Identification of a Novel Interaction Between Cell Division Proteins FtsK and MinD in *Escherichia coli*

by

Mitchele Demelo

A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Molecular and Cellular Biology

Guelph, Ontario, Canada

© Mitchele Demelo, April, 2019
Abstract

IDENTIFICATION OF A NOVEL INTERACTION BETWEEN CELL DIVISION PROTEINS FTSK AND MIND IN *ESCHERICHIA COLI*

Mitchele Demelo
University of Guelph, 2019

Advisor:
Dr. Cezar Khursigara

Cell division is a tightly regulated process essential for life. In bacteria, numerous protein-driven mechanisms work in concert to assure the proper distribution of cellular material into resulting daughter cells. Given the increasing threat of antibiotic resistant bacteria, these essential mechanisms have become ideal targets for the development of new antibiotics. The protein FtsK (Filamentous temperature sensitive protein K), a multi-domain inner-membrane protein, is essential for cell division in *Escherichia coli* however its function is not fully understood. To gain insight into this function, we probed interactions between FtsK and other proteins in the cytoplasm. This was done using *in vivo* UV cross-linking, which captured proteins in close association with FtsK. Cross-linked proteins were then digested into peptides and analyzed by mass spectrometry, which identified the captured proteins. The results identified MinD, a protein involved in the topological regulation of division site placement, as a potential FtsK interaction partner. This interaction was tested *in vitro* by combining purified FtsK and MinD in a pulldown assay. The result showed immobilized FtsK was sufficient to bind MinD despite stringent washing. Taken together, these findings provide strong evidence for an interaction between FtsK and MinD, implying a previously unknown link between FtsK and the division regulation machinery.
Dedication

This thesis is dedicated to my dearly departed mother,

Lori J. Demelo
Acknowledgements

There are many people to acknowledge regarding the completion of this thesis but first and foremost, I want to thank my advisor Dr. Cezar Khursigara. I never would have made it through without his constant encouragement and seemingly endless patience. Despite the numerous rough patches I encountered, his support and positivity never waivered and I am truly grateful for his mentorship. I would also like to thank my former co-advisor Dr. Reggie Lo and Dr. Janet Wood for serving on my advisory committee. Their expertise and guidance helped shape this project and kept me going in the right direction. Their generous patience in receiving this thesis has been extremely appreciated.

A special thanks goes out to Dr. Alison Berezuk for laying the entire foundation for this project. Her advice and ingenuity were essential to any progress I made with this tricky protein and I learned a tremendous amount thanks to her. I’d like to thank Dr. Elyse Roach for passing on some of her vast technical expertise and for many thoughtful discussions. Thank you to Dr. Jennifer Geddes-McAlister for her direct bioinformatics contributions and for generously sharing her proteomics expertise. I would also like to acknowledge Dr. Dyanne Brewer and Dr. Armen Charchoglyan for educating me in the ways of mass spectrometry even though their direct contributions did not make it into this thesis.

Thank you to all the members of the Khursigara lab for fostering a supportive research environment and for making the day-to-day work pleasurable. Special thanks to Nicole Garnier for being my loyal office mate and encouraging my shenanigans. I would like to acknowledge all the friends I made throughout this experience and the support they provided, be it scientific or otherwise. Finally, I would like to thank my family and friends for being there through all my successes and failures and for keeping my spirits lifted.
Author’s Declaration of Work Completed

I declare that all work presented in this thesis is my own, with the following exceptions:

The three single-cysteine FtsK_N mutants were constructed by Dr. Alison Berezuk. The image for Figure 3.2 was completed by Dr. Jennifer Geddes-McAlister. Mass Spectrometry was conducted by Dr. Jonathan Krieger at the SPARC Biocentre, The Hospital for Sick Children, Toronto, ON, while Mass Spectrometry sample preparation and data analysis was performed by myself.
# Table of Contents

Abstract .......................................................................................................................... ii  
Dedication .................................................................................................................... iii  
Acknowledgements ..................................................................................................... iv  
Author's Declaration of Work Completed .................................................................... v  
Table of Contents ........................................................................................................ vi  
List of Tables ................................................................................................................ viii  
List of Figures ............................................................................................................... ix  
List of Abbreviations .................................................................................................... x  

Chapter 1: Introduction ............................................................................................... 1  
1.1 Cell Division in *Escherichia coli* ........................................................................ 1  
1.2 Regulation of Cell Division .................................................................................. 2  
1.2.1 Nucleoid Occlusion ....................................................................................... 3  
1.2.2 The Min System ............................................................................................. 4  
1.3 Essential Proteins of the Divisome ...................................................................... 7  
1.3.1 Early Divisome Proteins ............................................................................... 8  
1.3.2 Late Divisome Proteins ............................................................................... 11  
1.4 The Role of FtsK in Cell Division ....................................................................... 13  
1.4.1 Functional Domains of FtsK ........................................................................ 14  
1.4.2 *FtsK* and the Divisome ............................................................................. 19  
1.5 Research Rationale ............................................................................................. 21  
1.6 Research Hypothesis and Outcomes ................................................................... 22  

Chapter 2: Materials and Methods ........................................................................... 24
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Bacterial Strains and Growth Conditions</td>
<td>24</td>
</tr>
<tr>
<td>2.2 Plasmid Construction</td>
<td>24</td>
</tr>
<tr>
<td>2.3 p-Azidophenacyl Bromide In Vivo Cross-linking Assay</td>
<td>25</td>
</tr>
<tr>
<td>2.4 Protein Digestion and Liquid-Chromatography Tandem Mass Spectrometry</td>
<td>26</td>
</tr>
<tr>
<td>2.5 Mass Spectrometry Data Organization and Statistical Analysis</td>
<td>28</td>
</tr>
<tr>
<td>2.6 In Vitro Pulldown Assay</td>
<td>29</td>
</tr>
<tr>
<td>2.7 Western Immunoblot Analysis</td>
<td>32</td>
</tr>
<tr>
<td>Chapter 3: Results</td>
<td>34</td>
</tr>
<tr>
<td>3.1 Single-Cysteine FtsK_N Variants Cross-link with Numerous Proteins In Vivo</td>
<td>34</td>
</tr>
<tr>
<td>3.2 His10-FtsK_N Binds FLAG-MinD in an In Vitro Pulldown Assay</td>
<td>46</td>
</tr>
<tr>
<td>Chapter 4: Discussion</td>
<td>50</td>
</tr>
<tr>
<td>Future Directions</td>
<td>58</td>
</tr>
<tr>
<td>References</td>
<td>60</td>
</tr>
<tr>
<td>Appendix A</td>
<td>74</td>
</tr>
</tbody>
</table>
List of Tables

Table 2.1 Bacterial Strains and Plasmids.................................................................................. 33

Table 3.1 *E. coli* Cell Division Proteins Deemed Potential FtsK₅ Interaction Partners by In Vivo Cross-linking and Mass Spectrometry ................................................................. 45

Table A.1 List of Putative FtsK₅ Interaction Partners Determined by In Vivo Cross-linking and Mass Spectrometry ........................................................................................................... 74
List of Figures

Figure 1.1 The Essential Proteins of the *Escherichia coli* Division Apparatus ...................... 2
Figure 1.2 Oscillation Dynamics of the Min System in *Escherichia coli.* .......................... 5
Figure 1.3 Diagram of FtsK and its Three Functional Domains ........................................ 14
Figure 1.4 Topology of the N-terminal Transmembrane Domain of FtsK .......................... 16
Figure 3.1 *In Vivo* Cross-linking using the Bifunctional Reagent *p*-Azidophenacyl Bromide. ... 35
Figure 3.2 Western Blot Analysis of Single-Cysteine FtsKN Variants R20C, I107C and Y217C Cross-linked *In Vivo* with *p*-Azidophenacyl bromide (*pAZP*) ........................................ 37
Figure 3.3 Column Correlation and Reproducibility Analysis of *In Vivo* Cross-linking Samples and Non Cross-linked Controls ................................................................. 39
Figure 3.4 STRING Network of Putative FtsKN Interaction Partners as Determined by *In Vivo* Cross-linking and Mass Spectrometry ................................................................. 42
Figure 3.5 Schematic Diagram of FLAG-MinD *In Vitro* Pulldown Assay ............................. 47
Figure 3.6 Results of FLAG-MinD and His$_{10}$-FtsKN *In Vitro* Pulldown Assays ................ 49
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Ammonium Bicarbonate</td>
</tr>
<tr>
<td>AGC</td>
<td>Automatic Gain Control</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Dnase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FT</td>
<td>Flow Through</td>
</tr>
<tr>
<td>HCD</td>
<td>Higher-energy Collisional Dissociation</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized Metal Affinity Chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1 thiogalactopyranoside</td>
</tr>
<tr>
<td>KOPS</td>
<td>FtsK-orienting Polar Sequences</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography-Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LDAO</td>
<td>Lauryldimethylamine N-oxide</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>pAZP</td>
<td>(p)-Azidophenacyl Bromide</td>
</tr>
<tr>
<td>PBP3</td>
<td>Penicillin-binding Protein 3</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered Saline</td>
</tr>
<tr>
<td>TER</td>
<td>Termination Macrodomain</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>ZAP</td>
<td>FtsZ-associated Protein</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Cell Division in *Escherichia coli*

Cell division is a tightly regulated process that is essential for life. The molecular mechanisms of cell division have long been of interest for researchers seeking to both understand and disrupt this essential process. The model organism *Escherichia coli* divides by binary fission, a process characterized by cell elongation, formation of a septum at the mid-cell and separation of the cell into two equally sized daughter cells. Early efforts to understand the mechanisms behind this process focused on uncovering the genetic basis for division. A common discovery method involved the use of mutagenic compounds to generate temperature-sensitive mutants which, when grown at the non-permissive temperature would display a distinct filamentous phenotype, suggesting an essential cell division function had been disrupted (Van de Putte *et al.*, 1964; Hirota *et al.*, 1968). As such, many of the genes found to be associated with cell division were designated Filamentous Temperature Sensitive (*fts*). Characterization of the products of these genes revealed that cell division in *E. coli* is facilitated by a complex macromolecular structure of interacting proteins, which taken together are called the Divisome (Figure 1.1) (Wang and Lutkenhaus, 1998; Buddelmeijer and Beckwith, 2002; Goehring and Beckwith, 2005). The process of divisome assembly begins with the formation of a dynamic ring structure at mid-cell, mainly composed of the protein FtsZ, which serves as a scaffold for the rest of the divisome (Bi and Lutkenhaus, 1991). Following FtsZ, a large number of proteins are recruited to the divisome, ten of these are known to be essential for division. The essential proteins localize in a hierarchical manner: FtsZ, FtsA, ZipA, FtsK, FtsQ/L/B, FtsW/I and FtsN.
Upon maturation of the divisome, the peptidoglycan layer around the site of division is remodeled into a septum that constricts until the inner- and outer-membranes are separated and the cell pinches in two.

Figure 1.1 The Essential Proteins of the *Escherichia coli* Division Apparatus.  
(A) Schematic representation of the essential divisome proteins in *E. coli*. Each representation depicts the approximate cellular location of each protein. FtsZ is displayed as a bundle of filaments to depict the mature Z-ring. Z represents FtsZ, A represents FtsA, etc. *Adapted from Huang et al. (2013).*  
(B) Diagram showing direct interactions between essential divisome proteins reported in the literature. Solid black lines depict an interaction between the connected proteins and curved arrows represent self-interaction. The protein-protein interactions shown in the diagram are supported by experimental data from bacterial two hybrid assays or *in vitro* pulldown assays. *Re-drawn from Goehring and Beckwith (2005).*

1.2 Regulation of Cell Division

In order to assure successful division in *E. coli*, the formation of the Z-ring must be carefully timed and spatially regulated. Premature division can interfere with the preceding
phases of chromosome replication and segregation, leading to uneven distribution of genetic material or even the chromosome being guillotined by the closing septum (Schumacher, 2017). It is also important to assure the cell has reached sufficient length during the growth phase given elongation and division utilize much of the same cell wall precursors and machinery (Cho et al., 2016). In addition to timing, the placement of the Z-ring at mid-cell is essential for the equal distribution of cellular material after division (Adler et al., 1967; de Boer et al., 1989). There are several mechanisms in *E. coli* that assure such aberrations are avoided and knowledge of these mechanisms is essential for a complete understanding of cell division.

1.2.1 Nucleoid Occlusion

Z-ring formation is tightly linked to the placement and orientation of the bacterial chromosome. This comes from the observation that the Z-ring is unable to form wherever the tightly packed nucleoid is present. This phenomenon is termed Nucleoid Occlusion (NO) (Woldringh et al., 1990; Woldringh et al., 1991). A number of models have been proposed to explain the mechanism of NO, however it is not fully understood. One model suggests NO may be the product of transertion, a process in which genes are transcribed into mRNA then translated directly into the inner leaflet of the plasma membrane, forming a sort of DNA-membrane tether (Woldringh et al., 1990; Norris, 1995; Zaritsky and Woldringh, 2003; Sun and Margolin, 2004). The sheer amount of nucleic acids and proteins may crowd out protofilaments of FtsZ and prevent the Z-ring from forming around the nucleoid.

Another model centers on the protein SlmA as the main driver of NO. SlmA was shown to bind specific sites on the nucleoid and to stimulate the depolymerization of FtsZ filaments *in vitro* (Bernhardt and de Boer, 2005; Cho et al., 2011). However, microscopy studies have
revealed that SlmA does not localize to the cytoplasmic membrane in vivo, and other studies have shown SlmA is not required for nucleoid positioning relative to the division machinery (Bernhardt and de Boer, 2005; Männik et al., 2012; Bailey et al., 2014). Overall, whether it is the result of molecular crowding by transertion, the action of SlmA, or a combination of redundant mechanisms, further study is required to build a complete and comprehensive model of NO in E. coli.

1.2.2 The Min System

Another essential regulatory mechanism in E. coli is the Min system. The Min system is comprised of the proteins MinC, MinD and MinE (de Boer et al., 1989). Early studies showed mutations within these loci resulted in a distinct mini-cell phenotype (for which the proteins were named), in which aberrant divisions near the cell poles produced tiny daughter cells lacking genetic material (de Boer et al., 1989). Later studies revealed that all three Min proteins are required for correct division placement, with MinC and MinD acting together as a Z-ring inhibitor and MinE acting as a topological regulator of MinCD (Figure 1.2) (Raskin and de Boer, 1999b; Hale et al., 2001; Dajkovic et al., 2008).

