Study of mechanism and regulation of a nuclear dynamin in *Tetrahymena thermophila*

By

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Usha Pallabi Kar entitled "**Study of mechanism and regulation of a nuclear dynamin in *Tetrahymena thermophila***" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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Usha Pallabi Kar

DECLARATION

I, hereby declare that the investigation presented in this thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Usha Pallabi Kar

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1. "Regulation and oligomerization status of a nuclear dynamin related protein in *Tetrahymena thermophila".* **Usha Pallabi Kar** and Abdur Rahaman. Poster presented in XXXV All India Cell

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3. "Understanding the Structure and Function of a Nuclear Dynamin Related Protein in *Tetrahymena thermophila."* **Usha Pallabi Kar** and Abdur Rahaman**.** Poster presented in XXXVII All India Cell Biology (AICB) Conference and Symposium on Cell Dynamics and Cell Fate. 22-24th December 2013, organized by National Center for Biological Sciences, inStem, C-CAMP and IISc, Bangalore, India.

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2. Workshop on Electron Microscopy (WEM). 23-25th November 2011, organized by Institute of Physics and IIT, Bhubaneswar, India.

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Usha Pallabi Kar

Contents

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Synopsis

Biological membranes serve as a barrier to the interior of the cell from external environment and helps in compartmentalization of distinct sub-cellular organelles inside the cell. Membranes are dynamic structures and undergo continuous remodeling during cellular growth and development. Membrane remodeling is brought about by changes in the membrane curvature. Intricate biological machineries have evolved to orchestrate the change in membrane curvature and dynamin family of proteins is one of the prominent players in membrane remodeling. The dynamin proteins constitute a family of large GTPases which carry out essential cellular functions with their nomenclature reflecting dynamic, molecular motor like properties. They are involved in various processes like vesicle scission, organelle fission/ fusion, vacuolar trafficking and viral resistance [1, 2]. These proteins couple GTP hydrolysis with membrane alterations required for fission, fusion or tubulation by undergoing repeated cycles of self-assembly/disassembly on target membranes [1, 3].

The unicellular eukaryote *Tetrahymena thermophila* harbors 8 dynamin-related proteins (Drps), correlating with its complex membrane organization. While Drp1 and Drp2 have functions in endocytosis, Drp7 and Drp8 show mitochondrial distribution. Remaining four dynamin related proteins, Drp 3 – 6 form a lineage-specific clade [4] and one of the members, Drp6, is localized on nuclear envelope and has evolved a novel role in nuclear remodeling [5]. A remarkable feature of *Tetrahymena* is that it harbors two nuclei, a germline micronucleus (MIC) and a somatic macronucleus (MAC). Drp6 is essential at a stage where a small MIC expands to form new MAC during conjugation. Loss of Drp6 protein or inhibition of its activity leads to an inhibition in nuclear expansion [5]. Assembly and disassembly of Drp6 on the nuclear envelope are regulated by physiological and developmental cues. Although the role of Drp6 in MAC development has been established, the mechanism of its function is unknown [5]. Therefore, the current study aims to understand the mechanism and regulation of Drp6 function. We have studied the structure and function of Drp6 protein along with the identification of its post-translational modifications and their effects on cellular distribution. Towards this aim, the following objectives were framed and the work carried out under each objective is presented as different chapters in this thesis.

Objectives:

1. Biochemical characterization of Drp6.
2. Identification of lipid binding domain in Drp6.
3. Investigation on Drp6 nuclear recruitment.
4. Identification of post-translational modifications of Drp6 and their effects on its cellular distribution.

Chapter 1:

This chapter deals with the review of literature about dynamins and dynamin related proteins. Dynamins are a family of large GTPases with essential functions in diverse cellular processes [1]. Dynamin was first discovered as a microtubule-interacting protein and its role in endocytosis was revealed later when the mutations in the protein translated from *shibire* locus were correlated with a conditional temperature sensitive paralytic phenotype in *Drosophila melanogaster* [6,7]. Subsequently, dynamins have been implicated in many cellular processes including receptor-mediated endocytosis, caveolae internalization and membrane trafficking from late endosomes and Golgi [8]. Dynamins play a central role in scission of vesicles during endocytosis by assembling into helical polymers at the necks of budding vesicles. GTP hydrolysis-dependent conformational changes promote fission of the underlying tubular membrane to generate a free endocytic vesicle. Other members of this family are involved in a multitude of cellular functions such as fission of mitochondria, chloroplasts and peroxisomes [1,9]. Some members of this family also catalyze homotypic fusion of mitochondria and endoplasmic reticulum [1]. A group of dynamin proteins tubulate golgi vesicles to form cell plate during cell division in plants. Apart from these canonical membrane-remodelling functions, some members such as MxA and MxB confer viral resistance [10–12].

Although the dynamin-family proteins perform diverse arrays of function, the underlying mechanism is similar. The members of this family can be categorized into two groups, classical dynamins and dynamin-related proteins (Drps). Classical dynamins harbor five distinct functional domains namely GTP-hydrolysis domain (GTPase domain), middle domain, pleckstrin homology (PH) domain, GTPase effector domain (GED), and proline-rich domain (PRD). Drps, although lack a defined PH and PRD, retain other three core domains [3]. The GTPase domain is highly

conserved within the members of this family [13]. The middle domain and GED play crucial role in oligomerization and regulation of GTPase activity; the PH domain is responsible for membrane interaction and PRD is implicated in protein-protein interaction. The nuclear dynamin in *Tetrahymena thermophila,* Drp6, has a domain organization similar to that in other Drps harboring the GTPase domain, middle domain and GED. Drp6 is localized on nuclear envelope and has a role in nuclear remodeling where it is essential for the development of macronucleus (MAC) from micronucleus (MIC) during conjugation [5]. The process of conjugation initiates a series of complex nuclear events resulting in the loss of the parental MAC and the formation of a new MIC and MAC through the fusion of haploid nuclei produced from parental micronuclei [14]. The precursors of the new MIC and MAC are identical in size and genome content at the initial stage of nuclear differentiation. However, two out of four new MICs subsequently enlarge rapidly increasing its volume 10–15 fold to accommodate the increased nuclear content [14]. This stage of nuclear differentiation involves rapid membrane expansion and either loss or inhibition of Drp6 function leads to an inhibition in nuclear expansion at this stage. Drp6 shows differential localization and activity at different physiological conditions and developmental stages [5]. Although the role of Drp6 in MAC development has been established, the mechanism by which it performs its function is not known. With this background, an attempt has been made to understand the mechanism and regulation of Drp6 function.

Chapter 2:

Self-assembly on the target membranes is one of the important properties of all dynamin family proteins. Biochemical analyses established that dynamin exists as a tetramer and polymerizes into rings and helices when incubated in solutions with low ionic strength or in the presence of lipid templates [15]. The intra and inter molecular interactions among dynamin protomers promote its

self-assembly into higher order rings and filamentous structures and triggers its GTPase activity [13]. Cycles of assembly and disassembly of dynamin oligomers at endocytic points are important for vesicle scission. This assembly/disassembly cycle is crucial for functions of other Drps as well. It is important for Drp1 during mitochondrial fission, for mitofusins/Mgm1/OPA1/Fzo1 during mitochondrial fusion and for MxA in antiviral activity. Disruption of assembly by mutations that interfere with oligomerization results in functional inhibition of dynamins and DRPs. Drp6 being a dynamin family member, shares three canonical domains with other dynamins [5]. In order to characterize the GTPase activity and the oligomeric status of Drp6, a recombinant form of the protein was expressed in bacteria and purified to near homogeneity using Ni-NTA affinity column. Analytical size-exclusion chromatography showed that Drp6 remains as a higher order oligomer at non assembly condition (300mM NaCl) in contrast to other known dynamins. To understand the precise oligomeric status of Drp6, chemical cross-linking experiment was performed. Appearance of high molecular weight bands and disappearance of monomeric Drp6 with increasing concentration of BS^3 (bis (sulfosuccinimidyl) suberate) with increasing time suggested that Drp6 remains as a self-assembled structure in high salt conditions (300 mM NaCl). All the dynamin-related proteins form higher order structures into rings and helical spirals important for their function. Analysis of Drp6 ultrastructure by transmission electron microscopy revealed the presence of rings and helical spirals of varying diameters and sizes in the micrographs of negatively stained Drp6. Presence of such rings and spirals in Drp6 strongly suggests a mechanism for its function similar to other dynamin family proteins. Both self-assembly and GTPase activity are essential for the function of all dynamins. The GTPase activity of Drp6 was measured using a malachite-based colorimetric assay and the results showed that Drp6 has high basal GTPase activity and low GTP binding affinity with the K_{cat} of 1.62±0.11 min^{-1} and the apparent K_m of ~395μM±5. Surprisingly, unlike other Drps, there was no significant increase in GTPase activity

of Drp6 in low salt condition. These results suggest that, in contrast to dynamins and similar to MxA, Drp6 self-assembles in absence of membrane templates and its GTPase activity is not enhanced upon membrane association.

Chapter 3:

All members of the dynamin family associate with membranes. Classical dynamins interact with lipids via the PH domain and many other DRPs have either trans-membrane helices or exposed loops for membrane binding. This chapter deals with the identification of the lipid-binding domain of Drp6 and its role in nuclear recruitment and macronuclear development. Although Drp6 lacks a recognizable PH domain, it is localized on nuclear envelope. Results from lipid overlay assay with total lipid extracted from *Tetrahymena* suggested that full-length Drp6 interacts with lipid without the requirement for GTP or any other factor. To identify the specific lipids that interact with Drp6, the assay was performed with commercial lipid strips containing 15 different lipids. Results from this assay showed that Drp6 specifically binds to three lipids, namely Cardiolipin (CL), Phospatidylserine (PS) and Phosphatidic acid (PA). In order to identify the domains responsible for lipid interaction, different fragments of Drp6 were cloned, expressed in bacteria and purified with affinity tags. Results from the lipid overlay assay using these purified fragments led to the conclusion that the fragment containing amino acid residues 517-600 is sufficient for interaction with lipids comparable to full-length Drp6.

To understand the position of the lipid-binding domain on the full-length Drp6 structure, a 3-D model of Drp6 was generated using the software I-TASSER [16]. The comparison of this modeled structure with the crystal structure of dynamin and MxA revealed that the lipid-binding domain is located in an equivalent position as that of PH domain of dynamin and membrane-binding loop of

MxA. These results suggest that, despite the amino acid sequence diversity, members of dynamin family proteins harbor the lipid binding domain structurally at similar positions.

In order to evaluate the role of Drp6 lipid-binding domain in nuclear recruitment, either the lipid-binding domain (GFP-Drp6 517-600) or the fragment lacking this domain (GFP-Drp6 Δ 517-600) was over-expressed as GFP-fusion tag in *Tetrahymena*. Confocal microscopy of the cells expressing these constructs demonstrated that the lipid-binding domain, though essential, is not sufficient for nuclear recruitment of Drp6. Moreover, the fragments containing the amino acid residues 1-600, 253-600 or 517-710 did not localize on nuclear envelope, suggesting that both the GTPase domain (1-253 residues) and the C-terminal end of Drp6 are important for nuclear recruitment. Conjugating *Tetrahymena* cells either expressing the lipid-binding domain or the fragment lacking lipid-binding domain showed inhibition in MAC development, underscoring the importance of this domain in nuclear expansion. Taken together, these results suggest that Drp6 harbors a lipid binding domain structurally located in equivalent position of the PH domain of dynamin and is important for nuclear recruitment and nuclear expansion.

Chapter-4:

As discussed in the previous chapter, importance of the lipid-binding domain of Drp6 in nuclear recruitment has been established. In order to understand the mechanism of nuclear recruitment, a Drp6 variant with Methionine substituting Isoleucine at position 553 (Drp6 I553M) was generated based on previous reports demonstrating the importance of an I533 in dynamin1 [17]. I533 in dynamin1 plays a crucial role in stable insertion of the PH domain into the lipid bilayer for generating membrane curvature and subsequent membrane fission during endocytosis [17]. Mutation of I553 of Drp6 (Drp6 I553M) resulted in a complete loss of nuclear localization when expressed as GFP-tag. To understand the basis of the loss of nuclear localization, the biochemical

properties of Drp6 I553M were compared with those of wild type Drp6. Oligomerization state of the mutant was not affected as analyzed by size-exclusion chromatography. GTP hydrolysis of Drp6 I553M was also found to be similar to that of wild type Drp6. Since, the mutation affected neither GTP hydrolysis nor the oligomeric state, the ultra-structure of Drp6 I553M was analyzed by negative staining transmission electron microscopy. Similar to wild type Drp6, the mutant protein also formed rings and helical spirals. Taking together, these results suggest that the loss of nuclear localization of the mutant is not due to the defect in GTP hydrolysis, oligomeric state and ultra-structure of Drp6. Therefore, it was speculated that it was due to a change in the membrane interaction. Results from floatation assay using liposomes prepared from total *Tetrahymena* lipid supplemented with either CL or PS demonstrated that, while both the mutant and the wild-type Drp6 interact with PS-containing liposomes, the mutant Drp6 specifically failed to interact with liposomes containing CL. This suggests that cardiolipin interaction is important for the nuclear recruitment of Drp6. To further assess the role of CL in nuclear recruitment of Drp6, CL was depleted using Pentacholorophenol (PCP) in *Tetrahymena* cells expressing either GFP-Drp6 or GFP-Nup3 (nuclear pore protein). It was observed that Drp6 dissociates from nuclear envelope without affecting nuclear localization of GFP-Nup3, strongly suggesting that cardiolipin is important for nuclear association of Drp6.

To examine if Drp6-CL interaction is important for MAC development, CL was either depleted using PCP or its interaction with Drp6 was interfered by using the CL-binding dye Nonyl Acridine Orange (NAO). In both the cases, MAC development was significantly inhibited without affecting earlier stages of nuclear differentiation. These results suggest that CL is important for mediating nuclear recruitment of Drp6 and for nuclear expansion.

Drp6 has high basal level GTPase activity, comparable to classical dynamins. The GTPase activity of dynamin proteins is enhanced by lipid templates. To determine if lipid-binding also stimulates GTPase activity of Drp6, its rate of GTP hydrolysis was assayed in the presence of liposomes containing either CL or PS and compared with that of wild type Drp6. Unlike other dynamins, the GTPase activity of Drp6 did not increase in the presence of liposomes. Interestingly, the Drp6 GTPase activity was specifically inhibited by CL containing liposomes but not by PS-containing liposomes. It is interesting to note that, though the GTPase activity of Drps such as MxA and BDLP is not enhanced upon lipid association, their activity is not inhibited [18]. This result suggests that Drp6 has a property distinct from other Drps and may have different requirement, in spite of having a similar overall mechanism for its function.

Chapter-5:

Drp6 shows differential localization under different physiological conditions and developmental stages, suggesting a regulation by post-translational modifications or by interaction with other factors. Other dynamin proteins are known to be regulated by various post-translational modifications including phosphorylation, ubiquitylation, S-nitrosylation and SUMOylation. *In vitro* phosphorylation assay with bacterially expressed purified Drp6 showed that it undergoes phosphorylation, which was further confirmed by mass spectrometry analysis of TAP-tag Drp6 purified from *Tetrahymena*. The analysis showed that four serine residues at positions 86, 248, 701 and 705 of Drp6 are phosphorylated. In addition to phosphorylation, the mass spectrometry analysis also identified ubiquitylation of six lysine residues at positions 250, 385, 392, 400, 533 and 655. In order to evaluate the effect of phosphorylation on Drp6 localization and function, phosphorylation mutants of Drp6 in various combinations were generated by site-directed mutagenesis. Expression of these mutant proteins as GFP-tag did not alter the nuclear localization

of Drp6 significantly. However, when all four serine residues were mutated to alanine, the protein was almost exclusively localized to nuclear envelope, indicating that phosphorylation at multiple sites regulates the dynamics of Drp6 in the nuclear envelope.

Conclusion:

In the present study, we have demonstrated that Drp6 self-assembles to form rings and helical spirals similar to other dynamins. However, unlike other dynamins, it does not require membrane association for self-assembly. We have identified the lipid binding domain of Drp6 which is located in the equivalent position of the PH domain of classical endocytic dynamin. The lipid binding domain is essential but not sufficient for nuclear recruitment and is also important for nuclear remodeling. An isoleucine in the lipid binding domain is required for nuclear recruitment possibly by its interaction with cardiolipin, and this interaction is important for nuclear expansion. Drp6 undergoes phosphorylation at four serine residues and ubiquitylation at six lysine residues. Loss of phosphorylation on these four serine residues shifts Drp6 localization from ER vesicles to nuclear envelope, indicating that phosphorylation regulates Drp6 dynamics between these two compartments. In conclusion, the present study provides a mechanistic basis for the novel function of a dynamin related protein in nuclear remodeling.

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Publications:

1. **Kar, U. P**., Dey, H., and Rahaman, A. (2017) Regulation of dynamin family proteins by post-translational modification. J. Biosci. (In press) *

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3	Dr. Praful Singru Reader-F, SBS	Member		21.2.2017
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List of figures and Tables:

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ABBREVIATIONS

ATP: Adenosine Triphosphate

ATL: Atlastin

BAR: Bin/Amphiphysin/Rvs

BDLP: Bacterial dynamin like protein

BSA: Bovine Serum Albumin

BSE: Bundle signaling element

CL: Cardiolipin

CME: Clathrin mediated endocytosis

DAPI: 4', 6-diamidino-2-phenylindole

DLP: Dynamin like proteins

DMC: Dryl's Medium concentrate

DNA: Deoxyribonucleic acid

DRP: Dynamin related proteins

dNTP: Deoxy nucleotide phosphates

EDTA: Ethylenediaminetetraacetic acid

EHD: Eh domain

EM: Electron microscopy

ER: Endoplasmic Reticulum

GAP: GTPase activating protein

GED: GTPase effector domain

GFP: Green fluorescent protein

GTP-Guanosine Triphosphate

GTPγS: guanosine 5'-O-[gamma-thio] triphosphate

HEPES: Hydroxyethyl piperazineethanesulfonic acid

hGBP: Human guanylate binding protein

His: Histidine

HIV-Human Immune deficiency virus

HRP: Horse radish peroxidase

IgG: Immunoglobulin G

IPTG: Isopropyl beta-D-1thiogalactopyranoside

kDa: Kilo Dalton

LB: Luria Bertani

LUV: Large unilammelar vesicles

MAC: Macronucleus

MBP: Maltose binding protein

MIC: Micronucleus

MTT: Metalothionine

NAO: Nonyl acridine orange

NE: Nuclear Envelope

NPC: Nuclear pore complex

OD: Optical density

PCP: Pentachlorophenol

PCR: Polymerase Chain Reaction

PH: Plecstrin homology

PRD: Proline rich domain

PS: Phosphatidylserine

PTM: Post-translational modifications

rDNA: Ribosomal DNA

RPM: Rotations per minute

SDS-PAGE: Sodium Dodycyl Sulphate Poly Acrylamide Gel Electrophoresis

SNARE: Soluble NSF Attachment Protein Receptor"

SPP: Super Proteose Peptone

TBST: Tris buffer saline tween 20

TAP: Tandem affinity purification

TGN: Trans golgi network

WT: Wild type

CHAPTER ONE:

Introduction and Review of Literature

1.1 Membrane remodeling:

Most biological processes involve changes in cell shape accompanied by changes in the membrane bilayer. Membrane remodeling and topological changes in membranes are fundamental processes in cellular physiology. Cellular membranes are dynamic structures and are constantly being remodeled to perform various biological functions. They adopt complicated three-dimensional shapes of varying curvatures. Cellular membranes change conformation in striking ways during processes such as cell movement, division, extension of neuronal structures and vesicle trafficking. Membrane remodeling serves as an active means to create membrane domains and to organize centers for membrane trafficking. Intricate biological machineries are evolved to bring about these changes in membranes. These machineries comprise of the proteins that are associated with cellular membranes and play crucial role in mediating membrane curvature [1]. Curvature can be dynamically modulated by changes in lipid composition, oligomerization of curvature inducing proteins and the reversible insertion of protein domains that act like wedges in membranes (Fig. 1.1) [2]. Each protein that bind to membrane, induces a small change in the local curvature of membrane bilayer. Interaction of multiple proteins can globally restructure membrane bilayers into new three-dimensional shapes, such as tubules or small vesicles. The interplay between curvature-sensing and curvature-generating proteins is critical for the formation of membrane vesicles. On a larger scale, changes in membrane curvature plays a central role in cellular growth, division and movement, and is regulated in a complex manner that involves the composition as well as distribution of lipids and assembly of proteins on the membrane.

Membrane curvature generation process can be divided into two stages. The first stage is membrane deformation induced by either changes in lipid composition of the bilayer (shapes,

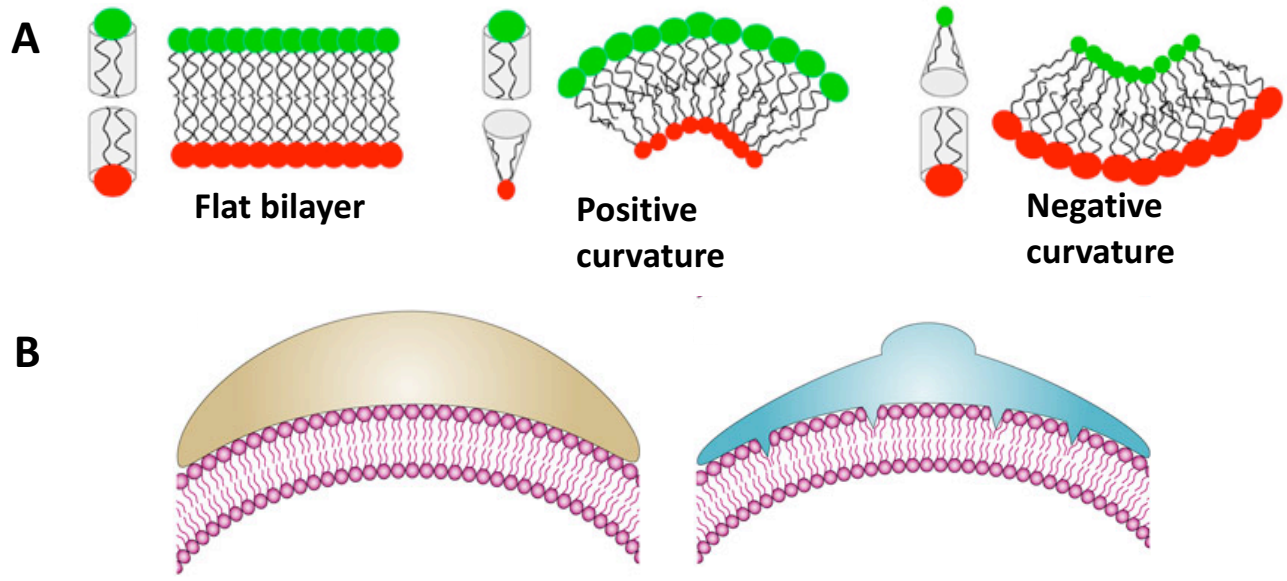


Figure 1.1. Membrane curvature is dynamic. (A) Regulation of membrane curvature by change in lipid compositions. Curvature is generated by differential distribution of lipids between two leaflets of the membrane bilayer. (B) Change in membrane curvature by protein interaction. Curvature change can be induced either by interaction of scaffolding proteins with the membrane surface (left) or by insertion of a protein domain into the hydrophobic core of the bilayer (right). The images are reproduced from Yandrapalli et al. 2014 (A) and from Zimmerberg and Kozlov 2006.

species and numbers of phospholipids), insertion of small hydrophobic or amphipathic protein domains into the leaflets, or by interaction of specific protein domains with intrinsic curvature (e.g., BAR domain containing proteins) to the membrane surface. This induces the membrane to take the shape of the protein domain (Fig. 1.1). The second stage is sensing the curvature, which involves recognition and stabilization of already curved membranes by protein domains. However, these two stages are not always separate events and may occur together in a complex manner. Thus, generation of membrane curvature in the context of membrane trafficking and organelle dynamics is a complex process, involving changes in the composition and distribution of lipids, assembly of proteins, and the coordinated activities of these regulatory proteins.

Moreover, maintenance of membrane integrity is vital for cellular membranes in order to function as specific barriers. A weak bilayer structure would lead to leakage during vesicle transport resulting in the loss of cargos. However, during membrane fission and fusion, the bilayer structure is transiently and locally breached by the formation of nonbilayer intermediates necessary to mediate membrane remodeling without leakage. Formation of these intermediates from stable bilayers is energetically unfavorable; thus requiring specialized protein machineries to maintain the geometry and composition during membrane remodeling process.

Although a number of proteins are involved in this process, dynamins and dynamin related proteins (DRPs) (collectively called as dynamins), a family of large GTPases are one of the prominent players in catalyzing membrane remodeling reactions [3]. These proteins have unique properties of self-polymerization, and ability to bind and tubulate lipids making them competent for the membrane fission and fusion function. Dynamin, the founding member of the family plays a critical role in endocytic vesicle fission events. Members of this family are associated with several important cellular functions ranging from scission of membrane vesicles, cytokinesis, maintaining organelle dynamics to conferring innate anti-viral immunity (Fig. 1.2) [3,4]. The list of their cellular functions are ever increasing. These proteins are mechano-chemical enzymes and couple free energy of GTP hydrolysis with membrane deformation. Classical dynamins perform mainly scission of vesicles from plasma membrane, where as other members such as dynamin like proteins (DLPs) or dynamin related proteins (DRPs) participate in fission and fusion of cell organelles such as mitochondria, chloroplasts and peroxisomes. They are also known to tubulate membranes which is required for cell plate formation during cell division [3]. Although membrane remodeling remains as the basic underlying mechanism of action for all dynamins and DRPs, amino acid sequence diversity could be attributed to their functional diversity on different membranes across phylogenetic groups.

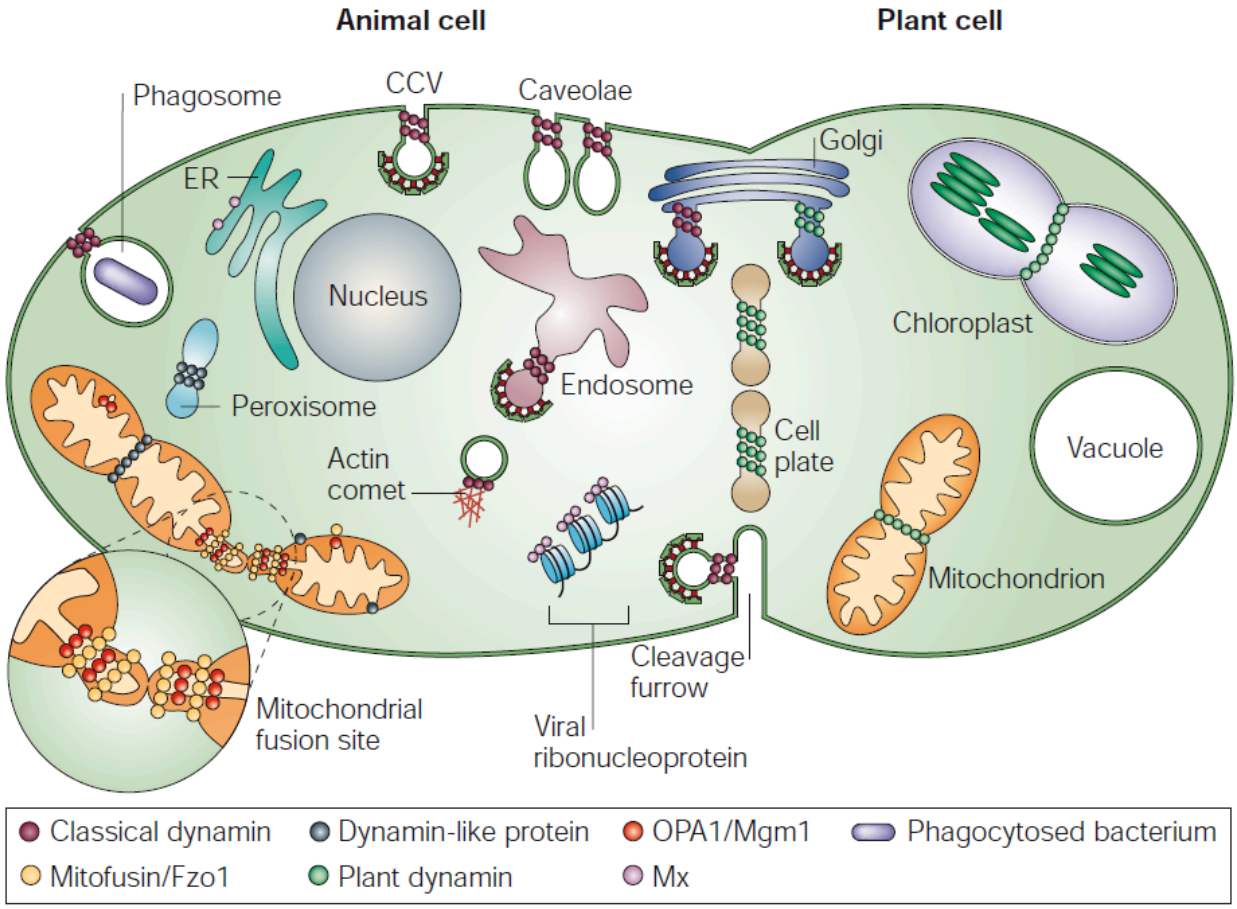


Figure 1.2: Members of dynamin superfamily in animals and plants. Taken from Praefcke and McMahon 2004a.

1.2 Dynamin family proteins:

1.2.1 Discovery of dynamin:

Dynamin, the founder member of this family was originally discovered in the brain and was identified as a microtubule associated protein [5]. Subsequently, its role in endocytosis was established when mutation in the protein translated from *shibire* locus was correlated with a conditional temperature sensitive paralytic phenotype in *Drosophila melanogaster*. The locus was called *shibire* after the Japanese word for 'paralysed'. The temperature-sensitive *shibire* fly exhibited a rapid and reversible paralysis at non-permissive temperature. These paralytic

mutation resulted in depletion of synaptic vesicles and showed accumulation of arrested 'collared' endocytic pits at the pre-synaptic plasma membrane [6,7]. Subsequent investigations in non-neuronal cells revealed dynamin to be a general component of clathrin-coated endocytic pits and its GTPase activity is essential for endocytosis since dynamin mutants impaired for GTP binding and/or hydrolysis showed dominant-negative effects on endocytosis [8–11].

1.2.2 Classical dynamins and Dynamin related proteins (DRPs):

Dynamin is a 100 kDa GTPase enzyme that plays a crucial role in controlling several endocytic pathways. Its role is best studied in clathrin mediated endocytosis (CME) so far (Fig. 1.3). These dynamin controlled endocytic pathways are critical in many aspects of cell and organismal physiology including uptake of nutrients, immune regulation, synaptic vesicle recycling, regulation of signal transduction and regulation of surface transporters [12]. Mammalian genomes contain 3 dynamin genes namely dynamin-1, dynamin-2 and dynamin-3 [13]. Although the three isoforms of dynamin share same domain organization and overall 80% amino acid identity, they have distinct expression patterns. Dynamin-1 is selectively expressed at high level in neurons specifically at the presynapse and is generally not present

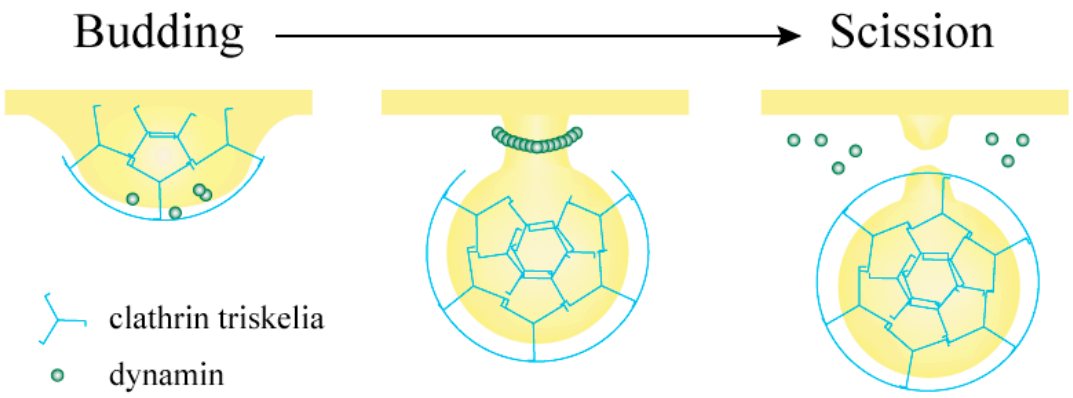


Figure 1.3: Role of dynamin in vesicle scission. Dynamin polymerizes on the neck of budding clathrin coated vesicles and pinches off the vesicle from the membrane.

in non-neuronal tissues [14,15]. However, dynamin-1 was also detected in many cultured cell lines [16,17]. While dynamin-2 is expressed ubiquitously, dynamin-3 is found predominantly in the brain, testis, and also in some other tissues such as the lung [14]. Dynamin diversity is further increased by the presence of multiple splice variants for each of these three isoforms. Dynamin-1 has 8 splice variants, dynamin-2 has 4 splice variants and dynamin-3 has 13 splice variants [13,18]. A large number of evidences suggest that different isoforms and splice variants function at distinct locations within the cell. Invertebrates such as *C. elegans* and *D. melanogaster* possess only a single dynamin gene [6,19]. The existence of three dynamin genes and their differential pattern of expression in mammals suggests different roles of dynamin during housekeeping clathrin-mediated endocytosis, mediated by dynamin-2, and specialized forms of endocytosis in cells that additionally express dynamin-1 and/or dynamin-3. Although different dynamin isoforms display unique protein-protein interactions, the major differences among isoforms are quantitative such as affinity for SH3-domain-containing proteins, GTPase activities, oligomerization and lipid binding properties [20–25].

