INVESTIGATING THE FUNCTIONAL ROLE OF DRP1 IN MITOCHONDRIAL FISSION

by

CHRISTOPHER A FRANCY

Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy

Dissertation Advisor: Dr. Jason A Mears

Department of Pharmacology
CASE WESTERN RESERVE UNIVERSITY

January 2017
CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the dissertation of

Christopher A Francy

Candidate for the Doctor of Philosophy degree *.

(Thesis Advisor) Jason Mears Ph.D.
(Committee Chair) Derek Taylor Ph.D.
(Committee Member) Johannes Von Lintig Ph.D.
(Committee Member) Paul Park Ph.D.
(Committee Member) Philip Kiser Ph.D.

Date of Defense
10/11/16

* We also certify that written approval has been obtained for any proprietary material contained therein.
DEDICATION

I dedicate this work to my family and friends. My family has always supported me to pursue science to the highest degree, and I would never have reached this point without their support. My friends here at Case and Cleveland have always been there to support me and get me out of the lab when I needed it. We had so many great memories together that I will never forget.
TABLE OF CONTENTS

LIST OF TABLES vii
LIST OF FIGURES viii
ACKNOWLEDGEMENTS xi
LIST OF ABBREVIATIONS xv
ABSTRACT 1

Chapter 1: Mitochondrial Dynamics and the Role of Drp1 in Mitochondrial Fission

1.1 Mitochondrial Origins 4
1.2 Mitochondrial ATP Synthesis 6
1.3 Mitochondrial Reactive Oxygen Species 7
1.4 Mitochondrial Lipid Synthesis 8
1.5 Mitochondrial Calcium Homeostasis 9
1.6 Mitochondrial Heat Generation 9
1.7 Mitophagy 10
1.8 Intrinsic Apoptosis 10
1.9 Mitochondrial Dynamics 11
1.10 The Dynamin Superfamily of GTPases 12
1.11 Mitochondrial Fusion Proteins 14
1.12 Mx Antiviral Dynamin 16
1.13 Mitochondrial Fission 17
1.14 Dynamin Related Protein 1 (Drp1) 17
Chapter 2: The Mechanoenzymatic Core Of Dynamin-Related 
Protein 1 Comprises the Minimal Machinery Required for 
Membrane Constriction

2.1 Abstract 44
2.2 Introduction 45
2.3 Results 48
2.4 Discussion 57
2.5 Materials and Methods 62
2.6 Acknowledgements 67

Figures and Legends 68

Chapter 3: Distinct Splice Variants of Drp1 Affect Membrane Curvature

3.1 Abstract 83
3.2 Introduction 84
3.3 Results 86
3.4 Discussion 90
Chapter 4: Cryo-EM Studies of Drp1 Reveal Cardiolipin Interactions that Activate the Helical Oligomer

4.1 Abstract
4.2 Introduction
4.3 Results
4.4 Discussion
4.5 Materials and Methods
4.6 Acknowledgements
Figures and Legends

Chapter 5: Discussion and Future Directions

5.1 Summary
5.2 Additional Drp1 Isoform Studies
5.3 Recapitulating In Vitro Membrane Fission
5.4 Variable Domain Conformation
5.5 Improving Cryo-EM Resolution
5.6 Additional Drp1 Cryo-EM Structural Targets
5.7 Conclusion

References
List of Tables

Table 2.1: The effect of nucleotide on Drp1-lipid tube morphology 80
Table 2.2: The VD modulates Drp1 curvature on lipid templates 81
List of Figures

Figure 1.1: Mitochondrial Architecture and Lipid Composition 32
Figure 1.2: Mitochondrial Generated Reactive Oxygen Species 34
Figure 1.3: Mitochondrial Intrinsic Apoptosis 35
Figure 1.4: Dynamin Family Architecture 36
Figure 1.5: The Mitochondrial Dynamics Balance of Fission and Fusion 37

Figure 1.6: Drp1 Mediated Mitochondrial Fission 38
Figure 1.7: Drp1 Oligomeric Properties 39
Figure 1.8: Lipid Structure 40
Figure 1.9: Post Translational Modification, Alternative Splicing and Key Mutations of Drp1 41
Figure 1.10: A Schematic Diagram of the Iterative Helical Real Space (IHRSR) Algorithm 42

Figure 2.1: Drp1-lipid tubules constrict upon GTP addition 68
Figure 2.2: Drp1-lipid tubules constrict only upon GTP hydrolysis, while a conformational stabilization occurs upon GTP binding. 70
Figure 2.3 The variable domain regulates Drp1 assembly, but is not required for lipid constriction. 72
Figure 2.4 WT and ∆VD Drp1 associate with DOPS and CL-mix liposomes to form ordered protein-lipid tubes. 74
Figure 2.5 The mechanoenzymatic core of Drp1 is sufficient for lipid-stimulated activity, which leads to oligomer disassembly. 76
Figure 2.6 Schematic representations of Drp1 recruitment, constriction and recycling on lipid bilayers.

Figure 3.1 Drp1 isoforms exhibit differential GTPase activities.

Figure 3.2 Drp1 isoforms exhibit differential oligomerization propensities in solution and distinct helical geometry on CL-containing membranes.

Figure 3.3 Drp1 A- and B-inserts differentially affect membrane curvature.

Figure 4.1 Drp1 helical polymers are more uniform on lipid nanotubes.

Figure 4.2 3D structure of Drp1 associated with a phosphatidylserine (PS) lipid template.

Figure 4.3 Drp1 geometries and handedness used for 3D reconstructions.

Figure 4.4 Comparison of fitted Drp1 G-domain dimers in the GC/PS density.

Figure 4.5 Drp1 recruitment and activation is enhanced with cardiolipin (CL) nanotubes.

Figure 4.6 3D structure of Drp1 bound to a cardiolipin (CL) lipid template.

Figure 4.7 Removal of the variable domain (VD) abolishes Drp1 sedimentation with CL nanotubes.

Figure 4.8 Comparison of the lipid-bound Dynamin and Drp1 polymers.

Figure 4.9 Cardiolipin interactions trigger active Drp1 assembly.
ACKNOWLEDGEMENTS

I would first like to acknowledge my Principal Investigator, Dr. Jason A Mears. Coming to CWRU in the summer of 2011, I was nervous, and I wasn’t sure how this whole graduate school thing was going to work out. I was uncertain if being interested in science and pharmacology was going to be enough to get me through. Luckily, I first rotated in Jason’s lab. I quickly recognized his lab was going to be a good fit for me. I was very interested in the structural biology research, but more importantly I felt he would be a good mentor. I could tell he would take the time to teach me the important aspects of research: developing experiments, critically analyzing data, making your own decisions and conclusions, effectively writing and public speaking. Jason is an excellent communicator, and he taught me many of those skills while in his lab. Jason and I are also big sports fans, so we have spent countless hours talking about various teams and games. This has allowed us to bond on levels many graduate students don’t experience. Although Jason is my boss, I also see him as a great friend.

I’d also like to thank Dr. Charles Hoppel. I would consider him my second mentor, as he has spent much of his time critiquing my work and giving me excellent feedback. Early on when I participated in mitochondrial data and journal clubs, I spoke up very little. After observing the scientific discussions Dr. Hoppel initiated, and the passion he displayed in his questions and explanations, I changed my outlook. I was no longer hesitant to ask questions, to speak my opinion and to challenge other’s theories as well as my own. This training helped me become a much better scientist.

Frances Alvarez was a postdoc in our lab, and I owe so much to her. She trained me how to physically prepare and conduct reproducible experiments. She also taught me
that being honest with your data is crucial. If the experiment didn’t work, or the data was messy, accept it, devise a plan of action and move on. Her two years in the lab were invaluable for my development as a successful scientist.

I’d also like to thank Dr. Katsu Murakami, my undergrad Principal Investigator at Penn State University. He first introduced me to research in 2010, and showed me what it would take to be a graduate student. He was firm at times, but also very caring when it came to teaching. Dr. Samantha Stubblefield was also critical is my development as a graduate student. As a postdoc in the Levine lab, she spent most of her time telling me what I was doing wrong in cell culture. However, this friendly criticism was important, as it molded me into a much better scientist, and gave her and I more than a few laughs. We’ve been playing volleyball together since then, and have been great friends.

Some of the best friends I will ever have in my life will be from graduate school here in the Department of Pharmacology and at Case Western Reserve University. My best friends include Chairut Vareechon, Mitchell Lakner and Deoye Tonade. We have enjoyed so many great times together playing intramural sports, going to blue collar bars, exploring new neighborhoods in Cleveland and going on outrageous camping trips (Deoye’s not much of an outdoors guy though). We will be such good friends for the rest of our lives, no matter where life may take us.

I’d also like to take this time to acknowledge Michael Mullins. He was a good friend of mine, and a graduate student here in the Department of Pharmacology. He tragically committed suicide in September of 2014. He also played sports, went out to the bars and camped with us all around the great lakes region. Mike had a passion for
science, biking and the outdoors. He was a great person, and I miss him dearly. Finishing my PhD will be in honor of you Mike, rest in peace.

As some may know, I have a strong passion for athletics. What made my graduate school experience so fulfilling, was that I didn’t have to give up this passion of mine. The first friends I made were from the pharmacology softball team in the summer of 2011. From that point on our team ended up winning 5 softball championships, and we had a great time in the process. I also played flag football, basketball, volleyball and dodgeball at mulberry’s downtown. I met so many friends this way from around Cleveland and in other CWRU departments. Specifically, I met two of my good friends Mark Holland and Rakim Tyler from macromolecular science. We combined teams and enjoyed destroying the case undergrads, medical students and dental students in basketball and flag football.

I must never forget to acknowledge my faith. I am so fortunate to have been blessed by God with so many talents. Without my health and great family I would never have gotten here. He has given me intelligence, athleticism, height, humor and a passion for life. I have done my best to utilize these talents to be a better person, scientist and to mentor others.

I’d like to take this time to acknowledge my parents and my brother in this last section (I know, it’s long). As a scientist, we know that many things turn out the way they do because of a complex relationship between genes and the environment. Without both it is difficult to succeed. Besides giving me excellent genes, my parents have provided me so much growing up, and continue to do so. They taught me hard work, competitiveness and courage. Whatever I wanted to do, they encouraged me to go for it and always gave me the tools or financial support necessary. They have taught me so many life skills that I
will one day pass down to my children. I can’t forget to acknowledge my older brother Billy. He taught me how to throw balls in every single sport hard and accurately, a skill I didn’t fully appreciate until coming to Case. I learned that to beat him in a sport, I had to be smart, because he was bigger and faster than me most of the time. We’ve always had a great relationship, and this has helped me accomplish my scientific goals as well. He said to me once back in college, “So, you want to be a scientist like Dr. Doak in James Bond?” (This is a fictitious Russian scientist in the James Bond video game, Goldeneye). I said, “Yeah, actually I do”. From that point forward, I never looked back.
LIST OF ABBREVIATIONS

Abbreviations:

CL   Cardiolipin
CTF  Contrast Transfer Function
DED  Direct Electron Detector
Drp1 Dynamin related protein 1
EM   Electron Microscopy
ETC  Electron Transport Chain
Fis1 Fission protein 1
GC   Galactosyl Ceramide
GED  GTPase Effector Domain
GUV  Giant Unilamellar Vesicle
HDX  Hydrogen Deuterium Exchange
IHRSR Iterative Helical Real Space Reconstruction
IMS  Inner Membrane Space
Mff  Mitochondrial fission factor
Mfn1 Mitofusin 1
MiD49/51 Mitochondrial dynamics protein of 49 and 51 kDa
MIM  Mitochondrial Inner Membrane
MnSOD Manganese Superoxide Dismutase
MOM  Mitochondrial Outer Membrane
MOMP Mitochondrial Outer Membrane Permeabilization
mtDNA Mitochondrial DNA
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opa1</td>
<td>Optic atrophy protein 1</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic Acid</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTM</td>
<td>Post Translational Modification</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>VD</td>
<td>Variable Domain</td>
</tr>
</tbody>
</table>
Investigating the Functional Role of Drp1 in Mitochondrial Fission

Abstract

by

CHRISTOPHER A FRANCY

Mitochondria are dynamic organelles that continually undergo cycles of fission and fusion. Maintaining this fission/fusion balance is extremely important in sustaining cellular health. Mitochondrial fission serves to separate daughter mitochondria during division, segregate damaged mitochondria for autophagy and initiate apoptosis during disease conditions. In fact, in neurodegenerative, heart and liver disease, increased mitochondrial fission is observed, leading to cell death. The main mediator of mitochondrial fission is Drp1, a large GTPase of the dynamin superfamily. Drp1 can form oligomers capable of remodeling mitochondrial membranes, and this can lead to membrane partitioning. However, the mechanism of this process is not well understood. This work aimed to investigate the role of Drp1 in membrane constriction, the function of the uncharacterized variable domain (VD) of Drp1 and the structure of Drp1 on lipid membranes mimicking mitochondrial interactions. We assembled Drp1 on negatively charged membranes and induced constriction upon the addition of GTP. Moreover, we found that only GTP hydrolysis induces full constriction of the Drp1 lipid oligomer. Furthermore, the unstructured VD was found to keep Drp1 in a more active cytosolic
conformation, as VD removal induced a hyperoligomeric state. We also found that alternatively spliced sequence insertion in the VD similarly reduces enzyme activity and narrows the diameters of Drp1-lipid polymers. In order to further investigate the function of these oligomers, we solved the 3D structure of Drp1 complexed on distinct lipid templates. Interestingly, we discovered that the mitochondrial specific lipid cardiolipin directly interacts with the VD and induces an activating conformational change to the rest of the Drp1 molecule. The helical assembly of Drp1 on CL templates is mediated exclusively through stalk and GTPase domain interaction sites. The VD has previously been termed a domain of unknown function, however our work characterizes the VD as a critical regulator of Drp1 self-assembly. We also find that the VD conformation influences stalk and novel G-domain orientations, which directly influence Drp1 enzymatic activity. This work advances our understanding of the Drp1 domains involved in the assembly and constriction of lipid bilayers. Overall, these findings will help us better understand Drp1 mediated mitochondrial fission.
CHAPTER 1: MITOCHONDRIAL DYNAMICS AND THE ROLE OF DRP1 IN MITOCHONDRIAL FISSION
1.1 Mitochondrial Origins

How did complex, eukaryotic life arise from single-celled organisms? While prokaryotes have demonstrated extraordinary biochemical ingenuity, they have not evolved substantially greater complexity in their 4 billion-year existence. Eukaryotes are generally larger in size, more highly structured, contain bigger genomes and produce more proteins. However, the underlying characteristic mediating this difference remained elusive. In fact, many eukaryotic traits are found in prokaryotes, including nucleus like structures, recombination, internal membranes, dynamic cytoskeleton and even endosymbionts (Lane and Martin, 2010). The mitochondrion turned out to be the key component in the development of eukaryotes. With mitochondria, cells had the ability to produce more ATP and expand their energy reserve. With this additional energy supply, cells had the luxury to increase their genome and synthesize many more proteins. Therefore, the advent of the mitochondrion opened up new avenues in biology.

The dawn of mitochondria in the cell was a turning point in life science, and there are several hypotheses as to how this occurred. Many believe mitochondria arose from an alpha-proteobacterium engulfed by a eukaryotic progenitor (Lane and Martin, 2010). Mitochondria originating as a bacterium is logical, as mitochondria possess many of the same characteristics. Similar to bacteria, mitochondria contain two functionally distinct membranes, the mitochondrial outer membrane (MOM) and the mitochondrial inner membrane (MIM). The MOM encapsulates the MIM, the inner membrane space and the mitochondrial matrix (Fig 1.1). The MOM is distinct from MIM is several respects. First, the mitochondrial outer membrane contains a higher percentage of neutral charged lipids, such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-
glycero-3-phosphocholine (DOPC) (Fig 1.1b). The charged component of the MOM includes 13 mol % phosphoinositides (PI), 2% 1,2-dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (DOPS) and 1 % cardiolipin (CL) (Horvath and Daum, 2013). The negative charged lipids interact with proteins on the MOM (Francy et al., 2015; Macdonald et al., 2014). The MIM is similar in composition, except it contains additional cardiolipin (18%). Contact sites represent membrane regions where the MIM and MOM directly interact. The MIM can also be anchored to the MOM, and these structures are defined as contact sites. Interestingly, CL is an ancient phospholipid that is enriched in contact sites and is believed to originate from bacteria, as it is found in bacterial cell membranes (Paradies et al., 2014). In the mitochondria, CL is essential in maintaining the activity of the mitochondrial complexes involved in the electron transport chain. It is also found at sites of curvature in the cristae. Therefore, mitochondrial membranes are in many ways similar to their proposed bacterial ancestors.

Similar to bacteria, mitochondria also undergo division events. During bacterial replication, the bacteria must divide rapidly to separate essential components such as DNA, proteins, lipids and other necessary materials. As one might expect, mitochondria also divide in order to separate mitochondria into daughter cells during cell replication, although the mechanism of division is distinct. Moreover, mitochondrial division is responsible for many other cellular roles as well. As the focus of this thesis, we will continually discuss the additional functions and the mechanism of mitochondrial fission in more detail.

Unlike most organelles, mitochondria contain their own DNA. This mitochondrial DNA (mtDNA) takes shape as a circular genome. In mammalian mitochondria, it only
encodes for 13 genes, all of which encode for proteins in mitochondrial electron transfer chain (ETC) complexes. Therefore, almost all of the 1,500 proteins in the mitochondria are nuclear encoded, and must be imported and directed to the correct mitochondrial compartments (Neupert and Herrmann, 2007; Schmidt et al., 2010). It is believed that the mitochondrial genome has been reduced during evolution through gene transfer, as the mitochondrion became less reliant on producing their own proteins. Furthermore, the close relatives of many mtDNA modifying enzymes, such as mtDNA polymerase, closely resemble bacteriophage proteins (Nunnari and Suomalainen, 2012). This indicates that viral infection further aided in bacterial derived mitochondrial evolution. The bacterial origins and bacteriophage related mtDNA maintenance systems also make mitochondria susceptible to many antibiotics targeting microbial ribosomes, including aminoglycosides and tetracyclines. Interestingly, in mammals, mtDNA inheritance is almost exclusively maternal, as paternal mtDNA is destroyed following fertilization (Al Rawi et al., 2011; Sato and Sato, 2011). Overall, the bacterial origins of mitochondria are further supported by the unique characteristics of their mtDNA.

1.2 Mitochondrial ATP Synthesis

The mitochondria play many important and diverse roles within the cell. Most notoriously, mitochondria are known for producing ATP. Mitochondria do this by using high-energy electron carriers from the TCA cycle such as NADH and FADH$_2$ in order to donate electrons to the inner membrane localized ETC complexes. Using this energy, complexes I, III and IV drive protons into the inner membrane space, generating a proton gradient (Nicholls et al., 2013). Protons then move through ATP synthase into the matrix, and the coupled energy is used to generate ATP. Maintaining membrane potential is not
only essential for ATP generation, but other processes such as mitochondrial protein import and quality control. Loss of membrane potential signals the mitochondria for degradation. Thus, maintaining ATP levels is of utmost importance in the cell, but in the process mitochondria can also release harmful byproducts.

1.3 Mitochondrial Reactive Oxygen Species

In addition to ATP production, mitochondria also generate reactive oxygen species (ROS). In the mitochondria, ROS occurs when electrons leak from the ETC and reduce molecules within the organelle. Most commonly oxygen is reduced to the superoxide anion (O$_2^-$, Fig 1.2). This nucleophilic anion will attack and damage proteins, lipids or nucleic acid (Cadenas and Davies, 2000). Superoxide will spontaneously dismutate into hydrogen peroxide (H$_2$O$_2$), which can also yield damaging hydroxyl radicals. These species of ROS will cause damage, and this is believed to contribute to the accumulated loss of mitochondrial function, often referred to as the mitochondrial free radical theory of aging (Forster et al., 1996; HARMAN, 1956; Harman, 1972). In fact, high levels of ROS correlate with increased mitochondrial fission events (Fan et al., 2010; Wu et al., 2011), supporting the role of mitochondrial fission in cellular homeostasis.

Fortunately, there are many enzymes in the mitochondria whose function is to eliminate ROS. Mangenese superoxide dismutase (MnSOD) detoxifies superoxide by converting it to hydrogen peroxide (H$_2$O$_2$). Hydrogen peroxide is less reactive and more stable than superoxide, and it can diffuse out of the mitochondria and act as a second messenger. In the mitochondrial inner membrane space (IMS), superoxide dismutation is similarly performed by cytosolic copper-zinc-SOD (Rimessi et al., 2016). Although
hydrogen peroxide is more stable, it can also damage the cell, so additional detoxifying mechanisms exist.

Hydrogen peroxide is further broken down by catalases (Kirkman and Gaetani, 2007). Catalase is an Fe-heme enzyme, and it exhibits one of the highest turnover rates among all proteins. Catalase decomposes 2 molecules of H₂O₂ to H₂O and O₂. Alternatively it can use hydrogen peroxide to oxidize various metabolites and toxins. Glutathione peroxidases and peroxiredoxins also cooperate to detoxify H₂O₂. Glutathione peroxidases use GSH to directly reduce H₂O₂ to H₂O, rendering the enzyme inactive. Moreover, thioredoxin restores peroxidase activity through the donation of electrons (Rimessi et al., 2016). Therefore, there are several mechanisms in place to reduce ROS produced by the mitochondria.

1.4 Mitochondrial Lipid Synthesis

Mitochondria play a critical role in lipid homeostasis and synthesis. They direct the synthesis of some key phospholipids, such as PE and PC. However, many lipids are obtained from the endoplasmic reticulum (ER), through mitochondrial-associated ER membranes (Clancey et al., 1993; Trotter et al., 1993). This connection mediates the transfer of lipid precursors such as PS and phosphatidic acid (PA), which are required for the synthesis of PE and the mitochondrial specific lipid CL. Mitochondria can also utilize lipids to produce ATP through the fatty acid B-oxidation pathway. This process converts long chain fatty acids to acetyl-CoA, which can be fed into the citric acid cycle to produce electron carriers like NADH to drive the ETC.
1.5 Mitochondrial Calcium Homeostasis

Mitochondria further interact with the ER and the plasma membrane to regulate Ca\textsuperscript{2+} homeostasis. Moreover, mitochondria are capable of fast Ca\textsuperscript{2+} uptake and slow Ca\textsuperscript{2+} extrusion. This blunts sudden spikes in Ca\textsuperscript{2+} concentrations and allows mitochondria to maintain long-term Ca\textsuperscript{2+} elevations (Walsh et al., 2009). Mitochondria can be strategically positioned in certain areas of the cell such as the ER, plasma membrane and nucleus in order to modulate calcium concentrations and thus signaling in those specific regions. Through modulation of calcium levels, mitochondria influence signal transduction pathways, neurotransmitter release from neurons, contraction of muscle cells, enzyme activity and voltage potential across membranes. Calcium signaling in the mitochondria is also essential in maintaining oxidative phosphorylation (Rizzuto and Pozzan, 2006). Several studies have demonstrated links between ER-mitochondrial contacts and mitochondrial fission (Friedman et al., 2011). It appears the ER reshapes an area of the mitochondria into a narrow tubule before fission proteins assemble to induce division (Rowland and Voeltz, 2012). However, it’s unclear what role calcium may be playing in relation to mitochondrial fission.

1.6 Mitochondrial Heat Generation

In addition to generating ATP, mitochondria can utilize energy from the proton gradient to produce heat, in a process called thermogenesis. In non-exercise thermogenesis, mitochondria in brown adipose tissue leak protons down their electrochemical gradient to yield heat. Uncoupling protein 1 (UCP1) is the proton channel responsible for the leakage of protons into the mitochondrial matrix without producing ATP (Nicholls et al., 2013). Brown fat is generally found at the highest levels
early in life and in hibernating mammals, while adult humans only possess small amounts of brown fat throughout their body. Plants such as skunk cabbage also utilize the UCP1 protein in their mitochondria to produce heat when environmental temperatures drop. Interestingly, mitochondrial fission is also essential in mediating thermogenesis. Moreover, norepinephrine activates free fatty acid release and triggers proteins (Drp1) responsible for inducing mitochondrial division (Gao and Houtkooper, 2014).

1.7 Mitophagy

Similar to other organelles, mitochondria must be recycled in a process termed mitophagy. During mitochondrial fission, occasionally the daughter mitochondria have low respiration and membrane potential. These defective mitochondria are rapidly eliminated by mitophagy in order to maintain overall mitochondrial quality (Twig et al., 2008). The Parkinson’s disease-related protein kinase (PINK1) and the ubiquitin ligase Parkin are involved in the mitophagy process. PINK1 is steadily degraded on healthy mitochondria, but upon membrane depolarization it is stabilized on the MOM. Parkin is then recruited to ubiquitinate the damaged mitochondria and initiate autophagosome formation (Otera et al., 2013). Many of the mitochondrial fission proteins are key to this process, as mutation of them decreases mitophagy. However, if the mitochondrial damage is too extensive, this can lead to apoptosis.

1.8 Intrinsic Apoptosis

Apoptosis is highly regulated by mitochondria through the intrinsic cell death pathway. This apoptotic pathway only proceeds if the MOM becomes permeabilized and releases cytochrome c into the cytosol (Fig 1.3). After an event such as DNA damage, BCL2 family proteins BAX and BAK can cause mitochondrial outer membrane
permeabilization (MOMP). The released cytochrome c activates caspases and initiates the apoptotic cascade (Tait and Green, 2010). Specifically, cytochrome c binds cytosolic protein Apaf-1, triggering formation of an activated caspase-9 complex called the apoptosome (Fig 1.3). The apoptosome then cleaves and activates executioner caspases-3 and -7, ultimately leading to cell death (Monian and Jiang, 2012). Studies have shown that cells deficient in BAX and BAK are resistant to all forms of intrinsic apoptosis. Further studies have also shown that a cytochrome c point mutant (K72A), which retains ETC function but reduces its ability to activate caspases also fails to initiate apoptosis (Hao et al., 2005; Wei et al., 2001). During apoptosis BAX forms rings on the MOM, and it appears to colocalize with mitochondrial fission proteins, such as Drp1 (Grosse et al., 2016). Interestingly, both BAX and Drp1 are required for full cytochrome c release. Therefore, mitochondrial fission plays a key role in the apoptotic response.