MinC is the main effector of the Min system, binding directly to FtsZ and inhibiting filament polymerization and bundling (de Boer et al., 1990; de Boer et al., 1992b; Hu et al., 1999). MinD belongs to the large and diverse Walker A Cytoskeletal ATPase family of proteins and is responsible for activation of MinC (de Boer et al., 1991; Koonin, 1993; Lutkenhaus and Sundaramoorthy, 2003). In the presence of ATP, MinD dimerizes and binds the membrane via a C-terminal amphipathic helix (Hu et al., 2002; Hu and Lutkenhaus, 2003; Szeto et al., 2003).
Figure 1.2 Oscillation Dynamics of the Min System in *Escherichia coli*.

In the presence of ATP, MinD binds the membrane at one of the cell poles and forms a dimer. MinC binds MinD-ATP and exhibits its FtsZ inhibitory function, creating a zone of Z-ring inhibition near that cell pole. MinE displaces MinC and activates the ATPase function of MinD, causing it to dissociate from the membrane and separate into MinD-ADP monomers. MinD-ATP binds the membrane at the opposite pole and recruits MinC, creating a new zone of Z-ring inhibition until MinE follows and drives the Min oscillation to the opposite pole again. This protein oscillation assures cell division is not initiated near the cell poles. *Adapted from Lutkenhaus, (2007)*

Fluorescence microscopy experiments have shown that MinD preferentially localizes to the cell poles rather than the cell middle (Raskin and de Boer, 1999a). This is partly due to its higher affinity for anionic phospholipids, such as cardiolipin, which cluster at the cell poles.
(Mileykovskaya and Dowhan, 2000; Koppelman et al., 2001; Mileykovskaya et al., 2003). The membrane binding and dimerization of MinD creates overlapping binding sites for both MinC and MinE (Hu et al., 2003; Lackner et al., 2003; Wu et al., 2011). The binding of MinC to membrane-bound MinD activates the Z-ring inhibitory function of MinC (by recruiting it to the cell membrane), thus preventing division from occurring at the cell poles (de Boer et al., 1992b; Szeto et al., 2003; Johnson et al., 2004). Interestingly however, early experiments showed the MinCD complex only pools in one pole at a time and that these proteins exhibit a dynamic pole-to-pole oscillation with a period of ~30-60 seconds (Raskin and de Boer, 1999b). Subsequent studies revealed this oscillation is driven by the protein MinE, which acts to spatially regulate the MinCD complex (Raskin and de Boer, 1997; Raskin and de Boer, 1999b; Hale et al., 2001; Hu et al., 2002). MinE is a small 88 amino acid protein with two functional domains (de Boer et al., 1989; King et al., 2000). The C-terminal domain is important for MinE dimerization and the N-terminal domain activates the ATPase activity of MinD (Zhao et al., 1995; Pichoff et al., 1995). MinE drives the protein oscillation characteristic of the Min system first by binding MinD at a site overlapping the binding site for MinC, thus displacing MinC from the complex (Hu et al., 2003; Wu et al., 2011). MinE then activates the ATPase function of MinD, causing the MinD dimers to separate and dissociate from the membrane and a conformational change in MinE that allows it to persist at the membrane (Hu et al., 2002; Park et al., 2011). This causes MinD to pool at the opposite cell pole and recruit MinC, after which MinE dissociates the complex and the oscillation cycle repeats.

Additionally, MinE forms a ring-like structure around mid-cell that is essential for correct division site placement (Raskin and de Boer, 1997). This MinE ring prevents nucleation of the
MinCD complex toward the cell middle, assuring the time-averaged concentration of MinCD is highest at the poles.

Although the Min proteins and their remarkable oscillations have been well studied, efforts are still being undertaken to get a more complete understanding of the Min system and its connection to other cellular processes. A recent study showed that the Min oscillation drives a counter oscillation of the early divisome proteins ZapAB, ZipA and FtsZ with a similar period as the Min oscillation (Bisicchia et al., 2013). These proteins form complexes near the pole opposite to the Min proteins until they are recruited to mid-cell during division initiation. Another study showed that in cells expressing a MinD-GFP fusion, MinD briefly pauses at mid-cell between oscillations and the frequency of pausing increases as the cell approaches division (Juarez and Margolin, 2010). The authors suggest this pausing may be important for the distribution of Min proteins into each daughter cell following division, given that a normal Min oscillation could be observed shortly after cell division, however the cause of mid-cell pausing is unknown (Juarez and Margolin, 2010). Another more recent study showed that in addition to MinC, MinD binds directly to FtsZ (Taviti and Beuria, 2017). This offers a more detailed model of MinC activation by MinD in which membrane-bound MinD binds FtsZ, bringing it in direct contact with MinC. This also offers a potential explanation for the mid-cell pausing of MinD reported by Juarez and Margolin (2010), though further work is needed.

1.3 Essential Proteins of the Divisome

Numerous proteins are involved in the cell division process, however 10 are known to be essential. The localization of these proteins to the division site is not immediate however, with
FtsZ, FtsA and ZipA considered early division proteins, FtsQ/L/B, FtsW/I and FtsN considered late and FtsK considered to be somewhere in the middle (Goehring and Beckwith, 2005).

1.3.1 Early Divisome Proteins

FtsZ is a filamentous GTPase located in the cytoplasm and is the first of the essential division proteins to localize to the division site (Begg and Donachie, 1985; Taschner et al., 1988; Mukherjee et al., 1993). Amino acid sequence and structure analyses revealed FtsZ to be a homolog of eukaryotic tubulin, and to contain two functional domains essential for cell division (Mukherjee et al., 1993; Löwe and Amos, 1998; Ma and Margolin, 1999). The first is a C-terminal domain involved in recruitment of some downstream division proteins and the second is an N-terminal GTP-binding domain (Mukherjee et al., 1993; Ma and Margolin 1999). In the presence of GTP FtsZ polymerizes into short protofilaments, which in turn activates its GTPase activity leading to protofilament disassembly (de Boer et al., 1992a; Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1998). FtsZ functions in cell division by forming the Z-ring, a cytoskeletal scaffold composed of bundles of FtsZ protofilaments localized at mid-cell (Bi and Lutkenhaus, 1991). Although much is known about the structure of FtsZ, the spatial organization and dynamics of the Z-ring are debated.

Immuno-electron and fluorescence microscopy studies showed the Z-ring as one continuous FtsZ-containing ring spanning the width of the cell (Bi and Lutkenhaus, 1991; Ma et al., 1996). In vitro and cryo-electron tomography studies also supported this model, showing evidence that the Z-ring may be composed of continuous FtsZ polymers (Thanedar and Margolin, 2004; Szwedziak et al., 2014). The use of super-resolution microscopy has led some researchers to propose a conflicting model in which the Z-ring is actually composed of a
patchwork of randomly bundled, discontinuous FtsZ filaments (Fu et al., 2010; Strauss et al., 2012; Rowlett and Margolin, 2014). This patchwork model aligns better with what is known regarding the highly dynamic nature of FtsZ filaments, however research is ongoing to provide definitive validation of either model.

Another debated aspect of the Z-ring is the mechanism of force generation required for septum constriction. Multiple studies have shown that FtsZ protofilaments can take on a slight curvature following GTP hydrolysis, however this has not been proven to be sufficient for constriction (Erickson et al., 1996; Lu et al., 2000; Osawa et al., 2008). A recent study showed FtsZ filaments making up the Z-ring display a directional “treadmilling” movement around the cell circumference, driven by GTP hydrolysis (Yang et al., 2017). Coupled with the septal peptidoglycan synthesis machinery, it is believed this treadmilling drives the formation of the septum in increasingly smaller rings until complete septum closure is achieved (Yang et al., 2017). Therefore, the constriction force may be generated by a combination of FtsZ filament curvature and concentric septal peptidoglycan synthesis. Interestingly however, Söderström and colleagues showed that FtsZ begins to dissociate from the divisome before the final stages of division are complete, suggesting a limited role for FtsZ in constriction force generation (Söderström et al., 2014).

The other essential, early stage divisome proteins FtsA and ZipA, are recruited to the division site by FtsZ and function to tether the Z-ring to the cytoplasmic membrane (Hale and de Boer, 1997; Pichoff and Lutkenhaus, 2005). ZipA is an inner-membrane protein required for the recruitment of divisome proteins FtsK, FtsQ, FtsL and FtsN (RayChaudhuri, 1999; Hale and de Boer, 2002). However it is not well conserved among bacterial species (Liu et al., 1999). FtsA
differs from ZipA mainly in that its membrane interaction is facilitated by an amphipathic α-helix and as a homolog of eukaryotic actin, it is highly conserved (van den Ent and Löwe, 2000; Pichoff and Lutkenhaus, 2005). Interestingly, a gain-of-function mutant designated \textit{ftsA*} was shown to bypass the need for ZipA, suggesting FtsA may be the primary membrane tether for the Z-ring (Geissler \textit{et al.}, 2003; Geissler \textit{et al.}, 2007). Additionally, recent studies have shown polymerized FtsA enhances Z-ring stability while monomeric FtsA is important for the initiation of septum constriction (Shiomi and Margolin, 2007; Loose and Mitchison, 2014; Pichoff \textit{et al.}, 2015).

In addition to the essential early divisome proteins, the early Z-ring is further stabilized by the recruitment of FtsZ-associated proteins (Zap), which help facilitate lateral interactions between FtsZ protofilaments (Aarsman \textit{et al.}, 2005). \textit{E. coli} has five Zap proteins (ZapA-E) and while none are singularly essential for cell division, it is believed that the combined action of these Zaps greatly contributes to Z-ring stability (Goehring and Beckwith 2005). ZapA forms a tetramer and binds FtsZ via an N-terminal charged α-helix (Galli and Gerdes, 2012; Roach \textit{et al.}, 2014). It was shown to compete with the Z-ring inhibitor MinC for FtsZ binding and also to bind the N-terminal region of ZapB (Galli and Gerdes, 2012; Huang \textit{et al.}, 2013). ZapB forms a homodimeric anti-parallel coiled-coil (Ebersbach \textit{et al.}, 2008). In addition to strengthening lateral interactions between FtsZ filaments, ZapA and B also participate in a Z-ring spatial regulation mechanism called the “Ter linkage”. The Ter linkage refers to the protein bridge that connects the termination macrodomain (Ter) of the chromosome to the developing Z-ring. During the segregation of replicated chromosomes, the Ter macrodomain localizes to mid-cell (Bailey \textit{et al.}, 2014). The DNA-binding protein MatP then binds both the Ter macrodomain and
the C-terminal domain of ZapB, creating a DNA-MatP-ZapB-ZapA-FtsZ linkage (Mercier et al., 2008). This linkage is believed to help localize the Z-ring to mid-cell and strengthen Z-ring stability (Galli and Gerdes, 2010; Espéli et al., 2012).

ZapC localizes to mid-cell independent of ZapA/B and binds FtsZ at its large globular core, rather than the C-terminal tail (Schumacher et al., 2016). ZapC binding reduces FtsZ GTPase activity, thus inhibiting filament de-polymerization and promoting bundling (Durand-Heredia et al., 2011; Hale et al., 2011). Similarly, ZapD binding also reduces GTPase activity of FtsZ and in vitro ZapD was shown to stimulate FtsZ filament bundling (Durand-Heredia et al., 2012; Roach et al., 2016). ZapE is an ATPase that binds FtsZ and is required for growth of E. coli in low-oxygen conditions (Marteyn et al., 2014). Unlike the other Zap proteins, ZapE localizes to the Z-ring at a late stage of division coinciding with cell constriction. It was also shown to interact with the division proteins FtsQ,L,I and N, suggesting it may play a role in late division (Marteyn et al., 2014).

1.3.2 Late Divisome Proteins

After the Z-ring has formed at the division site and is stabilized by the recruitment of the other early divisome proteins, the cell must begin remodeling of the peptidoglycan layer to generate the division septum. The bitopic protein complexes of the late divisome are believed to play essential roles in this process, beginning with the localization of FtsQ, L and B (Buddelmeijer and Beckwith, 2004). These three proteins are the first of the late division proteins recruited to the Z-ring, however they have been shown to form a stable complex prior to localization (van den Ent and Löwe, 2000; Buddelmeijer and Beckwith, 2004). FtsQ, L and B are all integral to the cytoplasmic membrane and the FtsQ/L/B complex is believed to interact
with other proteins of the divisome (Buddelmeijer and Beckwith, 2002; Di Lallo et al., 2003). The function of the FtsQ/L/B complex is not yet known but it is believed to be involved in the metabolism of peptidoglycan and connecting the later division proteins involved in peptidoglycan remodeling, to the divisome (Lutkenhaus et al., 2012). Recently, Condon and colleagues reported the structures of FtsL/B, revealing a transmembrane tetramer (FtsL_2FtsB_2). The study showed the FtsB transmembrane helix gives way to an unstructured periplasmic linker, whereas FtsL forms a continuous helix that is essential for function (Condon et al., 2018). It was also shown the periplasmic regions of the FtsL/B complex form a binding site for FtsQ (Condon et al. 2018).

The next complex to be recruited to the division site is FtsW/I (Mercer and Weiss, 2002; Fraipont et al., 2011). FtsW is a large inner membrane glycosyltransferase with 10 predicted transmembrane segments and is a member of the SEDS protein family (Khattar et al., 1994; Cho et al., 2016). FtsW shares homology with RodA and SpoVE from Bacillus subtilis, which are involved in cell elongation and sporulation respectively (Ikeda et al., 1989). FtsW is also required for the localization of FtsI, also known as penicillin-binding protein 3 (PBP3), to the divisome (Mercer and Weiss, 2002). FtsI is a bitopic inner-membrane transpeptidase that, in tandem with its SEDS partner FtsW, synthesizes the septal peptidoglycan essential for division (Begg et al., 1992; Eberhardt et al., 2003). Additionally, FtsW has been proposed as the Lipid II flippase that translocates peptidoglycan precursors from the cytoplasm into the periplasm, however this is debated (Mohammadi et al., 2011; Mohammadi et al., 2014; Young, 2014; Meeske et al., 2015).
The last essential protein to localize to the divisome is FtsN (Addinall et al., 1997). FtsN is a bitopic protein with a C-terminal domain capable of recognizing septal peptidoglycan (Yang et al., 2004). The function of FtsN is unknown, however its binding to the divisome is believed to be the final trigger of septation (Yang et al. 2004). Recently, FtsN was proposed to induce conformational changes in FtsA and the FtsQ/L/B complex, aiding in the completion of septum formation (Liu et al., 2015).