Dynamins, other than classical dynamins, are grouped under the category of Dynamin related proteins (DRPs) or Dynamin-like proteins (DLPs). Although these proteins have similar catalytic mechanisms and mode of action as that of classical dynamins, they lack highly conserved pleckstrin homology (PH) domain and proline rich domain (PRD) [3,26] (Fig. 1.4). However, they harbor different lipid binding motifs for recruitment to their target membranes. While DRPs like mitofusins, OPA1/Mgm1 and atlastins have trans-membrane helices, an exposed loop in Mx proteins, Drp1 and bacterial dynamin like proteins (BDLP) perform membrane binding function [3,26–41]. Unlike regulatory GTPases, dynamins are large in their size, show lower affinity for GTP binding and have higher basal GTP hydrolysis rates. The GTPase domain of dynamins exhibit cooperativity of GTP hydrolysis upon dynamin oligomerization. These proteins play both

regulatory and mechano-chemical roles. In case of regulatory role, the binding and hydrolysis of GTP mediates recruitment and release of effector

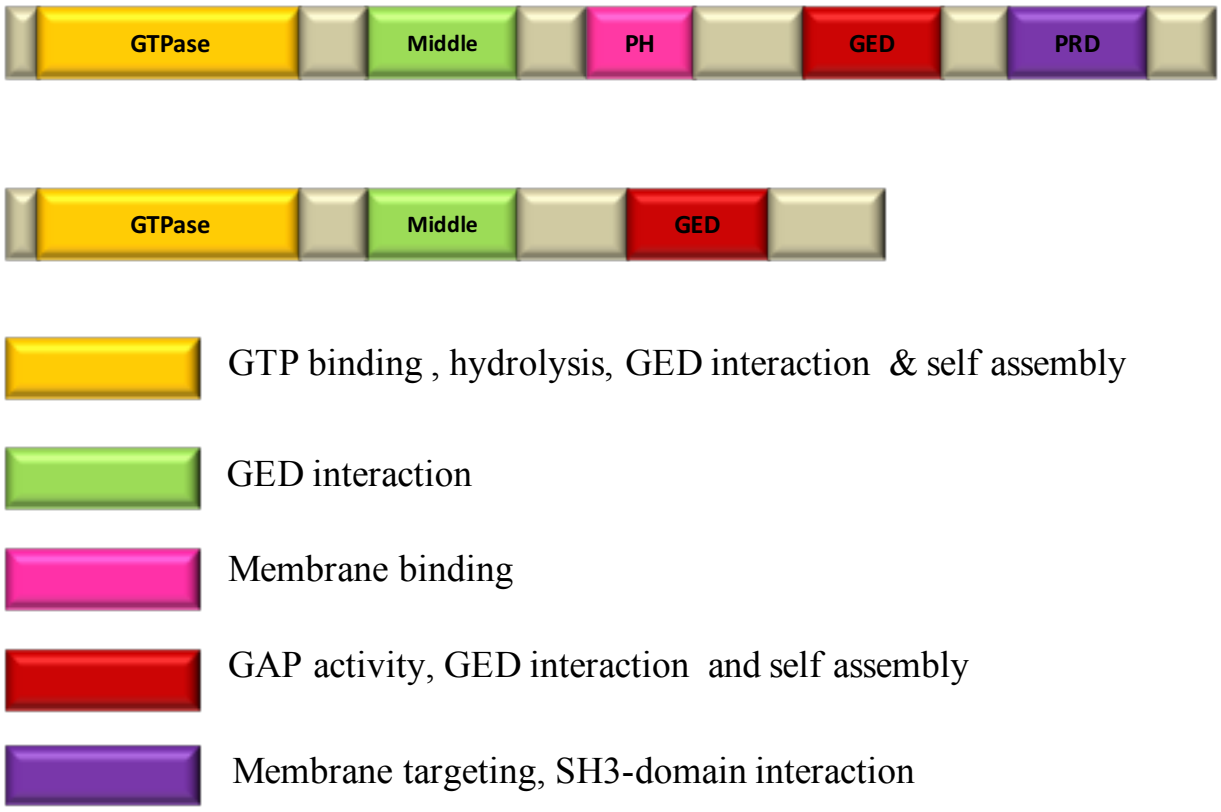


Figure 1.4: Domain structure of dynamins. Classical dynamin (top) and dynamin-related proteins (bottom). Drps lack PH and PRD. Domains are indicated in different colours.

proteins, and dynamin polymerization. The structural changes induced by GTP binding and hydrolysis in the G domain of DRPs are transduced into a movement, propagated through the polymerized structure, exerting a force on membranes during its mechano-chemical role [42,43]. Thus the cycle of GTP binding and hydrolysis is coupled to its membrane deformation activity [11].

1.2.3 Domain organization of dynamin family proteins:

Three domains namely an N-terminal GTPase domain (G domain), a middle domain or stalk region (MD) and a GTPase-effector domain (GED) form the core of all dynamins (Fig. 1.4). The classical dynamins harbor two additional domains, a highly conserved pleckstrin-homology (PH) domain and a proline-rich domain (PRD) which are lacking in dynamin related proteins (DRPs) (Fig. 1.4). A detail structural information on the molecular organization of various DRPs has provided new perspectives to understand their functions in detail. The human guanylate binding protein (hGBP) was the first dynamin like protein whose structure was solved [44]. Recently, information on structural organization of mammalian endocytic dynamin-1 [42,43] and other dynamin DRPs such as bacterial dynamin like proteins (BDLPs), mitochondrial Drp1, the interferon induced human MxA stalk domain, atlastin, and mitofusin-1 [41,45–49] has allowed better understanding of their domain architecture. This has led to a discernable conclusion that despite low sequence similarity, the structural motifs of dynamin related proteins are conserved.

1.2.3.1 The GTPase domain:

The GTPase domain is the key signature of dynamins and resemble that of Ras-like GTPases. It contains four conserved elements, the P-loop (G1 motif), switch-1 (G2), Switch-II (G3) and the nucleotide binding motif (G4) [3,50,51]. However, the dynamin GTPase domain contains additional sequences between G2 and G3, and downstream of G4 which contribute to the regulatory function of this domain. The G-domain resides on a helical bundle or neck, also known as the bundle signaling element (BSE). It is formed by 3 helices derived from sequences at the N and C terminal sides of the G-domain and the C-terminal region of the GED. The BSE channelizes nucleotide-dependent conformational changes from the G-domain to the stalk and regulates membrane binding during membrane fission. Although dynamins display a rather high

basal GTP turnover rate, the activity is stimulated 10–100-fold in some dynamin family members upon self-assembly and lipid-binding [52,53]. Conformational changes induced by GTP binding and hydrolysis are necessary for their function as mechano-chemical enzymes.

1.2.3.2 The middle domain (MD):

The middle domain comprises of the stalk and the bundle signaling element (BSE) [54–57]. The BSE has been proposed to propagate nucleotide-dependent conformational changes from the GTPase domain to the stalk, and to regulate dynamin activity. The middle domain along with the amino-terminal portion of the GED form an antiparallel four-helix bundle known as the stalk of these proteins [41–43]. The stalk mediates dimerization, tetramerization, and subsequent formation of higher-ordered structures into rings or spirals. The stalk dimerizes in a criss-cross manner to yield a dynamin dimer in which the two G-domains are oriented in opposite directions. This dimer represents the basic structural unit of dynamins [42,43]. The middle domain of dynamin lacks sequence homology to any known structural motif. Based on proteolysis results, the middle domain is further divided into N-terminal and C-terminal halves [58], with the N-terminal half being more conserved than the C-terminal half among dynamin isoforms (92% compared with 72% between dynamin-1 and 2) [53,59].Within the C-terminal half lies alternate splice sites for all the three dynamin isoforms. In the N-terminal half there is a predicted coiled-coil region (residues 320–350) that has been implicated in dynamin self-assembly [60,61].

1.2.3.3 Pleckstrin homology (PH) domain:

Pleckstrin homology (PH) domains are found in a number of membrane-associated proteins and have been implicated in protein-protein and protein-phospholipid interactions (Lemmon, 2007; Musacchio, Gibson, Rice, Thompson, & Saraste, 1993; Saraste & Hyvnen, 1995). In classical dynamin, PH domain links MD and GED and interacts directly with membrane bilayer

[57,65,66]. This domain is highly conserved and is essential for binding to negatively charged head group of phosphatidylinositol-4-5-biphosphate (PIP2) [67]. It binds to PIP2 in the cytosolic leaflet of the plasma membrane via a positively charged surface at the foot of the dynamin hairpin. Mutations in the PH domain that impair phosphoinositide binding exert dominant negative effects on clathrin-mediated endocytosis [68,69] [70]. PH domain of dynamin contains a hydrophobic variable loop (VL1) which mediates membrane binding by penetrating partially into the outer leaflet of a PIP2 -containing lipid bilayer [71–73]. This interaction is also important to generate membrane curvature required for vesicle scission during clathrin mediated endocytosis [74]. The binding of PIP2 to dynamin stimulates its GTPase activity [67]. This lipid binding is significantly enhanced upon dynamin oligomerization [65]. Although DRPs lack a recognizable PH domain, they have evolved different membrane-binding mechanisms. DRPs such as mitofusins, OPA1, Mgm1, and atlastins have trans-membrane helices that anchor the proteins to the target membranes. MxA and BDLP bind to membranes via an exposed loop [75,76] [77].

1.2.3.4 GTPase effector domain (GED):

The GED is also referred to as coiled-coil domain based on presence of two predicted small coiled-coil segments [61,78]. GED stimulates the GTP hydrolyzing function of the protein and thus acts as a GTPase activating protein (GAP). This domain interacts with GED domains from other monomers via an arginine finger motif during polymerization of dynamins into rings and spirals [58] [79] [60].

1.2.3.5 Proline rich domain (PRD):

The C-terminal domain of dynamin is rich in proline and basic residues, and contains several SH3-domain binding sites. SH3-binding domains play an important role in protein-protein interactions in many cellular processes, including clathrin-mediated endocytosis [80]. For

example, SH3 domain containing proteins such as Grb2, amphiphysin, and PLC bind to PRD domain of dynamin [81–84]. An array of PXXP motifs in the PRD interact with many SH3 domain-containing proteins and localizes dynamin at endocytic sites coordinating dynamin's function with other factors during endocytosis [84–87]

1.3 Cellular functions of dynamin family proteins:

1.3.1 Vesicle scission:

In animals, classical dynamins facilitate vesicle budding in clathrin mediated endocytosis (CME) from plasma membrane and are also required for budding of vesicles from the recycling endosome and Golgi. They are also required for the internalization of caveolae [3,26]. Dynamins are recruited by protein-protein and protein-lipid interactions through their PRD and PH domains respectively. In CME, clathrin assembly, the recruitment of dynamins and other membrane remodeling proteins lead to invagination of clathrin-coated buds and vesicle formation. Conformational changes of dynamin oligomers induced by GTP hydrolysis drives the constriction of the neck of the bud resulting in scission of the vesicle. The dynamin-1 homolog of plant, dynamin-related protein 2A (DRP2A) mediates Golgi-vacuole trafficking and localizes to the cell plate during cell division [88]. Though DRP2A interacts with clathrin binding partners, its direct role in endocytosis has not yet been demonstrated. *Arabidopsis* DRP1C, which lacks both the PH domain and the PRD, localizes to the division plane and the plasma membrane, and is thought to take part in clathrin-mediated membrane dynamics [89]. In yeast, a dynamin family member Vps1 (vacuolar protein sorting) controls trafficking between the *trans*-Golgi network (TGN) and endosomes. Vps1 localizes to the Golgi and is involved in clathrin-mediated vesicle formation at the TGN during transport of proteins to the vacuole and protein transport from the Golgi to the endosomal system [90–94]. Vps1 is the only yeast dynamin-like protein known to date that is involved in vesicular transport. Vps1 is also involved in the fission

of yeast vacuoles [90]. Recently, Vps1 has been shown to play an important role during endocytosis in yeast [95]. The important differences between CME events that are observed in animals, plants and yeast suggest that the role of dynamins in this process is not universal.

1.3.2 Mitochondrial fission

Maintenance of mitochondria requires continuous membrane fission and fusion. In animals and budding yeast, the dynamin-related proteins DRP1/Dnm1 mediate fission of mitochondria (Fig. 1.5). These proteins lack a PRD and PH domain but contain a B-domain located between the middle domain and the GED. Despite the absence of a PH domain, DRP1, similar to dynamins, binds to lipids and assembles into helical structures around lipid tubes [37]. The diameter of DRP1 helical assemblies on lipid tubes (~110 nm) are similar in dimension as those of mitochondrial constriction sites [38], suggesting that DRP1 spirals could assemble on mitochondrial sites destined for fission. Mitochondrial fission signals trigger the recruitment of the cytoplasmic DRP1 to prospective fission sites on the mitochondria [96].

Peroxisomes are highly dynamic single membrane bound organelles that increases in number in response to a variety of environmental stimuli. In yeast, dynamin related proteins Dnm1 and Vps1 mediate the fission of peroxisomes [97]. Dnm1 has an overlapping role in both mitochondrial and peroxisome fission. Mutation in Vps1 leads to formation of one or two giant peroxisomes or tubulated peroxisomes along cytoskeletal actin cables, suggesting its role in peroxisomal fission [93].

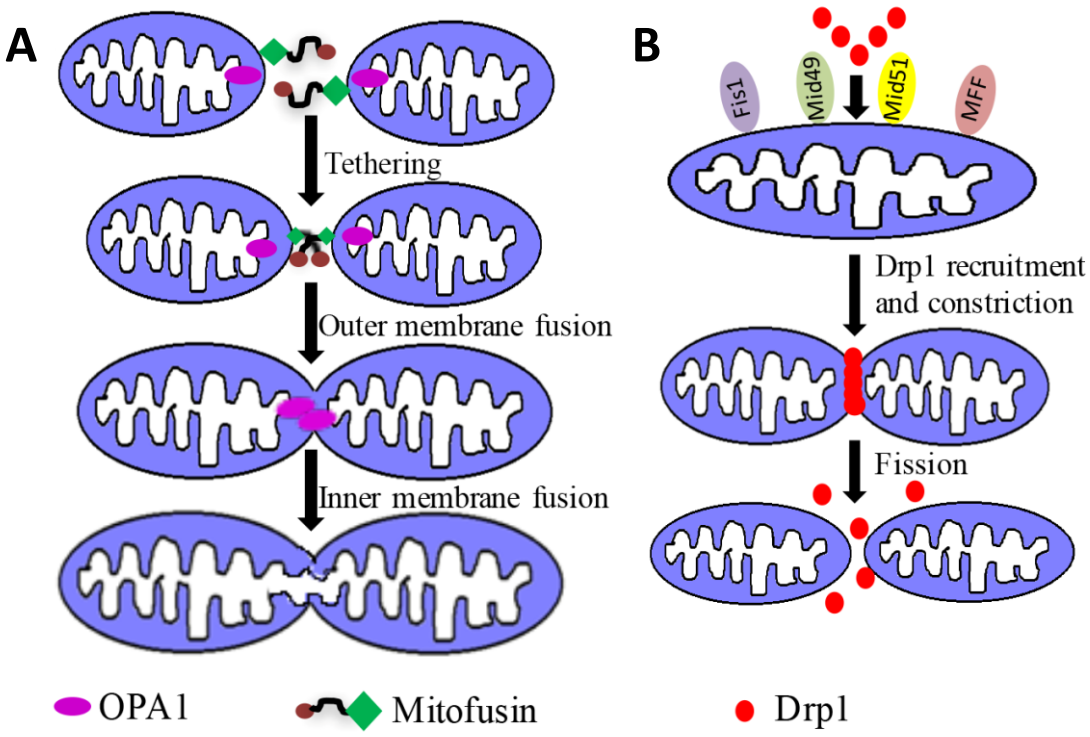


Figure 1.5: Maintainance of mitochondrial morphology by dynamins. (A) Mitochondrial fusion by mitofusins and OPA1. Mitofusins mediate tethering and fusion of outer mitochondrial membrane where as OPA1 brings about inner mitochondrial membrane tethering and fusion. (B) Mitochodrial fission by Drp1. Drp1 is recruited to fission sites by adapter proteins such as Fis1, Mff, Mid49, Mid51 and fragments mitochondria by constriction of mitochondrial membrane. Taken from Kar *et al*. 2017.

1.3.3 Peroxisome fission:

The mammalian counterpart is Drp1 which localizes in peroxisomes [98]. The finding that dynamins are involved in the division of both mitochondria and peroxisome organelles corroborates the notion that the activities of peroxisomes and mitochondria are fundamentally connected [99]. The dynamin related proteins DRP3A and DRP3B are involved in peroxisome fission in plants [100,101]. Though DRP3A and DRP3B share 77% sequence identity, they are not functionally redundant [102]. ARC5 (or DRP5B), another DRP family protein in *Arabidopsis* is also believed to participate in the peroxisomal fission, in addition to its major role in plastid division [103].

1.3.4 Chloroplast fission:

There are more numbers of dynamin like proteins present in plants than in animals and have been implicated in many cellular processes unique to the plant cell such as cell plate formation, cytokinesis, division of plastids. In plants, a combination of proteins of both prokaryotic and eukaryotic origins assists in the division of chloroplasts. DRP5B is the eukaryotic dynamin-related protein that facilitates division from the outside of chloroplasts [104]. Another dynamin like protein ARC5 (Accumulation and replication of chloroplast mutant 5) – is a dynamin like protein that was identified in *Arabidopsis thaliana*, and has been known to play a role in chloroplast division [103]. A similar protein has also been discovered in the red alga *Cyanidioschyzon merolae* and is named Dnm2 which is involved in the division of chloroplast. In *C.merolae,* patches of Dnm2 translocate from the cytosol to organize into rings around the middle of the chloroplast along with the FtsZ (a protein of prokaryotic origin) [105]. A constriction of this ring decreases in ring diameter and leads to fission of chloroplast.

1.3.5 Mitochondrial fusion:

Fusion of mitochondrial membranes is mediated by a different set of Dynamin family members (Fig. 1.5). The fusion of mitochondria involves at least two steps, fusion of the outer mitochondrial membrane and fusion of inner mitochondrial membrane. In animals, the membrane-anchored dynamins mitofusin-1 (MFN1)/ and MFN2 mediates outer membrane fusion, whereas fusion of the inner membrane is carried out by optic atrophy-1 (OPA1). In budding yeast, while Fzo1(mitofusin homolog) mediates fusion of outer mitochondrial membrane, the inner membrane fusion is carried out by Mgm1 (the OPA1 homolog) [106,107]. It was proposed that Mitofusins/Fzo1 form homo- or hetero-oligomers through C-terminal heptad repeat regions, and tether adjacent mitochondria during the initial steps of mitochondrial fusion [108]. However, recent report demonstrates that oligomerization during mitochondrial

tethering occurs through the interaction between GTPase domains [109]. Mammalian OPA1 and yeast Mgm1 exist in two forms, a membrane-bound form and a diffusible form, and both are required for mitochondrial fusion [110]. Although Mgm1 lacks a PH domain, it binds to negatively charged phospholipids that are typically found in mitochondrial membrane suggesting that Mgm1 contains a lipid-binding motif [110]. Moreover, the dynamins of the inner mitochondrial membrane, together with scaffolding proteins such as prohibitins, are involved in the maintenance of cristae morphology, possibly by assembling into higher-order structures [111,112]. Dynamins that are associated with mitochondrial fusion have not been found in plants. Although, a Fzo1-like protein (FZL) has been identified in plant, it was shown to be involved in determining thylakoid and chloroplast morphology, without affecting mitochondrial morphology suggesting that mitochondrial fusion in plant occur by a different mechanism [113,114].

1.3.6 Endoplasmic reticulum fusion:

Fusion of the endoplasmic reticulum (ER) is mediated by a class of dynamin-like GTPases known as atlastin (ATL). Depletion of or mutations in ATL causes fragmentation of the ER network whereas its over-expression results in the formation of expanded ER [30]. Atlastin localizes on adjacent membranes and form *trans*-oligomeric complexes and its GTPase activity is required to drive membrane fusion.

1.3.7 Cytokinesis:

Cytokinesis is the final stage of the cell cycle in which a single cell is physically separated into individual daughter cells. In eukaryotes, this process leads to the division and partitioning of chromatin, organelles, cytoplasmic components and the construction of new membrane between daughter cells. Animal cells divide by constricting plasma membrane and targeting membrane along the newly formed cleavage furrow whereas plant cells divide by forming a unique

cytoskeletal apparatus, the phragmoplast, across the division plane. Dynamin and dynamin-related proteins have been shown to play essential roles in cell division in animals, plants and the protest like *Dictyostelium discoideum* [115–118] [119]. Dynamin localizes to cleavage furrow membranes and is required for cytokinesis in a number of organisms. In animals, dynamin-I/ II along with clathrin, syntaxin, and endobrevin are thought to play a role in furrow-specific endocytosis and fusion events during daughter cell separation [120,121]. In *Drosophila,* dynamin localizes to the sites of membrane invagination and plays an important role in cleavage furrow formation [122]. In *C. elegans,* dynamin homolog Dyn-I is shown to participate in cytokinesis by regulating the final stages of cleavage furrow elongation and completion of cell division [123].

In plants, dynamins are involved in the formation of the tubulo–vesicular network (precursor for the cell plate) and in the retrieval of excess membrane by vesicle budding from the maturing cell plate during cytokinesis. Phragmoplastin, the soybean DRP1 homolog, was the first dynamin shown to be involved in cytokinesis [124]. Phragmoplastins are concentrated at the cell plate and help in transforming the vesicles into tubules [125,126]. Formation of a tubular network allows the generation of a two-dimensional structure that is subsequently flattened to form the membrane of the dividing cell.

Studies in the model plant *Arabidopsis* revealed two subfamilies of dynamins, DRP1 and DRP2 localize to the division plane during cytokinesis. The function of DRP1 in cytokinesis has been studied in more detail. DRP1 localizes to the spindle and phragmoplast where they are believed to provide anchor for Golgi-derived vesicles to attach to microtubules and in turn direct the vesicles to the forming cell plate during cytokinesis [117,127]. DRP2, which is closely related to mammalian dynamin I, localizes to the cell plate and is thought to be involved in membrane trafficking in the cell plate via clathrin-dependent recycling of vesicles between the *trans*-Golgi network and the vacuole [128].

1.3.8 Antiviral activity:

Mx proteins (Myxovirus resistance) form a separate group within the dynamin superfamily and are evolutionarily conserved [129]. Their expression is induced by interferon treatment. While Mx proteins are distributed both in nucleus and cytoplasm in rodents, they accumulate exclusively in the cytoplasm in other vertebrates. Most of the mammals possesses two *Mx* genes, *MxA* and *MxB*. The expression of *Mx* genes is controlled by type I and type III interferons. MxA confers resistance to a variety of viruses such as orthomyxovirus, paramyxoviruses, bunyaviruses, picornaviruses, rhabdoviruses *and* togaviruses [4]. The antiviral activity of MxA depends on both oligomerization and GTPase activity [41] [130]. MxB has been shown to be involved in nuclear import and cell cycle progression [131]. Similar to MxA, the expression of human MxB is also induced by type I IFN. Although MxB was reported to have anti-HIV-1 activity by reducing the level of integrated viral DNA, a recent finding contrasts this anti-HIV role of MxB [132–134].

Mx proteins directly interact with viral particles in infected cells leading to their sequestration. The antiviral activity of MxA was shown to be dependent on two essential features, namely GTP hydrolysis performed by the G domain and oligomerization mediated by the MD and GED [41,135–138]. As typical members of the dynamin superfamily, Mx proteins tubulate liposomes and oligomerize in ring-like structures around these tubuled membrane.

The mechanism of Mx proteins is less understood and has been studied only in few of the viruses. These proteins bind to essential viral components and block their function. MxA directly interacts with the nucleoprotein component of viral nucleocapsids of LACV (La Crosse virus) and THOV (Thogotovirus), and depletes nucleoprotein from viral replication sites [139–141]. Similarly, MxA inhibits the replication of influenza A virus [142]. Therefore it has been proposed that the MxA oligomeric rings represent antiviral molecular machines that oligomerize

around the viral nucleocapsid structure. Conformational changes upon GTP binding and/or GTP hydrolysis might lead to disintegration of the infecting nucleocapsids.

1.3.9 Bacterial dynamins:

Many bacterial species harbor genes encoding dynamin-like proteins (BDLP) closely related to the Fzo1/mitofusins [76,143,144]. Although known for more than a decade, cellular functions of bacterial dynamin counterparts are not well established. The first report about a bacterial dynamin is the elucidation of BDLP structure in *Nostoc punctiforme* (Low and Lowe, 2006). BDLP shares a similar domain architecture with other DRPs and oligomerizes at the surface of liposomes inducing membrane tubulation. It was suggested to have a role in membrane fusion [45]. Similarly in *Bacillus subtilis*, DynA has been shown to promote membrane tethering and is speculated to mediate membrane fusion [145]. Although structural and biochemical properties of BDLPs are known, their cellular functions are not known. The overall conservation of these large GTPases in many bacterial genomes suggests an important role in bacterial membrane remodeling. The homology of BDLPs to mitofusin/Fzo makes bacterial dynamins an ideal tool to unravel the mechanism of membrane fusion.

1.3.10 A novel function: Role of dynamins in nuclear membrane remodeling:

A new and interesting role of dynamin related protein has been found in the ciliate protist *Tetrahymena thermophila*. In this organism, a dynamin related protein, Drp6 is seen to be specifically upregulated and translocated to the nuclear membrane in a developmental stage specific manner [146]. *Tetrahymena* harbors eight dynamin related proteins reflecting the presence of complex endomembrane system. While some of the DRPs function in endocytosis and some localizes on mitochondria, Drp 3, 4, 5 and 6 don't have any homolog in other organisms, forming a lineage specific clade suggesting a lineage specific function [147]. Drp6 localizes on the nuclear envelope and ER derived cytoplasmic puncta. *Tetrahymena* exhibits

nuclear dimorphism and harbors two structurally and functionally distinct nuclei; a silent germline micronucleus (MIC) and a transcriptionally active somatic macronucleus (MAC) [148]. During conjugation, the sexual mode of reproduction, *Tetrahymena* MIC undergoes a series of nuclear divisions and differentiates to become the MAC in the exconjugants. The differentiation of MIC to MAC requires several fold expansion of the nuclear membrane. Both localization studies and functional analysis shows that Drp6 is involved in nuclear remodeling during this MIC to MAC development. Drp6 expression is specifically upregulated at this stage and is recruited to the nuclear envelope of newly developing nuclei. Either absence of Drp6 or inhibition of Drp6 function results in profound defect in the formation of polyploid MAC [146]. It has been suggested that the specific requirement for Drp6 localization to nuclear envelopes might be required for facilitating rapid expansion and consequent remodeling of the nuclear membrane.

1.4 Common biochemical and structural features of dynamin family proteins:

1.4.1 Self-assembly:

Dynamins are universal membrane remodeling molecules that have been adapted to function at many different compartments in the cell. The first essential property of dynamin is its capacity to oligomerize into lock-washer-like rings or a cylindrical helix [149]. Such oligomers were first observed at the non-permissive temperature in electron micrographs around the neck of plasma membrane buds in the temperature-sensitive *shibire* mutant in *Drosophila* [150]. Subsequent studies revealed that dynamin exists as a tetramer under physiological salt conditions [58,149]. The tetramers are capable of further self-assembly into higher-ordered structures that resemble rings and spirals [149] (Fig. 1.6). The tetrameric form, thus represents an intermediate in higher order assembly of dynamin. Purified dynamin spontaneously polymerizes into rings and helices when incubated at low ionic strength solutions [149]. or in the presence of negatively charged

narrow tubular templates (such as membrane tubules, microtubules or actin bundles) [5,151–153]. Investigation of the individual domains of dynamin confirmed that GED is essential for self-assembly and interacts strongly with the GTPase domain [58] [79] . Consistent with a role for GED in dynamin self-assembly, removal of the GED or one or both of the coiled-coil segments within GED impaired dynamin-dynamin interactions [61]. GED also binds to itself in the homodimers or homotetramers, and also interacts with the middle domain [36,61]. Although removal of PRD does not impair dynamin self-assembly completely, few stretches of amino acids within PRD are involved in dynamin-dynamin assembly, suggesting that the C terminus of dynamin is not

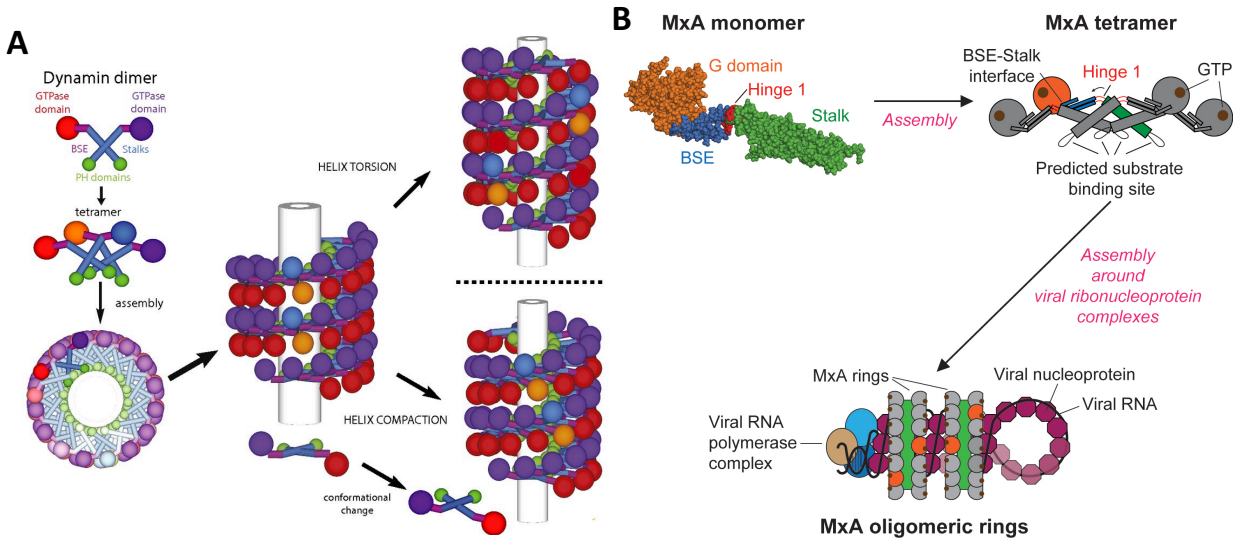


Figure 1.6: Mechanism of dynamin functions. (A) Model showing dynamin helical spirals constricting the target membrane resulting in membrane tubulation and fission. (B) Model showing MxA oligomeric rings around the viral nucleoprotein. Assembly and disassembly cycle regulate their functions. Taken from Colom *et al*. 2017.

essential but participates in dynamin assembly. Dynamin polymerization resulting from the side-by-side apposition of dimers via stalk tip interactions occurs at an angle that determines the diameter of the ring [42,43,55,130,154]. Thus, the stalks form the core of the ring, whereas the BSE and G-domains of each dimer project towards the adjacent rings of the dynamin helical spiral (Fig 1.6). A similar domain organization and mode of self-assembly is predicted to be shared by all DRPs involved in membrane fission. Structural studies of dynamin related proteins such as BDLP, Drp1/Dnm1, Mgm1/OPA1 and MxA have revealed that, even though the exact interactions between subunits are not conserved throughout the dynamin superfamily, the basic assembly properties such as formation of helical polymers or ring-like structures are shared by all the members of this family [37,38,75,76,155,156] [157,158].

1.4.2 GTP hydrolysis and conformational changes:

An essential function of dynamins is to couple energy from GTP hydrolysis to membrane remodeling. The GTPase domain is most conserved among all dynamin family proteins [159,160]. GTP hydrolysis modulates the helical structure of dynamin and constriction of membrane tubes [153]. It generates a rotational force during constriction producing a conformational change in the dynamin polymer. Dynamins exhibit relatively high basal level GTP hydrolysis rate and their activity is stimulated upon dynamin assembly. For example, dynamin, MxA and GBP1 have basal GTP hydrolysis rates of 1-5 min^{-1}. These rates cooperatively increase with the protein concentrations, correlating with the increased assembled structure formation. In the presence of appropriate lipid templates, the GTPase activity can be further enhanced, as for example, more than 100-fold increase in GTPase rate of dynamin in the presence of tubular membrane templates. [41,52,53] [151,161,162].

The mechanism by which the energy of GTP hydrolysis is coupled to perform mechano-chemical work on the membrane remains unclear. Theoretically, the energy of

hydrolyzing one GTP molecule to GDP yields about 20 k_BT. It has been proposed that about 40% of this energy can be transformed into mechanical force, and thus hydrolysis of only a few GTP molecules would be sufficient to overcome the energy barrier required for membrane fission by constriction (35–70 $k_B T$). The energy barrier for fission further depends on the tension and rigidity of the membrane, which *in vivo* is influenced by the organization of certain lipid molecules and also other membrane interacting proteins, in addition to dynamin [163].

1.4.3 Mechanism of membrane remodeling:

Lipid bilayers are auto-sealable, and only forces greater than 10^{-3}-10^{-2} N/m can disrupt the membrane. The auto-sealable property of lipid bilayers makes them difficult to break, and thus membrane fission is not a spontaneously triggered process [164]. Dynamin binds to negatively charged lipids through positive amino acid residues in the PH domain. Upon dynamin interaction with membrane, the PH domain orients towards the inner part of the helix and polymerization drives membrane flow inside the helix. The power stroke derived from GTP hydrolysis pulls adjacent dynamin filaments along each other, resulting in constriction of the underlying membrane template [165]. Consecutive rounds of GTP hydrolysis leads to increase in the membrane curvature sequentially, causing the constriction of membrane (50nm to 20nm) by the dynamin coat. Constriction of dynamin helix imposes a strong curvature on the underlying membrane. As elasticity of membrane competes with the rigidity of dynamin coat, the drastic curvature change is energetically expensive. Kinetics of dynamin fission depends on bending rigidity, tension, and constriction torque [166,167]. The membrane fission process is completed by membrane insertion of hydrophobic residues from the PH domain and the dissociation of dynamin oligomers following GTP hydrolysis [167,168]. This mechanism of membrane fission is followed by other dynamin family members such as Drp1 [38,155,158]. Such membrane

constriction mechanism strictly requires a helical arrangement of the dynamin polymers. However, dynamin also forms ring like assembled structures which are believed to mediate the stabilization of certain membrane curvatures [169]. Electron microscopy (EM) studies have provided insight into the mechanisms of mitochondrial fission. Like Dynamin, the yeast Drp1 homolog Dnm1 can tubulate membranes by assembling into large multimeric spirals that wrap around those tubules. There are, however, two striking differences between dynamin spirals and Dnm1 spirals. First, the diameter is 50 nm for dynamin, but 120 nm for Dnm1 [38,158]. This large diameter of Dnm1is sufficient to wrap around a double membrane structure formed at constriction sites in mitochondria where both inner and outer membranes are still intact. The luminal diameter of these spirals reduces to less than 10nm upon GTP hydrolysis at constriction site and brings about mitochondrial fission.

