The role of TRAP-1 in mitochondria transfer between cancer cells

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Submitted in the partial fulfilment of the requirement for the award of the degree of

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IN

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Submitted by

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DEPARTMENT OF BIOTECHNOLOGY

THAPAR INSTITUTE OF ENGINEERING AND TECHNOLOGY, PATIALA

(Declared as Deemed-to-be-University u/s 3 of UGC Act, 1956)

DISSERTATION THESIS

(July 2023- June 2024)

THE ROLE OF TRAP-1 IN MITOCHONDRIA TRANSFER BETWEEN CANCER CELLS

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CERTIFICATE

I certify that the research work presented in the dissertation, 'The role of TRAP-1 in mitochondria transfer between cancer cells', has been carried out by Ms Soumya (Admission No. 602204018) under my supervision. The research work is original and has not been submitted for any other degree of this or any other University. Also, she was a regular student and worked under my guidance as a full-time dissertation student at CSIR-Centre for Cellular and Molecular Biology, Hyderabad, until the thesis submission to Thapar Institute of Engineering and Technology, Patiala. The work presented in this thesis belongs to CSIR-CCMB and cannot be reproduced without prior approval from the supervisor.

A S Sreedhar

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ABSTRACT

The intercellular exchange of mitochondria, a well-established phenomenon known as 'mitochondrial transfer', has emerged as a fascinating phenomenon with implications in various physiological and pathological conditions, including cancer. Understanding the mechanisms governing mitochondrial transfer within the intricate landscape of cancer biology can provide new insights into novel therapeutic interventions. This study investigates the role of TNF receptor-associated protein 1 (TRAP-1), a molecular chaperone localized within the mitochondria, in mediating mitochondrial transfer between cancer cells. Through a series of experiments, we investigated the multifaceted interplay between TRAP-1 expression levels and mitochondrial dynamics within the neuronal cancer cells. We used TRAP-1 overexpression and knockdown cells to probe the consequences of TRAP1 modulation on mitochondrial transfer. We report that cells with altered TRAP1 levels exhibit actin reorganization. With the help of inducing oxidative stress and co-culture experiments, we aimed to elucidate the potential involvement of tunnelling nanotubes (TNT) in facilitating mitochondria transfer between cancer cells. We extended to exploring alternative mechanisms of mitochondrial exchange, particularly through extracellular vesicles and exosomes. The mitochondrial gene expression analysis provided insights that cancer cells utilize multiple routes for mitochondria transfer between cells. Overall, our study deepens understanding of mitochondrial transfer between cancer cells.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine 5'- triphosphate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CCM	Culture Conditioned Medium
cDNA	Complimentary DNA
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribose nucleotide tri-phosphate
EDTA	Ethylenediamine tetra acetic acid
EtBr	Ethidium Bromide
EVs	Extracellular vesicles
EXO	Exosomes
FP	Forward primer
g	grams
GFP	Green Fluorescent Protein
hrs	hours
Hsp	Heat Shock Protein
HSR	Heat Shock Response
KD	Knockdown
kDa	Kilo Dalton
l	Litre

M	Molar
µg	Microgram
µM	Micro molar
µl	Micro litre
µm	Micrometer
mg	Milligram
min	Minutes
ml	Millilitre
MVs	Microvesicles
ng	Nanogram
OE	Overexpression
%	Per cent
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RCF	Relative Centrifugal Force
ROS	Reactive Oxygen Species
RP	Reverse primer
sec	seconds
shRNA	Short Hairpin RNA
TAE	Tris-acetate EDTA
TRAP-1	Tumour necrosis factor receptor-associated protein-1

About CSIR-CCMB, Hyderabad

The Centre for Cellular and Molecular Biology (CCMB) in Hyderabad, India, is a premier research organization in the field of cellular and molecular biology. Established in 1977 under the Council of Scientific and Industrial Research (CSIR), CCMB aims to conduct high-quality basic research and training in frontier areas of modern biology.

Research Focus

CCMB's research spans a broad range of topics in life sciences, including:

1. **Genetics and Genomics:** Investigations into the genetic basis of various diseases, population genetics, and evolutionary biology.
2. **Cell and Developmental Biology:** Studies on the mechanisms of cell growth, differentiation, and development.
3. **Molecular Biology:** Understanding the molecular mechanisms of cellular processes, including transcription, translation, and gene regulation.
4. **Structural Biology:** Elucidating the structure-function relationships of biomolecules.
5. **Biotechnology and Bioinformatics:** Applying biological knowledge to develop new technologies and computational tools for data analysis.

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CCMB has made significant contributions to various fields, such as:

- **Human Genome Variation Studies:** CCMB has been instrumental in cataloging genetic variations in Indian populations, which is crucial for understanding disease susceptibilities and drug responses.
- **Conservation Biology:** The center has played a key role in the conservation of endangered species through genetic analysis and assisted reproduction techniques.
- **Biomedical Research:** CCMB's research has led to advances in understanding the molecular basis of diseases like cancer, diabetes, and infectious diseases, contributing to the development of new diagnostics and therapeutics.

Facilities and Infrastructure

CCMB is equipped with state-of-the-art facilities that support cutting-edge research, including:

- **Advanced Imaging and Microscopy:** Tools for high-resolution imaging of cellular and molecular structures.
- **Genomics and Proteomics:** Technologies for high-throughput sequencing, gene expression analysis, and protein profiling.
- **Bioinformatics Resources:** Computational infrastructure for analyzing large-scale biological data.
- **Animal House and Plant Growth Facilities:** For conducting in vivo and in vitro experiments.

Training and Outreach

CCMB is also dedicated to training the next generation of scientists. It offers various programs, including:

- **PhD Programs:** Comprehensive training in modern biology through rigorous coursework and research.
- **Workshops and Courses:** Short-term training programs for researchers, students, and educators.
- **Collaborative Research:** Partnerships with national and international institutions to foster collaborative research and innovation.

In conclusion, CCMB Hyderabad stands as a beacon of scientific excellence in India, contributing significantly to the advancement of cellular and molecular biology. Its research, training, and outreach activities continue to drive scientific progress and address pressing challenges in health, agriculture, and conservation.

CHAPTER 1

INTRODUCTION

Mitochondria are vital intracellular organelles that play important roles in oxidative metabolism (OXPHOS), cell signaling, proliferation, metabolism, and death. The vital role of mitochondria within cells includes intercellular mitochondrial transfer. This phenomenon is mainly explored for its potential to initiate stem cell differentiation and reprogramming. This phenomenon, termed 'mitochondrial transfer', has sparked significant interest due to its implications in human pathologies despite its role in cellular protection.

Transferring mitochondria involves the integration of donated mitochondria into the existing network of recipient cells, leading to alterations in the recipient cell metabolism and recovery from stress. This phenomenon holds significance in various pathological conditions, such as tissue repair, inflammatory modulation, oncogenesis, tumor drug resistance, and the maintenance of tissue homeostasis. Research indicates that the transcellular transfer of mitochondria employs diverse mechanisms, including the formation of tunneling nanotubes (TNTs), extracellular vesicles (EVs), gap junctions, exocytosis, endocytosis of naked mitochondria, cytoplasmic fusion, etc. Moreover, mitochondrial transfer shows promise as a therapeutic approach for treating diseases associated with mitochondrial dysfunction, such as organ degeneration and cancer.

TNF receptor-associated protein 1 (TRAP-1) emerges as a pivotal player among the proteins implicated in regulating mitochondrial dynamics and function. As a mitochondrial chaperone, TRAP-1 is thought to be involved in protein folding, assembly, and degradation of mitochondrial proteins within the mitochondrial matrix, thereby regulating mitochondrial protein quality control. In light of these insights, the present work delves into the complex mechanism of intercellular mitochondrial transfer.

Uncovering molecular mechanisms involved in mitochondrial transfer may uncover precise therapeutic interventions against altered tumor metabolism and therapeutic resistance in diseases like cancer. Since TRAP-1 is implicated in metabolic rewiring, we speculate it may play a role in mitochondria transfer. Towards this, we examined the role of TRAP-1 in mitochondria transfer.

CHAPTER 2

REVIEW OF LITERATURE

This chapter deals with the review of pertinent literature on the different aspects related to the role of TRAP-1 in mitochondria transfer between cancer cells. It covers the biochemical and functional properties of TRAP-1 and mitochondria, as well as the mechanisms of intercellular mitochondria transfer. The aim is to provide a comprehensive understanding of the current state of research in this area, laying a solid foundation for the subsequent investigation of TRAP-1's involvement in cancer cell behavior and therapy resistance.

Mitochondria, the powerhouses that fuel cellular life, have amazed scientific researchers with their extraordinary ability to migrate between cells, sparking a profound interest in understanding the mechanism and implications of Mitochondria Transfer.

2.1 MITOCHONDRIA

Mitochondria are remarkable organelles that are double-membraned structures in nearly all eukaryotic organisms. Mitochondria are thought to have originated from primitive prokaryotes via endosymbiosis (Gray *et al*., 1999). The pivotal role of mitochondria in cellular energy production can help in understanding metabolic rewiring in disease models such as cancer.

Mitochondria are double membrane structures. It contains an outer membrane and an inner membrane which folds to form cristae. Mitochondria contain a small circular DNA, known as **mitochondrial DNA** (mtDNA). The size of mitochondrial DNA is 16 kb which codes for 37 genes. Nuclear-encoded mitochondrial proteins are transported through the TIM-TOM complex. The TIM (Translocase of the Inner Membrane) and TOM (Translocase of the Outer Membrane) complexes are crucial components of mitochondrial import machinery, facilitating the transport of proteins across the double membrane of mitochondria. The TOM complex, located in the outer mitochondrial membrane, recognizes and translocates precursor proteins from the cytosol into the intermembrane space. These proteins are then transported by the TIM complex, situated in the inner mitochondrial membrane, to their final destination within the mitochondria.

Mitochondria have their own transcriptional and translational machinery. Unlike nuclear DNA inherited from both parents, mtDNA is maternally inherited. Mitochondria plays a crucial role in regulating calcium homeostasis, redox homeostasis, cell death pathways, metabolism, and mitochondrial unfolded protein response.

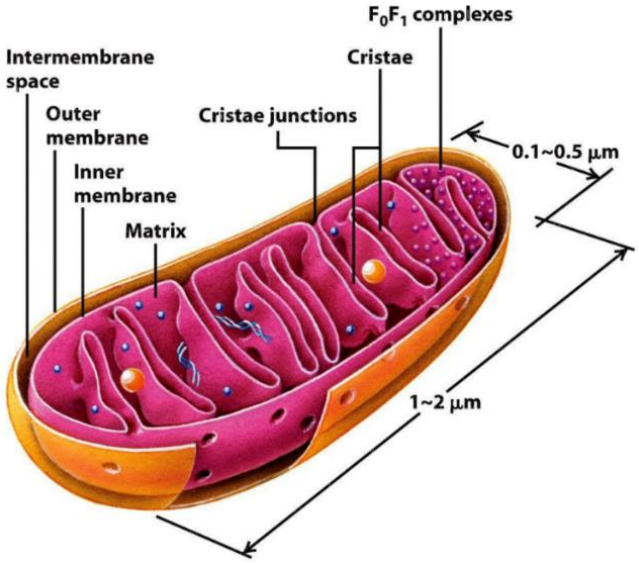


Figure 1: Cross-section of Mitochondrion (Source: Molecular Cell Biology, W H Freeman, 2000)

Mitochondria are the powerhouse of the cell that produces ATP through oxidative phosphorylation (OXPHOS). Glucose is metabolized to pyruvate through glycolysis, which is transported to mitochondria for further breakdown. Pyruvate is broken down to form acetyl CoA, which enters into the Krebs cycle or tricarboxylic acid cycle (TCA)(Hanna & Nelson, 1999). Reducing equivalents like $NADH_2$ and $FADH_2$ are utilized to generate ATP through the Electron Transport Chain (ETC). ETC consists of five respiratory complexes that are made up of protein subunits encoded by mitochondrial and nuclear genomes, namely

- NADH dehydrogenase (Complex I)
- Succinate dehydrogenase (Complex II)
- Cytochrome c reductase (Complex III)
- Cytochrome c oxidase (Complex IV)
- ATP synthase (Complex V)

Reduction of $NADH_2$ and $FADH_2$ produces reactive oxygen species (ROS) as a by-product which is further metabolized to form water by mitochondrial antioxidative machinery.

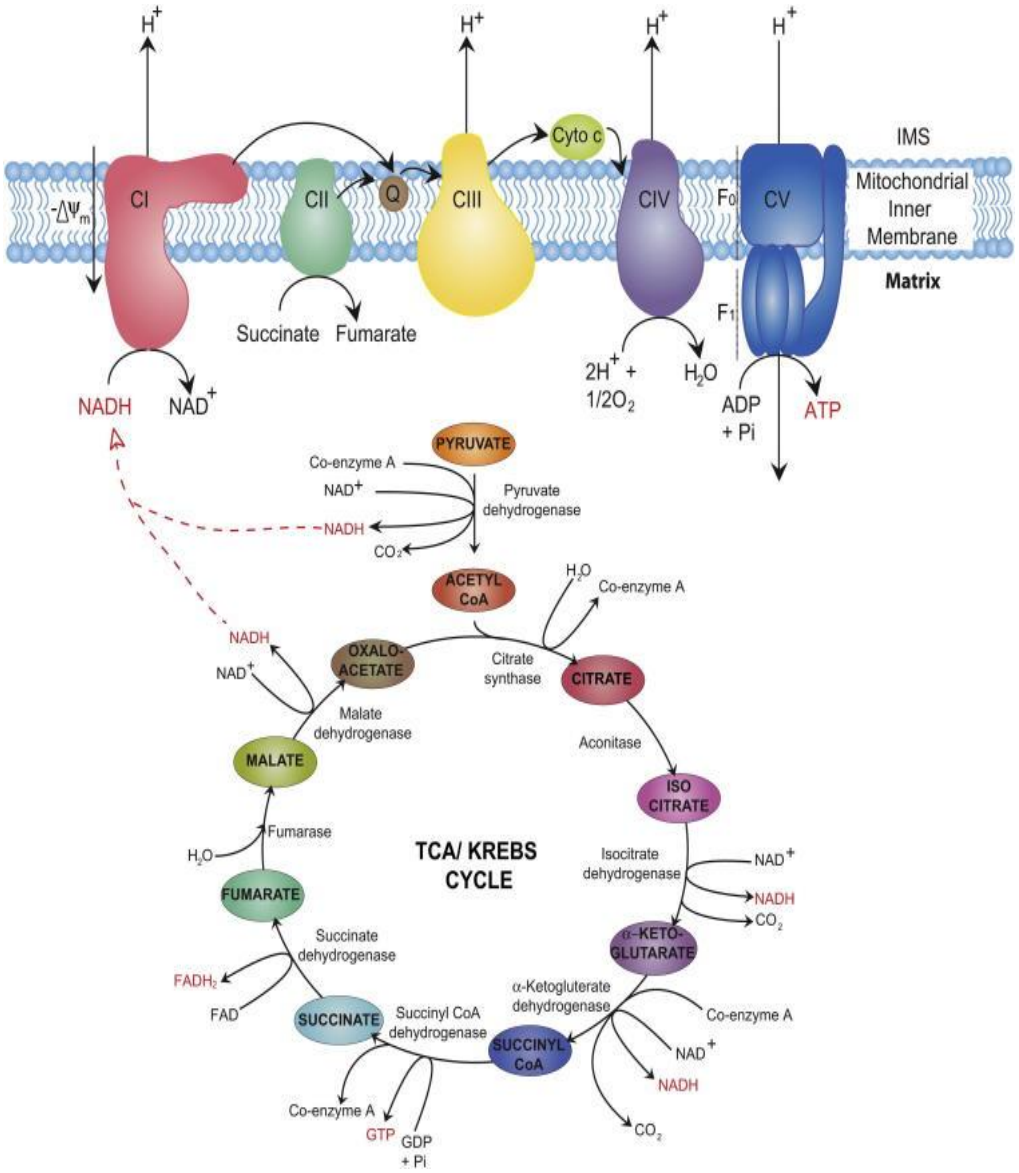


Figure 2: Schematic View of OXPHOS and TCA. Pyruvate is metabolized to form high-energy molecules like NADH, $FADH_2$, and GTP by catalyzing through the TCA cycle. NADH and FADH2 are transported to Complex I and II, respectively, where they play a key role in driving oxidative phosphorylation. (Source: (Osellame *et al*., 2012)).

2.2 CYTOSKELETON

The cytoskeleton is a remarkable and dynamic network of protein filaments that resides within the cells of all living organisms. (Fletcher, D. A., & Mullins, R. D. (2010)). It is a structure that maintains the shape and internal organization of the cell and provides it with mechanical support. It is essential for cell motility, intracellular transport, and cell division, making it a critical component for the proper functioning of cells.

The cytoskeleton comprises three main types of protein filaments: **microfilaments** (also known as actin filaments), **microtubules**, and **intermediate filaments**. Each type of filament is responsible for specific cellular functions, working collectively to form an intricately organized framework.