1.4 The Role of FtsK in Cell Division

The late proteins of the divisome appear to differ in function compared to the proteins of the early divisome, with the early being involved in Z-ring formation and stabilization and the late with peptidoglycan remodeling and septum formation. However, another essential divisome protein, FtsK, is believed to link the two stages of division (Begg et al., 1995). The ftsK gene was first discovered by Begg et al. (1995) using a temperature-sensitive mutant screen. The screen had generated a mutation in the ftsK gene that led to glycine 80 being substituted by alanine in the resulting protein. This mutation, termed ftsK44, produced an FtsK protein that was unstable at 42°C as evidenced by a dramatic filamentous cell phenotype. Such a phenotype is indicative of a cell division block and so FtsK was proposed as an essential cell division protein (Begg et al. 1995). Further studies confirmed FtsK to be a bifunctional protein composed of a large C-terminal DNA translocase domain and an N-terminal transmembrane anchor joined by a long unstructured linker (Yu et al., 1998a; Liu et al., 1998; Wang and Lutkenhaus, 1998) (Figure 1.3). These findings placed FtsK within the SpoIIE protein family – a class of membrane-bound ATPases present in a wide array of species. Proteins in this family share a
conserved DNA translocation domain and carry out functions ranging from conjugation to chromosome segregation to viral DNA packaging (Iyer et al., 2004).

Figure 1.3 Diagram of FtsK and its Three Functional Domains

The *E. coli* cell division protein FtsK is an inner membrane-bound ATPase composed of three domains. The N-terminal ~200 amino acids make up the domain that anchors FtsK to the inner membrane (FtsK\textsubscript{N}) and functions in cell division. The subsequent ~600 amino acids compose an unstructured linker (FtsK\textsubscript{L}) that connects to a ~500 amino acid domain that functions in chromosome segregation as a hexameric DNA translocase (FtsK\textsubscript{C}). *Adapted from Berezuk, (2018).*

1.4.1 Functional Domains of FtsK

The first ~220 N-terminal amino acids of FtsK make up the inner-membrane anchoring domain, designated FtsK\textsubscript{N} (Begg et al. 1995). This domain was predicted to contain four transmembrane helices, similar to SpoIIIE in *Bacillus subtilis* (Begg et al., 1995; Barre, 2007). Interestingly, a number of truncation experiments revealed that FtsK\textsubscript{N} is the only region of FtsK essential for cell division, however its precise role is not well understood (Yu et al., 1998a;
Experiments by Yu et al. showed that the N-terminal 15% (~200 amino acids) of FtsK was sufficient to localize GFP to the division septum, indicating the importance of this domain for mid-cell recruitment. (Yu et al. 1998). Additionally, two-hybrid analyses showed interactions between FtsK_N and both early (FtsZ) and late (FtsQ/L/W) divisome proteins (Di Lallo et al., 2003). Recent work in our lab has led to great strides in our understanding of this essential FtsK domain. First, Berezuk et al. used site-directed fluorescence labeling to show that FtsK_N is indeed composed of four transmembrane segments, with the second periplasmic loop being significantly larger than what was proposed in previous models (Figure 1.4) (Dorazi and Dewar, 2000; Berezuk et al., 2014). This study also showed there are at least four residues within said periplasmic loop that are essential for division and that mutations at these sites cause the outer membrane to become uncoupled from the greater division process. These findings seem to support the previously mentioned protein-protein interaction results reported by Di Lallo et al., suggesting FtsK interacts with both the Z-ring and the peptidoglycan re-modeling machinery and that it may serve as a checkpoint between early and late division (Di Lallo et al., 2003; Berezuk et al., 2014).

Additionally, Berezuk et al. used in vivo cross-linking combined with mass spectrometry to further explore potential FtsK_N interactions within the periplasm (Berezuk et al., 2018). This technique, followed by an in vitro pulldown assay, confirmed a novel interaction between FtsK_N and rare lipoprotein A (RlpA). Interestingly, this interaction causes an inhibition of septation, suggesting FtsK and RlpA may be implicated in a checkpoint mechanism that delays septation until complete divisome assembly has been achieved (Berezuk et al., 2018).
Figure 1.4 Topology of the N-terminal Transmembrane Domain of FtsK

Site-directed fluorescence labeling by Berezuk et al. revealed the N-terminal domain of FtsK (FtsK\textsubscript{N}) is composed of four transmembrane segments with a much larger periplasmic loop (residues 133-162) than previously reported (Dorazi and Dewar 2000; Berezuk et al., 2014). The diagram depicts a version of FtsK used to produce single-cysteine variants of the protein for \textit{in vivo} cross-linking. All native cysteine residues were replaced with alanine and the sites of single-cysteine substitutions are shown in yellow. ‘Other’ in the figure legend refers to amino acids lacking acidic, basic, polar or hydrophobic properties \textit{i.e.} glycine or proline. \textit{Adapted from Berezuk et al.,} (2014).

The next domain just downstream of FtsK\textsubscript{N} is a cytoplasmic linker approximately 600 amino acids in length, designated FtsK\textsubscript{L} (Begg et al. 1995). This proline- and glutamine-rich linker is unstructured and the length is highly divergent between species, with \textit{E. coli} FtsK having one of the longest known linker domains in the SpoIIIE family (Bigot et al., 2004). The
function of FtsK	extsubscript{L} in cell division is not fully understood, however Dubarry et al. showed there may be two distinct regions within FtsK	extsubscript{L} that are important for cell division, independent of the essential FtsK	extsubscript{N} domain (Dubarry et al., 2010). They did this by generating chimeric proteins containing different segments of FtsK	extsubscript{L}, fused with the transmembrane domain of FtsW. Two of these chimeras (FtsW::FtsK\textsubscript{179-331} and FtsW::FtsK\textsubscript{332-641}) were able to restore growth when expressed in ΔFtsK cells, suggesting these segments could independently restore cell division if anchored to the inner membrane. However, despite restoring growth, these chimeras were unable to restore normal cell morphology, instead causing aberrant filamentation. Both growth and morphology could only be rescued when FtsK	extsubscript{N} was present, thus further study is required to understand the importance of these FtsK	extsubscript{L} segments independent of FtsK	extsubscript{N} (Dubarry et al., 2010).

The C-terminal domain of FtsK (FtsK	extsubscript{C}) is a large cytoplasmic domain encompassing the final ~500 amino acids of the protein. Amino acid sequence analysis revealed FtsK	extsubscript{C} shared close similarity with the SpoIIIE family of DNA translocases, especially SpoIIIE from B. subtilis (Begg et al. 1995). Like SpoIIIE, FtsK	extsubscript{C} is composed of a DNA-orienting γ-domain and an αβ motor domain containing a RecA-type ATPase fold, which facilitates the ATP-dependent translocation of double stranded DNA (Barre, 2007). SpoIIIE plays an essential role in chromosome segregation during sporulation in B. subtilis. During sporulation, a septum forms near one pole of the cell dividing it into a small pre-spore and larger mother cell. SpoIIIE then pumps the spore-bound chromosome through the closed septum into the pre-spore compartment (Wu et al., 1995; Burton et al., 2007). Given the similarity, FtsK	extsubscript{C} was initially proposed to function the same way as SpoIIIE, however a study by Dubarry and Barre revealed it instead translocates the E. coli chromosome before the septum is fully closed (Dubarry and Barre, 2010).
FtsK\(_C\) also plays an important role in the resolution of chromosome dimers, which occur when two daughter chromosomes recombine end-to-end to form one doubly long chromosome (Steiner et al., 1999; Thanbichler, 2010). If the two chromosomes are not resolved, the molecule will be cut by the closing septum, eventually leading to activation of the SOS response followed by cell death (Capiaux et al., 2002). FtsK\(_C\) serves two functions in chromosome dimer resolution: the translocation of the chromosomal dif sites to the septum and the interaction with tyrosine recombinases XerC and XerD (Steiner et al., 1999; Aussel et al., 2002). DNA translocation begins with the formation of an FtsK\(_C\) homohexamer around the DNA strand (Massey et al., 2006). The FtsK\(_C\) \(\gamma\)-domain recognizes one of many short, conserved chromosomal sequences known as KOPS (FtsK-Orienting Polar Sequences), which serve to orient FtsK\(_C\) so that translocation occurs toward the dif site (Bigot et al., 2005). Translocation then occurs in an ATP-dependent manner at speeds upwards of 6.7 Kbp/s, the mechanism of which is not fully understood (Bigot et al., 2005; Massey et al., 2006). When both of the dif sites (one per sister chromosome) are aligned at the septum, FtsK\(_C\) binds XerD, which catalyzes a strand exchange followed by a second strand exchange by XerC, which is not FtsK\(_C\)-dependent (Aussel et al. 2002). In addition to its essential role in chromosome dimer resolution, FtsK\(_C\) may also be involved in chromosome decatenation controlled by the TopoIV topoisomerase (Thanbichler, 2010). FtsK\(_C\) was shown to interact with and stimulate TopoIV activity \textit{in vitro}, however decatenation can still occur in the absence of FtsK\(_C\) (Yu et al., 1998b; Espeli et al., 2003). Further study is required to fully understand the role of FtsK\(_C\) in chromosome decatenation.
1.4.2 FtsK and the Divisome

Studies of the divisome have largely centered on truncation/deletion mutant experiments or localization experiments to determine the hierarchy of divisome formation (Addinall et al., 1997; Wang and Lutkenhaus, 1998; Buddelmeijer and Beckwith, 2002; Pichoff and Lutkenhaus, 2007). Such experiments have shown localization of FtsK to the Z-ring requires early divisome proteins ZipA and FtsA, and that FtsK is required for the localization of FtsQ, FtsL and FtsI of the late divisome (Aarsman et al., 2005; Pichoff and Lutkenhaus, 2005). The fact that FtsK is purported to directly interact with many of these proteins (See subsection Functional Domains of FtsK) appears to highlight the importance of protein-protein interactions for the formation and maturation of the divisome. Unfortunately, given the inherent complexity of the divisome and the abundance of transmembrane proteins, detailed structural and functional information on these interactions remains elusive. Most of the information available to date was generated from two-hybrid analysis experiments, which are helpful for identifying potential interaction partners. However, they are unable to yield specific information about those interactions (Di Lallo et al., 2003; Karimova et al., 2005). In addition, two-hybrid experiments require the generation of protein-reporter chimaeras that alter the native form of the protein of interest, potentially leading to interactions that may not be physiologically relevant and yield a high false-positive rate (Di Lallo et al. 2003; Karimova et al. 2005). As such, many of the interactions proposed in these studies have yet to be validated by different methods.

The role of FtsK as a DNA translocase during the chromosome segregation process has been very well characterized. Given this role and the timing of its localization to the divisome (between early and late stage proteins), FtsK may serve a regulatory function, coupling
chromosome segregation, Z-ring formation (early) and septum initiation (late). However given the finding that only the N-terminal transmembrane domain (FtsK$_N$) is essential for cell division, and that many of the putative FtsK-divisome interactions described previously require FtsK$_N$, it is possible that DNA translocation is not the primary function of FtsK (Yu et al., 1998a; Draper et al., 1998; Dorazi and Dewar, 2000).

Studies investigating the suppression of the $ftsK44$ temperature-sensitive phenotype or the compensation of $ftsK$ deletion have revealed a further complex picture. First Begg et al. (1995) showed that deletion of the $dacA$ gene, encoding the peptidoglycan DD-carboxypeptidase PBP5, is able to rescue the 42°C division block exhibited by E. coli carrying the $ftsK44$ mutation (Begg et al. 1995). Geissler and Margolin then showed that expression of a gain-of-function $ftsA$ mutant or overexpression of $ftsQ$ suppressed the lethality of $ftsK$ deletion (Geissler and Margolin, 2005). Additionally, Goehring et al. proved overexpression of $ftsN$ could compensate for deleted $ftsK$, and recently, work in our lab uncovered a previously unknown $ftsA$ variant that bypassed the essential need for FtsK (Goehring et al., 2007; Berezuk, 2018). Given the varying functions of the proteins encoded by these genes, it is difficult to draw conclusions from these findings regarding the role of FtsK in cell division. However protein interactions appear to be important. Further biochemical analyses are required to verify these proposed interactions and the identification of specific interaction sites will be valuable for determining the essential role of FtsK in bacterial cell division.
1.5 Research Rationale

Cell division is an essential stage in the bacterial life cycle. Extensive research has led to the development of several robust cell division models. However, the field is lacking high-resolution evidence to support any of these at the molecular level. Investigating these mechanisms is particularly important today, given the mounting problem of antibiotic resistance in pathogenic bacteria. To date, almost all clinically approved antibiotics target the processes of protein synthesis, nucleic acid synthesis, cell-wall synthesis or folate synthesis (Lock and Harry, 2008). It is therefore believed that the development of antibiotics targeting different cellular processes is essential for combating the problem of antibiotic resistance (Stokes et al., 2005; Mantravadi et al., 2019). Although cell division is an essential process in all bacteria, it has largely been ignored as a target in the development of such novel antibiotics. This has partly been due to the lack of detailed, mechanistic information available for clinically relevant species (Vollmer, 2006). The study of cell division, therefore, has important implications in the fight against antibiotic resistance and the understanding of bacteria as a whole.

FtsK is a potentially promising target for cell division disruption. FtsK is present in almost all clinically important bacterial species and has no known homolog in eukaryotes (Begg et al., 1995; Lock and Harry 2008). In Escherichia coli, FtsK is essential for division, however the precise role it plays is unknown. The C-terminal domain of FtsK has been well characterized as a DNA translocase involved in chromosome segregation and dimer resolution (Yu et al., 1998; Di Lallo et al., 2003). However, truncation experiments have shown that of the three distinct FtsK domains, only the N-terminal transmembrane domain (FtsK$_N$) is essential for cell division (Draper et al., 1998). Recent work in our lab has led to number of breakthroughs
regarding the importance of FtsK\textsubscript{N} (described previously). As such, the results and techniques applied in said works helped to inform the design of the research presented here. Of particular importance was the study by Berezuk \textit{et al.}, which uncovered FtsK\textsubscript{N} protein-protein interactions within the periplasm (Berezuk \textit{et al.}, 2018). These findings led me to adopt similar strategies in studying the cytoplasmic interface of FtsK\textsubscript{N}. Overall, The goal of this research was to investigate the essential FtsK\textsubscript{N} domain and attempt to elucidate its purpose in cell division.

1.6 Research Hypothesis and Outcomes

Given the current findings relating to FtsK in \textit{E. coli}, I hypothesize that the N-terminal transmembrane domain of FtsK is involved in essential protein-protein interactions within the cytoplasm that are required for coupling the early and late stages of division.

