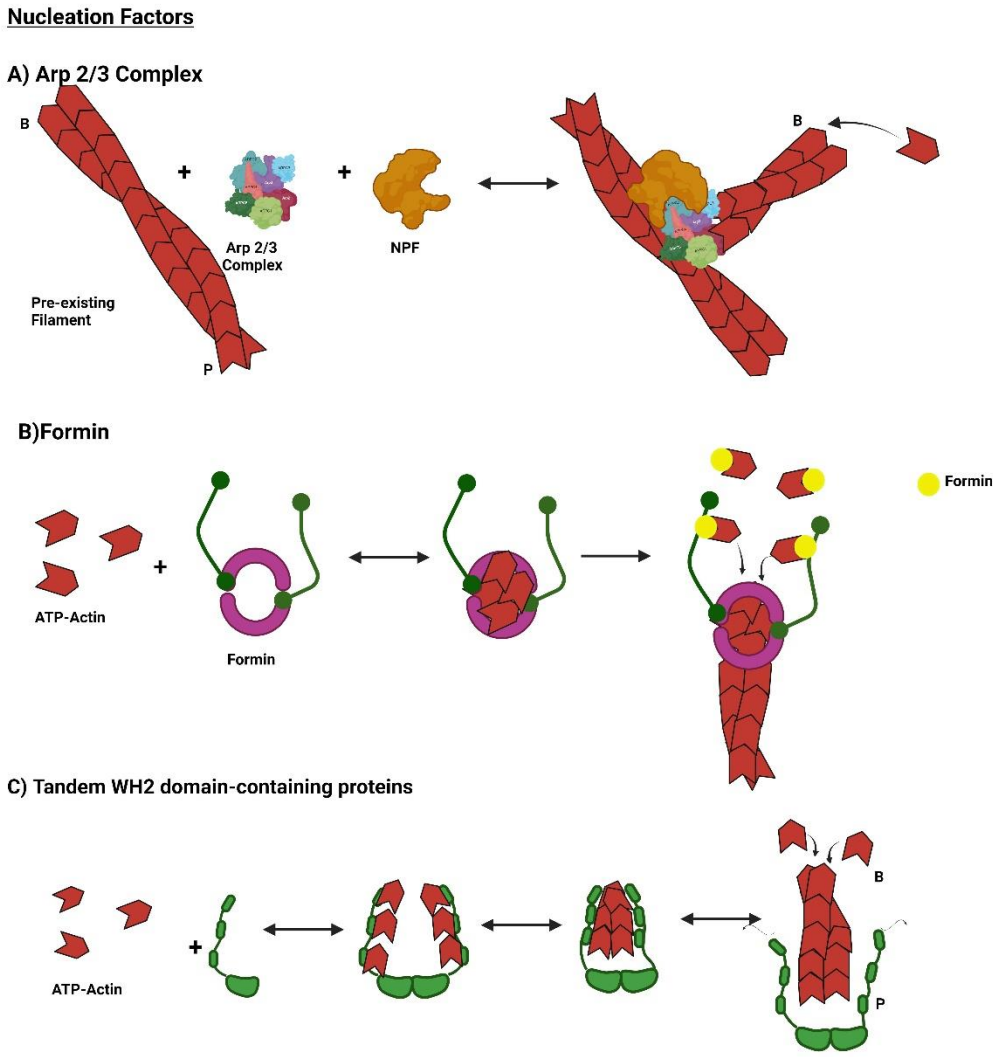


Figure 1 .6.3: Actin nucleation and elongation factors
A) Arp2/3 complex-mediated nucleation, requires NPF and an existing actin filament for activation. After activation, a branched filament is formed producing a B - barbed end. P - pointed end is attached to the existing branch B) Formin-mediated nucleation, with the formin remaining at the barbed end, and working with profilin in filament elongation. D) Tandem WH2 motif-containing protein nucleating a filament, first accumulates the monomer G-actin at their motifs and later adds monomer at the barbed end generating filaments. The figure was generated using biorender inspired from (305, 311).

3) ***Depolymerisation factors*** like

a. **Cofilin** preferentially binds to sections of actin filaments containing ADP (adenosine diphosphate), typically in the vicinity of the pointed end (263, 301). The severing action mediated by Cofilin in these regions often plays a pivotal role in filament depolymerization. However, there are instances where Cofilin-mediated severing actually promotes actin polymerization by generating new barbed ends. Cofilin-dependent depolymerization can be prevented by Cofilin

phosphorylation, as it inhibits its ability to bind to actin (317, 318). There are three mammalian cofilins: Cofilin1, Cofilin2 and Destrin. Cofilin1 may have a role in modulating mitochondrial dynamics in regulating mitochondrial fission (319, 320).

b**. Gelsolin** is an actin-capping protein, which, by attachment to the growing end of F-actin, prevents further monomer binding and eventually depolymerizes actin filament. Gelsolin (GSN), exists as cytoplasmic (cGSN) and secretory plasma (pGSN) isoforms (321). cGSN exists in the mitochondrial fractions and preserves mitochondrial integrity by blocking the loss of mitochondrial membrane potential (322). pGSN works as part of extracellular actin-scavenging system, a homeostatic mechanism responsible for depolymerization of actin and its removal from circulation(323).

4) ***Nucleation preventing factors,*** proteins that prevent actin nucleation.

a. **Profilin**, a small protein of 13 kDa, is highly abundant and known for its affinity for actin monomers. It facilitates the exchange of ADP for ATP on actin monomers, promoting the availability of ATP-bound actin for filament elongation. It binds to barbed ends of the unpolymerized actin, hindering nucleation and elongation at pointed ends while allowing elongation at barbed ends. Additionally, profilin exhibits low binding affinity to ATP–actin on the barbed end of filaments, leading to rapid dissociation of the profilin–actin complex, releasing the end for subsequent elongation (324, 325). Nevertheless, high concentrations of unbound profilin can slow elongation and even promote dissociation of the terminal subunit. On the contrary to its nucleation prevention, profilin has the capacity to bind to polyproline sequences in elongation factors, such as formins and Ena/VASP to promote elongation of actin filament barbed ends.

b. **Thymosins** are remarkably small proteins, with a size of approximately 5 kDa, that bind to actin monomers and essentially "sequester" actin, preventing it from nucleating or attaching to either end. In some cell types, thymosins have been found at higher concentrations than actin, while their role remains mysterious (326). Mammals possess at least three thymosins: thymosin β4 (encoded by the TMSB4 gene), the most extensively studied; thymosin β10 (TMSB10); and thymosin β15 (TMSB15).

1.6.3 Actin cytoskeleton regulators

Nucleation-promoting factors (NPFs):A range of proteins within the Wiskott-Aldrich syndrome protein family can activate the Arp2/3 complex and initiate actin polymerization (327). This protein family currently encompasses 16 members, including well-known members such as WASp, N-WASp, WAVE1-3 (WASP family verprolin-homologous), WASH (Wiskott-Aldrich syndrome protein and SCAR homolog), and newer additions such as WHAMM (WASP homolog

associated with actin, membranes, and microtubules) and JMY (327-329). They share a conserved C-terminal domain, termed the VCA or WCA (WASP homology 2-Connector-Acidic) involved in Arp2/3 complex activation, and a unique N-terminal domain that governs their assembly into macromolecular complexes, subcellular localization, and interactions with regulatory proteins (327). Various proteins involved in cytoskeleton signaling pathways, such as Rho GTPase Cdc42/Rac, regulate NPFs, such as WAVE and WASp (315, 330). Conversely, some NPFs can self-regulate through intramolecular interactions of WCA and other upstream domains of the NPF (328, 331). Activation of the Arp2/3 complex occurs when it binds to NPFs via the central/acidic regions, leading to F-actin nucleation. This diversity allows for precise spatial and temporal regulation of branched F-actin formation within the cell.

The WASp family proteins are also subject to different regulatory mechanisms. WASp and N-WASp are initially auto-inhibited by intramolecular interactions and require activation by upstream proteins. In contrast, WAVE proteins remain constitutively active and their regulation downstream of Rac relies on the formation of protein complexes involving intermediary proteins. These distinct regulatory modes correspond to different biological roles of these proteins (331, 332). For example, WAVEs are primarily involved in the creation of lamellipodial protrusions and circular ruffles (137, 333). In contrast, WASp and N-WASp play essential roles in the formation of specialized adhesive and invasive structures called podosomes or invadopodia, as well as in the initial stages of clathrin-dependent internalization and intracellular vesicle movement (327).

1.6.4 Actin and mitochondria coming together: what we know so far

Over the past two decades, there has been a substantial surge in interest concerning the relationship between actin and mitochondria. It is now well-established that actin plays a role in various aspects of mitochondrial biology, which we can categorize as follows:

1.6.4.1 Actin Cytoskeleton and Mitochondrial Dynamics

The role of actin in mitochondrial fission was first proposed almost 20 years ago (334). In recent years, researchers have identified key molecular players in this process. Specifically, the ER-anchored formin INF2 interacts with the actin nucleator Spire1C on the OMM, followed by polymerization of F-actin at the MERCs, causing pre-constriction of the OMM. This constriction is caused by the combined effects of ER, actin, and nonmuscular Myosin II. Actin polymerization mediated by INF2 is referred to as 'calcium-induced actin' because it is triggered by elevated levels of cytoplasmic calcium. Actin then stimulates the recruitment of DRP1 to mitochondria (136, 152, 335). DRP1 binds to actin filaments, and actin collaborates with mitochondrial receptors like MFF, MiD49/51, or Fis1 to enhance DRP1's GTPase activity (336, 337). This leads to constriction and

division of mitochondria through GTP hydrolysis. Interestingly, human cellular models lacking ABPs like Spire1C, Myosin II, and cofilin have revealed abnormalities in mitochondrial structure and altered DRP1 recruitment to the OMM (134, 135, 320, 338), suggesting its dual role in both the pre-constriction of the OMM and the recruitment of proteins involved in mitochondrial fission at the MERCs. Furthermore, it is worth noting that two distinct modes of mitochondrial division have been identified under stress induced conditions: "midzone division," which depends on the presence of associated ER and INF2, and "peripheral division," which does not require these factors (156).

1.6.4.2 Actin Cytoskeleton and Mitochondrial movement

In neurons, long-distance mitochondrial transport (339, 340) involves the attachment of mitochondria to microtubule motor proteins, specifically kinesins and dyneins, and the formation of a motor complex with the OMM GTPase Miro and the adaptor protein Milton/TRAK, which mediate mitochondrial movement (341, 342). This mitochondrial transport is regulated by intracellular calcium levels, where elevated calcium levels can hinder both kinesin-dependent forward and dynein-dependent backward movements (343). This regulatory mechanism is further influenced by the PINK1/Parkin pathway, which controls the turnover of Miro (344, 345), and nerve growth factor (NGF), resulting in the accumulation of mitochondria in the axon region and at the synapses. These processes involved PI3 kinase and actin (346). Recently, it was revealed that elevated cytoplasmic glucose levels also lead to post-translational modification of milton through N-acetylglucosamine (O-GlcNAcylation) (347). The modified milton subsequently recruits and binds to FHL2 (Four and a Half LIM Domains 2). Together, the Milton-FHL2 complex promotes the formation of actin structures around mitochondria, leading to a cessation of the microtubule-based movement (347).

Additionally, Miro play a pivotal role in recruiting and stabilizing the ABP Myosin19 (Myo19) in mitochondria (348, 349). In human cells, Myo19 regulates the equal distribution of mitochondria in daughter cells during mitosis (350). During interphase, Arp2/3 complex dependent actin filaments form ‘clouds’ around mitochondria, dynamically encircling around the cell. The presence of these clouds is associated with an increase in DRP1-dependent mitochondrial fission (351). Furthermore, during the mitotic phase, a distinct actin cloud emerges, characterized by a higher speed and increased actin density. This cloud completes a full revolution in 6 min, blocking mitochondrial motility (352). In a few instances, a mitochondrion manages to break free and propels away with an actin 'tail' (353). Although actin-driven motility does not play a critical role in achieving an overall symmetrical distribution of mitochondria during mitosis, it facilitates

mixing of mitochondrial populations. In yeast, the mitochondria undergo poleward movement before cell division, mirroring chromosome segregation. Actin-dependent mechanisms ensure equal segregation of mitochondria in both daughter cells (198, 354), leading to the process being called "mitokinesis (355).

1.6.4.3 Actin Cytoskeleton on mtDNA Expression and Maintenance

The 37 genes present in mtDNA genome are organized into dense DNA-protein complexes known as nucleoids (21). In yeast, the ERMES complex governs their stability and organization of mtDNA within nucleoids in an actin-dependent manner (354). In mammalian cells, MERCs, along with DRP1, are intimately linked to mitochondrial nucleoids, regulating their distribution (356), division, and active transport through microtubules (158). Although the presence of actin inside mitochondria has been debated, recent studies utilizing super-resolution microscopy have provided evidence of structures containing β-actin within the mitochondrial matrix (357). The absence of β-actin in human cells results in disruption of mtDNA mass and nucleoid organization due to stress induced by the loss of mitochondrial membrane potential (199). Additionally, Myosin II has been associated with purified mitochondrial nucleoids, and its suppression leads to mtDNA abnormalities (358). These findings imply that actin and actin-binding proteins are involved in the segregation of mitochondrial nucleoids and play a role in mtDNA transcription and maintenance.

1.6.4.4 Actin cytoskeleton and Metabolism

Actin filaments have also been identified as regulators of cellular metabolic pathways. For example, the direct binding of glycolytic enzymes, such as aldolase A or glyceraldehyde phosphate dehydrogenase, to F-actin can lead to their activation (359). Aldolase A remains inactive when trapped by the actin cytoskeleton, however, its release under PI3K signaling leads to its activation and increases aldolase activity, which in turn enhances glycolysis (360). Additionally, inducing damage to actin can drive glycolysis in normal, hypoglycemic and hypoxic (361) conditions.

Cells cultured in stiff ECM are subjected to high levels of physical tension, which leads to remodeling of their actin cytoskeleton. F-actin bundling and stress fiber formation due to actin cytoskeleton modeling spatially sequester the E3 ligase TRIM21(Tripartite Motif-containing Protein 21), which reduces its access to substrates such as phosphofructokinase (PFK). Consequently, glycolysis remains high. However, when cells are transferred to soft ECM conditions, the actomyosin cytoskeleton relaxes, releasing the ligase and allowing for the degradation of PFK, which in turn reduces glycolytic rates (362). In contrast, cancer cells on a soft ECM have also been demonstrated to generate actin stress fibers that increased DRP1 dependent mitochondrial fission and redox activity (219). On the other hand, a stiff matrix regulates

cytoskeletal activity through mechanical means and is dependent on OXPHOS activity(363). Recent studies have demonstrated that changes in actin structure can affect mitochondrial OXPHOS. Specifically, the rearrangement of mitochondrial actin filaments (mtF-actin) facilitated by fascin has been found to enhance mitochondrial OXPHOS by boosting the production of respiratory Complex I. During metabolic stress, fascin is directly recruited to mitochondria to enhance the stability of mtF-actin. Fascin and mtF-actin jointly regulate the balance of mtDNA, facilitating the promotion of mitochondrial OXPHOS (364). Altogether the impact of actin on mitochondrial metabolism is evident, but its regulatory mechanisms require further exploration and study.

1.6.4.5 Actin cytoskeleton in Apoptosis and Mitophagy

As discussed previously, we know that mitochondria plays a key role in cellular apoptosis (357). Increased OMM permeabilization and disorganization of mitochondrial cristae triggers the release of pro-apoptotic factors, such as Cyt C or AIF (Apoptosis-Inducing Factor) from the IMS into the cytosol. This leads to the formation of an apoptosome protein complex, caspase activation, and chromatin fragmentation (365). Actin is a caspase substrate, and its cleavage produces a 15-kDa fragment that can be N-myristoylated and targeted to mitochondria (366). This interaction regulates OMM permeabilization and ROS production. In yeast, monomeric actin interacts with voltage-dependent anion channels (VDAC) to modulate apoptosis (367, 368). Disruption of actin dynamics results in loss of mitochondrial $\Delta\Psi$, increased ROS, and cell death (369).

During apoptosis, ABPs such as cofilin lose their actin-binding ability and translocate to mitochondria, promoting Cyt C release (370-372). This process may be mediated by interactions with DRP1 (373), although binding to G-actin is sufficient to induce mitochondrial translocation (374). Oxidized cofilin is also translocated to mitochondria under oxidative stress, triggering Cyt C release and apoptosis (375). Other ABPs, such as gelsolin and villin, together target mitochondria in the early steps of the apoptotic pathway, controlling the trafficking of anti- and pro-apoptotic signals (371).

Furthermore, Peri-mitochondrial actin polymerization (N-WASP, Arp2/3 complex and formin(s) dependent) around damaged mitochondria leads to loss of $\Delta\psi m$ (361, 376, 377) and prevent fusion of damaged mitochondria with healthy ones (376). Myosin VI-mediated actin cages isolate damaged mitochondria from neighboring populations (376) and the caged mitochondria are sealed for destruction in mitophagy (377). Therefore, actin closely function along with mitochondria in organizing apoptosis and mitophagy.

1.7 Summary

From the literature, we have by now discussed the substantial contribution of the ECM to outside-in signaling, particularly in its role as a regulator of the actin cytoskeletal pathway. This observation underscores the intricate and multifaceted interplay between the ECM and various cellular processes.

Furthermore, as we delve deeper into research, it becomes increasingly apparent that the influence of the actin cytoskeleton extends beyond its established functions in cellular structure and motility. It also significantly impacts mitochondrial fission and their overall functional dynamics. This intricate relationship between the actin cytoskeleton and mitochondrial dynamics emerges as a central theme in our scientific inquiry.

The complex cellular signaling is a mystery on its own. For our study, we wanted to understand how cells communicate with their environment or ECM in modulating their cell behavior through actin cytoskeletal pathways. Concurrently, the crucial role of the actin cytoskeleton in shaping mitochondrial dynamics and function, and the intricate nature of their interactions within the cellular context builds the interest of this study.

1.8 Research Question:

It is believed that actin cytoskeleton interacts with the mitochondria during mitochondrial fission (134, 152) and distinct types of actin filaments have been reported to be serving specific functions in mitochondrial dynamics and mitophagy. The abundance of actin protein types suggests the possibility of numerous interactions with organelles; however, the mechanisms governing these interactions remain unclear. Therefore, it would be informative to determine whether a specific signaling pathway exists to guide these interactions or if distinct pathways are employed organising various cellular activities. And how are extracellular signals transduced within the actin cytoskeletal frameworks to regulate organelle function? Since mitochondrial dynamics serve as indicators of cellular health, understanding the signaling mechanisms that enable communication from the cellular environment to mitochondria is of great interest. Hence, with this promising vision, our research aims to answer **"how actin polymerisation pathway regulates mitochondrial dynamics and functions in response to ECM cues".** This has implications for understanding the physiological processes and developing treatments for diseases such as cancer.

In the context of cancer research, recent focus has been on cancer stem cells (CSCs), a subset of cancer cells that play a crucial role in tumor initiation, metastasis, and chemotherapy resistance, owing to their ability to self-renew and generate various tumor cell lineages. The dynamic nature

of CSCs, capable of transitioning between the epithelial and epithelial-mesenchymal states, underscores their importance in both tumor growth and metastasis (378). Metabolic flexibility further complicates the targeting of their dependencies owing to environmental influences, nutrient availability, and energy demands. The tumor microenvironment (TME) has emerged as a critical factor, providing signals that shield CSCs from immune surveillance and enhance their recurrence potential. The TME, comprising specific stromal cells, extracellular matrix (ECM) proteins, and signaling factors, supports CSC self-renewal and survival (98, 100). Given the profound impact of the microenvironment on the metabolic functions of CSCs, understanding this interplay is highly beneficial, particularly in addressing the intricate relationship between the ECM and mitochondria. Therefore, our approach involves utilizing the breast cancer stem cell (BrCSC) system to study this interplay comprehensively and address the following objectives.

Objective 1: To demonstrate the modifications in mitochondrial structure and function in response to varying microenvironments. For this demonstration, we will be utilizing Breast CSCs (MDA-MB 231 and MCF-7 cell lines) subjected to different culture conditions (Adherent, non adherent and ECM induced) mimicking changes in ECM and we will

1. Analyze the potential alterations in their mitochondrial morphology using the Immunofluorescence technique and capturing images with confocal microscopy.
2. Examine the changes in mitochondrial metabolism by performing Oxygen Consumption Rate (OCR) analysis using the Mitostress assay (by Seahorse analyser) and assess ATP and lactate levels by spectrophotometric assays
3. Determine changes in the mitochondrial protein levels by western blot.

Objective 2: To identify the potential mechanism by which the actin cytoskeleton interacts with mitochondria and affects their dynamics. Using the same cellular tools, we will

1. Determine the changes in polymerized actin arrangement by Immunofluorescence and confocal microscopy.
2. Investigate the impact of actin polymerization and depolymerization on mitochondrial dynamics using inhibitors and inducers, and analyzing structural changes through Immunofluorescence and confocal microscopy.
3. Identify the integrin pathway activated by cellular attachment to ECM (fibronectin) that leads to actin polymerization by blocking the pathway at each step and study changes in actin and mitochondrial structures through Immunofluorescence and confocal microscopy.

Objective 3: To illustrate the significant role played by the actin cytoskeleton in mitochondrial dynamics, with a particular emphasis on mitochondrial fusion. For this

objective we will use a primary human cell line derived from a healthy individual, owing to its larger size and ease in recording the mitochondrial movements under microscopy. To further simplify the imaging of actin localisation, we will utilize Actin chromobodies that specifically show signals for actin interacting regions on mitochondria and ER. These chromobodies were provided by our collaborator Prof. Uri Manor, UCSD, CA, United States. Hence using these plasmids, we will

1. Examine the localization of the polymerized actin at sites of mitochondrial fusion and fission by performing live cell imaging under confocal microscopy.
2. Investigate the impact of actin depolymerization (by treating with inhibitors, siRNA, and deACT probes) on mitochondrial fusion and fission.

This study will define the pathway regulating mitochondrial dynamics in diverse microenvironments, specifically focusing on BrCSC metabolism. Additionally, it will provide a comprehensive insight into the potential mechanisms through which actin modulates both the structure and function of the mitochondria.

CHAPTER II

EXTRACELLULAR MATRIX SIGNALS PROMOTE ACTIN-DEPENDENT MITOCHONDRIAL ELONGATION AND ACTIVITY

Priya Gatti[1,2,3], Pritha Mukherjee[4], Priyanka Dey Talukdar[4], Wesley Freppel[2,3,5,6], Joseph Kanou[1], Laurent Chatel-Chaix[2,3,5], Urmi Chatterji[4], Marc Germain[1,2,3#]

Under review; Pre-print available in bioRxiv (2024)
DOI : 10.1101/2024.01.22.576703

[1]Groupe de Recherche en Signalisation Cellulaire and Département de Biologie Médicale, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada
[2]Centre d'Excellence en Recherche sur les Maladies Orphelines - Fondation Courtois, Université du Québec à Montréal, Montréal, Québec, Canada
[3]Réseau Intersectoriel de Recherche en Santé de l'Université du Québec (RISUQ)
[4]Cancer Research Laboratory, Department of Zoology, University of Calcutta, India
[5]Institut National de la Recherche Scientifique, Centre Armand-Frappier Santé Biotechnologie, Laval, Québec, Canada
[6]Current address: Institute for Glycomics, Gold Coast Campus, Griffith University, Southport, QLD 4222, Australia

Correspondence:
Marc Germain: marc.germain1@uqtr.ca

Author contributions

Conceptualization, PG, PM, UC, and MG; Methodology, PG, PM, PDT, WF, LCC, UC and MG; Investigation, PG, PM, PDT, JK, UC, and MG; Resources, LCC; Writing–Original Draft, PG and MG; Writing – Review and Editing, all the authors; Supervision, LCC, UC, and MG; Funding Acquisition, MG

Abstract

Mitochondria are crucial metabolic organelles that are regulated by both intracellular and extracellular cues. The extracellular matrix (ECM) is a key component of the cellular environment that controls cellular behavior and metabolic activity. Here, we determined how ECM signalling regulates mitochondrial structure and activity. To distinguish mitochondrial regulation from the general survival cues generated by the ECM, we used breast cancer-derived spheres (mammospheres) because of their ability to grow in suspension culture in the absence of ECM. Using this system, we demonstrate that the association of mammospheres with the ECM results in dramatic mitochondrial elongation, along with enhanced mitochondrial respiration and ATP production. This remodeling occurs independently of DRP1 activity, but relies on integrin signaling and actin polymerization. Therefore, our findings demonstrate that ECM-driven actin polymerization plays a crucial role in remodeling mitochondrial networks to promote OXPHOS, which represents a vital step for migrating cells to enhance cellular adhesion and facilitate cell growth.

Key Words: Mitochondria, Actin polymerization, Metabolism, ECM, Cancer Stem Cells

Introduction:

Cells are highly responsive to their microenvironment, with cells grown in different environments exhibiting distinct phenotypic and functional characteristics (Baharvand et al., 2006, Baker et al., 2010). Factors such as the extracellular matrix (ECM) and nutrient availability, shape cellular behavior and determine cellular fate (Hynes, 2009, Mierke, 2019). In this context, the ECM provides structural support and biochemical cues to cells, controlling their adhesion, migration, proliferation, and differentiation (Dzobo and Dandara, 2023). The attachment of cells to specific ECM components (collagen, laminin, fibronectin) regulates cell morphology, gene expression, and even stem cell fate (Frantz et al., 2010, Discher et al., 2009, Buxboim et al., 2010, Rais et al., 2023). ECM properties, including stiffness (Romani et al., 2022, Baker et al., 2010), growth factors, nutrients and oxygen levels (Case and Waterman, 2015, Baker et al., 2010) thus have an important incidence on cell behavior.

While the ECM alters cellular responses, mitochondria play a crucial role in the regulation of cellular metabolism and apoptosis. This in turn affects cellular behaviours including cell differentiation and stem cell maintenance (Kasahara and Scorrano, 2014, Liesa and Shirihai, 2013). Mitochondrial activity is regulated through changes in mitochondrial structure, including changes in their length and connectivity (Ren et al., 2020). For example, mitochondria elongate in response to amino acid starvation to sustain cellular ATP levels (Gomes et al., 2011, Patten et al.,

2014, Thomas et al., 2018, Endo et al., 2020, Rambold et al., 2011). These alterations in mitochondrial structure are controlled by the balance between mitochondrial fusion and fission, which are both dependent on large GTPases of the dynamin family. Mitochondrial fusion requires mitofusins (MFN1/2, outer membrane) and optic atrophy 1 (OPA1, inner membrane) (Chen et al., 2007, Chen et al., 2010, Song et al., 2009, Ban et al., 2017, Cao et al., 2017, Qi et al., 2016), while mitochondrial fission is regulated by Dynamin-related protein 1 (DRP1) (Chakrabarti et al., 2018). Maintaining the balance between mitochondrial fusion and fission is crucial for content exchange (Chen et al., 2010, Dong et al., 2022, Adebayo et al., 2021), mitochondrial DNA (mtDNA) integrity (Sabouny and Shutt, 2021, Silva Ramos et al., 2019), removal of damaged mitochondria (Onishi et al., 2021) and ATP production (Yao et al., 2019, Yang et al., 2023, Hofmann et al., 2023).

Interestingly, recent studies have uncovered an intricate relationship between mitochondrial energy metabolism and the ECM. For example, downregulation of mtDNA-encoded respiratory chain subunits leads to altered ECM composition and stiffness (Bubb et al., 2021). In return, ECM stiffness affects mitochondrial length with softer ECM favoring shorter mitochondria (Romani et al., 2022), and stiffer ECM inhibiting DRP1-dependent mitochondrial fission (Chen et al., 2021). This impacts oxidative phosphorylation (OXPHOS), glutamine metabolism (Papalazarou et al., 2020) and glycolysis-related gene expression (Morris et al., 2016). As ECM stiffness depends on its composition and changes in response to the microenvironment (Yanes and Rainero, 2022), microenvironment alteration can significantly impact cell function, including mitochondrial activity.

The complex interplay between cells and their environment is a key feature of tumours, where cancer cells alter and respond to the tumour microenvironment to promote their survival and immune suppression. A small subset of cancer cells, known as tumour-initiating cells (TICs) or cancer stem cells (CSCs), have stemness features such as self-renewal, clonal proliferation, regeneration, metastasis, and drug resistance (Aponte and Caicedo, 2017, Baharvand et al., 2006). These cells are thought to generate the bulk of the cancer cells within the tumour. Importantly, these cells can be isolated through their ability to form spheres when grown in suspension. Nevertheless, the characteristics of CSCs, including their metabolic profile, mitochondrial status and responses to their environment remains debated.

Here, we demonstrate that breast cancer cells grown as spheres (mammospheres) alter their mitochondrial structure and metabolic state in an actin-dependent manner upon attachment to the ECM. Specifically, mammospheres exhibit fragmented mitochondria and lower ATP production, but dramatically elongate their mitochondria and increase OXPHOS upon ECM attachment. This

remodeling occurs independently of DRP1 activity, but relies on integrin signaling and actin polymerization as interfering with either process prevents it. Therefore, our findings demonstrate that ECM-driven actin polymerization plays a crucial role in remodeling mitochondrial networks to promote OXPHOS, which represents a vital step for migrating cells to enhance cellular adhesion and facilitate cell growth.

Results:

Breast cancer cells grown as mammospheres have a distinct mitochondrial structure

Some cancer cells can be cultured both as monolayer and suspension cultures, where they adopt distinct metabolic and proliferative phenotypes. For example, a subset of cells within a cancer cell population can grow in suspension as spheres with stem-like properties. These CSCs or TICs have metabolic properties that are distinct from their parent cells, although their study have yielded conflicting results (Andrzejewski et al., 2017, Endo et al., 2020, Pasto et al., 2014). As ECM stiffness alters mitochondrial structure in adherent cells and CSCs are known to be metabolically flexible, we tested the possibility that the interaction between spheres and the ECM controls their metabolic state.

To address this question, we used the aggressive metastatic breast cancer cells MDA-MB-231 (triple negative) that can be grown as an adherent monolayer (AM) and as spheres in suspension (mammospheres, MS)(Wang et al., 2014). Compared to their counterpart grown as a monolayer, MDA-MB-231 mammospheres had decreased ATP and lactate levels (Figure 1A-B), consistent with these cells being less metabolically active (Chen et al., 2021b, Cheung and Rando, 2013). As mitochondrial structure is a key determinant of mitochondrial function, we then determined whether the metabolic differences we observed were associated with changes in mitochondrial structure. MDA-MB-231 cells grown as a monolayer or mammospheres were labelled with the mitochondrial outer membrane protein TOM20 and imaged by confocal microscopy. Mitochondrial morphology was then manually quantified as long, intermediate, or fragmented (see methods). MDA-MB-231 cells grown as an adherent monolayer displayed a mixed mitochondrial phenotype, with the majority of mitochondria exhibiting an intermediate phenotype (Figure 1C-D). In contrast, mammospheres showed highly fragmented mitochondria (Figure 1C-D), correlating with the decrease in ATP levels.

To gain further insights into the status of mitochondrial dynamics, we then quantified the expression of fission and fusion proteins. Consistent with the observed mitochondrial fragmentation in mammospheres, we noted a significant decrease in the expression of the fusion proteins MFN1 and OPA1 in these cells (Figure 1E). This was accompanied by a decrease in

OPA1 oligomerization, which is required for OPA1 fusion activity (Figure 1F). Nevertheless, mammospheres also exhibited low levels of the fission protein DRP1, both in its active phosphorylated state (pS616) and in terms of total protein levels (Figure 1E), suggesting an overall suppression of mitochondrial dynamics in these cells.

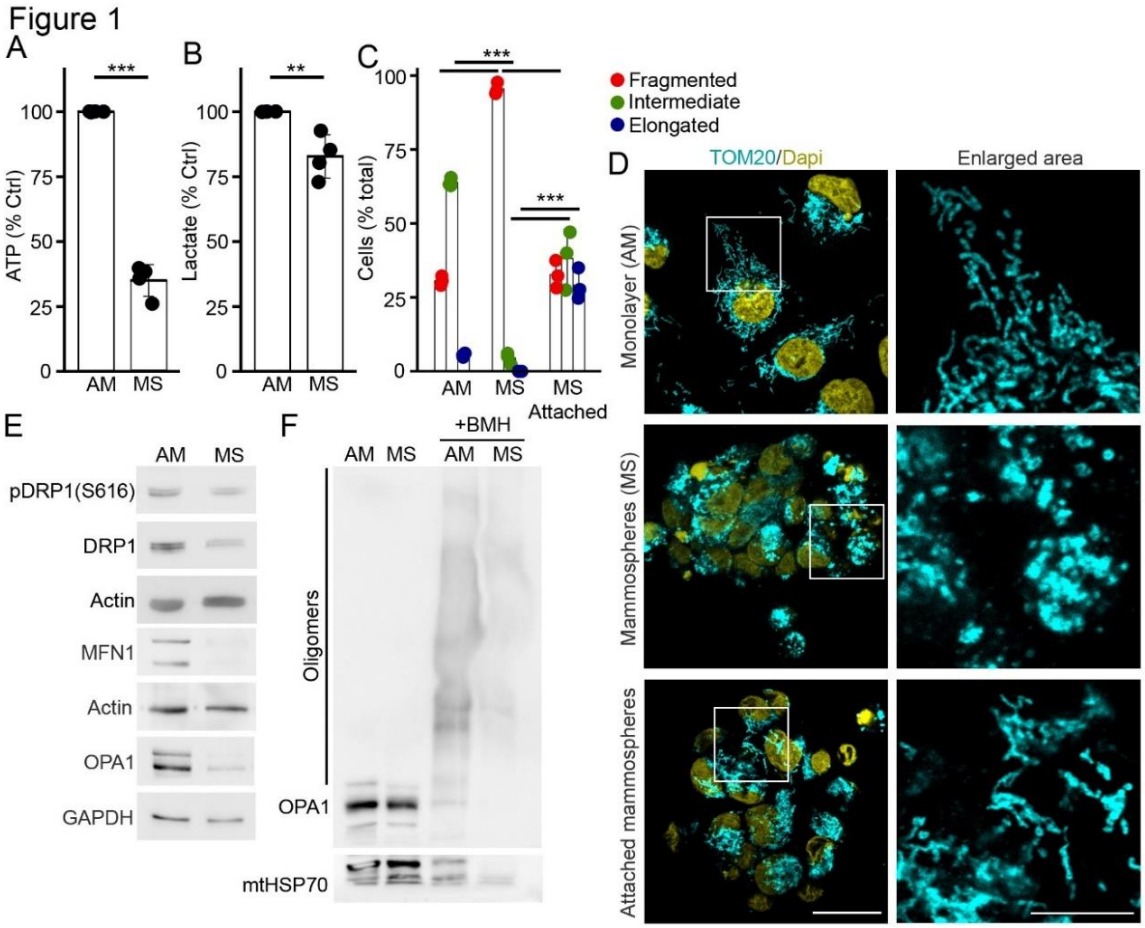


Figure 1. Mammospheres have decreased metabolism and fragmented mitochondria. (A-B) Measure of ATP (A) and lactate (B) levels in MDA-MB-231 cells grown as an adherent monolayer (AM) or as mammospheres in suspension (MS). Each point represents an individual experiment. Bars show the average of 4 independent experiments ± SD. *** $p<0.001$, ** $p<0.01$. Two-sided t-test (C-D) Mitochondrial fragmentation in mammospheres in suspension. MDA-MB-231 were grown as AM, MS or MS attached for 6 hours on glass coverslips, and their mitochondria were marked with an antibody against TOM20 (mitochondria, cyan; with nuclei stained with DAPI, yellow). Quantification of 3 independent experiments is shown in (C), with each point represents an individual experiment. Bars show the average ± SD. *** $p<0.001$. Two-way ANOVA. Representative images are shown in (D). Scale bar 10 µm. (E-F) Western blot showing the expression of mitochondrial dynamics GTPases (E) or OPA1 oligomerization (F). Actin (E) or mtHSP70 (F, lower band – the upper band represents leftover OPA1 signal) were used as loading controls.

ECM attachment drives mitochondrial elongation

The above data is consistent with mammospheres exhibiting a mitochondrial profile that is distinct from adherent monolayer cells. As one difference in culture conditions between adherent cells and mammospheres is the absence of attachment signals in the latter, we tested whether attachment alters mitochondrial dynamics in mammospheres. We thus let mammospheres attache onto coverslips for 6 hours while maintaining other culture parameters identical, and measured their mitochondrial structure. Compared to mammospheres in suspension, attached mammospheres exhibited elongated mitochondrial structures (Figure 1C-D). A similar pattern was also observed in the less invasive MCF-7 breast cancer cells (estrogen receptor-positive) (Supp Figure 1). Overall, these results indicate that attachment signals promote mitochondrial elongation in mammospheres.

While mammospheres were attached directly onto glass coverslips in this experiment, cells normally interact with components of the ECM that promote their adhesion. We thus tested the impact of adhesion molecules on mitochondrial elongation using three conditions: 1) Direct attachment to an uncoated plate without ECM, 2) Attachment to a plate coated with the cationic polymer Poly-D-Lysine (PDL) as an ECM control (Lu et al., 2009) and 3) Attachment to a plate coated with the ECM component fibronectin. Mammospheres were transferred to these plates and mitochondrial length quantified over a 6-hour time course. While it took 6 hours of attachment to reach 50% of cells with elongated mitochondria in the absence of substrate, the presence of PDL reduced this time to 2 hours (Figure 2A). Importantly, the presence of the ECM adhesion molecule fibronectin significantly expedited mitochondrial elongation, with most cells showing elongated mitochondria within one hour of attachment (Figure 2A). Similar results were also observed in MCF-7 mammospheres upon attachment to fibronectin and PDL (Figure 2B). Attachment to the ECM thus triggers mitochondrial elongation in mammospheres.

ECM attachment does not alter mitochondrial dynamins

To define the mechanism through which attachment to the ECM triggers mitochondrial elongation, we first verified that this is not the result of changes in their stem-like properties. For this, we measured the expression of the stem cell marker SOX2, which is expressed at high levels in mammospheres (Liu et al., 2018). Consistent with this, SOX2 levels were high in mammospheres compared to adherent monolayer cells and, importantly, SOX2 expression was maintained upon attachment (Figure 2C), indicating that these attachment conditions do not alter the stem-like characteristics of mammospheres.

The dynamins MFN1/2 and OPA1 play a key role in the regulation of mitochondrial fusion. Nevertheless, the expression level of MFN1 and OPA1 did not increase upon attachment to fibronectin (Figure 2D). Similarly, attachment to fibronectin did not promote OPA1 oligomerisation (Figure 2E), suggesting that ECM-dependent mitochondrial elongation is not caused by a rescue of MFN1 or OPA1 expression. Nevertheless, some processing of OPA1 to shorter isoforms did occur (Figure 2D), which has been linked to both mitochondrial fission and facilitated fusion of the inner mitochondrial membrane (Ban et al., 2017, Wang et al., 2021).