1.9 Mitochondrial Dynamics

Mitochondria are not static organelles in the cytosol; rather, they are highly dynamic in nature (Chen and Chan, 2005; Suen et al., 2008). In addition to mobility, these membrane-bound compartments are dynamic because they undergo many division (fission) and joining (fusion) events (Hoppins et al., 2007). Mitochondrial dynamics serves several functions, including sequestering daughter cells during cell division since mitochondria cannot be made de novo, the sharing of mtDNA, mitochondrial quality control (mitophagy) and regulating apoptosis (Chan, 2006; Detmer and Chan, 2007).

Mitochondrial dynamics is a very delicate process, and it must be carefully regulated, and many diseases result from an imbalance in mitochondrial dynamics (Chen and Chan, 2009; Herzig and Martinou, 2008; Karbowski and Youle, 2003; Ong et al.,
Excessive mitochondrial fusion occurs under starvation conditions or mild oxidative stress (Gomes et al., 2011b; Rambold et al., 2011b; Shutt et al., 2012). More commonly, excessive mitochondrial fission is seen in many neurodegenerative disorders such as Alzheimer’s, Parkinson’s and Huntington’s disease. Excessive mitochondrial fission has been termed mitochondrial fragmentation, and contributes to the intrinsic cell death pathway (Fig 1.5). Defects are not limited to the brain, as similar phenotypes are seen during heart disease, optic atrophy, liver disease and several others. Therefore, to better understand these diseases we need to discover the mechanisms and proteins involved in the mitochondrial fission and fusion processes.

1.10 The Dynamin Superfamily of GTPases

The proteins involved in the mitochondrial fission and fusion processes originate from the dynamin superfamily of GTPases. These proteins utilize the energy of GTP hydrolysis to actively remodel cellular membranes (Praefcke and McMahon, 2004) (Daumke and Praefcke, 2016). Thus, they have been termed mechano-chemical enzymes. In 1989, dynamin was first discovered as a microtubule associated protein (Shpetner and Vallee, 1989). The name dynamin originates from the greek word, “dynamis”, which means force or power. Originally thought to mediate microtubule sliding, further studies demonstrated its importance in synapse function. Temperature sensitive mutants of dynamin blocked the release of synaptic vesicles in Drosophila, resulting in paralysis (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). These studies further concluded that dynamin catalyzes the scission of clatherin-coated vesicles at the plasma membrane. Therefore, the discovery of dynamin revealed a new dynamin superfamily of GTPases that contribute to various membrane remodeling events throughout eukaryotic cells.
A common feature of dynamin superfamily members is their ability to assemble into regular oligomers on appropriate templates. One example includes dynamin itself, which can assemble into helical oligomers at the necks of clatherin-coated pits (Takei et al., 1995). Other dynamin family members involved in mitochondrial fission, Drp1, and mitochondrial fusion, Opa1, can also assemble into helical oligomers on membranes (Ingerman et al., 2005; Mears et al., 2011; Ban et al., 2010). These proteins additionally have low affinity for nucleotide; therefore, they do not need guanine nucleotide exchange factors (GEFs) for nucleotide release. However, compared to other GTPases, they display high basal rates of GTP hydrolysis. Most dynamins have basal rates between 1 and 5 min\(^{-1}\) at 37\(^\circ\)C, and these rates cooperatively increase with protein concentration (Daumke and Praefcke, 2016). Additionally, the GTPase rates can be substantially increased in the presence of lipid templates such as DOPS liposomes, sometimes yielding 50-100 fold increases in GTPase activity (Francy et al., 2015; Tuma et al., 1993). Moreover, dynamin superfamily proteins are often inhibited in solution, while assembly on lipid templates relieves those restraints (Francy et al., 2015).

Crystal structures of many dynamin superfamily members, including dynamin, Mx and Drp1 have revealed valuable architectural information about these proteins. The most conserved region of this family is the GTPase domain, in which the core structure resembles the Ras-like G-domain (Niemann et al., 2001a) (Fig 1.4). Additionally, these family members contain alpha helical stalk domains comprised of the middle domain and the GTPase effector domain (GED). Conserved interfaces between adjacent stalks mediate higher order assembly, and several stalk interactions were observed in dynamin (Ramachandran et al., 2007; Reubold et al., 2015a; Zhang and Hinshaw, 2001). These
interactions are believed to be conserved in all other dynamin family members; however, this has not been clearly demonstrated. Lastly, many dynamin family members contain lipid binding domains at the base of the stalk. Dynamin for example, contains a lipid binding pleckstrin homology (PH) domain that inserts into PI containing membranes (Fig 1.4). Other family members like Drp1 possess flexible loops at the base of the stalk, which may associate with only specific lipids like cardiolipin (Bustillo-Zabalbeitia et al., 2014; Macdonald et al., 2014; Stepanyants et al., 2015). In dynamin and Drp1, the VD or PH domain not only play roles in lipid association, but they also regulate Drp1 activity (Clinton et al., 2016; Francy et al., 2015; Strack and Cribbs, 2012). Dynamin also contains a proline rich domain (PRD), which drives interaction with SRC homology 3 (SH3) domains of partner proteins (Fig 1.4). Therefore, it appears that different proteins in the family contain specific regulatory motifs around the mechanochemical core comprised of GTPase-middle-GED interactions. These specific attributes contribute to their cellular function, but it is currently unclear whether, or how, these changes affect the structures and activities of these distinct family members.

1.11 Mitochondrial Fusion Proteins

The known mitochondrial fusion proteins include mitofusin 1 and 2 (Mfn1/2) and optic atrophy protein 1 (Opa1). For complete fusion to occur, both the outer and inner membranes must fuse. Specifically, mitofusins initiate fusion of the MOM where they reside. Mitofusins contain a GTPase domain, middle domain, 2 transmembrane segments and a C-terminal tail (Fig 1.4). Mfn 2 is slightly larger than Mfn 1, with additional sequence present in the GTPase domain. Mutations in Mfn2 are implicated in Charcot-Marie Tooth (CMT) Syndrome type 2A, characterized by neuronal degeneration of long
sensory and motor neurons, while mutations in Mfn1 have not been implicated in disease to this date (Mishra, 2016). There is some redundancy in Mfn1 and 2 function, as single cell line knockouts of either still have residual fusion activity. However, genetic ablation of both results in the complete absence of fusion, and causes clear defects, including the loss of cristae ultrastructure, decreased mitochondrial membrane potential and decreased mtDNA copy number (Mishra, 2016). Thus, Mfn fusion activity is critical for cell health, and defects lead to deleterious consequences associated with excessive fission.

Fusion of the MIM is mediated by Opa1, which can be found in the intermembrane space. The name comes from mutations in the protein that cause autosomal dominant optic atrophy (ADOA), characterized by progressive blindness and the degeneration of retinal ganglion cells and the optic nerve (Alexander et al., 2000). Although Opa1 is also part of the dynamin superfamily, there are some differences in its domain structure. First, it contains an N-terminal mitochondrial targeting signal (MTS) which directs import into the intermembrane space, where it is then cleaved. The N-terminal transmembrane (TM) segment then anchors the protein to the MIM, and this is considered the long form of Opa1. Moreover, 2 proteases (Oma1 and Yme1L) can cleave Opa1 from the N-terminal TM segment, producing a short soluble form. Studies have suggested the long form plays a bigger role in fusion, but this remains unclear (Anand et al., 2014). In addition to fusion, Opa1 has been implicated in apoptosis, cristae ultrastructure and stability of the ETC complexes. Therefore, it is clear that Opa1 plays a critical role in maintaining a healthy balance in fission and fusion events contributing to mitochondrial dynamics.
1.12 Mx Antiviral Dynamin

Another interesting dynamin member is the Mx family of proteins. Mx genes exist in nearly all vertebrate genomes, and they are active mainly against RNA viruses. The discovery of the Mx1 gene was reported 50 years ago (LINDENMANN, 1962) and was based on the resistance of an inbred mouse strain to an influenza virus infection. The resistance of this mouse strain was mapped back to the inheritance of a single gene called Mx1 located on chromosome 16. Therefore, this gene was found to encode for an important antiviral protein termed Mx. Nuclear Mx proteins (Mx1) protect against viruses that replicate in the nucleus, such as influenza virus (Verhelst et al., 2013). There are also cytoplasmic forms of Mx (Mx2) that inhibit the replication of viruses in the cytosol, such as vesicular stomatitis virus. The human MxA variant is cytoplasmic and has a broad antiviral activity. Due to their ability to interact with many viruses, including HIV, Mx proteins are of high physiological interest.

Similar to other dynamins, Mx proteins also contain a GTPase, middle and GTPase effector domain. The GTPase domain is the most conserved portion of the protein, and is believed to function similarly to dynamin. The stalk region, composed of the middle and GED domain, largely mediate oligomerization of Mx proteins (Fig 1.4). Oligomerization of Mx is very important in their antiviral activity, where, upon viral recognition they can oligomerize around the virus and facilitate its expulsion and/or degradation (Verhelst et al., 2013). Although Mx does not contain a consensus lipid-binding domain similar to dynamin (PH domain), it contains viral binding loops in the same position at the base of the stalk. Lysine residues in this stalk region associate with negatively charged lipid membranes, due to electrostatic interactions. This activity is
interesting, because it is consistent with the lipid binding properties of the variable
domain of Drp1, which is also a flexible loop domain. In the cell, Mx proteins utilize
their dynamin oligomeric properties to inhibit viral replication and serve a critical role in
immunology.

1.13 Mitochondrial Fission

As discussed previously, mitochondrial fission is an essential process within the
cell. It functions to segregate daughter mitochondria during cell division, distribute
mtDNA, segregate damaged mitochondria for degradation (mitophagy) and initiate
apoptosis when necessary. Too little mitochondrial fission leads to large interconnected
collapsed mitochondria, which results in lower ATP production and eventual cell death.
Conversely, too much mitochondrial fission leads to mitochondrial fragmentation, and
subsequent apoptosis. Therefore, a mitochondrial fission/fusion equilibrium must be
maintained within the cell (Fig 1.5). In fact, mitochondrial fragmentation is observed in
several diseases as explained earlier. The main mediator of mitochondrial fragmentation
is dynamin related protein 1 (Drp1, Fig 1.6). Moreover, it was shown that inhibition of
Drp1 with chemical inhibitors or peptides reduced over-active mitochondrial fission in
disease cell lines and even improved the motor skills of Huntington’s mouse models
(Cassidy-Stone et al., 2008; Lackner and Nunnari, 2010; Qi et al., 2013; Su and Qi,
2013). Despite these exciting discoveries, we still do not understand the mechanism
driving Drp1-mediated mitochondrial fission.

1.14 Dynamin Related Protein 1 (Drp1)

Drp1 is the key regulator of mitochondrial and peroxisomal fission (Fig 1.6). Drp1 was first discovered in a drosophila genetic screen, where deletion of the gene
product caused paralysis and additional defects in tissues outside the nervous system (van der Bliek and Meyerowitz, 1991). From this work Drp1 was first believed to be involved in vesicular traffic. Later, Drp1 was more accurately identified to play a role in mitochondrial dynamics, specifically mitochondrial fission (Smirnova et al., 1998; Smirnova et al., 2001). The oligomeric properties of Drp1 were described soon after, and this polymeric nature was thought to be critical for Drp1 function in mitochondrial division (Yoon et al., 2001). These initial discoveries sparked an exciting field of research in mitochondrial fission.

The domain architecture provides further clues to Drp1 function. If we take a look at its domain architecture, we notice that Drp1 is an 80 kDa protein that also contains a GTPase domain (Fig 1.4). Here, GTP is hydrolyzed and that energy is essential for membrane remodeling events. Lysine 38 within the GTPase domain was found to critically coordinate the gamma phosphate of GTP for hydrolysis, and mutation of this residue to alanine was found to ablate Drp1 GTPase activity completely (Daumke and Praefcke, 2016; Francy et al., 2015; Yoon et al., 2001) (Fig 1.9). As expected, this lysine is conserved in dynamin (K44A) as well as other small GTPases (van der Bliek et al., 1993). This mutant (K38A) is often used as a dominant negative construct for in vitro and in vivo studies.

While the GTPase domains hydrolyze GTP, the stalk domains are critical for higher order oligomerization of Drp1. Unlike the fusion family members, Drp1 is mostly cytosolic in the cell. In the cytosol it is found as dimers, tetramers or larger oligomeric species (Macdonald et al., 2014). The dimer is likely mediated through a conserved interface between stalks of adjacent Drp1 monomers (Frohlich et al., 2013). Similar to
dynamin and Mx, Drp1 utilizes its stalk domain for initial polymerization. This oligomerization can be initiated in the presence of GTP, negatively charged lipids or both (Fig 1.6B). Two patient mutations in the stalk domain have been linked to neonatal lethality. The first, A395D, was identified in the middle domain (Fig 1.9). This lethal mutation caused microcephaly, abnormal brain development, optic atrophy and lactic acidemia in the patient, who died 37 days after birth (Waterham et al., 2007). Moreover, the patient’s skin fibroblasts revealed hyperfused mitochondria, a hallmark of defective mitochondrial fission. Further investigation of the Drp1 A395D mutant unveiled deficiencies in oligomerization and GTPase activity (Chang et al., 2010). An additional mutation in the middle domain, R403C was recently discovered in patients who experienced normal development for several years before they presented with neurological decline (Fahrner et al., 2016) (Fig 1.9). This mutation reduced Drp1 oligomerization, fission activity and recruitment to the mitochondria, but to a lesser extent than A395D. Nevertheless, disruption of stalk interactions can inhibit Drp1 activity, and severely impact cellular health.

The stalks of Drp1 were believed to initiate several interactions necessary for oligomerization, similar to dynamin. Drp1 self-assembly also initiates stimulated GTPase activity (10-100 fold stimulation) (Francy et al., 2015; Frohlich et al., 2013; Macdonald et al., 2014; Ugarte-Uribe et al., 2014), which can occur in the presence of liposomes. This is believed to occur as a result of G-domain dimerization upon Drp1 oligomerization. However, without a canonical lipid-binding domain like dynamin, the mechanism of Drp1 association with membranes was unclear.
Drp1 contains a VD in place of the PH domain in dynamin. The VD is approximately 100 amino acids and is unstructured compared to the PH domain. Initially, the role of the VD was believed to be a similar lipid-binding motif; however, recent data has demonstrated its role as an autoinhibitory domain as well (Strack and Cribbs, 2012). When a ∆VD construct was transfected into cells, it hyper-polymerized in the cytosol. Therefore, the VD appeared to regulate Drp1 activity and oligomerization in solution, and further studies in our lab explored this phenomenon (Francy et al., 2015).

### 1.15 Cardiolipin Activation of Drp1

Despite the role of the VD in regulation, it also appears to interact with different lipids. Several different liposome compositions were tested for their ability to stimulate Drp1 activity. Previous studies showed that PS stimulates Drp1 activity and polymerization, similar to dynamin (Francy et al., 2015; Frohlich et al., 2013). However, more recently other groups have shown the ability of other lipids, such as CL and phosphatidic acid (PA, precursor to CL), to more robustly stimulate Drp1 GTPase activity (Bustillo-Zabalbeitia et al., 2014; Macdonald et al., 2014; Ugarte-Uribe et al., 2014). It was determined that CL interacts with a lysine patch on Drp1, and mutation of these four lysines ablates stimulated activity (Bustillo-Zabalbeitia et al., 2014). Drp1 was also found to actively cluster CL into microdomains, which may represent localized regions of mitochondrial fission (Stepanyants et al., 2015). In lieu of these exciting findings, the biggest question in the field remained: how does a MIM lipid such as CL translocate to the MOM in order to interact with the cytosolic protein Drp1?

CL can indeed translocate to the MOM. Under normal conditions, approximately 1% of CL is exposed on the MOM. However, under stress conditions CL content on the
MOM can increase up to 15 fold. Specifically stress conditions such as mitophagy, increase CL externalization and increase mitochondrial fission events (Chu et al., 2013). CL undergoes translocation to the MOM through an enzyme called phospholipid scramblase. Knockout of this enzyme ablates the externalization of CL and reduces mitochondrial fission events (Chu et al., 2013). Additionally, CL has been shown to reside at contact sites between the MOM and MIM, where lipid exchange could also occur (Ardail et al., 1990). This raises the possibility that Drp1 is recruited to specific sites on the MOM enriched for CL as a molecular recruitment cue. Therefore, CL is likely accessible for Drp1 interactions under normal and stress conditions. This research raised some interesting questions concerning the ability of CL to stimulate Drp1 activity more robustly than other negatively charged lipids.

CL has unique lipid architecture and is essential for mitochondrial function (Fig 1.8). Moreover, unlike most lipids that contain 2 acyl chains, CL contains 4. Also, most lipids like PS and PA have a single negatively charged head group, while CL has two negatively charged head groups (Fig 1.8). Moreover, CL is known as a cone shaped lipid since it is wider at the base of the molecule due to the splaying of its four acyl chains. As one might expect, cone shaped lipids such as CL induce natural curvature in a membrane (Li et al., 2015b). For example, this is why CL is found concentrated at highly curved regions within the cristae. On the MOM, CL externalization could potentially also induce curvature, which may provide a more suitable template for Drp1 induced mitochondrial fission. In fact, CL externalization on the MOM may be similar to PS externalization on the plasma membrane (Li et al., 2015b). CL could likewise act as a recruitment signal for Drp1 to induce fission, and potential cell death through mitochondrial fragmentation if
necessary. Therefore, there is strong evidence that CL directly interacts with Drp1, however the structural changes associated with this interaction were unknown until recently.

1.16 Dynamin Constriction Mechanism

Many dynamin family members are known as mechanochemical enzymes capable of constricting lipid templates. For dynamin, it has been shown that GTP hydrolysis is required for lipid constriction. Despite this, a dynamin mutant deficient in GTPase activity (K44A) can still achieve a superconstricted state when GTP is added (Sundborger et al., 2014). If we examine the yeast homolog of Drp1, Dnm1, GTP binding stabilizes the protein oligomer while GTP hydrolysis leads to membrane constriction (Mears et al., 2011). Dnm1 is slightly larger than Drp1 (85 kDa), and shares about 45% sequence homology. Furthermore, Drp1 has been shown to constrict membranes upon the addition of GTP (Frohlich et al., 2013; Koirala et al., 2013). However, constriction was also found in conditions where GDP and a non-hydrolyzable GTP analogue (GMPPCP) were added. Therefore, it was unclear whether GTP binding, GDP binding or GTP hydrolysis caused Drp1 constriction of lipid membranes. In our manuscript, we sought to clarify the mechanism of Drp1 induced constriction of lipid membranes. (Francy et al., 2015).

1.17 Drp1 Alternative Splicing

While conducting experiments to determine the constriction mechanism of Drp1, we realized we could choose from several different Drp1 isoforms. In fact, there are up to 8 different Drp1 splice variants that exist in the mammalian cell. These Drp1 isoforms range from 699 to 755 amino acids and are by-products of alternative splicing (Fig 1.9). Drp1 is encoded by 20 exons, and alternative splicing occurs in exons 3, 16 and 17.
Strack et al., 2013; Uo et al., 2009a). Not surprisingly, exons 16 and 17 are found in the dynamic VD. Drp1 isoform 1 contains exons 16 and 17 and is enriched in neurons. Both of these exons together comprise the “B-insert”. Exon 3 is a 13 amino acid region in the GTPase domain that is specifically localized to a flexible region called the 80 loop. Drp1 isoform 6, or Drp1-long, contains exon 3 as well as exon 16 and 17. This isoform is expressed almost exclusively in the post-mitotic neurons of the brain. On the other hand, Drp1 isoform 3 or Drp1-short does not contain any of the alternative exons, and it is ubiquitously expressed in the body. With the understanding that these isoforms likely play distinct functional roles in mitochondrial fission, we sought to characterize the Drp1 isoforms biochemically (Macdonald et al., 2016).

1.18 Drp1 Post Translational Modifications

In addition to alternative splicing, Drp1 is regulated by post-translational modifications. Simple overexpression of Drp1 is not believed to induce mitochondrial fission alone. Instead, post-translational modifications (PTMs) are believed to regulate Drp1 properties such as mitochondrial translocation, higher order assembly and GTPase activity by fine tuning Drp1 activity. Studies have found Drp1 is phosphorylated, nitrosylated, sumoylated, ubiquitinated and modified by O-GlcNACylation (Fig 1.9). Some modifications serve to increase Drp1 activity and mitochondrial fission, while others will inhibit fission activity. Interestingly, almost all modifications occur in the flexible VD domain, further supporting its important regulatory role.

Phosphorylation is the most well studied PTM in Drp1. Cdk1/cyclin dependent kinases phosphorylate Ser616 in the VD during mitosis to induce fission and segregation of mitochondria into daughter cells (Taguchi et al., 2007). Other than endogenous
functions, phosphorylation of Ser616 is also seen in hypertension induced brain injury. Protein kinase Cδ phosphorylates Ser616, which leads to mitochondrial fragmentation and neuronal cell death. Conversely, cAMP-dependent protein kinase A (PKA) phosphorylates Ser637 in the VD and inhibits mitochondrial fission (Chang and Blackstone, 2007; Cribbs and Strack, 2007). This modification was shown to interfere with intra-molecular interactions between GTPase and GED domains, which leads to decreased GTPase activity and decreased Drp1 recruitment to mitochondria. This is observed under starvation conditions, leading to mitochondrial elongation (Gomes et al., 2011a; Rambold et al., 2011a). In some instances both modifications will work together (Ser616 phosphorylated and Ser637 dephosphorylated) to further augment mitochondrial fission through Drp1 regulation.

Other PTMs that have also been characterized are S-nitrosylation, sumoylation, ubiquitination and O-GlcNAcylation. In Alzheimer’s disease, beta-amyloid proteins stimulate NO production and cause S-nitrosylation of Drp1 at Cys644 within the GED domain (Cho et al., 2009; Nakamura et al., 2010) (Fig 1.9). This enhances Drp1 GTPase activity, oligomer formation and causes excessive mitochondrial fission in neurons, leading to neuronal damage. Mutation of this Cys644 prevents mitochondrial fragmentation and the associated neurotoxicity. Furthermore, sumoylation of Drp1 by SUMO E3 ligase stimulates mitochondrial fission (Wasiak et al., 2007), while the consequences of ubiquitination on Drp1 remain unclear. Recently a new Drp1 PTM was discovered, as O-GlcNAcylation was identified as Thr585 and Thr586 in the VD of Drp1 when cells were subjected to (Gawlowski et al., 2012) high glucose conditions (type 2 diabetes), which induced mitochondrial fragmentation. Overall, researchers are still
trying to understand how these multi-site PTMs coordinate with one another to regulate mitochondrial fission.

1.19 Drp1 Structural Studies

Fortunately, over the last 5 years, some very insightful structural studies of Drp1 have been published. The first cryoelectron microscopy (cryoEM) structure of the yeast homolog of Drp1, Dnm1 was discovered in 2011 (Mears et al., 2011). It unveiled the overall domain architecture of Drp1, to a resolution of about 30 Å. Similar to dynamin, Dnm1 formed a characteristic T-structure on the surface of the lipid, with the strong peripheral density pertaining to the G-domains, and the strong middle density representing the stalk domains. The helical structure consisted of 24 subunits per turn, and it was a 2-start helix. This was surprising as dynamin was determined to form a 1-start helical structure (Zhang and Hinshaw, 2001), although a 2-start symmetry was later observed for a super-constricted state of the dynamin polymer (Sundborger et al., 2014). The 2-start helix in Dnm1 was believed to enable additional G-domain contacts between rungs of the helix, but the significance of the 2-start geometry was largely unknown. Interestingly, a gap between the protein and the lipid surface was clearly evident, and this was attributed to the flexible VD transiently interacting with the lipid bilayer (Mears et al., 2011). Overall, the gap was a puzzling observation that suggested a relatively weak interaction between Dnm1 and the lipid bilayer, so additional studies were required to provide further insight.

The crystal structure of Drp1 offered more molecular detail for the protein. A Drp1 tetramer was found as the repeating subunit, and with high resolution they were able to discover critical interaction interfaces within the multimer (Frohlich et al., 2013).
When compared to previous dynamin structures (Chappie et al., 2011), a conserved interface (Interface 2) was discovered, which consists of hydrophobic interactions between the stalk of two Drp1 monomers. This interface was shown to be essential to Drp1 function, as mutagenesis in this region abolished Drp1 oligomerization and activity. Additional interfaces in the stalk, which where found in dynamin, were not observed in the Drp1 structure. However, a novel interface 4 was discovered and was proposed to mediate an additional stalk interaction between dimers. Mutagenesis of interface 4 did greatly reduce mitochondrial fission in cells, but \textit{in vitro}, the effect on Drp1 oligomerization was less clear. Therefore, the relevance of this interface is still under debate.

In order to crystallize Drp1, considerable mutagenesis was necessary. Most notably, the VD (~100 aa) was deleted, as the flexibility of this domain caused considerable crystallization issues (Frohlich et al., 2013). Next, a region near the bottom of the stalk was also mutated to a polyalanine stretch (GPRP-AAAA). This limited Drp1 to a dimer, and did not allow further oligomerization. Therefore, these deletions significantly altered the self-assembly properties of Drp1, and the need for structural insight with the full-length molecule was apparent.

Using the Dnm1 cryo-EM density map, the same group proposed a helical Drp1 model by fitting the crystal structure. The model was very dynamin-like, in the sense that it was tightly packed. Drp1 stalks were juxtaposed amongst one another to accommodate several stalk interfaces, and due to the limiting resolution, it was difficult to make these assumptions (Frohlich et al., 2013). GTPase domains dimerized across rungs of the helix much like dynamin; therefore, it was assumed Drp1 would have a similar architecture
because it is a related family member. However, our cryo-EM structures with Drp1 have demonstrated a very novel helical architecture, where interface 2 and G-domain dimers are the minimal requirements for Drp1 lipid polymerization (see Ch. 4).

