Understanding the role of B-insert in Dynamin related protein 1(Drp1)-cardiolipin interactions

A Thesis

submitted to

Indian Institute of Science Education and Research Pune

in partial fulfillment of the requirements for the

BS – MS Dual Degree Programme

by

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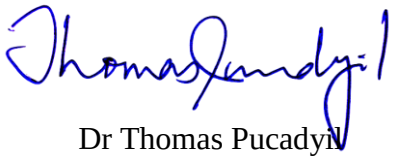
Certificate

This is to certify that this dissertation entitled **'Understanding the role of B-insert in Dynamin related protein 1(Drp1)-cardiolipin interactions'** towards the partial fulfillment of the BS-MS dual degree programme at the **Indian Institute of Science Education and Research, Pune** represents study/work carried out by **V P Gokul** at Indian Institute of Science Education and Research under the supervision of **Dr Thomas Pucadyil,** Associate Professor, Department of Biology , during the academic year 2017-2018.



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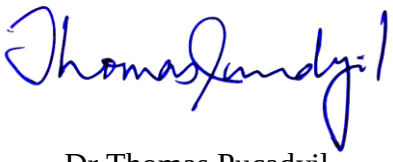
Declaration

I hereby declare that the matter embodied in the report entitled '**Understanding the role of B-insert in Dynamin related protein 1(Drp1)-cardiolipin interactions**' are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune under the supervision of **Dr Thomas Pucadyil,** and the same has not been submitted elsewhere for any other degree.

V P Gokul



Dr Thomas Pucadyil

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To,

Mom and Dad.

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Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr Thomas Pucadyil for his guidance, support and criticism during the course of the project. I thank all the professors and scientists with whom I had an enthralling time working with, for the training and experiences provided in my five years in IISER.

I would like to express my gratitude especially to Sukrut, and all my lab members for the entertaining conversations.

My five year in IISER was insane which I owe to my friends for making this trip, captivating and inspiring.

Abstract

Mitochondria exhibits dynamic morphology which is maintained by constant cycle's dynamic fission and fusion. Dynamin related protein 1 (Drp1), a large multidomain GTPase, is a crucial player involved in mitochondrial fission. Cardiolipin is the signature mitochondrial lipid and is implicated in regulating various mitochondrial function. Cardiolipin-Drp1 interactions are necessary for promoting its self-assembly and influences stimulated GTPase activity of Drp1. B-insert region in Drp1 is speculated to be the cardiolipin binding domain. Drp1 B-insert deletion mutant fails to localise to mitochondria, indicating the importance of B-insert for membrane binding in Drp1.

In this study, we investigate B-insert lipid binding specificity to understand role of B-insert in Drp1 recruitment to the membranes. We show that B-insert displays preferential specificity in binding to cardiolipin and propose that B-insert is required for Drp1-cardiolipin interactions. B-insert shows better specificity to cardiolipin than Nonyl-Acridine Orange(NAO) described in literature to be a cardiolipin specific probe. We also considered the possibility of using B-insert as a probe for cardiolipin and expressed it in-vivo to observe membrane localisation.

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Introduction

Mitochondria are complex double membraned cellular organelles present in eukaryotic cells. Mitochondria are present in varying morphologies from hyperfused interconnected networks to short fragmented form, which are maintained by fission and fusion cycles(Yaffe, 1999a). As mitochondria are not generated de novo, proliferation and replication during cell division is by growth and division of pre-existing mitochondria(Westermann, 2010; Yaffe, 1999b). Mitochondrial fission is essential for fragmentation and allows effective sorting of mitochondria to daughter cells (Taguchi et al., 2007). Mitochondrial fission, also enables cells to transport appropriately sized mitochondria within the cell, excises dysfunctional mitochondria and escorts it towards mitophagy (Westermann, 2010)(Ramachandran, 2017).

Mitochondrial fission is mediated by large GTPases in dynamin superfamily. Dynamin related GTPase Drp1 (Dynamin-related protein) in mammals is involved in catalysing fission of mitochondria and peroxisomes (Smirnova et al., 1998) (Smirnova et al., 2001) (Li and Gould, 2003).

Drp1 is essential for embryonic and brain development in mice and loss of Drp1 impairs mitochondrial fission and causes embryonic lethality in mice (Wakabayashi et al., 2009). Drp1 knockout cells have elongated networks of mitochondria due to loss of mitochondrial fission activity (Wakabayashi et al., 2009). **(Fig.1 a).**

Drp1 is majorly localised to the cytoplasm and exists as dimers/tetramers in cytosol(Smirnova et al., 2001)(Zhu et al., 2004)(Ugarte-Uribe et al., 2014). On getting recruited to the outer membrane of mitochondria, Drp1 forms higher order oligomers (Bui and Shaw, 2013).(Michalska et al., 2016). Further assembly on membranes leads to Drp1 forming rings on membrane tubes and catalyses fission by GTP hydrolysis (Smirnova et al., 2001) (Basu et al., 2017). The mechanism of how Drp1 interacts and binds to membranes is still unclear.

Domain architecture of Drp1:

Drp1 is a large GTPase of 736 amino acid residues (~80kDa) (Kamimoto et al., 1998). Like most members of the dynamin superfamily, Drp1 has an N terminal GTPase domain, Middle region or Stalk, Variable domain or B-insert and GTPase effector domain (GED) (Bustillo-Zabalbeitia et al., 2014;(Fröhlich et al., 2013; Smirnova et al., 2001)) **(Fig. 1 b)**. Dynamin has a well-defined pleckstrin homology (PH) domain which binds to PIP_2 and acts as the membrane binding domain of dynamin (Chappie and Dyda, 2013)(Timm et al., 1994).

In Drp1, the PH domain is absent and is replaced by a ~100 amino acid (~10kDa) region known as the variable domain or B-insert **(Fig. 1 b).** The structure and the function of B-insert is not completely understood.

The 3D reconstructed cryo-electron microscopy image of Dnm1 (yeast Drp1), indicates a 3-4nm gap between the protein and the membrane tubes, and does not reveal a membrane interacting domain for Dnm1 (Mears et al., 2011) **(Fig.1 c).** In case of Dynamin1, the PH domain is present at the gap and interacts with the membrane (Fig1 c). B-insert has been speculated, to be the probable membrane binding domain of Drp1 based on the similarity of domain architecture to the PH domain of Dynamin **(Fig. 1 b).** The absence of a membrane interacting domain in the 3D reconstruction EM image of Drp1, has also been articulated because of the B-insert being predicted to be unstructured and disordered, and therefore is undetectable in the averaged 3D structure (Fröhlich et al., 2013)(Mears et al., 2011).