In case of the fusion dynamins, GTPase domain dimerization tethers membranes which are in close apposition. As for example, in atlastin, GTPase domain dimerization tethers opposing ER tubules [29,170]. In this case, GTP hydrolysis is proposed to pull opposing membranes towards each other by catalyzing the transition from the open to the cross-over helical conformation. Fusion of the closely juxtaposed membranes is believed to be facilitated by disturbance of the lipids by trans-membrane motifs and the amphipathic helix in the C-terminal tail of atlastin [171,172]. In addition, it has been suggested that the GTP hydrolysis function may be used to recycle atlastin dimers from the cross-over conformation to the open conformation to allow new rounds of tethering. The bacterial dynamin, DynA localizes at the septa of dividing cells suggesting a role in membrane fusion of the invaginating septum [173]. BDLP shows GTP-dependent assembly on membranes resulting in the formation of a highly curved membrane tubule. These highly stressed membrane tubules are suggested to be .fusogenic. Membrane

tubules generated by BDLP are proposed to undergo fusion following GTP hydrolysis and the removal of the BDLP coat [75,76].

Mitochondria are double membrane organelles and fusion of mitochondria requires coordinated fusion of four sets of lipid bilayers. Unlike SNARE or AAA-ATPases, which are universal fusion mediators, mitochondrial fusion dynamins are specialized fusion machineries dedicated solely for mitochondrial fusion [174]. Mitochondrial fusion process is a homotypic membrane fusion. The fusion of outer and inner membrane are two mechanistically distinct processes, both requiring specialized dynamin proteins [112]. The most accepted model suggest that dynamins on the opposing membranes dimerize and then pull the membranes together with a SNARE-like mechanism [175]. During this process GTP binding and hydrolysis brings about the necessary conformational changes required for tethering of two apposing membranes [106].

The outer mitochondrial fusion dynamin, Mfn/Fzo1 require GTP-binding and hydrolysis for dimerization [176,177]. The *cis* and *trans* dimers of GTP-bound Fzo1 interact to tether mitochondrial outer membranes. GTP hydrolysis followed by ubiquitylation of Fzo1 is necessary for efficient membrane fusion in yeast [178]. Mfn also appears to follow a similar mechanism since mutation in the dimerization interface of Mfn2 leads to defect in mitochondrial fusion as seen in case of Charcot-Marie-Tooth disease patients [179]. Additionally, it has been found that ubiquitinylation also controls the fusion activity of mitofusins [180]. Earlier it was believed that trans-dimerization of Mfn1 occurs through its C terminus heptad repeats during mitochondrial tethering. In contrast, a recent report on the crystal structure of Mfn1 suggests that dimerization occurs through the G domain and is regulated by GTP binding and hydrolysis [48,109].

The mitochondrial inner membrane fusion is carried out by OPA1 (mammals)/Mgm1 (yeast). Alternate splicing and proteolytic processing generates long and short isoforms of both OPA1

and Mgm1 and are present in the mitochondrial intermembrane space. Results from genetic and biochemical studies suggest that homo and hetero-oligomers of short and long isoforms mediate inner mitochondrial membrane fusion by locally deforming the inner membrane [181,182]. It has been proposed that the GTPase inactive long isoforms tether the inner and out mitochondrial membranes and subsequent conformational changes in membrane by GTPase hydrolysis by the short isoforms facilitates the fusion process.

Alternative mechanisms of mitochondrial fusion propose that assembly of dynamins into spirals on individual mitochondria tubulates the underlying mitochondrial membrane. Protruding membrane tubules formed by this process subsequently might be tethered by contacts between opposing dynamin spirals [182] and this forced contacts between the convex lipid surfaces at the tips of the dynamin spirals could bring about the fusion of opposing membranes resulting in fusion of mitochondria.

1.5 Nuclear envelope remodeling during closed mitosis:

In eukaryotes, the genome is surrounded by a double membrane structure called nuclear envelope. Nuclear envelope fulfils a critical role in shielding the genome from cytoplasm and also represents a highly specialized membrane that provides anchoring sites for chromatin and cytoskeleton [183]. The outer nuclear membrane is continuous with the endoplasmic reticulum. In contrast to metazoans, where the nuclear envelope disassembles during mitosis, many fungi including budding yeast undergo closed mitosis, where the nuclear envelope remains intact throughout the cell cycle. In closed mitosis, the NE does not disassemble and the chromosome segregation happens inside the confined NE. During this process, the nuclear envelope expands to accommodate the movement of the segregating chromosomes. Followed by chromosome

segregation, the NE needs to be cleaved, resealed and restructured to form two round daughter nuclei. This process requires continuous remodeling of nuclear membrane. Since the nuclear envelope is continuous with the ER, it is speculated that the additional membrane could come from the ER, and if this is the case then the cell would have a mechanism for allocating membrane from the ER to the nuclear envelope specifically during nuclear membrane expansion. Thus, the mechanisms driving nuclear envelope dynamics, and in particular nuclear envelope expansion, remain to be uncovered.

Ciliates like *Tetrahymena thermophila* also undergo closed mitosis. The ciliate protozoa *Tetrahymena* are unicellular organisms, and they contain two functionally and structurally distinct nuclei in the same cytoplasm: a somatic macronucleus (MAC) and a germline micronucleus (MIC) [184]. The MAC is transcriptionally active during all stages of *Tetrahymena* life cycle and the MIC is transcriptionally inert. The NEs of both the MAC and MIC stay intact throughout life cycle. *Tetrahymena* undergo sexual conjugation under starvation, during which the MICs of each cell of the conjugating pair produce gametic nuclei by meiosis, subsequently gerating the zygotic nuclei after reciprocal fertilization. The zygotic nucleus (originated from the MIC) subsequently differentiates into two MACs and two MICs after two rounds of post-zygotic mitotic divisions (Fig. 1.7). During this stage of nuclear differentiation, the NE expands 10 to 15 folds and requires addition of membrane into already existing nuclear envelope. However, the mechanism of this expansion is not explored.

1.6 Objective of the current study:

Tetrahymena maintains a highly complex network of membrane trafficking pathway and has accommodated expansion of many gene families encoding essential proteins of membrane trafficking pathway [185–187]. Several membrane trafficking pathways in *Tetrahymena*, and

proteins playing key roles in these pathways share remarkable conservation with higher eukaryotes such as yeast and mammals

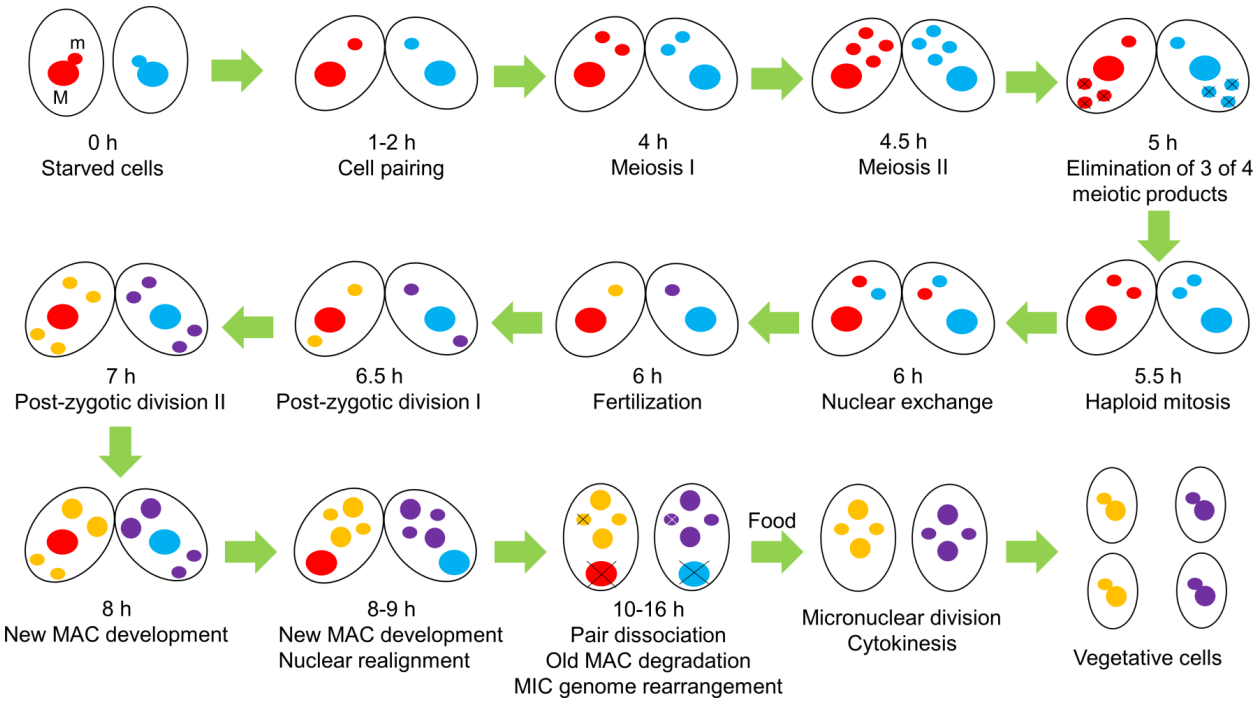


Figure 1.7: Conjugation stages of *Tetrahymena*. The small circle is micronucleus (m) and large circle is macronucleus (M). Taken from Aslan *et al.* 2017.

[188]. *Tetrahymena* genome encodes a large number of (63) of Rab GTPases, proteins that control key steps in membrane trafficking pathway, which is greater in number than Drosophila (33) and C. elegans (29) reflecting the complexity of membrane trafficking pathways in this organism [188]. Another example of complex membrane networking of *Tetrahymena* comes from the presence of eight dynamin related proteins. This is again a number significantly large for a unicellular organism. Drp1 has an essential role in endocytosis [147]. Although the function of Drp2 has not been studied in detail, similar to Drp1, it is also localized on parasomal sacs suggesting a role in endocytosis. Other two dynamins Drp7 and Drp8 localize to subcortical mitochondria and might have mitochondrial functions. The cluster of Drp3-6 represents a lineage specific group of DRPs with no homology with any other dynamin proteins and might have

evolved lineage specific function. In vegetatively growing *Tetrahymena* cells, Drp6 localizes on nuclear envelope and ER derived vesicles, and plays a role in nuclear expansion [146]. Its function is essential during nuclear differentiation where germline MIC undergoes several fold expansion to form somatic MAC. The localization of Drp6 shows stage-specific regulation. During starvation, Drp6 dissociates form nuclear envelope and a large proportion of Drp6 shifts to cytoplasmic puncta. During early conjugation, Drp6 localizes almost exclusively as cytoplasmic puncta and remains so until the MAC development stage. It reassociates strongly with the new MICs and the developing MACs, but not with the old degenarating MAC [146]. Either deletion or expression of dominant negative form of Drp6 leads to failure of development of MAC from MIC at this stage. Furthermore, the assembly of Drp6 at the nuclear envelopes during starvation and conjugation increases dramatically as compared to vegetative cells [146]. Together, these observations strongly suggested that Drp6 plays an essential role in macronuclear expansion in *Tetrahymena*. Drp6 represents an example of a novel role for DRPs. However, the mechanism and regulation of its function is not known. Therefore, the present study aims at understanding the mechanism of Drp6 function. To gain insight into the structure and function of Drp6, the biochemical and biophysical properties of the protein were characterized. Part of this study addressed investigation of the self-assembly property of Drp6. To understand its membrane remodeling function, the membrane binding domain of Drp6 was identified and the basis of its nuclear envelope recruitment was studied. Drp6 localization and activity is differentially regulated by physiological states and developmental stages. In order to understand the mechanism of Drp6 regulations, the post-translational modifications and their roles were also investigated. In order to understand the mechanism and regulation of Drp6 function, the objectives of the present study are:

1. Biochemical characterization of Drp6.
2. Identification of lipid binding domain in Drp6.
3. Investigation on Drp6 nuclear recruitment.
4. Identification of post-translational modifications of Drp6 and their effects on its cellular distribution.

Results are presented in four different chapters of this thesis.

CHAPTER TWO

Biochemical Characterization of Drp6

2.1 Introduction:

The *Tetrahymena* dynamin-related protein 6 (Drp6) localizes on nuclear envelope and plays a role in nuclear remodeling in a stage specific manner. Its function is essential during nuclear differentiation where the germline micronuclei undergo several-fold expansion to form somatic macronuclei [146]. Drp6 is a member of a family of large GTPase proteins that include classical dynamins and dynamin-related proteins (DRPs). Members of this family perform several important cellular functions such as endocytosis, cytokinesis, maintenance of mitochondrial dynamics and antiviral activity [160]. They are mechanochemical enzymes that couple the free energy of GTP hydrolysis with membrane remodeling. Classical dynamins are involved in membrane trafficking during endocytic vesicle scission at the plasma membrane and secretory vesicle scission at golgi [160]. Mitochondrial DRPs are involved in maintenance of mitochondrial dynamics by mediating both fission and fusion [189,190]. While fission DRPs like Drp1/Dnm1/Dlp1 oligomerize on mitochondrial fission sites and bring about fission, fusion DRPs such as Mfn1/Mfn2/Fzo1 and Mgm1/OPA1 mediate fusion of outer and inner mitochondrial membranes, respectively [191]. A group of interferon-induced DRPs such as MxA and MxB exhibit antiviral activity against a wide range of viruses by forming complexes around the viral nucleoproteins, different from the canonical membrane remodeling function [4,192]. Dynamins and DRPs are multi-domain proteins, and share similar domain architecture. Classical dynamins harbor five functionally distinct domains: a GTPase domain, middle domain (MD), GTPase effector domain (GED), pleckstrin homology (PH) domain, and proline rich domain (PRD) (Fig. 2.1A) [159,160]. Dynamin-related proteins retain three characteristic domains but lack PRD (involved in protein-protein interactions) and the PH domain (responsible for membrane binding). Although DRPs lack a defined PH domain, an exposed lipid binding loop in MxA, an alpha helical membrane penetrating paddle domain in BDLP and trans-membrane

helices in Mitofusins, OPA1, Mgm1 and atlastins function as membrane binding motifs [75,77,193–196].

Though involved in diverse functions, the members of this family share common biochemical properties such as low affinity GTP binding, a high propensity to oligomerize around lipid templates, and oligomerization-stimulated GTP hydrolysis [197]. A characteristic feature of dynamin family proteins is their ability to polymerize into regular structures on membrane templates. Dynamin self assembles into rings or helices around microtubules or the neck of clathrin-coated vesicles [149,198]. Other DRPs such as MxA, Dnm1, OPA1 and BDLP also form rings and helical oligomers on their target membrane [75,77,157,199] .

The intra- and inter-molecular interactions that promote self-assembly of dynamins into higher-order rings and filamentous structures stimulate GTP hydrolysis, and are important for membrane-remodeling functions [160,197]. Cycles of assembly and disassembly of dynamin oligomers at sites of endocytosis are important for vesicle scission. This cycle of assembly/disassembly is important for functions of other DRPs as well, for example Drp1 during mitochondrial fission, mitofusins/Mgm1/OPA1/Fzo1 during mitochondrial fusion and MxA in antiviral activity [77,160,200–202] . Disruption of assembly by mutations interfering with oligomerization results in functional inhibition of dynamins and DRPs [200,203,204].

Although role of Drp6 in *Tetrahymena* macronuclear development has been established, the mechanism of its function is not known. Like other DRPs, Drp6 also harbors three domains, the G domain, MD and GED (Fig.2.1A) [146] . In an attempt to understand the mechanism of Drp6 function, its GTPase activity and oligomeric state were evaluated.

2.2 Materials:

Bacterial Strains:

E. coli-DH5α strain was used for propagation of plasmids and for all recombinant DNA cloning steps. C41 and C43 strains of BL21 (DE3) used for expression were obtained from J.E.Walker, The Medical Research Council Laboratory of Molecular Biology, Cambridge, UK.

Plasmids:

pRSETB vector was obtained from Prof.M.S. Shaila's Lab, MCB, IISc, Bangalore.

pET15b Hdyn1 (His tagged Human dynamin 1) was a kind gift from Prof. Mark Lemmon, University of Pennsylvania, USA.

Gene Synthesis:

The codon optimized *DRP6* gene for bacterial expression and purification was commercially synthesized by Eurofins Scientific Ltd.

All restriction enzymes and DNA modifying enzymes for cloning purpose were purchased from New England Biolabs, UK unless specified otherwise.

Culture media:

Luria bertani broth and agar for bacterial culture were purchased from HiMedia. IPTG and other antibiotics were purchased from MP Biomedicals.

Chemicals and reagents:

Ni-NTA Agarose was purchased from Qiagen, USA. Protease inhibitor cocktail was purchased from Roche, Switzerland. BS^3 was purchased from Pierce, Thermo Scientific and Carbon coated grids for electron microscopy was purchased from Ted Pella,Inc. BIOMOL green used for GTP hydrolysis measurement was from Enzo Life Sciences, USA.

All other chemicals and reagents used were purchased from Sigma-Aldrich, USA, unless mentioned otherwise.

2.3 Methods:

2.3.1 Cloning, expression and purification of recombinant Drp6:

The codon optimized *DRP6* gene was synthesized (Eurofins, MWG) and obtained in the pUC57 vector. The insert was released and cloned into the pRSETB expression vector (a kind gift from Prof. M.S.Shaila, IISc, Bangalore) using KpnI and EcoRI sites. The pRESTB-*DRP6* construct was transformed into BL21 (DE3), C41 (DE3) and C43 (DE3) strains of E. coli to express the protein bearing an N-terminal His_6 tag. The expression of Drp6 was optimized by inducing the transformants for 5 hours at 37°C and 16 hours at 18°C. Maximum expression was obtained in C41 cells when induced with 0.5mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) (MP biomedical) at 18°C. For large scale expression, 6ml of overnight grown culture from single colony was innoculated into 1 l LB broth supplemented with 100µg/ml ampicillin and grown at 37°C at 220 RPM till the OD_{600} reached 0.4 - 0.5. The culture was shifted to 18°C, 0.5mM IPTG was added after 1 hour and protein expression was induced for 16 hours at the same temperature. For protein purification, cell pellets were resuspended in 50 ml of ice cold buffer containing 25mM HEPES pH 7.5, 300mM NaCl, 2mM $MgCl_2$, 2mM β-mercaptoethanol and 10% glycerol (buffer A) supplemented with EDTA-free protease inhibitor cocktail (Roche) and 100mM phenylmethyl sulfonyl fluoride (PMSF, Sigma). The cells were ruptured by sonication and the crude lysate was centrifuged at 15,000 RPM for 45 min at 4°C. The supernatant was incubated with pre-equilibrated Ni-NTA resin (Qiagen) for 2 hours, the resin was washed with 100 bed volume buffer A supplemented with 50mM imidazole and the protein was eluted with 250mM imidazole in the same buffer. The purified protein was checked by Coomassie-stained SDS-PAGE gels and the purity was assessed by Image J analysis (NIH). The fractions containing the purified protein were pooled, dialyzed with buffer A to get rid of imidazole and concentrated using Amicon ultra-15 centrifugal filters (Millipore) with a molecular cut-off of 10kDa. The

purified protein was confirmed by Western blot analysis using anti-His antibody (Sigma). Concentration of purified proteins were determined by Bradford assay (BioRad). Expression and purification conditions of histidine tagged human dynamin 1 (pET15b-Hdyn1) were similar to that of His-Drp6.

2.3.2 Size Exclusion chromatography:

For size exclusion chromatography, 500µl of concentrated recombinant Drp6 (0.5mg/ml) or Hdyn1 (0.5mg/ml) was loaded onto a Superdex 200 GL 10/300 (GE Life Sciences) pre-equilibrated with buffer A without glycerol. The column was run at a flow rate of 0.5ml/min and 0.5ml fractions were collected. The column was calibrated by using standard molecular weight markers from Sigma. The peak fractions were checked on SDS-PAGE followed by silver staining.

2.3.3 Chemical cross-linking:

Cross-linking of recombinant Drp6 was performed using bis(sulfosuccinimidyl)suberate (BS^3,Pierce, Thermo Scientific) in buffer containing 25mM HEPES pH 7.5, 150mM KCl and 2mM $MgCl_2$ (Buffer B) [58] . The protein (2µM) was incubated in presence of 0.5mM or 1mM BS^3 at room temperature for 5 min to 30 min. The reactions were stopped by adding glycine to 100mM and the cross-linked products were analyzed on 6% SDS-PAGE followed by silver staining.

2.3.4 Negative stain electron microscopy:

Purified recombinant Drp6 (1µM) in buffer B was incubated with 0.5mM GTPγS (Sigma) in a 20µl reaction volume for 20 min at room temperature and was adsorbed for 5 min onto a 200 mesh carbon coated Copper grid (Ted Pella, Inc.). The grid was stained with a drop of 2% freshly prepared uranyl acetate (MP Biomedical) for 2 min followed by washing with another

drop of 2% uranyl acetate for 6 sec [149]. The grid was dried at room temperature for 10 min and observed on a FEI Tecnai G2 120KV electron microscope.

2.3.5 Measurement of GTP hydrolysis activity:

The GTP hydrolysis activity of recombinant Drp6 was measured by colorimetric assay using a Malachite Green-based phosphate assay kit (BIOMOL Green, Enzo Life Sciences). Typically, a 90µl reaction volume containing 1µM Drp6/0.5 µM Hdyn1 and 1mM GTP (Sigma) in buffer B was incubated at 37°C unless mentioned otherwise. Aliquots of 20µl were collected at different time intervals and the reactions were stopped by adding 5µl of 0.5 mM EDTA. To this, 1ml BIOMOL green solution was added and the colour was allowed to develop for 20 min at room temperature before measuring the absorbance at 620nm. The amount of phosphate released was quantified by using phosphate standards provided with the kit. To measure GTPase activity at low salt, the assay was performed under same conditions except the KCl concentration of the buffer was kept at 40mM. For measuring K_m and K_{cat}, reactions were performed in triplicate with varying GTP concentrations ranging from 50 µM to 2000 µM for 10 min at 37° using 2µM Drp6. The values obtained from three independent experiments were plotted and analyzed using Origin Pro8.0 program.

2.4 Results:

2.4.1 Expression and purification of recombinant Drp6:

In order to understand the structure and function, Drp6 in *E. coli* C41 (DE3) cells was expressed and purified as soluble Drp6 bearing an N-terminal His_6 tag. The growth at low temperature was used for optimal expression of the protein to avoid formation of inclusion bodies. The protein purified by Ni^{2+} affinity chromatography migrated at 87 kDa by SDS-PAGE, corresponding to its calculated molecular mass (Fig. 2.1B). The identity of the protein was further confirmed by

Western blot analysis with anti-His antibody (Fig. 2.1B). The purified protein was estimated to be ~ 90% pure as determined by Image J analysis. Since several attempts to purify Drp6 further by ion exchange chromatography resulted in substantial loss of the protein without significant improvement in purity, Ni-NTA-purified Drp6 was used for subsequent experiments.

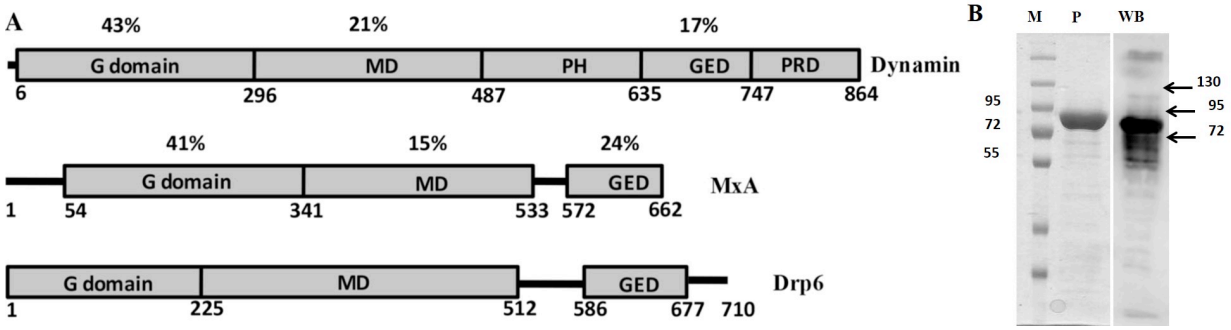


Figure 2.1: Domain structure and purification of recombinant Drp6. (A) Schematic representation of domains of dynamin 1, MxA and Drp6. Dynamins contain five distinct domains indicated as G domain, MD, PH domain, GED and PRD. MxA and Drp6 retain three core domains but lack PH domain and PRD. The numbers represent the position of amino acids in the protein. (B) SDS-PAGE analysis of purified Drp6 (left panel). Drp6 expressed in *E coli* C41 strain was purified using Ni-NTA column and separated on a 10% SDS PAGE gel. M and P denotes molecular weight standards and purified protein respectively. WB denotes western blot analysis of purified Drp6 with anti-histidine antibody (right panel). The molecular weight markers are indicated.

2.4.2 Drp6 exists as a self-assembled structure:

Classical dynamins exist as dimers and tetramers under non-assembly conditions such as high ionic strength solutions. They self-assemble into rings and spiral structures at low ionic strength or at physiological ionic strength on lipid templates [58,149,201,205]. Self-assembly is facilitated by membrane association and promotes enhanced GTPase activity; this is also true for DRPs including Dnm1,Dlp1, Mgm1, Mfn1,Fzo1 and MxA [53,59,77,203,206–208] .

To ask whether Drp6 also self-assembles into larger structures, its oligomerization state was assessed by using analytical size exclusion chromatography and chemical cross-linking. In gel

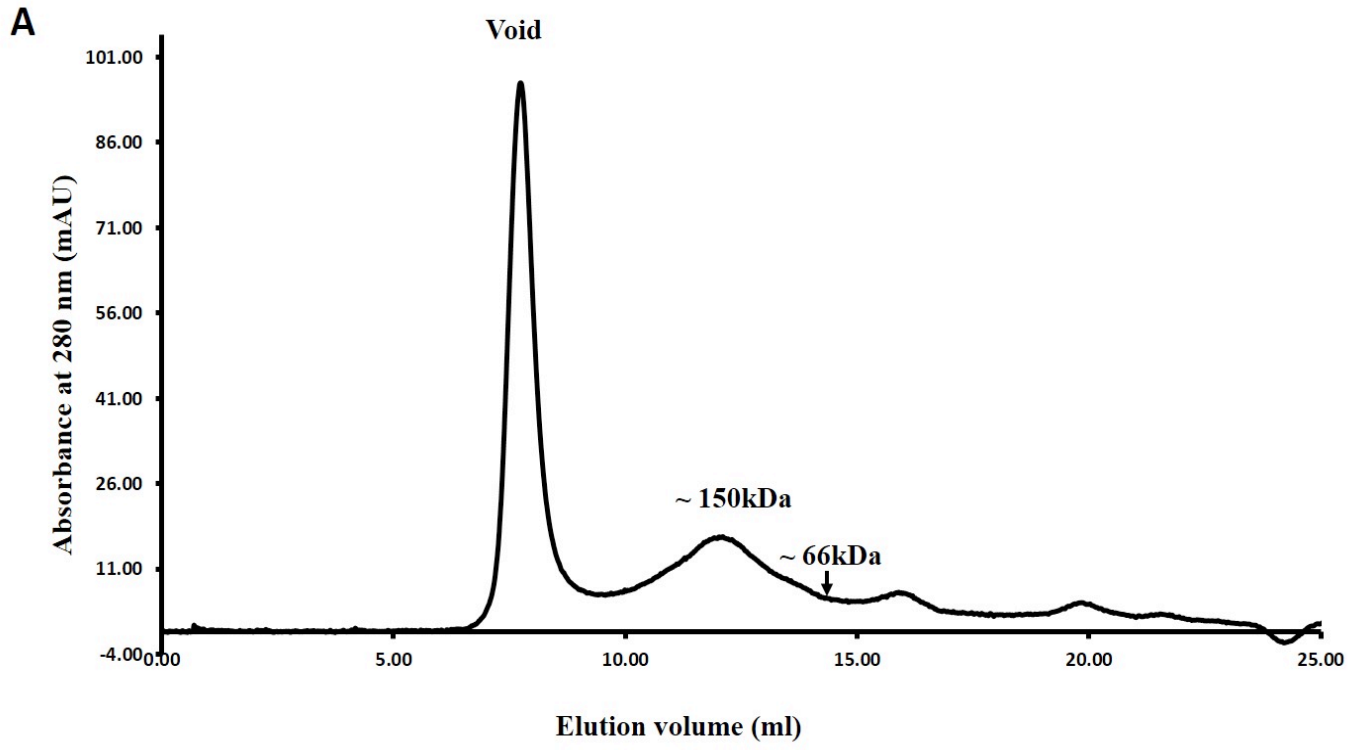
filtration experiments, purified Drp6 eluted as a high molecular weight species in the void volume (7.8 ml) even under non-assembly condition (300 mM NaCl) (Fig. 2.2A). This suggests that Drp6 exists as an oligomeric complex whose molecular mass exceeds 660 kDa, the exclusion limit of the gel filtration column. The exact size of Drp6 oligomers could not be determined by gel filtration analysis. However, since monomeric Drp6 has a molecular mass of ~ 87 kDa, we infer that the oligomeric complex contains at least 6 monomeric subunits. To exclude the possibility that self-assembly is influenced by bacterial environment, we have expressed human dynamin 1 in bacteria, purified under same conditions, and performed gel filtration experiments. As reported previously, human dynamin did not form self-assembled structure under same buffer conditions (Fig.2.2B). Moreover, similar to Drp6, another dynamin-related protein MxA, when expressed and purified in bacteria, formed self-assembled structure even at high salt concentration [141]. Therefore, it appears that some of the dynamin family proteins have self-assembly properties different from classical dynamin.

It is possible that recombinant protein purified from *E. coli* is contaminated with DNA or RNA and the peak observed in the void volume is due to protein bound with nucleic acid. To exclude this possibility, the presence of nucleic acid contamination (if any) in the purified protein was examined by measuring the ratio of A_{260}/A_{280}. A ratio of 0.8±0.05 eliminates the possibility of nucleic acid contamination in the purified protein and suggests that the protein in the void peak is due to large oligomeric species of Drp6. Since the high ionic strength buffer used in these experiments mimics the membrane-free state, these results imply that Drp6 does not require membrane association for self-assembly, and therefore differs from other known dynamin-family members.

Importantly, the gel filtration experiments were performed in the absence of added nucleotide. For classical dynamin and characterized DRPs, self-assembly at physiological salt conditions is

stimulated by addition of GDP or GTP analogs [205]. Our results suggest that Drp6 self-assembly, in contrast, may be less dependent on nucleotide binding.