### 1.20 Additional Mitochondrial Fission Partner Proteins

Although Drp1 is considered the main mediator of mitochondrial fission, there are other adaptor proteins involved in the fission process. In yeast mitochondrial fission studies, Dnm1 requires a 17 kDa MOM protein, Fission protein 1 (Fis1), as well as the adaptor proteins Mitochondrial division 1 (Mdv1) and CCR4-associated factor 4 (Caf4), to initiate mitochondrial scission (Suzuki et al., 2003). Therefore, Fis1, which is conserved in mammals, was also believed to play a key, singular role in mammalian mitochondrial fission. However, knockout of Fis1 only slightly reduces mitochondrial fission in mammalian cells, and there are no homologs of Mdv1 or Caf4 (Richter et al., 2015). Further studies showed that Fis1 might play a more important role in mitophagy rather than canonical fission (Shen et al., 2014). Subsequently in mammals, additional partner proteins involved in the fission process were discovered.

In an attempt to find other partner proteins, an RNAi library search was conducted, and Mitochondrial fission factor (Mff) knockdown caused mitochondrial elongation (Breckenridge et al., 2003; Richter et al., 2015). Conversely, overexpression of Mff led to increased mitochondrial fission, thus leading to the discovery of the first adapter protein that recruits Drp1 independently of Fis1. Mff is a ~28 kDa protein that contains a C-terminal TM segment, while the rest of the protein is largely unstructured based on secondary structure analyses. Studies in our lab show that Mff stimulates Drp1 GTPase activity under certain conditions (Clinton et al., 2016). Cellular studies also show
that Mff colocalizes with Drp1 foci on the mitochondria (Breckenridge et al., 2003). Therefore, it is clear that Mff plays a key role in mammalian mitochondrial fission with Drp1, and more biochemical and structural data are needed to further elucidate this interaction in the future.

Mitochondrial dynamics proteins of 49 and 51 kDa (MiD49, MiD51) were also discovered as part of a mitochondrial dynamics screen (Gad et al., 2000). MiD49 and MiD51 share 45% sequence homology and are N-terminally anchored to the MOM through a TM segment. The cytosolic portion consists of a disordered region and a cytosolic domain. Within the cytosolic loop there is a region responsible for the interaction with Drp1, termed the Drp1 recruitment region (DRR). The role of both proteins in augmenting or blunting mitochondrial fission was unclear initially, however recent studies have demonstrated the contribution of both MiD49/MiD51 in initiating Drp1 driven mitochondrial fission (Loson et al., 2013). Another group has shown that binding ADP promotes Drp1 assembly into spirals and enhances GTP hydrolysis (Loson et al., 2014). Thus, mitochondrial metabolism may influence the association of both proteins and ATP/ADP levels may thereby regulate mitochondrial fission efficiency. Research on the individual Drp1 partner proteins has been insightful, but how all these proteins work together on the mitochondria to initiate Drp1 driven mitochondrial fission remains to be answered.

1.2 Electron Microscopy of Drp1

Due to the propensity of Drp1 to oligomerize into large polymeric complexes, Electron Microscopy (EM) is a well-suited technique for observing Drp1 structural features. First, negative stain EM is used to screen samples using a Transmission Electron
Microscope (TEM). A negative image in photography refers to the object of interest appearing white on a dark background, and this holds true for the corresponding EM image as well. A heavy metal stain, such as uranyl acetate, coats the carbon support around the protein, resulting in a high contrast image. The oligomeric propensity of Drp1 is clearly seen using negative stain EM. Individual rungs of protein in the helix can be observed, and the diameters of nucleotide induced spirals and protein lipid tubules can be measured (Fig 2.1). Negative stain EM is also a relatively quick method for evaluating various conditions as sample preparation and insertion into the microscope takes little time (10 minutes total), making it optimal for screening grids. However, negative staining can alter the sample through flattening, drying artifacts and uranyl acetate crystal contamination.

Cryogenic-EM ameliorates many of the problems observed with negative stain EM. In cryo-EM, there is no stain present, as the sample is prepared and frozen in its initial buffer. Without the drying of a heavy metal stain, the protein-lipid tubes stay hydrated and will not flatten. These images provide much more detail, as there is no stain obscuring the sample. Therefore, the best helical diffraction patterns are obtained with cryo-EM images. This technique also allows us to accurately distinguish the lipid bilayers from the associated protein, leading to more precise diameter measurements. The drawbacks of cryo-EM include increased time before imaging (30-60 minute sample preparation, 90 minute sample insertion and microscope cooling), ice contamination (from local warming) and lower contrast due to the low electron dose conditions required to prevent beam-induced damage to the sample. Therefore, low contrast cryo-images must be averaged to overcome the low signal-to-noise ratio. Numerous cryo-EM images
of Drp1 oligomerized on lipid nanotubes allowed us to use helical processing software to determine the 3-D structure of these complexes.

1.22 Helical Reconstruction Image Processing

Image processing is the next step in the 3D reconstruction process. It begins with Contrast Transfer Function (CTF) correction of all suitable cryo micrographs using TOMO-CTF (Fernandez et al., 2006). CTF correction allows us to obtain lost information based on the defocus setting of the microscope. The wave function of the electrons interacting with the sample can be inverted to help obtain this lost information. With the images CTF corrected, the Drp1 helices are boxed and these helices are further partitioned into isolated square boxes representing particle images obtained from overlapping segments of each helix. The particles can then be sorted using supervised or non-supervised classification methods in an effort to obtain uniform datasets with reduced heterogeneity, as the diameters of the protein lipid tubules have been shown to vary throughout these samples.

Once a uniform dataset is obtained, an Iterative Helical Real Space Reconstruction (IHRSR) method can be used to reconstruct the 3-D helical structure (Egelman, 2007; Egelman, 2010). IHRSR is a program that can analyze and reconstruct single-particle EM images of helical segments into a 3D structure. The limitation is that an initial best guess for the helical symmetry is required, though the program does refine these helical parameters over iterative cycles to best match the data. In our case, we use a featureless cylinder as our starting model, from which reference projections, or images, are generated (Fig 1.10). The raw data (particles) are then aligned to these reference projections, and images within each projection class are averaged. These average
projections are then back projected to create a non-symmetrized 3D volume. Predetermined helical parameters, including the angular rise/subunit (Δφ) and axial rise distance between subunits (Δz), are applied to the 3D volume during initial iterations, and these values are refined in subsequent steps to sample new helical symmetries. Once these values converge, the final helical symmetry is imposed to create a symmetric 3D volume (Fig 1.10).

Furthermore, IHRSR can also be run to account for out of plane tilt. This assumes that protein helices are not perfectly flat in the ice, and tilts in the polymer can cause distortions in the images. To correct for out of plane tilt, additional reference projections of a tilted starting volume are used. The additional reference projections add a considerable amount of computational time, however, experts in the field have noted that out of plane tilt is a critical component of the helical reconstruction process (Egelman, 2014). Not implementing out of plane tilt can cause smearing of data, and lead to incorrect reconstructions. All of these EM techniques were utilized to explore the function and structure of Drp1.

It is clear from previous research that Drp1 plays a critical role within the cell. Despite this, there are still many questions concerning the biochemical and structural properties of Drp1. In chapter 2, we discuss our findings on the Drp1 constriction process as well as novel functions of the Drp1 variable domain. In chapter 3, we explored the functional consequences of endogenous sequence insertions in the VD and the GTPase domain. Lastly, in chapter 4, we explored the helical structural properties of full-length Drp1 on different lipid templates. This research answers many of the critical questions in the field, and offers new insights for future studies.
FIGURE 1.1

A

Mitochondrial outer membrane

Mitochondrial intermembrane space

Contact site

MtDNA

Mitochondrial matrix

Mitochondrial inner membrane

B

Mitochondrial Outer Membrane

PE

PC

PI

PS

CL

Mitochondrial Inner Membrane

3%

18%

5%

40%

34%
FIGURE 1.1 Mitochondrial Architecture and Lipid Composition

(A) The main structural components of the mitochondria. (B) The lipid composition of the outer and inner mitochondrial membranes (Daum). Note the increased cardiolipin (CL) content in the inner mitochondrial membrane. PE- phosphoethanolamine PC- phosphocholine PI- phosphoinositides PS- phosphoserine. Adapted from Horvath and Daum, 2013.
FIGURE 1.2 Mitochondrial Generated Reactive Oxygen Species

High energy electrons from the ETC (complex I and III) generate reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) which can cause damage in the cell. Manganese superoxide dismutase (MnSOD) converts O_2^- to H_2O_2, and enzymes such as catalase and glutathione peroxidase convert hydrogen peroxide to water and dioxygen.
FIGURE 1.3

Mitochondrial Intrinsic Apoptosis

Cellular insult such as DNA damage can activate the intrinsic apoptosis pathway. This leads to BH3-only activation, oligomerization of Bax and Bak, mitochondrial outer membrane permeabilization (MOMP), cytochrome c release and activation of the apoptosome. This signalling cascade initiates cell death. Drp1 has been shown to colocalize with Bax.
FIGURE 1.4 Dynamin Family Architecture

Separate dynamin family members and their domains are shown. The family members shown here all contain GTPase (green) and middles domains (blue). All except Mfn 1/2 also contain GTPase Effector Domains (GED, blue). Dynamin initiates vesicle endocytosis, and contains a lipid interacting pleckstrin homology (PH) domain, as well as a protein interacting proline rich domain (PRD). Drp1 is involved in mitochondrial and peroxisomal fission, and contains a regulatory and lipid interacting variable domain (VD). MxA oligomerizes around viruses to inhibit their activity. Opa1 is involved in MIM fusion and contains a mitochondrial targeting sequence (MTS) and a transmembrane (TM) domain. Mfn 1 and 2 are closely related MOM fusion proteins and contain two transmembrane segments.
FIGURE 1.5 The Mitochondrial Dynamics Balance of Fission and Fusion

In healthy cells, mitochondrial fission and fusion is balanced. Mitochondrial fission is primarily driven by Drp1 (green circles), while mitochondrial fusion is driven by Mfn 1/2 (dark blue circles) and Opa 1 (magenta circles). Overactive fission leads to cytochrome c release and cell death in many diseases, while excessive fusion leads to large interconnected dysfunctional mitochondria.
FIGURE 1.6 Drp1 Mediated Mitochondrial Fission

A cartoon demonstrating Drp1 oligomerization around the mitochondria and subsequent fission.
FIGURE 1.7 **Drp1 Oligomeric Properties**

(A) The crystal structure of Drp1 (PDB ID= 4BEJ) with the GTPase (green), stalk (blue) and variable domain (VD) labelled. (B) A cartoon of a Drp1 dimer forming helical complexes in the presence of GTP and lipid.
FIGURE 1.8 Lipid Structure

(A) A comparison of PS, PE and CL lipid structure. CL contains a net charge of -2, as well as a conical shaped architecture (wider base) due to its four acyl chains. PS is negatively charged, while PE is net neutral. (B) Membrane topologies formed by each lipid. PS forms malleable bilayers due to charge repulsion, while PE forms more stable bilayers without significant charge repulsion. Due to the charge and conical shape CL forms curved bilayers.
FIGURE 1.9 Post Translational Modification, Alternative Splicing and Key Mutations of Drp1

In the GTPase domain, the K38A mutation renders Drp1 inactive, there is also an alternatively spliced exon located here. In the middle domain the patient mutations A395D and R403C inhibit Drp1 fission activity, and lead to developmental defects. In the VD there is a larger alternatively spliced region. O-GlcNAcylation of T585/586 and phosphorylation of S616 activates Drp1 fission activity in cells, while phosphorylation of S637 inactivates Drp1 fission activity. Nitrosylation of cysteine 644 is believed to activate Drp1 activity in Alzheimer’s disease.
FIGURE 1.10 A Schematic Diagram of the Iterative Helical Real Space (IHRSR) Algorithm

A starting volume (grey featureless cylinder), is rotated by 4° increments to create 90 reference projections. The helical particles are then aligned to the reference projections according to certain parameters. The reference projections are then back projected to create an asymmetric 3D volume. Helical parameters are then applied and IHRSR searches close to these parameters to create a helically symmetric 3D volume. This cycle is repeated until there are no changes in helical symmetry. Figure adapted from Egelman, 2007.
CHAPTER 2: THE MECHANOENZYMATIC CORE OF DYNAMIN-RELATED PROTEIN 1 COMPRIS ES THE MINIMAL MACHINERY REQUIRED FOR MEMBRANE CONSTRUCTION

This chapter was previously published:

Christopher A. Francy#, Frances J.D. Alvarez#, Louie Zhou,
Rajesh Ramachandran and Jason A. Mears.

The Mechanoenzymatic Core Of Dynamin-Related Protein 1 Comprises The Minimal Machinery Required For Membrane Constriction.

J Biol Chem. 2015, 290, 11692-11703
2.1 ABSTRACT

Mitochondria are dynamic organelles that continually undergo cycles of fission and fusion. Dynamin-related protein 1 (Drp1), a large GTPase of the dynamin superfamily, is the main mediator of mitochondrial fission. Like prototypical dynamin, Drp1 is composed of a mechanoochemical core consisting of the GTPase, middle and GED regions. In place of the pleckstrin homology (PH) domain in dynamin, however, Drp1 contains an unstructured variable domain (VD), whose function is not yet fully resolved. Here, using time-resolved EM and rigorous statistical analyses, we establish the ability of full length Drp1 to constrict lipid bilayers through a GTP hydrolysis dependent mechanism. We also show the variable domain limits premature Drp1 assembly in solution and promotes membrane curvature. Furthermore, the mechanoochemical core of Drp1, absent of the variable domain, is sufficient to mediate GTP hydrolysis-dependent membrane constriction.
2.2 INTRODUCTION

Mitochondria are responsible for many cellular processes, including the generation of ATP and mediating apoptosis. These roles are directly linked to the dynamic nature of mitochondria (Chen and Chan, 2005; Suen et al., 2008), which undergo continual cycles of fission and fusion in healthy cells (Hoppins et al., 2007). Mitochondrial fission is necessary to evenly distribute and segregate mitochondria into daughter cells during cell division, to remove dysfunctional segments of mitochondria targeted for mitophagy, and to initiate apoptosis (Chan, 2006; Detmer and Chan, 2007). Moreover, excessive fission is associated with the release of apoptotic proteins that promote cell death in several human diseases (Herzig and Martinou, 2008; KARBOWSKI and YOULE, 2003; Perfettini et al., 2005), including cardiovascular and neurodegenerative disorders (Chen and Chan, 2009; Ong et al., 2010). Small compounds and peptides that directly inhibit mitochondrial fission can limit cell death and these have been proposed as future therapies (Cassidy-Stone et al., 2008; Lackner and Nunnari, 2010; Qi et al., 2013; Su and Qi, 2013). To further advance our knowledge of mitochondrial dynamics, the fundamental mechanism of mitochondrial fission must be more thoroughly understood.

In the mammalian cell, Drp1 is the main mediator of mitochondrial fission (Smirnova et al., 1998; Smirnova et al., 2001). Drp1 is part of the dynamin family of proteins and contains four distinct domains: the GTPase domain, middle domain, variable domain (VD) and the GTPase effector domain (GED) (Fig 2.1A). GTPase activity is essential for Drp1 function, and an alanine mutation at the conserved lysine in the GTPase domain (K38A) inhibits Drp1 activity and mitochondrial division (James et al., 2003; Yoon et al., 2001). The middle and GED domains promote self-assembly through oligomerization
interaction interfaces (Frohlich et al., 2013). The role of the VD is currently debated, but recent studies have identified a role in lipid interactions (Bustillo-Zabalbeitia et al., 2014; Ugarte-Uribe et al., 2014). Collectively, these domains work in concert to promote cycles of protein assembly and disassembly at sites of membrane remodeling. The enzymatic activity of Drp1 is proposed to mediate conformational changes within these oligomeric protein complexes that promote membrane scission.

In solution, Drp1 exists as a mixture of predominantly dimers and tetramers, which correspond to the cytosolic forms of the protein (Macdonald et al., 2014). *In vitro* interactions with GTP and lipids lead to the assembly of larger oligomers that represent the macromolecular fission machinery (Ingerman et al., 2005). Specifically, Drp1 forms large oligomers in the presence of non-hydrolyzable GTP analogues (Ingerman et al., 2005; Koirala et al., 2013; Macdonald et al., 2014; Yoon et al., 2001). The addition of Drp1 to negatively charged liposomes also leads to oligomerization, forming protein-lipid tubules (Frohlich et al., 2013; Yoon et al., 2001).

It is unclear how Drp1 associates with membrane bilayers, as bioinformatics analyses find no apparent lipid-binding domain in the Drp1 sequence when compared to the other dynamin family members. For dynamin, a pleckstrin homology (PH) domain interacts with phosphatidylinositol-4,5-bisphosphate (PIP2) at the plasma membrane. An analogous lipid-interacting role has been proposed for the Drp1 VD (Zhang et al., 2011), but no structural information is available for this unique sequence. The VD has also been proposed to function as an autoinhibitory domain of oligomerization based on cellular studies (Zhang et al., 2011). Therefore, Drp1 assembly and subsequent fission events are
likely regulated by interactions between the VD and the mitochondrial outer membrane. Still, it is not clear whether the VD is a vital component of the fission machinery.

As stated, the GTPase activity of Drp1 is essential to mediate mitochondrial fission. Several dynamin family members, including Drp1, have the ability to constrict lipid bilayers upon the addition of GTP (Koirala et al., 2013; Mears et al., 2011; Sweitzer and Hinshaw, 1998). For dynamin, it has been shown that GTP hydrolysis is required for lipid constriction (Danino et al., 2004; Sweitzer and Hinshaw, 1998). However, it has also been shown that a dynamin mutant deficient in GTPase activity can achieve a super-constricted state when GTP is added (Sundborger et al., 2014). For the yeast homolog of Drp1, Dnm1, it has been shown that GTP binding stabilizes the protein oligomer on lipid bilayers and GTP hydrolysis leads to membrane constriction (Mears et al., 2011). Similarly, Drp1 has been shown to constrict lipid bilayers upon the addition of GTP (Frohlich et al., 2013; Koirala et al., 2013). However, “constriction” was also noted in conditions where either GDP or a non-hydrolysable GTP analogue was added (Frohlich et al., 2013). Therefore, the separate roles of nucleotide binding and hydrolysis in mediating Drp1 constriction remain undefined.

In this study, we examine the cycle of Drp1 assembly, constriction and release from lipid bilayers. We show that Drp1 interactions with lipid templates yield tubular structures with a broad distribution of diameters that are stabilized upon GTP binding. To address the independent roles of GTP binding and subsequent hydrolysis in Drp1 constriction, we use time-resolved EM to examine changes to Drp1-lipid tube morphologies. While GTP binding stabilizes the protein oligomer, GTP hydrolysis is required for maximal constriction of the underlying lipid bilayer. These studies also
suggest that Drp1 undergoes cycles of disassembly and reassembly on the lipid template. We also found that removal of the VD does not impair membrane-dependent Drp1 self-assembly nor membrane constriction, which suggests that the mechanoenzymatic core of Drp1 is sufficient for membrane remodeling \textit{in vitro}. Moreover, we show that the VD acts as a negative regulator of Drp1 self-assembly by preventing the formation of large, functionally diminished oligomers in solution. The VD also regulates the curvature of Drp1 oligomers formed on lipid templates, which affects subsequent constriction. Collectively, these results highlight distinct conformational states of Drp1 oligomers that drive cycles of protein assembly and constriction to promote mitochondrial membrane fission.

\textbf{2.3 RESULTS}

\textit{Drp1-lipid oligomers constrict within minutes of GTP addition.}

Full-length human Drp1 was expressed with a CBP-tag and purified for our studies. Arginine was included in the purification buffer to suppress aggregation (Tsumoto et al., 2004). This modification of the purification protocol improved the amount of protein in the soluble fraction and also aided in resolving a homogenous Drp1 population from larger aggregates during gel filtration. The protocol was critical in isolating a soluble and active pool of protein for all Drp1 constructs in this study. WT Drp1 was examined using negative stain EM (Fig 2.1A), and electron densities corresponding to protein complexes were found uniformly distributed on the EM grid.

Consistent with previous studies (Bustillo-Zabalbeitia et al., 2014; Macdonald et al., 2014), we found that Drp1 favored oligomerization in the presence of negatively charged lipid preparations. In our studies, DOPS was found to induce the formation of more
extended and abundant Drp1 oligomers when compared to other preparations with negatively charged lipids. In fact, the CBP-tagged Drp1 exhibited a high propensity to oligomerize on DOPS liposomes (Fig 2.1B), and also displayed a higher basal and lipid stimulated activity (Fig 2.1C) compared to untagged Drp1 (Frohlich et al., 2013). This was advantageous as it afforded us the opportunity to examine a large population of polymers and perform rigorous statistical analyses to evaluate changes in polymer morphology. Therefore, DOPS was a suitable template for our time resolved EM measurements.

Although Drp1 has previously been demonstrated to constrict synthetic membrane bilayers in a nucleotide dependent manner *in vitro* (Frohlich et al., 2013; Koirala et al., 2013), a rigorous statistical examination of the membrane curvatures generated under various nucleotide-bound states has not been performed. Interactions with GTP have been proposed to promote conformational changes in Drp1 assemblies on lipid templates (Frohlich et al., 2013; Koirala et al., 2013; Mears et al., 2011; Yoon et al., 2001). To examine the specific role of nucleotide interactions in Drp1 constriction and disassembly, a series of experiments were performed using various guanine nucleotides.

To begin, EM images were acquired and the diameters of protein-lipid tubules were measured at discrete time points before and after nucleotide addition. Before GTP was added (0 min), lipid-bound Drp1 oligomers displayed a broad distribution of diameters that averaged $60 \pm 12$ nm (Fig 2.1D, Table 2.1). Upon further examination, WT Drp1 is capable of sampling larger diameters (Figure 2.1D). This trend was calculated as a measure of skewness (Table 2.1), which defines the asymmetry of a distribution of variables about its mean. For skewed distributions, the median can be a more accurate
measurement of central tendency. Therefore, we have also included median values in Table 2.1. For WT Drp1-lipid tubes, the distribution is left-tail limited because the lipid template resists curvature generated by the protein. In fact, diameters under 40 nm were not observed (highlighted by dashed gray lines, Fig 2.1).

Upon the addition of GTP, immediate constriction of Drp1-lipid oligomers was observed (a 11 nm decrease on average after 1 min, Fig 2.1G). Five minutes after addition of GTP, the diameter of the Drp1-lipid oligomers decreased considerably to an average diameter of $39 \pm 9$ nm (a 22 nm decrease, Fig 2.1, E & G). It should be noted that roughly two-thirds of the measured diameters were less than 40 nm (dashed lines, Fig 2.1), a diameter sufficiently narrow to place juxtaposed lipid bilayers under considerable strain (Kozlovsky and Kozlov, 2003; Sundborger et al., 2014). Therefore, the addition of GTP to Drp1 oligomers promotes significant constriction of the underlying lipid template.

Overall, these measurements represent a distribution of the total population of oligomers observed during the experiment. Qualitatively, there appeared to be fewer tubes to measure after GTP addition, which is consistent with Drp1 dissociation after GTP hydrolysis. Therefore, Drp1 oligomers constrict upon GTP hydrolysis to narrow the underlying lipid tubule diameter to promote membrane fission. Subsequent conformational rearrangements lead to Drp1 release, initiating additional dynamic cycles of Drp1 assembly and disassembly, which has been observed previously with other dynamin family members (Danino et al., 2004; Mears et al., 2011). This trend is evident as Drp1-lipid oligomers decrease in diameter initially, and then recover over time (Fig
After 60 minutes, Drp1-lipid oligomers recovered to a larger average diameter of 53 ± 11 nm (Fig 2.1, F & G).

**GTP hydrolysis is required for Drp1 induced constriction of lipid bilayers.**

To date, it is unclear whether GTP binding, GTP hydrolysis or GDP binding are required for Drp1 constriction of lipid bilayers. Therefore, we tested each distinct condition and examined Drp1-lipid tube morphologies.

The post-hydrolysis product, GDP, was added to preformed Drp1-lipid tubules. GDP interactions appeared to have little effect on WT Drp1-lipid tube morphology (average diameter of 59 ± 7 nm 15 min after addition, Fig 2.1E). Fewer large Drp1-lipid tubes were observed compared to WT Drp1 + DOPS alone, which results in a more symmetric distribution (decreased skewness value, Table 1).

To mimic the effect of GTP binding, GMPPCP, a non-hydrolyzable analog of GTP, was added to preformed lipid tubes. Fifteen minutes after GMPPCP addition, the protein-lipid oligomers averaged 51 ± 7 nm in diameter (Fig 2.1, D & F, Table 2.1). This decrease in average diameter was in part due to the lack of larger protein-lipid tubes, and also due to a conformational stabilization around 50 nm that may prime the polymer for constriction. This observed stabilization is further supported by the measured decrease in standard deviation (Table 2.1).

While this average size is significantly less than the 60 nm starting diameter, it did not approach the narrow diameters observed when GTP hydrolysis was permitted (Fig 2.2G). Indeed, none of the measured diameters were less than 40 nm (dashed line, Fig 2.2F). Comparatively, 15 minutes after GTP addition, preformed WT Drp1 lipid tubes achieved maximal constriction that was 13 nm narrower than when GMPPCP was added.
(38 ± 7 nm average diameter, Fig 2.2, D & G). Therefore, GMPPCP stabilized the conformation of the Drp1-lipid oligomers over time, but a sizable constriction of the lipid template was not observed (Fig 2.2D).

To better understand the mechanism of Drp1 constriction, Drp1 K38A, a GTPase defective mutant was utilized. The well-characterized alanine mutation at lysine 38 (K38A) results in a GTP hydrolysis-deficient mutant due to an inability to coordinate the γ-phosphate of the nucleotide (Yoon et al., 2001). Analogous mutations in other dynamin family members have a similar defect (Damke et al., 1994), and hydrolysis is prevented even though GTP binding occurs under saturating conditions (Damke et al., 1994; Naylor et al., 2006). In this study, Drp1 K38A was found to interact with GTP-agarose beads similar to WT, which is consistent with these previous findings that Drp1 K38A can bind but not hydrolyze GTP (data not shown).

When examined by EM, K38A in solution forms small oligomers that are distributed throughout the EM grid similar to WT (Fig 2.2B). In the presence of DOPS, K38A readily oligomerized and tubulated the membrane template as well (Fig 2.2C). The protein-lipid tubes were nearly identical in size when compared to WT (60 nm average for WT vs. 61 nm average for K38A lipid tubes, Table 2.1).