It was recently suggested that changes in mitochondrial length triggered by alterations in ECM stiffness in adherent cells is dependent on the mitochondrial fission protein DRP1 (Romani et al., 2022). As mammospheres grown in suspension versus attached could be functionally similar to a change from soft to stiff ECM, we then addressed the role of DRP1 in ECM-dependent mitochondrial elongation. Total DRP1 and its active phosphorylated version (pS616) remained very low following attachment to fibronectin (Figure 2D), and most cells had elongated mitochondria (Figure 2A). While this suggested that DRP1 is not involved in ECM-dependent mitochondrial elongation, we sought to confirm this by knocking down DRP1 using siRNA. Knockdown of DRP1 in adherent monolayer cells caused an elongated mitochondrial phenotype (Figure 2F-H), consistent with its role in promoting mitochondrial fission. In contrast, silencing of DRP1 had no effect on mitochondrial structure in mammospheres (Figure 2F-H), with most cells still exhibiting fragmented mitochondria. This suggested that ECM-triggered mitochondrial elongation in mammospheres operates through a DRP1-independent mechanism, implying the involvement of factors other than expression levels of fission/fusion dynamins in promoting mitochondrial elongation.

Actin polymerization drives mitochondrial elongation

Given that rearrangement of the actin cytoskeleton is a key event downstream of ECM attachment and that actin is required for mitochondrial fission (Chakrabarti et al., 2018, Hatch et al., 2014, Korobova et al., 2013, Li et al., 2015), we then tested whether actin regulates mitochondrial elongation in attached mammospheres. We first labeled MDA-MB-231 mammospheres in suspension with the filamentous actin (F-actin) stain phalloidin and the mitochondrial marker TOM20. Most mammospheres showed a faint/absent signal for F-actin, except for a small subset of cells displaying strong F-actin signal and elongated mitochondria compared to F-actin low cells (Figure 3A-B). In contrast, attached mammospheres exhibited a prominent F-actin signal (Figure 3C). Specifically, PDL and fibronectin treatments resulted in rapid cell spreading accompanied by well-developed actin structures (Figure 3C) that correlated with the rapid changes in mitochondrial network that occur in these cells (Figure 2A). As MCF7 mammospheres did contain some cells

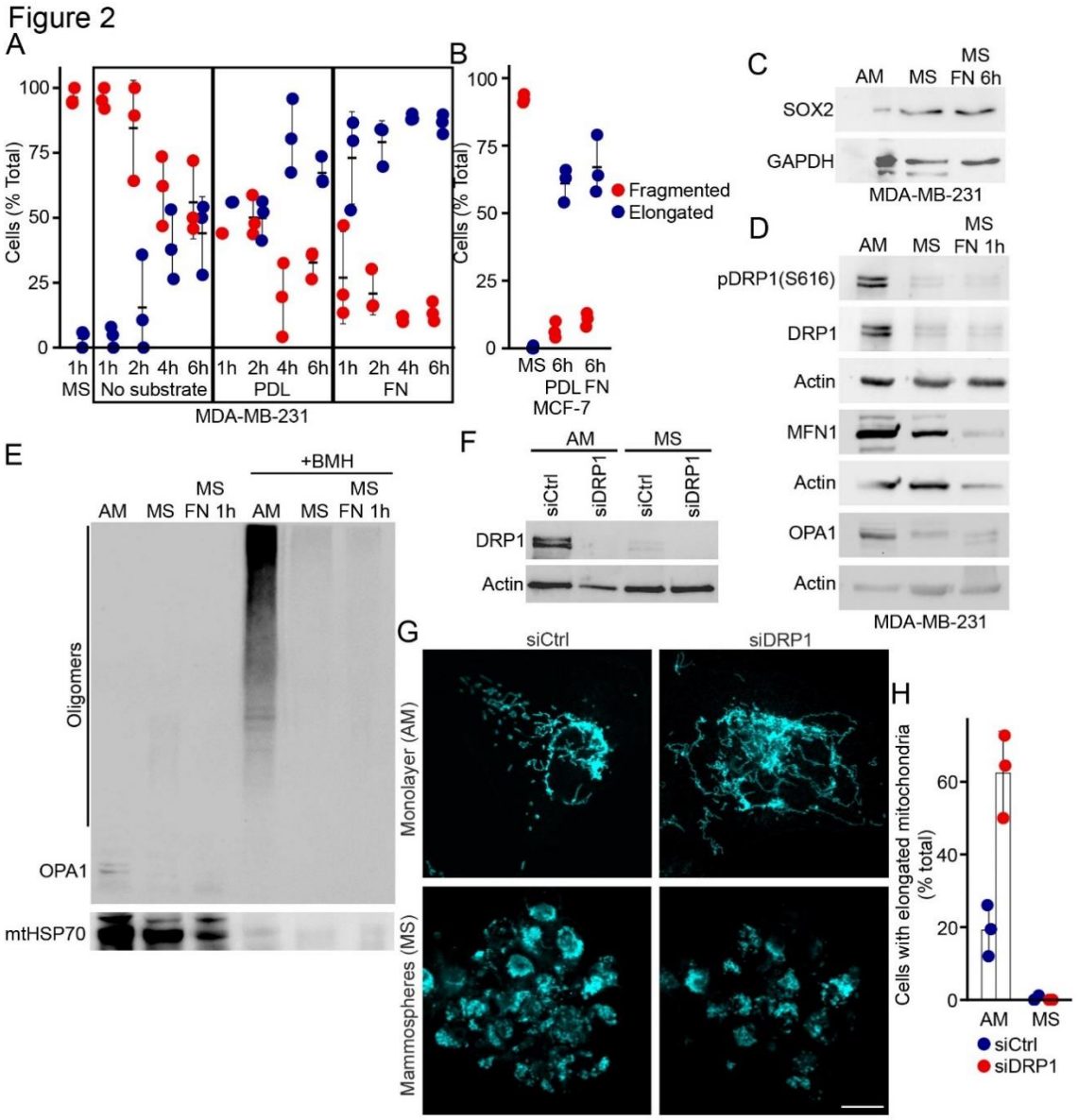


Figure 2. Cell attachment to the ECM promotes mitochondrial elongation. (A-B) Mitochondrial elongation in response to cellular attachment to ECM substrates. MDA-MB-231 (A) and MCF7 (B) were spun down on coverslips either not coated or coated with poly-D-lysin (PDL) or fibronectin (FN) and incubated for the indicated times. mitochondria were marked with an antibody against TOM20 and mitochondrial length quantified in 3 independent experiments. Each point represents an individual experiment. Bars show the average ± SD. (C-D) Western blot showing the expression of the stem cell marker SOX2 (C) and mitochondrial dynamics GTPases in MDA-MB-231 cells grown as AM, MS or MS attached for 6 hours on fibronectin. Actin (C) or GAPDH (D) were used as loading controls. Western blot showing the lack of OPA1 oligomerization in MS cells in the absence or the presence of fibronectin. mtHSP70 was used as a loading control. (F-H) DRP1 knockdown does not affect mitochondrial structure in MDA-MB-231 mammospheres. (F) Western blot showing the knockdown of DRP1 in MDA-MB-231 cells. (G) Representative images showing mitochondrial structure (marked with an antibody against TOM20) in MDA-MB-231 cells knocked down for DRP1. Scale bar 10 µm. Mitochondrial length

was quantified in 3 independent experiments (H) Each point represents an individual experiment. Bars show the average ± SD.

with intermediate mitochondria (Sup. Figure 1), we also assessed actin status in these cells. In contrast to MDA mammospheres, where actin was barely detectable in most cells, the majority of MCF7 cells in mammospheres had weak but clearly visible phalloidin signal that correlated with the presence of intermediate mitochondria (Figure 3A-B, images of MDA and MCF7 cells were taken together using the same settings, allowing comparisons).

Given the correlation between actin polymerization and mitochondrial elongation, we then inhibited actin polymerization using Cytochalasin D (CytoD) and determine its effect on mitochondrial networks. Inhibiting actin polymerization efficiently prevented fibronectin-activated mitochondrial elongation (Figure 3D) in attached mammospheres, consistent with an important role for actin in this process. However, CytoD did not significantly affect cells in adherent monolayers (Figure 3D), suggesting that actin is especially required during the elongation of the network (as opposed to steady-state maintenance).

Mitochondrial Elongation is dependent on signalling downstream of integrins

Cellular attachment to fibronectin is mediated by integrins, which subsequently activates Focal Adhesion Kinase (FAK) and Src, ultimately promoting actin polymerization (Sup. Figure 2A). Consistent with this, while levels of phophorylated FAK (pY397-FAK) were undetectable in mammospheres in suspension, its levels dramatically increased following one hour of attachment to fibronectin (Figure 4A). To ascertain whether FAK activation is required for mitochondrial elongation following cell attachment, we inhibited FAK using the small molecule inhibitor PF-573228 (PF). Consistent with the role of FAK in cellular attachment and actin polymerization, FAK inhibition led to a significant decrease in cell adhesion to fibronectin coated plates (Figure 4B) and impaired their ability to spread and form lamellipodia-like actin features (Figure 4C). Importantly, FAK inhibition caused a profound impairment of mitochondrial elongation in the cells that did attach to fibronectin-coated plates (Figure 4C-D), consistent with this pathway being required for fibronectin-driven mitochondrial elongation. Src is a tyrosine kinase found within the integrin adhesion complex that facilitates FAK activation and promotes maximal adhesion induced by FAK activation (Calalb et al., 1995). In fact, inhibition of Src in attached mammospheres using Src inhibitor-1 (SI-1) inhibited attachment and actin polymerization similarly to FAK inhibition (Figure 4B-C). More importantly, Src inhibition significantly inhibited mitochondrial elongation (Figure 4D), further supporting a role for integrin signalling in fibronectin-driven mitochondrial elongation.

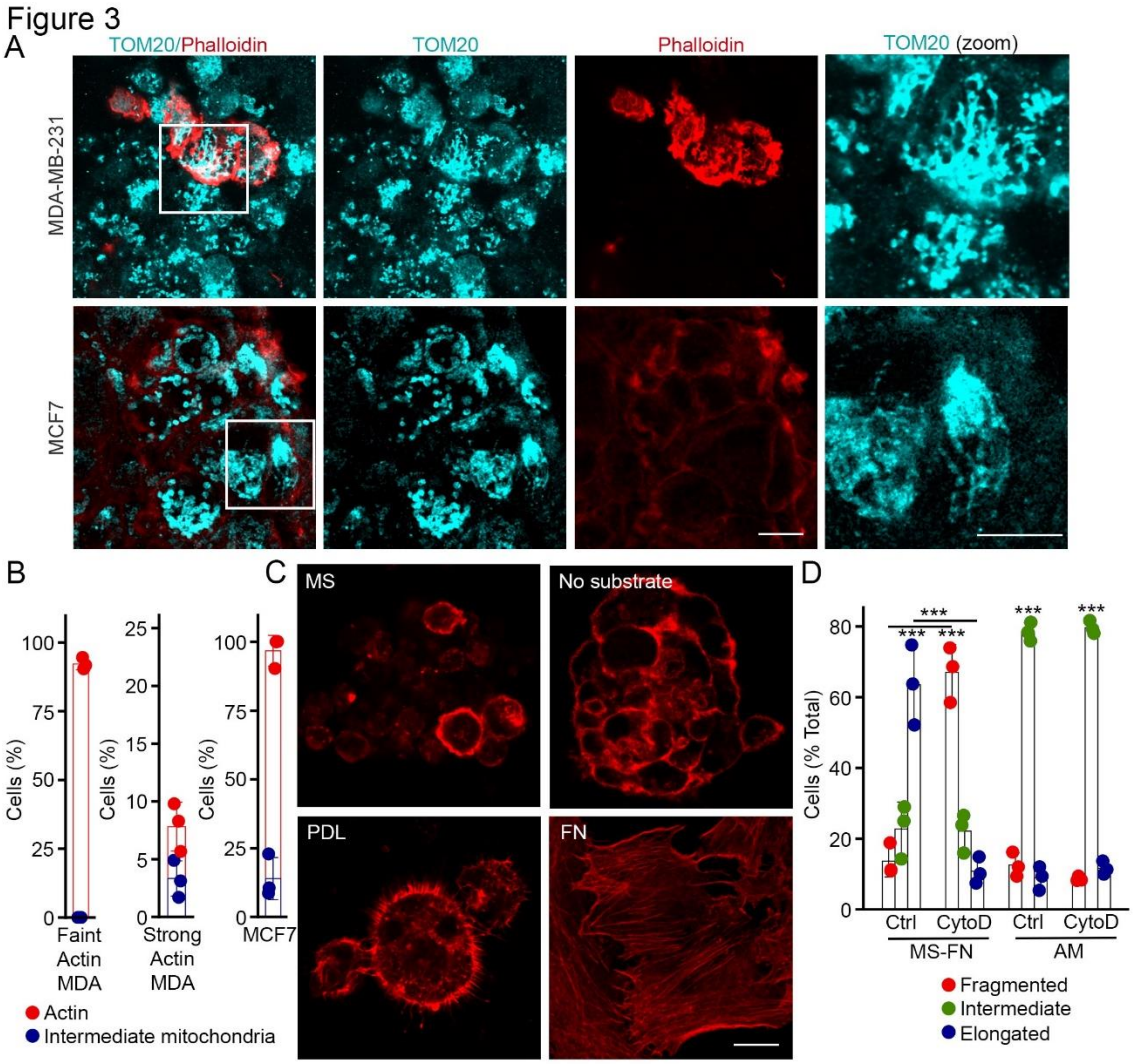


Figure 3. Actin polymerization drives mitochondrial elongation. (A-B) F-Actin staining in MDA-MB-231 and MCF7 mammospheres in suspension. Cells were marked for F-Actin (phalloidin, red) and mitochondria (TOM20, cyan). Scale bar 10 µm. The number of cells with intermediate mitochondria was quantified in MDA-MB-231 cells with faint (B, left) or strong (B, middle) actin staining. As most MCF7 cells had similar F-Actin staining, they were all grouped together for mitochondrial length analysis (B, right). Each point represents an individual experiment. Bars show the average of 3 independent experiments ± SD. (C) Representative images showing F-Actin structures (labelled with phalloidin) present in MDA-MB-231 mammospheres under different attachment conditions. Scale bar 10 µm. (D) Actin depolymerization prevents mitochondrial elongation in mammospheres. MDA-MB-231 cells were grown as an adherent monolayer (AM) or as mammospheres attached for 1 hour on fibronectin (MS-FN) in the absence or the presence of the actin depolymerizing agent Cyto D (0.1 µg/ml) and mitochondria were analysed as in (B). Each point represents an individual experiment. Bars show the average of 3 independent experiments ± SD. *** $p<0.001$.

Rho GTPases such as RhoA, Cdc42, and Rac1 are the key signalling proteins that act downstream of FAK/Src to promote actin cytoskeleton organization. Among these, Rac1 regulates the

formation of lamellipodia-like actin structures (Horton et al., 2015, Price et al., 1998) and its expression is elevated in cancer stem cells (Ko et al., 2014, Sundberg et al., 2003). Consistent with this, inhibition of Rac1 (with Rac Inhibitor III) reduced attachment to fibronectin and actin rearrangements in the cells that did attach (Figure 4E-F, Sup. Figure 2B). Rac1 inhibition also completely prevented mitochondrial elongation upon attachment (Figure 4G), further supporting a crucial role for actin in promoting mitochondrial elongation. On the other hand, RhoA inhibition (with Rho Inhibitor I) did not significantly affect attachment or mitochondrial structure (Figure 4E-G), suggesting that actin reorganization and mitochondrial elongation is mainly Rac-dependent in our experimental setting.

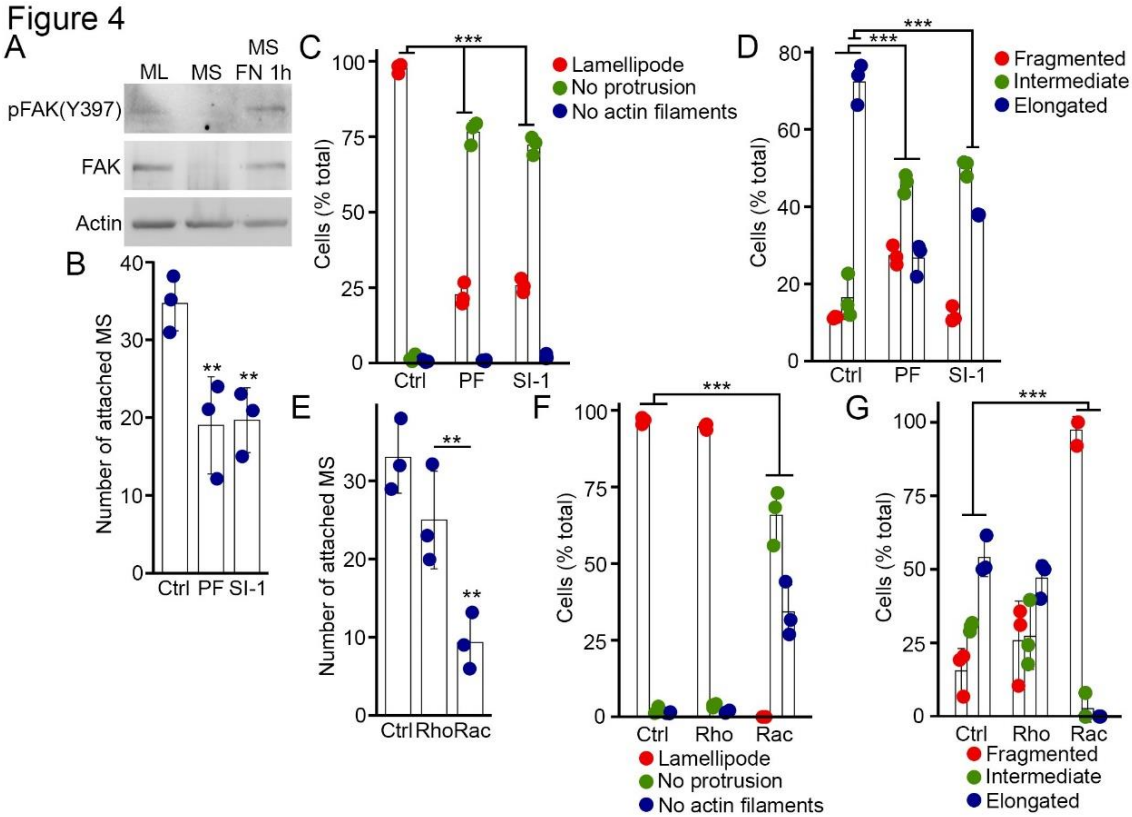


Figure 4. Inhibition of the signalling pathway downstream of integrins prevents mitochondrial elongation. (A) Western blot showing FAK activation (pFAK-Y397) in response to attachment to fibronectin. Actin was used as a loading control. (B-D) Effect of the FAK inhibitor PF-573228 (PF, 10 μM) and the Src inhibitor Src Inhibitor-1 (SI-1, 10 μM) on mammosphere attachment to fibronectin-coated plates (1 hour) (B) formation of F-actin structures (C) and mitochondrial structure (D). Each point represents an independent experiment. Bars show the average ± SD. *** $p<0.001$. (E-G) Effect of Rac1 (Rac Inhibitor III; 2 μM) and Rho (Rho Inhibitor I, 10μM) inhibition on mammosphere attachment to fibronectin-coated plates (1 hour) (E), formation of F-actin structures (F) and mitochondrial structure (G). Each point represents an independent experiment. Bars show the average ± SD. *** $p<0.001$. One-way ANOVA (B, E), Two-way ANOVA (C, D, F, G).

While our results indicate that a signalling pathway downstream of integrins is required for mitochondrial elongation upon attachment to fibronectin, the partial inhibition of cell attachment caused by the inhibition of this pathway could potentially influence this conclusion. As an alternative, we thus directly activated Rho GTPases in mammospheres in suspension culture using a small molecule known to activate RhoA, Cdc42, and Rac1 (Rho/Rac/Cdc42 Activator I). Following activation, we visualized the cells by labeling mitochondria (with TOM20) and actin (with phalloidin). While control mammospheres contained only a few cells with strong actin staining, the majority of cells treated with the activator displayed an intense F-actin signal (Figure 5A-B). More importantly, this was associated with an increase in the number of cells displaying elongated mitochondria (Figure 5A and C), further demonstrating that mitochondrial elongation occurs downstream of Rho family-driven actin polymerization.

Rac1 promotes lamellipodia formation by activating the Wiskott-Aldrich syndrome family of proteins (WASP, N-WASP, and WAVE1/2) that, in turn, activates actin-polymerizing proteins including the Arp2/3 complex and formins. Consistent with this, inhibition of Arp2/3 (using CK666) or formins (using SMIFH2) significantly reduced mitochondrial elongation in attached mammospheres (Figure 5D). On the other hand, the effect of the inhibitors was limited in adherent monolayer cells, with only CK666 causing an increase in cells with fragmented mitochondria relative to those with intermediate mitochondria (Figure 5E). While this is in line with the lack of effect of CytoD in these cells (Figure 3D), it suggests that actin plays a more prominent role during widespread changes in mitochondrial structure in response to changes in cellular environment.

Mitochondrial elongation promotes OXPHOS in mammospheres.

Alterations in mitochondrial architecture exert a profound influence on mitochondrial function, with mitochondrial fusion or elongation being vital in preserving mitochondrial function during starvation (Adebayo et al., 2021). We thus investigated whether the observed alterations in mitochondrial dynamics during cellular attachment impact mitochondrial activity. We first determined whether the changes in mitochondrial structure were associated with alterations in the expression of electron transport chain (ETC) proteins that are required for OXPHOS. In contrast to the decrease in ATP production observed in mammospheres in suspension (Figure 1A), these cells exhibited higher expression of all ETC components tested relative to their parental cells grown as an attached monolayer (Figure 6A). Interestingly, the expression of ETC components was partially decreased upon attachment to fibronectin (Figure 6A), suggesting a direct relationship between mitochondrial structure and ETC component expression. Nevertheless,

mitochondrial membrane potential remained similar across conditions (Figure 6B), suggesting that they remain functional irrespective of their morphology.

Figure 5

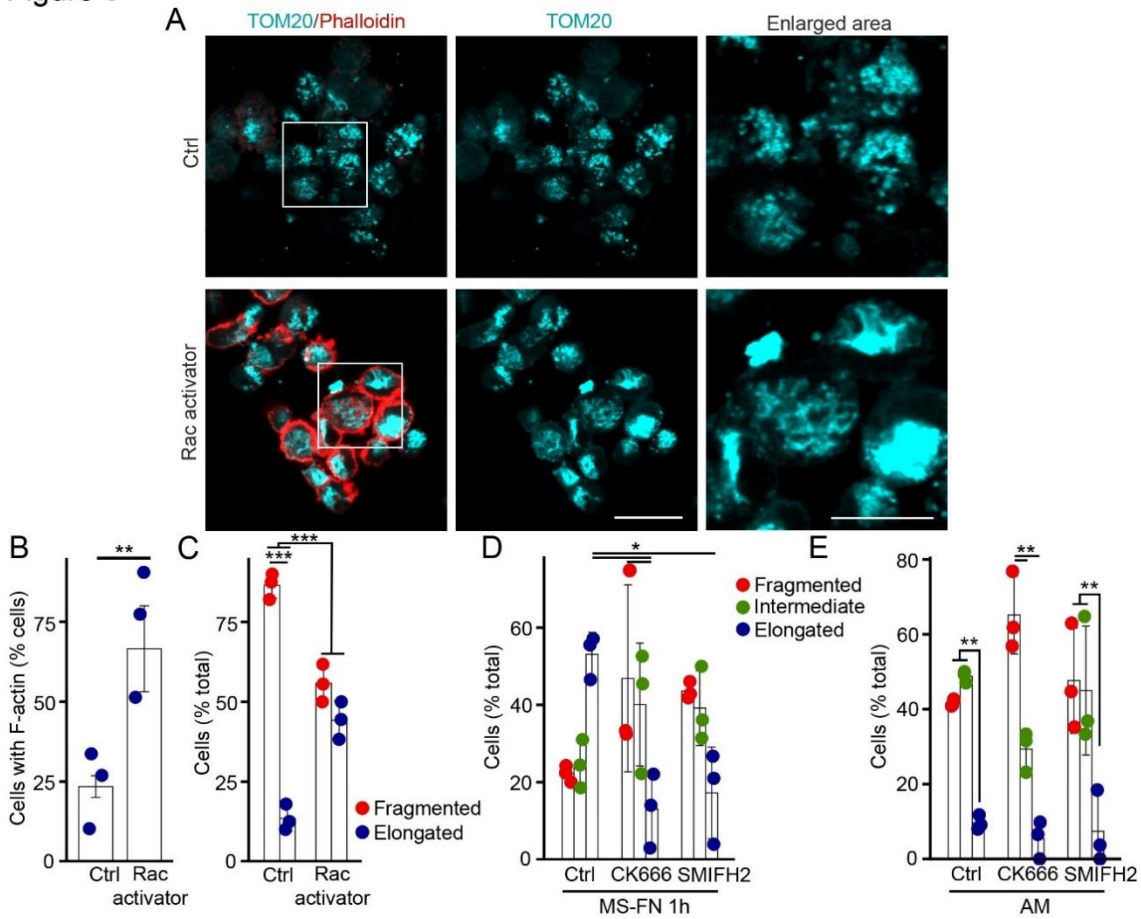


Figure 5. Actin polymerization promotes mitochondrial elongation. (A-C) Actin polymerization and mitochondria in mammospheres treated with a Rac activator (Rho/Rac/Cdc42 Activator I, 10 µM, 1 hour). (A) Representative images of F-actin (phalloidin, red) and mitochondria (TOM20, cyan) in MDA-MB-231 mammospheres. Scale bar 10 µm. The quantification of 3 independent experiments is shown in (B) for F-actin and (C) for mitochondrial length. Each point represents an individual experiment. Bars show the average ± SD. *** $p<0.001$. two-sided t-test (B), Two-way ANOVA (C). (D-E) Disruption of mitochondrial elongation in mammospheres treated with the Arp2/3 inhibitor CK666 (10 µM, 1 hour) or the formin inhibitor SMIFH2 (2 µm, 1 hour). Mitochondrial length was quantified in mammospheres attached to fibronectin (1 hour) (D) and attached monolayer cells (E). Each point represents an individual experiment. Bars show the average ± SD of 3 independent experiments. ** $p<0.01$, * $p<0.05$. Two-way ANOVA.

As previously noted, mammospheres are known to house a high proportion of CSCs, confirmed by the expression of the stem cell markers SOX-2 and ALDHIA1 in MDA-MB-231 and MCF-7 mammospheres (Figure 6C). We explored the relevance of these findings in an *in vivo* context by isolating ALDH-positive breast CSCs (brCSCs) from mouse mammary tumors. Consistent with

the mammosphere data, brCSCs displayed reduced expression of the fission protein DRP1 (both total and pDRP1; Figure 6D). These cells also had increased levels of the mitochondrial fusion proteins OPA1 and MFN1 (Figure 6D), likely reflecting their long-term interaction with the ECM within the tumour environment. Importantly, brCSCs also showed increased expression of the ETC proteins NDUFA9 and UQCRC2, mirroring our *in vitro* data (Figure 6D).

To better understand the consequences of ECM attachment on mitochondrial OXPHOS, we measured bioenergetic parameters (ATP and Lactate) in adherent monolayers, mammospheres, and attached mammospheres (FN-6 hours). Consistent with our previous results (Figure 1A-B), mammospheres exhibited lower ATP levels than adherent monolayer cells (Figure 6E). Importantly, attachment to fibronectin partially rescued ATP levels in mammospheres (Figure 6E), in line with the mitochondrial elongation observed following attachment. We then assessed the mitochondrial contribution to ATP levels using the ATP synthase inhibitor oligomycin. Oligomycin had little effect on adherent monolayers or mammospheres (Figure 6E), indicating limited reliance on mitochondria-derived ATP. In contrast, oligomycin significantly reduced ATP levels in attached mammospheres, suggesting increased mitochondria dependence. This correlated with a significant reduction in lactate levels upon attachment (Figure 6F). This indicates that, following attachment to fibronectin, cells stimulate their mitochondrial metabolism, promoting their dependency on OXPHOS for ATP production.

To further confirm that ECM attachment promotes mitochondrial dependency for energy production, we measured oxygen consumption rates (OCR). Consistent with mammospheres being less metabolically active, they showed lower basal OCR and minimal ATP-linked respiration compared to monolayer cells (Figure 6G-I). Importantly, attached mammospheres significantly increased both basal and ATP-linked OCR, consistent with mitochondrial elongation stimulating mitochondrial activity (Figure 6G-I). Altogether, our results indicate that the actin-driven mitochondrial elongation that occurs upon attachment to fibronectin stimulates mitochondrial respiration in previously less metabolically active mammospheres.

Discussion:

The ECM plays a crucial role in the modulation of cell survival and function. Previous studies have also suggested links between ECM signaling and mitochondria (Bubb et al., 2021, Romani et al., 2022, Visavadiya et al., 2016, Cai et al., 2023, Tian et al., 2023). While these observations could mechanistically link the migration, proliferation, and survival roles of ECM signaling with metabolic needs, the complex links between these events makes it challenging to identify clear causal links in adherent cells requiring ECM signals for their survival. Here, we took advantage of the fact that a subset of cells within a tumor can be grown in suspension in the absence of ECM.

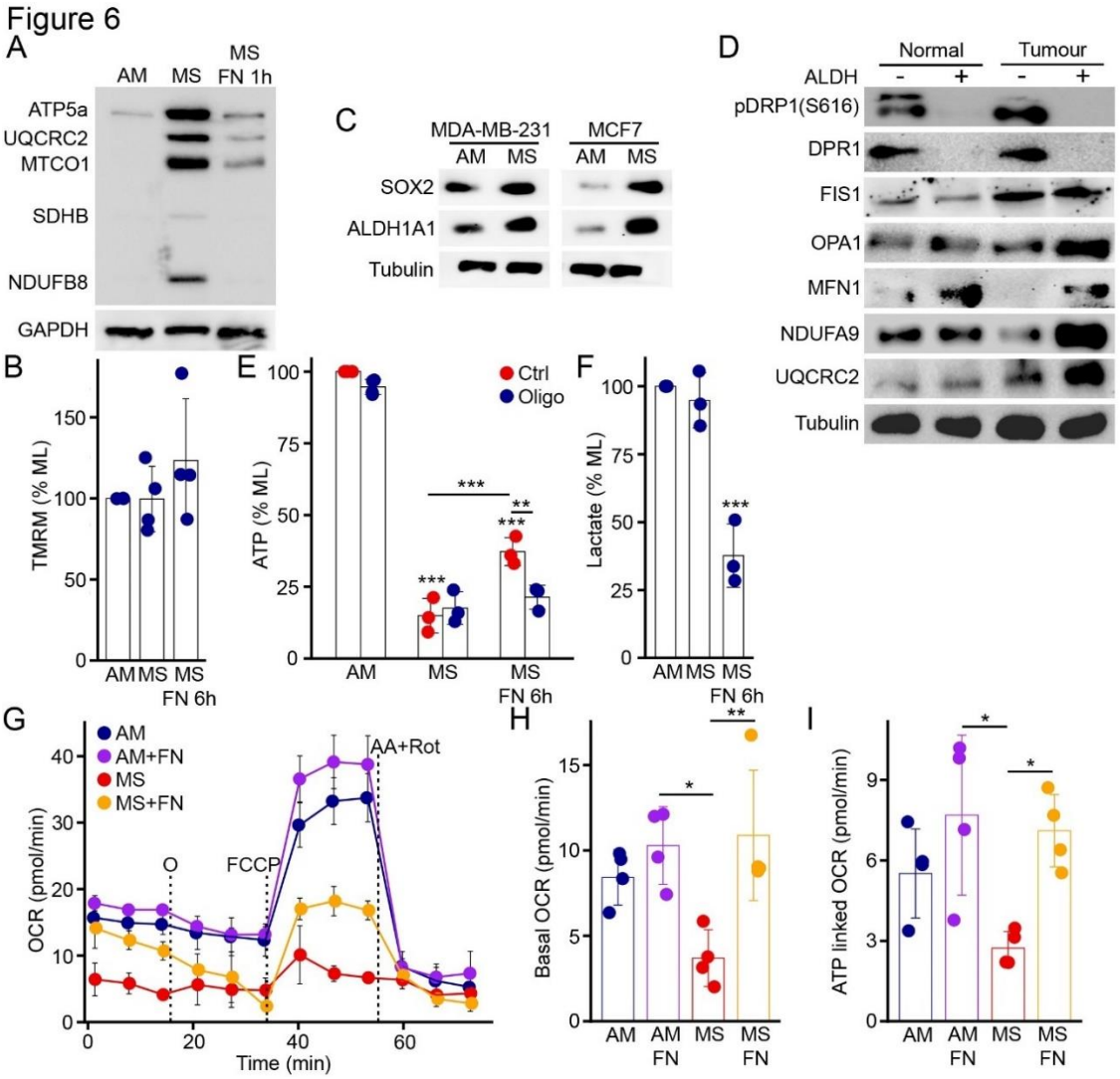


Figure 6. Mitochondrial elongation promotes OXPHOS in mammospheres. (A) Western blot showing the expression levels of ETC components in attached monolayer cells (AM), mammospheres (MS) and mammospheres attached to fibronectin for 1 hour (MS-FN). GAPDH was used as a loading control. (B) Mitochondrial membrane potential measured using TMRM in AM, MS and MS attached to fibronectin for 1 hour (MS-FN) in 3 independent experiments. Each point represents an individual experiment. Bars show the average ± SD. (C) (C) Western blot showing the expression levels of the stem cell markers SOX2 and ALDH1A1 in MDA-MB-231 and MCF7 cells grown as an adherent monolayer or as mammospheres in suspension. β-Tubulin was used as a loading control. (D) Western blot showing the expression levels of mitochondrial proteins in ALDH-negative and positive cells isolated from normal mammary gland and mammary tumour tissues. β-Tubulin was used as a loading control. (E-F) Metabolic changes in MDA-MB-231 mammospheres upon attachment to fibronectin. Total ATP (E) and lactate (F) were measured in 3 independent experiments. Each point represents an individual experiment. Bars show the average ± SD. *** $p<0.001$, ** $p<0.01$. one-way ANOVA, two-way ANOVA for (B). (G-I) Changes in Oxygen consumption in mammospheres upon attachment to fibronectin. (G) Representative oxygen consumption rate (OCR) curves. O, oligomycin; AA, antimycin A; Rot, rotenone. Basal OCR (H) and ATP-linked OCR (I) were calculated from 4 independent

experiments. Each point represents an individual experiment. Bars show the average ± SD. ** $p<0.01$, * $p<0.05$. One-way ANOVA.

These mammospheres have distinct metabolic properties and mitochondrial structure. As mitochondrial dynamics regulates mitochondrial activity, we used these cells to investigate how mitochondrial structure and function are regulated by the ECM. We show that even if mammospheres do not require an ECM for their survival, they modulate their mitochondrial structure and energy metabolism in response to ECM components. We also uncovered a key role for actin polymerization in this process.

Some studies have reported that the ECM regulates mitochondrial morphology in response to ECM stiffness. Stiff ECM has been found to block mitochondrial fission (Chen et al., 2021a), while soft ECM promotes it (Romani et al., 2022). Stiff ECM activates β1-integrin/PINCH1-kindlin-2 signaling, which inhibits DRP1 activity, thereby promoting mitochondrial elongation (Chen et al., 2021a). In contrast, soft ECM activates DRP1, leading to mitochondrial fission and metabolic alterations through the modulation of mitochondrial ROS and antioxidants (Romani et al., 2022). In contrast, the mitochondrial elongation we observed upon mammospheres attachment is unlikely to involve DRP1. Indeed, despite mammospheres in suspension (akin to soft ECM) having fragmented mitochondria and becoming elongated when plated on a fibronectin-coated adherent plates (stiff matrix), these mammospheres express very low levels of DRP1 compared to their parental adherent monolayer cells. Moreover, knockdown of DRP1, which typically causes mitochondrial elongation by blocking the fission process (Zhao et al., 2013, Kitamura et al., 2017), did not induce mitochondrial elongation in suspension cells. While it remains possible that DRP1 is required to generate the fragmented phenotype of mammosphere mitochondria, our results suggest that DRP1 does not play a crucial role in the regulation of mitochondrial networks once mammospheres are established. Instead, we propose that these cells modulate mitochondrial fusion to control the morphology of their mitochondria in response to ECM cues.

Cell attachment to the ECM component fibronectin stimulates integrin signaling (Buxboim et al., 2010). This signaling pathway activates a series of downstream processes that may regulate mitochondria (Sup. Figure 2A). We show that signalling events proximal to integrin activation, including FAK and Src activation, are required for mitochondrial elongation following mammosphere attachment. Further, downstream of FAK activation, the Rho GTPase Rac1 activates Arp2/3-dependent actin polymerization (Ko et al., 2014). Importantly, inhibiting Rac1 or Arp2/3 significantly disrupted mitochondrial elongation in attached mammospheres, supporting a key role for this pathway in the regulation of mitochondrial structure. Furthermore, activation of

Rac in suspension cells also leads to mitochondrial elongation, demonstrating a direct link between the two processes.

Actin polymerization has previously been linked with mitochondrial fission (Moore et al., 2016), mitophagy (Onishi et al., 2021, Li et al., 2018) and mitochondrial motility (Boldogh et al., 2001). During fission, the actin-nucleating mitochondrial protein Spire1C (Manor et al., 2015), associates with ER-anchored Inverted formin 2 (INF2) (Chakrabarti et al., 2018), regulating membrane constriction and DRP1 oligomerization at the fission site. Arp2/3 complex has been also shown to influence mitochondrial division, but the mechanism is not yet defined (Li et al., 2015). Our study reveals an additional role of actin in mitochondrial dynamics, specifically in regulating mitochondrial elongation through Arp2/3-dependent actin polymerization. This is consistent with our work showing that Arp2/3-dependent actin polymerization on mitochondria is required for mitochondrial fusion (Gatti et al., 2023), and the presence of shorter mitochondria in adherent monolayer cells treated with the Arp2/3 inhibitor (Figure 5E). These different roles of actin in the regulation of mitochondria likely depend of the specific signalling pathways activated in response to distinct cellular environments. Overall, we propose here that ECM-induced mitochondrial elongation is dependent on Arp2/3-driven actin polymerisation. This alternate pathway functions independently of DRP1 activity.