To better understand the oligomeric state of Drp6, chemical cross-linking experiments were performed using BS^3 as a cross-linker. Different concentrations of BS^3 were used to crosslink Drp6 for different time periods and the cross-linked products were separated on 6% SDS-PAGE gels. As shown in Fig. 2.2C, in addition to a band at its monomeric position (~ 87kDa), other high molecular weight bands are also visible around 250 kDa as well as at the top of the separating gel. We also observed very high molecular weight crosslinked products in the stacking gel, appearing as a smear (Fig. 2.2C). With increasing time and cross-linker concentrations, the lower molecular weight bands disappeared with concomitant increase in the



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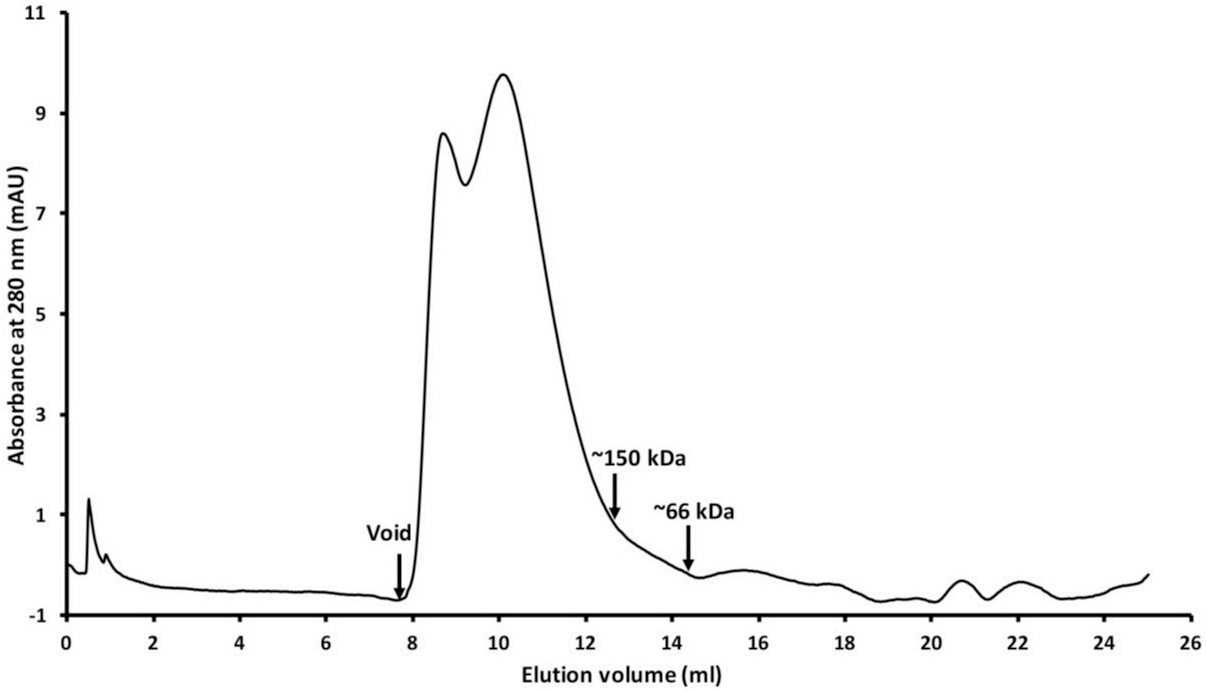
Absorbance at 280 nm (mAU)
11
9
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Elution volume (ml)
Void
~150 kDa
~66 kDa

C

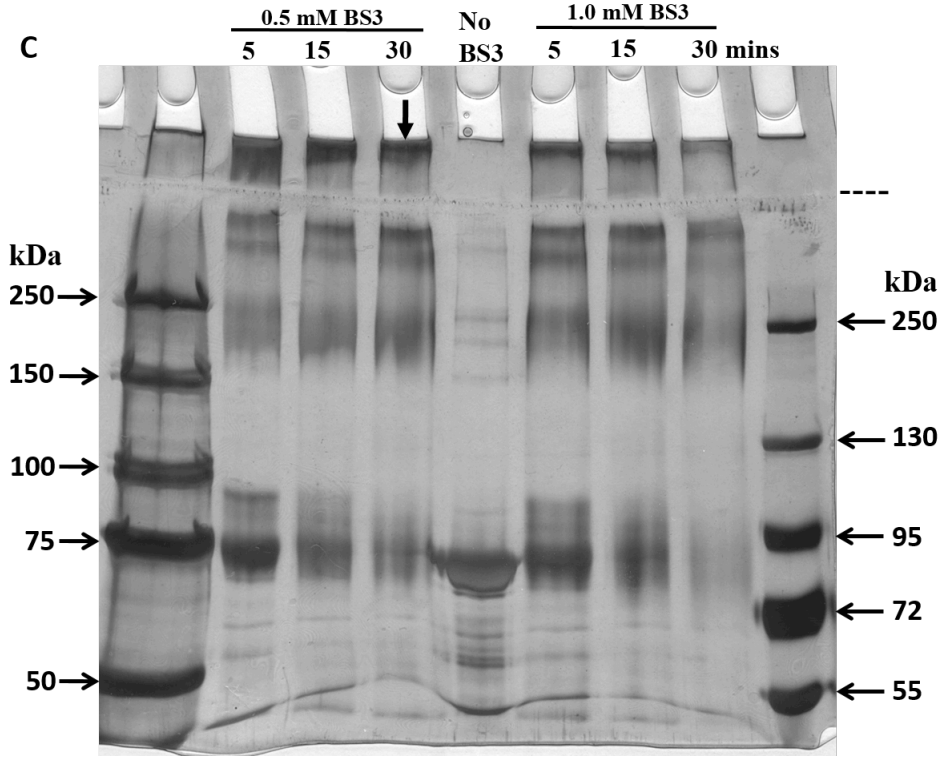
0.5 mM BS3
No BS3
1.0 mM BS3
5
15
30
5
15
30 mins
kDa
250
150
100
75
50
kDa
250
130
95
72
55

Figure 2.2: Drp6 self assembles into a high molecular weight structure. (A) Size exclusion chromatography profile of Drp6. Purified Drp6 (0.5mg/ml) was fractionated on a Superdex GL 10/300 column in buffer A containing 300mM NaCl and excluding glycerol. The void peak and positions of the standard molecular weight markers are indicated in the chromatogram. A broad peak around 150 kDa also contains Drp6 and may represent monomer or lower oligomeric state. (B) Size exclusion chromatography profile of human dynamin 1. Purified human dynamin 1 (0.5mg/ml) was fractionated on a Superdex GL 10/300 column in buffer A containing 300mM NaCl and excluding glycerol. Human dynamin 1 eluted as small oligomeric forms. Absence of any peak in the void volume suggests that human dynamin 1 does not form self-assembled structure under high ionic strength condition. Chemical crosslinking of Drp6 with BS^3. Silver stained 6% SDS PAGE gel showing purified Drp6 crosslinked with either 0.5mM (left lanes) or 1.0 mM BS^3 (right lanes) for 5, 15 and 30 min. The dotted line at right denotes the interface between stacking and separating gel.

appearance of very high molecular weight species at the top of the stacking gel (shown as vertical arrow in Fig. 2.2C). This result corroborates the presence of high molecular weight structures observed by size-exclusion chromatography. Taken together, the above results suggest that Drp6 self-assembles into very high molecular weight structures in high ionic strength buffer. This is independent of membrane association, and does not depend on addition of nucleotides.

2.4.3 Ultrastructure of Drp6:

A hallmark of dynamin superfamily proteins is their property to self-assemble to form rings and helical stacks of rings [42,57,149,197,203,205,209]. To find out whether the high molecular weight species of Drp6 are ordered assemblies, rather than amorphous aggregates, Drp6 ultrastructure was analyzed by electron microscopy. Importantly, in more than 200 electron micrographs taken from protein purified in three independent batches and then negatively stained, no disordered aggregates were observed, instead rings and helical spirals were noted in all the micrographs examined. The rings and helical spirals had varying sizes and diameters (Fig. 2.3). The size of these ring-like structures varied from 45 to 75 nm in diameter (Fig. 2.3), and

their appearance resembled that of structures formed by dynamin family members such as dynamin, MxA and Dlp1 [77,149,206]. The helical spirals varied in length and were generally of 60 nm diameter (Fig. 2.3). The formation of such ordered structures at high ionic strength was consistent with the results from size exclusion chromatography and chemical cross-linking (Fig. 2.2).

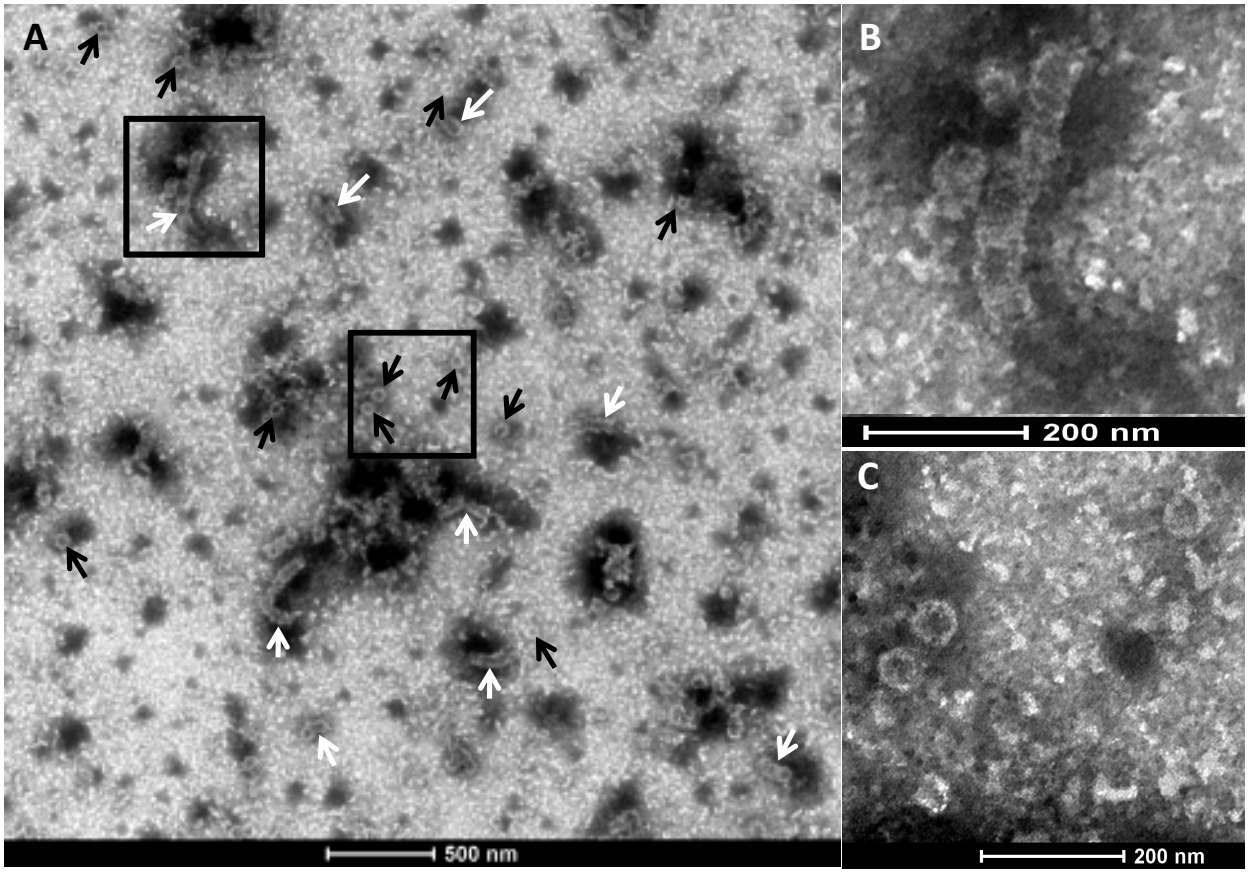

Figure 2.3: Drp6 forms rings and helical spirals. (A) Electron micrograph of negatively stained Drp6. Drp6 (1μM) at physiological salt concentration in presence of 0.5mM GTPγS was visualized under electron microscope after negatively stained with 2% uranyl acetate. Rings (black arrows) and helical spirals (white arrows) are indicated. The enlarged rings and spirals (boxes in A) imaged at higher magnification are shown in B and C. The scale bar is indicated at the bottom.

2.4.4 GTPase activity of Drp6:

All dynamin-family proteins that have been studied display relatively high basal rates of GTP hydrolysis, and that activity is enhanced by membrane association or by lowering the ionic strength (salt concentration <50 mM) [53,59,203,210–212]. The GTP hydrolysis activity of purified Drp6 was assessed

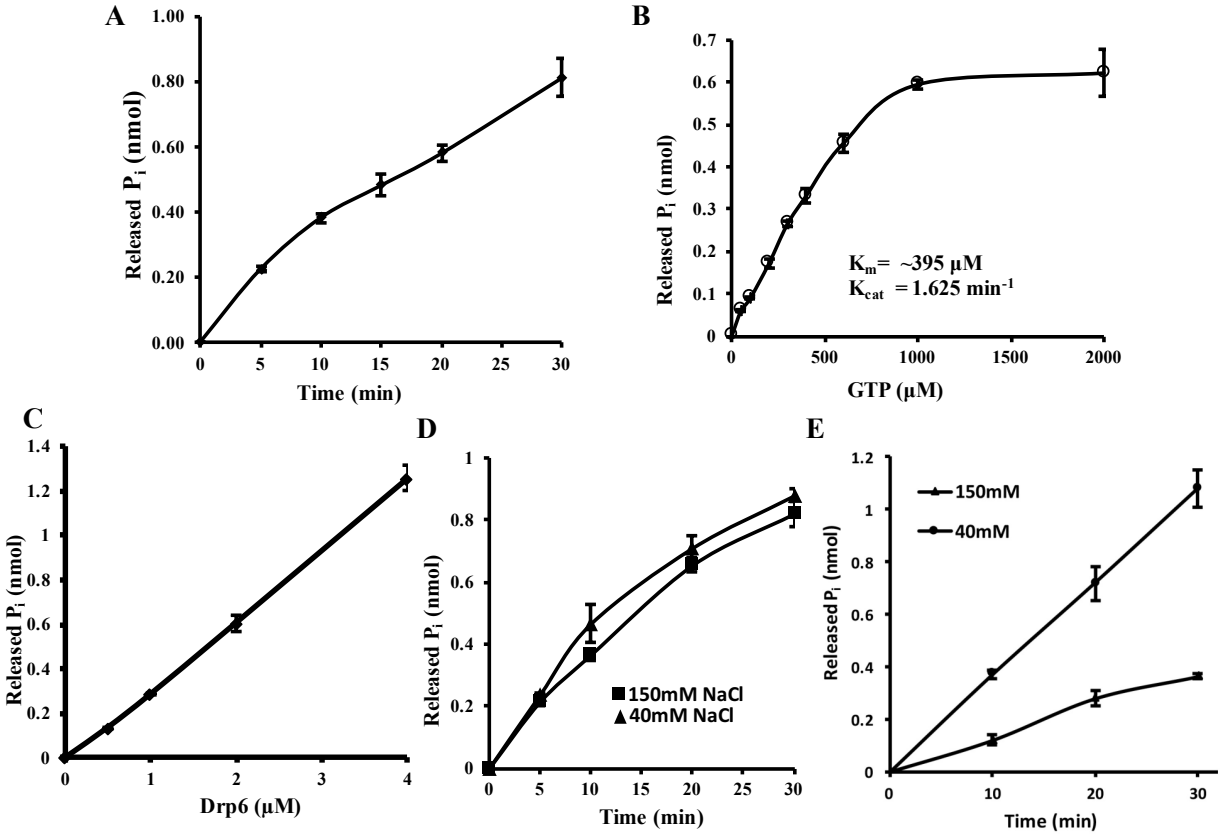

Figure 2.4: Recombinant Drp6 is catalytically active. (A) GTP hydrolysis activity of 1µM Drp6 with 1mM GTP in buffer B. (B) GTP hydrolysis activity using 2 µM Drp6 in buffer B for 10 min. (C) Effect of salt concentration on GTPase activity was analyzed by performing assays as in A, either at 150mM KCl (triangle) or at 40mM KCl (square). (D) Effect of salt concentration on GTPase activity of human dynamin 1 was performed as in (C) except 0.5 µM protein was used. (E) GTP hydrolysis activity using 0.5 µM to 4 µM Drp6 in buffer B for 10 min. The amount of P_i released (nmol) was plotted as a function of time (A and C) or GTP concentration (B) or Drp6 concentration (E). The mean of Pi released (nmol) from three independent assays (each with triplicate) is plotted as a function of time (A, C and D) or µM GTP (B) or µM protein (E). Error bars represent standard deviation. The plot was generated using Origin Pro 8.0. Statistical significance was calculated with Kruskal–Wallis test ($p<0.01$) and no significant difference between experiments was observed.

using a colorimetric assay. Drp6 showed high basal GTPase activity with a K_{cat} of ~ 1.62 min^{1}, V_{max} of 0.325 μM Pi/min/ μM protein and an apparent K_m of ~ 395μM (Fig. 2.4A, 2.4B). Similar GTPase activities were measured for total purified protein and for a fraction corresponding to the column chromatography void peak, indicating that the high molecular weight material does not represent inactive aggregated protein. Moreover, the measured GTPase activity was relatively insensitive to protein concentration (Fig. 2.4C), suggesting that the protein does not tend to form non-specific inactive aggregates.

To ask whether GTPase activity would be enhanced under canonical dynamin assembly conditions, the GTPase activity was measured in low salt. Surprisingly, unlike results for other DRPs no significant change in activity was observed upon lowering the ionic strength of the assay buffer (Fig. 2.4D). The Hdyn1 expressed and purified similarly was assayed in parallel under same assay conditions. As expected, the GTPase activity of Hdyn1 was enhanced more than threefold upon reducing the salt concentration (< 50mM NaCl) (Fig. 2.4E) confirming that lack of stimulated GTPase activity of Drp6 at low salt concentration is not due to the experimental conditions.

2.5 Discussion:

Dynamin-related protein 6 in *Tetrahymena* plays a role in nuclear remodeling [146]. As expected for a member of the dynamin family, Drp6 appears to undergo cycles of assembly and disassembly on the nuclear envelope, which are regulated by physiological and developmental cues [146].

In this study, the biophysical and biochemical properties of Drp6 were analyzed. Specifically, the oligomeric state, rate of GTP hydrolysis, and ultrastructure were assessed and the effect of salt concentration on these properties was evaluated. The bacterially expressed recombinant Drp6

used in this study hydrolyzes GTP, arguing that the protein is folded in its native structure. Results from size exclusion chromatography and chemical crosslinking clearly demonstrate self-assembly of Drp6 in absence of any membrane template. Drp6 assembled into rings and spirals as visualized by electron micrographs. While similar structures of dynamins and many dynamin related proteins represent the functional forms, they require lipid binding or low salt condition to form rings and helical spirals [77,149,197,213]. Interestingly, unlike dynamins and other DRPs, MxA exhibits self-assembly property similar to Drp6 where it assembles into rings and helical spirals in the absence of lipid templates [77]. MxA confers antiviral activity by associating with viral nucleoprotein, a function different from canonical membrane remodeling.

Dynamin family members show cooperative stimulation of GTPase activity upon assembly on lipid templates [58,197,200,214][200]. In contrast, we did not observe significant enhancement in GTPase activity of Drp6 under low salt concentration, a condition mimicking membrane bound state (Fig. 4B). Similar observation is also reported for MxA where assembly onto liposomes did not induce GTP hydrolysis [77]. The plausible explanation for this functional difference between MxA and dynamin is based on arrangement of G-domain in assembled structures [77,203,209]. In case of assembled dynamin, the stimulated GTP hydrolysis is a result of direct contact between the G domains of dynamin protomers in the neighboring turn [42,209]. Although dynamin and MxA form similar assembled structures, analysis of Cryo-TEM images revealed that oligomers of MxA are not positioned close enough in the assembled state and hence does not allow inter G domain interactions required for stimulated GTP hydrolysis [77,203]. It is possible that Drp6 follows a similar arrangement of G domain on target membrane as that of MxA and might explain the lack of enhanced GTPase activity in low ionic strength conditions. However, further studies are required to understand the domain arrangement of Drp6

in self assembled structure and basis of the difference in GTP hydrolysis property as compared to other dynamin family proteins.

Drp6 expressed in bacteria may not be fully active and histidine tag may influence its activity. However, classical endocytic dynamin, when expressed in bacteria as a histidine tag protein, shows activity comparable to that of the native protein. Therefore, we presume that Drp6 expressed similarly represent functional form and conclusion drawn is also relevant for the native protein. Though purified Drp6 catalyzed GTP hydrolysis, it could be argued that observed GTPase activity is due to small fraction of functional Drp6 and higher order structure is mainly amorphous aggregates. To exclude this possibility, we have isolated the void peak from gel filtration column and found it to be active. Moreover, increasing protein concentration did not stimulate GTPase activity supporting that the GTPase activity obtained is from high molecular structures and hence they represent functional form of Drp6. Analysis of more than two hundred electron micrographs obtained from three independent batches of purified proteins did not show presence of any amorphous aggregates, rather all the micrographs contained self-assembled structures in the form of rings and helical spirals (Fig. 2.3). All these results discussed above confirm that bacterially expressed purified Drp6 used in this study is functional and Drp6 exists as a self-assembled structure as rings and helical spirals without requiring membrane binding.

CHAPTER THREE

Identification of Membrane Binding Domain of Drp6

3.1 Introduction:

Biological membranes play a vital role for cells in separating the cytoplasm from the outside environment and providing sub-cellular compartmentalization. A hallmark of eukaryotic cells is the existence of an endomembrane system required for sophisticated compartmentalization of organelles such as the mitochondria, endoplasmatic reticulum, vacuoles, or plastids. However, there is a constant exchange of materials including lipids between different compartments requiring remodeling of the target membranes. Endocytosis, which is carried out by almost all eukaryotic cells, is the constitutive process by which the plasma membrane invaginates, captures extracellular components and ultimately buds off to form intracellular, membrane-bound vesicles. This process requires rapid membrane curvature dynamics. Dynamin family proteins are involved in membrane remodeling events in several biological processes such as endocytosis, fission and fusion of cell organelles. Lipid association is a hallmark of all dynamin proteins as membrane targeting and deformation is fundamental to their function. In classical dynamins, PH domain mediates membrane binding. PH domains are found in numerous membrane-associated proteins and have been implicated in mediating protein-protein and protein-phospholipid interactions. PH domain of dynamin binds to phosphoinositides (PIP2) and participates in the interaction between dynamin and the βγ subunits of heterotrimeric G proteins (Lemmon, 2007; Musacchio, Gibson, Rice, Thompson, & Saraste, 1993; Saraste & Hyvnen, 1995). Association of dynamin with lipid membrane is known to influence several aspects of cellular physiology as mutations in the PH domain exert dominant negative effects on endocytic process. Acidic phospholipids such as PIP2 interacts with positively charged surface of dynamin PH domain [215] [70]. Other DRPs, though lack PH domain, harbor either lipid binding loops or trans-membrane domains for membrane recruitment (Fig. 3.1) [3,26,27,35,39,40] [28–30,34,36,37,103] [31–33,112].

Drp6 also lacks a PH domain but it associates with nuclear envelope and ER derived vesicles. If recruitment of Drp6 to these membranes requires a membrane binding domain, the identification of membrane binding domain and its role in Drp6 function are investigated here.

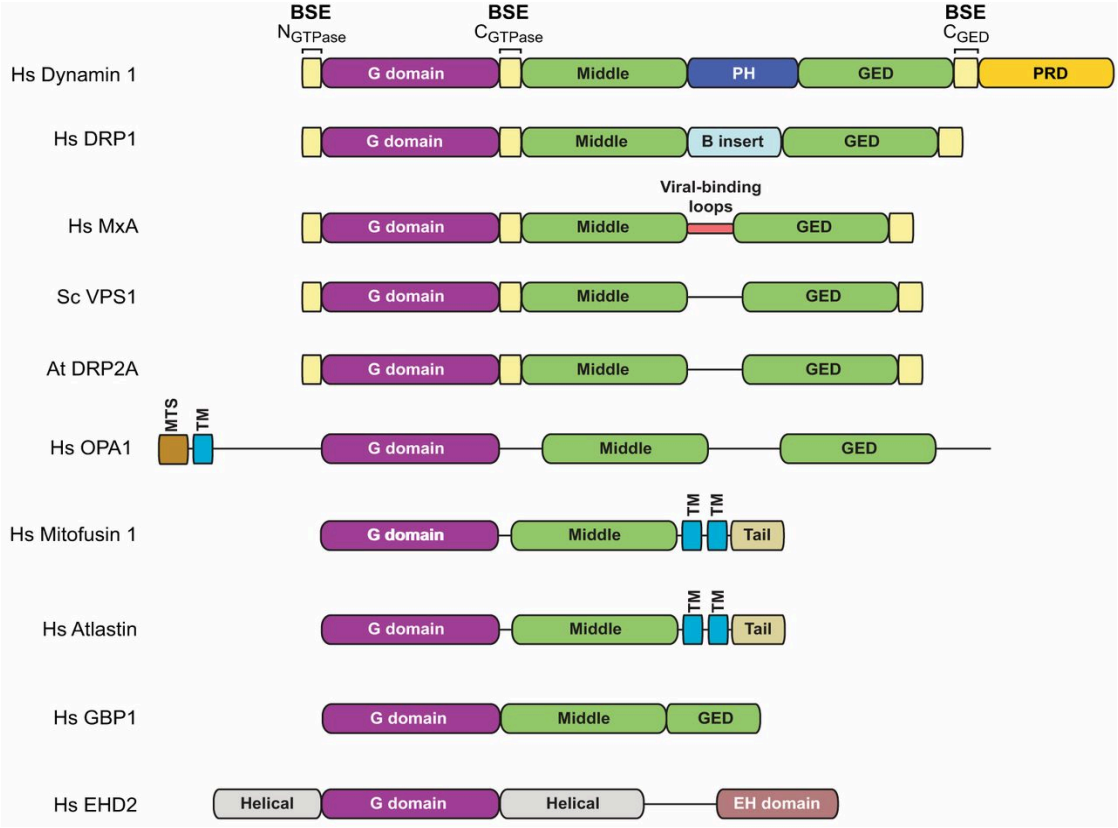

Figure 3.1: Domain organization of dynamin family proteins. Proteins of this family have 3 core domains namely GTPase domain (G domain), Middle domain and GTPase effector domain (GED). Classical dynamins additionally harbor a Pleckstrin homology (PH) domain and a Proline rich domain (PRD). Some members possess either trans-membrane (TM) domain or a membrane binding loop (B-insert/viral binding loop). MTS is mitochondrial target sequence and BSE is bundle signaling element. Taken from *Chappie et al.* 2013.

3.2 Materials:

Plasmids:

Plasmid vectors pRSET A, pGEX4T.1 and pMALP2 were obtained from Prof.M.S.Shaila, MCB, IISc, Bangalore. *Tetrahymena* expression vectors pVGF was from Meng-Chao Yao, FHCRC, Seattle, Washington.

***Tetrahymena thermophila* strains and culture media:**

Wild type *Tetrahymena thermophila* strains CU428.1 and B2086 were procured from *Tetrahymena* Stock Centre, Cornell University, USA. Proteose peptone, Yeast extracts were purchased from BD Biosciences, USA and Dextrose, Fe-EDTA was from Sigma, USA.

Other reagents:

Illustra NAP-5 columns were purchased from GE Healthcare. Lipids and lipid strips were from Avanti Polar Lipids, Inc. Alabama. DAPI was from invitrogen.

Other chemicals were from Sigma-Aldrich unless mentioned otherwise.

3.3 Methods:

3.3.1 Cloning of different fragments of Drp6:

The full length *DRP6* was cloned and expressed as mentioned earlier in chapter 2. For identification of lipid binding domain of Drp6, different fragments of *DRP6* were amplified by PCR and cloned into pRSETA expression vector. Table 3.1 lists the fragments and the oligonucleotides and table 3.2 mentions the PCR reactions and conditions used for amplification of these fragments. The amplified PCR products were cloned into pRSETA vector using XhoI and EcoRI sites. All the inserts were verified by DNA sequencing. These fragments were further sub-cloned as N-terminus MBP fusion constructs in pMALP2 vector. Due to lack of appropriate restriction sites, these deletion fragments were cloned into pGEX4T.1 vector using BamHI and EcoRI and subsequently into pMALP2 vector using BamHI and SalI. A schematic of the cloning

steps is given in Fig. 3.2. The positive constructs were screened by digestion with BamHI and SalI and constructs with appropriate release were selected for transformation and expression of the protein.

For cloning different fragments of Drp6 as N-terminus GFP fusion proteins, the inserts cloned in pRSETA vector were digested with XhoI and ApaI restriction enzymes. The released inserts were cloned downstream of MTT1 promoter in pVGF vector (an rDNA-based expression vector, from Meng-Chao Yao, FHCRC, Seattle, Washington).

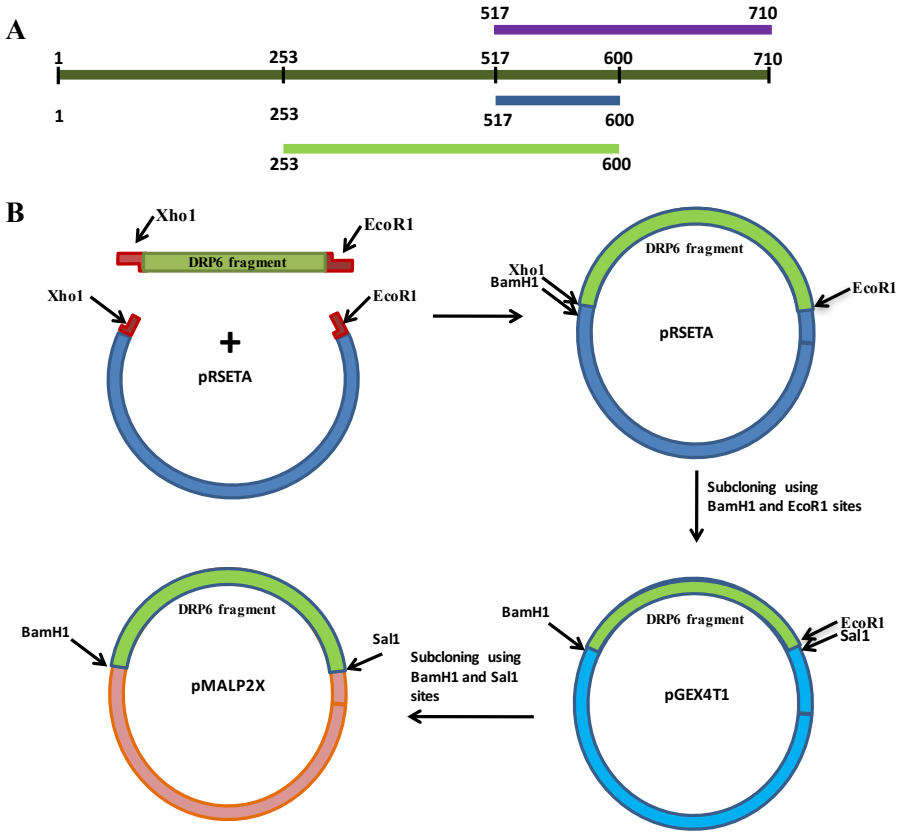

Figure 3.2: Cloning of different fragments of Drp6. (A) Diagram shows the fragments with their corresponding amino acid positions. (B) Scheme showing cloning of these fragments in different bacterial expression vectors with the restriction sites used for cloning.

Table 3.1

DRP6 Fragments	Primer	Oligonucleotide sequence
1-253	Forward primer	5' GCCTCGAGATGACCAACACCATTGTG 3'
	Reverse primer	5' GCGGGCCCGAATTCTCAGTTAATATCTTTCTGGCTGCG 3'
1-600	Forward primer	5' GCCTCGAGATGACCAACACCATTGTG 3'
	Reverse primer	5'GCGGGCCCGAATTCTCAGTTATCCATATATTTATAATAAATAATGCTATA 3'
253-600	Forward primer	5'GCCTCGAGAACGAAAACGTGAGCGT 3'
	Reverse primer	5'GCGGGCCCGAATTCTCAGTTATCCATATATTTATAATAAATAATGCTATA 3'
517-710	Forward primer	5'GCCTCGAGAAACTGAAACAGAACCATGTG 3'
	Reverse primer	5'GCGGGCCCGAATTCTCAATCATCAATGCCATCG 3'
517-600	Forward primer	5'GCCTCGAGAAACTGAAACAGAACCATGTG 3'
	Reverse primer	5'GCGGGCCCGAATTCTCAGTTATCCATATATTTATAATAAATAATGCTATA 3'

Table 3.2

PCR reaction		Temperature	Time
Template DNA (10ng/µl) (pUC57 DRP6)	1µl	98ºC	30 s
5X Phusion buffer	10µl	98ºC	10 s
dNTP mix (10mM)	1µl	56ºC	30 s
Forward primer	1µl	72ºC	60 s
Reverse primer	1µl	72ºC	300 s
MgCl2	1µl		
Phusion polymerase	0.5µl		
Water	34.5µl		

3.3.2 Generation of internal deletion construct of *DRP6* (*DRP6 Δ 517-600*):

The internal deletion of nucleotide sequences corresponding to amino acid residues 517 to 600 was achieved by performing overlap PCR as shown in the figure 3.3. For this purpose, the nucleotide sequences corresponding amino acids 1-516 and 601-710 were PCR amplified using

primers 1, 2 and primers 3, 4 respectively as shown in the table 3.3. The primer 2 (reverse primer) and primer 3 (forward primer) harbored 20 nucleotides overlapping sequences to enable annealing both the fragments in second round of PCR amplification to generate the final deleted construct using both the fragments (1-516 and 601-710) as template and 1,2,3 and 4 as primers. The final insert lacking nucleotide sequences (corresponding amino acids 517-600) was cloned into pRSET B vector using XhoI and EcoRI sites, and into pVGF vector using XhoI and ApaI sites.

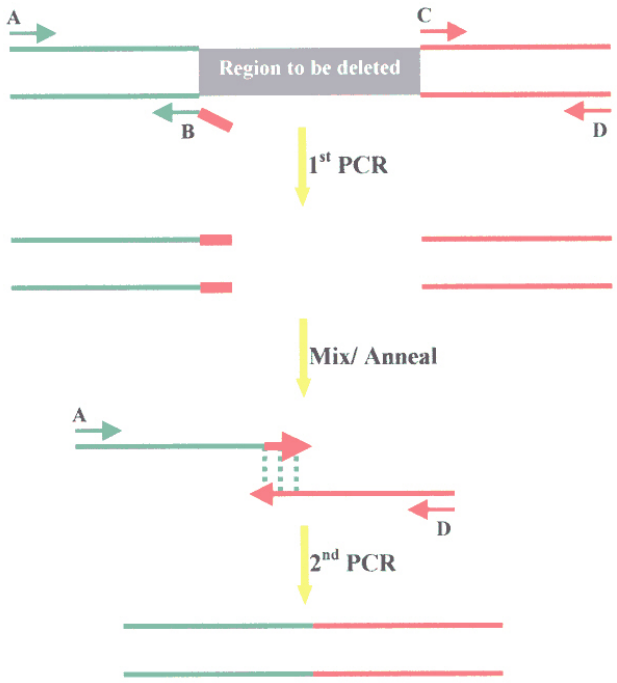

Figure 3.3: Schematic of overlap PCR for internal deletion. The internal deletion construct of Drp6 (DRP6 Δ 517-600) was generated by a two step PCR amplification using a set of four primers. In the first step, the upstream (green) and downstream (red) fragments of the deleted region were generated separately using primers A,B,C and D as indicated. The primer B has 20 nucleotide overhang from the 5' end of the downstream fragment (shown in red). The final deleted fragment was generated by a second step PCR using both amplified fragments and primers A and D.

Table 3.3

Primer 1	5' GCCTCGAGATGACCAACACCATTGTG 3'
primer 2	5' CTG TTT GCG AAA GCT TTC GGT TTC ATG AAT ATC 3'
primer 3	5' GAA ACC GAA AGC TTT CGC AAA CAG ATT AGC G 3'
primer 4	5'GCGGGCCCGAATTCTCAATCATCAATGCCATCG 3'

3.3.3 Expression and purification of deletion fragments of Drp6:

The constructs were transformed into *E.coli* DH5α cells. Single colonies were then inoculated in 3 ml of LB broth supplemented with 100µg/ml ampicillin and were grown over night. For large scale expression, 500µl of overnight grown culture was added to 1 L of same media and was grown at 37°C till the OD_{600} reached 0.4. The expression was induced by adding 0.5mM IPTG (final concentration) for 5 hours. The cells were harvested by centrifugation at 10,000 RPM for 5 min at 4°C and sonicated in buffer containing 25mM HEPES pH 7.5, 250mM NaCl and 10% glycerol supplemented with protease inhibitor cocktail and 1mM PMSF. The lysate was clarified by centrifugation at 14,000 RPM for 30 min at 4°C and was incubated with amylose resin at 4°C for 2 hours. The resin was washed with 100 bed volume lysis buffer and the proteins were eluted with 20mM maltose in the same buffer. The purified proteins were run on SDS-PAGE and were checked by coomassie staining. The buffer of purified proteins was exchanged with 25mM HEPES pH 7.5. 250mM NaCl and 2mM $MgCl_2$ using Illustra Nap-5 (GE Healthcare) columns according to manufacturer's protocol.