To test the hypothesis that GTP hydrolysis is required for constriction, Drp1 K38A-lipid tube diameter was monitored after addition of GTP. Drp1 K38A-lipid tubes did not constrict in the presence of GTP (62 ± 8 nm average diameter at 15 min; Fig 2.2, D & J and Table 2.1). GMPPCP was also added to K38A, and the conformational stabilization effect was not observed, as K38A lipid tubes maintained an average diameter of 59 nm (Fig 2.2I and Table 2.1).
Deletion of the variable domain (VD) leads to Drp1 hyperoligomerization.

In order to study the role of the VD in mediating membrane constriction, a ΔVD Drp1 mutant was isolated (Fig 2.3A). Again, the arginine purification protocol was critical in promoting the solubility of the mutant protein. We found that, at high concentrations, ΔVD began to precipitate in solution. Therefore, ΔVD was purified and analyzed at lower concentrations (1 µM) than had been used in previous studies (Frohlich et al., 2013).

A sedimentation assay was used to measure the oligomerization of WT, K38A and ΔVD Drp1 in solution and in the presence of lipids. Interestingly, ΔVD pelleted much more (76%) than WT Drp1 (16%) and K38A (12%) in solution (Fig 2.3B), which suggested that the mutant forms larger species. Negative stain EM confirmed this hypothesis as ΔVD was found to form ordered filamentous structures in solution (Fig 2.3C). Therefore, removal of the VD promotes premature Drp1 oligomerization in solution.

ΔVD mutant tubulates and constricts lipid bilayers.

Sedimentation of WT Drp1 (70%) and K38A (68%) was higher in the presence of DOPS than alone in solution, which is consistent with the formation of large protein-lipid tubes observed by EM (Figs 2.1B and 2.2C). Interestingly, ΔVD sedimentation was also elevated (96 ± 3%) when DOPS was added. To determine the cause of this increase, EM was used to visualize the sample and ΔVD-lipid tubes were observed (Fig 2.3D).

Interestingly, ΔVD Drp1 formed protein lipid tubules that were larger on average compared to WT (82 ± 25 nm, Table 2.1) and displayed an even broader distribution of diameters (Fig 2.3E), which is evident from an increased standard deviation and skewness value (Table 1).
First, the effect of GTP binding on the larger ∆VD lipid tubes was tested with GMPPCP incubation. After fifteen minutes, the protein lipid tube diameters decreased approximately 19 nm (63 ± 15 nm, Table 2.1). Similar to WT Drp1, we believe this change is due to a conformational stabilization, which is further supported by a large decrease in the measured standard deviation. Therefore, GTP binding stabilizes the ∆VD Drp1 oligomer on the lipid template.

Separately, GTP was added to determine whether this mutant contains the basic functional components needed to constrict a lipid template upon hydrolysis. Five minutes after GTP addition, the ∆VD-lipid tubes constricted to an average diameter of 47 ± 6 nm (Fig 2.3F). The magnitude of this constriction was larger than WT (a 30 nm difference versus a 22 nm change, respectively), but a large majority of oligomers did not constrict below 40 nm (94% greater than 40 nm; dashed line, Fig 2.3F), unlike WT Drp1 (30% greater than 40 nm). Therefore, the constriction machinery remained intact, but the final dimensions of ∆VD constriction were larger owing to the increased starting diameter.

**WT and ∆VD Drp1 Self-assemble on Liposomes Containing Cardiolipin.**

To ensure that Drp1 interactions with lipid are not exclusive to DOPS liposomes, WT and ∆VD Drp1 were added to cardiolipin (CL)-containing lipid templates (CL-mix). Both DOPS and CL-mix liposomes were found to be largely spherical, heterogeneous and featureless in solution (Fig 2.4, A-B). WT Drp1 tubulated the CL-mix liposomes to diameters comparable to those formed on DOPS (57 ± 6nm, Fig 2.4, C-D, Table 2.2). In contrast, the oligomers formed by ∆VD in CL-mix were much larger (266 ± 49 nm, Fig 2.4, E-F).
To further test the ability of the ΔVD mutant to induce curvature on lipid bilayers, the starting diameters of DOPS and CL-mix liposomes were measured using dynamic light scattering (DLS, Figure 2.4 G-H, gray shaded areas). Lipid extrusion through a 1 µm filter resulted in a broad distribution of liposome diameters, mostly between 300-450 nm. When WT and ΔVD Drp1 were added to the DOPS liposomes, both were able to tubulate the lipid template (Fig 2.4G), and the diameters were measured by EM (Table 2.2). When added to CL-mix liposomes, WT Drp1 was able to impose curvature, while ΔVD Drp1 was not. The diameters of ΔVD-CL-mix tubules were measured by EM, and these complexes were a similar size when compared to the starting diameters of the CL-mix liposomes measured by DLS (green line vs. gray shaded area, Fig 2.4H and Table 2.2). When the liposomes were filtered with a 0.2 µm filter, a similar trend was observed (Table 2.2). Therefore, WT and ΔVD Drp1 can associate with both lipid preparations in vitro, but the ΔVD Drp1 oligomer is deficient in generating membrane curvature, especially on CL-containing membranes.

**Drp1 ΔVD Exhibits Lipid Stimulated GTPase Activity.**

To assess protein function, a continuous GTPase assay (Ingerman and Nunnari, 2005) was used to measure the GTP hydrolysis activities of WT, K38A and ΔVD Drp1 (Fig 2.5A). As shown previously, WT Drp1 exhibited robust basal GTPase activity at 13 ± 1 min⁻¹. In the presence of DOPS liposomes, Drp1 activity was stimulated approximately 10 fold (123 ± 11 min⁻¹). This lipid-induced stimulation was similar in magnitude to what has been reported previously (Frohlich et al., 2013). As expected, K38A Drp1 exhibited no detectable GTPase activity in solution or in the presence of a lipid template (Fig 2.5A). At concentrations lower than had been studied previously (Frohlich et al., 2013),
ΔVD Drp1 exhibited GTPase activity in solution that was diminished compared to WT (a 2.6 fold reduction). Consistent with the ability of ΔVD to form ordered oligomers on a lipid template, a ~10 fold stimulated activity was observed when DOPS was added (30 ± 5 min⁻¹), although the total enzyme activity was less than WT Drp1 in the presence of DOPS liposomes (Fig 2.5A). Therefore, ΔVD Drp1 has the ability to form lipid-induced oligomers that stimulate GTPase activity, and this activity promotes constriction of the underlying membrane.

**GTP hydrolysis mediates Drp1 disassembly.**

After GTP-induced constriction, WT Drp1-lipid tube diameters appeared to recover to their starting value (53 ± 6nm). This suggests that Drp1 disassembles after GTP hydrolysis and subsequently rebinds lipid templates. An alternative interpretation is that the recovery of Drp1-lipid diameters was due to the relaxation of the Drp1 polymers on the membrane rather than recycling through disassembly and rebinding. However, this model is inconsistent with the observed decrease in the number of Drp1-lipid tubes observed by EM after GTP was added.

To monitor Drp1 polymerization dynamics on a lipid template, 90° light scattering experiments were conducted. Previous studies have established that the scattering intensity of a network of protein-decorated membrane tubules decreases as a function of both membrane constriction and protein disassembly. When GTP was added to preformed WT Drp1-DOPS lipid tubules, an immediate decrease in scattering intensity was observed (Fig 2.5B, red trace). A similar experiment with ΔVD Drp1 showed protein disassembly in much the same manner as WT (Fig 2.5B, green trace). Conversely, when GMPPCP was added to either preformed WT or ΔVD Drp1-lipid tubes, no such decrease
was observed (Fig 2.5B, blue and orange traces, respectively). Therefore, Drp1 disassembly is dependent on GTP hydrolysis.

The hyperoligomeric properties of the ΔVD mutant also provided the opportunity to use EM to observe disassembly of Drp1 polymers after the addition of GDP or GTP. As stated earlier, ΔVD alone in solution forms filamentous structures (Fig 2.5C). The addition of GDP did not induce any change in ΔVD oligomerization (Fig 2.5D). However, after the addition of GTP for 30 minutes almost no ΔVD filaments were observed on the grid (Fig 2.5E).

2.4 DISCUSSION

In this study, we examined the combined roles of guanine nucleotide and lipid interactions on the self-assembly properties of Drp1. In addition, the K38A and ΔVD mutants provided valuable insight into the roles of GTP hydrolysis and the largely uncharacterized VD in promoting membrane constriction.

Moreover, the ΔVD mutant offered valuable information regarding Drp1 self-assembly. Previous studies have characterized the formation of large protein aggregates in vitro and in cell culture when the VD was removed (Frohlich et al., 2013; Zhang et al., 2011). We have shown that removal of the VD yields a hyperoligomeric form of Drp1 that assembles into well-ordered filaments (Fig 2.3C) rather than disordered aggregates. Electron microscopy was used to identify the unique structural properties of the ΔVD mutant. The hyperoligomeric properties of this mutant are consistent with the hypothesis that the variable domain occludes a Drp1 self-assembly motif (Fig 2.6A). In this way, the variable domain serves to negatively regulate Drp1 oligomerization in solution and retain
Drp1 in a smaller, active state (Fig 2.6B). This may explain why removal of the VD leads to the formation of large filaments, which do not retain WT Drp1 activity (Fig 2.5A).

It should be noted that these results do not exclude the possibility of an interaction occurring between the VD and lipid. Rather, we propose that the variable domain of WT Drp1 likely conceals a self-assembly motif to limit the formation of larger assemblies in solution (Fig 2.6A). When the VD is removed, the negative regulation is alleviated and unopposed assembly commences. WT Drp1 interactions with lipid may expose the self-assembly motif to promote oligomerization. Therefore, the ΔVD mutant bypasses this regulatory mechanism as ΔVD oligomers in solution readily assemble on lipid bilayers.

A deficiency in protein oligomer curvature was apparent when ΔVD Drp1 was added to lipid templates as well. This result supports a model wherein the VD plays a role in promoting membrane curvature. ΔVD-lipid oligomers exhibit a larger average diameter compared to WT on DOPS liposomes (Fig 2.4G), and the diameter difference is even more profound on CL-mix liposomes (Fig 2.4H). In fact, ΔVD Drp1 was unable to significantly reshape the CL-mix liposomes. This difference could partially be due to the lipid template itself, as CL-mix liposomes are less pliable than DOPS liposomes (Lewis and McElhaney, 2009). This is in part due to the conical structure of cardiolipin, which favors negative membrane curvature and likely resists the positive curvature imposed by Drp1. In addition, the headgroups of DOPE and DOPC do not exhibit charge repulsion found in DOPS liposomes, so fewer membrane defects would be available for protein interactions. Very large protein-lipid tubes (>200 nm) were still observed when ΔVD was added to the DOPS template, which demonstrates a clear deficit in generating membrane
curvature. Therefore, VD interactions with the lipid bilayer and/or within the multimeric protein assembly influence curvature directly.

The VD has been proposed to interact directly with negatively charged lipid (Bustillo-Zabalbeitia et al., 2014; Ugarte-Uribe et al., 2014). Nevertheless, the ∆VD mutant was found to assemble on both negatively charged liposomes used in these studies. This suggests that Drp1 can associate with lipid independently of the VD, and weak electrostatic interactions near the base of the middle/GED stalk may be sufficient to promote protein-lipid tubule formation \textit{in vitro}. As such, the multimerization of the ∆VD protein likely provides an accumulation of charge that drives lipid association and bypasses specific regulatory interactions between the VD and lipid. This domain is also the site of several post-translational modifications that regulate Drp1 localization to mitochondrial membranes in cells (Chang and Blackstone, 2007; Cho et al., 2009; Cribbs and Strack, 2007; Taguchi et al., 2007). It is possible that the VD of Drp1 is analogous to the PH domain in dynamin and likely participates in membrane interactions. This interaction may target the protein to the mitochondrial surface, but our results suggest that membrane interactions mitigate the negative regulation of the VD and promote Drp1 self-assembly.

Interestingly, the removal of the PH domain in dynamin also leads to a hyperoligomeric form of the protein that readily sediments in solution (Vallis et al., 1999) and retains GTPase activity (Carr and Hinshaw, 1997; Muhlberg and Schmid, 2000). Therefore, it appears that the variable and PH domains both function to negatively regulate the premature, non-productive polymerization of their parent proteins in solution.
Following assembly of Drp1 on lipid bilayers, we have identified three additional steps in the Drp1 constriction process (Fig 2.6B). Before constriction, Drp1 undergoes a nucleotide-induced stabilization on lipid bilayers. GTP binding caused a slight but significant reduction in oligomer diameter as Drp1 assumes a more ordered conformation. This stabilization was disrupted by the K38A mutation, as adding GTP to preformed K38A-lipid tubes has no effect on membrane morphology. This result is surprisingly different from dynamin, as the homologous mutant, K44A, forms a superconstricted polymer in the presence of GTP. Drp1 K38A does not constrict to this intermediate state in the presence of GTP, which highlights the importance of lysine 38 in mediating conformational changes that promote this stabilization.

After GTP binding, GTP hydrolysis promotes Drp1 constriction of the lipid templates. Within seconds of GTP addition, highly constricted WT Drp1-lipid oligomers were observed with some diameters measuring less than 30 nm, a distance that would seem sufficient to mediate lipid mixing between juxtaposed membrane leaflets (Bashkirov et al., 2008; Kozlovsky and Kozlov, 2003). Moreover, because many of our diameter distributions were skewed (Table 1), the mean values are likely conservative representations of Drp1-induced membrane constriction.

Non-hydrolysable GTP analogs and GDP are incapable of mediating constriction to a significant degree. However, GDP appears to reduce the formation of much larger oligomers, which is evident in the lower skewness value (0.1, Table 2.1). This may explain the perceived constriction reported previously in the presence of GDP (Frohlich et al., 2013). Nevertheless, these data clearly demonstrate that GTP hydrolysis is essential for maximal constriction and eventual fission of the mitochondrial membrane.
Our results also showed that ΔVD Drp1 oligomers could undergo a conformational stabilization, and constrict lipid bilayers upon nucleotide addition (Fig 2.6B). Therefore, the mechanoenzymatic core of Drp1 (GTPase, Middle and GED) is sufficient for GTP-induced constriction of lipid bilayers. This minimal protein machinery is regulated by the VD sequence, which controls the oligomeric properties of Drp1 in solution and on membranes. These differences limit lipid tubule constriction, as the ΔVD oligomers were not able to constrict liposomes to the smaller diameters observed with WT Drp1 assemblies. Therefore, the VD ensures Drp1 oligomers attain a geometry that promotes full constriction of lipid tubules.

Lastly, we examined whether Drp1 GTPase activity mediates oligomer disassembly, which would then lead to successive membrane binding and constriction events. In agreement with the EM results, we found that WT Drp1 depolymerizes when GTP hydrolysis occurs, while GTP binding stabilizes the oligomer (Fig 2.6B). ΔVD Drp1 oligomers, both in solution and on lipid templates, disassemble after GTP addition. ΔVD oligomers are also stabilized by GTP binding, and a 19 nm decrease in average diameter was observed when GMPPCP was added. This may explain the slight decrease and subsequent plateau in the light scattering value (Fig 2.5A). Although removal of the VD enhances self-assembly, it does not prevent protein disassembly.

The EM time course experiments allowed for visualization of changes in Drp1-lipid morphology over time. However, a significant population of lipid fragments or vesicles was not observed in our studies. As with other dynamin family members, Drp1 likely requires several cellular factors to efficiently complete the fission process. These cellular factors would include partner proteins, which are responsible for the recruitment of Drp1.
to the outer mitochondrial membrane. The endoplasmic reticulum has also been implicated in inducing fission sites through extensive mitochondrial contacts and interactions with associated cytoskeletal structures (Friedman et al., 2011; Korobova et al., 2013; Li et al., 2015a).

Taken together, our data demonstrate the dynamic ability of Drp1 to accommodate different oligomeric conformations and sizes depending on ligand interactions. Our data support a model where the VD serves to limit Drp1 self-assembly in the cytosol, and membrane interactions relieve this inhibition to promote Drp1 oligomerization at mitochondrial fission sites (Fig 2.6A). GTP binding causes a conformational stabilization of these polymers, and hydrolysis is required to promote maximal constriction of the underlying membranes. This process leads to disassembly of Drp1 to allow for rebinding and successive cycles of membrane constriction in a processive manner (Fig 2.6B). Overall, this work identifies fundamental biochemical and structural characteristics of Drp1 and the core components that form the contractile apparatus to drive membrane fission.

2.5 MATERIALS AND METHODS

Drp1 expression and purification.

Human Drp1 isoform 1 (UniProtID: O00429-1, residues 1-736) was cloned into pCal-n-EK vector (Agilent) using BamHI and SalI as restriction sites resulting in a construct with an N-terminal calmodulin binding protein (CBP) tag. The Drp1 ΔVD and K38A construct were made using the Quikchange Mutagenesis Kit (Agilent Technologies). The VD (517-639) was deleted at sites described previously (Frohlich et al., 2013).
Drp1 was expressed and purified as described previously (Chang et al., 2010; Zhu et al., 2004) with some modifications. Briefly, CBP-Drp1 was transformed into *E. coli* BL21 DE3 cells. The cells were grown in LB broth and induced with 1.0 mM IPTG for ~19 hours at 18 °C. The overnight culture was harvested and cell pellets were resuspended in a cell lysis/binding buffer (500 mM Arginine pH 8, 300 mM NaCl, 10 mM B-mercaptoethanol (BME), 5.0 mM magnesium chloride, 1.0 mM imidazole and 2 mM CaCl₂) with 100 ng/µl lysosome (Sigma), 4 units/ml DNAse (Sigma), and complete EDTA-free protease inhibitor cocktail tablets (Roche). The resuspended cells were then passed through a micro fluidizer (M-110 Y, Microfluidics, Newton, MA, USA), and the lysate was centrifuged in a Beckman Coulter Optima L-look Ultracentrifuge (50.2 Ti rotor) at 40,000 rpm (184,048 x g) for 30 minutes at 4 °C. The supernatant containing CBP-Drp1 was mixed with pre-equilibrated calmodulin-affinity resin (Agilent Stratagene) and allowed to batch bind for 1 h at 4 °C, and then loaded onto a column fitted into the AKTA Purifier 10 (GE Pharmacia). The resin was washed with lysis/binding buffer until the absorbance at 280 nm returned to baseline and the target protein was eluted with elution buffer (500 mM Arginine pH 8, 300 mM NaCl, 10 mM BME, 5mM MgCl₂ and 2.5 mM EGTA). Fractions were collected, pooled and concentrated using centrifugal 50MWCO filter units (Millipore). The concentrated protein was then loaded onto a Superdex 200 gel filtration column (GE LifeSciences) equilibrated in HEPES column buffer (HCB300: 50 mM HEPES pH 8, 300 mM NaCl, 10 mM BME). CBP-Drp1 fractions were collected, aliquoted, and flash frozen in liquid nitrogen with 5% glycerol. Samples were stored at -60 °C.

*Liposome preparation.*
All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Liposomes were prepared by drying 100 mol% 1,2-dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (DOPS) or a cardiolipin- containing lipid mixture (CL-mix) in chloroform under a nitrogen stream. CL-mix contained 40 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 35 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 25 mol% bovine heart cardiolipin (CL). The dried lipid was stored in vacuum overnight and rehydrated in resuspension buffer (HCB100: 50 mM HEPES pH 8.0, 100 mM NaCl, 0.5 mM MgCl₂, 10 mM BME). The lipid was then extruded using a polycarbonate membrane of a defined size (Whatman) to generate uniform vesicles.

**Dynamic light scattering.**

DLS measurements of liposome size were performed in a Dynapro Nanostar (Wyatt Technologies) instrument according to previously published methods (Drin et al., 2008). Samples (50 µl) of liposomes (200 µM) prepared by extrusion were analyzed in an Eppendorf UVette at room temperature. Autocorrelation curves from a set of 10 acquisitions (10-s integration time each) were analyzed using Dynamics version 7.1.3 software (Wyatt Technologies) to resolve the relative size distribution of liposomes within each sample.

**EM analysis and time course experiments.**

Negative stain electron microscopy (EM) was used to visualize Drp1 structures. Sample mixtures were prepared using wild type (WT) or mutant Drp1 incubated with DOPS at room temperature for at least 30 minutes. To study the effect of nucleotides on the Drp1-lipid tubes, Drp1 samples were pre-incubated with DOPS to allow lipid-induced oligomerization. Subsequently, 1 mM nucleotide (GTP, GMPPCP or GDP) was added.
and aliquots of the sample reactions were taken at specified time points. For all samples, five microliters of reaction mixture were applied to carbon-coated grids, followed by 2% uranyl acetate stain (Electron Microscopy Sciences), and excess liquid was blotted in between applications. Images were acquired on a Tecnai T12 electron microscope (FEI, Co.) using a 4k x 4k CCD camera (Gatan).

**Drp1 lipid oligomer measurements.**

Tube outer diameters were measured using the ImageJ software (http://rsbweb.nih.gov/ij/). Diameters were measured to the outer edge of the protein layer on each side of the protein-lipid tubule. Extended or branched tubules were measured multiple times at increments no less than 300 nm apart. In order to account for heterogeneity within individual tubes, maximum and minimum widths were measured. Tubes that were not uniformly decorated with protein were omitted.

**Sedimentation assay.**

To quantify Drp1 oligomerization, a sedimentation assay was conducted similar to what has been described previously (Hinshaw and Schmid, 1995; Mears and Hinshaw, 2008). Large oligomers formed by Drp1 samples, in the presence of ligands, were found in the pellet after a medium-speed centrifugation. Specifically, protein was diluted in HCB100 to 0.2 mg/ml, and specified WT and mutant samples were incubated at room temperature with DOPS liposomes for at least 30 minutes. The mixtures were then spun at 13,200 rpm (16,100 x g) for 30 minutes in a tabletop centrifuge (Eppendorf). The supernatant and pellet fractions were carefully separated, collected and immediately mixed with SDS-PAGE loading dye (Bio-Rad) and heated briefly at 100 °C. These samples were subjected to reducing SDS-PAGE and the Coomassie stained gel was
Densitometry analysis was done using the ImageJ software (http://rsbweb.nih.gov/ij/).

**GTPase assay.**

Drp1 function was assessed using a continuous GTPase assay (Ingerman and Nunnari, 2005). A master mix was prepared to achieve the following final concentration in the assay solution: 50 mM HEPES-KOH pH 8, 150 mM NaCl, 0.5 mM MgCl₂, 10 mM BME, 4 mM Phosphoenolpyruvate (PEP), 0.3 mM NADH, 10 Units Pyruvate Kinase/Lactate Dehydrogenase and 1 mM GTP. Extruded DOPS liposomes were prepared as above (1 µm filter, Whatman). Drp1 was added to a final concentration of 0.2 µM, while DOPS was added to specified reactions at a final concentration of 198 µM (1:1, mass:mass). A 96 well quartz plate (Molecular Devices) was preheated and maintained at 37°C during the experiment. The decrease in the NADH absorbance at 340 nm was monitored (VersaMax, Molecular Devices), and the rate of NADH oxidation was measured and used to calculate the GTPase activity (kₚₒₛ) of Drp1 in each condition. The NADH oxidation was also monitored in the absence of protein using buffer alone with and without DOPS as controls. The experimental values were normalized to correct for background.

**90° light scattering.**

WT Drp1 and ΔVD (0.1-0.2 mg/ml) were pre-assembled on pure DOPS liposomes (198 µM; extruded 1µm diameter) in HCB100 buffer. Scattered light from these samples was measured continuously at 350 nm (0.5 nm bandpass; 0.5 sec integration time) using a Fluorolog 3-22 photon-counting spectrofluorometer (Horiba, NJ), before and after addition of nucleotide (1 mM final). Samples were contained in a 4 mm × 4 mm quartz
cuvette (Starna cells, CA) and temperature-equilibrated at 25 °C throughout the measurement. For comparison, the initial scatter obtained prior to addition of nucleotide under each condition was normalized.

2.6 ACKNOWLEDGEMENTS

The authors thank Craig Blackstone for providing a WT CBP-Drp1 construct. We also thank Heather Holdaway and Hisashi Fujioka for their expertise and advice with electron microscopy studies. We would like to acknowledge Ryan Clinton and Colleen Murphy for critically reading the manuscript and for providing feedback. The authors thank Natalia Stepanyants for assistance with DLS and Tom Radivoyevitch for his insight in statistical analyses. This work is supported by the American Heart Association (Grant ID: SDG12SDG9130039 for JAM and 13BGIA14810010 for RR), and CAF is supported by a NIH training grant (Grant ID: 2T32GM008803-11A1).
FIGURE 2.1 Drp1-lipid tubules constrict upon GTP addition. (A-B) Negative stain EM images of WT Drp1 in solution (A) and in the presence of DOPS (B) are shown. (C) WT Drp1 GTPase activities were measured in solution and in the presence of DOPS liposomes (n=3/sample). (D-F) Histograms show the distribution of measured diameters for WT Drp1-lipid tubes before addition of GTP (D, n=197), 5 minutes after GTP addition (E, n=173) and 60 minutes after GTP addition (F, n=207). The dashed gray line at 40 nm is provided for comparative reference. Representative EM images are shown for each time point. Scale bar = 50 nm. (G) A time course plot highlights the change in the average diameter of Drp1-lipid tubes after addition of GTP. Error bars indicate the measured standard deviation. A gray line is used to highlight the starting diameter of the Drp1-lipid tubes, which is used as a comparative reference of later time point diameters.
FIGURE 2.2
FIGURE 2.2 Drp1-lipid tubules constrict only upon GTP hydrolysis, while a conformational stabilization occurs upon GTP binding. (A) The position of the hydrolysis (K38A) mutation is shown in the domain architecture of Drp1 and within the 3D structure of Drp1 (PDB ID: 4BEJ). (B, C) Negative stain EM images of K38A Drp1 in solution (B) and in the presence of DOPS (C) are shown. (D) A time course plot highlights the change in the average diameter of WT and K38A Drp1-lipid tubes after addition of GTP and WT Drp1 after addition of GMPPCP. (E-J) Histograms show the distribution of measured diameters of protein-lipid tubes. The dashed gray line at 40 nm is provided for comparative reference. WT Drp1 distributions fifteen minutes after GDP (E, n=92), GMPPCP (F, n=150) and GTP (G, n=105) addition are shown. K38A Drp1 distributions alone in solution (H, n=300), fifteen minutes after GMPPCP (I, n=92) and GTP (J, n=73) addition are displayed. Representative EM images are shown for each sample. Scale bar = 50 nm.
FIGURE 2.3

A

[Diagram showing a protein structure with highlighted regions labeled GTPase, Middle, VD, and GED.]