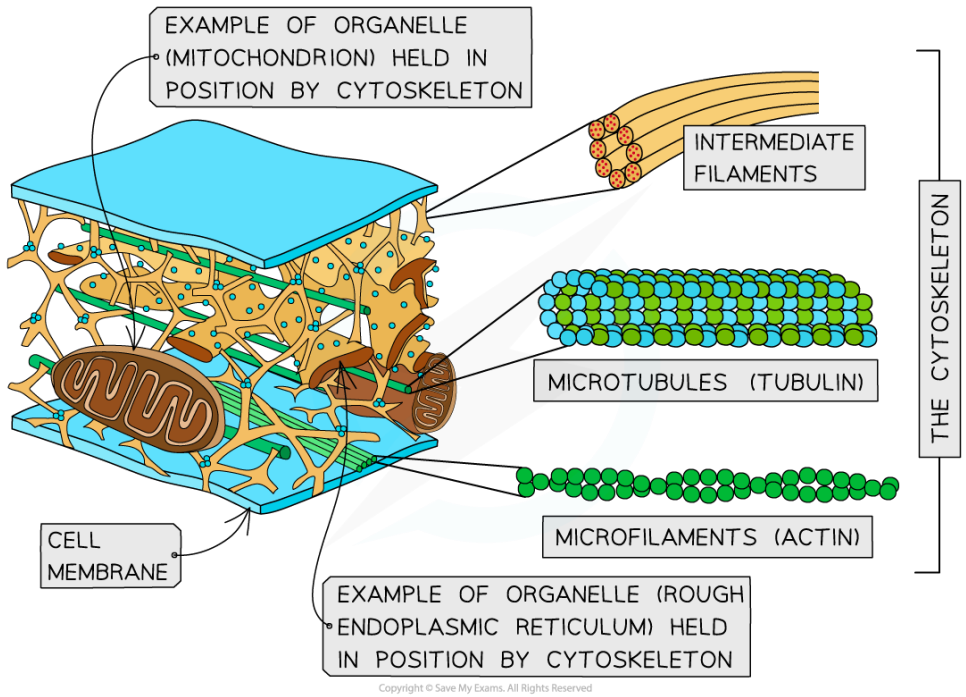


Figure 3: The cytoskeleton provides mechanical strength to cells, aids transport within cells, and enables cell movement (https://www.savemyexams.co.uk/a-level/biology/ocr/17/revision-notes/2-foundations-in-biology/2-1-cell-structure/2-1-8-the-cytoskeleton/)

1. **MICROFILAMENTS:** Microfilaments, composed of **actin** proteins, are thin and flexible, thread-like protein fibers. They have a diameter of 3-6nm and are particularly

found in muscle cells. They are involved in cell movement, such as muscle contraction and cell crawling, as well as providing mechanical support to the cell's membrane.

2. **MICROTUBULES:** Microtubules are small, hollow, and round tubes formed by **tubulin** proteins. They have a diameter of 24 nm. Also, they are very dynamic structures, facilitating the transport of various molecules within the cell. Microtubules are also essential during cell division, where they form the mitotic spindle to ensure accurate distribution of chromosomes.

3. **INTERMEDIATE FILAMENTS:** Intermediate filaments provide structural stability to the cell and play a vital role in maintaining cell shape. It has a diameter of 10 nm and unlike microfilaments and microtubules, they are more **stable** & are found in various cell types such as, skin cells which they contribute to tissue integrity.

In conclusion, the cytoskeleton stands as a remarkable and essential component within cells, providing the structural foundation and dynamic support required for cellular life.

2.3 MITOCHONDRIAL DYNAMICS

Advancements in live cell imaging techniques in the past few decades have dramatically changed the concept of mitochondria being static and isolated structures.

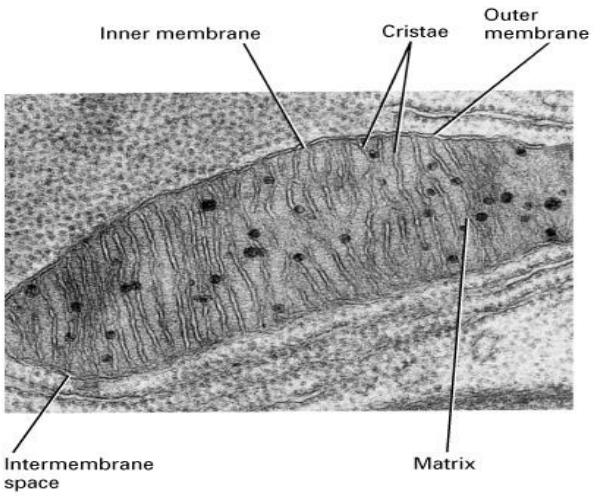


Figure 4: TEM of mitochondria.

(Source-http:// rachithscellanalogy.weebly.com/ mitochondira. html)

The dynamic nature of mitochondria explains the heterogeneity of mitochondrial morphology. Mitochondrial dynamics is a concept that includes modulation of their morphology to create a tubular network coordinated by fission and fusion events. The balance between mitochondrial fission and fusion events maintains the number and size of mitochondria (Liesa, Palacín *et al*. 2009).

'Mitochondrial fission' involves the division of one mitochondrion into two mitochondria, while **'Mitochondrial fusion'** refers to the fusion of two mitochondria to form a single mitochondrion. An imbalance between the two events results in small mitochondria or an intense tubular elongated mitochondria (Lee, H., & Yoon, Y.,2016). Dynamic alterations of mitochondria are important factors of mitochondrial function as well as sensing the metabolic needs of the cell (Nasrallah, C. M., & Horvath, T. L.,2014).

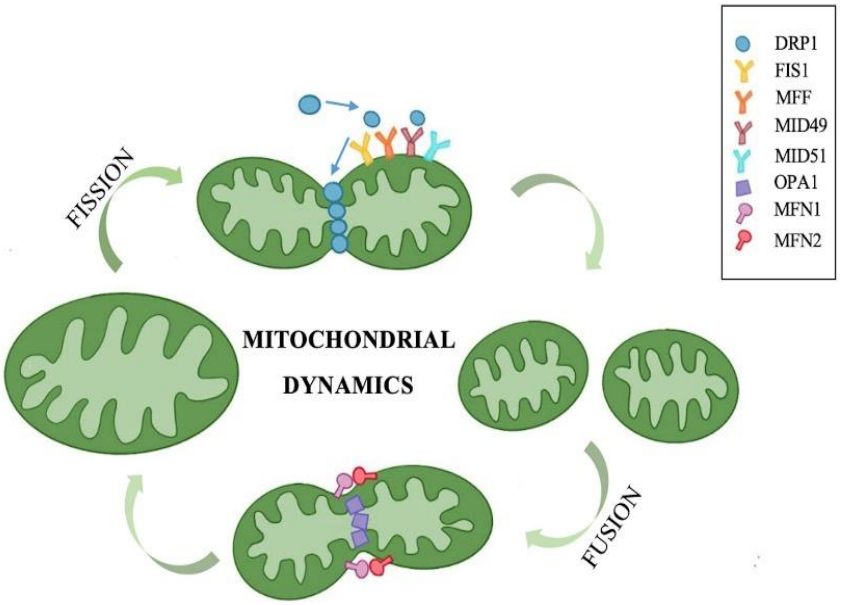


Figure 5: The figure illustrates Mitochondrial fission and fusion dynamics. Mitochondrial fission is mainly controlled by DRP1 in the cytoplasm. DRP1 is recruited by receptor proteins like FIS1, MFF, and MID49/51 on the outer membrane of mitochondria. Together they form a complex that induces mitochondrial fission. Fusion is governed by GTPases MFN1, MFN2 on the outer mitochondrial membrane forming along with OPA1 in the inner membrane that forms a complex orchestrating fusion event. (Source- Zou T *et al*., 2022)

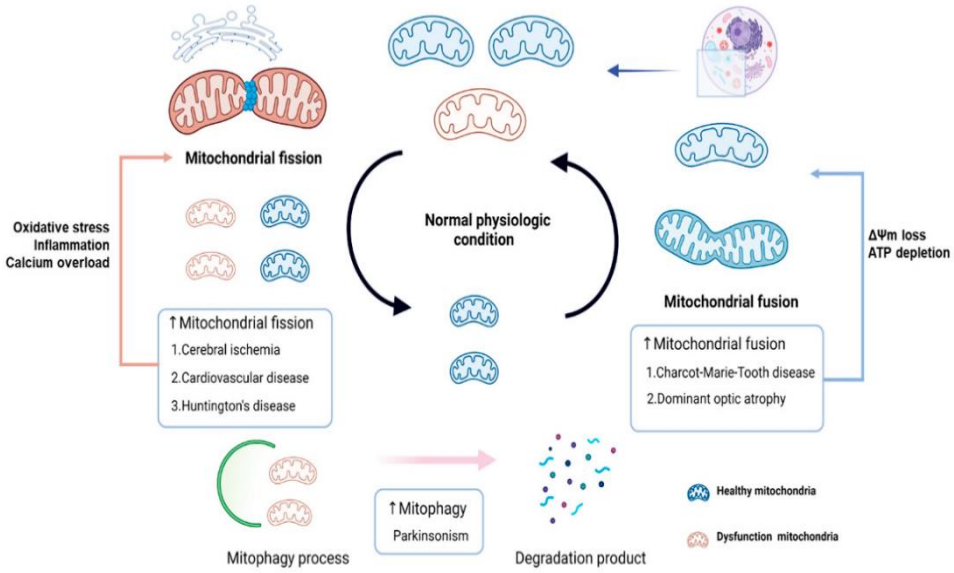


Figure 6: A schematic diagram representing the alteration of mitochondrial dynamic balance during pathology. Various diseases related to fusion, fission, and mitophagy dynamics in mitochondria such as Cerebral ischemia, cardiovascular disease, and Huntington's disease that shows increased mitochondrial fission via increased oxidative stress, inflammation, and calcium overload. Charcot–Marie–Tooth disease and dominant optic atrophy shows increased mitochondria fusion by stimulated mitochondrial membrane depolarization and ATP depletion. (Source- Vongsfak J *et al*., 2021)

2.3 MITOCHONDRIA TRANSFER

Mitochondria transfer involves the intercellular movement of whole or fragmented mitochondria from one cell to another. Initially discovered as a mechanism for cellular rescue and repair during stress, this phenomenon has now been recognized vital in various physiological and pathological contexts.

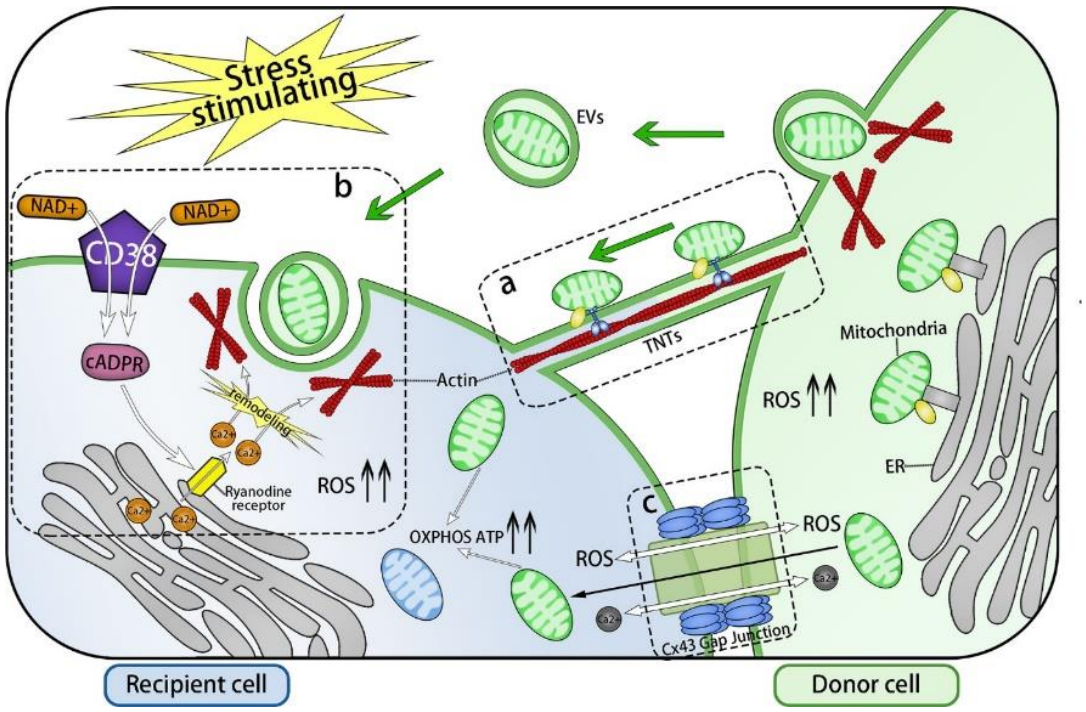


Figure 7: Diagram illustrates the three Main Forms of Intercellular Communication Related to the Transfer of Mitochondria. Under energy stress, inflammation, or DNA damage, intracellular ROS levels increase triggers mitochondria transfer via three major mechanisms: TNTs, EVs, and GJs. (A) TNTs which forms connection between two cells, whose main framework is F-actin. (B) EV uptake occurs through the NAD+/CD38/cADPR/Ca2+ pathway: During stress, intracellular NAD+ levels rise, transferring to the extracellular space. CD38, an activated transmembrane protein, catalyzes NAD+ to form cADPR, a second messenger regulating intracellular Ca2+ release. cADPR triggers Ryanodine receptors (RyRs) on the endoplasmic reticulum, elevating intracellular Ca2+ levels. This prompts actin cytoskeleton remodeling and cell membrane invagination, completing EV endocytosis. This pathway may also facilitate EV release and TNT formation. Additionally, transcellular mitochondrial transfer via the actin cytoskeleton enhances OXPHOS, ATP levels, and recipient cell viability. (C) Cx43 gap junction channels (GJCs) facilitate the transcellular mitochondrial transfer. This process can occur through three potential pathways: exchange of Ca2+ or ROS via Cx43 GJCs, influencing the formation of channels for mitochondrial transfer, or through direct mitochondrial transfer. ERMES, a complex, serves to anchor mitochondria to the endoplasmic reticulum. (Source- Qin Yiming *et al*.,2021)

This intercellular communication of mitochondria adds a new layer of complexity to cellular dynamics. It has been observed in diverse cell types, including immune cells, stem cells, neurons, and **cancer** cells, suggesting its relevance across biological systems. Understanding the mechanisms, signalling pathways, and functional roles of mitochondria transfer is key to uncovering the complexity of cellular biology. Cell-cell communication is a way that cells interact with each other by physically touching or being very close to one another. Through direct contact, cells can exchange information and signals without releasing signalling molecules into the surrounding environment.

This form of communication is vital for coordinating cellular activities, such as cell development, immune responses, and tissue repair. Examples of various structures that allow cells to physically interact and communicate directly are:

1. **Gap Junctions (GJ):** These are the structures crucially involved in direct communication and exchange of small molecules between adjacent cells. Gap junctions are pivotal in coordinating cellular activities, ensuring proper development, and maintaining tissue homeostasis.
2. **Tight Junctions:** They are vital structures that act as a barrier between adjacent cells and prevent the passage of molecules between them. These specialized junctions are crucial in maintaining the integrity of various tissues and organs throughout the body, ensuring selective permeability and regulating the movement of substances across cell layers.
3. **Tunnelling Nanotubes** (TNTs): Tunnelling nanotubes are thin, long, and filamentous structures that extend between cells, enabling direct cell-to-cell communication. These nanotubes form when one cell extends a thin tube-like structure towards another, establishing a direct physical connection. TNTs have been observed in various cell types, including immune cells, neurons, and cancer cells. They play a crucial role in the transfer of organelles (e.g. **Mitochondrial transfer,** etc.), ions, proteins, and even pathogens between cells, contributing to cellular cooperation, immune responses, and disease progression.
4. **Extracellular Vesicles** (EVs): Extracellular vesicles are small membrane-bound structures released by cells into the extracellular environment. These vesicles carry proteins, lipids, nucleic acids, and other bioactive molecules. They are found in various body fluids, such as blood, urine, and saliva, as well as in tissue microenvironments. EVs are produced by a wide range of cell types, including immune cells, stem cells,

and cancer cells. They act as messengers, delivering their cargo to nearby or distant cells, influencing cellular activities, and contributing to various physiological and pathological processes, such as cell-to-cell communication, tissue repair, and disease progression.

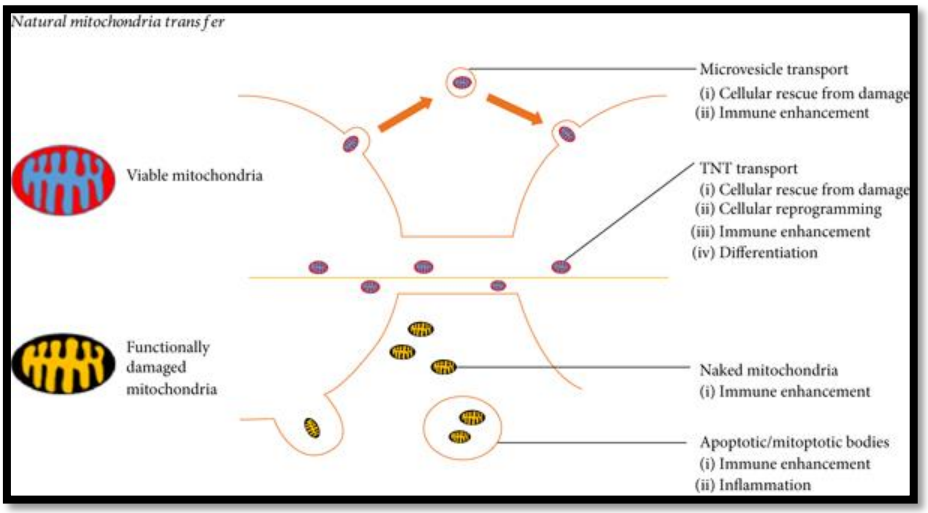


Figure 8: Figure illustrates the mechanisms of mitochondria transfer. By inducing cellular stress, the cell can share viable and nonfunctional mitochondria to rescue stressful conditions to promote inflammation. 1. The first method of transfer involves transporting mitochondria via microvesicles. 2. The second transfer mechanism occurs through tunneling nanotubes (TNTs), a capability shared by numerous cells. These TNTs facilitate mitochondrial transport, demonstrating significant effects in rescuing cells from damage, restructuring metabolism, enhancing immune responses, and even influencing cellular differentiation (Andrés Caicedo *et al.*, 2017)

2.3.1 MITOCHONDRIA TRANSFER THROUGH MICROVESICLES AND EXOSOMES

Extracellular vesicles encompass a heterogeneous group of membrane-bound vesicles released by cells into the extracellular space. This broader category includes microvesicles, exosomes, and apoptotic bodies.