In cancer cells, ECM-dependent promotion of adhesion and migration involves a metabolic shift towards increased OXPHOS, corelating with mitochondrial elongation (Wu et al., 2021, Tian et al., 2019, Papalazarou et al., 2020). FAK and PAK, two Integrin-activated signaling proteins, can alter mitochondrial structure, function and energy production (Kanteti et al., 2016) (Visavadiya et al., 2016, Yang et al., 2021). Specifically, FAK inhibition results in the loss of mitochondrial membrane potential, leading to impaired mitochondrial function. Additionally, Src and FAK-activated STAT3 can directly interact with mitochondria to stimulate OXPHOS activity. (Visavadiya et al., 2016, Djeungoue-Petga et al., 2019, Guedouari et al., 2021, Guedouari et al., 2020, Lurette et al., 2022). In contrast, our results show that actin polymerization downstream of these signalling events is required to modulate mitochondrial structure in response to ECM cues in mammospheres. We thus propose that actin polymerization will have an executional role in regulating mitochondrial structure (elongation) and metabolism (OXPHOS) in response to ECM cues. This suggests that the somewhat conflicting description onthe role of the ECM and downstream signalling pathways that have been described could arise, at least in part, through differences in the actin signalling pathways activated under distinct experimental conditions. This would in turn affect mitochondrial dynamics and metabolic modulation. For instance, CSCs that require upregulation of macromolecule biosynthetic pathways during differentiation may

fragment and switch to glycolysis (Serasinghe et al., 2015, Shiraishi et al., 2015), while energy demanding CSCs will gain fused mitochondria and induce OXPHOS (Rivadeneira et al., 2015). The difference in mitochondrial usage between distinct cellular conditions could also possibly explain the discrepancy we observed between ETC components expression and OCR and ATP levels. As one possibility, we think that mammospheres could use their mitochondria to synthesize other metabolites. For example, metastatic migratory cancer cells require a high production of mitochondrial superoxide (Porporato et al., 2014), which is obtained by an exaggerated TCA cycling (Porporato et al., 2014, Porporato and Sonveaux, 2015). It is also suggested that breast cancer cells colonizing lungs utilize the proline cycle to obtain $FADH_2$, which can be oxidized by ETC to produce mitochondrial ATP (Elia et al., 2017), or generate ATP synthesis by glycolysis and fatty acid-dependent OXPHOS, suggesting a selective rewiring of the energy substrate (Andrzejewski et al., 2017, Pascual et al., 2017).

Overall, our study reveals the complex interplay between mitochondrial dynamics and ECM-mediated actin regulation, providing insights into cellular adaptation process in changing environments. Further research is needed to comprehend actin's role in mitochondrial function, with potential implications for understanding cancer metastasis and guiding anti-cancer therapies.

Material and Methods

Cell culture and transfection:

MDA-MB-231 and MCF-7 cells were purchased from American Type Culture Collection. Cells in adherent monolayers were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, UT) at 37°C in a humidified 5% CO_2 incubator. Mammospheres were grown in DMEM supplemented with B-27 (Gibco™, 17504044), human EGF (10 µg/ml) and bovine insulin (10 µg/ml) in low-attachment plates (Nunclon sphera, Thermo Scientific™ #174932) and maintained at 37°C in a humidified 5% CO2 incubator for 3 days before harvesting. For attachment experiments, these 3-day mammospheres were spun down (1500g, 10 min) on coverslips that were previously coated with Fibronectin (Corning™ Fibronectin, Human Fischer Scientific, #CB-40008A) or Poly D Lysine (Sigma-Aldrich #P6403) where indicated. Cells were then kept at 37°C in a humidified 5% CO_2 incubator until they were fixed. The same procedure was followed for western blot except that cells were spun down on standard cell culture dishes for adherent cells.

siRNA treatment:

MDA-MB-231 cells were seeded onto 24-well plates and transfected with 15nM of DRP1 siRNA (Thermo Fisher Scientific, Silencer Select, #4390771) or negative siRNA (Thermo Fisher

Scientific, Silencer Select, #4390843) using siLenFect lipid reagent (Bio-Rad, #1703361). After 24 hrs, cells were collected for either western blotting or seeded onto coverslips for immunofluorescence.

Manipulation of actin polymerization:

The following proteins were manipulated using chemical inhibitors/activators: F-Actin depolymerization, Cyto D (0.1 µg/ml Sigma Aldrich #C8273); FAK, small-molecule inhibitor PF-573228 (10 µM; Sigma Aldrich #PZ0117); Src kinase, Src Inhibitor-1 (10 µM; Sigma Aldrich #S2075); Rho GTPase, Rho Inhibitor I (C3 Transferase from *Clostridium botulinum* covalently linked to a cell penetrating moiety; 10µM; Cytoskeleton, Inc #CT04-A); Rac GTPase, Rac Inhibitor III (2 µM; MilliporeSigma™ #55351310MG); Arp 2/3 complex, CK-666 (10 µM; Sigma Aldrich #182515); Formin, FH2 domain inhibitor SMIFH2 (2µM; Sigma-Aldrich #344092); Rac activation, Rho/Rac/Cdc42 Activator I (10 µM; Cytoskeleton, Inc #CN04-A). All treatments were applied for a duration of 1 hour prior to fixation with 4% PFA, followed by subsequent immunofluorescence and confocal imaging.

Immunofluorescence:

Cells were plated on glass coverslips (Fischer Scientific #12541000CA) and allowed to adhere overnight to establish monolayer cultures. Mammospheres, on the other hand, were anchored to coverslips through centrifugation at 3,000 rpm for 10 minutes, followed by incubation for a period ranging from 1 to 6 hours. Mammospheres in suspension were collected by centrifugation in a microtube. The cells were then fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). Cells were then 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 a primary antibody against TOM20 (Rb,1:250; Abcam #ab186735,) followed by fluorescent-tagged secondary antibodies (1:500; Jackson Immunoresearch). Cells were co-stained with Rhodamine-phalloidin (1:250; Sigma Aldrich #P1951), and DAPI (1:100; Invitrogen, Thermo Fisher #D1306).

Image processing and analysis:

The images were acquired using a Leica TSC SP8 confocal microscope with a 63×/1.40 oil objective using the optimal resolution for the wavelength (determined using the Leica software). All image manipulation and analysis were done in Image J/Fiji. The images shown are from single focal planes unless stated otherwise. For mitochondrial length analysis, a cell was binned in a specific category if at least 70% of mitochondria within that cell had the following length: less than 0.2 µm for fragmented, between 0.2 and 0.6µm for intermediate and more than 0.6 µm for elongated. Cells where mitochondrial networks were not clearly identifiable were excluded from the analysis.

Analysis of OPA1 oligomers:

OPA1 oligomerization within intact cells was done as described (Patten et al., 2014). Briefly, cells were treated with the cell-permeable cross-linking agent BMH (Thermo Scientific) at a concentration of 1 mM for 20 minutes at 37°C. Following the crosslinking procedure, BMH was quenched by rinsing twice using PBS containing 0.1% beta-mercaptoethanol (BME). Subsequently, the cells were collected and lysed in 10 mM Tris-HCl at pH 7.4, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100 with 0.1% BME, and the lysate was subjected to Western blot on NuPAGE Novex 3–8% Tris-acetate gradient gels (Invitrogen™ # EA0375BOX), transferred to a nitrocellulose membrane and blotted against OPA1 (Anti-Mouse, BD Biosciences, #612606) and mtHsp70 (Anti-Mouse, Clone: JG1, Invitrogen MA3028) antibody.

Western blots:

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 #11836170001) and phosphatase inhibitors (Sigma-Aldrich), kept on ice for 10 min and centrifuged at 16,000 x g for 10 minutes. Protein supernatants were collected, and protein concentration was estimated by DC protein assay (BioRad). For SDS-PAGE, 30 µg of proteins were mixed with 1X Lammeli buffer containing β-mercaptoethanol, then subjected to SDS-PAGE, transferred to a nitrocellulose membrane and blotted with the indicated antibodies (DRP1 (Anti-Mouse, 1:1000; BD Transduction Laboratories, #611112), Phospho-DRP1 (Ser616) (Anti-Rabbit, 1:1000; Cell signaling technology, (Clone D9A1) #4494S), MFN1 (Anti-Rabbit, 1:1000; Abcam, [EPR21953-74] #ab221661), OPA1(Anti-Mouse, BD Biosciences, #612606), NDUFA9(Anti-Rabbit, 1:1000; Abcam #ab128744), OSCP (Anti-Mouse, 1:1000; Santa Cruz Biotechnology (clone A-8) #sc-365162) UQCRC2 ((Anti-Mouse, 1:1000; Santa Cruz Biotechnology (clone G-10) #sc-390378) Phospho-FAK^{Y397} (Anti-Rabbit, 1:1000; ; Invitrogen, (clone 31H5L17) # 700255), FAK(Anti-Rabbit, 1:1000;) TOM20 (Anti-Rabbit, 1:1000; Abcam, ab186735), SOX-2 (Anti-Rabbit, 1:1000 #AB5603), ALDH1A1 (Anti-Mouse, 1:1000, # SC-166362), HRP-tagged Actin (1:10,000). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Jackson Immunoresearch) and visualized by enhanced chemiluminescence (Thermo Fisher scientific) using a Bio-Rad imaging system.

OCR measurements:

Oxygen Consumption Rates (OCR) were measured using the Seahorse XF Cell Mito Stress Test Kit (Agilent, Cat. 103708-100) with a Seahorse XFe96 analyzer (Agilent), following the instructions provided by the manufacturer. For mammospheres attached to fibronectin, a total of 40,000 cells from the 3D culture were placed onto a Seahorse XF96 culture plate coated with

fibronectin (10 µg/ml), in DMEM media supplemented with hEGF (10 ng/ml), insulin (10 µg/ml), and B-27 supplement (Dey et al., 2009). The cells were incubated for 6 hours at 37°C, 10% CO_2 promoting attachment of mammospheres. Following this, a transition to XF DMEM medium was carried out. Meanwhile, 40,000 AM and MS cells were seeded onto Seahorse XF96 plates in XF DMEM, supplemented as indicated by the manufacturer. AM cells were seeded and allowed to attach for 6 hours while MS were transferred at the time of the assay. The cells were then incubated at 37 °C without CO_2 for 1 hour, before proceeding immediately to perform OCR and ECAR analyses. OCR was monitored by sequentially introducing oligomycin (1 µM), FCCP (0.25 µM), and rotenone/antimycin A (0.5 µM) and non-mitochondrial respiration was subtracted for the calculation of mitochondrial respiration. Basal OCR and ATP-linked OCR were calculated according to manufacturer instructions.

ATP and Lactate assay:

ATP assays were performed using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega). Cells were seeded at a density of 20,000 cells per well in 96-well plates (transparent and opaque walled plates) and incubated overnight at 37ºC. For attached mammospheres, cells in suspension culture were seeded onto fibronectin-coated wells six hours prior to performing the assay. Where indicated, the cells in monolayer, mammospheres and mammosphere attached condition were then treated with Oligomycin (10 µM) for 1 hour before the end of the incubation period. CellTiter-Glo® Reagent was added to the cells in the opaque plates at a 1:1 ratio and incubated at room temperature for 10 minutes in a dark environment. The luminescence emitted was then measured at a 100% gain value in a plate reader.

The transparent plate was used for both lactate and protein estimation. For lactate estimation, a portion of the medium (10 µl) from each well was transferred to fresh wells in duplicate and the volume adjusted to 50 µl with PBS. Subsequently, 50 µl of Lactate reagent from Promega (# J5021) was added and incubated for 10 minutes at room temperature. The resulting absorbance was recorded at a wavelength of 450 nm in a plate reader. The media from the transparent plates was removed leaving the cells at the bottom. The cells were rinsed with PBS and subsequently lysed (10 mM Tris-HCl (pH 7.4), 1mM EDTA, 150 mM NaCl, and 1% Triton X-100). The protein concentration in this lysate was estimated using the DC protein assay (BioRad) and used to normalize ATP and lactate values.

TMRM experiments:

Mitochondrial membrane potential was evaluated using the potentiometric dye tetramethyl rhodamine methyl ester (TMRM; Sigma Aldrich) at a final concentration of 10 nm. For the assay, 3 x 10^5 cells/well of both adherent and suspension cells were seeded in a 6-well microplate and

left overnight in an incubator (5% CO_2, 37°C). Each experiment was performed in duplicate. The cells were then incubated 30 minutes with TMRM (10 nm) in DMEM. Note: for suspension cells, mammospheres were trypsinized to obtain single cells before TMRM treatment. Adherent cells were then washed with PBS, trypsinized, and brought to a single cell suspension. Suspension cells were collected and washed with PBS. The blank control cells untreated with TMRM were also collected. The cell pellets were then resuspended in PBS and analyzed using flow cytometry. The TMRM fluorescence was examined using a Cytoflex FACS analyzer (Beckman) with excitation at 514 nm and detection at 570 nm.

Animals:

All animal experiments adhered to the principles outlined in the guidelines of laboratory animal care (NIH publication no. 85-23, revised in 1985) and were conducted with the approval of the Institutional Animal Ethical Committee, Government of India (Registration Number 885/ac/05/CPCSEA). The experimental procedures strictly followed the regulations specified in the Indian Laws of Animal Protection (ILAP). Female BALB/c mice, weighing 20 ± 2g, were obtained and housed under standard laboratory conditions, maintaining a temperature of 25 ± 2°C, relative humidity of 50 ± 15%, and a 12-hour light-dark cycle throughout the experiment. The mice were provided with food pellets, and water was given *ad libitum* to facilitate acclimatization to the laboratory environment. (Elia et al., 2017).

Mice mammary tumor development:

Mammary tumors were developed in female BALB/c mice (n=3). Viable triple negative mouse mammary carcinoma cells 4T1 (10^5 cells) suspended in 200 µl of PBS were injected into the inguinal 4th mammary fat pad of mice. Tumors were allowed to develop for 14 days. Normal mice received only 200 µl of PBS. Eventually, the normal mammary gland and the mice-mammary tumors were excised from the control and mammary tumor-bearing animals respectively. The tissues were then processed for subsequent experiments (Elia et al., 2017).

Detection of $ALDH^+$ population:

The $ALDH^+$ population was detected by assessing aldehyde dehydrogenase (ALDH) enzyme activity using the ALDEFLUOR™ kit from STEMCELL Technologies, following the manufacturers guidelines. Briefly, single cell suspensions at a concentration of 1×10^6 cells/mL were incubated in aldefluor assay buffer with 5µL of activated BODIPY-aminoacetaldehyde (BAAA), the ALDH reagent, for 45 minutes at 37 °C. To serve as a negative control, 5 µL of diethylaminobenzaldehyde (DEAB), a specific ALDH1 enzyme inhibitor, was added along with BAAA. The fluorescence intensity, indicative of ALDH activity, was measured using FACSAria™ III, BD Biosciences. Subsequently, both $ALDH^-$ and $ALDH^+$ populations were

sorted and processed for protein extraction. Data analysis was performed using BD FACSDiva™ software.

Data analysis and statistics:

All graphs and statistical analysis were done using R. Immunofluorescence data were quantified and images representative of at least three independent experiments shown (exact "n" are in the quantification figures). Data is represented as average ± SD as specified in figure legends. Statistical significance was determined using Student's t test (between 2 groups) or one-way ANOVA with a Tukey post hoc test (multiple comparisons).

Conflict of Interest:

The authors have no conflict of interest to declare.

Acknowledgements.

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to MG, a team grant from Fonds de Recherche du Québec-Nature et Technologies (#2024-PR-326143) to MG and LCC., a Department of Science, Technology and Biotechnology, Government of West Bengal grant to UC (Sanction No.: 140 (Sanc.)-BT/P/Budget/RD-75/2017 dated 16.11.2018), as well as by the DST-FIST program of the Department of Zoology, University of Calcutta (UC). PG was supported by a Queen Elizabeth II Diamond Jubilee Scholarship and an FRQ-NT scholarship, PDT was supported by a fellowship from the Council for Scientific and Industrial Research, Government of India (File No.-09/028(1066)/2018-EMR-I). PG and PM received a Université du Québec à Trois-Rivières Programme d'aide à l'internationalisation de la science scholarship. WF received PhD fellowships from the Armand-Frappier Foundation and Fonds de la Recherche du Québec-Santé (FRQS). LCC is receiving a research scholar (Senior) salary support from FRQS.

References

ADEBAYO, M., SINGH, S., SINGH, A. P. & DASGUPTA, S. 2021. Mitochondrial fusion and fission: The fine-tune balance for cellular homeostasis. *FASEB J,* 35**,** e21620.

ANDRZEJEWSKI, S., KLIMCAKOVA, E., JOHNSON, R. M., TABARIES, S., ANNIS, M. G., MCGUIRK, S., NORTHEY, J. J., CHENARD, V., SRIRAM, U., PAPADOPOLI, D. J., SIEGEL, P. M. & ST-PIERRE, J. 2017. PGC-1alpha Promotes Breast Cancer Metastasis and Confers Bioenergetic Flexibility against Metabolic Drugs. *Cell Metab,* 26**,** 778-787 e5.

APONTE, P. M. & CAICEDO, A. 2017. Stemness in Cancer: Stem Cells, Cancer Stem Cells, and Their Microenvironment. *Stem Cells Int,* 2017**,** 5619472.

BAHARVAND, H., HASHEMI, S. M., KAZEMI ASHTIANI, S. & FARROKHI, A. 2006. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. *Int J Dev Biol,* 50**,** 645-52.

BAKER, E. L., LU, J., YU, D., BONNECAZE, R. T. & ZAMAN, M. H. 2010. Cancer cell stiffness: integrated roles of three-dimensional matrix stiffness and transforming potential. *Biophys J,* 99**,** 2048-57.

BAN, T., ISHIHARA, T., KOHNO, H., SAITA, S., ICHIMURA, A., MAENAKA, K., OKA, T., MIHARA, K. & ISHIHARA, N. 2017. Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin. *Nat Cell Biol,* 19**,** 856-863.

BOLDOGH, I. R., YANG, H. C., NOWAKOWSKI, W. D., KARMON, S. L., HAYS, L. G., YATES, J. R., 3RD & PON, L. A. 2001. Arp2/3 complex and actin dynamics are required for actin-based mitochondrial motility in yeast. *Proc Natl Acad Sci U S A,* 98**,** 3162-7.

BUBB, K., HOLZER, T., NOLTE, J. L., KRUGER, M., WILSON, R., SCHLOTZER-SCHREHARDT, U., BRINCKMANN, J., ALTMULLER, J., ASZODI, A., FLEISCHHAUER, L., CLAUSEN-SCHAUMANN, H., PROBST, K. & BRACHVOGEL, B. 2021. Mitochondrial respiratory chain function promotes extracellular matrix integrity in cartilage. *J Biol Chem,* 297**,** 101224.

BUXBOIM, A., IVANOVSKA, I. L. & DISCHER, D. E. 2010. Matrix elasticity, cytoskeletal forces and physics of the nucleus: how deeply do cells 'feel' outside and in? *J Cell Sci,* 123**,** 297-308.

CAI, L., SHI, L., PENG, Z., SUN, Y. & CHEN, J. 2023. Ageing of skeletal muscle extracellular matrix and mitochondria: finding a potential link. *Ann Med,* 55**,** 2240707.

CALALB, M. B., POLTE, T. R. & HANKS, S. K. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol,* 15**,** 954-63.

CAO, Y. L., MENG, S., CHEN, Y., FENG, J. X., GU, D. D., YU, B., LI, Y. J., YANG, J. Y., LIAO, S., CHAN, D. C. & GAO, S. 2017. MFN1 structures reveal nucleotide-triggered dimerization critical for mitochondrial fusion. *Nature,* 542**,** 372-376.

CASE, L. B. & WATERMAN, C. M. 2015. Integration of actin dynamics and cell adhesion by a three-dimensional, mechanosensitive molecular clutch. *Nat Cell Biol,* 17**,** 955-63.

CHAKRABARTI, R., JI, W. K., STAN, R. V., DE JUAN SANZ, J., RYAN, T. A. & HIGGS, H. N. 2018. INF2-mediated actin polymerization at the ER stimulates mitochondrial calcium uptake, inner membrane constriction, and division. *J Cell Biol,* 217**,** 251-268.

CHEN, H., MCCAFFERY, J. M. & CHAN, D. C. 2007. Mitochondrial fusion protects against neurodegeneration in the cerebellum. *Cell,* 130**,** 548-62.

CHEN, H., VERMULST, M., WANG, Y. E., CHOMYN, A., PROLLA, T. A., MCCAFFERY, J. M. & CHAN, D. C. 2010. Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell,* 141**,** 280-9.

CHEN, K., WANG, Y., DENG, X., GUO, L. & WU, C. 2021a. Extracellular matrix stiffness regulates mitochondrial dynamics through PINCH-1- and kindlin-2-mediated signalling. *Current Research in Cell Biology,* 2.

CHEN, K., ZHANG, C., LING, S., WEI, R., WANG, J. & XU, X. 2021b. The metabolic flexibility of quiescent CSC: implications for chemotherapy resistance. *Cell Death Dis,* 12**,** 835.

CHEUNG, T. H. & RANDO, T. A. 2013. Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol,* 14**,** 329-40.

DEY, D., SAXENA, M., PARANJAPE, A. N., KRISHNAN, V., GIRADDI, R., KUMAR, M. V., MUKHERJEE, G. & RANGARAJAN, A. 2009. Phenotypic and functional characterization of human mammary stem/progenitor cells in long term culture. *PLoS One,* 4**,** e5329.

DISCHER, D. E., MOONEY, D. J. & ZANDSTRA, P. W. 2009. Growth factors, matrices, and forces combine and control stem cells. *Science,* 324**,** 1673-7.

DJEUNGOUE-PETGA, M. A., LURETTE, O., JEAN, S., HAMEL-COTE, G., MARTIN-JIMENEZ, R., BOU, M., CANNICH, A., ROY, P. & HEBERT-CHATELAIN, E. 2019. Intramitochondrial Src kinase links mitochondrial dysfunctions and aggressiveness of breast cancer cells. *Cell Death Dis,* 10**,** 940.

DONG, F., ZHU, M., ZHENG, F. & FU, C. 2022. Mitochondrial fusion and fission are required for proper mitochondrial function and cell proliferation in fission yeast. *FEBS J,* 289**,** 262-278.

DZOBO, K. & DANDARA, C. 2023. The Extracellular Matrix: Its Composition, Function, Remodeling, and Role in Tumorigenesis. *Biomimetics (Basel),* 8.

ELIA, I., BROEKAERT, D., CHRISTEN, S., BOON, R., RADAELLI, E., ORTH, M. F., VERFAILLIE, C., GRUNEWALD, T. G. P. & FENDT, S. M. 2017. Proline metabolism supports metastasis formation and could be inhibited to selectively target metastasizing cancer cells. *Nat Commun,* 8**,** 15267.

ENDO, H., OWADA, S., INAGAKI, Y., SHIDA, Y. & TATEMICHI, M. 2020. Metabolic reprogramming sustains cancer cell survival following extracellular matrix detachment. *Redox Biol,* 36**,** 101643.

FRANTZ, C., STEWART, K. M. & WEAVER, V. M. 2010. The extracellular matrix at a glance. *J Cell Sci,* 123**,** 4195-200.

GATTI, P., SCHIAVON, C., MANOR, U. & GERMAIN, M. 2023. Mitochondria- and ER-associated actin are required for mitochondrial fusion. *bioRxiv*.

GOMES, L. C., DI BENEDETTO, G. & SCORRANO, L. 2011. Essential amino acids and glutamine regulate induction of mitochondrial elongation during autophagy. *Cell Cycle,* 10**,** 2635-9.

GUEDOUARI, H., OULD AMER, Y., PICHAUD, N. & HEBERT-CHATELAIN, E. 2021. Characterization of the interactome of c-Src within the mitochondrial matrix by proximity-dependent biotin identification. *Mitochondrion,* 57**,** 257-269.

GUEDOUARI, H., SAVOIE, M. C., JEAN, S., DJEUNGOUE-PETGA, M. A., PICHAUD, N. & HEBERT-CHATELAIN, E. 2020. Multi-omics Reveal that c-Src Modulates the Mitochondrial Phosphotyrosine Proteome and Metabolism According to Nutrient Availability. *Cell Physiol Biochem,* 54**,** 517-537.

HATCH, A. L., GUREL, P. S. & HIGGS, H. N. 2014. Novel roles for actin in mitochondrial fission. *J Cell Sci,* 127**,** 4549-60.

HOFMANN, A., MISHRA, J. S., YADAV, P., DANGUDUBIYYAM, S. V., BLESSON, C. S. & KUMAR, S. 2023. PFOS Impairs Mitochondrial Biogenesis and Dynamics and Reduces Oxygen Consumption in Human Trophoblasts. *J Environ Sci Public Health,* 7**,** 164-175.

HORTON, E. R., BYRON, A., ASKARI, J. A., NG, D. H. J., MILLON-FREMILLON, A., ROBERTSON, J., KOPER, E. J., PAUL, N. R., WARWOOD, S., KNIGHT, D., HUMPHRIES, J. D. & HUMPHRIES, M. J. 2015. Definition of a consensus integrin adhesome and its dynamics during adhesion complex assembly and disassembly. *Nat Cell Biol,* 17**,** 1577-1587.

HYNES, R. O. 2009. The extracellular matrix: not just pretty fibrils. *Science,* 326**,** 1216-9.

KANTETI, R., BATRA, S. K., LENNON, F. E. & SALGIA, R. 2016. FAK and paxillin, two potential targets in pancreatic cancer. *Oncotarget,* 7**,** 31586-601.

KASAHARA, A. & SCORRANO, L. 2014. Mitochondria: from cell death executioners to regulators of cell differentiation. *Trends Cell Biol,* 24**,** 761-70.

KITAMURA, S., YANAGI, T., IMAFUKU, K., HATA, H., ABE, R. & SHIMIZU, H. 2017. Drp1 regulates mitochondrial morphology and cell proliferation in cutaneous squamous cell carcinoma. *J Dermatol Sci,* 88**,** 298-307.

KO, H. S., KIM, J. S., CHO, S. M., LEE, H. J., AHN, K. S., KIM, S. H. & LEE, E. O. 2014. Urokinase-type plasminogen activator expression and Rac1/WAVE-2/Arp2/3 pathway are blocked by pterostilbene to suppress cell migration and invasion in MDA-MB-231 cells. *Bioorg Med Chem Lett,* 24**,** 1176-9.

KOROBOVA, F., RAMABHADRAN, V. & HIGGS, H. N. 2013. An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2. *Science,* 339**,** 464-7.

LI, G. B., ZHANG, H. W., FU, R. Q., HU, X. Y., LIU, L., LI, Y. N., LIU, Y. X., LIU, X., HU, J. J., DENG, Q., LUO, Q. S., ZHANG, R. & GAO, N. 2018. Mitochondrial fission and mitophagy depend on cofilin-mediated actin depolymerization activity at the mitochondrial fission site. *Oncogene,* 37**,** 1485-1502.

LI, S., XU, S., ROELOFS, B. A., BOYMAN, L., LEDERER, W. J., SESAKI, H. & KARBOWSKI, M. 2015. Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission. *J Cell Biol,* 208**,** 109-23.

LIESA, M. & SHIRIHAI, O. S. 2013. Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab,* 17**,** 491-506.

LIU, P., TANG, H., SONG, C., WANG, J., CHEN, B., HUANG, X., PEI, X. & LIU, L. 2018. SOX2 Promotes Cell Proliferation and Metastasis in Triple Negative Breast Cancer. *Front Pharmacol,* 9**,** 942.

LU, H., GUO, L., KAWAZOE, N., TATEISHI, T. & CHEN, G. 2009. Effects of poly(L-lysine), poly(acrylic acid) and poly(ethylene glycol) on the adhesion, proliferation and chondrogenic differentiation of human mesenchymal stem cells. *J Biomater Sci Polym Ed,* 20**,** 577-89.

LURETTE, O., GUEDOUARI, H., MORRIS, J. L., MARTIN-JIMENEZ, R., ROBICHAUD, J. P., HAMEL-COTE, G., KHAN, M., DAUPHINEE, N., PICHAUD, N., PRUDENT, J. & HEBERT-CHATELAIN, E. 2022. Mitochondrial matrix-localized Src kinase regulates mitochondrial morphology. *Cell Mol Life Sci,* 79**,** 327.

MANOR, U., BARTHOLOMEW, S., GOLANI, G., CHRISTENSON, E., KOZLOV, M., HIGGS, H., SPUDICH, J. & LIPPINCOTT-SCHWARTZ, J. 2015. A mitochondria-anchored isoform of the actin-nucleating spire protein regulates mitochondrial division. *Elife,* 4.

MIERKE, C. T. 2019. The matrix environmental and cell mechanical properties regulate cell migration and contribute to the invasive phenotype of cancer cells. *Rep Prog Phys,* 82**,** 064602.

MOORE, A. S., WONG, Y. C., SIMPSON, C. L. & HOLZBAUR, E. L. 2016. Dynamic actin cycling through mitochondrial subpopulations locally regulates the fission-fusion balance within mitochondrial networks. *Nat Commun,* 7**,** 12886.

MORRIS, B. A., BURKEL, B., PONIK, S. M., FAN, J., CONDEELIS, J. S., AGUIRRE-GHISO, J. A., CASTRACANE, J., DENU, J. M. & KEELY, P. J. 2016. Collagen Matrix Density Drives the Metabolic Shift in Breast Cancer Cells. *EBioMedicine,* 13**,** 146-156.

ONISHI, M., YAMANO, K., SATO, M., MATSUDA, N. & OKAMOTO, K. 2021. Molecular mechanisms and physiological functions of mitophagy. *EMBO J,* 40**,** e104705.

PAPALAZAROU, V., ZHANG, T., PAUL, N. R., JUIN, A., CANTINI, M., MADDOCKS, O. D. K., SALMERON-SANCHEZ, M. & MACHESKY, L. M. 2020. The creatine-phosphagen system is mechanoresponsive in pancreatic adenocarcinoma and fuels invasion and metastasis. *Nat Metab,* 2**,** 62-80.

PASCUAL, G., AVGUSTINOVA, A., MEJETTA, S., MARTIN, M., CASTELLANOS, A., ATTOLINI, C. S., BERENGUER, A., PRATS, N., TOLL, A., HUETO, J. A., BESCOS, C., DI CROCE, L. & BENITAH, S. A. 2017. Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature,* 541**,** 41-45.

PASTO, A., BELLIO, C., PILOTTO, G., CIMINALE, V., SILIC-BENUSSI, M., GUZZO, G., RASOLA, A., FRASSON, C., NARDO, G., ZULATO, E., NICOLETTO, M. O., MANICONE, M., INDRACCOLO, S. & AMADORI, A. 2014. Cancer stem cells from epithelial ovarian cancer patients privilege oxidative phosphorylation, and resist glucose deprivation. *Oncotarget,* 5**,** 4305-19.

PATTEN, D. A., WONG, J., KHACHO, M., SOUBANNIER, V., MAILLOUX, R. J., PILON-LAROSE, K., MACLAURIN, J. G., PARK, D. S., MCBRIDE, H. M., TRINKLE-MULCAHY, L., HARPER, M. E., GERMAIN, M. & SLACK, R. S. 2014. OPA1-dependent

cristae modulation is essential for cellular adaptation to metabolic demand. *EMBO J,* 33**,** 2676-91.

PORPORATO, P. E., PAYEN, V. L., PEREZ-ESCUREDO, J., DE SAEDELEER, C. J., DANHIER, P., COPETTI, T., DHUP, S., TARDY, M., VAZEILLE, T., BOUZIN, C., FERON, O., MICHIELS, C., GALLEZ, B. & SONVEAUX, P. 2014. A mitochondrial switch promotes tumor metastasis. *Cell Rep,* 8**,** 754-66.

PORPORATO, P. E. & SONVEAUX, P. 2015. Paving the way for therapeutic prevention of tumor metastasis with agents targeting mitochondrial superoxide. *Mol Cell Oncol,* 2**,** e968043.

PRICE, L. S., LENG, J., SCHWARTZ, M. A. & BOKOCH, G. M. 1998. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell,* 9**,** 1863-71.

QI, Y., YAN, L., YU, C., GUO, X., ZHOU, X., HU, X., HUANG, X., RAO, Z., LOU, Z. & HU, J. 2016. Structures of human mitofusin 1 provide insight into mitochondrial tethering. *J Cell Biol,* 215**,** 621-629.

RAIS, A., HUSAIN, A., HASAN, G. M. & HASSAN, M. I. 2023. A review on regulation of cell cycle by extracellular matrix. *Int J Biol Macromol,* 232**,** 123426.

RAMBOLD, A. S., KOSTELECKY, B., ELIA, N. & LIPPINCOTT-SCHWARTZ, J. 2011. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci U S A,* 108**,** 10190-5.

REN, L., CHEN, X., CHEN, X., LI, J., CHENG, B. & XIA, J. 2020. Mitochondrial Dynamics: Fission and Fusion in Fate Determination of Mesenchymal Stem Cells. *Front Cell Dev Biol,* 8**,** 580070.

RIVADENEIRA, D. B., CAINO, M. C., SEO, J. H., ANGELIN, A., WALLACE, D. C., LANGUINO, L. R. & ALTIERI, D. C. 2015. Survivin promotes oxidative phosphorylation, subcellular mitochondrial repositioning, and tumor cell invasion. *Sci Signal,* 8**,** ra80.

ROMANI, P., NIRCHIO, N., ARBOIT, M., BARBIERI, V., TOSI, A., MICHIELIN, F., SHIBUYA, S., BENOIST, T., WU, D., HINDMARCH, C. C. T., GIOMO, M., URCIUOLO, A., GIAMOGANTE, F., ROVERI, A., CHAKRAVARTY, P., MONTAGNER, M., CALI, T., ELVASSORE, N., ARCHER, S. L., DE COPPI, P., ROSATO, A., MARTELLO, G. & DUPONT, S. 2022. Mitochondrial fission links ECM mechanotransduction to metabolic redox homeostasis and metastatic chemotherapy resistance. *Nat Cell Biol,* 24**,** 168-180.

SABOUNY, R. & SHUTT, T. E. 2021. The role of mitochondrial dynamics in mtDNA maintenance. *J Cell Sci,* 134.

SERASINGHE, M. N., WIEDER, S. Y., RENAULT, T. T., ELKHOLI, R., ASCIOLLA, J. J., YAO, J. L., JABADO, O., HOEHN, K., KAGEYAMA, Y., SESAKI, H. & CHIPUK, J. E. 2015. Mitochondrial division is requisite to RAS-induced transformation and targeted by oncogenic MAPK pathway inhibitors. *Mol Cell,* 57**,** 521-36.

SHIRAISHI, T., VERDONE, J. E., HUANG, J., KAHLERT, U. D., HERNANDEZ, J. R., TORGA, G., ZARIF, J. C., EPSTEIN, T., GATENBY, R., MCCARTNEY, A., ELISSEEFF, J. H., MOONEY, S. M., AN, S. S. & PIENTA, K. J. 2015. Glycolysis is the primary bioenergetic pathway for cell motility and cytoskeletal remodeling in human prostate and breast cancer cells. *Oncotarget,* 6**,** 130-43.

SILVA RAMOS, E., MOTORI, E., BRUSER, C., KUHL, I., YEROSLAVIZ, A., RUZZENENTE, B., KAUPPILA, J. H. K., BUSCH, J. D., HULTENBY, K., HABERMANN, B. H., JAKOBS, S., LARSSON, N. G. & MOURIER, A. 2019. Mitochondrial fusion is required for regulation of mitochondrial DNA replication. *PLoS Genet,* 15**,** e1008085.

SONG, Z., GHOCHANI, M., MCCAFFERY, J. M., FREY, T. G. & CHAN, D. C. 2009. Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. *Mol Biol Cell,* 20**,** 3525-32.

SUNDBERG, L. J., GALANTE, L. M., BILL, H. M., MACK, C. P. & TAYLOR, J. M. 2003. An endogenous inhibitor of focal adhesion kinase blocks Rac1/JNK but not Ras/ERK-dependent signaling in vascular smooth muscle cells. *J Biol Chem,* 278**,** 29783-91.

THOMAS, M., DAVIS, T., LOOS, B., SISHI, B., HUISAMEN, B., STRIJDOM, H. & ENGELBRECHT, A. M. 2018. Autophagy is essential for the maintenance of amino acids and ATP levels during acute amino acid starvation in MDAMB231 cells. *Cell Biochem Funct,* 36**,** 65-79.

TIAN, C., CLAUSER, K. R., OHLUND, D., RICKELT, S., HUANG, Y., GUPTA, M., MANI, D. R., CARR, S. A., TUVESON, D. A. & HYNES, R. O. 2019. Proteomic analyses of ECM during pancreatic ductal adenocarcinoma progression reveal different contributions by tumor and stromal cells. *Proc Natl Acad Sci U S A,* 116**,** 19609-19618.

TIAN, G. A., XU, W. T., ZHANG, X. L., ZHOU, Y. Q., SUN, Y., HU, L. P., JIANG, S. H., NIE, H. Z., ZHANG, Z. G., ZHU, L., LI, J., YANG, X. M. & YAO, L. L. 2023. CCBE1 promotes mitochondrial fusion by inhibiting the TGFbeta-DRP1 axis to prevent the progression of hepatocellular carcinoma. *Matrix Biol,* 117**,** 31-45.

VISAVADIYA, N. P., KEASEY, M. P., RAZSKAZOVSKIY, V., BANERJEE, K., JIA, C., LOVINS, C., WRIGHT, G. L. & HAGG, T. 2016. Integrin-FAK signaling rapidly and potently promotes mitochondrial function through STAT3. *Cell Commun Signal,* 14**,** 32.

WANG, R., LV, Q., MENG, W., TAN, Q., ZHANG, S., MO, X. & YANG, X. 2014. Comparison of mammosphere formation from breast cancer cell lines and primary breast tumors. *J Thorac Dis,* 6**,** 829-37.

WANG, R., MISHRA, P., GARBIS, S. D., MORADIAN, A., SWEREDOSKI, M. J. & CHAN, D. C. 2021. Identification of new OPA1 cleavage site reveals that short isoforms regulate mitochondrial fusion. *Mol Biol Cell,* 32**,** 157-168.

WU, Y., ZANOTELLI, M. R., ZHANG, J. & REINHART-KING, C. A. 2021. Matrix-driven changes in metabolism support cytoskeletal activity to promote cell migration. *Biophys J,* 120**,** 1705-1717.

YANES, B. & RAINERO, E. 2022. The Interplay between Cell-Extracellular Matrix Interaction and Mitochondria Dynamics in Cancer. *Cancers (Basel),* 14.