B

[Bar graph showing percentage in pellet for different conditions: WT alone, WT with DOPS, K38A alone, K38A with DOPS, and ΔVD.]

C

[Two images of electron micrographs, labeled C and D, with a scale bar indicating 82 nm.]

E

[Histogram showing distribution of diameters for no GTP condition, with a scale bar indicating 82 nm.]

F

[Histogram showing distribution of diameters for + GTP 5 min condition, with a scale bar indicating 47 nm.]
FIGURE 2.3 **The variable domain regulates Drp1 assembly, but is not required for lipid constriction.** (A) The position of the VD deletion (residues 517-639, dotted red line) is shown in the domain architecture of Drp1 and within the 3D structure of Drp1 (PDB ID: 4BEJ). (B) Sedimentation analyses compare Drp1, K38A and ΔVD oligomerization in solution and with DOPS liposomes added (n=3/sample). (C-D) Negative stain EM images of ΔVD Drp1 in solution (C) and in the presence of DOPS (D) are shown. Scale bar = 100 nm. (E-F) Histograms show the distribution of measured diameters for ΔVD-lipid tubules before (E, n=146) and 5 minutes after GTP addition (F, n=79). The dashed gray line at 40 nm is provided for comparative reference. Representative EM images are shown. Scale bar = 50 nm.
FIGURE 2.4 **WT and ΔVD Drp1 associate with DOPS and CL-mix liposomes to form ordered protein-lipid tubes.** (A-B) DOPS and CL-mix liposomes were visualized using negative stain EM. Scale bar = 1 µm. (C-F) WT and ΔVD Drp1 form protein-lipid tubes on DOPS and CL-mix liposomes. WT Drp1-lipid tube morphology is largely unaffected by lipid composition (C-D), whereas ΔVD-lipid tubes exhibit larger diameters with DOPS and CL-mix liposomes (E-F). Scale bar = 100 nm. (G-H) Shaded gray areas represent the starting distribution of liposome diameters measured using dynamic light scattering. The diameters for WT and ΔVD Drp1 oligomers were measured by EM using DOPS (G, n=197, n=146) and CL-mix liposomes (H, n=152, n=86) as indicated.
FIGURE 2.5 The mechanoenzymatic core of Drp1 is sufficient for lipid-stimulated activity, which leads to oligomer disassembly. (A) WT Drp1 (n=3), K38A (n=3) and ΔVD (n=6) GTPase activities are measured in solution and in the presence of DOPS liposomes. K38A GTPase activities were undetectable, as indicated (#). (B) Protein oligomer disassembly was measured using 90° light scattering. WT Drp1-lipid tubes were evaluated in the presence GMPPCP (blue) and GTP (red). ΔVD Drp1-lipid tubules were also examined with GMPPCP (orange) and GTP (green). (C-E) Representative EM images are shown for ΔVD Drp1 alone (C), in the presence of GDP (D) and 30 minutes after GTP addition (E). Scale bar = 100nm.
FIGURE 2.6 Schematic representations of Drp1 recruitment, constriction and recycling on lipid bilayers. (A) The proposed role of the VD in regulating Drp1 self-assembly in the cytosol and on the outer mitochondrial membrane (OMM) is shown. Lipid interactions may selectively promote oligomerization at mitochondrial fission sites. (B) A schematic highlights the cycle of Drp1 assembly, constriction and disassembly. In solution, the variable domain (yellow) limits Drp1 assembly by obscuring a self-assembly motif(s). Without the variable domain, this interface is exposed leading to unopposed assembly of the ΔVD mutant (dark green filaments) in solution and on lipid templates (gray cylinder). Upon WT Drp1 lipid association, the VD undergoes a conformational change that drives oligomerization at that site. GTP binding stabilizes the oligomeric conformation of WT Drp1 on the lipid template. The K38A mutation prevents this stabilization and subsequent GTP hydrolysis. For WT and ΔVD Drp1, GTP hydrolysis mediates constriction of the underlying lipid bilayer. Lastly, Drp1 disassembles into solution, making it available for continuous rounds of constriction.
<table>
<thead>
<tr>
<th>Sample conditions</th>
<th>WT Drp1</th>
<th></th>
<th></th>
<th></th>
<th>K38A</th>
<th></th>
<th></th>
<th></th>
<th>ΔVd</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Std Dev</td>
<td>Skew*</td>
<td>Mean</td>
<td>Median</td>
<td>Std Dev</td>
<td>Skew*</td>
<td>Mean</td>
<td>Median</td>
<td>Std Dev</td>
<td>Skew*</td>
</tr>
<tr>
<td>DOPS alone</td>
<td>60.0</td>
<td>58.0</td>
<td>11.7</td>
<td>1.5</td>
<td>61.2</td>
<td>60.0</td>
<td>10.1</td>
<td>3.8</td>
<td>81.8</td>
<td>75.7</td>
<td>24.8</td>
<td>1.9</td>
</tr>
<tr>
<td>DOPS + PCP (15 min)</td>
<td>50.7</td>
<td>48.7</td>
<td>6.5</td>
<td>1.6</td>
<td>59.0</td>
<td>59.0</td>
<td>6.2</td>
<td>0.2</td>
<td>63.2</td>
<td>59.9</td>
<td>14.7</td>
<td>1.9</td>
</tr>
<tr>
<td>DOPS + GTP (15 min)</td>
<td>38.0</td>
<td>37.2</td>
<td>7.0</td>
<td>1.2</td>
<td>62.4</td>
<td>61.8</td>
<td>8.0</td>
<td>0.6</td>
<td>52.4</td>
<td>51.6</td>
<td>5.8</td>
<td>0.7</td>
</tr>
<tr>
<td>DOPS + GDP (15 min)</td>
<td>59.1</td>
<td>58.9</td>
<td>7.0</td>
<td>0.1</td>
<td>62.4</td>
<td>61.7</td>
<td>8.4</td>
<td>3.0</td>
<td>80.5</td>
<td>68.3</td>
<td>34.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*This value refers to the skewness (or asymmetry) of the distribution. Values closer to 0 are more symmetric (i.e. Gaussian), while those approaching +1 or -1 have more of a right-tailed or left-tailed distribution, respectively.

**TABLE 2.1** The effect of nucleotide on Drp1-lipid tube morphology.
TABLE 2.2 The VD modulates Drp1 curvature on lipid templates

<table>
<thead>
<tr>
<th>Sample conditions</th>
<th>WT Drp1</th>
<th></th>
<th></th>
<th></th>
<th>ΔVD Drp1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Std Dev</td>
<td>Skew*</td>
<td>Mean</td>
<td>Median</td>
<td>Std Dev</td>
<td>Skew*</td>
</tr>
<tr>
<td>CL mix (0.2 μm)</td>
<td>63.1</td>
<td>65.5</td>
<td>8.3</td>
<td>1.1</td>
<td>190.3</td>
<td>173.5</td>
<td>86.5</td>
<td>0.5</td>
</tr>
<tr>
<td>CL mix (1 μm)</td>
<td>56.6</td>
<td>61.9</td>
<td>5.5</td>
<td>0.9</td>
<td>266.1</td>
<td>269.1</td>
<td>49.2</td>
<td>-0.9</td>
</tr>
<tr>
<td>DOPS (0.2 μm)</td>
<td>61.9</td>
<td>61.3</td>
<td>5.6</td>
<td>0.6</td>
<td>75.3</td>
<td>61.0</td>
<td>35.5</td>
<td>2.5</td>
</tr>
<tr>
<td>DOPS (1 μm)</td>
<td>60.0</td>
<td>58.0</td>
<td>11.7</td>
<td>1.5</td>
<td>81.8</td>
<td>75.7</td>
<td>24.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*This value refers to the skewness (or asymmetry) of the distribution. Values closer to 0 are more symmetric (i.e. Gaussian), while those approaching +1 or -1 have more of a right-tailed or left-tailed distribution, respectively.
CHAPTER 3: DISTINCT SPLICE VARIANTS OF DRP1 AFFECT MEMBRANE CURVATURE

Data presented in this chapter were previously published in:

Patrick J. Macdonald, Christopher A. Francy, Natalia Stepanyants, Lance Lehman, Anthony Baglio, Jason A. Mears, Xin Qi, and Rajesh Ramachandran.

Distinct Splice Variants Of Dynamin-Related Protein 1 Differentially Utilize Mitochondrial Fission Factor As An Effector Of Cooperative GTPase Activity.

3.1 ABSTRACT

Multiple isoforms of the mitochondrial fission GTPase dynamin-related protein 1 (Drp1) arise from the alternative splicing of its single gene-encoded pre-mRNA transcript. Among these, the longer Drp1 isoforms, expressed selectively in neurons, bear unique polypeptide sequences within their GTPase and variable domains, known as the A-insert and the B-insert, respectively. Their functions remain unresolved. A comparison of the various biochemical and biophysical properties of the neuronally expressed isoforms with that of the ubiquitously expressed, and shortest, Drp1 isoform (Drp1-short) has revealed the effect of these inserts on Drp1 function. Utilizing various biochemical, biophysical, and cellular approaches, we find that the A- and B-inserts distinctly alter the oligomerization propensity of Drp1 in solution as well as the preferred curvature of helical Drp1 self-assembly on membranes. Consequently, these sequences also suppress Drp1 cooperative GTPase activity.
3.2 INTRODUCTION

The opposing processes of membrane fission and fusion govern mitochondrial dynamics in eukaryotic cells (Chan, 2012). Mediated by evolutionarily well conserved GTPases of the dynamin superfamily, the constant flux of mitochondria between fused and fragmented states is essential for the maintenance of mitochondrial size, shape, and distribution (Labbe et al., 2014). Distinct transmembrane domain (TMD)²-anchored dynamin-related proteins (DRPs), namely mitofusins (Mfn1/2) and OPA1 that reside in the mitochondrial outer and inner membranes, respectively, mediate the bilayer-specific fusion of this double membrane bound organelle. However, a cytosolic DRP, dynamin-related protein 1 (Drp1 in mammals/Dnm1p in yeast), catalyzes the apparent concerted fission of both membranes.

Further complicating this scenario, mammalian cells, unlike yeast, express multiple splice variants (isoforms) of Drp1 ranging from 699 to 755 residues that arise from the alternative splicing of its pre-mRNA transcript (Strack et al., 2013; Uo et al., 2009b). Encoded in its entirety by 20 exons, the alternative splicing of exons 3, 16, and 17, and several variations therein, gives rise to multiple Drp1 isoforms (Uo et al., 2009b). Among these, the full-length isoform (755 aa; hereafter referred to as Drp1-long) expresses exon 3 in addition to both exons 16 and 17. The exon 3-encoded polypeptide segment (13 aa), referred to as the “A-insert,” localizes to a loop within the Drp1 N-terminal GTPase domain (see Fig 3.1A), and is expressed as part of Drp1 selectively in the postmitotic neurons of the brain (Strack et al., 2013; Uo et al., 2009a; Yoon et al., 1998). On the other hand, shorter isoforms of Drp1 that lack the A-insert but alternatively exclude either exon 16 or 17, or both, are variably expressed in other cell types (Strack et
al., 2013; Uo et al., 2009a). Exons 16 and 17 together encode for the “B-insert” segment that constitutes a large part of the unstructured variable domain (VD) located between the middle domain and GED at the base of the Drp1 molecule (see Fig 3.1A) (Frohlich et al., 2013). The Drp1 isoform that lacks the A-insert but contains the B-insert in its entirety (736 or 742 aa; also known as isoform 1; hereafter referred to as Drp1-B-only) is also found enriched in neurons, whereas the shortest Drp1 isoform lacking both A-inserts and B-inserts (699 aa; also known as isoform 3; hereafter referred to as Drp1-short) is expressed ubiquitously. In contrast to Drp1- short, which has been widely studied and biochemically well characterized, very little is known about the A-insert-containing longer Drp1 variants, which have thus far only been partially characterized (Bustillo-Zabalbeitia et al., 2014; Frohlich et al., 2013; Macdonald et al., 2014; Yoon et al., 2001).

In this study, utilizing a host of sophisticated biochemical, biophysical, and cellular approaches, we have meticulously compared the various activities of Drp1-long with those of Drp1-short (Su and Qi, 2013). In addition, we have also characterized the activities of Drp1 isoforms that alternatively bear only the A-insert (Drp1-A-only) or the B-insert (Drp1-B-only), respectively. We reveal that the A-insert functions to enhance the propensity of Drp1 to form cooperative, higher-order polymers in solution, whereas the B-insert functions antagonistically to curtail it. Regardless, the presence of either or both sequences in Drp1 drastically reduces basal and assembly-stimulated cooperative GTPase activities. We further reveal that the B-insert functions to regulate membrane curvature by selectively promoting Drp1 polymerization of a preferred, narrow helical geometry. On the other hand, the A-insert, in the absence of the B-insert, promotes polymers of highly variable, non-uniform geometry with no set curvature preference. In the absence
of both A-inserts and B-inserts, Drp1 constitutes polymers of a uniformly large helical geometry. These data suggest that the differential oligomeric propensity and helical geometry of the various Drp1 isoforms may be uniquely tailored to the disparate mitochondrial size and distribution found in neuronal versus non-neuronal cell types.

3.3 RESULTS

**A- and B-inserts Suppress Drp1 GTPase Activity**

We compared the basal and lipid-stimulated GTPase activities of Drp1-short with that of Drp1-long, Drp1-A-only, and Drp1-B-only. As shown previously (Macdonald et al., 2014), the basal GTPase activity of Drp1-short was robustly stimulated upon its self-assembly on CL containing liposomes (Fig 3.1B). By contrast, both the basal and the lipid-stimulated GTPase activities of Drp1-long were dramatically reduced (Fig 3.1C). Characterization of the Drp1-A-only and Drp1-B-only variants revealed that the A- and B-insert sequences both suppressed Drp1 GTPase activity, with the B-insert exerting the most dominant inhibitory effect on these interactions (Fig 3.1, D–F). Based on the comparative data, we conclude that the A- and B-inserts conjunctly suppress cooperative GTPase activity in Drp1-long.

Previous studies demonstrated that the basal and lipid-stimulated GTPase activities of yeast Dnm1p are both cooperative with respect to GTP and protein concentration, and require higher-order Dnm1p self-assembly in solution or on membranes, respectively (Ingerman et al., 2005; Lackner et al., 2009). Therefore, our data indicated that either the self-assembly properties of Drp1-long and/or its GTP binding and cooperative GTP hydrolysis activities are significantly affected by the presence of the A- and B-inserts.
**A- and B-inserts Distinctly Affect Drp1 Oligomerization Propensity and Helical Geometry**

To determine whether Drp1- long possesses a diminished capacity to propagate into higher order polymers relative to Drp1-short, we next compared their respective oligomerization propensities in solution using SECMALS (Macdonald et al., 2014). Our SEC-MALS data had previously revealed that Drp1-short readily propagates into higher-order structures in solution in quantized dimeric increments (Macdonald et al., 2014). Remarkably, in contrast to Drp1-short, Drp1-long exhibited a relatively narrow and symmetric SEC elution profile when both proteins were examined at equivalent concentrations (Fig 3.2A, left panel). Furthermore, molar mass determination by MALS revealed that Drp1-long, under these conditions, exists predominantly as a monodisperse population of tetramers (Fig 3.2A, left panel). These data indicated that Drp1-long in solution is autoinhibited in its oligomerization properties relative to Drp1-short. To resolve the molecular determinants of this effect, we compared the oligomerization properties of Drp1-short and Drp1- long with Drp1-A-only and Drp1-B-only under similar conditions. Much to our surprise, Drp1-A-only exhibited a greater propensity to form higher-order polymers in solution relative to other Drp1 isoforms, indicating that the A-insert plays a significant role in promoting Drp1 self-assembly (Fig 3.2A, left panel). Based on its structural proximity to the bundle signaling element (BSE), a role for the A-insert-bearing loop (referred to as the “80-loop”) in Drp1 oligomerization has already been suggested (Wenger et al., 2013). On the other hand, Drp1-B-only exhibited a narrower elution profile relative to Drp1-short and Drp1-A-only, and appeared closer to
Drp1-long in oligomerization propensity (Fig 3.2A, left panel). These data revealed a role for the B-insert in restricting Drp1 self-assembly in solution akin to the role of the pleckstrin homology (PH) domain in prototypical dynamin function (Kenniston and Lemmon, 2010; Reubold et al., 2015b; Vallis et al., 1999). However, in the presence of the non-hydrolyzable GTP analog, GMP-PCP, all Drp1 isoforms formed short, structurally indistinguishable helical polymers in solution (~50 nm diameter by negative-stain EM), indicating a similar tendency to polymerize upon GTP binding (Fig 3.2A, right panel) (Macdonald et al., 2014).

Trp-Dansyl FRET was used to detect Drp1-membrane association, as described previously (Macdonald et al., 2014), and revealed similar binding of all Drp1 isoforms to 25 mol % CL-containing liposomes (Fig 3.2B). Distinct structural differences between the Drp1 isoforms, however, emerged upon EM examination of their membrane-bound polymers (Fig 3.2C). Although Drp1-short formed well ordered helical polymers of a relatively large diameter on CL-containing liposomes (~150–200 nm) (Fig 3.2C, left most panel), the polymers of Drp1-long were consistently smaller (~50–70 nm) in diameter (Fig 3.2C, second panel from left). Despite the equal association of Drp1-short and Drp1-long with CL-containing liposomes, membrane-bound polymers of Drp1-long were sparsely distributed, precluding further statistical analyses under these conditions (see below). Similar results were reported for Drp1-long previously (Yoon et al., 2001).

In contrast to Drp1-short and Drp1-long, Drp1-A-only formed helical polymers of variable geometry (~50–200 nm) (Fig 3.2C, right two panels), suggesting that the greater oligomerization propensity of this isoform (Fig 3.2A) offsets the curvature control of its
polymerization over CL-containing membranes. Despite repeated efforts, we were not successful in visualizing Drp1-B-only polymers on CL-containing membranes.

Therefore, to assess the preferred curvature of polymerization of the various Drp1 isoforms, their membrane-bound polymers on pure DOPS liposomes, over which Drp1 assembles more efficiently, were examined (Francy et al., 2015; Koirala et al., 2013). Unlike mixed lipid bilayers that present varying acyl chain content and charge distribution, pure DOPS liposomes offer little resistance to membrane bending and readily conform to the dimensions of the adsorbed helical Drp1 polymer (Bigay and Antonny, 2012; Francy et al., 2015; Fuller et al., 2003). Utilizing this approach, the structural differences between the Drp1 isoforms and their molecular determinants were resolved (Fig 3.3). Although Drp1-short and Drp1-A-only both gravitated toward polymers of a relatively large helical diameter (>100 nm), Drp1-A-only exhibited a wider distribution of tube diameters as observed earlier (Fig 3.3, A and C). On the other hand, Drp1-long and Drp1-B-only both constituted narrower helical polymers (<100 nm), with Drp1-long exhibiting the narrowest distribution (Fig 3.3, B and D). These data are also consistent with results obtained for Drp1-long on DOPS liposomes previously (Yoon et al., 2001). In agreement with previous findings (Francy et al., 2015), we conclude that the Drp1 B-insert within the VD is the major determinant of helical polymer curvature, and therefore, underlying membrane curvature.

We previously observed that helical polymers of Drp1-short formed over highly curved, preformed lipid nanotubes (~30 nm in diameter) exhibit a dramatically reduced cooperative GTPase activity (Macdonald et al., 2014). In conjunction with this finding, we observe that Drp1-long, Drp1-B-only, and a subpopulation of Drp1-A-only also form
narrow polymers, which similarly correlates with their reduced cooperative GTPase activities on membranes (Fig 3.1F). Therefore, it appears that larger polymer diameter accompanies greater assembly-stimulated GTPase activity on membranes. The A- and B-inserts establish the preferred curvature of polymerization, and thus appear to autoinhibit Drp1 enzymatic cooperativity.

3.4 DISCUSSION

The previously described 80-loop of the GTPase domain (Wenger et al., 2013) is common to all Drp1 isoforms. However, the 13-aa A-insert contained within is found only in select Drp1 variants, including Drp1-long and Drp1-A-only. Although they possess similarly extended structural counterparts in yeast Dnm1p and Vps1p, with which Drp1-long and Drp1-A-only share the greatest homology (42 and 40%, respectively) (Yoon et al., 1998), the role of the A-insert and its effect on Drp1 function have remained unexplored. However, based on its location on the outer periphery of the globular GTPase domain and the inclusion of a high proportion of charged amino acid residues, this region has been predicted to be a potential interaction site for protein binding partners (Niemann et al., 2001b; Strack et al., 2013). In addition, because of its proximity to the BSE, which putatively transmits conformational changes originating in the GTPase domain to the rest of the molecule (Mattila et al., 2015), this region has also been predicted to allosterically regulate BSE movement upon GTP hydrolysis and/or promote Drp1 oligomerization (Wenger et al., 2013). Here we demonstrate that the A-insert functions to promote Drp1 self-assembly by enhancing its oligomerization propensity. The means by which change is conferred remains to be characterized.
Furthermore, based on contacts formed upon crystallization of a minimal GTPase-BSE Drp1 construct for x-ray analysis, this loop has also been implicated in the creation of a novel polar interface between two GTPase domains at the opposite end of the expected GTPase domain dimerization interface (Wenger et al., 2013). Remarkably, mutation of two glutamate residues, Glu81 and Glu82, located at the tip of this loop suppresses both basal and lipid-stimulated GTPase activities, while decreasing the Km for GTP hydrolysis, in a Drp1 splice variant (isoform 2) that lacks the A-insert (Wenger et al., 2013). Collectively, these observations indicated that this Drp1-specific loop might play important roles in the regulation of Drp1 self-assembly, stimulation of assembly-dependent cooperative GTPase activity, and transmission of allosteric conformational changes upon Drp1 association with binding partners, e.g. Mff. Here we show that the A-insert, despite promoting Drp1 self-assembly, also suppresses Drp1 cooperative GTPase activity through uncharacterized mechanisms.

Similarly, the role of the 37-aa B-insert segment in Drp1 function has remained unclear. However, a role for the encompassing VD region in regulating membrane curvature has been previously ascertained (Francy et al., 2015). Drp1 VD has been shown to constitute helical polymers that are in excess of 250 nm in diameter on membranes, albeit under favorable circumstances (Francy et al., 2015). In Drp1-short that contains the VD but not the B-insert, the corresponding diameters are also large, although significantly reduced (200 nm) when compared with Drp1VD. These data indicate an essential role for the VD in governing Drp1 self-assembly as well as in establishing Drp1 helical geometry (diameter) on membranes. Here we demonstrate that the presence of the B-insert further reduces the diameter of Drp1 helical self-assembly on membranes to
substantially less than 100 nm in diameter. Via promoting narrow Drp1 helical geometry over highly constricted tubular membranes, the B-insert consequently also suppresses Drp1 cooperative GTPase activity as observed previously (Stepanyants et al., 2015). These data also suggest that neuronal isoforms of Drp1, Drp1-long and Drp1-B-only that contain the B-insert, unlike curvature-adaptable Drp1-short and Drp1-A-only, are morphologically constrained to adopt narrow geometries. The varied oligomerization propensities of the Drp1 isoforms in solution, their disparate GTPase activities and consequently, assembly-disassembly kinetics, and the distinct helical geometries of their membrane-bound polymers further suggest that when co-expressed together in vivo, they may retain isoform specificity by physically and kinetically precluding heterocopolymerization with other isoforms. Exogenous overexpression of select Drp1 isoforms over endogenous populations might nullify distinct isoform function by forcing heterocopolymerization in cell culture in vitro (Strack et al., 2013).

We find that the expression of the oligomerization promoting A-insert, in the absence of the regulatory B-insert, in Drp1-A-only impairs mitochondrial fission in vivo. These data are consistent with our previous interpretation (Macdonald et al., 2014) that higher-order polymers of Drp1 in solution are functionally impaired, exist primarily as reservoirs for the generation of functional Drp1 dimers, and are not engaged in mitochondrial fission in vivo. Indeed, the observed reductions in the efficacy of mitochondrial fission in cells expressing Drp1-long, Drp1-A-only, and Drp1-B-only relative to Drp1-short may be explained by their lower GTPase activities, and consequently, a lower rate of production of functional Drp1 dimers from the GTPase driven dynamic disassembly of higher-order polymers (Macdonald et al., 2014).
Alternatively, the narrower and/or non-uniform helical propensity and geometry found for these isoforms relative to Drp1-short may not easily conform to the larger dimensions of the mitochondria and mitochondrial fission sites found in nonneuronal cells despite the noted curvature adaptability of Drp1 (Chang and Reynolds, 2006; Chang et al., 2006; Friedman et al., 2011; Macdonald et al., 2014; Palmer et al., 2011; Schwarz, 2013).