- **Microvesicles:** Microvesicles, also known as shedding vesicles or ectosomes, are formed by the outward budding and shedding of the plasma membrane. They typically

range from 100 nm-1 μM in diameter and carry a cargo of proteins, lipids, nucleic acids, and organelles.

- **Exosomes:** Exosomes, on the other hand, are smaller vesicles, typically around 30-150 nanometres in diameter, originating from the endosomal system. The inward budding of endosomal membranes forms them to form multivesicular bodies (MVBs), which then release exosomes when they fuse with the plasma membrane.

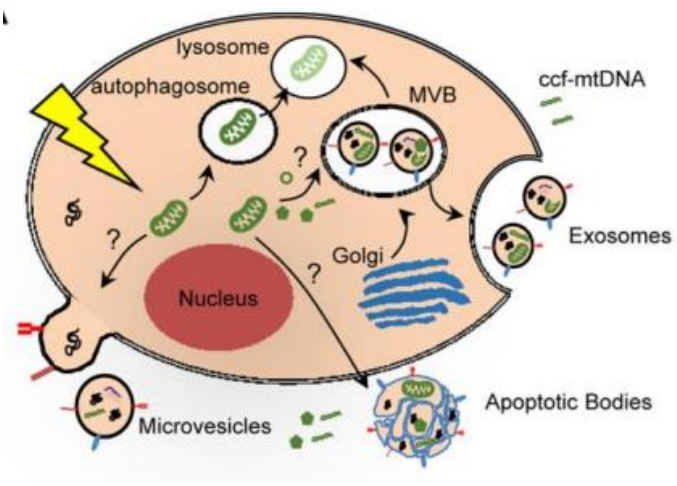


Figure 9: Mitochondrial DNA found in extracellular vesicles has been linked to human aging. When mitochondria suffer damage, they can release various components, including mitochondrial DNA (mtDNA). This mtDNA can either circulate freely in the bloodstream (ccf-mtDNA) or become enclosed within extracellular vesicles (EVs). Three main types of EVs are implicated, along with potential mechanisms for the inclusion of mitochondrial components. Alternatively, damaged mitochondria may undergo degradation through the mitophagy pathway. (Source-Noren Hooten *et al*.,2021)

2.3.2 ADVANTAGES OF MITOCHONDRIA TRANSFER

- ✓ **Enhanced cell survival:** Mitochondrial transfer may improve the survival of certain cell types, such as cells used in regenerative medicine or tissue engineering.
- ✓ **Protection against oxidative stress:** Healthy mitochondria effectively handle oxidative stress by recycling reactive oxygen species (ROS).
- ✓ **Neurological diseases:** Mitochondria transfer may improve neurological diseases like Parkinson's or Alzheimer's.
- ✓ **Cancer therapy:** Mitochondrial dysfunction is linked to tumor growth and progression. Mitochondrial transfer may enhance sensitivity to chemotherapeutic drugs.

- ✓ **Age-related Decline:** As we age, mitochondrial function declines, leading to reduced energy production and increased cellular damage. Mitochondrial transfer can be explored as a potential rejuvenation therapy to restore cellular function in aged cells, potentially slowing down the aging process or mitigating age-related degeneration.
- ✓ **Organ Transplantation:** During transplantation, organs can experience ischemic damage due to limited blood flow before implantation. Mitochondria transfer may reduce ischemic damage, leading to better outcomes of transplant procedures.
- ✓ **Regenerative diseases:** Mitochondria transfer could potentially enhance the functionality of cells in regenerative medicine, such as stem cell therapies. Introducing healthy mitochondria into stem cells before transplantation can improve their viability and differentiation ability.
- ✓ **Mitochondrial diseases:** Mitochondrial transfer could be used to treat individuals with mitochondrial diseases, such as Leigh syndrome, caused by mutations in their mitochondrial DNA.

2.3.3 MITOCHONDRIA TRANSFER & CELL FATE DETERMINATION

Cell fate determination implies a process by which a cell 'decides' what type of cell it will differentiate into or what kind of function it will perform in a multicellular organism. During development, cells undergo various signalling pathways that regulate their fate, differentiation, and specialization to particular cell types. Mitochondria transfer can influence cell fate determination and function. When healthy mitochondria are transferred into cells with dysfunctional or damaged mitochondria, several potential outcomes can be observed and as follows:

- ❖ **Enhanced cell viability:** The cells with compromised mitochondria have reduced cell viability and are prone to cell death. Mitochondria transfer can increase cell viability and survival by providing functional and healthy mitochondria.
- ❖ **Tissue Repair and Regeneration:** In the case of tissue damage or injury, mitochondria transfer might aid in repairing damaged cells, thus facilitating tissue regeneration.
- ❖ **Metabolic adaptation:** Different cell types have different metabolic demands. The mitochondria transfer can aid in meeting metabolic requirements.

- **Differentiation and specialization:** Transfer of healthy mitochondria can influence cell signalling pathways and cellular functions, potentially guiding the recipient cell differentiation into a specific cell type during development or tissue repair.
- **Epigenetic modifications:** Mitochondria are involved in epigenetic regulation, influencing gene expression patterns in the nucleus. Mitochondria transfer can induce epigenetic changes or alterations in recipient cells.
- **Cellular Reprogramming:** The mitochondria transfer could contribute to cellular reprogramming, a process where one cell type is transformed into another. By introducing a specific population of mitochondria into target cells, researchers can influence the characteristics and function of particular cell types.
- **Immuno-modulation:** Mitochondria play a role in immune response and inflammation. Transferred mitochondria could modulate the immune response of the recipient cell, affecting its ability to interact with the immune system and influencing cell fate in the context of inflammatory conditions.
- **Heterogeneousity:** Within a cell, mitochondrial populations can be heterogeneous, with varying levels of function and health. Mitochondria transfer may help to overcome consequences associated with cellular heterogeneity.
- **Mitochondrial quantity:** The number of transferred mitochondria and the ratio of transferred to endogenous mitochondria might influence cell fate. Optimal transfer protocols should be designed to achieve the desired outcomes without disrupting the recipient cell's machinery.
- **Safety and long-term stability:** Ensuring the safety and stability of mitochondria transfer within recipient cell is essential. The researchers need to carefully investigate the outcomes as well as risks like triggering unwanted immune responses or unintended effects on cellular function.
- **Ethical considerations:** Mitochondria transfer may raise ethical issues, especially relating to the germline editing and inheritable modifications. The potential long-term consequences on future generations must be carefully considered and debated before clinical applications are considered.

2.3.4 MITOCHONDRIAL TRANSFER AND TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) is a dynamic network of various cell types, extracellular matrix components, and signaling molecules that interact with cancer cells to influence tumor progression and metastasis. Recent studies have highlighted the importance of mitochondrial transfer between cells within the TME through mechanisms like tunneling nanotubes (TNTs), extracellular vesicles (EVs), and cell fusion. TNTs are actin-based cytoplasmic extensions that facilitate mitochondrial exchange over long distances, enhancing cancer cells' metabolic flexibility. EVs, including exosomes and microvesicles, carry mitochondria and other cargo, altering the metabolic state of recipient cells and supporting cancer cell survival under stress. Though less common, cell fusion results in direct cytoplasmic exchange, contributing to tumor heterogeneity and adaptation. Mitochondrial transfer helps cancer cells overcome mitochondrial dysfunction by acquiring healthy mitochondria from stromal cells, restoring respiration, enhancing ATP production, and improving redox balance. This process modulates metabolic reprogramming, allowing cancer cells to switch between glycolysis and oxidative phosphorylation (OXPHOS) based on environmental conditions. Understanding these mechanisms opens new avenues for therapeutic intervention, such as targeting pathways involved in mitochondrial transfer to disrupt the metabolic cooperation between cancer cells and their microenvironment, potentially impairing tumor growth and resistance to therapy. Thus, mitochondrial transfer in the TME is critical for cancer cell metabolism, survival, and proliferation, representing a promising target for innovative cancer treatments.

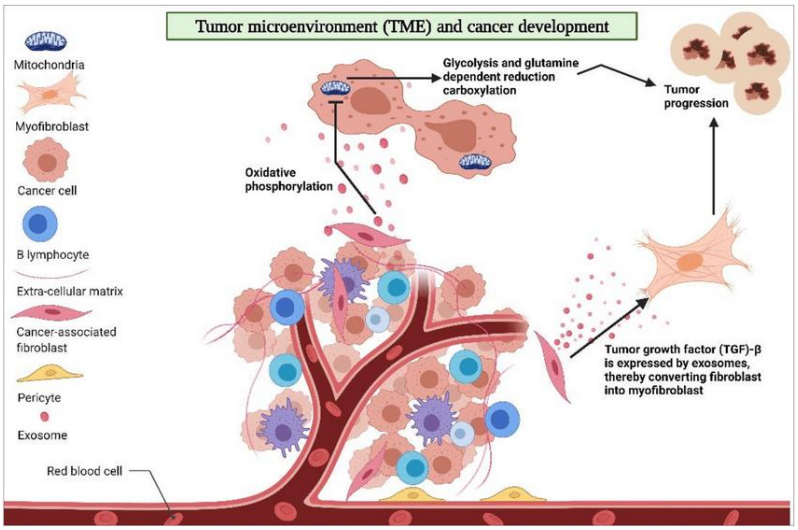


Figure 10: Illustration of the tumor microenvironment (TME) in cancer development. It shows cancer cells, myofibroblasts, B lymphocytes, and fibroblasts within the extracellular matrix. Key

processes include oxidative phosphorylation in mitochondria, glycolysis, and TGF-β expressed by exosomes converting fibroblasts to myofibroblasts, all promoting tumor progression (Source: Karami Fath, Mohsen, *et al*.,2022)

2.3.5 VARIABILITY IN DISEASE PROGRESSION DUE TO MITOCHONDRIAL DEFECTS

Disease progression can vary significantly depending upon the specific type and severity of the mitochondrial defect, as well as the affected tissues and organs. Mitochondrial dysfunction can impact various cellular processes and contribute to various diseases. Various such diseases can be:

- **Mitochondrial diseases:** These diseases are rare genetic diseases that arise from the mutations in mtDNA or nuclear genes involved in mitochondria function. These genetic defects or alterations impair the ability of mitochondria to synthesise ATP, the cells' energy currency. Hence, patients experience a broad spectrum of symptoms ranging from mild to severe, with varying degrees of tissue and organ involvement. Symptoms can affect organs such as liver, lung, heart, brain, muscles and nervous system leading to neuronal impairments, organ dysfunction, muscle weakness, developmental delays etc. Among them the most devastating disorder is 'Leigh Syndrome', characterised by progressive brain damage in early childhood, and Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), characterized by neurological and muscular symptoms with stroke-like episodes.

- **Neurodegenerative diseases:** These diseases are health draining diseases characterized by degeneration of nerve cells(neurons) in the brain and in spinal cord. Mitochondrial dysfunction significantly contributes to neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease. In these diseases, compromised mitochondria fail to provide sufficient energy for neurons leading to dysfunction and eventually death. As a result of these disorders, individuals experience memory loss, impaired motor skills and other neurological deficits. Searching for the role of mitochondria in neurodegeneration is shedding light on potential therapeutic targets for treating these devastating diseases.

- **Cardiovascular diseases:** The heart's pumping function requires a continuous and abundant supply of energy, making it heavily dependent on well-functioning mitochondria. Mitochondrial dysfunction in cardiac cells has been implicated in the development of various cardiovascular diseases. For example, 'cardiomyopathy', a condition characterized by a weakened and enlarged heart, can result from impaired energy production in cardiac mitochondria. Similarly, 'heart failure' and 'arrhythmias may arise due to mitochondrial dysfunction, leading to compromised heart function and life-threatening consequences. Hence, we can say that, understanding the role of mitochondria in cardiac health is crucial for advancing cardiovascular medicine and developing targeted treatments for heart-related disorders.

- **Cancer:** Based on the increased understanding in the past few decades, deregulated cellular energetics was recently added as one of the cancer hallmarks.1 Otto Warburg first proposed that tumor cells, unlike normal cells, exhibit increased glycolytic activity and reduced mitochondrial respiration even in the presence of oxygen. This phenomenon is known as the "Warburg effect." (Hsu, C. C., Tseng, L. M., & Lee, H. C. (2016)) Mitochondrial dysfunction and alterations in mitochondrial metabolism are key players in this metabolic reprogramming observed in various cancers. Dysfunctional mitochondria in cancer cells contribute to increased production of reactive oxygen species (ROS) and support the tumor's growth, invasion, and resistance to chemotherapy. Understanding the role of mitochondria in cancer biology may lead to the development of innovative therapeutic strategies targeting cancer cell metabolism and exploiting mitochondrial vulnerabilities (Luo, Y., Ma, J., & Lu, W. (2020)).

- **Metabolic disorders:** Mitochondria serves as the central unit in cellular metabolism, playing critical roles in energy production, fatty acid oxidation and glucose regulation. Dysfunctional mitochondria can disrupt these metabolic processes, contributing to various metabolic disorders. In the context of diabetes, impaired mitochondrial function is associated with insulin resistance and abnormal glucose metabolism, key factors contributing to the development of type 2 diabetes. Inherited

fatty acid oxidation disorders, on the other hand, arise from genetic defects in mitochondrial fatty acid metabolism, leading to an inability to break down fatty acids for energy production. So understanding mitochondrial involvement in metabolic diseases holds potential for developing novel strategies and improving disease management.

- **Age-related diseases:** The process of aging involves gradual decline in mitochondria function, leading to accumulation of cellular damage and impaired energy production. As mitochondria play a crucial role in cellular health and homeostasis, their decline with age can affect tissues and organs throughout the body. Unwinding, the relationship between mitochondria and the aging process may pave the way for interventions to promote healthy aging and combat age-related diseases. (Sun, N., Youle, R. J., & Finkel, T. (2016)).

- **Organ-specific diseases:** Mitochondrial dysfunction can result in diseases specific to certain organs, where energy-demanding tissues are particularly affected. For example, optic atrophy, characterized by degeneration of the optic nerve, is associated with mitochondrial dysfunction in the eyes. Hearing loss can also arise from impaired mitochondrial function in the inner ear. Additionally, mitochondrial dysfunction in the liver can contribute to liver diseases, affecting energy metabolism, detoxification processes, and overall organ health. Hence, understanding the link between mitochondria dysfunction and organ-specific disorders will lead towards novel interventions and therapeutics.