The first objective in testing this hypothesis was to identify potential FtsK\textsubscript{N} interaction partners using an exploratory \textit{in vivo} cross-linking method. \textit{E. coli} strains were engineered to express plasmid-encoded variants of FtsK consisting of only the N-terminal 220 amino acids. Additionally, each truncation contained only a single cysteine in the polypeptide. These strains were combined \textit{in vivo} with a thiol-specific, UV-inducible cross-linker to facilitate the capture of FtsK\textsubscript{N} interaction partners. Mass spectrometry was then used to identify the captured proteins and specific exclusion parameters were applied to separate putative interactors from false positives. The second objective was to validate potential interactions \textit{in vitro} using a pulldown assay. Purified FtsK\textsubscript{N} was immobilized on chromatography resin and combined with a purified putative interaction partner. The interaction was verified \textit{in vitro} if both proteins co-eluted after stringent washing steps. Unexpectedly, the \textit{in vivo} cross-linking assay revealed the protein
MinD, an essential component in the spatial regulation of division, to be a potential FtsK$_N$ interaction partner. Further analysis by *in vitro* pulldown assay confirmed this interaction, suggesting a previously unknown link between the divisome and the Min system. These results provide new insight into the essential N-terminal domain of FtsK and the protein interactions that may be involved in its function.
Chapter 2: Materials and Methods

2.1 Bacterial Strains and Growth Conditions

The bacterial strains used in this study are shown in Table 2.1. *E. coli* K12 W3110, DH5α and Lemo21 were cultured using Lysogeny broth - Miller (LB) (BD Biosciences) grown at 37°C in a rotary shaker at 200 rpm. *E. coli* Lemo21 medium was supplemented with 30 µg/mL chloramphenicol. The temperature sensitive *E. coli* strain LP11-1 was cultured using Complementation medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl) grown at 30°C (unless otherwise stated) in a rotary shaker at 200 rpm. The medium was supplemented with 15 µg/mL tetracycline. *E. coli* LP11-1 was kindly provided by Dr. K. Young (University of Arkansas).

2.2 Plasmid Construction

The plasmids used in this study are shown in Table 2.1. Plasmid pMD001 was constructed first by amplifying the *minD* gene from *E. coli* K12 W3110 genomic DNA by polymerase chain reaction (PCR), using iProof high fidelity DNA polymerase (Bio-Rad) and primers FLAG-*minD* F (5’ – TTAGCCATGGGAGATTATAAAGATGATGATAGTATAAAGCAGCTATGGCACGCA TTATTGTTG – 3’) and FLAG-*MinD* R (5’ – AAGTAAGCTTTTATCCTCGAACGAAGCG – 3’). These primers were designed to introduce an N-terminal FLAG®-tag and restriction sites NcoI and HindIII to the resulting DNA. The PCR products were purified using a Purelink™ quick PCR purification kit (Invitrogen). pET28a plasmid DNA and the PCR product were digested using FastDigest™ NcoI and HindIII restriction endonucleases (Thermo Scientific) and electrophorized in a 1% agarose gel. The digested products were excised from the gel using a
razor blade and purified using a GeneJET™ gel extraction kit (Thermo Scientific). The extraction products were ligated using T4 DNA Ligase (New England Biolabs) and transformed into *E. coli* DH5α by chemical heat shock. The gene construct was purified using a Purelink™ quick plasmid purification kit (Invitrogen) and verified by DNA sequencing (Genomics Facility, Advanced Analysis Centre, University of Guelph) to produce pMD001.

### 2.3 p-Azidophenacyl Bromide In Vivo Cross-linking Assay

Cross-linking with *p*-Azidophenacyl bromide (pAZP) (Sigma-Aldrich) was carried out as described by Lunn and Pigiet with some modifications (Lunn and Pigiet, 1986). *E. coli* LP11-1 strains carrying plasmids pAB006-21, pAB006-51 or pAB006-112 (encoding FtsK_N R20C, I107C and Y217C respectively) were cultured in Complementation media overnight. The OD_{600nm} was measured and the overnight culture was used to inoculate fresh Complementation media supplemented with 0.2% (w/v) L-Arabinose to an OD_{600nm} of 0.1. The cultures were incubated at 42°C for 2 hours then centrifuged at 4,416 x g for 10 minutes at 4°C. The cell pellets were suspended in Labeling Wash Buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.0) supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) and divided into two 5 mL aliquots per strain. The cross-linking reagent, *p*-Azidophenacyl bromide, was dissolved in methanol and added to the first aliquot to yield a final concentration of 10 mM. Methanol was added to the second aliquot to serve as a negative (not cross-linked) control. The aliquots were incubated for 20 minutes on a rocking platform at 4°C, then each was divided equally into two wells of a 6-well untreated culture dish (Thermo Scientific). Plates were placed without the lid in a G:BOX gel doc (Syngene) and irradiated at 365 nm for 5 minutes. Cells were then collected
by centrifugation at 4,696 x g for 10 minutes at 4°C. The supernatants were discarded and the cell pellets were frozen at -20°C for 1 hour. Frozen pellets were thawed at room temperature, resuspended in 3 mL Labeling Wash Buffer supplemented with 5 mM EDTA, 0.5% [w/v] SDS and 0.03% [v/v] Protease Inhibitor Cocktail Set III (Calbiochem) and sonicated using a tip Sonicator XL-2020 (Misonix) for 1 minute (10 seconds on, 10 seconds off). The resulting lysates were diluted in 27 mL Purification Wash Buffer (20 mM Tris-HCl, 300 mM NaCl, 25 mM Imidazole, pH 7.4). One hundred microliters of Profinity IMAC Ni-charged Resin (1:1 slurry resin:water) (BioRad) was added and the mixtures were incubated at 4°C on a rocking platform for 1.5 hours. The IMAC resin was then collected by centrifugation at 4,696 x g for 5 minutes at 4°C and washed 5x in 1 mL Purification Wash Buffer supplemented with 0.05% (w/v) SDS using a tabletop centrifuge at 13,523 x g for 1 minute. Protein was eluted by incubating the resin in 50 μL Elution Buffer (20 mM Tris-HCl, 300 mM NaCl, 1 M Imidazole, pH 7.4) supplemented with 0.05% (w/v) SDS for 15 minutes with occasional mixing. The eluate was collected by centrifugation at 13,523 x g for 2 minutes and used for Western Blot analysis. The same experiment was conducted in preparation for mass spectrometry, however in that case the elution step was skipped and the washed resin was immediately subjected to protein digestion. The entire experiment including subsequent mass spectrometry analysis was replicated once under the same conditions for each of the three FtsK_N variants.

2.4 Protein Digestion and Liquid-Chromatography Tandem Mass Spectrometry

The single-cysteine variants of FtsK_N used in the pAZP cross-linking assay were subjected to on-bead chymotrypsin digestion as described in Park et al., (2014). Briefly, protein bound
IMAC resins were washed 5 times in 50 mM ammonium bicarbonate (ABC) buffer (pH 8.0) and suspended in Denaturation buffer (6 M urea/2 M thiourea in 10 mM HEPES, pH 8.0). The resin-bound proteins were reduced by adding 10 mM dithiothreitol in ABC buffer and incubating for 30 minutes at 50°C. The proteins were alkylated by adding 55 mM iodoacetamide and incubating for 30 minutes in the dark at room temperature. The resins were then diluted in ABC buffer containing 0.01% (w/v) ProteaseMax (Promega), a surfactant used to improve membrane protein solubility and protease efficiency. The bound proteins were digested using 1 µg chymotrypsin (Princeton Separations) at 30°C overnight. Following digestion, the IMAC resins were separated by centrifugation and the peptide-containing supernatants were lyophilized in a vacuum concentrator in preparation for sample shipment. The following describes work done by Dr. Jonathan Krieger at the SPARC Biocentre, The Hospital for Sick Children, Toronto, ON: Samples were reconstituted in 1% trifluoroacetic acid in water and passed through a C18 stage tip to remove any trace detergent and salt from the purification process. Purified samples were lyophilized and resuspended in 0.1% formic acid prior to analysis by liquid chromatography tandem-mass spectrometry (LC-MS/MS) to identify cross-linked proteins.

Samples were analyzed on an Orbitrap analyzer (Q-Exactive, ThermoFisher) outfitted with a nanospray source and EASY-nLC nano-LC system (ThermoFisher). Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75 µm × 50 cm PepMax RS LC EASY-Spray column filled with 2 µm C18 beads (ThermoFisher) at a pressure of 800 Bar heated to 60°C. Peptides were eluted over 90 min at a rate of 250 nL/min using a 0 to 40% Solution A to Solution B gradient (Solution A: 0.1% formic acid; Solution B: 80% acetonitrile, 0.1% formic acid).
Peptides were introduced by nano-electrospray into the Q-Exactive mass spectrometer (ThermoFisher). The instrument method consisted of one MS full scan (400-1500 m/z) in the Orbitrap mass analyzer with an automatic gain control (AGC) target of 1×10⁶, maximum ion injection time of 120 ms and a resolution of 70,000, followed by 10 data-dependent MS/MS scans with a resolution of 17500, an AGC target of 1×10⁶, maximum ion time of 120 ms, and one microscan. The intensity threshold to trigger an MS/MS scan was set to 6.7×10⁴. Fragmentation occurred in the higher-energy collisional dissociation (HCD) trap with normalized collision energy set to 27. The dynamic exclusion was applied using a setting of 10 seconds.

2.5 Mass Spectrometry Data Organization and Statistical Analysis

Raw data files were loaded into PEAKS 7 software (Bioinformatics Solutions, Inc.) for peptide and protein analysis using the UniProtKB E. coli K12 database and organized in Scaffold (Proteome Software, Inc.) for filtering according to a number of exclusion parameters. First, samples that did not show FtsK among the list of detected proteins were excluded, as any proteins of interest should have resulted from cross-linking with FtsK_N. Proteins that did not meet a minimum protein and peptide threshold of 95% probability and/or displayed less than a minimum of 4 unique peptides were removed from the results. Additionally, periplasmic, outer membrane and ribosomal proteins were excluded from the list. Pairwise comparisons between the non-cross-linked (-pAZP) and cross-linked (+pAZP) samples were carried out for each FtsK_N variant (R20C, I107C, Y217C) and only proteins that were present in at least 4 of the 5 +pAZP samples (2 R20C replicates, 2 Y217C replicates, 1 I107C replicate) and absent in at least 2 of the
5-pAZP controls were added to a final list of “potential FtsK<sub>N</sub> interactors”. The protein MinD was selected from this list for subsequent analysis by <i>in vitro</i> pulldown assay based on its known involvement in cell division and lack of previous evidence of an interaction with FtsK.

Additionally, raw mass spectra data were processed using the MaxQuant computational platform version 1.5.5.5 for label-free quantification of mass spectra (Cox <i>et al.</i>, 2014). The resulting values were submitted to the bioinformatics software Perseus (version 1.5.5.5), which removed common contaminants (e.g., human keratin). The values were then log<sub>2</sub> transformed to reduce skew and missing values were imputed from a normal distribution (Tyanova <i>et al.</i>, 2016). Additionally, only proteins present in both replicates of at least one FtsK<sub>N</sub> variant were used, producing a list of 488 different proteins. To assess reproducibility and correlation between samples, column correlation analysis was carried out with this data using the Perseus software. Pearson correlation coefficients were assigned to each of the sample comparisons and organized by hierarchical clustering in a heat map.

### 2.6 In Vitro Pulldown Assay

The pulldown assay was carried out as described (with some exceptions) in Berezuk <i>et al.</i> (2018). <i>E. coli</i> Lemo21 carrying the plasmid MD001 was grown overnight in a rotary shaker in LB supplemented with 30 µg/mL chloramphenicol and 50 µg/mL kanamycin at 37°C. Twenty milliliters of overnight culture was used to inoculate 500 mL LB containing the same antibiotics and the culture was grown for 1 hour at 37°C. The expression of FLAG-MinD was induced by adding 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) followed by incubation for 2 hours
at 37°C. Cells were collected by centrifugation, washed with 10 mL Labeling Wash Buffer and the pellet was frozen for 1 hour at -20°C.

The pellet was thawed and suspended in Lysis Buffer (20 mM Tris-HCl, 100 mM NaCl, 5mM EDTA, 40 µg/mL DNase I, 300 µg/mL lysozyme and 0.1% (v/v) Protease Inhibitor Cocktail Set III) and incubated for 10 minutes at room temperature. The cells were sonicated on ice for 1 minute (10 seconds on, 10 seconds off) to complete cell lysis and cell debris was removed by centrifugation at 12,074 x g for 20 minutes at 4°C. Anti-FLAG M2 Affinity Resin (Sigma) was washed, prepared as a 1:1 (Resin:Labeling Wash Buffer) slurry according to the manufacturers instructions and added to the cell lysate. This mixture was incubated overnight at 4°C with shaking.

The FLAG-MinD bound resin was collected by centrifugation (94 x g for 2 minutes at 4°C) and washed 3x with 1 mL Labeling Wash Buffer. FLAG-MinD was eluted by suspending the resin in Tris-buffered Saline (TBS) supplemented with 100 µg/mL FLAG peptide (Sigma) and incubating for 30 minutes at 4°C with shaking. The resin was collected by centrifugation and the supernatant (containing the eluted protein) was stored at 4°C. The total purified protein was quantified using a Bicinchoninic Acid Protein Assay Kit (Pierce) according the manufacturer’s instructions, with bovine serum albumin (BSA) being used as a standard (Smith et al., 1985).

In parallel to FLAG-MinD purification, *E. coli* Lemo21 carrying plasmid pAB006-2 was grown in LB supplemented with 30 µg/mL chloramphenicol and 150 µg/mL ampicillin overnight at 37°C in a rotary shaker. Ten milliliters of overnight culture was used to inoculate 250 mL LB containing the same antibiotics, which was incubated for 1 hour at 37°C. Expression of WT
His$_{10}$-FtsK$_N$ was induced by adding 0.2% (w/v) L-Arabinose and incubating for another 2 hours at 37°C. Cells were collected by centrifugation and washed with Labeling Wash Buffer, then the pellet was frozen for 1 hour at -20°C.

The pellet was thawed and suspended in lysis buffer for 10 minutes at room temperature, sonicated on ice, and the resulting lysate was ultracentrifuged at 40,000 rpm for 1 hour at 4°C using a Beckman Coulter Optima XPN-90 Ultracentrifuge (Ti70 Rotor). The pellet was then suspended in Solubilization Buffer (20mM Tris-HCl, 300 mM NaCl, 25 mM imidazole, 10% [v/v] glycerol and 2% [v/v] Lauryldimethylamine N-oxide [LDAO] [Sigma]) and Profinity IMAC Ni-charged Resin (1:1 slurry resin:water) was added, followed by incubation for 1 hour at 4°C with shaking. The same amount of fresh resin was added to Solubilization Buffer to serve as a negative control.