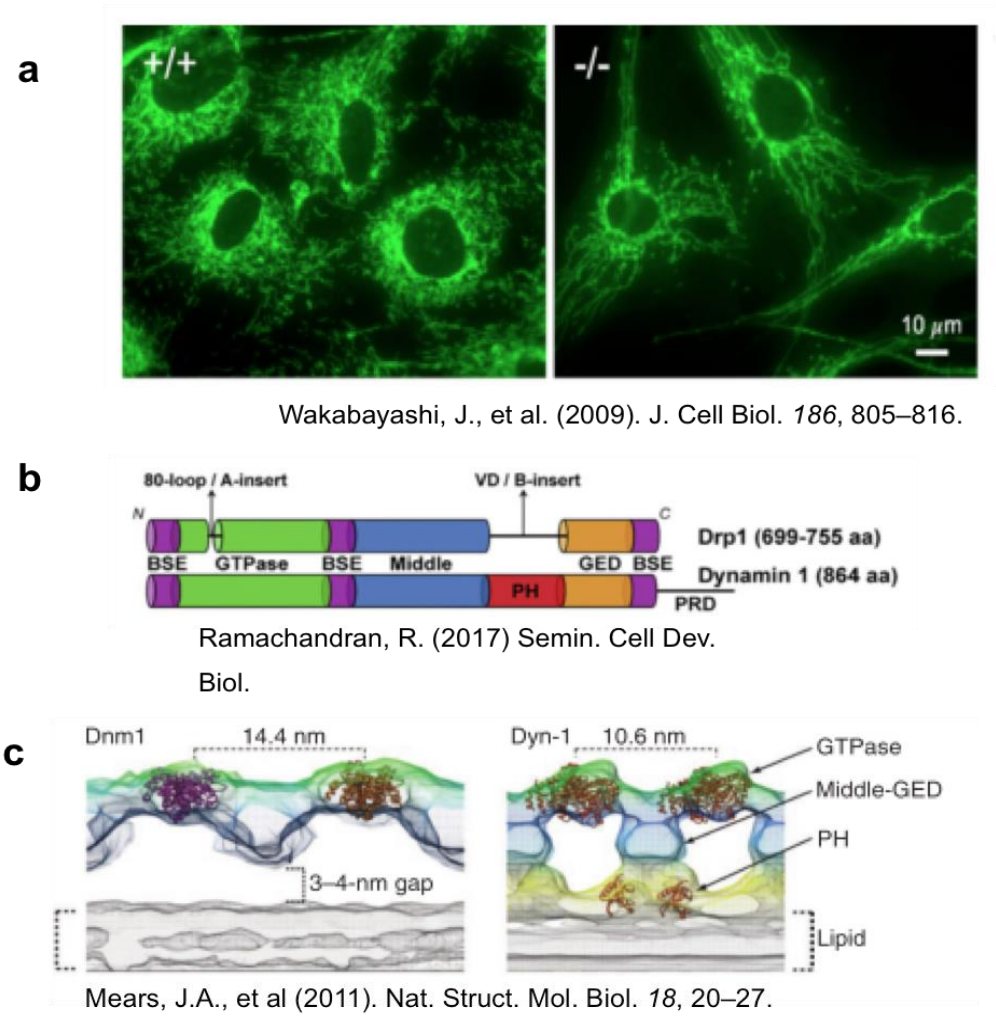


Figure 1 : Drp1 is a crucial mitochondrial fission protein a) Drp1 knockout mouse embryonic fibroblasts (MEFs) exhibit elongated mitochondrial morphology. Cells immunostained with anti-TOM20 as mitochondrial marker. b) Drp1 and Dynamin domain architecture c) 3D reconstructed images of Dnm1 and Dyn1.

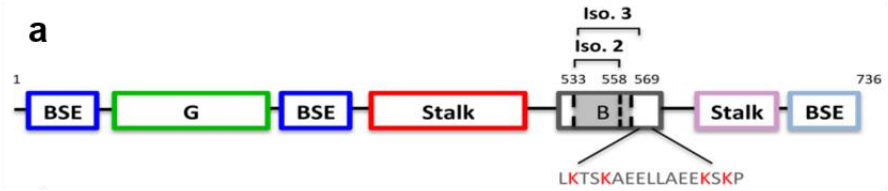
Expressing in DNM1L(Drp1) depleted COS-7 cells, DNM1L B-insert deletion mutant (DNML1Δ B-insert) failed to localise to mitochondria (Fröhlich et al., 2013).

Drp1ΔB-insert is not able to rescue mitochondrial fission in Drp1KD cells(Fröhlich et al., 2013). The effect of Drp1 knockout in cells **(Fig. 1 a)** is phenocopied on deletion of B-insert in Drp1 **(Fig. 2 a)**, with the cells displaying elongated networks of mitochondria (Fröhlich et al., 2013; Wakabayashi et al., 2009)

These observations suggest at B-insert being essential for Drp1 recruitment to mitochondrial membrane.

Drp B-insert:

Drp1 is known to have at least 8 isoforms due to alternate splicing of Drp1 gene(DNM1L), of which 5 of the isoforms are splice variants in the B-insert region. Isoform 1,2, and 3 of Drp1 is expressed in primarily in the brain and neurons, in testis (deletions from 533-558), and in skeletal muscles (deletions from 533-569) (Fig. 2 b). The differential expression of Drp1 isoforms in tissues suggests that B-insert is involved in regulation of Drp1 function. B-insert is highly variable across species but contains a cassette of four lysines which is conserved across metazoans, present in all Drp1 isoforms **(Fig. 2 c).** (Fig.2 c). Mutating the four conserved lysine residues to alanine (Fig. 2 b) (Drp1 4KA) in the B-insert lead to loss in binding of Drp1 to liposomes containing cardiolipin shown by Surface Plasmon Resonance; indicating that Drp1 interaction with cardiolipin is influenced by B-insert (Fig 2 d) (Bustillo-Zabalbeitia et al., 2014). DNM1LΔB fails to localise to mitochondria, probably indicating that B-insert is essential for cardiolipin binding and is necessary for Drp1 recruitment to mitochondria in vivo (Fröhlich et al., 2013)(Bustillo-Zabalbeitia et al., 2014).



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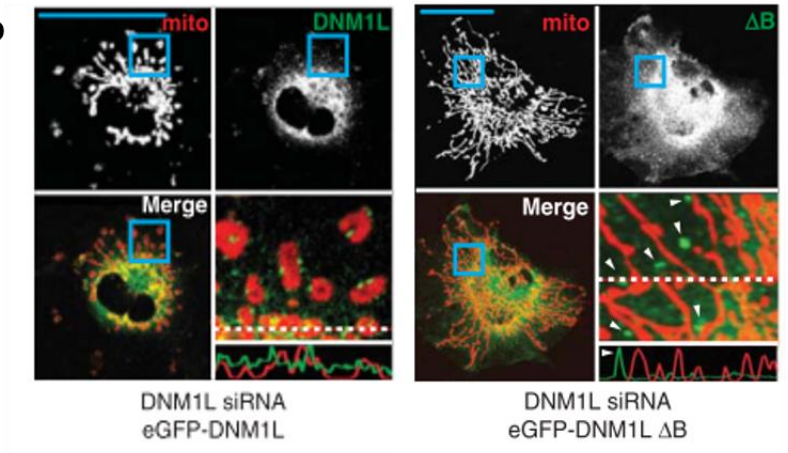
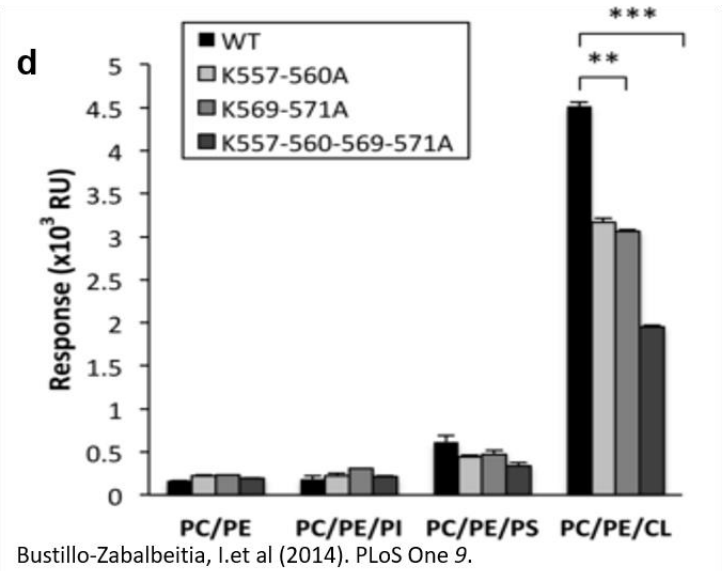


Fröhlich, C. *et al.* (2013). *EMBO J.* **32,** 1280–1292

c

H.sapiens (O00429-4)	GNWRGML**K**TS**K**AEELLAEE**K**S**K**PIPIMPASPQKGHAVNL
M.musculus (Q8K1M6-3)	GNWRGML**K**TS**K**AEELLAEE**K**S**K**PIPIMPASPQKGHAVNL
X.laevis (Q7ZWZ9)	GNWRGMM**K**-A**K**GEEASVEE**K**N**K**APPAFPASPLRGHAVNL
D.rerio (Q7SXN5)	GTWRGML**K**--**K**GDEGQGEE**K**T**K**LQSSIPASPQKGHAVNL

Bustillo-Zabalbeitia, I.et al (2014). PLoS One *9*.



Bustillo-Zabalbeitia, I.et al (2014). PLoS One *9*.

Figure 2 : Deletion of the B-insert in Drp1 affects recruitment to mitochondrial membrane. a)Drp1 domain architecture describing the isoforms. Isoform 1 , Isoform 2 (residue 533-558 deletion) and Isoform 3 (residue 533-569 deletion). b)DNM1L-depleted COS-7 cells expressing siRNA resistant eGFP-DNM1L , and eGFP-DNM1L ΔB-insert.

B-insert is required for Drp1 recruitment to cardiolipin containing membranes. c) Conserved lysine cassete (present in all Drp1 isoforms(a)). d).Drp1 4KA mutant shows decreased binding to cardiolipin

Cardiolipin:

Cardiolipin is the signature mitochondrial phospholipid important for a wide range of mitochondrial activities **(Fig. 3 b)**. Cardiolipin is present in abundance in the inner mitochondrial membrane(IMM) (~20% of lipid content) and present in low amounts (~3-10% of lipid content) in the outer mitochondrial membrane (Daum, 1985)(Horvath and Daum, 2013).