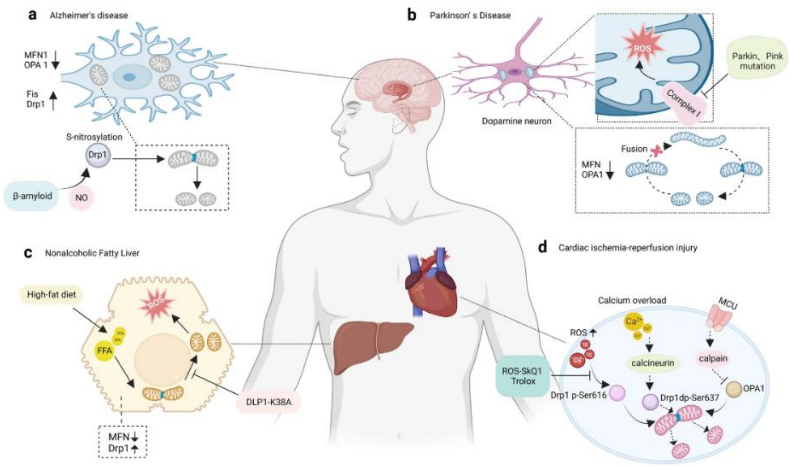


Figure 11: Mitochondrial dynamics and diseases. a. In neuronal cells of Alzheimer's disease patients, mitochondria appear small and fragmented. The expression levels of

Opa1, MFN1, and MFN2 are reduced, while Fis1 and Drp1 are increased. Beta-amyloid induces nitric oxide production, leading to neuronal injury and mitochondrial fission through S-nitrosylation of Drp1 b. Mitochondria in Parkinson's disease cells also exhibit small and fragmented structures c. The progression of non-alcoholic fatty liver disease is closely linked to mitochondrial fission and increased Drp1 protein expression. A high-fat diet can cause mitochondrial fragmentation, which precedes the generation of reactive oxygen species (ROS) d. Ischemia-reperfusion injury can cause mitochondrial fragmentation via the activation of Drp1 and downregulation of Opa1 (Source: Chen, W., Zhao, H., & Li, Y. (2023))

2.3.6 DISEASES ASSOCIATED WITH MUTATION IN MITOCHONDRIA

Mitochondrial disorders are a group of rare diseases caused by mutations in mtDNA or nuclear genes involved in mitochondrial function. Here are some diseases associated with mitochondrial mutations:

- **Leigh Syndrome (Subacute Necrotizing Encephalomyelopathy):** Leigh syndrome is a severe neurological disorder that typically appears in infant stage or early childhood. Leigh syndrome can be caused by mutations in mitochondrial DNA (mtDNA) or by deficiencies of an enzyme called pyruvate dehydrogenase (https://www.ninds.nih.gov/health-information/disorders/leigh-syndrome). These mutations in mtDNA interferes with the energy sources that run cells in an area of brain that plays role in motor movements. This syndrome is characterized by progressive degeneration of brain and spinal cord, leading to neurological impairments such as developmental delays, muscle weakness, seizures, and problems with movement and breathing.
- **Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS):** MELAS is a mitochondrial disease that primarily affects our nervous system and muscles. In MELAS, mutations in tRNA are believed to cause impairment of protein assembly into respiratory chain complexes, though the exact mechanisms have yet to be elucidated. MELAS is present in children or young adults as recurrent episodes of encephalopathy, headache, myopathy and focal neurological deficits etc. The neurological symptoms of MELAS are believed to result from a combination of impaired mitochondrial energy production, microvascular angiopathy, and nitric oxide

deficiency, which may cause impaired cerebral vasodilation. (Pia S, Lui F. Melas Syndrome, 2022)

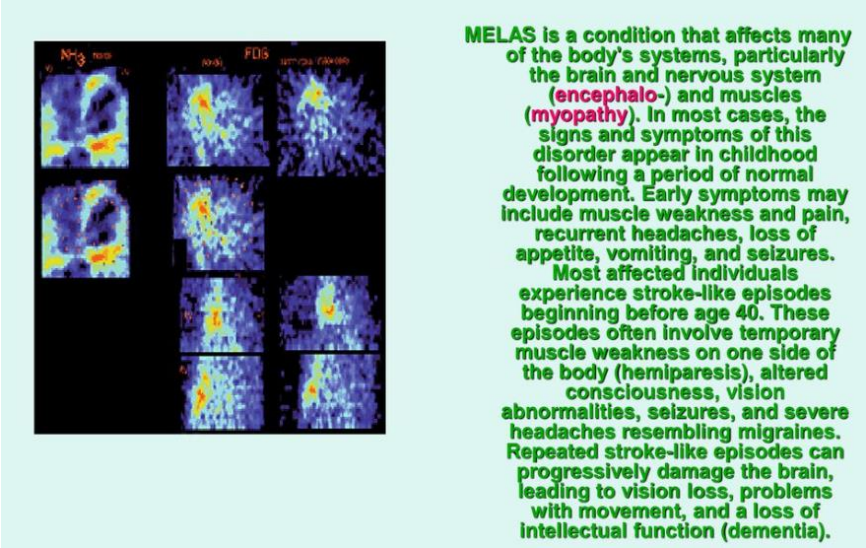


Figure 12: Brain scans and description of MELAS syndrome

- **Kearns-Sayre syndrome (KSS):** KSS is rare mtDNA deletion syndrome. It results from abnormalities in the DNA of mitochondria - small rod-like structures found in every body cell that produce the energy that drives cellular functions. This disease correlate with specific DNA changes that cause abnormalities with various tissues and organs of the body resulting in multisystem defects. Symptoms of this disease may start to appear at a variety of ages.

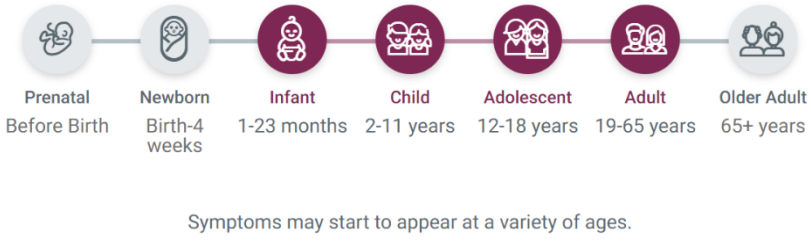


Figure 13: Illustration of KSS Syndrome and its symptoms at different ages (Source:Orphanet)

People with KSS experience muscle weakness, paralysis of eye muscles, drooping eyelids, pigmentary retinopathy, deafness, kidney problems etc.

- **Mitochondrial Cardiomyopathy:** It is described as a myocardial condition characterized by abnormal heart-muscle structure, function, or both, genetic defects, hypertension or congenital heart disease. It is a heterogeneous group of multisystemic diseases that develop consequent to mutations in nuclear or mitochondrial DNA. (Meyers DE *et al*.,2013). Mitochondrial cardiomyopathies varies in severity from asymptomatic status to severe manifestations, including heart failure, arrhythmias, and sudden cardiac death. Cardiomyopathy is estimated to occur in 20–40% of children with mitochondrial diseases. Therefore, screening for cardiomyopathy is a standard part of the management of children and adults with known or suspected mitochondrial disease. (Brunel-Guitton, C *et al*.,2015)

- **Leber's Hereditary Optic Neuropathy (LHON):** LHON is a mitochondrial genetic disease that causes blindness in males. It is characterized by bilateral subacute loss of central vision due to degeneration of ganglion cell layer and optic nerve. Over 95% of LHON cases are primarily the result of one of three mitochondrial DNA (mtDNA) point mutations, G3460A, G11778A, and T14484C, which all involve genes encoding complex I subunits of the respiratory chain. LHON is characterized by gender bias, with males more likely become affected than females. LHON carriers remain asymptomatic until they experience blurring or clouding of vision in one eye.

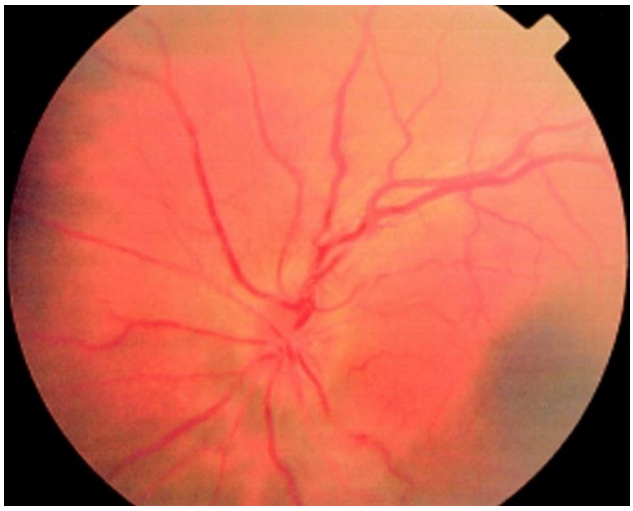


Figure 14: Acute fundal appearance in LHON (Man PYW *et al*., 2002)

- **Pearson Syndrome:** It is a rare mitochondrial disorder which is characterised by conditions such as sideroblastic anaemia (which leads to low red blood cell counts), liver disease, and exocrine pancreas dysfunction (causing diabetes). Pearson syndrome is caused by deletions in mtDNA. A common mtDNA deletion in this syndrome is about 4977bp. Diagnosing Pearson utilizes leukocyte DNA with the Southern Blot analysis. This kind mtDNA deletions are normally more abundant and easily isolated in the blood than in any other tissue type. Specifically, Pearson syndrome is a combination that involves both the bone marrow and the exocrine pancreas. (Pearson, Howard A *et al.*, 1979) Other symptoms may include lactic acidosis, liver problems, and neurological issues etc.

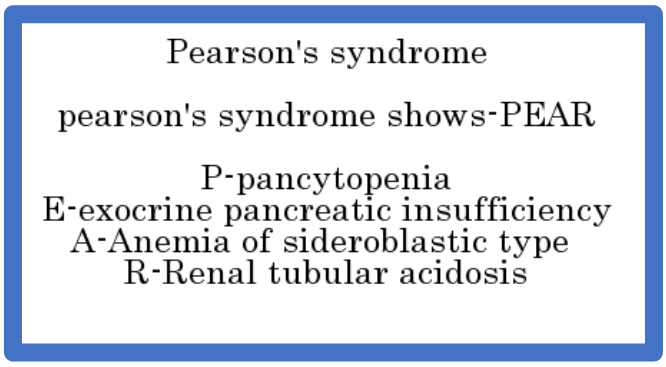


Figure 15: Pearson's syndrome representing its main clinical features

2.3.7 DISEASES ASSOCIATED WITH ALTERATION IN MITOCHONDRIAL DYNAMICS

- **Dominant Optic Atrophy (DOA):** Mutations in the OPA1 gene, responsible for mitochondrial fusion, lead to the progressive degeneration of the optic nerve and vision loss.

- **Charcot-Marie-Tooth Disease (CMT):** CMT type 2A is caused by mutations in the MFN2 gene, which is involved in mitochondrial fusion. This results in peripheral nerve damage and muscle weakness.

- **Mitochondrial Encephalopathy, Optic Atrophy, and Neuromyopathy (MEON):** This rare disorder is characterized by brain and muscle problems, as well as optic atrophy, and is caused by mutations in the OPA1 gene.

- **Autosomal Recessive Optic Atrophy (AROA):** Caused by mutations in the OPA3 gene, also involved in mitochondrial fusion, leading to optic nerve atrophy and vision impairment.

- **Cardiovascular Diseases:** Altered mitochondrial dynamics can impact cardiac health, leading to conditions such as heart failure and cardiomyopathies.

2.4 CANCER

Cancer arises due to the uncontrolled growth and division of normal cells. This relentless cellular proliferation can lead to the formation of tumours and their invasion. It is one of the leading causes of death worldwide and poses significant challenges to scientists, clinicians, and human health. It has been stated that spontaneous and progressive mutations in the cellular genome promote cancer. These mutations include chromosomal translocation, point mutation, deletion, amplification, and insertion activation (Hassanpour & Dehghani, 2017). Among cancer treatments, chemotherapy remains the most promising therapy. However, it is challenged by the severe side effects of the anticancer drugs and acquired drug resistance (Stortecky & Suter, 2010). Subsequent mutations acquired by these cells can further enhance their ability to spread to other tissues, known as metastasis. Hanahan (2022) has proposed a few cancer hallmarks contributing to proliferation and metastasis.

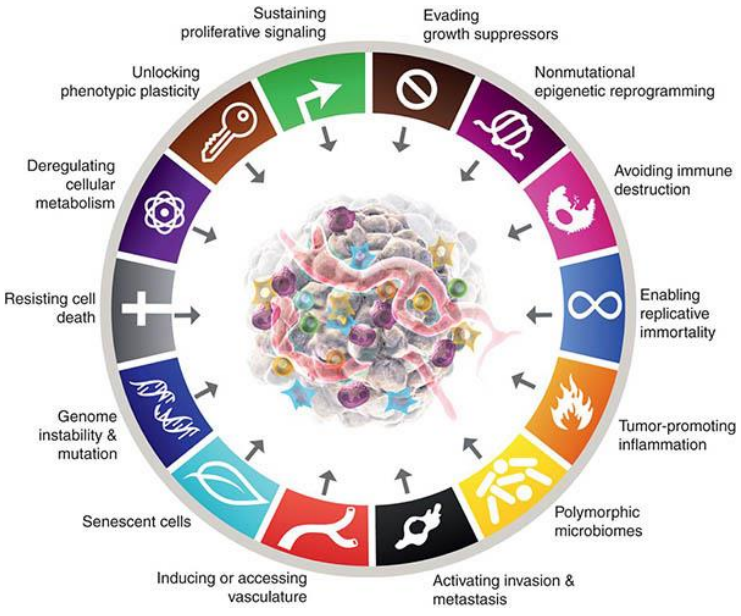


Figure 16: Hallmarks of cancer (Hanahan, 2022).

2.5 HSPS AND CANCER

Heats shock proteins (Hsps) are highly conserved and ubiquitously expressed proteins that form the most ancient cellular defence system. Several Hsps coordinate to maintain cellular protein homeostasis. A few studies have indicated that all Hsps overexpress in disease cells, including cancer. The overexpression of Hsps is correlated to higher tumour cell proliferation, invasion, metastasis, and immune recognition (Ciocca & Calderwood, 2005). Heat shock transcription factors tightly regulate the genes encoding Hsps, and between two major ones, HSF1 is involved in the inducible transcription of heat shock genes (Wu, 1995). Elevated levels of Hsps may implicate poor survival and response to therapy (Ciocca & Calderwood, 2005). Hsps protects the cell from spontaneous chemotherapy-induced apoptosis and promotes tumour progression (Ciocca *et al*., 2003; Mosser & Morimoto, 2004).

Hsp90, in its active conformation (obtained in response to stress conditions in normal cells and constitutively present in cancer cells), binds to mutated oncogenic gene products (called clients) and facilitates their proper functioning. More than 500 cellular proteins were shown to interact with Hsp90 for their functions, and the majority of them are kinases and transcription factors (Karagöz and Rüdiger 2015). Since Hsp90 is involved in the functional stabilization of cancer-causing genes, this is also referred to as cancer chaperone. Hsp90 inhibitors, such as geldanamycin, 17AAG, 17DMAG, *etc.,* can inhibit cancer growth. Therefore, Hsp90 inhibition disrupts the interaction between Hsp90 and its client proteins and is proposed as a novel treatment strategy (Li, Z. N., & Luo, Y. 2023). It can lead to the degradation of proteins involved in angiogenesis, proliferation, metastasis, and drug resistance.

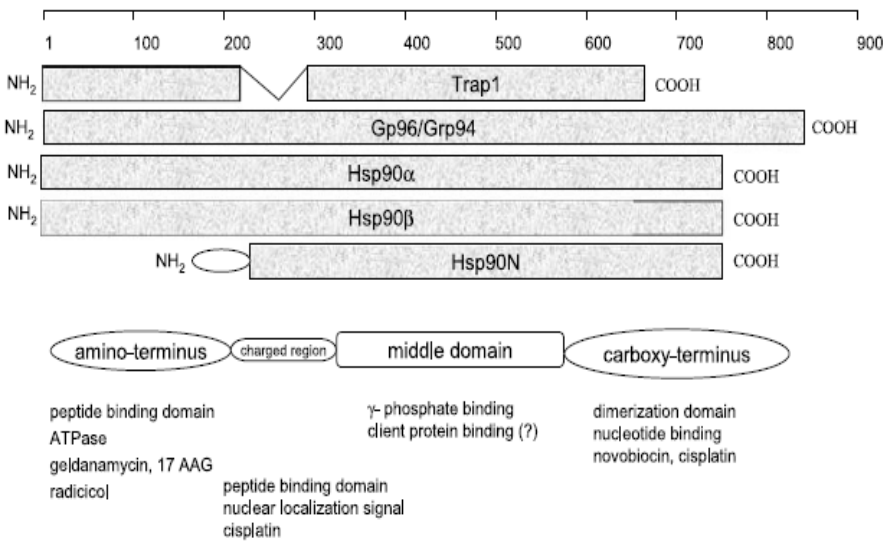


Figure 17: Schematic representation of Hsp90 and its isoforms (Sreedhar *et al*., 2004)

2.6 METABOLIC REPROGRAMMING IN CANCER

Cancer cells exhibit distinct alterations in their metabolism, enabling them to sustain their rapid growth and survival in harsh environments. Unlike normal cells, cancer cells rely on unique metabolic pathways to meet their energy demands and support anabolic processes essential for their proliferation. This metabolic shift, known as metabolic reprogramming, involves changes in glucose metabolism, amino acid metabolism, lipid metabolism, and alterations in mitochondrial function.

Metabolic reprogramming gives cancer cells an advantage, conferring resistance to apoptosis and promoting tumour progression. **The Warburg effect**, where cancer cells rely on glycolysis even in presence of oxygen, is a classic example of metabolic reprogramming (Warburg, O., Wind, F., & Negelein, E.,1927)

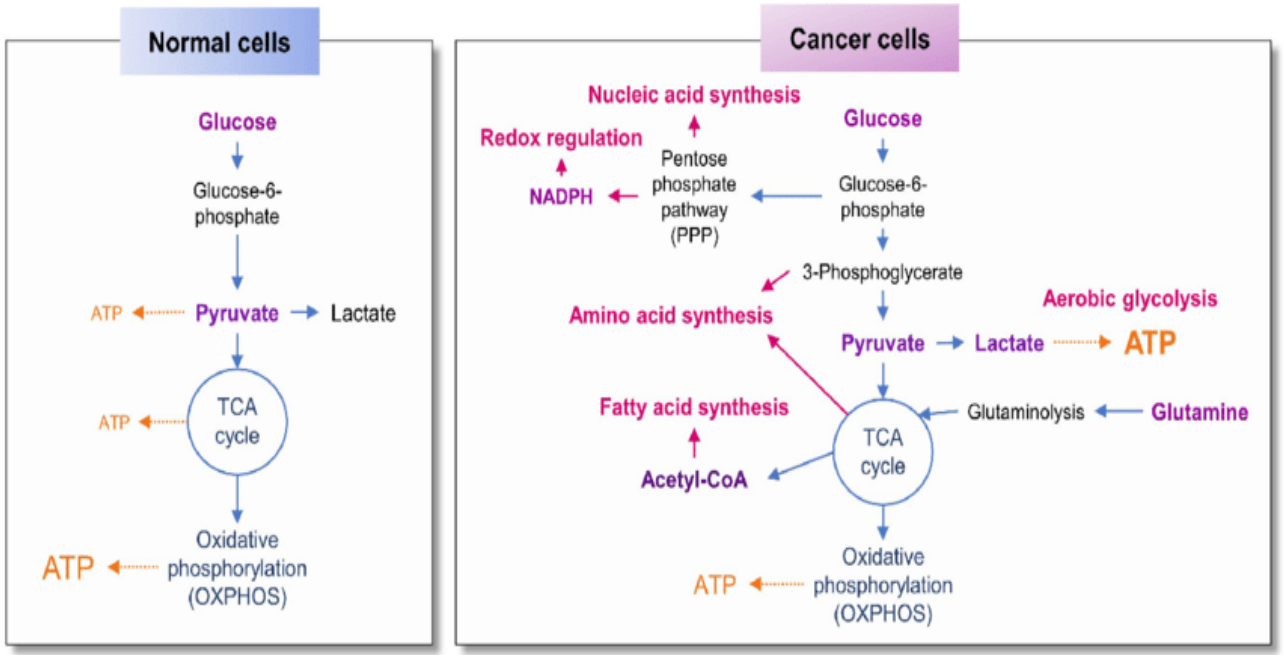


Figure 18: Metabolic alteration in cancer cells. Glucose is oxidised via glycolysis followed by TCA cycle in normal cells. While in cancer cells metabolic rewiring is observed utilising TCA cycle intermediates. (Source- Oncogene-Driven Metabolic Alterations in Cancer: Hye-Young Min & Ho-Young Lee-Dec 2017)

Recent studies show that mitochondria play an important role in tumor metabolism. Depleting mitochondrial has shown to decrease the tumorigenic potential of cancer cells. The upregulation of oxidative phosphorylation has been noted in cancer cells (Whitaker Menezes *et al.,* 2011, Magda *et al.,* 2008). Therefore, metabolic rewiring to aerobic glycolysis appears to help cancer cells proliferate by supplying the precursors of proteins, lipids and essential macromolecules. Thus, mitochondria play essential roles in bioenergetics and biosynthesis in cancer cells.