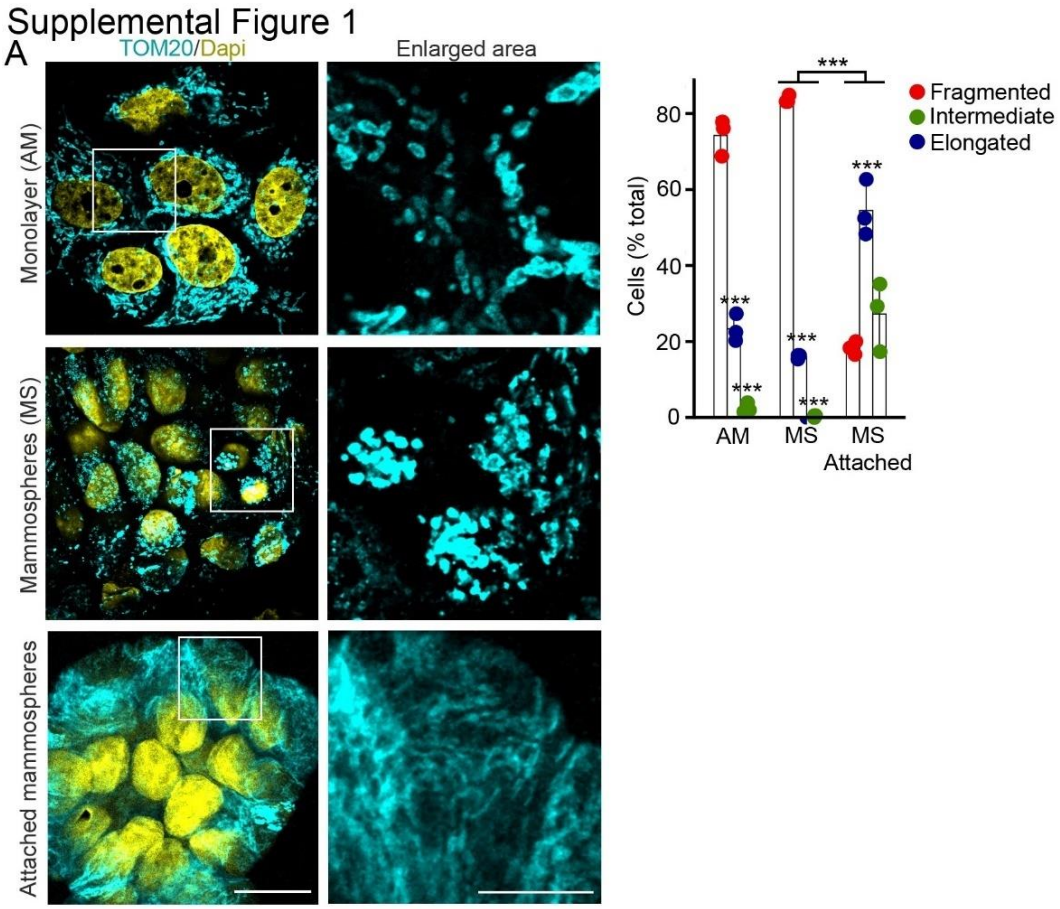
YANG, Q., WANG, L., LIANG, Y., HE, Q., SUN, Q., LUO, J., CAO, H., FANG, Y., ZHOU, Y., YANG, J., WEN, P. & JIANG, L. 2023. Loss of UCP2 causes mitochondrial fragmentation by OMA1-dependent proteolytic processing of OPA1 in podocytes. *FASEB J,* 37**,** e23265.

YANG, Y., WANG, Y., CHE, X., HOU, K., WU, J., ZHENG, C., CHENG, Y., LIU, Y., HU, X. & ZHANG, J. 2021. Integrin alpha5 promotes migration and invasion through the FAK/STAT3/AKT signaling pathway in icotinib-resistant non-small cell lung cancer cells. *Oncol Lett,* 22**,** 556.

YAO, C. H., WANG, R., WANG, Y., KUNG, C. P., WEBER, J. D. & PATTI, G. J. 2019. Mitochondrial fusion supports increased oxidative phosphorylation during cell proliferation. *Elife,* 8.

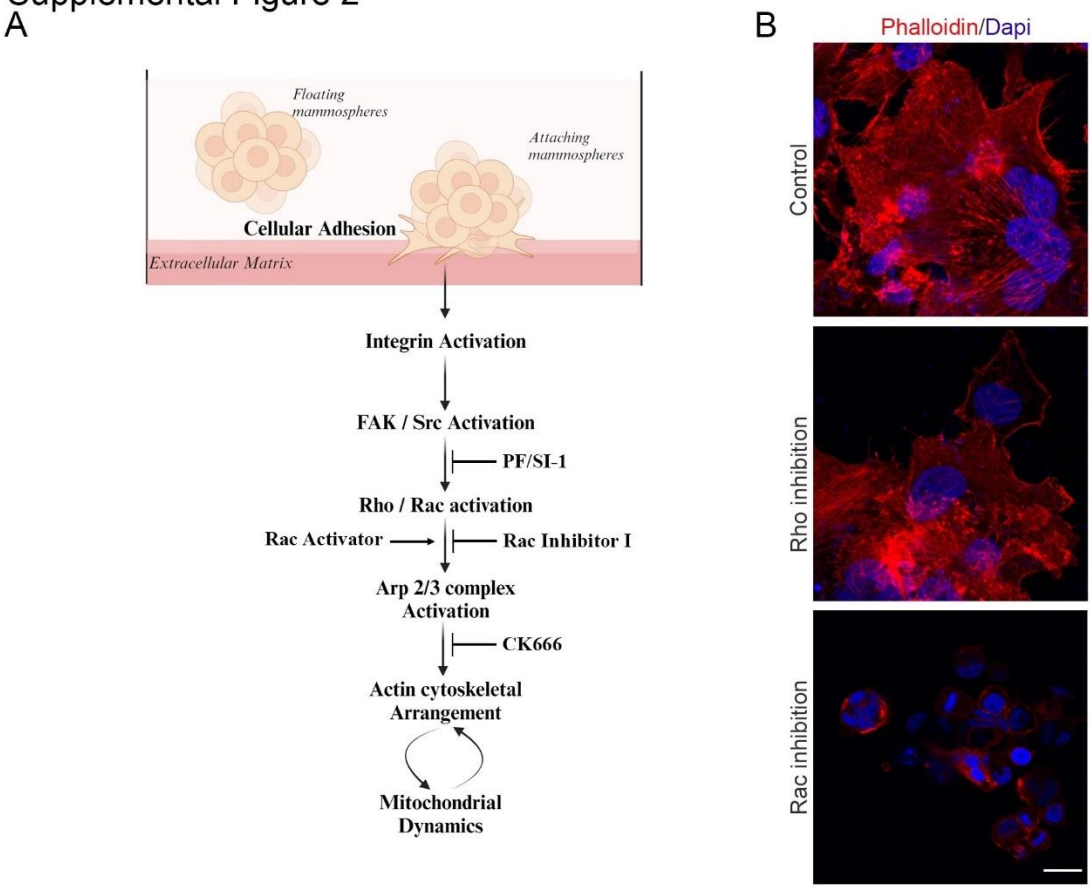
ZHAO, J., ZHANG, J., YU, M., XIE, Y., HUANG, Y., WOLFF, D. W., ABEL, P. W. & TU, Y. 2013. Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene,* 32**,** 4814-24.

Supplementary Figures



Supplemental Figure 1: Mitochondrial fragmentation in MCF7 mammospheres in suspension. (A) MCF7 were grown as AM, MS or MS attached for 6 hours on glass coverslips, and their mitochondria were marked with an antibody against TOM20 (mitochondria, cyan; with nuclei stained with DAPI, yellow). (B) Representative images. Scale bar 10 µm. (C) Quantification of 3 independent experiments, with each point represents an individual experiment. Bars show the average ± SD. *** $p<0.001$. Two-way ANOVA.

Supplemental Figure 2



Supplemental Figure 2. Mitochondrial fragmentation in attached MS upon Rac inhibition. (A) Model for the ECM-induced cytoskeletal pathway regulating mitochondrial dynamics in mammospheres. (B) Representative images of mammospheres attached to fibronectin for 1 hour in the absence or the presence of the Rac1 (Rac Inhibitor III; 2 μM) and Rho (Rho Inhibitor I, 10μM) inhibitors and marked for Actin (Phalloidin, Red) and nuclei (Dapi, Blue). Scale bar 10 μm.

CHAPTER III

MITOCHONDRIA- AND ER-ASSOCIATED ACTIN ARE REQUIRED FOR MITOCHONDRIAL FUSION

Priya Gatti[1,2,3], Cara Schiavon[4,5], Uri Manor[4,6#], Marc Germain[1,2,3#]

Under review; Pre-print available in bioRxiv (2023).
Doi: 10.1101/2023.06.13.544768

[1]Groupe de Recherche en Signalisation Cellulaire and Département de Biologie Médicale, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada
[2]Centre d'Excellence en Recherche sur les Maladies Orphelines - Fondation Courtois, Université du Québec à Montréal, Montréal, Québec, Canada
[3]Réseau Intersectoriel de Recherche en Santé de l'Université du Québec (RISUQ)
[4]Waitt Advanced Biophotonics Center, Salk Institute for Biological Studies, La Jolla, CA, United States.
[5]Molecular and Cell Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA, United States
[6]Department of Cell and Developmental Biology, School of Biological Sciences, University of California, San Diego, La Jolla, CA, United States.

Correspondence:
Uri Manor: urmanor@ucsd.edu
Marc Germain: marc.germain1@uqtr.ca

Abstract:

Mitochondria play a crucial role in the regulation of cellular metabolism and signalling. Mitochondrial activity is modulated by the processes of mitochondrial fission and fusion, which are required to properly balance respiratory and metabolic functions, transfer material between mitochondria, and remove defective mitochondria. Mitochondrial fission occurs at sites of contact between the endoplasmic reticulum (ER) and mitochondria, and is dependent on the formation of actin filaments that drive mitochondrial constriction and the recruitment and activation of the fission GTPase DRP1. The requirement for mitochondria- and ER-associated actin filaments in mitochondrial fission remains unclear, and the role of actin in mitochondrial fusion remains entirely unexplored. Here we show that preventing the formation of actin filaments on either mitochondria or the ER disrupts both mitochondrial fission and fusion. We show that fusion but not fission is dependent on Arp2/3, whereas both fission and fusion are dependent on INF2 formin-dependent actin polymerization. Together, our work introduces a novel method for perturbing organelle-associated actin filaments, and demonstrates a previously unknown role for actin in mitochondrial fusion.

Introduction:

Mitochondria play a crucial role in the regulation of cellular metabolism and signalling, thereby controlling key cellular processes including apoptosis, cell fate decisions, and inflammation. These activities are regulated by mitochondrial dynamics, the process of fusion and fission which controls mitochondrial shape, size, and function. Mitochondrial dynamics allows for the exchange of components, such as lipids and proteins, and the removal of damaged or defective mitochondria, maintaining the overall function and quality of mitochondria[1-5].

Mitochondrial dynamics are controlled by dynamin-related GTPases: mitochondrial fission requires Dynamin Related Protein 1 (DRP1), while mitochondrial fusion depends on mitofusins (MFN1 and MFN2) for the fusion of the outer membrane and OPA1 for the fusion of the inner membrane. In addition to this core machinery, mitochondrial fission requires specific contacts with the endoplasmic reticulum (ER), which marks fission sites[6]. These ER-mitochondria contact sites then work as platforms to recruit the fission machinery. One key event driving mitochondrial fission is the activation of the ER-anchored formin protein Inverted Formin 2 (INF2), resulting in actin polymerization at ER-mitochondria contacts[7-9]. These actin filaments have been proposed to further promote ER-mitochondria contacts in a Myo19-dependent manner [10] and are used by non-muscle myosin II to constrict the mitochondrion[11,12]. This partial constriction then allows the recruitment of the fission protein DRP1, followed by DRP1 GTPase-dependent scission of the

mitochondrion. Consistent with this, inhibiting actin polymerization, Myosin II activity, or INF2 decreases DRP1 oligomerization on mitochondria, suggesting that actin and myosin are involved in the recruitment and activation of DRP1[13-16].

Regulation of mitochondrial fusion is less well understood. Recent studies have demonstrated that the ER is present not only at mitochondrial fission sites but also at fusion sites[17] where it was suggested to accelerate the fusion process[18]. Consistent with this, loss of ER-mitochondria contact sites decreases the number of fusion and fission events[19]. Nevertheless, the exact role of the ER in mitochondrial fusion remains to be elucidated. One possibility is that it acts as a platform to recruit the fusion machinery, similarly to what occurs during fission[6,7,17].

Previous research supports important roles for mitochondria-associated actin ("mito-actin") aside from its role in mitochondrial fission. This includes mitochondria quality control[20] and the regulation of mitochondrial transport[21-24]. Similarly, actin plays an important role in recruiting and activating dynamin family GTPases to various cell compartments[25,26], and has been shown to act in concert with dynamin to promote cell-to-cell fusion[27]. As the ER likely acts as a platform to recruit the machinery for both mitochondrial fission and fusion, and ER-anchored INF2 and actin play crucial roles in GTPase-mediated mitochondrial fission, we hypothesize that mito- and ER-associated actin ("ER-actin") is important for GTPase-mediated mitochondrial fission and fusion.

Here, we uncover a previously unappreciated role for actin in the regulation of mitochondrial fusion, and provide new evidence for a role for both mito- and ER-actin in fission and fusion. Using fluorescent protein-tagged mitochondria- and ER-targeted actin nanobodies, we demonstrate that actin marks sites of mitochondrial fission and fusion. Importantly, we show that disrupting actin filaments specifically on mitochondria or the ER disrupts both mitochondrial fission and fusion. In addition, inhibiting the actin nucleation protein complex Arp2/3 or ER-anchored INF2 differentially impact the balance between mitochondrial fission and fusion. Our work also reveals two mechanistically distinct types of fusion events (tip-to-tip vs. tip-to-side) based on their requirement for mitochondrial actin. We show that mito-actin is associated with the immobilized "receiving" mitochondrion during tip-to-side fusion. Together, our data reveals a key organizational and regulatory role for mito- and ER-actin in both mitochondrial fission and fusion.

Results:

Actin accumulates at sites of mitochondrial fusion

To monitor the presence of actin at the site of mitochondrial fusion, we used GFP-tagged actin nanobodies (termed Actin Chromobodies (AC)) targeted to the outer mitochondrial membrane with the c-terminal transmembrane domain of Fis1 (AC-mito)[8]. AC-mito moves freely within the mitochondrial membrane giving a diffused signal. However, when the AC probe binds to mito-actin, it becomes immobilised, resulting in a higher intensity signal at the actin-associated site. We have previously used these probes to show the accumulation of actin at sites of mitochondrial fission[8].

We transfected primary human fibroblasts with AC-mito and a control mCherry construct targeted to mitochondria with the same Fis1 transmembrane domain as AC-mito (mCherry-mito) to label mitochondria (Figure 1A) and imaged them live with confocal microscopy. We then analysed the presence of the AC probe at the site of mitochondrial fission and fusion. Consistent with previous studies, we observed AC-mito accumulation at fission sites (Figure 1B), validating the approach. Importantly, we also observed AC-mito accumulation at fusion sites in most fusion events (Figure 1A-B, video 1). To confirm AC-mito accumulation at fusion and fission sites, we measured its specific enrichment relative to adjacent mitochondria. We observed a 2-fold increase in enrichment of AC-mito signal at fusion (Fig.1C) and fission (Fig. 1D) events, which was absent when measured using the mCherry-mito signal, the latter consistently spreading evenly across mitochondrial surfaces (Fig. 1C-D). Our data thus clearly demonstrates that AC-mito is enriched at sites of mitochondrial fusion, indicating the presence of mito-actin at these sites.

As sites of mitochondrial fusion are associated with actin, and fusion is mediated by the mitochondrial outer membrane dynamin-related GTPase MFNs, we asked whether promoting MFN-mediated fusion is sufficient to drive actin polymerization on mitochondria. To stimulate fusion, we overexpressed MFN1 in our primary fibroblasts. As predicted, MFN1 overexpression leads to clustering and fusion of mitochondria[28,29] (Figure 1E). These changes were accompanied by a large increase in AC-mito signal (Figure 1E-F). Thus, promoting MFN1-mediated mitochondrial fusion is sufficient to stimulate actin association with mitochondria.

Mitochondrial fusion occurs in two distinct ways: tip-to-tip fusion, where the end of one mitochondrion fuses with the end of another, and tip-to-side fusion, where the end of a mitochondrion fuses at the side of another mitochondrion[19] (Figure 2A). Notably, our data revealed that 75% of fusion events involved tip-to-side fusion (Figure 2B). Interestingly, actin was much more likely to be present at tip-to-side events (88%) than end-to end events (50%) (Fig. 2C), suggesting that actin predominantly favors tip-to-side fusion. We also observed that in most fusion events, one mitochondrion remains immobile while the other is mobile (Figure 2D, “Still”). This

was however not the case for tip-to-tip fusions, where both mitochondria involved were usually mobile (Figure 2E).

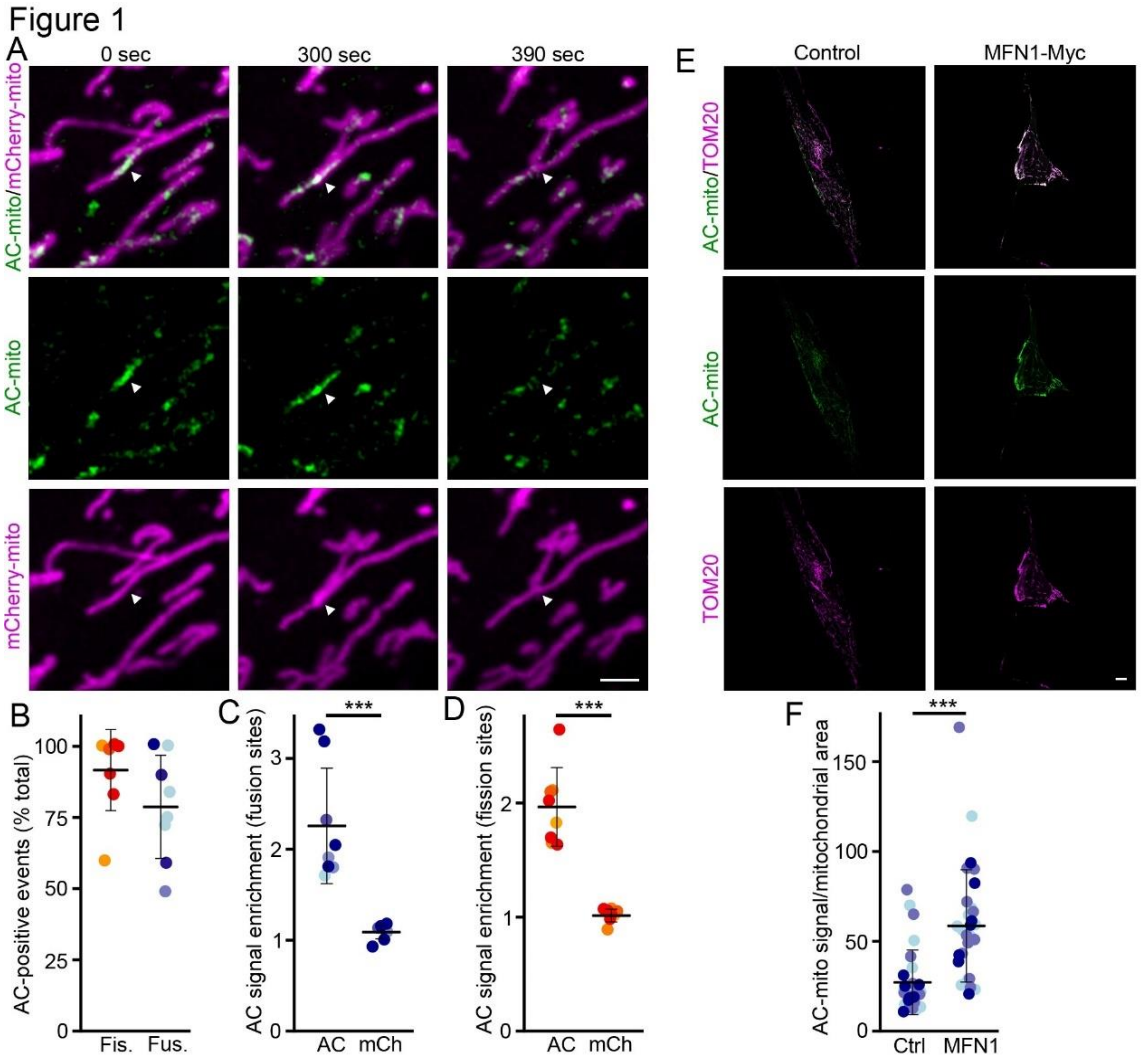
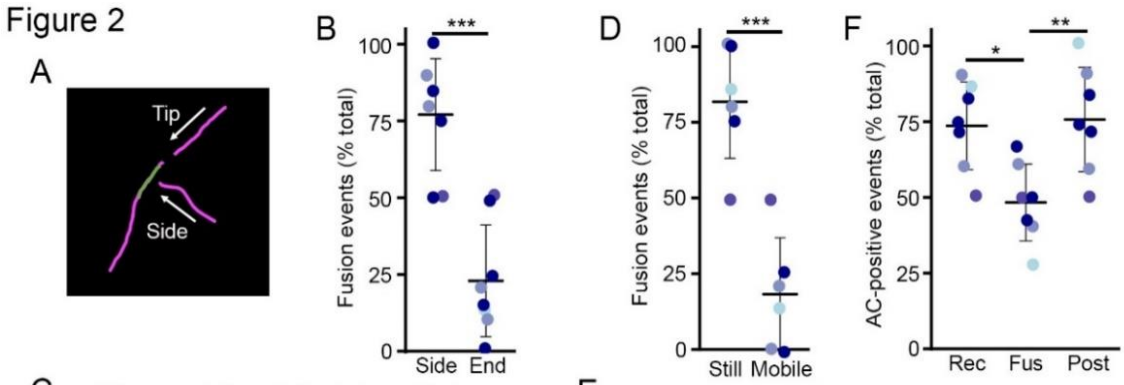


Figure 1. Actin marks the site of mitochondrial fusion. (A) Representative fusion event showing the enrichment of AC-mito at the fusion site (arrowhead). Cells were transfected with mCherry-mito (mitochondria, magenta) and AC-mito (actin, green). Scale bar 2µm (B) Quantification of fission (Fis.) and fusion (Fus.) events positive for AC-mito. Each point represents an individual cell, with 10 cells quantified in 4 independent experiments. Bars show the average ± SD. (C-D) AC-mito signal enrichment at fusion (C) and fission (D) sites in cells transfected as in A. Signal intensity at the event site relative to an adjacent site on the mitochondrial network was quantified for AC-mito (AC) and mCherry-mito (mCh). Each point represents an individual cell, with 8 cells quantified in 4 independent experiments. Bars show the average ± SD. *** $p<0.001$. (E-F) Representative image showing AC-mito signal (green) in cells transfected with mCherry-Fis1 (Control) or MFN1-Myc. Cells were then fixed, and mitochondria labeled with an antibody against TOM20 (magenta). Quantification of the AC-mito signal is shown in (F). Each point represents an individual cell, with 28 control and 32 MFN1-Myc cells quantified in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$. Scale bar 10 µm.

Our subsequent objective was to identify which of the two fusing mitochondria recruited actin. Since most fusion events involved one immobile mitochondrion, we selected this subset for further analysis. We referred to the immobile mitochondrion as the "receiving mitochondrion" and the moving one as the "fusing mitochondrion". By examining the AC-mito enrichment at the receiving and fusing ends of the mitochondrion, we discovered that actin is present to a greater extent on the immobilized receiving mitochondrion than on the fusing mitochondrion and that the AC-mito signal persists until the end of the fusion event (Figure 2F). Altogether, our findings indicate mitochondria-associated actin acts as a marker for the sites of mitochondrial fusion.



C AC accumulation relative to type of fusion

Type	AC-positive	Percent AC
Tip-to-tip	4/8	50%
Tip-to-side	30/34	88%

E Presence of one mobile end relative to type of fusion

Type	Mobile	Percent Mobile
Tip-to-tip	4/5	80%
Tip-to-side	0/26	0%

Figure 2. Actin is mainly associated with Tip-to-side mitochondrial fusion (A) Schematic representation of Tip-to-tip and Side-to-End fusion events. (B-C) Quantification of fusion events as end to-side (Side) or tip-to-tip (End)(B), and their corresponding association with AC-mito (C). Each point represents an individual cell, with 10 cells (E) quantified in 4 independent experiments. Bars show the average ± SD. *** $p<0.001$. Total events from the quantified cells are shown in (C). (D-E) Quantification of fusion events containing one immobile mitochondrion (Still) or two mobile mitochondria (Mobile) (D) and their corresponding association with AC-mito (E). Each point represents an individual cell 8 cells quantified in 4 independent experiments. Bars show the average ± SD. *** $p<0.001$. Total events from the quantified cells are shown in (E). (F) Quantification of AC-mito signal associated with each of the fusing mitochondria (the receiving mitochondrion (Rec) and the fusing mitochondrion (Fus)) as well as with the fusion site one frame post-fusion (Post). Each point represents an individual cell, with 9 cells quantified in 4 independent experiments. Bars show the average ± SD. * $p<0.05$, ** $p<0.01$

Actin-associated mitochondrial fusion sites recruit the endoplasmic reticulum (ER)

During mitochondrial fission, the ER acts as a platform to recruit actin-regulatory proteins (INF2) and actin-binding motor proteins (non-muscle myosin II) leading to mitochondrial constriction and DRP1-mediated scission of the mitochondrion[6,7,11,13,15,30]. To measure the presence of ER at fusion sites, we transfected primary human fibroblasts with mCherry targeted to the ER using the c-terminal domain of cytochrome b5 (mCherry-ER) and EGFP targeted to the mitochondrial

matrix (CCO-IRES), and imaged them live with confocal microscopy. Consistent with previous reports, most fusion and fission events were associated with the presence of ER (Figure 3A)[10,17-19]. We then tested whether ER-actin was also recruited at mitochondrial fusion sites. To specifically label ER-actin, we used our previously reported variation of the AC probe targeted to the ER using the c-terminal domain of cytochrome b5 (AC-ER)[8]. We simultaneously transfected cells with AC-ER and mCherry-mito and assessed the recruitment of AC-ER at fusion sites (Figure 3B). Similar to AC-mito, most fusion and fission events were positive for AC-ER (Figure 3C). The enrichment of AC-ER was confirmed at fusion (Figure 3D) and fission sites (Figure 3E) as for AC-mito. Like AC-mito, most receiving mitochondria were AC-ER-positive, while fusing mitochondria were less likely to show AC-ER signal (Figure 3F). Therefore, our results indicate that both mito- and ER-actin are present at mitochondrial fusion sites.

During mitochondrial fission, mito-actin accumulates first at the fission site, followed by ER-actin filaments prior to fission [8]. We thus investigated the temporal relationship between the recruitment of AC-ER and AC-mito at fusion sites. To determine when mito- and ER-actin filaments are recruited to the mitochondrial fusion site, cells were co-transfected with AC-Mito, a halo-tagged version of AC-ER, and mCherry-mito. We then measured ER- and mito-actin recruitment relative to fusion events. Similar to what we observed for mitochondrial fission, we found that AC-mito was recruited first to the future fusion site, followed by AC-ER (Figure 3G, fusion occurs at timepoint 0). Specifically, AC-ER was recruited at the fusion site at an average of 100 seconds after AC-mito (Fig. 3H), suggesting that recruitment of actin on mitochondria, not the ER, is the primary event for mitochondrial fusion.

Mitochondria-associated actin is required for fusion

As our results show that actin is associated with mitochondrial fusion, we then asked whether it was required for fusion. Actin depolymerizing drugs impact many subcellular processes and signaling pathways, making it difficult to distinguish primary from secondary effects. Thus, the ability to selective remove actin from subcellular compartments of interest would be enormously valuable for the cell biology research community. To address this gap and selectively remove actin from mitochondria, we modified a recently described Disassembly-promoting, encodable Actin tool (DeAct), a ~120 amino acid domain of the actin-regulating protein Gelsolin (Gelsolin Segment 1 - aa 53-176 – GS1) that sequesters actin monomers [31]. To specifically target GS1 to mitochondria or the ER, we fused it to the c-terminal transmembrane domain of Fis1 (DeAct-mito) or cytochrome b5 (DeAct-ER).

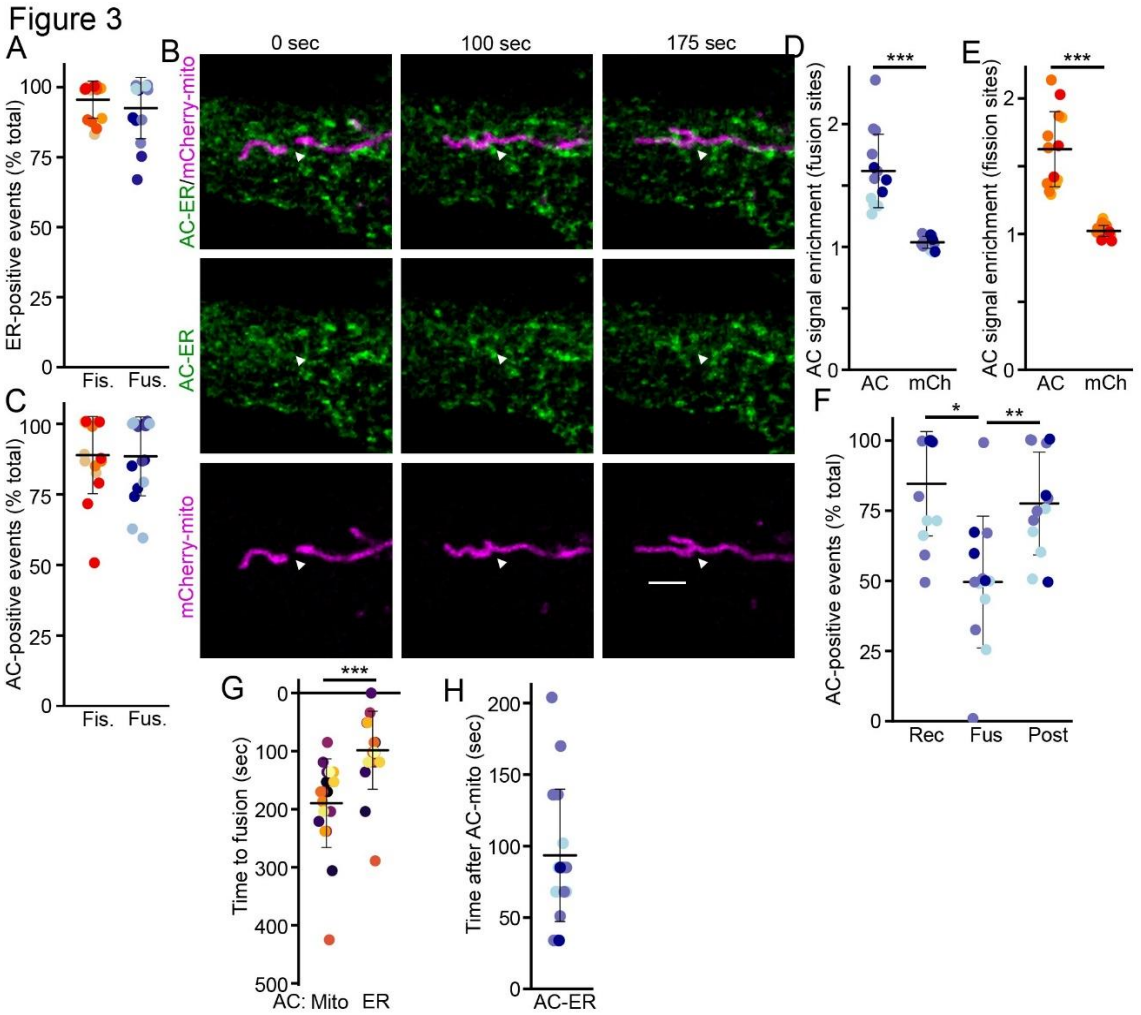


Figure 3. ER-associated actin is present at the site of mitochondrial fusion. (A) Quantification of fission (Fis.) and fusion (Fus.) events associated with ER. Each point represents an individual cell, with 15 cells quantified in 4 independent experiments. Bars show the average ± SD. (B) Representative fusion event showing the enrichment of AC-ER at the fusion site (arrowhead). Cells were transfected with mCherry-mito (mitochondria, magenta) and AC-ER (actin, green). Scale bar 2 µm. (C) Quantification of fission (Fis.) and fusion (Fus.) events positive for AC-mito. Each point represents an individual cell, with 13 cells quantified in 3 independent experiments. (D-E) AC-ER signal enrichment at fusion (D) and fission (E) sites in cells transfected as in B. Signal intensity at the event site relative to an adjacent site on the ER network was quantified for AC-ER (AC) and mCherry-ER (mCh). Each point represents an individual cell, with 13 cells quantified in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$. (F) Quantification of AC-ER signal associated with each of the fusing mitochondria (the receiving mitochondrion (Rec) and the fusing mitochondrion (Fus)) as well as with the fusion site one frame post-fusion (Post). Each point represents an individual cell, with 13 cells quantified in 3 independent experiments. Bars show the average ± SD. * $p<0.05$, ** $p<0.01$. (G-H) Kinetics of recruitment of AC-mito and AC-ER at the fusion site showing AC-ER recruitment relative to AC-mito (G) and recruitment of either marker relative to the time of fusion (H). Each point represents an individual event (20 events total in 4 cells within 3 independent experiments). *** $p<0.001$

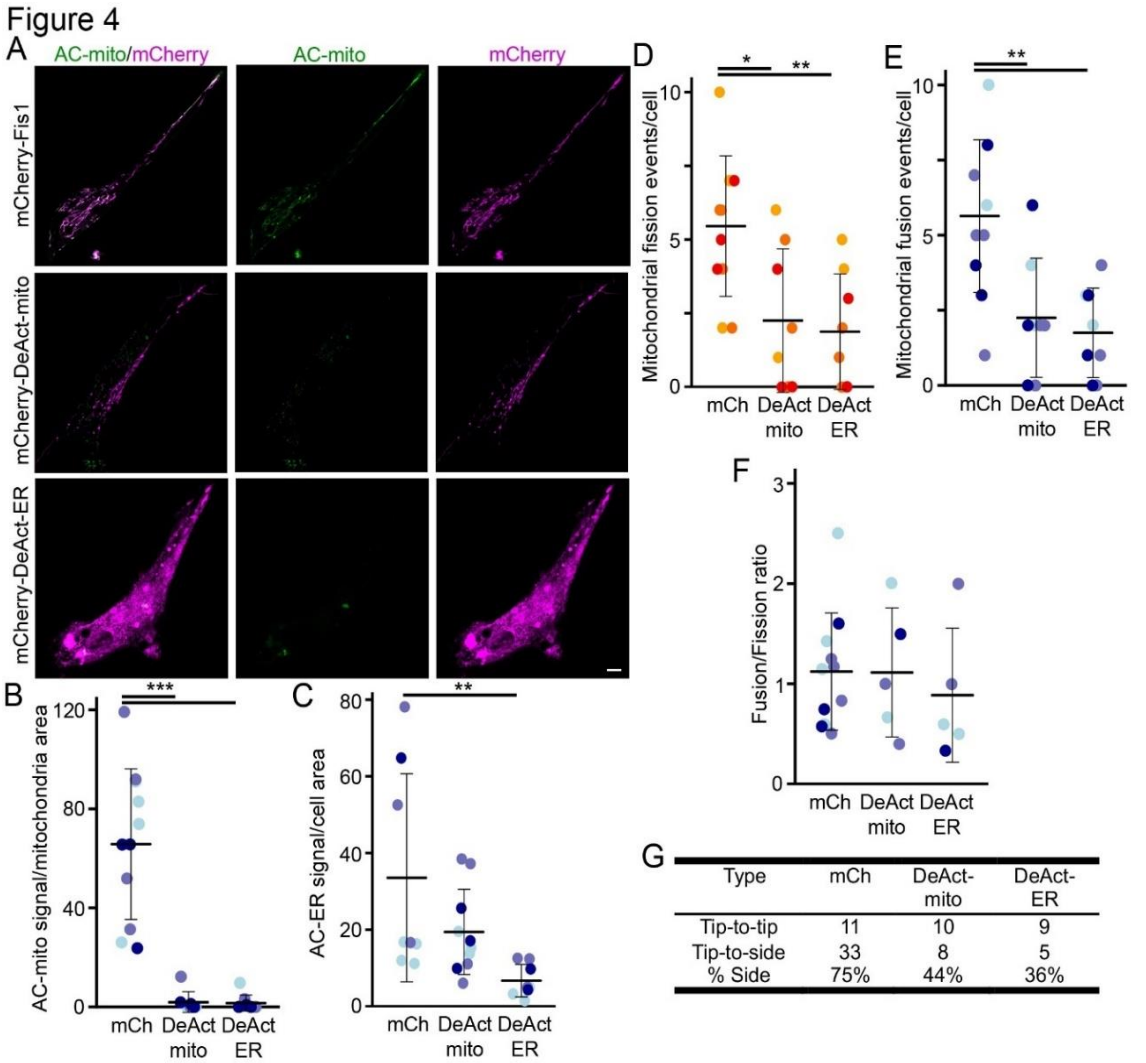
After confirming the proper localisation of the constructs (Supp. Figure 1A-B), we first determined if organelle-targeted DeAct prevented actin accumulation on mitochondria by co-transfecting DeAct-mScarlet (mito or ER) with AC-mito or AC-ER. Consistent with the ability of DeAct to locally disrupt actin filaments, DeAct-mito significantly decreased AC-Mito signal intensity (Figure 4A-B), while AC-ER showed a steep decline when transfected with DeAct-ER (Figure 4C, Sup. Figure 2). We also tested whether the organelle-targeted DeActs specifically affected their target organelle. DeAct-mito did not significantly affect AC-ER signal, highlighting the selectivity of the probe (Figure 4A-C). In contrast, DeAct-ER was also as efficient as DeAct-mito in preventing actin accumulation on mitochondria (Fig. 4A-B), possibly as a result of the ER making numerous contacts with mitochondria. Overall, both constructs remained selective for organelle-bound actin as no obvious alterations were found when actin filaments were labeled with phalloidin (Supp. Figure 1C).

Having validated the DeActs, we then evaluated their effect on mitochondrial fusion and fission events. Consistent with the proposed role for ER-associated actin in mitochondrial fission, both DeAct-mito and DeAct-ER significantly decreased mitochondrial fission rates (Fig. 4D). Importantly, mitochondrial fusion was also significantly decreased by both DeActs (Figure 4E), indicating that mitochondria-associated actin is required for mitochondrial fusion. We then determined whether fusion or fission was more affected by DeActs by taking the ratio of fusion over fission for each condition. There was no significant difference in the ratio compared to control cells (mCherry-mito) (~1), suggesting that DeActs blocked both processes (Figure 4F). These data therefore demonstrate an essential role for actin in the regulation of not only mitochondrial fission but also mitochondrial fusion.

As previously stated, the majority of fusion events that occurred in the presence of actin were of tip-to-side pattern (Figure 2A-C). We thus then asked whether one type of fusion event was more affected by DeActs. Consistent with tip-to-side events being mostly associated with actin, these were affected to a large extent by DeActs (Fig. 4G). In contrast, tip-to-tip events persisted in DeAct-mito and DeAct-ER transfected cells (Figure 4G), suggesting that the residual fusion observed with DeActs is largely the consequence of actin-independent tip-to-tip fusion events.

Arp2/3-dependent actin polymerization is required for mitochondrial fusion

Having demonstrated that actin is required for mitochondrial fusion, we next defined the role of actin-polymerizing proteins in this process. Most actin polymerization is regulated by one of two families of actin-regulatory proteins: Arp2/3 is responsible for the formation of branched actin filaments and actin patches, while formins promote the formation of parallel actin filaments.



Type	mCh	DeAct-mito	DeAct-ER
Tip-to-tip	11	10	9
Tip-to-side	33	8	5
% Side	75%	44%	36%

Figure 4. Mitochondrial actin is required for both fission and fusion. (A) Representative images showing the loss of AC-mito signal (green) in cells transfected with DeAct-mito or DeAct-ER (magenta). (B-C) Quantification of AC-mito (B) and AC-ER (C) signal in cells transfected as in (A). Each point represents an individual cell, with at least 8 cells quantified in 3 independent experiments for each condition. Bars show the average ± SD. *** $p<0.001$, ** $p<0.01$. (D-F) Quantification of the number of fission (D) and fusion (E) events, as well as the fusion/fission ratio (F) in cells transfected with the indicated DeAct probe, AC-mito and mCherry-Fis1 (to label mitochondria). Each point represents an individual cell, with 11 (mCh), 8 (DeAct-mito) and 9 (DeAct-ER) cells quantified in 3 independent experiments. Bars show the average ± SD. ** $p<0.01$, * $p<0.05$. (G) Quantification of fusion events as end to-side (Side) or tip-to-tip (End). The total number of events for 8 cells in 3 experiments is shown for each condition.