Cardiolipin is a lipid dimer formed by two phosphatidic acid groups being bridged by glycerol backbone (Claypool and Koehler, 2012) **(Fig.3 a)**. The two phosphates and the four alkyl chains of cardiolipin allows cardiolipin to interact with mitochondrial proteins by electrostatic and hydrophobic interactions (Joshi et al., 2009). Cardiolipin is shown to be essential for maintaining an electrochemical gradient in membrane during ATP synthesis. Interactions of cardiolipin with cytochrome c regulates the cytoplasmic pool of the protein and is necessary in regulating apoptosis (Joshi et al., 2009). Externalisation or movement of cardiolipin from inner to outer membrane has been linked to caspase and Bax activation in regulating cellular apoptosis (Francy et al., 2017). Alterations in cardiolipin function has been associated with numerous pathological disorder. Cardiolipin is also enriched at IMM-OMM contact sites (~25% of lipids), where it has been proposed to be where Drp1 gets recruited and promotes fission (Francy et al., 2017).

Multiple cardiolipin binding patches have been identified on proteins based on interaction via phosphate head group, hydroxyl group and the alkyl chain. These binding patches were characterised based on reported cardiolipin binding proteins, co-crystal structure with cardiolipin, etc (Planas-Iglesias et al., 2015). However, Drp1 was not considered in the study due to the lack of a cardiolipin binding PDB crystal structure as B-insert fails to crystallise. Results from our dot blot assay and previous studies indicating Drp1 interaction with cardiolipin containing membranes, elicits a need to identify the binding domain for cardiolipin and understand how Drp1 gets recruited to cardiolipin containing membranes.

Cardiolipin binding stimulates self-assembly of Drp1 on membnes. Drp1 shows stimulated GTPase activity on binding to membranes containing cardiolipin, inducing cardiolipin reorganisation and causes formation of local membrane constrictions (Bustillo-Zabalbeitia et al., 2014; Macdonald et al., 2014). Mitochondria forms

hyperfused mitochondrial networks in the perinuclear region on expressing a cardiolipin binding defective Drp1 4KA mutant, indicating that cardiolipin binding in Drp1 is required for fission activity (Macdonald et al., 2014; Stepanyants et al., 2015) **(Fig. 3 c)**.

a

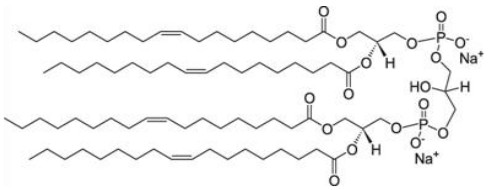
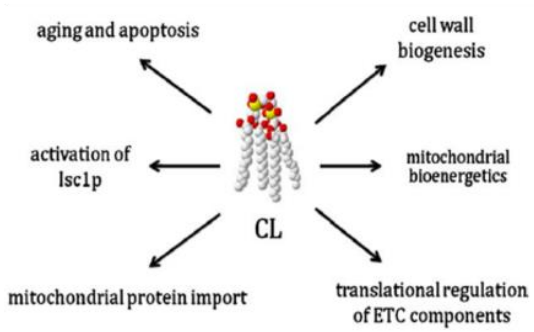


Figure obtained from: Avanti Polar Lipids

b



Joshi, A.S et al (2009). Biochim. Biophys. Acta - Mol. Cell Res. *1793*, 212–218.

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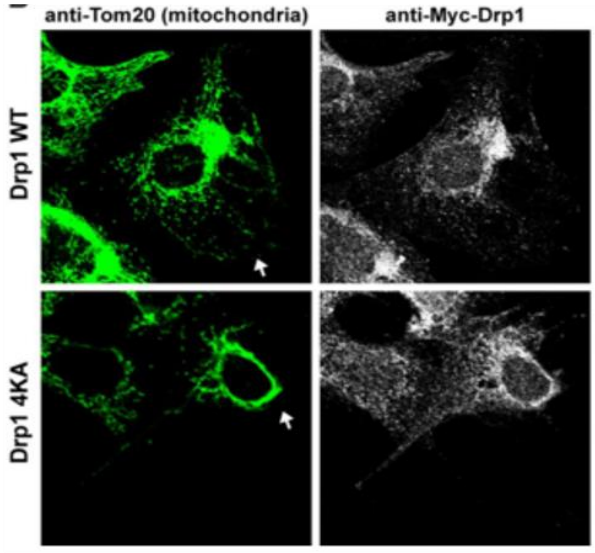

Stepanyants, N et al(2015). Mol. Biol. Cell *26*, 3104–3116.

Figure 3 : B-insert-cardiolipin interaction is necessary for Drp1 fission activity.
a) Cardiolpin (image obtained from Avanti Polar lipids b) Cardiolipin is essential for a wide range of mitochondrial activites c) Defect in cardiolipin binding in Drp1 4KA mutant affects the Drp1 fission activity. MEFs labelled with anti-Tom20 stains mitochondria, Myc tagged Drp1 constructs immunostained with anti-Myc-Drp1.

Interactions of B-insert with membrane has been looked by using Drp1 B-insert mutant constructs. Studies on B-insert and its membrane and cardiolipin binding have not been explored.

Thus, we aim to investigate and understand how the B-insert interacts and is involved in regulating Drp1-cardiolipin interactions and Drp1 recruitment to cardiolipin containing membranes.

Synopsis

In the thesis, we investigate the influence of B-insert of Drp1 in cardiolipin binding.

We start with looking at the lipid binding specificity of Drp1 which suggest that Drp1 binds preferentially to cardiolipin and PG than other anionic lipids. This is an accordance to Drp1 being able to localise to mitochondria and interact with cardiolipin.

Deletions in ~100 amino acid region with an undefined structure known as B-insert, regulates Drp1 fission activity and affects mitochondrial localisation in cells. We look at B-insert lipid specificity by dot blots which shows higher preferential binding to cardiolipin. We speculate that that the domain could confer the cardiolipin binding specificity to Drp1. So we went ahead with Drp1ΔB dot blots which indicated lower binding to cardiolipin when compared to Drp1. We suggest that the deletion of B-insert affected binding specificity and hence, B-insert can be the membrane binding domain of Drp1.

We went ahead to understand to understand B-insert structure by using heat stability as indication for structural properties. B-insert is shown to be heat stable and does not precipitate suggesting no effects on solubilisation of protein on heat denaturation. This suggests B-insert to be an unstructured disordered domain as proposed.

We expressed Drp1, B-insert in cells to understand membrane binding and localisation of Drp1 and B-insert. We seek to explore the possibility of using B-insert to detect cardiolipin *in vivo*.

Materials and Methods

- **Protein expression and purification**

Protein constructs used in the experiments

Isoform 3 of Drp1 is shown to be membrane active and causes fission of membrane tubes containing cardiolipin in presence of GTP (Kamerkar et al, manuscript in preparation). Hence, all constructs used for the experiments in the thesis are of Drp1 isoform3.

Drp1-GFP 6xHis-TEV cleavage site-Drp1 isoform3-mEGFP-StrepII

GFP-B-insert: 6xHis-Thrombin-mEGFP-TEV cleavage site-Drp1 Isoform3 B-insert-StrepII

Drp1ΔB-GFP: 6xHis -TEV cleavage site-Drp1 Isoform3 ΔB-mEGFP-StrepII

GFP: 6xHis-mEGFP

For *in vivo* experiments **GFP-B-insert** and **GFP** was cloned in mammalian expression vector pcDNA3

Drp1 was obtained from Sigma. Drp1ΔB was obtained as a gift from Prof. Jean-Claude Martinou, Department of Cell Biology - Faculty of Science, University of Geneva.

Expression of 6x His-StrepII tagged proteins:

BL21(DE3) *E. coli* cells were transformed with plasmid containing protein of interest on LB plates and selected against ampicillin resistance and were grown overnight at 37ºC. Colonies were picked and inoculated in a 10 mL LB broth containing ampicillin. Primary culture was grown at 37ºC on shaker till OD reaches 0.7. Primary culture of 10 mL was inoculated in 1L of auto induction media with ampicillin. Culture was grown at 18ºC shaking at 210 rpm for 40 hours using lactose as growth inducer. Cells were harvested by pelleting down in culture bottles at 6000g and resuspended in phosphate buffer saline (PBS) by mild vortexing. The vortexed mix was spun down

again at 6000g, supernatant was discarded and pellets were stored in 50 mL falcons at -40ºC.