2.7 TRAP-1 AND ITS ROLE IN METABOLIC REPROGRAMMING

TRAP-1 is a mitochondrial homologue of Hsp90 chaperone whose expression increases in diseases such as cancer. TRAP-1 has been shown to play an important role in tumour progression and altered metabolism (Ramkumar, B., Dharaskar, S. P., Mounika, G., Paithankar, K., & Sreedhar, A. S., 2020). Unlike Hsp90, TRAP-1 has a mitochondrial localization signal at its N-terminus. Unlike other Hsp90 members, TRAP-1 possesses a unique LxCxE motif (Felts, Owen *et al*. 2000). TRAP-1 is said to be dependent on stress kinases for its transcriptional activation (Lee, Park, *et al*. 2015). TRAP1 has the bacterial Hsp90 homolog, HtpG, where the highly charged hinge region of the N-terminal section is absent (Sreedhar, A. S., Kalmár, E., Csermely, P., & Shen, Y. F. 2004).

Studies have suggested that TRAP1 may play a cytoprotective role by buffering reactive oxygen species (ROS) mediated oxidative stress. It is speculated that TRAP-1 may have anti-oxidative properties thus protecting cells from oxidative stress (Hua, Zhang *et al.,* 2007). Since TRAP-1 regulates cell death pathways and metabolic rewiring in cancer cells, thus inhibiting TRAP-1 can help sensitize cancer cells to chemotherapeutic treatments.

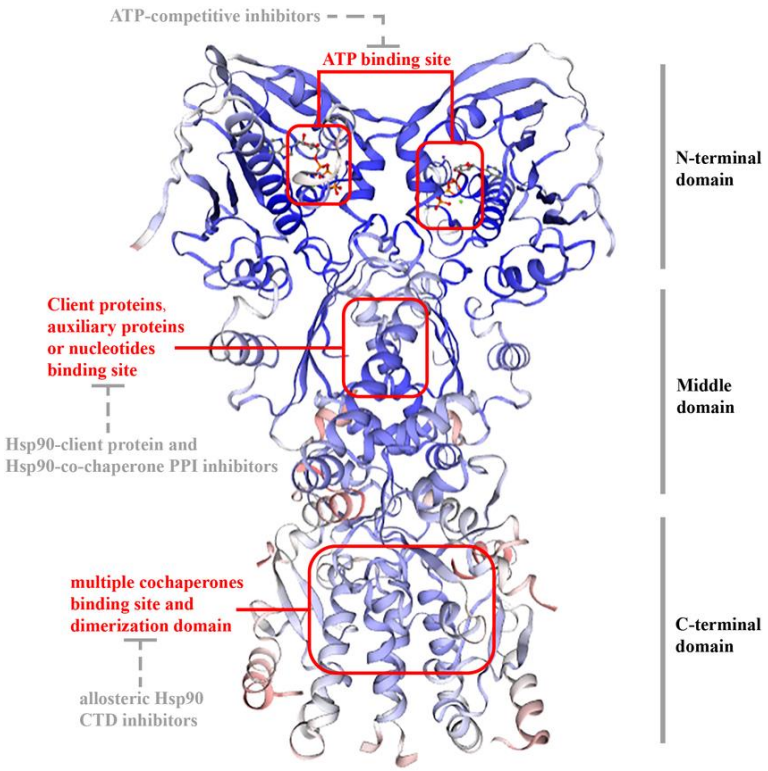


Figure 19: Structure of TRAP-1. The structure of TRAP-1 is similar to other Hsp90 proteins (Hsp90α, Hsp90β, and Grp94) in that they all have three sections: the N-terminal domain (NTD), the middle domain (MD), and the C-terminal domain (CTD). They also share an ATP-binding site in a deep pocket on the helical face of the NTD, which is targeted by ATP-competitive inhibitors. The MD has binding sites for client proteins, interacting with some client proteins and auxiliary proteins or nucleotides, and is targeted by Hsp90-client protein and Hsp90-co-chaperone PPI inhibitors. The CTD is responsible for binding multiple cochaperones and includes the dimerization domain, which is targeted by allosteric Hsp90 CTD inhibitors. However, unlike other Hsp90 proteins, TRAP-1 lacks a conserved amino acid sequence crucial for interactions with modulators at the end of the CTD. (Source: Xiang Y, Liu X, Sun Q, Liao K, Liu X, Zhao Z, Feng L, Liu Y and Wang B (2023)).

CHAPTER 3

HYPOTHESIS OF STUDY

3.1 HYPOTHESIS

Cancer cells do not rely on mitochondria for energy production. However, mitochondria are not obsolete, but exhibit decreased mitochondrial functions. Our earlier findings suggested that TRAP-1 maintained mitochondrial integrity and appeared involved in regulating mitochondrial integrity and function. We speculated that TRAP-1 is also involved in mitochondria transfer to facilitate mitochondria-independent cellular functions in the recipient cells.

3.2 AIM

To understand the role of TRAP-1 in mitochondria transfer in neuronal cancer cells (IMR-32).

3.3 OBJECTIVES

- To establish the crosstalk between cytoskeletal proteins and mitochondria in TRAP-1 KD (knockdown) and OE (overexpression) cells.
- To examine mitochondria transfer between TRAP-1 KD and OE cells.

3.4 SIGNIFICANCE OF STUDY

The significance of studying the role of TRAP1 in mitochondrial transfer between cancer cells lies in driving tumour progression and therapeutic resistance. Understanding how TRAP1 influences the exchange of mitochondria can shed light on the intricate interplay between cellular metabolism and cancer biology. This knowledge could lead to identifying new therapeutic targets to disrupt mitochondrial/ metabolic networks, offering innovative strategies to combat cancer progression and improve treatment outcomes. Moreover, elucidating TRAP1-mediated mitochondrial transfer may provide insights into developing precision therapies tailored to disrupt tumour metabolism, ultimately enhancing our arsenal against cancer.

CHAPTER 4

MATERIALS AND METHODS

4.1 CELL CULTURE REAGENTS

- **Dulbecco's Modified Eagle Medium (DMEM):** The DMEM powder of 6.85 g and Sodium bicarbonate (Na_2CO_3) of 1.85g were dissolved in 500mL of sterile ddH_2O. The pH was adjusted to 7.2, and the filter was sterilized with 0.45µm sterile filters and stored at 4°C.
- **Complete Medium:** The foetal bovine serum (FBS) in 10% concentration was added to an incomplete DMEM medium, filter sterilized, and stored at 4°C.
- **1X PBS:** Added 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of K_2HPO_4 to 700 mL of ddH20, adjusted the pH to 7.4, and the final volume was made to 1L and then autoclaved.
- **100X Antibiotic solutions:** 600 mg of penicillin, 500 mg of streptomycin are dissolved in 100 mL of 1X PBS and filter sterilized using 0.45 µm filters and stored at 4°C.
- **0.1% Trypsin-EDTA (TE):** 100 mg trypsin and 100 mg of EDTA were dissolved in 100 ml of 1X PBS, filter sterilized using 0.45 µm filters, and stored at 4°C.
  (The stock was diluted to 1X before use)
- **Lipofectamine 3000 transfection reagent:** Lipofectamine 3000 transfection reagent was purchased from Thermo Fischer Scientific (cat no. L3000001) to transfect recombinant plasmids to mammalian cells.

4.2 CELL LINES

The human neuroblastoma IMR-32 (CCL127^{TM}) was procured from the American Type Culture Collection (ATCC). After authentication, the cells were grown in DMEM (Thermo Fisher, cat no. 12491-023) supplemented with 10% FBS (Thermo Fisher, cat no. 12483-020) and antibiotics (500 µg/mL Streptomycin; 600 µg/ml Penicillin; 300 µg/ml Kanamycin) at 37°C and 5% CO_2. The cells were allowed to grow till 60% confluency and subsequently used for experiments.

4.3 METHODOLOGY

4.3.1 The TRAP-1 KD & OE systems:

- **TRAP-1 Knockdown (KD) Construction:** The shRNA against TRAP-1 (Acc No. NM_016292.2) was designed using online software like BLOCK-it RNA designer, Invivogen, and Biosettia and custom synthesized through Bioserve Biotechnologies, India.,

  Sense strand: 5'-TTTGGGGTTCCACTTCCAAACATGACGAATCATGTTT GGAAGTGGAACCCTTTTT- 3'

  Antisense strand:

  5'-CTAGAAAAAGGGTTCCACTTCCAAACATGATTCGTC ATGTTTGGAAGTGGAACCC – 3'

  This TRAP-1 shRNA was cloned in the mU6Pro vector, and then transfected into human neuroblastoma cells with this recombinant plasmid i.e., shRNA-TRAP-1-mU6Pro.

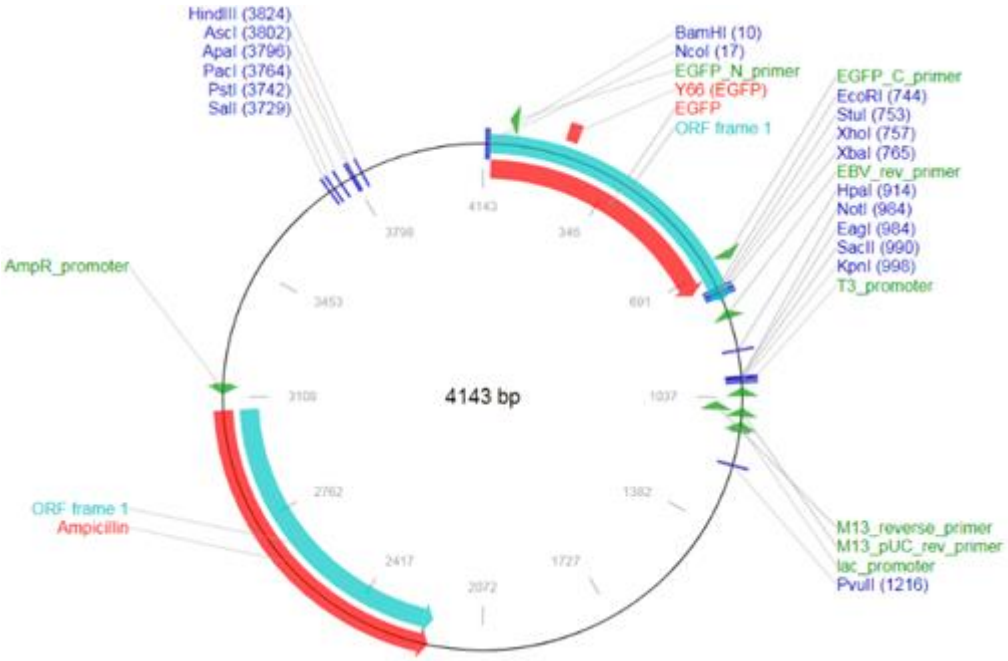


Figure 20: Vector map of mU6PRO (Turner, David *et al.*, 2002)

❖ **TRAP-1 Overexpression (OE) Construction:** The complementary strand of the TRAP-1 (Acc No. NM_016292.3) was used to amplify cDNA using specific primers. Then, the amplified gene was cloned into the pEGFP-N2 mammalian expression vector system using EcoR1 and BamH1 restriction sites (Clontech/Takara Bio USA, Inc.).

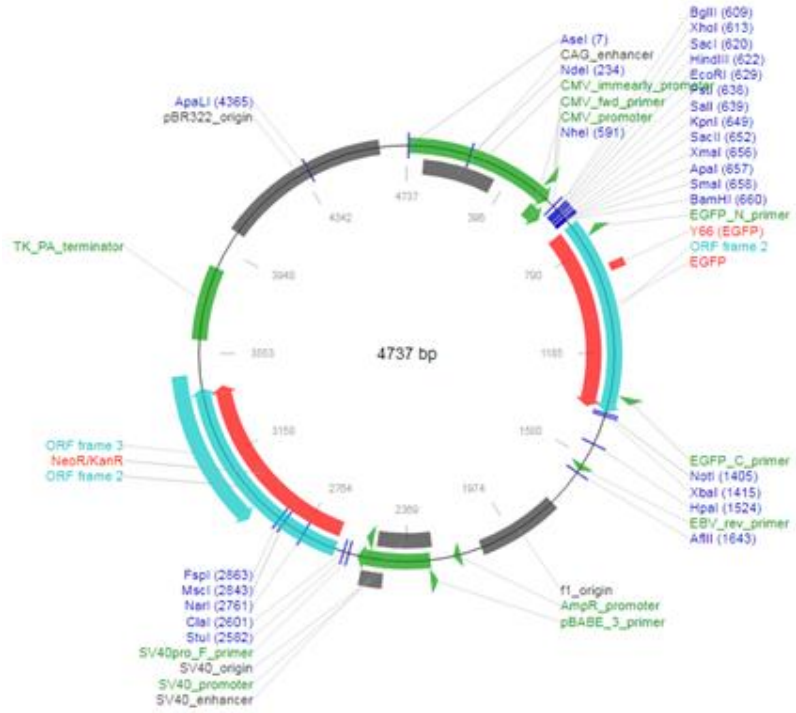


Figure 21: Vector map of pEGFP-N2 (Takara Biosciences USA, Inc.)

4.3.2 Transfection of KD & OE plasmids:

TRAP-1 OE or TRAP-1 KD recombinant plasmids were transfected in IMR-32 cells using lipofectamine 3000 (cat no. L3000008, Thermo Fisher Scientific). Before transfection, cells (1 X 10^5) were seeded and incubated at standard cell culture conditions. The used medium was discarded, and fresh 500 µl incomplete DMEM was added. The first tube contained 125 µl in incomplete DMEM and 6ul lipofectamine 3000 reagents are mixed well. The second tube had a mixture of 125 µl DMEM, 6 µl P3000^{TM} reagent, and 2 µg recombinant plasmid containing the gene of interest. Later on, the constituents from the second tube were mixed in the first tube, incubated for 20 min at room temp., and added to cells in a drop-wise manner and swirled gently to ensure homogeneity. Fresh complete media was added after 8 hrs of transfection. The

transfected cells were then subjected to 21 days of antibiotic selection using G418 (900 µg/mL, A1720 Sigma-Aldrich), expanded, and subsequently used for experiments.

4.3.3 FLoid- live cell imaging of all three phenotypes:

The optimal growth and viable cells were captured using a confocal scanning image of the microscope. The cells representing each phenotype are cultured in standard growth media unless otherwise indicated under controlled environmental conditions.

Figure 22: Life Technologies FLoid Cell Imaging Station

4.3.4 Laser scanning confocal imaging analysis of cytoskeleton architecture:

Cells ($0.4x10^6$/ml) of IMR-32 of phenotype PAR, KD, and OE were plated on cover glasses (18mm X 18mm) overnight at 37°C, 5% CO_2 supply. After reaching 50% confluency, the cells were washed with 1X PBS (3 times) and were fixed and permeabilized using 3.7% paraformaldehyde for 10 min and 0.1% triton X-100 for 30 sec, respectively. Then, after washing the cells with 1X PBS (3 times) cells were treated with 200 nM Rhodamine Phalloidin (cat no. R415, Invitrogen) and incubated for 30 min at 37°C in the dark to stain actin filaments. Cells were rinsed with 1X PBS, then mounted onto glass slides using the mounting medium containing DAPI (cat no. H1200, Vectashield). Sealed the edges with nail polish and stored the mounted slides for further use. Finally, actin network was observed using Laser scanning confocal microscope (Leica TCS SP8).

Figure 23: Leica TCS SP8 Microscope

4.3.5 Laser scanning confocal imaging analysis of mitochondria organization:

Cells ($0.4x10^6$/ml) of IMR-32 with PAR, KD, and OE phenotypes were seeded onto 18mm x 18mm cover glasses and incubated overnight at 37°C with 5% CO_2. Upon reaching the desired confluency, the cells underwent two washes with 1X PBS and were then treated with 200 nM MitoTracker Red (cat no. R237-10, CMX-Ros, Dojindo) for 20 min at 37°C in the dark to label mitochondria in all three phenotypes. Following staining, the cells were washed with 1X PBS (2-3 times) and fixed using 3.7% paraformaldehyde for 5-10 minutes and permeabilized using 0.1% triton X-100. After rinsing with 1X PBS the coverslips were mounted onto glass slides using a mounting medium (cat no. H1200, Vectashield). The edges were sealed with nail polish, and the mounting medium. The stained cells were visualized using a Laser scanning confocal microscope (Leica TCS SP8), and mitochondrial dynamics were captured using Mitotracker Red-labeled mitochondria.