3.3.4 *Tetrahymena* strains and culture conditions:

Wildtype CU428 and B2086 strains of *T. thermophila* (from Peter Bruns, Cornell University) were grown at 30°C and 90 RPM in SPP medium (2% proteose peptone (BD, USA), 0.2% glucose, 0.1% yeast extract and 0.003% ferric EDTA). The cell number was determined by using

Haemocytometer. For starvation, cells were grown to a cell number of 3-4 x 10^5/ml and were washed, starved in DMC media (0.17 mM sodium citrate, 0.1 mM NaH_2PO4, 0.1 mM Na_2HPO4, 0.65 mM $CaCl_2$, 0.1 mM $MgCl_2$) for 16-20 hours at 30°C. Conjugation was initiated by mixing equal number of starved cells of two different mating types.

3.3.5 Extraction of total lipid from *Tetrahymena*:

For lipid extraction, 100 ml CU428 cells were grown in SPP media till the cell density reached ~5.0 x 10^5/ml and the culture was rapidly chilled by dipping in a canister filled with liquid nitrogen followed by incubation on ice for 5 min. Cells were harvested by centrifugation at 10,000 RPM for 15 min and the cell pellet was resuspended in 9 ml of sterile water. The lipid was extracted following the protocol of Bligh and Dyer [216]. A mixture of 10 ml chloroform and 20 ml methanol was added to the cell suspension in a separating funnel capped with the ground glass stopper, shaken vigorously and allowed to stand for 10 min until phase separation. The trapped gasses were released by loosening the glass stopper for few seconds. The lower organic phase containing dissolved lipids was carefully collected, avoiding any contamination from interface. The addition of chloroform and water to the collected lipid suspension was repeated for three times in a separating funnel and the mixture was allowed to stand for 2 hours. The lower organic phase was collected carefully in a round bottom flask and was kept for drying in a rotary evaporator at 40 °C and 100 RPM. The dried lipid film was resuspended with a mixture of chloroform:methanol (6:1 volume/volume) and transferred into a smaller flask. The mixture was again dried in rotary evaporator under a stream of nitrogen gas. Finally the lipid was resuspended in chloroform and was stored at -20°C.

3.3.6 Lipid overlay assay:

For overlay assay ~ 3 to 4μg of lipids were spotted on nitrocellulose membrane and was allowed to dry completely before blocking with 3% fat free BSA (MP Biomedical) in TBST for 1 hour.

The membrane was washed 3 times with GTPase assay buffer prior to incubation with 1μM of Drp6 proteins in the same buffer for 1 hour at room temperature. The membrane was then washed 3 x 10 min with TBST and incubated with HRP cojugated anti-His antibody (Sigma) at 1:5000 dilution for 1 hour followed by washing with TBST 3 x 10 min. The protein was detected by chemiluminiscence. For overlay assay to identify lipids with which Drp6 interacts, same protocol was followed except a commercial strip spotted with 15 different lipids was used in place of total lipids from *Tetrahymena*. For detection of proteins with MBP tag, anti-MBP antibody (NEB) at 1:10,000 dilution as primary antibody and HRP conjugated goat anti-mouse secondary antibody (Imgenex) at 1:10,100 were used.

3.3.7 Liposome Preparation

Lipid mixtures (2.5 mg) containing total *Tetrahymena* lipids with additional 10% CL or PS were resuspended in 2 ml chloroform and were dried in a rotary evaporator at 40°C and 110 RPM in a 50 ml round bottom flask to obtain a lipid film. The film was further kept under vacuum for at least 2 hours before resuspending in GTPase assay buffer and subjected to repeated cycles of freezing in liquid nitrogen and thawing at 37°C in a water bath. The unilamellar vesicles were prepared by extruding the lipid suspension through a 100 nm polycarbonate membrane (Avanti Polar Lipids) 21 times. The size and density of the liposomes were checked by dynamic light scattering in a Malvern Zetasizer.

3.3.8 Floatation assay:

Floatation assay of Drp6 was performed with liposomes from *Tetrahymena* total lipids supplemented with 10% CL and 10% PS. The liposomes were prepared from a mixture of 2.0mg of *Tetrahymena* lipid extract, 0.25 mg of CL and 0.25mg of PS. Liposomes were prepared as mentioned in the previous section. For the assay, 2μM Drp6 was incubated with these liposomes for 1 hour at room temperature and it was brought to 40% sucrose before placing into the

centrifuge tube. It was layered with 2ml each of 35%, 30%, 25%, 20%, 15% and 1 ml of 10% sucrose on top and centrifuged at 35,000 RPM for 18 hours at 4ºC in Beckman SW41 rotor. 1ml fractions were collected from top and checked by western blot with anti-His antibody as mentioned earlier.

3.3.9 Prediction of 3-dimensional structure of Drp6 by I-TASSER:

The secondary and three dimensional structure of Drp6 was predicted using the iterative threading assembly refinement (I-TASSER) server [217]. For three dimensional modeling of Drp6, the Drp6 amino acid sequence was uploaded in the I-TASSER database. Estimation of accuracy of the predicted model was validated by analyzing the C-scores and RMSD values of the models generated.

3.3.10 Transformation of *Tetrahymena* cells:

3.3.10.1 Electroporation method

Transformation of *Tetrahymena thermophila* cells was carried out using Gene Pulser (Biorad) electroporation unit. Approximately 25 to 30 µg of DNA was resuspended in 125 µl of 10 mM HEPES pH 7.5. Conjugating culture of *Tetrahymena* cells (3 x 10^5 cells/ml) were washed with 10 mM HEPES buffer and concentrated to a final volume of 500 µl in the residual buffer. 125 µl of cells were mixed with 125 µl of DNA in an electroporation cuvette (Thermoscientific). Electropulsing using 250 V for 5 miliseconds was done at ten hours post-mixing of the culture. Transformed cells were diluted in SPP medium after one minute of pulsing and aliquoted in 96 well plates. The selection antibiotic paromomycin sulphate was added to a concentration of 100 µg/ml after 16 hours of transformation and positive transformants were selected.

3.3.10.2 Biolistic method

10 µg plasmid DNA resuspended in 5 µl water was coated onto 50µl of gold microcarriers in a microfuge tube placed on a vortex mixer. To this mixture, 125 µl of 2.5 M CaCl2 and 65 µL of

0.1 M spermidine were added and kept for mixing for 20 min at 4°C. The gold particles were pelleted down by centrifugation at 1200*g* for ~ 5 seconds, the supernatant was removed and the gold particles were washed successively with 70 % and 100 % ethanol. The gold particles were resuspended in 40µl of 100 % ethanol and spreaded uniformly onto the surface of a macrocarrier film. The dried macrocarrier was placed inside a desiccator to prevent moistening.

50 ml of *Tetrahymena* cells (starved or conjugted) was centrifuged at 1100 *g* for 2 min and concentrated in 500 µl of residual buffer. These cells were transferred to the centre of a sterile Whatman filter paper (cut into a circle of 7 cm diameter) kept on a petri-dish. The filter paper was soaked in DMC medium prior to adding cells. Biolistic transformation cells was carried out using PDS1000/He Biolistic particle delivery system (BioRad) according to manufacturer's instructions. The transformed cells along with the filter paper were transferred into 50 ml SPP media and were incubated at 25°C overnight. The selection antibiotics (either 100µg/ml paromomycin sulphate or 60µg/ml blasticidin) were added to the cells, dispensed into 96 well plates and incubated at 30°C. Transcription of transgenes from the *MTT1* promoter was induced by adding 1µg/ml cadmium chloride (Sigma) for 3-4h.

3.3.11 Microscopy:

Tetrahymena cells were fixed with 4% paraformaldehyde (PFA) in 50mM HEPES pH 7.5. The cells were spun down at 1100 RCF and the supernatant was discarded. The cell pellet was dislodged by tapping and PFA was added to the cells by dispensing quickly and incubated either at room temperature for 30 min or on ice for 45 min. Cells were recovered by centrifugation and washed with 10mM HEPES pH 7.5. The fixed cells were stained with DAPI (Invitrogen) and washed with 10 mM HEPES pH 7.5 before imaging.

The imaging was performed using a Zeiss LSM780 confocal microscope. For visualizing GFP tagged proteins, images were taken at excitation/ emission at 488 /500 nm and to visualize DAPI, images were taken at excitation /emission- 405/ 460 nm.

3.4 Results:

3.4.1 Drp6 interacts with *Tetrahymena* lipids:

Drp6 associates with NE and ER derived vesicles [146]. Drp6, like all other DRPs lacks a recognizable membrane binding domain. To investigate if Drp6 also possesses a membrane binding domain for membrane recruitment, a lipid binding assay was performed using total lipid extracted from vegetatively grown *Tetrahymena* cells and His_6-Drp6. For this purpose, total lipid extracted from *Tetrahymena* was spotted onto a nitrocellulose membrane and was overlaid with 0.5μM purified His_6-Drp6 either in presence or absence of GTP. The bound protein was detected by western blot analysis using anti-His antibody (Fig 3.4A). Bacterially expressed recombinant Drp6 was able to interact with total lipid from *Tetrahymena* both in presence and absence of GTP. This result suggests that lipid association properties of Drp6 is independent of any other cellular factors. This also indicate that although Drp6 does not have a recognizable PH domain, it possesses a lipid binding domain.

3.4.2 Drp6 interacts with specific lipids:

Specificity of membrane recruitment of a protein is often determined by its interaction with specific lipids. As Drp6 could interact with *Tetrahymena* lipids, to identify the specific lipids Drp6 interacts with, a lipid overlay assay was performed using commercially available lipid strips (Echelon Biosciences) and bacterially purified His_6-Drp6. The strip contains 15 different

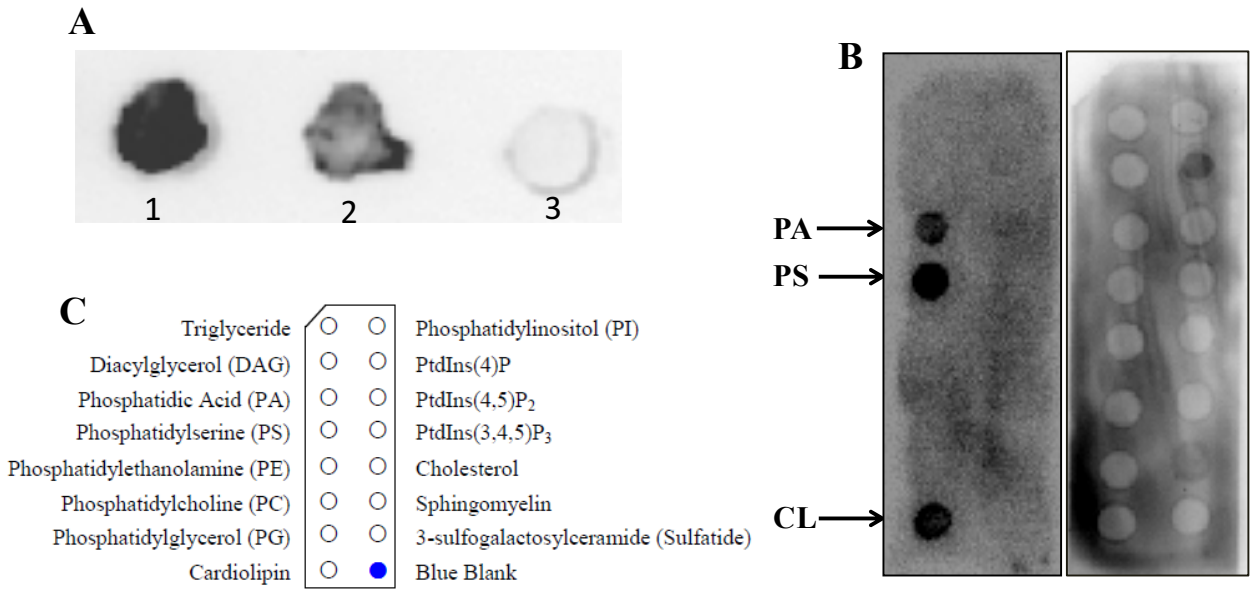

Figure 3.4: Lipid overlay assay of Drp6 (A) Western blot showing interaction of Drp6 with total *Tetrahymena* lipids either in absence (1) or in presence of GTP (2). Drp6 was replaced with BSA in the control (3).(B) Western blot showing interaction of Drp6 (left) or with methionine tRNA synthase as control (right) using commercial lipid strips containing 100 pmol each of the 15 lipids as shown in (C). HRP conjugated anti-His antibody was used to detect the histidine tag of recombinant Drp6 used in this assay.

lipids of 100 pmol each (Fig. 3.4C). The results of the assay showed that Drp6 specifically interacted with phosphatidic acid (PA), phospahtidylserine (PS) and Cardiolipin (CL) (Fig. 3.4B). While PA and CL are fusogenic lipids playing essential roles in generating membrane curvature, PS is known to interact with fusogenic effectors. Interaction of Drp6 with these lipids suggest that Drp6 might have a role in membrane remodeling, in particular in fusion.

3.4.3 Drp6 interacts with membrane *in vitro*:

Specific interaction of Drp6 with anionic phospholipids was detected where the lipids were spotted on nitrocellulose membranes. However, this condition does not represent the membrane

bilayer. To assess if Drp6 also interacts with membrane, an *in vitro* membrane binding assay was performed using floatation assay. In floatation assay, the liposomes are allowed to float over a gradient of sucrose and the bound proteins co-float with liposomes under high speed centrifugation (Fig.3.5A). For this assay, liposomes were prepared from total *Tetrahymena* lipids supplemented with CL and PS to mimic the membrane bilayer. Fractions of sucrose gradients were collected from top and were analyzed by western blotting. Drp6 was detected predominantly in the upper fractions when incubated with liposomes (Fig 3.5B). The protein itself failed to float since there was no detectable band of Drp6 in the corresponding fractions when liposomes were not added. These results indicate that Drp6 possesses a membrane binding domain and membrane interaction is independent of any other factor.

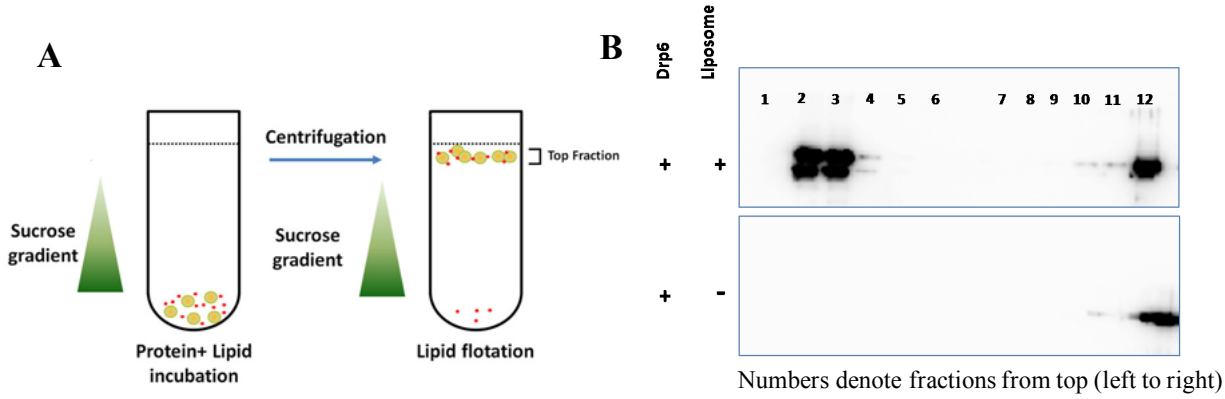

Figure 3.5: Liposome floatation assay. (A) Diagramatic representation of floatation assay in which a protein-liposome mixture is overlaid by a sucrose gradient and centrifuged at high speed to float liposomes. The protein if bound, co-floats with the liposomes. (B) Western blot showing interaction of Drp6 with liposomes in the floatation assay. The upper panel shows Drp6 in presence of liposomes and lower panel shows Drp6 in absence of liposomes. HRP conjugated anti-His antibody was used to detect Drp6. The numbers denote the fractions collected from top to bottom of the sucrose gradient.

3.4.4 Identification of membrane binding domain of Drp6:

Classical dynamins contain a Pleckstrin Homology (PH) domain responsible for membrane binding whereas DRPs lack PH domain or any recognizable lipid binding domain [218,219]. Drp6 being a dynamin related protein also lack a distinct membrane binding domain. However, it interacts with membrane *in vitro* and localizes on the nuclear envelope as well as on the ER derived vesicles *in vivo.* To identify the membrane binding domain of Drp6, a lipid overlay assay was performed with different fragments of Drp6 using lipid strips as mentioned in the earlier section. The fragments were generated based on an earlier study [147]. According to this study the part of Drp6 encompassing amino acids 517 to 600 is important for localization of Drp6. To see whether this fragment harbors the membrane binding domain of Drp6, it was purified as His_6 fusion protein (Fig 3.6) and was used for lipid binding assay. The result of this assay showed that similar to full length Drp6, Drp6 517-600 interacted with all the three lipids namely PA, PS and CL (Fig 3.7).

To rule out that other parts of Drp6 contain membrane binding domain, other fragments were expressed and purified as MBP tagged fusion protein (Fig. 3.6) since repeated attempts to purify them as soluble His_6 fusion proteins were not successful. The results showed that Drp6 1-253 failed to interact with any of the three lipids with which full length protein interacts. As expected, Drp6 517-710 and Drp6 253-600 (containing 517-600 within) interacted with these lipids. Taking together, these results suggest that residues 517-600 is sufficient for lipid binding.

3.4.5 Drp6 membrane binding domain is located at a position equivalent to PH domain of classical dynamin in 3-D model structure:

Classical dynamins associate with membrane via their PH domain. Crystallographic data indicates that the PH domain binds acidic phospholipids specifically phosphatidylinositol-4,5-bisphosphate through a positively charged surface at the foot of the dynamin hairpin [69,220].

The overall 3-dimensional structure of dynamin family proteins are similar. To see whether Drp6 can also form similar structure, a 3-D model was generated using.

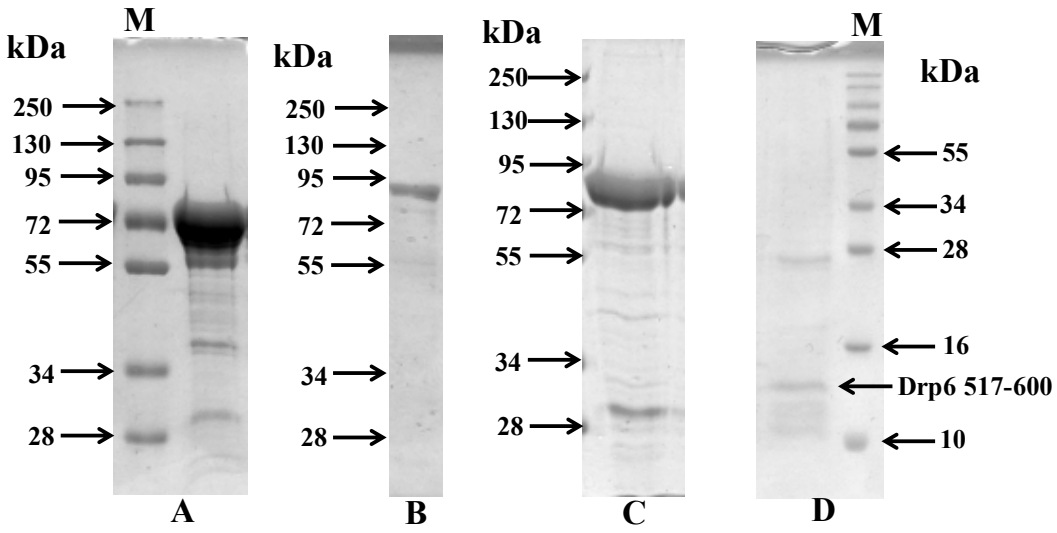

Figure 3.6: Purification of different fragments of Drp6. SDS PAGE showing the fragments of Drp6 corresponding to amino acids 1-253 (A), 253-600 (B), 517-710 (C) purified as MBP fusion tags in 10% gels and 517-600 (D) purified as His_6 tag in 15% gel. Molecular weight markers are either in left (A, B, C) or in right (D) of the gels.

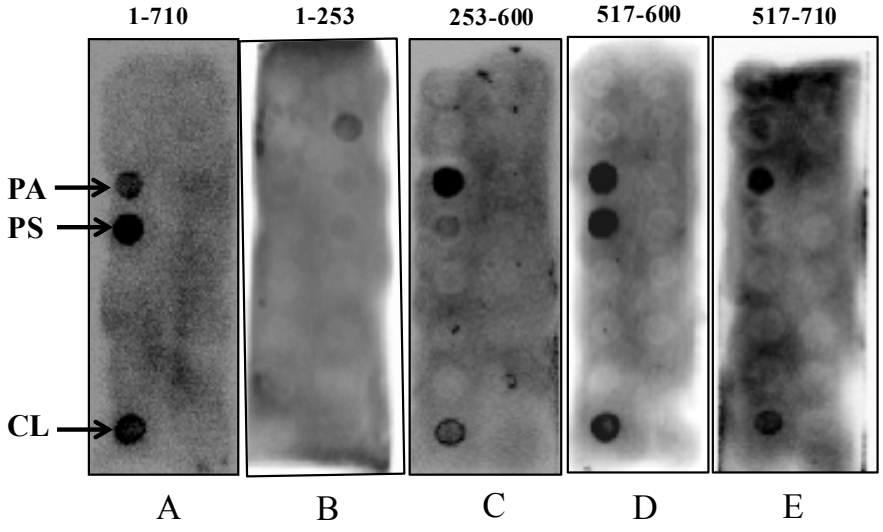

Figure 3.7: Lipid overlay assay of different fragments of Drp6. Western blot showing interaction of Drp6 (A), Drp6 1-253 (B), Drp6 253-600 (C), Drp6 517-600 (D) and Drp6 517-710 (E) with 15 different lipids using commercial strips. The fragment containing amino acids 517-600 interacted with PA, PS and CL similar to Drp6 full length (1-710).

software I-TASSER [217]. The comparison of this modeled structure (C-score of 0.45 and RMSD value of 3.1±0.8 Å) with the crystal structure of dynamin revealed that the Drp6 membrane binding domain is located at an equivalent position as that of PH domain of dynamin and membrane-binding loop of MxA (Fig 3.8). Therefore, it appears that although DRPs lack a PH domain, they harbor a membrane binding domain located at the same position as that of the PH domain. These results suggest that, despite the amino acid sequence diversity, members of dynamin family proteins harbor the lipid binding domain structurally at similar positions. The sequence diversity may be important for the functional diversity in different membranes in different organisms.

3.4.6 Membrane binding domain is essential but not sufficient for nuclear recruitment of Drp6:

To find out if membrane binding domain of Drp6 is required for nuclear recruitment, either Drp6 lacking the membrane binding domain (Drp6 Δ517-600) or the membrane binding domain (Drp6 517-600) were expressed in *Tetrahymena* as GFP tag fusion proteins. Drp6 Δ517-600 completely lost nuclear localization and was associated with cytoplasmic puncta suggesting that membrane binding domain is essential for nuclear recruitment. Interestingly, Drp6 517-600 also failed to associate with nuclear envelope indicating that membrane binding domain is not sufficient for nuclear recruitment. These results indicate that in addition to membrane binding domain, other domain/s is/are also important for nuclear recruitment (Fig 3.9). Expression of Drp6 1-600 (GTPase, middle and membrane binding domain) lacking C terminal domain, Drp6 517-710 (membrane binding domain and C terminal domain) lacking the GTPase domain and Drp6 253-600 (middle domain harboring membrane binding domain) also failed to localize on nuclear envelope (Fig 3.9). Taken together, these results indicate that GTPase and C terminal domains are also important for Drp6 nuclear recruitment.

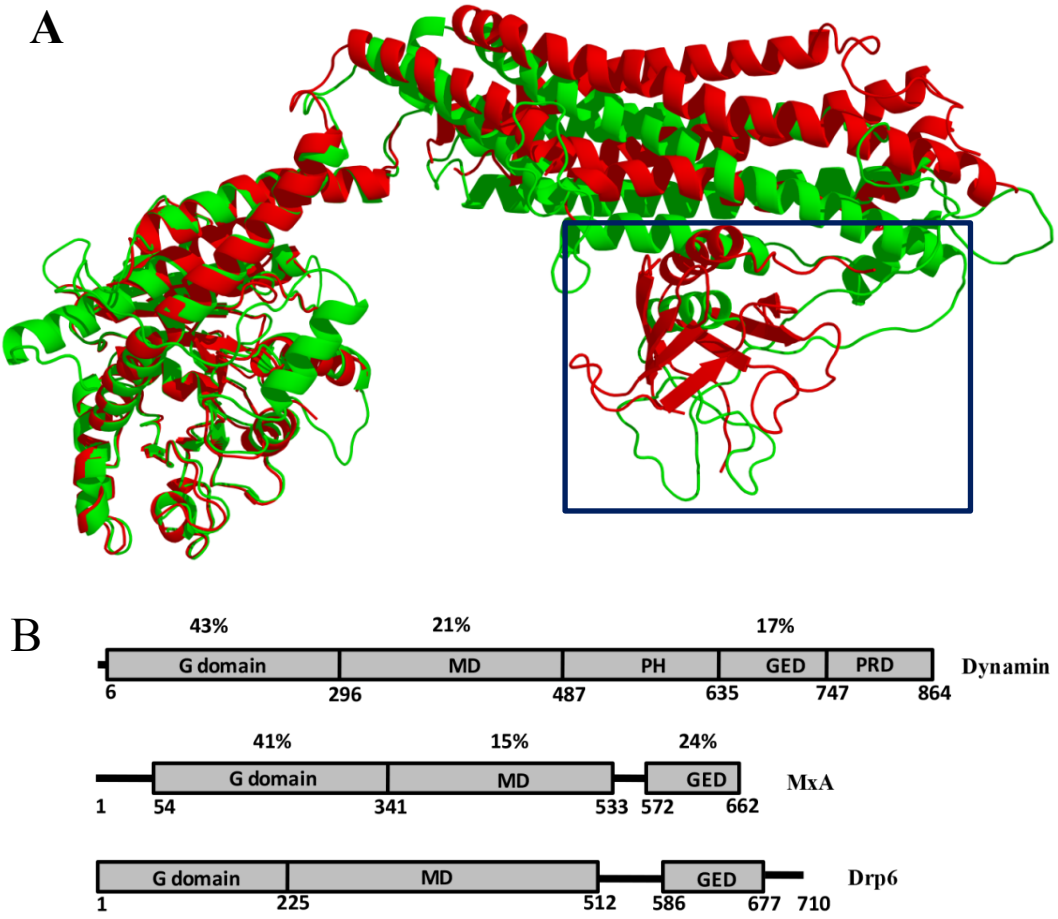

Figure 3.8: Predicted 3-dimensional model of Drp6. (A) The 3-D model of Drp6 (green) as predicted by I-TASSER using the human dynamin-1 (red) as template. PH domain of dynamin-1 is shown in the box. (B) Domain organization of dynamin, MxA and Drp6. Percent identity of Drp6 domains with MxA and dynamin are also shown. MxA and Drp6 lack PH domain but harbor a membrane binding region at the equivalent position. The numbers at the bottom indicate positions of amino acid residues.

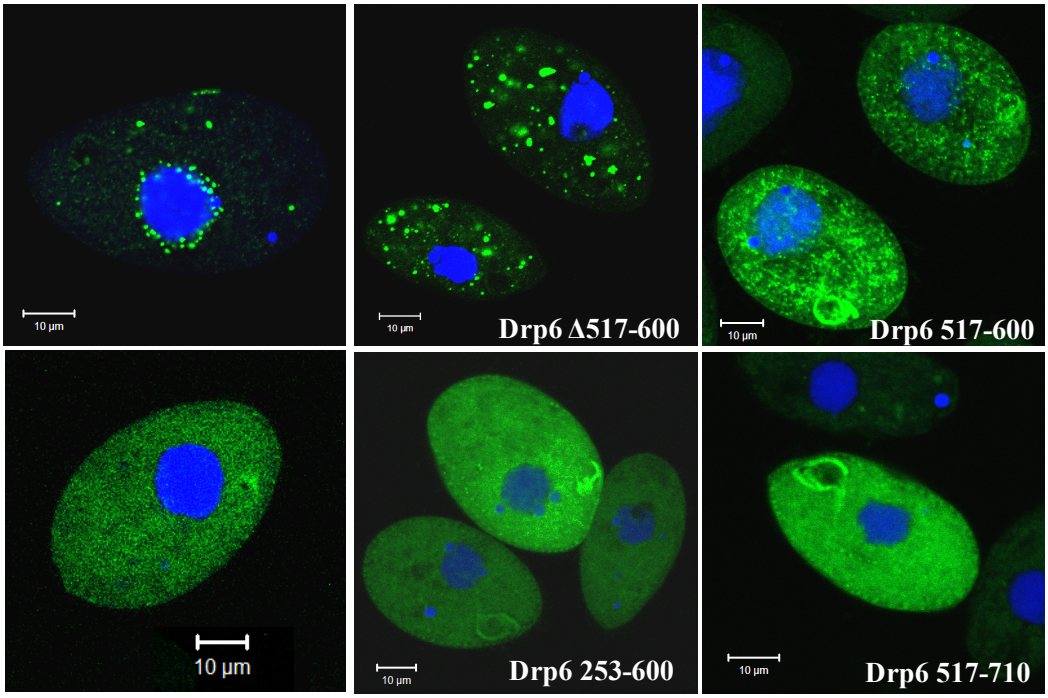

Figure 3.9: Localization of different fragments of Drp6. Confocal images of fixed *Tetrahymena* cells showing expression of GFP-Drp6 (top left), GFP-Drp6 Δ517-600 (top middle), GFP-Drp6 517-600 (top right), GFP-Drp6 1-600 (bottom left), GFP-Drp6 253-600 (bottom middle) and GFP-Drp6 517-710 (bottom right) after DAPI staining.

3.4.7 Membrane binding domain is essential for MAC expansion:

In order to evaluate the role of Drp6 membrane binding domain in nuclear recruitment of Drp6, either this domain (GFP-Drp6 517-600) or the protein lacking this domain (GFP-Drp6 Δ 517-600) was over expressed as GFP tag in *Tetrahymena.* Conjugation experiments of wild type x wild type, GFP-Drp6 Δ 517-600 x wild type and GFP-Drp6 517-600 x wild type were set up and MAC development was assessed in all these pairs. MAC development was significantly impaired in both GFP-Drp6 Δ 517-600 x wild type and GFP-Drp6 517-600 x wild type pairs as compared to wild type x wild type (Fig 3.10). However, the earlier stages such as MIC elongation and meiosis were unaffected. This signifies the importance of membrane binding domain in nuclear expansion.

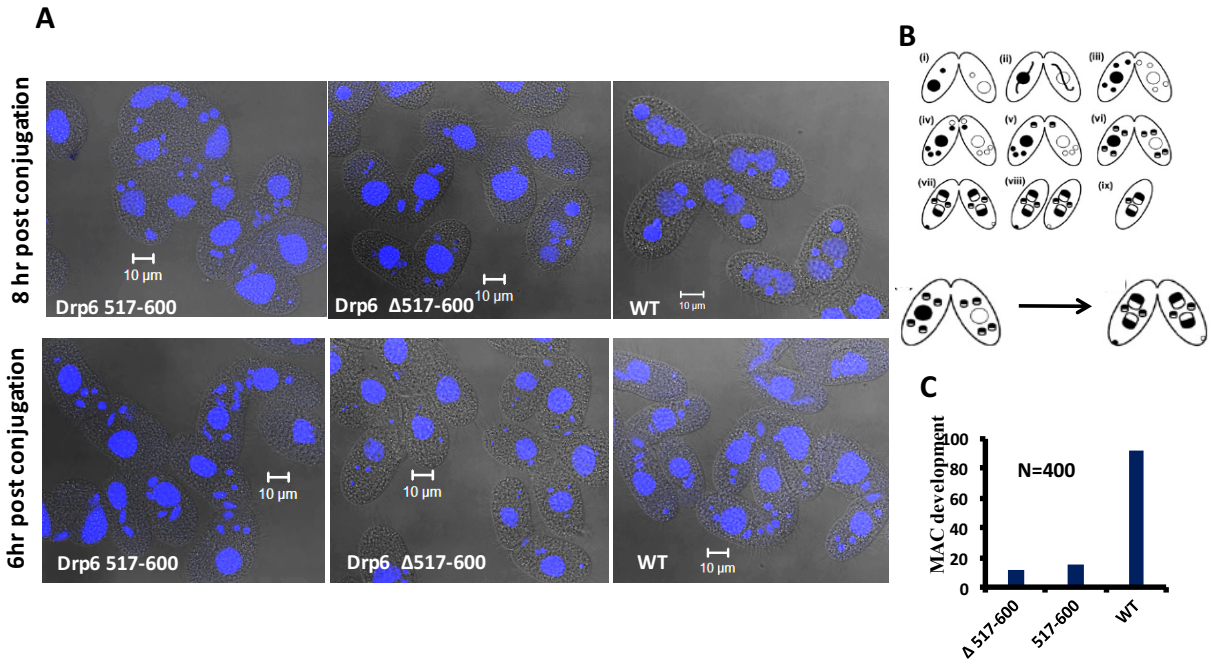

Figure 3.10: Membrane binding domain of Drp6 is important for macronuclear development. (A) Confocal images of fixed and DAPI stained *Tetrahymena* cells at 8 hours (top) or at 6 hours (bottom) post conjugation. Left: wild type cells conjugated with cells expressing GFP-Drp6 517-600; Middle: wild type cells conjugated with cells expressing GFP-Drp6 Δ517-600; Right: wild type cells conjugated with wild type cells. The newly developed MACs are indicated by arrow. (B) Conjugation stages in *Tetrahymena*. (i) pair formation, (ii) crescent stage (3 hr after mixing), (iii) meiosis (4.5 hr after mixing), (iv) pronuclear exchange (5 hr), (v) pronuclear fusion (5.5 hr), (vi) second postzygotic mitosis (6.5 hr), (vii) new MAC development (8 hr), (viii) pair separation (12 hr) and (ix) MAC resorption and MIC degradation (16 hr). Taken from Rahaman et al, 2008. (C) Quantification of MAC development in conjugated pairs as in A.

3.5 Discussion:

Dynamin and its related proteins are essential for many cellular functions, and play critical role in processes ranging from endocytosis to organelle fission and fusion. While classical dynamins are involved in endocytosis at plasma membrane, other members of this family including some in prokaryotes participate in membrane remodeling during organelle dynamics, cytokinesis and antiviral activity. Membrane recruitment is critical for their function of all dynamin family proteins. Classical dynamin is recruited to plasma membrane by its PH domain where it

catalyzes the scission of vesicles during endocytosis. Although DRPs do not contain a bonafide PH domain, they harbor either membrane binding loops or transmembrane helices for membrane interaction [3].