Tandem Affinity purification method (for Drp1-GFP, GFP-B-insert and Drp1ΔB-GFP):

Pellets were thawed on an ice-water mix. ~20 mL lysis buffer (20 mM HEPES.NaOH and 500 mM NaCl) with a protease inhibitor cocktail tablet (cOmplete Ultra tablet EDTA free, Roche) was added and the pellet was resuspended with mild vortexing. The pellets were sonicated for 10 minutes on ice-water mix with pulse: pause of 1s and 3s at 60%amplitude. Lysate was spun down at 18000 rpm in Oak Ridge tubes and the supernatant was collected. Supernatant was incubated with His-Pur cobalt resin beads already equilibrated in lysis buffer in a PD10 column for 1-hour rocking in a 50mL falcon at 4ºC. After pouring supernatant back to PD10 column, beads were washed with ~100mL of Hepes buffer saline (HBS) (**wash buffer 1**) (20 mM HEPES.NaOH and 150 mM NaCl). The beads were titurated repeatedly during washes to ensure to remove non-specific protein being bound. Protein bound to beads were eluted using 30 mL solution of HBS containing 250mM Imidazole. The column with beads was vortexed or titurated using pipette while eluting protein out.1mM DTT was added to the His-elution prior to loading to the StrepTrap column (GE Healthcare Life Sciences).

A 5mL StrepTrap column was connected to the ÄKTA Prime FPLC (GE Healthcare); washed with MilliQ ,0.2N NaOH and MilliQ (at 10mL/min) and equilibrated with HBS with 1mM DTT (**wash buffer 2**) (20 mM HEPES.NaOH , 150 mM NaCl and 1mM DTT). The His-elution was connected to the FPLC and protein was loaded to the StrepTrap column at the flow rate of 3-5 mL/min. The column was washed with 60mL of wash buffer 2 and flow through was collected for SDS PAGE. A high salt buffer wash of 30mL of 20 mM HEPES.NaOH and 300 mM NaCl was given to the column to further remove bound impurities. StrepTrap column was equilibrated back to wash buffer2. Protein was eluted using HBS with 1mM DTT and 2.5mM Desthiobiotin. 40 µL of elutions, His and Strep flowthrough and 5µL of supernatant, pellet were collected as samples for SDS PAGE (Holkar et al., 2015).

- **Dot blot Assay**

Pure lipid stocks (1mM) were prepared in chloroform of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and *E. coli* cardiolipin (CL). All lipid stocks used were obtained from Avanti Polar lipids.

Strips of Merck Millipore PVDF membrane were cut to dimensions of 7cm*1cm and spots were marked with pencils at 1cm separation at the base of the PVDF strip. Hamilton syringes were cleaned thoroughly with chloroform before spotting lipids. Using a clean 10µL Hamilton syringe, we spotted 1µL (1 nanomole) of lipid on PVDF membrane at equal interval of ~ 1cm. Blots were air dried. Blots were immersed for blocking in HKS (20 mM HEPES.KOH, 150 mM KCl) with 3%BSA for 1-hour shaking at RT. Incubate the blot with 1µM of bait protein in 3%BSA in HKS at RT for 3 hours. The blot was washed thrice with 10mL HKS buffer for 5 minutes each. The blot was imaged using G-Box in the specific excitation channel to detect protein fluorescence used as proxy for binding.

mEGFP tagged Drp1, B-insert, Drp1ΔB, and GFP were used for our dot blot assays, and hence we image the blots in eGFP channel (excitation wavelength 485nm, emission wavelength 530nm). All our dot blot assays are validated using (1µM) mEGFP as control.

- **Analysis softwares**

We used **ImageJ** for processing of dot blot images and microscopy images of in-vivo experiments.

GraphPad Prism was used for analysis of dot blot images and in plotting graphs.

- **Heat Denaturation Assay**

Heat denaturation assay was performed on B-insert to decipher the heat stability of B-insert.

10μM GFP-B-insert was digested at the TEV cleavage site (ENLYFQS) between GFP and B-insert by using 5μM of Tobacco Etch Virus (TEV) Protease. Complete digestion by TEV protease cleaves GFP-B-insert into GFP and B-insert. The protein mix (of ~200μL) is then subjected to a heat denaturation step for 10 minutes at 90ºC, followed by rapid flash cooling at 4ºC for 10 minutes in the Thermocycler PCR machine. The denatured mix is spun down at 20,000g for 20 minutes at RT to pellet down protein aggregated on denaturation. The supernatant is collected carefully to Eppendorf 1.5mL tubes and the pellet is resuspended in MilliQ. Samples were resuspended in 1X SDS dye and loaded to SDS PAGE gel. Experimental controls were B-insert and TEV digested B-insert which was not subjected to heat denaturation. Samples are run in a 15% SDS Poly acrylamide gel as follows **(Fig.3 a)** B-insert, heat denatured B-insert (supernatant and pellet), TEV digested B-insert, TEV digestion of B-insert followed by heat denaturation (supernatant and pellet), 5μM GFP and 5 μM TEV protease.

- **In-vivo PEI based transfection experiments in HeLa cells**

HeLa cells were maintained in DMEM medium with phenol red at 37ºC. Cells were passaged and split on the media achieving more than 75% confluency in terms of cell density.

Cells in 100mm dish cultures were washed with Invitrogen DPBS (Dulbecco's Phosphate Buffer Saline) twice with 2mL each. The washed media was sucked out using a vacuum pump, taking caution to have minimum contact with surface of the 100mm culture dishes where cells are adhered to. Cells were then treated with Invitrogen 0.5M Trypsin + EDTA carefully spread to surface evenly. The plate was then incubated at 37ºC for 1 minute to allow for trypsinization and allow cells to detach from the surface. Open sterile 35mm culture plates in the hood and add 1mL DMEM into the plates. Take out cells from incubator and tap the 100mm at the sides to ensure that cells are detached from the surface. Seed the 35mm plates with ~50μL of cells, and make sure to distribute cells evenly on the plate to avoid

clumping. Set plates at 37ºC incubator overnight to allow cells to adhere to plate surface.

Observe under microscope for healthy, adherent HeLa cells prior to setting up transfection.

To sterile 1.5mL Eppendorf tubes, add 450µl Invitrogen OptiMEM media. For transfection using PEI (Polyethylenimine) (Sigma 408727 from stock of 1mg/mL), add 2.4µL of PEI for every µg of DNA. In our transfection experiments we add 2µg of purified DNA plasmid in mammalian expression vector pcDNA3. Add 6µL PEI and 2µg of DNA to be transfected to Eppendorf tubes containing OptiMEM and incubate at RT for at least 30 minutes. Purified plasmids used for experiments were Drp1GFP in pcDNA3, GFP-B-insert in pcDNA3 and GFP as control.

Add the transfection mix (450µL OptiMEM, 6µL PEI and 2µg of DNA) to confluent 35mm plates containing HeLa cells. Cells were incubated overnight at 37ºC.

We proceeded to seed cells in Labtek chambers which were sterilised under UV. 400µL of DMEM was added to each well of the Labtek chamber. The media present in 35mm plates was removed by suction and the cells were washed with 500µL of DPBS twice. Trypsinisation of cells was done by adding 100µL of 0.5 MTrypsin + EDTA and placed in incubator at 37ºC for 1 minute. Cells were detached by tapping the sides of the plate vigorously. Trypsin was neutralised by adding 600µL (~4x volume of Trypsin) of DMEM. Labtek chambers were seeded with 40µL- 70µL of cells and incubated overnight at 37ºC.

After 48 hours of transfection of cells, we proceed to stain the cells with mitochondrial marker MitoTracker Red. The DMEM media was changed by removing media by pipetting from the edges of the wells of Labtek chambers followed by two washes of 500µL DPBS. Cells were incubated with 200µL DMEM media containing 500nM Mitotracker Red for 15 minutes and then observed under microscope.

B-insert and GFP localisation was observed for GFP fluorescence under FITC (Fluorescein Isothiocyanate) channel (Excitation at 490nm and emission at 525nm). Mitochondria was stained with Mitotracker Red (which shows excitation at 581nm and emits red fluorescence at 644nm) and was observed under Texas Red channel (Excitation max at 596nm and emission at 615nm).

Results

The B-insert in Drp1 is a Cardiolipin Specific Binding Domain

Drp1 binds to anionic lipids with a specificity in binding to cardiolipin

Dot blot assay was performed to identify the lipid specificity of Drp1.

For dot blot assays we used C terminal GFP construct of Drp1 isoform3 which is also shown to be membrane active and shows localisation to cardiolipin containing membrane tubes (Kamerkar et al, manuscript in preparation). The construct is 6xHis-TEV cleavage site-Drp1 isoform3-mEGFP-StrepII.

