TRAP1: MITOCHONDRIAL HOMOLOG OF CANCER CHAPERONE, HSP90 IN REGULATING MITOCHONDRIAL FISSION

M.Sc. Thesis
2016

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

For the partial fulfillment of the requirement for the
Master of Science in Biotechnology

Mitesh Shrestha
Roll No.: BT 109/069
T.U. Regd No.: 6-3-37-1128-2012

TRAP1: MITOCHONDRIAL HOMOLOG OF CANCER CHAPERONE, HSP90 IN REGULATING MITOCHONDRIAL FISSION

M.Sc. Thesis

2016

Submitted to

Central Department of Biotechnology
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

Submitted by

Mitesh Shrestha
Roll No.: BT 109/069
T.U. Regd No.: 6-3-37-1128-2012

Supervisor

Prof. Dr. Tilak R. Shrestha
Central Department of Biotechnology
Tribhuvan University

External Supervisor

Dr. Amere Subbarao Sreedhar
Scientist
Centre for Cellular and Molecular Biology

सी सी एम बी CCMB
सी.एस.आई.आर - कोशिकीय एवं आणविक जीवविज्ञान केन्द्र
CSIR - CENTRE FOR CELLULAR AND MOLECULAR BIOLOGY
उप्पल रोड, हैदराबाद - 500007 भारत
Uppal Road, Hyderabad - 500007, India.

CERTIFICATE

This is to certify that this dissertation entitled "**TRAP1: mitochondrial homolog of cancer chaperone, Hsp90 in regulating mitochondrial fission**" has been carried out by ***Mr. Mitesh Shrestha*** in my laboratory at the Centre for Cellular and Molecular Biology, Hyderabad, India in partial fulfillment for the degree of Master of Science in Biotechnology from the Central Department of Biotechnology, Tribhuvan University, Nepal. This work is original and has not been submitted in part or full to any other university or institute for any other degree or diploma.

A S Sreedhar

Supervisor

Dr. Amere Subbarao Sreedhar

Dr. A.S. Sreedhar, M.Sc., Ph.D.
Scientist
Centre for Cellular and Molecular Biology
Hyderabad - 500 007

फैक्स अंतर्राष्ट्रीय +91-40-27160591, 27160311 दूरभाष +91-40-27160222-41 तार बायासेन्टर
भारत 040-27160591, 27160311 Telephone
Fax International +91-40-27160591, 27160311 वेब साइट http://www.ccmb.res.in Telegram BIOCENTRE
India 040-27160591, 27160311 Website

ABSTRACT

Cancer is a polygenic disease and emerges from the deregulation of both genetic and epigenetic mechanisms. Two hallmarks of cancer, deregulating cellular energetics and evading apoptosis can be directly associated with mitochondria since mitochondria remain the hot spot for both. Mitochondria exist in a dynamic state between 'fusion' and 'fission' and these states regulate several cellular functions including ATP production. High molecular weight heat shock protein, Hsp90 is identified as a 'cancer chaperone' due to its involvement in regulating the functions of several cancer related signal transduction molecules. However, its mitochondrial homologue, TRAP1 does not show cancer associated molecular interactions like Hsp90, but its enhanced expression in cancer cells did correlate with disease progression. To understand TRAP1 involvement in regulating the mitochondrial dynamics between fusion and fission, we examined TRAP1 over expression in comparison with knockdown. We found that TRAP1 over expression does not kill tumor cells, but induces mitochondrial fission-like structural re-organization. To assess whether or not these structural alterations are comparable with fission, we cloned and over expressed fission genes, *Fis1*, *Drp1*, and *Mff* in normal (SRA01 and HEK293T) and metastatic cancer cells (IMR-32) and examined for cell and mitochondrial morphology. TRAP1 over expression alone showed enhanced fission compared to individual fission protein expression suggesting that one or all of the fission proteins requires TRAP1 for their regulation. Tumor cells showed more sensitivity to TRAP1 over expression compared to normal cells indicating tumor selective functions. Preliminary studies with protein-protein interaction suggested that TRAP1 interacts with these proteins however subsequent experiments need to be performed for further confirmation with respect to type of fission protein and mapping of the interacting region.

Keywords: Cancer, Mitochondrial dynamics, Heat shock proteins, Hsp90, TRAP1, Mitochondrial fission proteins

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is an abnormal growth of cells caused by mutations leading to multiple changes in gene expression resulting in deregulated cell proliferation and cell death mechanisms. Accumulation of such deregulated mechanisms ultimately evolves into metastatic and drug resistant cancers (Ruddon, 2007). Cancer does not arise from a single mutation but due to accumulation of several mutations in association with epigenetic alterations; hence, it is called as a polygenic disease resulting from both genetic (sporadic) and epigenetic deregulatory mechanisms (Bertram, 2001).

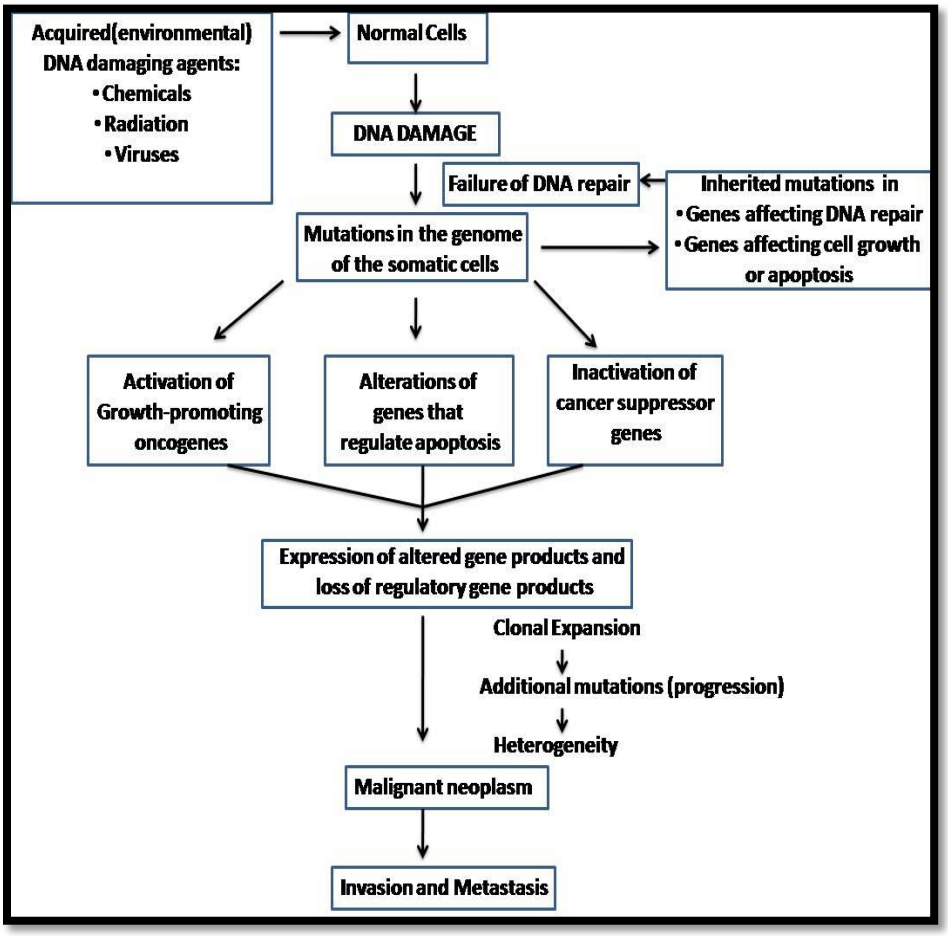

Figure 1.1 Cellular Transformation of normal cells into metastatic cells [Modified from Diaz-Cano, 2015].

1.2 Hallmarks of cancer

In addition to genetic mutations, alterations in the signal regulatory mechanisms (signaling pathways) decide the outcome and fate of cancer. After identifying signal transduction pathways as signatures of cancer, these mechanisms are considered as hallmarks of cancer (Hanahan and Weinberg, 2000; 2011).

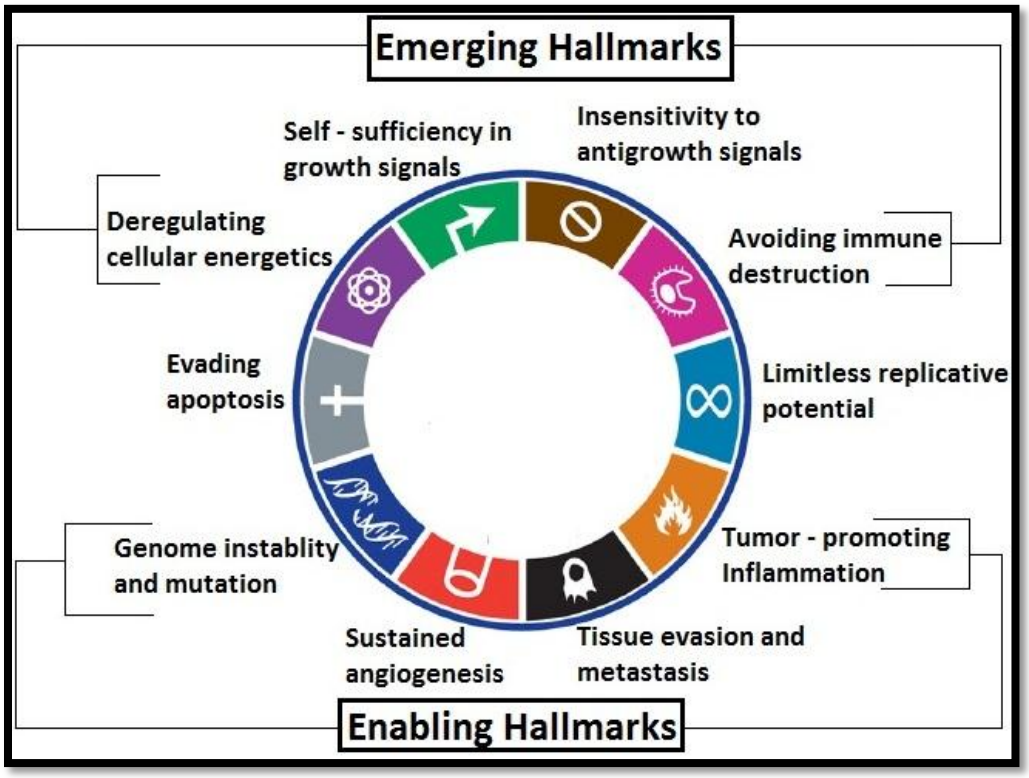

Figure 1.2 Hallmarks of Cancer [Adapted from Hanahan and Weinberg, 2011].

In addition to the emerging and enabling hallmarks, cancer growth majorly depends on cellular energy which actually acts as a driving force of cells and decides the fate of cancer. Mitochondria being at the center of cellular energy, it would be appropriate to examine the contribution of mitochondria in cancer progression and pathogenesis.

1.3 Cancer and mitochondria

Two hallmarks of cancer, deregulating cellular energetics and evading apoptosis can be directly associated with the mitochondria (Hanahan and Weinberg, 2011). The mitochondria in cancer cells were initially thought to be dysfunctional as per the discovery by Otto Warburg (1956) as because, higher amounts of lactate was produced in cancer cells even

under aerobic conditions. After more than half a century, it has been rediscovered that the mitochondria can still remain functional, however with a shift between glycolysis and oxidative phosphorylation. Several studies have now shown that this bioenergetic switch which occurs in many of the cancer cells results due to shift from high ATP production by OXPHOS to the process of energetically balanced production of substrates required for cellular biogenesis and reproduction. Alterations in enzymes such as succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) bring about the alterations in mitochondrial bioenergetics and metabolism mainly in OXPHOS and also initiate a cascade of events which not only initiates but also helps in sustaining the transformed state of cancer cells. (Wallace, 2012; Rehman et al., 2012).

1.4 Bioenergetics of normal and cancer cells

Tumor cells are able to thrive even in hostile environments where the normal cells would succumb to death because of selection of metabolic pathways that can generate sufficient ATP as well as the raw materials required for the maintenance of the cancer cells (Amoedo et al., 2013). Cancer cells tend to undergo aerobic glycolysis even under high oxygen environment whereas normal cells opt for ATP generation only through oxidative phosphorylation (Warburg, 1956). Besides glycolysis, it has also been observed that cancer cells use glutamine as source of energy (Amoedo et al., 2013). At present, the bioenergetic reprogramming has emerged as one of the hallmarks of cancer cells (Hanahan and Weinberg, 2011).

1.5 Mitochondrial dynamics and TRAP1

Mitochondria are static organelles; however exhibit dynamic transition between fusion and fission (Chen and Chan, 2004). The role of Tumor Necrosis Factor Receptor Associated Protein 1 (TRAP1) in the maintenance of mitochondrial dynamics (Takamura et al., 2012) has now emerged as a novel phenomenon which however, requires a thorough understanding.

TRAP1 has been shown to protect cancer cells from oxidative stress and apoptosis (Gesualdi et al., 2007). Differential expression of TRAP1 has been shown to be present in cancer cells in comparison to normal matched cells indicating specialized (Kang et al., 2007; Leav et al.,

2010). Further, TRAP1 expression correlated with cell motility and metastatic spread (Liu et al., 2010).

1.6 Rationale of the study

Since Hsp90 chaperone is pro-survival and indispensable for cell survival, the study gains importance with respect to examining its homologue, TRAP1 in the mitochondria. Despite showing chaperoning functions *in vitro*, TRAP1 does not have any potential for protein-protein interaction. However, TRAP1 is abundant in mitochondria that had raised interest in understanding the functional significance of this protein at the mitochondrial organization if not regulating cell fate. Therefore, the present study is aimed at understanding TRAP1 functions in regulating mitochondrial dynamics.

1.7 Objectives:

1.7.1 General Objectives

- To understand the TRAP1 functions in regulating the mitochondrial dynamics in normal and tumor cells

1.7.2 Specific Objectives

- Construction of TRAP1 mammalian expression system by sub-cloning TRAP1.
- Construction of fission gene mammalian expression system by sub - cloning Fis1 and cloning Drp1, and Mff.
- Transfection of TRAP1 and Fission genes into normal and tumor cells and examine cellular morphology and mitochondrial dynamics.

1.8 Research Hypothesis:

Null Hypothesis: Mitochondrial dynamics do not get affected by the overexpression of mitochondrial homolog of Hsp90, TRAP1.

Alternate Hypothesis: Mitochondrial dynamics get affected by the overexpression of mitochondrial homolog of hsp90, TRAP1.

CHAPTER 2

LITERATURE REVIEW

2.1 Stress

Cells must persistently adapt to the changes that occur in their exogenous and endogenous environment to maintain their structural and functional integrity for survival. The alterations could be in terms of physical parameters that include temperature, mechanical damage, osmotic pressure, exposure to harmful radiations, heavy metals and chemical signals such as ion concentrations, pH, oxygen tension, redox potential, metabolite accumulation or glucose starvation and disturbance of Ca^{2+} homeostasis. Besides these, the extracellular signals which include contact dependent signals, hormonal changes, cytokine levels, neural messengers or neurotransmitters, and infections as well as mental stress are also considered to play a vital role for proper cell functioning and survival (Csermely et al., 1998; Fulda et al., 2010; Kroemer et al., 2010; Kourtis and Tavernarakis, 2011). These changes in the cellular environment which disturb cellular homeostasis can be considered as stress for the cell. Whether it is extracellular or intracellular stress, stress in any form usually results in damaging of the cells unless and until they are equipped with the cytoprotective mechanisms such as heat shock/stress protein production. The production of these proteins is tightly regulated at the molecular level and is termed as "stress response". Hence, stress may be the product of any physical, chemical or biological changes which disrupts the homeostasis. Stress results in the initiation of a chain of cellular and systemic events which helps the cells to restore their homeostatic balance. (Kourtis and Tavernarakis, 2011).

2.2 Stress response and heat shock proteins (Hsps)

The Italian geneticist, Ritossa (1962), made a scrupulous observation of the change in the puffing pattern of the polytene chromosomes present in the salivary glands of *Drosophila busckii* with elevated temperature. This puffing of the chromosomes was subjected with limited parts of the chromosomes and thought to have cytoprotective roles (Ritossa, 1962) and is attributed to stress response. The stress response is therefore always associated with the transactivation of new set of genes, called as heat shock proteins (Hsps) (Lindquist and Craig, 1988; Ritossa, 1996). These proteins were initially termed as Hsps (Tissieres et al., 1974) since they were identified in response to heat stress, however Hsps can be induced by a diverse varieties of stimuli. Later it was found that Hsps are expressed in normal cells too and are highly

conserved and ubiquitously expressed in all cells and tissuses (Hartl, 1996), and that their expression is induced in response to stress stimuli (Benjamin and Mcmillan, 1998) although they also perform essential functions under normal physiological conditions. The stress response is highly conserved between prokaryotes and eukaryotes (Koga et al., 1999; Alexieva et al., 2001; Mary et al., 2003; Alsbury et al., 2004).

The basic function of Hsps is found to be chaperoning other proteins, hence they are also termed as "molecular chaperones". Chaperones interact with other proteins to help in the maintenance of cellular protein homeostasis (Hightower et al., 1994), especially helping native polypeptides to maintain in folding competent state, and refolding of denatured or damaged proteins to their native conformation under normal physiological conditions. Advanced/specialized functions of Hsps in subsequent years have identified that they even promote conformational stabilization and functional maturation of mutated gene products (Lindquist and Craig, 1988; Feder and Hoffman, 1999).

Mitochondrion is considered as a cell inside of a cell due to its ability to transcribe and translate its own metabolizing enzymes/proteins. Since mitochondria are thought to have emerged from the endosymbiosis thousand years ago, mitochondria related stress proteins were also thought to be present in bacteria from which they arise (Wheeler and Wong, 2007). Due to subsequent discovery of several homologues and analogues of Hsps in the cytosol (Hartl, 1996) and intracellular milieu, for convenience, Hsps are classified into families based on their molecular weights. These are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp10 and small Hsp families (Gething and Sambrook, 1992). If not all, some of the cytosolic Hsp analogues are present in mitochondria and are probably involved in performing similar functions.

Table 2.1 Heat shock protein families. [Modified and Compiled from Benjamin and Mcmillan, 1998; Wheeler and Wong, 2007; Altieri et al., 2012].

Hsp	size (kDa)	localization	co-chaperones	cellular functions
Hsp110	110	nucleolus, cytoplasm	Hsp40	protects nucleoli from stress; thermo tolerance
Hsp90	90	nucleus, cytoplasm	cdc37, p23	regulation of steroid hormone activity
Hsp75/TRAP1	80	mitochondria	?	protection against oxidative stress
Hsp70	72	cytosol, nucleus	Hsp40	highly stress inducible, cytoprotective
Hsc70	70	cytosol, nucleus	?	constitutively expressed chaperone
Hsp60	60	mitochondria	Hsp10	protein import and folding
Hsp47	47	cytosol	?	chaperone for procollagen
Hsp40	40	cytoplasm	-	?
Small Hsps				
Hsp32 (heme oxygenase)	32	ER, cytoplasm	-	antioxidative
Hsp27	27	cytosol, nucleus	-	Stabilization of cytoskeleton, cytoprotection, antioxidative
Hsp22 (αβ crystalline)	22	cytoplasm	-	Cytoskeletal stabilization, antioxidative

2.3 Heat shock protein 90

Among different Hsps, Hsp90 belongs to an ATPase/Kinase GHKL (Gyrase, Hsp90, Histidine Kinase and MutL) superfamily, whose members have been identified with unique attribute of having ATP binding cleft (Picard, 2002; Pearl and Prodromou, 2006). When compared to the total cellular proteins, Hsp90 contributes to 1 -2 % of the soluble protein (Lai et al., 1984), which is apart from its inducibility (Akner et al., 1992). Like any other chaperone, Hsp90 also has the ability to interact with other proteins either alone or taking help from the co-chaperones to facilitate assembly, stabilization, conformational maturation, activation and regulation of crucial signaling proteins such as protein kinases, hormone receptors, and transcription factors, and structural proteins such as actin and tubulin (Pearl and Prodromou, 2006). If Hsp90 interaction with the target protein is for folding or refolding, the target protein is termed as a "substrate". Similarly, if Hsp90 interaction with the target protein is for its conformational maturation and functional stabilization, the target is called as a "client". While all the proteins in a cell can be potential substrates of Hsp90, there are more than 300 client

proteins identified for Hsp90 (Li et al., 2012; http://www.picard.ch/downloads/Hsp90interactors.pdf; Whitesell and Lindquist, 2005; Trepel et al., 2010).

In eukaryotic cytosol Hsp90 exists in two forms, Hsp90α (inducible form) and Hsp90β (constitutive form). Its homologs in the endoplasmic reticulum (ER) are called Grp94 and in the mitochondria are called Trap1 (Csermely et al., 1998; Felts et al., 2000; Shiu et al., 1977). Since mitochondria are thought to have evolved from the bacteria, Trap1 must be similar to bacterial counterpart of Hsp90. The bacterial/prokaryotic Hsp90 is named HtpG (High Temperature Protein Gene) (Sato et al., 2010). Unlike Hsp90 in the eukaryotic system, HtpG expressions under normal physiological conditions are dispensable for survival (Bardwell and Craig, 1988). However, subsequent studies have indicated that HtpG play a role in cytoprotection under stress conditions, which needs further understanding (Hossain and Nakamoto, 2003).

Interestingly, both Hsp90 and HtpG exist as phospho-dimers and exhibit chaperoning activity *in vitro* (Iannotti et al., 1988; Wiech et al., 1992). The monomeric form of Hsp90 constitutes three major domains, N-terminal ATP binding domain (N- domain), middle domain (M-domain), and C-terminal dimerization domain (C-domain) containing the MEEVD motif (a tetratricopeptide repeat (TPR)) that helps in facilitating the protein-protein interactions. The N-terminal domain and the middle domain are joined by a highly charged hinge region (Li et al., 2012), which is thought to be involved in client protein interaction (Morra et al., 2012). However, Trap1 lacks this charged domain (Felts et al., 2000). Like any other chaperone, the N-terminus of Hsp90 also contains ATPase activity that helps in substrate binding and release in the chaperone cycle (Prodromou et al., 2000)

Accordingly, the endoplasmic reticulum homolog of Hsp90, Glucose-regulated protein 94 (GRP94) is present in its lumen and therefore chaperones secreted and membrane proteins. It plays vital role in development and physiology of multicellular organisms. GRP94 portrays domain organization similar to that of Hsp90 with certain unique features such as calcium binding, appropriated due to its localization in the endoplasmic reticulum. (Marzec et al., 2012).