Lastly, the selective need for B-insert-containing longer Drp1 isoforms in neurons may relate to the distinct architecture and dynamics of mitochondria found in this cell type, as well as the need for stringent regulation of its cooperative GTPase activity (Chang and Reynolds, 2006; Chang et al., 2006; Palmer et al., 2011; Schwarz, 2013). Unlike in other cell types, where the mitochondria are organized in complex reticular networks, axonal mitochondria exist as discrete entities ranging between 1 and 3 µm in length (Chang et al., 2006). The requirement for the rapid transport of mitochondria to regions of high ATP consumption, such as the synapse located at cellular extremities, under conditions of high turnover, necessitates that these organelles remain predisposed to fission in these cells (Ishihara et al., 2009). It is conceivable that neuronally enriched Drp1-long and Drp1-B-only, by virtue of their intrinsic abilities to generate narrower membrane curvatures even in the absence of GTP hydrolysis, poises sites of future mitochondrial division for rapid membrane fission.
3.5 MATERIALS AND METHODS

Protein Expression and Purification

Drp1-short (699 aa; human isoform 3; accession number NP_005681.2) and Drp1-long (755 aa; full-length clone from rat (Yoon et al., 1998); accession number AAB72197.1), were subcloned in pRSET C (Life Technologies) between BamHI and EcoRI MCS restriction sites. Drp1-A-only (712 aa; 699 aa + 13-aa A-insert) and Drp1-B-only (742 aa; 755 aa − 13-aa A-insert) were constructed by swapping BamHI and MfeI restriction fragments between Drp1-short and Drp1-long (a unique MfeI site occurs upstream of the B-insert coding region within the Drp1 ORF). Drp1-B-only is equivalent to human Drp1 isoform 1 (736 aa) that lacks the 6 additional amino acid residues (594–599, see Fig 2.1A) found in the VD of the rat clone. Human Drp1 isoform 1 exhibits similar enzymatic and assembly properties to Drp1-B-only under our experimental conditions and is not further characterized here (data not shown; also see the accompanying manuscript (Clinton et al., 2016)). All Drp1 isoforms were expressed and purified from Escherichia coli BL21 Star (DE3) as described in detail elsewhere (Macdonald et al., 2014). Mff (238 aa; human transcript variant isoform d corresponding to isoform 7 as illustrated that lacks two in-frame exon-encoded segments (from exons 5 and 6) and contains a shorter, truncated N terminus; accession number NP_001263993.1) and MffΔTM (1–217 aa) were subcloned in pGEX6P1 (GE Healthcare Lifesciences), and then expressed and purified as N-terminal GST fusion proteins from E. coli BL21 Star (DE3) using standard protocols. After buffer exchange by dialysis in 20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM EDTA, and 1 mM DTT, Mff and MffΔTM were excised from GST using PreScission Protease (GE Healthcare Lifesciences) or Pierce HRV 3C
protease (Life Technologies) using prescribed procedures. All proteins were aliquoted in buffer containing 10% (v/v) glycerol and frozen at −80 °C prior to use.

**Liposome Preparation**

All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Liposomes were by prepared by extrusion (21 times) through 400-nm-pore-diameter polycarbonate membranes as described previously (Macdonald et al., 2014). Unless noted otherwise, liposomes contained 25 mol % bovine heart CL, 35 mol % 1,2-dioleoylphosphatidylethanolamine (DOPE), and 40 mol % 1,2-dioleoylphosphatidylcholine (DOPC). In liposomes containing less than 25 mol % CL, a corresponding mole fraction of DOPC was substituted for CL. For Trp-dansyl FRET measurements, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (ammonium salt; 18:1 dansyl-PE) at 10 mol % replaced an equivalent concentration of DOPC. For the sucrose gradient flotation assay, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl (rhodamine-labeled PE or RhPE) was incorporated as a fluorescent lipid tracer at 1 mol %, replacing an equivalent concentration of DOPC. Pure DOPS liposomes contain 100 mol % 1,2-dioleoylphosphatidylserine (DOPS).

**GTPase Assay**

The basal and liposome assembly-stimulated GTPase activities of Drp1 were measured at 37 °C using a malachite green-based colorimetric assay as described previously (Leonard et al., 2005; Macdonald et al., 2014). Unless specified otherwise, the final Drp1 and total lipid concentrations were 0.5 and 150 µM, respectively. In experiments with externally added Mff, Mff at the indicated concentrations was
preincubated with liposomes for 15 min at room temperature prior to the addition of Drp1 and an additional 15 min of incubation to promote Drp1 interactions. Unless noted otherwise, GTP was used at 1 mM final concentration. Solutions were warmed up to 37 °C for 3 min prior to initiating the GTPase reaction. The initial burst rate of GTP hydrolysis was fit to a linear equation to obtain the turnover number (k_{cat}). Michaelis-Menten kinetics and constants (K_m or K_{0.5}) were determined using standard procedures.

**Size-exclusion Chromatography (SEC)-coupled Multi-angle Light Scattering (MALS)**

SEC-MALS measurements were performed at room temperature as described previously (Macdonald et al., 2014; Mehrotra et al., 2014). Briefly, Drp1 samples were each loaded at a concentration of 10 µM in a 500-µl volume onto a Superose 6 10/300 GL gel filtration column (GE Healthcare Lifesciences), pre-equilibrated in buffer containing 20 mM HEPES, pH 7.5, 150 mM KCl, and 1 mM DTT, and resolved at a flow rate of 0.3 ml/min on an ÄKTAexplorer™ (GE Healthcare Lifesciences) liquid chromatography system. The resolved samples were then passed inline through tandem differential refractive index and light scattering detectors (Wyatt Technology, Santa Barbara, CA). Data were analyzed and molar mass profiles were determined using the ASTRA 6.1 software package (Wyatt Technology).

**Negative-stain EM**

Drp1 (2 µM protein) was incubated with either nucleotide (1 mM final) or liposomes (50 µM total lipid final for 25 mol % CL-containing liposomes and 100 µM for pure DOPS liposomes) in buffer containing 20 mM HEPES, pH 7.5, 150 mM KCl, and 1 mM DTT for at least 30 min at room temperature prior to staining with 2% uranyl acetate and deposition on EM grids. For nucleotides, MgCl_2 was included in the buffer at a
concentration of 2 mM final. Images were obtained as described previously (Macdonald et al., 2014). The size distribution of lipid tube diameters was quantified and plotted as described elsewhere (Francy et al., 2015).

3.6 ACKNOWLEDGEMENTS

We thank Dr. Yisang Yoon (Georgia Regents University) for providing us with the full-length DLP1 cDNA clone, Dr. Hiromi Sesaki (Johns Hopkins University) for Drp1 KO MEFs, and Dr. David Chan (Caltech) for Mff KO MEFs.
FIGURE 3.1 **Drp1 isoforms exhibit differential GTPase activities.**

(A) graphic illustration of the Drp1 primary structure showing the locations of the A- and B-insert segments present in select Drp1 isoforms. The respective locations of the A- and B-inserts within the Drp1 structure (Protein Data Bank ID: 4BEJ) and their sequences are shown. The six extra amino acid residues (aa 594–599) found in the VD of the rat Drp1-
long clone are also indicated. GED, GTPase effector domain. (B–E) Time course of GTP hydrolysis for Drp1-short (B), Drp1-long (C), Drp1-A-only (D), and Drp1-B-only (E) (0.5 µM protein) in the absence and presence of 25 mol % CL-containing liposomes (150 µM total lipid) was measured as a function of P_i released over time using a malachite green-based colorimetric assay. The initial rates of GTP hydrolysis were fit to a linear equation. The calculated turnover number (k_{cat}) for each condition is indicated above. F, a bar plot summarizing the progressive reduction of cooperative basal and liposome-stimulated GTPase activities upon inclusion of either A-inserts or B-inserts or both in Drp1. Error bars indicate means ± S.D.
FIGURE 3.2 

Drp1 isoforms exhibit differential oligomerization propensities in solution and distinct helical geometry on CL-containing membranes. (A, left panel) SEC-MALS analyses of Drp1-short, Drp1-long, Drp1-A-only, and Drp1-B-only fractionated on a Superose 6 10/300 GL column at a loading concentration of 10 µM. Normalized differential refractive indices (left axis; line traces) and molar mass profiles underneath the peak regions (right axis; dots) are plotted against elution volume (in ml). The slopes of the molar mass profiles were fitted to an exponential trace (dotted line) to model oligomerization propensities. Right panel, magnified negative-stain EM images of
the corresponding Drp1 helical polymers (2 µM protein) formed in the presence of 1 mM GMP-PCP in solution. Scale bar, 100 nm. (B), left panel, representative FRET emission spectra of Drp1 Trp (0.5 µM protein) in the absence (Donor only) and presence of 10 mol % dansyl-PE (Donor + Acceptor) in 25 mol % CL-containing liposomes (150 µM total lipid). The acceptor-only trace for an equivalent concentration of dansyl-PE-containing liposomes in the absence of protein is also shown. FRET is characterized by the decrease in donor emission intensity in the presence of acceptor and an increase in the sensitized emission of the acceptor upon donor excitation. Right panel, Trp-dansyl FRET efficiency (E) for the various Drp1 isoforms (1 µM protein) in the presence of 25 mol % CL-containing liposomes that contain 10 mol % dansyl-PE (100 µM total lipid). E is expressed as a percentage. Error bars indicate means ± S.D. C, representative negative-stain EM images of Drp1-short, Drp1-long, and Drp1-A-only helical polymers (2 µM protein) tubulating 25 mol % CL-containing liposomes (50 µM total lipid). Scale bar, 200 nm. Histograms of tube diameter size distributions are shown below for Drp1-short and Drp1-A-only. The small sample size precluded statistical analyses for Drp1-long. No polymers were visualized for Drp1-B-only.
FIGURE 3.3 Drp1 A- and B-inserts differentially affect membrane curvature.

A–D, representative negative-stain EM images (right panels) and corresponding histograms of tube diameter size distribution (left panels) for Drp1-short (A), Drp1-long (B), Drp1-A-only (C), and Drp1-B-only (D) helical polymers (2 µM protein) tubulating and decorating unextruded pure DOPS liposomes (100 µM total lipid). Scale bar, 50 nm.
CHAPTER 4: CRYO-EM STUDIES OF DRP1 REVEAL CARDIOLIPIN
INTERACTIONS THAT ACTIVATE THE HELICAL OLIGOMER

This chapter has been submitted for publication:

4.1 ABSTRACT

Dynamins are mechano-chemical GTPases involved in the remodeling of cellular membranes. In this study, we have investigated the mechanism of dynamin-related protein 1 (Drp1), a key mediator of mitochondrial fission. To date, it is unclear how Drp1 assembles on the mitochondrial outer membrane in response to different lipid signals to induce membrane fission. Here, we present cryo-EM structures of Drp1 helices on nanotubes with distinct lipid compositions to examine liposome interactions with the fission machinery. These Drp1 polymers assemble exclusively through stalk and G-domain dimerizations, which generates an expanded helical symmetry when compared to other dynamins. Interestingly, we found the characteristic gap between Drp1 and the lipid bilayer was lost as Drp1 directly interacts with the membrane when the mitochondrial specific lipid cardiolipin was present. Moreover, this interaction leads to a change in the helical structure, which alters G-domain interactions to enhance GTPase activity. These results demonstrate how lipid cues at the mitochondrial outer membrane (MOM) can alter Drp1 structure to activate the fission machinery.
4.2 INTRODUCTION

Mitochondria are essential membrane-bound compartments in eukaryotic cells that generate ATP and play a central role in activating apoptosis. Concurrently, mitochondria undergo continuous cycles of fission and fusion, creating a dynamic population of organelles capable of responding to physiologic signals (Chen and Chan, 2005; Suen et al., 2008). Mitochondrial division is essential to maintain cell health, but excessive fission has been observed in several diseases (Herzig and Martinou, 2008; Karbowski and Youle, 2003; Perfettini et al., 2005; Chen and Chan, 2009; Ong et al., 2010). Despite this central role in mitochondrial health and disease, detailed insight into the fundamental mechanism of mitochondrial fission is lacking.

The key mediator of mitochondrial fission is dynamin-related protein 1 (Drp1), a large cytosolic GTPase (~80 kDa) belonging to the dynamin family of proteins. Drp1 localizes to mitochondrial fission sites and forms large oligomeric assemblies that are capable of inducing membrane constriction (Ingerman et al., 2005; Francy et al., 2015; Yoon et al., 2001; Koirala et al., 2013). *In vitro*, Drp1 assembles on lipid templates, which can be used to mimic molecular interactions with the mitochondrial outer membrane (MOM), and these interactions facilitate self-assembly and stimulated GTPase activity (Francy et al., 2015; Macdonald et al., 2014; Ugarte-Uribe et al., 2014; Mears et al., 2011). Based on previous studies with dynamin (Chappie et al., 2010; Chappie et al., 2011), this enhanced activity is believed to result from intermolecular G-domain dimerizations near the GTP-binding site, and a similar G-domain interaction was observed in Drp1 structural studies as well (H and S, 2013). Additionally, extra sequence within the G-domain of Drp1 has been found to mediate a novel G-domain dimer
(Wenger et al., 2013; Macdonald et al., 2016). This interaction is stabilized through an 18 amino acid region called the 80 loop that is conserved in homologous mitochondrial fission dynamins (Wenger et al., 2013). Despite this previous work, the functional implications and the cellular contexts for these distinct G-domain dimers remains unknown.

In addition to G-domain interactions, Drp1 self-assembly is primarily driven via intermolecular stalk interactions (Frohlich et al., 2013). The stalk is comprised of the middle domain and GTPase effector domain (GED, Fig 4.2A), and several mutations in these regions have been shown to disrupt Drp1 oligomerization (Frohlich et al., 2013; Chang et al., 2010) and are associated with developmental defects in humans (Waterham et al., 2007; Fahrner et al., 2016). Previously, crystallographic studies with dynamin, a related protein family member involved in endocytosis, revealed three important interfaces in the stalk. These interactions were also identified in cryo-EM structures of dynamin polymers on lipid templates (Zhang and Hinshaw, 2001; Chen et al., 2004; Mears et al., 2011; Chappie et al., 2011). However, within the Drp1 crystal lattice, only one of these interfaces was conserved (Frohlich et al., 2013). Consequently, it remains unclear how intermolecular Drp1 interactions drive assembly of the mitochondrial fission complex at membrane surfaces.

Between the middle and the GED sequences, an additional region called the variable domain (VD) bridges these domains similar to the pleckstrin homology (PH) domain in dynamin (Fig 4.2a). This sequence has been shown to be an important regulatory component of Drp1 function (Frohlich et al., 2013). Specifically, the VD serves as an autoinhibitory domain of Drp1 oligomerization as it maintains Drp1 in an
assembly-limited cytosolic state (Strack and Cribbs, 2012; Francy et al., 2015). The VD is largely unstructured, and it has previously been shown to associate with negatively charged membranes which relieves its inhibitory role to drive Drp1 self-assembly (Francy et al., 2015; Clinton et al., 2016). In this way, the VD mirrors the lipid interaction properties of the dynamin PH domain, which binds to phosphoinositol-4,5-phosphate (PIP2) enriched membranes (Zheng et al., 1996). For Drp1, several recent studies support a significant VD interaction with the mitochondrial specific lipid, cardiolipin (CL), which promotes Drp1 assembly and augments GTPase activity (Stepanyants et al., 2015; Bustillo-Zabalbeitia et al., 2014; Ugarte-Uribe et al., 2014; Macdonald et al., 2014). CL is most often found in the mitochondrial inner membrane (MIM), where it serves a critical role in electron transport chain function (Claypool, 2009; Paradies et al., 2014). Interestingly, CL translocation to the mitochondrial outer membrane (MOM) has been reported at contact sites with the MIM (Ardail et al., 1990) and under stress conditions that induce mitophagy (Chu et al., 2013). However, it is not known whether Drp1 stably associates with CL at the molecular level or how this interaction would convey enhanced activity to the GTPase domains of Drp1 polymers.

In order to address these critical questions about the mitochondrial fission complex, we have determined cryo-EM structures of human Drp1 helices on distinct membrane templates either containing the negatively charged phosphatidyl serine (PS) used in previous studies, or the mitochondrial specific CL. We found that distinct G-domain dimers were formed on each of these lipid templates, and that the magnitude of stimulated GTPase activity coincides with the mode of lipid-induced assembly of Drp1. In this way, changes in lipid composition can alter Drp1 structure and activity. In addition
to G-domain dimerization, we found that only one stalk interface was preserved within the lipid-induced Drp1 oligomers, and this produces a relatively extended polymer compared to dynamin. Lastly, we determined that the VD directly interacts with CL to induce functional assembly of Drp1 oligomers. In this way, CL microdomains at the surface of mitochondria could selectively trigger Drp1 activity to promote local constriction events. Collectively, these studies reveal the structural and functional consequences of Drp1 interactions with specific lipid membranes to advance our understanding of the mitochondrial fission complex.

4.3 RESULTS

Dimerization through Intermolecular G-domain and Stalk Interactions Drive Drp1

Helical Oligomerization on PS Lipid Templates

Drp1 has been shown to assemble on negatively charged lipid membranes (Francy et al., 2015; Macdonald et al., 2014; Yoon et al., 2001; Koirala et al., 2013), and PS has often been used as a model template given its ability to induce robust assembly of Drp1 and other dynamins. To build on these initial studies, we used negative stain electron microscopy (EM) to examine the structural properties of Drp1 polymers formed in the presence of PS liposomes. Although we observed robust formation of protein-lipid tubules, the diameters of these assemblies displayed a heterogeneous distribution (Fig 4.1a, 119 ± 49 nm), which highlights the inherent ability of Drp1 to accommodate diverse membrane geometries. As shown previously (Francy et al., 2015; Frohlich et al., 2013), the addition of a non-hydrolyzable GTP analogue, GMPPCP, led to a decrease in the average diameter (108 ± 44 nm), but a broad distribution of diameters was still apparent (Fig 4.1b). Therefore, galactosyl ceramide (GC) was used to generate lipid nanotubes
with a relatively fixed geometry. Incorporation of 30 mol% PS into the GC nanotubes (GC/PS) generated a uniform lipid template with sufficient negative charge to recruit Drp1. These templates enriched a population of Drp1-lipid tubules with smaller diameters that represented a subset of the distribution observed using PS liposomes (Fig 4.1c, 59 ± 4 nm). This enhanced homogeneity of Drp1 oligomers formed on GC/PS templates in the presence of GMPPCP provided an optimal sample for cryo-EM studies to examine protein-lipid interactions.

Drp1 oligomers on GC/PS templates were readily observed using negative stain and cryo-EM (Fig 4.2b,c, respectively). The laddering pattern displayed on the surface of the lipid nanotubes demonstrates Drp1 helical decoration on this membrane surface (closed arrow). Undecorated nanotubes were less abundant (Fig 4.2b-c, open arrow), but these were disregarded for structural studies. The 3D structure of Drp1 on a PS-containing membrane was determined using an Iterative Helical Real Space Reconstruction algorithm (IHRSR) (Egelman, 2007; Egelman, 2010) (Fig 4.2d). The helical lattice had a pitch of 13 nm and a right-handed symmetry (Fig 4.2d, Fig 4.3, more information available in Methods). Dimeric Drp1 forms the fundamental asymmetric unit, and 10 dimers comprised one turn of the helix, which represents a novel Drp1 helical conformation.

From the final reconstruction, the globular GTPase domains (green) are clearly discernable from the stalk regions (blue) of Drp1, and the protein assembly forms a characteristic T-shaped architecture similar to other dynamins (Fig 4.2f) (Zhang and Hinshaw, 2001; Chen et al., 2004; Mears et al., 2011). Interestingly, there appears to be no stable Drp1 contact with the lipid surface (Fig 4.2e-f, open arrow head), which is
consistent with previous studies using the yeast homolog of Drp1 (Mears et al., 2011). This “gap” may be due to helical averaging of flexible protein segments that weakly interact with the lipid surface. Still, the interaction between Drp1 and the PS template is not stable.

Upon docking the Drp1 crystal structures into the density, it was found that G-domain and stalk interactions were driving the helical assembly (Fig 4.2g-h). Interestingly, the canonical G-G interaction, proximal to GTP-binding sites, that has been demonstrated with dynamin did not fit well into the EM density (Fig 4.4). Rather, the G-domain dimer formed via the 80 loop (Wenger et al., 2013) closely matched the density when fitted (Fig 4.2h-i). This unique interaction is still consistent with the stalk occupying the middle radial density (blue) that dives towards the lipid membrane. Within this region, additional protein density was apparent (Fig 4.2h, dotted orange hexagon), and we attribute this to VD interactions near the stalk, comparable to interactions between the PH domain and stalk of dynamin (Srinivasan et al., 2016a). In this conformation, interactions between the VD and lipid would likely be transient, which is consistent with the weak membrane association.

**Cardiolipin Nanotubes Drive Functional Assembly of Drp1**

There have been several studies demonstrating the ability of CL to robustly stimulate Drp1 GTPase activity (Macdonald et al., 2014; Stepanyants et al., 2015) through direct interactions with Drp1 (Bustillo-Zabalbeitia et al., 2014). To further explore the recruitment and enzymatic activity using structural lipid templates, we utilized sedimentation assays, cryo-EM and GTPase assays to measure Drp1 interactions on several nanotubes with distinct lipid compositions. Alone in solution, Drp1 does not
sediment with medium-speed centrifugation (17% in pellet, Fig 4.5a), which is consistent with the smaller oligomers sampled in solution (Macdonald et al., 2014). Conversely, Drp1 sedimentation increases after the addition of GMPPCP (41% in pellet) (Fig 4.5a), which is consistent with the formation of larger protein spirals in solution (Fig 4.5b). The addition of neutral charged lipid nanotubes containing 12 mol% phosphatidylcholine (PC) did not increase Drp1 sedimentation when compared with the protein alone (22% and 45% in the pellet in the absence and presence of GMPPCP, respectively) (Fig 4.5a). Cryo-EM images revealed that Drp1 could not assemble on the GC/PC lipid nanotubes with or without GMPPCP (Fig 4.5a,e). Therefore, the curvature of the lipid nanotubes alone was not sufficient for Drp1 recruitment and polymerization. Conversely, the addition of GC/PS and GC/CL (12 mol % CL) initiated robust assembly of Drp1 oligomers as larger sedimentation values were observed (52% and 92% in the pellet, respectively). Addition of GMPPCP enhanced oligomerization with the PS nanotubes (an increase to 82% in the pellet), while the CL nanotube appeared to be saturated whether nucleotide was present or not (94% in the pellet), which demonstrates a stronger interaction for Drp1 with CL. Images of the Drp1-lipid tubule segments exhibited an ordered protein assembly around the lipid core (Fig 4.5d,g), and this pattern was noticeably absent for undecorated nanotubes (Fig 4.5c,e,f). Two dimensional class averages further illustrate these differences, as Drp1 uniformly decorated nanotubes containing PS and CL, while PC nanotubes were undecorated (Fig 4.5h). The protein lattice on the CL nanotubes also appears to adopt a distinct conformation when compared with the PS template.
To ensure that the lipid nanotubes were forming functional Drp1 complexes, we utilized a phosphate release GTPase assay (Leonard et al., 2005). Drp1 in solution displayed a basal activity (2.3 min⁻¹), and the addition of GC/PC lipid did not alter this measurement (2.3 min⁻¹), which is consistent with the lack of protein self-assembly (Fig 4.5i). Consistent with previous studies, GC/CL stimulated Drp1 activity (30 min⁻¹, a 13-fold enhancement) more than did GC/PS (16 min⁻¹, a 7-fold increase) (Fig 4.5i). Nevertheless, the negatively charged nanotube templates were able to induce functional Drp1 oligomerization. In addition, the increased stimulation in GTPase activity and the differences in 2D class averages suggested that CL formed a distinct Drp1 helical structure that could represent an alternative functional state dependent on the lipid composition of the membrane.

**Cardiolipin Interactions Stabilize an Active Helical Drp1 Conformation**

Since CL recruits and activates Drp1 more robustly than other negatively charged liposomes, we explored the structure of Drp1 helices on CL nanotubes to identify potential differences. Negative stain and cryo-EM images revealed that the Drp1-decorated CL nanotubes were largely comparable to the Drp1 + GC/PS sample (Fig 4.6b,c vs. Fig 4.2b,c). However, closer examination of the cryo images revealed that the Drp1-CL oligomer appeared to associate more strongly with the lipid surface (Fig 4.5h, Fig 4.6b right panel). Using the same IHRSR method described previously, we determined the 3D structure of Drp1 polymers on CL nanotubes in the presence of GMPPCP (Fig 4.6c). The oligomer was also found to have a right-handed symmetry (Fig 4.3) and a similar helical pitch of 13 nm. While the Drp1 dimer still formed the fundamental, repeating unit within the helical lattice, only 9 subunits were found to
comprise one turn of the helical lattice (vs. 10 subunits for the Drp1-PS helices). This change coincides with a different Drp1 helical conformation, and the G-domain (green) and stalk (blue) regions of the dimers assumed distinct orientations. Most strikingly, this altered assembly was promoted through changes at the lipid surface. Specifically, the Drp1 polymer forms a stable, direct interaction with the CL nanotube. In fact, no gap was found between the protein and membrane, and a strong protein density was observed proximal to the lipid tubule (Fig 4.6d,e, filled arrow head).

The density contacting the lipid surface was likely attributed to the VD, which was deleted in previous crystallographic studies (Fig 4.6h, orange dotted circle). To confirm the role of the VD in direct association with the CL nanotube, we utilized a ΔVD Drp1 mutant lacking this region (Frohlich et al., 2013). Sedimentation assays were performed with the ΔVD mutant to examine lipid association and protein oligomerization. Compared to WT, ΔVD sedimentation was greatly reduced on the GC/CL template (25% in the pellet, Fig 4.7) and this value only slightly increased when GMPPCP was added (32% in the pellet, Fig 4.7). This is consistent with previous studies that have shown diminished CL interactions when the VD was mutated (Bustillo-Zabalbeitia et al., 2014). Collectively, these data confirm that the VD is essential for Drp1 association with CL lipids leading to helical polymerization.

Docking of the Drp1 crystal structure revealed that interactions between the stalk dimers are maintained through a similar interaction, but each stalk was tilted toward the membrane surface, likely due to interactions with the lipid (Fig 4.6f-h). Interestingly, the G-domain interactions within the Drp1-CL oligomers were found to undergo a large conformational rearrangement when compared to the GC/PS model, which results in a
more extended dimer orientation. This orientation would support G-G interactions adjacent to the GTP-binding sites, which is consistent with structures observed in previous dynamin studies (Chappie et al., 2010) (Fig 4.6f,g). This interaction also correlated with enhanced GTPase activity, which implies that direct interaction with a CL membrane can rearrange the Drp1 polymer to promote GTP hydrolysis and subsequent constriction.