Both resin samples were washed 10 times in Pulldown Wash Buffer (20mM Tris-HCl, 300 mM NaCl, 25 mM imidazole, 10% [v/v] glycerol and 0.05% [v/v] LDAO) then suspended in TBS containing 100 mM NaCl, 25 mM imidazole, 0.05% (v/v) LDAO and 100 µg/mL FLAG-MinD (to a final concentration of 90 µg/mL). Both samples were incubated overnight at 4°C with shaking. Following incubation the resins were collected by centrifugation and samples of the initial supernatants were taken for Western Immunoblot analysis. The resins were washed 3 times with Pulldown Wash Buffer and samples of the first and third washes were collected. The resins were resuspended in His$_{10}$-FtsK$_N$ Elution Buffer (20mM Tris-HCl, 300 mM NaCl, 1 M imidazole, 10% [v/v] glycerol and 0.05% [v/v] LDAO) for 15 minutes at room temperature and the final eluents were collected. A second pulldown experiment was carried out using immobilized FLAG-MinD and His$_{10}$-FtsK$_N$ in the mobile phase. Culture and purification
conditions were the same, as mentioned previously, except His$_{10}$-FtsK$_N$ was eluted from the IMAC resin using His$_{10}$-FtsK$_N$ Elution Buffer. Anti-FLAG affinity resin with bound FLAG-MinD was washed 10 times in Pulldown Wash Buffer and suspended in TBS containing 1M NaCl and 25 µg/mL FtsK$_N$ then incubated overnight at 4°C with shaking. The pulldown steps were the same as previously mentioned but the wash buffer contained 20mM Tris-HCl, 300 mM NaCl, 10% [v/v] glycerol and 2.5 µg/mL FLAG peptide and the elution buffer contained the same with 100 µg/mL FLAG peptide.

2.7 Western Immunoblot Analysis

The samples collected from both the cross-linking assay and the in vitro pulldown assay were mixed with 5X SDS Sample Buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol and 0.1% bromophenol blue) and separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 10% SDS-polyacrylamide gel. Western blotting was carried out as described in Berezuk et al. (2018) with some changes. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad) for Western immunoblot analysis using a Trans-Blot® Turbo transfer system (Bio-Rad) and blocked using 2% BSA (Sigma) in TBS incubated overnight at 4°C with shaking. The blot was immuno-stained using either mouse anti-FLAG or mouse anti-His (Sigma) as the primary antibody and goat anti-mouse IgG (H+L) antibody conjugated with alkaline phosphatase (Sigma) as the secondary antibody. The blot was washed after each blocking and antibody incubation by submerging 3x in TBS + 0.05% (v/v) Tween 20 for 5 minutes with shaking. Protein bands were detected using 3.3 mg of nitro blue
tetrazolium (NBT) and 1.7 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 10 mL of alkaline phosphatase substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂).

Table 2.1 Bacterial Strains and Plasmids

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Descriptiona</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>rph-1IN (rrnD-rrnE)</td>
<td>Coli Genetic Stock Center</td>
</tr>
<tr>
<td>DH5α</td>
<td>F−Φ80lacZΔM15Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK, mK⁺) phoA supE44 λ-thi-1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Lemo21 (DE3)</td>
<td>fhuA2 [lon] ompT gal (λDE3) [dcm] ΔhsdS/pLemo(CamR) λ DE3 = λ.sBamH1o ΔEcoRIB int::(lacI::PlacUV5::T7 gene1) i21 Δnin5 pLemo = pACYC184-PrhaBAD-lys</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>LP11-1</td>
<td>W3110 fisK44 aroA::Tn10</td>
<td>K. Young (Potluri et al., 2012)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAB006-2</td>
<td>pBAD24 derivative encoding the N-terminal 220 amino acids of FtsK (His₁₀FtsK₅) from E. coli</td>
<td>A. Berezuk (Berezuk et al., 2014)</td>
</tr>
<tr>
<td>pAB006-21</td>
<td>pAB006-2 derivative encoding the single-cysteine His₁₀FtsK₅ variant R20C</td>
<td>A. Berezuk (Berezuk et al., 2014)</td>
</tr>
<tr>
<td>pAB006-51</td>
<td>pAB006-2 derivative encoding the single-cysteine His₁₀FtsK₅ variant 1107C</td>
<td>A. Berezuk (Berezuk et al., 2014)</td>
</tr>
<tr>
<td>pAB006-112</td>
<td>pAB006-2 derivative encoding the single-cysteine His₁₀FtsK₅ variant Y217C</td>
<td>A. Berezuk (Berezuk et al., 2014)</td>
</tr>
<tr>
<td>pMD001</td>
<td>pET28a derivative encoding N-terminally tagged FLAG-MinD from E. coli</td>
<td>This study</td>
</tr>
</tbody>
</table>

aCam – Chloramphenicol
Chapter 3: Results

3.1 Single-Cysteine FtsK<sub>N</sub> Variants Cross-link with Numerous Proteins In Vivo

A two-stage approach was used to investigate FtsK<sub>N</sub> interactions with potential protein partners in the cytoplasm. First, in vivo cross-linking was used to capture any proteins in direct contact with FtsK<sub>N</sub>. These proteins were then digested into peptides and identified using mass spectrometry. This approach was successfully used by Berezuk et al. to identify FtsK<sub>N</sub> interaction partners in the periplasm (albeit using a different cross-linking agent) and was adopted for use in this study (Berezuk et al., 2018). The cross-linking assay was designed to be site-specific so E. coli LP11-1 strains expressing single-cysteine variants of FtsK<sub>N</sub> were used in combination with the thiol-specific cross-linker p-Azidophenacyl bromide (pAZP). pAZP was chosen as the cross-linker due to its bi-functional chemistry (Figure 3.1-A). On one end, the reactive α-bromo ketone moiety binds the nucleophilic amino acid cysteine (Hixson and Hixson, 1975). Combining pAZP with cells expressing single-cysteine variants of FtsK<sub>N</sub> allowed us to target the cross-linker to specific locations on the protein. The opposite end of the cross-linker is comprised of an azide group that is photolyzed to an electrophilic nitrene group upon exposure to 365nm UV radiation. This facilitates the non-specific, covalent bonding of the FtsK<sub>N</sub> cysteine to any protein side chain within a 9Å radius (See Figure 3.1-B) (Hixson and Hixson 1975).
Figure 3.1 In Vivo Cross-linking using the Bifunctional Reagent p-Azidophenacyl Bromide.

(A) Chemical structure of p-azidophenacyl bromide (pAZP). Both reactive groups and the approximate length of the molecule are shown. The α-bromo ketone moiety binds the nucleophilic amino acid cysteine while the azide group can be photolyzed to an electrophilic nitrene group upon exposure to 365nm UV radiation. (B) Schematic diagram depicting the principle of the pAZP in vivo cross-linking assay. pAZP is added to E. coli cells expressing a single-cysteine version of FtsK$_N$, the α-bromo ketone moiety binds the cysteine side chain and following sufficient incubation time, the cells are exposed to 365nm UV radiation resulting in the activation of the cross-linker. This covalently binds pAZP to any protein within a ~9 angstrom radius resulting in an FtsK$_N$-pAZP-protein complex.

The temperature-sensitive LP11-1 strains (containing the fisk44 mutation) were grown at 42°C to minimize interference of chromosomally encoded FtsK (See Chapter 1: Introduction), while the simultaneously expressed, plasmid-encoded FtsK$_N$ variants (containing single cysteine residues) prevented the associated filamentous phenotype (Berezuk et al. 2014). It is important to note that FtsK$_N$ spans the cytoplasm, inner membrane and periplasm and thus protein-protein
interactions could be occurring in any of these cellular compartments. In this study I used FtsK\textsubscript{N} variants with cysteine residues engineered in the cytoplasmic segments of the protein. This was done to specifically target interactions within the cytoplasm, while another member of our laboratory investigated interactions within the periplasm (Berezuk \textit{et al.}, 2018). The selected variants contained engineered cysteine residues at positions 20, 107 and 217 (supplied by A. Berezuk) and were chosen to target the cross-linker to the N-terminal, middle and C-terminal cytoplasmic loops of FtsK\textsubscript{N} respectively (\textbf{Figure 1.4}).

To control for proteins that may co-purify with the FtsK\textsubscript{N} variants independent of cross-linking, two samples were prepared in parallel for each variant. Both were identical in preparation except one contained the cross-linker (+pAZP) and the other did not (-pAZP). This allowed us to remove potential protein artifacts from the list of FtsK\textsubscript{N} interaction partners generated in a later portion of the experiment. In order to assess the effect of the cross-linker, samples were initially analyzed using SDS-PAGE and Western blot (\textbf{Figure 3.2}). Cross-linking with pAZP had a noticeable effect on the band pattern (compared to pAZP-free controls) detected by Western blot for each FtsK\textsubscript{N} variant. These altered band patterns were likely due to the presence of FtsK\textsubscript{N}-containing cross-linked protein adducts in the pAZP-containing samples.

The initial strategy for identifying these cross-linked proteins was to perform in-gel digestion of specific FtsK\textsubscript{N}-containing protein complexes (verified by Western blot), which had been separated by SDS-PAGE. The resulting peptides would then be analyzed by mass spectrometry for protein identification. This strategy proved ineffective however, as attempts to discern which protein adducts contained FtsK\textsubscript{N} were unreliable.
Figure 3.2 Western Blot Analysis of Single-Cysteine FtsK\textsubscript{N} Variants R20C, I107C and Y217C Cross-linked \textit{In Vivo} with p-Azidophenacyl bromide (pAZP).

Temperature sensitive \textit{E. coli} strain LP11-1 expressing plasmid encoded WT FtsK\textsubscript{N} or one of three single-cysteine FtsK\textsubscript{N} variants was cultured at 42°C for 2 hours. Cells were incubated with (+) or without (-) the cross-linker pAZP and exposed to 365 nm UV radiation. FtsK\textsubscript{N} (WT or single-cys variant) was purified from the cell lysate by immobilized metal affinity chromatography, separated by SDS-PAGE and blotted onto a nitrocellulose membrane. These membranes were probed with mouse anti-His\textsubscript{6} antibodies followed by goat anti-mouse IgG conjugated with alkaline phosphatase. Cross-linking with pAZP noticeably affected the signal pattern detected for each FtsK\textsubscript{N} variant, likely due to the presence of various FtsK\textsubscript{N}-containing cross-linked adducts. WT FtsK\textsubscript{N} was included as a positive control for FtsK\textsubscript{N} detection.

This led us to devise a new strategy in which proteolytic digestion was carried out immediately following the affinity chromatography purification of FtsK\textsubscript{N} cross-linked products, thereby avoiding SDS-PAGE separation altogether. The resulting peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), generating a list of detected peptides and their associated proteins. The entire \textit{in vivo} cross-linking and LC-MS/MS experiment was performed twice under identical conditions for each FtsK\textsubscript{N} variant. This broader approach produced a large list of proteins, with hundreds being detected for each FtsK\textsubscript{N} variant.
in both +/-pAZP samples (data not shown). This outcome was expected given the unbiased approach previously described, and also because the experiment was carried out \textit{in vivo} and the UV-induced cross-linking was non-specific. The list of proteins detected was therefore subjected to statistical analyses and filtered according to a number of exclusion parameters.

First, raw LC-MS/MS data files were assessed using the computational platform MaxQuant (version 1.5.5.5) to further assess the effect of the cross-linker (Cox \textit{et al.}, 2014). The resulting values were inputted into the bioinformatics software, Perseus (version 1.5.5.5), in which common contaminants were removed (\textit{e.g.} human keratin). The values were log$_2$ transformed to produce a less skewed distribution and missing values were imputed from a normal distribution, allowing statistical analyses to be applied (Tyanova \textit{et al.}, 2016). Additionally, only proteins present in both replicates of at least one FtsK$_N$ variant were used for statistical analysis, producing a list of 488 different proteins in total.

Perseus (version 1.5.5.5) was used to compare the protein values of one sample to the values of every other sample. The software assigned a Pearson correlation coefficient to each sample comparison and organized the results by hierarchical clustering in a heat map shown in \textbf{Figure 3.3}. The first thing to note from these results is the +/-pAZP samples from one I107C replicate are absent from the dataset. These were the only samples in which FtsK$_N$ peptides were not detected (data not shown) and given that the cross-linking experiment was predicated on the presence of FtsK$_N$, and the experiment could not be repeated again due to time constraints, these samples had to be omitted from this and all further analyses. Interestingly, the column correlation heat map showed there was a stronger linear correlation between R20C, I107C and Y217C samples when pAZP was present compared to when it was absent.
Figure 3.3 Column Correlation and Reproducibility Analysis of *In Vivo* Cross-linking Samples and Non Cross-linked Controls.

Heat map of the Pearson correlation coefficients generated in the bioinformatics software Perseus (version 1.5.5.5) for comparison of each of the samples tested in the *in vivo* cross-linking experiment. Strain/Condition codes refer to the three single-cysteine variants of FtsK$_N$ used in the experiment and the presence of + or – denotes the respective presence or absence of the cross-linking agent pAZP. The blue-red gradient represents the Pearson correlation coefficient number between 0.4-0.8, with the higher the number (and thus the more red the square) suggesting a stronger linear correlation between the two samples. The +/-pAZP samples for each FtsK$_N$ variant were prepared in duplicate however one 1107C replicate (both +/-pAZP) was omitted due to the lack of FtsK$_N$ peptide detection in these samples. The data shows +pAZP samples show a stronger linear correlation than –pAZP samples, suggesting the proteins detected in each sample were more similar when the cross-linker was added. It also shows the addition of pAZP results in a higher level of reproducibility between replicates.
This was evidenced by the higher coefficient values of the +pAZP comparisons versus the –pAZP comparisons and also that hierarchical clustering by Euclidean distance automatically divided the samples into +pAZP clusters and –pAZP clusters. This suggests the proteins detected were more similar between FtsK\textsubscript{N} variants when cross-linked with pAZP. The heat map also showed a stronger correlation between replicates of the same FtsK\textsubscript{N} variant when pAZP was added, suggesting the addition of pAZP resulted in higher reproducibility between replicates. It is important to note, however; the highest correlation value is 0.8 out of 1 suggesting some notable variability even between +pAZP samples.