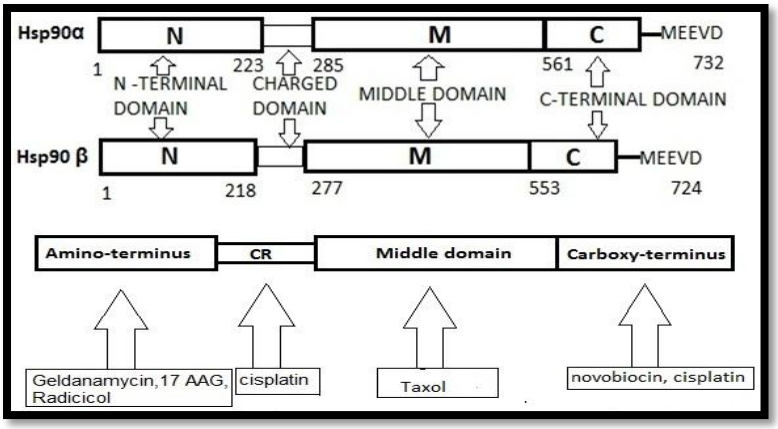

Figure 2.1 Domain organization of human Hsp90. Different drug binding sites on Hsp90 are also shown. The numbering refers to the amino acid sequence. CR – Charged Region. NLS – Nuclear Localization Signal. [Adapted from Sreedhar et al., 2004; Mollapour and Neckers, 2012].

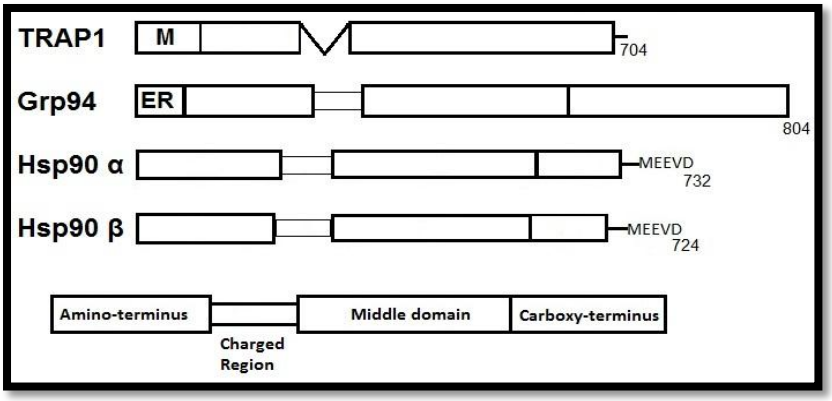

Figure 2.2 Comparison of domain organization of different isoforms of Hsp90 [Adapted from Felts et al., 2010; Gorza and Vitadello, 2012; Mollapour and Neckers, 2012].

Table 2.2 Hsp90 isoforms: localization, expression and functions.

Isoform	Localization	Expression	Functions
Hsp90 α	cytoplasm	Inducible	cytoprotection, cell cycle regulation, growth promotion, regulation of cell differentiation
Hsp90 β	cytoplasm	constitutive/inducible	early embryonic development, Long term cellular adaptation, cytoplasmic organization germ cell maturation
TRAP1	mitochondria	constitutive/inducible	cytoprotection against oxidative stress
GRP94	endoplasmic reticulum	constitutive/inducible	early embryonic development, quality control for proteins in the endoplasmic reticulum

2.4 TRAP1

Tumor Necrosis Factor Receptor-Associated Protein 1 (TRAP1), a member of Hsp90 family gained importance when it was found associated with the cytoplasmic domain of type I Tumor Necrosis Factor Receptor-1(TNFR1) in a yeast two-hybrid screening system (Song et al., 1995). In an independent study using the same model system, another protein was found to be translocated into the nucleus under heat shock where it was bound with the simian virus 40 T-antigen-binding domain of hypophosphorylated retinoblastoma using its unique LxCxE motif and was named Hsp75 (Chen et al., 1996). However, later sequence analysis showed that both proteins were identical.

TRAP1, due to the presence of mitochondrial localization signal at its N-terminal end is primarily a mitochondrial matrix residing protein (Felts et al., 2000). However, studies have shown that they can be present at specific extra-mitochondrial sites (Cechetto and Gupta, 2000). Although the functional aspect of non-mitochondrial localizations of TRAP1 is less characterized, a recent study has shown that TRAP1 interacts with TBP7 and regulates quality control of proteins destined to mitochondria (Amoroso et al., 2012).

TRAP1 has high sequence homology with members of Hsp90 protein family (Song et al., 1995) and forms a tight homodimer with significant increase in its ATPase activity upon heat shock condition (Leskovar et al., 2008). Due to this structural similarity with Hsp90, especially conserved in the N-terminal ATP binding region, TRAP1-ATPase activity can be inhibited by the Hsp90 inhibitors like geldanamycin and radicicol. In contrast, TRAP1 cannot bind with the Hsp90 co-chaperones and facilitate conformational maturation of progesterone receptor (Felts et al., 2000). Besides these, the TRAP1 structure also lacks the hinge region found in the Hsp90 (Song et al., 1995).

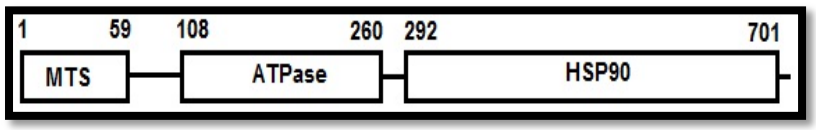

Figure 2.3 Domain organization of human TRAP1. The numbering refers to the amino acid sequence. MTS – Mitochondrial Targeting Sequence. [Adapted from Matassa et al., 2012].

TRAP1 has been shown to protect cancer cells from oxidative stress and apoptosis (Gesualdi et al., 2007). The TRAP1 level has been found to be higher in the mitochondria of breast, lung, colon, and pancreas and prostate cancer cells when compared to normal matched cells (Kang et al., 2007; Leav et al., 2010) indicating specialized functions. A microarray study suggested that TRAP1 can affect cell cycle too. Further, TRAP1 expression correlated with cell motility and metastatic spread (Liu et al., 2010).

2.5 Mitochondria

Mitochondria are often called as the power house of the cell due to their ability to produce ATP, the currency of the cell. They are semi-autonomous membrane bound organelles that are present in most of the eukaryotic cells. The size of the mitochondria can vary depending on the cell physiology (Kennady et al., 2004). Mitochondria are dynamic organelles, constantly undergoing fusion or fission processes, resulting in varying shapes like small spheres, long tubules or interconnected tubules (Chen and Chan, 2004), which otherwise also influences the mitochondrial energetics.

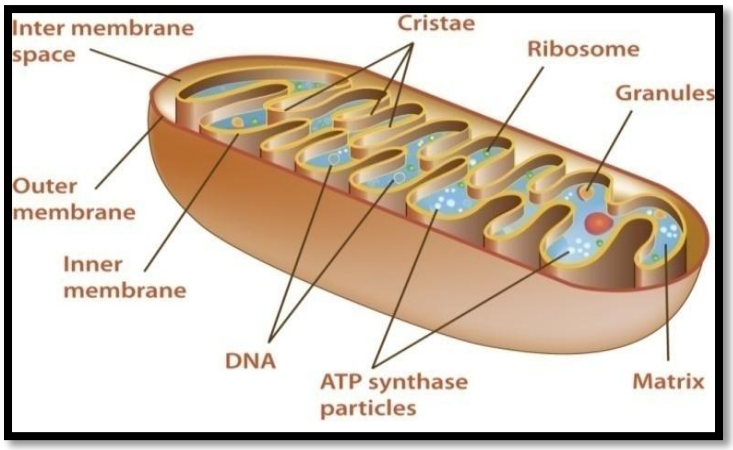

Figure 2.4 Mitochondria structure.

The human mitochondrial genome is a double stranded, circular consisting of 16,569 base pairs that code for 37 genes involved in mitochondrial oxidative phosphorylation (OXPHOS). These genes code for two rRNAs, 22 tRNAs and 13 polypeptides. OXPHOS system present in the mitochondria utilizes all the 13 polypeptides encoded by the mtDNA (Taanman, 1998).

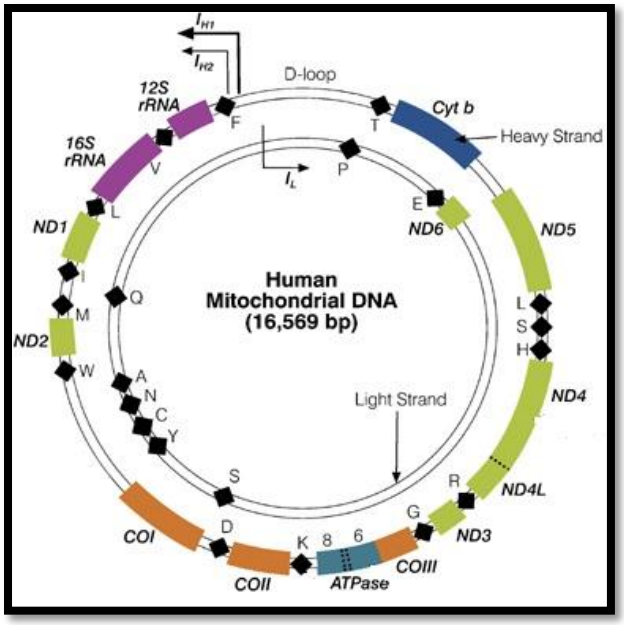

Figure 2.5 The Map of human mitochondria genome. I_{H1},I_{H2} – Promoter site in heavy strand, I_L – Promoter site in light strand; D-loop : Displacement loop; ND1 – 6 encoding members of NADH:ubiquinone oxido-reductase; Cytb encoding apocytochrome b of ubiquinol: cytochrome c oxido-reductase; Co I-III encoding members of cytochrome c oxidase; ATPase 6,8 encoding two members of F_0-F_1 ATP synthase. Single letter code is given for each mt-tRNA-encoding gene. [Adapted from Kyriakouli et al., 2008].

2.6 Mitochondrial dynamics

Mitochondrial dynamics deals with understanding the dynamic behavior of mitochondria in living cells. The shape, size and number of mitochondria in a cell are controlled by fusion or fission events. These actions also result in changes in morphology of mitochondria, their motility in the cytosol as well as their distribution during cell division. Recent advances in the visualization techniques have helped to study these dynamics in more detail (Scott and Logan, 2011). The dynamic nature of mitochondria can be attributed for maintaining the integrity of mitochondria, their physiological associations, and their number present in the cell as well as for the rejuvenation of mt-DNA (Berman et al., 2008). Mitochondrial fusion leads to elongated mitochondria especially found in metabolically active cells where they help in generation of energy (Skulachev, 2001). On the contrary, mitochondrial fission leads to numerous morphologically and functionally distinct small spheres and short rods, characteristic of quiescent cells (Collins et al., 2002). The mitochondrial dynamics i.e. both the fusion and fission events are regulated by dynamin related GTPases (Hoppins et al., 2007).

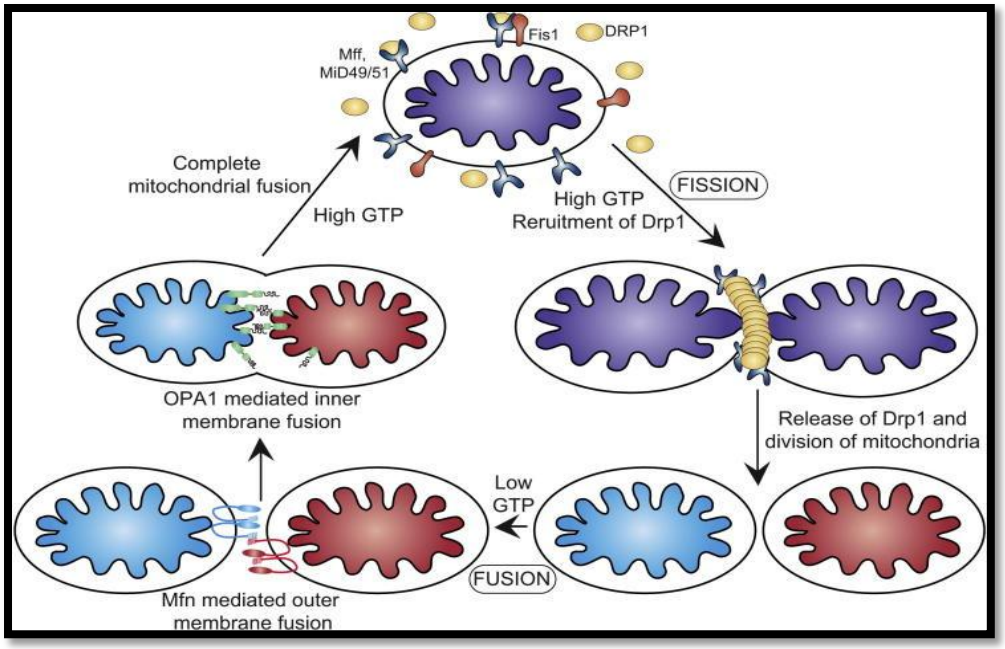

Figure 2.6 Schematic representations of mitochondrial dynamics. [Adapted from Osellame et al., 2012].

Table 2.3 Proteins regulating mitochondrial dynamics in mammals [Adapted from Westermann, 2010].

Components of fusion machinery	**Genes/proteins**	**Function**
Mitofusins	Mfn1 and Mfn2	outer membrane fusion
Optic Atrophy Protein 1	Opa1	inner membrane fusion
Components of fission machinery	**Genes/proteins**	**Function**
Dynamin related protein 1	Drp1	outer membrane division
Mitochondrial fission 1	Fis1	outer membrane receptor for Drp1
Mitochondria Fission Factor	Mff	putative alternative receptor for recruitment of Drp1

2.6.1 Mitochondrial fusion machinery

In mammals, the mitochondrial fusion is carried out by two highly conserved dynamin – related GTPases, Mitofusins (Mfn1 and 2) and Optic Atrophy Protein 1 (Opa1). Mitofusins are involved

in the fusion of outer mitochondrial membrane while Opa1 is involved in the fusion of inner mitochondrial membrane (Hoppins et al., 2007; Westermann, 2010).

2.6.1.1 Mitofusins (Mfn)

Mitofusins (Mfn1 and Mfn2) are large GTPases of approximately 90 kDa molecular weight ubiquitously present in the mitochondria (Rojo et al., 2002). They are the human homolog of *Drosophila* Fzo GTPase and *Saccharomyces cerevisiae* yFzo1p. The two isoforms are differentially expressed amongst the different tissues with Mfn1 expression being significantly higher in the heart tissues whereas, the transcript level of Mfn2 are higher in the heart and muscle tissues but lower in other tissues (Santel et al., 2003). Similarly, Mfn1 has higher capacity to induce mitochondrial fusion when compared to Mfn2 seemingly due to its higher GTPase activity (Ishihara et al., 2004). The domain organization of mitofusins include N-terminal GTPase domain facing towards the cytoplasm, two hydrophobic heptad repeat regions separated by a transmembrane repeat (Ong and Housenloy, 2010).

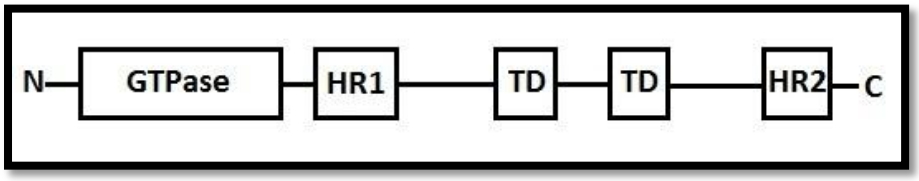

Figure 2.7 Domain organization of Mitofusins 1 or 2. HR – Heptad repeats. TD – Transmembrane domains. [Adapted from Ong and Housenloy, 2010].

2.6.1.2 Optic atrophy 1 (Opa1)

Opa1 is a 93 kDa protein either localized in the mitochondrial intermembrane space in soluble form or tightly attached to the inner mitochondrial membrane (Liesa et al., 2009). A total of eight mRNA isoforms has been described with variable level of expression in different tissues with retina, brain, testis, heart and muscle tissue showing the highest level (Delettre et al., 2001). The domain organization of Opa1 includes an N-terminal mitochondrial import motif sequence followed by transmembrane domain, GTPase domain, a middle domain and a C-terminal coiled coil domain which acts as a GTPase effector domain (GED) (Zanna et al., 2008). Over expression of Opa1 has been shown to result in elongated and tubulated mitochondria (Olichon et al., 2002). Genetic analysis has shown that Opa1 interacts with Mfn1 but not Mfn2 to bring about mitochondrial fusion (Cipolat et al., 2004).

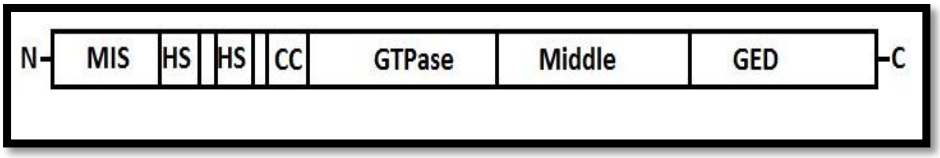

Figure 2.8 Domain organization of Opa1. MIS – Mitochodrial Import Sequence. HS – Hydrophobic Sequence. CC – Coiled Coil. GED – GTPase Effector Domain. [Adapted from Olichon et al., 2006].

2.6.2 Mitochondrial fission machinery

The mitochondrial division is carried out by two coupled mechanism. The first step initiated with the inhibition of fusion proteins followed by recruitment of mitochondrial fission protein, dynamin related protein–1 (Drp1) from the cytosol to the outer mitochondrial membrane with the help of several proteins such as mitochondrial fission 1 (Fis1), mitochondrial fission factor (Mff) and other accessory proteins (Hall et al., 2014).

2.6.2.1 Dynamin–related protein 1 (Drp1)

Drp1 is an 80 kDa cytosol protein with a small fraction of around 3 % found in mitochondria (Smirnova et al., 2001). It is a mammalian homolog of yeast Dnm1 which belongs to dynamin–related GTPase protein family that acts as a key regulator of mitochondrial fission in mammals (Westermann, 2010). The domain organization of Drp1 is characteristic of proteins belonging to the dynamin family. It has an N-terminal GTPase domain, a middle domain, a variable domain also referred to as insert B and a C-terminal GTPase effector domain (GED) (Praefcke and McMahon, 2004). Drp1 undergoes a series of posttranslational modifications such as phosphorylation, sumoylation, S-Nitrosylation and ubiquitination to mediate mitochondrial division. These modifications result in its translocation from cytoplasm to mitochondrial outer membrane, oligomerization into helical structures, GTP hydrolysis associated with conformational change and finally disassembly (Chang and Blackstone, 2010).

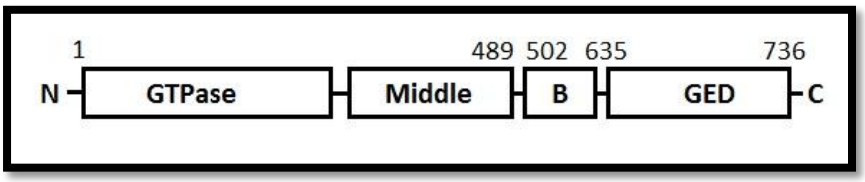

Figure 2.9 Domain organization of Drp1. The numbering refers to the amino acid sequence. B–variable domain. GED–GTPase effector domain. [Adapted from Chang and Blackstone, 2010].

2.6.2.2 Mitochondrial fission 1 (Fis1)

Fis1 is a 17 kDa integral mitochondrial outer membrane protein involved in the mitochondrial division. The domain structure consists of N–terminal end exposed to cytoplasm where it forms six α-helices which give rise to two TPR motifs (Hoppins et al., 2007). It is anchored to the mitochondrial outer membrane via its C-terminal end with a few amino acids exposed to the inter-membrane space. The two basic residues present at the C-terminal end are considered to be essential for its targeting to the mitochondria in humans (Stojanovsky et al., 2003). It is involved in the recruitment of Drp1 to the mitochondria for initiating mitochondrial division (Mozdy et al., 2000; Tieu and Nunnari, 2000).

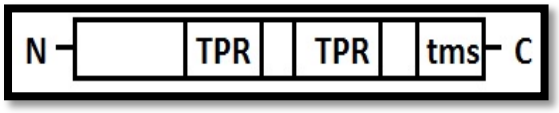

Figure 2.10 Domain organization of Fis1. TPR–tetratricopeptide repeats. tms–trans-membrane segment. [Adapted from van der Bliek et al., 2013].

2.6.2.3 Mitochondrial Fission Factor (Mff)

Mff is a 38 kDa novel tail anchored protein that is involved in the division of mitochondria and peroxisomes (Gandre-Babbe and van der Bliek, 2008; Koch and Brocard, 2012). It has been found to be highly expressed in heart, liver, kidney, muscle, brain and stomach and at low levels in other tissues. The domain organization of Mff includes a short amino acid repeat at the N-terminal end followed by a middle segment capable of forming a coiled coil structure and a C-terminal hydrophobic segment that helps in its anchorage to the mitochondrial outer membrane (Gandre- Babbe and van der Bliek, 2008). Mff has been found to physically interact with Drp1 and is capable of inducing mitochondrial fission independent of Fis1. It functions as an essential factor for recruitment of Drp1 onto the mitochondria (Otera et al., 2010).

Figure 2.11 Domain organization of MFF. R1/R2 –Short Repeats. CC–Coiled Coil domain. tms–transmembrane segment. [Adapted from Gandre–Babbe and van der Bliek, 2008].

2.6.3 Mitochondrial biogenesis

Mitochondrial biogenesis refers to the process of growth and division of pre-existing mitochondria. Mitochondrial biogenesis is a highly regulated process and requires coordinated expression of nuclear and mitochondria genes (Lee and Wei, 2005). The large number of nuclear encoded proteins synthesized on cytosolic ribosomes is imported into the mitochondria. The process also requires the mtDNA replication as well as mitochondrial fusion and fission. The output of the mitochondrial biogenesis depends upon the various internal and external stimuli such as energy demand, exercises, calorie restriction, oxidative stress, cell division and renewal and differentiation (Jornayvaz and Shulman, 2010).

In mammalian cells, the mitochondrion contains 2–10 copies of circular double stranded DNA molecule (Robin and Wong, 2008) which replicates predominantly in the late S and G_2 phases of the cell cycle but may occur throughout the cell cycle (Bogenhagen and Clayton, 1977). Peroxisome proliferator-activated receptor gamma co-activator 1–alpha (PGC1α) is considered to be the master regulator of mitochondrial biogenesis. PGC1α interacts and co–activates the nuclear respiratory factors (NRF1 and NRF2) whose downstream lays the mitochondrial transcriptional factor A (Ventura-clapier et al., 2008). Mitochondrial transcription factor A has been shown to regulate the mtDNA copy number in mammals (Ekstrand et al, 2004).