Figure 24: Leica TCS SP8 confocal microscope has been designed with optimal photon efficiency and high speed. This system has 3 detectors (1 sensitive Hybrid GaAsP and 2 PMT) and a motorized XYZ-stage

4.3.6 Laser scanning confocal imaging analysis of tunnelling nanotubes (TNT) formation between cancer cells:

Cells ($0.4x10^6$/ml) from IMR-32 cell lines expressing PAR, KD, and OE phenotypes in duplicates were plated onto 18mm x 18mm cover glasses and incubated overnight at 37°C. overnight grown cells were given two washes with 1X PBS and were subsequently one set (treated) out of duplicates treated with 200 µM Cobalt Chloride ($CoCl_2$) for 6 hrs and washed with 1X PBS (3 times) and then cells were suspended in 1 ml fresh media. On the other hand, the other set (untreated) was co-cultured with treated cells. After 20 hrs of co-culture, the cells were washed with 1X PBS followed by staining with 200 nM Mitotracker Red (cat no. R237-10, CMX-Ros, Dojindo) for 20 min at 37°C in the dark to label mitochondria. Again, cells were washed with 1X PBS (3 times) and fixed using 3.7% paraformaldehyde for 5-10 min followed by permeabilization with 0.1% triton X-100 for 30 sec. Then cells were washed with 1X PBS and then again exposed to 200 nM Alexa Fluor 488 Phalloidin (cat no. A12379, Invitrogen) for 30 min at 37°C in the dark to label actin filaments. Subsequently, the cells were washed with 1X PBS, and the coverslips were mounted onto glass slides with mounting medium (cat no. H1200, Vectashield). The edges were sealed with nail polish, and the slides were stored for future use. Finally, the stained cells were visualized by Laser scanning confocal microscope (Leica TCS SP8), capturing images of Oregon Green Phalloidin-labelled actin filaments, Mitotracker Red-labeled mitochondria, and DAPI-stained nuclei.

4.3.7 Laser scanning confocal imaging analysis for extracellular vesicles transfer:

Cells ($0.4x10^6$/ml) of IMR-32 of phenotype PAR and OE were plated on cover glasses (18mm X 18mm) for overnight at 37°C, 5% CO_2 supply. Then IMR-32 PAR cells were incubated with the cell conditioned media (CCM) of IMR-32 OE cells for 6 hr. Similarly, the IMR-32 OE cells were incubated in CCM of IMR-32 PAR cells for 6 hr interval. After incubation cells were washed with 1X PBS (2 times) and treated with 200nM of MitoTracker red (cat no. R237-10, Dojindo) and incubated for 20 min at 37°C in dark. Then cells were washed with 1X PBS (3 times) and fixed in 3.7% paraformaldehyde for 7 min. Again, after washing with 1X PBS, the cover glasses were mounted on glass slides using 10µl mounting medium and sealed with transparent nailpolish. Finally, the glass slides are observed using Laser scanning confocal microscope (Leica TCS SP8). The similar experiments were conducted for 12hr and 24hr incubation intervals in both IMR-32 PAR and OE phenotypes.

4.3.8 Isolation of microvesicles & exosomes:

4.3.8.1 Method: Ultracentrifugation

4.3.8.2 Principle: The principle of ultracentrifugation revolves around the differential sedimentation of particles within a sample subjected to high centrifugal forces. When a sample containing various particles is spun at high speeds in an ultracentrifuge, these particles experience outward movement from the axis of rotation due to the applied centrifugal force. This movement causes particles to sediment at different rates based on their physical properties, such as size, shape, and density. Larger and denser particles tend to sediment more rapidly and form a pellet at the bottom of the centrifuge tube. In comparison, smaller and less dense particles sediment more slowly or remain suspended in the supernatant. Adjusting the centrifugation parameters, such as speed and duration, can pellet specific particles while leaving others in the supernatant selectively. This principle allows for the isolation and purification of desired particles from complex biological samples, facilitating further analysis and experimentation.

4.3.8.3 Sample source: To obtain extracellular vesicles (EV), conditioned media was utilized, which serves as a rich reservoir containing a diverse array of exosomes, microvesicles, and other EVs. Cells release these vesicles into the surrounding media and play essential roles in intercellular communication, signalling, and cell-to-cell interactions.

4.3.8.5 Modified Procedure: The conditioned media was centrifuged at 300X g for 10 min to pellet out the cellular debris. The supernatant was collected and then transferred to fresh tubes for centrifugation at 2500X g for 20 min to pellet down the apoptotic bodies. Subsequently, the supernatant was carefully collected in polycarbonate tubes (Beckman Coulter) and spun at 40,000X g for 60 min in Ti 70 rotor (Beckman Coulter Optima XPN-100 Ultracentrifuge) to pellet down the microvesicles (MVs). The resulting supernatant from this step was then again centrifuged at 60,000X g for 60 min to pellet down the remaining MVs. The pellet from both rounds of centrifugation is stored for further use. Supernatant was collected after MVs isolation and filtered using 0.22 µm sterile filter into polycarbonate tubes to remove the larger vesicles and centrifuged at 1,20,000X g for 70 min in Ti 70 rotor to pellet down exosomes (EXOs). The pellets containing MVs and EXOs were washed once with sterile 1X PBS by centrifuging at 60,000X g for 60 min and 1,20,000X g for 70 min to wash the MVs and EXOs pellets respectively. The entire isolation was conducted at 4°C. All the EV samples were aliquoted and stored at -80°C for future experiments. Our EV isolation allowed us to separately isolate EXOs

and MVs in two different fractions, but we collectively refer to the cell-secreted, membranous EXOs and MVs as EVs.

Figure 25: Beckman Coulter Ultracentrifuge

Figure 26: Beckman Coulter TYPE 70 Ti Rotor & Polycarbonate tubes

4.3.9 Protein estimation of microvesicles & exosomes:

4.3.9.1 Method- BCA Assay: The BCA (Bicinchoninic Acid) assay is a commonly used method in biochemistry and molecular biology for quantifying protein concentration in a sample. This method offers high sensitivity and a wide linear range, making it a popular choice for protein quantification in biochemical research and various biotechnological applications.

4.3.9.2 Principle: The BCA assay operates based on the principle of colorimetric detection. It relies on the ability of proteins to reduce Cu^{2+} ions to Cu^{1+} ions under alkaline conditions. In the presence of proteins, the Cu^{2+} ions form a complex with bicinchoninic acid, forming a purple-coloured product. The intensity of this colour is directly proportional to the protein concentration in the sample. By measuring the absorbance of the coloured complex at a specific wavelength using a spectrophotometer and comparing it to a series of standards with known

protein concentrations, the concentration of proteins in the sample can be accurately determined.

4.3.9.3 Procedure: Firstly, we prepared a series of protein standards with known concentrations. These standards serve as crucial references for quantification. Next, the protein samples are prepared at an appropriate dilution that aligns with the protein standards range. The diluted samples and standards are then aliquoted into the wells of a 96-well plate, ensuring appropriate labelling for easy identification. Subsequently, the BCA working reagent, created by mixing BCA reagents A and B in the proper 50:1 ratio, is added to each well containing the standards and samples. Thorough mixing is essential to ensure uniform distribution of the reagent.

The plate is then appropriately covered and incubated typically at 37°C for 30 min to 1 hr, allowing the formation of a purple-coloured complex proportional to the protein concentration in each well. Using a microplate reader, the absorbance of the samples and standards is measured at around 562 nm. The absorbance readings then plot a standard curve, relating absorbance to known protein concentrations. This curve serves as a reference to determine the protein concentration of the samples based on their absorbance values, adjusting for any dilutions made during sample preparation.

Figure 27: Thermo Scientific™ Pierce™ BCA Protein Assay Kit

4.3.10 Complementary experiments:

4.3.10.1 PCR Amplification: Polymerase chain reaction (PCR) was performed using isolated exosomes and microvesicle samples using respective primers specific to mitochondria. By targeting a specific mitochondrial gene like ND1, we selectively amplified mtDNA fragments present within exosomes and microvesicles.

S. No.	Gene; Accession	Primer sequence	Product size
1.	NADH dehydrogenase subunit 1 (ND1); NM_001318827	**Forward primer** 5'-ATGGCCAACCTCCTACTCCTCA-3' **Reverse primer** 5'-TGGGGAGGGGGGTTCATAGTAG-3'	265 bp

4.3.10.2 Composition of PCR reaction mixture:

REAGENTS	AMOUNT
Template (EXO/MV)	5 µL
Forward primer (10 pmol)	0.5 µL
Reverse primer (10 pmol)	0.5 µL
2 X EmeraldAmp® GT PCR Master Mix	1 X
Total	20 µL

4.3.10.3 PCR conditions:

STEPS	TEMPERATURE	TIME
Hot start	95°C	2 min
Denaturation	95°C	30 sec
Annealing	62.8°C	30 sec
Extension	72°C	25 sec

No. of Cycles	Repeat the steps 2-4 29	-
Final extension	72°C	2 min
	4°C	∞

CHAPTER 5

RESULTS AND DISCUSSION

5.1 RESULTS:

5.1.1 FLoid- live cell imaging of all three phenotypes:

The TRAP-1 KD and OE plasmids were transfected into human neuroblastoma cells, IMR-32, and stably selected with G418 for 21 days. The figure below shows the Floid images of all three stable phenotypes for TRAP-1, *i.e.,* PAR, KD, and OE cells, which were utilized for further experiments.

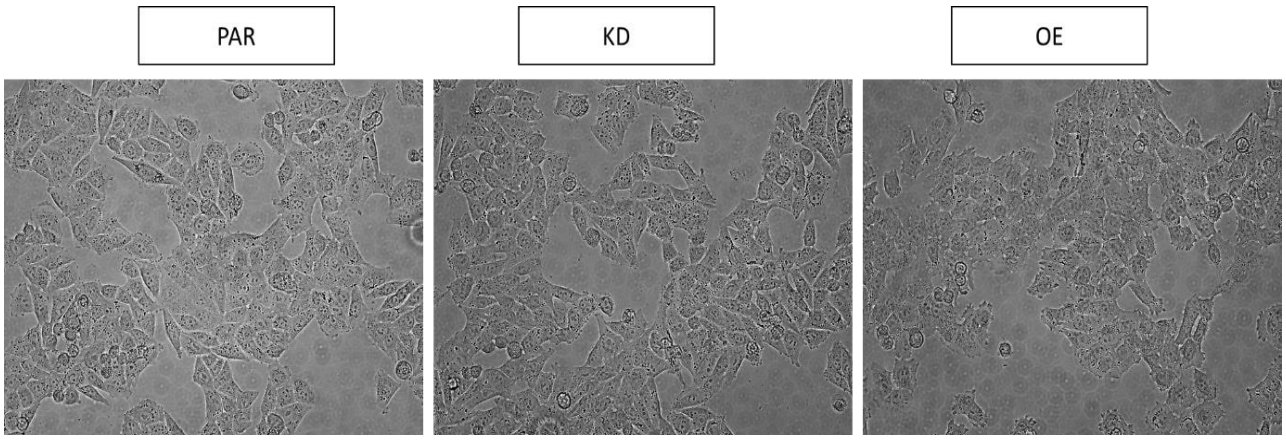


Figure 28: **DIC images of selected stable phenotypes PAR, KD, and OE**.

All the three phenotypes did not show remarkable morphological differences while showed functional competence.

5.1.2 Laser scanning confocal imaging analysis of cytoskeleton:

The cytoskeleton of actin filaments was observed in all three phenotypes, *i.e.,* PAR, KD and OE of IMR-32 cells. The stained cells were observed under Leica SP8 microscopy, and found variations in their cyto-architecture.

PAR cells showed actin filaments getting polymerized and maintained their cytoskeleton, whereas KD and OE showed alterations. The cytoskeleton reorganization and decreased actin polymerization in KD cells focuses on the TRAP-1 involvement in maintaining cyto-architecture. On the other hand, OE cells showed enhanced actin polymerization and extended filopodia on the periphery suggesting its ability to transfer mitochondria as it acquired more migratory potential.

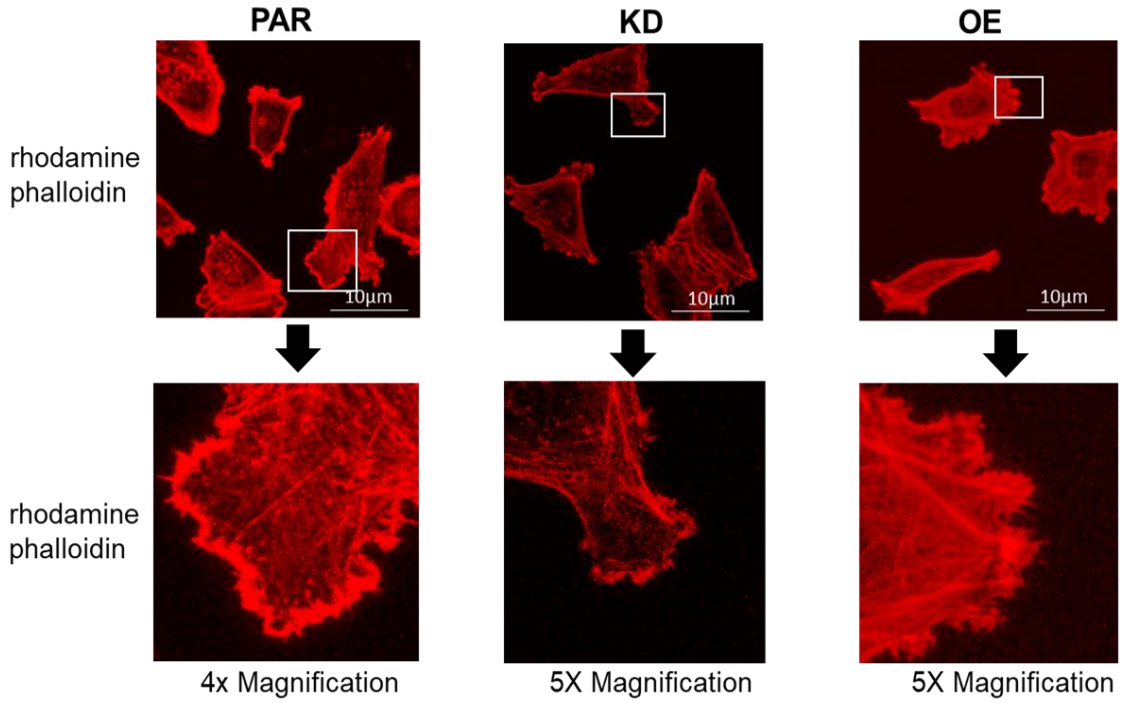


Figure 29: Confocal microscopy imaging analysis of cytoskeleton organisation in all three phenotypes PAR, KD, and OE

5.1.3 Laser scanning confocal microscopy imaging analysis of mitochondria organisation:

The mitochondria morphology for stained cells was observed under the Leica SP8 microscope for all three phenotypes i.e., PAR, KD, and OE.

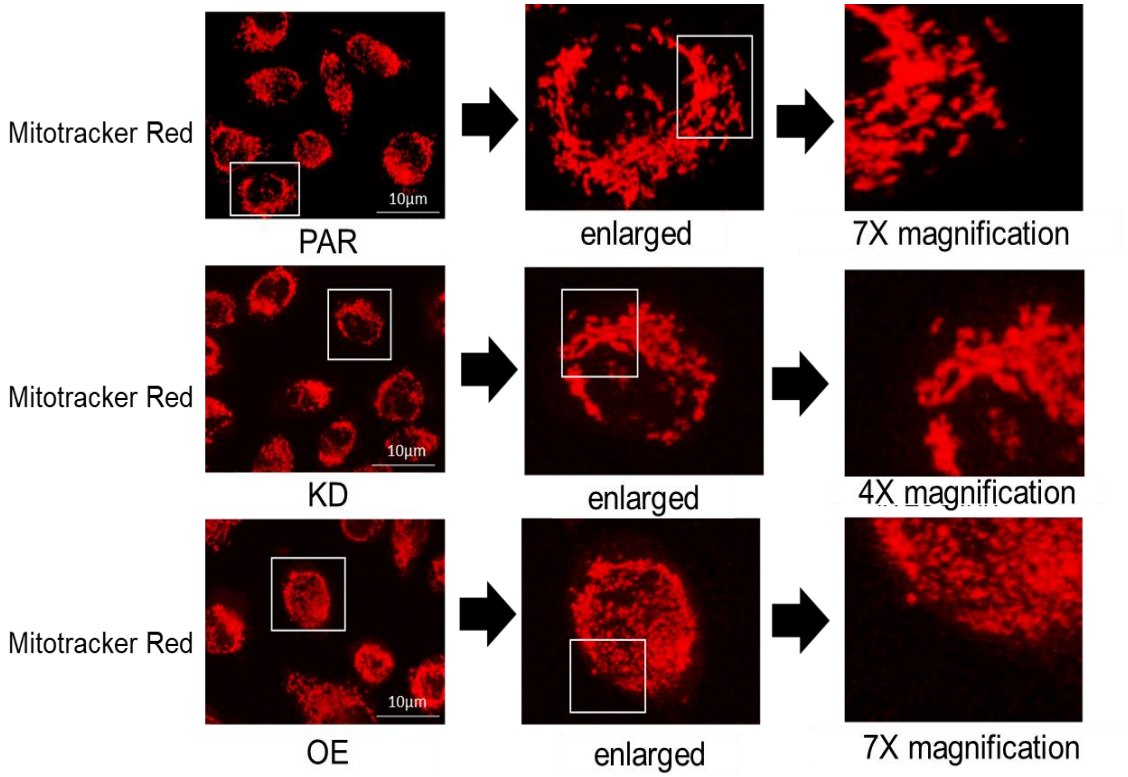


Figure 30: Confocal microscopy imaging analysis of distribution and size of mitochondria in all three phenotypes PAR, KD, and OE.