Next the LC-MS/MS peptide and protein data were organized in the Scaffold program (Proteome Software, Inc.) and stringently filtered to produce a smaller, more manageable list of potential FtsK\textsubscript{N} interactors. First, in order to maximize confidence that the spectra detected correspond to the correct peptides, only peptides with a probability threshold of >95% were considered. Next, all proteins with less than 4 unique peptides detected were removed. This was done to increase the threshold required for positive hits, thus increasing the level of confidence in the proteins identified. All periplasmic and outer membrane proteins were then removed from the list as well as all ribosomal proteins. This was done to focus on putative cytoplasmic proteins and because ribosomal proteins have been shown to be common contaminants in protein identification mass spectrometry experiments (Trinkle-Mulcahy \textit{et al.}, 2008). Finally, only proteins that were present in at least 4 of the 5 +pAZP samples (2 R20C samples, 2 Y217C samples, 1 I107C sample) and absent from at least 2 of the 5 -pAZP controls were added to the final list of potential FtsK\textsubscript{N} interactors.
These exclusion parameters produced a list of 163 proteins (Appendix A – Table A.1). The STRING v11 bioinformatics program was used to organize these proteins by function and known/predicted interactions, producing a STRING interaction network (Figure 3.4). According to this analysis, 121 proteins fell under the Gene Ontology (Biological Process) term Primary Metabolism (metabolism that is essential for survival and cell maintenance), 38 under Response to Stress, 7 under Cell Division, 1 under Cell Shape Regulation and 21 under various other terms relating to metabolism. 25 of these proteins showed overlapping functions, for example DnaJ was associated with both Primary Metabolism and Response to Stress.

It is important to note the 163 proteins are described as ‘putative’ FtsK\textsubscript{N} interaction partners, as it is unlikely all interact directly with FtsK\textsubscript{N}. This high false-positive rate is the result of a number of methodological limitations, similar to those described in the Ph.D. thesis of Dr. Berezuk (Berezuk, 2018). For example, immobilized metal affinity chromatography allows for the purification of FtsK\textsubscript{N} and its covalently cross-linked protein partners, however it is unable to remove all proteins associated with the cross-linked FtsK\textsubscript{N} complexes or those captured by non-specific binding to the chromatography resin. Combined with the high sensitivity of peptide detection that is characteristic of liquid chromatography tandem mass spectrometry, the detection of contaminating proteins is unavoidable (Urban, 2016). Due to these limitations, further verification is required to confirm any of the 163 putative FtsK\textsubscript{N} interaction partners as true interactors.
Mass spectrometry was used to identify proteins captured by \textit{in vivo} cross-linking of FtsK\textsubscript{N} variants R20C, I107C and Y217C. Analysis of the mass spectrometry data produced a list of 164 putative FtsK\textsubscript{N} interaction partners. The interactions between these proteins (including FtsK) were analyzed using STRING v11 (Szklarczyk \textit{et al.}, 2019). Each protein is represented by a labeled sphere with a diagram of its structure displayed within (if available). The lines represent interactions that are experimentally verified, predicted in curated databases or inferred from textmining of scientific journal articles. A minimum confidence score of 0.7 (high confidence) was applied, with darker lines corresponding to higher confidence scores. Sphere colour...
corresponds to function inferred from Gene Ontology terms (Biological Process). Colour legend: Purple – Primary Metabolism, Green – Stress Response, Red – Cell Division, Yellow – Cell Shape Regulation, Grey – Other. FtsK was added to show currently known interactions with proteins in the list and it is circled in red.

Ideal candidates from the putative interactors list were chosen based on conceptual likelihood of interaction, according to currently available FtsK literature. The majority of putative interactors were associated with primary metabolism, many with closely related functions (e.g. GatABD). However there is no evidence connecting FtsK to pathways associated with primary metabolism and so these proteins were not considered for further investigation.

The next largest protein group by Gene Ontology was related to stress response. Of particular interest were the proteins HflK and HflC. Both proteins are anchored in the inner-membrane in vivo and form a complex with the essential ATP-dependent metalloprotease FtsH (Kihara et al., 1996). FtsH itself is involved in proteome regulation and response to heat stress by degrading improperly folded proteins in the cytoplasm and cell envelope (Langklotz et al., 2012). HflKC functions to modulate FtsH-mediated degradation of specific proteins (e.g. λcII, a protein involved in the lysogeny phase of phage λ) and the expression of all three proteins is influenced by heat shock (Chuang and Blattner, 1993; Herman et al., 1995; Kihara et al., 1997). Given the in vivo cross-linking experiment was carried out at 42°C, it is conceivable the FtsH-HflKC complex could be involved in the heat shock-related quality control of FtsK. Findings from a similar cross-linking experiment carried out by Berezuk et al. lend additional support to this hypothesis. This experiment focused on interactions between FtsK_N and other proteins in the periplasm and identified YidC to be a putative interaction partner (Berezuk et al., 2018). Similar to HflKC, YidC also associates with FtsH and modulates its proteolytic activity, aiding in the
quality control of inner-membrane protein folding and translocation (van Bloois et al., 2008). Taken together, a connection between FtsK and the FtsH-HflKC-YidC complex seems plausible.

RodZ, the sole putative FtsK$_N$ interactor related to cell shape regulation is also of interest. RodZ is a bitopic inner-membrane protein essential for maintenance of rod shape and proper assembly of the cytoskeletal protein MreB (Shiomi et al., 2008; Bendezú et al., 2009). Recently it was shown that RodZ interacts directly with FtsZ and localizes to the division site in an FtsZ-dependent manner (Yoshii et al., 2019). Based on these findings, Yoshii et al. propose that RodZ is an important factor linking the cell elongation machinery with the divisome and that it helps regulate the early division process. Given its proven association with FtsZ and the early divisome, RodZ and FtsK likely share close spatial proximity for some amount of time during division. Its presence as a putative FtsK$_N$ interactor could therefore be due to a direct interaction with FtsK$_N$ or simply due to a tight association with FtsZ, which is known to interact directly with FtsK (Di Lallo et al., 2003; Grenga et al., 2008). Further biochemical validation is required to determine if RodZ directly interacts with FtsK$_N$.

Seven of the putative FtsK$_N$ interactors were associated with the Gene Ontology term ‘cell division’ and are listed in Table 3.1. Of these seven, only FtsZ and FtsI have been previously implicated in interactions with FtsK (Di Lallo et al., 2003; Grenga et al., 2008). FtsA however was also found to interact with FtsK, thanks to recent work in our lab (Berezuk, 2018). The fact that these proteins were also detected in this study, as a result of *in vivo* cross-linking and LC-MS/MS using FtsK$_N$ as opposed to the entire FtsK protein, provides further evidence for these proteins as biologically relevant FtsK interaction partners.
Table 3.1 *E. coli* Cell Division Proteins Deemed Potential FtsK\(_N\) Interaction Partners by *In Vivo* Cross-linking and Mass Spectrometry

<table>
<thead>
<tr>
<th>Protein(^a)</th>
<th>Function</th>
<th>R20C*</th>
<th>I107C*</th>
<th>Y217C*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClpX</td>
<td>ATP-dependent chaperone protein that complexes with ClpP, a serine protease. ClpXP helps to regulate Z-ring formation by degrading FtsZ and potentially other cell division proteins.</td>
<td>4</td>
<td>32.5</td>
<td>x</td>
<td>31</td>
</tr>
<tr>
<td>MinD</td>
<td>Helps determine the site of division in combination with MinC and MinE by inhibiting Z-ring formation at the cell poles. Essential for cell division.</td>
<td>7</td>
<td>30</td>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td>FtsA</td>
<td>Aids in Z-ring formation by tethering FtsZ filaments to the cytoplasmic membrane. Essential for cell division.</td>
<td>4</td>
<td>10.5</td>
<td>x</td>
<td>9</td>
</tr>
<tr>
<td>ZapD</td>
<td>FtsZ-associated protein. Helps to stabilize the Z-ring by inhibiting FtsZ protofilament depolymerization. Not essential for cell division.</td>
<td>x</td>
<td>6.5</td>
<td>x</td>
<td>8</td>
</tr>
<tr>
<td>FtsZ</td>
<td>Filamentous GTPase essential for cell division. Main component of the Z-ring, which forms at mid-cell and acts as a scaffold for the rest of the cell division apparatus.</td>
<td>x</td>
<td>6.5</td>
<td>x</td>
<td>7</td>
</tr>
<tr>
<td>MurE</td>
<td>UDP-N-acetylMuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase involved in peptidoglycan precursor biosynthesis.</td>
<td>x</td>
<td>6.5</td>
<td>x</td>
<td>4</td>
</tr>
<tr>
<td>FtsI</td>
<td>Peptidoglycan D,D-transpeptidase involved in the synthesis of septal peptidoglycan. Essential for cell division.</td>
<td>x</td>
<td>4.5</td>
<td>x</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Proteins listed in order of highest average unique peptides detected by LC-MS/MS

* Average number of unique peptides detected in samples with (+) and without (-) the cross-linking agent pAZP for each single-cysteine FtsK\(_N\) variant (*i.e.* R20C, I107C or Y217C). (x) denotes either zero peptides detected or number of peptides detected were below the minimum threshold described in Table A1.
Additionally, cell division proteins not previously associated with FtsK were present in the reduced list, such as the Z-ring stabilizing protein ZapD, the peptidoglycan precursor biosynthesis protein MurE, the Z-ring regulator ClpX and the septum site-determining protein MinD (de Boer et al., 1991; Gordon et al., 2001; Camberg et al., 2011; Durand-Heredia et al., 2012). The implication of these proteins as potential FtsK<sub>N</sub> interaction partners is interesting given they all (with the exception of MurE) associate in some way with the Z-ring during early division and could possibly come into close proximity with FtsK (Camberg et al., 2011; Durand-Heredia et al., 2012; Taviti and Beuria, 2017). The finding of MinD as a potential interaction partner with FtsK<sub>N</sub> is particularly interesting as it is essential for cell division and not considered part of the greater divisome protein complex. Due to its well-established role in cell division (detailed in Chapter 1: subsection 1.2.2), MinD was selected above the other proteins of interest for further investigation into its potential interaction with FtsK<sub>N</sub>. This investigation was focused on verifying the interaction in vitro using purified versions of both proteins combined in a pulldown assay.

3.2 His<sub>10</sub>-FtsK<sub>N</sub> Binds FLAG-MinD in an In Vitro Pulldown Assay

Design of the in vitro pulldown assay was based on that described in Berezuk et al. to confirm an interaction between FtsK and the outer membrane lipoprotein RlpA (Berezuk et al., 2018). The assay is predicated on the principle that a ‘bait’ protein immobilized on the appropriate chromatography matrix can be used to capture and immobilize a free ‘prey’ protein if the two proteins interact. To test for this capture, the prey protein is incubated with the
immobilized bait and the stationary chromatography phase is then washed until there is no prey present in the supernatant. The bait protein is eluted from the stationary phase and if the prey protein is also present in the eluate, capture has taken place and the proteins are said to interact in vitro. If the prey protein is not present in the eluate, it was likely removed completely during wash steps and the two proteins are said to not interact in vitro. In this study, His$_{10}$-FtsK$_N$ was used as the bait protein immobilized on IMAC resin and FLAG-MinD was used as the prey (Figure 3.5). Western blot analysis was used to determine the results of the pulldown assay.

**Figure 3.5 Schematic Diagram of FLAG-MinD In Vitro Pulldown Assay.**

Diagram depicting the experimental steps used to investigate the putative interaction between MinD and FtsK$_N$. Progressing from left to right, His$_{10}$-FtsK$_N$ was purified using immobilized metal affinity chromatography (IMAC) and left bound to the IMAC resin, which was then washed thoroughly and used as the stationary phase for subsequent steps. FLAG-MinD (previously purified by affinity chromatography) was added and incubated overnight, then subject to three rounds of stringent washing. After the third wash, elution buffer was added to remove all protein from the stationary phase. After each step, supernatant samples were collected and separated by SDS-PAGE. Western blotting was then used to probe the samples for the presence of FLAG-MinD. The diagram depicts a scenario where FLAG-MinD and His$_{10}$-FtsK$_N$
co-elute despite stringent washing steps removing all unbound protein from the stationary phase. This result would be indicative of an in vitro interaction between FLAG-MinD and His$_{10}$-FtsK$_{N}$.

The results of the in vitro pulldown assay are shown in Figure 3.6-A. The figure shows a Western blot image containing the unbound and FtsK$_{N}$-bound resin samples, as well as a protein standards ladder and FLAG-MinD standard. For the unbound resin control samples, Western blotting showed bands in the FT (flow through) and Wash #1 lanes corresponding to ~30 kDa, the known molecular mass of MinD. The presence of FLAG-MinD in these samples was expected as the flow through was taken directly from the overnight incubation and one wash was unlikely to completely remove FLAG-MinD from the mobile or stationary phase. The blot also showed FLAG-MinD was absent from both the Wash #3 and Eluate lanes, suggesting all FLAG-MinD was removed by the third wash from both mobile and stationary phases and thus did not cross-react with the IMAC resin. For the FtsK$_{N}$-bound resin samples, FLAG-MinD was present in the FT and Wash #1 lanes, which was expected for the same reasons previously mentioned. FLAG-MinD was also absent in the Wash #3 lane, suggesting unbound protein had been removed from the mobile phase by the third wash. Interestingly, FLAG-MinD was present in the Eluate lane, suggesting the protein had bound to the stationary phase and co-eluted with the previously bound His$_{10}$-FtsK$_{N}$. Given that the unbound resin control showed no cross-reaction between FLAG-MinD and the IMAC resin stationary phase, these results provide strong evidence for an in vitro interaction between MinD and FtsK$_{N}$.

To further verify this interaction, another pulldown assay was carried out with the bait and prey positions reversed. FLAG-MinD was immobilized on anti-FLAG affinity resin to act as the bait and incubated overnight with purified His$_{10}$-FtsK$_{N}$ (prey). The results of the His$_{10}$-FtsK$_{N}$
pulldown assay are shown in Figure 3.6-B. The figure shows a Western blot image similar to the previous pulldown assay, but with the unbound and MinD-bound resin samples and a purified His$_{10}$-FtsK$_N$ standard. For the unbound resin control samples, a number of bands were

**Figure 3.6 Results of FLAG-MinD and His$_{10}$-FtsK$_N$ In Vitro Pulldown Assays**

(A) Western blot of the samples attained from the pulldown assay using immobilized His$_{10}$-FtsK$_N$ as “bait” for the capture of FLAG-MinD. The blot was developed using mouse anti-FLAG (Sigma) as the primary antibody and goat anti-mouse IgG (H+L) antibody conjugated with alkaline phosphatase (Sigma) as the secondary antibody. The absence of FLAG-MinD in the “Wash #3” and “Elute” lanes of the unbound resin samples confirmed it did not cross-react with the IMAC resin used as the stationary phase. In the FtsK$_N$-bound resin samples, the absence of FLAG-MinD in “Wash #3” lane combined with its
presence in the “Elute” lane suggests FLAG-MinD bound the His\textsubscript{10}-FtsK\textsubscript{N} previously bound to the resin, providing evidence for an in vitro interaction between the two proteins. (B) Western Blot of the samples attained from the pulldown assay using immobilized FLAG-MinD as “bait” for the capture of His\textsubscript{10}-FtsK\textsubscript{N} (reverse of (A)). The blot was developed using mouse anti-His (Sigma) as the primary antibody and goat anti-mouse IgG (H+L) antibody conjugated with alkaline phosphatase (Sigma) as the secondary antibody. Similar to (A) the results showed evidence of an in vitro interaction between His\textsubscript{10}-FtsK\textsubscript{N} and FLAG-MinD with no cross-reaction of His\textsubscript{10}-FtsK\textsubscript{N} with the anti-FLAG affinity resin used as the stationary phase. FT – flow through; MinD Std. – FLAG-MinD standard; FtsK Std. - His\textsubscript{10}-FtsK\textsubscript{N} standard.