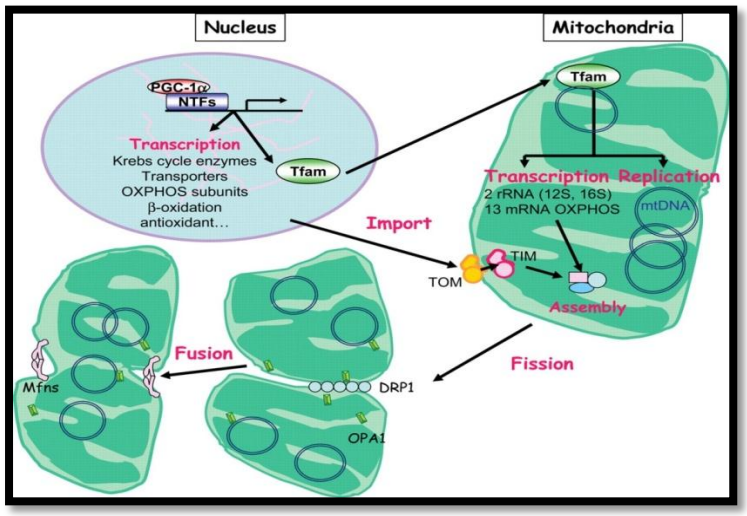

Figure 2.12 Mitochondrial biogenesis. PGC1α-Peroxisome proliferator-activated receptor gamma co-activator 1 – alpha. NTFs- Nuclear Transcription Factors. Tfam – Mitochondrial transcription factor A. TIM – Translocase of the inner membrane. TOM – Translocase of the outer membrane. Drp1 – Dynamin-related protein 1. Mfns – Mitofusins. [Adapted from Ventura-Clapier et al., 2008].

2.7 Mitochondrial dynamics in normal and cancer cells

In normal cells, it has been suggested that the fusion/fission cycle helps in the maintenance of quality of mitochondria. Fusion helps in the rejuvenation of mtDNA which might have been damaged due to exposure to ROS while fission helps in the elimination of damaged mitochondria which can no longer be repaired (Knorre et al., 2013).

Otto Warburg over 80 years ago discovered that tumor cells produce higher amount of lactate even in presence of oxygen, a state he termed as aerobic glycolysis. This occurred through increase in glycolysis and decrease in oxidative phosphorylation and is commonly referred to as Warburg effect (Warburg, 1956). Although initially interpreted as due to dysfunctional mitochondria, several studies have now shown that this bioenergetic switch which occurs in many of the cancer cells results due to shift from high ATP production by OXPHOS to the process of energetically balanced production of substrates required for cellular biogenesis and reproduction. Despite the seemingly passive role of mitochondria in cancer cells, interest in the mitochondrial metabolism came back into the limelight with the discovery of mutations in the TCA cycle enzymes such succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2). Alterations in these enzymes bring about the alterations in mitochondrial bioenergetics and metabolism mainly in OXPHOS and also initiate a cascade of events which not only initiates but also helps in sustaining the transformed state of cancer cells. Hence, contrary to the popular belief the mitochondria in cancer cells are functional but the reprogramming of bioenergetic system tilts the cells more towards the substrate generating glycolytic pathways than to energy production oriented pathways (Wallace, 2012; Rehman et al., 2012).

Although the reprogramming of bioenergetic pathways has been proven by several studies as a mechanism to explain the Warburg effect seen in many cancer cells, several recent studies have also been able to show the relation between mitochondrial dynamics and the cancer progression. In one such study, it has been shown that inhibition of mitochondrial fission results in cell cycle arrest in lung cancer. Rehman *et al.* discovered that there is an imbalance of Drp1/Mfn2 which favors the mitochondria in division state. They were able to demonstrate increased level of Drp1 and decreased level of Mfn2 in lung tumor tissue samples when compared to the corresponding healthy lung cells. Similarly, they were also able to reduce

tumor progression and increase apoptosis by restoring mitochondrial networking (Rehman *et al*., 2012).

Another study conducted by Zhao et al. (2013) was able to show the relation between the mitochondrial dynamics and migration and evasion of breast cancer cells. In this study, the authors were able to find the increased expression of Drp1 and decreased expression of Mfn1 in metastatic breast cancer cells when compared to non-metastatic breast cancer cells. They also found more number of fragmented mitochondria in metastatic breast cancer cells. While silencing Drp1 or over expressing Mfn1 inhibited the metastatic abilities of the breast cancer cells, silencing of Mfn proteins led to enhanced fragmentation subsequently leading to increased metastatic properties of the breast cancer cells.

In a similar set of study conducted recently, it has been demonstrated that when the mitochondria are in a hyper fused state, the cells become arrested at the G2/M stage of the cell cycle. This defect in cell cycle progression was observed after the loss of two mitochondrial fission regulators, Drp1 and Mff. In addition, it was observed that the persistent mitochondrial hyperfusion caused induction of programmed cell death (Westrate et al., 2014).

The level of Drp1 was also found to be higher in oncocytic thyroid tumors and regulated their migration. Ferreira-da-silva et al. were able to find the association between the over expression of Drp1 and malignant oncocytic thyroid tumors. Here the authors were able to present that the imbalanced mitochondrial dynamics characterized the malignant features of oncocytic thyroid tumor cells and acquisition of migrating phenotype (Ferreira-da-silva et al., 2015).

Hence, the differential level of mitochondrial fission and fusion proteins in the cancer cells leads to changes in the mitochondrial dynamics that can be observed in between the normal and cancer cells.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Cell culture and transfection

Human neuroblastoma cells (IMR-32), Human lens epithelial cells (SRA01) and Human Embryonic kidney cells (HEK293T) were procured from American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) in the presence of penicillin (60 µg /mL), kanamycin (30 µg/mL) and streptomycin (50 µg/mL) at 37 °C with 5% CO_2 supply. Cells were stably transfected using 2µg of plasmid with Lipofectamine LTX (Invitrogen) in serum-free DMEM as per the manufacturer' instructions and transfected cells were selected using Geneticin (400 µg/mL for Sra01/04 and 900 µg/mL for IMR-32 cells and HEK293T cells respectively) for selection.

3.1.1 Human Neurobloastoma Cells (IMR32)

The IMR-32 cell line was established by W.W. Nichols, J. Lee and S. Dwight in April, 1967 from an abdominal mass occurring in a 13-month-old Caucasian male. The tumor was diagnosed as a neuroblastoma with rare areas of organoid differentiation.

3.1.2 Human Lens Epithelial Cells (SRA01)

SRA01 is a human lens epithelial cell line capable of expressing an exogeneous immortalizing gene, which is established without infecting with a live virus.

3.1.3 Human Embryonic Kidney 293T (HEK293T)

The 293T cell line is a highly transfectable derivative of human embryonic kidney 293 cells, and contains the SV40 T-antigen.

3.2 Plasmids and cloning

Full length genes of *Fis1, Drp1, Mff* and *TRAP1* were amplified and cloned into mammalian expression vectors. *Fis1* was cloned into pCDNA 3.1 myc-his(-) A expression vector, *Drp1* and *Mff* was cloned into C2 FLAG expression vector and *TRAP1* was cloned into pCDNA 3.1(+) expression vector.

3.2.1 pCDNA™ 3.1/ myc-his(-) A expression system

The pCDNA™3.1/myc-his(-) A expression vector is 5522 bp long carrying ampicillin resistance gene as a selection marker for transformed bacteria and neomycin resistance gene as a selection marker for transfected eukaryotic cells.

- **Characteristics:** Vector size: 5522 bp. Human cytomegalovirus (CMV) immediate-early promoter/enhancer, T7 promoter/priming site, Multiple Cloning Site (MCS) in three reading frames, *myc* epitope (*c-myc*), C-terminal polyhistidine tag, BGH reverse priming site, Bovine growth hormone (BGH) polyadenylation signal, f1 origin, SV40 early promoter and origin, Neomycin (Geneticin®) resistance gene, SV40 polyadenylation signal, pUC origin, Ampicillin resistance gene (β-lactamase).

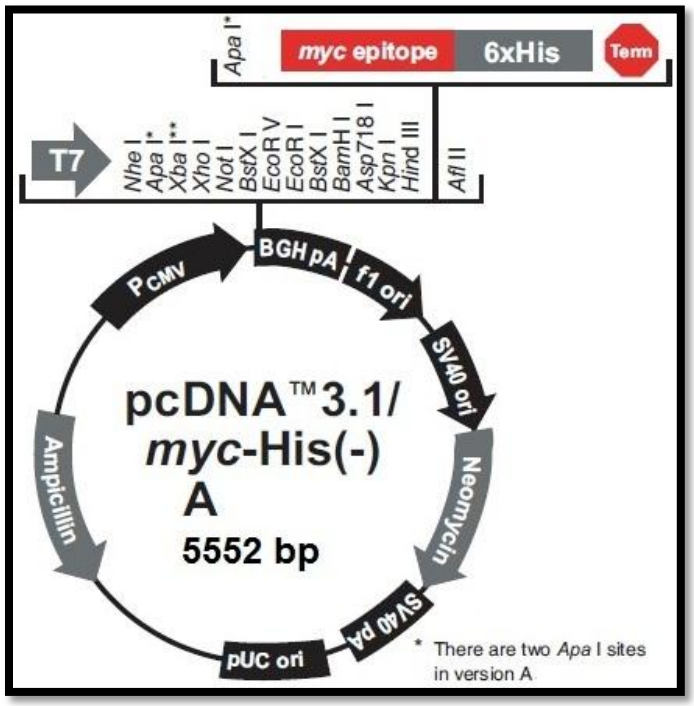

Figure 3.1 Vector Map for pcDNA3.1™/myc-his(-) A Mammalian Expression System.

3.2.2 C2-Flag expression system

The Flag expression vector is 4 Kb long carrying kanamycin resistance gene as a selection marker for transformed bacteria and neomycin resistance gene as a selection marker for transfected eukaryotic cells. C2-Flag mammalian expression vector is a customized vector made by replacing the C-terminal EGFP with Flag tag.

- **Characteristics:** Vector size: 4 Kb. SV40 early mRNA polyadenylation signal, SV40 origin of replication, SV40 early promoter, Kanamycin resistance gene, Neomycin resistance gene, Multiple Cloning Site (MCS), bacterial promoter for expression of kanamycin resistance gene, Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal, pUC plasmid replication origin.

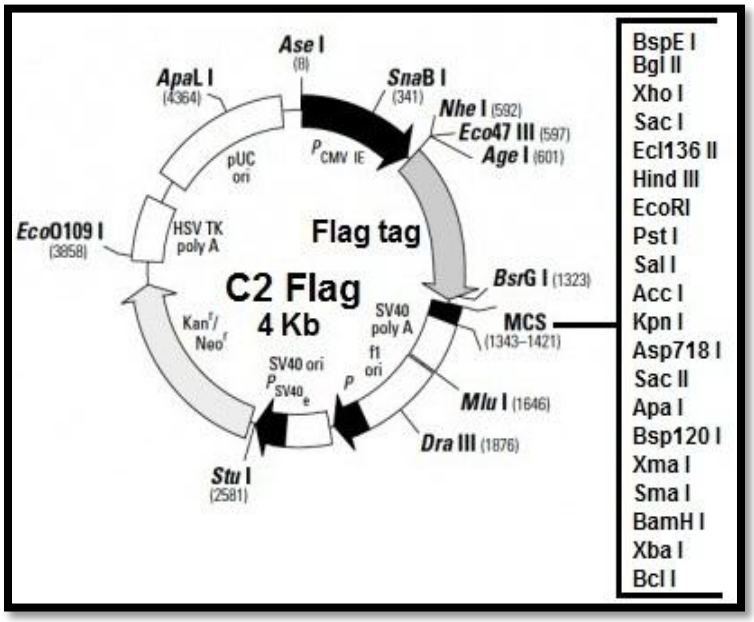

Figure 3.2 Vector Map for Flag Mammalian Expression System.

3.2.3 pcDNA^{TM} 3.1(+) expression system

The pcDNA^{TM} 3.1(+) expression vector is 5428 bp long carrying ampicillin resistance gene as a selection marker for transformed bacteria and neomycin resistance gene as a selection marker for transfected eukaryotic cells.

- **Characteristics:** Vector size: 5428 bp. Human cytomegalovirus (CMV) immediate-early promoter/enhancer, T7 promoter/priming site, Multiple cloning site in forward or reverse orientation, Bovine growth hormone (BGH) polyadenylation signal, f1 origin, SV40 early promoter and origin, Neomycin resistance gene, SV40 early polyadenylation signal, pUC origin, Ampicillin (*bla*) resistance gene (β-lactamase).

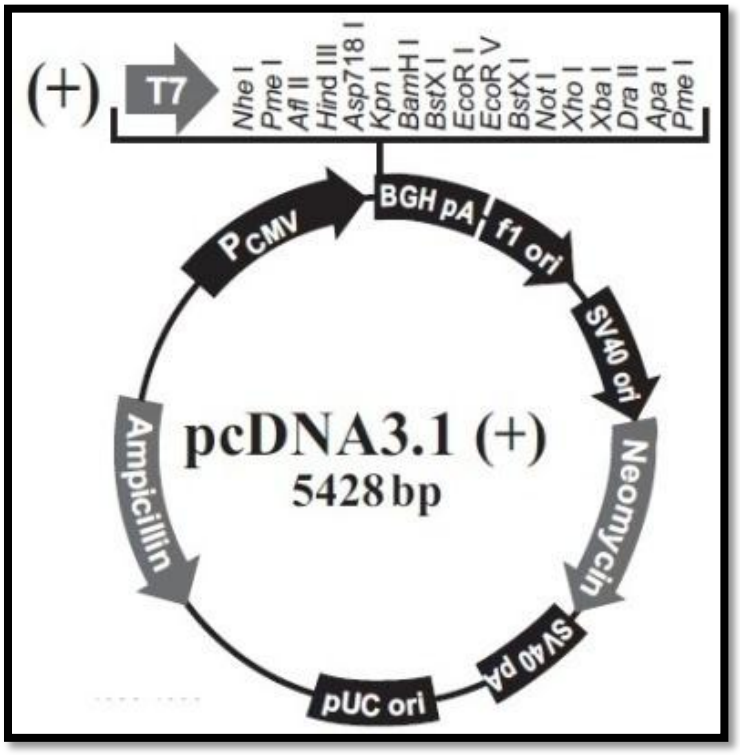

Figure 3.3 Vector Map for pcDNA3.1 (+) Mammalian Expression System.

3.2.4 pcDNA^{TM} 3.1(+) expression system with Fis1

Previously cloned Fis1 (459 bp) in the pcDNA^{TM} 3.1(+) (5428 bp) was procured from the laboratory stock and used as a template for sub cloning into the pCDNA^{TM} 3.1/ myc-his(-) A expression system.

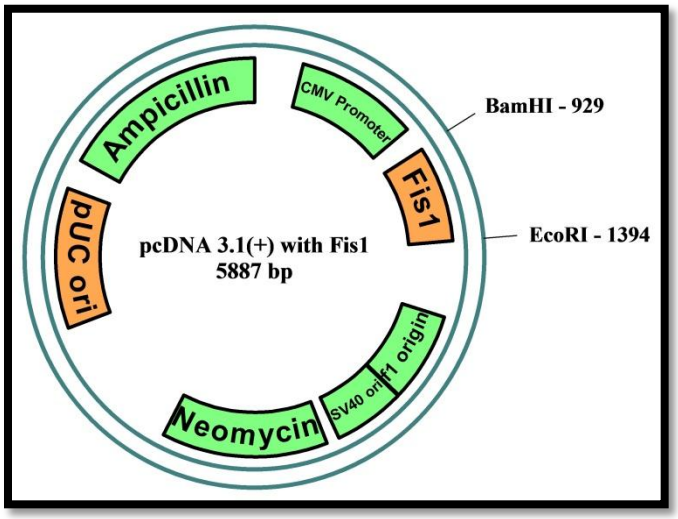

Figure 3.4 Vector Map for pcDNA3.1 (+) mammalian expression system with Fis1 insert between BamH I and EcoR I.

3.2.5 pEGFP-N2 expression system with TRAP1

Previously cloned TRAP1 (2112 bp) in pEGFP-N2 (4737 bp) was obtained from the laboratory stocks for further cloning into pcDNA3.1 (+) mammalian expression system**.** The pEGFP-N2 expression vector is 4737 bp long carrying kanamycin resistance gene as a selection marker for transformed bacteria and neomycin resistance gene as a selection marker for transfected eukaryotic cells.

- **Characteristics:** Vector size: 4737 bp. SV40 early mRNA polyadenylation signal, SV40 origin of replication, SV40 early promoter, Kanamycin resistance gene, Neomycin resistance gene, Multiple Cloning Site (MCS), bacterial promoter for expression of kanamycin resistance gene, Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal, pUC plasmid replication origin.

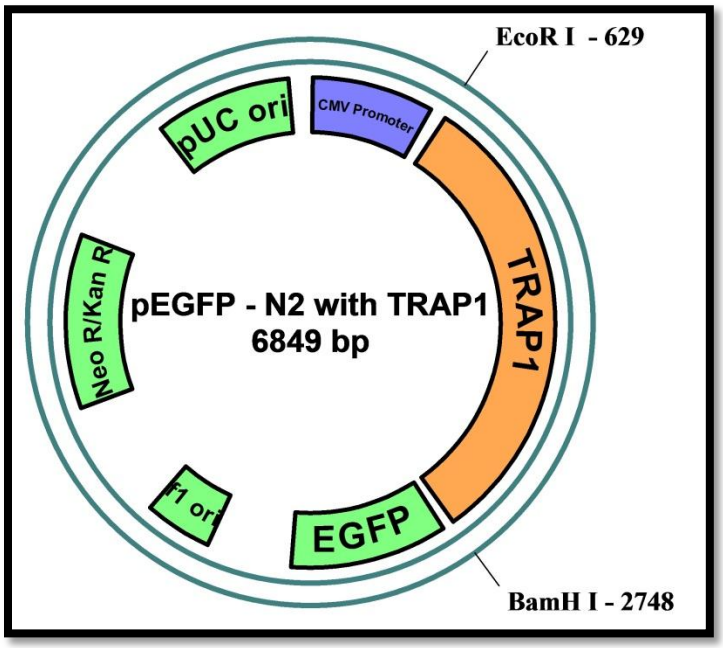

Figure 3.5 Vector Map for pEGFP-N2 mammalian expression system with TRAP1 insert between EcoR I and BamH I.

3.3 RNA isolation

Total RNA was isolated from human neuroblastoma cells (IMR-32) normal and Fis1 over expressing cells by TRIZOL method as per manufacturer's instructions (Invitrogen, USA). One mL of TRIZOL reagent was added to the cells and incubated at room temperature for 5 min. The cell lysate was transferred to 1.5 mL centrifuge tube and 0.2 mL of chloroform was added, mixed thoroughly and incubated at room temperature for 5 min. This mixture was subjected to

centrifugation at 12000 x g for 15 min at 4 °C. The aqueous phase containing total RNA was transferred to fresh tube and RNA was precipitated by adding 0.5 ml of isopropanol followed by incubation at room temperature for 10 min. The precipitate was centrifuged at 12000 x g for 10 min at 4 °C. The supernatant was discarded and RNA pellet was air dried for 10 min and resuspended in 20 µL of RNAse free water. The integrity of the RNA was checked on 1% agarose gel by electrophoresis.

3.4 Single strand cDNA synthesis (reverse transcription reaction)

The reverse transcription (RT) of RNA to cDNA was performed using Takara blue print cDNA synthesis kit as per manufacturer's instruction. 1 µg of total RNA was added to 1 µL of 10 mM dNTP, 1 µL of 50 µM oligo dT primers and the volume was made up to 10 µL with RNAse free water. This mixture was incubated at 65 °C for 5 min and was immediately transferred on to ice to make RNA single stranded without any loops. To the above reaction mixture, 4 µL of 5 x blue print first strand buffer, 0.5 µL of RNAse inhibitor, 1 µL of reverse transcriptase (40 U/ µL) were added and the volume was made up to 20 µL with RNAse free water. The mixture was mixed gently and incubated at 42 °C for 1 h. The RT enzyme was inactivated by incubating the tubes at 95 °C for 5 min. Finally 80 µL of nuclease free water was added to the RT reaction to make the final volume to 100 µL. The synthesis of the cDNA was confirmed by PCR with GAPDH primers.

3.5 Complementary DNA (cDNA) Sequence retrieval

Coding sequences for Homo sapiens *Fis1* (NM_016068.2), *Drp1* (NM_012062.4), *Mff* (NM_001277061.1) and *TRAP1* (NM_016292.2) were retrieved from the data base of National Center for Biotechnology Information (NCBI).

3.6 PCR Primer Designing

Primers were designed for *TRAP1* with mitochondrial localization signal (full length and internal), *Fis1* (full length)*, Drp1* (full length and internal) and *Mff* (full length) using gene tool software.

Table 3.1 PCR Primers.

Gene/ Accession No.	**Primer**	**Sequence**	**Tm (°C)**
Fis1 *(full length)* NM_016068.2	Forward primer	5'-ATTGGATCCATTATGGAGGCCGTGCTGAAC-3'	64.2
	Reverse primer	5'-ATTAAGCTTGGATTTGGACTTGGACACAGC-3'	61.5
Drp1 *(full length)* NM_012062.4	Forward primer	5'-ATTGTCGACGAGGCGCTAATTCCTGTC-3'	62.8
	Reverse primer	5'-ATTGGATCCTCACCAAAGATGAGTCTCCCGG-3'	65.5
Drp1 *(internal)*	Forward primer	5'-TACTGGTCCTCGTCCTGCTT-3'	56.3
	Reverse primer	5'-GTTGCCTGTGGTTGGTTCTT-3'	54.2
GAPDH (internal) NM_002046.5	Forward primer	5'-CCTCCCGCTTCGCTCTCTGCT-3'	68.5
	Reverse primer	5'-TGCAAATGAGCCCCAGCCTTC-3'	58.6
Mff (full length) NM_001277061.1	Forward primer	5'-ATTGTCGACATGAGTAAAGGAACAAGCAGTGA-3'	61.6
	Reverse primer	5'-ATTGGATCCCTAGCGGCGAAACCAGAG-3'	64.3
Mff (internal)	Forward primer	5'-GCGAATGAGGGTCCCAGAAA-3'	56.3
	Reverse primer	5'-CTGCTGGTATGCCCTACGAG-3'	58.3
TRAP1 with MLS *(full length)* NM_016292.2	Forward primer	5'-ATTGGATCCAATTATGGCGCGCGAGCTG-3'	64.3
	Reverse primer	5'-ATTGAATTCTCAGTGTCGCTCCAGGGCCT-3'	64.2
TRAP1 (internal)	Forward primer	5'-CATGGTGGCTGACAGAGTGGAG-3'	60.7
	Reverse primer	5'-GGCTGGCGTATTCTGAGAGGCT-3'	60.7

Table 3.2 Composition of PCR reaction mixture using individual components (25 µL).

S.No	**Reagents**	**Volume (µL)**
1.	Nuclease free water	17.3
2.	10 X PCR buffer	2.5
3.	25 mM $MgCl_2$	1.5
4.	10 mM dNTP	0.5
5.	Forward primer (10 pM)	1.0
6.	Reverse primer (10 pM)	1.0
7.	Template (100 ng)	1.0
8.	Taq DNA polymerase (5U/µL)	0.2

Table 3.3 Composition of PCR reaction mix using Phusion Master Mix.

S. No.	**Reagents**	**Volume (µL)**
1.	NFW	10.0
2.	Phusion Master mix	12.5
3.	Forward Primer	1.0
4.	Reverse Primer	1.0
5.	Template(100 ng)	1.0

Table 3.4 PCR conditions for different genes.