Drp6 localizes on nuclear envelope and ER derived vesicles [146]. Although it lacks a defined PH domain like classical dynamins, results of lipid overlay assay shows that Drp6 interacts with lipids independent of GTP and of any other factors suggesting that it harbors a lipid binding motif. It specifically interacts with three lipids namely phosphatidic acid, phosphatidylserine and cardiolipin. These lipids are anionic phospholipids and are known to play important roles in generation of membrane curvature. The physical properties of PA influence membrane curvature and it also acts as a signaling lipid recruiting cytosolic proteins to appropriate membranes. PA is also known to be involved in both vesicle fission and fusion [221,222]. Phosphatidylserine is distributed widely among animals, plants and microorganisms. It helps in docking of proteins with specific PS binding domains including several important signaling and fusogenic effectors on compartments such as endosomes and lysosomes. In addition, PS provides a negative surface charge to membranes to which polycationic proteins can bind. The effect is believed to facilitate redirection of certain proteins from one target membrane to another [223]. Cardiolipin is the signature lipid of mitochondria and constitutes almost 10% by weight of the total lipid content of mitochondria [224]. Although it is mostly present in mitochondrial inner membrane, CL is also found in mitochondrial outer membrane. In addition to mitochondria, cardiolipin is found to be present on nuclear membrane of *Tetrahymena* where it constitutes almost 3% of total nuclear lipid content [225]. These anionic phospholipids tend to perturb the packing density of lipid bilayer and facilitate penetration of hydrophobic protein motifs into the membrane bilayer [226].

Drp6 interacts with PS and CL containing membranes *in vitro*. Interaction of Drp6 with these lipids strongly indicate a membrane remodeling function of Drp6. As Drp6 is essential during

MIC to MAC transition in *Tetrahymena* it can be speculated that interaction of Drp6 with these lipids might have an essential role to play in nuclear recruitment and regulation of Drp6 function. The membrane binding domain of Drp6 was identified using different deletion fragments of Drp6. These fragments when assayed for lipid binding *in vitro* revealed that the fragment containing amino acids 517-600 interacts with all the three lipids the full length Drp6 interacts with, suggesting that Drp6 517-600 is the membrane binding domain of Drp6. A Drp6 variant lacking this motif (Drp6 Δ 517-600), when expressed in *Tetrahymena,* fails to localize on nuclear envelope suggesting that residues 517 to 600 are responsible for membrane interaction. However, this domain is not sufficient for recruiting Drp6 to nuclear envelope and requires presence of GTPase domain and C-terminal domain. This result is in line with the fact that isolated PH domain of dynamin has less affinity for membrane and oligomerization is a prerequisite for all dynamin proteins which requires the GTPase domain and the stalk comprising the middle domain and GED. Further, a predicted 3-dimensional model of Drp6 shows that region of Drp6 containing amino acids 517-600 is positioned at an equivalent place as that of the PH domain of dynamin and membrane binding loop of MxA, suggesting that the membrane binding domain in all dynamin proteins are located at the same position in the tertiary structure.

In conjugated *Tetrahymena* cells, Drp6 plays an important role in MIC to MAC transition. Assessment of MAC development in conjugants expressing either GFP-Drp6 517-600 or GFP-Drp6 Δ 517-600 revealed that in both the cases MAC development is inhibited significantly. This along with the previous results suggest that Drp6 membrane binding domain mediates the nuclear recruitment of Drp6 and is also required for macronuclear development during MIC to MAC transition in *Tetrahymena.*

CHAPTER FOUR

Investigation on Drp6 Nuclear Recruitment

4.1 Introduction:

Membrane recruitment is one of the most important steps for all dynamin proteins for their membrane remodeling functions. Either a PH domain or a lipid binding motif generally mediates membrane interaction with the target membrane [3,26,227]. Classical dynamin oligomers are recruited to its target membrane via interactions with their PH domain. Generation of high membrane curvature at the neck of the deeply invaginated coated pit is essential for membrane fission and vesicle release in intracellular membrane trafficking process. Dynamin self-assembly and resultant membrane constriction are required for membrane fission. Dynamin interacts directly with the lipid bilayer via its PH domain primarily through electrostatic interaction between a positively charged lipid binding pocket and the negatively charged lipid head group of PIP2 containing membrane [70]. The key residues involved in this association reside in the variable loops (VL)), and mutation of a critical lysine residue (K535A) in VL1 abrogates membrane binding [67,69,70]. Another isoleucine residue (I533) in the VL1 of dynamin has been shown to be critical for generation of membrane curvature by dynamin during vesicle endocytosis [72,228].

Other proteins of this family have well established roles in membrane remodeling during various cellular processes. These include mitochondrial maintenance DRPs involved in either fission or fusion of mitochondrial membrane. These dynamins do not possess a PH domain. Although recruitment of Drp1 (the mitochondrial fission dynamin) is believed to be mediated by interaction of its variable domain with mitochondrial receptor proteins, several evidences suggest that direct interaction of Drp1 with mitochondrial lipids have a prominent role in Drp1 recruitment and subsequent constriction of mitochondrial membrane driving fission of the organelle. Interaction with anionic phospholipids and subsequent stimulation of Drp1 GTPase activity has been shown to be crucial for Drp1 fission activity [229]. Moreover, a module of four

lysine residues present in the variable loop of Drp1 play crucial role in regulating interaction with cardiolipin, in its GTPase activity and subsequent mitochondrial fission [229]. The antiviral Mx proteins and distantly related EHD ATPases bind to membranes via exposed loops. In MxA, a stretch of four consecutive lysine residues in the L4 loop interacts with negatively charged phospholipids and mediates lipid binding [77]. Fusion dynamins such as mitofusins, OPA1/Mgm1 and atlastins have trans-membrane helices that anchor these proteins to the target membrane. The small isoform of Mgm1 (S-Mgm1), which is involved in mitochondrial inner membrane fusion, has a region in between the middle and GTPase effector domains which is responsible for binding to membrane and is essential for membrane fusion [230]. In case of bacterial dynamin, a paddle domain mediates the membrane binding by inserting itself into the lipid bilayer where two leucine residues and a phenylalanine residue contribute to the lipid binding [75]. Therefore, lipid interaction and membrane recruitment is a vital requirement for dynamin proteins. Mutations that impair membrane interaction have been implicated in defect in protein function in many proteins of dynamin family [3,69,73,74].

The *Tetrahymena* Drp6, also lacks a PH domain. However, it associates with nuclear envelope and ER derived vesicles [146]. In the previous chapter, a membrane binding domain of Drp6 was identified which represents the functional equivalent of dynamin PH domain. The present study addresses the understanding of mechanism of Drp6 nuclear recruitment and its importance in nuclear expansion.

4.2 Materials

pIGF was obtained from Dr. D.L. Chalker, University of Washington at St. Louis, USA.

pENTR/D-Topo vector and LR clonase kits, and Nonyl acridine Orange (NAO) were from Invitrogen.

Calmodulinagarose was from GE Healthcare.

All other chemicals and reagents were from Sigma-Aldrich or as mentioned in previous chapters unless otherwise mentioned.

4.3 Methods:

4.3.1 Incorporation of point mutations:

To generate *DRP6 I553M*, a point mutation corresponding to amino acid 553 in *DRP6* was incorporated by site directed mutagenesis using *DRP6* in pENTR/D-Topo vector as template and the primers (Forward: 5' GAA TGG ACC GTG CAG GAA **GCT** ATG ATT AAA GCG GAA AAC 3' and Reverse: 5' GTT TTC CGC TTT AAT CAT **AGC** TTC CTG CAC GGT CCA TTC 3') with the mutated codon underlined. The mutation was incorporated by using Quik Change Site-Directed Mutagenesis kit (Stratagene) and the presence of mutation was confirmed by DNA sequencing. The insert containing mutation was further cloned into pIGF vector under MTT1 promoter using LR clonase reaction (Invitrogen). The schematic of the cloning steps are shown in the figure 4.1.

4.3.2 Cloning of *TAP-DRP1* pVGF:

DRP1 gene was PCR amplified using primers (Forward: 5' CACC CTCGAG ATGGCTGAG CAAGAAAG3' and Reverse: 5' GC GGGCCC TCAAACAACTTGCTTTTAAGG 3') and *Tetrahymena* genomic DNA as template. The amplified PCR product was cloned into XhoI and ApaI sites of pVGF vector. The construct was confirmed by DNA sequencing.

4.3.3 Purification of TAP-Drp6 and TAP-Drp1:

For purification of TAP-Drp6 and TAP-Drp1, *Tetrahymena* cells harboring TAP-DRP6 or TAP-DRP1 pVGF plasmids were grown to a density of 3 x 10^5 cells /ml in SPP media at 30°C. The culture was induced with 1µg/ml cadmium chloride for 5 hours under same conditions and cells from 2 L cultures were collected by centrifugation. The cell pellet was resuspended in 30 ml lysis buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5%NP-40 and 10% glycerol

supplemented with a mixture of protease inhibitors (pepstatin, E-64, aprotinin, and protease inhibitor cocktail) and incubated on ice for 1 hour for cell lysis. All subsequent steps were carried out at 4^{0}C. The lysate was clarified by ultracentrifugation at 50,000 RPM for 1 hour. Rabbit-IgG agarose slurry (Sigma) pre-equilibrated with lysis buffer was added to the clarified lysate and was kept for binding for 2 hours. Resin was collected by centrifugation at 5,000RPM for 1 min and washed with 50 bed volumes of wash buffer containing 20 mM Tris-HCL pH 8.0, 2 mM MgCl2, 0.2 mM EGTA, 0.1 % Tween 20, 10 % glycerol, 1 mM DTT and 0.1mM PMSF. The resin was equilibrated with TeV cleavege buffer containing 10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 0.1%Tween 20, 0.5 mM EDTA and 1 mM DTT and was incubated with 2µl of TeV protease (Invitrogen) in 200 µL of the same buffer for 1.5 hour at room temperature followed by further incubation at 4^{o}C overnight. The eluate after proteolytic cleavage was adjusted to 3 mM $CaCl_2$ and mixed with 3 volumes of calmodulin binding buffer containing 10 mM Tris-HCl pH

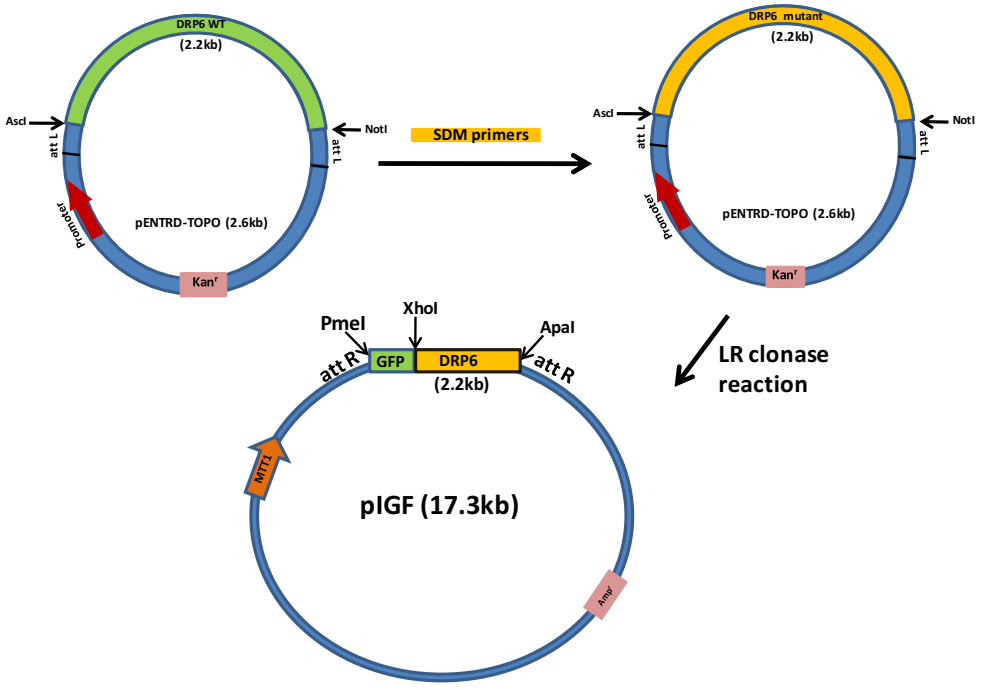

Figure 4.1: Schematic representation of site directed mutagenesis and cloning of *DRP6* variants as N-terminus GFP fusion constructs

8.0, 100 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM CaCl2, 0.1 % Tween 20 and 10 mM β-ME. This was incubated with 100 µl of calmodulin resin (GE Biosciences) for 1 hour and the resin was recovered by centrifugation at 5000 RPM for 1 min followed by washing with calmodulin binding buffer. The protein was eluted with calmodulin elution buffer containing 10 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1 mM Mg acetate, 1 mM imidazole, 10 mM EGTA, 0.1 % Tween 20 and 10 mM β-ME. Eluted fractions were loaded on 10 % SDS PAGE gel and the protein was detected by silver staining.

4.3.4 Liposome tubulation assay:

For tubulation assay, liposomes prepared from total *Tetrahymena* lipids supplemented either with 10% CL or PS. The liposomes were incubated with 1µM of purified protein in GTPase assay buffer in presence of 0.5 µM GTP-γ-S for 2 hours at room temperature. The samples were then adsorbed onto a 200 mesh carbon coated grid and was visualized under TEM as mention in chapter 2.

4.3.5 Fluorescence spectroscopy of proteins:

For measuring intrinsic fluorescence of proteins, 300µl (1µM) of proteins either in presence or absence of liposomes were placed in a quartz cuvette and the fluorescent spectra were recorded at excitation λ_{280} or λ_{295} using a spectrofluorimeter (Cary Eclipse Fluorescence Spectrophotometer, Agilent technologies). The emission spectra were analyzed and plotted using OriginPro 8.0 software.

4.3.6 Depletion of cardiolipin:

Depletion of CL either in vegetative or in conjugated *Tetrahymena* cells was performed by treating the wild type cells or cells expressing GFP-Drp6/ Nup3-GFP/ GFP-Nem1D with 10µM pentachlorophenol (PCP, Sigma) dissolved in DMSO for 30 min. Live cell imaging was performed to visualize the expressed proteins. For macronuclear inhibition assay, conjugating

cells at different stages of nuclear differentiation were collected by centrifugation and were fixed with 4% PFA as mentioned earlier. The control cells were treated with equivalent amount of DMSO. Cells were stained with DAPI before imaging.

4.3.7 Nonyl acridine orange (NAO) staining:

Tetrahymena cells (either vegetative or conjugated) were treated with 0.25-0.5μM NAO (Invitrogen) dissolved in DMSO for 30 min. The control cells were treated with equivalent amount of DMSO. Conjugating cells at different stages of nuclear differentiation were collected and were fixed using 4% PFA. The cells were stained with DAPI before imaging.

4.4 Results:

4.4.1 A single residue I553 in lipid binding domain is important for nuclear recruitment of Drp6:

Dynamin binds to membrane by its PH domain via interaction with PIP2 head group. [67,69]. An isoleucine at 533 in dynamin1 plays a crucial role in stable insertion of the PH domain into the lipid bilayer for generating membrane curvature and subsequent membrane fission during endocytosis [74]. In addition to dynamin, many other PH domain containing proteins utilize loop residing isoleucine residues for membrane insertion [231]. An isoleucine (I553) is present in the putative loop of the membrane binding domain in the predicted 3D structure of Drp6. In order to evaluate if this isoleucine residue is also important for membrane binding, a Drp6 variant with methionine substituting isoleucine at position 553 (*Drp6 I553M*) was generated and expressed as N-terminus GFP fusion protein in *Tetrahymena*. Confocal images of cells expressing GFP-Drp6 I553M showed that mutation of Isoleucine at 553 to methionine resulted in complete loss of nuclear localization and was present mostly as cytoplasmic puncta (Fig 4.2) suggesting that I553 is important for nuclear recruitment of Drp6.

4.4.2 I553M mutation does not affect GTPase activity and self assemly of Drp6

To understand the basis of loss of nuclear localization, various biochemical and biophysical properties of Drp6 I553M were compared with those of Drp6. For this purpose, both the wild type and mutant proteins were expressed in bacteria and purified as N-terminus His_6 fusion protein (Fig 4.3A). The GTPase activity of the mutant protein was measured and compared with the wild type protein. The result showed that the mutation of I553 did not affect GTPase activity significantly (Fig 4.3B). Moreover, the Km and Vmax of the mutant were also comparable to wild type protein (Fig. 4.3C) suggesting that loss of nuclear recruitment of Drp6 I553M is not due to defect in its GTP hydrolysis activity.

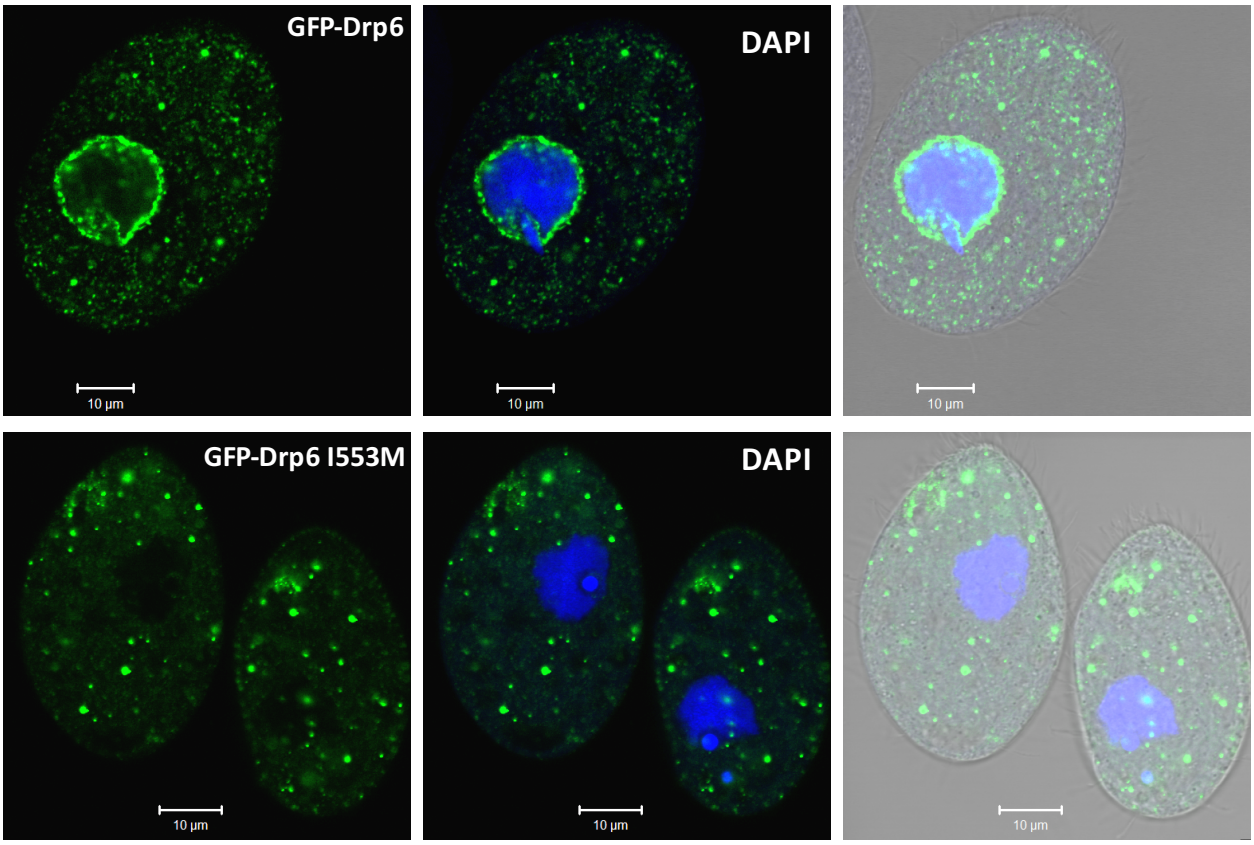

Figure. 4.2: Isoleucine 553 is important for nuclear localization of Drp6. Confocal images of fixed *Tetrahymena* cells either expressing GFP-Drp6 (top) or GFP-Drp6 I553M (bottom) after DAPI staining. Left panel, GFP; middle panel, GFP+DAPI and right panel, GFP+DAPI+DIC.

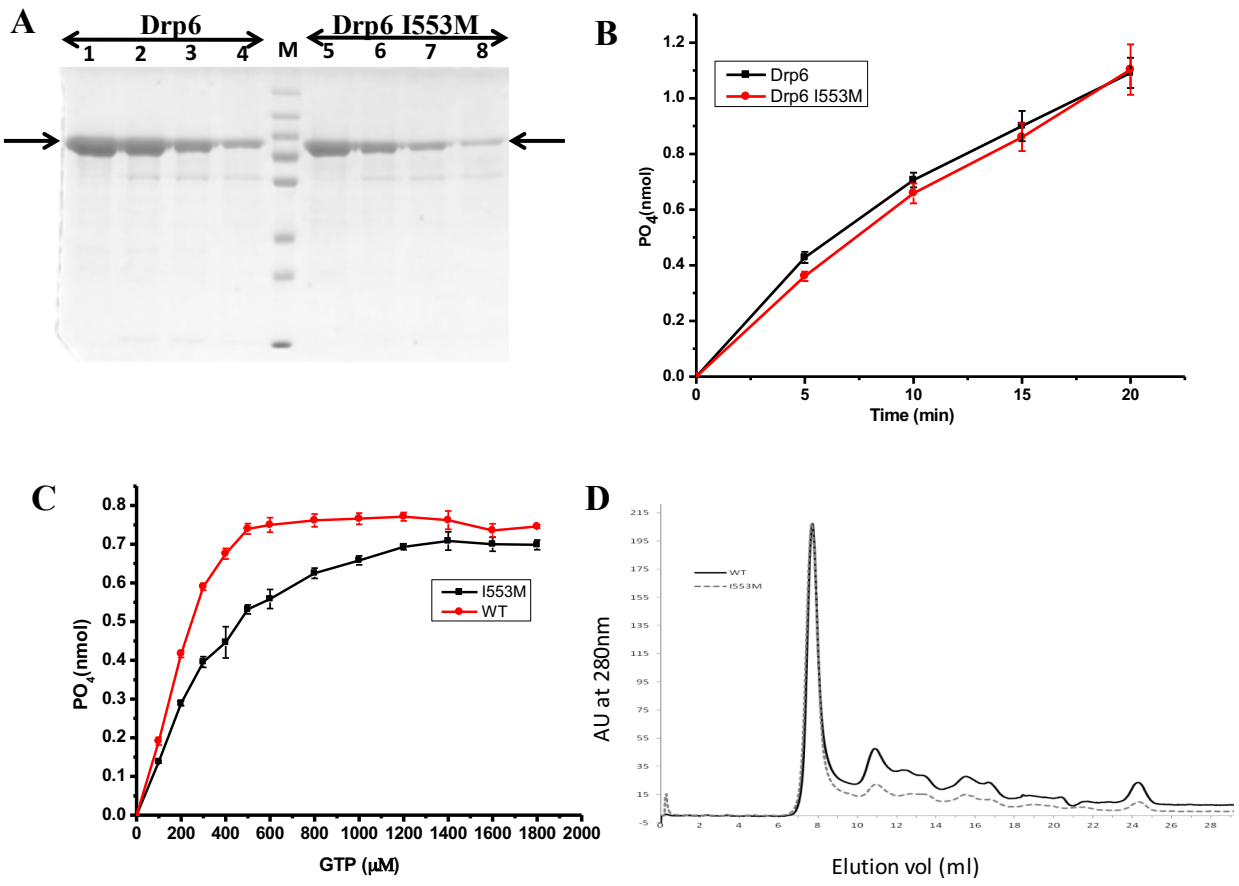

Figure 4.3: Mutation at Isoleucine 553 does not affect GTPase activity and oligomerization of Drp6. (A) 10% SDS PAGE gel showing different purified fractions of Drp6 (Lanes 1-4)and Drp6 I553M (Lanes 5-8) after Ni affinity purification. M denotes molecular weight marker. The position of purified proteins are shown by arrow. (B) GTP hydrolysis rates of Drp6 and Drp6 I553M using 1µM protein each. (C) GTPase assay performed for 20 min with different concentrations of GTP using 1µM protein. (D) Size exclusion chromatography of Drp6 (solid line) and Drp6 I553M (dotted line).

To see whether the mutation I553M disrupts Drp6 self-assembly, the oligomerization status of Drp6 I553M was assessed. Drp6 I553M, when subjected to analytical size exclusion chromatography, eluted in the void volume (Fig. 4.3D) and the elution profile was not significantly different from the wild type Drp6 (Drp6 WT). This suggests that the mutation I553M didn't perturb the self-assembly property of Drp6.

To further assess if the mutation affects Drp6 ultrastructure, both wild type and mutant proteins were analyzed by electron microscopy. Drp6 WT formed rings with diameters ranging from 45 to 75 nm and helical spirals of varying sizes. Similar to wild type Drp6, mutant protein also formed rings and helical spirals (Fig. 4.4). The rings and spirals of Drp6 I553M were measured to be of similar size as that of wild type suggesting that the mutation did not affect the higher order structure of the protein. Taken together, these results suggest loss of nuclear recruitment of Drp6 I553M is not due to defects in GTPase activity, self-assembly or ultrastructure.

4.4.3 Interaction with Cardiolipin is important for nuclear recruitment of Drp6

Although I553M resulted in complete loss of nuclear localization of Drp6, neither the enzymatic nor the assembly properties of the protein were affected by this mutation. To find out whether Drp6 I553M affects membrane association, a liposome floatation assay was performed. For this assay, both Drp6 WT and Drp6 I553M were incubated with liposomes prepared from total lipid extracted from *Tetrahymena* cells supplemented with either 10% cardiolipin or 10% phosphatidylserine. The fractions were collected and analyzed on a SDS PAGE followed by immunobloting. Results of this assay demonstrated that, while both Drp6 WT and Drp6 I553M interacted with PS-containing liposomes, the mutant failed to interact specifically with liposomes containing CL (Fig 4.5). This indicates that I553 is important for Drp6 interaction with CL and this interaction might be important for recruiting Drp6 onto nuclear envelope.

4.4.4 Cardiolipin depletion disrupts nuclear localization of Drp6

Pentachlorophenol (PCP) is a polychlorinated aromatic compound, a respiratory uncoupler and is a potent inhibitor of CL synthesis [232]. To assess if PCP depletes CL in *Tetrahymena*, the cells were treated with 10μM PCP. As shown in Figure 4.6A, the CL content in treated cells was reduced significantly as estimated by NAO staining (CL binding dye). NAO staining is widely used to monitor cardiolipin distribution in the cell [233, 234].

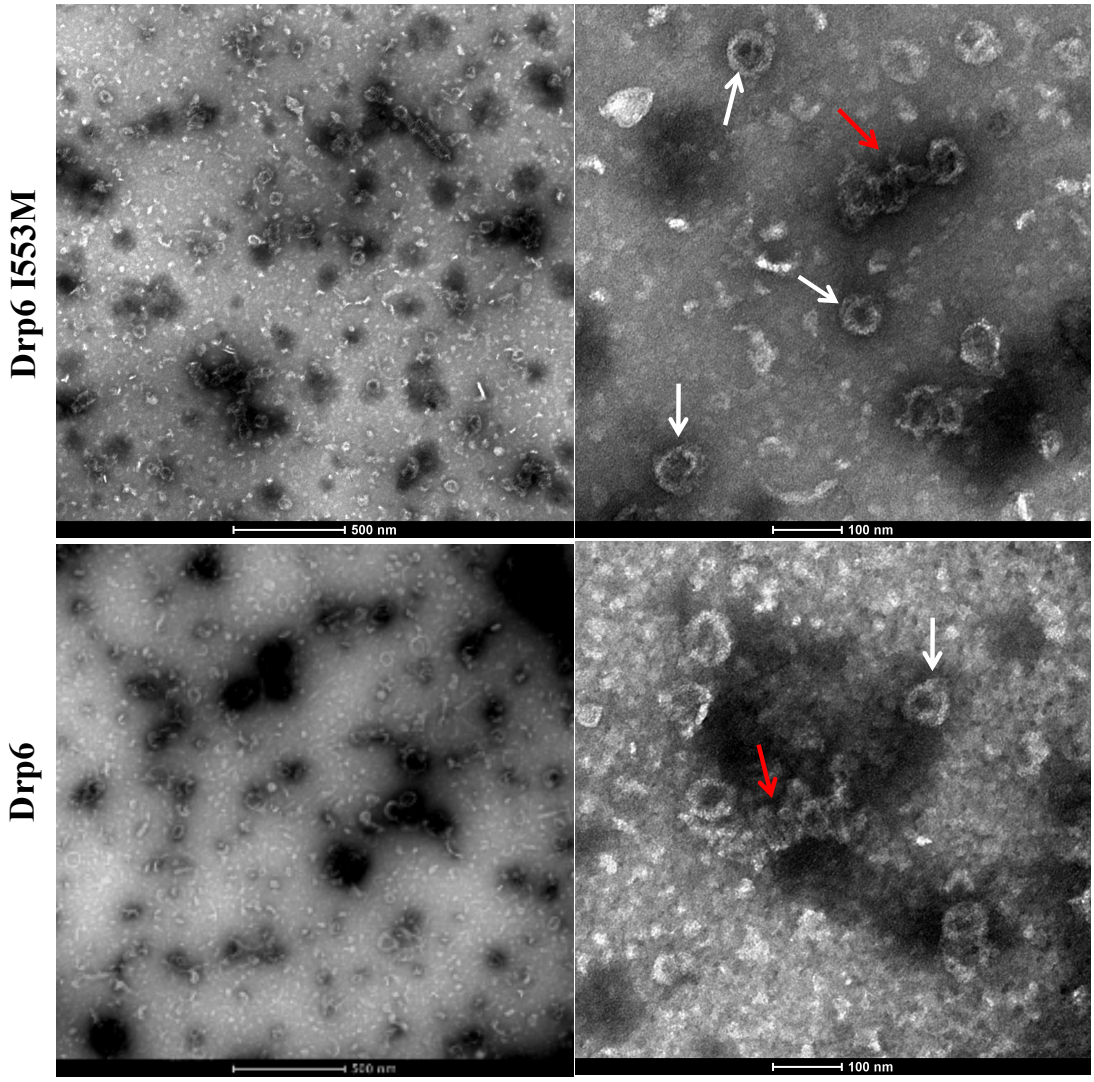

Figure 4.4: Mutation at Isoleucine 553 does not affect formation of rings and spirals of Drp6. Electron micrographs of negatively stained Drp6 I553M (top) and Drp6 (bottom). Rings and helical spirals are indicated by white and red arrows respectively. Micrographs at higher magnification are shown in right.

Drp6 mutant which fails to localize on nuclear envelope has reduced affinity for CL (Fig. 4.5). To further assess the role of CL in nuclear recruitment of Drp6 *in vivo*, distribution of Drp6 was evaluated in *Tetrahymena* cells where CL was depleted. CL was depleted using PCP in *Tetrahymena* cells expressing either GFP-Drp6 or GFP-Nup3 (nuclear pore protein). *Tetrahymena* cells expressing GFP- Drp6 treated with 50μM PCP for 30 minutes and distribution of GFP-Drp6 was analyzed by confocal microscopy. Upon PCP treatment, GFP-Drp6 readily dissociated from nuclear envelope and appeared as cytoplasmic puncta (Fig 4.6B, left panel) suggesting that CL is important for association of Drp6 with nuclear envelope. This effect was specific for Drp6 since PCP treatment did not affect the localization of another nuclear envelope protein GFP-Nup3 (Fig. 4.6B, middle panel). PCP treatment did not affect the overall membrane structure of the cell as distribution of another membrane binding protein Nem1D was also not affected by this treatment (Fig. 4.6B, right panel). Therefore, it can be concluded that dissociation of Drp6 from nuclear envelope is not due to disruption of nuclear envelope structure rather due to loss of interaction with CL. This suggested that Drp6-CL interaction is important for recruitment of Drp6 onto nuclear envelope.

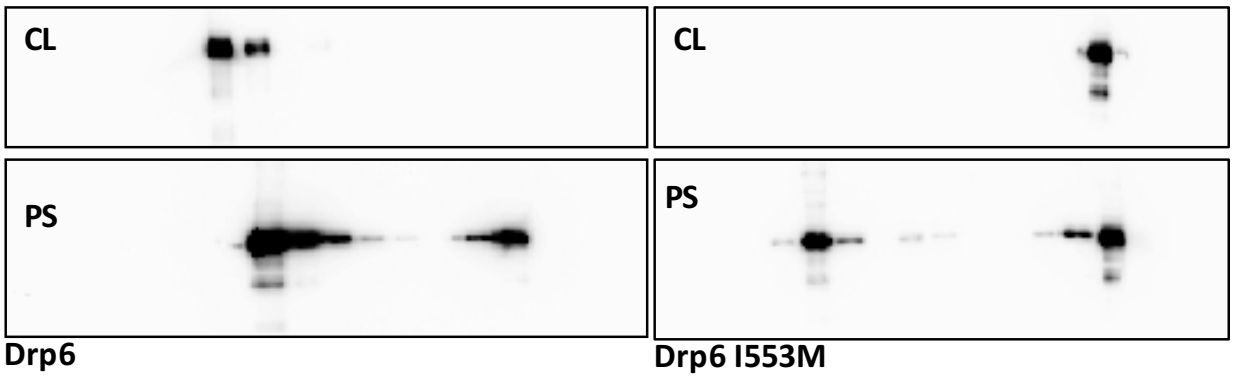

Figure 4.5: Drp6 I553M does not interact with cardiolipin. Western blot showing interaction of Drp6 (left) and Drp6 I553M (right) with liposomes containing cardiolipin (top) and phosphatidylserine (bottom). The successive fractions collected from top to bottom were loaded from left to right. Drp6 interacted with both cardiolipin containing liposomes and phosphatidylserine containing liposomes whereas Drp6 I553M although interacted with phosphatidylserine containing liposomes, it failed to interact with cardiolipin containing liposomes.