Comparing our cryo-EM reconstructions to other dynamin family members, one striking difference is the greater spacing within the helical lattice (Fig 4.8). In fact, the lipid nanotube is clearly visible beneath the protein coat. In the dynamin helical structure, the protein shell is much more densely packed (Zhang and Hinshaw, 2001; Chen et al., 2004; Sundborger et al., 2014) (Fig 4.8a,b). The lack of additional stalk interfaces within the Drp1 oligomer leads to an expanded assembly (Fig 4.8c,d). To this point, Drp1 dimers traverse a greater radial path length, as there are fewer subunits per turn (GC/PS-10 subunits/turn, GC/CL- 9 subunits/turn) when compared with ΔPRD-dynamin in a similar state (13 subunits/turn). This topology may be necessary for Drp1 to remain dynamic and accommodate various lipid curvatures. Nonetheless, stalk and G-domain dimerization appear to be the minimal interfaces required for active Drp1 polymerization.

4.4 DISCUSSION

In this study, we have identified key assembly factors that drive Drp1 helical oligomerization on lipid templates in the presence of nucleotide. As shown previously (Francy et al., 2015; Frohlich et al., 2013), Drp1 is capable of forming protein lipid tubules with various diameters (Fig 4.1). To limit heterogeneity for structural studies, we decorated lipid nanotubes with Drp1 in the presence of GMPPCP to maintain more
uniform helical diameters. These represent a subset of narrow oligomers that Drp1 formed when remodeling anionic liposomes (Fig 4.1). Drp1 interaction with negatively charged surfaces agrees with in vivo data, where Drp1 associates with several anionic membranes and structures within the cell, including mitochondria, peroxisomes, microtubules and actin (Frohlich et al., 2013; Strack et al., 2013; Ji et al., 2015; Korobova et al., 2013). Therefore, these structural studies reveal how Drp1 interactions with negatively charged membranes influence its structure and function.

Regardless of whether PS or CL was used to form Drp1-lipid structures, the fundamental building block within the Drp1 polymer is a dimer. This is consistent with previous studies demonstrating preferential assembly of dimers into larger oligomeric fission complexes (Macdonald et al., 2014). Moreover, within the GC/PS and GC/CL models, we can see that the stalk interface that drives dimerization in all dynamins is conserved, and mutagenesis in this region completely ablates Drp1 oligomerization (Frohlich et al., 2013). Interestingly, oligomerization of other dynamin family members are primarily driven through additional stalk interactions (Gao et al., 2010; Faelber et al., 2011; Ford et al., 2011; Chen et al., 2004; Sundborger et al., 2014; Mears et al., 2011). However, these other interfaces were not observed in our structures, owing to the greater spacing within the helical lattice (Fig 4.8a-d). We cannot exclude the important roles of additional Drp1 interfaces. Proposed interaction sites near the bottom of the stalk (previously termed interface 3) and between Drp1 dimers (interface 4) may be important for solution multimers or for distinct helical oligomers. It appears that Drp1 can assume different modes of assembly, which may rely on specific interactions at the surface of mitochondria.
Intermolecular G-domain contacts are formed in the presence of nucleotide to further build the helical polymer (Fig 4.8c,d). Despite the importance of the stalk domains in oligomerization, we found that the G-domain interactions play a significant role in driving Drp1 helical assembly as well. Intriguingly, the G-domain dimers formed in the GC/PS structure were distinct from those seen in the GC/CL structure. More specifically, we found that the 80 loop G-domain dimer, solved in previous crystal studies (Wenger et al., 2013), best fits in the G-domain density on the PS membrane (Fig 4.9a). Conversely, the G-domains are held in a “front-to-front” orientation in the GC/CL model, and this leads to an enhanced GTPase activity through a mechanism similar to G-domain dimerization seen in other dynamin structures (Sweitzer and Hinshaw, 1998) (Fig 4.9b). Specifically, these G-domain dimers are juxtaposed to allow for intermolecular interactions near the nucleotide-binding site. This conformational change in the presence of CL suggests that the VD can sense lipid microdomains and transduce a conformational change from the base of the molecule through the stalk to reorient G-domain interactions at the periphery of the contractile apparatus (Fig 4.9d). Drp1 interactions with CL in the cell would likely occur at specific sites, as CL is known to be enriched at contact sites between the MOM and MIM (Ardail et al., 1990) and externalized CL interacts with several cytosolic proteins on the MOM including Bax, tBid and LC3 proteins involved in mitophagy (Chu et al., 2013). Cardiolipin has also been shown to preferentially interact with flexible protein domains (Planas-Iglesias et al., 2015), just like the VD. Thus, Drp1 assemblies can respond to specific molecular cues, such as CL, to alter functional processivity (Fig 4.9e).
The Drp1 helical structure on GC/PS membranes is in a different conformation with limited activity compared to GC/CL. Strikingly, this complex does not strongly contact the lipid membrane and the G-domains dimerize through a distinct interface near the 80 loop. This stretch of 16 amino acids in the 80 loop is unique to Drp1 and other dynamin-related proteins involved in mitochondrial fission. We propose that this novel interaction may represent an additional regulatory interface. Specifically, Drp1 would assemble in a primed conformation until additional signals induce a conformational change in the VD that propagates through the stalk and G-domain to promote targeted Drp1 constriction. In cells, Drp1 has been shown to sample the MOM in oligomeric forms without inducing a fission event (Ji et al., 2015; Ugarte-Uribe et al., 2014). Therefore, the GC/PS polymer may represent a sampling assembly, and additional cues, including CL interactions, alter Drp1 conformation to promote GTP hydrolysis leading to Drp1 constriction and subsequent fission.

Mitochondrial fission partner proteins, including Mff, MiD49/51 and Fis1, may also act as physical cues and that promote functional assembly of mitochondrial fission complexes, similar to CL. The structure of the Drp1 helix has sufficient spacing to accommodate partner proteins within the polymer lattice, which could further explain the unique helical symmetry. Previous studies have shown interactions with Mff stimulate Drp1 GTPase activity (Clinton et al., 2016; Stepanyants et al., 2015), while interactions with MiD51 have been shown to reduce Drp1 GTPase activity (Osellame et al., 2016). Therefore, it is likely that this additional regulation with partner proteins coincides with lipid cues on the membrane surface to modulate Drp1 recruitment and function.
In the cell there are many physiologic cues that recruit specialized proteins so they can perform their distinct function. One example includes PS, which is externalized to the outer leaflet of the plasma membrane during apoptosis and acts as an ‘eat-me’ signal. PS-specific receptor proteins bind externalized PS and induce eventual apoptosis (Borisenko et al., 2003). In a similar fashion, CL may act as a ‘divide-me’ signal on the MOM, providing the cue for activation of the Drp1 polymer. In disease, CL externalization may be enhanced resulting in accelerated scission, increasing the efficiency of mitochondrial division. Thus, this could be an attractive therapeutic target.

Previously, nucleotide induced conformational changes of dynamin have been demonstrated, but in this study we show that CL can specifically induce conformational changes that enhance Drp1 function. Interestingly, dynamin may also undergo lipid specific conformational changes, as previous work has shown greater stimulation on PIP2 containing membranes compared to PS membranes (Ramachandran et al., 2007). Therefore, the ability of dynamins to respond to lipid signals may be conserved. Overall, this work advances our understanding of the potential cues and molecular mechanisms necessary to induce Drp1 mediated mitochondrial fission.

4.5 MATERIALS AND METHODS

Expression and Purification of Drp1

Human Drp1 (UniProtID: O00429-3, residues 1-710, isoform 2) was expressed and purified as described previously (Frohlich et al., 2013). Briefly, Drp1 was cloned into a modified pET28a vector as an N-terminal His6-tag fusion followed by a PreScission cleavage site, and specified mutations were inserted using standard protocols. The VD (aa 514–613) was deleted as previously described (Hansson et al., 2008). All constructs were
expressed in *Escherichia coli* host strain BL21 DE3 Rosetta2 (Novagen). Bacteria were cultured in TB medium at 37 °C to an OD₆₀₀ of about 0.4 followed by a temperature shift to 18 °C. The protein was expressed for 18 h by addition of 40 µM isopropyl β-d-1-thiogalactopyranoside (IPTG). Bacteria were collected by centrifugation and resuspended in buffer A (50 mM HEPES/NaOH (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 40 mM imidazole, 2.5 mM 2-mercaptoethanol, 1 mM DNase (Roche), 100 µM Pefabloc (Roth), followed by cell disruption in a microfluidizer (Microfluidics). Lysates were cleared by centrifugation at 40,000 x g for 30 min at 4°C, and the filtered supernatant was applied to a Ni-NTA column pre-equilibrated with buffer B (50 mM HEPES/NaOH (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 40 mM imidazole, 2.5 mM 2-mercaptoethanol). The column was extensively washed with buffer B, followed by buffer C (50 mM HEPES/NaOH (pH 7.5), 800 mM NaCl, 5 mM MgCl₂, 40 mM imidazole, 2.5 mM 2-mercaptoethanol, 1 mM ATP, 10 mM KCl) and buffer D (50 mM HEPES/NaOH (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 80 mM imidazole, 2.5 mM 2-mercaptoethanol, 0.5% (w/v) CHAPS). Bound Drp1 was eluted with buffer E (50 mM HEPES/NaOH (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 300 mM imidazole, 2.5 mM 2-mercaptoethanol) and dialysed overnight at 4°C (18 kDa cutoff) against buffer B without imidazole in the presence of PreScission protease to cleave the N-terminal His₆-tag. The protein was re-applied to a Ni-NTA column pre-equilibrated with buffer B without imidazole to which it bound under these conditions also in the absence of the His₆-tag. Subsequently, the protein was eluted with buffer B. In a final step, Drp1 was purified by size-exclusion chromatography on a Superdex-200 column (GE) in buffer F containing 20 mM
HEPES/NaOH (pH 7.5), 300 mM NaCl, 2.5 mM MgCl₂ and 2.5 mM dithiothreitol.

Fractions containing Drp1 were pooled, concentrated and flash frozen in liquid nitrogen.

**Liposome Preparation**

All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The liposomes used in this study included 1,2-dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (DOPS), heart bovine cardiolipin (CL), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and D-Galactosyl-β1-1’-N-Nervonoyl-D-erythro-Sphingosine (C24:1 β-D-Galactosyl Ceramide) (GalCer). Specific lipids in this study were dissolved in chloroform and dried under a nitrogen stream. For structural studies, 30 mol% DOPS was mixed with GalCer (GC/PS), and separately 12 mol % CL was mixed with GalCer (GC/CL). Additionally, 12 mol% DOPC in GalCer (GC/PC), GC/PS and GC/CL were used for sedimentation and GTPase assays. The dried lipid was stored in vacuum overnight and rehydrated in 37°C PBS buffer, followed by occasional vortexing. Assembly buffer was used to resuspend lipids for GTPase assays.

**Drp1 Lipid Tube Formation**

Drp1 was resuspended to 0.4 mg/ml in PBS buffer (10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, 10 mM BME, pH 7.4). Lipid nanotubes with a specific composition were added to the mixture to a final concentration of 0.4 mg/ml (450 uM). The suspension was incubated at RT for 5 minutes, then 1 mM GMPPCP (Sigma) was added. The GC/PS samples in the presence of GMPPCP were incubated overnight before making EM grids, while the GC/CL samples were incubated for 30 minutes.

**Electron Microscopy Sample Preparation**

For negative stain, samples were stained with 2% (w/v) uranyl acetate (Polysciences,
Inc.) on carbon-coated grids. For cryo-EM preparations, samples were applied to holey carbon grids (R3.5/1, Quantifoil). After a 1 minute incubation, the grid was blotted with filter paper and flash frozen in liquid ethane using a manual plunger. The samples were imaged at liquid nitrogen temperatures on a TF-20 FEG electron microscope (FEI Company) operating at 200 kV and recorded at 25,000x magnification. Images were collected on a Tvips Tietz 4k x 4k CMOS-based camera under low dose conditions with defocus values ranging from -2 to -6 um.

**Hand Determination**

To determine the handedness of the Drp1 tubes, negative stain EM samples were imaged at tilts of 0 and -40° or -50° on a TF-20 FEG electron microscope (FEI Co.) operating at 200 kV and recorded at 25,000x magnification using a TVIPS Tietz 4k x 4k CMOS-based camera. The striations of Drp1 oligomerized on GCPS and GCCL were more pronounced on the top of the tube (Fig 4.3b), confirming they are right-handed helices.

**Reconstruction of Drp1 Lipid Tubes**

Images were contrast transfer function corrected using the TOMO-CTF image processing package (Fernandez et al., 2006). Selected Drp1-lipid tubes with good diffraction and minimum astigmatism and drift were initially boxed into helical segments at a width of 180 pixels using the Helixboxer (Ludtke et al., 1999) program in the EMAN suite (Tang et al., 2007). Projection matching was used to sort boxed segments based on tube diameter (26,272 total particles for the GC/PS dataset and 26,138 for the GC/CL dataset). For this supervised classification, the particles were aligned to 17 cylindrical diameter classes ranging from 33 to 61 nm. Classes with average diameters centered on
~50 nm (Fig 4.3a) were used for the final 3D reconstructions of both structures. Based on this classification, 10,238 segments of the GC/PS filaments and 9,514 segments of the GC/CL segments were used in the final refinement. The iterative helical real space reconstruction (IHRSR) method (Egelman, 2007) was used to refine the structures. The particles were initially aligned to a featureless cylinder model, and several symmetries were tested with parameters consistent with the helical symmetry apparent in raw images (regular pitch of ~130 Å). Numerous subunit parameters were tested, ranging from 6-20 subunits/turn. During refinements, in-plane rotation was limited to ±5° and a cross-correlation cutoff was used to remove particles that did not align. For the final reconstruction, the datasets were aligned to 1,890 reference images to account for 4° helical rotations and out-of-plane tilts from +20° to -20° at 2° increments. The final GC/PS map converged to a rotation angle of 35.9° and rise of 12.7 Å per subunit. The resolution of the final map was determined to be 20.8 Å by Fourier shell correlation and was calculated using the new gold standard method (Henderson, 2013). The final GC/CL map merged to a rotation angle of 39.9° and rise of 14.4 Å per subunit, and the resolution of the final map was determined to be 21.0 Å.

**Docking All-Atom Structures into Cryo-EM Density**

Cryo-EM density was incorporated as a structural restraint to refine the relative positions of Drp1 GTPase and stalk regions using the YUP software package (Tan et al., 2008). All-atom Drp1 structures were placed in several distinct orientations within both the GC/PS and GC/CL maps to examine as many conformations that reasonably fit to the EM densities. The Yup.vlat method was used to implement a vector lattice (VLAT) force field term defines the cryo-EM density as a three dimensional potential, providing a score
for fitting the Drp1 structures to the density. Refinements were performed using rigid body Monte Carlo with simulated annealing, which allows for exhaustive sampling of conformational space while the structures move to orientations that best match the cryo-EM data. During these fittings, several reduced-representation models (Cα atoms) of the Drp1 crystal structure (Frohlich et al., 2013) were initially evaluated. For the GC/CL map, the GTPase and BSE regions were treated as a rigid unit with the stalk as a separate rigid body to achieve the best fit. From several starting models, the structures converged to a most common fit. This fit was then used to build multiple turns of the helix and a second fitting protocol was run to validate the placement. For the GC/PS map, a similar protocol was used. It became clear that the front-to-front orientation of the GTPase domains was not feasible based both on the lack of fit quality and topological limitations (Fig 4.4). Therefore, the 80 loop structure of the Drp1 GTPase and BSE domains (Wenger et al., 2013) was fitted to the peripheral radial density (green, Fig 4.2d) and the stalk region from the Drp1 crystal structure used previously (Frohlich et al., 2013) was treated as a separate rigid unit. The 80 loop dimer fit the GTPase density of the PS map, and the stalk regions fit into adjacent density diving toward the membrane. To match this topology, the BSE was also reoriented into an “open” conformation, consistent with the GTP-bound conformation seen with Drp1 (H and S, 2013) and other dynamin family members previously (Chappie et al., 2011). Again, a common fit was determined from several starting orientations for both the GTPase-BSE and stalk regions. This fit was then used to build a multiple turns of the helix and a second fitting protocol was run to validate the placement. After the final refinements, all-atom structures were superposed onto the refined Cα backbones for the Drp1 structures in both the PS and CL maps. The
depictions of cryo-EM helical structures and fitted structures were generated using UCSF Chimera (Pettersen et al., 2004).

**Sedimentation Assay**

To quantify Drp1 oligomerization, a sedimentation assay was conducted similar to what has been described previously (Francy et al., 2015; Mears and Hinshaw, 2008). Large oligomers formed by Drp1 samples, in the presence of ligands, were found in the pellet after a medium speed centrifugation. Specifically, protein was diluted in PBS buffer to 0.05 mg/ml (0.62 µM), and specified WT and ΔVD mutant samples were incubated at room temperature with lipid nanotubes (200 µM) and/or GMPPCP (1 mM) for at least 30 minutes. The mixtures were then spun at 13,200 rpm (16,100 x g) for 30 min in a tabletop centrifuge (Eppendorf). The supernatant and pellet fractions were separated, collected, and immediately mixed with SDS-PAGE loading dye (Bio-Rad) and heated briefly at 100˚C. These samples were run on an SDS-PAGE gel and stained with an InstantBlue Coomassie dye (Expedeon). Gels were scanned (HP Scanjet 8300) and densitometry analysis was done using the ImageJ software (Schneider et al., 2012).

**GTPase Assay**

Drp1 GTPase activity was determined using a colorimetric phosphate generation assay with some modifications (Leonard et al., 2005). Briefly, Drp1 (0.5 µM final) was diluted to 1.2x with Assembly Buffer (25 mM Hepes KOH, 150mM KCl, pH 7.5) in the presence or absence of lipid nanotubes (200 µM total lipid final) for 15 minutes at room temperature. 3x GTP/Mg²⁺ (1 mM and 2 mM final, respectively) in Assembly Buffer was added to Drp1/lipid mixtures to start reactions and samples were incubated at 37 ºC. At designated timepoints, EDTA (0.1 M final) was added to sample aliquots. Malachite
green reagent (1 mM malachite green carbinol, 10 mM ammonium molybdate tetrahydrate, and 1N HCl) was added to each sample and A_{650} was measured using a Versamax microplate reader (Molecular Devices). Using Excel (Microsoft), the obtained raw phosphate levels were converted into rates using all data that contributed to a linear trend. These rates were converted to k_{cat} by accounting for Drp1 concentration and were plotted in GraphPad Prism 6. Statistical significance was determined using an unpaired t-test.

4.6 ACKNOWLEDGEMENTS

We thank Frances Alvarez and Heather Holdaway for expertise and advice with electron microscopy studies. Oliver Daumke was extremely generous in sharing protein constructs, expertise and scientific discussions. We acknowledge Rajesh Ramachandran and Taylor Enrico for critically reading the manuscript and providing feedback. American Heart Association Grants SDG12SDG9130039 (J.A.M) and the National Institutes of Health Training Grant 2T32GM008803-11A1 (C.A.F.) supported this work.
FIGURE 4.1

(a) Drp1 + PS (apo)
   119 ± 49 nm
   n=126

(b) Drp1 + PS + PCP
   108 ± 44 nm
   n=157

(c) Drp1 + GC/PS + PCP
   59 ± 4 nm
   n=117

Diameter (nm)
FIGURE 4.1 Drp1 helical polymers are more uniform on lipid nanotubes.

The distribution of diameters measured from negative stain EM images are presented for Drp1 helical polymers formed in the presence of PS liposomes (a), PS liposomes with GMPPCP (b), and PS nanotubes with GMPPCP (c). Representative images are shown (right) and diameters are indicated. Scale bar, 100 nm. The average diameter is presented for each sample with the standard deviation and number of measurements (n) indicated.
FIGURE 4.2

(a) GTPase

(b) [Image of microscopic structure]

(c) [Image of microscopic structure]

(d) 80 loop interaction

(e) G-domain dimer

(f) Stalk dimer

(g) Stalk dimer

(h) [Image of molecular model]

(i) GTPase
FIGURE 4.2 3D structure of Drp1 associated with a phosphatidylserine (PS) lipid template.

(a) The primary sequence and tertiary structure (PDB ID: 4BEJ) of Drp1 highlights conserved domains: GTPase domain (green), middle domain (blue), variable domain (orange) and GTPase effector domain (GED, blue). (b-c) Negative stain (b) and cryo-EM (c) images of Drp1 oligomerized in the presence of GMPPCP on galactosyl ceramide (GC) nanotubes containing phosphatidylserine (PS) at low (left; scale bar, 100 nm) and high magnifications (right; scale bar, 50 nm). Filled arrows indicate Drp1 decorated tubes, while open arrowheads indicate undecorated GC/PS tubes. (d-e) The 3D reconstruction of Drp1 on a GC/PS nanotube is presented. The helical pitch (13 nm) and diameter (51.4 nm) are indicated. (f) Cross-section of the 3D structure demonstrates the T-shaped architecture, and a gap between the protein and lipid is highlighted (open arrowhead). (g) The fitted structures of Drp1 GTPase (green) and stalk (blue) dimers are shown. The 80 loop interface (red) mediating G-domain dimerization is highlighted. (h) A side view of multiple Drp1 dimers fitted into the helical density. Unoccupied density is highlighted (dotted orange hexagon). (i) An end-on view of the same fitted structures.
(a) The diameter distributions of Drp1 helical particles formed on PS and CL nanotubes in the presence of GMPPCP are shown. The two most populated classes were used for helical reconstructions. The total number of particles was 10,236 for the GC/PS sample and 9,514 for the GC/CL sample. (b) The handedness of the helical polymers was determined by tilting the sample during image acquisition, and a schematic highlights the changes observed for the helical Drp1 polymer (green) associated with lipid (grey). When a right-handed helix is tilted in the negative direction, the rungs on the top of the helix are more prominent. Both Drp1+GC/PS+GMPPCP (-40˚ tilt) and Drp1+GC/CL+GMPPCP (-50˚ tilt) displayed this phenomenon, which indicates that these helical polymers are right-handed.
FIGURE 4.3 **Drp1 geometries and handedness used for 3D reconstructions.**

(a) The diameter distributions of Drp1 helical particles formed on PS and CL nanotubes in the presence of GMPPCP are shown. The two most populated classes were used for helical reconstructions. The total number of particles was 10,236 for the GC/PS sample and 9,514 for the GC/CL sample. (b) The handedness of the helical polymers was determined by tilting the sample during image acquisition, and a schematic highlights the changes observed for the helical Drp1 polymer (green) associated with lipid (grey). When a right-handed helix is tilted in the negative direction, the rungs on the top of the helix are more prominent. Both Drp1+GC/PS+GMPPCP (-40° tilt) and Drp1+GC/CL+GMPPCP (-50° tilt) displayed this phenomenon, which indicates that these helical polymers are right-handed.
FIGURE 4.4 Comparison of fitted Drp1 G-domain dimers in the GC/PS density.

For orientation, the 3D reconstruction of Drp1 on a GC/PS nanotube is presented with an isolated G-domain density highlighted in green. Distinct G-domain dimers were fitted to this region, and the 80 loop orientation more closely matches the contour of the density. With the catalytic dimer, unoccupied density was observed (red arrowheads).
FIGURE 4.5

a

Drp1

- PCP + PCP

- PCP + PCP

S P S P

Drp1+PC

- PCP + PCP

- PCP + PCP

S P S P

Drp1+PS

- PCP + PCP

- PCP + PCP

S P S P

Drp1+CL

- PCP + PCP

- PCP + PCP

S P S P

Drp1+PC

Undecorated Decorated

d

e

Undecorated Decorated

f

g

h

Drp1+PC

Drp1+PS

Drp1+CL

Phosphate (μM)

0 25 50 75 100 125

0 1 2 3 4 5 6

Time (min)

+ GC/CL

+ GC/PS

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC

+ GC/CL

+ GC/PC
FIGURE 4.5 **Drp1 recruitment and activation is enhanced with cardiolipin (CL) nanotubes**

(a) Sedimentation analysis are presented for Drp1 alone, incubated with phosphatidylcholine nanotubes (GC/PC), phosphatidylserine nanotubes (GC/PS) and cardiolipin nanotubes (GC/CL) in the absence and presence of GMPPCP (-PCP and +PCP, respectively, n = 3/sample. Representative supernatant (S) and pellet (P) fractions are shown. Cryo-EM images are shown of Drp1 in the presence of GMPPCP (b), PS nanotubes (undecorated, c, and decorated, d), PC nanotubes (no protein decoration observed, e), and CL nanotubes (undecorated, f, and decorated, g). Scale bar, 50 nm. (h) 2-D class averages of Drp1 + GC/PC, Drp1 + GC/PS and Drp1 + GC/CL are presented. (i) A GTP hydrolysis assay displays the amount of phosphate released over time for Drp1 alone (black) and incubated with different nanotubes (GC/PC, gray; GC/PS, blue; GC/CL, orange, n = 3/sample). Measured GTPase activities (kcat) are shown (inset).
FIGURE 4.6 **3D structure of Drp1 bound to a cardiolipin (CL) lipid template.**

(a-b) Negative stain (a) and cryo-EM (b) images of Drp1 oligomerized in the presence of GMPPCP on galactosyl ceramide (GC) nanotubes containing cardiolipin (CL) at low (left; scale bar, 100 nm) and high magnifications (right; scale bar, 50 nm). Filled arrows indicate Drp1 decorated tubes, while open arrowheads indicate undecorated GC/PS tubes.

(c-d) The 3D reconstruction of Drp1 on a GC/CL nanotube is presented. The helical pitch (13 nm) and diameter (49.6 nm) are indicated. (e) A cross-section of the 3D structure is presented and the stabilized Drp1-lipid interaction is highlighted (filled arrowhead). (f) The fitted structures of Drp1 GTPase (green) and stalk (blue) dimers are shown. The GTPase domain dimers interact through a different catalytic interface when compared to the GC/PS structure. (g) A side view of multiple Drp1 dimers fitted into the helical density is presented. (h) An end-on view of the same fitted structures. Density contacting the lipid surface (orange dotted circle) likely represents VD interactions.
FIGURE 4.7

% in pellet

WT

ΔVD

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT (S)</th>
<th>WT (P)</th>
<th>ΔVD (S)</th>
<th>ΔVD (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC/CL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC/CL+PCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 4.7 Removal of the variable domain (VD) abolishes Drp1 sedimentation with CL nanotubes.