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Hold
Fis1	95 °C, 2 min	95 °C, 30 sec	63.9 °C, 30 sec	72 °C, 50 sec	72 °C, 5 min	4 °C
		29 cycles				
Drp1	95 °C, 2 min	95 °C, 30 sec	63.2 °C, 30 sec	72 °C, 230 sec	72 °C, 5 min	4 °C
		29 cycles				
Drp1 **(Internal)**	95 °C, 2 min	95 °C, 30 sec	58.3 °C, 30 sec	72 °C, 60 sec	72 °C, 5 min	4 °C
		29 cycles				
GAPDH **(Internal)**	95 °C, 2 min	95 °C, 30 sec	67.6 °C, 30 sec	72 °C, 50 sec	72 °C, 5 min	4 °C
		-0.5 °C/cycle , 14 cycles				
		95 °C 30 sec	60.6 °C, 30 sec	72 °C, 50 sec		
		19 cycles				
Mff	95 °C, 2 min	95 °C, 30 sec	63 °C, 30 sec	72 °C, 110 sec	72 °C, 5 min	4 °C
		29 cycles				
Mff **(Internal)**	95 °C, 2 min	95 °C, 30 sec	58.5 °C, 30 sec	72 °C, 60 sec	72 °C, 5 min	4 °C
		29 cycles				
TRAP1	95 °C, 2 min	95 °C, 30 sec	63 °C, 30 sec	72 °C, 220 sec	72 °C, 5 min	4 °C
		29 cycles				

TRAP1 **(Internal)**	95 °C, 2 min	95 °C, 30 sec	62.8 °C, 30 sec	72 °C, 90 sec	72 °C, 5 min	4 °C
		29 cycles				

3.7 PCR clean – up

The PCR clean - up was performed using Machery – Nagel NucleoSpin® Gel and PCR Clean-up kit as per the manufacturer's instruction with slight modification. Twice the volume of Buffer NTI was used for 1 volume of PCR sample. The mixture was then placed into the NucleoSpin® Gel and PCR Clean-up Column and subjected to centrifugation at 11,000 X g for 30 sec. The flow – through was discarded and the column was placed back into the collection tube. Seven hundred µL of buffer NT3 was added into the column and centrifuged at 11, 000 X g for 30 sec. The column was washed again with additional 700 µL of NT3 buffer and centrifugation at 11,000 X g for 30 sec. The flow – through was discarded and the column was kept back into the collection tube. The column was then dried by centrifugation at 11,000 X g for 1 min. The column was then placed at the dry bath at 70 ^{o}C for 5 min to remove any residual ethanol. Then, the spin column was placed into new 1.5 mL microcentrifuge tubes and 20 µL of warm Tris.EDTA buffer (TE, pH 8.0) (Appendix 1) was added onto the column and kept for incubation at room temperature for 5 min. After the incubation, the column was centrifuged at 11,000 X g for 1 min and the eluted PCR product was used for further downstream processes.

3.8 Restriction digestion of insert and vector

The PCR amplified gene product and the vector DNA were subjected to double digestion independently with appropriate restriction enzymes. All the restriction digestion mixtures were incubated in water bath at 37 °C for 90 min.

Table 3.5 Restriction digestion setup.

S.No	Components	Volume (µL)
1.	Nuclease free water	Made up to 25 µL
2.	10X NEB buffer	2.5
3.	Plasmid DNA (2 µg)	Based on DNA conc.
4.	Enzyme-1 (20 U/µL)	0.5
5.	Enzyme-2 (20 U/µL)	0.5

3.9 Gel Extraction

The double digested PCR product and the vector was purified by gel extraction using Machery – Nagel NucleoSpin® Gel and PCR Clean-up kit as per the manufacturer's instruction with slight modification. Once the gel run was complete, the gel was observed under UV transilluminator and the bands of interest were cut and transferred into the 1.5 mL microcentrifuge tubes. The weight of the tubes was taken before hand and then the tubes were weighed again with the cut gel inside so as to know the weight of the gel. For every mg of the gel twice the volume of buffer NTI was added. The sample was then incubated at 50 ^{o}C for 10 min with brief vortexing in between. Then, the sample was loaded into the NucleoSpin® Gel and PCR Clean-up Column and subjected to centrifugation at 11,000 X g for 30 sec. The flow – through was discarded and the column was placed back into the collection tube. 700 µL of buffer NT3 was added into the column and centrifuged at 11, 000 X g for 30 sec. The column was washed again with additional 700 µL of NT3 buffer and centrifugation at 11,000 X g for 30 sec. The flow – through was discarded and the column was kept back into the collection tube. The column was then dried by centrifugation at 11,000 X g for 1 min. The column was then placed at the dry bath at 70 ^{o}C for 5 min t remove any residual ethanol. Then, the spin column was placed into new 1.5 mL microcentrifuge tubes and 20 µL of warm TE buffer was added onto the column and kept for incubation at room temperature for 5 min. After the incubation, the column was centrifuged at 11,000 X g for 1 min and the eluted double digested vector and insert were used for further downstream processes.

3.10 Ligation

Double digested *FIS1* DNA was ligated with double digested pcDNA myc-his (-) A expression vector. Similarly, *Drp1* DNA was ligated with C2 Flag expression vector, full length TRAP1 DNA was ligated with pCDNA 3.1 (+) expression vector and *Mff* DNA was ligated with C2 Flag expression vector, using T4 DNA ligase at 4 °C for 16 h. A 1:6 molar ratio of vector: insert was used for ligation.

Table 3.6 DNA ligation.

S. No	Components	Volume (µL)
1.	Nuclease free water	Make up to 20 µL
2.	10X T4 DNA ligase buffer	2.0
3.	Double digested vector DNA	Based on the DNA conc.
4.	Double digested insert DNA	Based on the DNA conc.
5.	T4 DNA ligase (400 U/ µL)	1.0

3.11 Preparation of ultra-competent *E. coli* cells

Competent cells of *E. coli* DH5α strain were prepared by Inoue method (Inoue H *et al.* 1990). PIPES solution (piperazine-N,N'- bis 2-ethanesulfonic acid, 0.5 M, pH 6.7) was prepared by adding 15.1 g of PIPES in 50 mL double distilled water and the pH was adjusted to 6.7 using 5 M KOH. The volume was made up to 100 mL with double distilled water. The buffer was filter sterilized and was divided into aliquots and stored at -20 °C. Inoue transformation buffer was prepared by adding 2 mL of PIPES buffer with a final concentration of 10 mM, 1.088 g of $MnCl_2.4H_2O$ with a final concentration of 55 mM, 0.22 g of $CaCl_2.2H_2O$ with a final concentration of 15 mM, 1.865 g of KCl with a final concentration of 20 mM and 50 mL double distilled water. The volume was made up to 100 mL with double distilled water. The buffer was filter sterilized and stored in small aliquots.

A single *E. coli* colony was picked and transferred into a 250 mL flask containing 25 mL LB medium (Appendix 1). The bacterial culture was incubated for 8 h at 37 °C with shaking (250 rpm). This bacterial culture was used to inoculate in to 1 L flask with 250 mL of LB media (1:100 dilution of culture to LB medium). The bacterial culture was incubated overnight at 18 °C with shaking at 200 rpm. Cells were harvested when the OD_{600} reached 0.55 by centrifugation at 12000 rpm for 10 min at 4 °C. The supernatant was discarded and cells were resuspended in 80 mL of ice cold Inoue transformation buffer. The cells were harvested by centrifugation at 3900 rpm for 10 min at 4 °C. The supernatant was discarded and cells were resuspended in 20 mL of ice cold Inoue transformation buffer. To this, 1.5 mL of DMSO (dimethyl sulfoxide) was added and incubated on ice for 10 min. 100 µL aliquots of cell suspension were dispensed quickly into sterile microfuge tubes and the competent cells were snap frozen in liquid nitrogen and stored at -80 °C for subsequent usage.

3.12 Transformation

After ligation, 10 µL of the ligation mixture previously incubated with double digested vector and gene of interest was used for transforming ultra-competent *E. coli* DH5α strain. The ultra-competent cells were thawed on ice and ligation mix was added to 100 µL of competent cells and mixed properly. Cells were incubated on ice for 20 min. Heat shock was given for 90 sec at 42 °C followed by chilling on ice for 15 min. Nine hundred µL of LB medium was added to the transformation mix and cells were incubated at 37 °C for 1 h with shaking at 150 rpm. Cells were then centrifuged at 4000 rpm for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 100 µL of LB medium and spread on LB plate containing respective antibiotic and incubated overnight at 37 °C. The plates were examined on the next day for presence of transformed colonies.

3.13 Screening of recombinant clones

Several colonies were randomly picked from the selection plate and inoculated in LB medium with specific antibiotic and grown by incubating at 37 °C in a water bath with shaking at 200 rpm. Plasmid DNA was isolated from the culture by alkaline lysis method (Sambrook et al., 1989). The media containing the bacterial cells (1.5 mL) was transferred to the microcentrifuge tubes and the cells were harvested by centrifugation at 12000 rpm for 1 min. The supernatant was discarded and the cells were resuspended using 150 µL of the resuspension buffer from MN plasmid isolation kit. The cells were resuspended by vortexing vigorously. 200 µL of Lysis buffer (Appendix 1) was added onto the resuspended cells and the tubes were stored on ice. 150 µL of the Neutralization buffer (Appendix 1) was added into the tube and the tubes were inverted slowly so as to uniformly mix the solutions. The tubes then stores on ice for 5 min and then centrifuged at 12,000 X g for min at 4 ^{o}C. The supernatant was then transferred to a fresh tube and the plasmid DNA was precipitated with addition of 2 volumes of ethanol at room temperature. The contents of the tubes were mixed with vortexing and the mixture was kept at room temperature for 2 min. The tubes were then centrifuged at 12,000 X g for 5 min at 4 ^{o}C. The supernatant was discarded and the pellet was washed with 1 mL of 70 % ethanol and centrifuged at 12,000 X g for 5 min. The tubes were then allowed to air dry, at the end of which the plasmid DNA obtained was dissolved in 50 µL of TE buffer. The isolated plasmid was then subjected to double restriction digestion to confirm the release of the insert DNA.

3.14 Mini Preparation of Plasmid

The mini preparation of plasmid isolation was performed using modified alkaline lysis method. 1.5 mL culture of overnight grown culture was used for harvesting the cells at 12,000 rpm for 1 min. The supernatant was discarded and the cells were suspended in solution I. Thus resuspended cells were then lysed using solution II followed by neutralization with solution III. Then, the tubes were kept on ice for 5 min and centrifuged at 12,000 rpm for 5 min. the supernatant was transferred to a new tube and double the volume of absolute ethanol was kept for precipitating the plasmid. The tube was then centrifuged at 12,000 rpm for 15 min. the supernatant was discarded and the pellet was washed with 70 % ethanol to remove the salts. The tubes were centrifuged for 5 min and the supernatant was discarded. The pellet was then left for drying under the light and finally dissolved in TE buffer. The isolated plasmid was then subjected to Agarose gel electrophoresis to detect its presence and determine the quality.

3.15 Enrichment of plasmid DNA by midi-prep plasmid DNA isolation

The plasmid DNA isolation was performed using Machery – Nagel Plasmid DNA purification kit as per the manufacturer's instruction. The cells from the colonies with positive clones were inoculated into 50 mL of LB medium and grown at 37 °C with shaking in a water bath. The cells were harvested by centrifugation at 4000 rpm, for 20 min at 4 °C. The cell pellet was resuspended in 8 mL of re-suspension buffer containing RNAse A (20 µg/mL) by vortexing. To the cell suspension, 8 mL of lysis buffer was added and cell suspension was mixed well and incubated at room temperature for 5 min. Eight mL of neutralization buffer was added to the lysed cells and mixed gently by rolling as well as inverting the tube several times. The cell lysate was loaded on to the column (through a pre-filter) which had been equilibrated with 12 mL of equilibration buffer. Then the column was washed with 5 mL of equilibration buffer. The pre-filter was removed with the help of forceps and column was washed with 8 mL of wash buffer. The plasmid DNA was eluted from the column by using 5 mL of elution buffer and the eluate was collected in 50 mL tarson tube. Fresh Isopropanol (3.5 mL) was added to precipitate the plasmid DNA and the contents were mixed thoroughly by vortexing and subjected to centrifugation at 12000 x g for 30 min at 4 °C. Supernatant was discarded and DNA pellet was washed with 70% ethanol. The DNA was collected by centrifugation at 12000 x g for 10 min at 4 °C. The DNA pellet was air dried and dissolved finally in 100 µL of TE buffer. The DNA integrity was checked by agarose gel electrophoresis. The isolated plasmid was again validated either by PCR with gene specific internal primers or with single and double restriction digestion to see

the base shift of the recombinant plasmid when compared to the original empty plasmid as well as to see the insert release from the recombinant plasmid.

3.16 Immunoprecipitation

Immuniprecipitation is used to study the protein-protein interactions. The cell lysates of HEK293T and SRA01 cells (Control, *TRAP1*, *Fis1*, *Drp1* and *Mff* over expressed) from which the protein interaction studies was to be conducted were lysed with RIPA buffer without sodium deoxycholate(Appendix 1). The protein concentration was estimated by Bicinchoninic Assay (BCA) method as per the manufacturer's instruction and 100 µg of protein extracts were used. Twenty microlitres of 50 % Protein A slurry was added to each of the tubes containing the cell lysates from HEK293T and SRA01 cells for 2 h at 4 ^{o}C. The tubes were then centrifuged 2000 g at 4 ^{o}C for 10 min. The supernatant was transferred into the fresh tubes and 2 µg of primary antibodies for myc-tagged Fis1 protein (Santa Cruz Biotechnology Inc. Cat. # sc – 789), flag tagged with Drp1 and Mff proteins (Santa Cruz Biotechnology Inc. Cat. # sc – 807) were used for each of the cell lysates for pull down of the respective proteins. The tubes were then incubated overnight at 4 ^{o}C with gentle mixing. Fresh slurry was added onto the tubes and incubated for 60 min at 4 °C with gentle mixing. The tubes were then centrifuged at 3000 g for 2 min at 4 °C. the supernatant was discarded and the pellet was washed with 1 ml of washing buffer (RIPA buffer with Protease inhibitor cocktail) for three times and the tubes were centrifuged at 3000 g for 2 min at 4 ^{o}C. Supernatant was discarded and the pellet was resuspended in 20 µL of Laemmli sample buffer (Appendix 1). The samples were then heated at 95 ^{o}C for 5 min and centrifuged at 12,000 g for 1 min at room temperature. The supernatant was collected and used for immunoblotting.

3.17 Immunoblotting

The protein lysates obtained after immunoprecipitation were run in 12 % (w/v) SDS – PAGE and transferred onto nitrocellulose membrane (Millipore, Temecula, CA, USA). The blots were then probed with primary antibodies for TRAP1 protein (BD Transduction Laboratories. Cat. # 612345). HRP conjµgated anti – mouse/ anti rabbit antibody (Roche. Cat. #11520709001) was used as secondary antibody. The blots were then subjected to chemiluminescence using luminol as a substrate and the fluorescence was captured onto the X-ray film.

3.18 Mitotracker Red Staining

The untransfected and transfected cells were seeded on cover glass (22 x 22 mm, Fisher Scientifics) in DMEM complete medium grown at 37 °C for 48 h. The cells were stained with 250 nM CMX-*Ros* (Mitotracker red) dye in incomplete media and incubated at 37 °C, 5% CO_2 for 20 min. The staining solution was removed and cells were washed thrice with 1 x PBS at a time interval of 10 min each. The cells were fixed with 4% formaldehyde in 1 x PBS for 12 min, followed by three washes with 1 x PBS. The cover glasses were mounted on to glass slides along with Vectashield mounting medium containing DAPI (4', 6-diamidino-2-phenylindole,100 nM), sealed with nail polish and observed under laser scanning confocal imaging microscope (Leica, Germany).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 RNA isolation

Total RNA was isolated from the IMR-32 cells using Trizol reagent. The concentration was measured using NanoDrop and found to be 930 ng/µL. The quality of the isolated RNA was analyzed by running on 1% agarose gel containing 0.5 µg/mL ethidium bromide (Fig. 4.1A L1). Three bands were observed on the gel corresponding to 28S, 18S and 5.8S with the ratio of intensity of 28S:18S bands to be 2:1 which shows that the isolated RNA was intact. Using Takara Bluescript cDNA synthesis kit, cDNA was prepared from the RNA using oligodT primers and the quality of cDNA was checked by performing qualitative PCR for GAPDH using gene specific primers. Fig. 4.1B L2 shows the amplified product of GAPDH.

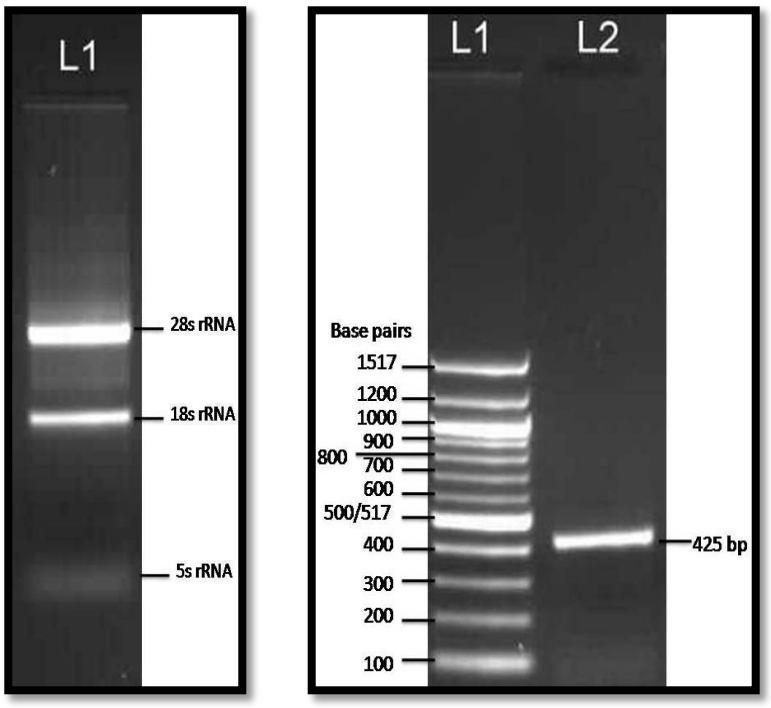

Figure 4.1 (A) L1–Agarose gel (1%) electrophoresis showing quality of RNA. (B) L1– 100 bp DNA ladder. L2– PCR amplified GAPDH amplicon (425 bp) with internal primers from the cDNA prepared from isolated RNA.

4.2 Sub-cloning *TRAP1* from pEGFPN2 expression vector into pCDNA3.1^{TM}(+) expression vector

Using the *TRAP1* insert in pEGFPN2 expression vector as template, PCR for *TRAP1* was carried out using primers for the full length *TRAP1*. The amplified PCR product was run on 1 % agarose gel and the band corresponding to 2112 bp (Fig. 4.3 L2, L3 and L4) corresponded to full length *TRAP1*. The PCR product was then cleaned free from unused primers and nucleotides using PCR clean up kit. The enriched pCDNA3.1^{TM}(+) expression vector and PCR cleaned up products were then subjected to double digestion using BamH I and EcoR I restriction enzymes for 2 h at 37 ^{o}C. Both the PCR cleaned up product and the pCDNA3.1^{TM}(+) expression vector was run on 0.8% agarose gel and extracted from the gel using the gel extraction kit. The purified vector and insert were ligated in presence of T4 DNA ligase in vector to insert molar ratio of 1:3. The ligation was carried out at 16 ^{o}C for overnight.

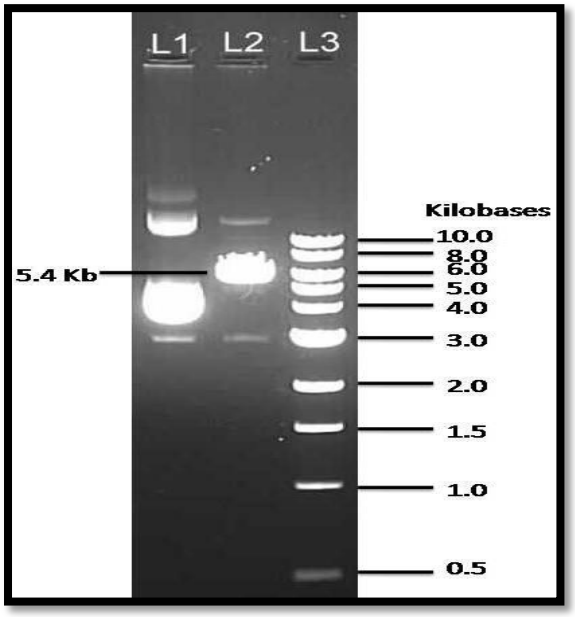

Figure 4.2 Agarose Gel (0.8 %) Electrophoresis of double digested pCDNA 3.1 (+) plasmid. L1– Undigested pCDNA 3.1 (+) plasmid. L2– pCDNA3.1^{TM}(+) double digested with BamH I and EcoR I. L3– 1 Kb Ladder.

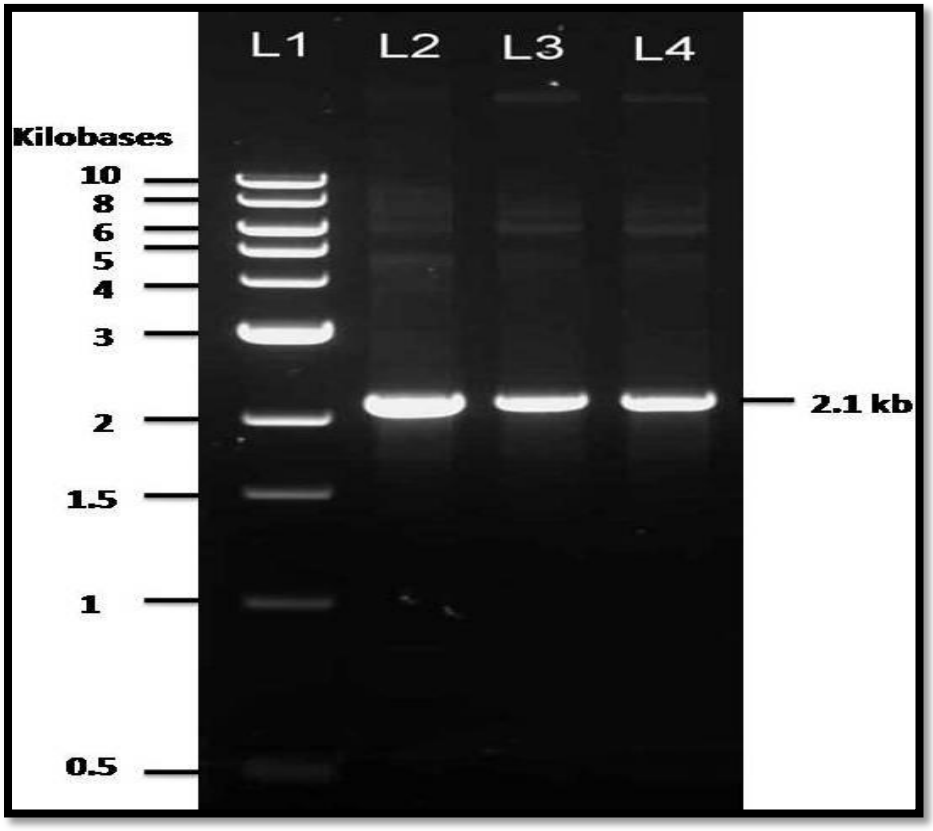

Figure 4.3 Agarose gel (1 %) electrophoresis of *TRAP1* amplified using high fidelity proof reading Taq polymerase enzyme. L1– 1 Kb Ladder. L2, L3, L4– *TRAP1* amplicon obtained after PCR with High fidelity Taq polymerase enzyme.