The darkest band from each lane matched the molecular mass of the band in the His\textsubscript{10}-FtsK\textsubscript{N} standard lane corresponding to His\textsubscript{10}-FtsK\textsubscript{N}. This band corresponded to a molecular mass of ~18 kDa, which is slightly lower than the ~24 kDa expected for His\textsubscript{10}-FtsK\textsubscript{N}, however this discrepancy is likely due to the abnormal gel migration commonly exhibited by membrane proteins (Rath et al., 2009). The other faint bands present in the FT and Wash #1 lanes also matched faint bands in the His\textsubscript{10}-FtsK\textsubscript{N} standard lane and were likely the result of non-specific protein binding to the IMAC resin used during purification. The presence of His\textsubscript{10}-FtsK\textsubscript{N} in these two lanes was expected due to the same reasons mentioned for the previous assay. His\textsubscript{10}-FtsK\textsubscript{N} was not detected in the unbound resin Wash #3 or Elute lanes, confirming there was no cross-reaction between His\textsubscript{10}-FtsK\textsubscript{N} and the anti-FLAG affinity resin. For the FLAG-MinD-bound resin samples, His\textsubscript{10}-FtsK\textsubscript{N} was detected in the FT and Wash #1 lanes and absent in the Wash #3 lane. As with the control, its presence in the FT and Wash #1 lanes was expected and its absence in the third wash suggests it was completely removed from the mobile phase. His\textsubscript{10}-FtsK\textsubscript{N} was detected in the Elute lane, which given its absence in the mobile phase suggests His\textsubscript{10}-FtsK\textsubscript{N} was bound to the stationary phase. Combined with the results of the unbound control, which showed no cross-reaction between His\textsubscript{10}-FtsK\textsubscript{N} and the anti-FLAG affinity resin, these results suggest there was an in vitro interaction between His\textsubscript{10}-FtsK\textsubscript{N} and FLAG-MinD. Importantly, these results corroborated the finding from the previous assay that also found an in
vitro interaction between the two proteins. Additionally, both assays were repeated under the same conditions and the results were confirmed to be reproducible (Data not shown). Taken together, these matching results provide even stronger evidence for a bona fide interaction between FtsK<sub>N</sub> and MinD in vitro.

**Chapter 4: Discussion**

FtsK is an inner membrane-bound protein essential for cell division in *E. coli*. Only the N-terminal trans-membrane domain (FtsK<sub>N</sub>) is required for this essential role, however its function is not fully understood (Begg *et al*., 1995; Draper *et al*., 1998; Yu *et al*., 1998). As part of the larger complex of division-related proteins known as the divisome, FtsK<sub>N</sub> is required for the localization of divisome proteins FtsQ, FtsL and FtsI to the division site (Aarsman *et al*., 2005; Pichoff and Lutkenhaus 2005). Additionally, it has been implicated in direct interactions with proteins FtsZ, FtsQ, FtsL and FtsI (Di Lallo *et al*., 2003). As such FtsK<sub>N</sub> is believed to be involved in the stabilization and maturation of the divisome through specific protein-protein interactions. In this work, I investigated these protein-protein interactions and present evidence of a novel interaction between FtsK<sub>N</sub> and MinD, a protein essential for determination of the division site in *E. coli*.

To capture and purify proteins in direct contact with FtsK<sub>N</sub>, cells expressing plasmid encoded, single-cysteine variants of FtsK<sub>N</sub> were combined with the UV-inducible cross-linker pAZP. Given the experiment was carried out *in vivo*, considerations were made to minimize potential interference of chromosomally encoded FtsK with the plasmid-encoded FtsK<sub>N</sub> variants. This was done using the temperature-sensitive *E. coli* LP11-1 strain, which contained the *ftsK44*
mutation. When grown at 42°C the chromosomally encoded FtsK is rendered non-functional, however this also results in cell division interruption. The resulting filamentous phenotype can be rescued by expressing a second copy of ftsK encoded on a plasmid as demonstrated by Berezuk et al. (2014), who showed that even a truncated version of FtsK containing only the N-terminal 220 amino acids (FtsK\textsubscript{N}) was sufficient for this rescue. Therefore, by expressing the second copy of the gene encoding FtsK at 42°C in \textit{E. coli} LP11-1, action of the chromosomal ftsK is minimized without compromising cell viability. Building on this, Berezuk et al. (2014) showed that a variety of single-cysteine variants of FtsK\textsubscript{N} could also rescue the temperature-sensitive phenotype. Plasmid constructs encoding three of these variants were supplied for use in this study due to the cysteine specificity of the cross-linker and because their cysteine residues are present in the cytoplasmic loops of FtsK\textsubscript{N} (R20C, I107C and Y217C) (Berezuk et al., 2014).

Following \textit{in vivo} cross-linking, the spectral data produced by LC-MS/MS were organized and interpreted using a number of analysis tools. In order to understand the effect of cross-linking with pAZP, data quantification and statistical analyses were conducted using the Maxquant and Perseus software suites respectively. The results showed that the mass spectra detected in samples with pAZP differed from the corresponding pAZP-free controls, suggesting the proteins captured by cross-linking were not artifacts of the FtsK\textsubscript{N} purification process (Figure 3.3). The results also showed that the proteins detected were more similar between sample replicates, and between different FtsK\textsubscript{N} variants when cross-linked with pAZP. Taken together these findings show cross-linking with pAZP had a statistically reproducible effect on which proteins were purified alongside single-cysteine variants of FtsK\textsubscript{N}, demonstrating the validity and usefulness of the method.
The bifunctional nature of the cross-linker (thiol-specific at one end and non-specific at the other) allowed for a broader, more exploratory approach to the study of FtsK interactions compared to related studies, which only investigated FtsK and certain suspected interaction partners (Di Lallo et al., 2003; D’Ulisse et al., 2007; Grenga et al., 2008). This also had the benefit of potentially capturing transient or weak interactions that could be important but otherwise difficult to detect. The main disadvantage to this method, however, is the high rate of false-positives and thus the need to further verify any interactions detected by additional experimentation. Given this, it was not surprising to find hundreds of different proteins detected in the cross-linked samples by LC-MS/MS. The initial results were therefore filtered according to a stringent set of exclusion parameters to produce a refined list of 163 proteins (Appendix A – Table A.1). The proteins on this list are involved in numerous cellular processes including cell division, elongation and metabolism. Given that all related studies have focused exclusively on interactions within the divisome, all proteins involved in cell division became the primary target of analysis. The cell division proteins found in the list of potential FtsK interactors were FtsZ, ZapD, FtsA, FtsI and MinD. The presence of FtsZ and FtsI support findings by Di Lallo et al. (2003) and Grenga et al. (2008) who showed evidence of FtsK-FtsZ and FtsK-FtsI interactions using bacterial two-hybrid systems. These results are particularly valuable, as the findings in the aforementioned studies had not been reproduced using an assay that did not require chimeric versions of the proteins in question. These results also support the prediction that FtsK functions to stabilize the divisome, as FtsZ and FtsI are both part of the divisome complex yet function in early and late stages of division respectively. Interestingly however, these results challenge other findings by Grenga et al. (2008) who used various truncated versions of FtsK in their two-
hybrid assay to elucidate which segments of the protein are required for said interactions. They found a segment of the FtsK linker domain was required for FtsZ interaction and the C-terminal DNA translocase was required for interaction with FtsI. In contrast, our findings suggest the N-terminal 220 amino acids of FtsK are involved in and may be sufficient for these interactions. This discrepancy could be due to the two-hybrid system used by Grenga et al. (2008), in which native interactions could be altered by the chimeric nature of the proteins used. It is also possible the cross-linking assay used in our study captured what would be transient or weak interactions under normal conditions. Further biochemical characterization of these interactions using different methods is required before any definitive conclusions can be drawn.

Di Lallo et al. (2003) and Grenga et al. (2008) also demonstrated interactions between FtsK and the divisome proteins FtsQ and FtsL, with the FtsK-FtsQ interaction being further verified by co-immunoprecipitation assay (D’Ulisse et al., 2007). It is possible FtsQ and FtsL were not implicated in our findings because the two proteins are largely membrane-bound while the FtsK_N residues involved in cross-linking were theoretically located in the cytoplasm. It is also possible the proteins were captured by cross-linking but could not be detected by LC-MS/MS as a result of low abundance or poor peptide fragmentation. Given the strong literature evidence for these interactions, it is unlikely the absence of FtsQ and FtsL in our data is indicative of a lack of interaction between these proteins and FtsK in vivo.

The presence of FtsA in our results also contrasts with the findings of Di Lallo et al. (2003) and Grenga et al. (2008). An interaction between FtsA and FtsK was not detected in their respective two-hybrid experiments, however it is possible this interaction was disrupted in the fusion proteins used to conduct these experiments. Recently, another member of our lab found
evidence of a direct interaction between FtsA and FtsK using an in vitro pulldown assay (Berezuk, 2018). This finding lends support to the putative FtsA-FtsK interaction implicated in our results, despite this interaction not being detected by Di Lallo or Grenga (and colleagues). While the biological significance of this interaction is not known, certain FtsA mutants have been shown to bypass the need for FtsK, suggesting there may be some functional overlap (Geissler and Margolin, 2005; Bernard et al., 2007). Recent work by Pichoff and colleagues showed FtsA might play a role in FtsN-mediated initiation of cell constriction, in addition to its role in Z-ring formation (Pichoff et al., 2012; Pichoff et al., 2015). They proposed a model in which the inhibition of FtsA oligomerization stimulates the recruitment of one or more downstream divisome proteins, ultimately leading to the recruitment of FtsN and the initiation of cell constriction. This lead Berezuk (2018) to postulate that FtsK may serve as said intermediate between FtsA and FtsN through its direct interaction with FtsA (Berezuk, 2018). While our results support the finding that FtsK interacts with FtsA, further biochemical characterization of this interaction is required before the aforementioned model can be proven or disproven.

The implication of ZapD as a potential FtsK interaction partner in our results is of interest, as ZapD has only been shown to interact with itself and FtsZ (Durand-Heredia et al., 2012). The fact that FtsZ is a mutual interaction partner between FtsK and ZapD, and is required for localization of both proteins to the division site, suggests they could be in close spatial proximity during Z-ring formation/maturation (Chen and Beckwith, 2001; Durand-Heredia et al., 2012; Roach et al., 2016). An interaction between FtsK and ZapD could therefore be plausible and is worth further investigation.
The presence of MinD in our results is also of interest as it functions in cell division and, like ZapD, has not been implicated in an interaction with FtsK in the literature. Unlike ZapD, MinD is essential for cell division and is not considered to be part of the mid-cell localized divisome complex. The role of MinD in division is described in Chapter 1: Introduction subsection Regulation of Cell Division. MinD is essential and could represent a previously unknown link between the divisome and the division site-regulating Min system. For this reason, primarily, MinD was selected from the list of potential FtsK\textsubscript{N} interactors for further investigation.

To test our results obtained by \textit{in vivo} cross-linking; we proceeded to validate the proposed FtsK\textsubscript{N}-MinD interaction \textit{in vitro}. This was done using a pulldown assay based on immobilized metal affinity chromatography (IMAC), which was chosen for its high level of sensitivity (Rao \textit{et al.}, 2014). A downside to this high sensitivity is false-positive interactions can be detected as a result of non-specific protein binding, therefore a number of controls were incorporated to assure the accuracy of the results. The results from this pulldown assay suggest MinD interacts directly with FtsK\textsubscript{N} \textit{in vitro} (Figure 3.6-A). Further verification was achieved using a similar pulldown assay based on immuno-affinity chromatography. The assay functioned similar in principal to the previous pulldown assay except the ‘bait’ and ‘prey’ roles were reversed. The results showed His\textsubscript{10}-FtsK\textsubscript{N} co-eluted with FLAG-MinD after being washed completely from the mobile phase and without cross-reacting with the anti-FLAG resin stationary phase (Figure 3.6-B). These results are in accordance with the previous assay showing an \textit{in vitro} interaction between FtsK\textsubscript{N} and MinD.
The *in vivo* and *in vitro* results presented here provide strong evidence that the essential cell division protein MinD directly interacts with FtsK and that the N-terminal transmembrane domain of FtsK is sufficient for this interaction. These findings have not yet been reported in the literature and may represent a previously unknown link between the divisome and the Min system. Although these results are not sufficient to determine the implications of this interaction, currently available studies can help us speculate as to its biological relevance.

A study by Juarez and Margolin (2010) tracked the oscillation patterns of MinD and showed that in *E. coli* cells expressing a MinD-GFP fusion, MinD briefly paused at mid-cell between oscillations in a MinC-independent manner and the frequency of pausing increased as the division septum developed (Juarez and Margolin 2010). The reason for this pausing has yet to be experimentally verified, however the authors proposed a number of potential models. Our findings support the model in which mid-cell pausing is facilitated by direct interaction with a divisome protein. It is possible FtsK localized to mid-cell temporarily ‘grabs’ MinD between oscillations, which, over the course of division, aids in the equal distribution of MinD between daughter cells (Juarez and Margolin 2010). In contrast there is also evidence to show MinD localization may be directed by its affinity for anionic phospholipids such as cardiolipin, which are enriched at the cell poles and division septum (Mileykovskaya and Dowhan 2000; Koppelman 2001; Mileykovskaya *et al*., 2003). Further study is required to determine if FtsK is responsible for MinD oscillation pausing, aiding in its distribution between daughter cells after division.

Draper *et al.* (1998) and Yu *et al.* (1998) found that overexpression of FtsK and FtsK$_N$-GFP respectively both caused a division block in *E. coli*, resulting in filamentous cells. Building
on the previously proposed model, I propose the FtsK-MinD interaction may play a role in this cell division inhibition. It is possible that overexpression of FtsK could enhance the normally transient hold of MinD at the division site, leading to the localization of MinC and thus formation of the MinCD division inhibitor. This would result in the FtsZ-inhibitory function of MinC acting on the Z-ring present at mid-cell and a block of normal division.