It was observed that PAR cells showed both smaller and larger mitochondria in equal distribution indicating they can act as either donor or recipient. In contrast, in KD a longer and lesser number of mitochondria was seen that matches with our previous studies where it was shown that KD cells induce fusion mitochondria (Dharaskar, S. P., Paithankar, K., Vijayavittal, A. K., Kara, H. S., & Subbarao, S. A.,2020). On the other hand, OE cells showed a smaller and higher number of suggesting fission mitochondria dynamics indicating its ability to get transferred to other cancer cells.

5.1.4 Laser scanning confocal imaging analysis of tunneling nanotubes (TNT) formation between cancer cells:

To examine the mitochondria transfer *via* TNT formation, co-culture experiments were performed. The F-actin was stained with Oregon Green phalloidin and mitochondria and nucleus were stained using Mitotracker Red and DAPI, respectively.

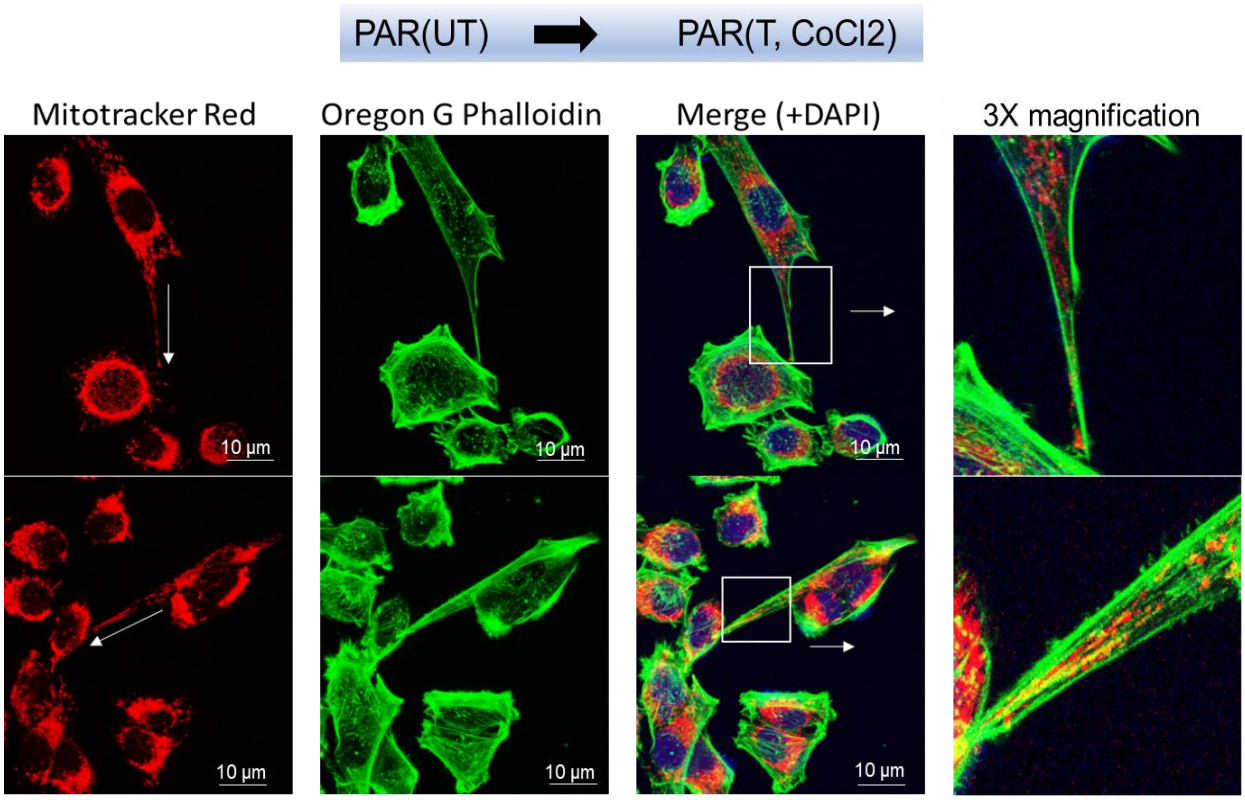


Figure 31: **Confocal microscopy imaging analysis of TNT formation in parental phenotype i.e., PAR untreated (UT) co-cultured with PAR treated (T) for mitochondria transfer**

It was observed that when one set of parental (PAR) cells were treated (T) with 200 nM cobalt chloride ($CoCl_2$) to induce chemical hypoxia and co-cultured with other untreated (UT) set of PAR cells. We observed TNT formation in both cells to facilitate mitochondria transfer.

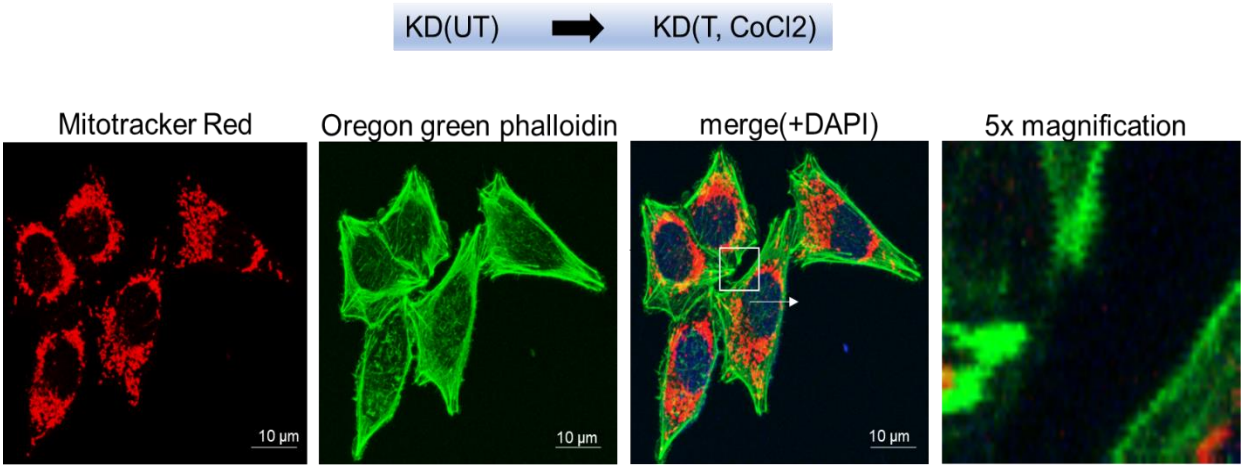


Figure 32: Confocal microscopy imaging analysis of TNT formation in knockdown phenotype i.e., KD untreated (UT) co-cultured with KD treated (T) with cobalt chloride for mitochondria transfer

Next, to examine if a similar phenomenon is observed in TRAP-1 compromised cells *i.e.,* KD cells, co-culture was conducted between KD (T) with 200 nM $CoCl_2$ to induce hypoxic stress and KD (UT) and then analyzed using confocal microscopy. There was no formation of TNT structures was observed and hence, these cells were incapable of transferring mitochondria.

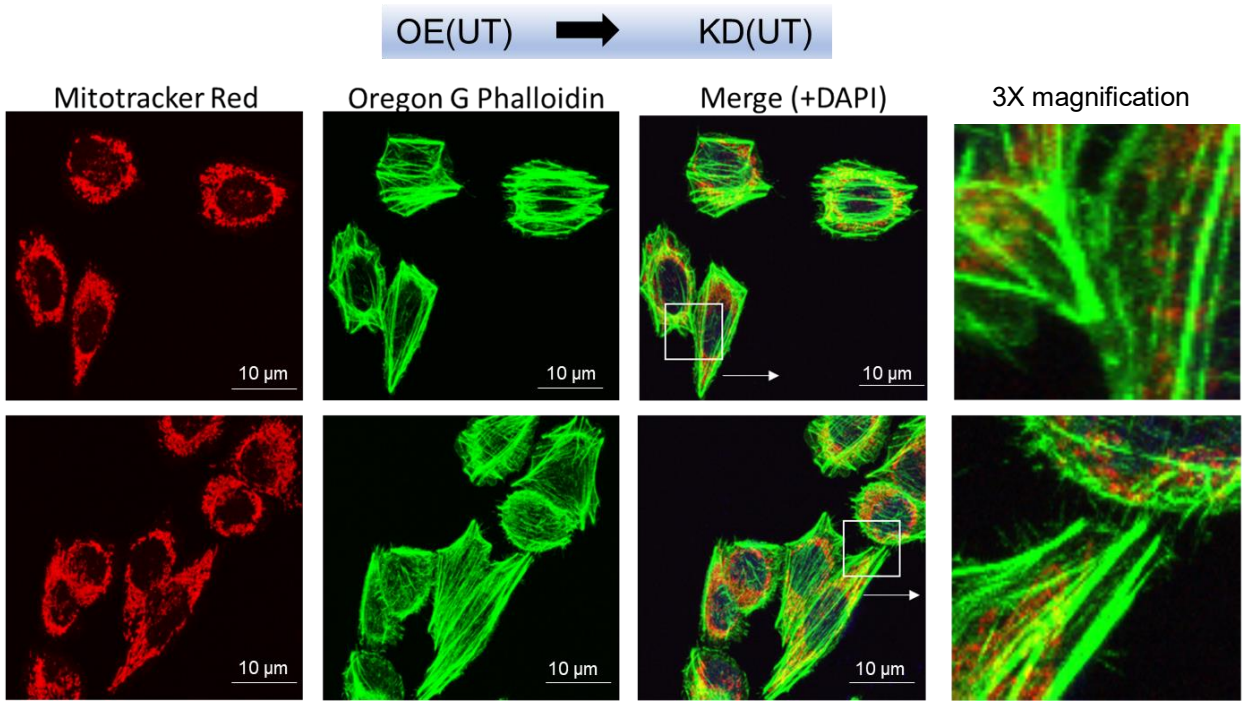


Figure 33: Confocal microscopy imaging analysis of TNT formation in overexpression and knockdown phenotype i.e., OE untreated (UT) co-cultured with KD untreated (UT) for mitochondria transfer.

Subsequently, both KD (UT) and OE (UT) cells were co-cultured and analyzed using confocal microscopy. It was observed that although few cells showed TNT-like structures no mitochondria transfer was observed suggesting that since, there was no stress-induced and both the cells were able to survive independently hence, this phenomenon of mitochondria transfer via TNT formation was not observed.

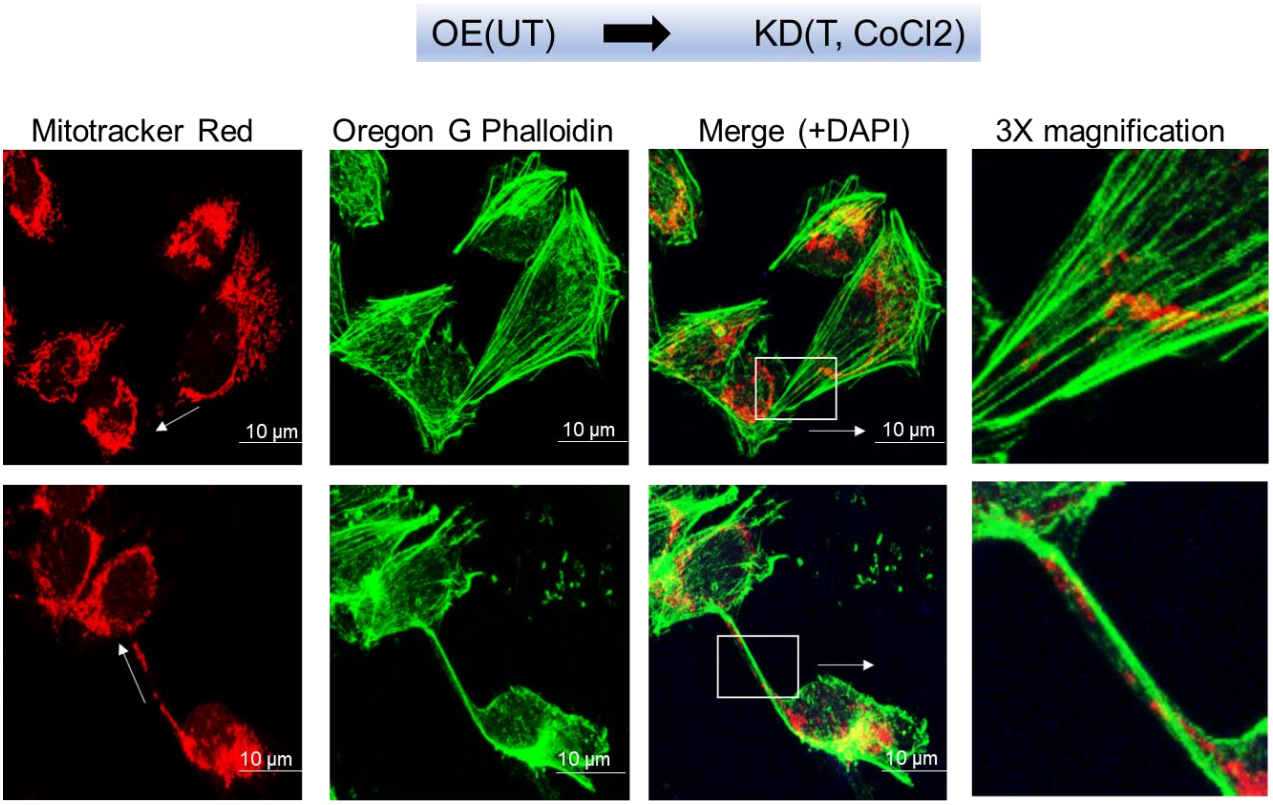


Figure 34: Confocal microscopy imaging analysis of TNT formation in overexpression and knockdown phenotype i.e., OE untreated (UT) co-cultured with KD treated (T) with cobalt chloride for mitochondria transfer

Later, the KD cells were treated with 200 nM cobalt chloride ($CoCl_2$) to induce chemical hypoxia and were co-cultured with OE(UT) cells and analyzed in confocal microscopy imaging. This experiment observed TNT formation, which also facilitated the mitochondria transfer as shown by white arrows in the above figure. Hence, it was speculated that stress may have induced the TNT formation in OE cells and resulted in mitochondria transfer.

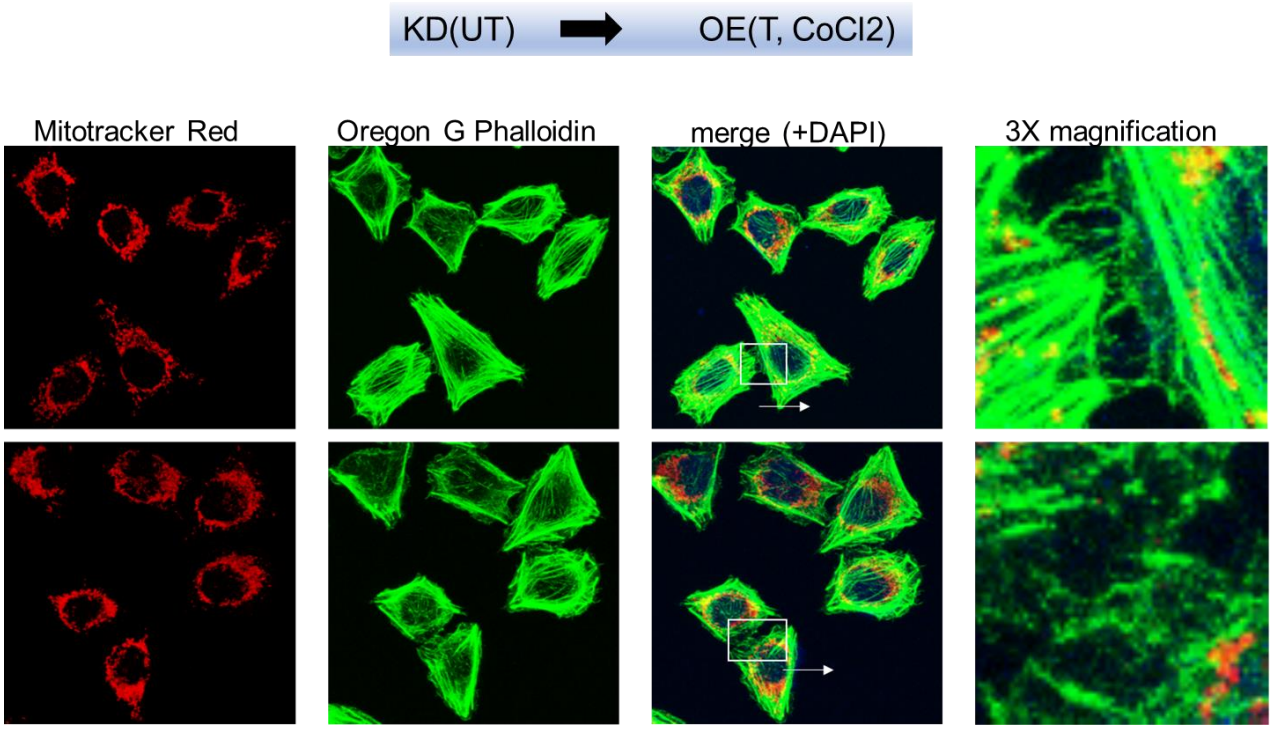


Figure 35: Confocal microscopy imaging analysis of TNT formation in overexpression and knockdown phenotype *i.e.,* KD untreated (UT) co-cultured with OE treated (T) with cobalt chloride for mitochondria transfer

Finally, to justify that OE cells can act as potential donors to facilitate mitochondria transfer, subsequently, the OE cells were given chemical hypoxic treatment and co-cultured with KD (UT) cells. It was concluded that since the OE cells were under hypoxic stress they couldn't favor the proper TNT formation as shown in the above figure and hence, failed to act as mitochondria donors.