The ligation mixture was then used for transformation of ultra competent DH5α *Escherichia coli* cells. Recombinant clones were selected by plating onto the LB agar plates containing Ampicillin as a selection marker. The plates were kept for incubation at 37 ^{o}C for overnight. The colonies were then screened for positive clones by isolating plasmids from them and subjected to double digestion with BamH I and EcoR I. The digested plasmids were then run on 0.8% agarose gel containing ethidium bromide and observed for release of TRAP1 insert. (Fig. 4.4 L7 and L9).

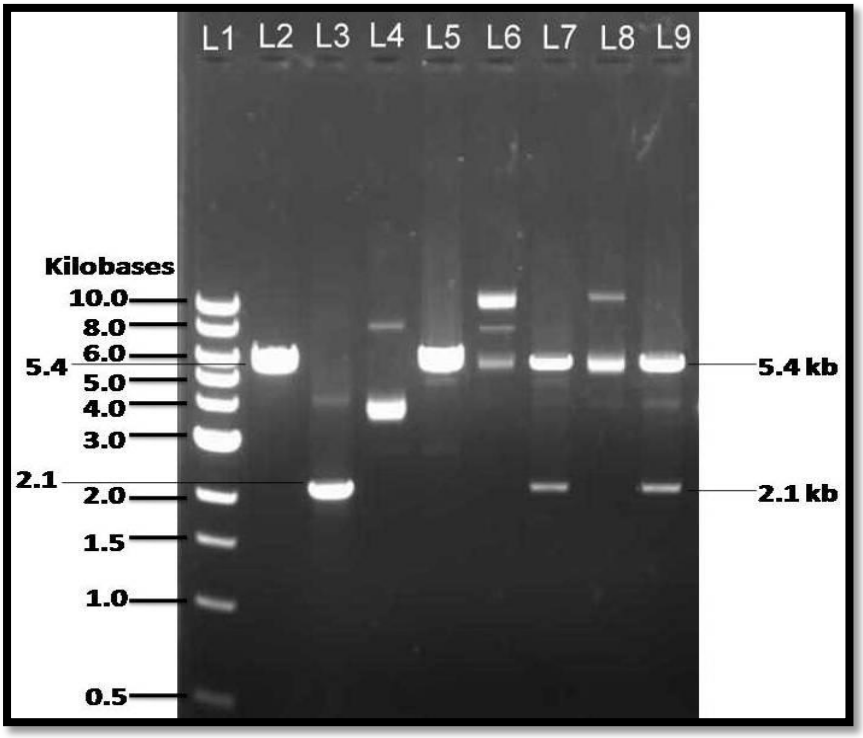

Figure 4.4 Agarose gel (0.8 %) electrophoresis for screening of the isolated plasmids from colonies obtained after transformation with ligation mix of pCDNA3.1^{TM}(+) vector and *TRAP1* insert by double digestion with BamH I and EcoR I. L1– 1 kb ladder. L2– double digested pCDNA 3.1 (+). L3– double digested *TRAP1* amplicon. L4/L6/L8– Undigested plasmid isolated from Colony I, II and III. L5, L7, L9– double digested plasmid isolated from colony I, II and III. L5 showed false positive result while L7 and L9 showed positive results with the plasmid containing the gene of interest.

The positive clone containing the recombinant plasmid was then enriched by growing a large scale culture and isolating plasmid from the culture using MN- Midi prep kit. Qualitative PCR was performed using the *TRAP1* internal primers for further validation of recombinant plasmids and the amplified PCR product (839 bp) could be seen on the gel (Fig. 4.6 L2).

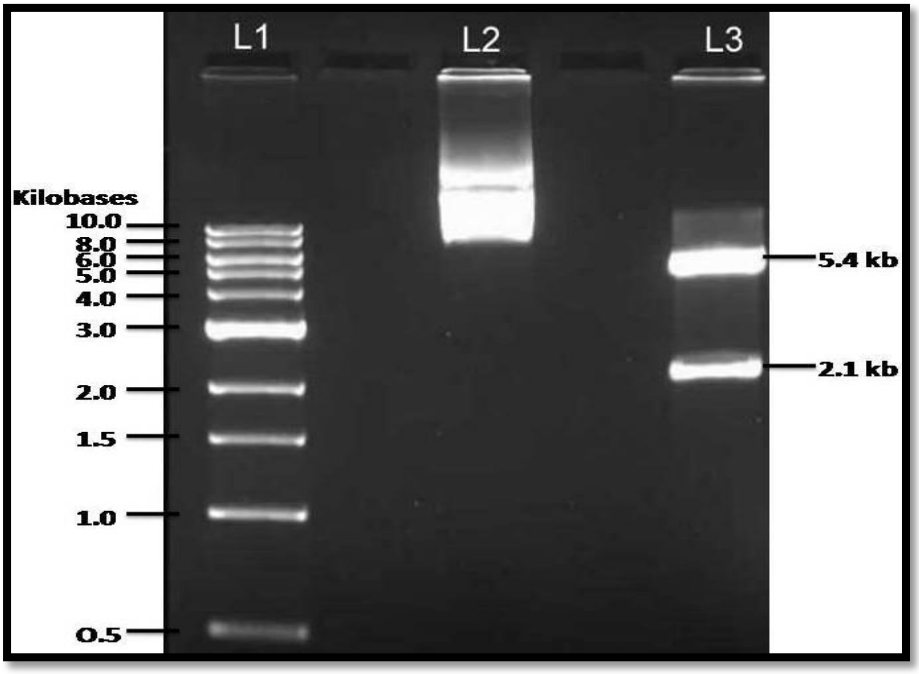

Figure 4.5 Agarose gel (0.8 %) electrophoresis of Enriched confirmed plasmid with TRAP1 insert followed by confirmation with double digestion By BamH I and EcoR I. L1– 1 Kb Ladder. L2– undigested recombinant plasmid with the TRAP1 insert. L3– double digested recombinant plasmid with release of *TRAP1* insert from the pCDNA3.1^{TM}(+) expression vector.

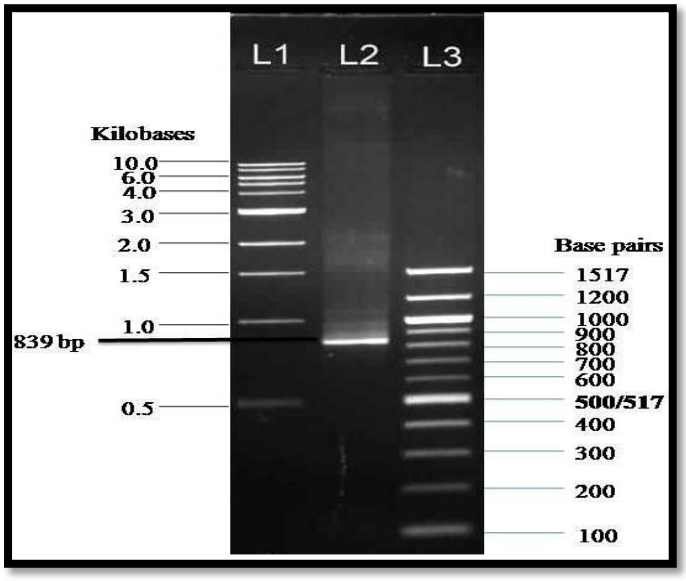

Figure 4.6 Agarose gel (1 %) electrophoresis for confirmation of *TRAP1* insert in pCDNA 3.1 (+) expression vector by PCR using *TRAP1* internal primers. L1– 1 Kb Ladder. L2– *TRAP1* amplicon with internal primers. L3– 100 bp Ladder.

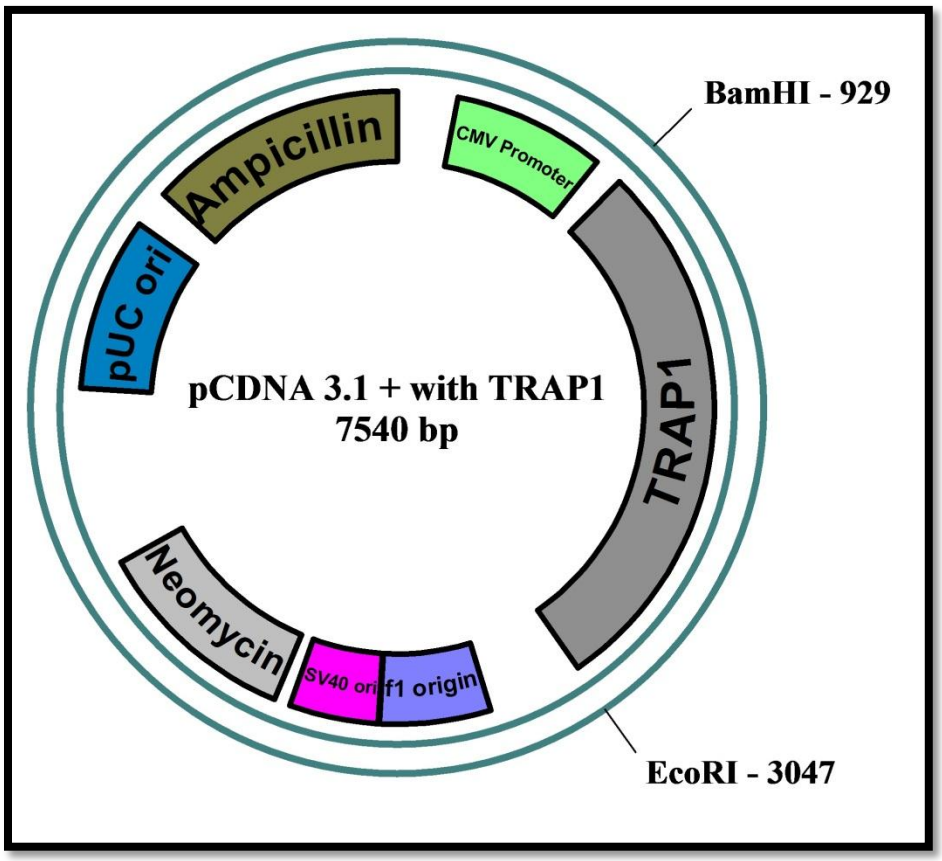

Figure 4.7 Vector map for recombinant plasmid pCDNA 3.1 (+) with TRAP1 (2112 bp) inserted between BamH I and EcoR I restriction sites.

4.2.1 Transfection into mammalian system

Recombinant TRAP1 plasmid cloned in pCDNA3.1 (+) mammalian expression vector was transfected into non-virally transformed lens epithelial cells- SRA01, SV40 Large T-antigen transformed kidney epithelial cells- HEK293T, and metastatic neuroblastoma cells- IMR-32 with the help of Lipofectamine LTX. Cells on 48 h post transfection were observed under inverted phase contrast microscope (10 x) and shown below.

Among the cells transfected with TRAP1, 1) SRA01 cells showed significant increase in cell volume with elongated morphology and enhanced cell-cell connections. 2) HEK293T cells did not show any morphological change. 3) IMR-32 cells exhibited slender elongated morphology. Compared to HEK293T and IMR-32 cells, SRA01 cells are non-virally

transformed and showed lower TRAP1 expression under normal physiological conditions (data not shown), which could one reason why cells could have become sensitive to enforced TRAP1 expression . Since HEK293T and IMR-32 cells already have increased TRAP1 (data not shown) at basal level, further expressing of TRAP1 in these cells might have influenced the cell proliferation potential (Fig 4.8).

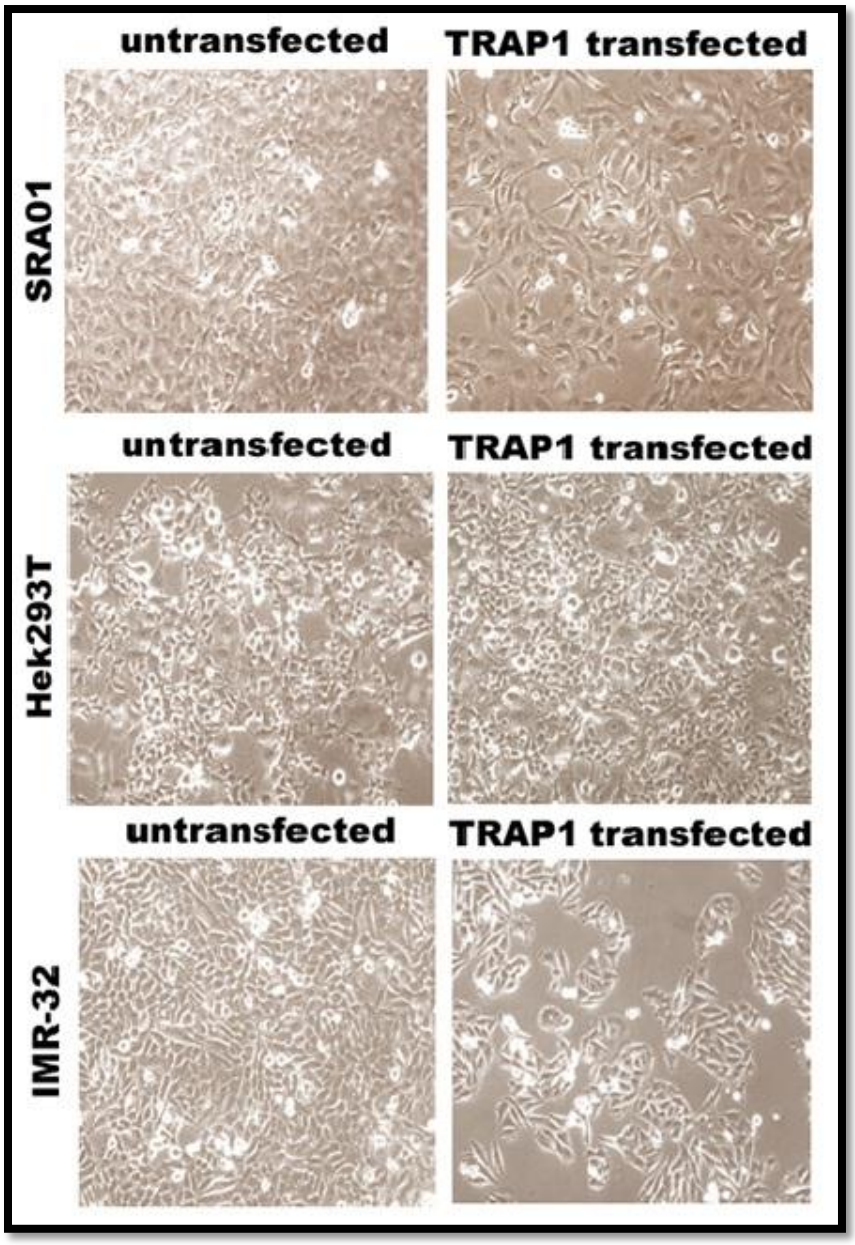

Figure 4.8 Cellular Morphology upon transfection with TRAP1

4.3 Sub-cloning of *Fis1* from pCDNA3.1^{TM}(+) expression vector into pCDNA^{TM}3.1/ myc-his(-) A expression vector

Fis1 in pCDNA3.1^{TM}(+) expression vector constructed earlier in the lab as template ws used as a source of DNA for *Fis1*. PCR was performed using primers specific for the full length CDS of *Fis1* and the PCR product was then run on 1% agarose gel electrophoresis. The amplified PCR product (459 bp) corresponding to *Fis1* CDS was observed (Fig. 4.10 L2-L5). The amplified PCR product was cleaned up using PCR clean up kit. The purified PCR product and pCDNA^{TM}3.1/ myc-his(-) A expression vector were subjected to double digestion with BamH I and Hind III for 2 h at 37 ^{o}C. The products were then ran on 1 % agarose gel and purified using Gel extraction kit mentioned in materials and methods. The double digested vector and insert were then ligated in the presence of T4 DNA ligase with ATP overnight at 16 ^{o}C.

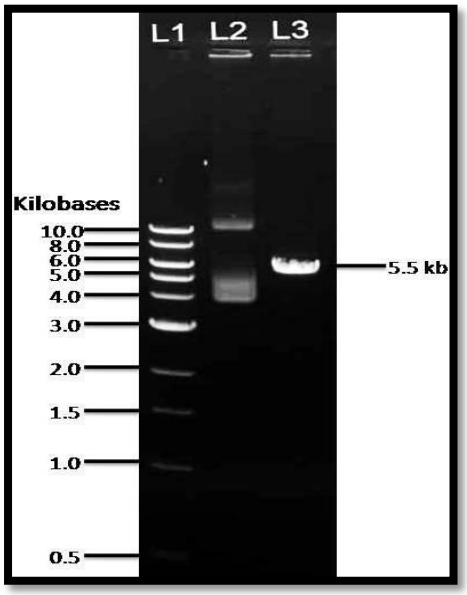

Figure 4.9 Agarose gel (0.8 %) electrophoresis for double digested pCDNA^{TM}3.1/ myc-his(-) A. L1– 1 Kb Ladder. L2– Undigested pCDNA^{TM}3.1/myc-his(-) A. L3– BamH I and Hind III Digested pCDNA^{TM}3.1/myc-his(-) A.

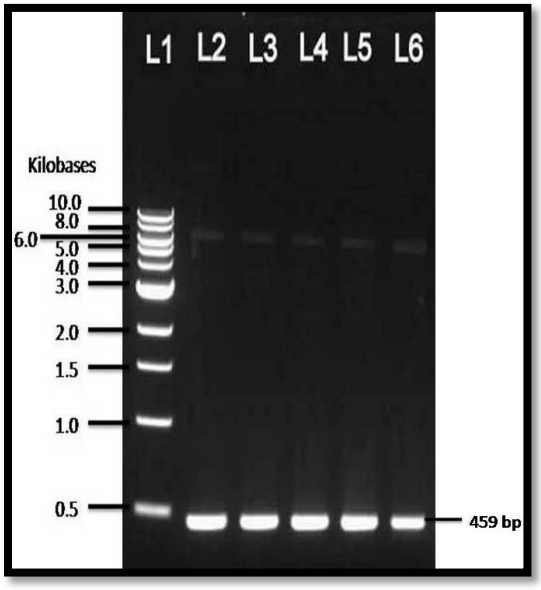

Figure 4.10 Agarose gel (1 %) electrophoresis for scale up PCR amplified *Fis1* amplicon. L1– 1 kb Ladder. L2 to L6– *Fis1* PCR amplicon obtained by Phusion High Fidelity Taq polymerase.

Ultra competent *E. coli* DH5α cells were transformed with the ligation mixture and the recombinant colonies were selected by plating the cells into the LB medium containing ampicillin (100 µg/mL) as selection marker and incubating at 37 ^{o}C overnight. The colonies were then screened for the recombinant plasmid by isolating plasmids from the cells and subjecting to double digestion with BamH I and Hind III. The plasmids showing the insert (459 bp) released were considered to be positive clones (Fig 4.11 L5, L7 and L9).

The recombinant plasmid was further enriched by growing a large scale culture at 37 ^{o}C overnight followed by plasmid isolation. The isolated plasmid was subjected to single digestion to see the base shift and double digestion for the confirmation of insert in the expression vector (Fig 4.12 L3 and L4). The presence of the *Fis1* insert was further confirmed by PCR using the *Fis1* gene specific primers (Fig. 4.13 L2 and L4).

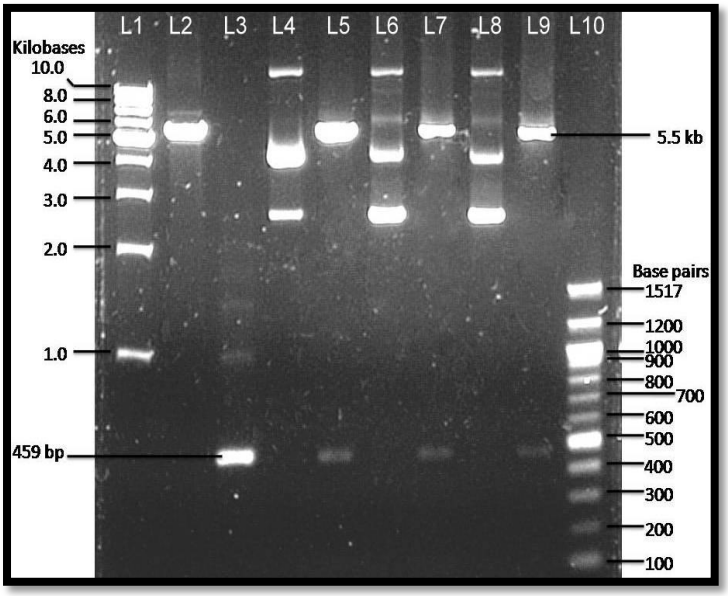

Figure 4.11 Agarose gel (0.8 %) electrophoresis for screening of the isolated plasmids from colonies obtained after transformation with ligation mix of pCDNA^{TM}3.1/myc-his(-) A expression vector and *Fis1* insert by double digestion with BamH I and Hind III. L1– 1 kb ladder. L2– double digested pCDNA^{TM}3.1/myc-his(-) A. L3– double digested *Fis1* amplicon. L4/L6/L8– Undigested plasmid isolated from Colony I, II and III. L5, L7, L9– double digested plasmid isolated from colony I, II and III. All the isolated plasmids gave positive results with insert band seen at 459 bp.

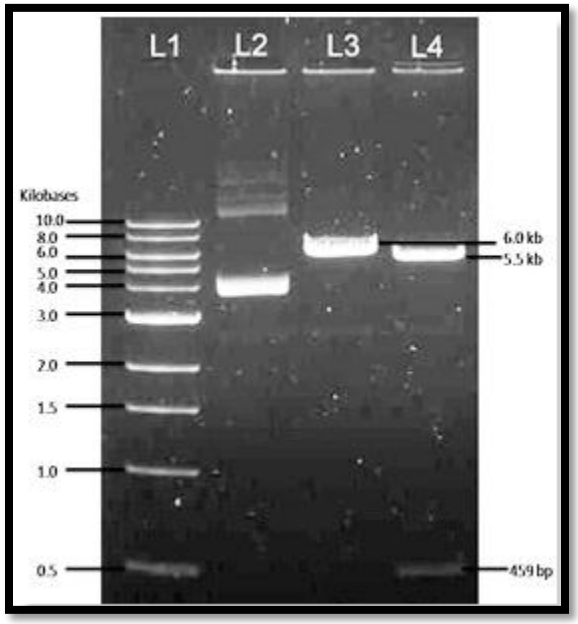

Figure 4.12 Agarose gel (1 %) electrophoresis for confirmation of recombinant plasmid (*Fis1* insert in pCDNA^{TM}3.1/myc-his(-) A after midi-prep isolation. L1– 1 Kb Ladder. L2–

undigested pCDNA^{TM}3.1/myc-his(-) A with *Fis1*. L3– single digested pCDNA^{TM}3.1/myc-his(-) A with *Fis1* insert by Hind III. L4– double digested pCDNA^{TM}3.1/myc-his(-) A with *Fis1* insert released by BamH I and Hind III.