To study the role of Arp2/3 in mitochondrial fusion, we selectively inhibited this nucleation complex using CK-666[32]. Firstly, there was a notable loss of AC-mito signal following a 60 min CK666 treatment (Fig. 5A-B), supporting a role for Arp2/3 in the generation of mitochondrial actin. The CK-666-dependent loss of mitochondria-associated actin was also associated with a

significant decrease in the number of mitochondrial fusion events (Figure 5C). In contrast, CK-666 did not significantly affect mitochondrial fission, resulting in a decrease in the fusion/fission ratio (Figure 5C). As the loss of mitochondrial actin due to the expression of DeAct-mito selectively inhibited tip-to-side fusion (Fig. 4H), we also examined the effect of CK-666 on tip-to-tip and tip-to-side fusion. Consistent with the DeAct data, CK-666 selectively prevented tip-to-side fusion (Fig. 5D). A similar decrease in mitochondrial fusion was also observed in the breast cancer line MDA-MB-231 following CK-666 treatment (Figure 5E). To further confirm our findings, we knocked down Arp2 in MDA-MB-231 cells (Figure 5F) and quantified mitochondrial fission and fusion. Consistent with the CK666 data, mitochondrial fusion was significantly reduced in cells where Arp2 was knocked down. In contrast, mitochondrial fission was not significantly affected, leading to a decrease in fusion/fission ratio (Figure 5G).

To further demonstrate that the Arp2/3 complex is required for mitochondrial fusion, we used a mitochondrial fusion assay based on a mitochondria-targeted photoactivable GFP (PA-GFP). In this assay, diffusion of the activated GFP in a subpopulation of mitochondria following mitochondrial fusion leads to a decrease in GFP fluorescence over time (Figure 5H, Supp. Figure 3). Consistent with Arp2/3 inhibition preventing mitochondrial fusion, PAGFP signal showed a lower rate of decrease in CK666-treated cells compared to control cells, leading to a significantly smaller decrease in GFP signal 3 minutes after activation (Figure 5H).

Altogether, our data shows that inhibition of the Arp2/3 complex significantly impairs mitochondrial fusion, indicating that Arp2/3-dependent actin polymerization plays a crucial role in regulating mitochondrial fusion.

Formin inhibition predominantly affects mitochondrial fission

While our results demonstrate a role for Arp2/3 in mitochondrial fusion, formin proteins are also known to be important for actin dynamics and can both collaborate and compete with Arp2/3 in forming dynamic actin structures[33-35]. In addition, both Arp2/3 and formin proteins are known to be important for normal mitochondrial fission dynamics[36-38]. We thus asked whether formin-mediated actin polymerization could also play a role in mitochondrial fusion. For this, we first used the Formin FH2 Domain Inhibitor (SMIFH2) to inhibit formin-mediated elongation of actin filaments. As with Arp2/3 complex inhibition, formin inhibition resulted in a significant loss of AC-mito signal, but this reduction in mitochondrial actin was less than for Arp2/3 inhibition (Figure 6A-C, Arp inhibition in 5A-B). Consistent with the role of formins in mitochondrial fission, fission was greatly affected by SMIFH2 (Fig. 6D). On the other hand, while SMIFH2 did reduce mitochondrial fusion, this effect was less important than for fission, leading to a significant

increase in the fusion/fission ratio (Fig. 6D). Nevertheless, as for Arp inhibition, SMIFH2 selectively prevented tip-to-side fusion events (Fig. 6E), consistent with actin being predominantly required for this type of fusion event.

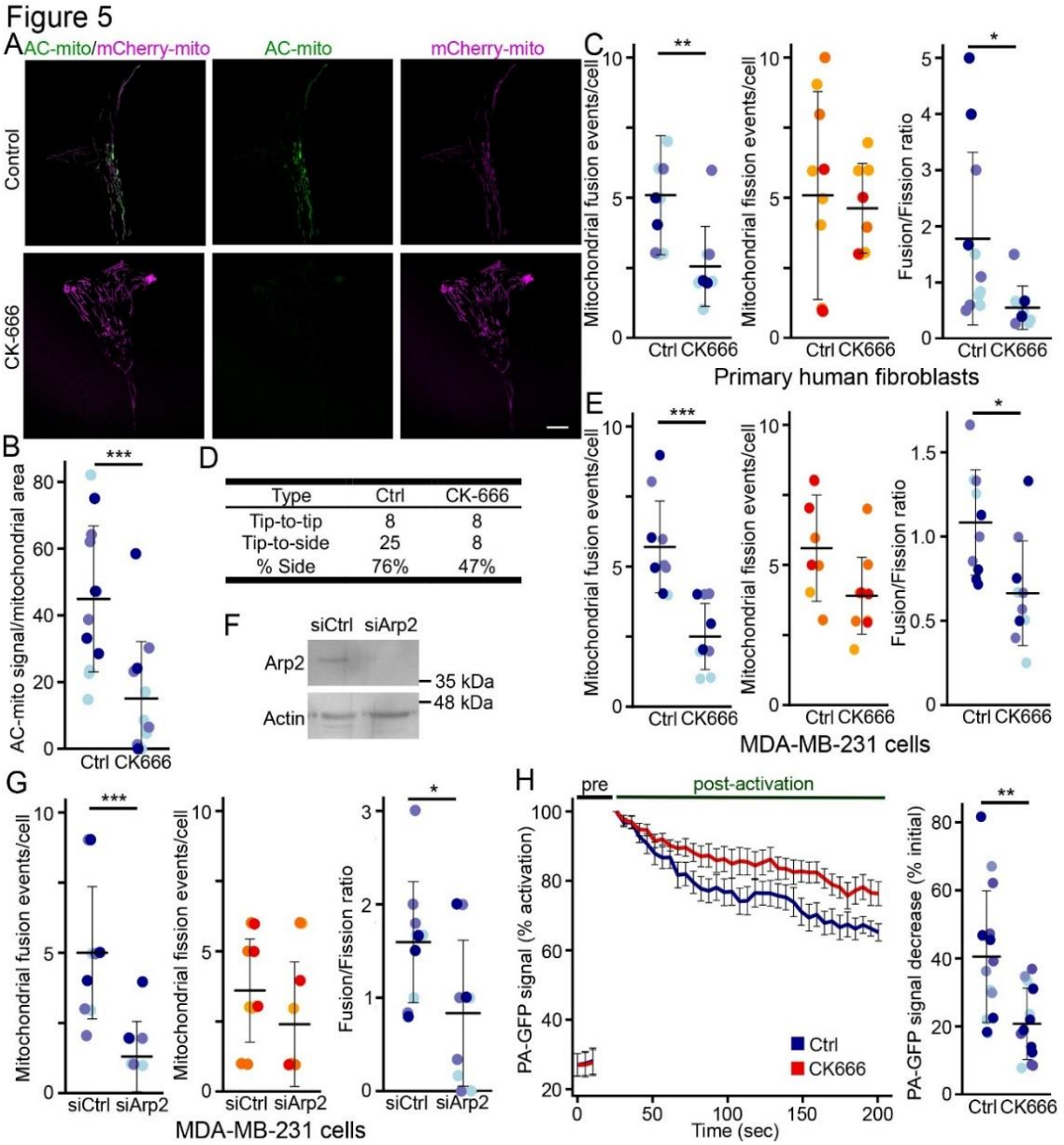


Figure 5. The Arp2/3 complex regulates mitochondrial fusion. (A) Representative images showing the loss of AC-mito signal in primary fibroblasts transfected with AC-mito (green) and mCherry-Fis1 (magenta), and treated with the Arp2 inhibitor CK666. Scale bar 10 µm (B) Quantification of AC-mito signal in cells transfected as in (A). Each point represents an individual cell, with 12 control and 13 CK666 cells quantified in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$. (C) Quantification of the number of fusion (Left, blue) and fission (Middle, orange) events, as well as the fusion/fission ratio (Right) in cells transfected as in (A) and treated as indicated. Each point represents an individual cell, with 12 (control) and 9 (CK666) cells quantified in 3 independent experiments. Bars show the average ± SD. ** $p<0.01$, * $p<0.05$.

(D) Quantification of fusion events as end to-side (Side) or tip-to-tip (End). The total number of events for 9 cells in 3 experiments is shown for each condition. (E) Quantification of the number of fusion (Left, blue) and fission (Middle, orange) events, as well as the fusion/fission ratio (Right) in MDA-MB-231 cells stably expressing mitochondria-targeted GFP and treated as indicated. Each point represents an individual cell, with 10 cells quantified for each condition in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$, * $p<0.05$. (F) Western blot showing Arp2 expression in cells treated with a control siRNA (siCtrl) or a siRNA against ARP2 (siARP2). Actin is used as a loading control. (G) Quantification of the number of fusion (Left, blue) and fission (Middle, orange) events, as well as the fusion/fission ratio (Right) in MDA-MB-231 cells stably expressing mitochondria-targeted GFP transfected with siRNAs as in (F). Each point represents an individual cell, with 10 cells quantified for each condition in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$, ** $p<0.01$. (H) Mitochondrial fusion assay. Human primary fibroblasts were transfected with photoactivatable-GFP (PA-GFP), treated as indicated and imaged before (pre) and after activation with the 405 nm laser. Fluorescence traces (Left) and the quantification of the loss of fluorescence at 3 min relative to the initial time post-activation (Right) are shown. Each point (Right) represents an individual cell, with 14 ctrl and 16 CK666 cells quantified in 4 independent experiments. Bars show the average ± SD. ** $p<0.01$

The formin protein INF2 is the only known ER-anchored actin regulatory protein and is required for mitochondrial fission[7,9]. We thus specifically examined the role of INF2 in fusion by measuring fission and fusion events in U2OS cells lacking INF2[9]. Consistent with our formin inhibitor data, INF2 deletion affected both fission and fusion (Figure 6F). However, contrary to the formin inhibitor, both processes were affected to a similar extent, resulting in a fusion/fission ratio that was similar between control and INF2 KO cells (Figure 6F). Importantly, as with our other manipulations of the actin cytoskeleton, INF2 deletion selectively affected tip-to-side fusion. Altogether, our results indicate that actin is required for tip-to-side events which constitute the majority of fusion events within cells, and that both Arp2/3 and INF2 play a role in this process.

Discussion

Actin plays a critical role in regulating organelle dynamics within cells, contributing to their movement, positioning, and organization[39-41]. Actin also plays an important role in mitochondrial fission, where it facilitates constriction of mitochondria, the recruitment of DRP1, and the activation of its GTPase activity [9,15,16,42]. The recruitment of actin to fission sites was originally shown under fission-inducing conditions, but has recently been demonstrated under unstimulated conditions by the use of organelle-targeted actin chromobodies[8]. It was also proposed that the requirement for actin filaments for mitochondrial fission is context-dependent, with only ~50% of fission events coinciding with actin polymerization[43]. However, Schiavon et al, 2020 and this current study revealed a much higher incidence of actin filaments at fission sites, likely as a result of using the more sensitive organelle-targeted actin probes.

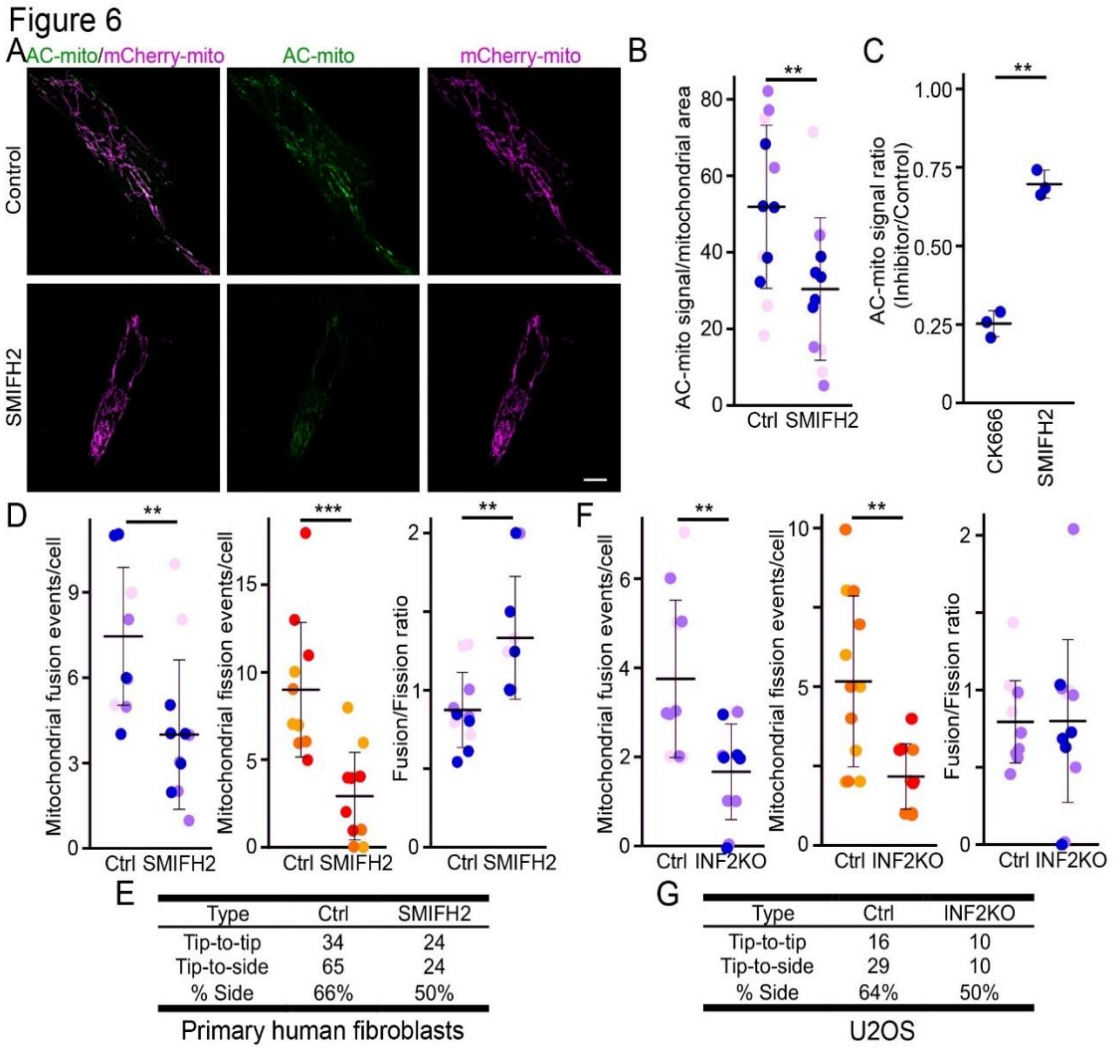


Figure 6. Formin-dependent regulation of mitochondrial fusion. (A) Representative images showing AC-mito signal in primary fibroblasts transfected with AC-mito (green) and mCherry-Fis1 (magenta), and treated with the formin inhibitor SMIFH2. Scale bar 10 µm (B) Quantification of AC-mito signal in cells transfected as in (A). Each point represents an individual cell, with 12 cells quantified in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$. (C) Loss of AC-mito signal caused by CK666 and SMIFH2 relative to control levels. CK666 data from Figure 5B, SMIFH2 data from panel (B) above. Individual points represent independent experiments. Bars show the average ± SD. ** $p<0.01$. (D) Quantification of the number of fusion (Left, blue) and fission (Middle, orange) events, as well as the fusion/fission ratio (Right) in cells transfected as in (A) and treated as indicated. Each point represents an individual cell, with 12 cells quantified in 3 independent experiments. Bars show the average ± SD. *** $p<0.001$, ** $p<0.01$. (E) Quantification of fusion events as end to-side (Side) or tip-to-tip (End). The total number of events for 12 cells in 3 experiments is shown for each condition. (F-G) Quantification of the number of fusion (Left, blue) and fission (Middle, orange) events, as well as the fusion/fission ratio (Right) in U2OS cells in which INF2 has been deleted using CRISPR/Cas9. Each point represents an individual cell, with 12 cells quantified in 3 independent experiments. Bars show the average ± SD. ** $p<0.01$. (G) Quantification of fusion events as end to-side (Side) or tip-to-tip (End). The total number of events for 12 cells in 3 experiments is shown for each condition.

In this study, we provide more direct evidence that ER- and mito-actin are required for both mitochondrial fission and fusion. Specifically, we used mitochondria- and ER-targeted AC probes[8] to show that mitochondria-associated actin is recruited to mitochondria at sites of both fusion and fission under unstimulated conditions. The ER and ER-actin were also present at fusion sites, but they were recruited just before or during fusion at pre-existing actin rich sites on mitochondria. Importantly, we showed that we can selectively disrupt organelle-associated actin filaments using our novel genetically-encoded DeAct-ER/mito tools. Disrupting mitochondria-associated actin with DeAct-mito expression disrupted mitochondrial fission and fusion, demonstrating the importance of mitochondria-associated actin in the regulation of both processes, and revealing a heretofore entirely unknown role for actin in fusion.

Mitochondria-associated actin has previously been reported in a number of settings in addition to mitochondrial fission. For example, actin clouds assemble around depolarised mitochondria to isolate them for autophagy[20] while mitochondrial actin comet tails promote mitochondrial redistribution during mitosis[38]. Distinct actin-polymerizing complexes are likely required depending on the cellular context. For example, two distinct populations of actin filaments have been suggested to regulate fission, the INF2-dependent pathway and a second, INF2-independent but Arp2/3-dependent pathway[9,37]. These mitochondria-associated actin polymerization processes differ in the cellular stimuli resulting in fission[7,9,37]. On the other hand, some studies showed that both formins and Arp2/3 are involved in complex-mediated actin assembly around mitochondria[9,36,44]. Overall, multiple mechanisms activated on either mitochondria or ER-mitochondria contact sites likely cooperate to stimulate the formation of mitochondria-associated actin filaments. As mito-actin has multiple roles, these processes likely play overlapping but distinct roles. Here, as our results show that mito-actin marks the site of fusion prior to the recruitment of ER-actin and mitochondrial fusion, we propose that Arp2/3-dependent actin polymerization on mitochondria acts upstream of INF2-dependent polymerization of ER-actin during fusion.

We also distinguished two types of mitochondrial fusion events with different requirements for actin. Tip-to-side fusion, which constitute the majority of fusion events and lead to the formation of branched mitochondria, predominantly occurred in the presence of actin. On the other hand, tip-to-tip fusion events, which elongates mitochondria, were much less likely to show actin recruitment. Importantly, disrupting actin polymerization significantly decreased the occurrence of tip-to-side fusion events, but not tip-to-tip events, demonstrating the selectivity of actin for tip-to-side fusion. Interestingly, deletion of ABHD16A, an ER protein regulating the formation of fission and fusion nodes at ER-mitochondria contact sites, results in the loss of tip-to-side fusion[19].

This suggests that actin could facilitate the formation of these nodes, therefore promoting tip-to-side fusion. These observations may potentially serve as the basis for new insights into genetic and environmental conditions that result in altered branching in mitochondrial networks.

Some studies have noted the presence of elongated mitochondria upon inhibition of actin-polymerizing proteins. While this was seen as evidence for a loss of fission relative to fusion, our results suggest that this could be the result of the lack of requirement for actin during tip-to-tip fusion promoting mitochondrial elongation rather than branching. Another point to take into consideration is the dynamic nature of mitochondrial fission and fusion and the requirement for cells to maintain a balance between the two. For example, INF2 deletion decreased both fusion and fission events. Under these conditions (steady state loss of INF2), it is possible that a decrease in fission rates led to a secondary decrease in fusion events to maintain a proper mitochondrial network. On the other hand, we did observe fission events in cells where the Arp2/3 complex was acutely inhibited (one hour treatment). This observation is likely due to the promotion of INF2-dependent fission events[7,9,30,37].

Changes in organelle architecture depend on both the inherent composition of membrane lipid bilayers[45,46] and the external energy necessary to overcome membrane resistance. These external forces include pulling or pushing forces that are generated by cytoskeletal components[47], including actin. For instance, branched actin assembled at the membrane exerts mechanical forces that alter its curvature[48]. Furthermore, actin-binding proteins containing a BAR (Bin-Amphiphysin-Rvs) domain can promote membrane curvature through direct membrane interaction and the formation of a scaffold that facilitates membrane curvature and shape cellular structures[41,49]. Consistent with this, recent studies have suggested that the actin cytoskeleton is crucial for maintaining the curvature of mitochondrial membranes[23]. We speculate that the interaction between the actin cytoskeleton and lipids could thus be crucial for the generation of membrane curvature required for tip-to-side fusion events.

A second potential role of actin in mitochondrial fusion relates to mitochondrial movement. Previous studies have suggested that actin-enriched regions exhibit reduced motility[7,9,50]. In this context, actin could stabilize or hold the receiving mitochondria in place to enable fusion to occur. Besides, in yeast[21,51] and higher plants[22,52], actin filaments can interact with specific motor proteins to regulate short-distance mitochondrial movement, which could also serve to properly align the two fusing mitochondria. As the role of actin filaments in mitochondrial movement is likely context-dependent, both processes could control the position and stability of the receiving mitochondrion which might be crucial for efficient mitochondrial fusion. In this context, it is

interesting to note that both mito- and ER-actin are recruited to fusion sites, as these two pools of actin may act at different steps in the fusion process.

A third potential role for actin in mitochondrial fusion relates to actin's well-established role in promoting the GTPase activity of dynamin-related proteins. For example, the dynamic interaction between actin and dynamin promotes the bundling of Arp2/3-dependent actin filaments in a manner that is dependent on cycles of GTP hydrolysis by dynamin[27]. Interestingly, this activity is required for cell-to-cell fusion. The promotion of the GTPase activity of DRP1 is also relatively well-established in mitochondrial fission. However, more work must be done to clarify whether actin also facilitates MFN1/2 GTPase activity. Nevertheless, our data showing increased mito-actin in cells overexpressing MFN1 certainly suggests some form of crosstalk between MFN1-mediated fusion and mitochondria-associated actin. Future studies will be needed to clarify these results and further elucidate the mechanisms by which actin facilitates mitochondrial fusion.

In summary, our findings emphasize the significant role of actin polymerization in organizing tip-to-side fusion events. However, unlike during fission, where actin is known to form constrictions, the functional aspect of actin during fusion remains yet to be elucidated. Further research is needed to gain a better understanding of the role of actin in fusion and its localization. Our study highlights the multifunctionality of actin in regulating organelle dynamics, emphasizing the need for continued investigation in this area.

Material and Methods

Cell culture:

Control Primary Human Fibroblasts were purchased from the Coriell institute and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with Penicillin/Streptomycin (100 IU/ml/100µL/mL). MDA-MB-231 cells (triple negative breast cancer) were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum. These cells were stably transfected with mitochondria-targeted GFP (CCOeGFP, addgene, pLVX-EF1a-CCO-IRES, #134861) using Metafectene (Biontex). Cell culture reagents were obtained from Wisent.

Plasmids and Transient transfection of primary cells:

Primary cells were trypsinized and centrifuged at 5000rpm for 5 minutes. Cell pellets were suspended in 10µl of the Neon transfection buffer R (ThermoFisher Scientific, MPK1096). Cells were transiently transfected with EF1a-CCO-IRES (addgene #134861), mito-PAGFP (addgene, #23348), MFN1-Myc (addgene #23212), mCherry-Fis1, mCherry-Cytb5, Halo-Fis1 and all custom actin nanobody probes that we previously reported[8] using the Neon transfection system (ThermoFisher Scientific, MPK5000) according to the manufacturer's protocol. For all

experiments, 1 µg of total DNA concentrations were transfected per 10ul of 10^6cells/ml (individually or combined for co-transfection). DeAct-ER and DeAct-Mito constructs where generated utilizing the "Gelsolin Segment 1 (GS1)" sequence described in Harterink et al[31]. CMV-DeAct-GS1 was a gift from Brad Zuchero (Addgene plasmid # 89445). The GS1 sequence was cloned into the pSIN vector under a CMV promoter, N-terminal to the mScarlet fluorescent protein sequence. Either a Cytb5ER or Fis1 localization sequence was fused to the mScarlet C-terminus to target expression to the ER (DeAct-ER) or mitochondrial outer membrane (DeAct-Mito), respectively. Cytb5ER and Fis1 targeting sequences are the same as those previously described in Schiavon et al.[8] to target AC-ER and AC-Mito to the ER or mitochondrial outer membrane.

siRNA treatment:

MDA-MB-231 were seeded onto 6 well dishes to reach 30–40% density in 24 hours. Then the cells were transfected with 10 nM of Arp2 siRNA (Dharmacon reagents, ACTR2 Gene 10097) and control siRNA (Thermo Fisher Scientific, Silencer Select, 4390843) using siLenFect lipid reagent (Bio-Rad,1703361). After 24 hours, the cells were imaged live or collected for western blotting.

Drug treatments:

Cells were incubated with 10 µM CK-666 (CAS 442633-00-3 – Calbiochem) or Formin FH2 Domain Inhibitor, SMIFH2 (CAS 340316-62-3 – Calbiochem) for 60 minutes to inhibit Arp2/3 complex and formins, respectively.

Immunofluorescence:

Transfected cells were seeded onto glass coverslips (Fisherbrand, 1254580) and allowed to adhere overnight. Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). Cells were then permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA / 0.1% Triton X-100 in PBS. The cells were then incubated with primary antibodies using following antibodies as per the experiment: TOM20 (Rb, Abcam, ab186735, 1:250), RTN4/NOGOA (Rb, Bio-Rad, AHP1799, 1:200), followed by with fluorescent tagged secondary antibodies (Jackson Immunoresearch, 1:500) and DAPI (Invitrogen, Thermo Fisher, D1306, 1:100).

Live imaging and confocal microscopy:

For Live cell microscopy imaging, cells were grown on glass bottom dishes in complete medium and stained for 30 min with 250 nM TMRM (Thermo fisher Scientific, T668) or 5 min with 100nM Mitotracker Deep Red (Thermo fisher scientific, M7512) where indicated. After staining, cells were washed 3 times with pre-warmed 1× phosphate buffered saline (PBS), and normal growth media was added prior to imaging. The plates were mounted onto Leica TSC SP8 confocal microscope fitted with a 63×/1.40 oil objective using the optimal resolution for the wavelength

(determined using the Leica software). Time-lapse images were acquired at a speed of (0.05-0.125 frames/s) for 10 minutes.

For the INF2 experiments, Wild-type and INF2 KO U2OS cells[9] were maintained in DMEM supplemented with 10% FBS at 37C with 5% CO2. Cells were plated on 8-well no. 1.5 imaging chambers (Cellvis) coated with 10ug/mL fibronectin. Cells were stained with 50nM MitoTracker Deep Red for 30min, washed with PBS, then imaged in FluoroBright medium (Thermo Fisher) supplemented with 10% FBS. Cells were imaged with a C-Apochromat x40/1.2 NA W Korr FCS M27 objective on an inverted Zeiss 880 LSM Airyscan confocal microscope with the environmental control system supplying 37C, 5% CO2, and humidity for live-cell imaging. MitoTracker Deep Red was imaged with a 647-nm laser line at ~250nW laser power at optimal resolution (determined by Zen Black software). Time-lapses were acquired at a speed of 0.152-0.357 frames/s for 5min. Following acquisition, images were Airyscan processed using auto-filter 2D-SR settings in Zen Blue.

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. For mitochondrial area, the images were segmented in ImageJ using Filter/Median (1.0), then thresholding and adjusting the resulting image using Binary/Erode. Total mitochondria area was then measured using the measure function.

Tracking of Fusion and Fission events:

Quantification of fusion and fission events was done manually. The images were tracked frame by frame to record movement of mitochondria that resulted in fusion or fission. Stable separation of the mitochondrial marker was counted as a fission event. Fusion events were defined as follows: two fusing mitochondria must retain their fused state for the following 10 frames or more (i.e 100sec). If there was a division at the fusion site within 10 frames, such an event is discarded. Also, the mean signal intensity of the mitochondrial marker at the site of fusion and a region elsewhere on mitochondria (basal) was recorded at the end of the 10 frames. If the ratio of signal intensity at the site of fusion and basal signal intensity was higher than 1, it was considered as an overlapping, not a fusion event. The presence of ER at the site of fusion or fission was also assessed manually, by looking for ER colocalizing with mitochondria at the site.

Tracking of AC-mito and AC-ER at fusion sites:

To identify when actin is accumulated on ER and mitochondria relative to a fusion event, we counted backwards from that fusion event to determine at which frame the AC probe accumulated on mitochondria and ER. Determining the time between the frames where AC-mito and AC-ER

appear at the fusion site gives us the information on the time taken by Actin to get accumulated on the organelle.

AC probe 'accumulation' calculation:

Using Fiji, a square selection was drawn around a region with obvious AC probe accumulation (0.8 μm^2 for mitochondria and ER). The mean pixel intensity of AC-GFP and mCherry channels was measured within the selection. Another square of equal dimensions was drawn in an adjacent, control area with mCherry signal and mean pixel intensity was measured. The mean pixel intensity in the accumulated region was then divided by the mean pixel intensity in the control region.

Mean AC Signal Intensity:

The loss or gain of AC probe signal was determined in cells transfected with DeActs, MFN1 or upon treatment with inhibitors for Arp2/3 complex and formins. The integrated density of AC-Mito/ AC- ER signal of individual cells was measured in Fiji. For AC-mito mean signal intensity, the integrated density signal was then divided by mitochondrial area. For AC-ER, the integrated density was divided by the cell area. The loss or gain of the AC probe signal was obtained by comparing the values to that of the control. The imaging parameters (laser intensity and gain values) were kept identical for control and test conditions for every experiment.

mtPA-GFP-based Mitochondrial Fusion Assay:

The photoactivation-based fusion assay[53] was conducted by expressing Mito-PAGFP (Addgene #23348) in primary human fibroblasts. The cells were co-stained with TMRM to monitor mitochondrial membrane potential. Photoactivation was performed in selected ROIs using the 405 nm laser with 100% power and 40 iterations. Upon activation of mito-PAGFP, a small portion of the mitochondrial network is photoactivated, and the spread or loss of the signal to the rest of the mitochondrial network was recorded in the ROIs. The signal of all ROIs with significant PAGFP activation within a cell was then averaged and normalized to the initial signal intensity post-activation.

Western Blot:

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 13000rpm for 10 minutes. Protein supernatants were collected, and protein concentration was estimated by DC protein assay kit (BioRad). For SDS-PAGE, 30 µg of proteins were mixed with 1X Lammeli buffer containing β-mercaptoethanol, then subjected to SDS-PAGE, transferred to a nitrocellulose membrane and blotted with the indicated antibodies (Arp2 (Santacruz, (E-12): sc-166103, 1:1000), HRP-tagged Actin (1:10,000). Membranes were then incubated with a 1:5000 dilution of horseradish

peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and visualized by enhanced chemiluminescence (Thermo Fisher scientific) using a Bio-Rad imaging system.

Data analysis and statistics:

All graphs and statistical analysis were done using R. Immunofluorescence data were quantified and images representative of at least three independent experiments shown (exact n are in the quantification figures). Data is represented as average ± SD as specified in figure legends. Statistical significance was determined using Student's t test (between 2 groups) or one-way ANOVA with a tukey post hoc test (multiple comparisons). Individual experiments are noted as different colour shades in the graphs.

Acknowledgements

The authors thank Tsung-Chang Sung from the Salk Transgenic Core for his assistance generating the DeActs-ER and DeActs-mito constructs. PG was supported by a Queen Elizabeth II Diamond Jubilee Scholarship and a FRQS scholarship. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to MG. The Waitt Advanced Biophotonics Core is supported by NIA P30AG068635 (Nathan Shock Center), the Waitt Foundation, and Core Grant application NCI CCSG (CA014195), and U.M. is supported by NIDCD R01 DC021075-01, NSF NeuroNex Award 2014862, L.I.F.E. Foundation, and the Chan-Zuckerberg Initiative Imaging Scientist Award. C.R.S. is supported by NIH 1F32GM137580.

References

1 Chandel, N. S. Evolution of Mitochondria as Signaling Organelles. *Cell Metab* **22**, 204-206, doi:10.1016/j.cmet.2015.05.013 (2015).

2 Chen, H. *et al.* Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol* **160**, 189-200, doi:10.1083/jcb.200211046 (2003).

3 Shirihai, O. S., Song, M. & Dorn, G. W., 2nd. How mitochondrial dynamism orchestrates mitophagy. *Circ Res* **116**, 1835-1849, doi:10.1161/CIRCRESAHA.116.306374 (2015).

4 Tan, J. X. & Finkel, T. Mitochondria as intracellular signaling platforms in health and disease. *J Cell Biol* **219**, doi:10.1083/jcb.202002179 (2020).

5 Detmer, S. A. & Chan, D. C. Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol* **8**, 870-879, doi:10.1038/nrm2275 (2007).

6 Friedman, J. R. *et al.* ER tubules mark sites of mitochondrial division. *Science* **334**, 358-362, doi:10.1126/science.1207385 (2011).

7 Korobova, F., Ramabhadran, V. & Higgs, H. N. An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2. *Science* **339**, 464-467, doi:10.1126/science.1228360 (2013).

8 Schiavon, C. R. *et al.* Actin chromobody imaging reveals sub-organellar actin dynamics. *Nat Methods* **17**, 917-921, doi:10.1038/s41592-020-0926-5 (2020).

9 Chakrabarti, R. *et al.* INF2-mediated actin polymerization at the ER stimulates mitochondrial calcium uptake, inner membrane constriction, and division. *J Cell Biol* **217**, 251-268, doi:10.1083/jcb.201709111 (2018).

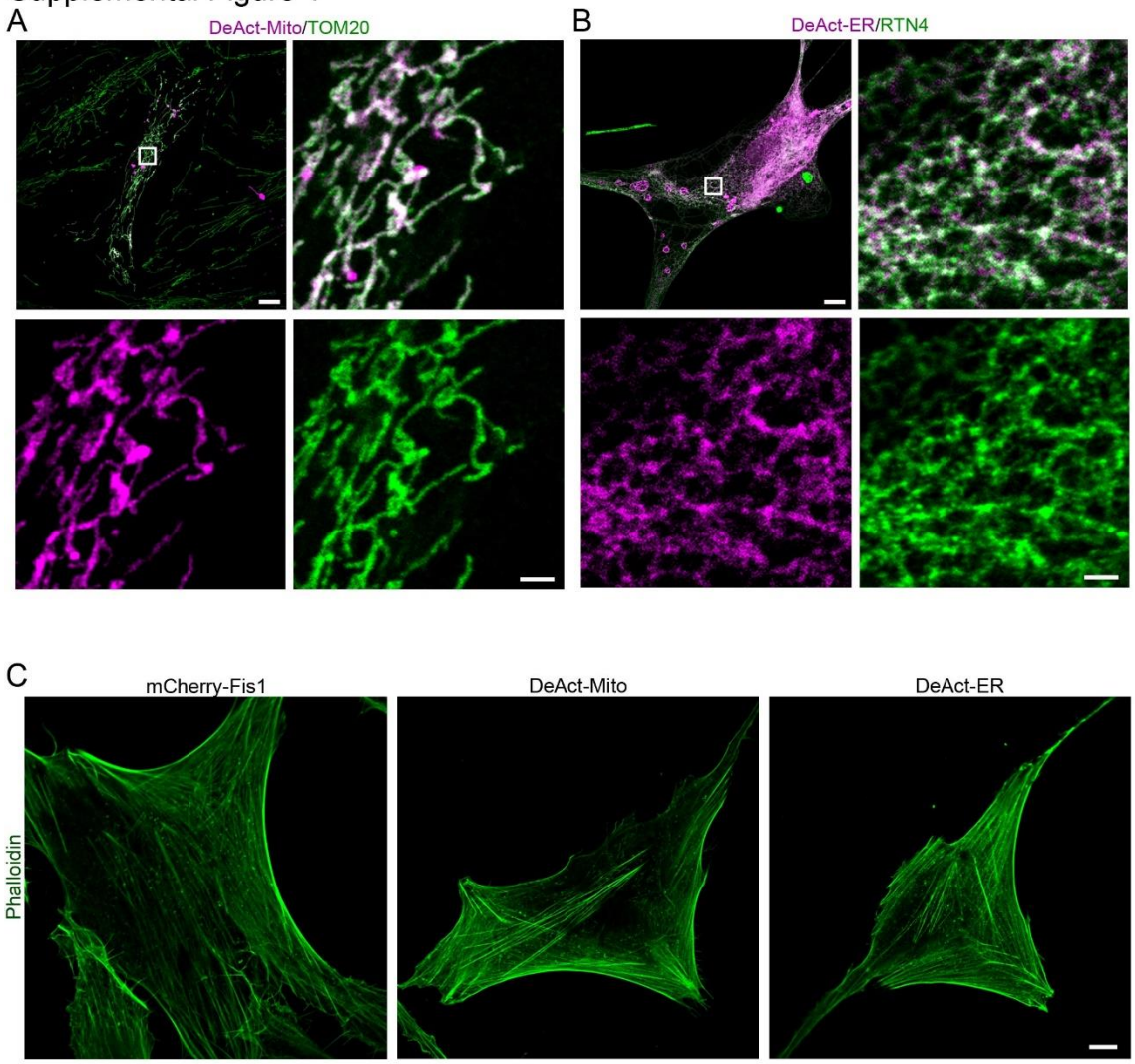
10 Coscia, S. M. *et al.* Myo19 tethers mitochondria to endoplasmic reticulum-associated actin to promote mitochondrial fission. *J Cell Sci* **136**, doi:10.1242/jcs.260612 (2023).
11 Korobova, F., Gauvin, T. J. & Higgs, H. N. A role for myosin II in mammalian mitochondrial fission. *Curr Biol* **24**, 409-414, doi:10.1016/j.cub.2013.12.032 (2014).
12 Yang, C. & Svitkina, T. M. Ultrastructure and dynamics of the actin-myosin II cytoskeleton during mitochondrial fission. *Nat Cell Biol* **21**, 603-613, doi:10.1038/s41556-019-0313-6 (2019).
13 Li, S. *et al.* Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission. *J Cell Biol* **208**, 109-123, doi:10.1083/jcb.201404050 (2015).
14 Hatch, A. L., Gurel, P. S. & Higgs, H. N. Novel roles for actin in mitochondrial fission. *J Cell Sci* **127**, 4549-4560, doi:10.1242/jcs.153791 (2014).
15 Ji, W. K., Hatch, A. L., Merrill, R. A., Strack, S. & Higgs, H. N. Actin filaments target the oligomeric maturation of the dynamin GTPase Drp1 to mitochondrial fission sites. *eLife* **4**, e11553, doi:10.7554/eLife.11553 (2015).
16 Liu, A., Kage, F. & Higgs, H. N. Mff oligomerization is required for Drp1 activation and synergy with actin filaments during mitochondrial division. *Mol Biol Cell* **32**, ar5, doi:10.1091/mbc.E21-04-0224 (2021).
17 Abrisch, R. G., Gumbin, S. C., Wisniewski, B. T., Lackner, L. L. & Voeltz, G. K. Fission and fusion machineries converge at ER contact sites to regulate mitochondrial morphology. *J Cell Biol* **219**, doi:10.1083/jcb.201911122 (2020).
18 Guo, Y. *et al.* Visualizing Intracellular Organelle and Cytoskeletal Interactions at Nanoscale Resolution on Millisecond Timescales. *Cell* **175**, 1430-1442 e1417, doi:10.1016/j.cell.2018.09.057 (2018).
19 Nguyen, T. T. & Voeltz, G. K. An ER phospholipid hydrolase drives ER-associated mitochondrial constriction for fission and fusion. *Elife* **11**, doi:10.7554/eLife.84279 (2022).
20 Fung, T. S. *et al.* Parallel kinase pathways stimulate actin polymerization at depolarized mitochondria. *Curr Biol* **32**, 1577-1592 e1578, doi:10.1016/j.cub.2022.02.058 (2022).
21 Boldogh, I. R., Fehrenbacher, K. L., Yang, H. C. & Pon, L. A. Mitochondrial movement and inheritance in budding yeast. *Gene* **354**, 28-36, doi:10.1016/j.gene.2005.03.049 (2005).
22 Doniwa, Y., Arimura, S.-i. & Tsutsumi, N. Mitochondria use actin filaments as rails for fast translocation in Arabidopsis and tobacco cells. *Plant Biotechnology* **24**, 441-447, doi:10.5511/plantbiotechnology.24.441 (2007).
23 Fernandez Casafuz, A. B., De Rossi, M. C. & Bruno, L. Mitochondrial cellular organization and shape fluctuations are differentially modulated by cytoskeletal networks. *Sci Rep* **13**, 4065, doi:10.1038/s41598-023-31121-w (2023).
24 Lopez-Domenech, G. *et al.* Miro proteins coordinate microtubule- and actin-dependent mitochondrial transport and distribution. *EMBO J* **37**, 321-336, doi:10.15252/embj.201696380 (2018).
25 Gu, C. *et al.* Regulation of dynamin oligomerization in cells: the role of dynamin-actin interactions and its GTPase activity. *Traffic* **15**, 819-838, doi:10.1111/tra.12178 (2014).
26 Gu, C. *et al.* Direct dynamin-actin interactions regulate the actin cytoskeleton. *EMBO J* **29**, 3593-3606, doi:10.1038/emboj.2010.249 (2010).
27 Zhang, R. *et al.* Dynamin regulates the dynamics and mechanical strength of the actin cytoskeleton as a multifilament actin-bundling protein. *Nat Cell Biol* **22**, 674-688, doi:10.1038/s41556-020-0519-7 (2020).
28 Legros, F., Lombes, A., Frachon, P. & Rojo, M. Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. *Mol Biol Cell* **13**, 4343-4354, doi:10.1091/mbc.e02-06-0330 (2002).