Taviti and Beuria, (2017) recently published work showing a MinD-FtsZ interaction via an in vitro pulldown assay. Their results also support our model. Given that FtsK has been shown to interact with FtsZ, these findings raise the possibility that the FtsK-MinD interaction could be facilitated or influenced by FtsZ. Further investigation is required to determine if FtsZ plays a role in the proposed model of FtsK and MinD.

**Future Directions**

The results presented in this thesis show a previously unreported interaction between the cell division proteins FtsK and MinD. Upon analysis of the current relevant literature we propose a model in which this interaction is important for the equal distribution of MinD proteins following division in *E. coli*. We also propose that the division blocks demonstrated by overexpression of either FtsK or MinD are, at least in part, caused by the improper localization of Min proteins to mid-cell during early stages of division, which would be facilitated by the interaction of FtsK and MinD. To test this model however, the FtsK-MinD interaction would require further characterization. This could be done by repeating the in vitro pulldown assay using different truncated versions of FtsK_N and MinD to determine which segments of each protein are essential for this interaction. Suspensions resulting from pulldown assays could also
be probed for the presence of FtsZ by Western blot using an FtsZ-specific antibody to determine if FtsZ is involved in the interaction. Additionally, site-directed mutagenesis could be used in combination with the in vitro interaction assay to determine essential interaction information at the residue level. If a variant of FtsKN could be found that does not interact with MinD, this could be used to determine if the interaction is indeed essential for cell division. Given the well-studied oligomerization dynamics of MinD (discussed further in Chapter 1: Introduction), it would also be important to use size-exclusion chromatography to determine if the interaction requires the monomeric or dimeric form of MinD.

In order to determine whether or not FtsK plays a role in the transient mid-cell pausing of the MinD oscillation, a co-localization assay could be developed. This would require the engineering of fluorescently labeled fusion proteins of FtsKN and MinD, which could be tracked in live E. coli cells by fluorescence microscopy. The confirmation of transient co-localization of MinD and FtsKN over the course of cell growth and division would provide initial evidence in support of our model. Verification that the FtsKN-MinD interaction is involved in the division block observed when overexpressing either protein would prove difficult given that both proteins are essential for division. However, the suppression of FtsK deletion lethality by overexpression of FtsA, FtsQ or FtsN could be used to develop an assay to test this prediction (Geissler and Margolin 2005; Goehring et al. 2007). For example, a strain of E. coli could be developed containing an ftsK deletion and a plasmid containing ftsA, which could be induced to overexpress the associated FtsA protein. This strain could then be transformed with a complimentary plasmid containing the minD gene. If the cells did not exhibit a phenotype indicative of a division block following the simultaneous overexpression of FtsA and MinD, this would confirm
the involvement of FtsK in the division inhibition caused by aberrant MinD expression. This would have to be accompanied by the appropriate controls to assure the rescue was not caused solely by the overexpression of FtsA. Overall, although significant study is still required, the discovery of the interaction between FtsK and MinD presented in this thesis could lead to a deeper understanding of these proteins and their role in cell division in *E. coli*.

References


Mercier, R., Petit, M.-A., Schbath, S., Robin, S., Karoui, M. El, Boccard, F., and Espéli, O.


Appendix A

Table A.1 List of Putative FtsK\textsubscript{N} Interaction Partners Determined by \textit{In Vivo} Cross-linking and Mass Spectrometry

Mass spectrometry was used to identify proteins captured by \textit{in vivo} cross-linking of FtsK\textsubscript{N} variants R20C, I107C and Y217C. Analysis of the mass spectrometry data produced a list of 164 putative FtsK\textsubscript{N} interaction partners. These proteins were selected according to the following parameters: A minimum of 4 unique peptides had to be detected for each protein and only peptides displaying a 95% or higher probability threshold were considered. Proteins meeting these criteria had to be present in at least 4/5 cross-linked samples and absent from at least 2/5 non cross-linked controls. Additionally, all non-cytoplasmic and ribosomal proteins were excluded.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene Ontology (biological process)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MinD</td>
<td>cell division</td>
</tr>
<tr>
<td>MasY</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>ClpX</td>
<td>cell division</td>
</tr>
<tr>
<td>Odo2</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>GuaA</td>
<td>glutamine metabolic process</td>
</tr>
<tr>
<td>DeoB</td>
<td>deoxyribonucleotide catabolic process</td>
</tr>
<tr>
<td>AraB</td>
<td>arabinose catabolic process</td>
</tr>
<tr>
<td>EnO</td>
<td>glycolytic process</td>
</tr>
<tr>
<td>SyD</td>
<td>aspartyl-tRNA aminoacylation</td>
</tr>
<tr>
<td>CdD</td>
<td>deoxycytidine catabolic process</td>
</tr>
<tr>
<td>RhO</td>
<td>DNA-templated transcription, termination</td>
</tr>
<tr>
<td>SyfB</td>
<td>phenylalanyl-tRNA aminoacylation</td>
</tr>
<tr>
<td>DnaJ</td>
<td>response to heat</td>
</tr>
<tr>
<td>AraC</td>
<td>arabinose catabolic process</td>
</tr>
<tr>
<td>MfD</td>
<td>DNA repair</td>
</tr>
<tr>
<td>ArcA</td>
<td>negative regulation of transcription, DNA-templated</td>
</tr>
<tr>
<td>HflK</td>
<td>response to heat</td>
</tr>
<tr>
<td>RpoA</td>
<td>transcription, DNA-templated</td>
</tr>
<tr>
<td>Kpyk1</td>
<td>glycolytic process</td>
</tr>
<tr>
<td>6pgD</td>
<td>D-gluconate catabolic process</td>
</tr>
<tr>
<td>EftS</td>
<td>guanyl-nucleotide exchange factor activity\textsuperscript{b}</td>
</tr>
<tr>
<td>PgK</td>
<td>glycolytic process</td>
</tr>
<tr>
<td>MbhM</td>
<td>hydrogenase (acceptor) activity\textsuperscript{b}</td>
</tr>
<tr>
<td>DksA</td>
<td>regulation of gene expression</td>
</tr>
<tr>
<td>FdoH</td>
<td>cellular respiration</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Biological Process</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Glrx4</td>
<td>cell redox homeostasis</td>
</tr>
<tr>
<td>LepA</td>
<td>positive regulation of translation</td>
</tr>
<tr>
<td>GcsH</td>
<td>glycine decarboxylation via glycine cleavage system</td>
</tr>
<tr>
<td>RpoD</td>
<td>response to heat</td>
</tr>
<tr>
<td>AdD</td>
<td>adenosine catabolic process</td>
</tr>
<tr>
<td>TdH</td>
<td>threonine catabolic process</td>
</tr>
<tr>
<td>HslU</td>
<td>proteolysis</td>
</tr>
<tr>
<td>AhpF</td>
<td>cell redox homeostasis</td>
</tr>
<tr>
<td>PtkB</td>
<td>galactitol metabolic process</td>
</tr>
<tr>
<td>HldD</td>
<td>carbohydrate metabolic process</td>
</tr>
<tr>
<td>MtlD</td>
<td>mannitol catabolic process</td>
</tr>
<tr>
<td>YkgF</td>
<td>lactate oxidation</td>
</tr>
<tr>
<td>YcjR</td>
<td>carbohydrate metabolic process</td>
</tr>
<tr>
<td>RcsD</td>
<td>phosphorelay signal transduction system</td>
</tr>
<tr>
<td>GstA</td>
<td>response to hydrogen peroxide</td>
</tr>
<tr>
<td>EttA</td>
<td>negative regulation of translational elongation</td>
</tr>
<tr>
<td>AcP</td>
<td>fatty acid biosynthetic process</td>
</tr>
<tr>
<td>YjiA</td>
<td>cellular response to DNA damage stimulus</td>
</tr>
<tr>
<td>SyM</td>
<td>methionyl-tRNA aminoacylation</td>
</tr>
<tr>
<td>DhaS</td>
<td>threonine biosynthetic process</td>
</tr>
<tr>
<td>GatD</td>
<td>galactitol catabolic process</td>
</tr>
<tr>
<td>FtsA</td>
<td>cell division</td>
</tr>
<tr>
<td>Ak2H</td>
<td>threonine biosynthetic process</td>
</tr>
<tr>
<td>RecA</td>
<td>SOS response</td>
</tr>
<tr>
<td>FumA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>GcsT</td>
<td>glycine decarboxylation via glycine cleavage system</td>
</tr>
<tr>
<td>SyQ</td>
<td>glutaminyl-tRNA aminoacylation</td>
</tr>
<tr>
<td>Mcp2</td>
<td>chemotaxis</td>
</tr>
<tr>
<td>SyS</td>
<td>selenocysteine biosynthetic process</td>
</tr>
<tr>
<td>YgfZ</td>
<td>tRNA processing</td>
</tr>
<tr>
<td>KbL</td>
<td>L-threonine catabolic process to glycine</td>
</tr>
<tr>
<td>IbpA</td>
<td>response to heat</td>
</tr>
<tr>
<td>NfuA</td>
<td>iron-sulfur cluster assembly</td>
</tr>
<tr>
<td>GalU</td>
<td>lipopolysaccharide core region biosynthetic process</td>
</tr>
<tr>
<td>WbbI</td>
<td>lipopolysaccharide biosynthetic process</td>
</tr>
<tr>
<td>AccD</td>
<td>fatty acid biosynthetic process</td>
</tr>
<tr>
<td>SlyD</td>
<td>protein maturation</td>
</tr>
<tr>
<td>DhnA</td>
<td>aerobic electron transport chain</td>
</tr>
<tr>
<td>GlyA</td>
<td>glycine biosynthetic process from serine</td>
</tr>
<tr>
<td>PepB</td>
<td>peptide catabolic process</td>
</tr>
<tr>
<td>IbpB</td>
<td>response to heat</td>
</tr>
</tbody>
</table>
RodZ regulation of cell shape
SdhL gluconeogenesis
DhaM glycerol catabolic process
T1mK DNA restriction-modification system
AsnB cellular amino acid biosynthetic process
HelD DNA recombination
UspA identical protein binding
PpiD chaperone-mediated protein folding
PntA NADPH regeneration
PepD peptide catabolic process
ProQ posttranscriptional regulation of gene expression
GlpD glycerol catabolic process
PgM glucose metabolic process
ThiI thiamine biosynthetic process
ThrC threonine biosynthetic process
KprS nucleotide biosynthetic process
GalF lipopolysaccharide biosynthetic process
Glrx2 cell redox homeostasis
NpD chemotaxis
SelB selenocysteine metabolic process
IscS iron-sulfur cluster assembly
ArcB regulation of transcription, DNA-templated
OsmC response to hydroperoxide
YebC regulation of transcription, DNA-templated
KdsA keto-3-deoxy-D-manno-octulosonic acid biosynthetic process
Mao1 gluconeogenesis
YkgG unknown
PmbA proteolysis
PtkA galactitol transport
HprT purine ribonucleoside salvage
CrL positive regulation of transcription, DNA-templated
YifE chromosome organization
OmpR positive regulation of transcription, DNA-templated
UbiE ubiquinone biosynthetic process
FrmA formaldehyde catabolic process
CrP transcription, DNA-templated
YceF nucleoside-triphosphate diphosphatase activity
SyC cysteinyl-tRNA aminoacylation
YcjS oxidoreductase activity
AroG aromatic amino acid family biosynthetic process
PflA glucose metabolic process
ZapD division septum assembly
RibA riboflavin biosynthetic process
PyrG CTP biosynthetic process
CapP tricarboxylic acid cycle
GlmS carbohydrate metabolic process
MasZ tricarboxylic acid cycle
Sdh8 tricarboxylic acid cycle
GlmM peptidoglycan biosynthetic process
YegQ peptidase activity
LdhD mixed acid fermentation
MutS mismatch repair
FabH fatty acid biosynthetic process
SyN asparaginyl-tRNA aminoacylation
PtfbC phosphoenolpyruvate-dependent sugar phosphotransferase system
AaT L-phenylalanine biosynthetic process
EfP translational elongation
SpeE spermidine biosynthetic process
Pt1P phosphoenolpyruvate-dependent sugar phosphotransferase system
IpyR phosphate-containing compound metabolic process
HsI0 response to heat
ThiO cell redox homeostasis
DnaA DNA replication
YgeW cellular amino acid metabolic process
AccC fatty acid biosynthetic process
PaaK phenylacetate catabolic process
LuxS quorum sensing
HflC response to heat
NusA transcription antitermination
HscA cellular response to cold
GpdA glycerophospholipid biosynthetic process
His7 histidine biosynthetic process
MurE peptidoglycan biosynthetic process
DeoC carbohydrate catabolic process
FdheI protein maturation
FtsZ cell division
McrB DNA catabolic process
FadA fatty acid beta-oxidation
WecC enterobacterial common antigen biosynthetic process
BcP response to oxidative stress
MalY negative regulation of DNA-binding transcription factor activity
YcdY bacterial-type flagellum-dependent swarming motility
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PpnN</td>
<td>pyrimidine-5'-nucleotide nucleosidase activity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glrx3</td>
<td>deoxyribonucleotide biosynthetic process</td>
</tr>
<tr>
<td>PfkA</td>
<td>canonical glycolysis</td>
</tr>
<tr>
<td>YbjX</td>
<td>cellular response to DNA damage stimulus</td>
</tr>
<tr>
<td>FtsI</td>
<td>cell division</td>
</tr>
<tr>
<td>QueC</td>
<td>queuosine biosynthetic process</td>
</tr>
<tr>
<td>MprA</td>
<td>response to antibiotic</td>
</tr>
<tr>
<td>Mcp3</td>
<td>chemotaxis</td>
</tr>
<tr>
<td>DapA</td>
<td>lysine biosynthetic process via diaminopimelate</td>
</tr>
<tr>
<td>KhsE</td>
<td>threonine biosynthetic process</td>
</tr>
<tr>
<td>UrK</td>
<td>cytidine triphosphate salvage</td>
</tr>
<tr>
<td>OxyR</td>
<td>response to oxidative stress</td>
</tr>
<tr>
<td>YeaK</td>
<td>positive regulation of translational fidelity</td>
</tr>
<tr>
<td>YihX</td>
<td>glucose-1-phosphatase activity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RmlC</td>
<td>lipopolysaccharide biosynthetic process</td>
</tr>
</tbody>
</table>

<sup>a</sup> Retrieved from the UniProtKB *E. coli* K12 database

<sup>b</sup> Gene Ontology (biological process) not available, used Gene Ontology (molecular function)