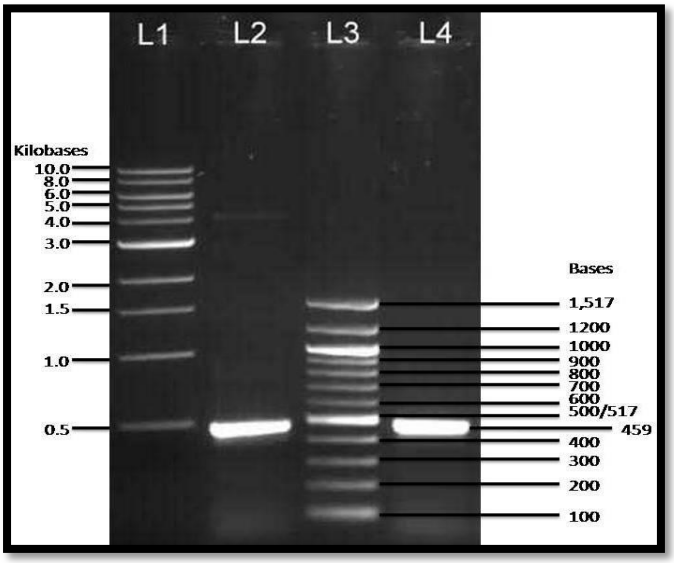

Figure 4.13 Agarose gel (1 %) electrophoresis for confirmation of *Fis1* insert in Recombinant plasmid [pCDNA^{TM}3.1/myc-his(-) A + *Fis1*]. L1– 1 Kb Ladder. L2– *Fis1* amplicon from the *Fis1* in pCDNA3.1^{TM}(+) expression vector. L3– 100 bp Ladder. L4– Fis1 amplicon from the newly sub - cloned *Fis1* construct in pCDNA^{TM}3.1/myc-his(-) A.

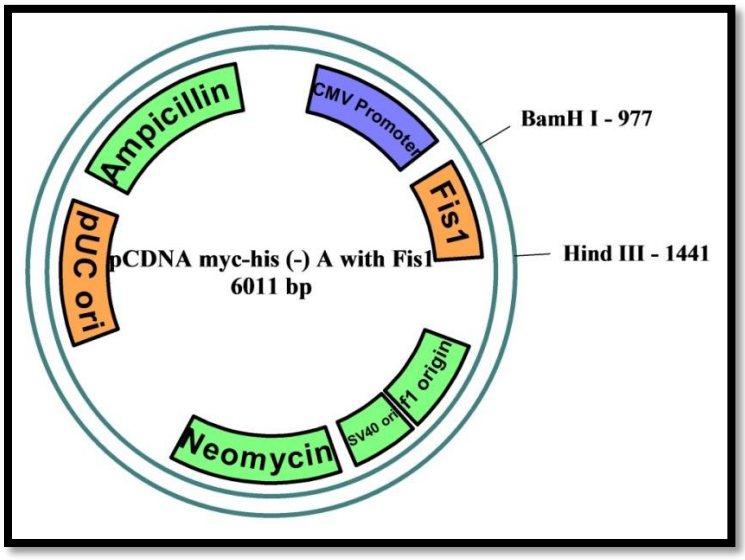

Figure 4.14 Vector map for recombinant plasmid pCDNA^{TM}3.1/myc-his (-) A with Fis1 (459 bp) inserted between BamH I and Hind III restriction sites.

4.3.1 Transfection into mammalian system

Recombinant *Fis1* plasmid cloned in pCDNA^{TM}3.1/myc-his(-) A mammalian expression vector was transfected into lens epithelial cells- SRA01, kidney epithelial cells- HEK293T, and metastatic neuroblastoma cells- IMR-32 with the help of Lipofectamine LTX. Cells on 48 h post transfection were observed under inverted phase contrast microscope (10 x) and shown below.

Among the cells transfected with *Fis1*, SRA01 and HEK293T did not show significant morphological changes. However, IMR-32 showed decrease in cellular volume and elongation with shrunken stubby morphology of cells. In comparison to control cells, the *Fis1* transfected IMR-32 cells were unable to proliferate.

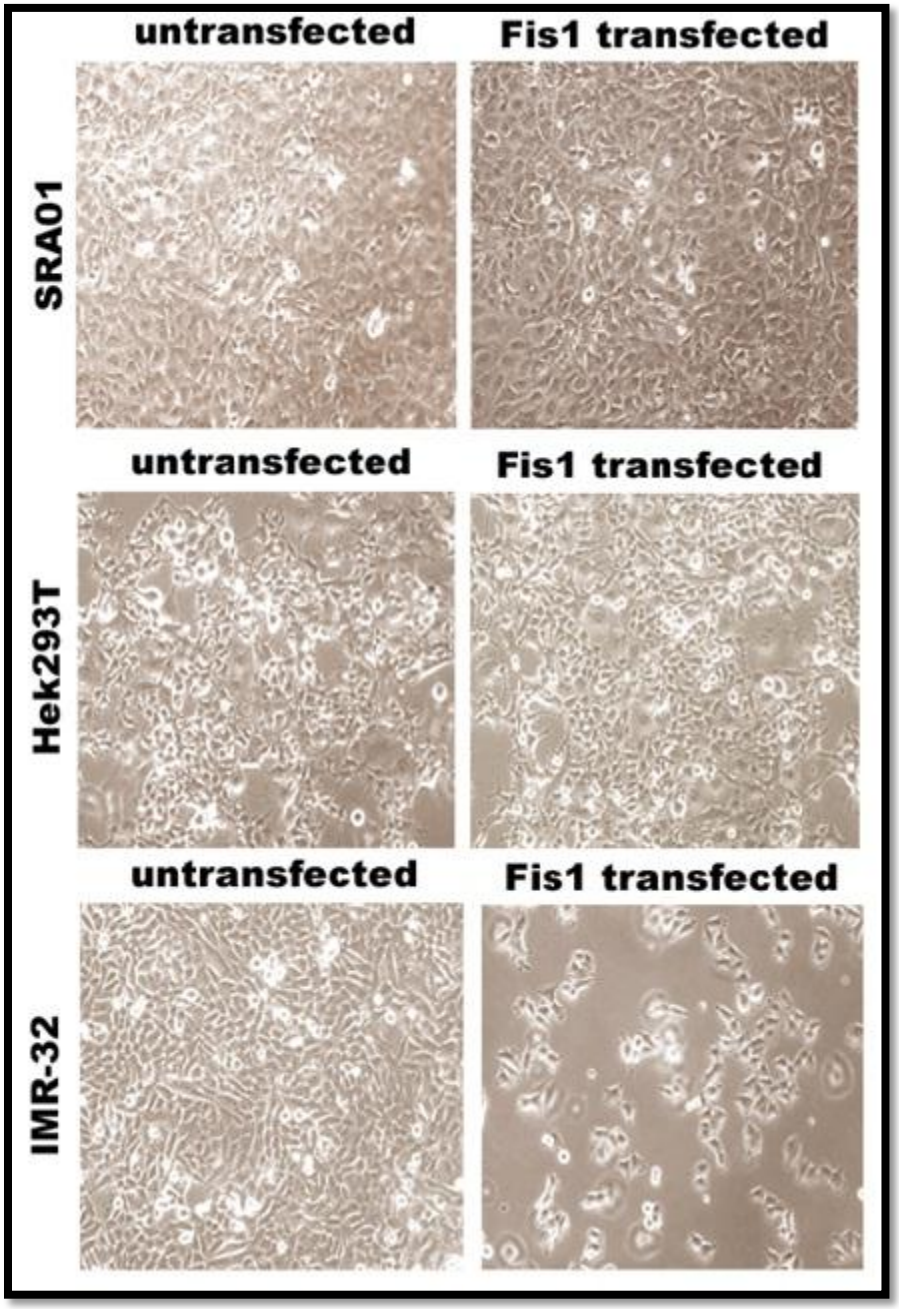

Figure 4.15 Cellular Morphology upon transfections with *Fis1*.

4.4 Cloning *Drp1* into FLAG-C2 expression vector

Using IMR-32 cDNA as template, PCR for *Drp1* was performed using primers specific for the full length CDS of *Drp1* and examined on 1 % agarose gel (Fig 4.17 L2). The PCR product was cleaned up using PCR clean up kit. FLAG-C2 expression vector (4 Kb) and purified PCR product were subjected to double digestion using BamH I and Sal I for 2 h at 37 ^{o}C. The products were then run on 1% agarose gel followed by gel extraction. The purified double digested vector and insert were then ligated in the presence of T4 DNA ligase at 16 ^{o}C overnight.

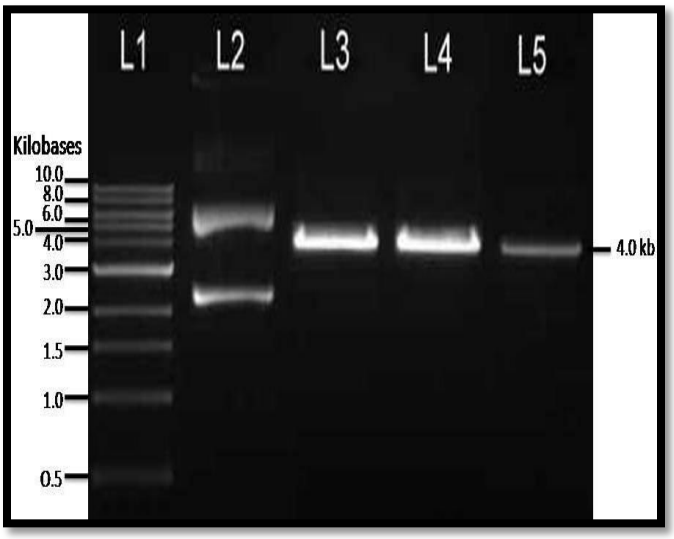

Figure 4.16 Agarose gel (0.8 %) electrophoresis of single and double digested C2 Flag Plasmid. L1 – 1 Kb Ladder. L2– undigested C2 Flag. L3– BamH I digested C2 Flag. L4– Sal I digested C2 Flag. L5– BamH I and Sal I digested C2 Flag expression vector.

Figure 4.17 Agarose gel (1 %) electrophoresis of *Drp1* Amplified using high fidelity proof reading taq polymerase enzyme. L1– 1 Kb Ladder. L2– *Drp1* amplicon obtained after PCR with High fidelity Taq polymerase enzyme.

Ultra competent *E. coli,* DH5α strain were transformed with the ligation mixture and the recombinant colonies were selected by plating the cells onto the LB medium containing kanamycin (50 µg/mL) as selection marker and incubating at 37 ^{o}C overnight. The colonies were then screened for the recombinant plasmid by isolating plasmids from the cells and subjecting to double digestion with BamH I and Sal I. The plasmids showing the insert (2.2 kb) released were considered to be positive clones (Fig 4.18 L6, L7, L12, and L16).

The recombinant plasmid was further enriched by growing a large scale culture at 37 ^{o}C overnight followed by plasmid isolation. The presence of the *Drp1* insert was further confirmed by PCR using the *Drp1* gene specific internal primers (Fig. 4.20).

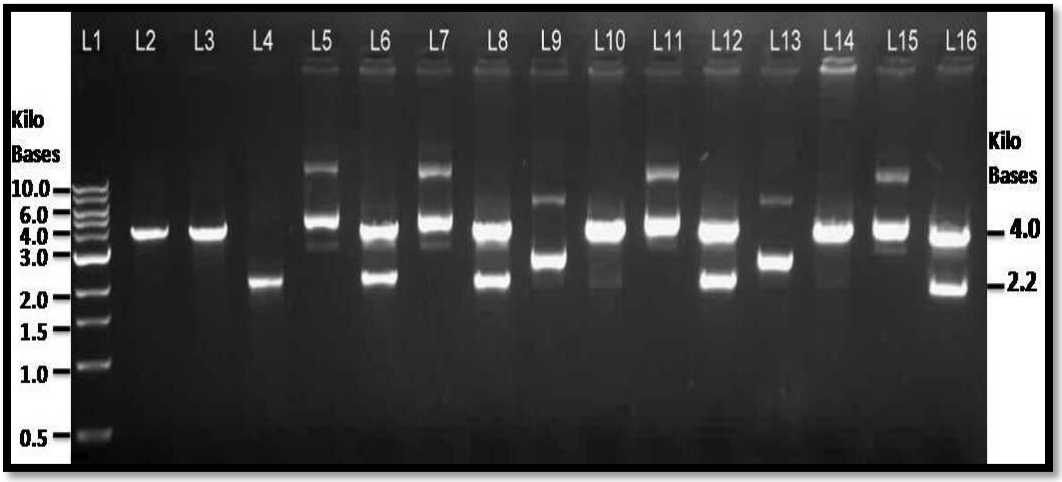

Figure 4.18 Agarose gel (0.8 %) electrophoresis for screening of plasmids obtained after transformation of E. coli DH5α with the ligation mixture of double digested C2 flag expression vector and double digested *Drp1* insert. L1– 1 Kb Ladder . L2– Single Digested C2 Flag. L3– double digested C2 Flag expression vector. L4– double digested *Drp1* amplicon. L5/L7/L9/L11/L13/L15– Undigested plasmid from colony I, II, III, IV, V, VI. L6/L8/L10/L12/L14/L16– double digested plasmid from colony I, II, III, IV, V, VI. Plasmids from Colony I, II, IV and VI showed positive result with release of insert upon double digestion while Plasmids from Colony III and V were pseudo positive colonies.

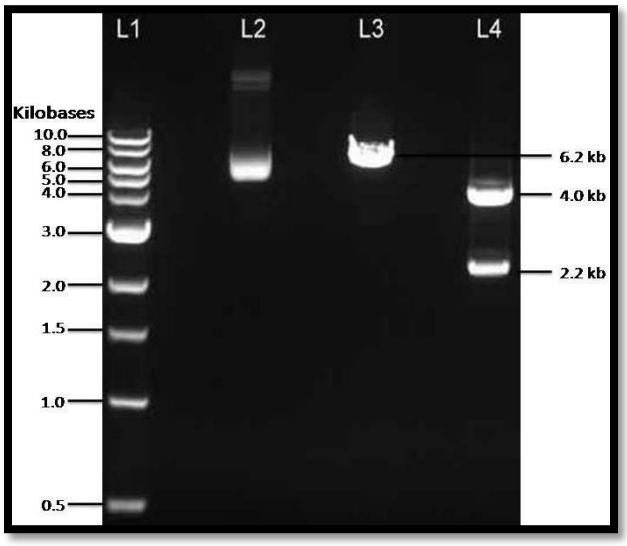

Figure 4.19 Agarose gel (1 %) electrophoresis for confirmation of recombinant plasmid (*Drp1* insert in C2 Flag expression vector) after midi prep isolation. Lane 1– 1 Kb Ladder. L2– Undigested C2 flag. L3– Single Digested C2 flag expression vector with *Drp1* insert with BamH I. L4– Double Digested C2 Flag expression vector with *Drp1* insert with BamH I and Sal I.

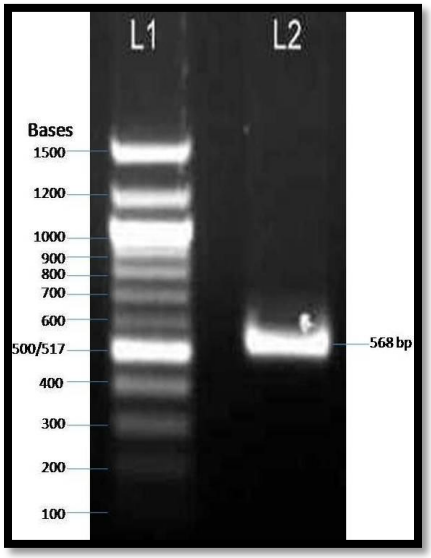

Figure 4.20 Agarose gel (1 %) electrophoresis for confirmation of *Drp1* insert in C2 Flag expression vector by PCR using *Drp1* internal primers. L1– 100 bp Ladder. L2– PCR product from *Drp1* in C2 flag expression vector construct with internal primers.

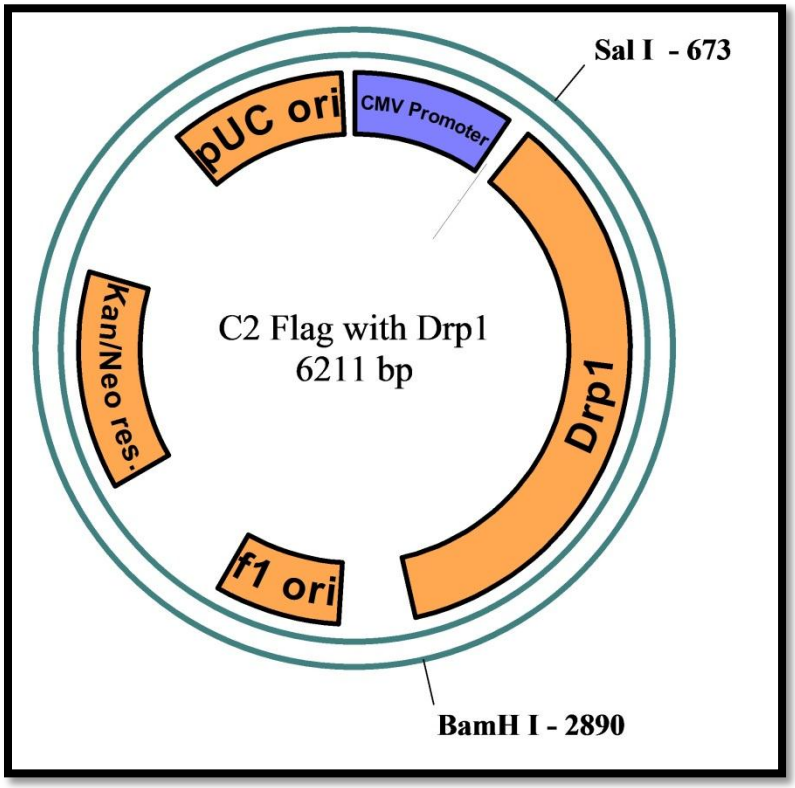

Figure 4.21 Vector map for recombinant plasmid C2 Flag with Drp1 (2211 bp) inserted between Sal I and BamH I restriction sites.

4.4.1 Transfection into mammalian system

Recombinant *Drp1* plasmid cloned in C2 Flag mammalian expression vector was transfected into lens epithelial cells- SRA01, kidney epithelial cells- HEK293T, and metastatic neuroblastoma cells- IMR-32 with the help of Lipofectamine LTX. Cells on 48 h post transfection were observed under inverted phase contrast microscope (10 x) and shown below.

Among the cells transfected with *Drp1*, SRA01 and HEK293T did not present significant morphological changes, however, IMR-32 cells showed extensive elongation with increase in cellular volume. The cells had tapered ends indicating the enhanced migration potential.

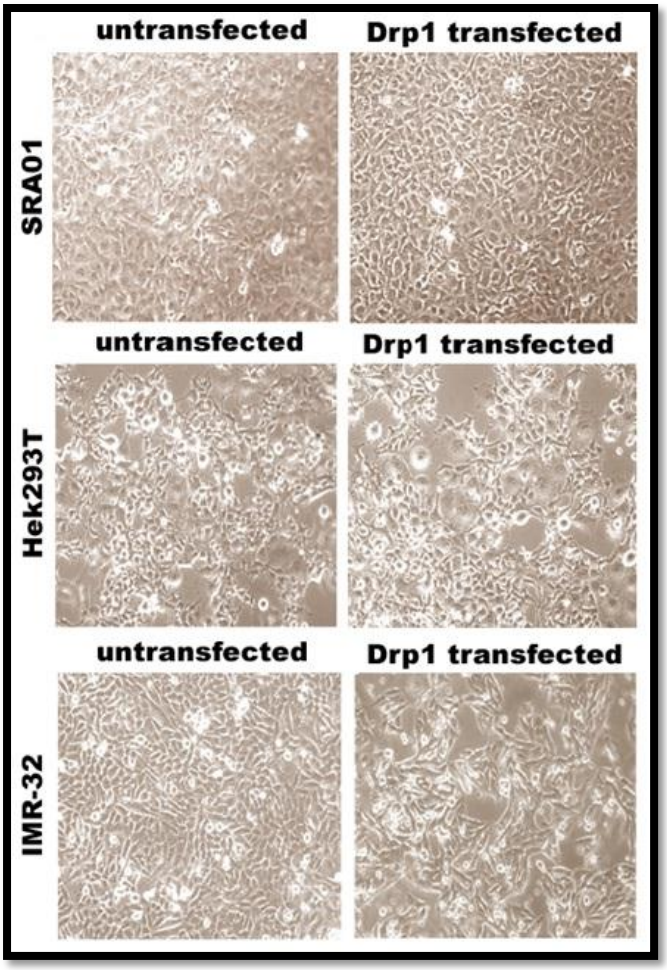

Figure 4.22 Cellular morphology upon transfections with Drp1.

4.5 Cloning *Mff* into FLAG-C2 expression vector

Using IMR-32 *Fis1* over expressed cDNA as template, PCR for *Mff* was performed using primers specific for full length CDS of *Mff* and examined on 1 % agarose gel. Non-specific bands could be observed on the gel, so the band closest to our gene of interest was cut out and reamplified using the same set of primers. The amplified PCR product (~ 1 Kb) (Fig. 4.24 L2) was obtained when compared with 1 Kb Ladder (Fig. 4.24 L1) indicating that *Mff* was amplified. The PCR product was cleaned up using PCR clean up kit. The purified PCR product and C2 flag expression vector were subjected to double digestion using BamH I and Sal I for 2 h at 37 ^{o}C. The double-digested products were then purified by gel elution using gel extraction kit. The purified double digested vector and insert were then ligated in the presence of T4 DNA ligase and ATP overnight at 16 ^{o}C.