29 Huang, P., Yu, T. & Yoon, Y. Mitochondrial clustering induced by overexpression of the mitochondrial fusion protein Mfn2 causes mitochondrial dysfunction and cell death. *Eur J Cell Biol* **86**, 289-302, doi:10.1016/j.ejcb.2007.04.002 (2007).
30 Manor, U. *et al.* A mitochondria-anchored isoform of the actin-nucleating spire protein regulates mitochondrial division. *Elife* **4**, doi:10.7554/eLife.08828 (2015).
31 Harterink, M. *et al.* DeActs: genetically encoded tools for perturbing the actin cytoskeleton in single cells. *Nat Methods* **14**, 479-482, doi:10.1038/nmeth.4257 (2017).
32 Hetrick, B., Han, M. S., Helgeson, L. A. & Nolen, B. J. Small molecules CK-666 and CK-869 inhibit actin-related protein 2/3 complex by blocking an activating conformational change. *Chem Biol* **20**, 701-712, doi:10.1016/j.chembiol.2013.03.019 (2013).
33 Chan, F. Y. *et al.* The ARP2/3 complex prevents excessive formin activity during cytokinesis. *Mol Biol Cell* **30**, 96-107, doi:10.1091/mbc.E18-07-0471 (2019).
34 Cifrova, P. *et al.* Division of Labor Between Two Actin Nucleators-the Formin FH1 and the ARP2/3 Complex-in Arabidopsis Epidermal Cell Morphogenesis. *Front Plant Sci* **11**, 148, doi:10.3389/fpls.2020.00148 (2020).
35 Dimchev, V. *et al.* Induced Arp2/3 Complex Depletion Increases FMNL2/3 Formin Expression and Filopodia Formation. *Front Cell Dev Biol* **9**, 634708, doi:10.3389/fcell.2021.634708 (2021).
36 Moore, A. S., Wong, Y. C., Simpson, C. L. & Holzbaur, E. L. Dynamic actin cycling through mitochondrial subpopulations locally regulates the fission-fusion balance within mitochondrial networks. *Nature communications* **7**, 12886, doi:10.1038/ncomms12886 (2016).
37 Fung, T. S., Ji, W. K., Higgs, H. N. & Chakrabarti, R. Two distinct actin filament populations have effects on mitochondria, with differences in stimuli and assembly factors. *J Cell Sci* **132**, doi:10.1242/jcs.234435 (2019).
38 Moore, A. S. *et al.* Actin cables and comet tails organize mitochondrial networks in mitosis. *Nature* **591**, 659-664, doi:10.1038/s41586-021-03309-5 (2021).
39 Fung, T. S., Chakrabarti, R. & Higgs, H. N. The multiple links between actin and mitochondria. *Nat Rev Mol Cell Biol*, doi:10.1038/s41580-023-00613-y (2023).
40 Lappalainen, P., Kotila, T., Jegou, A. & Romet-Lemonne, G. Biochemical and mechanical regulation of actin dynamics. *Nat Rev Mol Cell Biol* **23**, 836-852, doi:10.1038/s41580-022-00508-4 (2022).
41 Carlsson, A. E. Membrane bending by actin polymerization. *Curr Opin Cell Biol* **50**, 1-7, doi:10.1016/j.ceb.2017.11.007 (2018).
42 Hatch, A. L., Ji, W. K., Merrill, R. A., Strack, S. & Higgs, H. N. Actin filaments as dynamic reservoirs for Drp1 recruitment. *Mol Biol Cell* **27**, 3109-3121, doi:10.1091/mbc.E16-03-0193 (2016).
43 Kleele, T. *et al.* Distinct fission signatures predict mitochondrial degradation or biogenesis. *Nature* **593**, 435-439, doi:10.1038/s41586-021-03510-6 (2021).
44 Kruppa, A. J. *et al.* Myosin VI-Dependent Actin Cages Encapsulate Parkin-Positive Damaged Mitochondria. *Dev Cell* **44**, 484-499 e486, doi:10.1016/j.devcel.2018.01.007 (2018).
45 Hishikawa, D., Hashidate, T., Shimizu, T. & Shindou, H. Diversity and function of membrane glycerophospholipids generated by the remodeling pathway in mammalian cells. *J Lipid Res* **55**, 799-807, doi:10.1194/jlr.R046094 (2014).
46 Manni, M. M. *et al.* Acyl chain asymmetry and polyunsaturation of brain phospholipids facilitate membrane vesiculation without leakage. *Elife* **7**, doi:10.7554/eLife.34394 (2018).
47 Jarsch, I. K., Daste, F. & Gallop, J. L. Membrane curvature in cell biology: An integration of molecular mechanisms. *J Cell Biol* **214**, 375-387, doi:10.1083/jcb.201604003 (2016).
48 Baldauf, L., Frey, F., Arribas Perez, M., Idema, T. & Koenderink, G. H. Branched actin cortices reconstituted in vesicles sense membrane curvature. *Biophys J*, doi:10.1016/j.bpj.2023.02.018 (2023).

49 Kessels, M. M. & Qualmann, B. Interplay between membrane curvature and the actin cytoskeleton. *Curr Opin Cell Biol* **68**, 10-19, doi:10.1016/j.ceb.2020.08.008 (2021).
50 Bayraktar, S. *et al.* A Deregulated Stress Response Underlies Distinct INF2-Associated Disease Profiles. *J Am Soc Nephrol* **31**, 1296-1313, doi:10.1681/ASN.2019111174 (2020).
51 Fehrenbacher, K. L., Yang, H. C., Gay, A. C., Huckaba, T. M. & Pon, L. A. Live cell imaging of mitochondrial movement along actin cables in budding yeast. *Curr Biol* **14**, 1996-2004, doi:10.1016/j.cub.2004.11.004 (2004).
52 Romagnoli, S. *et al.* Microtubule- and actin filament-dependent motors are distributed on pollen tube mitochondria and contribute differently to their movement. *Plant Cell Physiol* **48**, 345-361, doi:10.1093/pcp/pcm001 (2007).
53 Karbowski, M., Cleland, M. M. & Roelofs, B. A. Photoactivatable green fluorescent protein-based visualization and quantification of mitochondrial fusion and mitochondrial network complexity in living cells. *Methods Enzymol* **547**, 57-73, doi:10.1016/B978-0-12-801415-8.00004-7 (2014).

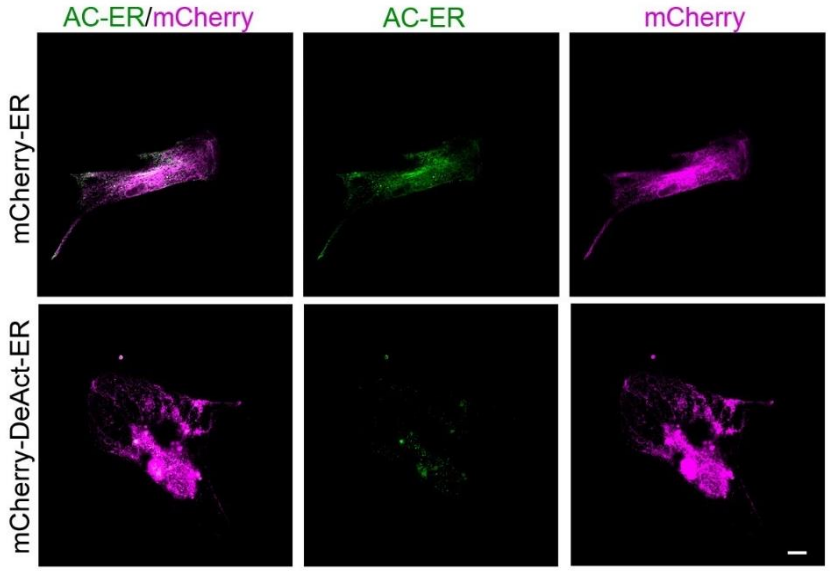
Supplemental Figures

Supplemental Figure 1



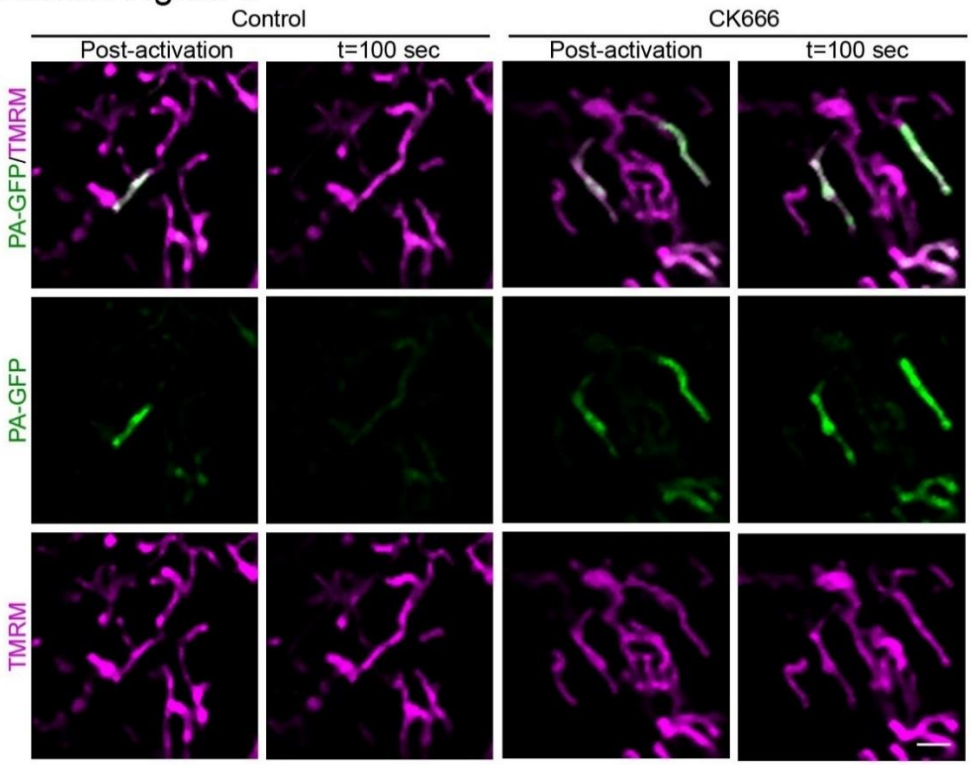
Supplemental Figure 1. Proper targeting of DeActs. (A) Representative images of primary human fibroblasts transfected with DeAct-mito (magenta) and marked with an antibody against TOM20 (mitochondria, green). The boxed area is shown enlarged below the main image. (B) Representative images of cells transfected with DeAct-ER (magenta) and marked with an antibody against RTN4 (ER, green). The boxed area is shown enlarged below the main image. (C) DeActs do not affect overall actin filaments. Cells were transfected as in (A-B) and stained with phalloidin (actin, green). Scale bar 10 µm, 2 µm for the enlarged images.

Supplemental Figure 2



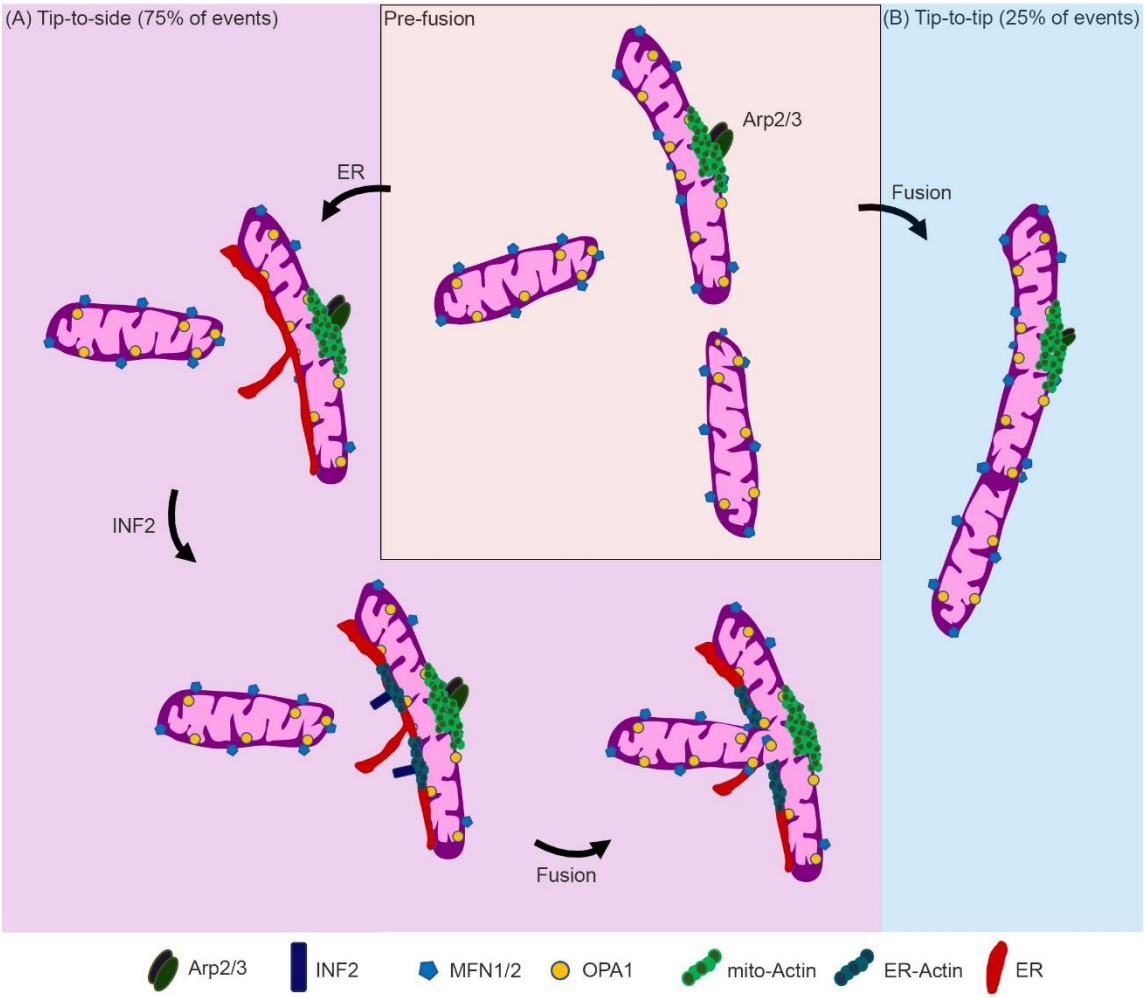
Supplemental Figure 2. DeAct-ER inhibits the polymerization of ER-associated actin. Representative images showing the loss of AC-ER signal (green) in cells transfected with DeAct--ER (magenta).

Supplemental Figure 3



Supplemental Figure 3. Representative images of the fusion assay. Representative images of human primary fibroblasts transfected with photoactivatable-GFP (PA-GFP) were treated as indicated and imaged immediately after activation (post-activation) and 100 sec following a fusion event. Scale bar 2 μm.

Supplemental Figure 4



Supplemental Figure 4. Graphical representation of mitochondrial fusion. 75% of mitochondria engage in tip-to-side fusions, wherein actin has a crucial role by localizing to the fusion site on mitochondria. Subsequently, the ER and ER-associated actin are recruited to this site. The second mitochondrion involved in the fusion process binds to this site, leading to the fusion of membranes. The remaining (25%) tip-to-tip fusion events occured independently without relying on actin.

CHAPTER IV

DISCUSSION

Mitochondria play a crucial role in the regulation of cellular metabolism and signaling. Mitochondrial fission and fusion control these processes by balancing respiratory and metabolic functions, transferring material between mitochondria, and removing defective mitochondria. Mitochondrial activity can be influenced by both internal and external signals. In this thesis, I investigated the role of the ECM, a key component of the cellular environment, in regulating mitochondrial structure and activity. We used breast cancer-derived spheres (mammospheres), which possess stem-like properties that enable them to proliferate in suspension culture without the need for ECM. Our observations revealed an astonishing phenomenon of mitochondrial elongation in these mammospheres upon interaction with ECM, which was accompanied by an increase in respiratory activity and ATP production. This remodeling occurred independently of DRP1 activity and relied on integrin signaling and actin polymerization. This thesis implies that actin has an additional role in regulating mitochondrial elongation, in other words, fusion. While the role of actin in mitochondrial fission is well established, it was unknown whether actin plays a role in the fusion process. Using specialized actin chromobody probes, we demonstrated for the first time that actin is recruited to both fission and fusion sites under unstimulated conditions and plays a key role in the fusion process by marking the fusion site. This adds an important role to actin in the context of mitochondrial dynamics suggesting that ECM-induced mitochondrial fusion is actin-dependent. In addition, the regulation of mitochondrial dynamics by actin may be a common phenomenon in cancer stem cells, enabling them to adapt and renew in response to new tumor microenvironments. In this discussion, I will further explore the relationship between actin and mitochondrial dynamics, as well as the factors that influence this relationship. Additionally, I will address the limitations of our research and highlight the questions and opportunities for further investigation in this field.

4.1 The microenvironment as a regulator of mitochondrial dynamics

The primary finding of this study was that mitochondria undergoes elongation upon cellular attachment to the ECM. Several factors might have caused such changes in mitochondrial structure. Recent research indicates that cellular conditions, such as nutrient levels, can influence

mitochondrial structure, causing elongation or fragmentation (206). For instance, cells experiencing nutrient deprivation exhibit elevated cyclic AMP levels, resulting in the activation of protein kinase A (PKA). Subsequently, PKA phosphorylates DRP1, causing its retention in the cytoplasm, thereby promoting unopposed mitochondrial fusion (205). Similarly, in budding yeast, exposure to nonfermentable carbon sources, such as glycerol, results in complex mitochondrial structures with numerous branches (379). Such adaptability of the cell to environmental changes might involve intricate signaling pathways, including AMP-activated protein kinase (AMPK), which monitors cellular energy levels and regulates mitochondrial shape according to nutrient availability (380). However, in our experimental design, despite having identical nutrient conditions for both suspension culture and ECM-attached conditions, mammospheres displayed changes in mitochondrial structure, becoming elongated in ECM attached condition. To promote cell attachment, we centrifuged cells onto plates either with or without fibronectin coating. Both conditions resulted in elongation of the cells, although fibronectin-coated plates facilitated faster attachment. Hence, nutrients were not a limiting factor that contributed changes in the mitochondrial morphology.

In addition to nutrients, the mechanical environment of the ECM, within which cells proliferate and adhere, is another important factor that can influence mitochondrial dynamics (362). The resistance forces exerted by the ECM are sensed as mechanical cues by the cells through a process called mechanotransduction (256). This process involves receptors, such as integrins or ion channels, which capture and convert mechanical stress into signals that trigger biological responses (258, 297). Cells grown in softer ECM display fragmented mitochondria due to heightened DRP1 activity (219). Conversely, a stiffer matrix impedes DRP1 activation, thereby promoting mitochondrial elongation (381). Surprisingly, our data indicated that changes in mitochondrial structure resulting from ECM alterations can occur independently of DRP1 expression. This is because mammospheres grown without ECM express very low levels of DRP1 despite having fragmented mitochondria. Moreover, silencing of DRP1 did not induce mitochondrial elongation. However, the same mammospheres would show elongated mitochondria upon attachment to the ECM, with no effect on dynamic proteins. This suggests that the observed elongation of mitochondria in mammospheres upon ECM attachment was not dependent on DRP1 expression, as the expression level of DRP1 remained unchanged. Instead, other factors may be responsible for inducing mitochondrial elongation upon attachment. It is possible that the altered behavior observed in our study is specific to the breast cancer cell lines MDA MB 231 and MCF-7, and further investigation is required to determine the universality of

these findings. Moreover, most of the understanding from the literature is from the adherent cancer cells, unlike the spheres in suspensions. Therefore, an extensive assessment of a broader range of cell lines is necessary to gain a deeper understanding of the dynamic behavior in spheres as well as adherent cells. While this represents a limitation of the study, the results emphasize that extracellular signals are sufficient to dictate changes in mitochondrial morphology.

4.2 ECM-mediated Integrin pathway in Mitochondrial dynamics

Our subsequent investigation aimed to determine the factors responsible for changes in mitochondrial structure upon attachment to the ECM. It is well established that cellular adhesion to fibronectin activates the integrin signaling pathway (242). Integrin-associated kinases (IACs) such as PAX, FAK, and Src are activated by integrins upon cellular adhesion (242, 244). In our study, attached mammospheres exhibited increased expression of phosphorylated and total FAK, indicating the activation of the integrin-driven FAK pathway. Moreover, when these attached mammospheres were treated with inhibitors targeting FAK/ Src, attachment-induced mitochondrial elongation was prevented. These results led us to infer that the integrin-driven FAK pathway plays a crucial role in the regulation of mitochondrial dynamics. Previous studies have presented conflicting findings, with some suggesting that FAK prevents DRP1 expression and promotes mitochondrial fusion (381), while others indicate that FAK-dependent DRP1 activation promotes mitochondrial fission (382). While it is uncertain whether FAK-dependent DRP1 activity relies on the cellular environment to inhibit or activate it, we maintained a neutral stance. This is because we have already demonstrated that mitochondrial elongation in mammospheres is independent of DRP1. Therefore, FAK-induced elongation can occur independent of DRP1 expression.

Furthermore, FAK and Src activate downstream pathways that link integrins to the actin cytoskeleton (247, 248). The Rho family of small GTPases (Rho, Rac, and CDC42), activated by FAK, regulates actin polymerization in response to extracellular stimuli, supporting cell motility and morphology. Activation of Rho in fibroblasts leads to the assembly of stress fibers and focal adhesions (296), whereas activation of Rac causes the extension of peripheral lamellipodia and assembly of small focal complexes (248). Cdc42 regulates the formation of peripheral filopodial extensions (294, 295). Mammosphere attachment to fibronectin showed lamellopedia-like structures along with mitochondrial elongation. While the inhibition of Rho had no effect on cells, Rac-1 inhibition prevented the ability of the spheres to attach to the ECM and did not show lamellipodia-like structures. Moreover, Rac-1 inhibition prevented mitochondrial elongation in attached mammospheres, and its activation promoted actin polymerization and mitochondrial

elongation in non-ECM-attached cells. This finding highlights the importance of Rac-induced actin polymerization in mitochondrial elongation. While the specific rationale behind the involvement of Rac-induced actin has not been addressed in our study, it could be linked to the pro-survival role of Rac GTPase activity in preventing mitochondrial apoptosis (382). Alternatively, FAK-activated STAT3 (transcription factor) can directly interact with mitochondria to stimulate OXPHOS activity and is essential to prevent mitochondrial depolarization (383). The role of RAC has not yet been studied in this context. However, considering that the primary objective of the adapting cell is survival, it is highly plausible that FAK-Rac signaling collaborates to prevent apoptosis and induce mitochondrial elongation, thereby promoting OXPHOS for cellular energy

4.3 Actin polymerization: regulator of mitochondrial dynamics

A noteworthy finding is the complementary relationship between actin polymerization and mitochondrial elongation in attached mammospheres. Inhibiting pathways that trigger actin polymerization prevented mitochondrial elongation. This prompted the intriguing question of whether actin plays a role in inducing mitochondrial elongation. Abundant evidence exists establishing a connection between actin polymerization and mitochondrial dynamics, particularly in the context of fission. In mammals, fission events are mainly regulated at MERCS, where INF2 localized on the ER (152) and spire1c on the mitochondria (134) form actin filaments around the mitochondrial membrane. Myosins are recruited to sites that cause mitochondrial constriction by applying tension on the actin network (355). Actin filaments then recruit DRP1 to initiate fission (151, 152, 261). Additionally, other actin regulatory proteins such as Arp2/3, cortactin, and cofilin also affect mitochondrial fission (136, 355). Nevertheless, an alternative study has documented that cofilin downregulation enhances the accumulation of DRP1 on mitochondria but leads to mitochondria hyperfusion (383). This implies that accumulated DRP1 may be inactive or non-functional, losing its ability to facilitate fission while still allowing for fusion activity. Our observations revealed that silencing DRP1 in mammospheres failed to induce fusion, likely due to the absence of polymerized actin. Moreover, Arp2/3 complex driven actin filaments have been noted to play a role in ensuring rapid mitochondrial fusion as an initial cellular survival response during starvation stress (384). This indicates that actin is not confined solely to the fission process but may also regulate the fusion process.

Several studies on fission have suggested that lack of actin prevents fission and promotes fusion (136, 261, 334, 354, 355, 383), assumed based on mitochondrial length. Many studies have implied that the disassembly of actin filaments hinders fission and induces fusion (217, 351).

However, the actual mechanism of action is poorly understood. We attribute this lack of knowledge to 1) The intricate structure of organelles, for example, actin filaments, are present as a dense meshwork throughout the cell. Hence, it is challenging to visualize the interaction signals between actin and mitochondria. 2) Necessity for high-resolution microscopy to enhance visualization 3) Difficulties in assessing mitochondrial dynamic events: Fission events are relatively straightforward to assess, involving mitochondria separating and moving away. However, determining fusion events is more challenging because they can be mistaken for overlap or close proximity without actual fusion. Identifying the exact fusion site requires specialized fusion assays. To overcome these limitations, we used fluorescent protein-tagged actin chromobodies (AC) developed by Schiavon *et al*. (385) to visualize sub-organellar actin dynamics with high spatiotemporal resolution. These ACs are fused to organelle membrane targeting sequences, allowing live cell imaging of actin-associated organelle dynamics.

Remarkably, through the use of AC probes, we demonstrated the presence of actin at both fission and fusion sites. Prior to fusion, actin associated with the mitochondria marks the fusion site. Subsequently, ER and ER-associated actin are recruited to this site. The second mitochondria involved in the fusion process then binds to this actin enriched site on the first mitochondria, followed by the fusion of membranes. We observed two types of fusion events: tip-to-side and tip-to-tip. 75% of the recorded events were tip-to-side fusions, which occurred at AC-accumulated mitochondrial sites. Conversely, the remaining tip-to-tip fusion events can occur even in the absence of actin. This suggests that actin is essential for the majority of fusion events, much like fission.

Inhibiting actin polymerization using genetically encoded tools (DeAct-ER/mito) effectively disrupted organelle-associated actin filaments, leading to suppression of both fission and fusion processes. Additional inhibition of actin nucleation factors, such as the Arp2/3 complex and formins suppressed tip-to-side fusion events. Furthermore, INF2 knockout (KO) significantly suppressed fission events, whereas the Arp2/3 complex had minimal effects on fission but strongly inhibited fusion. Building on our observation that mito-actin precedes ER-actin during fusion, we propose that mitochondrial-associated actin at fusion sites is Arp2/3 complex-dependent and acts upstream of INF2-dependent polymerization in the ER.

It was recently found that the ER protein ABHD16A regulates the formation of fission and fusion nodes at MERCs and is necessary for promoting tip-to-side fusion (170). Therefore, it can be assumed that both ER-actin and Mito-actin contribute to the formation of these nodes, which promote tip-to-side fusion and might be essential for branched mitochondrial networks. It has been

suggested that tip-to-tip fusion results in complete fusion, allowing for the sharing of both soluble and membrane components, whereas tip-to-side fusions have partial sharing. In such cases, fusion events last for a shorter time and fission activity is promoted by DRP1 at the same site (197). Although we have not verified transient and complete fusion using the parameters mentioned here, our PaGFP fusion assay has shown that the majority of fusion events in a cell are tip-to-side, and are true fusions as confirmed by the sharing of matrix GFP. Nevertheless, we considered fusion events lasting >100 seconds, categorizing them as complete fusion (while transient fusion occurs within a minute). Overall, tip-side complete fusion was observed in the presence of actin. However, the significance of such fusion requires further investigation. Voeltz et al. proposed that both fusion and fission machinery localize to the MERCS to organize their activity (170). If fusion and fission occur at the same site, it is essential that they occur at the same MERCS. However, additional research is required to substantiate the coexistence of both types of machinery at these sites. As a continuation of this project, we are currently investigating whether actin, fusion, and fission proteins coexist at the same sites during each process. It is likely that they coexist but function alternatively based on the trigger received by the ER and actin, regulating calcium homeostasis, lipid metabolism, mitochondrial dynamics, and metabolic functions (168).

As we demonstrate the presence of actin at the fusion site, what role does actin play in regulating fusion? One explanation that tip-to-side fusion will result in branching of mitochondria and it requires the formation of membrane curvature at the branch site. This is influenced by the composition of the membrane lipid bilayers and the external force required to overcome resistance. Such an external force can be provided by branched actin (386), and recent studies have shown that the actin cytoskeleton is significant in maintaining the curvature of mitochondrial membranes (387). Our observations of branched actin being present at the sites of tip-to-side fusion support the theory that actin forces may interact with membrane lipids, offering membrane curvature during the fusion process. Recent research has indicated that the actin cytoskeleton and its associated proteins play a role in regulating the structure of the mitochondrial cristae. Myosin 19 (Myo19) actin motors have been observed localize to mitochondria and interact with the SAM–MICOS supercomplex. Shi et al. (2022) discovered that Myo19 mediates the mechanical regulation of actin on cristae structure and affects mitochondrial metabolism, suggesting that the mechanical force generated by the Myo19 motor-actin tether contributes to the folding of cristae and maintains the cristae architecture (388). This finding supports the theory that membrane curvature is regulated by the actin structure, which is crucial during the fusion of membranes. Furthermore, through ongoing examination of actin's function, we are currently observing its

recruitment preceding that of fusion (MFN2) and fission (DRP1) proteins during dynamic events. This suggests that actin may serve as a scaffold for the recruitment of fusion and fission machinery (data not presented here).

On the other hand, we believe that tip-to-tip fusions may function as a default mechanism in a small population of mitochondria that work independently of actin. Such fusions would result in elongated mitochondrial structures, rather than branched ones. Some studies have noted the presence of elongated mitochondria upon inhibition of actin-polymerizing proteins, as evidence for a loss of fission relative to fusion (351, 383). However, our results suggest that this could instead be due to a lack of requirement for actin during tip-to-tip fusion, which promotes mitochondrial elongation rather than branching. This may address common misinterpretations in various studies reporting that depolymerizing actin promotes mitochondrial fusion based on the presence of longer mitochondria. In fact, our data establishes that depolymerizing actin prevents fission and tip-to-side fusion. Live imaging of mitochondrial dynamics helped us reveal actual influence of actin on mitochondrial fusion and fission, without limiting our understanding based solely on the overall length of mitochondria.

4.4 Polymerised Actin: Regulates GTPase activity of Dynamins

Actin is widely recognized for its role in enhancing the GTPase activity of dynamin-related proteins (389).Specifically, the promotion of the GTPase activity of DRP1 is well-documented in the context of mitochondrial fission (136). It has also been established that the interaction between actin and dynamin promotes the bundling of Arp2/3-dependent actin filaments in a manner dependent on cycles of GTP hydrolysis by dynamin (390). Interestingly, this activity is required for cell-to-cell fusion. Nevertheless, the actin-dependent GTPase activity of fusion proteins is yet to be demonstrated making it an area that warrants further investigation. Moreover, we have shown that the mitochondrial elongation observed in mammospheres upon attachment to the ECM is independent of DRP1 activity. To maintain fission fusion balance, DRP1 silencing would promote unopposed fusion. But silencing of DRP1 had no effect on mitochondrial structure, certainly due to lack of basal DRP1 in mammospheres or due to lack of polymerized actin. However, attachment-induced signals promoted actin polymerization, and subsequent mitochondrial elongation. Although our data do not demonstrate whether actin facilitates MFN1/2 GTPase activity (like that of DRP1 GTPase Activity during fission), we observed increased mito-actin in cells overexpressing MFN1, suggesting some form of crosstalk between MFN1-mediated fusion and mitochondria-associated actin. Further studies are needed to clarify these results and elucidate the mechanisms by which actin might be facilitating mitochondrial fusion.

4.5 Actin dependent motility, a possible role in fusion:

Mitochondrial dynamics involve the movement of mitochondria towards each other during fusion and away from each other during fission. This movement is dependent on the cytoskeletal network, with microtubules playing a significant role in long-distance transport (342). For shorter distances, actin may be utilized, as demonstrated in yeast, where mitochondria can bind to actin through outer membrane proteins and regulate mitochondrial transport and distribution (354). In mammals, actin-dependent mitochondrial transport has been observed in neuronal axons (269). Specific myosin motor proteins bind mitochondria to actin filaments to regulate short-distance movement (342, 349, 350). We speculated that this movement may facilitate the transport and alignment of the two fusing mitochondria. Meanwhile, in the majority of tip-to-side fusion events, we observed that one of the fusing mitochondria had an enriched actin site and remained stationary, while the second fusing mitochondrion moved towards this actin-rich site on the first mitochondrion for fusion. Given that actin-enriched regions exhibit reduced motility (347), we believe that actin may stabilize or anchor the receiving mitochondria to facilitate the fusion process. As the role of actin filaments in mitochondrial movement is likely context-dependent, both processes could control the position and stability of the receiving mitochondria, which might be crucial for efficient mitochondrial fusion.

4.6 Actin in fine tuning fission-fusion balance

The significance of maintaining a balance between mitochondrial fission and fusion for overall cellular health has been widely acknowledged. Research has shown that mutations in DRP1, inhibiting fission activity, lead to mitochondrial elongation due to sustained fusion (129, 132), and a lack of fusion proteins results in mitochondrial fragmentation, thereby countering the imbalance (155, 192). However, the precise mechanism by which this balance is achieved remains unclear. Our data suggests that actin, regulating both fission and fusion, emerges as a potential candidate for maintaining this equilibrium. Actin inhibition decreases the frequency of both fusion and fission, highlighting its essential role. While depolymerizing actin has been reported to cause mitochondrial elongation, our data imply that elongated mitochondria may result from tip-to-tip fusion in the absence of actin. This phenomenon may lead to an immediate survival response. In contrast, branched mitochondria resulting from tip-to-side fusion require actin. Under nutrient-deprived conditions, it has been reported that Ubl4A (ubiquitin protein) promotes such mitochondrial fusion process via the Arp2/3 complex, promoting cell survival (384). This is consistent with our data, where ECM induced Arp2/3 actin polymerisation induces mitochondrial elongation and acute inhibition of the Arp2/3 complex prevents fusion but not fission, likely due

to the promotion of INF2-dependent fission events. However, deletion of INF2 resulted in a reduction in both fusion and fission events, suggesting that a decrease in fission rates may have led to a decrease in fusion events to preserve a desirable mitochondrial network. Therefore, actin may have a deciding role in balancing fusion and fission, with actin proteins maintaining equilibrium under unstimulated conditions.

4.7 Cell-ECM induced regulation of Metabolism: The perspective of cancer

Cell adhesion to the ECM is essential for sensing the mechanical properties of the cellular environment. Cells typically depend on ECM adhesion for survival, and undergo cell death upon detachment. However, cancer stem cells (CSCs) are less sensitive to ECM detachment owing to oncogene activation, which enables their survival and contributes to metastasis (391). In *in vitro* conditions, cells cultured as mammospheres or spheroids exhibit loose connections with the ECM and extensive cell-cell contacts. A recent study revealed that cell detachment can lead to cadherin upregulation, promoting cell clump formation, and establishing a local hypoxic environment. This environment is associated with rewiring glutamine usage, indirectly supporting lactate and ATP production via glycolysis. Accordingly, our study model, mammospheres in suspension culture, relied on glycolysis with very low metabolic activity. However, they underwent a metabolic shift to OXPHOS dependence upon attachment to the ECM. Here, actin polymerization-induced mitochondrial elongation triggered OXPHOS-dependent activity in ECM-attached mammospheres. This increased metabolic activity was associated with increased FAK activation, which has a crucial role in maintaining mitochondrial membrane potential and OXPHOS activity (381). Altered cellular metabolism in response to changes in the cellular environment has been reported in various cancers. However, the precise mechanisms through which these changes induce alterations in the mitochondria have not been thoroughly investigated. Our study now provides evidence suggesting that ECM signaling triggering mitochondrial metabolism is regulated by the actin polymerization pathway.