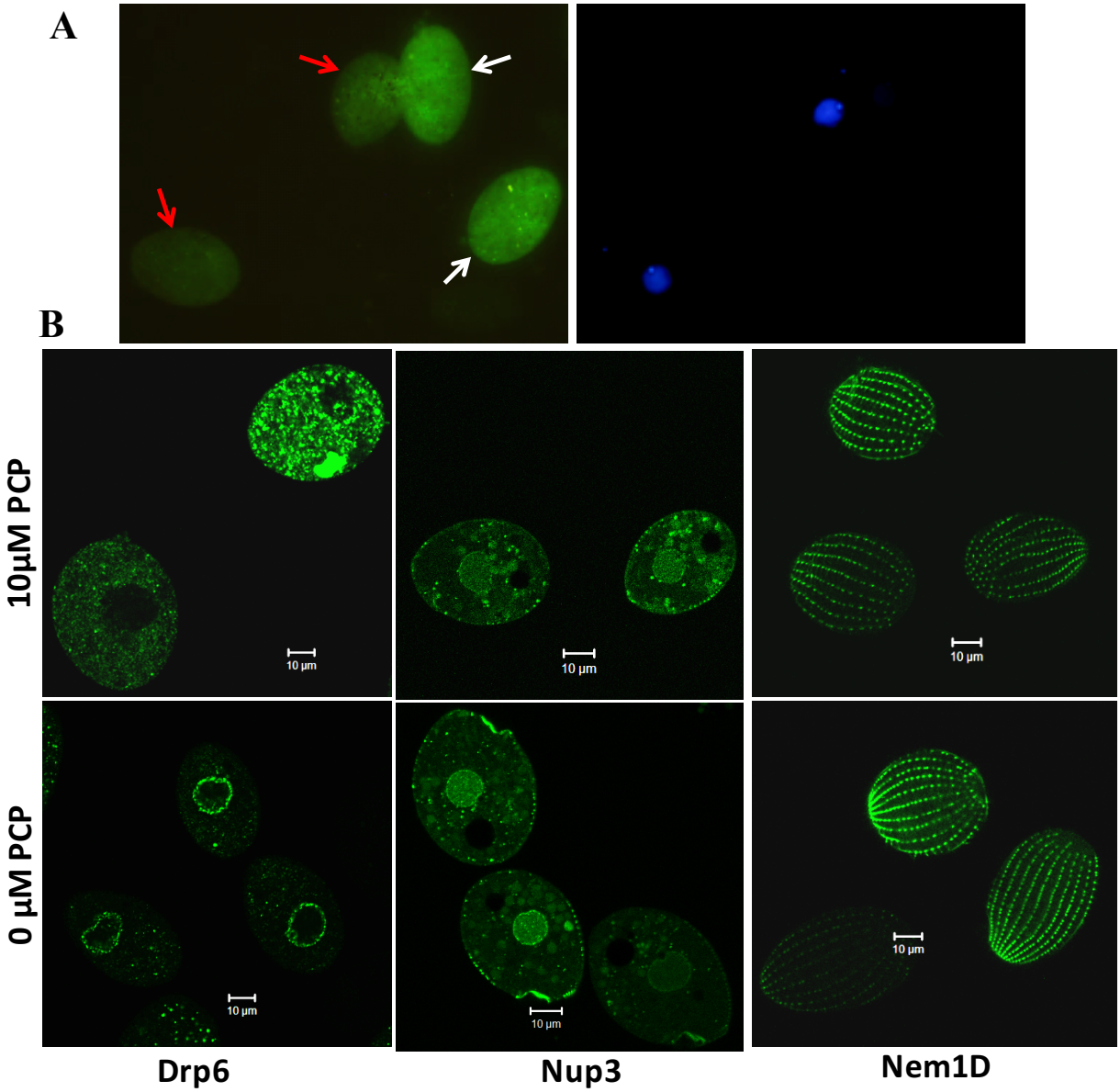

Figure 4.6: Cardiolipin is important for recruitment of Drp6 to nuclear envelope. (A) Images of NAO stained fixed *Tetrahymena* cells with (red arrow) or without (white arrow) 10µM PCP. Both treated and untreated cells were imaged simultaneuosly. Treated cells were stained with DAPI to distinguish them from untreated cells. The lower NAO signal in treated cells signifies depletion of cardiolipin by PCP treatment. (B) Images of *Tetrahymena* cells expressing either GFP-Drp6 (left) or Nup3-GFP (middle) or GFP-Nem1D (right). Top panel represents cells treated with 10µM PCP and bottom panel represents control where PCP was replaced with DMSO. Depletion of cardiolipin by PCP treatment dissociated GFP-Drp6 from nuclear envelope without affecting Nup3-GFP and GFP-Nem1D.

4.4.5 Cardiolipin is required for MAC expansion

Drp6 is essential for MAC development in *Tetrahymena* [146] and Drp6- CL interaction is important for nuclear recruitment of Drp6. To see if Drp6-CL interaction is important for MAC development, CL was either depleted using PCP or its interaction with Drp6 was interfered by using the CL-binding dye Nonyl Acridine Orange (NAO). NAO specifically interacts with CL and have been used for quantification of CL *in vivo* [232–234] In both PCP and NAO treated conjugated cells, MAC development was significantly inhibited (Fig. 4.7 and fig 4.8). Quantitative analysis showed that while 85% to 90% of the conjugants developed MAC

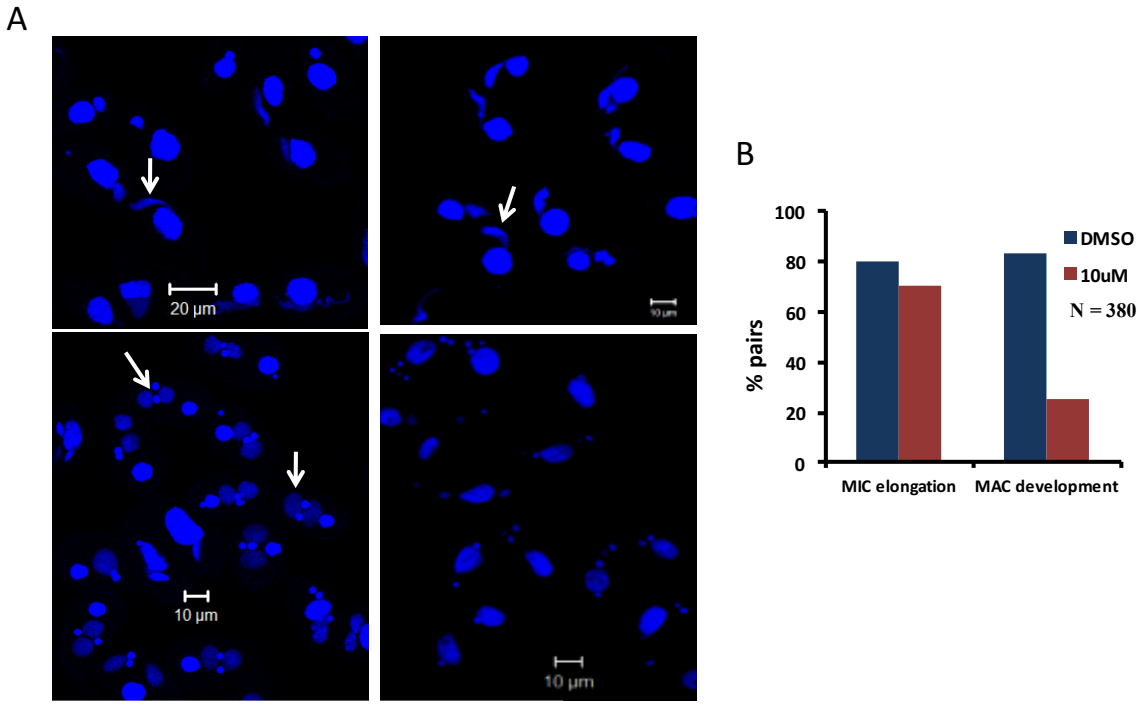

Figure 4.7: Depletion of cardiolipin inhibits MAC development. (A) Images of *Tetrahymena* pairs fixed at 3 hours (top) and 8 hours (bottom) post conjugation. The left panel is untreated control and right panel is cells treated with 10µM PCP. Elongated MICs and new MACs are indicated by arrow. (B) Quantification of MIC elongation and MAC development.

in the control, only 20% to 25% of the conjugants developed MAC when treated with PCP or NAO (Fig. 4.7B and fig 4.8B). This inhibition by PCP or NAO was specific to MAC development stage since other stages of nuclear differentiation such as MIC elongation and meiotic division were either unaffected or marginally affected. (Fig. 4.7 and fig 4.8). These results suggest that CL is important for macronuclear development. Since Drp6 is essential during macronuclear development and depletion of CL inhibits this process, it can be concluded that Drp6 mediated CL interaction is required for macronuclear development.

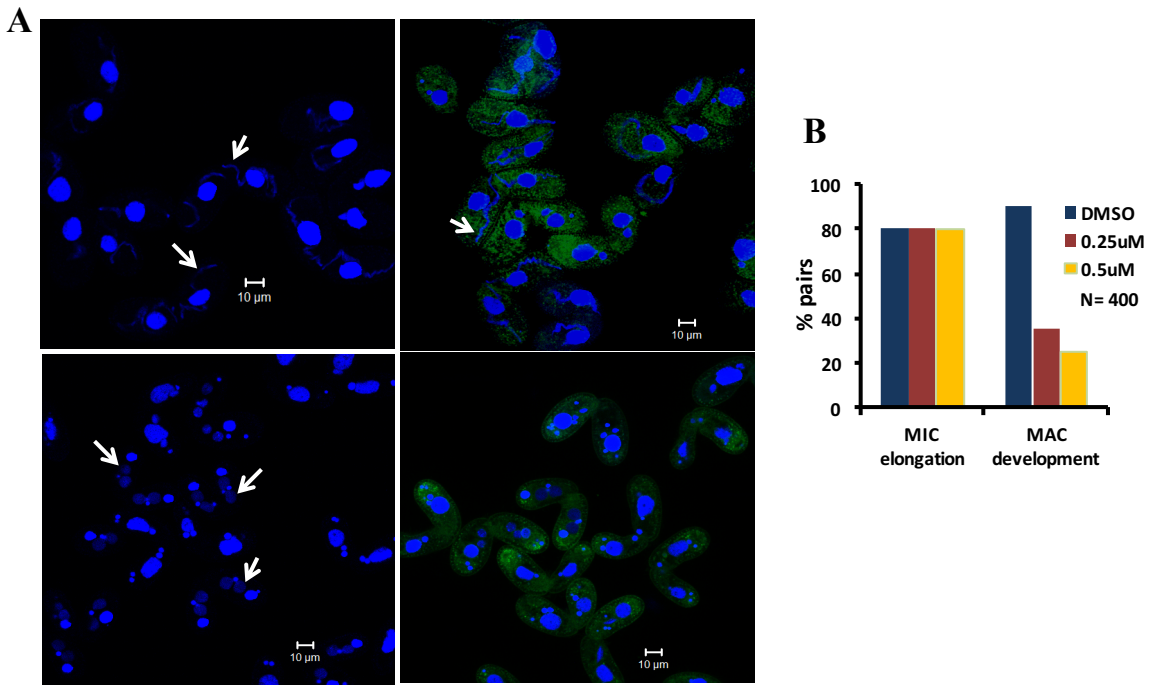

Figure 4.8: Cardiolipin binding dye NAO inhibits macronuclear development. (A) Images of *Tetrahymena* pairs fixed at 3 hours (top) and 8 hours (bottom) post conjugation. The left panel is untreated control and right panel is cells treated with 0.5µM NAO. Elongated MICs and new MACs are indicated by arrow. (B) Quantification of MIC elongation and MAC development. NAO staining is shown as green.

4.4.6 Cardiolipin interaction inhibits Drp6 GTPase activity:

Classical dynamins and many DRPs exhibit assembly stimulated GTPase activity upon membrane association [218,229,230,235]. Drp6 has high basal level GTPase activity, comparable to classical dynamins. To examine the effect of membrane binding on Drp6 GTPase activity, its rate of GTP hydrolysis was assessed in presence of liposomes containing either CL or PS. Recombinant Drp6 was incubated with liposomes prior to the GTPase assay. The results of the assay showed that GTPase activity of Drp6 was not stimulated by either CL or PS containing liposomes. Interestingly, activity was inhibited and the inhibition was more drastic (~ 3 fold) when Drp6 was incubated with CL containing liposomes than when incubated with PS containing liposomes (Fig.4.9A and B). Stimulation of GTP hydrolysis in presence of lipid is a characteristic feature of dynamin family members. Surprisingly, in case of Drp6, presence of lipid did not stimulate its GTPase activity, rather inhibited suggesting a different mechanism of Drp6 function in membrane remodeling. However, this lack of stimulation was not due to failure of membrane interaction, as in a floatation assay, Drp6 interacted with the liposomes under the assay conditions (figure 4.9C)

To further confirm that the inhibition of GTPase activity is also true for Drp6 purified from *Tetrahymena*, the protein was expressed and purified as a TAP tag fusion protein (TAP-Drp6) in *Tetrahymena* (Fig 4.10A). GTPase activity of TAP-Drp6 was also inhibited in presence of liposomes confirming that Drp6 when associates with membrane, its GTPase activity is inhibited. To rule out that inhibition is not an artifact, an endocytic dynamin (TAP-Drp1) was assayed under same experimental condition and as expected, its GTPase activity was enhanced 3 fold in presence of liposomes (Fig.4.10B). Together, this led to the conclusion that unlike other

dynamin proteins, GTPase activity of Drp6 is not enhanced, rather inhibited upon membrane association.

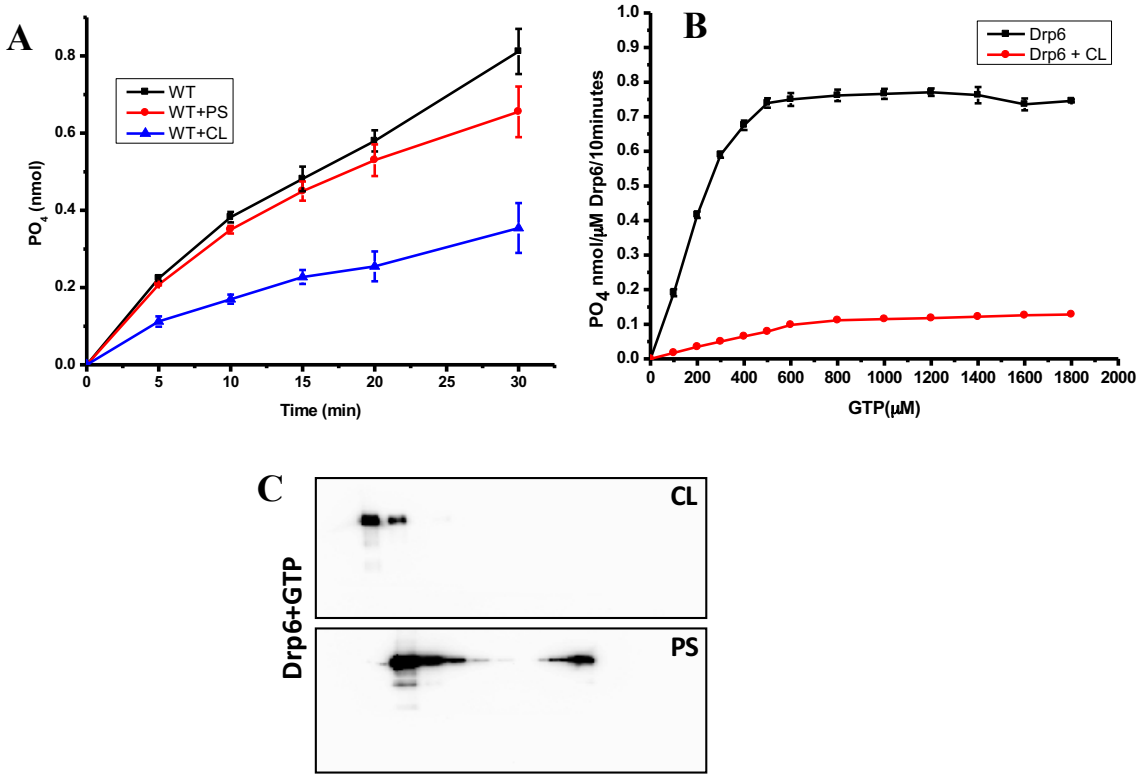

Figure 4.9: Cardiolipin inhibits GTPase activity of Drp6. (A) GTP hydrolysis rates of Drp6, Drp6 in presence of cardiolipin containing liposomes and Drp6 in presence of phosphatidylserine containing liposomes using 1µM protein. (B) GTPase assay performed for 20 min with different concentrations of GTP using 1µM protein either in presence or absence of cardiolipin containing liposomes. (C) Western blot showing interaction of Drp6 with cardiolipin liposomes in the assay conditions. The lack of inhibition in GTPase activity by phosphatidylserine containing liposomes is not due to lack of interaction with Drp6. The inhibition is specific for cardiolipin.

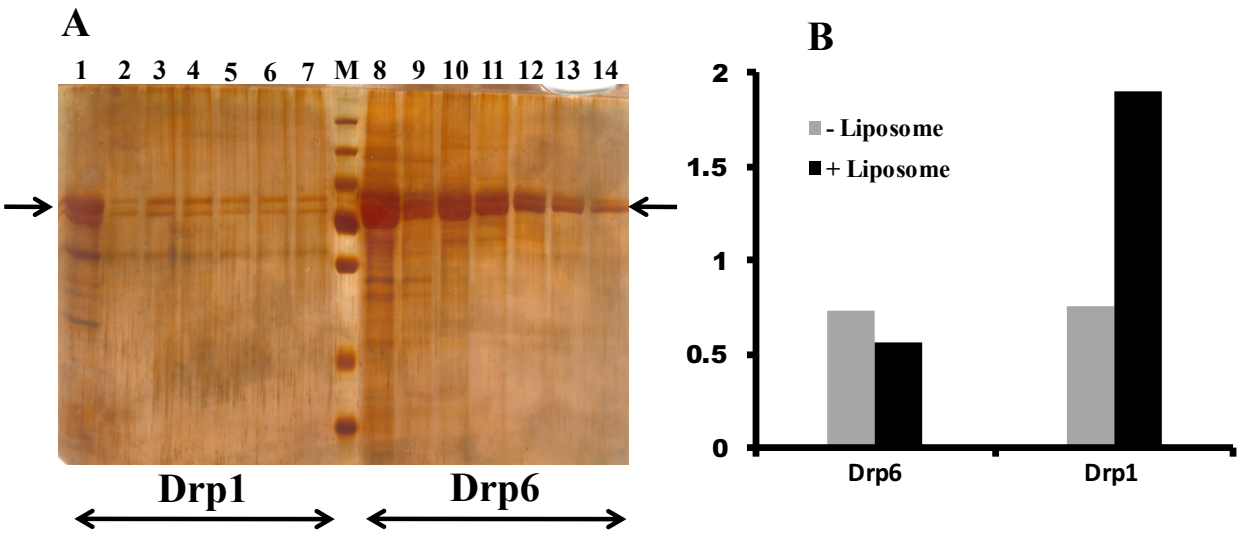

Figure 4.10: Inhibition of GTPase activity by liposomes is specific for Drp6: (A) Silver stained 10% SDS PAGE gel showing purified fractions of TAP-Drp1 (endocytic dynamin) (lanes 1-7) and TAP-Drp6 (lanes 8-14). Lane 1 and 8 show the fractions purified from IgG agarose column. Rest shows fractions further purified by calmodulin sepharose column. M denotes molecular weight marker. (B) GTPase activity of TAP-Drp1 and TAP-Drp6 either in presence (black) or absence (gray) of liposomes. GTPase activity upon membrane association was inhibited in case of TAP-Drp6 and was enhanced in case of TAP-Drp1.

4.4.7 Drp6 changes conformation upon interaction with cardiolipin:

Drp6 GTPase activity is inhibited in presence of CL containing liposomes. To assess if this inhibition was due to any change in protein conformation, the intrinsic fluorescence properties of Drp6 were assessed in presence of liposomes containing either CL or PS. Fluorescence spectra were recorded at 25°C on a spectrofluorimeter set at an excitation wavelength (λex) of 280 nm or 295 nm. The fluorescence intensity was reduced in presence of both PS and CL liposomes. However, the decrease in fluorescence intensity was more in case of CL (Fig 4.11A). The difference in change in fluorescence intensity in presence of CL liposomes and PS liposomes

might be due to change in protein structure since Drp6 formed relatively more SDS resistant oligomers when incubated with CL liposomes than with PS liposomes (Figure 4. 11B). Drp6 in presence of CL liposomes appeared as higher molecular weight bands with concomitant decrease in its monomeric form. Interestingly, presence of PS liposomes under similar assay conditions did not affect the oligomeric property. These results suggest that conformation of Drp6 is changed upon membrane binding and the conformation might be different for CL and PS containing membranes.

4.4.8 Drp6 tubulates membranes *in vitro*

Proteins of dynamin family catalyze membrane deformation by inducing high curvature stress. They have been shown to assemble onto the outer surface of liposomes *in vitro*, and to deform liposomes into tubules [152] [151] [236]. Drp6 assembles into ring like and helical spirals and also interacts with liposomes *in vitro*. To determine whether Drp6 polymers affect liposome structures similarly, liposomes pre-incubated with Drp6 were negatively stained with 2% uranyl acetate and visualized by electron microscopy. Liposomes were composed of total lipids extracted from *Tetrahymena* supplemented with either 10% CL or 10% PS. The protein free liposomes were spherical in shape and were of ~ 100 nm in diameter (Fig. 4.12). Incubation with Drp6 in presence of GTPγS resulted in deformation of the shape of liposomes containing both CL and PS. As shown in fig 4.12, liposomes treated with Drp6 appeared branched and tubulated. The results also showed that the branching and tubulation of CL containing liposomes were more profound than those of PS containing liposomes (Fig 4.12). These results clearly demonstrate that similar to other dynamin proteins, Drp6 can also tubulate membrane *in vitro* suggesting its role in membrane remodeling and possibly in regulation of nuclear membrane expansion.

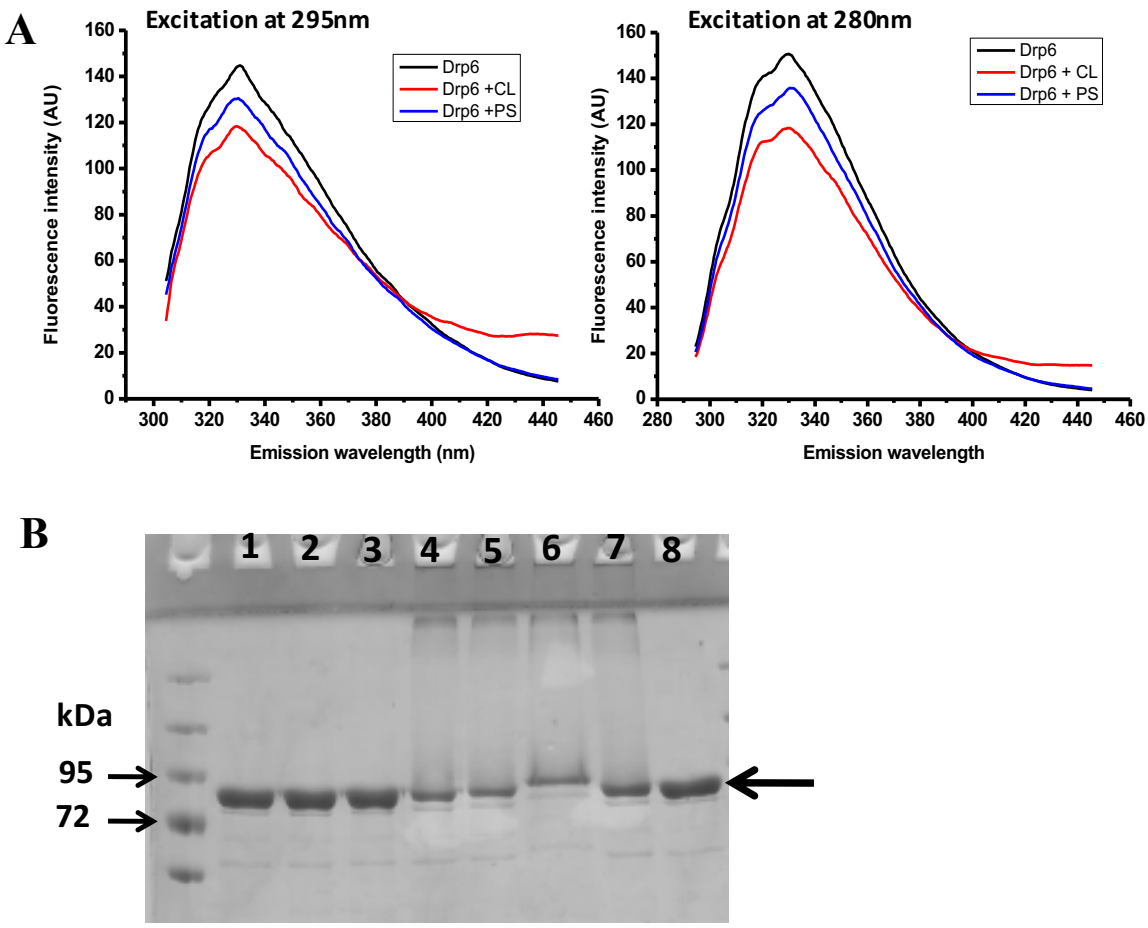

Figure 4.11: Drp6 changes conformation upon membrane association. (A) Emission spectra showing change in intrinsic fluorescence of 1µM Drp6 either in absence or in presence of liposomes containing cardiolipin or phosphatidylserine using excitation wavelength of 295 nm (left) and 280 nm (right). (B) Coomassie stained 10% SDS gel showing Drp6 (lane 1), Drp6 incubated with GTP (lane 2), Drp6 incubated with GTP and liposomes conataining phosphatidylserine (lane 3), Drp6 incubated with liposomes containing cardiolipin and GTP (lane 4). Lane 5 and 6 are same as lane 4 except 2X and 3X SDS loading dye respectively were used. Lane 7 is Drp6 incubated with GDP and liposome containing cardiolipin; lane 8 is Drp6 incubated with GDP. Approximately 7µg of Drp6 was used for all the samples. The presence of high molecular weight species above the monomeric band with concomitant decrease in monomeric species (indicated by arrow) specifically in presence of cardiolipin containing liposomes (lane 4, 5, 6 and 7) suggests formation of Drp6 oligomers relatively more resistant to SDS denaturation.

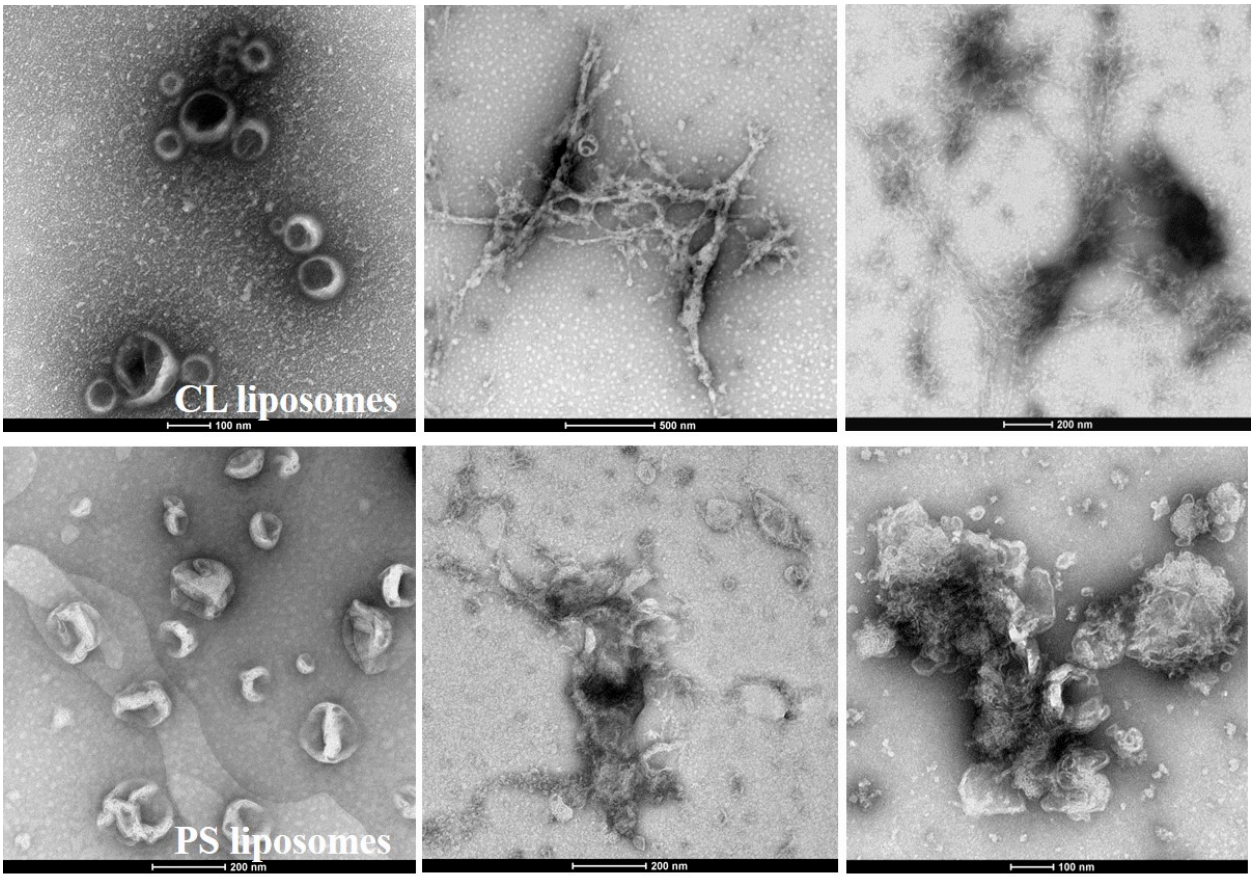

Figure 4.12. Drp6 tubulates membrane *in vitro*. Electron micrographs of cardiolipin containing (top) and phosphatidylserine containing liposomes (bottom) in absence (left) or in presence (middle and right) of Drp6. Branching and tubulation are more in case of cardiolipin.

4.5 Discussion:

In this chapter, an attempt was made to understand the mechanism of nuclear recruitment of Drp6. In case of dynamin-1, an isoleucine (I533) in the PH domain is required for stable insertion of hydrophobic loop VL1 and for generation of membrane curvature. Mutation of this isoleucine impairs vesicle scission during clathrin mediated endocytosis [73,74]. Based on this, a point mutation was incorporated at isoleucine 553 in the membrane binding domain of Drp6. The mutant Drp6 I553M completely lost nuclear localization and remained mostly as cytoplasmic

puncta suggesting that this residue is important for recruitment of Drp6 to nuclear membrane without affecting its recruitment to cytoplasmic puncta.

To understand the mechanism of nuclear recruitment of Drp6, biochemical and biophysical properties of Drp6 I553M were compared with Drp6. The results of these studies revealed that the mutation does not affect GTPase activity significantly. Moreover, Drp6 I553M self-assembles into higher order structure and forms rings and helical spirals like Drp6. Hence, loss of nuclear localization is not due to its defects in its catalytic activities, ultrastructure or self-assembly.

In vitro membrane binding assay demonstrated that Drp6 interacts with both CL and PS containing membranes. However, Drp6 I553M specifically lost interaction with CL containing membranes whereas its interaction with PS containing membranes was not affected. This loss of interaction suggests that CL mediated interaction is required for nuclear recruitment of Drp6. Although both CL and PS are anionic phospholipids, they have a differential distribution in cells. PS is predominantly found in plasma membrane and endosomes, whereas CL is abundant in the inner mitochondrial membrane. CL constitutes almost 3% of the *Tetrahymena* nuclear membrane [225] and depletion of CL by inhibiting its synthesis resulted in rapid dissociation of Drp6 from nuclear envelope. Hence, specific interaction of CL with Isoleucine at 553 appears to be critical for nuclear localization of Drp6. In conjugating cells, depletion of CL led to significant inhibition of MAC development. This inhibition was specific for a stage where Drp6 function is essential. Therefore, it could be envisaged that CL mediated Drp6 interaction is important for MAC development. Consistent with this, expression of membrane binding domain of Drp6 which interacts with CL, inhibited MAC development. Interaction of CL is known to affect the physical behavior of mitochondrial DRPs. In yeast and animals, CL promotes mitochondrial fusion by affecting the enzymatic processing and/or aggregation of fusion DRPs [182,237]. In conjugating

cells, depletion of CL led to significant inhibition of MAC development. This inhibition was specific for a stage where Drp6 function is essential. Therefore, it could be envisaged that CL mediated Drp6 interaction is important for MAC development. Consistent with this, expression of membrane binding domain of Drp6 which interacts with CL, inhibited MAC development. Interaction of CL is known to affect the physical behavior of mitochondrial DRPs. In yeast and animals, CL promotes mitochondrial fusion by affecting the enzymatic processing and/or aggregation of fusion DRPs. [182,235,237]. Interaction of CL containing membrane with DRP6 inhibits its GTPase activity. GTPase activity is required at the last step of membrane remodeling and dynamin disassembly. The slow rate of hydrolysis would favor self-assembly over disassembly and thus promote membrane tubulation, which in turn would promote membrane fusion. During membrane fusion, tethering of apposing membranes requires deformed and stabilized membrane, and hence it requires slow GTPase activity. Unlike fission dynamins, the fusion dynamins like BDLP and Mgm1 do not exhibit enhanced GTPase activity upon membrane association [77]. Therefore, it can be hypothesized that inhibition of Drp6 GTPase activity by CL interaction is required for membrane fusion. Notably, Drp6 could remodel spherical liposomes containing CL into branched tubules *in vitro* providing a direct evidence that it can remodel membrane curvature. Together, these results suggest that Drp6 could bring about membrane fusion by remodeling the target membranes. Drp6 is present on nuclear envelope and ER derived vesicles and these vesicles interact with nuclear envelope as observed in live cell imaging. Based on these results, a model can be proposed to explain the mechanism of nuclear expansion (MAC development) by Drp6 where Drp6 catalyzes the fusion of membrane between ER vesicles and nuclear envelope.