Our assays revealed that Drp1-GFP showed preferential specificity in binding to cardiolipin over other anionic lipids (DOPA and DOPS) and shows 2.5 fold more binding over DOPC **(Fig.4 a,b)**. This data is also consistent with reported Drp1 interaction via dot blot indicating specificity in binding to cardiolipin (Bustillo-Zabalbeitia et al., 2014). However, at concentrations of lipids (1.1 nanomoles) similar to employed in our dot blot analysis Drp1 shows specificity to PG, PI and PS as well (Bustillo-Zabalbeitia et al., 2014). Drp1-GFP also displayed similar binding preference (~2.4 fold) to DOPG in our dot blot assay **(Fig.4 b)**. It has been reported previously of cardiolipin binding protein to also display specificity affinity in binding to PG as well (Oliver et al., 2014)(Renner and Weibel, 2012).

B-insert shows preferential specificity in binding to cardiolipin over other anionic lipids

To understand whether B-insert has intrinsic membrane binding ability, dot blots of Drp1 isoform 3 GFP-B-insert (henceforth, referred to as B-insert) was set up to identify the lipid binding specificity. Identifying the lipid specificity would enable in understand how B-insert would influence membrane interactions in Drp1.

An N terminal mEGFP tagged B-insert construct engineered in the lab was used for our experiments with B-insert. B-insert showed ~10-fold higher binding specificity to cardiolipin normalised to DOPC **(Fig.4e)**. However, B-insert shows fairly lower binding affinity to PG, PA or PE, indicating that the interaction is not alone non-specific electrostatic interaction but also guided by specific interaction between B-insert and phospholipid. In comparison to Drp1 in our dot blot assays, B-insert displays higher affinity in binding to cardiolipin which allows us to speculate that B-insert could be the membrane interacting domain of Drp1 **(Fig.4e)**.

Drp1ΔB displays a lower cardiolipin binding affinity on deletion of B-insert

Studies conducted on a B-insert deletion mutant Drp1 (DNM1L ΔB-insert) expressed in endogenous DNM1L-depleted COS7 cells, affects the Drp1 localisation to mitochondria and renders the protein cytosolic (Fröhlich et al., 2013).

This guides us to pose questions at B-insert as the membrane binding domain, and to investigate whether Drp1 loses its lipid binding specificity on deletion of the B-insert. We looked at the membrane lipid specificity of Drp1ΔB in our dot blot assay. C terminal mEGFP construct of Drp1ΔB was used for the assay.

Our data indicates that Drp1ΔB-GFP(1μM) shows only around~1.5-fold affinity to cardiolipin over DOPC **(Fig.5b)**. The PG and cardiolipin binding affinity of Drp1 is lost in Drp1ΔB. Unpaired t-test indicates significant difference in cardiolipin binding between Drp1 and Drp1ΔB ($p= 0.0026$, <0.05). The deletion in B-insert might be a plausible reason for the reduction in cardiolipin binding affinity in Drp1ΔB.

Our assays only focus and allows to predict this, based on the head group affinity detected from the GFP tagged constructs, validated with a GFP control (1μM) for the same**(Fig.5c)**.

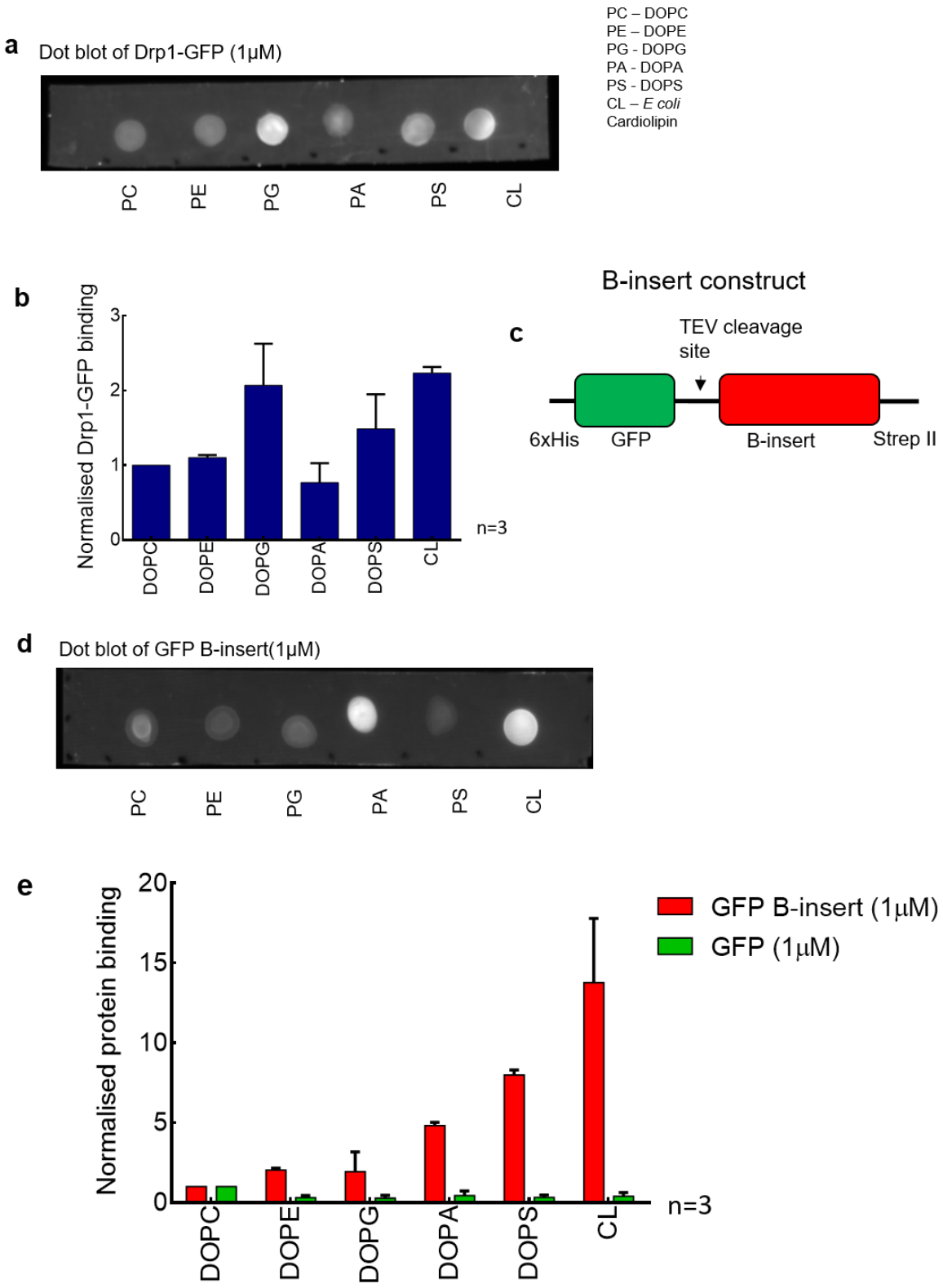

Figure 4 : **B-insert binds preferentially to cardiolipin over other anionic lipids** a) Dot blot image of Drp1(1µM) imaged in eGFP channel. The lipid head groups are marked below the representative blot image. b)Drp1 binding to anionic lipids normalised to DOPC binding. c) GFP B-insert construct. d) Dot blot image of B-insert imaged in eGFP channel. e) Analysis of phospholipid binding specificity of B-insert(1µM) with GFP (1µM) was taken as control for dot blot.