During metastasis, cancer cells encounter alterations in stiffness, transitioning from the rigid environment of primary tumors to softer sites of secondary metastases. Metastatic breast cancer cells exhibit varying ATP:ADP ratios based on collagen fiber density and orientation. Dense collagen matrices, which impede cell migration, show higher ATP:ADP ratios, whereas aligned collagen matrices, which promote migration, display lower ratios (392). This indicates the need for increased energy production to meet the demands of the cytoskeleton and focal adhesion remodeling in challenging environments. In pancreatic cancer cells, ECM stiffening induces a metabolic shift towards OXPHOS that promotes cell invasion and migration (51, 381). In BrCSCs,

Soft matrices promoted aerobic glycolysis, while stiffer substrates engaged in sustained ATP production through the TCA cycle and OXPHOS, which correlates with higher levels of reactive oxygen species (ROS). Increased ROS levels activate the transcription factor nuclear factor erythroid 2-related factor (NRF2), which exerts feedback control on ROS. The activation of DRP1 in the soft ECM regulates ROS levels and enables cells to overcome oxidative stress, ultimately facilitating cell migration (219).

The mechanical properties of the ECM, whether stiff or soft, play a crucial role in determining metabolic activity. However, several studies have reported conflicting metabolic phenotypes in different environments. This variability in results depends on the cell type or technique used. The cells in our study were grown in the absence of ECM for suspension culture, resulting in a phenotype distinct from that associated with attachment to a soft ECM. While both conditions lead to a reduction in cellular tension, detachment from the ECM elicits distinct effects. Additionally, detachment cultures maintain CSC properties, which can be compromised by adherent culture-induced differentiation. Because we wanted to study CSC behavior in changing environments, we chose these culture conditions for our study. A notable contrast between soft ECM and no ECM lies in DRP1 expression. While previous studies have reported increased DRP1 activity in cells cultured on soft ECM, we observed markedly low DRP1 expression in cells grown without ECM, and attachment to stiff ECM did not alter protein expression levels for up to 6 h. This disparity may stem from CSC behavior or distinct culture conditions. Furthermore, cells cultured in soft or stiff ECM may already have activated survival triggers, potentially influencing DRP1 expression over longer periods in soft ECM. Numerous variables could influence outcomes; therefore, conducting experiments that compare mitochondrial responses to a specific environmental cue (such as No ECM to Soft ECM) across diverse cell types using standardized techniques would be beneficial. We were limited to studying the mechanisms activated upon attachment; however, these experiments open up promising avenues for future studies in cancer research.

Cells can use multiple metabolic pathways to produce energy, antioxidant power, and intermediates for the biosynthesis of macromolecules. Cancer cells undergo metabolic reprogramming to support rapid growth, which involves various pathways beyond glucose and glutamine catabolism (31, 393). This reprogramming not only yields ATP but also furnishes building blocks for macromolecule biosynthesis and maintains redox homeostasis (31). In our study, we noticed increased expression of ETC proteins in mammmospheres despite their low ATP synthesis, suggesting a potential activity in metabolite production. Therefore, deriving these metabolites could have enhanced our understanding of CSC metabolism. This remains a limitation

of this study and presents a promising avenue for future research. The findings would provide additional proof on CSCs metabolic regulation.

Overall, the metabolic reprogramming exhibited by CSCs proves to be advantageous, aiding their metastatic potential. This study effectively demonstrates that the regulation of this adaptation is orchestrated by the ECM-driven actin pathway, modulating mitochondrial structure and function according to the CSC survival needs.

My PhD journey began with the exploration of the cellular adaptation pathways pursued by CSC. Along this journey, we discovered numerous pathways leading to actin, which plays a pivotal role in regulating both mitochondrial fusion and fission. This discovery holds significant importance in the realm of mitochondrial dynamics, and suggests a mechanistic role. We are optimistic about the future trajectory of mitochondrial dynamics, foreseeing an intricate and curated relationship between actin and mitochondria.

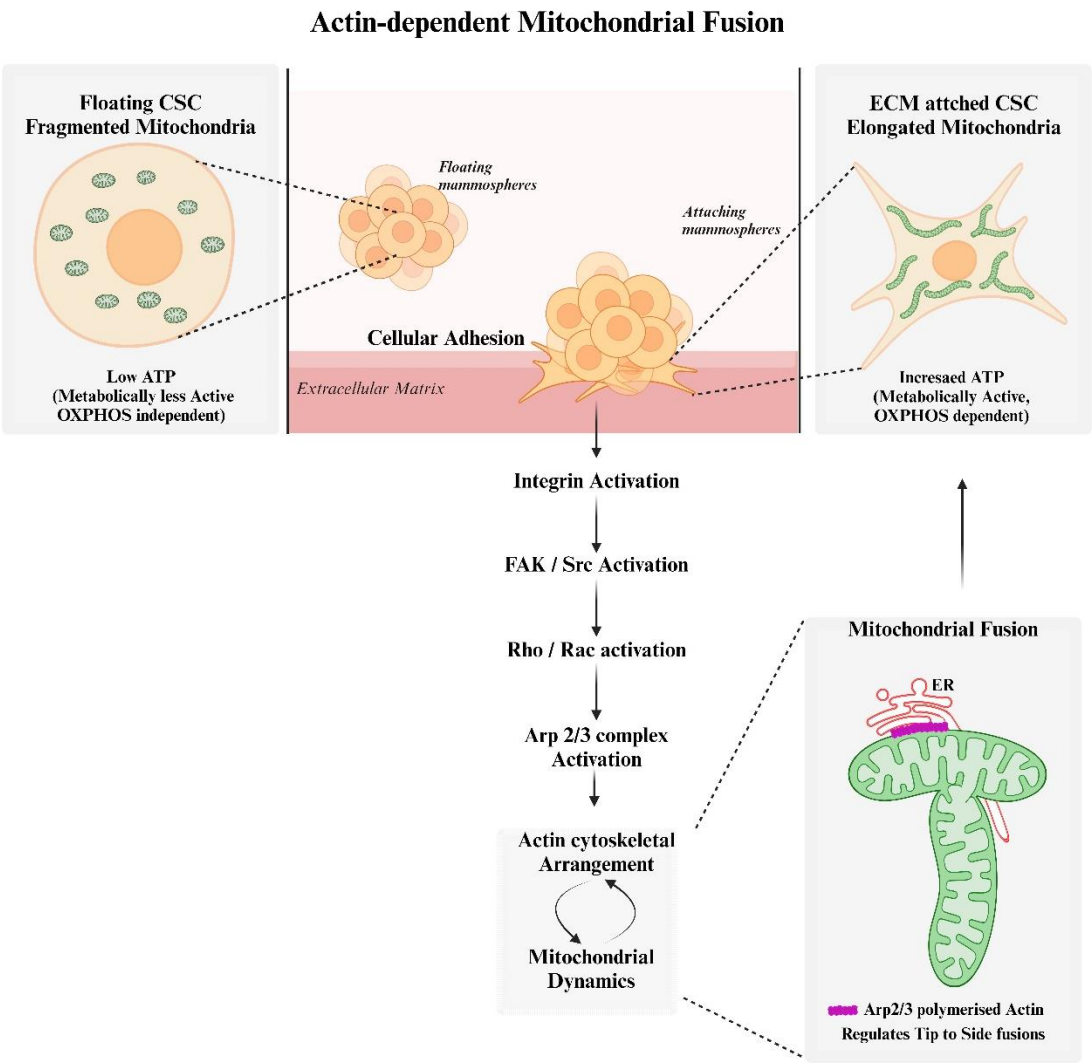


Figure 4.15: Proposed model for actin dependent mitochondrial fusion
Graphical illustration depicting actin-dependent mitochondrial fusion. Floating mammospheres (CSCs) with fragmented mitochondria are characterized by low metabolic activity and reduced ATP levels. Upon attachment to the ECM, they display elongated mitochondria, leading to increased OXPHOS dependency. The cell-ECM interaction triggers the integrin signaling pathway, activating FAK-Rac and promoting Arp2/3 actin polymerization, which plays a crucial role in facilitating tip-to-side mitochondrial fusion.

CHAPTER V

CONCLUSION

The work presented in this thesis contributes to the constantly growing body of evidence on the importance of cell-matrix interactions in guiding mitochondrial dynamics. Breast mammospheres showed a metabolic switch from a metabolically weak glycolytic state to an OXPHOS-dependent state, depending on the availability of the ECM. This switch resembles the dynamic switch in mitochondrial structure upon elongation from the fragmented state (Figure 4.1). This shows that environmental cues can drive growth strategies by regulating integrin signalling pathways.

We found that the integrin-mediated FAK-RAC actin polymerization pathway plays a crucial role in regulating mitochondrial dynamics, thereby regulating mitochondrial metabolism. This provides an answer to the long-standing question of metabolic plasticity in resistant CSC cells. We show that Rac-driven actin polymerisation was necessary to drive OXPHOS (Figure 4.1), hence representing potential targets for cancer therapy.

Moreover, the specific role of actin polymerization in promoting mitochondrial elongation led us to question the possible role of actin in the regulation of mitochondrial fusion, which has implications in balancing the dynamic process. Based on this question, we have now added to the existing knowledge of mitochondrial dynamics that actin is an essential regulator of mitochondrial fusion. We have shown that mitochondria-associated actin is localized to the site of mitochondrial fusion and is necessary to organize tip-to-side fusions (Figure 4.1). Specifically, Arp2/3 dependent actin polymerisation plays a key role in regulating the fusion process, while INF2 is essential for both fusion and fission.

In conclusion, the novel findings of this thesis revealed that CSCs acquire metabolic flexibility by regulating the actin polymerization pathway, promoting mitochondrial elongation, and enhancing OXPHOS to meet cellular energy demands. What adds an extra layer of significance to these findings is the robust evidence presented in this thesis, supporting the pivotal role of polymerized actin in mitochondrial fusion (Figure 4.1). This discovery unequivocally positions actin as the master regulator of mitochondrial dynamics. This thesis represents a significant advancement in our comprehension of the dynamic interplay between the ECM, cytoskeleton, and mitochondria, paving the way for exciting avenues in future research and potential therapeutic interventions.

References

1. Perfettini JL, Roumier T, Kroemer G. Mitochondrial fusion and fission in the control of apoptosis. Trends Cell Biol. 2005;15(4):179-83.
2. Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell. 2002;2(1):55-67.
3. A M, Fung TS, Francomacaro LM, Huynh T, Kotila T, Svindrych Z, Higgs HN. Regulation of INF2-mediated actin polymerization through site-specific lysine acetylation of actin itself. Proc Natl Acad Sci U S A. 2020;117(1):439-47.
4. Biswas G, Adebanjo OA, Freedman BD, Anandatheerthavarada HK, Vijayasarathy C, Zaidi M, et al. Retrograde Ca^{2+} signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk. EMBO J. 1999;18(3):522-33.
5. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell. 1996;86(1):147-57.
6. Martinus RD, Garth GP, Webster TL, Cartwright P, Naylor DJ, Hoj PB, Hoogenraad NJ. Selective induction of mitochondrial chaperones in response to loss of the mitochondrial genome. Eur J Biochem. 1996;240(1):98-103.
7. Moehlman AT, Youle RJ. Mitochondrial Quality Control and Restraining Innate Immunity. Annu Rev Cell Dev Biol. 2020;36:265-89.
8. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol. 2012;13(9):566-78.
9. West AP, Shadel GS. Mitochondrial DNA in innate immune responses and inflammatory pathology. Nat Rev Immunol. 2017;17(6):363-75.
10. Gray MW. Mitochondrial evolution. Cold Spring Harb Perspect Biol. 2012;4(9):a011403.
11. Rogers K. "mitochondrion." [Web article]. [October 14, 2023.:[Available from: https://www.britannica.com/science/mitochondrion.
12. Karnkowska A, Vacek V, Zubacova Z, Treitli SC, Petrzelkova R, Eme L, et al. A Eukaryote without a Mitochondrial Organelle. Curr Biol. 2016;26(10):1274-84.
13. Chandel NS. Mitochondria as signaling organelles. BMC Biol. 2014;12:34.
14. Giacomello M, Pyakurel A, Glytsou C, Scorrano L. The cell biology of mitochondrial membrane dynamics. Nat Rev Mol Cell Biol. 2020;21(4):204-24.
15. GM. C. Mitochondria. 2nd edition ed. Sunderland (MA): Sinauer Associates; 2000.
16. Cogliati S, Enriquez JA, Scorrano L. Mitochondrial Cristae: Where Beauty Meets Functionality. Trends Biochem Sci. 2016;41(3):261-73.
17. Gilkerson RW, Selker JM, Capaldi RA. The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett. 2003;546(2-3):355-8.
18. Mannella CA. Structure and dynamics of the mitochondrial inner membrane cristae. Biochim Biophys Acta. 2006;1763(5-6):542-8.
19. Poburko D, Demaurex N. Regulation of the mitochondrial proton gradient by cytosolic Ca(2)(+) signals. Pflugers Arch. 2012;464(1):19-26.
20. Herrmann JM, Riemer J. The intermembrane space of mitochondria. Antioxid Redox Signal. 2010;13(9):1341-58.
21. Spelbrink JN. Functional organization of mammalian mitochondrial DNA in nucleoids: history, recent developments, and future challenges. IUBMB Life. 2010;62(1):19-32.
22. Birky CW, Jr. The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. Annu Rev Genet. 2001;35:125-48.
23. Nath S, Villadsen J. Oxidative phosphorylation revisited. Biotechnol Bioeng. 2015;112(3):429-37.

24. Feher J. ATP Production II. Quantitative Human Physiology2017. p. 227-40.
25. Spinelli JB, Haigis MC. The multifaceted contributions of mitochondria to cellular metabolism. Nat Cell Biol. 2018;20(7):745-54.
26. Focusing on mitochondrial form and function. Nat Cell Biol. 2018;20(7):735.
27. Martin WF, Tielens AGM, Mentel M. Mitochondria and Anaerobic Energy Metabolism in Eukaryotes2020.
28. Dekhne AS, Hou Z, Gangjee A, Matherly LH. Therapeutic Targeting of Mitochondrial One-Carbon Metabolism in Cancer. Molecular Cancer Therapeutics. 2020;19(11):2245-55.
29. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab. 2017;25(1):27-42.
30. Figueira TR, Barros MH, Camargo AA, Castilho RF, Ferreira JC, Kowaltowski AJ, et al. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. Antioxid Redox Signal. 2013;18(16):2029-74.
31. Yoo HC, Yu YC, Sung Y, Han JM. Glutamine reliance in cell metabolism. Exp Mol Med. 2020;52(9):1496-516.
32. Haskins N, Bhuvanendran S, Anselmi C, Gams A, Kanholm T, Kocher KM, et al. Mitochondrial Enzymes of the Urea Cycle Cluster at the Inner Mitochondrial Membrane. Front Physiol. 2020;11:542950.
33. Dietz JV, Fox JL, Khalimonchuk O. Down the Iron Path: Mitochondrial Iron Homeostasis and Beyond. Cells. 2021;10(9).
34. Read AD, Bentley RE, Archer SL, Dunham-Snary KJ. Mitochondrial iron-sulfur clusters: Structure, function, and an emerging role in vascular biology. Redox Biol. 2021;47:102164.
35. Adebayo M, Singh S, Singh AP, Dasgupta S. Mitochondrial fusion and fission: The fine-tune balance for cellular homeostasis. FASEB J. 2021;35(6):e21620.
36. Chandel NS. Glycolysis. Cold Spring Harb Perspect Biol. 2021;13(5).
37. Ancey PB, Contat C, Meylan E. Glucose transporters in cancer - from tumor cells to the tumor microenvironment. FEBS J. 2018;285(16):2926-43.
38. Li Z, Ji BW, Dixit PD, Tchourine K, Lien EC, Hosios AM, et al. Cancer cells depend on environmental lipids for proliferation when electron acceptors are limited. Nat Metab. 2022;4(6):711-23.
39. Murphy MP, O'Neill LAJ. How should we talk about metabolism? Nat Immunol. 2020;21(7):713-5.
40. Houtkooper RH, Canto C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. Endocr Rev. 2010;31(2):194-223.
41. Martin JL, Costa ASH, Gruszczyk AV, Beach TE, Allen FM, Prag HA, et al. Succinate accumulation drives ischaemia-reperfusion injury during organ transplantation. Nat Metab. 2019;1:966-74.
42. Halestrap AP, Wilson MC. The monocarboxylate transporter family--role and regulation. IUBMB Life. 2012;64(2):109-19.
43. Gruszczyk AV, Casey AM, James AM, Prag HA, Burger N, Bates GR, et al. Mitochondrial metabolism and bioenergetic function in an anoxic isolated adult mouse cardiomyocyte model of in vivo cardiac ischemia-reperfusion injury. Redox Biol. 2022;54:102368.
44. Rabinowitz JD, Enerback S. Lactate: the ugly duckling of energy metabolism. Nat Metab. 2020;2(7):566-71.
45. Watson MJ, Vignali PDA, Mullett SJ, Overacre-Delgoffe AE, Peralta RM, Grebinoski S, et al. Metabolic support of tumour-infiltrating regulatory T cells by lactic acid. Nature. 2021;591(7851):645-51.
46. Karagiannis A, Gallopin T, Lacroix A, Plaisier F, Piquet J, Geoffroy H, et al. Lactate is an energy substrate for rodent cortical neurons and enhances their firing activity. Elife. 2021;10.
47. Brooks GA. Role of the Heart in Lactate Shuttling. Front Nutr. 2021;8:663560.

48. Zhang D, Tang Z, Huang H, Zhou G, Cui C, Weng Y, et al. Metabolic regulation of gene expression by histone lactylation. Nature. 2019;574(7779):575-80.
49. Mikkola S. Nucleotide Sugars in Chemistry and Biology. Molecules. 2020;25(23).
50. Ohashi K, Kawai S, Murata K. Identification and characterization of a human mitochondrial NAD kinase. Nat Commun. 2012;3:1248.
51. Zhu J, Schworer S, Berisa M, Kyung YJ, Ryu KW, Yi J, et al. Mitochondrial NADP(H) generation is essential for proline biosynthesis. Science. 2021;372(6545):968-72.
52. Campbell S, Mesaros C, Izzo L, Affronti H, Noji M, Schaffer BE, et al. Glutamine deprivation triggers NAGK-dependent hexosamine salvage. Elife. 2021;10.
53. Wellen KE, Lu C, Mancuso A, Lemons JM, Ryczko M, Dennis JW, et al. The hexosamine biosynthetic pathway couples growth factor-induced glutamine uptake to glucose metabolism. Genes Dev. 2010;24(24):2784-99.
54. Kennedy EP, Lehninger AL. Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. J Biol Chem. 1949;179(2):957-72.
55. Elia I, Rowe JH, Johnson S, Joshi S, Notarangelo G, Kurmi K, et al. Tumor cells dictate anti-tumor immune responses by altering pyruvate utilization and succinate signaling in CD8(+) T cells. Cell Metab. 2022;34(8):1137-50 e6.
56. Krebs HA, Eggleston LV. The oxidation of pyruvate in pigeon breast muscle. Biochem J. 1940;34(3):442-59.
57. Krebs HA, Johnson WA. Metabolism of ketonic acids in animal tissues. Biochem J. 1937;31(4):645-60.
58. Chandel NS. Mitochondria. Cold Spring Harb Perspect Biol. 2021;13(3).
59. Arnold PK, Jackson BT, Paras KI, Brunner JS, Hart ML, Newsom OJ, et al. A non-canonical tricarboxylic acid cycle underlies cellular identity. Nature. 2022;603(7901):477-81.
60. Rich PR, Marechal A. The mitochondrial respiratory chain. Essays Biochem. 2010;47:1-23.
61. Matlin KS. The Heuristic of Form: Mitochondrial Morphology and the Explanation of Oxidative Phosphorylation. J Hist Biol. 2016;49(1):37-94.
62. Birsoy K, Wang T, Chen WW, Freinkman E, Abu-Remaileh M, Sabatini DM. An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis. Cell. 2015;162(3):540-51.
63. Sullivan LB, Gui DY, Hosios AM, Bush LN, Freinkman E, Vander Heiden MG. Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. Cell. 2015;162(3):552-63.
64. Bauer DE, Hatzivassiliou G, Zhao F, Andreadis C, Thompson CB. ATP citrate lyase is an important component of cell growth and transformation. Oncogene. 2005;24(41):6314-22.
65. Hatzivassiliou G, Zhao F, Bauer DE, Andreadis C, Shaw AN, Dhanak D, et al. ATP citrate lyase inhibition can suppress tumor cell growth. Cancer Cell. 2005;8(4):311-21.
66. Sivanand S, Viney I, Wellen KE. Spatiotemporal Control of Acetyl-CoA Metabolism in Chromatin Regulation. Trends Biochem Sci. 2018;43(1):61-74.
67. Zhao S, Torres A, Henry RA, Trefely S, Wallace M, Lee JV, et al. ATP-Citrate Lyase Controls a Glucose-to-Acetate Metabolic Switch. Cell Rep. 2016;17(4):1037-52.
68. Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature. 1961;191:144-8.
69. Mitchell P. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. 1966. Biochim Biophys Acta. 2011;1807(12):1507-38.
70. Mitchell P. Keilin's respiratory chain concept and its chemiosmotic consequences. Science. 1979;206(4423):1148-59.
71. Racker E, Stoeckenius W. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. J Biol Chem. 1974;249(2):662-3.

72. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J. 2011;435(2):297-312.
73. Hunte C, Palsdottir H, Trumpower BL. Protonmotive pathways and mechanisms in the cytochrome bc1 complex. FEBS Lett. 2003;545(1):39-46.
74. Rieger B, Junge W, Busch KB. Lateral pH gradient between OXPHOS complex IV and F(0)F(1) ATP-synthase in folded mitochondrial membranes. Nat Commun. 2014;5:3103.
75. Santo-Domingo J, Demaurex N. Perspectives on: SGP symposium on mitochondrial physiology and medicine: the renaissance of mitochondrial pH. J Gen Physiol. 2012;139(6):415-23.
76. Wolf DM, Segawa M, Kondadi AK, Anand R, Bailey ST, Reichert AS, et al. Individual cristae within the same mitochondrion display different membrane potentials and are functionally independent. EMBO J. 2019;38(22):e101056.
77. Murphy MP, Chouchani ET. Why succinate? Physiological regulation by a mitochondrial coenzyme Q sentinel. Nat Chem Biol. 2022;18(5):461-9.
78. Gu J, Liu T, Guo R, Zhang L, Yang M. The coupling mechanism of mammalian mitochondrial complex I. Nat Struct Mol Biol. 2022;29(2):172-82.
79. Hirst J. Mitochondrial complex I. Annu Rev Biochem. 2013;82:551-75.
80. Brand MD. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. Free Radic Biol Med. 2016;100:14-31.
81. Fato R, Bergamini C, Bortolus M, Maniero AL, Leoni S, Ohnishi T, Lenaz G. Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen species. Biochim Biophys Acta. 2009;1787(5):384-92.
82. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. Elife. 2014;3:e02242.
83. Mitchell P. Possible molecular mechanisms of the protonmotive function of cytochrome systems. J Theor Biol. 1976;62(2):327-67.
84. Kaila VR, Verkhovsky MI, Wikstrom M. Proton-coupled electron transfer in cytochrome oxidase. Chem Rev. 2010;110(12):7062-81.
85. Katyal G, Ebanks B, Lucassen M, Papetti C, Chakrabarti L. Sequence and structure comparison of ATP synthase F0 subunits 6 and 8 in notothenioid fish. PLoS One. 2021;16(10):e0245822.
86. Nirody JA, Budin I, Rangamani P. ATP synthase: Evolution, energetics, and membrane interactions. J Gen Physiol. 2020;152(11).
87. Walker JE, Dickson VK. The peripheral stalk of the mitochondrial ATP synthase. Biochim Biophys Acta. 2006;1757(5-6):286-96.
88. Blum TB, Hahn A, Meier T, Davies KM, Kuhlbrandt W. Dimers of mitochondrial ATP synthase induce membrane curvature and self-assemble into rows. Proc Natl Acad Sci U S A. 2019;116(10):4250-5.
89. Symersky J, Osowski D, Walters DE, Mueller DM. Oligomycin frames a common drug-binding site in the ATP synthase. Proc Natl Acad Sci U S A. 2012;109(35):13961-5.
90. Hirst J. Open questions: respiratory chain supercomplexes-why are they there and what do they do? BMC Biol. 2018;16(1):111.
91. Milenkovic D, Blaza JN, Larsson NG, Hirst J. The Enigma of the Respiratory Chain Supercomplex. Cell Metab. 2017;25(4):765-76.
92. Han F, Hu Y, Wu M, He Z, Tian H, Zhou L. Structures of Tetrahymena thermophila respiratory megacomplexes on the tubular mitochondrial cristae. Nat Commun. 2023;14(1):2542.
93. Kohler A, Barrientos A, Fontanesi F, Ott M. The functional significance of mitochondrial respiratory chain supercomplexes. EMBO Rep. 2023;24(11):e57092.

94. Muhleip A, Flygaard RK, Baradaran R, Haapanen O, Gruhl T, Tobiasson V, et al. Structural basis of mitochondrial membrane bending by the I-II-III(2)-IV(2) supercomplex. Nature. 2023;615(7954):934-8.
95. Li JL, Lin TY, Chen PL, Guo TN, Huang SY, Chen CH, et al. Mitochondrial Function and Parkinson's Disease: From the Perspective of the Electron Transport Chain. Front Mol Neurosci. 2021;14:797833.
96. Pasteur L. The Physiological Theory of Fermentation: CreateSpace Independent Publishing Platform; 2018.
97. Racker E. Bioenergetics and the problem of tumor growth. Am Sci. 1972;60(1):56-63.
98. Zheng J. Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review). Oncol Lett. 2012;4(6):1151-7.
99. Corrado M, Pearce EL. Targeting memory T cell metabolism to improve immunity. J Clin Invest. 2022;132(1).
100. Zhao S, Peralta RM, Avina-Ochoa N, Delgoffe GM, Kaech SM. Metabolic regulation of T cells in the tumor microenvironment by nutrient availability and diet. Semin Immunol. 2021;52:101485.
101. Warburg O. ber den Stoffwechsel der Carcinomzelle. Die Naturwissenschaften. 1924;12(50):1131-7.
102. Crabtree HG. Observations on the carbohydrate metabolism of tumours. Biochem J. 1929;23(3):536-45.
103. Warburg O. On the origin of cancer cells. Science. 1956;123(3191):309-14.
104. Warburg O. On respiratory impairment in cancer cells. Science. 1956;124(3215):269-70.
105. Otto AM. Warburg effect(s)-a biographical sketch of Otto Warburg and his impacts on tumor metabolism. Cancer Metab. 2016;4:5.
106. Devic S. Warburg Effect - a Consequence or the Cause of Carcinogenesis? J Cancer. 2016;7(7):817-22.
107. Chandel NS, Schumacker PT. Cellular oxygen sensing by mitochondria: old questions, new insight. J Appl Physiol (1985). 2000;88(5):1880-9.
108. Li S, Li W, Yuan J, Bullova P, Wu J, Zhang X, et al. Impaired oxygen-sensitive regulation of mitochondrial biogenesis within the von Hippel-Lindau syndrome. Nat Metab. 2022;4(6):739-58.
109. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer. 2008;8(9):705-13.
110. Frankenfield DC, Muth ER, Rowe WA. The Harris-Benedict studies of human basal metabolism: history and limitations. J Am Diet Assoc. 1998;98(4):439-45.
111. Harris JA, Benedict FG. A Biometric Study of Human Basal Metabolism. Proc Natl Acad Sci U S A. 1918;4(12):370-3.
112. Folmes CD, Dzeja PP, Nelson TJ, Terzic A. Metabolic plasticity in stem cell homeostasis and differentiation. Cell Stem Cell. 2012;11(5):596-606.
113. Jia D, Park JH, Jung KH, Levine H, Kaipparettu BA. Elucidating the Metabolic Plasticity of Cancer: Mitochondrial Reprogramming and Hybrid Metabolic States. Cells. 2018;7(3).
114. Pelletier A, Stockmann C. The Metabolic Basis of ILC Plasticity. Front Immunol. 2022;13:858051.
115. Kondo H, Ratcliffe CDH, Hooper S, Ellis J, MacRae JI, Hennequart M, et al. Single-cell resolved imaging reveals intra-tumor heterogeneity in glycolysis, transitions between metabolic states, and their regulatory mechanisms. Cell Rep. 2021;34(7):108750.
116. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature. 2008;452(7184):230-3.
117. Potter M, Newport E, Morten KJ. The Warburg effect: 80 years on. Biochem Soc Trans. 2016;44(5):1499-505.

118. Liesa M, Palacin M, Zorzano A. Mitochondrial dynamics in mammalian health and disease. Physiol Rev. 2009;89(3):799-845.
119. Hoppins S, Lackner L, Nunnari J. The machines that divide and fuse mitochondria. Annu Rev Biochem. 2007;76:751-80.
120. McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. Curr Biol. 2006;16(14):R551-60.
121. Patten DA, Wong J, Khacho M, Soubannier V, Mailloux RJ, Pilon-Larose K, et al. OPA1-dependent cristae modulation is essential for cellular adaptation to metabolic demand. EMBO J. 2014;33(22):2676-91.
122. Joubert F, Puff N. Mitochondrial Cristae Architecture and Functions: Lessons from Minimal Model Systems. Membranes (Basel). 2021;11(7).
123. Smirnova E, Griparic L, Shurland DL, van der Bliek AM. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol Biol Cell. 2001;12(8):2245-56.
124. Labrousse AM, Zappaterra MD, Rube DA, van der Bliek AM. C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol Cell. 1999;4(5):815-26.
125. Sesaki H, Jensen RE. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. J Cell Biol. 1999;147(4):699-706.
126. Bhar D, Karren MA, Babst M, Shaw JM. Dimeric Dnm1-G385D interacts with Mdv1 on mitochondria and can be stimulated to assemble into fission complexes containing Mdv1 and Fis1. J Biol Chem. 2006;281(25):17312-20.
127. Ford MG, Jenni S, Nunnari J. The crystal structure of dynamin. Nature. 2011;477(7366):561-6.
128. Ramachandran R, Surka M, Chappie JS, Fowler DM, Foss TR, Song BD, Schmid SL. The dynamin middle domain is critical for tetramerization and higher-order self-assembly. EMBO J. 2007;26(2):559-66.
129. Chang CR, Blackstone C. Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem. 2007;282(30):21583-7.
130. Strack S, Cribbs JT. Allosteric modulation of Drp1 mechanoenzyme assembly and mitochondrial fission by the variable domain. J Biol Chem. 2012;287(14):10990-1001.
131. Frohlich C, Grabiger S, Schwefel D, Faelber K, Rosenbaum E, Mears J, et al. Structural insights into oligomerization and mitochondrial remodelling of dynamin 1-like protein. EMBO J. 2013;32(9):1280-92.
132. Dai CQ, Guo Y, Chu XY. Neuropathic Pain: the Dysfunction of Drp1, Mitochondria, and ROS Homeostasis. Neurotox Res. 2020;38(3):553-63.
133. Friedman JR, Lackner LL, West M, DiBenedetto JR, Nunnari J, Voeltz GK. ER tubules mark sites of mitochondrial division. Science. 2011;334(6054):358-62.
134. Manor U, Bartholomew S, Golani G, Christenson E, Kozlov M, Higgs H, et al. A mitochondria-anchored isoform of the actin-nucleating spire protein regulates mitochondrial division. Elife. 2015;4.
135. Korobova F, Ramabhadran V, Higgs HN. An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2. Science. 2013;339(6118):464-7.
136. Ji WK, Hatch AL, Merrill RA, Strack S, Higgs HN. Actin filaments target the oligomeric maturation of the dynamin GTPase Drp1 to mitochondrial fission sites. Elife. 2015;4:e11553.
137. Bisi S, Disanza A, Malinverno C, Frittoli E, Palamidessi A, Scita G. Membrane and actin dynamics interplay at lamellipodia leading edge. Curr Opin Cell Biol. 2013;25(5):565-73.
138. Loson OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell. 2013;24(5):659-67.

139. Palmer CS, Osellame LD, Laine D, Koutsopoulos OS, Frazier AE, Ryan MT. MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep. 2011;12(6):565-73.
140. Wang Z, Jiang H, Chen S, Du F, Wang X. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell. 2012;148(1-2):228-43.
141. Qi X, Disatnik MH, Shen N, Sobel RA, Mochly-Rosen D. Aberrant mitochondrial fission in neurons induced by protein kinase Cdelta under oxidative stress conditions in vivo. Mol Biol Cell. 2011;22(2):256-65.
142. Han XJ, Lu YF, Li SA, Kaitsuka T, Sato Y, Tomizawa K, et al. CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology. J Cell Biol. 2008;182(3):573-85.
143. Cereghetti GM, Stangherlin A, Martins de Brito O, Chang CR, Blackstone C, Bernardi P, Scorrano L. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc Natl Acad Sci U S A. 2008;105(41):15803-8.
144. Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, Gu Z, Lipton SA. S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science. 2009;324(5923):102-5.
145. Karbowski M, Neutzner A, Youle RJ. The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J Cell Biol. 2007;178(1):71-84.
146. Wang H, Song P, Du L, Tian W, Yue W, Liu M, et al. Parkin ubiquitinates Drp1 for proteasome-dependent degradation: implication of dysregulated mitochondrial dynamics in Parkinson disease. J Biol Chem. 2011;286(13):11649-58.
147. Harder Z, Zunino R, McBride H. Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr Biol. 2004;14(4):340-5.
148. Yamada S, Sato A, Ishihara N, Akiyama H, Sakakibara SI. Drp1 SUMO/deSUMOylation by Senp5 isoforms influences ER tubulation and mitochondrial dynamics to regulate brain development. iScience. 2021;24(12):103484.
149. Wasiak S, Zunino R, McBride HM. Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. J Cell Biol. 2007;177(3):439-50.
150. Lee JE, Westrate LM, Wu H, Page C, Voeltz GK. Multiple dynamin family members collaborate to drive mitochondrial division. Nature. 2016;540(7631):139-43.
151. Fonseca TB, Sanchez-Guerrero A, Milosevic I, Raimundo N. Mitochondrial fission requires DRP1 but not dynamins. Nature. 2019;570(7761):E34-E42.
152. Chakrabarti R, Ji WK, Stan RV, de Juan Sanz J, Ryan TA, Higgs HN. INF2-mediated actin polymerization at the ER stimulates mitochondrial calcium uptake, inner membrane constriction, and division. J Cell Biol. 2018;217(1):251-68.
153. Cho B, Cho HM, Jo Y, Kim HD, Song M, Moon C, et al. Constriction of the mitochondrial inner compartment is a priming event for mitochondrial division. Nat Commun. 2017;8:15754.
154. Wu W, Zhao D, Shah SZA, Zhang X, Lai M, Yang D, et al. OPA1 overexpression ameliorates mitochondrial cristae remodeling, mitochondrial dysfunction, and neuronal apoptosis in prion diseases. Cell Death Dis. 2019;10(10):710.
155. Baker MJ, Tatsuta T, Langer T. Quality control of mitochondrial proteostasis. Cold Spring Harb Perspect Biol. 2011;3(7).
156. Kleele T, Rey T, Winter J, Zaganelli S, Mahecic D, Perreten Lambert H, et al. Distinct fission signatures predict mitochondrial degradation or biogenesis. Nature. 2021;593(7859):435-9.
157. Chakrabarti R, Higgs HN. Revolutionary view of two ways to split a mitochondrion. Nature. 2021;593(7859):346-7.
158. Lewis SC, Uchiyama LF, Nunnari J. ER-mitochondria contacts couple mtDNA synthesis with mitochondrial division in human cells. Science. 2016;353(6296):aaf5549.

159. Yamashita SI, Jin X, Furukawa K, Hamasaki M, Nezu A, Otera H, et al. Mitochondrial division occurs concurrently with autophagosome formation but independently of Drp1 during mitophagy. J Cell Biol. 2016;215(5):649-65.
160. Wong YC, Ysselstein D, Krainc D. Mitochondria-lysosome contacts regulate mitochondrial fission via RAB7 GTP hydrolysis. Nature. 2018;554(7692):382-6.
161. Burman JL, Pickles S, Wang C, Sekine S, Vargas JNS, Zhang Z, et al. Mitochondrial fission facilitates the selective mitophagy of protein aggregates. J Cell Biol. 2017;216(10):3231-47.
162. Soubannier V, McLelland GL, Zunino R, Braschi E, Rippstein P, Fon EA, McBride HM. A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. Curr Biol. 2012;22(2):135-41.
163. Boutry M, DiGiovanni LF, Demers N, Fountain A, Mamand S, Botelho RJ, Kim PK. 2023.
164. Nagashima S, Tabara LC, Tilokani L, Paupe V, Anand H, Pogson JH, et al. Golgi-derived PI(4)P-containing vesicles drive late steps of mitochondrial division. Science. 2020;367(6484):1366-71.
165. Tabara LC, Morris JL, Prudent J. The Complex Dance of Organelles during Mitochondrial Division. Trends Cell Biol. 2021;31(4):241-53.
166. Meeusen S, McCaffery JM, Nunnari J. Mitochondrial fusion intermediates revealed in vitro. Science. 2004;305(5691):1747-52.
167. Malka F, Guillery O, Cifuentes-Diaz C, Guillou E, Belenguer P, Lombes A, Rojo M. Separate fusion of outer and inner mitochondrial membranes. EMBO Rep. 2005;6(9):853-9.
168. Abrisch RG, Gumbin SC, Wisniewski BT, Lackner LL, Voeltz GK. Fission and fusion machineries converge at ER contact sites to regulate mitochondrial morphology. J Cell Biol. 2020;219(4).
169. Guo Y, Li D, Zhang S, Yang Y, Liu JJ, Wang X, et al. Visualizing Intracellular Organelle and Cytoskeletal Interactions at Nanoscale Resolution on Millisecond Timescales. Cell. 2018;175(5):1430-42 e17.
170. Nguyen TT, Voeltz GK. An ER phospholipid hydrolase drives ER-associated mitochondrial constriction for fission and fusion. Elife. 2022;11.
171. Hoppins S, Edlich F, Cleland MM, Banerjee S, McCaffery JM, Youle RJ, Nunnari J. The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Mol Cell. 2011;41(2):150-60.
172. Brandt T, Cavellini L, Kuhlbrandt W, Cohen MM. A mitofusin-dependent docking ring complex triggers mitochondrial fusion in vitro. Elife. 2016;5.
173. Hales KG, Fuller MT. Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. Cell. 1997;90(1):121-9.
174. Hermann GJ, Thatcher JW, Mills JP, Hales KG, Fuller MT, Nunnari J, Shaw JM. Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J Cell Biol. 1998;143(2):359-73.
175. Santel A, Fuller MT. Control of mitochondrial morphology by a human mitofusin. J Cell Sci. 2001;114(Pt 5):867-74.
176. Yan L, Qi Y, Huang X, Yu C, Lan L, Guo X, et al. Structural basis for GTP hydrolysis and conformational change of MFN1 in mediating membrane fusion. Nat Struct Mol Biol. 2018;25(3):233-43.
177. Li YJ, Cao YL, Feng JX, Qi Y, Meng S, Yang JF, et al. Structural insights of human mitofusin-2 into mitochondrial fusion and CMT2A onset. Nat Commun. 2019;10(1):4914.
178. Koshiba T, Detmer SA, Kaiser JT, Chen H, McCaffery JM, Chan DC. Structural basis of mitochondrial tethering by mitofusin complexes. Science. 2004;305(5685):858-62.
179. Huang P, Galloway CA, Yoon Y. Control of mitochondrial morphology through differential interactions of mitochondrial fusion and fission proteins. PLoS One. 2011;6(5):e20655.