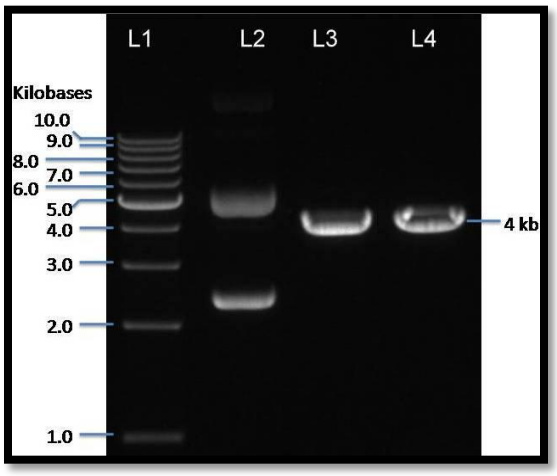

Figure 4.23 Agarose gel (0.8 %) electrophoresis of single and double digested C2 Flag Plasmid. L1– 1 Kb Ladder. L2– Undigested C2 Flag. L3– BamH I digested C2 Flag. L4– BamH I and Sal I digested C2 Flag.

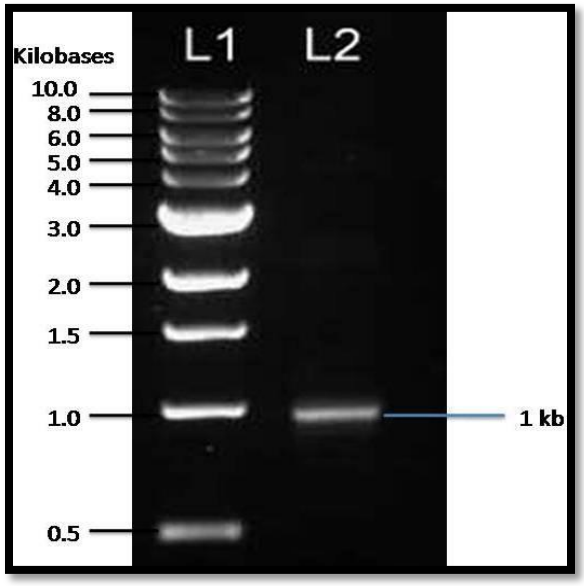

Figure 4.24 Agarose gel (1 %) electrophoresis of *Mff* amplified using high fidelity proof reading taq polymerase enzyme. L1– 1 Kb Ladder. L2– *Mff* amplicon obtained after PCR with High fidelity Taq polymerase enzyme.

Ultra competent DH5α *E. coli* cells were then transformed with the ligation mixture and the recombinant clones were selected by plating the transformed cells onto the LB-agar containing kanamycin (50 µg/mL) as selection marker. The plates were incubated at 37 ^{o}C overnight. Plasmid was isolated from a single colony obtained and then subjected to double digestion with BamH I and Sal I to release the cloned insert (1 kb) (Fig. 4.25 L6).

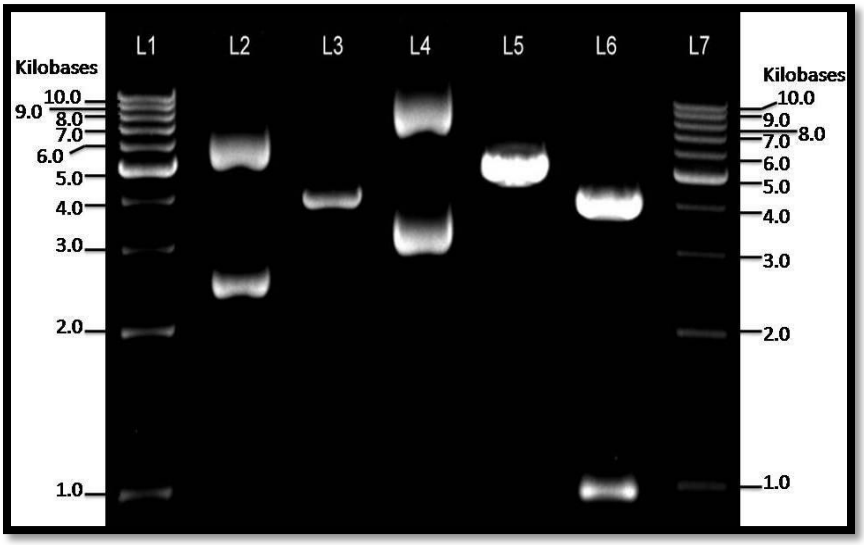

Figure 4.25 Agarose gel (0.8 %) electrophoresis for screening of plasmids obtained after transformation of E. coli DH5α with the ligation mixture of double digested C2 flag and double digested *Mff* insert. L1– 1 Kb Ladder. L2– undigested C2 Flag. L3– double digested C2 Flag. L4– undigested plasmid from the colony obtained after transformation. L5– Single digested plasmid from the colony obtained after transformation. L6– Double digested plasmid from the colony obtained after transformation. L7– 1 Kb Ladder.

The recombinant plasmid was then enriched by growing a large scale culture of the clone and isolated from the culture using MN- Midi. A Band of 589 bp obtained after PCR using specific internal primers further confirmed the presence of *Mff* insert in the C2 Flag expression vector (Fig. 4.26 L2).

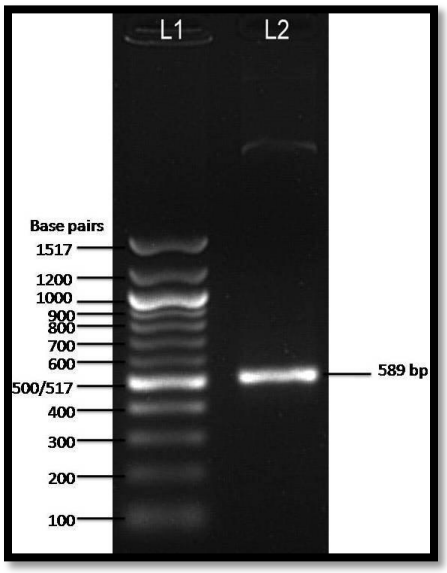

Figure 4.26 Agarose gel (1 %) electrophoresis for confirmation of *Mff* insert in C2 Flag expression vector by PCR using *Mff* internal primers. L1– 100 bp Ladder. L2– PCR product from *Mff* in C2 flag expression vector construct with internal primers.

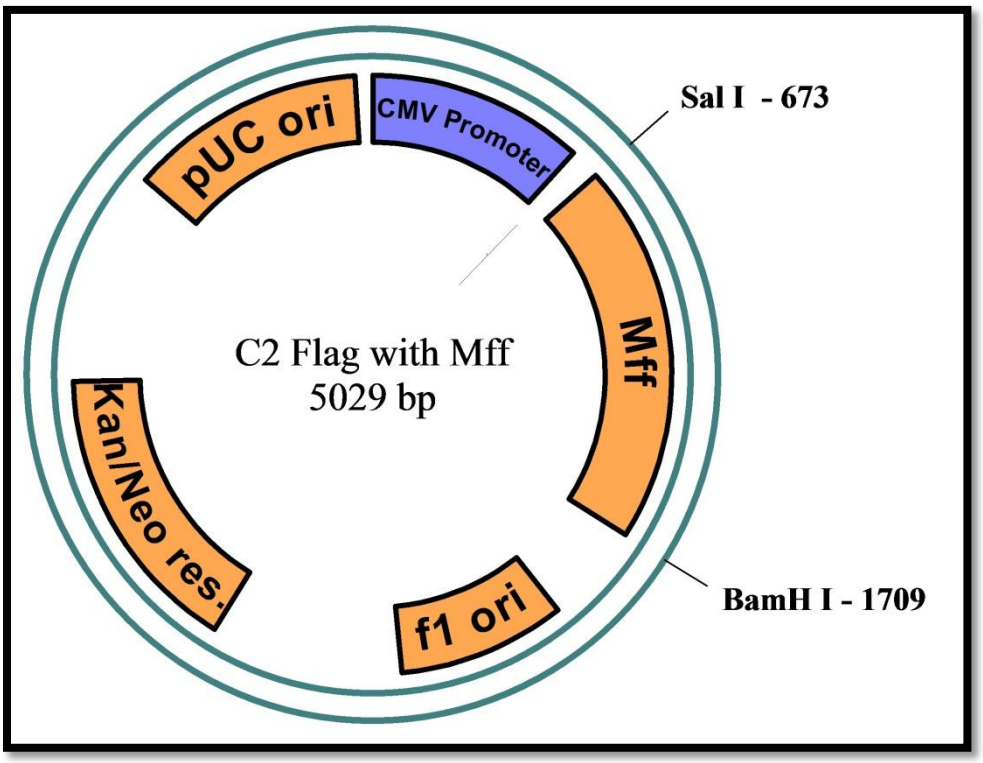

Figure 4.27 Vector map for recombinant plasmid C2 Flag with Mff (1029 bp) inserted between Sal I and BamH I restriction sites.

4.5.1 Transfection into mammalian system

Recombinant *Mff* plasmid cloned in C2 Flag mammalian expression vector was transfected into lens epithelial cells- SRA01, kidney epithelial cells- HEK293T, and metastatic neuroblastoma cells- IMR-32 with the help of Lipofectamine LTX. Cells on 48 h post transfection were observed under inverted phase contrast microscope (10 x) and shown below.

Among the cells transfected with *Mff*, significant change in the cellular morphology was not visible in any of the cell lines.

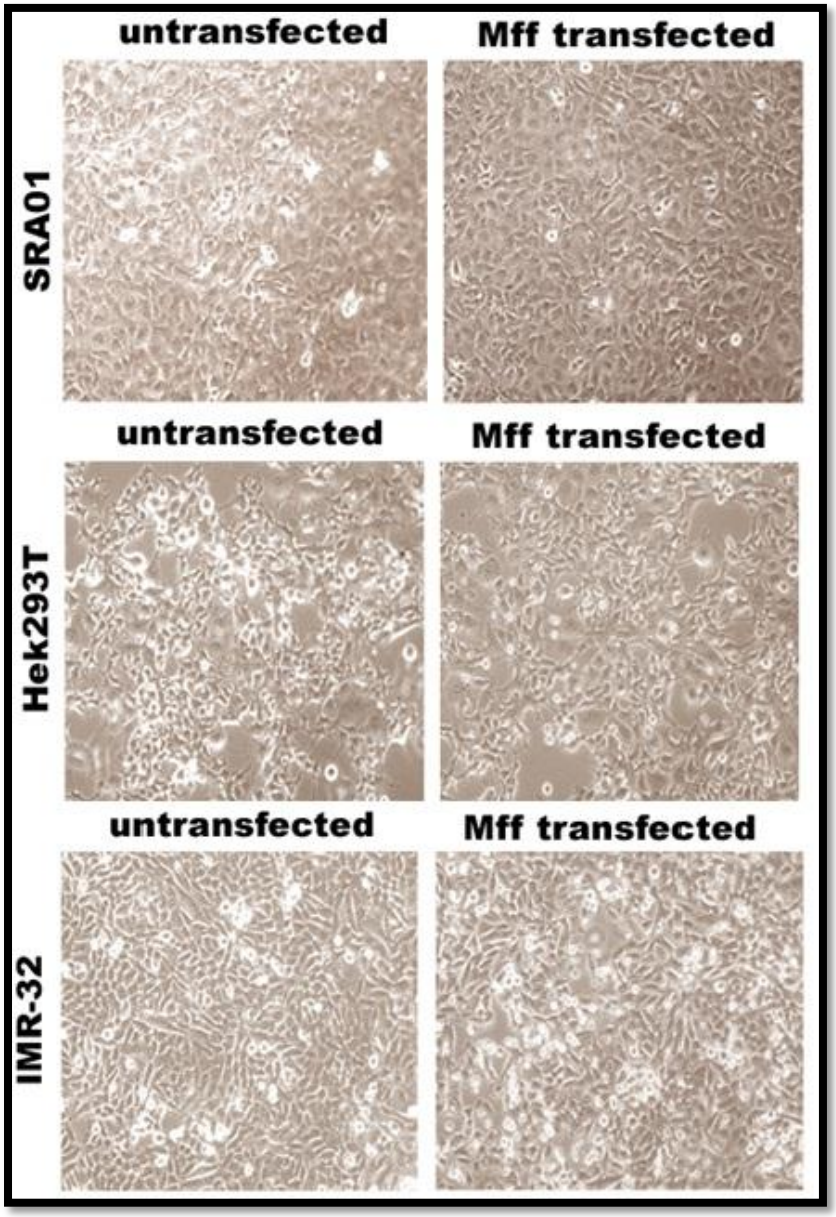

Figure 4.28 Cellular Morphology upon transfections with *Mff*.

4.6 CMX-ROS staining

The mitochondrial organization was studied using untransfected cells and cells transfected with different mammalian expression vectors. Upon over expression of TRAP1, IMR-32 cells showed enhanced mitochondrial circularization which is comparable with that of over expression of fission genes such as *Fis1*, *Drp1* and *Mff*. More interestingly, cells transfected with TRAP1 showed enhanced mitochondrial fission, than the over expression of individual fission genes.

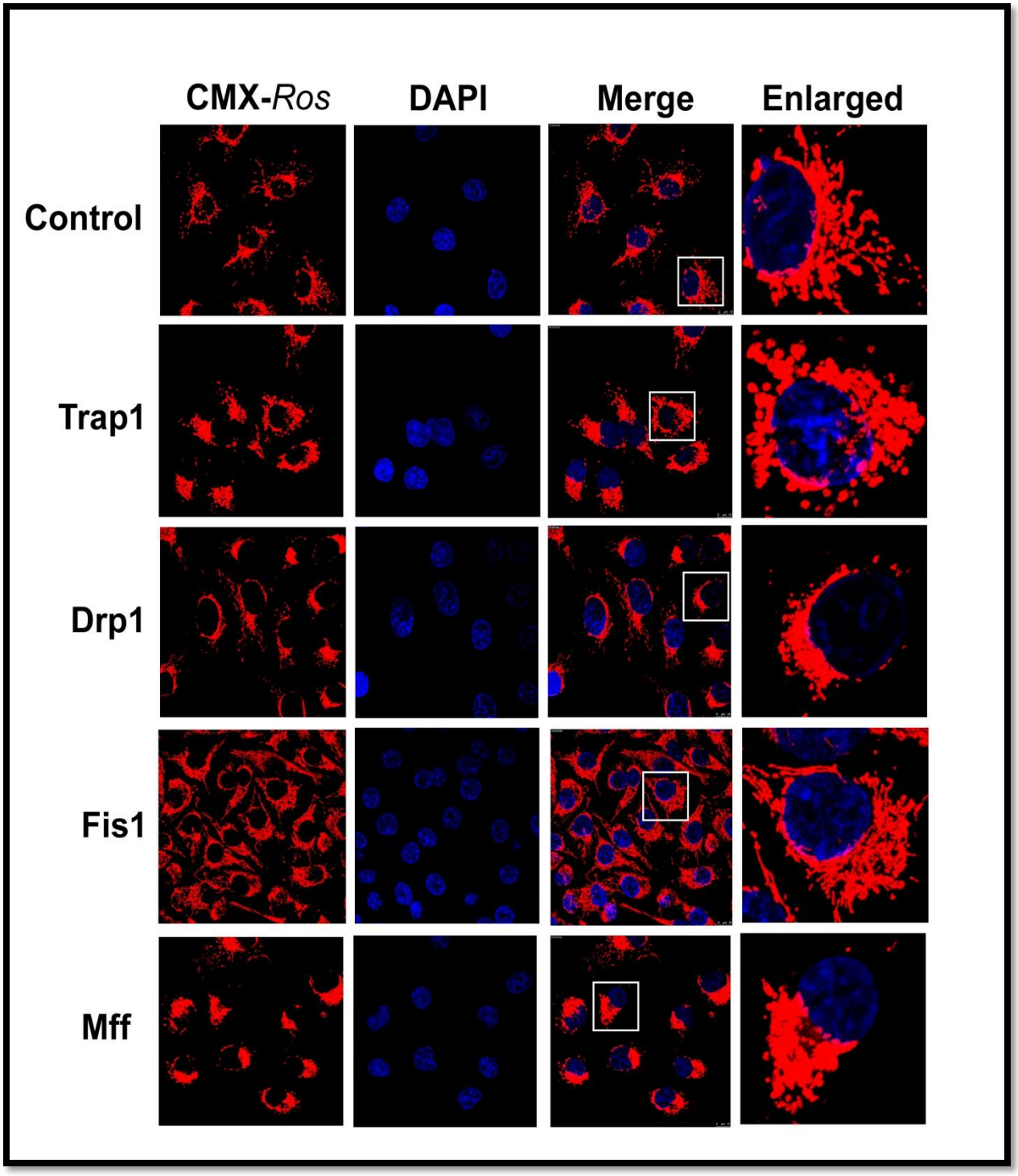

Figure 4.29 Mitochondrial morphology of untransfected IMR-32 cells, *TRAP1* transfected IMR-32 cells, *Drp1* transfected IMR-32 cells, *Fis1* transfected IMR-32 cells and *Mff* transfected IMR-32 cells stained with CMX-ROS and DAPI observed under scanning confocal imaging microscope (63x).

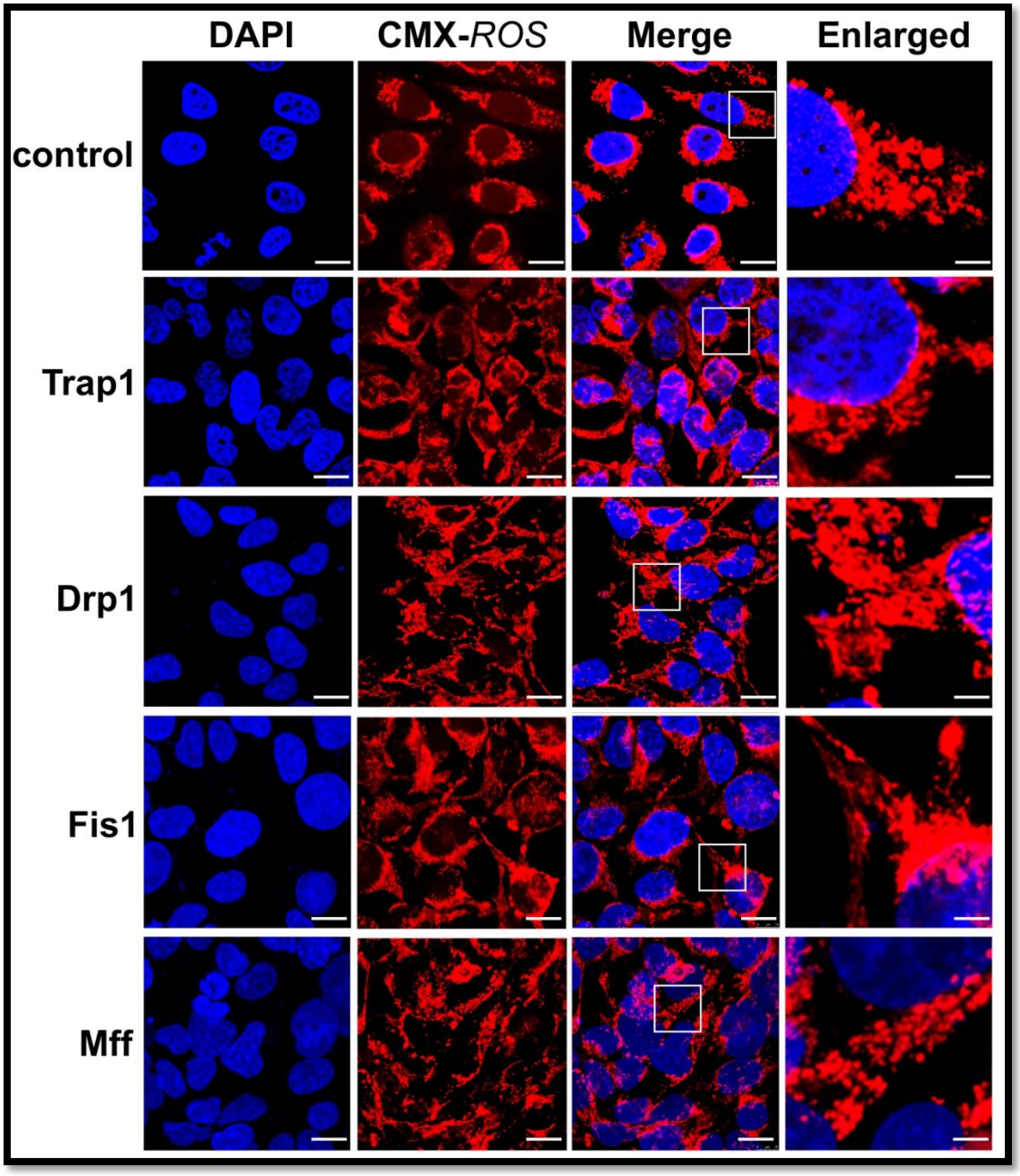

Figure 4.30 Mitochondrial morphology of untransfected HEK293T cells, *TRAP1* transfected HEK293T cells, *Drp1* transfected HEK293T cells *Fis1* transfected HEK293T cells and *Mff* transfected HEK293T cells stained with CMX-ROS and DAPI observed under scanning confocal imaging microscope (63x).

These results have indicated that TRAP1 probably facilitates mitochondrial fission by functional stabilization of either one or more of the fission proteins. Since none of the

transfected cells showed cell death or deformity with respect to nuclear integrity, the hypothesis that TRAP1 involvement in only promoting or facilitating the mitochondrial fission is being strengthened (Fig. 4. 29).

Unlike IMR-32, TRAP1 over expression in HEK293T failed to show significant changes in mitochondrial organization. Similar findings were observed upon *Drp1* or *Fis1* over expression. In comparison with *Drp1* and *Fis1, Mff* over expression showed distinct mitochondria. However, further studies are required to confirm whether these changes are related to mitochondrial fission or loss of mitochondrial integrity (Fig. 4.30).

It is known that mitochondrial dynamics influences the cell fate with respect to proliferation, growth and disease progression. Our preliminary studies have indicated that compared to normal cells, tumor cells have more number of mitochondria, which may be required for the increased rate of cellular metabolism (Wallace, 2012). In support of this, among the tumor phenotypes, metastatic and highly drug resistant cells showed further increases in TRAP1 expression suggested us that TRAP1 may be involved in enhanced mitochondrial energetics (Altieri et al., 2012). There are two major contradicting hypotheses which have emerged and are being equally agreed upon i. e 1) TRAP1 expression levels are sufficient for the functional stabilization of mitochondria while the other 2) TRAP1 differential localization is required for the functional stabilization of mitochondria (Amoroso et al., 2012). Subsequent studies have indicated that both the hypotheses were made with respect to TRAP1 extra mitochondrial functions, but not mitochondrial energetics (Liu et al., 2010). Otherwise, these hypotheses would actually weaken the Warburg's hypothesis (Warburg, 1956) that mitochondria may be dispensable for energy production in cancer cells as they depend on anaerobic metabolism but not on oxidative phosphorylation. However, Warburg's hypothesis has now become optional for cancer cells since they indeed opt for OXPHOS under a large variety of altered physiological conditions at least if not under normal physiological conditions.