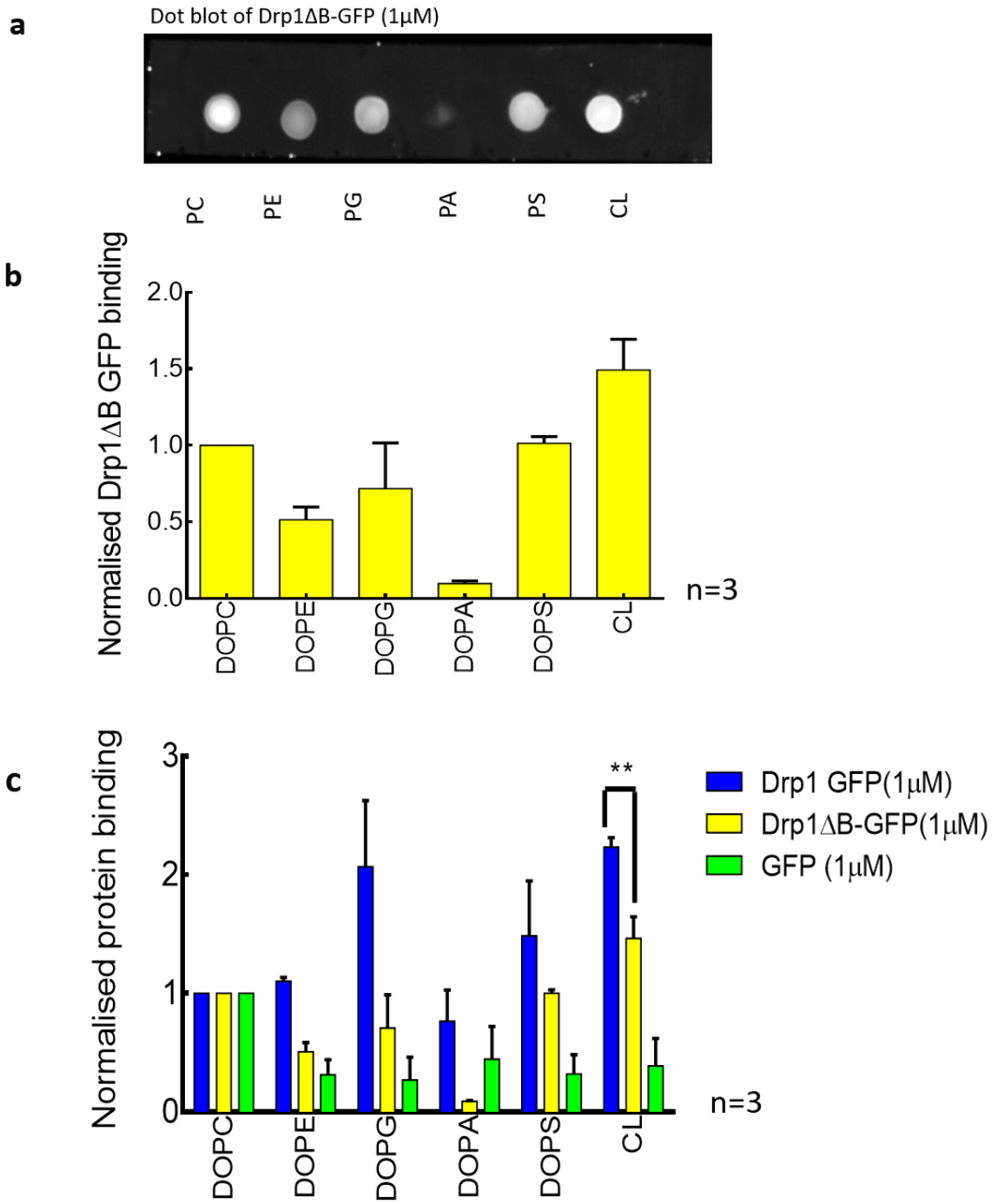

Figure 5 : B-insert deletion in Drp1 (Drp1ΔB) shows decrease in cardiolipin and PG binding specificity . a) Dot blot image of Drp1ΔB (1μM) imaged in eGFP channel. The lipid head groups are marked below the representative blot image. b)Drp1ΔB binding to anionic lipids normalised to DOPC binding. c) Analysis of phospholipid binding specificity of Drp1ΔB and Drp1. GFP (1μM) was taken as control for dot blot. Unpaired t-test shows significant difference in cardiolipin binding.

Heat Denaturation Assay indicates that B-insert is heat stable

To test for B-insert being an unstructured domain, we cleaved GFP from B-insert using TEV protease. On providing heat, a heat-labile protein would precipitate due to unfolding, denaturation and aggregation (Kim et al., 2000). Heat resistant proteins, are able to retrace or regain their protein conformations into native state after heat subjection. Protein either strictly do not change their structure, or move to an unfolded state, with potential to regain native structure at optimum temperature (Kim et al., 2000). Unstructured proteins however, are able to inherently access multiple conformations and having exposed domains to the buffer, heat denaturation does not affect the solubility of these protein which prevents aggregation.

In our assay, we subject B-insert to heat shock from 20ºC to 90ºC, heat denaturation extended for 10 minutes at 90ºC and then rapid cooling at 4ºC for 10 minutes. The protein is then centrifuged at 20,000g to separate out aggregated protein and then visualised on an SDS gel of supernatant and pellet **(Fig.6 a)**.

We observe that on TEV digestion and subsequent heat denaturation, B-insert (band at 10kDa) is only detected in the supernatant after centrifugation **(Fig.6 b)**. GFP(27kDa) and TEV(25kDa) in the mixture is present in the pellet possibly due to precipitation and separated out into the pellet after centrifugation **(Fig.6 b)**. A slight band of GFP is also detected in supernatant. Our results indicate that B-insert is heat stable and does not precipitate out on heat denaturation. This suggests that B-insert could be an unstructured protein as predicted in literature.

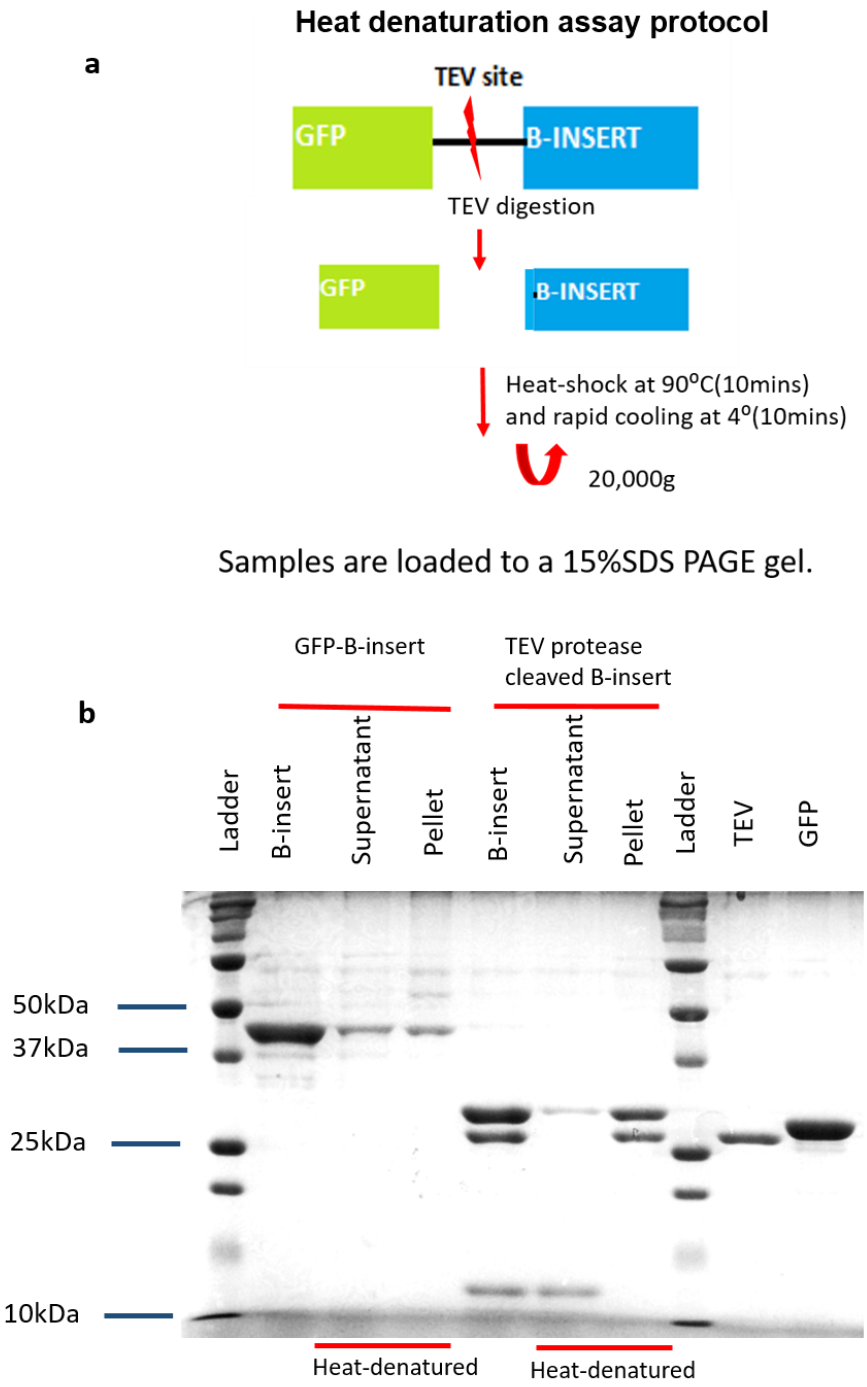

Figure 6 : **B-insert is heat stable and does not precipitate on heat denaturation .** a) Heat denaturation assay protocol described schematically. b) 15%SDS PAGE gel indicating presence of B-insert(band above 10kDa) only in supernatant after being spun down post heat denaturation treatment.