CHAPTER FIVE

Identification of Post-translational Modifications of Drp6 and Their Effects on its Cellular Distribution

5.1 Introduction

Post-translational modifications (PTMs) are essential mechanisms adopted by eukaryotic cells to regulate protein functions and to coordinate their signaling networks dynamically. Defects in modifications have been linked to numerous developmental disorders and human diseases, highlighting the importance of PTMs in maintaining normal cellular physiology. The vast array of protein post-translational modifications such as phosphorylation, ubiquitylation, SUMOylation, S-nitrosylation, acetylation provide the cell a dynamic and rapid way of regulating various essential processes in response to cellular stimuli.

The proteins of dynamin family perform wide range of biological functions across species and are associated with diverse cellular processes, including the release of transport vesicles, fusion and fission of mitochondria, division of chloroplasts and peroxisomes, cell division and resistance to viral infections [3,26]. Despite performing diverse functions, the members of this family exhibit similar underlying mechanisms [3,227]. They undergo cycles of assembly–disassembly on the target membrane, regulated by binding and hydrolysis of GTP, resulting in membrane curvature change [3,20,227,238]. The regulation of this cycle is critical for biological functions of all dynamin family proteins and is often driven by post-translational modifications. The proteins of dynamin family undergo a variety of post-translational modifications such as phosphorylation, nitrosylation, ubiquitylation, SUMOylation, acetylation and regulate their functional properties including membrane recruitment, self-assembly, GTPase activity and interactions with protein partners (Table 5.1). Loss of these regulations manifests several physiological disorders underscoring the importance of these modifications in various cellular processes [239–241]. Among all dynamins, the regulation of mitochondrial fission dynamin Drp1 is possibly understood in great detail. Drp1 undergoes several modifications

Table 1. Post-translational modifications and their effects on dynamin superfamily proteins

Modification	Protein	Residues	Effect on function	Mechanism
Phosphorylation	Dynamin 1	Tyr 597	Promotes clathrin-mediated endocytosis	Increases self-assembly and GTP hydrolysis
	Dynamin 2	Tyr 597	Promotes caveolae-mediated scission	Increases translocation to plasma membrane
		Tyr 231	-	Enhances Dyn2-Cav1 interaction
		Ser 774/778	Inhibit endocytosis	Impair interaction with Amphiphysin and Syndapin, inhibit its membrane recruitment
	Yeast Vps1	Ser 599	Promotes vesicle scission from trans golgi	Enhances interaction of Vps1 with Amphiphysin partner Rvs167
	Drp1	Ser 585(Rat) or Ser616(Human)	Promotes mitochondrial fission during mitosis.	Facilitates interaction with other adaptor proteins
		Ser 637	Inhibits mitochondrial fission	Inhibits interaction of GED and GTP binding domain and reduces GTPase activity
		Ser 600	Promotes mitochondrial fission	Facilitates Drp1 association with adapter Fis1
		Ser 635(Rat) or Ser617(human)	Promotes mitochondrial fission during apoptosis	Dissociates sequestered Drp1 from microtubule and recruits to mitochondria
	Mfn2	Ser 27	Promotes mitochondrial fission during apoptosis	Promotes ubiquitylation of Mfn2 by E3 ligase Huwe1 and targets for proteasomal degradation
		Thr 111/Ser442	Promotes mitochondrial fragmentation during apoptosis	Phosphorylated form acts as a receptor for E3 ligase Parkin
	Mfn1	Thr562	Promotes mitochondrial permeabilization and apoptosis	Promotes recruitment of BAK to mitochondrial membrane
S-nitrosylation	Dynamin1	Cys 86/Cys 607	Stimulates endocytosis	Induces self-assembly and GTPase activity
	Drp1	Cys 644	Promotes mitochondrial fission and causes synaptic damage	Promotes Drp1 dimerization, self-assembly andstimulates GTPase activity
Ubiquitylation	Drp1	-	Induces mitochondrial fragmentation	Promotes Drp1 association with mitochondria
	Fzo1	Lys 398	Inhibits mitochondrial fusion	Targets for proteasomal degradation
		Lys 464	Promotes mitochondrial fusion	Stabilizes Drp1 on the mitochondrial membrane
Sumoylation	Drp1	Lys 594,Lys 597, Lys 606, Lys 608	Promotes Cyt C release from inter mitochondrial membrane space during apoptosis	Stabilises Drp1 association with mitochondria and facilitates Bax oligomerization on mitochondria
Acetylation	OPA1	Lys 926, Lys 931	Promotes mitochondrial fragmentation	Inhibits GTPase activity of OPA1
	Mfn1	Lys 491	Inhibits mitochondrial fusion	Targets Mfn1 for MARCH-V-mediated proteasomal degradation

which include phosphorylation, sumoylation, ubiquitination, and *S*-nitrosylation [189]. These modifications can occur individually or they can also occur in combination and regulate the protein function by either regulating its GTP hydrolysis activity or assembly properties or interaction with its protein partners which in turn regulate mitochondrial fission. Besides Drp1, other members of this family are also known to be regulated by various PTMs [242].

Drp6 in *Tetrahymena* has been shown to be involved in nuclear envelope dynamics [146]. Drp6 showed most discernable activity during cell conjugation, at a stage when the diploid germline micronucleus (MIC) undergoes dramatic expansion to form polyploid (~45n) macronucleus (MAC). Drp6 is specifically upregulated at this stage and in the absence of Drp6 function, there is a profound deficiency in the formation of the polyploid macronucleus [146]. This protein represents the first cytoplasmic factor required for this important transition. Drp6 is also active in vegetative cells and perhaps performs the same function in vegetative cells as it does in

conjugating cells. Apart from being regulated transcriptionally, Drp6 appears to be regulated at multiple levels [146]. Drp6 shows at least three stage specific localization patterns. It is targeted to nuclear envelopes (both MAC and MIC) in growing cells as well as in cytoplasmic puncta. During starvation, a large proportion of Drp6 becomes associated with cytoplasmic puncta. At the early stage of conjugation, Drp6 no longer visibly associate with nuclear envelope. However, at a specific stage during conjugation, Drp6 re-associates with the envelopes of newly formed nuclei but not with the old MAC which is destined for degradation [146]. In addition to differential localization, Drp6 also exhibits differential activity. It is regulated at the level of its turnover rate on membranes. The protein assembly at the nuclear membrane increases dramatically in starved or conjugating cells as compared to vegetative cells [146]. All the dynamin proteins are known to function by performing rounds of assembly and disassembly on their target membrane and various post-translational modifications have been shown to play important role in regulating their localization and function [242]. Therefore, it can be hypothesized that the shift in localization and difference in activity of Drp6 is a consequence of post-translational modifications. This chapter addresses the identification of post-translational modifications of Drp6 and their effect on its localization.

5.2 Materials:

γ^{32} P ATP was obtained from Perkin Elmer

All other chemicals and reagents were from Sigma-Aldrich or as mentioned in previous chapters unless otherwise mentioned.

5.3 Methods:

5.3.1 *In vitro* phosphorylation assay:

The purified Drp6 expressed in bacteria was used for *in vitro* phosphorylation using *Tetrahymena* total cell lysate as kinase source. Cells were harvested from 50 ml culture of

Tetrahymena grown to a density of 2.5 x 10^5 cells/ml and resuspended in MOPs buffer containing 25mM MOPs pH 7.4, 100mM NaCl, 10mM $MgCl_2$ and 10mM DTT supplemented with protease inhibitor cocktail. The lysate was prepared by passing the cell suspension several times through a 12µm clearance sphere in a cell homogenizer (Isobiotech, Germany). Complete cell lysis was confirmed by observing the lysate under microscope. The lysate was clarified by centrifugation at 15,000 RPM for 45 min at 4°C.

The phosphorylation assay was performed in a 30 µl reaction mixture containing 10µl cell lysate, 17µl of Drp6, 0.6µl of NaF (500mM in H_2O), 1 µl γ^{32} P ATP (10 mCi) at 30°C for 30 min. The control reaction mixture contained 17 µl of MOPs buffer in place of Drp6. The samples were boiled in 1X SDS loading dye and separated on a 10% SDS PAGE gel. To purify Drp6 after phosphorylation, the reaction mixture was incubated with Ni-NTA agarose, the resin was washed with 100 bed volume of 50mM imidazole in MOPs buffer and eluted with 250mM imidazole in the same buffer. For the detection of phosphorylation signal, the gel was transferred to a phosphoimager cassette and was recorded using a phosphoroimager.

5.3.2 Mass-spectrometry:

TAP-Drp6 expressed in *Tetrahymena* was purified as mentioned earlier and run on a 10% SDS PAGE gel followed by silver staining. The bands were excised from the gel and were sent for mass-spectrometric analysis (W.M. Keck Biomedical Mass Spectrometry Laboratory, Univerisity of Virginia, USA) to identify post-translational modifications. The samples were subjected to MS and MS/MS in a Thermo Electron Velos Orbitrap ETD mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm x 75 µm id Phenomenex Jupiter 10 um C18 reversed-phase capillary column. The results were analyzed using SEQUEST search algorithm.

5.3.3 Generation of phospho-mutants of DRP6:

Point mutations were incorporated in the *DRP6* gene corresponding to amino acid residues S86, S248, S701 and S702 (serine to alanine) by using site directed mutagenesis kit as mentioned earlier using pENTRD-TOPO DRP6 as template and primers listed in table 5.2. Incorporation of mutations was verified by DNA sequencing and the mutant genes were cloned into pIGF vector as N-terminal GFP fusion constructs by LR clonase reaction. The schematic of cloning is given in figure 4.1.

Table 5.1

Mutation	Primers	Phosphorylation mutation primers
S86A	Forward primer	5' C ATA GAA GTT ACT AAT TAA GAC GCT GAT TTT AAG CCT TAC GCT TAC 3'
	Reverse primer	5' GTA AGC GTA AGG CTT AAA ATC AGC GTC TTA ATT AGT AAC TTC TAT G 3'
S248A	Forward primer	5' G TAT TAC GGT ATG AAA CCT CGT GCC TAA AAA GAC ATC AAC GAA AAT G 3'
	Reverse primer	5'C ATT TTC GTT GAT GTC TTT TTA GGC ACG AGG TTT CAT ACC GTA ATA C 3'
S701AS705A	Forward primer	5'AAT TAT ATT CAC GAG GCT TTA GAA GAC GCT GAT GGT ATT GAT GAT TG 3'
	Reverse primer	5'CA ATC ATC AAT ACC ATC AGC GTC TTC TAA AGC CTC GTG AAT ATA ATT 3'

5.3.4 Expression of *DRP6* variants in *Tetrahymena*:

The GFP-fusion constructs of *DRP6* variants (*GFP-DRP6*, *GFP-DRP6-S86A*, *GFP-DRP6-S248A, GFP-DRP6-S701A/S705A* and *GFP-DRP6-S86A/S248A/S701A/S705A*) were transformed in *Tetrahymena* by electroporation as mentioned earlier. Expression was induced by adding 1μg/ml $CdCl_2$ for 4 to 5 hours. The cells were fixed in 4% PFA and imaging of DAPI stained cells was performed as mentioned earlier.

5.4 Results:

5.4.1 Expression and purification of TAP-Drp6.

The plasmid harboring *TAP-DRP6* gene was transformed into conjugating *Tetrahymena* by electroporation and the positive transformants were selected by using paromomycin sulfate. TAP-Drp6 expression was induced by adding cadmium chloride to the growing *Tetrahymena* culture and was checked by western blot using rabbit IgG as the primary antibody which detects the protein A of the TAP tag. For purification of TAP-Drp6, approximately 3 L *Tetrahymena* cultures harboring *TAP-DRP6 pVGF* were induced with 1µg/ml $CdCl_2$ for 5h at 30^0C and purified using IgG speharose followed by calmodulin agarose column. The purified protein was analysed on a silver stained gel (figure 5.1). The purified TAP-Drp6 appeared as two bands in the silver stained gel and the identity of the protein was confirmed by mass-spectrometry. The upper band might correspond to the post-translationally modified form of Drp6.

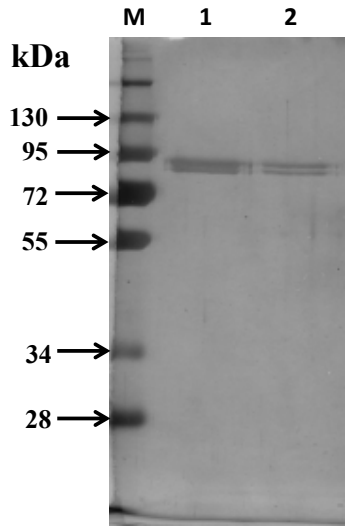

Figure 5.1: Purification of TAP-Drp6. 10% SDS PAGE gel showing purified fractions of TAP-Drp6. *Tetrahymena* cells expressing TAP-Drp6 were lysed and the clarified lysate was purified by IgG sepharose, cleaved with TEV protease and the cleaved product was further purified using calmodulin agarose. M is molecular weight markers; lane 1 and 2 corresponds to eluted fractions 1 and 2 respectively. Presence of two bands suggests that upper band may correspond to Drp6 with post-translational modifications.

5.4.2 *In vitro* phosphorylation of Drp6:

Phosphorylation is the most prevalent post-translational modification associated with the regulation of cellular functions of many proteins. To assess the presence of phosphorylation in Drp6, an *In vitro* phosphorylation assay was performed using bacterially purified Drp6, $\gamma\ ^{32}P$ labelled ATP and *Tetrahymena* cell lysate. The result shows that bacterially purified Drp6 is phosphorylated by *Teytrahymena* cell lysate (figure 5.2A). There are multiple bands which are phosphorylated *in vitro* (Fig 5.2A). To confirm that recombinant Drp6 is also phosphorylated, the reaction mixture was subjected to nickel affinity chromatography. The presence of phosphorylated protein in the purified fraction was detected only when Drp6 was added. This result strongly suggest that is phosphorylated *in vitro*.

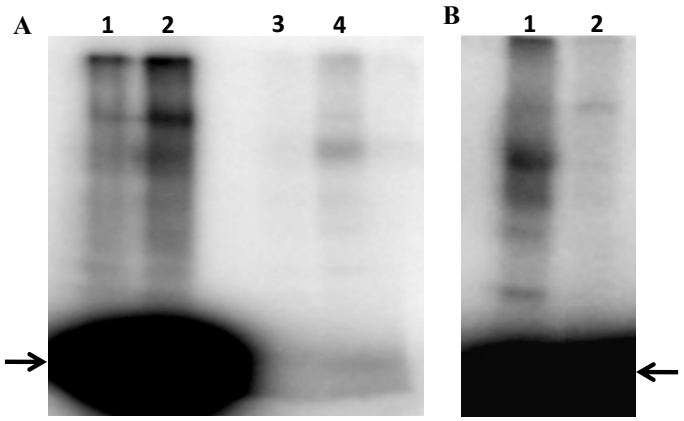

Figure 5.2: *In vitro* phosphorylation of Drp6. (A) Bacterially expressed purified protein was used for *in vitro* phosphorylation using *Tetrahymena* total cell lysate and γ^{32}P ATP. Lane1 is without Drp6; lane 2 is with Drp6; lane 3 and 4 same as lane 1 and 2 except that the reaction mixtures were used for purification of Drp6 using Ni-NTA agarose resin. The phosphorylation signal after purification was detected only in the reaction where Drp6 was present (lane 4). Additional bands in lane 1 and 2 may correspond to *Tetrahymena* proteins present in total cell lysate and undergoing phosphorylation. (B) *In vitro* phosphorylation of Drp6 either in absence (lane 1) or presence (lane 2) of 50 mM staurosporin. Inhibition of phosphorylation in presence of staurosporin suggests inhibition of protein phosphorylation *in vitro.*

In vitro phosphorylation assay was also carried out in presence of staurosporine, a kinase inhibitor that inhibits protein kinases by preventing binding of ATP [243]. The results of the inhibition assay showed that staurosporine treatment significantly inhibited Drp6 phosphorylation as compared to the control (Fig 5.2B), suggesting that Drp6 undergoes phosphorylation by cellular kinases.

5.4.3 Identification of post-translation modifications:

To further confirm presence of phosphorylation and any other modifications *in vivo*, Drp6 was expressed and purified in *Tetrahymena* as a TAP tag fusion protein and was subjected to tandem mass spectrometry and the results were analyzed by using the SEQUEST search algorithm. As expected, purified Drp6 appeared as two bands in the SDS-PAGE gel (fig 5.3A). Both the bands were analyzed for presence of post-translational modifications. Analysis of the mass spectrometry data showed that while both the upper and lower bands contained phosphorylations, the upper band additionally contained ubiquitylation. The mass spectromectric analysis also of identified four phosphorylation sites at S86, S248, S701, S705 and six ubiquitylation sites at K250, K385, K392, K400, K533 and K655 (Fig 5.3B).

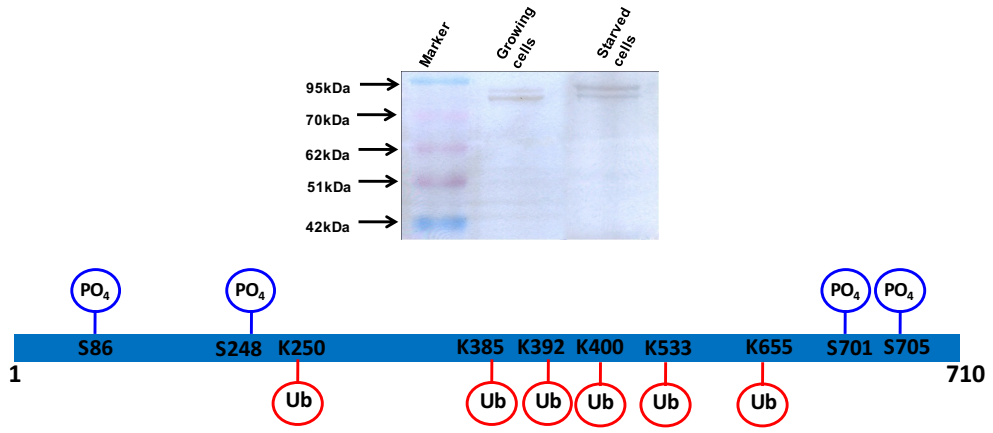

Figure 5.3: Post-translational modifications of Drp6. (A) 10% silver stained gel showing TAP-Drp6 purified either from vegetative cells (lane 1) or from starved cells (lane 2). Presence of two bands suggests that the upper band may correspond to post-translationally modified protein. (B) Both upper and lower bands were analysed by mass spectrometry and four phosphorylation and six ubiquitylation sites were identified. The residues undergoing modifications and their positions are indicated. PO_4 is phosphorylation and Ub is ubiquitylation.

5.4.4 Effect of phosphorylations on Drp6 localization:

To evaluate the role of phosphorylations on Drp6 localization, the four serine residues identified were mutated to alanines and expressed in *Tetrahymena* as GFP tagged fusion protein. For this purpose, site directed mutants of these four serine residues either individually or in combination (S86A, S248A, S701A/S705A, S86A/S248A/S701A/S705A) were generated by using Stratagene site directed mutagenesis kit. The mutants were generated in an entry vector and were confirmed by sequencing. From the entry vector, all the mutant genes were cloned into *Tetrahymena* expression vector pIGF which has an N-terminal GFP tag and were transformed into *Tetrahymena* cells. The wild type Drp6 and phosphomutants were expressed and their localizations were analyzed by confocal microscopy. Images of both live cells and fixed cells were analyzed. As expected, wild type GFP- Drp6 was distributed mainly on nuclear envelope and as few cytoplasmic puncta. Similar to wild type GFP-Drp6, all the phosphomutants were also localized to nuclear envelope and cytoplasmic puncta (Fig 5.4). Although there was no

detectable difference in distribution pattern of most of the mutants and wild type Drp6, the mutant containing all the four mutations (Drp6-S86A/S248A/S701A/S705A) appeared to be distributed more on nuclear envelope with lesser cytoplasmic puncta when compared with wildtype Drp6 (Fig 5.4 panel 4). This result suggests that individual phosphorylation, though not critical for regulation of Drp6, absence of all four phosphorylations appears to regulate Drp6 dynamics between nuclear envelope and ER vesicles.

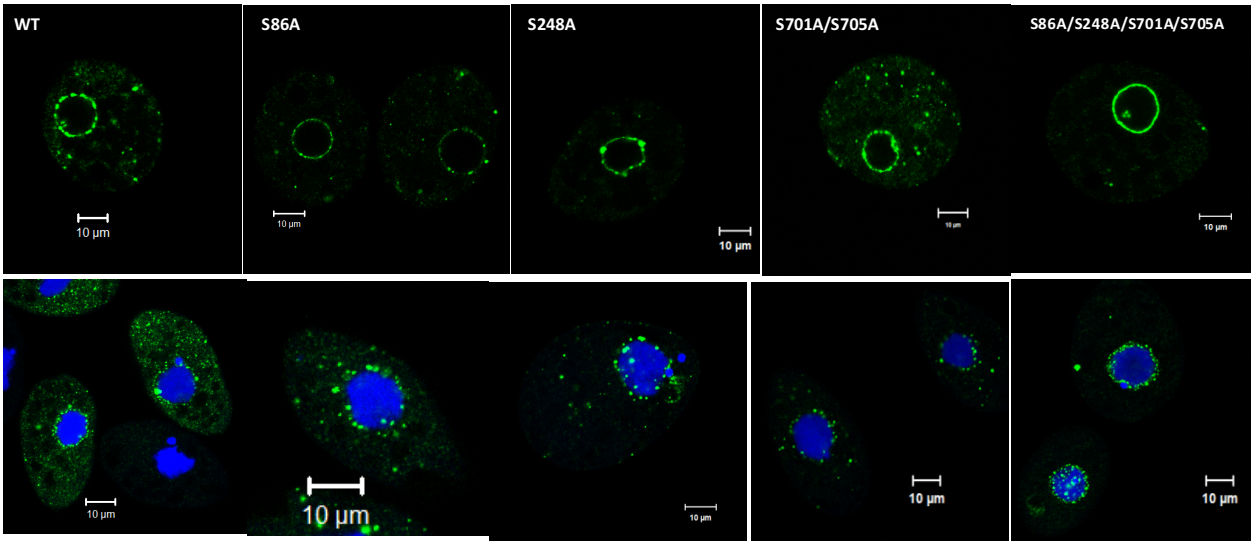

Figure 5.4: Effect of phosphorylation on Drp6 localization. Confocal images of live *Tetrahymena* cells (top panel) or DAPI stained fixed cells (bottom panel) expressing GFP-Drp6 (WT), GFP-Drp6 S86A (S86A), GFP-Drp6 S248A (S248A), GFP-Drp6 S701A/S705A (S701A/S705A) and GFP-Drp6 S86A/S248A/S701A/S705A (S86A/S248A/S701A/S705A). All the expressed mutant proteins, similar to wild type Drp6, associated with nuclear envelope and cytoplasmic puncta. However, Drp6 variant where all the four serine residues are mutated localized more on nuclear envelope than cytoplasmic puncta.

5.5 Discussion:

Drp6, a nuclear dynamin related protein exhibits differential localization and turnover rate in different physiological conditions and developmental stages. The mechanism of this regulation is not known. Post-translational modifications are major regulators of dynamin functions in various cellular processes. In an attempt to understand the mechanism of Drp6 regulation, the post-translational modifications were investigated. Drp6 was found to be phosphorylated and ubiquitylated suggesting a possible role in its regulation. However, loss of phosphorylations either singly or in combination did not affect its localization significantly. It is possible that phosphorylation along with other modifications might regulate Drp6 localization. Interestingly, in addition to phosphorylation, Drp6 also undergoes ubiquitylation which might contribute in regulation of Drp6 dynamics. Further studies are required to understand the role of ubiqutylation and the mechanism of Drp6 regulation.

Summary and Conclusions:

Summary:

Membrane curvature is an important parameter for many essential physiological processes and is regulated by a number of proteins. Dynamin superfamily proteins are multidomain mechano-chemical GTPases which are implicated in nucleotide-dependent membrane remodeling events [3,26]. They are broadly divided into two categories based on their domain organization, classical dynamins and dynamin related proteins (DRPs). DRPs, although retain three core domains such as GTPase domain, middle domain and GED, typically lack the PH domain and PRD which are present in classical dynamins. Classical dynamins are involved in endocytosis whereas DRPs have been shown to be involved in many cellular events such as organelle maintenance, cytokinesis and innate antiviral immunity. The functions of these proteins are widespread and they are ubiquitously distributed in animals, plants and prokaryotes [144]. Although a large number of dynamin family proteins are involved in many different processes, the mechanism of their functions are similar. A hallmark of these proteins is their self-assembly on the target membrane and assembly stimulated GTPase activity.

Tetrahymena thermophila, a unicellular protist harbors eight dynamin related proteins (Drps) indicating presence of a complex endomembrane system. Four of the Drps, Drp 3-6, are exclusive to ciliate lineage suggesting a lineage specific expansion to perform specialized functions [147]. Among these, dynamin related protein 6 (Drp6) is targeted to nuclear envelope and ER derived vesicles, and plays a role in nuclear expansion [146]. *Tetrahymena* harbors two nuclei namely, macronucleus (MAC) which contains the somatic genome, and the micronucleus (MIC) which houses a silent germline copy. Drp6 function is essential for nuclear differentiation where MIC undergoes several fold expansion to form MAC during conjugation, the sexual phase of the life cycle [146]. At the beginning of nuclear differentiation process, the precursors of the new MIC and MAC are identical in size and genome content, but the new MAC enlarges rapidly,

and its volume increases 5–10-fold in order to prepare for genome rearrangement and polyploidization. Thus, macronucleus development clearly involves rapid membrane expansion. Loss of DRP6 slows or blocks nuclear expansion, which suggests that Drp6 is involved in this process [146]. Moreover, Drp6 shows differential localization and activity in different stages of *Tetrahymena* life cycle. The mechanism of Drp6 function and regulation of its localization and activity are not known. The present study addressed the understanding of biochemical, biophysical and functional properties of Drp6. It also addressed the post translational modifications to understand the regulation of its localization and activity.

A unifying feature of dynamin superfamily protein is their ability to assemble into regular oligomers. Dynamin exists as a tetramer and polymerizes into rings and helices when incubated in solutions with low ionic strength or in the presence of lipid templates [149]. Whether Drp6 also polymerizes into self-assembled structures, various biophysical and biochemical properties were assessed. Analytical size exclusion chromatography results showed that Drp6 remains as a higher order oligomer, but in contrast to other dynamins it does not require lipid template or assembly conditions.

Chemical cross-linking experiments confirmed formation of large oligomeric complexes of Drp6. Electron microscopic analysis further revealed the presence of rings and helical spirals. Such structures also formed by other dynamins and are important for their functions. This suggests a similar mechanism of Drp6 function. GTPase activity is essential for functions of all dynamins. Drp6 exhibits a high basal GTPase activity with a K_{cat} of ~ 1.62 $min^{-1}$and K_m of ~ 395μM. Surprisingly, unlike other Drps, there was no significant increase in GTPase activity of Drp6 in low salt condition. Taken together, these results suggest that, in contrast to dynamins and similar to MxA, Drp6 self-assembles in absence of membrane templates and its GTPase activity is not enhanced upon membrane association.

All members of dynamin family associate with membranes. Classical dynamins interact with lipids via PH domain and many other DRPs have either trans-membrane helices or exposed loop for membrane binding. Although Drp6 lacks a recognizable PH domain, it localizes on nuclear envelope. Drp6 was able to interact with lipid directly, specifically with phosphatidic acid (PA), phosphatidylserine (PS) and cardiolipin (CL). It also associates with liposomes indicating presence of a membrane binding domain. Lipid binding assay with different fragments of Drp6 revealed that the fragment containing amino acids 517 to 600 is sufficient for interaction with lipids comparable to full length Drp6 suggesting that this fragment harbors the membrane binding domain. Analysis of a predicted 3-D model structure of Drp6 further revealed that the membrane binding domain is located at an equivalent position as that of PH domain of dynamin and membrane binding loop of MxA. These results suggest that members of dynamin family proteins harbor the membrane binding domain structurally at the same position.

Cycle of assembly-disassembly on the target membrane is the hallmark of all dynamin proteins. The recruitment of dynamin proteins is mediated by their membrane binding domain. The positive charged residues in the membrane binding domain often interact with the negatively charged lipids in the target membrane and this interaction is important for recruitment of dynamins onto their target membrane. To understand the mechanism of nuclear recruitment of Drp6, an isoleucine in the membrane binding domain was mutated to methionine and was expressed in *Tetrahymena* as a GFP fusion protein. The mutant lost the nuclear localization almost completely. Comparison of various biochemical and biophysical properties did not exhibit any significant defect in GTPase activity, self-assembly property and ultrastructure due to the mutation. Analysis of membrane binding using liposomes containing either CL or PS demonstrated that mutation impaired the interaction with CL specifically, without affecting its interaction with PS. These results suggested that CL mediated membrane interaction is important

for nuclear localization of Drp6. To evaluate if the dissociation of Drp6 I553M from the nuclear envelope is due to loss of its interaction with the CL in nuclear envelope, CL was depleted in the *Tetrahymena* cells expressing GFP-Drp6. As expected, depletion of CL resulted in rapid dissociation of GFP-Drp6 from the nuclear envelope. To rule out the possibility that this localization shift is not due to any major change in overall membrane structure, specifically nuclear envelope, the similar experiments were carried out using a Nem1D (cortical membrane protein) and Nup3(nuclear pore protein). Depletion of CL did not affect their localization on the target membrane confirming that dissociation of Drp6 was specifically due to loss of CL. Taken together, these results led to the conclusion that CL acts as a molecular switch for Drp6 localization and isoleucine at 553 in the membrane binding domain is important for its nuclear recruitment. Drp6 is required for MAC development in conjugating cells (Rahaman et al., 2008). Nuclear membrane of *Tetrahyemena* contains 3% CL [225] and CL depletion in these cells inhibited MAC development without affecting earlier stages of nuclear differentiation. Therefore, it can be proposed that Drp6 recruitment to the nuclear envelope by CL interaction is important for MAC expansion. This is further supported by the inhibition of MAC development by overexpression of the membrane binding domain of Drp6. Drp6 membrane binding domain which interacts with CL might competitively inhibit Drp6 interaction with CL and thereby its recruitment to nuclear envelope. CL is a fusogenic lipid and is enriched at sites where changes in membrane curvature are drastic. CL appears to be essential for fusion of mitochondrial inner membrane. Similar to mitochondria, Drp6-CL interaction might have a role in membrane fusion during MAC expansion.

All the fusion dynamins perform membrane fusion by tethering of opposing membranes by homotypic interactions [38,42,96,112,244]. Models for mitochondrial fusion propose that dynamins bring about fusion either by tethering mitochondrial membrane or by constricting the

membrane into protruding tubules which subsequently fuse upon GTP hydrolysis. The former model proposes that tethering of mitochondrial membrane occurs by dimerization between dynamins on the opposing membranes where as the later model requires tethering of protruding membrane tubules by interaction between opposing dynamin spirals [191]. In addition to nuclear envelope, Drp6 localizes on ER derived vesicles and these vesicles are observed to merge with the nuclear envelope in live cells (Rahaman A, unpublished). Based on this, it can be proposed that Drp6 mediates fusion of incoming ER vesicles with nuclear membrane in order to supply additional membranes on nuclear envelope required for macronuclear expansion. Drp6 might tether the membranes of ER vesicles with the nuclear membrane and brings about the fusion of both these membranes. Alternatively, it might assemble on both nuclear and ER membrane and tubulate the underlying membranes. These tubular membranes subsequently contact each other and fuse by further interaction of assembled Drp6 on closely positioned ER membrane and nuclear membrane. Based on the results of the present study, the later model appears more plausible as Drp6 assembles into rings and helical spirals and tubulates liposomes *in vitro*. Inhibition of GTPase activity by CL might stabilize the tethering of ER membrane with nuclear membrane and additional factor(s) might trigger the stimulation of GTPase activity resulting in membrane fusion.

The localization and activity of Drp6 is regulated at different stages of *Tetrahymena* life cycle. Most of the cellular processes are regulated by post-translational modifications. Dynamin family proteins also regulate their function by various modifications such as phosphorylation, ubiquitylation, S-nitrosylation and SUMOylation. To see if differential localization and activity of Drp6 are also regulated by post-translational modifications, presence of post-translational modifications were assessed. Drp6 when expressed in *Tetrahymena* appeared as two independent bands in SDS PAGE suggesting one of them is modified. To examine presence of

phosphorylation, purified Drp6 expressed in bacteria was used for an *in vitro* phosphorylation and was found to be phosphorylated. Mass spectrometric analysis of Drp6 expressed in *Tetrahymena* revealed presence of phosphorylations at four serine residues (S86, S248, S701 and S705) and ubiquitylations at six lysine residues (K250, K385, K392, K400, K533 and K655) sites suggesting that it undergoes phosphorylation and ubiquitylation *in vivo*. To see the effect of these phosphorylations on the localization, the four serine residues were mutated to alanine and the mutants were evaluated for their localization. Although, none of the mutation either singly or in combination affected distribution pattern of Drp6 on nuclear envelope and ER vesicles significantly. This lack of regulation by phosphorylation may be due to requirement of additional modifications such as ubiquitylation for regulating its localization.

Conclusions:

Although dynamin family proteins are extensively studied for their roles in endocytosis, organelle maintenance and viral resistance, their role in nuclear remodeling remained unexplored. The present study addressed to understand the mechanism of a nuclear dynamin related protein, Drp6 and its regulation in *Tetrahymena* thermophila, a unicellular eukaryote. Similar to other dynamin family members, Drp6 self-assembles into rings and spirals suggesting a similar mechanism of function in membrane remodeling. Consistent with this, Drp6 could also remodel membrane *in vitro*. However, the assembly requirements of Drp6 is similar to MxA but different from other dynamins, as it does not require membrane template or low salt condition. Drp6 harbors a membrane binding domain structurally located at the equivalent positions of membrane binding domains of other dynamins. It is recruited to nuclear membrane via interaction with cardiolipin and this interaction is important for macronuclear development. Based on the results from this study, a model explaining the mechanism of Drp6 function can be

proposed where ER vesicles serve as the membrane donors and Drp6 mediates fusion of these vesicles with nuclear membrane to facilitate rapid nuclear expansion during macronuclear development. This study provides a basis to understand the novel function of a dynamin family protein to perform specialized function on nuclear envelope. Understanding the regulation of Drp6 function would not only help in unravel the less explored mechanisms of nuclear expansion but would also enrich information on the basic properties of dynamin family proteins in general.

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