None of the studies have found actual correlation of mitochondrial dynamics with that of TRAP1 despite the hypothesis that mitochondrial dynamics regulate the cell fate through regulating the mitochondrial energetics. The present study therefore, is aimed at understanding the role of TRAP1 in regulating mitochondrial dynamics; especially the fusion and fission states attributing to the functional/disease state of the cell. As per our hypothesis, we found alterations in mitochondrial re-organization with TRAP1 over

expression (from the present data) and TRAP1 knockdown (previous unpublished data). While TRAP1 knockdown promoted mitochondrial fusion, in the present we show that TRAP1 over expression is leading to enhanced mitochondrial fission. The intriguing feature of mitochondrial fission is a cross-talk between cytosolic ligands such as Drp1 and mitochondrial resident receptors, Fis1 and Mff. Over expression of ligand/receptors showed a marginal increase in mitochondrial fission, however over expression of TRAP1 showed significant enhancement of fission indicating that TRAP1 may be acting as chaperone for these fission proteins or TRAP1 interaction with the proteins may interfere with the fusion process. Fis1 contain the TPR motif; hence one would definitely expect its interaction with chaperone like TRAP1 that belongs to Hsp family of proteins (Song et al., 1995). But, unlike other chaperones, TRAP1 does not contain the TPR recognition domain (Felts et al., 2000), hence TRAP1 interaction with fission proteins if promoting fission may be through interaction with unexplored regions/domains. The only way one can demonstrate these events is to extend the study to protein-protein interaction both under over expression as well as knockdown backgrounds. At this point in time, we do not have antibodies to any of the fission/fusion proteins in the lab. Although, I have started working on understanding these interactions with the help of affinity tag-based immunoprecipitation, it is very difficult to map these interactions with respect to a specific domain or signature. Due to time constraint, although I am unable to extend my studies to protein-protein interaction, I am sure the next student who will be taking up this project may look for such an interaction and explore the mechanism of TRAP1 mediated mitochondrial fission.

Chapter 5

Conclusion

The present study has provided clues that TRAP1 regulates mitochondrial dynamics, especially the fission events. Further, our results have indicated that over expression of TRAP1 in tumor cells (IMR-32) has enhanced effect on mitochondrial fission compared to normal cells (HEK293T). This is actually coinciding with the hypothesis that tumor cells express high amounts of TRAP1; hence a small increase immediately overloads the expression pattern and imparts the desired effect. Whereas in normal cells, TRAP1 levels are low and hence transient transfection could not overload cells with TRAP1, hence mitochondrial fission appeared may be low. Mitochondrial fission increases the number of mitochondria thus, is directly proportional to the increased ATP levels. Since increased energy favors enhanced cell metabolism to promote diverse cellular mechanisms that in turn favor tumor growth/progression, TRAP1 may be directly favoring tumor growth. Since cancer cells are good at adapting to the tumor micro-environment they must be equipped with the fission proteins and further loading with TRAP1 might have resulted in promoting fission events. Since normal cells lack this kind of adaptation, over expressing TRAP1 alone in the absence of increased fission genes might have resulted in decreased mitochondrial fission. However, further studies are required to demonstrate how and when TRAP1 facilitates such mechanism. Subsequent cellular energetic analysis under different physiological states probably provides additional information and mechanistic insights of TRAP1 role in tumor cells in mitochondrial dynamics and energetics.

Recommendations:

1. The present study focused on the overexpression of individual genes in the cells i.e. TRAP1 and genes required for mitochondrial fission. The next plausible step would be to overexpress these genes in combination (TRAP1 + Fis1, TRAP1 + Drp1, TRAP1 + Mff and other possible combinations) and study the mitochondrial morphology.
2. With the combined overexpression, the cellular energetics can be studied with respect to the functional and structural state of mitochondria.
3. Study of mitochondrial dynamics in presence of drugs like shepherdin which inhibits the functions of TRAP1 can also be done.
4. Study of domain-domain interaction between the TRAP1 and proteins required for mitochondrial fission.

References

Akner G, Mossberg K, Sundqvist KG,Gustafsson JA, and Wikstrom, AC (1992) Evidence for reversible, non-microtubule and non-microfilament-dependent nuclear translocation of hsp90 after heat shock in human fibroblasts. Eur. J. Cell Biol. 58(2): 356 - 364

Alexieva V, Sergiev I, Mapelli S, and Karanov, E(2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ. 24 (12): 1337 - 1344

Alsbury S, Papageorgiou K, and Latchman, DS (2004) Heat shock proteins can protect aged human and rodent cells from different stressful stimuli. Mech. Ageing Dev. 125(3): 201 - 209

Altieri DC, Stein GS, Lian JB and Languino LR (2012) TRAP1, the mitochondrial Hsp90. Biochimica et Biophysica Acta 1823(3): 767-773

Amoêdo ND, JP, Rodrigues MF, Galina A, and Rumjanek FD (2013) How does the metabolism of tumour cells differ from that of normal cells. Biosci Rep. 33(6): e00080

Amoroso MR, Matassa DS, Laudiero G, Egorova AV, Polishchuk RS, Maddalena F, Piscazzi A, Paladino S, Sarnataro D, Garbi C, Landriscina M, Esposito F (2012) TRAP1 and the proteasome regulatory particle TBP7/Rpt3 interact in the endoplasmic reticulum and control cellular ubiquitination of specific mitochondrial proteins. Cell Death Differ. 19(4): 592 - 604

Bardwell JCA and Craig EA (1988) Ancient heat shock gene is dispensable. J. Bacteriol. 170(7): 2977–2983

Benjamin IJ and McMillan DR (1998) Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circ Res. 83(2): 117 - 132

Berman SB, Pineda FJ, Hardwick JM (2008) Mitochondrial fission and fusion dynamics: the long and short of it. Cell Death Differ. 15(7): 1147 - 1152

Bertram JS (2000) The molecular biology of cancer. Mol Aspects Med. 21(6): 167 - 223

Bogenhagen D and Clayton DA (1977). Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. Cell. 11(4): 719–727

Cechetto JD and Gupta RS (2000) Immunoelectron microscopy provides evidence that tumor necrosis factor receptor-associated protein 1 (TRAP-1) is a mitochondrial protein which also localizes at specific extramitochondrial sites. Exp Cell Res. 260(1): 30 - 39

Chang CR and Blackstone C (2010) Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. Ann N Y Acad Sci. 1201: 34 - 39

Chen CF, Chen Y, Dai K, Chen PL, Riley DJ and Lee WH (1996) A new member of the hsp90 family of molecular chaperones interacts with the retinoblastoma protein during mitosis and after heat shock. Mol Cell Biol. 16(9): 4691–4699

Chen H and Chan DC (2004) Mitochondrial dynamics in mammals. Curr Top Dev Biol. 59: 119 - 144

Cipolat S, de Brito OM, Dal Zilio B, and Scorrano L (2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A. 101(45): 15927–15932

Collins TJ, Berridge MJ, Lipp P and Bootman MD (2002) Mitochondria are morphologically and functionally heterogeneous within cells. EMBO J. 21(7): 1616 - 1627

Csermely P, Schnaider T, Soti C, Prohászka Z and Nardai G (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther.79(2): 129 – 168

Diaz-Cano SJ (2015) Cancer: Etiologic Agents and General Mechanism [Powerpoint Presentation]. Retrieved from http://www.powershow.com/view/3b0b00-NWZjO/CANCER_ETIOLOGIC_AGENTS_AND_GENERAL_MECHANISMS_powerpoint_ppt_presentation.

Delettre C, Griffoin J-M, Kaplan J, Dollfus H, Lorenz B, Faivre L, Lenaers G, Belenguer P and Hamel CP (2001) Mutation spectrum and splicing variants in the OPA1 gene. Hum Genet. 109 (6): 584-91

Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, Rustin P, Gustafsson CM and Larsson N (2004) Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum. Mol. Genet.* 13 (9): 935-944

Feder ME and Hofmann GE (1999) HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. Annual Review of Physiology. 61: 243 - 282

Felts SJ, Owen BA, Nguyen P, Trepel J, Donner DB and Toft DO (2000) The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. J Biol Chem. 275(5): 3305 - 3312

Ferreira-da-Silva A, Valacca C, Rios E, Pópulo H, Soares P, Sobrinho-Simões M, Scorrano L, Máximo V and Campello S (2015) Mitochondrial Dynamics Protein Drp1 Is Overexpressed in Oncocytic Thyroid Tumors and Regulates Cancer Cell Migration. PLoS One. 10(3): e0122308

Fulda S, Gorman AM, Hori O and Afshin Samali (2010) Cellular Stress Responses: Cell Survival and Cell Death. International Journal of Cell Biology. Volume 2010 , Article ID 214074, 23 pp

Gandre-Babbe S and van der Bliek AM (2008) The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell. 19(6): 2402 - 2412

Gesualdi NM, Chirico G, Pirozzi G, Costantino E, Landriscina M and Esposito F (2007) Tumor necrosis factor-associated protein 1 (TRAP-1) protects cells from oxidative stress and apoptosis. Stress. 10(4): 342 - 350

Gething MJ and Sambrook J (1992) Protein folding in the cell. Nature. 355(6355): 33–45

Gorza L and Vitadello M (2012) Grp94 (HSP90B1). Encyclopedia of Signaling Molecules. pp 821-830

Hall AR, Burke N, Dongworth RK and Hausenloy DJ (2014) Mitochondrial fusion and fission proteins: novel therapeutic targets for combating cardiovascular disease. Br J Pharmacol. 171(8): 1890 – 1906

Hanahan D and Weinberg RA (2000) The Hallmarks of Cancer. Cell. 100 (1): 57 - 70

Hanahan D and Weinberg RA (2011) Hallmarks of Cancer: The Next Generation. Cell. 144(5): 646–674

Hartl FU (1996). Molecular chaperones in cellular protein folding. Nature. 381(6583): 571–579

Hightower LE, Sadis SE and Takenaka, IM (1994) Interactions of vertebrate hsc70 and hsp70 with unfolded proteins and peptides. In Morimoto RI, Tissieres A, and Georgopoulos, C (ed.) The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 26: 179-207

Hoppins S, Lackner L and Nunnari J (2007) The machines that divide and fuse mitochondria. Annu Rev Biochem. 76: 751 - 780

Hossain MM and Nakamoto H (2003) Role for the cyanobacterial HtpG in protection from oxidative stress. Curr Microbiol. 46(1): 70-76

Iannotti AM, Rabideau DA and Dougherty, JJ (1988) Characterization of purified avian 90,000-Da heat shock protein. Arch. Biochem. Biophys. 264(1): 54–60

Ishihara N, Eura Y and Mihara K (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J Cell Sci. 117(Pt 26): 6535-46

Jornayvaz FR and Shulman GI (2010) Regulation of mitochondrial biogenesis. Essays Biochem. 47: 10.1042/bse0470069

Kang BH, Plescia J, Dohi T, Rosa J, Doxsey SJ and Altieri DC (2007) Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. Cell. 131(2): 257 - 270

Kennady PK, Ormerod MG, Singh S and Pande G (2004) Variation of mitochondrial size during the cell cycle: A multiparameter flow cytometric and microscopic study. Cytometry A. 62(2): 97-108

Knorre DA, Popadin KY, Sokolov SS, and Severin (2013) Roles of Mitochondrial Dynamics under Stressful and Normal Conditions in Yeast Cells. Oxid Med Cell Longev. 2013: 139491

Koch J and Brocard C (2012) PEX11 proteins attract Mff and human Fis1 to coordinate peroxisomal fission. J Cell Sci. 125(Pt 16): 3813 - 3826

Koga T, Sakamoto F, Yamoto A and Takumi K (1999) Acid adaptation induces crossprotection against some environmental stresses in Vibrio parahaemolyticus. J. Gen. Appl. Microbiol. 45(4): 155 - 161

Kourtis N and Tavernarakis N (2011) Cellular stress response pathways and ageing: intricate molecular relationships. EMBO J. 30(13): 2520 - 2531

Kroemer G, Mariño G and Levine B (2010) Autophagy and the integrated stress response. Mol Cell. 40(2): 280 - 293

Kyriakouli DS, Boesch P, Taylor RW and Lightowlers RN (2008) Progress and prospects: gene therapy for mitochondrial DNA disease. Nature. 15(14): 1017 - 1023

Lai BT, Chin NW, Stanek AE, Keh W and Lanks, KW (1984) Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. Mol Cell Biol. 4(12): 2802 -2810

Leav I, Plescia J, Goel HL, Li J, Jiang Z, Cohen RJ, Languino LR and Altieri DC (2010) Cytoprotective mitochondrial chaperone TRAP-1 as a novel molecular target in localized and metastatic prostatecancer. Am J Pathol. 176(1): 393-401

Lee HC and Wei YH. (2005) Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int J Biochem Cell Biol. 37(4): 822-34

Leskovar A, Wegele H, Werbeck ND, Buchner J and Reinstein J (2008) The ATPase cycle of the mitochondrial Hsp90 analog Trap1. J Biol Chem. 283(17): 11677 - 11688

Li J, Soroka J and Buchner J (2012) The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. Biochim Biophys Acta. 1823(3): 624 - 35

Liesa M ,Palacín M and Zorzano A (2009) Mitochondrial Dynamics in Mammalian Health and Disease. Physiological Reviews. 89(3): 799-845

Lindquist S and Craig EA (1988) The heat-shock proteins. Annu Rev Genet. 22: 631– 677.

Liu D, Hu J, Agorreta J, Cesario A, Zhang Y, Harris AL, Gatter K and Pezzella F (2010) Tumor necrosis factor receptor-associated protein 1(TRAP1) regulates genes involved in cell cycle and metastases. Cancer Lett. 296(2): 194 - 205

Mary P, Sautour M, Chihib NE, Tierny Y and Hornez JP (2003) Tolerance and starvation induced cross-protection against different stresses in Aeromonas hydrophila. Int. J. Food Microbiol. 87(1-2): 121 - 130

Marzec M, Eletto D and Argon Y (2012) GRP94: An HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. Biochim Biophys Acta. 1823 (3): 774-787

Matassa DS, Amoroso MR, Maddalena F, Landriscina M and Esposito F (2012) New insights into TRAP1 pathway. Am J Cancer Res. 2(2): 235 - 248

Mollapour M and Neckers L (2012) Post-translational modifications of Hsp90 and their contributions to chaperone regulation. Biochim Biophys Acta. 1823(3): 648 - 655

Morra G, Potestio R, Micheletti C and Colombo G (2012) Corresponding functional dynamics across the Hsp90 Chaperone family: insights from a multiscale analysis of MD simulations. PLoS Comput Biol. 8(3):e1002433

Mozdy AD, McCaffery JM and Shaw JM (2000) Dnm1p GTPase mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J. Cell Biol. 151(2): 367 - 380

Olichon A, Emorine LJ, Descoins E, Pelloquin L, Brichese L, Gas N, Guillou E, Delettre C, Valette A, Hamel CP, Ducommun B, Lenaers G and Belenguer P (2002) The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. FEBS Lett. 523(1-3): 171 - 176

Ong SB and Hausenloy DJ (2010) Mitochondrial morphology and cardiovascular disease. Cardiovasc Res. 88(1): 16-29

Osellame LD, Blacker TS and Duchen MR (2012) Cellular and molecular mechanisms of mitochondrial function. Best Pract Res Clin Endocrinol Metab. 26(6): 711 - 723

Otera H, Wang C, Cleland MM, Setoguchi K, Yokota S, Youle RJ and Mihara K (2010) Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol. 191(6): 1141 - 1158

Pearl LH and Prodromou C (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery, Annu. Rev. Biochem. 75: 271 - 294

Picard D (2002) Heat-shock protein 90, a chaperone for folding and regulation, Cell Mol. Life Sci. 59(10): 1640 - 1648

Praefcke GJ, McMahon HT (2004) The dynamin superfamily: Universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol 5(2): 133 - 147

Prodromou C, Panaretou B, Chohan S, Siligardi G, O'Brien R, Ladbury JE, Roe SM, Piper PW and Pearl LH (2000) The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. EMBO J. 19(16): 4383–4392

Rehman J, Hannah J., Zhang HJ, Toth PT , Zhang Y, Marsboom G, Hong Z, Salgia R, Husain AN, Wietholt C, and Archer SL (2012) Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. FASEB J. 26(5): 2175–2186

Ritossa F (1996) Discovery of the heat shock response. Cell Stress and Chaperones. 1(2): 97 - 98

Ritossa F (1962) A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia. 18(12): 571 - 573

Robin ED and Wong R (1988). Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. Journal of Cell Physiology. 136(3): 507–513

Rojo M, Legros F, Chateau D and Lombès A (2002) Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. J Cell Sci. 115(Pt 8): 1663-1674

Ruddon RW (2007) Cancer Biology, Fourth edition, Oxford University Press, Inc. 198 Madison Avenue, New York, New York 10016: 3-4

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: A Laboratory Manual, Vol I, Cold Spring Harbor Laboratory Press, New York 11724: 1.25-1.28

Santel A, Frank S, Gaume B, Herrler M, Youle RJ and Fuller MT (2003) Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. J Cell Sci. 116(Pt 13): 2763-2774

Sato T, Minagawa S, Kojima E, Okamoto N and Nakamoto H (2010) HtpG, the prokaryotic homologue of Hsp90, stabilizes a phycobilisome protein in the cyanobacterium Synechococcus elongatus PCC 7942. Mol Microbiol. 76(3):576-89

Scott I and Logan DC (2007) Mitochondrial Dynamics: The Control of Mitochondrial Shape, Size, Number, Motility, and Cellular Inheritance, in Annual Plant Reviews Volume 31: Plant Mitochondria (ed D. C. Logan), John Wiley & Sons, Inc., Hoboken, NJ, USA 31 - 40 pp

Shiu RPC, Pouyssegur J and Pastan, I (1977) Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. USA 74(9): 3840 - 3844

Skulachev, VP (2001) Mitochondrial filaments and clusters as intracellular power-transmitting cables. Trends Biochem. Sci. 26(1): 23 - 29

Smirnova E, Griparic L, Shurland DL and van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol Biol Cell.12(8): 2245 - 2256

Song HY, Dunbar JD, Zhang YX, Guo D and Donner DB (1995) Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor. J Biol Chem. 270(8): 3574 - 3581

Taanman JW (1999) The mitochondrial genome: structure, transcription, translation and replication. Biochim Biophys Acta. 1410(2): 103 - 123

Tieu Q, and Nunnari J (2000) Mdv1p is a WD repeat protein that interacts with the dynamin-related GTPase, Dnm1p, to trigger mitochondrial division. J. Cell Biol. 151(2): 353 - 366.

Tissieres A, Mitchell HK and Tracy UM (1974) Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. J Mol Biol. 84(3): 389 - 398

Trepel J, Mollapour M, Giaccone G and Neckers, L (2010) Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer. 10(8): 537 - 549

van der Bliek AM, Shen Q and Kawajiri S (2013) Mechanisms of mitochondrial fission and fusion. Cold Spring Harb Perspect Biol. 5(6). pii: a011072

Ventura-Clapier R, Garnier A and Veksler V (2008) Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovasc Res. 79(2): 208-217

Wallace DC (2012) Mitochondria and Cancer. Nature Reviews Cancer 12 (10): 685-698

Westermann B (2010) Mitochondrial fusion and fission in cell life and death. Nature Reviews Molecular Cell Biology 11(12): 872-884

Westrate LM, Sayfie AD, Burgenske DM, and MacKeigan JP (2014) Persistent Mitochondrial Hyperfusion Promotes G2/M Accumulation and Caspase-Dependent Cell Death. PLoS One. 9(3): e91911

Wheeler DS and Wong HR (2007) Heat Shock Response and acute lung injury. Free Radical Biology and Medicine. 42(1): 1-14

Whitesell L and Lindquist SL (2005) HSP90 and the chaperoning of cancer. Nat Rev Cancer. 5(10): 761 - 772

Wiech H, Buchner J, Zimmermann R and Jakob, U (1992) Hsp90 chaperones protein folding in vitro, Nature 358 (6382): 169 - 170

Zanna C, Ghelli A, Porcelli AM, Karbowski M, Youle RJ, Schimpf S, Wissinger B, Pinti M, Cossarizza A, Vidoni S, Valentino ML, Rugolo M and Carelli V (2008) OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. Brain. 131(Pt 2): 352-367

Zhao J, Zhang J, Yu M, Xie Y, Huang Y, Wolff DW, Abel PW and Tu Y (2012) Mitochondrial dynamics regulates migration and invasion of breast cancer cells. Oncogene. 32(40): 4814-4824

Appendix

1 x PBS(pH 7.4)

8 gm NaCl

0.2 gm KCl

1.44 gm Na_2HPO_4

0.24 gm KH_2PO_4

Final volume made upto 1000 mL with Distilled water

5 % stacking gel

H_2O – 4.1 mL

30 % Acrylamide Mix – 1.0 mL

1.0 M Tris (pH 6.8) – 0.75 mL

10 % SDS – 0.06 mL

10 % APS – 0.06 mL

TEMED – 0.006 mL

5 x TGS

15.1 gm Tris-base

94 gm Glycine

50 ml of 10 % SDS

Final volume 1000 ml with distilled water

10 x TBS(pH 7.4)

80 gm NaCl

0.2 gm KCl

3 gm Tris

Final volume 1000 ml with distilled water

12 % resolving gel

H_2O – 3.3 mL

30 % Acrylamide mix – 4.0 mL

1.5 M Tris (pH 8.8) – 2.5 mL

10 % SDS – 0.1 mL

10 % APS – 0.1 mL

TEMED – 0.004 mL

50 x Tris Acetate – EDTA (TAE) buffer

242 g Tris base

57.1 mL Glacial Acetic acid

100 mL 0.5 M EDTA (pH 8.0)

Final volume 1000 ml to be made with Distilled Water

Alkaline Lysis Solution I

50 mM Glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

The solution was sterilized by autoclaving and stored at 4^{o} C.

Alkaline Lysis Solution II

0.2 N NaOH

1 % (w/v) SDS

Alkanline Solution III

5 M Potassium Acetate 60.0 ml

Glacial Acetic Acid 11.5 ml

H_20 28.5 ml

Laemlli Buffer

0.1% 2-Mercaptoethanol

0.0005% Bromophenol blue

10% Glycerol

2% SDS (electrophoresis-grade)

63 mM Tris-HCl (pH 6.8)

LB medium (Luria Bertani Medium)(pH 7.2)

Tryptone – 10 grams

Yeast Extract – 5 grams

Sodium Chloride – 10 grams

Lysis Buffer for Alkaline Lysis

0.2 N NaoH

1 % SDS

Neutralization Buffer for Alkaline Lysis

5M Potassium acetate – 60 mL

Glacial acetic acid – 11.5 mL

Autoclaved water – 28.5 mL

RIPA Buffer

50 mM Tris.Cl (pH 7.5)

150 mM NaCl

1 % Nonidet P-40

0.1 % SDS

RIPA Lysis Buffer

10 mM Sodium Fluoride

1 mM Phenylmethanesulfonylfluoride

1 mM Sodium orthovanadate

50 x Protease Inhibitor cocktail

Final volume 1000 ml with RIPA buffer

TBST

1 ml of Tween 20 added to 1000 ml of 1 x TBS

Transfer Buffer

72 gm Tris

15 gm Glycine

200 ml methanol

Final volume 1000 ml with distilled water

Tris - EDTA

10 mM Tris (pH 8.0)

1 mM EDTA

Phosphate Buffered Saline