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B-insert as a cardiolipin binding probe:

Comparison of B-insert binding specificity against cardiolipin binding probe NAO

10-nonyl acridine orange(NAO) has been used widely as a cardiolipin binding probe to detect cardiolipin in *E. coli* cells (Mileykovskaya et al., 2001). NAO inserts into the phospholipid with its alkyl chain guiding interactions with cardiolipin. NAO has an excitation wavelength of 495nm and emits fluorescence at 519nm and 640nm. We used NAO at a concentration of 1µM for our dot blot analysis and the probe binding was imaged in EGFP channel.

The dot blot reveals that NAO does not show high binding specificity to cardiolipin over other anionic lipids **(Fig. 7 c)**. B-insert on comparison to NAO displays better binding specificity to cardiolipin **(Fig. 7 d)**.

We speculate that B-insert can potentially be used as a cardiolipin domain sensor and expressing in cells may allow us to visualise whether B-insert exhibits membrane binding property.

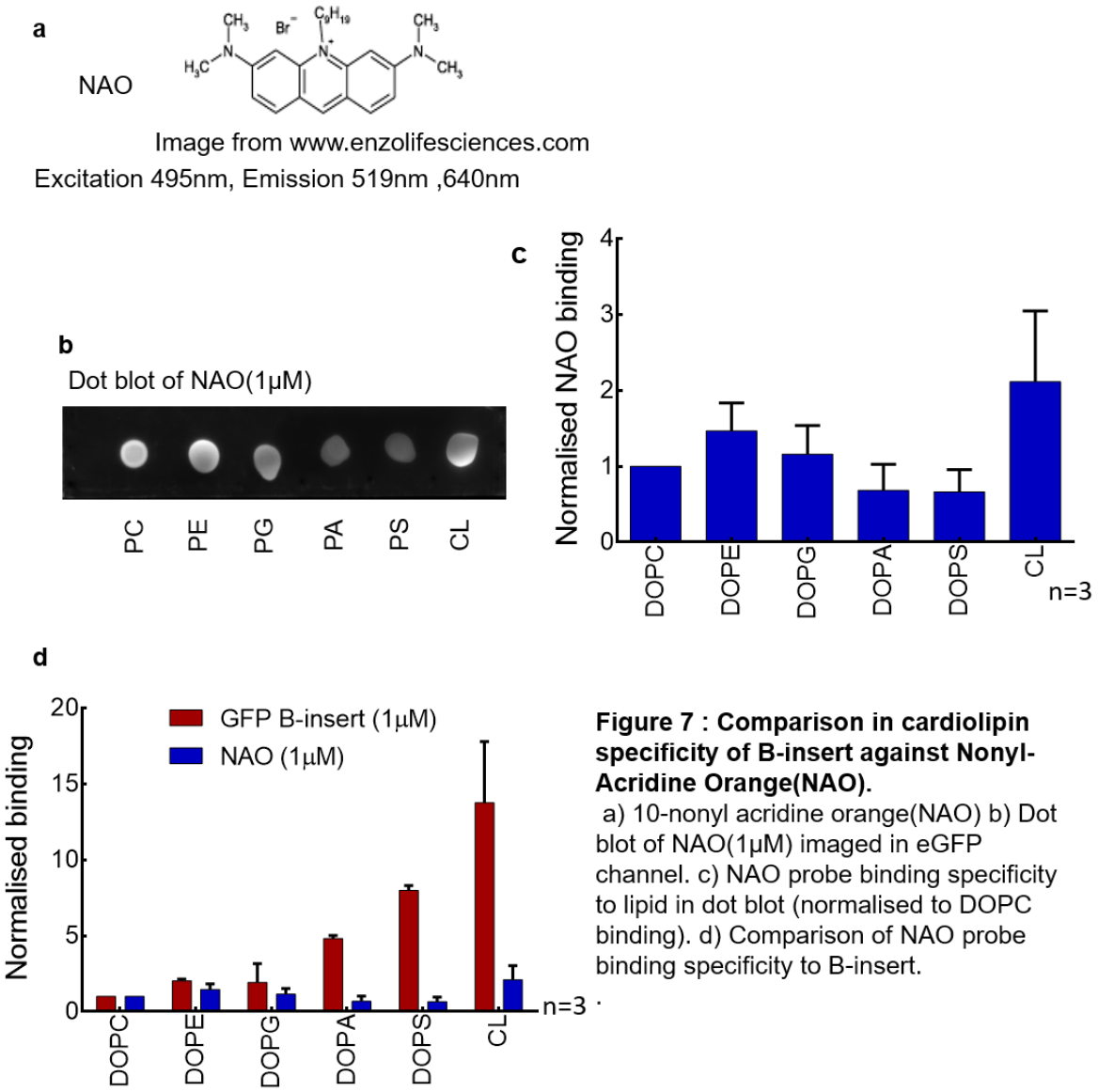


Figure 7 : Comparison in cardiolipin specificity of B-insert against Nonyl-Acridine Orange(NAO).
a) 10-nonyl acridine orange(NAO) b) Dot blot of NAO(1μM) imaged in eGFP channel. c) NAO probe binding specificity to lipid in dot blot (normalised to DOPC binding). d) Comparison of NAO probe binding specificity to B-insert.

Expression of GFP-B-insert in HeLa cells to observe localisation and membrane binding

To ascertain that B-insert can be the cardiolipin sensing membrane binding domain of Drp1 which allows to localise to mitochondria, we expressed B-insert in HeLa cells. GFP-B-insert would allow us to understand and visualise the mitochondrial membrane binding, localisation and the associated morphology of the mitochondria on expression of the B-insert *in vivo*. The presence of a fluorescent tag (mEGFP) allows us to detect the protein localisation and membrane binding. Mitotracker Red was used as mitochondrial marker. We employ GFP as the negative control for *in vivo* experiments with HeLa cells

Cells were stained with 500nM Mitotracker Red and observed under the microscope at 60x. Images of cells were captured in FITC channel for GFP-B-insert and GFP localisation and in Texas Red channel to observe mitochondrial morphology stained by Mitotracker Red **(Fig.8).**

We observe that B-insert is cytosolic and does not show enrichment to mitochondrial membrane **(Fig.8 a)**. Mitotracker staining reveals the mitochondria to be fragmented forming small clusters around the nucleus **(Fig.8 a)**.

GFP also appears to be cytosolic in its localisation, enriched in and around the nuclei and gets excluded from mitochondria **(Fig.8 b)**.

Long networks of mitochondria are observed in cells transfected with GFP **(Fig.8 b)**.

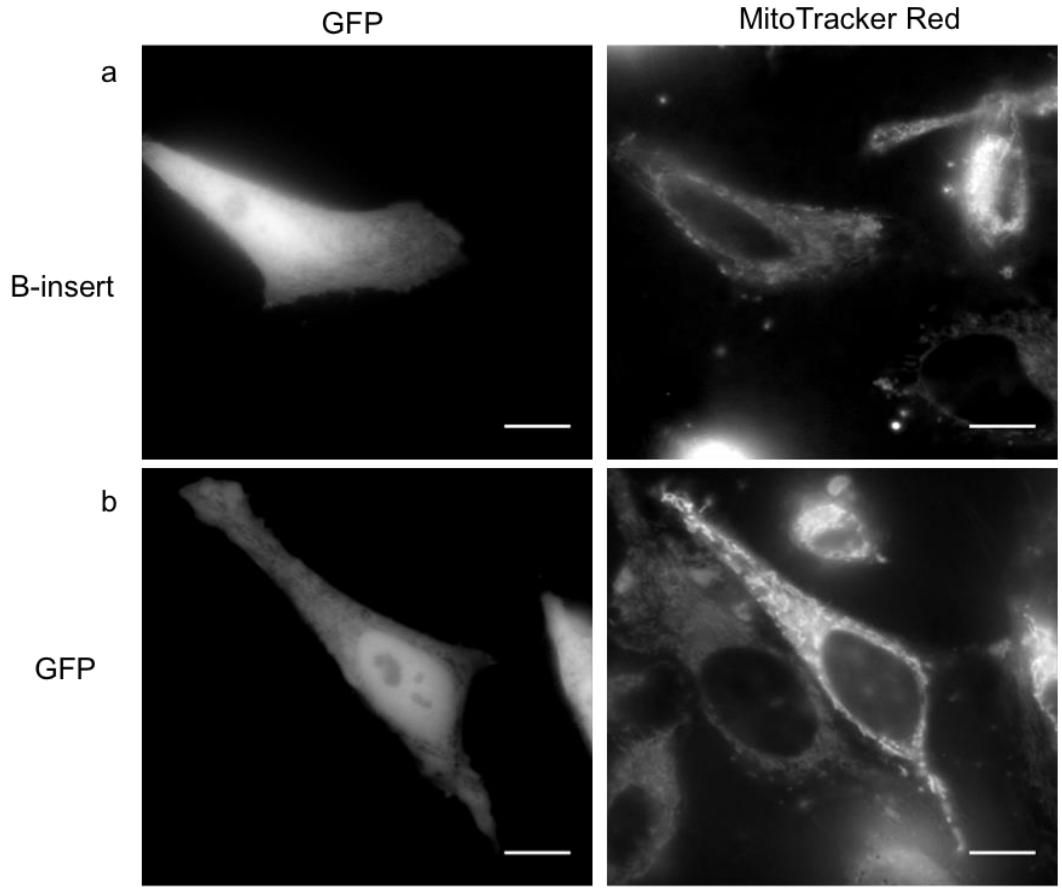


Figure 8 : *In vivo* expression of GFP-B-insert and mEGFP in HeLa cells.
HeLa cells were transfected with a) B-insert and b) GFP. Cells were imaged in FITC (for GFP fluorescence) and in Texas Red channel (for Mitotracker Red fluorescence).Cells Scale bar – 5 µm.