Sedimentation was measured to assess the oligomeric state of wild-type (WT, black) and ΔVD (grey) Drp1 alone, in the presence of GMPPCP, in the presence of CL nanotubes (GC/CL) and in the presence of CL nanotubes with GMPPCP (GC/CL+PCP).

Representative supernatant (S) and pellet (P) gel samples are shown.
FIGURE 4.8

a

ΔPRD Dynamin + PS + PCP

Diameter 40 nm
Subunits/Turn 13
Radial spacing 9.5 nm/subunit

c

Drp1 + GC/CL + PCP

Diameter 49.6 nm
Subunits/Turn 9
Radial spacing 17.3 nm/subunit
FIGURE 4.8 Comparison of the lipid-bound Dynamin and Drp1 polymers.

(a) The 3D cryo-EM reconstruction of the ∆PRD dynamin helical oligomer formed in the presence of PS liposomes and GMPPCP is presented with the GTPase (green), stalk (blue) and PH (yellow) domains fitted [Chappie et al., 2011, #14454]. (b) The tight helical packing of dynamin is illustrated and the helical parameters are shown. (c) The 3D cryo-EM reconstruction of the Drp1 helical oligomer formed in the presence of CL-containing nanotubes and GMPPCP is presented with the GTPase (green) and stalk (blue) domains (PDB ID: 4BEJ) fitted. (d) The more expanded helical packing of the Drp1 helix is illustrated and the helical parameters are shown.
FIGURE 4.9 **Cardiolipin interactions trigger active Drp1 assembly.**

(a) A model illustrates the Drp1 architecture on a GC/PS template. Stalk and 80 loop (purple) G-domain dimerization drive helical assembly. (b) A separate model illustrates the Drp1 architecture on a GC/CL template. Stalk and distinct G-domain dimerizations near the GTP binding pocket (orange) promote assembly of a more active polymer in the presence of CL. (c) On a GC/PS template, the variable domain of Drp1 interacts weakly with the membrane surface. (d) On a GC/CL template, the variable domain is stabilized on the membrane surface, which induces a pivot at the stalk interface to transmit a specific lipid signal at the membrane to the peripheral GTPase domains where an activate conformation is formed. (e) A model depicts Drp1 interactions with CL at the surface of mitochondria where this unique lipid activates Drp1 function at defined sites primed for mitochondrial fission.
CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS
5.1 Summary

From our studies, we have discovered several new functional and structural characteristics of Drp1. First, we elucidated the role of nucleotide interactions in the constriction mechanism of Drp1. Specifically, we determined that GTP binding induced a conformational stabilization, while GTP hydrolysis initiated full constriction of the protein lipid polymer. We also showed that the VD acts as a regulatory domain and keeps Drp1 in a more active, soluble conformation. Similar studies determined that the VD regulates Drp1 curvature on liposomes. Specifically, removal of the VD generated large, helical Drp1 lipid polymers. Endogenously, Drp1 also contains alternatively spliced exons in the VD, and we determined the shortest isoform analogously formed larger protein lipid polymers. Our next step was to explore the structural details of these Drp1 lipid structures using cryo-EM.

Our cryo-EM structural studies yielded novel results concerning Drp1 oligomerization on lipid templates modeling the mitochondrial outer membrane. We found that Drp1 is forms a helical lattice on our lipid nanotubes with a greater spacing than any observed with other dynamin proteins previously. This packing is mediated by G-domain dimerization and a single intermolecular stalk interaction, and this represents the minimal contacts necessary to propagate formation of a functional helical oligomer. When PS lipid was mixed into the nanotubes, we found that Drp1 was recruited and activated. We also found the GTPase domain of adjacent Drp1 molecules dimerized through an 80 loop interaction; however, there was no stable interaction of Drp1 with the membrane surface. Upon the addition of CL into the nanotubes, Drp1 was recruited and activated more robustly, and adopted a distinct helical structure. Most notably, the VD
stably interacted with the CL nanotube, and it appeared that this interaction drove a conformational change to the G-domains on the periphery of the oligomer. Moreover, the G-domains in this instance developed a different dimerization motif, termed the catalytic dimer.

Although this work contributed new insights in Drp1 and mitochondrial fission research, it raises many new questions. I only observed constriction of Drp1 isoform 1, would other isoforms that prefer distinct curvatures have different constriction properties? Can we recapitulate complete mitochondrial fission, and not just constriction? Concerning structural studies, there is still much to be learned concerning Drp1. Can we determine the conformation of the VD in different Drp1 structural states? With more advanced EM technology could we improve the resolution of our lipid structures and resolve the VD and the exact G-domain orientation? Can we solve the solution structure of Drp1? Finally, can we assemble Drp1 on native mitochondrial membranes for functional and structural analysis? In the next few sections I will discuss these questions, possible experiments and their potential outcomes. Although our work has moved the field forward, it only serves as a foundation for new discoveries.

5.2 Additional Drp1 Isoform Studies

Although we discovered key components concerning the mechanism of Drp1 induced constriction of lipid bilayers, several questions remain. In our initial study (Chapter 2), we utilized Drp1 isoform 1, which contains the full 37 amino acid B insert. Using this splice variant, we noticed a constriction from 60 nm to 39 nm (Francy et al., 2015). Would a similar range of constriction be observed with other isoforms of Drp1? In subsequent studies (Chapter 3), we showed that Drp1 isoform 3, the shortest Drp1
isoform, does indeed form larger protein lipid oligomers in the absence of nucleotide compared to Drp1 isoform 6, the longest Drp1 variant (Macdonald et al., 2016). Adding GTP to these oligomers of distinct size would likely yield different magnitudes of constriction, and the rates could be altered as well. This could easily be tested using the negative stain EM time course assay with pure PS liposomes or CL mix liposomes. The results would provide insight into how different Drp1 isoforms operate in distinct tissues with different mitochondrial morphologies. Additionally, protein cofactors (or Drp1 adapters) may provide an additional scaffold to help shape and regulate Drp1 polymerization in these different tissues, as the expression of these partner proteins varies between different organs.

5.3 Recapitulating In Vitro Membrane Fission

A lingering question in the field is, how does one recapitulate complete fission of lipid membranes with Drp1? From our research, we have never shown complete fission of lipid bilayers. Therefore, it appears Drp1 alone on lipid bilayers with GTP added is sufficient to induce constriction, but not complete membrane scission. As a result, additional components may be required for a processive fission machinery. One of these components could be tension. In theory, it is much easier to break something when it is tightly tethered on both ends. In the cell this is true, as mitochondria are attached to cytoskeletal elements such as microtubules through kinesins and other molecular motor proteins. To model tension, we could use a technique called mechanical pipet aspiration. In this technique, a glass pipet with suction is applied to a giant unilamellar vesicle (GUV). The force pulls a portion of the GUV radially, producing a lipid tube with considerable tension between the GUV and the glass pipet (Morlot et al., 2012; Roux et
al., 2010). The force can be adjusted to produce lipid tubes of variable diameters. A fluorescently labeled Drp1 could then be added along with GTP, and the results could be recorded continuously. We would observe Drp1 decoration of a negatively charged GUV, but we might also observe fission when tension is applied in this manner.

The other main component that may make a difference is additional lipids such as CL. We have shown previously that local constriction events occur when Drp1 and GTP are added to CL liposomes (Stepanyants et al., 2015). Although they don’t appear to undergo complete fission after 5 minutes, the measured diameter of local constriction sites can be very narrow (~20 nm). Additional incubation time or the application of tension may be enough to drive membrane fission. Because of CL’s physical properties, this theory is possible. When CL is concentrated in highly curved membranes by Drp1, it becomes destabilized due to its conical lipid structure. This destabilization could lead to spontaneous membrane division. Additionally, the presence of other partner proteins, such as Mff, could further destabilize the membrane through their TM domain insertion. Studies also show that Mff is recruited to CL patches, placing it in the correct proximity (Macdonald et al., 2016).

Although partner proteins may destabilize the membrane through wedging effects, they also bind Drp1, which has been shown in several fluorescence and biochemical studies (Clinton et al., 2016; Loson et al., 2013; Loson et al., 2014; Macdonald et al., 2016). Some of these studies have shown the ability of Mff to stimulate Drp1 GTPase activity when the VD is removed (Bustillo-Zabalbeitia et al., 2014; Clinton et al., 2016; Karbowski and Youle, 2003). Therefore, Mff likely induces a conformational change in Drp1, and this conformational change may prime Drp1 for a full fission event. A similar
EM time course experiment could be conducted using a 6-His-tagged Mff construct and nickel liposomes mixed in with CL, as utilized in our lab previously (Clinton et al., 2016). Drp1 would be added along with GTP and grids could be prepared at time points up to ten minutes. Initial screening can be done with negative stain electron microscopy to see if any small lipid vesicles form. Cryo-EM would then be used to further validate the lipid architecture, and whether the lipids are fully decorated with protein. We would expect Drp1 to disassemble from the membrane post GTP hydrolysis and constriction. This experiment could further be tested using fluorescent CL liposomes decorated with Mff. Using this procedure, the liposomes can be viewed in real time to determine whether tubulation or complete fission is occurring. Besides Mff, other partner proteins such as MiD49/51 or Fis1 could also be tested.

5.4 Variable Domain Conformation

Recent work from our lab has shown that the VD plays a very important regulatory role in Drp1 function (Clinton et al., 2016; Francy et al., 2015). We have proposed that under cytosolic conditions, the VD interacts with the bottom of the stalk, and precludes this interface from specific intermolecular interactions (Francy et al., 2015). However, we are not sure what specific residues or interfaces are responsible for the formation of hyperoligomeric Drp1 ΔVD species. To determine this, we could use hydrogen deuterium exchange (HDX) or a comparable proteomics method. HDX can identify solvent exposed amino acids by exchanging hydrogens found in the amide backbones of proteins. Therefore, HDX could be conducted using a full length WT Drp1, and a ΔVD Drp1 construct. Residues in the base of the stalk could therefore be inspected for the absence of deuterium exchange, which would indicate VD interaction with that
portion of Drp1. This method has been successfully used for dynamin in investigating the conformation of the PH domain (Srinivasan et al., 2016b), and therefore could likely be used successfully for Drp1.

As discussed previously, there are also several PTMs in the VD of Drp1. These phosphorylations, sumoylations and GlcNACylations could also induce different conformational sampling of the VD. These mutant constructs, many of which have been purified in our lab, could be tested and compared with WT Drp1. We would expect the activating PTMs, such as phosphorylation at Ser616, glycosylation at Thr585/Thr?586 and nitrosylation of Cys630, induce conformational changes in the VD. On the other hand, the inactivating phosphorylation at Ser637 may further stabilize the VD, and provide additional solvent protection. Therefore, HDX experiments have the potential to provide significant insight into the inter and intramolecular interacts that regulate Drp1 multimerization in solution.

We have also shown the VD interacts with CL nanotubes. From our model, CL acts as a factor that alters the VD conformation and thereby exposes additional interfaces for Drp1 oligomerization. A similar HDX experiment could be conducted with Drp1 in the presence of CL liposomes, and Drp1 in the presence of PC liposomes as a negative control. If additional residues are in fact exposed, these amino acids can be identified and compared with the previous experiment. Other negatively charged lipids could also be tested, such as PS. From our model, it appears PS does not form a stable interaction with the VD; therefore, we would not expect as much solvent accessibility. Another lipid called phosphatidic acid (PA), a precursor of CL, has recently been shown to potentially sequester Drp1 to the MOM, and keep it from forming active fission sites (Adachi et al.,
Thus, the interaction with PA may induce yet another VD conformational sampling that could be tested using HDX. The promising Drp1 lipid oligomers found in this study could be further validated with high-resolution cryo-EM. High-resolution cryo-EM structural studies could reveal the diverse conformational states of the VD and the rest of the molecule when exposed to different liposomes.

### 5.5 Improving Cryo-EM Resolution

Our cryo-EM structures provided valuable insight regarding Drp1 assembly on lipid bilayers. However, the moderate resolution (~20 Å) of our structures limits insights into specific interactions that are altered when comparing distinct assemblies. Some helical proteins, including tobacco mosaic virus (TMV), have been solved at close to 3 Å resolution (Fromm et al., 2015). Then why were our structures solved at a lower resolution? Resolution can be attributed to several parameters, including the structural characteristics of the protein (i.e. stability), the number of particle images being averaged in the final map, and the technology utilized. TMV is structurally very stable, as is characteristic of many viruses. A rigid, stable protein will always allow you to reconstruct to a higher resolution. In our case, Drp1 is a relatively flexible protein. As we know, the VD is the most flexible portion of the molecule, therefore making this region difficult to resolve. Moreover, other regions of the molecule are flexible, including the basal signaling element, located between the G-domain and the stalk. This region likely pivots upon nucleotide binding or hydrolysis, similar to dynamin. There are several other flexible loops within the protein, one of these being the 80 loop. Globally, we know Drp1 is flexible because it can accommodate several different geometries ranging from 20 to 400 nm diameters. To accommodate these vast differences, Drp1 may also pivot at the
critical stalk interface, effectively allowing the protein to “stretch” or “compress” over varying distances. This structural flexibility is likely inherent to Drp1 activity, and interchangeable conformations will limit structural resolution. This is the main reason why we utilized GC nanotubes, in an attempt to limit Drp1 structural sampling. Similarly, we found that GTP binding stabilizes the complex, and the helical lattice affords a regular packing of the repeating protein units in a defined geometry. These attributes suggest that a homogeneous subset of the imaged sample is obtainable.

To achieve this homogeneous dataset, additional imaging would provide additional statistical power. For our structures, we obtained ~26,000 particles from ~400 images. And of these, a subset of ~10,000 particles was used to generate a 3D reconstruction. With advances in automated image acquisition, we can obtain hundreds of thousands of images with automated data collection software such as Leginon (Suloway et al., 2005). Leginon will automatically and continuously image grids where there is appropriate ice thickness. This eliminates user error and fatigue, which often limits sessions to 100-150 micrographs. Conversely, Leginon can take several hundreds to thousands of images in a single session. Automatic image acquisition can also be set up on serial EM, another software interface installed on our JEOL microscope. More micrographs mean more particles, which can improve resolution by providing greater statistical power for identifying a homogeneous subset.

The last parameter, EM technology, is equally important when it comes to resolution. For our structures, lipid-bound Drp1 was imaged using a 200 kV TF20 microscope with a Tietz 4k x 4k (pixels) CCD camera. Although this equipment was state-of-the-art 10 years ago, EM technology has advanced so rapidly that this setup is
now relatively outdated. A higher voltage microscope, such as a 300 kV would likely improve resolution. The higher voltage emits higher energy electrons with a shorter wavelength, which translates to higher resolution. In addition to voltage, the type of detector used can greatly enhance the resolution that can be obtained. For my work, I used a TVIPS Tietz 4k x 4k CMOS-based camera, which will convert electrons from the sample into photons, then hit the detector and form an image. During this conversion process, some of the signal from the electrons can be lost. To improve imaging, Direct Electron Detectors (DED) were developed to directly detect single electrons, thus bypassing the conversion step, which provides higher resolution information. The DED camera can also correct for beam-induced motion by taking images at a faster rate. In cryo-EM, the electron beam will slightly damage the sample upon imaging, causing drift. This can be corrected by taking several images in succession in a high frame rate movie. Therefore, one can collect a series of frames with minimal electron dose, rather than one blurry image with a higher electron dosage. Recent software advances are also able to track beam or stage induced motion and correct it (Scheres, 2014). Fortunately, we recently have installed a DED camera on our 200 kV JEOL microscope. A new EM consortium has also been established at Florida State University, Baylor University, and the University of California Los Angeles. Here, users have access to cutting edge technology, including 300 kV electron microscopes equipped with DEDs. Therefore, in the future we should collect data at these sites in order to obtain larger datasets with better images. Collectively, the resolution of our samples will be greatly improved.

Image resolution can also be improved during the helical reconstruction process. We used IHRSR to generate our helical reconstructions, but there are other helical
reconstruction software available. Moreover, Relion, or Regularized Likelihood Optimization, has been implemented in the IHRSR method to improve image alignment and averaging (Clemens et al., 2015). Relion uses a Bayesian approach to find a model with the highest probability of being correct in respect to the observed data and available prior information (Scheres, 2012). Unlike conventional refinement procedures that employ sequential parameters that need to be adjusted by an expert, the Bayesian approach iteratively learns most parameters of the statistical model from the actual data. Since Relion is generally used for single particle reconstruction, it’s implementation for single-particle helical reconstructions provides a distinct sorting protocol to improve the resolution of our cryo-EM maps.

Another new helical processing software called Single Particle Reconstruction from Images of Known Geometries (SPRING), has also been utilized by some structural biology groups (Desfosses et al., 2014). This program is a class-based helical reconstruction approach that enables simultaneous exploration and evaluation of several helical symmetry combinations at low resolution. This step would essentially allow one to prescreen reconstructions before investing large amounts of computational time in each symmetry. Next, favorable symmetry solutions can be further assessed and refined by single particle helical reconstruction by correlating simulated and experimental power spectra. Utilizing additional processors from the Case Western Reserve Computing Cluster would also speed up the helical evaluation process. SPRING has yielded helical reconstructions up to 4 Å resolution with ideal samples (Fromm et al., 2015), and can be downloaded onto our linux workstation and implemented side by side with our IHRSR protocol.
Subnanometer resolution is certainly possible with this new technology, and it would allow us to make more definitive conclusions from our structures. At our current resolution, we are only able to discern protein domains within the 3D model. With subnanometer resolution, we will be able to observe secondary structure, which would identify the orientation of the alpha helices within the stalk and any associated conformational changes upon nucleotide or lipid binding. Distinguishing the conformation of the VD would be possible, as it should be stabilized to the membrane surface with GC/CL, and likely docked onto the stalk region within the helical polymer in the GC/PS structure. Furthermore, we could more confidently determine the orientation of the G-domains, as the interfaces drastically differ between the 80 loop dimer and the catalytic dimer structure. Improved resolution below 10 Å would certainly reveal additional structural features important in Drp1 lipid binding.

5.6 Additional Drp1 Cryo-EM Structural Targets

In our cryo-EM models, we have determined the structures of Drp1 on GC/CL or GC/PS nanotubes in the presence of non-hydrolysable nucleotide (GMPPCP). However, we are not sure how much of a role nucleotide or lipid plays in inducing the observed helical structure. To distinguish between the two, we would need to collect cryo-EM data on an apo model, or Drp1 on lipid nanotubes in the absence of GMPPCP. A side-by-side comparison would reveal any nucleotide induced conformational changes from lipid induced conformational changes. Also, we have been working on solving the GMPPCP induced Drp1 spiral structure. This helical oligomer of Drp1 is solely oligomerized through nucleotide binding, and I believe its structure would be very similar to our GC/PS structure. Overall, it appears that along with stalk dimerization, G-domain
Dimerization between Drp1 molecules is one of the major interfaces necessary in propagating the helical structure. All these structures would help reveal the mechanism of nucleotide induced Drp1 conformational rearrangements.

Another project worth pursuing is the solution structure of Drp1. Although this project has been worked on in the past, it was never completed. A preliminary structure of Drp1 dimers displayed an S-shape configuration, and this was done using negative stain EM. This Drp1 conformation was drastically different from the crystal structure orientation, further underlying the importance of EM structures in determining the physiologic interactions within and between proteins. The preliminary model based on negative stain data is at approximately 30 Å resolution, but could be improved drastically by using cryo-EM. Although the Drp1 dimer is small (160 kDa), we should be able to visualize it using the JEOL 2200 microscope with an added phase plate (adds contrast). In this case, the Drp1 dimer was isolated using a sucrose differential centrifugation technique, which separated Drp1 oligomers based on molecular mass differences. The problem with this technique is that Drp1 dimers could further oligomerize upon addition to the grid. A cleaner experiment would be to utilize the Drp1 G363D mutant, which limits Drp1 assembly as the protein is exclusively dimeric (Clinton et al., 2016). This could then be imaged side by side with Drp1 ΔVD, which is also an obligate dimer. If reconstructions could be obtained for both proteins, additional density in the G363D Drp1 would represent the VD. These structures could also tell us how much the VD influences Drp1 conformation in solution. A solution structure of Drp1 would greatly advance our understanding of Drp1 function and its propensity to oligomerize in the cytosol.
Other groups may claim that GC lipid nanotubes are not representative of the native MOM environment. Although GC is not found in the mitochondria, Drp1 forms similar helical assemblies of 50-60 nm with a multitude of different liposomes that are more representative of the MOM, such as CL-mix. However, the majority of Drp1 lipid tubes are much larger in diameter (~200 nm). Despite this, it brings up an interesting question; does Drp1 form dissimilar helical structures on different lipid geometries? Collecting cryo-EM data of Drp1 on CL-mix liposomes could answer this question. There would certainly be more sample heterogeneity, since this lipid template is much more flexible. Yet, diameter sorting could be used to generate different classes, even though more data collection would be required. GC nanotubes could also be used, but by titrating in other lipids such as PE and PC with CL, the diameters of these nanotubes could be increased. I would hypothesize that the overall helical architecture would not change depending on the diameter of the tube, however it will change depending on the lipid composition. Therefore, I believe the GC/PS structure would be similar to the structure of Drp1 on pure PS liposomes. The helical pitch may change to accommodate the wider template, but the intermolecular architecture will remain largely intact.

Testing Drp1 association with intact mitochondria is one way to physiologically model this interaction. In the past, I have obtained purified liver and skeletal muscle mitochondria from mice, and added Drp1 to each preparation. Sedimentation analysis with skeletal muscle mitochondria showed Drp1 sedimenting along with the mitochondria. Negative stain electron microscopy of the mitochondria alone displayed their overall architecture with substantial detail. Grids with Drp1 and GMPPCP added did not display striking differences in mitochondrial morphology, thus it was difficult to tell
if Drp1 associated or not. To continue these experiments, we could label Drp1 with an antibody and conjugate this to a gold-labeled secondary antibody. Negative stain EM would reveal the localization of Drp1, and we would expect to see a significant fraction near the MOM.

A simpler way to look at physiologic Drp1/mitochondria interactions could be done by obtaining isolated MOM, or mitochondrial ghosts. These membranes are separated from isolated mouse or rat mitochondria using proteases and differential centrifugation. Drp1 could then be added directly to these fractions, and we could examine the GTPase, sedimentation and EM data. Contact sites (Fig 1.1 A) could also be isolated, which contain increased CL levels. These contact site membranes could then be incubated with Drp1 as well. I would hypothesize that we would see similar formation of Drp1 lipid tubules, although they may be less ordered. In other words, I would not expect decoration on isolated MOM to be as complete as with our \textit{in vitro} prepared lipids. These experiments could add further physiologic relevance to our work.

It is also unclear what state of the Drp1 fission process we are representing on the GC nanotubes. Although the decorated nanotubes are relatively narrow in diameter (~50 nm), I don’t believe they represent a constricted conformation. As we know from our Drp1 constriction time course assays, Drp1 lipid tubes can constrict down to approximately half the size of our decorated nanotubes (~25 nm in diameter). Therefore, I believe our cryo-EM structures represent the preconstricted or stabilized state (Francy et al., 2015). More specifically, this represents lipid-bound Drp1 with GTP bound, but not hydrolyzed. In order to capture the constricted state, we could utilize the C452F Drp1 python mutant. According to a recent study, this mutation in mice caused
cardiomyopathy and dysregulated Drp1 disassembly by GTP (Cahill et al., 2015). Therefore, we could test this mutant by adding it to pure PS liposomes and CL-mix liposomes, then add GTP and examine the protein lipid oligomers using EM. If there is in fact a disassembly defect, Drp1 should constrict fully but not fall off the lipid template. This could potentially trap Drp1 in a fully constricted state, offering us a valuable structural candidate. The conformational changes in a fully constricted state would offer valuable insight into the mechanism of mitochondrial fission.

Drp1 also assembles on cytoskeletal structures within the cell, such as microtubules and actin (Hatch et al., 2016; Strack et al., 2013). As a result, we tested whether Drp1 could assemble on microtubules isolated from rat brain. We found that Drp1 weakly sedimented on preassembled microtubules, and Drp1 GTPase activity was also not significantly stimulated. Negative stain EM analysis displayed what appeared to be mostly undecorated microtubules, although some regions were coated with GMPPCP induced spirals. It was unclear whether Drp1 was partially decorating the microtubules or not, therefore secondary gold labeling of Drp1 could again be useful to give a more definitive answer. We hypothesized that Drp1 cycles on the microtubules in an active primed dimer state, ready to be released into the cytosol upon certain cues. This could be PTMs, or calcium bursts which may be present at ER/mitochondrial contacts. Drp1 phosphomimics could be added to the microtubules to see if there is additional stabilization. Calcium could also be spiked in to see if it affects Drp1 localization or oligomerization in a microtubule environment.
5.7 Conclusion

How has my work contributed to the field of mitochondrial dynamics?
Fortunately, my work has led to several new findings on Drp1 structure and function. The work described within this dissertation revealed the constriction cycle of Drp1, the regulatory properties of the VD and the helical structure of Drp1 with CL. We have found that Drp1 is not as similar to dynamin as previously thought. It forms fewer intermolecular interactions in the helical lattice, and adopts a much more extended geometry. In my opinion, many other groups will also find functional and structural differences between Drp1 and dynamin. In fact, I believe other dynamin family members like Mx will also adopt dissimilar helical geometries. The data described here are the first steps to better understanding the role of Drp1 in mitochondrial fission. Moreover, I believe it is important to continue to explore the Drp1-CL interaction, as this could be enhanced in disease states. Therefore, this could be a compelling therapeutic target in the future. Due to the role of Drp1 in so many diseases, continued research of its function in endogenous and disease mitochondrial fission is paramount.
References


dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane
permeabilization. Dev Cell 14, 193-204.


17) Chan, D. C. (2012). Fusion and fission: interlinked processes critical for

phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J
Biol Chem 282, 21583-21587.

19) Chang, C. R., Manlandro, C. M., Arnoult, D., Stadler, J., Posey, A. E., Hill, R. B.,
and Blackstone, C. (2010). A lethal de novo mutation in the middle domain of the
dynamin-related GTPase Drp1 impairs higher order assembly and mitochondrial division.


domain dimerization controls dynamin’s assembly-stimulated GTPase activity. Nature
465, 435-440.