180. Cao YL, Meng S, Chen Y, Feng JX, Gu DD, Yu B, et al. MFN1 structures reveal nucleotide-triggered dimerization critical for mitochondrial fusion. Nature. 2017;542(7641):372-6.
181. Mattie S, Riemer J, Wideman JG, McBride HM. A new mitofusin topology places the redox-regulated C terminus in the mitochondrial intermembrane space. J Cell Biol. 2018;217(2):507-15.
182. Huang X, Zhou X, Hu X, Joshi AS, Guo X, Zhu Y, et al. Sequences flanking the transmembrane segments facilitate mitochondrial localization and membrane fusion by mitofusin. Proc Natl Acad Sci U S A. 2017;114(46):E9863-E72.
183. Cohen MM, Tareste D. Recent insights into the structure and function of Mitofusins in mitochondrial fusion. F1000Res. 2018;7.
184. Sesaki H, Southard SM, Yaffe MP, Jensen RE. Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. Mol Biol Cell. 2003;14(6):2342-56.
185. Olichon A, Emorine LJ, Descoins E, Pelloquin L, Brichese L, Gas N, et al. The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. FEBS Lett. 2002;523(1-3):171-6.
186. Head B, Griparic L, Amiri M, Gandre-Babbe S, van der Bliek AM. Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J Cell Biol. 2009;187(7):959-66.
187. Griparic L, Kanazawa T, van der Bliek AM. Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol. 2007;178(5):757-64.
188. Tondera D, Grandemange S, Jourdain A, Karbowski M, Mattenberger Y, Herzig S, et al. SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO J. 2009;28(11):1589-600.
189. Ban T, Heymann JA, Song Z, Hinshaw JE, Chan DC. OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation. Hum Mol Genet. 2010;19(11):2113-22.
190. DeVay RM, Dominguez-Ramirez L, Lackner LL, Hoppins S, Stahlberg H, Nunnari J. Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. J Cell Biol. 2009;186(6):793-803.
191. Rujiviphat J, Meglei G, Rubinstein JL, McQuibban GA. Phospholipid association is essential for dynamin-related protein Mgm1 to function in mitochondrial membrane fusion. J Biol Chem. 2009;284(42):28682-6.
192. Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, et al. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell. 2006;126(1):177-89.
193. Pernas L, Scorrano L. Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. Annu Rev Physiol. 2016;78:505-31.
194. Kushnareva YE, Gerencser AA, Bossy B, Ju WK, White AD, Waggoner J, et al. Loss of OPA1 disturbs cellular calcium homeostasis and sensitizes for excitotoxicity. Cell Death Differ. 2013;20(2):353-65.
195. Lee H, Smith SB, Yoon Y. The short variant of the mitochondrial dynamin OPA1 maintains mitochondrial energetics and cristae structure. J Biol Chem. 2017;292(17):7115-30.
196. Elhamdani A, Azizi F, Artalejo CR. Double patch clamp reveals that transient fusion (kiss-and-run) is a major mechanism of secretion in calf adrenal chromaffin cells: high calcium shifts the mechanism from kiss-and-run to complete fusion. J Neurosci. 2006;26(11):3030-6.
197. Liu X, Weaver D, Shirihai O, Hajnoczky G. Mitochondrial 'kiss-and-run': interplay between mitochondrial motility and fusion-fission dynamics. EMBO J. 2009;28(20):3074-89.
198. Scott I, Youle RJ. Mitochondrial fission and fusion. Essays Biochem. 2010;47:85-98.
199. Xie X, Venit T, Drou N, Percipalle P. In Mitochondria ?-Actin Regulates mtDNA Transcription and Is Required for Mitochondrial Quality Control. iScience. 2018;3:226-37.

200. Pipis M, Feely SME, Polke JM, Skorupinska M, Perez L, Shy RR, et al. Natural history of Charcot-Marie-Tooth disease type 2A: a large international multicentre study. Brain. 2020;143(12):3589-602.
201. Yu-Wai-Man P, Bailie M, Atawan A, Chinnery PF, Griffiths PG. Pattern of retinal ganglion cell loss in dominant optic atrophy due to OPA1 mutations. Eye (Lond). 2011;25(5):596-602.
202. McCormick EM, Zolkipli-Cunningham Z, Falk MJ. Mitochondrial disease genetics update: recent insights into the molecular diagnosis and expanding phenotype of primary mitochondrial disease. Curr Opin Pediatr. 2018;30(6):714-24.
203. Song Z, Chen H, Fiket M, Alexander C, Chan DC. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J Cell Biol. 2007;178(5):749-55.
204. Chen Z, Borek D, Padrick SB, Gomez TS, Metlagel Z, Ismail AM, et al. Structure and control of the actin regulatory WAVE complex. Nature. 2010;468(7323):533-8.
205. Gomes LC, Di Benedetto G, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol. 2011;13(5):589-98.
206. Rambold AS, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc Natl Acad Sci U S A. 2011;108(25):10190-5.
207. Breckenridge DG, Stojanovic M, Marcellus RC, Shore GC. Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. J Cell Biol. 2003;160(7):1115-27.
208. Ban-Ishihara R, Ishihara T, Sasaki N, Mihara K, Ishihara N. Dynamics of nucleoid structure regulated by mitochondrial fission contributes to cristae reformation and release of cytochrome c. Proc Natl Acad Sci U S A. 2013;110(29):11863-8.
209. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J. 2001;20(23):6627-36.
210. Jezek J, Cooper KF, Strich R. Reactive Oxygen Species and Mitochondrial Dynamics: The Yin and Yang of Mitochondrial Dysfunction and Cancer Progression. Antioxidants (Basel). 2018;7(1).
211. Song M, Franco A, Fleischer JA, Zhang L, Dorn GW, 2nd. Abrogating Mitochondrial Dynamics in Mouse Hearts Accelerates Mitochondrial Senescence. Cell Metab. 2017;26(6):872-83 e5.
212. de Brito OM, Scorrano L. An intimate liaison: spatial organization of the endoplasmic reticulum-mitochondria relationship. EMBO J. 2010;29(16):2715-23.
213. Lang AB, John Peter AT, Walter P, Kornmann B. ER-mitochondrial junctions can be bypassed by dominant mutations in the endosomal protein Vps13. J Cell Biol. 2015;210(6):883-90.
214. Murley A, Lackner LL, Osman C, West M, Voeltz GK, Walter P, Nunnari J. ER-associated mitochondrial division links the distribution of mitochondria and mitochondrial DNA in yeast. Elife. 2013;2:e00422.
215. de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature. 2008;456(7222):605-10.
216. Nagashima S, Tokuyama T, Yonashiro R, Inatome R, Yanagi S. Roles of mitochondrial ubiquitin ligase MITOL/MARCH5 in mitochondrial dynamics and diseases. J Biochem. 2014;155(5):273-9.
217. Moore AS, Holzbaur ELF. Mitochondrial-cytoskeletal interactions: dynamic associations that facilitate network function and remodeling. Curr Opin Physiol. 2018;3:94-100.
218. Tilokani L, Nagashima S, Paupe V, Prudent J. Mitochondrial dynamics: overview of molecular mechanisms. Essays Biochem. 2018;62(3):341-60.

219. Romani P, Nirchio N, Arboit M, Barbieri V, Tosi A, Michielin F, et al. Mitochondrial fission links ECM mechanotransduction to metabolic redox homeostasis and metastatic chemotherapy resistance. Nat Cell Biol. 2022;24(2):168-80.
220. Suen DF, Norris KL, Youle RJ. Mitochondrial dynamics and apoptosis. Genes Dev. 2008;22(12):1577-90.
221. Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell. 2011;21(1):92-101.
222. Garcia-Saez AJ. The secrets of the Bcl-2 family. Cell Death Differ. 2012;19(11):1733-40.
223. McArthur K, Whitehead LW, Heddleston JM, Li L, Padman BS, Oorschot V, et al. BAK/BAX macropores facilitate mitochondrial herniation and mtDNA efflux during apoptosis. Science. 2018;359(6378).
224. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. 2008;9(1):47-59.
225. Westphal D, Kluck RM, Dewson G. Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. Cell Death Differ. 2014;21(2):196-205.
226. Iwasawa R, Mahul-Mellier AL, Datler C, Pazarentzos E, Grimm S. Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction. EMBO J. 2011;30(3):556-68.
227. Mishra P, Carelli V, Manfredi G, Chan DC. Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. Cell Metab. 2014;19(4):630-41.
228. Roy M, Reddy PH, Iijima M, Sesaki H. Mitochondrial division and fusion in metabolism. Curr Opin Cell Biol. 2015;33:111-8.
229. Song M, Mihara K, Chen Y, Scorrano L, Dorn GW, 2nd. Mitochondrial fission and fusion factors reciprocally orchestrate mitophagic culling in mouse hearts and cultured fibroblasts. Cell Metab. 2015;21(2):273-86.
230. Ishihara N, Nomura M, Jofuku A, Kato H, Suzuki SO, Masuda K, et al. Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol. 2009;11(8):958-66.
231. Wang Q, Zhang M, Torres G, Wu S, Ouyang C, Xie Z, Zou MH. Metformin Suppresses Diabetes-Accelerated Atherosclerosis via the Inhibition of Drp1-Mediated Mitochondrial Fission. Diabetes. 2017;66(1):193-205.
232. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 2008;27(2):433-46.
233. Cogliati S, Frezza C, Soriano ME, Varanita T, Quintana-Cabrera R, Corrado M, et al. Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. Cell. 2013;155(1):160-71.
234. Liesa M, Shirihai OS. Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. Cell Metab. 2013;17(4):491-506.
235. Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. J Pathol. 2003;200(4):423-8.
236. Trebacz H, Barzycka A. Mechanical Properties and Functions of Elastin: An Overview. Biomolecules. 2023;13(3).
237. Harburger DS, Calderwood DA. Integrin signalling at a glance. J Cell Sci. 2009;122(Pt 2):159-63.
238. Leiss M, Beckmann K, Giros A, Costell M, Fassler R. The role of integrin binding sites in fibronectin matrix assembly in vivo. Curr Opin Cell Biol. 2008;20(5):502-7.
239. Turino GM, Cantor JO. Hyaluronan in respiratory injury and repair. Am J Respir Crit Care Med. 2003;167(9):1169-75.

240. Souza-Fernandes AB, Pelosi P, Rocco PR. Bench-to-bedside review: the role of glycosaminoglycans in respiratory disease. Crit Care. 2006;10(6):237.
241. Jaumouille V, Cartagena-Rivera AX, Waterman CM. Coupling of beta(2) integrins to actin by a mechanosensitive molecular clutch drives complement receptor-mediated phagocytosis. Nat Cell Biol. 2019;21(11):1357-69.
242. Juliano RL. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Annu Rev Pharmacol Toxicol. 2002;42:283-323.
243. Chastney MR, Conway JRW, Ivaska J. Integrin adhesion complexes. Curr Biol. 2021;31(10):R536-R42.
244. Cary LA, Han DC, Guan JL. Integrin-mediated signal transduction pathways. Histol Histopathol. 1999;14(3):1001-9.
245. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123(Pt 24):4195-200.
246. Soles A, Selimovic A, Sbrocco K, Ghannoum F, Hamel K, Moncada EL, et al. Extracellular Matrix Regulation in Physiology and in Brain Disease. Int J Mol Sci. 2023;24(8).
247. Barczyk M, Carracedo S, Gullberg D. Integrins. Cell Tissue Res. 2010;339(1):269-80.
248. Downey-Biechler C, Craig DH, More SK, Basson MD. Inside-out signaling through FAK-integrin axis may regulate circulating cancer cell metastatic adhesion. Proc Natl Acad Sci U S A. 2019;116(40):19795-6.
249. Sharma A, Mayer BJ. Phosphorylation of p130Cas initiates Rac activation and membrane ruffling. BMC Cell Biol. 2008;9:50.
250. Di P, Camacho Leal MdP, Bisaro B, Tornillo G, Repetto D, Pincini A, et al. p130Cas and p140Cap as the Bad and Good Guys in Breast Cancer Cell Progression to an Invasive Phenotype. Breast Cancer - Focusing Tumor Microenvironment, Stem cells and Metastasis2011.
251. Galbraith CG, Yamada KM, Sheetz MP. The relationship between force and focal complex development. J Cell Biol. 2002;159(4):695-705.
252. Kechagia JZ, Ivaska J, Roca-Cusachs P. Integrins as biomechanical sensors of the microenvironment. Nat Rev Mol Cell Biol. 2019;20(8):457-73.
253. Riveline D, Zamir E, Balaban NQ, Schwarz US, Ishizaki T, Narumiya S, et al. Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol. 2001;153(6):1175-86.
254. Rossier O, Octeau V, Sibarita JB, Leduc C, Tessier B, Nair D, et al. Integrins beta1 and beta3 exhibit distinct dynamic nanoscale organizations inside focal adhesions. Nat Cell Biol. 2012;14(10):1057-67.
255. Bharadwaj M, Strohmeyer N, Colo GP, Helenius J, Beerenwinkel N, Schiller HB, et al. alphaV-class integrins exert dual roles on alpha5beta1 integrins to strengthen adhesion to fibronectin. Nat Commun. 2017;8:14348.
256. Wang N, Butler JP, Ingber DE. Mechanotransduction across the cell surface and through the cytoskeleton. Science. 1993;260(5111):1124-7.
257. Ingber DE, Wang N, Stamenovic D. Tensegrity, cellular biophysics, and the mechanics of living systems. Rep Prog Phys. 2014;77(4):046603.
258. Chen Y, Ju L, Rushdi M, Ge C, Zhu C. Receptor-mediated cell mechanosensing. Mol Biol Cell. 2017;28(23):3134-55.
259. Shannon N, Gravelle R, Cunniff B. Mitochondrial trafficking and redox/phosphorylation signaling supporting cell migration phenotypes. Front Mol Biosci. 2022;9:925755.
260. Kage F, Vicente-Manzanares M, McEwan BC, Kettenbach AN, Higgs HN. Myosin II proteins are required for organization of calcium-induced actin networks upstream of mitochondrial division. Mol Biol Cell. 2022;33(7):ar63.

261. Hatch AL, Ji WK, Merrill RA, Strack S, Higgs HN. Actin filaments as dynamic reservoirs for Drp1 recruitment. Mol Biol Cell. 2016;27(20):3109-21.
262. Valencia DA, Quinlan ME. Formins. Curr Biol. 2021;31(10):R517-R22.
263. Pollard TD. Actin and Actin-Binding Proteins. Cold Spring Harb Perspect Biol. 2016;8(8).
264. Cleveland DW, Lopata MA, MacDonald RJ, Cowan NJ, Rutter WJ, Kirschner MW. Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. Cell. 1980;20(1):95-105.
265. Gunning PW, Ghoshdastider U, Whitaker S, Popp D, Robinson RC. The evolution of compositionally and functionally distinct actin filaments. J Cell Sci. 2015;128(11):2009-19.
266. Gardiner J, McGee P, Overall R, Marc J. Are histones, tubulin, and actin derived from a common ancestral protein? Protoplasma. 2008;233(1-2):1-5.
267. Falzone TT, Lenz M, Kovar DR, Gardel ML. Assembly kinetics determine the architecture of alpha-actinin crosslinked F-actin networks. Nat Commun. 2012;3:861.
268. Pollard TD. Assembly and dynamics of the actin filament system in nonmuscle cells. J Cell Biochem. 1986;31(2):87-95.
269. Aiken J, Holzbaur ELF. Cytoskeletal regulation guides neuronal trafficking to effectively supply the synapse. Curr Biol. 2021;31(10):R633-R50.
270. Mattila PK, Batista FD, Treanor B. Dynamics of the actin cytoskeleton mediates receptor cross talk: An emerging concept in tuning receptor signaling. J Cell Biol. 2016;212(3):267-80.
271. Dominguez R, Holmes KC. Actin structure and function. Annu Rev Biophys. 2011;40:169-86.
272. Holmes KC, Popp D, Gebhard W, Kabsch W. Atomic model of the actin filament. Nature. 1990;347(6288):44-9.
273. Sept D, Elcock AH, McCammon JA. Computer simulations of actin polymerization can explain the barbed-pointed end asymmetry. J Mol Biol. 1999;294(5):1181-9.
274. Kudryashov DS, Reisler E. ATP and ADP actin states. Biopolymers. 2013;99(4):245-56.
275. Levitsky DI, Pivovarova AV, Mikhailova VV, Nikolaeva OP. Thermal unfolding and aggregation of actin. FEBS J. 2008;275(17):4280-95.
276. Spinardi L, Witke W. Gelsolin and diseases. Subcell Biochem. 2007;45:55-69.
277. Blanchoin L, Boujemaa-Paterski R, Sykes C, Plastino J. Actin dynamics, architecture, and mechanics in cell motility. Physiol Rev. 2014;94(1):235-63.
278. Shao X, Li Q, Mogilner A, Bershadsky AD, Shivashankar GV. Mechanical stimulation induces formin-dependent assembly of a perinuclear actin rim. Proc Natl Acad Sci U S A. 2015;112(20):E2595-601.
279. Wales P, Schuberth CE, Aufschnaiter R, Fels J, Garcia-Aguilar I, Janning A, et al. Calcium-mediated actin reset (CaAR) mediates acute cell adaptations. Elife. 2016;5.
280. Li TD, Bieling P, Weichsel J, Mullins RD, Fletcher DA. The molecular mechanism of load adaptation by branched actin networks. Elife. 2022;11.
281. Achard V, Martiel JL, Michelot A, Guerin C, Reymann AC, Blanchoin L, Boujemaa-Paterski R. A "primer"-based mechanism underlies branched actin filament network formation and motility. Curr Biol. 2010;20(5):423-8.
282. Akin O, Mullins RD. Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex. Cell. 2008;133(5):841-51.
283. Bieling P, Hansen SD, Akin O, Li TD, Hayden CC, Fletcher DA, Mullins RD. WH2 and proline-rich domains of WASP-family proteins collaborate to accelerate actin filament elongation. EMBO J. 2018;37(1):102-21.
284. Clark AG, Miller AL, Vaughan E, Yu HY, Penkert R, Bement WM. Integration of single and multicellular wound responses. Curr Biol. 2009;19(16):1389-95.
285. Mooren OL, Galletta BJ, Cooper JA. Roles for actin assembly in endocytosis. Annu Rev Biochem. 2012;81:661-86.

286. Yamaguchi H, Lorenz M, Kempiak S, Sarmiento C, Coniglio S, Symons M, et al. Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. J Cell Biol. 2005;168(3):441-52.
287. Wagner B, Tharmann R, Haase I, Fischer M, Bausch AR. Cytoskeletal polymer networks: the molecular structure of cross-linkers determines macroscopic properties. Proc Natl Acad Sci U S A. 2006;103(38):13974-8.
288. Kasza KE, Broedersz CP, Koenderink GH, Lin YC, Messner W, Millman EA, et al. Actin filament length tunes elasticity of flexibly cross-linked actin networks. Biophys J. 2010;99(4):1091-100.
289. Courson DS, Rock RS. Actin cross-link assembly and disassembly mechanics for alpha-Actinin and fascin. J Biol Chem. 2010;285(34):26350-7.
290. Schmoller KM, Lieleg O, Bausch AR. Cross-linking molecules modify composite actin networks independently. Phys Rev Lett. 2008;101(11):118102.
291. Kohler S, Schaller V, Bausch AR. Collective dynamics of active cytoskeletal networks. PLoS One. 2011;6(8):e23798.
292. Revenu C, Athman R, Robine S, Louvard D. The co-workers of actin filaments: from cell structures to signals. Nat Rev Mol Cell Biol. 2004;5(8):635-46.
293. Bartles JR. Parallel actin bundles and their multiple actin-bundling proteins. Curr Opin Cell Biol. 2000;12(1):72-8.
294. Ferrer JM, Lee H, Chen J, Pelz B, Nakamura F, Kamm RD, Lang MJ. Measuring molecular rupture forces between single actin filaments and actin-binding proteins. Proc Natl Acad Sci U S A. 2008;105(27):9221-6.
295. Vignjevic D, Yarar D, Welch MD, Peloquin J, Svitkina T, Borisy GG. Formation of filopodia-like bundles in vitro from a dendritic network. J Cell Biol. 2003;160(6):951-62.
296. Yang C, Svitkina T. Filopodia initiation: focus on the Arp2/3 complex and formins. Cell Adh Migr. 2011;5(5):402-8.
297. Burridge K, Wittchen ES. The tension mounts: stress fibers as force-generating mechanotransducers. J Cell Biol. 2013;200(1):9-19.
298. Hotulainen P, Lappalainen P. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. J Cell Biol. 2006;173(3):383-94.
299. Laporte D, Ojkic N, Vavylonis D, Wu JQ. alpha-Actinin and fimbrin cooperate with myosin II to organize actomyosin bundles during contractile-ring assembly. Mol Biol Cell. 2012;23(16):3094-110.
300. Schwarz US, Gardel ML. United we stand: integrating the actin cytoskeleton and cell-matrix adhesions in cellular mechanotransduction. J Cell Sci. 2012;125(Pt 13):3051-60.
301. Carlier MF, Laurent V, Santolini J, Melki R, Didry D, Xia GX, et al. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. J Cell Biol. 1997;136(6):1307-22.
302. Ingerman E, Hsiao JY, Mullins RD. Arp2/3 complex ATP hydrolysis promotes lamellipodial actin network disassembly but is dispensable for assembly. J Cell Biol. 2013;200(5):619-33.
303. Reymann AC, Suarez C, Guerin C, Martiel JL, Staiger CJ, Blanchoin L, Boujemaa-Paterski R. Turnover of branched actin filament networks by stochastic fragmentation with ADF/cofilin. Mol Biol Cell. 2011;22(14):2541-50.
304. Ishikawa R, Sakamoto T, Ando T, Higashi-Fujime S, Kohama K. Polarized actin bundles formed by human fascin-1: their sliding and disassembly on myosin II and myosin V in vitro. J Neurochem. 2003;87(3):676-85.
305. Gautreau AM, Fregoso FE, Simanov G, Dominguez R. Nucleation, stabilization, and disassembly of branched actin networks. Trends Cell Biol. 2022;32(5):421-32.
306. Muller J, Oma Y, Vallar L, Friederich E, Poch O, Winsor B. Sequence and comparative genomic analysis of actin-related proteins. Mol Biol Cell. 2005;16(12):5736-48.

307. Rouiller I, Xu XP, Amann KJ, Egile C, Nickell S, Nicastro D, et al. The structural basis of actin filament branching by the Arp2/3 complex. J Cell Biol. 2008;180(5):887-95.
308. Mullins RD, Heuser JA, Pollard TD. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc Natl Acad Sci U S A. 1998;95(11):6181-6.
309. Svitkina TM, Borisy GG. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. J Cell Biol. 1999;145(5):1009-26.
310. Pantaloni D, Boujemaa R, Didry D, Gounon P, Carlier MF. The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins. Nat Cell Biol. 2000;2(7):385-91.
311. Amann KJ, Pollard TD. The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. Nat Cell Biol. 2001;3(3):306-10.
312. Weaver AM, Karginov AV, Kinley AW, Weed SA, Li Y, Parsons JT, Cooper JA. Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr Biol. 2001;11(5):370-4.
313. Higgs HN, Peterson KJ. Phylogenetic analysis of the formin homology 2 domain. Mol Biol Cell. 2005;16(1):1-13.
314. Breitsprecher D, Goode BL. Formins at a glance. J Cell Sci. 2013;126(Pt 1):1-7.
315. Dominguez R. The WH2 Domain and Actin Nucleation: Necessary but Insufficient. Trends Biochem Sci. 2016;41(6):478-90.
316. Faix J, Rottner K. Ena/VASP proteins in cell edge protrusion, migration and adhesion. J Cell Sci. 2022;135(6).
317. Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, Caroni P. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature. 1998;393(6687):805-9.
318. Wiggan O, Bernstein BW, Bamburg JR. A phosphatase for cofilin to be HAD. Nat Cell Biol. 2005;7(1):8-9.
319. Hoffmann L, Rust MB, Culmsee C. Actin(g) on mitochondria - a role for cofilin1 in neuronal cell death pathways. Biol Chem. 2019;400(9):1089-97.
320. Rehklau K, Hoffmann L, Gurniak CB, Ott M, Witke W, Scorrano L, et al. Cofilin1-dependent actin dynamics control DRP1-mediated mitochondrial fission. Cell Death Dis. 2017;8(10):e3063.
321. Le HT, Hirko AC, Thinschmidt JS, Grant M, Li Z, Peris J, et al. The protective effects of plasma gelsolin on stroke outcome in rats. Exp Transl Stroke Med. 2011;3(1):13.
322. Koya RC, Fujita H, Shimizu S, Ohtsu M, Takimoto M, Tsujimoto Y, Kuzumaki N. Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. J Biol Chem. 2000;275(20):15343-9.
323. Wang H, Cheng B, Chen Q, Wu S, Lv C, Xie G, et al. Time course of plasma gelsolin concentrations during severe sepsis in critically ill surgical patients. Crit Care. 2008;12(4):R106.
324. Mockrin SC, Korn ED. Acanthamoeba profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5'-triphosphate. Biochemistry. 1980;19(23):5359-62.
325. Vinson VK, De La Cruz EM, Higgs HN, Pollard TD. Interactions of Acanthamoeba profilin with actin and nucleotides bound to actin. Biochemistry. 1998;37(31):10871-80.
326. Safer D, Elzinga M, Nachmias VT. Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J Biol Chem. 1991;266(7):4029-32.
327. Burianek LE, Soderling SH. Under lock and key: spatiotemporal regulation of WASP family proteins coordinates separate dynamic cellular processes. Semin Cell Dev Biol. 2013;24(4):258-66.
328. Rottner K, Stradal TEB, Chen B. WAVE regulatory complex. Curr Biol. 2021;31(10):R512-R7.

329. Rotty JD, Wu C, Bear JE. New insights into the regulation and cellular functions of the ARP2/3 complex. Nat Rev Mol Cell Biol. 2013;14(1):7-12.
330. Koronakis V, Hume PJ, Humphreys D, Liu T, Horning O, Jensen ON, McGhie EJ. WAVE regulatory complex activation by cooperating GTPases Arf and Rac1. Proc Natl Acad Sci U S A. 2011;108(35):14449-54.
331. Takenawa T, Suetsugu S. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol. 2007;8(1):37-48.
332. Stradal TE, Scita G. Protein complexes regulating Arp2/3-mediated actin assembly. Curr Opin Cell Biol. 2006;18(1):4-10.
333. Oikawa T, Yamaguchi H, Itoh T, Kato M, Ijuin T, Yamazaki D, et al. PtdIns(3,4,5)P3 binding is necessary for WAVE2-induced formation of lamellipodia. Nat Cell Biol. 2004;6(5):420-6.
334. De Vos KJ, Allan VJ, Grierson AJ, Sheetz MP. Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. Curr Biol. 2005;15(7):678-83.
335. Ji WK, Chakrabarti R, Fan X, Schoenfeld L, Strack S, Higgs HN. Receptor-mediated Drp1 oligomerization on endoplasmic reticulum. J Cell Biol. 2017;216(12):4123-39.
336. Francy CA, Alvarez FJ, Zhou L, Ramachandran R, Mears JA. The mechanoenzymatic core of dynamin-related protein 1 comprises the minimal machinery required for membrane constriction. J Biol Chem. 2015;290(18):11692-703.
337. Liu A, Kage F, Higgs HN. Mff oligomerization is required for Drp1 activation and synergy with actin filaments during mitochondrial division. Mol Biol Cell. 2021;32(20):ar5.
338. Pagliuso A, Tham TN, Stevens JK, Lagache T, Persson R, Salles A, et al. A role for septin 2 in Drp1-mediated mitochondrial fission. EMBO Rep. 2016;17(6):858-73.
339. Hirokawa N, Takemura R. Molecular motors and mechanisms of directional transport in neurons. Nat Rev Neurosci. 2005;6(3):201-14.
340. Schwarz TL. Mitochondrial trafficking in neurons. Cold Spring Harb Perspect Biol. 2013;5(6).
341. van Spronsen M, Mikhaylova M, Lipka J, Schlager MA, van den Heuvel DJ, Kuijpers M, et al. TRAK/Milton motor-adaptor proteins steer mitochondrial trafficking to axons and dendrites. Neuron. 2013;77(3):485-502.
342. Kruppa AJ, Buss F. Motor proteins at the mitochondria-cytoskeleton interface. J Cell Sci. 2021;134(7).
343. Rintoul GL, Filiano AJ, Brocard JB, Kress GJ, Reynolds IJ. Glutamate decreases mitochondrial size and movement in primary forebrain neurons. J Neurosci. 2003;23(21):7881-8.
344. Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, et al. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell. 2011;147(4):893-906.
345. Birsa N, Norkett R, Wauer T, Mevissen TE, Wu HC, Foltynie T, et al. Lysine 27 ubiquitination of the mitochondrial transport protein Miro is dependent on serine 65 of the Parkin ubiquitin ligase. J Biol Chem. 2014;289(21):14569-82.
346. Verburg J, Hollenbeck PJ. Mitochondrial membrane potential in axons increases with local nerve growth factor or semaphorin signaling. J Neurosci. 2008;28(33):8306-15.
347. Basu H, Pekkurnaz G, Falk J, Wei W, Chin M, Steen J, Schwarz TL. FHL2 anchors mitochondria to actin and adapts mitochondrial dynamics to glucose supply. J Cell Biol. 2021;220(10).
348. Shneyer BI, Usaj M, Henn A. Myo19 is an outer mitochondrial membrane motor and effector of starvation-induced filopodia. J Cell Sci. 2016;129(3):543-56.
349. Lopez-Domenech G, Covill-Cooke C, Ivankovic D, Halff EF, Sheehan DF, Norkett R, et al. Miro proteins coordinate microtubule- and actin-dependent mitochondrial transport and distribution. EMBO J. 2018;37(3):321-36.

350. Rohn JL, Patel JV, Neumann B, Bulkescher J, McHedlishvili N, McMullan RC, et al. Myo19 ensures symmetric partitioning of mitochondria and coupling of mitochondrial segregation to cell division. Curr Biol. 2014;24(21):2598-605.
351. Moore AS, Wong YC, Simpson CL, Holzbaur EL. Dynamic actin cycling through mitochondrial subpopulations locally regulates the fission-fusion balance within mitochondrial networks. Nat Commun. 2016;7:12886.
352. Moore AS, Coscia SM, Simpson CL, Ortega FE, Wait EC, Heddleston JM, et al. Actin cables and comet tails organize mitochondrial networks in mitosis. Nature. 2021;591(7851):659-64.
353. Lamason RL, Welch MD. Actin-based motility and cell-to-cell spread of bacterial pathogens. Curr Opin Microbiol. 2017;35:48-57.
354. Boldogh IR, Pon LA. Interactions of mitochondria with the actin cytoskeleton. Biochim Biophys Acta. 2006;1763(5-6):450-62.
355. Hatch AL, Gurel PS, Higgs HN. Novel roles for actin in mitochondrial fission. J Cell Sci. 2014;127(Pt 21):4549-60.
356. Ilamathi HS, Ouellet M, Sabouny R, Desrochers-Goyette J, Lines MA, Pfeffer G, et al. A new automated tool to quantify nucleoid distribution within mitochondrial networks. Sci Rep. 2021;11(1):22755.
357. Dadsena S, King LE, Garcia-Saez AJ. Apoptosis regulation at the mitochondria membrane level. Biochim Biophys Acta Biomembr. 2021;1863(12):183716.
358. Reyes A, He J, Mao CC, Bailey LJ, Di Re M, Sembongi H, et al. Actin and myosin contribute to mammalian mitochondrial DNA maintenance. Nucleic Acids Res. 2011;39(12):5098-108.
359. Arnold H, Pette D. Binding of glycolytic enzymes to structure proteins of the muscle. Eur J Biochem. 1968;6(2):163-71.
360. Hu H, Juvekar A, Lyssiotis CA, Lien EC, Albeck JG, Oh D, et al. Phosphoinositide 3-Kinase Regulates Glycolysis through Mobilization of Aldolase from the Actin Cytoskeleton. Cell. 2016;164(3):433-46.
361. Fung TS, Chakrabarti R, Kollasser J, Rottner K, Stradal TEB, Kage F, Higgs HN. Parallel kinase pathways stimulate actin polymerization at depolarized mitochondria. Curr Biol. 2022;32(7):1577-92 e8.
362. Park JS, Burckhardt CJ, Lazcano R, Solis LM, Isogai T, Li L, et al. Mechanical regulation of glycolysis via cytoskeleton architecture. Nature. 2020;578(7796):621-6.
363. Wu Y, Zanotelli MR, Zhang J, Reinhart-King CA. Matrix-driven changes in metabolism support cytoskeletal activity to promote cell migration. Biophys J. 2021;120(9):1705-17.
364. Lin S, Huang C, Gunda V, Sun J, Chellappan SP, Li Z, et al. Fascin Controls Metastatic Colonization and Mitochondrial Oxidative Phosphorylation by Remodeling Mitochondrial Actin Filaments. Cell Rep. 2019;28(11):2824-36 e8.
365. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. Biochim Biophys Acta. 2011;1813(1):238-59.
366. Utsumi T, Sakurai N, Nakano K, Ishisaka R. C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and targeted to mitochondria. FEBS Lett. 2003;539(1-3):37-44.
367. Xu X, Forbes JG, Colombini M. Actin modulates the gating of Neurospora crassa VDAC. J Membr Biol. 2001;180(1):73-81.
368. Roman I, Figys J, Steurs G, Zizi M. Direct measurement of VDAC-actin interaction by surface plasmon resonance. Biochim Biophys Acta. 2006;1758(4):479-86.
369. Gourlay CW, Ayscough KR. The actin cytoskeleton: a key regulator of apoptosis and ageing? Nat Rev Mol Cell Biol. 2005;6(7):583-9.
370. Chua BT, Volbracht C, Tan KO, Li R, Yu VC, Li P. Mitochondrial translocation of cofilin is an early step in apoptosis induction. Nat Cell Biol. 2003;5(12):1083-9.

371. Roy S, Esmaeilniakooshkghazi A, Patnaik S, Wang Y, George SP, Ahrorov A, et al. Villin-1 and Gelsolin Regulate Changes in Actin Dynamics That Affect Cell Survival Signaling Pathways and Intestinal Inflammation. Gastroenterology. 2018;154(5):1405-20 e2.
372. Roh SE, Woo JA, Lakshmana MK, Uhlar C, Ankala V, Boggess T, et al. Mitochondrial dysfunction and calcium deregulation by the RanBP9-cofilin pathway. FASEB J. 2013;27(12):4776-89.
373. Hu J, Zhang H, Li J, Jiang X, Zhang Y, Wu Q, et al. ROCK1 activation-mediated mitochondrial translocation of Drp1 and cofilin are required for arnidiol-induced mitochondrial fission and apoptosis. J Exp Clin Cancer Res. 2020;39(1):37.
374. Rehklau K, Gurniak CB, Conrad M, Friauf E, Ott M, Rust MB. ADF/cofilin proteins translocate to mitochondria during apoptosis but are not generally required for cell death signaling. Cell Death Differ. 2012;19(6):958-67.
375. Lapena-Luzon T, Rodriguez LR, Beltran-Beltran V, Beneto N, Pallardo FV, Gonzalez-Cabo P. Cofilin and Neurodegeneration: New Functions for an Old but Gold Protein. Brain Sci. 2021;11(7).
376. Kruppa AJ, Kishi-Itakura C, Masters TA, Rorbach JE, Grice GL, Kendrick-Jones J, et al. Myosin VI-Dependent Actin Cages Encapsulate Parkin-Positive Damaged Mitochondria. Dev Cell. 2018;44(4):484-99 e6.
377. Hsieh CW, Yang WY. Omegasome-proximal PtdIns(4,5)P(2) couples F-actin mediated mitoaggregate disassembly with autophagosome formation during mitophagy. Nat Commun. 2019;10(1):969.
378. Pascual G, Avgustinova A, Mejetta S, Martin M, Castellanos A, Attolini CS, et al. Targeting metastasis-initiating cells through the fatty acid receptor CD36. Nature. 2017;541(7635):41-5.
379. Turcotte B, Liang XB, Robert F, Soontorngun N. Transcriptional regulation of nonfermentable carbon utilization in budding yeast. FEMS Yeast Res. 2010;10(1):2-13.
380. Toyama EQ, Herzig S, Courchet J, Lewis TL, Jr., Loson OC, Hellberg K, et al. Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. Science. 2016;351(6270):275-81.
381. Keng Chen YW, Xiaoying Deng Ling Guo, Chuanyue Wu, . Extracellular matrix stiffness regulates mitochondrial dynamics through PINCH-1- and kindlin-2-mediated signalling. Current Research in Cell Biology. 2021;2(100008).
382. Chang YW, Song ZH, Chen CC. FAK regulates cardiomyocyte mitochondrial fission and function through Drp1. FEBS J. 2022;289(7):1897-910.
383. Li S, Xu S, Roelofs BA, Boyman L, Lederer WJ, Sesaki H, Karbowski M. Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission. J Cell Biol. 2015;208(1):109-23.
384. Zhang H, Zhao Y, Yao Q, Ye Z, Manas A, Xiang J. Ubl4A is critical for mitochondrial fusion process under nutrient deprivation stress. PLoS One. 2020;15(11):e0242700.
385. Schiavon CR, Zhang T, Zhao B, Moore AS, Wales P, Andrade LR, et al. Actin chromobody imaging reveals sub-organellar actin dynamics. Nat Methods. 2020;17(9):917-21.
386. Liu AP, Fletcher DA. Actin polymerization serves as a membrane domain switch in model lipid bilayers. Biophys J. 2006;91(11):4064-70.
387. Baldauf L, Frey F, Arribas Perez M, Idema T, Koenderink GH. Branched actin cortices reconstituted in vesicles sense membrane curvature. Biophys J. 2023;122(11):2311-24.
388. Shi P, Ren X, Meng J, Kang C, Wu Y, Rong Y, et al. Mechanical instability generated by Myosin 19 contributes to mitochondria cristae architecture and OXPHOS. Nat Commun. 2022;13(1):2673.
389. Taylor MJ, Lampe M, Merrifield CJ. A feedback loop between dynamin and actin recruitment during clathrin-mediated endocytosis. PLoS Biol. 2012;10(4):e1001302.

390. Mooren OL, Kotova TI, Moore AJ, Schafer DA. Dynamin2 GTPase and cortactin remodel actin filaments. J Biol Chem. 2009;284(36):23995-4005.
391. Lee P, Chandel NS, Simon MC. Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. Nat Rev Mol Cell Biol. 2020;21(5):268-83.
392. Zanotelli MR, Goldblatt ZE, Miller JP, Bordeleau F, Li J, VanderBurgh JA, et al. Regulation of ATP utilization during metastatic cell migration by collagen architecture. Mol Biol Cell. 2018;29(1):1-9.
393. Barros LF, Ruminot I, San Martin A, Lerchundi R, Fernandez-Moncada I, Baeza-Lehnert F. Aerobic Glycolysis in the Brain: Warburg and Crabtree Contra Pasteur. Neurochem Res. 2021;46(1):15-22.