Our results hint that B-insert might have a low binding affinity in cells, which could account for the protein being not enriched to mitochondrial membranes.

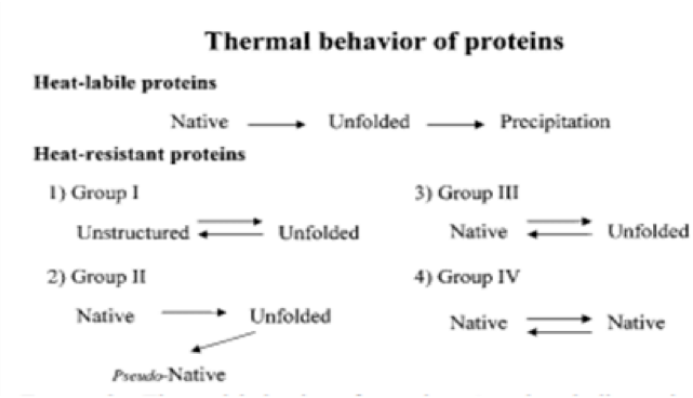
Discussion

Drp1-cardiolipin interactions are crucial in membrane interaction, binding and regulation of fission by Drp1.

Our dot blot assays looking at lipid binding, shows that B-insert shows higher binding to cardiolipin over other anionic lipids.

Drp1-GFP shows a 30% drop in cardiolipin binding in our dot blots on deletion of the B-insert indicated by Drp1ΔB-GFP. The B-insert, we speculate to be important as a membrane binding domain via interactions with cardiolipin. Unpaired t-tests on Drp1 and Drp1ΔB indicates significant difference in cardiolipin binding ($p=0.026$, <0.05). Deletion of the B-insert does not show a complete loss in binding to lipids provided in our assays. On expressing purified domains of Drp1 , the stalk domain also displays binding to lipids in lipid co-floatation assay (Adachi et al, 2016). Thus, lipid binding and interaction in Drp1 is not completely exhaustive and restricted to the B-insert.Similarly, mutation of conserved lysine residue in B-insert in Drp14KA displays a drop and not a complete loss, in binding to cardiolipin containing liposomes (Bustillo-Zabalbeitia et al, 2014).

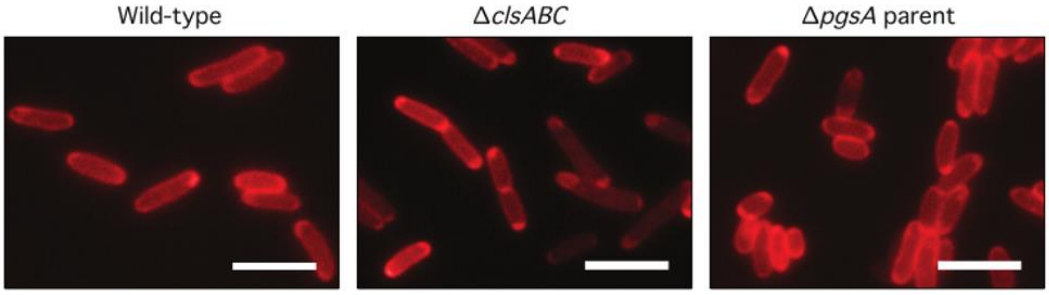
Binding to PG is affected as well in Drp1ΔB-GFP compared to Drp1-GFP showing significant (~65%) drop in PG binding in our dot blot assays. Most mitochondrial proteins which show affinity in binding to cardiolipin, also displays hig affinity in binding to PG present on membranes (Bustillo-Zabalbeitia et al, 2014)(Oliver et al, 2014; Renner & Weibel, 2012). Hence, it is proposed that on deletion of a putative membrane binding domain of mitochondrial proteins would also show loss in PG binding.



T.D. Kim et al, Biochemistry. 39 (2000) 14839–14846.

Figure 9 : Thermal behaviour of proteins:
Heat denaturation assay validates hypothesis that B-insert is intrinsically unstructured.

Heat denaturation assay on B-insert reveals that B-insert is heat stable and could possibly be unstructured. Heat denaturation does not induce precipitation in B-insert and suggests that B-insert is likely unstructured **(Fig. 9)** (Kim et al., 2000). Drp1ΔB dot blots revealed that Drp1 loses its PG binding affinity primarily but also shows partial loss in affinity in binding to cardiolipin. Literature suggests deletion of variable domain allows Drp1 to form higher order oligomers (Clinton et al., 2016)(Macdonald et al., 2016), hence used for crystallisation. Having an unstructured B-insert allows Drp1 to bind to cardiolipin on recruitment to membranes. B-insert dot blots indicate pronounced affinity to cardiolipin, implying the requirement of B-insert for cardiolipin binding and interactions regulating Drp1 activity.



Oliver, P.M., *et al* (2014). J. Bacteriol. *196*, 3386–3398.

Figure 10: NAO binds non-specifically to anionic phospholipids and is enriched at the poles in cardiolipin mutant *E. coli* cells: NAO displays polar localisation similar to wildtype (MG1655 cells) in cardiolipin mutant *E. coli* strains ΔclsABC (MG1655 BKT12 cells)(triple CL synthase gene mutant) and in ΔpgsA (UE53 cells) (mutant for PG synthesis)
Scale bar – 5μm

Cardiolipin was detected in cells earlier using NAO as a probe. However, NAO displays non-specific interactions with other anionic lipids. *E. coli* mutants which are devoid of cardiolipin does not exhibit altered cellular physiology and the cellular localisation of cardiolipin binding protein is unaffected. In *E. coli* K-12 cells where cardiolipin and PG is localised to poles, NAO shows fluorescence in polar regions even in PG synthase mutant (CL and PG deficient strain) of E coli UE54(UE53 Δpgs) **(Fig. 10)**. This suggests that NAO does not label cardiolipin specifically *in vivo* (Oliver et al., 2014).

B-insert exhibits better specificity to cardiolipin in our dot blots, and we speculated that it can bind to cardiolipin in-vivo.

Preliminary experiments on transiently overexpressing GFP-B-insert in HeLa cells, shows cytosolic localisation of B-insert. We suggest that this could be due to fairly low affinity of B-insert binding to cardiolipin on membranes and in-vivo. This could also suggest that B-insert might not be necessary in Drp1 recruitment to mitochondria, sidelined by binding to mitochondria via interactions with adaptor proteins like mitochondrial fission factor (Mff), Mid49 and Mid51 (Gandre-Babbe et al, 2008)(Palmer et al, 2011) We suggest that B-insert is necessary as a cardiolipin binding domain in Drp1.

Mitochondria stained by MitoTracker-Red indicates fragmented mitochondria in cells expressing B-insert. As our in-vivo experiments were not yet standardised and

results shown are preliminary, we are unable to explain the underlying possible causes for the fragmented mitochondrial morphology observed. We could only observe a few healthy cells displaying protein expression, with majority of the cells showing extensive blebbing indicating that cells could also be unhealthy.

Hence, we cannot draw necessary conclusions from our in-vivo experiments.

To note, mitochondrial morphology is not an accurate readout for protein functions. Cells under different nutrient media and nutrient-stressed conditions show tubular mitochondria compared to untreated cells(Rambold et al, 2011). Taking this into consideration, we cannot explain the mitochondrial morphology observed in our experiments.

We hypothesise that B-insert could possibly act as cardiolipin binding domain, and on Drp1 recruitment to membranes by adaptor proteins to outer membrane of mitochondria, B-insert-cardiolipin interactions influences Drp1 function (Macdonald et al., 2016).

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