5.1.5 Laser scanning confocal imaging analysis for extracellular vesicles transfer:

Laser scanning confocal microscopy revealed distinct mitochondrial dynamics between IMR-32 cells of PAR and OE phenotypes when incubated with conditioned media (CCM) from each other. After incubating IMR-32 PAR cells with the CCM of IMR-32 OE cells for 6 hours, MitoTracker red staining showed significant mitochondrial fission, indicating active mitochondrial division. Conversely, IMR-32 OE cells incubated in CCM of IMR-32 PAR cells exhibited signs of mitochondrial engorgement

and autophagy, as evidenced by changes in mitochondrial morphology and increased autophagic vesicles. Extending the incubation periods to 12 and 24 hours intensified these observations, with more pronounced mitochondrial fission in PAR cells and enhanced autophagy in OE cells

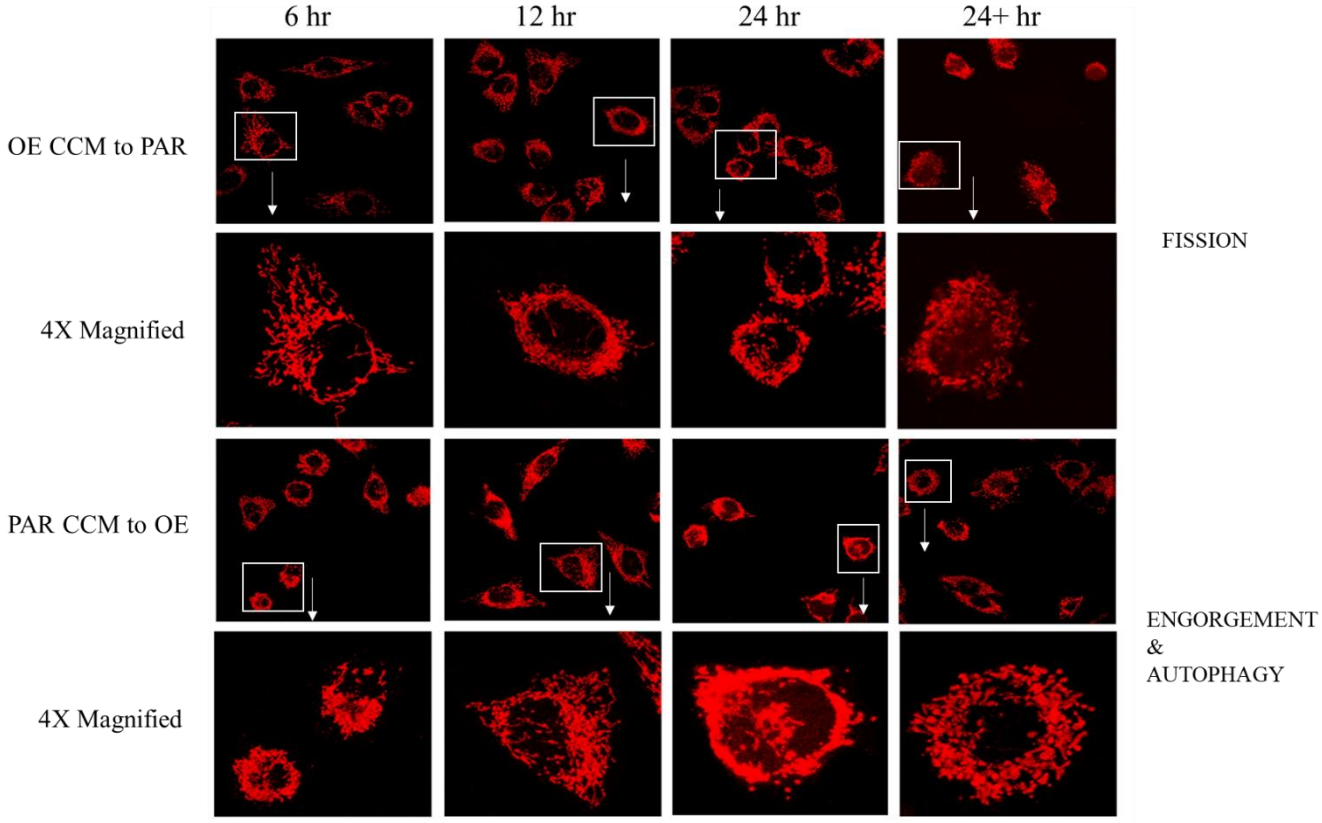


Figure 36: Confocal microscopy imaging analysis of extracellular vesicles transfer between IMR-32 cells of PAR and OE phenotypes when incubated with conditioned media (CCM) from each other

5.1.6 PROTEIN ESTIMATION OF MICROVESICLES & EXOSOMES:

The protein concentrations of exosomes (EXO) and microvesicles (MV) were measured in duplicates for each sample of PAR, KD, and OE.

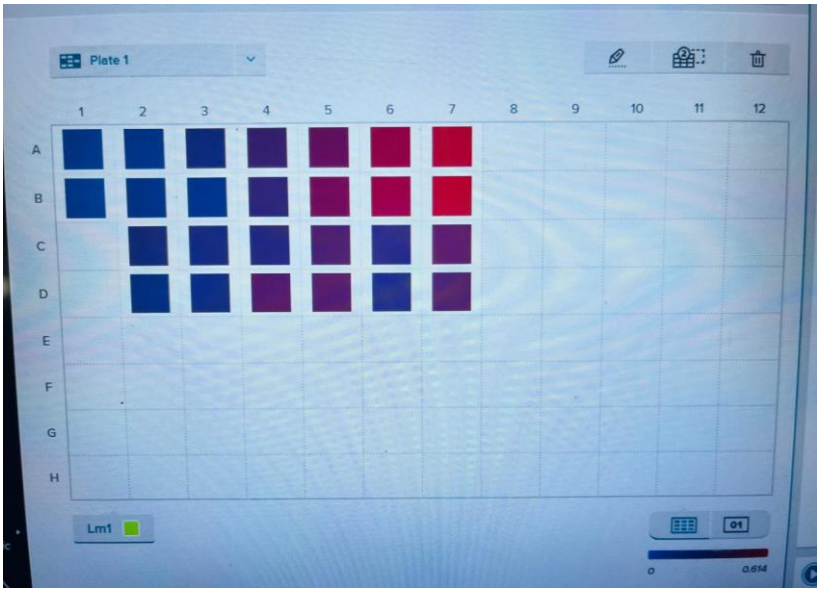


Figure 37: Visualization of protein content from low (blue) to high (red) protein expression levels in MVs and EXOs samples along with BSA standard levels at 562 nm.

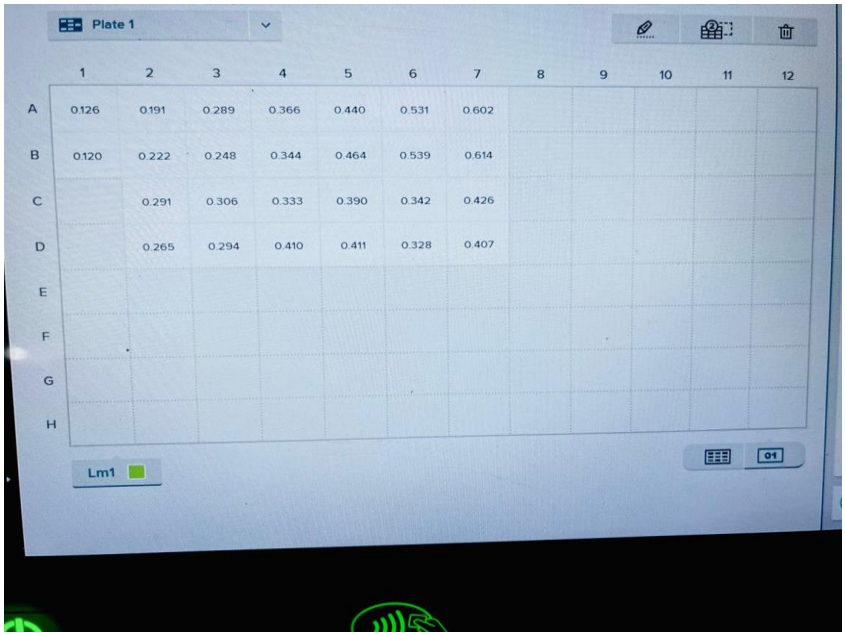


Figure 38: Visualization of protein concentration in MVs and EXOs samples along with BSA standards concentration at 562 nm.

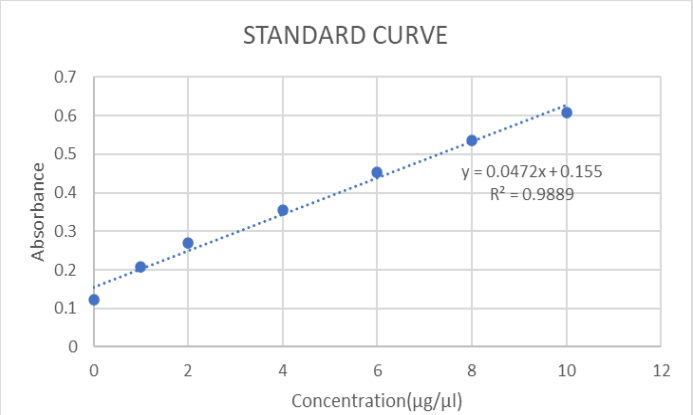
A

Conc.(ug/ul)	0	1	2	4	6	8	10
BSA(ul)	0	0.5	1	2	3	4	5
A+B(ul)	100	99.5	99	98	97	96	95
	0.126	0.191	0.289	0.366	0.44	0.531	0.602
	0.12	0.222	0.248	0.344	0.464	0.539	0.614
Average	0.123	0.2065	0.2685	0.355	0.452	0.535	0.608

B

Conc.(ug/5ul)	Absorbance
0	0.123
1	0.2065
2	0.2685
4	0.355
6	0.452
8	0.535
10	0.608

C



D

		MVs				EXOs	
		Absorbance				Absorbance	
	PAR	KD	OE		PAR	KD	OE
	0.291	0.306	0.333		0.39	0.342	0.426
	0.265	0.294	0.41		0.411	0.328	0.407
Average	0.278	0.3	0.3715		0.4005	0.335	0.4165
		Concentration [y=mx+c]				Concentration [y=mx+c]	
TOTAL per µl	2.60µg/µl	3.07µg/µl	4.58µg/µl	TOTAL per µl	5.20µg/µl	3.81µg/µl	5.54µg/µl
90ul	234µg	276.3µg	412.2µg	190ul	988µg	723.9µg	1052.6µg
	0.234mg	0.2763mg	0.4122mg		0.988mg	0.7239mg	1.0526mg

Figure 39: BCA assay for protein estimation of MVs and EXOs samples A. represents the absorbance values for BSA (Bovine Serum Albumin) standard from 0-10µg/µl protein concentration B. table illustration of absorbance of BSA standard at respective concentration (µg/5 µl) C. illustrates the standard curve generated from known concentrations of BSA standards using the BCA assay. Absorbance values (y-axis) are plotted against known concentrations of BSA (x-axis). A linear regression(R) line is fitted to the data points, allowing for the calculation of protein concentrations D. represents the absorbance values and protein concentration of MVs & EXOs samples in all 3 phenotypes i.e., PAR, KD & OE for 90 µl and 190 µl volumes respectively.

5.1.7 COMPLEMENTARY RESULTS:

The PCR analysis confirmed the presence of the *ND1* gene in exosome (EXOs) and microvesicle (MVs) samples, demonstrating that mitochondrial DNA is a component of these extracellular vesicles. The consistent detection across samples highlights the implications for their functional roles in intercellular communication and potential diagnostic applications.

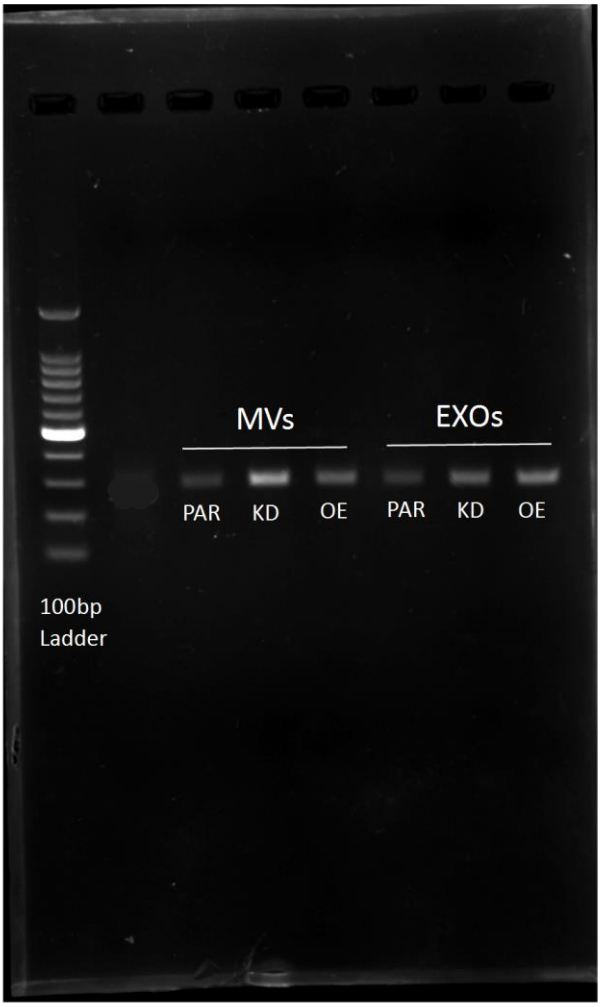


Figure 40: Gel Doc image for ND1 gene in MVs and EXOs samples

5.2 DISCUSSION:

Cancer cells exploit diverse communication mechanisms to sustain their survival and proliferation within the tumor microenvironment, with direct organelle exchange emerging as an essential mode of intercellular crosstalk. We chose human neuroblastoma cells as a model system since these cells exhibit high metabolic demand to meet their requirements. Among cellular organelles, mitochondria play a central role in metabolic cooperation and adaptation. We observed mitochondria organization in all the three phenotypes and observed that KD cells show a smaller number of mitochondria due to fusion mitochondria whereas OE cells show higher number and fission mitochondria which also relates with our previous findings. Heat shock proteins (Hsps) specifically Hsp90 which is also known as cancer chaperone, regulates cellular proteostasis and have been implicated in cancer progression. Elevated levels of Hsps, including TRAP-1, are frequently observed in cancer and are associated with aggressive phenotypes and therapeutic resistance. TRAP-1, a mitochondrial homologue of the Hsp90, influences various cellular processes, including apoptosis, metabolism, and signaling pathways, thereby promoting tumorigenesis. Hence, we speculated TRAP-1's role in facilitating mitochondria transfer between cancer cells. The transfer of functional mitochondria between cancer cells occurs through various mechanisms, including tunneling nanotubes (TNTs), dynamic membranous structures enriched with F-actin. Therefore, we looked at cytoskeletal architecture of all the different phenotypes by staining F-actin and observed that in TRAP-1 compromised cells there is less actin polymerization on the other hand, OE cells show high actin bundling and extended filopodia structures which reiterates the role of TRAP-1 in regulating cytoarchitecture. TNTs facilitate long-range intercellular communication, enabling the exchange of mitochondria and other cellular components. So, we performed co-culture experiments to examine the phenomenon of mitochondria transfer between all the three phenotypes. It was observed that PAR cells were

able to facilitate this phenomenon under stress but KD cells were not able to form TNT structures and hence, failed to transfer mitochondria. Subsequently, when OE cells were co-cultured with KD cells under stress it was observed that OE cells were able to form TNT and mitochondria transfer was observed. In agreement with this, OE cells having smaller and higher number of mitochondria and also, high F-actin bundling can act as potential donors to regulate the phenomenon of mitochondria transfer. We correlated TRAP-1, may modulate the formation and functionality of TNTs, potentially enhancing the intercellular transfer of mitochondria within the tumor microenvironment. In the context of understanding the interplay between TRAP-1, TNT-mediated mitochondria transfer, and cancer progression, elucidating novel therapeutic strategies targeting intercellular communication mechanisms in cancer, our study gained importance.

CHAPTER 6

FUTURE DIRECTIONS

Several promising avenues for research on the role of TRAP-1 in mitochondrial transfer between cancer cells present themselves. Firstly, there's a need for a deeper mechanistic elucidation of how TRAP-1 precisely facilitates mitochondrial transfer, including its interactions with other proteins or signalling pathways governing mitochondrial dynamics. Exploring the clinical implications of TRAP-1-mediated mitochondrial transfer in cancer progression and therapy resistance holds significant potential. Investigating the influence of the tumour microenvironment on TRAP-1 expression and mitochondrial transfer, along with its impact on metabolic reprogramming, could provide further insights. *In vivo,* studies using relevant animal models or patient samples are essential for validating findings from in vitro experiments and understanding the complexities of the tumour microenvironment. Moreover, therapeutic targeting of TRAP-1 or mitochondrial transfer warrants exploration in terms of efficacy and safety, possibly in combination with existing cancer therapies. Biomarker discovery, multi-omics approaches, crosstalk with other cellular processes, and the integration of emerging technologies are all avenues that can enrich our understanding of TRAP-1-mediated mitochondrial dynamics in cancer cells.

CHAPTER 7

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